EMERGENCE OF CYCLIC GUANOSINE 3':5'-MONOPHOSPHATE-DEPENDENT PROTEIN KINASE IMMUNOREACTIVITY IN DEVELOPING RHESUS MONKEY CEREBELLUM: CORRELATIVE IMMUNOCYTOCHEMICAL AND ELECTRON MICROSCOPIC ANALYSIS¹

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Abstract

The appearance of cyclic guanosine 3':5'-monophosphate-dependent protein kinase (cGK), an enzyme that may be involved in the regulation of various aspects of neuronal function and that is highly concentrated in cerebellar Purkinje cells, was studied in the developing and adult monkey cerebellum by indirect immunofluorescent staining. The appearance and distribution of cGK immunoreactivity were then correlated with the stages of Purkinje cell differentiation and with the establishment of synaptic inputs to Purkinje cells as revealed by electron microscopy and by the presence of synapsin I, a specific nerve terminal marker. In the adult monkey, in analogy with previous observations in the adult rat, cGK immunoreactivity was detected throughout the cytoplasm of all Purkinje cells and was not seen in other neurons. During ontogenesis, cGK immunoreactivity appeared for the first time in Purkinje cells at the 97th embryonic day (E97). On this day it was detectable in Purkinje cells situated in the posterior lobe concurrently with the emergence of synapsin I immunoreactivity surrounding their somata. The cGK-positive cells had entered the phase of rapid dendritic growth and had begun establishing axosomatic synapses. By E102, Purkinje cells in the posterior lobe and in most of the anterior lobe were cGK positive. By E125, all Purkinje cells had received synaptic contacts and had become cGK positive.

In addition to typical Purkinje cells situated in the cortex, we found another population of cGK-positive neurons present transiently in the prospective cerebellar white matter. These neurons, which were observed only during the second half of gestation, had morphological similarities to Purkinje cells. The emergence of cGK in these neurons also coincided with their dendritic proliferation and with the appearance of synapsin I immunoreactivity around their cell bodies. Subcortical cGK-positive cells were not observed in postnatal animals. Such neurons may be Purkinje cells which, failing to reach the cortical plate, subsequently degenerate.

The close temporal correlation between appearance of cGK immunoreactivity, onset of synaptic inputs, and dendritic proliferation, both in typical Purkinje cells and in the Purkinje cell-like cells in subcortical areas, suggests that expression of high levels of cGK may be an important aspect of the neuronal differentiation of these cells.

Cyclic adenosine 3':5'-monophosphate (AMP)-dependent, cyclic guanosine 3':5'-monophosphate (cGMP)-dependent, and Ca²⁺-dependent protein kinases are enzymes that catalyze the

phosphorylation of specific substrate proteins. Some of these substrate proteins appear to be involved in the regulation of various aspects of neuronal function (Greengard, 1980). In the rodent brain, cGMP-dependent protein kinase (cGK) is particularly highly concentrated in Purkinje cells of the cerebellum (Schlichter et al., 1980; Lohmann et al., 1981; De Camilli et al., 1984a). Immunocytochemical methods applied to the adult rat brain have revealed the presence of cGK within the cytoplasm of the perikaryon, dendrites, axons, and axon terminals of all Purkinje cells, while other neurons appear cGK negative (Lohmann et al., 1981; De Camilli et al., 1984a).

Prenatally, the rat cerebellum does not contain detectable levels of cGK, as measured by a histone phosphorylation assay (Bandle and Guidotti, 1979). The concentration of this enzyme remains relatively low during the first postnatal week, increases steadily during the second week, and reaches adult values by the fourth week (Bandle and Guidotti, 1979). It would be difficult, however, to correlate the expression of cGK in the rat

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cerebellum with the stages of Purkinje cell differentiation and with the establishment of various synaptic inputs to Purkinje cells, due to the rapid and overlapping manner in which these developmental events take place in this species. Yet such information is needed in order to help define the role of cGK in neuronal function.

The monkey cerebellum offers an excellent model with which to address these issues. In this species, development of the cerebellum occurs over a protracted prenatal and postnatal period (Rakic, 1971, 1972, 1973, 1980) and is, in many respects, similar to development of the human cerebellum (Rakic and Sidman, 1970; Zecevic and Rakic, 1976). In the rhesus monkey, Purkinje cells are generated in a relatively short period of time during the first quarter of the 165-day gestation and subsequently undergo a protracted differentiation that proceeds throughout the second half of fetal life and continues into early infancy. The slow development of the monkey cerebellum allows a precise temporal delineation of various cellular events, including the attainment of cell position, the sequential development of dendrites and axons, and the establishment of various classes of synapses (Rakic, 1971, 1972, 1973). Thus, use of the developing monkey cerebellum should facilitate the analysis of relationships between morphologically defined cell differentiation and the expression of cell proteins.

