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BRIEF COMMUNICATION TO NEUROBIOLOGY OF AGING

Selective white matter pathology induces a specific impairment in spatial working memory

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Disclosure statement

a) All authors certify that they do not have any actual or potential conflicts of interest, including any financial, personal or other relationships with other people or organizations within 3 years of beginning this work, that could inappropriately influence (bias) this work.

b) All experiments were performed under an appropriate Home Office Licence with the approval of the University of Edinburgh Ethical Review Panel and subject to the Animals (Scientific Procedures) Act 1986.

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Abstract:

The integrity of the white matter is critical in regulating efficient neuronal communication and maintaining cognitive function. Damage to brain white matter putatively contributes to age-related cognitive decline. There is a growing interest in animal models from which the mechanistic basis of white matter pathology in ageing can be elucidated but to date there has been a lack of systematic behaviour and pathology in the same mice. Anatomically widespread, diffuse white matter damage was induced, in three different cohorts of C57Bl/6J mice, by chronic hypoperfusion produced by bilateral carotid stenosis. A comprehensive assessment of spatial memory (spatial reference learning and memory (Cohort 1) and serial spatial learning and memory (Cohort 2) using the watermaze and spatial working memory (Cohort 3) using the 8-arm radial arm maze) was conducted. In parallel, a systematic assessment of white matter components (myelin, axon, glia) was conducted using immunohistochemical markers (MAG, dMBP, APP, Iba-1). Ischaemic neuronal perikarya damage, assessed using histology (H&E), was absent in all shams but was present in the hypoperfused group (2/11 in Cohort 1, 4/14 in Cohort 2 and 17/24 in Cohort 3). All animals with neuronal perikaryal damage were excluded from further study. Diffuse white matter damage occurred, throughout the brain, in all hypoperfused mice in each cohort and was essentially absent in sham-operated controls. There was a selective impairment in spatial working memory, with all other measures of spatial memory remaining intact, in hypoperfused mice with selective white matter damage. The results demonstrate that diffuse white matter pathology, in the absence of grey matter damage, induces a selective impairment of spatial working memory. This highlights the importance of assessing parallel pathology and behaviour in the same mice.

Key Words: Cognitive decline, axonal damage, myelin damage, hypoperfusion

1. Introduction

Cognitive performance declines with advancing age and a number of measures are especially impaired such as processing speed, executive function and episodic memory. These measures are increasingly linked to pathological changes in white matter *in vivo* using diffusion tensor imaging (Bucur et al., 2008; Deary et al., 2003). White matter lesions, at post-mortem, are defined by a loss of white matter integrity and an inflammatory response, often associated with chronic cerebral hypoperfusion resulting from small vessel pathology (Fernando et al. 2006). Although there is a statistical association between the white matter pathology and age-related cognitive decline (Bucur et al, 2008), a causal relationship remains to be established. This is further confounded by the presence of other vascular factors such as atherosclerosis, hypertension and diabetes which influence the presence of white matter lesions.

Experimental animal models have been developed in which white matter pathology is precipitated in response to chronic cerebral hypoperfusion. They allow the study of hypoperfusion, in isolation, on the development of white matter lesions and cognitive abilities, and provide a powerful tool to study the neurobiological basis of white matter lesions occurring in human brain in the absence of other confounding factors such as hypertension and diabetes. In rat models of chronic hypoperfusion (induced by permanent carotid ligation), deficits in spatial reference learning and memory are observed, although the presence of ischemic neuronal perikaryal damage complicates interpretation of the behavioural data (see Farkas et al 2007). A mouse model of chronic cerebral hypoperfusion, produced by carotid stenosis using microcoils, has been developed (Shibata et al., 2004) which produces selective and delayed damage to the white matter. Separate behavioural studies of this mouse model demonstrated an impairment of spatial working memory, with intact spatial reference memory, and intact contextual and cued fear conditioning (Shibata et al., 2007). Thus, this model could provide a basis to define whether selective white matter pathology parallels cognitive decline. However, as yet, there has been no parallel assessment of cognition and pathology in the same animals. To address this we assessed spatial learning and memory in parallel with a systematic assessment of white and grey matter pathology in the same animals in the mouse model of chronic cerebral hypoperfusion.

2. Materials and Methods

Animals and experimental design Chronic cerebral hypoperfusion was induced as previously described (Shibata et al., 2004) by applying microcoils (0.18mm internal diameter) to both common carotid arteries in male 3-4month

old C57Bl/6J mice (25-30g) under isoflurane anaesthesia. Sham mice underwent an identical surgical procedure to hypoperfused mice except the microcoils were not placed on the arteries. Mice were coded and randomised for surgery, and subsequent behavioural and pathological studies were conducted with investigators blind to the surgical status of the mice. Following behavioural testing, the mice were sacrificed under deep isoflurane anaesthesia by transcardiac perfusion fixation, the brains removed and processed for paraffin embedding. In Cohort 1, cued learning and spatial reference learning and memory were assessed using a water maze task, and the subsequent pathological examination was conducted at one month after the onset of hypoperfusion. In Cohort 2, cued learning and serial spatial learning and memory were assessed, also in a water maze, and the subsequent pathological examination was conducted at two months after the onset of hypoperfusion. In Cohort 3, spatial working memory was assessed using an 8 arm radial arm maze and pathological examination was conducted at two months after the onset of hypoperfusion.

