Sexually dimorphic effects of ancestral exposure to vinclozolin on stress reactivity in rats

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How an individual responds to the environment depends upon both personal life history as well as inherited genetic and epigenetic factors from ancestors. Using a 'two-hit, 3 generations apart' model, we tested how F3 descendants of rats given in utero exposures to the environmental endocrine-disrupting chemical (EDC) vinclozolin reacted to stress during adolescence in their own lives, focusing on sexually dimorphic phenotypic outcomes. In adulthood, male and female F3 vinclozolin- or vehicle-lineage rats, stressed or non-stressed, were behaviorally characterized on a battery of tests, then euthanized. Serum was used for hormone assays, and brains for qPCR and transcriptome analyses. Results showed that the effects of ancestral exposure to vinclozolin converged with stress experienced during adolescence in a sexually dimorphic manner. Debilitating effects were seen at all levels of the phenotype, including physiology, behavior, brain metabolism, gene expression, and genome-wide transcriptome modifications in specific brain nuclei. Additionally, females were significantly more vulnerable than males to transgenerational effects of vinclozolin on anxiety but not sociality tests. This fundamental transformation occurs in a manner neither predicted by the ancestral exposure or the proximate effects of stress during adolescence, an interaction we refer to as synchronicity.

The legacy of exposures to environmental chemicals, including endocrine-disrupting chemicals (EDCs), has permanently altered the present and future health of humans and wildlife 1–4. Direct exposure to EDCs affects the life history of individuals and their descendants. Such 'context-dependent' epigenetic modifications are not heritable per se as the germ cells are not affected. Other epigenetic modifications can affect the germline ('germline-dependent') and thus manifest each generation even in the absence of the causative agent (1–9). This is the case for several EDCs, notably, vinclozolin (10–12), bisphenol A (BPA; 13, 14), and tributyltin (15). Such transgenerational modifications affect all levels of biological organization, from gene regulation to behavioral interactions of conspecifics (13, 16–18).

Environmental and social stressors are an immediate and primary source of context-dependent epigenetic modifications (19–22). Chronic or excessive stress during sensitive periods such as prenatal development or the early postnatal period can predispose for disease and disorder later in life, a phenomenon known as the fetal basis of adult disease (23, 24). Later developmental periods, including adolescence, also have enduring effects that include neural remodeling, sensitivity to drugs of abuse, impaired learning and memory, and emotional disorders in adulthood (24–31).

Most neurobehavioral, neurological, and addiction disorders exhibit significant sex differences in relative risk level and severity. Women have higher levels of diseases and disorders such as Alzheimer's disease, dementia, major depressive disorder, posttraumatic stress disorders, anxiety and panic disorders (32); whereas autism-spectrum disorder and attention deficit hyperactivity disorder predominate in men (33). Developmental sex differences

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Abbreviations:

in adrenal and reproductive hormones must play a role in their etiology because, in many instances, disorders manifest following adrenarche/puberty, fluctuate during menstrual cycles, and change prevalence/severity at menopause or during times of stress, suggesting involvement of adrenal and gonadal steroid hormones (34–38). Environmental exposures to compounds that disrupt these hormonal systems have had a major impact on health and are believed to be a contributing factor to the increased incidence of obesity, illness, and affective disorders in humans (1, 39, 40).

Vinclozolin is a commonly used fungicide with demonstrated antiandrogenic properties (41, 42). When administered to human cancer cell lines in vitro it prevents the androgen receptor (Ar) from binding androgen response elements (ARE), thereby inhibiting the androgen receptor's ability to act as a transcription factor (43). Vinclozolin administered by either intraperitoneal injection or orally is metabolized into two primary metabolites (M1 and M2), both of which are effective against preventing the Ar from binding its targeted AREs (42). Exposure to vinclozolin during organizational periods of embryonic development results in feminized males with reduced anogenital distance and hypospadias (41); as adults these males exhibit reduced number of testis cords, increased apoptosis of immature sperm, and decreasing sperm motility, all of which result in decreased fertility (44). Some aspects of this disease phenotype persist for up to four generations in the absence of further exposure (10).

Previous work from our laboratories reveal significant interactions of transgenerational vinclozolin effects inherited from the great-grandparents with stress experienced in adolescence (45). That work was limited to males, overlooking fundamental sex differences in reproductive investment, behavior, and survival. In addition to the individual effects of ancestral exposure to vinclozolin and stress during adolescence, we were particularly interested in the combination of these two events, a phenomenon we refer to as synchronicity. We demonstrate here that the sexes differ markedly in their responses to these two types of epigenetic modifications, and that the synchronicity of ancestral exposure and proximate stress during one's own lifetime results in profound sex differences in the scope and nature of reactivity.

Materials and Methods

Animals. Male and female Sprague-Dawley (SD) rats, vinclozolin- and vehicle (DMSO)-lineage, were bred at Washington State University (10). An F0 generation of pregnant mothers (vinclozolin, n = 3; control n = 2) was injected i.p. with vinclozolin (100 mg/kg/d) or DMSO (vehicle) on embryonic days 8 – 14 (**Figure 1A**). Subsequent generations were bred such that there was no sibling or cousin inbreeding. F3 generation pups with no chemical body burden were weaned at postnatal day (PND) 21, implanted s.c. with an identification microchip (AVID, Norco, CA) and shipped to the University of Texas at Austin the following day. All animal protocols were approved at both Washington State University and the University of Texas at Austin. Upon arrival, animals were pair-housed with a same sex individual of the opposing lineage (eg, a control-lineage male with a vinclozolin-lineage male). Cage-mates were all within one-day separation in age. Dyads were housed in standard translucent polycarbonate rat cages (46 cm x 24 cm x 20.5 cm) with ad libitum access to tap water and standard rat chow (RMH 1800 from Purina) and environmental enrichment (7 cm diameter PVC pipe). The colony room was on a 14:10 light:dark cycle with lights off at 0830. Animals were handled and weighed twice a week (**Supplemental Figure 1**).

Chronic Restraint Stress in Adolescence and Behavioral Testing in Adulthood. Chronic restraint stress (CRS) during adolescence and behavioral testing of adults were conducted in a separate room as described in detail (45), transported in covered cages, and tested under dim red light or white light depending on the test. CRS was conducted for 6 hours daily for 21 days from PND 23–44 (**Figure 1A**).

Behavioral tests were conducted in a counterbalanced order and were administered to each individual beginning on PND 90. Behavioral tests consisted of open-field, sociability, and social novelty as described previously in earlier vinclozolin work (45–Supplemental Methods). In addition, the light-dark box and elevated plus maze were added to provide more thorough behavioral characterization. The test for sociability was performed to measure social approach, anxiety, and exploration. The social novelty test immediately followed, enabling animals to differentiate between the now familiar conspecific and a novel, unfamiliar conspecific, as a test for a rat's preference for social novelty (46, 47). The open field test is a standard measure of anxiety, taking advantage of a rat's natural aversion to exploration of a central lit area and the preference to spend time around the edges (48, 49). Similarly, a light-dark box test measures anxiety-like behaviors and aversion to exploration in a brightly lit compared to a dark chamber (50). The elevated plus maze allows a rat to spend time in an enclosed runway compared to an open runway that is raised off the ground, again providing information about anxiety-like behaviors (51). Details for how these standard behaviors were performed are provided in Supplemental Methods. Stimulus rats used for social tests were naïve, intact, same-sex and age-matched SD rats (Harlan, Indianapolis, IN). All behaviors were quantified using the video tracking system (ANY-maze) and apparatus from Stoelting (Wood Dale, IL). After all behavioral testing was completed, between PND 118–124, animals were euthanized by decapitation as per approved protocols.

Brain Collection and Selection of Brain Regions. The brain was quickly removed and processed as previously described (45). Briefly, each brain was split in the sagittal plane, with one sagittal half frozen for sectioning and cytochrome oxidase histochemistry (CO), and the other cut (2 mm) on a chilled rat brain matrix

for later micropunching of specific nuclei for mRNA and transcriptome analyses. The following regions of the amygdala, hippocampus, and hypothalamus were the focus of this study, selected for their interconnectivity and functional relationships in the controls of the behaviors studied herein: basolateral amygdaloid nucleus (BLA), central amygdaloid nucleus (CeAmy), medial amygdaloid nucleus (MeAmy), anterior cortical amygdaloid nucleus (CoAmy), CA1 and the CA3 of the hippocampus (CA1 and CA3, respectively), medial preoptic area (mPOA), ventromedial hypothalamic nucleus (VMN) and bed nucleus of the stria terminalis (BnST).

Brain Metabolic Activity. Cytochrome oxidase histochemistry, a measure of brain metabolic activity (52) was measured in 13 discrete neural nuclei (**Supplemental Table 1**). CO histochemistry is well-established as relating to overall metabolic history within a cell, as has been shown for learning and memory, sexual activity, and developmental experience (53). As published previously (45), in brief, fresh frozen brain tissue was cryosectioned and mounted. Slices containing nuclei of interest were incubated in a heated bath containing 3,3′-Diaminobenzidine (DAB) and saturated with oxygen. Cytochrome C within the tissue oxidizes DAB, which turns from clear to light brown. The slices were imaged (Javelin) using a constant intensity light box (Northern Light R95) and the optical density of defined nuclei was measured. The abundance of cytochrome C in brain tissue has been tightly linked to metabolic activity and therefore neuronal activity. Thus, the amount of DAB that is oxidized and its resulting optical density is used as an index of metabolic history.

