Chapter 2

Cerebral Hypoperfusion Yields Capillary Damage in the Hippocampal CA1 Area that Correlates with Spatial Memory Impairment

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Abstract

The impact of chronic cerebral hypoperfusion on cognitive function and cerebral capillary morphology in the hippocampus was examined. Young adult Wistar rats were subjected to permanent ligation of both common carotid arteries (two-vessel occlusion). One month after vascular occlusion, a small but non-significant impairment in the acquisition of spatial information was registered compared with sham-operated controls. Two months after surgery, the occluded animals displayed an impaired performance throughout the training period. One year after surgery, the acquisition curves demonstrated a significant attenuation of the learning rate in the occluded rats group, whereas no significant differences in long-term retention were observed. Thus, chronic hypoperfusion induced by two-vessel occlusion gave rise to impairment of spatial memory. Following behavioural testing, the rats were killed at the age of 17 months, and capillaries in the CA1 and dentate gyrus were examined using transmission electron microscopy. Typical age-related capillary abnormalities such as degenerative pericytes and thickened basement membranes (with or without fibrosis) were detected in the hippocampus of sham animals. In occluded rats, the occurrence of capillaries displaying such abnormalities almost doubled in the CA1 region, but was similar in the dentate gyrus, compared with sham controls. A highly significant correlation was found between the last Morris maze performance and the percentage of capillaries with deposits in the basement membrane in the hippocampal CA1 area of occluded rats, which was not present in the sham animals. We conclude that a long-term hypoperfusion accelerated the development of age-related ultrastructural aberrations of capillaries in the hippocampal CA1 area, but not in the dentate gyrus. Thus, not only neurons, but also capillaries in the hippocampal CA1 area are sensitive to an impaired microcirculation. Moreover, the cognitive performance of hypoperfused rats correlated closely with the condition of the capillaries in the CA1 area, suggesting that capillary integrity is one of the important determinants of brain function in conditions that compromise cerebral microcirculation.

Key words: chronic hypoperfusion, carotid artery ligation, rat, capillary morphology, transmission electron microscope, spatial learning.
Introduction

The impact of decreased cerebral blood flow on brain function has received increased scientific interest in recent years. Disturbances of the cerebral circulation have been associated with the decline of cognitive function in elderly subjects, as well as with the development of several types of dementia (Habert et al., 1991; Kalaria, 1996; Donnemiller et al., 1997). The bulk of this evidence indicates that cerebral hypoperfusion may fail to satisfy the metabolic demands of the neuronal tissue because of suboptimal delivery of vital nutrients to the brain. Owing to the inadequate energy supply, cognitive loss and memory deficits may develop.

Memory function, particularly spatial information processing, has been consistently associated with the hippocampal formation (Poucet and Benhamou, 1997). Non-invasive scanning studies performed on Alzheimer’s disease (AD) subjects, multi-infarct dementia patients and Parkinson’s disease patients with dementia showed that regional cerebral blood flow was reduced most severely in the temporal cortex, including the hippo-campus, where the observed hypoperfusion strongly correlated with the degree of dementia (Eberling et al., 1992; Ohnishi et al., 1995). Furthermore, animal studies revealed that the hippocampus, and particularly its CA1 area, was selectively vulnerable to the consequences of hypoperfusion (Schmidt-Kastner and Freund, 1991). All of these findings support the theory that cerebral hypoperfusion plays a significant role in cognitive disturbances and is likely to be an important risk factor in the development of several types of dementia, including AD (de la Torre and Mussivand, 1993; de la Torre, 1994; De Jong et al., 1997).

