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Adult Responses to an Ischemic Stroke in a Rat Model of Neonatal Stress and Morphine Treatment

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Abstract

Critically ill newborn infants experience stressors that may alter brain development. Using a rodent model, we previously showed that neonatal stress, morphine, and stress plus morphine treatments each influence early gene expression and may impair neurodevelopment and learning behavior. We hypothesized that the combination of neonatal stress with morphine may alter neonatal angiogenesis and/or adult cerebral blood vessel density and thus increase injury after cerebral ischemia in adulthood. To test this, neonatal Lewis rats underwent 8 h/d maternal separation, plus morning/afternoon hypoxia exposure and either saline or morphine treatment (2 mg/kg s.c.) from postnatal day 3 to 7. A subset received bromodeoxyuridine to track angiogenesis. Adult brains were stained with collagen IV to quantify cerebral blood vessel density. To examine vulnerability to brain injury, postnatal day 80 adult rats underwent right middle cerebral artery occlusion (MCAO) to produce unilateral ischemic lesions. Brains were removed and processed for histology 48 hours after injury. Brain injury was assessed by histological evaluation of hematoxylin and eosin, and silver staining. In contrast to our hypothesis, neither neonatal morphine, stress, nor the combination affected cerebral vessel density or MCAO-induced brain injury. Neonatal angiogenesis was not detected in adult rats possibly due to turnover of endothelial cells. Although unrelated to angiogenesis, hippocampal granule cell neurogenesis was detected and there was a trend ($P = 0.073$) toward increased bromodeoxyuridine incorporation in rats that underwent neonatal stress. These findings are discussed in contrast to other data concerning the effects of morphine on cerebrovascular function, and acute effects of morphine on hippocampal neurogenesis.

Keywords

perinatal stress; middle cerebral artery occlusion; stroke; cerebrovasculature

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1. Introduction

During critical care, preterm infants experience prolonged stress and undergo painful procedures that may have adverse effects on brain development and behavior (Anand and Scalzo, 2000). To alleviate pain and stress, opiates such as morphine are commonly prescribed. However, there is both clinical and experimental evidence that early opiate exposure alters brain development, angiogenesis and cerebrovascular responses (Armstead et al., 1990; Boyle et al., 2006; Hall et al., 2005; Pasi et al., 1991; Rao et al., 2007; Traudt et al., 2012; Van Woerkom et al., 2004). Neonatal factors such as stress may contribute to increased risk for stroke (Barker, 2000; Craft et al., 2006). Together, these data raise concern that the deleterious effects of neonatal morphine exposure may be enhanced by neonatal stress.

Previously, we created a multi-factorial model of neonatal stress by combining maternal separation with exposure to hypoxia/hyperoxia in order to simulate the stressors premature neonates often experience after birth. That neonatal stress protocol altered short-term patterns of mouse hippocampal gene expression (Juul et al., 2011). In addition, neonatal morphine exposure impaired adult rat avoidance learning (McPherson et al., 2007) and produced adult rat hyperglycemia in response to cerebral ischemia (McPherson et al., 2009). We now examine the possibility that neonatal stress and morphine may interact to exacerbate stroke-induced brain injury. We tested the hypothesis that neonatal morphine treatment during neonatal stress exposure may disrupt early cerebral blood vessel proliferation and thereby reduce adult cerebrovascular density and consequently increase brain injury after an ischemic stroke.

2. Material and methods

2.1 Animals

The Institutional Animal Care and Use Committee at the University of Washington approved all procedures. Time-mated, pregnant Lewis rats (n=43) were purchased from Charles River (Hollister, CA and Portage, MI) and arrived 1 week prior to delivery. Animals were housed under SPF conditions with a 12:12-hour light:dark cycle and fed *ad libitum*. On postnatal day (P) 3, newborn rats were culled to n=8/dam. Weights and mortality were monitored.

2.2 Neonatal treatments

Subcutaneous treatment injections (50 μ L) were given twice daily (08:00 and 16:00h) from P3 to P7. Five treatment groups were compared: uninjected controls, saline, morphine sulfate (2 mg/kg), *neonatal stress* + saline, and *neonatal stress* + morphine. As described previously (McPherson et al., 2007), neonatal stress combined 8 h/day of maternal separation (individual isolation in a veterinary warmer) with twice daily (08:30 and 16:30h) hypoxia/hyperoxia exposure (100% N₂ 5 min then 100% O₂ 5 min). Neonatal rats were gavage fed rat milk substitute during isolation. To track cell division, all rats received injections of bromodeoxyuridine (BrdU, 50 mg/kg, s.c.) on P3 and P5. After P7, rats remained housed under standard care in a vivarium until approximately P80. Rats were weaned and separated by sex on P24.

