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A behavioural study of neuroglobin-overexpressing mice under normoxic and hypoxic conditions☆

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ABSTRACT

Neuroglobin (Ngb), a neuron-specific heme-binding protein that binds O_2, CO and NO reversibly, and promotes in vivo and in vitro cell survival after hypoxic and ischaemic insult. Although the mechanisms of this neuroprotection remain unknown, Ngb might play an important role in counteracting the adverse effects of ischaemic stroke and cerebral hypoxia. Several Ngb overexpressing mouse models have confirmed this hypothesis; however, these models were not yet exposed to in-depth behavioural characterisations. To investigate the potential changes in behaviour due to Ngb overexpression, heterozygous mice and wild type (WT) littermates were subjected to a series of cognitive and behavioural tests (i.e., the SHIRPA primary screening, the hidden-platform Morris water maze, passive avoidance learning, 47 h cage activity, open field exploration, a dark–light transition box, an accelerating rotarod, a stationary beam, a wire suspension task and a gait test) under normoxic and hypoxic conditions. No significant behavioural differences were found between WT and Ngb-overexpressing mice at three months old. However, one-year-old Ngb-overexpressing mice travelled more distance on the stationary beam compared with WT littermates. This result shows that the constitutive overexpression of Ngb might counteract the endogenous decrease of Ngb in crucial brain regions such as the cerebellum, thereby counteracting age-induced neuromotor dysfunction. This article is part of a Special Issue entitled: Oxygen Binding and Sensing Proteins.

1. Introduction

Neuroglobin (Ngb), a vertebrate protein that belongs to the globin superfamily, is predominantly expressed by neurons. Ngb exhibits all the determinants of a genuine globin with specific adaptations [1]. Unlike the best-known globins, haemoglobin and myoglobin, which are penta-coordinated, the heme iron atom of Ngb is hexacoordinated. The intracellular concentration of Ngb is low (~1 μM) [2], and its O_2-binding affinity under physiological conditions is comparatively weak (P_{50} = 7.5 Torr) [3]. Thus, enhanced O_2 delivery is an unlikely function.

Increasing evidence has indicated that Ngb plays an endogenous neuroprotective role against hypoxic and ischaemic insults. To confirm this observation, different Ngb-overexpressing transgenic (Ngb-Tg) mouse models were developed via pronuclear injections with constitutive expressions in different tissues and the central nervous system [4–7]. To date, four models were developed using a chicken β-actin promoter and distal CMV enhancer (Ngb-Tg-1) [4], the human ubiquitin C promoter (Ngb-Tg-2) [6], the CMV promoter (Ngb-Tg-3) [7] and the synapsin I rat promoter (Ngb-Tg-4) [5].

Ngb-Tg-1 mice overexpressed Ngb using a chicken β-actin promoter and distal CMV enhancer. This combination of promoter and enhancer is most often used for widespread high expression levels. Ngb RNA and protein levels were overexpressed in the brain, heart and other tissues. Specifically, Ngb overexpression was confirmed in the cerebral cortex, striatum, olfactory bulb, hippocampus, cerebellum and brainstem [4]. An initial study found that the infarct size was reduced in Ngb-Tg-1 mice after cerebral and myocardial ischaemia compared with wild type (WT) mice [4]. Because myoglobin is present in both WT and Ngb-Tg-1 mice, the reduction of myocardial infarcts in the latter is due to the overexpression of Ngb in the heart and not the presence of myoglobin. Furthermore, this finding implies that the protective effect of Ngb in ischaemia operates through pathways that are also present in non-neuronal cells. No obvious phenotypic abnormalities were reported in Ngb-Tg-1 mice [4].

Ngb-Tg-2 mice overexpress Ngb under the human ubiquitin C promoter. This promoter leads to Ngb expression in both neuronal and non-neuronal tissues. In fact, Ngb was not only expressed in the brain

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but also in the heart and kidneys of Ngb-Tg-2 mice. Moreover, Ngb mRNA and protein expression in hippocampus increased by 4 and 2.5 times, respectively. The expression was also increased in other brain regions [6]. Production of reactive oxygen species and reactive nitrogen species (ROS/RNS), lipid peroxidation and neuronal injury markedly decreased in Ngb-Tg-2 mice compared with WT mice after hippocampal ischaemia-reperfusion [6].

A non-tissue-specific CMV promoter was used for the Ngb-Tg-3 model. Ngb mRNA levels increased by 2.6 times in the brains of Ngb-Tg-3 mice compared with WT mice [7]. Ngb-Tg-3 mice were used in a larger study that focused on long-term physiological and behavioural outcomes. The reduction in brain infarct size was confirmed and could even be sustained up to 14 days after a 1 h focal cerebral ischaemia in Ngb-Tg-3 mice compared with WT controls. The effect on behavioural outcome was studied using a sensorimotor function assessment. All scores were close to pre-injury baselines again 14 days after the onset of ischaemia. No significant differences were found between Ngb-Tg-3 and WT mice [7]. In addition, less conventional hypoxia/ischaemia models reveal Ngb-based neuroprotection. Zhao et al. used a standard controlled cortical impact model with a pneumatic cylinder to induce traumatic brain injury (TBI) in Ngb-Tg-3 mice. However, these authors did not confirm the 2.6-fold increase found in their previous study [7]; rather, they observed a 1.5-fold increase compared with WT controls [12]. However, lesion volume remained significantly reduced in Ngb-Tg-3 mice compared with WT mice 21 days after TBI. The significant reduction of 3-nitrotyrosine production (an oxidative tissue damage biomarker) in the brain of Ngb-Tg-3 mice suggested that Ngb overexpression at least partially might protect mice from brain damage by decreasing oxidative stress. These results match in vitro results [9–11]. In addition, behavioural outcome was tested using a 10-point neurological severity score, a standard wire-grip test (to test grip strength and endurance) and the Morris water maze (MWM) task (to test visual–spatial hippocampus-dependent learning and memory). Significant differences were not found with regard to the recovery of sensorimotor and spatial memory functional deficits between Ngb-Tg-3 and WT control mice up to three weeks after TBI [12].

