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Effect of ethanol on the cerebellar cortex of the chick embryo

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Summary. The effect of ethanol on the cerebellar cortex of chick embryos was studied in semi-thin sections of material prepared for electron microscopy. The embryos were injected with ethanol on the 3rd or 6th day of incubation and observed until days 13, 15, 17 and 21 of development. A decrease was seen in the number of germinal cells generated, together with defects in neuronal migration and the existence of a lower quantity of cells due to a generalised process of cell death. At the same time, a progressive neuronal degeneration was observed until the 15th day of incubation, the tissue recovering progressively on days 17 and 21. On the other hand, the embryos treated with ethanol on the 3rd day were less affected than those injected on the 6th day.

Key words: Ethanol, Cerebellum, Granule cells, Chick

Introduction

The morphology of the cerebellar neurons and their intracortical connections is that part of the central nervous system most studied in recent years. Ramón y Cajal showed in 1890, using argent impregnations, that the cerebellar cortex has a limited number of neuron types and a stereotyped pattern of architectonic organization. This latter is characterized by its structural uniformity in the whole extension of the cortical mantle (Ramón y Cajal, 1890; Palay and Chan-Palay, 1974).

The cerebellar plaque arises from young neurons which surround the IV ventricle and emigrate out of the germinal zone (Rakic and Sidman, 1970). In the chick embryo, this migratrion takes place before the fourth day of incubation (Saetersdal, 1959; Forstronen, 1963; Hanaway, 1967).

The ventricular germinal zone gives rise to the cells of Purkinje, Golgi and the cerebellar glia (Miale and Sidman, 1961; Fujita, 1964; Altman, 1972a,b). The other neurons of the cerebellar cortex, such as the star cells, basket cells, granular cells, and other glial elements, derive from original germinal cells in the rhombic heights. The germinal cells emigrate over the surface of the cerebellar plaque to form a transitory germinal zone situated in the most external part of the cerebellar cortex called the external granular layer (Miale and Sidman, 1961; Hanaway, 1967; Altman, 1972a,b).

The morphological transformations of the cerebellar cortex cells during their differentiation have been repeatedly analysed using argent impregnation, electron microscopy and autoradiography techniques (Saetersdal, 1959; Purpura et al., 1964; Hanaway, 1967; Larramendi, 1969; Mugnaini, 1969; Altman, 1972a,b; Zecevic and Rakic, 1976; Quesada and Génis-Gálvez, 1983).

The central nervous system can easily be altered by distinct teratogenic substances, and is particularly affected under the conditions of alcoholism (Courville, 1955; Freund, 1973; Begleiter, 1981; Lieber and Schenkers, 1982). It is known that of all the drugs used by man that are able to produce tolerance, ethyl alcohol is the one that presents the most devastating effects on this system.

Of the neurologically originated alterations most frequently resulting from alcoholism, the cerebellar syndrome is one of the most evident. This has raised the idea of the existence of a marked vulnerability of the cerebellum to the action of this neurotoxin (Romano et al., 1940; Neubuerger, 1957; Victor et al., 1959), characterised by great cerebellar lesions, such as neuronal degeneration, the granular layer being the most affected (Allsop and Turner, 1966; Ferrer et al., 1984).

The first studies on alcoholic embryofetopathia were due to Lemoni et al. (1968) and to Jones et al. (1973), who described the typical features and characteristics of the syndrome, giving them the name of the «fetal alcoholic syndrome», by which it is known today.

The majority of the research groups that have studied

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the effects of ethyl alcohol on the central nervous system give most importance to the physiological, psychological and biochemical questions (Walker and Hunter, 1971; Jarlstedt, 1972; Tewari and Noble, 1979; Abraham and Hunter, 1982) and give less value to the morphological aspects.

For this reason, on one hand, neither the mechanisms which condition the appearance of the pathological indications, nor their morphological and ultrastructural scope, is known today, and on the other, there is a lack of knowledge of the alterations which the ingestion of alcohol can cause to the processes of cell migration and differentiation. Thus, in the present work we analyse the effect of ethanol on the cell populations of the cerebellar cortex, as many clinical signs of the different pathological indications, such as motor uncoordination, trembling, slowness of movement, lack of precision, could be due to defects in the migration and differentiation of the neurons of the different nerve centres.

Materials and methods

75 fertilised White Leghorn eggs were injected with 15 µl ethanol 96% and 20 controls eggs with 15 µl saline solution. The injections were made at two different times, before the initiation of morphological differentiation, at 3 days of incubation (HH-18), or before initiation of the process of synaptogenesis, at 6 days of incubation (HH-29).

Later the embryos were extracted and the cerebellums dissected at 13, 15, 17 and 21 days of incubation respectively. The dates of extraction of the embryos were chosen close together with the aim of detecting morphological events occurring over brief periods of time.

The development at the moments of injection and extraction was determined systematically by means of the tables of Hamburger and Hamilton (1951).

The embryos were processed according to the technique of Palay and Chan-Palay (1974), cut to 1 µm with a glass blade, stained with 1% toluidine blue in 1% sodium borate (Richardson et al., 1960), and photographed in a Leitz orthoplan microscope. Copex Pan 35 mm film was used.

