A Vasopressin and Oxytocin Containing Nucleus in the Pig Hypothalamus That Shows Neuronal Changes During Puberty

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ABSTRACT

A vasopressin and oxytocin containing nucleus is described for the first time in the pig hypothalamus. It is located near the third ventricle, just dorsal to the suprachiasmatic nucleus, and consists of magnocellular neurons, similar to those of the supraoptic nucleus and paraventricular nucleus. Morphometric analysis of neuronal number, size, density, and volume was performed at four different ages: 1 day, 7 weeks, 16 weeks, and 30 weeks postnatally. No sex difference in these parameters was observed. In this period the volume of the nucleus increased gradually from 6.6×10^{-3} to $54.2 \times 10^{-3} \text{ mm}^3$. One day after birth $1,215 \pm 191$ (mean $\pm$ SEM) neurons were present in the vasopressin and oxytocin containing nucleus, followed by a decrease to 771 ± 80 neurons at 7 weeks and 697 ± 116 at 16 weeks. Between 16 and 30 weeks (puberty) there was a dramatic increase in neuron number up to $1,765 \pm 214$ neurons. This increase in the number of vasopressin and oxytocin containing neurons in the pig hypothalamus is much later in development than has ever been reported so far.

Key words: brain development, neuropeptides, hypothalamo-neurohypophyseal system, morphometry, sexual differentiation

The rapid perinatal phase of pig brain development (Dobbing, '74) and the gestational and neonatal peaks of testosterone (Colenbrander et al., '78; Ford et al., '80) make this species a suitable model for the study of the interaction of sex hormones and brain development. Therefore, the goal of our research is to determine the development of various nuclei of the pig hypothalamus in relation to periods when sex hormone levels fluctuate. Since very little information on the topography of the pig hypothalamus is available (Solnitzky, '39; Welento, '64; Sztayn et al., '80; Salinas-Zeballos et al., '86; Seeger, '87) a first aim was to describe the chemico-neuroanatomical organization of the pig hypothalamus. In the present paper a nucleus (VON), containing vasopressin (VP) and oxytocin (OT), is described in the pig for the first time. Neuronal number appeared not to differ between the two sexes but was found to increase during puberty. This observation may extend the period of postnatal brain development as mentioned in other reports (Altman, '69; Swaab and Hofman, '88) considerably.

MATERIALS AND METHODS

Forty-one crossbred Yorkshire $\times$ Dutch landrace pigs were used for morphometric analysis. Three males and two

females of 1 day old, five males and five females of 7 weeks, five males and four females of 16 weeks, eight males and four females of 30 weeks were studied. In addition five female animals of 30 weeks old were used for immunocytochemical studies. The animals were anesthetized with Azaperone 2 mg/kg bodyweight i.m. (Stressnill® Janssen Pharmaceutica) and Methomidate i.v. (Hypnodil® Janssen Pharmaceutica). The brains were first cleared of blood by an infusion through both carotid arteries with saline containing heparine, and subsequently perfused either with GPA fixative [glutaraldehyde (Merck) 25%, picric acid (Merck) (saturated solution) 74%, acetic acid (Brocacef) 1%] when used for morphometry, or with 4% paraformaldehyde (Merck) diluted in phosphate buffered saline, pH 7.4, when used for immunocytochemistry. Perfusion fluids were administered by a gravity fed system that did not require pumps or forced infusion. Following removal from the cranium, lateral and dorsal photographs were made of the whole brain. Estimations of brain size were made with a computer-assisted method that measured the surface area of the cerebral hemispheres on photographs. Dorsal and lateral contours were drawn and surface areas measured.

Accepted July 27, 1990.

These areas were summed to provide an estimation of total surface area. The hypothalamus was dissected and post-fixed in GPA for 24 hours when used for morphometry, or 48 hours in 4% paraformaldehyde solution when used for immunocytochemistry. Tissues used for morphometric analysis were subsequently dehydrated and embedded in paraffin and sectioned in 10 μ m sections. For morphometric analysis each tenth section was stained with cresyl violet and measured by a computer assisted method (Van Eerdenburg et al., '89) that will be described extensively elsewhere. Briefly, this method averages the distance between two cells in the 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. When a circle crosses two other ones, the neuron is considered to belong to the nucleus. To establish a contour around the nucleus the outer contour of all the connected circles is drawn.

