Acta Microscópica, Vol. 2, Nº 2, November 1993, pp 188 - 194

Ultrastructural Evidence of a Sexually Dimorphic Contribution from the Fornix to the Ventromedial Hypothalamic Nucleus: A Quantitative Study.

Jorge Larriva-Sahd, Julian Arista-Nasr, Adrian Rondan, Hector Orozco Estevez, Maria R. Sanchez Robles*.
Departamento de Patología, Instituto Nacional de la Nutrición SZ. Calle Vasco de Quiroga No. 15. Tlalpan, México D.F. CP 14000, México. Fax 525-655-1076
*Laboratorio de Cirugía Experimental de la Asociación Para Evitar la Ceguera en México.

ABSTRACT

The mammalian central nervous system undergoes a process of sexual structural differentiation, which is primarily determined by early interaction of gonadal sex steroids with the neuronal genome. The present study, confirmed that the neuropil of the ventrolateral part of the ventromedial hypothalamic nucleus (VL-VMHN) of the rat is sexually dimorphic in the density of axospinous and axodendritic types of synapses, which are numerous in the intact male. Perinatal exposure of the female to exogenous testosterone or castration of the newborn male "inverted" the difference, demonstrating the hormonal dependence of this sexual dimorphism. Another finding was, that in adult rats with transection of the fornix, the density of degenerating fibers terminating in the VL-VMHN is also sexually dimorphic. Suppression of endogenous testosterone by castration of the new born male decreased the density of degenerating fornical fibers terminating in the VL-VMHN to a number comparable to that found in the females' nucleus. It is concluded that: 1. The fornix gives a neural input to the VL-VMHN as proven by orthograde degeneration. 2. The number of fornical terminals in the VL-VMHN is greater in the male than in the female. 3. This dimorphism depends of the organizational effect of gonadal sex steroids.

KEYWORDS

Sexual dimorphism, fornix, hypothalamus, synapses.

INTRODUCTION

Thus far, it is widely accepted that the mammalian central nervous system undergoes a period of sexual structural differentiation, which is determined by the interaction [1] of gonadal sex steroids with the neuronal genome. Furthermore, the structure of certain brain areas is determined by this interaction. In the case of rodents in which it is possible to modify the titers of gonadal steroids, experimentally, it has been demonstrated that most if not all the sexual structural differences in the number of synapses of some nuclei, are determined by the organizational effects of sex steroids. Examples of such sexual differences include medial amygdala [2], bed nucleus of the stria terminalis [3], 'strial part' of the medial preoptic area [4], medial preoptic nucleus (MPN)[5], suprachiasmatic nucleus [6], arcuate hypothalamic nucleus [7], and the ventrolateral part of the ventromedial hypothalamic nucleus (VL-VMHN)[8]. In a previous study in which a larger density of synapses was found in the males' MPN with respect to the female homologue, it was commented that the source of these synapses remained unknown [5]. In fact, to our knowledge only the pioneer study of Raisman and Field has succeeded in demonstrating that the origin of the synaptic boutons which ended up in the sexually dimorphic 'strial part' of the medial preoptic area is the stria terminalis [9]. The present study was conducted in order to: 1. determine whether the fornix provides with synaptic terminals to the VL-VMHN by orthograde degeneration; 2. confirm a sexual dimorphism on the density of synaptic boutons in the VL-VMHN; 3. determine a possible sexual dimorphism in the density of fornical synaptic terminals within the neuropil of the V-VMHN.

MATERIALS AND METHODS

Sixty albino rats sacrificed at ten weeks of age were divided into ten groups (n=6) as follows: Group 1. Control males (CM) Group 2. Control females (CF). Group 3. Males castrated as

newborns (GxM). Group 4. Males castrated as newborns and treated with daily subcutaneous injections of 1 mg. of testosterone propionate (TP) for ten days (GxM+T). Group 5. Females whose mothers received daily subcutaneous injections of 7 mg. of TP from day 16 postfertilization. On day 21, the mothers underwent cesarean surgery and the obtained female pups received additional 1 mgr. TP treatment for ten days (F+T). Group 6. Males with transection of the fornix (MFx). Group 7. Females with transection of the fornix (FFx). Group 8. Similar to Group 3 but with transection of the fornix (GxMFx). Group 9. Similar to Group 4 but with transection of the fornix (GxMTFx). Group 10. Similar to Group 5 but with transection of the fornix (FTFx). Castrations of rats from Groups 3, 4, 8, and 9, were performed under cryo-anesthesia, using a suprapubic approach. The effectiveness the gonadectomy was confirmed by serial sections of the testes as well as by postmortem inspection.

