Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 5489–5493, July 1990
Neurobiology

Monoclonal antibody R2D5 reveals midsagittal radial glial system in postnatally developing and adult brainstem

(raphe nuclei/tanycytes/cerebrospinal fluid)

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Contributed by Osamu Hayaishi, May 11, 1990

ABSTRACT **Radial glial cells and their processes play critical roles in organizing the spatial arrangement of the nervous system in the embryonic brain. It has been thought that following completion of their roles in the embryo, most of the radial glial processes disappear before or shortly after birth. Here we use R2D5, a monoclonal antibody to a soluble cytosolic protein, to demonstrate that a specific system of midsagittal radial glial cells persists in postnatal and adult brain. In the brainstem of postnatally developing and adult rabbits and cats, the R2D5-positive processes of radial glial cells were observed to be arranged in a precisely parallel array at the midsagittal seam. These radial glial processes formed a continuous palisade separating the right and left brainstem. In early postnatal animals, R2D5-positive radial processes were found to reach the pial surface and to cover the entire midsagittal seam of the brainstem. These processes embraced dendrites and somata of neurons in almost all of the midsagittal nuclei, including the raphe nuclei, suggesting that the radial glial cells may interact with the midsagittal groups of neurons. In addition, the palisade of R2D5-positive radial processes formed loose openings for crossing axonal bundles at the midline decussations of fiber tracts. In more mature brains, somata of R2D5-positive radial glial cells that had migrated ventrally were observed within the palisades, and in adult cats, most of the R2D5-positive radial processes were found to have retracted from the ventral parts of the midsagittal seam. The spatial arrangement of R2D5-positive processes suggests that they may have persistent functional roles as an interface between ventricular humoral signals and midsagittal groups of neurons in the postnatally developing brainstem and in the adult brainstem. The structure of the midline glial system suggests also that it plays a role in organizing the spatial arrangement of decussating axons during development.**

Radial glial cells are process-bearing ependymal cells lining the surface of brain ventricles and the central canal of the spinal cord. These are the first class of glial cells to appear during embryonic development and they are thought to play critical roles in organizing the spatial arrangement of the nervous system (1, 2). In lower vertebrates, for example, radial processes of ependymal cells have been reported to form channels for growing axons during both regeneration and embryonic development and thus have been suggested to display on their surfaces trace pathways that the axons follow toward their destination (3, 4). In the mammalian embryo, ependymal cells project their processes (radial glial fibers) to almost all regions of the brain and are thought to supply pathways guiding the migration of newly generated neurons (1, 5). Most of these mammalian radial glial cells disappear before birth (5) and process-bearing ependymal cells (tanycytes) have been reported to be present only in restricted portions of the adult brain (6).

Using an antibody to S-100 protein, Van Hartesveldt *et al.* (7) found a massive radial glial structure distributed in the midline raphe of the midbrain, hindbrain, and cervical spinal cord of newborn rat. This midline radial glial structure was thought to be transient and to regress because the immunoreactivity to S-100 begins to disappear at postnatal day 5 (P5) and is no longer visible by P7 and P8 (7). We demonstrate here using monoclonal antibody (mAb) R2D5 that the midline radial glial structure persists in postnatally developing and adult brain and that it undergoes a structural change during postnatal development. Since mAb R2D5 labels even fine processes of radial glial cells, it enabled us to examine in detail the distribution and spatial arrangement of the radial glial processes in the whole brain and spinal cord of rabbits and cats. We report here a characteristic organization of the R2D5-positive radial glial system in the brainstem and describe its relation to midsagittal nuclear structures (e.g., raphe nuclei) and axonal decussations.

MATERIALS AND METHODS

mAb. The procedure for production of the hybridoma secreting mAb R2D5 has been described (8, 9). R2D5 is a mouse IgG1 and binds to a soluble cytosolic protein in olfactory receptor cells and ependymal cells in rabbits (9). R2D5 reacts also with olfactory receptor cells of cats, but not with those of rats.

