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Prenatal exposure to vinclozolin disrupts selective aspects of the gonadotropin-releasing hormone neuronal system of the rabbit

B.C. Wadas,

Department of Biomedical Sciences, Colorado State University, Fort Collins, CO 80523

C.A. Hartshorn,

Department of Biomedical Sciences, Colorado State University, Fort Collins, CO 80523

E.R. Aurand,

Department of Biomedical Sciences, Colorado State University, Fort Collins, CO 80523

J.S. Palmer,

Department of Physiology and Pharmacology, Oregon Health and Science University, Portland, OR 97239

C.E. Roselli,

Division of Pharmacology & Toxicology, The University of Texas at Austin, Austin, TX 78712

M.L. Noel,

Division of Pharmacology & Toxicology, The University of Texas at Austin, Austin, TX 78712

A.C. Gore,

Department of Biomedical Sciences, Colorado State University, Fort Collins, CO 80523

D.N.R. Veeramachaneni, and

Department of Biomedical Sciences, Colorado State University, Fort Collins, CO 80523

S.A. Tobet

Department of Biomedical Sciences, Colorado State University, Fort Collins, CO 80523

Abstract

Developmental exposure to the agricultural fungicide vinclozolin can impair reproductive function in male rabbits and was previously found to decrease the number of immunoreactive-gonadotropin-releasing hormone (ir-GnRH) neurons in the region of the organum vasculosum of the lamina terminalis (OVLT) and rostral preoptic area (rPOA) by postnatal week (PNW) 6. To further examine the disruption of GnRH neurons by fetal vinclozolin exposure, in the current study, pregnant rabbits were dosed orally with vinclozolin, flutamide, or carrot paste vehicle for the last two weeks of gestation. Offspring were euthanized at birth (males and females), PNW6 (females), PNW26 (adult males), or PNW30 (adult females) of age. At birth and in adults, brains were sectioned and processed for immunoreactive GnRH. The numbers of immunoreactive GnRH neuronal perikarya were significantly decreased in vinclozolin-treated rabbits at birth and in adult littermates. By contrast, there was an increase in GnRH immunoreactivity in the terminals in the region of the median eminence. Analysis of PNW6 female brains by radioimmunoassay (RIA) revealed a two-fold increase in GnRH peptide content in the mediobasal hypothalamus in vinclozolin-treated rabbits. This finding was complemented by immunofluorescence analyses that

showed a 2.8-fold increase in GnRH immunoreactivity in the median eminence of vinclozolin compared to vehicle-treated females at PNW30. However, there was no difference between treatment groups in the measures of reproduction that were evaluated: ejaculation latency, conception rates or litter size. These results indicate that subacute, prenatal vinclozolin treatment is sufficient to create perdurable alterations in the GnRH neuronal network that forms an important input into the reproductive axis. Finally, the effect of vinclozolin on the GnRH neuronal network was not comparable to that of flutamide, suggesting that vinclozolin was not acting through anti-androgenic mechanisms.

Keywords

reproduction; gonadotropin-releasing hormone; hypothalamus; neuroendocrinology; toxicology

Introduction

A number of mental disorders and altered physiological states are increasingly being associated with fetal antecedent events [1-3]. Disruptions of normal development can result from a number of sources, two of which are exposure to exogenous chemicals or to abnormal levels of endogenous hormones. Around the time of birth, the sex steroid hormone environment is important for brain sexual differentiation and the development of neuroendocrine systems; however, the precise timing of critical periods differs between species. During this critical developmental time, endocrine-disrupting compounds have the potential to be potent disruptors as alterations in hormone levels may lead to secondary anatomical and physiological changes in developing hormone-sensitive systems [4].

