## Title

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# Hypothermia Treatment after Hypoxia Ischemia in Glutathione Peroxidase 1 Overexpressing Mice

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**Abstract**
The developing brain is uniquely susceptible to oxidative stress, and endogenous antioxidant mechanisms are not suf cient to prevent injury from a hypoxic-ischemic challenge. Glutathione peroxidase GPX1) activity reduces hypoxic-ischemic injury. Therapeutic hypothermia HT) also reduces hypoxic-ischemic injury, in the rodent and the human brain, but the bene t is limited. Here, we combined GPX1 overexpression with HT in a P9 mouse model of hypoxia-ischemia HI) to test the effectiveness of both treatments together. Histological analysis showed that wild-type WT) mice with HT were less injured than WT with normothermia. In the GPX1-tg mice, however, despite a lower median score in the HT-treated mice, there was no signi cant difference between HT and normothermia. GPX1 protein expression was higher in the cortex of all transgenic groups at 30 min and 24 h, as well as in WT 30 min after HI, with and without HT. GPX1 was higher in the hippocampus of all transgenic groups and WT with HI and normothermia, at 24 h, but not at 30 min. Spectrin 150 was higher in all groups with HI, while spectrin 120 was higher in HI groups only at 24 h. There was reduced ERK1/2 activation in both WT and GPX1-tg HI at 30 min. Thus, with a relatively moderate insult, we see a bene t with cooling in the WT but not the GPX1-tg mouse brain. The fact that we see no bene t with increased GPx1 here in the P9 model unlike in the P7 model) may indicate that oxidative stress in these older mice is elevated to an extent that increased GPx1 is insuf cient for reducing injury. The lack of bene t of overexpressing GPX1 in conjunction with HT after HI indicates that pathways triggered by GPX1 overexpression may interfere with the neuroprotective mechanisms provided by HT.



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## Introduction

The developing brain is uniquely susceptible to oxidative stress and subsequent injury [1–3]. Hydrogen peroxide, in particular, accumulates in the neonatal, but not the adult, brain after hypoxia-ischemia (HI) [4]. Endogenous antioxidant mechanisms, such as catalase and glutathione peroxidase (GPX1), are not suf cient to prevent injury from a hypoxic-ischemic challenge. GPX1 activity has been shown to peak at day 1 (P1) of life in the mouse brain, decrease at P4, again at P7, and decline to adult levels by P14 [5]. Increased GPX1 at P7, however (via GPX1 overexpression in the mouse), reduces HI injury [6, 7]. Hypoxia preconditioning has been shown to protect the brain from subsequent insults, but we found that the GPX1-tg mice lost the protection afforded by GPX1 overexpression when given hypoxia preconditioning. WT littermates of these mice were protected by hypoxia preconditioning, however [7]. We also showed that ERK1/2 is transiently activated by hypoxia preconditioning in WT P7 mice, but this activation is blocked by GPX1 overexpression [8]. This indicates that redox balance depends on ERK1/2 activity, which subsequently protects against oxidative injury.

We previously determined GPX1 activity at P7 to be approximately 1.2-fold higher in the cortex of these GPX1-tg mice than in wild-type (WT) mice [6]. In addition, 24 h after HI, GPX1 activity rose 1.3-fold over naïve GPX1-tg cortex [6]. The P9-10 mouse brain is now considered by many to be more representative of the term-gestation human brain [9]. In the P9 GPX1-tg mouse, GPX1 protein expression was several times higher than WT in the cortex and hippocampus [10]. Since GPX1 targets hydrogen peroxide, its mechanism of action is presumably in the early, or acute, phase of HI injury, while hypothermia (HT), applied after injury onset, acts on the subsequent phase.

As a therapy, HT acts after injury is underway and has been shown to reduce HI injury, in the rodent [11] and the human [12] brain, but the bene t is limited and many questions remain regarding optimal treatment of newborns with HIE [13, 14]. The mechanisms of protection are not fully understood but are wide-ranging and include reduced metabolic rate, decreased production of free radicals, suppression of inflammation, and inhibition of excitotoxicity [14]. Consequently, additional treatments to HT may prove more effective, but they must be synergistic. Indeed, the challenge of inhibiting cell death without interfering with overall recovery indicates therapies should be timed to target the phases of injury and, ultimately, repair [15]. Here, we combined GPX1 overexpression with HT in a P9 mouse model of HI to test the effectiveness of enhanced antioxidant capacity with therapeutic cooling.

