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ORIGINAL ARTICLE

Neuronal Nitric Oxide Synthase as a Substrate for the Evolution of Pseudosexual Behaviour in a Parthenogenetic Whiptail Lizard

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The evolution of neuroendocrine mechanisms governing sex-typical behaviour is poorly understood. An outstanding animal model is the whiptail lizard (*Cnemidophorus*) because both the ancestral and descendent species still exist. The ancestral little striped whiptail, *Cnemidophorus inornatus*, consists of males and females, which exhibit sex-specific mating behaviours. The descendent desert grassland whiptail, *Cnemidophorus uniparens*, consists only of females that alternately exhibit both female-like and male-like pseudosexual behaviour. Castrated male *C. inornatus* will mount a conspecific in response to exogenous androgen, although some are also sensitive to progesterone. This polymorphism in progesterone sensitivity in the ancestral species may have been involved in evolution of progesterone-mediated male-typical behaviour in the descendant unisexual lizards. We tested whether progesterone activates a typically androgenic signalling pathway by investigating hormonal regulation of neuronal nitric oxide synthase (nNOS) using *in situ* hybridisation and NADPH diaphorase histochemistry, a stain for nNOS protein. NADPH diaphorase is widely distributed throughout the brain of both species, although only in the periventricular nucleus of the preoptic area (pvPOA) are there differences between mounting and non-mounting individuals. The number of cells expressing nNOS mRNA and NADPH diaphorase is higher in the pvPOA of individuals that mount in response to progesterone or androgen. Furthermore, the nNOS promoter has both androgen and progesterone response elements, and NADPH diaphorase colocalises with the progesterone receptor in the pvPOA. These data suggest that a polymorphism in progesterone sensitivity in the sexual ancestor reflects a differential regulation of nNOS and may account for the male-typical behaviour in unisexual whiptail lizards.

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The evolution of neural mechanisms that govern social decision-making are poorly understood. However, insight into the proximate mechanisms underlying behavioural outputs to social stimuli is of primary importance for understanding the evolution and selection of behavioural traits (1). Whiptail lizards (genus *Cnemidophorus*) are an excellent model system for the study of the evolution of neuroendocrine mechanisms mediating sexually dimorphic behaviour (2) because both ancestral and descendant species still exist. In the present study, we explore the neuroendocrine mechanisms governing reproductive behaviours of whiptail lizards in an effort to delineate the molecular substrates for the evolution of pseudocopulatory behaviour in a parthenogenetic species.

The all-female whiptail lizard, *Cnemidophorus uniparens*, consists of clonal descendants (3), and displays both male-like mounting behaviour and female-like receptivity during discrete phases of the ovarian cycle (4,5). This male-typical behaviour is indistinguishable from the mounting behaviour observed in males of their direct ancestor, *Cnemidophorus inornatus* (4). This behavioural adaptation serves to stimulate and synchronise reproductive activity in conspecific females (6). The display of male-typical behaviour in these

females is associated with the postovulatory phase of the ovarian cycle when progesterone levels are high (5,7). During the preovulatory phase when oestrogen levels are high, *C. uniparens* are receptive to mounting from conspecifics, similar to the ancestral *C. inornatus* females. Androgens are not detectable in the circulation at any time of the ovarian cycle in females of either species (8). Unlike *C. uniparens*, the ancestral *C. inornatus* females do not display mounting behaviour during the postovulatory phase when progesterone levels are high.

The regulation of male-typical mounting behaviour by progesterone in *C. uniparens* has an evolutionary precursor in *C. inornatus* males, where progesterone, acting either alone or synergistically with androgens, stimulates mounting behaviour (9). This effect of progesterone is mimicked by R5020, a non-metabolisable progesterone receptor (PR) agonist, and abolished by the anti-progestin RU486, suggesting that progestins mediate this behaviour at the level of PR rather than via progestin metabolites such as androgens (10). In both *C. inornatus* and *C. uniparens*, PR is expressed in two regions of the preoptic area: the medial preoptic area (mPOA) and the periventricular nucleus (pvPOA) (11). Implantation of progesterone directly into the POA elicits the full repertoire of mounting and intromission in *C. inornatus* males that had previously been established as sensitive to progesterone (12). However, the molecular actions of the ligand-activated PR that promote male-typical behaviour are not known.

To understand how progesterone may facilitate male-typical mounting behaviour in both the ancestral and descendent whiptail species, we first searched for a candidate molecule that facilitates male-typical behaviours in other vertebrates and is regulated by both progesterone and androgens in different physiological contexts. One model suggests that androgenic up-regulation of neuronal nitric oxide synthase (nNOS) may be a key cellular event that allows the expression of male-typical mounting behaviour in rodents (13). It has been established that the expression of male-typical copulatory behaviour in male *C. inornatus* is associated with an androgen-driven increase in nNOS mRNA and protein levels in the POA, and that the mounting behaviour is suppressed when nNOS is inhibited in *C. uniparens* (14,15). In addition to being regulated by androgens, nNOS is also regulated by progesterone in the context of rat female-typical sexual behaviour (16).

In the present study, we investigated the role of nNOS in facilitating male-typical copulatory behaviour, and its regulation by progesterone in the ancestral and descendant whiptail lizard species. To better understand where nNOS may be acting to facilitate male-like copulatory behaviour, we first mapped its distribution in the whiptail brain using NADPH diaphorase histochemistry, a marker for nNOS protein activity (17). We then analysed nNOS mRNA and protein levels in hormonally manipulated *C. inornatus* males and *C. uniparens* by *in situ* hybridisation and quantified NADPH diaphorase-positive cells in the pvPOA. We next determined whether progesterone could potentially regulate nNOS expression by analysing the nNOS promoter for progesterone receptor response elements and colocalised nNOS and PR in the pvPOA. Finally, to investigate species differences in mating behaviour, we compared the number of NADPH diaphorase-positive cells in the pvPOA of *C. inornatus* females and *C. uniparens* across the ovarian cycle. The findings obtained indicate that nNOS is up-regulated in individuals that mount in response to progesterone on a level comparable to testosterone-induced levels and that PR may directly regulate nNOS transcription. These data suggest that progesterone-mediated mechanisms of facilitating male-typical copulatory behaviour are similar to testosterone induction of mounting behaviour and further suggest that nNOS may have been recruited in the evolution of pseudocopulatory behaviour in *C. uniparens*.