2.3 Evaluation of blood vessel density

A subset of adult rats that had undergone neonatal treatments were transcardially perfused with buffered 4% formaldehyde saline. Brain tissues were paraffin embedded, sectioned, and slide mounted. Immunolabeling of antibodies directed against proliferating cells (BrdU,

Chemicon kit) or blood vessels (Collagen IV, Millipore AB756) was detected using standard peroxidase staining methods.

2.4 Middle Cerebral Artery Occlusion (MCAO)

To create a temporary ischemic stroke, MCAO surgeries were performed on a subset of adult rats ($P80 \pm 3$) that had undergone neonatal treatments. The animals were anesthetized using 3% Isoflurane in 30% oxygen and then placed supine. Local anesthesia was administered to the midline neck area (1 mg/kg, 0.25% Bupivacaine, Hospira, Lake Forest, IL), and then a 1.5 cm midline incision was made between the jaw and the sternum. The right common carotid artery was isolated, then the pterygopalatine, external carotid and superior thyroid arteries were ligated. An incision was then made in the right common carotid artery, and a nylon microfilament with a silicone-coated tip was inserted and advanced 20 mm into the right middle cerebral artery and secured. Two hours later, animals were re-anesthetized for filament withdrawal and final incision closure. Animals were euthanized 48 h after surgery with a 1 mL/kg i.p. injection of Phenobarbital-phenytoin (Euthasol, Delmarva Laboratories, Midlothian, VA). Brains were immersion fixed in 4% phosphate-buffered formalin. Brain tissues were paraffin embedded, sectioned, and slide mounted. Brain injury was evaluated using hematoxylin and eosin (H&E), and silver staining (Vogel et al., 1999).

2.5 Image analysis

Digital images were photographed (40x objective, ~400x magnification) using an Olympus BX41 microscope (Olympus America Inc., Center Valley, PA, USA). Multiple images from each animal were evaluated by two blinded observers, inter-rater reliability was calculated and data were included if Cronbach's alpha was $> 80\%$. For corroboration, collagen IV-positive blood vessel staining was quantified using two separate methods: 1. manual density counts from cortical coronal images, or 2. computer-automated analysis of the entire brain using a scanning microscope (NanoZoomer System and Visiopharm software, Hamamatsu/Olympus America). BrdU-positive neurons were evident in hippocampus and were counted. Slides with coronal sections through the massa intermedia of the thalamus were stained with H&E and the extent of cortical-subcortical tissue compromise (e.g. cortex, hippocampus, thalamus) and lateral spread of cortical encephalopathy in the lesioned hemisphere were evaluated and scored using a 4-point ordinal scale to represent the severity of gross injury (1-none, 2-mild, 3-moderate, 4-severe). Silver-stained tissues were photographed at 15x to capture the entire hemisphere and infarcted areas were outlined and quantified using software (UTHSCSA Image Tool, Dental Diagnostic Science, USA).

2.6 Statistical analysis

SPSS software (SPSS, Chicago, IL) was used to analyze data. For parametric data, ANOVA was followed by post hoc tests. Chi-squared was used for comparisons of proportions. The alpha criterion was $P = 0.05$ and tests were two tailed. To balance statistical comparisons, uninjected and saline-injected rats were combined into a single control group because they did not differ.

3. Results

3.1 Mortality

Neonatal mortality during the treatment period (14/218) differed by group with 1/82 deaths in the *control* group (uninjected plus saline injected), 1/38 in the morphine group, 5/45 in the stress group, and 7/53 in the stress plus morphine group ($\chi^2_{3,1} = 10.3$, $P = 0.016$). There were 10 unexplained vivarium deaths in juvenile rats prior to adult MCAO surgery with 2/81 deaths in the control group, 1/37 in the morphine group, 2/40 in the neonatal stress

group, and 5/46 in the stress plus morphine group ($\chi^2_{3,1} = 4.9$, $P = 0.177$). Adult mortality after MCAO surgery was substantial (50/156) but did not differ by neonatal treatment group, with 25/66 control, 6/28 morphine, 7/30 neonatal stress and 13/32 stress plus morphine group ($\chi^2_{3,1} = 4.5$, $P = 0.21$). There were no deaths in the 38 non-MCAO lesioned adult rats which were used only for analysis of cerebral blood vessel density. There were no sex differences in mortality.

3.2 Weight

There were no lasting effects of neonatal treatments on rat growth as previously reported. As expected, there were sex differences in the adult weights ($P = 0.001$). The mean weight ($\pm$ SD) for adult rats prior to MCAO surgery was 289 ± 36 g for males ($n = 71$), and 196 ± 29 g for female rats ($n = 85$). The mean ($\pm$ SD) 48 h post-MCAO surgery weight loss was -38 ± 13 g for male, and -29 ± 16 g for female rats with no effect of neonatal treatment.