Results

The control cerebellum of the chick embryos at 13 days of incubation has an external granular layer of considerable thickness (Fig. 1 EGL). The incipient molecular layer is small and occupied by a large quantity of cells in emigration towards the internal granular layer, and by the cells which differentiate in that layer: star cells and basket cells (Fig. 1 ML). The young Purkinje cells from three rows of nuclei from whose perikariums the principal dendritic trunks begin to individualize (Fig. 1 P). The disalinement of the Purkinje cells results in the inexistence of a clear boundary between the molecular layer and the internal granular layer (Fig. 1 IGL). Those granule cells which reach the internal granular layer (Fig. 1 g) are very dispersed, intercalated with Golgi cells (Fig. 1 G), and form a layer with little compactness, extensive fibrillar zones and little thickness.

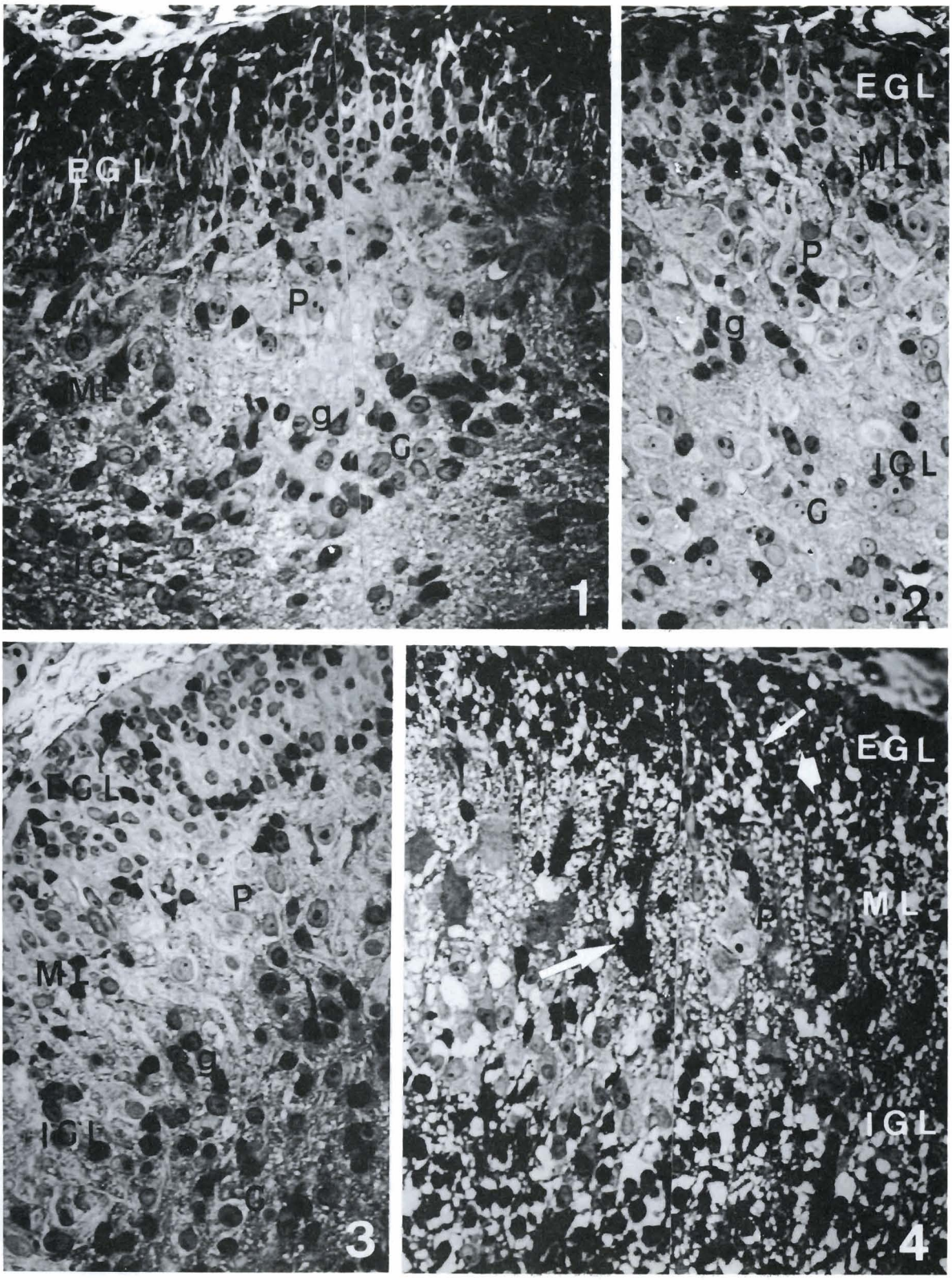
The cerebellums of chick embryos injected with ethanol on the 3rd day of incubation and sacrificed at 13 days of development (Figs. 2, 3), show an external granular layer less dense than in the control (Figs. 2 EGL, 3 EGL). The molecular layer is of little importance, and is more highly populated than in the control (Figs. 2 ML, 3 ML). The Purkinje cells show cytoplasmic destructuration, such as swellings, spaces and vacuoles (Figs. 2 P, 3 P).

