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Naturally occurring neuronal death during postnatal development of the cerebellar nuclei in the rat

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Summary

Naturally occurring neuronal death during the postnatal development of the cerebellar nuclei in the rat was analysed using classical cell counts and the labelling of fragmented DNA. Cell counts did not demonstrate a clear decrease in the number of cerebellar nuclei neurons along the first thirty days of postnatal life. However, the TUNEL technique revealed the existence of apoptotic cells with their DNA fragmented from the first to twentieth postnatal days. Apoptotic cells were randomly distributed throughout the cerebellar nuclei with no topographic preference and, in all cases, their numbers were very low, ranging from one to eight labelled cells per nucleus and section. The large amount of target (i.e., granule cells) that cerebellar nuclei neurons have could readily explain the absence of an important decrease in neuronal numbers during the postnatal development of these nuclei. The existence of apoptotic cells could be the expression of the withdrawal of neurons erroneously emplaced during the establishment of the refined topographic pattern of the cerebellar nucleo-cortical and cerebellofugal projections.

Key Words: DNA fragmentation - Neuronal death - Cerebellar projections - Target size - Numerical matching

Introduction

Naturally occurring neuronal death has been proposed as a developmental event after which the correct matching between the definitive number of neurons and the size of their appropriate target is attained (Oppenheim, 1991; Oppenheim et al., 1992; Raff et al., 1993; Homma et al., 1994). Naturally occurring cell death takes place in restricted time windows during development and is a consequence of new gene expression that gives rise to the apoptotic process (Milligan et al., 1995).

Naturally occurring neuronal death has been extensively studied in the cerebellar cortex and related precerebellar nuclei (Armstrong and Clarke, 1979; Bourrat and Sotelo, 1984; Delhaye-Bouchaud et al., 1985; Herrup and Sunter, 1987; Geoffroy et al., 1988; López-Román and Armengol, 1996). Thus, neuronal death coincides with the establishment of the adult projection of the olivocerebellar system (Bourrat and Sotelo, 1984; Delhaye-Bouchaud et al., 1985; Geoffroy et al., 1988, Fuhrman et al., 1995; López-Román and Armengol, 1996) and, as in other systems, depends on the availability of target (Armengol and López-Román, 1992). However, no attention has been paid to the possible existence of neuronal death during the development of the cerebellar nuclei (CN). CN are the main output of the information processed within the cerebellar cortex (Ramón y Cajal, 1904; Ito, 1984; Voogd, 1995) and also play a key role in the control of the cerebellar influence of the inferior olive

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(Angaut and Sotelo, 1989). The cerebellum and related precerebellar nuclei develop synchronously (Altman, 1982; Altman and Bayer 1985, 1987 a-d) and the precise pattern of nucleo-cortical and cerebellofugal circuitries is attained at the same time that the highly refined topography of the cerebellar system of afferent is established. Therefore, the existence of a similar synchronism would also be expected in the development of regressive phenomena during the establishment of the adult cerebellar circuitry.

The aim of this study was to analyse the presence of naturally occurring neuronal death during the postnatal development of the CN using the labelling of fragmented DNA (TUNEL method) (Gavrieli et al., 1992) and a classical cell count procedure, which helped to estimate the numerical evolution of CN neurons during the apoptotic period.

Materials and Methods

Experimental animals

Wistar rat pups bred in the colony of the Faculty of Medicine of Seville were used on postnatal (P) days 1, 3, 5, 10, 15, 20, 25 and 30. The day of birth was considered as postnatal day 0 (P0). Rat pups were deeply anaesthetised with ether (those aged between P1 and P10) or with sodium pentobarbital (30 mg/kg i.p., those aged from P15 to P30). All experiments were carried out in accordance with the guidelines established by the Spanish Royal Decree 223/1988.

TUNEL method

Rat pups (two animals per age) were perfused with 4% paraformaldehyde in 0.12M phosphate buffer. Brains were removed from the skull, and blocks of tissue comprising the cerebellum and attached brainstem were paraffin-embedded. Sections were cut at 6 µm thick in the coronal plane, mounted on stubbed slides and processed according to a modification of the TUNEL technique (Gavrieli et al., 1992) performed by Ferrer et al. (1994). Briefly, after proteinase K pre-treatment the sections were incubated with terminal deoxynucleotidyl transferase (TdT) and biotinylated 16-dUTP (Boehringer-Mannheim). The reaction product was amplified with the ABC kit (Vector) and revealed using 3,3'-diaminobenzidine as chromogen. Labelled cells were quantified as follows: (i) one section of every five was examined under a 40X objective, (ii) only those cells clearly exhibiting an extremely dark nucleus or nuclear masses were counted in both hemispheres, (iii) no correction procedures were used, due to the small size of the apoptotic cells, and (iv) the statistical analysis was the same as that employed for Nissl cell counts (see below).

