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GENETIC AND EXPERIENTIAL EFFECTS ON DOPAMINERGIC SYSTEMS

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GENETIC AND EXPERIENTIAL EFFECTS ON DOPAMINERGIC SYSTEMS

by

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Dedication

This dissertation is dedicated to Jon Sakata, my parents, and my brother for their continued love and support.

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Genetic and Experiential Effects on Dopaminergic Systems

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Successful reproduction requires the coordination of relevant sensory inputs with motivational and motor systems primed by sex steroid hormones to produce an appropriate hierarchical sequence of movements. Both behavioral and neural phenotypes can be altered by social interactions that, in turn, can produce long term changes in cellular activity and signaling, neural circuitry, and sexual behavior. There is considerable variability in the type and direction of neural and behavioral change in response to social interactions, and the degree of plasticity may depend on intrinsic or genetic individual differences. Dopaminergic systems modulate the expression of social and sexual behaviors in a number of vertebrate species and intrinsic differences in dopaminergic systems may underlie intrinsic individual differences in the display of sexual behavior. Here, I present data on how social interactions, genotype, and steroid hormones can affect dopamine

synthesis in limbic and midbrain nuclei. I investigated this in three model systems including knockout mice and two related species of whiptail lizard. The knockout mice have a targeted deletion of the progesterone receptor and display higher mount and intromission frequencies than wild-type males. Male whiptail lizards (*Cnemidophorus inornatus*) have natural variation in the display of courtship behaviors: some males are more sexually vigorous than others. Finally, individuals of the parthenogenetic species *C. uniparens*, which arose from two hybridization events involving the sexual species *C. inornatus*, display both male- and female-like sexual behaviors depending on reproductive state. In contrast, *C. inornatus* females only display receptive behavior, and this only during when pre-ovulatory. In all three species, individuals that displayed greater levels of mounting behaviors had greater numbers of cells expressing tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis, in the substantia nigra pars compacta. In addition, in male whiptail lizards and the related parthenogen, dopamine production in the dorsal hypothalamus was correlated with the propensity to display mounting behaviors. Dopamine can increase the display of mounting behavior in mice as well as in male and parthenogenetic whiptail lizards. My dissertation indicates that not only is dopamine sufficient to elicit mounting behavior, but differences in dopamine production may contribute to individual differences in behavioral phenotype.

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Chapter 1

Introduction

Successful reproduction requires the coordination of relevant sensory inputs with motivational and motor systems primed by sex steroid hormones to produce an appropriate hierarchical sequence of movements. There is considerable intrasexual variability in the display of sexual behaviors with some individuals showing greater intrinsic levels of sexual vigor than others. Sexual experience can produce long term changes in cellular activity and signaling, neural circuitry, and sexual behavior and the degree to which individuals are behaviorally plastic may depend on the degree of intrinsic sexual vigor. Thus, the study of individual differences in sexual behavior can provide insight into both the neural substrate underlying behavior as well as the neural substrate underlying behavioral plasticity.

Catecholamines have been implicated in an array of psychomotor, motivational, and attentional behaviors. In particular, dopamine released into cortical, striatal, and preoptic areas modulates decision making, reward and motor

learning, and sexual behaviors. Dopaminergic cells in the substantia nigra *pars compacta* (SNpc), ventral tegmental area (VTA), and preoptic area (POA) increase activity in response to salient or arousing stimuli (Damsma et al., 1992; Hull et al., 1995; Pfaus et al., 1990; Schultz, 1998; Schultz et al., 1997), while stimulation of post-synaptic dopamine receptors results in long-term, cell specific effects through the phosphorylation of the 32 kilodalton dopamine and cAMP response phosphoprotein (DARPP-32; Greengard et al., 1999). Dopaminergic systems may modulate both the motivational and motor aspects of copulation (reviewed in Melis and Argiolas, 1995) and can be altered by copulatory stimuli and sexual experience. However, the degree to which intrinsic differences in dopaminergic systems are correlated with intrinsic individual differences in the display of sexual behavior has not been rigorously investigated, and is the focus of this dissertation.

DOPAMINE AND SEXUAL BEHAVIOR

Dopamine release and dopamine receptor stimulation, particularly in limbic nuclei, is significant in the control of sexual behaviors in male mammals and birds. Intraperitoneal injections as well as microdialized infusions of certain dopamine agonists facilitate, while dopamine antagonists inhibit, various aspects

of male sexual behavior in male rats and mice (Bitran and Hull 1987; Bignami, 1966; Butcher et al., 1969; Warner et al., 1991). Dopaminergic drugs also affect the sexual behavior of male Japanese quail, with dopamine D1 agonists increasing both sexual motivation and copulatory efficiency (Absil et al., 1993; Balthazart et al., 1997). Dopamine release into the medial preoptic area (mPOA), a nucleus integral in the display of male copulatory behaviors, is a critical part of the neural substrate underlying male sexual behavior in mammals. Sexually active males that are presented with a receptive female behind a barrier increase dopamine release into the mPOA. Interestingly, males that fail to show increases in dopamine release into the mPOA when presented with an inaccessible receptive female, also fail to copulate when the barrier is removed (Hull et al., 1995). Thus, activation of dopamine receptors may not only be sufficient to initiate copulatory behaviors, dopamine release into the mPOA may be necessary for the display of copulatory behaviors.

Though preoptic and hypothalamic areas have traditionally been the focus of research on understanding neural control of sexual behavior, recent work highlights that dopamine release into the dorsal and ventral striatum (nucleus accumbens) is also significant in the display of male copulatory behaviors in mammals. Both the striatum, which receives afferent input from the SNpc, and the nucleus accumbens, which receives afferent input from the VTA, have often

been the focus of research on motor control. A number of diseases resulting in movement-based disorders, most notably Parkinson's syndrome, result from a decrease in the number of dopamine producing cells in the SNpc. Likewise, through a series of pharmacological studies, both the dorsal and ventral striatum have been demonstrated to mediate certain types of locomotor activity and stereotypy (Gold et al., 1989; Kuczenski and Segal, 1997; Kuczenski et al., 1991; Segal and Kuczenski, 1997). However, while locomotion does affect dopaminergic tone in areas such as the striatum, the effects of movement on dopamine synthesis and release, as well as the effects of dopamine on movement appear to be context dependent. For example, whether movement results in increases or decreases in dopamine synthesis and release is strongly affected by the behavioral paradigm (e.g. spontaneous activity vs. strenuous exercise) (Elam et al., 1987; Emerich et al., 1993; Hattori et al., 1994; Tumer et al., 2001). Moreover, stimulating the SNpc or VTA often increases particular movements in a context dependent manner (Okada et al., 1991). One hypothesis is that mesolimbic and mesostriatal dopamine do not instigate or control all movement but rather may mediate motor responses directed toward primary or secondary incentive stimuli (Salamone, 1991).

Pharmacological, lesion, and dialysis studies of dopamine in the striatum during sexual behavior support the notion that striatal dopamine may prepare

individuals for movement in response to salient, arousing stimuli. Infusion of dopamine agonists and antagonists into the nucleus accumbens and dorsal striatum, and lesions of the VTA and SNpc, affect the display of anticipatory and consummatory aspects of copulatory behaviors respectively (Brackett et al., 1986; Caggiula et al., 1976; Everitt, 1990; Everitt et al., 1989; Hull et al., 1990, 1991; McIntosh and Barfield, 1984; Moses et al., 1995). Sexually active males have greater dopamine synthesis in the dorsal striatum and nucleus accumbens than males that do not copulate when presented with a receptive female as well as than males who only perform a locomotion task (Ahlenius et al., 1987; Vega-Matuszczyk et al., 1993). Stimulation of the SNpc in primates in the presence of an estrous female increases the frequency with which the male touches and mounts the female. However, in the presence of a subordinate male or human, no increases in sexual behavior are produced, indicating that the changes in behavior are context specific (Okada et al., 1991). Moreover, copulation alone is not necessary for the increases in either the dorsal or ventral striatum. Presenting males with an inaccessible estrous female increases dopamine synthesis in both the dorsal striatum and nucleus accumbens relative to presentation with an empty cage (Ahlenius et al., 1987; Vega-Matuszczyk et al., 1993). Dopamine release in these areas is also affected by the presentation of a female. Males that are exposed to a receptive female show increases in dopamine release in the nucleus

accumbens, and males that copulate with females show increases in dopamine release in both the accumbens and the striatum (Damsma et al., 1992; Pfaus et al., 1990; Wenkstern et al., 1993). The level of dopamine release in response to a female as well as during copulation are greater than the level of release on a locomotor task (Damsma et al., 1992)

Dopamine neurons can serve as predictors of rewards. The activity of cells in the SNpc and VTA has been found to increase in response to rewards and reward-related stimuli during learning episodes (reviewed in Shultz, 1998; Schultz et al., 1997). For example, neurons in the SNpc and VTA show phasic activation when animals encounter a hidden food reward (Romo and Shultz, 1990) as well as when they receive an unexpected food reward while learning a task (Ljungberg, et al., 1991, 1992; Mirenowicz and Schultz, 1994; Schultz et al., 1993). Cells in the SNpc and VTA have been shown to shift their responses from rewarding stimuli to conditioned stimuli that predict rewards, or even to stimuli that predict conditioned stimuli (Schultz et al., 1997). Sexual experience reinforces a similar stimulus-response paradigm, as the sensory stimuli associated with a female become predictors of the act of copulation (reviewed in Pfaus et al, 2001). Sexual experience is thought to shape subsequent sexual interactions via the increased dopamine transmission in the striatum and nucleus accumbens during copulation, thereby sensitizing animals to sexual cues, such as a receptive female (Damsma et al., 1991). Thus, sexual experience, and the associated changes in brain and behavior, can be considered a form of reward-related

incentive learning involving long-term synaptic change (reviewed in Beninger and Miller, 1998).

DOPAMINE AND SEX STEROID HORMONES

Sex steroid hormones regulate dopamine synthesis and release in the incertohypothalamic, tuberoinfundibular, mesolimbic, and nigrostriatal dopamine systems. Castration affects the number of TH-ir cells (Brawer et al, 1986; Chu and Wilczynski, 2002), dopamine content and dopamine turnover (Gunnet, Lookingland, and Moore, 1986), and dopamine release (Hull et al., 1997; Mitchell and Stewart, 1989) throughout the preoptic area and hypothalamus as well as in the nucleus accumbens (Mitchell and Stewart, 1989). Implanting castrated males with testosterone restores dopaminergic function to intact levels.

In females, reproductive cycle affects the content and activity of dopamine neurons in areas that receive incertohypothalamic, mesolimbic, and nigrostriatal inputs. The dopamine content of the mPOA is lower during proestrus and estrus, when females are sexually receptive, and dopamine has been found to increase the display of lordosis in females rats and mice through the activation of the progesterone receptor in a ligand independent manner (Mani et al., 1995). The activity and spontaneous release of dopamine from nigrostriatal and mesolimbic

dopaminergic neurons is also altered by reproductive state and changes in sensorimotor function across the reproductive cycle may be due to changes in dopaminergic inputs to the striatum (Becker, 1999).

TYROSINE HYDROXYLASE REGULATION AND DISTRIBUTION

Tyrosine hydroxylase (TH) is the rate limiting enzyme in dopamine synthesis and is therefore highly regulated. The primary means of post-translational regulation is through phosphorylation at three Serine residues (Ser19, Ser31, and Ser40). However, as with other enzymes, TH is also regulated through changes in transcription and translation. Levels of TH mRNA in the striatum are affected by locomotion as well as by salient stimuli and have been correlated with changes in dopamine synthesis and release at target sites. Similarly, decreases in levels of TH protein have been correlated with decreases in tissue levels of dopamine, decreases in basal extracellular dopamine levels as measured by microdialysis, and decreased responsiveness to amphetamine challenge (Skutella et al., 1997). Thus, alterations of TH protein levels may have significant effects on both dopamine synthesis and release as well as on behaviors modulated by dopaminergic inputs.

There is similarity in the topography and distribution of dopamine producing cells and their projections across taxa (Smeets and Reiner, 1994). In vertebrates, there are cell populations in the midbrain, including the SNpc (A9), VTA (A10), and retrorubral fields (A8), the diencephalon, including subthalamic (A11), zona incerta (A13), incertohypothalamic (A14 and A15) and tuberoinfundibular populations (A12), and in the olfactory bulb (A16), and retina (A17). Considerable work has focused on documenting the details of the locations and cell types of these populations within different species, in particular to understand the evolution and homology of different catecholaminergic populations (Smeets and Gonzalez, 2000; Smeets and Reiner, last chapter). However, while there is substantial evidence that similar populations exist between species, it is not apparent whether similar populations are truly homologous or whether they underlie similar behavioral functions. Moreover, the degree to which individual differences in these populations are related to behavioral differences, either within or between species, have not been rigorously investigated.

DISSERTATION SUMMARY

In my dissertation, I address whether individual differences in the display of sexual behaviors are correlated with differences in catecholamine production in

a number of limbic and midbrain nuclei. I investigate this in three model systems: male transgenic mice with a targeted deletion of the progesterone receptor, male whiptail lizards in which there is natural variation in the display of courtship behaviors, and females of two related species of whiptail lizards which differ in their display of sexual behaviors across the ovulatory cycle. These systems afford the opportunity to look at how genotype, hormonal condition, and sexual experience affect both behavioral and neural differences. In addition, in all three systems, behavioral differences between groups may be due to individual differences in the sensitivity or response to the steroid hormone progesterone.

In Chapter two I present data on the effects of genotype and sexual experience on the number of TH-ir cells in limbic and midbrain nuclei in wild-type (WT) and progesterone receptor knockout (PRKO) mice. When sexually naive, WT males had a greater number of cells in the periventricular preoptic area than PRKO males, and this difference disappeared with sexual experience. In contrast, WT males had marginally more TH-ir cells in the SNpc or VTA than PRKO males when sexually naïve, however, sexually experienced PRKO males had a greater number of cells in both nuclei than sexually experienced WT males. There are behavioral differences between the genotypes as well, and genotype differences in the number of TH-ir cells may be associated with genotype differences in behavior.

In Chapter three I approach a similar question in a species with natural variation in progesterone sensitivity and sexual vigor, *Cnemidophorus inornatus*. I investigated whether the number of TH-ir cells in the preoptic area, hypothalamus, or SNpc was affected by long-term housing with a female in males showing different levels of intrinsic sexual vigor. There was a greater number of TH-ir cells in the dorsal hypothalamus of high courting males relative to low courting males. In the SNpc, the number of cells was affected by both sexual vigor and sexual experience. High courting males that were housed with females had a greater number of TH-ir cells in the SNpc than low courting males housed with females. There was no difference in isolate males. Thus, in *C. inornatus* males, midbrain and hypothalamic nuclei are differentially affected by sexual vigor and behavioral experience.

In Chapter four I describe a study looking at the number of TH-ir cells in the preoptic area, hypothalamus, and SNpc of two different species of whiptail lizard. One, *C. uniparens*, is a triploid parthenogen and a hybrid descendent of the second species *C. inornatus*. The study compares cell size and cell number in females from both species across reproductive states. *C. uniparens* individuals have larger somal areas than *C. inornatus* females in all nuclei. However, the effect of cell number was nucleus specific. In the PvPOA and AH, the number of TH-ir cells is higher in *C. inornatus* females than in *C. uniparens* individuals.

However, the species have similar numbers of TH-ir cells in the DH, while in the SNpc the number of TH-ir cells is greatest in post-ovulatory *C. uniparens*. The two species differ behaviorally only when they are post-ovulatory indicating that the difference in the SNpc may be associated with species differences in behavior.

In Chapter five I describe the first study to date investigating whether dopamine affects male-copulatory behavior in reptiles. I injected *C. inornatus* males and *C. uniparens* individuals with different doses of a dopamine agonist and tested them for sexual behavior. The agonist induced mounting behavior in both species. Interestingly, there was a difference in the effective dose: *C. uniparens* required a lower dose of the agonist to increase the display of mounting behavior than did *C. inornatus* males. There was also a species difference in the level of sexual behavior. Overall, *C. inornatus* males were more sexually vigorous when given the effective dose of the D1 agonist than were *C. uniparens* individuals.

In Chapter six I discuss the studies as a whole, providing insight on the similarities in brain-behavior relationships seen across studies. In part, Chapter 6 discusses the finding, demonstrated in all three models, of a relationship between the expression of male-typical sexual behaviors and the number of TH-ir cells in the SNpc.

Chapter 2

Effects of genotype and sexual experience on tyrosine hydroxylase expression in progesterone receptor knockout mice

INTRODUCTION

Dopamine release into limbic nuclei, such as the medial preoptic area (mPOA), and into the striatum and nucleus accumbens, modulates the display of copulatory behaviors in male mammals. Intraperitoneal injections as well as microdialized infusions of dopaminergic drugs affect various aspects of male sexual behavior in mammals, birds, and reptiles (Balthazart, 1997; Bignami, 1966; Bitran and Hull 1987; Butcher et al., 1969; Warner et al., 1991; Woolley et al., 2001). In addition, when presented with a sexually receptive female, male rats release dopamine into the mPOA, striatum, and nucleus accumbens, and levels of dopamine increase even more as males are allowed to interact with and copulate with females (Damsma et al., 1992; Hull et al., 1995; Pfaus et al.,1990). Moreover, males that fail to show the precopulatory surge in dopamine in the mPOA do not copulate (Hull et al., 1995). These data indicate that dopamine is

both sufficient and necessary for the display of sexual behaviors in male mammals.

Progesterone is also involved in the modulation of male sexual behaviors. Exogenous progesterone can reinstate the display of sexual behaviors in castrated male rats (Witt et al., 1995) and lizards (Lindzey and Crews, 1986; 1993). In addition, progesterone also affects cellular morphology and neural differentiation during development (Sakamoto et al., 2001, 2002). Progesterone receptors (PRs) are expressed perinatally in the substantia nigra and ventral tegmental area, and may affect the differentiation of dopaminergic cells (Beyer et al., 2002). In fetal and neonatal rats, males express high levels of PR in the mPOA and the periventricular preoptic area (PvPOA) whereas PR expression is virtually absent in females (Quadros et al., 2002; Wagner et al., 1998), and this difference may, in part, be responsible for the sexual differentiation of those nuclei. Progestrogenic stimulation during development is also important for the masculinization of adult behaviors of male rats and mice. Administration of a PR antagonist to neonatal males abolishes masculine sexual behavior in adulthood (Lonstein et al., 1999). Likewise, male progesterone receptor knockout (PRKO) mice show behavioral deficits on their first sexual encounter relative to wild-type (WT) males (Phelps et al., 1998).