Vinclozolin is a widely used dicarboximide fungicide previously described as an anti-androgenic compound in mammals [5]. As a result of its widespread use on lettuce, grapes, sod, and numerous other agricultural and horticultural products, vinclozolin is common in the environment. Endocrine-disrupting effects of vinclozolin are largely attributed to its two primary metabolites, 2-[[[(3,5-dichlorophenyl)-carbamoyl]oxy]-2-methyl-3-butenic acid (M1) and 3',5'-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2), which competitively antagonize androgen receptor (AR)-dependent transcriptional activation in vitro [5-8]. Vinclozolin treatment altered gene expression and urogenital differentiation in rats [6], and resulted in altered serum hormone levels, spermatogenic defects and sexual dysfunction in rabbits [9]. In that study, along with peripheral reproductive measures, we found that neuroendocrine systems in postnatal week (PNW) 6 rabbits exposed to vinclozolin perinatally exhibited a region-specific decrease in the number of neurons immunoreactive for gonadotropin-releasing hormone (ir-GnRH). Studies in rats have shown that vinclozolin altered sexual differentiation and play behavior in male rats [5,10], and recent work has shown transgenerational endocrine disruption of brain and behavior in third-generation descendants of rats that had been fetally exposed to vinclozolin [11,12]. Together these experiments provide evidence for neuroendocrine disruption of exposed animals, and their descendants, by prenatal vinclozolin.

The current study expands upon these previous reports, particularly our earlier report in the rabbit [13], in three important ways. First, to further determine the period of vulnerability to vinclozolin, exposure was restricted to a two-week fetal period. Second, to investigate potential mechanisms of vinclozolin action in the rabbit hypothalamus, vinclozolin- and vehicle-treated rabbits were compared with a set of rabbits similarly exposed to the well-characterized antiandrogenic compound flutamide [14]. Finally, to investigate a possible indirect antiandrogenic mechanism of vinclozolin, enzymatic activity of aromatase was analyzed.

Materials and Methods

Animals

Pregnant, timed-mated, Dutch-Belted rabbits were obtained from Myrtle's Rabbitry (Thompson Station, TN) and individually housed in stainless-steel cages. All animals were primiparous when mated at 6-7 months of age. All animal procedures were approved by the Colorado State University Institutional Animal Use and Care Committee.

Rabbits were maintained on a 12L:12D cycle, at 19-21°C and 40 percent humidity, were fed Prolab Hi-Fiber rabbit diet (5P25, Purina Mills) and water was provided *ad libitum*. Pregnant dams were dosed orally once daily with vinclozolin (10 mg in 1 ml vehicle/kg body weight/day; PS-1049, ChemService Inc, West Chester, PA) or certified organic carrot puree vehicle (1 ml/kg body weight; Earth's Best Organic First Carrots, Hain Celestial Group, Inc, Melville, NY) beginning on gestation day 15 (GD15; gestation length = 30-31 days). Vinclozolin was delivered as a homogenous suspension in carrot puree. As previously reported [13], vinclozolin-treated dams completed full-term pregnancies. There were no differences in body weight among dams in the different treatment groups prior to delivery and no differences (within sex) in body weight among the offspring at birth or prior to euthanasia in adulthood (data not shown).

Following birth, littermate offspring were randomly separated and assigned one of five fates with three age groups perfused transcardially, one dissected for aromatase, and one dissected for RIA. For postnatal day 1 (PND1) pups were perfused by hand-held syringe with 10-ml heparinized saline followed by 10 ml 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Littermates were raised until adulthood, killed by an intravenous injection of 0.25 ml Beuthanasia-D Special (390 mg pentobarbital sodium/ml; Schering-Plough, Union, NJ), and perfused at PNW26 (males) or, PNW30 (females) using 50 ml heparinized saline followed by 400-600 ml of 4% paraformaldehyde (males) or 4% paraformaldehyde with 0.5% glutaraldehyde (females) in 0.1 M phosphate buffer. Following perfusion, the brains were removed, postfixed in the same fixative overnight, and stored in 0.1 M phosphate buffer at 4°C until sectioning. Brains were sectioned (70 µm thick) into chilled phosphate buffered saline (PBS; pH 7.5) using a cooled vibrating microtome (Leica VT1000s) beginning at the diagonal band of Broca and ending at the median eminence. Sections were stored at 4°C for no more than 18-36 h before processing for immunocytochemistry.

