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Temporal Pattern of Neurogenesis in Spinal Cord: Cytoarchitecture and Directed Growth of Axons

(rat/neuroepithelium/autoradiography/selective fasciculation)

HOWARD O. NORNES* AND GOPAL D. DAS

Department of Biological Sciences, Purdue University, Lafayette, Indiana 47907

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ABSTRACT **Neurogenesis was studied in rat spinal cord by correlation of the observations from [^{3}H]thymidine autoradiography and Golgi techniques. The temporal pattern of the origin of neurons systematically lagged along the ventro-dorsal and rostro-caudal axes. The temporal pattern was correlated with the topographic pattern of the release and segregation of the newly formed neuroblasts from the neuroepithelium. These temporal and topographic patterns of neuroblast genesis imparted an order to the settling of the neuroblasts. Further, the emerging cytoarchitecture of the differentiating neuroblasts resulted in the formation of oriented interfaces along which the axons grew. The "pioneering" fibers grew along the interfaces formed by the regressed neuroepithelium and the assembled neuroblasts. As development progressed, the axons formed later "selectively fasciculated" along the "pioneering" fibers in a staggered pattern as a result of their differential times of origin. These temporal and topographic patterns occurred in a manner such that the preexisting structure served as an "organizing structure" or foundation upon which the axonal processes formed later in the developmental sequence were oriented and assembled.**

In earlier studies of the development of spinal cord at gross, as well as at cytological, levels (1–4), the emergence of cytoarchitecture and directed growth of axons have not been adequately treated. In the present investigation, some of these problems have been analyzed in rat spinal cord.

MATERIALS AND METHODS

Time of Origin. At least one pregnant albino rat (Holzman) for each day between days 9 and 21 of gestation was given a single intraperitoneal injection of [^{3}H]thymidine. (5 μCi/g of body weight; 6.7 Ci/mmol; 1 mCi dissolved in 1 ml of isotonic saline). The day after a 12-hr mating period was considered day 1 of gestation. The litters from these animals were allowed to develop to 6 weeks of age; the pups were then deeply anesthetized and perfused with 10% neutral formalin. The perfused spinal cord was removed, blocked, embedded in Paraplast, cut serially at 10-μm thickness, processed through the autoradiographic dipping and exposure process (5), and poststained with cresyl-violet. The labeled neurons were plotted on graph paper with the aid of an ocular grid. From the cytological viewpoint, the neurons were classified into large-sized (30 μm or more in diameter), medium-sized (20–30 μm), and small-sized (20 μm or less) neurons. The findings are presented in terms of this classification for two main reasons: to correlate cytology and time of origin of neurons, and to analyze the spinal cord as a whole.

Regression and Settling Pattern. Pregnant albino rats of 11–15 days of gestation were laparotomized and [^{3}H]thymidine was injected into their uteri at selected implantation sites (5–10 μCi/embryo). Embryos from each group were collected 12, 24, and 48 hrs after injection. Only those embryos with positive heart beat were saved and processed for histology and autoradiography.

Axon Growth and Fasciculation. Embryos from pregnant rats of days 11–19 of gestation were removed, blocked, and stained according to Valverde (6) for Golgi preparations. For thick sections, the embryos were embedded in Epon–Araldite and the sections were stained with Azure-B.

RESULTS

Time of origin of neurons

The day of injection of [^{3}H]thymidine that yields heavily labeled neurons is considered to be the time of origin of that particular group of neurons (7). In the present study, only heavily labeled nerve cells were evaluated. Neuronogenesis in rat spinal cord began on day 11 and terminated on day 16 of gestation. The overall pattern of labeling revealed that in any given region of the spinal cord, the earliest forming neurons were located in the ventral region of the gray matter; they were the motor neurons.

The medium- and small-sized neurons originated over a period of about 3 days after the appearance of the large motor neurons. They occupied the intermediate gray region of the spinal cord, and were evaluated as the neurons that give rise to the long ascending and propriospinal systems and the interneurons. Finally, the small-sized neurons of the substantia gelatinosa of Rolandi came into existence (Fig. 1). These observations helped to establish that neuronogenesis in a given region of the spinal cord progressed along the ventro-dorsal axis. Furthermore, when the spinal cord was viewed as a whole, it was observed that the initiation and cessation of neuronogenesis progressed along the rostro-caudal axis. These two axes of neuronogenesis—dorso-ventral and rostro-caudal—operating simultaneously, were also found in relation to the pattern of release of neuroblasts from neuroepithelium and to the pattern of their settling in the mantle layer.

