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THE NITRIC OXIDE–GUANOSINE 3′,5′-CYCLIC MONOPHOSPHATE PATHWAY REGULATES DOPAMINE EFFLUX IN THE MEDIAL PREOPTIC AREA AND COPULATION IN MALE RATS

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Abstract—Dopamine in the medial preoptic area (MPOA) plays a significant role in regulation of male copulation. One mediator of the MPOA dopamine level is nitric oxide. In the current study, we investigated the role of the nitric oxide–guanosine 3′,5′-cyclic monophosphate (cGMP) pathway in the regulation of MPOA dopamine and copulation in male rats. The reverse-dialysis of a membrane-permeable analog, 8-Br-cGMP, increased, while a soluble guanylyl cyclase inhibitor, 1*H*-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ), significantly reduced basal dopamine and its metabolite levels. ODQ successfully blocked a nitric oxide donor-induced increase in dopamine levels, while a neuronal nitric oxide synthase (nNOS) inhibitor was ineffective in blocking an 8-Br-cGMP-induced increase in dopamine, indicating that cGMP is "downstream" of nitric oxide. Furthermore, 8-Br-cGMP facilitated, while ODQ inhibited copulation. Given the steroid-sensitive nature of nNOS functions and the multiple roles nitric oxide plays in the MPOA, we propose that nitric oxide provides important integration of various neurochemical and neuroendocrine signals. The involvement of the central nitric oxide–cGMP pathway in the regulation of copulation also raises an interesting therapeutic possibility, as the manipulation of the same pathway in peripheral tissue is already utilized in treatment of male sexual dysfunction. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: male sexual behavior, nitric oxide, dopamine, cGMP, sGC, MPOA.

The medial preoptic area (MPOA) has been implicated in various physiological functions, including male copulatory behaviors (Hull et al., 2002). In the MPOA, dopamine (DA) plays an important role in mediating copulation (Melis and Argiolas, 1995; Hull et al., 2004). DA agonists in the MPOA have been shown to facilitate (Hull et al., 1986), while DA antagonists have been shown to inhibit copulation (Pehek et al., 1988; Warner et al., 1991). More recently, increases in extracellular DA levels in response to an estrous female, as well as during copulation, have been reported in rats (Hull et al., 1995; Sato et al., 1995) and hamsters (Triemstra et al., 2005). One mediator of this DA response is nitric oxide (NO). A nitric oxide synthase (NOS) inhibitor reduced, while the NO precursor L-arginine increased MPOA DA levels; the inactive isomer of the inhibitor was ineffective (Lorrain and Hull, 1993). Furthermore, a NOS inhibitor, but not its inactive isomer, abolished the DA response during copulatory testing (Lorrain et al., 1996). A NOS inhibitor also interfered with copulation when administered into the MPOA in both sexually naïve and experienced rats (Sato et al., 1998; Lagoda et al., 2004). Therefore, the DA–NO interaction in the MPOA is an important neurochemical process that regulates male copulation.

The importance of this DA–NO interaction has been also demonstrated in studies on the endocrine regulation of male copulation. Castration results in lower extracellular DA levels in the MPOA (Du et al., 1998), as well as reduced numbers of neuronal nitric oxide synthase immunoreactive (nNOS-ir) cells (Du and Hull, 1999). In addition, testosterone (T)-induced restoration of copulation in castrates was accompanied by reemergence of the DA response (Putnam et al., 2001) and increased nNOS-ir (Sato et al., 2005). More recent studies have shown that 17*β*-estradiol (E_2) is the main metabolite of T that mediates the effects of T on MPOA DA and nNOS. Removal of E_2 resulted in lower basal extracellular DA levels and loss of the MPOA DA response to a female (Putnam et al., 2003), as well as increased tissue content of DA, and fewer nNOS-ir cells (Scordalakes et al., 2002; Putnam et al., 2005). Thus, we have hypothesized that E_2, via its influence on nNOS, modulates DA release in the MPOA and copulation.

Although glutamate-induced DA release in the MPOA is mediated by NO (Dominguez et al., 2004), little is known about the biochemical pathways that mediate the effects of

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Abbreviations: aCSF, artificial cerebrospinal fluid; BL, baseline sample; cAMP, cyclic AMP; cGMP, guanosine 3′,5′-cyclic monophosphate; COP, copulation testing sample; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; EF, ejaculation frequency; EL, latency to ejaculation; EST, estrous female sample; E_2, 17*β*-estradiol; GnRH, gonadotropin releasing hormone; HPG, hypothalamic–pituitary–gonadal; HPLC, high performance liquid chromatography; HVA, homovanillic acid; IFT, total intromission frequency; ir, immunoreactivity/immunoreactive; L-NMMA, N^G-monomethyl-L-arginine acetate; MAPK, mitogen-activated protein kinase; ML, latency to the first mount; MPOA, medial preoptic area; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, 1*H*-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one; PDE, phosphodiesterases; PEI, postejaculatory interval following the first ejaculation; PKG, guanosine 3′,5′-cyclic monophosphate-dependent protein kinase; POST, post-treatment sample; PR, progestin receptor; sGC, soluble guanylyl cyclase; SNP, sodium nitroprusside; T, testosterone.

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NO on DA. A major effector of NO is the soluble guanylyl cyclase (sGC), and subsequent production of guanosine 3′,5′-cyclic monophosphate (cGMP) (Garthwaite et al., 1988; Bredt and Snyder, 1989). In the MPOA, the NO–cGMP pathway is known to play a role in regulation of gonadotropin releasing hormone (GnRH, Pu et al., 1996, 1998). Thus, it is possible that the NO–cGMP pathway in the MPOA regulates, not only endocrine function, but also neurochemical signaling involved in male copulation. We hypothesized that the NO–cGMP pathway mediates NO-induced MPOA DA release and facilitation of copulation. In the current study, we examined the effects of cGMP manipulation on MPOA DA as well as on copulation.

EXPERIMENTAL PROCEDURES

Subjects

Adult male Long-Evans Blue Spruce rats (250–300 g), purchased from Harlan (Indianapolis, IN, USA), were individually housed in a temperature- and humidity-controlled colony room. Food and water were available *ad libitum*. The colony room was maintained on a 14:10 reversed light/dark cycle with lights off at 11:00 h. All procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the local Institutional Animal Care and Use Committee (IACUC). Efforts were made to minimize the number of animals used and their suffering.

All animals were screened for copulatory ability 1 week after arrival. Ovariectomized and hormone-primed [10 μg E_2 benzoate and 400 μg progesterone (sc, in olive oil vehicle, Sigma-Aldrich, St. Louis, MO, USA), 48 h and 4 h, respectively, prior to testing] females of the same strain were used for all copulation testing. Only the animals that achieved at least three ejaculations during a maximum of four 30-min testing sessions were included.

Behavioral testing

The following behavioral measures were recorded during 30 min testing sessions: mount frequency during the first copulatory series (MF1), total mount frequency (MFT), intromission frequency during the first copulatory series (IF1), total intromission frequency (IFT), total ejaculation frequency (EF). In addition, latency to the first mount (ML) and first intromission (IL) following introduction of the female, latency to the first ejaculation (EL1) following the first intromission, and the duration of the postejaculatory interval following the first ejaculation (PEI1, latency from ejaculation to the next intromission) were recorded. When available, MF, IF, EL, and PEI for the subsequent copulatory series were recorded as well.

