Androgen receptor immunoreactivity in rat occipital cortex after callosotomy

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Gonadal steroidogenesis can be influenced by direct neural links between the central nervous system and the gonads. It is known that androgen receptor (AR) is expressed in many areas of the rat brain involved in neuroendocrine control of reproduction, such as the cerebral cortex. It has been recently shown that the occipital cortex exerts an inhibitory effect on testicular stereoidogenesis by a pituitary-independent neural mechanism. Moreover, the complete transection of the corpus callosum leads to an increase in testosterone (T) secretion of hemigonadectomized rats. The present study was undertaken to analyze the possible corticocortical influences regulating male reproductive activities. Adult male Wistar rats were divided into 4 groups: 1) intact animals as control; 2) rats undergoing sham callosotomy; 3) posterior callosotomy; 4) gonadectomy and posterior callosotomy. Western blot analysis showed no remarkable variations in cortical AR expression in any of the groups except in group I where a significant decrease in AR levels was found. Similarly, both immunocytochemical study and cell count estimation showed a lower AR immunoreactivity in occipital cortex of callosotomized rats than in other groups. In addition, there was no difference in serum T and LH concentration between sham-callosotomized and callosotomized rats. In conclusion, our results show that posterior callosotomy led to a reduction in AR in the right occipital cortex suggesting a putative inhibiting effect of the contralateral cortical area.

Key words: androgen receptor, callosotomy, right occipital cortex, corticocortical connections, rat.

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Paper accepted on July 4, 2008

European Journal of Histochemistry
2008; vol. 52 issue 3 (July-September): 163-168

Androgen receptor (AR) is a member of a superfamily of nuclear transcription factors mediating the action of steroid hormones (Prins, 2000). Traditionally, it is known to be expressed in many areas of the rat brain involved in neuroendocrine control of reproduction and growth, including the periventricular, paraventricular and arcuate nuclei, the ventral mamillary, dorsomedial nuclei and lateral hypothalamus (Simerly *et al.*, 1990; Stumpf and Sar, 1978). Recent investigations demonstrated that AR can act as part of a neuroprotective and antioxidant system when activated by male steroid hormones (Ahlbom *et al.*, 2001).

In addition to the classical intranuclear location, AR was also detected in axons and dendrites of rat forebrain suggesting a non-genomic mechanism of sex steroids that may not be attributed to transcriptional regulation (DonCarlos *et al.*, 2003). There is considerable evidence to show that AR immunoreactivity is largely present in rat cerebral cortex where the majority of receptor-bearing cortical cells were found to be pyramidal neurons or, in a smaller amount, glial cells (DonCarlos *et al.*, 2006). More precisely, that study highlighted the presence of AR-positive neurons in the occipital regions Oc1 and Oc2 according to Paxinos and Watson (1997), as well as in other cortical areas such as sensory and motor regions (Kritzer, 2004).

There is growing evidence that brain areas, such as the caudal raphe nuclei, locus coeruleus, periaqueductal grey matter, are transneuronally connected with the gonads (Gerendai *et al.*, 2000a; Gerendai *et al.*, 2000b) and their involvement in a complementary control mechanism independent of the pituitary gland has been suggested. The role of this neural influence in the control of gonadal functions is consistently shown by studies based on transections or lesions in the peripheral or central nervous system (Gerendai *et al.*, 1997; Banczerowski *et al.*, 2001) and by recent observations showing an inhibitory effect of the right occipital cortex on tes-

ticular steroidogenesis (Lepore *et al.*, 2006).

Furthermore, it has been recently demonstrated that the complete transection of the corpus callosum leads to an increase in T secretion of hemigonadectomized rats (Banczerowski *et al.*, 2000), suggesting that the callosotomy-induced upregulation of testicular steroidogenesis is pituitary-independent. If so, it can be hypothesized that brain hemispheres, through callosal fibers, can reciprocally modulate the extrapituitary control of sex steroid production.

Therefore, the present work aimed to clarify how the right occipital brain cortex can be influenced by the contralateral cortical area. The hemisphere of the right side was chosen since most observations suggest that in both male and female rats there is a higher involvement of the right-sided brain structures in the control of gonadal functions (Gerendai *et al.*, 1995).

