The supraoptic nucleus in the pig hypothalamus: postnatal development and the effect of gonadal steroids

F. J. C. M. Van Eerdenburg, C. M. J. E. Lugard-Kok and D. F. Swaab*

Department of Functional Morphology, Faculty of Veterinary Science, University of Utrecht, Yalelaan 1, 3508 TD Utrecht, The Netherlands

*Netherlands Institute for Brain Research, Meibergdreef 33, 1105 AZ Amsterdam, The Netherlands

(Requests for offprints should be addressed to F. J. C. M. Van Eerdenburg who is now at the Netherlands Institute for Brain Research, Meibergdreef 33, 1105 AZ Amsterdam, The Netherlands)

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ABSTRACT

Postnatal development of the supraoptic nucleus (SON) in the pig hypothalamus was studied morphometrically. The volume of the SON increased from 6.2 ± 0.45 (S.E.M.) mm³ at 7 weeks postnatally to 18.5 ± 1.35 mm³ at 2.5 years of age. A sex difference was found at the development point when the SON volume increased, with earlier SON enlargement in males. This sex difference was 30% at 30 weeks and 50% at 1 year of age. At 2.5 years of age no difference in volume was apparent between the sexes. The number of SON neurones was similar for all age groups concerned ($43\,500 \pm 1475$), except for the 2.5-year-old females where 40% more were found ($55\,500 \pm 3285$). No significant difference was found in neurone number between gonadectomized and sham-operated animals, but the operation caused a

30% reduction in the number of neurones and SON volume. Testosterone supplementation following gonadectomy, during the first 4 weeks postnatally, resulted in sexual dimorphism, the males having more SON neurones than the females. The volume showed only a trend in the same direction. Testosterone supplementation at other ages did not result in any difference when compared with controls.

The results of this study show that the postnatal development of the SON of the pig is sexually dimorphic, and that it continues after puberty in females. In contrast to the vasopressin- and oxytocin-containing nucleus, the development of the SON was not influenced by gonadectomy and only slightly by gonadal steroids.

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INTRODUCTION

The supraoptic nucleus (SON) is an anterior hypothalamic structure belonging to the hypothalamoneurohypophysial system (HNS). The SON is characterized by a high density of large neurones with abundant Nissl substance (Scharrer & Scharrer, 1954) that produce vasopressin or oxytocin (Swaab, Nijveldt & Pool, 1975) (see Plate, figs 1 and 2; Text-fig. 1). The large vasopressin- and oxytocin-containing neurones of the hypothalamus are considered to be formed early in fetal life (Altman & Bayer, 1978) and to remain as a very stable cell population thereafter (Goudsmit, Hofman, Fliers & Swaab, 1990). Recently we have described a vasopressin- and oxytocin neurone-containing nucleus (VON) in the pig hypothalamus, situated between the suprachiasmatic nucleus, paraventricular nucleus (PVN) and

SON, in which the cytoarchitecture was similar to the SON and PVN. The VON appeared to show a 250% increase in neurone number and volume during puberty, i.e. between 16 and 30 weeks of age, in both males and females (Van Eerdenburg, Poot, Molenaar *et al.* 1990). After puberty an additional twofold increase in the number of neurones and volume of the VON was observed in adult females of 2.5 years of age, resulting in a sexual dimorphism of this nucleus (Van Eerdenburg & Swaab, 1991). Neonatal gonadectomy appeared to have a marked effect on the development of the VON in both sexes (Van Eerdenburg, Lugard-Kok, Dieleman *et al.* 1991). Since the neurones of the VON closely resemble those of the SON, both cytoarchitecturally and in their vasopressin and oxytocin content (Van Eerdenburg *et al.* 1990), the present study examined whether the SON has a

similar late development that could also be influenced by gonadal steroids.

MATERIALS AND METHODS

The same 50 crossbred Yorkshire × Dutch Landrace pigs that had been used previously for VON measurements (Van Eerdenburg *et al.* 1990; Van Eerdenburg & Swaab, 1991) were studied morphometrically in order to describe the normal development of the SON. The number of animals used in each group for the developmental study is shown in Text-fig. 2. In this study, all animals were intact, healthy and exactly the age given except for the 2.5-year-old group, where ages differed by a few weeks. None of the females was pregnant at the time of death and only the animals of the 2.5-year-old group had had several litters (the latest at least 6 weeks before killing). Under general anaesthesia the animals were decapitated and perfused (for details see Van Eerdenburg *et al.* 1990) with a mixture of 25% (v/v) glutaraldehyde (E. Merck, Amsterdam, The Netherlands), 74% (v/v) picric acid (E. Merck; saturated solution) and 1% (v/v) acetic acid (Brocacef, Utrecht, The Netherlands). The hypothalami were dissected, embedded in paraffin wax and sectioned rostrocaudally (10 µm sections).