Gene expression analysis. Brain nuclei were punched from frozen coronal hemisections using a 1 mm diameter Palkovits punch. Ten regions were used for qPCR, and of these, four were used for further transcriptome analysis (**Supplemental Table 1**). RNA extraction, purification, and preparation for the cDNA reaction were performed based on standard procedures, as detailed in Supplemental Methods and as published (54). Gene expression analysis was performed using a 48-gene qPCR platform, the Taqman low-density PCR array (TLDA; Life Technologies, Foster City, CA) that enables detection of all 48 genes in microdissected brain regions of individual rats (54). The choice of genes for the qPCR panel was based on prior work from this laboratory and others, and are listed in **Table 1**, with full gene names in **Supplemental Table 2** (55, 56). Specific gene selection was based on a priori hypotheses about molecules involved in the neurobiological responses to EDCs; predicted sex differences; and relationships between selected genes and behaviors measured in the same rats. Selected genes fell in several functional groups including epigenetic modification, stress signaling, steroid hormones, and growth factors. TLDA qPCR results were quantified using a ViiA7 PCR machine (Life Technologies, Foster City, CA) as published (54). As a secondary level of analysis, transcriptome analysis was conducted on pooled samples in 4 of the brain regions (BLA, CeAmy, BnST, CA3) using the Affymet-

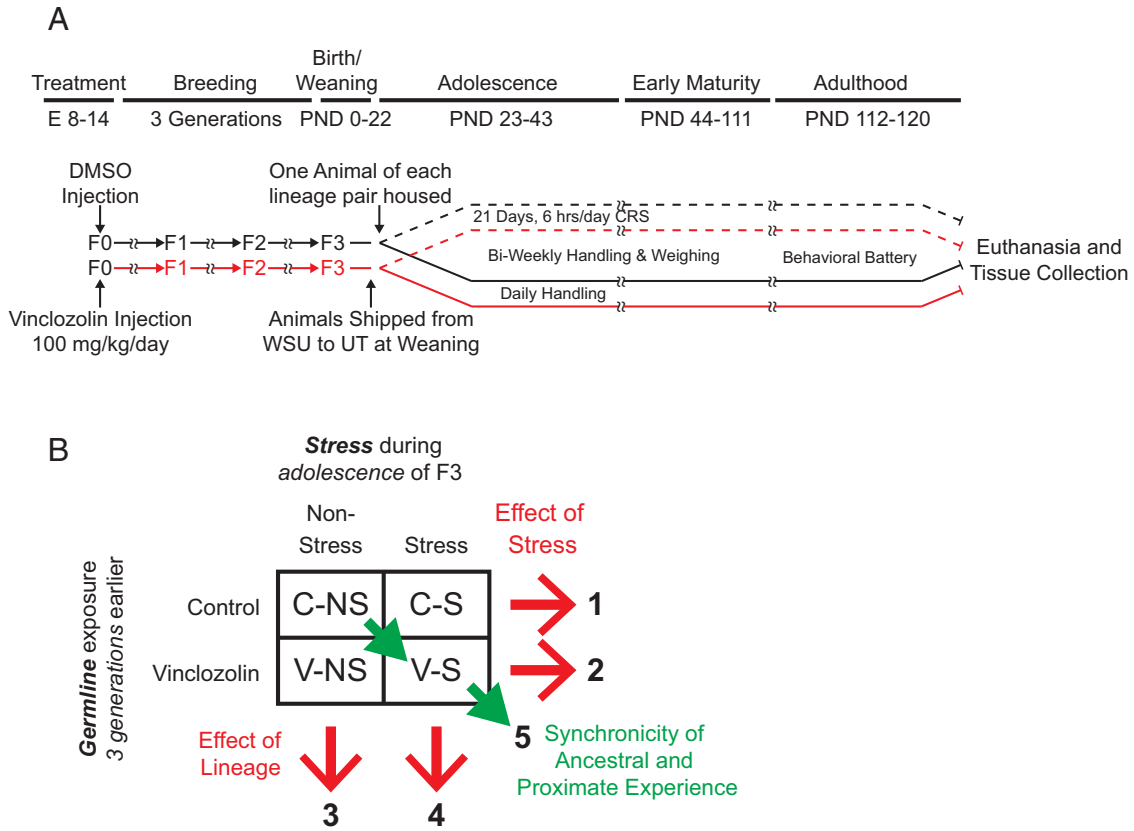


Figure 1. Experimental model and categorization of effects of ancestral vinclozolin vs. control lineage and chronic restraint stress in rats. A) Experimental flowchart. Rats were exposed to vinclozolin or vehicle (DMSO) control three generations previously, and the F3 generation used for chronic restraint stress during adolescence or no stress. All animals were then behaviorally tested as adults. B) Experimental groups and comparisons. Planned comparisons are shown, with *Comparisons 1, 3 and 5* the focal group of the study.

Table 1. Genes on the TLDA.

Housekeeping genes: *18S, Gapdh*
Epigenetic Modifiers: *Dnmt1, Dnmt3a, Dnmt3b, Dnmt3l, Hdac1, Mbd2*
Stress Signaling: *Crh, Crh1, Gmeb2, Mc3r, Mcr4r, Mc5r, Nr3c1, Pomc*
Steroidogenic Enzymes: *Cyp19a1, Hsd11b2, Srd5a1*
Sex Steroid Hormone Receptors: *Ar, Esr1, Esr2, Gnrhr, Gper, Pgr*
Dopaminergic: *Comt, Drd2, Drd4, Th*
Serotonergic: *Slc6a4*
Glutamatergic: *Gria1, Gria2, Grik2, Grin1, Grin2a, Grin2b, Grin2c, Grin2d*
GABAergic: *Gad1, Gad2*
Neuropeptides and Receptors: *Avp, Avp1a, Kiss1, Kiss1r, Lepr, Oxt, Oxtr, Tac2*
Growth Factors: *Bdnf, Ctgr, Igf1, Igf1r, Igfbp2, Igfbp5, Negr1, Ptgds, S100a4, Tgfa, Tgfb1*
Transcription Factors: *Nfkb1, Nrf1, Per2*

The genes measured by qPCR on a custom designed TLDA listed by functional group. Full gene names can be found in **Supplemental Table 2**.

ric Rat Gene 1.0 ST arrays, with all methods and analyses identical to those published by our laboratories (45 – Supplemental Material; *Supplemental Methods*).

Hormone Assays. A terminal trunk blood sample was collected, centrifuged, and frozen for hormone analyses, each performed following manufacturers' protocols. The three sex steroid hormones (estradiol, progesterone, and testosterone) were selected based on previous work for their disruption by vinclozolin (45, 57, 58). Corticosterone was also measured because a primary dependent endpoint of this study was chronic restraint stress, which affects adrenal glucocorticoid levels and is sexually dimorphic (59–61). For each assay, standards were processed in duplicate and samples in triplicate. In a few cases, outliers within a triplicate were removed using Grubb's test. Serum corticosterone was measured in 10 μl of serum from individual rats in a single RIA (MP Biomedicals, Santa Ana, CA). Intra-assay CV was 3%, and assay sensitivity was 25 ng/mL. Serum estradiol was measured in 200 μl of serum in a single RIA (Beckman-Coulter, Brea, CA). Intra-assay CV was 6.7% and assay sensitivity was 5 pg/mL. Progesterone serum concentrations were determined via an EIA in 1 μl serum for females and 25 μl for males (Cayman Chemical, Ann Arbor, MI). Intra-assay CV was 4.9%, and assay sensitivity was 7.8 pg/mL. Serum testosterone was measured by EIA (Cayman Chemical) using 200 μl serum for females and 6 μl for males. Intra-assay CV was 7.6% and assay sensitivity was 3.9 pg/mL.

Statistics. Initial analyses of individual datasets were performed first, and this revealed that datasets had non-normal distributions and heterogeneous variance, determined by Shapiro-Wilk test for normality and Levene's test for the equality of variance, respectively. Therefore, Kruskal-Wallis (KW) nonparametric analysis was performed to determine a main effect of EDC lineage (vinclozolin vs. control) and of stress (vs. nonstress) for each endpoint. Subsequent comparisons within sex and between groups were performed via a pair-wise Mann-Whitney U tests between independent groups and appropriately controlled for using a Benjamini-Hochberg correction form multiple comparisons where appropriate. Functional landscape analysis of correlated traits was performed as per (62) to provide a visual representation of the relationships among multiple variables between groups of individuals, allowing for a more concise presentation of data. As such, this captures the nature of relationships pictorially. However, this analysis in no way intended to replace individual statistical analyses. It is important to note that in the behavioral tests each node within the landscape represents a composite of several measures, whereas the bar graphs represent an individual measure within a task. This is not the case for the functional landscapes of brain metabolism.

Results

Five sets of comparisons were performed as shown in **Figure 1B**: control nonstress (C-NS) vs. control stress (CS; *Comparison 1*); C-NS vs. V-NS (*Comparison 3*); and C-NS vs. V-S (*Comparison 5*). *Comparisons 1, 3 and 5* are considered first order effects. *Comparison 1* gives an index of stress vs. nonstress in control rats; *Comparison 3* gives an index of control vs. vinclozolin lineage in nonstressed rats; and *Comparison 5* represents the synchronicity of stress and vinclozolin, c.f. control nonstressed rats. For graphed data, statistical details are presented in the figures and tables; for data not shown, p-values are indicated in the text.

Hormones and Adrenal Weight. Stress and ancestral vinclozolin had effects on serum testosterone and progesterone in a sex-dependent manner (**Figure 2**). Estradiol was unaffected (**Figure 2B**). For testosterone (T), in the nonstressed females, vinclozolin lineage rats had lower T than control-lineage females (*Comparison 3*, **Figure 2A**). Serum progesterone (P4) showed a main effect of stress in females only, with higher P4 in stressed than nonstressed females of both lineages (**Figure 2C**). Finally, adrenal weight was measured because it is sexually dimorphic, has been shown to be affected by vinclozolin treatment (57, 63), and because CRS is well-established as affecting adrenal weight as we previously reported in an earlier study on males (45). In both sexes there were main lineage effects, with adrenal weights lower in vinclozolin- than control-lineage rats (females $P = .008$ and males $P = .025$,

Figure 2E). Further, in both sexes, V-S rats had significantly lower adrenal weights compared to C-NS (*Comparison 5*) counterparts.

Behaviors. The Stoelting Any-Maze apparatuses allow for the observation and quantification of multiple measures within each specific behavioral test. While numerous behaviors were quantified, the large number precludes presentation of every aspect. Therefore, the measure that best discriminated the experimental groups was chosen for purposes of graphic presentation and used in the functional landscapes.

Open field, light-dark transition, and elevated plus maze behaviors were differentially affected in males and females by stress and lineage. Representative data of one individual component for each behavior are shown in **Figure 3**. For corner time in the open field test, the synchronicity of stress and lineage (*Comparison 5*) was affected in females, with V-S spending more time in corners compared to C-NS (**Figure 3A**). Corner time in males was affected only in the control lineage (*Comparison 1*), with C-S showing lower corner time than C-NS.

For the light-dark box, number of entries into the light chamber was greater in male C-S than C-NS (*Comparison 1*, **Figure 3B**). Vinclozolin-lineage males and females showed a delayed initial entry into the dark chamber, compared to control counterparts (data not shown; $P < .01$). Also, V-S males exhibited a longer latency to enter the dark chamber compared to C-NS males (*Comparison 5*; data not shown, $P = .03$).

