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Evidence That Pioneer Olfactory Axons Regulate Telencephalon Cell Cycle Kinetics to Induce the Formation of the Olfactory Bulb

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Summary

Early olfactory axons follow a specific pathway to reach the developing telencephalon. We observed that a subpopulation of these axons, the pioneer olfactory axons, penetrate into the ventricular zone of a highly restricted region of the telencephalon at E13 and E14. At E15, this same telencephalic region evaginates to form the olfactory bulb. To investigate the possibility that the pioneer olfactory axons induce the olfactory bulb by influencing precursor cell populations, we compared cell cycle kinetics and differentiation in the olfactory bulb primordium and the adjacent neocortex using cumulative bromdeoxyuridine labeling. The results showed that, 24 hr after the arrival of the first pioneer axons, the duration of the cell cycle is prolonged significantly in the olfactory bulb primordium. In addition, twice as many cells have exited the mitotic cycle in the olfactory bulb primordium versus the adjacent cortex. These findings suggest that pioneer olfactory axons play a role in the induction of the olfactory bulb by selectively modulating cell cycle kinetics in the olfactory bulb primordium. Afferent axons may influence target morphogenesis by modulating target precursor cell proliferation in other developing neural structures.

Introduction

Several recent studies of invertebrate nervous system development suggest that peripheral axons may regulate proliferation and/or differentiation of neurons during CNS development. Lesions of growing optic axons in Daphnia magna reduce the number of their CNS target cells, the laminar neurons (Macagno, 1979). Retinal innervation regulates the number of neuronal precursors in the optic ganglion of Drosophila melanogaster (Selleck and Steller, 1991). Retinal axons appear to influence retinal ganglion precursors at the G1-S phase of the cell cycle (Selleck et al., 1992). Studies of leech genitalia development also indicate that peripheral organs may regulate central neurogenesis (Baptista et al., 1990). By contrast, only a few reports of vertebrate development have suggested a role for peripheral afferents in the regulation of CNS neurogenesis. For example, eye removal during early frog development results in lower mitotic activity in rostral parts of the tectum that are innervated by optic fibers (Kollros, 1982). However, there is no evidence that peripheral axons influence cell cycle parameters of CNS cells in the vertebrate nervous system.

Olfactory bulb development is dependent on the olfactory placode (Burr, 1924, 1930; Byrd and Burd, 1991; Stout and Graziadei, 1980). The olfactory placode develops into the olfactory epithelium, the peripheral sensory organ of the olfactory system. Axons of receptor neurons in the olfactory epithelium make direct contact with the developing telencephalon around the time of olfactory bulb morphogenesis. The olfactory bulb develops from the rostral part of the telencephalon, the olfactory bulb primordium. The olfactory bulb primordium evaginates into a structure morphologically distinct from the neocortex and generates specific populations of olfactory bulb neurons.

Complete removal of the olfactory placode prevents the formation of the olfactory bulb in Xenopus laevis embryos; partial lesions of the placode result in a smaller olfactory bulb (Kosciuszko, 1958; Stout and Graziadei, 1980). Transplantation of an ectopic olfactory placode into different locations in the brain results in local hyperplasia (Burr, 1924, 1930; Stout and Graziadei, 1980). The latter authors reported that supernumerary olfactory axons contact the olfactory bulb and induce a 40%–60% increase in bulb volume. However, a more recent study showed that a supernumerary olfactory placode did not influence bulb volume significantly but found that increased olfactory axons correlated with increased numbers of mitral cells (Byrd and Burd, 1993). Thus, there are contradictory findings as to the role of olfactory axons in the induction of the olfactory bulb.

There are also potential problems in interpreting these lesion studies. When removing or surgically transplanting the olfactory placode, it is difficult to avoid damaging the telencephalon; such damage might influence subsequent olfactory bulb formation. Furthermore, though placode removal may prevent the development of the olfactory bulb, it is not clear whether this is due to the loss of diffusible factors emanating from the placode or the loss of the olfactory axons innervating the developing telencephalon.

Thus, either the olfactory axons or the placode or both may play a critical role in the induction of the olfactory bulb. Consistent with the possibility that axons are involved, Hinds (1972) showed that some early olfactory axons grow into the proliferative zone of the telencephalon. This was confirmed recently by DiI tracing experiments (Santacana et al., 1992). However, the temporal relationship between the contact of the olfactory axons with the telencephalon and the morphogenesis of the olfactory bulb is not known.

To investigate the possible role(s) of the olfactory axons in the induction of the olfactory bulb, we have characterized the development of the earliest olfactory axons and measured cell cycle kinetics in the olfactory bulb primor-

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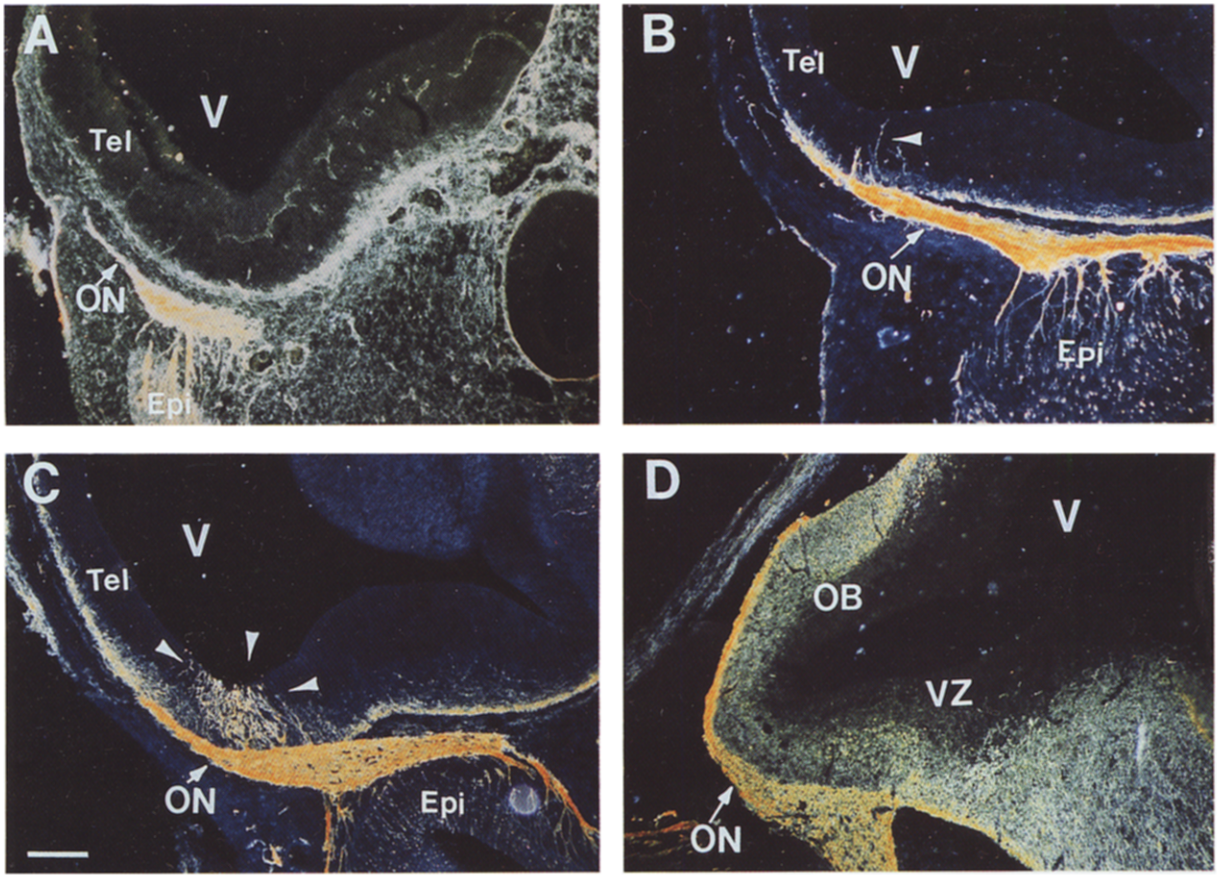

