Effect of Destruction of Germinal Matrix by Intracerebral Hemorrhage in the Immature Rodent Brain

By
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Abstract

Germinal matrix hemorrhage (GMH) refers to bleeding that arises from the subependymal (or periventricular) germinal region of the immature brain. Clinical studies have shown that infants who experience GMH/IVH may develop hydrocephalus or suffer from long-term neurological dysfunction including cerebral palsy, seizures, and learning disabilities. The goals of the experiments described in this thesis are to investigate the genomic, cellular, and behavioral changes following GMH. The brains of newborn rats were used because they are developmentally comparable to human fetuses of 24-26 gestation and have a prolonged period of postnatal neurological development. We have described three important findings at the molecular, cellular, and neurodevelopmental levels that occur following injection of blood in the neonatal brain respectively. In the gene expression study we found that rat brains subjected to blood infusion overexpressed many genes compared to aged-matched controls and compared to brains of older rats that received blood injection. At the cellular level, following blood injection, germinal cell proliferation was suppressed. Using Ki-67 we have shown this suppression started at 8 hours and lasted up to 2 days. The suppression was also observed at the contralateral germinal matrix suggesting that a diffusible molecule is responsible. In the long-term behavioral study we also showed that blood injection into the periventricular tissue of neonatal rat results in both immediate and long-term behavioral abnormalities. We believe that these results are in the early stages of providing answers regarding the mechanism or consequences following blood injection.

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Abbreviations

BCL	B-cell leukemia/lymphoma
BNP	brain natriuretic peptide
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
cdks	cyclin dependent kinase
cDNA	complementary DNA
CNP	2',3'-cyclic nucleotide 3' phosphodiesterase
CNS	central nervous system
CP	cerebral palsy
CXN	connexin
DAB	3,3-diaminobenzidine
dATP	2'-Deoxyadenosine-5'-triphosphate
dCTP	2'-Deoxycytidine-5'-triphosphate,
dGTP	2'-Deoxyguanosine-5'-triphosphate,
DNA	deoxyribonucleic acid
DTT	1,4-Dithio-DL-threitol
dTTP	2'-Deoxythymidine-5'-triphosphate
ECM	extracellular matrix
EST	expressed sequence tags
GABA	gamma-aminobutyric acid
GFAP	glial fibrillary Acid Protein
GluR1	glutamate receptor 1

GluR2	glutamate receptor 2
GM	germinal matrix
GMH	germinal matrix hemorrhage
H&E	hematoxylin and eosin
HLA	human leukocyte antigen
ICH	intracerebral hemorrhage
IVH	intraventricular hemorrhage
LPS	lipopolysaccharide
MAG	myelin associated glycoprotein
MBP	myelin basic protein
MMPs	Matrix metalloproteinases
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance image
mRNA	messenger ribonucleic acid
NMDA	N-methyl-D-asparate
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PVH	periventricular hemorrhage
RT-PCR	realtime polymerase chain reaction
SCIP	transcriptional repressor of myelin-specific genes
SDS	sodium dodecylsulfate solution
SEH	subependymal hemorrhage

SSC	saline Sodium citrate Buffer
SVZ	subventricular zone
TGF-beta	transforming growth factor-beta
TUNEL	terminal deoxynucleotidyl transferase end labeling
VEGF	vascular endothelial growth factor
VZ	ventricular Zone

Chapter 1 General Introduction

Chapter 1 General Introduction

1.1 Introduction and definition of germinal matrix hemorrhage

The germinal matrix (GM), also known as subventricular zone (SVZ) is located along the walls of the ventricles of the developing brain. The last part of the GM to regress during the development is the anterior ganglionic eminence, a thick region of the SVZ over the caudate nucleus. This area receives arterial input from the recurrent artery of Huebner and the anterior choroidal and lateral striate arteries. It has conventional capillaries, but the post-capillary veins are unusually large. These veins connect with deep and superficial venous systems of the cerebrum. Following premature birth germinal matrix hemorrhage (GMH) may arise in the germinal matrix region adjacent to the lateral ventricles. GMH is often unilateral, or asymmetric, and is often associated with intraventricular hemorrhage (IVH) (Larroche, 1964; Kuban et al., 2001). Lateral extension of the germinal matrix hemorrhage is associated with destruction of maturing parenchyma, including the head of the caudate and internal capsule (Del Bigio, 2004). Clinical studies have shown that infants who experience GMH/IVH may develop hydrocephalus or suffer from long-term neurological dysfunction including cerebral palsy and seizures (Kakita and Goldman, 1999; Volpe, 2001b; Volpe, 2001a).

1.2 Germinal matrix hemorrhage in the clinical setting

GMH occurs primarily, but not exclusively, in premature infants of very low birth weight. Studies have shown that improved obstetrics and neonatology techniques have improved the survival of very low birth weight infants. However, such infants are at high risk for GMH. GMH is graded according to the extent of hemorrhage: Grade I, isolated

subependymal hemorrhage (SEH); Grade II, SEH with IVH but no ventricle enlargement; Grade III, IVH with ventricles enlarged; and Grade IV, IVH and SEH with extension into the brain beyond the ganglionic eminence which includes an extension of hemorrhage laterally into the striatal region (Papile et al., 1978).

Studies have shown that gestational age at the time of birth and survival period after birth are the some of the major determinants of GMH/IVH. A large-scale study by the National Institute of Child Health and Human Development reported a 45% overall occurrence rate for GMH in premature infants with low birth weights. Of the 2% of extremely premature infants, GMH occurs in ~40% to 50% of infants born at <26 weeks, in ~20% of infants born at 26-32 weeks, and in <5% of those born at 32 weeks and older (Berger et al., 1997; Larroque et al., 2003). Furthermore, GMH in premature infants usually develops during the first days of life especially in very preterm children. The highest risk periods are within the first 3 hours after birth, on day 2, and day 10 (Paneth et al., 1993).

1.3 Pathogenesis of germinal matrix hemorrhage

GMH/IVH has been attributed to multiple factors, including hypoxia, hypocarbia, circulatory failure resulting in cerebral venous congestion, arterial hypotension, hypertension, loss of autoregulation of cerebral blood flow, alkaline therapy, hyperosmolality, and intravascular volume expansion (Goddard-Finegold, 1989; Volpe, 2001b; Del Bigio, 2004). Although these factors play an important role in provoking bleeding, the germinal matrix is particularly vulnerable to insults because it contains immature blood vessel walls and supporting structures (Volpe, 2001b). Endogenous fibrinolytic activity in the GM has also been shown to play a role in vascular fragility

(Gilles et al., 1971). Ventricular enlargement due to obstruction that has been caused by clotted blood or debris can lead to complications such as hydrocephalus. In addition, a secondary white matter injury may occur in GMH/IVH infants (Volpe and Cosentino, 2000; Perlman and Wu, 2001).

1.4 Long-term consequences of germinal matrix hemorrhage

GMH can lead to severe neurological sequelae including seizures, mental retardation, and cerebral palsy (CP) (Hagberg and Hagberg, 2000). CP is a diagnostic term used to describe a group of motor syndromes resulting from disorders of early brain development. CP is normally caused by a broad range of developmental, genetic, metabolic, ischemic, infectious, and other acquired etiologies (Shapiro, 2004).

1.5 Animal Models of Germinal Matrix Hemorrhage.

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1.5.1 Introduction

Germinal matrix hemorrhage (GMH) refers to bleeding that arises from the subependymal (or periventricular) germinal region of the immature brain. The most prominent germinal zone in the second trimester of human fetal development is the ganglionic eminence, which lies over the head of caudate nucleus. GMH is a clinical problem in children born prematurely. Intraventricular hemorrhage (IVH) refers to bleeding that extends into the ventricles; these are usually extensions of GMH (Volpe, 2001a). Clinical studies have shown that infants who experience GMH/IVH may develop hydrocephalus or suffer from long-term neurological dysfunction including cerebral palsy, seizures, and learning disabilities (Allen and Capute, 1989; Rubin et al., 1992).

Gestational age at the time of birth is the most important determinant of GMH/IVH. Approximately 15% of all births are premature (<37 weeks gestational age) and ~2% are extremely premature (<31 weeks). The incidence and magnitude of detectable periventricular hemorrhage is an inverse function of gestational age (50.0% at 24-30 weeks of gestation compared to 1.6% at 38-43 weeks) (Berger et al., 1997). Diseases of prematurity such as respiratory distress syndrome, pneumothorax, and seizures are associated with GMH/IVH, which has in turn been attributed to multiple factors including hypoxia, hypocarbia, circulatory failure resulting in cerebral venous congestion, arterial hypotension/hypertension, loss of autoregulation of cerebral blood flow, alkaline therapy, hyperosmolality, and circulatory volume expansion (Volpe and Pasternak, 1977; Goddard-Finegold, 1989; Del Bigio, 2004). The germinal matrix is particularly vulnerable to insults because the venous structures are large, thin-walled and lacking in supporting matrix. Endogenous fibrinolytic activity in GM has also been

shown to play a role in vascular fragility (Gilles et al., 1971). Imaging studies, at the present time most commonly ultrasound, are the primary tools for diagnosis and grading of brain hemorrhage. The most commonly used grading system takes into account hemorrhage within the ganglionic eminence, blood in the ventricles and extension into brain tissue: Grade I, isolated subependymal hemorrhage (SEH); Grade II, SEH with IVH but no ventricle enlargement; Grade III, IVH with ventricles enlarged; Grade IV, IVH and SEH with extension into brain beyond the ganglionic eminence (Papile et al., 1978). GMH is typically unilateral and located in the anterior ganglionic eminence, medial to the head of the caudate nucleus. IVH can be complicated by hydrocephalus if the cerebral aqueduct or fourth ventricle outlets are occluded. Lateral extension of the matrix hemorrhage is associated with destruction of maturing parenchyma including the head of the caudate and internal capsule (Del Bigio, 2004). Hypoxic-ischemic damage in the white matter (i.e. periventricular leukomalacia) can also be complicated by periventricular hemorrhagic infarction (Volpe, 2001a).

1.5.2 Animal models of germinal matrix hemorrhage

Understanding the causative factors and the pathogenesis of subsequent brain damage is important if GMH/IVH is to be prevented or treated. Appropriate animal models are necessary to achieve this understanding. This literature review critically evaluates the animal models of GMH/IVH. As a starting point for the review we used the following PubMed search strategy: "(newborn OR neonatal) AND (animal [MH]) AND germinal AND (hemorrhage OR intraventricular OR bleed*)". We screened abstracts and used the "Related articles" search tool to find additional papers. Finally, after

reading the original papers, relevant cited papers that had not been revealed by the PubMed search were collected. Interspecies comparisons as well as the merits and limitations of the models are described below. There are several published abstracts that never evolved into full manuscripts or that could not be acquired {Feldman, 1982 #114; Goske, 1984 #112}; they are therefore not discussed below. It is important to note that hemorrhagic and ischemic conditions often coexist, indicating that one insult may predispose to the other or that the underlying risk factors are the same (Del Bigio, 2004). Although ischemia is a component, it is not a singular factor in GMH/IVH of preterm infants. Most animal models of neonatal brain ischemia are not associated with significant hemorrhage and therefore are not reviewed here. The reader is referred to previous reviews for discussions of these models (Raju, 1992; Painter, 1995; Roohey et al., 1997; Inder et al., 1999).

A number of animal species including rodents, rabbits, sheep, pigs, dogs, cats, and primates have been used to model GMH/IVH (Goddard-Finegold, 1989). Although GMH/IVH is may not an isolated lesion in humans, in order to reduce the number of variables in the experimental setting, it is useful to have isolated lesions in animal models. In these models, GMH/IVH has been induced via physiological manipulation or by blood injections. Pre- and postnatal animals have been used in accordance with their maturity at birth.

1.5.3 Rodents

Newborn rodent brains are developmentally comparable to 24 to 26 week gestational age human brains. As in the preterm infant at the end of the second trimester, neuronal genesis is complete in most regions (Dobbing and Sands, 1970b). A large amount of literature concerning rodent brain development is available. Importantly,

the GM region is well studied in rodents, making them an attractive model to study GMH (Sturrock and Smart, 1980; Kakita and Goldman, 1999; Zhu et al., 1999; Levers et al., 2001). Moreover, rodents do not exhibit spontaneous brain hemorrhage at birth. Rats are highly suitable for studying long-term behavioral changes, as their neurological outcomes are well documented (Fox, 1965; Altman and Sudarshan, 1975). We have developed GMH/IVH models in newborn mice and rats by injection of autologous blood into the subependymal region. Using these models we have been able to study the molecular and cellular processes that contribute to brain damage, as well as the long term neurological abnormalities that occur following GMH/IVH (Xue et al., 2003). Magnetic resonance images obtained immediately following the blood injection are used to assess the site and size of the hematoma. As in the human GM, the rodent GM shows a significant decrease in cell proliferation following GMH/IVH. This might have consequences on the subsequent production of glial progenitor cells. Newborn mouse brain damage caused by blood injection is aggravated when the systemic immune system is prestimulated with lipopolysaccharide (LPS) (Xue and Del Bigio, 2005a). This may have relevance to the epidemiologic association between amnionitis and cerebral palsy (Dammann and Leviton, 1997). The 2-day old mouse brain is also relatively more sensitive to the thrombin and plasmin components of blood than 10 day or young adult mouse brain, suggesting that extracellular proteolysis might be important in the pathogenesis of brain damage after GMH/IVH (Xue and Del Bigio, 2005b). Recently, we have assessed the long-term neurological status in rats following blood injection into the periventricular tissue of the neonatal rat; this results in developmental delay and long-term behavioral abnormalities (Balasubramaniam et al., 2006). This may represent a reasonable model of the cerebral palsy that follows GMH after premature birth.

Rats have also been used to study other disorders such as posthemorrhagic hydrocephalus, created by injection of blood into the lateral ventricles of 7-day old rats {Cherian, 2003 #3445; Cherian, 2004 #4345}. With respect to GMH, a potential limitation of this model is that 7 day old rats are more comparable to ~32 week gestation infants in which the GM has involuted. Mutant mice can be used to address the hypotheses concerning specific mechanisms of GMH. For example, alpha V integrin knockout mice develop spontaneous intracerebral hemorrhage (ICH) in utero, likely because endothelial cell adhesion is impaired (McCarty et al., 2002).

There are shortcomings in using newborn rodents as experimental models. The physiological and anatomical differences between rodent and human brains are substantial and it is very difficult to monitor the former. Furthermore, diffusion kinetics of blood components potentially differ between the small rodent brain and human brain. Nevertheless, this model is relatively inexpensive and is suitable to study long-term behavioral outcomes. Moreover, the presence of a prominent GM in the postnatal period make it an appropriate model to study cellular aspects of the pathogenesis of post-GMH brain damage.

1.5.4 Rabbits

Rabbits have been used because some aspects of the postnatal development of brain and GM are similar to that seen in fetal humans (Harel et al., 1972; Dawood et al., 1988). As in the human, the GM blood vessels are immature (Sotrel and Lorenzo, 1989). The 28-day gestation pre-term rabbit (full term 31-32 days) can survive outside the uterus because the respiratory system is fairly mature. A complicating factor is that rabbits may exhibit spontaneous IVH. For instance, rabbit pups delivered via cesarean

section 3-5 days before term showed bleeding in 20% (Lorenzo et al., 1982; Coulter et al., 1984). In contrast, another lab found that none of the 167 normal rabbits nor 45 rabbits with induced hypertension developed IVH (Coulter et al., 1984). Factors such as mode of delivery, clinical status, and breed differences may account for this discrepancy.

Conner and coworkers have shown that intracranial hypotension, induced by intraperitoneal injection of a hypertonic solution of glycerol, leads to GMH/IVH in 70% of gestational day 28 rabbits (Conner et al., 1983). The major flaw of this model, however, is the resulting diffuse hemorrhage in the subarachnoid and subdural compartments, choroid plexus, white matter, ventricles, cerebral cortex, and striatum. GMH has been created in minority of preterm and term rabbits following furosemide diuresis (Lorenzo et al., 1982; Greene et al., 1985; Lorenzo et al., 1989). Sodium bicarbonate hyperosmolality also causes ICH including GMH in a high proportion of term rabbits (Sugimoto et al., 1981)

1.5.5 Sheep

In comparison to humans, the sheep brain at birth, following a gestation of ~147 days, is relatively more mature. The germinal layer of the sheep fetus, at mid-gestation, resembles that of the 26 to 30 week human fetus (Astrom, 1967; Barlow, 1969). The vascular pattern of the germinal layer also resembles that of the human infant (Reynolds et al., 1979). Reynolds and coworkers temporarily exteriorized sheep fetuses at 58 to 85 days of gestation (Reynolds et al., 1979). GMH developed in 16/52 sheep after periods of arterial and venous hypertension with asphyxia. Many sheep also had multiple foci of hemorrhage including regions of white matter and cortex. Because the

sheep fetuses were still connected via the umbilical cord to the dam, the physiological changes might not be comparable to those that occur in preterm human infants; the sheep cannot survive independently at this age. Another group failed to create GMH or IVH in mid-gestation sheep subjected to hypoxia, hypovolemia, or hypervolemia, although they did observed marked hypoxic-ischemic injury (Ting et al., 1984). When pregnant sheep were subjected to 1-hour hypotension at 135/144 days gestation, 5/19 fetuses developed subarachnoid hemorrhages but not deep hemorrhages (Wheeler et al., 1979). Since most GMH occurs postnatally in premature infants, it is apparent that the use of surviving, prematurely delivered animals would constitute a more exact model. In this respect, it is difficult to use sheep.

Another potential limitation of the sheep, as in beagle pups (see below), is the presence of a carotid rete mirables, which is a plexus of arterial channels near the skull base that alters perfusion pressures in the brain. A lower proportion of cardiac output perfuses the brain in these animals than in humans (Cohn et al., 1974). Physiological studies on newborn sheep, while purported to yield information of value for understanding GMH (Ong et al., 1986; Gleason et al., 1989), are probably of minimal relevance to human GMH because the term sheep brain is quite mature with no GM and considerable myelin.

1.5.6 Pigs

Like the sheep, the pig cerebrum at birth is quite mature with no germinal matrix and extensive myelin deposition (Pond et al., 2000). Therefore, the experiments described below are likely more relevant to the pathogenesis of brain injury in infancy (i.e. up to 1 year of age) than to GMH and premature birth. Intraventricular injection of

autologous blood into neonatal pig brain was used by one research group to test spectroscopic diagnostic tools (Stankovic et al., 1998). However, histopathology was not presented in the publications; the one photograph simply shows blood in the anterior frontal white matter. Newborn pigs have been used to study the effect of subdural / subarachnoid hematomas on brain metabolism and function (Parfenova et al., 1993; Yakubu et al., 1994, 1995; Shaver et al., 1996; Yakubu and Leffler, 1996). The effects of brain hemorrhage on pulmonary function have also been studied. For example, artificial IVH in newborn pigs leads to suppressed respiration at least in part through elevated intracranial pressure (Farstad et al., 1994). Systemic vascular reactivity is impaired in this model (Pourcyrous et al., 1997a; Pourcyrous et al., 1997b).

1.5.7 Dogs

Beagle pups were used extensively in the 1980's to explore the pathogenesis acute of GMH/IVH. Dogs represent an appropriate model for two reasons. First, like the premature human, the GM layer, including the immature blood vessels, is substantial in the first few days of life (Goddard et al., 1980a; Goddard et al., 1980b; Ment et al., 1982b). It matures rapidly and involutes between 4 and 10 days after birth (Leuschen et al., 1984; Leuschen and Nelson, 1986; Trommer et al., 1987; Ment et al., 1991). Dogs have been used to study the physiologic factors that lead to GMH/IVH (Goddard et al., 1980a; Goddard et al., 1980b; Goddard-Finegold et al., 1982; Ment et al., 1982a; Johnson et al., 1987). Using term pups from 12 to 48 hours, Goddard's group showed that rapidly induced, moderate hypertension (one or two infusions of 0.1mg/ml phenylephrine hydrochloride) without hypercarbia produced GMH/IVH (Goddard et al., 1980b). Mean arterial blood pressure increased from 54 to ~ 82 mm Hg. Among the

hypertensive pups, 4 of 9 developed IVH and 7 of 9 had subependymal hemorrhages. In addition, 5 of 9 animals had hemorrhages at subependymal sites other than the ganglionic eminence. Whether hypertension is complicated by the coexistence of hypoxia, hypercarbia, acidosis, and ischemia was not clearly demonstrated. Goddard's group later used hypercarbia to cause GMH/IVH (Goddard et al., 1980a; Goddard et al., 1980b). The rationale was that increased CO_2 tension produces significant vasodilatation and increased cerebral blood flow in mature animals. They used 12 to 72 hour pups postnatally and subjected them to slow (10% CO_2) and rapid (75 to 79% CO_2) hypercarbia for 1 hour. They were able to demonstrate GMH/IVH (9/9 pups in both the slow and rapid groups developed GMH, 2 of 5 in the rapid hypercarbia group developed IVH) in the dogs. Goddard and coworkers then created another model of IVH, which involves acute hypovolemic hypotension followed by rapid autotransfusion of previously withdrawn blood (Goddard-Finegold et al., 1982). They correlated GMH with increased cerebral blood flow. However, a subsequent study refuted the role of ischemia or hypoxia in this model (Johnson et al., 1987). Johnson and coworkers claimed that pCO_2, blood pressure, and average temperature were the key discriminating factors that predicted whether an animal would hemorrhage or not. PVH appears to be mediated by regional increases in GM blood flow (Pasternak et al., 1983; Goddard-Finegold and Michael, 1984; McPhee et al., 1985; Pasternak and Groothuis, 1985; Leuschen and Nelson, 1989). Leuschen and coworkers also showed that premature (delivered 6 days early) beagles exposed to asphyxia developed periventricular hemorrhage PVH (Leuschen and Nelson, 1987).

Ment's group has used the dog model to study interventions to prevent GMH; these include indomethacin (Ment et al., 1983b; Ment et al., 1983a; Ment et al., 1984c),

ethamsylate (Ment et al., 1984b), and superoxide dismutase (Ment et al., 1985). Other groups have used the model to study phenobarbital (Goddard-Finegold et al., 1990)} and high frequency oscillatory ventilation (Tamura et al., 1992). In this regard the dog model is extremely useful. The large size and consistency allow dissection of the physiological variables.

There are several limitations to this dog model. Foremost is the inherent problem of standardization, as the precise time of bleeding is not known. Ment and coworkers showed that 15-20% of the control animals developed spontaneous hemorrhage (Ment et al., 1982b). Potentially one cannot determine whether the bleeding occurred during the baseline period or as a consequence of the physiological manipulation. Hemorrhage occurs at multiple sites in the GM; this is similar to the situation in humans in some circumstances (Del Bigio, 2004) but it makes quantitation of the bleeding difficult. In addition, as with all animal models, there are issues related to the human comparability. Histological studies show that the GM is actually quite meager in 2 to 3 day-old beagle pups, perhaps comparable to the GM of 32 week gestation human fetuses (Goddard et al., 1980a; Goddard et al., 1980b). There is also exists a carotid rete mirables, which modifies systemic effects on brain blood flow. Finally, because these experimental preparations include carotid and jugular cannulation and ligation, the model is somewhat impractical for long-term behavioral outcome studies despite the fact that dogs are good subjects in which to study motor and sensory behaviors (Breazile, 1978).

1.5.8 Cats

Although there is a considerable literature describing postnatal neuronal development in cats, especially using Golgi type preparations (Adinolfi, 1977), the GM of cats has not been clearly described. One may assume that cat and dog brains

probably develop in a similar tempo. Tuberville and coworkers showed that intraperitoneal sodium loading and resultant hypernatremia were associated with IVH (Turbeville et al., 1976). However, the applicability of this information to neonatal GMH is limited. This study did not demonstrate whether kittens bleed spontaneously, and the sites of hemorrhage were not clearly documented. More importantly, although this study claimed to model a neonatal disease, it reportedly used a range of animal weights that did not include the neonatal period (from 550 to 2500 g; i.e. >6 weeks of age to young adulthood).

1.5.9 Primates

The GM of the ~14- week gestational aged baboon is similar to that of a 24-week gestation human; it involutes by ~162 days (Bass et al., 1992; Dieni et al., 2004). Similar developmental changes were documented in rhesus monkey (Brand and Rakic, 1979; Lenn and Whitmore, 1985). In studies done several decades ago, premature monkeys inconsistently developed secondary periventricular hemorrhage in ischemic white matter (Brann and Myers, 1975; Myers, 1975). More recently, Dieni and coworkers studied 16 baboons that were delivered at 125 days gestation (full term ~184 days) and kept alive for 14 days in a neonatal intensive care environment similar to that used for humans; 2/16 baboons developed PVH and 3/16 had multifocal white matter bleeding. Despite the complexity and expense, this experiment is of obvious direct relevance to humans (Dieni et al., 2004).

1.5.10 Concluding remarks

The following points should be kept in mind when planning experiments of germinal matrix hemorrhage.

1) Developmental brain anatomy and physiology are not well documented in all species that are used to model GMH, making interspecies extrapolation of information difficult. The paucity of knowledge concerning the biochemical, neurological and functional aspects of animal models results in disagreement among researchers. Different parameters need to be considered. For instance, the peak growth (i.e. the rate of weight change) was considered by Dobbing and Sands as a basis for comparison three decades ago (Dobbing and Sands, 1970a). However, a review by Romijn showed that the relative rates of synaptogenesis and maturation of biochemical and electrophysiological properties in rat and human brain differ slightly; overall he considered that 12 to 13 day old rats are comparable to term infants (Romijn et al., 1991). One must consider carefully the state of maturity of the periventricular tissues if one desires to model GMH. Animal models should have a prominent GMH in locations comparable to that of humans i.e. the ganglionic eminence. There are obvious advantages to studying species those whose brains are less mature at the normal time of birth than humans, because their brains resemble those of premature humans who are at risk for GMH. However, there are discrepancies between the relative maturity of other organ systems, most obviously pulmonary, making one consider that other less obvious differences (e.g. endocrine / hormonal) might influence the presentation and pathogenesis of GMH in those animal models. Large animals with gyrencephalic brains probably have greater similarity to humans with respect to brain damage pathogenesis; however they are expensive and more complex than rodents.

2) Experimental GMH/IVH is induced under extreme physiological conditions, which are imposed on healthy animals to mimic the clinical scenarios of premature infants. The physiological parameters are controlled in order to understand the variables, but they may oversimplify the human situation. Hematomas created by stereotaxic injection of blood into the GM region do not mimic the human situation, but they do offer a simple way of understanding the pathogenesis of cellular brain damage that follows GMH. Ideally, hemodynamic changes and blood gases should be documented so that the complicating factors of hypoxia / ischemia can be considered. This is not possible in very small animals.

3) There is a general lack of understanding of the pathogenesis of brain damage that follows GMH in the immature brain. Our recent work (Xue and Del Bigio, 2005b), as well cortex aspiration models of brain injury (Kolb and Tomie, 1988), clearly show that the neonatal rodent brain responds differently than the 10 day rodent brain or the mature brain. In the period comparable to that at which premature infants can survive, the brain is not as adaptive to injury as was once believed.

4) Hemostasis and hemostatic proteins differ between the fetus (and premature infant) and the term infant (Manco-Johnson et al., 2002; Kuhle et al., 2003; Manco-Johnson, 2005). To date, there has been little consideration of how these differences might impact on the ultimate brain damage after hemorrhage.

5) Standardization of the initial magnitude of the brain damage makes long-term outcome studies easier. Initial hemorrhage sites and sizes should be documented and quantified by imaging modalities such as magnetic resonance or computed tomography when possible. Given the major concern of human neurological disability resulting from

GMH/IVH, the paucity of long-term behavioral and neuropathological outcomes assessed in animal models is surprising.

The perfect animal model of human GMH does not exist. It is clear that no single animal model will be suitable for the study of all physiological, developmental, pathological, and molecular aspects of GMH/IVH. Nevertheless, these models can, in combination, provide knowledge of the pathogenesis of GMH and be used to study preventative and ameliorative measures.

1.6 Cell Proliferation and Migration in the Germinal Matrix during Normal Brain Development

During the prenatal period of 22-32 weeks, when GMH commonly occurs, many important events take place in the central nervous system (CNS). These include a vast array of genes involoved in developmental events like cell proliferation, cell migration, synaptic formation, and myelination. In addition, very few studies have looked at pre- and postnatal neurological development, assessed by behavior alterations following GMH. In the following three sections, a focused review of cell proliferation, cell migration, and gene expression in relation to the normal germinal matrix region will be discussed. In addition, neurobehavioral changes that occur during early normal brain development will also be discussed.

1.6.1 Temporal Profile of CNS Development

In human fetal brain development, the first half of gestation corresponds to the period of neurulation, neurogenesis, and differentiation. The second half is characterized by growth of the cerebral hemisphere. The germinal matrix appears during 5th week and the ventricular zone appears in the 7th week (O'Rahilly and Muller, 1994; Bayer et al., 1995). Neuronal proliferation occurs from weeks 12-20. Glial cell generation begins around 25 weeks and continues into infancy (Dobbing and Sands, 1970a). The volume of the subventricular zone along the lateral ventricle peaks at 23-25 weeks gestation (Kinoshita et al., 2001). Preoligodendrocytes are the predominant cell type of the oligodendrocyte lineage in the early developmental period of 23-32 weeks gestation. SVZ involutes by ~38-40 weeks gestation (Volpe, 2001a; Volpe, 2001b).

Between 24-36 weeks, when the gross brain architecture is established, growth and differentiation events predominate and persist into postnatal life. Between 26-28 weeks, periventricular white matter is vulnerable and toward the end of this trimester gray matter seems to be more vulnerable to insults. Most neurons have been generated during 18-20 weeks gestation in humans, but neither their migration nor their synapse formation is complete. Between 26-28 weeks the GM continues to give rise to precursors of oligodendrocytes, which produce myelin, and astrocytes, which in turn support maturation of neurons, until it involutes (Volpe, 2001a; Volpe, 2001b).

