Neonatal factors influence adult stroke outcome

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Summary Neonatal environment can have important, life-long influences on stress-reactivity and hypothalamic-pituitary-adrenal (HPA) axis regulation. In rodents, brief mother-infant separations have been shown to improve efficiency of the HPA axis, decrease stress-reactivity, and decrease age-related declines in cognitive function. Here, we provide evidence that there are potential costs associated with improved HPA axis regulation, including increased sensitivity to cerebral inflammation and glucocorticoid-mediated neuronal death following stroke. Specifically, brief mother-infant separation decreases the initial corticosteroid response to experimental stroke in adult mice, but increases post-stroke pro-inflammatory cytokine expression, edema, and infarct volume compared to ischemic controls. Brief maternal separation also compromises functional recovery and long-term survival following stroke. In addition, adrenalectomy reverses the effects of brief maternal separation on stroke outcome when corticosterone is replaced at baseline, but not ischemic, concentrations; thus, neonatally separated mice are more sensitized as adults to the detrimental effects of elevated corticosterone during ischemia. Taken together, these data provide the first direct evidence that neonatal environment can substantially influence adult cerebrovascular health.

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1. Introduction

Glucocorticoid release following exposure to a stressor is often an adaptive response that enables an individual to respond appropriately to changes in the environment (McEwen, 2000; Sapolsky, 1999).
However, the effect of cumulative, life-long exposure to stressors and mediators of the stress response, such as glucocorticoids, is increased vulnerability to a wide array of physical and psychological pathologies (McEwen, 1998; McEwen & Wingfield, 2003). Neonatal environment may be a particularly important determinant of the onset of psychopathology and age-related disease (McEwen, 2002). Indeed, in rodents, prenatal stress and extended maternal separation is associated with exaggerated physiological and behavioral responses to stressors, decreased neurogenesis, and a greater age-related decline in cognitive function compared to controls (Ladd et al., 2004; Lemaire et al., 2000; Maccari et al., 2003; Mirescu et al., 2004; Parfitt et al., 2004; Plotsky & Meaney, 1993; Romeo et al., 2003; Yamazaki et al., 2005). In contrast, brief maternal separation (i.e., the neonatal handling paradigm), is associated with dampened physiological and behavioral responses to stressors (Francis et al., 1999; Liu et al., 1997; Meaney et al., 1991; Parfitt et al., 2004; Vallee et al., 1997), and improved hypothalamic-pituitary-adrenal (HPA) axis regulation and cognitive function in aged animals (Meaney et al., 1988), but increased sensitivity to NMDA-induced neurodegeneration relative to controls (Horvath et al., 2004). Importantly, the initial findings of extended and brief maternal separation, which were conducted with rats, have been more recently replicated in mouse studies, suggesting a cross-species effect of the postnatal manipulation paradigms (Parfitt et al., 2004; Romeo et al., 2003). Furthermore, perinatal programming of the HPA axis also occurs among humans (reviewed in Matthews, 2000) and is thought to influence the development of cardiovascular disease and insulin resistance (Phillips et al., 1998; 2000; Reynolds et al., 2001).

The goal of the current study was to determine if neonatal programming of the HPA axis, achieved through brief maternal separation, is associated with altered outcome following cerebral ischemia. Activation of the HPA axis is among the first measurable physiological responses to cerebral ischemia, with elevated post-stroke corticosteroid concentrations surpassing a typical stress response and persisting for days, or months, beyond the ischemic attack (Fassbender et al., 1994; Johansson et al., 1997; Slowik et al., 2002). Several clinical studies have documented that elevated post-stroke serum concentrations of cortisol are associated with increased morbidity and mortality (Christensen et al., 2004; Feibiel et al., 1977; Murros et al., 1993; Olsson, 1990). Experimental evidence also suggests that elevated post-stroke corticosterone concentrations increase infarct size in mice and rats (Devries et al., 2001; Madrigal et al., 2003; May et al., 2002; Rami et al., 1998; Sugo et al., 2002). In contrast, adrenalectomy and treatment with compounds that decrease circulating corticosterone concentrations decrease infarct size in rodents (Antonawich et al., 1999; Risedal et al., 1999; Sapolsky & Pulsinelli, 1985; Smith-Swintosky et al., 1996). Thus, glucocorticoid exposure is a critical determinant of survival and functional outcome following ischemic attacks.

