Inhibitory neurons from fetal rat cerebral cortex exert delayed axon formation and active migration in vitro

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Summary
Inhibitory and excitatory neurons exhibit distinct patterns of development in the mammalian cerebral cortex. The morphological development of inhibitory and excitatory neurons derived from fetal rat cerebral cortex has now been compared in vitro. Inhibitory neurons were identified by immunofluorescence staining with antibodies to γ-aminobutyric acid, and axon formation was detected by staining with antibodies to phosphorylated neurofilaments. In chemically defined, glia-free and low-density cultures, excitatory neurons formed axons within three days of plating. By contrast, inhibitory neurons required more than six days to form axons. Time-lapse analysis over six days revealed that most inhibitory neurons were bipolar and that their two processes exhibited alternate growth and retraction without giving rise to axons. Movement of the cell body towards the growing process was apparent in about one-half of inhibitory neurons, whereas such movement was never seen in excitatory neurons. The migratory behavior of neurons was further investigated by culture on a glial cell monolayer. Inhibitory neurons migrated over substantially larger distances than did excitatory neurons. The centrosome of inhibitory neurons translocated to the base of the newly emerging leading process, suggesting the existence of a force that pulls intracellular organelles towards the leading process. Centrosome translocation was not detected in excitatory neurons. These observations suggest that the developmental programs of excitatory and inhibitory neurons differ. Inhibitory neurons thus possess a more effective cytoskeletal machinery for migration than excitatory neurons and they form axons later.

Movies available online

Key words: Axon formation, Migration, Interneuron, Time-lapse microscopy

Introduction
The morphogenesis of projection neurons precedes that of local circuit neurons during development, and this is a basic feature of maturation of the mammalian central nervous system. (Jacobson, 1991). The morphogenesis of pyramidal and of nonpyramidal cells in the rat cerebral cortex has been compared by Golgi staining. Although the overall patterns of dendritic and perikaryal growth are similar between these two types of neuron, the emergence and differentiation of axons differ (Parnavelas and Uylings, 1980). Pyramidal neurons begin to form axons while they are still migrating, whereas axons of nonpyramidal neurons develop after these cells have completed their migration (Shoukimas and Hinds, 1978; Parnavelas and Lieberman, 1979; Miller, 1986). Synaptogenesis by the axons of nonpyramidal neurons also begins later than that by pyramidal neuron axons (Blue and Parnavelas, 1983; Miller, 1986). Given that almost all nonpyramidal neurons in the cerebral cortex contain γ-aminobutyric acid (GABA) (Meinecke and Peters, 1987), the later development of these cells is responsible for the delayed formation of inhibitory systems in the cortex, providing a window in which enhanced excitation confers neuronal plasticity in young animals (Hensch et al., 1998; Huang et al., 1999).

Time-lapse analysis has revealed differences in the migratory behavior of inhibitory and excitatory neurons. Excitatory neurons originate in the ventricular zone of the cortex and migrate radially toward the cortical plate at a relatively constant rate of about 50 μm/hour (Nadaraja et al., 2001). By contrast, inhibitory neurons originate in the basal forebrain and migrate tangentially into the cortex. Although their average rate of migration is similar to that of excitatory neurons, inhibitory neurons often stop, turn and reverse their direction of movement (Nadaraja et al., 2002; Polleux et al., 2002), and they can achieve speeds of up to 140 μm/hour for periods of up to 30 minutes (Polleux et al., 2002). Furthermore, histological studies of the distribution of inhibitory neurons have suggested that these cells continue their migration as late as the second postnatal week in rats (Wolff et al., 1984; Seress and Ribak, 1988; Dupuy-Davies and Houser, 1999; Frahm and Draguhn, 2001).

Evidence thus indicates that inhibitory neurons in the developing cerebral cortex migrate over a longer distance, in a more active manner and for a longer period of time than do excitatory neurons, and that they form axons after excitatory neurons have already done so. It remains unclear, however, how the development of these two types of neurons is regulated. Cell development is often controlled by extracellular signals produced by other cells in situ, but it is also sometimes executed according to cell-intrinsic programs. Such intrinsic
programs of cell development have been studied by isolating cells and analyzing their maturation in vitro in the absence of influences from other cell types.