In the present study, we have used immunocytochemistry to study the emergence and distribution of cGK at different stages of prenatal and early postnatal development in the rhesus monkey cerebellum. In addition, we have correlated these findings with the appearance of synaptic connections as revealed by immunostaining for synapsin I, a protein specific to nerve terminals (De Camilli et al., 1983a, b; Huttner et al., 1983) and by transmission electron microscopy.

Materials and Methods

Immunohistochemical procedures. Cerebella of 12 rhesus monkeys (*Macaca mulatta*), ranging in age from 47 embryonic (E) days to 2 postnatal years, were analyzed for cGK immunoreactivity. Fetuses were obtained from pregnant females by hysterotomy and were perfused transcardially with 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M sodium phosphate (pH 7.4). Postnatal animals were deeply anesthetized with sodium pentobarbital before perfusion with the same fixative. The cerebella from all animals were cut sagittally into three to five blocks and postfixed overnight at 4°C. Tissue blocks were rinsed five times with 20% sucrose in 0.1 M sodium phosphate buffer (pH 7.4) and stored at 4°C in this solution until sectioned. Sodium azide was added to the storage medium at 0.1 mg/ml to prevent bacterial growth.

Tissue slabs were frozen by immersion in isopentane chilled with liquid nitrogen and equilibrated in a cryostat at -20°C. Sections, 10 µm thick, were collected on gelatin/chrome alum-coated slides, air dried, and stored at 4°C until stained. Immunocytochemical staining for cGK was performed as described previously for the rat (Lohmann et al., 1981), with the exception that the staining procedure was preceded by a 3-min incubation of the sections in freshly prepared 1% sodium borohydride. This step was required to quench the glutaraldehyde-induced autofluorescence of the tissue. After the borohydride step, sections were incubated for 3 hr at 37°C in the presence of either rabbit antiserum to cGK or preimmune serum. The sera were diluted 1:25 in Triton buffer (Lohmann et al., 1981) containing 10% normal swine serum. After incubation with the first antibody, the sections were washed extensively for 30 min with Triton buffer and incubated for 1 hr at 37°C in the presence of rhodamine-conjugated swine anti-rabbit IgG (Dako-Accurate Scientific) diluted 1:50 in Triton buffer containing 10% normal swine serum. After a 30-min rinse in Triton buffer, the sections were mounted in a 3:1 (v/v) mixture of glycerol:0.1 M sodium phosphate and examined on a Zeiss Universal photomicroscope equipped with epifluorescence optics and a rhodamine filter set.

In some experiments, alternate sections were stained by an antiserum to the nerve terminal marker protein synapsin I (formerly referred to as protein I) (De Camilli et al., 1983a, b; Huttner et al., 1983) in order to assess qualitatively, at the light microscopic level, the distribution and density of synapses in all areas of the developing cerebellar

cortex. The staining protocol was identical to that described for cGK localization, with the exception that the primary antiserum to synapsin I was diluted 1:50.

Electron microscopic analysis. The cerebella of five additional animals, at ages E91, E95, E97, E105, and E125, were analyzed by electron microscopy to determine the stage of differentiation of Purkinje cells and of the surrounding neuropil. Cerebellar tissue was prepared as described previously (Rakic, 1971, 1973). Following perfusion and fixation with 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4), 1 × 2 × 0.5 mm blocks of the cerebellar cortex were cut out with a razor blade, washed in 0.1 M sodium phosphate buffer (pH 7.4), osmicated, stained *en bloc* with 1% uranyl acetate, dehydrated, and embedded in a 1:1 mixture of Epon/Araldite. Silver thin sections were collected on 150 mesh grids and examined with a JEOL 100S electron microscope. Numerous random photographs of Purkinje cells and their dendrites were taken of each specimen.

Specificity of antisera. The antiserum to cGK was made in rabbit against native cGK purified from bovine lung as described by Walter et al. (1980). The specificity of the antiserum for bovine and rat cGK was demonstrated as described by Walter et al. (1980), Walter (1981), and Lohmann et al. (1981).

