Experimental Observations on the Development of Polarity by Hippocampal Neurons in Culture

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Abstract. In culture, hippocampal neurons develop a polarized form, with a single axon and several dendrites. Transecting the axons of hippocampal neurons early in development can cause an alteration of polarity; a process that would have become a dendrite instead becomes the axon (Dotti, C. G., and G. A. Banker. 1987. *Nature (Lond.).* 330:254–256). To investigate this phenomenon more systematically, we transected axons at varying lengths. The greater the distance of the transection from the soma, the greater the probability for regrowth of the original axon. However, it was not the absolute length of the axonal stump that determined the response to transection, but rather its length relative to the lengths of the cell's other processes. If one process was >10 μm longer than the others, it invariably became the axon regardless of its identity before transection. Conversely, when a cell's processes were nearly equal in length, it was impossible to predict which would become the axon. In these cases, axonal outgrowth began only after a long latency. During this interval, the processes appeared to be in dynamic equilibrium, some growing for short distances while others retracted. When one process exceeded the others by a critical length, it rapidly elongated to become the axon.

The establishment of neuronal polarity during normal development may similarly involve an interaction among processes whose identities have not yet been determined. When, by chance, one exceeds the others by a critical length, it becomes specified as the axon.

THE establishment and maintenance of polarity is of fundamental importance to the function of many cell types. In this regard, nerve cells offer a particularly striking example. They have two distinct classes of processes, axons and dendrites, which differ from one another in form, in growth pattern, in molecular composition, and in synaptic polarity (Peters et al., 1976; Banker and Waxman, 1988; Lasek, 1988). Despite many advances in our knowledge of the mechanisms underlying cell polarity in general, surprisingly little is known about the development of polarity in neurons.

To gain insight into this question, we have investigated the formation of axonal and dendritic processes 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 embryonic hippocampal cultures differ from one another in light and electron microscopic morphology (Bartlett and Banker, 1984*a*,*b*) and in the macromolecular composition of their cytoskeletons and membranous constituents (Shaw et al., 1985; Caceres et al., 1986; Matus et al., 1986; Dotti et al., 1987; Goslin et al., 1988). As in situ, axons are presynaptic and dendrites postsynaptic (Bartlett and Banker, 1984*b*). These aspects of neuronal polarity are expressed to a lesser extent by neurons cultured from the peripheral nervous system, and, for the most part, are not expressed by continuous nerve cell lines.

In culture, hippocampal neurons acquire their characteristic polarized form by a stereotyped sequence of developmental events (Dotti et al., 1988). Shortly after the cells have attached to the substratum, they become surrounded by flattened lamellipodia (referred to as developmental stage 1). Within the course of several hours, the lamellipodia condense at several discrete points along the cell's circumference; from these condensations short processes emerge that are roughly equal in length and cannot be identified as axonal or dendritic (developmental stage 2). These "minor processes" are dynamic, extending or retracting for short distances, but they typically undergo little net elongation for a period of 12–24 h. Then, rather abruptly, one of the minor processes begins to grow rapidly, becoming many times longer than the others within just a few hours and acquiring axonal characteristics. At this stage (developmental stage 3), the cell has become polarized. While the axon continues to grow, the remaining minor processes show little net elongation for several days. Then they also begin to grow (developmental stage 4) but at a significantly slower rate than the axon; they become the cell's dendrites.

These observations raised the possibility that, at the time when processes first emerge, they are not determined as axons or dendrites. To explore this possibility, Dotti and Banker (1987) transected the axons of young hippocampal neurons shortly after they had acquired their distinct characteristics. Often this caused an alteration of polarity. One of the cell's minor processes, which would otherwise have become a dendrite, became the axon. Frequently the stump of

the original axon persisted and ultimately became a dendrite. In the experiments of Dotti and Banker, axons were transected as close to the cell body as possible in an attempt to maximize the probability that cells would undergo respecification. It seemed likely, however, that if transections were made at greater distances from the cell body, leaving a longer axonal stump, the axon might retain its identity and simply regenerate; an alteration in polarity would not occur.

In the present study, we set out to examine this possibility systematically, by making axonal transections at varying distances from the cell body. As expected, when a sufficiently long axonal stump remained, polarity was not changed. Beyond this, however, the results of these experiments provided unexpected insights into the mechanisms that determine which process will become the axon. They suggest that the relative length of a cell's processes after transection, independent of the axonal or dendritic identity of the individual processes before transection, largely determines which will become the new axon and which the dendrites. If one process is significantly longer than the others, it will become the axon, even if it initially were destined to become a dendrite. If the processes remaining after transection are about the same length, they appear to undergo a period of competition until one exceeds the others by a sufficient length to become the axon.

Materials and Methods

Cell Culture

Methods for preparing the hippocampal cell cultures have been previously described (Banker and Cowan, 1977; Bartlett and Banker, 1984*a*). Special culture dishes were used to facilitate micromanipulation and relocation of individual cells after lesioning. These were prepared from plastic petri dishes (model No. 1006; Falcon Labware, Becton, Dickinson & Co., Oxnard, CA) by drilling a 16-mm hole in the bottom and attaching an acid-cleaned glass coverslip (22 mm in diameter) to the outer surface of the dish with a mixture of paraffin and vaseline (3:1). A glass ring, 18 mm in diameter and 5 mm deep (Thomas Scientific, Philadelphia, PA), 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, rinsed with water, and then the well was filled with MEM, containing 10% horse serum.

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 2,500 cells were plated into the well of each dish. After 3–4 h at 37°C, when the cells had attached to the substrate, the medium was replaced 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%). To provide trophic support (Banker, 1980), a coverslip containing a confluent monolayer of astroglial cells (Booher and Sensenbrenner, 1972) was then placed on the top of the glass ring, with the glia facing the neurons.

Lesions

Lesions were performed between 24 and 48 h after the cells were plated. Cells at stage 3 of development, whose axons were longer than 100 μm, were chosen for study. 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 prepared on a pipette puller (model 700C; David Kopf Instruments, Tujunga, CA) and held in a micromanipulator attached to the stage of a Zeiss IM-35 inverted microscope. The micropipette was positioned near the process to be transected, brought into contact with the substrate, and then rapidly drawn across the axon at right angles to its direction of growth. In preliminary experiments it was observed that regenerating axons frequently contacted and grew along the distal axonal stumps. Therefore, in most experiments, the entire distal portion of the axon was pulled off the substrate after transection to eliminate any possible influences from this interaction and to permit clear visualization of the regenerating axon. After completing all of the intended lesions, the glass ring and glial coverslip were replaced, and the dish was returned to the incubator.

