

Rapid Changes in the Distribution of GAP-43 Correlate With the Expression of Neuronal Polarity during Normal Development and under Experimental Conditions

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Abstract. Hippocampal neurons growing in culture initially extend several, short minor processes that have the potential to become either axons or dendrites. The first expression of polarity occurs when one of these minor processes begins to elongate rapidly, becoming the axon. Before axonal outgrowth, the growth-associated protein GAP-43 is distributed equally among the growth cones of the minor processes; it is preferentially concentrated in the axonal growth cone once polarity has been established (Goslin, K., D. Schreyer, J. Skene, and G. Banker. 1990. *J. Neurosci.* 10:588–602). To determine when the selective segregation of GAP-43 begins, we followed individual cells by video microscopy, fixed them as soon as the axon could be distinguished, and localized GAP-43 by immunofluorescence microscopy. Individual minor processes acquired axonal growth characteristics within a period of 30–60 min, and GAP-43 became selectively concentrated to the growth cones of

these processes with an equally rapid time course. We also examined changes in the distribution of GAP-43 after transection of the axon. After an axonal transection that is distant from the soma, neuronal polarity is maintained, and the original axon begins to regrow almost immediately. In such cases, GAP-43 became selectively concentrated in the new axonal growth cone within 12–30 min. In contrast, when the axon is transected close to the soma, polarity is lost; the original axon rarely regrows, and there is a significant delay before a new axon emerges. Under these circumstances, GAP-43 accumulated in the new growth cone much more slowly, suggesting that its ongoing selective routing to the axon had been disrupted by the transection. These results demonstrate that the selective segregation of GAP-43 to the growth cone of a single process is closely correlated with the acquisition of axonal growth characteristics and, hence, with the expression of polarity.

THE development of distinct axonal and dendritic domains is essential for the functional polarization of neurons. The differential outgrowth of these two types of processes can be readily observed in dissociated cell cultures derived from the hippocampal region of the cerebral cortex. Like their counterparts in situ, the axons and dendrites that develop in hippocampal cultures differ from one another in light and electron microscopic morphology, in the macromolecular composition of their cytoskeletons and membranes, and in synaptic polarity (Banker and Waxman, 1988; Goslin et al., 1988). Early in development, hippocampal neurons extend several short "minor processes," which are approximately equal in length (stage 2 of development). These stage 2 neurons show no indication of polarity as judged by their growth characteristics, light and electron microscopic appearance, and immunostaining for axonal and dendritic markers (Dotti et al., 1988; Goslin et al., 1990; Deitch, J., and G. Banker, unpublished observations). Moreover, experimental evidence indicates that at this stage the minor processes are not irreversibly specified as axons or dendrites (Dotti and Banker, 1987; Goslin and Banker,

1989). Polarity is first expressed when, rather abruptly, one minor process begins to grow rapidly and acquires axonal characteristics (stage 3 of development). The transition between stage 2 and stage 3 is thus a critical point in the expression of neuronal polarity. Somewhat later, during stage 4 of development, the remaining minor processes begin to elongate and take on the properties of dendrites.

To investigate possible mechanisms underlying the establishment of polarity, it is essential to identify molecular differences between axons and dendrites that precede or accompany the transition between stage 2 and stage 3, when hippocampal neurons first express a polarity. One protein of potential interest in this regard is the nervous system-specific phosphoprotein GAP-43 (also termed B-50, F1, P57, or pp46), a protein with possible growth regulatory functions (for reviews see Benowitz and Routtenberg, 1987; Skene, 1989; Gordon-Weeks, 1989). GAP-43 is an important constituent of axonal growth cones (Skene et al., 1986; Meiri et al., 1986, 1988) but is not detectable in dendrites or dendritic growth cones (Goslin et al., 1988). We have recently shown that GAP-43 becomes selectively distributed to the

axon early in the development of neuronal polarity (Goslin et al., 1990). These observations raise the possibility that the selective accumulation of GAP-43 in one process may be one of the events responsible for its axon-like rate of elongation and hence for the development of polarity.

In the present study, we sought to define more accurately the temporal correlation between the selective distribution of GAP-43 and the expression of neuronal polarity, both during normal development and under experimental conditions. In one set of experiments, we followed individual cells during the transition between stage 2 and stage 3 and determined the localization of GAP-43 at the earliest stage of axonal outgrowth. In a second set of experiments, we examined the distribution of GAP-43 after axonal transection. Because transection of the axon results in removal of the axonal growth cone, where GAP-43 is most concentrated, its rate of accumulation in the newly formed growth cone could be assessed. The results of both experiments demonstrate a very close correlation between the selective accumulation of GAP-43 in one process and the acquisition by that process of axonal growth characteristics.

Materials and Methods

Cell Culture

Methods for preparing the hippocampal cell cultures follow those previously described (Banker and Cowan, 1977; Bartlett and Banker, 1984). Special culture dishes were used to facilitate micromanipulation and relocation of individual cells after lesioning (for details see Goslin and Banker, 1989). These were prepared from plastic petri dishes by drilling a 16-mm-diameter hole in the bottom and attaching an acid-cleaned glass coverslip to the outer surface of the dish. A glass ring was sealed to the inside of the dish with silicone grease, forming a well above the coverslip sufficient to hold 1 ml of medium. The inner surface of the coverslip was treated with polylysine to enhance cell adhesion, and then the well was filled with MEM containing the N2 supplements of Bottenstein and Sato (1979), together with sodium pyruvate (0.01 mg/ml; Selak et al., 1985) and ovalbumin (0.1%).

Cell suspensions were prepared by trypsin treatment of hippocampi (dissected from the brains of 18-d rat fetuses) and trituration using a fire-polished Pasteur pipette. Approximately 10,000 cells were plated into the well of each dish. To provide trophic support (Banker, 1980), a coverslip, containing a confluent monolayer of astroglial cells (Booher and Sensenbrenner, 1972) was placed on the top of the glass ring, with the glia facing the neurons.

Video Recording and Process Transection

Observations of living cells were made by phase-contrast and differential interference contrast microscopy and recorded on videotape or an optical memory disk recorder. Measurements of process length, accurate to within a few micrometers, were made using a digitizing tablet on drawings taken from the monitor.

For studies of cells during the transition between stage 2 and stage 3, cells were recorded at intervals of 30–60 min. Only typical stage 2 cells, with three to six minor processes that were relatively equal in length, were chosen for study. The location of each of the cells was marked by inscribing a circle on the bottom of the dish with a diamond object marker. To determine the distribution of GAP-43 in such neurons, cells were followed until one process was at least 10 μm longer than the others. The cultures were then fixed and stained for GAP-43.

The responses of cells to process transection were also observed. Transections of minor processes of stage 2 cells were performed between 10 and 15 h after the cells were plated. Axonal transections were performed between 18 and 36 h after plating, when the axon was at least 100 μm in length. The location of each of the cells selected for lesioning was marked by inscribing a circle on the bottom of the dish with a diamond object marker. Once all of the desired cells from one dish had been selected, the lid, glial coverslip, and glass ring were removed to permit access for lesioning, and

additional medium, previously conditioned by exposure to glial cells (Banker, 1980), was added to minimize pH changes.

The transections were performed using a fine-tipped micropipette held in a micromanipulator attached to the stage of an inverted microscope, as described in detail by Goslin and Banker (1989). The tip of the micropipette was positioned near the process to be transected, brought into contact with the substrate, and then rapidly drawn across the process at right angles to its direction of growth. In the case of stage 2 cells, to eliminate any possible selection bias, the minor process that emerged from the cell body nearest to 12:00 o'clock was always chosen for transection. All manipulations after removing the glial coverslip were performed as rapidly as possible to minimize changes in pH. After transection, cells were recorded at frequent intervals. They were fixed and stained for GAP-43 at varying times after process transection.