The development of hippocampal neurons in vitro has been well characterized. Time-lapse video microscopy has thus revealed that these neurons undergo a stereotypical sequence of morphological changes that include the formation of lamellipodia around the circumference of the cell, growth of four or five minor processes, rapid extension of one process until it becomes an axon, elongation of the remaining minor processes and their acquisition of the morphological features of dendrites, and continued maturation of both axonal and dendritic arbors (Craig and Banker, 1994). This sequence of events proceeds without any stimulation, indicating that it is intrinsically programmed. Only about 6% of neurons in the hippocampal cultures used for such studies are inhibitory (Benson et al., 1994), however, and a comparison of the developmental sequences of inhibitory and excitatory neurons has not been performed.

We have therefore now compared axon formation by and the migratory behavior of inhibitory and excitatory neurons in vitro. In a chemically defined and glia-free culture, most inhibitory neurons derived from the fetal rat cerebral cortex remained bipolar and did not form axons within three days, in contrast to excitatory neurons, which did extend axons during this time. Time-lapse analysis revealed the motility of inhibitory neurons to be markedly greater than that of excitatory neurons. Our results thus show a pronounced difference in developmental programs between excitatory and inhibitory neurons.

Materials and Methods

Isolation and culture of cortical neurons

Neurons were dissociated from the cerebral cortex of rats at embryonic day 20 by digestion for 20 minutes with papain (9 U/ml) (Worthington Biochemical, Lakewood, NJ) (Hayashi and Shirao, 1999). For examination of axon formation under chemically defined and glia-free conditions, the dissociated neurons were plated at a density of 2000 cells/cm² in tissue culture dishes that had been coated with poly-D-lysine (30 to 70 kDa; 0.25 mg/ml) (Sigma, St Louis, MO) and fetal bovine serum. The cells were maintained in Neurobasal medium (Gibco) supplemented with G28 (Invitrogen, Carlsbad, CA).

Brain-derived neurotrophic factor (BDNF) was obtained from Sigma. Medium (Gibco) supplemented with 10% horse serum and arabinosylcytosine (5 μg/ml) (Sigma) (Hayashi et al., 2002). The cultures were kept in a humidified incubator at 37°C with 5% CO₂. The cells were allowed to adapt to the new conditions for 1 hour before imaging. A sequence of PICT images was acquired at 6 minute intervals with a CoolSNAP charge-coupled device digital camera (Roper Scientific, Trenton, NJ) controlled by IPLab software (Scanalytics, Fairfax, VA). An Optiscan x-y-z motorized microscope stage (Prior Scientific, Rockland, MA) was used to monitor multiple sites and to adjust the focus automatically. Images were converted to a QuickTime movie file with a Hyper Card stack written by Lawrence D’Oliveiro (University of Waikato, Hamilton, New Zealand), which was obtained from the NIH Image web site (http://rsb.info.nih.gov/nih-image). Measurement of migration distances was performed with NIH Image software. We determined the distance moved by the cell body during 10 hours at a sampling rate of 12 minutes.

Immunostaining of neurons

Neurons were fixed for 30 minutes on ice with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) and then exposed for 15 minutes to 0.1% Triton X-100 in phosphate-buffered saline (PBS). They were incubated at room temperature for 1 hour with 2.5% normal donkey serum and 0.05% Triton X-100 in PBS and then for 1 hour with primary antibodies diluted in the same solution. After washing with 0.1% Triton X-100 in PBS for 30 minutes, the neurons were incubated with secondary antibodies for 1 hour and then washed again for 30 minutes. The primary antibodies included mouse monoclonal antibodies to phosphorylated neurofilaments (Sternberger Monoclonals, Lutherville, MD), rabbit polyclonal antibodies to GABA (Sigma), rabbit polyclonal antibodies to pericentrin (Babco, Richmond, CA), mouse monoclonal antibodies to the Golgi 58K protein (Sigma) and mouse monoclonal antibodies to class III β-tubulin (TUJ1, Babco). Secondary antibodies included Rhodamine RedX-conjugated donkey antibodies to rabbit or mouse immunoglobulin G and Alexa488-conjugated donkey antibodies to mouse or rabbit immunoglobulin G (Jackson, West Grove, PA). Fluorescence was observed with an Olympus AX70 microscope equipped with a PXL cooled CCD camera (Olympus Optical, Tokyo, Japan). The intensity of fluorescence was measured with IPLab software. Standardized values were obtained with Excel software (Microsoft).

