University of Nevada, Reno

The Effect of Testosterone on Neurogenesis in the Hippocampus of the Side-blotched Lizard, *Uta stansburiana*

A thesis submitted in partial fulfillment of the requirements for the degree of Bachelor's of Science in Biology and the Honors Program

by

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Abstract

The hippocampus is the area of the brain that is responsible for learning in vertebrates. Having adequate spatial learning ability is critical for survival because it enables animals to remember resource locations, find mates, and navigate. Various spatial learning demands placed on the brain through different tasks have been shown to affect hippocampal morphology. Hormones have been implicated as one potential mechanism that can affect hippocampal morphology and consequently spatial learning ability. Manipulation of testosterone has been shown to affect spatial use and home range size in side-blotch lizards (*Uta stansburiana*), known for their variation in territory size and mating strategies. However, it remains unclear if these changes were mediated through the effects of testosterone on hippocampal morphology or processes. Adult neurogenesis in the hippocampus has been previously reported to occur in the adult vertebrate brain and has been linked with spatial learning. We hypothesized that testosterone-mediated changes in spatial use strategies occur via changes in adult hippocampal neurogenesis rates. We specifically predicted that experimental increases in testosterone levels should directly affect hippocampal neurogenesis rates, yet our results indicated no testosterone related changes in neurogenesis rates.

Acknowledgements

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Introduction

The adaptive specialization hypothesis refers to specific demands placed on the brain and their effects on the morphology within corresponding or specific areas utilized to process those demands (Krebs et al. 1989; Krebs J.R. 1990; Jacobs & Spencer 1994; Jacobs 1996; Lucas et al. 2004). In terms of spatial use and learning in vertebrates, the area of the brain responsible is the hippocampus, and increased spatial demands select for a larger hippocampus (Sherry et al 1989). The hippocampus is integral for survival because it enables an animal to navigate territories, locate potential mates, and acquire food (Brennan et al. 1990; Shettleworth 1990; Godard 1991; Menzel et al. 2000). It has been shown that animals with a larger hippocampus have higher demands placed on their spatial abilities to navigate their environment for the acquisition of resources needed for survival and reproduction (Brennan et al. 1990; Shettleworth 1990). This positive relationship has been demonstrated comparing variation in the different aspects of hippocampal morphology and increased spatial use patterns employed among a variety of species (Rehkämper et al. 1988; Krebs et al. 1989; Sherry et al. 1989).

The aspects of hippocampal morphology and processes that have shown the effects of increased spatial demands include: hippocampal volume, neuron count, and the rate of adult neurogenesis. Changes in hippocampal morphology affect certain types of animals more than others due to a higher demand in spatial use in order to survive in a particular environment. The animals with higher demands for spatial use are often animals that must navigate their environment for foraging (Day et al. 1999). Animals that store or cache food and animals that have a large home territory to defend are examples of animals that fit this type (Pravosudov & Clayton 2002; Roth & Pravosudov

2008). Conversely, animals that do not have to employ these methods for survival do not have modified hippocampal regions.

Although the relationship between hippocampal morphology and increased demands through spatial use has been demonstrated in numerous species, it still remains unknown what mechanism is responsible for this relationship. It has not been experimentally investigated whether variation is due to internal mechanisms or external factors. Here we were interested in the internal effect of hormones on the hippocampal processes in the side-blotched lizard (*Uta standburiana*).

The side-blotched lizard serves as a model system in this study for a variety of reasons. Male side-blotched lizards exhibit three morphs controlled by a single gene locus and are distinguished by one of three colors: orange, yellow, or blue (Sinervo et al. 2001, 2006b). Each color is displayed on the underside of the throat area, and corresponds to a strategy of spatial use. The orange morph males reside over large territories and defend many females for mating purposes. The blue morph males reside over smaller territories and have only a single female. The yellow morph males do not defend territories instead sneaking into territories of the other morphs. Orange males are able to gain territories from the blue males, another morph that maintains a much smaller territory compared to the orange morph. The yellow morph males are able to gain matings by sneaking into large territories of orange morphs. Because the orange morph males keep such large home ranges it is easier for the yellow morph males to sneak into their territories. However, the yellow morph males cannot as easily take resources from the blue males' territories because of the decreased territory size and increased

surveillance by the dominant blue males (Sinervo & Lively 1996; Sinervo et al. 2000b, 2006b; Zamudio & Sinervo 2000).

