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B-vitamin and choline supplementation increases neuroplasticity and recovery after stroke

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ABSTRACT

Folates are B-vitamins that play an important role in brain function. Dietary and genetic deficiencies in folate metabolism result in elevated levels of homocysteine which have been linked to increased risk of developing a stroke. Reducing levels of homocysteine before or after a stroke through B-vitamin supplementation has been a focus of many clinical studies, however, the results remain inconsistent. Animal model systems provide a powerful mechanism to study and understand functional impact and mechanisms through which supplementation affects stroke recovery. The aim of this study was to understand the role of B-vitamins in stroke pathology using *in vivo* and *in vitro* mouse models. The first objective assessed the impact of folate deficiency prior to ischemic damage followed by B-vitamins and choline supplementation. Ischemic damage targeted the sensorimotor cortex. C57Bl/6 wild-type mice were maintained on a folic acid deficient diet for 4 weeks prior to ischemic damage to increased levels of plasma homocysteine, a risk factor for stroke. Post-operatively mice were placed on a B-vitamin and choline supplemented diet for a period of four weeks, after which motor function was assessed in mice using the rotarod, ladder beam and forepaw asymmetry tasks. The second objective was to determine how a genetic deficiency in methylenetetrahydrofolate reductase (MTHFR), an enzyme involved in folate metabolism, increases vulnerability to stroke. Primary cortical neurons were isolated from *Mthfr*$^{+/+}$, *Mthfr*$^{+/-}$ and *Mthfr*$^{-/-}$ embryos and were exposed to *in vitro* models of stroke which include hypoxia or oxygen glucose deprivation. Cell viability was measured 24-h after exposure stroke like conditions *in vitro*. In supplemented diet mice, we report improved motor function after ischemic damage compared to mice fed a control diet after ischemic damage. Within the perilesional cortex, we show enhanced proliferation, neuroplasticity and anti-oxidant activity in mice fed the supplemented diet. A genetic MTHFR deficiency resulted in neurodegeneration after exposure to *in vitro* models of stroke, by activating apoptosis promoting p53-dependent mechanisms. These results suggest that one-carbon metabolism plays a significant role in recovery after stroke and MTHFR deficiency contributes to poor recovery from stroke.

1. Introduction

Deficiency in folate a type of B-vitamin, leads to increased plasma homocysteine which is linked to increased risk of cardiovascular disease (Castro et al., 2006), such as stroke (Han et al., 2015). Methylenetetrahydrofolate reductase (MTHFR) is an enzyme involved in folate metabolism. In all tissues including in the brain, MTHFR catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (5-methylTHF). The methyl group from 5-methylTHF is a substrate in the vitamin-B12-dependent methylation of homocysteine to form methionine by methionine synthase. Dietary folate and vitamin B12 deficiencies can limit this reaction leading to the accumulation of homocysteine in plasma. Dietary choline can also influence plasma homocysteine concentrations because it provides an alternate source of methyl groups for the remethylation of homocysteine to methionine *via* the enzyme betaine homocysteine methyltransferase in liver and kidney.

Genetic impairments of folate metabolism can also cause hyperhomocysteinemia. A polymorphism in *MTHFR* (677C ➔ T) has been identified in 5–15% of North American and European populations (Schneider

Abbreviations: BDNF, Brain derived neurotrophic factor; CD, Control diet; FADD, Folic acid deficient diet; OGD, Oxygen glucose deprivation; Nrf-2, Nuclear factor (erythroid-derived 2)-like2; MTHFR, Methylenetetrahydrofolate reductase; SOD2, Superoxide dismutase 2; SD, Supplemented diet.

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et al., 1998). Individuals with the TT genotype tend to have elevated homocysteine concentrations compared to the CT and CC genotypes (Frosst et al., 1995) and increased risk of stroke (Castro et al., 2006; Song et al., 2016). Furthermore, supplementation with folic acid has been reported to have beneficial effects after a stroke (Huo et al., 2015; Wang et al., 2007) as well as reduce the risk of a stroke (Saposnik et al., 2009); however, not all the data are consistent, possibly due to mandatory fortification in some but not all countries (Spence, 2007). Investigations using model systems of stroke are required since the mechanisms through which folates might exert their effects on the brain remain unknown.

A mouse model of MTHFR deficiency has been developed which facilitates the study of the *in vivo* effects of genetic deficiencies in folate metabolism (Chen et al., 2001). *Mthfr*$^{+/-}$ mice mimic the human polymorphism with the characteristic elevation in levels of plasma homocysteine (~5 μM) compared to ~3 μM in wildtype mice, whereas *Mthfr*$^{-/-}$ mice have severely elevated levels (~32 μM) of homocysteine (Chen et al., 2001). The aim of this study was two-fold, first we investigated whether dietary folic acid deficiency prior to ischemic damage and supplementation after with folic acid, vitamin B12, riboflavin and choline promoted recovery after photothrombosis ischemic damage to the sensorimotor cortex. The photothrombosis model has been reported to be a reproducible model of ischemic damage in mice, since it creates a well-defined damage similar in size between animals enabling the study of specific behaviors (Fluri, 2015; Kumar et al., 2016). Second, we assessed vulnerability of *Mthfr*$^{+/-}$ and *Mthfr*$^{-/-}$ primary neurons to damage using *in vitro* models of stroke. We found that when hyperhomocysteinemic mice were supplemented with B-vitamins and choline, they showed significant improvement in ischemia-induced sensorimotor deficits. Within the perilesional cortex, we also show enhanced proliferation, neuroplasticity and anti-oxidant levels in mice fed supplemented diet. The results suggest that the improvement in motor ability parallels enhanced neuroplasticity in the perilesional cortex. In support of this finding, we report that MTHFR deficiency results in greater vulnerability to neurodegeneration *in vitro* through activation of p53-dependent mechanisms.