Reduced cerebral blood flow appears to coincide with the well-described microvascular aberrations, although it is still undetermined whether capillary distortions are the cause and/or a result of altered blood flow. Most probably, the process works in both ways in a chronic, progressive fashion. Supporting evidence for both alternatives has been reported. When considering the relationship between vascular morphology and function, it is important to distinguish the cerebral arterial system and the fine capillary network. Arterioles regulate blood pressure with the help of their supporting smooth muscle, while capillaries provide the surface for nutrient transport (Kalaria, 1996). The attention of the present study is focused on the cerebral capillaries due to their prominent role in nutrient exchange via the blood–brain barrier and because reduced brain perfusion in AD is associated with structural capillary deformities (Scheibel and Duong, 1986; Perlmutter and Chui, 1990; Claudio, 1996; De Jong et al., 1997).
The angioarchitecture of the cerebral microvasculature in AD subjects was investigated at the light microscopic level. Several larger scale pathological alterations have been defined, such as atrophic, thin vessels, glomerular loops, fragmentation, and twisting or tortuous vessels (Scheibel and Duong, 1986). The fine ultrastructure of cerebral capillaries in aging and dementia was analysed at the electron microscopic level (De Jong et al., 1990; Perlmutter and Chui, 1990; De Jong et al., 1991; De Jong et al., 1992; De Jong et al., 1997; Claudio, 1996). Major categories of capillary aberrations encountered in these studies include basement membrane abnormalities and degenerative pericytes. The anatomical distortions in the vascular walls may interfere with fluid hemodynamics and alter normal flow pattern. The decreased local blood flow is apt to reduce the efficiency of nutrient delivery and deprive the brain of vital resources (de la Torre and Mussivand, 1993; de la Torre, 1994).

The decreased blood flow and compromised cerebrovascular microanatomy, together with the predictive consequences of reduced nutrient transport, may contribute to cognitive disturbances by causing impaired neuronal metabolism. In the present study, we used an animal model to investigate the relationship between pathological cerebrovascular microanatomy and functional deficits of cognitive processes. We subjected rats to chronic cerebral hypoperfusion and challenged the animals in a learning task. The animals were monitored for one year after two-vessel occlusion (2VO) surgery, after which they were killed to examine capillary ultrastructure in the hippocampus. Particular attention was aimed at investigating the presumed correlation between microvascular pathology and learning performance.

Experimental Procedures

Surgery

In this study, 12 male Wistar rats (Harlan) were used. The animals were group-housed, received food and water ad libitum, and were kept on a regular 12-h/12-h light–dark cycle. At the age of three months, rats were anaesthetized with a combination of sodium pentobarbital (30 mg/kg, i.p.) and Hypnorm (Solvay–Duphar, Weesp, The Netherlands; 0.4 mg/kg, i.m.). Chronic hypoperfusion was induced in seven animals by the earlier described (Ni et al., 1994; Pappas et al., 1996) permanent bilateral occlusion of the common carotid arteries (2VO). After ventral cervical incision, the carotid arteries were carefully separated from their sheath and vagal nerves, and double ligated with silk sutures. The remaining five rats received the same surgical procedure without actual ligation and served as sham-operated
controls. Local analgesics were given by applying Xylocaine gel on the sutures. After surgery, rats were left to recover for a period of one month. All rats were checked daily on their physical health condition.

**Morris water maze learning**

One month after surgery, the animals were trained in the Morris water maze to locate a hidden platform. The water maze consisted of a polyester circular pool (diameter: 140 cm; height: 35 cm) with a featureless black inner surface. Prominent extra maze cues were positioned on the wall of the testing room to enable the rats to learn the platform’s location. Swimming paths were registered by a computerized video imaging analysis system (EthoVision, Noldus Information Technology BV, Wageningen, The Netherlands). The pool was filled with water at 27 °C to a height of 25 cm. The hidden escape platform (diameter: 9 cm) was submerged 2 cm below the water surface and was invisible from the water level. All rats received two trials, with a constant intertrial interval of 1 h, for five consecutive days. The animals were gently placed in the water in one of four quadrants, facing the wall of the pool; the starting quadrant was varied randomly over the trials. Rats were allowed 180 s to find the escape platform. Rats, which failed to locate the platform, were placed on the platform for 30 s. For all trials, escape latency, swim speed and distance travelled before reaching the platform were measured.

Two months after surgery, all rats were again tested in the water maze. Special care was taken to maintain exactly the same extra maze cues as in the first training period. On the first day of this second training period, the platform was located in the same quadrant as in the first training period. On day 2, the location of the platform was changed. Thereafter, animals were tested for another three days (six trials) with the platform at the same location as on the second day.

