UNIVERSITAT DE BARCELONA

DEPARTAMENT DE MEDICINA

ISQUEMIA CEREBRAL TRANSITORIA EN EL JERBU COM A MODEL DE MALALTIA ISQUEMICA CEREBRAL: ESTUDI DE NEURONES INHIBIDORES CORTICALS DE CIRCUIT LOCAL I DE MECANISMES DE MORT CEL·LULAR.

Memòria per a optar al Grau de Doctor, presentada per Avelina Tortosa i Moreno.
III.2.5 DISCUSSION

Programmed cell death (Horvitz et al., 1982; Chalfie and Wolinsky, 1990, Ellis et al., 1991; Altman, 1992; Driscoll and Chalfie, 1992), naturally occurring cell death in the developing nervous system (Cowan et al., 1984; Oppenheim 1981; -91; Ferrer et al., 1992; Raff, 1992), and apoptotic cell death in other tissues (Wyllie, 1981; Wyllie et al.,1984; Kerr et al., 1987; Tomei and Cope, 1991) are characterized by a primary degeneration and fragmentation of the nucleus which occurs through the activation of killer proteins, or through the inhibition of survival genes. These actually activate an endonuclease cascade that produces the cleavage of the chromatin into regular fragments.

In contrast, delayed neuronal death is a form of localized necrosis characterized by a primary degeneration of the cytoplasm which is belatedly followed either by punctate chromatin condensation or by uniform condensation of the nucleus. Degeneration is first recognized in the peripheral regions of dendrites, later in the soma and finally in the nucleus (Kirino and Sano, 1984 b; Yamamoto et al., 1986; -90;Deshpande et al., 1992; Hara et al., 1993). Chromatin cleavage is never observed (Ito et al., 1992). In the present study, this pattern is further supported by the demonstration of the early reduction of MAP-2 immunoreactivity which precedes the development of nuclear anomalies. Similar early degeneration of tubulin, microtubules and neurofilaments has been reported in other studies (Yoshimine et al., 1985; Yanagihara et al., 1985; Yamamoto et al., 1986; Hatakeyama et al., 1988; Ogata et al., 1989; Kitagawa et al., 1989; Nakamura et al., 1992).
Naturally occurring and experimentally-induced cell death during development are massively arrested following protein synthesis inhibition (Martin et al., 1988; Oppenheim et al., 1990; Inouye et al., 1992; Ferrer, 1992). In the present study, a slight decrease in the number of dying cells is found in the CA1 area of the gerbil following low doses of cycloheximide administered after reperfusion. However, the limited benefit of cycloheximide injection on delayed neuronal death is in striking contrast with the massive reduction, after cycloheximide injection, of X-ray-induced apoptotic cell death in the developing rat hippocampus (Ferrer et al., 1993).

On the other hand cell death can be produced, under certain conditions, with high doses of cycloheximide in several tissues (Columbano et al., 1985; Ijiri and Potten, 1987; Baxter et al., 1989; Martin et al., 1990; Rotello et al., 1991; Bansal et al., 1991), including the cerebral cortex (Ferrer, 1992). However, the number of dying cells in the CA1 area of the ischemic gerbil following the injection of high doses of cycloheximide is only slightly increased when compared with cycloheximide-induced apoptotic cell death.

The present results indicate that the sequence of involvement of the different intracellular compartments and the morphology of the nuclear degeneration serves to differentiate delayed neuronal death from apoptosis (see Clarke, 1990 for a detailed discussion). Furthermore, the response to cycloheximide is different in delayed neuronal death and experimentally-induced apoptosis.
Our knowledge about the relationship between protein synthesis and delayed neuronal death is still fragmentary. It is known that a global decrease of protein synthesis occurs after ischemia (Kleihues and Hossmann, 1971; Takahashi et al., 1984; Dienel et al., 1985; Kiessling et al., 1986; Dwyer et al., 1987). However, increased synthesis of stress/heat-shock protein (hsp70), which has been correlated with increased resistance to injury (Lindquist and Craig, 1988; Kirino et al., 1991), has been found at the early postischemic stages (Nowak, 1985; Jacewicz et al., 1986; Dienel et al, 1986; Wass et al., 1988; Nowak, 1991; Kirino et al., 1991; Chopp et al., 1991; Welsh et al., 1992; Deshpande et al., 1992). These features point to the likelihood that the synthesis of certain proteins at early postischemic stages may prevent some nerve cells from dying.

It is possible that low doses of cycloheximide reduce the protein turnover and balance a deprived protein synthesis after ischemia, thus allowing nerve cells to survive. It is also feasible that high doses of cycloheximide block the synthesis of protective proteins and promote cell death. However, the present study has failed to demonstrate that heat shock proteins play a significant role on the effects of low and high doses of cycloheximide on delayed neuronal death. Further studies are needed to elucidate the impact of dose-related protective or inductive effects of cycloheximide on protein synthesis in delayed neuronal death.
III.2.6 REFERENCES


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Capítol 3

FRUCTOSE-1,6-BISPHOSPHATE FAILS TO AMELIORATE DELAYED NEURONAL DEATH IN THE CA1 AREA AFTER TRANSIENT FOREBRAIN ISCHAEMIA IN GERBILS

Avelina Tortosa, Rosa Rivera, Santiago Ambrosio, Ramon Bartrons, Isidro Ferrer.

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III.3 FRUCTOSE-1,6-BISPHOSPHATE FAILS TO AMELIORATE DELAYED NEURONAL DEATH IN THE CA1 AREA AFTER TRANSIENT FOREBRAIN ISCHAEMIA IN GERBILS

Avelina Tortosa M.D. (*), Rosa Rivera Tech.D (*), Santiago Ambrosio M.D, Ph.D (**), Ramon Bartrons M.D, Ph.D (**), Isidro Ferrer M.D, Ph.D (*)

From the Unit of Neuropathology, Department of Pathological Anatomy, Hospital "Prínceps d’Espanya",(*) Unit of Biochemistry. Dep. Ciencies Fisiològiques (**), University of Barcelona, Hospitalet de Llobregat.