Social experience alters both the expression of copulatory behavior (e.g.,

Dewsbury, 1969) and the neurobiological circuit underlying social behaviors. Sexually experienced males initiate copulation sooner (Dewsbury, 1969), continue to copulate longer after castration (Beach, 1970; Emery and Larson, 1979; Lisk and Heiman, 1980; Manning and Thompson, 1975;Rosenblatt and Aronson, 1957), and are less affected behaviorally by a novel testing environment (Pfaus and Wilkins, 1995) than sexually naive males. Interestingly, the behavioral differences between WT and PRKO mice when sexually naïve disappear with sexual experience (Phelps et al., 1998). Sexually experienced males are also more robust to lesions of neural areas involved in sexual behavior, including the vomeronasal organ (Merideth et al., 1986), medial and centromedial amygdala (Harris and Sachs, 1975; Kondo, 1992), and sexually dimorphic nucleus of the preoptic area (Arendash and Gorski, 1983; de Jonge et al., 1989). One means by which sexual experience could affect both neural circuitry and behavior may be through alterations of the dopaminergic system. Sexual experience reinforces a stimulus-response paradigm, as the sensory stimuli associated with a female become predictors of the act of copulation (reviewed in Pfaus et al, 2001). Moreover, experience may shape subsequent sexual interactions via the increased dopamine transmission in the striatum, nucleus accumbens, or preoptic area during copulation, thereby sensitizing animals to sexual cues (Damsma et al., 1992).

In this study, I assessed differences in the number of dopamine producing cells between NAIVE and EXPERIENCED WT and PRKO males. I hypothesized that the absence of PR in nuclei where dopamine production is significant in the control of sexual behavior, in particular the PvPOA, substantia nigra *pars compacta* (SNpc), and ventral tegmental area (VTA), would produce genotype differences in the expression of tyrosine hydroxylase (TH), the rate limiting enzyme in dopamine synthesis. In addition, I predicted that genotype differences in behavior and TH expression when males were naive would lead to differential effects of experience on TH expression between the genotypes. I found that WT males has significantly more TH cells in the PvPOA and marginally more TH cells in the SNpc and VTA than PRKO males when naïve. In addition, I found that changes in the number of TH-ir cells with experience were both genotype and nucleus dependent, with an overall trend toward fewer TH-ir cells in experienced WT males and more TH-ir cells in experienced PRKO males.

MATERIALS AND METHODS

Animals:

All animals were 50-90 day old sexually naive WT and PRKO male mice

obtained from the colony of Drs. Bert O'Malley and John Lydon at Baylor College of Medicine. Generation of PR deficient mice has previously been described (Lydon et al., 1995). Animals used in this study were approximately F10 of a 129SvEv X C57BL6 background from an initial cross of an F0 male chimera, generated by gene targeting, and a C57BL6 female. This initial cross generated 50% 129SvEv and 50% C57BL6 F1 heterozygotes that were subsequently crossed to generate F2. Generations subsequent to the F2 resulted from matings of either cousins or siblings.

Individuals were housed in separate polyvinyl cages on a 12:12 light:dark cycle with food and water *ad lib*. Experimental protocols adhered to institutional guidelines and NIH Guidelines for the Use of Animals in Research.

Behavior Testing

All males were sexually naive prior to behavior testing. Males were divided into two groups. One group (EXPERIENCED; n= 12/ genotype) received four behavior tests with a sexually receptive female each separated by one week. The second group (NAIVE; n= 8/ genotype) remained sexually naïve during the four-week test period.

Behavioral testing was conducted in the male's home cage under red lights

during the first third of the dark portion of the light:dark cycle. Males in the Experienced group were tested once per week for four weeks with a sexually receptive, prepubertal, CD-1 female mouse (Charles River Labs). Female receptivity was induced with an injection of 3 IU of pregnant mares serum gonadotropin (PMSG; Sigma Chemicals) administered 48 hrs prior to testing and a second injection of 1 IU of human chorionic gonadotropin (HCG, Sigma Chemicals) administered 8-14 hrs prior to testing. On the day of testing, females were screened for receptivity with sexually experienced CF-1 males. Only receptive females, those that were mounted and intromitted by at least two different stimulus males, were used as stimulus females. Males were sacrificed 1 to 3 hours after the fourth behavior test.

Tests lasted either 90 minutes or until the male ejaculated, and for each test we recorded the number of and latency to mount, intromit, and ejaculate. If a male did not mount, intromit, or ejaculate he did not receive a latency score and was given a frequency score of zero for that behavior.

NAÏVE males were housed in the same room as the EXPERIENCED males, were handled in a manner similar to EXPERIENCED males, and were sacrificed at the same time as EXPERIENCED males. However, NAIVE males did not have the opportunity to interact with females between weaning and sacrifice.

Tyrosine Hydroxylase (TH) Immunocytochemistry

At sacrifice, males from all groups were given a lethal dose of sodium pentobarbital (0.65g/kg) and transcardially perfused with 0.9% heparinized saline (50 ml at 8 ml/min) followed by 4% paraformaldehyde in 0.1M PBS, pH 7.4 (250 ml at 8 ml/min). Brains were removed and soaked overnight in 4% paraformaldehyde then soaked for 24 hours in 20% sucrose. Thereafter, they were frozen in isopentane and stored at -80 C. Serial 50 µm sections were cut on a cryostat and four sets of tissue were collected and stored in antifreeze at -20 °C. Immunocytochemistry was performed on one set of free-floating 50 µm sections and therefore sections were 200 µm apart. Sections were rinsed overnight in 0.05 M TBS (pH 7.7), then incubated in 3% hydrogen peroxide and 4% normal goat serum in TBS for 20 min. After blocking for 1 hour in 4% normal goat serum with avidin, sections were incubated for 72 hours at 4 C in primary antibody (1:1000; rat anti-TH, Chemicon International, Temecula, CA) with 4% goat serum, biotin, and TBS. Sections were then incubated in goat anti-rat secondary antibody (1:500; Vector Labs, Burlingame, CA) for 2 hours followed by an incubation in avidin-biotin complex (Vector Labs ABC kit) for 2 hours. Immunoreactivity was visualized using 3,3 diaminobenzedine (DAB, Vector

Labs). Sections were then mounted and dehydrated onto slides and counterstained with cresyl violet. Sections incubated in 4% goat serum in the absence of primary antibody were used as negative controls.

Cell Counting and Analysis

Slides were randomized and coded so that we were blind to the experimental groups (Genotype and Experience). Sections were imaged using a Zeiss microscope fitted with a Ludl Electronic Products MAC 2002 motorized stage (LEP, New York), an Optronics DEI 750 camera (Optronics, California), and a Dell Pentium III XPS B733r computer. We counted the number of TH-immunoreactive (TH-ir) cells in two nuclei in the midbrain, the ventral tegmental area (VTA), and substantia nigra pars compacta (SNpc), and three nuclei in the incertohypothalamic system, the periventricular preoptic area (PvPOA), paraventricular hypothalamus (PVH), zona incerta (ZI). The VTA, SNpc, and PvPOA express PR both perinatally as well as in adulthood and we expected to see genotype differences in these nuclei. Although both the PVH and ZI have been implicated in the display of male sexual behavior, they do not express PR and therefore we did not expect genotype differences in either nucleus. Nuclei were delineated based on cresyl violet staining using Paxinos and Franklin (2001).

For each indivdual, we counted cells unilaterally at 40X in each nucleus on all sections where the nucleus was present (2 to 4 sections per nucleus) using StereoInvestigator software (MicroBrightfield, Vermont). The number of cells on each section were averaged across all sections for each individual.

Statistical Analyses

Behavior

Behavior for the four behavior tests was analyzed using a multivariate analysis of variance (MANOVA) with Genotype (WT or PRKO) and Test (1 to 4) as the independent variables. Male ID was also included as a random variable nested within Genotype; adding this factor eliminates the variability among subjects due to individual differences from the error term (Sokal and Rohlf, 1995; Stevens, 1996). Behavior was the dependent variable, with one MANOVA performed for the latency (mount, intromission, and ejaculation) and one for the frequency (mount and intromission) measures. When the interaction between Genotype and Behavior was significant we performed two-way univariate ANOVAs for each behavioral measure. When there was a significant effect of Test day we performed planned post-hoc contrasts using Student's t-tests

comparing Tests 2, 3, and 4 to Test 1 and set our α to 0.0125 (Bonferroni correction 0.05/4) to account for the increased number of comparisons. Males were only allowed to ejaculate once per test, making the ejaculation frequency an ordinal variable. Consequently, the ejaculation frequency on each test was analyzed separately from the repeated measures MANOVA of other frequency measures. Specifically, we analyzed the proportion of males that ejaculated on each test day using Likelihood ratio tests.

Number of Tyrosine Hydroxylase Immunoreactive Cells in Midbrain and Limbic Nuclei

The number of TH-ir cells was analyzed using a MANOVA with Genotype (WT or PRKO) and Experience (EXPERIENCED or NAIVE) as the independent variables and Nucleus as the dependent variable. Midbrain (SNpc and VTA) and incertohypothalamic (PvPOA, PVH, ZI) nuclei were analyzed separately. When there was a significant Genotype X Experience X Nucleus interaction we performed two-way univariate ANOVAs for each nucleus. When there was a significant Genotype X Experience interaction with either the univariate or multivariate ANOVA we performed planned post-hoc contrasts (EXPERIENCE vs. NAIVE within each genotype and WT vs. PRKO within each experience group). To account for the increased number of comparisons, we

adjusted our α for the post-hoc contrasts with a Bonferonni correction to 0.0125 (0.05/4). For all multivariate tests we used Pillai's trace as our multivariate test statistic because it is robust to deviations in normality (Olson, 1974) and unless otherwise noted we set α= 0.05.

RESULTS

Behavior

There was a significant Behavior X Genotype interaction (Fig. 1; $F(2,36)= 18.58$, $P< 0.001$). Univariate ANOVAs of each behavioral parameter found that there was a significant effect of Genotype only on the ejaculation latency ($F(1,37)= 16.84$, $P< 0.001$). The ANOVA of the intromission latency found an effect of Test day. The intromission latency on test 1 was significantly greater than on tests 2 ($t(3,47)= 3.25$, $P= 0.002$), test 3 ($t(3,47)= 4.01$, $P< 0.001$), and test 4 ($t(3,47)= 3.27$, $P= 0.002$). In the Overall MANOVA, there was also an effect of Genotype ($F(1,37)= 16.64$, $P< 0.001$) on the behavioral latencies. Relative to WT males, PRKO males had higher latencies, driven primarily by differences in ejaculation latency. There was also a significant effect of Test day (F $(3,37)= 5.71$, $P= 0.003$), and post-hoc contrasts revealed that the latencies on

test 1 were significantly greater than on test 2 (F (1,37)= 10.27 P= 0.011), test 3 (F (1,37)= 15.69 P< 0.001), and test 4 (F (1,37)= 10.21 P= 0.003). Finally, there was a significant effect of Behavior (F (2,36)= 49.59, P< 0.001) on the latency measures: ejaculation latencies were greater than intromission or mount latencies.

For the analysis of the frequency measures (the number of mounts and intromissions) there was a significant effect of Genotype (Fig. 2; F (1,66)= 13.36 P< 0.001). Relative to WT males, PRKO males mounted and intromitted more frequently. There was also a significant interaction between Behavior and Test Day (F (3,66)= 5.61, P= 0.002) so we analyzed the effect of time for each behavior separately. Post-hoc contrasts indicated that the number of mounts on test 1 was significantly greater than on test 2 (F (1,66)= 15.10, P< 0.001) and test 3 (F (1,66)= 8.18, P= 0.006). There were no genotype differences on any of the four tests in the proportion of males that ejaculated.

Number of Tyrosine Hydroxylase Immunoreactive Cells in Limbic and Midbrain Nuclei

In the midbrain nuclei, there was a significant Genotype X Experience interaction (Fig. 3; F (1,30)= 10.92, P= 0.003). Post-hoc contrasts, with the adjusted α of 0.0125, revealed that EXPERIENCED PRKO males had marginally more cells than NAIVE PRKO males (F (1,30)= 6.62, P= 0.015), and

significantly more than EXPERIENCED WT males (F (1,30)= 7.23, P= 0.012). NAIVE WT males had greater numbers of TH-ir cells than EXPERIENCED WT males (F (1,30)= 4.48, P= 0.043) and NAIVE PRKO males (F (1,30)= 4.13, P= 0.051) but neither of these reached significance with the adjusted α.

In the analysis of the incertohypothalamic nuclei there was a significant Genotype X Experience X Nucleus Interaction. Univariate ANOVAs analyzing each nucleus individually revealed that there were significant effects only in the PvPOA. In the PvPOA, there was a marginally significant Genotype X Experience (F (1,32), 4.12, P= 0.051) interaction (Fig. 4). Post-hoc contrasts revealed that when naive, WT males had more TH-ir cells than PRKO males (F (2,30)= 8.11, P= 0.008), however, there were no differences between the genotypes with experience. There was also a significant Genotype X Nucleus interaction (F (2,29)= 7.45, P= 0.003) where WT males had a greater number of cells than PRKO males in the PvPOA, but had similar numbers of TH-ir cells in the ZI and PVH. Finally, there was a significant effect of Genotype (F (1,32)= 5.40, P= 0.027) where WT males had greater numbers of TH-ir cells than PRKO males and a significant effect of Nucleus (F (2,29)= 22.54, P< 0.001). The ZI had a greater number of cells than the PVH or the PvPOA.

DISCUSSION

Social and sexual experience can both alter and be altered by an individual's neural and behavioral phenotype. In this study, I investigated behavioral and neural changes in response to interacting with a female in PRKO mice. I found that the absence of PR affected the number of dopamine synthesizing cells in the SNpc, VTA, and PvPOA. In addition, social and sexual experience differentially affected the number TH-ir cells in wild-type (WT) and PRKO males. Taken together, these data indicate the absence of PR, either during development or adulthood, not only results in differences in TH production, but also appears to alter the plasticity of cells in the PvPOA, SNpc, and VTA. Thus, differences in genetic background or compensatory changes in response to gene deletion seem to have resulted in dramatically different neural responses to social environment and behavioral experience. It will be interesting to determine whether it is the absence of PR in adulthood or another mechanism, perhaps in compensation for PR deletion during development, which results in these divergent neural profiles.

Across all four behavior tests, PRKO males had higher copulatory latencies and frequencies than WT males. In addition, WT males decreased, while PRKO males increased the number of TH-ir cells in the SNpc, VTA, and PvPOA with sexual experience. Sexual experience can result in the consolidation

of neural circuits such that the sexual behavior of experienced males is less disrupted by lesions of the vomeronasal pathway than is the behavior of naïve males (Arendash and Gorski, 1983; de Jonge et al., 1989; Harris and Sachs, 1975; Kondo, 1992; Merideth et al., 1986). One possibility is that the trend toward a decrease in TH-ir cell number in the SNpc, VTA, and PvPOA of WT males, as well as the concurrent increase in behavioral efficiency, reflects such neural consolidation. Sexual experience also engenders a robustness to castration, enabling sexually experienced males to copulate longer after castration than sexually naïve males. Unlike WT males, sexually experienced PRKO males do not copulate after castration (Phelps et al., 1998). Taken together, these data imply that while PRKO males show neural changes in response to sexual experience, those changes do not result in the increased copulatory efficiency or the androgen independence of sexual behavior following castration that is typically associated with experience. Whether PR activation is necessary for the neural or behavioral changes seen in WT males in response to sexual experience remains to be investigated.

The PvPOA provides dopaminergic input to the mPOA, a nucleus integral for the display of male sexual behaviors in vertebrates and dopamine release into the mPOA seems to be necessary for the display of male sexual behaviors (Hull et al., 1995). Male rats show increases in dopamine release in response to sexually

receptive but inaccessible females and show even greater increases when allowed to copulate with those females. Males who fail to show increases in dopamine in the mPOA also fail to copulate (Hull et al., 1995). Infusion of dopaminergic drugs into the mPOA can affect both appetitive and consummatory aspects of copulation in rats and quail (Balthazart et al., 1993, 1997; Bitran and Hull, 1987; Pfaus and Phillips, 1991). Lower numbers of TH-ir cells in the PvPOA of sexually naïve PRKO males may be associated with lower levels of dopamine synthesis or lower levels of dopamine release into the mPOA. However, the differences in the PvPOA may not be functionally relevant. Relative to WT males, PRKO males continue to exhibit longer copulatory latencies when sexually experienced, despite similar numbers of TH-ir cells in the PvPOA. Thus, while TH-ir cells in the PvPOA respond to experience in males of both genotypes, the role of these changes in the display of male sexual behavior is still unclear.

Based on the current study we cannot discern the time course or amount of experience necessary for the TH changes. Changes in protein expression, for example the expression of the immediate early gene FOS, can occur with a single opportunity to copulate with a female. The TH gene has a cAMP response element and TH transcription is affected by cAMP *in vivo* (Douglass et al., 1994; Lewis et al., 1987). Because signal transduction pathways can rapidly alter neuronal signalling, it is possible that through the effects of such second messenger systems, changes in TH expression could occur after only minimal

sexual experience. Additional manipulations of social and sexual experience, for example providing males with fewer behavior tests, would provide insight into how rapidly changes in TH expression occur, while increasing the time from the final sexual experience to sacrifice would indicate how persistent the changes in TH are. Finally, it is possible that social or other aspects of interacting with a female, outside of copulation, may have affected dopamine synthesis. Because all males ejaculated on at least one test, we cannot determine which aspects of interacting with a female are paramount to the changes in TH between NAÏVE and EXPERIENCED males.

Progesterone receptor is expressed perinatally in the midbrain (Beyer et al., 2002) and PR peaks at a time when the number and activity of midbrain TH neurons are increasing to adult levels and beginning to form functional contacts in the striatum (Baker et al., 1982). Thus, genotype differences in TH-ir cell number may result as a consequence of the absence of PR during this developmental epoch. Estrogen receptor (ER) expression also increases during this time (Raab et al., 1999), and ER is regulated by and often requires the cooperative action of PR (Fitzpatrick et al., 1999). Estrogen stimulation of midbrain neurons during development affects the differentiation of dopaminergic cells and increases TH mRNA expression (Raab et al., 1995), alters dopamine uptake (Engele et al., 1989), and regulates neurite outgrowth of TH-ir neurons (Reisert et al., 1987).

Thus, in the absence of regulation by PR, ER may differentially affect TH expression or cell morphology during development.

In the PvPOA, PR expression is sexually dimorphic during development, with higher levels in males than in females (Quadros, et al., 2002) and is hypothesized to underlie estrogen-dependent neural masculinization. The differentiation of TH-ir cells is estrogen dependent and estrogen during development decreases the number of TH-ir cells in adulthood (Simerly, 1989; Simerly et al., 1997). Given the role of PR in masculinization, we would predict that PRKO males would have a feminized phenotype, with more TH-ir cells in the PvPOA. Interestingly, PRKO males have lower numbers of TH-ir cells than WT males when sexually naïve, indicating a less feminine phenotype. From the data we cannot discern the role that PR takes in the normal development and differentiation of TH-ir cells. However, it appears that the phenotype resulting from females with naturally low expression of PR during development in the PvPOA is quite different from the phenotype resulting from the deletion of PR within an otherwise male phenotype.

