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Neurogenesis and Morphogenesis in the Cerebellar Cortex*

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Abstract. The cerebellum presents the best site in the central nervous system for defining fundamental problems concerning the origin and differentiation of neurones, and their growth and development. The many recent experimental investigations of these problems are reviewed, and hypotheses based upon them are developed in relation to neurogenesis, morphogenesis, and synaptogenesis.

In the mammalian central nervous system neurones can be subdivided into two main classes—inhibitory and excitatory.[1] No ambivalent neurones are known. A fundamental problem in neurogenesis concerns the manner of production of these two classes, which in the adult are distinguished by differences in their chemistry, in their synaptic connections and in their synaptic actions.

The cerebellar cortex gives particularly favorable conditions for investigations on neurogenesis and morphogenesis because it is constructed in simple geometrical manner as a rectangular lattice,[2] and the histology and physiology of the constituent neurones are better understood than elsewhere in the central nervous system.[3, 4] For our present purpose the structure of a folium of the mammalian cerebellar cortex is shown in the perspective drawing of Figure 1. The principal neurones are the Purkyně cells (PC), one being drawn in full with its elaborately branched dendritic tree that extends as a thin leaflet in espalier fashion transversely across the folium. The longitudinal section displays the characteristic flattened form of the dendritic trees for two Purkyně cells, and also shows the ascending axons from the granule cells (GrC) with their T-shaped dichotomy in the molecular layer to form the parallel fibers (PF) that run in each direction in a strictly parallel fashion. Two other kinds of cells in the molecular layer, the basket (BC) and stellate (StC) cells, have planar dendritic trees resembling those of Purkyně cells, but are much simpler, and an axon transversely directed in one or other direction that gives *inhibitory* synapses to the somata and dendrites of the Purkyně cells. By contrast the parallel fibers give numerous *excitatory* synapses on the dendritic spines of the Purkyně, basket, and stellate cells, there being in this manner the very efficient and economical arrangement of the so-called "crossing-over synapse."[4–6] The axons of the Purkyně cells are shown leaving the cortex, and also shown are the two types of afferent fibers—mossy fibers (MF) and climbing fibers (CF).

Neurogenesis. Figure 2 is modified from two drawings by Ramón y Cajal[7] in order to give an initial illustration of the manner of formation both of the excitatory granule cells and of the inhibitory basket and stellate cells. It is in

the form of a perspective drawing as in Figure 1. Similar observations have been made in the chick.[8, 9] On the surface there is the external granular layer where the primitive stem or matrix cells (SC) exhibit a wealth of mitotic figures and then differentiate into granule cells (left side of diagram) or stellate and basket cells (right side of diagram). The granule cell neuroblasts are seen just below the external granular layer giving off branches, initially being sometimes unipolar (*a*), but always becoming bipolar (*b*) with long branches in parallel. Then, in (*c,d*) are progressive stages of the growth of a perpendicular process from the perinuclear area, and the passage of the nucleus down this process (*e–i*), eventually to form granule cells (GrC) as in Figure 1, with the T-shaped dichotomy of its axon giving the parallel fibers in the molecular layer. Meanwhile, the same cells of the external granular layer are producing stellate cell neuroblasts (StN), as shown to the right of the diagram, with their single sprout orthogonal to the parallel fibers. Much deeper there is an already-developed basket cell with its axon already searching for the Purkyně somata that are shown lightly dotted. Two primitive Purkyně cells with their bushy dendrites are shown to the left.

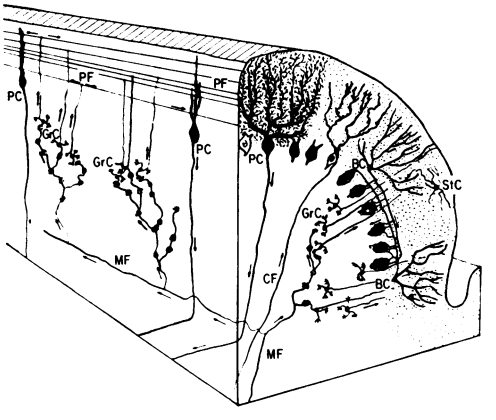

FIG. 1.—Perspective drawing of a section of a cerebellar folium.

