Research Report

Investigation the endocrine disruptor effect of zearalenone in the hypothalamus

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Background information

Zearalenone (ZEA, previously known as F-2 toxin) is mycotoxin produced by several *Fusarium spp*. (*F. graminearum (Gibberella zeae), F. culmorum, cerealis, F. equiseti, F. crookwellense, F. semitectum*) (Zinedine, Soriano et al. 2007). ZEA is found worldwide in a number of cereal crops; such as maize, barley, oats, rice and shorgum; moreover, ZEA is heat stable, hence processing of raw materials in food industry not eliminate completely the toxin. Accordingly ZEA can appear in the food and fodder product (Kuiper-Goodman, Scott et al. 1987) (Aziz, Attia et al. 1997). Trade of these commodities may contribute to the worldwide dispersal of this mycotoxin. In Hungary, (Fazekas, Kis et al. 1996) reported the contamination of mould and stored corn with ZEA that ranged between 0.01 and 11.8 mg/kg.

Effect of the Zearalenone in animals and human

ZEA is rapidly absorbed after oral administration and can be metabolised in intestinal cells where it is transformed into α-, β-zearalenol and α-, β-zearalanol compounds subsequently conjugated with glucuronic acid. ZEA binds to estrogen receptors, perturbs the hormonal balance of animals, and can cause serious problems of the reproductive system. ZEA and some of its metabolites have been shown to competitively bind to estrogen receptors (ERα and ERβ) in a number of *in vitro* or *in vivo* systems (Kuiper, Lemmen et al. 1998). Female reproductive system is more sensitive to ZEA than males, mainly in prepubertal age. Prepubertal ZEA administration can cause accelerated vaginal opening, persistent estrus and sterility (Ito and Ohtsubo 1994). Although ZEA and its metabolites are ubiquitous, they are generally considered a potential danger for human health, particularly when they are absorbed in high amounts or over a long time of exposure. In addition to their toxic effects, experimental data support the view that ZEA mycoestrogens are suspected as triggering factor for central precocious puberty at least in prepubertal girls (Massart and Saggese 2010).

Puberty and the Central Nervous System

The effects of the ZEA in the central nervous system are remained to elucidate. The estrogen receptors (ERα and ERβ) are widely expressed in different brain region, for example in the hypothalamus. Hypothalamic brain regions are the integrative regulator sites of metabolic control and the major regulator of the sexual maturation as well (Ojeda, Urbanski et al. 1986, Aubert, Pierroz et al. 1998).

Several publication have focused on the role of kisspeptin in puberty (Messager 2005, Tena-Sempere 2006). Humans and mice lacking a functional kisspeptin receptor do not progress normally to achieve puberty (de Roux, Genin et al. 2003). Many species exhibit a marked increase in *Kiss1* and/or *Kiss1r(Gpr54)* expression in association with the onset of puberty, suggesting that kisspeptin acts as gatekeeper for puberty (Han, Gottsch et al. 2005, Shahab, Mastronardi et al. 2005).

A detailed distribution of Kiss1 (transcript and protein) has been mapped in the murine hypothalamus. In this species, *Kiss1* mRNA and KISS1-immunoreactive (ir) cell bodies are expressed in areas of the hypothalamus implicated in the neuroendocrine regulation of gonadotropin secretion, including the anteroventral periventricular nucleus (AVPV), the periventricular nucleus (PeN), and the arcuate nucleus (ARC) (Clarkson and Herbison 2006, Clarkson, d'Anglemont de Tassigny et al. 2009). Although the overall distribution of KISS1-ir cells is similar between male and female mice, there is a remarkable sex difference in the number of cell bodies in the AVPV/PeN [as is the case with *Kiss1* mRNA-expressing cells in the rat (Kauffman, Gottsch et al. 2007)], with adult females exhibiting 10-fold greater numbers of kisspeptin-ir cells than males (Clarkson and Herbison 2006). KISS1-ir neurons in the ARC, DMH, paraventricular nuclei, VMH, caudoventrolateral reticular nucleus, lateral reticular nucleus, nucleus of the solitary tract, and spinal trigeminal tract (Dun, Brailoiu et al. 2003, Brailoiu, Dun et al. 2005). KISS1 act via activation of the KISS1 receptor (GPR54).

