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Sex differences in synaptic density in the prenatal rat striatum

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SUMMARY

In order to elucidate the developmental mechanisms responsible for sexual differentiation of the striatum, the ultrastructure of the striatum of embryonic day (E) 18 and 21 rats was examined for sex differences in densities of synapses. While the E18 striatum displayed very few contacts resembling mature synapses, the E21 striatum contained numerous synapses enabling clear distinctions to be made between asymmetrical Gray type I and symmetrical Gray type II synapses. The vast majority of all synapses counted were asymmetrical Gray type I. In both sexes, the synaptic densities exhibited large regional variations. The highest densities were present in the ventrocentral and the lowest in the medial regions of the E21 striatum. Marked sex differences were detected in the ventral and lateral portions throughout the rostrocaudal extent of the striatum. With both synaptic subtypes, the sex ratio was 1.5 in favor of females. We argue that these sex differences reflect sexually dimorphic inputs from the cortex as well as from the midbrain. Since the sex differences occur at the very beginning of the critical period for sex differentiation of the rat brain, they are presumably instrumental in establishing a sexually dimorphic circuitry in the striatum.

Key words: Electron microscopy - morphometry - development - critical period - basal ganglia.

INTRODUCTION

Experimental as well as clinical data suggest that the adult extrapyramidal system is characterized by functional sex differences. Tissue levels (Crowley et al., 1978), release (Becker and Ramirez, 1981; Becker, 1990) and uptake (Morissette and Di Paolo, 1993) of dopamine differ between male and female rat striatum. Sex affects signal transduction in striatal neurons (Castner and Becker, 1996). Sexually dimorphic responses have further been reported concerning long-term adverse effects of nicotine (Fung and Lau, 1989), methamphetamine (Wagner et al., 1993), and stress (Fameli et al., 1994) on striatal dopamine levels. Behavioral studies demonstrate sex differences in motor control. In rats, initiation of stereotype behavior by administration of amphetamine differs between sexes (Beatty and Holzer, 1978; Becker et al., 1982) and, in healthy humans, there are sex differences in capability of throwing and intercepting (Watson and Kimura, 1991) and in turning bias (Mead and Hampson, 1996). Finally, also diseases characterized by disturbances of motor control, such as Parkinson's disease, Tourette syndrome, tardive dyskinesia, hereditary progressive dystonia, and attention-deficit/hyperactivity disorder show a sex-specific prevalence (Kane and Smith, 1982; Minde, 1985; Lilienfeld et al., 1990; Segawa and Nomura, 1995).

It is generally thought that sex differences in neural circuitry are generated during a critical phase of brain development which, in the rat, coincides with the perinatal period (Dörner, 1980; MacLusky and Naftolin, 1981). The present work therefore examines the developing rat striatum immediately before birth for morphological evidence of the presence of a sexually dimorphic circuitry.

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Materials and Methods

Sprague-Dawley rats (Charles River, Sulzfeld, Germany) were mated from 8 to 11 a.m. (day of insemination = E 0). Dams were anesthetized in the morning of E18 or E21 with 4% chloral hydrate (1ml/100 g b.wt.) and embryos removed by Cesarean section. Embryos were anesthetized (0.1 ml of the above solution) and perfused through the heart with i) 1.0 ml saline (0.9% NaCl) containing 1 % $NaNO_2$ and 50 IU heparine (4°C) and ii) 2 % paraformaldehyde, 3 % glutaraldehyde, 1 % acrolein, 2.5 % dimethyl sulfoxide (v/v), and 6.8 M $CaCl_2$ in 0.1 M Na-cacodylate buffer (pH 7.4, 4°C). Male and female embryos were separated by inspection of the gonadal adnexa (Reisert et al., 1996). The brains were postfixed in the same fixative at 4°C overnight.