Figure 1. Development of Pioneer Olfactory Axons

Parasagittal sections through the olfactory epithelium and telencephalon showing olfactory axons stained for GAP43.

(A) E12: Olfactory axons initially extend directly toward the ventral telencephalon (Tel), then turn sharply to grow toward the rostral telencephalon. At E12, olfactory axons do not contact the brain.

(B) E13: Olfactory axons first contact the rostral telencephalon. The majority of these axons remain on the surface of the brain. A few pioneer axons (see text) penetrate deep into the telencephalic wall to the ventricular zone (arrowhead).

(C) E14: Additional olfactory axons reach the telencephalon and many more pioneer axons (arrowheads) grow into the ventricular zone.

(D) E15: The olfactory bulb evaginates from the rostroventral telencephalon. Thick arrow indicates the ventricular zone (VZ). Olfactory axons contact the entire surface of the evaginated bulb. Note, however, that few pioneer axons remain in the VZ. ON, olfactory nerve; OB, olfactory bulb; Epi, olfactory placode or olfactory epithelium; V, ventricle. Bar, 100 μm.

dium before and after the arrival of the earliest axons. Prior to the arrival of the pioneer olfactory axons, the cell cycle kinetics of the olfactory bulb primordium and the adjacent neocortex are equal. Subsequent to the arrival of the pioneer olfactory axons, there are significant differences in the cytokinetics in these two telencephalic compartments. In addition, the number of cells exiting the mitotic cycle is doubled in the olfactory bulb primordium. These findings suggest that pioneer olfactory axons may regulate proliferation and/or differentiation of CNS precursor cells to induce the morphogenesis of the olfactory bulb.

Results

Pioneer Olfactory Axons and the Development of the Olfactory Bulb Primordium

Consistent with previous findings, our results indicated that growth-associated protein 43 (GAP43) is one of the first molecules expressed in immature olfactory receptor neurons and their axons (Verhaagen et al., 1989, 1990). The vast majority of growing olfactory axons, if not all, are labeled with anti-GAP43. As they mature, these neurons and their axons lose GAP43 antigenicity.

E12

At embryonic day 12 (E12), the olfactory placode is a single invagination of the rostroventral cranial ectoderm. GAP43-labeled olfactory axons were first observed at this age. These axons emerge from the olfactory placode and grow dorsocaudally to reach the telencephalic vesicle. Olfactory axons did not bundle tightly at this stage, but they did grow along the same trajectory. As they approached to within 50 μm (47 ± 0.03 μm) of the telencephalon, the axons abruptly turned 90° and grew rostrally, parallel to the surface of the telencephalic vesicle, for a distance of approximately 450 μm toward the rostrodorsal part of the telencephalon. Olfactory axons did not contact the telencephalon at this age (Figure 1A). The telencephalon at this age was a uniform, multilayered neuroepithelium

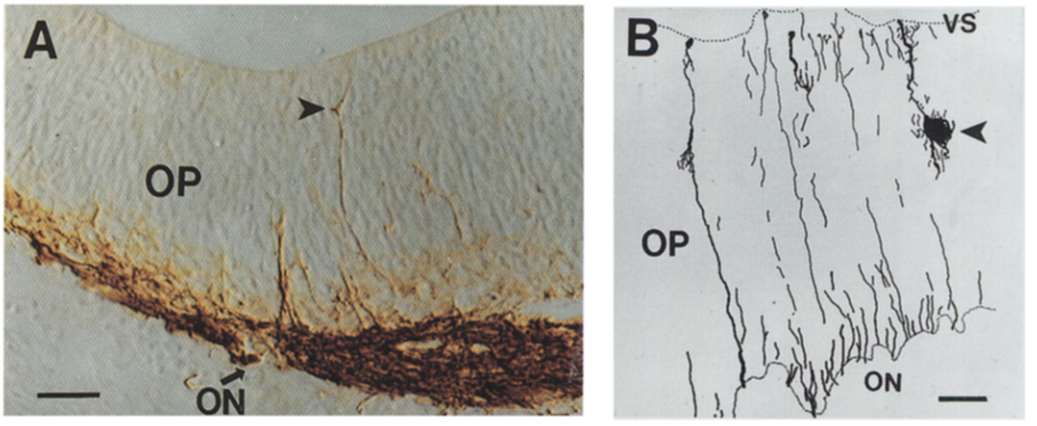

Figure 2. Olfactory Axons in the Ventricular Zone

(A) Parasagittal section of E13 olfactory primordium (OP) demonstrates GAP43-positive olfactory nerve (ON) axons (stained brown) in the ventricular zone. A growth cone filopodium reaches the cell layer near the ventricular surface (arrowhead).

(B) Camera lucida drawing of the DiI-labeled olfactory axons and growth cones in the OP. DiI-positive fibers were found in the ventricular zone of the OP. Some of the fibers reach the ventricular surface (VS). Growth cones of the olfactory axons are visible (arrowhead). Bars, 25 μm (A), 15 μm (B).

throughout the hemisphere (the thickness of the neuroepithelium is 130 ± 0.08 μm). There were no distinctive histological differences between the olfactory bulb primordium and the adjacent neocortex; both lacked a mantle layer.

E13

By E13, many more olfactory nerve axons had grown out of the olfactory epithelium, which at this stage had developed two to three turbinates. The newer olfactory axons followed the same trajectory as those seen at E12. The axons formed a more tightly bundled tract stretching from the olfactory epithelium to the telencephalon. Olfactory axons first contacted the rostral part of the telencephalon at this stage. The majority of the olfactory axons remained on the surface of the telencephalon. These axons were confined to a highly restricted rostral zone comprising ~6% of the surface area of the hemisphere. However, in addition to the superficial axons, several GAP43-positive olfactory axons extended below the surface to penetrate deep into the telencephalon. Indeed, a few of these fibers were present in the ventricular zone below the surface fibers. Many of these deep axons had well defined growth cones (Figure 1B; Figure 2). It is important to emphasize that these deep-penetrating olfactory axons were only a small subpopulation of the olfactory axons, the majority of which were deployed on the surface of the hemisphere. These deep axons were located in the ventricular zone that contains proliferating cells. Because these deep axons differ from the majority of the early olfactory axons and because no such deep-penetrating axons are seen in embryos after E15–16 (see below), we define them as *pioneer olfactory axons* and the telencephalic compartment that contains these pioneer axons as the *olfactory bulb primordium*.

