Research Report

Reproductive experience facilitates recovery from kainic acid-induced neural insult in female Long–Evans rats

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ABSTRACT

The hormones of pregnancy and lactation (e.g., estrogen, progesterone, and oxytocin) have been shown to modulate learning, memory, and the restructuring of brain areas not traditionally associated with maternal behavior. Given the impact of reproductive experience on plasticity of brain areas such as the hippocampus, kainic acid (KA) was used in the current study to induce hippocampal-specific neurotoxic insult in adult multiparous and virgin Long–Evans rats. In Experiment I, Fluoro-Jade B, an indicant of degenerating cells, revealed significant neuronal damage in KA-treated hippocampi at 16 h post-injection in both maternal and virgin rats. In Experiment II, maternal and virgin rats were assessed in spatial and novel object preference tasks to determine the effects of KA on subsequent behavioral and cognitive responses. Twenty-four hours post injection, saline maternal animals exhibited superior memory in a spatial task. Further, maternal saline-injected rats were more similar to maternal KA-injected rats than both the virgin groups. Forty-eight hours following the KA or saline injection, compared to virgins, maternal animals demonstrated enhanced memory in the novel object memory test, regardless of type of injection. Further, neurobiological assessments in Experiment II indicated that virgin KA exposed rats had significantly more glial fibrillary acidic protein (GFAP)-immunoreactivity in the hippocampus, suggesting that they were in an earlier stage of neural recovery compared to maternal animals or, alternatively, may have exhibited more trauma than maternal animals. Together, these data suggest that the previously reported plasticity of the maternal brain may facilitate neural and behavioral recovery from neural insults.

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1.   Introduction

Maternal experience, and its accompanying exposure to various reproductive hormones such as prolactin, progesterone and estrogen for extended durations, has dramatic effects on the brains of mammals, effects which may extend beyond stimulating the onset of maternal behavior (Kinsley and Lambert, 2006, 2008; Lambert and Kinsley, 2008). Past research, for example, indicates that maternal experience alters hippocampal plasticity, which may contribute to brain regions and behaviors that support care of young. Compared to virgins, late pregnant and lactating rats have denser populations of hippocampal dendritic spines (Kinsley et al., 2006). Further, reproductive status affects morphological changes in hippocampal astrocytes (Salmaso et al., 2005). Lower levels of amyloid precursor protein (associated with cognitive decline) have also been observed in the hippocampi of senescent rats with prior maternal experience, indicating that certain aspects of reproduction-induced modifications persist throughout the animal's life (Gatewood et al., 2005). Together, these results confirm that maternal experience alters hippocampal plasticity, an effect that, in some cases, leads to the expression of neuroplasticity and complementary neuroprotective effects across the lifespan.

Hippocampus-dependent behavioral change has also been assessed in maternal rats. Multiparous rats perform better than virgins in the radial-arm maze; further, both maternal and pup-sensitized rats exhibited a stronger spatial memory than virgins in a version of the Morris water maze known as the dry land maze (Kinsley et al., 1999). In a competitive spatial foraging task, multiparous rats exhibited shorter latencies to reach food rewards than age-matched primiparous and virgin rats (Love et al., 2005). In corroboration with the long-lasting effects observed in relevant neuroanatomy studies, as animals age, maternal rats have been shown to continue to outperform their virgin counterparts in spatial tasks (Gatewood et al., 2005; Love et al., 2005).

Explorations of responses to acute brain injuries have also indicated the importance of reproductive hormones in recovery from neural insults. When male and female rats, for example, were exposed to traumatic brain injury, females displayed less cerebral edema than males; additionally, females with the highest levels of progesterone exhibited the lowest incidence of secondary edema from the neural insult (Roof et al., 1993). These findings suggest potential neuroprotective and neuroregenerative effects of progesterone, a steroid hormone with largely estrogenic interactions (e.g., Carroll et al., 2007; He et al., 2004; Nilsen and Brinton, 2002, 2003). Furthermore, contact between astrocytes and neurons represent another essential component of the neuroprotective role of estrogens. For instance, astrocytes express estrogen receptors and participate in the regulation of synaptic plasticity, and estrogens modulate the release of neurotrophic factors and inflammatory molecules by astrocytes (Azcoitia et al., 2010; Barreto et al., 2009). Progesterone's effects include the facilitation of myelination (e.g., Baulieu and Schumacher, 2000; Schumacher et al., 2007), increased mitochondrial activity (e.g., Irwin et al., 2008), suppressed inflammation (Gibson et al., 2005) and protection against the breakdown of cell membranes, perhaps due to anti-oxidant properties (Stein, 2008). Progesterone receptors are found in many areas throughout the brain including the hippocampus, a particularly sensitive region for recovery of normal function in individuals suffering from acute brain injuries (Guerra-Araiza et al., 2000, 2001, 2003). Increased hippocampal cell death and hippocampal-dependent neural activity, for example, have been observed in animal models of traumatic brain injury (Barha et al., 2011). Recently, it was reported that male rats with bilateral frontal cortex contusions exhibited a more normalized pattern of cell proliferation and degeneration following progesterone treatment (Barha et al., 2011). Alternatively, though, recent studies have reported that progesterone may interfere with the neuroprotective action of other steroids, such as estradiol (Azcoitia et al., 2011).