The internal granular layer is more immature than in the control, the cellular elements in it are seen to be less abundant and more disperse (Figs. 2 g G, 3 g G).

The destructurating and noxius effect of ethanol on the cerebellar cortex is much more evident in the case of the embryos of 13 days of incubation which have been injected with alcohol on the 6th day of incubation (Fig. 4). The external granular layer (Fig. 4 EGL) conserves the same thickness but is occupied by a great number of picnotic cells (Fig. 4 EGL, white arrow head), between which there are large extracellular spaces (Fig. 4, thin arrow) with remains of cytoplasmic structures and lacking in defined organules. The molecular layer (Fig. 4 ML) is broader, crossed by orifices and vaculised canals, and scarcely traversed by migratory neuroblasts. In the Purkinje cell layer there are abundant cells with degenerative signs, turgidity, vacuolisation, large cellular spaces, disorientation, and numerous picnotic cells (Fig. 4 P and broad arrow). The internal granular layer (Fig. 4 IGL) is not free of this great destructurating effect of ethanol: the same aberrant characteristics being observed as in the superior layer, with the greater abundance of cell-free spaces standing out.

After 15 days of incubation, the cerebellums of the chick embryo controls (Fig. 5) show a slightly thinner external granular layer, formed by rows of some 8 to 10 cells deep. The subproliferative zone is somewhat greater than at 13 days of incubation. The molecular layer (Fig. 5 ML) increases in size considerably, and is composed of neurons which migrate towards the deeper layer, and the Purkinje cells begin to develop dendrites through the complex framework of its thickness (Fig. 5 P). The Purkinje cells are still arrayed in two rows, but the greater organization of their dendritic trunks is noteworthy if compared with that of the previous control. The internal granular layer (Fig. 5 IGL) begins to be a conglomeration of granule cells (Fig. 5 g) grouped in rosettes or clusters, in which the fibrillar connections have considerably reduced their extent, giving way to an increase in the number and distribution of the Golgi cells, which are easily distinguishable from the granule cells by the light perikariums and great size of the former.