Transection of the fornix

Three days prior to sacrifice each rat in Groups 6 to 10 underwent a lesion in the fornix. The interruption of the totality of the fornix was performed with a Halaz-type of knife [9]. Under deep barbiturate anesthesia each animal was held by the auditive meatus and placed in a stereotaxic apparatus. The knife measuring 1.4 mm was bent at 90° in the horizontal plane and the tip of the knife was placed 2.0 mm rostral to the bregma, which descended 5.0 mm from the longitudinal superior sinus. The knife was then rotated laterally 90° to both sides. The knife was again oriented to a sagittal position and removed. In Groups 1 to 5 a sham lesion was made by descending the knife without lateral rotation. After the surgery each animal was left until spontaneous recovery; no antibiotics were administered.

In all cases animals were sacrificed at ten weeks of age, three days after the surgery, according to the protocol described previously [10]. Briefly, the procedure consisted in perfusing Karnovsky's fixative (4% paraformalehyde/2% glutaraldehyde) through the left ventricle. From each brain appropriate tissue blocks were taken from the area which contained the ventromedial hypothalamic nucleus (VMHN). The tissues were postfixed in 1% osmium tetraoxide dissolved in 0.15 M sodium cacodylate buffer, pH 7.3. Following ethyl-alcohol dehydration all tissues were flat embedded in epoxy resins. In order to obtain appropriate thin sections through the VL-VMHN, 1μm thick sections were stained with alkaline toluidine blue and some anatomical landmarks were drawn with the aid of a camera lucida. This procedure allowed an unambiguous identification of the VMHN and trimming out of the surrounding tissue prior to ultrathin sectioning. Our observations were performed in the area of the VL-VMHN with the densest neuronal occurrence (Fig 1.). The thin sections were obtained with diamond knives, mounted in copper grids and double stained with uranium and lead salts. Observations were performed in Zeiss EM-10C electron microscopes operated at 60kV.

Morphometry

To study an area of 10, 500 μ2m per animal, ten 6,300x micrographs were randomly taken at the corners of each grid, being framed so that no neuronal somata were photographed. Each negative gas enlarged 2.7 times at printing. From each print the number of axo-dendritic (ADS) and axo-spinous synapses (ASP) were separately recorded and collected according to the experimental group. These counts were performed for Groups 1 to 5. In those groups with transection of the fornix (i.e. Groups6 to 10), the number of degenerating synapses (DS) were counted in 10,500μ^2m. The results of the quantitative analysis were compared by ANOVA; differences of $p < 0.05$ were considered significant.