In addition to reports indicating that the reproductive hormone progesterone facilitates recovery from neural insults, research suggests that lactating animals exhibit protection against neural insult, as they show less excitotoxicity-induced cell damage in the dorsal hippocampus than nulliparous rats following an acute neural insult (Morales, 2011).

Considering the accumulating evidence that reproductive experience influences the brain's ability to recover from various insults, the purpose of the current set of experiments was to determine the effects of maternal experience on both neural and behavioral recovery from neural insult in Long–Evans rats caused by kainic acid (KA), a naturally-occurring analog of the excitatory amino acid neurotransmitter glutamate—known to induce seizures and subsequent damage to the CA3 area of the hippocampus (Benkovic et al., 2004; Kesslak and Gage, 1986; Krajewska et al., 2011). Thus, KA was used to generate an acute brain injury; that is, a neurotoxic lesion (Hilton et al., 2006; Nadler et al., 1978; Olney et al., 1979; Sperk et al., 1983). The sustained excitatory amino acid exposure and associated hippocampal neurodegeneration precede a reactive gliosis which parallels the secondary effects observed in other models of acute brain injuries such as traumatic brain injury (Genarelli and Graham, 2005). Secondary injuries occur during the brain's recovery attempts following a brain injury and include such compensatory events as edema, apoptosis/degeneration of nerve cells, decreased cranial blood pressure, generation of free radicals, and the release of cytokines (Bouma and Muizelaar, 1992; McIntosh, 1994; Panter and Faden, 1992).

Here, the neurotoxic effect of KA was initially confirmed using Fluoro-Jade B (FJB), a fluorescent marker with a high affinity for degenerating cells (Schmued and Hopkins, 2000). Glial fibrillary acidic protein (GFAP) immunohistochemistry was also employed in maternal and virgin animals to determine the extent of kainate-induced reactive gliosis. In a second experiment, both spatial and non-spatial memory in maternal and virgin rats was assessed pre- and post-insult to determine the regulatory impact of maternal experience and neurotoxin-induced neural insult on recovery of function following KA exposure and the subsequent brain injury.

2.   Results

2.1.   Seizure scoring

In Experiments I and II, maternal experience failed to affect the mean seizure intensity (Experiment I: $F_{1,15}=0.68$, p=0.42

[4.75 (±0.25 SEM) and 2.75 (±1.31 SEM) for the maternal and virgin animals, respectively; Experiment II: $F_{1,34}=0.57$, $p=0.46$; [4.3 (±0.235 SEM) and 3.38 (±0.77 SEM) for the maternal and virgin animals, respectively). Rats from both reproductive groups exhibited seizures ranging from 1 to 5 on the Racine Scale (Racine, 1972). Two rats experienced level 5 seizures and expired following KA treatment—one mother and one virgin—both within 12 h of injection. Neither animal's brain was salvageable for analyses.

2.2. Experiment I

2.2.1. Neural analysis at 16 h (Fluoro-Jade B and GFAP)

Neural degeneration resulting from the KA injection was analyzed using the fluorochrome Fluoro-Jade B (FJB). As expected, KA treatment resulted in an increase in cell death in the CA3 hippocampal area ($F_{1,13}=7.31$, $p=0.018$—Fig. 1). No main effect of maternal experience was observed following KA lesions as similar levels of degeneration in the CA3 subregion were observed in the hippocampus of both mothers and virgins ($F_{1,13}=0.34$, $p=0.86$). These data, however, clearly demonstrate that KA was a potent neurotoxin in the current study, sufficient for neural degeneration.

Glial activity in the hippocampus of KA and saline treated animals was also evaluated at 16 h post-injection. In three subregions of the hippocampus—medial and lateral regions of the hippocampal fissure, and the CA3—no differences in GFAP-IR were found at this time point (all p-values>0.592). See Table 1 for GFAP-IR means.

