A Ferret Model of Encephalopathy of Prematurity

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Abstract

There is an ongoing need for relevant animal models in which to test therapeutic interventions for infants with neurological sequelae of prematurity. The ferret is an attractive model species as it has a gyrified brain with a white-to-gray matter ratio similar to that in the human brain. A model of encephalopathy of prematurity was developed in postnatal day 10 (P10) ferret kits, considered to be developmentally equivalent to infants of 24–26 weeks' gestation. Cross-fostered P10 ferret kits received 5 mg/kg of lipopolysaccharide (LPS) before undergoing consecutive hypoxia-hyperoxia-hypoxia (60 min at 9%, 120 min at 60%, and 30 min at 9%). Control animals received saline vehicle followed by normoxia. The development of basic reflexes (negative geotaxis, cliff aversion, and righting) as well as gait coordination on an automated catwalk were assessed between P28 and P70, followed by ex vivo magnetic resonance imaging (MRI) and immunohistochemical analysis. Compared to controls, injured animals had slower overall reflex development between P28

and P40, as well as smaller hind-paw areas consistent with "toe walking" at P42. Injured animals also displayed significantly greater lateral movement during CatWalk assessment as a result of reduced gait coordination. Ex vivo MRI showed widespread white-matter hyperintensity on T2-weighted imaging as well as altered connectivity patterns. This coincided with white-matter dysmaturation characterized by increased intensity of myelin basic protein staining, white-matter thinning, and loss of oligodendrocyte transcription factor 2 (OLIG2)-positive cells. These results suggest both pathological and motor deficits consistent with premature white-matter injury. This newborn ferret model can therefore provide an additional platform to assess potential therapies before translation to human clinical trials.

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Introduction

There is an ongoing need for large-animal models that reflect the pathophysiology of preterm brain injury in which therapeutic interventions for infants with neurological sequelae of prematurity can be tested. In 2016, 9.85% of infants born in the USA were premature, and,

for those born extremely preterm (<28 weeks' gestation), around 50% will have a poor outcome [1–3]. Preterm birth is commonly initiated by maternal infection (chorioamnionitis) and is also often associated with perinatal insults such as hypoxia and ischemia [4]. Subsequent intermittent iatrogenic hyperoxia is commonplace in the neonatal intensive care unit (NICU) [5]. These combined factors are thought to contribute to developmental and physiologic vulnerability of the brain, and to result in or exacerbate the encephalopathy associated with poor developmental outcomes in preterm infants [5–7].

Recently, because of the physical and developmental similarities in the ferret and human brain, the ferret has emerged as an attractive species in which to model brain injury [8–11]. Unlike rodents and rabbits, the ferret has a gyrencephalic cerebral cortex and a ratio of white-to-gray matter that is similar to humans. Ferrets are ideal candidates to model preterm brain injury as they are born lissencephalic, developing gyrencephalic brains postnatally. Postnatal white-matter maturation and complex cortical folding in ferret kits occur in a similar pattern to that observed in the human brain during the third trimester [12]. This includes development of the cortical subplate (a transient scaffolding for the evolving cortex), prominent in human brain development but minimal in rodents [13]. At birth, ferret brain development is similar to that of a 13-week-old human fetus, with postnatal day 10 (P10) kits considered to be equivalent to an infant of 24–26 weeks' gestation [12].

We have previously examined the white matter and motor development in the newborn ferret, with preliminary data suggesting that a lipopolysaccharide (LPS)-sensitized hypoxic and hyperoxic insult could result in short-term inflammation in the P10 ferret brain, including the activation of microglia and possible astrogliosis [11]. Here, we describe the long-term behavioral and pathological outcomes of inflammation-sensitized hypoxic/hyperoxic brain injury in the P10 ferret as a model of encephalopathy of prematurity.

Methods

Animals

Eight time-mated pregnant jills were acquired from Marshall BioResources (North Rose, NY, USA) at ≤ 28 days of gestation, and allowed to kindle naturally (typical gestation 41–42 days). Pregnant jills were acquired in pairs, and kits were cross-fostered at P8/P9 in order to balance litter sizes and sex distribution. Animals were maintained in a centralized vivarium with ad libitum access to food and water before and during experimental procedures. Standard housing ferret conditions included a 16-h light/8-h dark

cycle with a room temperature (RT) range of 61–72°F (16–22°C), humidity of 30–70%, and 10–15 fresh air changes per hour.