After the first set of perfusions (PND1), two samples per group were collected to measure aromatase activity. Aromatase activity was assayed using a tritiated water release method in vitro [15] with homogenized tissue from regions dissected and frozen on dry ice. Block dissections were performed as previously described for rats [16] with blocks taken for analysis of preoptic area, medial basal hypothalamus, cerebral cortex, and temporal lobe (containing amygdala). A preliminary experiment confirmed that brain aromatase activity in rabbit brain exhibited Michaelis-Menton enzyme kinetics ($K_m = 0.03 \mu M$) under the assay conditions employed. Thus, a single assay that used a saturating substrate concentration (i.e., $0.3 \mu M$; $10 \times K_m$) allowed us to estimate enzyme concentration as a function of activity.

A fourth set of littermates was sacrificed at PNW6 by an intravenous overdose of Beuthanasia-D (390 mg pentobarbital sodium/ml; Schering-Plough Animal Health, Omaha, NE). The sex composition of the available rabbits provided only sufficient numbers of females in each group for this component to measure tissue levels of GnRH peptide by RIA. However, based on previous data at this age, there was no basis for anticipating sex differences in GnRH neuronal characteristics [13]. Brains were rapidly removed and block-dissected on ice. Dissected samples were weighed and stored at -20°C in 15 ml conical polypropylene tubes containing 3 ml 10% formic acid in methanol until use. Each dissected

hypothalamus was homogenized in its storage medium and GnRH was extracted and assayed using established procedures [17].

Immunocytochemistry

Immunocytochemistry (ICC) was performed as previously reported [13]. Free-floating sections were incubated in 0.05 M PBS with 0.1 M glycine for 30 min, washed in PBS, incubated in 0.5% sodium borohydride for 15 min, and washed in PBS again. Sections were blocked for at least 30 min in 5% normal goat serum (NGS; Sigma-Aldrich, St. Louis, MO) with 1% hydrogen peroxide in 0.3% Triton-X-100 in PBS (TxPBS; for PND1), or in 0.5% TxPBS for adult tissue. Following block, sections were incubated at 4°C for 48 h in anti-GnRH (1:500; QED Bioscience, San Diego, CA) mouse monoclonal primary antibody. All washes were at 4°C in 0.05 M PBS.

After primary antiserum incubation, sections were rinsed in 0.05 M PBS with 1% NGS and 0.02% TxPBS at room temperature (RT), then incubated in secondary antibody for 2 h at RT (anti-mouse raised in goat diluted 1:2500; Jackson ImmunoResearch, West Grove, PA). Tissue was washed in 0.02% TxPBS and then incubated in Vectastain Elite ABC reagent for 1 h at RT (3 µl/ml each of reagents A and B mixed 30 min prior to use). Following incubation, sections were rinsed at RT in 0.05 M tris-buffered saline (TBS; pH 7.5), and developed by 5 min RT incubation in 0.025% diaminobenzidine/0.02% nickel (DAB/Ni; Sigma-Aldrich) with 0.02% H₂O₂ in TBS. DAB/Ni reactivity was quenched by washes in TBS and sections were stored in TBS at 4°C until mounting. Sections were mounted on gelatin-coated slides, dried, and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ).

Immunofluorescence for confocal microscopy

Tissue sections (100 µm) of PNW30 females were taken at the level of the median eminence as described previously [18,19]. All steps were performed on a shaking platform, with the exception of the primary antibody incubation done at 4°C, all incubations were at RT. Sections were washed in buffer (PBS), incubated in 10% normal goat serum (S-1000, Vector Laboratories, Burlingame, CA) and 0.1% bovine serum albumin (A9085, Sigma, St. Louis, MO) for 1 hour. Sections were then incubated in mouse anti-GnRH antibody (HU11b, 1:500 dilution, kindly provided by Dr. Henryk Urbanski; [20]) in 2% normal goat serum for 24 h. Following primary antibody incubation, sections were washed in buffer and incubated in 5% normal goat serum, anti-mouse FITC IgG (5 µg/mL; Vector Labs) for 1.5 h. Sections were washed, mounted on Superfrost Plus Premium microscope slides (12-550-15 Fisherbrand, Pittsburgh, PA), dried, and coverslipped with Vectashield mounting media (H-1000, Vector Labs). Previous preabsorption experiments show that all immunoreaction product is eliminated using either immunofluorescence or immunogold for electron microscopy [18].