The embryos treated with ethanol on the 3rd day of incubation and sacrificed at 15 days of incubation (Figs. 6, 7), show a greater effect than those injected under the same conditions and sacrificed at 13 days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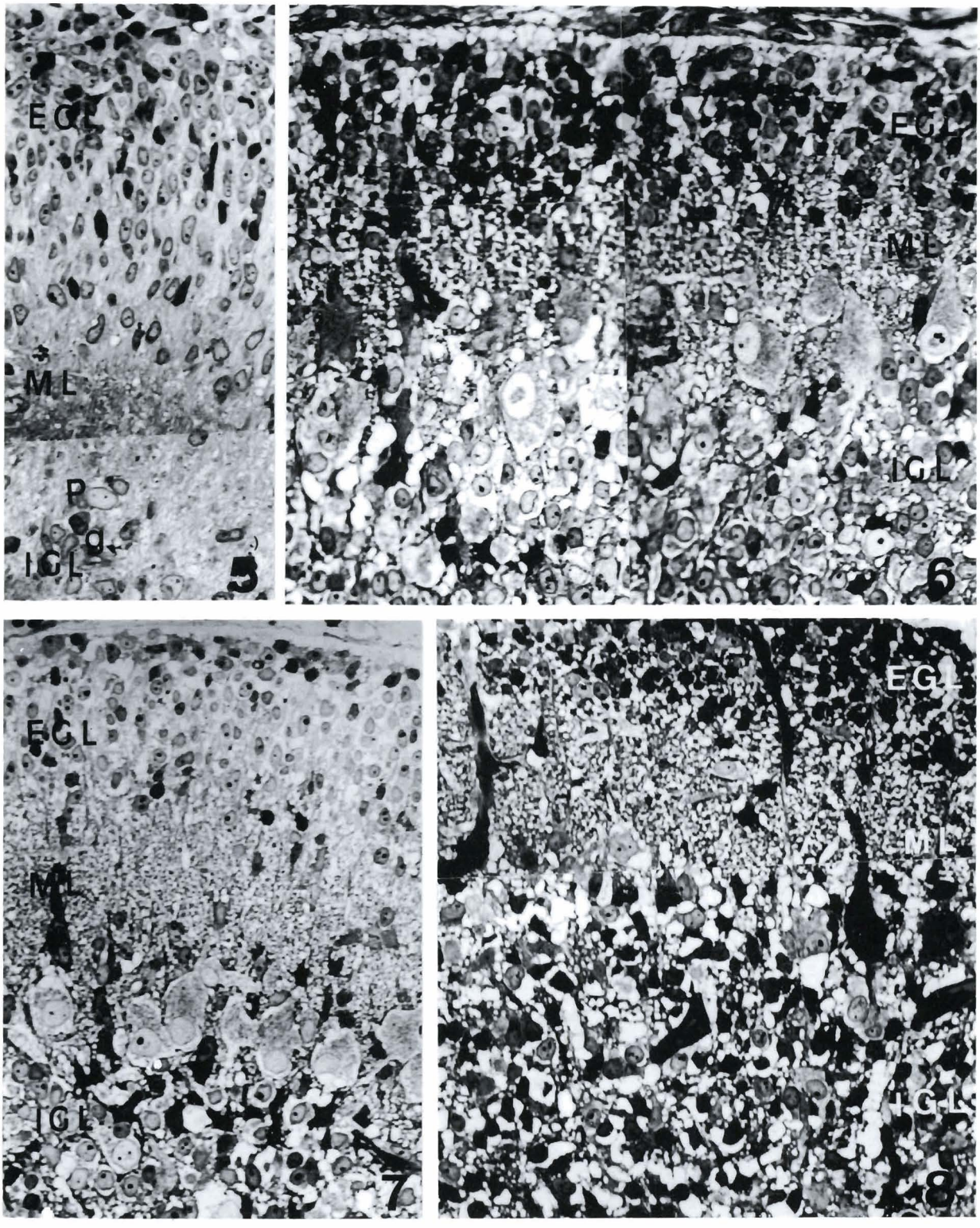
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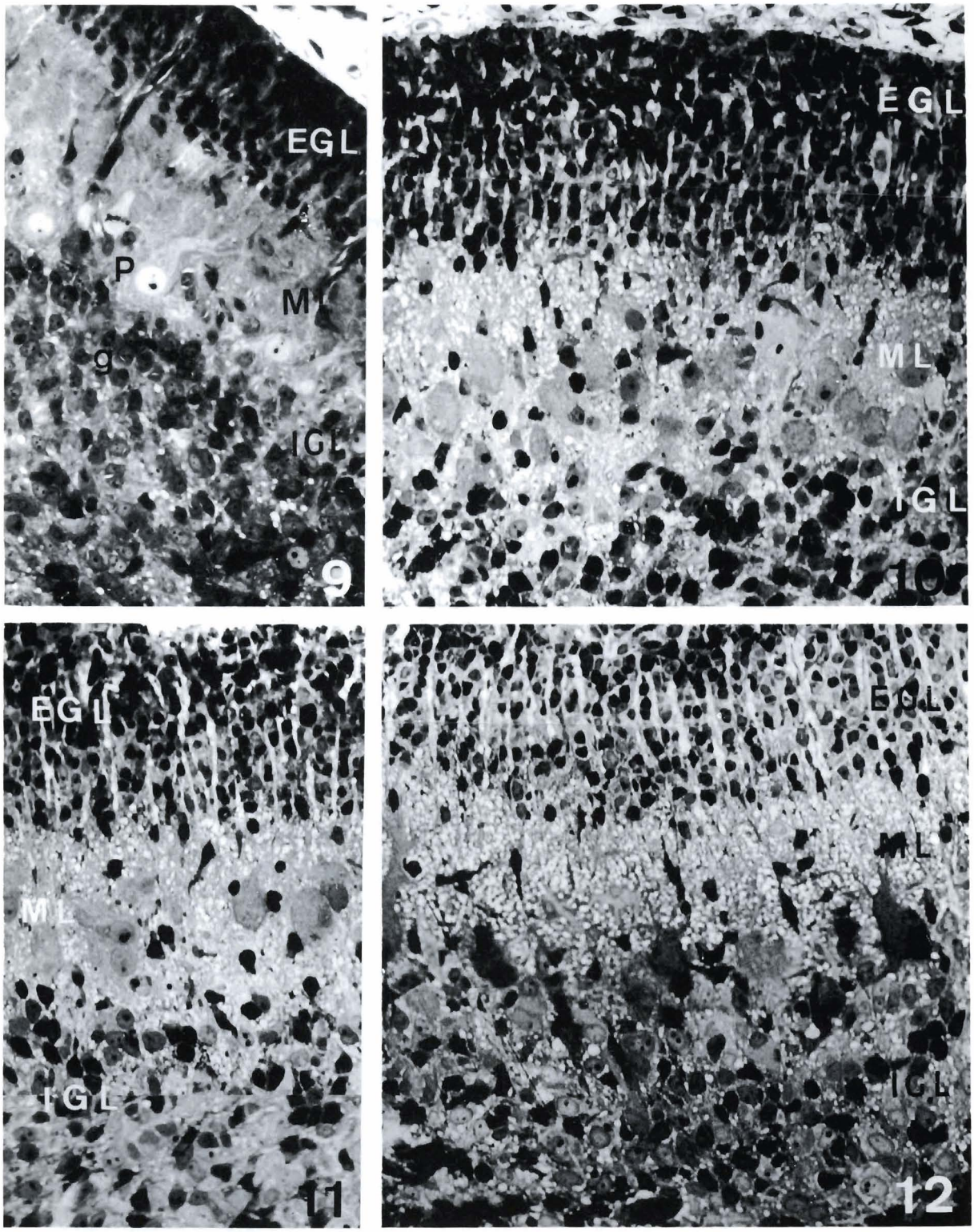
