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Investigating the mouse brain for sex differences

Gene dependent sex differences in the expression of the Kdm5d protein in SF-1 knockout and wild type mice

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Abstract

Genes located only on the Y chromosome are male specific and are thus not expressed in female cells, which results in gene dependent sex differences. One of the genes located on the Y chromosome and expressed only in male cells is Kdm5d. In our study, the hypothalamus was compared between female and male SF-1 KO, intact WT and gonadectomised WT mice to investigate the genetically and hormonally dependent differences between the expression of the protein encoded by the Kdm5d gene. Standard immunohistochemical staining on floating brain sections was used to visualize the Kdm5d protein and was further analysed under the microscope. Immunoexpression of Kdm5d protein was observed only in WT intact males, but was not detected in any other groups. This observation suggests that the expression of the Kdm5d protein is both genetically and hormonally conditioned. Further experiments with testosterone supplementation should be carried out to confirm our findings.

Introduction

Sex differences can be observed since childhood. The way we dress, the way we talk, what toys we play with- sex differences accompany us on every step. However, not only are our behaviours different, but our physiologies are also distinguishable. With the help of modern science, we realize that some organs, even the male and female brain, are different. Such differences first appear as the sperm carrying the X or Y chromosome fertilizes the female egg containing the X chromosome. Genes located on the Y chromosome are male specific and will not be expressed in female organisms, which results in some differences between sexes. Such differences are said to be gene dependent. One of the genes that are assumed to be on the Y chromosome and thereby produce a gene-dependent sex difference is Kdm5d. We investigated whether there are differences between sexes in the expression of the protein encoded by the Kdm5d gene and if so, whether the differences are gene-dependent.

Literature Review

Sex differences may result from differences in the effect of gonadal hormones, differences in gene expression that are independent of the effect of gonadal hormones, or the combination of both[1]. Sex differences in mammals occur from the Y chromosome containing the sex-determining region called SRY gene, which enables the undifferentiated gonads to develop into testis; otherwise the gonad develops into ovaries[2]. While there are many genes required for complete sexual differentiation, one of the most important is Stereoidogenic factor 1 (SF-1)[3]. SF-1 is a transcription factor involved in the production of steroid hormones, the antimullerian hormone, gonadotropins, LH and FSH[4]. Its effects can be seen in primary steroidogenic tissues as well as in the hypothalamus, pituitary and spleen[4].Mice with the inactivated SF-1 gene, so called SF-1 knockout (KO) mice, do not undergo sexual differentiation because their gonads regress in early stages of development so that they never produce their own endogenous sex hormones, which would result in masculinization or feminization. All SF-1 KO mice are phenotypically female and can be used as a unique model for investigation of sex differences independent of hormones, such as for gene dependent sex differences.

Sexual differentiation of the nervous system occurs mostly during secondary sexual differentiation and is therefore greatly influenced by steroid sex hormones[3]. This phenomenon can be especially seen in the brain, which exhibits sexual dimorphism in areas such as the medial preoptic area[5], anterior hypothalamus[6],ventromedial nucleus of the hypothalamus[7], medial amygdala[8] or amygdala[9]. Lack of SF-1 morphologically affects VMH and therefore this part of the brain is suitable for the investigations of the effect of SF-1 on neuron development. However, SF-1 KO mice are also born without gonads, which makes them an excellent model to study hormone independent sex differences in the brain[10].

KDM5D gene

Kdm5d gene encodes for a histone H3 lysine 4 (H3K4) demethylase that forms a protein complex with the MSH5 DNA repair factor during spermatogenesis[11]. This complex is involved in male germ cell chromatin remodelling. KDM5D is located in the germ cell nucleus during meiotic prophase I, suggesting its involvement in meiotic orchestration[12]. The expression of the gene

was detected in pools of mouse preimplantation embryos as early as the 2-cell stage[13]. KDM5D is one of the key azoospermia factor (AZF) spermatogenic determinants, but this gene alone is not sufficient to restore spermatogenesis, given that the AZF region on the Y chromosome is missing [14]. In mice Kdm5d encodes for an epitope, such as part of a antigene, that is defined by the peptide Tenksgidi. Expression of KDM5D was detected in all male tissues and expression of its homolog on the X-chromosome was present in all male and female mice tissues tested[13]. DNA microarray test showed equally high expression of KDM5D in both SF-1 KO and WT males [15], which suggests that the expression of the Kdm5d gene is genetically conditioned.