The presence of *Kiss1r* in the brain was first reported in 1999 by Lee *et al.* (Lee, Nguyen et al. 1999), who used *in situ* hybridization and found expression in the pons, midbrain, thalamus, hypothalamus, hippocampus, amygdala, cortex, frontal cortex, and striatum *Kiss1r* is expressed in GnRH neurons (Han, Gottsch et al. 2005), located in the preoptic area of the hypothalamus (Messager, Chatzidaki et al. 2005). More than 90% of GnRH neurons express *Kiss1r* transcript, thus providing evidence that, in the rodents, kisspeptin neurons provide direct synaptic input to GnRH neurons.

An important study by Goodman *et al.* (Goodman, Lehman et al. 2007) describes a subpopulation of ovine kisspeptin neurons in the ARC that coexpress dynorphin A and neurokinin B (NKB), and ERα and PR, because these steroid hormone receptors are expressed in nearly all dynorphin and NKB neurons in the ARC (Goubillon, Forsdike et al. 2000, Foradori, Coolen et al. 2002). This is the first study to provide direct evidence that kisspeptin neurons contain additional neuropeptides involved in reproductive control. Other work provide implicit evidence for a similar coexpression phenomenon in other species, including the rat, mouse, and human. For example, there is extensive colocalization of NKB and dynorphin in the ARC of the rat (Burke, Letts et al. 2006). Because these neurons all express ERα (Burke, Letts et al. 2006), we can reasonably infer coexpression of kisspeptin, NKB, and dynorphin in the ARC.

SUMMARY OF RESULTS

1. **Determination of ZEA induced hypothalamic RNA expression profile on rat and mice**

Rats (Wistar) and mice (CD1) were treated with 17α-ethinyl estradiol (EE2) (10 μg/kg body weight) or zearalenone (ZEA) (5 mg/kg bw) for 10 consecutive days starting at postnatal day 18 according to the protocol used for the uterotrophic assay developed and validated by OECD 440. The other groups of rats were treated with ZEA and EE2 until the vaginal opening was appeared. Furthermore a normal puberty group was introduced (naïve).

The dose of the ZEA was based on our previous study (Kriszt, Krifaton et al. 2012), while the dose of EE2 was selected in a pilot uterotrophic assay (Kriszt, Winkler et al. 2015). On PND 28, vaginal smears were taken then animals were sacrificed by decapitation, and trunk blood was collected for hormone measurement. The brain was rapidly removed from the skull, placed into acrylic coronal matrix and was dissected to isolate an AVPV/POA-enriched region and an arcuate-enriched region (ARC) or hypothalamic block were removed for collecting samples for NGS and quantitative real time PCR experiments. The NGS was carried out on Illumina platform.

Both ZEA and EE2 advanced puberty as measured by the number of rats showing vaginal opening, an external sign of puberty onset in this rodent. 100% of rats showed vaginal opening at postnatal day PND 24 or 25 in case of EE2 or ZEA treated rats, respectively. None of the vehicle-treated control animals displayed vaginal opening before the end of the experiment. Vaginal opening in the naïve group occurred at 30-31 days, postnatal.

