Effect of Incubation Temperature and Androgens on Dopaminergic Activity in the Leopard Gecko, *Eublepharis macularius*

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ABSTRACT: **Male leopard geckos that hatch from eggs incubated at a female-biased temperature (T_f) behave differently when compared with males hatching at a temperature which produces a male-biased sex ratio (T_m). We investigated the effect of incubation temperature and androgen implantation on aspects of the dopaminergic system of T_f and T_m males. Our data suggest that more dopamine (DA) is stored in the nucleus accumbens of naive T_f males compared with naïve T_m males when they encounter a receptive female conspecific across a barrier. No difference was measured in the preoptic area and the ventral tegmental area (VTA). This difference in intracellular DA levels in a motivation-related brain nucleus might be correlated with differences in sociosexual behavior observed between the two morphs. There were no differences in tyrosine hydroxylase (TH) expressing cell numbers in the VTA of cholesterol (CH)-implanted naive castrated T_f and T_m males. Only T_f males implanted with testosterone had significantly higher TH immunopositive cell numbers in the VTA compared with CH- and dihydrotestosterone-implanted T_f males. These data indicate that both the embryonic environment as well as the circulating hormonal milieu can modulate neurochemistry, which might in turn be a basis for individual variation in behavior.**

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INTRODUCTION

Individual variation in behavior serves as a substrate for the evolution of adaptive behavioral outcomes. While the importance of such variation is often noted, the underlying mechanisms are still unclear. A suitable model system to probe variation in behavioral repertoires must address between- (intersexual) and within-sex (intrasexual) differences as well as the effect of environmental variables. While between-sex differences in behaviors have received considerable attention (Godwin and Crews, 1997), relatively little work has been carried out to explain proximate factors that might play a role in behavioral differences observed within the same sex (Moore, 1991; Crews, 1998; Rhen and Crews, 2002; Ryan and Vandenbergh, 2002).

The leopard gecko, *E. macularius*, affords us an opportunity to investigate behavioral variation that might be triggered by the environment and observed within a sex. Gonadal sex in leopard geckos is determined by the temperature at which the egg is incu-

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bated (Viets et al., 1993). Eggs incubated at 30°C causes 25% of the eggs to hatch as male and 75% as female, whereas incubation at 32.5°C results in 75% of the eggs hatching as male and 25% as female. The skewed sex ratios produced from these incubation temperatures are termed T_f (for temperature female-biased) and T_m (for temperature male-biased), respectively.

In addition to altering sex ratios, incubation temperature affects the composition of the circulating hormonal environment, thereby establishing differences in the propensity to express sociosexual behaviors (Tousignant and Crews, 1995; Crews, 1996; Crews et al., 1998; Pieau et al., 2001). Such an interaction between incubation temperature and the hormonal milieu is thought to organize underlying neuronal circuits that lead to differences in sociosexual behaviors expressed by the two temperature morphs (T_f males vs. T_m males) (Sakata and Crews, 2004b; Crews et al., 2006). Comparisons between T_f and T_m males in sociosexual settings indicate that T_m males are more aggressive and less sexually active than T_f males (Flores et al., 1994; Rhen and Crews, 1999) and have different mate preferences (Putz and Crews, 2006). In addition, T_f males show greater anticipatory behavior when presented with a stimulus female on consecutive days when compared with T_m males (Sakata and Crews, 2003). These data illustrate that the environment (incubation temperature) might be a critical factor in establishing profound differences in behavior within the same sex.

One mechanism by which environment–hormone interactions might translate into behavioral differences between the temperature morphs involves individual differences in neurotransmission. Since dopamine (DA) has been implicated in sociosexual behaviors such as courtship and motivation (Pfaus and Phillips, 1991; Dominguez and Hull, 2005) across several taxa, we have examined the dopaminergic system of T_f and T_m males to determine if it is differentially responsive to the same stimulus as well as differentially organized and/or activated by embryonic and adult hormonal environments. Comparing between the temperature morphs, we first investigated if differing amounts of DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were stored in brain nuclei involved in sexual motivation and male sexual behavior upon observing a female conspecific. Next we addressed whether there are differences in DA-synthesizing cell numbers in the ventral tegmental area (VTA) as a function of incubation temperature and hormonal environment, which might be responsible for behavioral variation observed between these morphs.