Immunohistochemistry. Ten rabbits (Japanese White, ranging in age from P2 to adult) and six cats (from P8 to adult) were deeply anesthetized with urethane (>1.5 g/kg) and sodium pentobarbital (Nembutal, >50 mg/kg), respectively. The animals were then perfused via the left ventricle, first with isotonic saline and then with 10% formalin in 0.07 M sodium phosphate buffer (pH 7.4). Brains and spinal cords were removed, postfixed for about 10 hr in the same fixative, and then kept in cold (4°C) 0.07 M phosphate buffer (pH 7.4) containing 30% sucrose. Coronal or sagittal sections of the brainstem (40 μm in thickness) were cut using a freezing microtome.

For immunofluorescence staining, sections were incubated overnight (at 4°C) with undiluted hybridoma culture supernatant and then washed for 20 min with 10 mM sodium phosphate-buffered saline (P_i/NaCl, pH 7.4). They were then incubated for 20 min with fluorescein-conjugated goat anti-mouse IgG (reactive with both heavy and light chains; Cappel Laboratories) diluted 1:70 in P_i/NaCl. Sections were washed with P_i/NaCl, mounted on gelatinized glass slides, and coverslipped with 90% glycerol.

For immunoperoxidase staining, sections were incubated for 3 hr at room temperature or overnight at 4°C in hybridoma culture supernatant. Sections were washed in P_i/NaCl and

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Abbreviations: mAb, monoclonal antibody; P*n*, postnatal day *n*.

processed with the avidin–biotin-peroxidase complex kit (Vector Laboratories). The peroxidase was visualized by immersing the sections for 10–15 min in 0.07% 3,3′-diaminobenzidine tetrahydrochloride solution containing 0.005% H_2O_2.

For immunofluorescence and immunoperoxidase procedures, control sections were treated as described above, except that hybridoma culture supernatant was omitted or replaced with myeloma culture supernatant. No staining was observed under these conditions.

RESULTS

In the medulla and pons of postnatal (P2–P30) rabbits and cats, mAb R2D5 intensively labeled an array of radially oriented glial processes extending outward from the midline surface of the fourth ventricle. The R2D5-positive radial glial processes ran in parallel with one another and formed a relatively continuous palisade at the midsagittal seam (Fig. 1). The midline palisade was flanked by a number of blood vessels (indicated by arrows with an asterisk in Fig. 1) running in parallel with the R2D5-positive processes. In the central and ventral parts of the medulla, the palisade wound sinusoidally in a mediolateral direction. The palisade of R2D5-positive processes extended caudally to the floor plate region of the spinal cord.

Fig. 2 shows in detail the distribution of R2D5-positive radial glial processes in coronal sections through several representative portions of the brainstem of P8 cat. In the medulla and pons (Fig. 2*A*), R2D5-positive somata of ependymal cells at or near the midline of the ventricular surface gave off ventrally directed radial processes. The R2D5-positive processes gathered closely in a narrow (about 60 μm wide) midline region and formed a dense palisade. A large number of R2D5-positive processes extended to the pial surface of the medulla and pons and terminated as endfeet (Fig. 2 *D* and *E*). Nissl staining of the sections showed that the neuronal somata of the raphe nuclei (raphe pallidus, raphe obscurus, raphe magnus, and raphe pontis) were distributed mostly within the palisade of the R2D5-positive processes. In cats, but not in rabbits, a subset of neurons in these raphe nuclei were also labeled by mAb R2D5 (Fig. 2*E*).

In the midbrain of P8 cat, R2D5-positive radial glial processes extended from the ependymal layer of the cerebral aqueduct in virtually all directions (Fig. 2 *B* and *C*) and were distributed principally within the periaqueductal central gray. In addition, two different midsagittal palisades of R2D5-positive processes were observed. One was distributed in the midline dorsal to the cerebral aqueduct and formed a rostrocaudally extended boundary between the right and left colliculi (Fig. 2*B*). Within this palisade, ependymal cells at the dorsal midline of the aqueduct extended R2D5-positive processes to the dorsal surface of the midbrain. The other was distributed in the ventral midline and was continuous with the palisade of the pons. The ventral palisade of the midbrain was relatively wide and was formed by an aggregation of ventrally directed R2D5-positive processes (Fig. 2*C*). R2D5-positive processes of the ventral palisade extended into the dorsal raphe nucleus, median raphe nucleus, Edinger–Westphal nucleus, and interpeduncular nucleus. In the midline region of cat midbrain, mAb R2D5 labeled also a subset of neurons in the raphe nuclei and in the oculomotor nucleus (data not shown).

