Exploring the vomeronasal organ transcriptome in Rasa Aragonesa rams with different sexual behaviour

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Abstract

The vomeronasal organ (VNO) has long been of interest regarding the sensory control of social and sexual behaviour. In this study, high-throughput RNA sequencing was utilized to investigate transcriptional changes in the VNO between rams of Rasa Aragonesa with different sexual behaviour (active and not active). The RNA-Seq analysis generated 812,065,820 clean reads and identified six differentially expressed genes, of which *GFRA3*, *CEACAM*, and *LRIT3* were upregulated, whereas *SERPINB12, TP53INP2* and *CES4A* genes were downregulated in the active compared to the not active rams group. The Gene Set Enrichment Analysis (GSEA) of all the expressed genes in the VNO detected 128 overrepresented pathways at FDR 5%, predominantly related to biological process such as positive regulation of protein phosphorylation and cellular response to growth factor stimulus pathways. Studying these genes and the processes they control will improve our understanding of the genomic regulation of sexual behaviour in rams.

Introduction

In small ruminants, the vomeronasal organ (VNO) is morphologically constructed for the detection of pheromones, which are related to social and reproductive behavioural responses (Villamayor et al., 2021). The sensory information interferes with the nervous control of gonadotropin secretion (Signoret, 1991). These chemosensory signals stimulate male sexual arousal and behaviour (Keverne, 2004). In fact, Ungerfeld et al. (2006) pointed out that the perception of pheromones by the VNO stimulates the sexual performance of rams, which determine a faster response to the stimulus of oestrous ewes. In addition, mountings and ejaculations increased without the need of more courtship behaviour. However, the blockage or removal of the VNO leads to a reduction of number of mounts and services in bulls (McGrath, 1981) and a depression of sexual activity in male rats (Saito and Moltz, 1986). In sheep, the ram effect is commonly used to improve the out of season reproduction. It has been observed that males with greater sexual behaviour produce a greater stimulus during seasonal anoestrous, which lead into a higher percentage of mated ewes and higher fertility. However, there is a considerable variation in sexual behaviour between rams, hypothesizing that VNO features may differ not only among species but also within the same species at mating (Villamayor et al., 2021). In this study, we carried out an RNA sequencing approach to investigate transcriptional changes in the VNO by comparing two groups of Rasa Aragonesa rams with different sexual behaviour (active and not active).

Materials & Methods

Ethics statement. All experimental procedures including care of animals and euthanasia were performed in accordance with the guidelines of the European Union regulations for the use and care of animals in research (Directive 2010/63/EU) and approved by the Animal Ethics Committee of the Research Centre (protocol number 2017/02).

Animals, tissues samples and RNA extraction. The experiment was conducted with 59 rams, submitted to individual sexual behavioural pen tests, twice for each ram. Each ram was exposed to three synchronized adult ewes for 20 minutes to observe their behaviour, and count the frequency of mounts and services. Three serial blood samples were taken one hour apart the day before, in lithium heparin tubes, to measure testosterone. In addition, individual live weight (LW) and body condition score (BCS) were assessed as well as testicular size. Two ram homogeneous groups in LW and BCS were identified after carrying out a decision tree using the package "party" in R project, considering all the variables for the mounts and services phenotypes: active (A) (7.93 ± 3.56, average mounts ± sd) and not active (NA; without any mount) rams. Then, six rams of each group were sacrificed. VNO tissue samples were extracted and stored at −80°C until RNA isolation by RNeasy Lipid Tissue mini kit (QIAGEN, Madrid, Spain).

RNA-Seq data processing. Sequencing was carried out generating Illumina paired-end reads of 151 bp. Quality control of sequences was checked by FastQC. The Trimmomatic program was used to remove adaptors, low quality reads and overrepresented sequences. Then, the trimmed reads were mapped to the reference genome Oar_rambouillet 1.0 (GCA_002742125.1) using STAR v2.7.8a and gene level quantification was estimated using HTSeq. Differential expression analysis were performed with EdgeR using the threshold of CPM =0.5 in at least five samples. Multiple-testing corrections by Benjamini and Hochberg false-discovery rate (FDR 5%) controlling procedure were performed. Genes with an adjusted p-value ≤ 0.05 were identified as DEGs. Enrichment Analysis were examined by GSEA. All the pipeline analysis was done using the OmicsBox platform v2.0.36 from BioBam (https://www.biobam.com/omicsbox).

Results

Active and non-active rams had similar LW and BCS, and no significant differences were found in testosterone levels or testicular size. RNA-seq data were obtained for VNO tissue samples of Rasa Aragonesa rams (N=12), ranging the number of total raw reads per sample from 60,537,894 to 83,449,630. After filtering by Trimmomatic, the total number of clean reads ranged from 41,240,636 to 78,958,466. The reads uniquely mapped by STAR to the reference genome varied from 55.4% to 84.5% among samples. These findings indicated good quality data that were suitable for subsequent analysis. After counting the reads mapped in each gene by HTseq, we next investigated the differences in gene expression data between active and not active rams by EdgeR software. As a result, 15,227 genes were expressed in the VNO. EdgeR identified six DEGs at FDR 5%. The *GFRA3*, *CEACAM* and *LRIT3* were up-regulated in the active rams whereas the *SERPINB12*, *TP53INP2* and *CES4A* were found down-regulated (Table 1). Gene set enrichment analysis included the 15,227 expressed genes. We detected 128 overrepresented pathways at FDR q-value 5%, predominantly related to biological process (BP) and molecular function (MF). The top 30 GO are shown in Figure 1.