METHODS

Animals

Two-year-old adult male leopard geckos (*Eublepharis macularius*) from our animal colony were used in all experiments. All animals were sexually inexperienced (naive). These animals hatched from eggs incubated at either 30°C (T_f) or 32.5°C (T_m). After hatching, geckos were housed in a solitary environment as outlined in Sakata and Crews (2004a), fed with mealworms, and provided water three times a week. All animal protocols were carried out in accordance with UT-IACUC and NIH guidelines.

Experimental Design and Tissue Collection

Experiment 1: DA Levels in Brain Nuclei of Naive T_f and T_m Males After Observing a Receptive Stimulus Female Gecko. Three male geckos from each incubation temperature were habituated to a plexiglass chamber on 3 consecutive days, and then exposed to a receptive stimulus female across a wire-mesh barrier for 10 min on 3 consecutive days, with the same female being used for all males to control for stimulus quality (see Putz and Crews, 2006 for further details). On completion of testing on the last day, animals were sacrificed, brains rapidly dissected, fresh frozen, and stored at −80°C. Using a cryostat (Microm HM 500 OM), 200-μm coronal sections were thaw-mounted on to Superfrost Plus slides (Erie Scientific, USA). Sections were then rapidly frozen using a cooling block set at −20°C (Physitemp Instruments, USA) and the nucleus accumbens (NAc), preoptic area (POA), and VTA dissected using a 300-μm-diameter micropunch, as per Smeets and Steinbusch (1988) and Young et al. (1994). Tissue samples from each animal were assayed independently of each other and not pooled. Punched tissue was stored in 70 μL of ice-cold homogenization solution [a mixture of 60 μL homogenization buffer: 0.1 *M* perchloric acid (Sigma-Aldrich) containing 347 μM sodium bisulphate (Sigma-Aldrich) and 134 μM EDTA disodium salt (Fluka, USA), and 10 μL of 100 n*M* Epinine – internal standard (Sigma-Aldrich)]. Tissue samples in homogenization solution were frozen at −80°C overnight and thawed after 24 h. Thawed samples were centrifuged at 14,000 rpm at 4°C for 20 min, after which the supernatant was collected and used for HPLC analysis. Protein content in the resulting pellet was determined by resuspending and agitating the pellet in 45 μL of ice-cold 0.3 *N* NaOH for 24 h at 4°C, and a modified Bradford assay was performed thereafter (Pierce Biotechnology, USA).

Experiment 2: Tyrosine Hydroxylase Immunopositive Cell Numbers in the VTA of Naive T_f and T_m Male Geckos Receiving a Silastic Implant Containing Cholesterol, Dihydrotestosterone, or Testosterone. Fourteen male geckos from each incubation temperature were castrated and implanted with 20-mm Silastic capsules containing

cholesterol (CH; $n = 5$), dihydrotestosterone (DHT; $n = 5$), or testosterone (T; $n = 4$); all hormones were purchased from Sigma-Aldrich, USA. Two weeks after surgery and implantation, geckos were deeply anesthetized using hypothermia prior to transcardial perfusion using 0.9% saline and 4% paraformaldehyde (PFA). Animals were decapitated and the heads immersed in 4% PFA for 8 h at 4°C. Brains were then dissected and stored in 4% PFA for 24 h at 4°C, before being cryoprotected in 20% sucrose/1× PBS at 4°C for 24 h. Coronal sections (30 μm) were cut on a cryostat and thawed on to Superfrost Plus GOLD slides (Erie Scientific) and stored at −80°C until immunohistochemistry was performed.