Finally, 12 months after surgery, the animals were exposed for the third time to the water maze. The rats again had to learn the location of the hidden platform (which was situated in a different position to that in the initial learning trials) in a series of two daily trials for five consecutive days.

**Electron microscopic preparation**

Fourteen months after surgery, sham-operated and 2VO rats were deeply anaesthetized with 60 mg/kg pentobarbital (i.p.) and initially perfused transcardially with 0.1 M phosphate buffer with 0.4% heparin, immediately followed by 1% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.38). Brains were removed and cut into 50-
mm sections with a Vibratome. Vibratome sections were routinely embedded in glycid ether (the equivalent of Epon Serva 812) for ultrastructural examination.

**Morphometric procedure**

After light microscopic examination of the embedded Vibratome sections, selected regions of the dorsal hippocampus were collected for semithin and ultrathin sectioning. Selection of the hippocampal region was made from transverse sections at Bregma -3.60 according to the Paxinos and Watson rat brain atlas. The areas selected were trimmed *en bloc*, cut into semithin sections and stained with Toluidine Blue. Guided by these survey sections, the blocks were further trimmed to the desired proportions to match the size of the carrier mesh grids. Ultrathin sections were collected on 200-mesh grids and contrasted with 5% aqueous uranyl acetate and Reynolds’ lead solution. The ultrastructure of the hippocampal capillaries was examined in a Philips 201 electron microscope. Based on previous examinations (De Jong et al., 1990; De Jong et al., 1991; De Jong et al., 1992; De Jong et al., 1997), capillaries were classified into the following categories: (i) intact capillaries, (ii) capillaries with degenerative pericytes, (iii) capillaries with local depositions of basement membrane material with either fibrous (fibrosis) or homogeneous (basement membrane thickening) appearance and (iv) a miscellaneous group of capillaries with rare deviations of the endothelial cytoplasm. The number of intact microvessels or those displaying one of the above abnormalities was counted directly from the electron microscope screen of the CA1 and dentate gyrus (DG) region, by an investigator blind to the identity of the rat. This quantification was repeated in three adjacent ultrathin sections in order to compensate for fortuitous distribution patterns as a consequence of using mesh grids. A total of approximately 90 capillaries per CA1 and 45 capillaries per DG region were quantified per brain. From these quantitative data, we calculated the percentage of capillaries that displayed structural degeneration.

**Statistical analysis**

The Morris maze learning curves were expressed as medians, and the significance of differences was tested with the non-parametric Mann–Whitney U-test. The percentage of intact or aberrant capillaries was also evaluated using the Mann–Whitney U-test. The correlation between capillary ultrastructure and the Morris maze performance was tested using linear regression analysis. Statistical significance was defined as $P < 0.05$. 

44
Results

Morris water maze learning

One month after the occlusion of both carotid arteries, sham-operated animals quickly learned to locate the hidden platform, which is shown in Figure 2.1.A. In the analysis of the water maze data, the distance travelled to find the platform was taken as a measure of cognitive performance because of its independence of swimming speed. Major acquisition of the spatial information in sham rats took place in the first two days, as indicated by a sharp decrease in distance travelled before finding the platform. This acquisition levelled off after two days. The 2VO rats also learned to locate the hidden platform, but the levelling off occurred after four days (Fig. 2.1.A). On day 5 of the first training period, sham animals immediately swam towards the platform when placed in the maze, while the 2VO rats swam a significantly longer distance before finding the platform.

Two months after surgery, sham animals swam a relatively short distance to find the platform (Fig. 2.1.B), indicating a good retention of spatial memory. On the first day of training, 2VO rats travelled a significantly longer distance than sham animals (Fig. 2.1.B; \( P < 0.02 \)). On day 2, the location of the platform was altered, a situation that prompted both groups to travel a longer distance (Fig. 2.1.B). The magnitude of this response was not significantly different for the two groups. On days 3–5, both groups found the platform at its new location, although the performance of the 2VO animals remained significantly impaired compared with the sham controls on days 3 and 5 (\( P < 0.02 \) and 0.01, respectively).

