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Tandem insults of prenatal ischemia plus postnatal raised intrathoracic pressure in a novel rat model of encephalopathy of prematurity

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Abstract

Object—Encephalopathy of prematurity (EP) is common in preterm, low birth weight infants who require postnatal mechanical ventilation. The worst types of EP are the hemorrhagic forms, including choroid plexus, germinal matrix, periventricular, and intraventricular hemorrhages. Survivors exhibit life-long cognitive, behavioral, and motor abnormalities. Available preclinical models do not fully recapitulate the salient features of hemorrhagic EP encountered in humans. In this study, the authors evaluated a novel model using rats that featured tandem insults of transient prenatal intrauterine ischemia (IUI) plus transient postnatal raised intrathoracic pressure (RIP).

Methods—Timed-pregnant Wistar rats were anesthetized and underwent laparotomy on embryonic Day 19. Intrauterine ischemia was induced by clamping the uterine and ovarian vasculature for 20 minutes. Natural birth occurred on embryonic Day 22. Six hours after birth, the pups were subjected to an episode of RIP, induced by injecting glycerol (50%, 13 µl/g intraperitoneally). Control groups included naive, sham surgery, and IUI alone. Pathological, histological, and behavioral analyses were performed on pups up to postnatal Day 52.

Results—Compared with controls, pups subjected to IUI+RIP exhibited significant increases in postnatal mortality and hemorrhages in the choroid plexus, germinal matrix, and periventricular tissues as well as intraventricularly. On postnatal Days 35–52, they exhibited significant abnormalities involving complex vestibulomotor function and rapid spatial learning. On postnatal Day 52, the brain and body mass were significantly reduced.

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Disclosure

Author contributions to the study and manuscript preparation include the following. Conception and design: Simard. Acquisition of data: Koltz, Tosun, Kurland, Coksaygan, Ivanova. Analysis and interpretation of data: Simard, Koltz, Tosun, Kurland, Castellani, Ivanova, Gerzanich. Drafting the article: Simard, Koltz, Tosun, Kurland. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Simard. Statistical analysis: Simard, Kurland, Gerzanich. Administrative/technical/material support: Simard.

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Conclusions—Tandem insults of IUI plus postnatal RIP recapitulate many features of the hemorrhagic forms of EP found in humans, suggesting that these insults in combination may play important roles in pathogenesis.

Keywords

encephalopathy of prematurity; germinal matrix hemorrhage; choroid plexus hemorrhage; intraventricular hemorrhage; periventricular leukomalacia; rat model; vascular disorders

Encephalopathy of prematurity encompasses a broad constellation of nonhemorrhagic and hemorrhagic brain lesions encountered in preterm infants, including cystic and noncystic periventricular leukomalacia, and choroid plexus, germinal matrix, and periventricular hemorrhages that may extend intraventricularly, as well as neuronal and axonal pathology and hydrocephalus.^{4,29,52–54} The sequelae of EP are due not only to pathological tissue destruction but also to failed or aberrant development of selectively vulnerable progenitor cells.⁵³ Survivors of EP exhibit a wide range of cognitive, behavioral, and motor abnormalities that persist for life, at immeasurable cost to patients and society.^{1,9,11,18}

Arguably, the worst forms of EP are those that involve brain hemorrhages (choroid plexus, germinal matrix, periventricular, and intraventricular hemorrhages), as well as periventricular venous infarctions. Hemorrhagic lesions are closely associated with the degree of prematurity and lead to qualitatively poorer long-term outcomes.^{33,41,47} Hemorrhagic lesions have become more prevalent as the mean age of survivors of preterm birth has declined.⁵³ Hemorrhages not only destroy tissues directly, but they initiate an insidious inflammatory response that causes widespread “bystander” damage, especially to white matter. The principal factor determining outcome in these patients is the magnitude of the intracranial hemorrhage.^{33,41}

A major perinatal factor predisposing to EP is acute or intermittent episodes of hypoxia/ischemia.²⁸ Mild to severe hypoxia/ischemia may occur prior to birth due to maternal or fetal factors, as well as postnatally due to the abnormal pulmonary function (immature lungs, respiratory distress syndrome, or pneumonia) typically found in premature infants. Not only are the cells that are located periventricularly selectively vulnerable to insult,^{5,35} but also their location places them at additional risk, since this region is farthest downstream from vascular sources and there is no collateral blood supply.

Other factors predispose to the development of hemorrhagic lesions, among which transient episodes of RIP may be particularly important.^{27,30} Most periventricular hemorrhages occur postnatally, and the onset of bleeding often corresponds to the occurrence of hypoxia and respiratory distress requiring mechanical ventilation.^{25–27} Mechanical ventilation raises intrathoracic pressure, which is transmitted via thoracic veins directly to cerebral veins via valveless jugular veins. Veins are the source of most periventricular hemorrhages.^{24,37} Among cerebral veins, those located periventricularly are especially fragile due to high angiogenic activity.^{3,7} In addition, the innately fragile periventricular veins may be further weakened by prenatal or postnatal hypoxia/ischemia.⁵⁰ Thus, it is hypothesized that postnatal episodes of RIP can result in rupture of weak periventricular veins, leading to choroid plexus, germinal matrix, and periventricular hemorrhages, which can expand into intraventricular hemorrhages or can compromise venous outflow, leading to venous infarction.