Data Collection and Analysis

GnRH neuron numbers—Sections were examined on an Olympus BH-2 microscope using brightfield optics. The number of ir-GnRH neurons was counted in every fourth 70 µm thick section from the diagonal band of Broca through the median eminence by an investigator unaware of treatment group, resulting in a span of 280 µm between data points. Sections were aligned rostral to caudal for each animal and then between animals using the organum vasculosum of the lamina terminalis (OVLt) as an anatomical marker. Manual cell counts were analyzed by repeated measures ANOVA (fetal treatment × coronal plane as the repeated measure) using SPSS statistical software (SPSS Inc., Chicago, IL).

Confocal Microscopic Analysis and Metamorph

An SP2 AOBS Laser Scanning Confocal Microscope (Leica Microsystems Inc, Exton, PA) was used to determine GnRH fluorescence intensity in the ME. Subsections of the ME measuring $748\ \mu\text{m} \times 748\ \mu\text{m} \times 15\ \mu\text{m}$ were imaged with a $20\times$ (0.7 NA) objective at 1.00 zoom. Image stacks were acquired at excitation wavelengths of 488 nm and emission was collected between 500 nm and 510 nm for FITC-labeled GnRH with a $1.0\ \mu\text{m}$ z-axis step size. The $15\ \mu\text{m}$ depth was chosen to ensure equal penetration of both antibodies across the entire scan. Scans were conducted over a 4 h period in order to minimize photobleaching of antibodies, and to avoid “drift” in the confocal, with laser parameters remaining constant.

Image stacks obtained from the confocal were analyzed for fluorescence intensity using Metamorph Offline 6.1 (Universal Imaging Corp., Downingtown PA) as described in Yin et al. [18]. Individual images were analyzed and the threshold levels determined in order to subtract background autofluorescence. In addition, a secondary antibody-only control was performed in the same immunocytochemical run to determine any nonspecific immunoreactivity, and showed negligible labeling.

Results

GnRH Immunocytochemistry

Postnatal day 1—As previously reported [13], the GnRH neuron distribution extended from the diagonal band of Broca through regions of the basal hypothalamus with a peak in the region of the OVL. Analyzing the distribution of GnRH neurons by section suggested a selective decrease in the number of ir-GnRH somas only in the sections immediately surrounding the OVL (figure 1 and figure 2). Posthoc analysis of the data showed that the number of cells in the vinclozolin-treated animals was only lower than in vehicle or flutamide-exposed rabbits beyond the 95% confidence interval for $280\ \mu\text{m}$ rostral to the OVL through $280\ \mu\text{m}$ caudal (figure 2). Although there was significant interaction between treatment exposure and location ($F(16,64) = 2.87$, $p < 0.01$), the between-groups ANOVA was not statistically significant ($F(2,8) = 0.43$, $p = 0.64$). With no apparent sex difference in ir-GnRH neuronal numbers in this or previous analyses [13], the sexes were combined for the ANOVA ($n = 5$ per group). To illustrate the lack of a sex difference, figure 2B graphs the OVL section with individual animals and separate symbols for male ($n = 3$ per group) and female ($n = 2$ per group). The data show that vinclozolin-exposed offspring had notably fewer ir-GnRH neurons in the OVL section than vehicle or flutamide-exposed animals ($F(2,12) = 5.30$, $p < 0.03$) (figure 2B). Posthoc analysis showed that this difference was attributable to the significant difference between vehicle and vinclozolin-exposed offspring ($p < 0.01$, Dunnett's test).