HPLC Analysis

Levels of DA, and its metabolite DOPAC in the NAc, POA, and VTA were determined by HPLC-EC using modifications of Bai et al. (1999). Details are provided in Dias and Crews (2006). In brief, 50 μL of sample was injected into an HPLC system that comprised a Shimadzu SCL-10A system controller, LC-10AD pump, an SIL-10A auto-sampler (Shimadzu, Columbia, MD), and coupled with a four-channel CoulArray electrochemical detector (ESA, Chelmsford, MA). The isocratic mobile phase contained 4 m*M* citric acid, 8 m*M* ammonium acetate, 120 μM 1-octanesulfonic acid sodium salt, 60 μM EDTA disodium in water, and 5% MeOH, pH 3.5. The flow rate of the mobile phase remained at 1 mL/min. Separation was achieved by a 4.6 mm × 80 mm reverse-phase HR-80, 3-μm particle-size column (ESA). The potential of channels 1 through 4 of CoulArray was set at −50, 0, 300, and 400 mV, respectively. Peak area (nC) of DA and DOPAC at the corresponding retention time on the chromatogram resulted from 300 mV, and was used to quantify the amount based on the standard curve of each neurotransmitter. Recovery of internal standard was consistently high across all experimental runs (95–100%) making it unnecessary to correct for recovery. Levels of DA and DOPAC in the brain nuclei were expressed as pg/μg of protein in the microdissected tissue extract.

Tyrosine Hydroxylase Immunohistochemistry

Tyrosine hydroxylase (TH)-immunopositive cells in the gecko brain were stained using the following immunohistochemical protocol with all steps being carried out at room temperature (Fig. 1). Slides containing gecko brain tissue were removed from −80°C and allowed to dry completely. They were then immersed in 4% PFA for 10 min, and washed twice in 1× PBS for 5 min. To block endogenous peroxidase activity, the sections were exposed to 0.3% hydrogen peroxide in 1× PBS for 20 min and washed twice in 1× PBS for 5 min. Antigen retrieval was accomplished by incubation in 1% sodium borohydride in 1× PBS for 20 min, and 1× PBS was used to wash-off the excess sodium borohydride. A blocking step using 4% normal goat serum, 0.3% Triton X-100, and 1× PBS was carried out for 1 h to prevent nonspecific binding. Sections were then incubated overnight with a primary antibody solution that contained 2% normal goat serum, 0.3% Triton X-100, 1× PBS, and a mouse monoclonal antibody that detects TH (MAB 318; 1:250; Chemicon International, USA). After washing with 1× PBS, the slides were incubated for 2 h with a secondary antibody solution that contained 2% normal goat serum, 0.3% Triton X-100, 1× PBS, and biotinylated goat anti-mouse antiobody (1:200; Vector Laboratories, USA), followed by 1-h exposure to horseradish peroxidase-conjugated avidin–biotin complex (Vector Laboratories). TH-immunoreactive cells were visualized using the DAB substrate kit (Vector Laboratories). Sections incubated in the absence of a primary antibody served as controls and resulted in no staining of TH-immunopositive cells.

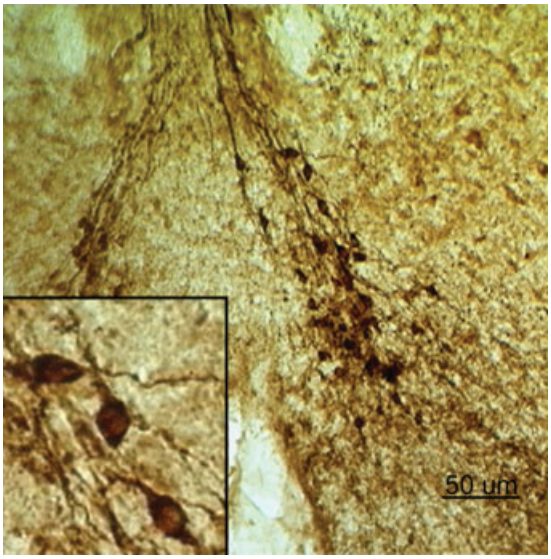

Figure 1 Representative photomicrograph of TH-immunopositive staining in the VTA of a naive adult male T_m leopard gecko (*Eublepharis macularius*). Immunohistochemistry was conducted on cryosectioned gecko nervous tissue using a mouse monoclonal antibody against TH, and DAB staining was used to visualize TH-expressing cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Cell Number Quantitation

The optical fractionator module of StereoInvestigator software (Microbrightfield, USA) was used as an unbiased estimator of TH-immunopositive cells in the VTA of the adult gecko. The VTA was outlined as per Smeets and Steinbusch (1988), and TH-immunoreactive cells were counted from four sections per brain. Cells were counted in sample frames placed at fixed stepping distances within the outlined region using the 40× objective on a Zeiss microscope. Total cell number was calculated using a formula enumerated in West et al. (1991).