Experimental Procedure

On P10, cross-fostered ferret kits were randomized to receive an intraperitoneal 5 mg/kg dose of LPS (from *Escherichia coli* O111:B4, List Biological, CA, USA) or saline vehicle, before being returned to their jills for 4 h. LPS-injected animals were subsequently placed in a humidified chamber within a water bath, and underwent consecutive hypoxia, hyperoxia, and hypoxia (60 min at 9% O₂, 120 min at 60% O₂, and 30 min at 9% O₂). Rectal temperature in a sentinel animal was monitored continuously throughout the insult (Precision 4000A thermometer, YSI, Yellow Springs, OH, USA), with a target intrahypoxic rectal temperature of 37°C. Saline controls received an identical period of normoxia, after which all animals were returned to their jills. A number of experimental protocols that did not produce any evidence of short-term injury were attempted in the development of the current approach. A summary of these is listed in online supplemental Table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000498968). The final protocol was developed based on this preliminary data as well as on evidence from the preclinical literature. An LPS dose of 5 mg/kg was chosen because preliminary experiments (online suppl. Table 1) showed greater histological injury in kits exposed to 3 doses of 5 mg/kg LPS before hypoxia/hyperoxia compared to doses of 2 mg/kg LPS, but the 5 mg/kg dose was similar to 10 mg/kg (online suppl. Fig. 1). A single LPS dose was then chosen due to concerns about potential preconditioning caused by multiple doses [11]. A 4-h delay between LPS exposure and hypoxia was used based on rodent literature, as this is the time frame associated with the greatest systemic inflammatory response and sensitization of the brain to hypoxia-ischemia [14–16]. A similar time course of inflammatory activation after LPS exposure has been shown in isolated ferret peripheral blood mononuclear cells [17]. The resulting final protocol published here was the first to show sustained pathological and neurobehavioral deficits in exposed P10 ferret kits. One iteration of the protocol (3 doses of LPS every 12 h at P9/P10 before 45 min at 6% oxygen and 6 h at 100% oxygen) was included in a previous publication [11].

Early Behavioral Testing

Beginning on P28, kits underwent reflex testing 3 times/week until P40. This included negative geotaxis (NG), cliff aversion (CA), and righting reflex (RR), as previously described [11]. Briefly, NG was performed on inclined planes at 45°. Kits were placed head-down in the middle of the plane, and time taken to rotate 90° and 180° was recorded. For CA, kits were placed with both front paws on the edge of a shelf. Time to spontaneously retreat away from the edge and rotate away was recorded. For RR, animals were placed on their backs and time to righting was recorded; they were required to show full-paw placement and begin coordinated locomotion to complete the righting task. A total of 3 runs of the NG and CA tests and 5 runs of the RR test were performed on every testing day. For all tests, failure was considered to be an inability to complete the task within 60 s. Total time (TT) score, i.e., the sum of the mean time for each of the 3 tasks on each day and area under the curve (AUC) across the whole testing period (as a measure of skill development over time) for each test were also calculated.

CatWalk Analysis

From P42 to P70, kits underwent weekly catwalk gait analysis (CatWalk XT, Noldus, Leesburg, VA, USA). At each time point, 3 compliant runs (lasting <10 s with a maximum speed variation of 60%) were collected from each animal. Paw placement pressure, paw area, stride length, swing speed, and base of support (BOS) were analyzed over time as measures of gait development. Due to variability in size between both litters and sexes, all CatWalk measures were either adjusted by weight, or used to generate ratios (i.e., between the forepaws and hind paws) within a single animal. Measures of paw pressure intensity were derived from CatWalk XT and are expressed in arbitrary units (au). A Python package was developed to analyze the lateral movement component of ferrets during the catwalk task. Raw images were extracted from the CatWalk dataset for postanalysis with a self-developed software package and an ImageJ macro (<https://github.com/ccurtis7/ferretfit>). Paw print coordinates were extracted from raw output images. Trajectory features were extracted from each run that gave measures of the lateral spread of the paw prints, including range and standard deviation in the lateral direction as well as the amplitude and period of a sinusoidal curve fitted to the trajectory of the animal's path (both measured in pixels).

Ex vivo Magnetic Resonance Imaging and Network Connectivity

At P70, kits underwent euthanasia and perfusion fixation with PBS followed by 10% neutral buffered formalin (NBF). Subsequently, the brains were removed and immersion-fixed in NBF for at least a further 72 h, before being rinsed and submerged in PBS at 4°C to rehydrate for 72 h. Brains were then mounted on agarose gel sleds inside 50-mL Falcon tubes and immersed in Fomblin (perfluoropolyether [PFPE]; Solvay Specialty Polymers, GA, USA). Diffusion-weighted magnetic resonance imaging (MRI) data were collected on the Bruker Avance III, 4.7-tesla (200 MHz, ¹H), 20-cm horizontal-bore magnet with ParaVision v6.0.1. The magnet is fitted with custom (Resonance Research, Billerica, MA, USA), 9-cm i.d., high-performance gradients achieving an average of 750 mT/m gradient with a 100-μs slew rate. A TurboRARE 3-dimensional (3D) T2 sequence provided anatomical images using the following settings: field-of-view (FOV) 30 mm, slice thickness 0.23 mm, sagittal-slice orientation matrix with a 1.6-s recycle time and 39-ms echo time accomplished via a rare factor of 16 and acquired into a 128 points per axis cubic volume. Diffusion tensor images (DTIs) were obtained using a 2D collection with the same FOV as the T2. Slice thickness was 0.6 mm for 30 diffusion directions (plus 5 A0 images), and 8 averages for 64 slices (128 × 128) each with a thickness of 0.6 mm. The recycle time was set to 5.6 s with an echo time of 48 ms. Diffusion weighting was set to a 4-ms gradient duration (δ) and 10-ms gradient separation (Δ) with a maximum B value of 4,320 to result in 30 diffusion directions. Diffusion-weighted images were motion- and distortion-corrected using the latest version of FMRIB Software Library (FSL v5.0, Oxford, UK) eddy software (<https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/eddy>). FSL's dtifit was then applied, and the resulting DTI median was filtered using the fslmaths-fmedian option. The resulting output files were fractional anisotropy (FA), L1, L2, L3 (the 3 eigenvalue maps), MD (mean diffusivity), and MO (dti mode) maps. Radial diffusivity was calculated by combining the L2 and L3 maps. Coregistration of the FA maps from all subjects was performed using Tract-Based Spatial Statistics (TBSS). This procedure builds a template from all