Table 1. Differentially expressed genes between the active and not active rams in the vomeronasal organ.

Gene ID	Gene name	logFC[1]	logCPM[2]	P-Value	FDR[3]
ENSOARG00020014530	*GFRA3*	1.3	3.40	4.46E-06	0.03
ENSOARG00020022556	*CEACAM*	2.8	0.92	7.65E-06	0.03
ENSOARG00020023342	*LRIT3*	2.4	-0.03	7.64E-06	0.03
ENSOARG00020025883	*SERPINB12*	-2.2	0.04	9.69E-06	0.03
ENSOARG00020013838	*TP53INP2*	-1.6	1.78	2.09E-05	0.05
ENSOARG00020018190	*CES4A*	-1.7	0.91	2.02E-05	0.05

[1]Log2 fold changes [2]The average log2 counts per millions[3]False Discovery Rate

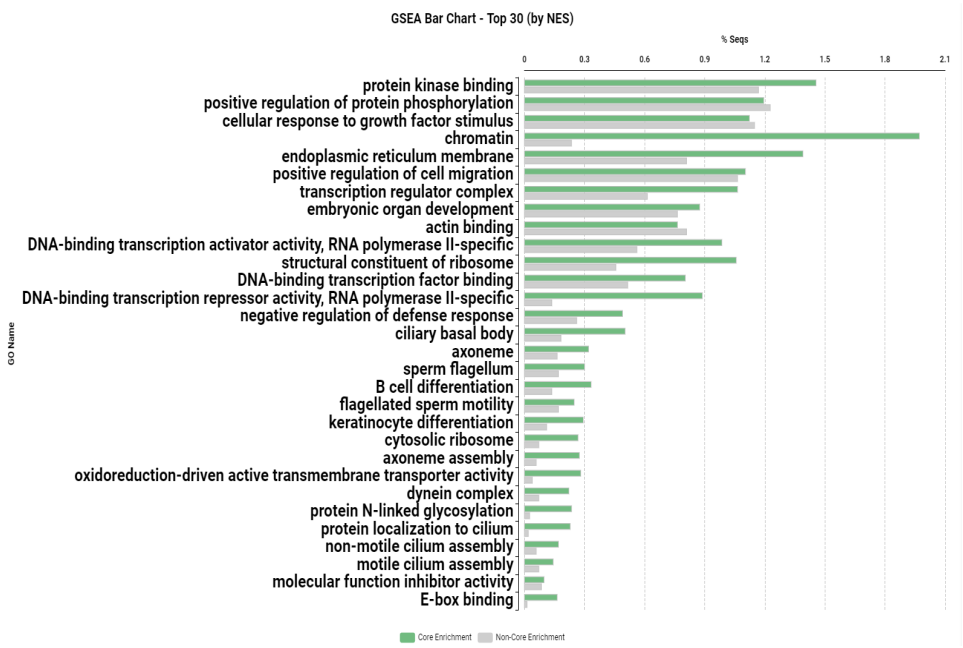

Figure 1. Top 30 GO between the A vs NA comparison in the VNO.

Discussion

The principal goal of this study was to determine the possible molecular mechanisms underlying the sexual behaviour of Rasa Aragonesa rams by investigating transcriptional changes in the VNO between active and not active rams. The analysis revealed fewer DEGs, suggesting little difference in the VNO. The three up-regulated DEGs identified belong to signal term and seems to affect the sexual behaviour via network interaction with other genes. In fact, *GFRA3* correspond to the glial-cell line-derived neurotrophic factor Family Receptor Alpha 3 (GDNF), among its related pathways are RET signaling. GDNF is recognized to regulate the development of small-diameter nociceptors and may be involved in modulating mechanosensation (Albers et al., 2006). Low expression of this gene deteriorates reproductive potential via accelerated neurodegeneration (Vasiliev et al., 2021). However, GFRA3 aberrant expression can increase human reproductive potential because of improvements in neural regeneration (Jankowski et al., 2010). For the *CEACAM* gene, Gu et al., (2020) reported that CEACAM proteins disrupt transforming growth factor beta (TGFB) signaling pathway. Kim et al. (2013) indicated that *CEACAM* was one of the de-repressed gene families in the brain of *Peg3* heterozygous embryos and adult mice. This gene was reported to play important roles in controlling foetal growth rates and sexual behaviour in males and females. Swaney et al. (2008) indicated that the *Peg3* regulates sexual experience dependent preferences for oestrous

odours. This is to say that mutation in *Peg3* could affect the expression of *CEACAM* gene family, which might result in a defect in reproduction or ability for mating. Similarly, *LRIT3* was reported as a modulator of Fibroblast Growth Factor Receptor 1 (*FGFR1*) that is essential for the development of the gonadotropin-releasing hormone (GnRH) system (Tata et al., 2012). Furthermore, the GSEA identified 128 overrepresented pathways, predominantly related to BP such as positive regulation of protein phosphorylation pathway, involved in the developing male and female rat brain as well as normal endocrine and behavioural processes in adulthood (Auger, 2003). Another pathway of interest was related to MF such as the protein kinase-binding pathway whose signaling pathways have been implicated in the estradiol regulation of sexual receptivity (Dewing et al., 2008). These findings encourage deeper investigation towards the importance of these genes and their associated mechanisms to understand better the genomic regulation of sexual behaviour in Rasa Aragonesa rams.

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