The antiserum to synapsin I was made in rabbit against synapsin I purified from bovine brain as described by De Camilli et al. (1983a), and its specificity for rat and bovine synapsin I was demonstrated as described by De Camilli et al. (1979, 1983a).

Results

Localization of cGK immunoreactivity in adults. Examination of the entire cerebellar hemisphere of the adult rhesus monkey reveals that Purkinje cells are the only cGK-immunoreactive neurons (Figs. 1 and 2, A and B). In agreement with previous studies performed in the adult rat (Lohmann et al., 1981; De Camilli et al., 1984a), all Purkinje neurons appear cGK positive (Fig. 2, A and B). Immunofluorescence is distributed uniformly throughout the cytoplasm, including the perikaryon and dendritic and axonal processes, while the nucleus remains unstained. In sagittal sections, the staining exquisitely displays the details of the large, profusely branched Purkinje cell dendritic tree within the molecular layer (Fig. 1). Although at low magnification dendritic shafts appear smooth, at high power dendritic spines can be seen.

Immunofluorescent axons can be seen emerging from the bottoms of Purkinje cell somata, penetrating the granule cell layer (Fig. 2C) and entering the subcortical white matter, from which they can be traced into the deep cerebellar nuclei (Fig. 2D). The network of Purkinje cell recurrent axon collaterals, which form a plexus in the granule cell layer beneath the Purkinje cells, is also visible. cGK-positive axons enter all of the deep cerebellar nuclei and form an exceptionally dense network (Fig. 2E). Their terminals often outline the silhouettes of large neurons (Fig. 2E) that have been shown in previous studies (Chan-Palay, 1977) to receive the majority of their axosomatic synapses from Purkinje cells.

Emergence of cGK immunoreactivity during development. No detectable levels of immunoreactivity of cGK are found in any cerebellar section obtained from fetuses sacrificed at E47, E61, E70, E86, and E94 (Fig. 3A). The first cGK-positive Purkinje cells to be seen in the monkey appear in the posterior lobe of the cerebellum at E97 (Fig. 4A). Although all Purkinje cells are generated before E45 (Rakic, 1980), no immunoreactive Purkinje cells are seen at E97 in most of the anterior lobe. Intermediate levels of immunoreactivity are observed in Purkinje cells between these two regions, with a negative posteroanterior, slightly mediolateral gradient. These findings are in agreement with the posteroanterior gradient of cerebellar cortex differentiation in the monkey (P. Rakic, unpublished observations). In fact, at E97, cGK-positive Purkinje cells are arranged in a single layer at the caudal end of the posterior lobe, whereas in the more anterior areas, Purkinje cell bodies still form a layer

Figure 1. Photomicrograph of a parasagittal section of the adult cerebellar cortex stained for cGK by immunofluorescence. cGK immunoreactivity is localized within the cytoplasm of the Purkinje cell and allows visualization of the entire dendritic tree residing in the molecular layer. Note the homogeneous distribution of immunoreactivity throughout the perikaryon and dendrites. Most dendritic spines are poorly resolved, but some can be seen distinctly (*arrows*). The Purkinje cell nucleus is unstained. The brightly stained dots seen in the ganglionic layer among Purkinje cell perikarya represent autofluorescence. These fluorescent dots, unlike the cGK immunoreactivity, persist when a fluorescein filter system is substituted for the rhodamine filter system. Bar = 40 μ m.

Figure 2. Distribution of cGK immunoreactivity in the mature monkey cerebellum. **A**, Low-power photomicrograph of a parasagittal section illustrating Purkinje cell bodies (*arrows*), their dendrites in the molecular layer (*m*), and their axons in the granular layer (*g*) and in the subcortical white matter (*w**m*). *Bar* = 100 μ m. **B**, Coronal section illustrating two opposing folia in which all Purkinje neurons are cGK positive. In this section, Purkinje cell dendrites appear as dots and segments lying in parallel rows in the molecular layer (*m*). *g*, granular layer. *Bar* = 100 μ m. **C**, A single immunofluorescent axon traced from its origin at the Purkinje cell perikaryon (*p*) through the granule cell layer (*g*), finally

two to four cells thick. The gradient of cGK immunoreactivity in Purkinje cells of different cerebellar regions suggests that cGK appears in all Purkinje cells at the same stage of differentiation.