Materials and Methods

All the experiments here described were performed according to the principles set out in the Declaration of Helsinki and approved by the Bioethics Committee of the University of Sassari, Italy. Adult male Wistar rats weighing 340±20g were used. Animals were housed in a temperature-controlled room (22±2°C) with a 12-hr. light/dark cycle and fed on a standard diet and water. The animals were divided into 4 groups and underwent the following procedures: I) intact animals (n=6), II) sham callosotomized rats (n=6), III) posterior callosotomized rats (n=6), IV) gonadectomized rats posterior callosotomized 14 days later (n=6).

Rats from groups I, II and III were killed seven days post-surgery and those from groups IV seven days after the last intervention. For groups II and III, serum T and LH levels were determined by radioimmunoassay (RIA) as previously described in detail (Lepore *et al.*, 2006). To perform posterior callosotomy, rats were anesthetized with zolazepam and tiletamine (Zoletil, Virbac, 50 mg/kg) and placed on a stereotaxic frame with the head in a 3.3 mm dorsoventral nose-down position relative to the interauricular line (Paxinos and Watson, 1997). The scalp was cut along the midline and a 4 mm hole was drilled 3 mm lateral to the midline and 3 mm posterior to the lambda. The corpus callosum was transected according to the method previously described in the mouse by Schalomon and Wahlstein (1995) and modified by Banczerowski *et al.* (2000) for the rat. Co-ordinates of posterior callosotomy were as follows: 6 mm posterior and 0.4 mm lateral to the bregma; angle 0° from vertical; depth of the incision 2.9 mm. The sham brain operation included anesthesia, immobilization of the head in the stereotaxic frame as well as opening of the skull and the dura mater. In order to check the location and extent of callosotomy, brains were fixed in 10% formaldehyde, dehydrated in alcohol and paraffin embedded. Serial sections, 5 μm thick, were stained according to Nissl's method. Bilateral gonadectomy was performed according to the surgical procedures performed by Turvin *et al.* (2007).

Three animals from groups I, II and III were anesthetized according to the same protocol described above, then perfused via the left cardiac ventricle with 100 U/10 mL heparinated saline followed by 300 mL of 4% buffered paraformaldehyde. Brains were postfixed in the same fixative solution for 4 hrs., dehydrated in ethanol and paraffin embedded. Serial sections of the forebrain, 10 μm thick, were treated according to the immunoperoxidase method for AR detection. In detail, sections were blocked for 20 mins. with normal goat serum and incubated overnight at 4°C with a polyclonal anti-androgen receptor antibody (20 μg/mL, Sigma) diluted 1:50, then for one hour in a secondary antibody (biotinylated goat anti-rabbit, Vector) diluted 1:250. After several rinses in PBS, sections were incubated with avidin-biotin complex system (ABC, Vector) for 30 mins. The slides were then incubated with diaminobenzidine (DAB) for 1-5 mins.w and finally dehydrated and coverslipped. Slides were concomitantly processed by omitting the primary antibody or incubating sections with non-immune rabbit serum (negative control sections). Afterwards, sections were observed by means of a Olympus microscope and captured in bitmap format with a F-View digital camera. The occipital cortical regions (Oc1 e Oc2) were selected and their area estimated by means of the validated Scion Image computer software (Scion Corporation). Computer-assisted counts of the immunoreactive cells were determined and expressed as number of positive cells/mm^2.

Brain occipital cortical areas from all groups of animals were immediately removed and homogenized in lysis buffer with protease inhibitor Roche Complete Mini according to Tabori *et al.* (2005). In

