Ejaculatory abnormalities in mice with targeted disruption of the gene for heme oxygenase-2

ARTHUR L. BURNETT[1], DEVIN G. JOHNS[2], LANCE J. KRIEGSFELD[3], SABRA L. KLEIN[3], DAVID C. CALVIN[1], GREGORY E. DEMAS[3], LAWRENCE P. SCHRAMM[2], SUSUMU TONEGAWA[4], RANDY J. NELSON[3], SOLOMON H. SNYDER[5–7] & KENNETH D. POSS[4]

[1]*Department of Urology,* [2]*Department of Biomedical Engineering,* [3]*Department of Neuroscience,* [5]*Department of Pharmacology and Molecular Science, and* [7]*Department of Psychiatry, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205, USA*

[3]*Department of Psychology, College of Arts and Sciences, Johns Hopkins University, Charles & 34th Streets, Baltimore, Maryland 21218, USA*

[4]*Center for Cancer Research, Massachusetts Institute of Technology, Building E17-110, 77 Massachusetts Avenue, Cambridge, Massachusetts 62139, USA*

Correspondence should be addressed to S.H.S.

Nitric oxide (NO) is well established as a neurotransmitter in the central and peripheral nervous systems[1]. More recently, another gas, carbon monoxide (CO) has also been implicated in neurotransmission. In the nervous system CO is formed by a subtype of heme oxygenase (HO) designated HO2 (ref. 2). HO2 is localized to discrete neuronal populations in the brain resembling localizations of soluble guanylyl cyclase, which is activated by CO (ref. 3). CO may also function in the peripheral autonomic nervous system, in conjunction with NO. The majority of ganglia in the myenteric plexus possess both HO2 and neuronal NO synthase (NOS)[4]. Defects in myenteric plexus neurotransmission occur both in mice with targeted deletion of genes for HO2 and neuronal NOS (ref. 5). HO2 also occurs in other autonomic ganglia including the petrosal, superior cervical and nodose ganglia[4]. Neuronal NOS is localized to neurons regulating male reproductive behavior, such as penile erection, and NOS inhibitors prevent erection[6]. Because of the other parallels between NO and CO, we speculated that CO may play a role in male reproductive behavior. In the present study we describe HO2 localization in neuronal structures regulating copulatory reflexes. Reflex activity of the bulbospongiosus muscle, which mediates ejaculation and ejaculatory behavior, is markedly diminished in mice with targeted deletion of the gene for HO2 ($HO2^-$).

Immunohistochemical staining reveals that HO2 protein is localized to the major pelvic ganglion and its nerve distributions to the penis, urethra, bladder neck, vas deferens and prostate, and to pudendal nerve branches in the penis and urethra, as well as to vascular endothelium and epithelium within genitourinary structures of wild-type mice (Fig. 1). The occurrence of HO2 in these autonomic ganglia parallels previously reported localization of HO2 in petrosal, superior cervical, nodose and myenteric ganglia, while genitourinary endothelial localization for HO2 resemble distributions in other blood vessels[4]. HO2 staining is absent in $HO2^-$ mice.

Because HO2 is localized to neuronal systems that mediate ejaculation, we monitored reflex activity of the bulbospongiosus muscle in response to urethral distention (Fig. 2). McKenna and associates[7] have shown that bulbospongiosus electrical activity following increased urethral pressure reflects firing of the pudendal motor neurons that mediate contraction of perineal musculature during ejaculation. Increased urethral pressure produces progressively increased activity of the bulbospongiosus muscle in wild-type mice. This activity is almost obliterated in $HO2^-$ animals with no consistent effect of increased urethral pressure. The reduction in bulbospongiosus reflex is evident in two different background strains of $HO2^-$ animals compared with the appropriate wild-type controls, that is, a 129/SvEv coisogenic and a C57BL6×129/SvEv hybrid strain (coisogenic data not shown).