In recent years there has been intensive investigation of cerebellar neurogenesis in chickens,[8–13] mice,[13–19] rats,[20–22] kittens,[23] and monkeys,[24, 25] using new technical procedures—electron microscopy, injection of tritiated thymidine and subsequent autoradiography, and the selective destructive actions of X rays[23, 26–28] or viruses.[29] In the first place these investigators have provided remarkable corroboration of the original descriptions of Ramón y Cajal and other members of the classical school. There is almost general agreement that the neurogeneses displayed on the two sides of Figure 2 are produced by the same

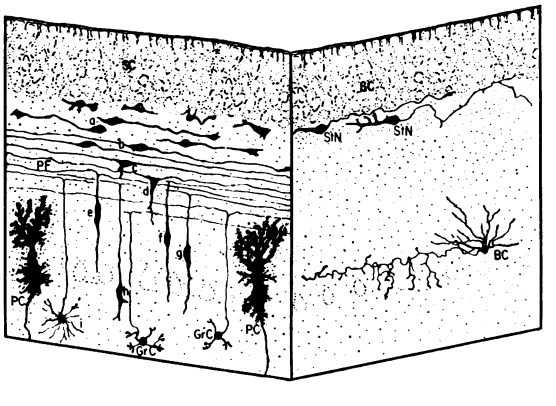

FIG. 2.—Montage perspective diagram composed from several drawings by Ramón y Cajal[8, 7] in order to show the various stages of neurogenesis and morphogenesis for the cerebellar cortex both along a folium (*left*) and across it (*right*). It could represent approximately postnatal day 10 for the rat.[22]

stem cells of the outer granular layer, and also that even the earliest sproutings of the two classes of neurones are arranged orthogonally.[22] Much of this recent investigation is concerned with later stages of development—the formation of synapses and cell maturation.[19, 23] The Purkyně cells, Golgi cells, and most of the glia of the cerebellar cortex are produced in a deep cell matrix earlier than the events of Figure 2.[7, 9, 12–14]

The earliest stages of neurogenesis have been studied with great advantage by high-resolution autoradiography in the chick,[10–13] the mouse,[10, 13–16] and the rat.[20–22] It is generally accepted that, once they are formed by some differentiating mitosis, nerve cells never again enter into a mitotic division.[12, 13, 20, 22] When injected intraperitoneally, ^{3}H-thymidine is rapidly absorbed into the circulation and is within an hour or two incorporated into the DNA that is being duplicated in the nuclei of cells preparatory to their mitosis, i.e., the incorporation of labeled thymidine constitutes a "declaration of intention to divide."[14] In an animal killed within a few hours, there is labeling of the nuclei of all the cells that have just been formed by mitosis. If the animal is killed many days later, this labeling is diluted even below detection for these cells that have been continuously dividing since the injection. The original quantity of label is shared between all the cells of the clone stemming from any originally labeled cell. On the other hand, once formed, the primitive neuroblasts never again divide, and hence retain the label with which they were born. Thus, in the mature cerebellum it is possible to recognize by their heavy labeling the neurones that were "born" just after a single injection of ^{3}H-thymidine.

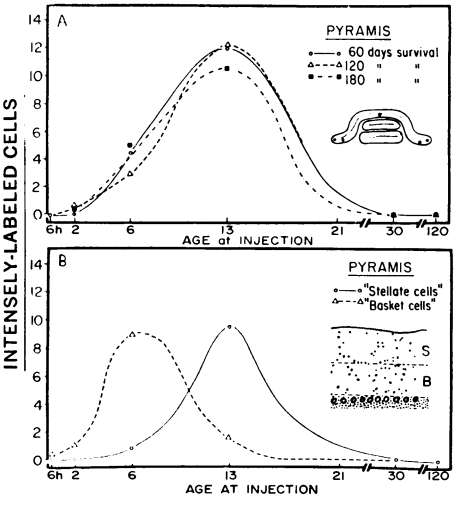

FIG. 3.—Mean numbers of intensely labeled neurones in pyramis region of rat cerebellar cortex in animals injected at various ages (indicated by abscissae) and examined some months later. (*A*) Granule cells after the indicated days of survival; inset shows sampling rates. (*B*) Presumed stellate and basket cells, as indicated in inset, after 120 days survival (Altman[22]).

