A Novel Preclinical Model of Germinal Matrix Hemorrhage Using Neonatal Rats

Tim Lekic, Anatol Manaenko, William Rolland, Jiping Tang, and John H. Zhang

Abstract *Background*: Germinal matrix hemorrhage (GMH) is a neurological disorder associated with very low birth weight premature infants. This event can lead to post-hemorrhagic hydrocephalus, cerebral palsy, and mental retardation. This study developed a novel animal model for pre-clinical investigations.

Methods: Neonatal rats underwent infusion of clostridial collagenase into the right germinal matrix (anterior caudate) region using stereotaxic techniques. Developmental milestones were evaluated over 10 days, cognitive function at 3 weeks, and sensorimotor function at 4 weeks after collagenase infusion. This was accomplished by anthropometric quantifications of cranial, cerebral, cardiac, and splenic growths.

Results: Collagenase infusion led to delays in neonatal developmental milestones, followed by cognitive and sensorimotor dysfunctions in the juvenile animals. Cranial growth was accelerated during the first week after injury, and this was followed by significant brain atrophy, splenomegaly, and cardiac hypertrophy 3 weeks later.

Conclusion: This study characterized the developmental delays, mental retardation, and cerebral palsy features resembling the long-term clinical course after germinal matrix hemorrhage in premature infants. Pre-clinical testing of therapeutics in this experimental model could lead to improved patient outcomes while expanding upon the pathophysiological understanding of this disease.

Keywords Animal models · Neurological deficits · Stroke, experimental

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Introduction

Germinal matrix hemorrhage (GMH) is the rupture of immature blood vessels within the subventricular (anterior caudate) progenitor cell region of neonatal brains [1] during the first 7 days of life [2]. GMH occurs in 20–25% of very low birth weight (VLBW ≤ 1,500 g) premature infants [3–5] and affects 3.5/1,000 births in the United States each year [6]. This is an important clinical problem, since the consequences are hydrocephalus (post-hemorrhagic ventricular dilation), cognitive and motor developmental delay, cerebral palsy, and mental retardation [4, 7]. However, available animal models to study the pathophysiological basis of these outcomes are lacking [8].

An important research priority is the development and validation of experimental models of brain hemorrhage for translational studies of human conditions [9]. Elevated MMP-2 and MMP-9 are associated with GMH induction in humans [10, 11]. Stereotaxic collagenase infusion is one of the most commonly used methods in adult experimental intracerebral hemorrhage (ICH) studies [12, 13] and functions as an MMP to lyse the extracellular-matrix around blood vessels to cause vascular rupture [13, 14]. This approach enables investigations of neurological and brain injury outcomes [12–19].

In this study, we hypothesized that unilateral germinal-matrix collagenase infusion in neonatal rats would model features similar to clinical GMH [4, 7]. With this approach, applications of therapeutic strategies can be tested to improve outcomes and to gain a better pathophysiological understanding of this disease [9].

Methods and Materials

Animal Groups and General Procedures

This study was in accordance with the National Institutes of Health guidelines for the treatment of animals and was approved by the Institutional Animal Care and Use Committee

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at Loma Linda University. Timed pregnant Sprague-Dawley rats were housed with food and water available *ad libitum*. Postnatal day 7 (P7) pups were blindly assigned to the following ($n=8$/group): sham (naive), needle (control), and collagenase infusion. All groups were evenly divided within each litter.

Experimental Model of GMH

Using an aseptic technique, rat pups were gently anesthetized with 3% isoflurane (in mixed air and oxygen) while placed prone on a stereotaxic frame. Betadine sterilized the surgical scalp area, which was incised in the longitudinal plane to expose the skull and reveal the bregma. The following stereotactic coordinates were determined: 1 mm (anterior), 1.5 mm (lateral) and 3.5 mm (ventral) from bregma. A bore hole (1 mm) was drilled, into which a 27-gauge needle was inserted at a rate of 1 mm/min. A microinfusion pump (Harvard Apparatus, Holliston, MA) infused 0.3 units of clostridial collagenase VII-S (Sigma, St Louis, MO) through a Hamilton syringe. The needle remained in place for an additional 10 min after injection to prevent "back-leakage." After needle removal, the burr hole was sealed with bone wax, the incision sutured closed, and the animals were allowed to recover. The entire surgery took an average of 20 min. Upon recovering from anesthesia, the animals were returned to their dams. Needle controls consisted of needle insertion alone without collagenase infusion, while naïve animals did not receive any surgery.

