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Androgens coordinate neurotransmitter-related gene expression in male whiptail lizards

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Sex steroid hormones coordinate neurotransmitter systems in the male brain to facilitate sexual behavior. Although neurotransmitter release in the male brain has been well documented, little is known about how androgens orchestrate changes in gene expression of neurotransmitter receptors. We used male whiptail lizards (*Cnemidophorus inornatus*) to investigate how androgens alter neurotransmitter-related gene expression in brain regions involved in social decision making. We focused on three neurotransmitter systems involved in male-typical sexual behavior, including the *N*-methyl-D-aspartate (NMDA) glutamate receptor, nitric oxide and dopamine receptors. Here, we show that in androgen-treated males, there are coordinated changes in neurotransmitter-related gene expression. In androgen-implanted castrates compared with blank-implanted castrates (control group), we found associated increases in neuronal nitric oxide synthase gene expression in the nucleus accumbens (NAcc), preoptic area and ventromedial hypothalamus, a decrease of NR1 gene expression (obligate subunit of NMDA receptors) in the medial amygdaloid area and NAcc and a decrease in D1 and D2 dopamine receptor gene expression in the NAcc. Our results support and expand the current model of androgen-mediated gene expression changes of neurotransmitter-related systems that facilitate sexual behavior in males. This also suggests that the proposed evolutionarily ancient reward system that reinforces sexual behavior in amniote vertebrates extends to reptiles.

Keywords: Androgens, dopamine receptors, glutamate, neuronal nitric oxide synthase, sexual behavior

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Sexual behavior by most vertebrate males is dependent on androgens, being eliminated by castration and reinstated by testosterone (Hull 2011). The preoptic area (POA) is necessary and sufficient for reinstatement, as lesions abolish sexual behavior in intact males while preoptic testosterone implants reinstate sexual behavior in castrated mammals (Hull & Rodríguez-Manzo 2009) and reptiles (Godwin & Crews 2002; Kingston & Crews 1994). Hull and Dominguez (2006) suggested that sexual behavior in male rodents is mediated by glutamate-induced dopamine release in the POA. This glutamatergic information from the medial amygdala stimulates dopamine release in the POA via *N*-methyl-D-aspartate (NMDA) receptors (Dominguez *et al*. 2007). This dopaminergic response is facilitated by nitric oxide in the POA, whose production is determined by neuronal nitric oxide synthase (nNOS) levels (Dominguez *et al*. 2004). Thus, androgen-induced mating behavior in male rats seems to depend on the coordinated actions of nNOS, the NMDA receptor and dopamine receptors in the POA. To what extent the action of neurotransmitters involve reinforcement in other brain regions, or how well conserved these mechanisms are across vertebrates, is unclear.

Whiptail lizards (genus *Cnemidophorus*) are a model system for studying the evolution of sexual behavior (Crews 2005). The neural mechanisms of sexual behavior in male whiptails are similar to those observed in mammals (Woolley *et al*. 2001). For example, nNOS is necessary for sexual behavior in male whiptail lizards (Sanderson *et al*. 2006), similar to male mammals (Sato *et al*. 2005). There has been little work, however, outside of the hypothalamus in this species, despite the importance of other forebrain structures in mediating sexual motivation (O'Connell & Hofmann 2011; Wade 2011).

This study tests the hypothesis that nNOS, dopamine receptors and NR1 (obligate NMDA receptor subunit) are differentially expressed between androgen-implanted and blank-implanted (control) males in androgen-sensitive brain regions important for sexual behavior and motivation (Young *et al*. 1994). We analyzed gene expression in the medial amygdaloid area and POA, as sensory information from the medial amygdala is integrated in the POA to regulate copulatory behavior in male rats (Hull & Dominguez 2007). In mammals, the POA projects to the ventral tegmental area (VTA) (Hull & Dominguez 2007), which along with the nucleus accumbens (NAcc) comprises the main mesocorticolimbic dopamine axis. To determine whether this reward pathway also plays a role in mating behavior in male reptiles, we measured candidate gene expression in the NAcc. We also measured gene expression in the ventromedial hypothalamus (VMH), as lesions of the VMH increase copulatory behavior in male rats (Christensen *et al*. 1977) but decrease courtship in the green anole lizard (*Anolis carolinensis*; Farragher & Crews 1979). Finally, we measured candidate gene expression in the basolateral amygdaloid

