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The Influence of Mechanical Injury on the Metabolic Activity of Transplanted Cerebral Cortex

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Summary

The authors studied the metabolic activity of rat embryonic cerebral cortex grafts (ED 15–16) implanted into rat brains immediately (TR_0) and 14 days (TR_{14}) after cavity formation. Over a period of two months, the ATP, lactate and glucose concentration in TR_0 transplants remained at the same level as observed in the intact cortex, whereas in TR_{14} transplants the ATP and glucose concentration fell significantly and the lactate concentration rose. The DNA concentration rose in both types of transplants, but the increase was more pronounced in TR_0 grafts. Choline acetyltransferase activity (a neuron marker) fell significantly in both cases, but the decrease was greater in TR_{14} transplants. The results indicate that grafts implanted into the brain immediately after cavities had been formed have better metabolic activity and are capable of longer survival than grafts implanted 14 days after cavitation.

Key words:
Cerebral cortex – Transplants – Metabolic activity

Introduction

The interaction of implanted nervous tissue with the brain is a complex and intricate process involving the participation of a whole series of different factors and reactions whose final effect is the acceptance or rejection of the transplant. On the one hand, successful with the surrounding tissue, but it also requires growth of the host tissue – in particular of the relevant axons – into the transplant. In other words, as well as suitable conditions for growth and maturation of the transplanted embryonic nerve cells, the process also requires proper conditions for the functional regeneration of the damaged part of the nervous tissue and the surrounding area. From the clinical aspect, transplantation to mechanically injured tissue, but also transplantation aimed at the replacement of non-functioning or otherwise altered regions of the brain, would correspond to this situation. Successful resolution of neurotransplantation problems could furnish information on mechanism of regeneration of the CNS and help to resolve certain neurobiological problems. The regeneration process in the CNS is influenced by a whole series of various

substances described as growth and trophic factors (Dekker *et al.* 1987, Varon *et al.* 1988) of either neuronal or some other (chiefly glial) origin (Müller and Seifert 1982). Some of these substances, produced by the brain after 7–14 days as a part of the long-term response of nervous tissue to injury, act as trophic stimulators promoting the viability of neurons (Nieto-Sampedro *et al.* 1982, Gage *et al.* 1984) and influencing the survival and growth of brain grafts (Nieto-Sampedro *et al.* 1984 ,1986). This would seem to favour transplantation into the nervous tissue 1–2 weeks after injury.

On the other hand, during the first hours, up to the third day after injury, there is an increase in the concentration of the acidic fibroblast growth factor (a-FGF) and the glial maturation factor (GMF), which evidently initiate processes leading to greater proliferation of the neuroglia (Nieto-Sampedro *et al.* 1988). Further processes also develop in the brain within a few hours after injury. After only two hours, for instance, the brain produces opioid peptides (McIntosh *et al.* 1987a) causing constriction of the blood vessels and increasing the extent of secondary post-traumatic degenerative changes in the brain (McIntosh *et al.* 1987b) After a few days, "reactive" gliosis also develops (Miller *et al.* 1986), the astrocytes proliferate (Latov *et al.* 1979) and in 1–2 weeks a glial scar is formed (Rice and Houle 1988). The presence of developing brain tissue can be very beneficial in this situation, since the graft releases substances with a trophic effect on the injured neurons (Sharp and Gonzales 1986, Cunningham *et al.* 1987, Sorensen *et al.* 1989) and at the same time prevents a glial scar from being formed (Kromer 1980). These data, together with the finding that long-term improvement of some cerebral functions occurred only after immediate transplantation, but not following transplantation 14 days after brain damage (Valoušková and Macias-Gonzales 1989), is evidence in favour of transplantation as soon as possible after injury.

Since the data in the literature on the ability of "immediate" and "delayed" grafts to survive and retain their function are somewhat at variance (Nieto-Sampedro *et al.* 1986, Bragin *et al.* 1988), we investigated in this study some of the metabolic properties of grafts transplanted into the brain immediately or 14 days after the formation of cavities in the cerebral cortex of adult rats. The aim was to determine objectively the ability of these two types of grafts for their long-term morphological and functional integration with the brain tissue of an adult host.