Myelination occurs in a systematic fashion, beginning with the medial longitudinal fasciculus in the brain stem at the end of the first trimester and continuing until the end of second decade (Brody et al., 1987; Kinney et al., 1988). Myelination continues at least through the first decade of life, with intracerebral association bundles only appearing fully myelinated in the second decade (107).

In rodents, the brains of both 1 day old mouse and a 2-3 day-old rat are at an equivalent level of maturity to a 24-26 week-old human (rat and mouse gestational duration: 21-24 days). Unlike the human brain where neuron formation occurs between 12 and 20 weeks of gestation, in rats the neurons are mainly generated from days 10-20 of gestation. Oligodendrocyte precursors appear by 14 days gestation (Rao and Mayer-Proschel, 1997) ((Levine and Hoenig, 1968; LeVine and Goldman, 1988; LeVine and Macklin, 1990) and are predominant between postnatal day 1 and 5 (Gard and Pfeiffer, 1989).

1.6.2 Cell Proliferation

In the telencephalon two major proliferating zones are present: ventral (medial) and dorsal (lateral) ganglionic eminence which is a region of the subventricular zone (SVZ) of the ventral telencephalon, and ventricular zone of the dorsal telencephalon. Unlike the ventricular zone which reduces progressively and disappears after the completion of neuronal migration (~40 weeks), the SVZ remains longer and disappears by the age of 1 (Kriegstein and Noctor, 2004; Kriegstein, 2005).

The ventricular zone (VZ), which lines the lateral ventricles, is replaced at the end of neurogenesis by ependymal cells with limited proliferative capacity. The SVZ cells proliferate in situ without nuclear translocation. Unlike the VZ which generates mainly neurons, the SVZ generates predominantly glial cells and olfactory interneurons.

In the perinatal period the SVZ can be subdivided into several anatomical regions. The anterior SVZ is located (1.5 mm anterior to bregma) in the frontal horn of the lateral ventricle and it is the major source of the cells in the rostral migratory stream. The dorsolateral SVZ is a large group of cells situated at the dorsolateral aspect of the lateral ventricles at the level of the anterior commissure (0.8 mm anterior to bregma) . Cells from this area predominantly give rise to oligodendrocytes and astrocytes (Levison and Goldman, 1993; Luskin, 1993; Luskin et al., 1993).

Many of the anterior SVZ cells that are committed to become neurons become interneurons of the olfactory bulb (Luskin, 1993; Luskin et al., 1993). Some cells of the SVZ remain pluripotent in origin. Almost all of the dorsal SVZ cells that settle in the white matter differentiate into oligodendrocytes (Levison et al., 1993; Levison and Goldman, 1993). Some of the SVZ cells become specified as astroblasts and oligodendroblasts while others remain as glioblast until they migrate into the lineage of astrocytes or oligodendrocytes (Zerlin et al., 1995). Precursors of oligodendrocytes of

SVZ can generate three morphologically distinct types of oligodendroglia in the cerebrum: perineuronal satellite oligodendrocytes, perivascular oligodendrocytes and myelinating oligodendrocytes.

1.6.3 Cell Migration

Studies using retroviral markers have shown that most cells from the VZ, migrate using a radial pathway (Gray and Sanes, 1992). The radial glia has been established in all regions of the developing CNS. Approximately 80-90% of the post mitotic neurons migrate using the radial glia fibers which provide a scaffold for migrations in the brain (Sidman and Rakic, 1973; Rakic, 1978).The remaining 10-20% of the neurons move tangentially, but their function seems unclear (O'Rourke et al., 1997). Recent studies have shown that many of these cells become GABA-ergic inhibitory interneurons (Menezes et al., 2002).

The next question arises as to how axon growth and the migration pathways are established. This process involves a complex interaction between cell surface protein, extracellular proteins, and attractant and repellent signals secreted by target tissues (Dodd and Jessell, 1988; Tessier-Lavigne and Placzek, 1991). At the terminal end of developing axons are specialized structures called growth cones that exhibit a filamentous process, which extends and retracts continuously. The direction of the axon pathway is determined by the outgrowth of these processes. In the the process of developing axo-axo or axo-glial connections, there is an overabundance of neurons and synapse formed in the prenatal brain. A tremendous amount of pruning and reduction in neurons occur during maturation. For instance, 25-50% of the cortical neurons seen in a given layer die during the postnatal period (Windrem et al., 1988). Why some neurons

die and others survive is still unclear. It is possible that one reason is the failure to establish a proper connection leading to a lack of ability to understand the signal or to obtain growth factors.

During the rodent postnatal period (1-15 days) progenitors migrate radially out of the SVZ to the white matter and cortex as well as laterally through the white matter and radially into the lateral cortex and striatum to develop into glial cells. A proportion of the glial progenitors that migrate along radial glia remain in the white matter. Most cells generated in the SVZ migrate dorsally into the cortex and the ventral areas and to the thalamus via the internal capsule (Anderson et al., 1997). The migration of cells from the dorsal SVZ between day postnatal day 2-14 has been characterized using a retroviral-mediated gene transfer (Levison et al., 1993; Zerlin et al., 1995; Kakita and Goldman, 1999). At postnatal day 2, approximately 20% of cells preferentially colonize gray matter. Approximately 80% of their daughter cells migrate to the neocortex, 10% migrate to the white matter, and the remaining labeled cells migrate to either the striatum or to the border of the white matter and neocortex or striatum. Between a postnatal period of 2 and 7 days, another 40% migrate from the SVZ, and the remainder migrate over the following 2 weeks (Levison et al., 1993; Levison and Goldman, 1993). At postnatal day 14, cells predominantly colonize white matter. Of those postnatal day 2 SVZ cells migrating into the corpus callosum, the majority differentiate into cells with the morphology of myelinating oligodendrocytes. Of the retrovirally labeled progenitors that exit the postnatal day 2 SVZ and migrate into the neocortex, approximately 45% produce homogeneous clonal clusters of protoplasmic astrocytes, and approximately 30% produce homogeneous clonal clusters of oligodendroglia. Approximately 15% produce clonal clusters that contain both oligodendrocytes and protoplasmic astrocytes

(Levison et al., 1993; Levison and Goldman, 1993). Approximately half of the oligodendrocytes form early myelin sheaths within 1 month (Levison et al., 1993; Levison and Goldman, 1993; Parnavelas and Nadarajah, 2001).

The development oligodendrocytes and neurons are intertwined, as are the behaviors that are classically assigned to each cell type. For instance, a recent study has shown that when oligodendrocyte protein 2',3'-cyclic nucleotide 3' phosphodiesterase (CNP) was deleted, ultrastructurally normal myelin was seen, but progressive neuropathy developed (Lappe-Siefke et al., 2003). In another study, virally mediated oligodendrocyte ablation had a profound impact on neurons, including defects in migration, dendritic arborization, and axon fasciculation. These studies and others suggest that oligodendrocyte-neuronal interaction is important.

Transformation of radial glia into astrocytes is a complex process. Radial glia, a highly differentiated cell, transformed into astrocyte, which is a different cell type with its own morphology and function. This conversion process called transdifferentiation, which is considered a rare phenomenon in the developing brain and following injury (Landis et al., 1990; Chanas-Sacre et al., 2000). However, the true transformation of radial glia to astrocytes is still unclear. Some studies show that radial glial transformation into astrocytes occurs when the radial glia begin expression of intermediate filaments. Vimentin expresses prenatally in radial glial cells, whereas GFAP appears at a later fetal or early postnatal stage; it is relatively specific for astrocytes (Pixley and de Vellis, 1984; Voigt, 1989). Other studies have shown that if an immature SVZ cell leaving the germinal matrix encounters an endothelial cell in the first few days after birth, when angiogenesis occurs at it peak rate, it differentiates into a protoplasmic astrocyte. It

then makes a connection with the blood vessel and begins to ensheath the blood vessel, forming the classic end-foot of an astrocytes (Zerlin et al., 1995).

Astrocytes play significant roles in the CNS. These include the control of fluid movements between the intracellular and extracellular space, the ability to take up glutamate and reduce excitotoxicity, and a role in spatial buffering potassium, neurotransmitters, and debris (Anderson and Nedergaard, 2003). Astrocytes are interconnected via gap junctions that allow the redistribution of potentially detrimental ions and metabolites over long distances. The interchange of many metabolites and intercellular messengers between astrocytes and neurons is complex and remains incompletely understood (Fields and Stevens-Graham, 2002). It has also been shown that astrocytes interact closely with oligodendrocytes. For instance, astrocytes promote oligodendrocyte precursor proliferation. Signals that regulate the terminal differentiation of oligodendrocytes and astrocytes are poorly understood but significant progress has been made in recent years.

The origin of microglia is probably the most controversial issue in glial development. Cajal (1913) first described the existence of a cellular "third element" that exists in the central nervous system besides neurons and macroglia. The view that microglial cells are derived from monocytes was proposed by Santha and Juba in 1933 (Rezaie and Male, 2002). An alternate, less well accepted view is the hypothesis that microglial cells are derived from neuroepithelium. Using lipocortin-1, which labels the neuroepithelial cells they were able to label microglia. (McKanna, 1993; Fedoroff et al., 1997). Other immunohistochemical studies using different antibodies that labeled microglial cells as well as astroglia of neuroectodermal origin also favored the neuroectodermal origin of microglia (Dickson and Mattiace, 1989). It has been shown

that human brains between 4.5-30-weeks are associated with negligible microglial reaction in the germinal region (Rakic and Zecevic, 2003), thus microglial proliferation, migration, and differentiation will not be discussed here.

1.7 Gene Expression Analysis

1.7.1 Methods of Gene Analysis

Although gene expression can be analyzed using standard molecular biology techniques such as Northern blot, RNase protection assay, semiquantitative RT- PCR, and real-time/quantitative PCR to examine the individual genes, a global analysis of mRNA is needed to appreciate fully the complex pathophysiology of disease states. In 1985, Polsky-Cynkin et al, published the first study of immobilized DNA for large scale hybridization (Polsky-Cynkin et al., 1985). Although this methodology had several limitations, it was a landmark study which opened the possibility of studying gene expression in a global scale using techniques such as microarray. Unlike older assays, DNA microarrays offer the possibility of simultaneously interrogating thousands of genes which can offer a genome-wide snapshot of gene expression levels in a given condition. This technique is consists of a library of genes, immobilized on a membrane or a glass slide.

The basic protocol involves the hybridization of complementary strands of labelled RNA from tissue with representations of known genes spotted onto a solid support (nylon, glass). Labelling can be radioactive ($^{32}p/^{33}P$), by a hapten group (biotin, digoxigenin, aminoallyl) or by fluorescent (Cy3, Cy5 etc.) nucleotides. Detection is performed by autoradiography, chemiluminescence or fluorescence scanning. Ideally, the intensity of the signal can be correlated with the relative expression of a known gene and allows the comparison with a standard.

1.7.2 Gene Expression Profiling of Developing Brain

It is important to establish a strong relationship between gene expression and brain development. There are about 30,000 different genes in higher mammals, and of them, 30-60% are unique to the brain. The developing brain requires numerous signals for the correct spatial and temporal expression of transcription factors and other genes that regulate the birth and migration of the neurons, oligodendrocytes, and astrocytes of the SVZ (Dirks and Rutka, 1997). Gene expression is important in brain development, especially in the germinal region where constant cell proliferation occurs. However, very few studies have looked at the gene expression profile of this region.

A close tight control of cell cycle progression is a pre-requisite for the generation of appropriate cell fates during the many developmental steps that transform proliferating, undifferentiated tissues into fully differentiated and functional brain structures. The cell cycle in brain can be divided into a number of distinct phases: mitosis (M), first gap (G_1), DNA synthesis (S), and second gap (G_2). These phases are regulated by key molecules to check the advancement of proliferation. During the cell cycle, a cell passes sequential checkpoints to determine whether the process of cell division should occur or not, when to divide and when not to divide. Almost all cell cycle proteins are controlled at the transcription level (i.e. at the mRNA level) rather than at the translation level (i.e at the protein level). However, during S phase of the cell cycle, proteins associated with DNA synthesis are fairly stable and persist throughout the cell cycle. Entry into, and progression through the cell cycle is determined by members of two distinct families of protein, i.e. cyclins and cyclin dependent kinases (cdks). The genomic expression of cyclins and cdks are highly regulated as these proteins are rapidly destroyed before the new cycle begins (Pines and Hunter, 1989). Gene products of cdks can be grouped

according to their roles at different phases of the cell cycle. G1 cdks are cdk2, cdk4, and cdk6; the S phase cdk is cdk2; and the M phase cdks include cdk2 and cdk1. Cyclins DI, D2, D3, C, and E seem to control the key transition of a cell to the G_1–S interface specifically Cyclin E plays the gatekeeper role for this transition and is essential for movement from G_1 to S phase (Evans et al., 1983). These checkpoints are highly controlled by gene expression. For example, yeast studies have shown that the product of the cdc2 gene, called p34 cdc2, must be phosphorylated to pass from the G1 to the S phase. Numerous genes, however, regulate the phosphorylation of these proteins (Lee and Nurse, 1988; Lee et al., 1988). In addition to the cell cycle regulators that are subject to phase-specific restriction of transcription, many of the structural and enzymatic activities that are involved in the cell duplication process are also regulated at the transcription level. For instance, transcription of histone genes, the products of which form nucleosome complexes on newly synthesized DNA, are tightly coordinated and confined to S phase of the cell cycle.

There are very few data available concerning the transcription regulation of cell cycle and migration of germinal matrix cells. Gene expression mechanisms of cell proliferation of other CNS regions, e.g. spinal cord, has been studied extensively. As in germinal matrix, spinal neurons are generated early during neural tube development; glial cells are generated later. The progenitors of different species of neurons—motor neurons or interneurons—are formed within their own specialized neuroepithelial domains. Since in many ways the properties of cell proliferation are similar to that of germinal matrix, it is a good system to understand genomic regulation of cell proliferation. During the early development of the spinal cord, several homeodomain transcription factors are expressed in the ventral region, where most of the neurons and

oligodendrocytes are generated. The process of their regulation in a time dependent manner might explain the spatial and temporal complexity of gene expression during cell proliferation. For example, bHLH transcription factors, Olig1 and Olig2, were identified in connection with oligodendrocyte progenitor production (Lu et al., 2000; Takebayashi et al., 2000; Zhou et al., 2000). It has also been shown that Olig2 is crucial for neuronal proliferation. Switching from neuronal to glial cell production is complicated and occurs mainly at the transcriptional level. Shortly after neuronal production is completed, the pro-neuronal bHLH factors are repressed. Then bHLH transcription factors undergo frame shift transcription to produce oligodendrocytic precursor specific Olig 2 (Miller, 1996). There is also little information on what triggers a neuron to become post-mitotic. Hippocampal studies have shown that from embryonic day 16 to postnatal day 1, almost all of the neurons switch from a highly active proliferation state to a post-mitotic state. As such, expression of proliferative genes involved in cell cycle progression are highly expressed at embryonic day 16, then subsequently become silent or reduced significantly after birth (Mody et al., 2001). It is possible the regulation of the germinal cells undergo similar mechanisms during early development.

Gene expression related to neuronal integration and synapse functions is extremely complicated in early brain development. For instance, to form and release a vesicle, some of the known gene expression changes that occur are as follows. For forming a synaptic transmitter, neurons need to produce components of vesicles such as clathrin, which is part of the coat that surrounds vesicles, and synaptogamin, a vesicle-associated protein involved in the calcium-mediated release of neurotransmitters. The process of forming a neurotransmitters also needs carrier proteins such as VAMP2, synaptophysin, and UNC-18. In addition, what controls the

release of these vesicles is also complicated. For vesicular trafficking, the gene expression of several presynaptically-released neuromodulators includes chemokines and neurotrophins are needed (Walton et al., 1999). Furthermore, in order to convey a message from one neuron to another, these vesicles need expression and translation of appropriate receptors on the post-synaptic terminal. For the neurotransmitter glutamate, transcription and translation of post-synaptic receptors include glutamate receptor 1 (GluR1), glutamate receptor 2 (GluR2), and the NMDA receptor are required. Many studies have shown that these receptors are not constitutively produced, but rather are controlled by developmental gene regulation (Dingledine et al., 1999; Myers et al., 1999).

Gene expression related to migration has been studied to some extent. A number of studies have examined molecular relationship between the radial glia and migrating neurons. In the mouse that lacks astrotactin a gene which is involved in neuro-glial interaction and provides a neural receptor for the migration along the glia, migration and the neuronal interaction to radial glia is absent (Fishell and Hatten, 1991). In addition, astrotactin mRNA has been shown to be developmentally regulated with high levels in the early developmental period (Kuhar et al., 1993; Zheng et al., 1996). Using real-time video microscopy, Suzuki and Goldman have shown that neuronal progenitors and glial progenitors migrate in distinct pathways to exit SVZ (Suzuki and Goldman, 2003). This implies that different cells require different cues to migrate. Some of these migration signals have been characterized and they include the sonic hedgehog, bone morphogenic protein, Olig 2, and Pax6 (Kessaris et al., 2001). To date, only scant information about the gene expression, regulation, and processing of these genes in relation to migration is known. It has also been shown that genes that are expressed

during cell proliferation can affect migration. Interruption to gene cdk transcription can lead to abnormal corticogenesis in mice (Ohshima et al., 1996). Another study has shown that mice with abnormal p53 gene transcription showed abnormal cell migration (Fulci and Van Meir, 1999).

Lists of known genes that are involved in cell cycle, migration, and integration are plentiful and cannot be reviewed here. Considering, however, the complications involved in the cell cycle and migration as well as other important components of development, such as cell death, synapse and myelin formation, it is safe to speculate that gene expression during brain development does not occur one gene at a time, but rather by an plethora of genes that are expressed and regulated in an accurate temporal and spatial manner. DNA microarray studies have opened the way to understanding molecular and genetic programs underlying the mammalian brain development.

1.7.3 Gene expression changes after brain injury

Prior microarray studies of the adult rat hippocampus 4 to 72 hours after global ischemia revealed changes in genes related to protein synthesis, pro- and anti-apoptosis, ion channel and receptor expression, repair of enzymes, and injury response (Jin et al., 2001). The adult rat brain 24 hours after ischemia or ICH exhibited upregulation of genes related to immune processes, cytokines, and heat shock proteins among others (Tang et al., 2001). We have previously shown that the genomic responses of rat brain to hydrocephalus differ according to age (Balasubramaniam and Del Bigio, 2002).

Although adult brain studies have looked at gene expression using DNA microarray technology, assessment of gene expression using this technology following

neonatal brain development or injury has been largely ignored. In hope of finding information with regards to gene expression in the neonatal brain development, we used the following PubMed search strategy: "(Microarray or affimetrix) AND (rat or mouse or rodent) AND brain AND (neonatal or postnatal or embryo)." We found 18 papers, only 6 of which were remotely related to our interest (Labudova et al., 1999; Mody et al., 2001; Poguet et al., 2003; Hu et al., 2004; Kisby et al., 2005; Matsuki et al., 2005). These papers examined topics ranging from the gene expression of embryonic stem cells as well as developmental changes of hippocampus and cerebellum to the effects neurotoxins and thyroid hormones on brain development. This emphasizes the lack information on normally developing brains; without that information, gene expression following injury is difficult to study.

1.8 Behavioral aspects of normal brain development

Behavior learning is observed very early on in the development of human infants and in animals. Since behavior is the aspect of postnatal brain development that is largely monitored in the clinical scenario, it is important to understand the corresponding behaviors in laboratory models. Human infants have a prolonged postnatal developmental period, unlike most laboratory models such as rodents, kittens, dogs, and rabbits. Therefore comparing human behavioral development to these models is complicated. However, considering that most mechanisms of injury are studied using rodent animal models, it is important to establish a base line connection between early human and rodent behavior. In order to make a fair comparison between newborn humans and rodents, we have gathered information on sensorymotor and cognitive development, as summarized below.

1.8.1 Behavior in preterm infants

Although my interest is to understand the long-term consequences of premature infants, as a starting point we needed to establish behavior in preterm infants. Of all endogenously generated movement patterns in fetuses and infants, spontaneous gross motor movements occur frequently and are the most complex. These include complicated movements of the arm, leg, head and trunk, and rotations superimposed on flexion and extension, all of which are varied by speed, amplitude, force and intensity (Cioni et al., 1997). Gross motor movements can be observed in fetuses as early as 9 weeks of gestation (de Vries et al., 1982, 1986) and continue in a similar pattern until about the end of the second month postnatally.

Gross motor movement has shown to be reduced or abnormal following perinatal injuries such as periventricular leukomalacia and germinal matrix hemorrhages (grade 1 or 2). In these infants the gross motor movements have a monotonous character with reduced complexity and variability (Ferrari et al., 1990; Prechtl, 1990, 1997). It has been shown that two specific abnormal gross motor movements reliably predict cerebral palsy in later life: firstly, a persistent pattern of cramped and synchronized gross motor movement, i.e. movements that tend to be rigid and abnormal in character, with the limb and trunk muscles contracting and relaxing almost simultaneously. Secondly, "fidgety movements" — small movements of moderate speed with variable acceleration of neck, trunk, and limbs in all directions—are absent. Serial neuroimaging techniques have also been used to diagnose neurological behavior and are routinely performed as part of medical care (Bozynski et al., 1985; Pierrat et al., 2001; Hyman et al., 2005). However, these studies have been criticized in that none of the developmental scales or

instruments truly measures developmental delay and predicts long-term consequences in preterm and term infants. (Illingworth, 1987; Majnemer and Mazer, 2004).

1.8.2 Postnatal human behavior

Between 3-7 months, human infants can hold their shoulders and abdomens off the floor with their forearms, and by 7 months they can balance their weight on one hand. They develop crawling behavior by 9 months, and walking begins by 13 months and develops into an adult-like motor pattern by 3 years (Illingworth, 1986b, a).

Between 0-2 months, reflexive behavior is used as a major measure of developmental milestones (Bower et al., 1979; Sheppard and Mysak, 1984; Connolly, 1985). These reflexes include response to touch, palmar finger grasp, and automatic walking when held upright. Palmar reflex grasping is present at birth and disappears at around 2 months of age. By 5 months infants develop reaching behavior, and by 8 months they can accurately grasp an object. Stable manual dexterity begins at 15 months.

Human infants begin cognitive development a few weeks after birth. Between 1-2 months, infants begin to smile and vocalize at 3 months, open their mouths in response to the feeding bottle by 6 months (Illingworth, 1986a, b). Studies have also observed cognitive and learning behaviors early on in the infant's life. In a study where it uses condition stimuli to stimulate blinking, when neonates were re-tested, significant retention was observed in neonates trained at 20 and 30 days of age, but not at 10 days (Illingworth, 1986a, b).

1.8.3 Early Rat Behavior

Ambulation, surface righting, and negative geotaxis are among the earliest motor developmental milestones in rats (Bolles and Woods, 1964; Smart and Dobbing, 1971; Altman and Sudarshan, 1975; Westerga and Gramsbergen, 1990). By postnatal day 4, rat pups are able to ambulate and pivot, using their forelimbs, torsos, and heads. This behavior peaks at postnatal day 7 and disappears by postnatal day 15. Reflex behaviors such as the righting response emerge by postnatal days 1 and 3 days and mature during the first week of postnatal development (Smart and Dobbing, 1971; Bignall, 1974). Between postnatal days 9 and 11, rats develop negative geotaxis where they gain the ability to orient themselves upward on an inclined plane. The air righting reflex (free fall) can be detected between postnatal days 9 and 18 (Unis et al., 1991; Vorhees et al., 1994). Rats develop adult-like locomotion shortly after developing full vision around postnatal day 16. Around the same period rats can stand on their hind limbs while leaning on their forelimbs for support (Bolles and Woods, 1964).

Fine manual dexterity is difficult to assess in immature rats and is considered an adult behavior. Techniques such as skilled reaching and wire climbing are used to study fine motor tasks and are in the early stages of providing insight. Whishaw et al. have studied a skilled reaching task that involves single pellet retrieval through a narrow slit. The trained rats have shown long-range of limb movement and accurate ability to reach for different distances and directions to grab tiny pellets (Ballermann et al., 2000; Whishaw, 2000).

Learning capabilities in rats have been shown as early as in the fetal period. Smotherman and Robinson (1994) demonstrated that exteriorized rat fetuses have the capability for rapid associative learning (Smotherman and Robinson, 1994). Johnson and Hall demonstrated that 1 day-old pups are capable of discriminating between 2

paddles with different smells when they are given rewards (Johanson and Hall, 1979). Learning and memory seemed to improve in an age-dependent manner. For example, in an active avoidance test for neonatal rats aged between 7-12 days, older rats showed superior retention while 7 day-old pups underachieved in all performances (Spear and Smith, 1978). This is possibly attributed to the lack of development of a sensory system, such as auditory and visual capabilities, by 7 days.

1.9 Summary

Clinical evidence shows that germinal matrix hemorrhage can lead to severe neurological dysfunction. When it develops during a critical period of brain development (22-32 weeks), it possibly interrupts normal brain development, including complex long-term neurological processes. Understanding the causative factors and the pathogenesis of subsequent brain damage is important if GMH/IVH is to be prevented or treated. Appropriate animal models are necessary to achieve this understanding. Newborn rat brains are developmentally comparable to the 24 to 26-week gestational age of human brains. As in the preterm infant at the end of the second trimester, neuronal genesis in these rat brains is complete in most regions. Importantly, the germinal matrix region and cell proliferation / migration are well studied in rats. In addition, rats are highly suitable for the study of long-term behavioral changes, as their neurological outcomes are well characterized.

1.10 General Hypothesis and Specific Aims

Since the level of maturity of the one to three day-old rat brain is comparable to that of 24-26 gestaional week humans (Sturrock and Smart, 1980; Kakita and Goldman, 1999; Zhu et al., 1999; Levers et al., 2001), these animals were used to characterize GMH. We have previously studied the effects of blood products and immune responses in the periventricular hematoma model of neonatal mice (Xue et al., 2003; Xue and Del Bigio, 2005c). Here, we studied rats, because the brain is larger than that of mice and because the behavioral outcomes are more readily assessed. Using the rat model of periventricular hematoma we wanted to further our knowledge of cerebral injury

following blood injection. We predicted that blood injection into the periventricular region would lead to genomic changes leading in turn to cellular changes which would lead to long-term neurological changes.

1.10.1 HYPOTHESIS 1: The genomic response of the newborn rat brain to blood infusion differs from that of the mature brain.

Rationale for Hypothesis 1

Considering that the germinal matrix continues to give rise to precursors of oligodendrocytes, which produce myelin, and astrocytes (Volpe, 2001b) and that infants who had GMH develop severe neurological deficits, it is important to understand the genomic cellular responses following blood injection. Prior microarray studies of adult rat hippocampus 4 to 72 hours after global ischemia revealed changes in genes related to protein synthesis, pro- and anti-apoptosis, ion channel and receptor expression, repair enzymes, and injury response (Jin et al., 2001). The adult rat brain 24 hours after ischemia or ICH exhibited upregulation of genes related to immune processes, cytokines, and heat shock proteins, among others (Tang et al., 2001). Immature brain hemorrhages have not been studied.

Considering the fact that germinal tissue in the newborn brain is undergoing rapid changes associated with development, the consequences of hemorrhage in the newborn brain may differ from those in the mature brain. The reason for this may be that the newborn rat brain is undergoing anatomical organization and physiological initiation of various brain systems, which have already been completed in the more mature brain.

Aim 1: To determine age-dependent gene expression changes following ICH in newborn, young, and adult rats.

Previously, we have shown age-dependent histological responses to blood products using our periventricular hematoma in the mice model. We predict that at the genomic level, response to hemorrhage by the newborn brain will differ from the mature brain.

Aim 2: To correlate the newborn rat brain gene expression changes with protein changes.

The proteins are the functional counterparts to genomic sequence information, in this study we used antibody microarray to correlate gene expression changes with protein changes in the newborn rat brain following blood injection.

1.10.2 HYPOTHESIS 2: Blood infusion causes regional damage to germinal brain tissue with permanent effects on the brain.

Rationale for Hypothesis 2

In mice (Xue et al., 2003) and in human infants (Del Bigio et al., 2003), we observed decreased proliferation in the germinal matrix near the site of GMH. We predicted that periventricular GMH will prevent proliferation and migration of glial precursors, and have deleterious effects on myelin formation and neuronal development. The germinal tissue, where cell proliferation occurs and the majority of brain cells are born, involutes before maturity. Furthermore, myelin production, cell migration, and synapse formation are not complete until maturity. In the rat, myelin formation begins after the period of cellular proliferation and migration and extends into

adulthood. Jacobson has shown that myelin content increases 1500% between 15 days and 6 months after birth in the rat brain (Jacobson, 1963).

Periventricular hemorrhage (PVH) in the premature infant brain is often associated with developmental delay and persistent motor deficits CP. CP is a diagnostic term used to describe a group of motor syndromes resulting from disorders of early brain development. Considering the fact that perinatal brain injury is among the most prevalent and costly form of neurological disabilities (Rubin et al., 1992; MacDonald et al., 2000), it is important to understand the brain damage mechanism and behavioral consequences that follow PVH.

Aim 1: To determine the effect of neonatal ICH on germinal matrix cell proliferation in rats and in mice.

The proliferating germinal cell layers are of interest because they are vulnerable to a variety of neonatal brain injuries. Although most neurons have been generated during 18-20 weeks gestation in humans, neither their migration nor their synapse formation is complete. The germinal matrix continues to give rise to precursors of oligodendrocytes, which produce myelin, and astrocytes, which support maturation of neurons (Volpe, 2001b; Volpe, 2003), until it involutes at approximately 30-32 weeks of gestation (Del Bigio, 2004). Pre-oligodendrocytes are the predominant cell type of the oligodendrocyte lineage in the early developmental period of 23-32 weeks gestation, when most GMH/IVH occurs. The brains of both a day-old mouse and a 2-3 day-old rat, are at an equivalent level of maturity to a 24-26 week-old human. Rodent brains are therefore useful for the study of prenatal injuries such as GMH.

Aim 2: To determine the effect of neonatal ICH on long-term behavioral and structural outcome in rats.