Materials and methods

Animals

Adult *C. inornatus* were captured in the vicinity of Sanderson, TX, in May 2008 and transported to the University of Texas at Austin campus. Adult *C. uniparens* were captured in the vicinity of Portal, AZ, in June 2008, housed for no more than 3 weeks at the Southwestern Research Station, and then transported to the University of Texas at Austin. Lizards are housed in environmentally controlled chambers in terraria with water and food available *ad lib.* in the form of crickets (18,19).

Behavioural tests and surgeries

C. inornatus: After allowing 1–2 weeks to acclimatise to the laboratory environment, sexually active males were identified as previously described (14,19). These sexually active males were castrated and returned to their original tanks as previously described (20). Seven weeks after castration, they were given three 10-min tests with receptive females to verify the absence of courtship behaviour, and then implanted s.c. with 12-mm Silastic capsules (diameter 1.47 mm, outer diameter 1.96 mm) packed with crystalline testosterone, progesterone or with empty implants (controls), as described previously (18,19), resulting in physiologically relevant levels of testosterone and progesterone in whiptail lizards and the induction of male-like copulatory behaviour. All surgical procedures were carried out under hypothermic anaesthesia. Eighteen days later, males were tested once a day for 5 consecutive days with receptive females in the home cage of the focal male for reinstatement of copulatory behaviour. After testing, they were immediately anaesthetised by hypothermia, and killed by decapitation. Males were killed within five min of the final behavioural observation to avoid the effect of recent behaviour on nNOS induction. This has been shown to be an appropriate time-window because egr-1 expression, an immediate early gene marker for neuronal activity, has a delay of 10–30 min from the last behavioural experience (21). Males whose sexual behaviour was reinstated with systemic progesterone were classified as progesterone-sensitive; those in which sexual behaviour was not reinstated were classified as progesterone-insensitive. Reinstatement of sexual behaviour was considered mounting and taking a stereotyped doughnut-posture in three out of five tests. Males classified as progesterone-insensitive did not mount in any of the five tests. Approximately 40% of tested males were progesterone-sensitive. In all cases, castrated males implanted with blank capsules did not display mounting behaviour to a receptive female. One male implanted with testosterone did not mount in response to a receptive female and was excluded from further analysis. All other castrated males implanted with testosterone mounted a receptive female. At the time of death, all males were inspected to confirm complete castration.

Female *C. inornatus* and *C. uniparens*: Preovulatory and postovulatory ovarian states were determined by abdominal palpation. Female-like receptivity was tested as described previously (22). Ovarian morphology was noted after killing the experimental animal. All preovulatory animals were

characterised by the presence of developing follicles and receptive behaviour. Postovulatory animals had corpora lutea and were not receptive.

In hormonally manipulated groups, adult *C. uniparens* were ovariectomised and implanted with an empty 12-mm Silastic capsule (OVX + Blank) or with a capsule packed with testosterone (OVX + T). Animals were behaviourally tested 18 days after surgery. In all cases, OVX + Blank animals failed to show either male-like mounting behaviour or female-like receptive behaviour. Some OVX + T animals failed to display mounting behaviour to a receptive individual (n = 3) and were excluded from further analysis.

We adopted the neuroanatomical nomenclature of Young *et al.* (11). All procedures were approved by the University of Texas IACUC.

nNOS promoter cloning and analysis

Genomic DNA from whiptail livers was isolated by phenol/chloroform extraction. The whiptail nNOS promoters were cloned using GenomeWalker (Clontech, Palo Alto, CA, USA) in accordance with the manufacturer's instructions. The outer and nested gene specific primer used for nNOS promoter cloning were 5′-TGGAAGATTCAAGCCTTTAGCCTCAGTAAG and 5′-AACCATTGGACACACACTTTGGGCGG, respectively. The promoter sequence was submitted to Genbank (FJ517552). Putative steroid hormone receptor binding sites were identified using MatInspector (Genomatix, Ann Arbor, MI, USA).

In situ hybridisation

Brains were removed, embedded in OCT and stored at −80 °C until sectioning. Brains were then sectioned on a cryostat at 20 μm and thaw-mounted onto Super-Frost Plus slides (Erie Scientific Co., Portsmouth, NH, USA) in three series. Sections were fixed as previously described (14) and stored at −80 °C. Riboprobes were reverse-transcribed in the presence of fluorescein-labelled UTP (Roche, Indianapolis, IN, USA) using a T7/SP6 Maxiscript *in vitro* transcription kit (Ambion, Austin, TX, USA) to produce antisense or sense fluorescein-labelled riboprobes. The template used to make the nNOS probe was the same as that described by Sanderson *et al.* (14) and was 641 bp in length (Genbank accession DQ141603). Slides were then warmed to room temperature, air dried and pre-equilibrated in hybridisation buffer (50% formamide, 5× SSC, 5× Denhardt's solution, 125 mg/ml Baker's yeast tRNA, 250 mg/ml denatured herring sperm DNA) for 2 h at 60 °C. Sections were then incubated in riboprobe overnight at the same temperature. Experimental slides were exposed to anti-sense fluorescein-labelled probe, whereas control slides were incubated with sense fluorescein-labelled probe. After RNase A treatment at 37 °C for 15 min, sections were washed in a decreasing series of SSC and equilibrated in 150 mM NaCl/100 mM Tris (pH 7.5) at room temperature before incubation in 1 : 1000 anti-Fluorescein-alkaline phosphatase Fab fragments (Roche) in 0.5% Tween 20/phosphate-buffered saline (PBS) for 2 h at room temperature. Sections were then washed in 100 mM Tris (pH 7.5). Chromogenic product was formed using BM Purple (Roche) at room temperature until the desired darkness was achieved and was terminated simultaneously for all slides. The sections were then dehydrated, delipidated, and coverslipped under Permount (Fisher Scientific, Itasca, IL, USA).