2.3. Experiment II

2.3.1. Spatial memory tasks at 24 h

Twenty-four hours following KA injections, all animals were subjected to a second probe test in the dry-land maze (DLM) to gauge the spatial memory abilities of animals that had

Table 1 – Mean values (and SEMs) of GFAP-IR in Exp. I.

Treatment	HiF (media)	HiF (lateral)	CA3
Kainic acid	2.1 (.6)	2.1 (.6)	1.8 (.4)
Saline	2.1 (.3)	2.5 (.4)	1.9 (.3)
Total	2.1 (.3)	2.3 (.3)	1.9 (.2)

recently experienced a neural insult. Using repeated measures ANOVA, it was found that the latency scores were significantly higher in the post-KA trial compared to pre-injury (127 s post-KA vs. 77 s pre-injection – $F_{1,33}=5.05$, $p=0.032$), indicating a decrease in memory for the task following KA exposure. The GLM model performed in the post-injection period, however, indicated a significant interaction between treatment (KA vs. saline) and reproductive status (maternal vs. virgin) ($F_{1,31}=5.65$, $p=0.029$): planned group comparisons revealed that post-injection latencies were not significantly different between saline-virgins (143 s) and KA-injected virgins (134 s); KA-injected mothers (123 s), however, exhibited significantly slower latencies approaching the previously baited well in the probe task than saline mothers (78 s). See Fig. 2.

The second measure, time spent in proximity to the previously baited well, was not significantly different between the pre- and post-injection trials (repeated measures ANOVA: $F_{1,33}=0.04$, $p=0.84$). The GLM model performed in the post-injection period, however, indicated a significant interaction effect between treatment (KA vs. saline) and reproductive status (maternal vs. virgin) ($F_{1,31}=4.68$; $p=0.051$). Planned comparison LSD tests indicated that saline maternal rats spent significantly more time near the previously baited well than the saline virgins (19.2 s and 1.9 s, respectively); alternatively, no difference was observed in KA-injected maternal and virgin animals (7.3 vs. 2.5 s, respectively) (Fig. 3). Further, the saline maternal rats were significantly different than both
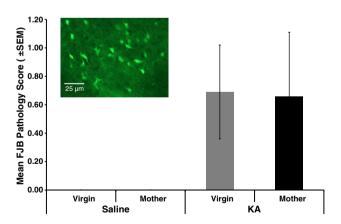

Fig. 1 – Cell death as measured by Fluoro-Jade B (FJB) staining in hippocampal subregion CA3 of virgin and maternal rats 16 h after injection. Saline injections had no effect on cell death whereas both reproductive groups exhibited neural degeneration following kainic acid (KA) injections; reproductive experience, however, failed to affect neurodegenerative processes. Photomicrograph insert of KA-reactive cells (40×).
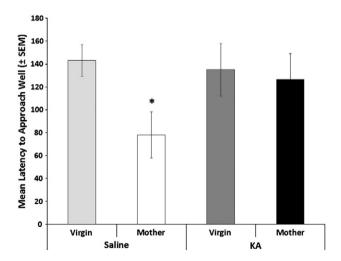

Fig. 2 – Twenty-four hours following KA injections, all animals were subjected to a second probe test in the dry-land maze (DLM) to gauge the spatial memory abilities of animals that had recently experienced a neural insult. A significant interaction between treatment (KA vs. saline) and reproductive status (maternal vs. virgin) was found. *p<0.05.
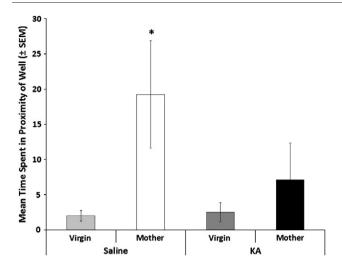
Fig. 3 – Time spent in proximity to previously baited well (probe trial) 24 h after injection with either saline or kainic acid (KA). A significant interaction between treatment (KA *vs.* saline) and reproductive status (maternal *vs.* virgin) was found. *p<0.05.
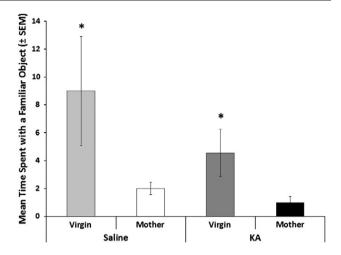

Fig. 4 – Forty-eight hours after injection, all animals were subjected to a novel object preference (NOP) test. A significant main effect of reproductive status was found: mothers spent less time with the familiar object than virgins. *p<0.05.

saline virgin and KA virgin groups yet the difference between saline maternal and KA maternal groups failed to reach statistical significance.

