STEROID TRANSFER BETWEEN CONSPECIFICS AND ITS POTENTIAL IMPACTS ON THE REPRODUCTIVE ENDOCRINOLOGY OF FEMALE MICE

STEROID TRANSFER BETWEEN CONSPECIFICS AND ITS POTENTIAL IMPACTS ON THE REPRODUCTIVE ENDOCRINOLOGY OF FEMALE MICE

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree Doctorate of Philosophy

McMaster University DOCTORATE OF PHILOSOPHY (2012)

Hamilton, Ontario (Psychology, Neuroscience & Behaviour)

TITLE: Steroid transfer between conspecifics and its potential impacts on the reproductive endocrinology of mice

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NUMBER OF PAGES: xv, 178

Abstract

Sex steroids are critical for the post-natal development of the female reproductive system, and are involved in ovulatory cycling and pregnancy. In the mouse, *Mus musculus*, female development, cycling, and pregnancy can be affected by the urine of conspecifics, which is known to contain active steroids. Specifically, reproductive development can be accelerated (the Vandenbergh effect), cycling can be prolonged (the Lee-Boot effect) or synchronized (the Whitten effect), and blastocyst implantation can be disrupted (the Bruce effect). Since steroids alone can affect females in ways that are indistinguishable from these social reproductive effects, I hypothesized that urinary steroids of conspecifics may be absorbed by females, arrive in the reproductive system, and thereby affect females through known mechanisms. First I showed that tritium-labelled 17β-estradiol (^{3}H-E_2) injected into males is excreted in their urine, that application of ^{3}H-E_2 to the nose of an inseminated female results in its arrival in her brain and reproductive tissues, and that application of urine from males injected with ^{3}H-E_2 to the nose of an inseminated female results in detectable levels in her uterus. When I paired inseminated females with non-sire males injected with ^{3}H-E_2, radioactivity was detected in the brain and reproductive tissues of the females. This is the first demonstration of inter-individual steroid transfer that directly enters the body of the recipient. Similar results were found when I indirectly exposed juvenile females to adult males injected with ^{3}H-E_2. Untreated adult females that I exposed to same-strain ^{3}H-E_2- or ^{3}H-progesterone- (^{3}H-P_4) treated

adult males or females had significant amounts of radioactivity in brain and reproductive tissues, with greater amounts in the ^{3}H-E_2 condition than in the ^{3}H-P_4 condition. Taken with the existing literature, these results suggest that steroid transfer may underlie various social reproductive phenomena in mice, with potential implications for many other species.

Acknowledgements

Thank you to my supervisor, Dr. Denys deCatanzaro, for your almost infinite patience with me. Without your understanding and sympathy, I simply would not have completed any of these studies. You were always available when I needed you the most, and your constant encouragement will keep me looking up for decades to come.

When I was just starting out in graduate school, I was fortunate enough to enjoy the mentorship of Dr. Robert Berger. Rob, you showed me how to do everything and anything in the lab, and by doing so, you set me off on the right foot. I was always happy to celebrate with you in our successes, and commiserate with you in our frustrations. You are an amazing person, and I have no doubt that you will only continue to succeed in everything that you do.

Many thanks also go to the rest of my dear lab mates, both past and present. Dr. Ayesha Khan, thank you for always helping me talk things through, and for always having that infectious smile. Jordan Shaw, you helped keep me active and grounded in that first overwhelming year. Nazanin Rajabi and Brent Crawford, your determination and energy helped prop me up and gave me the fuel I needed to push through that last little bit of data collection and writing. And of course, the soon-to-be Dr. Joelle Thorpe: You have put up with me for an unimaginably long time. Thank you, Joelle, for supplying me with an endless amount of entertaining and thought-provoking conversations, and for handing me important opportunities I never would have otherwise considered. You are a

better scientist than you will ever admit to, and a better friend than you will ever know.

Thanks also to my committee members, Dr. Paul Faure and Dr. Deda Gillespie. You both helped me in small ways that ended up being very significant, while keeping those foreboding committee meetings down to a fun and functional minimum. Special thanks to Dr. Jim Quinn for stepping up at the last minute to help see me through the end.

Now comes the part where I have the privilege of listing the excellent people who were left under my care in the lab. Emmanuel Awuah, you kindly and cooly helped me figure out everything I needed to know to start collecting good data. Sean Pearce, thank you for putting in the hard work needed to keep that data flowing so smoothly. Jihwan Jheon, our seemingly endless days in the lab without so much as a hint of the sun probably did us more harm than good, but we pulled through together, and the results were outstanding. Faizan Imtiaz, your incredible spirit had a profoundly positive effect on Jihwan and me; thank you for lending us your remarkable talent and personality. Yubin Ha, you only got to come in when things were at their strangest in the lab, but you adapted quickly to everything we threw your way and made it through unscathed. And finally, thank you, Tyler Pollock, for sticking with me over the home stretch, and for being a good friend even if, deep down, you really (and justifiably) wanted to strangle me all of the time. As you would say to me at the end of every work day: “well, if I

don’t see you again, best wishes and good luck with all of your future endeavours.”

To the people that made this line of research safe, humane, and even possible: Thank you, April Scott and Heather Couture, for managing my animal subjects and for training me on how to do the best possible work with them. Thanks also to Wendy Whittaker, Dawn Graham, Kathleen Barber, and Tamara Kearns, all of whom have done the hard dirty work that can never be appreciated enough. It was a pleasure getting to know each of you, and I will forever treasure the thoughts you each shared with me on the nature of this kind of work.

And a final thank you to my lovely wife, Dr. Janice Calzavara. You kept me going when everything seemed too challenging to continue. You mean the world to me, and I am only too lucky to get to spend the rest of my life with you.

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List of Abbreviations

Estradiol (17β-estradiol, oestradiol)	E_2, E2
Progesterone	P_4, P4
Testosterone	T
Tritium	^3H, [^3H]
Tritium-labelled estradiol	^3H-E_2, [^3H]E2
Tritium-labelled progesterone	^3H-P_4, [^3H]P4
Tritium-labelled testosterone	^3H-T
Hydrogen peroxide	H_2O_2
Mesencephalon and diencephalon	MC + DC
Olfactory bulbs	OB
Disintegrations per minute	DPM, d.p.m.
Counts per minute	CPM
Pico	p
Nano	n
Micro	µ
Milli	m
Centi	c
Kilo	k
Litres	l
Grams	g

Gauge	GA
Gravitational force	***g***
Metres	m
Hours	h
Minutes	min
Seconds	s
Degrees Celsius	°C
Curies	Ci
Molar	M, mol
Daltons	Da
Vomeronasal organ	VNO
Standard error of the mean	S.E.M.
Intraperitoneal	i.p.
Subcutaneous	s.c.
Analysis of variance	ANOVA
Major histocompatibility complex	MHC
Major urinary protein	MUP
Luteinizing hormone	LH
Follicle-stimulating hormone	FSH

Chapter 1

General Introduction

Overview

Steroid hormones, such as 17β-estradiol (E_2), progesterone (P_4), and testosterone (T), control the biochemistry and physiology of the reproductive system throughout mammalian life. Steroids function at very low concentrations in females, and so reproductive events such as puberty, estrous cycling, and pregnancy, are each susceptible to interference by factors that affect internal steroid concentrations. Endocrine-altering factors can include environmental toxins, synthetic and natural steroid-like substances, psychological and physiological stressors, smells in the environment, many foods, and plain unmodified exogenous steroids. Exposure to exogenous steroids can occur in many ways, and one of the most overlooked sources that can lead to significant steroid exposure comes from the excretions of other members of the same species (*i.e.*, conspecifics).

Steroids are present and viable in several mammalian excretions. In mice (*Mus musculus*) specifically, urinary steroids are particularly relevant, as the quantities are high enough and the inter-individual exposure significant enough that steroids from conspecifics could impact all aspects of a female's reproductive system throughout life. Concerning the extent to which urinary steroids can invade the female reproductive system, this thesis addresses the excretion of circulating steroids, the uptake of steroids via plausible routes of entry, and the absorption and binding of steroids that originate from conspecifics. Throughout

this research, tritium-labelled (^{3}H) steroids were used, as tritium allows for the tracing of steroids without hindering any of their biological properties.

Steroids and the mammalian female reproductive system

Steroid hormones are small lipophilic molecules that are metabolically derived from cholesterol in the gonads, adrenal glands, and some other tissues. Steroids include estrogens, androgens, progestins, and other groups of bioactive chemicals. Estrogens, androgens, and progestins are collectively referred to as sex steroids. Sex steroids are so named because their actions in an individual are responsible for sexual differentiation, sexual development, reproductive behaviour, and many elements of pregnancy (Arnold 2009). Since sexual reproduction is, for better or worse, the naturally selected mode of replication for mammals, sex steroids are universally produced within each mammal and act as common ligands for receptors that are highly conserved among species (Mangelsdorf *et al*. 1995). Despite the reliance animals have on sex steroids in order to successfully reproduce, much of the reproductive system can be adversely affected by nearly undetectable variations in sex steroid levels (Aksglaede *et al*. 2006), or even by chemicals that can act as steroid receptor ligands, molecules that are collectively referred to as xenosteroids (Berger *et al*. 2010, Tena-Sempere & Aguilar 2005, Tyler *et al*. 1998).

The mammalian female reproductive system develops prenatally in the absence of the *Sry* gene, which leads to an absence of testes and the hormones that

they produce (Koopman *et al*. 1991). As a result, pre- and peri-natal development of the female phenotype occurs in the relative absence of sex steroids from the gonads (Wilson *et al*. 1981). Typically, female secondary sex characteristics and sexual behaviour do not develop until puberty, which is a genetically timed event marked by the production of estrogens and progestins in the ovaries, at which point sex steroids and their receptors are essential (Nelson *et al*. 1990). In puberty, estrogen receptors within the female are activated primarily by estrogens, including 17β-estradiol (E_2), estriol, and estrone. Progestin receptors are activated primarily by progesterone (P_4). When these receptors bind with the E_2 or P_4 secreted by the ovaries, they create receptor-ligand complexes that act as DNA transcription factors (Mangelsdorf *et al*. 1995). Estrogen and progestin receptor-ligand complexes are then responsible for the development of the uterus and changes in adipose distribution and accumulation (Wilson *et al*. 1981). But steroid receptors are present throughout the body prior to their activation by gonadal sex steroids, and they will bind with exogenous steroids and xenosteroids at various efficiencies (Blair *et al*. 2000). An increase in estrogen receptor ligands in a pre-pubertal female thereby accelerates transcription throughout her reproductive system, and when this happens the female undergoes precocious puberty, *i.e.*, the female begins estrous cycling at a younger than normal age (Alonso & Rosenfield 2002, Beaton *et al*. 2006).

Following puberty, the female reproductive system remains susceptible to atypical levels of steroid receptor ligands due to the involvement of steroids in the

ovarian cycle. In interaction with the pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), E_2 and P_4 control the timing of ovulation in most species of mammals. In mice, E_2 acts in the follicular phase of the estrous cycle, causing cell proliferation in the uterus, and also causing a release of LH that later results in physiological and behavioural estrus (Mahesh & Brann 1998). P_4 is involved in selectively inhibiting estrogen receptor activity in certain cell types, which controls the effect of E_2 to prevent generalized cell proliferation throughout all estrogen receptor rich cell types (Tibbetts *et al*. 1998). Behavioural estrus, as defined by sexual proceptivity and receptivity, can even be stimulated by elevations in circulating E_2 as a result of E_2's non-genomic activational effects on the ventromedial nucleus of the hypothalamus (Rainbow *et al*. 1984), an area of the brain known to be involved in female sexual behaviour (Pfaff 1980).

Once mated, an inseminated female's ova, oviducts, and uterus all must synchronize in their stages of preparedness in order for blastocyst implantation to occur (Dey *et al*. 2004, Harper 1992, Paria *et al*. 1993). First, the fertilized ova must divide until they reach the blastocyst stage, a process that involves E_2 (Paria *et al*. 1993). While this occurs, the uterus responds to endogenous E_2 and P_4, and becomes capable of adhering with and supporting the blastocysts (Potter *et al*. 1996). The blastocysts then pass through the oviducts at a rate that is controlled by E_2 and possibly P_4 (Orihuela *et al*. 2001), entering the uterus just as both the blastocysts and uterus are in respective stages that allow for adherence of the

blastocysts to the uterine epithelium. This cellular adhesion between a blastocyst and the uterine epithelium is referred to as implantation. If the balance of E_2 and P_4 is disrupted before or around the time that implantation is supposed to occur, the blastocysts will be reabsorbed by the uterus, decay in the oviduct, or pass through the uterus in a halted state (Banik & Pincus 1964, Fossum *et al*. 1989).

Puberty acceleration in male-exposed females (the Vandenbergh effect)

In the presence of a male or his chemical emissions, a female mouse tends to exhibit sexual behaviour earlier in life than do her isolated counterparts, and she also demonstrates the uterine, ovarian, and vaginal physiology indicative of sexual maturity (Vandenbergh 1967, 1969). Because of the extensive work done by John Vandenbergh on this phenomenon, it is often referred to as the Vandenbergh effect. The timing of puberty in female mammals is controlled by the interplay of several hormones, including leptin, luteinizing hormone, follicle-stimulating hormone, estrogens, and progestins. For female mice, puberty acceleration in the presence of males can be mimicked by injections of estrogens and progestins, or by repeated injections of estrogens without progestins (Bronson 1975).

Although estrogens and progestins were once thought to be produced mainly by the ovaries, the metabolic pathways required to endogenously synthesize all steroids are present in males as well. Testosterone (T) is produced in male testes, or more specifically, in the Leydig cells within the testes. The

enzyme aromatase converts a small but critical fraction of circulating T into E_2 throughout the body of a male. Much of this aromatase activity is local, occurring in adipose tissue, bone, smooth muscle tissue, and specific regions of the brain, but aromatase activity also occurs in Leydig cells, which could account for much of the E_2 found in male urine (deCatanzaro *et al*. 2006, Jones *et al*. 2006). When exposed to peri-pubertal females through a wire mesh grid, males urinate more frequently, with more dilute urine that contains more overall E_2 when adjusted for volume, compared to isolated males (deCatanzaro *et al*. 2009, Khan *et al*. 2009). Coinciding with increased male urinary E_2 production, young females that are exposed to adult males show early behavioural and physiological signs of sexual maturation (deCatanzaro *et al*. 2009, Khan *et al*. 2008a, 2008b, 2009), indicating that male urinary E_2 may be involved in inducing the Vandenbergh effect.

Adding to this idea, males that have been castrated cannot induce the Vandenbergh effect (Thorpe & deCatanzaro 2012). Castration necessarily takes out the Leydig cells contained within the testes. Castration thereby affects male in several ways, but most importantly, it drastically lowers T and E_2 production (Spironello-Vella & deCatanzaro 2001). A reduction in urinary E_2 naturally follows, which corresponds to males losing their ability to accelerate puberty (Thorpe & deCatanzaro 2012). However, injections of E_2 alone are sufficient to restore a castrated male's puberty accelerating abilities (Thorpe & deCatanzaro 2012), and such injections would not affect T levels in any known way. So although non-steroidal androgen-dependent substances may be involved in the

ability of a male to induce the Vandenbergh effect (Novotny 2003), it is possible that exposure to male urinary E_2 could alone drive puberty acceleration in females.

Induction of prolonged estrus or pseudopregnancy in grouped females (the Lee-Boot effect)

Females that are housed together in large groups demonstrate prolonged estrous cycles, pseudopregnancy, or anestrus. This is known as the Lee-Boot effect after the discoveries made by S. van der Lee and L. M. Boot. In the original observations by Lee and Boot (1955), as few as four females housed together resulted in spontaneous pseudopregnancy in some of the females over time. Pseudopregnancy is a non-reproductive state in which the diestrous phase of the ovarian cycle is maintained, and this state requires consistently elevated levels of prolactin (Ryan & Schwartz 1977). Prolactin production can be induced by injecting females with estradiol benzoate (a modification of the E_2 molecule made to resist degradation) alone or in combination with P_4 (Chen & Meites 1970), which allows for the possible involvement of female urinary steroids in the induction of the Lee-Boot effect. However, caution must be exercised in evaluating the role of exogenous steroids, as some evidence suggests that substances from the adrenal gland, specifically 2,5-dimethlpyrazine, may be necessary for this effect to occur (Ma *et al*. 1998).

Synchronization of estrous cyclicity in male-exposed females (the Whitten effect)

Following discovery of the Lee-Boot effect, Wesley Whitten (1956) showed that when females housed in groups were exposed to a male, the timing of their estrous cycles synchronized back to a three to four day period. Accordingly, this is often cited as the Whitten effect in the literature. Not unlike the Vandenbergh effect, the key chemosignal involved in the Whitten effect is found in the urine of intact males (Marsden & Bronson 1964). The role of steroid exposure in the Whitten effect is still poorly understood, but considering how closely estrus is linked to E_2 and P_4 levels, it is not unreasonable to predict the involvement of these steroids. But as with the Lee-Boot effect, non-steroidal male urinary compounds, in this case 2-(*sec*-butyl)-4,5-dihydrothiazole, dehydro-brevicomin, E,E-α-farnesene, and E-β-farnesene, may be involved in the elicitation of the effect (Jemiolo *et al*. 1986, Ma *et al*. 1999). Estrogens and progestins administered to animals are also known to induce estrus (Edwards *et al*. 1968, Mahesh & Brann 1998), so if urinary steroids can enter into circulation in a female, it is plausible that they could then induce estrus.

Disruption of blastocyst implantation by non-sire males (the Bruce effect)

In 1959, Hilda Bruce showed that albino-strain females inseminated by same-strain males failed to give birth when these females were paired with unfamiliar wild-type males 24 hours following insemination. Placing a mesh box containing an unfamiliar wild-type male in the same cage as an inseminated

albino-strain female was equally as effective in blocking pregnancy as was pairing, indicating that direct behavioural interactions are not required for males to induce the Bruce effect. Pairing an inseminated female with an unfamiliar female or with the original stud male did not block pregnancy at all, and pairing an inseminated female with either an intact or castrated unfamiliar same-strain male led to some pregnancy block, but much less than that arising from exposure to a wild-type male. These results have been widely replicated within and across species since Bruce's initial report (*e.g.*, deCatanzaro 2011, Marashi & Rülicke 2012, Parkes & Bruce 1962, Pillay & Kinahan 2009, Rohrbach 1982), and the general pregnancy blocking effect of an unfamiliar male is now referred to as the Bruce effect.

The Bruce effect is now known to be caused by something specific to the urine of an unfamiliar male (deCatanzaro *et al*. 1999, Dominic 1965, Peele *et al*. 2003). The bigger challenge has been in determining which exact chemical or chemicals in urine impact which biological mechanisms in order to prevent implantation from occurring. Two non-exclusive possibilities presently exist. The first possibility is that unfamiliar males may excrete non-steroidal chemosignals associated with their major urinary proteins (MUPs) or major histocompatibility complexes (MHCs) (Brennan & Peele 2003). These chemicals, which are known to be involved in social recognition, bind in the vomeronasal organ of an inseminated female. Once bound, these chemicals have been suggested to set off a neural cascade that causes a reduction of prolactin release

from the pituitary, and thereby a reduction of progesterone release from the corpora lutea. In the absence of sufficient levels of progesterone, pregnancy cannot occur. The second possibility, which is the focus of my research, is that urinary steroids could pass from an unfamiliar male to an inseminated female, directly opposing implantation by altering the hormonally-controlled timing of events required for pregnancy to occur (deCatanzaro 2011). Such steroid transfer would result in an excess of steroid hormones at the uterus and oviducts, and even within fertilized ova. Excess systemic E_2 could thereby bind to and directly affect a female's reproductive system during the peri-implantation period, preventing implantation from occurring, which in turn would prevent pregnancy.

Initial studies on the properties of the pregnancy blocking chemosignal in male urine demonstrated that the substance was likely non-volatile, as inseminated females housed over novel males are less likely to exhibit implantation failure than those housed under novel males (deCatanzaro *et al*. 1996, Gangrade & Dominic 1984). The urine of ovariectomized females given testosterone implants can block pregnancy almost as consistently as can male urine, which indicates that the chemosignal is androgen-dependent but not sex-specific (Dominic 1965). MUP and MHC fragments each possess many of the properties required of a potential pregnancy blocking chemosignal, and so these compounds have been heavily investigated (Marchlewska-Koj 1977, Thompson *et al*. 2007). MUPs alone can block pregnancy, but only when the animal from which they came has a level of circulating androgens typical of an adult male

(Marchlewska-Koj 1977). Even then, only the low molecular mass ligands (< 14 kDa) bound to the MUPs induce pregnancy block, meaning that MUPs must act as a transport mechanism for the active chemosignals (Peele *et al*. 2003). For MHCs, attached peptide ligands are considered to be the necessary factors in eliciting pregnancy block (Leinders-Zufall *et al*. 2004, Thompson *et al*. 2007). In both cases, the ligands are thought to activate the accessory olfactory system via the vomeronasal organ, as opposed to activating the main olfactory system via the olfactory epithelium (Lloyd-Thomas & Keverne 1982). However, the active ligands in question are poorly understood, and the system said to be involved in pregnancy block requires the same inter-individual interactions as does the system involved in social recognition (Brennan 2009), which may not be entirely ecologically plausible.

