Age influences aggressive behaviour: an RNA-seq analysis in bovines

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Abstract

Aggressiveness is one of our most basic behaviors, characterized by targeted intentional actions oriented to cause harm or damage. The reactive type of aggression is regulated mainly in the brain prefrontal cortex; however, the molecular changes underlying aggressiveness in adults have not been fully characterized. Here we used a RNA-seq approach to investigate differential gene expression in the prefrontal cortex of bovines from the aggressive Lidia breed at different age stages: young three-year old and adult four-year-old bulls. The number of up and down-regulated genes in the adult group were 50 and 193, respectively. These genes were associated with diverse functions, including neural plasticity and behavior. A cross-species comparative analysis retrieved 29 genes in common with previous studies on aggressive behaviors. Particularly, we

detected changes in the regulation of networks such as the synaptogenesis, involved in the synapses maintenance and refinement, and the glutamate receptor pathway, which acts as excitatory driver in aggression responses. Furthermore, the Wnt//β- catenin signaling pathway was present in both up and down-regulated genes in the adult group, and has been implicated in adult altered behaviors and cognitive disorders. At the regulatory level, we identified four upstream regulators, all involved in behavioral processes and neuropsychiatric disorders including aggressive demonstrations. Our results on an animal model in which the aging process enhance the intensity of aggressive responses provide, for the first time, insights into candidate genes and networks involved in the molecular mechanisms leading to the maturation of the brain, with a focus on cattle agonistic components.

Introduction

Aggression in animals, including humans, serves important purposes in securing mates, territory and food and, therefore, is one of our most basic behaviors. We understand aggression as an overt behavior directed at an object or a subject with an intention to cause harm or damage (Gannon et al. 2009). This definition is assumed to be a heterogeneous and multifaceted construct, where the actor ultimate intention makes to get a distinction between two different subtypes of aggression, reactive and proactive (Smeets et al. 2017). Reactive aggression is the impulsive or “hot blooded” response to provocations, frustrations or threats from the environment. Proactive aggression, also called “cold-blooded”, is conscious and goal-directed, planned with a specific goal (e.g. resources). While both subtypes can co-occur, different neurobiological structures have been suggested to underlie reactive and proactive aggressions (Blair, 2013).

Animal studies have shown that reactive aggression is mediated by the limbic system, through a circuit that runs from the amygdala to the hypothalamus, and from there to the periaqueductal grey. This circuitry is assumed to be regulated by frontal cortical regions, particularly by the prefrontal cortex (PFC) and the anterior cingulate cortex (Blair, 2013). There is strong evidence that reactive aggression often appears earlier in life than proactive aggression, and that heightened levels of reactive aggression at early stages are associated with increased levels of anxiety in adulthood. In humans, childhood reactive aggression can be found in different neuropsychiatric disorders including attention-deficit/hyperactivity disorder, oppositional defiant disorder or conduct disorder during their adult life (Fite et al. 2010). Therefore, a comparison of gene expression profiles in PFC tissues at different age stages may be relevant to reveal some of the molecular characteristics and mechanisms involved in aggressive-related perturbations that characterize early stages of aggression and its progression into adulthood.

The Lidia cattle breed is a promising and natural animal model for studying reactive aggression, given that its agonistic responses have been exacerbated through long-term selection of aggressive behaviors. The Lidia is a primitive breed with its origins in the Iberian Peninsula, where these bovines have been raised for centuries with the exclusive purpose of taking part in socio-cultural and anachronistic events grouped under the term of "*tauromaquias*" (Eusebi et al. 2018). At these festivities, male bovines are placed on a bullring in solitary with a human subject, resulting in a series of aggressive reactions that measures the animal´s fighting capacities by means of reactive responses, such as fighting, chasing and ramming. Different types of *tauromaquia* take place at the bullrings, perhaps the most popular are those known as major festivities: the "*corrida*" and the "*novillada*" (Domecq, 2009). The difference between these two events is the age of the bovines. In a "*corrida*", the protagonists are 4-year-old bulls, while a

"*novillada*" uses 3-year-old bovines (Maudet, 2010) (Maudet, 2010). This age discrimination between events is not random; 3-year-old animals are smaller, less strong and their reactive responses tend to be less explosive and intense than those of 4 year-old bovines.

