Available online at www.sciencedirect.com

SCIENCE DIRECT®

Developmental Biology 296 (2006) 150–163

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Chronic cocaine exposure in *Drosophila*: Life, cell death and oogenesis

Stacey Sedore Willard [a,b], Cara M. Koss [a], Claire Cronmiller [a,*]

[a] *Department of Biology, University of Virginia, P.O. Box 400328, Charlottesville, VA 22904-4328, USA*
[b] *Department of Cell Biology, The Johns Hopkins University, Baltimore, MD 21205, USA*

Received for publication 22 November 2005; revised 30 March 2006; accepted 12 April 2006
Available online 21 April 2006

Abstract

Developmental signaling cascades that can be perturbed by cocaine and other drugs of abuse have been difficult to study in humans and vertebrate models. Although numerous direct neural targets of cocaine have been elucidated at the molecular level, little is known about the specific cellular events that are impacted indirectly as a result of the drug's perturbation of neural circuits. We have developed oogenesis in *Drosophila melanogaster* as a model in which to identify downstream biochemical and/or cellular processes that are disrupted by chronic cocaine exposure. In this model, cocaine feeding resulted not only in expected reductions in viability, but also in unanticipated developmental defects during oogenesis, including aberrant follicle morphogenesis and vitellogenic follicle degeneration. To identify mechanisms through which cocaine exerted its deleterious effects on oogenesis, we examined candidate components of neural and hormonal signaling pathways. Cocaine-induced disruptions in follicle formation were enhanced by juvenile hormone exposure and phenocopied by serotonin feeding, while cocaine-activated follicle apoptosis was enhanced by concomitant dopamine feeding. HPLC analysis of dopamine and serotonin in the ovary suggests that these neurotransmitters could variably mediate cocaine's effects on oogenesis indirectly in the brain and/or directly in the ovary itself. We confirmed the involvement of hormone signaling by measuring ecdysteroids, which increase following cocaine exposure, and by demonstrating suppression of cocaine-induced follicle loss by hormone receptor mutants. Cocaine-induced ovarian follicle apoptosis and adult lethality appear to be caused by modulation of dopamine levels, while morphological defects during follicle formation likely result from perturbing serotonin signaling during cocaine exposure. Our work suggests not only a new role for juvenile hormone and/or serotonin in *Drosophila* ovarian follicle formation, but also a cocaine-sensitive role for dopamine in modulating hormone levels in the female fly.

Keywords: Drosophila; Oogenesis; Cocaine; Ecdysone; Juvenile hormone; Dopamine; Serotonin

Introduction

Although the impact of cocaine on the nervous system has been extensively characterized, with many direct molecular targets identified, the cellular and developmental effects of long-term exposure to the drug have been difficult to ascertain. In most cases, for example, it is not known whether cocaine-induced perturbations of neurotransmitter signaling also account for drug-induced phenotypes in non-neural tissues. At least for the increases in heart rate and blood pressure that are associated with cocaine usage in humans, a neural link is evident from the drug-induced elevation in norepinephrine signaling (reviewed in Egred and Davis, 2005). However, the sources of any phenotypic effects of chronic cocaine exposure on human fetal development are not at all clear (reviewed in Addis et al., 2001; Fajemirokun-Odudeyi and Lindow, 2004). Key insights have been provided by the study of vertebrate models, in which cocaine-induced changes in dopamine signaling have been linked to altered neural development in the fetus (reviewed in Harvey, 2004). What remain to be addressed are specific non-neural cellular effects of cocaine-induced changes in neurotransmitter signaling where the physiological connection is not as obvious. For example, apoptosis in rat and rabbit testes is induced following cocaine administration subcutaneously (Li et al., 1999, 2003); whether activation of this cell death program results directly from cocaine-sensitive neurotransmitter signaling is not known. Similarly, the drug-hormone signaling connection that is inferred from cocaine's deleterious effects on spermatogenesis and sexual maturation in rats has not been

* Corresponding author. Fax: +1 434 982 5626.
E-mail address: crc2s@virginia.edu (C. Cronmiller).

0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.04.448

elaborated (George et al., 1996). In addressing these and other questions of mechanism, invertebrate models have become valuable not only for reinforcing, but also expanding our understanding of cocaine's action.

Drosophila melanogaster is an established behavioral model for molecular responses to cocaine in the nervous system, with substantial mechanistic similarity to vertebrates (reviewed in Hirsh, 2001; Wolf and Heberlein, 2003). Cocaine binds the transporters of dopamine (DA), serotonin (5HT) and, in mammals, norepinephrine (NE); this results in the decreased reuptake of these monoamines into presynaptic cells (Ritz et al., 1987; Porzgen et al., 2001; Rothman and Baumann, 2003). The consequent increase of neurotransmitter binding to cognate receptors on postsynaptic cells is what leads to the elevated neural signaling that is associated with cocaine's stereotyped behavioral responses (reviewed in Rocha, 2003). Increased receptor stimulation could similarly lead to cocaine's cellular and/or developmental effects, either by direct innervation of the target tissue or by indirect long-range hormonal signaling. We have used *Drosophila* oogenesis as a cellular/developmental model for identifying such downstream effects of chronic cocaine exposure.

Drosophila oogenesis, which begins with the formation of a follicle and proceeds with the growth and specialization of the oocyte, requires the coordination of multiple cell types, their division, their migration and their organization. Each ovary is made up of 15–20 egg-producing units, called ovarioles. Follicle formation occurs in the germarium at the anterior end of the ovariole, where the germline and somatic stem cells reside (for reviews, see King, 1970; Spradling, 1993; Horne-Badovinac and Bilder, 2005) (Fig. 2A). The germline component of each follicle consists of 16 interconnected cells that are derived by mitotic divisions from a single founder cell; one of the cells in this germline cyst will differentiate as the oocyte, while the remaining 15 become polyploid nurse cells. The somatic component of each follicle is organized as an epithelial monolayer that surrounds the germline cyst. Within that epithelium, specialized somatic cells at the anterior and posterior poles of the follicle direct the placement of the interfollicular stalk, which is a stack of somatic cells that separate adjacent follicles. Once a follicle is formed, the remainder of oogenesis is marked by the growth and maturation of the oocyte. At approximately stage seven (stages as defined by King, 1970), vitellogenesis (i.e. yolk protein synthesis and uptake) begins, with yolk proteins being synthesized by somatic follicle cells, as well as by more distant fat body cells. Following release into the hemolymph from their sites of synthesis, yolk proteins are taken up by the maturing oocyte through the final stages of oogenesis.

Successful coordination of the elaborate cellular events described above requires control both within and from outside the ovary and involves signal transduction pathways, hormone signaling and developmental regulators. A few of the notable signal transduction pathways that function in the ovary during follicle formation are *Notch/Delta*, *Egf* and *JAK/STAT* (for more inclusive reviews of ovary signaling pathways, see Roth, 2001; Hombria and Brown, 2002; Lin, 2002; Lopez-Schier, 2003); however, specific genetic targets of many of these regulators have not been identified. Follicle formation has also been shown to involve at least two extraovarian controls, namely that provided by *stall* gene function and also insulin-like signaling (Willard et al., 2004; LaFever and Drummond-Barbosa, 2005). At least for the insulin-like signaling, it has also been shown that some of this hormone must originate in the nervous system and that it also influences follicle maturation (LaFever and Drummond-Barbosa, 2005; Richard et al., 2005). Indeed, links between hormone regulation and follicle maturation have been well established (reviewed in Gilbert et al., 2000, 2002). In newly eclosed females, initiation of vitellogenesis requires interactions between the sex peptide and juvenile hormone (JH) signaling pathways (Postlethwait and Giorgi, 1985; Postlethwait and Kunert, 1986; Soller et al., 1997). Moreover, follicle survival and egg production are maintained throughout the life of the female by tightly controlled levels of the steroid hormone, 20-hydroxyecdysone (20E) (for reviews, see Gilbert et al., 2000, 2002). In fact, the onset of vitellogenesis has been well defined as a 20E-sensitive checkpoint to monitor follicle progression: either too much or not enough 20E results in apoptosis of follicles at this stage, leading to markedly decreased egg production (Shwartz et al., 1985; Soller et al., 1999). Although a significant source of regulatory 20E is probably the ovary itself, the nature and origins of the factors that modulate its synthesis are uncertain (Richard et al., 2005).

Our studies of *Drosophila* oogenesis as a model for discovering long-term effects of cocaine exposure have identified dose-dependent deleterious effects of the drug on adult and embryonic lifespan, as well as on ovarian follicle formation and maturation. Furthermore, we demonstrate that cocaine's ovary phenotypes likely result from differential effects of at least two of the drug's neurotransmitter signaling targets, namely 5HT and DA, suggesting that these monoamines normally provide distinct regulatory functions during oogenesis. Finally, we show that cocaine and DA treatments synergistically lead to elevated steroid hormone levels, thereby suggesting not only how primary neural modulations by the drug may be translated into deleterious effects on non-neural tissues, but also identifying DA signaling as a normal modulator of ecdysteroids.

Materials and methods

Drosophila stocks and culture

Flies were reared on molasses–cornmeal–yeast medium at 25°C. The WT strain used was *Oregon-R* (*OR*, Bloomington Stock Center) in all experiments, unless otherwise noted. Flies were collected 24–48 h post-eclosion under carbon dioxide anesthesia to a density of 40–50 per vial; they were allowed to recover from anesthesia for 24 h on standard food before any treatment. Two additional WT strains were used: *Canton-S* (*CS*) was obtained from the Bloomington Stock Center and *Dahomey* (*DH*) was a gift from Nick Priest. *Ecdysone receptor*^{M5545fs}/*SM6b* and *ry*^{506} *P{ry[+t7.2]=PZ} Insulin-like receptor*^{05545}/*TM3, ry*^{RK}, *Sb*^{1}, *Ser*^{1} were obtained from the Bloomington Stock Center.

Pharmacological agent administration

All feeding experiments were carried out in glass *Drosophila* culture vials prepared with 2 ml of drug or vehicle solution and 0.4 g dry instant fly food (Carolina Biological Supply). The food was supplemented with 10–20 dry

yeast pellets, and flies were transferred to the prepared food without anesthesia. To control for room temperature variations, all vials were kept in a 25°C incubator, and to maintain moisture and nutritional conditions, flies were transferred to fresh food vials every 3–4 days. Throughout the course of an experiment, females were incubated in cocaine-containing vials with equal numbers of males, allowing for normal mating conditions. Food coloring was added in preliminary cocaine feeding experiments to ensure that flies continued to eat throughout the experiment and were not starving. In addition, we performed control experiments to ensure that the amount of yeast we provided did not result in effects on oogenesis besides those caused by drug feeding (data not shown).