Results

Axon formation by inhibitory neurons occurs later than that by excitatory neurons

To investigate axon formation by inhibitory neurons, we cultured dissociated cells from the fetal rat cerebral cortex under chemically defined, glia-free and low-density conditions. After 3 days in vitro (DIV), cells were fixed and stained with antibodies to GABA (Fig. 1A) and with antibodies to phosphorylated neurofilaments (Fig. 1B) to identify inhibitory neurons (red chromophore) and axons (green chromophore), respectively. After examination, the same cultures were then stained with antibodies (TUJ1) to the neuron-specific class III β-tubulin (Fig. 1C) in order to distinguish neurons (green chromophore) from other cell types such as glial cells and fibroblasts.

Antibodies to GABA stained cells with a variety of intensities (Fig. 1D). Most cells were stained weakly (standardized value <0.4), but a small population of cells exhibited a relatively high staining intensity. We defined those cells with a standardized staining intensity value of ≥0.4 as GABA positive. On the basis of the pattern of staining with anti-GABA and TUJ1, we classified cultured cells as GABA-positive cells (14.4% of a total of 167 cells examined), GABA-negative and TUJ1-positive cells (79%), or TUJ1-negative cells (6.6%). These categories correspond to inhibitory neurons, excitatory neurons and non-neuronal cells, respectively. We did not detect any GABA-positive, TUJ1-negative cells in this particular experiment, although we did observe a few such cells in other experiments. The percentage of inhibitory neurons in this study (14.4%) is
Development of inhibitory neurons in vitro

Consistent with that previously described for the adult rat visual cortex (15%) (Peters and Kara, 1985; Lin et al., 1986; Meinecke and Peters, 1987) and for cultures of rat cerebral cortical neurons (10.5 to 12.6%) (Stichel and Muller, 1991), about 71% of inhibitory neurons were bipolar after 3 DIV (Fig. 1A; see also Fig. 3B-D), whereas only 10% of excitatory neurons were bipolar at this time (Table 1). One of the two processes of the inhibitory neurons was thick, with a growth cone at its tip, whereas the other was thin, short and tapered. The antibodies to phosphorylated neurofilaments stained axons clearly (Fig. 1B). Of 132 excitatory neurons examined after 3 DIV, 116 (88%) possessed a phosphorylated neurofilament-positive axon (Table 1, Fig. 1B). By contrast, only 2 (8%) of 24 inhibitory neurons manifested an axon at this time (Table 1). After culture for 6 days, the percentage of inhibitory neurons bearing an axon had increased to 55% (Fig. 2A,B; Table 1). Most inhibitory neurons thus required 6 days in culture to form axons, in marked contrast to excitatory neurons, which formed axons within 3 DIV.

BDNF was recently shown to promote the differentiation of GABAergic neurons in vitro (Yamada et al., 2002). We therefore examined the possible effect of BDNF on axon formation by inhibitory neurons (Fig. 2C-E). Treatment with BDNF (5 μg/ml) for 3 days did not increase the percentage of inhibitory neurons bearing an axon (Table 1), even though it induced extensive branching of the axons of excitatory neurons (Fig. 2D) and increased the staining intensity for GABA in inhibitory neurons (Fig. 2E). These results thus indicated that BDNF promotes the differentiation of inhibitory neurons without affecting axon formation by these cells.

We investigated cell behavior during culture by time-lapse video analysis. After collecting images for 6 days, we stained the cells with anti-GABA, antiphosphorylated neurofilaments and TUJ1 to identify neuronal types. Axon formation by

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**Table 1. Percentages of axon-bearing cells and of bipolar cells among excitatory and inhibitory neurons under various culture conditions**

| Culture condition    | Neuronal type | With axon (%) | Bipolar (%) | n  |
|---------------------|--------------|---------------|-------------|----|
| Glia-free (3DIV)     | Excitatory   | 88            | 10          | 132|
|                      | Inhibitory   | 8.3           | 71          | 24 |
| Glia-free+BDNF (3DIV)| Excitatory   | 84            | ND          | 153|
|                      | Inhibitory   | 8.8           | ND          | 34 |
| Glia-free (6DIV)     | Excitatory   | 100           | ND          | 148|
|                      | Inhibitory   | 55            | ND          | 31 |
| Glial layer (2DIV)   | Excitatory   | 85            | 6.1         | 149|
|                      | Inhibitory   | 30            | 38          | 53 |