Figure 2.1.

Mount, Intromission, and Ejaculation latencies (minutes) of WT (black bars) and PRKO (open bars) males averaged across all four behavior tests. While the mount and intromission latencies are similar between the genotypes, PRKO males have significantly longer ejaculation latencies. Mean ± SEM.

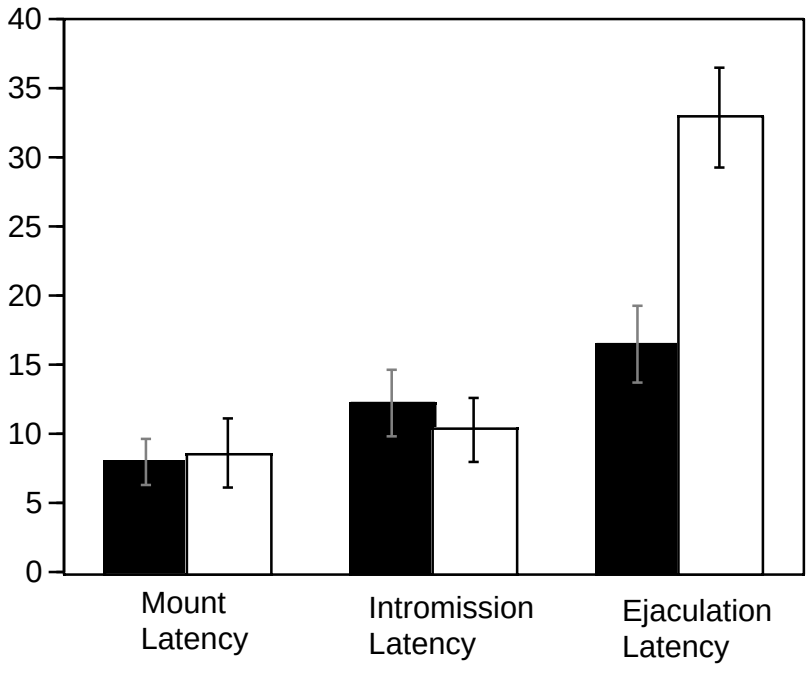


Figure 2.2.

Mount and Intromission frequencies of WT (black bars) and PRKO (open bars) males averaged across all four behavior tests. Relative to WT males, PRKO males mount and intromit significantly more frequently. Mean ± SEM.

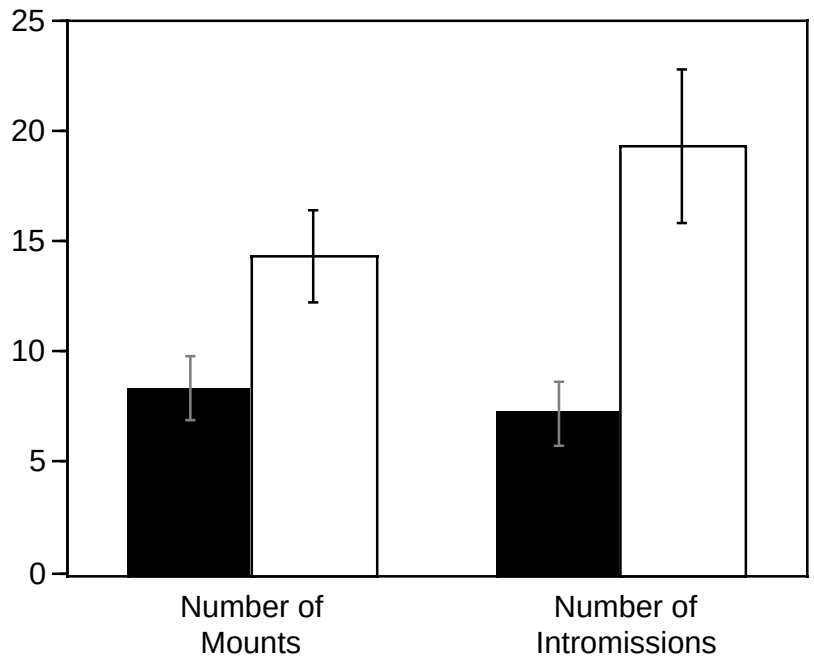


Figure 2.3.

When sexually naïve, WT males (black bars) had a greater number of TH-ir cells in the SNpc than did PRKO males (open bars), although the difference is not significant. However, when sexually experienced, PRKO males had significantly greater numbers of TH-ir cells than WT males. Experienced PRKO males also had greater numbers of TH-ir cells than sexually naïve PRKO males, while sexually experienced WT males had fewer TH-ir cells than sexually naïve WT males. A similar pattern is seen in the VTA (data not shown). Mean $\pm$ SEM. * indicates $P < 0.0125$

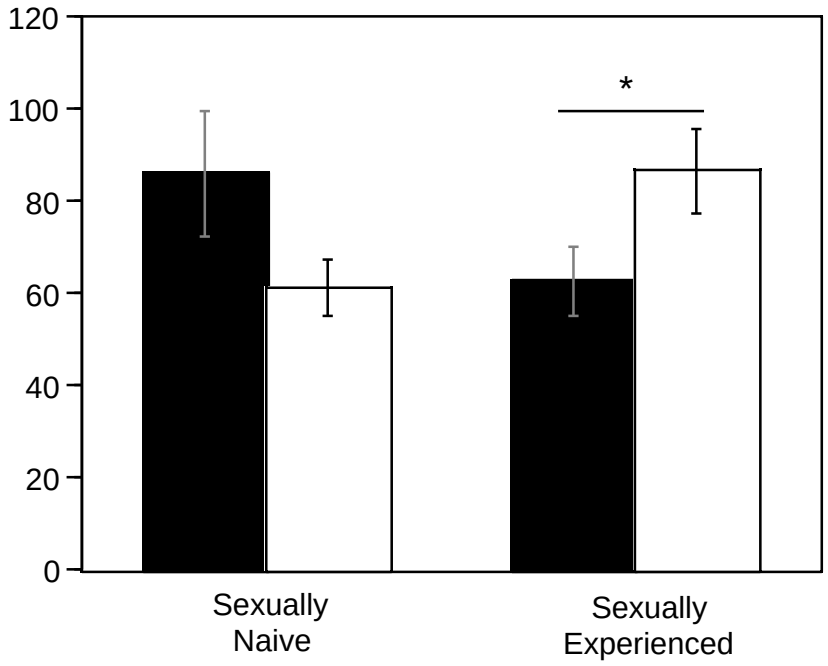
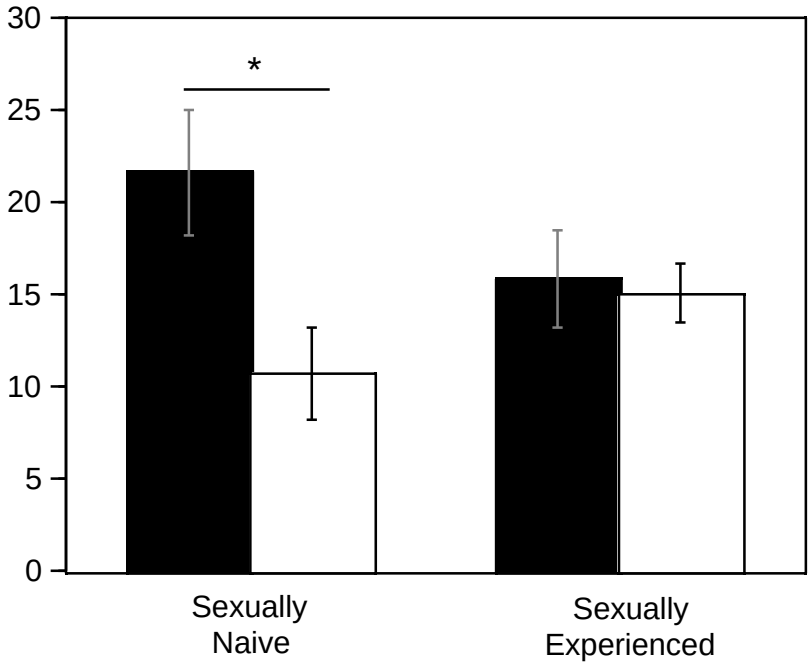


Figure 2.4.

Sexually naïve WT males (black bars) had a greater number of TH-ir cells in the PvPOA than sexually naïve PRKO males (open bars). There was no genotype difference in the number of TH-ir cells between males when sexually experienced. In addition, the decrease in the number of TH-ir cells between sexually naïve and sexually experienced WT males and the increase in the number of TH-ir cells between naïve and experienced PRKO males, are not significant. Mean $\pm$ SEM, * indicates $P < 0.0125$.



Chapter 3

Tyrosine hydroxylase expression is affected by sexual vigor and social environment in male *Cnemidophorus inornatus*

INTRODUCTION

Catecholamines have been implicated in the display of sexual and aggressive behaviors in many vertebrate species. For example, dopaminergic agonists and antagonists, respectively, facilitate and inhibit the display of courtship and copulatory behaviors in rats (Bitran and Hull), quail (Absil et al., 1994; Balthazart et al., 1997), and lizards (Woolley et al., 2001), and dopamine release into the medial preoptic area (mPOA) is critical for the display of copulatory behavior in male rats (Hull et al., 1995). In addition, across taxa there are catecholamine-synthesizing cells in brain areas critical for the expression of sexual behavior, including a number of limbic nuclei (Smeets and Gonzalez, 2000), and catecholamine synthesis is also affected by steroid hormones. In birds, frogs, and rodents, the expression of tyrosine hydroxylase (TH), a rate limiting enzyme in catecholamine synthesis, as well as catecholamine content and

turnover are affected by androgen deprivation and replacement in limbic nuclei implicated in the expression of social behaviors. (Balthazart et al., 1992; Barclay and Harding, 1988, 1990; Gunnet, Lookingland, and Moore, 1986; Mitchell and Stewart, 1989; Simerly, 1989; Chu and Wilczynski, 2002). Adrenal steroid hormones also influence TH expression (Van Loon et al., 1977; Ortiz et al., 1995). Consequently, across a variety of taxa, the production of catecholamines both influences the display of social behaviors and is regulated by steroid hormones.

Changes in dopaminergic systems have been hypothesized to underlie behavioral changes with social experience as well as intrinsic differences between sexually active and sexually inactive males, and *Cnemidophorus* whiptail lizards offer a useful model system in which to investigate these differences. Interactions with females alter the neural phenotype as well as the display of post-castration sexual behavior in *C. inornatus* males (Sakata et al., 2002). Housing males with females also increases corticosterone and decreases androgen concentrations in male whiptail lizards (Lindzey and Crews, 1988) without significantly affecting courtship behavior in intact males (Sakata et al., 2002). Because of these endocrine differences between social housed and isolate males and because social and sexual experiences affect TH expression in mammals (Wommack and

Delville, 2002; Filipenko et al., 2001; Watanabe et al., 1995), it is possible that heterosexual housing can alter the dopaminergic system in whiptail lizards.

In this experiment, we investigated the relationship between sexual vigor, social environment, and TH expression in limbic and midbrain nuclei. We housed sexually active and inactive males either in isolation or with 3-4 intact, cycling females for several months, then looked at the size and number of TH-immunoreactive (TH-ir) cells in three limbic and one midbrain nucleus. Though the distribution of catecholamine-synthesizing cells in a number of reptiles has been described, the functional significance of these populations remains unknown. We anticipated that differences between sexually vigorous and sluggish males as well as between socially housed and isolated males would lend insight into the function of specific cell populations.

MATERIALS AND METHODS

Animals, Housing, and Behavioral Testing

Cnemidophorus inornatus males were collected near Sanderson, Texas during the summer of 2001 under a license from the State of Texas. Males were taken to the lab at the University of Texas at Austin and either housed in isolation

(25 x 32 x 32 cm)(ISOLATE: n=23) or housed with 3-4 intact, cycling females (75 x 32 x 32 cm)(HWF: n=12). In each cage there was a water dish and at least one wood block to allow for retreat from the light. During the summer, individuals were housed on a 14:10 L:D light cycle with temperatures fluctuating from 33 ˚C during the day to 23 ˚C during the night. In November, all individuals were acclimated to conditions resembling hibernation by decreasing photoperiod and temperatures on a weekly basis. During hibernation, males were kept on a photothermal cycle of 8:16 L:D with temperatures fluctuating from 12.5 ˚C during the day to 10 ˚C during the evening. After 10 weeks in hibernation, photoperiod and daily temperatures were gradually increased on a weekly basis until reaching the summer photothermal regime.

Three males were housed with females beginning immediately before emergence from hibernation, and the neural phenotype of these males was not significantly different from those housed with females from the summer. Therefore, we pooled males housed with females during emergence from hibernation together with males housed with females since the summer in our statistical analyses.

Beginning two weeks after the onset of the summer schedule, ISOLATE and HWF males were given five daily tests with a receptive female. Because group cages were substantially larger than the cages in which ISOLATE males

resided, for each screening test HWF males were taken from their group cage and placed into a cage the same dimensions as those of ISOLATE males. To minimize the effects of handling stress on behavior, testing did not commence for at least two hours after the transfer of HWF males. To minimize the effect of novelty on courtship behavior (e.g., Crews, 1974) HWF males were also put in these cages for several hours on two consecutive days before the first day of screening tests to habituate the males to the cage. ISOLATE males were tested in their home cage.

At least 10 minutes before each test, wood blocks and water dishes were removed from the cage. Thereafter, a receptive female was introduced into the cage, and males were watched for three min. Females were first screened for receptivity with a sexually vigorous male. In this species, courting males first approach the female, then mount, and then proceed to grip the neck of the female with their jaws while rapidly undulating their pelvis laterally on top of the female. After one to three minutes of riding the female, males will intromit (Lindzey and Crews, 1986). If the male failed to mount, tests were terminated at three min, but if the male mounted, tests were stopped before the male intromitted. In our lab we have consistently used three minute tests to screen for sexual activity under a variety of hormonal states (e.g., Lindzey and Crews, 1988; Sakata et al. 2002), and in most cases, sexually active males will mount females within one min of the female's introduction (J.T. Sakata and D. Crews, unpublished data). Males that

courted and mounted females on at least 50% of the screening tests were considered sexually active (n=11 for ISOLATE males and n=6 for HWF males), and those that courted and mounted on fewer than 50% of the tests were categorized as sexually inactive (n=12 for ISOLATE males and n=6 for HWF males).

Three weeks following the screening tests, all males were killed by rapid decapitation after mild anesthesia (~1 min in ice). Brains were removed and placed in 4% paraformaldehyde in phosphate buffered saline for 48 hours at 4 °C, then transferred to a 20% sucrose solution overnight. Thereafter, brains were frozen in isopentane and kept at –80 °C until processing.

Tyrosine Hydroxylase (TH) Immunocytochemistry

Serial 60 µm sections were cut on a cryostat and two sets of tissue were collected and stored in antifreeze at -20 °C. One set was used for TH immunocytochemistry performed on free-floating sections. Sections were rinsed overnight in 0.05 M Tris buffered saline (TBS; pH 7.7), then incubated in 3% hydrogen peroxide and 4% normal goat serum in TBS for 30 min at 4°C. After blocking for 1 hour in 4% normal goat serum, sections were incubated for 72 hours at 4°C with a monoclonal primary antibody (1:600, mouse anti-TH,

Chemicon International, Temecula, CA) in 4% goat serum. The antibody has been used in other reptiles (Lopez et al.,1992) and has been demonstrated to react with TH in lizards. Sections were then incubated for 2 hours in a horseradish peroxidase conjugated goat anti-mouse secondary antibody (1:350, Vector Labs, Burlingame, CA) at room temperature. Immunoreactivity was visualized using 3,3 diaminobenzedine (DAB, Vector Labs). Sections were then mounted and dehydrated onto slides and counterstained with a Nissl stain. Sections incubated in 4% goat serum in the absence of primary antibody were used as negative controls.

Cell Counting and Analysis

Slides were randomized and coded so that we were blind to housing condition and behavioral profile. Sections were imaged using a Zeiss microscope fitted with a Ludl Electronic Products MAC 2002 motorized stage (LEP, New York), an Optronics DEI 750 camera (Optronics, California), and a Dell Pentium III XPS B733r computer. We counted the number of TH-ir cells in the periventricular hypothalamus (PvPOA), anterior hypothalamus (AH), dorsal hypothalamus (DH), and substantia nigra pars compacta (SNpc). Nuclei were delineated using Young and Crews (1995) and Smeets and Reiner (1994). All

labeled cells within the nucleus were counted at 40X. For each individual, we counted all cells unilaterally in each nucleus on all sections where the nucleus was present. Two to four sections were counted per nucleus per individual. The number of cells was averaged across all sections for each individual. The somal area of each cell was measured using a nucleator program (MicroBrightfield, Vermont). The Nucleator program requires the user to identify a point associated within the cell, for example the nucleus. From that point a set of rays are extended, and the intersection of each ray with the boundaries of the cell is marked. The somal area is calculated based on these parameters. We marked the center point of eight randomly chosen cells per section, located throughout the entire nucleus, and used eight rays per cell to indicate the boundaries of the cell. We measured cells on two sections per nucleus.

Statistical Analysis

All data on TH-ir cell number and size were normally distributed. Therefore, for each parameter in each nucleus, we analyzed the data using a two-way analysis of variance (ANOVA) with sexual vigor (active vs. inactive) and housing condition (ISOLATE vs. HWF). If there was an interaction between sexual vigor and housing condition, planned post-hoc contrasts were performed

using Studentized t-tests. All statistics were done using JMP 3.2 (SAS Institute) for the Macintosh, and for all analysis, $\alpha = 0.05$.

RESULTS

Description of tyrosine hydroxylase-immunoreactive cells in the whiptail brain

The number of TH-ir cells was highest in the SNpc (X= 49.8 $\pm$ 3.60) and AH (X= 50.9 $\pm$ 2.69) and lower in the DH (X= 26.6 $\pm$ 1.45) and PvPOA(X= 17.2 $\pm$ 0.95). Soma sizes of TH-ir cells were largest in the SNpc (X= 149.0 $\pm$ 3.16), moderate in the DH (X= 113.1 $\pm$ 2.97) and AH (X= 105.9 $\pm$ 1.53), and smallest in the PvPOA (X= 93.2 $\pm$ 1.60).

In the preoptic area, most TH-ir cells were located medially near the third ventricle in the PvPOA. In addition, some cells were found in more lateral positions within the mPOA. Cells in the PvPOA were oriented along the dorsoventral axis and sent TH-ir fibers ventrally toward the supraoptic nucleus, medially toward the third ventricle, or dorsally (Fig. 1 A, 1B).

