Excretion and binding of tritium-labelled oestradiol in mice (*Mus musculus*): implications for the Bruce effect

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Abstract

Male mouse urine contains 17 β -oestradiol (E₂) and other steroids. Given that males actively direct urine at proximate females and intrauterine implantation of blastocysts is vulnerable to minute amounts of exogenous oestrogens, males' capacity to disrupt early pregnancy could be mediated by steroids in their urine. When male mice were implanted with osmotic pumps containing tritium-labelled E₂ (³H-E₂) or injected i.p. with ³H-E₂, radioactivity was reliably detected in their urine. Following intranasal administration of ³H-E₂ to inseminated females, radioactivity was detected in diverse tissue samples, with there being significantly more in reproductive tissues than in brain tissues. When urine was taken from males injected with ³H-E₂, and then intranasally administered to inseminated females, radioactivity was detected in the uterus, olfactory bulbs, and mesencephalon and diencephalon (MC + DC). When inseminated and ovariectomised females were perfused at the point of killing to remove blood from tissues, more radioactivity was detected in the uterus than in muscle, olfactory bulbs, MC + DC and cerebral cortex. Pre-treatment with unlabelled E₂ significantly reduced the uptake of ³H-E₂ in the uterus. Taken with evidence that males deliver their urine to the nasal area of females, these results indicate that male urinary E₂ arrives in tissues, including the uterus, where it could lead to the disruption of blastocyst implantation.

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Introduction

Exposing inseminated female mice to novel males or their urine around the point of blastocyst implantation can terminate pregnancy (Bruce 1959, 1960, Labov 1981, deCatanzaro & Storey 1989, deCatanzaro *et al.* 1996). This phenomenon, commonly referred to as the 'Bruce effect' (Schwagmeyer 1979, Becker & Hurst 2008), is mediated by chemosignals in the urine of novel males (Dominic 1965, Marchlewska-Koj 1977, Brennan *et al.* 1999, Peele *et al.* 2003). Sex steroids are known to play a critical role in the Bruce effect. The capacity of males to disrupt pregnancy is diminished by castration (Bruce 1965, Vella & deCatanzaro 2001), but not by the removal of major androgen-dependent glands, the preputial and vesicular-coagulating glands (deCatanzaro *et al.* 1996, Zacharias *et al.* 2000). This capacity is restored when castrated males are given testosterone (deCatanzaro & Storey 1989, deCatanzaro *et al.* 1995c) or 17 β -oestradiol (E₂; deCatanzaro *et al.* 1995b).

There is a broad consensus that the Bruce effect is mediated by male urinary chemosignals acting on the nasal region of the inseminated females (e.g. Bruce & Parrott 1960, Lloyd-Thomas & Keverne 1982, Brennan *et al.* 1999, Leinders-Zufall *et al.* 2004, deCatanzaro *et al.* 2006). Increasingly, evidence suggests that the critical chemosignals in male urine include androgens

and oestrogens, with E₂ being especially important. Male mouse urine reliably contains unconjugated E₂ and testosterone (Muir *et al.* 2001, deCatanzaro *et al.* 2006). Intact novel males given the aromatase inhibitor anastrozole and a low phyto-oestrogen diet subsequently produce less urinary E₂ and show a diminished capacity to disrupt pregnancy (Beaton & deCatanzaro 2005). Recently-inseminated females given daily doses of E₂ antibodies maintain pregnancy despite exposure to novel males (deCatanzaro *et al.* 1995a). Administration of minute quantities of exogenous oestrogens to inseminated females, either intranasally or subcutaneously, terminates pregnancy in a manner that mimics the Bruce effect (deCatanzaro *et al.* 1991, 2001, 2006). Following a few days of exposure to inseminated or developing females, adult males develop polyuria and target urine at these females (Reynolds 1971, Maruniak *et al.* 1974, Hurst 1990, Arakawa *et al.* 2007, deCatanzaro *et al.* 2009), and the ratio of E₂ to creatinine rises in male urine (deCatanzaro *et al.* 2006, 2009).

In the initial days of gestation, endogenous E₂ binds to the uterus, preventing epithelial cell loss prior to blastocyst invasion and afterwards disrupting epithelial integrity to allow for implantation (Potter *et al.* 1996). However, an excess of circulating E₂ prior to blastocyst invasion causes the uterus to advance to a refractory state, preventing implantation (Ma *et al.* 2003).

