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Strain-Related Differences in Mouse Neonatal Hypoxia-Ischemia

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Abstract

Neonatal hypoxic-ischemic brain injury is commonly studied by means of the Vannucci procedure in mice or rats (unilateral common carotid artery occlusion followed by hypoxia). Previously, we modified the postnatal day 7 (P7) rat procedure for use in mice, and later demonstrated that genetic strain strongly influences the degree of brain injury in the P7 mouse model of hypoxia-ischemia (HI). Recently, the P9 or P10 mouse brain was recognized as the developmental equivalent of a term neonatal human brain, rather than P7. Consequently, the Vannucci procedure has again been modified, and a commonly used protocol employs 10% oxygen for 50 min in C57Bl/6 mice. Strain differences have yet to be described for the P9/P10 mouse model. In order to determine if the strain differences we previously reported in the P7 mouse model are present in the P9 model, we compared 2 commonly used strains, CD1 and C57Bl/6J, in both the P7 (carotid ligation [in this case, right] followed by exposure to 8% oxygen for 30 min) and P9 (carotid ligation [in this case left] followed by exposure to 10% oxygen) models of HI. Experiments using the P7 model were performed in 2001–2012 and those using the P9 model were performed in 2012–2016. Five to seven days after the HI procedure, mice were perfused with 4% paraformaldehyde, their brains were sectioned on a Vibratome (50 μm) and alternate sections were stained with Perl's iron stain or cresyl violet. Brain sections were examined microscopically and scored for the degree of injury. Since brains in the P7 group had been scored previously with a slightly different system, they were reanalyzed using our current scoring system which scores injury in 11 regions: the anterior, middle, and posterior cortex; the anterior, middle, and posterior striatum; CA1, CA2, CA3, and the dentate gyrus of the hippocampus and thalamus, on a scale from 0 (none) to 3 (cystic infarct) for a total score of 0–33. Brains in the P9 group were scored with the same system. Given the same insult, the P7 CD1 mice had greater injury than the C57Bl/6J mice, which agrees with our previous findings. The P9 CD1 mice also had greater injury than the C57Bl/6J mice. This study confirms that CD1 mice are more susceptible to injury than C57Bl/6J mice and that strain selection is important when using mouse models of HI.

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Introduction

Hypoxic-ischemic injury is a major cause of perinatal brain injury in term infants [1]. The Vannucci model of hypoxia-ischemia (HI) has evolved since it was first established in postnatal day 7 (P7) rats nearly 40 years ago, but remains the most widely used model of neonatal HI [2]. The model was adapted for mice, allowing for the use of genetic manipulation to study the role of single genes in the setting of HI [3]. Subsequently, we found the background strain of genetically altered mice itself had an effect on outcome. Specifically, we showed that CD1 mice are highly susceptible to injury and require 30 min of 8% oxygen to attain moderate injury, while the C57Bl/6J and SV129 strains are more resistant to injury, requiring 45 and 90 min, respectively, to achieve a similar degree of injury, but have a higher mortality rate [4].

Age has also been modified in the Vannucci model to recreate different types of injury based on developmental stage [5–7]. In rats, for example, very young (P1–P3) rats are used to mimic very immature injury that manifests largely as white-matter injury [8–11]. In mice, P9 or P10 is now considered a better rodent equivalent to the term human newborn than P7, and the Vannucci model has been adjusted accordingly, with different percentages of oxygen and different durations of hypoxia being deployed, depending on the type and degree of injury being modeled [6, 12–14].

With the widespread adoption of the P9 model of murine HI, we sought to map strain influences and degree of injury in this variation of the procedure. With our current scoring system, we first reanalyzed P7 model data collected over 11 years, and then compared wild-type (WT) CD1 and C57Bl/6J mice. We hypothesized that the P9 model would show susceptibility to injury based on strain, as previously found in the P7 model. For this, we summarized data using the P9 model collected over 5 years, and also compared CD1 and C57Bl/6J mice.