Elevated plus maze behavior was affected only in males (**Figure 3C**), with V-S spending more time in the closed arm than C-NS (*Comparison 5*). Furthermore, V-S males

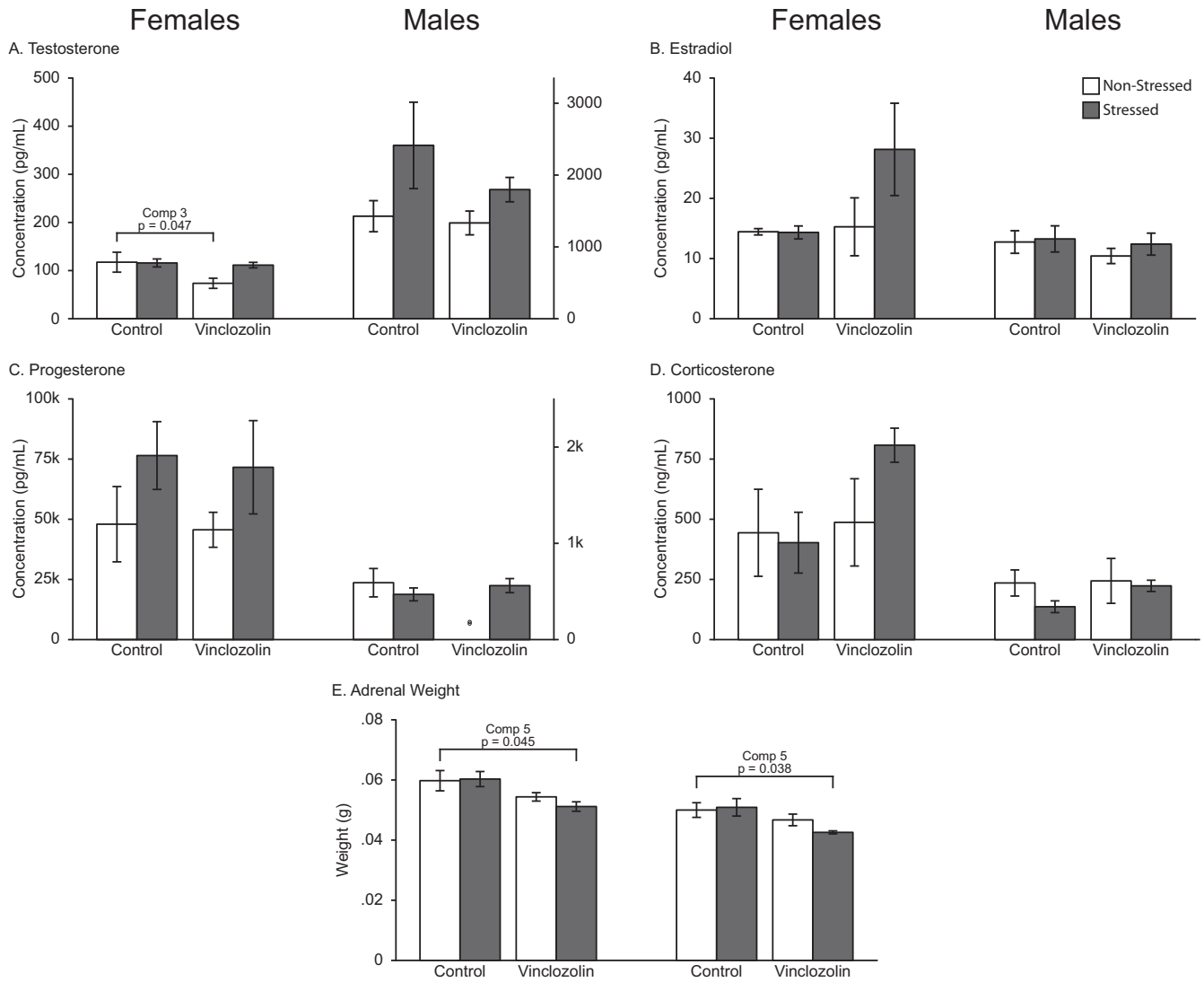


Figure 2. Serum testosterone (A), estradiol (B), progesterone (C), corticosterone (D), and adrenal weights (E) are shown. Significant pair-wise comparisons are shown within the figure. Main effects: progesterone (C) was increased in females due to CRS ($P = .048$), adrenal weight was decreased in both females and males due to vinclozolin lineage ($P = .008$ and $P = .025$ respectively).

spent more time in the closed arm of the elevated plus maze ($P = .02$), displayed a slower mean speed ($P = .02$), and covered a shorter distance ($P = .02$), than their C-NS counterparts (*Comparison 5*, data not shown).

Behaviors in the sociability and social novelty test were also quantified (**Supplemental Figure 2**). In the test for sociability, both males and females preferred to associate with an animal as opposed to an empty cage ($P < .001$, **Supplemental Figure 2A**). In a test for social novelty, females preferred to spend more time with a novel animal opposed to a familiar one ($P = .005$). Males showed no preference for social novelty (**Supplemental Figure 2B**).

Functional landscape analysis was performed to provide an integrative perspective of behaviors related to anxiety, activity, and sociability for the first-order *Comparisons 1, 3 and 5* (**Figures 4A, 4C, and 4E**, respectively). For *Comparison 1*, there are few differences between C-S and C-NS females, whereas males differ for composite behaviors for light-dark and open field tests. More effects of stress were seen in females compared to males for *Comparison* 3. Finally, females and males were differentially affected in the synchronicity model of *Comparison 5*.

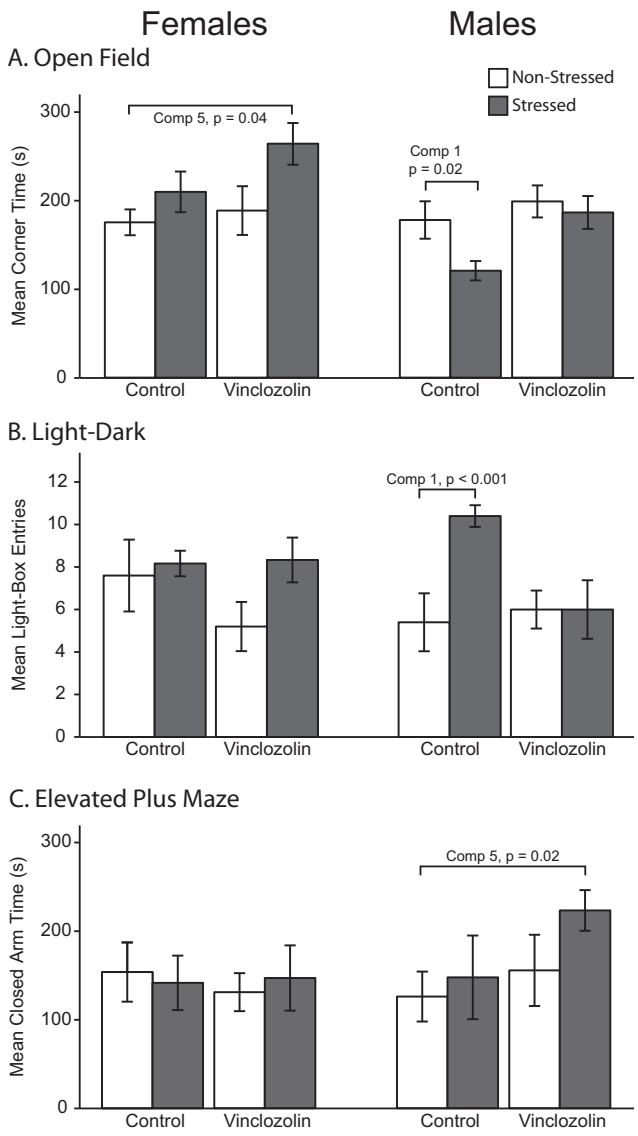


Figure 3. Behavioral measures of interest. A) Open Field: time spent in the corner of the arena indicating decreased exploration and increased anxiety. B) Light-Dark Box: number of entries into the light box indicating increased exploration and decreased anxiety. C) Elevated Plus: Time spent in the closed arm indicating decreased exploration and increased anxiety.

Brain Metabolism. Cytochrome oxidase histochemistry was used as an index of brain metabolism (45, 52) and data are graphed as functional landscapes in **Figure 4B, 4D, and 4F** (*Comparisons 1, 3, 5* respectively). For *Comparison 1*, which focuses on effects of stress in control rats (C-S vs. C-NS; **Figure 4B**), C-S males showed increased metabolic activity compared to C-NS males in the BnST ($P = .02$), while females (C-S) showed increased activity compared to C-NS in the MPOA ($P = .03$) and BnST ($P = .04$). For *Comparison 3*, effects of vinclozolin in non-stressed rats (C-NS vs. V-NS; **Figure 4D**), C-NS males showed decreased activity compared to V-NS males in the CA1 and CA3 ($P = .04$ and $P = .02$), while V-NS females showed increased activity compared to C-NS females in the MeAmy ($P = .04$), CoAmy ($P = .02$), and VMN ($P = .04$).

Gene Expression and Transcriptome. Brain micropunches were analyzed by TLDA and the relative expression of 48 selected genes was analyzed (listed in **Supplemental Table 2**) in each of 10 selected brain regions (**Supplemental Table 1**). Results of affected genes are shown for the female CA3, CA1 and BnST (**Figure 5**), chosen because these regions had most robust effects for *Comparisons 1, 3 and 5*. A summary of identified genes and directionality of effect for these three comparisons is presented in Table 2 and showing sex- and region-specific differences in affected genes. Additional gene expression data are shown in **Supplemental Figure 3** for the male BLA, and for the male and female lateral hypothalamus.

In the female CA3 (**Figure 5A**), expression of a large number of genes were significantly different between V-S and C-NS (*Comparison 5*): *Crhr1*, *Drd2*, *Tgfa*, *Esr1*, and *Esr2* (gene abbreviations are listed in **Supplemental Table 2**). In all cases, expression was higher in V-S than C-NS. In addition, there was significantly higher expression of 3 genes in V-NS than C-NS (*Comparison 3*): *Esr1*, *Gnrhr*, and *Ptgds*.

In the female CA1 (**Figure 5B**), expression of *Ar* was lower in C-S than C-NS (*Comparison 1*), and for *Esr1*, expression was higher in V-NS than C-NS (*Comparison 3*). Finally, 3 genes were affected in the female BnST (**Fig-**

ure 5C). *Mc4r* and *Pomc* were lower in V-S than C-NS females (*Comparison 5*) and *Th* was higher in V-NS than C-NS females (*Comparison 3*).