E₂ is also involved in preparing blastocysts for implantation, but it is deleterious to them when present in supraphysiological levels (Roblero & Garavagno 1979, Safro *et al.* 1990, Paria *et al.* 1993), both slowing the growth and increasing the mortality of the blastocysts (Valbuena *et al.* 2001). Transport of pre-implantation embryos from the oviduct to the uterus is facilitated by E₂ (Roblero & Garavagno 1979), but the administration of oestrogens to inseminated animals can result in a rapid acceleration of embryo transport, further contributing to oestrogen-induced implantation failure (Burdick & Whitney 1937, Banik & Pincus 1964, Greenwald 1967, Ortiz *et al.* 1979).

If E₂ is to be considered the primary urinary component responsible for pregnancy disruption in the Bruce effect, it would be expected that E₂ in male urine will travel to and bind in the uterus of a recently inseminated female. Here, we investigated urinary tritium-labelled E₂ (³H-E₂) excretions in non-sire males implanted with osmotic pumps. Next, we studied the time course of urinary ³H-E₂ excretion in i.p. injected males. We then tested the binding of intranasally administered ³H-E₂ in inseminated female mice, examining diverse tissues that vary in oestrogen receptor concentration (Eisenfeld & Axelrod 1966, Pfaff 1968, Stumpf 1969, Pfaff & Keiner 1973, Gorzalka & Whalen 1974, Sar & Parikh 1986, Simerly *et al.* 1990), including areas in the reproductive system and brain. We also intranasally administered male urine collected from males injected with ³H-E₂ to inseminated females, and target tissues were examined for ³H-E₂ uptake. Subsequently, in order to control for circulating ³H-E₂ that might not be bound in tissues, animals were perfused with saline at the point of killing. Finally, as natural E₂ would bind competitively with ³H-E₂, we examined uptake in ovariectomised females and intact inseminated females pre-treated with unlabelled E₂.

Results

Radioactivity was detected in the urine of males implanted with osmotic pumps containing ³H-E₂, both on day 1 post surgery (231.5 ± 22.3 DPM/ μ l urine) and on day 6 post surgery (173.1 ± 15.3 DPM/ μ l urine). As determined by a paired samples *t*-test, there was significantly more ³H-E₂ excreted on day 1 than on day 6 post surgery, $t(5)=3.79$, $P=0.01$. Background radioactivity levels obtained from males implanted with ethanol-containing pumps were not significantly different from zero by one-sample *t*-tests on both day 1 post surgery (0.05 ± 0.02 DPM/ μ l urine), $t(3)=1.73$, $P=0.18$, and day 6 post surgery (0.06 ± 0.01 DPM/ μ l urine), $t(3)=3.00$, $P=0.06$.

Figure 1 shows the radioactivity detected over time in the urine of males i.p. injected with ³H-E₂. Despite all males receiving the same quantity of ³H-E₂, the timing and quantity of ³H-E₂ excretion were clearly

Figure 1 Radioactivity detected in urine of five males over the time following a single i.p. injection of 50 μ Ci tritium-labelled oestradiol.

idiosyncratic. Most urinations (81%) occurred within the first 8 h of the 12-h observation period.

Figure 2 shows radioactivity in the target tissues of females intranasally administered ³H-E₂ or ethanol (negative control). Initial statistical analyses indicated that there were no significant differences among doses of ³H-E₂, therefore the four doses were pooled in subsequent statistical analysis, yielding mean ($\pm$ S.E.M.) values in DPM/mg tissue: uterus, 272.5 ± 39.0 ; ovaries, 242.4 ± 28.2 ; mesencephalon and diencephalon (MC+DC), 110.8 ± 16.5 ; olfactory bulbs, 155.3 ± 25.2 and cortex, 104.5 ± 13.3 . An ANOVA, treating condition (negative control versus ³H-E₂) as a between-subjects variable and tissue as a within-subjects variable, showed significant main effects of condition, $F(1,10)=11.09$, $P=0.0076$, and of tissue, $F(4,40)=5.46$, $P=0.0016$, and a significant interaction, $F(4,30)=5.49$, $P=0.0016$. Newman-Keuls multiple comparisons showed that all tissues from ³H-E₂-treated females differed from all control tissues ($P<0.01$). In addition, the quantities of

Figure 2 Mean ($\pm$ S.E.M.) radioactivity detected in the uterus, ovaries, mesencephalon and diencephalon (MC+DC), olfactory bulbs and cortex of inseminated females intranasally administered 20 μ l 95% ethanol (negative control), or 18, 24, 27 or 37 μ Ci tritium-labelled oestradiol. All animals were killed 1 h following the administration procedure.