NADPH diaphorase histochemistry

Brains were removed, fixed in 4% paraformaldehyde at 4 °C overnight, cryoprotected in 30% sucrose overnight at 4 °C, and then embedded in OCT and stored at −80 °C until sectioning. Brains were then sliced on a cryostat at 20 μm and thaw-mounted onto Super-Frost Plus slides (Erie Scientific Co.) in three series. Slides were taken from the −80 °C, air-dried briefly, and processed for NADPH diaphorase histochemistry as described previously (14). Briefly, slides were fixed for 20 min in chilled 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB). After two washes in PB, slides were incubated for 2 h at 37 °C in PB containing 0.3% triton-X, 0.1 mg/ml nitro blue tetrazolium and 0.25 mg/ml β-NADPH. Samples intended for co-localisation were immediately processed for PR immunohistochemistry. All other sections were then rinsed, dehydrated and cover-slipped with Permount. For control sections, all procedures were the same, except that β-NADPH was omitted.

Dual immunohistochemistry for PR

After processing for NADPH diaphorase histochemistry, sections were then rinsed in PBS, and incubated in 3% hydrogen peroxide in PBS for 20 min. After washing in PBS, antigen retrieval was performed by incubating in boiling citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0). After 2 min, the boiling citrate buffer was replaced two times and incubated for 5 min each, followed by a PBS wash. After blocking for 1 h in blocking solution (5% normal goat serum and 0.3% TritonX-100 in PBS), sections were incubated in primary antibody (PR 1 : 500, abcam 2767, monoclonal antibody raised against chicken PR) in PBS with 2% normal goat serum and 0.3% Triton-X at room temperature overnight. Sections were then rinsed, incubated for 2 h in a biotinylated goat anti-mouse secondary antibody (dilution 1 : 200; Vector Laboratories, Inc., Burlingame, CA, USA), rinsed again and, after treatment with the ABC peroxidase staining kit (Vector Laboratories) in accordance with the manufacturer's instructions, immunoreactivity was visualised using 3,3′-diaminobenzidine substrate (Vector Laboratories). Sections were then dehydrated and cover-slipped with Permount (Fisher Scientific). For control sections, all procedures were the same except that primary antibody was omitted.

Western blot characterisation of the PR antibody

To determine whether this antibody would bind specifically to the lizard antigens, we extracted protein from whole brain using a Mammalian Cell Lysis kit (Sigma, St Louis, MO, USA) in accordance with the manufacturer's instructions. Whole brain protein extract was run on an sodium dodecyl sulphate-polyacrylamide gel electrophoresis gel in replicate, in which one half of the gel was used for downstream western blotting and the other half was exposed to Coomassie stain to verify protein presence. Whole brain extract on the gel was transferred onto a nitrocellulose membrane overnight. The membrane was then blocked in 5% dry milk in wash buffer [0.5% TritonX-100, 0.1% Tween-20 in 1× Tris-buffered saline (TBS)] and incubated in primary antibody (1 : 2000 PR in 1× TBS and 2% NaN3) for 1 h. After incubation, the membrane was washed five times for 3 min each in wash buffer, and then incubated in goat-anti-mouse horseradish peroxidase (HRP)-conjugated antibody (Southern Biotech, Birmingham, AL, USA) in blocking solution for 30 min. After washing five times for 3 min each with wash buffer, the membrane was exposed to HRP substrate (Immobilon Western; Millipore, Billerica, MA, USA) and exposed to film for 5 min. Using the PR antibody, one band was visualised at the predicted size of 130 kDa, putatively representing PR.

Densitometric analysis of nNOS expression

Densitometic analysis of expression levels was measured as in Zhao *et al.* (23) and Zhang *et al.* (24). Briefly, images of nNOS *in situ* hybridisation slides were captured using a Nikon Eclipse 80i microscope with a Nikon 12-bit 2-megapixel monochrome camera (DS-2MBWc) and analysed using Nikon NIS-Elements BR 2.30 program (Nikon Corp., Tokyo, Japan). An investigator blind to group status measured the mean optical density of a defined region using grid sizes of 100 × 100 μm obtained from two to three individual sections from each animal. The background optical density was measured simultaneously to account for nonspecific hybridisation

using a grid size of 100 × 100 μm. The background was defined on each section as an adjacent area of tissue that did not contain a specific hybridisation signal. The specific hybridisation signal was obtained by subtracting the background optical density from the optical density in the specifically labelled nuclei. The mean optical density of the target POA subnuclei was averaged for each section per individual. The values from each treatment group were averaged to obtain the final mean ± SEM. Nonradioactive and radioactive *in situ* hybridisation has been shown to give similar results in previous studies (25).

Cell counting

Cells labelled by NADPH diaphorase histochemistry were counted using the Fractionator routine of the STEREO INVESTIGATOR software package (Microbrightfield, Williston, VT, USA). A region of interest was defined under low power, and then, under higher magnification, positive cells were counted that fell within 50-μm square counting frames. The computer placed counting frames systematically every 80 μm (i.e. with 30 μm between each counting frame) within the region, after a randomly chosen start-site. Cell cytoplasm containing nNOS protein was clearly marked by dark purple staining after diaphorase histochemistry, and was counted using a × 20 objective. Slides were coded and processed by a blinded observer.

