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Fetal Stress and Neurogenesis in *Thamnophis sirtalis parietalis*

by

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Introduction

Maternal Stress

The question of whether and how stress impacts brain development is of great interest to developmental and biomedical researchers, as well as to the general public. Particularly relevant to those interested in the study of disease prevention and the study of conservation, there is evidence that prenatal stress hormone exposure due to stressors experienced by a pregnant mother can have a negative impact on fetal development in-utero that continues to affect development post-parturition and across the life span (McCormick et al. 1995; Meaney et al. 2007; de Vries et al. 2007), including effects on human intellectual and language function that become evident in toddlerhood (LaPlante et al. 2004). A stressful maternal environment could, therefore, predispose offspring to health or behavioral problems, many of which might not become apparent until later in the life span. The effects of environmental stressors thus have the potential to impact species' reproductive fitness as well as individual survival. For example, if brain development is affected in ways that produce maladaptive behaviors, individual members of a species that is experiencing stress from habitat reduction may have reduced fitness due to the effects of maternal stress, on top of survival pressures due to reduced or degraded habitat for foraging, mating, and raising young of their own.

One of the primary physiological hallmarks of stress that researchers can easily examine are glucocorticoids. Glucocorticoids are released from the adrenal

glands and activate energy mobilization in the form of glucose when an animal is under acute stress, as well as having significant impact on the metabolism of lipids. The immediate physiological response to elevated blood glucocorticoids includes rapid heartbeat, elevated skin temperature, and inhibited digestion. There is significant evidence that repeated or chronic exposure to high levels of glucocorticoids can result in damage to an organism's brain (Gray et al. 2013), particularly in a developing fetus. Synthetic glucocorticoids have also been used for decades to halt preterm labor in human mothers, with the result being significant behavioral developmental differences in babies born at full-term after this treatment, as compared to full-term babies whose mothers were not treated with synthetic glucocorticoids (Davis et al. 2013). Hence, we have ample evidence that leads us to expect differences in physical brain development, but for obvious reasons, it is extremely difficult to examine the physical structure of the brain in human children. Therefore, animal models are essential for expanding our knowledge in this area.

Brain development and neurogenesis

Little is yet known about normal development of the brain, let alone abnormal development, in most branches of the animal kingdom, and research so far has been conducted almost exclusively using mammalian model organisms. The red-sided garter snake, *Thamnophis sirtalis parietalis*, may tell us a great deal about

understanding this process from a comparative evolutionary perspective.

Neurogenesis is an important part of normal brain development in young animals; new neurons must proliferate and migrate to regions that are important for tasks that enable survival, such as locating and capturing food. Neurogenesis may also play a role in neuroplasticity, such as in healing from brain injury or mapping new territories (Taupin, 2006), and looking at neurogenesis patterns in postnatal brain development in reptiles may help us to also better understand these processes. While it is generally understood to be important, as brain functions tend to be both energetically expensive and highly evolutionarily conserved, the evolutionary and functional significance of postnatal and adult neurogenesis is not well understood.

This research seeks to shed light on this process by looking at neurogenesis in the nucleus sphericus, which is the reptile homologue of the mammalian amygdala. This region of the brain plays a role in regulating social behaviors, including reproduction. The nucleus sphericus is also a major secondary vomeronasal region and therefore important in the processing of odor signals (Lanuza & Halpern, 1997).

It is likely that stress hormones play a role in brain development and neurogenesis, and in order to better understand the effects of stress in prenatal development in ways that are relevant to human physiology, it is helpful to be able to make direct comparisons across genera using animal models in which young are

gestated internal to the mother, with a placenta or placenta-like structure to act as an interface between her vascular system and that of her young. Garter snakes offer us one such animal model system.

Natural history

Other than mammals, snakes are the only widespread group of animals in which many species are viviparous, giving live birth to live young that are gestated within the mother. This is a helpful analogue to mammalian gestation, in terms of making more direct comparisons of the effects of maternal stress. Because many species of snakes are readily kept in captivity, have large litter sizes, and give birth to live young, a compelling argument can be made for the value of using snakes as an animal model in developmental research (Guerreiro & Duboule, 2014). One important aspect to consider is that including reptiles in our repertoire of animal models enables us to better understand whether developmental responses to stress are mammal-specific, or are evolutionarily conserved across phyla.