The requirement of HO2 for the bulbospongiosus reflex suggests that HO2 and CO may normally play a contributory role in ejaculation even though substantial defects in fecundity were not evident during the breeding of $HO2^-$ mice[8]. However, fertility and overall reproductive success have not been systematically evaluated, and initial observations suggest some reduced breeding efficiency in $HO2^-$ animals. We evaluated mating in $HO2^-$ males and females paired with wild-type mating partners. We observe no abnormalities in female $HO2^-$ mating behavior in terms of the ability of $HO2^-$ females to elicit mounts from wild-type males and the number of lordotic postures in response to mounting (data not shown). However, we do observe abnormalities in male mating behavior (Table 1). In a 30-min test period, 60% of wild-type males mount, consistent with the known slow mating activity of C57BL6 mice[8,9]. The percent of $HO2^-$ mice mounting is significantly less than wild-type, although there are no differences between the groups in mounting latency. $HO2^-$ mice also display significantly less intromission activity. Most striking is our observation that none of the $HO2^-$ animals ejaculate, whereas one-third of wild-type mice that intromit do ejaculate, a proportion typically observed in mice of this strain[8,9].

To ascertain whether the copulatory abnormalities are related to a defect in the erectile mechanisms, we employed electrical stimulation of the cavernous nerves in a paradigm that reflects physiological erection[6]. In $HO2^-$ mice ($n = 3$) erection following nerve stimulation is as robust as in wild-type mice ($n = 18$) (data not shown).

Copulatory behavior is influenced by serum testosterone. We find no significant difference in serum testosterone between $HO2^-$ and wild-type animals (data not shown). Moreover, McKenna *et al.*[7] have shown that castration does not alter the bulbospongiosus reflex. To ascertain that the abnormal sexual

behavior in HO2⁻ animals does not derive from defects in sensorimotor activity, we evaluated olfactory ability, motor coordination, motor strength and visual acuity as well as behavior in an open field arena (Table 2). In the open-field test, which assesses the "anxiety" of animals monitored by their reluctance to move about in an open field area, HO2⁻ mice display increased activity. We do not detect abnormalities in any of the other tests except for some decrease in apparent forelimb strength. In the test of forelimb strength mice are suspended by their forelimbs from a wire and latency to fall is monitored. Conceivably, the increased tendency to fall displayed by the HO2⁻ animals reflects less "fear of falling" as is implied by the decreased fear responses in the open-field test. Thus, the overall sensorimotor behavior of HO2⁻ animals does not seem to be notably impaired and is not likely to account for the deficient intromission and ejaculation. To evaluate aggressive behavior, we utilized an intruder-resistant model in which we previously reported increased aggressive behavior in male mice with targeted deletion of the gene for neuronal NOS (ref. 10). We observe no abnormal aggressive behavior in HO2⁻ mice (data not shown).

Table 1 Mating behavior of HO2⁻ male mice

Behavior	Wild-type strain			HO2– strain		
Mounting	129	B6/129	Total	129	B6/129	Total
Fraction mounting	14/25	10/15	24/40 (60%)	6/22	5/8	11/30 (37%)
Latency to mount (s)	681 ± 142	496 ± 128	–	857 ± 172	767 ± 181	–
Intromission						
Fraction intromitting	12/14	8/10	20/24 (83%)	3/6	3/5	6/11 (54%)
Latency to intromit (s)	978 ± 140	835 ± 152	–	919 ± 180	868 ± 83	–
Ejaculation (of those intromitting)	4/12	3/8	7/20 (35%)	0/3	0/3	0/6 (0%)*

Data are means ± s.d. with numbers of animals in each group as the denominator and the number of performing animals as the numerator; s, seconds.
*Significantly different from wild-type mice, $P << 0.05$ by chi-square analysis.

In summary, our behavioral experiments reveal a reduction in intromission and mounting and an absence of ejaculation in HO2⁻ animals. These behavioral abnormalities probably do not reflect a defect in erection, since induced erection by nerve stimulation appears to be normal in the HO2⁻ animals. Recent studies indicate that the "coital reflex" comprises closely coordinated systems that mediate both intromission and ejaculation[11]. Thus, the same or closely related abnormalities may underlie both the reduced number of intromissions and the defective ejaculation. The reduced frequency of mounting in HO2⁻ animals may be secondary to motivation, owing to diminished positive feedback from intromission and ejaculation activity.