At E97, cGK immunostain labels both the somata of Purkinje cells and their immature primary dendrites which at this stage are very rudimentary. Immunostained Purkinje axons and terminals are not discernible at this fetal age, although corticodentate synaptic contacts have been observed by electron microscopy in this species as early as E75 (Kornguth et al., 1968). Lack of staining of axons and axon terminals at this early stage may well be due to their small dimension rather than to a concentration of cGK lower than in cell bodies and dendrites; low concentrations of cGK may be undetectable in processes of small caliber whereas they are already clearly visible in larger structures.

By E102, the population of cGK-positive Purkinje cells extends throughout the posterior cerebellar lobe (Fig. 3*B*) and most of the anterior lobe as well. The posteroanterior gradient of staining intensity is not as evident as it was at E97. Although older by only 5 days, Purkinje cells of the posterior lobe are considerably larger and more differentiated at E102 than at

E97. Secondary dendrites are beginning to form and both primary and secondary dendrites are cGK positive. At this stage, Purkinje cell axons are detectable in the white matter (Fig. 3*C*), and a faint punctate pattern of immunoreactivity is detectable in the dentate nucleus and other deep cerebellar nuclei. Electron microscopic analysis reveals that Purkinje cell axon terminals at this stage form well defined synaptic terminals consisting of membrane specializations and numerous round vesicles (Kornguth et al., 1968).

About 3 weeks later, at E125, all Purkinje cells in the monkey cerebellum are cGK positive (Fig. 4*C*). Except for the differences in size of the cell somata and in the extent of dendritic arborization, the Purkinje cell population at this time, as revealed by cGK immunoreactivity, appears similar to that observed in the adult. The immunoreactive neurons are now aligned regularly in a monolayer throughout the entire cerebellar cortex. The primary and secondary dendrites, forming the characteristic espalier-shaped dendritic arbor, are well illustrated in parasagittal sections. Brightly stained immunofluorescent axons, arising from the bottom of Purkinje cell somata, can be seen penetrating the granule cell layer and entering the subcortical white matter. These axons can be traced to the deep

Figure 3. Localization of cGK immunoreactivity in fetal monkey cerebellar cortex. Parasagittal sections. *A*, Cerebellum of the E94 fetus. No cGK-positive cells or processes are present in any region. *B*, Section through the posterior lobe of the cerebellum at E102 demonstrating the specific localization of cGK immunofluorescence in Purkinje cells. The lack of staining on the left side is due to the absence of Purkinje neurons in this region, as could be seen by counterstaining the section with cresyl violet (not shown). *C*, Interstitial neurons (large arrows) in the prospective white matter of the posterior lobe of the E102 fetus. These neurons are similar in appearance to Purkinje cells at this age, except that their primary dendrites (small arrows) are oriented randomly. *egl*, external granule cell layer; *g*, internal granule cell layer; *m*, molecular layer; *p*, Purkinje cell layer. Bars = 50 μ m.

turning (arrowhead) into the subcortical white matter (*w*m). The axon (large arrow) has a recurrent collateral (small arrow) which ascends through the granule cell layer. Bar = 50 μ m. *D*, Purkinje cell axon bundles in the cerebellar medulla. These fibers are coursing toward their termination in the deep cerebellar nuclei. Bar = 50 μ m. *E*, A neuron innervated by Purkinje cell terminals in one of the deep cerebellar nuclei. This target neuron (*n*) is completely surrounded by cGK-positive terminals (arrows). Purkinje cell axon bundles cut longitudinally (arrowheads) and in cross-section (open arrows) are also visible. Bar = 25 μ m.

Figure 4. Pairs of adjacent parasagittal sections of cerebellar cortex at two different embryonic ages, stained for cGK (A and C) and synapsin I (B and D). A, Section from E97 fetus showing the earliest appearance of cGK immunoreactivity in the Purkinje cell layer (arrow). Note the decrease in staining intensity as one proceeds rostrally (upper right of micrograph) in parallel with the negative posteroanterior gradient of cerebellar differentiation. The lack of cellular details in the stained sections at this age is related to the difficulty of fixing fetal material. B, Adjacent section stained for synapsin I. Staining of terminals in the granule cell layer (g) is evident (these are probably mossy fiber terminals). In addition, some staining is also visible in the Purkinje cell layer and in the still very shallow molecular layer. In some cases, immunoreactive dots outline the profile of Purkinje cell somata (arrow). Synapsin I staining decreases as one proceeds rostrally (upper right of micrograph). C, This section from an E125 fetus shows that Purkinje cells (large arrows) at this age are intensely stained for cGK. The dendritic tree (small arrows) extends into the narrow molecular layer, terminating just below the external granule cell layer. D, Adjacent section which demonstrates the synapsin I-positive terminals in the granule cell layer and the very dense synapsin I immunoreactivity around Purkinje cell somata (large white arrows) and dendrites (small black arrows) in the molecular layer. The large white arrows designate the same cells in C and D. egl, external granule cell layer; g internal granule cell layer; p, Purkinje cell layer; wm white matter. Bars = 50 μ m.

cerebellar nuclei, where they form a dense plexus. As in the adult animal, immunofluorescence is particularly prominent around the somata of innervated neurons in these nuclei, indicating an abundance of axosomatic synapses.