Pathological assessment of white matter integrity Sections were stained with haematoxylin and eosin to determine the presence of neuronal perikarya damage. Adjacent sections were immunostained using standard techniques with different antibodies to visualise the cellular components of white matter. The loss of myelin integrity, assessed using anti-myelin-associated glycoprotein (MAG, Santa Cruz Biotechnologies), was identified by the presence of disorganised white matter fibres and myelin debris and graded from 0-3 (none-extensive). Degraded myelin, assessed using anti-dMBP (Millipore), was identified by the presence of intensely stained irregular myelin sheaths which were graded from 0-3 (none-extensive). Axonal damage, visualised using anti-amyloid precursor protein (APP, Millipore), was identified as intense APP immunoreactivity in swollen or bulbous axons and graded as 0-3 (none-extensive). Microglial activation was assessed using anti-ionised calcium-binding adaptor molecule (Iba-1, Menarini) and activated cells per mm² defined. Cellular changes were assessed in corpus callosum, external capsule, internal capsule, fimbria hippocampus, optic tract and the fibre bundles of the caudate nucleus and the grading score summated from these 6 regions.

Cue task The motivation and ability to swim were assessed using a cued task in the water maze for one day prior to surgery and at 3 weeks after surgery for five days (Cohort 1) or four days (Cohort 2).

Spatial reference learning and memory task Animals from Cohort 1 were trained to find a hidden platform over 5 days with 4 trials per day (10 min inter trial interval (ITI)), beginning 3 days after the cue task ended. The platform location remained constant throughout testing. To test memory retention, probe trials were run 10 min and 24 h after the final training trial.

Serial spatial learning and memory task Animals from Cohort 2 were trained on this task as described previously (Chen et al. 2000; Daumas et al. 2008) beginning 3 days after the cue task ended. Each animal was first trained to swim to a hidden platform for up to eight trials per day (10 min ITI; maximum of 32 trials), to a criterion of three successive trials with an escape latency of less than 20s. The platform location remained the same on each trial. Once this criterion was achieved, the platform was moved to a new location on the following day, and the mouse commenced training on the second location, again until it reached criterion. This procedure was repeated for at least five different platform locations, and for at least 10 days. The number of trials taken to reach criterion for each of the first five platform locations (trials to criterion), and the number of platform locations learned within 10 days (learning capacity) were analysed. To assess memory for each platform location, two probe tests were conducted 10 min and 3 h after reaching criterion on each of the first five platform locations.

Spatial working memory Animals from Cohort 3 were trained on this task as described previously (Shibata et al. 2007). Mice were trained over 16 days on an 8 arm radial arm maze in which, on each daily testing session, each arm was baited with a single reward. Each animal was placed in the centre of the maze, and allowed to explore the maze until it retrieved all 8 rewards. Between arm choices each mouse was confined to the central platform for 5 s. The number of entries into baited arms during the first 8 arm choices, and the number of visits into unbaited arms during the whole session (revisiting errors) were analysed. Full details of all behavioural methods are available in the supplementary methods.

Statistics Statistical comparisons of myelin and axonal damage between shams and hypoperfused groups were made using Fisher's exact test. Microglial activation was analysed with Student's *t*-test. Behavioural data were analysed using 2-way ANOVA with the Greenhouse-Geisser correction. Student's *t*-test with Bonferroni adjustment for multiple comparisons was used *post hoc*. Learning capacity data in the serial spatial task were analysed using an unpaired Student's *t*-test.

3. Results

Pathology There was no evidence of neuronal perikaryal damage in any brain region in any of the sham-operated mice. Ischaemic neuronal perikaryal damage was seen in 2 out of 11 hypoperfused animals in Cohort 1, in 4 out of 14 hypoperfused mice in Cohort 2 and in 17 out of 24 hypoperfused mice in Cohort 3. The mice in Cohort 3 were food-deprived for the radial arm maze task which may contribute to the higher frequency of ischemic neuronal damage in this Cohort compared to Cohorts 1 and 2. Mice exhibiting ischemic neuronal damage were excluded

from the subsequent analysis of white matter pathology and behaviour (Fig. 1 A, B). In sham operated mice, minimal damage to the myelin or axons was detected in each region examined (corpus callosum, external capsule, internal capsule, fimbria, optic tract, and myelinated tracts within the caudate nucleus, Fig. 1K). In contrast, in hypoperfused animals, marked anatomically-widespread damage to myelinated fibres was observed. There was no difference in the extent of white matter damage between the three cohorts ($p < 0.05$, Kruskal Wallis). In each cohort, there was extensive damage to the myelin (myelin debris, vacuolation, disorganisation of fibres) (Fig. 1 C, D, L) in addition to an increased presence of degraded myelin after hypoperfusion as compared to sham (Fig. 1 E, F, M). Axonal damage was observed in some hypoperfused mice but absent in shams (Fig. 1 G, H, N). Upregulation of microglia was also observed in response to hypoperfusion (Fig. 1 I, J, O)