Since fecundity is at least grossly preserved, most HO2⁻ mice presumably ultimately ejaculate. In gene knockout experiments, one can never definitively ascertain the magnitude of the gene's contribution to a given function, because the gene has been deleted from early embryonic stages, usually leading to compensatory mechanisms. Thus, we cannot judge the relative contribution of HO2 to ejaculatory behavior. It is likely that the crucial role of reproductive physiology in species survival leads to multiple regulatory mechanisms for each aspect of reproductive physiology, including ejaculation.

The ejaculatory defect in HO2⁻ animals may derive from their profoundly defective bulbospongiosus reflex. Loss of HO2 localized to pudendal nerve branches and pelvic autonomic neurons that innervate the bulbospongiosus and related muscles presumably accounts for the defective reflex. The exact ganglia that regulate the reflex have not been identified. Besides its neuronal localization in the genitourinary pathway, HO2 occurs in vascular endothelium and epithelial layers, and its presence there might have some chemosensory influence on ejaculatory mechanisms. However, the rapid reflex character of the ejaculatory process fits best with a predominantly mechanically stimulated neural regulation. It is interesting that neuronal NOS is also localized to autonomic neurons and vascular endothelium of the genitourinary tract[6]. NOS inhibitors block penile erection[6] and penile smooth muscle relaxation[12], indicating a role for NO in the erectile process. The normal erections observed in mice with genetic deletion of neuronal NOS have been linked to upregulation of endothelial NOS (ref. 13).

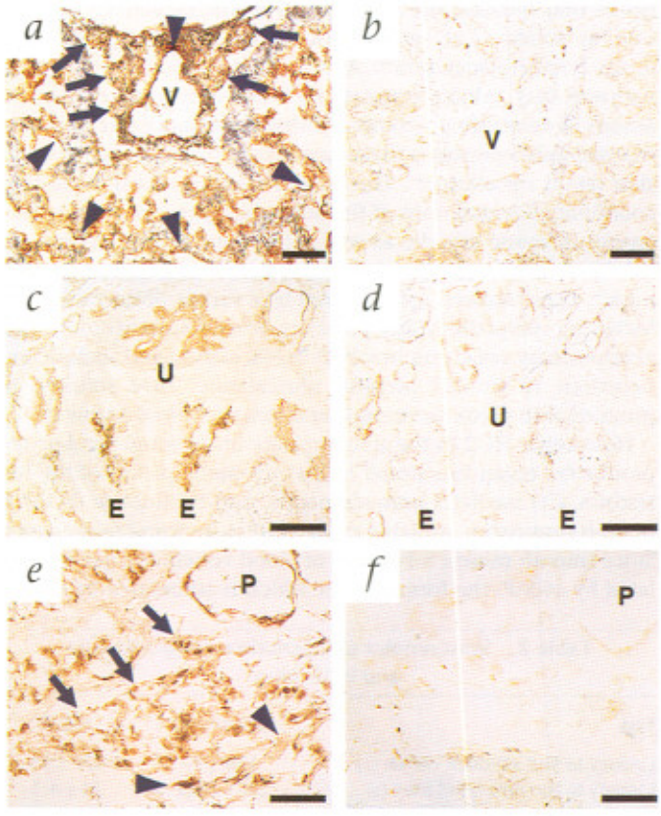

Fig. 1 Heme oxygenase-2 (HO2) immunostaining of genitourinary tissues from wild-type and HO2⁻ mice. In sections of the dorsal aspect of the penis (***a***) and proximal urethra (***c***) of the wild-type mouse, HO2 localization includes nerve trunks of the dorsal nerve of the penis (arrows), endothelium (arrowheads), and the epithelium of the ejaculatory ducts (E) and urethra (U). Corresponding sections from HO2⁻ mice (***b*** and ***d***) exhibit only nonspecific background staining. In the major pelvic ganglion of the wild-type mice (***e***), HO2 immunoreactivity is apparent in ganglion cells (arrows) and nerve fibers (arrowheads), as well as in the glandular epithelium of the adjacent prostate (P). Preincubation of HO2 antiserum with HO2 peptide abolishes immunoreactivity (***f***). V, dorsal vein of the penis. Scale bars, 250 μm.