Our previous studies using Lidia bovines as an aggressive-selected model revealed large changes on their PFC genetic expression in comparison with tamed Wagyu bovines, a meat production breed known by their docile-temperament, providing insights into potential molecular components implicated in structural and functional processes that may work together in the cortical expression of aggressiveness (Eusebi et al. 2020). Furthermore, genome scans seeking selection signatures in the Lidia cattle breed have uncovered the existence of several genes strongly associated with aggressive behaviors and pathologic conditions previously reported in humans and other animal models (Eusebi et al. 2019). Particularly, we have associated a novel polymorphism in the promoter of the monoamone oxidase A (*MAOA*) with different levels of aggressiveness among cattle breeds, a gene that has been clearly linked to aggression in humans (Brunner et al. 1993; Eusebi et al. 2019).

Although studies characterizing gene expression differences between juveniles and adults at PFC have shown promising results in mice (Agoglia et al. 2017;, Lander et al. 2017), to our knowledge, there is a lack of such studies using cattle as a model. Thus, the aim of the present study is to explore the differences in gene expression at two age stages of Lidia cattle, "young" three-year-old and "adult" four-year-old bovines, given the marked differences in the intensity of their agonistic responses. For this purpose, gene expression profiles from the PFC tissue of eight adult and eight young Lidia bovines were compared. The results of the present study will give insights into the molecular modulation involved in the complex mechanisms leading to the maturation of the brain, with a focus on aggressive behaviors.

Material and Methods

Ethics statement

No special permits were required to conduct the research. All animals were sacrificed for reasons other than their participation in this study. Harvesting of bovines´ brains at the cutting room followed standard procedures approved by the Spanish legislation applied to abattoirs (B.O.E., 1997).

Animals

Post mortem PFC tissue samples were retrieved from 16 non-castrated bovine males of the Lidia breed, eight three-year-old and eight four-year-old, for the young and adult groups respectively. In standard RNA-seq studies, this level of replication is 2.6-fold higher than the minimum required (3 individuals/group). Similarly to a classic resident-intruder laboratory test, where an unfamiliar animal (intruder) is placed in the territory of another animal (resident) resulting frequently in an aggressive conflict (Koolhas et al. 2013), each bovine was placed in a bullring and incited to develop reactive aggressive responses for approximately thirty minutes prior to its sacrifice, to measure their fighting abilities (Domecq, 2009). Samples of PFC were collected less than an hour post-mortem and immediately submerged in RNA-later™ (Thermo Fisher Scientific, Madrid, Spain), followed by 24 hours' storage at 5°C and long-term conservation at -80°C.

RNA isolation and sequencing

Total RNA was extracted from PFC samples using the RNeasy Lipid Tissue Mini Kit (QIAGEN, Madrid, Spain), and total RNA concentration and purity were assessed with a Nanodrop ND-1000 spectophotometer (Thermo Fisher Scientific, Spain). To guarantee its preservation, RNA samples were treated with RNAstable (Sigma-Aldrich, Spain), and shipped at ambient temperature to DNA-link Inc. sequencing laboratory (Seoul, Korea) to perform high throughput sequencing using a Novaseq 6000 sequencer (Illumina, San Diego, CA, USA). For quality check, the OD 260/280 ratio was determined to be between 1.87 and 2.0. All these procedures were conducted according to the respective manufacturers´ protocols.

Individual libraries for each of the analyzed bovines (*N*=16) were processed using the TruSeq Stranded mRNA Preparation kit (Illumina, San Diego, CA, USA) according to manufacturer´s instructions. Each library was pair-end sequenced (2x100 bp). Individual reads were de-multiplexed using the CASAVA pipeline (Illumina v1.8.2), obtaining the FASTQ files used for downstream bioinformatics analysis. Illumina reads generated from all samples have been deposited in the NCBI GEO / bioproject browser database (Accession Number: GSE149676).