$^3\text{H}\text{-E}_2$ in the uterus and ovaries did not differ significantly, but both the uterus and ovaries had significantly greater ($P < 0.01$) quantities of $^3\text{H}\text{-E}_2$ than did the MC+DC, olfactory bulbs and cortex. Significantly ($P < 0.05$) more $^3\text{H}\text{-E}_2$ was detected in the olfactory bulbs than in MC+DC and cortex, but radioactivity in the MC+DC and cortex did not differ significantly.

Figure 3 shows radioactivity recovered in tissues of females intranasally administered either male urine containing $^3\text{H}\text{-E}_2$ (treatment) or negative control male urine. An ANOVA, treating condition (negative control versus treatment) as a between-subjects variable and tissue (uterus, olfactory bulbs, and MC+DC) as a within-subjects variable, indicated a significant main effect of condition, $F(1,8) = 31.64$, $P = 0.0008$, but no main effect of tissue, $F(2,16) = 1.38$, $P = 0.2797$, and no interaction, $F(2,16) = 0.56$, $P = 0.5889$. Multiple comparisons of radioactivity between conditions and tissues showed that each tissue extracted from females in the treatment condition had significantly greater levels of radioactivity than the same tissue taken from the negative control condition, $P < 0.05$, but that within conditions there was no significant difference in $^3\text{H}\text{-E}_2$ quantities between tissues.

Figure 4 shows radioactivity in the tissues of ovariectomised females intranasally administered ethanol (negative control) or $^3\text{H}\text{-E}_2$, followed by perfusion. Tissue samples from negative control females demonstrated background levels of radioactivity in a distinct and non-overlapping range from that of $^3\text{H}\text{-E}_2$ -treated animals, and were excluded from statistical analyses. An ANOVA, treating tissue (uterus, muscle, olfactory bulbs, MC+DC and cortex) as a within-subjects variable indicated a significant difference in radioactivity between the tissues sampled, $F(4,12) = 9.48$, $P = 0.0014$. Multiple comparisons of radioactivity showed that uterine samples were significantly different from all other samples, $P < 0.01$, and that the other tissues did not differ significantly from one another, $P > 0.05$.

Figure 3 Mean ($\pm$ S.E.M.) radioactivity detected in the uterus, olfactory bulbs and mesencephalon and diencephalon (MC+DC) of inseminated females intranasally administered either 25 μl male urine collected from untreated males (negative control) or 25 μl male urine collected from males that were i.p. injected with 50 μCi tritium-labelled oestradiol (treatment).

Figure 4 Mean ($\pm$ S.E.M.) radioactivity detected in the uterus, muscle, mesencephalon and diencephalon (MC+DC), olfactory bulbs and cortex of ovariectomised females intranasally administered either 10 μl 95% ethanol (negative control) or 10 μCi tritium-labelled oestradiol ($^3\text{H}\text{-E}_2$).

Figure 5 shows radioactivity in the tissues of inseminated females intranasally administered ethanol (negative control) or $^3\text{H}\text{-E}_2$, as well as of females pre-treated with E_2 and then intranasally administered $^3\text{H}\text{-E}_2$, where all animals were subsequently perfused to flush blood from tissues. Tissue samples from negative control females demonstrated background levels of radioactivity in a distinct and completely non-overlapping range from that of all other animals, and were excluded from further analyses. An ANOVA, treating condition as a between-subjects variable and tissue (uterus, ovaries, muscle, olfactory bulbs, MC+DC, and cortex) as a within-subjects variable, did not show a significant effect of E_2 pre-treatment, $F(1,6) = 2.07$, $P = 0.1992$, but there was a main effect of tissue, $F(5,30) = 13.58$, $P < 0.0001$, and a significant interaction between condition and tissue, $F(5,30) = 3.41$, $P = 0.0147$. Multiple comparisons showed that uterine samples taken from females given $^3\text{H}\text{-E}_2$ without E_2 pre-treatment were significantly more