At E13, the telencephalon had increased in volume compared with E12, but the thickness of the neuroepithelium had not changed appreciably (139 ± 0.3 μm at E13 compared with 130 ± 0.08 μm at E12; $p > .1$). The histological organization of the olfactory bulb primordium was still indistinguishable from that of the adjacent neocortex at E13. The ventricular zone remained the dominant component of both the olfactory primordium and the adjacent neocortex. However, a thin mantle layer, one or two cells thick, was present in both the olfactory bulb primordium and the adjacent neocortex.

E14

At E14, additional olfactory axons grew out of the newly forming turbinates of the olfactory epithelium. These new axons joined the olfactory nerve, which at this stage connected the olfactory epithelium with the olfactory bulb primordium. The vast majority of the GAP43-immunoreactive olfactory axons were densely aggregated in the mantle layer and on the surface of the olfactory bulb primordium. Many more pioneer olfactory axons were observed in the ventricular zone at E14 than at E13. Most of the pioneer olfactory axons appeared to be separated from each other in the ventricular zone, although occasional fascicles were also observed. These pioneer olfactory axons in the ventricular zone remained confined to a highly restricted region of the rostral telencephalon, the olfactory primordium (Figure 1C; Figures 3A and 3B).

To confirm further that the axons penetrating into the ventricular zone of the telencephalon were olfactory axons, fast DiI (a lipophilic molecule that diffuses within the plasma membrane) was injected into the olfactory cavity of fixed E14 embryos to label olfactory axons. The results show that the pattern of DiI labeling was the same as that of GAP43 immunoreactivity (Figure 2B): DiI-labeled olfactory axons and growth cones were present within a restricted compartment of the ventricular zone of the telencephalon.

A subtle evagination of the olfactory bulb primordium, making it morphologically distinguishable from the adjacent neocortex, was first observed at E14. In addition, the mantle layer in the olfactory bulb primordium had become much wider than that in the adjacent neocortex, which remained only two to three cells thick. These morphologi-

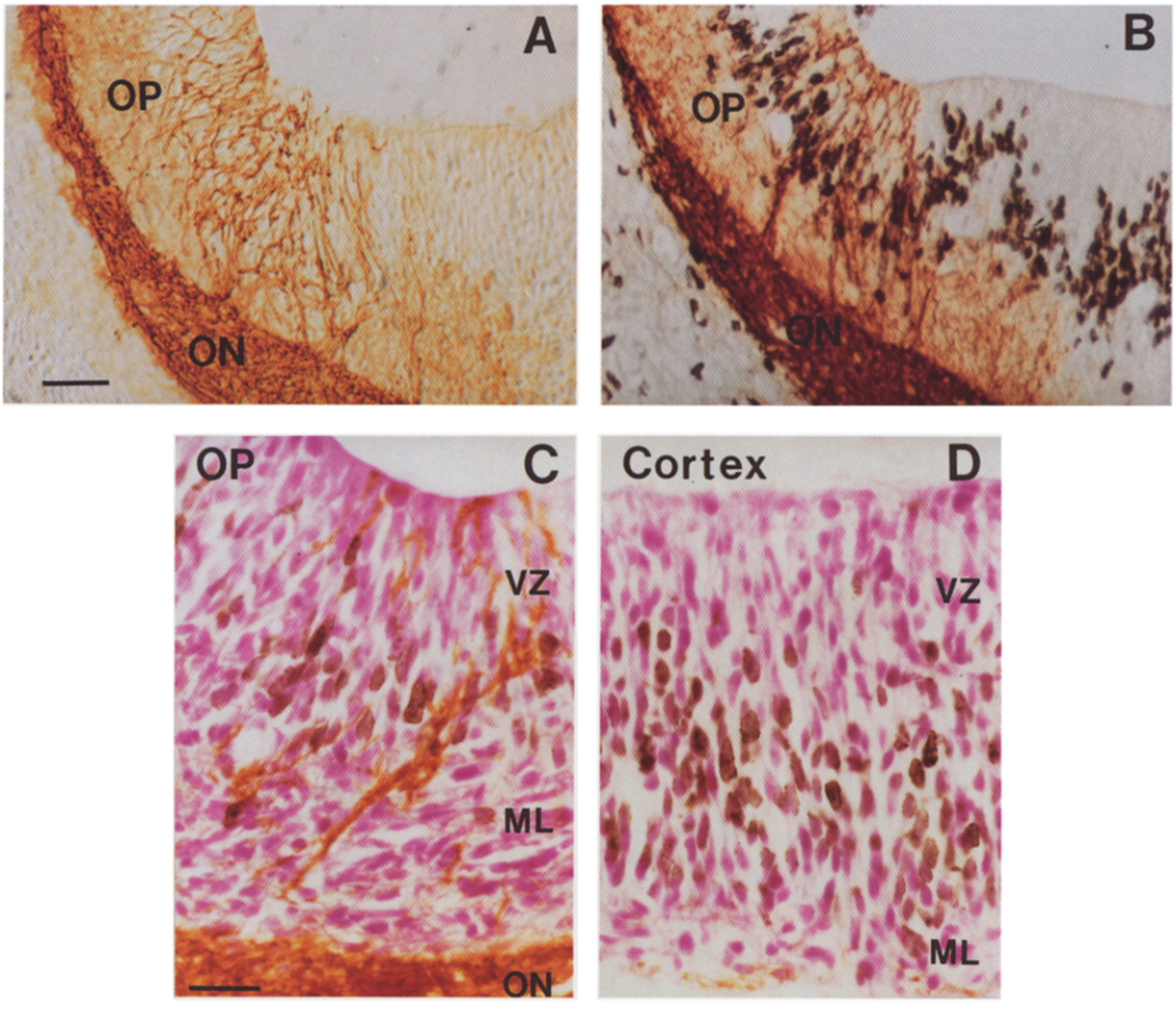

Figure 3. Pioneer Olfactory Axons and Proliferating Cells

GAP43 immunoreactivity (stained brown) labels olfactory axons in the olfactory nerve (ON) and in the olfactory bulb primordium (OP).

(A) Parasagittal view of GAP43-labeled deep-penetrating olfactory axons in the ventricular zone of the OP at E14.

(B) GAP43 and BrdU double labeling on E14 parasagittal section 30 min following a single pulse of BrdU. BrdU immunoreactivity (black) labels proliferating cell nuclei. No GAP43-positive cells were found in the ventricular zone of the telencephalon.

(C and D) Pink basic fuschin–counterstained E14 OP and adjacent neocortex (Cortex), respectively, from an animal sacrificed 30 min after a single pulse of BrdU labeling. High magnification micrographs demonstrate that the OP has a thicker mantle layer compared with the adjacent neocortex region.