Cocaine HCl (received from the National Institute on Drug Abuse Drug Supply Program) was dissolved in 10 mM potassium acetate buffer (pH 4.8) to make a stock solution, which was then diluted with sterile dH_2O to provide the desired concentration. Dopamine (DA) HCl, serotonin (5HT) HCl and fluoxetine (Sigma-Aldrich) solutions were prepared fresh in sterile dH_2O. When DA and cocaine were co-fed, the cocaine stock solution was diluted with dH_2O to a final volume of 2 ml, and DA HCl was added. Methoprene (Sigma-Aldrich) was diluted in acetone to a final concentration of 1 μg/μl. Methoprene or vehicle control (acetone) was volatilized by transferring the desired amount to a 1 cm^2 of filter paper inside a glass fly culture vial.

To assay for survival

Vials were prepared as described above, and for each time point, the number of deceased females was counted. The data were imported into the program, JMP, which was used to calculate mean survival, standard error of the mean, and survival curves. Statistical tests (Log-Rank and Wilcoxon tests) were also performed with this program, and a cutoff *P* value of 0.01 was used to determine statistical significance of survival curves.

To assay for vitellogenic follicles per ovariole (VF/O) and to quantify defects during ovarian follicle formation

Vials were prepared according to standard protocol, and five females were dissected from drug treatments and vehicle controls on days one, three, six, ten and fourteen. Ovaries were prepared as described below. To score vitellogenic follicles, dark-field illumination was used in combination with DAPI fluorescence (see below), which allowed simultaneous visualization of yolk granules (which reflect light under dark-field microscopy) and nuclei. We first calculated an average number of VF/O for four females per time point and finally an average of all time points over the course of each 14-day experiment for each treatment. In calculating this average, we did not include the 24-h time point since we were interested in documenting the chronic effects of cocaine. Defects during ovarian follicle formation were also counted, and the average number of ovarioles containing defects was determined for each female. Error bars indicate standard error between averages in independent experiments. Statistical significance was determined using either Student's *t* test or ANOVA two-factor with replication (Microsoft Excel) with a cutoff *P* value of 0.01. At least 100 ovarioles were scored for every time point in each experiment.

Ovary staining and visualization

Ovaries were dissected in 1× phosphate-buffered saline (PBS), fixed for 15 min in 4% paraformaldehyde and stained with DAPI, as described previously (Cummings and Cronmiller, 1994). TUNEL staining was carried out according to the protocol outlined by the manufacturer (Roche Molecular Biochemicals) with the following exceptions: ovaries were incubated in TUNEL enzyme and labeled nucleotide overnight at 37°C and, after washing in 1× PBS for 30 min, were stained with DAPI according to standard protocol. Monoclonal anti-phospho-histone H3 (Upstate Biotechnology, 1:1000) was detected with a FITC-conjugated secondary antibody (Jackson Immunoresearch, 1:300). Stained ovarian tissue was visualized on a Zeiss Axiophot/Axioskop, and images were captured in black and white by either a Pixera or an Olympus Magnafire digital camera. Images were false-colored for fluorescein in Adobe Photoshop. In double-labeled preparations, FITC is depicted as green and TRITC as magenta (overlap white) to make the images colorblind accessible.

High performance liquid chromatography (HPLC)

Ovaries were dissected from *OR* or *white*^{1118} (*w*^{1118}) flies in 1× PBS and immediately transferred to a vial on ice, containing 75 μl mobile phase buffer (50 mM citrate/acetate, pH 4.5, 20.25% acetonitrile and 11 mM decanesulfonic acid). Samples were homogenized with a pestle and passed through a 0.22 μm centrifugal filter device (Millipore Corporation). Standards of 100 pg of DA and 5HT (Sigma) were run for comparison. Since a typical injection volume was 20 μl, the HPLC trial illustrated in Fig. 9 represents approximately 13 ovaries. Replicates containing 10 μl (6.6 ovaries) were assayed with identical results (data not shown). HPLC chromatography and electrochemical detection were performed according to parameters previously determined to maximize the separation and detection sensitivity of the biogenic amines DA and 5HT (Cole et al., 2005; Hardie and Hirsh, in press). Standards that included OA, DA, TA and 5HT were used to search for these amines. Data from the detector were collected and analyzed by Jasco ChromPass chromatography software.

Radioimmune assay (RIA) for ecdysteroids

OR females were collected and treated with drug according to standard protocol. After 3 days of drug treatment, 100 female flies were collected and centrifuged at 14,000 rpm for 20 min to produce whole-fly extracts. Extracts were immediately chilled on ice and extracted once with several volumes of cold methanol. Methanol extracts were then passed through a 0.22 μm centrifugal filter device (Millipore Corporation), and the extract was dried thoroughly in a SpeedVac to remove the methanol. The resulting pellet was resuspended in 20 μl sterile water, and the RIA was carried out according to the protocol of Warren and Gilbert (1988). The antibody against ecdysteroids (H22, generously provided by Dr. Lawrence Gilbert) was used at a dilution of 1:1000. ^{3}H-Ecdysone was obtained from Perkins Elmer and used at a concentration of 16,000 dpm/100 μl, diluted in 100 mM borate buffer, pH 8.5. Separation of bound and free ecdysteroids was achieved using 20% heat-killed, formalin-treated *Staphylococcus aureus* Cowan1 (a gift from the Gilbert laboratory). Scintillation counting was performed on a Beckman counter. Samples were normalized to not only a scintillation fluid-only blank, but also a no-antibody blank. Serial dilutions of 20-hydroxyecdysone (Sigma) were used to produce a standard curve, and the equation of the best-fit trendline was used to calculate the concentration of ecdysteroids in each sample. Control values were set at 100%, and percent difference between control and unknown samples was calculated. An ANOVA (Microsoft Excel) was used to calculate statistical significance with a cutoff *P* value of 0.01.

Results

Chronic exposure to cocaine decreases Drosophila adult and embryonic survival

Cocaine feeding resulted in concentration-dependent deleterious effects on both adult female lifespan and embryo survival and development. Adult *Oregon-R* (*OR*) flies fed cocaine exhibited concentration-dependent shorter lifespans, compared to no-drug controls (Fig. 1A). All three of the administered cocaine doses resulted in significantly decreased lifespans (Table 1). To ensure that the effect of cocaine feeding on fly survival was independent of genetic background, we also tested two additional wild-type (WT) strains [*Canton-S* (*CS*) and *Dahomey* (*DH*)] on the middle dose (1.5 mg/ml) of cocaine: the survival of cocaine-fed *CS* and *DH* flies was dramatically reduced (Fig. 1B). Interestingly, these wild-type strains varied in their mean lifespans regardless of the presence of cocaine (Table 1), indicating that genetic background had a significant impact on lifespan; however, in both WT strains, survival was significantly reduced after cocaine feeding. We also observed

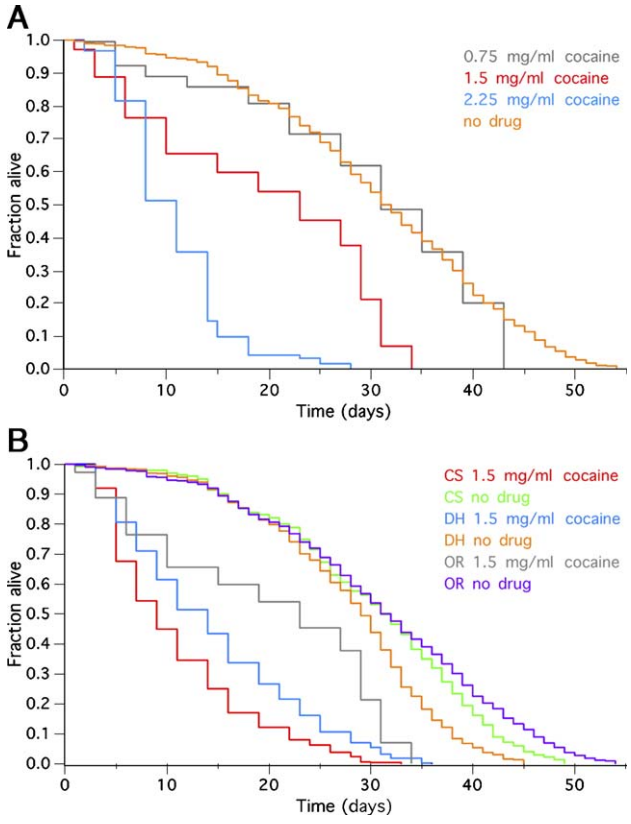

Fig. 1. Cocaine feeding reduces adult lifespan. (A) Survival curves for *OR* females fed various doses of cocaine. Each line graph plots the fraction of *OR* female flies alive each day on plain food (orange line) or food containing the indicated concentration of cocaine. Low (0.75 mg/ml, gray line), medium (1.5 mg/ml, red line) and high (2.25 mg/ml, blue line) doses of cocaine were administered. See Table 1 for statistical analysis. (B) Survival of different WT strains fed cocaine. Each line plots the fraction of flies alive on each day for three WT strains (*OR*, *CS*, *DH*) fed plain food (purple, green and orange lines) or food containing 1.5 mg/ml cocaine (gray, red and blue lines, respectively). See Table 1 for statistical analysis.

deleterious effects on embryonic survival, when we placed 0–2 h old wild-type embryos from untreated mothers on a cocaine-containing substrate: Exposure to 1.5 mg/ml cocaine resulted in embryonic arrest prior to cuticle deposition (data not shown). Finally, during the course of our survival assays, we noted that females fed any dose of cocaine showed progressive decreases in fecundity, prompting us to examine the structure of the ovaries of drug-treated females.

Cocaine feeding disrupts oogenesis

Chronic feeding of cocaine to females produced dramatic defects in ovarian follicle formation and maturation (Fig. 2). Morphological defects included mispackaging of germline cysts, such that more than the normal 16 germline cells were packaged into one epithelium (Fig. 2B), mispositioning of the oocyte to follicular locations other than the normal posterior site (data not shown) and missing and/or aberrantly formed interfollicular stalks (Fig. 2B, arrow). Disrupted ovarioles often displayed multiple follicle defects, especially following longer exposures to cocaine. Significant ($P < 0.01$) effects became apparent at treatments of 10 days or longer (Fig. 2D); the most consistently severe defects were also associated with these longer exposures. Females who were fed the highest cocaine dose for the longest time showed the most extreme ovarian phenotypes. For example, in females exposed to 2.0 mg/ml cocaine for 2 weeks, every ovary included numerous follicular abnormalities, with an average of 24% of ovarioles containing such defects; individual females were scored with as many as 62% disrupted ovarioles. In contrast, similar exposure to 0.75 mg/ml cocaine produced an average frequency of 17% defective ovarioles and a per female maximum of 19%. Although cocaine treatment per se was associated with these ovarian effects, we could not show a significant effect of dose, presumably because the more severe effects of higher cocaine doses on viability precluded observing higher accumulations of ovarian defects.