The result of the NGS

Hypothalamic samples were obtained from vehicle, ZEA, EE2 treated and naïve rats and processed for global analysis of transcriptional changes detected by Next Generation Sequencing technology (NGS, Illumina). Based on different biostatistical evaluation of top affected genes were analysed. The new gene interactions were analysed using Ingenuity Pathway software. The results are summarized in Fig1. We firstly identified the ZEA and EE2 induced interaction between the *Gnrh* and *Tgfbr2* genes. Moreover up regulation of the *Gpr54* mRNA levels were identified in both xenoestrogen treated group and the naïve (NVO) animal's hypothalamic samples. Increased *Npy* mRNA expression level was detected in the ZEA treated and naïve animal's hypothalamic samples. The naïve animal's hypothalamic samples showed more complex expressions alterations compared the xenoestrogen treated groups. Our experiments were identified numerous novel puberty related genes in the hypothalamus. We have revealed the *Prkcd, Prkcg, Cck, Slc17a1* and *Gabard* downregulation in the naïve (NVO) animal's hypothalamic samples. On the other hand the Sim1 mRNA levels were increased in the naïve group alone. The canonical puberty related genes such as *Mkrn3* was decreased in the naïve group alone. We firstly demonstrated that the zearalenone provoked precocious puberty is *Mkrn3* independent phenomenon. The result of the mice hypothalamic samples NGS was similar to the rat hypothalamic block transcriptional changes detected by NGS.

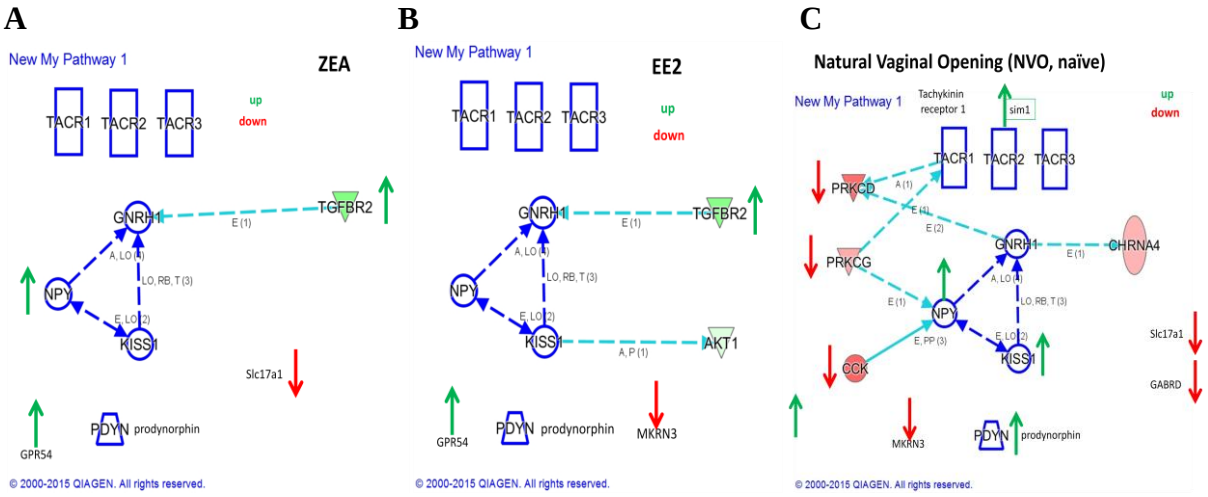

Fig. 1: **The new gene interactions were revealed using Ingenuity Pathway software**

New interactions were revealed based on the result of NGS in zearalenone (A), ethinyl estradiol (B) treated and naïve (C) prepubertal rat's hypothalamus (Ferenczi et al., under publication).

2. **Validation the mRNA expression levels in the hypothalamus**

Kisspeptin (KISS1) and its receptor GPR54 have been recognized as a key regulator of GnRH secretion during puberty and adulthood, conveying the feedback influence of endogenous gonadal steroids onto the GnRH system. These results were confirmed by the result of NGS. *Gnrh* expression was significantly increased following EE2 or ZEA treatment as measured by qPCR from AVPV/POA-enriched brain tissues (p=0.0012 for both xenoestrogens) relative to vehicle-treated rats.