Histology and cell counts

The brains of rats were dissected and immersed in Clarke's solution (absolute ethanol-acetic acid 3:1). The cerebellum plus attached brainstem was embedded in paraffin, cut serially at 10 µm in the coronal plane and stained with cresyl violet-thionine.

Twenty-four rats (three per age) were used. The boundaries of the CN were less defined in postnatal animals than in the adults (Altman and Bayer, 1985). Therefore, for the counting procedure the general division of three CN [i.e., lateral nucleus (LN), interposed nucleus (IN) and medial nucleus (ML)] was used. The cell count procedure has been reported elsewhere (López-Román and Armengol, 1996). Briefly, in each animal the CN neurons of both hemispheres were counted as follows: (i) one section out of every three was studied under a 40X objective; (ii) each nucleus was counted independently; (iii) only those cells clearly exhibiting a nucleus containing one nucleolus and a neuron-like cytoplasm were counted; (iv) the definitive number of CN neurons was obtained using the corrections for double counting (Abercrombie, 1946) and multinucleolation (Delhaye-Bouchaud et al., 1985); (v) the total number of CN neurons was obtained by adding the results of the three subnuclei per age; (vi) all cell counts were performed by the same investigator (LN and IN, F.P.; MN, C.M.) to minimise the effect of variables that might influence the results; (vii) Student's *t*-test was used to compare the mean values of the different ages.

Results

Although CN neurons are already located within their definitive domain at birth (Fig. 1A), these nuclei do not attain their characteristic cytoarchitectural subdivision until the fifth or seventh postnatal day, on which the different nuclei can be readily distinguished, as in adult animals (Fig. 1E). Immature CN comprise small cell bodies distributed throughout the incipient neuropil (Fig. 1B), in which mitotic figures can be frequently observed (Fig. 1B). From P10 to P30, the neuropil increases in size, and cerebellar neurons, larger than on preceding days, have triangular or rounded somata with evident Nissl bodies within their cytoplasm, and rounded nuclei with a pale chromatin from which the nucleolus is evident (Fig. 1C-D). In contrast with previous observations carried out in the inferior olive (Bourrat and Sotelo, 1984; Armengol and