A number of axonal tracts decussate at the midline of the brainstem (10). Immunohistochemical examination with mAb R2D5 demonstrated that the palisade of R2D5-positive processes formed loose openings for decussating axonal bundles. At the pyramidal decussation (Fig. 2*F*), for example, the midline palisade of R2D5-positive radial processes bulged out and formed a number of large meshes. The bundles of pyramidal tract fibers passed through these meshes to cross the midline. Similar bulgings of the palisade were observed in

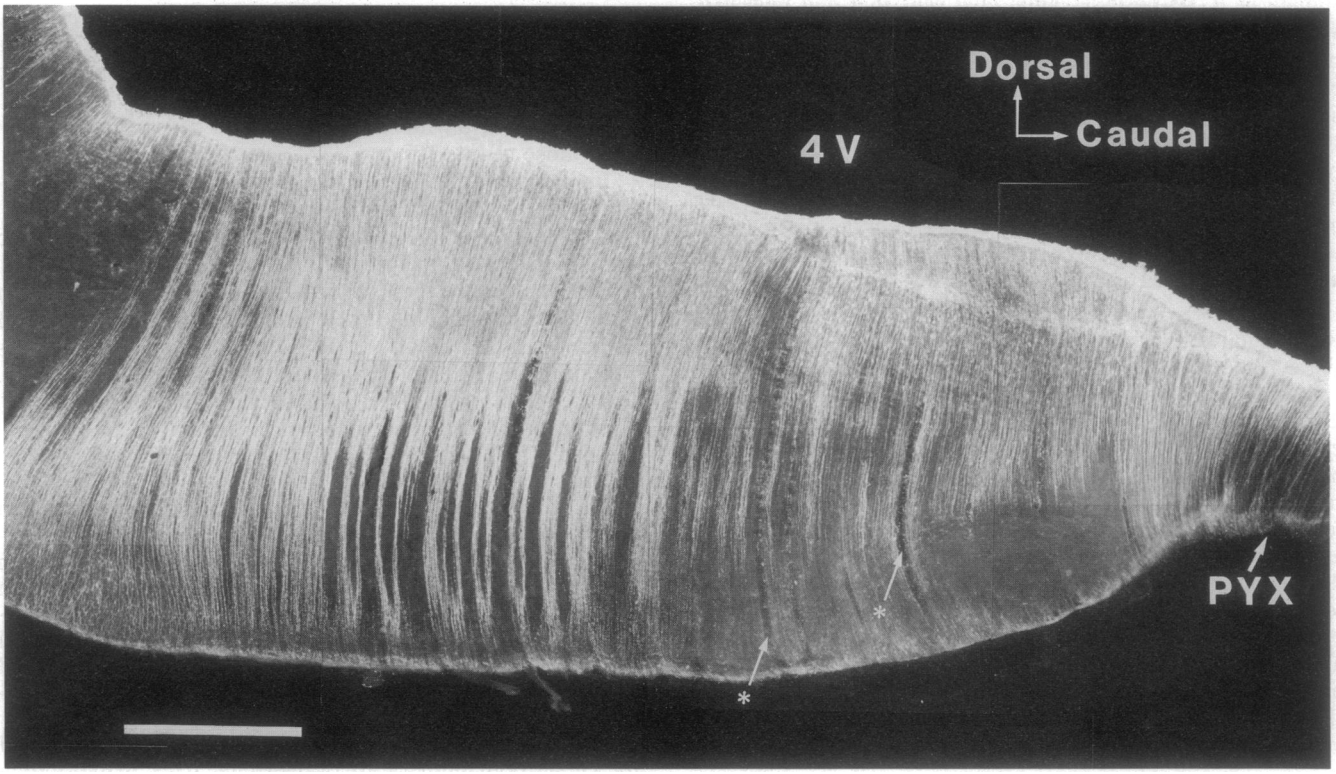

FIG. 1. Immunofluorescence staining with mAb R2D5 of a midsagittal section through the brainstem in P11 rabbit. Thin arrows with asterisk indicate large blood vessels running in parallel with R2D5-positive radial glial processes. The cerebellum has been removed. PYX, pyramidal decussation; 4V, fourth ventricle. (Bar = 1 mm.)