At the level of the AH, there were two populations of TH-ir cells. One was a ventral population located immediately adjacent to the ventricle that has been hypothesized to be part of the A12 tuberoinfundibular dopamine system. A

second population, which we term the AH population, spread from ventral to dorsal and medial to lateral in a line that followed the lateral edge of the anterior hypothalamic nucleus (Fig. 1C, 1D). Tyrosine hydroxylase-ir cells in this large AH population sent dense projections ventro-laterally into the lateral hypothalamic area. This population is very similar to the group described as the anterior hypothalamus - periventricular hypothalamus – lateral hypothalamic area in *Anolis carolinensis* (Lopez et al., 1992) and to the periventricular hypothalamus of *Gekko gecko* described by Smeets (1986; 1994).

The DH population is located just caudal to the AH population and dorsal to the ventromedial hypothalamus. The population consists of cells located dorsolateral to the ependymal or periventricular organ (PVO) (Fig.1E, 1F). The cells in the DH send TH-ir fibers both medially toward the PVO and laterally toward the lateral hypothalamus. The DH population has been described as the cells "adjacent to the periventricular organ" by Smeets (1994). More recently, it has been argued that the population is the periventricular nucleus of the zona incerta or a caudal periventricular hypothalamic population (Sanchez Camacho et al., 2000, 2001; Smeets and Gonzalez, 2000).

In the midbrain, TH-ir cells were visible near the ventricle, in what is presumed to be the reptilian ventral tegmental area, and lateral to the VTA cells was the SNpc population. In the SNpc, both cells and projections were oriented

ventromedially to dorsolaterally. Tyrosine hydroxylase-ir fibers were visible within and between the SNpc and adjacent VTA and also projected lateral to the SNpc (Fig. 1G, 1H). There was a dense accumulation of TH-ir fibers and varicosities in the striatum and nucleus accumbens, which we presume were fibers of SNpc and VTA neurons (Gonzalez et al., 1990).

Tyrosine hydroxylase-immunoreactive cell counts

In the DH, sexually active males had significantly more TH-ir cells than inactive males ($F(1,31) = 11.0$, $P = 0.002$; Fig. 2). In the SNpc, there was a significant interaction between sexual vigor and housing condition ($F(1,22) = 6.21$, $P = 0.021$; Fig. 2). Sexually vigorous HWF males had significantly more TH-ir cells than sexually sluggish HWF males ($P= 0.021$) and there was a trend for greater numbers of THp-ir cells in sexually active HWF males than sexually active ISOLATE males ($P = 0.08$). There were no significant effects of sexual vigor or housing condition on the number of TH-ir cells in the PvPOA or AH (Fig. 2).

Tyrosine hydroxylase-immunoreactive cell size

In the PvPOA, the somal area of TH-ir cells was significantly greater in ISOLATE males than in HWF males ($F(1,26) = 5.2$, $P = 0.020$; Fig. 3). There was also an interaction between sexual vigor and housing condition on somal area ($F(1,26) = 4.2$, $P = 0.051$; Fig. 3). Sexually inactive males had greater TH-ir somal areas than sexually active males only among HWF males ($P = 0.032$), and only among sexually active males did ISOLATE males have greater somal areas than HWF ($P = 0.006$). There was no significant effect of sexual vigor or housing condition on TH-ir cell soma size in the AH, DH or SNpc, though the effect of sexual vigor approached significance in the AH (inactive > active: $F(1,26) = 3.3$, $P = 0.080$ Fig. 3).

DISCUSSION

Dopamine production and release in limbic brain areas has been found to be critical in the expression of sexual behavior in a variety of vertebrates, including whiptail lizards (Balthazart et al., 1997; Bitran and Hull, 1987; Hull et al., 1995, 1997; Woolley et al., 2001). Therefore, differences in the expression of the rate-limiting enzyme in dopamine production, tyrosine hydroxylase (TH), can have profound implications for the display of social behavior. Here we analyzed

differences in the number and somal area of TH- immunoreactive (TH-ir) cells between sexually vigorous and sluggish male whiptail lizards, *C. inornatus*, either housed in isolation or with females. The number of TH-ir in the dorsal hypothalamic area (DH) and the substantia nigra pars compacta (SNpc) was greater in sexually vigorous males than in sexually sluggish males. In the SNpc, the difference between vigorous and sluggish males was greater among males housed with females (HWF males), suggesting that sexual experience with females drives the difference in TH expression. We also report that HWF males had smaller TH-ir cells in the periventricular preoptic area (PvPOA) than males housed in isolation (ISOLATE males), and this difference was greater in sexually active males.

In the SNpc, sexually vigorous HWF males had more TH-ir cells than sexually sluggish HWF males, and the difference between vigorous and inactive males was minimal in ISOLATE males. Though the SNpc is traditionally thought of as an area primarily controlling motor output, there is evidence that the SNpc and its efferents to the striatum are also involved in the expression of sexual behavior as well as stimulus-reward associations. In rats, dopamine synthesis and release in the striatum increase more with copulation than with locomotion alone (Ahlenius et al., 1984; Damsma et al., 1992). Activity of neurons in the SNpc increases both with the presentation of rewards as well as in response to stimuli

that predict rewards (Schultz 1998; Schultz et al., 1997). Sexually vigorous *C. inornatus* males initiate copulation sooner and are more likely to copulate with a sexually receptive female and vigorous HWF males continue to copulate longer after castration than vigorous ISOLATE males (Sakata et al., 2002). It is possible that sexual experience with females increases the expression of TH in the SNpc and that this increase enhances the reward value of copulatory experiences with females. Further, this heightened reinforcement value with females could lead to the increase in the capacity to court females in the absence of androgens.

Because the difference between sexually vigorous and sluggish males in the SNpc is enhanced when males are housed with females, we propose that this difference might be driven by copulatory interactions with females. HWF males had the opportunity to mount, intromit and ejaculate, whereas ISOLATE males were only allowed to mount females during screening tests. Thus, the difference between high courting males may result from differences in whether males were able to intromit and ejaculate. Whereas sluggish HWF males also had ample opportunity to copulate with females, given their lower level of sexual vigor, they may have copulated less frequently than sexually vigorous males. Intromission and ejaculation experience induce greater changes in motivational and sexual behaviors than mounting alone (Kagan, 1955; Whalen, 1968; Sheffield et al., 1951; Ware, 1968; Lopez et al., 1999) and may have greater effects on neural

phenotype in the SNpc. Copulation increases dopamine synthesis in the striatum and nucleus accumbens (Ahlenius et al., 1984), however it is currently unknown whether dopamine synthesis is differentially affected by certain components of sexual behavior. It is possible that the increase in TH-ir cell number in the SNpc reflect greater copulatory activity, in particular the experience of ejaculation, in high courting HWF males.

The DH is an area that has not previously been implicated in the expression of courtship behavior in reptiles, but here we report that sexually vigorous males, regardless of housing condition, have more TH-ir cells in the DH. In quail, copulation increases FOS expression in the periventricular organ and periventricular hypothalamus, two nuclei that may be similar to the DH in lizards (Meddle et al., 1999). Based on the current results we cannot entirely dissociate whether differences in the number of TH-ir cells in the DH are related to differences in recent behavioral experience acquired during the screening tests or intrinsic differences in sexual vigor. Though we attempted to minimize the influence of recent experience by killing males three weeks after the screening tests, we do not know how long these effects can last. However, despite the dramatic differences in social and sexual experience that result from being housed with females, sexually vigorous HWF and ISOLATE males did not differ in TH expression in the DH. This supports the alternative hypothesis that differences in

TH-ir cell number reflect differences in intrinsic sexual vigor rather than recent experience.

The DH topographically resembles the TH cell population adjacent to the ependymal organ, originally referred to as the "accompanying cell group of the periventricular organ" by Gonzalez and Smeets (1991). This population has been found in birds (Appeltants et al., 2001), other reptiles (Medina et al., 1994), and amphibians (Gonzalez and Smeets, 1991). In amphibians, the TH-ir cell population near the periventricular organ (PVO) has more recently been proposed to be homologous to the mammalian zona incerta (ZI), or A13 population, based on topology, cellular constitution, and similar afferent projections to the spinal cord (Sanchez-Camacho et al., 2000, 2001a, 2001b, 2002; Milan and Puelles, 2000). A similar population in reptiles, termed the rostrodorsal periventricular hypothalamic region, is thought to be homologous either to the caudal portion of the A14 cell population or to the A13 population in mammals (Smeets and Gonzalez, 2000). Lesions of the ZI in male mice result in decreased copulatory behavior without affecting sexual motivation (Maillard and Edwards, 1991; Maillard et al., 1994), and FOS expression in the ZI steadily increases as males are allowed greater copulatory opportunities (Heeb and Yahr, 1996).

Based on sex steroid hormone receptor expression, the DH is more similar to the mammalian dorsomedial hypothalamus (DMH), a nucleus spatially adjacent

to the ZI and part of the A14 cell population. In whiptails, the DH expresses both estrogen and progesterone receptor (ER and PR, respectively) mRNA but not androgen receptor (AR) mRNA (Young et al., 1994). The mammalian ZI expresses high levels of AR and ER mRNA but no PR mRNA (Simerly et al., 1990; Hagihara et al., 1992; Shughrue et al., 1997). In contrast, the DMH expresses ER and PR mRNA but not AR mRNA (Simerly et al., 1990; Hagihara et al., 1992; Shughrue et al., 1997). Interestingly, electrical stimulation of the DMH in male rhesus monkeys results in hypersexual behavior (Perachio et al., 1979). Additional information on the development, chemoarchitecture, connectivity, and function of the DH will provide much needed insight into the proposed homology of the DH, as well as enable interspecies comparisons on the role of dopaminergic cells in the nucleus.

In mammals, the size of the anteroventral periventricular preoptic area (AVPV) as well as the number of TH-ir cells is greater in females than in males (Simerly, 1985, 1989; Simerly et al., 1985a, 1985b, 1997); therefore, less catecholamine synthesis is associated with masculine behavioral phenotypes. Further, repeated sociosexual experiences with females decrease the volume of the AVPV in sexually active but not sluggish males (Prince et al., 1998). This interaction is reminiscent of our finding that the somal area of TH-ir cells in the PvPOA was reduced in HWF males, particularly in sexually vigorous HWF

males. Because there was no difference between sexually sluggish HWF and ISOLATE males, we propose that heightened copulatory experiences with females drive the decrease in cell size. This finding is consistent with the notion that decreased catecholaminergic synthesis is correlated with a masculinized phenotype.

Hormonal differences have been documented between HWF and ISOLATE males (Lindzey and Crews,1988) but not between sexually vigorous and sluggish males (J.T. Sakata and D. Crews, unpublished data). Therefore, differences between HWF and ISOLATE but not between vigorous and sluggish males might be attributed to endocrine differences. Consequently, the diminished somal area of TH-ir cells in the PvPOA in HWF males could be due to higher concentrations of corticosterone and lower concentrations of androgens (Lindzey and Crews, 1988). However, there was also a significant interaction between housing condition and sexual vigor on somal area of TH-ir cells in the PvPOA (as well as cell number in the SNpc), and it is unknown whether there is an interaction between sexual vigor and housing condition on steroid hormone concentrations. Therefore, the role of hormones in generating these neural phenotypes is unknown.

Figure 3.1

Photomicrographs of TH-ir (brown) cells and fibers in male whiptail lizards in the PvPOA (Panels A and B), AH (Panels C and D), DH (Panels E and F), and SNpc (Panels G and H).

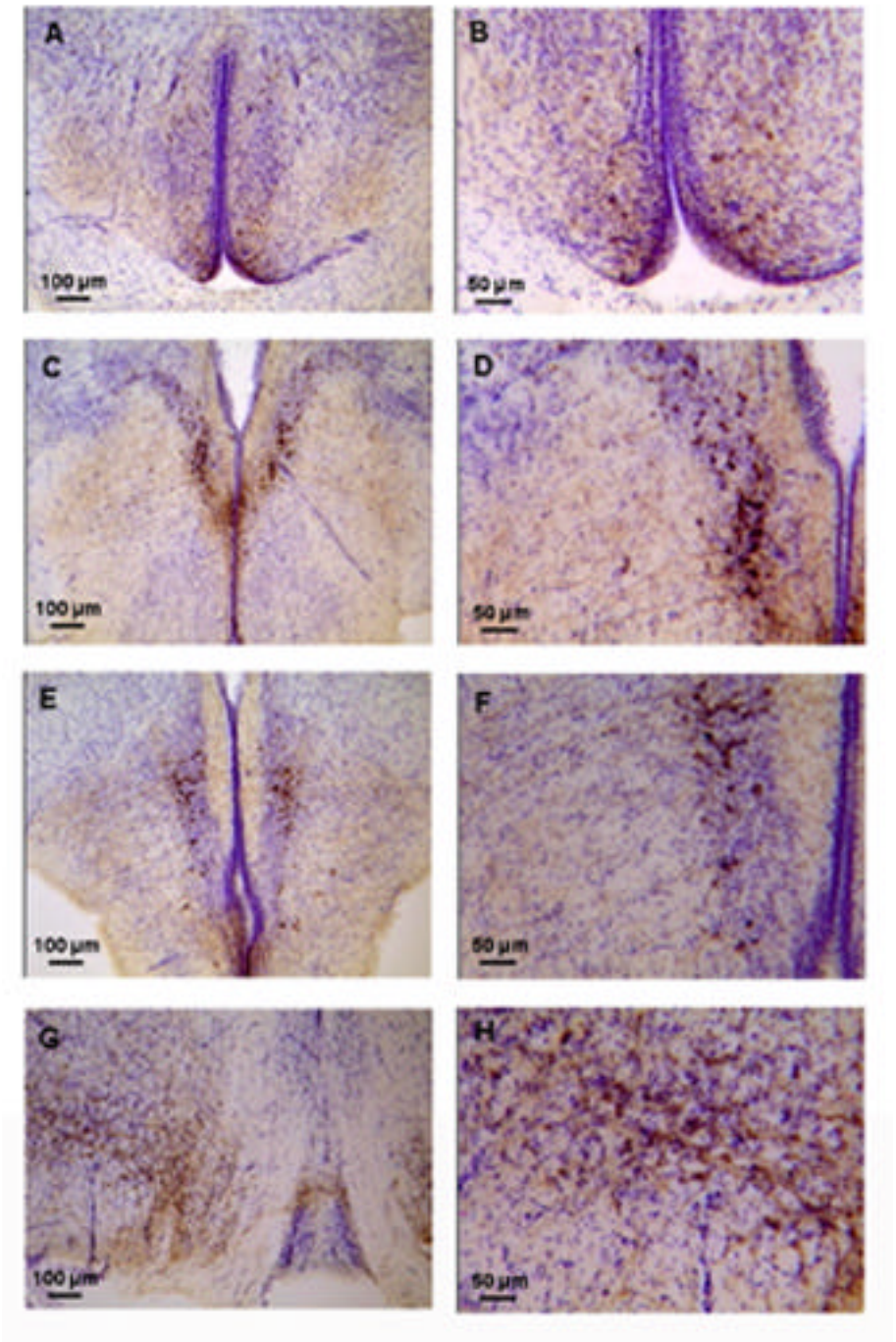

A
100 µm
B
50 µm
C
100 µm
D
50 µm
E
100 µm
F
50 µm
G
100 µm
H
50 µm

Figure 3.2

Effects of social housing (ISOLATE, black bars versus HWF, open bars) and sexual vigor (High versus low courting) on the number of TH-ir cells in the DH. Males that are more sexually vigorous have more cells in the DH than less vigorous males, regardless of housing condition. Mean ± SEM.

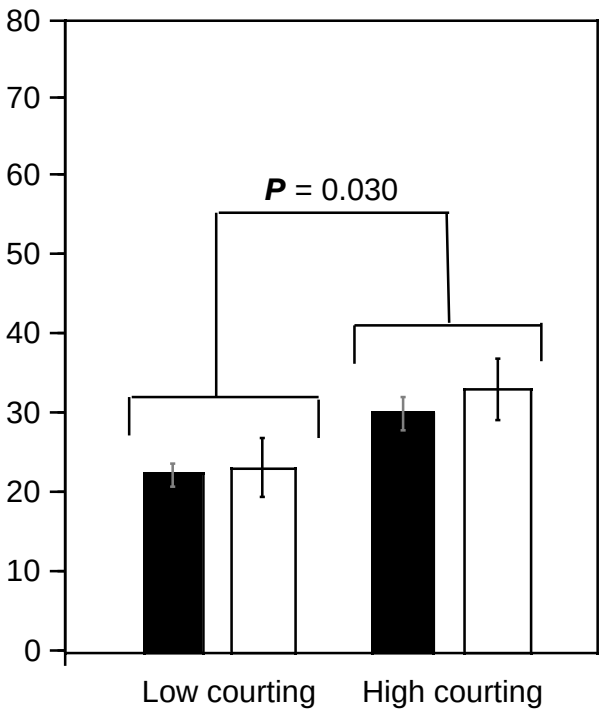


Figure 3.3

Effects of social housing (ISOLATE, black bars versus HWF, open bars) and sexual vigor (High versus low courting) on the number of TH-ir cells in the of TH-ir cells in the SNpc. Sexually vigorous males housed with females have more cells than ISOLATE males or less vigorous HWF males. Mean ± SEM.

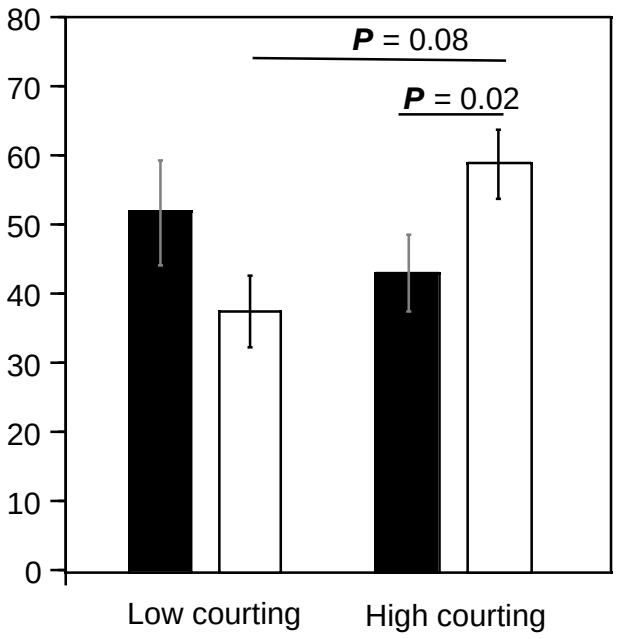
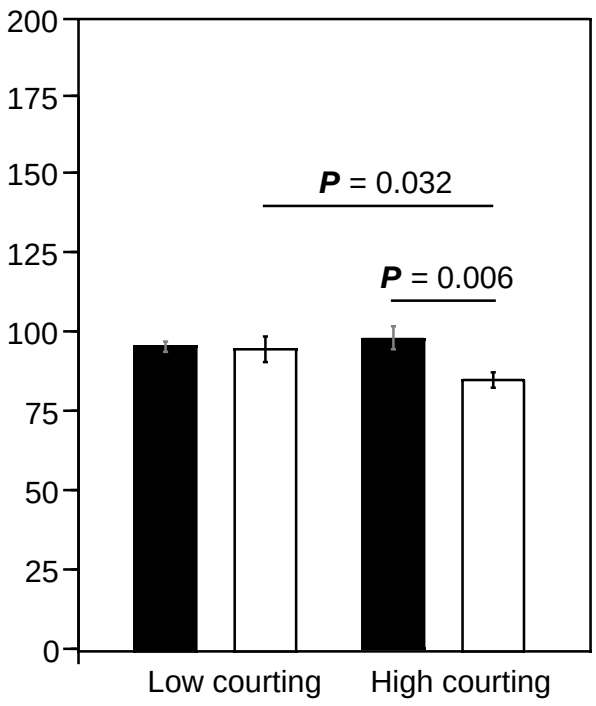


Figure 3.4

Effects of social housing (ISOLATE, black bars versus HWF, open bars) and sexual vigor (High versus low courting) on the somal area of TH-ir cells in the PvPOA. Sexually vigorous HWF males have smaller somal areas in the PvPOA than less vigorous males or ISOLATE males. Mean ± SEM.