When tested one year after carotid occlusion, sham rats improved their performance in a similar fashion as during the first two training periods, with the largest improvement on day 2. The performance of the 2VO animals on day 2 was not ameliorated when compared with day 1, and differed significantly from the sham animals (\( P < 0.02 \)). A small improvement of 2VO rats was detected on days 3 and 4, but on the last day of testing these rats scored even lower than on day 1. The retention seen at the beginning of the third trial session showed no difference between the two groups. During all three training periods, no differences were observed between the two groups with regard to swim speed, leading to the conclusion that motor functions were not disturbed by the surgical procedure.

Capillary ultrastructure in the hippocampus

The ultrastructure of hippocampal capillaries was examined exactly 14 months after surgery, when the animals were actually 17 months of age. From earlier studies, it is known that, at this chronological stage, capillaries start to manifest age-related ultrastructural
Figure 2.1. The median values of sham-operated and 2VO groups that were trained (two trials per day on five consecutive days) one month (A), two months (B) or one year after surgery.

alterations (De Jong et al., 1990; De Jong et al., 1991; De Jong et al., 1992). As described in detail previously (De Jong et al., 1990; De Jong et al., 1991; De Jong et al., 1992), we distinguish two basically different categories of capillary degeneration that occur during aging: (i) degenerative pericytes (Fig. 2.2.B) and (ii) deposits in the capillary basement membrane (Fig. 2.2.C, D).

The first category is characterized by membranous degenerative material enclosed by a basement membrane. Within these structures, cytoplasmic elements such as mitochondria can be recognized. The category of microvascular deposits includes microvascular fibrosis (i.e. banded collagen fibrils deposited within the basement membrane; Fig. 2.2.C) and local basement membrane thickening (Fig. 2.2.D).

Approximately 50% of all hippocampal capillaries were ultrastructurally intact in the sham-operated animals. In the DG, no significant difference was found between sham and 2VO rats (Fig. 2.3.A; $P < 0.27$), whereas in the CA1 region, significantly fewer intact capillaries were encountered in the 2VO animals (Fig. 2.3.A; $P < 0.03$).

The incidence of capillaries with degenerative pericytes was higher in the 2VO animals in both hippocampal regions examined (Fig. 2.4.B), which was significant for the CA1 ($P < 0.05$) and strongly suggested a trend for the DG ($P < 0.07$).

The occurrence of capillaries with deposits, the term we use as a collective phrase for basement membrane thickening and/or collagen accumulation in the basement membrane, was prominently and significantly increased exclusively in the CA1 area (Fig. 2.4.C; $P < 0.03$), and not in the DG ($P < 0.34$).
Correlation between the Morris water maze performance and capillary ultrastructure

Correlations between morphological capillary pathology and behavioural performance were based on the last training period in the water maze prior to the fixation of the brains. It would appear that the difference in spatial memory recorded on day 2 of the last training period was the most important to feature the cognitive impairment of the 2VO animals, since on this day the sham animals improved their performance considerably, unlike the 2VO rats. For this reason, we correlated the integrity of hippocampal capillaries with the distance travelled in the water maze by the 2VO animals on day 2.

No correlation between capillary integrity and water maze performance was found for the DG (Fig. 2.4.D–F). However, we observed a strikingly negative correlation between the percentage of intact capillaries in the hippocampal CA1 area and the distance travelled in
Figure 2.3. Median values of the percentage of intact microvessels (A), capillaries with degenerative pericytes (B) and capillaries with deposits within the basement membrane (C) in sham and 2VO rats 14 months after surgery in the hippocampal CA1 area and DG. *P, 0.05.

the maze on day 2 (Fig. 2.4.A). This correlation ($r = 0.952$) was highly significant ($P < 0.0009$), indicating that within the 2VO group the performance in the Morris maze is better in rats with the fewest capillary abnormalities.

When regression analysis was performed on the different categories of capillary abnormalities, it became clear that the observed correlation was based on the percentage of capillaries with deposits percentage of capillaries with degenerative pericytes (Fig. 2.4.B; $r = 0.935$, $P < 0.002$) and not on the (Fig. 2.4.C; $r = 0.44$, $P < 0.321$).