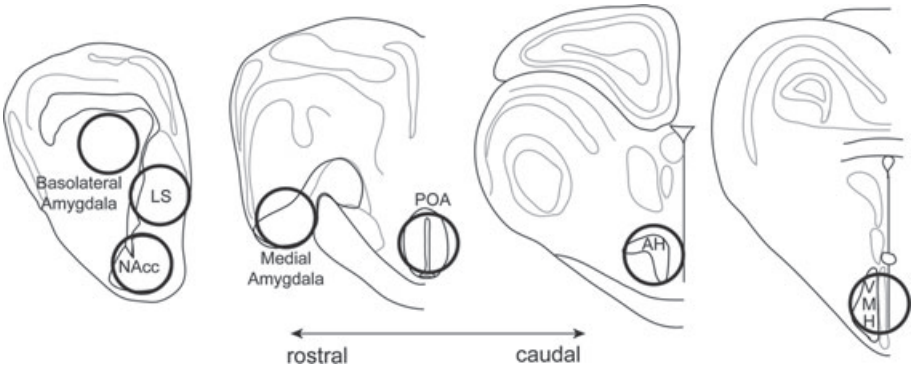

Figure 1: Transverse sections of the whiptail brain are shown with circles indicating micro-punches excised for analysis of gene expression.

area, lateral septum (LS) and anterior hypothalamus (AH). Although these regions are important in mediating social behavior (O'Connell & Hofmann 2011), they are not crucial to copulatory behavior in male rats and, thus, serve as neutral brain regions to determine how androgens induce gene expression specifically in regions that regulate sexual behavior rather than social behavior in general.

Methods

Behavior

Adult *Cnemidophorus inornatus* were captured near Sanderson, TX in May 2008 and transported to the University of Texas at Austin, where they were individually housed in environmentally controlled chambers in terraria with *ad libitum* water and fed two to three crickets every other day. All males used in this study were sexually active upon arrival, and thus we cannot control for sexual experience prior to field capture. Every care was taken to minimize animal discomfort, and all procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Following hibernation, male *C. inornatus* were castrated as previously described (Lindzey & Crews 1986) and returned to their original enclosures. After 7 weeks, castrated males were screened for absence of copulatory behavior by testing with receptive female *C. inornatus* in their home tanks three times over 5 days. Copulatory behavior was defined as mounting and taking a stereotyped 'doughnut posture' (Crews & Fitzgerald 1980) in all tests. Castrated males that had ceased copulatory behavior were then randomly assigned to one of two groups and implanted with Silastic tubing (Helix Medical, Carpinteria, CA, USA) containing testosterone (T, $n = 10$) or a blank (CTL, $n = 6$) as previously described (Lindzey & Crews 1986). Six weeks later, males were tested for reinstatement of sexual behavior in their home tank once a day for 3 days with receptive females (Sanderson *et al*. 2008). All CTL males failed to mount in all three tests. One T male did not mount in response to a receptive female in any of the tests and was excluded from further analysis. Males were immediately anesthetized by hypothermia and killed by decapitation within 10 min of the final test to avoid immediate gene expression changes due to behavioral testing. At the time of death, all males were inspected to confirm complete castration. Brains were removed, embedded in Tissue-Tek Optimal Cutting Temperature medium (Fisher Scientific, Pittsburg, PA, USA) and stored at −80°C until sectioning.

Brain region punches and measuring gene expression

Brains were sectioned at 100 μm onto Superfrost Plus slides (Fisher Scientific). Circular punches 50 μm in diameter were taken of the AH, basolateral amygdaloid area, LS, NAcc, medial amygdaloid area, POA and VMH according to O'Connell and Hofmann (2011) (Fig. 1). Punches of the NAcc may have included some ventral pallidum and the POA punches include both the medial POA and periventricular POA. Tissue punches were immediately placed in Trizol (Life Technologies, Grand Island, NY, USA) and stored at −80°C. RNA was extracted with Trizol according to the manufacturer's instructions and was reverse transcribed using Superscript III reverse transcriptase (Life Technologies) and gene-specific primers (Table 1). Excess primers and salts from the transcription reaction were removed in Microcon YM30 columns (Millipore, Billerica, MA, USA). Quantitative polymerase chain reaction (qPCR) primers for 18S, nNOS, NR1 and D1 and D2 dopamine receptors (D1R and D2R, respectively) (Genbank accession numbers: AY217941, DQ141603.2, EU358568.1, EU124512.1 and EU124516.1) were designed to flank exon boundaries using the *A. carolinensis* genome as a reference. Target gene abundance was measured as previously described (Sanderson *et al*. 2008) and normalized to 18S ribosomal RNA abundance. Not all brain regions were measured for all individuals because of the lack of sufficient tissue in some cases.