Animal models of periventricular hemorrhage have been reported, but none captures the salient features of prenatal hypoxia/ischemia coupled with postnatal RIP, and none fully recapitulates the spectrum of neuropathological findings observed in neonates with

hemorrhagic forms of EP.^{6,20} Fortuitously, the newborn rat brain is developmentally comparable to the 24–26-week gestational age human brain and, as in preterm infants at the end of the second trimester, neurogenesis is complete in most regions.⁶ Here, we report that a rat model that recreates the common clinical presentation of transient global ischemia in utero followed postnatally by transient RIP faithfully recapitulates many of the pathological features encountered in infants with hemorrhagic forms of EP, and leads to developmental delay and abnormalities in complex vestibulomotor and learning paradigms. Our findings are consistent with the hypothesis that these insults in tandem may play important roles in pathogenesis.

Methods

Transient IUI

Animal experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore. Timed-pregnant Wistar dams were obtained from Harlan Laboratories (embryonic Day 1 corresponds to the day of sperm-positivity following overnight mating). Pregnant dams underwent laparotomy on embryonic Day 19. Dams were anesthetized with 3% isoflurane delivered with 75% air + 25% O₂; anesthesia was maintained with 2% isoflurane for the duration of surgery. Pulse oximetry (Mouse-Ox; STARR Life Sciences Corp.) was used to maintain an O₂ saturation of 90%–95%. A heating pad was placed beneath the dam to maintain body temperature at approximately 37°C. A laparotomy was made, the uterus was externalized, and the uterine and ovarian vasculature was clamped for 20–60 minutes to induce transient IUI, as described,² using vascular clamps with low closing pressure (5–15 g/mm², Fine Science Tools). Care was taken to clamp both the uterine and ovarian vasculature bilaterally, to ensure global ischemia in all pups (for more detail, please refer to the figure on page 125 of the book by Cook¹⁴). Laser Doppler flowmetry confirmed the reduction in blood flow (Fig. 1 upper). The uterus was reinternalized, the abdominal incision was closed, and the dam was allowed to recover from anesthesia. Controls included dams left untouched (naive) or dams subjected to sham surgery, which consisted of anesthesia and laparotomy without manipulation of the uterus. The duration of anesthesia was approximately 35 minutes in all cases.

Spontaneous, unaided vaginal delivery usually occurred 2–3 days later (embryonic Days 21–22) after no intrauterine insult (naive), sham insult, or IUI; the day of birth is defined as postnatal Day 0. Newborn pups were allowed 6 hours undisturbed to bond with the mother. At 6 hours, the pups were assigned randomly to receive either an injection of 50% glycerol (13 µl/g intraperitoneally) to cause RIP (see *Results*), or no second insult. Care was taken to minimize the time that pups were removed from the mother. The various insults to which the pups were subjected are listed in Table 1.

Experimental Series

The effects of the various insults were assessed in 2 series of pups. In Series 1, surviving pups were killed on postnatal Day 1, approximately 30 hours after birth, to determine the incidence and severity of intracranial hemorrhages. These pups were not studied behaviorally and were not counted in the analysis of mortality. In Series 2, pups were assessed for mortality (see below) in response to the various insults, and surviving pups underwent extensive neurobehavioral testing (see below) between postnatal Days 1 and 49. The sequence of interventions and tests implemented for pups in Series 2 is depicted in Fig. 1 lower.

Measurement of Intrathoracic Pressure

Mice (C57/b6) were anesthetized (ketamine, 100 mg/kg, plus xylene, 10 mg/kg, intraperitoneally) and, using microscopic technique, the probe (1 mm in diameter) of the pressure transducer was introduced into the pleural cavity. Intrathoracic pressure was measured using a factory-calibrated system (model PA-C10 PhysioTel Transmitter, model RPC-1 PhysioTel Receiver, and Dataquest A.R.T. system for acquisition and analysis; Data Sciences International).

Scoring Brain Hemorrhage

Coronal sections of postnatal Day 1 brains stained with H & E were used to determine the incidence, location, and magnitude of brain hemorrhages. An unbiased scoring system was used for grading hemorrhages as follows: 1) One point was assigned for hemorrhage in any of the following 3 locations: choroid plexus, and intraventricular and periventricular regions, with no more than one point per location being scored. 2) One point was scored if hemorrhage could be observed grossly during cryosectioning. Thus, a score of 0–4 was assigned for each pup. Data were collected by 3 blinded and independent investigators, with the final score being the average of the 3 investigators.

Mortality

Mortality was measured during the first 48 hours after birth; pups that survived this initial period rarely died later. For the groups subjected to a postnatal insult, mortality was based on the number of live pups subjected to the insult. For pups exposed to IUI alone (IUI-N group), deaths were also counted as the difference between the number of fetuses observed at laparotomy and the number of pups observed after birth, a method necessitated by the fact that the dam often ate her sick or dead pups during unsupervised hours after giving birth. For pups without in utero injury (N-N group) and those exposed to sham injury in utero (S-N group), in utero counts were not made, and only observed postnatal deaths were counted.