* Department of Anatomy, Colorado State University, Fort Collins, Colorado, 80521.

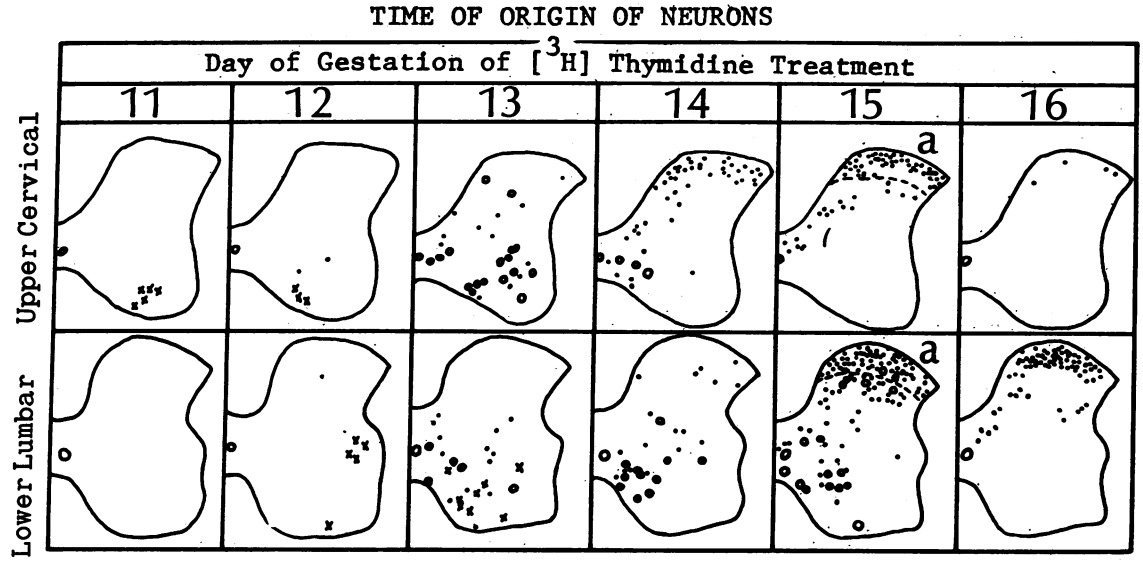


FIG. 1. Plots of the location of heavily labeled neurons in 6-week-old rats that had received a single treatment of [^{3}H]thymidine on day 11, 12, 13, 14, 15, or 16 of gestation. Note the ventro-dorsal gradient in the neuronogenesis in the cervical, as well as the lumbar, regions. Comparing the cervical and lumbar regions comprehensively, one may note that the lumbar region lags behind the cervical region, suggesting the presence of a rostro-caudal gradient in the neuronogenesis of spinal cord. Each plot represents the large motor neurons pooled from five unilateral sections, and the medium- and small-sized neurons pooled from four unilateral sections. *a*, substantia gelatinosa; ×, large motor neurons; O, medium-sized neurons; ●, small-sized neurons. ×200.

Pattern of release of neuroblasts

After having established the "time table" of origin of the various neurons of spinal cords of adult animals, we could now evaluate the changes within the neural tube of rat embryos. Since the release of the neuroblasts on their specific day of genesis involved loss of cells in the neuroepithelium, such release may be directly related to the regression of the neuroepithelium. Correlated with the ventro-dorsal pattern of neuronogenesis in any given region of the spinal cord, the neuroepithelium regressed along the ventro-dorsal axis (Fig. 2). For instance, in the 14-day-old embryos, motor neuroblasts were clustered in the ventral aspect of the mantle layer and, correspondingly, the neuroepithelium in the ventral half of the basal plate appeared regressed (*b* in Fig. 2*A*). Dorsal to this region, by contrast, the mantle layer had not acquired any mass of neuroblasts nor did the neuroepithelium appear regressed. On subsequent days of development, as the mantle layer appeared to acquire neuroblasts, the corresponding region of the neuroepithelium appeared to regress increasingly in the ventro-dorsal direction (Fig. 2*B*, *C*, *D*). This observation suggested that a specific region of the neuroepithelium could give rise to only a specific group of neurons. When the spinal cord was looked at as a whole, the regression of the neuroepithelium progressed along the rostro-caudal axis.