Discussion

This study aimed to investigate the effects of chronic cerebral hypoperfusion on cognitive function and the anatomical integrity of hippocampal capillaries. Therefore, we have monitored the cognitive capacity of young adult Wistar rats that underwent permanent ligation of both common carotid arteries (2VO) for a period of one year. One month after surgery, a minor but non-significant impairment in the acquisition of spatial information was registered in the 2VO rats. After two months, the 2VO group showed an impaired spatial memory that persisted throughout the training period. One year after surgery, acquisition curves demonstrated a small, significant impairment in the 2VO group. In conclusion, chronic hypoperfusion induced by 2VO gave rise to impairment of spatial memory throughout the entire 12-month observation period following surgery.

2VO surgery in rats leads to a chronic reduction in cerebral blood flow to 70% of the original flow rate. Such chronic hypoperfusion in rats has been reported to impair spatial memory in the Morris maze as early as seven days after surgery (Pappas et al., 1996; de la
Figure 2.4. The distance travelled in the Morris water maze on day 2 of the final training period plotted against the percentage of capillaries in either the hippocampal CA1 area (A–C) or DG (D–F) that were intact (A, D) or displayed capillary deposits (B, E) or degenerative pericytes (C, F) in sham (open circles) and 2VO (filled circles). For the calculation of the linear regression coefficient r, only the values of the 2VO rats were considered.

Torre et al., 1997), whereas radial maze performance was compromised several months post-surgery (Ni et al., 1994; Pappas et al., 1996). Pappas et al. (1996) performed surgery on middle-aged rats, indicating that the age of the animal at the time of surgery is relevant to strong cognitive impairment, whereas an extended survival period (one year in the present study) did not noticeably aggravate cognitive failure. Cerebral hypoperfusion was demonstrated to induce memory impairment in additional, different memory tasks. Chronic hypoperfusion with cerebral blood flow of 70–80% of original values yielded impaired learning in the passive avoidance paradigm two months after vascular intervention in gerbils (Kudo et al., 1990; Kudo et al., 1993). Sekhon et al. (1997b) observed an increased exploratory behaviour in the open field and an impaired working memory in the T-maze in rats six months after surgery, by using a very elegant model (an arterio-venous fistula in the neck of rats, by which cerebral blood flow is reduced to 25–50%).

In the present study, we assessed the occurrence of well-defined ultrastructural abnormalities of the capillary wall in the rat hippocampus after 12 months of hypoperfusion, and found that chronic hypoperfusion induced the degeneration of pericytes and the development of capillary deposits in the hippocampal CA1 area but not in the DG.
Previously, other studies described microvascular changes following either reversible occlusion of the middle cerebral artery or the common carotid arteries in rats, leading to a period of post-ischemic hypoperfusion. Under these experimental conditions, damaged surfaces of arteriolar smooth muscle cells and tortuous capillaries were observed using scanning electron microscopy (Takahashi et al., 1997). The same study also demonstrated that postschaemic hypo-perfusion induces non-reversible damage and separation of pericytic cell bodies from the capillary wall. Degeneration of capillary pericytes is known to be age related (Farrell et al., 1987; De Jong et al., 1990; De Jong et al., 1991; De Jong et al., 1992; De Jong et al., 1997) and also occurred in the presently studied sham animals (aged 17 months) in both the CA1 and DG. It is reasonable to assume that the pericytic degeneration observed with transmission electron microscopy (TEM) in this study is the morphological correlate of the aberrations which Takahashi et al. (1997) detected using scanning electron microscopy. It seems likely that pericytes leave the capillary wall in order to act as microglia cells in the neuropil. This hypothesis corroborates the separation of pericytic cell bodies from the wall and indicates that the TEM images of degenerative pericytes in the present study are the remnants of cellular debris of pericytic origin. A similar suggestion was put forward after careful examination of cortical capillaries from the Alzheimer patients, where capillaries seemed to be activated by nearby deposited amyloid (Zarow et al., 1997). Chronic hypoperfusion yielded more hippocampal capillaries with degenerative pericytes, which, however, did not correlate with cognitive impairment. The latter suggests that pericytic pathology does not directly interfere with memory formation.