100 µm thick coronal sections of the whole brain were cut on a Vibratome (Oxford Instruments) in 0.1 M Na-cacodylate buffer containing 6.8 M $CaCl_2$ (pH 7.4). Osmolarity was set to 560 mosm with sucrose. Striata (caudate-putamen, CP) were cut out from sections that lay between the frontal pole of the CP and the rostral pole of the globus pallidus (figs. 31-38 and 77-86, respectively, in the atlas of Paxinos et al., 1991). To facilitate orientation, each section included the ependyma of the lateral ventricle and the corpus callosum (Fig. 1). Thus, at E21, the resulting CP tissue block yielded 24-25 Vibratome sections which were divided into 3 portions (rostral, intermediate, caudal, 8 sections each). Sections were postfixed with 2% OsO_4 in 0.1 M Na-cacodylate buffer (pH 7.2) for 2 hours, rinsed twice in cacodylate buffer and once in ddH_2O, block-stained with 2% uranylacetate in ddH_2O, dehydrated in ethanol and hydroxypropylmethacrylate and flat-embedded in Epon 812 between a glass slide and coverslip. The slides were precoated with 2% dimethyldichlorosilane. Ultrathin sections were collected on single-slot grids. The slot was covered with polyvinyl formvar (0.5% dissolved in chloroform) film. Sections were stained with 0.2% lead citrate in ddH_2O and 10% uranyl acetate in 70% methanol.

Ultrathin sections were made of a few E18 striata for preliminary studies of synaptogenesis. Systematic sampling was confined to E21 animals. 5 male and 5 female E21 fetuses (2 litters) were included in the analysis. Sampling of the material for morphometry was carried out as follows: Ultrathin sections were prepared from 2 randomly chosen tissue blocks of the rostral, intermediate, or caudal portion of the CP of each animal with no regard to right or left-hand sides of the brain. Per portion and tissue block, 2 ramdomly chosen ultrathin sections were examined for numbers of synapses making up a total of 6 ultrathin sections per animal. The cross-sectional area of the CP was divided in 5 times 5 squares with a size of 0.04 mm^2 each. Synapses were systematically counted in 9 of the 25 squares (Fig. 1). The criteria for identification of synaptic contacts were at least one of the following: presence of pre- and/or postsynaptic membrane thickenings, clustering of synaptic vesicles close to a plasma membrane. Mean synaptic densities were calculated for each of the nine squares per plane and animal. Subsequently, means of the means were calculated per animal group (5 males, 5 females). Sex differences were analyzed for statistical significance by the t test.

Results

Synaptic morphology in the developing striatum

In the E18 rat striatum, almost no intercellular contacts could be detected that fulfilled all criteria of mature synapses, i.e., the presence of synaptic vesicles as well as pre-and postsynaptic membrane densities. Most of the contacts resembled the "intermediary contacts" described by Schuster (1990). They were characterized by absence of vesicles and symmetric thickenings of the opposed membranes that were more dense and broader than those encountered with symmetrical synapses. Such contacts were frequently seen between cellular somata and presumably represent a transient developmental phenomenon since they were less frequently seen in E21 striatum and are not known to occur in adulthood. In contrast to E18, many mature synapses were encountered in the E21 striatum. In most cases, these could be classified according to Gray and Guillery (1966) and subdivided into asymmetrical Gray type I (Fig. 2b, c, d) and symmetrical Gray type II synapses (Fig. 2a, c). Gray type I synapses contained round, oval or pleomorphic clear vesicles. Occasionally dense core vesicles were seen. The thickness of the pre- and postsynaptic membrane and the width of the synaptic cleft were 7.1 ± 1.5, 15.1 ± 4.7, and 10.3 ± 2.0 nm, respectively. Gray type II synapses contained the same types of vesicles as type I synapses. The thickness of pre- and postsynaptical densities was identical at 7.9 ± 1.9 nm, and the width of the of the synaptic cleft measured 9.9 ± 2.9 nm. In 22% of the symmetrical synapses, the postsynaptic thickenings were interrupted 2-4 times by sections of plasma membrane of regular width giving them the appearance of so-called perforated synapses. No sex difference was noted with respect to synaptic morp-