Statistical analysis

Statistical analysis was conducted using PASW v18.0.3 (IBM, Somers, NY, USA). As the data were not normally distributed and sample sizes for some brain regions for the CTL group were

Table 1: Primers for reverse transcription and qPCR

Target gene	RT primer	Forward qPCR primer	Reverse qPCR primer
D1R	5′-CTCGGGGTCATTTTCCTCTC	5′-TTGGCAGTTTCAGACCTTTTGGTAGC	5′-GCAACCCAGATGTTACAGAACGATCC
D2R	5′-AGTTCTCGTCTTGCCGTTGG	5′-CCTCTGTGCCAGTTCAATCCTCCG	5′-GACCGTTTTGGGGTTGTCTGTCG
NR1	5′-AGTCCCAAATGAAGGCGTGG	5′-TGCGAAATCCCTCCGACAAGTTC	5′-TGTGCCGATACATGGTGCTCAGC
nNOS	5′-TCAAACTTGGGGTGC	5′-TGGGTACAAGCAACCAGATG	5′-GCAGGACATCAAATCGACCT
18S	5′-ACGCCACTTCTGCCTCTAAG	5′-CTCAACACGGGAAACCTCA	5′-CAAATCGCTCCACCAACTAAG

Primers used for gene-specific reverse transcription (RT) and qPCR are listed for dopamine D1R and D2R, NR1, nNOS and 18S ribosomal RNA (reference gene).

small, non-parametric Mann–Whitney *U*-tests were used with gene expression data as the dependent variable and hormonal state as the independent variable for each brain region. To account for multiple hypotheses testing of differential gene expression, we applied Benjamini–Hochberg *post hoc* false discovery rate corrections (Benjamini & Hochberg 1995) within each brain region.

Results

NMDA receptor (NR1 obligate subunit)

Androgen-implanted sexually active (T) *C. inornatus* males had lower NR1 mRNA levels (Fig. 2) in both the NAcc ($U=0$, $P=0.02$) and medial amygdaloid nucleus ($U=0$, $P=0.003$) compared with blank-implanted controls (CTL) that do not mount. NR1 gene expression did not differ between T and CTL groups in other regions, including the basolateral amygdaloid area ($U=11$, $P=0.40$), LS ($U=12$, $P=0.45$), POA ($U=11$, $P=0.13$), AH ($U=14$, $P=0.54$) and VMH ($U=22$, $P=0.85$).

Dopamine D1 and D2 receptors

Gene expression of both dopamine receptor subtypes in the NAcc was lower in T males compared with CTL males (D1R, $U=0$, $P=0.02$; D2R, $U=0$, $P=0.02$) (Fig. 3). There were no differences in dopamine receptor gene expression between T and CTL males in other target brain regions including the basolateral amygdaloid area (D1R, $U=8$, $P=0.17$; D2R, $U=8$, $P=0.17$), medial amygdaloid area (D1R, $U=20$, $P=0.889$; D2R, $U=20$, $P=0.889$), LS (D1R, $U=14$, $P=0.73$; D2R, $U=14$, $P=0.73$), POA (D1R, $U=14$, $P=0.26$; D2R, $U=14$, $P=0.26$), AH (D1R, $U=8$, $P=0.12$; D2R, $U=14$, $P=0.54$) and VMH (D1R, $U=18$, $P=0.44$; D2R, $U=17$, $P=0.37$).