2.4.   Non-spatial memory task at 48 h

In the novel object preference (NOP) task, no significant difference was observed between the pre- and post-injection trials (repeated measures ANOVA: $F_{1,33}=0.02$, $p=0.91$). After the rats were allowed to recover from KA injections for 48 h, each animal was given a second NOP assessment. GLM analysis indicated that in the post-injection period, no significant interaction effect between treatment (KA *vs.* saline) and reproductive status (maternal *vs.* virgin) ($F_{1,31}=0.56$; $p=0.46$) was found. However, a significant main effect of reproductive status was observed ($F_{1,31}=7.04$; $p=0.017$): mothers spent less time with the familiar object than virgins (Fig. 4), regardless of neural insult condition.

2.5.   Evaluation of reactive gliosis at 48 h

GLM assessments of GFAP immunoreactivity revealed significant main effects for both treatment (KA *vs.* saline) and reproductive status (maternal *vs.* virgin) throughout the hippocampal fissure medial (HFm—Fig. 5A), hippocampal fissure lateral (HFl—Fig. 5B), and CA3 (Fig. 5C).

Analysis of the HFm indicated a main effect for treatment as KA-injected animals had more GFAP-IR than saline injected animals; in this case a three-fold increase was observed in KA treated animals ($F_{1,31}=11.74$; $p=0.002$). Further, a main effect was observed for parity; specifically, maternal rats had significantly less gliosis than virgins ($F_{1,31}=9.46$; $p=0.004$). More importantly, a significant interaction between reproductive status and KA treatment was observed ($F_{1,31}=3.19$; $p=0.014$). Planned comparisons revealed no differences in saline animals; however, virgin KA-treated animals exhibited more

GFAP-IR than maternal KA-treated rats. In the HFl, significant effects of both reproduction ($F_{1,31}=5.72$; $p=0.023$) and drug treatment were also observed ($F_{1,31}=15.01$; $p<0.01$), with the effects following the same direction as the HFm data. However, no significant interaction effect was found in this brain area ($F_{1,31}=0.04$; $p=0.84$).

Focusing in CA3, main effects of reproduction ($F_{1,31}=4.19$; $p=0.049$) and drug treatment ($F_{1,31}=6.71$; $p=0.014$) were observed; once again, maternal animals exhibited lower basal immunoreactivity levels than virgins and KA injected animals exhibited elevated immunoreactivity compared to saline injected animals. As observed in the HFl, no significant interaction effect between treatment (KA *vs.* saline) and reproductive status (maternal *vs.* virgin) was observed in this brain area ($F_{1,31}=0.21$; $p=0.65$).

3.   Discussion

The process of becoming a mother—becoming pregnant, giving birth, and caring for young—has been shown to have a long-lasting impact on learning and memory abilities, as well as various neuroplasticity measures associated with the hippocampus (Gatewood et al., 2005; Kinsley et al., 2006; Lambert et al., 2005). These neuronal changes are likely an evolutionary adaptation that facilitates the mother's ability to successfully protect, feed, and otherwise care for her offspring (Kinsley et al., 2008). Our findings in the current study extend this interpretation by suggesting that the restructuring of the maternal brain, likely through the effects of pregnancy hormones such as progesterone and estrogen, may also lead to a buffering effect in response to a neural insult, thereby adding another layer of protection to the maternal arsenal.

The mechanisms, therefore, underlying the natural maternal experience may activate various processes that contribute to enduring neuroprotection for the maternal animal when faced with future neural challenges and insults. Such change could provide an opportunity to extend or complete the
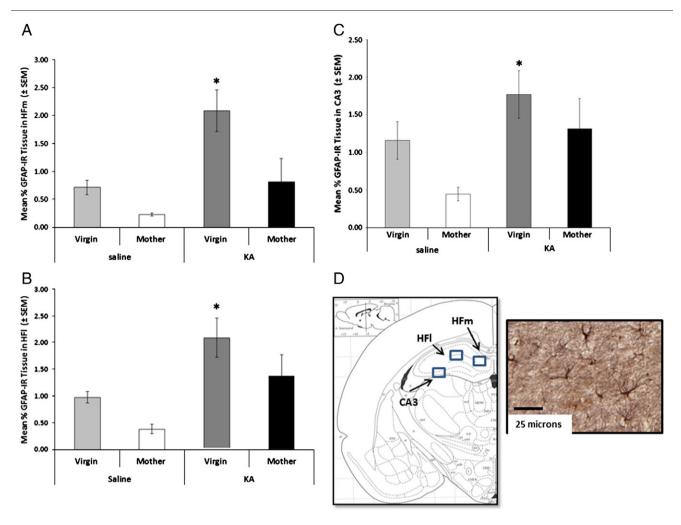
Fig. 5 – GFAP immunoreactivity revealed significant main effects for both treatment (KA *vs.* saline) and reproductive status (maternal *vs.* virgin) throughout the hippocampal fissure medial (HFm) (A), hippocampal fissure lateral (HFl) (B), and the CA3 (C). Further, a significant interaction was found in the HFm. Areas quantified in the hippocampus [hippocampal fissure medial (HFm) and lateral (HFl) as well as CA3; Paxinos and Watson, 1986], as well as a photomicrograph of GFAP immunoreactive cells in the CA3 areas are depicted in D. *p < 0.05.