In the current study, a brief maternal separation paradigm was used to produce mice with blunted corticosteroid responses to stressors (Meaney et al., 1987; Parfitt et al., 2004). Both clinical and rodent data provide evidence that peri-ischemic glucocorticoid concentration influences stroke outcome (as reviewed above). Thus, it was predicted that neonatal manipulations that cause life-long alterations in HPA axis reactivity would affect neuronal integrity and survival following an ischemic insult.

2. Methods

This study was conducted in accordance with the National Institute of Health guidelines for the care and use of animals in research, and under protocols approved by the local Institutional Animal Care and Use Committee.

2.1. Animals and housing

The adult male C57 BL/6 mice (approximately 15 weeks of age) used in this study were bred on-site using stock obtained from Harlan Laboratories, Inc. (Indianapolis; IN). The mice were maintained in a temperature-controlled (~20 °C) vivarium on a 14 h/10 h light/ dark cycle, with ad libitum access to food and water.

2.2. Postnatal manipulation

The brief maternal separation (BMS) group consisted of offspring given two weeks of postnatal handling beginning one day after birth, a paradigm adapted from previous literature (Meaney et al., 1987). The maternal separation was initiated each day during the light cycle between the hours of 10:00 am and 1:00 pm (dark cycle begins at 2:00 pm). First, the mother was removed from the home cage and placed in a clean cage. Then, the pups were removed gently from the home cage, one at a time, and placed together on a paper towel in a separate clean cage. Besides nesting the pups together on the paper towel during the
manipulation, no other means of thermoregulation were provided during the brief separation. After 15 min of separation was complete, the litter and then the mother were returned to the home cage. Following completion of the BMS procedure on postnatal day 14, the dam and pups were provided their first bedding change in their home cage, and then were left undisturbed until weaned (postnatal day 21). The undisturbed (UD) mice were not disturbed during the first 14 days of life except for routine feeding. As with the BMS litters, UD mice also received their first and only bedding change on postnatal day 14. All mice were weaned at 21 days of age and housed in iso-sexual groups of up to 5 littermates. At 40-50 days of age, the mice were placed in individual housing and weighed weekly. Once within the desired weight range of 24.5-28.0 g (on average, two months following individual housing), male mice (females were not included in this study) were enrolled in pre-surgical behavioral testing and pseudo-randomly assigned to either the MCAO or SHAM-MCAO groups (see section 2.4 for description). To prevent a possible litter effect, no more than two mice from the same litter were included in an experimental group.

2.3. Behavioral testing

Experiments 2 and 3 used two behavioral tests performed at various times pre- and post-surgery. All tests were performed during the light phase.

2.3.1. Locomotor activity

General locomotor activity was assessed during a one-hour session. The locomotor apparatuses were enclosed in individual sound attenuating chambers equipped with a 15-W florescent white light and a ventilation fan. The monitors consisted of a clear 60 cm wide $\times$ 60 cm long acrylic box that was cleaned between trials and placed in a metal frame that was lined with 16 equally spaced photobeams along two sides of the box. Total distance traveled (cm) was determined using PAS software (San Diego Instruments, San Diego, CA).

2.3.2. Cylinder test

This test evaluated paw preference. The mice were placed in a clear Plexiglas cylinder (8 cm internal diameter, 12 cm height). Rearing frequency and total time spent grooming were recorded during 5 min. In addition, initial placement of the contralateral versus ipsilateral paw (relative to the ischemic hemisphere) on the cylinder during rearing was recorded to assess preferential paw use (Karhunen et al., 2003). Only the first paw placement was recorded for each rear. Paw preference data were presented as a frequency of contralateral paw placement/total rearing frequency. A decrease in contralateral paw use suggested a functionally significant impairment of the ipsilateral sensorimotor cortex. Mice that escaped the apparatus (1 BMS and 1 UD) or did not rear at least once during the post-MCAO session (1 UD) were not included in the statistical analyses for this test.

