IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN: 2278-3008, p-ISSN:2319-7676. Volume 9, Issue 5 Ver. V (Sep -Oct. 2014), PP 14-18
www.iosrjournals.org

Effect of Melanocortin 3 receptor (MC_3R) on Adult male mouse reproductive capacity

Eluwole O.O[1] Titilayo O.E[1] And Arinola O.T[2]
Department Of Medicalmedical Laboratory Sciences, Acheiver's University Owo, Ondo State1
Funbell Medical Dianostics Egbeda ,Akowojo .Lagos State Nigeria

Abstract: *Breeding difficulties have been noted in the Melanocortin 3 receptor(MC_3R) knockout mouse. The melanocortin 3 receptor(MC_3R) is a 7-transmembrane G- protein coupled receptor which signals through the activation of adenylase cyclise andits its characterised by possession of similar binding affinities for α-, β-, γ-MSH, and ACTH. Breeding difficlties observed in MC_3R knockout mouse was investigated through examination of the semniferous tubules of the mouse testis and detection of 3βHSD which is a known marker of Leydig cell of semniferous tubules. Leydig cells was known to perform vital endocrine functions in the adult mice testis by secretion of testosterone, a male sex hormone that affects reproductive capacity.In this study, Haematoxylin and Eosin staining was used to examine the morphology of the semniferous tubules in wild type and MC_3R Knockout mouse. Result shows morphological abnormalities exist in testicular histology of MC_3R knock out mouse when compared with the wild type. The diameter of the somniferous tubules and thickness of the cell layer lining the tubules were measured and compared, there were no any significant difference found (P value for diameter and thickness of the semniferous tubules was found to be 0.27 and 0.37 respectively. Immunoflourescence staining using green fluorescein goat anti 3βHSD antibody and Texas red rabbit anti MC_3R antibody was also performed on tissue section from wild type and MC_3R knockout mouse. Result shows significant staining for 3βHSD in wild type and to a lesser extent in MC3R knock out .No significant staining was observed for MC_3R in wildtype even though the conditions for immunostaining for were optimized. MC3R knock out mouse which also served as the negative control did not show staining for MC3R as expected.*

Abbrevation: *MC_3R: Melanocortin 3 receptor ,H and E: Haematoxylin and eosin, 3βHSD: 3β hydroxysteroid dehydrogenase, MSH: Melanocyte-stimulating hormone* ACTH: Adrenocorticotropic hormone.

Key words: *Melanocortin 3 receptor(MC_3R), semniferous tubules, Leydig cells, 3βHSD: 3β hydroxysteroid dehydrogenase*

I. Introduction

The melanocortin system regulates different forms of physiological functions .This include pigmentation, adrenocortical steroidogenesis, energy homeostasis, natriuresis, erectile responses, energy homeostasis,exocrine gland secretion anti-inflammatory and immunomodulatory properties (Cone, 2006) It is best known for its role in melanogenesis and regulation of energy homeostasis. The melanocortin 3 receptor(MC_3R) is a 7-transmembrane G- protein coupled receptor which signals through the activation of adenylase cyclise and characterised by possession of similar binding affinities for α-, β-, γ-MSH, and ACTH. The MC_3R has similar binding affinities to all melanocortin peptides and its activation can lead to signaling via cAMP as well as inositol–phospholipid pathways MC_3R is expressed in the CNS; for example within the hypothalamus, thalamus, hippocampus, anterior amygdale and cortex . It is detectable in the testis, placenta, stomach, duodenum, pancreas, ovary, mammary gland, skeletal muscle, and kidney (Gantz et al., 1993). Melanocortin 3 receptor is suggested to play a role in regulating cardiovascular functions and thermoregulation, as well as in the control of feeding behaviour (Santoro et al., 2007).It plays an important role in mediating some of the anti- inflammatory effects of MSH (Getting et al., 2003). Also, natural as well as synthetic agonists of MC_3R have also been shown to possess anti-inflammatory properties (Getting et al., 2001). Characterisation of MC3R in the mouse testis is important because MC_3R could be hypothesized to play a contributory role in immunological privilege nature of the testis through down regulation of the host immune system against the germ cells. This may then play a part in breeding difficulties observed in MC_3R Knockout mouse as against the wild type by Wotherspoon and colleagues(2008). At the moment melanocortin receptors on the fetal-type Leydig cells which persist into early neonatal life have been shown to directly mediate effects of adrenocorticotropic hormone (ACTH) on fetal/neonatal androgen production. ACTH stimulates androgen production by the fetal/neonatal mouse testis through the melanocortin type 2 receptor (MC_2R) (O′Shaugnessy et al., 2007).Therefore, the objectives of this research is to demonstrate the effect of MC_3R on the development and morphology and reproductive capacity of the adult mouse testis. This can be examined experimentally in the laboratory by demonstrating the histology of the wild type and MC_3R knock out mouse and the detection of 3βHSD which is a known marker of Leydig cell by immunoflourescent staining. Leydig