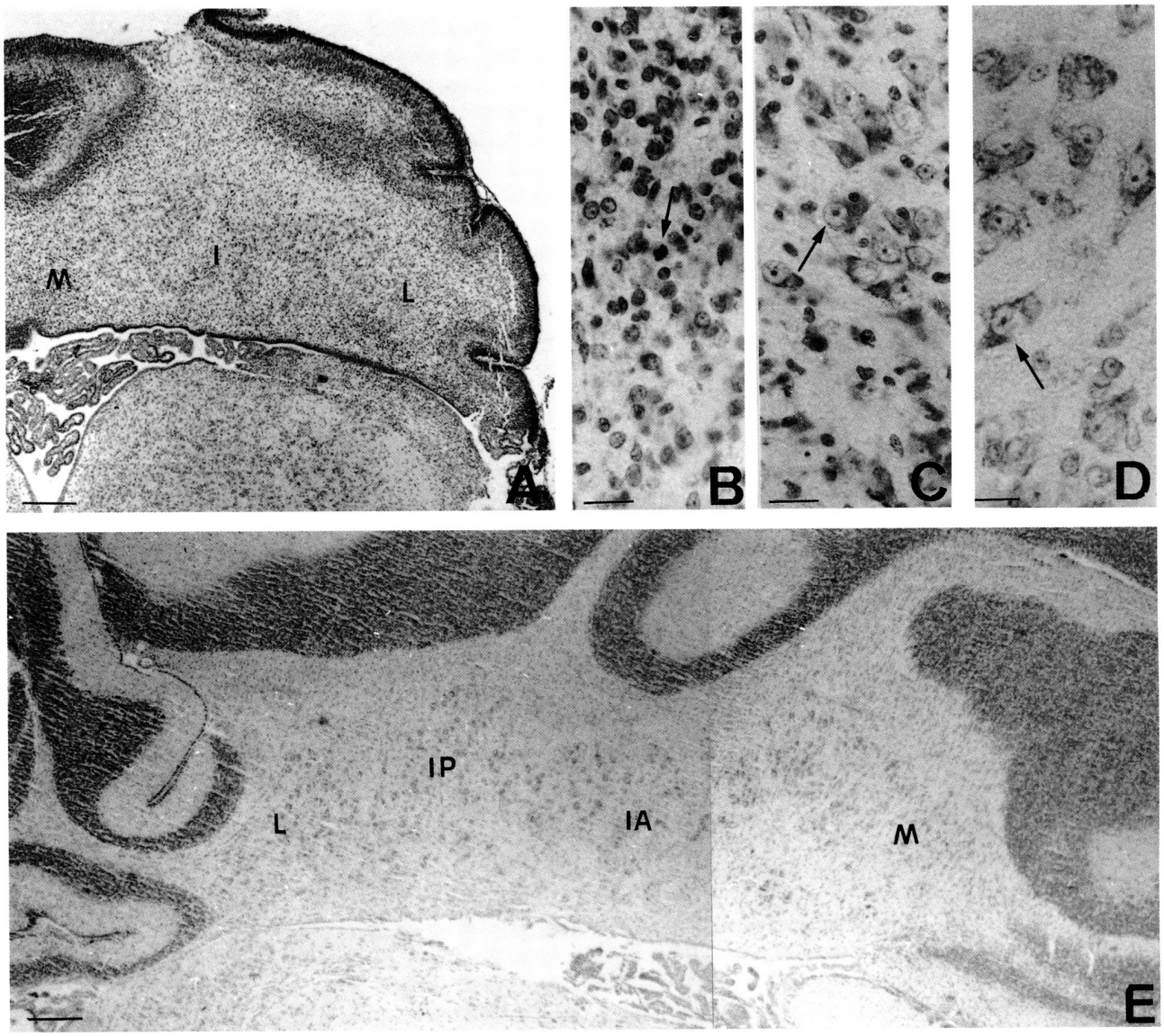

Fig. 1.- Nissl-stained coronal sections through the cerebellum of P1 (**A-B**), P10 (**C**) and P20 (**D-E**) rats. Although it is possible to distinguish the three main cerebellar nuclei in young animals, their adult morphology becomes completely delineated as from P5-7. In young animals cerebellar nuclei neurons are very small and densely packed (B). Mitotic figures are frequently observed at this date (B, arrow). C and D illustrate the morphological criteria followed for neuronal cell counts (arrows). I, interposed nuclei. IA and IP anterior and posterior interposed nucleus respectively. L, lateral nucleus. M, medial nucleus. The bar marks 200 μm in A and E and 20 μm in B-D.

López-Román, 1992; López-Román and Armengol, 1996), in Nissl-stained sections of the CN it was possible to observe the presence of dark stained condensed nuclear masses, strongly suggesting the existence of apoptotic phenomena (Fig. 2 D-E)(Clarke, 1990).

The labelling of fragmented DNA revealed the presence of labelled cells within the three CN from P1 to P15 animals, the period in which a slight decrease in the number of CN neurons was found in classic cell counts (see below). Labelled cells are randomly distributed throughout the CN (Fig. 2A,C) and are characterised by a dark brown reaction product that was confined as a homogeneous mass within the cell nuclei and was often fragmented (Fig. 2 F-H). On all days, the number of labelled cells was very small, ranging from none to eight cells per section in the most profusely labelled case. Cell counts revealed that the evolution of the number of labelled cells was quite similar to that defined by classic cell counts (see below). Labelled cells are observed from P1 to P20 in three clearly defined phases (Fig. 3): (i) from P1 to P10 the number of labelled cells remains almost constant ($p = 0.37$); (ii) the bulk of labelled cells is present between P10 and P15 [$p < 0.05$ ($p = 0.01$)]. After this period (iii) the presence of dying cells decreases until they disappear from P25 onwards (Fig. 3).

The classic cell counts performed from P1 to P30 (Table 1, Fig. 4) show that the neuronal cell number varies in each nucleus (Fig. 4A-C), as seen in the whole CN (Fig. 4D), according to