Figure 5 Mean ($\pm$ S.E.M.) radioactivity detected in the uterus, ovaries, muscle, mesencephalon and diencephalon (MC+DC), olfactory bulbs and cortex of inseminated females intranasally administered 10 μl 95% ethanol (negative control), 10 μCi tritium-labelled oestradiol ($^3\text{H}\text{-E}_2$), or 10 μCi tritium-labelled oestradiol following an s.c. injection of 2 μg 17 β -oestradiol in 0.05 cm^3 peanut oil two days prior ($\text{E}_2 + ^3\text{H}\text{-E}_2$).

radioactive than all other samples, $P < 0.01$, and that none of the other samples from $^3\text{H-E}_2$ -treated subjects, regardless of E_2 pre-treatment, differed significantly from one another.

Discussion

These data indicate that males' systemic E_2 readily enters their urine. Radioactivity was reliably detected in the urine of males implanted with osmotic pumps containing $^3\text{H-E}_2$. In males injected intraperitoneally with $^3\text{H-E}_2$, radioactivity in urine peaked within 8 h after administration, with patterns of $^3\text{H-E}_2$ excretion varying among subjects. These data also indicate that nasal exposure of inseminated females to $^3\text{H-E}_2$ leads to detectable radioactivity in diverse areas including the uterus and brain. In inseminated females given a single intranasal administration of $^3\text{H-E}_2$, radioactivity was detected in all tissue samples, and binding was greater in reproductive organ samples than in brain samples. In inseminated females intranasally administered urine from males injected with $^3\text{H-E}_2$, radioactivity was detected in all samples. When ovariectomised females were nasally administered $^3\text{H-E}_2$, and were then perfused with saline to flush tissues of blood, substantially greater radioactivity was observed in the uterus than in muscle tissue, olfactory bulbs, MC+DC and cerebral cortex. The uterus similarly showed greater radioactivity than in all other tissues including ovaries in inseminated females perfused with saline; uterine $^3\text{H-E}_2$ uptake was significantly reduced when inseminated females were pre-treated with unlabelled E_2 .

E_2 is reliably measurable in urine of adult male mice through enzyme immunoassays employing antibodies displaying low cross-reactivities to other steroids and their conjugates (Muir *et al.* 2001, Vella & deCatanzaro 2001, Beaton & deCatanzaro 2005, Beaton *et al.* 2006, deCatanzaro *et al.* 2006, 2009). Oestrogen conjugates, which are measurable but sparse compared to unconjugated E_2 in female mouse urine, are not detectable in male mouse urine (Muir *et al.* 2001). Accordingly, it seems likely that the radioactivity detectable in male urine subsequent to systemic $^3\text{H-E}_2$ delivery arrives in urine without metabolic alteration. Some limitations to the present methodology arise from the fact that $^3\text{H-E}_2$ is dissolved in ethanol. The quantity of ethanol that is deliverable to adult male mice without causing death or obvious toxicity provides an upper limit to the concentration of $^3\text{H-E}_2$ that can be harvested from male urine. This limits experimentation involving the administration of urine from $^3\text{H-E}_2$ -treated males to females. The $^3\text{H-E}_2$ observed in male urine is only a minute fraction of the E_2 that naturally exists in urine in these animals, where typical values of E_2 in adult male urine typically range from about 2 to 15 ng/ml urine (or 3–30 ng/mg creatinine) depending on the age and social condition of the males (deCatanzaro *et al.* 2006, 2009).

The nasal area of female mice housed in proximity to males is substantially exposed to the urine of these males. When housed across wire grid from stimulus females, adult males actively direct their urine to females (Maruniak *et al.* 1974, Hurst 1990, Arakawa *et al.* 2007, deCatanzaro *et al.* 2009). Females in these circumstances do not avoid novel males (deCatanzaro & Murji 2004); instead, they make nasal contact to male genitals, excretions and urine droplets (deCatanzaro *et al.* 2006). Adult males display polyuria and polydipsia when exposed to both male and female conspecifics, using this urine in social communication (*cf.* Reynolds 1971, Maruniak *et al.* 1974, Hurst 1990, Drickamer 1995, Arakawa *et al.* 2007, deCatanzaro *et al.* 2009). Steroid content of male urine is dynamic in the presence of females, as is creatinine, which is measured in this context as an index of an animal's hydration (deCatanzaro *et al.* 2009). Over the initial days of exposure to females, male urinary creatinine decreases in conjunction with greater dilution of urine, while the ratio of E_2 to creatinine significantly rises (Beaton *et al.* 2006, deCatanzaro *et al.* 2006, 2009, Khan *et al.* 2009).