Postnatal week 26/30—Littermates raised until PNW26 (males) and PNW30 (females) revealed similar decreases in ir-GnRH neurons to that identified at PND1. In males, there was a significant decrease in the number of ir-GnRH cell bodies spanning from approximately $280\ \mu\text{m}$ rostral to the OVL through $560\ \mu\text{m}$ caudal to the OVL (figure 3; vehicle, $n = 4$; vinclozolin, $n = 5$; flutamide, $n = 5$). Repeated measures analysis indicated that the number of ir-GnRH neurons was dependent on position ($F(8, 88) = 32.9$, $p < 0.001$), and altered by treatment in a position-dependent manner ($F(16, 88) = 3.14$, $p < 0.001$). The between groups ANOVA also revealed a significant effect of treatment on the number of ir-GnRH neurons ($F(2,11) = 4.99$, $p < 0.05$). However, posthoc analysis of the data showed that the number of ir-GnRH cells in the vinclozolin treated animals were only lower than in vehicle or flutamide-exposed rabbits beyond the 95% confidence for the restricted interval from $280\ \mu\text{m}$ rostral to the OVL through $560\ \mu\text{m}$ caudal (figure 3D). Although inconsistent/varying section angles for the females (PNW30) made quantitative analyses

more difficult, qualitatively they also showed a similar decrease in a restricted region close to the OVLT (figure 4; $p = 0.064$ for sections 2 and 3 combined as a repeated measure; vehicle, $n = 3$; vinclozolin, $n = 4$). In both sexes, there were a notable number of ir-GnRH cells in the medial basal hypothalamus (MBH) in sections that also contained arcuate nuclei and median eminence. Qualitatively, however, there was no evidence of a diminution in ir-GnRH cell number or fiber density (by brightfield ICC) in rabbits exposed prenatally to either vinclozolin or flutamide in this region.

GnRH Immunofluorescence in the Median Eminence

To determine effects of vinclozolin on GnRH peptide immunoreactivity in the median eminence more quantitatively, immunofluorescence and semi-quantitative confocal microscopy was performed (figure 5). Immunoreactive GnRH was punctate and distributed through the median eminence (ME), most concentrated in the external zone, but also present in the vicinity of ependymal cells of the third ventricle consistent with other reports in rabbits [21]. Comparisons were limited to the adult PNW30 females (vinclozolin and vehicle groups) due to limitations in availability of animals and tissues. The results showed a significant ($F(1,6) = 9.68$, $p = 0.02$; $n = 4$ per group) 2.8-fold higher level of GnRH immunofluorescence in the vinclozolin compared to the vehicle group (vehicle 121.9 ± 41.0 versus vinclozolin 342.5 ± 57.9)(arbitrary units).

GnRH Peptide Content in MBH

Because of the disparity in results on GnRH cell bodies (loss of ir-GnRH neuronal cell bodies in vinclozolin-treated rabbits at 3 different ages (this study and [13])), compared to the immunofluorescence results at the median eminence (increase in GnRH immunofluorescence in adult females), radioimmunoassays were performed at the level of GnRH processes and terminals in the MBH of vinclozolin- or vehicle-treated female littermates. The results showed that the vinclozolin-exposed females had significantly increased levels of GnRH peptide at PNW6 in the MBH, as detected by RIA (figure 6; $n = 6$ per group). More specifically, GnRH peptide levels were increased by 2-fold in vinclozolin-treated females as compared to vehicle-treated females, without a significant change in GnRH peptide content in the more rostral OVLT and anterior hypothalamic (AH) regions. In contrast, GnRH levels were not significantly altered in flutamide-treated rabbits compared to vehicle in any region. By ANOVA, this result revealed a significant group $\times$ region interaction ($F(4,45) = 4.31$, $p < 0.01$; figure 6), for which posthoc testing showed the difference to be due solely to the larger value in the MBH of vinclozolin-exposed rabbits.

Aromatase

Similar to the findings of George et al., [22], significant levels of aromatase were observed at birth (PND1) in several brain regions. In particular, aromatase activity was elevated in the block-dissected temporal lobe containing amygdala (mean $\pm$ range = 870 ± 0.2 fmol/h-mg protein) and in the preoptic area (1368 ± 27 fmol/h-mg protein). Aromatase activity levels were particularly low in the cerebral cortex (35.6 ± 0.4 fmol/h-mg protein). There was no indication of an influence of either vinclozolin or flutamide on aromatase activity in any region studied (temporal lobe = 861 ± 130 for flutamide and 777 ± 104 for vinclozolin; pre-optic area = 1695 ± 581 for flutamide and 1798 ± 335 for vinclozolin; cortex = 30 ± 13 for flutamide and 28 ± 1.6 for vinclozolin; data not shown).