The alternative explanation invokes the potential for male-excreted E_2 to bear upon nearby females. E_2 is non-volatile, small, and androgen-dependent, and it can be produced and excreted by either sex. These properties of E_2 are analogous to the known properties of the pregnancy blocking chemosignal (see Brennan 2009, deCatanzaro 2011). Unlike other androgen-dependent urinary compounds, only E_2 would be affected by estrogen antibodies. Females injected with estrogen antibodies are much less susceptible to the Bruce effect than are untreated females (deCatanzaro *et al*. 1995). Furthermore, when inseminated females are intranasally given quantities of E_2 that are reflective of the quantities they would be exposed to when housed under a novel male, they exhibit a similar

rate of implantation failure (deCatanzaro *et al*. 2006). Finally, castrated males that are given repeated injections of E_2, which would not restore androgens or influence androgen-dependent MUP and MHC ligands, can significantly disrupt implantation in inseminated females (Thorpe & deCatanzaro 2012). Taken together with evidence on the negative impact of excess E_2 on intrauterine implantation (Ma *et al*. 2003, Roblero & Garavagno 1979, Safro *et al*. 1990, Valbuena *et al*. 2001), male urinary E_2 alone could be responsible for blocking pregnancy under the conditions of the Bruce effect. However, it is possible that MUP and MHC ligands attract inseminated females to novel males (Brennan & Zufall 2006, Moncho-Bogani *et al*. 2004), thereby allowing a male to transfer a large enough quantity of his steroid-laden excretions to disrupt implantation and thereby block pregnancy in an inseminated female.

Urine as a mediator of social reproductive phenomena

Urine is a complex solution that contains dozens of organic molecules, some of which are non-steroidal but are similarly bioactive at very low concentrations. This makes urine useful for social communication in mice and other rodents. As an attractant urine can draw females toward males, and as an idiosyncratic scent urine can be used to identify individuals (Boehm & Zufall 2006, Spehr *et al*. 2006). Many researchers have suggested that the chemicals involved in scent and recognition directly interplay with the olfactory system, resulting in a neural cascade that affects the reproductive system via the

hypothalamic-pituitary-gonadal axis (Marchlewska-Koj *et al*. 2000, Novotny *et al*. 1999, Peele *et al*. 2003). The suggested putative pheromones may each play a role in social endocrine regulation, possibly accounting for the increased effect of non-sire, different-strain males in the Bruce and Vandenbergh effects. However, the steroid content of mouse urine and the susceptibility of the female reproductive system to even slight elevations in circulating steroids cannot be overlooked.

The most common, natural source of endocrine disrupting chemicals that females are likely to encounter are the unconjugated sex steroids found in the excretions of conspecifics (deCatanzaro *et al*. 2004, 2006, Muir *et al*. 2001, 2008, Ziegler *et al*. 1989). Still, in order for urinary steroids to affect females in any way, there are a few necessary conditions that must be met. First, the urine of conspecifics must contain detectable levels of steroids in quantities similar to those known to have reproductive effects. This condition is met at least in the context of the Bruce and Vandenbergh effects, as demonstrated by enzyme-linked immunosorbent assay (ELISA) quantification of male urinary E_2 (deCatanzaro *et al*. 2006, 2009, Khan *et al*. 2009, Thorpe & deCatanzaro 2012). Second, the steroids in urine need a pathway into the circulation of a female. Once in circulation, urinary steroids and their metabolites must arrive in the brain and reproductive organs of the female, where these hormones could then affect local levels of endogenous steroids.

Research goals

My research was aimed to establish steroid transfer as a mechanism of endocrine disruption and regulation in mice. Since the onset of estrus, ovarian cyclicity, and pregnancy can each be affected by small changes in circulating E_2 and P_4 levels, females were selected as the recipient sex in all studies. Administration of radiolabelled steroids to stimulus animals allowed examination of the direct and indirect transfer of steroids among mice. Radioactivity was analyzed in various tissues including uterus, ovaries, muscle, olfactory bulbs (OB), mesencephalon and diencephalon (MC + DC), and cerebral cortex as indicators of exogenous steroid uptake.

Chapter 2: Guzzo AC, Berger RG & deCatanzaro D 2010 Excretion and binding of tritium-labelled oestradiol in mice (*Mus musculus*): implications for the Bruce effect. *Reproduction* **139** 225-263.

Abstract: Male mouse urine contains 17β-oestradiol (E_2) 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 E_2 (^{3}H-E_2) or injected i.p. with ^{3}H-E_2, radioactivity was reliably detected in their urine. Following intranasal administration of ^{3}H-E_2 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 ^{3}H-E_2, 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 E_2 significantly reduced the uptake of ^{3}H-E_2 in the uterus. Taken with evidence that males deliver their urine to the nasal area of females, these results indicate that male urinary E_2 arrives in tissues, including the uterus, where it could lead to the disruption of blastocyst implantation.

Chapter 3: Guzzo AC, Jheon J, Imtiaz F & deCatanzaro D 2012 Oestradiol transmission from males to females in the context of the Bruce and Vandenbergh effects in mice (*Mus musculus*). *Reproduction* **143** 539-548.

Abstract: Male mice actively direct their urine at nearby females, and this urine reliably contains unconjugated oestradiol (E_2) and other steroids. Giving inseminated females minute doses of exogenous E_2, either systemically or intranasally, can cause failure of blastocyst implantation. Giving juvenile females minute doses of exogenous E_2 promotes measures of reproductive maturity such as uterine mass. Here we show that tritium-labelled E_2 (^{3}H-E_2) can be traced from injection into novel male mice to tissues of cohabiting inseminated and juvenile

females. We show the presence of ^{3}H-E_2 in male excretions, transmission to the circulation of females and arrival in the female reproductive tract. In males, ^{3}H-E_2 given systemically was readily found in reproductive tissues and was especially abundant in bladder urine. In females, ^{3}H-E_2 was found to enter the system via both nasal and percutaneous routes, and was measurable in the uterus and other tissues. As supraoptimal E_2 levels can both interfere with blastocyst implantation in inseminated females and promote uterine growth in juvenile females, we suggest that absorption of male-excreted E_2 can account for major aspects of the Bruce and Vandenbergh effects.

Chapter 4: Guzzo AC, Pollock TJ & deCatanzaro D (submitted) Transfer of [^{3}H]estradiol-17β and [^{3}H]progesterone from males or females to the brain and reproductive tissues of cohabiting female mice.

Abstract: Estradiol-17β and progesterone play critical roles in female reproductive physiology and behaviour. Given the sensitivity of females to exogenous sources of these steroids, their presence in interacting conspecifics' excretions is of potential interest. We first established that estradiol and progesterone are reliably present in urine of both male and female CF1 mice. We subsequently paired individual adult female mice with a stimulus male or female conspecific given daily injections of [^{3}H]estradiol or [^{3}H]progesterone. Following 48 hours of direct interaction with the stimulus animal, we measured radioactivity in the uterus, ovaries, muscle, olfactory bulbs, mesencephalon and diencephalon,

and cerebral cortex of the untreated female cohabitant. When females were each exposed to a stimulus male given either [^{3}H]estradiol or [^{3}H]progesterone, radioactivity was observed in all tissues. Radioactivity was similarly found in all tissues of females that were each exposed to a stimulus female given either of these [^{3}H]steroids. Following exposure to stimulus animals of either sex, [^{3}H]estradiol transfer was more prominent than was [^{3}H]progesterone transfer. We suggest that steroid transfer among individuals has implications for the understanding of various forms of pheromonal activity.

Chapter 2

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

Adam C. Guzzo, Robert G. Berger, and Denys deCatanzaro (2010)

Reproduction, 139, 255-263.

Authors' Contributions

Adam C. Guzzo: Experimental design, data collection, data analysis, literature review, manuscript preparation, writing, and editing.

Robert G. Berger: Data collection, assistance with experimental design and methods development.

Denys deCatanzaro: Assistance with experimental design, data analysis, manuscript preparation, writing, and editing.

Abstract

Male mouse urine contains 17β-oestradiol (E_2) 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 E_2 (^{3}H-E_2) or injected i.p. with ^{3}H-E_2, radioactivity was reliably detected in their urine. Following intranasal administration of ^{3}H-E_2 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 ^{3}H-E_2, 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 E_2 significantly reduced the uptake of ^{3}H-E_2 in the uterus. Taken with evidence that males deliver their urine to the nasal area of females, these results indicate that male urinary E_2 arrives in tissues, including the uterus, where it could lead to the disruption of blastocyst implantation.

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 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*. 1995*c*) or 17β-oestradiol (E_2; deCatanzaro *et al*. 1995*b*).

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 E_2 being especially important. Male mouse urine reliably contains unconjugated E_2 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 E_2 and show a diminished capacity to disrupt pregnancy (Beaton & deCatanzaro 2005). Recently-inseminated females given daily doses of E_2 antibodies maintain pregnancy despite exposure to novel males (deCatanzaro *et al*. 1995*a*). 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 E_2 to creatinine rises in male urine (deCatanzaro *et al.* 2006, 2009).

In the initial days of gestation, endogenous E_2 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 E_2 prior to blastocyst invasion causes the uterus to advance to a refractory state, preventing implantation (Ma *et al*. 2003). E_2 is also involved in preparing blastocysts for implantation, but 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 E_2 (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 E_2 is to be considered the primary urinary component responsible for pregnancy disruption in the Bruce effect, it would be expected that E_2 in male urine will travel to and bind in the uterus of a recently inseminated female. Here, we investigated urinary tritium-labelled E_2 (^{3}H-E_2) excretions in non-sire males implanted with osmotic pumps. Next, we studied the time course of urinary ^{3}H-E_2 excretion in i.p. injected males. We then tested the binding of intranasally administered ^{3}H-E_2 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 ^{3}H-E_2 to inseminated females, and target tissues were examined for ^{3}H-E_2 uptake. Subsequently, in order to control for circulating ^{3}H-E_2 that might not be bound in tissues, animals were perfused with saline at the point of killing. Finally, as natural E_2 would bind competitively with ^{3}H-E_2, we examined uptake in ovariectomised females and intact inseminated females pre-treated with unlabelled E_2.

Results

Radioactivity was detected in the urine of males implanted with osmotic pumps containing ^{3}H-E_2, both on day 1 post surgery (231.5±22.3 DPM/µl urine) and on day 6 post surgery (173.1±15.3 DPM/µl urine). As determined by a paired-samples *t*-test, there was significantly more ^{3}H-E_2 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/µl urine), $t(3)=1.73$, $P=0.18$, and day 6 post-surgery (0.06±0.01 DPM/µl 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 ^{3}H-E_2. Despite all males receiving the same quantity of ^{3}H-E_2, the timing and quantity of ^{3}H-E_2 excretion was clearly idiosyncratic. Most urinations (81%) occurred within the first 8 h of the 12-h observation period.

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

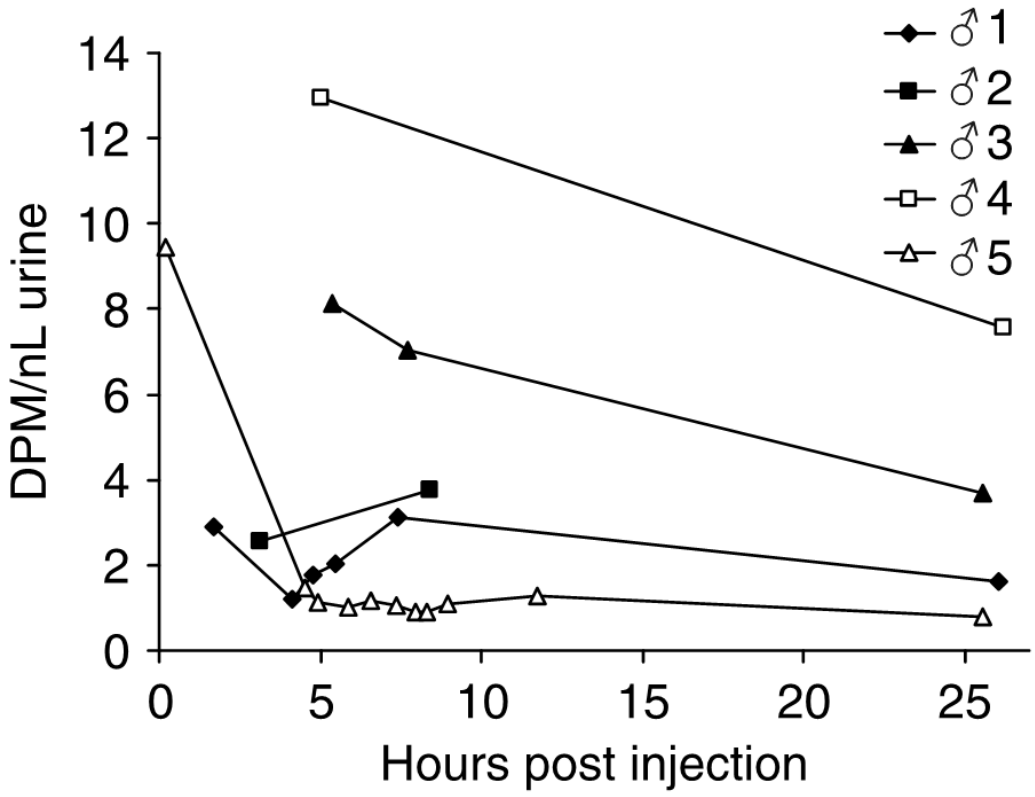


Figure 2 shows radioactivity in the target tissues of females intranasally administered ^{3}H-E_2 or ethanol (negative control). Initial statistical analyses indicated that there were no significant differences among doses of ^{3}H-E_2, therefore the four doses were pooled in subsequent statistical analysis, yielding mean (±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 vs. ^{3}H-E_2) 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 ^{3}H-E_2-treated females differed from all control tissues ($P<0.01$). In addition, the quantities of ^{3}H-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}H-E_2 than did the MC + DC, olfactory bulbs, and cortex. Significantly ($P<0.05$) more ^{3}H-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 2 Mean (± S.E.M.) radioactivity detected in the uterus, ovaries, mesencephalon and diencephalon (MC + DC), olfactory bulbs and cortex of inseminated females intranasally administered 20 µl 95% ethanol (negative control), or 18, 24, 27, or 37 µCi tritium-labelled oestradiol. All animals were killed 1 h following the administration procedure.

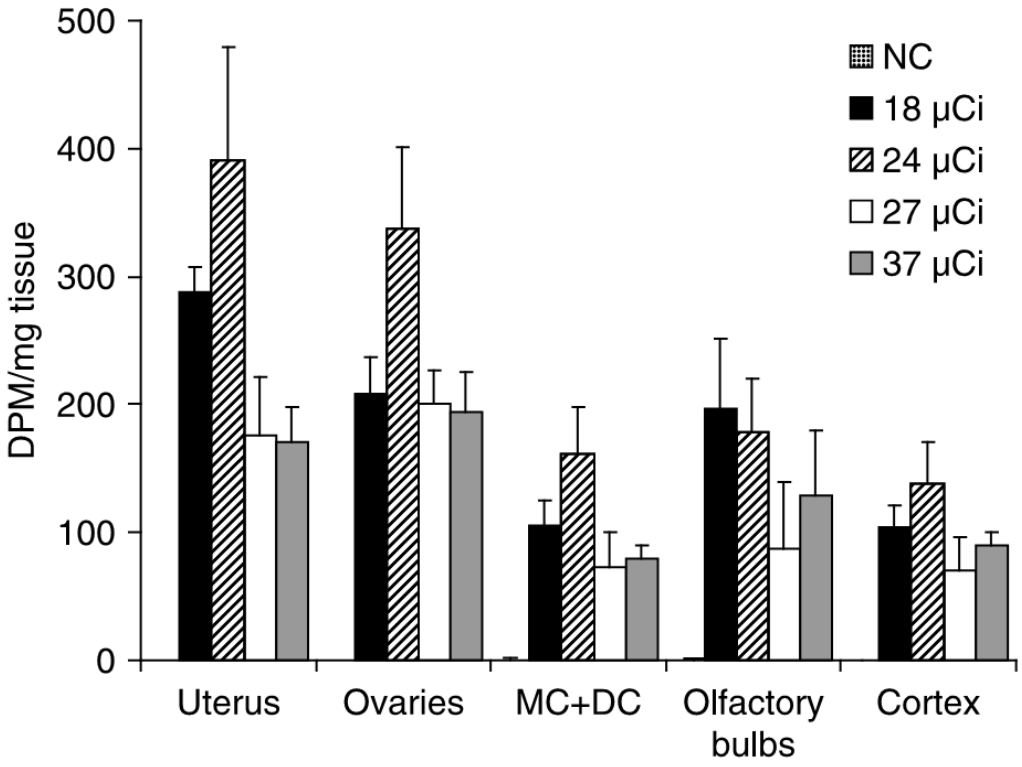


Figure 3 shows radioactivity recovered in tissues of females intranasally administered either male urine containing $^{3}H\text{-}E_2$ (treatment) or negative control male urine. An ANOVA, treating condition (negative control versus treatment) as a between-subjects variable and tissue (uterus, olfactory 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 $^{3}H\text{-}E_2$ quantities between tissues.

Figure 3 Mean (± 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).

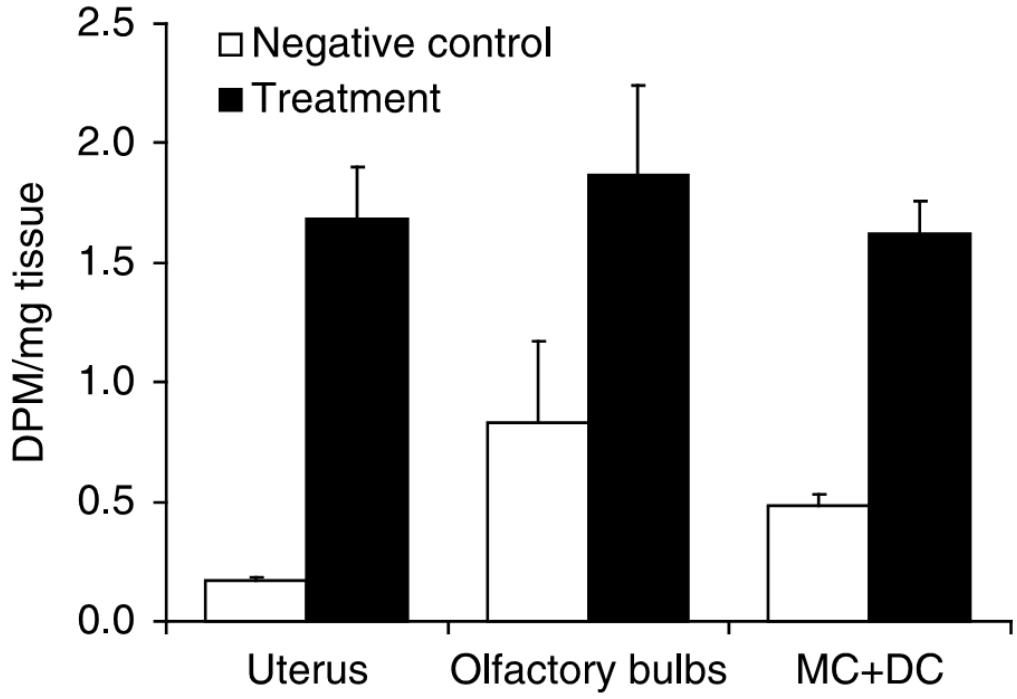


Figure 4 shows radioactivity in the tissues of ovariectomised females intranasally administered ethanol (negative control) or ^{3}H-E_2, followed by perfusion. Tissue samples from negative control females demonstrated background levels of radioactivity in a distinct and non-overlapping range from that of ^{3}H-E_2-treated animals, and were excluded from statistical analyses. An ANOVA, treating tissue (uterus, muscle, olfactory bulbs, MC + DC, 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 4 Mean (± 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}H-E_2).