Except for the generally smaller size of the Purkinje cells, the distribution and intensity of cGK staining in the cerebellum

at birth (E165) is basically the same as that described for the adult (see above). The dendritic arbor of Purkinje cells extends to the border of the external granule cell layer, which, at this age, is reduced to a thickness of less than 30 μ m.

Other cGK-positive neurons in the developing cerebellum. In the adult monkey, Purkinje cells are the only cGK-positive

neurons visible in the entire cerebellum. In the developing monkey cerebellum, however, there is an additional population of immunoreactive cells. In the three oldest fetuses sacrificed and analyzed for cGK immunoreactivity (E97, E102, and E125), a number of cells residing in the prospective white matter below the cerebellar cortex are clearly cGK positive. These neurons resemble Purkinje cells in many respects. They are usually monopolar, and the branching characteristics of their dendrites appear similar to those of Purkinje cells in the cortex (Fig. 3C). For a given age, these cGK-positive neurons appear more differentiated than do the Purkinje cells in the supra-adjacent cortex. However, they do not undergo the extensive and characteristic arborization of Purkinje cells during the period between E97 and E125.

cGK-positive neurons located in the prospective white matter well below the cortex appear for the first time at E97. These interstitial cells are situated mostly in the posterior lobe at this age. It may be significant that their cell somata lie directly below the cortical region that contains cGK-positive Purkinje cells at this age. The number of cGK-positive interstitial cells increases by E102, since more of these cells appear in the anterior portions of the cerebellum. They are usually aggregated in groups of up to 10 cells, although some solitary cells are scattered throughout the prospective white matter of the anterior and posterior lobes at this age. By E125, the number of interstitial neurons containing cGK immunoreactivity has begun to decline in all areas of the cerebellar white matter. No cGK-positive cells are detectable in the white matter of young postnatal monkeys. Sections counterstained with cresyl violet demonstrate that the number of interstitial neurons in the white matter is greatly diminished by the time of birth.

Temporal correlation between the formation of synaptic contacts and appearance of cGK. Since cGK may be involved in mediating certain actions of synaptic inputs on Purkinje cells (Greengard, 1980), it seemed reasonable to carefully examine the temporal correlation between the appearance of cGK immunoreactivity and the development of synaptic connections by Purkinje cells. To do so, we first used synapsin I immunoreactivity as a general marker for synapses. This procedure has been shown to be valid in developing rat and chick cerebellum (De Camilli et al., 1983a). Once we identified synapsin I around Purkinje cells, we performed a more detailed electron microscopic analysis to determine the types of synapses present and their distribution at specific times during fetal development.

Immunocytochemical localization of synapsin I was performed on sections adjacent to those immunostained for cGK. In early fetal monkeys (i.e., prior to the appearance of cGK in Purkinje cells at E97), there is a virtual absence of synapsin I staining in the Purkinje cell layer. Only at high magnification can a few spots of synapsin I immunofluorescence be discerned in the Purkinje cell layer. In the same specimens, however, synapsin I-positive boutons, probably mossy fiber terminals, are clearly evident in the granule cell layer, particularly in the posterior cerebellar lobe. This finding can serve as an indicator of the validity of the staining and of the appropriateness of the antibody dilutions used. At E97, spots of synapsin I immunoreactivity of moderate intensity surround Purkinje cell somata situated in the posterior lobe and in the caudal sector of the anterior lobe (Fig. 4B). As seen in an adjacent section (Fig. 4A) stained with cGK antiserum, this is the region that contains cGK-positive Purkinje cells. On the other hand, in regions where Purkinje cells are not yet cGK positive, there is little synapsin I staining in the Purkinje cell layer. In contrast, dots of synapsin I immunoreactivity are visible in all regions of the internal granule layer at this age. Synapsin I staining can also be visualized around cGK-positive cells situated in the prospective white matter of the posterior lobe.