Figure 3*A* is a population plot so determined by Altman[22] for the birthdays of granule cells in the pyramis region of a rat cerebellum. The time of survival (60–180 days) is shown to have no significant influence. Figure 3*B* is a similar plot for basket and stellate cells. It shows that basket cells tend to be produced earlier than stellate and granule cells, though there is considerable overlap in this latter case, e.g., at 6 days.

The process whereby cells become differentiated is still very poorly understood,[30] but we may consider that in the process of its creation by a differentiating mitosis, a neuroblast suffers some serious and irreversible loss in the DNA of its genome. It may be that there is a deletion of DNA or there could be a permanent repression. It can further be postulated that the re-

maining effective DNA is different for different classes of neurones, and in particular for the classes of excitatory and inhibitory neurones. This genetic difference would endow them with their different chemical properties, not only in production of the specific transmitter substances, but also in their surface properties whereby they give and receive the appropriate synaptic connections. Figure 4*A* repre-

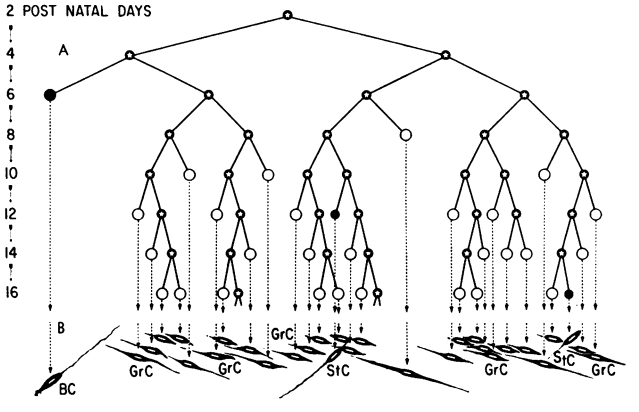

FIG. 4.—(*A*) Clonal diagram for progeny of a single stem cell of the external granular layer of cerebellar cortex of a rat at postnatal days indicated by scale to left. Stem cells are shown with a central star. Neuroblasts to form granule cells are indicated by open circles, whereas those to form basket cells and stellate cells are shown by large and small solid circles, respectively. Actual rate of multiplication would be at least twice that represented, and production of granule cells would be much greater relative to basket and stellate cells.

(*B*) This part of the diagram is a perspective drawing of the development of the various neuroblasts. Granule cell neuroblasts (excitatory) are shown to grow in parallel array, as in the left side of Fig. 2. Basket and stellate cell neuroblasts (inhibitory) are shown growing orthogonally thereto, as in right side of Fig. 2.

sents, in the light of the birthday sequences of Figure 3, an attempt to diagram in clonal form the generation of basket, stellate, and granule cells, and to take into account, but inadequately, the great numerical discrepancy, the granule cells being over 100 times more numerous than the combined basket and stellate population.[4] Because of this discrepancy it is not possible to entertain the postulate that in the differentiating mitosis the stem cell divides into an excitatory and an inhibitory neuroblast. Figure 4*A* has been diagrammed on the basis of a random production of neuroblasts by stem cells, but with the recognition of the great numerical discrepancy and also of the observations in Figure 3*A* showing the temporal courses of the respective birthdays. Incorporated in the design is the further important feature that in the rat there is a progressive decline in the population of the stem cells of the external granular layer from about 12 days onward to its eventual disappearance by 21 days.[22] It is assumed that all cells have by then become differentiated into neuroblasts. In the later stages of Figure 4*A* there are several examples of a stem cell dividing into two granular neuroblasts.