Prior published research on a similar experimental model has reported in-depth transcriptome analysis in response to ancestral vinclozolin (64), and CRS in males (45). In the current study we performed transcriptome analysis in aliquots of RNA from the same samples used for TLDA, in four selected brain nuclei: BLA, BnST, CeAmy, and CA3 (**Supplemental Table 1**) to compare with TLDA results (**Figure 6**). A cut-off of a 1.2-fold change (transcriptome) and a 1.5-fold change (TLDA) was applied for identified genes with the goal of supporting the results obtained from the more targeted TLDA analysis and to identify candidate networks for future analysis. Because samples were pooled for transcriptome, but were individually assayed by TLDA, only general information about directionality of change with reference to C-NS can be drawn. Patterns of expression were very different between the sexes (**Figure 6C**). The TLDA analysis identified the female CA3 as having an over represented number of affected genes, especially for *comparisons 3 and 5*, an effect that was largely confirmed by transcriptome analysis, albeit with the caveat that samples were pooled for transcriptome analysis.

Discussion

Vinclozolin is a well-established endocrine disruptor when administered during critical developmental life stages. Due to its antiandrogenic properties, male offspring of dams treated with vinclozolin during pregnancy show a demasculinized morphological (reduced anogenital distance, nipple retention, ectopic testis and vaginal pouches, and agenesis of the prostate) and behavioral (failure to attain intromission or ejaculate with a receptive female) phenotype (42). Female rats display reduced anogenital distance (41); later in life they exhibit uterine hemorrhaging and anemia during pregnancy, and increased tumor formation (65).

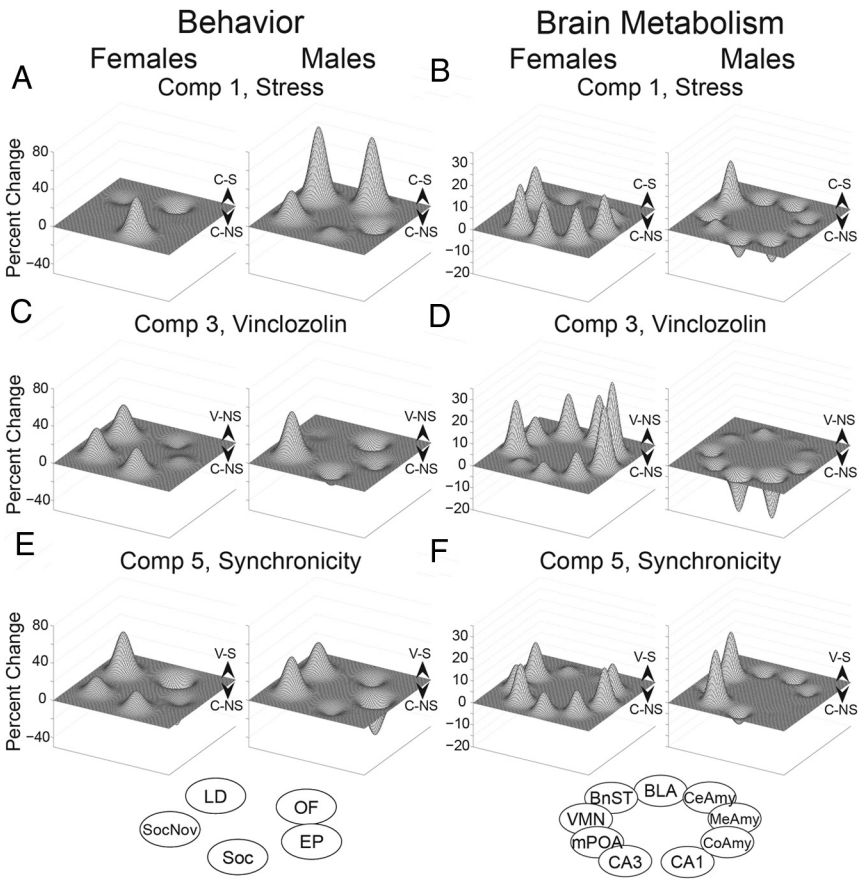


Figure 4. Functional landscapes of behaviors and brain metabolism (CO activity) are shown for Comparison 1 (4A and 4B), Comparison 3 (4C and 4D) and Comparison 5 (4E to 4F), respectively, in male and female rats. A relative increase for one group over the other is shown by the directionality and height of a peak or valley. For behaviors (4A, 4C, and 4E), the clockwise nodes are: Light:Dark Box (LD), Elevated Plus Maze (EP), Open-Field (OF), Sociability (SOC), and Social Novelty (SOCNOV). For brain metabolism (4B, 4D, and 4F), the clockwise nodes shown are: basolateral amygdaloid nucleus (BLA), central amygdaloid nucleus (CeAmy), medial amygdaloid nucleus (MeAmy), anterior cortical amygdaloid nucleus (CoAmy), CA1 and the CA3 of the hippocampus (CA1 and CA3, respectively), medial preoptic area (mPOA), ventromedial hypothalamic nucleus (VMN) and bed nucleus of the stria terminalis (BnST).

In addition to its direct effects, vinclozolin was the first environmental EDC shown to have transgenerational effects (10, 16, 64, 66). Although another laboratory reported transgenerational actions of vinclozolin on expression of imprinted genes (12), other laboratories have not replicated these findings (67, 68), underscoring the need for more research and careful attention to experimental differences. However, interand transgenerational effects of other endocrine disruptors, such as bisphenol A (13, 69), diethylstilbestrol (mouse –70; human –71) and PCBs (72) have been reported, adding further evidence that EDCs affect multiple generations, up to and beyond the F3 generation.

Previously, we showed that ancestral exposure to vinclozolin caused behavioral differences in the F3 male descendants when challenged with

CRS (45). The present study was predicated on our understanding of fundamental sex differences in the developing nervous system governed by steroid exposures and functional outcomes on brain structure, neurochemistry, and numerous behaviors, both reproductive and otherwise (73). While many effects of prenatal EDCs are sex-specific (74–77), little is known about sex-specific transgenerational EDC effects. This, together with observations that CRS elicits sex-specific responses in physiology and behavior, and expression of genes and proteins in related brain regions (78–80), led to our current

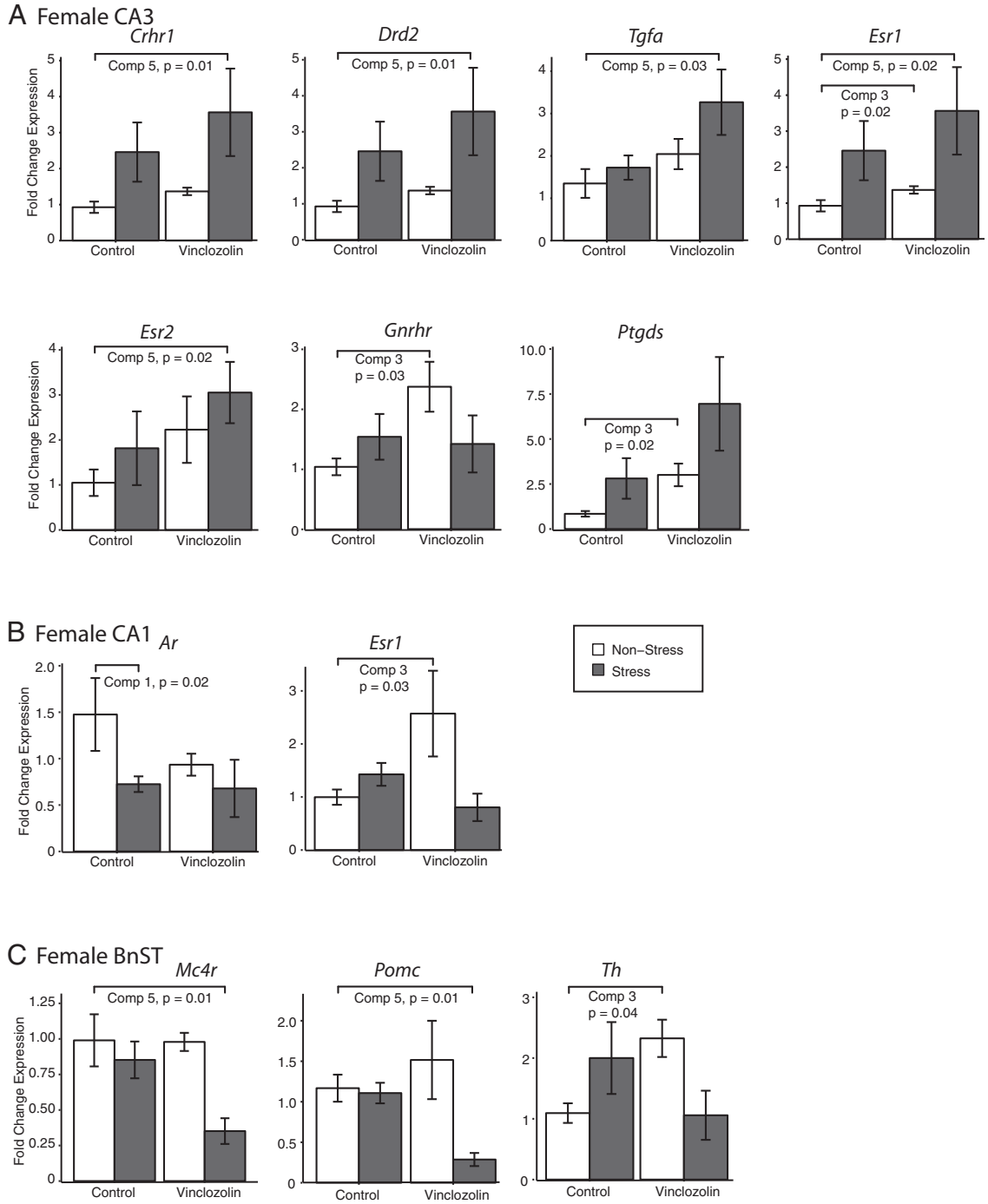


Figure 5. Gene expression results of affected genes identified by TLDA qPCR assays are shown for the female CA3 (A), CA1 (B), and BnST (C). A) In the CA3, 5 genes had higher expression in V-S than C-NS females (*Comparison 5*) and 3 genes had higher expression in V-NS than C-NS females (*Comparison 3*). B) In the CA1, *Ar* mRNA levels were lower in C-S than C-NS females (*Comparison 1*) and *Esr1* mRNA was higher in V-NS than C-S females (*Comparison 3*). In the BnST, *Mc4r* and *Pomc* mRNA levels were lower in V-S than C-NS females (*Comparison 5*) and for *Th*, mRNA levels were higher in V-NS than C-NS females (*Comparison 3*).