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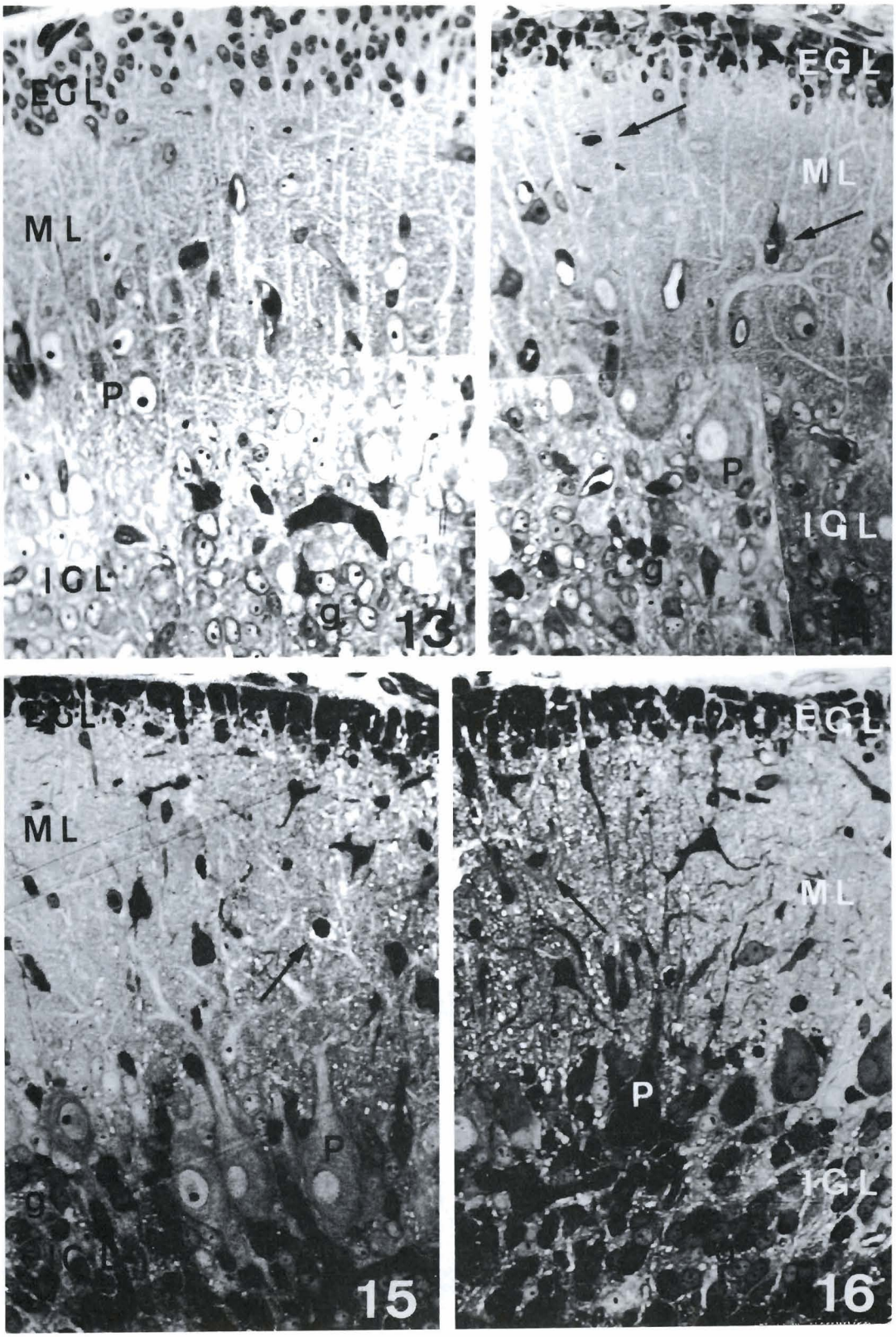
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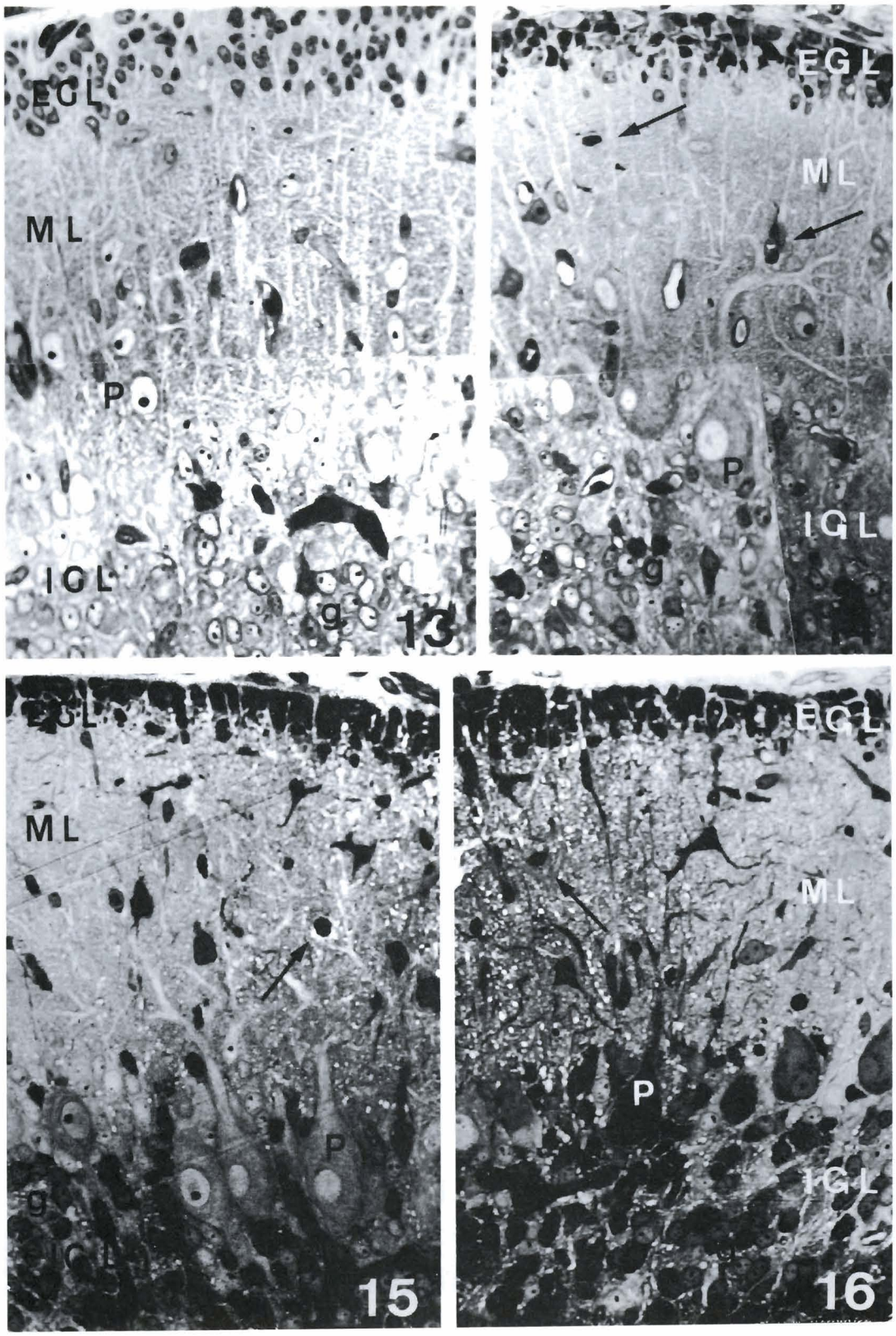
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Fig. 1. Semi-thin section of control cerebellum after 13 days of incubation. EGL: external granular layer. ML: molecular layer. IGL: internal granular layer. P: Purkinje cell. g: granule cell. G: Golgi cell. × 400