Statistical Analysis

SPSS v12.0 was used for statistical tests with significance set at $p < 0.05$. In Experiment 1, a multivariate analysis of variance (MANOVA) was conducted with DA, DOPAC levels, and DOPAC/DA ratio being the dependent variables, while region (NAc, POA, VTA) and incubation temperature (T_f, T_m) were independent variables. When a region by temperature interaction was found to be significant for DA levels, individual ANOVA tests were conducted on every region to analyze statistical differences in DA levels between T_f and T_m animals within a region. In Experiment 2, a univariate ANOVA that included both the T_f and T_m groups was used to compare mean TH-ir cell numbers with Tukey *post hoc* analysis.

RESULTS

Experiment 1: DA, DOPAC Levels, and DOPAC/DA Ratio in Discrete Brain Nuclei of Naive T_f and T_m Male Leopard Geckos After Observation of a Receptive Stimulus Female

A significant interaction between region and incubation temperature was observed when DA levels were measured in the NAc, POA, and VTA of T_f and T_m naive intact male geckos ($F_{2,12} = 27.310$, $p < 0.001$) (Fig. 2). Specifically, DA levels were significantly elevated in the NAc of T_f males compared with those in T_m males ($F_{1,4} = 29.771$, $p = 0.005$). DA in the POA and VTA did not differ between T_f and T_m males. DOPAC levels as well as the DOPAC/DA ratio remained unchanged in all regions across both incubation temperatures (Table 1).

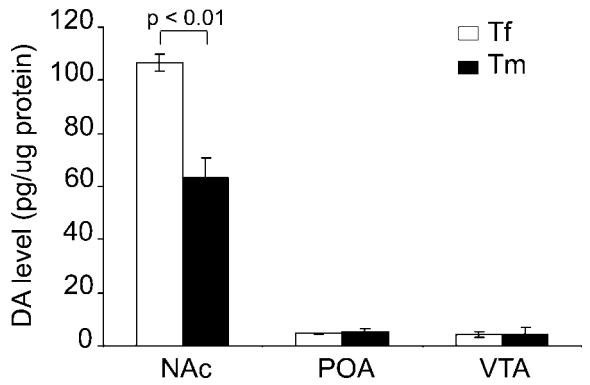

Figure 2 DA levels in the NAc, POA, and VTA of naive T_f and T_m male leopard geckos (*Eublepharis macularius*). Data expressed as average pg/μg of protein expressed $\pm$ SEM. T_f males had significantly higher DA levels in the NAc compared with T_m males. POA and VTA levels were not significantly different between the two groups.

Table 1 DA, DOPAC Levels, and the DOPAC/DA Ratio in the NAc, POA, and VTA of T_f and T_m Male Leopard Geckos, *E. macularius*, After Observation of a Receptive Stimulus Female Across a Barrier

Morph	DA	DOPAC	DOPAC/DA
T_f			
NAc	106.6 (3.32)	3.56 (0.58)	0.033 (0.005)
POA	4.65 (0.30)	1.69 (0.13)	0.36 (0.04)
VTA	4.23 (0.89)	1.28 (0.53)	0.30 (0.09)
T_m			
NAc	63.27 (7.21)	3.22 (0.51)	0.05 (0.008)
POA	5.53 (0.68)	1.88 (0.23)	0.34 (0.03)
VTA	4.41 (2.45)	0.96 (0.54)	0.21 (0.15)

Data expressed as pg/μg of protein [Mean (SEM)]. Only DA levels in the NAc were significantly different across the temperature morphs (see Fig. 2). DOPAC levels and DOPAC/DA ratio were unaltered in any region in either temperature morph.

Experiment 2: Number of TH-Immunopositive Cells in the VTA of Naive CH-, DHT-, and T-Implanted T_f and T_m Male Geckos

A significant incubation temperature by hormone interaction was obtained using a univariate ANOVA with TH cell count as the dependent variable ($F_{2,22} = 5.647$, $p = 0.01$). Tukey *post hoc* analysis indicated that T_f-T males had a significantly higher number of TH-immunopositive cells in the VTA than T_f-CH and T_f-DHT males (T_f-CH vs. T_f-T, $p < 0.01$; T_f-DHT vs. T_f-T, $p = 0.02$) (Fig. 3).