Table 2. Gene Expression **Males**

	CA3			CA1			CeA			BLA			BnST			LH		
	Stress	Vincl	V&S	Stress	Vincl	V&S	Stress	Vincl	V&S	Stress	Vincl	V&S	Stress	Vincl	V&S	Stress	Vincl	V&S
Comparison	1	3	5	1	3	5	1	3	5	1	3	5	1	3	5	1	3	5
Gene																		
Avp																	↑ 0.03	↑ 0.03
Bdnf											↑ 0.02							
Oxt																		↑ 0.02
Negr1												↑ 0.02						
Pgr												↑ 0.02						

Females

	CA3			CA1			CeA			BLA			BnST			LH		
	Stress	Vincl	V&S	Stress	Vincl	V&S	Stress	Vincl	V&S	Stress	Vincl	V&S	Stress	Vincl	V&S	Stress	Vincl	V&S
Comparison	1	3	5	1	3	5	1	3	5	1	3	5	1	3	5	1	3	5
Gene																		
Ar				↓ 0.02														
Crhr1			↑ 0.01															
Drd2			↑ 0.01															
Esr1		↑ 0.02	↑ 0.02		↓ 0.03													
Esr2			↑ 0.02															
Gnrhr		↑ 0.03																
Lepr							↓ 0.02											
Mc4r															↓ 0.01			
Pomc															↓ 0.01	↓ 0.03		↓ 0.03
Ptgds		↑ 0.02																
Tgfa			↑ 0.03															
Th														↑ 0.04				

Summary of mRNA expression of targeted genes, selected for their roles in social, affiliative, and anxiety-related behavioral tests, and predicted to be regulated by endocrine disruptors and/or stress, for Comparisons 1, 3 and 5. Six hypothalamic and hippocampal nuclei with significant gene expression effects are shown. Differences are shown relative to the C-NS group and are at least 2-fold in magnitude. Note that each region has unique gene expression changes by sex, treatment and stress. Region abbreviations: CA3 and CA1 – areas of the hippocampus, CeAmy – central amygdaloid nucleus; BLA – basolateral amygdaloid nucleus; BnST – bed nucleus of the stria terminalis; LH – lateral hypothalamic nuclei. Gene abbreviations: *Ar* – androgen receptor, *Avp* – arginine vasopressin, *Bdnf* – brain-derived neurotrophic factor, *Drd2* – dopamine receptor D2, *Esr1* – estrogen receptor α, *Esr2* – estrogen receptor β, *Gnrhr* – gonadotropin releasing hormone receptor, *Lepr* – leptin receptor, *Mc4r* – melanocortin 4 receptor, *Negr1* – neuronal growth factor, *Oxt* – oxytocin prepropeptide, *Pomc* – proopiomelanocortin, *Pgr* – progesterone receptor, *Ptgds* – prostaglandin D2 synthase, *Tgfa* – transforming growth factor α, *Th* – tyrosine hydroxylase. Up-arrows indicate a significant increase, and Down-arrows represent a significant decrease, relative to the C-NS group.

work combining transgenerational effects of vinclozolin with CRS in males and females.

Behavioral effects of proximate adolescent stress and ancestral exposure to vinclozolin

In our study, males exposed to CRS in adolescence showed decreased anxiety behaviors in adulthood. Results of the open field and light-dark box tests demonstrated greater exploration and decreased aversion to potentially dangerous and stressful situations. No such differences caused by CRS were found in females. These results suggest that males and females differ in the compensatory strategies acquired with early life stress.

By contrast, ancestral exposure to vinclozolin was associated with few behavioral effects in either control (non-stressed) males or females, a finding that was surprising as previous work showed that ancestral vinclozolin increased anxiety behaviors in young females in an elevated plus maze and decrease anxiety behaviors of young males in a light dark box (64). Differences between the laboratory environments (current work conducted at UT-Austin, past work at WSU) and age at testing may contribute to divergent experimental outcomes. Additionally, the limited number of animals used in the current study, derived from relatively few litters, likely play a role in differences.

Other endocrine disruptors have been implicated in modifying anxiety responses. BPA consistently increases anxiety behavior in rats as measured by the elevated plus and open-field, regardless of sex, in F1 animals (81, 82). These results have been replicated in mice and extended to social behaviors and the light-dark box (83). While BPA has a different mechanism of action, targeting different steroid hormone receptors, these results provide further support for transgenerational effects of EDCs on a suite of behaviors.

The combined effect of ancestral vinclozolin and proximate CRS, or their "synchronicity", fell into two general behavioral outcomes: first, measures that were influenced by CRS that were abolished by vinclozolin, and second, those measures that neither CRS nor ancestral exposure to vinclozolin alone predicted. In the case of the open-field test, synchronicity was limited to females, with increased anxiety behavior in the V-S group compared to C-NS group. Synchronicity in males was seen in the elevated plus maze, again with increased levels in the V-S group. Neither of these outcomes could be predicted by either ancestral EDC exposure or CRS.

Social behavior was not strongly affected by vinclozolin or CRS, nor was there evidence of synchronicity. In the

sociability test in which rats distinguished between a conspecific and an empty cage, both males and females showed increased time in the social chamber compared to the empty cage. A sex difference was seen in the social novelty test, where females showed a strong preference for the novel rat while males spent equal time investigating a familiar and novel animal. No robust effects were seen with either lineage or CRS. These results are in contrast to our previous report where C-NS males showed a preference for social novelty (45), likely due to sample size.

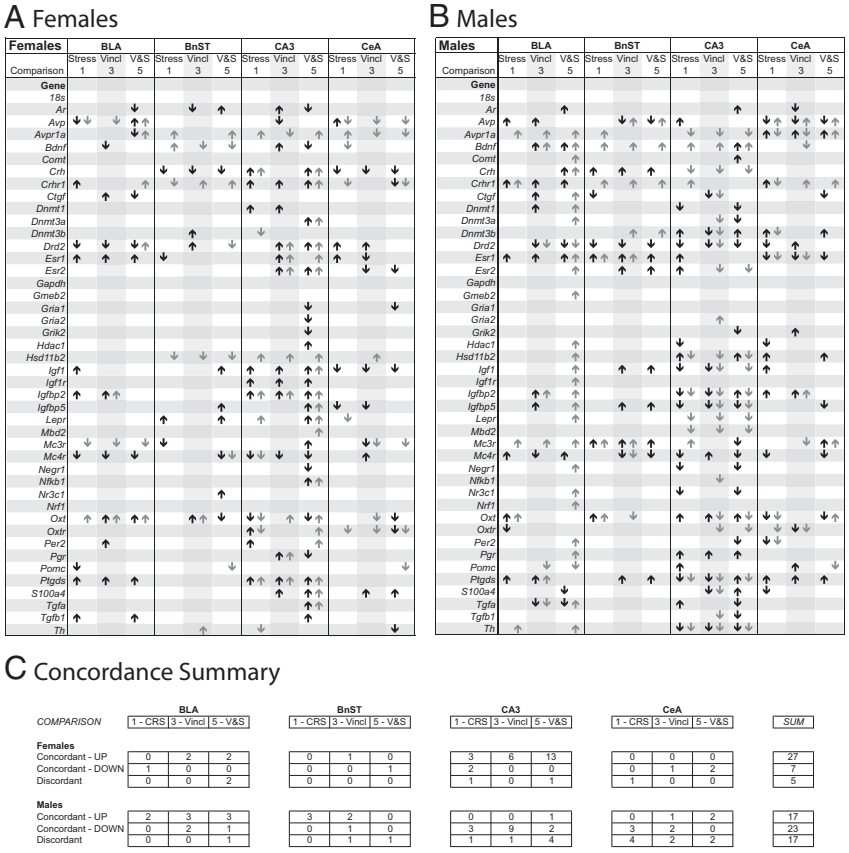
Social behavior alterations have been noted for other EDCs like BPA which affects both directly exposed animals (F1) and transgenerational lineages (F3-F4) but there has been little investigation into the social effects of vinclozolin (69, 84). The effect of vinclozolin on social affiliation has been limited to the F1 generation, where vinclozolin increased juvenile play behavior in male rats (85). It is likely that social affiliation behavior contains aspects of anxiety and risk taking. Considering that stress has lasting effects on anxiety behavior in both sexes, it is interesting that investigation of the stimulus animals in the sociability and social novelty tests was not affected. Anxiety plays a role in social interactions, but not when stimulus animals are confined and cannot engage in complete tactile interactions. Because of this, it is possible to deconstruct interest and motivation to investigate conspecifics from sociality.

Metabolic Brain Activity

We found substantial effects of ancestral vinclozolin and CRS as determined by CO histochemistry, as well as substantial sex differences. Throughout the brain nuclei measured, females showed an increased metabolic profile due to stress and vinclozolin treatment while males showed a decrease of lesser magnitude than the increase seen in females. Stress increases activity of the BnST in males and females, a brain area essential in the communication between the amygdala and hypothalamus, likely representing a long-term compensatory mechanism to deal with future stress. Ancestral vinclozolin exposure affects distinct but related groups of nuclei in males (hippocampal) and females (amygdaloid). These results demonstrate that males and females show a difference in how the activity of neural circuitry within each sex compensates for both ancestral and life challenges.

Gene Expression

Gene expression results demonstrated sex differences in patterns of expression and regulation by vinclozolin ancestry and/or CRS, with far more effects in females, especially in CA3, CA1, and BnST. Of these, the CA3 of the female hippocampus were usually up-regulated by vinclozolin. Among these were the estrogen receptors alpha and beta that mediate effects of estradiol on neuronal activity in the hippocampus (86, 87). The *Crhr1* and *Drd2* receptors were also up regulated, genes that are involved in cognitive function and stress reactivity in the hippocampus (88, 89). Last, the growth



C Concordance Summary

COMPARISON	BLA 1 - CRS	BLA 3 - Vincl	BLA 5 - V&S	BnST 1 - CRS	BnST 3 - Vincl	BnST 5 - V&S	CA3 1 - CRS	CA3 3 - Vincl	CA3 5 - V&S	CeA 1 - CRS	CeA 3 - Vincl	CeA 5 - V&S	SUM
Females													
Concordant - UP	0	2	2	0	1	0	3	6	13	0	0	0	27
Concordant - DOWN	1	0	0	0	0	1	2	0	0	0	1	2	7
Discordant	0	0	2	0	0	0	1	0	1	1	0	0	5
Males													
Concordant - UP	2	3	3	3	2	0	0	0	1	0	1	2	17
Concordant - DOWN	0	2	1	0	1	0	3	9	2	3	2	0	23
Discordant	0	0	1	0	1	1	1	1	4	4	2	2	17

Figure 6. Taqman low density PCR array (TLDA) and transcriptome comparisons. Correspondence between expression patterns in genes identified by PCR-based TLDA (Black) and genome-wide transcriptome (Gray) is shown for affected genes in the four brain regions used for both TLDA and transcriptome (Basolateral Amygdala, Bed nucleus of the Stria Terminalis, Central Amygdala, and CA3 of the hippocampus). Data are shown for females (A) and males (B). Columns show differences with C-NS individuals as the reference group. Up arrows indicate up-regulation while down arrows indicate down-regulation. Cut-off criteria were > 1.5 fold change (TLDA) and > 1.2 fold change (transcriptome). C) Summary of concordance, defined as measures that were in the same direction (upregulated or downregulated). Discordant changes were those for which the two measures did not agree.

factors *Ptgds* and *Tgfa* were up-regulated suggesting altered neuronal growth and remodeling (90, 91). Together, these expression changes suggest wholesale reprogramming of neurobiological gene expression, an effect that is manifested 3 generations later.