2.4. Surgery

Transient focal cerebral ischemia was induced in the mice by middle cerebral artery occlusion (MCAO). The mice were anesthetized with 1-1.5% halothane in oxygen-enriched air delivered through a facemask. Occlusion of the right middle cerebral artery was achieved by using the intraluminal filament insertion technique. A 6-0 nylon monofilament was inserted into the internal carotid artery, via the external carotid artery. Then the filament tip (approximate 1.0 mm length and 0.25 mm width) was positioned for occlusion at a distance of 6 mm beyond the internal carotid artery-pterigopalatine artery bifurcation. Once the filament was secured, the incision was sutured and the animal was allowed to emerge from the anesthesia in its home cage. After 60 min of occlusion, the animal was briefly re-anesthetized with halothane in oxygen-enriched air, and reperfusion was initiated via withdrawal of the filament. This surgical protocol typically results in a core infarct limited to the parietal cerebral cortex and caudate putamen of the right hemisphere. For the SHAM-MCAO surgery, the internal carotid artery was exposed but not disturbed. All other aspects of the surgery were similar for the MCAO and SHAM-MCAO groups. Throughout the surgery, rectal temperature was maintained at 37 ± 0.5°C through the use of a homoeothermic blanket system. All animals were given a 0.5 ml s.c. injection of lactated Ringer’s solution at the conclusion of the surgical procedure, and returned to their home cage for recovery.

2.5. Corticosterone determination

All blood samples were collected, and then centrifuged for 20 min at 10°C and 3500 rpm. The plasma was stored at −72°C until the assay. Corticosterone concentrations were determined using a radioimmunoassay kit (ICN Biomedicals, Irvine, CA). The standards were measured in triplicate and the samples were measured
in duplicate. Baseline, intra-ischemic and post-ischemic blood samples were collected in separate cohorts of animals.

2.6. Determination of stroke volume

Brains were rapidly removed, placed in a −70 °C freezer for 2 min, and then sectioned into five 2-mm-thick coronal sections. Sections were incubated for 15 min in 2,3,5-triphenyltetrazolium (TTC), with rotation every 2 min to allow uniform tissue staining. The TTC solution was maintained at 37 °C throughout the staining process. Following staining, the sections were fixed in 10% buffered formalin solution. The brain slices were photographed and analyzed using Inquiry software (Loats Associates, Inc., Westminster, MD); infarct size was calculated as a percentage of the contralateral hemisphere after correcting for edema, as previously described (DeVries et al., 2001).

2.7. Procedure

2.7.1. Experiment 1. Determination of intra-ischemic blood flow and corticosterone concentrations

Relative cerebral blood flow, blood gases, glucose concentrations, and blood corticosteroid concentrations were determined in a non-surviving cohort of animals (BMS n = 5; UD n = 5). To assess blood flow, a closed cranial window was made in the parietal skull for placement of a Laser Doppler flowmetry probe (DRT4, Moor Instruments, LTD, Devon, England). Laser Doppler flowmetry readings were taken at 15 min intervals beginning 30 min prior to occlusion, and continuing through 60 min of ischemia and 30 min of reperfusion. Blood samples were collected via a femoral artery catheter at baseline and following the intra-ischemic blood flow and corticosterone sample (15 μl) was collected at 30 min of ischemia for assessment of PaCO2, PaO2 and glucose (I-STAT Portable Clinical Analyzer with CG8 + cartridge, Heska, Fort Collins CO). A separate blood sample (15 μl) was collected at 30 min of ischemia for assessment of corticosterone concentration. One intra-ischemic corticosterone sample (BMS) was excluded from statistical analysis because it was greater than 2 SD from the mean. The baseline corticosterone samples were collected from a cohort of unmanipulated animals and the post-ischemic samples were collected from animals used in the 24 and 72 h survival experiments described below.

2.7.2. Experiment 2. Evolution of infarct: 24 h, 72 h and 7 day survival

24 h Survival. Baseline behavioral testing of locomotor activity occurred 24 h prior to surgery. MCAO or SHAM-MCAO surgery was performed the following day, and animals were returned to their home cage. Post-surgical behavioral testing was conducted 24 h later. Three hours after the conclusion of behavioral testing, blood samples and brains were collected via cervical dislocation and rapid decapitation within two minutes of removing the home cage from the colony.

72 h Survival. Behavioral testing was performed 24 h before and 72 h after MCAO surgery, and included locomotor activity and paw preference. Blood samples were collected 3 h following the post-surgical behavioral testing via rapid cervical dislocation and decapitation. Post-MCAO locomotor data was lost for one animal due to an equipment failure.