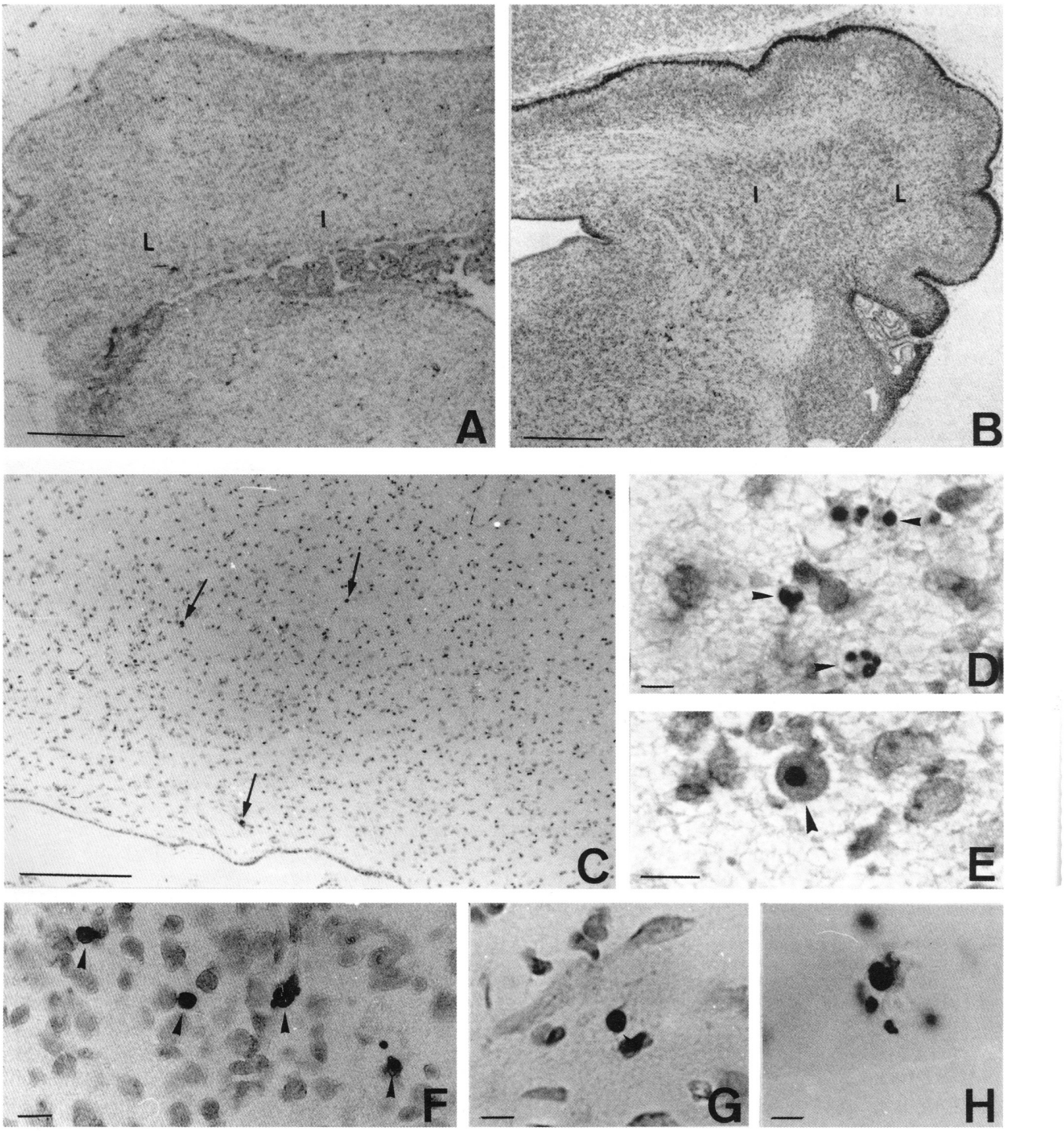

Fig. 2.- Coronal sections through the cerebellum of P1 (**A-B, F**) and the interposed nucleus of P10 (**C**) rats. A and B show the morphology of cerebellar nuclei in both TUNEL and Nissl-stained sections. In Nissl-stained sections is possible to observe the clusters of condensed chromatin characteristic of apoptotic figures (**D-E**, arrowheads). The detection of fragmented DNA reveals the random distribution of labelled cells (C, arrows). **F-H** illustrate the characteristic morphology of the condensed nuclear masses observed with the TUNEL technique (arrowheads). I, interposed nucleus. L, lateral nucleus. The bar marks 300 μm in A and B, 200 μm in C and 15 μm in D-H.

three different phases: (i) during the first ten days of postnatal life, there is an increase in cell numbers; however, in each nucleus and throughout the CN this increase is below the level of significance ($p > 0.05$); ii) from P10 to P15, a 13% reduction in neuron numbers occurs which, with the exception of the interposed nucleus (Fig. 4B), is statistically significant ($p<0.05$), and iii) a continuous increase is seen in cell number values from P15 to P30, finally reaching a peak cell number on P30, similar to that seen on P10 ($p > 0.05$).

Table 1.- Corrected values ± S.E.M. of the neuronal cell counts in the rat cerebellar nuclei.

Age	MN	IN	LN	CN
P1	2698±77	2091±62	5745±207	10533±268
P3	2462±255	2150±67	5886±238	10498±475
P5	2478±189	2821±319	6533±219	10833±461
P10	2671±178	2846±156	5525±196	11235±57
P15	2108±125	2707±85	4906±451	9741±450
P20	2043±181	2428±63	5293±308	9765±350
P25	2372±276	2330±53	5221±254	9926±337
P30	2460±223	2400±65	5624±170	10502±180

The nucleolar diameter of the cerebellar nuclei neurons used here for the Delhaye-Bouchaud et al. (1985) corrections was 1.72 ± 0.13. CN, cerebellar nuclei; IN, interposed nucleus; LN, lateral nucleus; MN, medial nucleus.

Discussion

In situ labelling of fragmented DNA within the CN reveals the existence of naturally occurring cell death during the postnatal development of CN. Classic cell counts have recently been replaced by dissector methods (for a review, see West, 1999), a more accurate method to determine the exact numbers of cell populations. An example of this is clearly illustrated in Figure 4, in which the first increase in the number of neurons could be due to mistakes in the counting procedure owing to the small size of young CN neurons. However, here we chose a cell counting procedure used in previous works addressing the analysis of cell death in the cerebellum and cerebellar afferents, because our aim was to observe the evolution of CN populations during postnatal development, rather than to quantify exactly the number of neurons comprising the CN. Therefore, the cell counts reported here should be considered as a complement to the study of the apoptotic phenomenon detected by the TUNEL technique.

