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The proliferation of astrocytes around a needle wound in the rat brain

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INTRODUCTION

It is well known that, as in other tissues, cells increase in numbers around a wound in the brain. There is, however, little exact information about which cells respond by proliferation or about the factors that initiate and control the response. Thus, Hortega & Penfield (1927) noted an increase in astrocytes around a needle wound, but detected no mitotic figures in them and because of this assumed that they underwent a process of amitotic devision. Lapham (1962), studying the DNA content of human astrocytes reacting to injury, found microspectrophotometric evidence of DNA duplication in their nuclei but again found no evidence of their mitotic division.

Konigsmark & Sidman (1964) made a careful study of the origin of macrophages around a needle wound in mouse brain using ^{3}H-thymidine. They concluded that the majority of reactive macrophages were of haematogenous origin and they found little positive evidence for proliferation of astrocytes. The large nucleate cells that they saw taking up the label they interpreted as being 'transitional' cells of haematogenous origin. Only Sjöstrand (1965) has reported hyperplasia as well as hypertrophy of astrocytes in a study of the rabbit's hypoglossal nucleus reacting to section of its nerve, while Adrian (1968) in a methodical autoradiographic study of the reaction to a small needle wound in the mouse spinal cord, concedes that local cells proliferate in response to the injury but fails to identify clearly astrocytes as taking part in the process.

There are certain major disadvantages to the autoradiographic technique for studying proliferation in neuroglial cells. There is, firstly, the objection that a pulse label demonstrates that nuclei are synthesizing DNA, but does not indicate whether, or how, the cell divides. Secondly, the identification of the cell type beneath the autoradiographic film is a universally admitted difficulty, and this is particularly acute in small rodents where resting astrocytes are often difficult to distinguish by their size alone from other cells. Both these objections are adequately met by studying the mitotic activity of cells, and, in addition, often it is possible to identify the dividing cell more readily than its resting counterpart (Cavanagh & Webster, 1955). The purposes of this study, therefore, were to determine the numbers, distribution and types of cells that divide around a simple brain wound paying particular attention to the role of the astrocyte in this response.

MATERIALS AND METHODS

Animals. Adult rats (200–250 g) of various strains and of both sexes were used. They were housed in plastic boxes and fed Chardex and water *ad libitum.*

The brain wound. Under ether anaesthesia the scalp was clipped and cleansed with alcohol (70%). A mid-line incision was made and the pericranium scraped off the surface of the skull. With a spherical dental burr a hole was drilled in the skull 3 mm to the right of the mid-line and 2 mm behind the coronal suture, the hole being slightly larger than a Bashford transplantation needle (2·4 mm external diameter). This needle with its plunger withdrawn into the barrel for about 0·5 cm was thrust gently into the brain vertically downwards until the point just touched the base of the skull. The needle on withdrawal contained a short cylinder of brain tissue. Bleeding from the wound occurred for a short while. The skin wound was closed with silk sutures and the animals quickly recovered. No obvious functional disturbances or other complications occurred from this wound.

Preparation of tissues. The animals were killed at 1, 2, 3, 4, and 6 d under chloroform anaesthesia. When deeply anaesthetized they were pinned out onto a cork board and the chest was widely opened by reflecting the anterior wall. The inferior vena cava and then the left ventricle were quickly cut open and a polythene cannula was thrust into the aorta and tied there. Normal saline from a perfusion apparatus was run through for 10 s, and then formal-acetic acid (10%–1%) was run into the vascular tree. The fixative began to flow about 20–30 s after the chest had been opened and was associated with marked muscular activity in the face and upper limbs. The fixative was left to run for 20–30 min, and then the skull was opened and the brain removed. In successful perfusions the brain was firm and uniformly white at this time, and it was further fixed overnight in formol-acetic acid. In unsuccessful perfusions grey patchy areas were seen in the brain and the meningeal vessels contained blood. These animals were discarded.

The hardened brain was sliced horizontally at the level of the olfactory lobes by a single cut and the two halves were dehydrated and embedded side by side. The slides, therefore, contained pairs of sections at two different levels that cut horizontally across the circular wound. The wound lay in the lateral part of the thalamus at about its mid part. In the earlier stages after wounding a small amount of blood filled the circular cavity.

Sections 10 μm in thickness were cut from paraffin blocks and stained routinely with Haematoxylin and eosin or cresyl fast violet, at least three slides being stained by each method. Selected sections were stained with Feulgen's stain, the PAS method for glycogen, Unna Pappenheim stain, or iron haematoxylin-van Gieson stain.

Microscopical methods. All observations were made with a square Erhlich ocular; the sides of the field, when an oil immersion $\times 95$ objective was used, measured 100 μm and this field size and magnification were used throughout the measurements. The area around the wound was systematically scanned square by square in a radial direction as near as possible perpendicular to the wound edge. The numbers of mitoses, their type and the stage of the cycle were recorded for convenience on squared paper. Twelve squares were taken stepwise from the edge wherever this was

possible, i.e. in a lateral, anterior or posterior direction (Fig. 1). The medial side of the wound was usually too close to the ventricle to be useful. Between 25 and 35 rows of twelve squares each could usually be sampled from each section, the first square in each radial row being taken where the square is maximally filled with cells at the wound edge. From four to eight sections were sampled in this way, the upper and the lower part of the cylindrical wound being studied alternately. In the normal series it was important to make sure that an adequate sample at different levels of the wound was taken, so that a larger number of sections were studied than was probably necessary.