Bioinformatics analyses

Quality control and pre-processing of genomic datasets was carried out with the PRINSEQ v. 0.20.4 software (Schmeider & Edwards, 2011). To improve the downstream analysis, low quality (Q < 20) and ambiguous bases (N) were first trimmed from both ends of the reads and, then, the trimmed reads were filtered by a Phred quality score (Q ≥ 20 for all bases) and read length of ≥ 68 bp. The obtained FASTQ files were mapped to the bovine reference genome (Bos taurus ARS.UCD 1.2) with the STAR Alignment v.2.7.3a software (Dobin et al., 2013), using default parameters for pair-end reads and including the Ensembl *Bos taurus* ARS-UCD 1.2 as

reference annotation. To characterize PFC gene expression in each sample, we used the FeatureCounts tool (Liao et al. 2014). FeatureCounts assigns reads to features by comparing the genomic location of each base in the read with the chromosomal region empompased by each figure.

Once reads were mapped, the expression of each mRNA and their relative abundance in fragments per kilobase of exon per million fragments mapped (FKPM) were measured with Cufflinks v.2.2.1 (Trapnell et al. 2010). Only expressed transcripts with FKPM values < 0 were used for downstream gene expression analyses, leaving a total of 14 animals (eight young and six adults) (Supplementary Figure 1).

The analysis of differential gene expression between young and adult groups was performed using Cufflinks software (Trapnell et al. 2010). the assembled transcripts from all samples were merged using the Cuffmerge command. Cuffdiff was then used applying default parameters and the option "fr-firststrand" to define pair-end reads. A Benjamini-Hochberg False Discovery Rate (FDR), which defines the significance of the Cuffdiff output, was set as threshold for statistical significant values of the Differentially Expressed Genes (DEG). The results of the Cufdiff RNA-seq analysis were visualized with the R software application CummeRbund v.2.28.0 (Goff et al., 2012).

Additionally, we performed a complementary analysis to avoid the risk of statistical artifacts that Cufdiff may produce when managing limited fold change variations. We used EdgeR software, (Robinson et al., 2010), which uses an exact test with a negative binomial distribution for differential expression testing.

To study the relationships between differences in PFC gene expression among groups and its biological function, we separated the results of DEG in two independent gene-lists according to

their Log_2 Fold Change (FC) obtained with Cufdiff, in the adult group: up-regulated for those transcripts displaying a $Log_2FC \geq 0.1$; and down-regulated for those with a $Log_2FC \leq 0.1$.

Cross Species Comparative Analysis (CSCA)

We conducted a comparison between our DEG and a compendium of genes associated with aggressiveness and previously identified in different genomic studies in humans, rodents, foxes, dogs and bovines, as proposed by Zhang-James et al. (Zhang-James et al., 2019). This list of genes is based on four categories of genomic evidence: a) genome wide association studies (GWAS) in humans of different age groups, children and adults (Ferbández del Castillo & Cormand, 2016); b) genes showing selection signatures previously identified in the Lidia breed (Eusebi et al., 2018; Eusebi et al., 2019); c) RNA-seq studies in rodents (Clinton et al., 2011; Malki et al. 2014) and silver foxes (Kukekova et al.,2011; Kukekova et al., 2018); and d) genes identified in studies with causal evidence of the Online Mendelian enheritance in Man (OMIM) and the Online Mendelian Inheritance in Animals (OMIA) databases, and a report of knockout mice (KO) (Våge et al., 2010; Veroude et al., 2016; Zhang-James et al., 2019). The gene-list and details of the different studies are described in Supplementary Table 1. Bovine official gene names were converted to its human orthologues using biomaRt (Durinck et al. 2005) in order to homogenize our DEG with the cross-species gene list. Finally, we assigned a weight value (weighted ranking, WR) with the same conditions proposed by Zhang-James et al. (2019).