The postnatal period from day 1 to day 14 involves corresponding rapid changes in brain structure (Eayrs and Goodhead, 1959a; Davison and Dobbing, 1966). Thus, it is likely that insults, such as blood injection, can interrupt the developmental processes necessary for the timely acquisition of behavioral skills. To correlate function with structure, rats were subjected to a variety of behavioral studies. Very young animals were examined for pivoting, righting response, and negative geotaxis as an index of motor developmental delay (Altman and Sudarshan, 1975). When selecting adult behavior tests, the following points were considered: 1) this model produces a predominantly unilateral lesion, therefore behavioral tests sensitive to lateralization were chosen, 2) considering the site of injury, we expected sensorimotor deficits with minimal adverse effect on learning and 3) because training could affect brain recovery, behavioral tests were organized so that non-trained "automatic" behaviors were assessed first (e.g. cylinder exploration and tape test), followed by more complex behaviors that require minimal learning (walking on a rotating cylinder and ladder) and finally the skilled reaching test, which requires training.

Chapter 2.1 (See hypothesis 1, aim 1 and 2)

Analysis of newborn rat striatum following intracerebral hemorrhage/germinal matrix hemorrhage using cDNA microarray and antibody array techniques.

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Note:. Blood injections in this project were done by Dr. Xue

ABSTRACT

INTRODUCTION: GMH is a common occurrence in premature infants. Neurological outcome following brain injury is dependent on the age at the time of injury. We hypothesized that the newborn rat brain would react differently than mature rat brain in response to GMH at the level of gene expression. We used an antibody-based protein detection array to corroborate the findings.

METHODS: Newborn (24-36 hour), young (7 day) and adult (9-10 week) male Sprague-Dawley rats underwent injection of autologous blood into the striatum. Striatal tissue adjacent to the hematoma was subjected to cDNA microarray analysis after 24 hours and to antibody array analysis after 48 hours.

RESULTS: After blood injection, newborn brains overexpressed 547 genes, many of which are important in brain development, inflammation, proteolytic enzyme activity, and extracellular matrix dynamics. Diverse protein overexpression was confirmed in newborn blood injection rats using antibody microarray analysis. There was no comparable overexpression in young and adult brains. However, young and adult underexpressed 462 genes that was common to both groups after blood injection.

CONCLUSION: At the level of gene expression the newborn rat brain reacts very differently than 7 day and adult brains in response to blood injection. These findings demonstrate the need to study the newborn brain separately from the mature brain.

INTRODUCTION

In premature infants GMH/IVH is a common occurrence (Robertson et al., 1998). Lesions of this type can be associated with poor developmental outcome including hemiplegic cerebral palsy and mental retardation. Furthermore, prognosis following traumatic brain injury appears to be worst in children under the age of 4 years (Levin et al., 1992). Experimental studies have shown that plasticity following cortical ablation is not very effective in newborn rodent brains, likely because normal developmental processes are interrupted (Kolb et al., 1986).

Intracerebral hemorrhage causes brain damage through a cascade of events that initially involves direct tissue destruction. This is followed by secondary damage to tissue through (a) proteolytic activity of enzymes that are involved in blood clot formation and lysis, (b) inflammation that includes lymphocytes, neutrophils, activated microglia, and cytokines, and (c) edema. Although some of these mechanisms have been experimentally studied in adult brains, neonatal brain hemorrhage has been largely ignored experimentally with the exception of studies that focus on physiological factors that predispose to hemorrhage (Goddard-Finegold, 1989). Considering the fact that germinal tissue in the newborn brain is undergoing rapid changes associated with development, the consequences of hemorrhage in the newborn brain may differ from the mature brain. Because molecular changes following blood injection might serve as potential targets for therapeutic intervention we assessed the gene expression changes using DNA microarray screening. One to three day old rats are comparable to 24-26 week gestational age human brains (Sturrock and Smart, 1980; Kakita and Goldman, 1999; Zhu et al., 1999; Levers et al., 2001), a state of maturation prone to periventricular hemorrhage. Prior microarray studies of adult rat hippocampus 4 to 72

hours after global ischemia revealed changes in genes related to protein synthesis, pro- and anti-apoptosis, ion channel and receptor expression, repair enzymes, and injury response (Jin et al., 2001). Adult rat brain 24 hours after ischemia or ICH exhibited upregulation of genes related to immune processes, cytokines, and heat shock proteins among others (Tang et al., 2001). We hypothesized that at the level of gene expression newborn rat brain would react differently from young and adult brains following blood injection. We have previously shown that the genomic responses of rat brain to hydrocephalus differ according to age (Balasubramaniam and Del Bigio, 2002). Typically, cDNA microarray studies need to be confirmed using in situ hybridization or RT-PCR of a few selected gene products. Because proteins are the functional counterparts to genomic sequence information, in this study we used newly developed antibody microarrays to correlate gene expression changes with protein changes in the newborn rat brain following ICH.

MATERIALS AND METHODS

Animals

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. Newborn (24-36 hour), young (7 day) or young adult (9-10 week) male Sprague–Dawley rats were used. The approximately comparable human ages are 24 weeks gestation, 32 weeks gestation, and second decade respectively (Bayer et al., 1995). Rats were anesthetized with pentobarbital (50 mg/kg intraperitoneal). Adults and 7-day-old rats were placed in a stereotactic frame. For adults, a midline scalp incision was made and a hole was drilled in the skull (3 mm lateral to midline, 0.02 mm anterior

to coronal suture). Seven-day old rats were injected percutaneously. Newborn rats, which could not be secured in the frame, were injected freehand with the needle inserted percutaneously with adjusted coordinates using the eye as a reference point. Autologous whole blood was collected in a sterile syringe by placing the tail in warm water for 5 minutes, cleansing the skin with 70% alcohol, and cutting the tail tip. A 25-gauge needle was attached and quickly introduced into the striatum. Blood was injected (newborn 15µl, young 25µl and adult 50µl), over 1-2 minutes; except in the newborns, the needle was left in the place for 3 minutes and then removed slowly. Although a 15ul blood injection into neonatal brain may seem large in proportion to brain weight (15µl in 0.25g vs. 50µl in 2g), much of the injected blood escapes into ventricles and therefore the quantity retained in the striatum was considered comparable. The bone hole was sealed with bone wax, the scalp wound was sutured, and the animal was placed in a cage with free access to food and water. Newborn and young rats were returned to the mother. Although the observed responses are due to a combination of injury by needle insertion, space-occupying effect of injected blood, and the "irritant" or "toxic" effect of the blood itself, we very deliberately chose not to use sham (needle insertion or saline injected) controls because we have observed using magnetic resonance imaging (data not shown) that these procedures are associated with a small amount of bleeding, especially in the young brains, which are very soft. Furthemore, our mouse studies have shown no significant effect of intact vs saline controls (Xue et al., 2003). In this study we were interested in understanding the effect of hemorrhagic injury in relation to normal brain and comparing the changes at different ages. Therefore, control animals were anesthetized only. At 24 or 48 hours after blood injection rats were anesthetized and perfused with ice-cold 0.1 M phosphate-buffered saline (PBS). Their brains were quickly

removed and dissected on a cooled surface. The brain was sectioned in the coronal plane through the needle insertion site. With a dissecting microscope, localization of the hematoma in the striatum was confirmed. Rats with improperly localized or small hematomas were discarded. Striatal tissue abutting on the hematoma and including petechial hemorrhage but not including the mass of the hematoma was dissected (~5mm X 5mm) and quickly frozen.

cDNA Microarray Analysis

Brain samples were homogenized in TRIzol reagent (Gibco BRL, Carlsbad, CA.). RNA was isolated according to the manufacturer's instructions and RNA concentrations were determined spectrophotometrically. RNA samples of blood injected rats (newborn n=5; young 24h post blood injection n=4; young 48h post blood injection n=3; adult n=3 24h post injection) and those of age-matched controls (n=5, n=4, n=3, and n=3 respectively) were used. For the preliminary studies we had pooled RNA to decrease variability. For the actual experiment we decided to use individual samples in triplicate trials to confirm those findings. RNA integrity was checked by spectrophotometry and gel electrophoresis. Gene expression patterns were determined using rat GeneFilter GF300 microarrays (Research Genetics, Huntsville, AL). Control and ICH samples were hybridized in parallel on pairs of membranes to reduce processing variation. All steps were done according to the manufacturer's recommended protocols. Briefly, 1 mg of total RNA was incubated with 2 mg of oligo(dT) (Research Genetics, Huntsville, AL), 1.5 ml of reverse transcriptase (Superscript II; LifeTechnologies, Carlsbad, CA), 20 mM dATP, dGTP, and dTTP (Amersham Pharmacia, Piscataway, NJ), 6.0 ml of 53 first strand buffer, 0.1M DTT (Life Technologies, Carlsbad CA), and 10 mCi/ml of [^{33}P]dCTP

in 30 ml of diethyl pyrocarbonate-treated water for 90 min at 37°C. After purification through a Bio-Spin 6 chromatography column (Bio–Rad Hercules, CA), labeled probe was mixed with prehybridization solution and hybridized to GeneFilter membranes overnight at 42°C. Filters were then washed twice at 50°C in 2X SSC, 1% SDS for 20 min and once at room temperature in 0.5X SSC, 1% SDS for 15 min. Membranes were exposed to a phosphorimager screen (Bio–Rad, Hercules, CA) for 3–4h and 16–18 h, the latter to look for genes expressed at relatively low levels. The screen was scanned using a phosphorimager apparatus (Bio–Rad, Hercules, CA) and membrane images were stored as PWF files for analyzing using Pathways 3 software.

mRNA Data Analysis

Expression data from the membranes were quantified using Pathways 3 software (Research Genetics, Huntsville, AL). Each rat GeneFilter membrane contains approximately 3500 known rat genes and 1500 expressed sequence tags (EST) considered similar to named genes in other organisms. For each trial, control and ICH membrane images were superimposed and the corresponding intensities were normalized against background levels automatically. The data sets were analyzed using the software algorithm known as Condition Pair Analysis where ratios are determined using the averaged normalized intensities across all array trials within a given condition. For example, the data sets from all 5 newborn brains with ICH were normalized, combined, and averaged, then compared to averaged data from aged matched control brains. This method consistently yields a normal distribution pattern of gene expression change. To determine which genes had changed expression levels we used the statistical data filter in the software to include only the 95% confidence intervals for each group. i.e. the altered ratio of gene expressions between groups is correct at the $p<0.05$

probability level. We manually checked several dozen spots and found that the statistical algorithm reliably excluded false positive readings that were due to adjacent bright spots associated with highly expressed genes. Data were exported to Microsoft Access and Excel for management and analysis. For comparative purposes we arbitrarily assigned overexpression ratios of 180-200% and >200% and underexpression ratios of 60-50%, and <50%. We searched the Internet accessible PubMed and protein databases extensively in order to arrive at a "best" assignment for gene product function. We acknowledge that placement in more than one of our functional clusters is probable. Approximately 40% of the detected changes were in ubiquitous genes such as those related to ribosomal, mitochondrial, and basic metabolic or structural functions. Interpretation of such changes is difficult, therefore we have not reported them in detail.

Antibody Microarray Analysis

A set of newborn rats separate from those used for RNA extraction was used. Brain tissue dissections were performed in the same manner. Protein expression of newborn rat brain 48h post ICH was determined using monoclonal antibody microarray (Product: Ab microarray 382, catalog #K1847, Lot #2060681; BD Biosciences / Clontech, Palo Alto, CA). The glass slides have 378 monoclonal antibodies (all in duplicates), 4 positive control spots, and 2 negative control spots. Antibody microarrays provide a relative measure of protein abundance where the protein samples of one sample (i.e. ICH sample) are compared to those of second (control) sample. The rationale for choosing 48h following ICH is that many genes were overexpressed 24 hours post ICH, and we predicted that much of the protein translation should occur within the following 24 hours.

Total protein was extracted from brain tissue according to the manufacturer's instructions. Protein samples of rat brain with ICH (n=5) were pooled and those of age-matched controls (n=4) were pooled in equal amounts. The reason for pooling the protein sample was to minimize the effect of inter-individual variability. Protein concentrations were measured using BCA protein assay kit (Pierce, Rockford IL). All steps were done according to the manufacturer's recommended protocols. Briefly, blood injected and control samples were split into two equal portions. A portion from each group was then labeled with either Cy5 or Cy3 (Amersham Biosciences Piscataway, NJ) and incubated at 4°C for 90 minutes, the reaction was stopped by incubating 4ul of blocking buffer at 4°C for 30 minutes. Unbound dye was removed by gel filtration using PD-10 desalting columns (Amersham Biosciences Piscataway, NJ). Protein concentrations were measured again using BCA protein assay kit (Pierce, Rockford IL.). Antibody microarray slides were incubated with 20mg of labeled sample from each group combined (i.e. slide 1 with Cy3 labeled control and Cy5 labeled blood infusion; slide 2 with Cy5 labeled control and Cy3 labeled blood infusion) for 30 minutes at room temperature. Using two slides with proteins labeled in reverse manner minimizes unbalanced results due to selective binding of certain proteins with either of the two labels. Effectively, the final result is based upon 4 competitive assays for each antibody. Slides were washed seven times using the washing buffers provided by the manufacturer.

Protein Data Analysis

Slides were scanned using a glass microarray scanner (GenePix 4000B, Axon Instruments, Union City, CA) and analysis was done using GenePix Pro software 4.1 (Axon Instruments). Following alignments Cy3/Cy5 fluorescent signal ratios were

calculated for all spots on each array. Data were transferred to a manufacturer-supplied Microsoft Excel file that converts fluorescence intensity data into an internally normalized ratio (INR). Combining the INR from each of the two arrays mathematically, a relative expression ratio between the two groups for individual protein is determined. Clustering of the results was done in a manner similar to gene expression clustering as described above. It is critical to note that the protein name and gene name nomenclature on the cDNA and antibody microarray respectively, was often not the same. We did our best manually searching the protein, cDNA, and Pub Med databases to come to a consensus identity. We arbitrarily assumed that a 40% increase is substantial enough to conclude that there is true overexpression.

RESULTS

In newborn rat brains 627 genes were overexpressed and 48 were underexpressed after blood injection. In contrast, blood injection caused relatively more underexpression of genes in young and adult rat brains (Table 1). Young and adult brains exhibited a large number of changes in common between the two groups, and there were many changes that persisted from 24 hours to 48 hours in the blood infused young group. However, the newborn rat brain exhibited few changes in common with either the young or adult brain 24 hours after blood injection(Table 2).

We will specifically discuss only those genes that changed in newborn rats 24 hours following ICH and that have an obvious relevance to brain function. Newborn brains following blood infusion underexpressed 48 genes below the ratio of 0.7 (Table 3), and 627 genes were overexpressed above ratio of 1.8. Among these are genes related to endothelium and brain water regulation (Table 4), astrocytes, oligodendrocytes and myelin (Table 5), striatal neuron function and structure (Table 6),

brain / neuron development and trophic factors (Table 7), cell cycle, cell survival and cell death (Table 8), oxidative and chemical stress (Table 9), extracellular matrix (Table 10), proteolytic and lipolytic enzymes (Table 11), inflammation (Table 12), cytoskeleton (Table 13), signal transduction (Table 14), and others (Table 15). In the tables the absolute mRNA expression levels are expressed as low or high as judged by the normalized intensity of the hybridized spots. We arbitrarily designated a normalized intensity above 0.3 as high (maximum value of 2.17) and a normalized intensity between 0.02-0.3 as low.

Protein analysis

Following blood injection 81/378 proteins were expressed at >140% of control levels. None were expressed at <90% of control levels. Among those that were increased, 27/81 were also present on the cDNA membrane and 20/27 showed overexpression both at the mRNA and protein levels. The other 7/27 proteins that showed overexpression exhibited no significant change in the cDNA study. The remaining 54/81 overexpressed proteins did not have corresponding spots on the cDNA membrane. There was an additional 71/378 proteins corresponding to probes on the cDNA membrane that did not exceed 140% overexpression. Of those, 41/71 were overexpressed on the cDNA membranes and the remaining 30/71 exhibited no significant change.

Table 1. Changes in gene expression after blood injection in newborn, Young, and adult rats.

EST: Expression sequence tag; Known: known gene sequences

Rat group (age; time post blood injection)	# of genes underexpressed (<50%)		# of genes underexpressed (50-60%)		# of genes over expressed (180-200%)		# of genes overexpressed (>200%)	
	Known	EST	Known	EST	Known	EST	Known	EST
Newborn, 24h	8	5	40	91	171	661	346	857
Young, 24h	46	422	33	183	36	44	140	71
Young, 48h	100	303	37	104	17	66	15	121
Adult, 24h	237	1126	112	317	34	59	80	114

Table 2. Comparison of specific gene alterations after blood injection between rats of different ages.

	Common decrease (<60%)		Common increase (>180%)	
	Known	EST	Known	EST
Newborn and young, 24h	0	35	10	17
Newborn and adult, 24h	0	7	0	3
Young, 24 and 48h	26	155	2	7
Young and adult, 24h	59	462	20	18

Legend : Tables 3-12 H=high abundance, L=low abundance, Blank = cDNA or antibody was not present on the array studied, and xxx = did not make the 95% confidence level.

Table 3. Underexpressed genes in newborn rat striatum 24 hours after blood injection

Gene product	mRNA expression ratio (relative to control)	mRNA Abundance
11-beta-hydroxylsteroid dehydrogenase type 2	0.58	H
acetyl-coenzyme A carboxylase (EC 6.4.1.2.)	0.67	H
acyl-CoA synthetase 5	0.68	H
alpha II spectrin	0.54	L
branched chain alpha-keto acid dehydrogenase E1-subunit	0.58	H
casein kinase I delta (Blanquet, 2000)	0.56	H
CDK110	0.65	H
ceruloplasmin (ferroxidase)	0.67	H
CLP36 (Wang et al., 1995)	0.56	L
mitochondrial ATP synthase complex coupling factor 6	0.50	H
cytochrome c oxidase subunit VIa	0.62	L
cytochrome c oxidase subunit VIIa (EC 1.9.3.1)	0.46	L
DNA polymerase alpha	0.66	H
pancreatic trypsinogen II	0.63	H
Fc gamma receptor	0.62	H
fetuin-like protein IRL685 (Terkelsen et al., 1998)	0.68	H
G10 protein homolog (edg2)	0.65	L

Table 3 (continued). Underexpressed genes in newborn rat striatum 24 hours after blood injection

Gene product	mRNA expression ratio (relative to control)	mRNA Abundance
kynurenine 3-hydroxylase (EC 1.14.13.9)	0.69	H
MIPP65	0.60	H
mitochondrial ATP synthase beta subunit	0.42	L
N-acetylglucosaminyltransferase I	0.68	H
neuronatin beta	0.37	L
nuclear receptor binding factor 1	0.62	H
nuclear RNA helicase	0.66	L
paired-like homeodomain transcription factor (DRG11)	0.67	H
peroxisome assembly factor-1 (PAF-1)	0.62	H
phosphofructokinase isozyme (EC 2.7.1.11)	0.48	H
phospholipase C delta-4	0.53	H
preprocathepsin D (EC 3.4.23.5)	0.62	L
prostacyclin synthase (EC 5.3.99.4)	0.59	L
protein inhibitor of neuronal nitric oxide synthase (PIN)	0.55	L
ribosomal protein L10	0.36	L
ribosomal protein L30	0.59	L
ribosomal protein L41	0.59	L
rS-Rex-b (neuronal secretory protein) (Baka et al., 1996)	0.54	L
RT1.Ma (MHC class2) (Kurth et al., 1997)	0.65	H
Smad2 protein	0.50	L

Table 3 (continued). Underexpressed genes in newborn rat striatum 24 hours after blood injection

Gene product	mRNA expression ratio (relative to control)	mRNA Abundance
Smad4 protein	0.64	H
thrombin receptor (Niclou et al., 1998)	0.47	L
thymosin beta-10	0.36	L
triadin 95 kD (TRISK 95)	0.68	L
type III adenylyl cyclase	0.68	H
xanthine dehydrogenase	0.56	H
$zinc^{2+}$ binding protein	0.69	L

Table 4. Overexpression of genes and proteins related to endothelium and brain water regulation in newborn rat striatum 24 hour after blood injection.

Gene product	mRNA expression ratio (relative to control)	mRNA abundance	Protein expression ratio (relative to control)
angiotensin receptor 2	2.07	L	
brain natriuretic peptide (BNP)	1.95	L	
dipeptidyl peptidase 4	2.07	H	
endothelin-converting enzyme	2.71	L	
GATA-binding protein 1	2.28	L	
multidrug resistance protein	2.77	L	
organic cation transporter	2.38	H	
retinol binding protein (RBP)	2.03	H	1.24
solute carrier family 9 (NHE-1)	2.33	L	1.24
solute carrier 16 family (monocarboxylic acid transporter)	3.00	L	
tight junction protein (ZO-1)			1.46
vascular endothelial growth factor (VEGF)	2.27	L	
vascular protein tyrosine phosphatase-1 rDEP-1	1.85	L	

Table 5. Overexpression of genes and proteins related to astrocytes, oligodendrocytes, and myelin in newborn rat striatum 24 hours after blood injection.

Gene product	mRNA expression ratio(relative to control)	mRNA abundance	Protein expression ratio (relative to control)
10-methyltetrahydrofolate dehydrogenase	2.39	L	
connexin (CXN-31.1)	1.94	H	
folate binding proteins	3.46	H	1.72
GFAP (glial fibrillary acidic protein)			1.50
glycogen phosphorylase	2.38	L	
integrin beta-1	1.85	L	
kynurenine aminotransferase II	2.62	H	
lipophilin	2.42	H	
Myelin-oligodendrocyte glycoprotein (MOG)	2.67	L	
myelin-associated glycoprotein (MAG)	2.25	H	
parathyroid-like peptide	2.45	H	
proton-coupled peptide transporter PEPT2	2.10	H	
Rab3B (astroglial) (Madison et al., 1996)	1.82	H	
S100 calcium-binding protein	2.58	H	
S6 protein kinase (Rsk-1)	1.85	L	
transcriptional repressor of myelin-specific genes	3.00	L	
TGF-beta-masking protein subunit	2.12	H	

Table 6. Overexpression of genes and proteins related to neuron function and structure in newborn rat striatum 24 hours after blood injection.

Gene product	mRNA expression ratio(relative to control)	mRNA abundance	Protein expression ratio (relative to control)
A2b-adenosine receptor	2.40	H	
acetylcholine receptor b	2.63	L	1.21
acetylcholine receptor gamma-2a chain	2.63	H	
Agrin	2.03	H	
aryl hydrocarbon receptor	2.78	L	
benzodiazepine receptor (peripheral)	2.14	H	
beta-galactoside-binding lectin (Hynes et al., 1990)	2.89	H	
brain specific Na+-dependent inorganic phosphate cotransporter	1.84	L	
brain specific peptide	2.28	H	
Cain (inhibitor of calcineurin) (Lai et al., 1998)	2.59	H	
calcium channel alpha 1A	1.89	H	
calcium-activated potassium channel rSK3 (SK)	2.42	L	
catecholamine-O-methyltransferase	5.53	H	1.21
chemokine receptor LCR1 40	3.55	H	
chloride channel (ClC-2)	1.95	L	
cysteine-rich protein 1 (CRP1)	1.93	H	1.50
D-dopachrome tautomerase	2.53	L	
Dopa decarboxylase	1.90	L	

Table 6 (continued). Overexpression of genes and proteins related to neuron function and structure in newborn rat striatum 24 hours after blood injection.

Gene product	mRNA expression ratio(relative to control)	mRNA abundance	Protein expression ratio (relative to control)
Enhancer-of-split and hairy-related protein 1 (SHARP-1)	2.41	L	
EP4 prostanoid receptor	1.78	L	
FK506-binding protein (FKBP 51)			1.40
fragile X receptor 2			1.60
GABA b receptor	3.26	H	1.50
GABA transporter protein	1.82	H	
gamma-glutamyl transpeptidase	2.35	H	
gamma-preprotachykinin A	2.11	H	
Glucagons receptor	2.03	H	
glutamate receptor (GluR-B)	1.79	L	
glutamate transporter	1.94	H	
glutamate receptor (metabotropic) (GLUR4)	1.94	H	
glutamate/aspartate transporter protein	1.85	L	
glycine receptor alpha 2	1.93	L	
inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	2.07	H	
mint 3 (Okamoto and Sudhof, 1998)	3.01	L	
neuraminidase 2	2.11	L	
neurexin II-beta	3.00	L	
neuroendocrine-specific protein (RESP18)	2.28	H	
oxytocin / neurophysin (Oxt)	2.06	L	

Table 6 (continued). Overexpression of genes and proteins related to neuron function and structure in newborn rat striatum 24 hours after blood injection.

Gene product	mRNA expression ratio(relative to control)	mRNA abundance	Protein expression ratio (relative to control)
P2X6 receptor	2.12	L	
paranodin	1.85	H	
parathyroid hormone/ parathyroid hormone related-peptide receptor	2.95	L	
peptide/histidine transporter	2.37	L	
phosphatidylethanolamine-binding protein	1.85	H	
postsynaptic density protein (citron)	1.96	H	
postsynaptic protein CRIPT	2.69	L	1.57
Potassium channel (KCNQ2)	1.93	L	
Presenilin-2	3.02	H	
proopiomelanocortin (POMC)	3.15	H	
protein kinase C iota			1.50
rabaptin-5	2.57	L	1.16
RGS8 (regulator of G-protein) (Saitoh et al., 1997)	1.73	L	
RHS2 class III POU protein (RHS2)	2.86	L	
Serotonin receptor (5-HT2CR)			1.49
Serotonin transporter (solute carrier family 6)	2.76	H	
SH3 domain binding protein (CR16)	2.33	H	
SH3-containing protein p4015	2.27	L	
sip9	2.04	L	

Table 6 (continued). Overexpression of genes and proteins related to neuron function and structure in newborn rat striatum 24 hours after blood injection.