A second experiment also studied animals that had been used previously for VON measurements (Van Eerdenburg *et al.* 1991). The SON of 27 pigs were studied at 7 and 16 weeks postnatally, in order to establish the effect of the gonads on the development of the SON. The gonads have a remarkable effect on the development of the VON during this period (Van Eerdenburg *et al.* 1991). Animals were gonadectomized or sham-operated within 12 h of birth under general inhalation anaesthesia (N₂O, O₂, Hoekloos NV, Amsterdam, The Netherlands and Fluothane, Trofield Surgicals, Zug, Switzerland) without premedication (for details see Van Eerdenburg *et al.* 1991). All animals in this second experiment were killed and perfused under general anaesthesia as described above. The number of animals in each group is given in Text-fig. 3. After embedding the hypothalami in paraffin wax, 10 µm serial coronal sections were cut.

A third experiment was intended to investigate the effect of testosterone on SON development during puberty compared with its effect on the development of the VON (Van Eerdenburg *et al.* 1991). Forty-three animals born between 17 and 22 December 1987 were killed at 38 weeks of age. The animals (Dutch Landrace × Duroc) × Great York, also used in a previous experiment (Van Eerdenburg *et al.* 1991), were divided into four groups: (1) gonadectomized, no testosterone supplementation (four females and nine

males); (2) gonadectomized, testosterone supplementation during the first 4 weeks neonatally (three females and six males) (GXNT); (3) gonadectomized, testosterone supplementation from 16 weeks onwards (five females and seven males) (GXPT); (4) sham-operated (four females and five males). Treatment of the four groups is described in detail in a previous paper (Van Eerdenburg *et al.* 1991).

The animals of the GXNT group received testosterone supplementation during the first 4 weeks. From 16 weeks onwards, the GXPT group received testosterone supplementation within the physiological range (Colenbrander, de Jong & Wensing, 1978; FlorCruz & Lapwood, 1978). The animals of the other groups received sham supplementation. For details on treatment and testosterone and levels, see Van Eerdenburg *et al.* (1991). The animals were killed and perfused under general anaesthesia as described above. After embedding the hypothalamus in paraffin wax, 10 µm serial coronal sections were cut.

For morphometric analysis, every tenth section was stained with cresyl violet. Of each nucleus, seven sections were selected using the systematic sampling technique (Cruz-Orive, 1985a,b; Uylings, Van Eden & Hofman, 1986) and measured by a computer assisted method which is described briefly here. First, the distance between two cells in the central part of the nucleus was measured (Van Eerdenburg, Pool, Hoogedijk & Swaab, 1989; Van Eerdenburg *et al.* 1990). This distance was taken as the radius of a circle, which was used to determine the lateral endpoint of the SON in each section. The circle was moved between the main part of the SON and small groups of neurones that lay in straight lines on a video image (see Plate, figs 1 and 3). The lateral end of the SON was considered to occur when the circle could be moved between the perikarya without touching them. When the lateral end of the SON was defined, a contour was drawn around all the SON neurones. Because of the considerable length of the SON in rostrocaudal sections (see Plate, fig. 1 and Text-fig. 1), it was necessary to divide each section into four or five images. These areas were then summed in order to obtain the total area per section. Only one SON was measured for each animal. There was no preference for the left or right side. Section thickness was estimated through differential focusing (40 × dry objective), see Uylings *et al.* (1986). The volume of the measured structure was then calculated as the product of the area within the contour of the nucleus and the section thickness. Multiplying the mean volume of the SON in each section with the number of sections resulted in the total volume. Neurone number was estimated by counting the nucleoli that were visible within a frame of 10 000 µm². This frame was placed randomly

TEXT-FIGURE 1. Schematic drawings of the supraoptic nucleus in the pig hypothalamus. Distances in the rostrocaudal direction to the first drawings are given (in μm) in each lower right corner. Abbreviations: ac, anterior commissure; Bst, bed nucleus of the stria terminalis; cc, corpus callosum; Ci, capsula interna; DB, diagonal band of Broca; f, fornix; GP, globus pallidum; LS, lateral septum; MS, medial septum; OVLT, organum vasculosum laminae terminalis; ox, optic chiasm; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus; st, stria terminalis; 3V, third ventricle.

within the SON, but always so that it was filled completely by this nucleus. The number of double nucleoli was less than 1% at all ages studied. Ten frames were counted for each SON. Neurone density was calculated by dividing the sum of the counted nucleoli by the volume of the nucleus in the measured frame. The total number of neurones was obtained by multiplying the neurone density by the total volume of the nucleus (Uylings *et al.* 1986). Since our main interest was the number of neurones, no correction was made for shrinkage. All computer assisted image analysis routines were performed using standard software (TEA Image Manager; TEA, distributed by DIFA, Breda, The Netherlands) extended with routines developed by the author, a detailed description of which is available upon request.