ZEA and EE2 differentially affect *kiss1* and *gpr54* mRNA levels in the hypothalamus

A peripubertal increase of *gnrh* is attributable, at least in part, to estrogen-sensitive inputs to the GnRH neurons. Among these, kisspeptinergic innervation plays a pivotal role in pubertal timing. Because *kiss1* expression is regulated reciprocally by estrogen in the anteroventral periventricular (AVPV)/rostral periventricular (PeV) and arcuate (ARC) regions in the rodent brain, *kiss1* and kisspeptin receptor *gpr54* mRNA levels were measured in these brain areas separately. Both ZEA and EE2 treatment resulted in a significant increase in *kiss1* expression in the AVPV/PeV (ZEA: 2.83-fold increase; EE2: 2.82-fold increase), while in the ARC region, significant alterations were not observed. The expression of *Gpr54* increased significantly only in the ARC (ZEA: 1.68-fold increase; EE2: 1.89-fold increase).

Within the arcuate block, the relative quantity of neurokinin B (*Nkb*) mRNA levels decreased in ZEA and EE2 treated rats (p=0.013). However, the expression of dynorphin (*Dyn*) did not differ in xenoestrogen treated animals compared to the vehicle-treated controls (p=0.84). No changes were detected in the hypothalamic mRNA levels of estrogen receptors (ER) *Erα* (p=0,11) or *Erβ* (p=0,30).

Ring finger transcription factor makorin 3 (*Mkrn3*) has been implicated in sexual development as a break of puberty (Macedo, Abreu et al. 2014). *Mkrn3* mRNA was elevated exclusively in the AVPV/PeV following EE2 treatment. A small increase in the mRNA level of *Eed* was also detected in the anterior hypothalamic block. The relative quantities of *Cbx7, Cux1, Eap1* and *Ttf1* transcription factor mRNA remained unaltered in ZEA and EE2 treated animals compared to vehicle controls. These transcription factors play pivotal role on the normal puberty but then the ZEA toxicities did not show any influences in their transcription activity.

Our experiments were identified numerous novel puberty related genes in the hypothalamus. We have revealed and validated the expression levels by qRT-PCR the *Prkcd, Prkcg, Cck, Slc17a1* and *Gabard* down regulation in the naïve (NVO) animal's hypothalamic samples. On the other hand we firstly demonstrated that the *Sim1*and *Ddr2* mRNA levels were increased in the naïve group alone. Increased *Npy* mRNA expression level was validated in the ZEA treated and naïve animal's hypothalamic samples by qRT-PCR measurment. These findings suggest that the timing of puberty depends on the metabolic resources of the animals. The daily body weight data was indicated that EE2-treated premature female rats gained significantly less body weight during the treatment period than vehicle, naïve or ZEA treated animals. Daily and cumulative food and water intake of the three groups were not significantly different. The unaltered hypothalamic *Npy* expression level in the EE2 treated animals is a putative explanation for the reduced body weight gain in this group.

3. **Mapping the anatomical distribution of ZEA-regulated validated transcripts in the rat brain.**

To monitor kisspeptin and *Gpr54* mRNA neuroanatomical localisation, radio labeled riboprobes were used. Results of our experiments showed that zearalenone can influence the reproductive system. Animals was transcardially perfused for *in situ* hybridization and immunocytochemistry. Antisense cRNA probes labeled by ^{35}S-uridine triphosphate were used to mRNA expression anatomical localisation. To evaluate changes in Kispeptin fiber density and kisspeptinergic inputs to GnRH neurons, double labeling immunofluorescence was performed. Confocal imaging was performed on Olympus FluoView FV1000 (Olympus, Japan) laser scanning confocal microscope. The vehicle treated female rats show *Kiss1* in situ hybridization signals exclusively over the neurons in the anteroventral periventricular (AVPV), the rostral periventricular (PeV) and the arcuate nuclei (ARC). In response to ZEA or EE2 administration, increased kisspeptin mRNA expression was revealed in the AVPV/PeV (Fig.2), without detectable changes in the ARC. Extrahypothalamic expression of *Kiss1* was not detected following xenoestrogen treatment. ^{35}S-UTP labeled riboprobes corresponding to *Gpr54* mRNA revealed a specific pattern of kisspeptin receptor expression in the rat brain. In vehicle treated animals, intense hybridization signals were detected in cortical layers as well as in the CA1-4 areas and the dentate gyrus of the hippocampal formation. Strong labeling corresponding to *Gpr54* mRNA has been revealed in the lateral habenula, in the subfornical organ, bed nucleus of stria terminalis and in the mammillary region. Within the hypothalamus, the preoptic area, suprachiasmatic-, supraoptic-, caudal paraventricular-, ventromedial- and arcuate nuclei were moderately labeled. In response to ZEA and EE2 there was a selective increase in the intensity of the hybridization signal in the medial basal hypothalamus (Fig.2). The strength and distribution of the hybridization signal at the extra hypothalamic areas also remained unchanged after ZEA and EE2 treatment.