All manipulations after removing the glial coverslip were performed as rapidly as possible to minimize changes in pH and to reduce the possibility of microbial contamination. Typically <10 min was required to lesion the axons of 10 cells on one dish. Under these circumstances, >90% of the cells survived transection and could be relocated 24 h later.

Video Recording and Data Analysis

Observations were made by phase-contrast microscopy and recorded on video tape, using a camera (model 67M equipped with a Newvicon tube; Dage-MTI Inc., Wabash, MI) and a video recorder (model AG-6050; Panasonic Company, Secaucus, NJ). Recordings were begun just before transection and continued until lesioning had been completed. To determine which process became the axon after repolarization, cells were recorded about 24 h after lesioning. In a second series of experiments, cells were recorded every 2–4 h to examine the time course of axonal outgrowth. In all, >250 cells chosen from over 20 separate culture preparations were observed in the course of this work.

The video tapes were replayed at the desired timepoints and the cells were traced directly from the monitor onto transparent sheets of plastic and then onto paper. From these drawings the length of each process was measured using a digitizing tablet (GTCO Digi-pad 5A) and appropriate software (Sigmascan; Jandel Corporation, Sausalito, CA). These procedures may have introduced errors of a few micrometers into the measurements of process length, but the relative length of processes from individual cells is unlikely to have been affected.

Results

The Determination of Process Identity after Axonal Transection

To determine how the length at which an axon was transected influenced the probability that a reorganization of polarity would be induced, we transected the axons of cells at varying distances from the soma. Only typical stage 3 cells (see Dotti et al., 1988), with a single axon longer than 100 μm, were included in these experiments. No differences were observed in the responses of 1- and 2-d-old cells, and the results of both will be considered together.

Fig. 1 illustrates the responses of two cells whose axons were transected at different distances from the cell body. The cells are shown just before (*A* and *E*) and just after transection (*B* and *F*), after the axonal stump had regenerated its growth cone (*C* and *G*), and after polarity had been reestablished (*D* and *H*). There was little or no retraction or degeneration of the axonal stump of either cell and within 35 min after transection, both cells had reacquired growth cones. Wessells et al. (1978) have also reported that new growth cones form rapidly after transection of the neurites of ciliary ganglion cells. Within 24 h after lesioning, both cells had regained the morphology typical of polarized hippocampal neurons, with a single long axon and several, shorter minor processes. However, the two cells became repolarized in two, quite different ways. In one case (Fig. 1, *A–D*), a new axon arose from one of the minor processes, which otherwise would have become a dendrite. This was especially common after close transections, like the one shown. In the other case (Fig. 1, *E–H*), after a more distal transection, the axon simply regenerated; the cell's polarity was not altered.