Exogenous E_2 can disrupt blastocyst implantation in mice in doses as low as 37 ng/day on days 1–5 of gestation through s.c. administration (deCatanzaro *et al.* 1991, 2001) or 140 ng/day on days 2–4 of gestation through intranasal administration (deCatanzaro *et al.* 2006). The current data clearly demonstrate that once a female's nasal system is exposed to E_2 , this steroid rapidly enters circulation and is detectable in diverse tissues (*cf.* Anand Kumar *et al.* 1974). The uterus was especially affected, which was particularly clear when tissues had been flushed of blood via saline perfusion. This reflects the exceptional density of E_2 receptors in uterus relative to other tissues (Eisenfeld & Axelrod 1966, Gorzalka & Whalen 1974). Administration of unlabelled E_2 to females prior to nasal $^3\text{H-E}_2$ delivery diminished detectable radioactivity, most clearly in uterine tissue, implicating receptor binding. Binding of male-originating E_2 at the uterus could well contribute to the Bruce effect given the established adverse influences of minute elevations in E_2 on blastocyst implantation (Burdick & Whitney 1937, Banik & Pincus 1964, Greenwald 1967, Ortiz *et al.* 1979, Roblero & Garavagno 1979, Safro *et al.* 1990, Paria *et al.* 1993, Valbuena *et al.* 2001, Ma *et al.* 2003).

In addition to its disruptive effects at the uterus, exogenous E_2 could have influences in the central nervous system which affect the female's behaviour, and might indirectly influence the uterus via hypothalamic control of pituitary gonadotrophins. The ventromedial hypothalamus is very rich in oestrogen receptors (Pfaff 1980), where it is established to play a critical role in the onset of behavioural oestrus (Mathews & Edwards 1977, Pfaff & Sakuma 1979, Pfaff *et al.* 2000). Our evidence that nasally administered $^3\text{H-E}_2$ in male

urine leads to detectable radioactivity in the MC+DC suggests that novel male urine could have influences on brain and behaviour.

Evidence indicates that the inseminated female's vomeronasal organ is critical for the Bruce effect (Bellringer *et al.* 1980, Lloyd-Thomas & Keverne 1982). It has generally been assumed that chemical signals there are transduced to neural events (Brennan 2004), and indeed evidence from vomeronasal slice preparations indicates action potentials in single neurons in response to six putative male pheromones with thresholds near 10^{-11} M (Leinders-Zufall *et al.* 2000, 2004). Nevertheless, exposed vasculature in the nasal system also presents a mode via which small molecules might enter directly into circulation. The vomeronasal system is highly vascularised, and indeed constitutes a vascular pump that sucks stimulus substances into the organ in response to novel situations and stimuli that catch the animal's attention (Meredith & O'Connell 1979, Meredith *et al.* 1980, Meredith 1994). The current data support the notion that small molecules can directly enter circulation via nasal absorption and arrive at receptors where they are established to disrupt blastocyst implantation. E_2 's very low molecular weight (272.4 kDa) and lipid solubility facilitate ready passage across biological membranes and rapid arrival at sites with abundant oestrogen receptors.