Chapter 4

Effects of species and reproductive state on tyrosine hydroxylase expression in a parthenogenetic and a sexual species of *Cnemidophorus* whiptail lizard

INTRODUCTION

Genome duplication has been proposed to release constraints on selection allowing evolution in novel directions (Otto and Whitton, 2000). By virtue of carrying more alleles, polyploid individuals have a greater chance of carrying new, beneficial mutations (Paquin and Adams, 1993). Genome size is positively correlated with cell size across taxa and is inversely correlated with a number of life history traits, including rates of growth (Fankhauser, 1945; Lowcock, 1994), cell division (Bennett, 1972) and metabolism (Goin et al., 1968; Licht and Lowcock, 1991). Thus, genome size may directly affect aspects of cellular morphology either through increases in the quantity of DNA and the necessary transcriptional or translational machinery or through gene dosage effects on the quantity of cytoskeletal, ribosomal or other genes (discussed in Olmo, 1983). Along with effects on cell size, increases in ploidy also alter other aspects of

neural morphology, including the size and amount of branching of dendritic arbors (Szaro and Tompkins, 1987). Such neural morphological changes may ultimately affect brain structure and function and serve as a substrate for the evolution of novel neural or behavioral phenotypes.

Neural and behavioral evolution are often difficult to study because ancestral species are no longer extant. *Cnemidophorus* lizards enable the study of evolutionary processes because new species arise through multiple hybridizations. *Cnemidophorus uniparens*, for example, is a triploid parthenogen that arose through two hybridization events, both involving the sexual species *C. inornatus* (Wright, 1993). Though females of both species show identical hormonal changes across the reproductive cycle, there are considerable behavioral differences between the two. Females of both species display female-like receptive behaviors during vitellogenesis when estrogen levels are rising, however, following ovulation when there is a surge of progesterone, females of the ancestral, sexual species become unreceptive while the parthenogens display male-like copulatory behaviors (reviewed in Crews and Sakata, 2000). By investigating the neural correlates of this behavioral difference, we can elucidate the mechanism underlying the evolution of this behavior.

There is considerable homology in the distribution of catecholaminergic systems across vertebrates (Smeets and Reiner, 1994) and there may be similarity

in the function of those systems, as catecholamines modulate reproductive behaviors in a number of vertebrate species. For example, dopamine agonists affect the display of sexual behaviors in mammals and birds (Absil et al., 1994; Balthazart et al., 1997; Melis and Argiolas,) and stimulation of D1 receptors increases the display of male-like mounting behavior in *C. inornatus* males and in *C. uniparens* individuals (Woolley et al., 2000), and dopamine release into the striatum, nucleus accumbens, and limbic nuclei increases during copulation in rats (Damsma et al., 1992; Hull et al., 1995; Pfaus et al., 1990). Catecholamine systems are also steroid sensitive, with steroid hormones affecting transmitter synthesis and release (Gunnet, Lookingland, and Moore, 1986; Mitchell and Stewart, 1989; Simerly, 1989) as well as the expression of pre- and post-synaptic receptors (Becker, 1999; Hruska and Nowak, 1988; Lammers et al., 1999; Lee and Mouradian, 1999;). Thus, one means by which steroid hormones may regulate the display of reproductive behaviors may be through changes in the synthesis, release, or reception of catecholamines. Because *C. uniparens* and *C. inornatus* differ in the regulation of steroid hormone receptors across the reproductive cycle, species differences in the display of reproductive behaviors could result from the differential regulation of dopaminergic systems by steroid hormones.

We investigated differences in the expression of tyrosine hydroxlase (TH), a rate-limiting enzyme in catecholamine synthesis, in limbic and midbrain nuclei across the reproductive cycle in *C. uniparens* and *C. inornatus* under the hypothesis that ploidy and reproductive state would interact to affect the size and number of TH-ir cells. Work on amphibians has found that cell size predicts cell number in the optic tectum (Roth et al., 1994). Thus, we predicted that there would be larger but fewer TH-ir cells in the triploid parthenogen than in the diploid ancestral species. In addition, we predicted that cell number would be affected by reproductive state, especially in preoptic and hypothalamic nuclei that contain steroid sensitive neurons and are involved in control of the reproductive cycle. We report that while ploidy affected the size of catecholaminergic cells similarly across nuclei, with larger cells in the parthenogen, species differences in the number of cells was nucleus dependent. Interestingly, there were no significant effects of reproductive state on the preoptic or hypothalamic nuclei. Instead, the only nucleus to show both species differences and changes with reproductive state was the substantia nigra *pars compacta*. Moreover, the species differences in catecholamine production in the substantia nigra *pars compacta* were correlated with species differences in the display of male-typical behaviors, implying that this nucleus may serve a novel function in the descendent species.

MATERIALS AND METHODS

Housing

Cnemidophorus uniparens indivduals were collected near Portal Arizona under state permits from Arizona and New Mexico in the summer of 2001. After being brought into the lab at the University of Texas individuals were housed in groups of 4 to 5 in 75 x 32 x 32 cm aquaria. *Cnemidophorus inornatus* females were collected outside Sanderson Texas in the summer of 2001 and 3 to 4 females and 1 male were housed together in 75 x 32 x 32 cm aquaria. Individuals received crickets or mealworms dusted with vitamin powder 2-3 times/week and water was provided *ad libitum.* Each group cage contained wood blocks for shelter. During the summer, all individuals were housed in environmental chambers on a 14:10 L:D light cycle with temperatures fluctuating from 33˚C during the day to 23˚C during the night. In October, individuals were gradually introduced to conditions resembling hibernation by decreasing photoperiod and temperatures on a weekly basis. During hibernation, individuals were kept on a 8:16 L:D photothermal cycle with temperatures fluctuating from 25˚C during the day to 12.5˚C at night. After 10 weeks in full hibernation, photoperiod and daily temperatures were gradually increased on a weekly basis until returning to the

summer photothermal regime. Individuals were housed at summer temperatures for 4 weeks prior to sacrifice to allow sufficient exposure to summer temperature and light cycles for females to become reproductively active.

Animals

Brains were collected from 10 females of each species in each of three reproductive states: pre- or early vitellogenic, mid or late vitellogenic, or post ovulatory. Reproductive state was determined by palpating the ventral side of each individual and was confirmed after sacrifice. The diameters of vitellogenic follicles were measured after sacrifice. Individuals with follicles less than 2 mm in diameter or no follicles were classified as pre-vitellogenic, individuals with follicles 6 to 10 mm in diameter were classified as vitellogenic, and individuals with eggs were classified as post-ovulatory. The snout-vent length (the distance from the end of the nose to the cloaca) of each individual was also recorded prior to sacrifice. Individuals were anesthetized on ice prior to decapitation and all procedures were performed in accord with NIH and institutional guidelines on the care and use of animals and.

Tyrosine Hydroxylase Immunocytochemistry

Brains were soaked in 4% paraformaldehyde in phosphate buffered saline for 48 hours at 4 °C then soaked in 20% sucrose overnight and then frozen in isopentane and stored at –80 °C until sectioning. Serial 60 µm sections were cut on a cryostat and two sets of tissue were collected and stored in antifreeze at –20 °C. One set was used for the tyrosine hydroxylase (TH) immunihistochemistry described in this study.

Immunocytochemistry was performed on free-floating sections. Sections were rinsed overnight in 0.05 M Tris buffered saline (TBS; pH 7.7), then incubated in 3% hydrogen peroxide and 4% normal goat serum in TBS for 20 min. After blocking for 1 hour in 4% normal goat serum, sections were incubated for 72 hours at 4 °C in a monoclonal primary antibody (1:600, mouse anti-TH, Chemicon International, Temecula, CA) with 4% goat serum. The antibody has been used in a number of other studies on reptiles (Lopez et al.,1992) and has been demonstrated to react with TH in lizards. Sections were then incubated for 2 hours in a horseradish peroxidase conjugated goat anti-mouse secondary antibody (1:350, Vector Labs, Burlingame, CA). Immunoreactivity was visualized using 3,3 diaminobenzedine (DAB, Vector Labs). Sections were then mounted and dehydrated onto slides and counterstained with a Nissl stain. Sections incubated in 4% goat serum in the absence of primary antibody were used as negative

controls.

Cell Counting and Analysis

Slides were randomized and coded so that we were blind to species and reproductive state. Sections were imaged using a Zeiss microscope fitted with a Ludl Electronic Products MAC 2002 motorized stage (LEP, New York), an Optronics DEI 750 camera (Optronics, California), and a Dell Pentium III XPS B733r computer. We counted the number of TH-ir cells in the periventricular hypothalamus (PvPOA), anterior hypothalamus (AH), dorsal hypothalamus (DH), and substantia nigra *pars compacta* (SNpc). Nuclei were delineated based on cresyl violet staining using Young and Crews (1995) and Smeets and Reiner (1994). The area of each nucleus was outlined at 4X and the thickness of the section was measured at 40X using StereoInvestigator software (MicroBrightfield, Vermont). All labeled cells within the volume outlined were counted at 40X. For each individual, we counted all cells in each nucleus on all sections where the nucleus was present. Because some sections were lost during staining or were of poor quality, this meant that 2 to 4 sections were counted per nucleus per individual. The number of cells and the volume of the nucleus on each section were averaged across all sections for each individual.

The somal area of each cell was measured using a nucleator program from MicroBrightfield. The Nucleator program requires the user to identify a point associated with the cell, for example the nucleus. From that point a set of rays are extended and the intersection of each ray with the boundaries of the cell are located and marked and the somal area is calculated based on those parameters. We marked the center point of 8 randomly chosen cells per section, located throughout the entire nucleus, and used 8 rays per cell to indicate the boundaries of the cell. We measured cells on at least two sections per nucleus.

Statistical Analysis

We performed two-way analysis of variance (ANOVA) tests with Species and Reproductive State as independent variables and the number of cells in each nucleus (PvPOA, AH, DH, and SNpc) as the dependent variables. To analyze species and reproductive state effects on cell size we performed two-way ANOVAs with Species and Reproductive State as the independent variables and the average somal size as the dependent variable for each nucleus. However, because body, and hence brain size, may affect the size of cells or nuclei, we also included snout-vent length as a covariate for analysis of the size of cells. When the interaction between Species and Reproductive State was significant for either

the analyses of the number or size of cells, we performed planned contrasts within species, between reproductive states, and within reproductive states between species. We used Pillai's trace as our test statistic because it is more robust to deviations in normality (Olson, 1974). For all tests, $\alpha = 0.05$. Data were analyzed using JMP version 3.2 statistical software for the Macintosh.

RESULTS

In all four nuclei, there was a significant effect of Species on the size of cells (Fig. 1 and Fig. 2). *C. uniparens* had larger somal areas in the PvPOA ($F_{1,29} = 13.76$, $p= 0.0092$), the AH ($F_{1,31} = 47.56$, $p= 0.0008$), the DH ($F_{1,34} = 31.33$, $p= 0.0075$), and the SNpc ($F_{1,30} = 21.09$, $p= 0.0007$) than did *C. inornatus* females with body size (snout-vent length) taken into account.

There was a significant effect of Species on the number of cells in the PvPOA and AH (Fig. 2). Overall, *C. inornatus* females had a larger number of cells in both nuclei than *C. uniparens*. In the PvPOA this effect was only marginally significant ($F_{1,36} = 3.67$, $p= 0.063$) while in the AH, there was a significant difference between the species ($F_{1,40} = 8.21$, $p= 0.007$). There was no effect of Species on the number of cells in either the DH or in the SNpc.

There was a significant effect of Reproductive State on the number of cells in the SNpc (Figure 3, $F_{2,34} = 4.28$, $p= 0.022$) as well as a trend toward an interaction between Species and Reproductive State ($F_{2,34} = 2.55$, $p= 0.093$).

Planned contrasts revealed that post-ovulatory *C. uniparens* had significantly more TH-ir cells than pre-vitellogenic ($F_{1,?}$ = 12.03, p= 0.001) and marginally more TH-ir cells than vitellogenic ($F_{1,?}$ = 3.83, p= 0.059) *C. uniparens* individuals and significantly more TH-ir cells than post-ovulatory *C. inornatus* females ($F_{1,?}$ = 5.00, p= 0.032).

DISCUSSION

We found that whereas there was an overall effect of ploidy on the somal area of TH-ir cells, with larger cells in *C. uniparens* in all nuclei measured, the species differences in TH-ir cell number were nucleus dependent. The parthenogen had fewer cells than the sexual species in the PvPOA and AH, a similar number of cells in the DH, and in the SNpc, post-ovulatory *C. uniparens* had a greater number of cells than post-ovulatory *C. inornatus*. These data indicate that ploidy does not have similar effects on the number of TH-ir neurons across different brain areas. Consequently, the process of speciation appears to have differentially affected neurochemical circuits as well as neural morphological parameters. The two species may achieve behavioral differences,

and even behavioral similarities, through variations in connectivity resulting from differences in cell size and cell number.

The SNpc was the only nucleus to show a significant effect of reproductive state on the number of TH-ir cells. *Cnemidophorus. uniparens* and *C. inornatus* females differ behaviorally only during the post-ovulatory state, when *C. uniparens* display male-like mounting behaviors while *C. inornatus* females become unreceptive and more aggressive. The number of TH-IR cells in the SNpc was highest in post-ovulatory *C. uniparens.* While preoptic and hypothalamic nuclei are often implicated in the control of sexual behaviors, recent work indicates that the SNpc is also involved in sexual and motivated behaviors in mammals. In mammals, cells in the SNpc increase activity during locomotion, during copulation, and in response to salient and arousing stimuli and one hypothesis for the function of the SNpc is that it mediates motor responses to primary or secondary incentive stimuli. Thus, the SNpc may shape learning and performance of sexual behaviors in a context-specific manner. To a post-ovulatory *C. uniparens* individual, a sexually receptive female may represent a salient or arousing stimulus. The increase in dopamine production in post-ovulatory *C. uniparens* females in the SNpc may provide either an active or permissive effect on the increase in mounting behavior in *C. uniparens*.

Under this experimental design, we cannot differentiate between whether behavioral differences are driving differences in cell number in the SNpc or whether differences in cell number are producing behavioral differences. Individuals of both species were group housed prior to the study. *Cnemidophorus uniparens* individuals were housed with other *C. uniparens* individuals of different reproductive states and *C. inornatus* females were housed with other females as well as one sexually active *C. inornatus* male. Thus, individuals of both species had the opportunity to display social and reproductive behaviors prior to inclusion in the study. In rats, interacting with a sexually receptive female results in much greater increases in dopamine synthesis and release into the striatum and nucleus accumbens than do either general activity or performance on a running wheel (Ahlenius et al., 1987; Damsma et al.,199?). Thus, we believe that the differences in TH-ir cell number do not reflect differences in general activity between the species but rather are related to more specific behavioral differences, in particular the propensity of the parthenogen to display male-like behaviors.

Studies of the relationship between the estrous cycle and dopaminergic function in the striatum of mammals have found changes in dopamine synthesis and release depending on reproductive state. Female rats have greater dopamine synthesis and release during proestrus and estrous, when females display

proceptive and receptive sexual behaviors, than at other times during the ovulatory cycle. During proestrus estrogen and progesterone levels peak, indicating that changes in dopaminergic tone may result from effects of steroid hormones on dopaminergic cells. Circulating concentrations of estrogen are lower in *C. uniparens* than in *C. inornatus* females and there are species differences in estrogen regulation of ER and PR in limbic nuclei. Additionally, there are differences in the behavioral responses of *C. inornatus* and *C. uniparens* to physiological doses of progesterone. While the distribution of PR and ER in limbic nuclei are similar between the species, the distribution of both receptors in the SNpc is unknown. The difference in the number of cells in the SNpc between *C. inornatus* females and *C. uniparens* individuals in response to reproductive state may result from differences in sensitivity to progesterone, differences in PR expression in the SNpc, or species differences in the PR promoter or transcriptional targets.

While the DH, located just caudal and dorsal to the paraventricular hypothalamus, is clearly recognizable in birds, reptiles and amphibians, which mammalian population is homologous to the reptilian DH is unclear. Moreover, the connectivity and function of the DH are currently unknown in reptiles, making interspecies comparisons difficult. However, one interesting finding is that the number of TH-ir cells in the DH is higher in sexually active *C. inornatus* males

than in males that are less sexually active (Woolley, Sakata, and Crews in preparation). Whether the lack of a difference in cell number between *C. uniparens* and *C. inornatus* females represents an upregulation of TH as a consequence of sexual vigor, behavioral experience, or the utility of a neural circuit underlying male-typical sexual behavior is unknown. It would be interesting to determine whether the number of TH-ir cells in the DH can be affected by recent social or sexual experience in *C. uniparens* and whether the DH is involved in the display of male-typical sexual behaviors in either *C. inornatus* males or *C. uniparens* individuals.

Although there was a trend toward a species difference in the number of TH-ir cells in the PvPOA, there was no effect of reproductive state. Estrogen receptor activation in the PvPOA of adult female rats downregulates TH-mRNA and protein expression (Simerly, 1989). That reproductive state did not influence the level of TH-ir in either species is intriguing and raises the possibility that TH activity in the PvPOA is regulated at some other level, for example through changes in phosphorylation, in order to affect hormonal feedback and gonadotropin secretion. Alternatively, it may be the case that dopamine released from PvPOA cells is not involved in neuroendocrine integration in reptiles as it is in mammals. The reproductive physiology and regulation of steroid hormone receptors and reproductive behaviors by steroid hormones is considerably

different between whiptail lizards and rats and mice (reviewed in Crews and Sakata, 2000). Consequently, it is perhaps not surprising that TH expression does not show similar changes over the reproductive cycle in whiptail lizards and rats.