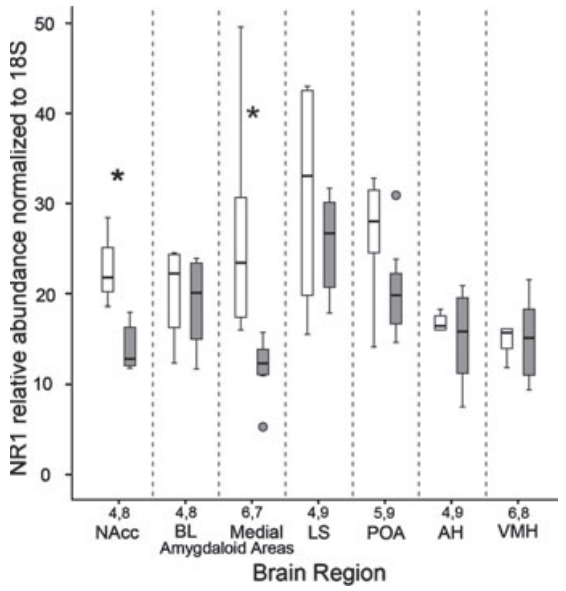

Figure 2: The NMDA receptor gene expression differences between blank- and testosterone-implanted male whiptails. Box and whisker plots (boxes show first and third quartiles, line in box represents median, whiskers indicate minimum and maximum value and circles indicate outliers) show gene expression changes in NR1 (obligate NMDA receptor subunit) between castrated males implanted with either blank (white boxes) or testosterone (gray boxes). Sample sizes are indicated below each group. Abbreviation: BL, basolateral. Asterisks indicate statistical significance with a Mann–Whitney *U*-test of $P<0.05$.

Neuronal nitric oxide synthase

Gene expression of nNOS in NAcc was higher in T males compared with CTL males ($U=0$, $P=0.012$) (Fig. 4). In the hypothalamus, both the POA and VMH of T males also expressed more nNOS compared with CTL males (POA, $U=3$, $P=0.01$; VMH, $U=3$, $P=0.01$). However, nNOS gene expression did not differ between T and CTL males in the basolateral amygdaloid area ($U=13$, $P=0.61$), medial amygdaloid area ($U=8$, $P=0.06$), LS ($U=12$, $P=0.50$) or AH ($U=12$, $P=0.36$).

Discussion

Hull and Dominguez (2006) present a model with preoptic nNOS, NMDA receptors and dopamine receptors as main players in facilitating sexual motivation and mating in male mammals. The data presented here suggest the coordination of gene expression by androgens impinges on a circuit involving the medial amygdaloid area, the POA and the mesolimbic dopamine tract from the VTA to the NAcc, whereas the VMH seems to serve a modulatory role. We did not find evidence for regulation by androgens of gene expression of nNOS, NR1 or dopamine receptors outside of these brain regions, suggesting a circuit involving the medial amygdaloid area, POA, NAcc and VMH serve to gate and reinforce sexual behavior in male whiptails as in male rodents (Hull & Dominguez 2006).

Integrating information from the present results with work in mammals, we can now expand our current understanding of the neural circuitry promoting sexual behavior in amniote males (Fig. 5). We find that NR1 mRNA is downregulated in androgen-treated males in the medial amygdaloid area, although we do not know whether this change in gene expression was due to exposure to a female or to androgen treatment. On the basis of the research in the green anole, the latter is more likely as testosterone treatment but not exposure to a female decreased immediate early gene (IEG) induction in the medial amygdaloid area compared with blank-implanted anole males (Neal & Wade 2007). This is in agreement with our finding that compared with controls, NR1 gene expression in this same region is decreased in T-implanted male whiptails, possibly because of negative feedback regulation.

The presence of NMDA and dopamine receptors in the POA is crucial to male copulation (Hull & Dominguez 2006). However, we did not find differences in gene expression of these receptors in the POA of T males compared with CTL males, as only nNOS gene expression differed, as described