A longstanding hypothesis concerning the Bruce effect has invoked females' discrimination among distinctive odours of the sire and novel males, and signalling from olfactory mechanisms to hypothalamus to pituitary to uterus (e.g. Thomas & Dominic 1988, Brennan *et al.* 1990, 1999, Kaba *et al.* 1994). Some individual recognition may occur among mice (Baum & Keverne 2002, Bakker 2003), at least partly mediated by major histocompatibility complex (MHC) proteins (Singh *et al.* 1987, Singer *et al.* 1997). Large urinary proteins from male urine cannot disrupt pregnancy on their own, unlike low molecular weight constituents (Peele *et al.* 2003). Short peptides that serve as ligands for MHC molecules and which stimulate a subset of basal zone vomeronasal sensory neurons can disrupt pregnancy when added to male urine (Leinders-Zufall *et al.* 2004). The pregnancy-disrupting effect of familiar males' urine can be enhanced by adding a cocktail of peptides associated with unfamiliar males, although peptide mixtures were ineffective in mimicking the Bruce effect when applied in water (Kelliher *et al.* 2006). Actions of short, strain-specific peptides may help to explain why males of a distinctive genetic strain are more effective in inducing the Bruce effect than are males of the same strain as the inseminating males (Parkes & Bruce 1962, Marsden & Bronson 1965, Spironello & deCatanzaro 1999).

On the other hand, the hypothesis that male urinary E_2 is absorbed by females and influences blastocyst implantation directly at the uterus is consistent with

the current data, as well as with much of the previous data indicating steroid dependency of the effect (Bruce 1965, Rajendren & Dominic 1988, deCatanzaro *et al.* 1991, Vella & deCatanzaro 2001, Beaton & deCatanzaro 2005, deCatanzaro *et al.* 2006). It is also concordant with clear mechanisms by which elevated oestrogens at the uterus disrupt blastocyst implantation (Burdick & Whitney 1937, Banik & Pincus 1964, Greenwald 1967, Ortiz *et al.* 1979, Roblero & Garavagno 1979, Safro *et al.* 1990, Paria *et al.* 1993, Valbuena *et al.* 2001, Ma *et al.* 2003). This hypothesis and previous notions implicating olfactory memory are not necessarily mutually exclusive, and efforts are needed to clarify their potential interactions.

Materials and Methods

Animals and housing

Except where stated otherwise, all animals were housed in standard polypropylene cages measuring $28 \times 16 \times 11$ (height) cm with wire bar tops allowing constant access to food and water. Sexually experienced male mice, aged 10–15 months and in good health, were of heterogeneous strain, a vigorous hybrid of C-57, DBA, Swiss and CF-1 strains. Males of such a background are known to produce a robust Bruce effect (Spironello & deCatanzaro 1999, Beaton & deCatanzaro 2005, deCatanzaro *et al.* 2006). CF-1 female mice were of a stock from Charles River Breeding Farms of Canada (LaPrairie, Québec, Canada) and were aged 3–4 months. Females were inseminated by being singly paired with same-strain adult males. Hindquarters were inspected three times daily. Upon sperm plug detection, females were identified as being on day 0 of pregnancy. The animal colony was maintained at 21 °C with a reversed 14 h light:10 h darkness cycle. All research was approved by the Animal Research Ethics Board of McMaster University, conforming to the standards of the Canadian Council on Animal Care.

Osmotic pump administration of $^3\text{H}\text{-E}_2$ in males exposed to inseminated females

Alzet model 1007D micro-osmotic pumps (Durect Corporation, Cupertino, CA, USA) were filled with solutions consisting of either 16 μCi $^3\text{H}\text{-E}_2$ (E_2 -[2,4- ^3H]; in ethanol, 1.0 mCi/ml, 35.0 Ci/mM, Sigma) added to 91 μl distilled water, or 16 μl ethanol added to 91 μl distilled water as a control condition. Pumps were weighed before and after filling to confirm that they were at 95–100% of their rated capacity. Pumps had a mean fill volume of $107 \pm 6 \mu\text{l}$, and a mean pumping rate of $0.50 \pm 0.02 \mu\text{l/h}$.

Males were anaesthetised under a gaseous 1:1 isoflurane: oxygen mixture, and were given a local injection of 0.05 cm^3 2% lidocaine hydrochloride (Xylocaine, Astra-Zeneca, Wilmington, DE, USA). A region between the scapulae was shaved and cleaned, and a small incision was made. Using a haemostat, a pocket was formed by spreading the subcutaneous tissues apart, and a pump containing either the $^3\text{H}\text{-E}_2$ solution ($n=6$; treatment condition) or the ethanol

solution ($n=4$; negative control condition) was inserted. Animals were given 12–16 h to recover from the surgery. The day following the surgery, males were placed in a compartment in the upper level of a two-tier exposure apparatus (deCatanzaro *et al.* 1996). Each compartment measured $14.5 \times 21 \times 13.5$ (height) cm and was separated by an opaque plastic barrier that prevented contact between the males. Wire grid cage tops allowed constant access to food and water. During days 1–5 after the surgery, males were exposed to a single inseminated female on day 2 of pregnancy placed in the lower compartment of the apparatus measuring $30 \times 21 \times 13.5$ (height) cm. Each male's compartment had a stainless steel wire grid floor (squares of 0.5 cm^2) that allowed excretions to pass to the lower compartment and limited behavioural interaction between the male and female.