Correspondance to: Isidro Ferrer M.D., PH.D, Unit of Neuropathology, Department of Pathological Anatomy, Hospital "Prínceps d’Espanya, L’Hospitalet de Llobregat, Spain
FAX-34-3-2045065

Running title: Fructose-1,6-bisphosphate and delayed neuronal death.

Key words: delayed neuronal death, fructose-1,6-bisphosphate, ischaemia, gerbil.
III.3.1 SUMMARY

Fructose-1,6-bisphosphate has been shown to reduce ischaemic-induced brain damage in rabbits and gerbils. In view of these findings, we investigated the effects of fructose-1,6-bisphosphate on delayed neuronal death, following bilateral forebrain ischaemia, in the gerbil hippocampus at the fourth day of reperfusion.

We subjected gerbils to bilateral forebrain ischaemia for 20 minutes. Fructose-1,6-bisphosphate was administered: intraperitoneally at a dose of 1 g/kg in saline one hour before the occlusion or at a dose of 1 g/Kg one hour before the occlusion and every 24 hours for 3 days; or intraventricularly at a dose of 0.1 g/Kg just after the carotid occlusion. No significant differences in the number of dying cells in the CA1 area were found between each group of treated animals when compared with controls.

This study suggests that fructose-1,6-bisphosphate, administered according to these three different schedules, fails to ameliorate delayed neuronal death after 20 minutes of bilateral forebrain ischaemia in the CA1 area of the gerbil hippocampus.
III.3.2 INTRODUCTION

Treatment with fructose-1,6-bisphosphate (FBP) has been associated with a reduction of tissue damage after bilateral forebrain ischaemia in gerbils (Trimarchi et al., 1990) and rabbits (Farias et al., 1990). FBP has also been reported to protect, in vitro, astrocytes from hypoxic damage (Gregory et al. 1989; -90), and to maintain normal intracellular levels of Ca\(^{2+}\) during hypoxia in rats (Bickler and Kelleher, 1992). Moreover, FBP reduces the extent of tissue damage in ischaemic kidneys (Didlake et al., 1985) and galactosamine induced-hepatitis (De Oliveira et al., 1992), and, prevents and reduces biochemical and histological changes in the hypoxic myocardium in experimental animals (Markov et al., 1980; Farias et al., 1986) and in humans (Jones et al., 1980; Marchioni et al., 1985). However, these results have not been reproduced in other studies of cerebral and myocardial ischaemia (Eddy et al., 1981; LeBlanc et al., 1989), in adults and foetuses.

Several studies in rats and gerbils have demonstrated that transient cerebral ischaemia results in a characteristic pattern of delayed neuronal death (DND) with highly reproducible lesions in the CA1 area of the hippocampus (Ito et al, 1975; Kirino, 1982; Petito and Pulsinelli, 1984; Yamamoto et al., 1986; -90; Crain et al., 1988; Araki et al., 1989; Deshpande et al., 1992; Tortosa i Ferrer, in press).

In the present work we investigate whether intraperitoneal or intracerebral administration of FBP can preserve CA1 neurons from dying in gerbils subjected to bilateral transitory forebrain ischaemia.
III.3.3 MATERIALS AND METHODS

Mongolian gerbils (*Meriones unguiculatus*), of both sexes and weighing 50 to 70 g, were anesthetized with halothane mixed with room air (3% for induction; 1.5% for maintenance). An anterior midline cervical incision was made and both common carotid arteries were exposed and isolated from the vagus nerve. Two miniature aneurysm clips were placed on the carotids and blood flow was interrupted for 20 minutes. Anesthesia was discontinued when the clips were still in their place. Occlusion and reperfusion of the carotid arteries were verified by visual observation. During the occlusion, the rectal temperature was maintained at 37°C by placing the gerbil on a heating blanket.

Animals treated with FBP were categorized into three groups. Group I (n = 8) received 0.1 ml of FBP at a dose of 1 g/kg intraperitoneally one hour **before** the occlusion. Animals of group II (n = 9) received the same dose one hour **before** the occlusion and every 24 hours for 3 days. Animals of group III received 10 μl of FBP intraventricularly at a dose of 0.1 g/Kg just **after** the carotid occlusion. Control animals (n = 10) received saline either intraperitoneally or intraventricularly. Intraventricular injection was done into the right lateral ventricle (1 mm anterior to the bregma, 1.25 mm lateral to the midline, 2.25 mm deep from the cortical surface).

Four days after transient ischaemia, the animals were re-anesthetized with diethyl-ether and fixed transcardially with 150 ml of 4% paraformaldehyde in 0.1M phosphate buffer (PBS) after briefly washing out the blood vessels with heparinized
saline. The brains were removed from the skull and kept in a similar solution of 4% paraformaldehyde in PBS overnight at 4°C. Finally, the brains were embedded in paraffin, and dewaxed sections, 4-micron-thick, were stained with hematoxylin and eosin.

Quantitative studies were focused on the rostral hippocampus where the number of dying cells were counted. The counts were arbitrarily made on segments of 290 microns long in the CA1 area through the ocular micrometer of the microscope at a magnification of x 400. Results were expressed as mean values ± SE. Statistical processing was carried out with the Mann-Whitney U test.
III.3.4 RESULTS

After 20 minutes of forebrain ischaemia the animals either controls or treated with FBP, exhibited a restless behavior with torsion of the neck and continuous circling for 3 to 6 hours. After this time, the gerbils had no abnormal manifestations. Mortality was not found in any group through the 4 days after ischaemia.

Microscopic examination of the CA1 area four days after bilateral carotid occlusion in the control groups revealed identical and extensive necrosis of the pyramidal cell layer with preservation of a few neurons. Most dying cells were characterized by their swollen cytoplasm and punctate chromatin condensation but a few neurons exhibited a dark and uniformly condensed nucleus. A similar morphology was observed in the CA1 area in the three groups of animals treated with FBP (Figure 1).