For statistical analyses, the animals were grouped according to age and treatment. Analysis of variance was used to compare for sex and age. Homogeneity of variance was tested using Bartlett's test. Subsequent

multiple comparisons were performed using the Kruskal-Wallis procedure and Mann-Whitney U test. A P value < 0.05 was considered to be significant.

RESULTS

Development (Text-fig. 2)

No significant differences were found for SON neurone number between the sexes within each group or between different age groups, except for the 2.5-year-old females which had a 40% higher number than was found at 1 year ($P < 0.01$). The number did not differ significantly from that found in males of the same age. The measurements of volume showed delayed growth in females, whereas the males showed continuous growth, resulting in a significant sex difference in volume at 30 weeks of age ($P < 0.05$) and 52 weeks of age ($P < 0.01$) of 33% and 55% respectively. This sex

TEXT-FIGURE 2. Mean $\pm$ S.E.M. (a) neurone number and (b) volume of the supraoptic nucleus (SON) in the pig hypothalamus during development. The numbers in parentheses represent the number of animals in each group. Note the significant difference in neurone number between males (shaded bars) and females (solid bars) at 130 weeks and the delay in development as expressed in the SON volume of the females at 30 and 52 weeks.

difference in volume completely disappeared at 2.5 years.

Neonatal gonadectomy (Text-fig. 3)

The controls were the same animals as were used in the development study. There were no significant differences between the treated groups of any age. There was, however, a significant ($P < 0.02$) difference between the non-treated controls and all the treated (including sham-operated) animals, indicating that neonatal operation had a significant effect.

Testosterone supplementation after neonatal gonadectomy (Text-fig. 4)

There were no significant differences in neurone number or SON volume among the four groups. A significant ($P < 0.05$) sexual dimorphism was only

TEXT-FIGURE 3. Mean $\pm$ S.E.M. (a) neurone number and (b) volume of the supraoptic nucleus (SON) in the pig hypothalamus after neonatal gonadectomy (GX) at the ages of 7 and 16 weeks postnatally. The numbers in parentheses represent the number of animals in each group. Note the difference between treated and control (C) animals. S = sham treatment.

observed in the GXNT group for neurone number, the males having 50% more neurones than the females. The difference in SON volume was not significant.

DISCUSSION

Since this is the first morphometric description of the SON in the pig hypothalamus, there are no data available for comparison. In the SON of the rat, Bandarayanake (1971) found an average of 6800 neurones, Swaab *et al.* (1975) approximately 6500 neurones and Crespo, Ramos, Gonzalez & Fernandez-Viadero (1990) about 5000 neurones, while in man Goudsmit *et al.* (1990) measured some 40 000 neurones. The last is comparable with the number of neurones which we found in the pig (43 500). In the present study the SON of the pig hypothalamus showed an almost constant number of neurones during the first year of development. These findings are in agreement with those of Sartin & Lamperti (1985)

TEXT-FIGURE 4. Mean $\pm$ S.E.M. (a) neurone number and (b) volume of the supraoptic nucleus (SON) of the animals in the four groups of the testosterone supplementation experiment. GX, gonadectomized, no testosterone supplementation; GXNT, gonadectomized, testosterone supplementation in the first 4 weeks; GXPT, gonadectomized, testosterone supplementation from 16 weeks; SHAM, sham operated, no testosterone supplementation. Note the significant ($P < 0.05$) sex difference in neurone number between females (solid bars) and males (shaded bars) in the GXNT group.

and Crespo *et al.* (1990) in rat and Goudsmit *et al.* (1990) in man, where the neurone population of the SON appeared to be very stable during postnatal development. The increase in neurone number previously found in the VON during puberty (Van Eerdenburg *et al.* 1990) was not observed in the SON. However, the ($\pm 50\%$) variation in neurone number between 1 and 2.5 years of age which was found in the VON in females (Van Eerdenburg & Swaab, 1991) was also observed in the SON ($\pm 50\%$). This is especially remarkable since in the rat the cytoarchitectonically identical neurones of the SON arise relatively early in development (fetal day 12–14 (Altman & Bayer, 1978; Anderson, 1978)) and the population

remains constant between 3 and 30 months of age (McNeill, Clayton & Sladek, 1980). Sartin & Lamperti (1985) reported similar results. Only Bentura Remacha & Lazcano Camarero (1988) reported a decrease from 4692 to 3696 neurones in the SON of the rat between 3 and 30 months of age. In man, Goudsmit *et al.* (1990) reported no changes in neurone number between 10 and 93 years of age. Until now the underlying mechanism of the increase in neurone number in the SON, and especially in the VON of the pig, has not been clarified. Theoretically, there are several possible explanations.