Ingenuity pathway analysis

Up and down regulated DEG lists were imported into the Ingenuity Pathway Analysis (IPA) (QIAGEN, www.qiagen.com/ingenuity) software to assess Gene Ontologies (GOs) and canonical pathways enrichment scores. Additionally, up and down-regulated DEGs in common

with the CSCA were imported together to build biological networks and identify upstream regulators. Thus, we ran three Core Analyses with the following criteria: genes and endogenous chemicals, direct and indirect interactions, and 35 molecules per network and 25 networks per analysis.

For the canonical pathway analyses, a right-tailed Fisher's exact test was performed for the enrichment of the DEGs in IPA´s hand-curated canonical pathway database. Here, the P-value calculated for a pathway measures the probability of being randomly selected from all of the curated pathways. To control the error rate in the analysis, IPA also provide the corrected P-values to identify the most significant results in IPA's canonical pathways based on the Benjamini-Hochberg method (Benjamini & Hochberg, 1995). This tool allowed us to identify the signaling pathways in which the DEGs were enriched. We used the predetermined cut-off of the corrected P-value < 0.05 to define the significant pathways.

For network enrichment and detection of upstream regulators, the genes in common with the CSCA were overlaid onto a global molecular network (GMN) developed based on the Ingenuity Pathways Knowledge Base, in which functional relationships such as activation, chemical-protein interaction, expression, inhibition and regulation of binding were manually searched. Sub-networks of genes were then extracted from the GMN based on their connectivity using the algorithm developed by IPA. The sub-networks were visualized using IPA´s Path Designer tool.

Results

Sequencing and read assembly

For the 16 individually sequenced samples, we generated an average of 61.6 million pairs of 100-bp paired-end reads per sample after filtering by quality score. For each sample, an average

of 91.6% reads were uniquely mapped to the bovine reference genome, ranging from 86.8 to 95.2% (Supplementary Table 2). The transcripts expressed in both the young and adult groups reaching FKPM values < 0 (*N*=8 young and *N*=6 adult) were included in the differential expression and subsequent IPA analyses (Supplementary Figure 1).

Gene expression differences revealed a total of 27,153 and 16,384 DEGs between young and adult groups with Cufflinks and EdgeR programmes respectively. Of those genes, only 243 were statistically significant; 50 up-regulated ($\log_2 FC \geq 0.1$) and 193 down-regulated ($\log_2 FC \geq -0.1$) (Table 1 and Figure 1A and B). For the detailed list of significant and not significant up and down-regulated DEG and FPKM values obtained with Cufflinks sotfare see Supplementary Tables 3 and 4, respectively. Furthermore, the biological variation of DEGs per group was explored and, as expected, both the young and adult groups displayed similar values (Figure 1C).

Genes in common with the cross-species comparative analysis (CSCA)

The cross-reference analysis between our up and down-regulated DEGs in the adult group and the gene-list associated with aggressive behavior, overlapped numerous loci. Notably, the down-regulated DEGs shared 21 genes, while the up-regulated displayed only 8 genes in common with the gene reference list (Table 2). The subset of DEGs included in the CSCA are shown in the expression bar plot of Figure 2 in relation to their standard deviation of FPKM values.

Ontological analysis of the differentially expressed genes (DEG)

The up and down regulated DEGs in the 4-year-old adult group were subject to a Fisher's exact test within IPA to obtain Ingenuity ontology annotations. IPA annotations follow the GO annotation principle, but are based on a proprietary knowledge base of over a million protein-

protein interactions. Significant results are summarized in Supplementary table 5. The most relevant results for the up-regulated DEGs were obtained under the *physiological system development* and the *disease and disorders* categories. Within the first one, the top of the list gathered terms related with *behavior* (highest p-value range of 5.51E-08 and 15 DEG) and *nervous system development and function* (highest p-value range of 7.30E-07 and 24 DEG), while within the category of *diseases*, terms related to *cancer* (highest p-value range of 1.21E-07 and 44 DEGs), *neurological diseases* (highest p-value range of 8.07E-07 and 21 DEGs) and *psychological disorders* (highest p-value range of 3.35E-06 and 12 DEGs) occupied the top positions.