By E102, the synapsin I staining surrounding Purkinje cells

has spread anteriorly, parallel with the posteroanterior spreading of cGK immunoreactivity. At this age, synapsin I immunofluorescence is localized specifically around the somata and the base of the primary dendrites of Purkinje cells. The change in staining distribution from E97 to E125 is illustrated in Figure 4. At E125, synapsin I staining has become more intense in the molecular layer along the dendrites of the Purkinje cells (Fig. 4D). Immunoreactivity around Purkinje cell somata is still abundant.

In order to define more specifically the types and locations of synapses being formed with Purkinje cells at the time during fetal development when cGK immunoreactivity emerges, electron microscopic examination was performed on embryos before, during and after the appearance of cGK immunoreactivity. At E91, a few days before cGK becomes detectable, Purkinje cells appear relatively immature, with only a few or no secondary dendritic branches. The molecular layer contains numerous parallel fibers—one of the two major types of extrinsic Purkinje cell afferents—which run in a course perpendicular to that of the Purkinje cell dendrites. Parallel fiber axonal membranes are closely apposed to Purkinje cell dendrites, but neither presynaptic nor postsynaptic membrane specializations are detected at this time. Nor are any morphologically well defined synaptic junctions observed along the soma of the Purkinje cell at this fetal age (Fig. 5A).

At E97, when cGK is first detected in Purkinje cells, the organization of the molecular layer remains similar to that at the E91 stage. Thus, although parallel fibers are more numerous at this age, they still do not form synapses with the Purkinje cell dendrites. However, a few axosomatic synapses now appear along Purkinje cell bodies (Fig. 5B). Most of these synapses are formed with transient Purkinje cell somatic spines, which are evident in favorable sections (Fig. 5C). Axosomatic and axospinous synapses on somata appear to be similar ultrastructurally. Both classes of synapses have asymmetrical membrane specialization, clear round vesicles, and a light cytoplasmic matrix. A few synaptic junctions can be found on the primary dendrites close to their origin from cell soma. Such synapses are also asymmetrical and occur directly on the dendritic shafts.

By E105, a larger number of axospinous synapses are present on Purkinje cell somata (Fig. 6B). They are similar in size and ultrastructural characteristics to the synapses present on Purkinje cell somata at E97. In addition, at E105, some synapses are observed between parallel fibers and Purkinje cell dendritic spines (Rakic, 1973). It should be mentioned, however, that some parallel fiber-Purkinje cell synapses may occur directly on dendritic shafts at this age (Fig. 6A). In parallel with the increase in number and type of synapses during the period between E97 and E105, there is substantial growth and differentiation of the dendritic arbor. By E125, when cGK has reached its adult distribution and intensity, the maturing Purkinje cells have acquired a full complement of their synaptic repertoire (Rakic, 1973).

Discussion

Correlation between the emergence of cGK immunoreactivity and developmental events in Purkinje cells. Immunohistochemically detectable levels of cGK in the developing monkey cerebellum can first be observed around E97, i.e., 2 weeks after midgestation. This is approximately 1½ months after generation of all Purkinje cells, as revealed by [³H]thymidine labeling (Rakic, 1980). At E97, only Purkinje cells of the posterior lobe are cGK immunoreactive. Subsequently, cGK gradually becomes detectable in more anterior portions of the cerebellum until, around E125, all Purkinje cells are cGK positive.

During the first half of gestation, prior to the appearance of cGK immunoreactivity, Purkinje cells are in their initial stage

Figure 5. Earliest synapses to appear on Purkinje cells of fetal monkey. **A**, Electron micrograph of a Purkinje cell from an E91 fetus. No synapses are present along the cell soma at this age, which is prior to the appearance of cGK immunoreactivity in Purkinje cells. Magnification $\times 14,000$. **B**, A few axosomatic synapses (arrowheads) appear on the Purkinje cell soma at E97. One synapse on a Purkinje cell somatic spine (arrow) is also evident in this field. Magnification $\times 10,000$. **C**, Higher-power micrograph of a different Purkinje cell at E97 revealing a classical axospinous synapse (arrow). Magnification $\times 16,000$. *n*, nucleus.

of differentiation, designated "stage I" by Zecevic and Rakic (1976). The neurons are aligned in a layer several cells thick and have relatively few processes. Immunostaining for synapsin I in the present study demonstrated that few, if any, synapsin I-positive terminals are present in the Purkinje cell layer at this stage, whereas synapsin I-positive processes, probably mossy fiber terminals, are already visible in the granule cell layer. Electron microscopic examination confirmed that Purkinje cells at this stage lack morphologically definable synaptic

inputs, although climbing fibers, which comprise one of the two major extrinsic inputs to Purkinje cells, have entered the cerebellar hemispheres by this time (Fig. 7, *A* and *B*).