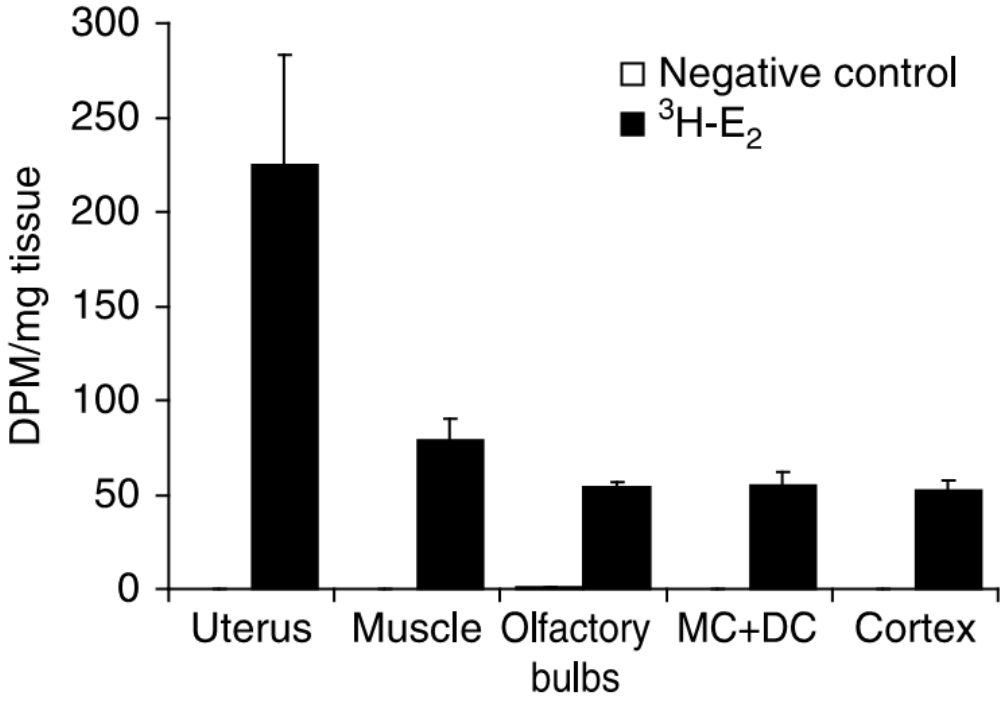
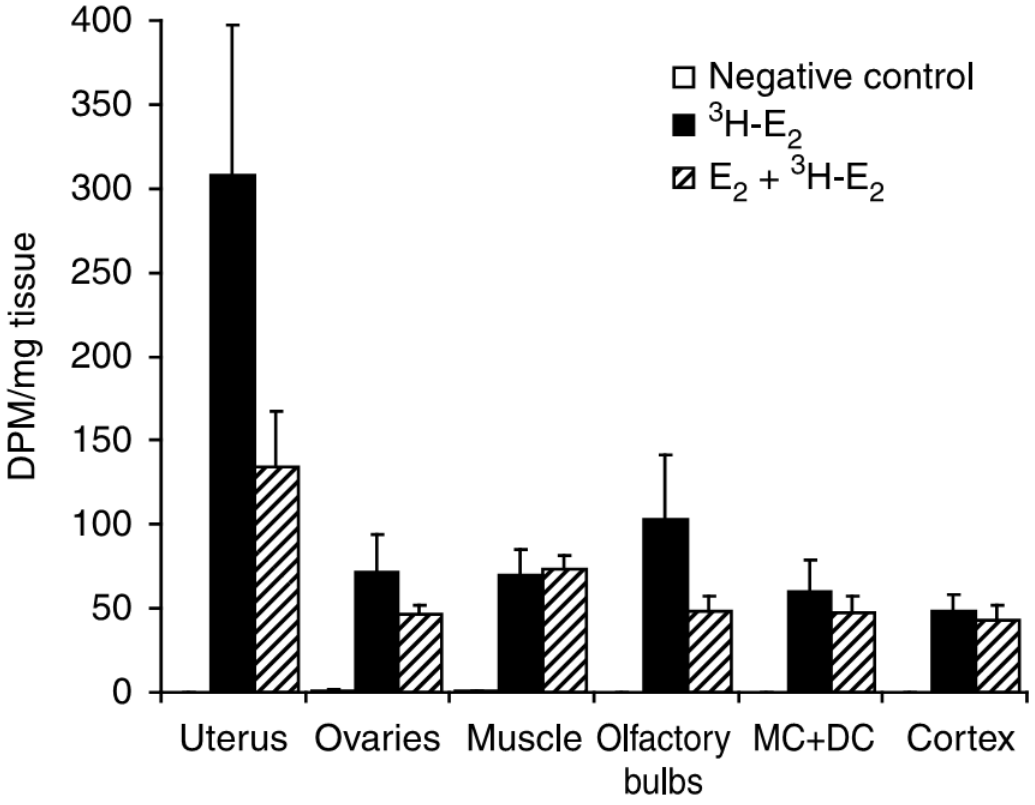


Figure 5 shows radioactivity in the tissues of inseminated females intranasally administered ethanol (negative control) or ^{3}H-E_2, as well as females pre-treated with E_2 and then intranasally administered ^{3}H-E_2, where all animals were subsequently perfused to flush blood from tissues. Tissue 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, 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}H-E_2 without oestradiol pre-treatment were significantly more radioactive than all other samples, $P<0.01$, and that none of the other samples from ^{3}H-E_2-treated subjects, regardless of E_2 pre-treatment, differed significantly from one another.

Figure 5 Mean (± 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}H-E_2), or 10 µCi tritium-labelled oestradiol following an s.c. injection of 2 µg 17β-oestradiol in 0.05 cm^3 peanut oil two days prior (E_2 + ^{3}H-E_2).



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}H-E_2. In males injected intraperitoneously with ^{3}H-E_2, radioactivity in urine peaked within 8 h after administration, with patterns of ^{3}H-E_2 excretion varying among subjects. These data also indicate that nasal exposure of inseminated females to ^{3}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}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}H-E_2, radioactivity was detected in all samples. When ovariectomised females were nasally administered ^{3}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 the ovaries in inseminated females perfused with saline; uterine ^{3}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 oestradiol 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}H-E_2 delivery arrives in urine without metabolic alteration. Some limitations to the present methodology arise from the fact that ^{3}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}H-E_2 that can be harvested from male urine. The ^{3}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 at 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}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 that 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 oestrus (Mathews & Edwards 1977, Pfaff & Sakuma 1979, Pfaff *et al*. 2000). Our evidence that nasally-administered ^{3}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 1980, Meredith *et al*. 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 male (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 much previous data indicating steroid dependency of the effect (Bruce 1965, Rajendren & Dominic 1988, deCatanzaro *et al*. 1991, Vella & deCatanzaro, 2001, Beaton & deCatanzaro 2005, deCatanzaro 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 28×16×11 (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 stock from Charles River Breeding Farms of Canada (LaPrairie, Québec) 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}H-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}H-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 they were at 95-100% of their rated capacity. Pumps had a mean fill volume of 107±6 µl, and a mean pumping rate of 0.50±0.02 µl/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}H-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 surgery. The day following surgery, males were placed in a compartment in the upper level of a two-tier exposure apparatus (deCatanzaro *et al.* 1996). Each compartment measured 14.5×21×13.5 (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 30×21×13.5 (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 wax paper lining a clean Teflon-coated stainless steel tray measuring 29.8×44.5×2 (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 was used for each collection. Samples were pooled by day and frozen until processed.

Injected ^{3}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 µCi ^{3}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 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}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}H-E_2 (E_2-[2,4-3H]; in ethanol, 1.0 mCi/ml, Sigma; 18 µCi, *n*=3; 24 µCi, *n*=3; 27 µCi, *n*=2; 37 µCi, *n*=2) or 95% ethanol (20 µl, *n*=2; negative control group). Two different ^{3}H-E_2 preparations were used in the 18 and 24 µCi groups (35.0 Ci/mM) and the 27 and 37 µCi groups (52.7 Ci/mM). However, the absolute quantities of ^{3}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 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}H-E_2 in inseminated female mice

Inseminated females were intranasally administered either 25 µl male urine containing ^{3}H-E_2 (*n*=6) or 25 µl negative control male urine containing no radioactivity (*n*=4) using the technique described above. Male urine containing ^{3}H-E_2 was prepared by pooling urine from males injected with ^{3}H-E_2 as described above, excluding samples with <7 DPM/nl urine. Negative control male urine was prepared by pooling urine from six untreated males. Dissections were performed as above, however cortical and ovarian tissues were not analysed in this experiment.

Intranasal administration of ^{3}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 µCi ^{3}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 µl 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, females were dissected as above. Additionally, a sample of muscle tissue was taken from an inner rear thigh to be processed.

Intranasal administration of ^{3}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 µg 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 µCi ^{3}H-E_2 (E_2-[2,4,6,7-3H]; in ethanol, 1.0 mCi/ml, 70 Ci/mM, PerkinElmer) 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 in 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 ($E_x=[CPM_1–CPM_0]\div DPM_T$), 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 information provided by the manufacturer. DPM for each sample (DPM_x) was calculated using Equation 2 ($DPM_x=CPM_0\div E_x$).

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.

Funding

This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) awarded to D deCatanzaro.

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Received 28 August 2009

First decision 23 September 2009

Accepted 30 September 2009

Chapter 3

Oestradiol transmission from males to females in the context of the Bruce and Vandenbergh effects in mice (*Mus musculus*)

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Reproduction, 143, 539-548.

Authors' Contributions

Adam C. Guzzo: Experimental design, data collection, data analysis, literature review, manuscript preparation, writing, and editing.

Jihwan Jheon: Data collection, assistance with experimental design and methods development.

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Denys deCatanzaro: Assistance with experimental design, data analysis, ELISA measures, manuscript preparation, writing, and editing.

Abstract

Male mice actively direct their urine at nearby females, and this urine reliably contains unconjugated oestradiol (E_2) and other steroids. Giving inseminated females minute doses of exogenous E_2, either systemically or intranasally, can cause failure of blastocyst implantation. Giving juvenile females minute doses of exogenous E_2 promotes measures of reproductive maturity such as uterine mass. Here we show that tritium-labelled E_2 (^{3}H-E_2) can be traced from injection into novel male mice to tissues of cohabiting inseminated and juvenile females. We show the presence of ^{3}H-E_2 in male excretions, transmission to the circulation of females and arrival in the female reproductive tract. In males, ^{3}H-E_2 given systemically was readily found in reproductive tissues and was especially abundant in bladder urine. In females, ^{3}H-E_2 was found to enter the system via both nasal and percutaneous routes, and was measurable in the uterus and other tissues. As supraoptimal E_2 levels can both interfere with blastocyst implantation in inseminated females and promote uterine growth in juvenile females, we suggest that absorption of male-excreted E_2 can account for major aspects of the Bruce and Vandenbergh effects.

Introduction

Mammalian males and their excretions can have major impacts on the reproductive state of nearby conspecific females. Termination of early gestation by novel (non-sire) males around the time of intrauterine blastocyst implantation (the Bruce effect; Bruce (1960)) and advancement of sexual maturation in nearby juvenile females (the Vandenbergh effect; Vandenbergh (1967)) have been extensively studied in mice and in a number of other mammals (e.g. Bronson & Eleftheriou 1963, Izard & Vandenbergh 1982, Drickamer 1983, Lepri & Vandenbergh 1986, Storey 1996). These phenomena share a number of qualities, especially their dependency upon male urinary constituents that bear upon the female's nasal area (Parkes & Bruce 1962, deCatanzaro *et al.* 2006, 2009). During the initial days of exposure to females, males develop polyuria and polydipsia, with urination occurring in small droplets that may be directed at nearby females (e.g. Maruniak *et al.* 1974, Hurst 1990, deCatanzaro *et al.* 2006, 2009). These males will attempt to mate with nearby females and may become extremely agitated if there is a barrier between them and the females (deCatanzaro *et al.* 1996, 2000, 2006). It has been hypothesised that the critical male urinary constituents responsible for implantation failure in the Bruce effect (deCatanzaro *et al.* 2006, deCatanzaro 2011) and reproductive tract growth in the Vandenbergh effect (Beaton *et al.* 2006, deCatanzaro *et al.* 2009) could simply be oestrogens. There is evidence that small molecules such as sex steroids can be absorbed into circulation following nasal exposure (e.g. Bawarshi-Nassar et a l. 1989, Arora *et*

al. 2002, Türker *et al.* 2004, Guzzo *et al.* 2010), and it is conceivable that male urinary oestrogens could be absorbed by females and have impacts directly on blastocyst implantation and maturation of the reproductive tract. Giving inseminated females exogenous oestradiol (E_2), either systemically or intranasally, disrupts implantation and mimics the Bruce effect (deCatanzaro *et al.* 1991, 2001, 2006). Also, giving juvenile females exogenous E_2 can mimic the Vandenbergh effect (Bronson 1975) and endogenous E_2 in developing females changes after male exposure (Bronson & Desjardins 1974). Male mouse urine consistently contains substantial quantities of unconjugated E_2, and levels rise while males are in the presence of females (Muir *et al.* 2001, deCatanzaro *et al.* 2006, 2009). Castrated males cannot induce the Bruce effect (Bruce 1965, Vella & deCatanzaro 2001) or the Vandenbergh effect (Colby & Vandenbergh 1974). Treatment with testosterone (Dominic 1965, Lombardi *et al.* 1976) or E_2 (Thorpe & deCatanzaro 2012) restores the ability of castrated males to induce these effects.

Evidence shows clear mechanisms by which E_2 can disrupt blastocyst implantation and promote growth of the juvenile reproductive tract. Although E_2 actions are essential for preparation of the uterus for implantation, very small elevations above optimal levels can impede implantation (Ortiz *et al.* 1979, Paria *et al.* 1993, Potter *et al.* 1996, deCatanzaro *et al.* 2001, 2006, Valbuena *et al.* 2001, Ma *et al.* 2003, Dey *et al.* 2004). Also, it is well established that uterine growth in developing females is driven by endogenous E_2 (Ogasawara *et al.* 1983,

Quarmby & Korach 1984, Kahlert *et al.*2000, Sato *et al.* 2002). It is also clear that various exogenous oestrogenic substances can promote the growth of uterine mass in laboratory animals (e.g. Tinwell & Ashby 2004).

A necessary condition for the male urinary E2 hypothesis of the Bruce and Vandenbergh effects would be that this steroid is transmissible from male urine directly to the systems of nearby females. Previously, Guzzo *et al.* (2010) showed that when tritium-labelled E_2 (^{3}H-E_2) or male urine containing it is administered to a female's nasal area, radioactivity is measurable in her uterus, but this involved manual application rather than direct exposure to ^{3}H-E_2-treated males. Here we demonstrate that ^{3}H-E_2 injected into a novel male arrives in a female's reproductive tract when the male interacts directly with the female. We show this in both inseminated peri-implantation females and post-weanling juvenile females. These direct exposure procedures match those that are established to cause the majority of females to lose their initial pregnancies (deCatanzaro *et al.* 1996) and those that produce significant growth of the reproductive tract in developing females (Beaton *et al.* 2006). We also show that, following an injection of ^{3}H-E_2 to male mice, radioactivity is observed in the reproductive tissues and fluids of these males. Furthermore, we examined percutaneous entry of ^{3}H-E_2 into the female system and compared radioactivity in female organs after nasal exposure to ^{3}H-E_2, tritium-labelled testosterone (^{3}H-T) and tritium-labelled progesterone (^{3}H-P_4).

Results

Direct exposure of inseminated females to ^{3}H-E_2-treated males

In the first experiment, we inquired whether radioactivity could be measured in reproductive and other tissues of inseminated females after they cohabited for a few days with males that were either administered ^{3}H-E_2 in ethanol or ethanol alone (controls). Liquid scintillation counting on solubilised tissues and blood revealed radioactivity in all samples from females exposed to ^{3}H-E_2-treated males on both days 3 and 4 of gestation, whereas all samples from females exposed to control males showed levels close to background radiation (Table 1). Statistical analysis was conducted for each tissue and blood, comparing females exposed to ^{3}H-E_2-treated males and control females, pooling the similar data from the 2 days for reasons of sample size. The effect of condition was significant for each measure: uterus, $t(10)=6.11$, $P=0.0002$; ovaries, $t(10)=4.83$, $P=0.0005$; muscle, $t(10)=7.02$, $P < 0.0001$; blood, $t(10)=7.31$, $P < 0.0001$. Samples of the males' urine were collected the day after removal from the females; these showed values of 688.2±298.4 d.p.m./µl for day 3 males, 587.4±88.1 d.p.m./µl for day 4 males and 0.433±0.219 d.p.m./µl for pooled control males.

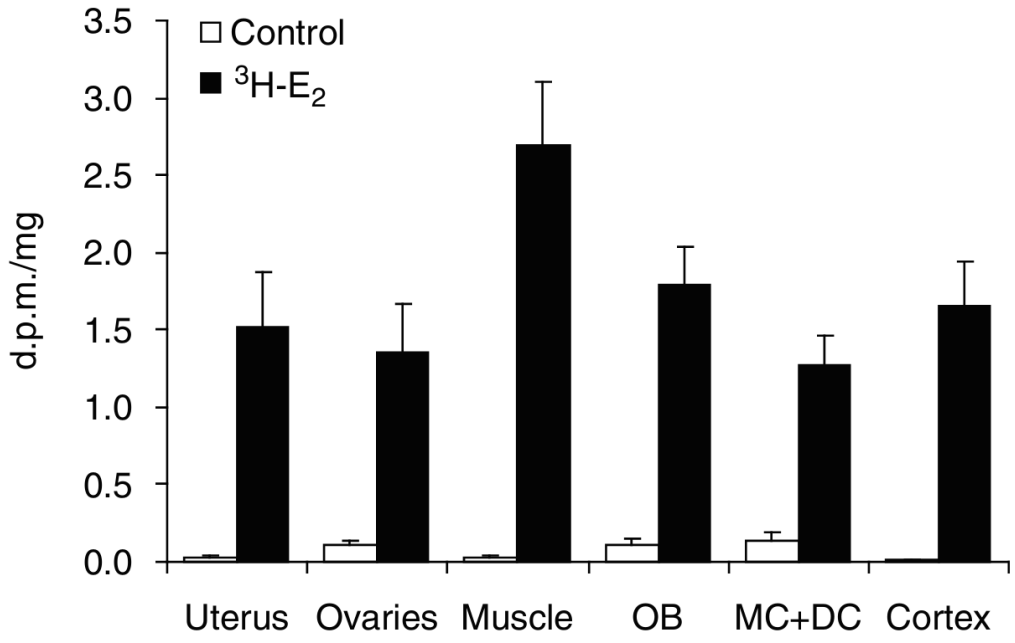
Table 1 Mean (±S.E.M.) radioactivity (d.p.m./mg) in the uterus, ovaries, muscle, and blood of inseminated females following exposure to a sexually-experienced HS male for 3 days or 4 days, where the stimulus male was given daily injections of 10 µl ethanol (control) or 10 µCi ^{3}H-E_2.

Condition (*n*)	**Uterus**	**Ovaries**	**Muscle**	**Blood**
Day 3 Control (2)	0.026 ± 0.005	0.019 ± 0.019	0.039 ± 0.007	0.076 ± 0.001
Day 4 Control (2)	0.033 ± 0.005	0.138 ± 0.046	0.096 ± 0.033	0.096 ± 0.000
Day 3 ^{3}H-E_2 (4)	2.609 ± 0.434	2.346 ± 0.375	3.823 ± 0.337	4.240 ± 0.714
Day 4 ^{3}H-E_2 (4)	4.107 ± 0.313	3.397 ± 0.646	5.530 ± 0.599	5.979 ± 0.135

Replication of direct exposure of inseminated females to ^{3}H-E_2-treated males

In the second experiment, we examined several tissues of inseminated females on day 3 of gestation after cohabitation with either ^{3}H-E_2-treated males or ethanol-treated males (controls). Tissues of females on day 3 of gestation after 3 days of exposure to ^{3}H-E_2-treated males showed measurable radioactivity in all samples, whereas tissues of females exposed to ethanol-treated males showed negligible radioactivity at background levels (Fig. 1). Each tissue showed significance in separate tests: uterus, $t(8)=4.20$, $P=0.0017$; ovaries, $t(8)=4.01$, $P=0.0021$; muscle, $t(8)=6.42$, P=0.0002; olfactory bulbs, $t(8)=7.15$, $P=0.0001$; mesencephalon and diencephalon (MC + DC), $t(8)=5.65$, P=0.0004; cortex, $t(8)=5.75$, $P=0.0004$. Although within-subject ANOVA comparing tissues among females exposed to ^{3}H-E_2-treated males showed significance, $F(5,20)=6.46$, $P=0.0013$, there were no significant differences among tissues identified by multiple comparisons.

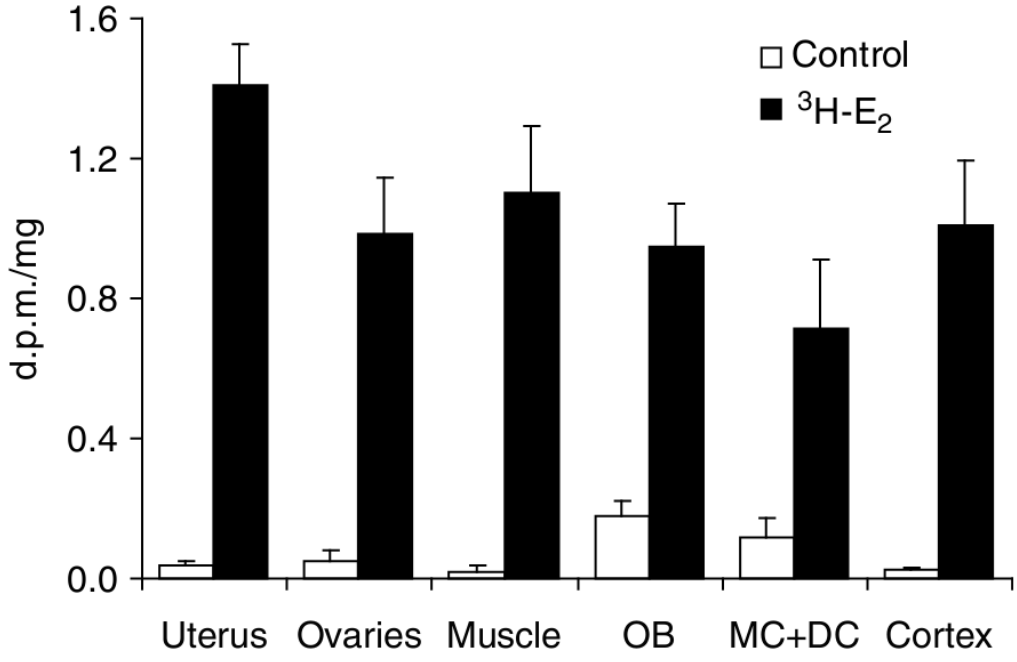
Figure 1 Mean (±S.E.M.) radioactivity in the uterus, ovaries, muscle, olfactory bulbs (OB), mesencephalon plus diencephalon (MC + DC), and cerebral cortex of inseminated females following exposure to sexually-experienced males for 3 days. Stimulus males were each given daily injections of either 10 µl ethanol (control) or 10 µCi tritium-labelled oestradiol (^{3}H-E_2), starting on the day insemination was detected in the experimental female (day 0 of gestation) and ending the day before tissues were obtained. Each inseminated female was paired with a stimulus male on day 1 of gestation, and sacrificed on day 3 of gestation. *n*=5 for each condition.



