

## ONTOGENESIS OF OLIVOCEREBELLAR RELATIONSHIPS

### II. Spontaneous Activity of Inferior Olivary Neurons and Climbing Fiber-mediated Activity of Cerebellar Purkinje Cells in Developing Rats<sup>1</sup>

JEAN MARIANI<sup>2</sup> AND JEAN-PIERRE CHANGEUX

*Neurobiologie Moléculaire et Laboratoire Associé au Centre National de la Recherche Scientifique, Interactions Moléculaires et Cellulaires, Institut Pasteur, Paris, France*

#### Abstract

During development of vertebrate cerebellum, a multiple innervation of each Purkinje cell (PC) by several climbing fibers (CFs) (Crépel, F., J. Mariani, and N. Delhay-Bouchaud (1976) *J. Neurobiol.* 7: 567-578; Mariani, J., and J.-P. Changeux (1981) *J. Neurosci.* 1: 696-702) precedes the one-to-one relationship characteristic of the adult stage. In the present study, the spontaneous activity of inferior olivary neurons (whose axons constitute the CFs) and the spontaneous activity elicited in PCs by the CF system were investigated in developing rats.

Extracellular unitary recordings were obtained in medial and dorsal accessory olives from 249 neurons from 1-day-old to adult animals. The mean discharge rate varied from 0.19 spike · sec<sup>-1</sup> on day 1 to 1.69 spikes · sec<sup>-1</sup> on postnatal day 21 and 1.78 spikes · sec<sup>-1</sup> in adult animals. Although the spontaneous discharge was low, interspike interval histograms were constructed for a sample of cells. Their modal value varied from a mean value of 660 msec on day 1 to 250 msec in the adult with, however, marked variations at each age in the young animals.

Intracellular recordings from PCs were performed in rats ranging in age from 5 to 15 postnatal days. The spontaneous (i.e., asynchronous) discharge of excitatory postsynaptic potentials mediated through climbing fibers (CF-EPSPs) was measured and compared to the discharge of inferior olivary neurons at the same age. The main results are the following: (1) In PCs innervated by one CF, the mean frequency of CF-EPSPs was similar to the discharge of inferior olivary neurons; (2) in PCs innervated by several CFs, the mean discharge of CF-EPSPs was much higher than the discharge of inferior olivary neurons; (3) as a whole, the CF-mediated activity of PCs decreased from day 5 to day 11, while the multiple innervation disappeared; from then on, the activity increased with the maturation of inferior olivary neurons until adult values. The ratio of the frequencies of CF-EPSPs and olivary neuron discharges gives an approximate mean number of CF collaterals per PC at each age; it varied from 3.6 on day 5 to values close to 1 on day 11 and the following days.

The climbing fibers (CFs) which originate mainly, if not exclusively, from the contralateral inferior olivary (IO) nucleus, at least in rodents (Desclin, 1974), represent one of the two main afferent systems of the cerebellar cortex (Ramon y Cajal, 1911) and exert a powerful excit-

atory action on cerebellar Purkinje cells (PCs) (Eccles et al., 1966).

Since each PC receives only one CF in the adult mammal (Ramon y Cajal, 1911; Eccles et al., 1966), the classical climbing fiber response (CFR) of PC discharge reflects the discharge of one olivary cell at the adult stage. Some reports of the spontaneous discharge of IO cells in adult cats (Armstrong, 1974), rats (Headley et al., 1976), and mice (Mariani, 1980) exist, although the spontaneous activity of PCs has been studied more extensively (review in Armstrong, 1974; see also Belcari et al., 1977; Mariani and Delhay-Bouchaud, 1978; Armstrong and Rawson, 1979).

During the development of olivocerebellar relationships, a transient multiple innervation of each PC by several CFs has been described in rats (Crépel et al., 1976; Mariani and Changeux, 1980). Thus, the CF-me-

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<sup>2</sup> Chargé de Recherche, Institut National de la Santé et de la Recherche Médicale. To whom correspondence should be addressed at Institut Pasteur, Département de Biologie Moléculaire, 25, rue du Dr. Roux, 75724 Paris Cedex 15, France.

diated activity of these multiply innervated PCs is not identical to the spontaneous discharge of IO cells. Until now, data were available on neither the spontaneous activity of IO neurons during development nor the CF-mediated activity of the PCs innervated by one or several CFs. The aim of the present work was to describe and to compare these two spontaneous patterns of activity in the developing rat.

### Materials and Methods

Intracellular recordings of the spontaneous discharge of excitatory postsynaptic potentials mediated through climbing fibers (CF-EPSPs) from PCs in developing rats were obtained from cells used in the experiments presented in the accompanying paper (Mariani and Changeux, 1981).