7 Day survival. Pre-surgical behavioral testing began 4 days prior to surgery. Day 1 included locomotor activity, and Day 3 included paw preference. Animals underwent MCAO or MCAO-sham surgeries on day 5. Six days after surgery, all behavioral tests were repeated. However, because only one BMS-MCAO mouse survived the full seven days, hormonal, behavioral and histological comparisons could not be made.

2.7.3. Experiment 3. Adrenalectomy and Corticosterone replacement

In order to standardize peri-ischemic corticosteroid concentrations in BMS and UD mice, a subset of mice were adrenalectomized and implanted with corticosterone pellets (50 mg; 20 or 40% corticosterone wt/wt) 24 h prior to ischemia. Behavioral tests were performed 4 h prior to ischemia, and included locomotor activity and paw preference. The lower dose pellet produced circulating corticosterone concentrations of approximately 170 ng/ml (BMS n = 5, 163.4 ± 24.60 ng/ml; UD n = 6, 176.6 ± 15.03 ng/ml; t(9) = −13.15, P < 0.05). The higher dose pellet produced circulating corticosterone concentrations of approximately 290 ng/ml (BMS n = 7, 300.4 ± 35.33 ng/ml; UD n = 9, 279.7 ± 21.98 ng/ml; t(14) = −0.52, P > 0.05). The pellets produced corticosterone concentrations within the intra-ischemic (low dose) and post-ischemic (high dose) ranges observed in Experiment 2. All adrenalectomized animals were provided with a water bottle containing 1% saline in addition to tap water. Data from two trials (1 BMS and 1 UD) were excluded because the animals repeatedly escaped from the cylinder.

2.7.4. Experiment 4. Quantification of post-stroke inflammation and edema

PCR sample preparation and quantitative real-time PCR. A separate cohort of UD and BMS MCAO mice
were survived 12 h following 60 min MCAO. Total RNA was extracted from <10 mg striatal tissue collected from the ischemic and non-ischemic hemispheres using a homogenizer and an RNeasy Micro Kit (according to manufacturer's protocol; Qiagen, Valencia, CA); RNA concentration was determined by spectrophotometer (SmartSpec 3000, Bio-Rad, Hercules, CA). Amplification of cDNA from individual samples was performed on an ABI 7000 Sequencing Detection System by using Taqman PCR Master Mix (Applied Biosystems, Foster City, CA). The universal two-step RT-PCR cycling conditions used were: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Relative mRNA expression was calculated by comparison to relative standard curve consisting of serial dilutions of pooled *M. musculus* cDNA followed by normalization to 18S mRNA gene expression. Data are presented as mRNA expression in ischemic hemisphere/mRNA expression in non-ischemic hemisphere.

**Determination of post-stroke edema.** In a separate cohort of 60 min UD and BMS MCAO mice, brain tissue was collected at 48 h reperfusion immediately following transcardial perfusion with 20 ml saline. The brain tissue was immediately divided into right (ischemic) and left (non-ischemic) hemispheres and weighed. The tissue was then dried at 70 °C for 72 h, with each hemisphere reweighed at 24 h intervals. Once fully dehydrated, an index of edema was calculated using the following equation, as previously described (Tanaka et al., 1997):

\[ l = \frac{(W/D_R - W/D_L)}{(W/D_L)} \times 100. \]

### 2.8. Statistical analyses

Blood gases and infarct size were analyzed using Analysis of Variance (ANOVA) followed by post hoc analysis using Fisher's PLSD Test. Corticosterone concentrations were analyzed using *t*-test at each individual timepoint, because the samples were collected from different cohorts of animals that had been exposed to slightly different procedures (i.e. behavioral testing paradigms); however, within-timepoint preparations were identical for all experimental groups. The behavior was analyzed by two-way ANOVA for repeated measures. Within group analysis of paw preference was analyzed via *t*-test. The edema and cytokine expression data did not meet the assumptions of ANOVA, and were therefore analyzed using the Mann-Whitney Rank Sum Test. Effects were considered statistically significant at *P* ≤ 0.05.