The side-blotched lizard system lends itself nicely to testing how holding a territory or not having this demand, might affect the hippocampus, the area of the brain responsible for spatial cognition. The orange and blue morphs fall under the territorial category, while the yellow is the sole morph comprising the non-territorial category. The hippocampus of orange males has been documented as being larger in both the dorsal and medial cortexes compared to the yellow males, which exhibited smaller hippocampal volumes (LaDage et al. 2010). This relationship connects increased spatial usage effects on the morphology of a specific area of the brain and the spatial use strategies used by different morphs of the side-blotched lizard. The morphs of the side-blotched lizard that require increased spatial memory capacity have been shown to have a larger hippocampal areas and the morphs using less spatial memory have been shown to have smaller hippocampal areas (LaDage et al. 2010).

This variation between morphs of the side-blotched lizard in hippocampal morphology and space use strategies could be due to a variety of factors. Hormones, in general have been shown to modify the structure of the hippocampus, and the changes have been shown to persist through maturation into adulthood (Gould et al. 1990; Roof & Havens 1992; Woolley & McEwen 1992; Cooke et al. 1998; Galea et al. 1999). In the side-blotched lizard specifically, testosterone (T) has had been implicated as a hormone that directly affects spatial use. Thus if the spatial use strategies have been altered or modified and a change in the spatial demands has occurred, then it is possible that this change could result in a change in the hippocampal morphology (LaDage et al. 2009).

Spatially, when testosterone levels were increased in blue and yellow males, they acquired a larger territory (DeNardo & Sinervo 1994; Sinervo et al. 2000b). These sort of changes in spatial use strategies observed behaviorally could then translate into changes in the hippocampus, based on previous findings. The larger territories were similar in size to the oranges males that were left un-manipulated. If testosterone levels were supplemented in identical pairs of males in both the yellow and blue morphs, and in orange males, and the males were allowed to mature, then this could indicate that testosterone has an effect on hippocampal characteristics, independent of spatial use (DeNardo & Sinervo 1994; Sinervo et al. 2000b).

Previous research has only considered the morphology changes in terms of volume and neuron count. However, the volume change should be due to an increase or decrease in neuron numbers comprising that area, and thus volume and neuron numbers should both be considered as measurements for such change. What is of interest in this experiment specifically is the rate of adult neurogenesis occurring in the hippocampus.

Neurogenesis is a process of generating and recruiting of new neurons. These new neurons are generated beyond replacing old neural connections, but to adapt and accommodate increased spatial demands that are being placed on the brain. There has been evidence supporting the idea that increased spatial demands enlarge the volume and increase the number of neurons in the hippocampus because of a possible increase rate of neurogenesis (Barnea & Nottebohm 1994; Gould et al. 1999; Kempermann 2002; Patel et al. 1997).

Here we hypothesize that testosterone-mediated changes in spatial use strategies might occur via changes in adult hippocampal neurogenesis rates. More specifically, we

predicted that experimental increases in testosterone levels should directly affect adult hippocampal neurogenesis rates. In order to locate and mark the new cells resulting from neurogenesis, doublecortin protein was used. Doublecortin is protein that is expressed in new neural cells that have not fully matured. After a neural cell matures it expresses a different protein and the expression of doublecortin is halted (Brown et al. 2003; Couillard-Despres et al 2005). Thus by marking the production of this protein we will be able to differentiate between newly born neural cells and pre-existing mature neurons.

