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Quantitative changes in neuroglia in the white matter of the mouse brain following hypoxic stress

R. R. STURROCK
Department of Anatomy, The University, Dundee, Scotland

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INTRODUCTION

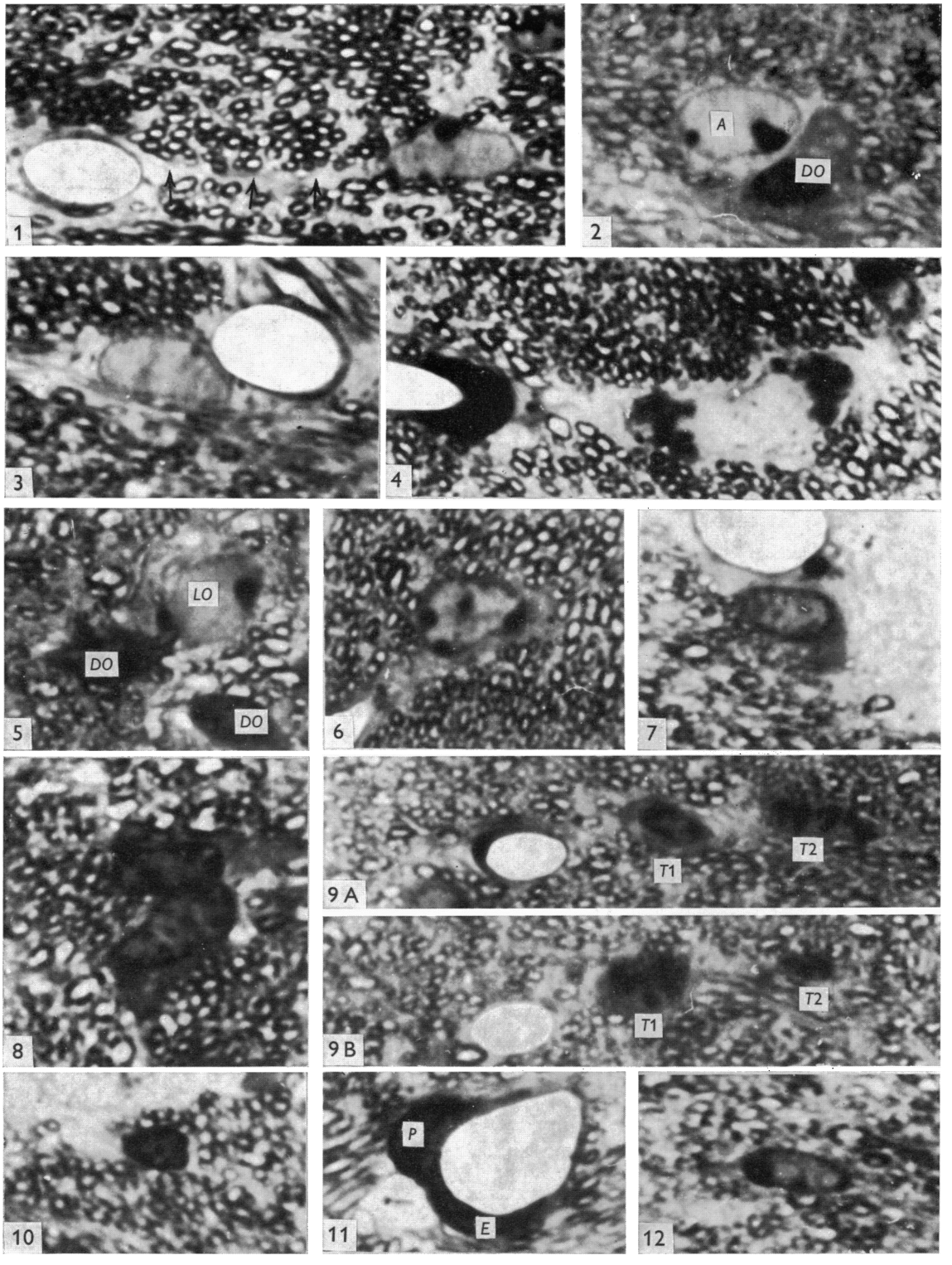
The normal neuroglial population of the anterior limb of the anterior commissure of the mouse brain during development has been studied by both light microscopy (Sturrock, 1974*a*, *b*, *d*, 1975*c*) and electron microscopy (Sturrock, 1974*c*). Using this work as a basis, the effects of environmental and pathological changes can be assessed, and the aim of the present study was to find whether quantitative changes occur in the neuroglia of the anterior limb of the anterior commissure following hypoxia, using semithin, toluidine blue stained sections as a means of identifying the different glial cell types. The use of semithin sections for glial identification was first suggested by Mori & Leblond (1969*a*, *b*, 1970) and its efficacy confirmed by Griffin, Illis & Mitchell (1972). Detailed criteria for glial identification in semithin sections have recently been drawn up by Ling *et al.* (1973). These criteria have been used in the present study except that, for brevity, the cells referred to by Ling *et al.* (1973) as 'free subependymal cells', will be termed glioblasts.