Tonic and Postural Reflexes

Postnatal activity was assessed using a combination of well-characterized tests.¹⁹ We tested for developmental milestones of forelimb placement in response to a noxious stimulus, response to negative geotropism, and ability of a pup to roll from its back onto its abdomen (righting reflex). The pups were scored 0–3 for each test, based on performance. These tests were carried out on postnatal Days 1, 3, 7, and 14.

Motor Assessment

Vestibulomotor function was tested using a beam (1.5 cm × 2 m, 30 cm above the floor) balance task⁵⁵ and using the Rotarod (Series 8, IITC Life Science). On postnatal Day 28, the rats were tested on the apparatus at a constant speed of 6 rpm for 3 separate trials (maximum trial time 180 seconds). On postnatal Day 35, animals were tested using an accelerating protocol (starting with 4 rpm and accelerating to 45 rpm in 100 seconds); they were allowed to stay on for a maximum of 180 seconds. For both paradigms, the latency to fall off the wheel was recorded for each trial.

Morris Water Maze Testing

The MWM was used to assess spatial learning and memory.^{15,36} The device and the specific paradigms used for MWM testing in this laboratory have been described.⁴⁰ As an index of open-space anxiety-related behavior, we also analyzed thigmotaxis. A rat was considered to be in thigmotaxis if it swam at a distance within 10 cm of the wall of the pool. For each cohort, the time spent in thigmotaxis was calculated for the first trial of acquisition learning in the MWM.

Immunolabeling

Pups (postnatal Day 1) were processed to minimize freeze artifact.⁴⁵ Brains were perfusion-fixed with 10% neutral buffered formalin. After fixation, brains were cryoprotected using 30% sucrose in PBS for 48 hours at 4°C, or were placed into 70% ethanol prior to paraffin embedding.

Cryosections (10 µm) were collected on slides, blocked in 5% goat serum with 0.2% Triton X-100 in PBS for 1 hour, then incubated overnight with primary antibodies directed against ED-1 (CD68) (dilution 1:500, MAB1435), cleaved caspase-3 (dilution 1:200, Cell Signaling Technology), or laminin (dilution 1:200; FA-2404-1, EY Laboratories, Inc.) at 4°C. Sections were washed 3 times in PBS, then incubated in the dark with the appropriate fluorescent-labeled secondary antibodies (dilution 1:500; goat anti-mouse AlexaFluor555 and goat anti-rabbit AlexaFluor555, Invitrogen) or, for double-labeling for erythrocytes, with an anti-erythrocyte antibody (dilution 1:400, 88R-M001; Fitzgerald Industries International). Slides were washed and coverslipped with Prolong Antifade reagent with DAPI (Molecular Probes, Invitrogen). TUNEL staining was performed using an in situ cell death detection kit (Roche, Applied Science) according to the manufacturer's instructions. Control experiments involved omission of the primary antibody. Sections were examined using epifluorescence microscopy, and images were captured using a CoolSNAP camera (Photometrics).

To double label for laminin and alkaline phosphatase, 40-µm floating cryosections were incubated in 0.3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. After 3 washes in PBS, sections were blocked as above and incubated overnight with primary antibody against laminin (dilution 1:20,000). Sections were washed in PBS and incubated with biotinylated secondary antibody (BA-1000 dilution 1:500 goat anti-rabbit, Vector Laboratories) for 2 hours. After washing in PBS, sections were incubated in avidin-biotin solution (Vector Laboratories), and the color was developed in diaminobenzidine chromogen solution (0.02% diaminobenzidine in 0.175 M sodium acetate) activated with 0.01% hydrogen peroxide. Sections were rinsed in PBS before proceeding to the alkaline phosphatase reaction, as described.²¹ Sections were rinsed, mounted, dehydrated, and cover-slipped with DPX mounting medium (Electron Microscopy Services).

Quantitative Immunohistochemical Analysis

Manual cell counting was carried out in the CA3 region and hilus of the hippocampus separately. Unbiased measurements of signal intensity within ROIs were obtained using NIS-Elements AR software (Nikon Instruments) from sections immunolabeled in a single batch, as previously described.^{23,40} All ROI images for a given signal were captured using uniform parameters of magnification, area, exposure, and gain. For each ROI (411.8 × 329.4 µm), we examined 3 noncontiguous sections (anterior, posterior, and midway) containing supra- and infrapyramidal limbs of the hippocampal granule cell layer in the corner of the field for dentate gyrus, and the hippocampal CA3b pyramidal cell layer in the corner of the field for CA3. Specific labeling was defined as pixels with signal intensity greater than 2 times that of background. For TUNEL, the number of positive nuclei with specific labeling in the granule or pyramidal cell layer of the ROI were manually counted with the use of Adobe Photoshop software (Adobe Systems) and a cell counter for precision. For cleaved caspase-3, the number of cells with specific labeling in the granule or pyramidal cell layer of the ROI were manually counted.