Understanding susceptibility or resistance to injury based on genetic background in mice should not only improve research methods but also our understanding of human variation in injury outcome, and it may ultimately lead to novel targets for intervention [15, 16].

Methods

Hypoxia-Ischemia

Experiments using the P7 model were performed in 2001–2012. After ligation by electrical coagulation of the right carotid artery under isoflurane anesthesia and a 1-h recovery period with the dam, both CD1 and C57Bl/6J mice received 30 min of 8% oxy-
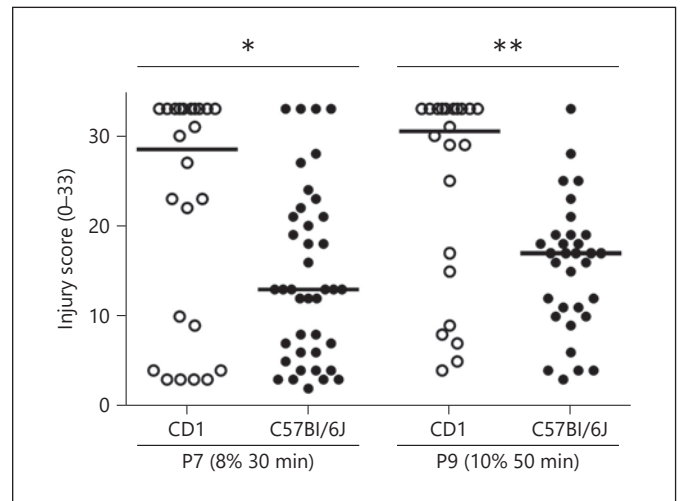
Fig. 1. Histological injury scores for the entire hemisphere after HI (scale 0–33). CD1 brains were compared to C57Bl/6J brains after HI at P7 with 30 min of 8% oxygen and after HI at P9 with 50 min of 10% oxygen. Median injury score is higher in CD1 than in C57Bl/6J in both the P7 (* $p < 0.03$) and P9 (** $p = 0.0025$) models. The horizontal line indicates median score.

gen while in chambers maintained at 36.5°C. Experiments for the P9 model were performed in 2012–2016. After carotid ligation as above (except that now the left artery was ligated), CD1 and C57Bl/6J mice received 50 min of 10% oxygen.

Mice from all groups were anesthetized with Euthasol (Henry Schein) and perfused with 4% paraformaldehyde 5–7 days after HI (i.e., when edema has lessened and injured regions are histologically distinct) and brains were postfixed in the same fixative overnight. Brains were sectioned on a Vibratome (50 μm) and alternate sections were stained for Nissl (cresyl violet) and iron (Perl's stain modified by enhancement with diaminobenzidine). The brains in the P7 group were reanalyzed using our current scoring system (a scale of 0 [none] to 3 [cystic infarct] for a total score of 0–33), which scores injury in 11 regions: the anterior, middle, and posterior cortex; the anterior, middle, and posterior striatum; CA1, CA2, CA3, and the dentate gyrus of the hippocampus and thalamus, as previously described [17]. The brains in the P9 group were analyzed with the same system.

Statistical Analysis

Injury scores were analyzed by the Mann-Whitney U test, Mortality was analyzed by contingency tables with Fisher's exact test. Analysis was done with Prism v7.0 (Carlsbad, CA, USA). $p < 0.05$ indicated significance.