DISCUSSION

In the leopard gecko, the embryonic environment affects dopaminergic activity in the brain of naive adult males. Specifically, males from an incubation temperature that produces mostly females (T_f males) had higher levels of DA stored in the NAc than did males from an incubation temperature that produces mostly males (T_m males) after observation of a receptive stimulus female. Further, naive T_f males implanted with testosterone had a greater number of TH-immunopositive cells in the VTA than did CH- and DHT-implanted T_f males. No significant differences in DA levels were noted in the POA and VTA, and DOPAC levels and DOPAC/DA ratios were not different between the temperature morphs in the examined brain regions. Such differences in the dopaminergic systems of T_f and T_m male geckos might influence behavioral differences observed in sociosexual settings.

Hormones recruit neurotransmitter-mediated signaling mechanisms to elicit behavioral responses. It

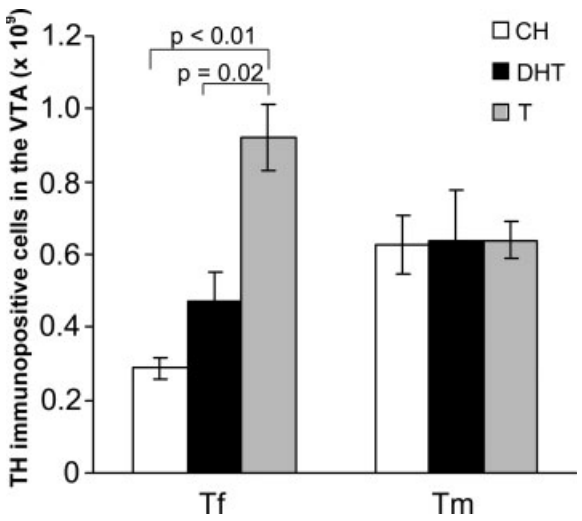

Figure 3 Number of TH-immunopositive cells in the VTA of castrated naive T_f and T_m male leopard geckos (*Eublepharis macularius*) implanted with cholesterol (CH), dihydrotestosterone (DHT), or testosterone (T). Data are represented as mean ± SEM. Testosterone-treated T_f males had a significantly higher number of TH-immunopositive cells in the VTA compared with CH- and DHT-implanted males.

is well established that embryonic and neonatal environments have a profound effect on behavioral responses in adulthood. For example, in litter-bearing mammals, female embryos that develop between two male embryos in the uterus show masculinized morphology, physiology, and behavior in adulthood when compared with female embryos that do not develop between males. Such a phenomenon has been attributed to differences in hormonal exposure *in utero* (reviewed in Ryan and Vandenbergh, 2002) and *in ovo* (reviewed in Crews and Groothuis, 2005). Thus, extending the organizational/activational paradigm of sexual differentiation from hormones to neurotransmitter systems suggests that such systems are also organized perinatally and/or activated in adulthood to yield distinct responses.

The mesoaccumbens dopaminergic system that consists of projections from the VTA into the NAc is involved in reward and motivation in several species (Ikemoto and Panksepp, 1999; Franken et al., 2005). Dopaminergic receptors in the NAc are involved in the formation and maintenance of pair bonds (sociosexual behavior) in voles (Aragona et al., 2006). In male rats, an increase in extracellular DA level in the NAc by sexually relevant stimuli is known be positively correlated with enhanced motivation to engage in sexual behavior (Damsma et al., 1992; Wenkstern et al., 1993; Pfaus et al., 1995). The existence of the mesoaccumbens dopaminergic pathway has been documented in another gekkonid lizard, *Gekko gecko* (Smeets et al., 1986). In addition, T_f male geckos learn to associate a testing environment with the presentation of a female by showing greater anticipatory behavior than T_m males when tested on consecutive days (Sakata and Crews, 2003). In the present study, when T_f and T_m male geckos had been repeatedly exposed to a receptive female conspecific, more DA was stored in the NAc of T_f males than T_m males. These data also suggest a specificity of response since no differences in DA levels were observed in the VTA and the POA. This observation reinforces the concept that the same stimuli elicit different responses by potentially affecting the way a neurotransmitter system is organized. While it is not known if the observed differences in intracellular dopaminergic reserves in the present study are mirrored by differences in the release of DA, such a mechanism might underlie the morph differences in motivation and anticipation.