Pattern of settling of neuroblasts

Autoradiograms obtained from embryos that had received a single dose of [^{3}H]thymidine on a particular day of gestation, and had been killed 48 hr after the injection, provided information on the pattern of settling of the neuroblasts. 48 Hr was sufficient for the labeled cells to be released from the germinal layer and to become settled in the mantle layer. In spinal cords of embryos that received [^{3}H]thymidine on day 12 of gestation, and were killed 48 hr later, a cluster of unlabeled neuroblasts was found in the most ventral region of the mantle layer. This was the cluster of motor neuroblasts that had come into existence on day 11 of gestation (*d* in Fig. 2*A*). The motor neuroblasts that came into existence on day 12 of gestation (heavily labeled cells, indicated by *c* in Fig. 2*A*) settled dorsally and medially in relation to their predecessors. In the embryos that had received the [^{3}H]thymidine on day 13 of gestation and had survived for 48 hr after the injection, the early-forming unlabeled neuroblasts were located in the periphery of the ventral and lateral portions of the mantle layer (*d* in Fig. 2*B*), and the later-forming heavily labeled cells (*c* in Fig. 2*B*) had settled medially and dorsally upon the contours of their predecessors. In the embryos that had received the radiochemical on day 14 of gestation and survived for 48 hr after the injection, nearly all the neuroblasts arising out of the basal plate had already been formed (unlabeled cells indicated by *d* in Fig. 2*C*). In the dorsal region, two groups of labeled neuroblasts were distinguished, one of which had settled medially, while the other had migrated through and settled distally upon the early-formed unlabeled neuroblasts (*c* in Fig. 2*C*). In the embryos that had received the radiochemical on the next day (day 15) and survived for 48 hr after the injection, more neuroblasts were added to the two groups, and these two groups had become more sharply delineated (*c* in Fig. 2*D*).

These findings suggested that, with the exception of clusters of neuroblasts in the dorsal horn, neuroblasts in spinal cords may not undertake extensive migrations. In particular, the motor neuroblasts forming on days 11 and 12 of gestation appeared to settle wherever the regressing neuroepithelium deposited them. The settling pattern in the case of the subsequently forming neuroblasts appeared to be determined by the time and pattern of their release from the neuroepithelium, regression of the neuroepithelium, and cytological organization of the mantle layer. Of the three, the last factor seemed to be the most potent one in the emergence of the cytoarchitecture

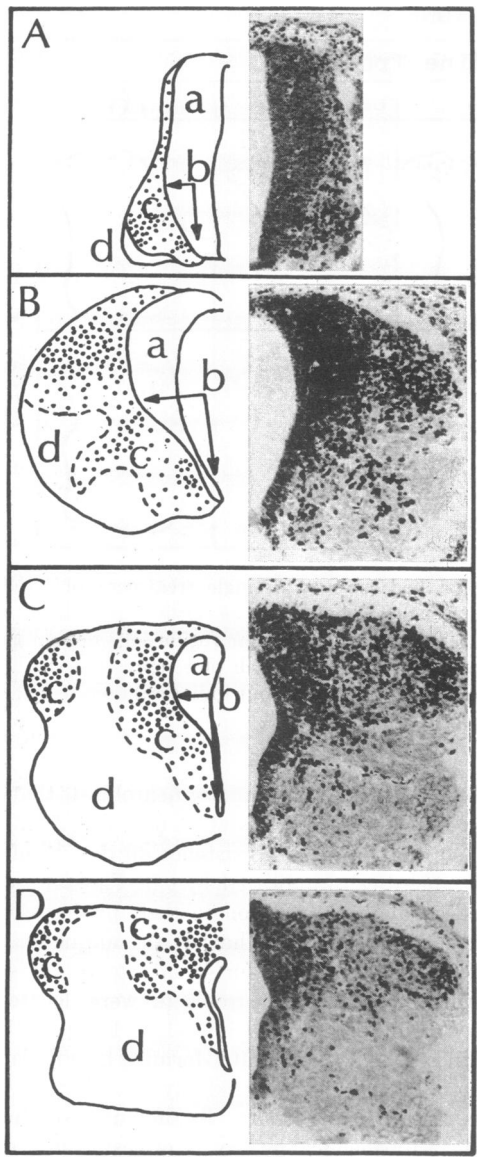


FIG. 2. Autoradiograms from embryos that received a single treatment of [^{3}H]thymidine on day 12(*A*), 13(*B*), 14(*C*), or 15(*D*) of gestation and were killed 48 hr after injection. Note the systematic loss of cells from the neuroepithelium (*a*) along the ventro-dorsal axis. In the case of embryos that were injected on day 15 of gestation and killed 48 hr later (*D*), note that the entire neuroepithelium appears to have regressed. *a*, neuroepithelium; *b*, region of regression of neuroepithelium; *c*, predominantly later-formed, heavily-labeled neuroblasts; *d*, predominantly earlier-formed, unlabeled neuroblasts. ×60.

of adult spinal cord. Finally, the pattern of settling of the neuroblasts followed the temporal lag along the rostro-caudal axis.