Figs. 2 and 3. Semi-thin section of cerebellum treated on the 3rd day of incubation with 15 µl ethanol and sacrificed at 13 days of incubation. A lower cellular density of the external granular layer (EGL), and a very narrow molecular layer (ML) is observed. The Purkinje cells (P) are vacuolised and the internal granular layer (IGL) is seen to be more immature than the control. g: granule cell. G: Golgi cell. × 400

Fig. 4. Semi-thin section of cerebellum treated on the 6th day of incubation with 15 µl ethanol and sacrificed after 13 days of incubation. There are large extracellular spaces (thin white arrow) and abundant picnotic cells (white arrow head) in the external granular layer (EGL). The picnoses are very evident in the Purkinje cells (broad white arrow): these cells show distinct degrees of effect (P), as do the other cellular components of the internal granular layer (IGL). × 400

Fig. 5. Semi-thin section of control cerebellum after 15 days of incubation. EGL: external granular layer. ML: molecular layer. IGL: internal granular layer. P: Purkinje cell. g: granule cell. × 400

Fig. 6. Semi-thin section of cerebellum injected on the 3rd day of incubation with 15 µl ethanol and sacrificed after 15 days of incubation. EGL: external granular layer. ML: molecular layer. IGL: internal granular layer. × 400

Figs. 7 and 8. Semi-thin section of cerebellum injected on the 6th day of incubation with 15 µl ethanol and sacrificed after 15 days of incubation. EGL: external granular layer. IGL: internal granular layer. ML: molecular layer. × 400

Fig. 9. Semi-thin section of control cerebellum after 17 days of incubation. P: Purkinje cell. g: granule cell. EGL: external granular layer. ML: molecular layer. IGL: internal granular layer. × 400

Fig. 10. Semi-thin section of cerebellum injected on the 3rd day of incubation with 15 µl ethanol and sacrificed after 17 days of incubation. EGL: external granular layer. ML: molecular layer. IGL: internal granular layer. × 400

Figs. 11 and 12. Semi-thin section of cerebellum injected on the 6th day of incubation with 15 µl ethanol and sacrificed after 17 days of incubation. EGL: external granular layer. ML: molecular layer. IGL: internal granular layer. × 400

Fig. 13. Semi-thin section of control cerebellum after 21 days of incubation. EGL: external granular layer. ML: molecular layer. IGL: internal granular layer. P: Purkinje cell. g: granule cell. × 400