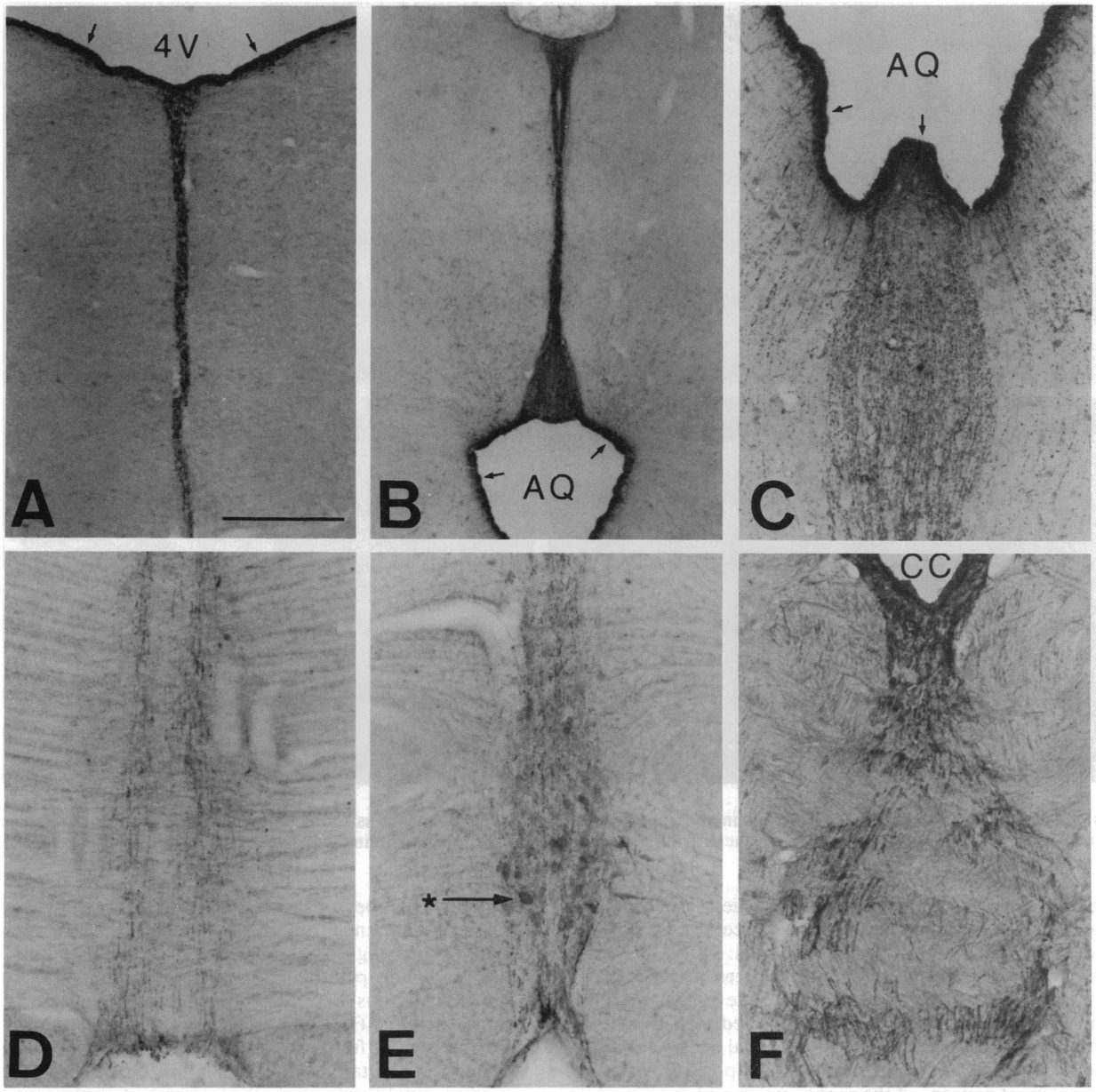

FIG. 2. Immunoperoxidase staining with mAb R2D5 of coronal sections through the brainstem of P8 cat. (*A*) Section through the dorsal part of the medulla showing R2D5-positive ependymal cells (arrows) lying at the surface of fourth ventricle (4V) and dense aggregations of R2D5-positive radial fibers at the midline seam. (*B* and *C*) Sections through the dorsal midline (*B*) and ventral midline (*C*) regions of the midbrain. R2D5-positive ependymal cells (arrows) covered the surface of the cerebral aqueduct (AQ). (*D* and *E*) Sections through ventral midline regions of the medulla demonstrating relatively sparse aggregation of R2D5-positive processes at the midline of the trapezoid body (*D*) and R2D5-positive raphe neurons (arrow with a star) distributed within the palisade (*E*). (*F*) Coronal section through the pyramidal decussation. CC, central canal. (Bar = 500 μm in *A* and *B* and 200 μm in *C–F*.)

the regions of the posterior commissure, the commissure of the inferior colliculus, and the decussation of the brachium conjunctivum.

Fig. 3 shows structural changes of the midsagittal radial glial system occurring during the postnatal development of the cat medulla. At P8, the somata of radial glial cells were distributed in or near the ventricular surface, and their palisade consisted mostly of radial processes. At P21 and P35, a large number of glial cell bodies were observed within the palisade (Fig. 3*B*), and the number of radial fibers reaching up to the ventral surface had decreased. In adult cat, R2D5-positive radial glial fibers were restricted to the dorsal and middle parts of the midline seam, and only occasional R2D5-positive processes were observed in the ventral parts of the medulla. A similar migration of R2D5-positive somata of radial glial cells during postnatal development was observed in rabbits. However, even in adult rabbits many R2D5-positive radial glial fibers projected to the pial surface. Besides the brainstem radial glial system, mAb R2D5 labeled a subset of astrocytes, epithelial cells of the choroid plexus, and various types of ependymal cells, including third-ventricle tanycytes, in adult rabbit and cat brains.

DISCUSSION

The raphe, or midsagittal seam, of the mammalian brainstem forms a characteristic unpaired structure composed of midline groups of neurons (the system of raphe nuclei, ref. 11) and a number of decussations of axonal tracts (10). In the present study, we have used mAb R2D5 to show that the