Previous work on EDCs has demonstrated effects on hippocampal gene expression, albeit mostly in the F1 generation. PCB exposure during gestation caused a delayed growth profile in the hippocampus indicated by dampened expression of neural proliferation genes (92). BPA affects the expression profile of neural proliferation genes multiple generations after exposure (93). Additionally, PCBs modulate neurotransmitter abundance in the hippocampus, including dopamine (94). Together, these results suggest that EDCs have particularly potent effects on the hippocampus and its growth and proliferation profile, with possible effects on memory, learning, and spatial navigation.

Far fewer gene expression effects were found in the male. In the BLA, three genes were up-regulated in the vinclozolin lineage (V-NS or V-S) compared to C-NS. The BLA is involved in modulating fear and anxiety responses (95). Two of the three genes altered in the BLA are growth factors (*Bdnf* and *Negr1*). Increased growth factor expression in the BLA has been implicated in stress-induced structural plasticity, which may relate to the increased anxiety behaviors observed in V-S males.

Summary and Conclusions

Animals and humans are exposed to a milieu of man-made and natural EDCs that can alter the trajectory of development and cause detrimental behavioral deficits through multiple generations. How an individual is able to respond to challenges during its lifetime can dictate the probability of reproductive success and survival. Here we show males and females differ in their behavioral response to these challenges. Identified alterations in metabolic history and gene expression may give clues as to the mechanisms responsible and provide targets for more focused analysis.

The process of life has a past and a present that, together, establish the parameters of future potential. Most experiments in this realm manipulate a single, or limited number of factors to explore how challenges disrupt the individual. A large subset of these experiments have employed a "two-hit" model fashioned after the classic 'organizational-activational' paradigm from developmental biology in which a perturbation early in development is followed by a second perturbation, usually later in life, to explore the impact of these sequential events on physiological and/or behavioral parameters. Our studies are unlike these latter studies in that that the separation between events is generations, not days or months within a lifetime. Our studies invite comparison to the classic nature-nurture problem or Gene X Environment studies. That is, vinclozolin exposure during embryonic life of the F1 generation becomes incorporated into the germline by epigenetic modifications, thereby becoming heritable or the 'nature' or 'G' component. In the third generation offspring, who completely lack any body burden of vinclozolin, some individuals experience of CRS during adolescence that lead to epigenetic modifications that affect the nature and quality of later life; these are the 'nurture' or 'E' component.

The novelty of this study lies not in the nature of the effects (first order or main effects of ancestral exposure to vinclozolin or CRS). Rather it identifies how they transform the individual when they occur together (second order effects or interactions and a third order effect, synchronicity). Further, the divergent effects seen among the sexes of F3 adults is dramatic, indicating that the consequences of global contamination and stressful experiences encountered by living descendants is likely to have its own specific risk for males and females for a given spectrum of adverse outcomes. Importantly, the gene expression pat-

Table 3. Genes on the TLDA.

Housekeeping genes: *18S, Gapdh*
Epigenetic Modifiers: *Dnmt1, Dnmt3a, Dnmt3b, Dnmt3l, Hdac1, Mbd2*
Stress Signaling: *Crh, Crh1, Gmeb2, Mc3r, Mcr4r, Mc5r, Nr3c1, Pomc*
Steroidogenic Enzymes: *Cyp19a1, Hsd11b2, Srd5a1*
Sex Steroid Hormone Receptors: *Ar, Esr1, Esr2, Gnrhr, Gper, Pgr*
Dopaminergic: *Comt, Drd2, Drd4, Th*
Serotonergic: *Slc6a4*
Glutamatergic: *Gria1, Gria2, Grik2, Grin1, Grin2a, Grin2b, Grin2c, Grin2d*
GABAergic: *Gad1, Gad2*
Neuropeptides and Receptors: *Avp, Avp1a, Kiss1, Kiss1r, Lepr, Oxt, Oxtr, Tac2*
Growth Factors: *Bdnf, Ctgr, Igf1, Igf1r, Igfbp2, Igfbp5, Negr1, Ptgds, S100a4, Tgfa, Tgfb1*
Transcription Factors: *Nfkb1, Nrf1, Per2*

The genes measured by qPCR on a custom designed TLDA listed by functional group. Full gene names can be found in **Supplemental Table 2**.

terns generally support the functional behavioral differences that were observed. The implications of these results for the protection of human health and endocrine-based questions target questions of morbidity and the quality of life (QOL).

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References

1. **Crews D, McLachlan JA.** Epigenetics, evolution, endocrine disruption, health, and disease. *Endocrinology*. 2006;147(6 Suppl):S4–10. doi:10.1210/en.2005–1122.
2. **Diamanti-Kandarakis E, Bourguignon J-P, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC.** Endocrine-disrupting chemicals: an Endocrine Society scientific statement. In: Vol 30. 2009:293–342. doi:10.1210/er.2009–0002.
3. **Landrigan PJ.** What causes autism? Exploring the environmental contribution. *Curr Opin Pediatr*. 2010;22(2):219–225. doi:10.1097/MOP.0b013e328336eb9a.
4. **Landrigan PJ, Miodovnik A.** Children's health and the environment: an overview. *Mt Sinai J Med*. 2011;78(1):1–10. doi:10.1002/msj.20236.
5. **Crews D.** Epigenetics and its implications for behavioral neuroendocrinology. *Front Neuroendocrinol*. 2008;29(3):344–357. doi:10.1016/j.yfrne.2008.01.003.
6. **Whitehead A, Crawford DL.** Variation within and among species in gene expression: raw material for evolution. *Mol Ecol*. 2006;15(5):1197–1211. doi:10.1111/j.1365–294X.2006.02868.x.
7. **Whitehead A, Crawford DL.** Neutral and adaptive variation in gene expression. *PNAS*. 2006;103(14):5425–5430. doi:10.1073/pnas.0507648103.
8. **Whitehead A, Roach JL, Zhang S, Galvez F.** Genomic mechanisms of evolved physiological plasticity in killifish distributed along an environmental salinity gradient. *PNAS*. 2011;108(15):6193–6198. doi:10.1073/pnas.1017542108.
9. **Kidd KA, Blanchfield PJ, Mills KH, Palace VP, Evans RE, Lazorchak JM, Flick RW.** Collapse of a fish population after exposure to a synthetic estrogen. *PNAS*. 2007;104(21):8897–8901. doi:10.1073/pnas.0609568104.
10. **Anway MD, Cupp AS, Uzumcu M, Skinner MK.** Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science*. 2005;308(5727):1466–1469. doi:10.1126/science.1108190.
11. **Skinner MK, Manikkam M, Guerrero-Bosagna C.** Epigenetic transgenerational actions of environmental factors in disease etiology. *Trends Endocrinol Metab*. 2010;21(4):214–222. doi:10.1016/j.tem.2009.12.007.
12. **Stouder C, Paoloni-Giacobino A.** Transgenerational effects of the endocrine disruptor vinclozolin on the methylation pattern of imprinted genes in the mouse sperm. *Reproduction*. 2010;139(2):373–379. doi:10.1530/REP-09–0340.
13. **Wolstenholme JT, Edwards M, Shetty SR, Gatewood JD, Taylor JA, Rissman EF, Connelly JJ.** Gestational exposure to bisphenol A produces transgenerational changes in behaviors and gene expression. *Endocrinology*. 2012;153:3828–3838. doi:10.1210/en.2012–1195.
14. **Salian S, Doshi T, Vanage G.** Impairment in protein expression profile of testicular steroid receptor coregulators in male rat offspring perinatally exposed to Bisphenol A. *Life Sci*. 2009;85(1–2):11–18. doi:10.1016/j.lfs.2009.04.005.
15. **Chamorro-García R, Sahu M, Abbey RJ, Laude J, Pham N, Blumberg B.** Transgenerational Inheritance of Increased Fat Depot Size, Stem Cell Reprogramming, and Hepatic Steatosis Elicited by Prenatal Exposure to the Obesogen Tributyltin in Mice. *Environ Health Perspect*. 2013;121(3):359–366. doi:10.1289/ehp.1205701.
16. **Crews D, Gore AC, Hsu TS, Dangleben NL, Spinetta M, Schallert T, Anway MD, Skinner MK.** Transgenerational epigenetic imprints on mate preference. *PNAS*. 2007;104(14):5942–5946. doi:10.1073/pnas.0610410104.
17. **Crews D.** The (bi)sexual brain. Science, Society Series on Sex and Science. *EMBO Rep*. 2012;13(9):779–784. doi:10.1038/embor.2012.107.
18. **Kundakovic M, Gudsnuk K, Franks B, Madrid J, Miller RL, Perera FP, Champagne FA.** Sex-specific epigenetic disruption and behavioral changes following low-dose in utero bisphenol A exposure. *PNAS*. 2013. doi:10.1073/pnas.1214056110.
19. **Hunter RG, McEwen BS.** Stress and anxiety across the lifespan: structural plasticity and epigenetic regulation. *Epigenomics*. 2013;5(2):177–194. doi:10.2217/epi.13.8.
20. **Karatsoreos IN, McEwen BS.** Resilience and vulnerability: a neurobiological perspective. *F1000prime reports*. 2013;5:13. doi:10.12703/P5–13.
21. **McEwen BS.** Brain on stress: how the social environment gets under the skin. *PNAS*. 2012;109 Suppl 2(Supplement 2):17180–17185. doi:10.1073/pnas.1121254109.
22. **McEwen BS.** Neuroscience. Hormones and the social brain. *Science*. 2013;339(6117):279–280. doi:10.1126/science.1233713.
23. **Heindel JJ.** The fetal basis of adult disease: Role of environmental exposures—introduction. *Birth Defects Research Part A: Clinical and Molecular Teratology*. 2005;73(3):131–132. doi:10.1002/bdra.20119.
24. **Meaney MJ.** Epigenetics and the biological definition of gene x environment interactions. *Child Dev*. 2010;81(1):41–79. doi:10.1111/j.1467–8624.2009.01381.x.
25. **Jankord R, Solomon MB, Albertz J, Flak JN, Zhang R, Herman JP.** Stress vulnerability during adolescent development in rats. *Endocrinology*. 2011;152(2):629–638. doi:10.1210/en.2010–0658.
26. **Romeo RD.** Adolescence: a central event in shaping stress reactivity. *Dev Psychobiol*. 2010;52(3):244–253. doi:10.1002/dev.20437.
27. **Romeo RD, Tang AC, Sullivan RM.** Early life experiences: Enduring behavioral, neurological, and endocrinological consequences. In: *Hormones, Brain and Behavior, Second Edition*.Vol Second Edition. New York: Elsevier Inc; 2009.
28. **McCormick CM, Mathews IZ.** Adolescent development, hypothalamic-pituitary-adrenal function, and programming of adult learning and memory. *Progress in neuro-psychopharmacology, biological psychiatry*. 2010;34(5):756–765. doi:10.1016/j.pnpbp.2009.09.019.
29. **McCormick CM, Mathews IZ, Thomas C, Waters P.** Investigations of HPA function and the enduring consequences of stressors in ad-