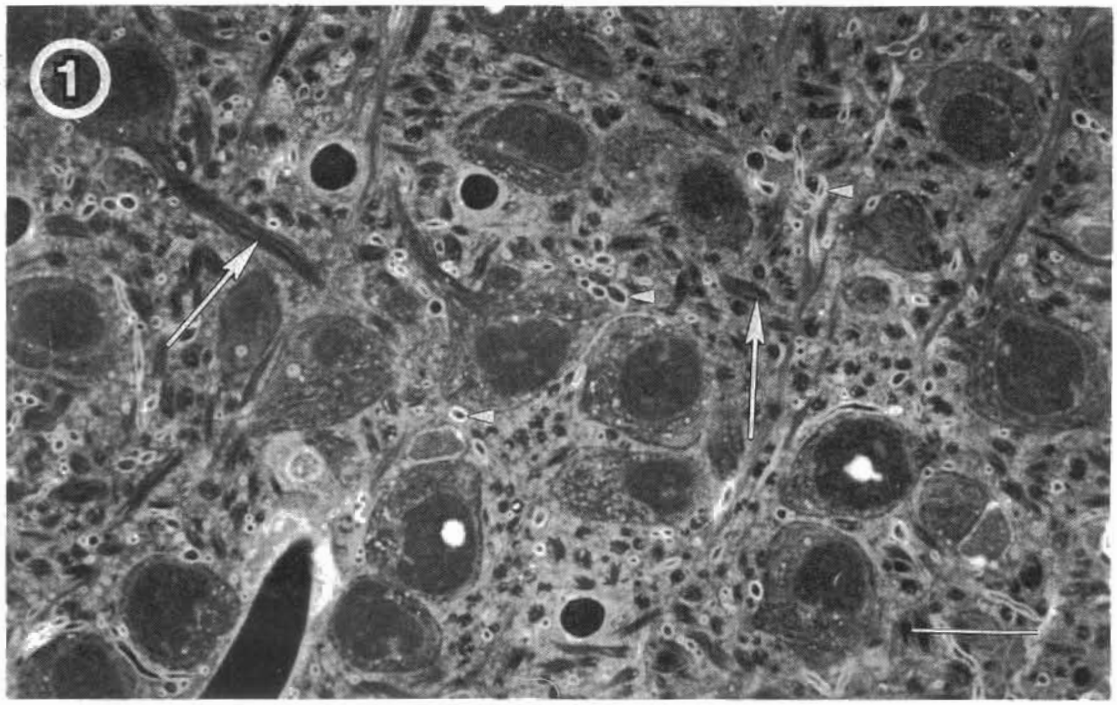
RESULTS

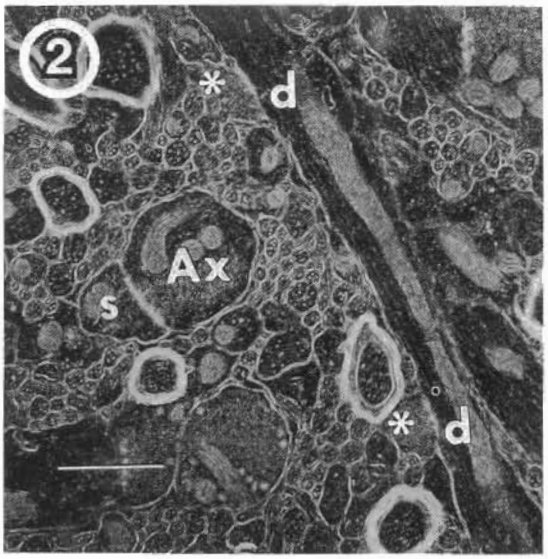
Animals without lesion of the fornix

At the light microscope the VL-VMHN showed round or oval neurons with a central nucleus, containing a distinctive nucleolus. The neuropil consisted of multiple proximal dendrites and thin myelinated axons (Fig. 1). At the electron microscope no degenerating terminals were found in rats from Groups 1 to 5. The neuropil of normal rats of each sex showed no apparent qualitative differences. The neuropil was characterized by numerous synaptic boutons which fell into two categories: ADS and ASP. While in ADS no appreciable modifications were seen in that part of the dendrite in contact with its axonal counterpart (Fig. 2), in the ASP type a fungiform protuberance budding off from the dendrite was seen (Fig. 2). In both synaptic types the presynaptic element contained abundant round vesicles, some dense-cored granules and a few mitochondria. Few myelinated axons passed through the neuropil (Figs. 1, 2). There was a paucity of glial cells and if seen these consisted of oligodendrocytes.

Morphometry

Figure 3 summarizes the quantitative analysis made in Groups 1 to 5. As can be seen,





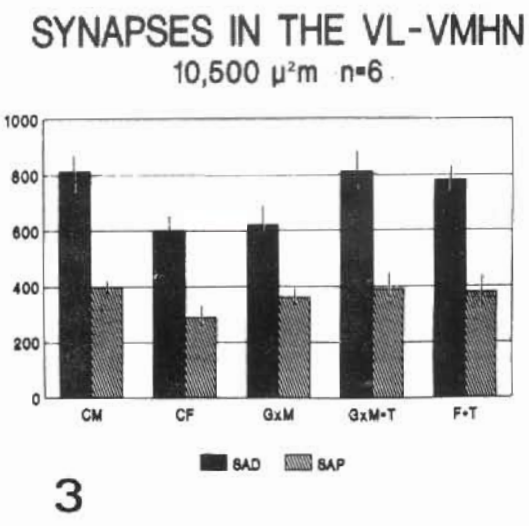


Figure 1. Light micrograph of the ventrolateral part of the ventromedial hypothalamic nucleus of a control male. The neuropil shows numerous round or oval-shaped neurons surrounded by proximal dendrites (arrows) and thin myelinated axons (arrow heads). Barr= 10 µm.