Gene product	mRNA expression ratio(relative to control)	mRNA abundance	Protein expression ratio (relative to control)
sodium/hydrogen exchanger 3 (solute carrier family 9, NHE3)	2.34		1.48
Somatostatin	2.48	H	
survival motor neuron (smn) (Battaglia et al., 1997)	3.77	L	1.26
synaptic vesicle protein (SV2)	2.59	H	
synaptobrevin 2 (vesicle-associatedmembrane protein, VAMP-2)	1.73	H	
synaptojanin II	3.90	H	
Synaptotagmin		XXX	1.60
synGAP-b1	1.95	L	
syntaxin 5	1.97	H	1.48
Synuclein	2.85	L	1.32
transcription factor (Olf-1)	2.18	H	
transferrin (also in oligodendrocytes)	2.42	H	
transketolase	1.82	H	
two period homologs: Per2			1.54
tyrosine hydroxylase	2.10	L	
vesicle associated protein-(VAP1)(synapsin 1)	3.30	H	
Vesl-2 (Kato et al., 1998)	1.89	H	1.57

Table 7. Overexpression of genes and proteins related to brain/neuron development and trophic factors in newborn rat striatum 24 hours after blood injection

Gene product	mRNA expression ratio(relative to control)	mRNA abundance	Protein expression ratio (relative to control)
bone morphogenetic protein 2	1.89	L	
bone morphogenetic protein 4	1.96	H	
ciliary neurotrophic factor receptor alpha		XXX	1.50
Collapsing response mediator protein 1	1.90	H	
cystathionine beta synthase	1.90	H	
decidual prolactin-related protein	2.72	L	
distal-less homeobox (DLX1)	2.60	L	
fatty acid amide hydrolase	2.09	H	
f-spondin (neurite outgrowth and adhesion) (Klar et al., 1992)	2.21	H	
growth hormone receptor/binding protein (GHR/BP)	1.79	H	
HNF-3/forkhead homolog-1 (HFH-1)	2.06	H	
insulin-like growth factor binding protein complex	1.80	H	

Table 7 (continued). Overexpression of genes and proteins related to brain/neuron development and

trophic factors in newborn rat striatum 24 hours after blood injection

Gene product	mRNA expression ratio(relative to control)	mRNA abundance	Protein expression ratio (relative to control)
insulin-like growth factor I (IGF-I)	2.12	L	
lim-2 (Tsuchida et al., 1994)	2.13	L	
MAS1 oncogene	2.15	L	
MEGF5 (multiple EGF-like motifs) (Nakayama et al., 1998)	1.89	H	
meprin 1 beta	2.58	H	
neural cell adhesion molecule L1 (NCAM-L1)			1.42
neuritin	2.19	L	
neurogenin	1.83	L	1.67
neuropilin	2.05	L	1.40
Ninjurin	2.90	H	
Nogo-A (neurite outgrowth inhibitor) (Chen et al., 2000)	3.30	H	
Norbin	2.73	L	
orphan nuclear receptor OR-1 (Nur77)	1.72	L	1.49
plakophilin 2a	1.70	L	1.43
protein tyrosine phosphatase (PTPNE6)	1.98	L	
thyroid stimulating hormone receptor	1.95	L	
thyrotroph embryonic factor (TEF-1)			1.52

Table 7 (continued). Overexpression of genes and proteins related to brain/neuron development and

trophic factors in newborn rat striatum 24 hours after blood injection

Gene product	mRNA expression ratio(relative to control)	mRNA abundance	Protein expression ratio (relative to control)
thyrotropin releasing hormone receptor	2.62	H	
transthyretin (thyroid hormone binding protein)	9.48	H	
Wilms' tumor (Sharma et al., 1992)	2.34	H	1.29

Table 8. Overexpression of genes and proteins related cell cycle, cell survival, and cell death in newborn rat striatum 24 hours after blood injection

Gene product	mRNA expression ratio(relative to control)	mRNA abundance	Protein expression ratio (relative to control)
AATF protein (inhibitor of apoptosis)	1.90	L	
ADP-ribosyltransferase (NAD+; poly (ADP-ribose) polymerase)	1.92	L	1.56
apolipoprotein E gene: ECL (Shen and Howlett, 1992)	2.07	H	
baculoviral IAP repeat-containing protein 6 (BRUCE)			1.41
Bcl-2 associated death promoter (BAD)	1.82	H	
Bcl-2-associated transcription factor			1.50
Bcl-x			1.46
C7-1 protein (C7-1)	1.90	H	
caspase-8 / FADD-homologous ICE / CED-3-like protease (FLICE)			1.50
CCAAT binding transcription factor-B subunit (CBF-A1)	2.25	H	
CCAAT/enhancer binding, protein (C/EBP) delta	1.83	L	
cdc2 promoter region	1.82	L	
cdc25B	2.01	H	
cdc34 (Ubiquitin carrier protein)			1.41
cdc37	2.64	L	

Table 8 (continued). Overexpression of genes and proteins related cell cycle, cell survival, and cell death in newborn rat striatum 24 hours after blood injection

Gene product	mRNA expression ratio(relative to control)	mRNA abundance	Protein expression ratio (relative to control)
cdc 42 (GTPase-activating protein)			1.53
cdk1			1.47
cdk4			1.52
cdk-activating kinase assembly factor MAT1	2.05	L	1.49
centrosomal NEK2-associated protein 1(C-NAP1)			1.40
cyclin B	2.50	L	
cyclin D2	1.85	L	1.35
cyclin G-associated kinase	1.82	L	
DNA helicase p50	2.15	L	
DNase gamma	3.13	L	
Fanconi anemia group C	2.40	L	
FAS-associating death domain-containing protein (FADD / Mort-1)			1.50
frizzled related protein frpAP (DDC-4)	1.80	H	
growth factor-inducible serine-threonine phosphatase FIN13			1.51

Table 8 (continued). Overexpression of genes and proteins related cell cycle, cell survival, and cell death in newborn rat striatum 24 hours after blood injection

Gene product	mRNA expression ratio(relative to control)	mRNA abundance	Protein expression ratio (relative to control)
H2A and H2B histones	2.06	H	
H2A histone family, member Y	2.31	L	
H3.3 histone	2.90	L	
high molecular weight DNA polymerase beta (rnpolb)	2.43	L	
max protein	2.61	H	1.43
Mismatch repair protein (MLH1)	1.81	L	
Nedd 2 / Ich-1 (Caspase 2)	1.76	L	
Nedd-4 protein	2.59	H	1.38
nuclear oncoprotein p53	2.10	L	
p27	2.47	L	
p53-like transcription factor p73a			1.50
par-4 (prostate apoptosis response)	2.77	L	
protein-tyrosine phosphatase PTEN	2.97		1.28
serine/threonine-protein kinase PLK (PLK1)			1.48
TEGT (Bax-1 inhibitor) (Jean et al., 1999)	1.80	L	
telomerase protein component 1 (TLP1)	1.87	H	
TRADD (CD95)			1.45
tuberous sclerosis 2 (tuberin)	2.52	L	

Table 9. Overexpression of oxidative and chemical stress-inducible genes and proteins in newborn rat striatum 24 hours after blood injection

Gene product	mRNA expression ratio (relative to control)	mRNA abundance	Protein expression ratio (relative to control)
Alpha B crystallin-related protein (HSP20)	1.87	L	
alpha-crystallin B chain	1.89	H	
beta-B3-2-crystallin	2.97	L	
calcium-binding protein	2.25	H	
cysteine dioxygenase	1.80	L	
glutathione S-transferase 1 (theta)	2.27	L	
glutathione-S-transferase	3.60	H	
glutathione-S-transferase, mu type 2 (Yb2)	2.05	L	
HBP23 (heme-binding protein 23 kD)	2.54	H	
heat shock 10 kD (chaperonin 10)	2.10	L	
heme oxygenase 1		XXX	1.44
metallothionein-III	1.86	H	
phospholipid hydroperoxide glutathione peroxidase	1.96	L	
S-100-like protein	2.10	H	
stress activated protein kinase alpha	2.37	H	1.27
superoxide dismutase 3 (SOD3)	2.79	H	
YT521 splicing-related protein (astrocytes)	1.89	L	

Table 10. Overexpression of genes and proteins related to extracellular matrix in newborn rat striatum 24 hours after blood injection

Gene product	mRNA expression ratio (relative to control)	mRNA abundance	Protein expression ratio (relative to control)
Biglycan	2.19	H	
Caldesmon	3.32	H	1.40
chondroitin sulfate proteoglycan core protein	1.99	L	
collagen alpha 2 type V	1.86	H	
embigin (immunoglobulin family)	2.36	L	
extracellular matrix protein 2	2.11	H	
Fibromodulin	3.15	L	
Fibronectin	1.95	H	1.19
glypican 3	1.88	L	
integrin alpha VLA	2.16	H	1.48
laminin chain beta 2	1.86	L	
laminin chain beta 3 kalinin	1.89	L	1.32
Lumican	1.89	L	
pro alpha 1 collagen type III	1.93	H	
procollagen II alpha 1	2.10	L	1.32
prolyl 4-hydroxylase alpha subunit (Hopkinson et al., 1994)	2.33	L	
tenascin X	1.88	H	

Table 11. Overexpression of proteolytic (including blood clotting cascade) and lipolytic enzymes-related genes and proteins in newborn rat striatum 24 hours after blood injection

Gene product	mRNA expression ratio (relative to control)	mRNA abundance	Protein expression ratio (relative to control)
alpha-1-macroglobulin	3.35	L	
calpain Lp82	2.35	L	
calpain-like protease (Capa6)	1.91	L	
μ calpain large subunit (cls1)	1.75	H	
Cathepsin B	1.89	H	
Cathepsin K	2.10	L	
Cathepsin L	2.24	H	1.30
Cathepsin S	2.50	H	
dual specificity phosphate	1.84	H	
insulin degrading enzyme	1.87	L	
Leuserpin-2	2.27	L	1.29
MMP-2 (gelatinase A)	2.07	L	
MMP-23 (MIFR)	2.32	H	
PACE-4 convertase	2.10	L	
phospholipase A1 (Sato et al., 1997)	2.57	H	
phospholipase A2	1.72	H	
phospholipase A2 (Ca^{2+} independent)	2.24	H	
phospholipase D	2.80	L	1.39

Table 11 (continued). Overexpression of proteolytic (including blood clotting cascade) and lipolytic enzymes-related genes and proteins in newborn rat striatum 24 hours after blood injection

Gene product	mRNA expression ratio (relative to control)	mRNA abundance	Protein expression ratio (relative to control)
serine proteinase rPC7 precursor (convertase)	2.09	H	
serine/threonine kinase (gamma-PAK) (thrombin activated)	2.10	L	
tissue inhibitor of metalloproteinase 3 (TIMP3)	1.98	H	
trypsin inhibitor type II (PSTI-II)	2.00	H	
urinary plasminogen activator receptor 2 (uPAR-2)	1.96	L	
urinary plasminogen activator (urokinase)	1.79	H	

Table 12. Overexpression of genes and proteins related to inflammation in newborn rat striatum 24 hours after blood injection

Gene product	mRNA expression ratio(relative to control)	mRNA abundance	Protein expression ratio (relative to control)
Attractin			1.42
CD1 antigen precursor	2.86	H	
CD3 antigen delta polypeptide	2.45	H	1.48
CD28 (T cells)			1.45
CD37	1.85	L	
CD38 antigen (ADP-ribosyl cyclase / cyclic ADP-ribose hydrolase)	3.55	L	
CD81 antigen (target of antiproliferative antibody 1)	1.86	H	
complement factor I (CFI)	1.93	H	
complement protein C1q beta chain	2.24	L	
complement protein C4	1.81	H	
complement regulation binding binds protein (C4BP)	2.86	H	
cytokine-induced neutrophil chemoattractant-2 (Shibata et al., 1998)	1.98	H	
Gal/GalNAc-specific lectin	2.01	L	
high mobility group 1	1.87	H	
interleukin 1 beta (IL-1β)		XXX	1.44
interferon tau-5			1.51
Leukocyte common antigen	1.78	H	1.34

Table 12 (continued). Overexpression of genes and proteins related to inflammation in newborn rat striatum 24 hours after blood injection.

Gene product	mRNA expression ratio(relative to control)	mRNA abundance	Protein expression ratio (relative to control)
Leukocyte-specific SH2- and SH3-containing adaptor-like protein-grap2 (MONA)			1.54
Ly6-B antigen	2.33	L	
Ly6-C antigen	2.43	L	
mast cell protease 1 precursor (RMCP-1)	2.23	H	
mast cell protease 8	2.70	H	
mast cell, chymase 1	2.33	H	
MHC class I	2.69	H	
natural killer cell protease 4 (RNKP-4)	2.07	L	
natural resistance-associated macrophage protein 2	2.08	L	
prostaglandin E receptor 1 (subtype EP1)	1.80	L	
PU.1(Spi-1)			1.60
T-cell receptor active beta-chain C-region	3.09	L	
T-cell receptor gamma	2.24	H	
tryptase 2 (lymphocytes)	1.94	L	
tumor necrosis factor alpha (TNF□)	2.13	H	

Table 13. Overexpression of genes and proteins related to cytoskeleton in newborn rat striatum 24 hours after blood injection.

Gene product	mRNA expression ratio (relative to control)	mRNA abundance	Protein expression ratio (relative to control)
ankyrin B	1.71	L	1.29
b-tubulin		XXX	1.49
clathrin heavy chain	2.02	H	1.43
cypher1 (PDZ-LIM domain Z-line protein)			1.47
dynamin I	2.63	L	1.30
ezrin	1.89	L	1.24
katanin p80	2.19	H	1.32
moesin (membrane-organizing extension spike protein)	2.50	H	1.25
nestin	1.82	L	1.24
tensin	1.92	H	1.34
VASP (ligand for profilin p140mDIA)	1.89	H	1.53

Table 14. Overexpression of genes and proteins related to signal transduction in newborn rat striatum 24 hours after blood injection.

Gene product	mRNA expression ratio (relative to control)	mRNA abundance	Protein expression ratio (relative to control)
Bruton tyrosine kinase-associated protein-135 (TFII-I /BAP-135)			1.47
CAM kinase kinase	1.72	L	1.27
casein kinase II	2.10	H	1.30
casein kinase II alpha subunit (CK2□)	1.93	H	
cGMP-specific 3',5'-cyclic phosphodiesterase cGB-PDE	2.43	L	1.34
DGKq	2.69	H	1.46
disabled homolog 2 (doc-2 (p96)	1.87	H	1.48
JNKK1			1.41
mammalian STE20-likeprotein kinase 1 (MST1)			1.42
mitochondrial import stimulation factor L (14-3-3e)			1.43
mitogen-activated protein kinase 13 (p38d / SAPK4)	2.26	H	1.44
mitogen-activated protein kinase kinase 7 (MKK7)			1.55

Table 14 (continued). Overexpression of genes and proteins related to signal transduction in newborn rat striatum 24 hours after blood injection.

Gene product	mRNA expression ratio (relative to control)	mRNA abundance	Protein expression ratio (relative to control)
PAK-interacting exchange factor beta b-PIX	2.01	H	1.30
phosphatidylinositol 3-kinase catalytic beta	2.97	H	1.30
protein kinase C alpha (PKC □)			1.41
protein kinase C theta (PKC □)			1.42
protein-kinase C-related kinase 2 (gamma PAK)	2.39	H	1.44
rhoA - binding serine/threosine kinase alpha (ROK alpha)			1.50
signal recognition particle 54 kD protein (SRP54)			1.51
V-1/myotrophin			1.42

Table 15. Overexpression of proteins (not found on cDNA array) in newborn rat striatum 24 hours after blood injection.

Protein	Protein expression ratio (relative to control)
adapter-related protein complex 1 (adaptin 1gamma-clathrin / Golgi)	1.42
adaptin d	1.65
AMPK b (AMP activated protein kinase- fatty acid synthesis)	1.62
calnexin (endoplasmic reticulum, Ca^{+2} binding)	1.42
Flotillin 2 / (caveolae)	1.48
GAGE	1.54
Golgi snare GS15	1.48
Karyopherin b	1.44
PEX1(Peroxisome biogenesis factor 1,Peroxin-1)	1.45
proteosome assembly factor PA28-g	1.54
Ran-specific GTPase-activating protein (RanBP1)	1.41
Transportin (nuclear protein importer)	1.45
XIN (Wang and Pevsner, 1999)	1.43

DISCUSSION

In this study using the DNA microarray approach, we have shown that changes in gene expression in the rat brain following acute hemorrhage are age dependant. Newborn brains react very differently from young (7 day) or adult brains. In general, newborn brains tend to overexpress many genes that are likely important for brain development and function. This finding was generally confirmed at the protein level using antibody microarray analysis. The reason for this may be that the newborn rat brain is undergoing anatomical organization and physiological initiation of various brain systems, which have already been completed in the more mature brain. For example, the periventricular germinal matrix, which in the newborn rat gives rise to glial precursors (Zerlin et al., 1995), has involuted by 7 days. Thus, following injury such as blood injection, as part of a compensatory or perhaps protective mechanism the newborn brain is capable of overexpressing many specific genes that are not as well expressed in the more mature brain.

Neurons, Glia and Endothelial Cells

In this study all three age groups (newborn, young, and adult) showed changes in gene expression related to neurons, glia and endothelial cells following blood injection. Newborn brains tended to overexpress many of these genes following blood injection. For instance, newborn brains overexpressed several transporters of glutamate and GABA. This may be a mechanism used to clear toxic neurotransmitters that accumulate following injury. Newborn brain also showed overexpression of genes related to postsynaptic proteins such as P2X6 purine receptor, A2b adenosine receptor, and glutamate receptor, as well as genes related to presynaptic proteins such as SV2, citron, VAMP2, and CRIPT. CRIPT over expression was further confirmed at protein

level. In the young and adult brain many of the above-mentioned genes were either not detected either in control brains or following blood injection or were down regulated after blood injection, e.g. synaptotagmin V, a postsynaptic density protein. In glial clusters, genes and proteins related to reactive astrocytes including S100 and GFAP, were overexpressed in response to damage. Interestingly, many genes related to myelin such as MOG, MAG, SCIP were also overexpressed despite the fact that myelin is not normally produced in the striatum at this age. This could reflect reactive changes in immature oligodendrocytes. Genes related to endothelial cells including VEGF and endothelin converting enzyme were also overexpressed. This may be due to damage of blood vessels following blood injection or as preparation for growth of new vessels.

Growth Factors / Brain Development

In newborn rat brain the hematoma is in close proximity to the periventricular germinal matrix. Hence it might affect the generation or differentiation of glial precursors (Volpe, 2001a) with subsequent deleterious effects on myelination and maturation of neurons. Overexpression of many of these genes could be a compensatory mechanism in germinal tissue, which would not be possible in 7 day old or adult brains. Many genes related to trophic factors and brain development were overexpressed in newborn brain following blood injection (see Table 7). At the protein level, neurogenin and neuropilin showed over expression following blood injection. In young and adult brain, insulin-like growth factor 2 was also overexpressed, but genes such as MEGF and GHR/BP were underexpressed.

It is worth noting that the newborn brain overexpressed several genes related to thyroid hormone including thyrotropin releasing hormone receptor, thyroid stimulating

hormone receptor, and transthyretin. Such changes were not seen in young and adult brain. Thyroid hormone is known to play a critical role in early brain development (Anderson, 2001). Although only one thyroid related antibody was present on the array (thyrotroph embryonic factor-1), it was also overexpressed following blood injection. The newborn brain may overexpress these genes to compensate for the developmental interruption. Newborn brains, in addition, showed overexpression of many genes related to cell cycle, including cyclins. Increase in cell cycle related genes may reflect germinal cell proliferation or glial cell proliferation, which has been observed following many forms of brain injury. In spite of this apparent compensation at the genetic level, newborn rodent brains heal poorly after injury to the neocortex (Kolb et al., 1998; Kolb and Cioe, 2000; Kolb et al., 2000b; Kolb et al., 2000a). Whether the same is so for striatal lesions is not known.

Cell Death Following Blood Injection

Following blood injection, newborn rat brains not only overexpressed genes related to apoptotic cell death (e.g. BAD at the cDNA level and Bcl-x at the protein level), they also overexpressed genes involved in inhibition of apoptosis (e.g. AATF protein and p53). This was paralleled by an increase in the expression of genes related to DNA repair such as Fanconi anemia group C and mismatch repair proteins. At the protein level p53 and DNA repair proteins were overexpressed. While triggering of apoptotic pathways in response to injury is predictable, overexpression of genes involved in cell survival, cell proliferation, and inhibition of apoptosis is more surprising. We do not, however, know if the same cell populations exhibit both changes. In newborn mouse brains, striatal and germinal tissue cells exhibit increased cell death and decreased cell proliferation after autologous blood injection (data not published). In

the young and adult rat counterparts, genes related to cell death were often down regulated e.g. BAX, and Maxp1 (a tumor suppressor).

Extracellular matrix, Proteolytic Enzymes, and Lipolytic Enzymes

Proteolytic enzymes associated with blood clotting, inflammation, and intercellular signaling play an important role in normal brain development. These enzymes are found within and on the surfaces of neurons, macroglia, microglia, and endothelial cells. Plasminogen activators are involved in remodeling of cells including synapse formation and reactive changes (Krueger et al., 1997). Previously we have shown that thrombin and plasmin, when injected into the adult brain, cause edema and cell death (Xue and Del Bigio, 2001). Recently, we have shown that thrombin also plays a role in neonatal periventricular brain damage following PVH (Xue et al., 2005).In this study genes involved in proteolyic activity such as urokinase type plasminogen activator and cathepsins, which are potentially involved in degenerative processes, were overexpressed. Furthermore, newborn brain with blood injection downregulated thrombin receptors, which are normally present on immature neurons (Mahajan et al., 2000). This downregulation may be important in averting damage that could be caused by entry of thrombin into the brain from plasma. Newborns also overexpressed endogenous inhibitors of proteolytic activity such as alpha 1 macroglobulin, a broad-spectrum proteinase inhibitor (Eggertsen et al., 1991), and trypsin inhibitor type II. In the young and adult brains, however, similar genes either were down regulated (e.g. alpha 2 macroglobulin) or did not change in expression. Lipolytic enzymes such as phospholipases A1, A2 and D, which are involved in the synthesis of prostaglandins and production of arachidonic acid, were overexpressed in the newborn brain.

Phospholipase D was also overexpressed at the protein level following blood injection. This could have adverse consequences through generation of oxidation products. In the mature brain phospholipase C was overexpressed.

The extracellular matrix is a complex aggregate of glycoproteins and proteoglycans that together serve a multitude of roles including structural support, axon guidance, synapse isolation, and the capacity for binding of diffusible substances, including growth factors. A variety of genes related to extracellular matrix (ECM) were overexpressed in newborn brain. Matrix metalloproteinases (MMPs), a family of proteolytic enzymes with relative specificity for ECM components, are usually present in developing brain where they play a role in cell migration, growth and myelination (Canete-Soler et al., 1995; Uhm et al., 1998; Del Bigio and Seyoum, 1999). Many studies have shown that following brain injury MMPs are produced by infiltrating inflammatory cells, microglia, and astroglia (Rosenberg, 1995). In the newborn brain following ICH we found that MMP 2 and 23 were overexpressed along with TIMP 3, which is an inhibitor of MMPs. In the mature brain, however, TIMP 3 was down regulated.

Inflammation

The intensity of brain inflammation is greater in hemorrhagic lesions than in non-hemorrhagic lesions (Xue and Del Bigio, 2000). In this study we found that newborn brain overexpressed many antigens (CD1, CD3, CD37, CD38, CD81, LY6-B, LY6-C, and T cell receptors) that are expressed on lymphocytes. This is consistent with previous studies, which have shown T lymphocyte infiltration into brain following ICH

(Hickey et al., 1991). Cytokines can be produced by activated microglia, neutrophils, or lymphocytes. TNFα was overexpressed in response to ICH in newborn brain. This is complementary to a prior experiment, where TNFα was found to be overexpressed by microglia following ICH in mature rat brains (Mayne et al., 2001). A DNA array study of peripheral blood from adult rats 24h post ICH showed overexpression of protease enzymes, transferrin receptor, and vascular protein tyrosine phosphatase 1 (rDEP1) (Tang et al., 2001). In our study rDEP1 was also overexpressed. We observed in the adult brain that annexin 1 (lipocortin), a calcium binding protein with the potential to inhibit proinflammatory cytokines and neutrophil infiltration, was over expressed after blood injection. A recent microarray study following adult rat with blood injection by another group yielded similar results where interleukin-1, caspase-1, and annexin 1 were over expressed (Tang et al., 2002).

Shortcomings of This Study

There are several shortcomings in our methodology. 1) For the cDNA microarray study we might have ignored some genes of interest that are expressed at only low levels because we focused our analyses on short exposure phosphor images. 2) We do not exclude the possibility that changes of lesser magnitude than our arbitrary cut off (over 180% or under 60% for cDNA and over 140% for protein arrays) might be biologically important. 3) We created an arbitrary clustering system to group genes and proteins using available published information, therefore we may have ignored genes or gene products that are potentially important yet not studied. 4) In order to understand the pathophysiological process, detection of changes in gene and protein expression must be followed by studies to determine the cellular localization and activity of the gene product. 5) Because tissues from different parts of the brain have differential gene

expression (Geschwind, 2000) our results are likely skewed by the presence of the periventricular germinal tissue. 6) Many of the gene protein products were not present on the antibody chip. 7) We did not study many time points to assess mRNA or proteins that might change earlier or later times.

In conclusion, in this study using microarray analysis of gene expression, newborn rat brains were shown to react very differently than young and adult rat brains following blood injection. Generalized overexpression of genes by the newborn brain following blood injection was corroborated at the protein level using antibody microarray. This study emphasizes the need to study newborn brain separately and not to extrapolate information from studies in mature brain. This study is of potential relevance to human neuropathological processes because the newborn rat brain is developmentally comparable to a 24-week human brain, at which age premature infants are prone to germinal matrix hemorrhage. We present an array of gene products that can potentially lead to further understanding of the pathogenesis of blood injection -related damage in immature brain. These findings should be considered a means of focusing further questions into studies concerning blood injection.

Chapter 2.2 (See hypothesis 2 -aim 1)

Germinal Matrix Hematoma Suppresses Cell Proliferation in Premature Humans and Neonatal rats

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Summary

In premature infants born at 24-28 weeks gestational age, germinal matrix hemorrhage (GMH) is a common occurrence with adverse outcomes on brain development. We hypothesized that GMH suppresses germinal matrix cell proliferation and the subsequent production and migration of glial precursors. We assessed the status of human germinal matrix cell proliferation following GMH using Ki-67 nuclear antigen. In comparison to control brains without hemorrhage, the brains of premature human infants with GMH were found to have significantly decreased cell proliferation, particularly if survival was >24 hours. In the neonatal model of periventricular hematoma, we used Ki-67 immunoreactivity, flow cytometry, and bromodeoxyuridine (BrdU) incorporation to do the same. In addition, we studied the migratory fates of cells using BrdU incorporation. In the neonatal rodent blood injection model, suppression of cell proliferation using the Ki-67 marker, BrdU incorporation, and flow cytometry was demonstrated. Differentiation studies showed that the distribution of $BrdU^+$ cells was altered 5 and 21 days post blood injection. This study shows that extravasated blood in the ganglionic eminence is associated with suppression of cell proliferation in premature human infants and in newborn rats. This may help to explain smaller brain size and cognitive problems in children who suffer GMH after premature birth.

Keywords: cerebral palsy, preterm infants, ganglionic eminence, intraventricular hemorrhage

INTRODUCTION

In premature infants born at 24-28 weeks gestational age, germinal matrix hemorrhage (GMH) is a common occurrence (2.5/1000 live births) (Robertson et al., 1998) following the rupture of delicate immature blood vessels. It is often unilateral, located in the ganglionic eminence (germinal matrix) beside the frontal horn of the lateral ventricle. Blood can either extend into the ventricles and or it can cause damage to the adjacent tissue (Volpe, 2001a). A potential consequence of GMH is a permanent neurological deficit, such as cerebral palsy (Hagberg and Hagberg, 2000). Considering that prenatal brain damage is among the most prevalent causes of costly neurologic disabilities (Rubin et al., 1992; MacDonald et al., 2000), it is important to understand both the mechanism of injury and the brain response following neonatal GMH.

The proliferating germinal cell layers and developmental processes are of interest because they are vulnerable to a variety of neonatal brain injuries. Although most neurons have been generated by 18-20 weeks gestation in humans, neither their migration nor their synapse formation is complete (Lemire et al., 1975). The germinal matrix continues to give rise to a considerable quantity of precursors of oligodendrocytes, which produce myelin, and astrocytes, which support maturation of neurons until it involutes at approximately 30-32 weeks of gestation (Del Bigio, 2004). Preoligodendrocytes are the predominant cell type of the oligodendrocyte lineage in the early developmental period of 23-32 weeks gestation (Back et al., 2001b) when most GMH/IVH occurs. The brains of both the 1-day old mouse and the 2-3-day old rat, are at an equivalent level of maturity to a 24-26 week old human (Kakita and Goldman, 1999; Levers et al., 2001). Postnatal rodent brains may therefore be useful for the study of

human prenatal injuries such as GMH. In a blood infusion neonatal mouse model (Xue et al., 2003) and in a preliminary study of human infants (Del Bigio et al., 2003), reduced cellular proliferation has been observed in the germinal matrix near the site of the hematoma. Thus, we hypothesized that GMH prevents germinal matrix proliferation and the migration of glial precursors. Studies using bromodeoxyuridine (BrdU) or tritiated thymidine pulse-labeling have yielded valuable information about the nature of cell proliferation in the germinal layers (Takahashi et al., 1995). BrdU is a thymidine analog incorporated into newly synthesized DNA of dividing cells, rendering them detectable by immunohistochemical means (del Rio and Soriano, 1989). Ki-67 immunohistochemistry technique was used in the periventricular hematoma model in neonatal rats. In addition, migratory fates were studied in this model using BrdU and specific cellular markers for double immunostaining. Mature oligodendrocytes were studied using myelin basic protein (MBP) and transferrin (Bloch et al., 1985). Reactive astrocytes and microglia were labeled using GFAP and lectin Bandeiraea simplicifolia respectively.

METHODS

Animal studies

All experimental procedures were done in accordance with guidelines of the Canadian Council on Animal Care and protocols were approved by the local experimental ethics committee. Newborn (24-36 hours) Sprague–Dawley rats (n= 36 for Ki-67 study and n=65 for BrdU study) were used. They were anesthetized by cooling on an ice bed. Autologous blood was collected in a sterile syringe by placing the tail in warm water for 1 minute, cleaning the skin with 70% alcohol, and cutting 2 mm off the tail tip as previously described (Xue et al., 2003). After blood collection (15μL), a 28-

gauge needle was attached to the syringe and introduced percutaneously into the right periventricular region (adjacent to the caudate-putamen) using a custom-made guide (2.5 mm deep to the skull surface, 1.5 mm lateral to midline, and 0.5 mm posterior to the outer canthus of the right eye). Sham rats received an injection of 15μL saline. As needle insertion alone can cause brain damage, intact control animals were anesthetized only. Identification was made by ear punch, and then the animals were warmed on a 25˚C blanket and returned to the mother.

Magnetic Resonance Imaging (MRI)

MRI was used to assess the size and location of the brain hematomas as previously described (Xue et al., 2003). Briefly, the pups were anesthetized with 1.5-2 % halothane in a 70:30 mixture of N_2O:O_2. MRI was performed using a Bruker Biospec/3 MR scanner equipped with a 21cm bore magnet operating at a field of 7T (Karlsruhe, Germany) to obtain T2-weighted images of brain. Newborn rats were imaged 20-30 minutes after injection of blood or saline. Sham injections that resulted in substantial hemorrhage and misplaced blood injections (typically too lateral) were excluded. Observers blinded to the condition of the animal made all subsequent observations.

BrdU injection

BrdU (50mg/kg intraperitoneal) was administered within 10 minutes after blood or saline injection to label cells that were undergoing mitosis at that time (for each time point blood n = 4; sham control n = 4; intact control n = 4). BrdU was also administered at 24 hours and 48 hours after initial blood or saline injction. Rats were sacrificed 2 hours, 24 hours, 72 hours, 5 days or 21 days following blood or saline injections. Rats

sacrificed at 2 and 24 hours were used to confirm the Ki-67 findings concerning acute changes in cell proliferation. For the longer-term studies, we used three BrdU doses to increase the likelihood of labeling and detecting proliferating cells that subsequently migrate.

Tissue processing

Rat were overdosed with ketamine / xylazine after blood injections and perfused through the heart with 5 ml ice-cold 4% paraformaldehyde in 0.1M PBS. Brains were removed, immersion fixed in 4% paraformaldehyde for 8 hours, cut into 3-5 mm slices, and postfixed in 4% paraformaldehyde for an additional 20 hours. Tissue was then dehydrated through an ethanol series and embedded in paraffin wax. As a positive tissue control for the BrdU incorporation, samples of small intestine and muscle were embedded beside the brain slices. Paraffin embedded tissue was sectioned to 5μm thickness and mounted on glass slides. From each group, sections were stained with hematoxylin and eosin (H&E) and the coronal levels with damage were identified.

Detection of proliferating cells by Ki-67

Rats were studied 8, 24, 48 hours and 7 days after blood injection (blood n=3; sham control n=3; intact control n=3 at each time point). Paraffin sections were dewaxed and rehydrated, slides were microwaved in 0.6M citric acid buffer for 15 minutes, cooled in 0.1M PBS for 20 minutes, blocked with 10 % normal donkey serum, and incubated with anti-rabbit Ki-67 polyclonal antibody (Novocastra, U.K, diluted 1/500) at 4˚C overnight. Slides were then washed, incubated with Cy3 donkey anti-rabbit IgG

(1: 500), and nuclei were counterstained with bisbenzimide (Hoechst 33342, Sigma) prior to coverslipping. Negative controls were processed without the primary antibody.

Ki-67 cell counting

The proportion of Ki-67 positive proliferating cells was calculated by dividing the number of Ki-67 immunoreactive nuclei by the total number of nuclei stained with bisbenzimide. These proportions were determined in the germinal matrix and white matter over a 100 x 100 μm area and counted at 400x magnification. Cells were also counted in striatum, superficial and deep cerebral cortex within a 250 x 250 μm area at 200x magnification. The presence of blood and the size of the ventricles precluded blinding between groups.

