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Mitochondrial regulation of reactive oxygen species (ROS) production—Unexpected observations in early postnatal cerebral vasculature

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ABSTRACT

Nicotinamide-nucleotide-transhydrogenase (Nnt) is a mitochondrial protein. It is altered and functionally lacking in the C57BL/6J sub-strain. This leads to the generation of more radical oxygen species than in the C57BL/6N sub-strain. During studies on the effect of Nnt in perinatal hypoxia the cerebral vasculature was investigated in postnatal day 9 mice using post mortem arterial filling with silicone rubber compounds. Surprisingly, the tiny vessels were no longer uniformly filled and a bleb-like pattern occurred in both sub-strains. Furthermore, considerably more bleb-like spots were observed in the C57Bl/6J sub-strain than in the C57Bl/6N sub-strain. These blebs might be the result of feathery vessels bursting. It remains unclear how the mechanisms in the used strains differ. Nnt might influence the vascular structure or its development and mechanisms and should be investigated further.

1. Introduction

In the course of investigations into the influence of nicotinamide-nucleotid-transhydrogenase (Nnt) on the formation of postnatal hypoxic lesions the cerebral vasculature was visualized in postnatal day 9 mice (P9). Nnt is a mitochondrial protein that promotes the reduction of ROS by catalyzing the generation of NADPH. The lack of Nnt results in increased reactive oxygen species (ROS) levels (Ronchi et al., 2013; Nickel et al., 2014). Interestingly, in a very recent publication Nnt was shown to be a therapeutic target and reduced mortality in an animal model of heart failure (Nickel et al., 2015).

Birth-related hypoxia often leads to brain lesions and is a major disorder in neonatology with severe life-time consequences. A hypoxia/ischemia-model is applied in mouse pups with combined disruption of the left carotid artery, hypoxia and hyperthermia (Vannucci and Hagberg, 2004). Interestingly, this technique is necessary since the disruption of the carotid artery alone has no significant effect.

Postnatal C57BL/6N mice with an intact Nnt gene and C57BL/6J mice in which exons 7–11 of the Nnt gene were lacking (which leads to non-formation of the Nnt protein (Mekada, 2009)) were used in this study. The structure of the brain arteries in animals with normal intracellular ROS production was compared to that of those displaying increased intracellular ROS production. Silicone rubber filling was applied to demonstrate the cerebral arterial vessels (Dai et al., 2015; Yuan et al., 2012). In this study, the vascular morphology of early postnatal animals lacking the Nnt protein was investigated.

2. Materials and methods

For the experiments offspring of C57BL/6N and C57BLl/6J mice were used (Charles River, Sulzfeld, Germany): Seven normal P9C57BL/6N mice and six normal P9C57BL/6J mice. In addition, the hypoxia/ischemia protocol was performed on three C57BL/6N pups. Briefly, at postnatal day 7 the animals were narcotized with an isoflurane-oxygen inhalation narcosis and the left common carotid artery of the mice was severed. After surgery the pups were allowed to recover with their dams for 2 h, after which they were exposed to systemic hypoxia for 15minutes in a ventilated and temperature-controlled chamber (8% oxygen, 92% nitrogen, 37°C) (Hugo Sachs, March-Hugstetten, Germany) with a flow rate of 3 l/min.

The silicone rubber injection was performed in all pups at postnatal day 9 as follows: In deep ketamine anesthesia the left ventricle was punctured and flushed with saline/heparin (1:100)

Abbreviations: P9, postnatal day 9; Nnt, Nicotinamide-nucleotide-trans hydrogenase; NADPH, Nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species.

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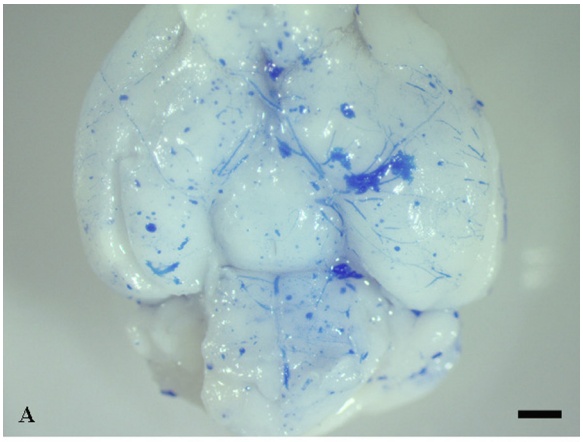
followed by Microfil® (Flow Tech Inc., Carver, MA, USA) infusion. In brief: per mouse 1000 μl color, 1000 μl diluent, 200 μl glue were mixed and gently injected into the left ventricle of the beating heart. The results were noted and then further approaches with reduced viscosity were tested (900 μl, 666 μl and 500 μl color with the appropriate volume of diluent to reach a final volume of 2000 μl plus 200 μl glue). After 20 min the brain was obtained and fixed in ethanol (24 h 50%, 24 h, 80%, 24 h 90%, 24 h 100%). Photographs were taken from the basis of the brain. Bleb-like formations per quadrant were counted, mean and standard error of the mean (SEM) were calculated and the Mann–Whitney test was applied (Prism 6 software, GraphPad, La Jolla, USA). For histology small pieces of those brains (silicone filled vessels, immersion-fixation) were embedded in resin (epon mix) using standard protocols. Semi-thin sections were made and stained with hematoxyline. To check the barrier function of the brain vessels Evans Blue filling was applied in separate experiments. For this, Evans Blue (2% in saline) was injected into the left ventricle of 3 N-strain and 3 J-strain mice at postnatal day 7 as described above. Five minutes later the animals were killed and the brains obtained. Photographs were taken and cryostat sections were analyzed using fluorescence microscopy.

