

Injections of Blood, Thrombin, and Plasminogen More Severely Damage Neonatal Mouse Brain Than Mature Mouse Brain

Mengzhou Xue, MD; Marc R. Del Bigio, MD, PhD, FRCPC

Department of Pathology, University of Manitoba and Manitoba Institute of Child Health, Winnipeg, MB, Canada

Corresponding author:

Marc R. Del Bigio, MD, PhD, FRCPC, Department of Pathology, University of Manitoba, D212-770 Bannatyne Ave, Winnipeg, MB, R3E 0W3, Canada (E-mail: delbigio@cc.umanitoba.ca)

The mechanism of brain cell injury associated with intracerebral hemorrhage may be in part related to proteolytic enzymes in blood, some of which are also functional in the developing brain. We hypothesized that there would be an age-dependent brain response following intracerebral injection of blood, thrombin, and plasminogen. Mice at 3 ages (neonatal, 10-day-old, and young adult) received autologous blood (15, 25, and 50 μ l respectively), thrombin (3, 5, and 10 units respectively), plasminogen (0.03, 0.05, and 0.1 units respectively) (the doses expected in same volume blood), or saline injection into lateral striatum. Forty-eight hours later they were perfusion fixed. Hematoxylin and eosin, lectin histochemistry, Fluoro-Jade, and TUNEL staining were used to quantify changes related to the hemorrhagic lesion. Damage volume, dying neurons, neutrophils, and microglial reaction were significantly greater following injections of blood, plasminogen, and thrombin compared to saline in all three ages of mice. Plasminogen and thrombin associated brain damage was greatest in neonatal mice and, in that group unlike the other 2, greater than the damage caused by whole blood. These results suggest that the neonatal brain is relatively more sensitive to proteolytic plasma enzymes than the mature brain.

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INTRODUCTION

Intracerebral hemorrhage (ICH) is a consequence of hypertension, bleeding into an ischemic infarct, rupture of abnormal blood vessels, or trauma. A significant proportion of premature births are also accompanied by bleeding into the brain. The incidence of periventricular/intraventricular hemorrhage (PVH/IVH) increases progressively with decreasing gestational age, eg, from 1.6% at 38 to 43 weeks to up to 50.0% at 24 to 30 weeks of gestation (5). Periventricular hemorrhage that extends into the adjacent brain parenchyma has a poor outcome (16). Following ICH, prothrombin and plasminogen, which are the precursors of thrombin and plasmin in plasma, enter the brain substance. In vitro, thrombin induces apoptosis (11), causes morphological changes of astrocytes (39), and causes neurite retraction in cultured neurons (76) likely through protease activated receptors (PARs) (12, 58). In adult rodent brains, injected thrombin causes brain edema perhaps by digestion of vascular basement membrane (23, 33), as well as

inflammation, brain necrosis and apoptosis (72). Plasmin is produced after cleavage of plasminogen by tissue type (tPA) or urokinase-type plasminogen activators (uPA) (66). Injected plasmin also causes considerable brain edema, inflammation, and brain cell death (72). Following brain injury and ischemia, endogenous tPA and thrombin are capable of potentiating neuronal degeneration through various mechanisms (27, 49, 55).

There are developmental changes in brain with regard to proteases. In mouse brain, uPA and its receptor are maximally expressed by neurons at 8 to 10 days (17). Neurons and glia are capable of expressing thrombin receptors (ie, protease activated receptors [PARs]) (76), which are most abundant in the neonatal rodent brain (14, 47, 51). Endogenous brain thrombin and plasmin (3, 20) are implicated in migration of immature neurons, synaptogenesis, and formation of myelin. Endogenous inhibitors of plasmin and thrombin, including neuroserpin (32, 75), plasminogen activator inhibitors (PAI) 1 and 2 (17), α 2-mac-

roglobulin (37, 38), and protease nexin-1 (PN-1) (41), are present in developing brain in general at higher concentrations than mature brain.

Because immature migrating cells normally use and respond to proteolytic enzymes, we hypothesized that brain damage related to plasmin and thrombin would be greater in young brains than in mature brains. To test the hypothesis we assessed the brain damage and inflammation following intracerebral injections of autologous blood or plasma enzymes in three ages in mice. Our previous data in adult rats showed that inflammatory cell infiltration and cell death peak 2 to 3 days after injection of autologous whole blood into the brain, therefore we chose a survival period of 48 hours (71, 73). Neonatal (1-2 day-old) mice have a maturational state of the subependymal zone/ganglionic eminence roughly comparable to 24-26 week gestational age human brains (61). Ten-day-old mice are roughly comparable to neonatal human brains. Adult mice (7 weeks) are roughly comparable to young adult human brains (4, 36, 61).