Immunofluorescence Microscopy

After the video recordings were completed, the medium was gently removed from the dishes and replaced with 4% formaldehyde in PBS containing 0.12 M sucrose. Cells were fixed for 40 min at 37°C, permeabilized in ethanol, rinsed in PBS, and blocked with 10% BSA for 1 h at 37°C. They were then exposed to mouse monoclonal anti-GAP-43 antibody (clone 9-IE12; diluted 1:4,000 in 1% BSA in PBS) overnight at 4°C. This antibody, which was kindly provided by Drs. D. J. Schreyer and J. H. P. Skene (Stanford University, Stanford, CA), reacts monospecifically with GAP-43 in immunoblots of fetal rat brain and cultured hippocampal neurons (Goslin et al., 1990). Cells were then rinsed with PBS and incubated for 1 h at 37°C with FITC-labeled anti-mouse IgG (1:400; Cappel Laboratories, Cochranville, PA). Finally, cells were rinsed with PBS and mounted in PBS/glycerol (1:1) containing 0.05% *p*-phenylenediamine (Johnson et al., 1981). Cultures were photographed with Tri-X film, which was developed in D19.

Cycloheximide Treatment

To determine if growth cone regeneration or the appearance of GAP-43 in regenerating growth cones required the synthesis of new proteins, some cultures were treated with cycloheximide at a final concentration of 10 $\mu\text{g}/\text{ml}$ (from a stock solution of 1 mg/ml ethanol). Cultures were incubated with cycloheximide for 1 or 2 h before axonal transection, maintained in cycloheximide for an additional 1 h, and then fixed and stained for GAP-43 as described above. In a quantitative study, this concentration of cycloheximide was found to inhibit protein synthesis in cultured nerve cells by ~95% (Daniels, 1972). To verify the effectiveness of this drug in hippocampal cultures, we used light microscopic autoradiography to assess the extent of labeling after incubation of cultured neurons with [³H]leucine for 45 min. We estimate that [³H]leucine incorporation was inhibited by ~75–90%.

Results

The Initial Outgrowth of the Axon

To examine the distribution of GAP-43 during the initial expression of polarity, it was first necessary to establish criteria for identification of the axon at the earliest stage possible. Previous studies of the response of hippocampal neurons to axonal transection suggest that axonal identity is determined once one minor process exceeds the others by a length of ~10 μm or more (Goslin and Banker, 1989). To determine if this criterion could be applied to normal cells undergoing the transition between developmental stages 2 and 3, individual cells were recorded at intervals of ~30 min, beginning during stage 2 and continuing until their axons could be clearly identified using previously established criteria (Dotti et al., 1988). Selected frames from a representative record are shown in Fig. 1. A pronounced change in the pattern of growth of this cell, signaling the transition from stage 2 to stage 3, occurred in the interval between 21:46 and 22:21 (Fig. 1, *D* and *E*). Before this transition (Fig. 1, *A–D*), the minor processes underwent little net elongation but were

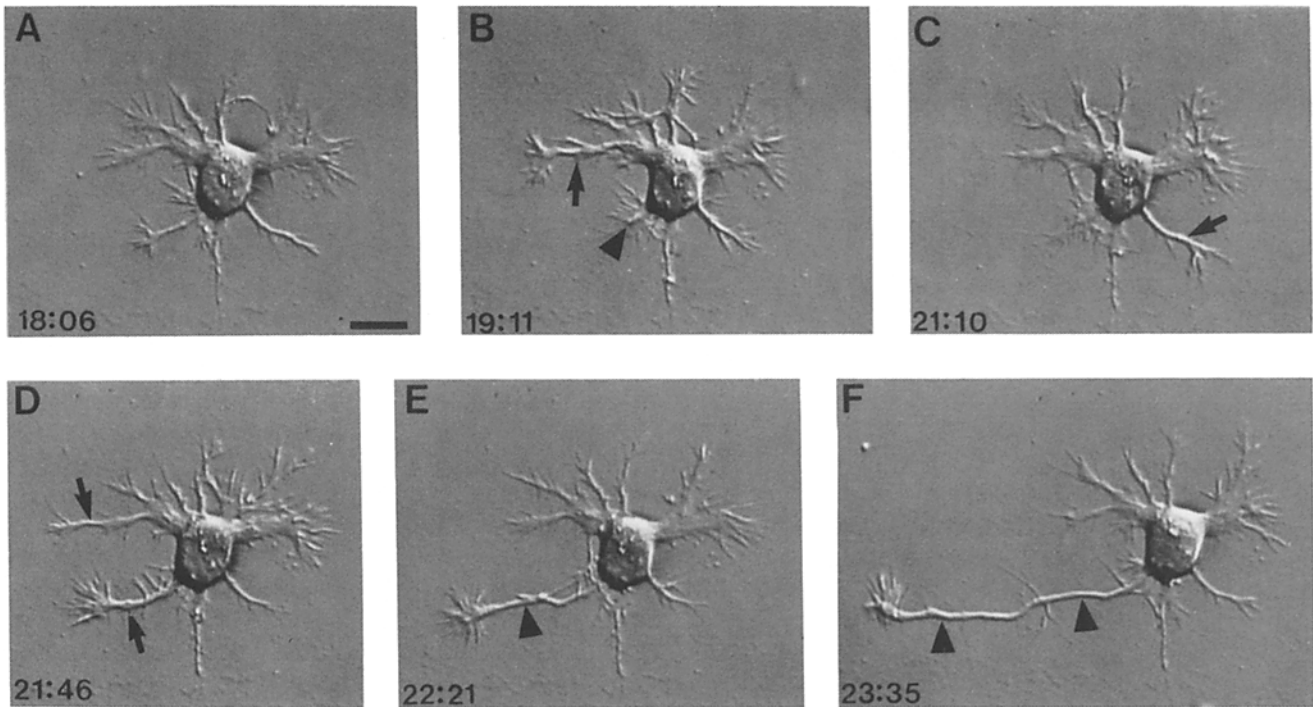


Figure 1. The initial outgrowth of the axon during the development of hippocampal neurons in culture. These differential interference contrast micrographs illustrate a representative cell during the transition from stage 2, when the cell was symmetric in appearance, to stage 3, when the cell's axon was clearly defined. In the long period preceding axonal outgrowth, several of the minor processes underwent brief periods of elongation, often followed by retraction (*B*, *C*, and *D*, arrows). Once one process exceeded the others in length by $\sim 10 \mu\text{m}$ (*E*, arrowhead), it grew continuously at a rapid rate for >3 h, becoming the definitive axon (*F*, arrowheads). At one point during stage 2, the process that ultimately became the axon retracted almost entirely (*B*, arrowhead). These illustrations are taken from a longer series, that began at 11:58 and continued for 25 h. Bar, $10 \mu\text{m}$.

highly dynamic (see also Goslin and Banker, 1989). In succession, three of the minor processes elongated, and then retracted. In some cases, 2 minor processes elongated during the same time interval (compare Fig. 1, *C* with *D*). During the transition between stages 2 and 3, growth became largely confined to one process only, the emerging axon. The axon grew rapidly and continuously for >3 h after its emergence. Then its growth became intermittent, as is typical of axons during stage 3 (as described by Dotti et al., 1988). Most remarkably, the profound change in this cell's pattern of growth that marked the emergence of its axon occurred within an interval of only 35 min.

Based on observations of 11 cells, the axon could be reliably identified once it had become $10\text{--}15 \mu\text{m}$ longer than the other processes. Until this degree of asymmetry developed, we were unable to predict which process would become the axon. Indeed, we observed instances when a process retracted even when it was as much as $8\text{--}12 \mu\text{m}$ longer than the cell's other processes.

The Distribution of GAP-43 during the Transition between Stage 2 and Stage 3 of Development

We have previously described the distribution of GAP-43 during stage 2 and stage 3 of development (Goslin et al., 1990). Because this distribution is critical for the interpretation of the present results, it was confirmed in this study (Fig. 2). At stage 2, GAP-43 was concentrated in the growth cones

of the minor processes and distributed relatively equally among them. At stage 3, it was preferentially distributed to the axonal growth cone. At both stages of development, the content of GAP-43 in growth cones of minor processes varied. In some cells, the growth cones of minor processes were virtually devoid of GAP-43 immunoreactivity (Fig. 2, *B* and *F*). In others, GAP-43 was present in the growth cones of minor processes (Fig. 2, *D* and *H*) but was clearly less concentrated than in axonal growth cones. At both stage 2 and stage 3, the intensity of GAP-43 staining in growth cones did not necessarily correlate with their size. Most important for the results that follow, at stage 2 of development GAP-43 was never observed to be selectively concentrated in the growth cone of a single minor process.