Data Analysis

Nonparametric data sets were rank-transformed prior to analysis.¹³ Statistical analysis was performed using a 1-way ANOVA with Bonferroni post hoc comparisons. Mortality data

were analyzed using a 2×2 contingency table and Fisher exact test (2-tailed). Significance was accepted at $p < 0.05$.

Results

Model Development

In Utero Ischemia—We first determined the duration of IUI (performed on embryonic Day 19) that would result in an appropriately low mortality rate in rat pups born naturally 2–3 days later. We evaluated the effect of 20, 40, and 60 minutes of IUI (Fig. 2 upper). Unmanipulated naive (N-N group) and sham-injured (S-N group) pups had 0% (0 deaths in 28 pups) and 2% (1 death in 47 pups) mortality rates, respectively. Pups exposed to 20, 40, and 60 minutes of IUI had mortality rates of 6.8% (7 deaths in 102 pups), 50% (5 deaths in 10 pups), and 100% (12 deaths in 12 pups), respectively. Since 20 minutes of IUI on embryonic Day 19 yielded the most acceptable mortality rate, this duration of IUI was used in all subsequent experiments.

Transient Postnatal RIP—Administration of glycerol (50%; 13 μ l/g intraperitoneally) to premature rabbits results in germinal matrix hemorrhages^{12,22} by a mechanism that has not been fully characterized. We hypothesized that intraperitoneal injection of an osmotically active substance would increase intraabdominal fluid volume, resulting in upward displacement of the diaphragm and raised intrathoracic pressure. Direct assessment of this hypothesis was not feasible in newborn rat pups because, in our hands, newborn (postnatal Day 0) pups were too frail to readily withstand anesthesia and surgery. Instead, we studied this question in young mice of the same mass (approximately 5 g) which, because they were older (approximately postnatal Day 10), were more tolerant to anesthesia and surgery. Mice were anesthetized, and the probe of a pressure transducer was introduced into the pleural cavity to measure intrathoracic pressure. Injection of 50% glycerol, 13 μ l/g intraperitoneally, resulted in an increase in pressure of approximately 30 mm Hg, which did not occur with sham injection (Fig. 2 lower).

We evaluated the effect of the same dose of glycerol, injected 6 hours after birth, in rat pups subjected to 20 minutes of IUI. Examination of the brains 24 hours after the glycerol injection revealed frequent hemorrhages (Fig. 3). To determine the source of the hemorrhages, cryosections were double labeled for laminin, which identifies both arterioles and venules, and for alkaline phosphatase, which stains only arterioles, not venules.^{3,24} In the cortex, where a regular pattern of penetrating arteriole alternating with a penetrating venule makes it easiest to see, hemorrhages were located at the terminus of laminin-positive, alkaline phosphatase-negative venules that were interrupted, and away from or between alkaline phosphatase-positive arterioles that were continuous (Fig. 3A and B). In other regions as well, hemorrhages were located near laminin-positive structures, away from alkaline phosphatase-positive structures (Fig. 3C and D). Intraparenchymal, subependymal, and intraventricular hemorrhages near laminin-containing structures were confirmed using an antibody directed against erythrocytes (Fig. 3E and F).

Pups with tandem insults (20 minutes IUI plus postnatal transient RIP) exhibited hemorrhages in the choroid plexus, intraventricularly as well as in periventricular tissues, including the subventricular zone, which is analogous to the human germinal matrix, and in white matter such as the internal capsule and corpus callosum (Fig. 4A–C).

Hemorrhage into the brain incites an endogenous inflammatory response that includes widespread activation of microglia.⁵⁶ To confirm an inflammatory response to extravasated blood at this early age, we immunolabeled tissues for ED1, a marker of activated microglia and of macrophages.³⁹ At 24 hours after the second insult, areas of hemorrhage were

surrounded by ED-1-positive cells, consistent with a robust inflammatory response (Fig. 4D).

We assessed the incidence and severity of brain hemorrhages in pups with tandem insults (IUI+RIP group) versus a variety of controls, including pups with no insult (N-N group) or with sham prenatal insults (S-N group), IUI alone and no postnatal insult (IUI-N group), and sham prenatal insult plus postnatal glycerol injection (S-RIP group). Hematoxylin and eosin-stained coronal sections were scored by 3 blinded investigators.

No brain hemorrhages were identified in pups from groups without frank insult (N-N group, 11 rats; or S-N group, 5 rats) or in pups with a postnatal insult alone (S-RIP group, 5 pups) (Fig. 4E). Several pups with prenatal insult alone (IUI-N group, 14 pups) exhibited mild hemorrhage, but scores were not statistically different from controls (Fig. 4E). By contrast, the majority of pups exposed to tandem insults (IUI+RIP group, 16 pups) exhibited moderate-to-severe hemorrhages, with hemorrhage scores that were significantly different from those in other groups (Fig. 4E).

The anatomical distribution of the hemorrhages resulting from tandem insults (IUI+RIP group; 16 pups) is shown in Fig. 4F, demonstrating a predominant occurrence in the choroid plexus, as well as intraventricularly and periventricularly. Together, these data suggest that the tandem insults of prenatal ischemia of modest duration, combined with postnatal RIP, recapitulated in the rat many of the key pathological features of hemorrhagic forms of EP encountered in humans.