Activity of IO neurons was recorded in Wistar rats from day 1 (day of birth = day 0) until adulthood at the ages indicated under "Results." Extracellular recordings from inferior olivary neurons were performed as described with *staggerer* mice (Mariani, 1980). The occipital bone was exposed, the atlanto-occipital ligament was removed, and unitary extracellular recordings of IO neurons were performed through a dorsal approach near the midline. Glass micropipettes filled with 3 M potassium acetate and methyl blue were used and their DC resistance measured *in situ* was 15 to 30 megohms. The physiological state of the animals was controlled during all of the experiments as described in the companion paper (Mariani and Changeux, 1981). The data were photographed with a camera and stored on a magnetic tape for further statistical analysis.

**Identification of IO cells.** During preliminary experiments, IO neurons were identified by their antidromic response after stimulation of the surface of the contralateral vermal surface as already described in mice (Mariani, 1980). However, since medial and dorsal accessory olives (MAO and DAO) constitute the main olivary projection to the cerebellar vermis (see, for example, Brodal, 1980), we decided to study only the neurons of these subnuclei for comparison with the activity of vermal PCs. Thus, it became necessary to identify the position of the cells recorded. In each experiment, one dye spot was made on each side by iontophoretic ejection of methyl blue and a maximum of 5 or 6 cells were recorded from a given animal; the unmarked cells were always located in the same sagittal plane as one of the spots, and their position was reconstructed from the depth indicated by the micromanipulator. Further identification of IO neurons was achieved from the shape of the responses (see "Results"). Moreover, in 1- and 3-day-old animals, the position of 80% of the cells recorded was marked by a dye spot.

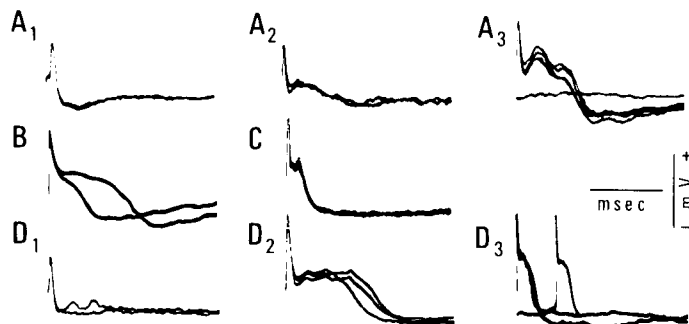
**Analysis of the data.** Mean spontaneous discharge and interspike interval histograms of IO neurons were studied with a small computer (Intertechnique, Didac 800). Bin widths used were 10 sec for mean discharge and 10 or 20 msec for interspike interval.

During the study of CF-EPSPs of PCs, a simple threshold system could not be used for counting, especially in the case of multiply innervated PCs where steps of different amplitude coexisted in the discharge, because of the multiwave shape of many of the spontaneous EPSPs.

A simple method was used: spontaneous EPSPs of a given PC were visualized on a memory scope and monitored through the audio system, with each sweep of the scope triggered by the rising edge of a given step. Each visualized and heard step was counted with a small manual counter. Whenever it was necessary (especially for multiply innervated PCs), the tape was used at a speed 2 or 4 times slower than the speed used for recordings; the appropriate correction of time scale was made later on.

### Results

**Ontogenesis of the spontaneous activity of inferior olivary neurons.** Extracellular unitary recordings were obtained from a total of 249 neurons located in medial and dorsal accessory olives (MAO and DAO) of Wistar rats from 1 day old to adulthood. The shape of the unitary responses recorded extracellularly was nearly the same at all of the stages studied. As the microelectrode was advanced progressively toward the cell, the unitary action potential, initially negative, rapidly became positive-negative (Fig. 1A<sub>1</sub>). The spontaneous activity of the cell was studied with this spike configuration. At the end of the recording, the microelectrode was advanced further and the response became purely positive with a sharp rising spike followed by a plateau on which wavelets sometimes appeared (Fig. 1, A<sub>2</sub>, A<sub>3</sub>, C, and D<sub>2</sub>). After this plateau, a negative wave appeared whose duration was about 80 to 150 msec (Fig. 1, A<sub>3</sub> and C). This positive configuration was observed, in our sample of cells, as early as postnatal day 1; its amplitude was usually 5 to 10 mV, even in young animals, but could reach 20 to 40 mV, especially at older stages (after 11 to 13 days). As already discussed (Mariani, 1980), this configuration resembles the intracellular responses obtained in the cat (Crill, 1970; Llinas et al., 1974) and probably corresponds to an electrode tip closely apposed to the cell membrane. This was confirmed in some cells from which DC poten-



