Influence of Gonadectomy and Testosterone Supplementation on the Postnatal Development of the Vasopressin and Oxytocin-Containing Nucleus of the Pig Hypothalamus

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Abstract. We have studied the effects of gonadectomy and testosterone supplementation on the development of the vasopressin- and oxytocin-containing nucleus (VON) of the pig hypothalamus that shows a decrease in neuron number during the first weeks postnatally, followed by an increase during puberty. Neonatal gonadectomy caused a 2.5-fold increase in VON volume and neuron number in males and 3-fold in females at the age of 16 weeks, the onset of puberty. However, the difference between gonadectomized and nongonadectomized animals disappeared after puberty (38 weeks). Testosterone replacement from 16 weeks onwards induced a decrease in neuron number and volume of the VON. The present study indicates that the development of the VON is influenced by gonadal steroids although it seems improbable that these hormones affect the VON directly.

Recently a vasopressin- and oxytocin-containing nucleus (VON) has been described in the hypothalamus of the pig, situated lateral from the third ventricle, dorsal to the suprachiasmatic nucleus and rostroventral from the paraventricular nucleus. This nucleus shows an increase in neuron number and volume during puberty (between 16 and 30 weeks) in both males and females [1]. Since the periods, during which the number of neurons increase, coincide with higher levels of testosterone (T) and luteinizing hormone (LH) in the male, the present study was aimed at investigating whether sex steroids might influence the size of the VON during development. In this respect it is interesting to note that T levels in the male pig follow a similar pattern as in man during early development, i.e. a prenatal surge at about the end of the first trimester of gestation, followed by a second surge directly after birth, to rise again around puberty [2–10]. Estradiol levels in female pigs are low until puberty [11–14]. In both male and female pigs there is a perinatal LH surge, which in the male results in a T surge postnatally [5, 15]. The present studies indicate that manipulation of the hypothalamo-pituitary-gonadal axis during development influences the size and neuron number of the VON.

Materials and Methods

In the first experiment the VON of 55 pigs was studied at 7 and 16 weeks postnatally. All animals were operated on within 12 h after birth under complete inhalation anesthesia (N₂O, O₂ and Fluothane) without premedication. Males were gonadectomized through two incisions in the perineum which were closed by suture (Vicryl®, Ethicon). Sham-operated males received only skin incisions which were closed by suture. Females were gonadectomized by laparotomy. The wound was sutured in three layers (Vicryl). Sham-operated females were treated in the same way, except for the ovaries, which were left intact. No antibiotics were administered either pre- or postoperatively. For this study 5 intact male and 5 intact female (controls), 5 sham-operated male, 7 sham-operated female, 7 gonadectomized male and 7 gonadectomized female pigs (Dutch landrace × Great York) were sacrificed at 7 weeks postnatally. At 16 weeks 5 intact male, 4 intact female, 6 gonadectomized male and 4 gonadectomized female animals of the same breed were sacrificed. All animals of this first experiment were sacrificed in summer (i.e. in June–August).

The animals were sacrificed and perfused under complete anesthesia by means of azaperone (Stresnil®) intramuscularly and methomidate (Hypnodil®) intravenously. For perfusion, first a physiological saline solution containing heparin was used, followed by a solution of 25% glutaraldehyde, 74% picric acid and 1% acetic acid, has been described previously [1]. After embedding in paraffin, 10-µm serial sections were cut and Nissl-stained with cresyl violet.