order to rule out any possible difference in protein estimation and loading, protein concentrations were determined by a colorimetric assay (DC Protein Assay, Bio-Rad) setting the spectrophotometer (LKB) to 750 nm. Aliquots (80 µg of protein) were prepared in sample buffer (62.5 mM Tris-HCl pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.5% bromophenol blue). Proteins were separated on SDS-PAGE in 10% gel at 60 mA for one hour and then transferred onto nitrocellulose membranes (Trans-Blot transfer medium, 0.45 µm, Bio-Rad) using a semi-dry transfer unit at a constant current of 20 volts for 20 mins. The nitrocellulose membranes were blocked for one hour in 5% skim milk PBS and incubated overnight with the same anti-androgen receptor antibody (2 µg/mL) used in the immunocytochemical study. The membranes were then incubated with a 1:15,000 IgG anti-rabbit alkaline phosphatase-conjugated antibody (Sigma) for one hour and the reaction developed using nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate, toluidine salt (NBT/BCIP, Roche) as chromogen. A molecular weight marker (Color Burst Electrophoresis Marker, Sigma) was used to ascertain the molecular weight of the band observed. It ran between 210 (violet) and 90kD (pink), consistent with the 110kD molecular weight of AR, as reported in the literature (Prins *et al.*, 1991). Finally, densitometric values of protein bands were quantified using the Scion Imaging computer software. Results of both densitometric and cell count analyses were statistically processed by analysis of variance (ANOVA) followed by Student-Newman-Keuls multiple comparison method. $p<0.05$ were considered significant.

Results

Nissl's staining of the site of the surgical lesions (groups III and IV) showed that posterior transection of the corpus callosum was performed according to the scheduled protocol and involved the whole thickness of the corpus callosum. The lesions produced a limited extracallosal inflammation (Figure 1). There was no difference in serum T and LH levels determined by radioimmunoassay between sham-callosotomized (group II) and callosotomized rats (group III), whereas serum T levels were 4.5 nmol/L for group I and 4.2 nmol/L for group II, and LH concentrations were 1.5 nmol/L for both these two latter groups.

Western blot technique revealed one band at 110kD in each sample examined. The right cerebral cortex from intact (group I), sham-operated (group II) and gonadectomized callosotomized animals (group IV) displayed approximately equal amounts of AR. By contrast, rats who underwent callosotomy (group III) showed a noticeable decrease in AR expression of about 60% of the maximum value, i.e. that of intact animals (Figure 2).

Qualitative analyses showed a difference in immunoreactivity in occipital areas Oc1 and Oc2. Indeed, intact rats (group I) showed a high number

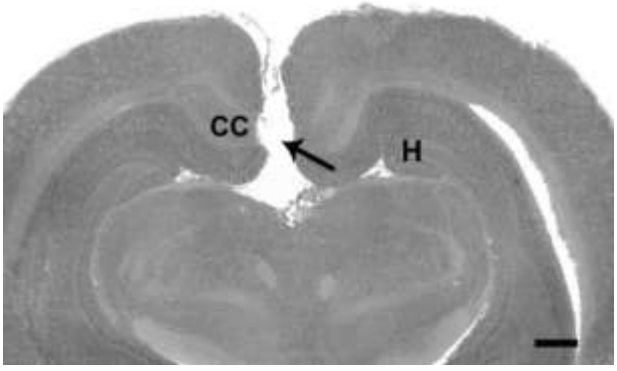

Figure 1. Rat brain coronal section showing the surgical lesion (arrow) strictly confined to the posterior part of the corpus callosum without involvement of other brain structures. Nissl's staining. CC=corpus callosum; H= hippocampus. Bar = 1 mm.

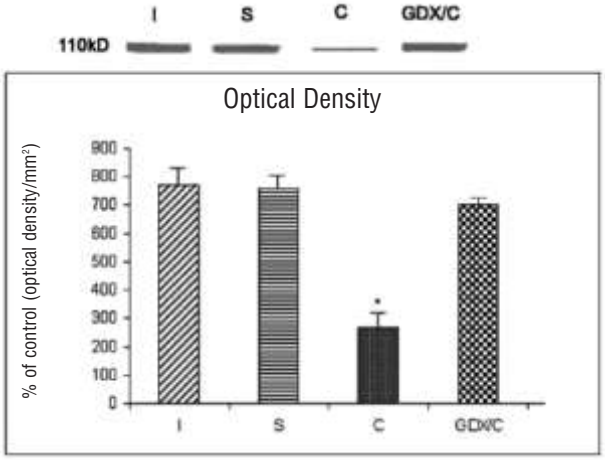

Figure 2. Western blot and densitometric analyses showing AR content in right occipital cortex of all experimental groups. Data are expressed as mean ±SEM. Asterisk indicates significant difference ($p<0.05$) from all other groups. I=intact animals; S=sham callosotomized rats; C= posterior callosotomized rats; GDX/C= gonadectomized rats posterior callosotomized 14 days later.