On days 1 and 6 post surgery, immediately prior to and following female exposure, males in the two-compartment apparatus were placed 1 cm above the wax paper lining a clean Teflon-coated stainless steel tray measuring $29.8 \times 44.5 \times 2$ (depth) cm, permitting excretions to collect on the wax paper. Urine was collected every 15 min for 4 h after the onset of the darkness phase of the light:darkness cycle. Pooled urine was aspirated via 1 ml syringes with 26-gauge needles. A fresh syringe and needle were used for each collection. Samples were pooled by day and frozen until processed.

Injected $^3\text{H-E}_2$ in male mice

Prior to the onset of the darkness phase of the light:darkness cycle, males ($n=5$) were i.p. injected with $50 \mu\text{Ci } ^3\text{H-E}_2$ (E_2 -[2,4- ^3H]; in ethanol, 1.0 mCi/ml, 52.7 Ci/mM, Sigma). Each male was individually placed in a urine-collection apparatus similar to that described above. Urine samples were collected as described above by monitoring animals continuously for 12 h, immediately collecting any urine produced. A final collection was made the following morning at the onset of the darkness phase of the light:darkness cycle. Samples were labelled and analysed immediately.

Intranasal administration of $^3\text{H-E}_2$ in inseminated female mice

Following intranasal procedures of deCatanzaro *et al.* (2006), on day 2 of pregnancy, inseminated females were lightly anaesthetised under a gaseous 3:2 isoflurane:oxygen mixture in a sealed chamber. Upon immobilisation, a micropipette tip was placed into one nostril of a female, and a micropipetter was used to administer either $^3\text{H-E}_2$ (E_2 -[2,4- ^3H]; in ethanol, 1.0 mCi/ml, Sigma; $18 \mu\text{Ci}$, $n=3$; $24 \mu\text{Ci}$, $n=3$; $27 \mu\text{Ci}$, $n=2$; $37 \mu\text{Ci}$, $n=2$) or 95% ethanol ($20 \mu\text{l}$, $n=2$; negative control group). Two different $^3\text{H-E}_2$ preparations were used in the 18 and $24 \mu\text{Ci}$ groups (35.0 Ci/mM) and the 27 and $37 \mu\text{Ci}$ groups (52.7 Ci/mM). However, the absolute quantities of $^3\text{H-E}_2$ ranged between 140 and 190 ng, doses known to disrupt pregnancy (deCatanzaro *et al.* 2006). All females recovered within 2 min following the procedure. One hour after the intranasal administration procedure, females were killed with a lethal dose of sodium pentobarbital.

Each subject was decapitated, and the brain was removed. Gross dissection of the brain involved first making an incision at the posterior base of the olfactory bulbs to separate them from the rest of the brain, then removing the cerebellum and medulla by cutting along the transverse sinus, and finally excising the entire region enclosed by the left and right posterior communicating arteries extending from the basal aspects to the dorsal aspects of the brain. The section enclosed by the posterior communicating arteries was labelled as MC+DC, which included (but was not limited to) the hypothalamus, pituitary and amygdala. The uterus and ovaries were removed through an incision made near the abdominal cavity. The ovaries were then cut from the uterus. Excess fat and mesentery were carefully removed from the uterus. All excised tissues were placed into pre-weighed vials and re-weighed to determine tissue wet mass.