MATERIALS AND METHODS

Male ASH/TO mice aged 70–80 days postnatum were used in these experiments. Fifteen animals were selected at random, five being used as controls, and ten as experimental animals. The experimental animals were subjected to hypoxia for 2 days in a decompression chamber, at a pressure of 390 mmHg, as described by Hunter & Clegg (1973).

Fortyeight hours after the animals were first placed in the decompression chamber, five were selected at random for immediate killing, and five were allowed to recover for 1 week before being killed. (Therefore the former group will be referred to as the hypoxic group and the latter as the recovery group.) The control animals were killed at once. All animals were killed, while under ether anaesthesia, by perfusion of physiological saline at 37 °C through the ascending aorta, followed by perfusion-fixation with a solution of 2 % paraformaldehyde and 3 % glutaraldehyde, as described by Ling *et al.* (1973).

After fixation the mice were placed in polythene bags and left overnight at 4 °C, before the brains were removed. The anterior limbs of the anterior commissures, obtained intact from the left halves of the brains by careful microdissection, were rinsed in cacodylate buffer for 1 hour, post-fixed in buffered 1 % osmium tetroxide for 1 hour, dehydrated in graded alcohols, and embedded in Spurr's resin.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Coronal sections were cut at 1 μm using a Spencer microtome fitted with a glass knife. Fifteen consecutive sections were cut and placed on a drop of a 10% acetone solution on a microscope slide with the aid of an artist's brush (Weakley, 1972). The slide was passed rapidly through a Bunsen flame 12–15 times to remove wrinkles from the sections, placed on a hot plate at 90 °C until all the acetone solution had evaporated, and stained with 1% toluidine blue in a saturated borate solution for 1 minute. One section on each slide was used for glial counts. After each slide had been prepared as described above, a further fifteen sections were cut and discarded, to ensure that no cell was counted twice.

The differential counts were carried out under oil immersion at × 1000 using a Zeiss Photomicroscope I with a yellow filter, and employing the criteria of Ling *et al.* (1973). The percentage of each type of glial cell present was corrected using Abercrombie's (1946) correction as recommended by Ling & Leblond (1973). A minimum of 300 cells was examined in each anterior limb, i.e. at least 1500 cells in each group.

The number of glial cells per unit area of the anterior limb was estimated by photographing each section, and measuring the area of each × 800 print with a Beck metric planimeter.

Fig. 1. Astrocyte. Note the pale nucleus with a small chromatin clump against the nuclear envelope, which is the characteristic site of such clumps in astrocytes. The pale cytoplasm is the most important single distinguishing feature of astrocytes. A cytoplasmic process (arrows) can be traced from this astrocyte to a nearby capillary. × 2400.

Fig. 2. Astrocyte (*A*) demonstrating the pale nucleus and cytoplasm with peripheral chromatin clumping. The adjacent cell is a dark oligodendrocyte (*DO*). × 2400.

Fig. 3. Astrocyte adjacent to a capillary. Note the 'ground-glass' appearance of the nucleus, described by Ling *et al.* (1973) as commoner in material fixed by glutaraldehyde alone. This astrocyte has a few dense bodies in the cytoplasm. × 2400.

Fig. 4. Mitotic astrocyte. The pale cytoplasm and nucleoplasm enables this cell to be positively identified as an astrocyte (cf. Figs. 9A and B). × 2400.

Fig. 5. Light oligodendrocyte (*LO*). Light oligodendrocytes differ from astrocytes in having a cytoplasm which is darker than that of the myelinated axons. The other cells are dark oligodendrocytes (*DO*). × 2400.

Fig. 6. Light oligodendrocyte. This cell has more clumping of chromatin and a slightly darker cytoplasm than the light oligodendrocyte in Fig. 5. It is probably in the process of differentiating into a medium oligodendrocyte. × 2400.