By the time cGK immunoreactivity appears in the posterior lobe of the cerebellum (E97), Purkinje cells have entered "stage II" of differentiation (Zecevic and Rakic, 1976). This period is characterized by an enormous increase in the surface area of Purkinje cells which is unparalleled in any other neuronal class and which reflects the rapid dendritic growth beginning rather

suddenly at midgestation (Zecevic and Rakic, 1976; Rakic, 1980). This growth is clearly illustrated by Golgi staining (Rakic, 1980), as well as by cGK immunofluorescence.

The first well defined synapsin I-positive terminals in the Purkinje cell layer appear in the posterior lobe of the cerebellum at E97, concurrently with the emergence of cGK immunoreactivity. At the electron microscopic level, synapses on Purkinje cells are observed for the first time. They are predominantly axosomatic, but a few synapses on the dendritic shafts of primary dendrites can be seen. Without the use of appropriate

tracing methods, it is not possible to classify these synapses as belonging to a single class of afferents, but there are at least two possibilities. One, widely accepted, is that these early synapses are formed with immature climbing fibers that transiently contact Purkinje cell bodies in monkey embryos (Kornuth and Scott, 1972; Rakic, 1973). These terminals are ascribed to the immature climbing fibers on the basis of their ultrastructural characteristics and subsequent shift to the dendritic tree, as has been elegantly demonstrated by Larramendi (1970) and Mugnaini (1970). The second possibility is that

Figure 6. Electron micrograph of cerebellar cortex at E105, 1 week following the emergence of cGK immunoreactivity. **A**, Examination of the molecular layer in a section perpendicular to the major axis of the folium reveals the presence of parallel fibers (*pf*) coursing by a Purkinje cell dendrite (*d*). Axodendritic synapses between parallel fibers and Purkinje cell dendrites are rarely encountered. One (*arrow*) is visible in this electron micrograph. **B**, In contrast, numerous axosomatic and axospinous synapses (*arrows*) are present along the maturing Purkinje cell body. *n*, nucleus. Magnification $\times 16,000$.

Figure 7. Schematic diagram depicting the relationship between the level of maturation of Purkinje cells, the establishment of their basic connectivity, and the appearance of cGK immunoreactivity during development in the rhesus monkey cerebellum. Prior to E97, no cGK-positive cells are seen. Around E95 (A), the Purkinje cell body contains somatic appendages and a few immature dendrites but does not receive any synapses. It has already established initial contacts with the dentate nucleus (DN) or one of the other Purkinje cell target nuclei. Early-generated granule cells (G) have well developed parallel fibers, but no synapses with Purkinje cell dendrites can be observed. By E97, cells in the anterior lobe (B) change only slightly. However, in the posterior lobe (C), Purkinje cells have matured beyond those in the anterior lobe. These posterior lobe Purkinje cells are the first to express immunohistochemically detectable levels of cGK (black areas). Their immature primary dendrites have grown and extend into the molecular layer (ML). In addition, the first axosomatic synapses, presumably belonging to climbing fibers (CF) and monoamine fibers (MA), appear at this age. The Purkinje cell axons and their terminals in the deep cerebellar nuclei do not exhibit immunofluorescence as yet. Lack of axonal staining at this stage, however, might be due to their small caliber rather than to a lack of cGK. Their axonal cytoplasm is therefore labeled by stippling in this figure. By E102, all Purkinje neurons in the cerebellar cortex have cGK-positive somata. They have undergone substantial dendritic growth and their axons and axon terminals in the dentate nucleus are now cGK positive. At about the same time (E105), the number of axosomatic and axospinous synapses has increased, but axodendritic synapses with parallel fibers have not yet appeared. By E125, the Purkinje cells appear morphologically well differentiated and possess numerous tertiary dendritic branches that are studded with spines. One class of spines receives climbing fiber inputs and/or monoamine fiber inputs; the other, more elongated spines receive granule cell inputs. GL, granule cell layer; PL, Purkinje cell layer; WM, prospective white matter.

some of these early synapses arise from the monoamine inputs. Neurons of both the locus ceruleus and the raphe nuclei of the brainstem are generated very early in monkey embryos (Levitt and Rakic, 1982). Their axons are known to invade the cerebellar primordium during the first half of pregnancy in the human fetus (Olson et al., 1973) and are present in the rat cerebellum at an early developmental stage as well (Olson and Seiger, 1972).