We next examined the distribution of GAP-43 at the earliest stage that the axon could be identified. As shown in Fig. 1, the initiation of axonal growth occurs very rapidly (i.e., in ≤ 1 h). Does the selective concentration of GAP-43 to the axonal growth cone occur this rapidly? To answer this question, we used video microscopy to record cells at 1-h intervals, beginning at stage 2 and continuing until one process was at least $10 \mu\text{m}$ longer than all of the cell's other processes. Such cells were then fixed and stained for GAP-43.

Several representative examples of cells recorded during this study are shown in Fig. 3. The cell in Fig. 3, *A-G*, was observed for 6 h before its axon began to emerge. During this interval, the dynamic properties of the minor processes were evident. When one process had become $>10 \mu\text{m}$ longer than

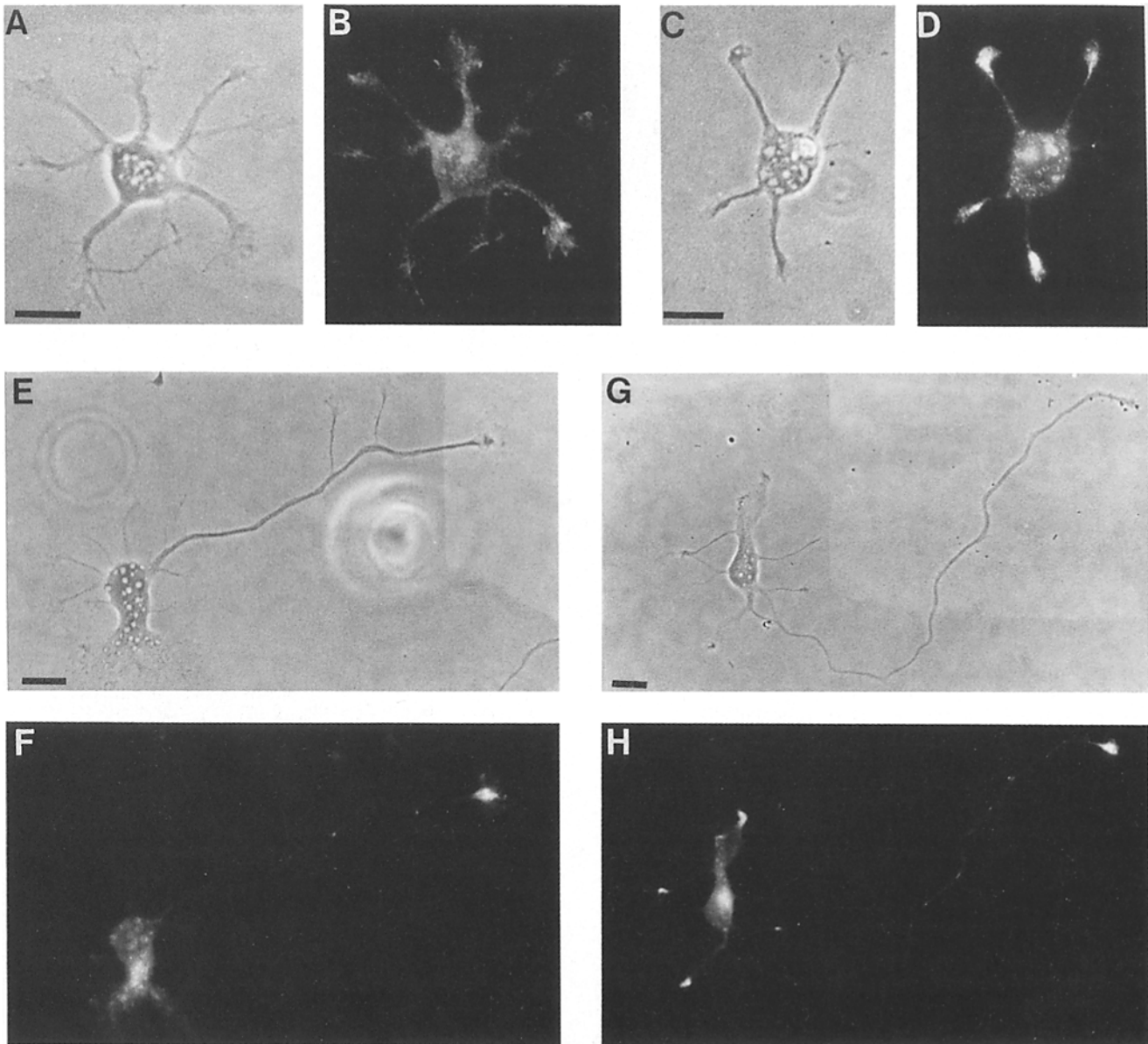


Figure 2. Representative examples of the distribution of GAP-43 during stage 2 and stage 3 of development. Hippocampal neurons in culture initially extend several short minor processes that are relatively equal in length (*A* and *C*). GAP-43 is concentrated in the growth cones of the minor processes at this stage and is distributed relatively equally among them (*B* and *D*). Stage 3 neurons have a polarized morphology with a single axon and several minor processes (*E* and *G*). At this stage, GAP-43 is selectively concentrated in the axonal growth cone (*F* and *H*). At both stages of development, the content of GAP-43 in minor process growth cones is variable. In some cells, the growth cones of minor processes are virtually devoid of GAP-43 immunoreactivity (*B* and *F*). In others, GAP-43 is noticeably concentrated in these growth cones (*D* and *H*). Bar, 10 μm .

all of the others (Fig. 3 *F*), the cell was fixed and stained. Just 42 min before fixation (Fig. 3 *E*), the cell gave no indication of a polarized morphology, but, at the time of fixation, GAP-43 was selectively concentrated in the growth cone of the emerging axon (Fig. 3 *G*). Very little GAP-43 immunoreactivity could be detected in any of the cell's other processes, which had not elongated during the transition interval before fixation. Since the preferential concentration of GAP-43 in a single process was never observed in stage 2 cells, its selective accumulation in the growth cone of the young axon must have occurred within the 42-min interval immediately preceding fixation.

The cell in Fig. 3, *H-I*, is shown ~ 1 h before fixation, when it was entirely symmetric (Fig. 3 *H*), and at the time of fixation, when its axon was first identifiable (Fig. 3 *I*). Within this interval, GAP-43 had become selectively concentrated in the growth cone of the young axon (Fig. 3 *I*); very little GAP-43 was observed in the cell's other processes. The two cells shown in Fig. 3, *K-M*, were both symmetric when the recordings were begun. After 8 h, their appearance was relatively unchanged (Fig. 3 *K*). Then, just 1 h later one of the minor processes of the cell on the right had elongated to become the axon (Fig. 3 *L*). After fixation and staining, GAP-43 was found to be highly concentrated in the growth