Statistical analysis

All statistical analysis was conducted using JMP (SAS Inc., Cary, NC, USA). $P < 0.05$ was considered statistically significant. For *in situ* hybridisation and cell counting data, Kruskal–Wallis was conducted using hormonal state as the independent variable and mRNA level or cell number as dependent variables. Kruskal–Wallis was chosen because most of the data sets were not normally distributed and such nonparametric tests are more conservative for small sample sizes. Mann–Whitney U-tests were applied post-hoc.

Results

General distribution of nNOS

Because the distribution of nNOS within the whiptail brain is unknown, we used NADPH diaphorase histochemistry to visualise nNOS cells and fibres (26). To our knowledge, there are no commercially available antibodies that recognise the reptilian nNOS protein. In the present study, we report the distribution of NADPH diaphorase histochemistry (Fig. 1) throughout the brain of the whiptail lizard. Accordingly, we used *C. uniparens* individuals displaying either female-like or male-like sexual behaviours and *C. inornatus* males. The only qualitative (total presence or absence) difference between the groups was in the pvPOA.

In the rostral telencephalon, there are NADPH diaphorase positive cell bodies dorsal to cortex medialis and cortex dorsalis, as well as in the dorsal ventricular ridge (DVR) (Fig. 1A). More caudally, the number of NADPH diaphorase positive cells increase in the DVR. There are also some cell bodies and fibres positive for NADPH diaphorase in the nucleus accumbens and the ventral striatum (Fig. 1B). Caudal to the striatum, these cell bodies and fibres appear to wrap around the lateral forebrain bundle (LFB; Fig. 1C). There are a few cells positive for NADPH diaphorase staining in the external amygdaloid nucleus (AME), but no fibres. Interestingly, the pvPOA contains many cells and fibres positive for NADPH diaphorase (Fig. 1C1), but staining is completely absent from the mPOA. The caudal portion of the pvPOA only has cells positive for NADPH diaphorase histochemistry in the dorsal part surrounding the third ventricle (Fig. 1D). Near this portion of the caudal POA, NADPH diaphorase-positive cells and fibres are no longer alongside the LFB, although there are cells in both the central amygdaloid nucleus and AME and fibres only in the AME. There are also a few cells and fibres positive for NADPH diaphorase staining in the spheris lateralis nucleus (NSL), although no reactivity was seen here in the more rostral regions of the NSL.

In the diencephalon, there are few cells and fibres positive for NADPH diaphorase histochemistry in the lateral hypothalamic area and dorsolaterais anterior nucleus (Fig. 1E). More caudally, there are cells and fibres positive for NADPH histochemistry in the lentiformis thalami pars plicta (Fig. 1F). In the hypothalamic regions, the ventromedial nucleus of the hypothalamus (Fig. 1F1) only has fibres, whereas the dorsal hypothalamus only has cells positive for NADPH diaphorase histochemistry.

Hormonal modulation of nNOS and facilitation of male-typical behaviour

To determine the relationship between nNOS and progesterone-facilitated mounting behaviour, we quantified nNOS mRNA levels in individuals with different hormonal conditions. *In situ* hybridisation for nNOS in the pvPOA was performed in both *C. inornatus* males and *C. uniparens* animals that displayed mounting behaviour in response to progesterone or testosterone compared to non-mounting individuals. Progesterone-sensitive (n = 8) and testosterone-treated (n = 3) male *C. inornatus* have comparable nNOS mRNA levels in the pvPOA that are significantly greater than progesterone-insensitive (n = 10) and castrated + blank-implanted (n = 10) *C. inornatus* males, none of which mount (Kruskal–Wallis test: $\chi^2 = 21.624$, d.f. = 3, $P < 0.0001$, post-hoc Mann–Whitney U-test, $P < 0.004$) (Fig. 2A). Similarly, intact postovulatory *C. uniparens* (n = 14) have nNOS expression levels in the pvPOA comparable to ovariectomised individuals implanted with testosterone (OVX + T, n = 10) (Fig. 2B). Both postovulatory and OVX + T groups have significantly higher levels of nNOS in the pvPOA than preovulatory (n = 12) and OVX + Blank (n = 10) groups (Kruskal–Wallis test: $\chi^2 = 25.931$, d.f. = 3, $P < 0.0001$, post-hoc Mann–Whitney U-test, $P < 0.004$).

Colocalisation of nNOS with PR

We next examined the number of cells positive for nNOS protein in the pvPOA by NADPH diaphorase histochemistry in preovulatory (n = 13) and postovulatory (n = 13) *C. uniparens*. nNOS-positive cell number within the pvPOA is significantly higher in postovulatory *C. uniparens* compared to preovulatory individuals (Wilcoxon Rank Sum: $\chi^2 = 8.153$, d.f. = 1, $P < 0.0001$, Fig. 3C), which supports the results found with mRNA. We also colocalised cells in the pvPOA with nNOS cells by immunohistochemistry for PR. We found that almost 90% of nNOS-positive cells colocalised with PR in the pvPOA of *C. uniparens* (Fig. 3B). Taken together, this