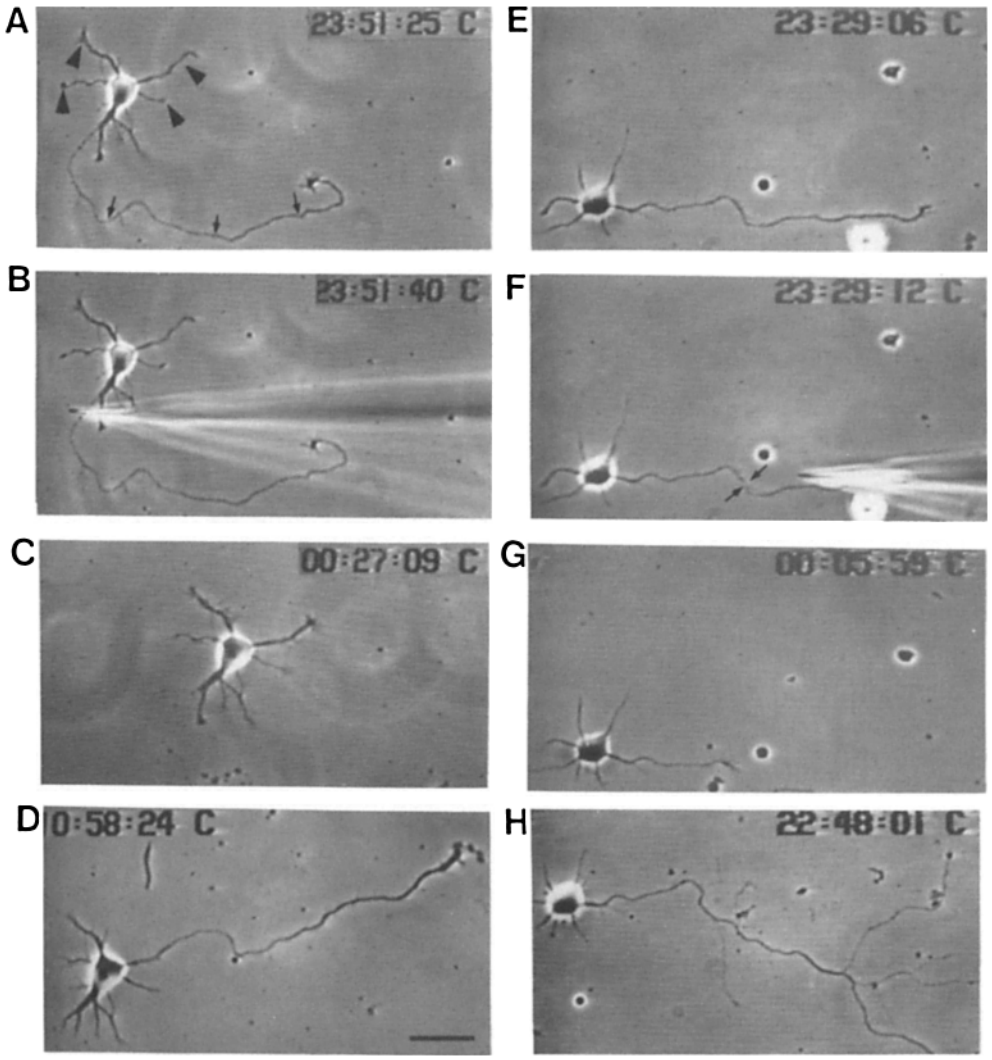

Figure 1. Two patterns of response after axonal transection. Each set of phase-contrast micrographs illustrates a cell just before and immediately after transection, ~35 min after transection, and after the cell had reestablished its polarity. The axon of the cell illustrated in *a–d* was transected at a length of 15 μm, close to the cell body. Before transection (*A*), this cell had a single, long axon (*arrows*) and four short, minor processes (*arrowheads*). *B* illustrates the same cell during transection. After transection, there was no distal-to-proximal degeneration of the axonal stump and, within 35 min, a new growth cone had formed (*C*). After 11 h (*D*), a new axon had developed from a minor process. The axonal stump, which did not increase in length during this interval, is indistinguishable from the remaining minor processes. The axon of the cell illustrated in *E–H* was transected somewhat farther from the cell body, at a distance of 60 μm (*F*, *arrows*). Again, there was no significant degeneration or retraction of the proximal axonal stump, which rapidly acquired a new growth cone (*G*). After 23 h (*H*), the original axon had regenerated, extending for a length of 420 μm (far beyond the field shown). Bar, 30 μm.

A different, and unexpected, phenomenon occurred when axons were transected near their growth cones (data not shown). Lesions within 10–20 μm of the growth cone caused pronounced distal-to-proximal degeneration along the axon. In about half of the cases, this progressed proximally to the cell body, resulting in cell death. These degenerative changes occurred rapidly. Beading of the proximal stump began almost immediately after the transection, and cell death sometimes occurred within 30 min. Even when such cells survived for 24 h after lesioning and became repolarized, the extent of degeneration that occurred proximal to the transection prevented meaningful assessment of the effective lesion site. Therefore these data have been omitted from this analysis.

Fig. 2 summarizes the responses of 175 cells after lesions at distances ranging from 2–200 μm from the soma. In ~6% of cases, the lesioned cells developed two axons (see Dotti and Banker, 1987; Dotti et al., 1988); these data were excluded. To assess the extent of axonal retraction or degeneration, selected cells were examined between 30 and 60 min after transection. In general, little retraction occurred so that measurements of the site of transection, as shown in Fig. 2, also reflect the length of the axonal stump at the time a new growth cone appeared.

The results of this experiment clearly show that the greater the distance of the lesion from the soma, the greater the probability for regrowth of the original axon (Fig. 2). At lesion distances of ~40 μm or more from the soma, the probability of regrowth approached 100%. In contrast, when transections were made close to the soma, the probability that a minor process became the axon increased markedly. There appeared to be a transition between these two response patterns at a lesion distance of ~30 μm. Axons transected more distally regenerated in >75% of the cases; after more proximal transections, regeneration occurred in <40% of the cases.

The data presented in Fig. 2 might suggest that transections at distances greater than ~30 μm leave a sufficient length of axonal stump so that its axonal properties are retained and the probability of its regeneration is greatly increased. However, observation of individual cells suggested that the length of the axonal stump relative to that of the other processes, rather than its absolute length, determined the re-

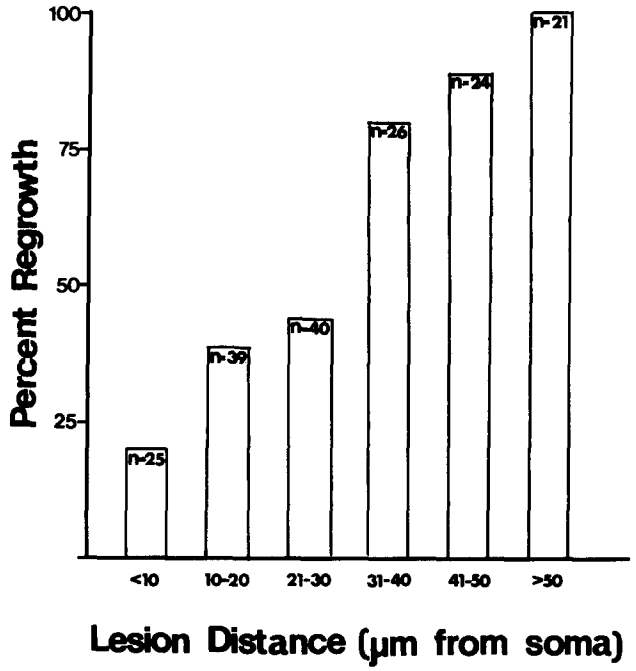

Figure 2. Relationship between the site of axonal transection and the probability of axonal regrowth, with maintenance of polarity. The axons of neurons that had been maintained in culture for 1 or 2 d were transected at varying distances from the soma, ranging from 2 to 200 μm. The more distal the site of transection, the greater the likelihood that the axonal stump regenerated. With more proximal transections, the frequency of regrowth declined (i.e., the cell's polarity was altered). A transition in the response of the cells was observed at transection lengths of ~30 μm. Beyond this distance, regrowth of the original axon occurred almost invariably.

sponse to axotomy. In particular, we noted a tendency for the axon to arise from the longest process remaining after axotomy, regardless of whether this was the axonal stump or a minor process. Since at this stage of development minor processes seldom exceeded 30 μm in length, this could explain the results illustrated in Fig. 2.

To examine this tendency more closely, the data in Fig. 2 were reanalyzed on a cell-by-cell basis, as shown in Fig. 3. Each vertical line represents an individual cell. The length of each of a cell's minor processes is indicated by a circle along this line and the length of its axonal stump, just after transection, by a square. The open symbol denotes the process that became the axon, as determined 24 h after axonal transection. Thus, open circles indicate cells in which a minor process became the axon, and open squares indicate cells whose original axon regenerated. The data are arranged from left to right in order of increasing length of the axonal stump.

There was a striking tendency for the axon to arise from one of the longest processes remaining after transection, regardless of whether this process was originally the axon or one of the minor processes. In 74% of cases, the new axon arose from the longest process; in 89% of cases, the process that became the axon was within 5 μm of the longest process, and in 96% of cases, within 10 μm of the longest. Since minor processes seldom exceeded 30 μm in length, if the transection was made at 30 μm or more, the axonal stump was nearly always longer than any of the minor processes, and it regrew (as indicated in Fig. 2). If the transection was made at 20 μm or less, the axonal stump was often significantly shorter than the remaining minor processes, one of which became the axon.