Mortality

Pups exposed to tandem insults had a 48-hour mortality rate of 16.7% (IUI+RIP group; 5 deaths in 30 pups), which was significantly different from the rate in naive and sham pups (Fig. 2A). The rate with tandem insults (16.7%) was twice the rate with 20-minute IUI alone (6.8%; IUI-N group; 7 deaths in 102 pups), although this difference did not reach statistical significance ($p = 0.1$).

Hippocampus

The brains of surviving pups in 5 insult groups (N-N, S-N, S-RIP, IUI-N, and IUI+RIP) were studied on postnatal Day 1 with specific focus on the hippocampus. In pups subjected to tandem insults (IUI+RIP), considerable cell injury was evident in dentate and CA3 regions of the hippocampus, with the cell death markers, TUNEL and cleaved (activated) caspase-3, both being much more prominent than in any other group (Figs. 5A–D and 6A–D). The number of cells with TUNEL staining and cleaved caspase-3 labeling in the 2 hippocampal regions was quantified in various injury groups, which showed that the most prominent elevations were in the CA3 region of the hippocampus following tandem insults (Figs. 5E and 6E).

Basic Vestibulomotor Tests

On postnatal Day 1 and continuing through postnatal Day 14, pups in 3 groups (N-N, IUI-N and IUI+RIP; 11, 16, and 16 pups, respectively) were evaluated using a battery of 3 well-established tests to assess for the development of vestibulomotor reflexes, including forelimb placement in response to a noxious stimulus, response to negative geotropism, and ability of a pup to roll from its back onto its abdomen (righting reflex). In all 3 tests, uninjured pups (N-N group) and all surviving pups from the IUI-N and IUI+RIP groups reached maximum scores by postnatal Day 14, indicating no overt deficits by this time. On each test, a transient deficit or a delay in performance achievement was observed on

postnatal Day 3 in pups from the IUI-N and IUI+RIP groups; however, only the data for the righting reflex in pups from the IUI+RIP group reached statistical significance (Fig. 7A).

On postnatal Day 28, rats in 3 groups (N-N, IUI-N, and IUI+RIP; 21, 16, and 28 rats, respectively) were tested using a Rotarod to evaluate basic vestibulomotor performance. Rats were tested using a constant speed protocol (6 rpm) on 3 successive trials. All rats, regardless of group, were able to remain on the apparatus for the maximum time of 180 seconds in at least 1 of the 3 trials. Statistical analysis indicated that performance was not different between groups (Fig. 7B).

On postnatal Day 35, rats in 3 groups (N-N, IUI-N, and IUI+RIP; 11, 16, and 9 rats, respectively) were evaluated on the beam balance task. Both groups that had sustained IUI performed significantly worse than controls (Fig. 7C).

Complex Neurobehavioral and Motor Tasks

On postnatal Day 35, rats in 3 groups (N-N, IUI-N, and IUI+RIP; 21, 16, and 28 rats, respectively) were tested using an accelerating Rotarod protocol, which is more challenging than the constant speed protocol used on postnatal Day 28. Each rat was tested in 3 separate trials, with the best time taken for statistical analysis. Rats from the IUI+RIP group performed poorly compared with rats from the N-N or IUI-N groups. Rats from the N-N and the IUI-N groups were able to remain on the apparatus for 61.9 ± 5.6 and 52.75 ± 3.5 seconds, respectively, whereas rats from the IUI+RIP group remained on for only 38.5 ± 2.9 seconds before falling off (Fig. 7D). This finding is consistent with tandem insults resulting in a deficit in performance in a complex vestibulomotor task.

On postnatal Day 35, rats in 3 groups (N-N, IUI-N, and IUI+RIP; 21, 16, and 28 rats, respectively) began testing in the MWM. During vision testing, there were no differences in average swimming speed between groups ($p > 0.05$; data not shown). All rats eventually reached the visible platform. However, during the first trial of vision testing, there was a nonsignificant trend for rats in the IUI+RIP group to take longer to reach the platform, compared with rats in the N-N group (Fig. 8A). The difference in latencies to reach the target during this trial was accounted for by a significantly longer time spent swimming around the periphery of the pool, that is, thigmotaxis (Fig. 8B). Half of the rats (14 of 28) in the IUI+RIP group spent more than 60 seconds in thigmotaxis during Trial 1 of vision testing, compared with 4 of the 16 and 1 of 11 of the rats in the IUI-N and N-N groups, respectively. The high incidence of thigmotaxis is consistent with tandem insults producing an abnormal state of anxiety.⁴⁹

After the vision test, the rats were trained to find a hidden platform kept in a constant location. No differences were observed during the incremental learning period, and rats in all groups were able to learn the location of the platform, with performance reaching steady-state for all rats by Day 3 (Fig. 8C). On postnatal Day 42, 1 day following the fifth and final day of training, a memory probe was performed in each rat, and the total time spent in the correct quadrant, the one that had contained the platform, was measured. Rats in all groups exhibited the correct preference, spending longer than chance (25% of the time, over 60 seconds) in the quadrant that had contained the platform (Fig. 8D). These data suggested that the ability for incremental acquisition of spatial memory during successive trials was intact, even in rats that had been subjected to tandem insults.