Material and Methods

Experimental animals and operations

Three-month-old male rats (Long Evans strain, n=24) were anaesthetized with Nembutal and cavities about 3 mm in diameter were formed bilaterally in their cerebral cortex. In some of these animals (n=12), fragments (about 2 mm^3) of the cortex dissected from the wall of the forebrain vesicle of 15-day-old rat embryos of the same strain were implanted in these cavities as soon as bleeding had been arrested (Fig. 1). In the rest (n=12), the skin was sutured when bleeding stopped and brain tissue was not transplanted (bilaterally) until 14 days later. The transplantation method has already been described in detail elsewhere (Valoušková 1989).

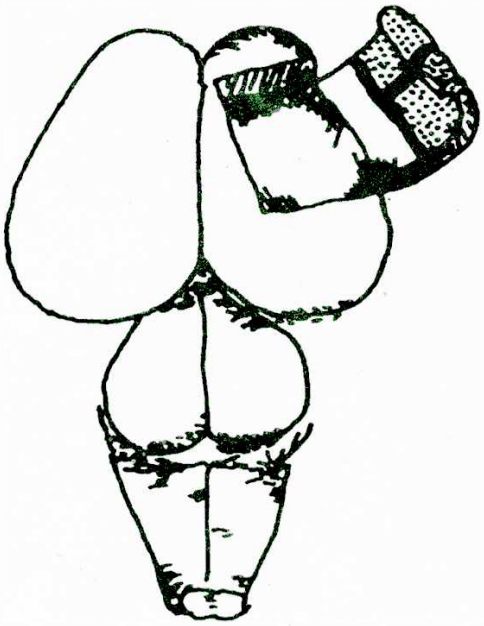

Fig. 1.
Method of collecting cerebral cortex from rat embryos (ED 15)

Biochemical analysis

The tissues from 20 "immediate" transplants (TR_o) and from 18 "delayed" transplants (TR_{14}) and homotopic sensorimotor cortex fragments from 10 intact rats (C) were analysed biochemically. Tissue samples from five TR_o, five TR_{14} and five C animals were used for each analysis.

Determination of the ATP, phosphocreatine, glucose and lactate concentration

After exsanguination, the head was plunged whole into liquid nitrogen. Keeping the head still frozen, bones were removed and transplants (or, in intact animals, homotopic fragments of the cortex) were excised, weighed and pulverized in liquid nitrogen. The resultant powder was transferred into test tubes containing 0.1 mol. l^{-1} HCl in 99 % methanol. After partly thawing to 0 °C, four parts of 0.3 mol. l^{-1} $HClO_4$ containing 1 mmol. l^{-1} EDTA were added to the mixture, which was then vortexed for a short time. The pellet obtained by 20 min centrifugation at 9.81×10^4 $m.s^{-2}$ was re-extracted with 0.5 ml of the same solution. The supernatants were combined and 2 ml portions were neutralized by adding 300 μl solution containing 1.5 mol.l^{-1} KOH, 0.4 mol.l^{-1} imidazole and 0.3 mol.l^{-1} KCl. The precipitate was separated by centrifugation for 20 min at 4.9×10^4 $m.s^{-2}$ and the supernatant was stored at −30 °C until required (Folbergrová *et al.* 1972). The ATP, phosphocreatine, glucose and lactate concentration in the brain extracts was determined fluorometrically by the method of Lowry and Passonneau (1972).

Determination of the DNA and RNA concentration

Fragments of grafts and intact cortex were homogenized in 10 volumes of 10 mmol. l^{-1} phosphate buffer solution at pH 7.5 containing 1 mmol. l^{-1} $CaCl_2$, 1 mmol. l^{-1} $MgCl_2$ and 0.14 mol. l^{-1} NaCl. The DNA and RNA concentration in the homogenates was determined by the fluorescence method, using ethidium bromide (Karsten and Wollenberger 1977). Fluorescence of the solutions was measured at 360 nm (excitation light) and 580 nm (emission light) on a Spekol (Zeiss, Jena) apparatus with a fluorometric adapter and digital output (TEC-1).