Spatial learning and memory

Cue task In the cued-navigation task, there were no significant differences in performance between hypoperfused and sham groups in either Cohort 1 (sham $n=9$ and hypoperfused $n=9$, $p=0.97$; Fig 2a) or Cohort 2 (sham $n=11$; hypoperfused $n=10$, $p=0.82$; Fig 2D). In both cohorts, performance improved significantly across days (Cohort 1 $p < 0.001$; Cohort 2 $p < 0.001$) and escape latencies averaged less than 10 s in all groups by the end of training. Swim speeds did not differ significantly between the groups (supplementary data).

Spatial reference learning and memory task There were no significant differences between hypoperfused ($n=9$) and sham ($n=9$) mice in the latency to reach the platform across training ($p=0.66$; Fig. 2B). Both groups improved significantly over the training period ($p < 0.001$). Both groups also showed a similar preference for the quadrant in which they had been trained when memory for the platform location was probed at 10 min ($p=0.97$; Fig 2C) and 24 h ($p=0.53$; supplementary Fig. 1A) after the end of training.

Serial spatial learning and memory task There were no significant effects of hypoperfusion on any measure on this task. Sham ($n=11$) and hypoperfused ($n=10$) mice took a similar number of trials to reach criterion on the first 5 platform locations ($p = 0.85$; Fig. 2E). There were also no significant differences between the groups on the 10 min ($p=0.89$; Fig. 2F) and 3 h ($p=0.70$; supplementary Fig. 1B) probe trials. The learning capacity, defined as the number of platform locations learned in 10 days, did not differ significantly between the groups ($p=0.46$; Fig. 2G).

Spatial working memory The hypoperfused mice ($n=7$) committed significantly more revisiting errors than the sham group ($n=17$) ($p = 0.015$; Fig 2H). In addition, the hypoperfused mice visited significantly fewer new arms in the first 8 arm entries compared to shams ($p=0.027$; Fig 2I). There was a significant improvement in learning across days, with the number of errors committed by both groups decreasing significantly over the training period

($p < 0.001$) and the number of new arms visited in the first 8 arm entries increasing significantly over the same period ($p < 0.001$). Full details of statistical analyses and additional behavioural data are provided in the supplementary results.

4. Discussion

The development of a mouse model of chronic cerebral hypoperfusion has provided a powerful tool to study the effects of hypoperfusion, in isolation, on the development of white matter lesions and their impact on cognition. In human disease this type of study has been limited by the presence of multiple factors such as hypertension, atherosclerosis and diabetes, which may influence the development of white matter lesions. The present study provides a robust demonstration that diffuse white matter pathology, 1-2 months after chronic hypoperfusion, selectively impairs spatial working memory and does not affect a number of other different measures of spatial memory. Analysis of the behavioural consequences of white matter damage in rat models of chronic cerebral hypoperfusion are often limited by the presence of neuronal ischaemic perikaryal damage (Farkas et al, 2007). In the mouse model of chronic cerebral hypoperfusion, in which selective white matter pathology occurs in a subset of animals, there has been an absence of parallel pathology and behavioural analysis in the same animals. Behavioural analysis, conducted in isolation, has indicated that chronic hypoperfusion results in deficits in spatial working memory in the absence of changes in spatial reference memory, or in contextual and cued fear conditioning (Shibata et al. 2007). Although separate cohorts of hypoperfused mice revealed the presence of selective white matter pathology in this model it is impossible to directly correlate pathology to behaviour between different cohorts. Miki et al., (2009) recently addressed the impact of the severity of white matter abnormalities, as a result of hypoperfusion, on cognitive and motor abilities. They found a significant association between white matter integrity and performance on a probe test in a spatial reference memory task in the water maze and concluded that the deficits in spatial memory were caused by white matter lesions. However, all of the hypoperfused mice in their study exhibited a degree of hippocampal perikaryal damage which could have contributed to the behavioural deficit. In the present study we measured a range of spatial memory abilities. We hypothesised that the white matter lesions, as a result of cerebral hypoperfusion, would lead to disconnection and impair spatial learning and memory. We were able to demonstrate that selective white matter pathology is sufficient to impair spatial working memory. However, mice with the same degree of selective white matter pathology were able to learn the spatial reference memory task and demonstrated a similar degree of improvement