Reproductive Competence

Adult male and female rabbits were evaluated for reproductive competence. All males mounted females over 6 trials, tested once every three days in the presence of a teaser female. Ejaculation latency for the first test was the only episode in which there was even a

slight trend for increased latencies in the treated groups (control 14.1 $\pm$ 1.8 sec (n = 11); vinclozolin 33.7 $\pm$ 13.1 sec (n = 12); flutamide 26.5 $\pm$ 8.0 sec (n = 11)); however, due to high variability this was not close to being statistically significant ($p > 0.40$). Female offspring from all 3 treatment groups were bred with control males and the conception rates were similar across groups; for the vehicle females 5 of 6 became pregnant, while for both vinclozolin (n = 7) and flutamide (n = 5) all rabbits became pregnant. Litter sizes were also similar across treatments (range: 6-9 pups per litter, except in one flutamide-exposed female which had only one pup).

Discussion

In the current study, gestational vinclozolin exposure was sufficient to create a sustained and consistent reduction in a select subset of GnRH immunoreactive perikarya. The decrease in number of ir-GnRH neurons is reported herein after a specific 2-week developmental exposure window, and observed both at PND1 and in adulthood. It was similar in both magnitude and spatial location to the decrease previously described at PNW6 after a much more substantial exposure (two-week gestational exposure plus a four-week lactational exposure) to vinclozolin [13]. As the selective decrease in detectable neurons was consistent and maintained into adulthood in littermate males and females, it is likely that a subpopulation of GnRH neurons is particularly susceptible to a developmental influence of vinclozolin during fetal development.

The GnRH neuronal population is remarkably heterogeneous [23,24] and immunocytochemistry provides only a semi-quantitative means to determine the size of a population containing a detectable amount of immunoreactive peptide/protein. To begin to address how a decrease in the size of the ir-GnRH neuronal population might relate to hypophysiotropic GnRH physiology, two different methods were used and complementary results were obtained. In adult female rabbits, fetal vinclozolin exposure led to an increase in fluorescence intensity in ir-GnRH terminals of the median eminence compared to vehicle-exposed. RIA was also performed to more accurately quantify the amount of GnRH peptide in the hypothalamus. In six-week-old vinclozolin-exposed females there was a two-fold increase in the RIA-detectable amount of GnRH peptide in the region of the MBH. The apparent discrepancy between the decreased number of ir-GnRH cell bodies and increased peptide content in the MBH may be a result of several possible factors. On a basic level, the decrease in number of ir-GnRH neurons may be a result of cell loss (e.g., aberrant generation, migration, or cell death) or altered cellular function (e.g., decreased production, altered processing, or transport of GnRH peptide). Increased GnRH peptide content detected by RIA may be a result of either overproduction of GnRH paired with normal secretion, or normal production of GnRH that accumulates as a result of impaired secretion. The latter hypothesis would be supported by accumulation of GnRH primarily in the region of the GnRH nerve terminals. Although no changes were discernible in immunoreactive density at the median eminence of vinclozolin-treated rabbits when examined by light microscopy (data not shown), there was a significant increase in fluorescence intensity for ir-GnRH in terminals at the median eminence by high magnification confocal microscopy. This strongly implicates a defect in GnRH neuronal function. Further, although this remains to be proven, we speculate that there may be a deficiency in the ability of GnRH terminals to release the peptide under non-stimulated conditions, based on the apparent accumulation in the terminal region. At this point, a complete description of the mechanisms controlling GnRH secretion remains elusive despite their important role in reproductive function [25], and further investigation is necessary.

Subpopulations within the GnRH neuronal network may have distinct functional roles, and it has been proposed that the pulsatility underlying GnRH secretion may originate from a

subset of cells in the GnRH neuron population [26]. Under these circumstances, disruptions of even small subsets of GnRH could have deleterious effects for reproductive fitness at the level of the individual or species. Studying the GnRH neuronal network in endocrine-disrupted systems may provide useful insight into the differential functions of subpopulations within the GnRH neuronal network. Additionally, hypothalamic nuclei are particularly sensitive to endocrine-disruption during development [27], yet the effects of vinclozolin on these essential components of the mammalian neuroendocrine axis largely remain to be studied.