2. Materials and methods

2.1. Animals and experimental design

All experiments were conducted in accordance with animal welfare regulations, and the protocols were approved by local ethics committees. Experiments were performed in a fully randomized and blinded fashion.

Dietary supplementation of folic acid and other B-vitamins as well as choline after ischemic damage was investigated in 32 wild type C57Bl/6 male mice (Charles River Laboratories). At one-month of age, animals were placed on a control (CD; Envigo) or folic acid deficient diet (FADD) for a period of 4 weeks prior to ischemic damage (Fig. 1A). After ischemic damage, mice on the FADD were placed on the supplemented diet (SD). CD mice continued to be fed the same diet for the remainder of the experiment. The CD contained 2 mg/kg of folic acid whereas the FADD contained 0.3 mg/kg of folic acid (Table 1), with all other nutrients the same, in order to increase levels of homocysteine prior to ischemic damage (Jadavji et al., 2015a; Jadavji et al., 2015b). The specific components that were increased in the SD are shown in Fig. 1B with an asterisk. Folic acid was increased to 5 mg/kg from 0.3 mg/kg, riboflavin to 10 mg/kg from 6 mg/kg, vitamin B12 0.5 mg/kg from 0.025 mg/kg and choline bitrate was increased to 4950 mg/kg from 1150 mg/kg (Table 1). These levels of vitamins were chosen because they have previously been reported to be beneficial *in vivo* (Agte et al., 1998; Craciunescu et al., 2003; Wolff et al., 1998). Animals were maintained on the SD for 4 weeks after ischemic damage, after which behavioral testing and tissue collection was conducted.

2.2. Photothrombosis

To induce ischemic damage, animals were anesthetized in an isoflurane chamber (4–5% in O_2 for induction), where their heads were clean-shaven and disinfected and tear gel applied to their eyes, before being moved to a stereotaxic frame (Harvard Apparatus). Anesthesia was maintained using a face mask at 1.5%–2% in O_2. A temperature probe and heating pad were employed to maintain body temperature at 37 °C. A photosensitive dye, 10 mg/kg of Rose Bengal (Sigma) was injected (i.p.) 5 min prior to laser exposure. The skull of the animal was then exposed, and a laser light (532 nm) was positioned 2 cm above the sensorimotor cortex (mediolateral + 0.24 mm from Bregma) for 15 min (Beta Electronics). The corresponding sham procedure involved an i.p. injection of Rose Bengal, but no laser exposure. All animals received 20 mg/kg tramadol 6 h after damage to assist with post-operative pain.

2.3. Behavioral analysis

Four weeks after ischemic damage to sensorimotor cortex, motor function was assessed using the rotarod, ladder beam walking and cylinder tasks.

2.4. Rotarod

Animals were tested on a standard rotarod (Omnitech Electronic Inc.). The metal rotarod cylinder was 3.0 cm in diameter and 6 cm wide. The cylinder was positioned 30 cm from the ground by a Plexiglas container and moved by a rubber belt connected to a small motor. Animals were tested on an accelerating rotarod (4 to 60 rpm) over 8 min and the latency to fall was recorded. Animals were tested on a single day with three separate trials and an inter trial interval of 5 min (Jadavji et al., 2015b).

2.5. Ladder beam

The ladder rung apparatus was composed of two Plexiglas walls. Each wall contained holes located at the bottom edge of the wall; the holes could be filled with metal bars. The entire apparatus was placed atop two standard mouse housing cages. The performance was video-recorded from the side, with the camera positioned at a slight ventral angle so that all 4 limbs could be recorded at the same time (Farr et al., 2006).

All video recordings were analyzed frame-by-frame. Each step was scored according to the quality of limb placement as previously described (Farr et al., 2006). For analysis of foot placement accuracy, the number of errors in each session was counted. The error score was calculated from the total number of errors and the number of steps for each limb (Farr et al., 2006).

2.6. Forepaw placement

The cylinder (19 cm high, 14 cm diameter) was made of thick glass. The mouse was placed in a vertical glass cylinder for 10 min and video recorded from above. Contacts of the forepaws with the wall of the cylinder were counted upon replay of the videotape. The first 20 movements were recorded during the 10-min test. The final score was calculated as follows: final score = (number of non-impaired forelimb movement − number of impaired forelimb movement)/(number of non-impaired forelimb movement + number of impaired forelimb movement + number of movements). A positive score indicated favored use of the non-impaired forelimb; a negative score indicated favored use of the impaired forelimb and a score of zero indicated equal use of both non-impaired and impaired forelimbs upon rearing and exploration of the cylinder (Theoret et al., 2015).