In order to learn more about the origins of cocaine-induced effects on oogenesis, we examined whether the follicular defects resulted from modulation of germline or somatic cell division in the germarium; they did not. The possibility that cocaine exposure might lead to disruption of follicular morphology via perturbation of cell proliferation was suggested by the similarities between the cocaine-induced ovarian defects and those seen in *daughterless* (*da*) mutants. In that case, the balance between germline cyst production and somatic epithelial division is lost, resulting in fused and compound follicles that are similar to those observed after cocaine exposure (Cummings and Cronmiller, 1994; Smith et al., 2002). In addition, a *da*-regulated germline cyst survival checkpoint exists in the germarium, such that if environmental conditions are not favorable, cysts undergo programmed cell death (Drummond-Barbosa and Spradling, 2001; Smith et al., 2002). To determine whether cocaine feeding similarly altered division rates in the germarium, we stained ovaries with an anti-phospho-histone H3 antibody to mark mitotic cells and then counted the number of dividing clusters of cells (both germline and somatic) in the germarium after 48 h of exposure to either plain or cocaine-containing food (for example, Fig. 2C). Ovaries from control (no

Table 1
Mean survivals and statistical analyses of treated females

Genotype	Treatment	Mean survival (days ± SE)
Oregon-R (OR)	Vehicle	31.1 ± 0.56
OR	0.75 mg/ml cocaine	29.9 ± 0.80[a, b]
OR	1.5 mg/ml cocaine	19.7 ± 0.57[a,b]
OR	2.25 mg/ml cocaine	10.7 ± 0.23[a,b]
OR	5 mg/ml DA	32.1 ± 0.74
OR	5 mg/ml DA ± 0.75 mg/ml cocaine	17.3 ± 0.67[a, c]
Canton-S (CS)	Vehicle	30.2 ± 0.58
CS	1.5 mg/ml cocaine	11.0 ± 0.41[a]
Dahomey (DH)	Vehicle	27.8 ± 0.41
DH	1.5 mg/ml cocaine	14.4 ± 0.49[a]

n = at least 141 flies per treatment.
[a] P value less than 0.01 when compared to vehicle control of the same genotype using Log-Rank and Wilcoxon statistical analysis.
[b] $P < 0.01$ when compared to all other cocaine doses within the same genotype.
[c] $P < 0.01$ when compared to either drug treatment alone.

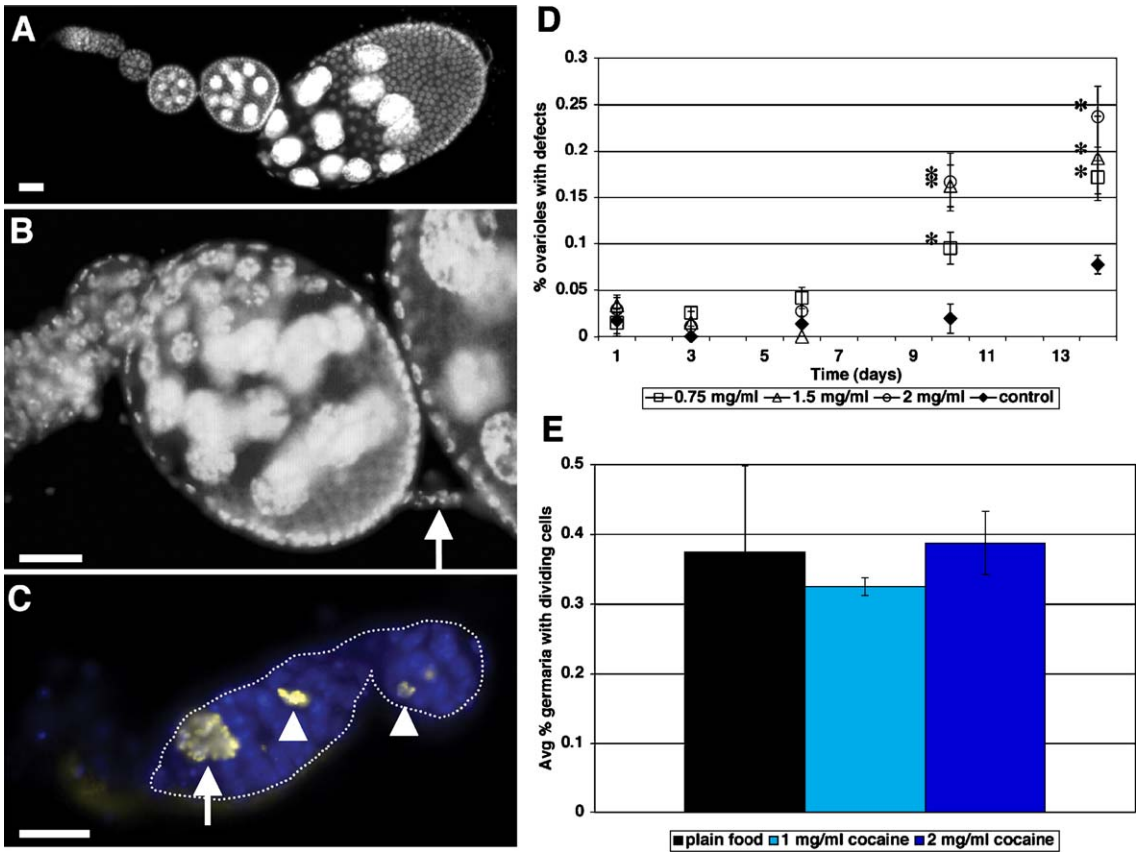

Fig. 2. Cocaine feeding results in morphological defects in ovarian follicles. (A) A normal ovariole, stained with the nuclear dye, DAPI. Each follicle consists of 15 nurse cells and one oocyte, surrounded by an epithelial monolayer and separated from neighboring follicles by an interfollicular stalk. (B) An example of an aberrant ovariole after cocaine feeding (1.5 mg/ml, 8 days). The arrow indicates an interfollicular stalk containing 16 cells. Note that there are 30 nurse cells in this follicle. (C) An example of a WT germarium stained with anti-phospho-histone H3 (green) and DAPI (blue, overlap: yellow). The arrow indicates a germline cyst positive for phospho-histone H3 during mitosis; arrowheads indicate somatic cell division. (D) The incidence of cocaine-induced follicle formation defects. Plotted are the average percentages of morphologically defective ovarioles per female on each day and dose of cocaine. For each time point, three independent experiments were performed, each containing at least 4 females per time point and representing, on average, 283 ovarioles. Asterisks indicate significance, relative to plain food treatment by a two-factor ANOVA with replication, $P \leq 0.01$. Error bars represent standard error between independent experiments. (E) The number of dividing germline and somatic cells in the germarium. This bar graph illustrates the percentage of germaria that contain dividing clusters of germline and/or somatic cells. Error bars represent standard deviation between females. A–C: anterior is to the left; scale bar = 100 μm.

drug), moderate (1.0 mg/ml) and high (2.0 mg/ml) cocaine treatment females all contained comparable cell division frequencies (37%, 32% and 39% of germaria, respectively) (Fig. 2E). In addition to demonstrating that cocaine-induced follicle defects did not result from changes in germline or somatic division rates in the germarium, these data also indicate that cocaine feeding did not arrest germline cyst production.

To determine whether cocaine interfered with follicle maturation by activation of late stage cyst degeneration, we quantified ovariole and vitellogenic follicle survival following cocaine exposure. While cocaine feeding had no effect on the number of ovarioles per female (data not shown), it did result in a measurable degradation and loss of individual maturing follicles, even at the lowest dose and shortest exposure time tested (Fig. 3). After only 24 h of cocaine exposure, the ovaries of treated females contained significant numbers of degenerating follicles at maturation stages 7–8, which corresponds to the onset of vitellogenesis. Degenerating follicles were easily recognized by the pycnotic appearance of DAPI-stained nuclei (Fig. 3A, arrows). Overall, three types of ovary phenotypes were observed after cocaine feeding; these appeared to represent progressive stages in the degeneration process. First, ovaries included multiple degrading chambers; no ovariole contained healthy follicles later than stage 8 (Fig. 3A). Second, ovaries were found in which pycnotic cellular debris was accumulated at the posterior of the tissue, although no follicular structure was discernible (data not shown). Finally, some ovaries contained neither degenerating follicles nor follicular debris but simply showed a deficit in the number of vitellogenic follicles (data not shown). The ovaries in this group could represent cases in which (1) follicle remnants had been cleared from the tissue prior to our inspection or (2) follicle maturation had stalled without/prior to subsequent degeneration. We quantified the effect of cocaine on follicle maturation by counting the number of vitellogenic follicles (VF) in each ovariole (O), termed VF/O. We used VF/O as an indirect measure of the number of follicles that either underwent degeneration in response to cocaine or were prevented from completing oogenesis: a decrease in VF/O could represent more apoptosis and/or the disappearance or absence of normally maturing follicles. First, we analyzed the