Developmental Milestones

Animals were assessed over 10 days after collagenase infusion. For the righting reflex, time needed for the rat pups to completely roll over onto all four limbs after being placed on their backs was measured [20]. For negative geotaxis, the time needed for complete rotation (180°) after being placed head down on a slope (20° angle), was recorded [20]. The maximum allotted time was 60 s/trial (two trials/day).

Cognitive Measures

Higher order brain function was assessed during the third week after collagenase infusion. The T-Maze assessed short-term (working) memory [21]. Rats were placed into the stem (40 cm × 10 cm) of a maze and allowed to explore until one arm (46 cm × 10 cm) was chosen. From the sequence of ten trials, of left and right arm choices, the rate of spontaneous alternation (0% = none and 100% = complete, alternations/trial) was calculated, as routinely performed [22, 23]. The Morris water maze assessed spatial learning and memory on four daily blocks, as described previously in detail [16, 17]. The apparatus consisted of a metal pool (110 cm diameter), filled to within 15 cm of the upper edge, with a platform (11 cm diameter) for the animal to escape onto, that changed location for each block (maximum = 60 s/trial), and was digitally analyzed by Noldus Ethovision tracking software. Cued trials measured place learning with the escape platform visible above water. Spatial trials measured spatial learning with the platform submerged, and probe trials measured spatial memory once the platform had been removed. For the locomotor activity, in an open field, the path length in open-topped plastic boxes (49 cm-long, 35.5 cm-wide, 44.5 cm-tall) was digitally recorded for 30 min and analyzed by Noldus Ethovision tracking software [17].

Sensorimotor Outcome

At 4 weeks after collagenase infusion, animals were tested for functional ability. Neurodeficit was quantified using a summation of scores (maximum = 12), given for (1) postural reflex, (2) proprioceptive limb placing, (3) back pressure towards the edge, (4) lateral pressure towards the edge, (5) forelimb placement, and (6) lateral limb placement (2 = severe, 1 = moderate, 0 = none), as routinely performed [22]. For the rotarod, striatal ability was assessed using an apparatus consisting of a horizontal, accelerated (2 rpm/5 s), rotating cylinder (7 cm diameter × 9.5 cm wide), requiring continuous walking to avoid falling recorded by photobeam circuit (Columbus Instruments) [16, 17]. For foot fault, the number of complete limb missteps through the openings, was counted over 2 min while exploring over an elevated wire (3 mm) grid (20 cm × 40 cm) floor [23].

Assessment of Growth

Over 28 days after collagenase infusion, the head (width and height) and rump-to-crown (length) measurements were performed using a Boley Gauge (Franklin Dental Supply, Bellmore, NY), as previously described [24]. Head width was measured anterior to the side of the ears, head height from posterior to the adjacent mandible, and rump-to-crown was the greatest cranial (caudal) to tail (rostral) extension. At the completion of experiments, the brains were removed, and hemispheres separated by a midline incision (loss of brain weight has been used as the primary variable to estimate

brain damage in juvenile animals after neonatal brain injury [25]). For organ weights, the spleen and heart were separated from surrounding tissue and vessels. The quantification was performed using an analytical microbalance (model AE 100; Mettler Instrument Co., Columbus, OH) capable of 1.0 µg precision.

Statistical Analysis

Significance was considered at $P<0.05$. Data were analyzed using analysis of variance (ANOVA), with repeated measures (RM-ANOVA) for long-term neurobehavior. Significant interactions were explored with conservative Scheffe *post hoc* and Mann-Whitney rank sum tests when appropriate.