**Figure 1.** Unitary recordings of spontaneous discharge of inferior olivary neurons in developing rats. Several sweeps are superimposed in all traces and each sweep is triggered by the rising edge of the response. A<sub>1</sub> to A<sub>3</sub>, IO neuron recorded extracellularly in a 1-day-old rat. The morphology of the response changed from A<sub>1</sub> to A<sub>3</sub> while the microelectrode was advanced toward the cell (see text). B and C, IO neurons recorded with the electrode presumably very close to the cell in 5- (B) and 10- (C) day-old rats. D<sub>1</sub> to D<sub>3</sub>, Olivary discharges obtained from an IO neuron in a 15-day-old rat. D<sub>1</sub> and D<sub>2</sub> were extracellular recordings. D<sub>3</sub> was obtained after penetration of the same cell. Calibration: horizontal bar, 20 msec in A<sub>1</sub> to A<sub>3</sub>, B, D<sub>1</sub>, and D<sub>2</sub>; 40 msec in C; and 80 msec in D<sub>3</sub>. Vertical bar, 8 mV in A<sub>1</sub> to A<sub>3</sub>, B, C, and D<sub>1</sub>; 20 mV in D<sub>2</sub>; 40 mV in D<sub>3</sub>.

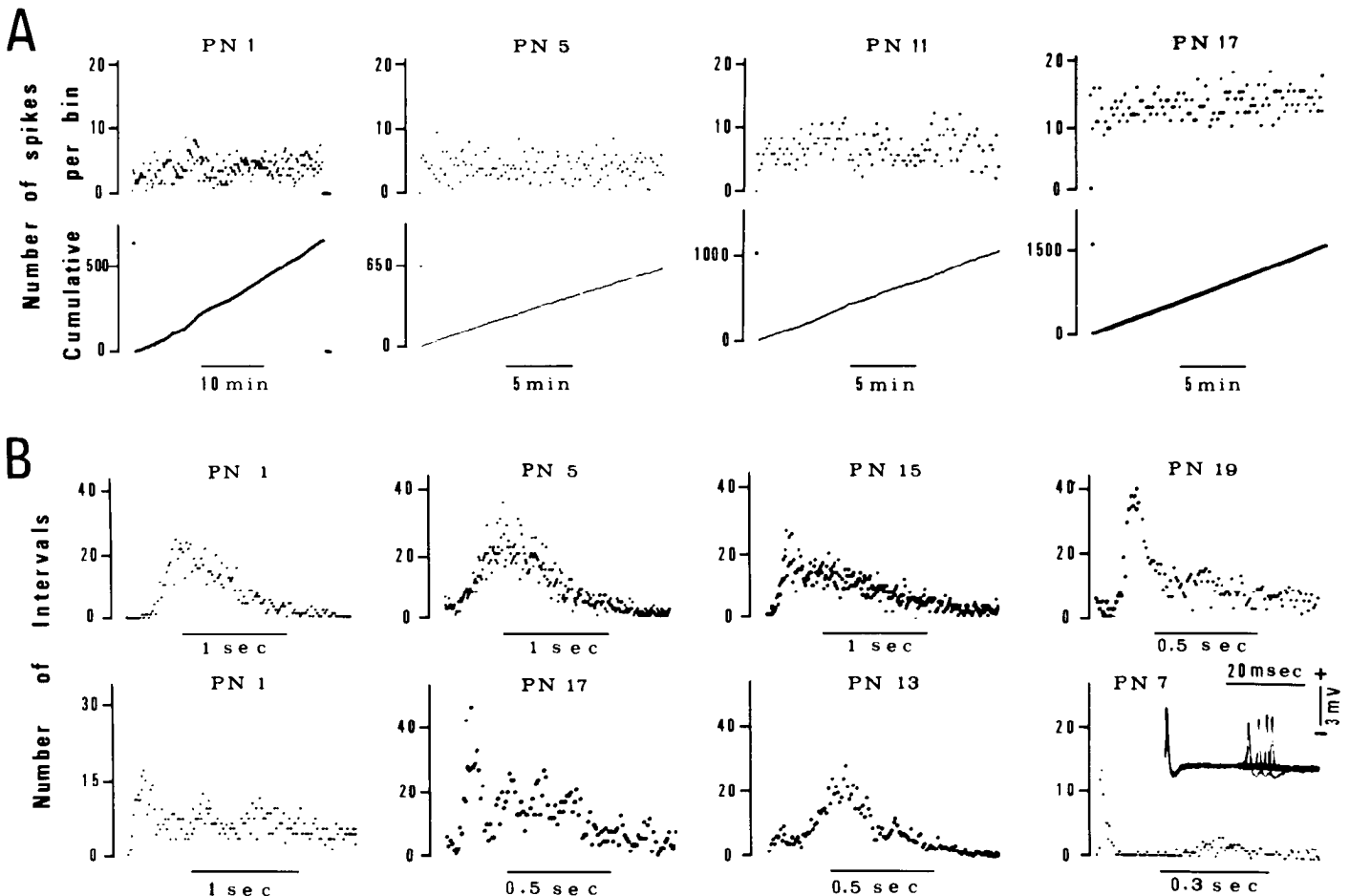
tial shift and full spike intracellular response were obtained with a further small advance of the microelectrode (Fig. 1D<sub>3</sub>).