MATERIALS AND METHODS

Experimental group design. All experimental procedures were done in accordance with guidelines of the Canadian Council on Animal Care. Protocols were approved by the local experimental ethics committee. Seventy-four CD-1 mice (Charles River Canada, St. Constant, Quebec) at 3 ages (neonatal 1-2 days weighing 1.42-1.95 grams; 10 days weighing 5-7 grams; 8 week young adult weighing 22.0-26.5 grams) were used for this study. Young mice (5 litters) were hosted with their mothers in litter sizes of 10. There were 12 groups (3

	Saline	Blood	Thrombin	Plasminogen
Neonatal	15 μ l (n=6)	15 μ l (n=6)	3 units in 15 μ l (n=6)	0.03 units in 15 μ l (n=6)
10-day old	25 μ l (n=6)	25 μ l (n=6)	5 units in 25 μ l (n=6)	0.05 units in 25 μ l (n=6)
Young adult	50 μ l (n=6)	50 μ l (n=6)	10 units in 50 μ l (n=6)	0.1 units in 50 μ l (n=6)

Table 1. Experimental intracerebral injections into CD1 mouse brains.

ages \times 4 treatments). The treatment groups included saline injection as control, autologous blood injection, thrombin injection, and plasminogen injection (Table 1). Each experimental group consisted of 6 mice (no more than 2-3 mice from a given litter).

Animal model. Neonatal mice were anesthetized by cooling on an ice bed. Ten-day old and adult mice were anesthetized by intraperitoneal injection with ketamine/xylazine (9.0/0.5 mg/kg). Autologous blood was collected in a sterile syringe by placing the tail in warm water for one minute, cleaning the skin with 70% alcohol, and cutting 2 mm off the tail tip as previous described (68). In the neonatal mice, the brain injections were done with the needle inserted percutaneously. In 10-day-old and adult mice, a midline scalp incision was made and a small hole was drilled in the skull. A 27-gauge needle was attached to the syringe and quickly introduced into the right striatum of the mouse brain with a custom-made guide to help stabilize the needle and guide it to the correct depth (in neonatal mice, 1.5 mm lateral to midline, 0.5 mm posterior to the outer canthus of the right eye, 2.5 mm deep to the skull surface; in 10-day-old mice, 2 mm lateral to midline, 1.0 mm posterior to the outer canthus of the right eye, 3.0 mm deep to the skull surface; in adult mice, 2.0 mm lateral to midline, 1.5 mm posterior to the outer canthus of the right eye, 3.5 mm deep to the skull surface). The sites of injection were more lateral than those used in our PVH/IVH models (68) because we wanted to avoid blood escape into the lateral ventricles. The volume of injected blood was 15 μ l in neonatal, 25 μ l in 10-day-old, and 50 μ l in adult mice. Saline, thrombin, and plasminogen solutions were in the same volumes respectively. We chose these volumes in an attempt to create a hematoma that was of equal proportion to the total brain weight in all ages. We based our calculation on prior studies that reported brain weights of approximately 150 mg in neonatal mice, 250 mg in 10-day-old mice, and 420 mg in adult mice (44, 52). Saline, thrombin, and

plasminogen solutions were injected in the same volumes as blood in each age. The total dose of thrombin (from bovine plasma; T-6634, Sigma Chemical Company) (3, 5, 10 units in 3 ages respectively) and plasminogen (from bovine plasma; P-9156, Sigma) (0.03, 0.05, 0.1 units in 3 ages respectively) administered was the amount calculated to be in the blood volume injected at each age (Table 1). We chose plasminogen rather than plasmin because in a previous study showed that plasmin potency seemed to be reduced (72). We calculated the units of prothrombin and plasminogen in whole blood based on the following assumptions: plasma volume is ~60% total blood volume, each ml plasma contains 260 to 360 units of prothrombin and 200 μ g (1.95 units) plasminogen (7, 13). Coded syringes were loaded by the senior investigator with thrombin, plasminogen, and saline; the junior investigator responsible for the analysis was thereby blinded to the identity of the injected agent. The plunger of the syringe containing blood or other solutions was depressed gradually over 40 to 60 seconds to prevent excessive pressure gradients; the needle was left in place for 30 seconds, and then removed slowly. The scalp (in 10-day-old and adult mice) was sutured. Physiological monitoring was not practical due to the small size of the mice. All mice were returned to the cages after the procedure, which took approximately 3 to 5 minutes. Adult mice were placed in a cage in groups of 4 to 5 with free access to food and water.

Histological evaluation. Mice were overdosed with ketamine/xylazine at 48 hours after brain injections and perfused through the heart with ice cold 4% paraformaldehyde in 0.1 mol/L phosphate buffered saline (PBS). The brain was removed and stored in the same fixative for 1 to 10 day(s). Fixed brains were cut coronally to surround the injection sites, and the slices were dehydrated and embedded in paraffin. Sections (6 μ m) were cut serially through the whole slice, and each 10th section was stained with hematoxylin and eosin (H&E). Near the

lesion center, where the brain damage was maximal, a variety of histological and histochemical stains were performed on adjacent sections.