Pattern of growth of axons

This systematic release and settling of the neuroblasts appeared to determine the direction of growth of axons within an embryonic spinal cord. At early stages of embryonic development, the release and segregation of motor neuroblasts from the neuroepithelium seemed to result in the formation of interfaces. These provided a growth surface for ventral growth of axons arising from subsequently forming neurons, which settled medially and dorsally to the motor neuroblasts (Fig. 3*A*, *A'*). The interface between the periphery of the cluster of motor neuroblasts and the external limiting membrane of the spinal cord provided a growth surface for their longitudinal growth (Fig. 3*B*, *B'*). Thus, these early-growing axons did not have to venture about, but were guided by these interfaces. This phenomenon, when viewed for spinal cord as a whole, appeared to follow a temporal lag along the rostro-caudal axis; it is precisely this factor that regulated the rostral direction of growing axons. As development progressed, the later-growing axons, which grew in a sequential pattern as a result of their differential time of origin, accumulated upon these early axons. The early-forming axons extended along the most ventro-medial aspect of the longitudinal interface (*a* in Fig. 4*A*) and, as development progressed, the later-forming axonal processes systematically accumulated upon these early axons such that the fasciculus became thicker and also extended farther around the periphery of the mantle layer (*b* in Fig. 4*B*).

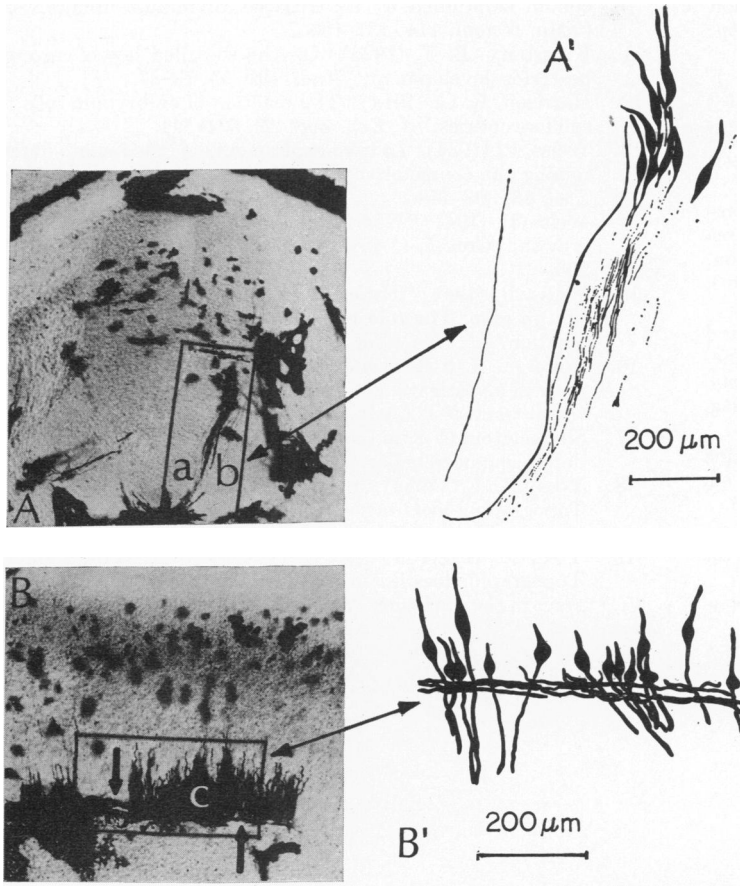
DISCUSSION

Embryonic development of nervous system is singularly characterized by differential times of origin of different neurons (3, 5, 8–10); this sequence appears to impose an order upon various events of neurogenesis, such as release, migration, and settling of the neuroblasts, their differentiation, emergence of cytoarchitecture, and directed growth of axonal processes. In the case of spinal cord, neurogenetic events followed a definite topographic order in addition to the temporal order. The topographic order determined the development of spinal cord along ventro-dorsal and rostro-caudal axes, as described in detail by early investigators including Coghill (1), Kingsbury (11), and Hamburger (2).