Figure 2. Electron micrograph of the neuropil of the ventrolateral part of the ventromedial hypothalamic nucleus. Two synaptic types are shown: axo-spinous in which an spine (s) is contacted by its corresponding synaptic terminal (Ax). The axo-dendritic synapses (asters) contact a dendrite (d) without an appreciable modification of its contour. Barr= 0.5 µm.

Figure 3. Comparative histogram illustrating the densities of axodendritic (SAD) and axospinous synapses (SAP) in the ventrolateral part of the ventromedial hypothalamic nucleus (VL-VMHN). When SADs are compared significance resulted between control males (CM) and control females (CF) as well as between CM and gonadectomized males (GxM); comparison between CM and gonadectomized males supplemented with testosterone (GxM+T) and with masculinized females (F+T) led to no statistical difference. Notice that SAP of GxM and CF have a comparable density of synapses. Likewise no difference was detected between GxM+T and F+T respect to CM.

when CM were compared with CF, the density of ADS and ASP was statistically larger in the male ($p< 0.05$). It is interesting that castration of the male at birth (MGx) or perinatal masculinization of the female (F+T) "inverted" the difference in the densities of ADS and ASP, i.e. F+T and MGx behaved similarly to CM($p<0.9$) and CF ($p< 0.7$) Furthermore, the treatment of the castrated male with testosterone "restored" the number of ADS and ASP to that recorded in the intact male (CM).

Animals with transection of the fornix

The most striking finding in the neuropil of animals submitted to transection of the fornix consisted in the presence of degenerating terminals (DT) resulting of an orthograde degenerative process. These DTs were characterized by clumps of packed filaments surrounded by thinner fibrils (Figs. 4 and 5). Another degenerating element were myelinated axons, which showed disruption of the myelin covering and axoplasm (Fig. 5).

Morphometry

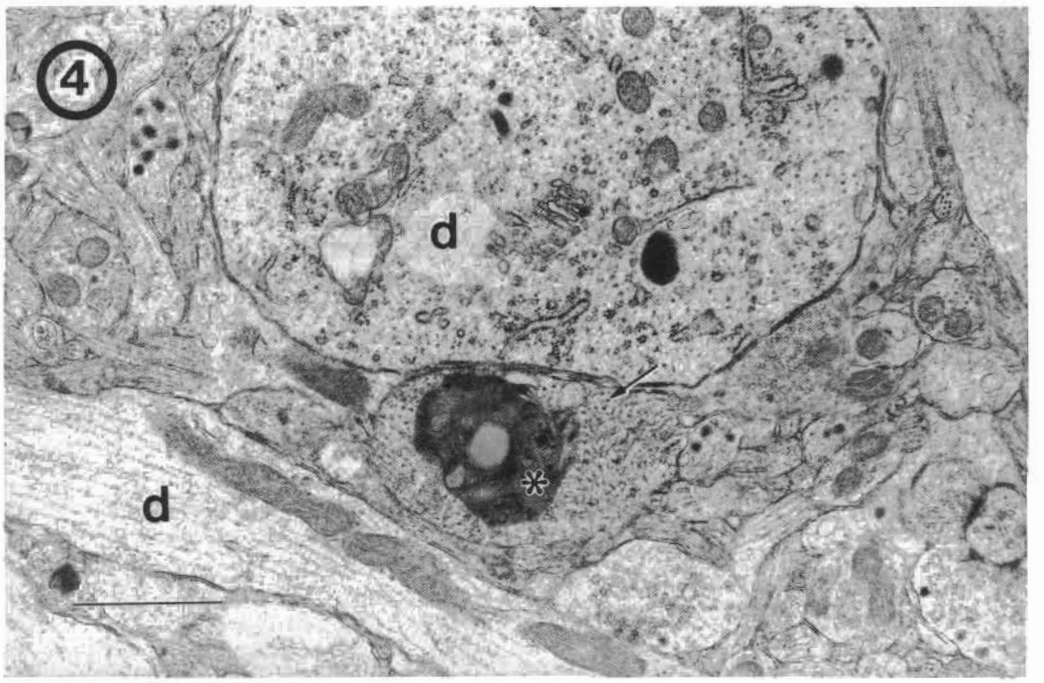
Figure 6 shows the results of the quantitative analysis of animals with transection of the fornix. When the totals of DT were compared with those rats without transection, the DTs were an order of magnitude smaller, i.e. at least one tenth of the synapses of the V-VMHN was provided by the fornix. In addition, the numbers of DTs varied in a parallel fashion with respect to the ADS and ASP counted in Groups 1 to 5. Likewise, when differences among Groups were tested by ANOVA there was a significant difference in DTs between MFx and FFx. The same was true when GxMFx were compared with MFx, as well as between FFx and F+TFx. The difference disappeared when MFx were compared to MGxFx, which was restored by testosterone (i.e. GxMTFx). Finally, there was no statistical difference between the neuropil of the FFx and that of the GxMFx.