3.3 Brain immunostaining

Figure 1 illustrates brain immunostaining of collagen IV to label blood vessels (top panel), and BrdU immunostaining (bottom panel) to label cells that divided during the neonatal treatment period. To examine whether neonatal treatments altered adult cerebrovascular density, collagen IV-positive blood vessels were quantified in brains from the 38 non-lesioned adult rats. Table 1 lists the mean density counts and computer scanned proportional areas for cerebral blood vessels. There were no significant effects of neonatal treatments on cerebral blood vessel density and there were no sex differences. These brains were also stained for BrdU, however, BrdU-positive blood vessels were extremely rare and thus could not be reliably quantified. In contrast, BrdU-positive hippocampal granule cell neurons were abundant (Figure 1). BrdU-positive cells in the dentate gyrus were quantified and Table 2 lists the density counts for each treatment group. Two-way ANOVA using neonatal stress and morphine as factors detected a significant effect of neonatal stress on BrdU incorporation ($P = 0.05$), but there was no effect of morphine and no interaction. However, subsequent post hoc testing of the combined group means suggested only a trend ($P = 0.073$) toward neonatal stress-induced enhancement of hippocampal granule cell proliferation.

3.4 MCAO-induced injury

Neonatal treatments did not change the degree of injury after unilateral MCAO. Figure 2 is a histogram quantifying MCAO-induced brain injury as indicated by gross injury scoring of the lesioned (R) hemisphere (top panel), and by L/R ratios of silver stained areas (bottom panel). A pattern of slightly more gross brain injury and less silver staining was evident in the rats subjected to neonatal stress plus morphine, however, the differences were not significant. There were no sex differences.

4. Discussion

We hypothesized that neonatal morphine and stress would decrease neonatal angiogenesis and thereby alter adult cerebral blood vessel density and exacerbate stroke-induced brain injury. In contrast, neonatal treatments did not alter adult cerebral blood vessel density or MCAO-induced brain injury. Any evidence of neonatal angiogenesis was below detection in adult rats, possibly because BrdU-positive blood vessels have undergone turnover by P60. Although unrelated to the blood vessel hypothesis, evidence of neonatal neurogenesis was detected and so we chose to count BrdU-positive granule cell neurons in the hippocampal dentate gyrus. Below, the results of that analysis are contrasted with recent data describing effects of neonatal morphine on neurogenesis.

For critically ill neonates, pain and stress must be carefully managed. Although, several clinical trials have found morphine effective for reducing stress in ventilated preterm infants (Anand, 2001; Orsini et al., 1996; Quinn et al., 1993), more recent trials have found no benefit of morphine infusion on stress or neurological outcomes in preterm infants (Anand et al., 2004; Simons et al., 2003) and a subsequent Cochrane systematic review concluded that there was insufficient evidence to support routine use of opioids in ventilated neonates (Bellu et al., 2005). And the risks of neonatal morphine may outweigh the benefits. For example, animal data indicate that prolonged exposure may delay brain growth and motor development, or induce neuronal apoptosis and alter nociception (Handelmann and Dow-Edwards, 1985; Hu et al., 2002; Kirby et al., 1982; Seatriz and Hammer, 1993). Opiates are also known to regulate angiogenesis (Blebea et al., 2000; Gupta et al., 2002; Pasi et al., 1991; Poonawala et al., 2005) and cerebral hemodynamics (Armstead, 1996; Armstead et al., 1990; Van Woerkom et al., 2004). There is a concern that neonatal morphine may increase risk for adult stroke (Hanson et al., 2004). These observations prompted us to examine directly whether neonatal morphine exposure altered cerebrovascular density and response to adult stroke.

Although we examined cerebrovascular density, there are mixed data suggesting that cerebrovascular *function* may change in response to neonatal morphine. In ventilated premature infants, one study found no effect of morphine on cerebral blood flow or perfusion pressure (Sabatino et al., 1997), but another reported that morphine altered both cerebral oxygenation and hemodynamics (van Alfen-van der Velden et al., 2006). Using data from animals, one experiment suggested that adrenal enkephalins may regulate blood flow to both hypothalamus and pituitary to modulate the stress response (Dora et al., 1992). If true, it is plausible that exogenous morphine exposure could interfere with endogenous regulation of hypothalamic blood flow during neonatal stress. Future experiments measuring cerebral blood flow in neonatal animals would be needed to address that hypothesis directly.