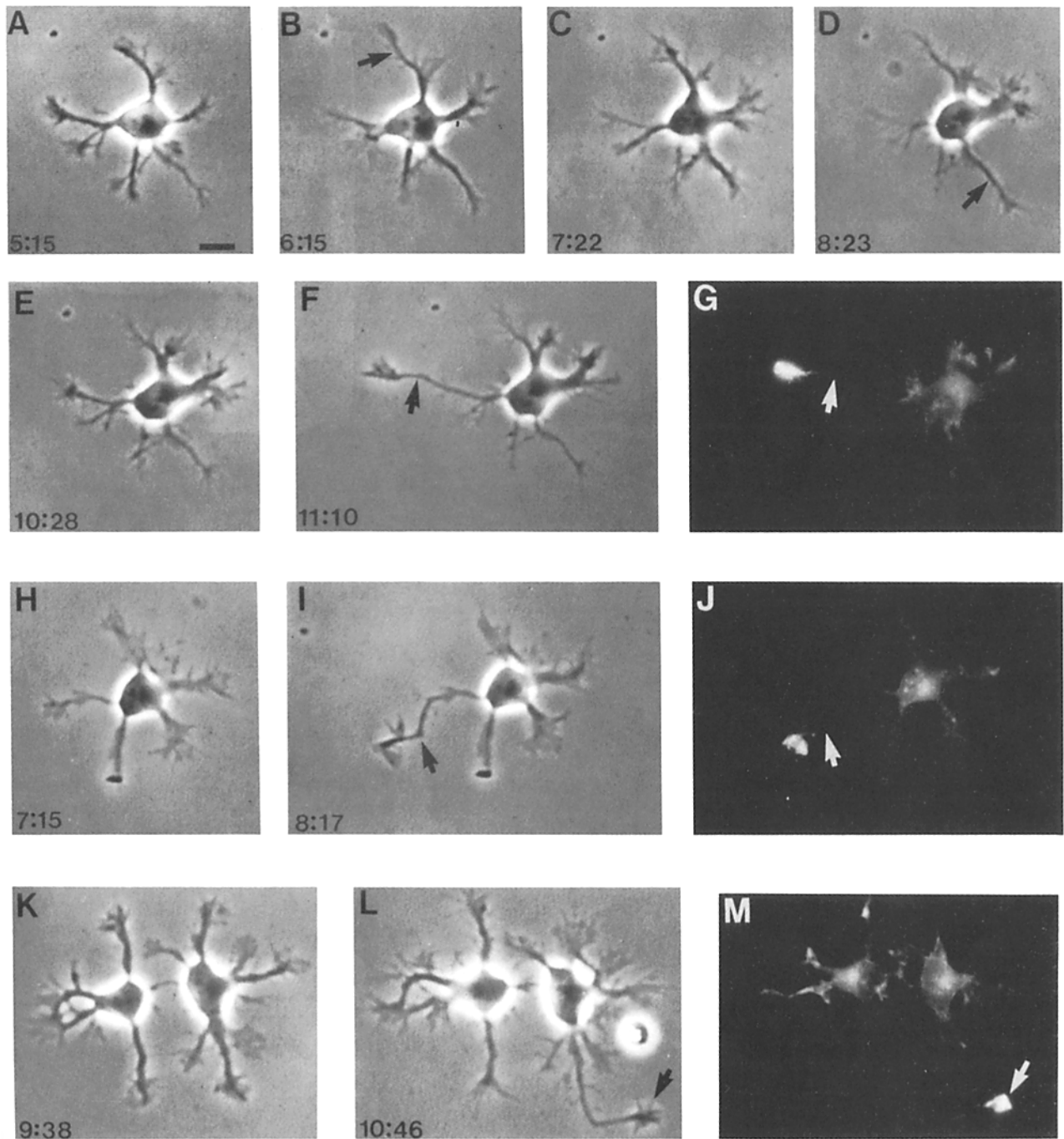


Figure 3. The distribution of GAP-43 in hippocampal neurons during the transition between stage 2 and stage 3 of development. In the cell shown in the first series of micrographs (A–F), the dynamic growth properties of the minor processes during stage 2 are evident. Several minor processes alternately extended and retracted (B and D, arrows), but after 5 h (E) the appearance of the cell was strikingly similar to its appearance at the beginning of the recordings. Just 42 min later, one of the minor processes had elongated at a rapid rate, until it was 18 μm longer than the other processes (F, arrow). At this time, the cell was fixed and stained for GAP-43 (G). GAP-43 was extremely concentrated in the growth cone of the emerging axon (G, arrow) and was barely detectable in the minor processes. A second cell is shown during stage 2 (H), when it was entirely symmetric, and 1 h later, when one process was 20 μm longer than the others (I, arrow). GAP-43 was selectively concentrated in the growth cone of this process (I, arrow). The two cells shown in K were initially very similar in appearance, with all processes about equal in length. Approximately 1 h later (L), one of the minor processes of the cell on the right had elongated to become the young axon (L, arrow), while the cell on the left was essentially unchanged. GAP-43 was highly concentrated in the growth cone of the young axon (M, arrow) but undetectable in the growth cones of this cell's other processes. In contrast, GAP-43 was present in the growth cones of several of the minor processes of the cell on the left. Bar, 10 μm .

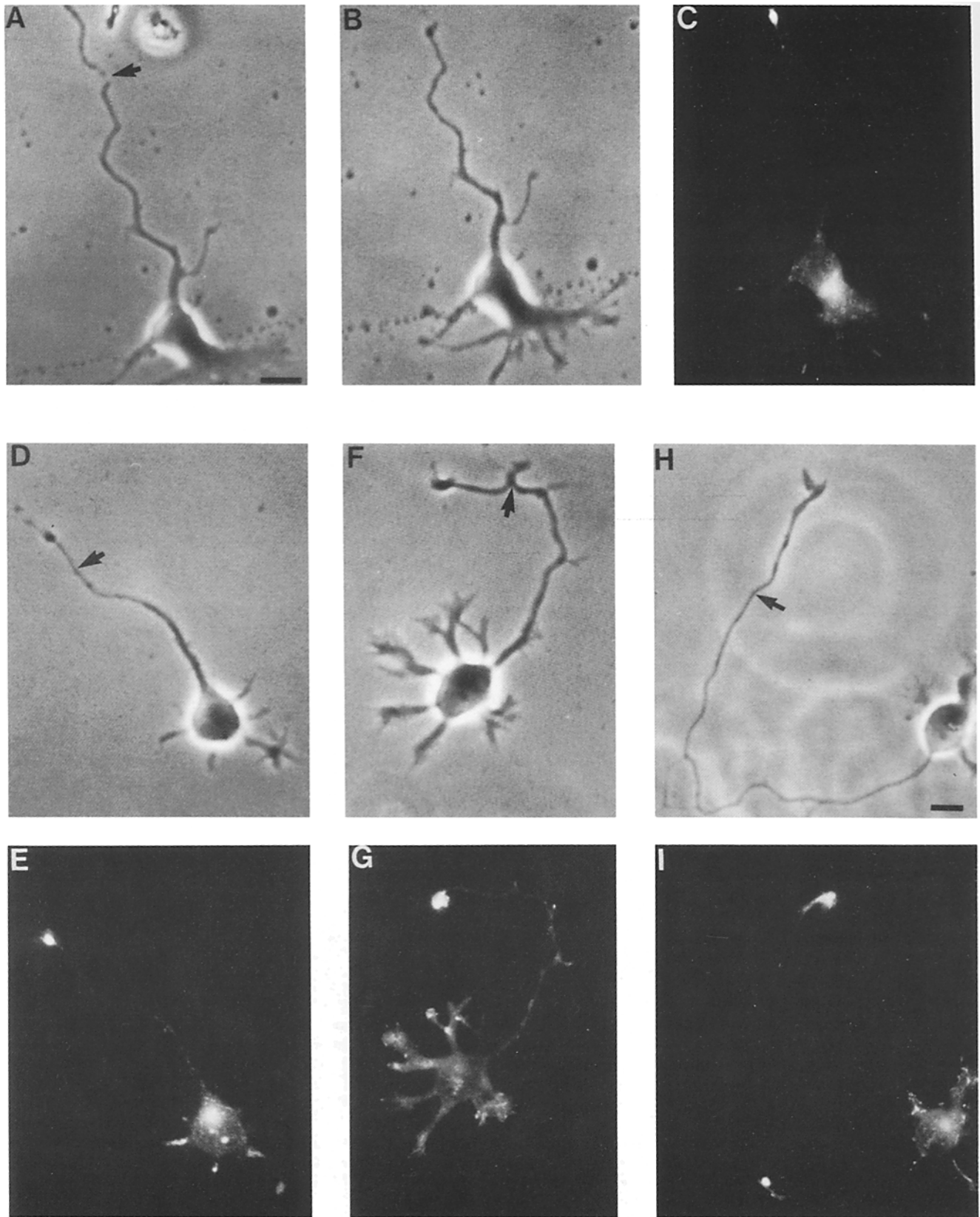


Figure 4. The redistribution of GAP-43 after distal axonal transections during stage 3 of development. Phase-contrast micrographs (*A*) show a cell just after its axon was transected (*A*; site of transection indicated by arrow) and 17 min later, when a new growth cone had formed (*B*, arrow). Though the new growth cone was still smaller than normal, it already contained a marked concentration of GAP-43 (*C*, arrow). Other stage 3 neurons fixed at progressively later times after axonal transection are shown in *D* (27 min), *F* (60 min), and *H* (150 min), with the sites of axonal transection indicated by arrows. GAP-43 was highly concentrated in the new axonal growth cones of each of these cells (*E*, *G*, and *I*, arrows). Note that the axons of the cells shown in *F* and *H* had elongated significantly in the interval between axonal transection and fixation. Bar, 10 μ m.