Method

Detailed procedure from breeding to obtaining the brain of mice

Brains of adult mice Mus Musculus (stain C57Bl/6J) were used. Mice were bred in standard laboratory conditions; 20-25°C, 40-60% humidity, light: darkness exposure 12:12; in line with directive 2010/63/EU on the protection of animals used for scientific purposes [16]. They were fed food without fitoestrogenes (Harlan Teklad 2016, Great Britain) and water with 0.2% HCl. To obtain SF-1 KO mice, the model proposed by Luo et al. was used[17]. Mice have been genetically modified by having a part of the SF-1 gene cut out and replaced with the gene for resistance to neomycine. By breeding two SF-1 heterozygous mice, (SF-1-/+) SF-1 homozygous mice (SF-1-/- or SF-1+/+) were obtained. If the mice had two copies of inactive SF-1, they are considered as SF-1 KO (SF-1-/-). Due to the lack the adrenal gland in SF-1 KO all mice were injected with 50?L of corticosteroid mixture (400?g/mL hydrocortisone, 40ng/mL Dexamethasone, 25ng/mL fludrocortisone, dissolved in corn oil) for the first 7 days of their birth. Later were SF-1 KO mice transplanted the adrenal gland, so no further injections were required[18]. Control mice (WT) were exposed to the same procedure (the cut), except they were not inserted the adrenal gland. To provide a good control model, sex glands from WT mice have been removed between the age of 21 and 25 days.

When animals were 3 to 4 months old, they were euthanised according to directive 2010/63/EU and Recommendations of the European Comission for euthanasia of experimental animals: Parts 1 and 2[16][19][20]. After animals were anaesthetized with a mixture of ketamine, acetylpromazine and xylazine, tissue perfusion took place. A needle was inserted in the heart (in the left ventricle) and a peristaltic pump was used to wash of the blood with cold 0.05 M PBS followed by perfusion with cold 4% paraformaldehyde in 0.05 M PBS. When fixation was completed, brains were removed and stored in 4% paraformaldehyde overnight. From the next day they were stored in 0.1M PB at 4°C. We compared the expression of protein Kdm5d encoded by the KDM5D gene throughout the hypothalamus (from medial preoptic area (rostraly) to the VMH (caudally) from Bregma 0.38mm to (-2.30) mm) of six groups of brain samples; SF-1 KO male, SF-1 KO female, intact WT male, intact WT female, gonadectomised WT female and gonadectomised WT male.

Immunohistochemical staining

Standard immunohistochemical staining on floating sections was used. Brains have been embedded in 5% agarose (Sigma) and sliced on 50?m thick slides (Leica, VT1000 S, Leica Biosystems Nussloch, Germany). Sections were then stored at 4?C in 0.05 M phosphate buffered saline (PBS) over the night. Then, brain slices were incubated in 0.1 M glycine (Sigma) for 30 minutes at 4°C, rinsed three times for 5 minutes in 0.05 M PBS at 4°C and incubated in sodium borohydride on a shaker (Tehtnica, Domel, Slovenia) at 4°C. After 15 minutes the samples were rinsed four times for 5 minutes in PBS at 4°C. Next, the samples were put in a mixture of 5% normal goat serum (NGS; Chemicon, Temecua, CA, USA), 10% Triton X-100 (Tx; Sigma), 1% H_2O_2 (Merck, Germany) and PBS for 30 minutes at 4°C. Finally, they were incubated with primary antibodies Anti-Kdm5d (Anti-JARID1; ABE203; Merck Millipore, MA, USA); 1:2500) in 20% BSA, PBS and 10%Tx buffer for 2 days. Sections were washed four times for 15 minutes with buffer 0.5 M PBS + 0,02%Tx + 1% NGS at room temperature. The following the primary antibodies, we applied secondary antibodies (Biotinylated donkey anti-rabbit antibody (Immunoresearch, PA, USA); 1:500) for 2 hours and again rinsed sections four times for 15 minutes with a mixture of 0.5 M PBS and 0.02 Tx at room temperature. We then incubated the brain tissue for 1 hour in peroxidase (Jackson Immunoresearch) with 0.05 M PBS, 0.5% Tx; (1:2500) at room temperature. Next, we did four rinsings for 15 minutes each in Tris buffered saline (TBS; 0.05 M Tris, 0.9% NaCl; pH 7.5; Sigma). A solution of 0.025% 3,3?-Diaminobenzidine (DAB; Sigma) 0.02% H_2O_2 and TBS was used at room temperature for 5 minutes to color antibody-epitope complexes. This reaction was stopped by triple TBS rinsing for 10 minutes. Brain sections were then placed on microscope slides, covered with Pertex mounting media (Pertex Medite, Burgdorf, Germany) and covered by cover slips. We observed the results under a microscope. To get a clearer picture we have edited brightness and contrast of photos using Photoshop CS (Microsoft, USA).