On postnatal Day 49, an additional MWM experiment was used to test a rat's ability to learn rapidly a new platform location, which is a hippocampus-specific task.^{31,32,34,38} During the rapid learning task, each rat was given a single acquisition trial, which was followed by a Memory Probe after a 30-minute interval. For this experiment, the percentage time spent in

the correct quadrant was calculated for the first 30 seconds of the memory probe because after this, rats tended to give up in their search. Rats from both the N-N and the IUI-N groups showed a preference for the new quadrant (more than 25% of the first 30 seconds), whereas rats from the IUI+RIP group showed no such preference (Fig. 8E and F). This finding is consistent with tandem insults producing a deficit in hippocampus-specific rapid spatial acquisition or spatial working memory.

Body and Brain Mass

To characterize the long-term growth and development of rats subjected to different insults (N-N, IUI-N, and IUI+RIP groups), we measured body mass from birth until the time of euthanasia on postnatal Day 52. Between postnatal Days 1 and 21, the percentage gain in body mass was similar between all groups. After this time, values for the IUI-N and IUI+RIP groups diverged from those of the N-N group (Fig. 9 upper). Body and brain mass were significantly less at the time of euthanasia in rats in the IUI+RIP group, compared with other groups (Fig. 9 lower).

Discussion

The principal finding of the present study is that tandem perinatal insults consisting of a late-term intrauterine ischemic event of moderate severity combined with an early postnatal episode of raised intrathoracic pressure recapitulated salient features of the hemorrhagic forms of EP encountered in premature infants. By itself, IUI of 20 minutes was associated with low mortality, modest pathological findings, and modest neurobehavioral deficits. Similarly, in rats without in utero ischemia, a postnatal event of RIP was harmless. However, in combination, these modest insults resulted in numerous, clinically relevant abnormalities, including choroid plexus, periventricular, and intraventricular hemorrhages; elevated mortality; developmental abnormalities in complex, but not in simple, vestibulomotor and spatial learning tasks; and decreased brain and body mass. Faithful replication of many features of the human condition by the tandem insults in this animal model is consistent with the hypothesis that one or more episodes of ischemia of moderate severity, combined with one or more episodes of RIP several days later, may play critical roles in hemorrhagic forms of encephalopathy of prematurity encountered in preterm low birth weight infants.

Several animal models have been described that reproduce different aspects of the pathophysiology encountered in human EP, including models with hypoxia/ischemia alone and models with germinal matrix or intraventricular hemorrhage. Models with hypoxia/ischemia alone may deliver insults of varying severity at different times prenatally and postnatally. Rat models in which transient global ischemia is induced prenatally have been well characterized,^{10,46,51} as have models in which the hypoxia/ischemia insult is delivered postnatally.^{17,42,48} Of the latter type, the Rice-Vannucci model,⁴⁴ wherein neonatal rats undergo common carotid artery ligation (unilateral or bilateral) combined with hypoxia, has been extensively studied. In addition, several models of intraventricular hemorrhage have been described,⁶ including a model of germinal matrix hemorrhage in which rabbit fetuses that are delivered prematurely undergo injection of glycerol intraperitoneally.^{6-8,22} By emphasizing different aspects of pathophysiology, these various models have greatly advanced our understanding of the etiology and pathophysiology of EP.

Although much attention rightfully has been directed to damage caused by hypoxia/ischemia, brain injury also may be associated with mechanical ventilation.²⁷ Infants who require ventilation due to prematurity are at risk for fluctuations in arterial blood pressure and impairments of cardiac output, cerebral oxygenation, and blood flow.^{53,54} Moreover, ventilating poorly compliant (“stiff”) immature lungs may require high inspiratory pressures,

resulting in RIP that can be transmitted to cerebral veins. Most periventricular hemorrhages occur postnatally, with the onset of bleeding often corresponding to the onset of mechanical ventilation.^{25–27} Veins are the source of most periventricular hemorrhages,^{24,37} and periventricular veins are especially fragile due to high angiogenic activity.^{3,7} We previously hypothesized that elevated venous pressures transmitted from the thorax may cause veins or venules in the brain to rupture, especially if they were weakened by a preceding insult such as ischemia.⁵⁰ This hypothesis is supported by our findings in the present study. The tandem insult model that we describe demonstrates how a single episode of RIP occurring several hours after birth can be completely innocuous in the context of a normal pregnancy, but can result in devastating hemorrhagic lesions when preceded by IUI of modest duration 2–3 days previously. In addition, we provide data demonstrating that intraparenchymal venules, not arterioles, are the source of bleeding in the context of RIP. This finding points to a vulnerability of cerebral venules to hypoxia/ischemia during gestation that heretofore may not have been appreciated.