Results

Collagenase infusion delayed the developmental acquisition of eye opening, negative geotropism and righting reflex by 2–3 days (Fig. 1a–c, $P<0.05$). Three weeks after GMH, significant deficits were discovered in spatial learning and memory (Fig. 2a, b, $P<0.05$), T-maze (working) memory (Fig. 2c, $P<0.05$), and hyperactivity, in the open field (decreased corner time and increased center crossings, Fig. 2d, e, $P<0.05$). Juvenile animals had significant sensorimotor dysfunction, as revealed by the neurodeficit score, accelerating rotarod and foot fault (Fig. 3a–c, $P<0.05$). These dysfunctions were associated with increased cranial size at 7 days (Fig. 4a, $P<0.05$), and dysfunctional growth of the body, brain, heart, and spleen (Fig. 4b–e, $P<0.05$) 3 weeks later.

Discussion

Germinal matrix hemorrhage (GMH) is an important problem affecting approximately 12,000 births in the United States each year [6]. The clinical consequences of GMH are developmental delay, cerebral palsy, and mental retardation [4, 7]. In this study collagenase was infused into the germinal matrix of neonatal rats as an approach to model these features, since animal models to study the basis of these outcomes are lacking [8].

This neonatal rat model of GMH resembles the neurological consequences seen in the pediatric population after hemorrhagic brain injury. Collagenase infusion led to developmental delays in the neonates that were followed by cognitive and sensorimotor dysfunction in the juvenile

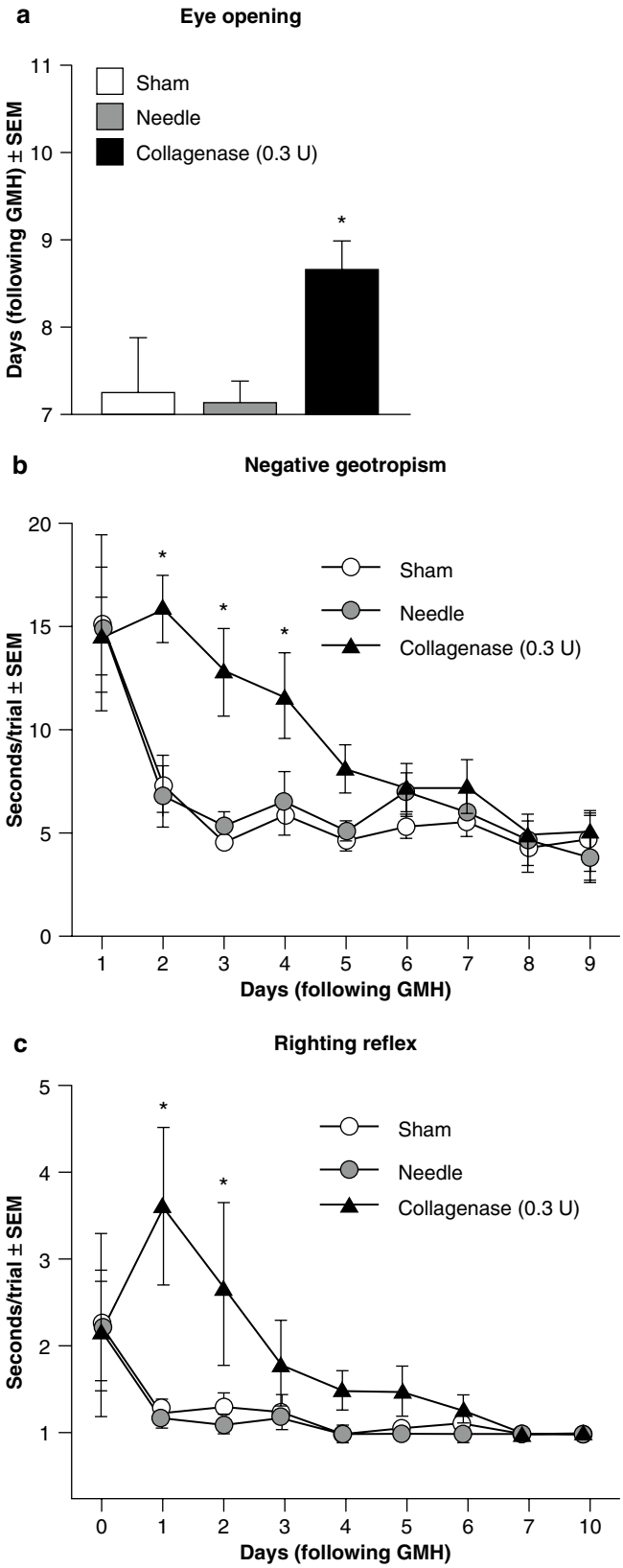

Fig. 1 *Developmental delays:* Neonates were assessed for (**a**) eye opening latency, (**b**) negative geotropism, and (**c**) righting reflex, over 10 days after collagenase infusion. Values expressed as mean ± SEM, $n=8$ (per group), $^*P<0.05$ compared with controls (sham and needle trauma)