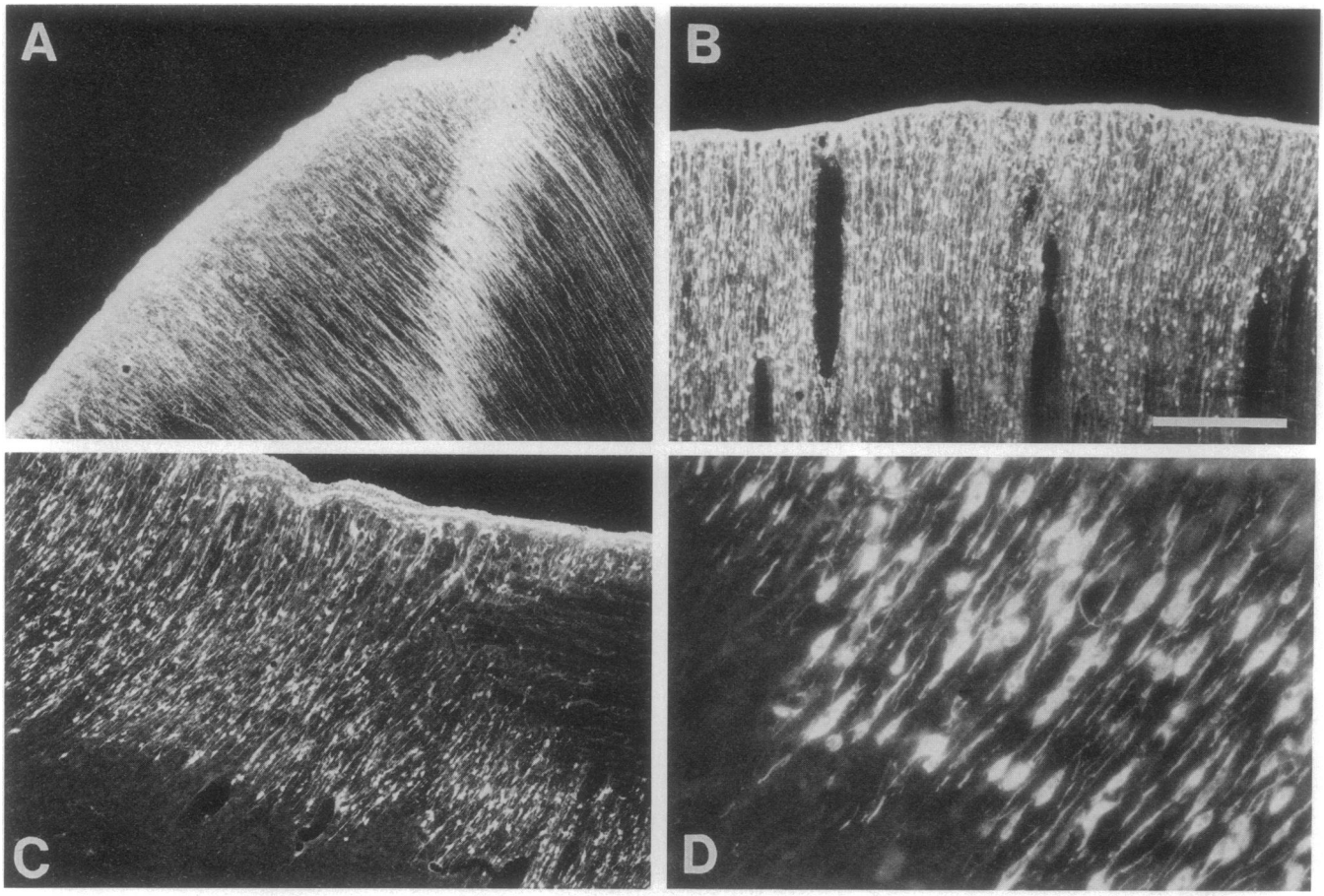

FIG. 3. Changes in the structure of the midline palisade during postnatal development as shown by midsagittal sections of the medulla of P8 (*A*), P35 (*B*), and adult (*C* and *D*) cat. Note the distribution of R2D5-positive somata within the palisade of R2D5-positive processes in *B–D*. (Bar = 500 μm in *A–C* and 100 μm in *D*.)

midsagittal seam has another major constituent, a continuous palisade of R2D5-positive radial glial processes, in postnatally developing and adult rabbits and cats.

Possible Communication Channel Between Cerebrospinal Fluid and Midline Groups of Neurons. The raphe neuronal system extends from the level of the interpeduncular nucleus in the midbrain to the level of the pyramidal decussation (6). In the brainstem of P8 cats, the R2D5-positive palisade embraced most neurons of the raphe nuclei, and even in adult cat, a large number of neurons in the dorsal parts of the raphe neuronal system were located within the palisade of the R2D5-positive processes. In Golgi studies of developing and adult rabbit brainstem, Felten and coworkers (12–14) found that raphe nuclei neurons frequently abutted radial processes of tanycytes (which presumably correspond to R2D5-positive radial processes) lining the floor of the fourth ventricle, and suggested that these tanycytes constitute a communication channel between the cerebrospinal fluid of the fourth ventricle and medullary raphe neurons. However, with the Golgi method, which randomly labels a small number of cells, it is difficult to examine the spatial arrangement of the entire midline radial glial system. The present finding of the continuous palisade of R2D5-positive processes covering extensive regions of the midline seam suggests that this radial glial system provides an effective means for chemical substances released in the cerebrospinal fluid to exert widespread effects simultaneously on various neuronal functions mediated by the midline groups of neurons. Neurons in the raphe nuclei have been implicated in diverse functions including the regulation of sleep–wakefulness, control of pain, locomotor activity, and control of neuroendocrine activity (15–17). When injected intaventricularly or in the periventricular region, a number of molecules have been shown to exert powerful influences on these functions. These molecules include prostaglandins D_2 and E_2 (18, 19) and delta-sleep-inducing peptide (20), regulating sleep–wakefulness, and endogenous opioid peptides, modulating pain-related neural activity (17). It is tempting to speculate that these molecules have functional roles requiring signal transmission through the putative communication channel between the cerebrospinal fluid and midline groups of neurons.