Intracranial guide cannula implantation

Animals were implanted with a guide cannula, ending 2 mm above the left MPOA (mm from bregma: AP, +2.3; ML, 0.4; DV, −6.3; (Pellegrino et al., 1979), as previously described (Putnam et al., 2003). A photomicrograph of a typical probe placement is shown in Fig. 1, and a summary of probe placements for all animals in this study is shown in Fig. 2 (modified from Swanson, 2004). All animals were tested 7±1 days after surgery.

In vivo microdialysis

Probe construction. Microdialysis probes with a concentric flow design were constructed in house as previously described (Renno et al., 1998). The finished probe had a 1 mm long active dialyzing membrane (MW cut off 13,000 kD, 210 μm o.d., Spectrum Laboratories, Rancho Dominguez, CA, USA).

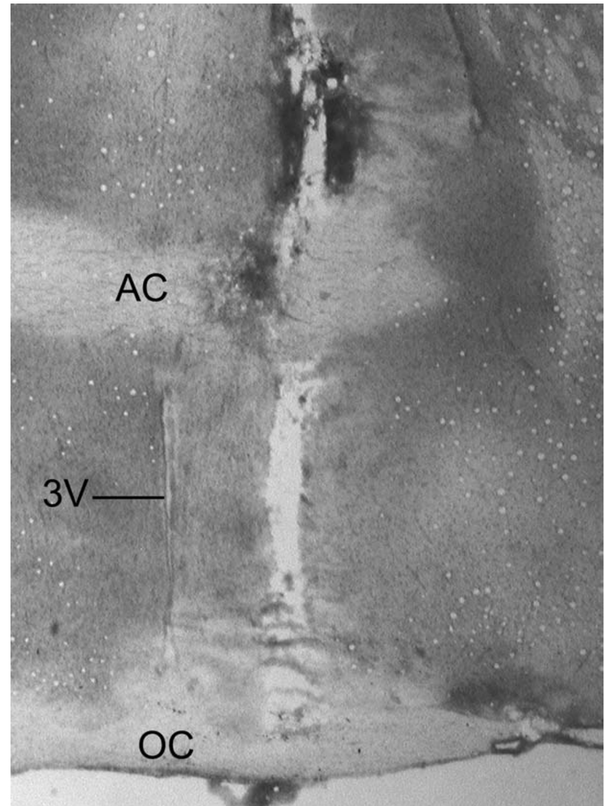

Fig. 1. A photomicrograph showing a representative microdialysis probe placement. AC, anterior commissure; OC, optic chiasm; 3V, third ventricle.

Microdialysis setup. On the day of testing, subjects were lightly anesthetized with ketamine hydrochloride (12.5 mg/kg) and xylazine hydrochloride (1 mg/kg), and a microdialysis probe was inserted into the guide cannula. Flow of the artificial cerebrospinal fluid (aCSF; Dulbecco's solution, Sigma-Aldrich; in mM: 138 NaCl, 2.7 KCl, 0.5 $MgCl_2$, 1.5 KH_2PO_4, and 1.2 $CaCl_2$, pH=6.8, filtered and degassed before use) began immediately upon the insertion of the probe. aCSF was delivered to the microdialysis probe with a Harvard Syringe pump (PHD2000) through a dual-channel swivel (Instech Laboratories, Plymouth Meeting, PA, USA) at 0.5 μl/min. Samples were collected into a sample vial attached to the tether line through a fused silica capillary. Following a minimum of 3 h stabilization period, samples were collected every 6 min, immediately frozen in powdered dry ice, and stored in a −80 °C ultracold freezer until analysis. For the experiment using sodium nitroprusside (SNP), deoxygenated aCSF was used throughout the experiment to prevent degradation of SNP. Upon the conclusion of dialysis testing, animals were killed, the brains removed, and the placement of probes histologically verified under a projection microscope.

High performance liquid chromatography (HPLC)

Monoamines and their metabolites were assayed with HPLC with electrochemical detection (HPLC-EC). Mobile phase (pH. 3.8), consisting of 30 mM citric acid, 50 mM Na^+ acetate, 0.027 mM ${Na^+}_2$ EDTA, 0.5 mM octane sulfonic acid, 2.5% (v/v) acetonitrile, and 0.2% (v/v) tetrahydrofuran, were delivered by a Gilson (Middleton, WI, USA) model 307 pump with a 5 ml piston head at 0.5 ml/min. The pump was equipped with a Valco (Houston, TX, USA) flow splitter to deliver 8 μl/min to an LC-Packings (San Francisco, CA, USA) Fusica II reverse-phase capillary column

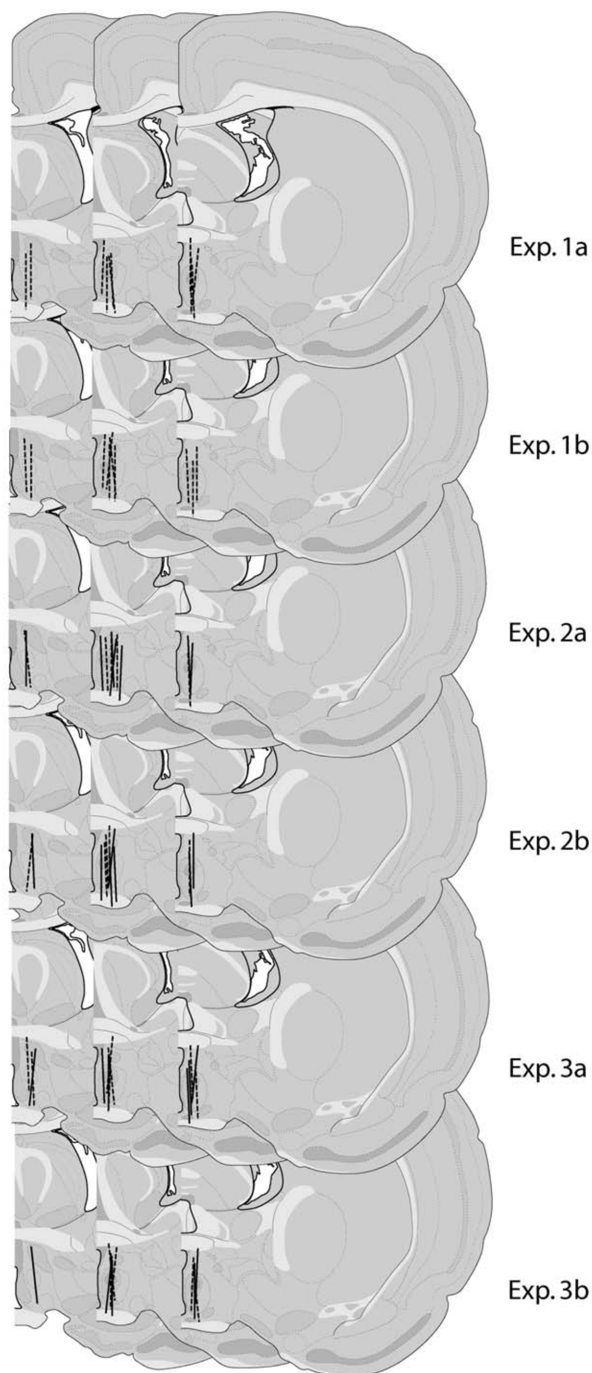

Fig. 2. Microdialysis probe placement (figures modified from Swanson, 2004). For exp. 2 and 3, placements for control animals are in dotted lines, while those of drug-treated animals are shown in solid lines.