Table 1. Accumulation of GAP-43 in Newly Formed Growth Cones after Distal Transections of the Axon during Stage 3 of Development

Time after transection	Growth cones examined	Concentrated*	Selectively concentrated‡
<i>min</i>	<i>n</i>	%	%
15–30	14	100	86
31–60	10	100	80
61–90	13	100	70
>90	7	72	72

* GAP-43 was considered to be concentrated in the new growth cone of the transected process if it was clearly more concentrated than along the process, thus resembling the GAP-43 staining typical of the growth cones of minor processes.

‡ GAP-43 was considered to be selectively concentrated if the staining in the new growth cone was significantly greater than in any of the cell's other growth cones, thus resembling the GAP-43 staining typical of stage 3 axonal growth cones.

cone of the young axon but almost completely absent from the other processes of this cell (Fig. 3 *M*). By comparison, there was no suggestion of a selective concentration of GAP-43 in any of the processes of the other cell which had not yet formed an axon.

In all, we followed 27 cells in transition between stages 2 and 3, fixing them as soon as a morphological asymmetry developed. In every case, at the time of fixation there was a pronounced concentration of GAP-43 in the growth cone of the emerging axon while little GAP-43 was detected in the other processes. The interval between observations averaged 64 min and, in several cases, was as little as 30 min. At the time of fixation, the young axons were on average 17 μm longer than the longest of the other processes. At the immediately preceding recording interval, their appearance was still symmetric; the minor process that became the axon was on average only 2 μm longer than the others (ranging from 12 μm

shorter to 10 μm longer than the others). Thus, in a brief period the localization of GAP-43 changed from a relatively uniform distribution in all growth cones to a highly selective concentration in the growth cone of the young axon. This occurred concurrently with the acquisition of an asymmetric morphology, the onset of rapid growth, and the restriction of growth to a single process.

We also fixed a few cells after a minor process had elongated, exceeding other minor processes by only 6–8 μm in length. In these cases, GAP-43 was present in the growth cone of the process that had elongated but, unlike the cells described above, was not selectively concentrated there.

The Redistribution of GAP-43 after Axonal Transection

The results so far described are consistent with the possibility of a link between the selective distribution of GAP-43 and the development of polarity. To address this possibility experimentally, we examined the time course of the accumulation of GAP-43 in the new growth cone that forms after transection of the axon. At stage 3 of development, GAP-43 is concentrated primarily in growth cones, with little GAP-43 distributed along the shafts of the processes. Since such transections remove the original growth cones, it is possible to follow the dynamics of GAP-43 accumulation as new growth cones form.

When the axon of a stage 3 neuron is transected at a distance from the cell body, the cell's polarity is preserved; the original axon invariably regrows, beginning its regrowth almost immediately (Goslin and Banker, 1989). Examples of cells fixed and stained for GAP-43 at different times after such transections are shown in Fig. 4. GAP-43 accumulated in the new growth cones about as rapidly as they formed (10–30 min). Moreover, GAP-43 became selectively concentrated in these growth cones; they contained distinctly more GAP-43 than the growth cones of the cells' other minor pro-

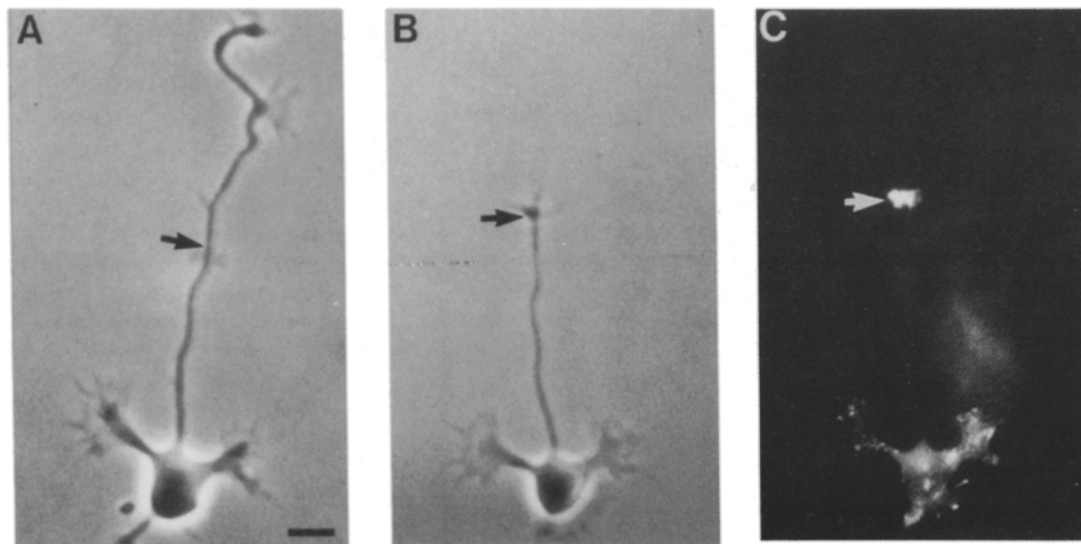


Figure 5. Inhibition of protein synthesis does not prevent the rapid accumulation of GAP-43 after a distal transection of the axon. The phase-contrast micrographs show a stage 3 neuron just before its axon was transected (*A*; site of transection indicated by arrow) and 1 h later, when the new growth cone had formed (*B*, arrow). GAP-43 was markedly concentrated in the new axonal growth cone (*C*, arrow). Cycloheximide (10 $\mu\text{g}/\text{ml}$) was added to the medium 1 h before transection. Bar, 10 μm .