Axonal Decussations. Another interesting feature of the midsagittal palisade of the R2D5-positive processes is that it forms a continuous wall-like structure separating right and left brainstem and a number of gate-like openings for decussating axons. These structures are striking in view of the radial glial guidance of axonal projection (4). Snow *et al.* (21) indicated that during development, cells at the roof plate of the spinal cord and the dorsal midline seam of the midbrain form a wall that inhibits growing axons from crossing the dorsal midline. Since the palisades of R2D5-positive radial processes are located in these dorsal midline regions, the R2D5-positive processes may compose the "wall" in these regions. The structure of the palisade, including a dense aggregation of R2D5-positive processes at the ventral midline seam of the brainstem, suggests that the ventral midline seam also forms a wall inhibiting axons from crossing the ventral midline.

In both the dorsal and ventral midline seams, the palisade of R2D5-positive processes formed mesh-like openings in specific regions, through which decussating axons crossed the midline. Cells in the embryonic spinal cord at the ventral midline seam, the floor plate, have been shown to secrete a diffusible substance(s) that guides axons of commissural

neurons toward the floor plate (22). It has been suggested that cells in the floor plate interact with the decussating axons of commissural neurons and control the expression of commissural axon surface glycoproteins TAG1 and L1 (23, 24). The structure and the strategic position of the R2D5-positive dorsal and ventral palisades seem to be advantageous for guiding the decussation of growing axons at specific positions of the midline of the brainstem.

Topographic Arrangements of the Radial Glial System. The parallel trajectory of R2D5-positive processes within the midsagittal plane of the brainstem (cf. Fig. 1) clearly demonstrates a topographic relationship between the radial glial processes and various structures, including the raphe nuclei and axonal decussations, located within the midline seam. Structures located at different places in the rostrocaudal axis receive projections from different groups of radial glial cells. For example, the pyramidal decussation received R2D5-positive radial processes from a specific group of ependymal cells lying at the surface of the most rostral part of the central canal (Fig. 2*F*). Neurons in the dorsal raphe nucleus received radial processes selectively from those ependymal cells that were distributed at the ventral midline of the cerebral aqueduct (Fig. 2*C*). It is thought that in the developing cerebral cortex, radial glial fibers provide a scaffold for migration of neurons and specify the spatial arrangement of somata and dendrites of cortical neurons (1, 2, 25). Similarly, the midline radial glial processes in the brainstem may serve as guides for migration of midline groups of neurons and for their spatial arrangement in the midsagittal seam (26). The midline radial glial system may also provide a scaffold not only for instructing growing axons to decussate at specific positions but also for reorganizing transient axonal projections during embryonic and postnatal development (27). In contrast to most other radial glial fibers in the central nervous system, which disappear before or shortly after birth (5), the midline radial glial system persists in the postnatally developing and in the adult brain, although undergoing structural changes during development. This suggests that the midsagittal radial glial system has functions required in postnatal and adult brain as well as in embryonic brain. Use of mAb R2D5 and characterization of the R2D5 protein may provide insights into the interactions of the radial glia and neurons at the midline seam of the brainstem.

We thank Dr. M. Connolly and Dr. K. Imamura for reading the manuscript. This work was supported in part by Special Coordination Funds from the Science and Technology Agency of the Japanese Government.

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