Fig. 7. Medium oligodendrocyte. This cell lacks the characteristic chromatin clumps of dark oligodendrocytes and the cytoplasm is less dense. × 2400.

Fig. 8. A pair of dark oligodendrocytes. Note the fairly extensive dark cytoplasm and the characteristic chromatin clumping in the nuclei. × 2400.

Fig. 9(A) and (B). These two figures show the same late telophase (*T1*, *T2*) at different levels. The dark nucleoplasm and cytoplasm enable this cell to be identified as an oligodendrocyte. 9(B) was taken from the same print as Fig. 6 and allows a comparison of cytoplasmic densities to be made. This suggests the mitotic cell may be a medium oligodendrocyte. × 2400.

Fig. 10. Interstitial microglia. These cells can be recognized by the densely clumped chromatin around the nuclear membrane, their small size and irregular shape. × 2400.

Fig. 11. Pericytial microglia (*P*). These cells have dark nuclei usually crescentic in shape, with small dark clumps of chromatin. They are closely opposed to capillaries but their blunt-ended nuclei enable them to be distinguished from endothelial cells (*E*). × 2400.

Fig. 12. Glioblast. These cells have a moderately pale nucleus, usually ellipsoidal in shape, with one or two clumps of chromatin. The cytoplasm is usually extremely scanty. × 2400.

Table 1. *Percentage of each glial cell type present in control, hypoxic and recovery groups*

Group	Light oligo-dendrocytes	Medium oligo-dendrocytes	Dark oligo-dendrocytes	Astrocytes	Microglia	Glioblasts
Control	1·8±0·6	8·0±1·7	63·0±1·7	14·2±1·6	5·5±0·8	7·7±1·9
Hypoxic	1·1±0·5	11·7±1·0	65·3±1·8	9·0±2·6	5·8±1·8	7·0±2·1
Recovery	0·8±0·4	9·3±2·0	68·7±2·1	11·1±1·0	3·5±1·2	6·6±2·2

Table 2. *Significance of 't' test values between control and hypoxic, control and recovery, and hypoxic and recovery groups*

Groups	Oligodendrocytes	Astrocytes	Microglia	Glioblasts
Control–hypoxic	$P<0·001$	$P<0·005$	$P>0·3$	$P>0·3$
Control–recovery	$P<0·005$	$P<0·005$	$P<0·01$	$P>0·2$
Hypoxic–recovery	$P>0·3$	$P<0·1$	$P<0·02$	$P>0·3$
	$P<0·05$ (5 %) taken as significant.			

RESULTS

Observations

The following glial types were identified in control, hypoxic and recovery group animals: astrocytes (Figs. 1–3); light, medium and dark oligodendrocytes (Figs. 5–8); interstitial (Fig. 10) and pericytial microglia (Fig. 11); and glioblasts (Fig. 12). While oligodendrocytes, in general, were easily identified, some difficulty was experienced in identifying the three classes of oligodendrocytes, particularly in deciding whether a cell should be classified as a medium or dark oligodendrocyte, as many transitional types were present. Thus, to a certain extent, the classification of oligodendrocyte sub-types was subjective. Recognition of astrocytes, microglia and glioblasts was straightforward and presented no difficulties.

No morphological differences were apparent between neuroglia in control and experimental animals in semithin sections. Two mitotic figures were observed (Figs. 4, 9*A* and *B*), both in control animals. One was an astrocyte in anaphase, the other an oligodendrocyte in late telophase.

Differential counts

The results of the differential counts are shown in Table 1. The mean percentage of astrocytes was lower in the hypoxic and recovery groups than in the control group, and these differences were shown by Student's '*t*' test to be significant (Table 2). The mean percentage of astrocytes was higher in the recovery group than in the hypoxic group but this difference was not great enough to be statistically significant. The fall in the percentage of astrocytes in the hypoxic and recovery groups was balanced by a highly significant increase in the mean percentage of total oligodendrocytes in both groups (Table 2). There was no difference between the percentage of oligodendrocytes in the hypoxic group and in the recovery group.

There was no significant difference between the percentage of glioblasts in the

control, hypoxic and recovery groups. There was no difference between the percentage of microglia in the control and hypoxic groups, but the percentage of microglia in the recovery group was significantly lower than in either the control or the hypoxic groups (Table 2).

Cell density

No significant difference was detected between the number of cells per unit area in the control and hypoxic groups or in the hypoxic and recovery groups. The number of cells per mm^2 in control, hypoxic and recovery groups was 1009 ± 87, 1063 ± 104 and 1232 ± 189 respectively.