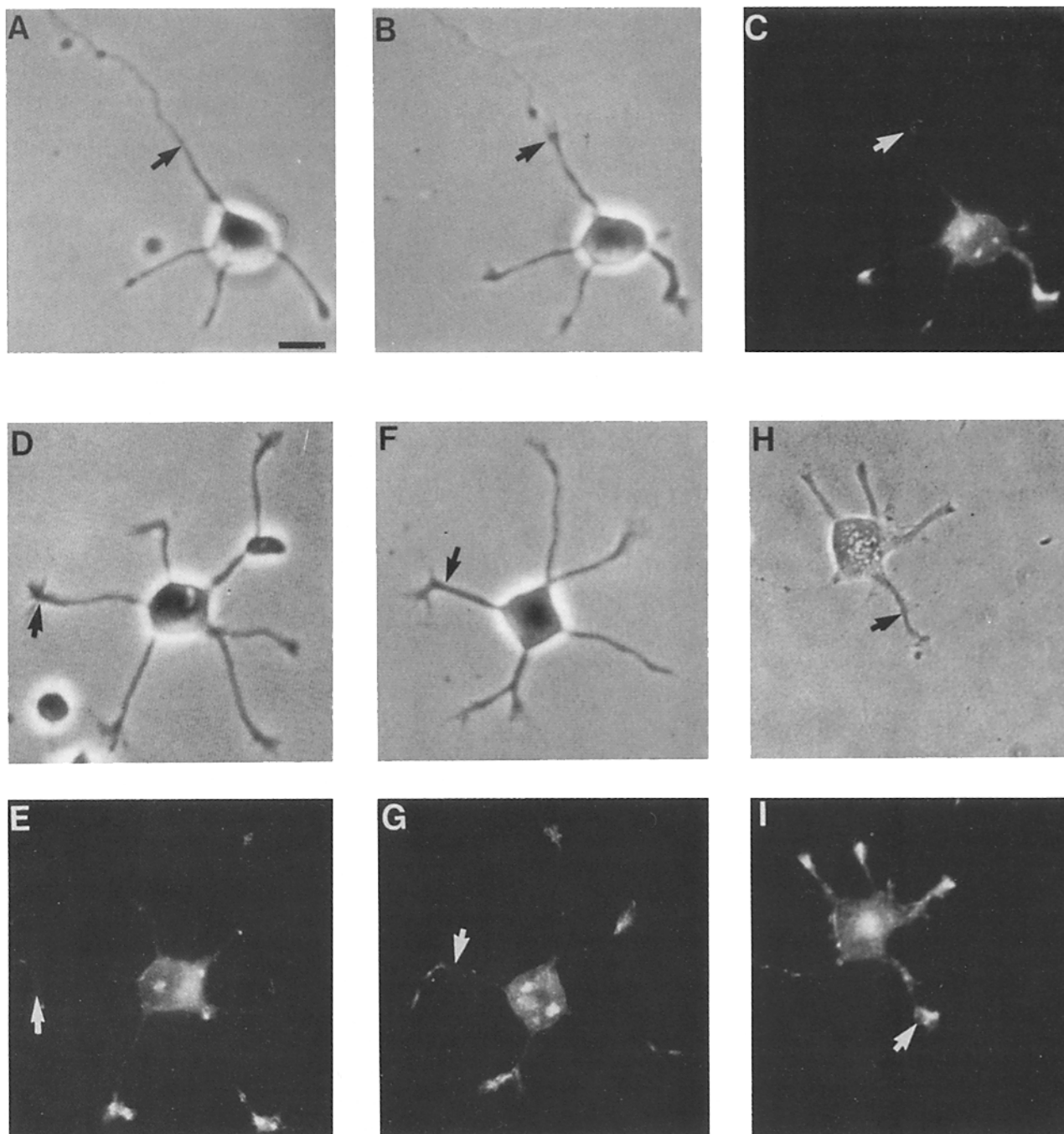


Figure 6. The redistribution of GAP-43 after axonal transections close to the cell body. The phase-contrast micrographs in *A* and *B* show a stage 3 neuron just before its axon was transected (at the site indicated by the arrow) and 26 min later, at the time of fixation. As shown by the fluorescence micrograph (*C*), GAP-43 was not detectable despite the large size of the new growth cone (*B* and *C*, arrows). The phase-contrast micrographs in *D*, *F*, and *H* show cells fixed at progressively later times after close axonal transections (80, 150, and 210 min, respectively). The distribution of GAP-43 in these cells is shown in the corresponding fluorescence micrographs (*E*, *G*, and *I*). Even after 80 min, GAP-43 was not detectable in the growth cone of the axonal stump (*E* and *D*, arrow). In the two cells fixed at longer times after transection, GAP-43 was present in the new growth cones (*F-I*, arrows). By 210 min, GAP-43 had reached about the same concentration as in the growth cones of the cells' minor processes. Bar, 10 μ m.

cesses. In the examples shown in Fig. 4, *A-E* (17 and 27 min, respectively, after transection), GAP-43 was selectively concentrated in the axonal growth cones, even though they were still quite small. GAP-43 was never concentrated at the tip of a severed axon that had not formed a new growth cone at the time of fixation ($n = 9$; data not shown). Similar results

were obtained at longer times after axonal transection (Fig. 4, *F-I*). At this stage, the new growth cones had become indistinguishable from normal axonal growth cones in size and morphology.

The results from observations of 44 stage 3 cells fixed and stained for GAP-43 at various times after a distal axonal tran-

Table II. Accumulation of GAP-43 in Newly Formed Growth Cones after Proximal Transections of the Axon during Stage 3 of Development

Time after transection	Growth cones examined	Concentrated*	Selectively concentrated‡
<i>min</i>	<i>n</i>	%	%
15–30	13	8	0
31–60	20	15	5
61–90	20	20	10
>90	16	63	12

* GAP-43 was considered to be concentrated in the new growth cone of the transected process if it was clearly more concentrated than along the process, thus resembling the GAP-43 staining typical of the growth cones of minor processes.

‡ GAP-43 was considered to be selectively concentrated if the staining in the new growth cone was significantly greater than in any of the cell's other growth cones, thus resembling the GAP-43 staining typical of stage 3 axonal growth cones.

section are summarized in Table I. GAP-43 was considered to be "concentrated" in the new growth cone of the transected process if it was clearly more abundant there than along the process. GAP-43 was considered to be "selectively concentrated" if the staining in the growth cone that formed on the transected process was significantly greater than in any of the other growth cones. Thus, in untreated cells GAP-43 typically is concentrated in the growth cones of each of the processes but selectively concentrated only in the axonal growth cone. At the earliest time points examined after distal transections (12–30 min), GAP-43 had already become concentrated in the new growth cones in every case. More significantly still, GAP-43 was selectively concentrated in the new axonal growth cone in 77% of the cases.

The rapid time course of the reappearance of GAP-43 in axonal growth cones after a distal transection suggests that it must originate from a pool of GAP-43, either soluble or membrane bound, that already exists in the cell at the time of transection. To confirm this, we used the protein synthesis inhibitor cycloheximide to block the synthesis of new GAP-43 after transection. Stage 3 neurons were preincubated in cycloheximide for 1–2 h, and then their axons were cut and maintained in cycloheximide for 1 h more. They were then fixed and stained for GAP-43. In all cases ($n = 17$), a new growth cone formed, and GAP-43 was selectively concentrated in the new growth cone (Fig. 5 B). Although under these conditions protein synthesis was profoundly inhibited, the concentration of GAP-43 in the growth cones was not noticeably different from untreated cells subjected to axonal transection.

Transection of the axons of stage 3 neurons close to the soma, so that all of their processes are about equal in length, results in a loss of polarity (Goslin and Banker, 1989). Any of the remaining processes can become the axon, and there is a long delay, averaging 17 h, before axonal growth begins. The time course of GAP-43 accumulation after such proximal transections differed significantly from the time course observed after more distal lesions (Fig. 6). Even 80 min after transection (Fig. 6, D and E), the new growth cone that formed was almost completely devoid of GAP-43 immunoreactivity, despite its large size. At later times, the amount of GAP-43 in the new growth cone reached a level about equal

to the amount in the growth cones of the other processes in the same cell (Fig. 6, H and I).

The results from observations of 61 cells whose axons were transected close to the cell body are summarized in Table II. Within the first 90 min after transection, GAP-43 was rarely concentrated in the new growth cones. Moreover, GAP-43 seldom became selectively concentrated in the new growth cones (<8% of the cases). It is important to note that the delay in the accumulation of GAP-43 that occurred after close axonal transections was not due to a delay in the formation of new growth cones.

For comparison, we examined the accumulation of GAP-43 after transection of a minor process of neurons at stage 2, before the expression of polarity (Fig. 7 and Table III). After transection, a delay of at least 90 min occurred before GAP-43 became concentrated in a significant number of the new growth cones. Only rarely (3% of the total cases) did GAP-43 accumulate selectively in the new growth cone of a transected process, even after 2.5 h.

The data from the different sets of experiments are summarized in Fig. 8, which compares the time courses of the accumulation of GAP-43 in the growth cones that form on transected processes. It is evident that the rate and pattern of the redistribution of GAP-43 was essentially the same after transection of a stage 2 minor process and of a stage 3 axon close to the cell body, while both differed significantly from the redistribution that occurred after transection of a stage 3 axon distant from the cell body. After the first two types of transections, the cells resembled unpolarized stage 2 neurons in which GAP-43 is more or less equally distributed among the minor process growth cones. In keeping with their unpolarized morphology, GAP-43 accumulated slowly in the new growth cones of the transected processes (Fig. 8 A) and, in most cases, it never became selectively concentrated in these growth cones (Fig. 8 B). After distal transections that preserved the cells asymmetric morphology, GAP-43 rapidly and selectively accumulated in these growth cones.

The Distribution of GAP-43 in Minor Processes

Although minor processes undergo little net elongation, they are highly dynamic, growing rapidly for short distances and then retracting while other minor processes elongate (Dotti et al., 1988; see also Goslin and Banker, 1989). In the course of the present studies in which we followed single cells for extended periods of time, we observed a correlation between the elongation of minor processes and the concentration of GAP-43 in their growth cones. Minor processes that had elongated rapidly in the interval immediately preceding fixation frequently had high concentrations of GAP-43 in their growth cones (Fig. 7, C and E), although there were exceptions. By comparison, minor processes that had not elongated for a significant period of time often had much less GAP-43 in their growth cones (Fig. 7 C, double arrows).