The red-sided garter snake has many advantages as an animal model for this type of research. One advantage is that it is viviparous and proto-placental, giving birth to live young which are gestated within the mother and hence exposed to her glucocorticoid levels. Another advantage is that red sided garter snakes are both numerous and widespread, and it is possible to obtain a large number of animals for experimental purposes without compromising natural populations. For experimental purposes, the primary glucocorticoid in reptiles, corticosterone

Another chemical called metyrapone can be used to suppress glucocorticoid synthesis, allowing us to compare development among treatment groups. It is expected that there will be significant differences in cell proliferation in the brain of juvenile *Thamnophis sirtalis parietalis* whose mothers were treated with metyrapone or corticosterone, as compared to those who were not.

Methods and Materials

These experiments were conducted in the laboratory at Portland State University with animals collected from a field site in Inwood, Manitoba, Canada. All protocols were approved by PSU's Institutional Animal Care and Use Committee and performed under the authority of a Manitoba Department of Conservation Scientific Wildlife Collecting permit.

The purpose of this study was to investigate the effects of elevated prenatal glucocorticoid levels on neurogenesis in the red-sided garter snake, *Thamnophis sirtalis parietalis*, as well as to collect data on normal brain development in this species. To this end, we collected 75 recently-mated female snakes during the spring breeding season and transported them to our laboratory at Portland State University.

Animal Husbandry

Animals were housed individually in 40-liter aquaria under summer-like conditions (16 h: 8 h LD photoperiod, 24 C: 18 C thermoperiod). Snakes were fed twice weekly with vitamin-fortified fish and earthworms; water was provided ad libitum. Females gave birth to 182 live neonates from August 18 - September 3, of which 113 survived to 15 weeks post-parturition. Juveniles were fed earthworms and chopped raw fish 2x/week. Water was provided ad libitum.

Maternal hormone treatment

Female snakes were implanted with capsules releasing either corticosterone (n=25), metyrapone (n=25), or no hormone (blank capsule, n=25). Metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) inhibits 11 β -hydroxylase, an enzyme necessary for the synthesis of glucocorticoids in vertebrates (Sampath-Kumar, 1997), and previous investigators have used it effectively to inhibit corticosterone and cortisol synthesis (Bordone et al., 1997; Lutterschmidt and Maine, 2014; Masid-de-Brito et al., 2014; Moore and Miller, 1984; Rodela et al., 2012; Thaker et al., 2010). Metyrapone has no other effects as far as is currently known.

The treatment implant capsules were made as in Lutterschmidt and Maine (2014), using a two-part prosthetic silicone elastomer (item A-101/6382; Factor II, Inc., Lakeside, AZ) along with approximately 5 μ g food dye to ensure homogeneity of the mixture. The silicone elastomer mixtures were extruded through a 10 cm³ syringe into heat shrink tubing that had been molded to fit the plunger of a syringe-

cured, removed from the tubing, and cut into individual 1.75mm x 5mm implants.

CORT capsules:

For the CORT treatment, 134.8mg corticosterone (Sigma-Aldrich, St. Louis, Mo., USA) was mixed with 3g silicone elastomer and 50 drops (approximately 1.25g) catalyst, for a result of approximately 43 μ g CORT/mg elastomer mixture. Each corticosterone implant contained approximately 992 μ g per capsule, and each female snake received two capsules for a total of 1.984mg. The capsules were replaced every 21 days, for a dose rate of 94.48 μ g/day.

Metyrapone capsules:

For the metyrapone treatment, 1074.8mg metyrapone was mixed with 3g silicone elastomer and 50 drops (approximately 1.25g) catalyst, for a result of approximately 202 μ g metyrapone/mg elastomer mixture. Each metyrapone implant contained approximately 5.9mg per capsule. The capsules were replaced every 21 days, for a dose rate of 281 μ g/day.