cells perform vital endocrine functions in the adult mouse testis by secretion of testosterone a male sex hormone that affects reproductive capacity.

II. Materials and Methods

This research work was carried out at Department of Medical Laboratory Science, Acheiver's University Owo, Ondo State, Nigeria. The procedures and methods of the study were as follow.

2.1) Tissue histology and Image analysis

Paraffin embedded tissue sections from 4 wild type (C57 B1.6) and 4 MC3R Knockout mice on a homogenous C57B16 background mouse at age 16 weeks were obtained from UCL research laboratory UK .

2.2) Haematoxylin and Eosin

Paraffin embedded testes sections (5µm thick) were dewaxed in xylene (BDH) and taken back to water by hydration in descending grade of alcohol. Sections were stained with Gills haematoxylin and eosin stain, dehydrated in ascending grades of alcohol, cleared in xylene(BDH) and mounted using DPX(Bios Europe).The sections were then examined using a Leica microscope. Image capture of two semniferous tubules per section was done. This was placed on Microsoft PowerPoint and the height was scaled.

2.3) Immunofluorescent

Paraffin embedded testes tissue section (5µm thick) was selected from two wild type and two MC_3R Knockout mouse and processed for immunoflourescent staining using 1/200 dilution of previously characterise anti MC_3R antibody and anti 3βHSD antibody in 0.1% Tx 100PBS as the primary antibody. This was incubated for 1hour at room temperature and overnight at 4 ºC(in fridge) after which 1/500 dilution of flourescent Texas red in 0.1%Tx 100 in PBS was used as the secondary antibody. It was mounted with vectasheild mounting medium and coverslip was sealed with nail vanish and cover with foil to avoid light induced fading. Image capture of the sections was done using Axiovert S100 flourescent microscope. Stain was further optimiised by reducing the dilution factor for anti MC_3R to 1/100

2.4) Statistical analyses

For each cross section of a semniferous tubules the diameter of the seminiferous tubules and the thickness were measured in two different places. The mean of mean for each animals (Wild type and MC_3R knockout) was taken and compared using Microsoft excel unpaired t-test (P values of <0.05 were considered to be significant).

III. Result

Fig 1a is H&E stained cross section of wild mouse testicular tissue, showing how the diameter of the somniferous tubules and the thickness was measured since the diameter and the internal thickness of the semniferous tubules is a reflection of the level and functionality of the germ cell which are maintain by the testosterone (Kennet et al., 1992)

Fig2a is a cross section of an MC_3R Knock out semniferous tubules demonstrate apparent loss of the sertoli and the germ cells, large vacuolation and reduce thickness in cell layer lining of the semniferous tubules. This is not observed in the wild type in fig 2(b)

3.1) Localization of MC3R and 3βHSD

3βHSD was immunolocalized in the cell of the interstitial tissue in both the wild type(fig 3b) and MC_3R knock out mice(fig3d) . The MC_3R knock out mouse show no staining for MC_3R which is appropriate for a negative control. However, MC_3R in the wildtype testis could not immunolocalized even though the stains was optimised