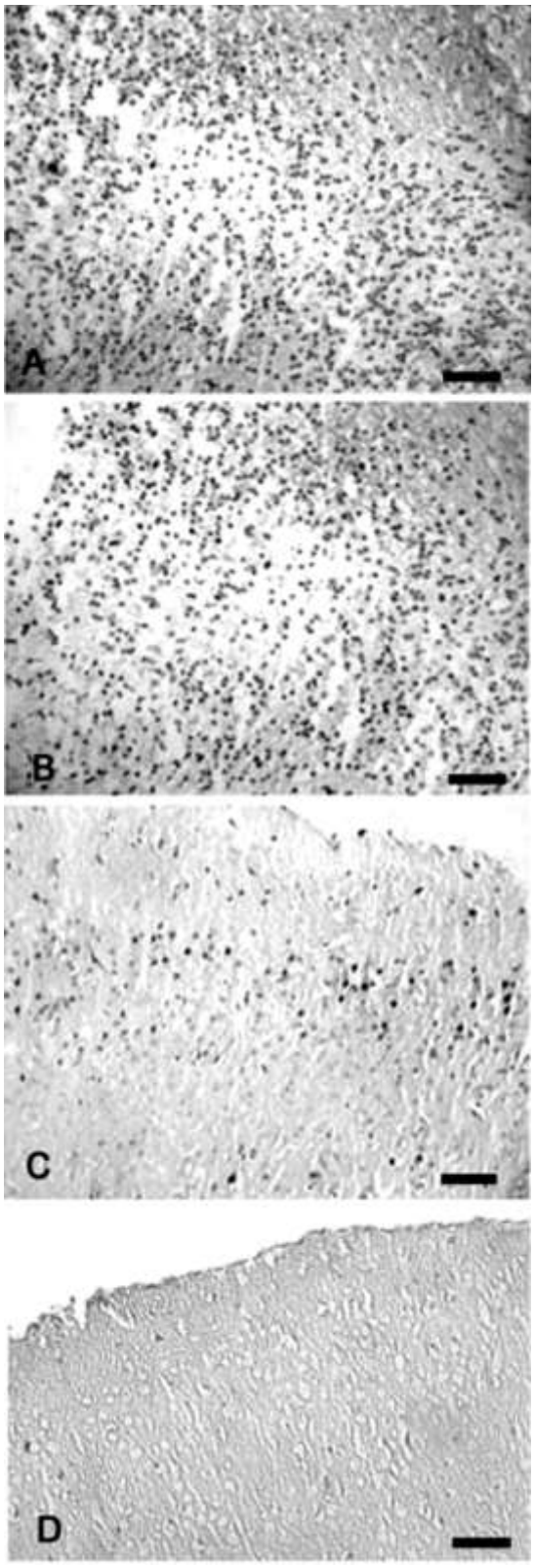

Figure 3. Qualitative analysis. AR immunoreactivity in sections from right occipital cortex of intact (A), sham-callosotomized (B) callosotomized (C) rats and negative control sections (D). The lowest signal is detected in callosotomized rats, whereas intact and sham operated groups display the same AR immunoreactive pattern. Bar = 100 µm.

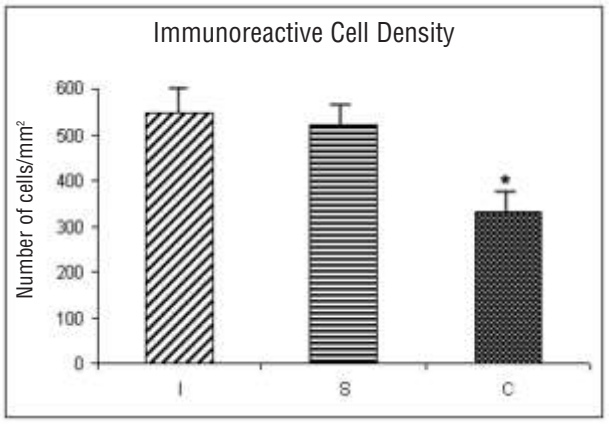

Figure 4. Quantitative analysis. The number of AR immunoreactive cells/mm² is significantly lower in callosotomized rats than intact and sham operated subjects. Asterisk indicates significant difference ($p<0.05$). I=intact animals; S=sham-callosotomized rats; C= posterior callosotomized rats.