(1) Activation of inactive, glial-like cells that are not recognized as neurones in earlier stages of development. This is rather improbable as neurones can generally be recognized very early in their development (Leuba, Heuman & Rabinowicz, 1977).

(2) Delayed migration. Autoradiographic studies in the rat (Ifft, 1972; Altman & Bayer, 1978; Anderson, 1978) have shown that the neurones of the SON arise near the PVN and migrate to the area where they can be found after birth. A number of neurones fail to reach their definitive location and become 'displaced neurosecretory cells', forming the accessory SON. Theoretically, this process could become activated again after 1 year of age so that migrating neurones, halted halfway, can complete their migration. However, migration is generally thought only to take place early in development, immediately after cell formation.

(3) Neurogenesis is also improbable since it is generally believed that the neurones of the SON only arise early in development (Ifft, 1972; Altman & Bayer, 1978; Anderson, 1978; McNeill *et al.* 1980; Crespo *et al.* 1990). On the other hand, postnatal neurogenesis has been reported in other regions of the rat brain, in infants and even in adulthood, i.e. in the olfactory bulb, cerebellum and dentate gyrus (Altman, 1966), and in the rat visual cortex at 3 months of age (Kaplan, 1981). Further research is necessary to examine these options for the pig SON and VON.

This study revealed another indication that perinatal stress can be of major influence on the development of the brain. Prenatal stress has already been shown to be associated with an abnormal ontogenetic pattern of testosterone secretion from the fetal testes in the rat (see review by Ward, 1984) which is due to differences in fetal luteinizing hormone levels (Salisbury, Reed, Ward & Weisz, 1989). The effect of neonatal operation, which was observed in the SON after neonatal gonadectomy, was not seen in the VON (Van Eerdenburg *et al.* 1991). It is perhaps relevant that a decrease in the number of neurones in the VON occurs during the first 7 weeks after birth (Van

Eerdenburg *et al.* 1990), a phenomenon which was not observed in the SON. The two nuclei seem to react quite independently in this respect and in others (see below).

The increase in volume of the SON occurred at a different rate in males and females, resulting in a sex difference at 30 and 52 weeks. This may be interpreted as a delay in the development of the SON in the female pig because at 2.5 years of age the sex difference in volume no longer exists. The period in female development when the SON volume increases drastically coincides with the postpubertal increase in VON volume and neurone number (Van Eerdenburg & Swaab, 1991). The factor or factors which are involved in these changes are still unknown. However, it is unlikely that gonadal steroids mediate the changes in the SON. Testosterone supplementation only has an effect on the neurone number and volume of the SON when it is administered during the first 4 weeks after birth. So testosterone does not have the same suppressing influence on the development of the SON as it exerts on the VON between 16 and 38 weeks of age. Furthermore, neonatal gonadectomy does not lead to an increase in volume and cell number in the SON as it does in the VON between 7 and 16 weeks of age (Van Eerdenburg *et al.* 1991). Other putative mediators in the development of the SON and VON are gonadotrophic hormones (Van Eerdenburg *et al.* 1990). The possibility that the activity of SON and PVN can be influenced by gonadotrophic hormones (Swaab & Jongkind, 1971) has yet to be tested in the pig. Another possible factor in the increase in size of the SON and VON in 2.5-year-old females is their production of several litters. Large litters of fast growing piglets may indeed require an adaptation of the female brain, perhaps in oxytocin production. In the rat, the female HNS is clearly stimulated during pregnancy, labour and lactation (Swaab & Jongkind, 1970), but cell counts have not been studied in these conditions.

The sexual dimorphism observed in the SON in the GXNT group was not present to a significant degree in the VON (Van Eerdenburg *et al.* 1991). This is the first observation of a morphological effect related to neonatal testosterone in the pig (Colenbrander *et al.* 1978). The different effect of neonatal testosterone supplementation on males and females indicates that there is already some degree of sexual differentiation present in the hypothalamus of the pig at birth. The biological significance of this difference, however, remains unclear, since the testosterone surge occurs only in the male during normal development.

The results of this study show that the postnatal development of the SON of the pig is sexually dimorphic, and that it continues after puberty in females. In contrast to the VON, the development of

the SON was not influenced by gonadectomy and only slightly by gonadal steroids.

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DESCRIPTION OF PLATE

FIGURE 1. The supraoptic nucleus (SON) in the pig hypothalamus in a rostrocaudal section after staining with cresyl violet. For names of structures etc. see Text-fig. 1 (lower left section). Bar indicates 250 µm.

FIGURE 2. Neurones of the SON in the pig hypothalamus after staining with cresyl violet. Bar indicates 20 µm.

FIGURE 3. The lateral part of the SON. The lateral endpoint is considered to occur when the circle can be moved between neurones without touching them; for details see Materials and Methods (detail from fig. 1). Bar indicates 100 µm.