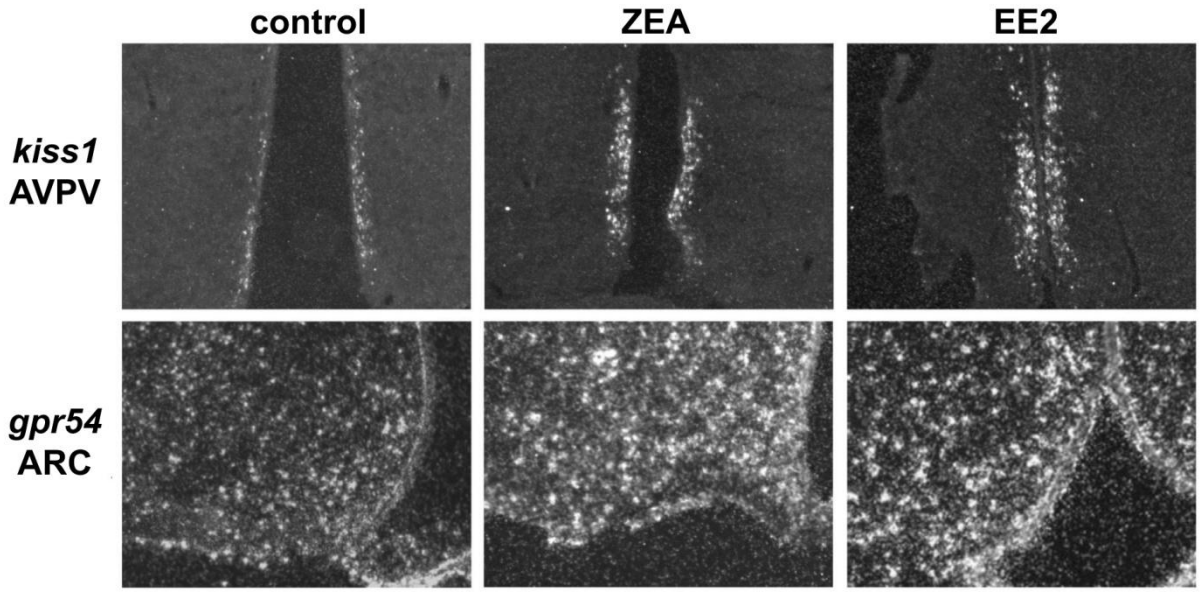

Fig.2: **Effects of xenoestrogens on *Kiss1* and *Gpr54* mRNA signals in the rat hypothalamus.** Representative dark field autoradiograms showing signals obtained following *in situ* hybridization with cRNA probes. Top row: *Kiss1* mRNA in the anterior periventricular region Bottom row: *Gpr54* mRNA signals in the arcuate nucleus following vehicle- zearalenone (ZEA) or ethinyl-estradiol (EE2) treatments. Photomicrographs 75x magnifications (Kriszt et al., Endocrinology, 2015).