subjects and coregisters individual FA maps to the same template. Comparisons of TBSS between groups were threshold-free cluster enhancement (TFCE) adjusted for multiple comparisons. DTI connectivity values were measured using FSL's probtrackx2 software with the network option enabled and using 71 different anatomical seed points and associated regions of interest (ROIs) spread throughout the brain. Matlab "Brain Connectivity Toolbox" (<https://sites.google.com/site/bctnet/construction>) was used to perform the complex network analysis/graph theory analysis, as described by Rubinov and Sporns [18]. The clustering coefficients of the regions were based on the full-brain connectivity network regions thresholded at 10% sparsity. Graph-based network analysis allows for the visualization of connectivity patterns among all the elements of the brain (e.g., brain regions) as well as the quantitative characterization of global organization. The utility of graph-based techniques has been proven by an increasing number of studies probing potential mechanisms involved in normal development [19–21], aging [22, 23], and various brain disorders [24–26].

Immunohistochemistry

After performing MRI, coronal slices at the level of the caudate nucleus were taken from each brain, embedded in paraffin, and 4-μm sections were prepared for hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC). Myelin basic protein (MBP) and oligodendrocyte transcription factor 2 (OLIG2) IHC was performed at the University of Washington Histology and Imaging Core. For MBP, slides were baked for 30 min at 60 °C and deparaffinized on the Leica Bond automated immunostainer (Leica Microsystems, Buffalo Grove, IL, USA). Antigen retrieval was performed by placing slides in EDTA for 20 min at 100 °C. The primary antibody (rat anti-ferret MBP, 1:500 dilution, Abcam, AB7349) in Leica primary antibody diluent was applied for 30 min. A secondary antibody, unconjugated rabbit anti-rat IgG (1:300 + 5% NGS in TBS, Vector, AI-4001) was then applied for 8 min. Goat anti-rabbit horseradish peroxidase Leica Bond polymer was added for 8 min. Antibody complexes were visualized using Leica Bond mixed refine (DAB, 3,3'-diaminobenzidine) detection 2× for 10 min at RT. For OLIG2, staining was performed using rabbit polyclonal anti-OLIG2 (Millipore, cat. No. AB9610.) on formalin-fixed paraffin-embedded sections. Antigen retrieval was performed by placing slides in citrate for 20 min at 100 °C. The primary antibody, OLIG2 (1:500) or rabbit IgG (1:1,000) in Leica primary antibody diluent, was applied for 30 min at RT. In both the MBP and OLIG2 protocols, tissues were counterstained with hematoxylin for 4 min. GFAP IHC was performed at the University of Washington Harborview Medical Center histology IHC lab. For GFAP, staining was performed using rabbit polyclonal antigial fibrillary acidic protein (GFAP; Agilent, Dako) at a dilution of 1:300, using the Leica Bond III IHC stainer, the polymer refine detection kit, and the Bond epitope retrieval 1 solution for 20 min. These antibodies had not previously been optimized in the ferret. Images of representative brain regions were acquired from digitally scanned images or glass slides using NIS-Elements BR 3.2 64-bit and plated in Adobe Photoshop Elements. Image brightness and contrast were adjusted using auto smart fix and/or auto white balance, with manipulations applied to the entire image. The original magnification and/or scale bar is stated.

Fig. 1. Weight gain. On the day of the insult (P10), mean (SD) weight of the kits was 41.4 (8.8) g for males and 38.8 (7.2) g for females. Injured animals lost, on average, 8.5% (10.4%) body weight between P10 and P11 as a result of the insult. By P12, injured animals had gained 9.2% (13.7%) of their P10 body weight, compared to a 24.4% (5.2%) weight gain in controls at P12. By the 6th week of age (P35–P42), injured animals had caught up in terms of weight to control animals, and males began to become heavier than females. No difference in weight between injured and control animals was seen at P70.