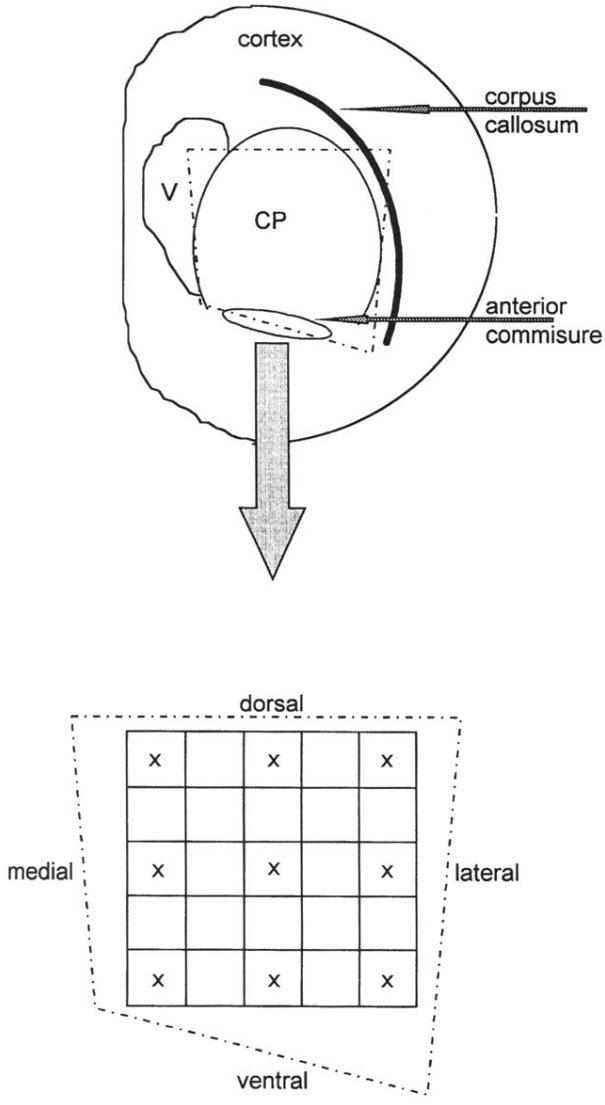

Fig. 1.– Orientation of striatal tissue excised from Vibratome sections and sampling procedure for morphometry. V lateral ventricle, CP caudate-putamen

hology. This concerns especially the size of the contact zone. The mean length of synaptic contacts was 358.7 ± 196.7 nm in females and 332.2 ± 155.1 nm in males (p = 0.1).

Numbers of synapses per area

Because of the absence of mature synapses from E18 striatum, the evaluation of the distribution of synapses and the measurements of synaptic numbers per area of tissue (density of synapses) were restricted to E21 male and female rats. Fig. 3 summarizes the results. In both sexes, the numbers of synapses exhibited large regional variations. In all three frontal planes investigated, few synapses were present in the medial and dorsal portions of the striatum. Higher numbers were found in its central, ventral, and lateral subdivisions. The highest densities were present in the ventrocentral and the lowest in the medial regions. Marked sex differences were detected in all three frontal planes with females consistently showing more synapses than males. These sex differences most often reached statistical significance in the ventral and lateral portions of the striatum. Table 1 gives the total numbers of all type I and type II synapses counted in all subdivisions of the male and female striatum. It can be seen that the vast majority were asymmetrical Gray type I. Independently of the subtype, there was a constant sex ratio of about 1.5 in favor of females.