Histochemistry. Ricinus communis agglutinin lectin (RCA-1) labeling was used to demonstrate reactive microglial cells (40). Paraffin sections were dewaxed and rehydrated, washed, quenched with 0.3% H₂O₂, blocked with 10% normal sheep serum, and incubated with biotinylated lectin (diluted 1/2000, Vector Laboratories, Inc., Burlingame, Calif). Slides were then washed, incubated with streptavidin-peroxidase (1/400, Dako Corporation, Carpinteria, Calif), colored with diaminobenzidine-H₂O₂ solution, washed and coverslipped. Control sections were processed with omission of the biotinylated lectin. TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling) was used to identify dying cells with damaged DNA. Paraffin embedded sections were dewaxed and rehydrated, then incubated in 20 μ l/ml proteinase K for 15 minutes. TUNEL was accomplished using *Apoptag in situ* kit (Intergen; Purchase, NY). After immersion in equilibration buffer for 10 minutes, sections were incubated with TdT and dUTP-digoxigenin in a humidified chamber and then incubated in the stop/wash buffer. Sections were washed before incubation in anti-digoxigenin-peroxidase solution (1/500 in PBS), and colored with diaminobenzidine-H₂O₂ solution. Sections were counterstained with methyl green. Negative control sections were treated similarly but incubated in the absence of TdT enzyme or dUTP-digoxigenin. Sections of brain were stained with Fluoro-Jade to show dying neurons (54) by incubating sections in 0.06% potassium permanganate for 15 minutes while gently shaking. Then 0.001% Fluoro-Jade (Histo-Chem Inc.; Jefferson, Ark) staining solution was applied for 30 minutes, following by PBS wash, drying in air, and coverslip application.

Determination of damaged brain volume and cell counts. Using H&E stained sections of all affected levels, a "camera lucida" was used to trace the brain damage area, which was defined by the presence of blood, tissue rarefaction, or necrosis. Com-

puterized planimetry was used to measure the cross sectional areas of damage and of brain. The volumes of damaged brain were calculated by adding the areas of damage on all levels multiplied by the distance between sections. Using an ocular reticule and 400× magnification (objective magnification ×40), Fluoro-Jade positive neurons, TUNEL positive dying cells, extravascular neutrophils (identified on H&E sections by characteristic nuclear morphology), and RCA-1 binding cells were counted in 4 fields (each area 540 μm × 540 μm) immediately adjacent to the needle injection/damage site as previously described (68, 71-73). Areas with large blood vessels were avoided. Counts were made near the edge of the lesion because the necrotic cores were devoid of viable cells. The cell counts and damaged brain areas were blindly assessed by the experienced junior author to minimize the observation bias.

Statistical analysis. All data are expressed as mean ± SEM. Data were analyzed to ensure normal distribution. The initial test was 2-way analysis of variance (ANOVA) using age and treatment as independent variables. Intergroup comparisons within a given age and between ages were made using Scheffé test, which allows for differences in variation between groups. The differences were considered significantly different when $p < 0.05$. We used StatView 5.01 software (SAS Institute; Cary, NC).

RESULTS

Most mice tolerated the surgical procedure well. There were no overt neurological deficits on casual observation. No seizures were observed. There were 2 deaths among the 10-day-old mice; these occurred immediately after injection of whole blood and plasminogen (1 each) and were thought to be complications of anesthesia.

Following saline injection (Figure 1A) the brains exhibited small collections of blood and negligible edema around the needle tract in the striatum. Following autologous blood injection, an irregular hematoma characterized by edema, blood debris, and necrosis was located in striatum (Figure 1B). Following injections of plasminogen and thrombin, the volume of damaged brain was large, especially in the neonatal mouse, where hemorrhagic infarction consistently extended into adjacent white