At the early time point illustrated here (30 min after a single BrdU pulse), 48% of the cells in the ventricular zone of the OP were labeled with BrdU versus 52% in the adjacent cortex. Following repeated injections of BrdU to produce cumulative labeling, the two regions shown in (C) and (D) were analyzed by the model of Nowakowski to measure cell cycle kinetics and proliferation rates in the OP and adjacent cortex. The results of this analysis are summarized in Figure 4. In the case shown here, the proliferation rate, which is the ratio of BrdU-positive cells to the whole population (ventricular zone plus mantle layer), is 27% in the OP versus 49% in the cortex. ML, mantle layer; VZ, ventricular zone. Bars, 30 μm (A and B), 20 μm (C and D).

cal changes in the olfactory bulb primordium were correlated spatially and temporally with the accumulation of pioneer olfactory axons at E14 (Figure 3C).

E15

By E15, the olfactory bulb primordium had evaginated to the degree that a recognizable olfactory bulb had formed. The olfactory bulb was significantly thicker than the adjacent cortex owing to its much thicker mantle layer. GAP43-positive olfactory axons contacted most of the surface of the newly formed olfactory bulb. However, a clear difference was that there were significantly fewer GAP43-positive fibers in the ventricular zone at E15 than at E14. The vast majority of the olfactory axons at E15 terminated on the surface and in the mantle layer of the olfactory bulb (Figure 1D). By E16 (data not shown), axons were virtually absent from the ventricular zone.

In summary, a small subpopulation of the early olfactory axons penetrate deep into the ventricular zone of the telencephalon where the germinal cells are located. Many more of these pioneer olfactory axons are present in the ventricular zone of the telencephalon at E14. At E15 (24 hr later), the olfactory bulb clearly bulges out of the telencephalon,

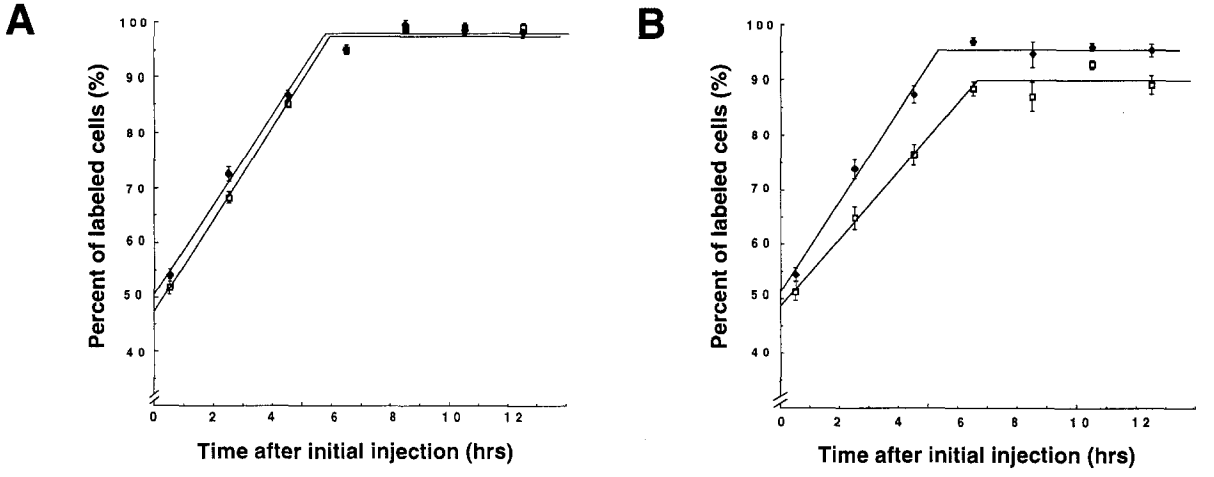

Figure 4. Comparison of Cell Cycle Kinetics in the Olfactory Primordium and Adjacent Cortex at E13 and E14

Cell cycle kinetics were determined by the cumulative BrdU labeling method of Nowakowski. At E13 (A), the cell cycle kinetics in the olfactory primordium and the adjacent neocortex were the same. The y-intercept is 47.5% versus 50.6%, and the slope is 8.4 versus 8.2 in the olfactory primordium and the adjacent neocortex, respectively. At E14 (B), the y-intercept is 48.8% versus 51.5%, and the slope is 6.1 versus 8.2 in the olfactory primordium and the adjacent neocortex, respectively. The cell cycle duration is longer and the growth fraction is lower in the olfactory primordium (open circles) than in the adjacent neocortex (closed circles).

and few, if any, deep-penetrating olfactory axons remain in the ventricular zone. These findings demonstrate that the presence of the pioneer olfactory axons in the ventricular zone correlates spatially and temporally with morphological induction of the olfactory bulb.

Cell Cycle Kinetics

Based on these findings, it seemed reasonable that the arrival and transient appearance of the pioneer olfactory axons might be associated with changes of cell cycle kinetics in the olfactory bulb primordium region, as local changes in cell cycle kinetics can have a significant effect on morphogenesis (Alberch and Alberch, 1981). Thus, we hypothesized that interactions between pioneer olfactory axons and germinal cells in the ventricular zone could influence the cytokinetics of the precursor population and thus induce the olfactory bulb.

To test this hypothesis, we measured the cell cycle parameters in the ventricular zone of the olfactory bulb primordium using a cumulative S-phase labeling method, prior and subsequent to the arrival of the pioneer olfactory axons. We reasoned that, if the pioneer olfactory axons influenced cell cycle kinetics, then the cell cycle parameters in the olfactory primordium and the adjacent neocortex should be similar at E13 and different at E14.

To test this prediction, it was necessary to distinguish between proliferating cells in the olfactory bulb primordium and the adjacent neocortex. To do this we developed a double labeling immunohistochemical procedure to visualize the pioneer olfactory axons and the dividing cells in the same histological section simultaneously. In addition, we needed to counterstain the section so that we could visualize the total population of the cells in the telencephalon. We used 2-bromo-5′-deoxyuridine (BrdU) to label proliferating cells. BrdU is incorporated into the DNA of the cells in S phase and thus labels proliferating cell nuclei. Pioneer olfactory axons were labeled with anti-GAP43. With this double label method, the pioneer axons are morphologically distinguishable from the nuclear staining of the proliferating cells, but to further distinguish pioneer axons and proliferating cells, we used a dual chromogen technique: BrdU staining was visualized by cobalt- and nickel-intensified 3,3′-diaminobenzidine (DAB) reactions, which appeared black, and GAP43 staining was visualized by the conventional DAB reaction, which is brown (Figure 3B). Finally, sections were counterstained with 0.1% basic fuschin, which is a light pink, and thus clearly distinguishable from the BrdU and GAP43 staining. With this triple labeling method, it was possible to count the BrdU-labeled cells and the total cell population in regions of the telencephalon with and without pioneer axons. Although the fuschin counterstain somewhat obscures the BrdU labeling in the photomicrograph shown (Figures 3C and 3D), the labeled cells were easily recognized and counted under the microscope.