(300 μm I.D. and 25 mm in length, packed with Hypersil C18 BDS, Sunnyvale, CA, USA). Samples were loaded via a Rheodyne (Rohnert Park, CA, USA) 7520 manual injector equipped with a 0.5 μl sample loop. Electrochemically active compounds were detected with an Antec (Leiden, The Netherlands) microcell (VT-03 EC flowcell with a 25 μm spacer) with a glassy carbon working electrode maintained at a potential of +0.7 V relative to a Ag/AgCl reference electrode on the Antec Decade.

Daily injections of 1 pg/μl of external standards (0.5 pg on column) were used to verify sensitivity, reproducibility, and the retention time. Using the Gilson Unipoint program, the amount of DA in a sample was measured as the area under the curve of the appropriate peak in the chromatogram. The changes in DA levels were expressed as percent change relative to the average of the last three baseline samples (BL).

Drugs

1*H*-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ), 8-Br-cGMP, and SNP were obtained from Sigma-Aldrich, and N^G-monomethyl-L-arginine acetate (L-NMMA) was obtained from Tocris-Cookson (Ellisville, MO, USA). All drugs that were reverse-dialyzed were dissolved in the aCSF and were tested on the HPLC to ensure that they did not interfere with the transmitter analysis. The doses for each drug were determined through pilot studies and previous experiments (Lorrain and Hull, 1993). In experiment 1, we initially tested 8-Br-cGMP at doses higher than 100 μM. However, the doses higher than 100 μM resulted in extremely unstable DA levels and were not tested further. We did not test ODQ at doses higher than 100 μM, due to its limited solubility in our aCSF.

Experimental design and statistical analysis

Experiment 1: effects of 8-Br-cGMP and ODQ on extracellular DA and its metabolites in the MPOA. In order to test whether cGMP can increase efflux of DA and its metabolites in the MPOA, a membrane-permeable analog, 8-Br-cGMP, was reverse-dialyzed into the MPOA of 10 males in experiment 1A. To test whether inhibition of sCG decreases endogenous MPOA DA levels, ODQ, an inhibitor of sGC, was reverse-dialyzed into the MPOA of 10 males in experiment 1B (Fig. 3). Following the BL collection, two to three doses of each drug were tested per animal in a random order. Each dose was reverse-dialyzed for 30 min, with a 15 min wash-out period between doses. The average percent change relative to the average baseline was used for statistical analysis. The neurochemical data were analyzed with a one-way ANOVA, followed by planned comparisons with BL with independent sample *t*-tests with Bonferroni's procedure ($P<0.05$).

Experiment 2; is cGMP "downstream" from NO? To verify that NO's facilitation of MPOA DA efflux is mediated by cGMP, we first reverse-dialyzed a sGC inhibitor before and during reverse-dialysis of a NO donor (experiment 1A). Inhibition of sGC should block the ability of NO to increase extracellular DA and metabolites. We also tested whether inhibition of NOS could decrease the facilitative effects of a cGMP analog (experiment 1B). If cGMP is downstream from NO, then NOS inhibition should not affect cGMP's facilitation of efflux. Following the BL collection, the inhibitor of sGC or NOS was reverse-dialyzed for 30 min into the MPOA of 12 and 10 males, respectively, followed by co-administration of the appropriate inhibitor with SNP (experiment 2A, $n=7$, Fig. 4) or 8-Br-cGMP (experiment 2B, $n=5$, Fig. 5). The percent changes from the baseline values for DA and its metabolites were analyzed with a two-way mixed ANOVA (Drug$\times$sample period), followed by a one-way repeated measures ANOVA and planned pair-wise comparisons with BL, using paired sample *t*-tests with Bonferroni's procedure ($P<0.05$).

Experiment 3: effects of 8-Br-cGMP and ODQ on copulation and MPOA DA. Finally, we determined whether the facilitative effects of 8-Br-cGMP and the inhibitory effects of ODQ on MPOA DA efflux were correlated with enhancement and inhibition, respectively, of male copulation. Following the BL collection, 10 μg 8-Br-cGMP ($n=7$) or 10 μg ODQ ($n=5$) was reverse-dialyzed for 30 min. Control animals ($n=6$ and $n=5$, respectively) received aCSF throughout the test. An estrous female was then introduced behind a barrier for 24 min (four samples, EST), after which the male was allowed to copulate for 30 min (five samples, COP). The