To address whether vinclozolin might be acting on the GnRH neuronal network through an antiandrogenic mechanism in the current study, vinclozolin-treated rabbits were compared with rabbits exposed to the well-characterized antiandrogenic compound flutamide [14]. Offspring from flutamide-treated rabbits had ir-GnRH neuronal populations that were normal in size and distribution when analyzed at birth and in adulthood independent of sex. Furthermore, analysis of female flutamide-exposed littermates revealed similar hypothalamic GnRH peptide content to control animals when measured by RIA at PNW6, in contrast to the two-fold increase seen in vinclozolin-exposed rabbits. Previous reports have indicated that normal GnRH neuron differentiation occurs in other species in the presence of excess androgens, including sheep [28], rat [29,30] and rhesus monkey [31]. The present findings in the rabbit further suggest that the androgenic environment of developing GnRH neurons is not a determinant of the final number or distribution of GnRH neurons in the mammalian forebrain.

Several lines of evidence suggest that androgen-mediated changes in the GnRH neuronal network would likely be indirect. First, neither excess androgens [32], nor the potent antiandrogenic compound flutamide (current study) disrupt the final positions of GnRH neurons. Second, there is little evidence for the presence of androgen receptor (AR) expression on GnRH neurons in other species [33-35]. One possible indirect route could have been through an alteration in aromatase activity, an enzyme known to be androgen-regulated in adult brain [36], and in some cases for fetal brain [37], but not always [38,39]. However in the current study, there was no indication that fetal rabbit brain aromatase is either androgen-regulated (i.e., influence by flutamide) or influenced by vinclozolin. Thus, it is most likely that vinclozolin's influence on the GnRH neuronal network is through a non-antiandrogenic mechanism; the plausibility of non-hormone mediated events in other systems has not been ruled out even though it is not considered likely [40].

Although initial mechanistic investigations reported that vinclozolin had no binding affinity for estrogen receptors and demonstrated no estrogenic activity *in vitro* [41-43], recent steroid profiling suggests that vinclozolin and its metabolites may have mixed agonist/antagonist actions at estrogen receptors, progesterone receptor, glucocorticoid receptor, and mineralocorticoid receptor [44]. Subsequently, it was reported that subacute oral administration of vinclozolin disrupts the neuroendocrine axis in adult rats by altering serum hormone levels and mRNA expression levels of sex steroid receptors via both androgenic and estrogenic mechanisms [45]. Taken together with the current study, these data warrant further investigation of potential non-antiandrogenic mechanisms of action on the GnRH neuronal network.

GnRH neurons represent only one hypothalamic cell population that may play a role(s) in regulating the reproductive axis that could contribute to physiological phenotypes described previously [9]. None of the female rabbits in the current study were reproductively compromised. Thus the approximately 30% reduction in ir-GnRH neuronal number in the region of the OVLT, along with the 2-3 fold increase in GnRH immunoreactivity in the terminals, were not sufficient to cause reproductive failure. In one sense, this agrees with the

recent observation that a much larger percentage of the GnRH neuronal network needs to be impaired to cause complete reproductive failure [24]. The reproductive competence of vinclozolin treated females may also be due in part to the nature of rabbits as reflex ovulators. Perhaps coital stimulation was sufficient to overcome whatever secretory block may have existed in the vinclozolin-exposed females. The reflex ovulatory system is likely particularly primed to release GnRH in response to coital stimulation as previously shown in ferrets [46]. Further, the current studies differ from prior one [13] in that vinclozolin exposure was restricted to fetal exposure and this may have produced less powerful functional deficits. However, the decline in ir-GnRH neuronal number may be a fetal antecedent, prerequisite, or synergistic factor for whatever changes four additional weeks of vinclozolin exposure might cause (e.g., alterations in behavior – [9]).