Fig. 14. Semi-thin section of cerebellum injected on the 3rd day of incubation with 15 µl ethanol and sacrificed after 21 days of incubation. The arrows show degenerative structures in the thickness of the molecular layer (ML). EGL: external granular layer. IGL: internal granular layer. P: Purkinje cell. g: granule cell. × 400

Figs. 15 and 16. Semi-thin section of cerebellum injected on the 6th day of incubation with 15 µl ethanol and sacrificed after 21 days of incubation. The arrows show degenerative structures in the thickness of the molecular layer (ML). EGL: external granular cell. IGL: internal granular layer. P: Purkinje cell. g: granule cell. × 400

(comparing Figs. 2 and 3 with Fig. 6). When injected at 6 days and sacrificed at 15 days, the modifications are even greater (Fig. 8). In both cases the signs of effect are those described above: large empty spaces, turgidity, vacuolisation, disorientation and picnosis.

After 17 days of incubation the structuration of the cerebellar cortex in the control embryos is revealed by an increase in thickness of the molecular layer (Fig. 9 ML), which coexists with a compact external granular layer (Fig. 9 EGL). The perikariums of the Purkinje cells already form a single row arrayed similarly to that of the adult cerebellum (Fig. 9 P). The internal granular layer has a considerable thickness, in which the perikariums of the Golgi cells (Fig. 9 G) and those of the granule cells (Fig. 9 g) are observed.

The embryos of this age which have been injected with ethanol on the 3rd day (Fig. 10) and the 6th day (Figs. 11, 12) of incubation, show a lesser effect than on earlier days. On the other hand, in these 17 days there are no marked differences between the cerebellums injected on the 3rd day and those injected on the 6th day (comparing Fig. 10 with 11 and 12). This decrease in the ethyllic effects, which begins to become noticeable after 17 days, is well shown at 21 days of incubation, where the cerebellar tissue is almost totally repaired, as can be observed on comparing the control cerebellum of 21 days (Fig. 13) with those of 21 days injected on the 3rd day (Fig. 14) and the 6th day (Figs. 15, 16) of incubation.

After 21 days of incubation, the external granular layer in the control cerebellums is already quite reduced (Fig. 13) with respect to the periods previously studied. Meanwhile the molecular layer has increased considerably in size and complexity. As in the previous control studies (Quesada and Génis-Gálvez, 1983), the Purkinje cells form a layer of cells in a single row which is clearly differentiated. The granule cells, together with the Golgi cells, occupy the whole of the definitive internal granular layer.

After 21 days of incubation, the external granular layer in the cerebellums injected with ethanol on the 3rd day (Fig. 14) and the 6th day (Figs. 15, 16) presents a greater size than in the control, and its cells are very basophile. The molecular layer is quite compact, somewhat more in the embryos injected on the 3rd day than in those injected on the 6th, without the empty extracellular spaces of the previous days. Cells with degenerative characteristics are seen in its thickness (Figs. 14, arrow, 15, arrow and 16, arrow). The Purkinje cells are alined, but the majority, are turgid, with an apparently normal dendritic tree (Figs. 14 P and 15 P). The granular layer presents, as a sign of ethyllic effect, quite basophilic granule cells which are much more abundant in the embryos treated on the 6th day (Figs. 15 g, 16 g) than in those treated at on the 3rd day (Fig. 14 g).

Discussion

Jones and Smith (1975) were the first to study the brain of patients with Fetal Alcohol Syndrome. These authors restrict their observations to the cerebrum, describing gross abnormalities in neuronal and glial migration over the surface of the cerebral hemispheres. Clarren (1981) also observes great abnormalities in cell migration in the cerebral hemispheres, and describes this abnormality as the «hallmark of alcohol's teratogenic effect on the brain». Peiffer et al. (1979) observe a delay in the neuronal maturation of the cerebellum. Functional

disorders of the cerebellum are implicated in the symptoms of affected babies (Ford, 1960; Pierog et al., 1977), and disordered motor movement is a characteristic that persists in older children (Clarren, 1981).

One of the most common effects of exposure to alcohol during development is a generalised delay in maturation and growth. Chick embryos exposed to the effect of alcohol show a delay in at least two stages of Hamburger and Hamilton (Clarren and Smith, 1978). This fact as well as the existence of microcephalia, have been proven by ourselves to be predominant features of the effect of ethanol on the central nervous system (Jones et al., 1973; Jones and Smith, 1973, 1975; Ellis and Pick, 1980; Phillips and Cragg, 1982).

It is of interest to point out that the time of exposure to alcohol is an important variable which affects the vulnerability of the neurons; in particular the specific periods of vulnerability to alcohol of Purkinje cells have been studied (Volk, 1984; Pierce and West, 1985). On the other hand, we know that the changes following exposure to alcohol take place more frequently in undifferentiated cells (Majdecki et al., 1976) or during their process of differentiation (West and Pierce, 1986). Bauer-Moffet and Altman (1975, 1977) suggest that the action of ethanol is dependent on neuronal age. Thus our interest in beginning our study observing the early days of development.