The earliest neurons to come into existence in spinal cord were the motor neurons. In their neuroblastic stage, they segregated from the neuroepithelium of the basal lamina, and occupied the most ventral zone of the mantle layer. As the later-forming neuroblasts systematically formed from progressively more dorsal regions of the neuroepithelium, they settled medially and dorsally upon their predecessors. These factors seemed to contribute to the lateral displacement of some of the motor neuroblasts. This pattern of genesis and release of neuroblasts along the ventro-dorsal axis, their settling along the medial and dorsal aspects of the preexisting clusters of the neuroblasts, and the lateral displacement of some neuroblasts were among the major events observed in the emergence of the cytoarchitecture. However, in the dorsal horn the emergence of the substantia gelatinosa involved active migration of neuroblasts through the preexisting clusters of neuroblasts.

The differential times of origin of neuroblasts were related to an appropriately staggered pattern of their differentiation. Thus, the early-forming neuroblasts grew their axons earlier than the late-forming neuroblasts. These observations, when related to the emergence of the mantle layer and its cytoarchitecture, seemed to provide an understanding of the directed growth of the individual axons and their subsequent fasciculations.

Harrison (12), in his pioneering studies using tissue culture techniques, observed that "solid objects are an important and even necessary factor in the movement of embryonic cells, such as mesenchyme and epithelium". Weiss (13–15), using *in vitro* techniques for the study of embryonic nervous tissue,



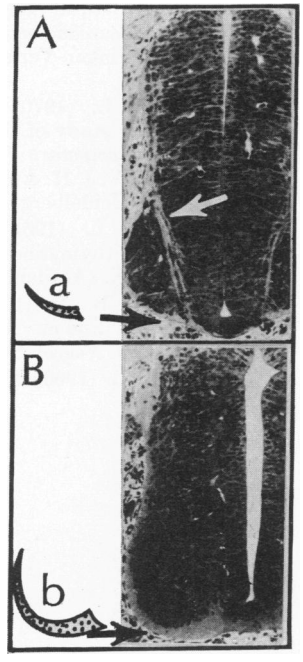


FIG. 3. (*Left*) Coronal (*A*) and sagittal (*B*) sections of the embryonic spinal cord of 14-day-old embryos. *A'* and *B'* are the drawings made from the sections shown in *A* and *B*, respectively. In the coronal plane (*A*,*A'*), note how the axonal processes course along the interface between the regressed neuroepithelium (*a*) and the cluster of neuroblasts (*b*) in the mantle layer. In the sagittal section (*B*,*B'*), note how the axonal processes (*arrow*) course longitudinally along the base of the column of neuroblasts (c). Golgi stain ×75.

FIG. 4. (*Right*). Coronal sections of the embryonic spinal cord of 13(*A*)- and 14(*B*)-day-old embryos. In *A*, note the cluster of axonal processes extending ventrally along the interface between the regressed neuroepithelium and the cluster of motor neuroblasts (*arrow*). Along the longitudinal interface, note that the early processes accumulated along its most ventro-medial aspect (*a*), and that, as development progressed, this bundle became thicker and extended farther around the periphery of the mantle layer (*b*). 1-μm thick sections; Azure-B stain. ×75.

established that neuronal processes require a solid surface upon which to advance. On the basis of these and other studies, he formulated the fundamental principles underlying the directed growth of axons individually or in bundles. He observed that the early fibers, appropriately called "pioneering" fibers, advanced along the contours of the interfaces (principle of contact guidance) and that the later-forming processes advanced along and accumulated upon their predecessors (principle of selective fasciculation). These principles provide a foundation for the interpretation of our findings reported in this study.

Oriented interfaces were observable within embryonic spinal cord at the time the early axons formed. One existed between the regressed neuroepithelium and the column of motor neuroblasts, and another between the column of motor neuroblasts and the external limiting membrane. It was along these interfaces that the early-forming axons, which might truly be called "pioneering" fibers, initially extended ventrally and then longitudinally. As development progressed, following the principle of "selective fasciculation", the later-forming axonal processes extended along and accumulated upon their predecessors. Thus, with time, the longitudinal fasciculus progressively became thicker and extended farther around the periphery of the mantle layer.

This pattern of growth of the axonal processes, together with the progressive rostro-caudal lag in the time of origin of neuroblasts, could account for the layering that has been observed in the ascending fiber tracts of adult spinal cords (16–19). The axons originating from the most rostral segments are located in the most ventral and medial aspect of the fasciculus, and those from progressively more caudal levels are sequentially layered laterally and dorsally to their more rostral counterparts. These observations suggest a systematic layering of the later-forming processes upon their predecessors.

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