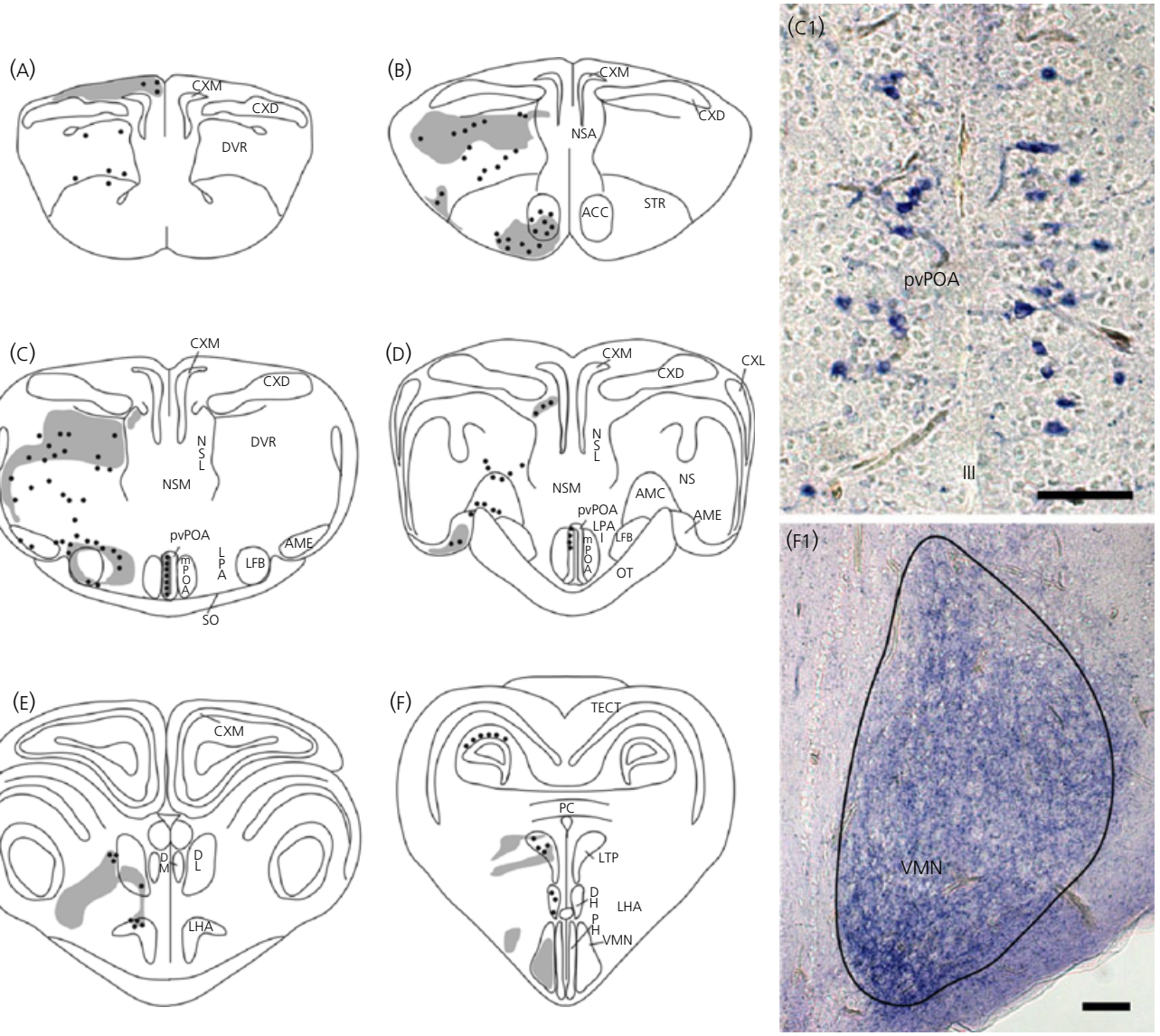

Fig. 1. Distribution of NADPH diaphorase histochemistry in the brain of whiptail lizards. Representative sections of the whiptail brain are depicted on the left (A–F) with nitric oxide synthase-positive cell bodies represented by dots and fibres depicted as grey shading. Micrographs on the right panel indicate NADPH diaphorase staining in the anteroventral periventricular nucleus of the preoptic area (pvPOA; C1) and the ventromedial nucleus of the hypothalamus (VMN; F1). Scale bars = 50 μm. III, third ventricle ACC, nucleus accumbens; AMC, nucleus centralis amygdalae; AME, nucleus externus amygdalae; CXD, cortex dorsalis; CXL, cortex lateralis; CXM, cortex medialis; DH, nucleus dorsalis hypothalami; DL, nucleus dorsolateralis anterior; DM, nucleus dorsomedialis; DVR, dorsal ventricular ridge; LFB, lateral forebrain bundle; LHA, lateral hypothalamic area; LPA, lateral POA; LTP, lentiformis thalami pars plicta; mPOA, medial preoptic area; NS, nucleus sphericus; NSA, nucleus sphericus anterior; NSL, nucleus sphericus lateralis; NSM, nucleus sphericus medialis; OT, optic tract; PC, posterior commissure; PH, nucleus periventricularis hypothalami; SO, nucleus supraopticus; STR, striatum; TECT, optic tectum.

suggests that nNOS plays a role in progesterone-mediated facilitation of mounting behaviour in a similar mechanism to testosterone-facilitation.

Hormonal regulation of the nNOS promoter

To determine whether PR can regulate transcription of nNOS, we analysed the promoter regions of both the rat and the whiptail lizard nNOS gene, and found several putative steroid hormone receptor binding sites (Fig. 4), which are relatively conserved between rats and whiptails. In addition to many putative androgen and oestrogen receptor response elements, two putative progesterone response elements (PREs) are located upstream of the whiptail translation start site, whereas the rat nNOS promoter has a single putative PRE. To our knowledge, this is the first analysis of the nNOS promoter region in any species, and supports our hypothesis that transcription of nNOS may be directly controlled by steroid hormone receptors in whiptail lizards.