Detection of proliferating cells by BrdU

For BrdU single labeling, slides were dewaxed, rehydrated, incubated in 50% formamide/2x saline–sodium citrate (SSC) for 2 hours at 65°C, washed in 2x SSC for 5 minutes, incubated in 2N HCl for 30 minutes at 37°C, washed with borate buffer (0.1M, pH 8.5) for 12 minutes at room temperature, washed in 0.1M PBS (pH 7.4) with 1% Triton X 100, blocked with 10% donkey serum for 30 minutes, and incubated with mouse anti-BrdU antibody (1:200, Sigma) overnight at 4˚C in a humidifying chamber. Cy3 donkey anti-mouse antibody (Jackson ImmunoResearch Lab, West Grove, PA) was used to visualize BrdU labeling. For BrdU double labeling we modified the pre-antibody step slightly. Slides were washed in 0.1M PBS with 1% BSA for 15 minutes, microwaved in 0.6M citric acid buffer (pH 6) for 15 minutes, cooled in 0.1M PBS for 20 minutes, incubated in HCl (1N) for 5 minutes on ice followed by HCl (2N) for 10 minutes

at room temperature followed by 20 minutes at 37˚C, washed in PBS with 0.1% Triton-X 100 for 10 minutes, blocked in a solution that contained 0.1% BSA, 0.2% Triton-X 100, and appropriate blocking serum for 20 minutes. Sections were incubated with the following antibodies in combination with the anti-BrdU at 4˚C overnight: rabbit anti-GFAP to label reactive astrocytes (1:500, Sigma, USA), rabbit anti-MBP (1:400, DAKO, USA) or rabbit anti-transferrin (1:400, Cappel, USA) to label mature oligodendrocytes. The slides were incubated in the following secondary antibodies for 1 hour and cover slipped: Cy3 donkey anti-mouse, FITC goat anti-rabbit, Cy3 rabbit anti goat, and FITC sheep anti-mouse. All secondary antibodies came from Jackson ImmunoResearch Lab (West Grove, PA). For the demonstration of reactive microglia, after detecting the BrdU cells slides were quenched with 0.3 % H_20_2 for 10 minutes and washed in 0.1M PBS for 10 minutes. Slides were blocked with 1% BSA for 20 minutes, incubated with lectin Bandeiraea simplicifolia (1:100, BS-I, Sigma) at room temperature for 1 hour, washed in PBS for 10 minutes, incubated in streptavidin-conjugated peroxidase (1:400) for 30 minutes, then 3,3-diaminobenzidine (DAB) solution was used to identify positive cells.

BrdU+ cell counting

BrdU+ cells were counted in the same regions as Ki-67. As in previous studies (Price et al., 1997), BrdU+ cells were defined as cells in which more than half the nucleus was labeled. Three areas of the germinal matrix and white matter (100 x 100 μm, 400 x magnification), striatum and cortex (250 x 250 μm area at 400x magnification) were counted. In order to understand the spatial and temporal distribution, BrdU+ cells in the ipsilateral brain sections of 5 and 21 day post-blood injection rats and age-matched controls were mapped onto a diagram at 200x ocular magnification. Each point on the map represented 10 cells in the region. In addition,

BrdU+/GFAP+ and BrdU+/transferrin+ cells on brain sections from rats 5 and 21 days after blood injection as well as corresponding aged-matched controls were counted in three areas of the germinal region, white matter, striatum, and cortex at 400x magnification.

Statistics

Results are presented as mean ± standard error of the mean (SEM). Statistical analysis for both BrdU and Ki-67 labeled cells was performed using ANOVA followed by Scheffé's (unequal sample size) or Fisher (equal sample size) post-hoc tests respectively. Statistical analysis for BrdU-GFAP labeled and BrdU-transferrin labeled cells was performed using Student's t test. $P < 0.05$ was considered statistically significant (StatView 5.1 software, SAS Institute Inc., Berkeley CA).

RESULTS

Animal studies

All rats tolerated the injection of 15 μl blood into the periventricular region and there was no surgical mortality. On T2-weighed MR images, blood appeared dark in the periventricular region and in the ventricles. Saline injection was associated with only very small foci of blood accumulation (Figure 2). In the normal postnatal brain, the thickness of the germinal matrix decreased in an age- dependent manner: 0-8 hours ~12-15 cell layers, 24-48 hours ~8-10 layers, and 5-7 days ~2-3 layers. H&E staining showed that 8, 24 and 48 hours after blood injection, a small hematoma occupied the germinal matrix and adjacent striatum. Blood cells mixed with germinal cells at the angle

of the lateral ventricles and small amounts were present in the ventricles (Figure 1B). Twenty-four and 48 hours after the blood injection, a penumbra surrounding the hematoma appeared pale due to edema. By day 7, edema and the hematoma had disappeared. Twenty-one days after the blood injection, the ventricles were minimally enlarged.

Cell proliferation

In the normal brain, the majority of cells in the newborn germinal matrix were Ki-67+ (Figure 4) at 8 , 24, and 48hours. Many cells were also positive in the white matter, striatum, and cerebral cortex. There was a significant decrease in Ki-67 immunoreactivity in the germinal matrix 8 hours following blood injection. This suppression persisted up to 48 hours (Figure 4). Suppression was also observed on the contralateral side for up to 48 hours. Reduced Ki-67 immunoreactivity was observed in the white matter up to 7 days after blood injection on the ipsilateral side and up to 24 hours on the contralateral side (Figure 4). Suppression was observed in the ipsilateral striatum and deep cortex at 24 hours post blood injection (not shown).

In the normal brain, BrdU+ cells were observed throughout the germinal region (Figure 4), white matter, striatum, and cerebral cortex, similar to the Ki-67 pattern. The quantity of BrdU+ cells was significantly decreased 24 hours after blood injection in the germinal matrix ipsilateral to the hematoma (Figure 5). In the white matter, striatum, and cortex there was a decrease in proliferating cells that was not statistically significant.

Cell Maturation and Distribution

To understand the migration process following blood injection, we compared rat brains 5 and 21 days after blood injection to age-matched intact controls (Figure 6). In the 5-day control group, cells were distributed evenly in the cortex, white matter, and striatal region. By 21 days, more BrdU+ cells were apparent in the cerebral cortex than the white matter and striatal region. In the blood-injected brains at 5 days, fewer BrdU+ cells were observed in the dorsal striatum and BrdU+ cells were unevenly distributed in the cerebral cortex. There was a conspicuous paucity of cells in the dorsal cortex along the needle injection tract. There were also fewer cells in the external capsule and corpus callosum. At 21-days, these abnormalities persisted.

Transferrin labeling of oligodendrocytes was absent in white matter of 5-day controls. At 21 days, abundant transferrin+ cells were co-labeled with BrdU+ in the white matter (Figure 7) and striatum. Several BrdU+/transferrin+ cells were observed in the deep layers of cerebral cortex and but not in the superficial layers of cerebral cortex. BrdU+ nuclei were intermingled with the MBP+ myelin at 21 days (Figure 7). However, the absence of selective labeling of the oligodendrocyte cell bodies precluded definitive identification of BrdU+ cells with this antibody.

In control and blood injected brains at 5 days we observed GFAP+ cells in the germinal region with radial glial morphology. GFAP+ cells were also present in periventricular white matter around blood vessels. By 21 days, GFAP+ cells were present in the subcortical white matter and deep cortical regions. At 21 days, in comparison to control brains, blood injected brains exhibited more intense GFAP immunoreactivity throughout the ipsilateral white matter and striatum. However, the majority of GFAP + cells were not BrdU+. Using the lectin BS1, only a few reactive microglia were identified in the periventricular region of 21 day blood injected rats.

Labeling was absent in the intact and sham control groups. Furthermore, we did not find any cells that were co-labeled for BrdU+. Not all BrdU+ cells could be accounted for by our double labeling strategies. A substantial proportion of those in the cerebral cortex and white matter were elongated and aligned with small blood vessels, suggesting that they represent endothelial cells. These and other small cells with unidentifiable morphology were not included in the quantification. We quantified the co-localization of BrdU/transferrin and BrdU/ GFAP in three regions of cerebral cortex. There were significantly more BrdU+/transferrin+ cells than BrdU+/GFAP+ cells. Following blood injection, there was a 30% decrease in BrdU+/transferrin+ cells, although this did not reach statistical significance (P=0.156) (Figure 8).

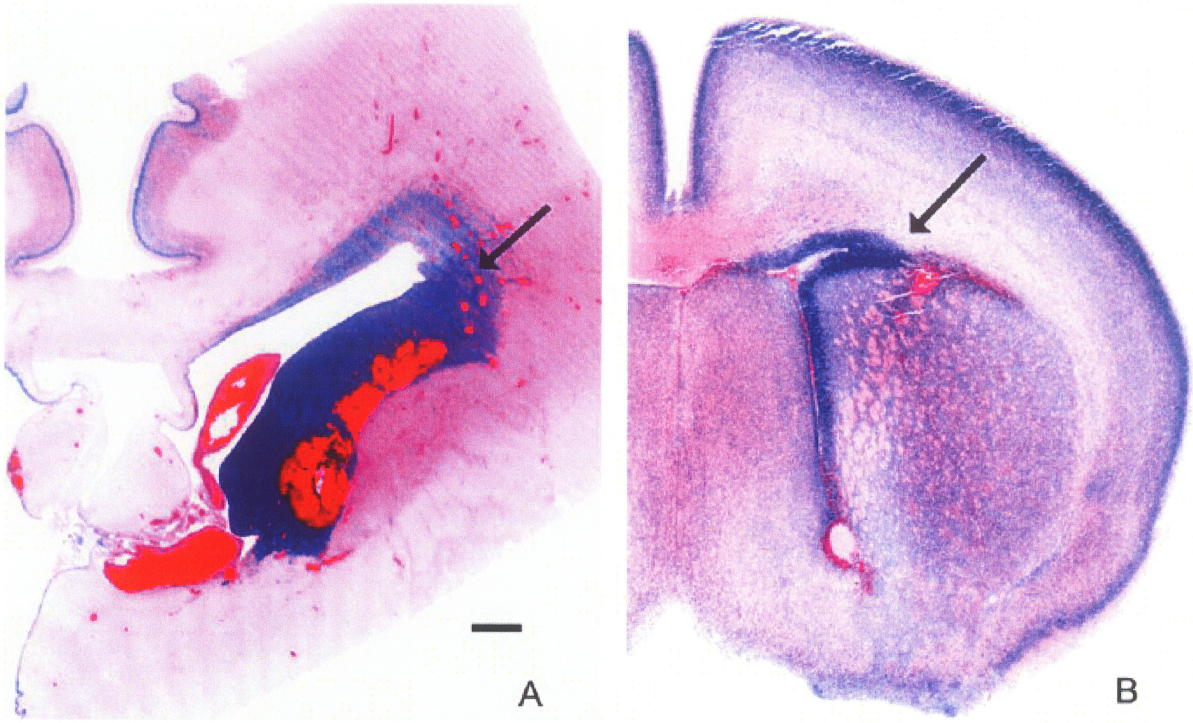

Figure 1. Hematoxylin and eosin stained coronal brain sections of periventricular germinal tissue from a human fetus (~25 weeks gestational age) obtained after several hours survival (A), and a neonatal rat brain obtained 24 hours after blood injection (B). the hematoma (red) is seen in the germinal matrix (blue, arrow) and in the lateral ventricles (white space) of both. Bar = 1mm for human brain sections and 300µm for rat brain section.

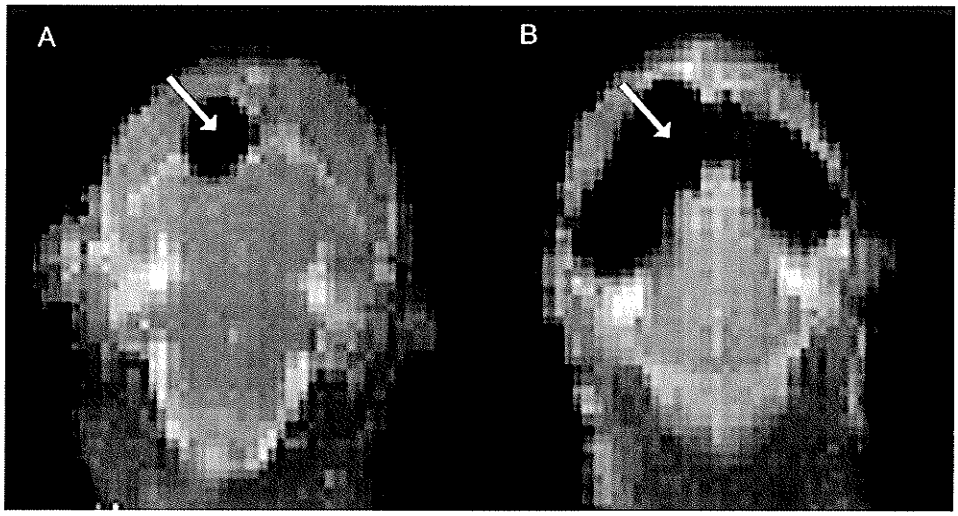

Figure 2. T2-weighted magnetic resonance images showing horizontal slices of 1-day old rat brain. Fresh blood collections have hypointense (dark) signal. Ten minutes after saline injection (15µl) there is small blood collection due to needle damage (arrow) in the striatum (A). Ten minutes after autologous blood injection (15µl) the hematoma spreads from the striatum (arrow) into the ventricles (B).

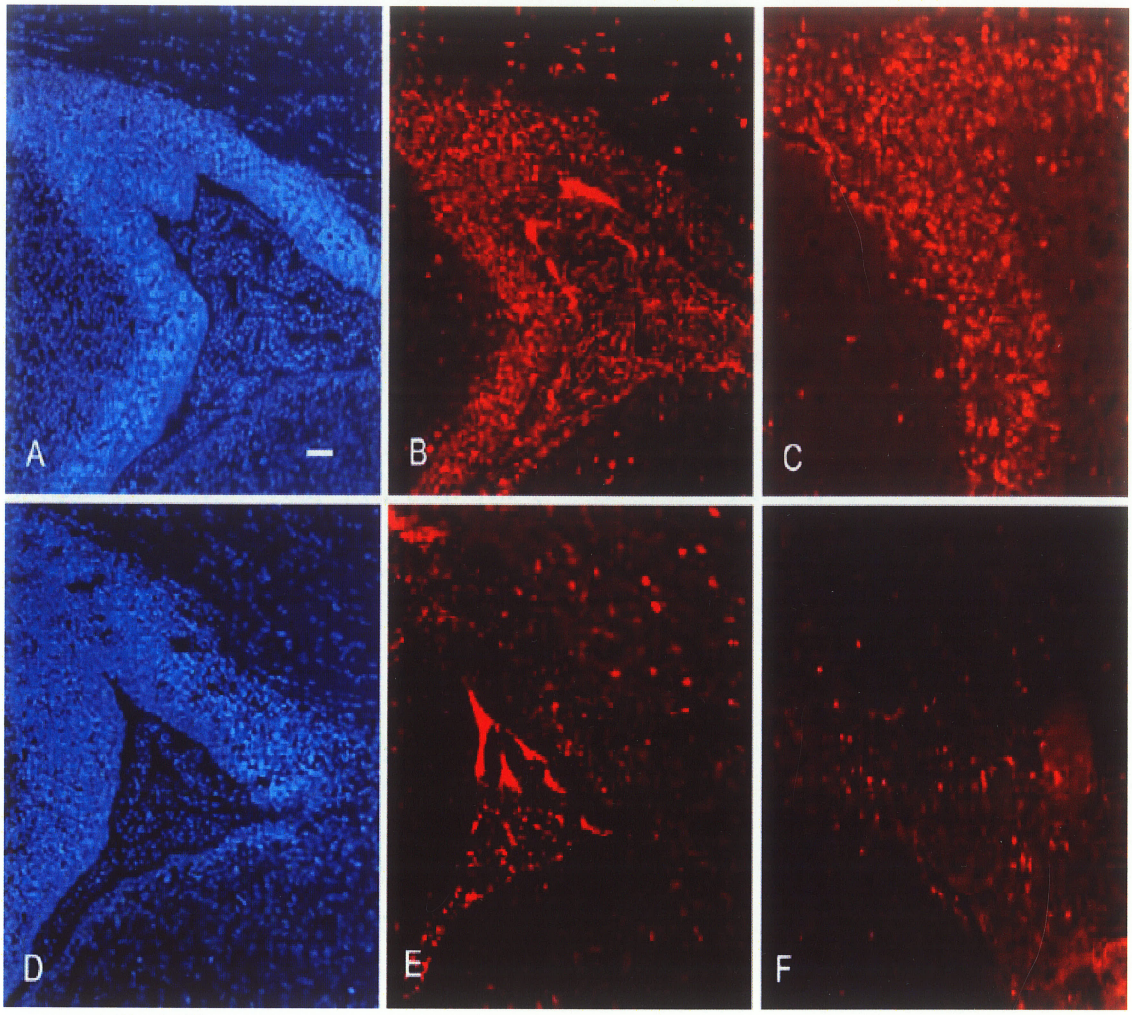

Figure 3. Photomicrographs showing Ki-67 immunolabeling (B and E) and corresponding bisbenzimide counterstaining of nuclei (A and D) in the germinal matrix at the angle of the lateral ventricle of control rat (upper row) and rat that received blood injection 24 hours earlier (lower row). BrdU incorporation is shown in corresponding location (C and F). In controls there are abundant proliferating cells in the germinal matrix After blood injection far fewer Ki-67+ and BrdU+ cells are seen than in the respective age-matched brain sections. Bar = 30μm for A, B, D, E and 15μm for C and F.

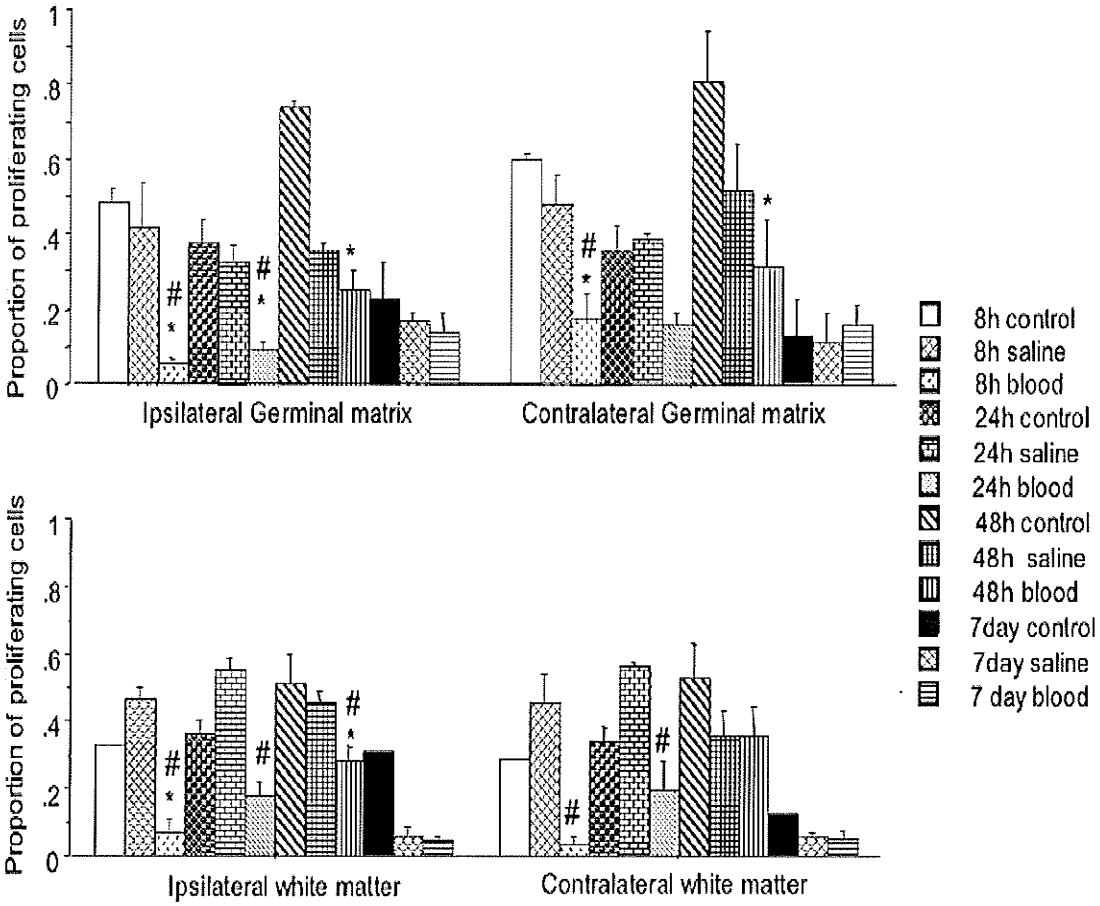

Figure 4. Bar graphs showing the proportion of proliferating cells (calculated by the number of Ki-67 immunoreactive nuclei / total bisbenzimide stained nuclei) in the germinal matrix and white matter regions of newborn rat brain at sequential times after blood injection. * indicates intact control vs. blood $p<0.05$, and # indicates saline control vs. blood $p<0.05$.

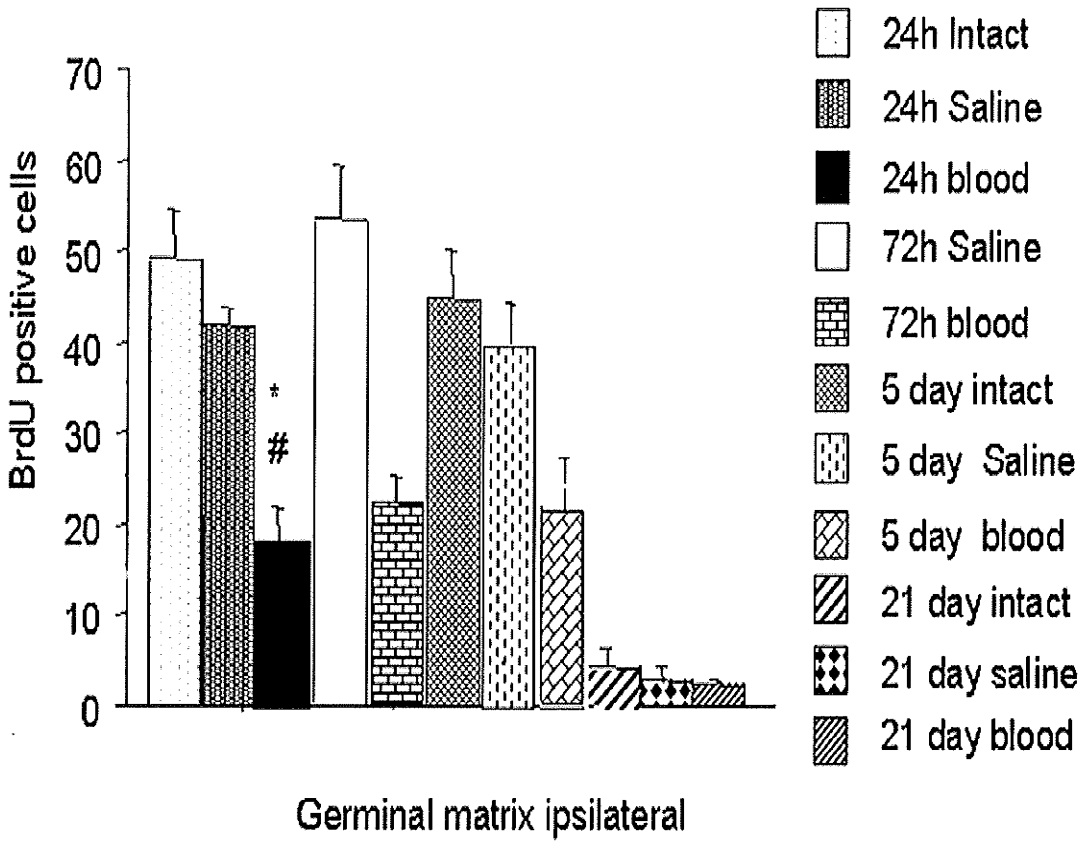

Figure 5. Bar graph showing BrdU+ cells in the germinal matrix at sequential times after blood injection. * Indicates intact control vs. blood $p<0.05$ and # indicates saline control vs. blood $p<0.05$.

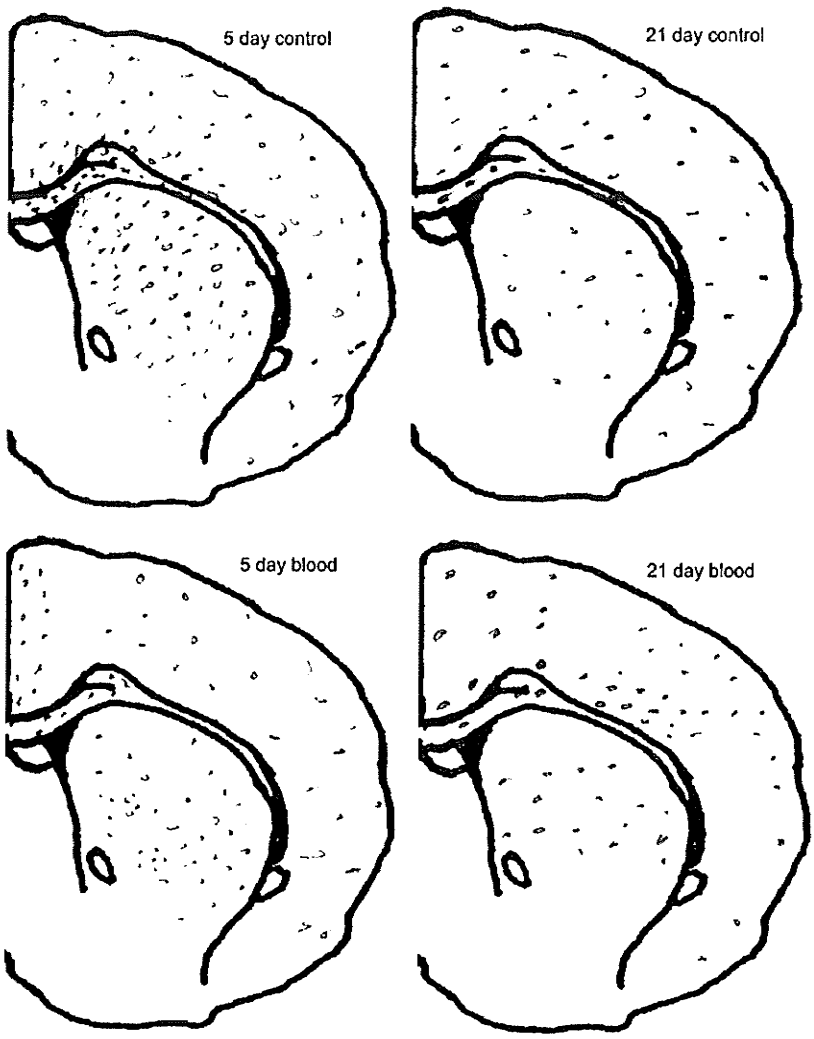

Figure 6. Schematic drawings of coronal rat brain sections showing the locations of BrdU+ cells in individual 5 and 21 day old control rats and rats that had received blood injection. Each dot represents approximately 10 cell clusters (x 400 magnification). Note the paucity of labeled cells in the dorsal striatum and foci of the cerebral cortex after blood injection.

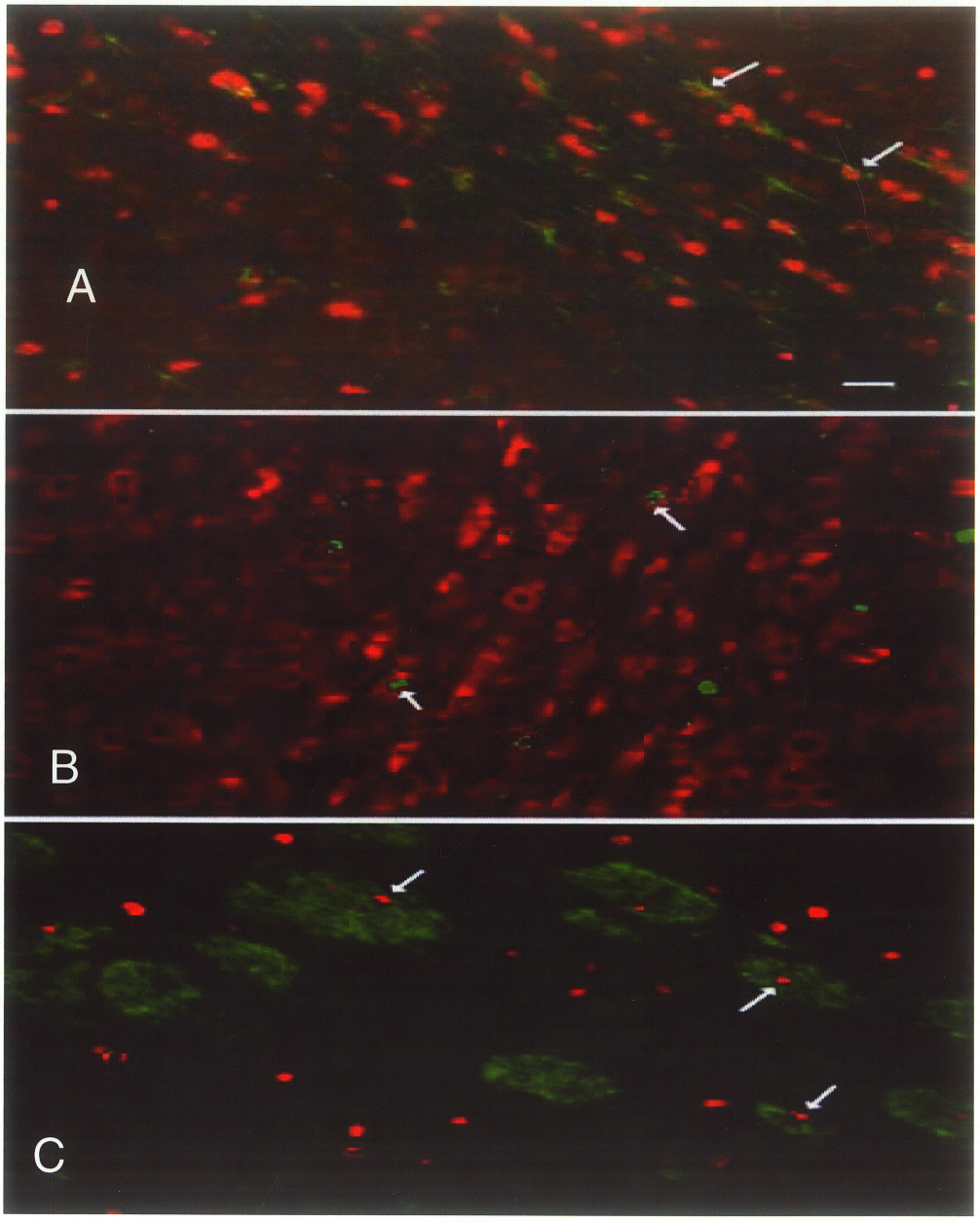
A
B
C

Figure7. Photomicrographs showing differentiation of cells that have incorporated BrdU in 21-day cerebrum. Only rare astrocytes in the white matter are double immunolabeled for GFAP+ (green) and BrdU+ (red) (A, arrows). Scattered oligodendrocytes are also transferrin+ (red) and BrdU+ (green) (B, arrows). In the striatal white matter bundles, myelin basic protein (MBP)+ (red) surrounds BrdU+ nuclei (green) (arrows), but individual cell colocalization is not possible (C). Bar = 15μm.