Quantitative studies revealed similar numbers of dying cells (mean values ± SE) in the CA1 area in the three groups of treated animals and controls; Group I: 29.3 ± 3.2; Group II: 30.4 ± 3.1; Group III: 27.1 ± 6.4; Controls: 28.8 ± 3.5. Statistical analysis did not show significant differences between the control group and three groups of animals treated with FBP.
Figure 1: CA1 area of gerbils subjected to 20 minutes of bilateral forebrain ischemia and killed four days later. A: control receiving saline alone intraperitoneally. B: Group 1 treated with FBP at a dose of 1g/kg intraperitoneally one hour before the occlusion. C: Group II treated intraperitoneally with 1g/kg of FBP one hour before the occlusion and every 24 hours for 3 days. D: Group III treated with FBP intraventricularly at a dose of 0.1g/kg just after ischemia. No differences were found between animals treated with FBP and controls. H.E x 400.
III.3.5 DISCUSSION

Information about the mechanism by means of which FBP can protect cells from damage are contradictory. Several authors (Galzigna et al., 1977; Markov et al., 1985; Markov, 1986; Giacosa et al., 1987) have suggested that the bisphosphorylated sugar crosses the cell membrane and restores the depressed glycolytic activity, intervening in the glycolytic pathway not only as a metabolic regulator but also as a substrate. Exogenous supply of a high-energy intermediary metabolite could, in this case, favor greater cellular energy production in conditions under which the glycolytic flux is limited. However, these explanations have been previously challenged, mainly on the basis that sugar phosphates cannot cross the cell membrane (Eddy et al., 1981; Hassinen et al., 1991). Reports suggesting that they do are based on indirect evidence (Gregory et al., 1989). Other possible explanations could be that FBP interacted with cell membranes, modifying the ion permeability (Cattani et al., 1980; Hassinen et al., 1991).

Previous studies have reported that intravenous infusion of FBP reduces ischaemic-induced brain damage in gerbils (Trimarchi et al., 1990) and rabbits (Farias et al., 1990). However, our results indicate that intraperitoneal or intraventricular administration of FBP does not ameliorate delayed neuronal death in the CA1 area after 20 minutes of bilateral forebrain ischaemia in gerbils. Differences between these experimental models and the present model, could account in part for the different results. Trimarchi et al (1990) subjected gerbils to 15 minutes of bilateral forebrain ischaemia and examined the brains levels of putrescine 24 h later. Although putrescine levels were significantly reduced after
FBP administration, no histopathological examination was carried out. On the other hand, the study of Farias et al. (1990) was performed in rabbits. The animals were subjected to hypotension, hypoxia and bilateral common carotid artery occlusion during five to eight minutes. FBP injection produced a decrease in the extent of the necrosis in treated animals when compared with controls. Species differences as well as differences in the timing and schedules of ischaemia in rabbits and gerbils (present results) could explain these discrepancies.

The present results suggest that FBP does not protect DND after transient ischaemia in gerbils. Further studies are needed to elucidate whether, and under which conditions, FBP can protect ischaemic cells from dying.
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III.3.6 REFERENCES


PARVALBUMIN AND CALBINDIN D-28K IMMUNOREACTIVITY, AND POSTISCHEMIC CELL DEATH IN THE DEVELOPING HIPPOCAMPUS OF THE GERBIL

A. Tortosa, I. Ferrer

Dev Brain Res (submitted)
III.4  PARVALBUMIN AND CALBINDIN D-28K IMMUNOREACTIVITY, AND POSTISCHEMIC CELL DEATH IN THE DEVELOPING HIPPOCAMPUS OF THE GERBIL

A. Tortosa, I. Ferrer

Unidad de Neuropatologia, Servicio de Anatomia Patológica; Hospital Príncepes de España, Universidad de Barcelona, 08907
L’Hospitalet de Llobregat, Spain

Key words: ischemia, cell death, parvalbumin, calbindin D-28K

Correspondance: I. Ferrer
III.4.1 SUMMARY

Vulnerability following transient forebrain ischemia for 20 minutes was examined in the hippocampal formation of gerbils during postnatal development. No cellular damage was seen in animals aged 7 days. Dying cells were observed at the base of the granule cell layer of the dentate gyrus in animals aged 15, 21 and 30 days. Pyramidal cells in the CA3 subfield were also sensitive to ischemia in gerbils aged 15 days, and less frequently in animals aged 21 days. The adult pattern of cellular damage, characterized by selective vulnerability of the CA1 subfield, was seen from day 30 onwards. Parvalbumin immunoreactive neurons first appear in the *stratum pyramidale* of CA3 at postnatal day 15 (P15), and in CA2 and hilus of the dentate gyrus from P21 onwards. Immunoreactive terminals also follow the same sequence from CA3 to CA1 to reach adult patterns by the end of the first month. Calbindin D-28k immunoreactivity is seen in the external part of the upper blade of the *dentate gyrus* at P5, and progress to the granule cell and molecular layers of the whole gyrus by P15, except a thin band of immature cells located at the base of the granule cell layer which are calbindin negative. Calbindin immunoreactivity in mossy fibers progresses from the external to the hilar region of CA3 during the same period. A few immunoreactive cells are also found in the stratum radiatum/lacunare of the CA3, but no calbindin-immunoreactive cells are observed in the CA1 and CA2 subfields. The adult pattern of calbindin immunoreactivity is reached at P21. These findings show that the pattern of selective vulnerability following transient forebrain ischemia is different in young and adult gerbils, and suggest that no correlation exists between resistance to ischemic damage and calcium-binding protein content.
III.4.2 INTRODUCTION

Delayed neuronal death in rats and gerbils occurs in the CA1 subfield of the hippocampus a few days after transient forebrain ischemia (15, 16, 18-20, 27, 28, 30). The rise of intracellular calcium levels following ischemia has given support to the hypothesis that calcium overload may play a pivotal role in postischemic cell death (2, 5, 13, 14, 38). Pyramidal neurons are the most severely affected, whereas GABAergic, parvalbumin-immunoreactive local-circuit neurons are largely spared (23-25, 36). The reason of the resistance to ischemia in this latter group of neurons is not known, but it has been suggested that parvalbumin may act as a cytosolic calcium-buffer which regulates calcium influx, and may prevent neurons from calcium-dependent cell death (34).