Exposure of juvenile females to ^{3}H-E_2- or ethanol-treated males

In the third experiment, we examined radioactivity in tissues of juvenile females after housing underneath males during postnatal days 22–24. The males were given either ^{3}H-E_2 or ethanol and were separated from the females by a wire mesh grid. Radioactivity was clearly evident in all tissues of all females exposed to ^{3}H-E_2-treated males (Fig. 2). Each tissue showed significance in separate tests: uterus, $t(8)=11.96$, $P<0.0001$; ovaries, $t(8)=5.79$, $P=0.0003$; muscle, $t(8)=5.66$, $P=0.0004$; olfactory bulbs, $t(8)=6.03$, $P=0.0003$; MC + DC, $t(8)=2.89$, $P=0.0098$; cortex, $t(8)=5.30$, $P=0.0005$. Within-subject analysis comparing tissues among the females only exposed to ^{3}H-E_2-treated males indicated significance, $F(5,20)=4.68$, $P=0.0056$; multiple comparisons ($P<0.05$) showed greater amounts of radioactivity in the uterus than in any other tissue.

Figure 2 Mean (±S.E.M.) radioactivity in the uterus, ovaries, muscle, olfactory bulbs, mesencephalon and diencephalon (MC + DC), and cerebral cortex from juvenile females each exposed to two males during postnatal days 22-24. Tissues were extracted on postnatal day 24. Stimulus males were each given daily injections of either 10 µl ethanol (control) or 10 µCi tritium-labelled oestradiol (^{3}H-E_2) starting on postnatal day 21 of the corresponding female. *n*=5 for each condition.



Tracing injected ^{3}H-E_2 within males

In the fourth experiment, we probed the dispersion of ^{3}H-E_2 in the reproductive tissues and fluids of injected males. Sexually experienced males were first administered ^{3}H-E_2 or ethanol and then killed after a 1 h absorption period. Radioactivity was clearly present in all substrates of ^{3}H-E_2-treated males in a range completely different from that of controls (Table 2). Tests comparing ^{3}H-E_2-treated and control males on each substrate showed significance for all measures: preputial fluid, $t(6)=3.16$, $P=0.0097$; bladder urine, $t(6)=2.34$, $P=0.0283$; testes, $t(8)=7.41$, $P=0.0001$; epididymides, $t(8)=5.71$, $P=0.0004$; seminal fluid, $t(8)=9.01$, $P<0.0001$.

Table 2 Mean ±S.E.M. (*n*) radioactivity in the testes, epididymides, coagulated seminal fluid, bladder urine, and preputial fluid of sexually experienced HS males 1 h after a single i.p. injection of 10 µl ethanol or 10 µCi ^{3}H-E_2.

Substrate	**Control**	**^{3}H-E_2**
Testes (d.p.m./mg)	0.067 ± 0.012 (5)	256.1 ± 34.5 (5)
Epididymides (d.p.m./mg)	0.100 ± 0.036 (5)	262.6 ± 46.0 (5)
Seminal fluid (d.p.m./mg)	0.304 ± 0.191 (5)	158.9 ± 17.6 (5)
Preputial fluid (d.p.m./µl)	0.350 ± 0.132 (4)	5.83 ± 1.73 (4)
Bladder urine (d.p.m./µl)	0.337 ± 0.120 (3)	3506 ± 1124 (5)

Enzyme immunoassay of natural male urinary steroids

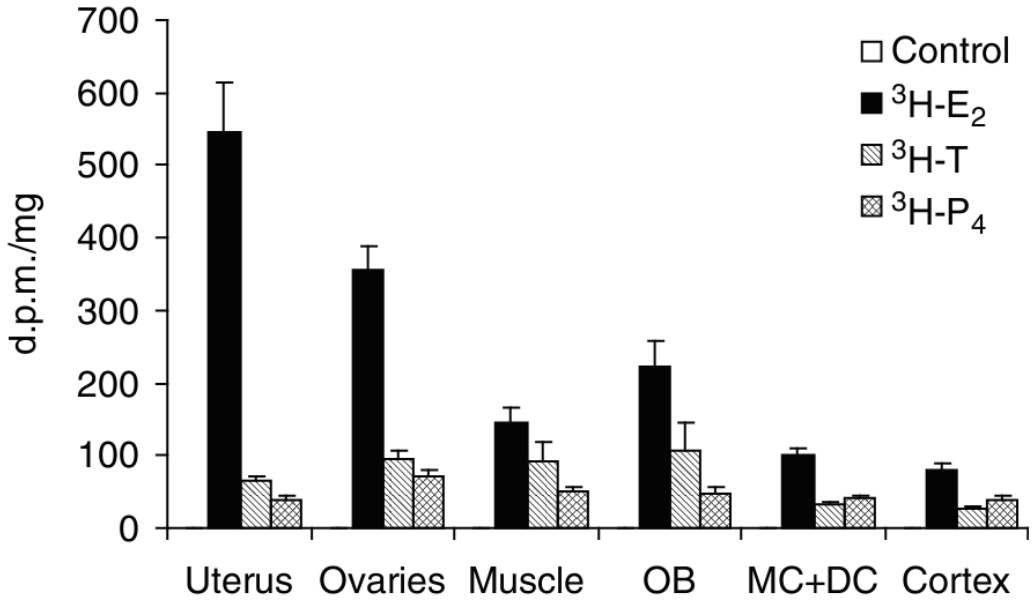
We also conducted enzyme immunoassays of natural E_2, testosterone and progesterone on urinary samples taken from seven outbred males that were housed beside previously inseminated females across a wire grid since day 1 of gestation. The urinary samples were collected non-invasively during the peri-implantation period on days 3, 4 and 5 of gestation, and values were collapsed across this 3 day period. Detectable levels of each steroid were found in all samples from all individuals. Mean (±S.E.M.) creatinine-adjusted values were: E_2, 17.19±5.97; testosterone, 80.34±22.25; and progesterone, 25.17±5.57 ng/mg.

Tracing intranasally administered ^{3}H-steroids within inseminated females

In a subsequent experiment, we examined the uptake of ^{3}H-E_2, ^{3}H-T, ^{3}H-P_4 and ethanol delivered by intranasal administration to inseminated females on day 2 of gestation. Following a 1 h absorption period, females were killed and their tissues were weighed and analysed for radioactivity. Liquid scintillation counting on all prepared tissues revealed radioactivity among ^{3}H-steroid-treated females in a completely different range from the background levels in control animals (Fig. 3). Females that received ^{3}H-E_2 clearly showed greater radioactivity in all comparable tissues than ethanol-treated controls, and also more than those treated with ^{3}H-T or ^{3}H-P_4. Each tissue showed significance in separate one-way analyses of variance: uterus, $F(3,22)=63.12$, $P<0.0001$; ovaries, $F(3,22)= 87.65$, $P<0.0001$; muscle, $F(3,22)=11.27$, $P=0.0002$; olfactory bulbs, $F(3,22)=11.74$, $P=0.0002$; MC + DC, $F(3,22)=55.35$, $P<0.0001$; cerebral cortex, $F(3,22)=45.74$,

$P<0.0001$. Multiple comparisons indicated that the $^{3}H\text{-}E_2$ condition differed from all other conditions for all tissues, and that controls also differed from all other conditions for the ovaries, muscle, MC + DC and cortex. Separate within-subject ANOVA was conducted comparing tissues for subjects given each hormone. Among females given $^{3}H\text{-}E_2$, tissues differed significantly in radioactivity, $F(5,25)=39.17$, $P<0.0001$; multiple comparisons indicated greater radioactivity in uterus and ovaries than in other tissues. Among females given ^{3}H-T, radioactivity again varied between tissues, $F(5,30)=3.22$, $P=0.0190$, but multiple comparisons showed no specific significant differences among tissues. Among females given $^{3}H\text{-}P_4$, tissues again differed in radioactivity, $F(5,30)=17.14$, $P<0.0001$, and multiple comparisons showed more radioactivity in ovaries than in other tissues.

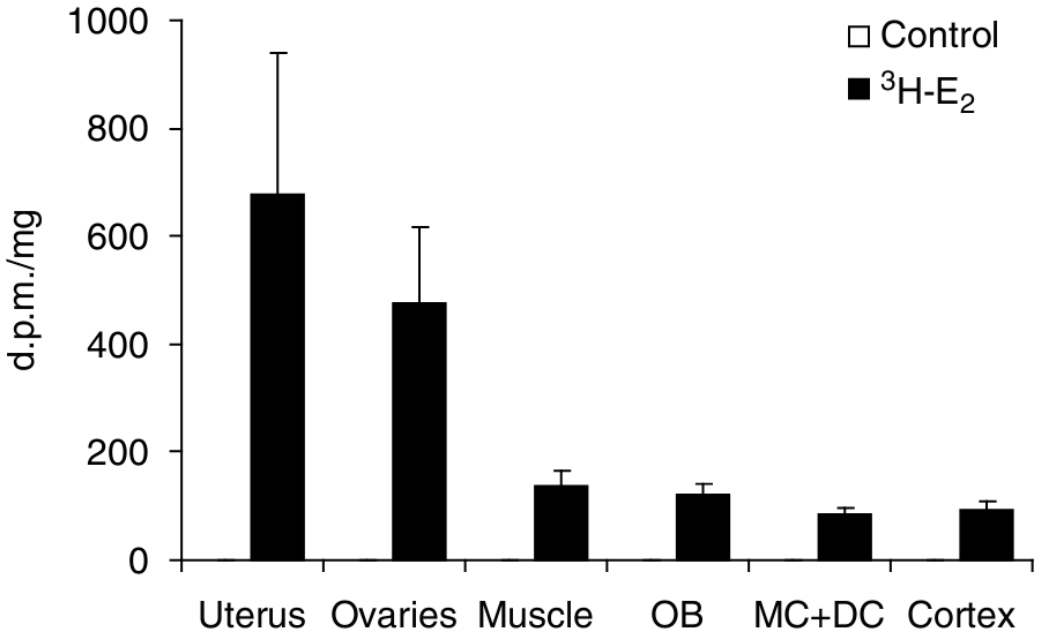
Figure 3 Mean (±S.E.M.) radioactivity in the uterus, ovaries, muscle, olfactory bulbs (OB), mesencephalon plus diencephalon (MC + DC), and cerebral cortex of inseminated females intranasally administered 10 µl ethanol (control), 10 µCi tritium-labelled oestradiol (^{3}H-E_2), 10 µCi tritium-labelled testosterone (^{3}H-T), or 10 µCi tritium-labelled progesterone (^{3}H-P_4), on day 2 of gestation. Tissues were extracted 1 h following treatment. *n*=6 for each of the control and ^{3}H-E_2 conditions; *n*=7 for each of the ^{3}H-T and ^{3}H-P_4 conditions.



Tracing intranasally administered ^{3}H-E_2 within juvenile females

On postnatal day 28, juvenile females were weaned and then intranasally administered either ^{3}H-E_2 or ethanol. Following a 1 h absorption period, females were killed and their tissues were weighed and analysed for radioactivity. Radioactivity was greater in all tissues of all females given ^{3}H-E_2 than in those of females given ethanol (Fig. 4). This was significant in tests for each tissue: uterus, $t(8)=2.08$, $P=0.0348$; ovaries, $t(8)= 2.76$, $P=0.0120$; muscle, $t(8)=3.78$, $P=0.0029$; olfactory bulbs, $t(8)=4.38$, $P=0.0014$; MC + DC, $t(8)=5.87$, $P=0.0003$; cortex, $t(8)=4.67$, $P=0.0010$. Within-subject ANOVA applied across tissue in only ^{3}H-E_2-treated animals showed significance, $F(5,25)=6.01$, $P=0.0011$; multiple comparisons indicated that the uterus differed from all other tissues except the ovaries.

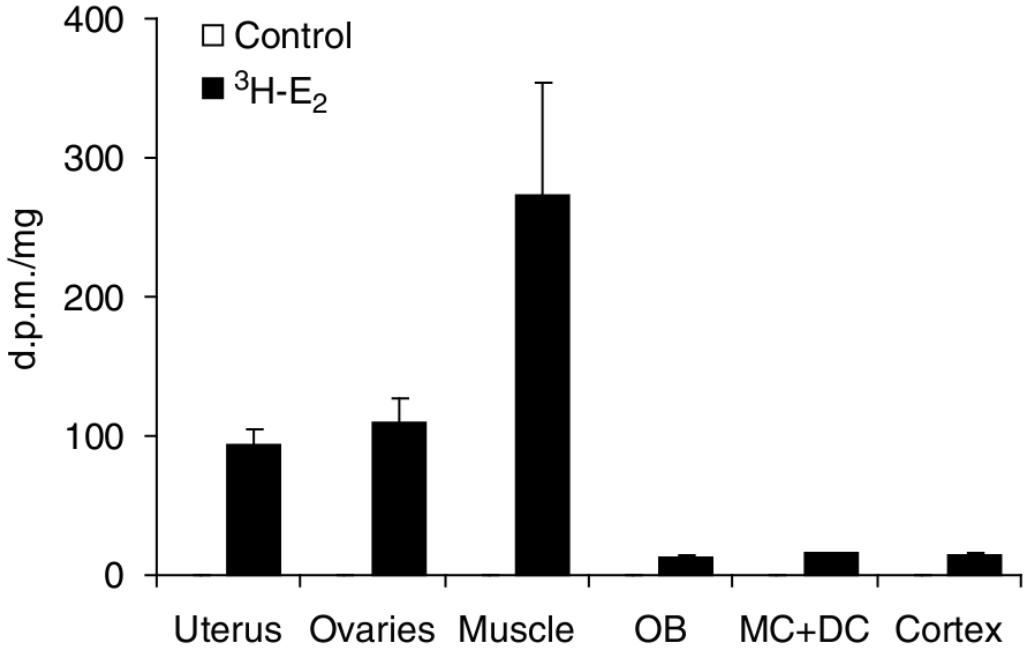
Figure 4 Mean (±S.E.M.) radioactivity detected in the uterus, ovaries, muscle, olfactory bulbs, mesencephalon and diencephalon (MC + DC), and cerebral cortex of juvenile females intranasally administered 10 µCi tritium-labelled oestradiol (^{3}H-E_2) or 10 µl ethanol (control). *n*=6 for the ^{3}H-E_2 condition; *n*=4 for the control condition.



Tracing cutaneously administered ^{3}H-E_2 within inseminated females

A further experiment examined percutaneous ^{3}H-E_2 absorption in females. Inseminated females on day 2 of gestation were each anaesthetised and received either ^{3}H-E_2 or ethanol, applied on the lower left quadrant of abdomen skin by micropipette. Following a 1 h absorption period, females were killed, perfused with saline, and then their tissues were collected, weighed and examined for radioactivity. Females exposed to ^{3}H-E_2 had more radioactivity in all tissues than the controls (Fig. 5). Each tissue showed significance in separate tests: uterus, $t(6)=8.07$, $P=0.0003$; ovaries, $t(6)=6.02$, $P=0.0007$; muscle, $t(6)=3.40$, $P=0.0073$; olfactory bulbs, $t(6)=7.48$, $P=0.0003$; MC + DC, $t(6)=11.05$, $P=0.0001$; cerebral cortex, $t(6)=10.23$, $P=0.0001$. Within-subject ANOVA applied across tissue in only ^{3}H-E_2-treated animals showed significance, $F(5,15)= 8.43$, $P=0.0008$; multiple comparisons indicated greater radioactivity in muscles than in other tissues.

Figure 5 Mean (±S.E.M.) radioactivity in the uterus, ovaries, muscle, olfactory bulbs (OB), mesencephalon plus diencephalon (MC + DC), and cerebral cortex of inseminated females cutaneously administered 10 µl ethanol (control) or 10 µCi ^{3}H-oestradiol (^{3}H-E_2), on day 2 of gestation. Tissues were extracted 1 h following treatment. *n*=4 for each condition.



Discussion

Conventionally, steroid hormones have been understood to operate within the individual, originating primarily from gonadal and adrenal sources and affecting the individual's own receptors. The current data suggest that certain steroids, at least E_2, may also have impacts across individuals, being excreted by one and absorbed by another. We found that males' systemic E_2 can be transmitted via his excretions and arrive in the uterus and ovaries of both inseminated and juvenile females that interact directly with these males.

Much evidence indicate that small lipophilic molecules can enter circulation after nasal or dermal exposure. Nasal absorption of small molecules into circulation is facilitated by characteristics of the nasal membrane including a large surface area, absorbent endothelium, and a highly vascularised mucosa (Türker *et al*. 2004). Although molecules under 300 Da may be absorbed into circulation after nasal exposure with little influence of their physicochemical properties, lipophilicity facilitates absorption of larger molecules (Arora *et al*. 2002). There is also evidence that lipophilic substances can pass from the nasal cavity directly into cerebrospinal fluid (Sakane *et al*. 1991). Percutaneous absorption of small molecules into circulation is well established (Wester & Maibach 1983), and is affected by several factors including molecular mass, solubility, and polarity (Schaefer *et al*. 1982). More specifically, percutaneous absorption has previously been observed for several steroids (Scheuplein *et al*. 1969, Hueber *et al*. 1994, Waddell & O'Leary 2002).

The present study converges with previous work (*e.g.* Scheuplein *et al*. 1969, Waddell & O'Leary 2002, Guzzo *et al*. 2010) showing that small, lipophilic sex steroids can enter circulation following nasal or dermal exposure. Moreover we show clear arrival at the female reproductive tract. E_2 readily enters circulation from external sources. Testosterone and progesterone can also be absorbed, although we found less radioactivity in various tissues after ^{3}H-T and ^{3}H-P_4 exposure than we did for ^{3}H-E_2, which could be due to other metabolic and binding factors in addition to differential absorption. Absorbed testosterone from male excretions may be aromatised and thus enhance actions of absorbed E_2. As progesterone is the precursor of many adrenocortical and gonadal steroids, measurement of radioactivity induced by ^{3}H-P_4 administration may reflect its metabolites as well as progesterone *per se* (Brittebo 1982). With respect to E_2, metabolites include other unconjugated oestrogens, which can also disrupt implantation (deCatanzaro *et al*. 2001) and promote uterine growth (Hisaw 1959). Conjugated oestrogens are not detectable in male mouse urine (Muir *et al*. 2001). In the rat, the bioavailability of intranasally administered E_2 is similar to that following intravenous administration, with the majority of E_2 remaining in the unconjugated form in both cases (Bawarshi-Nassar *et al*. 1989).

Once at the female reproductive tract, E_2 can induce the Bruce effect through known mechanisms. As little as 37 ng/day of E_2 given systemically on days 1-5 of gestation can terminate pregnancy in inseminated female mice (deCatanzaro *et al*. 1991, 2001). One study (Ma *et al*. 2003) has shown that the

implantation window is closed by a single E_2 dose as low as 10 ng *in uteri* previously primed by E_2 and progesterone. Supraoptimal E_2 levels can alter the rate of transport of pre-implantation embryos from the oviduct to the uterus (Ortiz *et al*. 1979), render the uterine epithelium entirely refractory to blastocyst implantation (Ma *et al*. 2003), and threaten survival of the blastocysts themselves (Roblero & Garavagno 1979, Safro *et al*. 1990, Valbuena *et al*. 2001). Similarly, once at the female reproductive tract, E_2 can promote uterine growth through known mechanisms. Injections of 100 ng E_2 to immature females for two successive days induced vaginal opening similar to that seen in immature females exposed to adult males (Bronson 1975). Endogenous E_2 is one of the principal factors in the development of the female mammalian reproductive tract, stimulating growth of the uterus by increasing DNA synthesis and cell proliferation (Mukku *et al*. 1982, Ogasawara *et al*. 1983, Quarmby & Korach 1984, Cooke *et al*. 1997).

Selective damage to the female's olfactory system, especially the vomeronasal organ (VNO) can prevent her vulnerability to the Bruce effect (Lloyd-Thomas & Keverne 1982). Previously it has been assumed that such damage does so by disturbing transduction of novel male odours to neural signals (see review by Brennan (2004)). Vomeronasal neural receptors respond distinctively to chemical factors associated with males of different genotypes, particularly short peptides related to the major histocompatibility complex (Leinders-Zufall *et al*. 2004, Kelliher *et al*. 2006). Although this may account for

aspects of the Bruce effect, especially differences among genetic strains in the capacity to induce it, the transduction of such neural events to survivorship of fertilised ova is not yet clearly established (*cf.* Brennan 2009, deCatanzaro 2011). There is another possible explanation of the effects of VNO ablation. This organ is a vascular pump that sucks in substances in response to stimuli catching the animal's attention (Meredith 1994), and its ablation would disrupt proximate vascular networks and thereby interfere with nasal absorption of E_2 into circulation. This latter possibility requires no mediation by neural events.