olescence in animal models. *Brain and Cognition*. 2010;72(1):73–85. doi:10.1016/j.bandc.2009.06.003.
30. **McEwen BS.** Stress, sex, and neural adaptation to a changing environment: mechanisms of neuronal remodeling. *Ann N Y Acad Sci*. 2010;1204 Suppl(s1):E38–59. doi:10.1111/j.1749–6632.2010.05568.x.
31. **Wei L, Meaney MJ, Duman RS, Kaffman A.** Affiliative Behavior Requires Juvenile, But Not Adult Neurogenesis. *J Neurosci*. 2011; 31(40):14335–14345. doi:10.1523/JNEUROSCI.1333–11.2011.
32. **Solomon MB, Herman JP.** Sex differences in psychopathology: of gonads, adrenals and mental illness. *Physiol Behav*. 2009;97(2): 250–258. doi:10.1016/j.physbeh.2009.02.033.
33. **Baron-Cohen S, Knickmeyer RC, Belmonte MK.** Sex differences in the brain: implications for explaining autism. *Science*. 2005; 310(5749):819–823. doi:10.1126/science.1115455.
34. **Goel N, Bale TL.** Examining the intersection of sex and stress in modelling neuropsychiatric disorders. *J Neuroendocrinol*. 2009; 21(4):415–420. doi:10.1111/j.1365–2826.2009.01843.x.
35. **Shors TJ.** Significant life events and the shape of memories to come: a hypothesis. *Neurobiol Learn Mem*. 2006;85(2):103–115. doi: 10.1016/j.nlm.2005.09.004.
36. **Weinstock M.** Gender differences in the effects of prenatal stress on brain development and behaviour. *Neurochem Res*. 2007;32(10): 1730–1740. doi:10.1007/s11064–007–9339–4.
37. **Wood BL, Haque S, Weinstock A, Miller BD.** Pediatric stress-related seizures: conceptualization, evaluation, and treatment of nonepileptic seizures in children and adolescents. *Curr Opin Pediatr*. 2004; 16(5):523–531. doi:0.1097/01.mop.0000140997.24408.53.
38. **Gillies GE, McArthur S.** Estrogen actions in the brain and the basis for differential action in men and women: a case for sex-specific medicines. *Pharmacol Rev*. 2010;62(2):155–198. doi:10.1124/pr.109.002071.
39. **Grandjean P, Landrigan PJ.** Developmental neurotoxicity of industrial chemicals. *Lancet*. 2006;368(9553):2167–2178. doi:10.1016/S0140–6736(06)69665–7.
40. **Landrigan PJ, Lambertini L, Birnbaum LS.** A research strategy to discover the environmental causes of autism and neurodevelopmental disabilities. *Environ Health Perspect*. 2012;120(7):a258–60. doi:10.1289/ehp.1104285.
41. **Gray LE, Ostby JS, Kelce WR.** Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex differentiation of the male rat. *Toxicol Appl Pharmacol*. 1994;129(1): 46–52. doi:10.1006/taap.1994.1227.
42. **Kelce WR, Monosson E, Gamcsik MP, Laws SC, Gray LE.** Environmental hormone disruptors: evidence that vinclozolin developmental toxicity is mediated by antiandrogenic metabolites. *Toxicol Appl Pharmacol*. 1994;126(2):276–285. doi:10.1006/taap.1994.1117.
43. **Wong C, Kelce WR, Sar M, Wilson EM.** Androgen receptor antagonist versus agonist activities of the fungicide vinclozolin relative to hydroxyflutamide. *J Biol Chem*. 1995;270(34):19998–20003. doi: doi:10.1074/jbc.270.34.19998.
44. **Uzumcu M, Suzuki H, Skinner MK.** Effect of the anti-androgenic endocrine disruptor vinclozolin on embryonic testis cord formation and postnatal testis development and function. *Reprod Toxicol*. 2004. doi:doi:10.1016/j.reprotox.2004.05.008.
45. **Crews D, Gillette R, Scarpino SV, Manikkam M, Savenkova MI, Skinner MK.** Epigenetic transgenerational inheritance of altered stress responses. *PNAS*. 2012;109(23):9143–9148. doi:10.1073/pnas.1118514109.
46. **Moy SS, Nadler JJ, Perez A, Barbaro RP, Johns JM, Magnuson TR, Piven J, Crawley JN.** Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice. *Genes Brain Behav*. 2004;3(5):287–302. doi:10.1111/j.1601–1848.2004.00076.x.
47. **Tõnissaar M, Herm L, Eller M, Kõiv K, Rinken A, Harro J.** Rats with high or low sociability are differently affected by chronic variable stress. *Neuroscience*. 2008;152(4):867–876. doi:10.1016/j.neuroscience.2008.01.028.
48. **Klenerova V, Krejci I, Sida P, Hlinak Z, Hynie S.** Modulary effects of oxytocin and carbetocin on stress-induced changes in rat behavior in the open-field. *J Physiol Pharmacol*. 2009;60(2):57–62.
49. **Bouwknecht JA, Spiga F, Staub DR, Hale MW, Shekhar A, Lowry CA.** Differential effects of exposure to low-light or high-light open-field on anxiety-related behaviors: Relationship to c-Fos expression in serotonergic and non-serotonergic neurons in the dorsal raphe nucleus. *Brain Res Bull*. 2007;72(1):32–43. doi:10.1016/j.brainresbull.2006.12.009.
50. **Bourin M, Hascoët M.** The mouse light/dark box test. *Eur J Pharmacol*. 2003;463(1–3):55–65. doi:10.1016/S0014–2999(03)01274–3.
51. **Walf AA, Frye CA.** The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat Protoc*. 2007;2(2):322–328. doi:10.1038/nprot.2007.44.
52. **Crews D, Rushworth D, Gonzalez-Lima F, Ogawa S.** Litter environment affects behavior and brain metabolic activity of adult knockout mice. *Front Behav Neurosci*. 2009;3:12. doi:10.3389/neuro.08.012.2009.
53. **Sakata JT, Crews D, Gonzalez-Lima F.** Behavioral correlates of differences in neural metabolic capacity. *Brain Res Brain Res Rev*. 2005;48(1):1–15. doi:10.1016/j.brainresrev.2004.07.017.
54. **Walker DM, Kermath BA, Woller MJ, Gore AC.** Disruption of Reproductive Aging in Female and Male Rats by Gestational Exposure to Estrogenic Endocrine Disruptors. *Endocrinology*. 2013;154(6): 2129–2143. doi:10.1210/en.2012–2123.
55. **Siegel A, ed.** Anxiogenic effects of developmental bisphenol A exposure are associated with gene expression changes in the juvenile rat amygdala and mitigated by soy. *PLoS ONE*. 2012;7(9):e43890. doi:10.1371/journal.pone.0043890.
56. **Walker DM, Goetz BM, Gore AC.** Dynamic Postnatal Developmental and Sex-Specific Neuroendocrine Effects of Prenatal Polychlorinated Biphenyls in rats. *Mol Endocrinol*. 2014;28(1):99–115. doi:10.1210/me.2013–1270.
57. **Matsuura I, Saitoh T, Ashina M, Wako Y, Iwata H, Toyota N, Ishizuka Y, Namiki M, Hoshino N, Tsuchitani M.** Evaluation of a two-generation reproduction toxicity study adding endpoints to detect endocrine disrupting activity using vinclozolin. *J Toxicol Sci*. 2005;30 Spec No.:163–188. doi:doi:10.2131/jts.30.S163.
58. **Loutchanwoot P, Wuttke W, Jarry H.** Effects of a 5-day treatment with vinclozolin on the hypothalamo-pituitary-gonadal axis in male rats. *Toxicology*. 2008;243(1–2):105–115. doi:10.1016/j.tox.2007.09.025.
59. **Gądek-Michalska A, Spyrka J, Rachwalska P, Tadeusz J, Bugajski J.** Influence of chronic stress on brain corticosteroid receptors and HPA axis activity. *Pharmacol Rep*. 2013;65(5):1163–1175. doi:doi:10.1016/S1734–1140(13)71474–9.
60. **Hernandez ME, Martinez-Mota L, Salinas C, Marquez-Velasco R, Hernandez-Chan NG, Morales-Montor J, Pérez-Tapia M, Streber ML, Granados-Camacho I, Becerril E, Javier B-H, Pavón L.** Chronic Stress Induces Structural Alterations in Splenic Lymphoid Tissue That Are Associated with Changes in Corticosterone Levels in Wistar-Kyoto Rats. *BioMed Research International*. 2013;2013(3): 1–6. doi:10.1155/2013/868742.
61. **Kim J-G, Jung H-S, Kim K-J, Min S-S, Yoon B-J.** Basal blood corticosterone level is correlated with susceptibility to chronic restraint stress in mice. *Neurosci Lett*. 2013;555:137–142. doi:10.1016/j.neulet.2013.09.031.
62. **Scarpino SV, Gillette R, Crews D.** multiDimBio: An R Package for the Design, Analysis, and Visualization of Systems Biology Experiments. *arXiv preprint arXiv:14040594*. 2014.
63. **Dumm ME, Laken B, Ralli EP.** Factors influencing adrenal weight and adrenal cholesterol in rats following stress. *J Nutr*. 1955;56(4): 517–531.
64. **Skinner MK, Anway MD, Savenkova MI, Gore AC, Crews D.**

Transgenerational Epigenetic Programming of the Brain Transcriptome and Anxiety Behavior. *PLoS ONE*. 2008;3(11):e3745. doi: 10.1371/journal.pone.0003745.s008.