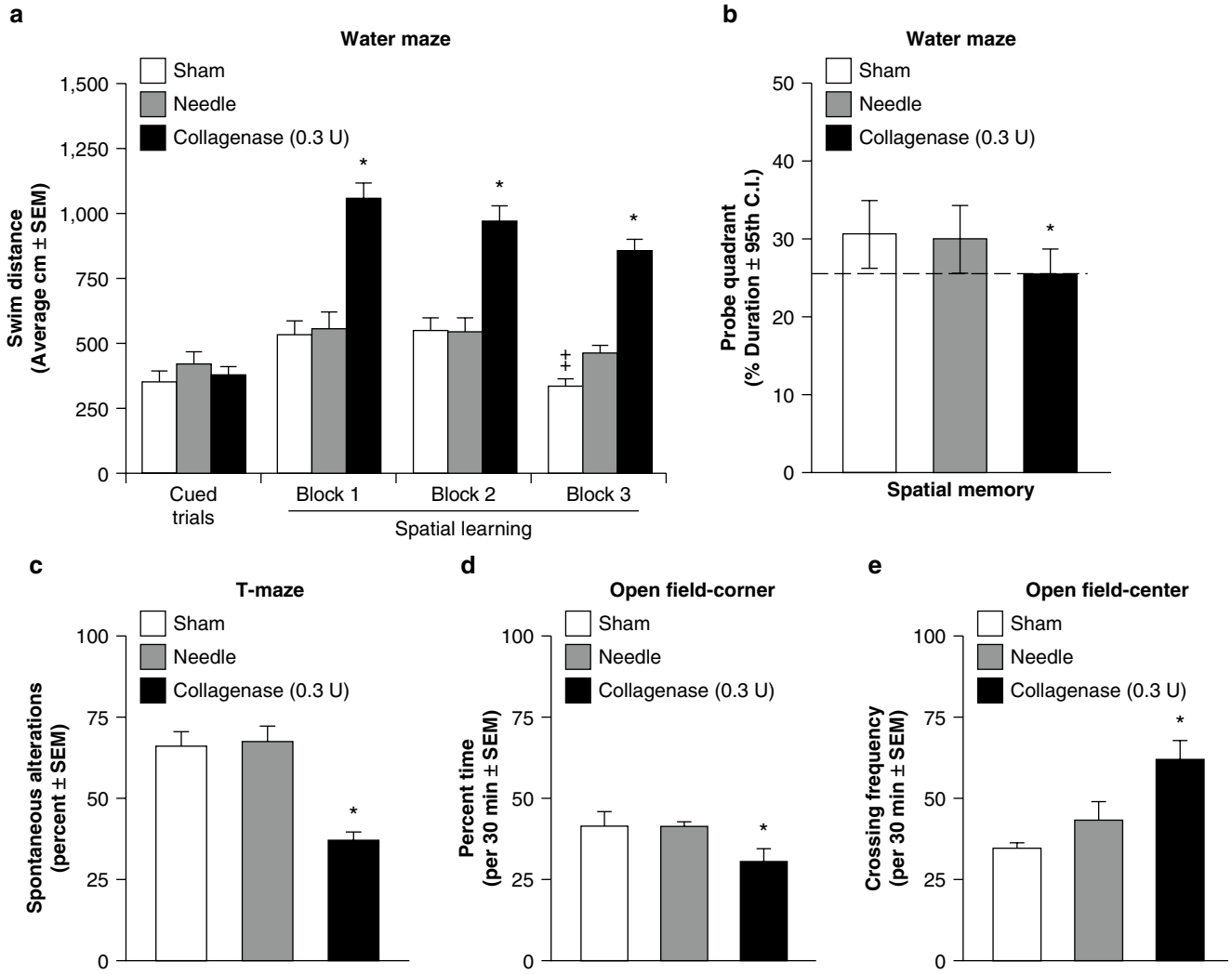

Fig. 2 *Cognitive dysfunction:* Higher order function was measured at the 3rd week after collagenase infusion. (**a**) Cued and spatial learning water maze, (**b**) probe (spatial memory) water maze, (**c**) T-maze, (**d**) open field (percent time in corner), and (**e**) open field (center crossing frequency). Values expressed as mean ± 95th C.I. (probe quadrant) or mean ± SEM (all others), $n = 8$ (per group), $^{*}P < 0.05$ compared