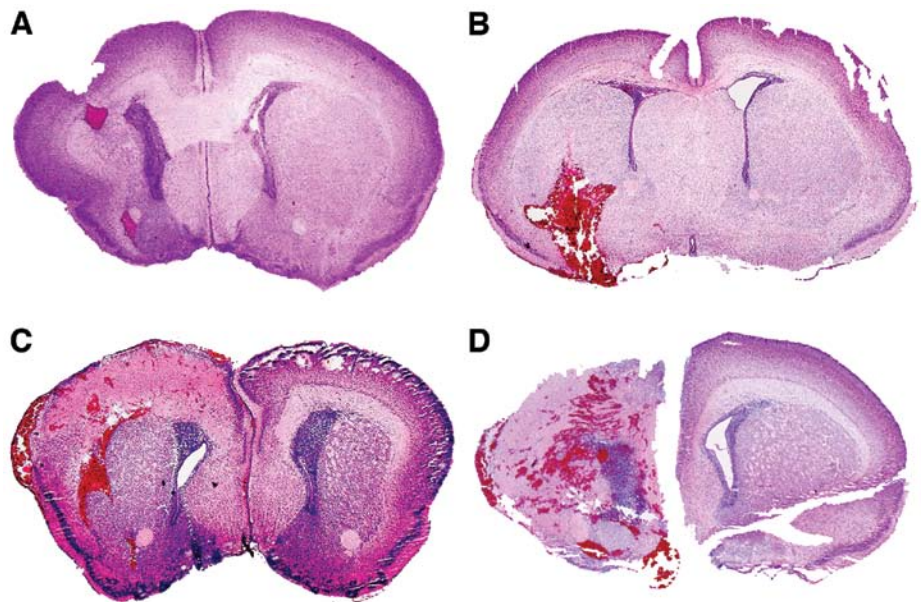


Figure 1. Low magnification photomicrographs of neonatal mouse brain coronal sections 48 hours after injection. After saline injection, there is small quantity of blood in the striatum along the needle tract (Figure 2A). Blood injection is associated with a localized hematoma in the striatum (Figure 2B). After plasminogen (Figure 2C) and thrombin (Figure 2D) injection, the damage areas are large; hemorrhagic infarcts extend to the white matter and lateral cerebral cortex.

matter and cerebral cortex (Figure 1C, D). Fluoro-Jade staining highlighted the dying neurons, which could also be seen on the H&E stained sections due to the hypereosinophilic staining of their cytoplasm. Large and small TUNEL positive dying cells were also present, but their identity was seldom obvious. Neutrophils were identifiable at the periphery of the necrotic/hemorrhagic lesion. Reactive microglia/macrophages with ramified processes and swollen bodies respectively were present adjacent to the damaged brain tissues, especially around blood vessels (Figure 2).

The quantitative comparisons are detailed in Figures 3 to 5. Both age and type of injection had a significant effect on brain damage volume, dying neurons, and TUNEL positive cells. At the cellular level, destructive and reactive changes were roughly proportionate to the total volume of injury. Total brain damage volume and damage area on the most severely affected level were highly correlated ($r = 0.897$, $p < 0.0001$). Saline injection was associated with only small volumes of brain damage. Injections of blood, thrombin, and plasminogen caused proportionally larger volumes of damage in neonatal mice than in 10-day old and adult mice (Figure 3). TUNEL positive dying cells were infrequently seen in the striatum of control brains in the neonatal group but never in the older mice.

Peripheral to the necrotic core of the lesion, the quantities of TUNEL positive cells and Fluoro-Jade stained dying neurons were greater following the injections of blood, plasminogen, and thrombin compared to saline. In addition, the quantity of dying neurons was significantly greater after plasminogen and thrombin injection compared to blood injection in the neonatal group. In contrast, dying neurons were significantly fewer following plasminogen and thrombin injection compared to blood injection in the 10-day-old and adult mouse groups. TUNEL positive cells were significantly more abundant after plasminogen injection compared to blood injection in the neonatal group (Figure 4).

Accompanying the destructive change was an inflammatory response. Both age and type of injection had a significant effect on neutrophil infiltration. Only treatment, but not age, had significant effect on the quantity of RCA-1 labeled microglia/macrophages. Neutrophils and microglia/macrophages were more abundant 48 hours after blood, plasminogen, and thrombin injection compared to saline in all three age groups. In the neonatal group, both neutrophils and microglia/macrophages were significantly greater after plasminogen injection compared to blood injection (Figure 5).