Endocrine disruptors increase kisspeptin fiber density in the preoptic area and the number of KISS1 (KP) appositions onto GnRH neurons

Analysis of immunofluorescent stained samples revealed an increased density of kisspeptin-immunoreactive fibers in the preoptic region of ZEA and EE2-treated animals. One way ANOVA revealed a significant treatment effect ($p=0.042$). Treatment with the endocrine disruptors resulted in 5-fold and 8-fold increases in KP fiber density in the case of ZEA and EE2, respectively ($p=0.0008$) (Fig.3).

Furthermore, using double-labeled immunfluorescence, we revealed KP fibers in close apposition to GnRH neurons in the preoptic area. Specifically, the number of KP appositions on GnRH neurons was significantly increased in EE2 and ZEA treated animals ($p=0.0009$) (Fig.3).

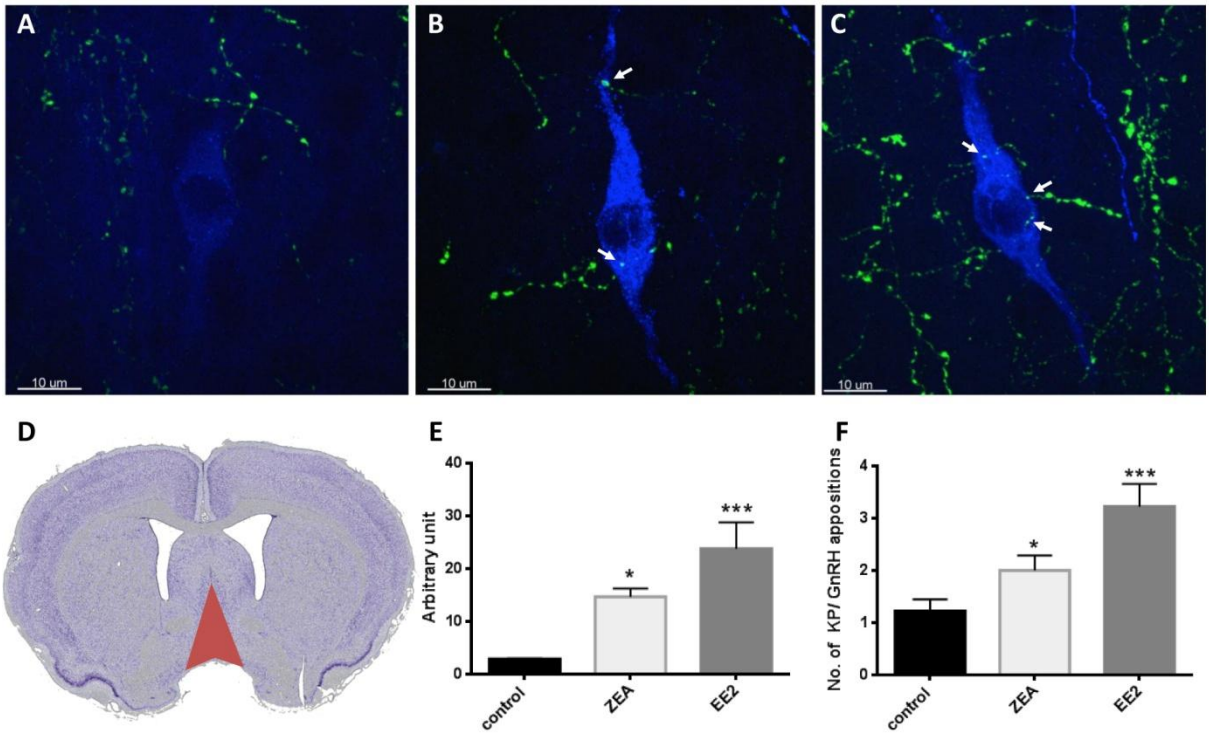

Fig.3: **Changes in GnRH and kisspeptin immunostaining in the preoptic area of xenoestrogen-treated prepubertal female rats**

Top: Representative fluorescence images showing double-labeling for GnRH (blue) and kisspeptin (green) in the preoptic area of vehicle treated control (A), ZEA-treated (B) and EE2-treated (C) female rats.