Methodology

Studies conducted with the patterns in space use by the side blotched lizard have previously shown that modification of testosterone (T) levels have resulted in variation of such space usage (DeNardo & Sinervo 1994; Sinervo 1994). We predicted that increased testosterone levels will affect hippocampal neurogenesis rates. This experiment entailed breeding males and females with known genotypes in laboratory. After the progeny were hatched and all three morphs (blue, orange, and yellow) were represented, the parents were released and the progeny were raised. The male offspring were raised in pairs of the same morph type. Forty eight males (n=48), 8 sibling pairs in each morphs were raised until adulthood, which was defined as approximately six months. By raising these pairs in the laboratory with each pair receiving the same treatment over the same amount of time other factors contributing to lizard development were controlled for, these factors being: maternal, genetic, and experimental. This way the effects, if any, were attributed to the changes mediated by the testosterone manipulation. Orange, blue and yellow males were subjected to a testosterone implantation procedure in which a randomly chosen male of each sibling pair in each blue and yellow pair received the testosterone implant treatment.

The testosterone implant were composed of a 3 mm Silastic medical grade tubing (Dow Corning 602-305), with a 1 mm testosterone implant enclosed. The control vehicle used for the other sibling in each pair was of the same length and composition but did not contain testosterone. All implants were soaked in a saline solution 24 hours prior to implantation. After the anethestic was used the implants were placed intracoelomically through an incision in the flank (DeNardo & Sinervo 1994). The testosterone implants provided testosterone to the males for three months after placement to ensure for enough

time for the increased testosterone to incorporated into the body and for changes (if any) to take effect and for those changes to be matriculated in the hippocampus (DeNardo & Licht 1993; DeNardo & Sinervo 1994),

After this three-month period all males were sacrificed so that the brain could be removed and sectioned for staining. With each of these treatments it was predicted that hippocampal neurogenesis would increase in the supplemented yellow and blue males, and a decrease would be observed in the orange males, and no change should occur in the control orange males. The blue and yellow males should have display new neurons in the hippocampal region at the same rate that the orange male would normally (Lancaster et al. 2008; DeNardo & Sinervo 1994; Sinervo et al. 2000b). If these changes are seen then this would indicate the effects testosterone can mediate on the process of neurogenesis in the hippocampus.

The dorsal and medial cortices of the hippocampus were analyzed separately using an Optical Fractionator. The two cortices have been identified as the areas of the hippocampus that are most involved with spatial processing (Grisham & Powers 1990; Petrillo et al. 1994; Reiman-Avigan & Schade-Powers 1995; López et al. 2003). The brains were sectioned at 40 µm and every 3rd tissue was subjected to the DCX (doublecortin) staining. DCX (doublecortin) only stains new immature neurons in the brain, which allows for estimation of the total number of new neurons prior to their maturation (Brown et al 2003; Couillard-Despres et al 2005). DCX staining thus provides a reliable measure of neurogenesis, but it provides a combined estimate of new neuron production and survival rates prior to maturation (LaDage et al. 2010). Stained tissue was mounted on slides and analyzed using the Optical Fractionator stereological method

(StereoInvestigator, MBF Bioscience). The Optical Fractionator stereological method employed a grid size of 70 µm by 70 µm and the step size was also 70 µm by 70 µm. The guard zone was two µm and the dissector height was five µm.

Results

After the experimentation was performed only 30 males were used for the analysis. We specifically estimated the total number of DCX-labeled cells in two hippocampal regions – medial and dorsal cortices. Six territorial and four non-territorial male lizards comprised the control groups and the testosterone supplemented group had 5 territorial and 4 non-territorial male lizards. Testosterone-supplemented lizards had significantly higher plasma levels of testosterone ($F_{1,16}=57.58$, $p< 0.001$;Fig. 1). However, there were no significant differences in the total number of DCX-stained neurons in either the dorsal or medial cortices of the hippocampus between control and testosterone-supplemented lizards, whether territorial or non-territorial. There were no significant differences in the number of new neurons produced between the control, non-supplemented lizards and the supplemented lizards in the dorsal cortex region of the hippocampus ($F_{1,15}=0.22$ $p=0.64$; Figure 2) or between the territorial and non-territorial morphs ($F_{1,16}=0.40$, $p=0.55$; Figure 2). Similarly no significant differences were observed between the control and supplemented testosterone implants in the medial cortex ($F_{1,15}=1.0$, $p=0.33$;Figure 3) or between the territorial and non-territorial morphs ($F_{1,15}=1.65$,$p=0.22$; Figure 3).