of nuclei with a marked immunoreactivity spreading all over the cortical layers. Such images (Figure 3a) mostly overlapped with those from sham operated animals (group II, Figure 3b). In contrast, the number of immunoreactive cells of callosotomized rats (group III) was significantly lower. Indeed, except for a few completely labeled neurons, immunostaining in those animals was confined to nuclei of cells mainly located in the II and III cortical layers (Figure 3c). Finally, no immunoreactivity was detected by omitting the primary antibody (Figure 3d).

Quantitative analysis carried out with the image software and expressed as immunoreactive-cell density/mm² confirmed the data obtained with Western blot and immunocytochemical methods. Indeed, in callosotomized rats the density of positive cells was reduced by approximately 40% in comparison with the values detected in intact and sham operated subjects respectively (Figure 4).

Discussion

The present work confirmed that AR immunoreactivity can be detected in the visual cortical areas Oc1 and Oc2 and, therefore, agrees with Kritzer (2004) who found AR expression in the pyramidal neurons of the visual areas of hormonally intact rats. Moreover, the results of our study show for the first time that posterior callosotomy leads to a decrease in the expression of AR in right occipital cortex (Oc1 and Oc2), as shown by both immunocy-

tochemical and Western blot analyses. The reduction in cortical AR immunoreactivity observed in callosotomized rats could be explained by the fact that the left hemisphere might exert a modulatory influence on AR expression in the contralateral occipital areas. If so, it can be postulated that androgens produced by occipital neurons may paracrinally regulate cortical AR expression. Such androgen synthesis is in accordance with studies showing the presence of the complete machinery for androgen production in hippocampal neurons (Mukai *et al.,* 2006). The right cortical disfacilitation determined by callosotomy may induce a significant increase in the locally produced androgens with a consequent downregulation of AR expression. Indeed, it has been demonstrated that androgen receptor mRNA is inversely regulated by T in adult mouse brain and an autologous downregulation of AR mRNA was found in several tissues such as prostate, epididymis, kidney and brain after androgen stimulation (Quarmby *et al.*, 1990). In contrast, castration of adult male rats significantly increases AR mRNA in brain structures involved in reproductive activities such as the anterior pituitary gland and hypothalamic preoptic area (Kumar and Thakur, 2004). This is in accordance with our data showing that castration reduced the decrease in AR after callostomy (Figure 2).

In conclusion, our results suggest that in the right occipital cortical areas Oc1 and Oc2 AR expression can be significantly influenced by the contralateral areas independently from the pituitary gland since LH serum levels did not change in either sham callosotomized or callosotomized rats. This is consistent with a previous study showing that right occipital decortication and posterior callosotomy resulted in changes in gonadal T production *in vitro* without any significant variation on serum T and LH levels (Lepore *et al.*, 2006). All these data are in accordance with other reports describing pure neural links between the nervous system and the gonads (Gerendai *et al.*, 1986; Lee *et al.*, 2002; Selvage *et al.*, 2006). Thus, occipital cortex could be considered a part of the direct neural fine-tuning mechanism involved in the production of male testosterone. On the other hand, it has been shown that the occipital cortex is interconnected through temporolimbic structures with hypothalamic centers and brain stem nuclei (Banczerowski *et al.*, 2003). Neural signals may then reach the testis via parasympathetic vagal fibers or via spinal cord and sympathetic nerves (Gerendai *et al.*, 1997).

Further investigation is currently being carried out on the left hemisphere in order to verify whether cortical asymmetries occur between the occipital cortical areas of the two sides.

Acknowledgments

Work supported by the Fondazione Banco di Sardegna.

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