Several neonatal conditions, including stress, have been associated with adult disease states. For example, Craft *et al* found that neonatal stress altered the corticosteroid response and increased injury due to experimental stroke in adult mice (Craft et al., 2006). Another experiment suggested that fetal growth retardation may increase risk for adult hypertension and stroke (Eriksson et al., 2000). Experimental neonatal inflammation increased adult injury due to global cerebral ischemia (Spencer et al., 2006). And prematurity has been directly related to increased mortality in adults (Crump et al., 2011). As mentioned earlier, we previously observed that adult rats exhibit significant hyperglycemia after a hypoxic-ischemic lesion, but only if they were previously exposed to prolonged stress as neonates (McPherson et al., 2009). The mechanisms by which neonatal stressors produce these permanent changes in adult physiology are likely mediated by changes in gene expression. In support of that hypothesis, we used microarray analysis of neonatal mouse hippocampal gene expression and reported that repeated neonatal stress produces changes in gene expression and that there is a complex interaction of neonatal stress with morphine treatment (Juul et al., 2011). In that particular experiment, we examined hippocampal gene expression, but it is likely that other loci are also responsive to neonatal stressors, and it is probably true that changes in multiple loci are responsible for the stress-induced increase in adult vulnerability to brain injury and disease. It is still unknown whether these acute neonatal stress-induced changes in gene expression are present in adult animals.

Finally, we observed a trend toward enhancement of hippocampal neurogenesis by neonatal stress. This discovery complements our reports describing effects of neonatal stress on hippocampal gene expression (Juul et al., 2011) and neuronal proliferation (Hays et al., 2012), as well as older studies describing effects of early opiate exposure on brain development (Hammer et al., 1989; Lapointe and Nosal, 1982; Seatriz and Hammer, 1993).

Given that glucocorticoids inhibit neurogenesis (Mirescu and Gould, 2006), our findings may result from a stress-induced decrease in hippocampal glucocorticoid receptor expression (Ladd et al., 2004). Regarding morphine exposure, a very recent study found that neonatal morphine reduced hippocampal neurogenesis in animals killed at P8 (Traudt et al., 2012) and this acute effect could be due to morphine-induced neuronal apoptosis (Hu et al., 2002). Collectively, these observations suggest opposite effects of neonatal stress and neonatal morphine on hippocampal neurogenesis and thus support continued concern that combined neonatal exposure may have long-term adverse effects on brain development and function. For the safety of critically ill newborns, it is important to identify the conditions and underlying mechanisms that increase risk for adult disease so that we may develop and refine therapies.

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Abbreviations

MCAO	Middle cerebral artery occlusion
P	postnatal day
BrdU	Bromodeoxyuridine
H&E	hematoxylin and eosin

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Highlights

- Newborn rats exposed to neonatal stress with/without neonatal morphine were later exposed to focal cerebral ischemia as adults.
- Cerebral vessel density and ischemia-induced brain injury were not altered by neonatal treatments.
- Neonatal stress promoted hippocampal neurogenesis.

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Figure 1. Photomicrographs of collagen-IV-immunolabeled blood vessels (top panel) and BrdU-labeled hippocampal granule cell neurons in adult rat brain. Tissues were counterstained with hematoxylin (blue). The magnification is 400x and the black bar is 100 μ M. These are typical of images used for the measurements presented in Tables 1 and 2.

Figure 2.

Unilateral cortical injury in adult rats after right-hemisphere MCAO lesion. Bars show the means ($\pm$ SEM) for measurements of gross injury (top panel) or for left/right ratios of silver staining area (bottom panel). L/R hemispheric ratios are expressed so that numbers >1 indicate increasing MCAO-induced injury.

Table 1

Mean ($\pm$ SEM) vessel density counts and computer-scanned proportional areas for collagen IV-immunoreactive cerebral blood vessels in groups of adult rats exposed to neonatal morphine and/or neonatal stress. There were no statistical differences.

Group (n)	Collagen IV-immunoreactive blood vessels	
	Density (#/mm ²)	Proportional Area (% of total)
Control (14)	42.4 $\pm$ 2.1	4.6 $\pm$ 0.4 %
Morphine (8)	39.3 $\pm$ 1.3	5.0 $\pm$ 0.7 %
Neonatal stress (8)	42.7 $\pm$ 3.3	4.6 $\pm$ 0.5 %
Neonatal stress & morphine (8)	37.6 $\pm$ 2.1	5.0 $\pm$ 0.2 %

Table 2

Mean $\pm$ SEM (n) density counts for BrdU-immunoreactive hippocampal granule cells in groups of adult rats exposed to neonatal stress with or without neonatal morphine treatment.

	Dam-reared	Neonatal Stress ^I
Control	279 $\pm$ 50 (13)	329 $\pm$ 37 (8)
Morphine	201 $\pm$ 53 (7)	352 $\pm$ 38 (8)

^I ANOVA (stress x morphine) revealed a significant effect of neonatal stress ($F_{1,36} = 4.1$, $P = 0.05$) but no effects or interactions due to morphine.