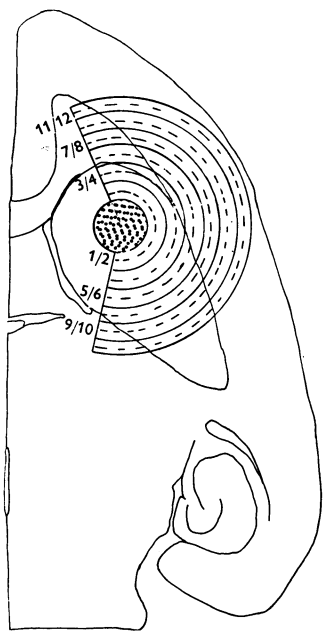


Fig. 1. Diagram of a horizontal section of rat brain showing the site of the wound (dotted) and the method of obtaining the figures for zones at different distances from the wound edge. After recording mitoses or nuclear numbers in the square fields, in a radial direction from the wound edge, the mean of each pair of zones was obtained at each distance.

At every 10th radial row of square fields the numbers of non-neuronal nuclei were counted in each square, so that a 10% population sample of potentially dividing cells was thus determined.

When the counts were completed the mitotic and nuclear numbers were calculated for each distance from the wound area, the unit area being 100 squares. Finally, the mean of each two adjacent zones, namely zones 1 and 2, zones 3 and 4 etc., were derived. Graphs of mitotic rates and nuclear populations per unit area could thus be drawn which provided, as it were, average profiles of these parameters in zones 200 μm wide from the wound edge to a distance of 1200 μm.

Mitotic figures were classified according to size and to phase. 'Large' divisions measuring more than 8 μm and usually 12–15 μm across as shown later were astrocytes. 'Small' divisions less than 8 μm in diameter were of macrophages,

microglia and other cells. Some of these were closely associated with capillaries, and of these a proportion were elongated and divided in the long axis of a vessel: these were probably vascular cells. In addition, a small proportion of the 'small' divisions may have been oligodendroglia, but since the primary purpose of this study was to specify astroglial proliferation from the remaining cells, no special steps were taken to try to subdivide these 'small' nuclei. In assessing meta- or anaphase in the 'large' cells particular care had to be paid to seeing whether the chromosomes were single or double, because the large size of the mitotic figures often did not allow them to be wholly included in one section.

RESULTS

Appearances around the brain wound

At 1 d after operation the centre of the wound was either filled with red blood cells or was oedematous and the cells within it showed pyknosis and other evidence of necrosis. At the wound edge there was a suggestion of increased microglial activity and numbers, but dividing cells were rare and all 'small'.

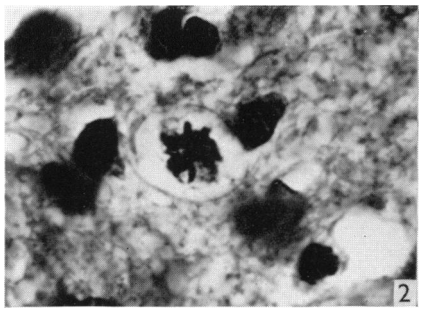
At 2 d the centre of the wound was quite necrotic and often contained red blood cells. At the edge, in a band about 200 μm wide, there was a marked increase in cells with small, often oval or lobed, nuclei and scanty slightly basophilic cytoplasm. Often they were concentrated around small blood vessels. The endothelial cells of the latter were conspicuously activated at this stage with enlarged nuclei and swollen basophilic cytoplasm. Evidence of growth of these latter towards the centre of the necrotic area was readily found at 2 and 3 d. Beyond this band of cells activation of microglia was seen in all zones up to 1200 μm from the wound edge. Frequently cell division was found in satellite cells of neurons of both cortex and basal ganglia. Cells identifiable as dividing astrocytes (on criteria discussed later) began to be seen on this day.

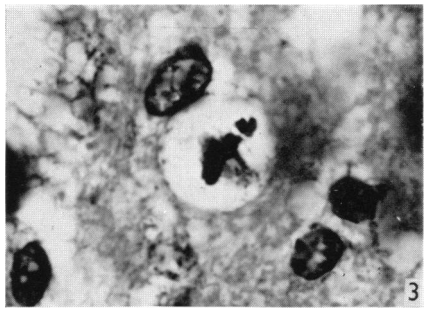
At 3 d after operation the band of mononuclear cells at the edge of the wound was increasing in density and dividing cells were most numerous here, but there was also a general increase in cell activity throughout the 1200 μm region around the wound. Foam cells also began to be a conspicuous feature of the zones near the necrotic core of the wound. Division of large cells recognizable as astrocytes was maximal on this day. Division of vascular endothelial cells and the growth of capillaries were also still active.

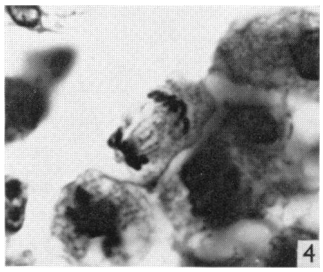
At 4 d after operation the appearances were substantially the same while at 6 d the necrotic centre was appreciably smaller and the density of mononuclear cells at the wound edge was even greater. Mitotic activity was still active in these border cells, but was greatly diminished in the zones removed from the wound edge.

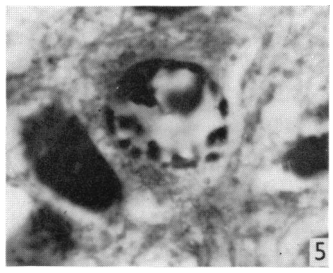
Evidence of mitotic activity in cells

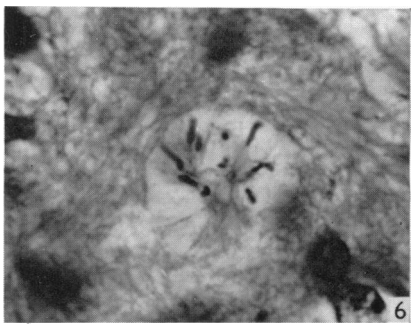
Macrophages/microglial cells (*Figs.* 2–4). For the purposes of this study these two classes of cells are considered to be indistinguishable except by virtue of their position. In the two 100 μm zones next to the wound the great majority of the cells in fact resemble blood mononuclear cells and are presumed to have arrived from this exogenous source. Mitoses occur among these cells from the second day onwards.