### 3. Results

#### 3.1. Experiment 1

Baseline corticosterone concentrations were equivalent in UD and BMS mice (UD *n* = 8 and BMS *n* = 7; *t*(13) = 0.29; *P* > 0.05). However, intra-ischemic corticosterone concentrations were...
significantly lower in BMS than UD MCAO mice ($t(8) = -2.879$, $P < 0.05$; Fig. 1). BMS and neonatally undisturbed (UD) mice responded to the stroke procedure by exhibiting similar decreases in mean relative cerebral blood flow in the cortex during MCAO (UD $n = 6$, 89.3 ± 1.2%; BMS $n = 6$, 91.0 ± 1.12%; $t(9) = 1.77$, $P = 0.31$) and similar intraischemic values for PaCO$_2$ ($t(9) = 0.55$, $P > 0.05$), PaO$_2$ ($H = 0.30$, $P > 0.05$), glucose ($t(4) = 0.79$, $P > 0.05$), MAP ($t(9) = 0.17$, $P > 0.05$) and pH ($t(9) = 0.65$, $P > 0.05$), based on blood samples collected after 30 min of occlusion. Furthermore, both groups returned to baseline cerebral blood flow (approximately 100%) upon withdrawal of the occluder ($t(9) = 1.69$, $P > 0.05$). These data suggest that the relative decrease in blood flow and all measured physiological parameters, except corticosterone concentration, were similar for BMS and UD mice during ischemia.

3.2. Experiment 2

At 24 h of reperfusion, infarct sizes were similar between UD MCAO mice ($n = 8$) and BMS MCAO mice ($n = 8$; $t(14) = -0.507$, $P > 0.05$; Fig. 2A). The difference in corticosterone concentrations at this timepoint was not significant between BMS and UD MCAO mice ($t(11) = 1.368$, $P > 0.05$; Fig. 1). There was a significant decrease in locomotor activity following MCAO ($F(1,14) = 97.7$, $P < 0.01$, an approximate 77% decrease relative to baseline), but no significant effect of experimental group ($F(1,14) = 0.03$, $P > 0.05$) on locomotor activity.

At 72 h of reperfusion, infarct size was significantly larger in the BMS MCAO mice ($n = 7$) than UD MCAO mice ($n = 8$; $t(13) = 2.151$, $P < 0.05$; Fig. 2B). Corticosterone concentrations were similar between BMS and UD MCAO mice ($t(13) = 0.580$, $P > 0.05$; Fig. 1). There was an approximate 50% decrease in general locomotor activity following MCAO ($F(1,12) = 12.53$, $P < 0.05$); however, there was no significant effect of experimental group ($F(1,12) = 1.22$, $P > 0.05$) on activity. There also was an approximate 50% decrease in rearing frequency in the cylinder test following MCAO ($F(1,12) = 15.37$, $P < 0.05$), but no overall effect of experimental group on rearing frequency ($F(1,12) = 0.70$, $P > 0.05$). However, there were significant effects of both experimental group ($F(1,10) = 6.18$, $P < 0.05$) and surgery ($F(1,10) = 7.41$, $P < 0.05$) on initial left paw use during rearing. Within group analysis indicated that following MCAO, BMS mice, but not UD mice ($P > 0.05$), exhibited a significant decrease in left (contralateral) paw use during rearing ($P < 0.05$; Fig. 3).

At 7 days of reperfusion, survival rate was significantly lower for BMS MCAO mice (1/10; 10%) than UD MCAO mice (8/11; 73%; Fig. 4A). Different survival trends for UD MCAO versus BMS MCAO groups are illustrated in the Kaplan-Meier Cumulative Survival Plot (Fig. 4B). Survival rates for SHAM surgery in BMS and UD mice were 100%. Because only one BMS MCAO mouse survived seven days poststroke, comparisons of infarct volume and behavior were not meaningful between BMS MCAO and UD MCAO mice. However, it is noteworthy that there were no significant differences ($P > 0.05$) between BMS SHAM and UD SHAM mice in any behavioral measure.