The spontaneous discharge of the units was studied during intervals as long as 40 min but never shorter than 10 min. Examples of the discharge of IO neurons at four different ages are illustrated in Figure 2A (PN 1, 5, 11, and 17). The evolution with age of the mean discharge rate is illustrated in Figure 3A. The mean discharge was very low in newborn animals and increased more or less regularly from 0.19 spike/sec on day 1 ( $n = 16$ ,  $SD = 0.168$ ) to 1.67 spikes/sec on day 21 ( $n = 18$ ,  $SD = 0.28$ ). This last value did not differ significantly from the value found in adult rats (i.e., 1.78 spikes/sec,  $n = 22$ ,  $SD = 0.36$ ).

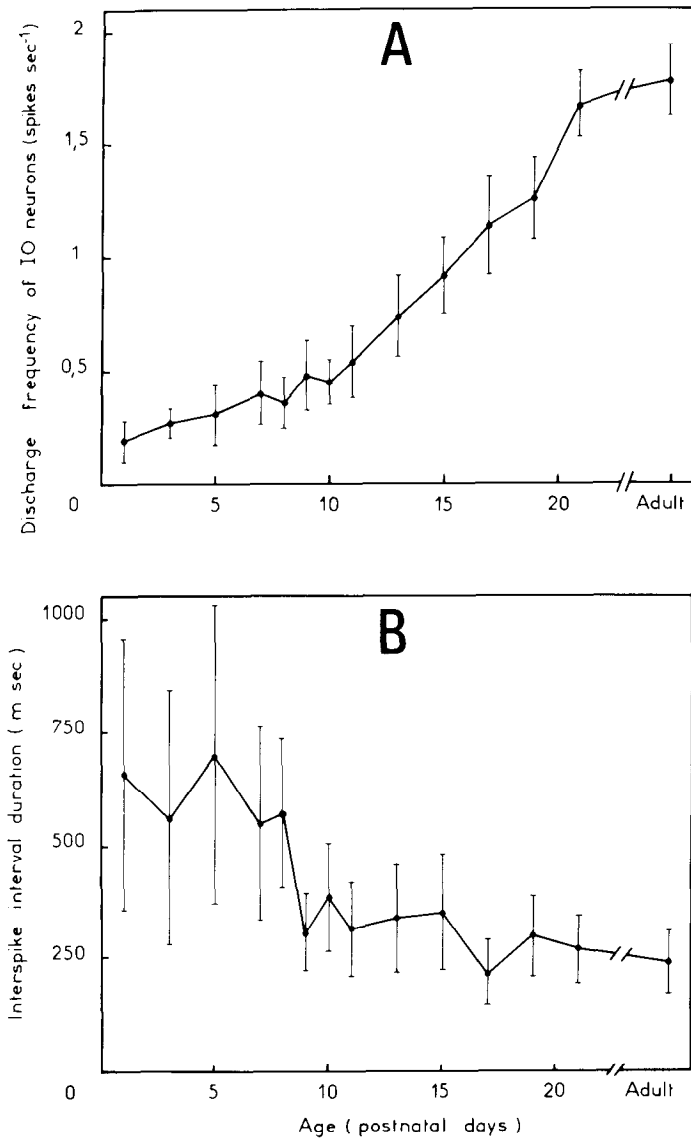
Interspike interval histograms were constructed from only some of the cells recorded, since a minimal number of events was necessary to obtain clear-cut peaks on the histograms and since the activity of the cells was low, especially until days 9 and 10. On postnatal days 1 and 3, most of the histograms were obtained from particularly

long recordings (30 to 40 min) of the most active IO cells; thus, the sample could be somewhat biased. A total of 137 histograms was studied through development; they were always skewed with a more or less pronounced tail to the right. Most of these histograms were unimodal as illustrated in Figure 2B, upper row. The remaining histograms exhibited two or sometimes three peaks, the highest one being most often the first or sometimes the second depending on the cells (Fig. 2B, lower row). The evolution with age of the mean values of the mode is illustrated in Figure 3B.

Large variability appeared especially from day 1 to day 8, around 600 to 700 msec, and an apparently abrupt decrease of the mean peak value occurred around postnatal days 9 and 10 when values similar to the adult ones were reached (around 300 msec). Furthermore, for a few cells recorded on postnatal day 7 and the following days, an early sharp peak appeared in the histograms (Fig. 2B, PN 7) and it corresponded to a repetitive activity of IO neurons at short intervals of about 20 to 40 msec as already described in adult mice (Mariani, 1980).