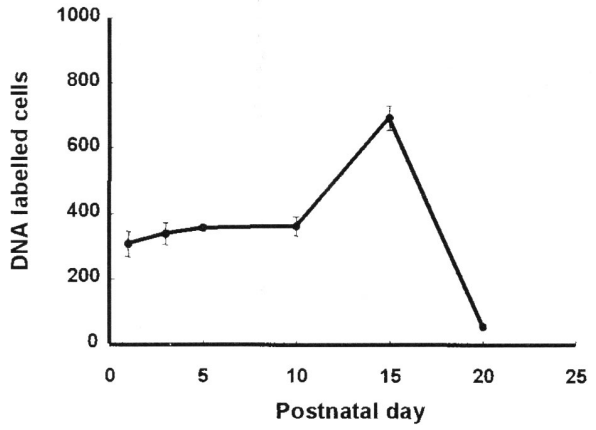

Fig. 3.- Evolution of the number of labelled cells from P1 to P20 throughout the cerebellar nuclei. The number of apoptotic cells is almost invariable during the first ten days of postnatal life. From P10 to P15 the number of dying cells increases. Very few labelled cells were encountered on P20.

Another point of discussion concerns the later increase in CN neuronal numbers as from P15, which could be related to a similar phenomenon found in cell counts of the inferior olive of both rodents and chicks (Caddy and Biscoe, 1979; Bourrat and Sotelo, 1984; Delhaye-Bouchaud et al., 1985; López-Román and Armengol, 1996). These observations have also been reported in the cerebellum (Diglio and Herrup, 1982) and other brain areas (Ruth and Goldsmith, 1983). No explanation is yet available for this increase, but the experimental data (see Armengol and López-Román, 1992) seem to suggest that such an increase would not be due to counting errors. However, as discussed above for the first postnatal days counted here the possibility of unbiased errors made during the cell counting procedure cannot be completely ruled out.

Naturally occurring neuronal death in CN extended from P1 to P15, a period when cortical cerebellar circuitry attains its adult organisation. Although no direct evidence is available on the date at which synaptogenesis between CN neurons and granule cells begins, in vitro horseradish peroxidase (Eisenman et al., 1991) and electrophysiological (Gardette et al., 1985) studies have indirectly revealed that cortico-nuclear and CN incoming afferents are present on the first days after birth. Further, in adults animals, mossy fibre terminals of nucleo-cortical fibres have been described as recurrent collaterals of projective nucleofugal fibres (Hamori et al., 1981). Nucleofugal fibres arise from the cerebellar nuclei towards their brainstem or thalamic targets during late embryonic life (Shirasaki et al., 1995; Song et al., 1995). Thus, it is possible to assume that nucleo-cortical projections would be present in the granular layer as from the beginning of granule cell migration. The period of time in which neuronal death took place in the CN is closely related to the presence of later regressive phenomena in cortical synaptogenesis as the elimination of redundant climbing fibres (Crepel, 1982; López-Román et al., 1993), which also coincides with the end of the molecular specification of the topographic maps of