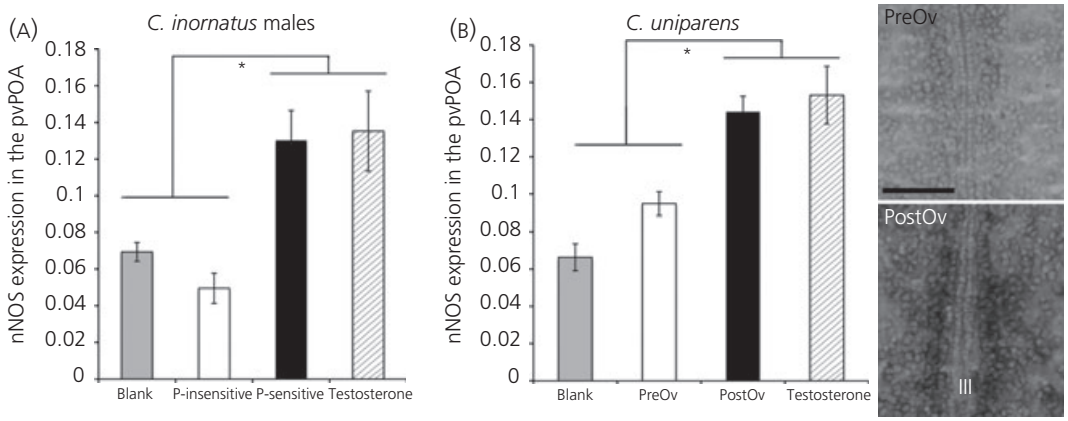

Fig. 2. Regulation of nitric oxide synthase (nNOS) mRNA expression in the periventricular nucleus of the preoptic area (pvPOA) by hormone state. (A) Progesterone (P)-sensitive males and testosterone implanted *Cnemidophorus inornatus* males, both of which display mounting behaviour, have significantly higher levels of nNOS mRNA in the pvPOA than progesterone-insensitive and castrated (Blank) males who do not mount (Kruskal–Wallis test, $P < 0.0001$, post-hoc Mann–Whitney U-test, $P < 0.004$). (B) Postovulatory (PostOv) and ovariectomised + testosterone-implanted *Cnemidophorus uniparens* have higher levels of nNOS mRNA in the pvPOA than preovulatory (PreOv) and ovariectomised + blank implanted animals (Kruskal–Wallis test, $P < 0.0001$, post-hoc Mann–Whitney U-test, $P < 0.004$). Data are shown as the mean ± SEM. In the third panel are representative micrographs of PostOv and PreOv *C. uniparens* pvPOA nNOS density. Scale bar = 100 microns. Abbreviations are as shown in Fig. 1. *$P < 0.004$.

Species differences in nNOS across the ovarian cycle

We next investigated the between-species differences of female whiptails in nNOS regulation in different ovarian stages because females of both the ancestral and descendent species have high levels of progesterone during the post-ovulatory phase of the ovarian cycle, although only the parthenogens respond to progesterone with male-like mounting behaviour. To better understand the molecular basis for this species difference, nNOS-positive cells were quantified within the pvPOA of naturally cycling *C. uniparens* and *C. inornatus* females. Postovulatory *C. uniparens* (n = 18) have significantly more NADPH diaphorase-positive cells in the pvPOA than preovulatory *C. uniparens* (n = 12) and both preovulatory (n = 9) and postovulatory (n = 9) *C. inornatus* (Kruskal–Wallis test: $\chi^2 = 24.222$, d.f. = 3, $P < 0.0001$; post-hoc Mann–Whitney U-test, $P < 0.002$) (Fig. 5). There was no significant difference between postovulatory and preovulatory *C. inornatus* females (post-hoc Mann–Whitney U-test, $P = 0.136$). These numbers in postovulatory *C. uniparens* are comparable to those found in testosterone-implanted *C. inornatus* males (14), suggesting that nNOS is up-regulated in postovulatory *C. uniparens* and facilitates male-like mounting behaviour.

Discussion

By investigating species differences in the regulation of a gene crucial for the display of male-typical mounting behaviour, we have shown that nNOS is up-regulated in individuals displaying mounting behaviour by both progestins and androgens. Although nNOS cell bodies and fibres are widely distributed throughout the whiptail brain, we found qualitative differences (absence/presence) between different reproductive states only in the pvPOA. Below, we discuss the role of the pvPOA in whiptail lizards, modulation of nNOS by steroid hormones and the evolutionary implications of these results.

nNOS distribution and role of the pvPOA

NADPH diaphorase and cells immunoreactive to nNOS are comparable both in brain and peripheral tissues (17). The distribution of NADPH diaphorase-positive cells and fibres was similar to that reported (27) for the gecko, with the exception that NADPH diaphorase cells were not observed in the whiptail cortex. The only region where we observed qualitative differences (i.e. a complete absence or presence) in the number of cells positive for NADPH diaphorase between sex, species and ovarian state was the pvPOA, which became the brain region of focus for the present study. However, it should be noted that quantitative differences in nNOS was not examined in other brain regions that could potentially be regulating reproductive behaviour. The presence of NADPH diaphorase cells in the POA appears to be highly conserved across vertebrates, including mammals (28), birds (29), frogs (30) and fish (31). We hypothesise that the pvPOA in whiptails is more similar to the mammalian mPOA as a result of its central role in mediating male-typical copulatory behaviour (32,33) and the neurochemical profile of steroid hormone receptors and nNOS expression (11,28, present study).