requisite maternal care toward vulnerable young, despite an otherwise debilitating injury to the mother. Accordingly, this proximal neuroprotection is similarly adaptive and of benefit to both offspring and maternal genomes.

Maternal animals, for example, have been shown to be resilient against the neural challenges accompanying aging (Gatewood et al., 2005). Previous studies on the effects of KA on the brains of lactating female rats have reached similar conclusions. Lactation, for example, is considered a natural model for neuroprotection, since it effectively prevents acute and chronic cell damage of the hippocampus induced by excitotoxicity (Morales, 2011; Vanoye-Carlo et al., 2008).

Although KA proved to be a potent neurotoxin sufficient to induce pathology in the hippocampus of both maternal and virgin rats as evidenced by an increase in degenerating cells 16 h following treatment, no effects of reproductive experience were observed at this early time point. Added to equal intensities of seizures in both groups, it appeared that the maternal and virgin animals experienced similar degrees of neural insult or, perhaps, primary injuries. Because increased

differences have been observed when animals are examined at longer time points from the KA injection (e.g., up to 72 h), however, it is possible that differential damage would have been observed at a later time (Cabrera et al., 2009). In contrast to Experiment I, the second experiment focused on behavioral and neural effects of KA exposure in rats previously trained in both a hippocampal-dependent spatial task and a recognition task that is less dependent on hippocampal functioning (Barker et al., 2007). At the time of the recovery assessments, i.e., 24 h post insult, the recovery process has likely commenced in the brains of both mother and virgin rats experiencing a neural insult. One component of neural recovery includes the infiltration of glial cells to remove degenerated cells and facilitate recovery processes (Benkovic et al., 2004). Providing strong evidence of the critical role of glial cells in the recovery process, increased vulnerability to kainate-induced damage was observed in GFAP knockout mice (Otani et al., 2006).

Focusing more closely on the results of Experiment II, as soon as the animals experiencing kainate-induced brain

injuries were able to move capably after the KA exposure, they were re-exposed to the dry land maze spatial memory task to assess the interruption of previously learned information in these animals. Results confirmed enhanced spatial memory effects previously found in maternal animals in our laboratory (Kinsley et al., 1999). Considering the latency to approach the previously baited well (a measure of memory strength), the saline maternal animals approached the previously baited well significantly faster than all other groups. Focusing, however, on the amount of time spent in proximity to the previously baited well (another measure of memory strength), both of the virgin groups exhibited a weaker memory of the previously baited well than the saline maternal rats. On the contrary, although the KA maternal rats spent less time in proximity to the previously baited well than the saline maternal rats, no significant difference was observed.

At 48 h after neurotoxic insult, the protective effect of reproductive experience was also evident in the novel object task. In this task, maternal experience enhanced memory of the familiar object—a main effect that was not diluted by kainate-induced brain damage; in fact, although not significantly different, the KA mothers performed better than the saline mothers. Interestingly, regardless of treatment condition, the virgin animals spent more time investigating the familiar object. The convergence of these behavioral findings suggests a heightened resilience against kainate-induced neural insult in maternal animals, a finding that deserves further investigation. These buffering effects could be due to either diminished cellular degeneration or enhanced facilitation of recovery—or some combination of these variables.