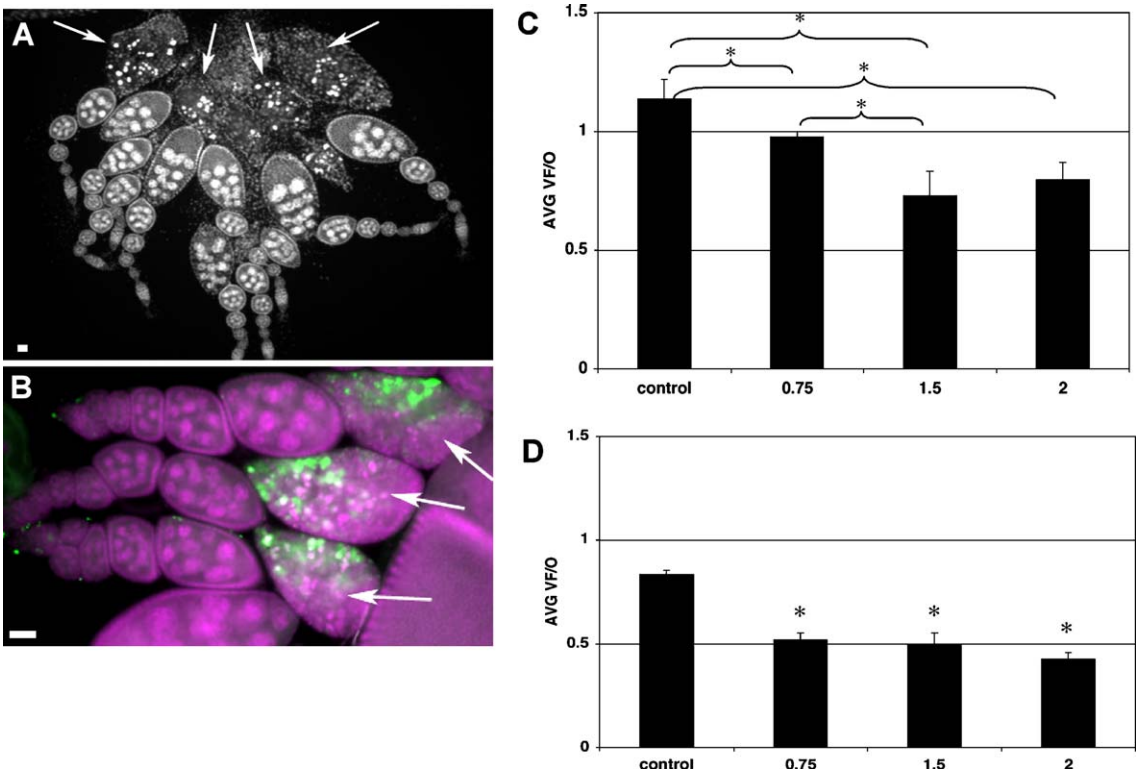

Fig. 3. Cocaine causes apoptosis of follicles at the onset of vitellogenesis. (A) A DAPI-stained ovary, dissected from a female fed a moderate dose (1.5 mg/ml) of cocaine for 24 h. Arrows indicate degenerating follicles. Follicles younger than stage 7 show no sign of degeneration. (B) DAPI (magenta)- and TUNEL (green)-stained ovarioles (overlap white), dissected from a female fed cocaine (1.5 mg/ml) for 24 h. Arrows indicate degenerating follicles with TUNEL labeling. (C) Acute effects of cocaine feeding on vitellogenic follicle number. The bar graph is a plot of the average number of VF/O per female after 24 h on either plain (control) or cocaine-containing (0.75, 1.5 or 2 mg/ml) food. Each bar represents three independent experiments, with at least 4 females per time point per day. Error bars show the standard error between replicate experiments; asterisks indicate significance between treatments, according to a two-factor ANOVA with replication ($P < 0.01$). (D) Chronic effects of cocaine feeding on vitellogenesis. This bar graph is a plot of 14-day VF/O averages for females fed either plain (control) or cocaine-containing (0.75, 1.5 or 2 mg/ml) food. The VF/O values for multiple daily time points over a 14-day experimental time course were combined (excluding the acute effects of day 1 displayed in panel C) to visualize the effect of chronic cocaine exposure. Asterisks indicate significance relative to the no-drug control (ANOVA, $P < 0.01$).

acute effects of cocaine exposure on vitellogenic follicle number (Fig. 3C). Low (0.75 mg/ml) and medium (1.5 mg/ml) doses of cocaine resulted in significant concentration-dependent decreases in the number of vitellogenic follicles, when assayed after 24 h of feeding; the effects of acute exposure to cocaine appeared to saturate at 1.5 mg/ml since a higher dose (2.0 mg/ml) produced a VF/O reduction that was as bad as, but not worse than, that observed for that lower dose. Following chronic exposure to cocaine, vitellogenic follicle number decreased at all drug doses tested (Fig. 3D). However, the effects were not concentration-dependent, indicating that chronic exposure to cocaine saturated the ovarian response even at the lowest drug level: For each concentration tested, the number of vitellogenic follicles per ovariole was reduced by about 50%, resulting in a severe reduction in female fecundity. The decrease in VF/O throughout 14 days of cocaine exposure resulted specifically from a loss of vitellogenic follicles at/around the onset of vitellogenesis since we never observed degeneration of earlier stages (data not shown).

Because cocaine-induced follicle degeneration appeared to coincide in timing with the known 20E-sensitive follicle survival checkpoint (Shwartz et al., 1985; Soller et al., 1999), we tested whether cocaine exposure resulted in the activation of programmed cell death in maturing follicles. Normally, this follicle maturation checkpoint contributes to the regulation of egg production in the adult female, such that insufficient/excess 20E triggers apoptosis of follicles at the onset of vitellogenesis with a consequent marked decrease in egg production (reviewed in Soller et al., 1999; McCall, 2004). We performed TdT-mediated dUTP nick end labeling (TUNEL) assays and found clear staining in the degenerating egg chambers of cocaine-fed females (Fig. 3B), indicating that vitellogenic follicles were lost via apoptosis, possibly associated with the follicle maturation checkpoint.

Cocaine's effects on oogenesis are mediated at least in part by hormones

Our demonstration of an apparent activation of the 20E-sensitive follicle survival checkpoint by chronic cocaine exposure, as well as the previously reported activation of vitellogenic follicle apoptosis by insulin-like signaling (Drummond-Barbosa and Spradling, 2001), prompted us to examine the involvement of hormones in the disruption of oogenesis following cocaine exposure, and we did this in three ways. First, we measured ecdysteroid levels following cocaine feeding.

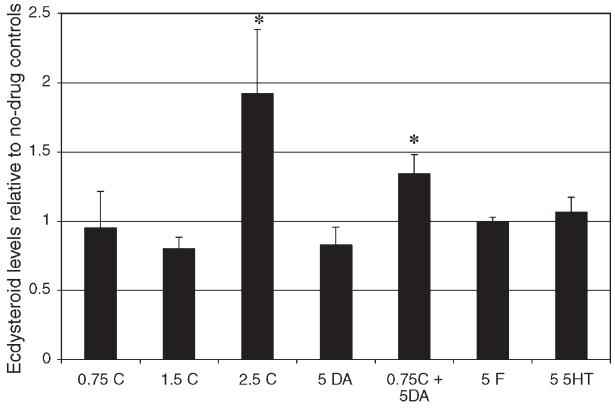

Fig. 4. Ecdysteroid equivalents after drug exposure, as determined by radioimmune assay. This bar graph plots fly extract ecdysteroid levels following various drug treatments, relative to no-drug controls. Each bar represents ecdysteroid equivalents in experimental samples as a fraction of steroid levels in control (no-drug) samples. Error bars indicate the standard error between females; n = at least three trials for each bar. Asterisks indicate significant increases (ANOVA, $P < 0.05$) in ecdysteroid levels. Drug treatment doses are mg/ml (C, cocaine; F, fluoxetine; DA, dopamine; 5HT, serotonin).

Second, we used genetic disruption of hormone signaling to suppress cocaine-induced follicle apoptosis. Finally, examining both follicle formation and maturation, we used hormone treatments to phenocopy the cocaine-induced effects on oogenesis.

To determine ecdysteroid equivalents in adult fly extracts, we used a highly sensitive radioimmune assay (RIA) (Warren and Gilbert, 1988). Extracts were prepared from batches of 100 female flies after 3 days of drug exposure and assayed immediately. Fig. 4 illustrates the ecdysteroid concentrations in cocaine-fed flies, relative to no-drug controls. Although lower doses of cocaine (0.75 and 1.5 mg/ml) had no significant impact on steroid hormone levels, exposure to the high dose of cocaine (2.5 mg/ml) resulted in a significant induction of ecdysteroids. Since even the lowest cocaine doses had been associated with dramatic effects on follicle survival (Fig. 3), their failure to produce significant elevation in ecdysteroid levels suggests that perturbation of ecdysteroid signaling may not be solely responsible for the cocaine-induced activation of the follicle survival checkpoint. Alternatively, a significant transient change could have been missed by this assay. The twofold increase in ecdysteroids that resulted from high cocaine exposure, however, is consistent with a cocaine-induced hormonal activation of that checkpoint.

Since elevated ecdysteroid levels suggested a link between cocaine's effects on the ovary and activation of the hormone-mediated follicle survival checkpoint, we used a genetic approach to demonstrate directly such a link. Ideally, we would have examined the effects of cocaine in ecdysone biosynthetic or receptor mutant genotypes; however, except for the conditional *ecdysoneless* mutant (ecd^1), those genotypes are lethal. Although a pilot experiment with ecd^1 mutant flies at permissive vs. restrictive temperatures did show a trend toward suppression of cocaine-induced follicle degeneration (data not shown), analysis of these samples was confounded by the high level of background ovarian follicle defects that were associated with both high temperature (29°C) and the ecd^1 genotype. We bypassed these nonspecific complications by examining whether simultaneous non-lethal reductions in both checkpoint-activating hormone pathways, i.e. ecdysone and insulin, diminished ovary sensitivity to cocaine. While the severity of cocaine-induced follicle degeneration in females heterozygous for loss of function mutations in either hormone receptor gene alone [*Ecdysone receptor* (*EcR*), *Insulin-like receptor* (*InR*)] was indistinguishable from that observed in WT, degeneration-associated reductions in VF/O did not occur in cocaine-treated females that were doubly heterozygous for *EcR* and *InR* (Fig. 5). The protective effect of this sensitized genotype in response to cocaine demonstrates the involvement of both hormone signaling pathways in mediating the drug's activation of the follicle survival checkpoint.

Phenocopy experiments also suggested hormone mediation of cocaine's other deleterious effects. Because of the extreme toxicity of 20E, we were unable to examine the direct effects of ecdysteroid overstimulation on the ovary; however, juvenile hormone (JH) proved to be a practical substitute for indirectly hyperactivating this steroid hormone pathway. Indeed, JH is a valid substitute for this purpose since it has been shown to play a role in the initiation of insect vitellogenesis, specifically through activation of ecdysteroid signaling (Richard et al., 1998, 2001). We used the JH analog, methoprene, to test JH's effects on cocaine-induced phenotypes and found that its administration strongly enhanced both the lethality and follicle formation defects associated with the drug (Fig. 6). Four days after a single dose of 10 μg of methoprene, females showed a typical increase in VF/O, as previously reported (Soller et al., 1999). The number of VF/O rose from the average vehicle control number of 1.0 to 1.38 with no deleterious effect on viability (data not shown). However, when flies were exposed to 10 μg methoprene, concomitant with a medium dose of cocaine (1.0 mg/ml), lethality reached 100% by 48 h. Moreover, after

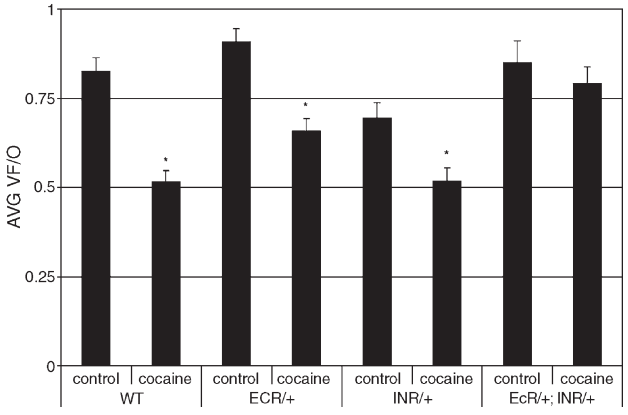

Fig. 5. The effect of reduced hormone signaling on cocaine-induced follicle loss. This bar graph illustrates the average VF/O over a 12-day cocaine feeding experiment at a moderate dose (1.5 mg/ml). Only relevant genotypes are shown; for complete stock genotypes, see Materials and methods. Error bars indicate the standard error between replicate experiments (at each time point, at least 18 females were scored and each bar includes data from at least 50 females); asterisks indicate significance between cocaine treatment and no-drug controls within a genotype, according to two-factor ANOVA with replication ($P < 0.01$).