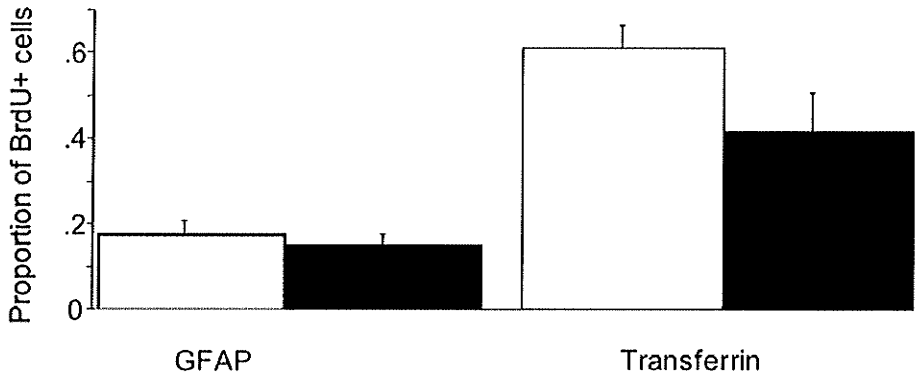

Figure 8. Bar graph showing proportion of GFAP+ astrocytes and transferrin+ oligodendrocytes that are co-labeled with BrdU in the rat cerebrum 21 days after blood injection (black bars) and in corresponding age-matched control brains (white).

DISCUSSION

Using both the Ki-67 and BrdU labeling, suppression of germinal cell proliferation was also observed following autologous blood injection into brains of neonatal rats and mice, which have brain maturity comparable to 24-26 week old humans (Kakita and Goldman, 1999; Levers et al., 2001).

By postnatal day 1 in rats and mice, most neurons have been generated and oligodendrocyte progenitors are the predominant product of the germinal matrix / subventricular zone (Levison and Goldman, 1993). Preoligodendrocytes are also the predominant cell in the oligodendrocyte lineage between 23-32 weeks of human gestation, when prenatal damage is common (Back et al., 2001a). Several animal and cell culture studies have shown the selective vulnerability of oligodendrocyte lineage cells following injury to immature brain associated with excitotoxicity (Matute et al., 1997), oxidative stress (Oka et al., 1993) and hypoxic ischemia (Back et al., 2002). Given the susceptibility of immature oligodendrocytes to injury in the immature brain, we expected that a decrease in oligodendrocyte quantity would be demonstrated as a consequence of germinal matrix suppression. We did observe a decrease in the number of BrdU+ oligodendrocytes, but the change was not statistically significant. One possibility is that the decrease is real but our sample size limited the power of the test. Alternately, because the germinal matrix was not completely suppressed and assuming that surplus cells are normally generated, sufficient preoligodendrocytes may have been available. This enhanced maturation phenomenon has been shown in other brain injury models (Nishiyama et al., 1997; Back et al., 2002). We also observed BrdU+ cells that were not double-labeled with any of the cell markers used. Some of these cells had

endothelial morphology and others were unidentifiable. It is possible that the unidentified cells were immature oligodendrocytes. We attempted to label these cells using antibodies to PDGF alpha-receptor, O4, and NG2. The potential non-specificity of these antibodies, however, has been previously established. The PDGF alpha receptor is also expressed in neurons (Oumesmar et al., 1997; Andrae et al., 2001), NG2 has been found in interneurons, astrocytes, and microglia (Butt et al., 2002; Aguirre et al., 2004), and O4 has localized to microglia (Yokoyama et al., 2004). As the unidentified cells represent a miniscule proportion of the quantified cells, it probably did not affect our quantitative analysis.

Rat and our prior mouse experiments (Xue et al., 2003) suggest that germinal matrix cell suppression becomes apparent at least several hours after blood exposure. In this study rat data using Ki67 and BrdU suggest that the suppression persists for at least 5 days. It is not clear that it rebounds because the germinal matrix involutes toward the end of the suppression period. A phenomenon of transient suppression has been previously documented. Three-day old mice subjected to 24-hour hypoxia exhibit significantly decreased brain subventricular zone cell proliferation on day 4 and 11, with normalization by day 18 (Fagel et al., 2005). Both the Ki67 and the BrdU methods of detection used in this experiment are considered to be specific for dividing cells, however the BrdU method used might be less sensitive for a few reasons. A consequence of only counting cells that were strongly labeled with anti-BrdU may have lead to an underestimation of labeling. Unlike Ki-67, which is expressed from late G1 to M phase of the cell cycle, BrdU is incorporated solely in the S phase. Thus Ki-67 can detect roughly 4 times as many cells that are undergoing mitosis. Finally, the first BrdU injection was made immediately following the blood injection, possibly before the onset

of suppression. If the onset of suppression is in fact delayed by hours, we might have labeled cells still undergoing normal development thereby reducing the likelihood of showing a delayed difference. Regardless, the germinal matrix cell cycle suppression that occurs following blood injection or hemorrhage has been clearly demonstrated by several methods. The onset and duration of the effect requires further clarification.

The mechanism of the suppression phenomenon is largely unknown. Suppression of proliferation following an insult has previously been studied in other model systems, such as in hypoxia-ischemia (Levison et al., 2001; Fagel et al., 2005), although none have specifically examined the germinal matrix. Several authors have postulated potential mechanisms. These include decreased blood flow, cytokines such as tumor necrosis factor alpha and interferon gamma (Wu et al., 2000), chemokines such as CXCL1 and CXCR2 (Tsai et al., 2002), and toxic neurotransmitters such as glutamate and GABA (Cameron et al., 1998; Luk and Sadikot, 2001). In mouse studies we have shown that systemic activation of the immune system aggravates the brain damage after neonatal blood injection (Xue and Del Bigio, 2005a). However, in regard to inflammation, we must note that our immunohistochemical studies in the human brains showed only very few activated microglia, neutrophils, or lymphocytes in the vicinity of the hematoma after GMH. While this does not rule out an inflammatory explanation, it does seem to reduce the likelihood that this plays the major role. In the neonatal rodent brain with experimental periventricular hematoma, we have shown that the proteolytic plasma protein thrombin likely plays a role in the damage (Xue et al., 2005). Injections of blood, thrombin, and plasminogen more severely damage neonatal mouse brain than mature mouse brain. It is important to note in this experiment that cell suppression was also observed contralateral to the blood injection site. Since the white

matter in the rats is in close proximity to the germinal matrix, it is likely that a diffusible substance plays a role. Due to the retrospective nature of the human component, we cannot answer the question of the range of the effect of a hematoma after premature birth. Because the volume of cerebral white matter in rodents is small, some aspects of the white matter damage will need to be studied in brains of larger animals.

There are a few limitations and confounding factors that should be mentioned. Because BrdU+ glial cells were quantified in only limited areas, immature cell differentiation in other brain regions may have been overlooked. Using both Ki-67 and BrdU labeling, a minor increase in cell proliferation following saline injection occurred in the white matter but not in the germinal matrix. This could be a result of injury during the needle insertion with subsequent reactive astrogliosis. It is difficult to separate the cellular induction from the suppression in the blood-injected group. Finally, as noted above, the timing of our BrdU injections might not have been optimal to detect changes after blood injection because suppression might take more than a few hours.

Even with the statistically significant reduction in germinal matrix cell proliferation in infants with GMH and in the rodent periventricular hematoma model, the consequences of suppression are not entirely clear. It has been suggested that changes in cerebral size and shape are attributable to the number of progenitor cells in the proliferative zones (Luskin et al., 1993; Kornack and Rakic, 1998). Human studies have shown that the volume of the cerebrum, white matter, and subcortical gray matter is reduced after GMH (Kuban et al., 1999; Kesler et al., 2004; Vasileiadis et al., 2004b; Inder et al., 2005). Our long-term behavioral and imaging studies in rats have also shown a decrease in brain volume following blood injection(Balasubramaniam et al., 2006). Therefore, our finding of reduced cell proliferation may be of clinical significance and

may be associated with impaired developmental outcomes. The ultimate goal of developing therapeutics for GMH will be feasible only if the cellular mechanism of injury is elucidated. Although many questions remain, the demonstration that blood in the germinal matrix causes suppression of germinal matrix proliferation in humans and in a neonatal rat model is a positive step in this regard. To our knowledge this is the first study that demonstrates that the suppression of proliferation in human GMH after premature birth. The fact that this finding can be replicated in animal models means that they can be used to study the mechanism.

Chapter 2.3 (hypothesis 2- aim 2)

Title: Persistent motor deficit following infusion of autologous blood into the periventricular region of neonatal rats.

Note : manuscript published in Experimental neurology 2006 Jan;197(1):122-32

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(Injections were done by Dr. Xue, MRI was done by Dr. Buist, and Golgi studies were done by Ms. S. Natuik.)

Abstract

Periventricular hemorrhage (PVH) in the brain of premature infants is often associated with developmental delay and persistent motor deficits. Our goal is to develop a rodent model that mimics the behavioral phenotype. We hypothesized that autologous blood infusion into the periventricular germinal matrix region of neonatal rats would lead to immediate and long-term behavioral changes. Tail blood or saline was infused into the periventricular region of 1-day old rats. Magnetic resonance (MR) imaging was used to demonstrate the hematoma. Rats with blood infusion, as well as saline and intact controls, underwent behavior tests until 10 weeks age. Blood infused rats displayed significant delay in motor development (ambulation, righting response, and negative geotaxis) to 22 days of age. As young adults they exhibited impaired ability to stay on a rotating rod and to reach for food pellets. MR imaging at 10 weeks demonstrated subsets of rats with normal appearing brains, focal cortical infarcts, or mild hydrocephalus. There was a good correlation between MR imaging and histological findings. Some rats exhibited periventricular heterotopia and/or subtle striatal abnormalities not apparent on MR images. We conclude that autologous blood infusion into the brain of neonatal rats successfully models some aspects of periventricular hemorrhage that occurs after premature birth in humans.

Key words: behavior, brain, cerebral palsy, magnetic resonance imaging

Introduction

Approximately 2% of human births are extremely premature (<31 weeks gestation). Periventricular hemorrhage (PVH; also called subependymal or germinal matrix hemorrhage) occurs in ~40% to 50% of infants born at <26 weeks and ~20% of infants born at 26-32 weeks gestation (Berger et al., 1997; Larroque et al., 2003). It arises in the germinal matrix region adjacent to the lateral ventricles, especially the ganglionic eminence. Fragile blood vessels in the germinal matrix and physiologic conditions (e.g. impaired cerebral autoregulation, variable blood pressure) are the major contributors to PVH (Larroche, 1977; Ghazi-Birry et al., 1997; Shalak and Perlman, 2002). PVH is usually graded according to the extent of hemorrhage; the most severe form (grade IV) includes extension of hemorrhage medially into the ventricles and laterally into the striatal region (Papile et al., 1978). PVH can lead to severe neurological sequelae including seizures, mental retardation, and cerebral palsy (Hagberg and Hagberg, 2000). Cerebral palsy is a clinical term used to describe a broad group of motor syndromes resulting from damage to the developing brain (Shapiro, 2004). There are few good animal models mimicking this. Derrick and coworkers (2004) demonstrated hypertonia and motor abnormalities in neonatal rabbits subjected to perinatal hypoxia, but this study extended to only a few days age (Derrick et al., 2004). An in utero inflammation model in rats was not associated with alteration in neurobehavioral development (Poggi et al., 2005), although it is not clear that the resulting brain damage in this model was significant. Considering the fact that perinatal brain injury is among the most prevalent and costly form of neurological disabilities (MacDonald et al., 2000) (Rubin et al., 1992) it is important to understand the brain damage mechanism and long term consequences that follow PVH.

Newborn rats were used because they are developmentally comparable to 24-26 weeks gestation humans (Dobbing and Sands, 1979; Bayer et al., 1995). We developed a model of autologous blood injections into the periventricular region of neonatal mouse cerebrum. We showed that there is infarction extending beyond the hematoma itself, that the damage is associated with suppressed germinal matrix cell proliferation, that the damage likely depends at least in part on the proteolytic activity of thrombin, and that the damage can be aggravated by pre-activation of the immune system (Xue et al., 2003; Xue and Del Bigio, 2003; Xue and Del Bigio, 2005a; Xue and Del Bigio, 2005b). However, the behavioral changes can be difficult to monitor in mice. Our goal was to create severe brain damage in newborn rats by injecting autologous blood into the germinal matrix region in order to model the motor abnormalities that develop after severe human PVH. We hypothesized that this would produce structural alterations as well as persistent behavioral deficits. We studied early developmental progression, long-term sensory motor abnormalities, and long-term changes in brain tissue using biochemical and histopathological techniques.

Materials and Methods

Animals

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. Neonatal (24-36 hours) Sprague–Dawley rats were used. Group sizes are described in Table 1 and testing schedule is shown in Figure 1. Unless otherwise specified, all experiments were done in a blinded manner. Rats were housed in standard cages, 12-hour light/dark schedule, with free access to rat chow and water.

Experimental blood infusion model

Neonatal rats were anesthetized by cooling on an ice bed. Autologous blood was collected in a sterile syringe by placing the tail in warm water for 1 min, cleaning the skin with 70% alcohol, and cutting 2 mm off the tail tip as previous described in mice (Xue et al., 2003). After blood collection (15μL), a 28-gauge needle was attached to the syringe and it was introduced percutaneously into the right periventricular region of the rat brain with a custom-made guide that helped to stabilize the needle and guide it to the correct depth (coordinates 3 mm deep to the skull surface, 1.5 mm lateral to midline, and 0.5 mm posterior to the outer canthus of the right eye). The tails of the sham and intact control rats were cut, maintaining experimental blinding. Sham rats received an injection of 15μL of saline. Intact control animals were anesthetized only. Animals were warmed in a 25˚C blanket, and then returned to the mother following recovery from anesthesia. Rat identification numbers were applied to the back with a felt-tip marker, and reapplied as needed. This was converted to a permanent ear punch at day 10.

Magnetic Resonance Imaging (MRI)

MRI was used to assess the hematoma size and location in the neonatal rats as previously described (Xue and Del Bigio, 2003). Briefly, the pups were anesthetized with 1.5-2 % halothane in a 70:30 mixture of N_2O:O_2. MRI was performed using a Bruker Biospec/3 MR scanner equipped with a 21cm bore magnet operating at a field of 7T (Karlsruhe, Germany) to obtain T2-weighted images of brain. Newborn rats were imaged immediately after the injection of blood or saline and the size of the hematoma or fluid collection was assessed. On MR images, the injection site was shown to be

anterior to the optic chiasm, roughly at the level of anterior commissure. Pups were only used for subsequent behavioral tests and histological analysis if MRI showed that the hematoma was in the correct location. Misplaced PVH injections (e.g. lateral extension to the brain surface) and sham injections that resulted in substantial hemorrhage due to accidental vascular injury were excluded. Observers blinded to the group identity made all subsequent observations.

The volumes of brain and hematoma were measured on T2-weighted images obtained immediately after blood injection (using locally developed software). Briefly, the perimeter of the hypointense area corresponding to the hematoma including ventricular extension, and that of the brain were traced and area values for the cross-sections were obtained. Values were multiplied by the slice thickness and summed to yield a total volume for the hematoma. Area/volume measurements were calculated for slices from the rostral cerebrum to the cerebrum/colliculus interface. A similar technique was used to obtain total brain volume. Using the MR images obtained prior to sacrifice, the volume of the left and right cerebral hemispheres was determined separately, subtracting the volume of the lateral ventricles.

Short-term histological evaluation

To ascertain that the histopathologic features of the acute brain damage are similar to those described previously in a similar model in mice (Xue et al., 2003), 22 newborn rats were killed 24 or 48 hours after saline (15μL, n=3 at each time point) or blood (15μL, n=4 at each time point) injections. Intact animals (n=4, at each time point) received anesthetic only. For logistic reasons, they did not undergo prior MR imaging. Rat were overdosed with ketamine / xylazine 24 and 48 hours after brain injections and

perfused through the heart with 15 ml of ice-cold 4% paraformaldehyde in 0.1M PBS. The brains were removed, fixed, and sliced in the coronal plane around the injection sites. Slices were dehydrated and embedded in paraffin. Sections (6 μm) were cut serially and each 30th section from the rostral to the caudal portion of the damage area was stained with hematoxylin and eosin (H&E).

Developmental behavior tests

Approximately 5 minutes before testing the dam was separated from the litter. Pups were individually removed from the cage and weighed. To study early motor development, the rats underwent behavioral testing starting on postnatal day 4 and ending on day 22. The observers were blinded to the condition of the rats. Table 2 summarizes the timing of the behavior tests. Briefly, the rats were observed for ambulation, surface righting, negative geotaxis, and forelimb grip (Altman and Sudarshan, 1975). Early ambulation was assessed on days 4, 8, 11, and 15. Each pup was observed for 5 minutes (triplicate trials) on a wood surface (50 cm x 50 cm). Movements were categorized as follows: No movement = 0, asymmetric limb movements = 1, slow crawling = 2, and fast walking = 3. The surface righting response was measured by gently placing rats onto their backs on a cotton sheet. The time to return to prone position and the direction of turning were recorded on days 4, 8, 11, and 15. The duration of the test was limited to 2 minutes and it was done in triplicate (Schroeder et al., 1995). Negative geotaxis reflects the pups' preference to face upward on a sloped surface. Each pup was placed head downward on an inclined plane 25° slope. Then the pup was held gently on the starting position for 5 seconds before being released. The time and direction to face incline position was recorded. The maximum

allotted time was 2 minutes and the test was done in triplicate. To assess forelimb grip strength and climbing ability, beginning at 11 days rats were allowed to grasp a wire (1.5-mm diameter, 70 cm long between two poles at a height of 40 cm. with a soft sponge placed underneath) with their forepaws. The time to falling was measured; the maximum allotted time was 2 minutes.

Adult rat behavior

Rats were housed with their mother until weaning on postnatal day 21. They were then placed in standard housing plastic cages, with 2-3 rats of the same gender in each cage. Table 3 summarizes the behavior tests. On weeks 6, 7, and 8 rats were examined weekly for spontaneous behaviors that did not require training; i.e. cylinder exploration and gait on rotorod. To assess forepaw preference, rats were placed in a transparent cylinder (20 cm diameter, 30 cm height) for 5 minutes (Schallert et al., 2000). The natural response is to explore for a route of escape. Two mirrors placed behind the cylinder at angles of 45° and 135° enabled the observer to record forelimb movements when the animal turned away from camera. The number of times the rat reared and the paw that it initially used to support its body against the cylinder wall was documented on videotape. Ambulatory agility was assessed using a rotating cylinder (7-cm diameter) (rotorod) (Economex, Columbus Instruments). First, endurance at a constant speed of 5 rpm was assessed for a maximum of 2 minutes. Second, the ability to stay on the cylinder at an accelerating speed was tested beginning at 2.5 rpm and increasing at a rate of 0.1 rpm every second for up to 2 minutes. The time was measured from the moment the rat was placed on the cylinder until it fell off. Each animal was given 3 trials.

Beginning on week 8, a subset of rats was trained for skilled reaching behavior (blood infused n=12, sham controls n=6); it was not practical to test skilled reaching on all rats because of the considerable training time required. Rats were randomly selected for training by the senior investigator who was aware of the treatment group but not the performance on prior tests. Food was restricted to ~80% of free feeding amount (~15 g / day) for up to 7 days to reduce the body weight by 5-15%. Rats were weighed daily and habituated to the special food (45 mg 3.5 mm diameter; Rodent "Dustless" Precision Pellets; Bio-Serv, Frenchtown NJ) during this period. Training for skilled reaching occurred during the 8th and 9th weeks. Rats were placed in a Plexiglas box (25 x 25 x 30 cm high) that has a narrow opening (10 mm wide slit) extending from the floor to a height of 15cm. On the outside wall, in front of the slit, 3 cm above the floor is a 2 cm wide by 4 cm long shelf. During the training period, rats were placed in the box until 20-25 pellets had been retrieved and eaten with the forepaw of their choice. During the second training week, the forelimb ipsilateral to the brain lesion was wrapped with an adhesive bandage (Waterblock-plus, Band-Aid) to force the rats to use the limb contralateral to the injection site (Whishaw et al., 1986). The testing period (10th week) consisted of exposure to similar feeding conditions on 5 consecutive days. Twenty pellets were offered. Reaches were only considered successful if the pellet was grasped and eaten on the first attempt. Failed attempts included all limb advances resulting in a dropped, missed, or displaced pellet. For the qualitative analysis, eating of 10-15 pellets was documented on videotape and detailed forelimb movements were scored as described in detail previously by Whishaw and coworkers (Whishaw et al., 1986; Vergara-Aragon et al., 2003). The single pellet retrieval task examines subtle impairments. Rats not selected for skilled reaching analysis (blood infused n=9, sham

controls n=13 and intact controls n=10) underwent gait analysis by placement on a 100 cm long, 15 cm wide ladder at a 45° incline with 3 mm diameter rungs spaced 2.5 cm apart. A dark box at the top is the goal. The speed to climb and the number of foot slips were recorded (Metz and Whishaw, 2002). Three trials were performed each on weeks 8 and 9. Cylinder exploration, ladder walking, and skilled reaching tests were video recorded and analyzed using a frame-by-frame analysis method (Whishaw et al., 1986).

Long-term histological evaluation

At the end of the behavioral experiments 10-week old rats were euthanized following MR imaging with pentobarbital (100mg/Kg of body weight) and transcardially perfused with ice-cold 0.1M phosphate-buffered saline. The brains were removed and split coronally at the site of the original needle insertion as shown in Figure 2. The middle slice of cerebrum (–1.30 mm to –0.26 mm relative to bregma) was dissected on a cooled surface (for potential biochemical analyses). The anterior portion of the brain (beginning –0.26 mm behind the bregma) was immersion fixed in 10% formalin, cut into coronal slices and embedded in paraffin. Paraffin sections (6 μm thick) were stained with hematoxylin and eosin, Perl's stain for iron, and solochrome cyanine for myelin. Reactive astroglial change was detected using polyclonal rabbit anti GFAP followed by detection with Envision System (DAKO).

Electron Microscopic Studies

A portion of the right medial corpus callosum was dissected from the fixed anterior cerebrum (n = 5 intact controls, n = 4 saline controls, and n = 7 blood infused) using a dissecting microscope (Figure 2B). It was washed, postfixed in 2.5%

glutaraldehyde in 0.1M PBS followed by 2% osmium tetroxide, dehydrated and embedded in epoxy resin so that the axons could be cut in cross section. Ultrathin sections were cut onto copper grids, contrasted with lead citrate and uranyl acetate, examined with JEOL 1010 microscope, and photographed for final printing at 27,000 X magnifications. On six non-overlapping photographs, myelinated axons that could be unambiguously identified were measured and minimum diameter (to exclude bias from oblique sections) was determined. Myelin thickness was also measured and the myelin thickness/ axon diameter was calculated for axons in the size ranges of <0.25 μm, 0.25-0.50 μm, 0.05-1.00 μm, and 1.0-2.0 μm.

Statistical Analyses

Data are expressed as mean ± standard error of the mean. The senior author decoded the data only after all analyses were complete. In the behavioral experiment, nonparametric data were compared using the Kruskal-Wallis test. For parametric data, 2-way ANOVA using treatment group and gender as independent variables were done followed by intergroup comparisons using Scheffé test, which is tolerant to differences in group sizes. The experiment was not originally designed to test for gender differences, however because we became aware of potential differences even in neonatal animals, we analyzed for this factor. Chi square test was used to compare the proportion of male and female rats with large structural defects. Paired t-test was used to compare left and right hemisphere volumes in individual rats. Regression analysis was used to determine if the initial hematoma size determined the final outcome. Differences were considered significantly different when $p<0.05$. We used StatView 5.01 software (SAS Institute; Cary NC).

Results

Neonatal rat blood infusion model

There were no deaths as a consequence of blood infusion into the neonatal brain. For the short-term experiment, we studied 8 rats with blood injection, 6 rats with saline injection, and 8 intact controls. For the long-term experiment, 21 blood infused rats were included in the final analysis. Of 26-saline infused control rats, 7 were removed from the study after the first MRI because there was excessive bleeding due to accidental vascular injury; 19 were included in the final analysis. Seventeen age matched intact controls were used for comparison (Table 1). Hair growth, eyelid opening, and weight did not differ between blood infused, saline, and control groups. There was a significant difference in weight at 9 weeks that was apparently attributable to gender ($F=7.197$, $p=0.0278$). A single brief seizure episode was noted during the training sessions of the skilled reaching test in one blood infused rat.

Measurement of hematoma volume using MRI

On T2-weighted MR images of neonatal rat brains, 20 minutes following whole blood injection (15 μL) the hematoma in the striatum and ventricles appeared hypointense (Figure 3). There was no significant difference in the initial cerebrum volumes of blood infused and control groups (mean volume 185 ± 29 mm^3). The initial apparent hematoma volume was 43 ± 5 mm^3. However, it must be recognized that MR imaging overestimates the blood volume in this model because of the large susceptibility effect of paramagnetic hemoglobin products (Xue et al., 2003).

Short-term histologic changes

Twenty-four (n = 4) and 48 hours (n = 4) after blood injection, a small hematoma occupied ~1/3 of the medial striatum, including the germinal matrix, and blood was apparent in the lateral ventricles, which were minimally enlarged. Five of 8 rats exhibited hemorrhagic infarction in the lateral cerebral cortex, approximately in the distribution of middle cerebral artery. This was remote from the needle insertion site and not necessarily contiguous with hematoma (Figure 4). No such damage was evident in the saline infused rat brains, which had only a small quantity of blood and edema along the needle tract (not shown).

Final MR imaging

MR imaging at the end of the experiment (Figure 3) revealed three discrete subsets of rats within the blood infused group. Among these rats, 13 had normal appearing brains. This includes mild ventricle asymmetry (right side larger n = 3; left side larger n = 2) similar to that seen in 1 intact and 4 saline infused (2 each side) rats. Focal atrophy involving the frontal cortex was identified in 5 rats (Figure 3H). On coronal slices atrophy extended ventrally to the piriform cortex and dorsally to within 3 mm of the midline, affecting the lateral aspect of the hemisphere. The rostrocaudal extent was roughly from the midbrain level (bregma –6 mm) to the anterior limit of the lateral ventricle (bregma +2 mm). This region includes the primary and secondary somatosensory regions. The hippocampus was not affected. Three rats had mild enlargement of the lateral ventricles (Figure 3J). One rat that received saline injection had a small focus of atrophy in the dorsal cortex at the needle entry site. Blood injected rats had smaller right hemisphere volume compared to intact control groups (680 ± 14

mm^3 vs. 713 ± 21 mm^3) and there was a significant interhemispheric volume difference in the blood infused group ($p = 0.002$, paired t-test). Regression analysis did not show a relationship between the initial hematoma volume and the final right hemispheric volume ($r^2 = 0.007$). In trying to explain the subset of rats with focal cortical atrophy we found that neither the initial hematoma size nor the position predicted whether the final MR appearance would be normal or abnormal. Of the blood infused rats with major structural abnormalities (cortical atrophy or hydrocephalus), 6/7 were males. This gender effect was not statistically significant (Chi square = 2.79, $p = 0.10$), and we cannot be certain whether this represents a random occurrence or a true gender effect that would be supported in a larger experiment.

Final histologic outcome

Ten weeks following injection, there was complete correspondence between the MR findings and the gross histologic features, for example the focal cortical atrophy that was the consequence of infarction (Figure 5A). The 5 infarcts extended from the pial surface to the external capsule. The hippocampus was not affected. These brains had scattered GFAP-immunoreactive hypertrophic astrocytes in the lateral striatum, the periventricular region, and the superficial cerebrum at the site of the infarct. Rare iron-containing macrophages were identified in the external capsule and subarachnoid compartment, but not along the ventricle wall. In the cerebral cortex dorsal to the atrophic regions, there were irregular radial columns of selective neuron loss alternating with columns of normal neuron density and myelin staining. Histologic examination also demonstrated subtle abnormalities that were not evident on the MR images; 11/21 rats had decreased myelin staining in the lateral and inferior striatum and external capsule

(Figure 5D and 5F), and 5 of these had subjective neuronal loss in the lateral and inferior striatum. An additional 4/21 blood infused rats had a subtle qualitative increase in GFAP immunoreactivity suggestive of reactive astrogliosis in the periventricular region without any other identifiable changes (not shown). Three of 21 rats exhibited periventricular cell collections that included GFAP positive astrocytes; these likely represent heterotopia (Figure 5G and 5H). Electron microscopic examination of the corpus callosum demonstrated moderately good morphological preservation of myelin, although small unmyelinated axons could not be identified with certainty. A total of 2384 myelinated axons ranging from 0.25 μm to 1.7 μm diameter were measured. For axons in the 0.5 μm to 1 μm diameter range, both blood infused and saline infused groups had increased myelin to axon ratios (0.145 ± 0.004 and 0.166 ± 0.007 respectively vs. controls 0.122 ± 0.005; $p = 0.025$). This change, of uncertain significance, might be due to the trauma of needle insertion.