Little is known of the effects of transient forebrain ischemia in young animals. However, very different pattern of vulnerability are observed in other experimental models of cerebral ischemia and hypoxia in young rodents when compared with adult animals (22, 26, 29, 31, 37). Therefore, it is feasible that the pattern of neuronal damage after transient forebrain ischemia also differs in young gerbils when compared with adults. The possible role played by different calcium-binding proteins in conditions damaging the nervous system during development is poorly documented, but the presence of calbindin D-28K, another calcium-binding protein, has been associated with survival of dentatus gyrus granule cells after hipoxic/ischemic injury in immature rats (11).
In the present study we have examined the developmental pattern of cell death after transient forebrain ischemia, and the maturation of parvalbumin or calbindin D-28K immunoreactivity in the hippocampal formation of the gerbil. It has been our intention to investigate whether a possible correlation exists between sensitivity to ischemia and calcium-binding protein content in the developing hippocampus.
III.4.3 MATERIAL AND METHODS

Mongolian gerbils (*Meriones unguiculatus*) of both sexes, bred in our own colony were used. For parvalbumin and calbindin D-28K immunohistochemistry, animals were killed at different postnatal ages from day 0 (P0) to day 30 (P30). A few adults (aged between 90 and 180 days) were also included. The animals were anesthetized with diethyl-ether, and the brains were perfused through the heart with 0.9% saline and 1% heparin, followed by 4% paraformaldehyde in phosphate buffer. Immediately afterwards, the brains were removed from the skull and immersed in a similar fixative solution for 24 h, later cryoprotected with 30% saccharose in phosphate buffer, frozen with liquid nitrogen and stored at -80°C until use. Sections 55 microns thick were obtained with a cryostat and processed free-floating following the avidin-biotin-peroxidase method (ABC procedure). After blocking endogenous peroxidases with metanol, and non-specific binding with 3% normal horse serum, the sections were incubated at 4°C overnight with well-characterized monoclonal anti-parvalbumin or anti-calbindin D28K antibodies (Sigma clone PA-235 and CL-300) used at dilutions of 1:2000 and 1:500, respectively, in PBS containing 0.2% triton x-100, 0.2% gelatin and 1% horse normal serum. Later, the sections were incubated in biotinylated horse anti-mouse IgG (Vector Labs) at a dilution of 1:200 for 1h, and avidin-biotin (ABC kit, Vectastain, Vector Labs) at a dilution of 1:100 for 1h. The peroxidase reaction was visualized with 0.05% diaminobenzidine and 0.01% hydrogen peroxide. False positive immunoreaction were ruled out by incubating a few sections without the primary antibody.
A group of gerbils aged 7, 15, 21, 30 and 120 days were subjected to transient forebrain ischemia. These animals were anesthetized with halothane mixed with oxygen (2% for induction and 1% for maintenance). Both common carotid arteries were exposed through a midline neck incision, and bilateral forebrain ischemia was induced by occluding the common carotid arteries with small clips for 20 minutes. After this time the clips were removed and the incision was sutured with 6-0 silk. One hour after reperfusion, the gerbils were returned to their cages. The absence of blood flow during the occlusion and the reperfusion after removal of the clips were controlled visually through a binocular microscope. Since hypothermia may reduce hippocampal injury (6, 12, 39, 40), the body temperature was maintained at 36-37°C with a heat blanket during the ischemic and postischemic (1h) periods. The animals were killed 4 days after ischemia under deep diethyl-ether anesthesia, and the brains were perfused through the heart with saline followed by 2.5% glutaraldehyde in phosphate buffer pH 7.3-7.4. Brain slabs containing the hippocampus were embedded in paraffin and cut with a sliding microtome. Sections 5 microns thick were stained with hematoxylin and eosin.

Twenty four animals were used for immunohistochemistry, and other twenty (four at every age) were subjected to ischemia.
III.4.4 RESULTS

a. Parvalbumin immunohistochemistry

The first parvalbumin-immunoreactive neurons in the hippocampal formation were seen at the postnatal day 15 (P15). The adult pattern of immunoreactivity was reached at the end of the first month.

At P15 small numbers of immunoreactive neurons were present in the stratum pyramidale of the CA3 subfield. The immunoreaction was limited to the cytoplasm and short dendrites, but immunoreactive terminals were absent (Fig. 1A). At P21, in addition to immunoreactive cells in the CA3 subfield, a few parvalbumin-containing neurons were observed in the CA2 and CA1 subfields, and in the hilus of the dentate gyrus. These cells were multipolar neurons and pyramidal-like cells with short smooth dendrites. Punctate immunoreactive terminals occurred in the CA3 subfield, but were absent in the other hippocampal areas (Fig. 1B, C and D).

At the age of 30 days, parvalbumin-immunoreactive neurons were found in the CA2 and CA3 subfields of the hippocampus and hilus of the dentate gyrus. However, immunoreactive cells were not longer present in the CA1 subfield. Immunoreactive punctate terminals decorated the pyramidal layer (stratum pyramidale) of the CA1, CA2 and CA3 subfields (Fig. 1E, F and G). At this age, the perforant path was also stained with anti-parvalbumin antibodies.
b. Calbindin-D28K immunohistochemistry

No immunoreactive cells were seen in the hippocampal formation in newborn gerbils (Fig. 2A). Immunoreactive cells were seen in the upper granular blade of the dentate gyrus at P5. In addition, mossy fibers were also moderately stained with anti-calbindin antibodies in the external region of the CA3 subfield (Fig. 2B and C). By the end of the first two-week period, the upper and inner granular cell layer, and the stratum moleculare of the whole dentate gyrus were calbindin immunoreactive (Fig. 2D). Mossy fibers, at this time, were highlighted with anti-calbindin antibodies from the external to the hilar region of CA3. However, the intensity of the staining was not uniform through the granule cell layer because immature neurons located at the base of this layer were only slightly immunoreactive or not at all so (Fig. 2E). A few immunoreactive cells were also seen in the stratum radiatum/lacunare of the CA3 subfield, whereas no immunoreactive cells were present in the CA1 and CA2 subfields.