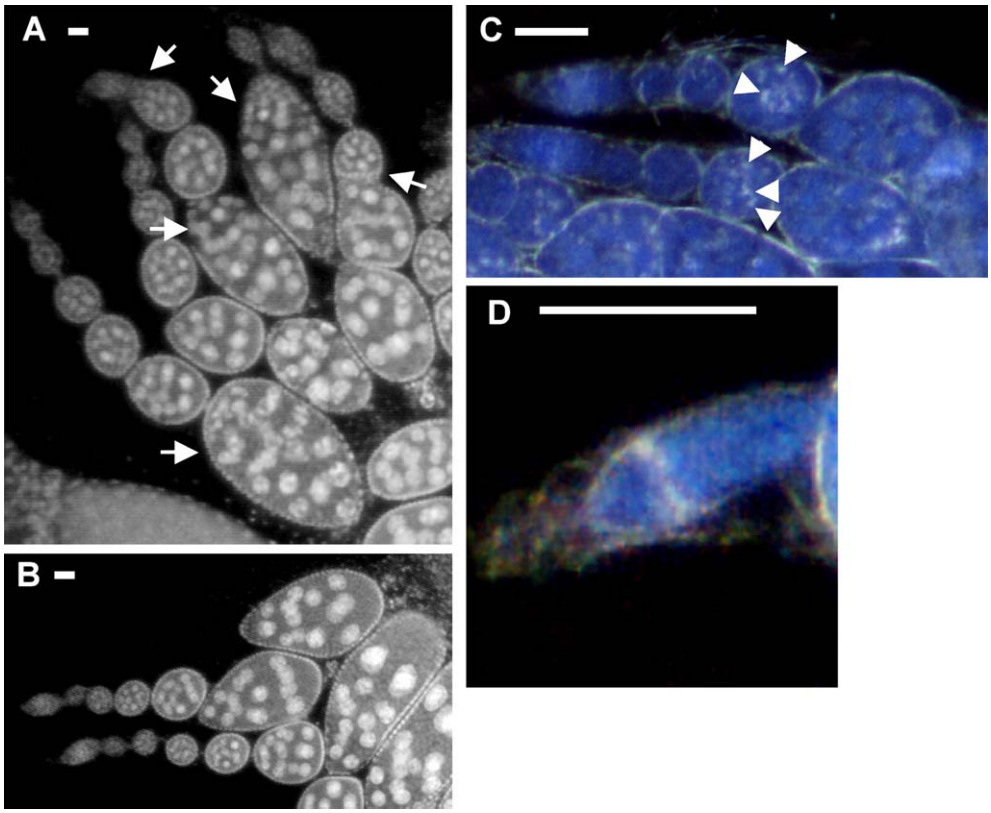

Fig. 6. The effects of methoprene and cocaine co-administration on oogenesis. (A) DAPI-stained ovarioles, dissected from a cocaine-fed female 24 h after a single exposure to 10 μg methoprene. This panel illustrates the effects of cocaine plus methoprene exposure on follicle structure. The arrows indicate follicle packaging defects that typically result (62% of ovarioles) from this combined drug treatment. (B) DAPI-stained ovarioles, dissected from cocaine-fed control females 24 h after a single vehicle only (acetone) treatment. Approximately 16% of ovarioles contain follicle defects. (C) Simultaneous exposure to cocaine and methoprene also results in inappropriate yolk uptake. The arrowheads indicate the inappropriate presence of yolk in nurse cells, as well as in oocytes of follicles that are too young for vitellogenesis. (D) This germarium, dissected from a female exposed to both cocaine and methoprene, contains a yolky germline cyst. Anterior is to the left in all panels; ovaries in C and D: DAPI-stained (blue) and simultaneously illuminated with fluorescence and dark field to visualize yolk granules (white). Cocaine concentration = 1.0 mg/ml; scale bar = 100 μm.

only 24 h of treatment, a marked increase in the occurrence of follicle formation defects (Fig. 6A) made it impossible to determine the number of VF/O. Abnormal follicle morphology included various germline cyst packaging defects, as well as fused follicles that resulted from the absence of the normal interfollicular stalk. After 24 h of JH and cocaine co-administration, 62% (n = 128) of ovarioles contained follicular defects, while 16% (n = 109) were defective in vehicle-only control ovaries (Figs. 6A, B); the occurrence of follicle defects following methoprene treatment alone was 19%. These data suggest that JH administration rendered flies hypersensitive not only to cocaine-induced lethality, but also to the drug's effects on follicle morphogenesis. It is possible that this JH-cocaine synergy reflects a previously unrecognized role for JH, if the observed increase in follicle defects resulted from cocaine's perturbation of a JH signaling system that normally regulates follicle formation.

In addition to the follicular structural defects described above, a new ovarian phenotype resulted from the simultaneous exposure to both methoprene and cocaine, namely ectopic yolk accumulation: yolk uptake occurred not only at inappropriate stages of folliculogenesis, but also in inappropriate cells (Figs. 6C, D). For example, germline cysts in the germarium contained yolk (Fig. 6D), as did young, normally previtellogenic follicles (Fig. 6C); in both cases, yolk accumulation was not restricted to the oocyte but aberrantly found also in nurse cells. According to current models, overstimulation of JH signaling would be expected to result in increased 20E production followed by increased yolk protein production and uptake (reviewed in Bownes, 1989); however, our experimental manipulations apparently activated JH signaling ectopically, resulting in ectopic yolk uptake. This conclusion is also consistent with our discovery of cocaine-induced elevation of ecdysteroid levels.

DA and 5HT feeding differentially affect cocaine-induced phenotypes

Because DA and 5HT have been shown to be involved in the regulation of steroid hormone signaling in other insects (Hirashima et al., 1999) and because direct effects of cocaine on the nervous system have been well documented (reviewed in Rocha, 2003; Rothman and Baumann, 2003), we expanded our analysis of cocaine-induced phenotypes to determine whether they resulted from perturbation of DA and/or 5HT signaling. We carried out individual feeding and drug interaction experiments

with these neurotransmitters and found that they contribute to different cocaine effects during oogenesis.

To determine whether perturbation of DA levels was capable of influencing cocaine-induced phenotypes, we fed flies DA, either alone or together with cocaine. At high doses of DA (20 mg/ml), the number of vitellogenic follicles plummeted to near 0 within 48 h and flies died within 4 days (data not shown). To circumvent the strong lethal effects of DA, we identified a low dose of DA (5 mg/ml) that had a small effect on viability (Fig. 7A), but no effect on VF/O (Fig. 7B), and we administered this dose in combination with the low dose of cocaine (0.75 mg/ml) that had previously been shown alone to have only small effects on lethality and oogenesis (Figs. 1–3). This combination of DA and cocaine significantly decreased the lifespan of *OR* flies, relative to that of flies exposed to either drug alone (Fig. 7A). The mean survival of doubly treated females was significantly different (Table 1) from that of singly treated or no drug control flies. Although no defects in follicle formation were detected (data not shown), the combined drug treatment resulted in a significantly ($P < 0.01$) decreased number of VF/O, relative to either individual drug treatment

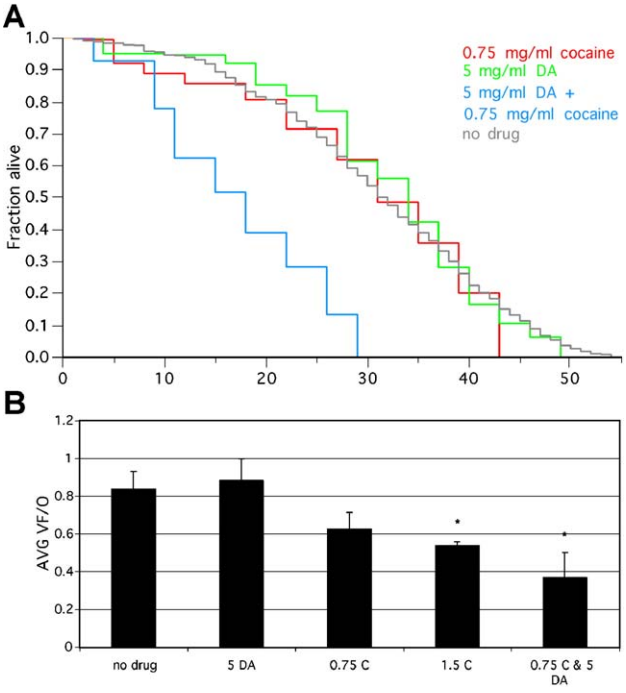

Fig. 7. Synergistic effects of DA and cocaine on female lifespan and follicle maturation. (A) Survival curves for *OR* females fed cocaine and/or DA. The treatments represented are: plain food (gray line), a low dose of DA (5 mg/ml, green line), a low dose of cocaine (reproduced from Fig. 1A, 0.75/ml, red line) or the combination of DA and cocaine (0.75 mg/ml and 5 mg/ml DA blue line). The combination drug treatment resulted in a significant reduction in lifespan relative to either drug alone (see Table 1, $P < 0.01$). (B) Chronic effects of DA and cocaine co-feeding on vitellogenic follicle number. This bar graph plots the 14-day average VF/O (excluding day 1) for females fed cocaine (0.75, 1.5 mg/ml) and/or DA (5 mg/ml). The combination drug treatment significantly reduced VF/O; DA alone did not. Error bars indicate standard error between replicate experiments; asterisks indicate significant comparisons (ANOVA, $P < 0.01$). In addition to those indicated by asterisks, both cocaine doses resulted in significant effects on the number of VF as compared to no-drug treatments.

(Fig. 7B). Administered together, cocaine and DA produced VF/O values that were as low as, and not significantly different from, those recovered following exposure to twice the dose of cocaine (1.5 mg/ml) alone (Figs. 3D, 7B). Thus, dopamine and cocaine acted synergistically in their effects on both lifespan and ovarian follicle survival. Although previous studies have implicated DA in controlling ovarian size (Neckameyer, 1996, 1998), this is the first evidence of a possible role for DA in regulating the progression of follicles through vitellogenesis. Moreover, these results suggest that DA mediates some, but not all, of cocaine's deleterious effects since we found no disruption of follicle morphogenesis associated with DA feeding. When we examined the impact of altered 5HT signaling on cocaine-induced phenotypes, we found that this neural target of cocaine action had the reciprocal effect on oogenesis: it disrupted follicle morphogenesis without obvious vitellogenic stage follicle degeneration.