Neurobehavioral development

The average ambulation score of blood infused rats was significantly ($p < 0.05$) lower than that of the age-matched intact and sham controls on days 4, 8, 11, and 15 (Figure 6). In the righting test, blood infused rats were significantly slower than sham or intact control rats at day 4 and day 8. But by day 11, when all rats righted very quickly, there was no difference (Figure 7). No directional preferences were observed. In the negative geotaxis test on day 4, most pups did not turn and were given the maximum score. On day 8 and day 15, blood infused rats took significantly ($p < 0.031$) longer to turn to the incline position (Figure 8). The wire hanging test revealed no significant differences between any of the groups on days 11, 15, 18, or 22 ($F < 0.668$, $p > 0.530$).

Blood infused rats did not exhibit significant forepaw preference in the cylinder exploration test (e.g. $F = 0.556$, $p = 0.59$ on week 7). In the rotating cylinder test, blood infused rats stayed on the cylinder for a significantly shorter duration than intact and sham control groups at both constant speed (week 6, $F = 2.66$, $p = 0.119$; week 7, $F = 1.408$, $p = 0.289$; week 8, $F = 7.90$, $p = 0.009$) and accelerating speed (week 6, $F = 0.886$, $p = 0.044$; week 7, $F = 5.744$, $p = 0.022$; week 8, $F = 0.625$, $p = 0.555$) (Figure 9). In the ladder-walking test, ipsilateral and contralateral foot slips did not differ between groups (e.g. $F = 0.874$, $p = 0.534$ at week 8). In the skilled reaching test on week 10, blood infused rats successfully retrieved significantly fewer ($p = 0.0452$) pellets than sham controls (Figure 10). Qualitative evaluation of the reaching movement showed that the rats with ventricular dilation or lateral cortical atrophy had marked impairment in advance, digit extension, grasp, supination, and release phases. Neither hematoma volumes nor final right hemispheric volumes correlated strongly with any of the behavioral outcomes (all R values were less than 0.2).

Table 1: Group sizes for long-term study

Groups	Intact controls	Sham controls	PVH
Initial number of rats	17	26	40
Final gender distribution	9 males, 8 females	6 males, 13 females	11 males, 10 females
Hydrocephalus			3 males, 0 female
Cortical atrophy			4 males, 1 female

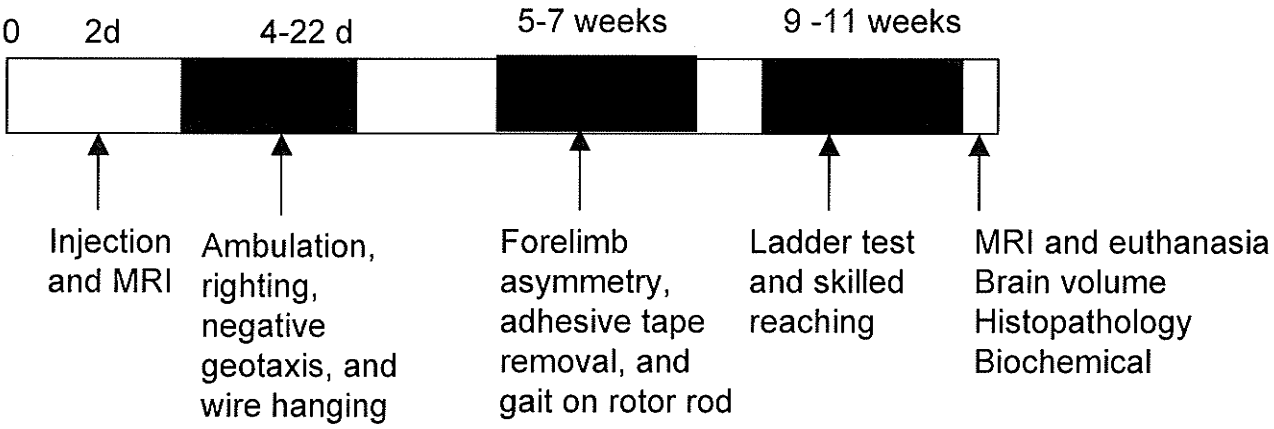
Table 2. Summary of the early developmental behaviors assessed

Early developmental behavior parameters	Test days (postnatal age)	Function
Ambulation	4, 8, 11, 15	Open field observation of quadruped locomotion
Righting on a surface	4, 8, 11, 15	Assessment of dynamic postural adjustment
Negative geotaxis	4, 8, 11, 15	Orientation response to a gravitational stimulus
Wire hanging ability	11, 15, 18, 22	Synergistic fore limb support to prevent falling

Table 3. Summary of the adult behaviors assessed

Adult behavioral parameters	Test weeks	Function
Cylinder exploration	5, 6, 7	Tests the level of preference for using non-impaired forelimb during vertical exploration
Adhesive tape removal	7,8	Tests lateralizing preference for responding to sensory stimuli placed on forelimb. Indicates sensorimotor integration
Rotating cylinder	5,6,7	Tests the ability for motor coordination and balance on a rotating cylinder moving at constant and accelerating speed
Ladder walking	8,9	Gait-coordination, sensorimotor
Skilled reaching	8,9,10	Assessment of motor coordination in a complex manipulative task using impaired forepaw

Figure 1. Testing schedule.



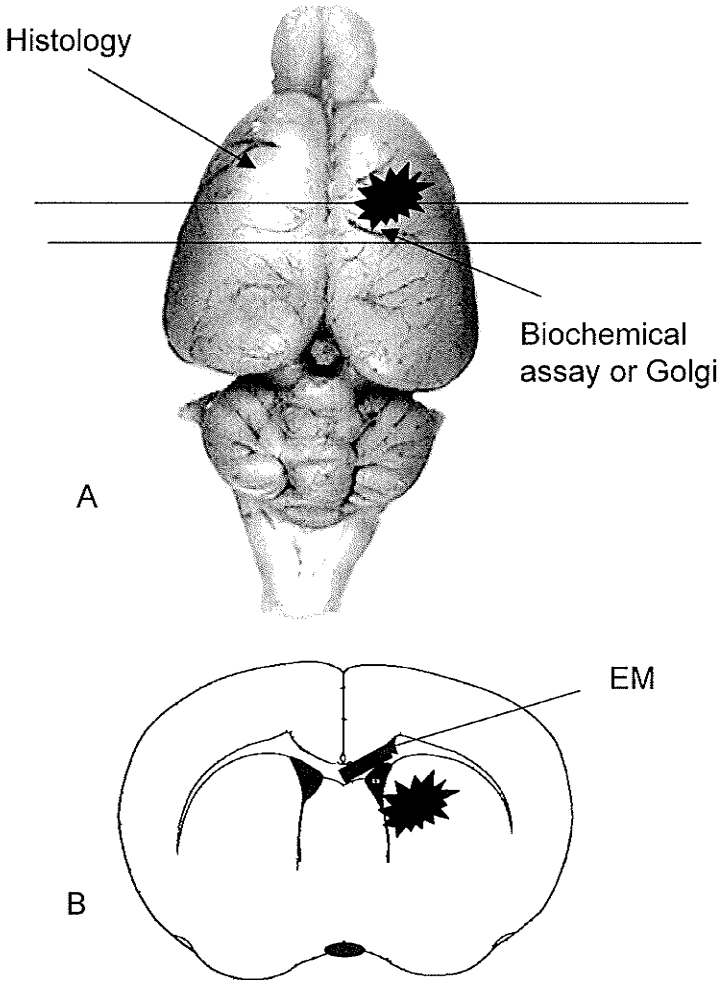


Figure 2. Schematic diagram of rat brain dissection. The coronal slice locations are shown in A. The anterior portion of the brain (beginning –0.26 mm behind the bregma, which was estimated to represent the approximate level of needle insertion) was for histologic studies. Coronal section of the cerebrum is shown in B. EM represents the area of right medial corpus callosum that was used for electron microscopic studies. The irregular star shape represents the assumed site of the hematoma, which was no longer evident at 10 weeks.

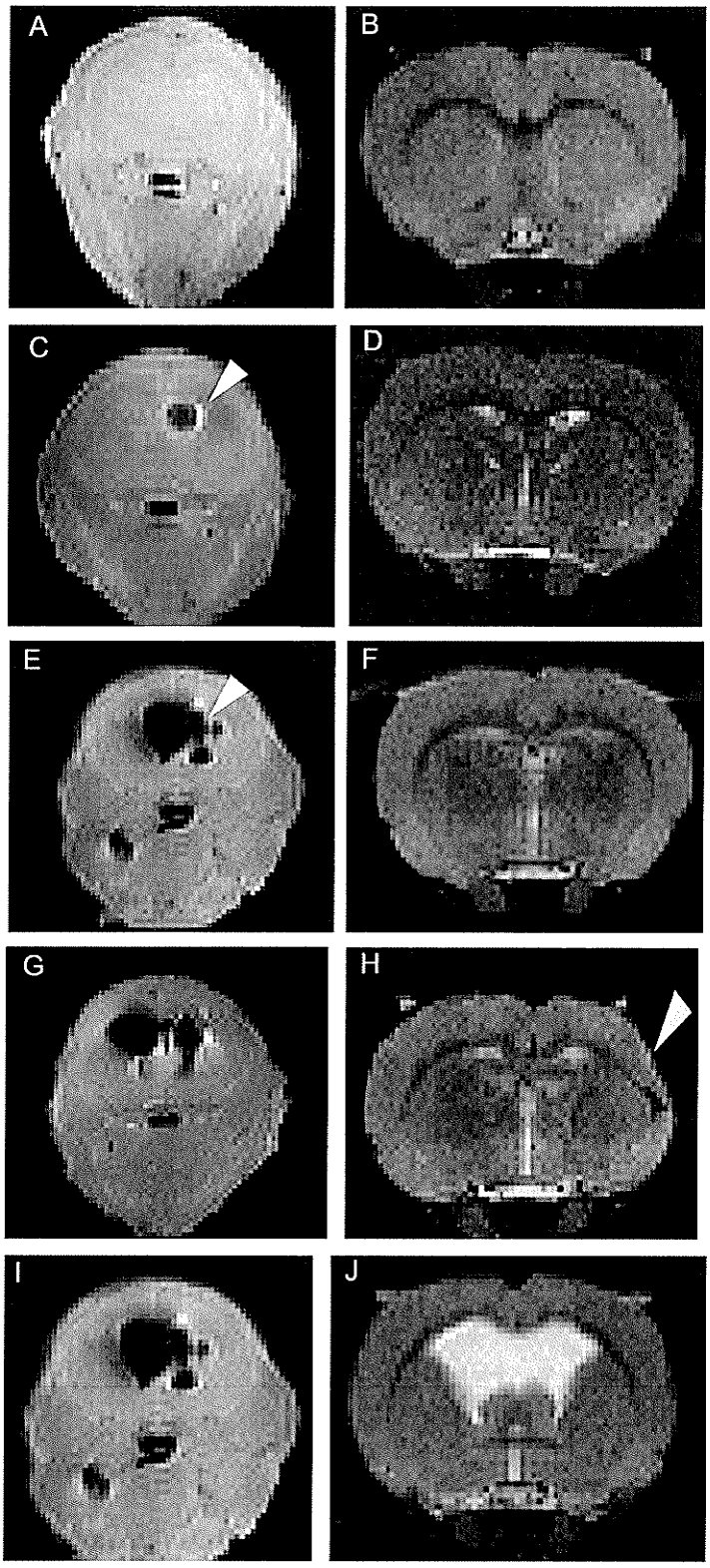

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Figure 3. T2-weighted magnetic resonance images of rat brain at early and late stages of the experiment. Normal neonatal and corresponding 10-week rat brain images are shown in panels A and B. Twenty minutes following saline injection a small hypointense area is seen (C, arrow); the brain appears normal at 10 weeks (D). Twenty minutes following blood injection, the hematoma in the striatum and ventricles appears dark (E arrow, G, and I). At 10 weeks, three outcomes were possible: most rats had normal appearing brains (F), focal cortical atrophy was identified in 5 rats (H, arrow), and 3 rats had bilateral ventricular dilation (J).

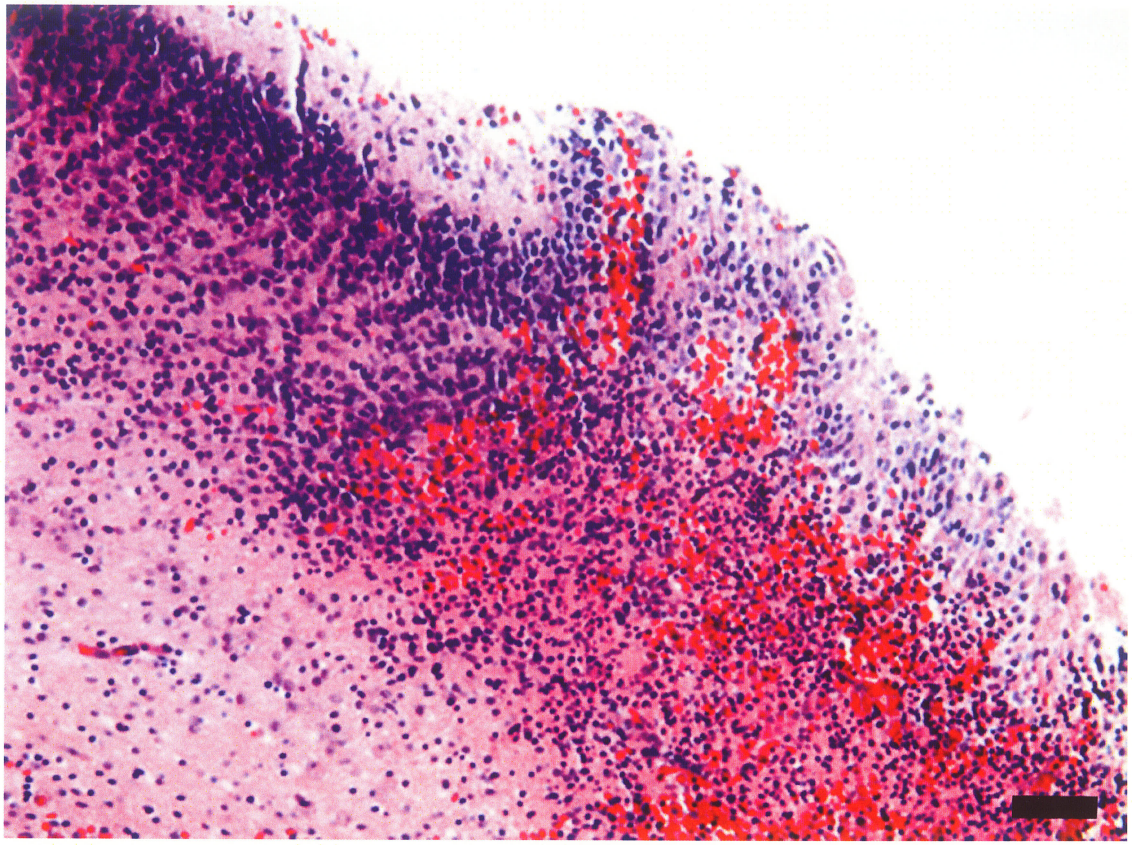

Figure 4. Cortical infarct 48 hours after blood injection into the periventricular region. Photomicrograph of a coronal section of rat brain showing hemorrhagic infarction in the lateral cerebral cortex. The cortical layers on the upper left have normal appearing nuclei. In regions with blood (lower right), the cell nuclei are pyknotic. This was remote from the needle insertion site. (hematoxylin and eosin stain, Bar = 50 μm)

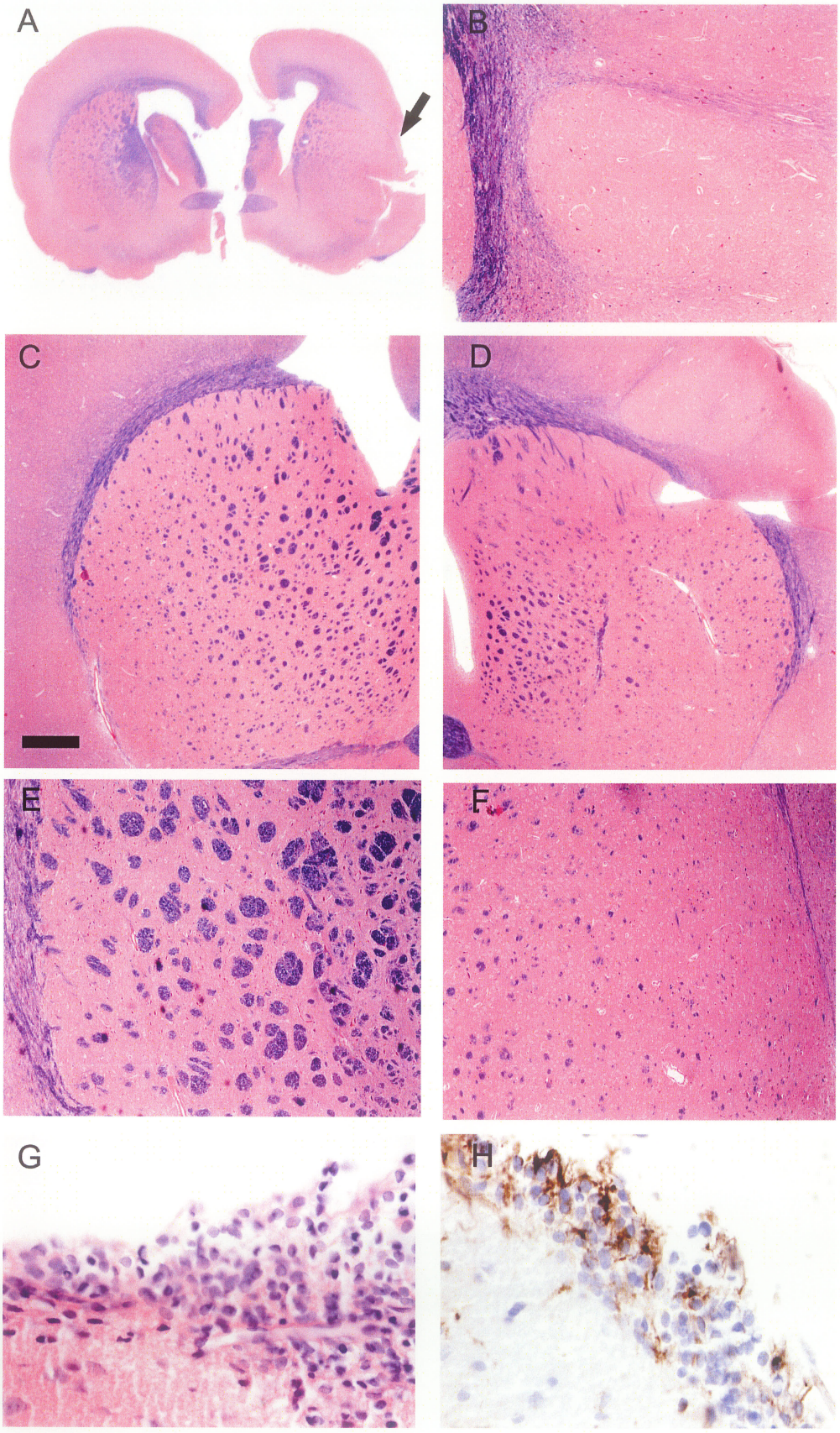
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Figure 5. Long-term histological outcomes. A - Low magnification photomicrograph showing coronal section of a rat brain that had been infused with blood; the arrow indicates cortical atrophy. Solochrome cyanine stains myelin purple. B - Cortical areas dorsal to the atrophic region exhibit columns of myelin staining rather than an even distribution. C to F - In another rat, decreased myelin staining in the striatum and external capsule is apparent (C and E, normal side contralateral to injection; D and F, atrophic striatum ipsilateral to injection). G- Irregular clusters of cells along the ventricle wall (hematoxylin and eosin stain) of rats that received blood injections included some GFAP immunoreactive astrocytes (H). Bar = 500 μm for C and D, 150 μm for B, E, and F and 30 μm for G and H.

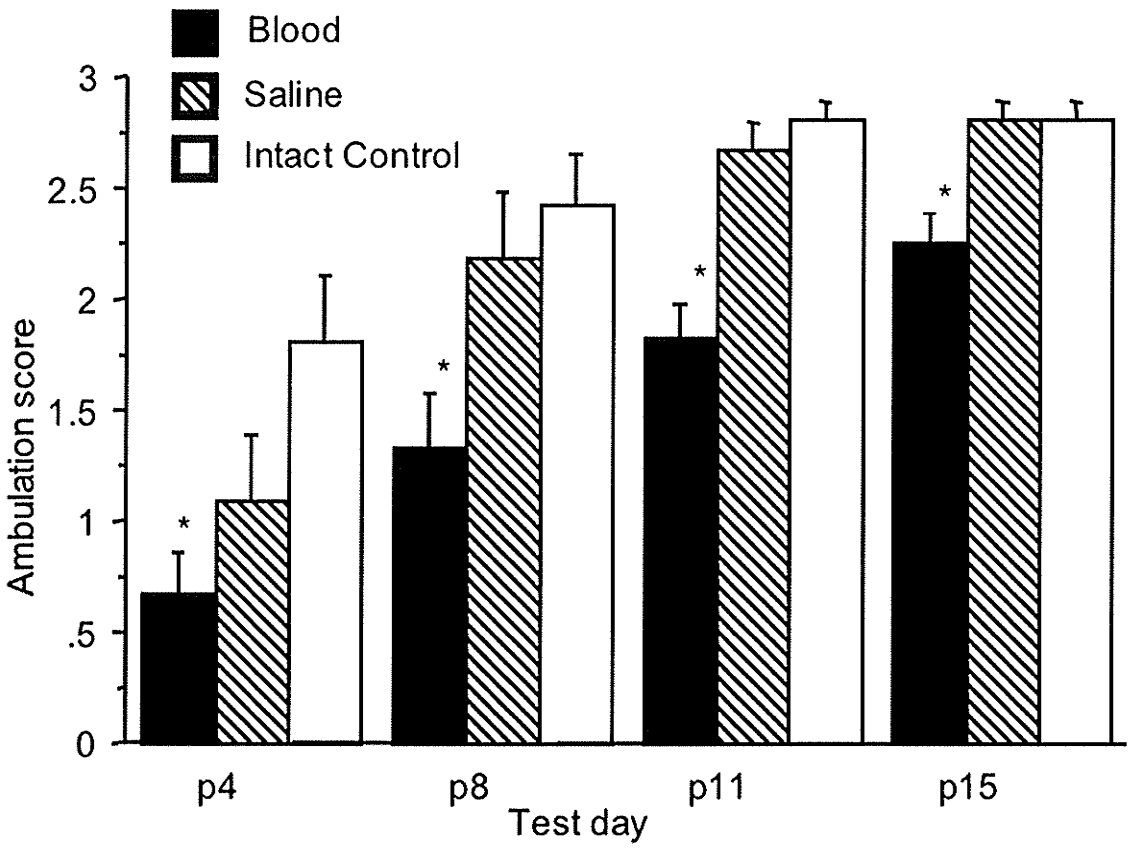

Figure 6. Mean ambulation score of blood infused rats. Open field observation of quadruped locomotion was categorized as follows: No movement = 0, asymmetric limb movements = 1, slow crawling = 2, and fast walking = 3. The average ambulation score of blood infused rats was significantly lower than in the age matched intact and sham controls. Data are expressed as mean ± SEM (Kruskal-Wallis test, * indicates $P < 0.05$).

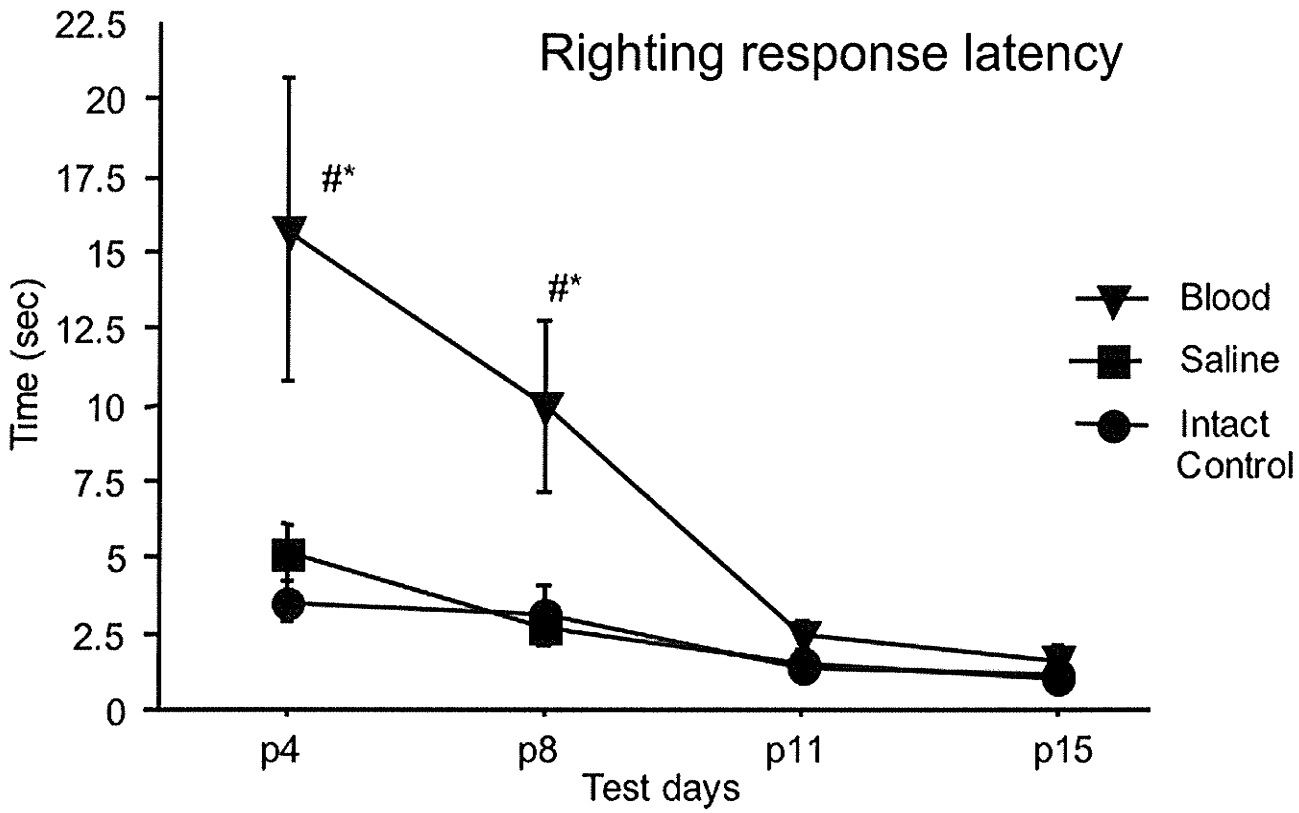

Figure 7. Righting response of blood infused rats relative to saline and intact control rats. Dynamic postural adjustments were assessed by placing rats onto their backs and documenting the time to return to prone position on days 4, 8, 11, and 15. Blood infused rats showed significantly longer latency than sham or intact control groups at day 4 ($F = 2.247$, $p = 0.032$) and day 8 ($F = 2.254$, $p = 0.016$). Data are expressed as mean ± SEM and were analyzed with 2-way ANOVA followed by Scheffé's post hoc test. * indicates intact control vs. blood infused groups (day 4 $p = 0.032$ and day 8 $p = 0.046$) and # indicates saline control vs. blood infused groups (day 4 $p = 0.076$ and day 8 $p = 0.035$).

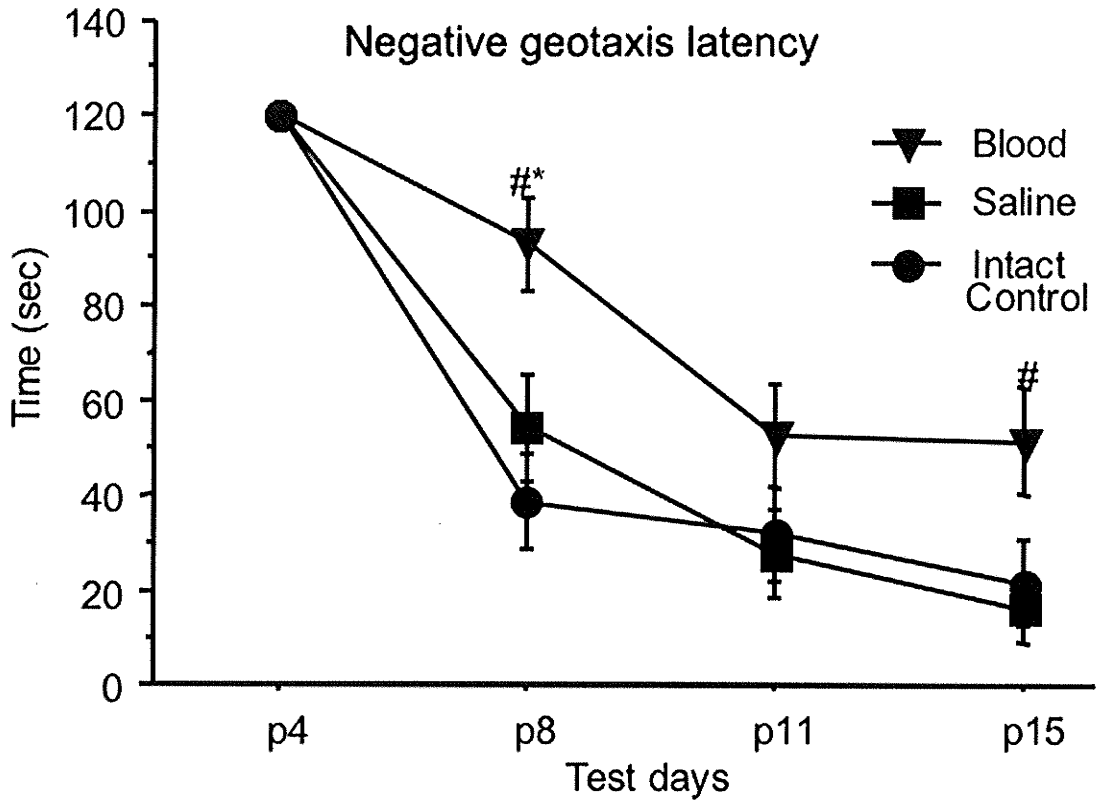

Figure 8. Negative geotaxis of blood infused and control rats. Negative geotaxis reflects the pups' preference to face upward on a sloped surface. On day 4 most pups did not turn and were given the maximal score of 120 seconds. The differences were significant on day 8 and day 15 ($F = 8.403$, $p = 0.001$ and $F = 3.524$ $p = 0.369$ respectively) with the blood infused rats being slower than saline controls ($p = 0.043$, $p = 0.220$, and $p = 0.032$ on days 8, 11, 15). Data are expressed as mean ± SEM and were analyzed with 2-way ANOVA followed by Scheffé's post hoc test. * indicates intact control vs. blood infused (day 8 $p = 0.019$) and # indicates saline control vs. blood infused (day 8 $p = 0.034$ and day 15 $p = 0.032$).