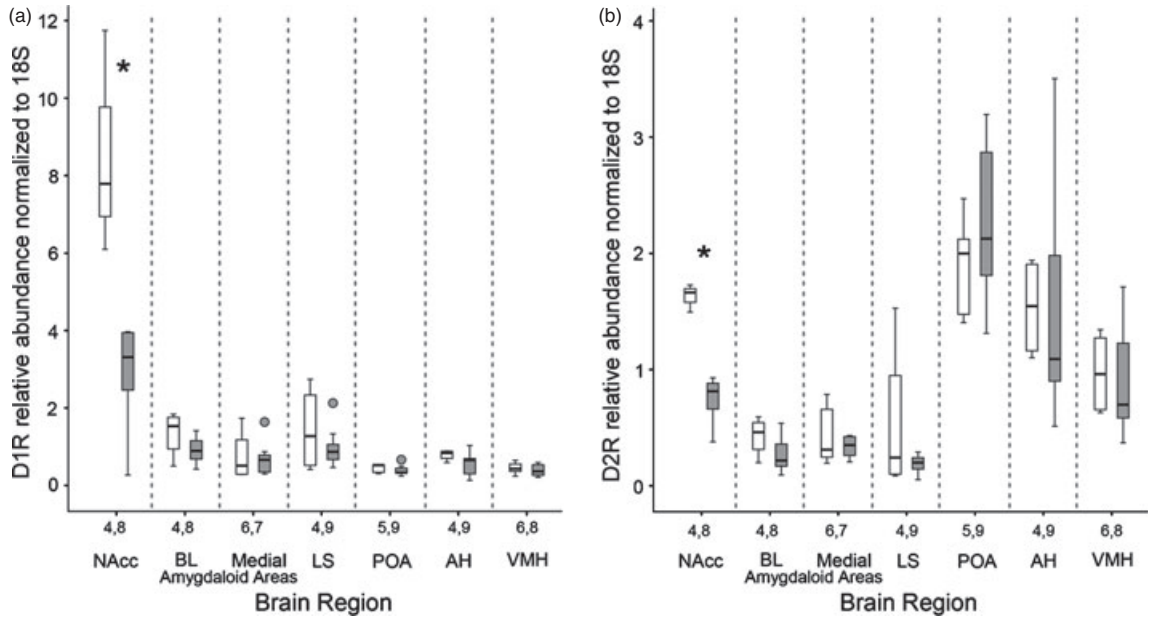

Figure 3: Dopamine receptor gene expression in the NAcc is different between blank- and testosterone-implanted male whiptails. Box and whisker plots show gene expression changes in dopamine D1R (a) and D2R (b) between castrated males implanted with either blank (white boxes) or testosterone (gray boxes). Sample sizes are indicated below each group. Abbreviation: BL, basolateral. Asterisks indicate statistical significance with a Mann–Whitney *U*-test of $P < 0.05$.

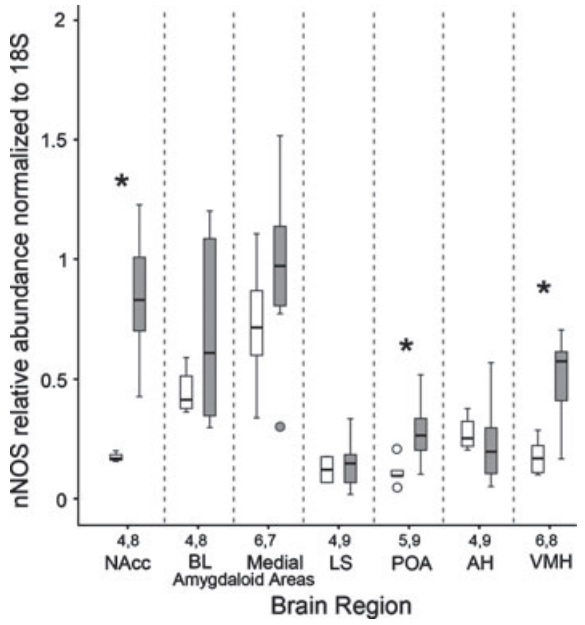

Figure 4: The nNOS gene expression differences between blank- and testosterone-implanted male whiptails. Box and whisker plots show gene expression changes in nNOS between castrated males implanted with either blank (white boxes) or testosterone (gray boxes). Sample sizes are indicated below each group. Abbreviation: BL, basolateral. Asterisks indicate statistical significance with a Mann–Whitney *U*-test of $P < 0.05$.

previously (Sanderson *et al*. 2008). We are not aware of any work on dopamine or NMDA receptor gene expression in the POA of males in any vertebrate, and thus it is unclear if this pattern is specific to reptiles or conserved across vertebrates. Consistent with the lack of a difference between T and CTL male whiptails in preoptic NR1, D1R or D2R mRNA levels, Neal and Wade (2007) found in green anole males that neither androgen treatment nor the presence of a female induced IEG expression in the POA. Importantly, nNOS gene expression depends on androgens (Sanderson *et al*. 2008) and is independent of sexual experience (Sato *et al*. 2005), providing further support for the notion that the elevated gene expression of nNOS in T-implanted whiptail males compared with CTL males is due to androgen treatment rather than exposure to a female during behavioral testing. Together with results from previous studies in whiptails and rodents, preoptic nNOS gene expression may be the androgen-driven gating mechanism by which sexual behavior in males can proceed (Hull 2011; Sanderson *et al*. 2008).