Bottom: Schematic drawing depicting the rostral preoptic area as the "region of interest" (ROI) for quantitative analysis of kisspeptin (KP)-immunoreactive fibers (D). Density of kisspeptin-immunoreactive fibers in the preoptic area (E) and number of kisspeptinergic profiles in close apposition with GnRH-ir neurons (F) in control, ZEA and EE2-treated female rats (Kriszt et al., Endocrinology, 2015).

4. **Effect of ZEA on the key steroid hormone production related genes on ovary**

We have characterized the gene expression level alterations of the key ovarian enzyme coding genes by qRT-PCR. ZEA an EE2 treatment significantly reduced the steroid hormone production related genes mRNA levels. The mRNA expression of *star, cyp19 (aromatase), cyp17a1, cyp1a1* and *hsd3b* was significantly reduced by the both xenoestrogene administration. However the *star, cyp19* and *hsd3b* were showed significant elevation in naïve animal's ovary.

5. **Effect of ZEA on plasma hormone levels**

Plasma LH (p=0,42) and FSH (p=0,87) concentrations did not change significantly after ZEA or EE2 treatment. EE2, but not ZEA, administration significantly reduced the 17β-estradiol concentration (p=0,006) by the end of the 10-day administration period. On the other hand the xenoestrogen treatment until the vaginal opening was appeared the plasma P4

and T concentrations did not change significantly. The naïve animal's plasma P4 and T concentrations were increased compared to vehicle group ($p<0.0001$ and 0.001 respectively) (Fig.4 C, B). The ZEA administration significantly elevated the E2 concentration ($p<0.05$) compared to control, but the EE2 treatment significantly reduced the 17β-estradiol concentration compared the ZEA treated group ($p<0.001$) (Fig.4 A). The E1 hormone levels were significantly reduced by the xenoestrogens treatment ($p<0.001$) (Fig.4 D). The ZEA and EE2 administration until the vaginal opening was appeared did not change significantly the Anti-Müllerian hormone (AMH) concentration in the plasma (Fig.5). The naïve animal's plasma E2, T, P4 and AMH levels was elevated significantly compared to the control (Fig.4 A, B, C, Fig.5, $p<0.01$, $p<0.001$, $p<0.0001$ and $p<0.01$ respectively).

We firstly analysed the ZEA induced complete ovarian gene expression and hormone profile. These results suggest that ZEA and EE2 blocked the ovarian sex steroid hormone synthesis and AMH secretion. These phenomenons lead to hormonal perturbation during the ZEA toxicities.