Two days post-insult, the brains of virgins exposed to KA exhibited higher levels of glial fibrillary protein (GFAP) immunoreactivity than both saline virgin and maternal rats, significantly so in the medial hippocampal fissure. Thus, these data confirm that KA had no impact on maternal animals but increased GFAP-IR in virgins, again suggesting that reproductive experience affected another level of regulation, and rendered the KA maternal rats indistinguishable from their healthy rodent counterparts. One possible explanation is that more efficient maintenance of hippocampal neurons in maternal rats via either enhanced neuroplasticity (Vanoye-Carlo et al., 2008) or, alternatively, progesterone exposure (Stein, 2008) provided the necessary buffer for the maternal animals, during both primary and secondary recovery phases. Further, these data suggest that, even at baseline, maternal rats have less GFAP-IR than virgins. If less GFAP-IR in control mothers is indeed an indicator of decreased neuronal death (or perhaps improved or more efficient neural maintenance), then it is reasonable to conclude that some neuroendocrine component of the reproductive process (e.g., altered progesterone exposure) is maintaining a neuroprotective process such as facilitating myelination, increasing mitochondrial activity, etc. in those animals (Irwin et al., 2008; Schumacher et al., 2007). Considering the complex role of astrocytes in secondary injury and recovery, it is also possible that the higher levels in KA virgins reflected more extensive damage, perhaps resulting from a less efficient inflammatory response (Chen and Swanson, 2003). Although reactive astrogliosis begins within a few hours of a brain injury, the 16 h time point in Experiment I may have preceded the peak activation of astrocytes

(Ridet et al., 1997). Consequently, it would be interesting to investigate various time points to determine, in accordance with behavioral data and FJB immunoreactivity, how the trajectory of reactive astrogliosis differs between virgin and maternal animals.

Focusing on progesterone, once a neural insult occurs, other beneficial effects such as inflammation suppression (Gibson et al., 2005) may provide an additional benefit to maternal neurons as compared to those in a virgin rat. Such neuronal maintenance could potentially facilitate recovery in maternal brains responding to neural insults. Beyond glial activity in the brain, the behavioral differences observed in the memory tasks reveal both adaptive and relevant responses in maternal rats; animals previously exposed to the enriching and challenging conditions associated with the full maternal experience.

A unique aspect of the current study is the suggestion that the neuroprotective effects of reproductive experience on neural insults extend to long-lasting post-lactational stages of the female's life. Past research has clearly indicated neuroprotection during lactation (Morales, 2011; Vanoye-Carlo et al., 2008); however, the current study suggests that this benefit is long-lasting. Additionally, the current study suggests that, although the initial impact of KA had similar effects in maternal and virgin animals (evidenced by similar stages of seizures and no differences in GFAP-immunoreactivity early in the recovery process in Experiment I), the maternal animals demonstrated an enhanced retention of spatial memory and formation of new memories of object familiarity (in Experiment II). Neural mechanisms underlying decreased signs of aging in maternal rats (Gatewood et al., 2005) may also influence the more efficient recovery from neural insults. As suggested in this study, behavioral recovery is also an important variable to consider in conjunction with neural mechanisms to determine functional recovery in animal models of acute brain injuries. In addition to the previous findings of multiple neurobiological adaptations in maternal animals, the current study suggests that prolonged neuroprotection is also a consequence of reproductive experience.

Because the emphasis of the current study was to explore the possibility of long-term protective effects in animals exposed to the full maternal experience, a limitation is the lack of specific information about individual relevant variables comprising the maternal experience including effects of progesterone, estrogen, oxytocin, pup exposure, stress hormones, etc. Identifying the specific mechanisms of the proposed neuroprotection is indeed a challenge considering the plethora of environmental, behavioral and neural events accompanying pregnancy, parturition, and lactation. Whether the neuroprotective effects are due to the effects of progesterone during pregnancy as discussed above, the direct exposure to offspring, or the complex environment accompanying caretaking strategies required to raise offspring, the current data indicate that maternal animals also exhibit resiliency in the face of a neural insult. Consequently, further investigations of neural degeneration and recovery in the maternal brain may lead to the identification of adaptive and efficient responses to neural insults, potentially yielding better treatments or strategies for recovery from acute brain injuries and other neurodegenerative conditions. Nevertheless, it is apparent that maternal

experience, broadly defined, contributes to a more plastic and re-sponsive brain, one with multiple layers of redundancy and potential.

4.   Experimental procedures

4.1.   Animals

This study consisted of two experiments. In Experiment I, animals were injected with kainic acid (KA) and sacrificed 16 h later to determine the impact of reproductive experience on the neurological impact of the neural insult early in the recovery process. In Experiment II, animals were trained and tested on behavioral tests, injected with KA or saline, and then tested again on behavioral tasks to assess post-trauma spatial and non-spatial memory abilities.