When axons were transected at intermediate lengths, a cell's response to transection depended on the length of its minor processes. The pairs of cells indicated by the arrows illustrate this point (*a* and *b* in Fig. 3). For both pairs, the axons were transected at identical distances from the cell body (18 μm in the case of pair *a*, 24 μm in the case of pair *b*). In one cell of each pair, the axonal stump was longer than any of the minor processes and it regenerated. In the other cell, two or three of the minor processes were longer than the axonal stump. The longest of these became the new axon.

Such examples suggested that it was not the absolute length of the remaining axonal stump that determined whether the axon retained its identity or the cell's polarity was altered, but rather the relative length of the processes remaining after transection. To analyze this, we measured the probability of axonal regrowth as a function of the difference in length between the axonal stump and the cell's longest minor process at the time of transection (Fig. 4). Positive length differences represent instances when the axonal stump was longer than the longest minor process, and negative differences instances when a minor process was longer than the axonal stump.

The frequency of regeneration increased markedly as the length difference between the axonal stump and the longest minor process increased. When the axonal stump was >10 μm longer than any minor process, it almost always regenerated (56 of 58 cases). When it was at least 10 μm less than a minor process, it seldom regenerated (2 of 35 cases). In those cases where none of the processes was 10 μm longer than the others (i.e., for length differences between −10 and 10 μm), the probability of regrowth averaged ~50%. Even for these small differences in length, the frequency of regrowth was strongly correlated with the difference in length between the axon and the longest minor process. When the axonal stump was only 5 μm longer than the longest minor process, it regrew in 15 of 21 cases. When it was 5 μm shorter than the longest minor process, it regrew in only 2 of 14 cases.

These results clearly show that the length of the axonal stump relative to the lengths of the cell's other processes is an important determinant of the cell's response to transection. Beyond this, the data indicate surprisingly little tendency for the original axon to retain its identity, independent of its length relative to the minor processes. The continuous curve in Fig. 4 shows an idealized relationship between the probability of regrowth and length difference. This relationship derives from two assumptions: (*a*) when the axonal stump is equal in length to a minor process, each is equally likely to become the axon; and (*b*) any process 10 μm longer than the others will invariably become the axon. The data do not differ significantly from this relationship although there may have been a slight tendency for the axon to retain its identity. For example, when the axonal stump was nearly equal in length to a minor process, the axon regenerated in 63% of cases (n = 19), compared with a predicted value of 50%. At most, there was a small bias toward maintenance of polarity.

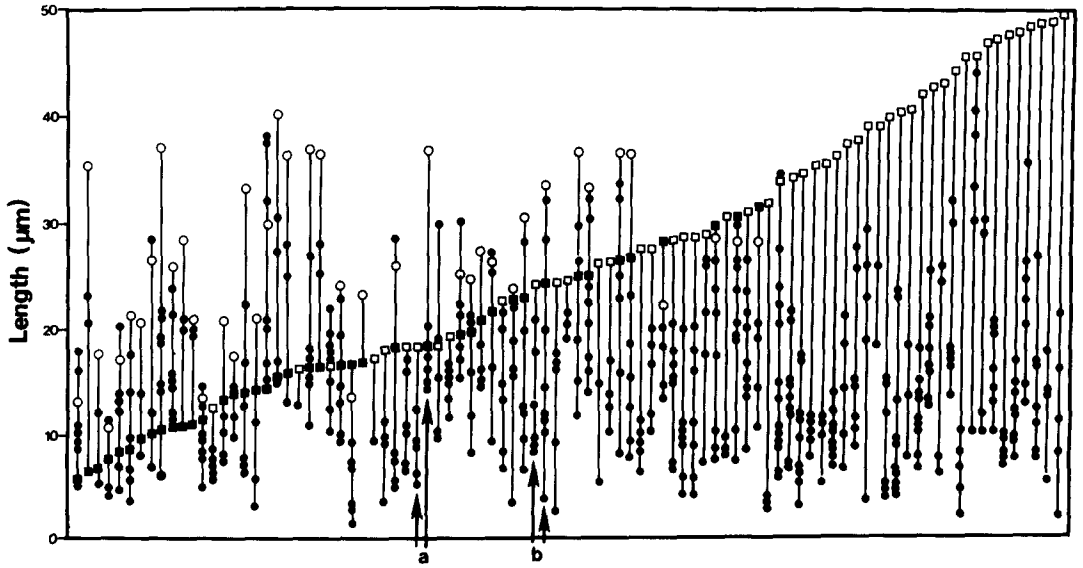

Figure 3. Process length at the time of transection, and its influence on the determination of the axon. Each vertical line corresponds to a single cell. The line extends from the position of the shortest process to the position of the longest process; the circles along the line represent the length of each of the cell's minor processes and the square represents the site of axonal transection. The process that became the axon, as assessed 24 h after transection, is indicated by the open symbol. The cells are arranged, from left to right, in order of increasing transection length. A cell's response depended not only on the site of axonal transection, but also on the length of the cell's other, minor processes. For example, the arrows (*a* and *b*) indicate two pairs of cells whose axons were transected at comparable lengths (pair *a* at 18 μm; pair *b* at 25 μm). In one cell of each pair, the axonal stump was the longest process and regenerated. In the other, a minor process, which was longer than the axonal stump, became the new axon. In general, the axon arose from the longest, or one of the longest, of a cell's processes, regardless of its original identity. These data derive from the same set of cells illustrated in Fig. 2. To simplify this figure, only a representative subset of the data is shown.

Time Course of Axonal Regrowth and Reorganization of Polarity

To gain further insight into the cellular events that lead to regrowth or to an alteration of polarity, we studied the behavior of cells in the interval between axonal transection and the beginning of new axonal outgrowth. Fig. 5 shows drawings of two cells that were followed for 24 h after axonal transection. Both cells were from the same culture and both were lesioned 36 h after plating. Nevertheless, their responses to axonal transection were quite different. Fig. 5 *A* illustrates the response of a cell whose axon was transected 45 μm from the soma. After transection, the cell retained an asymmetric appearance, the axonal stump being nearly 25 μm longer than the longest of its minor processes. As expected, its axon regrew. Indeed, there was no measurable delay between transection and the beginning of regrowth. The rate of axonal growth reached 35 μm/h almost immediately after axotomy, comparable to or somewhat faster than axonal growth in unlesioned cells (Dotti et al., 1988). Elongation of the regenerating axon was intermittent, as is axonal outgrowth in normal cells. For example, after growing rapidly in the interval between 5 and 12 h after axotomy, the axon did not elongate further during the next 2 h. During the course of axonal regeneration, there was little alteration in the length of any of the minor processes.