Interestingly, whiptail lizards, gecko (27) and turtle (34) show NADPH diaphorase fibres but not cell bodies within the ventromedial hypothalamus. This is in contrast to results obtained in birds (29), mammals (35) and frogs (30), where cell-bodies positive for NADPH diaphorase are clearly visible. This observation is interesting because the ventromedial hypothalamus is crucial for the display of female-typical receptivity in mammals and reptiles (36,37) and nNOS plays a role in facilitating female receptivity in rats (16). It will be interesting to determine whether nNOS plays a role in

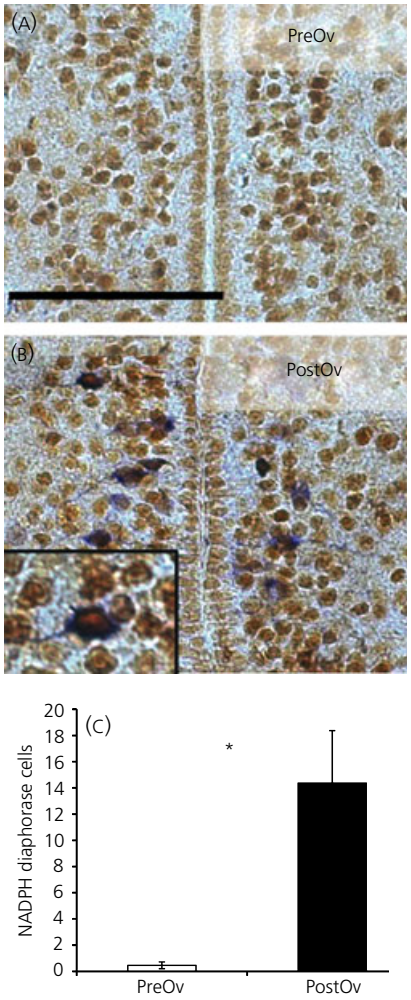

Fig. 3. Regulation of nitric oxide synthase (nNOS) in the periventricular nucleus of the preoptic area (pvPOA) of *Cnemidophorus uniparens* and colocalisation with progesterone receptor (PR). nNOS-positive cells detected by NADPH diaphorase histochemistry (blue staining) were counted in preovulatory (PreOv) (A) and postovulatory (PostOv) (B) *C. uniparens* females. PostOv animals have significantly more nNOS-positive cells in the pvPOA than PreOv animals (C; Wilcoxon rank sum test, *$P < 0.0001$). Slides were also processed for PR immunoreactivity (brown staining) and showed most nNOS-positive cells (approximately 90%) also contain PR. Data are shown as the mean ± SEM. Scale bar=100 microns. Abbreviations are as shown in Fig. 1.

female receptivity in whiptail lizards as well as male-typical copulatory behaviour.

Integration of behavioural and neuroendocrine mechanisms

The role of nitric oxide in male sexual behaviour has been extensively studied in mammals. nNOS expression in the rat POA is reduced by castration (38) and increased by either testosterone or oestradiol (39). Hull *et al.* (40) have proposed an elegant model of the hormonal gating of male sexual behaviour in which testosterone (after aromatisation to oestradiol) facilitates an up-regulation of nNOS. Sensory stimuli from the amygdala trigger glutamate release that stimulates NMDA receptors and, after an influx of calcium, calmodulin activates nNOS. Synthesis of nitric oxide then increases the level of dopamine in the POA, which increases the probability of a copulatory response. These same androgen-mediated mechanisms appear to play out in male *C. inornatus* as well (14) and over a time course that parallels reinstatement of copulatory behaviour after castration. Although work in rodent models with nNOS facilitating male sexual behaviour has mainly been limited to androgens and oestrogens, it would be interesting to investigate whether progesterone also plays a role in the nNOS pathway in rodents because progesterone also facilitates mounting behaviour in male mammals (41).

The regulation of nNOS by oestrogens and androgens has been well studied in rodents in the context of sexual behaviour. However, the regulation of nNOS by either androgens or oestrogens appears to be sex and steroid specific because oestradiol and progesterone will increase nNOS in the POA in females rats but not male rats (42). In female rats, both oestradiol and progesterone up-regulate nNOS protein in the POA and hypothalamus both *in vivo* and in *in vitro* hypothalamic cells (43). In male rats, androgens are required for the development of the nNOS system (44), whereas oestradiol appears to be necessary for the role of nNOS with respect to facilitating sexual behaviour in adults. (39).

On the basis of the insights gained from mammals, we can speculate regarding the synergistic actions of progesterone and nitric oxide in the whiptail lizards. It appears that progesterone via the progesterone receptor has taken on a new role in the parthenogens to facilitate male-typical mounting behaviour, and that this behaviour requires nitric oxide (15,45). Presumably, PR up-regulates nNOS, which, through a cascade of events, influences dopamine levels in the POA, and leads to an increased probability of mounting

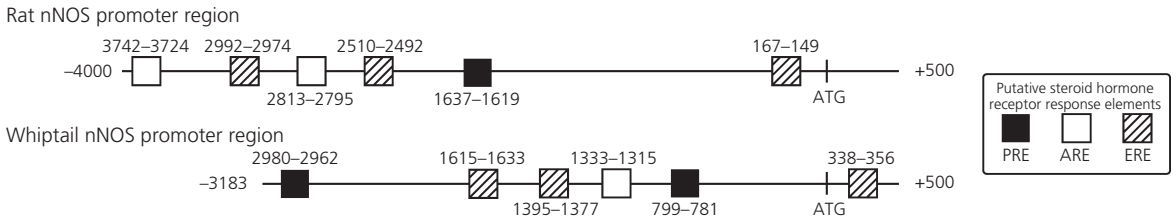

Fig. 4. Putative transcription factor binding sites in the rat and whiptail nitric oxide synthase (nNOS) promoters. The rat nNOS promoter (top) and whiptail nNOS promoter (bottom) show putative progesterone response elements (PRE, black boxes), putative androgen response elements (ARE; white boxes) and putative oestrogen response elements (ERE, hatched boxes).