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In a second experiment 43 animals born between 17 and 22 December 1987 were sacrificed at 38 weeks of age. For technical reasons it was not possible to use exactly the same breed as in the first experiment. The animals [(Dutch landrace × Duroc) × Great York] were divided into four groups: (1) gonadectomized, no T supplementation (4 females and 9 males) (= GX), (2) gonadectomized, T supplementation during the first 4 weeks neonatally (3 females and 6 males) (= GXNT), (3) gonadectomized, T supplementation from 16 weeks onwards (5 females and 7 males) (= GXPT), and (4) sham-operated (4 females and 5 males) (= Sham). Treatment of the four groups is summarized in figure 1. Neonatal gonadectomy was performed as described in experiment one. Each animal of the GXNT group received an oral dose of 10 mg T-undecanoate daily, for the first 4 weeks (Andriol®, kindly donated by Organon) [for pharmacology and characteristics, see 16, 17]. The other three groups in experiment two were given solvent oil only. In a pilot experiment [unpubl. data] this ester was found to give T levels in peripheral blood of neonatal pigs within the physiological range. In the same pilot experiment injectable preparations (Testoviron®, Schering; Durateston®, Intervet) have also been tested. These preparations gave a very high starting level that dropped within 2 days. Such preparations would thus have required frequent, i.e. daily, injections; for this reason they were not chosen.

From 16 weeks onwards the GXPT group received T supplementation by means of a subcutaneous silastic tube implant (SR 7 A, Talas) that provided T levels within the physiological range [5, 6, 15, 18]. Oral supplementation of these animals would have required a daily treatment and consequently considerable stress. Silastic tubing has proven to release steroids at a constant rate [19–22]. Each animal was implanted with 3 tubes of 0.5 m length (3.5 × 4.5 mm diameter) filled with crystalline T (Bufa-chemie, USP XXI). The animals of the other groups received at the same time 3 empty silastic tube implants each. During implantation the animals were under complete anesthesia by means of azaperone (Stresnil, Janssen) intramuscularly and methomidate (Hypnodil, Janssen) intravenously. Because of the high growth rate of the pigs the dosage provided by the tubing implants was not sufficient after 25 weeks of age (levels < 2.0 ng/ml). Therefore T-enanthate (Testoviron, Schering) injections were given in addition to the silastic implants. The GXPT group received extra injections of 500 mg T-enanthate (Testoviron) weekly. All other groups received sham injections with solvent at the same time. The T injections resulted in shortlasting high T levels that dropped in 1 week, with a tendency to accumulate, so that high levels (> 8 ng/ml) were evident by the end of the experiment.

T and LH levels were measured weekly in peripheral venous blood. Blood was obtained between 11.00 and 11.45 a.m. by puncture of the cranial vena cava (samples were taken just before administration of T or sham supplementation).

Plasma concentrations of porcine LH were estimated by a previously validated radioimmunoassay [23]. The intra- and interassay coefficients of variation were 2.3 and 16.4%, respectively. The lowest detectable amount was 0.2 µg/l.

Concentrations of T were estimated in plasma samples by a solid-phase ¹²⁵I radioimmunoassay method (Coat-A-Count TKTT; Diagnostic Products, Los Angeles, Calif., USA) according to the manufacturer. The main cross-reactivities were 3.3 and 0.5% for dihydrotestosterone and androstenedione, respectively, and < 0.1% for other steroids of interest according to the manufacturer. The sensitivity was 0.04 nmol/l and the interassay coefficient of variation was 12% (n = 10).

Morphometric analysis was performed on 10-µm paraffin sections for neuron number and volume of the VON as previously described [1,

Fig. 1. Schematic representation of the treatment of the experimental animals of experiment 2. GXN = Gonadectomized.

24]. Briefly this computer-assisted method averaged the distance between two neurons in a representative central part of the nucleus. This distance is used as the diameter of a circle. This circle is drawn around each neuron in all sections measured. Sections are selected by systematic sampling [see 25]. When a circle crossed two other ones, the neuron was considered to belong to the nucleus. To establish a contour around the nucleus, the outer contour of all circles is drawn. Within this contour the surface area and section thickness were measured to calculate the volume of the VON and the nucleoli were counted in order to estimate the total neuron number.

For statistical analysis the animals were grouped according to age and treatment. Analysis of the morphometric data was performed first for sex differences within the groups with a MANOVA, Kruskal-Wallis and a Mann-Whitney-U test. Because there were no significant sex differences in neuron number, data from both sexes were combined and Mann-Whitney-U tests were performed between all the groups in each study for neuron number. For volume a sex difference was found in experiment 2 and subsequent tests, in this experiment, were performed for each sex separately.