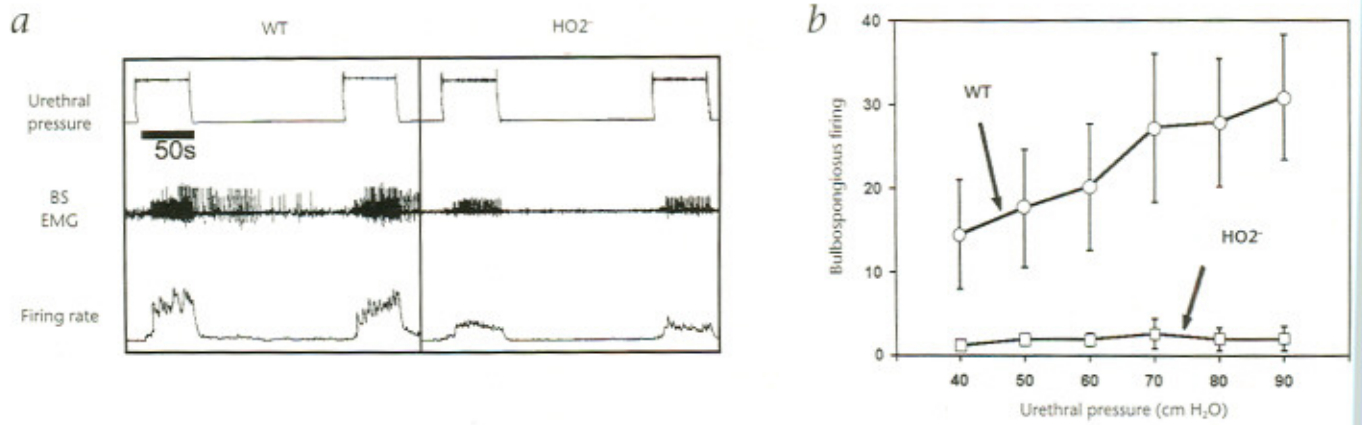

Fig. 2. Bulbospongiosus electromyographic activity in response to urethral pressure stimulation in wild-type and HO2⁻ mice. ***a***, An example of an experiment using an urethral pressure stimulus of 50 cm H_2O in which unprocessed and integrated voltage recordings were obtained. In order to provide clarity of EMG and firing rate depictions in HO2⁻ samples, the example is from a mouse with a lesser defect than the majority of HO2⁻ mice. ***b***, Bulbospongiosus firing (arbitrary units) at designated urethral pressures in wild-type and HO2⁻ mice. Reflex activity is significantly reduced in HO2⁻ mice ($n = 8$) compared with that of wild-type mice ($n = 7$) ($P < 0.005$, for all pressure levels).

Despite its clinical importance, ejaculation has not been well characterized in terms of fundamental neuromuscular mechanisms that mediate the response. Sympathetic activity plays a role, as ablations of sympathetic nerves and antisympathetic drugs impair ejaculation[14]. A descending serotonin neuronal pathway may inhibit ejaculation, as 5,6-dihydroxytryptamine lesions of descending serotonin pathways enhance ejaculation[15], whereas antidepressants that augment serotonin neurotransmission retard ejaculation[16]. The ejaculatory reflex itself comprises coordinated contractions of the bulbospongiosus and ischiocavernosus somatic muscles as well as the smooth musculature of the vas deferens, ejaculatory ducts, proximal urethra and bladder neck[14]. These muscles are regulated by a variety of neural inputs including sympathetic and parasympathetic innervation to the genitourinary structures involved in ejaculation and somatic innervation to striated perineal musculature. HO2 staining is prominent in pelvic autonomic and somatic neural pathways.