During the transition between stage 2 and stage 3, as the axon first emerges, the remaining minor processes are almost invariably stationary or retracting. This pattern of growth differs from that observed during both stage 2 and stage 3, when some minor processes advance while others retract. In cells fixed during this transition period, the growth cones of the remaining minor processes were virtually devoid of GAP-43 (Fig. 3).

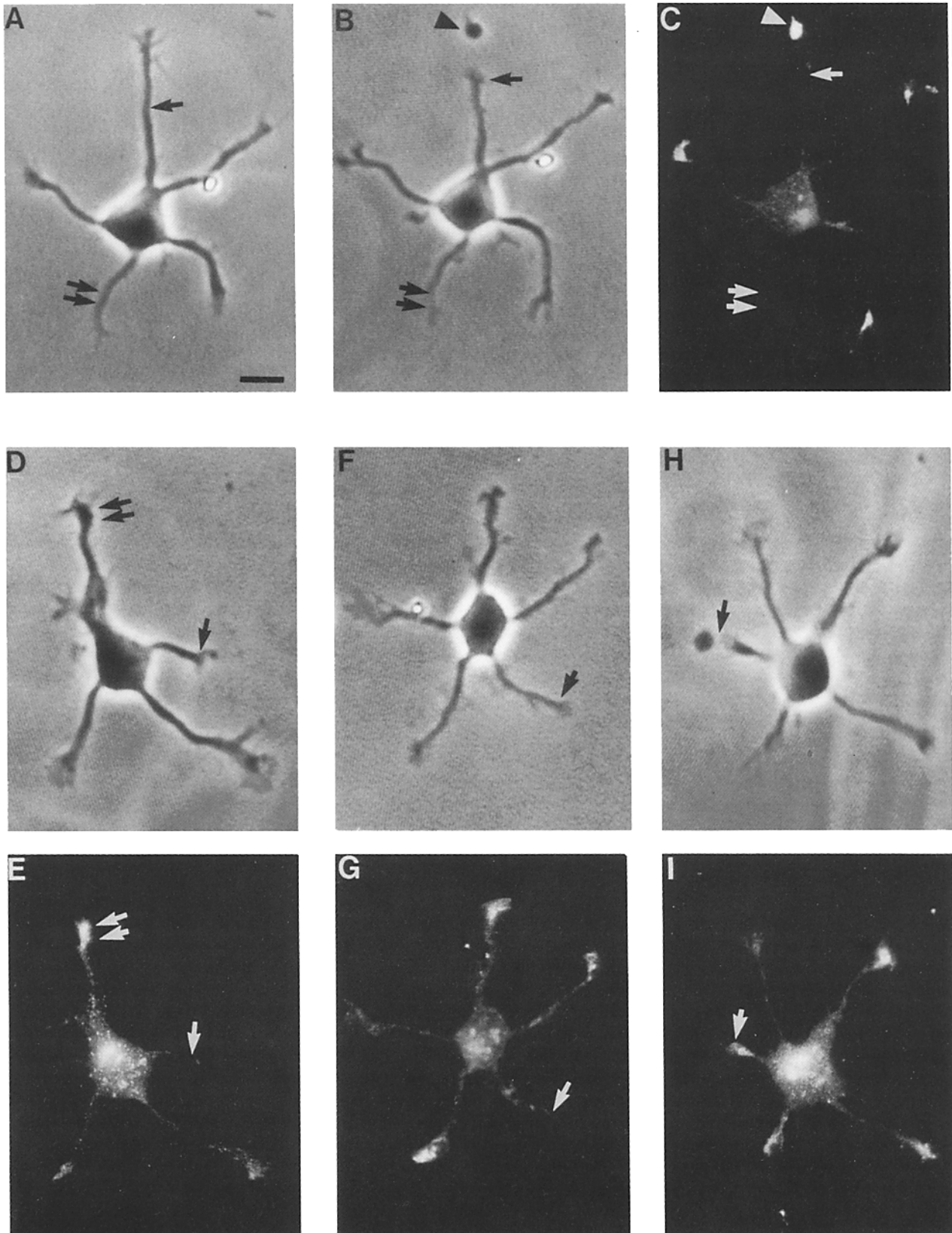


Figure 7. The redistribution of GAP-43 after transection of minor processes during stage 2 of development. In *A*, a stage 2 neuron is shown immediately before transection of one of its processes (at the site indicated by the arrow). At the time of fixation 22 min later (*B*), a new growth cone had formed at the tip of the transected process (arrow), while a remnant of the original growth cone remained attached to the substrate (arrowhead). As shown in *C*, the new growth cone was completely devoid of GAP-43 immunoreactivity, while the growth cone remnant was intensely stained. Three of the other minor processes elongated in the 22 min before fixation (unlabeled minor processes in *B*); all three contained a significant concentration of GAP-43 in their growth cones (unlabeled minor processes in *C*). The growth cone

Table III. Accumulation of GAP-43 in Newly Formed Growth Cones after Transections of a Minor Process during Stage 2 of Development

Time after transection	Growth cones examined	Concentrated*	Selectively concentrated†
<i>min</i>	<i>n</i>	%	%
15-30	15	13	0
31-60	13	31	0
61-90	13	31	8
>90	16	81	6

* GAP-43 was considered to be concentrated in the new growth cone of the transected process if it was clearly more concentrated than along the process, thus resembling the GAP-43 staining typical of the growth cones of minor processes.

† GAP-43 was considered to be selectively concentrated if the staining in the new growth cone was significantly greater than in any of the cell's other growth cones, thus resembling the GAP-43 staining typical of stage 3 axonal growth cones.

Discussion

The Selective Segregation of GAP-43 and the Expression of Polarity

In the present report, we examined two situations in which there are rapid and predictable changes in neuronal polarity to determine if these are accompanied by changes in the distribution of GAP-43, a protein that is preferentially distributed to the axon of stage 3 hippocampal neurons. In both situations, it was possible to monitor changes in the distribution of GAP-43 at the single cell level.

In the first case, during the transition between developmental stages 2 and 3, a single minor process begins to elongate at the rapid rate characteristic of axons. This change in the pattern of growth occurs rapidly, in no more than 30-60 min. In every cell that we followed during this transition, GAP-43 became selectively localized to the growth cone of the emerging axon within 1 h of the morphological expression of polarity. Because most cells were recorded only at 1-h intervals, the selective segregation of GAP-43 to the axon may occur even more rapidly than this.

The second case involved the response of hippocampal neurons to axonal transection. After distal axonal transections, which preserve polarity, GAP-43 rapidly and selectively accumulated in the new growth cone that formed on the axonal stump. Because of the speed with which this occurred and because it was not blocked by inhibition of protein synthesis, it most probably involves the redistribution of the pool of GAP-43 that remains within the cell. Although the greatest amount of GAP-43 is in the axonal growth cone, lesser concentrations are present in the cell body and in the growth cones of the minor processes. Some GAP-43 is also present along the axon, but at levels too low to be revealed

with normal photographic exposures (see Goslin et al., 1990; Fig. 4). A quite different pattern of GAP-43 accumulation was observed after axonal transections near the cell body, which cause a loss of polarity. GAP-43 accumulated much more slowly in the growth cone that formed on the axonal stump, and almost never became selectively concentrated there. Apparently the ongoing selective routing of GAP-43 into the axon is abruptly disrupted by axonal transections close to the cell body.

When processes were transected during stage 2, the accumulation of GAP-43 in the newly formed growth cone was similar to that observed after transections near the cell body. GAP-43 accumulated only slowly and did not become selectively concentrated. This result is consistent with the suggestion that transecting the axons of stage 3 neurons near the cell body returns them to the previous stage of development (Goslin and Banker, 1989). Apparently the intracellular trafficking of GAP-43, and probably of other proteins as well, differs at stage 2 and stage 3 of development.

In summary, under all of these circumstances there is a close correlation between the segregation of GAP-43 to the growth cone of a single process and the expression of neuronal polarity.