Somal area is an important determinant of connectivity and is likely to be related to the extent of the dendritic arbor and the size of the neuronal receptive field. In addition, increases in the size or number of neurons are likely to increase the amount of synaptic input to a particular nucleus (Rakic, 1975; Szaro and Tompkins, 1987; Tompkins et al., 1984). Thus, species differences in both the size and number of cells in limbic and midbrain nuclei may have dramatic functional consequences for both neural organization and behavior. The effects of changes in cellular or synaptic organization on behavior have been studied in a number of systems. For example, changes in the morphology, density, and innervation of dendritic spines in the hippocampus have been associated with changes in long-term potentiation, memory, and learning (Meng et al., 2002; Sandstrom and Williams, 2001; Woolley and McEwen, 1992, 1993;). Similarly, seasonal changes in the size, number of neurons, and dendritic and synaptic morphology of vocal control nuclei of songbirds (Brenowitz et al., 1991; Clower et al., 1989; DeVoogd et al., 1985; Hill and DeVoogd, 1991; Johnson and Bottjer, 1995; Nottebohm, 1981; Smith et al., 1995) are correlated with seasonal changes in the ability to produce song (Nottebohm et al., 1986; Smith et al., 1997). Thus,

differences in the size and number of TH-ir cells may contribute to behavioral differences between the species.

Allozyme analysis of polyploid whiptail lizards has found that each of the three sets of chromosomes is actively transcribed at rates proportional to gene dosage (Dessauer and Cole, 1984; Neaves and Gerald, 1968). Thus, it is assumed that there is not inactivation of one set of chromosomes or other compensation for the increase in gene dosage. Supporting this idea, basal transcription rates of progesterone receptor appear to be higher in *C. uniparens* than in *C. inornatus* (Young et al., 1995). Despite the species difference in cell size, the staining intensity of cells in *C. inornatus* and *C. uniparens* appeared similar, indicating that *C. uniparens* individuals likely produce larger quantities of TH. Consequently, changes in cell size, as well as cell number, may alter the level of catecholamine synthesis and release throughout the brain and such changes may be functionally relevant. Individual differences in the level of dopamine release from preoptic nuclei have been correlated with differences in the display of sexual behaviors. For example, in rats the release of dopamine into the medial preoptic area may be necessary for the display of male sexual behavior. Males that fail to increase dopamine release when presented with a sexually receptive female also fail to copulate (Hull et al., 1995).

Figure 4.1

Photomicrographs illustrating the differences in cell size and cell number between *C. uniparens* (A) and *C. inornatus* (B) in the AH. Brown DAB labeled cells are TH-ir while those in purple are stained with cresyl violet.

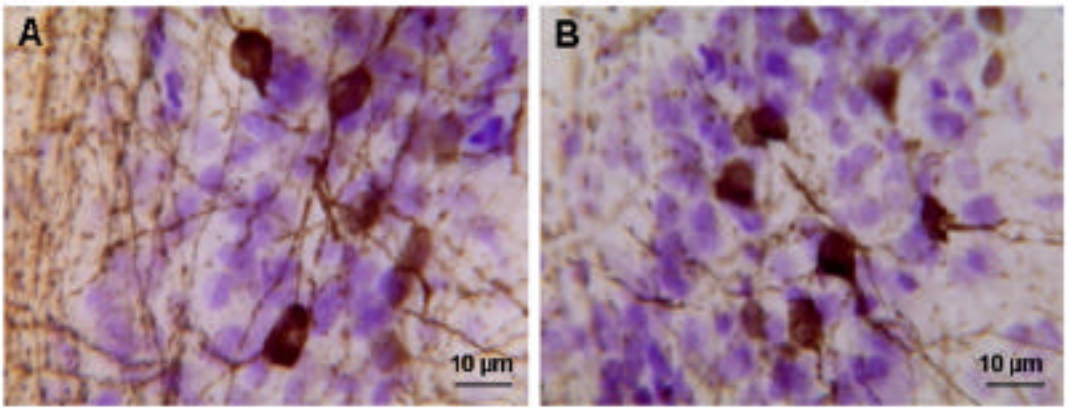


Figure 4.2

Species significantly affects cell size. *C. uniparens* individuals (black bars) have significantly larger somal areas than *C. inornatus* females (open bars) across all nuclei measured. Mean ± SEM. * indicates a significant difference between the species at $p < 0.05$.

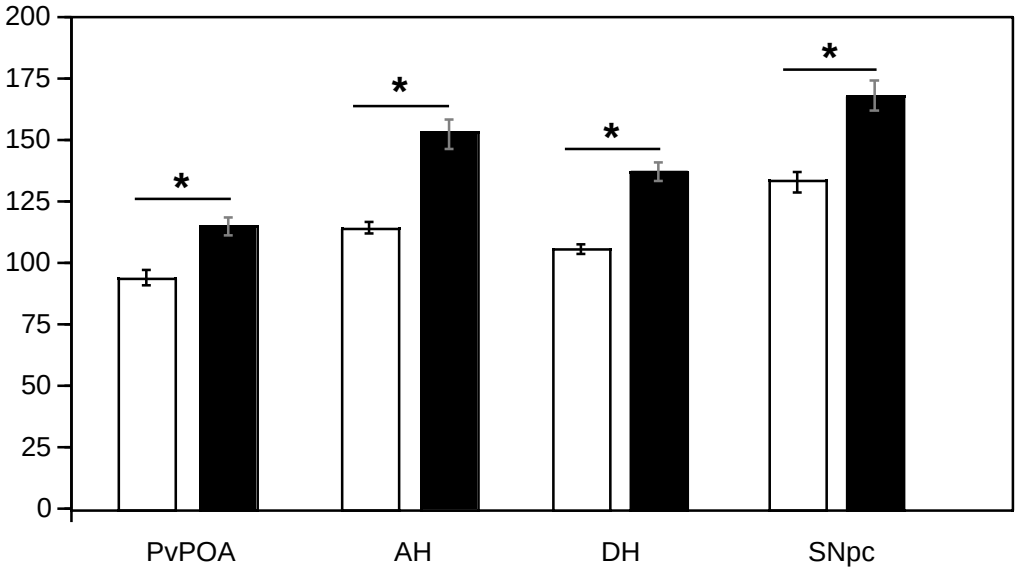


Figure 4.3

C. uniparens females (black bars) have a significantly fewer cells in the AH than do *C. inornatus* individuals (open bars). There is also a trend towards a difference in the number of TH-ir cells in the PvPOA ($F_{1,36} = 3.67$, $p = 0.063$), with more cells in *C. inornatus* females than in *C. uniparens* individuals. Mean $\pm$ SEM, * indicates significant difference at $p < 0.05$, + indicates a trend towards a difference at $p < 0.07$.

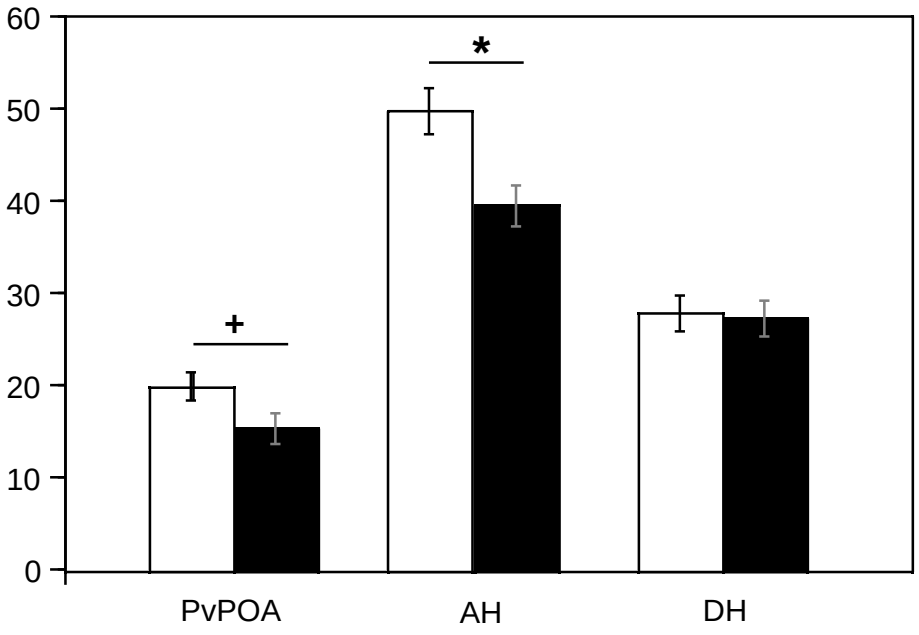
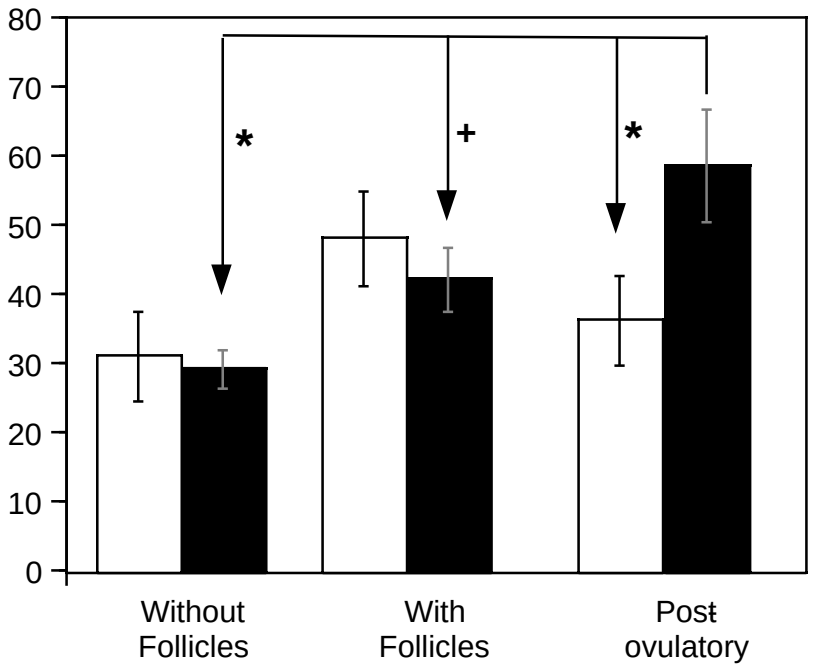


Figure 4.4

Reproductive state differentially affects the number of TH-ir cells in the SNpc. *C. uniparens* (black bars) have significantly higher numbers of TH-ir cells when post ovulatory than *C. inornatus* females (open bars) when post-ovulatory or than pre-vitellogenic *C. uniparens*. There is also a trend toward higher numbers of TH-ir cells in post-ovulatory *C. uniparens* relative to vitellogenic *C. uniparens* individuals ($F_{1,?}$ = 3.83, p= 0.059). Mean $\pm$ SEM, * indicates significant difference at p <0.05. + indicates a trend towards a difference at p < 0.06.



Chapter 5

Evolutionary changes in dopaminergic modulation of courtship behavior in *Cnemidophorus* whiptail lizards

INTRODUCTION

The neurotransmitter dopamine (DA) has been implicated in the regulation of male copulatory behaviors in rodents (reviewed in Melis and Argiolas, 1995). Studies on the role of DA in modulating sexual behaviors have focused on the mesolimbic system, mainly on projections from the A10 cell bodies in the ventral tegmental area to the nucleus accumbens, and the incertohypothalamic system (A12-A15) which sends projections into the hypothalamus and preoptic area (Moore and Lookingland, 1995). Dopamine transmission increases in both the nucleus accumbens (Damsma et al., 1992; Mas et al., 1990, 1995; Pfaus et al., 1990; Pfaus and Phillips, 1991) and medial preoptic area (Hull et al., 1995) when males are presented with a sexually receptive female as well as when males engage in copulation. The rate, efficiency, and probability of copulatory behavior is affected both by peripheral injections and by microinjections into the medial

preoptic area of DA agonists and antagonists (Bignami 1966; Butcher et al., 1969; Bitran and Hull 1987; Hull et al., 1986, 1992; Markowski et al., 1994; Warner et al., 1991).

There are five DA receptor subtypes that can be categorized into two types (D1 and D2) based on pharmacology and second messenger systems (Neve and Neve, 1997). In rodents, D1 and D2 receptor types affect different components of male sexual behavior: D1 type receptor activation facilitates the early stages of copulation while D2 type receptor activation facilitates ejaculation (Moses et al., 1995).

The only non-mammalian species in which the role of DA in copulatory behavior has been studied is the Japanese quail. As in rats (Hull et al., 1997), D1 receptor activation facilitates, while D2 receptor activation inhibits, pre-ejaculatory aspects of copulatory behavior (e.g., mount attempts) in the male quail (Balthazart et al., 1997). Furthermore, there are a number of similarities in the distribution of tyrosine hydroxylase (TH), the DA synthesizing enzyme, between quail and rats. For example, in quail, incertohypothalamic DA cells project into the medial preoptic nucleus, an area critical for the expression sexual behaviors (Bailhache and Balthazart, 1993). In summary, responses to dopaminergic drugs and neuroanatomical distribution of TH are similar between quail and rodents.

Research on DA in reptiles has thus far focused only on the distribution of TH, while the role of DA in sexual behaviors in reptiles is largely unknown. There are a number of dopaminergic nuclei that are potentially homologous to those in mammals. For example, in *Gekko gekko* TH-immunoreactive (TH-IR) cells in the ventral tegmental area project to the nucleus accumbens, and TH-IR cell bodies in both the periventricular preoptic area and the paraventricular hypothalamus send projections into the anterior hypothalamus and preoptic area (reviewed in Smeets 1994). However, relatively little is known about whether anatomical similarity or homology in neurotransmitter systems across vertebrates is correlated with similarity in function. The comparison between rats and quail suggest that this is the case. We hypothesized that, because of the substantial conservation in the role of preoptic, hypothalamic, and amygdalar nuclei in the control of sexual behaviors (Crews and Silver 1985; Meisel and Sachs 1994), similarities in neurochemical distribution are likely to be correlated with similarities in neurochemical function.

In this experiment we assessed the behavioral effects of the full, specific D1 receptor agonist, SKF 81297, on courtship behavior in *Cnemidophorus inornatus* males and *C. uniparens* individuals. Whiptail lizards (genus *Cnemidophorus*) represent a unique system to investigate the evolution of neural foundations of sexual behavior. Approximately one-third of the species in the

genus are parthenogenetic, or all-female, as a result of hybridization events and are direct descendents of extant, sexually reproducing species. In many of the parthenogenetic species, individuals reliably and consistently display both male- and female-typical sexual behaviors. *Cnemidophorus uniparens* is a triploid, unisexual species that arose from two hybridization events, both putatively involving males of the diploid sexual species *C. inornatus* (Wright, 1993). Therefore, through comparisons between these two species, we can directly assess how the brain changed due to this evolutionary transition and how changes in ploidy can affect brain-behavior relationships. We predicted that the agonist would increase the display in copulatory behavior in both species. Furthermore, given previous work that has found differences in sensitivity between the two species in response to steroid hormones, we also predicted that there would be species differences in the sensitivity to the agonist due to ploidy. That is, we hypothesized that *C. uniparens* individuals would require a lower dose of the agonist to induce courtship behavior.

MATERIALS AND METHODS

Animals and Housing

Lizards were captured either in Portal, Arizona (*C. uniparens*) and housed in groups of 4 or 5 in large aquaria (78 x 29 x 29 cm) or were captured in Sanderson, Texas (*C. inornatus*) and housed individually in aquaria divided into compartments (26 x 29 x 29 cm). Throughout the experiment animals were maintained in environmental chambers under breeding conditions as described previously (Wade and Crews, 1991). They were fed 3-5 crickets or mealworms 3 times a week and had water *ad lib*.

Behavior Testing

C. uniparens:

Shortly after arriving in the laboratory from the field (Portal, Arizona), 37 group-housed *C. uniparens* were ovariectomized under cold anesthesia as described in Wade and Crews (1991) and housed in isolation (26 x 29 x 29 cm). One week after surgery, individuals were injected intraperitoneally with 0.5 μg of estradiol benzoate (EB) in steroid suspension vehicle. This injection was given so as to mimic the preovulatory estrogen surge, which is postulated to prime the display of pseudocopulatory behavior (Godwin and Crews, 1999) Forty-eight hours later, individuals received an intraperitoneal injection of either 0.005 (n=8), 0.05 (n=6), or 0.5 (n=12) μg/kg of the full, specific D1 receptor agonist SKF

81297 (hereafter SKF) or vehicle (water; n=11). All doses were prepared immediately prior to injection. Beginning fifteen minutes after the injection we tested experimental individuals in their home cage for one hour with a sexually receptive stimulus *C. uniparens*.

C. inornatus:

Forty sexually active *C. inornatus* males were castrated under cold anesthesia after screening for sexual vigor while intact. Males that mounted females on 3 of 5 consecutive tests while gonadally intact were considered sexually active. After castration, we tested individuals for the loss of courtship behaviors, then implanted them with a Silastic implant (10 mm in length; 1.47 mm inner diameter; 1.96 mm outer diameter) containing progesterone (P) to determine each individuals capacity to display courtship behavior with P implants (i.e., P-sensitivity). Beginning 3 days after implantation, males were given 20 consecutive tests with a receptive female. Individuals were considered P-sensitive if they courted on 3 of 5 tests (floating tally) during the 20 daily tests following P implantation. Only P-sensitive males were used in this study because P-sensitive males are putatively involved in the hybridization processes leading to *C. uniparens* (reviewed in Crews and Sakata, 1999). After P-sensitivity screening, implants were removed, and beginning the following day males were screened for

the loss of sexual behavior. Ten daily tests were administered, and only males that failed to court on at least five consecutive tests were used in this study. Thus, all males in this study were P-sensitive, but sexually inactive when given the DA manipulation. Males were injected intraperitoneally with either 0.005 (n=6), 0.05 (n=8), or 0.5 (n=7) µg/kg of SKF or vehicle (n=6) 15 minutes prior to a one hour behavior test with a sexually receptive *C. inornatus* stimulus female.

Testing of individuals of both species occurred in the individual's home cage under heat lamps between 10:00 and 15:00 when animals are most active. Receptivity was induced in the stimulus animals of both species using 0.5 µg of EB injected 24 hours prior to testing. Stimulus animals of both species were screened for receptivity prior to testing with either a sexually experienced, testosterone-implanted *C. inornatus* male or *C. uniparens* individual. In all of the tests, we recorded the latency to the first approach, mount, and neckgrip. The sequence of courtship behaviors has been described in detail in Lindzey and Crews (1986). Briefly, individuals approach, then mount, then grip the stimulus animal's neck with their jaws while rapidly undulating their pelvis laterally on top of the stimulus animal (termed neck grip). After 1 to 3 minutes of remaining mounted on the female individuals will intromit the stimulus female. In this study, mounts were recorded only when individuals remained straddling the stimulus animal for at least 3 seconds. For each measure, if an individual failed to

perform that particular behavior the maximum latency score (3600 sec) was recorded.