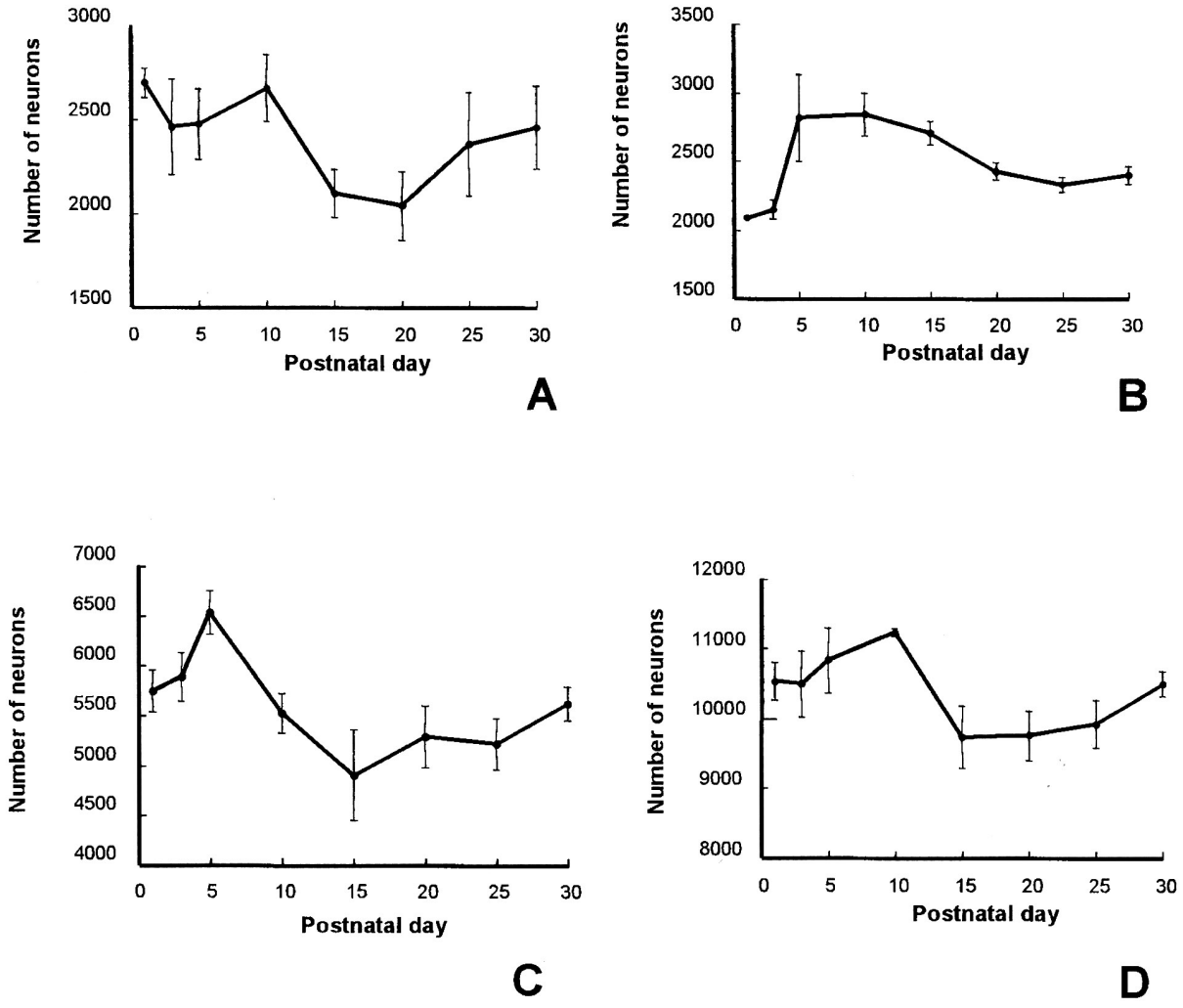

Fig. 4.- The number of neurons within the medial (A), interposed (B), lateral (C) and the whole cerebellar nuclei (D) of the rat as a function of age. Each symbol represents the mean corrected value ± S.E.M.

Purkinje cells (Hawkes et al., 1992; Wassef et al., 1992).

All this data, together with the large size of both cerebellar target cells available for CN neurons, whose ratio is 4.904 granules cells for each CN neuron [10.3 x 10^7 granules cells (according Harvey and Napper, 1988) : 21 x 10^3 CN neurons at P30, taking both sides)] and diencephalic and brainstem targets (Voogd, 1995), seem to exclude the possibility that CN neuronal death could be explained merely in terms of the numerical matching hypothesis (for a review, see Oppenheim, 1991; Oppenheim et al., 1992; Raff et al., 1993). An alternative explanation would be that qualitative differences of the targets would be responsible for CN neuronal death. In fact, adult cortico-nuclear, nucleo-cortical and cerebellofugal projections have a highly refined topographic pattern that allows the reciprocity of cortico-nuclear and nucleo-cortical projections (Buisseret-Delmas and Angaut, 1988; Umetani, 1991) as well as the segregation of CN efferent fibres (Song et al., 1993a; Song et al., 1995; Voogd, 1995). It can therefore be assumed that during development the complex final pattern of both nucleo-cortical and cerebellofugal projections induces competitive phenomena during synaptogenesis, and that these are based on qualitative factors of the target (i.e., positional cues for each cerebellar compartment, see Chedotal et al., 1997; Sotelo and Chedotal, 1997) as has been proposed for other brain regions (Catsicas et al., 1987).

In conclusion, the present results support the hypothesis of a general developmental schema for the cerebellum and precerebellar nuclei (Altman, 1982; Altman and Bayer 1985, 1987 a-d) in which both neuronal cell death (Bourrat and Sotelo, 1984; Delhaye-Bouchaud et al., 1985; Herrup and Sunter, 1987; López-Román and Armengol, 1996) and the elimination of axon collaterals (Crepel, 1982; López-Román et al., 1993; Song et al., 1993b) may play a crucial role in the establishment of the highly ordered pattern of connections of afferents to the adult cerebellar cortex.

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REFERENCES

ABERCROMBIE M (1946). Estimation of nuclear population from microtome sections. *Anat Rec*, 94: 239-247.

ALTMAN J (1982). Morphological development of the cerebellum and some of its mechanisms. In: Palay SL, Chan-Palay V (eds). *The Cerebellum. New Vistas*. Springer-Verlag, Berlin, pp 8-49.