At E13, cell cycle kinetics were indistinguishable in the ventricular zone of the olfactory bulb primordium and the adjacent neocortex (Figure 4A). The growth fraction (GF) in the ventricular zone of the olfactory bulb primordium was 97.7% ± 0.94% versus 97.9% ± 0.98% in the adjacent neocortex ($t = -0.13$, $df = 6$, $p > .05$). On the cell cycle kinetics curve, the y-intercept in the olfactory bulb primordium was 47.5% versus 50.6% in the adjacent cortex, and the slope of the olfactory primordium was 8.4 versus 8.2 in the adjacent neocortex. Therefore, the length of the cell cycle (Tc) was 11.6 hr in the olfactory bulb primordium versus 11.9 hr in the adjacent cortex. The length of the S phase (Ts) in the olfactory bulb primordium was 5.6 hr versus 6.2 hr in the adjacent cortex. Thus, at the time when the initial pioneer olfactory axons grow into the ventricular zone of the telencephalon, the cell cycle

parameters in the olfactory bulb primordium and the adjacent neocortex are identical.

By E14, the cell cycle kinetics in the olfactory bulb primordium and the adjacent neocortex were significantly different (Figure 4B). The GF in the olfactory bulb primordium was 89.5% ± 2.23% versus 95.8% ± 0.52% in the adjacent neocortex ($t = -3.75$, $df = 6$, $p < .001$). In the cell cycle kinetics graph, the y-intercept in the olfactory primordium was 48.8% versus 51.5% in the adjacent neocortex, and the slope in the olfactory primordium was 6.1 versus 8.2 in the adjacent neocortex. Therefore, Tc in the olfactory primordium was significantly longer (14.3 hr) than in the adjacent neocortex (11.7 hr). Ts in the olfactory bulb primordium was 8.0 hr versus 6.3 hr in the adjacent neocortex. Thus, 24 hr after the arrival of the pioneer olfactory axons in the ventricular zone, cell cycle parameters in the olfactory bulb primordium are altered significantly compared with the adjacent neocortex.

Cell Differentiation

At E14, the mantle layer of the olfactory primordium is significantly thicker than it is in the adjacent neocortex, suggesting that the pioneer axons may influence the production of postmitotic cells. This possibility cannot be assessed from cumulative labeling experiments alone, because the cell cycle kinetics analysis focuses only on the ventricular zone of the telencephalon. Therefore, we also analyzed the proliferation rate, i.e., the proportion of the proliferating cells in the total population of the cells in the ventricular zone plus the mantle layer of the olfactory primordium and the adjacent neocortex, after a single pulse of BrdU. We predicted that the presence of the pioneer olfactory axons would be correlated with increased differentiation of cells in the olfactory bulb primordium, i.e., that the proliferation rate would be lower in the olfactory bulb primordium compared with the adjacent neocortex.

To test this hypothesis, we gave a single pulse of BrdU to E14 timed pregnant rats and collected fetal tissues 0.5 hr after the pulse. The ratio of labeled to total number of cells in the olfactory bulb primordium is 25.4% ± 2.6%, compared with 50.2 ± 1.3% in the adjacent cortex ($p < 1 \times 10^{-7}$). Thus, approximately twice as many cells are postmitotic in the olfactory primordium as in the adjacent neocortex (Figures 3C and 3D). Most of the nonlabeled postmitotic cells are located in the mantle layer of the olfactory primordium. This suggests that the majority of these cells left the mitotic cycle and migrated from the ventricular zone into the mantle layer.

Discussion

The importance of the olfactory placode to the development of the olfactory bulb has long been suspected (Burr, 1924, 1930; Byrd and Burd, 1991, 1993; Kosciuszko, 1958; Stout and Graziadei, 1980), but there are conflicting notions about the specific role of olfactory axons. In the present experiments, we analyzed in detail the initial contact of the olfactory axons with the telencephalon and the dynamics of cell proliferation and differentiation in the telencephalon before and after the arrival of the earliest olfactory axons.

We used GAP43 to label early olfactory axons because this molecule is expressed in growing and regenerating axons and is particularly enriched in growth cones (Meiri et al., 1988; Skene, 1989; Skene et al., 1986; Skene and Willard, 1981a, 1981b). GAP43 is expressed strongly by immature olfactory receptor neuronal axons (Ramakers et al., 1992; Verhaagen et al., 1989, 1990). In our experiments, GAP43 identified olfactory axons at the early stages of the development and was expressed robustly by axons en route to and within the telencephalon. DiI tracing in fixed embryos confirmed observations made with GAP43 labeling.

Early Olfactory Axon Outgrowth

Early olfactory development in the rat can be considered as having four stages. First, olfactory axons grow out of the olfactory placode and follow a specific pathway to the telencephalon. Second, the olfactory axons fasciculate to form nerve bundles and make contact with the surface of the telencephalon. Third, a subpopulation of the olfactory axons (pioneer axons) penetrate into the ventricular zone of the olfactory bulb primordium, and the olfactory primordium starts to evaginate. Fourth, the pioneer olfactory axons disappear from the ventricular zone, and newly arriving olfactory axons stop in the mantle layer of the olfactory primordium. The olfactory primordium bulges out to form the olfactory bulb.

The trajectory of the early olfactory axons is surprising in that they do not take the most direct route to the rostral telencephalon. The axons emerge from the olfactory placode, which is located caudal and ventral to the olfactory primordium, and initially grow toward the closest region of the telencephalon, which is the primordium for the basal forebrain. However, as olfactory axons approach to within 50 μm of the telencephalon, they make a 90° turn and grow parallel to the surface of the telencephalon, toward the rostral telencephalon. This stereotyped trajectory was observed in every animal. Our previous study indicated that the low affinity nerve growth factor (NGF) receptor, which is considered an early marker for Schwann cells, is not expressed on the olfactory axon pathway at E12 (Gong et al., 1994). At E12–13, the low affinity NGF receptor is associated with cells flanking the developing olfactory nerve tract but not within the olfactory nerve bundles. Therefore, olfactory Schwann cells, at least as indicated by expression of the low affinity NGF receptor, may not provide the substrate for the initial olfactory axons. The cellular and molecular guidance cues used by early olfactory axons are unknown but merit further investigation, as these axons exhibit a highly directed trajectory.

A key observation of the present experiments was that a subset of the olfactory axons penetrate into the ventricular zone of the telencephalon at E13–14. These deep-penetrating olfactory axons were first seen in the classic ultrastructural examination of Hinds (1972) and were confirmed recently by DiI labeling (Santacana et al., 1992). The present investigation disclosed several features that distinguish these deep-penetrating axons from other olfac-

tory axons. First, they are a small subset of the olfactory axons; at the same time that the deep-penetrating axons are observed, the vast majority of the olfactory axons remain at the surface of the telencephalon. Second, they penetrate to the ventricular zone in a highly restricted region of the telencephalon. Third, they are a transient population; they are present in the ventricular zone at E13–14 and are not seen after E15–16. Fourth, the appearance of these axons is correlated spatially and temporally with the morphological induction of the olfactory bulb. Based on their unusual growth pattern and unique transient appearance, we refer to these axons as pioneer olfactory axons. To date, we have not found any molecular signature that distinguishes these pioneer axons from the rest of the olfactory nerve fibers.