Results and discussion

The use of silicone rubber compounds is an established technique and we have successfully implemented it in adult mice. Large and tiny vessels, arteries and arterioles can be made visible (not shown). Unexpectedly, the situation was completely different in the P9 brain samples. It must be mentioned that the handling of the extremely small, early postnatal brains was demanding. The brain vessels are tiny at day 9 after birth but the investigated samples showed that the vasculature could be filled with silicone. Interestingly, the continuity of the vessels was often interrupted (Fig. 1AB). Many bleb-like spots or little depots of silicone were found. It might be that the pressure or viscosity was too high in relation to the fragile vessel structures at distinct locations. The additional approaches using silicone with reduced viscosity were not successful. Interestingly, a clear difference was found between the N and J sub-strain of the C57BL/6 mice under investigation: In relation to the whole basal surface, the number of bleb formations was increased in all brain samples of the J sub-strain (mean/SEM: 87 + 4 blebs per quadrant, Fig. 2AB) as compared to the N sub-strain (32 + 7, significant with $p < 0.001$ in the Mann–Whitney test, Fig. 2CD). This was insofar unexpected as there is no knowledge of any structural differences between the two sub-strains. On the other hand, developmental differences are not unlikely as the Nnt gene influences both glucose and glucocorticoid regulation (Meimaridou et al., 2012; Toye et al., 2005; Heiker et al., 2013; Nicholson et al., 2010). The structure of the vessels was microscopically investigated using semi-thin sections of resin-embedded tissue (Fig. 3). The vessels and the capillaries were found to be intact and no morphological abnormality was found. Interestingly, micro-lesions or ruptures were found where the silicone had leaked. These are almost certainly the pendant of the blebs described macroscopically. Was ROS the reason for a weakening or degradation of the walls of vessels? The influence of ROS on endothelial integrity has mostly been investigated in cell culture experiments as summarized by Paul A. Fraser (Fraser , 2011). Increase in vascular permeability may be caused directly by ROS but also by ROS-induced mediators such as bradykinins and by inflammatory cytokines such as interleukin-1β, tumor necrosis factor-α or interferon-γ. Tumor necrosis factor influences the barrier between endothelial cells by activating different Rho GTPase's and other mechanisms (Marcos-Ramiro et al., 2014). In general, the vascular density or solidity might not only be influenced by ROS and its downstream cascades. A recent important and comprehensive study from Simon and co-workers showed many genetic differences and "significant phenotypic differences between the two lines" (Simon et al., 2013). For instance, the heart beating frequency was significantly higher in N than in J mice. Aqueous Evans Blue perfusion was performed over 5 min. The brain tissue remained unstained, indicating a tight vasculature at least after the short perfusion time of 5 min (Fig. 4A). This was supported by microscopic investigation, showing the vessel-surrounding tissue free of Evans Blue dye (Fig. 4B). The perfusion time might have been to short and a longer period could yield different results. In contrast, the pressure during perfusion with silicone was higher and probably led to leakage and blebs at distinct vulnerable locations.

Furthermore, in those three animals, in which the left carotid artery had been disrupted, no differences in the silicon filled vessels were detectable between the left and the right hemisphere (not shown). This suggests sufficient blood distribution via the contralateral carotid artery and the arterial circle of Willis.



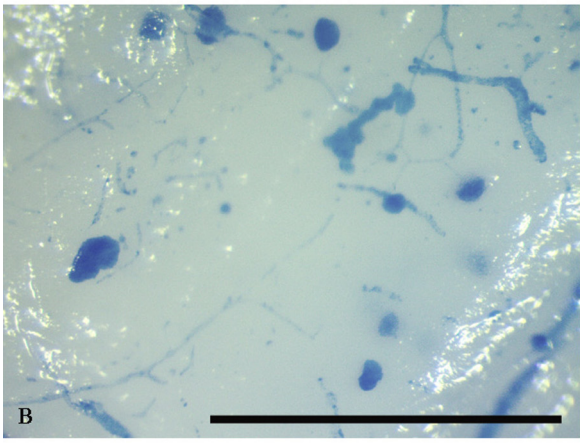


Fig. 1. (AB) Representative view of the basis of the brain of a P9 animal after microfil perfusion with filled vessels (blue). A part of the right cerebellum was lost during handling procedures (A). At higher magnification, bleb-like formations can be observed (B). The bar represents 100 μm.