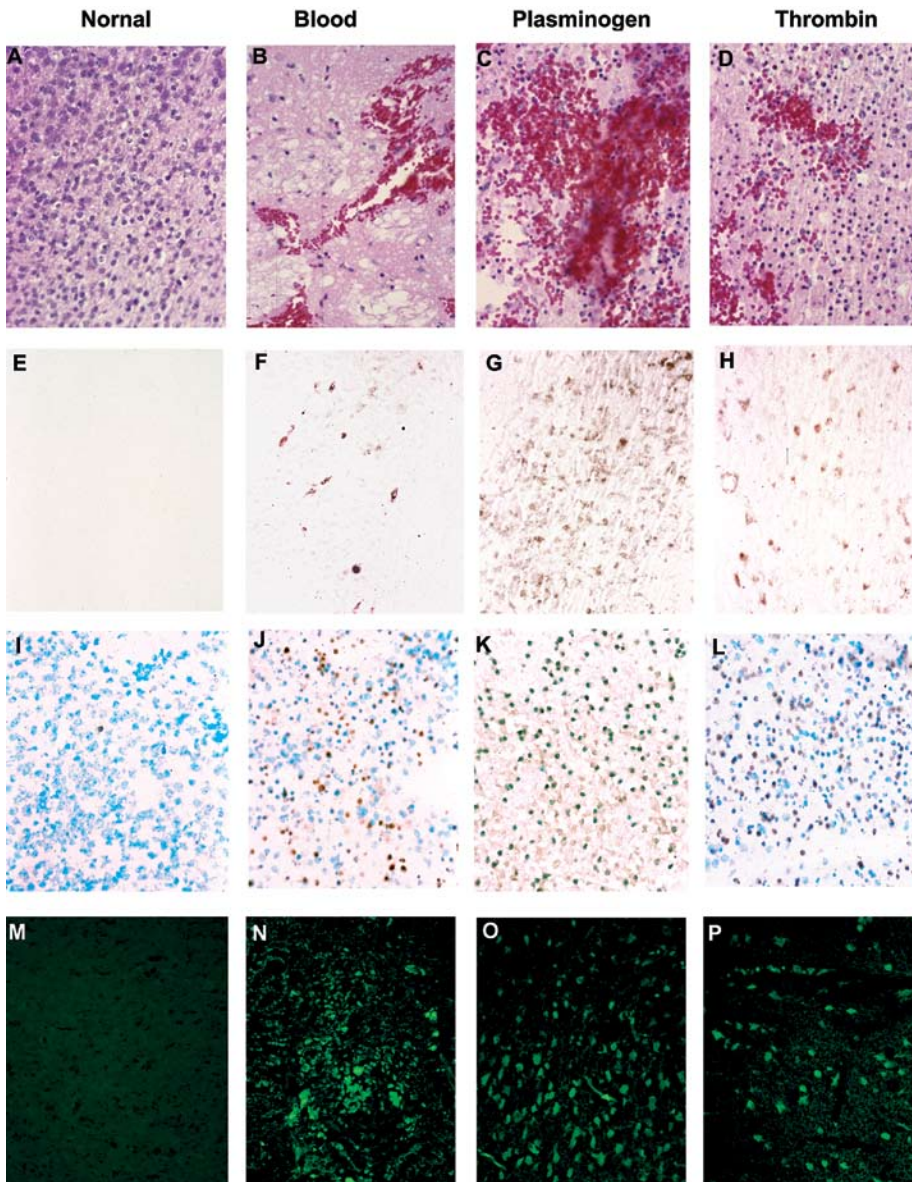


Figure 2. Photomicrographs showing histopathologic features 48 hours following injections into neonatal mouse brain. Intact neonatal medial striatum exhibits tightly packed immature neurons (A). Damaged striatum after blood (B), plasminogen (C), and thrombin (D) injection exhibits collections of erythrocytes, pallor due to edema, and fewer neurons. RCA-1 lectin staining demonstrates negligible microglial cells in intact striatum (E), mild accumulation of activated microglia/macrophages in striatum after blood injection (F), and more substantial collections associated with plasminogen (G) and thrombin (H) injection. TUNEL demonstrates rare dying cells (brown) in intact neonatal striatum (I), and more dying cells in striatum after blood (J), plasminogen (K), and thrombin (L) injection. Fluoro-Jade shows no dying neurons in normal brain (M), and an increased quantity of dying neurons adjacent to blood (N), plasminogen (O), and thrombin (K) injection sites. Note that the histologic features in 10-day-old and young adult mice were qualitatively similar. Bar = 50 μ m.

DISCUSSION

The pathogenesis of brain damage that follows ICH has largely been studied in mature brain systems. ICH causes brain damage through multiple mechanisms. Direct tissue destruction by dissection of blood along tissue planes occurs immediately. Delayed damage likely occurs through a variety of mechanisms including local ischemia, release of toxins by blood breakdown prod-

ucts, and inflammatory responses (43, 67, 69). Thrombin, which is involved in blood clotting, and plasmin, which is involved in clot lysis, cause damage and edema in adult rodent brain (34, 72). In the neonatal brain, thrombin inhibition is associated with reduced brain injury after blood injection (70). Thrombin can be indirectly toxic to neurons through microglia-mediated oxidative stress and can act as a chemoat-