ALTMAN J and BAYER SA (1985). Embryonic development of the rat cerebellum. II. Translocation and regional distribution of the deep neurons. *J Comp Neurol*, 231: 27-41.

ALTMAN J and BAYER SA (1987a). Development of the precerebellar nuclei in the rat: I. The precerebellar neuroepithelium of rhombencephalon. *J Comp Neurol*, 257: 477-489.

ALTMAN J and BAYER SA (1987b). Development of the precerebellar nuclei in the rat: II. The intramural olivary migratory stream and the neurogenetic organization of the inferior olive. *J Comp Neurol*, 257: 490-512.

ALTMAN J and BAYER SA (1987c). Development of the precerebellar nuclei in the rat: III. The posterior precerebellar extramural migratory stream and the lateral reticular and external cuneate nucleus. *J Comp Neurol*, 257: 513-528.

ALTMAN J and BAYER SA (1987d). Development of the precerebellar nuclei in the rat: IV. The anterior extramural migratory stream and the nucleus reticularis tegmenti pontis and the basal pontine gray. *J Comp Neurol*, 257: 529-552.

ANGAUT P and SOTELO C (1989). Synaptology of the cerebello-olivary pathway. Double labelling with anterograde axonal tracing and GABA immunocytochemistry in the rat. *Brain Res*, 479: 361-365.

ARMENGOL JA and LÓPEZ-ROMÁN A (1992). Left unilateral pedunculotomy prevents neuronal death during postnatal development of the remaining left inferior olivary complex in the rat. *Eur J Neurosci*, 4: 640-647.

ARMSTRONG RC and CLARKE PGH (1979). Neuronal death and the development of the pontine nuclei and inferior olive in the chick. *Neuroscience*, 4: 1635-1647.

BOURRAT F and SOTELO C (1984). Postnatal development of the inferior olivary complex in the rat. III. A morphometric analysis of volumetric growth and neuronal cell number. *Dev Brain Res*, 16: 241-251.

BUISSERET-DELMAS C and ANGAUT P (1988). The cerebellar nucleocortical projections in the rat. A retrograde labelling study using horseradish peroxidase combined to a lectin. *Neurosci Lett*, 84: 255-260.

CADDY KWT and BISCOE J (1979). Structural and quantitative studies on the normal CH3 and lurcher mutant mouse. *Philos Trans R Soc Lond (Biol)*, 287: 167-201.

CATSICAS S, THANOS S and CLARKE PGH (1987). Major role for neuronal death during brain development: Refinement of topographical connections. *Proc Natl Acad Sci USA*, 84: 8165-8168.

CHEDOTAL A, BLOCH-GALLEGO E and SOTELO C (1997). The embryonic cerebellum contains topographic cues that guide developing inferior olivary axons. *Development*, 124: 861-870.

CLARKE PGH (1990). Developmental cell death: morphological diversity and multiple mechanisms. *Anat Embryol*, 181: 195-213.

CREPEL F (1982). Regression of functional synapses in the immature mammalian cerebellum. *Trends Neurosci*, 5: 266-269.

DELHAYE-BOUCHAUD N, GEOFFROY B and MARIANI J (1985). Neuronal death and synapse elimination in the olivocerebellar system. I. Cell counts in the inferior olive of developing rats. *J Comp Neurol*, 232: 299-308.

DIGLIO TJ and HERRUP K (1982) A significant fraction of the adult number of Purkinje cells first appears between postnatal days 16 and 30 in the mouse. *Soc Neurosci Abstr*, 8: 636.

EISENMAN LM, SCHALEKAMP MPA and VOOGD J (1991). Development of cerebellar cortical efferent projec

FERRER I, TORTOSA A, BLANCO R, MARTÍN F, SERRANO T, PLANAS A and MACAYA A (1994). Naturally occurring cell death in the developing cerebral cortex of the rat. Evidence of apoptosis-associated internucleosomal DNA fragmentation. *Neurosci Lett*, 182: 77-79.

FUHRMAN Y, PIAT G, THOMSON MA, MARIANI J and DELHAYE-BOUCHAUD N (1995). Abnormal ipsilateral functional vibrisae projection onto Purkinje cells mutiply innervated by climbing fibres in the rat. *Dev Brain Res*, 87: 172-178.

GARDETTE R, DEBONO M, DUPONT J-L and CREPEL F (1985). Electrophysiological studies on the postnatal development of intracebellar nuclei neurons in rat cerebellar slices maintained in vitro. I. Postsynaptic potential. *Dev Brain Res*, 19: 47-55.

GAVRIELI Y, SHERMAN Y and BEN-SASSON SA (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol*, 119: 493-501.

GEOFFROY B, SHOJAEIAN H, DELHAYE-BOUCHAUD N and MARIANI J (1988). Neuronal death and synapse elimination in the olivocerebellar system. III. Cells counts in the inferior olive of developing rats X-irradiated from birth. *J Comp Neurol*, 267: 296-305.