Exposure to alcohol produces a reduction of some 20% in the number of granule cells (Bauer-Moffet and Altman, 1975, 1977) and some 40% in the number of Purkinje cells in the cerebellums of rats (Bauer-Moffet and Altman, 1977; Phillips and Cragg, 1982). We think that the teratogenic effect of alcohol on the number of neurons may be a mechanism which interferes not only with neuronal proliferation but also with differentiation.

In our research, in the cerebellums treated with ethanol and sacrificed at 13 and 15 days of development, the lesion is shown morphologically by the signs described in the results. If the lesion is not very severe, the neurons recover (Snell, 1982), return to their primitive size and form, the nucleus returns to the centre of the cell body and the Nissl bodies adopt their normal position. However, in other cerebellar cells the lesion is greater and they are not capable of overcoming the damage. Cell death is imminent, the cytoplasm turns hyperchromatic and the nuclear structure loses sharpness. The final stages take place after neuronal death: the cytoplasm become vacuolised and the nucleus and cytoplasmic organules disintegrate. The neuron dissolves and is eliminated by phagocyte activity, a task carried out in the CNS by the microglial cells.

We observed the above process in a generalised way in the cerebellums treated with ethanol. The effect is more drastic if these are injected on the 6th day of incubation, in place of the 3rd day. However, as accumulative doses of ethanol are not given, the cell damage is overcome and the tissue regenerates from the 17th day of incubation.

We know that ethanol acts in different ways according to the day of injection, independently of the dose. If we inject on the 3rd day of incubation, it acts on the histogenesis; if is treated on the 6th day of incubation, the effect is on the synaptogenic process. Perhaps this is the cause of the lesion being greater in the cerebellums treated later. Although the dose on the 3rd day acts on the number of cells, in part this could be overtaken by new waves of cells which leave the mitotic cycle, whereas the dose given on the 6th day intervenes not only quantitatively, but also qualitatively, on modifying the functional capacity of the cell. Any alteration in the microenvironment of a neuron during its development can compromise its later maturation (Griffin et al., 1977; Lauder, 1978; Pysh et al., 1979).

We have shown, as have other authors (Neubuerguer, 1957; Victor et al., 1959; Bauer-Moffet and Altman, 1977), that the granule cells, of all the cellular elements of the cerebellar cortex, are those most affected by ethanol. Their number decreases progressively up to day 15 of incubation, after which they recover, on not receiving successive doses of ethanol.

Degeneration of the granule cells is not uniform: there are areas which are more affected than others. At the same time, we observed that the death of the perikarium was preceded by the loss of its prolongations. This process has pronounced functional implications at the level of the cerebellar circuit, as cell number and integrity are vital factors for the normal depolarisation of the Purkinje cells. The level of depolarisation of the Purkinje cell is more related to the activity of the underlying groups of granule cells (Llinás, 1982) than to the distribution of the parallel fibres which cross its arborisations, as was believed until recently. This fact lends great importance to cell loss and to the pattern of degeneration of the granule cells, as activation of the Purkinje cells is more difficult in those areas with a lower number of granule cells, even though crossed by a high number of parallel fibres from non-lesioned granule cells.

We agree with Coyle (1977) that the development of differentiation and maturation of the CNS, and thus of effective synaptic contacts, depends greatly on the equilibrium between the diverse neurotransmitters existing in the differentiating neurons. This fact leads us to ask again: Why are the embryos treated on the 3rd day less affected, recovering sooner, than those injected on the 6th day? Perhaps the explanation is the one put forward by various authors (Appel, 1975; Black and Patterson, 1980): that the developing neuroblast has the possibility of changing its phenotype according to the production of neurotransmitters. Thus a greater time exposure to ethanol in the embryos injected on the 3rd day of incubation would induce a premature change, possibly reversible, which could modify the synaptic adhesion. This hypothesis should be put to test by means of the immunocytochemical and autoradiographic study of the diverse neurotransmitters which alternately appear during development. This would permit their quantification at a biochemical level, under both experimental and control conditions.

We know that although the loss of nerve cells is a

permanent state, damage in the neuropyl (dendrites, axon, synapsis and glial cell process) is capable of being repaired in the CNS of adult mammals. Sumner and Watson (1971) observed retraction and regrowth of the dendritic trees of cells in the hypoglossal nucleus of the rat following sectioning of the hypoglossal nerve. It has been suggested that such regrowth could take place following treatment with alcohol. For example, Bauer-Moffet and Altman (1977) believe that there may be a hypertrophy of the dendrites of Purkinje cells that survive alcohol treatment in the neonatal rats. This idea has been proven by Golgi techniques at electron microscopical level. Carlen et al. (1978) suggest that the dendrites of the Purkinje cells could regrow in the adult brain after an extensive period of abstinence.

For such a repair and regrowth of the dendritic tree, the production of structural proteins by the nerve cells is necessary. There is evidence in the rat of an increase in protein synthesis in the brain of experimental animals after a prolonged period following withdrawal of treatment with ethanol (Tewari et al., 1977; Hemmingsen et al., 1980; Peters and Steele, 1982).

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