The adult pattern of calbindin D28K immunoreactivity was reached by the end of the third week (Fig. 2F). All granule cells of the dentate gyrus, even those located at the base of this layer, were calbindin immunoreactive (Fig. 2G).

c. General effects of transient forebrain ischemia

No abnormal behavior was noted in gerbils aged 7 and 15 days once recovered from the anesthesia, following forebrain ischemia for 20 minutes. However, animals aged 21 and 30 days exhibited squatting position for 1 hour.
After this time, recovery was complete in gerbils aged 21 days, whereas continuous circling for about 6 hours occurred in animals one month old.

d. **Histological findings following transient forebrain ischemia**

No morphological abnormalities were seen in animals at P7 examined four days following bilateral forebrain ischemia of 20 minutes duration. However, dying cells, characterized by their extremely pyknotic and shrunken nuclei, were found at the base of the cell layer of the upper and inner blade of the dentate gyrus in gerbils aged 15 days. A few pyramidal cells in the CA3 subfield of the hippocampus also showed a contracted cytoplasm and dark nucleus, whereas no abnormalities were found in the CA1 and CA2 subfields (Fig. 3A, B and C).

Similar findings were observed in animals aged 21 days at the fourth day following transient ischemia for 20 minutes, although the CA3 subfield was less frequently affected in these animals (Fig. 3D, E and F).

Gerbils aged 30 days showed a distinct pattern of neuronal degeneration in the CA1 subfield of the hippocampus, which was similar to that observed in adults. Dying neurons had a shrunken or absent cytoplasm and punctate aggregates of chromatin in their nuclei. The CA3 subfield was spared, but a few dying cells were still observed at the base of the cell layer of the dentate gyrus (Fig. 3G, H and I).
FIGURA 1: Parvalbumin immunoreactivity in the hippocampal formation during postnatal development. Immunoreactive cells first appear in the CA3 subfield at P15 (A). This is followed by the CA2 and CA1 subfields, and hilus of the dentate gyrus by day 21 (B, C and D). The adult pattern is reached at the end of the first month (E, F and G). Immunoreactive terminals are present in the CA2 subfield by P21 (D), but only at P30 in the CA1 and CA3 subfields (F and G). A few parvalbumin-immunoreactive cells are found at P21 in CA1 (C); no immunoreactive neurons are found at P21 in CA1 (C); no immunoreactive neurons are observed in normal animals in this subfield from day 30 onwards (F). CA1, CA2 and CA3: subfields of the hippocampus; DG: dentate gyrus; OR, PYR, RAD: stratum oriens, pyramidale and radiatum of the hippocampus; PP: perforant path; C and F: CA1; A, D and G: CA3; B and E, bar = 350 microns; A, C, D, F and G, bar = 100 microns.
FIGURA 2: Calbindin-D28K immunoreactivity in the hippocampal formation during postnatal development. Immunoreactive cells are not seen in the newborn (A). Immunoreactive neurons are first observed in the external region of the upper blade of the dentate gyrus by postnatal day 5. At this stage, anti-calbindin antibodies also decorate mossy fibers in the external region of CA3 (B and C). At P15, most neurons in the granule cell layer, and stratum moleculare of the dentate gyrus are immunoreactive (D), but immature neurons located at the base of the cellular layer are still not immunoreactive (E). The adult pattern of immunoreactivity is reached in animals aged 21 days (F). At this age, all neurons in the granule cell layer are calbindin-immunoreactive (G). CA1 and CA3: subfields of the hippocampus; DG: dentate gyrus; MS: mossy fibers; UB, IB: upper and inner blades of the dentate gyrus; H: hilus; MOL, GR: molecular (stratum moleculare) and granule cell layers of the dentate gyrus. A, B, D and F, bar = 350 microns; C, E and G, bar = 100 microns.
FIGURE 3: Histological findings at the fourth day following bilateral forebrain ischemia for 20 minutes in developing gerbils. A, B and C: animals aged 15 days; D, E and F: 21-day-old gerbils; G, H and I: gerbils aged 30 days. Dying cells were seen at the base of the cellular layer of the dentate gyrus in every group (C, F and H). A few pyknotic cells were seen in the internal region of the CA3 subfield in animals aged 15 days (B) and 21 days (E), but not in gerbils aged 30 days. Although the CA1 subfield was spared in animals aged 15 and 21 days (A and D), typical lesions, similar to those found in adults, were observed in gerbils aged 30 days (G and I).

CA1, CA2 and CA3: subfields of the hippocampus; DG: dentate gyrus; H: hilus of the dentate gyrus; UB and IB: upper and inner blades of the dentate gyrus; MOL and GR: molecular and granular (cellular) layer of the dentate gyrus; OR, PYR, RAD: stratum oriens, pyramidale and radiatum of the CA1 subfield. Hematoxylin and eosin. A, D and G, bar = 350 microns; B, C, E, F, H and I, bar = 100 microns.
III.4.5 DISCUSSION

In the gerbil hippocampus, parvalbumin-immunoreactive cells first appear in the CA3 subfield at P15, followed by neurons in CA2. Parvalbumin-immunoreactive cells are transitorily observed in CA1 by P21. From this age onwards, only immunoreactive punctate terminals, probably basket cell terminals, are found in this subfield. The adult pattern including parvalbumin immunoreactivity in the perforant path (32, 36), is reached at the end of the first month. Calbindin D-28K-immunoreactive cells first appear at P5 in the external region of the upper blade of the dentate gyrus. Immunoreactivity extends to the whole granule cell layer and molecular layer in the following days, at the time that mossy fibers become heavily stained with the anti-calbindin D-28k antibodies. Very few, if any, calbindin-immunoreactive cells are observed in the cellular layer of the hippocampus at any stage of postnatal development. In the dentate gyrus of gerbils aged 15 days, calbindin-immunoreactivity concentrates in mature neurons located in the vicinity of the molecular layer rather than in immature neurons at the base of this layer. The adult pattern of calbindin immunoreactivity is reached by P21. These patterns are largely similar to those observed in the developing rat, although with a delay of 5 to 7 days in the gerbil (4, 33).

The functions of the different calcium-binding proteins during development are barely understood. It has been suggested that the presence of calbindin D-28k is associated with the early maturation of neurons (1, 3, 7, 8), whereas parvalbumin immunoreactivity in the cerebral neocortex and hippocampus
correlates with the maturation of particular subpopulations of local-circuit neurons and, most particularly, with effectiveness of intracortical inhibition (1, 21, 35).

Parvalbumin-immunoreactive cells in the CA1 subfield of the hippocampus of adult gerbils are resistant to degeneration and death following transient forebrain ischemia for 7 or 20 minutes (23-25, 36). However, other studies have failed to demonstrate a close relationship between calcium-binding protein content and cell survival in animals subjected to complete forebrain ischemia for 30 minutes (9, 10). Therefore, it is still not clear whether, and to what extent, parvalbumin content may preserve postischemic cells from dying in adult animals (17).