Discussion

Sex differences in synaptic density are a typical morphological correlate of a sexually differentiated brain circuitry (reviewed by Pilgrim and Hutchison, 1994). Presently we demonstrate for the first time a sex difference in densities of synaptic contacts in the rat striatum immediately before birth. Clearly, the results call for an extension of the investigation into the postnatal period in order to see whether the sex differences are of a transient or permanent nature. However, even if the differences were transient, the observation would be of considerable interest in its own right. With respect to the process of sexual differentiation of the rat brain, the late prenatal period plays a key role. The last days before birth are generally accepted as the start of the so-called critical period of sexual differentiation elicited by the surge of systemic androgen in the

Type of synapse	Total (%)	Male	Female	Ratio ♀ : ♂
asymmetrical*	13568 ± 641 (87.5)	5464 ± 400	8104 ± 883	1.48
symmetrical*	1938 ± 73 (12.5)	780 ± 71	1142 ± 76	1.48
total	15506 ± 357 (100.0)	6244 ± 235	9246 ± 479	1.48

Table 1.– Mean numbers (percentages) ± SEM and sex ratios of synapses in caudate-putamen (irrespective of regional variability).
* p male vs. female = 0.0001

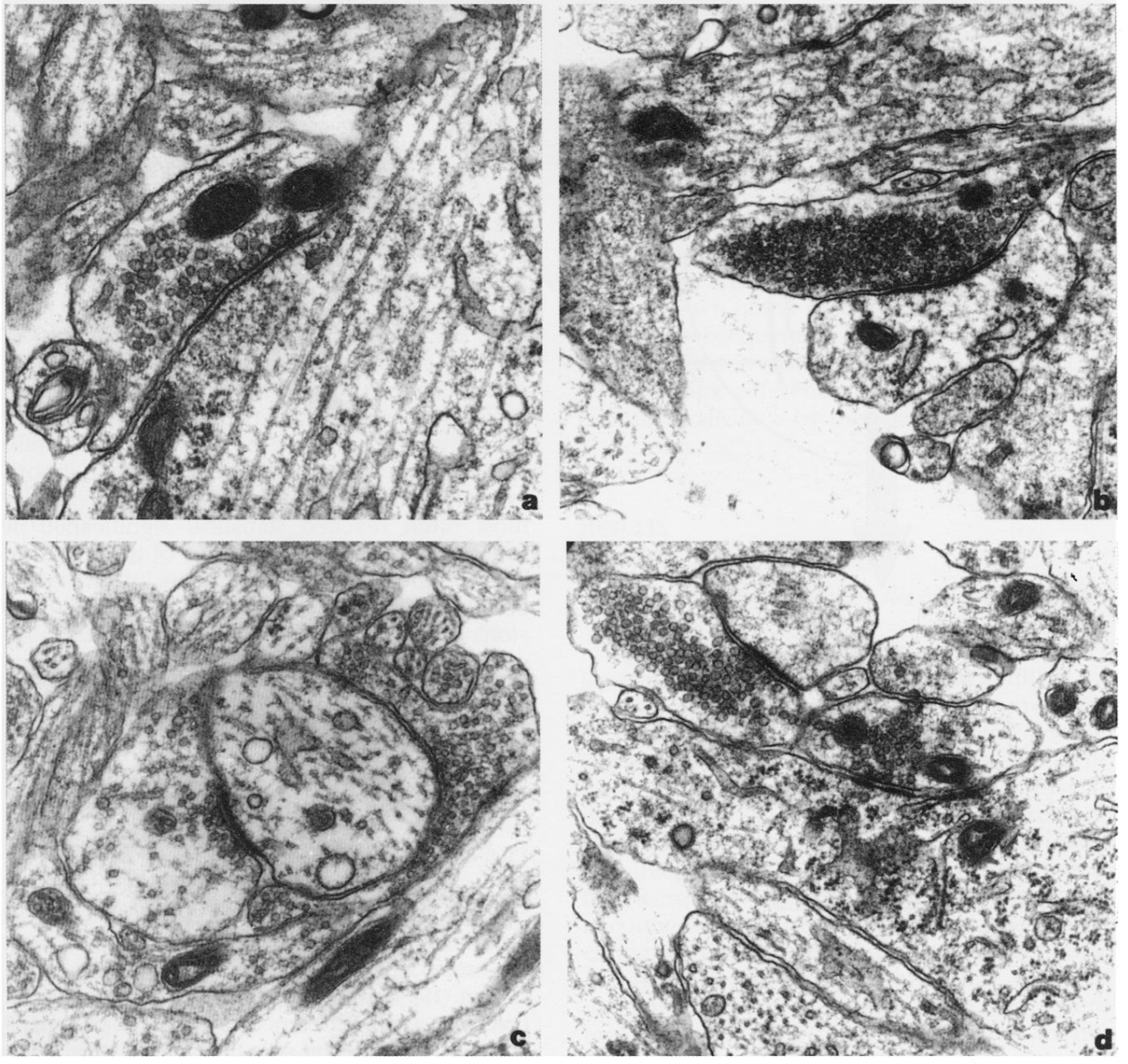

Fig. 2.– Ultrastructure of synaptic contacts observed in the E21 striatum.
a: axodendritic symmetrical Gray type II synapse. x 19,500.
b: axodendritic asymmetrical Gray type I synapse. x 12,500.
c: cross-sectioned dendrite contacted by an asymmetrical synapse and a symmetrical synapse en passant (axonal varicosity characteristic for dopaminergic fibers). x 12,500.