## Materials and Methods

*Mice*

The GPX1-overexpressing mice used here (GPX1-tg) were developed with 1 additional copy of the transgene, resulting in expression in all brain regions analyzed in adult mice, including the cortex and striatum, as previously described [16]. The GPX1 activity ratio in these mice was approximately 1.5 for mesencephalon, and protein expression was 2.4-fold in cortex and 1.9-fold in striatum compared to WT [16]. GPX1-tg mice were bred and maintained at the UCSF Laboratory Animal Resource Center. Male mice heterozygous for GPX1 were bred with female WT (CD1) mice, and the genotype of resulting litters was determined by PCR, using standard methods as previously described [6, 16]. While we have used these mice in several previous studies [6, 7, 17] and have con rmed increased GPX activity and expression in the P7 mouse [6], we felt that the brain of the P9 mouse was suf ciently more mature to warrant con rmation of degree of increased GPX activity.

*GPX Activity*

To con rm overexpression in P9 GPX1-tg mice, selenium-dependent GPX1 enzymatic activity was measured in naïve cortex and hippocampus as previously described [6, 18]. Briefly, brains were removed from anesthetized GPX1-tg ($n = 10$) and WT mice ($n = 13$); cortices and hippocampi were quickly dissected on a cold surface and immediately frozen in methylbutane cooled by dry ice. Brain samples were stored at 80°C until assay, at which time they were homogenized in 50 mM potassium phosphate buffer with 1 mM EDTA (pH 7.0). GPX activity was measured in a coupled test system in which reduced glutathione and tert-butyl hydroperoxide were used as the substrates, and oxidized glutathione produced by GPX activity was measured by kinetic spectrophotometry (340 nm) of glutathione reductase-mediated NADPH oxidation. Units of GPX activity were determined by a standard curve of GPX expressed as units GPX/mg/min, where 1 unit (U) is de ned as 1 nmol NADPH oxidized per minute. Protein was determined by Pierce BCA spectrophotometric assay (Pierce, Rockford, IL, USA).

*HI/HT*

Neonatal mice overexpressing GPX1 and their WT littermates (CD1 background) underwent HI on postnatal day 9 (P9) [11, 19, 20]. Briefly, under isoflurane anesthesia, the left common carotid artery was dissected and coagulated until severed. After recovery with the dam for 1 h, mice were exposed to hypoxia by exposure to 10 oxygen (balance nitrogen) for 40 min. After another 1 h period with the dam, mice were placed in chambers maintained at 36.5°C (normothermia [NT]) or 32°C (HT) for 3.5 h and gradually rewarmed over 30 min before returning to the dam. The temperature of each mouse was measured at the nape of the neck with a hand-held laser-guided remote thermometer which we have compared to a rectal probe and found this measurement to be 0.5°C less than the rectal temperature. Temperature was recorded every 30 min.

*Histopathologic Analysis of Injury*

For determination of degree of injury, 18 weeks after HI and HT or NT (a timepoint when injury is fully resolved), mice were anesthetized with Euthasol (Virbac AH, Fort Worth, TX, USA), perfused with 4 paraformaldehyde, brains were cut on a vibratome (50 m), and alternate sections stained with cresyl violet and Perl s iron stain. Brain injury in the Vannucci model is variable, from large areas of infarct to scattered areas of pyknosis. The Perl s iron stain reveals these smaller areas of injury and thus provides a complement to the Nissl stain of the cresyl violet. Consequently, all sections are examined, and 11 regions are scored on a scale of 0–3: anterior, middle, and posterior cortex; anterior, middle, and posterior striatum; CA1, CA2, CA3, and dentate gyrus of the hippocampus; and thalamus, with 0 = no injury and 3 = severe cystic infarction, for a cumulative score of 0–33 [21]. In addition, injury volume was measured with the cresyl violet-stained sections using ImageJ software (NIH).

*Protein Expression by Western Blot*

At 30 min or 24 h after sham surgery, HI and NT, or HI and HT, brains were removed from anesthetized mice, and cortices and hippocampi were quickly dissected on a cold surface and immediately frozen in methylbutane cooled by dry ice. Brain samples were stored at 80°C. For 30 min: WT sham NT ($n = 6$), WT sham HT ($n = 4$), WT with HI and NT ($n = 9$), WT with HI and HT ($n = 11$), GPX1-tg sham NT ($n = 6$), GPX1-tg sham HT ($n = 4$), GPX1-tg with HI and NT ($n = 10$), GPX1-tg with HI and HT ($n = 10$). For 24 h: WT sham NT ($n = 10$), WT sham HT ($n = 14$), WT with HI and NT ($n = 16$), WT with HI and HT ($n = 16$), GPX1-tg sham NT ($n = 10$), GPX1-tg sham HT ($n = 11$), GPX1-tg with HI and NT ($n = 18$), GPX1-tg with HI and HT ($n = 17$). It should be noted that the timepoints used for Western blots here, 30 min and 24 h after NT or HT exposure, are 5 and 29 h after hypoxia, respectively. Thirty minutes is the point of recovery from HT and is the early timepoint for analysis.