ND, not determined.
excitatory neurons (Fig. 3A) appeared similar to that described for hippocampal neurons (Dotti et al., 1988). The neurons thus extended several minor processes during the first several hours after plating. After an average of 24 hours in culture (n=59), one of the processes began to elongate (Fig. 3A). By contrast, most inhibitory neurons extended two processes that exhibited growth and retraction alternately for more than 3 days (Fig. 3B-D; see Movies 1-3 at http://jcs.biologists.org/supplemental/). It was not possible to determine the precise time of axon formation by inhibitory neurons because the events preceding axon formation, such as growth cone enlargement and rapid process elongation, were obscure in these cells. In many inhibitory neurons, the cell body moved toward the growing process (Fig. 3B,D); movement of the cell body for >20 μm was observed in more than half of inhibitory neurons (13 out of 24) but was not detected in >100 excitatory neurons examined.

Given that these experiments were performed with chemically defined and glia-free cultures, our data suggest that the observed differences in the mode of axon formation and in cell behavior between inhibitory and excitatory neurons are independent of influences of other cell types. These properties thus appear to be intrinsically programmed by the time the cells were placed in culture.

Rapid migration of inhibitory neurons on a glial cell layer
The movement of the cell body revealed by our time-lapse analysis suggested that inhibitory neurons are highly motile. Given that the poly-D-lysine-coated culture dishes used for the above experiments are not suitable for studies of neuronal migration, we plated dissociated cortical neurons onto a monolayer of glial cells. After 2 DIV, we stained the cells with anti-GABA (Fig. 4A), antiphosphorylated neurofilaments (Fig. 4B) and TUJ1 (data not shown). About 85% of excitatory neurons possessed a phosphorylated neurofilament-positive axon (Fig. 4B), whereas only 30% of inhibitory neurons did so (Table 1), indicating that axon formation by inhibitory neurons was also delayed in the presence of glial cells as it was in glia-free culture. More than half of the axon-less inhibitory neurons (38% of the total) were bipolar (Table 1); these cells possessed one thick process with a growth cone and one thin process (Fig. 4A; see also Fig. 5A). A similar morphology has been described for migrating inhibitory neurons both in vivo (De Carlos et al., 1996; Tamamaki et al., 1997) and in slice cultures (Nadaraja et al., 2002; Polleux et al., 2002).

Time-lapse video analysis revealed that cortical neurons migrated rapidly on glial cells (see Movie 4 at http://jcs.biologists.org/supplemental/). To quantify the migratory activity, we measured the distance traveled by the neurons during a 10 hour period after culture for 3 days. Such analysis revealed that inhibitory neurons migrated over substantially greater distances than did excitatory neurons (Fig. 5A). Migrating inhibitory neurons possessed a leading process with a growth cone and a trailing process (Fig. 5B). A prominent characteristic of the migrating neurons was that they often reversed their direction of movement. Such reversal was not initiated by turning of the leading process but by the disappearance of the
growth cone from this process and the emergence of a new growth cone at the tip of the other process. Similar behavior has been described for the tangential migration of inhibitory neurons in slice cultures (Nadaraja et al., 2002; Polleux et al., 2002).

Translocation of organelles of inhibitory neurons towards the leading process

Migrating inhibitory neurons differed from nonmigratory excitatory neurons in that the cell body of the former moved towards the leading process but that of the latter did not move towards the growing axon. To examine the force responsible for the movement of the cell body of inhibitory neurons toward the leading process, we monitored the Golgi apparatus, the centrosome and the nucleus during neuronal migration. After culture of the neurons on glial cells for 1 day, neuronal migration was observed by time-lapse analysis for 120 minutes and the Golgi apparatus, centrosome and nucleus were then stained with antibodies to the Golgi 58K protein, antibodies to pericentrin and 4',6-diamidino-2-phenylindole (DAPI), respectively (Fig. 6A,B). The Golgi apparatus and the centrosome were usually located near each other in the perinuclear region. The centrosome was located at the base of the leading process in 13 (36%) of 36 migrating neurons analyzed (Fig. 6C). Furthermore,
in most of the neurons that did not reverse the direction of migration during the 120 minute observation period, the centrosome was located at the base of the leading process (Fig. 6A). By contrast, in neurons that reversed the direction of movement, the centrosome was often located at the rear of the cell body (Fig. 6B). We therefore divided the migrating neurons into three groups on the basis of the time of the reversal of the direction of migration: of seven neurons that reversed the direction of movement in the last 60 minutes of recording, two (29%) contained the centrosome at the base of the leading process (Fig. 6D); of 12 neurons that reversed direction in the first 60 minutes of recording but not thereafter, three (25%) contained the centrosome at the base of the leading process (Fig. 6E); and of 17 neurons that did not reverse direction during the 2 hour recording period, 8 (47%) contained the centrosome at the base of the leading process (Fig. 6F). These results thus indicated that the centrosome translocated toward the base of the leading process within a few hours of the reversal of migration. However, in excitatory neurons cultured on glial cells for 1 day, the position of the centrosome did not appear to be related to that of the extending axon (Fig. 7). This result is compatible with the previous report that the position of the Golgi complex and the site of origin of the axon were not correlated in hippocampal neurons (Dotti and Banker, 1991).