In the mature olfactory system, olfactory axons terminate in the glomerular layer, which is the most superficial neuronal layer of the olfactory bulb. Olfactory axons synaptically terminate on periglomerular neurons and the mitral and tufted cells whose dendrites extend into the glomeruli and whose cell bodies are deeper in the olfactory bulb (Pinching and Powell, 1971; Willey, 1973). Although penetrating olfactory axons are observed deep into the glomerular layer during late embryonic and early postnatal development, these olfactory axons never penetrate as deep as the proliferative zone of the developing olfactory bulb (Monti-Graziadei et al., 1980; Santacana et al., 1992). It has been suggested that the early deep-penetrating olfactory axons are unable to form synapses and thus overgrow their future target as "exuberant" projections (Onoda, 1992). However, preliminary results from this laboratory indicate that synaptic vesicles are present in the terminals of the pioneer olfactory axons (Shipley et al., 1993, Soc. Neurosci., abstract). Whether pioneer olfactory axons form transient synapses with telencephalic cells is currently being examined by electron microscopic studies.

The present evidence suggests the possibility that the pioneer olfactory axons are not simply exuberant fibers that overgrow their targets. At this stage (E13–14), most of the olfactory axons terminate on the surface of the telencephalon. The growth of the pioneer axons into the ventricular zone at E13 occurs prior to the evagination of the olfactory bulb and prior to the birthdate of the mitral cells, which is around E14 in the rat (Hinds, 1968). Thus, the pioneer olfactory axons are present in the proliferative zone 24 hr prior to the morphogenesis of the olfactory bulb and the birth of the first olfactory bulb neurons. Based on these considerations, we reasoned that the pioneer axons might constitute a special subset of axons that function to contact, and perhaps influence, the germinal cells of the ventricular zone prior to the induction of the olfactory bulb from the olfactory primordium. Consistent with this, the olfactory bulb forms at E15, 24–48 hr after the appearance of the pioneer olfactory axons in the ventricular zone.

Cell Cycle Kinetics and Cell Differentiation

To assess the role of pioneer axons on the induction of the olfactory bulb, we analyzed telencephalic cell cycle kinetics and cell differentiation, before and after the arrival of the pioneer axons. Our results demonstrated a close spatial and temporal correlation of the pioneer olfactory axons with significant changes in the cell cycle kinetics of the cells, specifically in the olfactory bulb primordium.

The morphological differentiation of the olfactory bulb primordium from the neocortex may be directly related to these regional changes in cell cycle kinetics. GF and Tc are critical determinants of the proliferation output of the ventricular epithelium during neocortical histogenesis (Takahashi et al., 1993). Even slight changes of GF and Tc will significantly alter the number of cells in a given area and, therefore, determine the morphology of the different regions of the CNS (Jacobson, 1991). Thus, we determined GF and Tc in the olfactory primordium and the adjacent neocortex. At E13, when the neocortex and the olfactory bulb primordium are morphologically indistinguishable, GF and Tc are the same in these two zones. However, when the olfactory bulb primordium begins to evaginate at E14, GF and Tc are significantly different in these two compartments: Tc in the ventricular zone of the olfactory bulb primordium was significantly longer, and GF was significantly lower than that in the adjacent neocortex. Therefore, the cells in the olfactory bulb primordium proliferate more slowly than those in the neocortical region. This differential proliferation is coincident with the bulging of the olfactory bulb. A similar phenomenon was found during segmentation of the developing hindbrain, in which differential proliferation along the neural tube results in neuroepithelium bulging in different directions to form rhombomeres (Lumsden and Keynes, 1989). Thus, olfactory bulb induction might be the result of differential cytokinetics in the olfactory bulb primordium and the rest of the developing cerebral hemisphere.

By E15, few olfactory axons remain in the proliferative zone of the olfactory bulb. Hence, it is unlikely that the pioneer axons *continue* to exert an influence on proliferation in the bulb. Thus, the role of the pioneer axons may be to *trigger* changes in the cell cycle and/or differentiation of olfactory bulb precursor cells; these changes may subsequently be regulated by other factors such as cell–cell interactions among newly differentiated olfactory bulb cells.

We also determined whether there were differences in cell differentiation in the olfactory primordium and the adjacent cortex by measuring proliferation rates. Our results demonstrated that, in the 24 hr following the arrival of the first pioneer axons, the proportion of proliferating cells in the olfactory epithelium falls to half that in the adjacent neocortex. This indicates that twice as many cells exit the mitotic cycle and begin to differentiate and migrate out of the ventricular zone in the olfactory bulb primordium as do in the adjacent cortex. These postmitotic cells form the mantle layer, which is thicker in the olfactory bulb primordium than in the adjacent neocortex at E14. The thickening of the mantle layer locally increases the radial growth of the hemisphere and contributes to the bulging of the olfactory bulb primordium.

Morphogenesis of the Olfactory Bulb

The observation that the cell cycle lengthens in the olfactory bulb primordium at the same time that the primordium

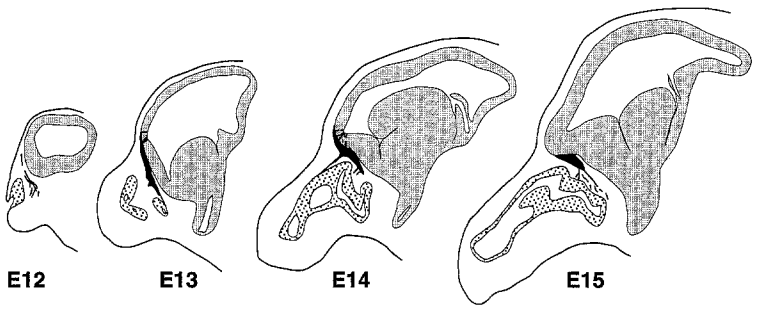

Figure 5. Growth of the Telencephalon and Morphogenesis of the Olfactory Bulb

At E12 and E13, the thickness of the telencephalic vesicle is uniform. The expansion of the telencephalon is rapid and uniform. At E14, the olfactory bulb primordium is thicker compared with adjacent neocortex, which continues to expand rapidly. At E15, the thickness of the olfactory bulb is more obvious compared with adjacent neocortex. The olfactory bulb primordium does not expand as much as the rest of the telencephalon. Dotted area indicates olfactory epithelium. The black area indicates the olfactory nerve, and the shaded area is the brain.

evaginates may be counter-intuitive. Bulging out seems to imply that a higher rather than lower mitotic rate is required. However, as the following analysis indicates, localized, differential rates of mitosis and/or differentiation can cause significant changes in morphology that are not immediately obvious.

At E13, the cell cycle is approximately uniform throughout most of the hemisphere. As a result, the hemisphere expands uniformly. In addition, most of the dividing cells re-enter the mitotic cycle to divide again. Because of this, the mantle layer remains fairly thin, and the thickness of the expanding hemisphere remains relatively uniform. At this point, the pioneer olfactory axons grow into the ventricular zone of the olfactory primordium. If the cell cycle length were to shorten in the olfactory primordium compared with the adjacent neocortex, i.e., if the cells were to divide faster and if there were no change in the proportion of cells leaving the cell cycle, then the surface area of the primordium would expand faster than the rest of the hemisphere, but it would retain the same thickness. Conversely, if the cell cycle length were to increase, as was observed, then the surface of the primordium would expand less rapidly than the rest of the hemisphere. As can be seen in Figure 3, many more postmitotic cells migrate into the mantle layer. This should lead to an increase in the thickness of the olfactory primordium relative to the rest of the hemisphere, which was also observed (Figure 5). Together, the reduced cell cycle length plus the increased rate of differentiation leads to a slowing of the expansion (tangential growth) and an increase in the thickness (radial growth) of a restricted part of the expanding hemisphere (the olfactory bulb primordium). This results in the morphogenesis of the olfactory bulb. Viewed in this way, the evagination of the bulb is not the result of rapid growth in the primordium but a consequence of the rapid growth of the rest of the hemisphere.