We suggest that transfer of excreted E_2 from novel males to females could provide a clear explanation of failure of blastocyst implantation in the Bruce effect and uterine growth in the Vandenbergh effect. This mechanism accounts for the established dependency of both of these effects upon the novel male's intact testes (Bruce 1965, Lombardi *et al*. 1976, Vella & deCatanzaro 2001). In contrast, the androgen-dependent preputial and vesicular-coagulating glands can be removed from males without influencing their abilities to induce these effects (deCatanzaro *et al*. 1996, Zacharias *et al*. 2000, Khan *et al*. 2009). It is notable that we observed relatively high concentrations of ^3H-E_2 in male urine and much lower concentrations in the fluids of the preputial and vesicular-coagulating glands.

These data clearly show direct transmission of ^3H-E_2 between males and females in the paradigms from this laboratory in which the Bruce and Vandenbergh effects have been demonstrated. This includes conditions involving

direct placement of the males with the females, where we previously have shown the Bruce effect (deCatanzaro *et al*. 1996), as well as exposure through a wire mesh grid with males above females, where we previously have demonstrated both the Vandenbergh effect (Beaton *et al*. 2006, Khan *et al*. 2009) and Bruce effect (*e.g.* deCatanzaro *et al*. 2006). This satisfies a necessary condition for the hypotheses that males' excreted oestrogens are absorbed by inseminated and juvenile females, arriving at the reproductive tract where actions on implantation and uterine growth can occur. These data likely have implications that go beyond the Bruce and Vandenbergh effects in mice. For example, male excreted androgens and E_2 in goldfish are established to impinge upon conspecific females (Sorensen *et al*. 2005). Also, there is evidence that unconjugated E_2, testosterone and progesterone are abundant in axillary perspiration of young men (Muir *et al*. 2008), potentially affecting females during intimacy.

Materials and Methods

Animals

Housing was in standard polypropylene cages measuring 28×16×11(height) cm with wire tops allowing access to food and water, except where otherwise stated. Sexually experienced heterogeneous strain (HS) mice, aged 3-8 months, were interbred from C57BL/6N, DBA/2, Swiss Webster, and CF1 strains were obtained from Charles River (Kingston, NY, USA). The colony was maintained at 21 °C with a reversed 14 h light:10 h darkness cycle. CF1

females aged 2-4 months were inseminated by pairing with same strain males and then checking for vaginal sperm plugs three times daily. The day of plug detection was designated as day 0 of gestation. CF1 juvenile females were weaned at either 21 or 28 days of age as indicated below. All research was approved by the Animal Research Ethics Board of McMaster University, conforming to standards of the Canadian Council on Animal Care.

Direct exposure of inseminated females to ^{3}H-E_2 injected males

Stimulus HS males were first gradually tolerated to ethanol for a period of 4-8 weeks by replacing their water with a water-ethanol solution (week 1, 2% ethanol; week 2, 3% ethanol; subsequent weeks, 4% ethanol). This treatment was necessary to prevent inactivity following injections. Inseminated females were each housed with a sexually experienced HS stimulus male on days 1-3 or 1-4 of gestation. On day 0 of a designated female's gestation, at 8 h following the onset of darkness, each stimulus male was prepared via i.p. injection of either 10 µl 95% ethanol or 10 µCi ^{3}H-E_2 ([2,4,6,7-^{3}H(N)]-E_2, in ethanol, 1.0 µCi/µl, 89.2 Ci/mmol, PerkinElmer, Waltham, MA, USA). This was achieved using a 50 µl syringe (705RN, Hamilton, Reno, NV, USA) with a 26 GA needle (19131-U Type 2-S, Hamilton). The injection site was swabbed with cotton doused in 95% ethanol to prevent external contamination. Each stimulus male was paired with its designated female at the onset of darkness on day 1 of gestation. The injection procedure was repeated daily for each stimulus male at 7 h following the onset of darkness. Stimulus males were temporarily housed in a separate cage for 1 h

following each additional injection to prevent direct contamination of the inseminated female or the shared bedding. On day 3 or 4 of pregnancy, the inseminated female was anaesthetised via sodium pentobarbital. Blood was drawn from the right atrium until 0.5 ml was collected, which was ejected into a pre-weighed 20 ml scintillation vial. The female was then perfused with 30 ml saline. The uterus and ovaries were removed from the abdomen, and a sample of muscle tissue was taken from one of the rear thighs. The ovaries and oviduct were isolated from the uterus. Fat and mesentery were removed, and each tissue was placed into separate pre-weighed 20 ml scintillation vials for sample processing. Vials were re-weighed to determine tissue wet mass.

A replication was conducted examining radioactivity in females on day 3 of gestation after exposure to males treated with either ^{3}H-E_2 or ethanol as described above. Instead of collecting blood, we examined three areas of the brain, while still including samples of the uterus, ovaries, and muscle. Following perfusion, each subject was decapitated and its brain removed. The olfactory bulbs were isolated. We then extracted the MC + DC, which is the region enclosed by the posterior communicating arteries extending from the basal to the dorsal aspects of the brain. The cerebral cortex was then extracted as a whole. Uterus, ovary, and muscle samples were taken as described above, and all tissues were separately placed in pre-weighed, pre-labelled vials and weighed to determine tissue wet mass.

Exposure of juvenile females to ^{3}H-E_2 injected males through a wire grid

On postnatal day 21, females were weaned and isolated to the lower level of a two-tier exposure apparatus (see deCatanzaro *et al*. 1996), measuring 30×21×13.5 (height) cm. On the day a female was weaned, two males that were previously tolerated to ethanol were assigned to that female, and both males were each subsequently injected i.p. with 10 µL 95% ethanol or 10 µCi ^{3}H-E_2 as described above. Following injection, both males were placed in the upper level of the two-tier exposure apparatus, each in one of two compartments measuring 14.5×21×13.5 (height) cm and separated from each other by an opaque plastic barrier in order to prevent contact between the males. Compartments had stainless steel wire grid floors (squares of 0.5 cm^2) that allowed excretions to pass to the lower level of the apparatus while limiting direct physical contact between the males and the female. The lower and upper levels of the apparatus were kept apart overnight so that the animals could acclimate to the new environment. On the morning of female postnatal day 22, the upper part of the apparatus was placed over the lower level, thereby exposing the female to the excretions of the stimulus males. Males were re-injected as described above on female postnatal days 22 and 23, for a total of three injections. For these subsequent injections, males were each isolated from the apparatus for a 1 h period. On female postnatal day 24, the female was anaesthetised and perfused and the uterus, ovaries, a sample of muscle tissue, the MC + DC, the olfactory bulbs, and cerebral cortex were extracted, processed and analysed for radioactivity as described above.

Tracing of injected ^{3}H-E_2 within males

HS male mice were injected i.p. with 10 µl 95% ethanol or 10 µCi ^{3}H-E_2 ([2,4,6,7-$^{3}H(N)$]-E_2, in ethanol, 1.0 µCi/µl, 89.2 Ci/mmol, PerkinElmer), and then set aside for 1 h. Each male was then killed with sodium pentobarbital. The preputial glands were exposed via a lateral incision through the skin of the lower abdomen, then removed and stored in a cryotube. After an incision through the abdominal muscles, bladder urine was collected with a syringe and 27 GA needle. The seminal vesicles were removed, and seminal fluid was collected into a weigh tray. Finally, the epididymides and testes were removed, isolated, and trimmed of fat. The preputial glands were sliced and spun at 6,000 ***g*** for 10 min to isolate the fluid. Seminal fluid was allowed to coagulate for 1 h, then was weighed and processed. Testes and epididymides were separately placed in pre-weighed vials, weighed to determine tissue wet mass and then processed.

Enzyme immunoassay of natural male urinary steroids

HS males were each paired with an inseminated CF1 female using a Plexiglas apparatus with two adjacent chambers each measuring 15×21×13 cm (height). The chambers were separated with wire-mesh grid with squares measuring 0.5 cm^2, and the floor consisted of wire-mesh raised ~1 cm above a Teflon stainless-steel surface covered with wax paper, which permitted non-invasive collection of urine. The female was introduced to the chamber on day 1 of gestation at the commencement of the dark phase of the cycle. The male was then immediately introduced into the adjacent chamber. Following the

commencement of the dark phase of the cycle of days 3, 4, and 5 of the female's gestation, urine was collected from each male, placed into a labelled vial and then frozen at -20 °C.

Validations and full procedures for enzyme immunoassays for mouse urine were previously reported (Muir *et al*. 2001, deCatanzaro *et al*. 2003). Steroids and creatinine standards were obtained from Sigma. Steroid antibodies and corresponding horseradish peroxidase conjugates were obtained from the Department of Population Health and Reproduction at the University of California (Davis). Cross-reactivities for anti-E_2 are: E_2 100%, oestrone 3.3%, progesterone 0.8%, testosterone 1.0%, androstenedione 1.0% and all other measured steroids <0.1%. Cross-reactivities for anti-testosterone are: testosterone 100%, 5α-dihydrotestosterone 57.4%, androstenedione 0.27%, androsterone, and DHEA, cholesterol, E_2, progesterone, and pregnenolone <0.05%. Cross-reactivities for anti-progesterone are: progesterone 100.0%, 11α-hydroxyprogesterone 45.2%, 5α-pregnen-3,20-dione 18.6%, 17α-hydroxyprogesterone 0.38%, 20α-hydroxyprogesterone 0.13%, 20α-hydroxyprogesterone 0.13%, pregnanediol <0.001%, pregnenolone 0.12%, E_2 <0.001% and oestrone <0.04%.

Nunc Maxisorb plates were first coated with 50 µl of antibody stock diluted at 1:10 000 in a coating buffer (50 mmol bicarbonate buffer/l, pH 9.6) and stored for 12-14 h at 4 °C. Wash solution (0.15 mol NaCl/l containing 0.5 ml of Tween 20/l) was added five times to each well using an ELx50 automated strip washer (Bio-Tek, Winooski, VT, USA) to rinse away unbound antibody, and then

50 µl phosphate buffer/well was added. The plates were incubated at room temperature (21 °C) for 2 h for E_2 determination, 30 min for testosterone, and 1 h for progesterone determination before adding standards, samples or controls. Urine samples were diluted 1:8 in phosphate buffer before they were added to the plate. Standard curves were derived by serial dilution from a known stock solution. For each hormone, two quality control urine samples at 30 and 70% binding (the low and high ends of the sensitive range of the standard curve) were prepared. For all assays, 50 µl E_2, testosterone, or progesterone horseradish peroxidase was added to each well, with 20 µl of standard, sample, or control for E_2 or 50 µl of standard, sample, or control for progesterone or testosterone. The plates were incubated for 2 h at room temperature. Subsequently, the plates were washed and 100 µl of a substrate solution of citrate buffer, H_2O_2 and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) were added to each well and the plates were covered and incubated while shaking at room temperature for ~30-60 min. The plates were read with a single filter at 405 nm on an ELx 808 microplate reader (Bio-Tek). Blank absorbance was subtracted from each reading to account for non-specific binding. In all assays, absorbance was obtained, standard curves were generated, a regression line was fit to the most sensitive range of the standard curve (typically 40-60% binding) and samples were interpolated into the equation to obtain a value in pg per well.

Urinary creatinine measures were also taken, with the average of duplicates taken for each sample. All urine samples were diluted 1:40.77

(urine:phosphate buffer). Using Dynatech Immulon flat bottom plates, 50 µl/well of standard was added with 50 µl distilled water, 50 µl 0.75 M NaOH and 50 µl 0.4 M picric acid. The plate was then shaken and incubated at room temperature for 30 min. The plate was measured for optical density with a single filter at 490 nm. Standard curves were generated, regression lines were fit and the regression equation was applied to the optical density for each sample. Steroid measures were adjusted for creatinine by dividing the obtained value by the measure of creatinine per millilitre of urine for the particular sample.

Intranasal or cutaneous administration of ^{3}H-steroids to inseminated or juvenile females

Inseminated females on day 2 of gestation were briefly anaesthetised with isoflurane delivered for 2 min in a sealed chamber. Each was delivered 10 µl 95% ethanol; 10 µCi ^{3}H-E_2 (E_2-[2,4,6,7-^{3}H(N)], in ethanol, 1.0 µCi/µl, 89.2 Ci/mmol, PerkinElmer); 10 µCi ^{3}H-T (testosterone-[1,2,6,7-^{3}H(N)], in ethanol, 1.0 µCi/µl, 70 Ci/mmol, PerkinElmer); or 10 µCi ^{3}H-P_4 (progesterone-[1,2,6,7-^{3}H(N)], in ethanol, 1.0 µCi/µl, 101.3 Ci/mmol, PerkinElmer) via micropipette with the tip placed in one nostril. Intranasal administration of ^{3}H-E_2 was also examined in juvenile females weaned at 28 days of age. Following the above procedure, females were [anaesthetised] with isoflurane and then intranasally administered either 10 µl 95% ethanol or 10 µCi ^{3}H-E_2. Cutaneous application was examined in inseminated females that were given a direct application of 10 µl

95% ethanol or 10 µCi ^{3}H-E_2 on the surface of the lower left quadrant of the abdomen, also by micropipette.

In all cases, following a 1 h absorption period, females were [anaesthetised] with sodium pentobarbital and then perfused through the heart via the left ventricle with 30 ml saline for inseminated females and 20 ml saline for juvenile females. Uterus, ovaries, muscle samples, olfactory bulbs, MC + DC and cortex were extracted as described above, and all tissues were separately placed in pre-weighed, pre-labelled vials and weighed to determine tissue wet mass.

Sample processing for scintillation counting

Female and male tissue samples, as well as coagulated male seminal fluid, were homogenised by adding 1-1.5 ml SOLVABLE (PerkinElmer) to each vial immediately after wet masses were determined. Each vial was placed in a 60 °C water bath for 2-4 h. Following the first 1 h, vials containing blood were set aside and allowed to cool for 10 min. After cooling, 0.4 ml 30% H_2O_2 was added in 0.1 ml aliquots (one aliquot every 5 min) to the blood and SOLVABLE solution to reduce colour quenching. Once the final aliquot of H_2O_2 was added, vials were placed back in the water bath for 1 h. Following cooling, 10 ml Ultima Gold scintillation cocktail (PerkinElmer) was added to each vial, with 15 ml Ultima Gold added to solubilised blood to further reduce quenching. Male urine and preputial gland fluid samples were processed by directly adding 10 µl of sample to 10 ml Ultima Gold scintillation cocktail in 20 ml scintillation vials. Vials were left undisturbed in a TriCarb 2910 TR Liquid Scintillation Analyzer

(PerkinElmer) for at least 12 h to eliminate background noise in the form of heat and luminescence. Radioactivity was measured for 5-10 min per sample, and counting efficiency was determined using QuantaSmart software (PerkinElmer).

Statistical analyses

Data are presented as means ± S.E.M. For each measure within an experiment, *t*-tests for independent samples were applied where two conditions were compared. For comparisons involving animals exposed to ^{3}H-E_2, one-tailed tests were applied because of a clear prediction about direction of the effect. One-way ANOVA was applied where there were more than two conditions. In order to compare tissues within a particular treatment group, within-subjects ANOVA was used. The threshold for statistical significance (α level) was set at $P<0.05$. Following significant ANOVA, pairwise multiple comparisons were conducted using the Newman-Keuls method with significance set at $P<0.01$ unless otherwise indicated.

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.

Funding

This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada awarded to D deCatanzaro (RGPIN/1199-2010, EQPEQ/3904072010).

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Received 7 October 2011

First decision 6 December 2011

Accepted 9 January 2012

Chapter 4

Transfer of [^{3}H]estradiol-17β and [^{3}H]progesterone from males or females to the brain and reproductive tissues of cohabiting female mice

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Journal of Endocrinology

Authors' Contributions

Adam C. Guzzo: Experimental design, data collection, data analysis, literature review, manuscript preparation, writing, and editing.

Tyler Pollock: Data collection, assistance with experimental design.

Denys deCatanzaro: Assistance with experimental design, data analysis, ELISA measures, manuscript preparation, writing, and editing.

Abstract

Estradiol-17β and progesterone play critical roles in female reproductive physiology and behaviour. Given the sensitivity of females to exogenous sources of these steroids, we examined the presence of estradiol and progesterone in conspecifics' excretions and the transfer of excreted steroids between conspecifics. We paired individual adult female mice with a stimulus male or female conspecific given daily injections of [^{3}H]estradiol or [^{3}H]progesterone. Following 48 hours of direct interaction with the stimulus animal, we measured radioactivity in the uterus, ovaries, muscle, olfactory bulbs, mesencephalon and diencephalon, and cerebral cortex of the untreated female cohabitant. Radioactivity was significantly present in all tissues of female subjects after individual exposure to a stimulus male or female given [^{3}H]estradiol. In females exposed to males given [^{3}H]progesterone, radioactivity was significantly present in the uterus, ovaries, and muscle, but not in other tissues. In females exposed to stimulus females given [^{3}H]progesterone, radioactivity was significantly present in all tissues except the mesencephalon and diencephalon. In mice directly administered [^{3}H]steroids, greater radioactivity was found in the urine of females than of males. Among females directly administered [^{3}H]steroids, greater radioactivity was found in urine of those given [^{3}H]progesterone than of those given [^{3}H]estradiol. When females were administered unlabelled estradiol before exposure to [^{3}H]estradiol-treated females, less radioactivity was detected in most tissues than was detected in the tissues of untreated females exposed to

[^{3}H]estradiol-treated females. We suggest that steroid transfer among individuals has implications for the understanding of various forms of pheromonal activity.

Key words: Estradiol; Progesterone; [^{3}H]steroids; Pheromones; Steroid transfer

Introduction

Conspecifics' excretions can impact mammalian females' reproductive state in many ways. Male excretions can accelerate female sexual development (Vandenbergh 1967, Harder & Jackson 2003), terminate pregnancies sired by other males (Bruce 1960, Rohrbach 1982, Marashi & Rülicke 2012, Thorpe & deCatanzaro 2012), and alter estrous cycling (Whitten 1956, Dodge *et al.* 2002, Rivas-Muñoz *et al.* 2007). In laboratory mice, males develop polyuria and polydipsia in the presence of females and progressively target their urine toward these females, while females in this context have nasal and dermal contact with male urine droplets (deCatanzaro *et al.* 2006, 2009). Female excretions can also have effects on the reproductive physiology of other females, prolonging or even completely disrupting estrus (Lee & Boot 1955, 1956, Ma *et al.* 1998). Female mice generally excrete less urine than do males, but their urinary output is greater while they are in estrus than in di-estrus, which coincides with a greater ability during estrus to affect the development and physiology of cohabiting females (Drickamer 1982, 1986, 1995). Although evidence indicates that some pheromonal effects could be mediated by females' responses to conspecifics' urinary proteins (Ma *et al.* 1999, Marchlewska-Koj *et al.* 2000, Leinders-Zufall *et al.* 2004), growing evidence also implicates steroids, especially estrogens, in conspecifics' excretions (Beaton *et al.* 2006, deCatanzaro *et al.* 2006, Khan *et al.* 2009, Thorpe & deCatanzaro 2012). Indeed, recent data suggest that small

lipophilic steroids such as estradiol-17β (E2) could transfer directly between cohabiting individuals (Guzzo *et al.* 2010, 2012).

The steroids that regulate many aspects of female reproductive state, E2 and progesterone (P4), are abundant in unconjugated form in mouse urine from both sexes (deCatanzaro *et al.* 2004, 2006, Khan *et al.* 2009, Thorpe & deCatanzaro 2012, Guzzo *et al.* 2012). Urinary E2 in male mice was previously detected at an average of 5-8 ng per mg creatinine, but estrone conjugates, if present, were below the threshold for detection (Muir *et al.* 2001). In females, urinary E2 was detected at an average of 9-13 ng per mg creatinine, and urinary estrone conjugates could be detected, but at 1-2% the level of E2 (Muir *et al.* 2001). Both E2 and P4 are lipophilic and have low molecular mass (272.4 and 314.5 Da respectively) and high chemical stability, allowing for cutaneous (Scheuplein *et al.* 1969, Hueber *et al.* 1994) and nasal (Bawarshi-Nassar *et al.* 1989) entry into circulation. When E2 or P4 is administered nasally to rats, the majority of the administered quantity is bioavailable as unconjugated E2 in circulation (Bawarshi-Nassar *et al.* 1989). Following cutaneous application of [^{3}H]E2 and [^{14}C]P4, as much as 44% of the applied quantity of each appears in urine within 72 hours (Goldzieher & Baker 1960).