Control capsules:

For the control capsules, 3g silicone elastomer was mixed with 50 drops (approximately 1.25g) catalyst, for an inert capsule.

The hormone implants were placed subcutaneously on the lateral side of each snake using a syringe-style planter with a 12-ga needle. The injection site was then sealed using tissue glue. Implants were placed June 11th-12th, and replaced at 21 days (July 5th-6th) and 42 days (July 26th), then removed Aug 17th.

The animals were checked daily to ensure integrity of the implants; there was no implant loss.

Bromodeoxyuridine labeling

To determine if maternal glucocorticoid treatment influences brain development in neonates, we treated juvenile snakes 15 weeks after birth with 5'-bromodeoxyuridine (BrdU; 100 mg/kg, item B5002; Sigma-Aldrich, St. Louis, Mo., USA), a thymidine analog that is incorporated into the DNA of newly proliferating cells. The BrdU was diluted with 25% DMSO in reptile Ringer's solution to a concentration of 20 mg/ml. As in Almlı and Wilczynski 2009 and Maine et al. 2014, each snake received 2 pulse injections intraperitoneally; the total injection volume for each snake was 1% of body mass. Five days post-treatment, juvenile snakes were euthanized with an overdose of sodium pentobarbital, and the brains collected for immunohistochemistry.

Tissue preparation

The brains of the juvenile snakes were fixed via immersion in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, at 4 ° C for 18 hours. They were then transferred to 0.1 M phosphate buffer and stored at 4 ° C until sectioning.

Brains were prepared for sectioning by immersing them in 30% sucrose solution until saturated, after which they were frozen at -15 ° C and sectioned on a cryostat (Leica 3050S) into 4 alternate series of 25- μ m coronal slices. The slices

were thaw-mounted onto slides (Fisherbrand Superfrost Plus) coated with a gelatin substrate. These brain tissues were then stored at -20°C until they were processed for BrdU immunohistochemistry.

Immunohistochemistry

Differences in cell proliferation among groups were assessed via immunohistochemistry for BrdU-positive cell nuclei. All slides were processed in a single assay. We followed the protocols described by Almlı and Wilczynski 2009 and Maine et al. 2014. Briefly, slides were defrosted and dried on a slide warmer at 50°C for 40 min, after which the tissues were outlined with a hydrophobic barrier (Liquid Blocker – Super Pap Pen; Electron Microscopy Sciences, Hatfield, Pa., USA). Tissues were then briefly incubated in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) for 5 min for better adherence of tissue to slides. Slides were washed 3 times for 5 min per wash with 0.1 M PBS, followed by fixative neutralization with 0.1% sodium borohydride (pH 8.5) for 20 min. The slides were washed again in PBS (3×5 min). DNA denaturation and antigen retrieval were performed by incubating the slides in 2 N HCl in PBS at 37°C for 30 min. The slides were neutralized by washing with 0.1 M boric acid buffer (pH 8.5; 2×5 min) followed by PBS (3×5 min). Endogenous peroxidase activity was quenched with 1% hydrogen peroxide in 0.1 M PBS for 30 min. The slides were washed in PBS (2×5 min) followed by PBS with 0.3% Triton X (PBS-T; 1×5 min) and then incubated for 60 min in PBS-T containing 10% normal goat serum (item G676, Sigma-Aldrich