We experimentally perturbed 5HT signaling either by feeding 5HT or by blocking serotonin transporter (dSERT) activity with the drug fluoxetine (Porzgen et al., 2001); both treatments resulted in aberrant follicle morphology (Fig. 8). As with dopamine, our initial high dose of 5HT (20 mg/ml) had a severe toxic effect on female lifespan (survival <4 days: data not shown). A lower chronic dose (5 mg/ml) did not significantly impact female lifespan, relative to vehicle controls (data not shown); however, the incidence of follicle formation defects was significantly increased (Fig. 8A). The results were similar for fluoxetine treatment (Fig. 8A and data not shown). In both cases, defects in follicle morphogenesis were identical to those produced by cocaine feeding: germline cyst packaging defects, mispositioning of oocytes and aberrant/missing interfollicular stalks (data not shown). Indeed, an assessment of the effect of these treatments on follicle survival was confounded by a high frequency of follicle formation abnormalities. Although chronic 5HT feeding did result in a significant reduction in the number of vitellogenic follicles in each ovariole ($P < 0.05$, relative to no drug control: Fig. 8B), related observations suggest that this decrease in the VF/O did not result from follicle degeneration. First, defective follicles often contained more than one germline cyst and therefore more than one oocyte. In some cases, multiple oocytes in a single follicle contained yolk, while in others none of the oocytes did, regardless of the follicle's maturation stage (data not shown). This is commonly the situation for similar compound follicle phenotypes in a wide variety of female-sterile mutants (unpublished observation). Furthermore, there was no sign of the late stage follicle degeneration or cellular debris that was characteristic of the cocaine-induced apoptosis in stage 7 and later follicles (data not shown). Thus, it is likely that the 5HT-induced reduction in the VF/O resulted from stalled early follicle progression because of structural defects rather than diminished vitellogenic follicle survival.

DA, but not 5HT, is detected in the ovary

Although the drug treatment studies described above demonstrated that both DA and 5HT were involved in mediating

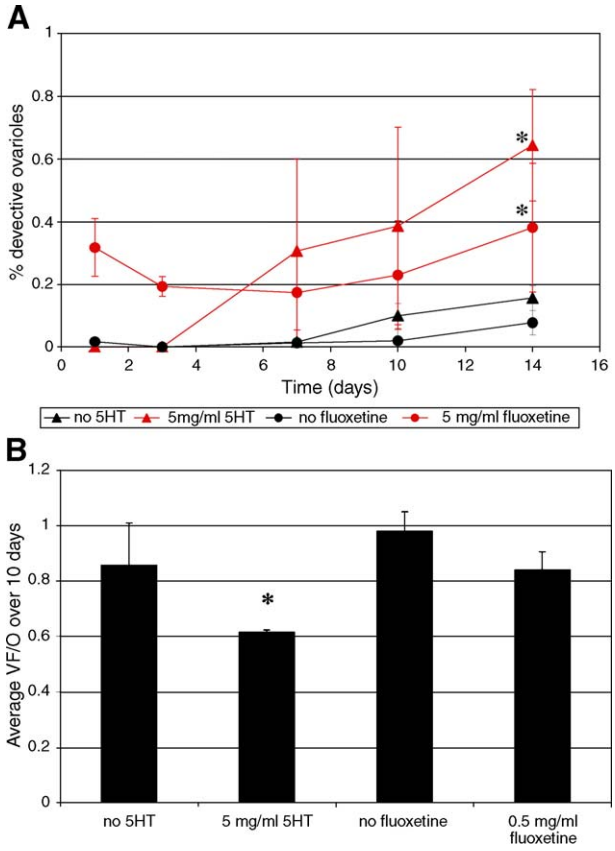

Fig. 8. Chronic effects of 5HT and fluoxetine feeding on follicle morphology and maturation. (A) Occurrence of follicle disruptions following chronic exposure to 5HT or fluoxetine. This graph displays the average percent of ovarioles with follicle formation defects at each time point after 5HT or fluoxetine feeding. Although a wide female-to-female variation in the frequency of follicle defects is evident through most of the time course of the drug treatments, significant occurrences resulted following 14-day exposures for both drugs (asterisks, Student's t test, $P < 0.05$ for fluoxetine feeding and $P = 0.05$ for 5HT feeding). (B) Chronic effects of 5HT and fluoxetine feeding on vitellogenesis. This bar graph plots the 10-day average VF/O for females fed plain food, 5 mg/ml 5HT or 0.5 mg/ml fluoxetine. 5HT treatment significantly reduced VF/O (asterisk, Student's t test, $P < 0.05$), while fluoxetine did not. Unlike the other instances of decreased VF/O (e.g. following cocaine or cocaine + DA exposure), 5HT-treated ovaries showed no sign of vitellogenic stage follicle degeneration (see text for explanation). Error bars indicate standard errors between replicate trials.

cocaine-induced ovarian phenotypes, albeit differentially, our experimental manipulations of DA and 5HT signaling could not address whether the effects of these neurotransmitters on the ovary were direct or indirect. Although some of the genes that encode enzymes of catecholamine biosynthetic pathways have been shown to be expressed in the ovary (Monastirioti et al., 1996; Neckameyer, 1998; Cole et al., 2005), a direct measurement of DA and 5HT in the ovary has not been reported. We used high performance liquid chromatography (HPLC) to search for biogenic amines in the ovary.

Using HPLC, we detected DA, but not 5HT in the ovary (Fig. 9). Each ovary contained, on average, 1.66 ± 0.23 pg DA. In contrast, ovary samples concentrated from as many as 13 ovaries did not contain any measurable 5HT. Thus, while DA is present in the ovary and could be acting there directly, 5HT signaling, either normally or in response to perturbation by cocaine, clearly originates outside of the ovary.

Chronic exposure to DA results in elevated ecdysteroid levels

Our failure to detect both of the classical neural targets of cocaine in the ovary suggested that hormonal communication between the nervous system and the ovary might be responsible for the effects of cocaine on oogenesis. And, since we had found that chronic cocaine exposure led to increased ecdysteroid levels, we used the RIA to measure ecdysteroid equivalents in whole fly extracts following DA or 5HT feeding. As had been found with low doses of cocaine alone, DA alone did not significantly change steroid hormone levels (Fig. 4). However, chronic simultaneous exposure to both DA (5 mg/ml) and cocaine (0.75 mg/ml) resulted in a significant increase in the levels of hormone. This was the same dosage combination of drugs that had significantly reduced follicle survival without disrupting follicle morphogenesis (Fig. 4), suggesting that DA is a link between cocaine exposure and the consequential effects on the ecdysone-sensitive checkpoint at vitellogenesis. Consistent with our conclusion that 5HT levels did not affect follicle progression through vitellogenesis, neither 5HT nor fluoxetine feeding resulted in detectable changes in ecdysone equivalents (Fig. 4). Thus, with respect to cocaine-sensitive long-range signaling, regulation of vitellogenesis appears to include DA together with steroid hormones, while regulation of folliculogenesis may require 5HT in combination with JH.

Discussion

Cellular and/or developmental effects of cocaine appear to result from distinct and separable perturbations of both DA and 5HT signaling, linked to coordinately distinct downstream hormone regulatory pathways. The individual cocaine-induced phenotypes described here were associated with the disruption of either DA or 5HT levels: decreased viability and activation of apoptosis at the vitellogenesis checkpoint were enhanced by increased DA, while follicle formation defects were phenocopied by increased 5HT. In contrast, cocaine's extensively studied and well-documented effects on behavior in vertebrates have pointed almost exclusively to the drug's effects on DA signaling (reviewed in Uhl et al., 2002). For example, DAT knockout mice show complete suppression of cocaine-induced hyperlocomotion (Giros et al., 1996), while a redundant function of SERT has been linked with reward behavior (Sora et al., 2001). Even in flies, DA has emerged as the most likely agent of cocaine's disruptive effects on locomotor behavior since DA-depleted flies show no signs of cocaine-induced hyperactivity (Bainton et al., 2000). Nevertheless, the involvement of 5HT or other amines has not been ruled out, and the fly model may provide an opportunity to separate subtle effects of 5HT modulations by cocaine from more dramatic phenotypes caused by changes in DA signaling. This is certainly the case in our developmental paradigm, where we successfully teased apart discrete effects of DA and 5HT and matched them with potential hormonal effectors. The elevated ecdysteroid levels that

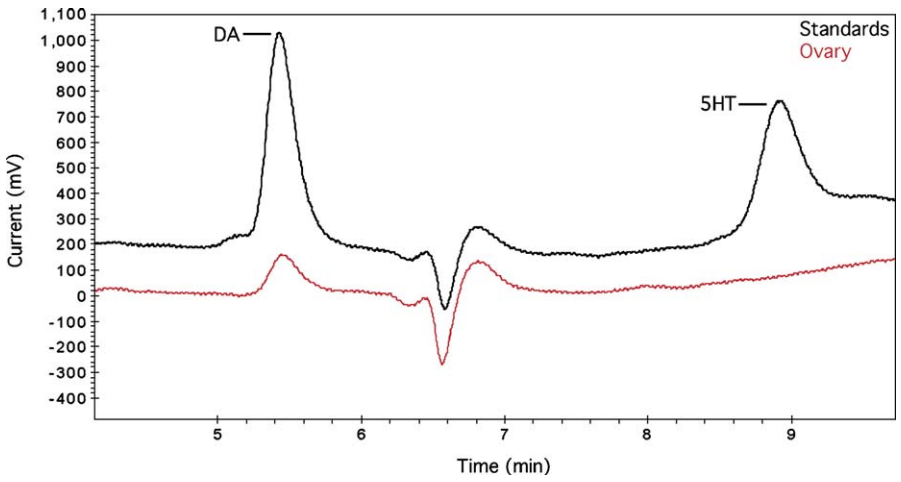

Fig. 9. HPLC detection of biogenic amines in the ovary. This trace was produced after subjecting ovary extracts to HPLC with electrochemical detection. This run represents approximately 13 ovaries. Standards: DA, dopamine; 5HT, serotonin.

resulted from concomitant chronic exposure to DA and cocaine identify this hormone as the likely effector of the DA-sensitive activation of apoptosis at the vitellogenesis checkpoint. Similarly, the fact that chronic cocaine exposure, 5HT feeding and blockage of dSERT by fluoxetine all showed phenotypes that were indistinguishable from the JH-enhanced cocaine phenotype may indicate that JH is the downstream effector of the 5HT-sensitive accumulation of follicle formation defects. These results suggest a generalized mechanism that could account for various cocaine-induced tissue/developmental effects in mammals. Such an explanation would be most obvious for tissues already known to rely on hormonal support, such as the reproductive organs. Previously described deleterious effects of cocaine on testes, spermatogenesis, sexual maturation and fertility (Chasnoff et al., 1985; Kaufmann et al., 1990; George et al., 1996; Walker et al., 2001; Li et al., 2005) are consistent with hormones being downstream effectors of the drug's actions for these phenotypes. Indeed, dramatic changes in steroid hormone levels have been measured following both acute and chronic cocaine treatments (Kaufmann et al., 1990; Walker et al., 2001). Although the precise mechanism by which cocaine modulates these hormone levels is not known, our results suggest the direct involvement of neurotransmitters, such as DA and 5HT.