DISCUSSION

The total number of neuroglia in the anterior limb of the anterior commissure of the mouse brain is constant after 35 days postnatum (Sturrock, 1974*a*), but after this time there is a slow turnover of neuroglia, as evidenced by a constant number of mitotic figures (Sturrock, 1974*b*) and pyknotic cells (Sturrock, 1974*d*). Differentiation of glia is largely complete by 45 days postnatum (Sturrock, 1975*c*). Animals aged 70–80 days postnatum were selected for the present study as the neuroglial population is steady by this age, and any changes which occurred can be attributed to the effects of hypoxia. Only the left anterior limbs from male animals were used, to avoid any possibility of right–left or sex differences obscuring the results.

Although the groups were small the fall in the percentage of astrocytes in the experimental animals was significant. The difference between the percentage of astrocytes in the hypoxic and recovery groups was not significant, but the slight increase in the recovery group may indicate that the percentage of astrocytes was slowly returning to normal. If the rate of increase had remained constant the percentage of astrocytes would have returned to normal levels within 3 weeks.

The fall in the percentage of astrocytes may have been due to a fall in the total number of astrocytes, or to an increase in the total number of the other types of glia. In the latter case the total number of cells, and therefore the cell density, would have had to increase. No such increase in cell density was observed in hypoxic animals. Furthermore, although the percentage of oligodendrocytes increased significantly there was no increase in the percentage of glioblasts or microglia. These facts suggest that there was probably a fall in the total number of astrocytes, which was balanced by an increase in the total number of oligodendrocytes. As the animals were subjected to hypoxia for only 2 days the effect on the neuroglial population was evidently fairly rapid. The fall in astrocytes is probably due to these cells being especially sensitive to hypoxia, which is perhaps surprising when one considers their preferential anatomical relationship with blood vessels.

If the total number of oligodendrocytes does increase this would suggest that these cells, as well as maintaining the integrity of the myelin sheath, may possible assist in the metabolic support of unmyelinated axons (see Smart, 1964). This metabolic role *vis-à-vis* unmyelinated axons might explain the remarkably constant astrocyte: oligodendrocyte ratio in tracts in which myelination varies from 18% to 100% (Sturrock, 1975*c*). In the anterior limb 70% of axons are unmyelinated (Sturrock, 1975*a*).

In the recovery group the fall of 2·3 % in the percentage of microglia was probably significant ($P < 0·02$), and was similar to the 2·1 % rise in the percentage of astrocytes. This raises the possibility that either interstitial microglia are multipotential glial precursors, as suggested by Vaughn & Peters (1968), or that some of the cells classified by light microscopy as microglia are dark glioblasts which have previously been proposed as astrocyte precursors (Sturrock, 1974*c*, 1975*b*). If either one is correct then the fall in the percentage of microglia might be due to part of the 'microglial' population differentiating into astrocytes.

SUMMARY

Two experimental and one control group of 70–80 day old mice were used in this study. The two experimental groups were subjected to hypoxia for 2 days in a decompression chamber at 390 mmHg. The animals in one experimental group were killed on removal from the chamber (hypoxic group) while those in the other (recovery group) were allowed to recover at sea-level atmospheric pressure for one week before being killed. Semithin, toluidine blue stained sections from the anterior limb of the anterior commissure were examined to find whether any quantitative changes occurred in the neuroglia with hypoxic stress.

The following changes were observed:

(1) The percentage of astrocytes in the hypoxic and recovery groups was significantly ($P < 0·005$) lower than in the control group.

(2) The percentage of oligodendrocytes in the hypoxic and recovery groups was significantly ($P < 0·001$) higher than in the control group.

(3) The percentage of microglia in the recovery groups was significantly ($P < 0·02$) lower than in either of the other two groups.

(4) The percentage of astrocytes in the recovery group was slightly (2·1 %) higher than in the hypoxic group, and although not statistically significant, this result suggested that a slow return to normal might be occurring.

(5) Little change was observed in cell density.

The possible significance of these changes is discussed.

I should like to express my indebtedness to Dr E. J. Clegg of the Department of Anatomy, Sheffield University, for the use of the decompression chamber, for his advice and help in the preparation of the control and experimental animals, and also for his hospitality throughout the duration of the experiments. Thanks are also due to Mrs Sheila Ramsay for her careful preparation of the perfusing fluids, and to Mrs Dawn Alexander for typing the manuscript.

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