Quantitative IHC

Image analysis was performed using whole-slide digital images and automated image analysis. All slides were scanned in bright field with a $\times 20$ objective using a Nanozoomer Digital Pathology slide scanner (Hamamatsu; Bridgewater, NJ, USA). Whole-slide digital images were imported into Visiopharm software (Hoersholm, Denmark) for analysis. The software converted the initial digital imaging into gray-scale values using 2 features, RGB-R with a mean filter of 5×5 pixels and an RGB-B feature. Visiopharm was then trained to label positive staining and the background tissue counterstain using a project-specific configuration based on threshold pixel values. Images were processed in batch mode using this configuration to generate the desired outputs (Ex. area of MBP and ratio of MBP to total tissue area). For quantitative measurements of MBP, OLIG2, and GFAP, the Visiopharm image analysis module was used to define ROIs. ROIs were selected by manually drawing around or placing a circle of equal size within the anatomic structure of interest. ROIs were selected, in part, based on areas noted to be potentially different between treated and control animals on MRI; they included half of the brain, a circular region of the corpus callosum (CC), a circular region of the dorsal internal capsule (IC), a circular level of the ventral IC, and the dorsal cerebrum (online suppl. Fig. 2). As described above, positively stained versus unstained tissue was segmented using a project-specific configuration to generate the desired outputs. After quantitative staining analysis, MBP images were imported into NDP.view2 (Hamamatsu Photonics, Bridgewater, NJ, USA), and the thickness of the CC and 3 levels of the IC (online suppl. Fig. 2) were measured by an operator blinded to the treatment groups.

Statistical Analysis

Statistics and images were generated in GraphPad Prism v7 (San Diego, CA, USA). Early reflex testing was analyzed by comparing the injured and control groups at each time point using a

Wilcoxon-Mann-Whitney U-test. CatWalk data were primarily compared between groups at P42, as visual inspection of the data showed similar results in both groups from P49 to P70. Bonferroni corrections were used to adjust for multiple comparisons in the behavioral testing data. For quantitative IHC analyses, the ratio of positive staining to total tissue area was calculated for each animal, and the median value was calculated by treatment group and sex. Comparisons between injured animals and controls were performed using a Wilcoxon-Mann-Whitney U-test with the Bonferroni correction for multiple comparisons. The outcomes of reflex and catwalk testing were compared using Spearman's rank correlations. Statistical results with a p value < 0.05 were considered statistically significant.

Results

Model Outcomes

Eight litters including 60 kits were initially included, with an insult mortality of 18.3% ($n = 11$). A subset of 15 animals (3 controls and 12 injured) was used for early time point studies to develop the model (data not shown). As a result, 34 animals (14 controls and 20 injured) survived to P70 and were included in the final analysis. The median (range) temperature of 7 nesting sentinel LPS-treated animals 4 h after injection (immediately before hypoxia) was 34.7°C (34.3 – 37.1°C), which was similar to littermate controls (35.1°C , 34.2 – 36.1°C ; $n = 8$). On the day of the insult (P10), mean (SD) weight of the kits was 41.4 (8.8) g for males and 38.8 (7.2) g for females. Injured animals lost, on average, 8.5% (10.4%) body weight be-

Fig. 2. Reflex development. Area under the curve (AUC) analysis for reflex development of negative geotaxis (a), cliff aversion (b), righting reflex (c), and total time across all 3 tests (d). Median (IQR) negative geotaxis was significantly slower to develop in the injured group than in the control group. No difference was seen in the AUC for cliff aversion or righting reflex. However, total time

AUC was significantly greater in the injured group compared to the control group. The pattern of the AUC results suggested a bimodal distribution of injury, with 6/18 animals scoring similarly to control animals, and 12 displaying delayed skill development suggestive of cerebral injury. * $p < 0.05$.

tween P10 and P11 as a result of the insult. By P12, injured animals had gained 9.2% (13.7%) of their P10 body weight versus 24.4% (5.2%) in controls. By week 6 (P35–P42), injured animals had caught up to control animals in terms of weight, and males began to become heavier than females (Fig. 1). No difference in weight between injured and control animals was seen at P70.

Early Reflex Testing

At all ages (P28–P40), median time to complete all tasks (NG, CA, RR, and TT) was longer in the injured group than in controls; however, the variability in scores was high and differences were not consistently statistically significant. In all of the individual reflex tests, and in the TT score, median time was slower in the injured group than in the control group on every test day; how-

ever, no significant difference was seen between groups in any given test at any age.

Analysis of the reflex AUCs showed that NG (median; IQR) was significantly slower to develop in the injured group (552 s • days; 446–660 s • days) than in the control group (397 s • days; 337–507 s • days) ($p = 0.03$; Fig. 2a). No difference was seen in the AUC for CA (Fig. 2b) or RR (Fig. 2c). However, TT AUC was significantly greater in the injured group (1,101 s • days; 663.5–1,338 s • days) than in the control group (656.4 s • days; 572.9–883.1 s • days) ($p = 0.048$). The pattern of the AUC results for CA, RR, and TT suggest a bimodal distribution of injury, with 6/18 animals scoring similarly to control animals (Fig. 2), and 12 displaying delayed skill development suggestive of cerebral injury. No difference between injured male and female animals was seen at any time point.

Fig. 3. Weight-adjusted paw areas. **a** At P42, adjusted area of the hind paws (RH and LH) of injured animals was significantly smaller than that of control animals, with no difference between forepaws (RF and LF). **b** Representative paw prints from the CatWalk

software show that the hind paws of injured animals have a smaller area than the control animals. The red box around the LH print is the same size, for comparison. * $p < 0.05$.