Young animals appear less vulnerable to transient forebrain ischemia, and the distribution of cellular damage is different when compared with adults. No abnormalities are seen in gerbils aged 7 days. Dying cells are largely restricted at the base of the granule cell layer of the dentate gyrus in animals aged 15 and 21 days, and also to the CA3 subfield in gerbils aged 15 days, and less often in animals aged 21 days. Selective involvement of the CA1 is found in animals aged 30 days and older.

Based on these findings, it is clear that no correlation exists between ischemic-induced cell death and parvalbumin content in the developing hippocampus.
As regards calbindin, Goodman et al. (11) observed that immature neurons located at the base of the granule cell layer of the dentate gyrus were the only neurons lacking this calcium binding protein, and the only ones which were vulnerable to the hypoxic/ischemic insult in rats aged 7-10 days. These findings suggest that lack of calbindin is causally related to vulnerability, and that the presence of this calcium-binding protein can protect nerve cells from dying. As pointed out by the same authors, another possibility is coincidental reflection of immaturity (11). The present study also indicates that there is a period of sensitivity to ischemia for granule cells of the dentate gyrus between days 15 and 30 in the gerbil, and that vulnerable cells at day 15 are devoid of calbindin. However, the putative role of calbindin as a neuroprotector can be questioned because immature granule cells at P7, which lack calbindin, are resistant to ischemia, whereas a few granule cells at P21 and P30, which are calbindin-immunoreactive, are sensitive. Furthermore, no correlations exists between lack of calbindin content and vulnerability to ischemia in the CA1, CA2 and CA3 subfields of the hippocampus at any time of development.

Taken together, the present results point to the likelihood that resistance to ischemic damage in the developing hippocampus is largely independent of calcium-binding protein content.
III.4.6 REFERENCES


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Capitol 5

X-RAY-INDUCED CELL DEATH IN THE DEVELOPING HIPPOCAMPAL COMPLEX INVOLVES NEURONS AND REQUIRES PROTEIN SYNTHESIS

Isidro Ferrer, Teresa Serrano, Soledad Alcantara, Avelina Tortosa, and Francesc Graus.

III.5 X-RAY-INDUCED CELL DEATH IN THE DEVELOPING HIPPOCAMPAL COMPLEX INVOLVES NEURONS AND REQUIRES PROTEIN SYNTHESIS

ISIDRO FERRER M.D., PH.D. (*); TERESA SERRANO M.D. (*); SOLEDAD ALCANTARA (*); AVELINA TORTOSA M.D. (*); and FRANCESC GRAUS M.D., PH.D. (**).

From the Unit of Neuropathology, Service of Pathological Anatomy, Hospital "Prínceps d’Espanya", University of Barcelona, Hospitalet de LLobregat (*); Service of Neurology, Hospital Clinic i Provincial, Barcelona (**); Spain

Correspondence to: Isidro Ferrer, M.D., PHD, Unit of Neuropathology, Service of Pathological Anatomy, Hospital Princeps d’Espanya, 08907 Hospitalet de Llobregat, Spain

FAX -34-3-2045065

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Sprague-Dawley rats aged 1 or 15 days were irradiated with a single dose of 200 cGy X-rays and killed at different intervals from 3 to 48 h. Dying cells were recognized by their shrunken and often fragmented nuclei and less damaged cytoplasm in the early stages. On the basis of immunocytochemical markers, dying cells probably represented a heterogeneous population which included neurons and immature cells. In rats aged 1 day the number of dying cells rapidly increased in the hippocampal complex with peak values 6 h after irradiation. This was followed by a gentle decrease to reach normal values 48 h after irradiation. The most severely affected regions were the subplate and the cellular layer of the subiculum, gyrus dentatus and hilus, and the stratum oriens and pyramidale of the hippocampus (CA1 more affected than CA2, and this more affected than CA3). X-ray-induced cell death was abolished with an injection of cycloheximide (2 microg/gi.p.) given at the time of irradiation. X-ray-induced cell death was not changed after the intraventricular administration of nerve growth factor (NGF; 10 microg in saline) at the time of irradiation. Cell death was not induced by X-irradiation in rats aged 15 days. These results indicate that X-ray-induced cell death in the hippocampal complex of the developing rat is subjected to determinate temporal and regional patterns of vulnerability; it is an active process mediated by protein synthesis but, probably, not dependent on NGF.

key words: cell death, cycloheximide, development, hippocampal complex, X-rays
III.5.2 INTRODUCTION

Cell death is a common phenomenon during the development of the nervous systems (1-6). Cell death and engulfment of selective cell populations in the nematode *C. elegans* depend on the activity of specific genes which program a suicidal cascade (7-12). In the vertebrate nervous system, many developing sensory, motor and sympathetic neuroblasts compete for access to neurotrophic factors necessary for their survival (5, 13-16). In these populations, nerve growth factor (NGF) not only prevents cell death during normal development, but also preserves cells from dying in determinate pathologic conditions (17). Since cycloheximide, puromycin or actinomycin D inhibit cell death induced by NGF deprivation (18, 19), it is likely that RNA translation and activation of protein synthesis are involved in this process. Similarly, induced cell death in the developing nervous system is observed in the absence of targets or markers derived from these targets (6, 16, 20, 21). Induced sensory and motorneuron death in the chick embryo are also reduced by cycloheximide or actinomycin D (22).