3.3. Experiment 3

When BMS MCAO and UD MCAO mice were adrenalectomized and implanted with low dose...
There was no effect of neonatal treatment on infarct size (BMS \( n=5 \); UD \( n=6 \); \( t(9)=0.18; P>0.05 \); Fig. 5A). There was an approximate 67% decrease in general locomotor activity following MCAO (\( F(1,9)=58.64; P<0.05 \)), but there was no significant effect of experimental group (\( F(1,9)=0.00; P>0.05 \)) on locomotor activity. There also was a significant decrease in rearing frequency in the cylinder test following MCAO (\( \sim 60\% \) of baseline; \( F(1,9)=27.72; P<0.05 \)), but no significant effect of experimental group (\( F(1,9)=0.001; P>0.05 \)). There was no effect of MCAO (\( F(1,6)=0.03; P>0.05 \)) or experimental group (\( F(1,6)=0.02; P>0.05 \)) on left paw placement during rearing (Fig. 5A).

In contrast, the high corticosterone replacement dose (producing approximate blood serum concentrations of 290 ng/ml) reproduced the disparate BMS and UD post-stroke histological and behavioral outcomes observed in the 72 h reperfusion group in Experiment 2. The BMS mice (\( n=7 \)) had a significantly larger mean infarct size than the UD mice (\( n=9 \); \( t(14)=-3.33; P<0.05 \). Fig. 5B). There was an approximate 48% decrease in general locomotor activity following MCAO (\( F(1,14)=26.27; P<0.05 \)), but no significant effect of treatment group (\( F(1,14)=0.03; P>0.05 \)) on locomotor activity. There also was an approximate 50% (UD) to 80%
(BMS) decrease in rearing frequency in the cylinder test following MCAO ($F(1, 12) = 82.44; P < 0.05$), but no overall effect of experimental group ($F(1, 12) = 0.65; P > 0.05$). There was a significant effect of MCAO ($F(1, 10) = 6.18; P < 0.05$) on initial left paw use during rearing. There was no overall significant effect of experimental group on left paw use ($F(1, 10) = 2.91; P > 0.05$), but there was a significant interaction between experimental group and surgical status ($F(1, 10) = 7.73; P < 0.05$). Within group analysis indicated that following MCAO, BMS animals, but not UD animals ($P > 0.05$), exhibited a significant decrease in left (contralateral) paw use during rearing ($P < 0.05$; Fig. 5B).

**3.4. Experiment 4**

Relative to the non-ischemic hemisphere, TNF-α and IL-1β mRNA was increased 4-fold and 7-fold, respectively, in the ischemic hemispheres of UD mice (Fig. 6A). BMS mice ($n = 6$) experienced significantly more TNF-α (224-fold increase) and IL-1β (290-fold increase) gene expression compared to UD mice ($n = 8$; $T = 62, P = 0.03; T = 52, P = 0.01$, respectively). In addition, BMS mice experienced more edema than UD mice following stroke ($n = 7$/group; $T = 70; P = 0.03$; Fig. 6B).

**4. Discussion**

BMS produced mice with lower intra-ischemic corticosterone concentrations, but increased susceptibility to ischemia-induced neuronal death and behavioral deficits compared to animals raised under typical colony conditions (UD; Figs. 1–3). Typically, following 60 min MCAO, infarct is limited to the caudate putamen; however, more extensive damage can continue into the cortex and hippocampus. Infarct sizes were equivalent between BMS and UD animals at 24 h of reperfusion (Fig. 2A). By 72 h of reperfusion, however, mean infarct size had nearly tripled among the BMS MCAO mice and was significantly larger than the mean infarct size of UD MCAO mice (Fig. 2B). The group difference in infarct size was functionally significant, as indicated by a significant decrease in use of the paw.
that was contralateral to the ischemic hemisphere in BMS, but not UD, mice (Fig. 3). In keeping with the group difference in infarct evolution and behavioral deficits, only 10% of the BMS mice survived seven days of reperfusion versus 73% of the UD mice (Fig. 4). Survival rates for SHAM surgery in BMS and UD mice were 100%. Taken together, these data indicate that modifying neonatal environment can substantially affect stroke recovery and survival.