How might HO2 in the genitourinary innervation regulate reproductive organ functions? In the myenteric plexus of the intestines, CO mediates non-adrenergic, non-cholinergic (NANC) neurotransmission, utilizing cyclic GMP as a second messenger[5]. Since smooth muscle relaxation of blood vessels and gut is regulated by cGMP, the formation of which is stimulated by CO, it appears most plausible that the product of HO2 regulating th neuromuscular reflex mechanisms involved in ejaculation is CO However, HO2 also forms Fe^{++} and biliverdin, which may be in volved, although neither has been demonstrated to influenc cGMP. Bulbospongiosus reflex activity is not influenced b adrenergic and cholinergic agents[7], and so may also reflec NANC transmission. Whether cyclic GMP is the second messen ger of CO in the urogenital system remains to be established.

Ejaculatory disturbances are common clinical problems, re ported in approximately 40% of adult males[17]. Except for th recent use of serotonin-specific reuptake-inhibiting antidepre sants, such as fluoxetine, sertraline and paroxetine, few trea ments are available for premature ejaculation. Treatmer options for delayed ejaculation are similarly lacking. If CO is transmitter mediating ejaculation, then peripherally selectiv HO2 modulators might be effective agents in treating these dy functions with minimal central nervous system side effects.

Table 2 Sensorimotor task performance by wild-type and HO2⁻ mice

Task	Wild-type	HO2⁻
Latency to find a hidden cookie (s)	386 ± 27	420 ± 41
Latency to turn in a blind alley (s)	20.1 ± 2.9	28 ± 4.3
Latency to walk one body length (s)	14.8 ± 5.2	20.7 ± 4.7
Latency to turn on an inclined screen (s)	13.9 ± 2.3	16.5 ± 1.7
Forelimb strength (latency to fall from a suspended wire (s)	76.1 ± 7.0	18.5 ± 6.2*
Latency to fall from a suspended pole (s)	37.7 ± 7.0	50.0 ± 14
Open field behavior:		
No. of external squares crossed	155 ± 17	210 ± 19*
No. of internal squares crossed	10.5 ± 3.2	20.1 ± 6.6
Duration spent in open field (s)	12.5 ± 2.6	18.13 ± 2.57

Data are expressed as means ± s.e.m. for groups of 15 wild-type and 8 HO2⁻ mice; s, seconds.
*Significantly different from wild-type mice ($P < 0.05$).

Methods

Adult male C57BL6 and Agouti 129 (wild-type) and HO2⁻ mice[8] (~30 were used. The HO2⁻ animals were derived from the wild-type strai Animals were killed by CO_2 inhalation, and the urinary bladder, urethra (i cluding the external urethral sphincter), and the penis with adjacent b bospongiosus musculature were removed *en bloc*. Tissue sections (10 μ thick) were obtained after liquid nitrogen immersion for immunohist chemistry and histology[4].

Affinity-purified rabbit anti-rat HO2 antibody was generated against synthesized peptide corresponding to amino acids 247–258 of rat HO2 (r 18). Immunohistochemical localizations of nerves were verified with rab anti-human protein gene product 9.5 antibody[19] (Accurate Chemical C Westbury, NY).

Behavioral testing involved a battery of sensorimotor tasks that appra capabilities necessary for copulatory success in rodents and mating stud as described previously[10]. Electrophysiologic induction of penile erecti was performed as described previously[6]. Serum testosterone levels w measured as described[20].

Monitoring of urethrogenital reflexes was conducted in a manner sim to that previously described[7]. In brief, animals were anesthetized with u thane (1.8 g/kg, i.p.) and underwent lower midline abdominal laparoto and cystotomy for antegrade proximal urethral cannulation with a PE catheter, distal penile urethra ligation, and bipolar electrode placement the surface of the bulbospongiosus muscle for electromyographic reco ings. Urethral pressures were generated by catheter infusion for 50-s du

tions in duplicate at 10-cm H_20 increments from 40 to 90 cm H_20 with 200-s intervals between stimuli. Action potential responses were detected by a window discriminator, and the resultant standardized pulses were averaged by a low-pass filter to produce a signal that is proportional to the frequency of the action potentials. This signal was sampled at 25 Hz and recorded on a computer for further processing. Experiments were done with the experimenter blind to the genotype of animals.

Where appropriate, results are expressed as mean values ± s.e.m. Variables were evaluated by analysis of variance with statistical differences determined by using Student's *t*-test.

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