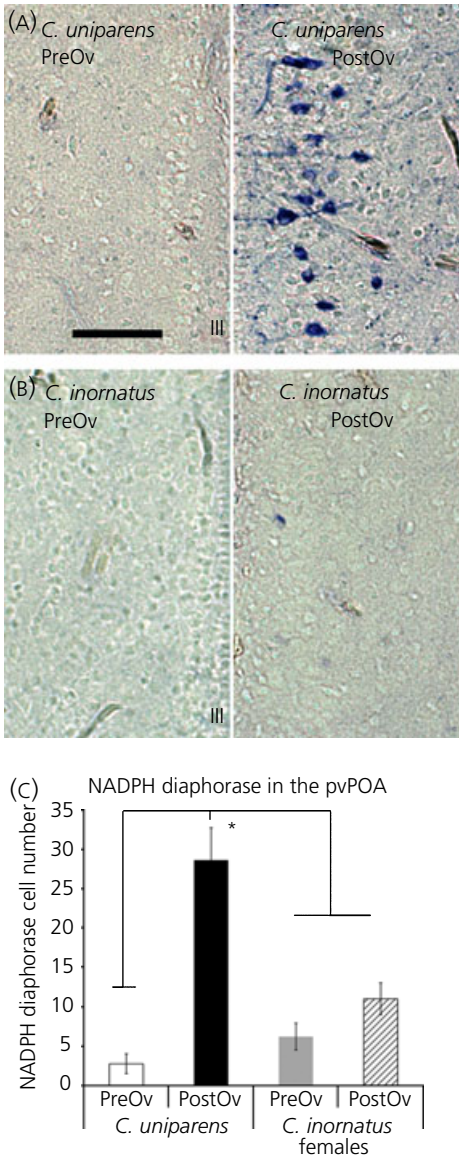

Fig. 5. Species differences in nitric oxide synthase (nNOS) regulation in the periventricular nucleus of the preoptic area (pvPOA) across the ovarian cycle. Micrographs of an NADPH diaphorase histochemistry showing nNOS-positive cells within the pvPOA of *Cnemidophorus uniparens* (A) and *Cnemidophorus inornatus* females (B). Postovulatory (PostOv) *C. uniparens* have significantly more cells within the pvPOA than all other groups (C), suggesting that the presence of nNOS facilitates mounting behaviour (Kruskal–Wallis test, $P < 0.0001$, post-hoc Mann–Whitney U-test, *$P < 0.002$). Data are shown as the mean ± SEM. Scale bar = 50 μm. Abbreviations are as shown in Fig. 1.

behaviour. This is important because the copulatory response to a receptive female is influenced by the dopamine availability in the POA of male rats (13). However, at this point, we cannot exclude possible local steroid synthesis that may be regulating nNOS in the whiptail pvPOA. We have shown that the nNOS promoter has putative steroid hormone response elements sensitive to oestradiol and androgens, as well as progestins, although these results need to be confirmed with promoter assays in future studies. The results reported in the present study strongly support the interaction between PR and nNOS in promoting male-typical sexual behaviour in the all-female whiptail species, *C. uniparens*, although, to firmly establish a functional relationship, pharmacological studies need to be conducted.

In addition to providing insights into the role of nNOS in sexual behaviour, nNOS knockout mice have also revealed the role of nNOS in regulating aggression, possibly via the serotonin system, because these mice are highly aggressive and will repeatedly mount unreceptive females (46,47). Serotonin metabolism is drastically decreased in nNOS knockout mice, and these animals also have deficits in serotonin receptor expression throughout several brain regions (48). Postovulatory *C. uniparens* individuals have lower serotonin levels in the POA compared to preovulatory individuals and infusions of serotonin directly into the POA increased mount latency in testosterone-implanted individuals (22). Given the interaction of serotonin and nitric oxide, it would be interesting to investigate the dynamics of these two molecules in the context of regulating sexual behaviour in whiptails.

Molecular substrates of evolution

Through the comparative analysis of the nNOS promoter region, we have found more putative PREs in the nNOS promoter region of whiptails compared to rats. Rodriguez-Trelles *et al.* (49) suggested that promoter sequences (more so than coding regions) are a key substrate for selection and the evolution of between-species differences in phenotype. Given that a transcription factor such as PR interacts with numerous promoters, it is more likely that the evolutionary novel regulation of genes required for the induction of male-typical mounting behaviours by PR is a result of changes in promoter sequences (i.e. a gain or loss of PREs) rather than the transcription factor specificity for its response element (50), which is supported by the data reported in the present study.

The findings of the present study have opened a new avenue of possible research into mechanisms that govern species differences in gene expression. *Cnemidophorus inornatus* females and *C. uniparens* both have a progesterone surge during the postovulatory phase of the ovarian cycle (5,7), and PR is present in the pvPOA of both species during the postovulatory phase (present study). However, only in *C. uniparens* does progesterone via PR elicit the male-typical mounting behaviour. Additionally, only *C. uniparens* has high levels of nNOS present in the pvPOA during the postovulatory phase. This observation begs the question of how PR up-regulates nNOS in *C. uniparens* but not the *C. inornatus* females? This question can also be asked of progesterone-sensitive and progesterone-insensitive *C. inornatus* males. We hypothesise that differences in regulation of the promoter sequences and the epigenome could be regulating the polymorphism to progesterone sensitivity and this will be thoroughly investigated in future studies.

Conclusions

Evolutionary change results from exploitation of variation leading to the emergence of new molecules and, as in this case, coopting endocrine signals for new functions (8). These data support the

hypothesis that a progesterone-sensitive *C. inornatus* individual played a major role in the hybridisation events that gave rise to the all-female *C. uniparens* species and, in the absence of androgens, the evolution of a novel neuroendocrine mechanism controlling pseudosexual mounting in response to high levels of progesterone. Future studies will investigate the role of nNOS in progesterone-facilitation of male-typical mounting behaviour using pharmacological manipulations of both nNOS and PR, as well as female receptivity in both the ancestral and descendant species.

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Supporting Information

The following supplementary material is available:

Fig. S1. PR immunohistochemistry control. Micrographs depict staining in the pvPOA using an antibody to PR (left) and a not antibody control (right).

Fig. S2. Western blot for PR confirms specificity for lizard antigens. One band is detected in each species of the appropriate size. Tubulin is shown below as a loading control. Ladder units are in kilodaltons.

This supplementary material can be found in the online article.

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