We examined two possible mechanisms that could account for the increased sensitivity of BMS mice to stroke: (1) increased corticosteroid-mediated neuronal death and (2) increased brain inflammation and edema. In rats, handling (brief maternal separation) is associated with increased glucocorticoid receptor (GR) expression (Ladd et al., 2004; Meaney et al., 1985; 1996; O'Donnell et al., 1994), which may magnify the effects of corticosteroids on neuronal death following cerebral ischemia. Indeed, the role of GRs in modulating stress or corticosteroid effects on ischemia-induced neuronal death is well-established (Scheuer & Mifflin, 1997; Sugo et al., 2002). In order to standardize peri-ischemic corticosteroid concentrations in BMS and UD mice, a subset of mice were adrenalectomized and implanted with corticosterone pellets (50 mg; 20 or 40% corticosterone wt/wt) 24 h prior to ischemia. When BMS MCAO and UD MCAO mice were adrenalectomized and implanted with the lower dose corticosterone replacement pellets (producing approximate blood serum concentrations of 170 ng/ml), there was no effect of neonatal treatment on infarct size (Fig. 5A) or post-stroke contralateral paw use (Fig. 5A). In contrast, the higher corticosterone replacement dose (producing approximate blood serum concentrations of 290 ng/ml) reproduced the disparate BMS and UD post-stroke histological and behavioral outcomes observed in the 72 h reperfusion group. Indeed, the adrenalectomized BMS mice that received the higher dose corticosterone replacement pellet had a significantly larger mean infarct size than the adrenalectomized UD mice that received a similar corticosterone replacement dose (Fig. 5B). Furthermore, following MCAO, BMS mice, but not UD mice, exhibited deficits in contralateral paw use (Fig. 5B). Data from the adrenalectomized mice used to repeat the 72 h reperfusion experiment support the hypothesis that BMS mice are more susceptible to glucocorticoid-mediated ischemic cell death than UD mice. When a corticosterone pellet was implanted in adrenalectomized mice to maintain low intra-ischemic corticosterone concentrations, and to prevent the corticosterone surge that typically occurs within 24 h of ischemia, the difference in infarct volume previously observed between the UD and BMS mice was eliminated. In contrast, the higher dose corticosterone pellet, which produced concentrations in adrenalectomized mice that were similar to those observed in intact BMS mice at 72 h post-stroke, resulted in a significant increase in infarct volume for the BMS, but not UD, mice. Therefore, it appears that sustained corticosterone concentrations greater than 170 ng/ml are critical for altering infarct evolution in the BMS mice. Mice that have not undergone neonatal manipulations also are sensitive to peri-ischemic corticosterone concentrations (DeVries et al., 2001; Sapolsky and Pulsinelli, 1985; Sugo et al., 2002); however, the circulating corticosterone concentrations achieved...

**Figure 6** Inflammatory markers in UD and BMS mice following MCAO. (A) BMS dramatically increased relative gene expression of the inflammatory cytokines, IL-1β and TNF-α at 12 h reperfusion compared to UD MCAO mice (p < 0.05). Data are expressed as a ratio of relative gene expression in the ischemic (right) hemisphere over relative gene expression in the non-ischemic (left) hemisphere. In addition, (B) BMS MCAO mice had significantly more edema than UD MCAO mice at 48 h reperfusion (p < 0.05). (Edema index = ((wet wt/dry wt R – wet wt/dry wt L)/((wet wt/dry wt L)) × 100). Together, these data suggest an exaggerated inflammatory response to stroke in BMS mice.

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by our high dose pellet was within the range typically experienced by UD mice following ischemia. An even higher dose corticosterone pellet most likely would have led to larger infarcts in the UD mice, but would have produced a ceiling effect in the BMS mice since it is unlikely that many would survive 72 h with larger infarcts than were produced by the high dose pellet in the current study.