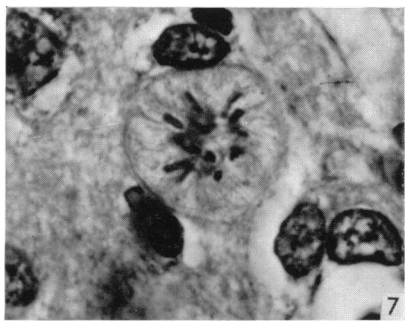


Fig. 2. A macrophage/microglial cell in metaphase showing the small clumped chromosomes, the watery or vacuolated cytoplasm and the clear circular cell border. From about 500 μm from the wound edge at 3 d. H and E $\times$ 1700.

Fig. 3. A macrophage/microglial cell in metaphase, the densely clumped chromosomes congregated at the metaphase plate and the spindle having formed. Some distance from the wound edge at 3 d. H and E $\times$ 1700.

Fig. 4. A macrophage/microglial cell in anaphase at the wound edge at 3 d and probably haematogenous in source. H and E $\times$ 1700.

Fig. 5. An astrocyte in prophase. The chromosomes are still contained within a nuclear membrane and the cytoplasm, not well seen here, is diffusely eosinophilic. 3 d. H and E $\times$ 1700.

Fig. 6. An astrocyte in metaphase. Note the large cell size, the well dispersed arrangement of the chromosomes, which are double, and the final radial striations in the cytoplasm. 3 d. H and E $\times$ 1700.

Fig. 7. An astrocyte in metaphase. Similar to Fig. 6, but with a more sharply outlined cell border. 3 d close to the wound edge. H and E $\times$ 1700.

All figures reduced $\times \frac{1}{2}$.

Their chromosomes are small and usually closely clumped together at metaphase so that it is difficult to identify individual chromosomes. The cytoplasm of such dividing cells is sometimes homogenous and basophilic. In addition, dividing cells with foamy, finely vacuolated cytoplasm become increasingly numerous from the second day onwards. The cells of this kind lie in the apparently undamaged parenchyma of the surrounding brain either solitary or as satellites of neurons. The nucleus, or clumped chromosomes, of these cells rarely exceeds 8 μm in diameter, the cell outline is round and clear and the spindle at anaphase usually very well seen.

Astrocytes (*Figs.* 5–7). The nucleus of the normal resting rat astrocyte has a mean diameter of about 6·5 μm, and is very finely granular, the chromatin being evenly scattered throughout. A small nucleolus is usually seen close to the nuclear membrane. On activation of the cell the nucleus becomes enlarged to 12 μm in diameter or more, the nucleolus becomes prominent and the chromatin more condensed. At this stage the nucleus may closely resemble in size and appearance the nuclei of small neurons. The cytoplasmic RNA of these cells is diffuse and not clumped as in neurons, and a further point of difference is that there is no nuclear cleft or groove visible such as can usually be seen in well-fixed neurons. Glycogen is also commonly present in the cytoplasm of these cells. Distinction between the two cells is in general not difficult on these grounds. The prophase stage of mitosis (Fig. 5) in these cells is quite characteristic both in the size of the nuclei and the conspicuousness of the chromosomes when they form up within the nuclear membrane. The cytoplasm at this stage is hyaline and eosinophilic with a slight bluish tinge in well-stained haematoxylin preparations, and in Unna–Pappenheim preparations there is light diffuse pyroninophilia.

Metaphase (Figs. 6 and 7) is most dramatic in these cells both because of the abundance of the eosinophilic cytoplasm and particularly because of the long, well dispersed arrangement of the chromosomes. The cell outline, when it lies in the tissue well removed from the wound edge, may be jagged due to processes running into the parenchyma which are preserved. Sometimes quite round forms occur, especially near the wound edge, which are 20–25 μm in diameter and show fine radial striations. Possibly because of the hydropic nature of the cytoplasm of this cell the chromosomes tend to be more widely dispersed than is usually seen in dividing cells of any other mammalian tissues. The form of the chromosomes is thus readily seen with great regularity, as though they had been dispersed by hypotonic treatment. Neither anaphases nor telophases were frequently seen in astrocytes, a feature that may, in large part, be explained by the size of the division in relation to section thickness. Unless the presence of single or double chromosomes is particularly looked for identification of these phases is very difficult.

Oligodendroglial cells. No definite evidence that these cells responded to an injury of this type by proliferation could be found. In those areas, i.e. white matter, where oligodendroglia were numerous mitotic activity either among astrocytes or other cells was never greater than in grey matter where these cells were fewer in numbers. Nor did they show activation of nuclei or increase in nuclear size at any time. It is conceivable that they did divide but in doing so they could well become indistinguishable from microglia.

Vascular endothelial cells. The division of these cells was invariably in a direction

parallel to the direction of the vessel. At prophase the nucleus was larger and more elongated than that of macrophages/microglia but was distinctly smaller than astrocytes. They proliferated only within the two 100 μm zones adjacent to the wound edge.