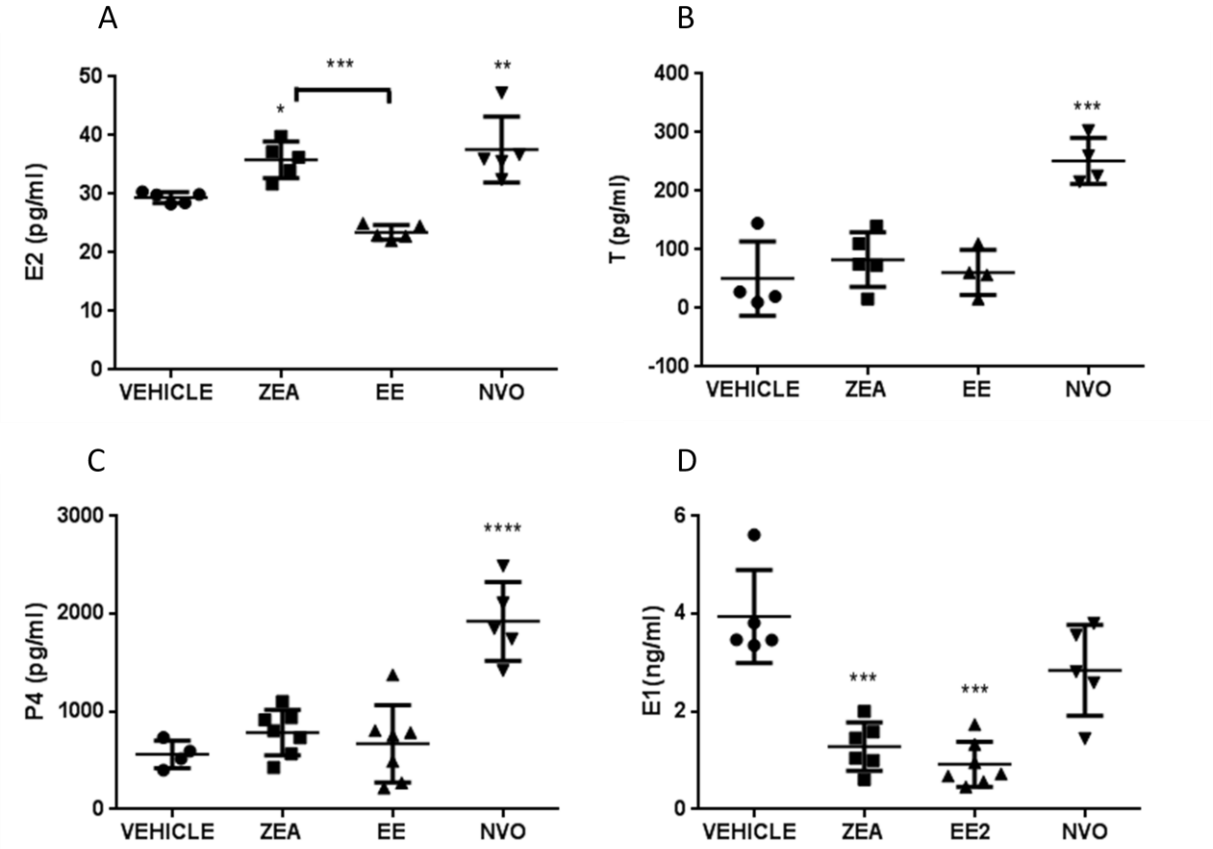

Fig.4: **Plasma sexual steroid hormone levels following xenoestrogen treatment**

Mean ± SD values of plasma 17β-estradiol (A), testosterone (B), progesterone (C) and estrone (D) levels in vehicle (control), zearalenone (ZEA), ethinyl estradiol (EE2) treated and naïve (NVO) prepubertal rats (Ferenczi et al., under publication).

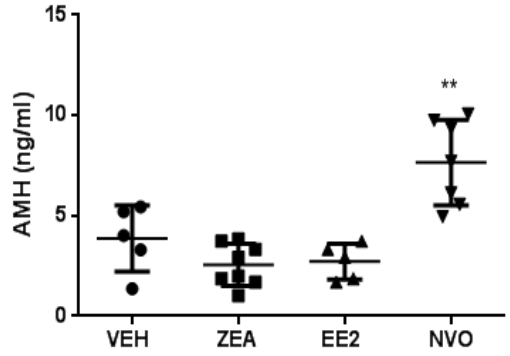

Fig.5: **Plasma Anti-Müllerian hormone (AMH) levels following xenoestrogen treatment**

Mean ± SD values of plasma AMH levels in vehicle (control), zearalenone (ZEA), ethinyl estradiol (EE2) treated and naïve (NVO) prepubertal rats (Ferenczi et al., under publication).

6. Effect of ZEA on estrogens degrading hepatic enzymes transcriptional activity

We have characterised the major estrogens degrading hepatic enzymes transcriptional activity. The *Cyp1b1* mRNA level was increased significantly in the ZEA treated animals liver samples. On the other hand the *Cyp1 a1, Cyp19 (aromatase), Comt, Ugt2b, Sta1* and *Ste1* did not change significantly after ZEA administration until vaginal opening was appeared. The naïve animal liver samples were showed reduced *Cyp19, Cyp1a1, Sta1* and *Ste1* transcriptional activity. Moreover *Cyp1b1, Ugt2b* were increased significantly at mRNA level in the naïve animal liver samples.

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