Determination of acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) activity

When determining AChE and ChAT activity, TR_0, TR_{14} and C tissue was homogenized in 40 mmol.l^{-1} phosphate buffer solution at pH 7.4, containing 200 mmol.l^{-1} NaCl, 10 mmol.l^{-1} $MgCl_2$ and 0.5 % Triton X-100. The enzymatic activities in the homogenates were determinated radiometrically after Fonnum (1969), as modified by Malátová *et al.* (1989).

Determination of the amino acid concentration

Frozen graft or intact cortex fragments were mixed with 10 volumes of sulphosalicylic acid and were sonicated 20 s in a Dynatech Sonic Dismembrator apparatus. After 5 h the suspension was centrifuged 10 min at 4.9×10^4 m.s^{-2}; the precipitate was washed with a small amount of sulphosalicylic acid and was then re-centrifuged. The combined supernatants were evaporized to dryness, which was dissolved in an acetate buffer at pH 2.2 (Martiniak *et al.* 1989). The free amino acid concentration in the samples was determined on a T 339 amino acid analyzer (Microtechna, Prague) in a Li^+ cycle.

All results were evaluated statistically by Student's t-test.

Results

The concentration of some substrates and products of energy metabolism in the two groups of cerebral cortex transplants as compared with intact cortex is illustrated in Fig. 2. It can be seen that no change (compared with the control) occurred in graft implanted in a host brain immediately after cavity formation. The decrease in the phosphocreatine concentration borders on statistical significance ($p = 0.05$). The situation in tissue transplanted into the cavities 14 days after the lesion is significantly different. In this case, the ATP, phosphocreatine and glucose concentration fell significantly compared with the control, while the lactate concentration rose (Fig. 2).

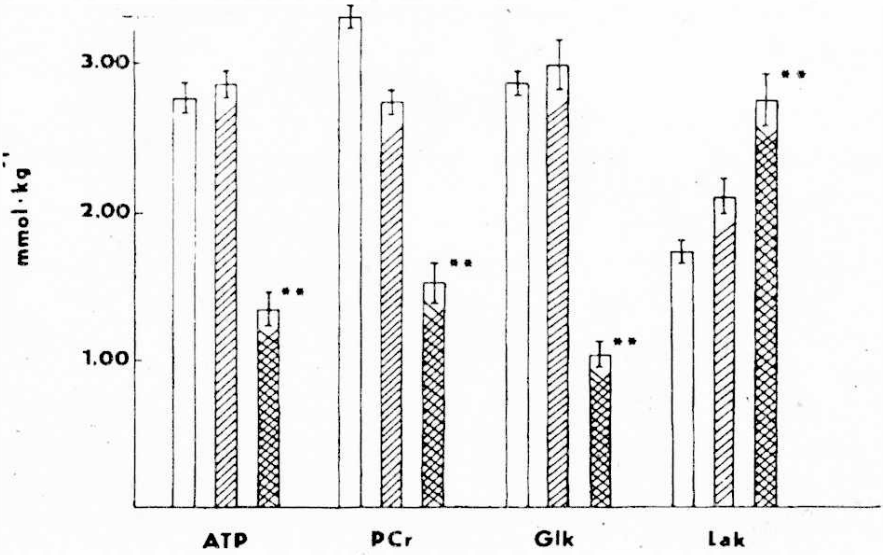

Fig 2.

The ATP, phosphocreatine (PCr), glucose (Glk) and lactate (Lak) concentration in transplanted and intact rat cerebral cortex. The results are expressed in mmol. kg^{-1} tissue wet weight ± SEM (means of 5 samples in each group). White columns – intact control; hatched columns – "immediate" transplants (TR_0); cross-hatched columns – "delayed" transplants (TR_{14}). ** $P<0.01$ compared with the intact control.