Quantitative aspects of mitotic activity

The mean mitotic activity of all cells in the zones 1/2 steadily increased from the first day to reach a peak at 4 d, and showed a beginning decline at 6 d (Fig. 8). In

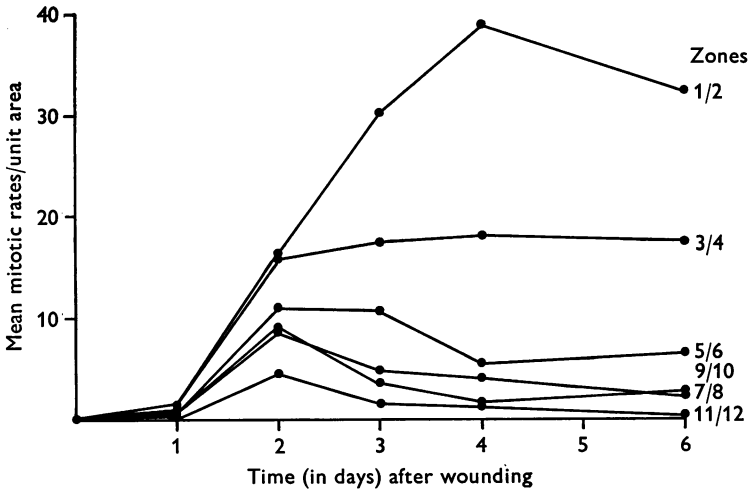


Fig. 8. Mean total mitotic rates per unit area at differing days after wounding in each zone at increasing distances from the wound edge. Zone 1/2 is at the wound edge, zone 11/12 is between 1000 and 1200 μm distant from it. The peak activity is reached soonest the more removed the zone is from the wound edge.

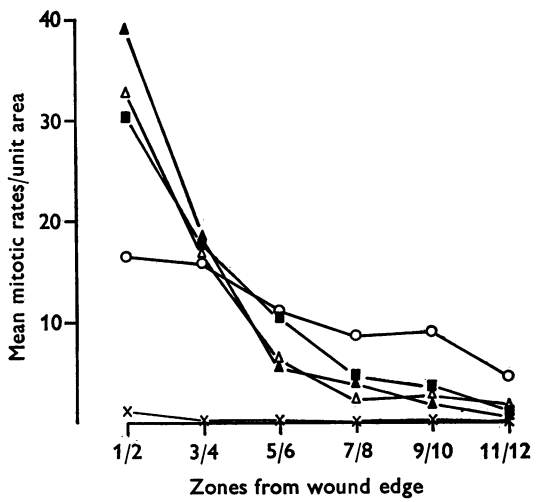


Fig. 9. Mean total mitotic rates per unit area at different distances from the wound edge. ×——×, One day; ○——○, 2 days; ■——■, 3 days; ▲——▲, 4 days; △——△, 6 days.

zones 3/4 a plateau was reached at 2–3 d which remained steady during this period, but with zones 5/6 to 11/12 the peak mitotic activity was reached at 2 d and was declining towards negligible values by the 4th d (Table 1).

If the mean mitotic rate per unit area for the zones is considered in relation to the distance from the wound edge, the rate is found to decline exponentially from the wound edge at all periods from the second day (Fig. 9). The logarithm of the mean mitotic rate/unit area correlates closely (Table 2) in a linear fashion with distance from the wound edge (Figs. 10*a–d*).

Table 1. *Mean mitoses/unit area at different days after a brain needle wound with range of figures and standard errors of the means where appropriate*

	1 d		2 d		3 d		4 d		6 d	
Zones	Mean (3)*	Range	Mean (2)	Range	Mean (7)	Range (±SEM)	Mean (5)	Range (±SEM)	Mean (2)	Range
1/2	1·2	0–2·7	16·6	14·2–19·0	30·3	21·7–40·3 (±5·13)	39·0	24·4–72·4 (±9·58)	32·5	26·1–39·0
3/4	0·6	0–1·4	16·2	12·6–19·5	17·5	14·4–20·8 (±1·03)	18·0	11·0–25·5 (±3·03)	17·4	9·2–25·6
5/6	0·4	0–0·7	11·0	11·0–11·1	10·7	8·1–17·0 (±0·54)	5·5	2·7–7·7 (±1·00)	6·5	3·9–9·1
7/8	0·3	0–0·7	8·5	6·5–10·5	4·9	1·7–8·4 (±0·96)	4·0	1·3–10·0 (±1·82)	2·3	1·6–3·0
9/11	0·3	0–0·5	8·9	4·6–13·2	3·4	1·1–6·4 (±0·82)	1·8	0·6–3·4 (±0·59)	2·6	1·5–3·7
11/12	0·0	—	4·6	3·9–5·3	1·5	0–3·9 (±0·53)	1·4	0·9–1·7 (±0·19)	0·6	0·6–0·7

* Number of animals

The numbers of large mitoses (astrocytes) was always maximal in zones 3/4, that is to say not immediately adjacent to the wound edge but in the two 100 μm zones beyond this (Fig. 11). Dividing astrocytes could be found, however, at all zones up to 1200 μm from the wound edge. The percentage of total mitoses in all regions that were formed by astrocytes was greatest on the third day (19·3%), at 2 d it was 13·7%, while at 6 d the proportion was 11·7% (Fig. 12). These figures are of limited value, however, because they are derived from the whole area under study. They demonstrate, however, that at the time of peak proliferation almost one division in five was likely to be an astrocyte while in the parenchyma beyond the wound edge the proportion of astrocytes dividing would be substantially greater than this.

Effects of delay in fixation on mitotic activity around a brain wound

Two animals were killed 3 d after making a brain wound exactly as before, but instead of perfusing the brain with fixative it was taken out and dropped into formal-acetic acid. No special attention was paid to doing this rapidly but the process took less than 5 min.

Mitotic counts (Table 3) showed a marked lowering at all points away from the

wound edge, the numbers being about half of the mean of the perfused-fixed brains. Each point in the immersion-fixed brains fell below the range of mitotic rates of the perfused brains, but the proportion of large to small dividing nuclei remained the same and so also did the proportion of the various phases of mitosis.