In Figure 4 it is assumed that by their repeated mitoses the stem cells initially form a clone. In the rat after about 4 days this clonal development is progres-

sively more and more eroded by differentiation into neuroblasts and so to its eventual extinction.[22] In the diagram it was assumed that, until the later stages of overwhelming neurogenesis, a differentiating mitosis of a stem cell resulted in one neuroblast and one stem cell that could continue with the clonal sequence. However, the essential features of the diagram would be preserved if, instead, it was assumed that stem cells had two distinctive kinds of mitosis, those of the usual clonal sequence, and those in which two neuroblasts were formed, which only rarely were both inhibitory or one excitatory and one inhibitory.

At the early stage of development depicted in Figure 4*A* it is not as yet possible to identify the excitatory and inhibitory neurones. However, as seen in Figure 2, the growth of sprouts soon makes this possible.[9] This phase of morphological differentiation is shown in the perspective plot of Figure 4*B* for the neuroblasts that were formed in Figure 4*A*. With granule cells the nucleus remains central in an elongated soma and a sprout grows from each end. With basket and stellate cells the nucleus is at one pole of the soma, and from that pole grows out the axon, whereas the organelles are at the other pole from which later grow the dendrites.[9]

The clonal diagram (Fig. 4*A*) serves to define several fundamental problems of neurogenesis. Firstly, are the functions of neuroblasts irreversibly determined from their birth as being excitatory or inhibitory? This would be expected if, as postulated above, the differentiation was due to some partial DNA deletion or irreversible repression, one type of surviving DNA competence resulting in excitatory neurones and another in inhibitory neurones. This raises the still more fundamental problems of the genetic mechanisms involved in the differentiating mitosis. There is an additional problem in attempting to account for the irreversible loss of mitotic ability by neuroblasts. Another problem illustrated in Figure 4*A* concerns the "aging" of the stem cells. At first they only produce stem cells; then, after a small beginning with production of basket cell neuroblasts, the clonal sequences of stem cells are eroded more and more by the production of granule cell neuroblasts and some stellate cell neuroblasts. Finally, as a terminal aging process, they suffer annihilation by their total involvement in neuroblast production. It can be recognized that the techniques of electron microscopy, autoradiography, and X radiation have a very important role to play in the further investigation of the problems here outlined. Many fundamental discoveries will be made by subjecting stem cells to various procedures, chemical or radiation treatment for example, and following their subsequent progeny by electron microscopy and autoradiography. In this way it should be possible to alter the normal production ratio of excitatory and inhibitory neurones, and even to discover the underlying genetic factors.

Hypotheses Relating to Morphogenesis. In the attempt to explain the remarkable construction of the cerebellar cortex as a rectangular lattice, it is first necessary to determine the prime factor in giving directionality. Ramón y Cajal[3, 7] suggested that the parallel fibers first set the direction that determined the orientation of other elements, and Mugnaini concurs.[9] As we have seen (Figs. 2 and 4), the parallel fibers grow along the folia that already are formed

by the transverse convolutions in the primitive cerebellum before neuroblasts are generated in the external granular layer. Possibly this direction is mechanically determined, growth occurring through the more open structure along the folium in contrast to the transverse compression resulting from the folding.

It is postulated that by a homotypic reaction the newly formed parallel fibers grow along those already formed,[9] so establishing the strictly parallel arrangement of the fibers in the adult molecular layer. Furthermore, growth occurs in both directions because of intrinsic (genetic) factors that cause the nucleus to be symmetrically placed in the elongated soma.[9] This central position of the nucleus later results in the outgrowth of the downwardly directed process (cf. Fig. 2), which presumably is sufficiently explained by the high rate of protein synthesis in the proximity of the nucleus. It has been postulated that the direction of this process is determined by its association with the upwardly growing Purkyně cell dendrites.[22] By homotypic recognition the downward growing sprouts of granule cells follow each other by a process that may be called homotypic fasciculation, and so eventually they reach the granular layer and the waiting mossy fibers.