Additionally, we explored the overrepresentation of specific enriched functions within the category of *behavior* and its relationship with diseases or function annotations, finding high overall significance levels of association between up-regulated DEGs and diverse behavioral conditions (p-values $< 10^{-5}$) (Table 3, Figure 3).

The Core Analysis of the down-regulated DEGs in the 4-year-old group revealed also genes related to the *physiological system development* and the *disease and disorders* categories. The top enriched genes in the first category were associated with *organismal* (highest p-value range of 2.38E-17 and 63 DEGs), *embryonic* (highest p-value range of 2.38E-17 and 58 DEGs) and *cardiovascular system* (highest p-value range of 1.08E-16 and 43 DEGs) development functions. Furthermore, at the *diseases and disorders* category the down-regulated DEGs were shown to be involved with *cardiovascular disease* (highest p-value range of 3.05E-11 and 34 DEGs), *cancer* (highest p-value range of 1.73E-10 and 84 DEGs), and *organismal injury and abnormalities* (highest p-value range of 1.73E-10 and 84 DEGs) (Supplementary Table 4).

Metabolic pathways affected by the up and down-regulated DEGs in the adult group

Within the up-regulated DEGs in the adult 4-years-old group, four pathways surpassed the significance threshold (p-values < 0.05, Supplementary Figure 2). The *synaptogenesis* and the *Wnt/β- catenin signaling* are among the most significant pathways (p-value = 3.08E-03). Besides, the *glutamate receptor pathway*, which determines the uptake of this primary excitatory neurotransmitter at the central nervous system (CNS), resulted also highly significant (p-value = 5.74E-03).

The analysis of down-regulated pathways revealed the involvement of the retrieved DEGs on 53 metabolic routes (Supplementary Table 6). Amongst the most significant, the *intrinsic prothtrombin activation pathway* (p-value =1-08E-08) and, noteworthy, again the *Wnt/β- catenin signaling* (p-values = 4.76E-05) route, are highlighted within the down-regulated DEGs in the adult group (Supplementary Figure 2).

Gene networks and upstream activator analyses of the genes in common with the CSCA

We analyzed the gene transactivation networks and upstream activators of the 29 DEGs in common with the CSCA, finding three highly interconnected networks related to organ morphology, organismal development, behavior, cancer, neurological disease and psychological disorders (Figure 4).

Finally, the upstream analysis tool of the IPA package was employed to identify potential upstream regulators that may explain the differential patterns of expression between young and adult groups. We explored the activation/inhibition states of the top upstream regulators by using IPA´s activation z-score tool, identifying four main upstream regulators: KRAS proto-oncogene (*KRAS),* Brain Derived Neurotrophic Factor *(BNDF),* CAMP Responsive Element Binding

Protein 1 *(CREB1)* and SRY-Box Transcription Factor 2 *(SOX2).* As shown in the graphic network in Figure 5, these genes are involved in an array of diverse biological functions related with behavioral development.

Discussion

The structural and molecular changes occurring in the PFC of diverse species at different age stages have been previously explored (Counotte et al., 2010; Moczulska et al., 2014; González-Lozano et al., 2016; ,Laeremans et al., 2017). The majority of these analyzes conducted in rodents focus on the study of the molecular changes occurring in post-natal and puberty-adolescence stages. However, studies on the specific changes of genetic expression during adulthood are still scarce (Agoglia et al., 2017; Lander et al., 201712). In the present study, we used RNA-seq to identify genes and gene networks that show differential expression in the PFC of adult bulls when compared to young bovines of the aggressive Lidia breed.