In Experiment I, 16 rats (8 multiparous; 8 virgin) were used. In Experiment II, 34 animals were used (9 saline multiparous; 9 KA multiparous; 9 saline virgins; 7 KA virgins). All animals were approximately 5 month old Long–Evans rats (Harlan Inc.; Indianapolis, IN); reproductive females had previously had two litters at similar ages, with the experiment beginning approximately 23 days following the last lactation period. Following the weaning of their pups at 21 days of age, maternal animals were assigned to either a KA or saline group along with age-matched virgins. All animals were individually housed in standard laboratory conditions with *ad libitum* food and water on a 12/12 light/dark cycle. In Experiment II, prior to training, rats were given 3 weeks to habituate to the laboratory (see Fig. 6 for timeline of methodological events during Exp. II). Two days prior to training, rats were subjected to mild food restriction and maintained at approximately 90% of initial body weight. All procedures were conducted in accordance with Randolph-Macon College IACUC.

4.2.   Neurotoxic insult: kainic acid (KA) treatment and seizure scoring

In both Experiments I and II, rats were given a single intraperitoneal injection of kainic acid (Sigma) at a relatively low dosage (8 mg/kg dissolved in sterile saline solution). On the day of injections, the weight of the animals ranged from 240 to 313 g. This dose was much lower than doses used in previous mouse studies (35 mg/kg; Benkovic et al., 2004) but comparable to rodent studies investigating the effect of reproductive experience on neural insult (5–7.5 mg/kg; Vanoye-Carlo et al., 2008). Control rats were injected with comparable volumes of sterile saline. All injections were administered between 1000 and

1200 h to minimize potential interactions with circadian patterns of endogenous hormones and neurotransmitters. A maximum of 12 animals (6 KA and 6 Saline) were subjected to injections per day to allow for adequate observation time.

Following injections, animals in both groups were continuously observed for 4 h. In addition to species typical behaviors, seizures were observed and scored according to the Racine Scale (Racine, 1972). After 4 h, animals were returned to their original housing conditions, with moistened food supplied during their continued recovery from the seizures.

4.3.   Assessment of spatial memory: dry land maze (DLM)

Research using the DLM, a dry land adaptation of the Morris Water Maze, has previously indicated that maternal experience enhances spatial memory in rats when compared to nulliparous animals (Kinsley et al., 1999). As previously described (Lambert et al., 2005) rats were initially exposed to a 3 day training period during which the rats became familiar with the apparatus and location of baited wells. During each day of training, the number of baited wells was systematically reduced from eight, then four and, on the third day, two baited wells. On the fourth day of testing, the Initial Acquisition Trial required the animals to locate the final single baited well—this food well served as the baited well throughout the remainder of testing. For the subsequent day of testing, latency to locate the baited well was recorded for three 3-minute test trials with 1-minute inter-trial intervals. On Day 6 of the assessment period, animals were exposed to a Probe test in which no well was baited; in this trial latency to approach the previously baited well and duration of time spent in proximity to the well were recorded as measures of spatial memory in the absence of any food-related cues. The specific nature of the DLM apparatus has been described elsewhere (Franssen et al., 2011).

One day following injections, animals in Experiment II completed a 3-minute DLM 'booster' trial in which the same single well was baited, followed by an additional 3-minute probe trial as described above. Both pre- and post-injection DLM assessments were conducted between 10:00 and 12:00 h.

4.4.   Assessment of non-spatial memory: novel object preference task

Both before and after KA injections, rats' memory for a familiar object was assessed in the novel object preference (NOP) task. During the NOP task, animals were placed in the open field apparatus (122 cm×91 cm×51 cm box with bedding) containing two identical objects (two red balls) for a 2-minute period.
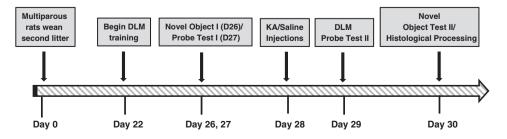

Fig. 6 – Timeline for maternal animals in Experiment II.

Following this initial exploration, animals were removed from the open field apparatus and placed back into their home cages for 5 min. Animals were then placed back into the arena for a 2-minute test period with two sample objects, one identical to the object utilized in the first exploration (red ball) and the other a novel object (plastic toy). Time spent exploring each object, frequency of contacts with each object, duration of proximity (defined as the animal placing its head or forelimbs in a 1 square radius around object) as well as latency to approach the objects were recorded. These behaviors were used to calculate a measurement of preference for both the original (ball) and novel (toy) objects as described in the Results section. The pre-injection novel object preference task was administered during the afternoon of the last day of DLM testing and, post-injection, 2 days following KA and saline exposure at the same time (with new stimuli). Both pre- and post-NOP tasks were conducted between 13:00 and 15:00 h.