**Figure 2.** Spontaneous activity of IO neurons in developing rats. *A*, Frequency histograms of discharges recorded from IO cells at the indicated postnatal days (PN 1, 5, 11, and 17) (bin width = 10 sec). Note the progressive increase in the level of activity throughout development. Under each histogram, the cumulative frequency distributions of the corresponding cells illustrate the regularity of the spontaneous discharge throughout development. *B*, Interspike interval histograms of IO neurons recorded at the indicated postnatal days (PN). The bin widths used were 10 msec in all histograms except those for PN 1 and PN 7 where they were 20 and 6 msec, respectively. The upper row corresponds to the unimodal type of histograms; the lower row relates to the multimodal one. In the first two examples (PN 1 and PN 17) of multimodal histograms, the highest peak is the first one, while in the third example (PN 13), it is the second one. PN 7 disclosed an early peak in the histogram at 24 msec, corresponding to the repetitive discharge illustrated in the inset. Sampling times for interval histograms were between 20 and 33 min except for PN 13 (14 min).



**Figure 3.** Evolution with age of the spontaneous discharge of IO neurons in developing rats. *A* represents the evolution of the mean discharge frequency; each value is the mean of 14 to 25 cells. *B* represents the evolution of the mode of interspike interval histograms; each value is the mean of 9 to 17 cells. Vertical bars represent the confidence limit interval of the mean at each age (at 95%).

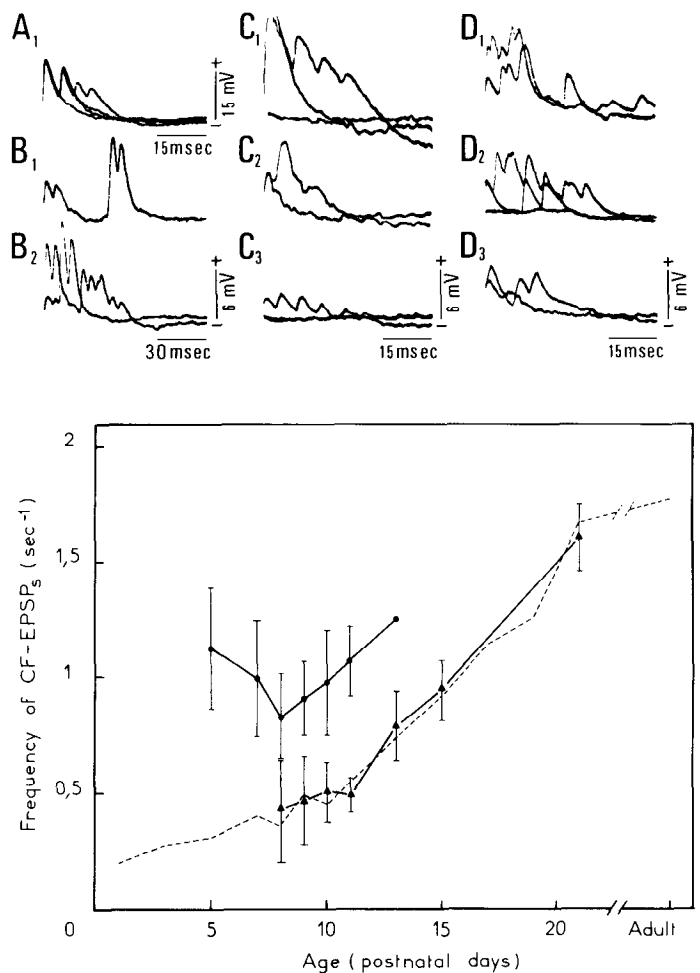
*Ontogenesis of climbing fiber-mediated activity of Purkinje cells.* The expression "spontaneous EPSPs" refers to EPSPs recorded from the background discharge of PCs while no electrical stimulation was applied to the CF pathway.

As already described in the companion paper (Mariani and Changeux, 1981), two populations of PCs were recorded, those in which spontaneous (and evoked) EPSPs had an all-or-none behavior (Fig. 4*A*) and the PCs whose EPSPs exhibited a stepwise variation in amplitude (Fig. 4, *B*, *C*, and *D*).

Interstep interval histograms were not constructed in developing rats, but it was clear from a qualitative point of view that the different CF-EPSPs tended to occur in a burst-like fashion in many cells (Fig. 4, *B*<sub>2</sub>, *D*<sub>1</sub>, and *D*<sub>2</sub>). It should be noticed that every effort was made to observe the small steps preceding higher ones (Fig. 4*B*<sub>1</sub>) since,

even in singly innervated PCs, when two EPSPs occurred with a short separation, the size of the second one often was reduced slightly (not illustrated). Although evoked inhibitory postsynaptic potentials (IPSPs) were observed first on day 7 and more commonly on day 9 and the following days (Mariani and Changeux, 1981), spontaneous CF-EPSPs were followed by slight hyperpolarization as early as day 5 (Fig. 4, *C*<sub>1</sub> and *C*<sub>3</sub>).