Section thickness was estimated as described by Uylings et al. ('86). The volume of the measured structure was then calculated as the area within the contour of the nucleus multiplied by the section thickness. Multiplying the mean volume of the VON in each section with the number of sections resulted in the total volume.

Neuron number was estimated by counting the nucleoli that were visible within the contour of the nucleus. Neuron density was calculated by dividing the sum of the counted nucleoli by the sum of the volumes of the nucleus in the measured sections. The total neuron number was obtained by multiplying the neuron density with the total volume of the nucleus (Uylings et al., '86). Since our main interest was in neuron number no correction was made for shrinkage.

In each animal the area and diameter of profiles of perikarya and cell nuclei were measured in 25 neurons from which the nucleolus was visible. These neurons were chosen randomly and measured by a computer assisted image analysis routine in which 10 diameters were drawn. The first diameter was drawn randomly through the center of gravity and the next nine were drawn at 18° intervals. Minimum, maximum, and mean diameter were subsequently calculated as well as the area.

All computer assisted image analysis routines were performed on an Olivetti M24 personal computer extended with a Microsoft mouse (bus version) and an Imaging Technology PCVision plus framegrabber. For the video images a CCD black-and-white camera (HTH MO) and a color monitor (Sony Trinitron) were used. Standard software used was TIM (TEA, distributed by DIFA, Breda, The Netherlands) extended with routines developed by the authors.

Seventy-five micron vibratome sections were used for immunocytochemistry. To inhibit pseudoperoxidase staining caused by erythrocytes, sections were pretreated with methanol and H_2O_2 according to Streefkerk ('72). The monoclonal antibody D-7 MAB (Hou-Yu et al., '86) (dilution 1:100 in 0.05 M Tris [Sigma], 0.5 M NaCl [Sigma], pH 8.6, containing 0.5% Triton [Sigma]) served as first antibody for VP staining. For OT staining, the monoclonal antibody A1 29 (Hou-Yu et al., '86) (dilution 1:100 in 0.05 M Tris, 0.5 M NaCl, pH 8.6, containing 0.5% Triton) was used (both antibodies were a generous gift of Dr. A. Hou-Yu, Columbia University, New York). As second antibody, biotinylated sheep anti-mouse antibody (Amersham) (dilution 1:200 in 0.05 M Tris, 0.5 M NaCl, pH 8.6) was used. The third step was Avidine-Biotin Complex-peroxidase (Amersham) (dilution 1:200 in 0.05 M Tris, 0.5 M NaCl, pH 8.6) followed by the incubation in diaminobenzidine (DAB) and intensified by the addition of 0.2% $NiNH_4SO_4$ (in Tris NaCl, pH 7.6). All sections were rinsed for at least 1 hour between each step with 0.05 M Tris 0.15 M NaCl, pH 7.6. Following application of the first antibody, sections were left at room temperature for 1 hour and then placed at 4°C overnight. The duration of subsequent incubations was at least 1 hour at room temperature. All incubations were performed on a rocking table.

Analysis of variance was used to compare for sex and age. Homogeneity of variance was tested using the Bartlett-Box test. Due to inhomogeneity of variance ANOVA was performed on ranked data rather than the original data (Conover, '80). Subsequent multiple comparisons were performed using Kruskal-Wallis 1-way ANOVA and Student-Newman-Keuls (SNK) procedure. A *P* value < 0.05 was considered to be significant.

RESULTS

A nucleus consisting of magnocellular neurons located laterally from the third ventricle, dorsal from the SCN, and ventro-rostral of the PVN was found (Figs. 1–3). The perikarya stained intensely with cresyl violet (Nissl staining) and the cell type appeared similar to that of the PVN and the SON. The nucleus was nearly ball-shaped, with a diameter of approximately 500 μ m at 30 weeks of age. Immunocytochemistry revealed that the VON contained neurons displaying VP or OT immunoreactivity. (Figs. 4, 5). Table 1 lists size of the profiles of perikarya and cell nuclei.

Since no significant sex difference was observed for neuronal number, volume or neuron density (ANOVA, *P* > 0.6), data from the two sexes were pooled per age for further statistical evaluation.