The second set of drawings (Fig. 5 *B*) illustrates the behavior of a cell whose axon was transected 23 μm from the soma. After axonal transection, several of its processes, including the axonal stump, were approximately equal in length. As expected from the data presented previously, the longest of this cell's minor processes elongated to become the axon. In marked contrast to the cell shown in Fig. 5 *A*, however, this occurred only after a long delay, amounting to >17 h. During this latent period before axonal outgrowth, the cell's processes were not inactive. Rather, several of them underwent spurts of growth followed by periods of retraction. This was a frequent phenomenon in cells whose processes were similar in length after axotomy, as commonly occurred after lesions within 30 μm of the soma.

49 cells were followed in a similar manner. Fig. 6 *A* illustrates 12 examples, representative of the behavior of the great majority of lesioned cells (40 of 49 cases). In half of the cells illustrated a minor process became the new axon (*squares*). In the remainder (*circles*), the initial axon regenerated. As Fig. 6 *A* shows, there was a pronounced dichotomy in the pattern of response to axotomy. After distal lesions, which led to regeneration with maintenance of polarity, axonal growth began almost immediately. After short lesions, when an alteration of polarity occurred, axonal outgrowth began only after a long delay, which averaged 17 h. However, once the new axon emerged, it grew at a rate comparable to that of the regenerating axons.

These results might suggest that the cellular events involved in the reorganization of polarity are more complex and require a longer time than is needed for the initiation of axonal regeneration when polarity is maintained. This was not invariably the case, as shown by the data in Fig. 6 *b*. In four cells a change in polarity occurred with minimal delay, and in five cells axonal regeneration occurred, but only after a long latent period. Closer examination of the data sug-

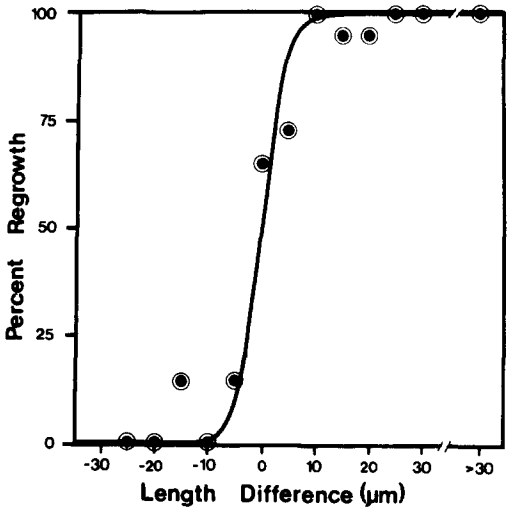

Figure 4. Relationship between the probability of axonal regrowth (with maintenance of polarity) and the difference in length between the axonal stump and the longest minor process. Positive length differences correspond to cases where the axonal stump was longer than the minor processes, negative values to cases where it was shorter. The symbols indicate the frequency of regrowth for length distances intervals of 5 μm (based on an average of 14 cells at each interval; range, 10–21). The longer the axonal stump compared with the minor processes, the greater the chance for axonal regeneration. The curve illustrates the relationship predicted if (*a*) the axonal stump always regenerates when it is 10 μm longer than any minor process; (*b*) the axonal stump never regenerates when it is 10 μm shorter than a minor process; and (*c*) there is no bias for the original axon to regenerate when it is the same length as a minor process. The data do not differ significantly from this relationship. Data were derived from the set of 175 cells illustrated in Fig. 2.

gested that the unusual behavior of these cells was related to the relative length of their processes after transection. Of the four cells whose polarity became rapidly reorganized, all had one minor process that was significantly longer than the other remaining processes and that rapidly acquired axonal characteristics. Of the five cells whose axons regenerated slowly, all were cut short, so that the remaining axonal stumps were comparable in length to the minor processes. These data can, therefore, be reconciled with the results described above. Axonal outgrowth from a cell whose processes are all about the same length after axotomy might occur only after a long latency, regardless of whether the axon arises from a minor process (Fig. 6 *A*, *squares*) or by regeneration of the original axon (Fig. 6 *B*, *circles*). Conversely, axonal outgrowth might occur rapidly if, after axotomy, one process were significantly longer than the others, regardless of whether this process was originally the axon (Fig. 6 *A*, *circles*) or a minor process (Fig. 6 *B*, *squares*).

To examine this possibility more fully, the data from all 49 cells were analyzed in terms of the difference in length between the process that became the axon and the longest of the cell's other processes (both measured at the time of transection). As a measure of the rate of initiation of axonal outgrowth, we determined the duration of the latent period between transection and the beginning of the rapid growth characteristic of axons. The results of this analysis are shown in Fig. 7. The latency to rapid axonal outgrowth clearly depends on the relative length of the processes remaining after axonal transection. If one exceeded the others by more than ~10 μm in length, axonal growth almost always began within 2 h. This held true not only when the original axon regenerated, but also in those few cases when a new axon arose from a minor process that was significantly longer than the others (*doubled squares*). If the difference in length between processes was less than ~10 μm, axonal outgrowth began only after a long delay, regardless of whether a change in polarity occurred or the axon regenerated. These results suggest that the relative length of processes after axonal transection is an important determinant of the rate of initiation of axonal outgrowth; the identity of individual processes before transection is not.

There is a striking parallel between these results concerning the rate of initiation of axonal outgrowth and results previously described concerning the determination of process identity after axonal transection (see Fig. 4). In both cases, there is a pronounced dichotomy in the response to axonal transection that depends on the relative lengths of the processes, and in both cases, the critical length difference at which this dichotomy arises is ~10 μm.