In the neocortex of the rat, naturally occurring cell death occurs during the first postnatal week, and predominates in layers II-III, Vlb, and in the future subcortical white matter (23-25). Exposure to low doses of X-rays in the rat during the first week of postnatal life produces a marked increase in the number of dead cells in the same neocortical layers (26). Since these effects are curbed with protein synthesis inhibitors, it can be suggested that X-rays enhance cortical cell death through the activation of killer proteins (26).
To further investigate early effects of X-irradiation on migratory and early postmigratory cells of the developing nervous system, we investigate the regional distribution and timing of X-ray-induced cell death in the hippocampal complex of the rat, and their possible regulation through protein synthesis. Since NGF receptors are present in the hippocampus during development (27, 28), as well as in adulthood (29), the possibility that NGF prevents X-ray-induced cell death has also been studied.
III.5.3 MATERIAL AND METHODS

Sprague-Dawley rats aged 1 day and 15 days were irradiated with a single dose of 200 cGy X-rays using a 300 Kvp Stabilipan with an HVL of 3.3 mm Cu. Animals aged 1 day were killed 3 (n = 5), 6 (n = 5), 24 (n = 5) or 48 (n = 5) h later. Another group of animals (n = 8) received cycloheximide (Actidione; ICN Biochemicals), at a dose of 2 microg/g b.wt. dissolved in saline immediately after irradiation, and was allowed to survive for 6 h. Finally, a third group of rats received an intraventricular injection in the right lateral ventricle of NGF (Sigma 7 S type) 10 microg/g in 10 microl of saline, or a similar quantity of saline alone immediately after irradiation. These animals were killed 6 (n = 5, NGF; n = 5, saline: S), 24 (n = 5, NGF; n = 5, S) or 48 (n = 5, NGF; n = 5, S) h later. Age matched rats (n = 12) were used as controls. Animals aged 15 days were irradiated with a similar dose of X-rays and killed 3 (n = 3), 6 (n = 3), 24 (n = 3) or 48 (n = 3) h later. Age-matched animals (n = 3) were used as controls.

The animals were killed with an overdose of diethyl ether and their brains were fixed with 2% buffered glutaraldehyde. Brain slabs containing the anterior hippocampus were embedded in paraffin, serially sectioned at 10 microns and stained with hematoxylin and eosin (H.E.). Dead cells were counted in the following areas: subiculum, CA1, CA2, CA3, gyrus dentatus and hilus. Three sections were examined in every rat and the number of dead cells was expressed as mean values +/- SD. Results were statistically processed with the Mann-Whitney U test.
A few animals were used for ultrastructural examination. After glutaraldehyde fixation for 24 h, small blocks of the hippocampal complex were postfixed with 1% osmium tetroxide for 2 h, dehydrated in ethanol and propylenoxid and embedded in durcupan. Selected ultrathin sections were stained with uranyl acetate and lead citrate.

Finally, the brains of rats aged 1 day, controls (n = 3) and irradiated, killed 3 (n = 5), 6 (n = 5), 24 (n = 3) or 48 (n = 3) h later, were fixed with Carnoy for 24 h and embedded in paraffin. Sections 7 microns thick were processed for Hu, glial fibrillary acidic protein (GFAP) and vimentin immunocytochemistry following the avidin-biotin-peroxidase procedure (ABC; Vectastain, Vector Labs, Burlingame, USA) or the PAP method (Sternberger-Meyer, Jarrettsville, USA). The polyclonal antibody against GFAP (raised in rabbit, DAKO, Dakopatts, Glostrup, Denmark) was used at a dilution of 1:250. The prediluted monoclonal antibody against vimentin (Biogenex, San Ramon, USA) was used according to the indications of the supplier. Biotinylated IgG containing the Hu antibody was obtained from the serum of a patient with small cell lung cancer and paraneoplastic encephalomyelitis, and prepared as detailed elsewhere (30). After blocking endogenous peroxidases with 0.3% hydrogen peroxide for 20 minutes, the sections were incubated with 10% normal goat serum for 20 minutes, biotinylated IgG diluted at 1:1000 overnight at 4°C and ABC complex. The peroxidase reaction was visualized with 0.05% diaminobenzidine and 0.01% hydrogen peroxide (31). A few sections were processed without the primary antibody to rule out false positive results. All sections were counterstained with hematoxylin.
Patients with small cell lung cancer (SCLC) and paraneoplastic encephalomyelitis and paraneoplastic sensory neuropathy harbor in their serum and cerebrospinal fluid high titers of a highly specific antibody called anti-Hu (32-34). The anti-Hu antibody reacts with a set of basic proteins of 38-40 kDa molecular weight expressed in neurons, SCLC cells and a few other neuroendocrine related tumors, especially neuroblastomas (34-36). The use of this antibody as a neuron marker is justified because previous studies have shown that Hu immunoreactivity during development is present before the appearance of immunoreactivity to other markers (30, 37).
III.5.4 RESULTS

Dying cells were recognized by their extremely dark and shrunken, often fragmented, nucleus. In control rats aged one day, small numbers of dying cells were found in the subplate (future subcortical white matter) and cellular layer (cortical plate) of the subiculum; stratum oriens (inner plexiform layer) and stratum pyramidale (cellular layer) of the areas CA1, CA2 and CA3 of the hippocampus, and hilus of the gyrus dentatus. Dying cells were seldom observed in the gyrus dentatus, but dying cells were almost completely absent in the molecular layer of the subiculum and upper plexiform layers of the hippocampus.

Dying cells dramatically increased in number in irradiated rats aged one day (Fig. 1). Electronmicroscopical examination revealed the primary condensation and fragmentation of the nucleus together with a better preservation of the cytoplasm in the early stages of naturally occurring and X-ray-induced cell death (Fig. 2). Immunocytochemical studies showed that about 20% of dying cells in the stratum pyramidale of the hippocampus, and 15% in the cortical plate of the subiculum were immunoreactive with the anti-Hu antibody, thus indicating their neuronal origin. Only 5% of dying cells in the future subcortical white matter of the subiculum and stratum oriens of the hippocampus were Hu-immunoreactive cells. Labeled cells were not seen in the hilus of the gyrus dentatus (Fig. 3). No GFAP-immunoreactive dying cells were found in any area, but about 10% dying cells in the future subcortical white matter of the subiculum and inner plexiform layer of the hippocampus (stratum oriens) were immunoreactive with the anti-vimentin antibody.
Dying cells in irradiated animals increased in number 3 h after X-irradiation in the subiculum and *stratum oriens* of the CA1 and CA2 areas of the hippocampus. Peak values of cell death were reached 6 h after X-ray exposure in every area of the subiculum, hippocampus, *gyrus dentatus* and *hilus*, with the exception of the *stratum pyramidale* of CA3. Dying cells were not significantly increased in the molecular layer of the subiculum and upper plexiform layers of the hippocampus. The number of dying cells decreased in the following hours, but significantly large numbers were still observed in the subiculum, *stratum oriens* of CA1, CA2 and CA3, *stratum pyramidale* of the CA1, and *gyrus dentatus* 24 h after X-rays exposure. Dying cells were rarely seen at the end of the second day (Fig. 4).