Catwalk

The greatest difference between the groups was seen at P42, with many deficits improving from P49 onwards. On P42, the weight-adjusted area (median; IQR) of the hind paws of injured animals ($1.68 \text{ cm}^2/\text{kg}$; $1.11\text{--}2.64 \text{ cm}^2/\text{kg}$) was significantly smaller than that of control animals ($2.36 \text{ cm}^2/\text{kg}$; $1.88\text{--}3.75 \text{ cm}^2/\text{kg}$) ($p = 0.05$; Fig. 3). Similarly, this resulted in a larger ratio of forepaw and hind paw areas in injured animals (2.34 ; $1.80\text{--}2.73$) than in controls (1.92 ; $1.39\text{--}2.29$) ($p = 0.04$). Intensity of pressure per unit area was also higher in the hind paws of injured animals at P42 ($89.8 \text{ au}/\text{cm}^2$; $53.2\text{--}149.1 \text{ au}/\text{cm}^2$) than in control animals ($50.1 \text{ au}/\text{cm}^2$; $37.9\text{--}81.3 \text{ au}/\text{cm}^2$) ($p = 0.04$; Fig. 4a). At P42, the BOS of the forepaws (relative to hind paws) was significantly wider in injured (0.83 ; $0.67\text{--}1.03$) animals than in control animals (0.62 ; $0.56\text{--}0.76$) ($p = 0.009$; Fig. 4b). However, the wider BOS was not associated with any significant differences in measures of ataxic gait patterns (couplings and phase dispersions; data not shown). Instead, the gait of injured animals was characterized by an inability to consistently maintain forward motion in a straight line (online suppl. Video 1) when compared to controls (online suppl. Video 2). As measurements of lateral movement are not as well-de-

fined in the CatWalk XT software, a custom Python package (FerretFit) was developed to analyze these movements from the paw print images extracted from CatWalk. Regarding paw print range, the lateral spread of prints relative to the midline was not significantly different between groups at any time point. However, the AUC (median; IQR) across the entire testing period was significantly greater in injured animals ($1,786 \text{ pixels} \cdot \text{days}$; $1,713\text{--}1,854 \text{ pixels} \cdot \text{days}$) than in control animals ($1,659 \text{ pixels} \cdot \text{days}$; $1,609\text{--}1,734 \text{ pixels} \cdot \text{days}$) ($p = 0.008$, Fig. 4c). Using the same software package, a sine curve was fitted to the extracted paw prints from each run, and amplitudes were compared. There was a trend towards a greater amplitude (median; IQR) in the injured group ($847.9 \text{ pixels} \cdot \text{days}$; $660.3\text{--}963.7 \text{ pixels} \cdot \text{days}$) than in the control group ($690.5 \text{ pixels} \cdot \text{days}$; $619.9\text{--}854.0 \text{ pixels} \cdot \text{days}$) ($p = 0.1$; Fig. 4d). Figure 5 depicts the method of image processing by the software package. To see whether early reflex performance predicted performance on the catwalk, reflex TT AUC was compared to the catwalk parameters that were significantly different in injured animals at P42. Reflex TT AUC was significantly correlated with P42 adjusted hind paw area ($p = 0.004$, $r = -0.64$), forepaw-to-hind paw area ratio ($p = 0.004$, $r =$

Fig. 4. Gait differences over time. Intensity of pressure per unit area was higher in the hind paws of injured animals than controls at P42 (**a**), and the base of support (BOS) ratio of the forepaws relative to the hind paws was also significantly wider in injured animals (**b**). However, these differences were absent in subsequent

weeks of testing. **c** Using a custom Python package to analyze paw print trajectories, paw print range AUC across the entire testing period was significantly greater in injured animals than in control animals. **d** In the injured group, a trend towards a greater median path amplitude than in the control group was seen. * $p < 0.05$.

0.65), hind paw mean pressure intensity per unit area ($p = 0.003$, $r = 0.66$), but not with BOS. No difference between injured male and female animals was seen at any time point.

MRI and Connectome

Greater FA values were seen in the control group in the right IC dorsolateral to the ventricle at the level of the thalamus (Fig. 6a). On T2-weighted imaging, significantly greater signal intensity was seen in the injured group throughout the white matter bilaterally (Fig. 6b). Network connectivity analysis showed 3/71 ROIs that were significantly different between injured and control animals (Fig. 6c, d). In ROI 3 (the right IC at the level of the mesencephalon), connectivity (mean; standard error) was significantly greater in injured females (0.13 au; 0.015 au) than in control females (0.088 au; 0.018 au) ($p = 0.03$). In the same ROI, connectivity in injured males (0.14 au;

0.010 au) was significantly decreased compared to in control males (0.18 au; 0.007 au) ($p = 0.008$). In ROI 19 (the left IC and associated white matter at the level of the caudate nucleus), connectivity was significantly greater in injured males (0.14 au; 0.006 au) than in control males (0.10 au; 0.009 au) ($p = 0.02$). Similarly, in ROI 20 (the left IC and associated white matter at the level of the caudate nucleus, ventral to ROI 19), connectivity was significantly greater in injured females (0.11 au; 0.003 au) than in control females (0.09 au; 0.002 au) ($p = 0.003$). Volumetric analysis from MRI outputs showed a trend towards greater cerebral volumes in control animals than in injured animals ($p = 0.09$; Fig. 6e). In the 9 injured females, the cerebral volume (median; IQR) was significantly decreased (5.61 cm³; 5.46–6.35 cm³) compared to in the 8 control females (6.53 cm³; 6.23–6.94 cm³) ($p = 0.002$). Cerebral volume was not significantly different in the 10 injured (7.06 cm³; 6.30–8.06 cm³) and 6 control (7.56 cm³;