Accordingly, we reasoned that small lipophilic steroids excreted by individuals could enter into and journey through the circulatory system of cohabiting conspecifics, where they could then bind to any available receptors throughout the body. We tested this for both E2 and P4 using tritium-labelled

versions ([^{3}H]E2 and [^{3}H]P4) injected into either male or female mice, and then measured the level of radioactivity in various tissues of untreated female mice after a few days of cohabitation. Previously (Guzzo *et al.* 2010), we showed that males injected i.p. with [^{3}H]E2 excrete it in their urine, and that females nasally administered [^{3}H]E2 had radioactivity in brain and reproductive tissues. Radioactivity was particularly evident in the uterus and significantly reduced by pre-administration of unlabelled E2 to females. We subsequently (Guzzo *et al.* 2012) demonstrated that [^{3}H]E2 injected into male mice is detectable in the brain and reproductive tissues of inseminated female and juvenile female cohabitants. Here we examined reproductive, brain, and other tissues of freely cycling females. We showed both male-to-female and female-to-female transfer of [^{3}H]P4 and [^{3}H]E2, and showed greater absorption of [^{3}H]E2 than [^{3}H]P4 when equivalent quantities were given in terms of radioactivity. We also demonstrated differential excretion over time of administered [^{3}H]E2 and [^{3}H]P4 between the sexes. Finally, we showed that directly administering unlabelled E2 to female subjects significantly reduced radioactivity in their tissues when they were exposed to [^{3}H]E2-treated stimulus females.

Materials and Methods

Animals

Mice (*Mus musculus*) were housed in standard polypropylene cages measuring 28×16×11(height) cm with wire tops allowing access to food and

water, except where otherwise stated. Sexually-experienced males aged 3-8 months and sexually-inexperienced females aged 2-6 months were of CF1 strain and were either bred from stock or obtained from Charles River (Kingston, NY, USA). Age was counterbalanced across conditions. The 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 standards of the Canadian Council on Animal Care.

Measurement of natural urinary E2 and P4

Individual males and females (10 per sex) were each placed in a Plexiglas apparatus measuring 15×21×13 (height) cm with a wire-mesh grid floor with squares measuring 0.5 cm^2. The floor consisted of wire-mesh raised approximately 1 cm above a Teflon stainless-steel surface covered with wax paper, which permitted non-invasive collection of urine. The animal was introduced to the chamber at the onset of darkness and left undisturbed for 2 days to acclimate to the apparatus. Following the onset of darkness on the subsequent 6 days, urine was collected from each animal, placed into a labelled vial, and then frozen at -20 °C.

Validations and full procedures for enzyme immunoassays for mouse urine were previously reported (Muir *et al.* 2001, deCatanzaro *et al.* 2003, 2004). Steroids and creatinine standards were obtained from Sigma (St Louis, MO, USA). Steroid antibodies and corresponding horseradish peroxidase conjugates were obtained from the Department of Population Health and Reproduction at the

University of California, Davis. Cross reactivities for anti-estradiol are: estradiol 100%, estrone 3.3%, progesterone 0.8%, testosterone 1.0%, androstenedione 1.0% and all other measured steroids <0.1%. Cross-reactivities for anti-progesterone are: progesterone 100.0%, 11α-hydroxyprogesterone 45.2%, 5α-pregnen-3,20-dione 18.6%, 17α-hydroxyprogesterone 0.38%, 20α-hydroxyprogesterone 0.13%, 20α-hydroxyprogesterone 0.13%, pregnanediol <0.001%, pregnenolone 0.12%, estradiol <0.001% and estrone <0.04%. Following convention, urinary steroid levels were considered with and without adjustment for urinary creatinine (deCatanzaro *et al.*, 2009).

Direct exposure of cycling females to [^{3}H]steroid-injected males

Stimulus males were gradually tolerated to ethanol as described previously (Guzzo *et al.* 2012). Briefly, the ethanol concentration in the water given to these males was increased by 1% v/v per week, from 1% to 4%, and then maintained at 4% until the time of the experiment. At 7 h following the onset of darkness on the first day of the experiment (day 1), each stimulus male was given an i.p. injection of 10 µl 95% ethanol (control), 10 µCi [2,4,6,7-[^{3}H](N)]-estradiol (in ethanol, 1.0 µCi/µl, 89.2 Ci/mM, PerkinElmer, Waltham, MA, USA), or 10 µCi [1,2,6,7-[^{3}H](N)]-progesterone (in ethanol, 1.0 µCi/µl, 101.3 Ci/mM, PerkinElmer). Injections were performed using a 50 µl syringe (705RN, Hamilton, Reno, NV, USA) with a 26 GA needle (19131-U Type 2-S, Hamilton). For animals given [^{3}H]E2, this is equivalent to 30.5 ng per dose, for a total of 91.5 ng per animal. For animals given [^{3}H]P4, this is equivalent to 31 ng per dose or 93 ng per animal.

The injection site was swabbed with cotton doused in 95% ethanol to prevent external contamination.

At the onset of darkness on day 2, randomly selected females with regular estrous cycles were each paired in a clean cage with a stimulus male. There were 5 male-exposed females prepared in each treatment. Stimulus males were each given additional i.p. injections identical to their first at 7 h following the onset of darkness on days 2 and 3 of the experiment, and were temporarily housed in a separate cage for 1 h following each additional injection in order to prevent direct contamination of the paired female or the shared bedding. On day 4 of the experiment, at 4 h following the onset of darkness, each female was heavily anaesthetized with sodium pentobarbital and then perfused with 30 ml saline. Whole uterus, ovaries, mesencephalon and diencephalon (MC+DC), olfactory bulbs, cerebral cortex, and a sample of muscle tissue, were extracted as previously described (Guzzo *et al*. 2012) and placed in pre-weighed scintillation vials. The vials were re-weighed to determine tissue wet mass, and then processed for measurement of radioactivity as described below.

Following previously published procedures (Guzzo *et al.* 2012), female tissue samples were homogenised by adding 1.0 (all tissues except cerebral cortex) or 1.5 (cerebral cortex) ml SOLVABLE (PerkinElmer) to each vial after re-weighing. Vials were then placed in a 60 °C water bath, swirled after 1 h, and then left in the bath for an additional 1-3 h until no visible pieces of tissue remained. After removal from the bath and at least 10 min of cooling, 10 ml

Ultima Gold scintillation cocktail (PerkinElmer) was added to each vial. Vials were stored in darkness in a TriCarb 2910 TR Liquid Scintillation Analyser (PerkinElmer) for at least 12 h to eliminate background noise in the form of heat and luminescence. Following this interval, radioactivity was measured for 5 min per sample, and counting efficiencies and final adjusted estimates for the amount of radioactivity per sample (in d.p.m.) were automatically calculated by the accompanying QuantaSmart software package (PerkinElmer).

Direct exposure of cycling females to [^{3}H]steroid-injected females

The procedures for this experiment replicated the procedures for the previous experiment, except that the stimulus animals were non-sibling females instead of males. There were 5 female subjects prepared in each of the control and [^{3}H]E2 treatments, and 4 subjects prepared in the [^{3}H]P4 treatment. All other details, including ethanol tolerance, timing of the injections, solutions used for the injections, timing of separation, timing of the surgeries, tissues analysed in the female paired with the stimulus animal, tissue processing, and scintillation counting were identical to those of the previous experiment.

Measurement of urinary radioactivity in [^{3}H]steroid-injected males and females

This experiment was conducted in order to determine the rates and time profiles of urinary excretion of [^{3}H]E2 and [^{3}H]P4 in injected males and females. At the onset of the darkness phase of the light:darkness cycle, 7 males and 4 females aged 4-5 months were each given an i.p. injection of 10 µCi [2,4,6,7-[^{3}H](N)]-estradiol (in ethanol, 1.0 µCi/µl, 72.1 Ci/mM, PerkinElmer), and 10

males and 4 females received 10 µCi [1,2,6,7-[^{3}H](N)]-progesterone (in ethanol, 1.0 µCi/µl, 101.3 Ci/mM, PerkinElmer). These doses provide a total of 37.7 ng [^{3}H]E2 and 31 ng [^{3}H]P4. Larger numbers of males were prepared than females as male urination patterns differ from those of females, rendering sample collection much more difficult (deCatanzaro *et al*. 2006, 2009). Immediately following injection, animals were individually placed in a Plexiglas apparatus measuring 15×21×13 (height) cm with a wire-mesh grid floor raised approximately 1 cm above a surface covered with wax paper, which permitted non-invasive collection of urine. Urine was first collected one hour after injection at about the commencement of the darkness phase of the animals' reversed lighting cycle, and subsequently every hour for a total of 10 h. Animals were left undisturbed through the light phase of the light:darkness cycle and a final urine sample was taken 24 h following injection. Urine was aspirated using 1 ml syringes with 26-guage needles and placed into a 1.5 ml microtube. 10 µl of each urine sample was subsequently transferred to vials containing 5 ml Ultima Gold scintillation cocktail (PerkinElmer). Vials were shaken for 10 min and then placed in the scintillation counter. Radioactivity was measured for 5 min per sample, and counting efficiencies and final adjusted estimates for the amount of radioactivity per sample (in d.p.m.) were calculated as described above.

[^{3}H]E2 transfer to females administered E2

We then tested whether or not administering unlabelled E2 to female subjects would alter the uptake of radioactivity in tissues resulting from exposure

to a [^{3}H]E2-treated conspecific. At the onset of the darkness phase of the light:darkness cycle, 10 stimulus females were each given an i.p. injection of 50 µCi [2,4,6,7-[^{3}H](N)]-estradiol (stock solution in ethanol, 1.0 µCi/µl, 72.1 Ci/mM, PerkinElmer) diluted in 0.15 ml saline to yield a 1:3 ethanol:saline solution. This dose provides a total of 188.6 ng [^{3}H]E2. Five other stimulus females for the control treatment were simply given 0.2 ml ethanol:saline solution. Within 15 min, 10 female subjects were given 0.05 ml peanut oil s.c., while 5 others were injected s.c. with 0.05 ml oil containing 5 µg E2 (Sigma). After 1 h, female subjects were each paired with a stimulus female in a clean cage, such that there were 5 oil-treated subjects matched with 5 ethanol-treated stimulus females in the control treatment, 5 oil-treated female subjects paired with 5 [^{3}H]E2-treated stimulus females, and 5 E2-treated female subjects paired with 5 [^{3}H]E2-treated stimulus females. After another 24 h, female subjects were anaesthetised as described above and perfused with 20 ml heparinised phosphate-buffered saline (1000 units heparin/ml). Uterus, ovaries, olfactory bulbs, and cerebral cortex were collected as in previous experiments. Additional tissues were also taken, including lateral slices of the cerebellum, a section of the hypothalamic region (bilateral sections posterior to the optic chiasm on the ventral surface of the brain above the pituitary stalk), a sample of the heart, a sample of one lung, a sample of the liver, and a sample of abdominal adipose. Tissues were processed and measured for radioactivity on the scintillation counter as described above.

Statistical analyses

Data are presented as means ± S.E.M. The threshold for statistical significance (α level) was set at $P < 0.05$, unless otherwise stated. Where possible, data were analysed by analysis of variance, and significant effects were followed by Newman-Keuls multiple comparisons on all pairs of treatment combinations. In experiments where female subjects were exposed to stimulus conspecifics given [^{3}H]steroids or the ethanol vehicle (controls), there were highly unequal variances among treatments by Bartlett's test due to very low variance in the control treatments. Therefore such data were analysed using a conservative nonparametric statistic, the Wilcoxon Rank-Sum test. Bonferroni adjustments were applied to the probabilities in order to correct for the fact that there were multiple tissues measured in each experiment. We focused statistical comparisons on differences between treatments within each tissue, omitting comparisons across tissues due to potentially differential impacts of perfusion upon these tissues.

Results

Natural urinary E2 and P4

E2 and P4 were present in every urine sample from both males and females, consistently over 6 successive days of measurement. An average value over days was taken for each subject, and the means (±S.E.M) of those values for each subject are presented (Table 1). The ranges among individual samples for

females were 0.8-66.2 ng E2/ml and 6.1-138.4 ng P4/ml, whereas those for males were 0.6-5.6 ng E2/ml and 4.0-66.7 ng P4/ml.

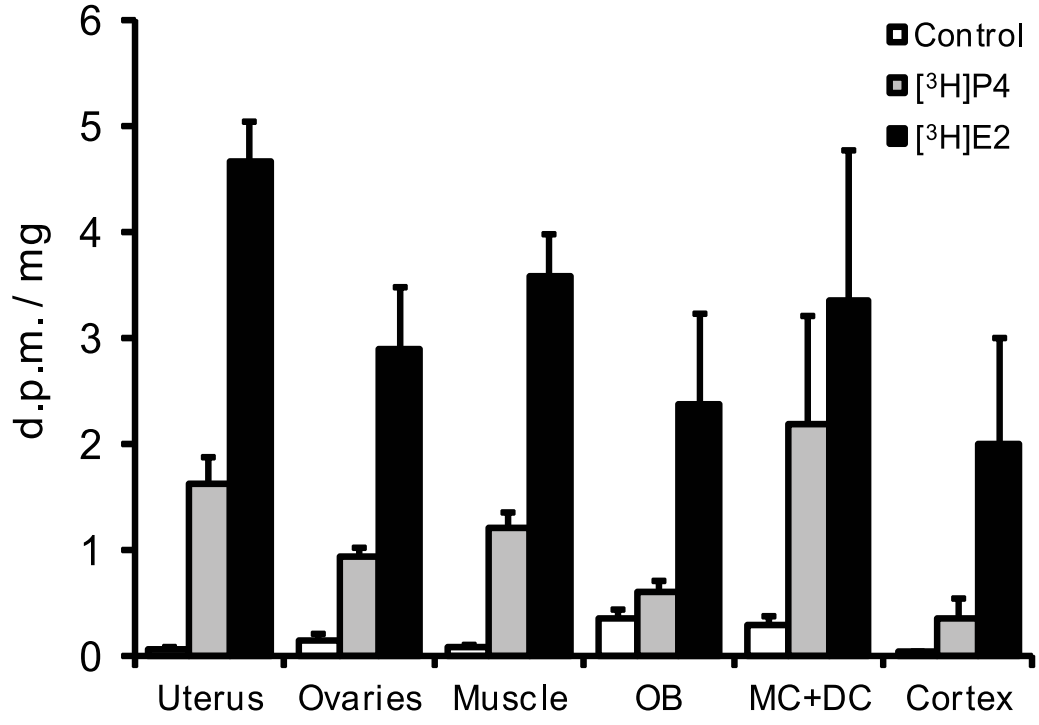
Table 1 Urinary progesterone (P4), estradiol (E2), creatinine-adjusted P4, creatinine-adjusted E2, and creatinine levels of female and male adult CF1 mice. Each subject was measured for 6 successive days and assigned the average value for all available samples over those days, and the mean ± S.E.M. of those values across subjects is presented.

	Females	**Males**
ng P4 / ml urine	28.54±5.78	13.54±2.75
ng P4 / mg creatinine	29.33±6.19	17.14±2.93
ng E2 / ml urine	13.23±1.74	4.14±0.50
ng E2 / mg creatinine	13.57±1.76	6.37±0.99
mg creatinine / ml urine	0.97±0.07	0.78±0.06

Direct exposure of cycling females to [^{3}H]steroid injected males

Following approximately 48 h of free interaction with stimulus males given ethanol (control), [^{3}H]E2, or [^{3}H]P4, female subjects were killed and their tissues were processed for liquid scintillation counting to assess radioactivity. Planned orthogonal comparisons were made for each tissue between controls and subjects exposed to [^{3}H]steroid-treated males. Considering that 6 tissues were measured, the Bonferroni-corrected threshold value for significance is $P < 0.0083$. Radioactivity (d.p.m./mg) among females paired with males injected with [^{3}H]E2 was in a completely non-overlapping range from that of control females (Figure 1); this comparison was significant for all tissues, $W_s = 15$, $P < 0.001$. Females paired with males injected with [^{3}H]P4 also had significantly more radioactivity (in a non-overlapping range) compared to controls for uterus, ovaries, and muscle, $W_s = 15$, $P < 0.001$. Radioactivity in other tissues showed some overlap in range between the [^{3}H]P4 and control treatments and did not reach significance. In comparing only the animals exposed to [^{3}H]steroid treated males through analysis of variance, there was a main effect of condition, $F(1,8)=10.62$, $P=0.0115$, indicating more radioactivity in females exposed to [^{3}H]E2-injected males than in those exposed to [^{3}H]P4-injected males.

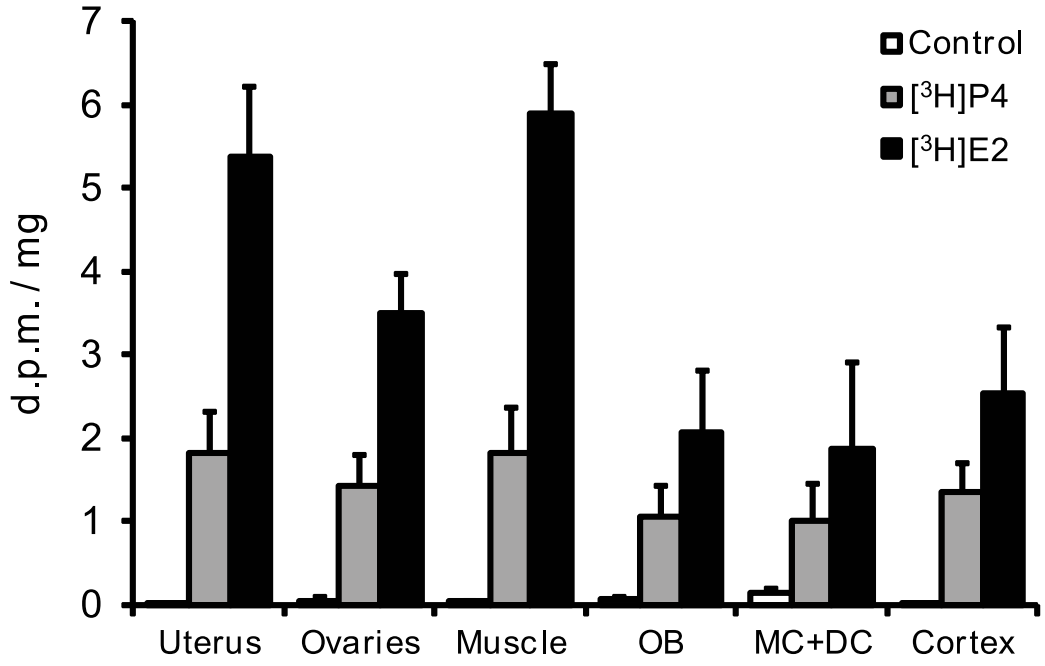
Figure 1 Mean (+S.E.M.) radioactivity in the uterus, ovaries, muscle, olfactory bulbs (OB), mesencephalon and diencephalon (MC+DC), and cerebral cortex of females following exposure to sexually-experienced stimulus males for 2 days. Stimulus males were injected with either 10 µl ethanol (control), 10 µCi tritium-labelled progesterone ([^{3}H]P4), or 10 µCi tritium-labelled estradiol ([^{3}H]E2) once a day for 3 days. Females were each paired with a stimulus male at the onset of darkness following the male's first injection.



Direct exposure of cycling females to [^{3}H]steroid injected females

Following 48 h of pairing and free interaction with stimulus females injected with ethanol (control), [^{3}H]E2, or [^{3}H]P4, radioactivity was measured in tissues of female subjects. Planned orthogonal comparisons were made for each tissue between controls and subjects exposed to [^{3}H]steroid-treated males. Considering that 6 tissues were measured, the Bonferroni-corrected threshold value for significance is $P < 0.0083$. Radioactivity among females paired with stimulus females injected with [^{3}H]E2 was in a completely non-overlapping range from that of control females (Figure 2); this comparison was significant for all tissues, $W_s = 15$, $P < 0.001$. Females paired with stimulus females injected with [^{3}H]P4 also had significantly more radioactivity (in a non-overlapping range) for uterus, ovaries, muscle, olfactory bulb, and cortex, $W'_s = 10$, $P < 0.005$; however the comparison for the MC+DC was not significant due to overlap in range between the treatments. In comparing only the animals exposed to [^{3}H]steroid treated females, there was a main effect of condition, $F(1,7)=13.89$, $P=0.0074$, indicating more radioactivity in females exposed to [^{3}H]E2-injected females than in those exposed to [^{3}H]P4-injected females.

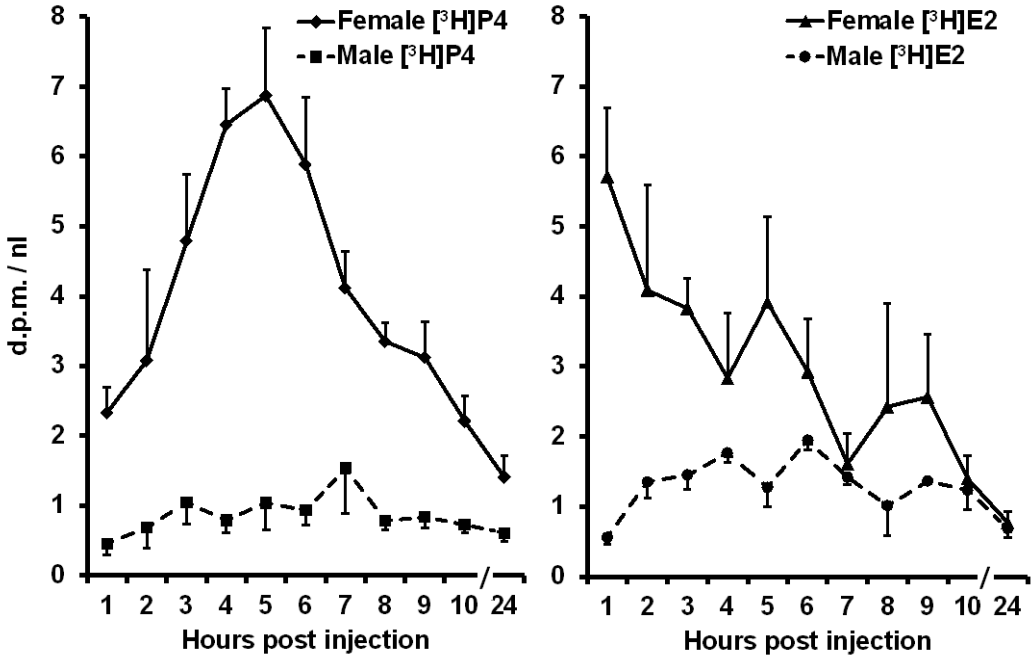
Figure 2 Mean (+S.E.M.) radioactivity in the uterus, ovaries, muscle, olfactory bulbs (OB), mesencephalon and diencephalon (MC+DC), and cerebral cortex of females following exposure to stimulus females for 2 days. Stimulus females were injected with either 10 µl ethanol (control), 10 µCi tritium-labelled progesterone ([^{3}H]P4), or 10 µCi tritium-labelled estradiol ([^{3}H]E2) once a day for 3 days. The females represented by the data were each paired with a stimulus female at the onset of darkness following the stimulus female's first injection.