The research presented here was approved by the Institute for Animal Care and Use Committee of the University of Texas at Austin and adhered to the National Institute of Health *Guide for the care and Use of Laboratory Animals.*

Statistical Analyses:

All analyses of the effects of dose were done within each species. The latencies to approach, mount, and neckgrip as well as the interval between approach and mount were analyzed using a nonparametric Kruskal-Wallis test, because of heteroscedasticity in the data, with dose as the independent variable. When there was an overall effect of DA treatment, each dose was compared to the vehicle control using Wilcoxon rank-sum tests. Likelihood Ratio tests were used to analyze the percent of individuals courting at each dose. Finally, to analyze species differences, the latencies to approach and mount were compared between *C. inornatus* and *C. uniparens* given the effective dose using Wilcoxon rank-sum tests. Likewise, the percent of individuals mounting in each species with the effective dose was analyzed with a Likelihood Ratio test. Significance was determined at an alpha level of 0.05.

RESULTS

C. uniparens

There was no significant effect of SKF on approach latencies in *C. uniparens*, suggesting that there were no robust motor side effects of the drug. However, SKF increased the display of mounting behavior. There was an overall effect of SKF on the absolute latency to mount (χ^2_3= 12.405, P= 0.006) as well as on the interval between first approach and first mount (χ^2_3= 12.405, P= 0.006). Post-hoc tests revealed that treatment with 0.005 µg/kg (effective dose) of SKF decreased the absolute latency to mount (Z= -2.489, P= 0.013) as well as the interval between the first approach and the first mount (Z= -2.489, P= 0.013; Fig. 1) relative to individuals treated with vehicle. Other doses of SKF were ineffective at decreasing mount latencies. Likewise, 0.005 µg/kg (effective dose) of SKF increased the proportion of individuals mounting relative to treatment with vehicle (χ^2_3= 8.466, P= 0.004; Fig. 2) while the other doses had no effect on the proportion of animals mounting. There was no significant effect of treatment on neck grip latencies.

C. inornatus

The latency to approach was not affected by SKF in *C. inornatus*. There was an overall effect of SKF on the absolute latency to mount (χ^2_3= 10.279, P= 0.016) as well as the interval between first approach and first mount (χ^2_3= 10.581, P = 0.014; Fig. 1). Post-hoc tests revealed that, relative to vehicle injections, treatment with 0.05 µg/kg of SKF (effective dose) decreased the latency to mount (Z= 2.284, P= 0.022) as well as the interval from first approach to first mount (Z= 2.150, P= 0.032). The other doses of SKF did not significantly affect mount latencies. The dose of 0.05 µg/kg (effective dose) also increased the proportion of individuals mounting relative to vehicle treatment (χ^2_1= 7.686, P= 0.006). Treatment with SKF did not affect neckgrip latencies.

To assess qualitative differences in courtship behaviors elicited by the D1 agonist, we compared the responses of both species at their effective doses (i.e., 0.005 µg/kg for the parthenogen and 0.05 µg/kg for *C. inornatus*). Approach, mount, and neckgrip latencies were not significantly different between the species at the effective doses. However, there was a trend for the interval from first approach to first mount to be shorter in *C. inornatus* males than in *C. uniparens* (Z= 1.652, P= 0.099; Fig. 1) and a trend towards a higher proportion of

C. inornatus males mounting relative to *C. uniparens* individuals (χ^2_1= 2.756, P= 0.097; Fig. 2).

DISCUSSION

In this study, we found that the D1 agonist SKF 81297 increased the display of mounting behavior in two related species of lizard: *C. inornatus*, the ancestral, diploid species and *C. uniparens*, the descendant, triploid species. This is the first experiment, to our knowledge, that demonstrates an effect of DA on courtship behavior in lizards. We found that SKF elicited mounting at a lower dose in *C. uniparens* than in *C. inornatus.* The species also differed in the robustness of their response to SKF, although these differences did not reach significance: overall, a greater percentage of *C. inornatus* males mounted and mounted sooner than did *C. uniparens* individuals. The increase in the display of courtship behaviors in response to a D1 agonist in these species indicates that DA is also significant in the control of sexual behaviors in lizards. Further, the behavioral responses support the hypothesis that similar distributions of dopaminergic cells between mammals, birds, and reptiles may have similar functions across taxa.

Of particular interest is the finding that the parthenogen required a lower dose of SKF to display mounting behavior than male *C. inornatus*. *C. uniparens* individuals are triploid hybrids, and it is speculated that two-thirds of their genome comes from *C. inornatus* (Wright, 1993). Previous work has found that they express higher levels of estrogen receptor (ER) and progesterone receptor (PR) mRNA in hypothalamic and preoptic regions than do either males or females of the ancestral species (Godwin and Crews 1995; Young, Nag, and Crews, 1995a, 1995b). Further, *C. uniparens* are more sensitive to the effects of estrogen on receptive behavior than are *C. inornatus* females: a lower dose of estrogen is required in *C. uniparens* to induce receptivity. It is hypothesized that this heightened sensitivity is due to elevated levels of ER in the parthenogen and that this is directly related to the addition of a third copy of the genome. We propose that the increase in ploidy in C. *uniparens* accounts for the difference in sensitivity to SKF and that D1 receptor expression may be elevated in areas like the preoptic area and nucleus accumbens in *C. uniparens*.

On the other hand, it is possible that the species difference is due to differences in experimental design between the species. For example, that *C. uniparens* individuals were group-housed prior to ovariectomy whereas *C. inornatus* males were always housed in isolation, could have contributed to the heightened sensitivity of the parthenogen. Furthermore, a priming dose of

estrogen was administered to *C. uniparens* but not to *C. inornatus* males 48 hours before the tests with SKF. It is possible that the estrogen injection up-regulated D1 expression in preoptic and hypothalamic areas as has been found in cell cultures (Lee and Mouradian, 1999). However, estrogenic stimulation may not be critical in the dopaminergic modulation of masculine behavior, as ERα knock-out males do not differ from wild-type males in their reactivity to apomorphine (Wersinger and Rissman, 2000).

The difference in DA sensitivity may also be due to the fact that *C. inornatus* males and *C. uniparens* individuals are of different sexes. In rats, males have fewer TH-IR cells in an area of the periventricular preoptic area (pvPOA) than do females (Simerly, Swanson, Handa, and Gorski, 1985). The pvPOA has been implicated in the evolution of male-typical pseudocopulatory behavior in *C. uniparens* (Godwin and Crews 1999; reviewed in Crews and Sakata, 1999), and it is possible that *C. inornatus* males have fewer TH-IR cells in the pvPOA relative to *C. uniparens* individuals.

In this study, we used only *C. inornatus* males that were sensitive to the activational effects of P on courtship behavior (i.e., P-sensitive males). This is because P-sensitive males are hypothesized to have been involved in the hybridization events leading to *C. uniparens* (Crews and Sakata, 1999). We have preliminary evidence that P-sensitivity modulates the capacity of SKF to elicit

mounting behavior, although the sample sizes are small. We found that the effective dose (0.05 µg/kg) used in P-sensitive males did not significantly decrease mount latencies in P-insensitive males (n= 5) relative to males treated with vehicle (n= 5). The effects of lower or higher doses, however, are not known, so we cannot definitively argue that P-sensitive males are more sensitive to SKF than P-insensitive males. Nevertheless, this suggests that P-sensitivity affects DA sensitivity. It is possible that, due to the hybridization events putatively involving P-sensitive males, *C. uniparens* are more sensitive to the D1 agonist because they possess three copies of the genes required to activate courtship behavior with P. Because DA-PR interactions, such as the ligand-independent activation of PR by DA, are known to occur in other species (reviewed in Mani, Blaustein, and O'Malley, 1997), it is possible there exists an interaction between these two systems in the modulation and evolution of male-typical courtship behavior. With the current investigations of the interactions between steroid hormones and neurotransmitters in rodents (e.g., Mani, Allen, Clark, Blaustein, and O'Malley, 1994; O'Malley, Schrader, Mani, Smith, Weigel, Coneely, and Clark, 1995) understanding the evolution of mechanisms for both DA and P sensitivity in whiptail lizards may provide insight into interactions between these systems in other species.

It was surprising that we failed to find a traditional dose response to the agonist in either species. Rather, in both species, only one of the doses was effective in increasing mounting behavior. However, work on other behaviors using a variety of dopaminergic drugs including amphetamine (Taylor and Snyder, 1971) as well as more specific D1 agonists (such as SKF 82958 and SKF 77434; Shelf and Stein, 1992) have found similar all-or-none behavioral responses when using a broad range of doses (e.g. doses separated by a factor of 10), but found more gradual responses in a narrower range. We predict that, for example, doses between 0.005 μg/kg and 0.05 μg/kg in *C. uniparens* and *C. inornatus* will also be somewhat effective in increasing mounting behavior.

Finally, although the SKF was capable of increasing mounting behaviors in both species, there were no increases in the expression of other copulatory behaviors. That there is such a sharp division between the ability of DA to increase mounting but not subsequent behaviors in the hierarchy raises questions about the involvement of other DA receptors or other neurochemical systems. For example, whereas D1 receptor stimulation is important in the expression of the early components of copulatory behavior, stimulation of D2 receptors is important in the expression of ejaculatory behavior in rodents (reviewed in Melis and Argiolas, 1995). Therefore, it is possible that D2 receptor stimulation could be required for neckgrip and intromission behaviors in whiptail lizards. An

additional possibility is that DA alone is not sufficient to increase the expression of all aspects of male copulatory behaviors but rather priming by steroid hormones is necessary to fully facilitate the expression of the entire suite of sexual behaviors.

Figure 5.1

Effects of the full, D1 receptor agonist SKF 81297 on the interval between the first approach and first mount on two related species of whiptail lizard: the ancestral *C. inornatus* and the descendent species *C. uniparens*. The (*) indicates that the treatment significantly decreased the interval from approach to mount relative to vehicle treated individuals at $\alpha < 0.05$.

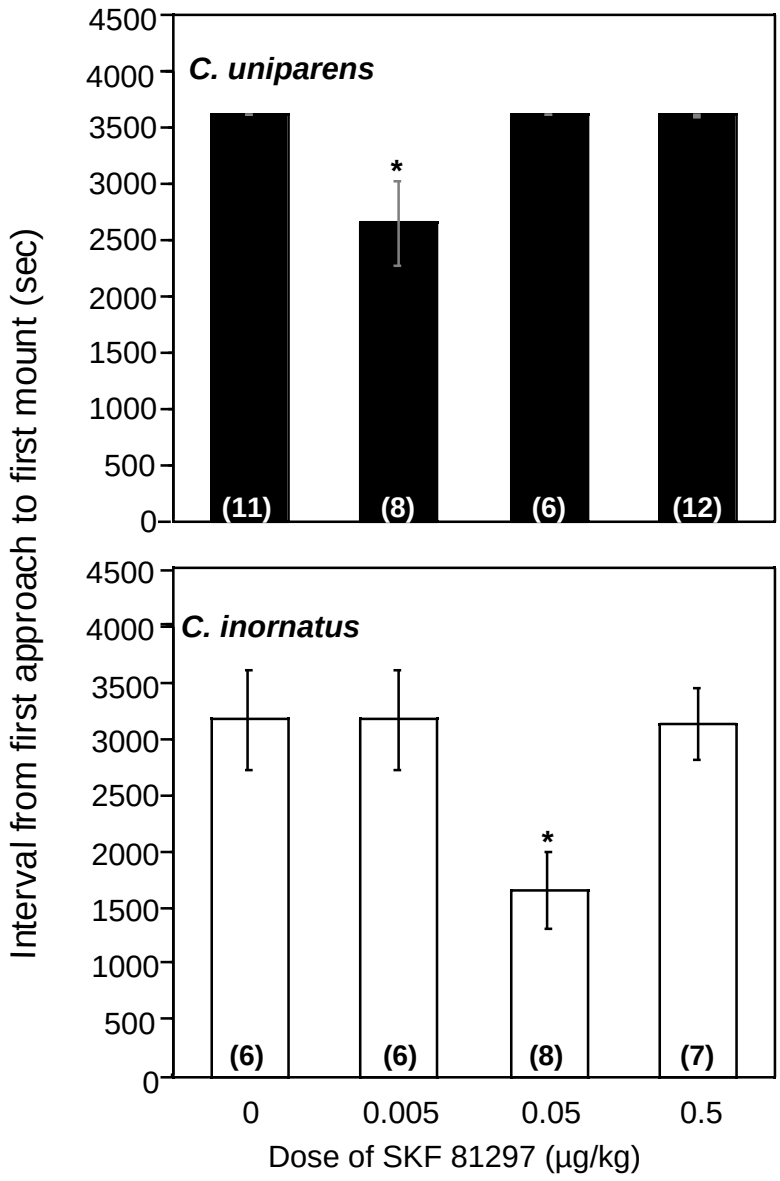
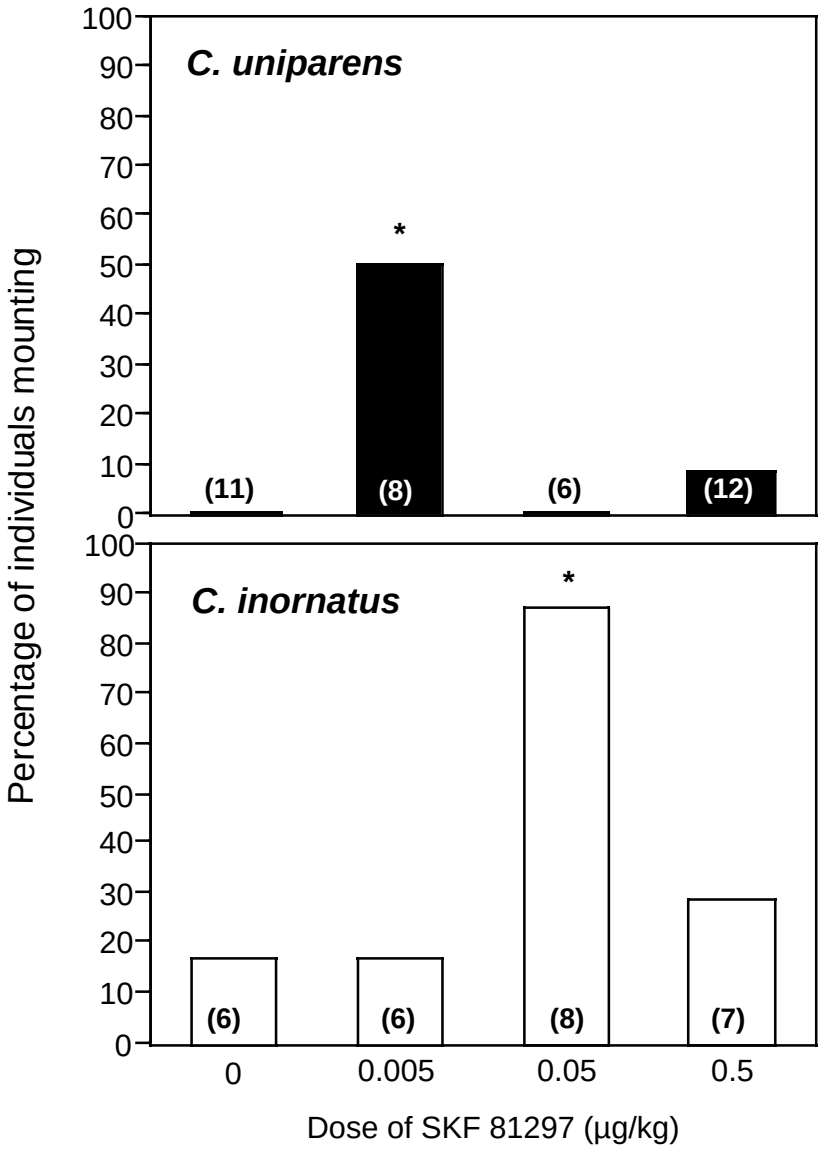

C. uniparens
C. inornatus
Interval from first approach to first mount (sec)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
*
(11)
(8)
(6)
(12)
(6)
(6)
(8)
(7)
0
0.005
0.05
0.5
Dose of SKF 81297 (µg/kg)

Figure 5.2

Effects of the full, D1 receptor agonist SKF 81297 on percentage of individuals mounting on two related species of whiptail lizard: the ancestral *C. inornatus* and the descendent species *C. uniparens*. The (*) indicates that significantly more individuals mounting in that treatment group relative to vehicle treated individuals at $\alpha < 0.05$.


C. uniparens
C. inornatus
Percentage of individuals mounting
Dose of SKF 81297 (µg/kg)
0
0.005
0.05
0.5
(11)
(8)
(6)
(12)
(6)
(6)
(8)
(7)
*
*
100
90
80
70
60
50
40
30
20
10
0

Chapter 6

Conclusion

Social experience can modify both neural and behavioral phenotypes. In general, I am interested in the mechanisms underlying neural and behavioral changes in response to social experiences. Catecholamines, in particular the neurotransmitter dopamine, are significant in the regulation of both social and sexual behaviors in a number of vertebrates. Moreover, through the interaction with steroid hormone systems as well as the activation of DARPP-32 and second messenger pathways, these neuromodulators have the potential to produce long-term changes in cellular responses. Differences in the dopaminergic system may be correlated with differences in behavioral phenotype and may underlie differences in neural and behavioral plasticity. Here, I report on how genotype or hormonal profile, and sexual experience can modify dopamine synthesis in transgenic mice and two species of lizard. There are a number of similarities between the three species in the distribution and regulation of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis. Thus, through comparisons between the three model systems we gain a broader understanding of the relationship between dopamine function and phenotypic plasticity.

SUMMARY OF DATA BY MANIPULATION

Effects of Genotype on Tyrosine Hydroxylase

There were two models where the effects of genotype were assessed: the progesterone receptor knockout (PRKO) mouse and the triploid, parthenogenetic lizard *C. uniparens*. The PRKO mouse enables the investigation of the neural and behavioral consequences of the specific and targeted deletion of a single gene. In contrast, the triploid parthenogen allows the study of the effect of having a third copy of the entire genome. However, despite the dramatic differences in the types of genetic manipulations each system represents, sexual experience and sexual vigor produce changes in similar nuclei in both models, perhaps indicating a similarity in the function or plasticity of those nuclei.

Progesterone Receptor Knockout Mice

When sexually naïve, wild-type (WT) males had more of cells in both the periventricular preoptic area (PvPOA) and Substantia nigra pars compacta (SNpc) than PRKO males. Both of these areas express progesterone receptor (PR) during development and PR may provide trophic support to developing neurons. While

these data implicate PR as significant in the differentiation of TH expression, the knockout model is limited in what we can glean about the mechanism of change. Because the gene is absent throughout development and adulthood, it is difficult to determine when the absence of PR is most deleterious. In addition, there are likely to be mechanisms compensating for the absence of the receptor. Thus is it not possible to disentangle which effects are due directly to the absence of the gene versus those effects that result from changes in response to gene deletion. However, despite the drawbacks of the transgenic model, these data do indicate that PR may be involved in the regulation of TH expression as well as the effects of sexual experience on copulatory behavior.