Fig. 5. FerretFit paw print analysis. **a** For each catwalk run, an image showing every paw print was manually extracted from the CatWalk XT software. Using a specially developed ImageJ macro, the paw print boxes were identified and separated out (**b**) before being analyzed using the FerretFit Python library (**c**) to determine the total range of paw prints, as well as the amplitude of a sine curve that best fit the trajectory of the prints. RF, right forepaw; LF, left forepaw; RH, right hind paw; LH, left hind paw.

7.44–8.00 cm³) males ($p = 0.26$). No difference in cerebral volumes was seen between injured and control animals (data not shown).

Immunohistochemistry

On IHC, MBP staining across an entire hemisphere as well as within the CC, was not significantly different between the 2 groups (data not shown). However, across 2 ROIs within the IC, the MBP staining ratio (median; IQR) was significantly greater and less variable in the injured group (0.92; 0.90–0.9) than in the control group (0.88; 0.84–0.93) ($p = 0.04$). This was particularly evident in male animals (Fig. 7a). The thicknesses of the CC and 3 IC areas at the base of consecutive sulci were then measured. Though no significant differences in thickness were seen within any individual region, a summary score based on the ranked weight-adjusted thickness of all 4 areas (a thinner IC or CC resulted in a lower rank) sug-

gested thinning of the white matter in injured males ($p = 0.026$; Fig. 7b). By comparison, OLIG2 staining intensity was decreased in the CC of injured animals (0.042; 0.032–0.046) compared to control animals (0.048; 0.042–0.053) ($p = 0.033$; Fig. 7c). Across 2 ROIs within the IC, OLIG2 staining ratio was also lower in injured males (0.031; 0.028–0.040) compared to in control males (0.037; 0.030–0.040) ($p < 0.05$), but differences between injured and control females were not significant (Fig. 7d). Figure 8 shows images taken from animals best representing median MBP thickness and OLIG2 staining for both control and injured animals. No differences in GFAP staining intensity were seen either globally or regionally (in the CC, IC, and corona radiata). H&E images at the level of the caudate nucleus and thalamus did not show substantial or consistent abnormalities in control or injured animals.

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(Figure continued on next page.)

Fig. 6. MRI and connectome. **a** Greater fractional anisotropy values were seen in the control group in the right internal capsule (IC) dorsolateral to the ventricle at the level of thalamus (marked in red). **b** On T2-weighted imaging, significantly greater signal intensity was seen in the injured group throughout the white matter bilaterally (marked in blue). **c** Network connectivity analysis showed 3/71 ROIs that were significantly different between injured and control animals. In ROI 3 (right IC at the level of the mesencephalon), connectivity was greater in injured females than in control females. In the same ROI, connectivity in injured males was significantly decreased compared to in control males. In ROI 19 (left IC and associated white matter at the level of the caudate

nucleus), mean connectivity was greater in injured males than in control males. In ROI 20 (left IC and associated white matter at the level of the caudate nucleus, ventral to ROI 19), mean connectivity was greater in injured females than in control females. **d** Overall connectivity projections show control (left panels) and injured (right panels) animals, with points of increased connectivity in controls compared to injured animals (bottom left panel), and increased connectivity in injured compared to control animals (bottom right panel). **e** Cerebral volumes in injured females were significantly decreased compared to control females, but no difference in cerebral volume was seen between injured and control males. * $p < 0.05$.

Fig. 7. Quantitative immunohistochemistry. **a** Within the internal capsule (IC), MBP staining ratio was significantly greater and less variable in the injured group than in the control group. This was particularly evident in male animals. **b** The thickness of the corpus callosum (CC) and 3 areas of the IC at the base of consecutive sulci were then measured, and a summary score based on the

ranked weight-adjusted thickness of all 4 areas suggested thinning of the white matter in injured males. **c** OLIG2 staining intensity was decreased in the CC of injured animals compared to in control animals. **d** OLIG2 staining ratio was also lower in injured males than in control males within the IC. * $p < 0.05$.

Discussion

We present here a novel model of encephalopathy of prematurity in an extremely preterm-equivalent ferret. Compared to control animals, injured ferrets displayed delayed reflex development and early gait characteristics consistent with white-matter injury (WMI). At P70, an early childhood-equivalent age (i.e., roughly equivalent to a child 4–6 years of age), widespread white matter changes were seen on MRI and histology, including altered cerebral network connectivity and evidence of possible dysmaturation on IHC.