Discussion

We have investigated factors that influence the development of neuronal polarity by transecting the axons of hippocampal

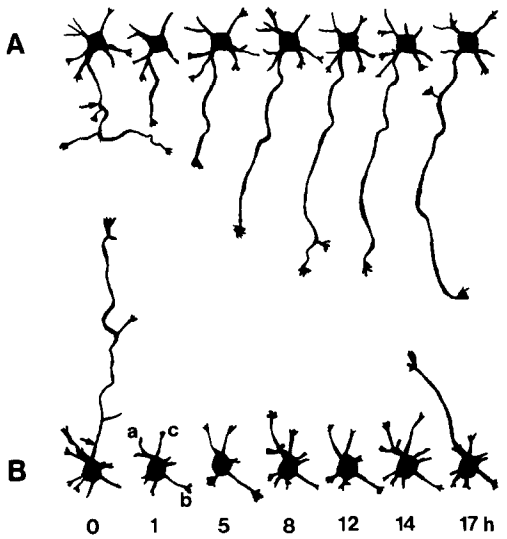

Figure 5. The sequence of events that precede axonal regeneration or reorganization of polarity. The drawings illustrate the responses of two representative cells between 1 and 17 h after lesioning (times, in hours, are shown at bottom). The axon of the cell in *A* was transected 45 μm from the cell body (*arrow*) and regrew rapidly. Within 1 h after axotomy, its axon had extended 18 μm, and, after 8 h, it had already grown to its original length. The polarity of the cell shown in *B* was altered after transection of its axon 23 μm from the soma (*arrow*). Its new axon arose from the minor process labeled *a*, the longest minor process at the time of transection—but this occurred only after a delay of 17 h. In the interval between transection and axonal outgrowth, two of its minor processes (*a* and *b*) alternately grew and retracted, as did the axonal stump (*c*). Both cells were from the same culture; their axons were transected 36 h after plating.

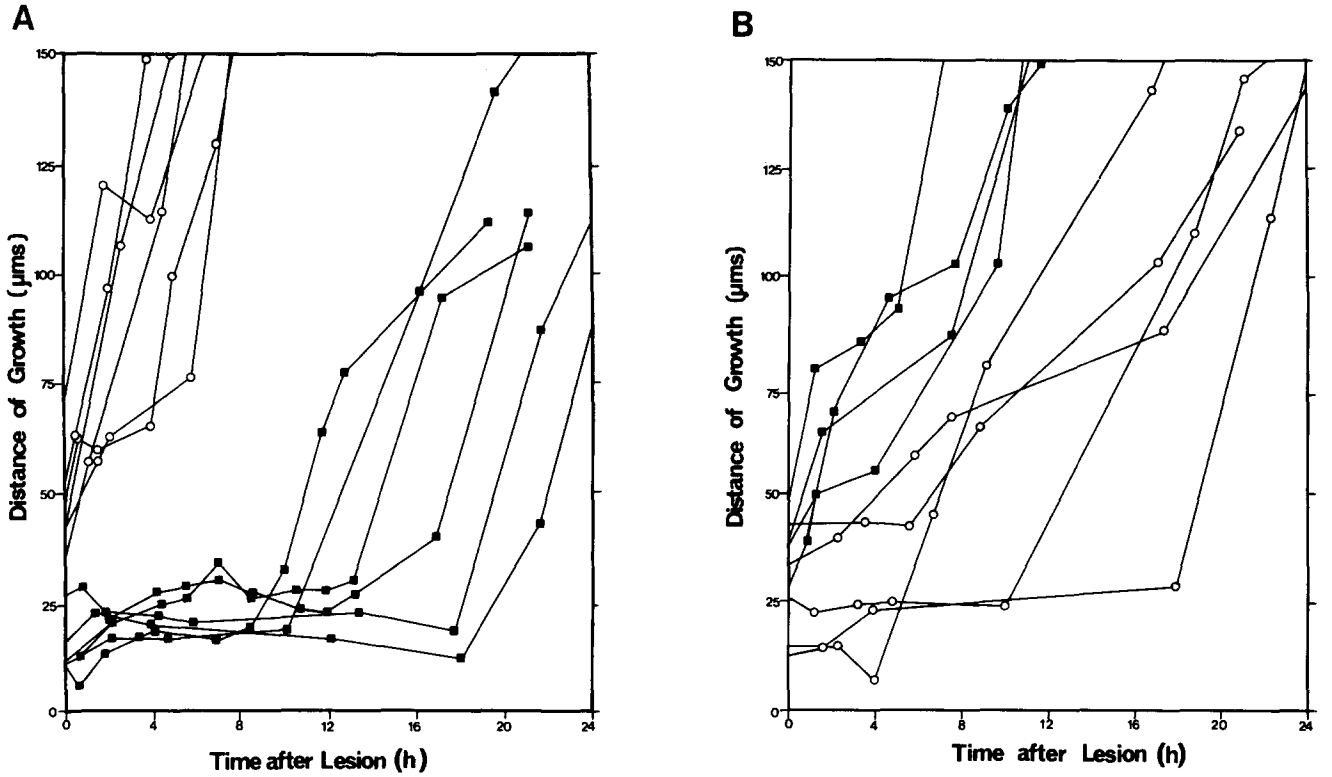

Figure 6. The rate of axonal growth after axotomy. *A* shows 12 examples, representative of the behavior of the majority of cells after axotomy. Cells in which the original axon regenerated are represented by open circles; cells in which the axon arose from a minor process are represented by closed squares. At each timepoint, the length of the process that became the axon is shown. Cells that regenerated their axons did so rapidly, without a significant delay. In contrast, there was a marked delay, ranging from 8 to 18 h, before the beginning of axonal growth in cells whose polarity was altered. Once axonal growth began, the rate of growth was similar for both populations of cells. *B* shows the rate of axonal growth for cells whose responses were atypical (9 of 49 cells, symbols as in *A*). Some underwent a change of polarity with little delay in the onset of axonal growth (*squares*); in others the initial axon regenerated after a long latent period (*circles*).

neurons after 1 or 2 d in culture. At this developmental stage, polarity has been expressed, but processes are not irreversibly specified as axons and dendrites (Dotti and Banker, 1987). Our results show that the cellular response depends primarily on the length of the processes that remain after axotomy, not on the identity of the processes before transection.

Competitive Interactions and Process Determination

After axonal transection, hippocampal neurons can be grouped into one of two classes, based on the relative length of the processes that remain: asymmetric cells, having one process that is clearly longer than the others, or symmetric cells, whose processes are nearly equal in length. The responses of these two classes of cells are quite different, as summarized in Fig. 8.