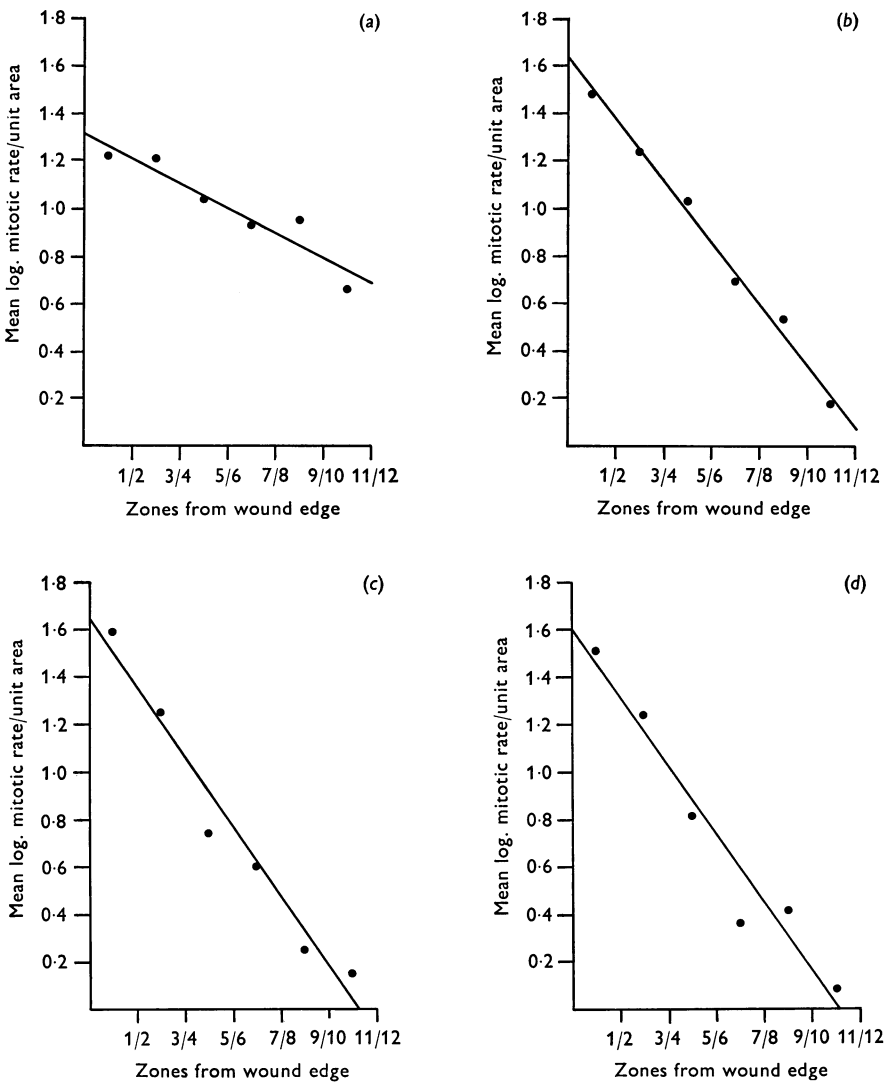


Fig. 10. Calculated regression lines relating the logarithm of the mean total mitotic rates/unit area at: (*a*) 2 d; (*b*) 3 d; (*c*) 4 d, and (*d*) 6 d after wounding. For details of the regression equations see Table 1.

Delay in fixation even for the short time required to remove the brain from the cranium and the penetration of fixative, therefore, plays a part in reducing the numbers of visible mitoses.

Effects of colchicine on the mitotic numbers around a brain wound

Two rats were given 2 mg/kg colchicine intraperitoneally in saline 4 h before killing and two rats were given 2 mg/kg colchicine intraperitoneally 6 h before killing, in every instance 3 d after making a brain needle wound. The brains were fixed by perfusion with formal-acetic acid.

The mean mitotic counts (Table 3) at each time fell within the range of those of

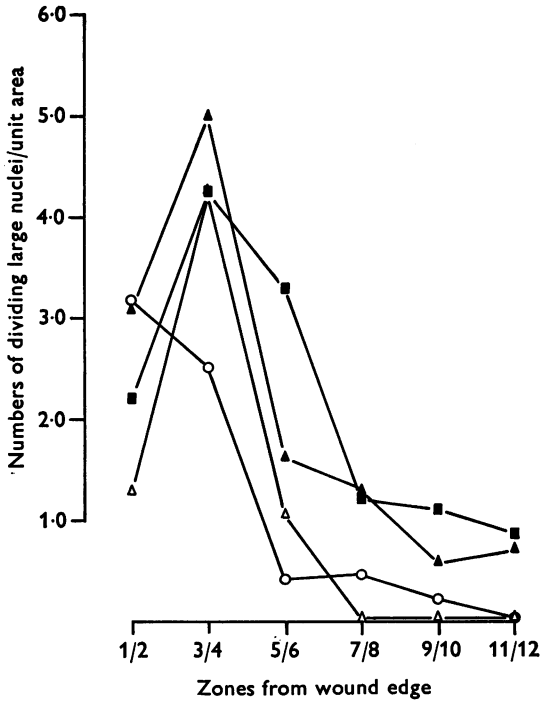


Fig. 11

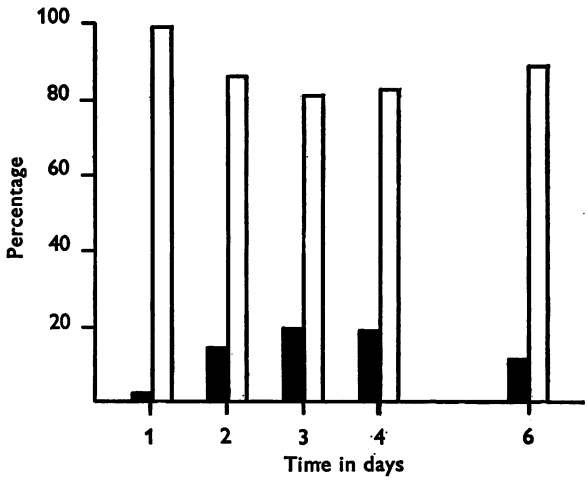


Fig. 12

Fig. 11. Mean numbers of dividing 'large' nuclei (astrocytes)/unit area in relation to distance from the wound edge at different days after injury. ○——○, two days; ■——■, 3 days; ▲——▲, 4 days; △——△, 6 days.