Hemorrhagic forms of EP include choroid plexus, germinal matrix, and intraventricular hemorrhages. Intraventricular hemorrhages are commonly ascribed to germinal matrix bleeding, but it has long been recognized that intraventricular hemorrhage also may be due to bleeding of the choroid plexus.^{4,43} One important observation to emerge from the tandem insult model described here is that the choroid plexus frequently demonstrated considerable hemorrhage with substantial intraventricular extension, reminiscent of sonographic and autopsy findings in infants with intraventricular hemorrhage.^{4,43}

The mortality observed in our tandem insult model mimicked observations in humans with EP. Pups exposed to tandem insults of ischemia on embryonic Day 19 and postnatal RIP on postnatal Day 0 (equivalent to infants of 24–26 weeks of gestational age⁶) had a 16% mortality rate, double the rate for pups exposed to IUI alone (7%). The mortality rate with tandem insults was similar to the rate of 15%–25% in infants with hemorrhagic forms of EP.^{53,54}

Rat pups that survived tandem insults exhibited impairments in growth and in early sensorimotor reflex development. Basic vestibulomotor function was relatively normal, as indicated by performance on the Rotarod task with constant speed. However, when task complexity was augmented using an accelerating protocol, deficiencies became apparent that distinguished the tandem insult group from IUI alone and from controls. Similarly, deficiencies in spatial learning became evident principally when task complexity was augmented. Morris water maze testing of 3 groups (naive, IUI only, tandem insult) of young rats showed that the mean speed and latency to find the visible platform were similar, and that all were able to learn the location of a hidden platform based on external cues. A single previous study documented only minor impairments in spatial memory tasks in rats subjected to somewhat longer prenatal ischemia (30 minutes at embryonic Day 17).¹⁰ However, there were 2 aspects in the MWM that distinguished rats that had been subjected to tandem perinatal insults. First, they spent significantly more time in thigmotaxic behavior when they were first placed into the pool, consistent with open-space anxiety.⁴⁹ Secondly, they showed significantly worse performance in rapid/reversal learning, indicating that they were not capable of quickly and efficiently learning a new task.¹⁶ Deficiencies in rapid/reversal learning reflect hippocampal injury specifically,^{31,32,34,38} which accords with our findings of a high incidence of cell death in CA3 and dentate regions of the hippocampus. In general, our observations on deficiencies in complex but not in simple vestibulomotor and spatial learning tasks are consistent with many of the abnormalities observed in the infants with EP.^{1,11,18,47,57}

Conclusions

Tandem perinatal insults consisting of IUI of moderate severity combined with a postnatal episode of RIP recapitulated salient features of the hemorrhagic forms of EP encountered in premature infants, including choroid plexus, periventricular, and intraventricular hemorrhages, and elevated mortality. Especially noteworthy were findings of decreased brain and body mass, cell death in regions such as hippocampus that are critical to learning, and abnormalities in complex vestibulomotor and cognitive functions. Future studies will be needed to elucidate specific cellular and molecular mechanisms of injury that render cerebral venules so vulnerable to ischemic injury and so susceptible to rupture after raised venous pressures during the perinatal period. We anticipate that this model of tandem insults will promote studies that will reveal novel therapeutic targets that could be inhibited safely in infants at risk for EP, to reduce the incidence and life-long impact of this devastating condition.

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Abbreviations used in this paper

EP	encephalopathy of prematurity
IUI	intrauterine ischemia
MWM	Morris water maze
PBS	phosphate-buffered saline
RIP	raised intrathoracic pressure
ROI	region of interest

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Fig. 1.

Upper: Laser Doppler flowmetry signal obtained from the externalized pregnant uterus prior to and during IUI. The *asterisk* indicates the time of initial clip placement; note the rapid return of flow when the clips are momentarily released. **Lower:** The sequence and timing of the experimental protocols for rat fetuses and pups in Series 2. Accel. = accelerating; AU = arbitrary units; Const. = constant; E = embryonic Day; P = postnatal Day.

Fig. 2.

Mortality induced by various perinatal insults and RIP. **Upper:** Fetuses were subjected to IUI lasting 20 minutes (in 102 pups), 40 minutes (in 10 pups), or 60 minutes (in 12 pups) alone, or 20-minute IUI plus RIP on postnatal Day 1 (30 pups); naive (N-N) and sham (S-N) cohorts consisted of 28 and 47 pups, respectively; mortality with tandem insult was significantly different compared with naive and sham (** $p < 0.01$). **Bold labels** on the x axis indicate the conditions that were used for the remainder of the study. **Lower:** Intrathoracic pressure (mean $\pm$ SE) following sham injection (*squares*) or injection of glycerol (50%; 13

μl/g intraperitoneally) (*circles*) given at the time indicated (*arrow*), measurements were made in 4 mice, approximately 5 g, on postnatal Day 10.

Fig. 3.