Distal transections of the axon produce asymmetric cells. In such cases, the new axon invariably arises by regeneration of the original axon and the minor processes differentiate, as they normally would, into dendrites (Fig. 8 *A*). With only a brief delay, the axonal stump begins to grow at the rapid rate characteristic of axons. As shown by the second example in Fig. 8 *A*, asymmetric cells can also result from a proximal, rather than distal, transection if one of the minor processes is significantly longer than the axonal stump and the other minor processes. For such cells, the axon also invariably arises from the longest process, and its outgrowth begins rapidly. This implies that the events leading to the initiation of axonal outgrowth may be similar in both instances, even though one involves regeneration of the original axon and the other an alteration of polarity.

Symmetric cells result from transections of the axon close to the soma such that all of the processes, including the axonal stump, are approximately equal in length (Fig. 8 *B*). The polarity of such cells is frequently altered by transection, one of the minor processes giving rise to the new axon (Fig. 8 *B*, example 1). Occasionally, as in the second example, the axonal stump itself will regrow. The events leading to axonal outgrowth from symmetric cells are similar in both cases.

In contrast to asymmetric cells, the identity of the process that will become the axon in symmetric cells appears to be determined probabilistically. The longer a process, the more likely it is to become the axon. Conversely, when a cell's processes are all nearly equal in length, it is impossible to predict which one will become the axon. These results extend the observations of Dotti and Banker (1987), whose data derived exclusively from transections of the axon near the cell body. Their findings suggested that each process, including the axonal stump, had an equal potential to become the new axon. Based on the present observations, this conclusion appears valid, but only for cells that are highly symmetric after lesioning. Even for such highly symmetric cells, there is at present no direct evidence that every minor process has the potential to become an axon.

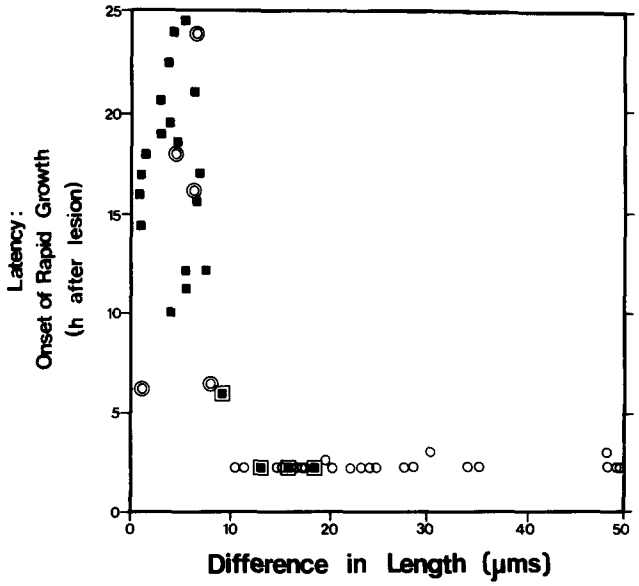

Figure 7. The relationship between relative process length after axonal transection and the latency before the initiation of axonal growth. The horizontal axis represents the difference in length between the process that became the axon and the next longest remaining process. The vertical axis indicates the interval between transection and the beginning of rapid, axonal growth (here defined as growth at a rate averaging >10 μm/h). Circles represent cells whose original axons regenerated. Squares represent cells whose polarity was altered by the lesion. Enclosed symbols correspond to the cells shown in Fig. 6 *B*, whose responses to transection were atypical. An abrupt increase in the latency of axonal outgrowth occurred when the difference in process length decreased below 10 μm. Note that the latency to axonal outgrowth was more closely related to the difference in process length than to whether polarity changed or not, as judged by the responses of the atypical cells. The behavior of the four cells whose polarity changed but whose axons began to grow soon after lesioning (*enclosed squares*) was appropriate to the difference in length of their processes, as was the behavior of the cells whose axons regenerated only after a long latent period (*enclosed circles*). Since cells were not observed continuously, but at intervals of 1–4 h, measurements of latency are not exact. The minimum possible latency was 2 h, the interval between lesioning and the first observation.

In addition, for all symmetric cells, a longer interval elapses before outgrowth of the axon begins. The events that occur during this latent period offer intriguing clues concerning the mechanism of axonal determination after lesions that produce symmetric cells. The processes of such cells appear to be in dynamic equilibrium; at any moment, some processes experience small growth spurts while others retract or are stationary. This competitive interaction between processes may well explain the long latency before axonal outgrowth begins. This equilibrium persists until one process exceeds the others by a critical length. This critical length difference, ~10 μm, appears to represent a threshold for rapid, axonal outgrowth. According to this view, cells that are asymmetric after axonal transection already exceed the critical length threshold. Rapid growth, restricted to the longest process, begins almost immediately.

We are aware of only a few examples of similar competitive interactions that occur in single cells. One phenomenon, referred to by Weiss and Garber (1952) as a "tug or war," sometimes occurs when fibroblasts first attach to a substrate. Initially lamellae (membrane ruffles) arise from two or three different parts of the cell, pulling in different directions, so that the cell is unable to migrate. With time, one lamellae becomes larger than the rest, dominating the others, and the cell begins to move in that direction. Bray (1979) observed an interactive effect among processes of sensory neurons in culture that may also be relevant. The growth and branching of one of a cell's processes appeared to be influenced by the length and orientation of the others, perhaps through the mechanical tension exerted by their growth cones. Finally, in the biflagellate *Chlamydomonas*, amputation of one flagellum can influence the length and growth of the other (Rosenbaum et al., 1969). In none of these examples are the underlying cellular mechanisms understood.