A significant main effect for age (ANOVA, *p* < 0.05) was observed in neuron number and volume of the VON. Subsequent multiple comparisons revealed that mean neuron number decreased from 1 day to 7 weeks and increased from 16 to 30 weeks (SNK; *P* < 0.05; Fig. 6). Volume of the VON increased gradually from the day of birth until the end of puberty (Fig. 7). Each group differed significantly (SNK, *P* < 0.05) from the previous one.

Neuron density decreased significantly (ANOVA, *P* < 0.05) from day 1 until 16 weeks postnatally (Fig. 8). There was no significant difference in neuron density between 16 and 30 weeks of age.

Abbreviations

AP	accessory part of paraventricular nucleus
CA	commisura anterior
GPA	glutaraldehyde-picric acid-acetic acid
LV	lateral ventricle
NC	nucleus circularis
OC	optic chiasm
OT	oxytocin
OVL	organum vasculosum laminae terminalis
PVN	paraventricular nucleus
SCN	suprachiasmatic nucleus
SNK	student-Newman-Keuls procedure
SON	supraoptic nucleus
VON	vasopressin and oxytocin containing nucleus
VP	vasopressin

Although the volume of the VON showed an increasing growth rate, brain growth rate decreased with age (see Fig. 9). No sex differences in brain size could be observed.

Figure 10 shows the VON at the four different ages that have been investigated.

DISCUSSION

The present study revealed a previously undescribed (Solnitzky, '39; Welento, '64; Szteyn et al., '80; Salinas-Zeballos, et al., '86; Seeger, '87) hypothalamic nuclear structure (VON) in the pig. One may presume that this structure may be structurally related to the PVN or SON, as an accessory part (AP). For instance, that part of the PVN that has been described by Kineman et al. ('88) might be identical to the VON. However, although the neurons of this nucleus contain also VP and OT, the VON is spatially clearly separated from the PVN and the SON. Moreover, in the pig hypothalamus the AP appear as a compact, thin layer of cells around relatively large blood vessels (Fig. 11). The VON has a less compact appearance, and the cells of the nucleus do not surround a single bloodvessel. Furthermore, recent unpublished results of measurements of the SON reveal no changes in neuron number in this nucleus during puberty. This is one additional argument to suggest that the VON is a distinct structure and is not an AP of the PVN or SON.

The VON, as described here in the pig, may be homologous to structures described in closely related species such as sheep and cow. In sheep, a similar nucleus has been described as part of the PVN (Vierling, '57). In the cow, the nucleus intrasupraoptico-paraventricularis (Vierling, '58) is a structure which also resembles the VON of the pig. Although Szteyn et al. ('81) called this cell group nucleus intrasupraoptico-paraventricularis in the cow, his description of the nucleus intrasupraoptico-paraventricularis in the pig fulfills the criteria of an AP rather than of the VON (Szteyn et al., '80).

In less related species such as horse, rabbit, cat, and mouse, nuclei have been described too at the same location. The ventral paraventricular nucleus as described by Melrose and Knigge ('89) in the horse, might be homologous to the VON, although it is not separated from the PVN.

The paraventricular anterobasal extension as described in the rabbit by Schimchowitsch et al. ('89) is located in the same area and is in size comparable to the VON. It is called anterobasal extension of the PVN because it is connected with the main part of the PVN. The shape of this nucleus is not round as is the VON.

In the cat (Caverson et al., '87) a possible homologous structure has been described as the anterior component of the PVN. This looks very similar to the paraventricular anterobasal extension of the rabbit (Schimchowitsch et al., '89) and has connections with the main dorsal part of the PVN.

Fig. 1. Schematic topography of the pig hypothalamus. The vasopressin and oxytocin containing nucleus (VON) is indicated by the arrows. CA: commissura anterior; LV: lateral ventricle; OC: optic chiasm; OVLT: organum vasculosum lamina terminalis; PVN: paraventricular nucleus; SCN: supraoptic nucleus; SON: supraoptic nucleus; III: third ventricle. The distance between the figures is 200 μm , except between the second and third figure, where it is 600 μm .

Fig. 2. Photomicrograph of the pig hypothalamus. The arrow indicates the VON. The bar indicates 400 μ m.

Fig. 3. Photomicrograph of a detail of the VON in the pig hypothalamus. The bar indicates 20 μ m.

Fig. 4. Photomicrograph of the VON in the pig hypothalamus after staining for Vasopressin. Adjacent section of Figure 5. The bar indicates 200 μ m.