There remains a significant need for an expanded repertoire of relevant large-animal models in which to test therapeutic interventions for infants with the neurological sequelae of prematurity. Rodent models often fail to recapitulate the WMI that is pathognomonic of premature brain injury, and few large-animal models of prematurity are available due to the relative brain development of model species at birth [27]. This is particularly evident when comparing the literature on premature brain injury to term hypoxic-ischemic encephalopathy, where a wide range of models allow for the sequential translation of therapies from in vitro work to rodent models and then

Fig. 8. MBP and OLIG2 immunohistochemistry (IHC). Images are taken from animals best representing median MBP thickness and OLIG2 staining for both control and injured animals. Anti-myelin basic protein (MBP) IHC at the level of caudate nucleus shows 2 areas of the internal capsule (IC): IC1 in a control animal (**a**) and an injured animal (**b**), and IC2 in a control (**c**) and treated animal (**d**). $\times 5$. Positive anti-MBP staining (brown); hematoxylin counterstain (blue). Anti-OLIG2 IHC at the level of the IC in a control animal (**e**) and an injured animal (**f**). $\times 20$. Positive anti-OLIG2 staining (brown); hematoxylin counterstain (blue).

large-animal models before their application in clinical trials. This approach led to therapeutic hypothermia becoming the standard of care for infants with hypoxic-ischemic encephalopathy, as well as the development of pharmacological agents such as erythropoietin and xenon [28–32]. Our work in the newborn ferret aims to address some of this gap in the preclinical literature of premature brain injury.

The ferret is an attractive species in which to model premature brain injury due to its altricial nature. At birth, ferret cerebral development is equivalent to the human brain towards the end of the first trimester, with the P10 and P21 ferret equivalent to 24–26 weeks' gestation and term, respectively. This allows for the potential to model premature brain injury along the entire spectrum of ages seen clinically. Additionally, the ferret is amenable to long-term behavioral testing methods similar to those used in rodents [11]. This allows for the analysis of more complex and long-term behavioral outcome data than is available for other larger model animals such as piglets and sheep. However, one interesting aspect of developing preclinical models in the newborn ferret is its relative resistance to brain injury. During the development of the model, it became clear that standard injury mechanisms used in rodents (including presensitization with LPS, hypoxia, hyperoxia, and unilateral carotid artery ligation) did not lead to significant long-term brain injury in ferrets unless applied in specific combinations. Important variables include maintaining core temperature at $\geq 37^{\circ}\text{C}$

during hypoxia, ensuring adequate hypoxia without producing significant intrahypoxic mortality, and moderating the oxygen concentration during hyperoxia; a hyperoxic oxygen concentration of 60% was used due to the fact that prolonged exposure to 80 or 100% oxygen after hypoxia resulted in acute pulmonary edema or hemorrhage. The final combination of LPS presensitization and alternating hypoxia/hyperoxia/hypoxia presented here results in a relatively mild but sustained injury detectable for up to 2 months after the initial injury.

In early reflex testing, injured animals showed slower median reflex times in every test on every day of testing. However, significant variability was seen within both groups, as well within individual animals across testing days, and these differences were not significantly different. Day-to-day variability in the performance of individual animals may be why individual comparisons were not different between groups. By comparison, AUC calculations of reflex development were significant between groups for NG and TT, perhaps because the accumulated differences over time became clear. This difference may have been due to delayed motor development in the injured animals, a delayed ability to learn the tasks, or a combination of the two. As with most small-animal models of neonatal brain injury, and indeed in premature infants seen clinically, there is a certain degree of variability seen in the injury that occurs as a result of the insult. This was particularly noticeable in the early reflex testing, where AUC calculations (as a measure of rate of reflex

development over time) showed a distinct bimodal distribution of injury. Roughly two-thirds of animals displayed delayed reflex development, with the remaining third performing as well as control animals. Overall reflex development (TT AUC) was also significantly correlated with a number of catwalk markers associated with injury, suggesting that early reflex testing may be able to identify injured animals early on.

Based on our experience translating reflex testing to the newborn ferret, some degree of prior stimulation is required for the animal to engage with the tasks. It is likely that highly standardized pretesting procedures, as well as collecting a greater number of parameters during reflex testing, will allow for more granular and consistent differences between injured and control animals. This will form part of our future work with the model as a platform to test potential neuroprotective agents.

Gait assessment showed multiple deficits in the injured animals compared to controls, particularly at the earliest time point (P42). This included a widened base of support in the forepaws, which appeared to be an artifact of an unstable gait characterized by significant lateral movement. At P42, injured animals displayed smaller paw print areas in the hind paws, with the pad of the paw often absent from the visible paw print, both of which are suggestive of the toe-walking gait characteristic of spastic diplegia noted in preterm infants (Fig. 3a) [33]. This is also described in rodent models of WMI [34]. Toe-walking was associated with a greater relative intensity of paw placement in the hind paws during locomotion, as well as greater lateral movement during locomotion (measured as range and amplitude of paw prints) over the entire testing period. Despite the initial differences in gait and paw placement in injured animals at P42, the majority of these deficits disappeared from P49 onwards. This may be due to the fact that the ferret is able to adapt in spite of ongoing macroscopic brain injury. Similar results have been seen in a controlled cortical impact (CCI) model of traumatic brain injury in the adult male ferret [9]. Additionally, as the ferrets remained housed with the littermates during the survival period, natural periods of play within the nest may have constituted some degree of environmental enrichment, which is known to have a neuroprotective benefit [35].