We were further interested in the DNA concentration in the grafts, since it furnishes information on cell density in a tissue. As seen from Fig. 3, during a period of two months the DNA concentration (expressed in mg protein) rose in both cortex transplantation groups compared with intact control. The increase in grafts implanted in the brain immediately after the cavities has been formed was greater than after delayed transplantation. The RNA concentration did not alter in any of the groups (Fig. 3).

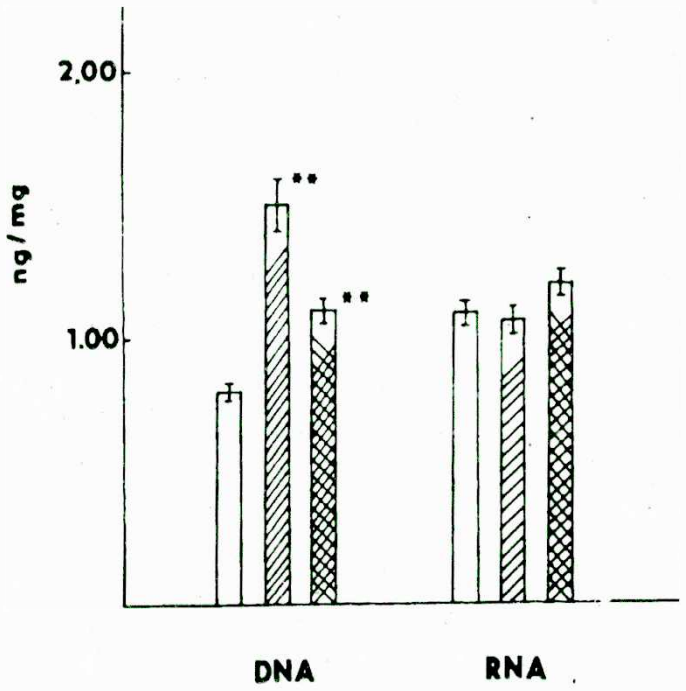

Fig. 3
The DNA and RNA concentration in transplanted and intact rat cerebral cortex. The results are expressed in ng.mg^{-1} tissue wet weight ± SEM (means of 5 samples in each group). ** $P<0.01$. Symbols as in Fig. 2.

The metabolism of certain amino acids is closely associated with the metabolic activity of the brain, and hence also with its function. Among these amino acids, the ASP concentration in cortex transplanted 14 days after cavitation fell and the GLN concentration rose (Fig. 4), whereas the GLU and GABA concentrations remained at the same level as in the intact control. The concentrations of the various amino acids in grafts implanted immediately after the formation of cavities did not alter compared with the control (Fig. 4).

Lastly, we studied the activities of enzymes in the grafts synthesizing and degrading the neurotransmitter, acetylcholine. In both types of grafts, both enzyme activites fell compared with the control (Fig. 5). The activity of AChE, the enzyme degrading acetylcholine, was far lower in "immediate" grafts, whereas ChAT, the synthesizing enzyme activity, was lower in tissues implanted in 14-day-old cavities