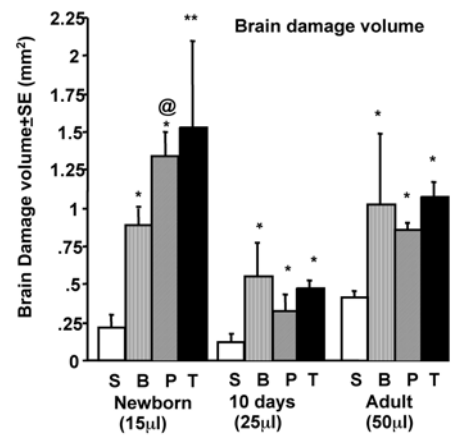


Figure 3. Bar graphs showing the brain damage volume in mouse brains 48 hours following saline, blood, plasminogen, and thrombin injections. Damage area (ANOVA, $F[\text{treatment}] = 7.658$, $p < 0.0005$; $F[\text{age}] = 6.605$, $p < 0.005$, respectively) and damage volume (ANOVA, $F[\text{treatment}] = 7.099$, $p < 0.0008$; $F[\text{age}] = 8.895$, $p < 0.0008$, respectively) were influenced by treatment and age, but there were no interaction effects (ANOVA, $F[\text{area}] = 2.036$, $p = 0.0877$, $F[\text{volume}] = 1.356$, $p = 0.2602$, respectively). There was a significant interaction effect between age and treatment (ANOVA, $F[\text{treatment}] = 9.557$, $p = 0.0001$; $F[\text{age}] = 16.288$, $P < 0.0001$; $F[\text{interaction}] = 5.843$, $p = 0.0003$). There is a significantly larger volume of brain damage following injection of plasminogen in newborn mice compared to 10-day-old and adult mice (@ $p < 0.001$, ANOVA using age as independent variable). The volumes of damaged brain were greater after blood, plasminogen, and thrombin injection compared to saline control in all three ages. (* $p < 0.05$; ** $p < 0.01$, ANOVA with Scheffé post hoc intergroup comparisons). Abbreviations: S = saline, B = blood, P = plasminogen, T = thrombin.

tractant agent for leukocytes (9, 45). Activated leukocytes can cause secondary brain damage through the release of cytokines, reactive oxygen species, nitric oxide, matrix metalloproteinases (MMPs), and other proteases (26). Systemic prestimulation of the immune system with lipopolysaccharide is associated with aggravation of brain injury that follows injection of blood into the neonatal mouse brain (74).

The main finding in this experiment was that injections of blood, thrombin, and plasminogen caused relatively more damage in neonatal mouse brain than in 10-day-old or young adult mouse brain. In blood, thrombin converts fibrinogen into fibrin, which causes the blood to clot. Plasmin digests fibrin to lyse blood clots. Thrombin receptors, PAR-1 and PAR-2 are located on neurons and endothelia of brain (2, 12, 47). These receptors can be activated by low concentrations of thrombin (58). It is im-

portant to note that the effect of thrombin is concentration dependent (51). Through these receptors, thrombin can cause retraction of cell processes on cultured neurons (24) and is toxic to neurons in brain slices in a dose-dependent manner (60). Thrombin and plasmin can induce endothelin synthesis by astrocytes; this could cause vasospasm and aggravate ischemia (21).

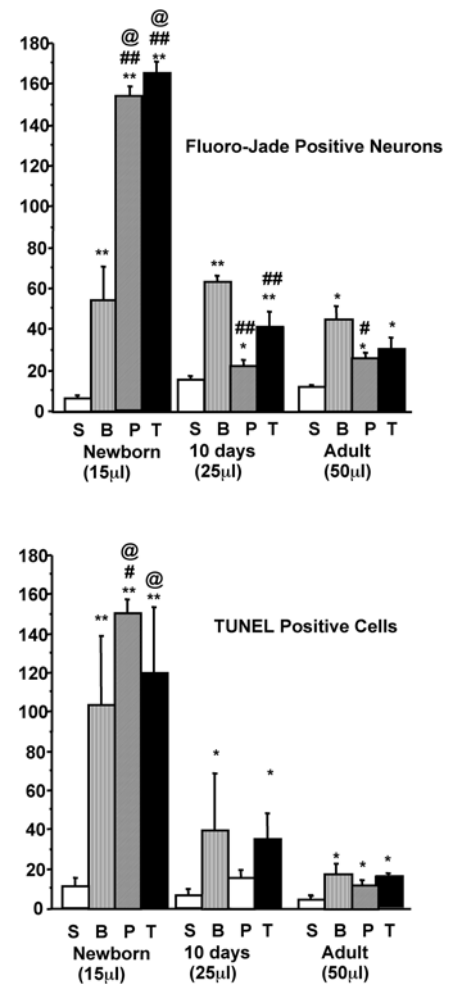
Why is the brain damage more widespread in neonatal mice? Several possibilities exist. The first relates simply to diffusion; toxic substances associated with the hematoma might diffuse more broadly in neonatal brain because the extracellular spaces are larger (35). Second, thrombin and plasmin are normally active in brain at low concentrations (76), especially during development and during reactive changes (47, 48). Immature cells in neonatal brain might be more sensitive to thrombin and plasminogen because they have more PARs (47, 48, 65). Third, the regulators of plasminogen activation and proteolysis such as PAI-1, protease nexin-1, and alpha 2 macroglobulin are present in developing brain only in very small quantities, albeit at higher concentrations than in adult brain perhaps to control the proteolysis involved in development (17, 32, 37, 38, 41, 53). They can be upregulated following experimental brain damage (1), however the quantity and response time are probably insufficient to deal with the large amounts of plasmin and thrombin that enter brain following hemorrhage. Finally, thrombin can cause endothelia to separate from basement membranes (63); and plasmin can directly degrade extracellular matrix proteins of vascular basal lamina and can activate matrix metalloproteinases, which can also digest matrix proteins (8, 25). Therefore, damage to immature vessels (64) might explain the widespread hemorrhagic infarct in the middle cerebral artery distribution of neonatal brain following thrombin and plasmin injections. Another possible explanation for hemorrhagic infarction is thrombosis of deep veins.