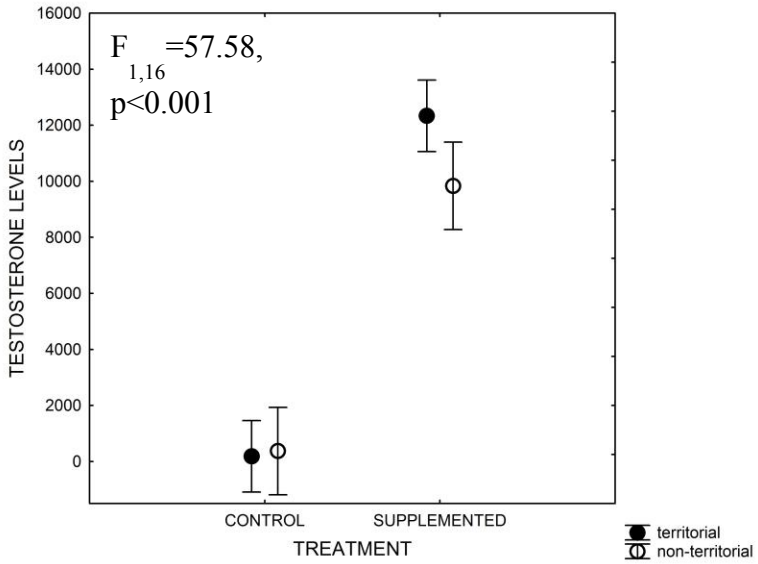

Figure 1. Testosterone Levels in control and supplemented lizards. Testosterone as measured in pg/mL.

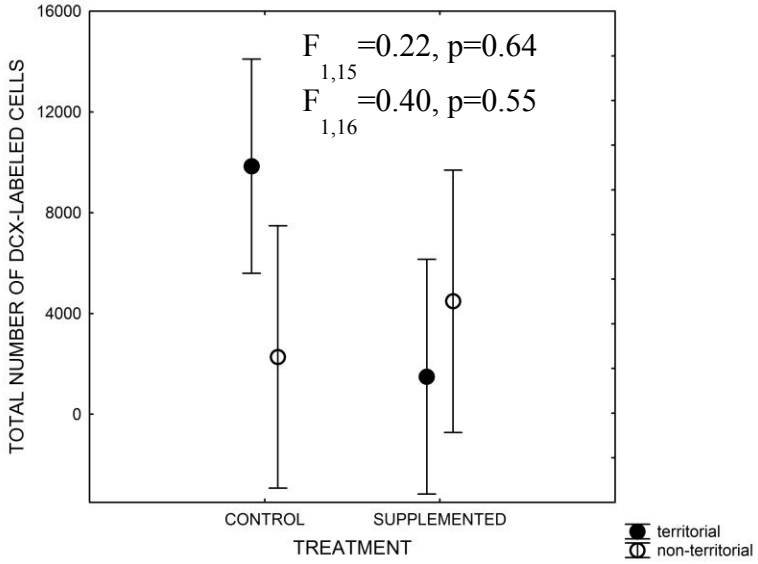

Figure 2. The total number of DCX –labeled neurons in the dorsal cortex in territorial and non-territorial morphs subjected to different levels of testosterone (control and supplemented).