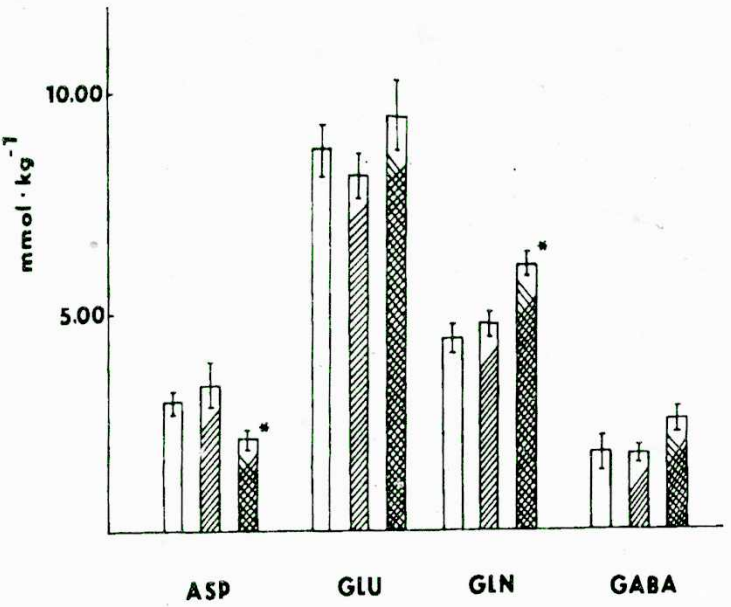

Fig. 4
The aspartic acid (ASP), glutamic acid (GLU), glutamine (GLN) and γ-aminobutyric acid (GABA) concentration in transplanted and intact rat cerebral cortex. The results are expressed in mmol.kg^{-1} tissue wet weight ± SEM (means of 5 samples in each group). * $P<0.05$. Symbols as in Fig. 2.

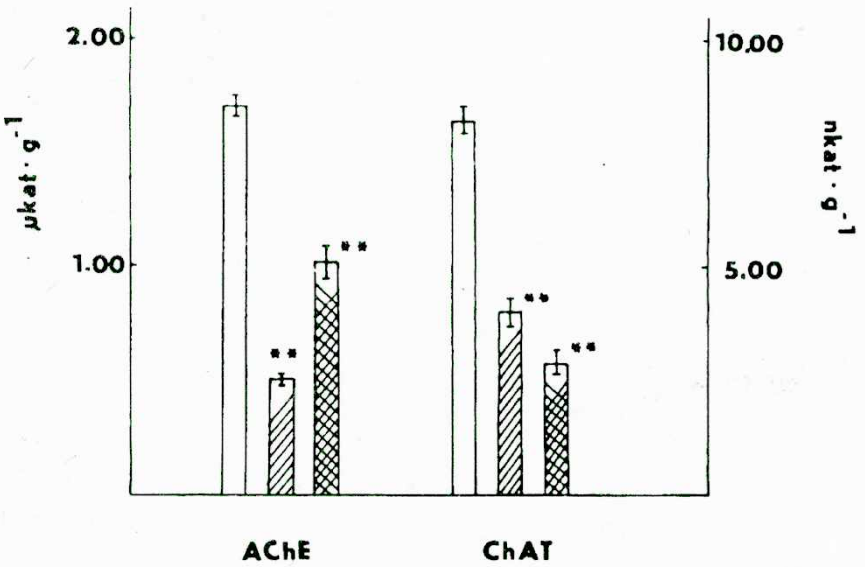

Fig 5.
Acetylcholinesterase (AChE) activity and choline acetyltransferase (ChAT) activity in transplanted and intact rat cerebral cortex. The results are expressed in kat. g^{-1} protein ± SEM (means of 5 samples in each group). ** $P<0.01$. Symbols as in Fig. 2.

Discussion

The formation of morphological and functional integrity of the transplanted and host tissue is a basic condition in neurotransplantation experiments. Successful neurotransplantation is unconditionally associated with the course of various metabolic reactions indispensable for the growth and functional properties of the grafts.

In the first place, an adequate supply of nutrients is essential for the growth and survival of neurotransplants. Determination of the glucose concentration shows a manifested decrease in TR_{14} compared with TR_o and the intact control. The lower glucose concentration in TR_{14} – assuming its metabolic consumption to be unchanged – could be due to the reduced density of the vascular network found in grafts by Smith and Ebner (1986) two months after transplantation. In transplantations carried out immediately after injury, trophic substances act on the implanted tissue; these include a-FGF, which stimulates neovascularization (Thomas and Giménez-Gallego 1986) and thus improves the graft's chances of survival.

Another factor which influences the graft's nutrients and oxygen supply is simple diffusion, as is also the case in surviving brain slices, in which diffusion maintains given pO_2 values to a depth of 150 μm (Bingman and Kolde 1982). In the smaller TR_o (Valoušková, unpublished results), diffusion can be a far more effective supplementary source of nutrition than in the bulkier TR_{14}.

Differences in the ATP and phosphocreatine concentration in TR_o and TR_{14} correspond to differences in the glucose concentration. The higher lactate concentration in TR_{14} than in the control shows that the decrease in ATP and phosphocreatine is probably due to a lack of the necessary substrates, including oxygen, and to a deficient course of oxidative phosphorylation in the mitochondria.