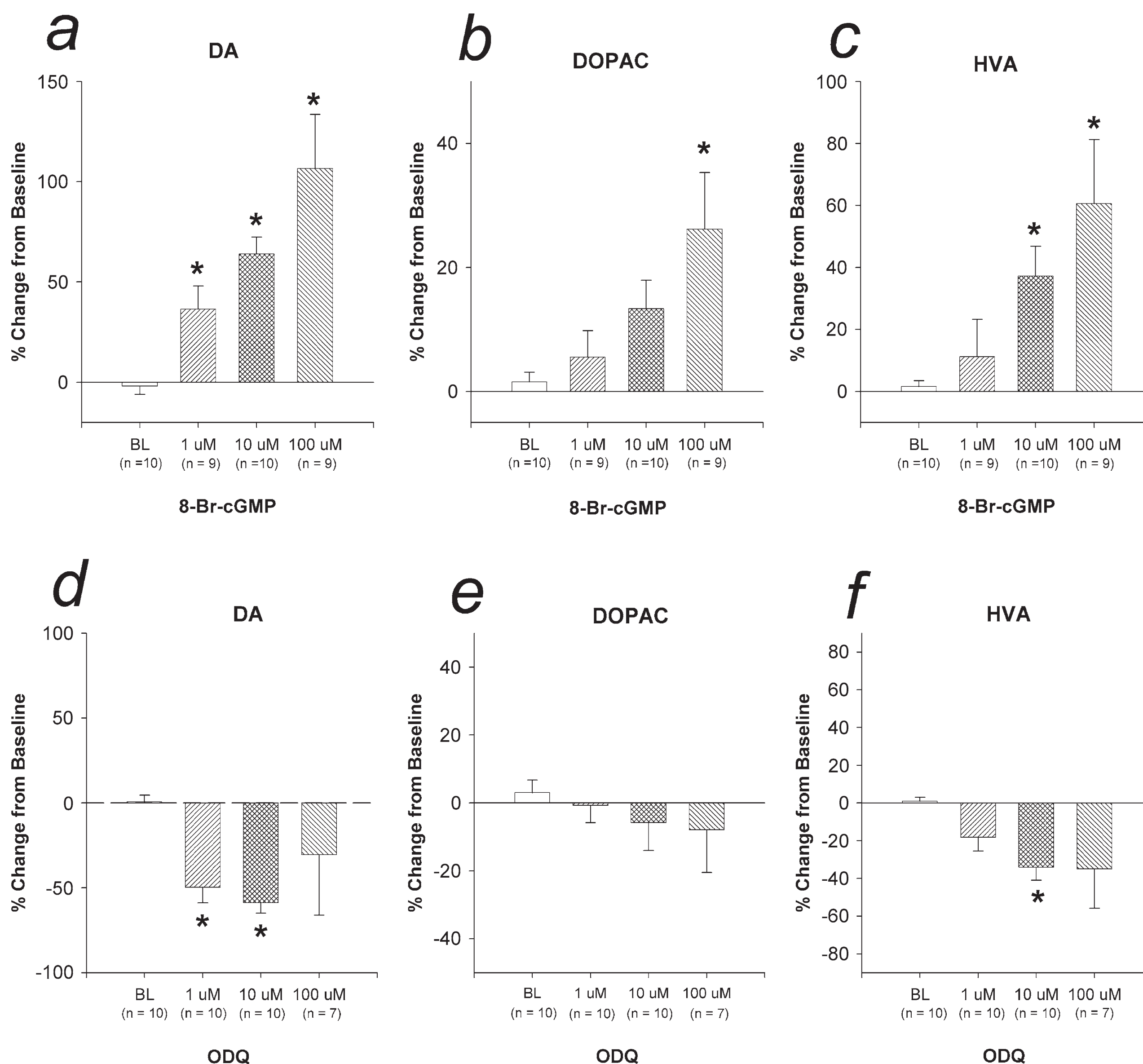

Fig. 3. Exp. 1a: Effects of 8-Br-cGMP reverse-dialysis on MPOA DA (a), DOPAC (b) and HVA (c). The BL immediately preceding the drug administration is shown as BL. Exp. 1b: Effects of ODQ reverse-dialysis on MPOA DA (d), DOPAC (e), and HVA (f). The BL immediately preceding the drug administration is shown as BL. * Significantly different from average of BL ($P<0.05$).

female was then removed and three post-testing samples were collected (POST, Figs. 5 and 6). The DA data were analyzed as in experiment 2. The behavioral data were analyzed with independent sample *t*-tests.

RESULTS

Experiment 1: effects of 8-Br-cGMP and ODQ on extracellular DA in the MPOA

Experiment 1A. 8-Br-cGMP increased extracellular DA levels in the MPOA. One-way ANOVA revealed a significant effect of 8-Br-cGMP on DA levels [$F_{(3,\ 34)}=9.57$, $P<0.001$, Fig. 3a]. Post hoc tests revealed significant increases in DA levels with 1, 10, and 100 μM 8-Br-cGMP administration. Similarly, there were significant effects of 8-Br-cGMP on 3, 4-dihydroxyphenylacetic acid (DOPAC) [$F_{(3,\ 34)}=3.98$, $P=0.016$] and homovanillic acid (HVA) [$F_{(3,\ 34)}=4.49$, $P=0.009$] (Fig. 3b and 3c). Post hoc tests revealed that 100 μM 8-Br-cGMP increased both DOPAC and HVA, and 10 μM 8-Br-cGMP increased HVA levels relative to the BL.

Experiment 1B: ODQ decreased extracellular DA levels in the MPOA. There was a significant effect of ODQ on DA [$F_{(3,\ 33)}=3.46$, $P=0.027$, Fig. 3d] and HVA [$F_{(3,\ 33)}=3.20$, $P=0.036$, Fig. 3f] levels, while the effect on DOPAC was not significant [$F_{(3,\ 33)}=0.423$, $P>0.05$, Fig. 3e] (Fig. 3a). Post hoc tests revealed that 1 μM ODQ significantly reduced DA levels, while 10 μM ODQ reduced both DA and HVA.

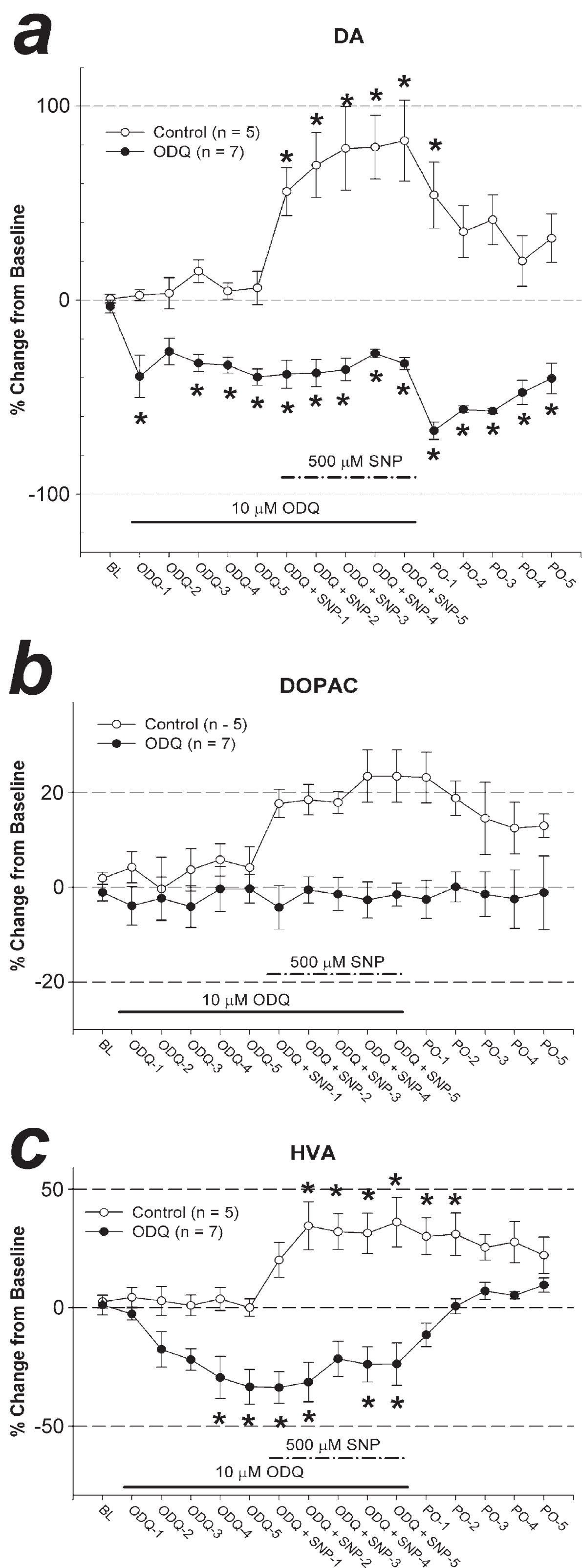

Fig. 4. Exp. 2a: Effects of ODQ pre-treatment on SNP-induced decreases in MPOA DA (a), DOPAC (b), and HVA (c). The data for the control animals (without ODQ-pretreatment) are shown as open circles, and the data for the ODQ animals (with ODQ-pretreatment) are shown as closed circles. The BL immediately preceding the drug administration is shown as BL. * Significantly different from average of BL ($P<0.05$).