65. **Nilsson EE, Anway MD, Stanfield J, Skinner MK.** Transgenerational epigenetic effects of the endocrine disruptor vinclozolin on pregnancies and female adult onset disease. *Reproduction*. 2008; 135(5):713–721. doi:10.1530/REP-07–0542.
66. **Anway MD, Leathers C, Skinner MK.** Endocrine Disruptor Vinclozolin Induced Epigenetic Transgenerational Adult-Onset Disease. *Endocrinology*. 2006;147(12):5515–5523. doi:10.1210/en.2006–0640.
67. **Schneider S, Kaufmann W, Buesen R, van Ravenzwaay B.** Vinclozolin - The lack of a transgenerational effect after oral maternal exposure during organogenesis. *Reprod Toxicol*. 2008;25(3):352–360. doi:10.1016/j.reprotox.2008.04.001.
68. **Inawaka K, Kawabe M, Takahashi S, Doi Y, Tomigahara Y, Tarui H, Abe J, Kawamura S, Shirai T.** Maternal exposure to anti-androgenic compounds, vinclozolin, flutamide and procymidone, has no effects on spermatogenesis and DNA methylation in male rats of subsequent generations. *Toxicol Appl Pharmacol*. 2009;237(2): 178–187. doi:10.1016/j.taap.2009.03.004.
69. **Wolstenholme JT, Goldsby JA, Rissman EF.** Hormones and Behavior. *Horm Behav*. 2013;64(5):833–839. doi:10.1016/j.yhbeh.2013.09.007.
70. **Newbold RR, Padilla-Banks E, Jefferson WN.** Adverse effects of the model environmental estrogen diethylstilbestrol are transmitted to subsequent generations. *Endocrinology*. 2006;147(6 Suppl):S11–7. doi:10.1210/en.2005–1164.
71. **Titus-Ernstoff L, Troisi R, Hatch EE, Hyer M, Wise LA, Palmer JR, Kaufman R, Adam E, Noller K, Herbst AL, Strohsnitter W, Cole BF, Hartge P, Hoover RN.** Offspring of women exposed in utero to diethylstilbestrol (DES): a preliminary report of benign and malignant pathology in the third generation. *Epidemiology*. 2008;19(2): 251–257. doi:10.1097/EDE.0b013e318163152a.
72. **Steinberg RM, Walker DM, Juenger TE, Woller MJ, Gore AC.** Effects of perinatal polychlorinated biphenyls on adult female rat reproduction: development, reproductive physiology, and second generational effects. *Biol Reprod*. 2008;78(6):1091–1101. doi: 10.1095/biolreprod.107.067249.
73. **Nugent BM, McCarthy MM.** Epigenetic underpinnings of developmental sex differences in the brain. *Neuroendocrinology*. 2011;93: 150–158. doi:10.1159/000325264.
74. **Gioiosa L, Parmigiani S, Saal vom FS, Palanza P.** Hormones and Behavior. *Horm Behav*. 2013;63(4):598–605. doi:10.1016/j.yhbeh.2013.02.016.
75. **Lilienthal H, Heikkinen P, Andersson PL, Viluksela M.** Sexually dimorphic behavior after developmental exposure to characterize endocrine-mediated effects of different non-dioxin-like PCBs in rats. *Toxicology*. 2013;311(1–2):52–60. doi:10.1016/j.tox.2013.01.025.
76. **Jones BA, Watson NV.** Hormones and Behavior. *Horm Behav*. 2012;61(4):605–610. doi:10.1016/j.yhbeh.2012.02.011.
77. **Carbone S, Ponzo OJ, Gobetto N, Samaniego YA, Reynoso R, Scacchi P, Moguilevsky JA, Cutrera R.** Hormones and Behavior. *Horm Behav*. 2013;63(5):692–699. doi:10.1016/j.yhbeh.2013.01.006.
78. **Bowman RE, Beck KD, Luine VN.** Chronic stress effects on memory: sex differences in performance and monoaminergic activity. *Horm Behav*. 2003;43(1):48–59. doi:10.1016/S0018–506X(02)00022–3.
79. **Kuipers SD, Trentani A, van der Zee EA, Boer den JA.** Neuropharmacology. *Neuropharmacology*. 2013;75.
79. **(c) :426–436. doi:10.1016/j.neuropharm.2013.08.018.**
80. **Lenglos C, Mitra A, Guèvremont G, Timofeeva E.** Sex differences in the effects of chronic stress and food restriction on body weight gain and brain expression of CRF and relaxin-3 in rats. *Genes Brain Behav*. 2013;12(4):370–387. doi:10.1111/gbb.12028.
81. **Patisaul HB, Bateman HL.** Neonatal exposure to endocrine active compounds or an ERβ agonist increases adult anxiety and aggression in gonadally intact male rats. *Horm Behav*. 2008;53(4):580–588. doi:10.1016/j.yhbeh.2008.01.008.
82. **Weinstein SD, Villafane JJ, Juliano N, Bowman RE.** Adolescent exposure to Bisphenol-A increases anxiety and sucrose preference but impairs spatial memory in rats independent of sex. *Brain Res*. 2013;1529(C):56–65. doi:10.1016/j.brainres.2013.07.018.
83. **Xu X, Hong X, Xie L, Li T, Yang Y, Zhang Q, Zhang G, Liu X.** Hormones and Behavior. *Horm Behav*. 2012;62(4):480–490. doi: 10.1016/j.yhbeh.2012.08.005.
84. **Wolstenholme JT, Taylor JA, Shetty SR, Edwards M, Connelly JJ, Rissman EF.** Gestational exposure to low dose bisphenol A alters social behavior in juvenile mice. *PLoS ONE*. 2011;6:e25448. doi: 10.1371/journal.pone.0025448.
85. **Colbert NKW, Pelletier NC, Cote JM, Concannon JB, Jurdak NA, Minott SB, Markowski VP.** Perinatal Exposure to Low Levels of the Environmental Antiandrogen Vinclozolin Alters Sex-Differentiated Social Play and Sexual Behaviors in the Rat. *Environ Health Perspect*. 2005;113(6):700–707. doi:10.1289/ehp.7509.
86. **Smejkalova T, Woolley CS.** Estradiol acutely potentiates hippocampal excitatory synaptic transmission through a presynaptic mechanism. *J Neurosci*. 2010;30(48):16137–16148. doi:10.1523/jneurosci.4161–10.2010.
87. **Huang GZ, Woolley CS.** Estradiol acutely suppresses inhibition in the hippocampus through a sex-specific endocannabinoid and mGluR-dependent mechanism. *Neuron*. 2012;74(5):801–808. doi: 10.1016/j.neuron.2012.03.035.
88. **Wang X-D, Chen Y, Wolf M, Wagner KV, Liebl C, Scharf SH, Harbich D, Mayer B, Wurst W, Holsboer F, Deussing JM, Baram TZ, MÃ¼ller MB, Schmidt MV.** Forebrain CRHR1 deficiency attenuates chronic stress-induced cognitive deficits and dendritic remodeling. *Neurobiol Dis*. 2011;42(3):300–310. doi:10.1016/j.nbd.2011.01.020.
89. **Sakata K, Duke SM.** Lack of BDNF expression through promoter IV disturbs expression of monoamine genes in the frontal cortex and hippocampus. *Neuroscience*. 2014;260(C):265–275. doi:10.1016/j.neuroscience.2013.12.013.
90. **Nagata A, Suzuki Y, Igarashi M, Eguchi N, Toh H, Urade Y, Hayaishi O.** Human brain prostaglandin D synthase has been evolutionarily differentiated from lipophilic-ligand carrier proteins. *PNAS*. 1991;88(9):4020–4024. doi:10.1073/pnas.88.9.4020.
91. **Fallon J, Reid S, Kinyamu R, Opole I, Opole R, Baratta J, Korc M, Endo TL, Duong A, Nguyen G, Karkehabadhi M, Twardzik D, Loughlin S.** In vivo induction of massive proliferation, directed migration, and differentiation of neural cells in the adult mammalian brain. *PNAS*. 2000;97(26):14686–14691. doi:10.1073/pnas.97.26.14686.
92. **Royland JE, Wu J, Zawia NH, Kodavanti PRS.** Gene expression profiles in the cerebellum and hippocampus following exposure to a neurotoxicant, Aroclor 1254: Developmental effects. *Toxicol Appl Pharmacol*. 2008;231(2):165–178. doi:10.1016/j.taap.2008.04.022.
93. **Jang YJ, Park HR, Kim TH, Yang W-J, Lee J-J, Choi SY, OH SB, Lee E, Park J-H, Kim H-P, Kim HS, Lee J.** High dose bisphenol A impairs hippocampal neurogenesis in female mice across generations. *Toxicology*. 2012;296(1–3):73–82. doi:10.1016/j.tox.2012.03.007.
94. **Honma T, Suda M, Miyagawa M, Wang R-S, Kobayashi K, Sekiguchi S.** Alteration of brain neurotransmitters in female rat offspring induced by prenatal administration of 16 and 64 mg/kg of 2,2′,4,4‘,5,5′-hexachlorobiphenyl (PCB153). *Ind Health*. 2009; 47(1):11–21. doi:doi:10.2486/indhealth.47.11.
95. **Campeau S, Davis M.** Involvement of the central nucleus and basolateral complex of the amygdala in fear conditioning measured with fear-potentiated startle in rats trained concurrently with auditory and visual conditioned stimuli. *J Neurosci*. 1995;15(3 Pt 2): 2301–2311.
96. **Galea LA, McEwen BS, Tanapat P, Deak T, Spencer RL, Dhabhar**

FS. Sex differences in dendritic atrophy of CA3 pyramidal neurons in response to chronic restraint stress. *Neuroscience*. 1997;81(3):689–697. doi:doi:10.1016/S0306–4522(97)00233–9.

97. **Anway MD, Rekow SS, Skinner MK.** Transgenerational epigenetic programming of the embryonic testis transcriptome. *Genomics*. 2008;91(1):30–40. doi:10.1016/j.ygeno.2007.10.002.