Fig. 12. Histograms of percentage of 'small' (white blocks) and 'large' (black blocks) mitoses over the whole area studied at different days after wounding.

Table 2. *Values for the correlation coefficients and the regression equations calculated for the lines relating the logarithm of the total mean mitotic rates per unit area against unit distances from the wound edge*

Days after injury	Correlation coefficient	Slope	X-intercept
2	−0·946	−0·053	1·318
3	−0·996	−0·128	1·627
4	−0·981	−0·148	1·649
6	−0·973	−0·144	1·600

control rats and there was no significant increase in numbers of cells in division during these periods. Moreover, the proportions of cells in the four phases of mitosis were the same in the animals given colchicine as in the controls. No cells were found with typical colchicine-arrested metaphases.

It must be concluded that colchicine when given systemically does not have the expected effect upon dividing cells in the brain. To exclude the possibility that for some unknown reason cells in the brain might be insensitive to the known action of colchicine, attempts were made to inject colchicine directly into the brain wound. It was found that when a saline solution containing 5 γ colchicine/ml was successfully injected into the wound in two animals and the brain perfused 4 h later, there was a substantial increase in mitotic figures in all zones from the wound edge (Table 3; Fig. 13). Large numbers of colchicine-arrested metaphases were seen in the sections from each animal both in small dividing cells and in large (astrocyte) cells. It must be concluded, therefore, that there is no inherent resistance in these cells to colchicine but that this substance fails to enter the area around the brain wound.

Table 3. *Mean mitotic rates/unit area 3 d after brain wounding showing diminution in mitotic numbers after immersion fixation, the absence of increased numbers of mitoses after Intraperitoneal (I/P) colchicine (2 mg/kg), and the occurrence of increased mitotic numbers after Intracerebral (I/C) colchicine*

	Perfusion fixation		Immersion fixation		I/P colchicine 4 h (perfusion fixation)		I/P colchicine 6 h (perfusion fixation)		I/C Colchicine 4 h (perfusion fixation)	
Zones	Mean (7)*	Range (± SEM)	Mean (2)	Range	Mean (2)	Range	Mean (2)	Range	Mean (2)	Range
1/12	30·3	21·7–40·3 (± 5·13)	14·6	12·6–16·7	26·3	21·7–32·8	35·0	29·6–40·3	56·5	38·1–74·9
3/4	17·5	14·4–20·8 (± 1·03)	7·6	7·1–8·1	15·1	14·4–15·9	16·1	15·6–16·7	27·2	26·7–27·7
5/6	10·7	8·1–17·0 (± 0·54)	3·2	2·5–4·0	8·1	8·1–8·2	9·8	8·1–11·5	20·9	17·6–24·2
7/8	4·9	1·7–8·4 (± 0·96)	2·1	0·7–3·6	2·5	1·7–3·2	6·3	5·4–7·3	9·9	9·1–10·7
9/10	3·4	1·1–6·4 (± 0·82)	0·9	0·7–1·1	5·4	1·4–4·0	4·6	2·7–6·4	5·3	4·5–6·1
11/12	1·5	0–3·9 (± 0·53)	0·1	0·0–0·3	1·0	0·0–2·0	2·0	0·0–3·9	7·3	3·9–10·8

* Number of animals

Nuclear population changes

The estimates of mitotic activity used in the above experiments have been related to unit areas of brain at different distances from the wound edge. A more accurate way of expressing this function would be to relate it to numbers of potentially divisible cells. In such a mixed tissue as brain this is a difficult parameter to estimate, and the numbers of cells with the potential for division cannot at present be gauged. Population estimates of non-neuronal nuclei are of interest in themselves, however, and were made on an approximately 10% sample of the square fields inspected. As with the mitoses, the numbers were related to units of 100 squares in each zone and

the means for each pair of zones were computed for different distances from the wound edge.

All non-neuronal nuclei were counted, including vascular and oligodendroglial nuclei. The numbers of the latter in white matter were about three times those found in grey areas, nonetheless the size of the mitotic population was not found to be in any way related to grey or white matter. Although negative evidence of this sort is difficult to evaluate, no indication was found to show that oligodendroglia of white