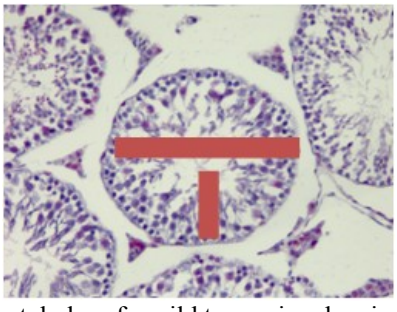


Fig 1a- Cross section of semniferous tubules of a wild type mice showing the measurement of thickness and diameter of the semniferous tubules.

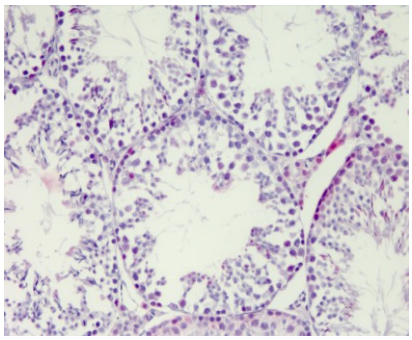


Fig-2a- Cross section of semniferous tubules of MC_3R Knockout mice showing morphological abnormalities.

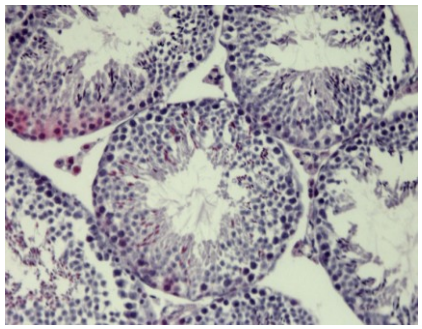


Fig2b Cross section of semniferous tubules of wild type mice showing the normal morphology.

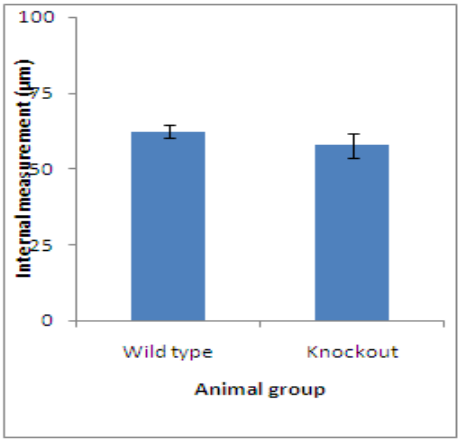


Fig 3a- Graphical comparison of the internal diameters of semniferous tubules in MC_3R knock out and wild type mice

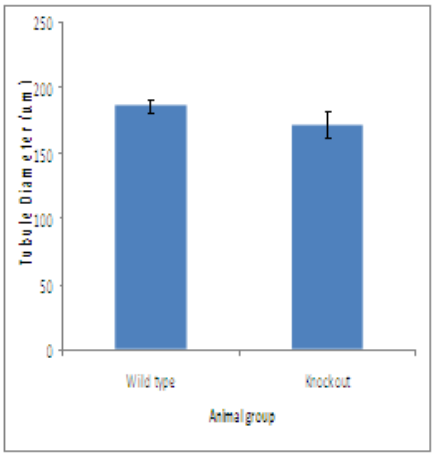


Fig3b-Comparison of the tubular diameter of semniferous tubules in MC_3R knock out and wildtype mice.