Intranasal administration of male urine containing $^3\text{H-E}_2$ in inseminated female mice

Inseminated females were intranasally administered either $25 \mu\text{l}$ male urine containing $^3\text{H-E}_2$ ($n=6$) or $25 \mu\text{l}$ negative control male urine containing no radioactivity ($n=4$) using the technique described above. Male urine containing $^3\text{H-E}_2$ was prepared by pooling urine from males injected with $^3\text{H-E}_2$ as described above, excluding samples with $<7 \text{ DPM/nl}$ urine. Negative control male urine was prepared by pooling urine from six untreated males. Dissections were performed as described above, however, cortical and ovarian tissues were not analysed in this experiment.

Intranasal administration of $^3\text{H-E}_2$ in ovariectomised female mice followed by perfusion

Ovariectomised females were prepared via bilateral removal of the ovaries under sodium pentobarbital anaesthesia, and were then group housed for a period of 21–28 days prior to the intranasal procedure. Females were intranasally administered either $10 \mu\text{Ci } ^3\text{H-E}_2$ (E_2 -[2,4,6,7- ^3H]; in ethanol, 1.0 mCi/ml, 70 Ci/mM, PerkinElmer, Waltham, MA, USA, $n=4$) or $10 \mu\text{l}$ 95% ethanol ($n=4$) as described above. One hour following intranasal administration, females were anaesthetised with sodium pentobarbital. Once an animal was unresponsive to tactile stimuli, the heart was exposed and the female was perfused with 30 ml saline using a syringe and needle. Once perfused, the females were dissected as described above. Additionally, a sample of muscle tissue was taken from an inner rear thigh to be processed.

Intranasal administration of $^3\text{H-E}_2$ in untreated and E_2 -treated inseminated female mice followed by perfusion

E_2 -treated inseminated females were prepared via a s.c. injection of $2 \mu\text{g } \text{E}_2$ (Sigma) in 0.5 cm^3 peanut oil on day 0 of pregnancy. Inseminated ($n=4$) and E_2 -treated inseminated ($n=4$) females were intranasally administered $10 \mu\text{Ci } ^3\text{H-E}_2$ (E_2 -[2,4,6,7- ^3H]; in ethanol, 1.0 mCi/ml, 70 Ci/mM, Perkin-Elmer) as described above. Negative control inseminated

females ($n=4$) were intranasally administered 10 μl 95% ethanol. Females were then perfused and dissected as described above.

Sample processing

Male urine samples were processed by adding 10–100 μl urine to 10 ml Ultima Gold LLT scintillation cocktail (PerkinElmer) in 20 ml scintillation vials. Female tissue samples were first homogenised by adding 1–1.5 ml Soluene-350 tissue solubiliser (PerkinElmer) to each vial immediately after tissue wet weights were determined. Vials were then placed in a 50 °C water bath for 2–4 h. Upon complete tissue homogenisation, vials and their contents were allowed to cool for 10 min, after which 0.2 ml 30% H_2O_2 was added in 0.1 ml aliquots to reduce colour quenching. Vials were then placed back into the 50 °C water bath for 30 min, and were then removed and cooled to 21 °C (room temperature). Following cooling, 10–15 ml Hionic-Fluor scintillation cocktail (PerkinElmer) was added to each vial. Vials were left in darkness overnight to permit light and temperature adaptation. Radioactivity was measured for 10 min/sample in a liquid scintillation counter (LKB 1217 RackBeta, Wallac, Turku, Finland). Quench correction was performed using an internal standardisation procedure. In experiments with perfused animals, radioactivity was measured in a liquid scintillation counter (LS 6500, Beckman Coulter, Fullerton, CA, USA) using external standards for quench correction.

Internal standardisation procedure

Following the procedure of Kessler (1989), in order to determine DPM for all urine and tissue samples, a known quantity of radioactivity was added to each vial after the sample counts per minute (CPM) had been determined. Vials were then swirled to ensure even distribution of the added radioactivity, and CPM was again measured for 10 min/sample in a liquid scintillation counter (LKB 1217 RackBeta). Counting efficiency for each sample (E_x) was determined using Equation 1 ($E_x = (\text{CPM}_1 - \text{CPM}_0) \div \text{DPM}_T$), where CPM_1 is the measured CPM of the sample after additional radioactivity was added, CPM_0 is the original CPM of the sample, and DPM_T is the theoretical DPM of the added quantity of radioactivity, calculated from the information provided by the manufacturer. DPM for each sample (DPM_x) was calculated using Equation 2 ($\text{DPM}_x = \text{CPM}_0 \div E_x$).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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