d: two asymmetrical synapses, one axo-somatic and the other axo-dendritic (presumably on a dendritic spine). x 15,600.

male rat embryo at E17/18 (Weisz and Ward, 1980). According to the classical "organizational" hypothesis, androgens (or, for that matter, estrogens synthesized from androgens within the male brain) "organize" a male-type brain circuitry (Arnold and Gorski, 1984). During the critical period, the steroid hormones appear to induce irreversible changes in the developing brain that ultimately lead to male-type brain function, i.e. male-specific neuroendocrine regulation and behavior (Dörner, 1980). Several ideas have been put forward about how the organizational effects of sex steroids are brought about. The mechanisms include steroid modulation of axon outgrowth, expression of growth- and synapse-associated proteins as well as synaptic reorganization (discussed by Pilgrim and Hutchison, 1994). Especially the latter mechanism is potentially relevant to the present findings. Transient changes in numbers of synapses are a frequently encountered phenomenon in neural development. Sculpting of neural circuitry often entrains a temporary overproduction of synapses which are subsequently eliminated (McDonald and Johnston, 1990; Wolff and Missler, 1992). Temporary changes in synapses relate to the trophic action which neurotransmitters exert during brain development (Lauder, 1993). Appe-

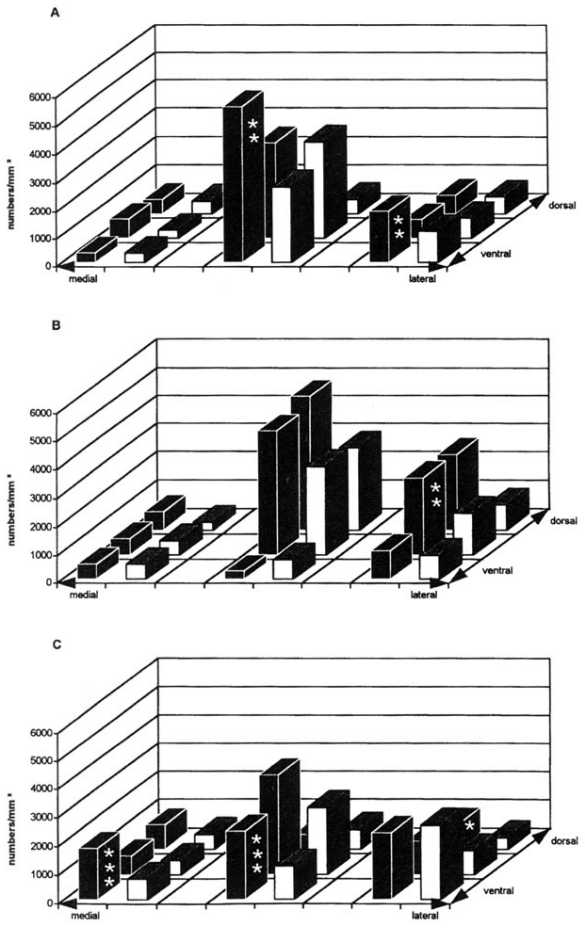

Fig. 3.– Synaptic densities in different regions of the E21 caudate-putamen. **A, B, C:** rostral, intermediate, caudal planes. Black and white columns represent data from 5 females and 5 males, respectively. Asteriks indicate statistically significant sex differences (* p = 0.02, ** p = 0.01, *** p = 0.001)