Venules are the source of hemorrhage. **A–D:** Cryosections immunolabeled for laminin (*brown*), which identifies both arterioles and venules, and stained for alkaline phosphatase (*blue*), which labels only arterioles, showing hemorrhages in the cortex (A and B), internal (int.) capsule (C), and hippocampus (D) located near laminin-positive venules (*arrows*), away from alkaline phosphatase-positive arterioles. **E and F:** Cryosections immunolabeled for laminin (*yellow*) and for erythrocytes (*red*); note extravasated erythrocytes near laminin-positive vascular structures in the internal capsule (E), and in subependymal and intraventricular locations (F). All experiments were performed in pups transcardially

perfused to remove intravascular blood; the data shown are representative of findings in 6 pups for each type of labeling. Bar = 100 μ m (A–D) and 50 μ m (E and F).

Fig. 4.

Brain hemorrhages induced by tandem insults. **A–C:** Hematoxylin and eosin-stained coronal brain sections demonstrating choroid plexus and intraventricular hemorrhages (A), and periventricular hemorrhages in the subventricular zone (SVZ) (B) and an intraparenchymal hemorrhage (*asterisk*) in the thalamus adjacent to the internal capsule (C). **D:** Immunolabeling for ED-1 (*red*), showing activated microglia surrounding a petechial hemorrhage (*asterisk*); staining for TUNEL is also shown (*green*); nuclei labeled with DAPI (*blue*); same section as panel C. **E:** Hemorrhage scores (see *Methods* for definitions) for individual pups (*black triangles*) 24 hours after the second insult; pups were from 5 injury

groups: naive (N-N) and sham (S-N), postnatal RIP alone (S-RIP), IUI alone (IUI-N), and tandem insult (IUI+RIP); median values for each group are also shown (*red triangles*). (**p < 0.01, ANOVA of rank-transformed scores). **F**: Location of hemorrhages induced by tandem insults; superimposed data compiled from 16 rats. Bar = 100 μ m (A–C).

Fig. 5. Hippocampal cell death induced by tandem insults. **A–D:** Staining for TUNEL in CA3 (A and B) and dentate (C and D) regions of pups subjected to sham injury (A and C), or tandem insult (B and D). **E:** Counts of TUNEL-positive cells (cells per ROI) in dentate and CA3 regions of pups subjected to various insults, including naive (N-N), sham (S-N), postnatal RIP alone (S-RIP), IUI alone (IUI-N), and tandem insult (IUI+RIP); 5 pups in each group. ** $p < 0.01$. Bar = 100 μ m (A and C).

Fig. 6. Hippocampal cleaved caspase-3 (Casp-3) induced by tandem insults. **A–D:** Immunolabeling for cleaved caspase-3 in CA3 (A and B) and dentate (C and D) regions of pups subjected to sham injury (A and C) or tandem insult (B and D). **E:** Counts of cleaved caspase-3-positive cells (cells per ROI) in dentate and CA3 regions of pups subjected to various insults, including sham (S-N), IUI alone (IUI-N), and tandem insult (IUI+RIP); 5 pups in each group. ** $p < 0.01$. Bar = 100 μ m (A and C).

Fig. 7. Vestibulomotor function in pups with tandem insults. Righting reflex from back to abdomen (**A**), performance on constant speed Rotarod (**B**), performance on beam balance (**C**), and performance on accelerating Rotarod (**D**), in pups subjected to various insults, including naive (N-N), IUI alone (IUI-N), and tandem insult (IUI+RIP). ** $p < 0.01$.

Fig. 8.

Cognitive function in pups with tandem insults. Time to acquire visible platform (**A**) or hidden platform (**C**) during successive trials in pups subjected to various insults, including naive (N-N), IUI alone (IUI-N), and tandem insult (IUI+RIP). Time spent swimming along the edge of the pool (thigmotaxis) during the first trial of vision testing in pups subjected to various insults, as indicated (* $p < 0.05$) (**B**). Memory probe following the last trial of incremental learning in pups subjected to various insults, as indicated; the *dotted line* at 25% represents chance alone (**D**). Memory probe following the single trial of rapid learning in pups subjected to various insults, as indicated; the *dotted line* at 25% represents chance

alone (** $p < 0.01$) (**E**). Typical swim patterns during the memory probe following the single trial of rapid learning in rats from the IUI-N versus IUI+RIP groups; the *black circle* denotes the platform position during previous incremental learning trials; the *white circle* denotes the new platform location during the single rapid learning trial (**F**).

Fig. 9.

Brain and body mass in rats with various insults. **Upper:** Body mass at various times after birth in pups subjected to various insults, including naive (N-N), IUI alone (IUI-N), and tandem insult (IUI+RIP) (* $p < 0.05$). **Lower:** Brain mass at postnatal Day 52 versus body mass at postnatal Day 49 (mean $\pm$ SE) in the 3 groups of rats; 9–16 rats per group. ** $p < 0.01$.

TABLE 1

Insults and their abbreviations *

In Utero Insult	Postnatal	
	None	RIP
none	N-N (naive)	
laparotomy of dam	S-N (sham)	S-RIP (RIP alone)
IUI	IUI-N (IUI alone)	IUI+RIP (tandem insult)

* N = none (naive); S = sham surgery (anesthesia and laparotomy of the dam without manipulation of the uterus); RIP = transient raised intrathoracic pressure (50% glycerol, 13 ml/g intraperitoneally).