Afferent Axons and Target Morphogenesis

Morphogenesis is a complex process that includes cell proliferation, differentiation, and migration as well as the formation of synapses to establish neural circuits. The rates of proliferation and differentiation of the cells in different regions of the CNS are critical for regional pattern formation. It has been suggested that heterochronic cell proliferation is a major factor in morphogenesis (Alberch and Alberch, 1981; Byrd and Burd, 1993). Segmentation during early hindbrain development is the result of differential proliferation in rhombomere mitotic zones (Lumsden, 1990; Lumsden and Keynes, 1989). The olfactory bulb is the product of regional differentiation of the rostral end of the telencephalon. The present findings suggest that differential proliferation and differentiation of telencephalic precursor cells play key roles in olfactory bulb morphogenesis.

Several studies suggest that afferent innervation influences the proliferation and differentiation of target neurons in the invertebrate CNS (Anderson et al., 1980; Baptista et al., 1990; Selleck and Steller, 1991). In the Drosophila visual system, innervation by the optic nerve stimulates target CNS neuron precursors to go through the G1-S phase of the cell cycle, and therefore, up-regulates neurogenesis. In insect appendages, the initial contact of the peripheral neuronal processes precedes the appearance of the first sensory neurons in the CNS (Bate, 1976; Edwards, 1977; Sanes et al., 1976). In the leech, a segmented hermaphrodite, two segmental CNS ganglia provide afferent and efferent innervation of the male genitalia; ablation of the genitalia early in embryogenesis prevents the birth of a subpopulation of neurons in those CNS ganglia (Baptista et al., 1990). The mechanisms by which afferent axons influence target neurogenesis in these invertebrates are unknown. Moreover, it is not known whether afferent axons influence proliferation and/or differentiation of target cells in vertebrate CNS development.

The present results are consistent with the possibility that olfactory axons influence proliferation and/or differentiation of target cells in the developing telencephalon. Morphogenesis of the olfactory bulb appears to be driven by differences in cell cycle kinetics and differentiation of daughter cells. These differences are correlated spatially and temporally with the arrival of pioneer olfactory axons. The coincidence of measured changes in cell cycle kinetics with the arrival of the pioneer olfactory axons suggests that pioneer olfactory axons may induce the olfactory bulb via diffusible or cell contact influences on proliferating cell populations. However, it is important to recognize that the present findings do not rule out the possibility that the observed changes in cell cycle kinetics are intrinsic to the olfactory primordium, and that the correlation of these changes with the arrival of the pioneer olfactory axons is coincidental. Indeed, it is possible that an intrinsically different program of proliferation and/or differentiation of

the olfactory bulb primordium distinguishes it from the adjacent neocortex and makes it an attractive target for the early olfactory axons. A final possibility is that the interaction of the pioneer olfactory axons and the olfactory primordium is mutual: the olfactory bulb primordium may be intrinsically different from the adjacent neocortex in the sense that it attracts the pioneer olfactory axons, but the presence of the pioneer olfactory axons in the ventricular zone may be necessary to trigger change in the cell cycle kinetics and cell fate to induce the formation of the olfactory bulb. We are currently using in vitro slice and explant cultures to investigate these possibilities.

In summary, olfactory axons penetrate into the ventricular zone of the telencephalon prior to the induction of the olfactory bulb and the birth of the earliest olfactory bulb neurons. Prior to the arrival of the pioneer axons, the cell cycle kinetics and the proliferation rates in the olfactory primordium and the adjacent neocortex are identical. However, 24 hr after the arrival of the pioneer axons, the cell cycle kinetics of the olfactory bulb primordium and the neocortex are significantly different. There is a longer duration of Tc and twice the number of postmitotic cells in the olfactory primordium. These factors may cause the induction of the olfactory bulb. In view of the suggestion that pioneer olfactory axons influence the production of olfactory bulb precursor cells, it may be worthwhile to consider the possibility that peripheral, and possibly even central, afferents also modulate the production and/or differentiation of precursors in other target structures. Modulation of precursor cells by early arriving afferents may be a common factor in neuro-morphogenesis.

Experimental Procedures

Animal Preparation

Timed pregnant Sprague–Dawley (Zivic Miller) rats were used in all the experiments. The presence of a vaginal plug was checked at 9 AM; E0 was defined as the day the plug was found. Embryos from ages E12–15 were used for morphological studies; approximately 80 embryos (E13–14) were used for the cell cycle kinetics analysis. Timed pregnant rats were anesthetized with sodium pentobarbital (71.5 mg/kg, intraperitoneally), and Cesarean sections were performed. The embryos were decapitated and immersion fixed overnight at 4°C in 4% paraformaldehyde made in 0.1 M phosphate buffer (pH 7.4). All tissues were cryoprotected in 30% sucrose for 48 hr (4°C) following postfixation.

Immunohistochemistry

Axons

The tissues were placed in embedding matrix (Lipshaw) and frozen in dry ice powder. Parasagittal cryostat sections (10 or 20 μm) were collected on chrome-alum-coated slides for immunocytochemistry processing. The sections were air dried for 10 min and postfixed on the slides in 4% paraformaldehyde for 10 min. The paraformaldehyde was washed out thoroughly after the fixation. To eliminate endogenous peroxidase staining, sections were incubated in 0.5% H_2O_2 for 10–15 min. Normal horse serum (2%) was used to block nonspecific staining. Sections were incubated in primary antibody overnight at 4°C. The mouse monoclonal antibody used to label growing olfactory axons is anti-GAP43. Anti-GAP43 (Boehringer and Mannheim) was used at a concentration of 1 μg/ml. The immunoreactivity was visualized with a biotinylated secondary antibody (horse anti-mouse, rat adsorbed, Vector Lab) and avidin/biotin–peroxidase complex (Vectastain Elite kit, Vector Labs) and reacted with DAB. Sections were dehydrated, cleared in Hemo-De, and coverslipped with DPX. The sections were examined by light- and dark-field microscopy.