Results

Injury was greater in the HI hemisphere (ipsilateral to the ligation) of the CD1 mouse brain than in the C57Bl/6J brain for both the P7 and P9 models, thereby confirming

Table 1. Injury scores for each brain region

Injured region	P7			P9		
	CD1 (n = 24)	C57Bl/6J (n = 40)	p value	CD1 (n = 22)	C57Bl/6J (n = 32)	p value
Whole hemisphere	28.5 (3–33)	13 (2–33)	<0.03[a]	30.5 (4–33)	17 (3–33)	<0.003[b]
Cortex	9 (3–9)	5 (2–9)	<0.01[a]	9 (0–9)	4 (1–9)	<0.0001[c]
Hippocampus	10 (0–12)	6 (0–12)	>0.10	11 (0–12)	7 (0–12)	<0.005[b]
Striatum	7.5 (0–9)	2 (0–9)	<0.02[a]	6 (0–9)	5 (0–9)	<0.03[a]
Thalamus	2 (0–3)	1 (0–3)	<0.03[a]	2 (0–3)	1 (0–3)	<0.003[b]

Values are presented as median (range). [a] ████; [b] ████; [c] ████

Table 2. Injury scores by sex

Strain	P7			P9		
	male	female	p value	male	female	p value
CD1	32 (3–33) (n = 18)	3.5 (3–33) (n = 6)	<0.03[a]	29 (4–33) (n = 11)	33 (5–33) (n = 11)	>0.5
C57Bl/6J	12.5 (3–33) (n = 20)	13 (2–33) (n = 20)	>0.97	16.5 (4–33) (n = 16)	15 (3–25) (n = 11)	>0.4[a]

Values are presented as median (range). [a] Sex was not determined in 5 P9 C57Bl/6J mice.

our previous findings for P7 and documenting the strain difference for P9 (Fig. 1; Table 1). When analyzed separately, the cortex also had greater injury in the CD1 mouse brains than in the C57Bl/6J brains in both the P7 and P9 groups (Fig. 2a; Table 1). In the hippocampus, however, injury scores were not significantly different between CD1 and C57Bl/6J mice in the P7 group (p > 0.10). In the P9 group, however, the CD1 mouse brains were more injured than the C57Bl/6J brains (Fig. 2b; Table 1). In the striatum, injury was greater in the CD1 mouse brains than in the C57Bl/6J brains in both the P7 and P9 groups (Fig. 2c; Table 1). In the thalamus, a region we did not analyze separately in our previous study, injury was greater in the CD1 mouse brains than in the C57Bl/6J brains in both the P7 and P9 groups (Fig. 2d; Table 1).

Comparison of Brain Injury Scores by Age at HI
Injury scores were not significantly different in the CD1 P7 and P9 groups or in the C57Bl/6J P7 and P9 groups, either overall or regionally.

Sex Differences
Some studies using HI have shown an effect associated with sex [18–20]. Here, when we compared injury scores of male and female mice, we found CD1 males to be more

Table 3. Mortality

Strain	P7			P9		
	male	female	p value	male	female	p value
CD1	10 (36)	5 (45)	0.72	2 (18)	2 (18)	1
C57Bl/6J	15 (43)	11 (35)	0.62	0 (0)	0 (0)	1

Values are presented as n (%).

injured than females at P7, but no difference between CD1 males and females at P9 (Fig. 3a; Table 2). However, there were far fewer females than males in the P7 group. In the C57Bl/6J mice, there were no differences associated with sex at either P7 or P9 (Fig. 3b; Table 2; note: data were missing for 5 P9 C57Bl/6J mice).