Co.) to reduce nonspecific binding. BrdU immunoreactivity was examined using a rat anti-BrdU antiserum (item OBT0030; Accurate Chemical, Westbury, N.Y., USA) at a dilution of 1: 5,000 in PBS-T containing 10% normal goat serum (item G676, Sigma-Aldrich Co.). Sections were incubated with the primary antibody for 48 h at 4 ° C in a humid chamber. The slides were then washed in PBS (2 × 5 min) followed by PBS-T (1 × 5 min) and the primary antibody signal amplified by incubation for 60 min with biotinylated goat anti-rat secondary antibody (item BA-9400; Vector Labs) diluted 1: 400 in 0.1 M PBS-T. The slides were washed in PBS (2 × 5 min) followed by PBS-T (1 × 5 min). Tissues were incubated for 60 min in avidin conjugated to horseradish peroxidase (Elite ABC peroxidase kit; Vector Labs) and rinsed in PBS (2 × 5 min) followed by PBS-T (1 × 5 min). Primary antibody binding was visualized using 0.25 mg/ml diaminobenzidine (item 0430-5G; BioExpress, Kaysville, Utah, USA) in 0.2% hydrogen peroxide in 0.05 M Tris-HCl buffer (pH 7.2). The reaction was terminated by immersion in nanopure H₂O (3 × 5 min). Tissues were counterstained for one minute in haematoxylin to identify regions of interest, then dehydrated in a graded ethanol series, cleared with Citrisolv (Fisher Scientific, Pittsburgh, Pa., USA), and covered with Permount and coverslips.

Immunoreactive cell counting

Newly-synthesized brain cells were identified as BrdU-labeled cell nuclei.
The

sphericus was determined to quantify neurogenesis. The count was performed at 200x and repeated at 400x using an Olympus BX40 microscope with a QIClick digital camera and QImaging software (QImaging; Surrey, B.C., Canada). Proliferating cells in the nucleus sphericus (NS) were counted manually in one hemisphere of the brain in each animal, with animals coded so as to ensure that the observer was blind to the treatment group of each animal.

Statistical Analysis

For this pilot study, 30 juvenile individuals, matched by sex, were selected randomly from 14 litters. Differences among groups were analyzed using natural log-transformed data in a two-way ANOVA with sex and maternal hormone treatment as between-subjects factors. Additional analysis was conducted using a Tukey's multiple comparisons test on raw cell count data, with unmatched individuals removed from the analysis.

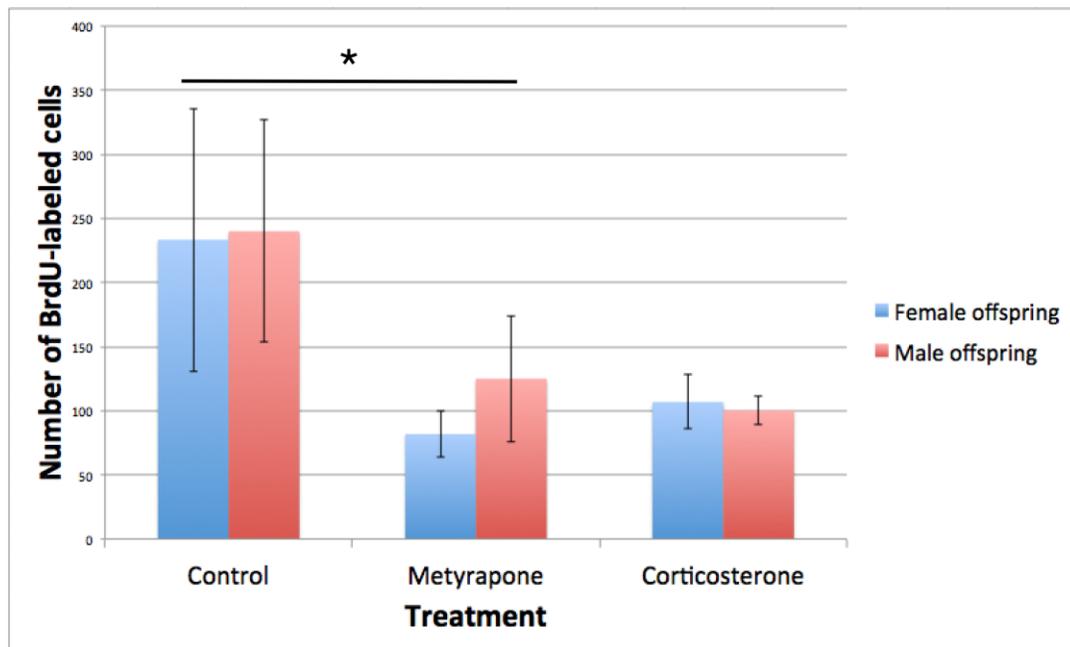
Results

Neurogenesis

Maternal hormone treatment during gestation significantly affected the number of newly-synthesized cells in the brains of juvenile red-sided garter snakes ($F = 4.143$, $df = 2$, $P = 0.028$; from a two-way ANOVA). There was not a significant effect of sex of the juvenile on responses to maternal hormone treatment ($F = 0.347$; $df = 1$; $P = 0.561$). There was no significant interaction between factors ($F = 0.133$; $df = 2$; $P = 0.876$).