TEM evaluation after middle cerebral artery occlusion revealed swelling of endothelial cells followed by an increased number of microvilli on the luminal site of the endothelial cell membrane (Sakaki et al., 1997). Similar microvilli were found to correlate with the duration of the ischemic episode (Dietrich et al., 1984). To our knowledge, only two other animal studies have examined the effect of chronic hypoperfusion on cerebral capillaries. Firstly, in cats, a period of up to 15 days of hypoperfusion yielded collapsed capillaries along with several features of neuronal and glial degeneration (Romanski and Stamenov, 1995). Secondly, using the aforementioned arteriovenous fistula model, Sekhon et al. (1997a) found that the capillary density increased significantly in the CA1 of rats that were subjected to 26 weeks of hypoperfusion. Combined with the observation that several capillaries lack astrocytic end-feet, the authors concluded that hypoperfusion leads to neovascularization. Others found an increased capillary density in the cerebral cortex after hypobaric hypoxia and suggested capillary segment elongation (Mironov et al., 1994). Such an increased capillary density may
serve as compensation for the originally weaker microvascular architecture of the CA1 (Coyle, 1978; Imdahl and Hossmann, 1986; Mossakwski et al., 1994).

The deposition of collagen or basement membrane-like material in the capillary basement membrane is an aging-related phenomenon (De Jong et al., 1990; 1990; De Jong et al., 1991; De Jong et al., 1992; De Jong et al., 1997). We found such pathological deposits in both the DG and CA1 of sham-operated animals. The incidence of capillary deposits in the DG of 2VO rats was comparable to that of sham animals, but in contrast they were significantly increased in the CA1 area. In fact, the number of capillaries displaying deposits was almost doubled. In view of the earlier mentioned increased capillary density (Sekhon et al., 1997a), it is important to emphasize that our data provide information about the integrity of the total capillary population in a given region, without taking into account eventual changes in capillary density. In other words, it may very well be that chronic hypo-perfusion yields more (Sekhon et al., 1997a), but also more damaged, capillaries in the CA1 region (present study). The present data suggest that the rat CA1 is a brain region where not only neurons, but also capillaries, seem to be extremely vulnerable to hypoperfusion.

The importance of intact hippocampal capillaries became clear from the remarkable correlation between the Morris water maze performance and the percentage of capillaries with basement membrane deposits. The present findings indicate a correlation between behavioural deficits and capillary deposits in 2VO rats. Although other factors (e.g., impaired visual abilities (Ohta et al., 1997)) may also contribute to poor performance on the water maze, it would appear from our data that the compromised microvasculature in CA1 affects spatial memory function. The striking correlation between impaired spatial memory and increased percentage of degenerated capillaries in the CA1 as found in the present study indicates that chronic hypoperfusion may accelerate aging-related behavioural and microvascular deterioration. This hypothesis supports the increased occurrence of aging-related lipofuscin pigment in CA1 neurons of chronically hypoperfused rats (Sekhon et al., 1997c). Moreover, the finding that chronic cerebral hypoperfusion in rats leads to increased accumulation or induction of amyloid precursor protein (Kalaria et al., 1993) suggests a relation to AD as well. Based on these and other data, de la Torre (de la Torre, 1994) formulated the hypothesis that an impaired cerebral microcirculation plays a key role in the development of AD, which is further supported by the present data. Finally, the observation that AD patients show significantly more capillaries with deposits in the basement membrane (De Jong et al., 1997), as do rats with chronic brain hypoperfusion (present study), strongly supports the microvascular theory of AD.
Conclusions

We found a small but consistent impairment in spatial memory after chronic 2VO. Long-term hypo-perfusion accelerated the development of aging-related ultrastructural abnormalities of capillaries that seemed to target the hippocampal CA1 area. We conclude that not only neurons, but also capillaries in the CA1 are extremely vulnerable to an impaired microcirculation. Moreover, the cognitive performance of hypoperfused rats correlated very closely with the condition of the capillaries in the CA1 region, implying that capillary integrity is one of the major determinants of brain functioning in conditions where cerebral circulation may be compromised.

References