Weight
Prior to HI, CD1 mice were heavier than C57Bl/6J mice at P7 (mean 4.54 ± 0.59 and 4.20 ± 0.55 g, respectively; p < 0.016) and at P9 (mean 5.91 ± 0.68 and 5.25 ± 0.85 g, respectively; p = 0.003).
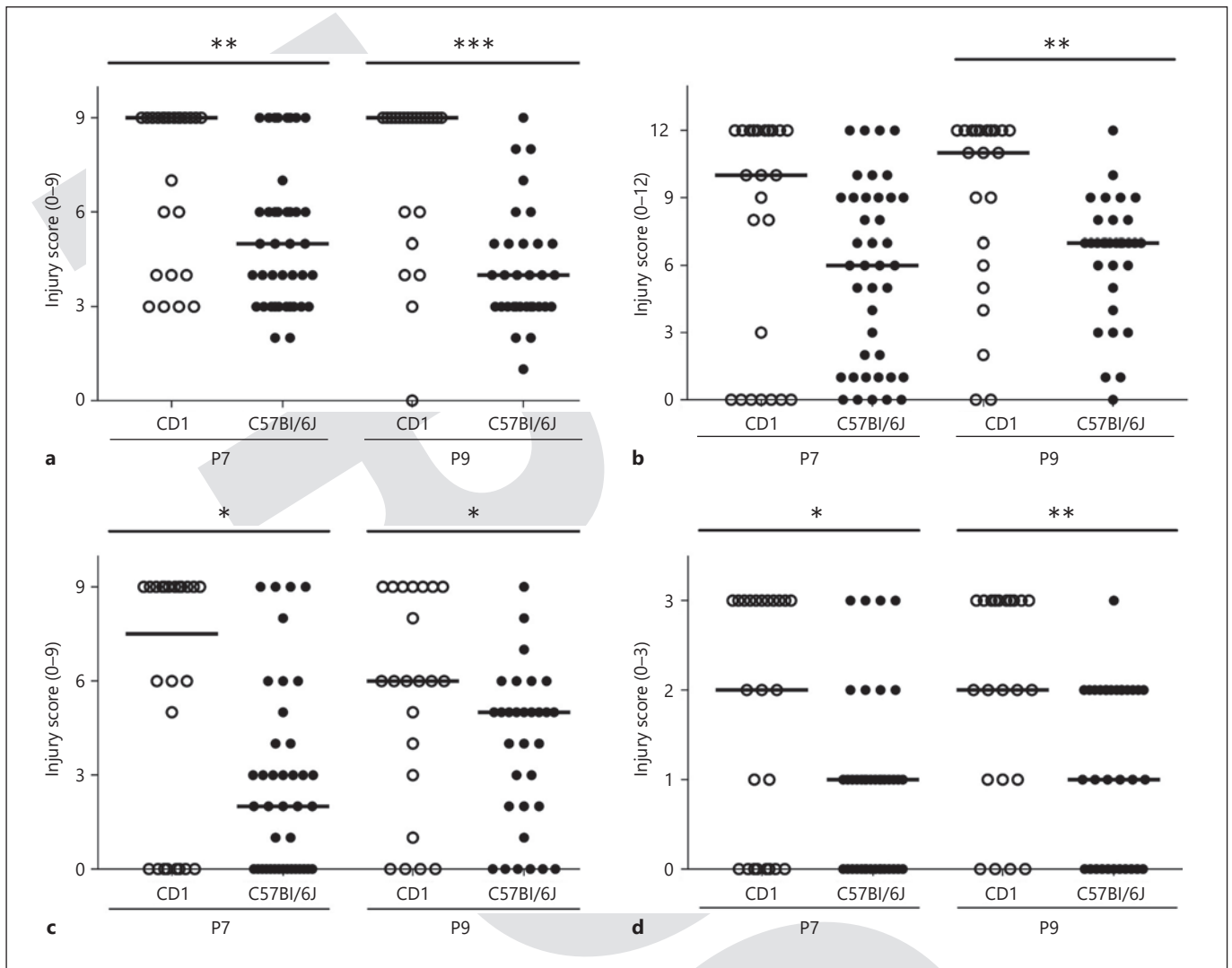
Fig. 2. Histological injury scores by region. CD1 compared to C57Bl/6J after HI at P7 with 30 min of 8% oxygen and after HI at P9 with 50 min of 10% oxygen. **a** Cortex (scale 0–9). Median injury score is higher in CD1 than C57Bl/6J in both the P7 (* $p < 0.03$) and P9 (** $p = 0.003$) models. **b** Hippocampus (scale 0–12). Median injury score is not different in CD1 and C57Bl/6J in the P7 model ($p > 0.10$), but injury is higher in CD1 than C57Bl/6J in the P9 model (** $p < 0.005$). **c** Striatum (scale 0–9). Median injury score is higher in CD1 than C57Bl/6J in both the P7 (* $p < 0.02$) and P9 (* $p = <0.03$) models. **d** Thalamus (scale 0–3). Median injury score is higher in CD1 than in C57Bl/6J in both the P7 (* $p < 0.03$) and P9 (* $p < 0.003$) models. Horizontal line indicates median score.