In the present study, cGK was found to emerge prenatally at the same time that Purkinje cells undergo the phase of rapid differentiation. It is noteworthy that cGK is not detectable by histone phosphorylation assay until after birth in the rat (Bandle and Guidotti, 1979), whereas it appears prenatally in the

monkey. However, this difference can be explained as a reflection of the later onset of cerebellar differentiation in the rat.

The protracted development of the primate brain allows one to establish a precise correlation of cGK appearance with specific cellular events during differentiation. Thus, in the present study, cGK emergence appeared to coincide with the formation of axosomatic synapses, the earliest synapses formed on Purkinje cells. Although axodendritic synapses were occasionally observed at E97 on Purkinje cells, such synapses were extremely rare. Furthermore, they were located in the smooth portion of dendritic shafts. Typical, adult-type parallel fiber-Purkinje cell synaptic junctions on dendritic spines were not observed until slightly later (Fig. 7).

Our results indicate that the emergence of cGK immunoreactivity, the establishment of synaptic inputs, and the onset of rapid dendritic growth occur at very similar times during development. It will be of interest to determine whether these cellular and biochemical phenomena are causally related or whether their concurrence reflects simultaneous but independent genetic expression. Recent studies demonstrate that the tempo of cGK appearance is maintained in heterotopic explants of developing cerebellar cortex (C. Sotelo, unpublished results). These explants are not reached by the proper extrinsic synaptic inputs such as climbing fibers, suggesting that cGK expression may not necessarily be triggered by such inputs. The latter experiments, however, do not rule out involvement of other synaptic inputs in cGK appearance in explants. In any case, even if the expression of cGK by Purkinje cells was not triggered by synaptic inputs but was simply co-programmed with synapse formation, the temporal coincidence of the two events may still be biologically meaningful. Thus, it is noteworthy that expression of cGK coincides with a phase of rapid neuronal differentiation indicated by the rapid dendritic growth and by the appearance of neuron-specific properties such as calcium conductances (Llinás and Sugimori, 1981). This supports the hypothesis that cGK-dependent protein phosphorylation is involved in neuron-specific processes (Nestler and Greengard, 1983).

Transient cGK-positive neurons in the prospective white matter of the developing cerebellum. In addition to Purkinje cells in the cortex, another population of cGK-positive neurons was transiently observed in the prospective white matter of the developing monkey cerebellum. In agreement with the observed relation between the onset of synaptic inputs and expression of cGK in Purkinje cells, appearance of cGK coincides with appearance of synapsin I immunoreactivity around the somata of noncortical cGK-positive neurons. These neurons had remarkable morphological similarities to Purkinje cells. However, at variance with Purkinje cells, they developed a stunted dendritic tree and were not observed at later stages of cerebellar ontogenesis. The hypothesis that they represent Purkinje cells still on their way to the cortex can be excluded for several reasons. First, all Purkinje cells are generated and have reached their position in the cerebellar cortex by E50, i.e., 1½ months before the cGK-positive neurons in the prospective white matter are first observed (Rakic, 1980; P. Rakic, unpublished observations). Second, these cGK-positive neurons already have multiple dendritic processes that would interfere with any significant translocation of their cell bodies. Possibly such neurons originate from the Purkinje cell precursors in the ventricular zone but fail to reach a cortical position. In spite of their ectopic location, these cells receive synaptic inputs and undergo an initial phase of differentiation (P. Rakic, unpublished observation). The disappearance of these cGK-positive cells at later stages of cerebellar ontogenesis may be due to their degeneration or to a reduction of cGK levels in their cytoplasm below the limit of detectability. The first possibility seems more likely for two reasons. First, the interstitial neurons that are found in the adult cerebellar white matter have dendritic trees remarkably different from Purkinje cells and from these cGK-positive cells (Ramón y Cajal, 1911). In contrast, at least in the rat, the dendritic trees of interstitial cells found in the adult cerebellum share important similarities with the dendritic trees of Golgi II cells and with deep cerebellar nuclei neurons (De Camilli et al., 1984b). Second, the total population of neurons present in the cerebellar white matter is greatly reduced in the adult. Since only a small fraction of cells in the prospective cerebellar white matter of the fetus are cGK positive, it is possible that these few cells undergo selective degeneration.

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