Patterns of urinary excretion after [^{3}H]-steroid injection

When adult males or females were given a single injection of [^{3}H]E2 or [^{3}H]P4, radioactivity was measured in all samples taken within the next 24 h. Mean values are given for all obtained samples at each collection time (Figure 3). As expected, urine collections were more readily obtained for females than for males, such that there were substantially more missing values for males. Radioactivity was generally more abundant in female samples than in male samples. In female samples, radioactivity was more evident in earlier samples than later ones for those given [^{3}H]E2, whereas there was a clear peak in radioactivity 4-6 h after administration in females given [^{3}H]P4. For males, excreted radioactivity appeared less variable across measurement times. Because of missing values, statistics were conducted only on a single average sample value for each mouse, collapsing all available samples. Factorial (2×2) analysis of variance indicated significant effects of sex, $F(1,21)=111.27$, $P<0.0001$; of [^{3}H]E2 *vs.* [^{3}H]P4, $F(1,21)=4.22$, $P=0.0500$; and of the interaction, $F(1,21)=4.26$, $P=0.0490$. Multiple comparisons indicated that urinary radioactivity for females given [^{3}H]P4 exceeded that from all other treatments, and that urinary radioactivity for females given [^{3}H]E2 exceeded that for both treatments of males.

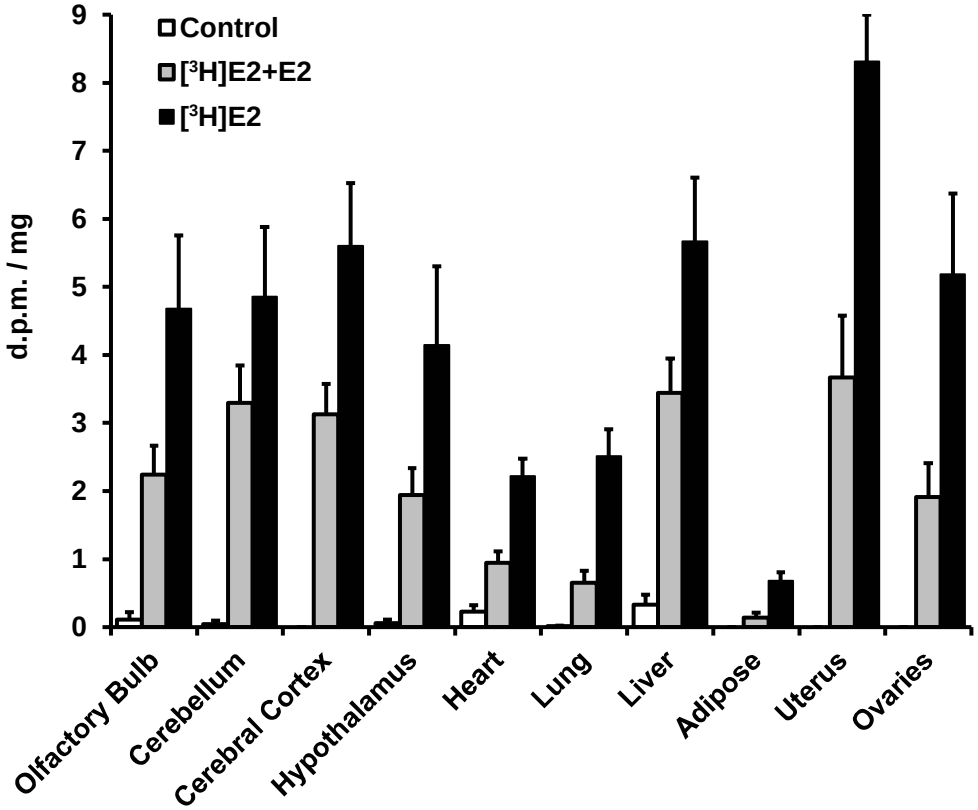
Figure 3 Mean radioactivity in urine of females and males given a single injection of either 10 µCi tritium-labelled progesterone ([^{3}H]P4) or 10 µCi tritium-labelled estradiol ([^{3}H]E2), measured in samples taken hourly for 10 hours during the animals' active period (dark phase of the light cycle) and also 24 h after the injection. S.E.M. is given above the mean for females and below the mean for males.



[^{3}H]E2 transfer to females administered E2

Following exposure to stimulus females given [^{3}H]E2, females pre-treated with unlabelled E2 had lower uptake of radioactivity from steroid transfer than did females pre-treated with oil vehicle (Figure 4). Ranges of the data for each tissue were completely non-overlapping between the control subjects and subjects exposed to [^{3}H]E2-treated stimulus females and given oil. There was some overlap in range between controls and subjects given E2 and exposed to [^{3}H]E2-treated stimulus females for the olfactory bulbs, heart, liver, and adipose, but not for the other tissues. Statistical analysis focused on planned comparisons between the two groups exposed to [^{3}H]E2-treated stimulus females. In comparison to a Bonferroni-corrected threshold of $P < 0.005$ for 10 tissues, Wilcoxon tests on this contrast in each tissue showed non-overlapping ranges and significant effects in the uterus, lungs, and adipose ($W_s = 15$, $P < 0.001$), and small overlap with nevertheless significant effects in the heart and ovaries ($W_s = 16$, $P < 0.005$). Other tissues did not show significance by the corrected threshold.

Figure 4 Mean (+S.E.M.) radioactivity in tissues of females exposed to ethanol-injected stimulus females (control) or[^{3}H]E2-treated stimulus females. Females exposed to [^{3}H]E2-treated stimulus females also directly received either an E2 or an oil injection before exposure to the stimulus females.



Discussion

These data show that [^{3}H]E2 and [^{3}H]P4 excreted by a mouse of either sex can be readily absorbed by an unmanipulated adult female cohabitant, with radioactivity observed in her reproductive, brain, and other tissues. Although identical amounts of radioactivity were injected in the [^{3}H]E2 and [^{3}H]P4 treatments, radioactivity was more evident in females exposed to [^{3}H]E2-treated conspecifics than among those exposed to [^{3}H]P4-treated conspecifics. Our results corroborate those of previous studies with regards to inter-individual steroid transfer in this species (Guzzo *et al.* 2010, 2012). That previous work was conducted in the context of the Bruce effect (disruption of blastocyst implantation by non-sire males) and the Vandenbergh effect (promotion of reproductive maturation of juvenile females by males). Those studies involved a distinct strain of outbred males (HS) that show substantially higher levels of urinary E2 than those observed here for CF1 males (deCatanzaro *et al.* 2006, 2009, Thorpe & deCatanzaro, 2012). The previous E2 measures from HS males were proven to be especially elevated after a few days of exposure to inseminated or juvenile females, which also causes males to show polydipsia, polyuria and direction of urine toward the stimulus females (deCatanzaro *et al.* 2006, 2009). Here we measured natural urinary E2 and P4 in isolated CF1 males and females. We also demonstrated for the first time that E2 and P4 transfer can occur from male to female and from female to female simply through two days of cohabitation.

In samples of urine taken from animals injected with [^{3}H]P4 or [^{3}H]E2, radioactivity was greater in urine of females than that of males. Nevertheless, comparable levels of radioactivity were evident in the tissues of non-injected females exposed to [^{3}H]steroid-injected males and females. This is consistent with the fact that males actively deliver their urine to females in their presence (deCatanzaro *et al*. 2009). Male mice tend to urinate more than do females (Drickamer 1995). Males also tend to disperse their urine in small droplets whereas females urinate in puddles (Desjardins *et al*., 1973; Maruniak *et al*., 1974, deCatanzaro *et al*. 2006, 2009), which makes male urine much more difficult to collect. Polyuria and polydipsia can make male urine more dilute (deCatanzaro *et al*. 2009), which could help to account for differences between males and females seen in Figure 3. The data presented here also indicate that females exposed to [^{3}H]E2-treated males or females show much greater radioactivity in their tissues than do those exposed to [^{3}H]P4-treated males or females. This is clearly not due to differences in excretory rates in injected animals, as radioactivity in urine is equal after injection of the two [^{3}H]steroids in males and somewhat greater for females given [^{3}H]P4 than for those given [^{3}H]E2. The superior levels of radioactivity in tissues of recipient females exposed to conspecifics given [^{3}H]E2 are accordingly due to parameters of absorption and metabolism in these recipients, as was also previously observed when [^{3}H]P4 and [^{3}H]E2 were directly given to females intra-nasally (Guzzo *et al*. 2012).

The quantities of [^{3}H]E2 and [^{3}H]P4 that were injected into the stimulus animals were miniscule relative to the endogenous levels of these steroids. In the experiments showing transfer to untreated females, the total quantity injected into each stimulus animal was 91.5 ng [^{3}H]E2 or 93 ng [^{3}H]P4 per dose. In contrast, a dose of 60 µg E2 every two days was necessary to restore normal urinary levels of E2 in castrated males (Thorpe & deCatanzaro 2012); that is almost one thousand times the dose of [^{3}H]E2 given in these experiments. E2 and P4 from endogenous sources each pass into urine in both sexes, where it is detectable by specific antibodies in enzyme immunoassay (see Table 1, also Muir *et al*. 2001, deCatanzaro *et al*. 2004, 2009). E2 from exogenous sources is similarly detectable in urine (Thorpe & deCatanzaro 2012), and the current data show this for both E2 and P4 by radioactive tracing. Small, lipophilic sex steroids can readily enter circulation following nasal or dermal exposure (Scheuplein *et al*. 1969, Waddell & O'Leary 2002, Guzzo *et al*. 2012), and we show here that females exposed to conspecific-excreted [^{3}H]E2 or [^{3}H]P4 have radioactivity dispersed throughout the females' body. Following nasal entry into the system in rats, the bioavailability of E2 has been shown to be at least 50% of an equivalent i.v. dose, with the remainder possibly being oxidized to the less active estrogen estrone (Bawarshi-Nassar *et al.* 1989). For P4, the bioavailability from nasal absorption has been shown to be equal to that from i.v. administration (Bawarshi-Nassar *et al.* 1989). Another indication that nasally-administered E2 remains biologically active is the fact that it can disrupt blastocyst implantation in

inseminated females, as does subcutaneous administration of E2 (deCatanzaro *et al*. 2001, 2006). The best indication that steroids excreted in urine remain biologically active is that restoration of E2 in castrated males via repeated injections restores their ability to disrupt implantation and accelerate puberty (Thorpe & deCatanzaro 2012).

We also demonstrated here that pre-administration of E2 to females exposed to [^{3}H]E2-treated conspecifics significantly lessens the uptake of radioactivity in at least some tissues of these females. This suggests that the injected E2 displaced [^{3}H]E2 at receptors and other binding factors (Sasson & Notides 1983, Terenius 1969). While some of the [^{3}H]E2 administered to the stimulus animals could have been metabolized, this provides further evidence that [^{3}H]E2 remains bioactive despite potential metabolism in both the stimulus animal and the recipient. We have not made direct comparisons among tissues given the likelihood that perfusion techniques differentially remove blood from these tissues. It is not technically feasible to remove all blood from tissues of mice, even when heparinised saline is used in perfusion as was the case in this last experiment. While many tissues were blanched after perfusion, muscle tissue for example remained relatively red, which, in addition to the involvement of E2 in glucose homeostasis in skeletal muscle and adipose (Barros *et al*. 2009), could account for the high radioactivity readings observed in this tissue. Estrogen receptors are also involved in blood vessel nitric oxide regulation (Rubanyi *et al*.

1997), and so E2 bound in capillary walls may factor in to the total levels of radioactivity detected in various tissues.

Estrogen receptors are diversely located throughout the body. Studies using reverse transcription PCR for estrogen receptors α and β showed heavy expression of both receptors in ovary and uterus, detectable expression of estrogen receptor α in liver and heart, and expression of estrogen receptor β in bladder, lung, cerebellum, and other areas of the brain (Kuiper *et al*. 1997). The expression of estrogen receptors imperfectly corresponds with the general distribution of [^{3}H]E2 following its i.v. administration (Eisenfeld & Axelrod, 1966). Upon nasal or cutaneous administration of [^{3}H]E2 to non-cycling females, radioactivity can be detected in every tissue examined, with generally greater levels in the uterus and ovaries (Guzzo *et al*. 2010, 2012). A steroid's route of entry into the body greatly affects its subsequent distribution (Anand Kumar *et al*. 1974, Guzzo *et al*. 2012), which could explain the relatively flat distribution among tissues that we detected here in our transfer experiments.

These data could have implications for the common laboratory practice of group housing of animals. Moreover, the data potentially have implications for a broad range of phenomena where males and other females influence a female's reproductive state. E2 and P4 play critical roles in control of the estrous cycle (Butcher *et al.* 1974, Hsueh *et al.* 1976, Ryan & Schwartz 1980). Exogenous E2 followed by exogenous P4 is sufficient to induce behavioural estrus in ovariectomized rats, and supraphysiological levels of P4 can inhibit female sexual

receptivity (Blaustein & Wade 1977). Across species, the estrous cycle is known to be sensitive to housing with conspecifics and exposure to their urine. Mice of some strains can exhibit pseudopregnancy when housed in groups of four per cage (Lee & Boot 1955, 1956), and exposure to males can induce regular estrous cycling in females (Whitten, 1956). Similarly, exposure to males will induce estrous behaviour in grouped anestrous female goats (Rivas-Muñoz *et al.* 2007), and female Siberian hamsters exposed to the excretions of intact males enter pro-estrus three days following the onset of exposure (Dodge *et al*. 2002). In mice, urine from female mice that are in estrus and under long photoperiods can accelerate the onset of first estrus in other females (Drickamer 1982, 1986). Steroid transfer could also be relevant to some human endocrine phenomena. Axillary glands have some enzymatic capacity to modify steroids and even synthesize them *de novo* (Rothardt & Beier 2001, Zouboulis *et al.* 2007) and very high levels of unconjugated E2 and P4 are found in axillary perspiration of young men (Muir *et al*., 2008). Women repeatedly exposed to the axillary extracts of men show more stable menstrual cycle lengths, with fewer aberrant cycles (Cutler *et al.* 1986). Axillary extracts from one woman may influence the recipient woman's menstrual cycle (Preti *et al.* 1986, Preti 1987, *cf.* Stern & McClintock 1998). Given the importance of steroids such as E2 and P4 in regulating the female reproductive cycle, we suggest that the possibility of inter-individual steroid transfer should not be overlooked in the design and analysis of endocrine research.

Acknowledgements

This research was supported by grants from the Natural Sciences and Engineering Research Council of Canada awarded to D deCatanzaro (RGPIN/1199-2010, EQPEQ/3904072010). We greatly appreciate the assistance of Yubin Ha, Joelle Thorpe, Morgan Afonso, Jihwan Jheon, and Brandon Tang.

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Chapter 5

General Discussion

Purpose

The research presented in this thesis shows the definite transfer of steroids between conspecifics. The tissues and measures used were selected specifically to determine whether steroids from one individual could arrive at and bind to sites of notable steroid activity in another individual. Although steroid absorption by skin or nasal mucosa is an established phenomenon (Bawarshi-Nassar *et al*. 1989, Hueber *et al*. 1994, Schaefer *et al*. 1982, Türker *et al*. 2004), the passage of urinary steroids from one animal to another should not be presumed and has never previously been demonstrated. Urinary steroids have several potential barriers that they must overcome in order to retain the properties necessary for absorption and binding. First, these steroids have to remain unconjugated by the liver, as conjugation changes a steroid's bioactivity by preventing its subsequent receptor binding. Second, these steroids must be able to exit the urine solution and enter into an exposed animal's body. Third, once transferred and absorbed, the steroids have to bind to target sites, instead of being immediately conjugated by the recipient's liver or absorbed into his or her adipose tissue. This research was the first to demonstrate inter-individual steroid transfer, and it suggests that such transfer could have effects on the reproductive endocrinology of a recipient mouse.

Summary of Results

In Chapter 2, I established the passage of administered radiolabelled E_2 into urine and into the body. Since urine is a known vector for chemosignals that affect female reproductive endocrinology (Dominic 1965, Marsden & Bronson 1964, Vandenbergh 1969), these experiments were critical for determining urinary E_2's potential as an endocrine modulator. The urine of outbred males intraperitoneally injected with 50 µCi ^{3}H-E_2 contained approximately 1-14 DPM of radioactivity per nl urine, which translates to approximately 2-32 fg of ^{3}H-E_2 per nl urine. While this calculation depends on many factors, it still provides a way to relate quantity of radioactivity to quantity of steroid. Tissue samples taken from females intranasally administered ^{3}H-E_2 contained 50-400 DPM of radioactivity per mg of wet tissue under a variety of conditions. Once saline perfusion was incorporated into the methodology, removing much of a subject's blood, uterine samples were consistently more radioactive than other tissue samples. Females pre-treated with E_2 likely showed no difference in radioactivity between uterine and other tissue samples due to the competitive binding of the unlabelled and labelled E_2 in the uterus (Sasson & Notides 1983, Terenius 1969). Significant radioactivity was detected in the tissues of inseminated females intranasally administered 25 µl urine that had been collected from outbred males injected with 50 µCi ^{3}H-E_2, demonstrating that male urinary E_2 has the potential to arrive at and bind to key sites involved in reproductive physiology.

In Chapter 3, I showed the definite transfer of ^{3}H-E_2 from males to inseminated females, and from males to juvenile females. Furthermore, I traced the distribution of intraperitoneally injected ^{3}H-E_2 in males, and also demonstrated the absorption of nasally and cutaneously administered steroids in females. Inseminated females paired with ^{3}H-E_2-injected males between days 1 and 3 following the detection of a sperm plug had 1-3 DPM/mg tissue in samples of uterus, ovaries, muscle, olfactory bulbs, MC + DC, and cerebral cortex. A similar, but slightly lower (0.5-1.5 DPM/mg tissue), trend was found for juvenile females that were housed beneath ^{3}H-E_2-injected males between postnatal days 22-24. Tracing of ^{3}H-E_2 in outbred males one hour following an intraperitoneal injection of 10 µCi ^{3}H-E_2 showed an average of 256 DPM/mg in testes, 263 DPM/mg in epididymides, 159 DPM/mg in seminal fluid, 6 DPM/µl in preputial gland fluid extract, and 3.5 DPM/nl in bladder urine. In terms of radioactivity per unit volume, bladder urine therefore contained much more ^{3}H-E_2 than any other sample tested. Tissue samples from inseminated females intranasally administered 10 µCi ^{3}H-E_2, ^{3}H-T, or ^{3}H-P_4 had varying levels of radioactivity despite the equivalent quantities administered. Samples from females given ^{3}H-E_2 had averages of 81-545 DPM/mg, samples from females given ^{3}H-T had averages of 27-106 DPM/mg, and samples from females given ^{3}H-P_4 had averages of 40-72 DPM/mg. Among conditions and tissues, samples of uterus and ovaries from females given ^{3}H-E_2 were the most radioactive. Tissues from juvenile females intranasally administered 10 µCi ^{3}H-E_2 averaged 85-678 DPM/mg, with the

greatest amount of radioactivity in the uterus. Finally, samples from inseminated females cutaneously administered 10 µCi ^{3}H-E_2 averaged 12-273 DPM/mg, but with muscle coming out on top. Combined, these results provide a complete circuit for E_2, from being excreted by a male to entering and binding in a female via the nose or skin. Given the conditions of the females in these experiments (recently inseminated or juvenile), the transfer of E_2 from male to female was suggested to play a significant role in the Bruce and Vandenbergh effects.