with controls (sham and needle trauma) and $^{\ddagger}P < 0.05$ compared with block 1 (spatial learning water maze)

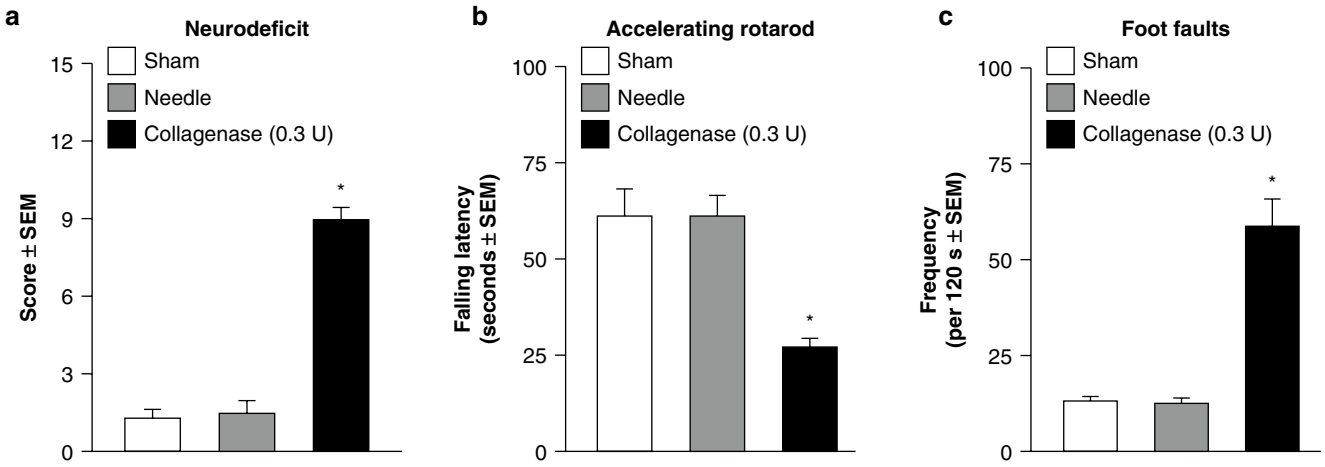

Fig. 3 *Sensorimotor dysfunction:* Cerebral palsy measurements were performed in the juveniles at 1 month after collagenase infusion. (**a**) Neurodeficit score, (**b**) rotarod, and (**c**) foot fault. Values expressed as mean ± SEM, $n = 8$ (per group), $^{*}P < 0.05$ compared with controls (sham and needle trauma)