Fig. 5. Photomicrograph of the VON in the pig hypothalamus after staining for Oxytocin. The bar indicates 200 μ m.

TABLE 1. Area and Diameters of the Profile of Perikarya and Cell Nucleus of the VON

Age (wk)	Area (μm ²)	Min. diam. (μm)	Max. diam. (μm)	Mean diam. (μm)	Mean nucl. diam. (μm)
0.2	76.04 (3.3)	4.98 (0.29)	12.45 (0.37)	9.03 (0.21)	5.49 (0.35)
7	200.85 (11.9)	7.86 (0.33)	21.33 (1.19)	14.90 (0.49)	8.00 (0.24)
16	279.34 (14.9)	9.04 (0.50)	24.33 (0.84)	17.62 (0.44)	8.86 (0.29)
30	344.24 (10.9)	10.31 (0.24)	26.53 (1.78)	19.66 (0.34)	10.08 (0.15)

Area represents the area of the profiles of the perikarya of the neurons in the VON. Min., Max., and Mean diameter represent diameters of the perikarya of the neurons of the VON; and Mean nucl. diameter represents the mean diameter of the neuronal nucleus. Nos. in parenthesis represent SEM.

The mouse accessory nucleus, as described by Castel and Morris ('88), is probably homologous to the VON. It consists of VP and OT immunoreactive cells and has a comparable location and shape. The number of neurons in mice is approximately 50 for each nucleus. The cytoarchitecture is similar to that of the VON and distinct from that of the nucleus circularis (NC) as described in the rat and hamster (Peterson, '66; Hatton et al., '76; Fisher et al., '78, '79; Ferris et al., '89). It is also separated from the PVN.

Fig. 6. Number of neurons (mean ± SEM) in the VON in the pig hypothalamus at four different ages. A decrease is found from day 1 to 7 weeks postnatally, whereas an increase is observed between 16 and 30 weeks (i.e., puberty). f = female, m = male.

Fig. 7. Volume (mean ± SEM) of the VON in the pig hypothalamus at four different ages. A gradual increase is seen from day 1 to 30 weeks postnatally. f = female, m = male.

Fig. 8. Neuron density (mean ± SEM number of neurons × 10³ mm⁻³) in the VON in the pig hypothalamus at four different ages. A decrease is seen from day 1 to 16 weeks postnatally. From 16 to 30 weeks it remains constant, although the number of neurons increases dramatically (cf. Fig. 6). f = female, m = male.

Castel and Morris ('88) come to the same conclusion as we do for the VON, that this is a separate nucleus, though probably closely related to the PVN or SON.

Compared to the NC in the rat and in the hamster the VON has more neurons and a lower neuron density than the rat NC and its location is more medial (G.I. Hatton and C.F. Ferris, personal comm.). Yet, the VON in the pig might be homologous to the NC. Three pieces of information which would help to confirm this homology are currently lacking in the pig: 1) all rat NC neurons send at least one axon to the posterior pituitary; 2) rat NC neurons are sensitive to dehydration and respond with an increase in multiple nucleoli and cell size, and production of ribosomes (Hatton and Walters, '73; Hatton, '76; Tweedle and Hatton, '76, '77); and 3) as Ferris et al. ('86, '89) reported, the number of vasopressin active neurons in the hamster NC is influenced by dominant-subordinate behaviour. These data are currently not available for the VON in the pig.

Fig. 9. Brain size (mean ± SEM) of the pig set against the volume of the VON (mean ± SEM). While there is a decrease in brain growth rate there is an increase in growth rate of the VON between 16 and 30 weeks.

Figure 10

Fig. 11. Accessory part of the PVN or SON of the pig hypothalamus. Note the compact appearance of a few cell layers around the central bloodvessel. The bar indicates 100 μ m.

Although the location of the VON is comparable to that of the intermediate (Braak and Braak, '87) or sexually dimorphic nucleus in man (Swaab and Fliers, '85), it is not homologous to this structure, since the intermediate nucleus is sexually dimorphic after puberty and does not contain VP or OT (Swaab and Fliers, '85; Swaab and Hofman, '88).

Since no structure can be called homologous to the nucleus described here without further investigation, the neutral name *vasopressin and oxytocin containing nucleus* is proposed.