The Significance of these Results for the Normal Development of Polarity

Competition between processes and a threshold length difference for axonal outgrowth may also underlie the establishment of polarity during normal development in culture. Symmetric cells after axonal transection resemble normal cells at Stage 2 of development. Both have several short processes that are approximately equal in length and appear to be indistinguishable from one another in terms of their potential to become axons. Based on electron microscopy (J. Dietch and G. Banker, unpublished observations) and on the distribution of several axonal and dendritic marker antigens (Goslin et al., 1988; our unpublished observations), no one process can be identified at stage 2 as the forerunner of the axon. Similarly, the results of the present study indicate that the processes of cells that are symmetric after axotomy have a roughly equal potential to become the axon. Finally, the events leading to the establishment of polarity are similar for both normal cells and for symmetric cells produced by

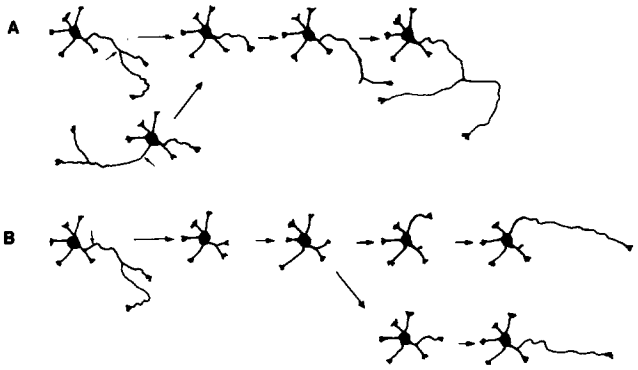

Figure 8. Drawings summarizing the responses of hippocampal neurons after axonal transection. When cells appear asymmetric after transection (*A*), that is when one process is significantly longer than the others, the longest process rapidly acquires axonal characteristics. Asymmetric cells can be produced in two ways: by distal transections that leave the axon longer than the minor processes (*upper row*), or by close transections of cells with one minor process that is longer than the others (*lower row*). When cells appear symmetric after transection (*B*), the axon emerges only after a long period of competitive interaction among the remaining processes. The axon can arise by respecification of a minor process (*upper row*), or by regeneration of the original axon (*bottom row*).

lesioning. For both, there is a considerable delay before one process, by its rapid growth, becomes the axon (Dotti et al., 1988; this paper). In the case of lesioned cells, a "tug of war" occurs during this period, indicative of a competitive interaction among processes. Preliminary observations using high resolution time-lapse video microscopy suggest that a similar "tug of war" may occur among the processes of normal cells before axonal outgrowth. According to this view, one might predict that the normal process of polarization in culture involves a competitive interaction that continues until, by chance, one minor process exceeds a critical length threshold and becomes specified as the axon.

This model of the development of neuronal polarity derives from observations in culture, where the growth of cells is largely a function of their endogenous properties. In situ, the axons of hippocampal pyramidal cells invariably emerge from the basilar aspect of the cell, the pole nearest the lateral ventricle. Under such circumstances, positional cues in the cells' environment may modulate these endogenous cellular mechanisms to determine the origin of the definitive axon.

Cellular Mechanisms Underlying the Development of Neuronal Polarity

The ability of hippocampal neurons to develop a polarized organization, with a single axon and several dendrites, is an endogenous cellular property that can occur in an apparently homogenous environment (Bartlett and Banker, 1984*a*; Caceres et al., 1984; Dotti et al., 1988). This is not, of itself, surprising, since endogenous determinants play an important role in governing the shape of many types of cells. The mechanisms that underlie the development of polarity in hippocampal neurons, however, appear to be quite different from any that have been previously described. This is exemplified by comparing our results with those of studies concerning the control of shape in neuroblastoma cells, which extend neuritic processes but are apparently not polarized. In the case of neuroblastoma cells, the daughters arising from a mitosis often resemble one another with regard to the number, position, length, and branching of their processes, suggesting that these aspects of shape are specified by cytoplasmic determinants within the cell body (Solomon, 1979, 1981). Likewise, neuroblastoma cells whose processes have been caused to retract by microtubule disassembly tend to recapitulate these aspects of their shape when allowed to recover (Solomon, 1980). In contrast, in the case of hippocampal neurons, endogenous cellular mechanisms appear to permit only a single process to become the axon, but do not specify which process will do so. In fact, which process becomes the axon is a function of relative process length and can be manipulated simply by varying the site of axonal transection.

What might be the cellular basis for competition and the importance of relative length in determining which process becomes the axon? Our observations raise the possibility that some substance, present in a limited amount, is itself limiting for growth. Based on studies of neurite growth in other cell types, it seems likely that the limiting factor is a regulatory protein rather than a structural component of the growing axon (Seeds et al., 1970; Yamada et al., 1971; Drubin et al., 1985). One might hypothesize that the concentration of such a regulatory protein in the growth cone is a function of process length. Such a relationship could arise if the protein were actively transported along processes in one direction but not the other. In such a case, its flux in the direction of active transport would be independent of length, but in the opposite direction would vary inversely with length. For example, a growth-promoting protein that was actively transported away from the cell body and returned by diffusion would reach its highest concentration in the growth cone of the longest process. An inhibitor of growth produced in the cell body would, by an analogous mechanism, reach its lowest concentration in the longest process. Alternatively, changes in surface-to-volume ratio that occur as processes elongate might, of themselves, induce the formation of gradients within the cell that could control the rate of process elongation. Any of these mechanisms would ensure that the regulatory protein reached the threshold concentration necessary for axonal growth only in the longest process.

Additional mechanisms are necessary to explain why rapid growth continues to be restricted to a single process, once it exceeds the critical length difference. We suspect that this involves a selective sorting of materials, including a specific population of membranous elements, into the growing axon. Preliminary electron microscopic and immunocytochemical evidence suggests that selective sorting and delivery of membranous elements to the axon and its growth cone begins at about the time the axon exceeds the length threshold necessary for the initiation of rapid axonal elongation (Goslin et al., 1988; our unpublished observations). One membrane protein in particular, the growth-associated protein GAP-43, which is initially present in the growth cones of all minor processes, rapidly and selectively accumulates in the axonal growth cone at about the time the axon becomes noticeably longer than the other minor processes (our unpublished observations). If this view is correct, it remains to be determined how selective sorting occurs, and how it is initiated when one process sufficiently exceeds the others in length.

These results, like those of Dotti and Banker (1987), suggest a surprising plasticity in the polarization of developing hippocampal neurons. At a stage when the axon is 100–200 μm long and axonal differentiation is already well developed, as judged by growth properties, ultrastructural appearance, and the selective sorting of membrane constituents, the processes are still readily capable of acquiring a different identity. It remains to be determined if neuronal processes become irreversibly specified and, if so, when and how this occurs. By transecting the axons of neurons in progressively more mature cultures, it should be possible to address these issues.

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