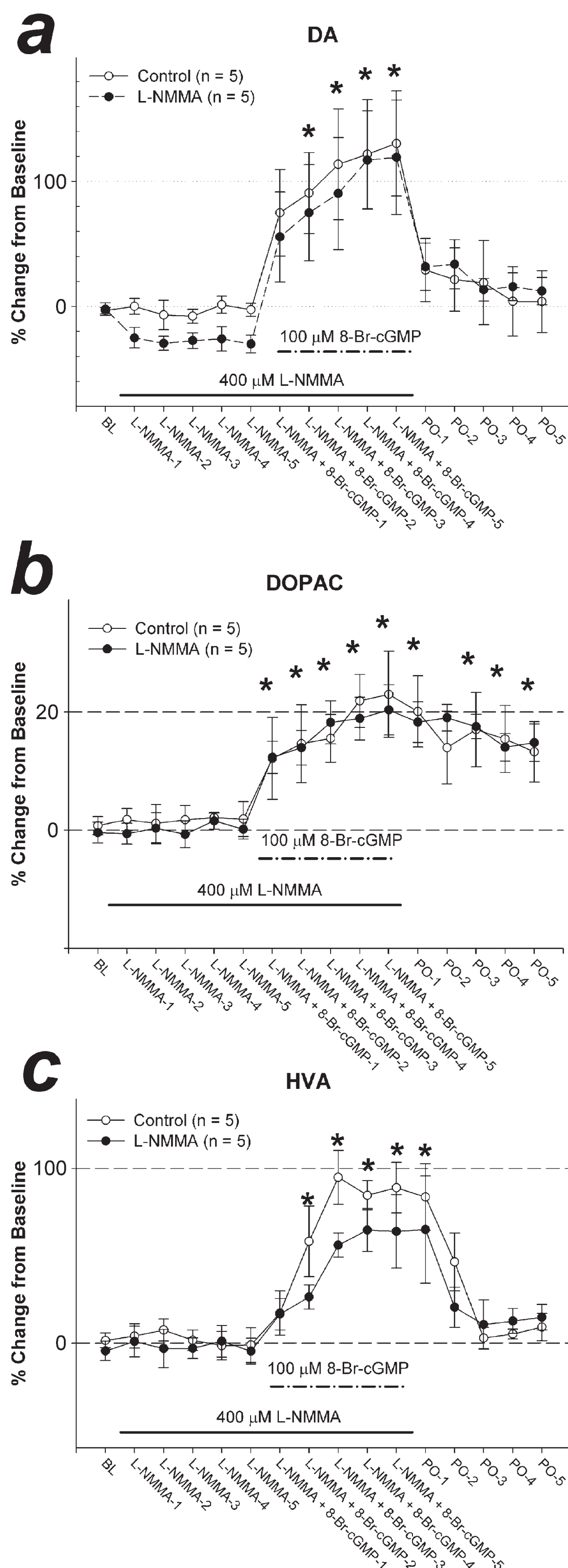

Fig. 5. Exp. 2b: Effects of L-NMMA pre-treatment on 8-Br-cGMP-induced increases in MPOA DA (a), DOPAC (b), and HVA (c). The data for the control animals (without L-NMMA-pretreatment) are shown as open circles, and the data for the L-NMMA animals (with L-NMMA-pretreatment) are shown as closed circles. The BL immediately preceding the drug administration is shown as BL. * Significantly different from average of BL ($P<0.05$).

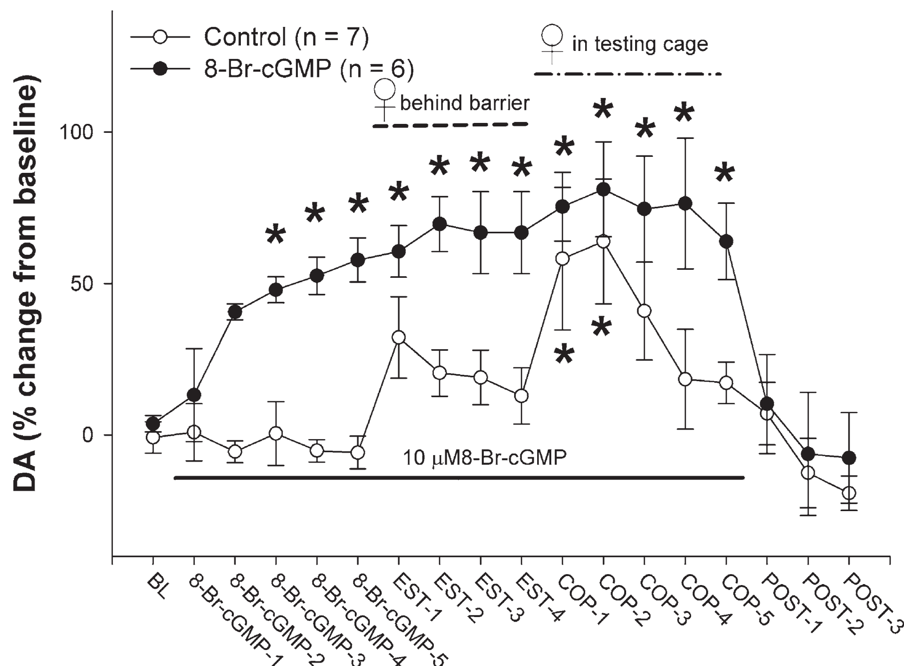

Fig. 6. Exp. 3a: Effects of 8-Br-cGMP reverse-dialysis on MPOA DA during copulatory testing. The data from control animals (without 8-Br-cGMP) are shown as open circles, while the data from the 8-Br-cGMP-treated animals are shown as closed circles. The solid line at the bottom of the figure indicates the duration of 8-Br-cGMP administration. The dotted lines at the top of the figure indicate the duration of the exposure to an EST behind a barrier and the duration of COP. The BL immediately preceding the drug administration is shown as BL. POST samples were taken after removal of the female. * Significantly different from average of BL ($P<0.05$).

Experiment 2: is cGMP "downstream" from NO?

Experiment 2A: sGC inhibition blocks NO-induced DA release in the MPOA. ODQ successfully blocked SNP-induced increases in DA and its metabolite levels. A two-way ANOVA revealed a significant interaction on DA levels [$F_{(15,\ 150)}=8.26$, $P<0.001$], as well as significant main effects of sample period [$F_{(15,\ 120)}=5.86$, $P<0.001$] and ODQ [$F_{(15,\ 150)}=45.53$, $P<0.001$] (Fig. 4a). Post hoc tests revealed significant increases in DA levels by SNP administration in the absence of ODQ, while ODQ reduced basal DA levels and blocked a SNP-induced increase, suggesting that cGMP mediates the NO-induced increase in extracellular DA.

The effects on DOPAC were less pronounced. Although there were main effects of sample period [$F_{(15,\ 120)}=1.73$, $P=0.05$] and ODQ [$F_{(15,\ 150)}=38.73$, $P<0.001$], the interaction was not significant [$F_{(15,\ 150)}=1.67$, $P>0.05$] (Fig. 4b). Post hoc tests revealed no significant increase in DOPAC levels from the BL. HVA, on the other hand, showed changes similar to those seen in DA levels. There were significant interaction [$F_{(15,\ 150)}=5.91$, $P<0.001$] and main effects of sample period [$F_{(15,\ 120)}=7.06$, $P<0.001$] and ODQ [$F_{(15,\ 150)}=30.58$, $P<0.001$] (Fig. 4c). Post hoc tests revealed that ODQ reduced HVA levels by itself and blocked a SNP-induced increase.