A total of 143 significant DEGs, 50 up and 193 down-regulated in the adult group were identified. The GO analysis of up-regulated DEGs in the adult group rendered highly significant results for behavioral and nervous system development features, while the down-regulated genes were mostly implicated in systemic developmental functions (organismal, embryonic and cardiovascular system). We detected a modest coincidence between these results and what has been published in previous analysis using high-throughput molecular data in PFC. For instance, Agoglia et al. (2017) used an unbiased proteomics approach to analyze the differential expression of proteins in adolescent compared to adult PFC in mouse. There is a concordance between the proportion of up and down-regulated DEGs in the adult groups reported by both us and Agoglia et al. (2017), as well as results from the GO analyses showing down-regulation of

organismal growth and development processes in adults. This means that both technologies, proteomics and RNA-seq are, as awaited, correlated, detecting similar sets of DE molecules (Kogenaru et al., 2012).

Canonical pathway analyses revealed also that metabolic routes involved in cellular signaling and neurotransmission impact in the adult PFC during the aging process (Figure 2). We detected an over representation of the synaptogenesis and the glutamate signaling reception pathways in the four-year-old bulls, both involved in brain´s synaptic plasticity; the synaptogenesis pathway is implicated in developmental processes of formation, maintenance and refinement of synapses (Cohen-Cory, 2002), while the glutamate signaling receptor acts as excitatory driver of the Hypothalamus-Pituitary-Adrenal (HPA) system, a complex system that triggers reactive aggression responses (Herman et al., 2004; Evanson & Herman 2015). Similar results were obtained by Lander et al. (2017); they detected an over expression in adults of glutamate markers comparing the cortical mRNA expression of mid-adolescent and adult mice.

Interestingly, we detected differences in the expression of genes included in the Wnt//β- catenin signaling pathway in both up and down-regulated DEGs in the adult group. The complex interactions of genes, with actors triggering the expression of the Wnt//β- catenin signaling pathway while others are needed to silence routes leading to its suppression, justifies the inclusion of genes from this network in the up and down-regulated cohorts. This pathway is considered one of the most strongly conserved routes among species and is involved in synaptic transmission activities (Maguschak et al., 2012). Malfunction of many components of the Wnt//β- catenin signaling pathway in the adult brain can result in altered behavior and cognitive disorders (Svenningsson et al., 2002).

In addition, the prothrombrin activation pathway acts as signaling route at the CNS, inducing the decrease of cyclic AMP (CAMP) and the activation of protein kinase C (PKC), both enzymes linked to catecholamine and serotonin receptors (David et al., 2004). It has been proposed that, during encounters between individuals, a de-synchronization of the top-down control at these receptors influence a lack of control of emotional reactivity (Kretschmer et al., 2014). As suggested by Egoglia et al. (2017), the increased expression on these routes observed in the 4-year-old PFC may contribute to the fine-tuning of synaptic transmission, resulting in a superior control of the PFC executive functioning in mature brains, intensifying the addressed aggressive behaviors.

Complementarily to the above-mentioned results, the IPA network tool applied to the 29 DEGs in common with the CSCA, retrieved three inter-connected networks associated with organismal morphology and development, behavior, cancer, neurological disease and psychological disorders (Figure 3). Particularly, the Mitogen Activated Protein Kinase (*MAPK*) and two Extracellular Signal Regulated Kinases (*ER*K) appear to be involved in diverse molecular mechanisms underlying aggression. Similar results were obtained in previous studies on PFC gene expression (Zhang-James et al., 2019; Eusebi et al., 2020), identifying a differential expression of the ERK/MAPK signaling pathway in their comparisons between aggressive and non-aggressive individuals.