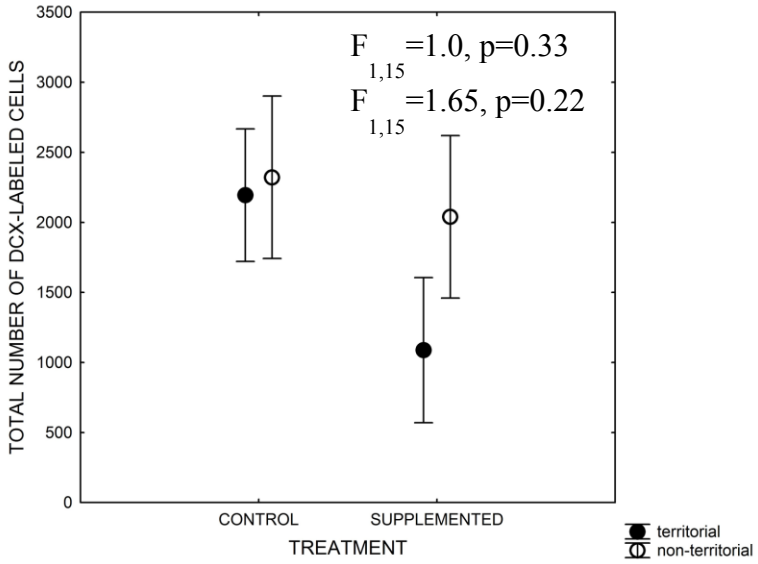

Figure 3. The total number of DCX-labeled neurons in the medial cortex in territorial and non-territorial morphs subjected to different levels of testosterone (control and supplemented).

Discussion

Although testosterone supplementation resulted in significant elevation of testosterone levels in supplemented lizards, we failed to detect any significant differences in hippocampal neurogenesis rates (measured as the number of DCX-labeled neurons) between control and supplemented lizards. Therefore, these results suggest that elevated testosterone might have no effect on hippocampal neurogenesis rates. Alternatively, it is also possible that we failed to detect significant differences because of relatively small sample sizes and high individual variation in neurogenesis rates. Furthermore, testosterone might affect either new neuron production or new neuron survival independently (Galea et al. 2006), in which case our methods may not have been sufficient as doublecortin (DCX) only allows combined measurement of neuron production and survival as it labels new neurons at all aging stages until they reach maturity (LaDage et al. 2010). For example, in rodents elevated testosterone appears to affect only new hippocampal neuron survival, but not new neuron production rates (Galea et al. 2006).

Additionally, it has been shown that neurogenesis could be directly affected by exposure to novel environments and through spatial use in those environments (Kempermann 2002). The purpose behind the need for new neurons is still debated, and their function in the hippocampus is still debated. The proliferation of neurons might not be for the purpose of increasing memory capacity but rather to form new memories. If the purpose is to form new memories than the necessity of forming new neurons would be tied to the pending formation of new memories. Those new memories would need to be created through the navigation of a new environment or the relocation of food resources.

If such needs are not apparent then the need for those connections to rerouted throughout the brain for storage is not necessary.

Through this reasoning we could extend an explanation for the absence of differences in cell proliferation after the testosterone implants were implanted. After implantation the lizards were not exposed to novel environments where navigation and resource locations were needed for survival. Thus there was no need for new spatial use or need to use this spatial learning to survive. Without this spatial use, the hippocampus did not need to re-route incoming spatial information to different storages in that area of the brain for later retrieval or usage. Thus the need for cell proliferation to complete this task was unnecessary.

Based upon this reasoning it would be interesting to see if after implantation of testosterone and exposure to novel environments for resource location and navigation there was a difference in neurogenesis. This could then be compared to the baseline data collected in this experiment when no exposure to new environments occurred. This extension could prove to be beneficial in determining if testosterone has any role in hippocampal neurogenesis.

Conclusion

In conclusion we find that there was no significant different in the rate of neurogenesis in either the dorsal or medial cortices due to the increased testosterone levels.

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