These observations suggest that the leading processes of migrating neurons generate a force via microtubules to pull centrosomes and other microtubule network in cell body. The growing axons of nonmigratory neurons appeared not to generate such a force.

The force exerted on the microtubule network in migrating neurons was evident when the movement of the nucleus was impeded. Fig. 8A (see Movie 5 at http://jcs.biologists.org/supplemental/) shows an inhibitory neuron

**Fig. 4.** Properties of cortical neurons cultured on a glial cell layer for 2 days. (A) An inhibitory neuron (arrow) revealed by anti-GABA staining. (B) Two excitatory neurons (arrows), each bearing an axon (arrowheads), were revealed by antiphosphorylated neurofilament staining. The antibodies to phosphorylated neurofilaments also stain nonspecifically the nuclei of cells including those of glia. Bar, 100 µm.

**Fig. 5.** Time-lapse analysis of the migration of cortical neurons after culture on a glial cell layer for 3 days. (A) Histogram of the distance migrated by inhibitory (solid bars) and excitatory (open bars) neurons over a 10 hour period. (B) Images collected at 30 minute intervals of migrating inhibitory neurons. The neurons each possessed a leading process with a growth cone at the tip as well as a trailing process. Reversal of the direction of migration was accompanied by the disappearance of the growth cone from the original leading process and the appearance of a new growth cone at the tip of the previous trailing process. Bar, 20 µm.
migrating toward the top of the field (asterisk). The leading process moved below the process of an adjacent neuron (arrowheads). Although the nucleus of the migrating neuron was not able to move beneath this adjacent process, cytoplasmic material was able to do so and progressed within the leading process towards the growth cone, resulting in swelling of the middle portion of the leading process (arrow). Immunostaining of similar swellings of other migrating neurons revealed the presence of the centrosome and the Golgi apparatus within the swelling (Fig. 8B,C).

**Fig. 6.** Translocation of the centrosome and the Golgi apparatus during the reversal of migration in inhibitory neurons. Cortical neurons were cultured for 1 day on a glial cell layer. The migration of inhibitory neurons was then monitored for 120 minutes before staining of the centrosome with anti-pericentrin (green), of the Golgi apparatus with anti-Golgi 58K protein (red) and of the nucleus with DAPI (blue). (A) A neuron that migrated without reversal of direction for 120 minutes. The centrosome and Golgi apparatus were located at the base of the leading process (arrowhead). The arrow indicates the direction of migration. (B) A neuron that reversed direction during the last 60 minutes of recording. The centrosome and the Golgi apparatus were located at the rear side of the nucleus (arrowhead). Bars, 10 μm. (C) The position of the centrosome relative to that of the leading process in individual migrating inhibitory neurons is indicated by dots. The nucleus is represented by the large open circle and the site of origin of the leading process is indicated by the arrow. In about 75% of neurons, the centrosome was located at the front side of the nucleus. (D-F) The data shown in (C) were divided into three groups based on the time of reversal of the direction of migration. (D) Neurons that reversed their direction of migration during the last 60 minutes of recording. (E) Neurons that reversed direction during the first 60 minutes of recording but not thereafter. (F) Neurons that did not reverse the direction of movement during recording. The centrosome thus translocated to the base of the leading process after the reversal of migration.

### Discussion

We have examined the differences in development in vitro between excitatory neurons and inhibitory neurons of the fetal rat cerebral cortex. We found that axon formation by inhibitory neurons was delayed for several days compared with that by excitatory neurons, and that, during this delay, inhibitory neurons were highly motile (Fig. 9).