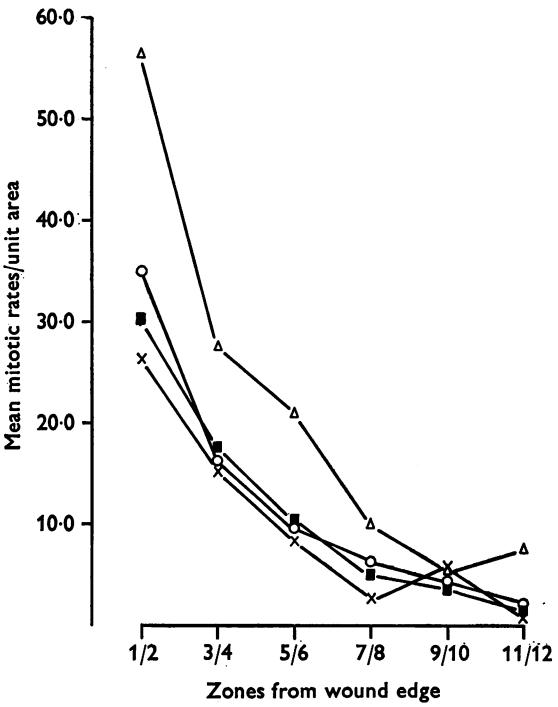


Fig. 13

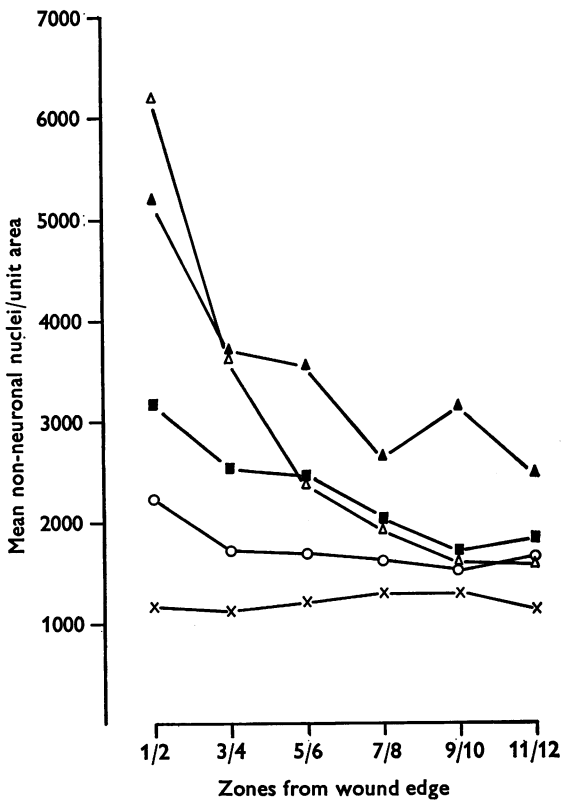


Fig. 14

Fig. 13. Mean total mitotic rates/unit area at 3 d after a brain needle wound. Intraperitoneal (I/P) colchicine (2 mg/kg) does not alter the expected rates, whereas intracerebral (I/C) colchicine (5γ/ml) causes an increase in mitoses all distances from the wound edge. Numbers of animals in parentheses. △——△, I/C colchicine 4 h (2); ×——×, I/P colchicine 4 h (2); ○——○, I/P colchicine 6 h (2); ■——■, normal (7).

Fig. 14. Mean non-neuronal nuclear populations/unit area taken from a 10% sample at different distances from the wound edge with days after wounding. ×——×, one day (3); ○——○, 2 days (2); ■——■, 3 days (7); ▲——▲, 4 days (5), △——△, 6 days (2).

matter played any part in the mitotic activity around a wound. If it is true that these cells have, like neurons, no capacity to respond to injury of this type by cell division, then all nuclear population estimates presented here should be lowered to take this fact into account. Fig. 14 shows that there was a steady increase in numbers of

nuclei in all areas up to the 4th d after injury. At the 6th d, while the cells at the wound edge were still tending to increase in density, at distances further from the wound edge their concentrations had fallen appreciably. Whether this means that cells were tending to move out of these areas, or were being lost by other means, cannot be said without sequential labelling studies. It is of interest, however, that the nuclear populations in areas not immediately adjacent to the wound edge only a little more than doubled over the period of study.

DISCUSSION

The hypertrophic changes in astrocytes after brain injury or around neurons whose axons have been damaged have been long recognized and many aspects of this change have been documented (Hortega & Penfield, 1927; Linell, 1929; Klatzo, Piraux & Laskowski, 1958; Rubinstein, Klatzo & Miguel, 1962; Friede, 1962; Sjöstrand, 1966). The exact purpose of this response is still by no means clear.

The chief purpose of this study was to resolve the puzzling failure of others who had studied the response of neuroglia to injury in Man and in animals to find mitotic activity in these cells, and particularly to determine whether there was any evidence for amitotic division in these cells. As mentioned earlier, Hortega & Penfield (1927), using metallic impregnation methods, found no cell divisions. Although they observed that the astrocyte nuclei became swollen and less regular than usual, the increase in cells was considered to be by an amitotic process (Penfield, 1932). Lapham (1962) and Lapham & Johnstone (1964) have found by microspectrophotometric measurements that reactive astrocyte nuclei in human brain commonly have twice the normal content of DNA and that these nuclei are larger in size than normal diploid nuclei. Because they found no mitoses, they too concluded that these cells underwent an amitotic process of division. Only Sjöstrand (1965) has claimed positive evidence of mitotic proliferation in astrocytes, although in small numbers, in the hypoglossal nucleus of the rabbit after nerve crush, and, moreover, he could find no evidence for amitosis or for DNA synthesis without subsequent mitosis.

The use of isotopically labelled thymidine has shown that cells identified as astrocytes around a wound in the spinal cord incorporate the label into their nuclei (Adrian & Walker, 1962; Adrian, 1968), and similarly Koenig, Bunge & Bunge, (1962) found the label being taken up by macroglia following selective demyelination in the cat spinal cord. As indicated in the Introduction, a study of the mitotic activity of cells has some advantages over the use of labelled DNA precursors, although the two methods are best looked upon as complementary. The main disadvantages of using mitotic activity is the shortness of its duration by comparison with the duration of the label in the cell. As Messier & Leblond (1960) have pointed out, the labelling index after ^{3}H-thymidine tends to be about ten times the mitotic index. Nonetheless the advantages of seeing the cell division and determining perhaps whether it is normal or not are very substantial. This method has made it possible to show that delay in fixation, as in this instance using immersion rather than perfusion fixation, can cause a considerable reduction in the numbers of visible mitotic figures even in such a small brain as that of the rat. In larger brains, where penetration of fixative will be even slower, the loss of mitoses would be expected to be even

greater, and sufficient to account for the total absence of mitoses commented upon by Lapham (1962) and others in the earlier literature. A similar reduction in mitotic activity in the subependymal plate of the adult rat has been found by Cavanagh & Lewis (1969) to occur unless perfusion fixation is practised. The quantitative approach to proliferation in this region and around a brain wound has also allowed us to determine with confidence the absence of effect of colchicine when given intraperitoneally on proliferating cells in the brain. As noted by Cavanagh & Lewis (1969), this finding confirms the observations of others that this substance, even though it is of small molecular weight, does not seem to be able to reach dividing cells in the brain.