over the period of training as shams. They also showed intact short- (10 min) and long-term (24 h) memory for the platform location. These data are consistent with those of Shibata et al (2007) who reported no deficits on a spatial reference memory task conducted on a radial arm maze in hypoperfused mice at 1 month. In aging humans, there are clear links between white matter integrity and episodic memory and task switching (Deary et al, 2003, Bucur et al., 2008). Studies in aging monkeys have also revealed that spatial reversal memory is impaired and associated with white matter volume (Lyons et al. 2004). A novel behavioural task has been developed to investigate serial spatial learning and memory in mice (Chen et al, 2000). This task, which is sensitive to aging and Alzheimer pathology (Chen et al, 2000; Daumas et al., 2008), is a form of serial spatial memory in which mice are required to learn a series of different platform locations successively. Thus, once an animal has learned one location, the platform is moved to a novel location, and so on. Successful learning and recall of the successive platform locations requires the ability to encode changes in the spatial representation of the environment, and also the selective recall of the appropriate platform location (which requires memory flexibility – an element of episodic-like memory). We found that the spatial memory flexibility, memory retention and learning capacity of the hypoperfused mice with selective white matter damage all remained intact in this task. Taken together, the behavioural data indicate an impairment only in the spatial task that requires the most behavioural flexibility, as the spatial working memory task requires constant updating of memory regarding which arms have been visited within a session, whereas the serial spatial learning task requires updating only across days, and the spatial reference memory task requires a consistent response to one rewarded location. Similar to previous studies, we determined that the mouse model of hypoperfusion is characterised by a predominantly white matter pathology (Shibata et al. 2004, 2007; Miki et al. 2009). Extending previous studies, we assessed the cellular vulnerability of white matter components using sensitive immunohistochemical approaches. In particular we found that the cellular distribution of MAG immunoreactivity is altered and that there is a significant increase in degraded MBP in hypoperfused mice as compared to shams. Interestingly, MAG, which may function in glia–axon interactions, when deficient in MAG-null mice does not affect spatial learning and memory assessed in the water maze (Montag 1994). Thus in the present model, disruption of components of the white matter are able to induce a deficit in spatial working memory but may be insufficient to cause a detectable impairment of less challenging measures of spatial learning and memory.

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Figure legends

Figure 1 Representative images to illustrate the integrity of the neuronal perikarya (CA1 region of the hippocampus; (A,B) and white matter integrity (optic tract) of myelin (C,D), degraded myelin (E,F), axons (G,H) and microglia (I, J) in sham and hypoperfused mice. Fig. K illustrates the regions that were examined for white matter pathology. The grade of pathology or number of microglia from these regions was summated and analysed between the groups. Data is shown from Cohort 1 which is representative of the three cohorts as there was no significant difference in the extent of white matter damage between the cohorts. There was no neuronal perikarya damage in any of the shams (A) or the hypoperfused mice that were used for behavioural analysis (B). Myelin debris (Fig D, arrows), and vacuolation, indicative of a loss of myelin integrity and damage, was significantly increased in hypoperfused animals (Fig L) and there was also a significant increase in the extent of degraded myelin (dMBP) (Fig M, arrows Fig F). There was evidence of axonal damage in hypoperfused mice (Fig N, arrows Fig H) and the number of activated microglial was also increased after hypoperfusion (Fig. O, arrows Fig J). Scale bar represents (15 μ m A-C and 30 μ m D-L).

Figure 2 Three different cohorts of mice underwent either a spatial reference learning and memory task (A-C) a serial spatial learning and memory task in the water maze (D-G) or a spatial working memory task in an 8-arm radial maze (H, I). In an initial cued platform task in the water maze, hypoperfused and sham groups in Cohorts 1 and 2 showed similar learning and asymptotic levels of performance, indicating intact motor ability and motivation (A, D). The ability to learn the location of a hidden platform by Cohort 1 was not affected by chronic cerebral hypoperfusion (B). Both groups of animals showed the same degree of preference for the quadrant in which they had been trained to find the platform when memory was probed 10 min after the task (C). In the serial spatial task (Cohort 2), both sham and hypoperfused groups took a similar number of trials to reach criterion on each of the 5 platform locations (E). Both groups of animals showed the same degree of preference for the most recently learned platform location when memory was probed 10 min after each task (F). The number of platform locations the animals learned in 10 days was not statistically different between the shams and hypoperfused mice (G). In the spatial working memory task, hypoperfused mice exhibited significantly more revisiting errors in the 8-arm radial maze than sham mice [$F(1, 22) = 6.981$; $p = 0.015$ (Fig H). They also visited significantly fewer new arms in the first 8 arm entries than shams [$F(1, 22) = 5.619$; $p = 0.027$] (Fig I)

APPENDIX

SUPPLEMENTARY MATERIAL

Methods

Animals and experimental design

Male C57Bl/6J mice (25-30g, approx 3-4 months old) were obtained from Charles River. All procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. Chronic cerebral hypoperfusion was induced as previously described by applying microcoils (0.18mm internal diameter constructed by Sawane Spring Co.) to both common carotid arteries (CCA) (Shibata et al., 2004) under isoflurane anaesthesia. A period of 30 min was left between the insertion of each coil. Sham-operated animals underwent the identical surgical approach except that microcoils were not applied to the CCA. The temperature of the mice was maintained between 36.5⁰C and 37.5⁰C. The recovery of the mice was monitored closely, and the weight of the mice, whether they were eating and drinking, and if there were signs of overt neurological dysfunction (eg. circling, rolling, hunching) was recorded.