HAMORI J, MEZEY E and SZENTAGOTHAI J (1981). Electron microscopic identification of cerebellar nucleo-cortical mossy terminals in the rat. *Exp Brain Res*, 44: 97-100 (1981).

HARVEY RJ and NAPPER RMA (1988). Quantitive study of granule cells in the cerebellar cortex of the rat. *J Comp Neurol*, 274: 151-157.

HAWKES R, BROCHU G, DORÉ L, GRAVEL C and LECLERC N (1992). Zebrins: Molecular markers of compartmentation in the cerebellum. In: Llinás R, Sotelo C (eds). *The Cerebellum Revisited*. Springer-Verlag, Berlin, pp 22-55.

HERRUP K and SUNTER K (1987). Numerical matching during cerebellar development: Quantitative analysis of granule cell death in staggerer mouse chimeras. *J Neurosci*, 7: 829-836.

HOMMA S, YAGINUMA H and OPPENHEIM RW (1994). Programmed cell death during the earliest stages of spinal cord development in the chick embryo: A possible means of early phenotypic selection. *J Comp Neurol*, 345: 377-395.

ITO M (1984). *The Cerebellum and Neural Control*. Raven Press, New York.

LÓPEZ-ROMÁN A, AMBROSIANI J and ARMENGOL JA (1993). Transient ipsilateral innervation of the cerebellum by

developing olivocerebellar neurons. A retrograde double-labelling study with fast blue and diamidino yellow. *Neuroscience*, 56: 485-497.

López-Román A and Armengol JA (1996). Naturally occurring neuronal death during the development of the inferior olive in the chick. *Neurosci Res*, 26: 171-179.

Milligan CF, Prevette D, Yaginuma H, Homma S, Cardwell C, Fritz LC, Tomaselli KJ, Oppenheim RW and Schwartz LM (1995). Peptide inhibitors of the ICE protease family arrest programmed death of motoneurons in vivo and in vitro. *Neuron*, 15: 385-93.

Oppenheim RW (1991). Cell death during development of the nervous system. *Annu Rev Neurosci*, 14: 453-501.

Oppenheim RW, Schwartz LM and Shatz CJ (1992). Neuronal death, a tradition of dying. *J Neurobiol*, 23: 1111-1115.

Raff MC, Barres BA, Burne JF, Coles HS, Ishizaki Y and Jacobson MD (1993). Programmed cell death and the control of cell survival: Lessons from the nervous system. *Science*, 262: 695-700.

Ramón y Cajal S (1904). *Textura del Sistema Nervioso del Hombre y de los Vertebrados*, Vol. 2. Imprenta y Librería de Nicolás Moya. Madrid. Re-ed. Gráficas Vidal Leuca, Alicante.

Ruigrok TJH and Cella F (1995). Precerebellar nuclei and red nucleus. In: Paxinos GE (ed). *The Rat Nervous System*. 2nd ed. Academic Press, Sydney. pp 277-308.

Ruth RE and Goldsmith SK (1983). Postnatal neuron death in the locus coeruleus of rats. *Soc Neurosc Abstr*, 9: 857.

Shirasaki R, Tamada A, Katsumata R and Murakami F (1995). Guidance of cerebellofugal axons in the rat embryo: directed growth toward the floor plate and subsequent elongation along the longitudinal axis. *Neuron*, 14: 961-972.

Song W-J, Kanda M, Ohno T, Kanda M, Oikawa H and Murakami F (1983a). Segregation of cerebellorubral synaptic inputs on rubrospinal neurons of fetal cats as demonstrated by intracellular recording. *Neurosci Lett*, 159: 99-102.

Song W-J, Kobayashi Y and Murakami F (1983b). An electrophysiological study of a transient ipsilateral interpositorubral projection in neonatal cats. *Exp Brain Res*, 92: 399-406.

Song W-J, Kanda M and Murakami F (1995). Prenatal development of cerebrorubral and cerebellorubral projections in cats. *Neurosci Lett*, 200: 41-44.

Sotelo C and Chedotal A (1997). Development of the olivocerebellar projection. *Perspect Dev Neurobiol*, 5: 57-67.

Umetani T (1991). Topographic organization of the cerebellar nucleocortical projection in the albino rat: and autoradiographic orthograde study. *Brain Res*, 507: 216-224.

Voogd J (1995). Cerebellum. In: Paxinos GE (ed). *The Rat Nervous System*. 2nd ed. Academic Press, Sydney, pp 309-350.

Wassef M, Angaut P, Arsenio-Nunes L, Bourrat F and Sotelo C (1992). Purkinje cell heterogeneity: Its role in organizing the topography of the cerebellar cortex. In: Llinás R, Sotelo C (eds). *The Cerebellum Revisited*, Springer-Verlag, Berlin, pp 5-21.

West MJ (1999). Stereological methods for estimating the total number of neurones and synapses: issues of precision and bias. *Trends Neurosci*, 22: 51-61.