One explanation for our HPLC results might be differing amounts of DA being synthesized by different numbers of DA producing cells (TH-immunopositive cells) in the VTA. Environmental factors such as housing conditions alter TH-immunoreactive cell numbers in discrete brain nuclei in a whiptail lizard species, *C. inornatus* (Woolley et al., 2004). In addition, physiological factors like the circulating hormonal milieu affect dopaminergic activity. Gonadectomized leopard frogs have fewer TH-immunopositive cells in forebrain and midbrain nuclei when compared with DHT- and T-implanted animals (Wilczynski et al., 2003). Our data suggest that both incubation temperature and the adult hormonal environment play roles in the modulation of dopaminergic activity by regulating TH cell numbers in the VTA. Only T_f male geckos appear to be responsive to hormonally mediated alteration of TH cell numbers in the VTA with androgen treatment increasing cell numbers. This differential responsiveness of TH cell numbers to the hormonal environment once again points toward variation in response of neuronal circuitry to physiological factors.

The absence of significant differences in TH-immunopositive cell numbers in the VTA between T_f and T_m males implanted with CH suggests that incubation temperature may not organize the dopaminergic system. However, it is important to keep in mind that TH cell numbers are only one aspect of the neurotransmitter system that might be modulated by the environment; rate of synthesis, storage, release, and response may also be differentially organized and deserve investigation. From an activational perspective, androgens appear to alter TH cell numbers only

in T_f males but not in T_m animals. Androgen levels in T_f and T_m male geckos do not differ significantly early in life (Rhen et al., 2005) or later in adulthood (Tousignant and Crews, 1995; Coomber et al., 1997; Rhen et al., 2000). However, T_f males have higher levels of estrogen in adulthood than do T_m males (Tousignant and Crews, 1995). It is possible that estrogenic regulation of TH cell numbers *in ovo* establishes differences in neurochemistry that might persist into adulthood, and further experiments are needed to investigate this question. The reported study does not address the possibility that there might be baseline differences in DA levels in the NAc across the temperature morphs that might be independent of repeated exposure to a receptive female. The investigation of such a scenario coupled with hormonal and pharmacological manipulations of the dopaminergic system within a sociosexual context presents an avenue for future research.

Between-sex differences in behavioral expression could result from factors that include but are not limited to sexual dimorphisms in neuroanatomy and neurochemistry (Simerly et al., 1984). However, within-sex differences in neuroanatomy and neurochemistry that might explain individual variation in behavior are not as widely investigated. Modulation of embryonic and neonatal neurotransmitter systems' activity has received considerable attention as being correlated with altered behavioral phenotypes in adulthood. For example, administration of serotonin to neonatal rats altered adult serotonin levels in discrete brain nuclei as well as affected sexual activity in adulthood (Csaba et al., 2003).

Our data suggest that androgens regulate TH cell numbers only in T_f males and not T_m males. Also, estimates of neurotransmitter turnover obtained by calculating the ratio of DOPAC/DA take into account synthesis and catabolism of the system. Although no significant differences in DOPAC/DA ratio were observed between T_f and T_m males, the DOPAC/DA ratio observed in the NAc of T_f males is lower than the ratio in T_m males. Such an observation may be indicative of lesser dopaminergic turnover in T_f males compared with T_m males in the NAc. These data raise the possibility of a compensatory mechanism in T_f males that would allow them to compete with their more masculinized T_m counterparts for a female conspecific by storing or releasing more DA than is being metabolized, and by being more sensitive to the hormonal environment with respect to TH cell number regulation.

Portions of these data have been reported in preliminary form in Crews et al., 2006. We thank Dr. Herng-Hsiang Lo in the CRED Analytical Instrumentation Facility Core at UT-Austin for his assistance with the HPLC analysis. This work would not have been possible without gecko care provided by Casey Wommack, Devra Heyer, Linh Tran, and Ryan Benett.

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