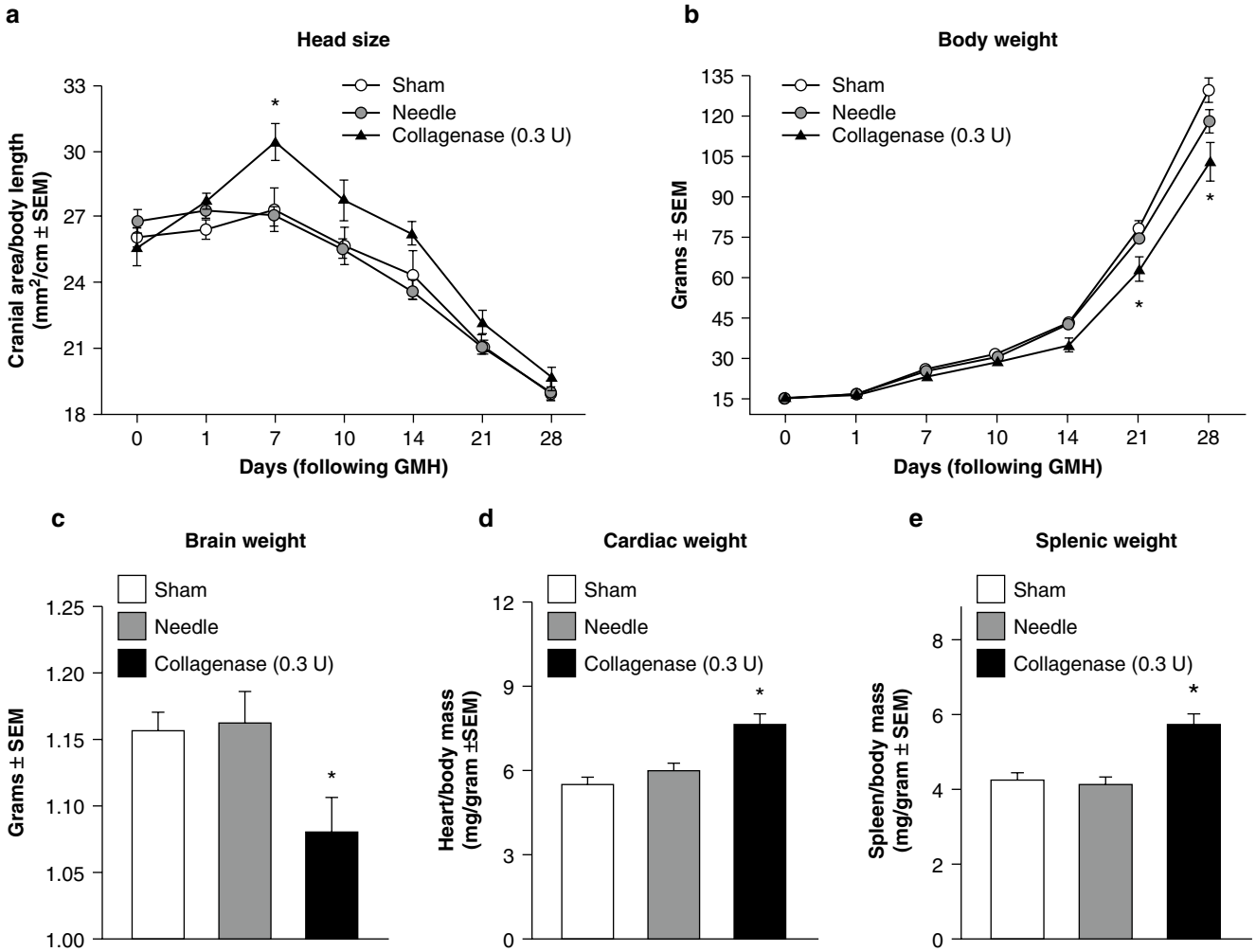

Fig. 4 *Cranial and somatic pediatric growth:* 1 month assessment of (**a**) head size (cranial area/body length), (**b**) body weight, (**c**) brain weight, (**d**) cardiac weight and (**e**) splenic weight. Values expressed as mean ± SEM, $n = 8$ (per group), $^*P < 0.05$ compared with controls (sham and needle trauma)

developmental stage. The cranium was enlarged compared to somatic growth during the first week, with significant brain atrophy 3 weeks later. This presentation is likely a reflection of hydrocephalic cerebrospinal fluid build-up, leading to cranial expansion and compression of the brain tissue into an atrophic developmental growth pattern. Splenomegaly and cardiac hypertrophy presented at 1 month after injury, and this could either be a reflection of the disproportionate somatic growth or of prolonged peripheral hemostatic or inflammatory consequences of the brain bleed.

In summary, we have characterized a highly reliable and easily reproducible experimental model of germinal matrix hemorrhage using neonatal rats. This provides the basis for studying the clinical and pathophysiological features of this disease, and establishes a foundation for performing further preclinical therapeutic investigations.

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Conflict of interest statement We declare that we have no conflict of interest.

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