Results

First Experiment (fig. 2)

The gonadectomized animals at 16 weeks had about a 3-fold larger number of neurons and VON volume ($p < 0.0001$) than all other groups of the first experiment. There were no significant sex differences observed in this first experiment ($p = 0.547$). The other groups did not differ significantly from each other for neuron number or volume.

Second Experiment (fig. 3, 4)

The T and LH levels of the animals in experiment 2 are given in figure 3. The GX group had low or undetectable T and high LH levels throughout the experiment. The LH levels of males were higher than those of the females in the first 10 weeks postnatally ($p < 0.01$). The GXNT group had high T levels in the

Fig. 2. Total neuron number and volume of the VON at 7 and 16 weeks postnatally in control (female and male), sham-operated and gonadectomized (GNX) pigs.

first 4 weeks postnatally, after which T levels dropped to below detection level. LH levels were slightly lower than those from the GX and GXPT group in the first 4 weeks. After this period they were not found to be different from the GX group. The GXPT group had low T levels until supplementation, medium high T levels after implantation of the silastic tubings and high levels after the start of the additional weekly injections. The LH levels followed a similar pattern to those of the GX group until supplementation. After supplementation started, LH levels dropped, to rise again after 23 weeks. When the additional injections were given LH levels dropped again. The females in the Sham group showed low or undetectable T levels throughout the experiment, while the male T levels tended to rise after 16 weeks. LH levels were high in the first 8 weeks, although the females had lower values than the males. After 8 weeks the LH levels of both males and females followed a similar curve to the GXPT group.

The results of the neuron counts of the second experiment are given in figure 4. There was no significant sex difference ($p < 0.42$). Group GX, GXNT and Sham did not differ significantly from each other (GX-Sham: $p < 0.57$, GX-GXNT:

$p < 0.63$, GXNT-SHAM: $p < 0.46$). GXPT differed significantly from GX ($p < 0.02$), GXNT ($p < 0.05$), and Sham ($p < 0.05$).

The results of the volume measurements of the second experiment are given in figure 4. A significant sex difference (MANOVA $p < 0.04$) was observed, so the differences between the groups were tested separately for the sexes. For the males GX and GXPT differed significantly ($p < 0.01$). The other groups did not differ significantly from each other (GX-GXNT: $p < 1.00$, GX-Sham: $p < 0.64$, GXNT-GXPT: $p < 0.12$, GXNT-Sham: $p < 0.58$, GXPT-Sham: $p < 0.17$). For the females none of the groups differed significantly from each other (GX-GXNT: $p < 0.72$, GX-GXPT: $p < 0.46$, GX-Sham: $p < 1.00$, GXNT-GXPT: $p < 0.56$, GXNT-Sham: $p < 0.72$, GXPT-Sham: $p < 0.14$). Testing for sex differences in volume within the four groups gave the following results GX: $p < 0.08$, GXNT: $p < 0.19$, GXPT: $p < 0.54$, Sham: $p < 0.67$. The small sex difference in volume found in the second experiment must thus be seen as an overall effect and not as the result of an extreme difference in one of the four groups. When looking at the groups individually this effect is not significant in one group, but it is most prominent in GX and GXNT.

Discussion

The VON shows a decrease in neuron number to 63% during the 1st weeks postnatally, followed by a 3-fold increase during puberty (16–30 weeks) [1]. The present study was aimed at determining whether T influenced the changes observed in the number of neurons and volume of the VON during development. However, since pig testes produce a considerable amount of estrogens [26], such steroids might play a role too.

Sexual dimorphism in volume of the VON was observed only at 38 weeks and was most prominent in the GX and GXNT group. Both groups did not receive T at the time of sacrifice. Postnatally males and females of each group received the same treatment, but prenatally the hormone environment was different. A major endocrinological difference between males and females during development in GX and GXNT occurred at the end of the first trimester of gestation, where males showed a T surge and females did not. The LH levels in the neonatal period are also sexually dimorphic, although they are elevated in both sexes [27]. The T feedback mechanism is considered to be not yet functional in the first weeks of life [28–30] and the neonatal rise of LH in the females might thus be considered as an indication that at that time the hypothalamus of the pig is still only partly differentiated functionally. Earlier studies [31, 32] revealed that the differentiation of the control of gonadotropin secretion in fetal pigs is influenced by androgens during the second trimester of the gestation period which is 115 days long. The sex difference in the volume of the VON of the GX and GXNT group might be an expression of this partial differentiation of the hypothalamus, already existent at birth.