**Delayed axon formation by inhibitory neurons**

Axon formation is an important event in neuronal development. The events leading to axon formation have been well characterized in cultured hippocampal neurons. Soon after plating, hippocampal neurons form several minor processes. Although all of these processes have the potential to become axons (Dotti and Banker, 1987; Goslin and Banker, 1989), only one begins to elongate rapidly and develops into an axon. The first indications of axon formation include cytoplasmic flow in the selected process (Bradke and Dotti, 1997) and enlargement of the growth cone at its tip (Bradke and Dotti, 1997; Bradke and Dotti, 1999). These changes are induced by uneven exposure of the neuronal processes to extracellular matrix molecules such as laminin (Esch et al., 1999). However, even under homogeneous conditions, such as in low-density culture, one process is stochastically chosen for axon formation within a certain period. Thus, the transition from the axon-less stage of neuronal development (stage 2 according to Dotti et al.) (Dotti et al., 1988) to axon formation (stage 3) appears to be a cell-autonomous event. The manner and time course of this transition have been assumed to be basically the same in most types of polarizing neurons (Craig and Banker, 1994), including hypothalamic neurons (Diaz et al., 1992) and cerebellar macroneurons (Ferreira and Caceres, 1989). However, axon formation by inhibitory neurons in hippocampal cultures has not been well characterized, probably because these neurons constitute only ~6% of the total neurons in these cultures (Benson et al., 1994).
We have now shown that, in cultures of cerebral cortical neurons, the time course of axon formation differs markedly between inhibitory and excitatory neurons. Most excitatory neurons extended axons within a day of culture, whereas inhibitory neurons required about six days to form an axon. This observation suggests that the mechanisms of axon formation may differ between the two types of neurons. It is unlikely that the later axon formation by inhibitory neurons was due to their younger age, because both types of neurons are born throughout the period of neurogenesis of the central nervous system, between embryonic days 14 and 20, with a peak production at day 17 (Miller, 1985).

Migratory behavior of inhibitory neurons
The migration of inhibitory neurons in the cerebral cortex is thought to occur in response to gradients of chemoattractants such as GABA (Behar et al., 1998), SLIT (Zhu et al., 1999) and hepatocyte growth factor (Powell et al., 2001). Our observation of the rapid migration of inhibitory neurons on a homogeneous layer of glial cells, however, suggests that the migratory activity of inhibitory neurons does not require such a gradient. Rather, it is possible that glial cells produce diffusible or membrane-bound molecules that induce motility in inhibitory neurons, similar to the migration-inducing activity described by Mason et al. (Mason et al., 2001) and that of astrotactin (Zheng et al., 1996). Our observations with chemically defined, glia-free cultures, however, suggest that the migration of inhibitory neurons does not require any factors produced by other cells. Although inhibitory neurons did not migrate extensively under these conditions, as a result of the artificial substrate of poly-D-lysine, the inhibitory neurons exhibited a bipolar morphology, a prominent characteristic of migrating cells in

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**Fig. 7.** Localization of the centrosome in axon-extending neurons. (A) Neurons cultured on a glial cell layer for 1 day were stained with antiphosphorylated neurofilaments (red), anti-pericentrin (green) and DAPI (blue). Centrosomes were located either at the base of the extending axon (left panel), at the side of the nucleus relative to the position of the axon (middle panel) or on the opposite side of the nucleus from the axon (right panel). Arrows indicate axons. Bar, 10 μm. (B) Summary of the position of the centrosome (dots) in 51 axon-extending neurons. The arrow represents the site of origin of the extending axon. There was no relation between the position of the centrosome and the site of axon extension.

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**Fig. 8.** Movement of intracellular organelles within the leading process of migrating neurons cultured on a glial cell layer. (A) The leading process of an inhibitory neuron (asterisk) moved beneath a process of an adjacent neuron (arrowheads). The images were collected at 10 minute intervals. The nucleus of the migrating neuron was not able to pass beneath the adjacent process; by contrast, cytoplasmic components moved into the leading process towards the growth cone, resulting in swelling (arrow) of the middle portion of the leading process. Bar, 20 μm. (B,C) Additional examples of neurons with a swelling (arrows) of the leading process. Immunostaining revealed the presence of the Golgi apparatus and the centrosome (arrowheads) within the swellings. Right-hand panels are merged images of staining with anti-pericentrin (green), anti-Golgi 58K protein (red) and DAPI (blue). Bar, 10 μm.
Inhibitory neurons.