In summary, the explanation for neonatal brain hypersensitivity is likely multifactorial. This conclusion presupposes that our modeling of proportionate volume injections was correct. We chose total brain volume because there are insufficient data concerning the postnatal growth of striatum in mice (22, 57). Rapid changes oc-

Figure 4. Bar graphs showing dying neurons (per $4 \times 540 \times 540 \mu\text{m}^2$ area) detected by Fluoro-Jade staining (upper panel) and cell death detected by TUNEL (lower panel), in mouse striatum 48 hours after saline, blood, plasminogen, and thrombin injections. Both age and treatment had significant effects on TUNEL positive cells (ANOVA $F[\text{age}] = 42.800$, $p < 0.0001$; $F[\text{treatment}] = 9.599$, $p < 0.0001$; $F[\text{interaction}] = 5.718$, $p < 0.0003$), on dying neurons (ANOVA $F[\text{age}] = 145.375$, $p < 0.0001$; $F[\text{treatment}] = 71.984$, $p < 0.0001$; $F[\text{interaction}] = 53.798$, $p < 0.0001$). There is a significantly larger number of dying neurons and TUNEL positive cells following the injection of plasminogen and thrombin in newborn mice compared to 10-day-old and adult mice (@ $p < 0.001$, 2-way ANOVA using age as independent variable). The quantities of dying neurons and TUNEL positive cells were greater adjacent to the blood, plasminogen, and thrombin injection sites compared to saline controls (* $p < 0.05$; ** $p < 0.01$, compared to saline, ANOVA with Scheffé post hoc intergroup comparisons). Dying neurons were significantly more abundant after plasminogen and thrombin injections compared to blood injection in the neonatal group, whereas there were significantly fewer dying neurons after plasminogen and thrombin injection compared to blood injection in 10 days and adult mice (# $p < 0.05$; ## $p < 0.01$, compared to blood, ANOVA with Scheffé post hoc intergroup comparisons). The number of TUNEL positive cells exhibited a similar pattern of differences. Abbreviations: S=saline, B=blood, P=plasminogen, T=thrombin.

cur in mouse striatum during the first few postnatal days. Corticostriatal connections form around day 2 (56), and the glia to neuron ratio drops rapidly between birth and day 10, after which it remains stable (61). We cannot exclude the possibility that our neonatal doses were too high relative to the number of cells; total brain protein at birth is disproportionately low relative to brain weight as a consequence of high water content (29). Regardless of the precision of comparative dosing, the response of the neonatal mouse brain was qualitatively different with large hemorrhagic infarctions occurring in contrast to localized areas of necrosis.

A secondary finding in the experiment was that the neonatal mouse brain appeared to be relatively more sensitive to thrombin and plasminogen than to whole blood quantities presumed to contain the same amounts of these proteins. This is somewhat more difficult to explain than the differential age sensitivity. We can speculate that thrombin and plasmin in whole blood clot are less active than when an equivalent quantity is injected into the brain. In whole blood, the protein C anticoagulation system, antithrombin, and others control the activity



of thrombin (15, 19, 59). Furthermore, the activity of thrombin at a particular site can be very difficult to predict because it is self-amplifying as well as rapidly inactivated. Plasmin activity is also regulated by several proteins, for example thrombin-activatable fibrinolysis inhibitor (TAFI) (42). Hence, all of the controls present in whole blood are not available when thrombin and plasminogen are injected alone.

In this study, the site of intracerebral blood injection was more lateral than that used in a previously reported model (68) because we wanted ensure that blood did not escape into the lateral ventricles. Because toxic substances were not diluted in cerebrospinal fluid, we observed more parenchymal damage and inflammation than in that experiment. In regard to the identification of dying cells we must offer the following comment. Although the quantities of TUNEL positive cells and Fluoro-Jade stained dying neurons were similar, they might not reflect the same cell populations. TUNEL positive immature cells are report-