Frozen brain tissue was homogenized in RIPA buffer (Sigma, St. Louis, MO, USA) with protease and phosphatase inhibitors (Thermo Fisher, Rockford, IL, USA) using dounce homogenizers. The homogenate was transferred to chilled Eppendorf tubes and left on ice for 30 min before centrifugation at 14,000 rpm for 15 min at 4°C. The supernatant was transferred to clean chilled tubes, and an aliquot was removed for determination of total protein by BCA assay (Thermo Fisher). 30 g protein were separated by SDS-PAGE and transferred to PVDF membranes. After blocking for 1 h in 5 non-fat dry milk in TBS with 0.5 TWEEN, membranes were incubated in the following antibodies: goat-actin 1:2,000 (Abcam, Cambridge, MA, USA); rabbit-GPX1, 1:2,000 (Abcam, Cambridge, MA, USA); mouse-spectrin 1:4,000 (Millipore, Temecula, CA, USA); mouse-ERK1/2 1:4,000, Invitrogen; rabbit-phospho-ERK1/2 (Cell Signaling, Danvers, MA, USA); and corresponding secondary antibodies, all 1:2,000. The signal was visualized with enhanced chemiluminescence (Thermo Fisher) and blots exposed to lm. Film was scanned, and mean optical densities were determined with ImageJ (NIH).

*Statistical Analysis*

GPX1 enzymatic assay results were analyzed by unpaired *t*-test and described as mean U GPX/mg protein ± SEM. Injury scores were evaluated by one-way ANOVA and Kruskal-Wallis with Dunn s multiple comparison test and are shown as scatter plots with the median value of each group a horizontal line. Injury volumes were compared by *t*-test and are described as percent injured hemisphere compared to contralateral hemisphere ± S.D. Western blots were normalized to -actin, evaluated with unpaired *t*-test, and presented as fold change relative to WT sham NT. Differences were considered signi cant at $p$ 0.05. Mortality was analyzed by contingency test and $\chi^2$. Graphpad Prism 7.0 (Carlsbad, CA) was used for all analysis except injury volumes which were by Excel.

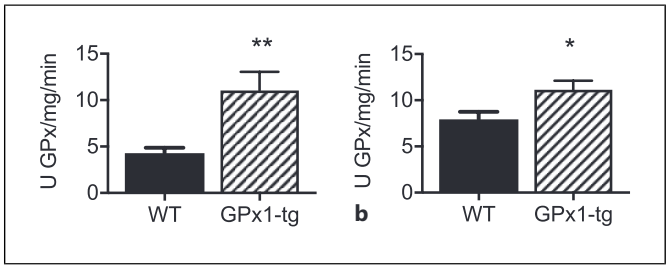


**Fig. 1.** GPX1 activity is higher in the GPX1-tg brain. **a** Cortex; $p$ 0.002. **b** Hippocampus; $p$ 0.02.

## Results

*Con rmation of GPX1 Overexpression in GPX1 tg Mice by Enzymatic Assay*

GPX1 activity was higher at P9 in the GPX1-tg ($n = 10$) compared to WT ($n = 13$) littermates in both the cortex (GPX1-tg = 11.0 ± 2.02 U/mg vs. WT = 4.28 ± 0.59 U/mg; $p$ 0.002; Fig. 1a) and hippocampus (GPX1-tg = 11.1 ± 0.98 U/mg vs. WT = 7.92 ± 0.83 U/mg; $p$ 0.02; Fig. 1b).

*Brain Injury after HI*

WT mice with HT were less injured than WT with NT (median scores 6 [$n = 13$] and 29 [$n = 15$], respectively; $p$ 0.03; Fig. 2a). In the GPX1-tg, however, despite a lower median score in the HT-treated mice, there was no signi cant difference between HT and NT (median scores 19 [$n = 12$] and 31 [$n = 10$], respectively; Fig. 2a). The pattern is similar in the brain regions analyzed separately: In the cortex, WT mice with HT were less injured than WT with NT (median scores 3 and 9, respectively; $p$ 0.03; Fig. 2b). In the GPX1-tg, there was no signi cant difference between HT and NT (median scores 5.5 and 9, respectively; Fig. 2b). In the hippocampus, WT mice with HT were less injured than WT with NT (median scores 3 and 10, respectively; $p = 0.05$; Fig. 2c). In the GPX1-tg, there was no signi cant difference between HT and NT (median scores 7 and 11, respectively; Fig. 2c). In the striatum, WT mice with HT were less injured than WT