The POA projects to the VTA to stimulate dopamine release in the NAcc (Damsma *et al*. 1992). We found that dopamine D1R and D2R mRNA is downregulated in the NAcc of androgen-implanted males compared with control males. To our knowledge, there are no studies reporting the effects of androgens on dopamine D1R gene expression in the vertebrate male brain, and thus we cannot conclude whether the observed differences in gene expression are due to androgen treatment or the display of copulatory behavior. However, Guivarc'h *et al*. (1995) showed that testosterone treatment decreased hypothalamic dopamine D2R gene expression in castrated male rats. Also, NAcc dopamine

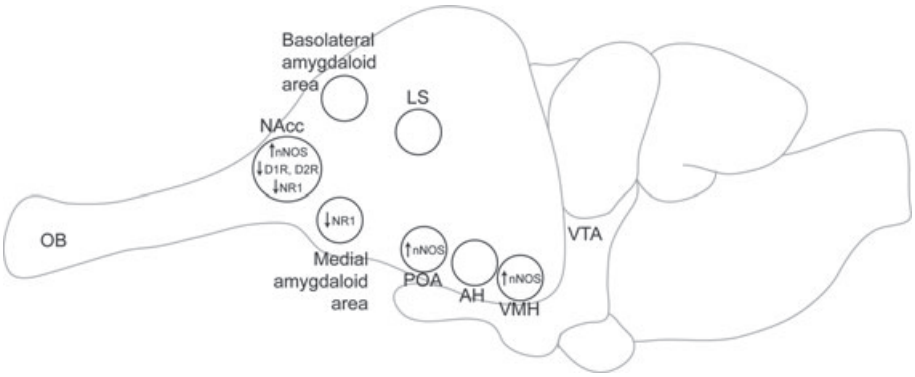

Figure 5: Summary/working model of neural circuitry promoting sexual behavior in male amniotes.

levels are decreased in castrated male rats, an effect that is rescued by androgen treatment but is independent of sexual experience (Mitchell & Stewart 1989). If androgens similarly increase NAcc dopamine levels in male whiptails, the decreased gene expression levels of dopamine receptors we observed may be due to a compensatory change in response to excess dopamine levels (Fauchey *et al*. 2000). We also found reduced NAcc NR1 gene expression in androgen-treated males. Studies in male rats have shown that androgenic compounds decrease NAcc NR1 gene expression (Le Grevès *et al*. 1997), suggesting a similar pattern of negative regulation of NR1 gene expression in the NAcc of male whiptails. Importantly, NMDA receptors in the NAcc are necessary for reinforcement learning (Kelley *et al*. 1997) and thus may also be important in reinforcing copulatory behavior in males. Finally, we found an androgen-induced increase in nNOS mRNA in the NAcc. This may function to reinforce sexual behavior given that nitric oxide, in conjunction with NMDA receptors, serves to facilitate the release of dopamine in the NAcc in rats (Kelley *et al*. 1997; Ohno *et al*. 1995).

Our work supports the hypothesis that, similar to the situation in mammals, the mesolimbic dopamine system reinforces sexual motivation in male reptiles, suggesting that the steroid hormone–neurotransmitter relationships that promote copulatory behavior in males are conserved across amniote vertebrates. However, it is important to note that we cannot exclude that some changes in gene expression may have been modulated by estrogen receptors, as aromatase is expressed in many of the brain regions we have investigated here (Dias *et al*. 2009). Additional studies will be required to fully separate the effects of androgen treatment from those of copulatory behavior by using an androgen-treated group of males that are exposed to a female but not permitted to display mounting behavior.

Consistent with the reports of a dynamic relationship between the POA and the VMH in regulating mounting behavior in male rodents (Crews 2010), we find elevation of nNOS gene expression in both the POA and VMH. To our knowledge, this is the first report of androgen-induced nNOS gene expression in the VMH of any vertebrate, although the behavioral implications of this result need to be explored further with site-specific manipulations of nNOS in the VMH. Another interesting avenue warranting further investigation relates to the actions of glutamate and nNOS in the NAcc, as little attention has been given to measuring glutamate or nNOS in the NAcc in response to androgen-induced copulation. Finally, more studies are needed in birds, amphibians and fish to determine to what extent these mechanisms are conserved across vertebrates.

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