Mortality

The highest mortality was among the P7 mice. Fifteen of 39 CD1 mice died and 26 of 66 C57Bl/6J mice died. Among the P9 mice, only 4 of 26 CD1 mice died and none of the C57Bl/6J died. There were no differences in mortality between males and females (Table 3).

Discussion

Given the same insult at P7, CD1 mice were more susceptible to HI injury than the commonly used inbred strain C57Bl/6J, confirming our previous results [4]. Our results also validated the reanalysis of HI data acquired over many years. Our previous study did not analyze brain regions separately, which we do here, showing the
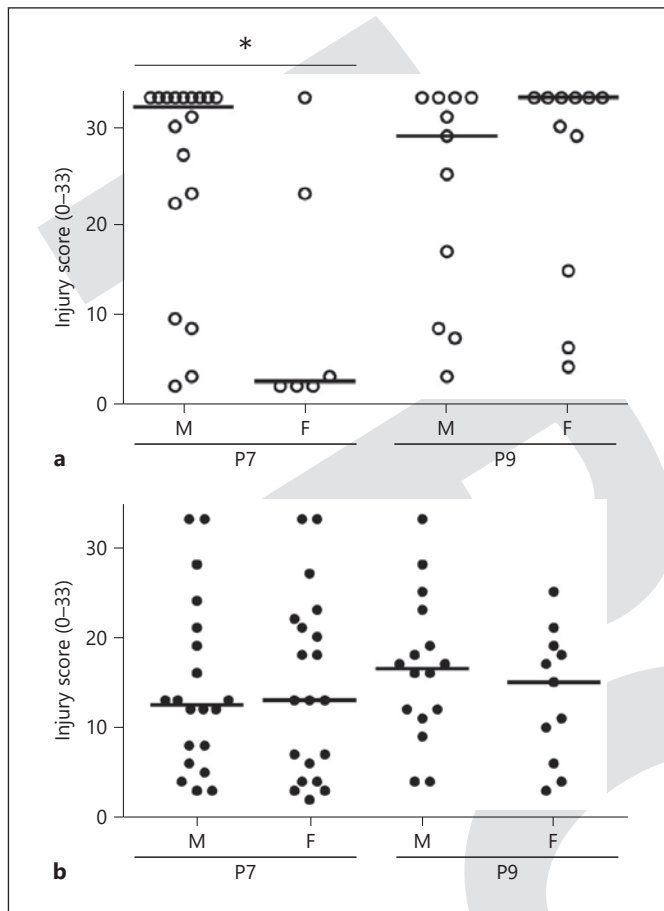
Fig. 3. Histological injury scores by sex (scale 0–33). **a** CD1. Male (M) mice have greater injury than females (F) at P7 (* $p < 0.03$) but not at P9 ($p = 0.5$). **b** C57Bl/6J. There is no difference between male and female C57Bl/6J mice at P7 and P9.

cortex, striatum, and thalamus to be the regions where the difference in injury is the greatest, with injury in the hippocampus demonstrating only a trend to difference.

We also looked for differences associated with sex and found male CD1 mice at P7 to have greater injury than females. However, there were relatively few females in this group, which likely influenced the result. We did not see differences due to sex in previous studies on CD1 mice and HI at P7 [21, 22]. Thus, while additional data on female CD1 mice might have reduced the overall median score of the CD1 P7 group, our experience indicates that significant sex differences are unlikely. However, it is not known how additional data on female CD1 mice affected the comparison of the CD1 and C57Bl/6 brains in the P7 model overall. A limitation of this study is that the data for P7 came from archived slides, with no new mice used to equalize the numbers.