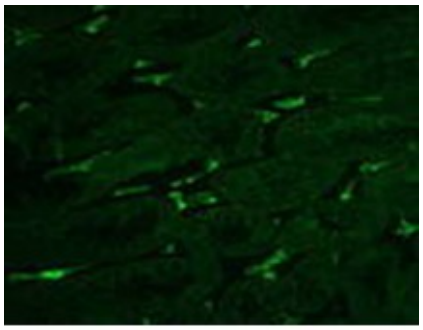
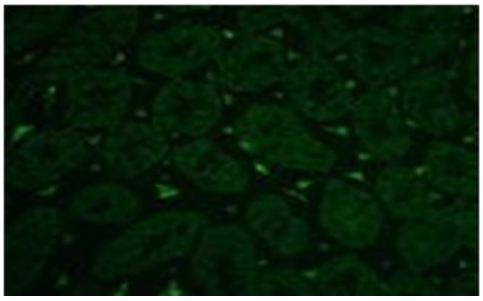


Fig 4 a Immunoflourescence staining for 3βHSD in wild type mice testis.

B- Immunoflourescence staining for MC_3R in wild type mice testis.



C- Immunoflourescence staining for 3βHSD in MC_3R Knock out mice testis.

D- Immunoflourescence staining for MC_3R in MC_3R Knock out mice testis

IV. Discussion/Conclusion

The melanocortin system is made up of the melanocortin peptides , five melanocortin receptors, two endogenous antagonists (agouti and agouti-related protein) and two ancillary proteins(mahogany and syndecan-3) (Gantz et al., 2003). The melanocortins are a family of bioactive peptides which are product of

proopiomelanocortin gene.They are synthesized in the central nervous system and various peripheral organs where they participate in various physiological functions (Abdel-Malek, 2001).

There are 5 known melanocortin receptors. Their functions are modulated by the endogenous peptides α-, β-, and γ-melanocyte stimulating hormone (α- β- and γ-MSH) and adrenocorticotropic hormone (ACTH). In addition, the endogenous antagonists agouti and agouti-related protein (AGRP) also regulate the receptors (www.palatin.com accessed July 2nd 2010

The germinal epithelium of the seminiferous tubules consists of cells which are made up of developmental stages of germ cells, namely spermatogonia, primary and secondary spermatocytes and spermatids located within the invaginations of Sertoli cells (Holstein et al., 2003)**.** Thus, the diameter and the internal thickness of the semniferous tubules is a reflection of the level and functionality of the germ cell which are maintain by the testosterone (Kennet et al., 1992)

In this study we evaluate the effect of MC_3R on adult mouse reproductive capacity through the analysis of Leydig cell function. Light microscopy, using H&E staining techniques demostrate variation in thickness and diameter of semniferous tubules between wild type and MC_3R knockout mouse.Therefore, morphological abnormality observed in the MC_3R knockout as compared with the wild type could be hypothesized to have been responsible for the breeding difficulties observed in the knock out mouse. However, although abnormalities were observed there were no statistically significant differences in measurements of the diameter and thickness of the cell layer lining the semniferous tubules of the two mice strains. The P value for diameter and thickness of the semniferous tubules was found to be 0.27 and 0.37 respectively. Further investigation should involve selection of morphological normal and abnormal somniferous tubules in both the wild type and the MC_3R knock out and comparing their diameters and thickness, measurement and comparison of the volume of the interstitial tissue and empty spaces within the somniferous tubules.

The melanocortin 3 receptor(MC_3R) was said to be expressed in peripheral organ such as the testis but its role is not very clear at the moment except that it has been observed that Mc3R knockout mouse exhibit difficulty in breeding (Wotherspoon et al., 2008).Therefore, Immunoflourescence staining was done to for evaluate known markers of testicular steriodogenesis in both the wild and MC_3R knock out mouse.This was done in the laboratory by localizing 3βHSD a marker of testicular steriodogenesis on testis tissue section from both wild and MC_3R knock out mouse

The result shows the presence of 3βHSD in the both the wild type and MC_3R knock although to a lesser extent in the latter. The MC_3R knock out mouse show no staining for MC_3R which is appropriate for a negative control .However, MC_3R did not show significant staining in the testis of wild type mouse even though immunostaining was optimized. This observation is against the discovery of Wotherspoon and colleagues(2008) although O'Shaughnessy et al., (2003) has previously reported that MC_3R is not expressed in the testes of adult mouse.

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