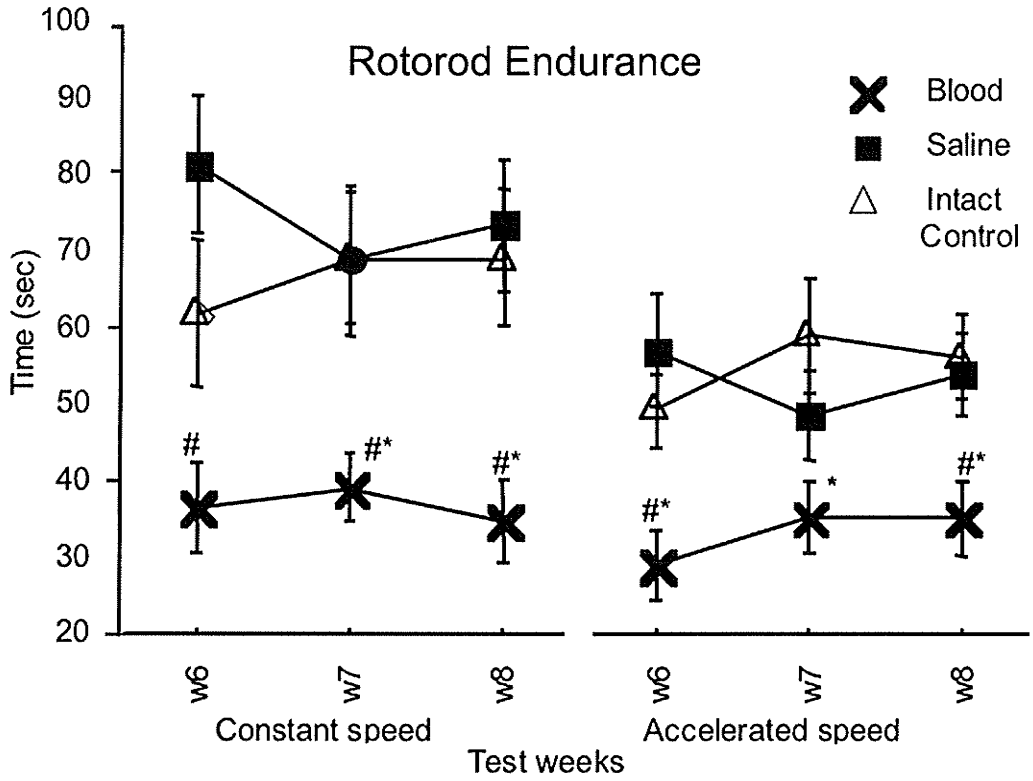

Figure 9. Rotorod endurance of blood infused rats. Motor coordination and balance on a rotating cylinder moving at constant and accelerating speed was assessed. Blood infused rats performed significantly less well than intact and sham control groups at both constant and accelerating speed. Data are expressed as mean ± SEM and were analyzed with 2-way ANOVA followed by Scheffé's post hoc test. * indicates intact control vs. blood infused and # indicates saline control vs. blood infused .

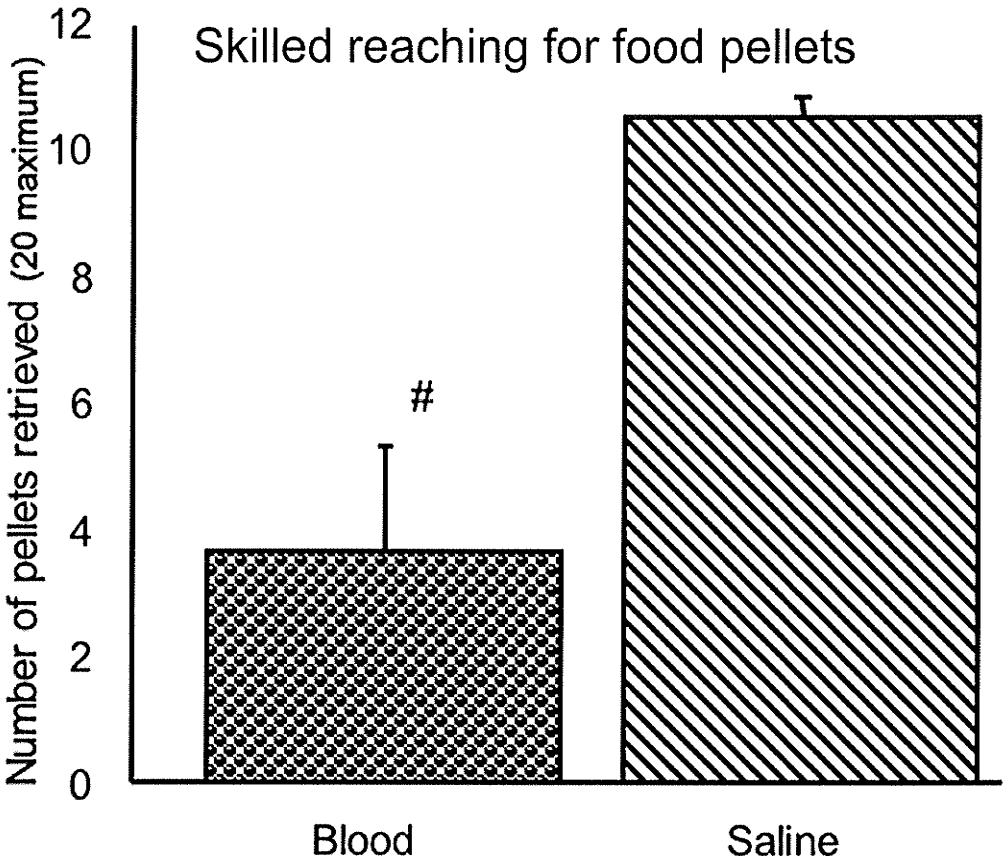

Figure 10. Skilled reaching in rats with blood injection. The skilled reaching test during 10th week measured success in retrieving food pellets through a narrow opening. Blood infused rats retrieved significantly fewer (# $p = 0.045$) pellets than sham controls. Data are expressed as mean ± SEM and were analyzed with unpaired Student's t test.

Discussion

Despite making a concerted effort through the use of MR imaging to minimize initial variability of the hematoma size and location in the blood infusion model in neonatal rats, morphological outcomes were somewhat varied. The most frequently observed abnormality adjacent to the site of the germinal matrix blood injection was atrophy in the striatum, including loss of white matter bundles. This could reflect direct damage to the developing tissue or secondary effects on the generation of precursor cells. In a mouse study (Xue et al., 2003) and in human infants (Del Bigio et al., 2003), we observed decreased proliferation in the germinal matrix near the site of the hematoma. Hypothesizing that oligodendrocyte precursors might be reduced, we had hoped to show in the corpus callosum loss of myelin that was independent of axon damage. However, electron microscopy revealed subtle abnormalities probably related to needle insertion; the experiment will have to be redesigned to test that hypothesis, perhaps with a more laterally placed blood injection that avoids penetration of the corpus callosum or with hypertension-induced hemorrhage.

The histopathologic features of this model need to be considered carefully. Shortly following blood injection into the periventricular region of neonatal rats a substantial proportion of rats developed diffuse hemorrhagic infarction in the lateral cortex. This corresponds to the focal cortical atrophy observed in ~25% of long-term survivors. In humans, white matter and striatal atrophy are often demonstrated following periventricular hemorrhage (Del Bigio, 2004) and cortical infarction is a rare outcome after PVH (Ment et al., 1984a). The larger proportion of neonatal rodents sustaining this lateral extension likely is a consequence of the different proportionate anatomy – the

white matter interface between the periventricular region and the cerebral cortex is much smaller in rodents. The pathogenesis of the damage following blood infusion is unclear. Widespread damage to immature vessel walls by blood-borne proteolytic enzymes (i.e. thrombin and plasmin) might be responsible. We have observed this type of damage following intrastriatal injection of thrombin in neonatal mice (Xue and Del Bigio, 2005b). Plasmin can also activate matrix metalloproteinases (Castellino, 1998; Indyk et al., 1999), which can damage blood vessels. Another possible explanation is thrombosis of deep veins, although usually in this circumstance the hemorrhage is patchy. In the hippocampus, we did not find any damage. This may reflect the anatomical distribution of the lesion or the fact that hippocampus is relatively resistant to insult at postnatal days 2 to 3 (Towfighi et al., 1997). A few rats exhibited periventricular glial heterotopia. Considering that damage was induced in the gliogenic and cell migration period of brain development (Zerlin et al., 1995), heterotopia may be the consequence of failed migration of periventricular progenitor cells. Even in areas of regional infarction, very few hypertrophic astrocytes were observed, supporting prior documentation of negligible astrogliosis in models of neonatal brain damage (Balasingam et al., 1994). Furthermore, despite the direct introduction of blood into the periventricular region and MR imaging proof of extension into the ventricles, very few iron-containing cells were identified in the blood-infused rats. We suggest that most of the hemoglobin-ingesting macrophages departed from the brain via the blood. Intraventricular blood extension likely explains the few cases of moderate bilateral ventricular dilation (Cherian et al., 2003), which was insufficient to cause myelin changes in the corpus callosum. However, in humans it has been shown that severe

ventricular dilation is strongly associated with white matter damage following PVH (Kuban et al., 1999).

In this study, blood injection into the periventricular region of neonatal rats produced both early and persistent behavioral deficits. Ambulation, surface righting, and negative geotaxis are among the earliest motor developmental milestones in rats (Altman and Sudarshan, 1975; Westerga and Gramsbergen, 1990). The postnatal period from day 1 to day 14 involves corresponding rapid changes in brain structure (Eayrs and Goodhead, 1959b; Davison and Dobbing, 1966). Thus, it is likely that insults, such as blood injection, can interrupt the developmental processes necessary for timely acquisition of behavioral skills. Similar delays in early development have been documented in premature humans (Allen and Capute, 1989). When selecting adult behavior tests, the following points were considered: 1) this model produces a predominantly unilateral lesion, therefore behavioral tests sensitive to lateralization were chosen, 2) considering the site of injury, we expected sensorimotor deficits but minimal adverse effect on learning, and 3) because training could affect brain recovery, behavioral tests were organized so that non-trained "automatic" behaviors were assessed first (e.g. cylinder exploration), followed by more complex behaviors that require minimal learning (walking on rotating cylinder and ladder) and finally the skilled reaching test, which requires training. Blood injection into the periventricular region appears to have damaged the cortico-striate pathways with adverse effects on motor performance. The striatum is activated at different stages of motor skill learning and function, depending on the behavioral paradigm used (Ungerleider et al., 2002) (Costa et al., 2004). Blood-infused rats retrieved significantly fewer pellets than controls in the skilled reaching test. This is consistent with previous findings in other unilateral lesions

(Whishaw et al., 1997; Ballermann et al., 2000). The quality of skilled reaching movements were abnormal only in rats that had severe focal cortical atrophy or increased ventricular size. This is also consistent with skilled- reaching studies that have shown a relationship between severity of lesion and functional deficit (Tomimatsu et al., 2002). Although others (Metz and Whishaw, 2002) have shown that unilateral cortical and subcortical lesions impair ladder walking, we did not detect any deficits in that test. This may be because ladders with evenly spaced rungs present a non-challenging task. Relative insensitivity of some of the tests might be explained by plasticity in this immature brain injury model (Kolb et al., 2000a, Benecke, 1991 #4274, Ono, 1990 #4275).

PVH uncomplicated by cortical infarcts produces motor disabilities and reduced cortical volume in human infants (Pinto-Martin et al., 1995, Vasileiadis, 2004 #4128) and imaging studies of humans born prematurely show cerebral volume reductions that correlate to some extent with late developmental outcome (Inder et al., 1999, Peterson, 2000 #2784, Vasileiadis, 2004 #4128, Weisglas-Kuperus, 1993 #4263, Whitaker, 1997 #4262). In the blood infused rats we observed a significant decrease in right hemispheric brain volume and atrophy in the striatum independent of large cortical lesions. However, we detected behavioral abnormalities only in rats with large structural defects but not subtle striatal damage. This might indicate that the tests we chose are not sufficiently sensitive. Also, the initial hematoma and the final brain volumes did not correlate strongly. This finding is consistent with human data showing that PVH size is not a good predictor of functional outcome (Dubowitz et al., 1981, Palmer, 1982 #3966).

There are several potential shortcomings in this experiment. First, we do not yet fully understand the mechanism of brain injury in this blood infusion model. As

discussed above, some aspects of it do not reflect the human situation. To date, we have shown that proteolytic enzymes, especially thrombin, are in part responsible for the damage (Xue and Del Bigio, 2005c), and that the damage can be aggravated by stimulation of the immune system (Xue and Del Bigio, 2005c). Our laboratory is in the early stages of understanding the mechanism and the consequences of this cerebral injury. Second, the damage introduced by needle insertion is a confounding factor. Therefore, alternate approaches, such as lateral injection that does not penetrate the primary sensorimotor cortex or a non-invasive approach such as the induction of transient neonatal hypertension should be utilized for some aspects of PVH research. The problem with the latter method is the known variability, as seen in dog and rabbit models (Lorenzo et al., 1982; Ment et al., 1982b; Johnson et al., 1987). Finally, similar to humans, the outcome of the blood infused group is variable in this model. Despite the use of MR imaging to screen rats for a uniform initial insult, we are unable to predict the ultimate damage and loss of function. This emphasizes the need for large sample sizes if the model is to be used for therapeutic testing. We also recognize that follow-up MR imaging did not reveal the subtle abnormalities detected histologically.

Using immature rabbits, dogs, cats, and sheep, the pathogenesis of periventricular hemorrhage has been elucidated (Goddard-Finegold, 1989). However, in none of these model systems have long-term behavioral outcomes been measured. Using autologous blood infusion, this is the first study to demonstrate in a neonatal animal model of periventricular hemorrhage that persistent motor deficits can occur. These are similar to the impairments observed in children who suffer PVH after premature birth, an important cause of cerebral palsy. Our findings are consistent with clinical PVH studies in which a broad range of behavioral and pathophysiological

outcomes occur (Ment et al., 1999). This model could have value for testing specific hypothesis concerning pathophysiology of brain damage as well as therapeutic studies.

Chapter 3 General Discussion

Germinal matrix hemorrhage (GMH) refers to bleeding that arises from the subependymal (or periventricular) germinal region of the immature brain. GMH is a clinical problem in children born prematurely. Considering the fact that perinatal brain injury is among the most prevalent and costly form of neurological disabilities (Rubin et al., 1992; MacDonald et al., 2000) we planned to understand the brain damage mechanism and consequences that follow periventricular hematoma. The data described in this thesis provide new information regarding the pathophysiology of brain damage following periventricular hematoma in the neonatal rats. In addition, they give insight into the early developmental and long-term behavioral consequences following blood injection into the periventricular region.

The brains of newborn rats were used because they are developmentally comparable to 24-26 weeks human gestation (Sturrock and Smart, 1980; Kakita and Goldman, 1999; Zhu et al., 1999; Levers et al., 2001), and have a prolonged period of postnatal neurological development (Dobbing and Sands, 1979). We have described three important findings at the molecular, cellular, and neurodevelopmental levels that occur following injection of blood in the neonatal brain respectively. In the gene expression study we found that rat brains subjected to blood infusion overexpressed many genes compared to aged-matched controls and compared to brains from older rats that received blood injection. At the cellular level, following blood injection, germinal cell proliferation was suppressed. Using Ki-67 we have shown this suppression started at 8 hours and lasted up to 2 days. The suppression was also observed at the contralateral germinal matrix suggesting that a diffusible molecule is responsible. In the

long-term behavioral study we also showed that blood injection into the periventricular tissue of neonatal rat results in both immediate and long-term behavioral abnormalities.

Since molecular changes following blood injection might serve as potential targets for therapeutic intervention we assessed the changes using DNA microarray screening. Using this technique we studied an array of gene products that can potentially lead to further understanding of the pathogenesis of hemorrhage in the immature brain. In addition, since proteins are the functional counterparts to genomic sequence information, we used antibody microarrays to correlate gene expression changes with protein changes following blood injection. Using the DNA microarray techniques, we have shown that changes in gene expression in the rat brain following blood injection are age-dependant. Newborn brains react very differently from young (7 day) or adult brains. In general, newborn brains tend to overexpress many genes following blood injection, that are likely important for brain development and function. That immature brains react differently from mature brain is not surprising as the newborn brain is undergoing anatomical organization and physiological initiation of various brain systems, which have already been completed in the more mature brain. We found that many cell cycle related genes (e.g. cdk and cyclins) were over-expressed, perhaps to compensate for the developmental interruption caused by blood injection. Increase in cell cycle related genes may reflect germinal cell proliferation or glial cell proliferation, which has been observed following many forms of brain injury. Such a response was not seen in the mature brains following blood injection. This study emphasizes the need to study newborn brain separately and not to extrapolate information from studies in mature brain. The findings in this study are as means of focusing further questions into studies concerning GMH.

Preoligodendrocytes are the predominant cell type of the oligodendrocyte lineage in the early developmental period of 23-32 weeks gestation (Back et al., 2001a), when most GMH/IVH occurs. The periventricular germinal matrix, is involved with constant expression of genes that are related to cell proliferation, maturation, and death. To assess the status of germinal matrix cell proliferation following blood injection, we used Ki-67 nuclear antigen to identify cells in the late G1 through M phases of the cell cycle (Hall et al., 1990; Hall and Coates, 1995). In addition, we studied the migratory fates using BrdU and specific cellular markers for double immunostaining. Mature oligodendrocytes were studied using myelin basic protein (MBP) and transferrin (Bloch et al., 1985). Reactive astrocytes and microglia were labeled using GFAP and lectin Bandeiraea simplicifolia respectively. Using both the Ki-67 and BrdU labeling, we observed a statistically significant suppression of germinal cell proliferation following blood injection. Ki-67 studies showed that suppression started at 8 hours and persisted up to 2 days. These findings were consistent with our previous mouse study. In the human study, we found that in comparison to control brains without hemorrhage, the brains of premature infants with GMH were found to have statistically decreased cell proliferation based upon immunoreactivity for Ki-67, particularly if post-birth survival was greater than 24 hours. This effect was most pronounced in the 25-28 week range. To our knowledge this is the first study that demonstrates that the suppression of proliferation observed in human GMH/IVH could be replicated in the animal model.

As preoligodendrocytes are the predominant cell type of the oligodendrocyte lineage during the blood injection period we expected that there would be fewer oligodendrocytes in the mature brain. We were unable to study preoligodendrocytes directly because of lack of reliable antibodies that detects antigens in vivo. At 21 days,

however, we did not observe any difference in the number of oligodendrocytes between control and blood injected rats. One possibility is that preoligodendrocytes underwent accelerated maturation to compensate for a loss. This phenomenon has been shown in other brain injury models (Nishiyama et al., 1997; Back et al., 2002). Along with the findings that changes to oligodendrocytes were absent, our long-term biochemical and electron microscopy studies have also shown no difference in the myelin levels. Moreover, in the gene expression studies we found that genes that are involved myelin formation were over expressed. Considering these findings, it is possible that following suppression large amount of cell proliferation and myelin production occurs to compensate for the loss.

It is worth noting that the newborn brain overexpressed several genes related to thyroid hormone, including thyrotropin releasing hormone receptor, thyroid stimulating hormone receptor, and transthyretin. Such changes were not seen in young and adult brain. Thyroid hormone is known to play a critical role in early oligodendrocyte development (Anderson, 2001). Overexpression of thyroid related genes may play a role in compensatory plasticity in the brains of blood injected rats.

The consequences of cell suppression are still not clear. It has been suggested that changes in cerebral size and shape are attributed to the number of progenitor cells in the proliferative zones (Parnavelas et al., 1991; Luskin, 1993; Luskin et al., 1993; Kornack and Rakic, 1998). Human studies have shown that decrease in cerebral volume occurs following GMH (Vasileiadis et al., 2004a). Our long-term behavioral studies have shown a decrease in brain volumes following blood injection (Balasubramaniam et al., 2006). Therefore, our finding of reduced cell proliferation may

be of clinical significance and may be associated with impaired developmental outcomes.

The mechanism of cellular suppression following blood injection is still not understood. In the neonatal mouse brain with experimental periventricular hematoma, we have already shown that thrombin likely plays a role in the damage (Xue et al., 2005) and that stimulation of the immune system can aggravate the damage (Xue and Del Bigio, 2003), but it is not clear whether thrombin or immune cells plays any role in cell cycle regulation. In the microarray study we found that following blood injection, newborn brain downregulated thrombin receptors, which are normally present on immature neurons (Smirnova et al., 1998; Mahajan et al., 2000). Newborns also overexpressed endogenous inhibitors of proteolytic activity such as alpha 1 macroglobulin, a broad-spectrum proteinase inhibitor (Eggertsen et al., 1991), and trypsin inhibitor type II. The downregulation of thrombin receptors and up-regulation of inhibitors may be important in averting damage that could be caused by entry of thrombin into the brain from plasma.

Since behavior is a major aspect of postnatal CNS development that is clinically monitored in humans, our objective was to understand the behavioral consequences following blood injection. We studied early developmental acquisition of motor skills, long-term sensory motor abnormalities, and long-term changes in brain tissue using biochemical and histopathological techniques. We observed persistent behavioral deficits, similar to the motor impairments observed in children with cerebral palsy. Moreover, we found a broad range of behavioral and pathophysiological outcomes occur (Ment et al., 1999) that are consistent with the clinical GMH .

It is interesting that in spite of this apparent reaction at the genetic level, newborn rodents performed poorly in the behavioral test. Previous studies have shown that newborn rodent brains heal poorly after ablation of part of the neocortex (Kolb et al., 1998; Kolb and Cioe, 2000; Kolb et al., 2000b; Kolb et al., 2000a). Whether the same is so for striatal lesions is not known. Considering in our long-term behavioral study where behavioral abnormalities were only seen with complex behaviors such as rotor rod balance, it is possible that integration of cells rather than migration that we need to be concerned about.

In studying adult behavior we found that tests used to detect limb use asymmetry such as cylinder exploration and adhesive tape tests were not sensitive, although these tests have been shown to be effective in other unilateral injuries (Schallert et al., 2000). In those studies, tests were done immediately after injuries. In our system the tests were done 70 days post injury. During the period it is possible that the brains could have compensated through plasticity.

It is important to relate the findings to human situation. As in human GMH, we have shown the presence of hematoma in the periventricular region. In addition, similar to humans we found cellular suppression following periventricular hematoma(Del Bigio et al., 2003). GMH can lead to severe neurological sequelae including seizures, mental retardation, and cerebral palsy (CP) (Hagberg and Hagberg, 2000), In our model we have also shown a broad range of behavioral abnormalities which starts early on and persists for a long period. Histological studies and behavioral have shown variability and variability of outcome is seen in the human state and therefore the model is realistic. We acknowledge these models do not represent every aspect of the human GMH. Such studies cannot be performed in a short span of study and is beyond the scope of this

thesis. However, our studies open doors to formulate many focused hypotheses in the future.

3.1 Future directions

As mentioned previously, we acknowledge that microarray studies have several limitation. However, such studies are crucial in the studies of brain injury. Until the expression patterns, and consequent functions, of the many genes involved are understood, the knowledge we currently have will not progress. Perhaps we should look into alternate techniques such as subtractive hybridization, which is a powerful technique for assesing large scale genetic changes that are specifically expressed in one tissue or cell type or at a specific stage. The advantages of subtractive hybridization include the need for only very small amounts of mRNA and the ability to detect rare mRNAs. In contrast to microarray, radioactivity is not incorporated during the hybridization procedure, obviating problems of probe decay and radioactive handling, enabling the preparation of more than one probe from one batch of subtracted product, and reduction of background signal in library screening, enabling detection of low abundance mRNA genes. The use of subtractive hybrization, have already been studied in the context of early brain development (Porteus et al., 1992).

It is important to note that cell suppression was also observed in the ipsi and contralateral white matter at 8 and 24 hours. Since the white matter in the rats is in close proximity to the germinal matrix, it is likely that the diffusible substance causing the cellular suppression at the germinal matrix may also play a role here. Rat brains are too small and the quantity of white matter is not large enough to study white matter

vulnerability alone. In future, we need to study cell proliferation using brains of larger animals such as pigs and canines.

Considering the cellular suppression begins within 8 hours of injection, it is possible that following blood injection biochemical events such as energy failure, membrane depolarization, brain edema, an increase of neurotransmitter release and inhibition of uptake, an increase of intracellular Ca(2+), production of oxygen-free radicals, lipid peroxidation, and a decrease of blood flow may have occurred. Therefore, we need to asses these important physiological parameters. To study cell volume changes that occur following suppression, radiographic studies such as positron emission tomography (PET), single photon emission computed tomography (SPECT), and most recently magnetic resonance spectroscopy (MRS) could be used.

The understanding of cell migration and fate needs to expand further. Cell transplantation may provide some benefit to replace injured cells and study their fate. Transplanted cells create many dilemmas that would probably add confusion to results in this study. Retroviral studies by labeling precursor cells by injecting the retrovirus into a spatially restricted region of the germinal zone would be more promising in our study. For instance, Levison and Goldman (1993) have demonstrated that both astrocytes and oligodendrocytes of the rat forebrain are descendants of SVZ progenitors labeled 2 days after birth (P2), SVZ cells appeared to migrate through adjacent white matter and into adjacent gray matter to colonize neocortex, white matter and striatum (Levison et al., 1993). Using the same technique we could study cell fate following blood injection in our model.

We need to study normal structure function correlation before attempting to test behaviors in experimental models. We explored large number of behavioral tests to

develop sensitive testing for our injury model. Anatomical pathways affected following blood injection are not entirely clear. Considering it was a unilateral injury, it was still not obvious why blood injected rats showed no laterlization in the cylinder test but had a deficit in the rotating rod test. In the structural study we found that many rats developed ventricular dilatation and lateral cortical atrophy. In addition, we found that these rats performed poorly in all the adult behaviors. We are not sure what anatomical structures are responsible for the tested behaviors as the anatomical sites are not clearly studied. The early developmental behavior deficit was not surprising since immediately upon injection we were able to see significant changes at the gene expression and cellular levels. Adult behaviors however are complex, perhaps we need to establish that structure function correlation in the normal brains before we attempt to test in the blood injection model.

3.2 Shortcomings

Understanding the causative factors and the pathogenesis of subsequent brain damage is important if GMH/IVH is to be prevented or treated. Appropriate animal models are necessary to achieve this understanding. However, in chapter 1.6 we have explored the available animal models and their potential problems. From the peer reviewed publications we realized that no single animal model will be suitable for the study of all physiological, developmental, and pathological aspects of GMH/IVH. These models can in combination provide knowledge of the pathogenesis of GMH, and be used to study preventative and ameliorative interventions.

There are a few limitations to our study using the newborn rat as an experimental model. The physiological and anatomical differences between rodent and human brains are substantial. Furthermore, diffusion kinetics of blood products potential differs between the small rodent brain and human brain. We realized our model has significant degree of variability. Nevertheless, this model is relatively inexpensive and is suitable to study long-term behavioral outcomes. Moreover, prominent postnatal germinal matrix make it an appropriate model to study cellular aspects of the pathogenesis of post-GMH brain damage. However, using brains of animal models that are phylogenetically closer to human would improve the understanding of this injury. One major problem is that the developmental brain anatomy and physiology are not well documented in large animals that have been used to model GMH, making interspecies extrapolation of information difficult.

For the microarray study we found that many of the gene protein products were not present on the antibody chip. In addition, there is large controversy as to common nucleotides that are present in between different microarray techniques (Jain, 2001; Knight, 2001; Kothapalli et al., 2002). We realize these type of studies do not produces answers to specific question, rather they open doors for many hypothesis. Genes from these data need to be followed using techniques such as quantitative PCR.

In the cell migration and differentiation study, we were uncertain if the migration of cells are interrupted following blood injection as we only looked at specific regions and not the total volume of brain region. For comprehensive understanding complete serial section of BrdU incorporation should be studied.

Despite the use of MR imaging to screen rats for a uniform initial insult, we are unable to predict the ultimate damage and loss of function. This emphasizes the need

for large sample sizes if the models are to be used for therapeutic testing. We also acknowledge that we were unable to do all behavioral tests on all rats due to technical issues. However, having these initial data we can design focused behavioral studies and improve our understanding of this model.

3.3 Conclusion

We have demonstrated that significant changes occur in rat brain at the molecular, cellular, and behavioral level following periventricular blood injection. Very few studies have looked at these parameters in relation to GMH/IVH. We believe that these results are in the early stages of providing answers regarding the mechanism or consequences following blood injection. Many medical treatments have been attempted to prevent initial or progressive GMH in preterm infants; phenobarbital, vitamin K, vitamin E, indomethacin, and ethamsylate appear to be ineffective. Without fully understanding the of mechanism periventricular hematoma, it is impossible to develop effective therapy. Further understanding of the brain damage after hemorrhage is necessary to expand treatment options.

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