There were no sex-related differences in injury in the C57Bl/6J mice at P7. Other study groups, however, have seen sex differences in neonatal brain injury [23]. Recently, in a stroke model with P9 C57Bl/6J mice, saline-treated males had greater tissue loss than females 3 months after the insult, but not at 3 or 8 days [24]. The outcome at adulthood is important, and the possibility of worsened injury, in particular, warrants further study.

We also demonstrated that CD1 mice are more susceptible to HI injury than C57Bl/6 in an age-adjusted P9 version of the Vannucci model. In the P9 group, the differences were seen in the entire hemisphere as well as in all regions analyzed: cortex, striatum, thalamus, and hippocampus. In a recent study using P9 mutant mice with a C57Bl/6J background, we compared 50 and 60 min of 10% oxygen in order to see if an increased duration of hypoxia increased injury, and, if so, how a greater degree of injury would affect outcome relative to the WT mice. Injury scores increased in WT mice with the additional minutes of hypoxia, demonstrating that injury is indeed increased with a small increase in duration of hypoxia in C57Bl/6J mice [17].

Other studies have compared CD1 to C57Bl/6 mice. For example, Li et al. [15] administered chronic hypoxia to both strains at P3; they found C57Bl/6 mice and the neural progenitor cells derived from them to have a "blunted" response to hypoxia in several measures. A model of ischemic brain injury and seizures with carotid ligation alone (no hypoxia) also showed CD1 mice to be far more susceptible to injury than C57Bl/6 [25] and C3HeB/FeJ [26] mice. Substrains of C57Bl/6 have also been compared in the P7 HI model. Wolf et al. [27] showed that C57Bl/6J mice had a decreased lesion size and score compared to C57Bl/6N.

Anatomical variations between mouse strains, particularly vascular differences, are often proposed to be the cause for differences in experimental outcome in stroke and hypoxic-ischemic encephalopathy [28]. An adult mouse study, for example, showed the C57Bl/6 strain to have the poorest development of the Circle of Willis of the 7 strains studied (not including CD1), as well as the highest mortality and greatest susceptibility to injury after bilateral common carotid artery occlusion [29].

Recently, another study of adult mice examined 32 inbred strains for variations in the Circle of Willis as well as the diameters of major cerebral arteries [30]. They found strain-related differences not only in vessel diameters, but also strain-related anomalies such as a unilateral or bilateral absence of the first segment of the posterior communicating artery, and the presence of accessory middle ce-

rebral arteries. They also corrected for weight and age, strengthening the argument for genetic causality. The same authors previously explored the genetic basis for these vascular differences and identified 4 SNPs associated with infarct volume in mice after middle cerebral artery occlusion, 2 of which were relevant to human stroke susceptibility [16].

There is need for more work on the genetic underpinnings of variation in neonatal models, given the variation in injury (and mortality) attributable to genetic strain, and the different mechanisms of cell injury and death and attempts at repair [31, 32].

An advantage of the P9 model of HI in C57Bl/6J mice is the lower mortality (than at P7), especially when attempting to achieve a moderate to severe degree of injury. It is not known why mortality is high in C57Bl/6J at P7, while the brain is relatively resistant to injury, but this may be due to cardiovascular differences. The neonatal rodent brain develops rapidly, and the 2 days from P7 to P9 encompass a period of substantial growth and maturation. Given that we rarely know precisely when our murine research subjects are born, several hours could conceivably make a difference in a mouse's response to HI.

The optimal duration of hypoxia should be determined for different strains and ages prior to their use in experiments. More importantly, the background strain must be carefully considered and controlled for when using mutant mice.

Acknowledgements

Statement of Ethics

All animal research was approved by the University of California San Francisco Animal Care and Use Committee (IACUC) and performed in accordance with the standards of humane care established by the NIH.

Disclosure Statement

The authors have no conflicts of interest to disclose.

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