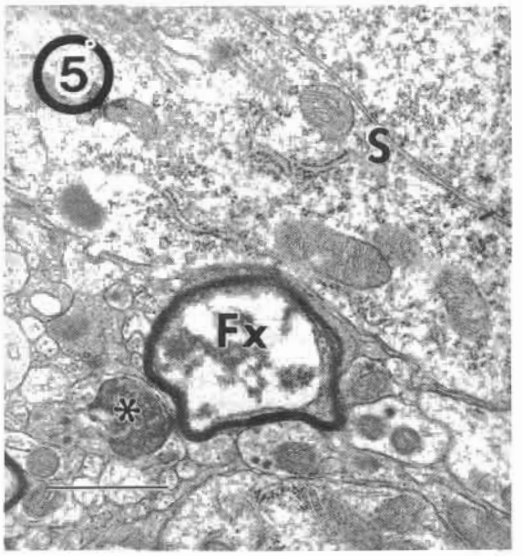
DISCUSSION

The present study provides support to the concept of the structural sexual differentiation of the mammalian brain [2-8]. Our data demonstrate the existence of a sexually dimorphic pattern in the number of ADS and ASP in the VL-VMHN, in that both synaptic types are significantly more numerous in the intact male than in the control female. Moreover, the perinatal exposure of the female to exogenous testosterone led to an increase in the number of ADS and ASP, making them comparable to those recorded in the normal male. Conversely, castration of the male as newborn produced a decrease in ADS and ASP making them comparable to those of the normal female. From these data can be concluded that the sex difference in the larger density of ADS and ASP observed in the intact male is determined by the early organizational effect of testosterone and its metabolites on the developing hypothalamus, rather than intrinsic genomic sex differences. This is consistent with Matsumoto and Arai study of the VL-VMHN [8] as well as with investigations of other sexually dimorphic nuclei of the mammalian diencephalon [2-7]

The contribution of fornical fibers to the VL-VMHN is demonstrated beyond doubt, since the transection of the fornix produced typical orthograde degeneration [11, 12] of synapses within the neuropil of the nucleus (Figs. 4 and 5). Previous light microscopic studies with lesions [13] and autoradiographic [14] approaches strongly suggested a contribution to the VMHN from the hippocampus via fornix. Our electron microscopic findings demonstrate that fornical fibers readily ended up as synaptic boutons in the neuropil of the V-VMHN, which underwent orthograde degeneration following fornical transection.

One of the central issues of the contemporary neuroendocrinology has been the demonstration of the existence of sexually dimorphic neuronal chains which presumably underlie the sex specific endocrine and behavioral functions [1, 4, 15-17]. Of particular interest for the present study is Raisman and Field's [4] which demonstrates that fibers from the stria terminalis give rise to sexually dimorphic numbers of synapses within the hypothalamus, and the dependence of such dimorphism on the perinatal titers of testosterone. This motivated us to search for a possible sexual dimorphism in the number of degenerating synapses following a complete interruption of the fornix. As shown, the number of DTs is indeed sexually dimorphic and dependent on the early exposure of the developing brain to testosterone. Although the functional significance of this new dimorphism requires elucidation, the following conclusions could be reached on the basis of available information. The VMHN has reciprocal connections with the limbic system, e.g. hippocampus [13-15], since the latter receives neural input from the olfactory system from the entorhinal area [18] and is considered as a site of integration of different modalities of endocrine and sexual behavior [19], it is plausible that the sexually dimorphic pattern in the density of fornical synapses terminating in the neuropil of the VL-VMHN, may represent the morphological signature of sexually dimorphic functions assumed