A general decrease in a metabolism in "delayed" grafts (7 days) was also found by measuring the uptake of 2-deoxyglucose by the tissue; higher 2-deoxyglucose transport was found in the less bulky frontal and medial part of the graft, where it was attributed to better interconnection of this part of the graft with the host's brain (Sharp and Gonzales 1984). Similarly, the differences we found in energy metabolism in TR_o and TR_{14} may also be associated with volume differences between the two types of grafts. The greater bulk of the grafts in TR_{14} is evidently a disadvantage, because the connections of large grafts with the host structures are concentrated mainly peripherally (Steedman *et al.* 1983).

The differences in choline acetyltransferase (ChAT) activity are also indicative of differences in the degree of innervation from the host's brain. ChAT occurs practically solely in cholinergic neurons and its concentration likewise depends on innervation from other structures. Reduced ChAT activity may be a sign of lower density of cholinergic innervation of the grafts from the surrounding structures of the host. From this aspect, TR_o ought to be better innervated than TR_{14}. This corresponds to histological findings confirming greater width of the zone of complete union of the graft neuropil with the host's tissue in TR_o (Němeček *et al.* 1989a).

The other enzyme whose activity we measured and which is responsible for the degradation of acetylcholine, is present in both the neurons and the glia cells. Its higher concentration in TR_{14} than in TR_o could reflect the increase in the amount of glia, found chiefly in the surface layers in TR_{14}. These zones of raised glial density

are probably the outcome of regression processes (Němeček *et al.* 1989b). The higher AChE activity in TR_{14} as against TR_o could likewise be a manifestation of differences in glial metabolism in the two types of grafts. The increase in the glutamine concentration (a glial marker) found in TR_{14} shows that the activity of the glia in delayed grafts is probably higher than in TR_o or in the control.

In both types of grafts, the DNA concentration was found to be higher than in the controls, in keeping with greater cell density in the graft tissue (Sharp and Gonzales 1984). The increase in the DNA concentration could also have been due to changes in the nuclei of the cells in the transplanted tissue, however. In addition to changes in the nuclear membrane, the nuclei of the graft neurocytes contained one or two nucleoli of different electronoptic density, evidently in association with the increase in the DNA concentration (Liška *et al.* 1990).

The results of this study show that "immediate" grafts are capable of better integration with the host's tissue, as seen from the course of a number of metabolic processes. Our results are in agreement with the data of Macias-Gonzales *et al.* (1988) on the viability of cortical grafts from 14-day-old embryos. Eleven months after transplantation, 60 % survival was found in TR_{14} and 95 % survival in TR_o. On the other hand, Gonzales and Sharp (1987) described 73 % survival in TR_7 and only 32 % survival in TR_o. One of the reasons for the low viability of these TR_o could have been the higher embryonic age of the donor (ED 18). On using tissues from younger embryos (ED 14 – 16), viability is much higher and if the operation is performed under strictly sterile conditions, survival could be close to 100 % (Bragin *et al.* 1988). This fact evidently has something to do with the degree of differentiation of the given nervous tissue and with its sensitivity to ischaemic conditions. The growth capacity of grafts also decreases with their embryonic age (Das *et al.* 1980). The changes that we found in the metabolism of "delayed" grafts, permit the assumption that their functional fitness is reduced. They are indicative of ischaemic regressive processes which, during prolonged survival, can give rise to degenerative changes or result in total necrosis (Němeček *et al.* 1989 b).

The reason why metabolism in TR_o is so much better than in TR_{14} is probably that, in immediate transplantation, the graft comes under the influence of trophic substances, whose concentration in the rat brain rises only a few hours after injury (Nieto-Sampedro *et al.* 1988). Of these, GMF stimulates growth and maturation of the glia, while a-FGF promotes neovascularization of the graft and thus contributes to better metabolism in TR_o than in TR_{14}. It can be concluded from the above findings that, from the aspect of survival of the grafts, and probably also of their functional fitness, transplantation should be undertaken as soon as possible after brain ijnury.

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