At the regulatory level, three of the four genes predicted to be activated display clear roles in aggressive behaviors, including *BDNF* (Kretschmer et al., 2014; Vigers et al., 2012), *CREB* (David et al.,2001) and *SOX2* (Gatewood et al., 2006). The behavioral functions affected by these regulators include learning and memory cognitive processes, along with behavioral conditions and neuropsychiatric disorders, such as fear, addictive behaviors, schizophrenia and

bipolar disorder, all displaying aggressiveness (Figure 4). In this regard, there is evidence of increased memory encoding during early adulthood (Rubin et al., 1998) and also, it has been observed that adults maintain elevated plasma levels of molecules associated with abnormal aggressive behaviors (i.e. corticosterone) longer than adolescents (Romeo et al.,2006). Furthermore, some neural receptors at adult PFC have shown heightened expression levels, conferring them higher risk of addictive and impulsive behaviors (Sonntag et al., 2014). This evidence suggest that behavioral and physiological functional processes of adults differs from that of adolescents, which may reflect the ongoing maturation of the limbic structures that regulate reactive aggression responses in adulthood.

Furthermore, the presence of *KRAS* gene as upstream regulator was also observed. Although *KRAS* is a proto-oncogene associated principally with cancer (Zhu et al., 2008), at the transcriptional level it acts as a short-term inductor to astrocytes in response to stimulus and as a sensor that adapts cells to metabolic needs and oxidative stress at the brain (Messina et al., 2017). The reactive aggression responses of the bulls during the *corrida* may trigger the mechanisms implicated in oxidative stress states, which have been observed to increase with age due to a disruption in the oxidant and anti-oxidant balance (Sies, 1991). Besides, in neuropsychiatric disorders such as bipolar disorder, elevated markers in blood of oxidative stress levels has been reported among adults, relating oxidative stress as mediator on neuropathological processes of adulthood (Andreazza et al., 2008).

Finally, it is worth also highlighting two genes implicated in functional development functions: the *NPAS4* gene, which participates in the structural and functional plasticity of neurons acting as regulator of the excitatory-inhibitory neural balance (Jaehne et al., 2015); and the *HTR2C* gene, modulator of the neural activity by functioning as down-stream effector of insulin

sensitivity and glucose homeostasis (Stam et al., 1994). Both genes have also been candidates in studies of human pathophysiology in mental disorders, including schizophrenia, bipolar and cognitive disorders (Iwamoto et al., 2009), and are up (*NPAS4*) and down-regulated (*HTR2C*) in the adult group of bulls (Table 2).

Interestingly, whereas Agoglia et al. (2017) reported enhanced protein alterations in networks that regulate neuronal signaling, anxiety-related behavior and neurological disease in the adolescent PFC when compared to adult mice, our gene expression results found similar associations but only in adults. This different outcome may be due to differences in the age-stage comparison between studies. While in mice the biological transition of adolescence to adulthood is well studied, defining maturation stages in cattle has proved more complex, as its lifespan depends on the production purpose of each breed, among other factors (Essl, 1998). Given that the average life of a Lidia sire is approximately 15 years, and the average generation sire interval pathway (sire-offspring) is 7,5 years (Fernández-Salcedo, 1990), perhaps here the comparison between early and middle age adulthood would be a more correct approximation.

In summary, a comparison of the differences in genomic expression between young and adult cattle of the aggressive selected Lidia breed at PFC was conducted, identifying 50 up and 193 down-regulated genes in the adult group. These genes were related to different functions such as neural plasticity and behavior. We also performed a cross-species comparison analysis (CSCA) identifying 29 genes in common with previous aggressiveness-related studies and investigated their regulatory networks and upstream regulators. Changes in the regulation of networks such as the synaptogenesis, involved in the synapses maintenance and refinement, and the glutamate receptor pathway, acting as excitatory driver in aggression responses were detected. In addition,

the Wnt//β- catenin signaling pathway was present in both up and down-regulated genes in the adult group, and has been implicated in adult altered behaviors and cognitive disorders.

The present findings suggest that the cortical gene expression in 4-year-old Lidia cattle is characterized by an enhancement of molecular mechanisms involved in the increase of aggressive behaviors and neurophysiological disorders. However, despite that our results suggest cortical enhanced genetic expression associated with reactive aggression in adults, this may not be representative of other brain regions within the limbic system reported also as important actors in this process. Future experiments to evaluate the role of these other brain regions, particularly in the context of aggressive responses, will shed further light on this age transition period.