Fig. 3. T and LH levels of the animals of experiment 2.

Fig. 4. Total neuron number and volume of the VON of the animals in the four groups of experiment 2.

The differences in the neuron number between the animals of experiment 1 and 2 might be due to the different breeds used and can therefore not be compared. A strong 2.5- to 3-fold increase in VON volume and neuron number was found following neonatal gonadectomy in both sexes (fig. 2). This effect was only observed at the age of 16 weeks, the onset of puberty. After puberty (38 weeks, experiment 2) the VON of the sham-operated animals had the same neuron number and size as the gonadectomized animals (fig. 4). However, at that age a significant decrease in neuron number was observed following the supplementation with T from 16 weeks onwards. One might expect that these animals had a high neuron number at 16 weeks, since until 16 weeks postnatally the animals of experiment 2 (i.e. GXPT: T supplementation from 16 weeks onwards and GX: no T supplementation) received the same treatment as the animals of experiment 1.

It is difficult to explain the enlargement of the VON during puberty in the intact animal by a direct action of steroids. T levels as well as VON size and neuron number rise in the intact male animal during puberty whereas T supplementation in the

same period (experiment 2) resulted in VON shrinkage. A similar phenomenon was described by Morishita et al. [33, 34] in the rat. They described in the paraventricular and supraoptic nucleus an increase in the size of the nucleus of neurons both during puberty and following prepuberal (30 days postnatally) gonadectomy. Unfortunately neuron numbers in these nuclei were not measured in their experiments. An additional problem in explaining the VON changes by direct T actions is that there are no significant sex differences in the volume or neuron number of the VON, in nontreated animals [1], in spite of the huge differences in sex hormone levels during development [5, 35, 36]. An alternative explanation might be that gonadotropic hormones are involved in the VON size alterations. Both female and male pigs have a similar pattern of LH levels during development, although the levels show some differences (fig. 3) [27]. As Swaab [37] and Swaab and Jongkind [38] described, gonadotropics may directly influence the activity of supraoptic nucleus and paraventricular nucleus neurons. The observation that receptors for gonadotropics are present in a number of hypothalamic nuclei in rat, including suprachiasmatic, periventricular and arcuate nucleus [39], reinforces this possibility. When the animals are gonadectomized luteinizing hormone-releasing hormone (LHRH) and LH levels rise and become more pulsatile [12, 23, 29, 31]. When supplemented with T, LHRH and LH levels drop and due to the negative feedback probably will stop pulsing. The size of the VON seems to follow this pattern. In the intact pig LHRH and LH levels are high and pulsatile around birth and the first weeks postnatally when the feedback mechanism is not functional yet [12, 23, 27, 29, 40]. The VON has a relatively large neuron number and volume at that time. Subsequently, LHRH and LH levels drop and the VON becomes smaller in both parameters too. During puberty LHRH and LH levels become pulsatile again [41] and the VON enlarges. In the intact pubertal animal LH levels fluctuate due to the negative feedback of steroids. In our experimental animals (experiment 2) we supplemented the animals with a relatively constant level of T. As expected the LH, and presumably the LHRH, levels were continuously low. So a stimulatory effect of gonadotropic hormones or their releasing hormones on the VON would explain why during puberty in an intact animal, although steroid levels increase, the VON enlarges. Because of the nonfunctional feedback mechanism for T during the first weeks of life [12, 27, 29, 40], the VON might still be small at 7 weeks of age to be enlarged at 16 weeks in the gonadectomized animals. Further studies should be performed to test the hypothesis that LH or LHRH might be a causal factor in the alterations of the VON.

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