All the mice underwent behavioural assessment at 3 weeks after the surgical procedure. In Cohort 1, cued learning and spatial reference learning and memory were assessed using a water maze task (n=12 sham and n=12 hypoperfused mice). Of these, 3 shams and 1 hypoperfused mouse were excluded as they were exhibited floating behaviour. The subsequent pathological examination was conducted at 1 month after the onset of hypoperfusion (n=9 sham and n=11 hypoperfused mice). In Cohort 2, cued learning and serial spatial learning and memory were assessed, also in a water maze (n=12 sham, n=15 hypoperfused mice). Of these, 1 sham and 1 hypoperfused mouse were excluded at the outset as they exhibited floating behaviour. The subsequent pathological examination was conducted at 2 months after the onset of hypoperfusion (n=11 sham, n=14 hypoperfused mice). In Cohort 3, spatial working memory was assessed using an 8-arm radial maze using the protocol used previously by Shibata et al. (2007) in the model (n=17 sham, n = 24 hypoperfused) and all these mice were included for pathological analysis.

Pathological assessment

At either 1 month (Cohort 1) or 2 months (Cohort 2, Cohort 3) after placement of the carotid microcoils, mice were deeply anaesthetised with 5% isoflurane and transcardially perfused with 20 ml 0.9% heparinised saline and then

20 ml 4% paraformaldehyde in 0.1% phosphate buffer. The brains were postfixed in 4% paraformaldehyde solution and processed for paraffin embedding. 6 µm thick sections were cut and mounted on poly-L-lysine coated slides. Sections from levels corresponding to 0.38mm and -1.70mm from bregma according to Franklin and Paxinos (1997) were selected for staining with haematoxylin and eosin (H + E) to determine the presence of neuronal perikaryal damage in the hippocampus and striatum, grey matter structures known to be particularly vulnerable to reductions in cerebral blood flow. Sections adjacent to those used for histological staining were selected for immunohistochemical staining using different markers to the cellular components of white matter. Immunohistochemistry was carried out according to standard procedures and underwent pre-treatment to remove paraffin, and for APP detection, antigen retrieval with microwaving in 10mM citrate buffer (pH 6) was employed. Primary antibodies were applied overnight after blocking. Myelin integrity was assessed using anti-myelin-associated glycoprotein (MAG) (1:250; Santa Cruz Biotechnologies). Degraded myelin was assessed using anti-dMBP (1:300; Millipore). Axonal damage was visualised using anti amyloid precursor protein (APP) antibody (1:1000; Millipore). Microglial activation was assessed using anti-ionised calcium-binding adaptor molecule (Iba-1) antibody (1:750; Menarini). Sections were incubated with biotinylated secondary antibody in PBS (1:100) followed by a solution of streptavidinbiotin-peroxidase complex. Peroxidase activity was localized using 3,3'-diaminobenzidine tetrahydrochloride as a chromogenic substrate.

Cellular changes were assessed in key regions with predominantly white matter tracts; the corpus callosum, external capsule, internal capsule, fimbria hippocampus, optic tract and the fibre bundles of the caudate. The grading/counting was conducted with the individual blind to the surgical treatment. Myelin damage identified with MAG was determined as the presence of disorganised white matter fibres and myelin debris. The scale was as follows; normal (grade 0), minimal myelin debris, vacuolation, and disorganisation of fibres (grade 1), modest myelin debris, vacuolation, and disorganisation of fibres (grade 2), and extensive myelin debris, vacuolation, and disorganisation of fibres (grade 3). dMBP positively immunostains degraded myelin which was graded as follows; no degraded myelin present (grade 0), minimal degraded myelin (grade 1), moderate areas of degraded myelin (grade 2) and extensive areas of degraded myelin (grade 3). Axonal damage was identified as intense APP immunoreactivity in swollen or bulbous axons and graded as normal (grade 0), minimal axonal damage (grade 1), moderate areas of axonal damage (grade 2) and extensive areas of axonal damage (grade 3). A global score of myelin (and axonal) damage in each animal was calculated by summation of the scores in the anatomical regions

examined. Statistical comparisons of the global scores of myelin and axonal damage in the sham-operated and hypoperfused groups was made using Fisher's exact test. Microglial activation was quantified by placing a box grid of defined size in randomly selected fields within each region of interest and then calculating the number of activated microglial cells per mm². Statistical comparisons between the sham and hypoperfused group were made using Student's t-test.

Assessment of spatial learning and memory

Behavioural testing for Cohorts 1 and 2 took place in an open-field water maze (Morris 1981, 1984) consisting of a 2 m diameter tank filled with water (depth 0.5m; temperature 25°C + 10°C made opaque with the addition of 400mL of liquid latex. An escape platform (20 cm diameter for cue tasks, 13 cm for reference memory and serial spatial learning task) was submerged 1.5 cm below the water surface. The testing room was equipped with a rich array of two- and three-dimensional extra-maze cues. The animal's swimming behaviour was monitored using a video tracking system via a camera fixed above the centre of the maze (Watermaze). Prior to testing, the animals were handled for 10 min per day for 5 days by an experimenter. For all tasks, the animals were placed into the water next to, and facing, the wall of the tank, at one of four locations (counterbalanced across trials). The maximum trial duration was 90 s, and after the animals reach the platform they remained on it for 30 s before being removed.