In Chapter 4, I showed the transfer of ^{3}H-E_2 and ^{3}H-P_4 to nulliparous adult females, either from a male or a female source. Tissues from females exposed to ^{3}H-P_4-injected males averaged 0.4-2.2 DPM/mg, and tissues from females paired with ^{3}H-E_2-injected males averaged 2.0-4.7 DPM/mg. Samples taken from females paired with ^{3}H-P_4-injected females averaged 1.0-1.8 DPM/mg, and finally, samples from females paired with ^{3}H-E_2-injected females averaged 1.9-5.9 DPM/mg. As with previous experiments, these results demonstrate that urine can certainly be a vector for steroids, and that urinary steroids can bind generally throughout the body of an exposed animal. Furthermore, ^{3}H-E_2 was again detected in greater quantities in tissues than was ^{3}H-P_4, which was likely a result of the differences between estrogen and progestin receptor densities in the tissues examined, although other factors could have been involved. In terms of steroid excretion, females excrete 2-4 times the amount of labelled or unlabelled steroid as do males in a given volume of urine. Finally, females injected with unlabelled E_2 and then exposed to ^{3}H-E_2-injected females had lower amounts of radioactivity

in most tissues than did females injected with a vehicle, indicating that the difference in detected radioactivity was due to intact, receptor-bound ^{3}H-E_2. The experiments in Chapter 4 help support the idea that steroid transfer may occur more generally, and such transfer could have implications for other reproductive phenomena.

Steroid Transfer and Reproduction

I suspect that steroid transfer could play a role in numerous reproductive effects. These effects include pregnancy disruption by unfamiliar males (the Bruce effect), puberty acceleration in females by males (the Vandenbergh effect), synchronization of estrus by males (the Whitten effect), and inhibition of estrus by females (the Lee-Boot effect). In Chapter 2, I showed that significant urinary excretion of administered ^{3}H-E_2 occurs in males. In Chapters 3 and 4, I showed the transfer of ^{3}H-E_2 between mice in the context of these effects. Social communication in mice is partially mediated by urine (Maruniak *et al*. 1974, Reynolds 1971), and environmental novelty, such as the introduction of unfamiliar conspecifics, increases the frequency of urination in males (deCatanzaro *et al*. 2009, Maruniak *et al*. 1974). Males actively direct their excretions towards unfamiliar females (deCatanzaro *et al*. 2009), providing a method of steroid passage from one animal to another.

Small variations in E_2 levels are known to interfere with implantation, thereby blocking pregnancy (Paria *et al*. 1993, Roblero & Garavagno 1979, Safro

et al. 1990). In some mammals, the vomeronasal organ (VNO) responds to novel stimuli, resulting in the insufflation and subsequent absorption of chemicals (Meredith 1994). The nasal regions of females exposed to unfamiliar males can be yellowed from the amount of contact these females have with the urine droplets of the males (deCatanzaro *et al*. 2006), which could lead to the absorption of urinary chemicals through nasal cutis. Absorption of male-originating ^{3}H-E_2 in inseminated females is evidence that the absorption of natural male urinary E_2 could at least be partially responsible for the Bruce effect. The consistent detection of radioactivity in the uteri and other tissues of inseminated females that were exposed to ^{3}H-E_2-injected males shows that male-originating E_2 can interfere directly with the tightly-controlled processes involved in the intrauterine implantation of fertilized blastocysts.

Similarly, excess E_2 can result in an earlier onset of puberty in juvenile females (Bronson 1975b). In the Vandenbergh effect, reproductive function occurs earlier in life for juvenile females exposed to adult, sexually-experienced males (Khan *et al*. 2008b, Vandenbergh 1967). In order for puberty acceleration to occur, the excretions of a male have to upregulate estrogen receptor-mediated transcription in an exposed female's body (Alonso & Rosenfield 2002, Quarmby & Korach 1984) and brain (Pfaff 1980). Previous reports suggest that male major urinary proteins (MUPs) may bind within the vomeronasal organ in order to affect the release of luteinizing hormone in the brain of a recipient juvenile female, which in turn is theorized to cause the release of E_2 from the ovaries (Bronson &

Desjardins 1974, Mucignat-Caretta *et al*. 1995). While activation of the hypothalamic-pituitary-gonadal axis through MUPs is possible, systemic absorption of male-excreted E_2 and direct activation of estrogen receptors is at least as likely to cause puberty acceleration. In Chapter 3, I showed that male urinary E_2 definitely arrives in the brain and reproductive tissues of juvenile females exposed to ^{3}H-E_2-injected males. Although this is only evidence of transfer and uptake, note that castrated males cannot induce the Vandenbergh effect, whereas castrated males given injections of E_2 can (Thorpe & deCatanzaro 2012).

In the Lee-Boot and Whitten effects, urinary E_2 and P_4 from conspecifics could control the estrous cycle of exposed females, delaying estrus or resulting in its synchrony. For the Lee-Boot effect, a female exposed to numerous other females tends to show delayed estrus and may even enter a non-reproductive state (Lee & Boot 1955), whereas in the Whitten effect, grouped females exposed to the urine of males tend to show regularly-timed and synchronized estrus (Whitten 1956). As with most aspects of reproduction, estrus is typically controlled by endogenous steroid levels (Edwards *et al*. 1968, Mahesh & Brann 1998). In Chapter 4, I showed that both males and females excrete substantial amounts of steroids in their urine, and that these steroids are transferred generally to adult nulliparous female cohabitants. Unlike the Vandenbergh and Bruce effects, there is no present direct evidence that levels of steroids are involved in the Lee-Boot and Whitten effects. However, given that steroids transfer freely among animals,

the impact of exogenous steroids on the estrous cycle should be examined more thoroughly in future studies. For the time being, it is important to note that transferred E_2 can induce an increase in progestin and estrogen receptors, while simultaneously binding to the estrogen receptors thereby created (Butcher *et al*. 1974, Hsueh *et al*. 1976). In the brain, transferred E_2 and P_4 could create the conditions necessary for the onset of sexual behaviour (Blaustein & Wade 1977). In terms of the suppression of estrus, excess E_2 resulting from exposure to large groups of females could cause a constant elevation in prolactin, resulting in pseudopregnancy across the group (Chen & Meites 1970, Ryan & Schwartz 1977).

Each of these effects can drastically affect how and when individuals reproduce. Effectively, steroid transfer may be the driving force behind the ability of male mice to accelerate puberty in juvenile females, to control estrus in adult females, and to compete with other males by blocking pregnancy in inseminated females. The degree to which steroids could be involved in the mechanisms of pheromone action for other species is presently unknown. Many actions of pheromones in animals are mediated by volatile chemicals produced by various glands, where these pheromones can then be released in urine, faeces, or other bodily excretions (Rekwot *et al*. 2001). However, where contact with a pheromonal substance is necessary to elicit an effect, and where the pheromonal effect is known to involve the reproductive endocrine system, it is possible that the direct transfer of excreted steroids could modify the recipient animal's steroid

levels. In some mammals, such as pigs, ponies, and sheep, the majority of steroids excreted in urine is conjugated and therefore rendered biologically inactive, whereas steroids excreted in faeces remain unconjugated (Palme *et al*. 1996). Some evidence even suggests that ingested conjugated steroids may be deconjugated in the guts of some mammals (Palme *et al*. 1996). At least in humans, but potentially in other mammals as well, unconjugated steroids can be excreted in perspiration (Muir *et al*. 2008), providing a route for steroid transfer upon cutaneous contact between two individuals. By directly influencing one another's steroid levels, the presence of additional conspecifics may have consequences for the reproductive traits of a population as that population's density increases.

The Mechanisms Underlying Reproductive Pheromones

There is a persistent argument in the published literature on pheromones favouring mechanisms of action in the olfactory system and their indirect effects on reproductive physiology (Brennan 2009, Brennan & Peele 2003). I have demonstrated another possible mechanism of action. Pheromones are demonstrably involved in attraction and social recognition in mice (Baum & Keverne 2002), as extensively reviewed elsewhere (Brennan & Keverne 1997, Spehr *et al*. 2006). However, the evidence in favour of vomeronasal receptor-transduced neural events altering luteinizing hormone release from the pituitary is weaker than the many reviews on the topic suggest (see deCatanzaro 2011). From

the experiments in Chapters 3 and 4, steroid transfer can and does occur in many social contexts. Furthermore, each reproductive phenomena reported in mice can be influenced or mimicked by picogram to nanogram changes in circulating steroids, in particular E_2 and P_4. I did not show that the administered ^{3}H-steroids themselves directly affected the reproductive physiology of the mice used in my experiments. Since I only showed the presence of steroids in tissues relevant to reproductive effects following transfer or administration via plausible natural routes of bodily entry, my work requires the context of many other reports.

First, urinary steroids must be unconjugated and free of other binding factors. This has been demonstrated for mouse urine: testosterone, P_4, and E_2 are each present in the urine of both males and females, as verified by enzyme immunoassays coupled with high-affinity, high-specificity anti-steroid antibodies, (Beaton *et al*. 2006, deCatanzaro *et al*. 2003, 2004, 2006, Khan *et al*. 2008b, Muir *et al*. 2001, Thorpe & deCatanzaro 2012). In Chapter 3, creatinine-adjusted quantification of unconjugated urinary steroids in outbred male mouse urine is shown, and in Chapter 4, unconjugated E_2 and P_4 in CF1 strain males and females is shown. Males exposed to developing females have higher concentrations of urinary testosterone and E_2 than do isolated males (Beaton *et al*. 2006), which is related to their ability to accelerate puberty. A male's ability to block pregnancy in unfamiliar inseminated females seems to be most related to the E_2 content of his urine (Beaton & deCatanzaro 2005). Finally, the ability of a male to accelerate puberty or block pregnancy is diminished by castration, and is restored

by E_2 treatment, which directly coincides with his urinary E_2 levels (Thorpe & deCatanzaro 2012). So not only are unconjugated steroids present in mouse urine, but also many reported pheromonal phenomena depend on these steroids.

Second, the direct transfer of steroids must be possible through known pathways. In Chapters 2 and 3, I showed that the nose and skin readily absorb ^{3}H-E_2 in ways that are similar to pharmacological reports (Bawarshi-Nassar *et al*. 1989, Hueber *et al*. 1994, Scheuplein *et al*. 1969, Türker *et al*. 2004). Although quantification and structural analysis of absorbed steroids as they were detected in tissue was not possible, there is evidence that steroids are uniquely absorbed into circulation, with varying levels of metabolism following nasal mucosa contact (Arora *et al*. 2002, Bawarshi-Nassar *et al*. 1989) and epidermal contact (Goldzieher & Baker 1960, Pannatier *et al*. 1978, Scheuplein *et al*. 1969). In the rat, nasally administered P_4 is not immediately metabolized, whereas a proportion of nasally administered E_2 is metabolized into estrone and estrogen conjugates (Bawarshi-Nassar *et al*. 1989). Similarly, P_4 applied to the epidermis enters circulation and is metabolized as it would be if injected intravenously (Waddell & O'Leary 2002), whereas E_2, which is more polar than P_4, is absorbed into circulation as well, albeit more slowly and with some metabolism to estrone (Goldzieher & Baker 1960). Despite its lesser efficiency in uptake, E_2 is biologically active at such low concentrations that any E_2 entry into circulation from an outside source could be physiologically consequential.

Third, the binding of transferred steroids in the reproductive tissues and related brain regions is necessary for the steroids to have physiological effects. In Chapters 2 and 4, I showed the competitive binding of unlabelled E_2 with ^3H-E_2 by administering E_2 to females prior to ^3H-E_2 exposure. In Chapter 4 specifically, unlabelled E_2 was given to females immediately before they were exposed to ^3H-E_2-treated females. Compared to control subjects that were given a vehicle before exposure to ^3H-E_2-treated females, the E_2 pre-treated females had much lower levels of radioactivity in samples of brain and reproductive tissues. This is partially due to the competition of E_2 and ^3H-E_2 for the same estrogen receptors (Terenius 1969), which is strong evidence that at least some transferred E_2 maintains its structure and binding properties. Furthermore, the largest overall differences in binding were found in the uterus, the prime site of action for reproductive effects. In many of the experiments throughout this thesis, ^3H-E_2 was also detected in the ovaries and in the mesencephalon and diencephalon of the brain, which includes the hypothalamus. From this, the transfer and binding of at least ^3H-E_2 was shown for the entire hypothalamic-pituitary-gonadal axis, which is crucial in the elicitation of female sexual receptivity (Musatov *et al*. 2006, Pfaff 1980).

The research in this thesis strongly supports the idea of steroid transfer as a mechanism of endocrine control in mice. Steroids in urine, known to be unconjugated and bioactive, enter into the bodies of exposed conspecifics, wherein they bind to their respective receptors and cause physiological changes to

the recipient animal. These changes include, but are not limited to, acceleration of puberty, modification of the estrous cycle, and disruption of intrauterine blastocyst implantation. Many of these effects appear to favour the biological fitness of males, and indeed, the proactive behaviour of males in inducing these effects suggests that male co-option of existing and ancient endocrine systems for a potential gain in reproductive fitness is an evolved strategy. Whether or not this is the case, the transfer of steroids between conspecifics should not be overlooked as an either incidental or evolved mechanism driving known pheromonal phenomena.

Limitations

Transfer levels were low in every experiment in which females were exposed to ^{3}H-E_2-injected conspecifics, with the quantity of ^{3}H-E_2 detected in females equating to the femtogram range. However, this low level of recovered ^{3}H-E_2 was anticipated. In one report, researchers intravenously injected mice with ^{3}H-E_2, waited 1 hour, and then decapitated the subjects (Gorzalka & Whalen 1974). An average of 0.007%[1] of the administered quantity of ^{3}H-E_2 was recovered in each mg of mouse uteri using this procedure, even though intravenous administration suffers from no barriers to entry into circulation. In a natural setting, urinary steroids are thought to enter circulation via absorption

[1] This number itself was not reported; however, Gorzalka & Whalen injected 4 µCi ^{3}H-E_2 and recovered an average of 618.89 DPM/mg wet uteri mass in Swiss-Webster strain mice. Since 1 DPM ≈ 4.504×10^{-13} Ci, 618.89 DPM ≈ 2.787×10^{-10} Ci = 2.787×10^{-4} µCi.
$(2.787 \times 10^{-4}$ µCi ÷ 4 µCi) × 100% = (6.97×10^{-3})% ≈ 0.007%.

through nasal mucosa and skin. In terms of absorption through nasal mucosa, in rhesus monkeys, radioactivity in uterine samples taken from females given ^{3}H-E_2 intravenously was found to be 21.75 times greater than that of subjects given the same dose intranasally (Anand Kumar *et al*. 1974). In terms of cutaneous uptake of steroids, P_4 has a permeability constant five times greater than that of E_2, since in containing two alcohols, E_2 is more polar than P_4 and is thereby less likely to pass through intrinsically non-polar dermis (Scheuplein *et al*. 1969). ^{3}H-P_4 applied topically to rats results in substantial uptake into circulation within the first hour following administration, and intact ^{3}H-P_4, as opposed to its metabolites, is detectable in uterus samples 3 hours following administration (Waddell & O'Leary 2002). Although E_2 does not permeate into cutis as effectively as does P_4, it can permeate enough to result in significant uptake following exposure to skin, and follows a pattern of diffusion similar to that of topically applied P_4 (Goldzieher & Baker 1960). Combining these barriers to entry with idiosyncratic rates of steroid excretion, competitive steroid binding, and the natural metabolism of steroids, any detection of radioactivity in an untreated subject resulting from interaction with a ^{3}H-steroid-injected conspecific is a strong indicator of steroid transfer.

Since all ^{3}H-steroids come from the manufacturer in an ethanol solution, intraperitoneal administrations of unmodified ^{3}H-steroid solutions are toxic to mice. A small, unpublished pilot study indicated that mice given daily injections of as little as 20 µl 95% ethanol urinate and defecate much less, become lethargic,

and may even die. The initial solution to this problem, included in Chapters 2 and 3, was to leave the ^{3}H-steroid solution unmodified, and instead slowly increase each subject's ethanol tolerance. While data indicate that tolerance was moderately effective, in the context of reproductive phenomena, a full range of stimulus animal behaviour is preferable. Preliminary data later indicated that it may be better to evaporate off the ethanol in the ^{3}H-steroid solutions, thereby recystallizing the steroids, or to dilute the stock solution with saline. Reconstituting the steroids into an ethanol-saline solution still allows for steroid solubility without affecting the structure of the steroid, and this solution is comparatively non-toxic. Indeed, a male injected s.c. with such a solution suffered no noticeable adverse effects from the injection, and the female paired with him for 24 hours showed uptake levels similar to those found in Chapter 4. Since steroids are chemically stable at room temperature and since the radioactivity in ^{3}H-steroids is maintained following recrystallization, researchers should use less toxic reconstituted ^{3}H-steroid solutions in future studies.

Whether the transfer of steroids was actively instigated by the injected mice or passively caused by general contamination of the bedding is unknown. The issue of contamination is not crucial to the inferences made about any of the reproductive phenomena investigated, as most of these phenomena can be replicated by exposing females to the soiled bedding of males (Dominic 1969, Parkes & Bruce 1962, Vandenbergh 1969, Whitten 1956). Although steroids are not volatile, it is possible that they are absorbed passively through skin as the

female interacts with the bedding, primarily by sleeping in it. As the liquid portion of urine evaporates, what remains may recrystallize, and the resulting residue could then be inhaled in dust. However, since female mice will make direct nasal contact with the urine droplets of males (deCatanzaro *et al*. 2006), it is probable that the majority of absorbed steroids come from the insufflation of urine or urinary contact with the nasal epidermis.

Future Directions

To address the possibility of bedding contamination as a source of radioactivity in a female paired with a ^{3}H-steroid-treated male, the daily urinary output of treated males could be quantified, and the amount of radioactivity in a given amount of urine could be determined. An equivalent quantity of radiolabelled steroid could then be mixed with bedding. Since the properties of urine could be a key factor in the uptake of steroids from bedding, other females would need to be placed in cages where the bedding is manually soiled with the determined amount of urine dosed with the determined amount of radiolabelled steroid. By simply comparing the tissues of females in these two settings with those of untreated controls and those of females exposed to treated males, one could determine the degree to which interaction between the male and female, the female and the bedding, and the female and the urine in the bedding are involved in reproductive phenomena.

In Chapter 2, I examined the competitive binding of E_2 and ^{3}H-E_2 *in vivo* and found a strong effect in uterine samples (see Figure 5). However, in that experiment, the unlabelled E_2 was subcutaneously administered two days prior to the intranasal administration of ^{3}H-E_2. This method was improved upon in Chapter 4, where females were injected with unlabelled E_2 and then immediately exposed to ^{3}H-E_2-injected females, showing significantly lower levels of radioactivity than did untreated females also exposed to ^{3}H-E_2-injected females. Although subcutaneous E_2 enters circulation and competes systemically with ^{3}H-E_2, follow-up work could be done on the simultaneous administration of unlabelled and labelled steroids combined into a single solution. Administering a mixture of labelled and unlabelled E_2 to male and then examining his transfer of radioactivity to a female may better demonstrate the competitive binding of male-excreted E_2 and E_2 metabolites with male-excreted ^{3}H-E_2 and ^{3}H-E_2 metabolites in the exposed female's reproductive tissues. Furthermore, since ^{14}C-labelled steroids are also commercially available, the activity of two steroids can be examined simultaneously in a dual-labelling procedure (*e.g.*, Goldzieher & Baker 1960). For example, ^{3}H-E_2 and ^{14}C-P_4 could both be injected into a male, with the addition of overwhelming quantities of unlabelled E_2 and P_4 for a control condition, and then the transfer dynamics of both of these reproductive hormones could be examined in females paired with these males, inherently controlling between-subject differences.

Tracing the pathway of chemicals through radiolabelling can also provide information regarding the absorption and metabolism of non-steroidal endocrine modulators. In the research of this thesis, I determined that exogenous steroids do enter into the body through vectors such as the excretions of conspecifics, and through nasal and cutaneous exposure. These vectors are also likely involved in the absorption of endocrine disrupting chemicals (EDCs), such as bisphenol-A. Bisphenol-A, a common manufacturing monomer used in the production of many consumer products, is known to adversely affect the endocrine systems of mice during development (Howdeshell *et al*. 1999) and pregnancy (Berger *et al*. 2007, 2008, 2010, Susiarjo *et al*. 2007). The current estimates for environmental exposure to bisphenol-A and other EDCs in humans and wildlife fail to account for all potential sources and interactive effects. One primary source of EDCs is manufacturing and consumer waste (Wang *et al*. 2012). Considering the direct exposure of wildlife to untreated environmental contaminants in combination with exogenous steroid exposure, the potential for adverse synergistic effects well beyond those already empirically supported (Crawford & deCatanzaro 2012, Mu & LeBlanc 2004, Yang *et al*. 2012) is an immediate concern (Casals-Casas & Desvergne 2011, Diamanti-Kandarakis *et al*. 2009). Given the availability of ^{3}H- and ^{14}C-labelled versions of steroids and potential EDCs, research could be conducted on the accumulation of radioactivity in all tissues of a model species following experimental exposure to any number of chemicals simultaneously. While information on the specific binding of any one chemical could be lost in

such a study, the absorption of an ecologically relevant cocktail would provide information on the extent to which environmental contaminants combine to invade an organism.

Conclusion

Steroid transfer may naturally occur between conspecifics, with steroids being excreted in urine by one animal and absorbed by the nasal mucosa and cutis of another. Following uptake, transferred steroids arrive at key sites involved in reproduction, where they likely bind to intracellular or cell-surface steroid receptors, adding to the recipient animal's endogenous steroid action. This phenomenon of steroid transfer may explain hidden endocrine interactions among animals in the wild or in laboratory settings. In the wild, steroid transfer may play a role in intrasexual competition or in controlling population growth. In the lab, steroid transfer may result in endocrine differences, and therefore physiological differences, between animals housed in groups and those housed alone.

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