In summary, this study provides evidence that prenatal vinclozolin may affect the GnRH network in a non-antiandrogenic manner. Our finding for a loss of GnRH cell bodies, concomitant with an increase in GnRH peptide in the terminals, suggests a potential disruption of transport and/or release of the GnRH decapeptide. Together with evidence that vinclozolin's effects are substantially different from those of the anti-androgen flutamide, and for little difference in aromatase activity in the brain of vinclozolin and control rabbits, our data suggest that the mechanism by which prenatal vinclozolin causes neuroendocrine disruption remains to be identified.

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Figure 1.

Gestational vinclozolin exposure disrupted the immunoreactive GnRH neuronal network in the organum vasculosum of the lamina terminalis region. Images from representative rabbits show that by postnatal day 1 (A-F) fewer immunoreactive GnRH neurons were detected in the immediate vicinity of the organum vasculosum of the lamina terminalis (OVLT) of vinclozolin-exposed rabbits as compared to vehicle and flutamide-exposed. A similar decrease in immunoreactive GnRH neuronal population was noted in both males (A,C,E) and females (B,D,F). V, third ventricle; Scale bars represent 100µm.

Figure 2.

Mean number of immunoreactive GnRH neurons detected by section with a decrease in the region surrounding the OVLT in vinclozolin-exposed PND1 (males and females) compared to vehicle and flutamide-exposed rabbits. For panel A, males and females were pooled together as no sex difference was observed ($n = 5$ per group). Bars denote mean immunoreactive GnRH neurons in the OVLT (one section) in both sexes. For panel B, each black diamond represents mean number of ir-GnRH neurons in OVLT region for one male, each grey circle represents mean number of ir-GnRH neurons in OVLT region for one female (B). Veh, vehicle; Vinc, vinclozolin; Flut, flutamide; OV, OVLT; negative numbers denote section rostral to OVLT and positive numbers denote section caudal to OVLT; Error bars represent $\pm$ SEM; * designate p value < 0.05 .

Figure 3.

Images from representative rabbits show a similar decrease in the immunoreactive GnRH neuronal population in PNW26 males (A-C). Fewer immunoreactive GnRH neurons were detected in the immediate vicinity of the organum vasculosum of the lamina terminalis (OVLT) of vinclozolin-exposed rabbits as compared to vehicle and flutamide-exposed. Mean number of immunoreactive GnRH neurons detected per section (D). Scale bars represent 100 μ m; Veh, vehicle (n = 4); Vinc, vinclozolin (n = 5); Flut, flutamide (n = 5); OV, OVLT; negative numbers denote section rostral to OVLT and positive numbers denote section caudal to OVLT; Error bars represent $\pm$ SEM; * denotes p value < 0.05.

Figure 4.

Mean number of immunoreactive GnRH neurons detected per section in PNW30 females. Veh, vehicle (n = 3); Vinc, vinclozolin (n = 4); OV, OVLT; negative number denotes section rostral to OVLT and positive number denotes section caudal to OVLT; Error bars represent $\pm$ SEM; *.

Figure 5.

Confocal micrographs of GnRH-immunopositive processes and terminals in the region of the median eminence show data from representative adult female rabbits with fetal exposure to vehicle (A) or vinclozolin (B). Tissues were labeled with a primary mouse monoclonal antibody directed toward GnRH followed by the FITC-labeled secondary antibody in a single immunocytochemical run, and images were acquired on the confocal microscope in a single session together with appropriate controls (see Methods for details). No software adjustments of the micrographs were made after image acquisition. Immunoreactive GnRH was detectable in the median eminence of all rabbits, with greater intensity seen in the vinclozolin than the vehicle treated rabbits. ME = median eminence; V = third ventricle. Scale bar = 100 μ m.

Figure 6.

Hypothalamic GnRH content was increased two-fold in prepubertal PNW6 female rabbits exposed gestationally to vinclozolin as detected by radioimmunoassay. AH, anterior hypothalamus; DBB/OVLT, diagonal band of Broca/organum vasculosum of the lamina terminalis; MBH, mediobasal hypothalamus; Error bars represent $\pm$ SEM (n = 6 per group); * denotes $p < 0.05$.