Cue task

We tested the motivation and the ability of the animals to swim by training the mice using a cued version of the water maze, for one day prior to and five days (cohort one) or four days (cohort 2) following surgery beginning 3 weeks after surgery. Each animal received 4 trials training per day with a 10 min ITI. The maze was surrounded by white curtains to occlude the sight of the extra-maze cues. The platform was placed pseudo-randomly in different locations on different trials, and was cued by means of a 20 cm tall object placed onto it.

Spatial reference learning and memory

Spatial reference memory began 3 days after the end of the cue task for Cohort 1. The platform was unmarked by any local cues, and the curtains surrounding the maze were opened, giving access to the extra-maze cues. Animals were trained over 5 days with 4 trials per day (10 min ITI), during which the platform location remained constant

for each animal throughout testing. For each trial, the escape latency, path length, and swimming speed were measured. To test spatial reference memory retention, probe trials were run 10 min and 24 h after the final training trial. During the probe trials, an “Atlantis” platform (Spooner et al, 1994) was positioned in the training quadrant and rendered unavailable for the first 60 s of the trial. After 60 s, the platform was raised automatically, and the animal was given a further 30 s to find it. The time spent in each quadrant of the pool during the first 60 s of each probe trial was analysed.

Serial spatial learning and memory

The animals from cohort 2 were trained on a serial spatial learning task (Chen et al. 2000; Daumas et al. 2008) beginning 3 days after the end of the cue task. This task involves learning a series of successive hidden platform locations in the water maze. Each animal was trained to swim to the first platform location, for up to eight trials per day (10 min ITI; maximum of 32 trials), to a performance criterion of three successive trials with an escape latency of less than 20s. The platform location remained the same on each trial. Once this criterion was achieved, the platform was moved to a different location on the following day, and the mouse commenced training on the second location, again until it reached criterion. This procedure was repeated for at least five different platform locations, and for at least 10 days. The platform locations were selected from 16 possible locations – eight on a virtual inner ring (1m diameter) and eight on an outer ring (1.5 m diameter) in a counterbalanced fashion within and across animals. Two measures of performance were analysed: the number of trials taken to reach criterion for each of the first five platform locations (trials to criterion), and the number of platform locations learned within 10 days (learning capacity).

In order to assess memory for each platform location, two probe tests were conducted 10 min and 3 h after reaching criterion on each of the first five platform locations, using the Atlantis platform as described above.

Spatial working memory

Behavioural testing for Cohort 3 took place on an 8-arm radial maze made of white plastic with transparent Plexiglas arm walls (20 cm tall). The maze consisted of an octagonal central platform (20 cm diameter) with 8 equally spaced arms radiating from it (47 cm long, 7 cm wide). At the distal end of each arm, a plastic food well (3.5 cm diameter, 2 cm deep) was located. Doors, located at the proximal end of each arm, were controlled

remotely using Any-Maze software (Stoelting, UK), which also recorded the behaviour and allowed for automation of door opening and closing based on tracking of each animal's position in the maze. The maze was elevated 1 m from the floor of the experimental room. A camera connected to a PC was fixed on the ceiling just above the central platform of the maze. The experimental room was dimly lit, and equipped with salient extra maze cues.

Three weeks after surgery the mice were submitted to a food deprivation procedure in order to reduce their initial body weight by 10-15% and the restricted feeding was maintained until the end of behavioural testing. Two pretraining days were undertaken in order to familiarize the animals with the experimental environment, the apparatus and the task itself. On pretraining day one, food pellets (45mg, Bio-Serv) were scattered around the maze and each animal was left to explore freely the maze for 5 min. On pretraining day 2, a single food pellet was placed at the end of each of the eight arms. The mouse was placed in the central platform and allowed access to each arm in turn (controlled using the doors), and allowed to retrieve the food pellet. For the training procedure one food pellet was placed at the end of each of the 8 arms of the maze. At the beginning of every trial (one trial/day) the animal was placed on the central platform with all arm doors open and allowed to make an arm choice. An arm choice was recorded when the centre of the mouse (as tracked by the *AnyMaze* software) was 5 cm into the arm. Once the mouse had entered one of the arms, the doors to the other 7 arms were closed automatically. When the animal exited the visited arm it was confined on the central platform for 5 sec by closing the remaining door. After the 5 sec delay it was allowed to make a new choice. A trial ended when the mouse had retrieved all 8 pellets or 25 min had elapsed. The behavioural testing lasted 16 days. For each trial, the number of correct arm entries within the first eight visits, the number of revisited arms (working memory errors) and the time taken to complete the task were recorded.