The spontaneous discharge of CF-EPSPs was studied from 130 PCs, 12 recorded in 21-day-old rats and 118 of the 310 cells used in the related research. The reduced



**Figure 4.** Intracellular recording of spontaneously occurring CF-EPSPs in developing rats. Several sweeps are superimposed in all traces and each sweep is triggered by the rising edge of the response. *A*<sub>1</sub>, All-or-none typical CF-EPSP recorded from an 11-day-old rat. *B*<sub>1</sub> and *B*<sub>2</sub>, CF-EPSPs recorded in a 9-day-old rat. Their sizes fluctuated in a stepwise manner. Note that, in *B*<sub>1</sub>, the small step precedes the high one. *C*<sub>1</sub> to *C*<sub>3</sub>, Different steps of CF-EPSPs recorded in a 5-day-old rat. Note that even the small step exhibits a multiwave shape. *D*<sub>1</sub> to *D*<sub>3</sub>, Spontaneous EPSPs in a 7-day-old animal. The superimposition of different steps (*D*<sub>1</sub> and *D*<sub>2</sub>) gives more complex shapes. The graph illustrates the evolution with age of the mean discharge of spontaneous CF-EPSPs in Purkinje cells. ●—●, Multiply innervated PCs; each value is the mean of 9 to 12 cells except for days 11 (4 cells) and 13 (2 cells). ▲—▲, Singly innervated PCs; each value is the mean of 10 to 18 cells except for day 8 (5 cells). The dashed line reproduces the evolution of the IO neuron discharge of Figure 3*A*. Vertical bars are as in Figure 3. No bar was indicated on day 13 for multiply innervated PCs because of the small number of these cells.

number of cells conserved for the measurement of the spontaneous activity is explained by the following: (1) We retained only cells in which at least 2 min of stable intracellular recording of spontaneous CF-EPSPs could be obtained; too few cells were counted on days 3 and 4 and these stages were discarded. (2) On the other hand, on postnatal days 13 to 15, we found that counting only 35 of the 100 cells recorded was sufficient for the purpose of the present study. (3) The PCs in which "dual" CF-EPSPs were recorded were not used for counting since, as already discussed by Puro and Woodward (1977a), dual responses might correspond either to one CF-EPSP or to several CF-EPSPs originating from different fibers.

The evolution of CF-evoked activity of PCs during development is summarized in Figure 4. The mean discharge rate in PCs that exhibited all-or-none CF-EPSP was studied from days 8 to 21 and fitted well with the spontaneous discharge of IO neurons at the same age. On the contrary, the activity presumably evoked through CFs in PCs whose EPSPs were graded stepwise was strikingly higher than the activity of IO neurons at the same age. The mean CF-EPSP discharge decreased slightly from days 5 to 8 and then increased regularly until postnatal day 13, the age at which the last multiply innervated PCs were recorded.

When the mean CF activities of singly and multiply innervated PCs of a given age were pooled, taking into account the respective percentage of multiple innervation at this age (see Mariani and Changeux, 1981), the result (Fig. 5A) gave a rough estimate of the CF-evoked activity of the whole population of PCs during development. This activity was clearly higher than the activity of the IO neurons until day 11; on day 11 and thereafter, the two activities became nearly identical, since very few cells exhibited CF-EPSPs with several steps. At each age, the ratio between IO and PC activities can be taken as an estimate of the mean number of functional CFs impinging