Indeed, exposure to corticosteroids prior to or following stroke is a key determinant of stroke outcome. Although glucocorticoids have often been used to treat inflammatory diseases within the central nervous system (Barnes and Adcock, 1993; Coyle, 1999; Filippini et al., 2000; Salaki et al., 2004), more recent data suggest that glucocorticoids actually exacerbate neuronal death in the context of a neurological insult (Dinkel et al., 2003; Sapolsky and Pulsinelli, 1985a; Sapolsky and Pulsinelli, 1985; Stein-Behrens et al., 1992). During cerebral ischemia, glucocorticoids may contribute to the frequency, severity, and extent of injury by aggravating neuronal vulnerability to neurotoxins such as excitatory amino acids and free radicals (Behl et al., 1997; Sapolsky and Pulsinelli, 1985), and suppressing neuroprotectors such as Bcl-2 (Alkayed et al., 2001; DeVries et al., 2001). Notably, our study suggests that early programming of the HPA axis can alter corticosteroid sensitivity, and ultimately exacerbate the effects of corticosteroids in ischemic cell death. Thus, neuronal sensitivity may be as important, if not more important than blood glucocorticoid concentrations in determining the effects of corticosteroids on stroke outcome. Glucocorticoid sensitivity during ischemia may be directly linked to GR concentration. Though exact mapping of GR expression differs among species, rodents have extensive distribution of GRs throughout the brain (Adams et al., 2003; Ahima and Harlan, 1990; McGimsey et al., 1991; Pepin et al., 1992; Pesini et al., 1998); specifically, GRs are highly concentrated in areas of the brain, such as the hippocampus and striatum, that also are susceptible to stress- and disease-induced neurodegeneration (Adams et al., 2003; Defiore and Turner, 1983; Haynes et al., 2001; Sapolsky, 1985b; Watanabe et al., 1992).

Indeed, the use of GR antagonists can prevent the negative effects of stress (Sugo et al., 2002) and exogenous corticosteroids (Antonawich et al., 1999; cf. Krugers et al., 2000) on post-ischemic neuronal death. Previous research has determined that brief periods of maternal separation during neonatal development lead to increased expression of GR (Ladd et al., 2004; McCormick et al., 2000; Meaney et al., 1985; 1996; O’Donnell et al., 1994). The surplus of GR increases efficiency of the negative feedback regulation of the HPA axis during an acute stressor (Meaney et al., 1991), ultimately minimizing exposure to excess glucocorticoids and potentially decreasing allostatic load (McEwen and Wingfield, 2003). However, during and following stroke, there is a sustained release of glucocorticoids. Thus, because the BMS animals express more GR in the most vulnerable areas of the brain to ischemic damage, the chronic exposure to elevated glucocorticoid concentrations following stroke may make them more susceptible to glucocorticoid-induced exacerbation of ischemic cell death.

Our second hypothesis was that the BMS mice had larger infarcts and lower survival rates than the UD mice after stroke because they were more susceptible to secondary brain damage caused by increased brain inflammation and edema. To test this hypothesis, we assessed TNF-α and IL-1β mRNA expression via quantitative PCR 12 h after stroke, and edema formation at 48 h following stroke, in separate cohorts of mice. Relative to the non-ischemic hemisphere, TNF-α and IL-1β mRNA was increased 4-fold and 7-fold, respectively, in the ischemic hemispheres of UD mice (Fig. 6A). BMS mice experienced significantly more TNF-α (224-fold increase) and IL-1β (270-fold increase) gene expression compared to UD mice. In keeping with increased stroke-induced pro-inflammatory cytokine expression, the BMS mice also experienced more edema than UD mice following stroke (Fig. 6B). Thus, the substantial increase in infarct size among BMS mice between 24 and 72 h following stroke may be due, in part, to secondary neuronal death associated with the increased expression of pro-inflammatory cytokines (Barone et al., 1997; Boutin et al., 2001; Dawson et al., 1996; Yang et al., 1998) and edema (Stoll et al., 2002). Indeed, glucocorticoid exposure following an excitotoxic neurological insult results in increased inflammatory cell and pro-inflammatory cytokine expression (Dinkel et al., 2003). Whether the HPA axis and immune system are acting independently or together to influence neuronal death in the BMS mice remains to be determined.

In summary, these data suggest that although BMS mice experienced a dampened corticosteroid response to ischemia initially, they were ultimately more susceptible to corticosteroid-induced exacerbation of ischemic damage following long-term exposure to elevated corticosteroids and increased inflammatory responses to stroke. Thus, a cost associated with increased efficiency of negative feedback regulation may be increased susceptibility of cells to glucocorticoid influences on cell death as a result of prolonged HPA axis activation following stroke. Although a large body of literature has
examined perinatal determinants of long term health (Graham et al., 1999; Welberg and Seckl, 2001), a direct physiological link between changes in the developing brain and adult cerebrovascular and cardiovascular disease has been lacking. This study clearly demonstrates, for the first time, that neonatal environment can drastically affect adult sensitivity to ischemic injury by altering the HPA axis and inflammatory responses.

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