Results

Pathology

Ischaemic neuronal perikaryal damage was seen in 2 out of 11 hypoperfused animals in Cohort 1, 4 out of 14 animals in Cohort 2 and 17 out of 24 mice in Cohort 3. The mice in Cohort 3 were food-deprived for the radial arm maze task which may contribute to the higher frequency of ischemic neuronal damage in Cohort 3 as compared to Cohorts 1 and 2 that were not food deprived. All animals in which neuronal perikaryal damage was detected were excluded from the quantitative analysis of white matter damage, and from the behavioural analysis. In addition, any

animals that exhibited floating behaviour in the water maze studies were excluded (Cohort 1, n=3 shams and n=1 hypoperfused mouse and Cohort 2, n=1 sham and n=1 hypoperfused mouse exhibited floating behaviour). This left final group sizes that were analysed for the white matter pathology and behavioural analysis as follows: Cohort 1, n=9 sham and n=9 hypoperfused, Cohort 2 n=11 sham and n=10 hypoperfused and Cohort 3 n=17 sham and n=7 hypoperfused mice.

Spatial Learning and Memory

Cue task

Average swim speed, in Cohort 1, for shams was 25.8 ± 0.6 m/s (average \pm sem) compared to 25.3 ± 0.7 m/s for hypoperfused ($p = 0.984$). In Cohort 2, average swim speed for shams was 25.5 ± 0.7 m/s compared to 26.4 ± 0.6 m/s for hypoperfused ($p = 0.379$). These were not significantly different for either cohort (Cohort 1: $[F(1, 16) = 0.002, p = 0.97]$; Cohort 2: $[F(1, 19) = 0.834, p = 0.37]$). A repeated measures ANOVA on the latency to reach the platform across days for the two groups in Cohort 1 revealed a significant main effect of day $[F(5, 80) = 21.961, p < 0.001]$, but no significant main effect of group $[F(1, 16) = 0.002, p = 0.97]$, and no significant interaction $[F(5, 80) = 1.333, p = 0.273]$. Similarly, for cohort 2 there was a significant main effect of day $[F(4, 76) = 34.538, p < 0.001]$ but no significant main effect of group $[F(1, 19) = 0.054, p = 0.82]$ and no significant interaction $[F(4, 76) = 0.160, p = 0.828]$.

Spatial reference learning and memory

A repeated measures ANOVA on the escape latencies across the five training days revealed a significant main effect of day $[F(4, 64) = 10.327, p < 0.001]$, but no significant main effect of group $[F(1, 16) = 0.206, p = 0.66]$ and no significant group \times day interaction $[F(4, 64) = 0.412, p = 0.80]$. This suggests that both groups learned at a similar rate and to a similar level. In the probe sessions, repeated measures ANOVAs assessing % of time spent in each of the four quadrants of the pool showed a significant main effect of quadrant at both the 10 min $[F(3, 48) = 44.996, p < 0.001]$ and 24 h probes $[F(3, 48) = 22.903, p < 0.001]$, but no significant main effect of group (10 min: $[F(1, 16) = 0.105, p = 0.75]$; 24 h: $[F(1, 16) = 1.80, p = 0.20]$) or group \times quadrant interactions (10 min: $[F(3, 48) = 0.088, p = 0.97]$; 24 h: $[F(3, 48) = 0.664, p = 0.53]$). Both groups spent significantly more time in the training quadrant than would be expected by chance at each probe (10 min: hypoperfused $|t| = 5.894, p < 0.001, df = 8$;

sham $|t| = 6.56$, $p < 0.001$, $df = 8$; 24 h: hypoperfused $|t| = 4.131$, $p < 0.003$, $df = 8$; sham $|t| = 4.70$, $p < 0.002$, $df = 8$).

Serial spatial learning and memory

A repeated measures ANOVA on the trials to criterion across the first five platform locations revealed no significant main effects of task number [$F(4, 76) = 1.566$, $p = 0.19$], or group [$F(1, 19) = 0.035$, $p = 0.85$] and no significant group x task number interaction [$F(4, 76) = 0.524$, $p = 0.72$]. This suggests that both groups learned each of the five platform locations at a similar rate. Analysis of the % time spent in each quadrant during probe tests at 10 min and 3 h for each of the first 5 platform locations revealed no significant main effects of group, and no significant group x quadrant interactions for any probe test (data not shown). A repeated measures ANOVA on the % time spent in the training quadrant in the probe tests for the first five platform locations revealed no significant main effects of task number or group, and no significant interactions between task number and group at either 10 min or 3 h. (10 min: task [$F(4,72) = 6.81$, $p = 0.55$]; group [$F(1,18) = 0.018$, $p = 0.89$]; task x group [$F(4,72) = 2.43$, $p = 0.85$]; 3 h: task [$F(4,76) = 1.943$, $p = 0.11$]; group [$F(1,19) = 0.151$, $p = 0.70$]; task x group [$F(4,76) = 1.004$, $p = 0.41$]. A t-test on the number of platform locations learned in the first 10 days (the learning capacity) revealed no significant differences between the groups [$|t| = 0.756$, $p = 0.459$, $df = 18$].