Experiment 2B: NOS inhibition fails to block cGMP-induced DA release in the MPOA. The NOS inhibitor failed to affect 8-Br-cGMP-induced DA release. A two-way ANOVA revealed a significant main effect of sample period on DA levels [$F_{(15,\ 120)}=8.01$, $P<0.001$], while the interaction [$F_{(15,\ 120)}=0.16$, $P>0.05$] and the main effect of L-NMMA [$F_{(15,\ 120)}=0.69$, $P>0.05$] were not significant (Fig. 5a). Post hoc tests revealed that there were significant increases in DA levels during 8-Br-cGMP administration. Similarly, there were main effects of sample periods on DOPAC [$F_{(15,\ 120)}=11.88$, $P<0.001$] and HVA [$F_{(15,\ 120)}=14.01$, $P<0.001$], while there was no interaction on DOPAC [$F_{(15,\ 120)}=0.20$, $P>0.05$] or HVA [$F_{(15,\ 120)}=0.75$, $P>0.05$] and no main effects of L-NMMA [$F_{(15,\ 120)}=0.03$, $F_{(15,\ 120)}=4.53$, respectively, both $P>0.05$] (Fig. 5b and 5c). Post hoc tests revealed increases in DOPAC and HVA levels during and after 8-Br-cGMP administration. Therefore, blocking NOS activity failed to affect 8-Br-cGMP's increases in DA and its metabolites, suggesting that NOS is "upstream" of cGMP.

Experiment 3: effects of 8-Br-cGMP and ODQ on copulation and MPOA DA

Experiment 3A: cGMP facilitates MPOA DA release and copulation. As in experiment 1, 8-Br-cGMP increased extracellular DA levels. There were significant interaction [$F_{(17,\ 187)}=2.11$, $P=0.008$] and main effects of sample period [$F_{(17,\ 187)}=12.05$, $P<0.001$] and 8-Br-cGMP [$F_{(1,\ 187)}=9.41$, $P=0.011$] on DA levels (Fig. 6). Post hoc tests revealed that DA increased only during early copulation samples (COP) in control animals; DA levels remained elevated during most of the 8-Br-cGMP administration period in drug-treated animals.

Table 1. Exp. 3a: effects of 8-Br-cGMP reverse-dialysis on copulation

Treatment	n	MFT	MF1	MF2	MF3
Control	6	23.83±8.34	10.50±3.97	8.50±2.79	4.83±1.76
10 μM 8-Br-cGMP	7	11.50±1.57	5.86±0.94	3.29±1.08	2.14±0.59
Treatment	n	IFT	IF1	IF2	IF3
Control	6	19.33±2.04	7.83±0.91	6.17±2.79	6.00±0.82
10 μM 8-Br-cGMP	7	17.67±2.66	5.86±1.03	3.72±1.08*	4.00±0.85
Treatment	n	EF	EL1	EL2	EL3
Control	6	3.00±0.00	323.50±46.99	221.67±27.03	212.33±44.25
10 μM 8-Br-cGMP	7	3.71±0.76*	207.14±48.58	125.00±27.09*	93.29±19.65*
Treatment	n	ML	IL	PEI1	PEI2
Control	6	10.00±2.31	55.33±28.02	391.83±22.51	481.67±32.29
10 μM 8-Br-cGMP	7	9.57±2.63	18.14±3.20	369.29±22.86	356.14±14.76*

* Significantly different from control animals ($P<0.05$). IF, intromission frequency; IL, intromission latency; MF, mount frequency; MFT, total mount frequency.

The effects of 8-Br-cGMP administration on copulatory measures are shown in Table 1. 8-Br-cGMP significantly increased the number of ejaculations [EF, $t_{(11)}=2.30$, $P=0.042$], while reducing the number of intromissions during the second copulatory series [IF2, $t_{(11)}=2.51$, $P=0.029$], latency to the second and third ejaculations [EL2, $t_{(11)}=3.72$, $P=0.003$; EL3, $t_{(11)}=2.42$, $P=0.034$], and post-ejaculatory interval following the second ejaculation [PEI2, $t_{(11)}=2.59$, $P=0.025$].

Experiment 3B: sGC inhibition reduces MPOA DA levels and inhibits copulation. As in experiment 1, ODQ inhibited MPOA DA efflux. There were significant interaction [$F_{(17,\ 136)}=8.47$, $P<0.001$] and main effects of sample period [$F_{(17,\ 136)}=2.68$, $P<0.001$] and ODQ [$F_{(17,\ 136)}=31.57$, $P<0.001$] (Fig. 7). Post hoc tests revealed that DA levels increased during early COP in control animals; DA levels remained below baseline throughout the duration of ODQ administration in ODQ-treated animals.

The effects of ODQ on copulatory measures are shown in Table 2. ODQ significantly reduced the number of ejaculations [EF, $t_{(8)}=4.95$, $P<0.001$] and number of intromissions during the 30 min test [IFT, $t_{(8)}=2.42$, $P=0.042$].

DISCUSSION

NO plays an important role in the regulation of DA levels in the MPOA and copulation. In the current study, we demonstrated that NO exerts its effects on MPOA DA and copulation via its stimulation of sGC and subsequent pro-

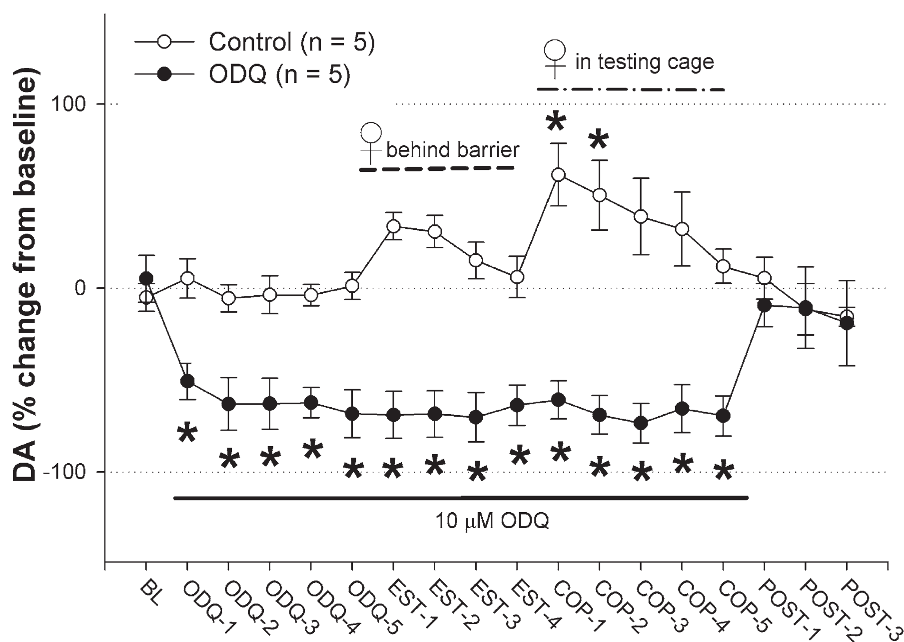

Fig. 7. Exp. 3b: Effects of ODQ reverse-dialysis on MPOA DA during copulatory testing. The data from control animals (without ODQ) are shown as open circles, while the data from the ODQ-treated animals are shown as closed circles. The solid line at the bottom of the figure indicates the duration of ODQ administration. The dotted lines at the top of the figure indicate the duration of the exposure to an EST behind a barrier and the duration of COP. The BL immediately preceding the drug administration is shown as BL. POST samples were taken after removal of the female. * Significantly different from average of BL ($P<0.05$).