The connection of both DA and ecdysteroids to cocaine-induced apoptosis of vitellogenic follicles fits well with the current understanding of the known 20E-sensitive follicle survival checkpoint. Normally, survival of vitellogenic follicles depends upon finely titrated levels of 20E, such that significant deviations in either direction trigger apoptosis at the onset of vitellogenesis (Shwartz et al., 1985; Soller et al., 1999). Although the vitellogenic checkpoint in *Drosophila* is sensitive to environmental sensors, such as temperature or nutritional status (reviewed in McCall, 2004), specific regulators of 20E titers in response to such environmental factors are not known. In other insect species, DA and/or other biogenic amines are responsible for modulating the levels of 20E, either positively or negatively (Hirashima et al., 1999); there may even be a feedback loop between 20E and DA to tweak the response (Gruntenko et al., 2005). Most likely, DA signaling normally regulates 20E levels in *Drosophila* as well. Thus, in the context of chronic cocaine exposure, increased DA signaling would lead to increased 20E synthesis with the subsequent activation of the follicle survival checkpoint. The results of our cocaine/DA feeding experiments and most of our RIA analyses are consistent with this interpretation. What appears to be inconsistent is our failure to detect significant changes in ecdysteroids at lower cocaine doses, despite their clear association with vitellogenic follicle loss. It is possible that our assay was not sensitive enough to detect the small changes in ecdysteroid titers that would have been induced in the lower cocaine dose feeding experiments. Alternatively, low doses of cocaine could activate the apoptosis checkpoint via an ecdysteroid-independent mechanism. Operation of the vitellogenesis survival checkpoint is known to be sensitive to insulin-like signals that are independent of both JH and 20E (Drummond-Barbosa and Spradling, 2001, 2004; LaFever and Drummond-Barbosa, 2005; Richard et al., 2005). Indeed, our demonstration that simultaneous reduction of both *EcR* and *InR* function protected against cocaine-induced follicle degeneration, while decreased signaling from either pathway alone did not, is consistent with a model in which these hormones both mediate cocaine's activation of the vitellogenesis checkpoint, albeit redundantly. Thus, at cocaine doses that do not appear to elevate ecdysteroids, drug-induced increases in insulin pathway activity could be responsible for the resulting loss of vitellogenic follicles.

The link to the cocaine-induced follicle phenotype that is shared by 5HT and JH not only suggests how cocaine exposure brings about those early morphological defects, but also reveals additional essential regulation of follicle formation and uncovers previously unrecognized regulatory functions for both of these molecules. Current models of follicle morphogenesis outline a complex array of interacting signal transduction and genetic regulatory pathways that originate and function primarily in the ovary itself (reviewed in Horne-Badovinac and

Bilder, 2005). However, there is clear evidence for extraovarian control through *stl* gene function, as well as from insulin-like signaling (Willard et al., 2004; LaFever and Drummond-Barbosa, 2005). Although the specific involvement of the *stl* gene product is unknown, some roles for insulin-like signaling have been identified. Just like 20E, insulin-like signaling has been noted as an indicator of environmental quality with respect to ovarian follicle maturation (Bownes, 1989), and it has also been shown to function in that capacity for ovarian follicle initiation (Drummond-Barbosa and Spradling, 2001). Both 20E and insulin-like signaling act long range to adjust oocyte production rates in response to unfavorable growth conditions: and, while both induce abortive apoptosis of vitellogenic follicles late (Drummond-Barbosa and Spradling, 2001; Soller et al., 1999), insulin also modulates stem cell division rates early (Drummond-Barbosa and Spradling, 2001, 2004). Although the insulin-like peptides that control those stem cell cycles have been shown to originate from neural secretory cells (LaFever and Drummond-Barbosa, 2005), the molecular control of peptide release is unknown. Our demonstration that cocaine causes an aberrant follicle formation phenotype that is enhanced by JH presents the possibility that neurotransmitter (5HT?) and/or hormone signaling modulate the balance between germline and somatic cell numbers. It is tempting to speculate that cocaine-sensitive monoamine signaling influences such long-range proliferation controls, perhaps including that provided by neural insulin.

Given the overlapping contributions to ovarian phenotypes that we have documented for biogenic amine and hormone signaling pathways, we propose that neural signals, such as DA and 5HT, normally fine tune hormone regulatory pathways that are important during follicle formation and maturation, and what remains to be established is where these neurotransmitters primarily act. Our detection of DA in ovary extracts suggests that DA signaling may contribute to the control of oocyte maturation by signaling to the ovary directly, with or without additional extraovarian activities. Current models postulate that neural projections direct to the ovary are responsible for aiding in ovulation and oviposition (Monastirioti et al., 1996; Monastirioti, 2003; Cole et al., 2005). We propose another function: DA released from these peripheral projections in response to neural signaling may lead to ecdysteroid biosynthesis and therefore allow proper follicle maturation. In accordance with this model, cocaine or DA-induced increases in ecdysteroids may have been caused by increased DA release from ovarian neural fibers and enhanced downstream signaling in the ovary. Importantly, ovarian samples prepared according to our HPLC preparative protocol included not only somatic and germline cells of the ovary, but also muscular epithelial and peritoneal sheath cells and the neural projections that contact them at the posterior of the ovary. And, although germline and/or somatic follicle cells appear to express several of the components of catecholamine biosynthesis (e.g. tyrosine hydroxylase and GP cyclohydroxylase: Neckameyer, 1996; Colas et al., 1999a), the general availability of downstream constituents of neurotransmitter signaling pathways (e.g. receptors) in this tissue has not been investigated. Thus, both the source(s) and the cellular target(s) of the ovarian DA discovered here remain to be identified. In contrast to the apparent "ovary-direct" involvement of DA during oocyte maturation, it appears to be extraovarian signaling by 5HT that is required for normal follicle morphogenesis since this neurotransmitter was undetectable in ovary extracts. Based on a recent report of antibody detection of 5HT in the cells of the muscular sheath that envelopes each ovariole (Heifetz and Wolfner, 2004), we might at least have expected to detect this neurotransmitter by HPLC since we did not remove the sheath prior to the preparation of the extracts for the assays. However, the isolated levels of 5HT in that one cell type might be insufficient to bring the tissue levels up to our detectable limit (>1 pg). Alternatively, 5HT might be taken up by or synthesized in sheath cells only transiently in response to stimulation, as from nearby neural projections (Cole et al., 2005). In other non-neural developmental processes, monoamine signaling has been clearly shown to participate similarly inexplicably in the regulation of morphogenetic events. For example, the 5HT receptor, $5HT_{2Dro}$, is critical for germband extension in the embryo (Colas et al., 1995, 1999b), and 5HT signaling has been implicated in morphogenetic movements and cell proliferation in a variety of other species (reviewed in Nebigil et al., 2001). However, the source of this early embryonic neurotransmitter has not been addressed in either flies or any other developmental system. Likewise for the regulation of follicle morphogenesis, the source(s) and cellular target(s) of 5HT signaling remain to be elucidated. For both DA and 5HT, however, since enhanced neurotransmitter activity can lead to deleterious effects on follicle formation or maturation, signaling is likely to be precisely modulated, regardless of where it originates or acts.

Finally, the effect of chronic cocaine exposure on adult and embryonic mortality could involve DA signaling. In *Drosophila* and many other organisms, aging is undoubtedly regulated by insulin-like signaling (reviewed in Tatar and Yin, 2001; Tatar, 2004). The removal of insulin-like receptor function in *Drosophila*, for example, dramatically extends adult lifespan (Tatar et al., 2001). Although our experiments did not address specific molecular mechanisms by which chronic cocaine exposure increases adult and embryonic mortality, the phenotypic links between DA and hormone regulatory pathways make it tempting to suspect mediation by insulin signaling. Future experiments will clarify this connection.

Acknowledgments

We are indebted to James T. Warren and Lawrence Gilbert for technical advice on the ecdysteroid RIA and for reagents, including the H22 antibody and the Cowan1 *S. aureus* strain. We also thank Shannon Hardie (Cole) and Jay Hirsh for assistance with HPLC, Michelle Neveklovska for performing the yeast control experiment, Catherine Wolkow for help with statistical analysis and Meghan Bernier for help with double-blind ovary scoring. This work was supported by a grant to C.C. from the National Institute on Drug Abuse. S.S.W. received

additional support from a National Institute for Child Health and Human Development training grant (HD07528-01) and a Ruth L. Kirschstein Memorial National Research Service Award (1 F31 DA06063-01).

References

Addis, A., Moretti, M.E., Ahmed Syed, F., Einarson, T.R., Koren, G., 2001. Fetal effects of cocaine: an updated meta-analysis. Reprod. Toxicol. 15, 341–369.

Bainton, R.J., Tsai, L.T., Singh, C.M., Moore, M.S., Neckameyer, W.S., Heberlein, U., 2000. Dopamine modulates acute responses to cocaine, nicotine and ethanol in *Drosophila*. Curr. Biol. 10, 187–194.

Bownes, M., 1989. The roles of juvenile hormone, ecdysone, and the ovary in the control of *Drosophila* vitellogenesis. J. Insect Physiol. 35, 409–413.

Chasnoff, I.J., Burns, W.J., Schnoll, S.H., Burns, K.A., 1985. Cocaine use in pregnancy. N. Engl. J. Med. 313, 666–669.

Colas, J.F., Launay, J.M., Kellermann, O., Rosay, P., Maroteaux, L., 1995. *Drosophila* 5-HT2 serotonin receptor: coexpression with fushi-tarazu during segmentation. Proc. Natl. Acad. Sci. U. S. A. 92, 5441–5445.

Colas, J.F., Launay, J.M., Maroteaux, L., 1999a. Maternal and zygotic control of serotonin biosynthesis are both necessary for *Drosophila* germband extension. Mech. Dev. 87, 67–76.

Colas, J.F., Launay, J.M., Vonesch, J.L., Hickel, P., Maroteaux, L., 1999b. Serotonin synchronises convergent extension of ectoderm with morphogenetic gastrulation movements in *Drosophila*. Mech. Dev. 87, 77–91.

Cole, S.H., Carney, G.E., McClung, C.A., Willard, S.S., Taylor, B.J., Hirsh, J., 2005. Two functional but noncomplementing *Drosophila* tyrosine decarboxylase genes: distinct roles for neural tyramine and octopamine in female fertility. J. Biol. Chem. 280, 14948–14955.

Cummings, C.A., Cronmiller, C., 1994. The *daughterless* gene functions together with *Notch* and *Delta* in the control of ovarian follicle development in *Drosophila*. Development 120, 381–394.

Drummond-Barbosa, D., Spradling, A.C., 2001. Stem cells and their progeny respond to nutritional changes during *Drosophila* oogenesis. Dev. Biol. 231, 265–278.