A synapsin promoter drives Ngb overexpression in Ngb-Tg-4 mice. This promoter was expected to drive robust transcription only into neuronal cells [8]. In fact, northern blotting revealed robust Ngb overexpression in the cerebrum and cerebellum but not the liver or pancreas of Ngb-Tg-4 mice. Ngb protein levels were significantly higher in the brains of adults compared with those of pups and embryos (E16) [5]. Neurological deficits can also be induced by the acute inhalation of combustion smoke. The inhalation of carbon monoxide and noxious gases creates a hypoxic environment. These gases disrupt oxygenation and generate free radicals. Ngb-Tg-4 mice showed reduced inhibition of respiratory complex I activity and reduced smoke-induced DNA damage in their brains compared with WT mice. A decline in DNA damage after the acute inhalation of combustion smoke might dramatically reduce the risk of subsequently accumulated damaged and/or ill-repaired DNA in the brains of Ngb-Tg-4 mice, thereby minimising the risk of delayed neuronal dysfunction [5].

The understanding of the physiological function of Ngb might be one step closer to curing (neurodegenerative) diseases besides ischaemic stroke. This was strengthened by a study that used an Alzheimer's disease (AD) mouse model crossed with Ngb-Tg-1. Ngb overexpression reversed the Y maze spatial learning deficit observed in $APP_{Sw,Ind}$ mice, an AD mouse model that overexpresses the Swedish (Sw) and Indiana (Ind) amyloid precursor protein (APP) mutations linked to familial AD. As such, Ngb overexpression protected against the transgenic Alzheimer phenotype in vivo [13].

Although neuroprotection was clearly shown across different experiments, the behavioural differences between Ngb-Tg mice and WT controls were mostly non-significant. However, only a few mouse models were subjected to a subset of behavioural tests; to our knowledge, no extensive and comprehensive behavioural baseline studies have been performed using an Ngb-Tg mouse model. The present study employed the Ngb-Tg-1 mouse model to assess the effects of Ngb overexpression on a wide variety of standardised behavioural tests. Furthermore, we investigated the effects of 48 h hypoxia and 24 h reoxygenation on general disturbance using the SHIRPA primary screening. In addition, we tested motor function using the gait test, the stationary beam task, the wire suspension task and an accelerating rotarod.

2. Materials and methods

2.1. Transgenic mouse model

Ngb-Tg-1 mice (B6.Cg-Tg(CAG-Ngb,-Efgp)1Dgrn/j, stock number 007575, the Jackson Laboratory, Bar Harbor, Maine, USA) were crossbred with C57Bl/6J mice (stock number 000664, Charles River, L'Arbresle Cedex, France) to produce heterozygous Ngb-Tg-1 mice and WT littermates. Three-month-old and one-year-old male heterozygous Ngb-Tg-1 mice and WT littermates were bred, grown and analysed in the same facilities. Genotypes were confirmed by measuring the presence of recombinant Ngb via the polymerase chain reaction as previously described [4]. Animals were housed in mixed genotype groups in standard mouse cages under conventional laboratory conditions with food and water available ad libitum. The room was kept at a constant temperature and humidity with a 12 h/12 h light–dark cycle. The researchers were blind to the genetic status of the animals. All experiments were conducted in compliance with the European Communities Council Directive (86/609/EEC). The Animal Ethics Committee of the University of Antwerp approved all protocols. The number of mice used for the different tests is described in Table 1.

2.2. SHIRPA primary screening

The SHIRPA primary screening identifies global disturbances in gait, posture and muscle tone as well as abnormalities in motor control and coordination. This screening includes simple measurements of physical and neurological health, weight and body condition as well as sensory and motor functions. The SHIRPA primary screening was conducted as previously described [14].

2.3. Cognitive performance assessments

2.3.1. The hidden-platform Morris water maze (MWM) test

The MWM setup consisted of a circular pool (diameter = 150 cm, height = 30 cm) filled with opaque water kept at 25 °C. A round, acrylic, glass platform was placed in one quadrant. Acquisition training consisted of eight blocks of four trials per day starting from four different positions in a semi-random order and a 15-min inter-trial interval. If the mice were unable to reach the platform in 120 s, then they were placed on the platform where they stayed for 15 s before being returned to their home cages. The acquisition phase was followed by a probe trial during which the platform was removed from the maze, and the animals were allowed to swim freely for 100 s. During acquisition training and the probe trials, the animals' trajectories were recorded using a computerised video-tracking system (Ethovision, the Netherlands). During the training trials, the escape latency to the platform and path lengths were measured. During the probe trial, performance was indexed as the percentage of

Table 1
The number of mice used in the behaviour tests.

Tests	Specification	Ngb-Tg-1	WT
Baseline performance	3 months	16	14
	1 year	13	16
SHIRPA primary screening	3 months normoxia	18	15
	3 months hypoxia	11	9
48 h hypoxia with 24 h reoxygenation	3 months	11	13
Stationary beam test 2	3 months normoxia	13	12
	3 months hypoxia	16	16