Table 2. Exp. 3b: Effects of ODQ reverse-dialysis on copulation

Treatment	*n*	ML	IL	EL1	PEI1	
Control	5	7.80±1.83	63.60±32.50	308.00±51.55	393.20±21.72	
10 μM ODQ	5	13.40±2.64	366.20±186.69	342.20±105.11	417.80±66.68	
Treatment	*n*	MF1	MFT	IF1	IFT	EF
Control	5	11.60±4.69	26.00±9.84	7.40±1.36	18.00±2.85	3.20±0.20
10 μM ODQ	5	13.20±3.12	17.20±2.46	5.20±0.58	9.40±2.14*	1.80±0.20*

* Significantly different from control animals ($P<0.05$). See Table 1 for abbreviations.

duction of cGMP. 8-Br-cGMP, a membrane-permeable analog of cGMP, increased basal DA levels in freely moving rats, while ODQ, a sGC inhibitor, reduced basal DA levels. Furthermore, ODQ successfully blocked the increase in DA induced by SNP, a NO donor. However, L-NMMA, a NOS inhibitor, failed to block the 8-Br-cGMP-induced increase in DA, indicating that 8-Br-cGMP had bypassed the need for NO to produce the effect. Therefore, cGMP is "downstream" of NO in the MPOA. Finally, 8-Br-cGMP increased DA during copulatory testing and facilitated copulation. On the other hand, ODQ reduced DA levels and inhibited copulation. Therefore, the current study demonstrates that the NO–cGMP pathway mediates the NO-induced increase in MPOA extracellular DA levels and facilitation of copulation in male rats.

Currently, we do not know how cGMP regulates DA levels. One possibility is through activation of guanosine 3′,5′-cyclic monophosphate-dependent protein kinases (PKGs), as seen in female sexual behaviors (Chu et al., 1999; Gonzalez-Flores and Etgen, 2004). In females, activation of the NO–cGMP-PKG pathway, possibly through mitogen-activated protein kinases (MAPKs), phosphorylates progestin receptors (PR), subsequently activating female sexual behaviors (Mani et al., 1994b; Gonzalez-Flores and Etgen, 2004). In addition to the NO–cGMP–PKG–MAPK–PR pathway, other mutually facilitative intracellular pathways have been shown to activate female receptivity. Both DA and P stimulate cyclic AMP (cAMP) production, which activates DA- and cAMP-related phosphoprotein (DARPP-32), which may in turn decrease dephosphorylation of the PR and its associated coactivators, allowing them to remain active for a longer time (Mani et al., 1994a, 2000). It is possible that similar pathways exist in males as well, converging on PR or other effectors to activate sexual behaviors; however P appears to have relatively little effect in males (Witt et al., 1994; Phelps et al., 1998). Another possible pathway for the effect of cGMP on DA is through cGMP-sensitive phosphodiesterases (PDEs, Beavo, 1995; McDonald and Murad, 1996). This could lead to changes in cAMP levels and subsequent biochemical changes that eventually result in DA release. Further studies are needed to examine these and other possibilities.

In contrast to these studies, cGMP does not appear to mediate the effects of NO in the paraventricular nucleus of the hypothalamus on penile erection (Melis and Argiolas, 1997). Instead, the authors suggested that NO is produced within oxytocinergic cells to increase extrahypothalamic oxytocin release. Other mediators of NO effects include ADP-ribosylation (reviewed in Schlossmann et al., 2003) and S-nitrosylation (reviewed in Ahern et al., 2002).

The current study and a previous report from our laboratory (Lorrain and Hull, 1993) suggest that NO enhances DA release, but not by inhibition of reuptake. Both studies show increases in DA, HVA and DOPAC levels with stimulation of the NO–cGMP pathway and decreases in all three with inhibition of that pathway. One would expect an NO-stimulated increase in DA and decreases in its metabolites, if the DA transporter were inhibited or reversed, since intracellular degradation would be delayed. However, we have observed DA and its metabolites to change in opposite directions with reverse-dialysis of glutamate in a study in anesthetized rats, suggesting that the DA transporter had been inhibited (Dominguez et al., 2004). It is not clear whether the urethane anesthetic or effects of glutamate on pathways other than NO gave rise to these differences. Given the wide spectrum of NO effectors including the DA transporter (Pogun et al., 1994; Kiss et al., 1999; Kiss, 2000; Kiss and Vizi, 2001), *N*-methyl-D-aspartate receptors (Manzoni et al., 1992; Manzoni and Bockaert, 1993; Fagni and Bockaert, 1996), and synaptic docking/fusion proteins (Meffert et al., 1996), it is quite possible that NO regulates both release and reuptake of DA through multiple pathways, depending on the level of NO production or other factors.

There are contradictory reports on the role of NO in regulation of DA release (see Prast and Philippu, 2001 for review). In the striatum, most report facilitative effects of NO on DA release (see West et al., 2002 for review), although some report inhibition of DA release (Ishida et al., 1994 for example). One of the most exhaustive series of studies by West and colleagues (West and Galloway, 1996, 1997, 1998; West et al., 2002) reports that the effects of NO on DA are mostly mediated by its effects on release, not on reuptake. Contrary to the current study, they observed potentiation of basal and NO-induced DA release with sGC inhibition (West and Galloway, 1996). This may be accounted for by a local increase in GABA levels (Guevara-Guzman et al., 1994) or the regulation of medium spiny neuron firing by the NO–sGC pathway (West and Grace, 2004), which could inhibit nigral DAergic neurons. Thus, there appeared to be an area-specific regulation of DAergic systems by the NO–cGMP pathway.

Both in the striatum and the MPOA, one striking feature of NO is its involvement in multiple cellular processes (Ahern et al., 2002). In the MPOA, NO regulates not only DA and copulation, but also GnRH release in males (Pu et

al., 1998) and females (Pu et al., 1996). NO has also been shown to inhibit aromatase (Snyder et al., 1996), an enzyme that synthesizes estrogens. In addition, several groups have shown that MPOA nNOS is sensitive to gonadal steroids. MPOA nNOS is co-localized with gonadal steroid receptors in rats (Sato et al., 2005), mice (Scordalakes et al., 2002), and hamsters (Hadeishi and Wood, 1996). nNOS-ir is reduced in castrated rats (Du and Hull, 1999) and hamsters (Hadeishi and Wood, 1996) and is restored by T or estrogen, but not by the non-aromatizable androgen dihydrotestosterone (Putnam et al., 2005). This reduction in nNOS-ir with castration does not appear to be a simple reduction in nNOS expression, as we did not observe any changes in nNOS protein levels in one-month castrates (S. Sato, unpublished observations). This is in contrast to the hormonal regulation of nNOS in females, where changes in nNOS mRNA expression are reported (Ceccatelli et al., 1996). Our previous reports on MPOA DA levels also indirectly suggest reduced nitrergic stimulation in the MPOA of castrates, since castration decreases extracellular DA, and we have shown NO to be a major regulator of both basal and female-stimulated DA release. Collectively, these data suggest that the lack of gonadal steroids leads to reduced NO levels in the male MPOA, although there is one contradictory report on the hormonal regulation of nNOS in the MPOA (Singh et al., 2000). It is possible that the apparent discrepancies may reflect steroid effects on protein–protein interactions of nNOS, since all of the studies showing castration-induced decreases in nNOS-ir (Hadeishi and Wood, 1996; Du and Hull, 1999; Scordalakes et al., 2002; Putnam et al., 2005) used the same antibody to the C-terminal end of nNOS, whereas the single