Spatial working memory

In the radial arm maze task, the hypoperfused mice committed significantly more revisiting errors compared to the sham group [$F(1, 22) = 6.981$; $p = 0.015$]. The effect of training day was statistically significant [$F(7, 154) = 10.687$; $p = 0.000$]. The group x training day interaction effect was not significant [$F(7, 154) = 1.059$; $p = 0.393$]. In addition, the hypoperfused mice visited significantly fewer new arms in the first 8 arm entries compared to sham mice [$F(1, 22) = 5.619$; $p = 0.027$]. There was a significant effect of training session [$F(7, 154) = 9.223$; $p = 0.000$], but the interaction between training session and group was not significant [$F(7, 154) = 1.676$; $p = 0.119$].

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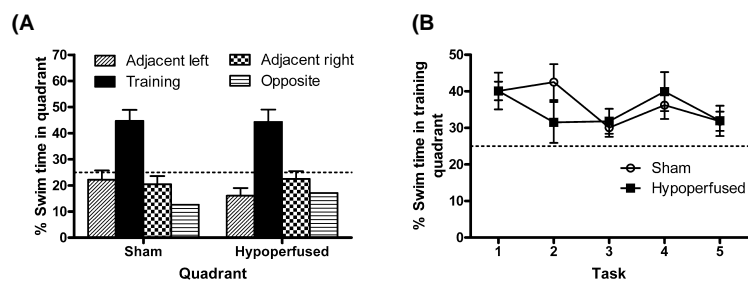
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Supplementary figure

Fig.1

Both groups of animals showed the same degree of preference for the platform when memory was probed 24 h after the end of training in the spatial reference memory task (Cohort 1; A). Similarly, both groups performed similarly when probed 3 h after reaching criterion on each of the first 5 platform locations in the serial spatial learning and memory task (Cohort 2; B).



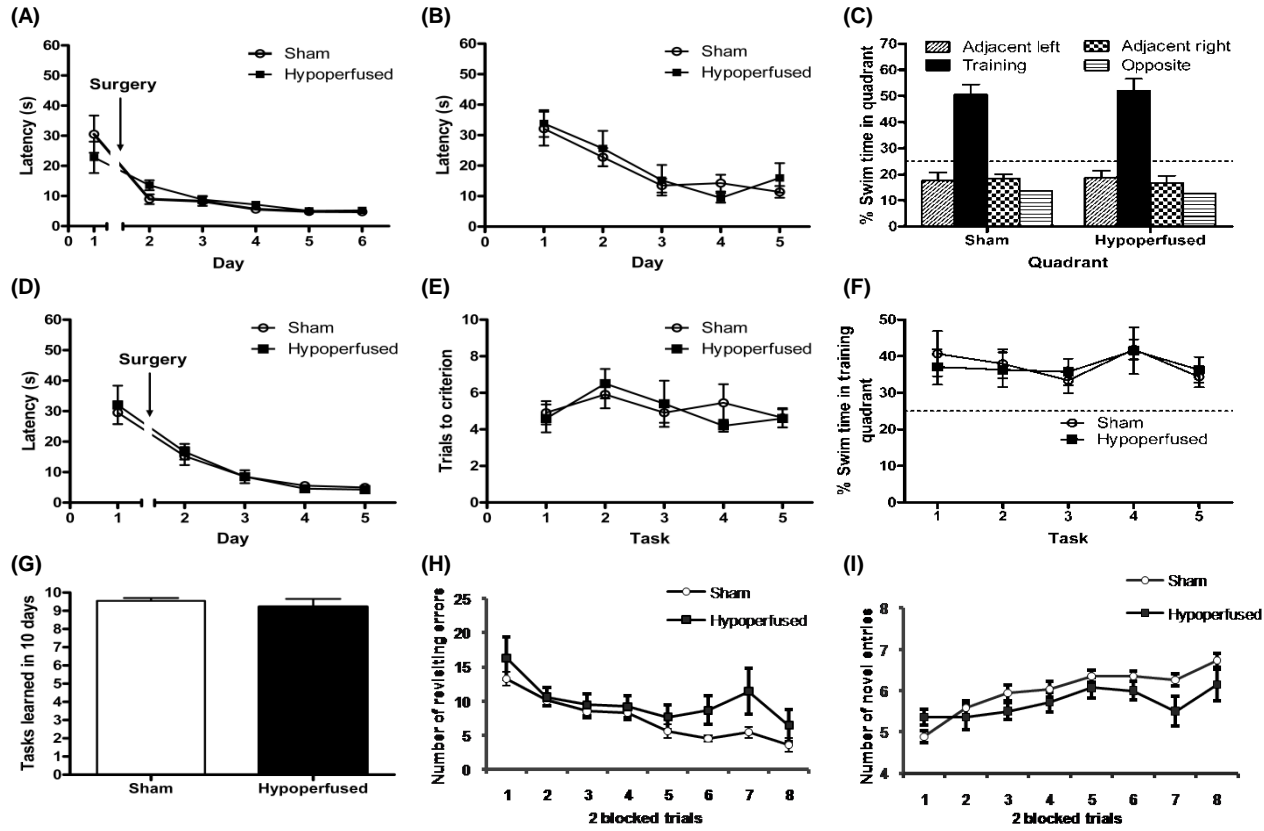


Figure 2 Coltman et al.