arance and disappearance of neurotransmitters in certain target regions may be instrumental in shaping their connectivity (Parnavelas and Cavanagh, 1988). It is highly probable that such mechanisms are also employed in the process of sexual differentiation. Reports of transient sex differences in synaptic densities in the preoptic area (Reier et al., 1977) and visual cortex (Muñoz-Cueto et al., 1991) of the developing rat point in that direction. In our case, it is conceivable that androgen action brings about a reduction of synaptic contacts in the male relative to the female striatum. This would then transiently alter the balance of neuroactive substances in the environment of growing striatal neurons in a sex-specific manner. Even small shifts in this balance, if they occur in sensitive time windows, may permanently affect neuronal survival and/or differentiation and thus lead to sex differences in adult neural circuitry.

The sex differences in synaptic densities were restricted to certain parts of the striatum and would have probably gone unnoticed if it had not been for a painstaking morphometrical analysis that included a careful dissection of the striatum into small subdivisions. Interpretation of these sex differences in terms of striatal connectivity would require more detailed knowledge about the time course of ingrowth of specific afferents and establishment of intrinsic connections during the perinatal period. However, an important clue as to the origin of the fibers exhibiting sexually dimorphic synapses is provided by the observation that almost 90 % of all synapses registered were asymmetric (Gray type I). With respect to the adult striatum, there is general agreement that these almost exclusively represent glutamatergic excitatory cortical afferents (Gerfen, 1988; Smith and Bolam, 1990). The sex differences therefore primarily reflect a sexually dimorphic input from the cortex because contributions from any other circuit, should it be sexually dimorphic, would have influenced the synapse counts only marginally. As to the topography of the cortical afferents, it remains to be seen why the sex differences were confined to the ventral portions of the developing striatum. One clue could come forward from the fact that there is a ventrodorsal gradient in maturation of striatal target neurons (Bayer, 1984). This would imply that sex differences in more dorsal regions might be found later in development, i.e. postnatally. Another possibility would be that the border between the so-called ventral (limbic) striatum and the dorsal striatum (caudate-putamen) runs more dorsally than assumed by most authors (Groenewegen et al., 1991). If this is the case especially around E21, we may have included samples from the former in our analysis. The sexually dimorphic circuitry would then largely concern the input from the limbic, i.e. the prefrontal cortex. This would have interesting implications as to the type of behavior hormonally imprinted during this period. The ventral striatum is believed to represent a key relay in the processing of affective information and control of motivated behavior (Robbins and Everitt, 1996).

In addition to Gray type I synapses, symmetric Gray type II synapses were also more frequently encountered in females than in males. We propose that these mainly represent mesostriatal dopaminergic afferents. Specifically, the sex differences observed in the lateral striatum could be attributed to dopaminergic afferents because these are known to form symmetric synapses (Freund et al., 1984) and the laterodorsal subregions have been described to receive the most dense mesostriatal innervation at E21 (Voorn et al., 1988; Reisert et al., 1990). Additional arguments may be derived from previous observations made in this laboratory. First, mesencephalic dopaminergic neurons develop

morphological and functional sex differences in cell cultures of embryonic rat mesencephalon (Reisert et al., 1994) and, second, throughout prenatal development from E16 to E21 in vivo, the female rat striatum exhibits higher densities of tyrosine hydroxylase-immunoreactive (dopaminergic) axons than in the male (Ovtscharoff et al., 1992).

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