A Tukey's multiple comparisons test showed that this difference was driven by a significant difference in the number of labeled cells between the control and the metyrapone treatment groups ($P = 0.024$). The difference was largely driven by the females ($P=0.081$) as compared to males ($P=0.196$), although the differences between sexes within the Metyrapone group was not statistically significant ($P=0.502$).

Fig. 1: Differences in neurogenesis between treatment groups



Discussion

Prenatal treatment with metyrapone significantly reduced the rate of postnatal neurogenesis in neonate *Thamnophis sirtalis parietalis*, as compared to the control group. Metyrapone inhibits the synthesis of endogenous corticosterone, and it is likely that decreased exposure to corticosterone during development may have altered patterns of normal brain development, seen here as reduced numbers of proliferating brain cells.

If normal brain development relies, at least in part, on optimal levels of corticosterone, then it would be reasonable to expect that brain cell proliferation in the corticosterone-treated group would also be disrupted due to exposure to unusually elevated levels of this hormone. Interestingly, although neurogenesis in the corticosterone treatment group was lower than in the control group, the difference was not statistically significant. This is most likely due to our currently low sample size of juveniles within each treated female garter snake. Another possible explanation is that organisms are known to be able to compensate for a continuously elevated level of exogenous glucocorticoids by downregulating their endogenous production, shifting their baseline of total glucocorticoids to within a normal range (Chiba et al., 2012; Meaney et al., 2013). Potential methods for circumventing this adaptation in future research is to introduce intermittent stressors or give shorter-duration corticosterone injections at random time intervals, rather than treat the animals with continuous exposure to the hormone via capsules.

As it is known that some organisms, including rats, mice, and humans, are capable of responding to continuously decreased or elevated levels of glucocorticoids during development by increasing or decreasing the expression of glucocorticoid receptors (Liu et al., 1997; Turecki & Meaney, 2016), one possible explanation for the reduced brain cell proliferation in the metyrapone treatment group is that the resulting reduction in corticosterone during prenatal development leads to neonates developing with an overabundance of glucocorticoid receptors in the brain and elsewhere, leading to unusual levels of sensitivity to their own endogenous glucocorticoids after birth. Because of physiological negative feedback loops which help to regulate glucocorticoid levels by decreasing glucocorticoid production when receptors detect the presence of that hormone, (Nelson, 2011), this sensitivity could in turn lead to suppression of corticosterone, continuing to perpetuate abnormal development postnatally.

Intriguingly, corticosterone receptors have been found to modulate the activity of other transcription factors (Ratman et al., 2013). In future studies, techniques that directly examine receptor density, such as Western blot, are suggested to address the question of whether higher expression of glucocorticoid receptors is indeed occurring in the metyrapone treatment group, and lower expression of glucocorticoid receptors occurring in the corticosterone treatment group.

Preliminary analyses using offspring sex and litter grouping as co-factors did not reveal a significant effect of these factors on cell proliferation in the brain. The lack of significant differences may be due to the small sample sizes. My future work on this project to add additional juveniles to this data set should clarify whether this is truly the case.

These results are intriguing because they contribute to the body of research that shows that disruptions in maternal glucocorticoid levels during pregnancy alter the path of brain development, in this case by suppressing proliferation of new brain cells in offspring. Such disruptions could translate into detrimental consequences to survival and reproduction for organisms affected by prenatal stress or other maternal disruptions of glucocorticoid expression. Further research into the effects of prenatal stress is urgently needed.

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