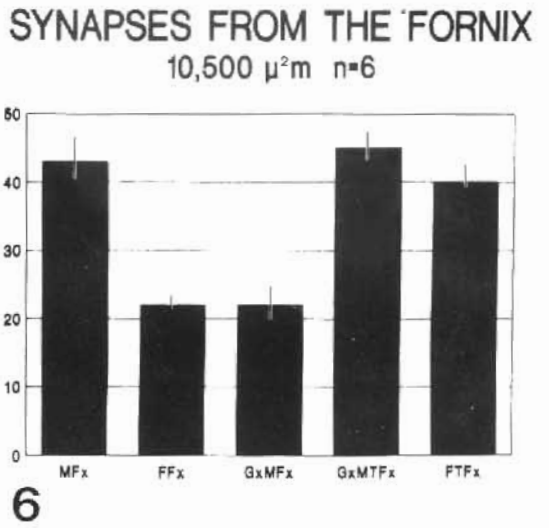


Figure 4. Electron micrograph of the neuropil of the ventrolateral part of the ventromedial hypothalamic nucleus of a male with transection of the fornix three days prior to sacrifice. Two proximal dendrites are seen (d). At the center a degenerating synapse is seen between the dendrites (aster). The degenerating synapse contains an electron-dense clump of amorphous material which is surrounded by a fine framework of neurotubules (arrow) and filaments. Barr= 0.9 µm.

Figure 5. Electron micrograph of the neuropil and a neuron soma (S) of the ventrolateral part of the ventromedial hypothalamic nucleus. Two degeneratingelements are illustrated, a thin myelinated axon containing clumps of amorphous electron dense material surrounded by empty areas (Fx). Another type of degenerating structure consists of a synaptic bouton which contain a clump of electron dense aggregates (aster). Barr= 0.5 µm.

Figure 6. Histogram comparing the number of degenerating synapses three days after transection of the fornix. Significance was found between MFx and FFx. Castration of the male as new born (GxMFx) suppersed the difference when compared with FFx. As can be seen the treatment of the castrated male with exogenous testosterone (GxMFx) led to a number comparable to that counted in the CM. Finally, perinatal treatment of the female (FTFx) matches the number of degenerating synapses to those of the MFx and GxMTFx.

by the VMHN [16, 17, 20, 21] and influenced by the hippocampus via fornix.

RESUMEN

El sistema nervioso central de los mamíferos atraviesa por un proceso de diferenciación sexual estructural determinado por la interacción temprana de las hormonas sexuales esteroides con el genoma neuronal. En el presente estudio, se demostró que el neuropilo de la porción ventrolateral del núcleo hipotalámico ventromedial (VLNHVM) de la rata es sexualmente dimórfico, ya que la densidad de sinapsis axodendríticas y axoespinosas, es mayor en el macho que en la hembra. La dependencia hormonal de este dimorfismo se demostró al "invertirlo" mediante el tratamiento de la hembra con testosterona o con la castración del macho recién nacido. En ratas adultas con interrupción completa del fórnix, se determinó que la densidad de fibras degeneradas que terminan en el VL-NHVM es también sexualmente dimórfica. La exposición perinatal de la hembra a testosterona exógena, o la supresión de testosterona endógena por castración del macho recién nacido "invirtió" la densidad de fibras de origen fornical en el VL-NHVM. Se concluye: 1. El fórnix inerva al VL-NHVM, demostrado por degeneración ortógrada; 2. El número de terminales del fórnix al VL-NHVM es mayor en el macho; 3. El dimorfismo sexual observado es dependiente de las hormonas sexuales esteroides de origen gonadal

AKNOWLEDGEMENTS

The present study was supported by Consejo Nacional de Ciencia y Tecnología (CONACYT), México, Grant No. 0056N.

The authors thanks Mr. Raúl Bernal for his valuable help in preparing the illustrations of this work.

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