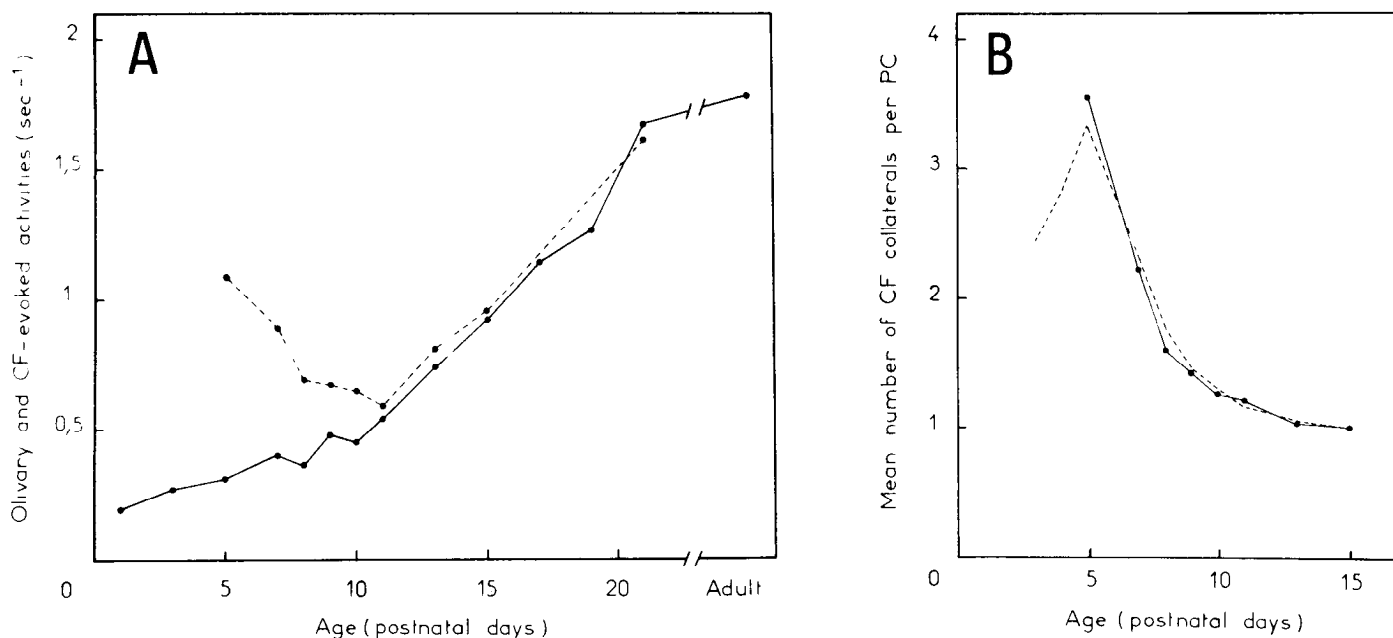
on the whole population of PCs. The mean number of CF collaterals decreased from 3.57 on day 5 to 1.46 on day 9 and finally reached 1 on day 15. This evolution fitted closely with the evolution of the mean number of steps in the EPSPs elicited by electrical stimulation of the CF pathway (Fig. 5B; Mariani and Changeux, 1981).

## Discussion

The spontaneous discharge of IO neurons and the spontaneous climbing fiber response (CFR) of PCs have been described already in adult animals from several species (Armstrong, 1974; Headley et al., 1976; Belcari et al., 1977; Mariani and Delhaye-Bouchaud, 1978; Armstrong and Rawson, 1979; Mariani, 1980). This paper deals with the spontaneous discharge of inferior olivary neurons and with the spontaneous synaptic activities recorded from PCs during postnatal development in rat.

The evidence indicating that the synaptic activities recorded from PCs throughout development were mediated through CFs has been presented in the companion paper (Mariani and Changeux, 1981) in which other modes of synaptic organization that might account for stepwise graded EPSPs have been discussed. It should be added that the excellent agreement between the mean discharge of EPSPs recorded in PCs innervated by one CF and the mean olivary discharge at the same age, even in the adult, strengthens the hypothesis that the synaptic activities recorded, indeed, were mediated through CFs.

*Spontaneous activity of olivary neurons in developing rats.* The cytological organization of the IO nucleus is already well established at birth (Altman and Bayer, 1978); nevertheless, the level of spontaneous activity in the accessory olives is very low at this age. The almost 10-fold increase of activity that leads to the adult value on postnatal day 21 might reflect both the development of the autorhythmic properties of olivary neurons (Llinas and Yarom, 1980) basically of dendritic origin (Llinas



**Figure 5.** Comparative study of olivary and Purkinje cell spontaneous discharges. *A*, Evolution with age of spontaneous discharge of IO neurons (●—●) versus CF-mediated discharge (●- -●) of the global population of PCs (see text for details). *B*, Mean number of CF collaterals per PC estimated by the ratio between IO and PC discharges (●—●). Note the good agreement with the value determined using the mean number of steps in the evoked response (dashed line).

and Sugimori, 1980b) and the maturation of the activity of afferent pathways to the IO nucleus. Morphological data on the maturation of such olivary afferences are scarce and concern only monkey (Robertson and Stotler, 1974) or opossum (Martin et al., 1980). However, Puro and Woodward (1977a) have demonstrated that, in the rat, transmission from both the periphery and sensorimotor cortex through the climbing fiber system to the Purkinje cells is found as early as postnatal day 3 but with immature features that change progressively during the first 2 postnatal weeks.