Thus demonstrated might be responsible for the motility of within the leading process toward the growth cone. The force obstacle, the centrosome and the Golgi apparatus translocated process moved beneath a process of another neuron. Although panel). Such tethering was evident in neurons in which this tether the centrosome to the leading process (see Fig. 9, lower panel). It is known that in mature neurons, microtubules are released from the centrosome by the severing activity of katanin (McNally and Vale, 1993; Ahmad et al., 1999). Katanin thus probably destroys the tether linking the centrosome to the leading process, explaining the lack of centrosome translocation in excitatory neurons. The released short microtubules are transported into the axon with the dynein-dynactin complex (Ahmad et al., 1998; Baas, 1999) and are thought to stabilize the leading edge of cells (Abal et al., 2002). These processes are thought to underlie the continuous growth of axons of excitatory neurons and of inhibitory neurons at the later stage.

**Fig. 9.** The proposed intrinsic developmental programs of excitatory and inhibitory neurons. In excitatory neurons, axon formation occurs soon after the emergence of minor processes. By contrast, inhibitory neurons undergo a migratory stage before axon formation. During this migratory stage, the centrosome is tethered to the leading process, whereas the centrosome is dissociated from the extending axon.

Slice cultures, and movement of the cell body was apparent in about half of the cells. Such movement was never observed in excitatory neurons. Inhibitory neurons thus appear to possess a machinery for cell migration that does not require signals from other cell types for its activity.

**Migratory machinery of inhibitory neurons**

In some types of cells, the centrosome and the Golgi apparatus translocate in the direction of cell migration (Gotlieb et al., 1981; Kupfer et al., 1983; Koonce et al., 1984). For example, relocalization of centrosomes to the site of newly emerging processes has been observed in cultured cerebellar granule neurons (Zmuda and Rivas, 1998). The location of the Golgi apparatus has been proposed to determine the direction of efficient membrane transport and thus the direction of cell migration (Singer and Kupfer, 1986). By contrast, other studies have shown that the extension of a new pseudopod precedes centrosome repositioning (Euteneuer and Schliwa, 1992; Ueda et al., 1997), suggesting that the location of the centrosome or the Golgi apparatus is not the determinant of the direction of migration. Instead, the centrosome, which is attached to the minus ends of microtubules, might be pulled by microtubules whose plus ends are anchored at the cell membrane of the pseudopod. Protein complexes such as APC-EB1 (Tirnauer and Bierer, 2000), dynein–β-catenin (Ligon et al., 2001) and CLIP-170–IQGAP1 (Fukata et al., 2002) are candidates for the molecular anchor of microtubules at the cell membrane.

Our demonstration of centrosome reorientation after the reversal of neuronal migration suggests that microtubules tether the centrosome to the leading process (see Fig. 9, lower panel). Such tethering was evident in neurons in which this process moved beneath a process of another neuron. Although the nucleus of such neurons was unable to migrate beyond this obstacle, the centrosome and the Golgi apparatus translocated within the leading process toward the growth cone. The force thus demonstrated might be responsible for the motility of inhibitory neurons.

**Development of inhibitory neurons in vitro**

In excitatory neurons, however, we did not detect any association between centrosomes and growing processes. This indicates that the centrosome does not link with microtubules in the processes (see Fig. 9, lower panel). It is known that in mature neurons, microtubules are released from the centrosome by the severing activity of katanin (McNally and Vale, 1993; Ahmad et al., 1999). Katanin thus probably destroys the tether linking the centrosome to the leading process, explaining the lack of centrosome translocation in excitatory neurons. The released short microtubules are transported into the axon with the dynein-dynactin complex (Ahmad et al., 1998; Baas, 1999) and are thought to stabilize the leading edge of cells (Abal et al., 2002). These processes are thought to underlie the continuous growth of axons of excitatory neurons and of inhibitory neurons at the later stage.

**Significance of the later development of inhibitory neurons**

Intracortical inhibition matures slowly compared with excitation (Blue and Parnavelas, 1983; Komatsu, 1983; Miller, 1986; Luhmann and Prince, 1991). This developmental mismatch provides a window of time – a critical period – when sensory input strongly influences the organization of cortical circuitry. In the visual cortex, the development of GABAergic systems probably plays an essential role in terminating the critical period for plasticity in ocular dominance (Hensch et al., 1998; Huang et al., 1999). We propose that the longer migratory period and later axon formation apparent for inhibitory neurons are not only necessary for the colonization of the cortex by these neurons, but are also responsible for the delayed maturation of inhibitory systems, which renders the young brain highly plastic.

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