Despite the variability and resolution of certain behavioral deficits, sustained WMI was seen throughout the white matter on both MRI and IHC. Ex vivo MRI showed extensive differences in white-matter signal on T2 imaging, especially in male animals. On IHC, this translated to a greater intensity of MBP staining within the IC, as well

as relative thinning of the white matter in the CC and IC. At the same time, OLIG2 staining within the CC of injured animals, and the IC of injured males, was decreased compared to controls. Overall, this suggests a degree of sexually dimorphic white-matter dysmaturation after the insult. In males particularly, the insult resulted in the loss of OLIG2-positive cells and reduced white-matter thickness. The remaining functional oligodendrocytes then appeared to undergo accelerated myelination, with greater MBP density in the thinned white matter. However, it must be noted that white-matter thickness was only assessed immunohistochemically at a single level (at the caudate nucleus), and minor section-to-section variability during trimming and processing may contribute to variations in the apparent thickness of the white-matter tracts on 2D images. We were also unable to ascertain whether the increased MBP intensity in male animals was associated with normal MBP structure, or the mechanisms underlying the final observed histopathology. Therefore, future studies will include in-depth examination of white-matter structure, including electron microscopy, as well as examination of the time course of the expression of white-matter markers after injury, and how this differs between the sexes.

Interestingly, and consistent with the resilience and plasticity of the ferret brain after injury, connectome changes on MRI included increased connectivity in the IC in both males and females, perhaps as a response to deficits resulting from more widespread WMI. This was particularly evident in injured female animals, where multiple areas of increased connectivity were seen despite smaller overall brain volumes than control female animals. Though the brains of injured female animals showed fewer white-matter abnormalities than injured males, overall cerebral volume was decreased in injured females despite no difference in body weight from control females.

This work does have some additional limitations. Based on our parameters of LPS dosing and hypoxia/hypoxemia exposure, we were unable to determine exactly which factor(s) contributed most significantly to the final injury. However, based on our iterative approach to developing the model, it is likely that all factors played an important role in the final injury observed. We previously showed that LPS exposure results in microglial activation in the P10 ferret brain [11], but LPS alone did not appear to produce lasting injury. LPS exposure in near-term-equivalent rodents resulted in a circulating inflammatory cytokine peak around 4 h after exposure, which corresponds with sensitization of the brain to hypoxia-

ischemia and a significant increase in brain injury [14–16]. A similar time course of inflammatory cytokine release (peak TNF- α and IL-6 release 2–4 h after LPS exposure) was seen in isolated ferret peripheral blood mononuclear cells [17]. This suggests that the ideal timing of LPS exposure for presensitization of the brain is likely to be similar in rodents and ferrets, and this was the final approach used here. However, our data do not allow for the comparison of the effects of hypoxia or hyperoxia alone without inflammatory presensitization.

Similar to the isolated effects of LPS and hypoxia, our work to date has not included multiple iterations of hyperoxia duration. As with any preclinical model, the goal was not to accurately reproduce the exposures encountered by preterm infants clinically, but to provide a confluence of the mechanistic factors thought to be involved in premature brain injury. These include inflammation, hypoxia, and the production of reactive oxygen species [7]. The last of these is exacerbated by hyperoxia, which is particularly problematic in premature infants due to a relatively underdeveloped antioxidative capacity [36, 37]. As such, hyperoxia was included in the protocol to exacerbate the production of reactive oxygen species after a period of inflammatory activation and hypoxia. We used a prolonged period of hyperoxia compared to what might be seen clinically to maximize hyperoxia-induced oxidative stress and increase the likelihood of sustained cerebral injury.

With respect to future use of this model, a greater initial injury severity would be desirable, such that the effects of any therapeutic intervention would be easier to detect in behavioral outcomes as well as on MRI and pathology. Though no difference was seen between the sexes on any behavioral measure at any time point, those seen on MRI suggest that differences may be detectable with larger group sizes, more severe injury, or a greater battery of testing procedures. As with other models of neonatal brain injury, it is also likely that males and females will respond differently to any neuroprotective therapies. Future studies will involve further iteration of the model to increase injury severity, as well as increasing the number of behavioral tests to include cognition and other sensory assessments [9, 38]. Mechanistic exploration of the ferret's resilience to brain injury may also allow for that resilience to be "reverse-engineered" as a way to develop potential therapeutic strategies for human preterm brain injury.

In summary, we present long-term characterization of the behavioral deficits, pathology, and changes in brain structure seen after an inflammation-sensitized hypoxic/

hyperoxic brain injury model in the P10 ferret. This newborn ferret model has the potential to provide an additional platform on which to assess potential therapies for encephalopathy in infants born extremely preterm.

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Statement of Ethics

The authors confirm that all animal experiments were performed in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and the NIH Guide for the Care and Use of Laboratory Animals and part of a protocol approved by the University of Washington Institutional Animal Care and Use Committee.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

TW, DM, KC, PP, and SEJ designed and performed the experiments, and collected and analyzed the data. JSM performed the IHC and pathological assessments. CT prepared samples for MRI and analyzed and interpreted the results. CC and EN developed the software package for paw print analysis. TW drafted and revised the manuscript. All authors contributed to the critical evaluation and revision of the manuscript, and approved the final version.

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