The organization of the spontaneous discharge of olivary neurons was investigated through the study of interspike interval histograms in adult and developing rats. In adult animals, the IO neurons tend to fire phasically, and one or sometimes more peaks of increased discharge probability appear at intervals that are multiples of a basic one, as already described in several other species (Armstrong, 1974; Belcari et al., 1977; Bell and Kawasaki, 1972; Mariani, 1980). Throughout development, the pattern of activity of IO neurons appears qualitatively similar to the adult one; the histograms were skewed toward longer intervals and showed one or more peaks of increased discharge probability. Although large variations existed in very young animals, the modal value of the interval histograms decreased rather abruptly on postnatal days 9 and 10 and reached values similar to the adult ones. At the same age, evoked repetitive climbing fiber inputs were recorded at intervals of 200 to 300 msec (Puro and Woodward, 1977a); these values were similar to the ones found for interspike interval peaks at the same stage. On day 7, repetitive discharges of IO cells at short intervals were first observed (Fig. 2B, PN 7) which might cause the "dual" EPSPs recorded from PCs from the same postnatal days (see Mariani and Changeux, 1981). It is also after the end of the 1st postnatal week that synchronization was noticed between high frequency bursts recorded from neighboring PCs (Dupont and Crépel, 1979) and that harmaline becomes able to elicit tremor and rhythmical olivocerebellar discharges in rats (Henderson and Wooley, 1970; Knowles and Phillips, 1980; J. Mariani, unpublished data) and rabbits (Barragan and Delhay-Bouchaud, 1980).

As a whole, these data suggest that the cellular mechanisms or the neural organization necessary for the rhythmical and synchronized discharges of inferior olivary neurons were established mainly at 7 to 10 days, although they could already be effective at earlier stages for some cells (see Fig. 2B, PN 1).

*Spontaneous CF-EPSPs in PCs of developing rats.* A salient feature of PCs which exhibited stepwise graded CF-EPSPs is a clear-cut increase in the discharge of spontaneously occurring synaptic potentials in comparison with the discharge of IO neurons or PCs with a one-step CF-EPSP at the same age. This increased activity was observed transiently during normal development from the earliest stages tested (5 days) until 11 to 13 days. The stepwise variation of spontaneously occurring CF-EPSPs provides additional evidence for a transient multiple innervation of PCs by CFs (Crépel et al., 1976; Mariani and Changeux, 1981). Stepwise variation of CF-EPSPs could result also from the innervation of a PC by several collaterals of the same climbing fiber with a

conduction failure occurring intermittently at the level of the branching, as already demonstrated for other axons (Parnas et al., 1969; Van Essen, 1973; Westerfield et al., 1978). In this situation, the mean discharge rate of the steps would not be higher than the discharge of IO neurons at the same age. The increased discharge of CF-EPSPs disclosed in this work supports the idea also suggested by former collision experiments that most of the CF collaterals converging on a PC belong to different IO neurons (Crépel et al., 1976).

The development of the overall spontaneous activity of PCs (Crépel, 1972) results from both possible endogenous properties of autorhythmicity (Llinas and Sugimori, 1980a, b) and synaptic activation of the PCs through their main afferences, first the CFs and then, after day 7, the mossy fiber-parallel fiber system (Puro and Woodward, 1977b). Our results indicate that, although the overall spontaneous activity of PCs augments during development (Crépel, 1972), the evolution of the activity through a given synaptic system might be more subtle. The evolution of CF-mediated synaptic activities in PCs during development results from two phenomena acting in opposite directions, the maturation of IO cells that acquire the properties of an oscillator (Llinas and Yarom, 1980) and the regression of supernumerary collaterals of CFs. It should be noticed that most of the regression occurs around days 7 to 10 during the appearance of the rhythmical features of the olivary discharge.

Throughout this regression, the comparison of CF-mediated spontaneous activity of PCs and IO neurons shows an excellent agreement with the data obtained from the study of the evoked CF-EPSPs (Mariani and Changeux, 1981). The mean number of PCs innervated by one IO neuron (the "olivary unit") decreases by a factor of about 4 during development. Since, in adult rat (Schild, 1970), the mean size of olivary unit is about 6 PCs and since no death of PCs has been described at the same period, our data suggest that the mean olivary unit is about 24 PCs when the multiple innervation is maximal.

In peripheral systems, recent experiments have shown that neural spontaneous activity might regulate several steps of synapse formation (see review in Changeux, 1979), including synapse elimination (Benoit and Changeux, 1975, 1978; O'Brien et al., 1978; Thompson et al., 1979). In the central nervous system, it has been well established that "peripherally evoked" activity plays a role during critical periods for the maintenance of functional characteristics of cortical visual cells (Wiesel and Hubel, 1965), although the underlying synaptic events have not been elucidated yet. It is tempting, although still speculative, to consider that the functional activity of the developing olivocerebellar network might regulate, directly or indirectly, the specification of the adult connectivity (Changeux and Danchin, 1976).

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