The identification of cells in the brain reacting to injury is a major problem that influences all interpretations of autoradiographic studies. In the present study there was clearly one cell that was outstandingly different in both size and morphology from other dividing cells. This cell has been termed here an astrocyte not on the usual basis of metallic impregnation methods, but (i) on the appearance of processes passing into the surrounding tissue, (ii) on the abundant homogeneous nature of its cytoplasm and the occurrence therein of glycogen, (iii) on its distribution around the brain wound both in white matter and in grey, and (iv) on the large size of its nucleus in prophase and the strikingly well displayed chromosomes in metaphase. This last feature is unusual in mammalian cells unless they have been placed in a hypotonic solution (Hsu, 1952). The well spread chromosomes in these paraffin-embedded preparations are likely to be a reflexion of the marked tendency for astrocytes to imbibe water from the extracellular space and to swell in the region of vascular injury (Klatzo, 1967; Blakemore, 1969). The reluctance of others to allow that these cells divide is certainly curious, and even Adrian (1968), who shows figures of three typical astrocyte nuclei in division, does not admit to the nature of these cells. It is possible, however, that the size of the wound made may affect the numbers of dividing astrocytes. Konigsmark & Sidman (1964) used a No. 27 gauge needle, while Adrian (1968) used a No. 30 gauge needle, both these being less than 1 mm in external diameter. According to Bullough & Laurence (1960), wounds of skin less than 1 mm in width produce little or no mitotic activity in the adjacent epidermis. The same could well happen around a brain wound and it was for this reason that a large transplantation needle (2·4 mm external diameter) was used.

The identification of other cell types is perhaps less clear. Microglia away from the wound edge were probably identifiable by their foamy cytoplasm, clumped chromosomes and well-rounded cell outlines. At the second and third days after wounding such cells commonly occurred as satellites to neurons, often at considerable (1000 μm or more) distances from the wound edge. Similar proliferation activity has been found by others for satellites of microglial nature (Kreutzberg, 1965; Sjöstrand, 1965; Watson, 1965) around neurons responding to injury at their axons. The meaning of this response at distances from the wound in the present study is not clear but could be a reflexion of a reaction to axon damage. On the other hand there was very little evidence for the activation or proliferation of oligodendroglia to this type of injury even when the edge of the wound involved white matter. A similar lack of reactivity of oligodendrocytes in spinal tract degeneration of nerve fibres has been observed by Lampert & Cressman (1964) and by Blakemore & Cavanagh (1969).

The time relationships of the peaks of mitotic activity at different distances from the brain wound are of interest in relation both to the nature of the cells and the stimuli causing the proliferation. The cells in the two 100 μm zones at the edge of the wound, with the characters of haematogenous cells (Konigsmark & Sidman, 1964), continue to proliferate freely up to the sixth day, whereas the more distally placed cells, more than 400 μm from the wound edge, reach their peak of proliferation at the second or third day. The time relationships of this latter curve of response is closely similar to that found by Adrian (1968) after a needle wound to the mouse spinal cord and to that following nerve section in the facial nucleus found by Cammermeyer (1965). It stands in marked contrast with the rate of glial response to a denervating lesion in the dorsal columns of the rat where the peak mitotic activity was between 1 and 2 *weeks* after the onset of denervation. The most characteristic feature of the needle wound as opposed to a denervation lesion is damage to the vascular bed and leakage of plasma into the brain (Klatzo, 1967) and the proteins that escape are known to be actively taken up into microglia and into astrocytic processes (Klatzo, Miquel & Otenasek, 1962; Blakemore, 1969). The relationship between the logarithm of the mean mitotic rate and the distance from the wound edge that has been demonstrated here suggests that the essential mitotic stimulant arises from the wound area and its effects are diminished with distance from the wound edge. Thus, the escape of protein, which is conspicuously absent in the degenerating spinal tract lesion, could be the essential stimulus to this proliferative process. Alternatively, the increased hydration of these cells due to their uptake of water from the extracellular space, as suggested by Bullough & Laurence (1960) for epidermal cells, may by the dilution of a mitotic inhibitor (chalone) produce the same effect. Chalones have not yet, however, been shown to occur in astrocytes as they do in other tissues (Bullough, 1967). Since the vascular leakage in a brain wound occurs only for the first few days after damage, the stimulus would be effective only so long as material from the plasma remained in the extracellular space. Electron microscopy has shown that following a cold lesion all visible material may be removed from the extracellular space by phagocytosis within 3 days (W. F. Blakemore, in preparation), a period that coincides within a day to the virtual cessation of cell division in the parenchyma beyond the immediate vicinity of the wound. In the degenerating spinal tract no vascular leakage can be detected either with trypan blue or ultrastructurally, and some other more sustained stimulus must be operative here. In the regenerating cranial nerve nuclei after nerve section again no increased vascular leakage can be detected (Sjöstrand, 1966), so that another factor, such as leakage of materials from the affected nerve cells into the extracellular space, must be postulated as the essential stimulus to the mild astrological proliferation in this lesion.

SUMMARY

A quantitative approach has been made to the cellular proliferation around a needle wound in the rat brain. The mitotic rates/unit area decline exponentially with distance from the wound edge, and the rate of fixation (perfusion vs. immersion) exerts a marked influence on the numbers of visible mitoses. Away from the immediate vicinity of the wound edge the peak of proliferation occurs at the second and third

days, and it is suggested that this may be a response to the diffusion of materials in the escaped plasma from the region of vascular damage. Mitoses in astrocytes were found to form up to one-fifth of the total cell divisions, and no evidence of an amitotic process of division could be detected.

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