GAP-43 and the Growth of Minor Processes

The association of GAP-43 with the growth cones of minor processes, which we have previously described (Goslin et al., 1990), at first seems paradoxical because minor processes ultimately become dendrites, which lack detectable GAP-43. However, before stage 4, when they lose their residual GAP-43 and acquire dendritic features (Goslin et al., 1990), minor processes share some characteristics with axons (e.g., Baas et al., 1989). In particular, they elongate at rapid rates, though only for brief periods (Goslin and Banker, 1989). In the present experiments, when individual cells were followed by video recording, the minor processes that were elongating at the time of fixation tended to have higher concentrations of GAP-43 in their growth cones than the minor processes that were stationary or retracting. In addition, during the transition between stage 2 and stage 3, when growth was restricted to the emerging axon, the remaining minor processes lacked detectable GAP-43 immunoreactivity. These observations are consistent with the possibility that GAP-43 is associated with the growth of minor processes, as well as of the axon.

The Possible Role of GAP-43 in the Development of Polarity

The function of GAP-43 in process elongation is not presently understood (for discussion see Benowitz and Routtenberg, 1987; Skene, 1989; Gordon-Weeks, 1989; Dekker et al., 1989; LaBate and Skene, 1989). Indirect evidence is consistent with the suggestion that the rate of growth of indi-

of the fourth minor process, which retracted, lacked GAP-43 (*A-C*, double arrows). The phase-contrast micrographs in *D*, *F*, and *H* show stage 2 cells that were fixed at successively later times after transection (39, 70, and 120 min, respectively; arrows indicate the site of transection). As shown in the corresponding fluorescence micrographs, GAP-43 was not concentrated in the new growth cones of the transected minor processes after 39 and 70 min (*E* and *G*, arrows). One of the other minor processes of the cell shown in *D* elongated rapidly in the 39-min interval before fixation and was brightly stained for GAP-43 (*D* and *E*, double arrows). By 2 h after transection, GAP-43 had attained roughly the same concentration in the new growth cone as in the growth cones of the other minor processes. Bar, 10 μ m.

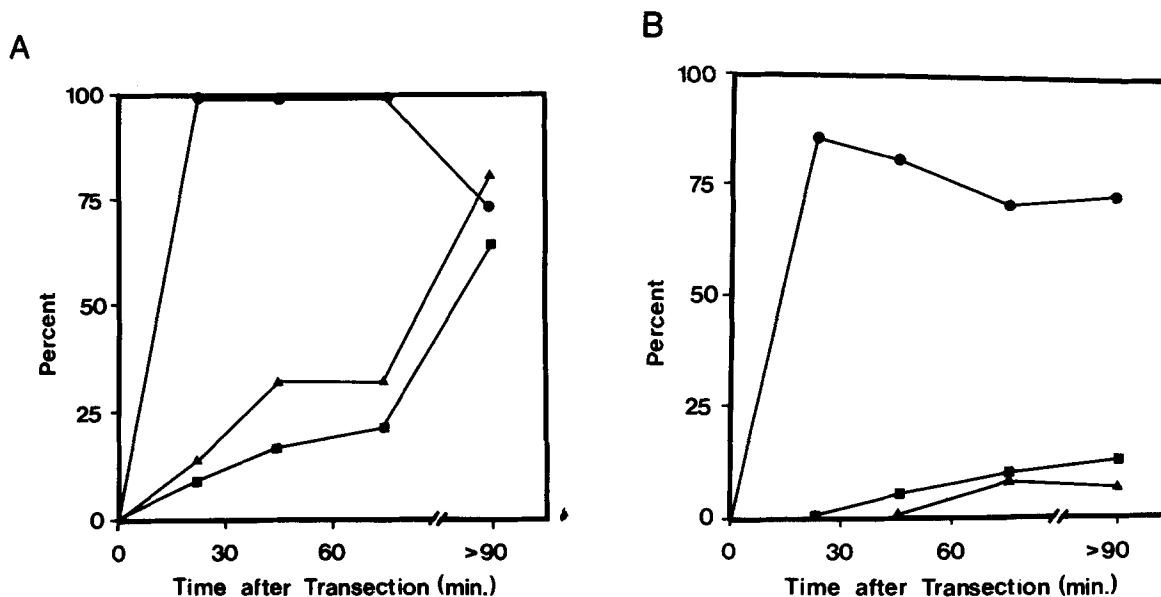


Figure 8. Summary figures comparing the time course of the accumulation of GAP-43 in the newly formed growth cones after the transection of processes under the three different experimental conditions. Axons were transected during stage 3 of development, at a site either far from the cell body (*circles*) or near to it (*triangles*). Minor processes were transected at stage 2 of development (*squares*). *A* illustrates the percent of cases at each time point in which GAP-43 was clearly more concentrated at the newly formed growth cone than along the process; *B* illustrates the percent of cases at each time point in which GAP-43 had become selectively segregated in the growth cone of the transected process (i.e., GAP-43 was clearly more concentrated in the new growth cone than in the growth cones of any of the other processes). After transections of the distal axon, GAP-43 rapidly and selectively accumulated in the new growth cone. In contrast, the time course of GAP-43 redistribution after close transections of the axon resembled that after transection of minor processes; GAP-43 accumulated slowly and only rarely became selectively concentrated.

vidual processes is correlated with their content of GAP-43. If the concentration of GAP-43 at the growth cone determines the rate of process growth, then the rapid changes in its distribution that we observed could account for many aspects of the development of polarity by hippocampal neurons in culture. During normal development, when one process becomes 10–15 μm longer than the others, GAP-43 becomes selectively segregated to this process. Subsequently, rapid growth is restricted to this process, which becomes the cell's axon. When an axon is transected distally, GAP-43 rapidly and selectively reaccumulates in its growth cone. This process then resumes its rapid rate of elongation, retaining its identity as the axon. On the other hand, when the axon is transected near the cell body, the selective routing of GAP-43 is disrupted. The axon ceases its rapid rate of growth, and the cell's polarity is lost. The possible validity of such interpretations hinges entirely on the functional significance of GAP-43. All of our observations could as readily be explained if the concentration of GAP-43 at the growth cone were a consequence of rapid growth, which was controlled by some other as yet unidentified factor.

The cellular mechanisms that underlie the selective sorting of proteins in neurons and the possible link between process length and the routing of specific proteins remain unknown (for discussion see Goslin and Banker, 1989). Presumably, a mechanism for the sorting of distinct populations of vesicles, analogous to that described in epithelial cells (Simons and Fuller, 1985), exists in neurons. However, the properties of GAP-43 suggest a mechanism by which this protein might accumulate preferentially in the longest process of a cell, even in the absence of signal-mediated sorting.

GAP-43 is actively transported in the anterograde direction in the rapid phase of axonal transport (Skene and Willard, 1981), presumably in association with membrane vesicles. The association of GAP-43 with membranes may be mediated by the fatty acylation of amino acid residues near the protein's amino terminus, a process that is reversible (Skene and Virag, 1989; Zuber et al., 1989). Thus, GAP-43 that had been actively transported to the tip of a process in association with a membrane vesicle could, after deacylation, dissociate from the membrane and return to the cell body by diffusion through the cytoplasm. Under such circumstances, the rate at which GAP-43 accumulated at the growth cone would be largely independent of process length. On the other hand, the rate at which GAP-43 returned to the cell body, from whence it might be transported into a different process, would vary inversely with length.

Based on studies of the response of neurons to axonal transection (Goslin and Banker, 1989), we have proposed a model to account for the emergence of polarity during the development of hippocampal neurons in culture, where spatially organized extracellular signals are apparently absent. This model is based on two assumptions: (*a*) that the rate at which a process elongates is a function of the concentration of some regulatory protein in its growth cone, and (*b*) that the distribution of this protein is in turn determined by process length. According to this hypothesis, if one process were slightly longer than the others, it would receive more of the growth controlling protein and would grow longer still. Small asymmetries in length or in the distribution of the regulatory protein, which might occur by chance, could, through such positive feedback, lead to the segregation of

this protein to a single process and cause its selective elongation. We have found it provocative to consider the possibility that GAP-43 is the regulatory protein posited in this model.

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