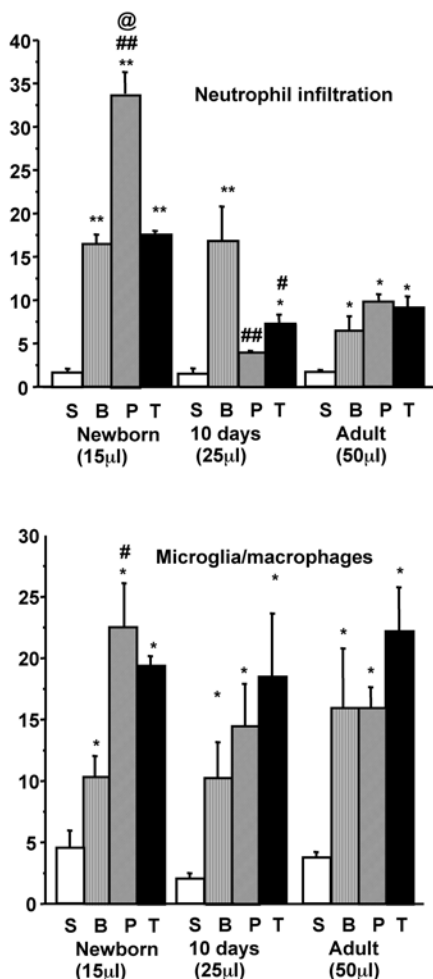


Figure 5. Bar graphs showing inflammatory cell infiltration (per $4 \times 540 \times 540 \mu\text{m}^2$ area) including neutrophils (upper panel) and microglia/macrophages (lower panel) in mouse striatum 48 hours after saline, blood, plasminogen, and thrombin injections. Rare inflammatory cells were apparent in saline control brains. Both age and treatment had significant effects on neutrophils (ANOVA $F[\text{age}] = 52.295$, $p < 0.0001$; $F[\text{treatment}] = 43.682$, $p < 0.0001$; $F[\text{interaction}] = 22.530$, $p < 0.0001$). RCA-1 labeling was influenced by treatment ($F = 13.620$, $p < 0.0001$) but not age ($F = 1.009$, $p = 0.375$) and there was no interaction effect ($F = 0.614$, $p = 0.717$). There was a significantly greater number of neutrophils following injection of plasminogen in newborn mice compared to 10-day-old and adult mice (@ $p < 0.001$, 2-way ANOVA using age as independent variable). There was no difference in the number of microglia/macrophages between the three ages. The quantities of neutrophils and microglia/macrophages were greater adjacent to the blood, plasminogen, and thrombin injection sites (* $p < 0.05$; ** $p < 0.01$, compared to saline, ANOVA with Scheffé post hoc intergroup comparisons). The number of neutrophils was significantly greater after plasminogen injection compared to blood injection in the neonatal group. The number of neutrophils was significantly lower after plasminogen and thrombin injection compared to blood injection in the 10-day-old group (# $p < 0.05$; ## $p < 0.01$, compared to blood, ANOVA with Scheffé post hoc intergroup comparisons). Abbreviations: S=saline, B=blood, P=plasminogen, T=thrombin.

brain in a matter of several hours (62) while dead neurons that appear hyper eosinophilic on H&E staining can persist for days or weeks (10). Furthermore, TUNEL positive cells include neurons, glia, and leukocytes (46). Following autologous blood injection into adult rat brain we found that most TUNEL positive cells are not neurons (18).

There are several caveats that must be considered in the interpretation of this experiment. First, we have no way of determining whether the proteolytic enzyme doses match the activity that actually occurs following hemorrhage. As indicated above, the purified enzymes were injected in the absence of control systems present in normal whole blood. Second, the plasminogen and thrombin that we used were bovine proteins; we cannot exclude the contribution of an immune response to foreign proteins, although we consider this unlikely. Third, the neonatal and young mice are very small and have soft skulls. Therefore, the method of blood injection was associated with some variability in terms of depth.

Nevertheless, our microscopic assessments of cellular changes were done in comparable regions of the striatum and therefore the quantification is likely valid. Fourth, we did not assess long-term outcomes to determine if the response in older animals is delayed, although based on our prior work we think that is unlikely (71, 72). Finally, the anesthetic methods differed between age groups. Ketamine used in the older animals is a potentially neuroprotective glutamate antagonist (50) while hypothermia used in the neonates is also potentially neuroprotective (6, 28). In the future we will likely use isoflurane anesthesia to improve consistency.

In summary, these results demonstrate that injections of blood, thrombin, and plasminogen into mouse brain are associated with cell death and inflammation in an age dependent manner. We postulate that the neonatal brain is more susceptible because the immature migrating brain cells and growing blood vessels, which normally utilize proteolytic mechanisms, are more sensitive to perturbations in the proteolytic environment. This observation complements Kolb's findings that the neonatal

rat brain recovers less well than juvenile or adult brains following cortical aspiration, presumably because developmental processes are interrupted (30, 31). Thrombin and plasmin in the blood might play an important role in premature neonatal brain injury that follows brain hemorrhage. They therefore represent potential targets for therapeutic intervention (70).

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