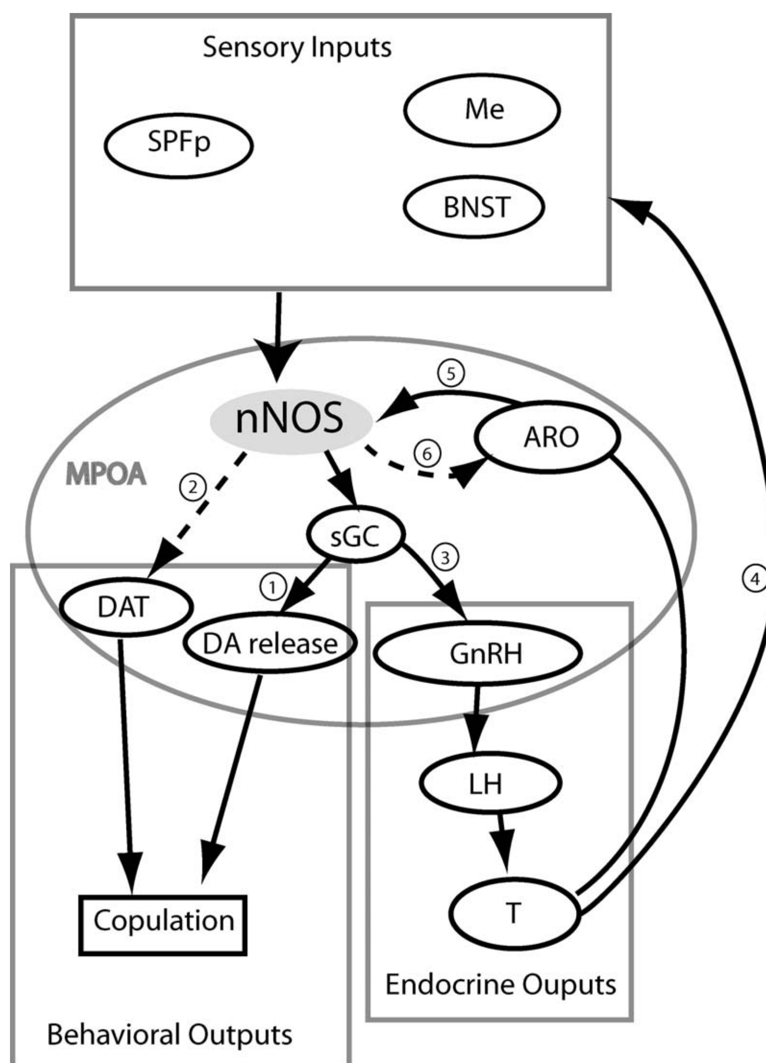

Fig. 8. Schematic representation of the male copulation circuit in relationship to NO. Solid lines indicate facilitative, and dashed lines indicate inhibitory influence. Sensory inputs activate nNOS, resulting in generation of NO. NO, in turn, induces 1) DA release via activation of sGC, 2) inhibits DA reuptake by inhibition of DAT, which facilitates copulation. In addition, NO stimulates the HPG axis (3), resulting in increased T secretion. T, in turn, (4) enhances sensory inputs by acting in structures that mediate sensory inputs (e.g. BNST), and (5) alters nNOS function after conversion to E_2. NO also inhibits ARO (6), acting as a part of direct negative feedback loop. ARO, aromatase; BNST, bed nucleus of stria terminalis; DAT, DA transporter; LH, luteinizing hormone; ME, medial amygdala; SPFp, subparafascicular nucleus.

study showing an increase (Singh et al., 2000) used an antibody to the N-terminal region. Furthermore, the latter study found very few nNOS-labeled cells in the MPOA, whereas the former studies reported numerous nNOS-ir cells.

The gonadal steroid-sensitive nature of nNOS in the MPOA provides us with two possible feedback mechanisms. First, increased NO levels might lead to inhibition of aromatase, and subsequently estrogen production, which in turn would reduce NO levels. This may serve as a local negative feedback loop. Second, an increase in NO production, via its effect on the hypothalamic–pituitary–gonadal (HPG) axis, would lead to an increase in gonadal steroid levels, which would further stimulate NO production. This positive feedback mechanism would serve the organism, when he encounters multiple mating partners within a relatively short time frame, by preparing the neural circuitry for optimum copulatory performance. We suspect, for example, that NO is involved in the endocrine responses (increased luteinizing hormone and T secretion) to the exposure to an estrous female or copulation (Bliss et al., 1972; Kamel et al., 1975, 1977; Macrides et al., 1975; Kamel and Frankel, 1978; Amistislavskaya and Popova, 2004). Such endocrine responses would enhance nitrergic stimulation of the MPOA via their effects on MPOA nNOS and facilitate the male's subsequent copulatory performance, increasing the chance of successful reproduction. If an estrous female is not available, the nitrergic (and DAergic) stimulation of the MPOA would remain low, and endocrine response would remain subdued. It appears that NO, by its diffusible and highly reactive nature, is well-suited to play a role in integration of sensory and endocrine inputs, as well as coordination of motor and endocrine outputs (Fig. 8).

Besides the elucidation of neurochemical pathways involved in regulation of male sexual behaviors, the current study also raises an interesting clinical implication. The role of the NO–cGMP pathway in the regulation of several physiological functions, including smooth muscle relaxation, is well known (McDonald and Murad, 1996). This has been utilized in the clinical setting in the use of PDE inhibitors to prolong NO-induced increases in cGMP and thereby treat erectile dysfunction (Corbin et al., 2002). The current study indicates that essentially the same signaling pathway is involved in the central regulation of erection and copulation, although the specific subtype of PDEs involved may differ (Van Staveren et al., 2003). Therefore, it may be possible to target central and peripheral loci involved in sexual functions with a single pharmacological agent. In females, the facilitation of sexual behaviors by central administration of PDE inhibitors is well-documented (Beyer and Canchola, 1981; Fernandez-Guasti et al., 1983). Clearly, further studies are needed to explore this possibility, since the data on the effects of PDE inhibitors on male sexual behaviors beyond erection are rather limited (Ottani et al., 2002).

CONCLUSION

In summary, we have demonstrated that the NO–cGMP pathway is involved in the regulation of MPOA DA and copulation. NO seems to play an important role in integration of sensorimotor and endocrine systems, given the steroid-sensitive nature of nNOS in the MPOA and the involvement of NO in endocrine systems closely linked to copulation. The data from the current study also suggest a possible avenue for more effective treatment for sexual disorders.

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