The effects of X-irradiation were largely suppressed after an intraperitoneal injection of cycloheximide (2 microg/gb.wt.). The number of dying cells in animals killed 6 h later was similar to or even smaller than that in control rats (Fig. 4).

The number of dying cells in irradiated rats was not modified in the ipsilateral hippocampus after an intraventricular injection of NGF or saline alone (Fig. 5).

Naturally occurring and X-ray-induced cell death were negligible in rats aged 15 days.
Figure 1: Normal subiculum at P1 (A), and increased numbers of dying cells (B, C and D) in the hippocampal complex in rats aged 1 day after X-irradiation with a single dose of 2 G. B: cellular layer of the subiculum; C: *stratum pyramidale* of the CA1 area; D: Hilus of the dentate gyrus. Dying cells show an extremely shrunken nucleus, often fragmented into chromatin granules (arrows). Dewaxed paraffin sections stained with H.E. Bar = 20 microns.
Figure 2: Dying cells, characterized by extremely condensed and dark, often fragmented, nucleus and variably preserved cytoplasm in the hippocampal complex of 1-day-old X-irradiated rats, killed 6 h later. A: subiculum; B and C: stratum pyramidale of CA1 (B) and CA2 (C); D: stratum oriens of CA1. Bar = 1 micron.
Figure 3: Dying neurons, as revealed by their nuclear immunolabelling with the anti-Hu antibody (arrows), in the CA1 (A and B) and CA2 (C) areas of hippocampus, and subiculum (D) in 1-day-old X-irradiated rats killed 6 h later. Carnoy-fixed, dewaxed paraffin sections counterstained with H.E. Bar = 20 microns.
Figure 4: Number of dying cells (mean values +/- SD) in the hippocampal complex in control and 1-day-old X-irradiated rats killed at different intervals (3, 6, 24 and 48 h) after irradiation, and in X irradiated rats receiving 2 microg/g b.wt. of cycloheximide at the time of irradiation, and killed 6 h later. GD: dentate gyrus; H: hilus; CA3, CA2, CA1: areas of the hippocampus; SUB: subiculum; or: stratum oriens; pyr: stratum pyramidalis; pl: cortical plate (cellular layer); subpl: cortical subplate (future subcortical white matter). Asterisks indicate p < 0.0001 in relation to the corresponding values in controls (Mann-Whitney U test).
Figure 5: Number of dying cells (mean values +/- SD) in the hippocampal complex in 1-day-old irradiated rats receiving an intraventricular injection of NGF (10 microg in 10 microl of saline) or saline (S) alone, and killed at different intervals. Counts, made in the ipsilateral hippocampal complex, are similar in both groups of rats and in non-treated irradiated rats (see Fig. 4, for comparison). Abbreviations are the same as in Fig. 4.
In the developing hippocampal complex of the rat, naturally occurring cell death predominates in the cortical subplate and cortical plate of the subicular complex, stratum oriens and cellular layer (starum pyramidale) of the CA1 and CA3 areas (38). Dead cells are also found in the suprapyramidal blade and infrapyramidal blade/hilus of the gyrus dentatus (39). X-irradiation in rats aged 1 day produces an increase in the number of dead cells in the same subicular and hippocampal areas, reaching peak values 6 h after irradiation and normal values of cell death 48 h later. In contrast, no effects were observed after X-ray exposure in animals aged 15 days.

Although the nature of most dying cells is not known, a substantial number of dying cells in the cellular layers of the subiculum and hippocampus are neurons, as demonstrated with the anti-Hu antibody. This indicates that, in addition to germinal cells (40-42), some neurons, probably postmigratory, can be killed by X-irradiation at very precise times of development. The morphology and regional distribution is similar in naturally occurring and X-ray-induced cell death in the developing hippocampal complex of the rat (38). Early nuclear shrinkage and fragmentation of the nucleus, which is a characteristic feature of apoptosis (43-48), is found in naturally occurring and X-ray-induced cell death. The varying regional vulnerability to irradiation is similarly found during normal development (38). Although the reasons of this regional vulnerability are not known, it is striking that naturally occurring (38) and X-ray-induced cell death in the cellular layers of the hippocampal complex of the rat predominate in the same hippocampal areas.
that are most affected in certain human involutive or degenerative conditions such as aging and Alzheimer’s disease (49-53).

X-ray-induced cell death in the external germinal layer of the cerebellum (54) and in the neocortex (26), subiculum and hippocampus (present data) during development can be curbed with cycloheximide. This feature indicates that X-ray-induced cell death is an active process mediated by protein synthesis. Whether this process occurs through the activation of killer genes or through the inhibition of survival genes remains to be elucidated.

Finally, NGF is synthesized at high levels in the hippocampus and neocortex during development (55, 56). In the adult rat brain, a rapid increase of NGF and brain-derived neurotrophic factor mRNA occur after hippocampal damage and kainic acid injection (57), and delayed neuronal death in the hippocampus of the adult gerbil after ischaemia is ameliorated by exogenous administration of NGF (58, 59). We failed to demonstrate a similar effect of NGF in relation to X-ray induced cell death. It can be argued that NGF did not reach substantial concentrations in the hippocampus to preserve cells from dying, and, therefore, the possibility that NGF might curbe X-ray-induced cell death under appropriate conditions cannot be ruled out. However, it is worth noting that a similar method of NGF injection has been employed in adult animals with good results (58).

The present findings indicate that X-irradiation enhances cell death through an active process that requires protein synthesis, but probably not depends on NGF. Neurons, glial cells and immature cells may be involved at the time in which
these populations are subjected to naturally occurring cell death. The relationship between naturally occurring, and induced, cell death and the expression of different "cellular immediate early-genes" is still obscure. However, transient c-fos expression occurs at the onset of cell death in the developing interhemispheric cortex of the rat (60), and this observation suggests a possible involvement of c-fos in certain forms of naturally occurring cell death (60).
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III.5.6 REFERENCES