Results

If results are positive, anti-Kdm5d antibodies have successfully bound to the Kdm5d protein, which means that the protein is expressed. The expressed protein with bound antibodies will be shown as dark dots on immunohistochemically coloured slides. If the protein was not expressed, such as when the results are negative, antibodies could not bind and there are no darker areas on brain slides.

Third ventricle of SF-1 KO female (a), SF-1 KO male (b), intact WT female (c), gonadectomised WT female (d), gonadectomised WT r

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Fig. 1: Third ventricle

Third ventricle of SF-1 KO female (a), SF-1 KO male (b), intact WT female (c), gonadectomised WT female (d), gonadectomised WT male (e) and intact WT male (f); magnification 1:100. Third ventricle of intact WT male (g); magnification 1:40. Arrows point to positive cells visible as dark dots.

Bed nucleus of stria terminals of SF-1 KO female (a), SF-1 KO male (b), intact WT female (c), gonadectomised WT female (d), gonad

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Fig. 2: Bed of nucleus of stria terminals

Bed nucleus of stria terminals of SF-1 KO female (a), SF-1 KO male (b), intact WT female (c), gonadectomised WT female (d), gonadectomised WT male (e) and intact WT male (f); magnification 1:100. Bed nucleus of stria terminals of intact WT male (g); magnification 1: 40. Arrows show positive cells visible as dark dots.

Preoptic area in SF-1 KO female (a), SF-1 KO male (b), intact WT female (c), gonadectomised WT female (d), gonadectomised WT m

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Fig. 3: Preoptic area

Preoptic area in SF-1 KO female (a), SF-1 KO male (b), intact WT female (c), gonadectomised WT female (d), gonadectomised WT male (e) and intact WT male (f); magnification 1:100. Medial preoptic nucleus (MnPO) of intact WT male (g); magnification 1:40. Arrow points to positive cells visible as dark dots.

Discussion

None of the females (SF-1 KO, WT and ovarectomized) were found to be expressing Kdm5d, therefore we are assuming that Kdm5d protein is not expressed in females. This shows that Kdm5d indeed is male specific. Immunopositive cells were found only in intact WT male mice, but in this case they were found in all observed areas of the brain. A comparison of intact WT male with SF-1 KO males and castrated males showed that differences occur due to endogenous sex hormones. In the case of the intact WT male being positive and SF-1 KO female, intact WT female and ovarectomized female the differences could be due to male specific endogenous hormones, the Y chromosome or both of these factors. Since no positive cells were found in SF-1 KO mice or in castrated mice, our study suggests that Kdm5d protein expression is not solely genetically conditioned, in that just the presence of Y chromosome is not enough for the full expression of this protein.

With this method we can determine only the expression of the protein, not the expression of the gene. Often, several other factors, such as hormones, have to be present for a protein to be expressed; therefore the absence of expression of the protein does not necessarily mean that the gene itself is not present. In order to confirm our findings, further experiments with testosterone implants should be conducted. Testosterone implants should be implanted under the skin 10 days before sacrificing in order to show that the expression of Kdm5d is both Y chromosome linked and hormonally dependent.

Conclusions

Immunohistchemical coloring with Anti-Kdm5d antibodies has shown positive results only in intact WT male mice. Our results show that the expression of Kdm5d is sex chromosome linked, but just the presence of Y chromosome is not enough for the expression of this protein. It can be assumed that testosterone is essential for the full expression of the protein encoded by this gene. Further investigations on SF-1 KO mice with testosterone implants are required to confirm the findings of this research.

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