Axons plus Proliferating Cells

To assess the possibility that the arrival of early olfactory axons correlated with changes of cell cycle kinetics in the olfactory primordium, it was necessary to visualize the olfactory axons, label the proliferating cells, and counterstain the total population of the cells, all in the same histological section. The procedure was to label the proliferating cells with BrdU, a thymidine analog, which is incorporated into DNA during the S phase of the cell cycle, to label the olfactory axons with anti-GAP43, and to counterstain the sections with basic fuschin. BrdU immunocytochemistry was performed before GAP43 staining. For the cytokinetics studies, 8 μm sections were cut. The sections were air dried, postfixed, and treated with H_2O_2 as described above. To facilitate the binding of the anti-BrdU antibody, sections were incubated in 2 N HCl for 45 min at 37°C to denature the DNA. The acid was rinsed out thoroughly with 0.1 M phosphate buffer saline (pH 7.4) before transferring sections into normal horse serum. Antibody against BrdU (mouse monoclonal; Boehringer and Mannheim) was used at 0.5 μg/ml. BrdU was visualized by biotinylated secondary antibodies (horse anti-mouse, rat adsorbed, Vector Lab) with an avidin/biotin–peroxidase complex and followed by DAB reaction. This reaction was intensified with 1% $CoCl_2$ and 1% $NiCl_2$ to produce a black reaction product. The sections were next rinsed with 0.1 M phosphate buffer, then incubated in 2% normal horse serum for 1 hr for GAP43 staining. The GAP43 immunocytochemistry procedure was as described above. Control experiments were done by omitting either one or both primary antibodies. Finally, the sections were counterstained with 1% basic fuschin to visualize all cells. With this double labeling strategy, there was no confusion of BrdU-labeled cells and GAP43-labeled axons: BrdU labeling consisted exclusively of small, black, round nuclei; GAP43 labeling consisted exclusively of long, thin, brown processes. Basic fuschin counterstaining was done by incubating the sections in 1% basic fuschin for 40 min at room temperature. Overstaining was differentiated by 50%, 70%, 95%, and 100% alcohol. The sections were then cleared in Hemo-De and coverslipped. Using this triple labeling method, we were able to visualize the proliferating cell nuclei with black BrdU staining, the olfactory axons with brown GAP43 reaction product, and all other cells with the pink fuschin staining.

DiI Injections and Photoconversion

E14 embryos were fixed by immersion with 4% paraformaldehyde as described above. The embryos were stored in fixative for several days or weeks. Fast DiI (Molecular Probes) was injected into the nasal cavity of embryos using a 1 ml syringe attached to a pressure injection system. Multiple injections were made for a total of 300–500 nl of Fast DiI. The embryos were then stored in fixative, in the dark at room temperature, for at least 3 weeks. Longer diffusion periods did not weaken the fluorescence.

E14 embryos were cryoprotected in 30% sucrose and cut on the cryostat at 50 or 60 μm. The sections were dried on the slides and washed with 0.1 M Tris buffer (pH 8.2). After confirming the presence of fluorescent axons in the telencephalon, the fluorescent DiI was photoconverted using a modified protocol from Sandell and Masland (1988). Several drops of DAB solution (1.5 mg of DAB per milliliter of Tris buffer) were placed on the section. The section was viewed with a Leitz epifluorescence microscope using a 25× immersion objective and rhodamine filters with a 100 W mercury arc lamp power source to maximize the fluorescent signal. DAB, kept cold, was changed every 10 min for a total of 30–40 min. Sections were rinsed with Tris buffer and then phosphate buffer, dehydrated, and coverslipped with Permount. These sections were analyzed with differential interference contrast microscopy, and reconstructions were made by image analysis.

Cell Cycle Kinetics Analysis

To analyze the cell cycle kinetics for the olfactory bulb and neocortex, we used the cumulative S-phase labeling method established by Nowakowski (1989). This analysis assumes that there is asynchronous homogenous proliferation in the ventricular zone of the telencephalon, and that the population growth is at steady-state. Following BrdU injection into timed pregnant rats, the proliferating cells in S phase take up BrdU and incorporate it into their DNA as thymidine. Multiple exposures of BrdU at regular intervals should label all the cells passing through S phase. If the labeling duration is significantly longer than

the duration of S phase, saturation is reached and all the proliferating cells in the ventricular zone are labeled by BrdU.

All cumulative labeling experiments started at 9 AM. Timed pregnant rats (E13 and E14) were injected with BrdU (50 mg/kg body weight) at 2 hr intervals up to 12 hr, i.e., 9 AM, 11 AM, 1 PM, 3 PM, 5 PM, 7 PM, and 9 PM. From previous studies of neocortical development of mice, the duration of the S phase was 3–6 hr at comparable embryonic ages (Caviness et al., 1991, Soc. Neurosci., abstract; Takahashi et al., 1993).

Embryos from at least two litters were collected 0.5 hr after each BrdU injection. Immunocytochemistry was performed with anti-BrdU and anti-GAP43 antibodies on 8 μm serial sections to visualize the proliferating cells and the growing olfactory axons as above. We defined the olfactory primordium as the zone containing deep-penetrating olfactory axons. The adjacent region, 200 μm rostrodorsal to the olfactory primordium, was examined to determine the cell cycle parameters in the neocortex, which is not contacted by olfactory axons. The areas examined were 1600 μm^2 for both the olfactory primordium and the adjacent neocortex. The proportions of BrdU-labeled and non-labeled cells in the olfactory epithelium and the adjacent cortex were counted in several nonadjacent sections from each animal. At least 4 embryos from two litters were analyzed at each time point for cell cycle kinetics study. Approximately 86,400 nuclei were counted.

Data were analyzed by plotting the labeling index (LI) as a function of time after the initial injection. The LI is the ratio of proliferating cells to the total population. According to Nowakowski's method, the LI at the first BrdU injection is $LI_0 = GF \times Ts/Tc$, where GF is the growth fraction, Tc is the duration of the cell cycle, and Ts is the length of the S phase. The linear increase in the proportion of labeled cells as a function of time after the initial exposure to BrdU is $f(t) = GF/Tc \times t + LI_0$. The rate of increase (i.e., the slope) of the proportion of labeled cells is GF/Tc. Data were least-square fitted to a curve by linear regression. The slope and the y-intercept were used to calculate Tc, Ts, and GF for the olfactory bulb primordium and the adjacent neocortex.

As it is possible that high dosages of BrdU are toxic (Bannigan, 1985, 1987), the amount BrdU used here was the lowest dose (50 mg/kg) used by other laboratories for cell cycle kinetics studies (Miller and Nowakowski, 1988; Nowakowski et al., 1989; Takahashi et al., 1992). In addition, we independently assessed potential cytotoxicity of this BrdU dosage with long-term labeling. BrdU was injected into E14 timed pregnant rats; at postnatal day 30, the distribution of the BrdU-labeled nuclei in the olfactory bulb was as expected: the majority of the BrdU-positive nuclei were located in the mitral cell layer (data not shown). Therefore, the amount of BrdU used in our experiments did not appear to have a substantial effect on neuronal proliferation, migration, differentiation, or survival. However, as we cumulatively labeled the proliferating cells with BrdU for 12 hr at 2 hr intervals in our cell cycle kinetics studies, there could have been some cumulative effect of BrdU. If the S phase of the proliferating cells were longer than 2 hr, cells passing through S phase could be labeled twice, and these cells might be more prone to BrdU cytotoxicity. Thus, we closely examined tissue sections from the cumulative labeling experiments but found no evidence of pyknotic cells either in the olfactory bulb primordium or in the adjacent neocortex. Furthermore, even if the BrdU did influence cell cycle kinetics, any effect should be the same in the olfactory primordium and the adjacent neocortex and could not obviously account for the observed differences in cell cycle kinetics in the two adjacent telencephalic compartments. Therefore, at the dose used, potential cytotoxic effects of BrdU probably did not influence our findings.

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