Both cell type and section thickness influence the way cell number is determined in hypothalamic sections. Available procedures such as the disector method (Sterio, '84) count without bias and shape assumptions. The only requirement for this method is that it has to be possible to identify the object counted in a lookup (in this case adjacent) section. In the VON this appeared to be very time consuming. In a study done with animals 30 weeks old (not published), only 1% out of 1,000 nucleoli counted was found in the adjacent section. Therefore we choose to simply count nucleoli in one section in order to estimate neuron number. The number of double nucleoli observed was less than 1% in all other groups studied except for the one day old animals, where 2 out of 5 had a relatively large number of double nucleoli ($\pm 25\%$). Since there is no accurate way of correcting for double nucleoli, this was not done. The decrease in neuron number between 1 day and 7 weeks may be at least partly be due to the double nucleoli, but the main conclusions of the present paper are not affected by this phenomena.

The relatively large brainstem and bulbi olfactorii of the pig brain cannot be dissected in a reproducible way. It was therefore impossible to give an unbiased brain weight or volume. However, when viewing the photographs, the border between bulbus olfactorius and cortex could accurately and consistently be delineated. Therefore the area of

the hemispheres on the photographs has been taken as index for brain size.

For the immunocytochemical experiments, monoclonal, mouse strain antibodies were used because rabbit immunoglobulins bind aspecifically to magnocellular components in the pig hypothalamus (Meijer et al., '86). Such monoclonal antisera, followed by incubation with goat-antimouse immunoglobulin-biotine and streptavidine-peroxidase, avoided the problem of aspecific binding of rabbit IgG's. Details on the distribution of the VP and OT immunoreactive neurons in the pig hypothalamus will be presented in a separate paper.

The cause of the increase in number of visible neurons of the VON around puberty remains unclear. Theoretically it might be due to 1) neurogenesis, 2) activation of inactive neurons (e.g., activation of cells with a glial-like appearance), or 3) migration. Since the small size of glial cells prevents them from being counted in the same section as the neurons with the disector method (Sterio, '84), total cell numbers have not been determined. But even if total cell counts would have been known, it would not allow a distinction between the three possibilities because the number of glial cells might change during development too (Altman, '66). Injection of animals with tritiated thymidine during the period from 16 to 30 weeks may provide data that would clarify some of these issues. Since the animals weigh over 100 kg at 30 weeks this is, however, not a practical way to proceed. The fact that cell size increases only slightly during puberty and the VON volume increases almost 3 times implies also that there must be more neurons present in the VON at the end of puberty than before. The differentiation between neurons and glia is easy in cresyl violet stained sections (Leuba et al., '77). Therefore, it is very improbable that cells will first resemble glia cells and subsequently neurons.

One may presume that due to the increase in volume neurons that were first lying outside the VON become part of the VON. If this was the cause of the increase in neuron number there should be many neurons lying in the direct environment of the VON in the earlier stages. Since we determine the border on the basis of the mean cell distance, these cells would have been considered to belong to the nucleus.

Fig. 10. Photomicrographs of the VON in the pig hypothalamus at the age of 1 day (A and B), 7 weeks (C and D), 16 weeks (E and F), and 30 weeks (G and H). The photomicrographs on the right side are details of those on the left side. The bar indicates 100 μ m.

Although the periods in which neuronal number is higher in the VON corresponds to surges of testosterone in the males (Meusy-Desolle, '75; Colenbrander et al., '78; Ford et al., '80) (i.e., perinatally and around puberty), similar neuronal numbers were found in the VON of females which lack these testosterone surges. Therefore, it is doubtful that testosterone is a critical factor influencing cell number in the VON. Because LH and FSH show similar curves in males and females throughout development (Colenbrander et al., '77), it might be worthwhile to consider a correlation between the increased levels of gonadotropic hormones and the increase in number of VON neurons.

ACKNOWLEDGMENTS

The authors like to thank Henk van Dijk, Carla Lugard-Kok, Ben Scheiberlich, Henk Stoffels, Onno van der Veen, and Hanneke de Waal for their technical assistance; Dr. Elmer Goudsmit, Dr. Harry B.M. Uylings, and Dr. John F. Axelsohn for their help with the statistics, morphometry, and reviewing the manuscript; and Dr. Glen I. Hatton and Dr. Craig F. Ferris for their advice.

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