With respect to basket and stellate neurones it is first postulated that genetically determined factors cause the nucleus to be at one pole with the organelles at the other.[9] As would be expected, the first outgrowth, the axon, occurs from the nuclear pole. Mugnaini[9] recognized that the failure of this axonal outgrowth to run along the parallel fibers indicated that there is no heterotypic affinity. It is now suggested that, if there is a heterotypic repulsion between the basket-stellate sprouting axons and the parallel fiber fasciculi, these axons will tend to grow transversely, being repelled from one contact only to find another and so on. Nor can they grow downwards, because in that direction also they find the repulsive fasciculi of the granule cell axons (cf. Fig. 2). Hence it is postulated that their rather irregular transverse course across the folium (Fig. 2) is explained by what we may term an "avoidance control." There seems to be no need to postulate any vertical motivation for the basket-stellate cells. Their progressive descent through the molecular layer is sufficiently explained by the growth of new elements above the level at which they were formed, these new elements being largely the parallel fibers formed by the later differentiating granule cells and the superficial branches of the Purkyně cell dendrites. Thus there is a depth gradation in the molecular layer from the earliest formed basket cells up to the more recent stellate cells (cf. Figs. 1, 2, and 4). As Ramón y Cajal[7] pointed out, the axons of the basket cells initially grow irregularly in an unguided manner (Fig. 2) until the somata of the Purkyně cells mature to the stage where they are attractive. Then all adjacent basket cell axons converge to form the dense basket-like concentrations around the somata and initial axon segments. The stellate cell axons are too superficial to be so attracted, but the large Purkyně cell dendrites provide a substitute target.

The most remarkable geometrical features of the molecular layer are the thin espalier dendritic trees of the Purkyně cells (Fig. 1)that are arranged orthogonally to the parallel fibers so as to give the maximum opportunity for crossing-over synapses.[4, 5, 31] Initially the apical dendritic trees of Purkyně cells are

bushy (Fig. 2, PC) and this espalier configuration seems to be induced as a consequence of their upgrowth to mesh with the developing parallel fibers.[7] It is postulated that this induction is due to an "exclusion principle" according to which a parallel fiber cannot enter into the formation of adjacent synapses. Larramendi[19] postulates that at developing synapses on parallel fibers there is a presynaptic trapping of freely flowing synaptic vesicles by "presynaptic vesicle glue." In this way it could come about that there is a minimum spacing of effective synapses. As a consequence the upgrowing Purkyně cell dendrites fail to make synapses with parallel fibers already engaged by precocious dendrites. The later growing dendrites become successful only when they spread laterally to territories of parallel fibers not yet engaged. If it be postulated in addition that unsuccessful dendrites atrophy, then it seems possible to account for the orthogonal espalier arrangement of the Purkyně dendrites simply by the prerequisite of achieving single synaptic contacts with the largest number of parallel fibers.

This principle of maximizing the number of single synaptic contacts with parallel fibers also will account for the espalier formation of the dendritic trees of basket and stellate cells (cf. Fig. 1). The dendritic tree of the Golgi cell is widely branched in all directions, so apparently provides an exception to this "exclusion principle." A possible explanation is that the branches are so widely spaced[4] that the same parallel fiber could make several synapses. Experimental support for the role of the parallel fibers in determining the espalier pattern of dendrites is provided by the extreme irregularity of dendritic branches when X radiation causes severe degranulation with failure of parallel fiber development.[23, 26]

The cerebellar cortex is a most favorable site for the investigation of many questions relating to synaptogenesis in the central nervous system. For example, is there a correlation between the time of development of synaptic receptiveness of a neurone and the time of the formation of synapses by its own axons?[19] Is there some sequential course of synaptic development along neural pathways? What is the sequence for the development of excitatory and inhibitory synapses on the same neurones?

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