That there are genotype differences in the SNpc is especially interesting since much of the work on the development of TH neurons in the SNpc has focused on the role of steroid independent genetic effects to set up sexual dimorphisms and at the role of the estrogen receptor in the subsequent neural differentiation (Beyer et al., 1991; Engele et al., 1989; Raab et al., 1995; Reisert et al., 1987, 1990). It has been hypothesized that the differentiation of limbic nuclei is dependent on estrogen regulation of PR (Quadros et al., 2002). Estrogen is thought to be significant in the differentiation of the SNpc (Beyer and Karolczak, 2000; Kuppers et al., 2001), and, given the effects of deleting PR on TH-ir expression in

the SNpc of PRKO mice, there is the possibility that ER and PR interactions are also significant in the SNpc.

Triploid, Parthenogenetic Whiptail Lizard

Ploidy had direct effects on cell size: the triploid parthenogen had larger cells in all nuclei measured than the diploid ancestral species. Previous work by Wade and Crews (1992) found a similar effect on the size of cells in the preoptic area and hypothalamus. Studies on amphibians have found correlations between neural complexity and cell size. That is, in species with larger somal areas, the overall number of cells, as well as the number of cell layers and cells per layer, is smaller (Roth et al., 1994). Such an effect makes intuitive sense, given only modest changes in brain size between species, accommodating larger cells would require decreasing the number of cells in a given space. Thus, I anticipated that if ploidy was the only factor affecting cell number in whiptails, given that somal areas are larger in *C. uniparens* individuals, the number of TH-ir cells in *C. uniparens* would be smaller than in *C. inornatus*. While this effect was true in the PvPOA and anterior hypothalamus (AH), it was not the case in the dorsal hypothalamus (DH) or SNpc. Consequently, while ploidy is clearly a significant factor affecting cell size and number, other factors such as hormonal state or behavioral performance also contribute to the number of TH-ir cells.

Effects of Hormonal State on Tyrosine Hydroxylase Expression

Hormonal changes are likely present in each of the models studied. However, only in the comparison of the two female species is there a direct indication that hormonal state may affect TH expression.

Endocrine Profile and Tyrosine Hydroxylase in Female and Unisexual Whiptails

I used animals in three reproductive states: previtellogenic, vitellogenic, and post-ovulatory. Previtellogenic females have either no follicles or very small follicles; such animals generally have low levels of both estrogen and progesterone and show no reproductive behaviors. Vitellogenic individuals have medium to large follicles, and tend to have high levels of estrogen but low levels of progesterone. Females of both species show receptive behaviors when vitellogenic. Finally post-ovulatory individuals, or individuals with eggs, generally have high levels of progesterone and low levels of estrogen. When post-ovulatory, *C. uniparens* individuals show male-like pseudocopulatory behaviors while *C. inorntus* females are unreceptive.

The only nucleus to show changes in TH expression correlated with reproductive state was the SNpc: post-ovulatory *C. uniparens* had more TH-ir cells than post-ovulatory *C. inornatus* or *C. uniparens* from other stages. Based on these data the mechanism through which progesterone is affecting TH expression is unclear. For example, there may be species differences in the localization or regulation of PR in the midbrain, or a species difference in the TH promoter. Progesterone could thereby differentially affect TH transcription in the SNpc between the two species. While it is known that PR is expressed in the mammalian midbrain (Beyer et al., 2002), we currently do not know whether PR is expressed in the SNpc in whiptails. Alternatively, progesterone may affect TH expression in *C. uniparens* indirectly through effects on behavior. While both species show similar increases in progesterone when post-ovulatory, *C. uniparens* also exhibit a dramatic upregulation of PR mRNA in the PvPOA with estrogen treatment while *C. inornatus* females do not (Young et al., 1995b). The increased expression of PR in the PvPOA has been hypothesized to underlie the display of male-like pseudocopulatory behaviors in *C. uniparens* (Crews and Sakata, 2000), and the increase in behavior could result in increases in TH expression in the SNpc.

Effects of Sexual Experience and Sexual Vigor on Tyrosine Hydroxylase Expression

In the male whiptails and PRKO mice, the two systems in which social experience was manipulated, there were dramatic effects of interacting with females on dopaminergic systems and, in particular, on the expression of TH in the SNpc. Moreover, in both systems, there was an interaction between sexual experience and sexual vigor in the SNpc.

Behavioral Changes and Tyrosine Hydroxylase Expression in the Substantia Nigra

Male WT and PRKO mice were given four opportunities to copulate with females. Across all four tests, PRKO males had higher ejaculation latencies and tended to mount and intromit more frequently. In contrast, WT males became more efficient copulators across the four tests, with lower behavioral latencies and frequencies by the third and fourth tests. The changes in the number of TH-ir cells with sexual experience in the SNpc were consistent with the behavioral differences between the genotypes. Sexually experienced PRKO males had higher numbers of TH-ir cells than sexually naïve PRKOs or than sexually experienced WT males. The converse was the case for sexually experienced WT males who had fewer TH-ir cells than sexually naïve WT males.

Among sexually vigorous *C. inornatus* males, those that were housed with females had more cells in the SNpc. While I did not monitor the sexually activity of males while they were group housed, it is likely that sexually vigorous males that were housed with females had more copulatory experience than sexually vigorous isolate males. In addition, although they had similar opportunities to copulate with females, it is likely that sexually vigorous males housed with females copulated more often than less vigorous group-housed males. Thus, as in male mice, in male whiptail lizards the difference in the SNpc may reflect a difference in the quality and quantity of copulatory experience.

Similarly, although they were not observed for behavior, or screened for sexual vigor, the increase in TH expression in the SNpc of post-ovulatory *C. uniparens* is consistent with results in the male mice and whiptails. Post-ovulatory *C. uniparens*, have a greater propensity to court receptive females and therefore the increase in TH may reflect an increase in the expression of male-like pseudocopulatory behaviors. There is individual variation in the vigor of *C. uniparens* individuals given exogenous steroid hormones and there are likely natural differences in intrinsic sexual vigor as well. It would be interesting to screen *C. uniparens* for sexual vigor and determine whether there is the additional correlation between sexual vigor, behavioral experience, and TH in the SNpc in the parthenogen.

Tyrosine hydroxylase Expression in the Preoptic Area

In the PvPOA, WT male mice had fewer TH-ir cells and therefore lower TH expression when sexually experienced than when sexually naïve. Sexually vigorous HWF male whiptails also had lower TH expression, however, the decrease resulted from a decrease in somal area rather than in the number of cells. The number of TH-ir cells in the AVPV of male rats and mice is dramatically lower than in females (Simerly, 1989). Likewise, the volume of the PvPOA is lower in sexually experienced males than sexually inactive males or female rats (Prince et al., 1998). Thus, the lower number of TH-ir cells in WT males is perhaps indicative of a more masculine neural phenotype.

It was a surprise to find that PRKO males had especially low numbers of TH-ir cells when sexually naïve, which suggests that they had a hypermasculinized neural phenotype. The PvPOA is involved in neuroendocrine control in female mammals, though its role in male mammals is less clear. While we do not know whether WT and PRKO males differ in circulating levels of steroid hormones, it is possible that the especially low number of TH-ir cells in PRKO mice may indicate or result in a perturbation of negative feedback of the hypothalamic-pituitary-gonadal axis. Additional work on the role of TH-ir neurons in either male neuroendocrine functioning or the display of sexual

behavior would provide useful insight into interpreting the difference in TH-ir expression between naïve male WT and PRKO mice.

Tyrosine Hydroxylase in the Dorsal Hypothalamus of whiptail lizards

In the DH, sexually vigorous male whiptails had more TH-ir cells than less vigorous males, regardless of housing condition. In males, therefore, the level of TH expression in the DH appears to reflect differences in sexual vigor independent of sexual experience. Comparing the parthenogen with females of the sexual species, *C. uniparens* also produced larger quantities of TH in the DH. While there was no difference in cell number between the parthenogen and females of the sexual species in the DH, the somal area of cells in *C. uniparens* was significantly larger. It is likely, therefore, that *C. uniparens* are producing, and potentially releasing, larger quantities of dopamine. Reproductive state did not affect the number of TH-ir cells in the DH of *C. uniparens* or *C. inornatus*. Thus, it appears that the level of dopamine production in the DH in *C. uniparens* was not affected by the display of behavior, but as in the male whiptails, is determined by the propensity or capacity to display male-like pseudosexual behaviors. The function of the DH has not been studied in reptiles previously, though these data indicate that greater dopamine production in the DH may be significant in the capacity to display of male-like copulatory behaviors. As the *C.*

uniparens individuals were not screened for sexual vigor, it is not possible to determine whether the relationship between the number of TH-ir cells in the DH and the capacity to display male-like copulatory behaviors would be more prominent in sexually vigorous *C. uniparens* is unknown.

SPECIES SIMILARITIES IN BRAIN-BEHAVIOR RELATIONSHIPS

Across the three species studied, there were correlations between sexual vigor and TH expression in the SNpc. Sexually experienced PRKO mice displayed higher mount and intromission frequencies than WT males and also had greater numbers of TH-ir cells in the SNpc than WT males. Similarly, sexually vigorous male whiptails that were housed with females had greater numbers of TH-ir cells in the SNpc than males housed in isolation or sexually inactive males housed with females. Finally, post-ovulatory *C. uniparens* individuals, which display male-like pseudocopulatory behaviors, that were housed with other *C. uniparens* individuals, had greater numbers of TH-ir cells in the SNpc than post-ovulatory *C. inornatus* females housed with a sexually active male.

However, whether the differences in the number of TH-ir cells in the SNpc result from or produce the differences in behavior in any of these systems is unclear. Copulatory experience has been demonstrated to affect dopamine

synthesis in the SNpc in rats (Ahlenius et al., 1987). At the same time, stimulation of dopamine receptors in the nucleus accumbens and striatum has been demonstrated to affect the display of sexual behavior (Pfaus and Phillips, 1991). One possibility is that behavior affects TH expression, and increases in TH expression alter subsequent behavior. Thus, we would postulate that individuals with greater TH expression would have stronger stimulus-reward associations than individuals with lower levels of dopamine synthesis.

The relationship between behavioral and neural phenotype is less consistent across species in the preoptic area and hypothalamus. Among PRKO males, there was no correlation between behavioral performance and changes in TH-ir cell number with sexual experience; the number of TH-ir cells in PRKO mice changed with sexual experience while the quality of the behavior did not. However, among male WT mice and male whiptail lizards, there were similar effects of behavioral experience on TH expression. In both cases, sexually active males that copulated with females had lower levels of TH than males with limited or no interactions with females. While the role of the PvPOA in the sexual behavior of male lizards has not been studied extensively, these data imply that the PvPOA may underlie similar functions in mice and lizards.

The expression of TH in the dorsal hypothalamus appears to be related to the capacity to display male-typical sexual behaviors. Sexually vigorous male

whiptail lizards have more TH-ir cells than less vigorous males, regardless of housing condition. Similarly, although *C. uniparens* individuals have the same number of cells as *C. inornatus* females, the cells are larger in the parthenogen. Unfortunately, it is still unclear what mammalian nucleus is most homologous to the reptilian DH because of the lack of information on the ontogeny, connectivity, and neurochemistry of the DH (Smeets and Gonzalez, 2000). It would be interesting to measure TH expression in a mammalian system in which the differences in sexual vigor are more pronounced than they are in WT and PRKO mice. For example, a mammalian system in which a subset of males fail to copulate may be more comparable to the differences in sexual vigor present in the whiptails. Such a system may prove useful in investigating nuclei related to the reptilian DH.

MECHANISMS OF CHANGE IN THE NUMBER OF TYROSINE HYDROXYLASE CELLS

Ontogeny

Steroid hormone receptors, acting as transcription factors, affect neural differentiation during development. Progesterone receptor is expressed as early as embryonic day 15 in mice (Beyer et al., 2002) and may affect neural

differentiation as well as provide trophic or protective support during development (Sakamoto et al., 2001, 2002). In the mouse model, there were genotype differences in the expression of TH in sexually naïve males. One possibility is that these differences arose as a consequence of the absence of PR during development. While I cannot pinpoint the time when the absence of PR produces genotype differences, given the role of PR in neural differentiation during development (Beyer et al., 2002; Quadros et al., 2002; Sakamoto et al., 2001, 2002), it is possible that the absence of PR early in development contributes to genotype differences in adults.

Puberty is thought to be a critical period for the fine-tuning of the organization of male reproductive behavior by steroid hormones. During puberty there are increases in steroid hormone production and release as well as neural structural changes that may be necessary for the display of sexual behaviors in adulthood (Romeo et al., 2002). Whether concentrations of steroid hormones are aberrant in PRKO males either in puberty or in adulthood is currently unknown. However, compromises in steroid hormone release due to the absence of PR, either during puberty or adulthood, could contribute to the genotype differences in TH expression.

Immediate Effects on Tyrosine Hydroxylase Regulation

Alterations of TH protein levels may have considerable effects on both dopamine synthesis and release as well as on behaviors modulated by dopaminergic inputs.

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in dopamine synthesis and is therefore highly regulated through transcriptional and translational mechanisms and post-translational phosphorylation. Levels of TH mRNA and dopamine synthesis and release in the striatum are affected by locomotion as well as by salient stimuli (Ahlenius et al., 1987; Damsma et al., 1992; Louilot et al., 1991; Pfaus et al., 1990). Similarly, the number of TH-ir cells in the SNpc is correlated with TH activity in the SNpc and striatum (Baker, 1982). Decreases in TH protein have been correlated with decreases in tissue levels of dopamine, decreases in basal extracellular dopamine levels as measured by microdialysis, and decreased responsiveness to amphetamine challenge (Skutella et al., 1997).

The performance of sexual behavior results in increased dopamine release into the striatum, nucleus accumbens, and mPOA, as well as increased dopamine synthesis in striatal neurons (Ahlenius et al., 1987; Damsma et al., 1992; Hull et al., 1995; Pfaus et al., 1990; Vega- Matuszczyk et al., 1993) . As levels of DA are depleted it may be the case that TH is upregulated to compensate for the increased

release. However, it is somewhat surprising that there are such robust changes in the level of TH protein. Relative to the rapid changes in TH activation through effects on phosphorylation, changes in TH protein levels would likely take longer to establish and erase. Thus, it is possible that the differences in TH described here represent long-term changes in the level of the enzyme. While mechanisms of TH regulation by changes in phosphorylation have been investigated in mammals (Lindgren et al., 2001), the mechanisms underlying the changes in TH translation or transcription, in reptiles are unknown. Given that social interactions often produce changes in steroid hormone levels, the changes in TH seen in these studies may, in some instances, reflect changes in endocrine systems.

In *C. uniparens* individuals and a subset of *C. inornatus* males, progesterone facilitates the display of male-like copulatory behaviors, while in *C. inornatus* females, progesterone does not elicit the display of male-like copulatory behaviors. One hypothesis has been that the hybridization events leading to the generation of the triploid parthenogen involved progesterone sensitive males (Crews and Sakata, 2000). Subsequent studies of sex and species differences in the volume of limbic nuclei as well as the regulation of steroid hormone receptors have found that *C. uniparens* individuals have a feminized phenotype, more similar to that of *C. inornatus* females than *C. inornatus* males (Crews et al., 1990; Wade and Crews, 1991a, 1991b; Young and Crews, 1995a, 1995b). The

studies presented here indicate nuclei in which there are similar changes with sexual vigor and sexual experience between *C. inornatus* males and *C. uniparens*. Thus, in addition to co-opting the surge in progesterone to increase the display of male-like pseudosexual behaviors, *C. uniparens* also appears to have acquired a masculinized expression of TH in areas that are significant in the display of sexual behaviors in males. In rats, it is known that progesterone can affect TH transcription in females through effects on phosphorylation of the cAMP response element binding protein (pCREB; Gu and Simerly, 1996). Whether progesterone is involved in the changes in TH expression in the SNpc of either whiptail species is unknown, but it would be interesting to investigate whether the similar changes in TH expression seen in the two species are regulated by similar mechanisms.

FUTURE DIRECTIONS

Hormonal Versus Behavioral Effects on Tyrosine Hydroxylase Regulation

In both the male and parthenogenetic whiptails, endocrine changes may have affected TH expression in the SNpc. Levels of corticosterone increase while levels of testosterone decrease in males housed with females (Lindzey and Crews,

1988), while in the parthenogen levels of estrogen and progesterone fluctuate across the ovulatory cycle. However, whether these endocrine changes directly affect TH expression, or affect behavior which in turn may affect TH expression is unknown. To distinguish between these possibilities, we could measure TH expression in males housed in isolation but given extensive opportunities to interact and copulate with females, thereby increasing behavior without altering endocrine state. To address this question in the unisexual species, we can implant *C. uniparens* individuals with either testosterone or progesterone, both of which elicit male pseudosexual behavior, and provide individuals with either extensive sexual experience, or leave them in isolation. Comparison of TH expression under the two hormonal treatments and housing condition would provide insight into whether behavioral performance or hormonal stimulation underlie changes in TH expression in intact animals.

Changes in Dopamine Synthesis and Learning

In mammals, the SNpc has been implicated in behavioral plasticity, in particular reward-related and motor learning (Schultz et al., 1997), and dopamine synthesis in the striatum and the activity of cells in the SNpc are affected by both locomotion and the presentation and acquisition of rewards (Ahlenius et al., 1987;

Schultz et al., 1997). However, whether the SNpc serves a similar function in non-mammalian species has received limited attention. In addition, whether the changes in TH that occur with sexual learning in the whiptail will affect the capacity for other forms of learning is unknown. It would be interesting to determine whether changes in TH expression through differences in sexual experience and sexual vigor affect not only the subsequent performance of sexual behaviors, but also other forms of reward-related learning. For example, whether the higher TH expression in sexually vigorous HWF males facilitates performance in conditioning paradigms involving food, heat, or other non-sexual rewards.

Regulation of Dopamine Receptors

While increases in the expression of TH are likely to be correlated with increases in dopamine synthesis or release, whether the TH changes that are described here result in changes in neurotransmission still needs to be tested. While voltammetry or micridialysis may not be feasible given the size of the brain in whiptails, tissue punches of discrete brain areas combined with high pressure liquid chromatography may provide insight into whether there are also changes in dopamine levels and release. In addition, there is often co-regulation of the pre-synaptic release of neurotransmitters and post-synaptic expression of receptors.

Investigation of the regulation of D1 receptors by social experience, sexual vigor, or hormonal state may provide better understanding of how changes in TH affects dopaminergic communication between cells. Furthermore, based on behavioral responses to treatment with a D1 agonist I would expect species differences in the expression of D1 receptors due to differences in ploidy.

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S. C. Woolley, J. Lydon, B. W. O'Malley, and D. Crews. Tyrosine Hydroxylase Expression Affected by Genotype and Sexual Experience in Mice Lacking Progesterone Receptor. Under Revision for Behavioral Neuroscience.

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