4.5. Histology and neuroimaging

Fixed brain tissue for immunocytochemical analysis was obtained following transcardial perfusion of the rats. Each rat was individually placed into an airtight chamber with 1 mL of Halothane gas (Sigma-Aldrich, Co; St Louis, MO) until respiratory rate slowed and animals were nonresponsive. Rats were then given an intraperitoneal injection of 0.2 mL sodium pentabarbitol (Fort Dodge Animal Health; Fort Dodge, IA) and transcardially perfused at 40 mL/min using a MasterFlex L/S perfusion pump (Cole-Parmer) with 100 mL phosphate-buffered saline solution (PBS) followed by 100 mL 4% paraformaldehyde solution. Brains were extracted and post-fixed in 4% paraformaldehyde overnight at 4 °C, then transferred to a 10% sucrose solution for 24 h at 4 °C, and were then stored in 20% sucrose solution at 4 °C until sectioning (at least 24 h).

Brains were sectioned via cryostat (Microm HM525) at 40 μm beginning at plate 54 (Paxinos and Watson, 1986), then assessed for cell death using Fluoro-Jade B (FJ-B) at 16 h post injection. Fluoro-Jade B is an anionic fluorescein derivative that has been utilized as a marker for neurodegeneration (Benkovic et al., 2004; Schmued and Hopkins, 2000) and was used to confirm the effectiveness of the selected dose of KA as a neurotoxin in this particular animal model. Based on Benkovic et al. (2004), sections were incubated in 0.0001% FJ-B dissolved in 0.1% acetic acid for 10–15 min, rinsed three times for 1 min each in distilled water, dehydrated through ethanol and covered with a non-aqueous, low fluorescence styrene-based mounting media (DPX). Tissue was examined using a Zeiss Axioskop with a FITC cube. FJB reactive cells were considered markers for neurodegenerating cells; using low magnification (10×), the entire CA3 was assessed. FJB stained tissue was quantified using a semi-quantitative analysis of degeneration consisting of a four point rating scale in which '0' indicates no pathology; '1' indicates little degeneration, '2' moderate degeneration, and '3' major degeneration (Benkovic et al., 2004).

Sections were also stained for activity of astrocytes using glial fibrillary acidic protein (GFAP) antibody in both Experiments I and II, i.e., 16 h and 48 h post-injection. Astrocytes have been implicated in neural repair following brain injuries (Sofroniew,

2005). Immunohistochemistry was performed for DAB visualization of the GFAP antibody. All brain tissue was incubated at room temperature overnight in rabbit anti-GFAP primary antibody (1:40,000 ImmunoStar, Inc., Hudson, WI) in phosphate buffered saline (PBS), washed, and incubated for 60 min in goat anti-rabbit biotinylated secondary antibody in PBS (1:200;Vector Laboratories, Burlingame, CA). GFAP proteins were visualized using Vector avidin–biotin complex and 3-3′ diaminobenzidine tetrahydrochloride (ABC and DAB kits; Vector, Burlingame, CA). GFAP-immunoreactive tissue was analyzed using thresholding with bright field microscopy (Bioquant Life Sciences, Nashville TN). A 640×480 visual field was used to threshold for GFAP-positive cells at a magnification of 40× to determine the percentage of the visual field occupied by GFAP tissue.

For both GFAP and FJB histological assessments, four brain sections were quantified to determine the overall average score for each particular animal. All sections were quantified by an observer blind to the experimental conditions; however, prior to the histological quantifications, the blind observer worked with a second observer to fine-tune rating consistency.

4.6. Statistical analysis

All behavioral data (number of lines crossed, latency to find previously baited well, time spent with the novel object, etc.) and levels of FJB and GFAP immunoreactivity were quantified and compiled in SPSS (SPSS 16.0, Chicago, IL). Repeated measures ANOVAs were used to assess the differences between pre- and post-injection. General Linear Models (GLMs) were computed to assess the effects of reproductive experience (mother *vs.* virgin) and treatment (KA *vs.* saline). GLM analysis is a flexible statistical procedure that enables researchers to assess hypotheses suited for either ANOVA or regression analysis. Specifically, GLM assesses the value of a dependent variable based on its relationships to categorical (factors) and scale (covariates) predictors. In its general form it can be written as $Y=b_0+bX+\varepsilon$, where Y is the dependent variable and X represents a set of independent predictors (either categorical or scale; Warner, 2007). In the current study, predictors were reproductive experience (2 levels) and treatment (2 levels). Significance was set for $\alpha=0.05$. In the case of a significant interaction, a Least Significant Difference (LSD) test was run as a planned comparison determination of individual group means; again significance was set for $\alpha=0.05$.

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