Drummond-Barbosa, D., Spradling, A.C., 2004. Alpha-endosulfine, a potential regulator of insulin secretion, is required for adult tissue growth control in *Drosophila*. Dev. Biol. 266, 310–321.

Egred, M., Davis, G.K., 2005. Cocaine and the heart. Postgrad. Med. J. 81, 568–571.

Fajemirokun-Odudeyi, O., Lindow, S.W., 2004. Obstetric implications of cocaine use in pregnancy: a literature review. Eur. J. Obstet. Gynecol. Reprod. Biol. 112, 2–8.

George, V.K., Li, H., Teloken, C., Grignon, D.J., Lawrence, W.D., Dhabuwala, C.B., 1996. Effects of long-term cocaine exposure on spermatogenesis and fertility in peripubertal male rats. J. Urol. 155, 327–331.

Gilbert, L.I., Granger, N.A., Roe, R.M., 2000. The juvenile hormones: historical facts and speculations on future research directions. Insect Biochem. Mol. Biol. 30, 617–644.

Gilbert, L.I., Rybczynski, R., Warren, J.T., 2002. Control and biochemical nature of the ecdysteroidogenic pathway. Annu. Rev. Entomol. 47, 883–916.

Giros, B., Jaber, M., Jones, S.R., Wightman, R.M., Caron, M.G., 1996. Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. Nature 379, 606–612.

Gruntenko, N.E., Karpova, E.K., Adonyeva, N.V., Chentsova, N.A., Faddeeva, N.V., Alekseev, A.A., Rauschenbach, I.Y., 2005. Juvenile hormone, 20-hydroxyecdysone and dopamine interaction in *Drosophila virilis* reproduction under normal and nutritional stress conditions. J. Insect Physiol. 51, 417–425.

Hardie, S.L., Hirsh, J. (in press) An improved method for the separation and detection of biogenic amines in adult *Drosophila* brain extracts by high performance liquid chromatography. J. Neurosci. Methods.

Harvey, J.A., 2004. Cocaine effects on the developing brain: current status. Neurosci. Biobehav. Rev. 27, 751–764.

Heifetz, Y., Wolfner, M.F., 2004. Mating, seminal fluid components, and sperm cause changes in vesicle release in the *Drosophila* female reproductive tract. Proc. Natl. Acad. Sci. U. S. A. 101, 6261–6266.

Hirashima, A., Hirokado, S., Ohta, H., Suetsugu, E., Sakaguchi, M., Kuwano, E., Taniguchi, E., Eto, M., 1999. Titres of biogenic amines and ecdysteroids: effect of octopamine on the production of ecdysteroids in the silkworm *Bombyx mori*. J. Insect Physiol. 45, 843–851.

Hirsh, J., 2001. Time flies like an arrow. Fruit flies like crack? Pharmacogenomics J. 1, 97–100.

Hombria, J.C., Brown, S., 2002. The fertile field of *Drosophila* Jak/STAT signalling. Curr. Biol. 12, R569–R575.

Horne-Badovinac, S., Bilder, D., 2005. Mass transit: epithelial morphogenesis in the *Drosophila* egg chamber. Dev. Dyn. 232, 559–574.

Kaufmann, R.A., Savoy-Moore, R.T., Sacco, A.G., Subramanian, M.G., 1990. The effect of cocaine on oocyte development and the follicular microenvironment in the rabbit. Fertil. Steril. 54, 921–926.

King, R.C., 1970. Ovarian Development in *Drosophila melanogaster*. Academic Press, New York, NY.

LaFever, L., Drummond-Barbosa, D., 2005. Direct control of germline stem cell division and cyst growth by neural insulin in *Drosophila*. Science 309, 1071–1073.

Li, H., Jiang, Y., Rajpurkar, A., Dunbar, J.C., Dhabuwala, C.B., 1999. Cocaine induced apoptosis in rat testes. J. Urol. 162, 213–216.

Li, H., Xu, L., Dunbar, J.C., Dhabuwala, C.B., 2003. Role of mitochondrial cytochrome c in cocaine-induced apoptosis in rat testes. Urology 61, 646–650.

Li, G., Xiao, Y., Zhang, L., 2005. Cocaine induces apoptosis in fetal rat myocardial cells through the p38 mitogen-activated protein kinase and mitochondrial/cytochrome c pathways. J. Pharmacol. Exp. Ther. 312, 112–119.

Lin, H., 2002. The stem-cell niche theory: lessons from flies. Nat. Rev., Genet. 3, 931–940.

Lopez-Schier, H., 2003. The polarisation of the anteroposterior axis in *Drosophila*. Bioessays 25, 781–791.

McCall, K., 2004. Eggs over easy: cell death in the *Drosophila* ovary. Dev. Biol. 274, 3–14.

Monastirioti, M., 2003. Distinct octopamine cell population residing in the CNS abdominal ganglion controls ovulation in *Drosophila melanogaster*. Dev. Biol. 264, 38–49.

Monastirioti, M., Linn Jr., C.E., White, K., 1996. Characterization of *Drosophila* tyramine beta-hydroxylase gene and isolation of mutant flies lacking octopamine. J. Neurosci. 16, 3900–3911.

Nebigil, C.G., Etienne, N., Schaerlinger, B., Hickel, P., Launay, J.M., Maroteaux, L., 2001. Developmentally regulated serotonin 5-HT2B receptors. Int. J. Dev. Neurosci. 19, 365–372.

Neckameyer, W.S., 1996. Multiple roles for dopamine in *Drosophila* development. Dev. Biol. 176, 209–219.

Neckameyer, W.S., 1998. Dopamine modulates females sexual receptivity in *Drosophila melanogaster*. J. Neurogenetics 12, 101–114.

Porzgen, P., Park, S.K., Hirsh, J., Sonders, M.S., Amara, S.G., 2001. The antidepressant-sensitive dopamine transporter in *Drosophila melanogaster:* a primordial carrier for catecholamines. Mol. Pharmacol. 59, 83–95.

Postlethwait, J.H., Giorgi, F., 1985. Vitellogenesis in insects. Dev. Biol. 1, 85–126.

Postlethwait, J.H., Kunert, C.J., 1986. Endocrine and genetic regulation of vitellogenesis in *Drosophila*. Prog. Clin. Biol. Res. 205, 33–52.

Richard, D.S., Watkins, N.L., Serafin, R.N., Gilbert, L.I., 1998. Ecdysteriods regulate yolk protein uptake by *Drosophila melanogaster* oocytes. J. Insect Physiol. 44, 637–644.

Richard, D.S., Jones, J.M., Barbarito, M.R., Cerula, S., Detweiler, J.P., Fisher, S.J., Brannigan, D.M., Scheswohl, D.M., 2001. Vitellogenesis in diapausing and mutant *Drosophila melanogaster*: further evidence for the relative roles of ecdysteroids and juvenile hormones. J. Insect Physiol. 47, 905–913.

Richard, D.S., Rybczynski, R., Wilson, T.G., Wang, Y., Wayne, M.L., Zhou, Y., Partridge, L., Harshman, L.G., 2005. Insulin signaling is necessary for vitellogenesis in *Drosophila melanogaster* independent of the roles of juvenile hormone and ecdysteroids: female sterility of the chico1 insulin

signaling mutation is autonomous to the ovary. J. Insect Physiol. 51, 455–464.

Ritz, M.C., Lamb, R.J., Goldberg, S.R., Kuhar, M.J., 1987. Cocaine receptors on dopamine transporters are related to self-administration of cocaine. Science 237, 1219–1223.

Rocha, B.A., 2003. Stimulant and reinforcing effects of cocaine in monoamine transporter knockout mice. Eur. J. Pharmacol. 479, 107–115.

Roth, S., 2001. *Drosophila* oogenesis: coordinating germ line and soma. Curr. Biol. 11, R779–R781.

Rothman, R.B., Baumann, M.H., 2003. Monoamine transporters and psychostimulant drugs. Eur. J. Pharmacol. 479, 23–40.

Shwartz, M.B., Kelly, T.J., Iberski, R.B., Rubenstein, E.C., 1985. The effects of nutrition and methoprene treatment on ovarian ecdysteroid synthesis in *Drosophila melanogaster*. J. Insect Physiol. 31, 947–957.

Smith Jr., J.E., Cummings, C.A., Cronmiller, C., 2002. *Daughterless* coordinates somatic cell proliferation, differentiation and germline cyst survival during follicle formation in *Drosophila*. Development 129, 3255–3267.

Soller, M., Bownes, M., Kubli, E., 1997. Mating and sex peptide stimulate the accumulation of yolk in oocytes in *Drosophila melanogaster*. Eur. J. Biochem. 243, 732–738.

Soller, M., Bownes, M., Kubli, E., 1999. Control of oocyte maturation in sexually mature *Drosophila* females. Dev. Biol. 208, 337–351.

Sora, I., Hall, F.S., Andrews, A.M., Itokawa, M., Li, X.F., Wei, H.B., Wichems, C., Lesch, K.P., Murphy, D.L., Uhl, G.R., 2001. Molecular mechanisms of cocaine reward: combined dopamine and serotonin transporter knockouts eliminate cocaine place preference. Proc. Natl. Acad. Sci. U. S. A. 98, 5300–5305.

Spradling, A., 1993. Developmental genetics of oogenesis. In: Bate, M., Arias, A.M. (Eds.), The Development of *Drosophila melanogaster*, vol. 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 1–71.

Tatar, M., 2004. The neuroendocrine regulation of *Drosophila* aging. Exp. Gerontol. 39, 1745–1750.

Tatar, M., Yin, C., 2001. Slow aging during insect reproductive diapause: why butterflies, grasshoppers and flies are like worms. Exp. Gerontol. 36, 723–738.

Tatar, M., Kopelman, A., Epstein, D., Tu, M.P., Yin, C.M., Garofalo, R.S., 2001. A mutant *Drosophila* insulin receptor homolog that extends life-span and impairs neuroendocrine function. Science 292, 107–110.

Uhl, G.R., Hall, F.S., Sora, I., 2002. Cocaine, reward, movement and monoamine transporters. Mol. Psychiatry 7, 21–26.

Walker, Q.D., Francis, R., Cabassa, J., Kuhn, C.M., 2001. Effect of ovarian hormones and estrous cycle on stimulation of the hypothalamo–pituitary–adrenal axis by cocaine. J. Pharmacol. Exp. Ther. 297, 291–298.

Warren, J.T., Gilbert, L.I., 1988. Radioimmunoassay: ecdysteroids. In: Gilbert, L.I. (Ed.), Immunological Techniques in Insect Biology. Springer-Verlag, New York, NY, pp. 181–214.

Willard, S.S., Ozdowski, E.F., Jones, N.A., Cronmiller, C., 2004. *stall*-mediated extrinsic control of ovarian follicle formation in *Drosophila*. Genetics 168, 191–198.

Wolf, F.W., Heberlein, U., 2003. Invertebrate models of drug abuse. J. Neurobiol. 54, 161–178.