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Conflict of interest statement None declared.

Author contributions P.G carried out the experiment. P.G and N.S. wrote the manuscript with support from S.D. M.P support the sample retrieving. S.D. helped supervise the project and final paper remarks. P.G. conceived the original idea.

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Tables

Table 1. Summary statistics of differentially expressed genes (DEG).

Classification	Transcripts
Significant DEG	243
Up-regulated DEG	50
Down-regulated DEG	193

Table 2. Up and down regulated DEGs in four-year-old bulls in common with the cross-species comparative analysis (CSCA).

Up-regulated DEGs

Gene symbol	Gene Name	Weighted Ranking (WR)
NPAS4	Neuronal PAS Domain Protein 4	1.5
PENK	Proenkephalin	1.5
FMN1	Formin 1	1.5
SLC24A2	Solute Carrier Family 24 member 2	1
SEL1L3	SEL1L Family Member 3	1
VGF	VGF Nerve Growth Factor Inducible	1
LGI2	Leucine Rich Repeat LGI Family Member 2	0.5
BRINP1	BMP/Retinoic Acid Inducible Neural Specific 1	0.5

Down-regulated DEGs

Gene symbol	Gene Name	Weighted Ranking (WR)
IGF2	Insulin Like Growth Factor 2	1
PAMR1	Peptidase Domain Containing Associated With Muscle Regeneration 1	1
ROBO1	Roundabout Guidance Receptor 1	1
SCARA5	Scavenger Receptor Class A Member 5	1
SPARC	Secreted Protein Acidic And Cysteine Rich	1
DACT2	Dishevelled Binding Antagonist Of Beta Catenin 2	1
ALCAM	Activated Leukocyte Cell Adhesion Molecule	0.5
KCNG1	Potassium Voltage-Gated Channel Modifier Subfamily G Member 1	0.5
TIMP3	TIMP Metallopeptidase Inhibitor 3	0.5
GJA1	Gap Junction Protein Alpha 1	0.5
ADGRA2	Adhesion G Protein-Coupled Receptor A2	0.5
TP53I11	Tumor Protein P53 Inducible Protein 11	0.5
DCN	Decorin	0.5

HES5	Hes Family BHLH Transcription Factor 5	0.5
HTR2C	5-Hydroxytryptamine Receptor 2C	0.5
FRZB	Frizzled Related Protein	0.5
HACD4	3-Hydroxyacyl-CoA Dehydratase 4	0.5
NUPR1	Nuclear Protein 1, Transcriptional Regulator	0.5
CRIM1	Cysteine Rich Transmembrane BMP Regulator 1	0.5
ZFP36L1	ZFP36 Ring Finger Protein Like 1	0.5
CRISPLD1	Cysteine Rich Secretory Protein LCCL Domain Containing 1	0.5

Table 3. Overrepresentation of enriched specific functions within the category of *behavior.* N*:* Number of up-regulated DEGs in the adult group.

Disease / function annotation	P-value	N	Genes
Emotional behavior	5.51E-08	9	*BRINP1,CBLN4,GRIN2A,PENK,SCN1B,SLC17A6, SCN4B,SCN1B,SCN1A,VGF*
Learning	3.27E-06	9	*ABCC8,BRINP1,DKK1,GRIN2A,NPAS4,SCN1A,SLC4A2,TAFA2,VGF*
Memory	7.70E-07	8	*ABCC8,BRINP1,DKK1,GRIN2A,NPAS4,SCN1A,SLC4A2,TAFA2*
Anxiety	1.75E-05	6	*BRINP1,CBLN4,GRIN2A,PENK,SCN1A,TAFA2*
Spatial learning	7.09E-05	5	*ABCC8,GRIN2A,SLC24A2,TAFA2,VGF*
Irritable behavior	3.35E-06	4	*GRIN2A,SCN1A,,SCN1B,SCN4B*

Color Figures

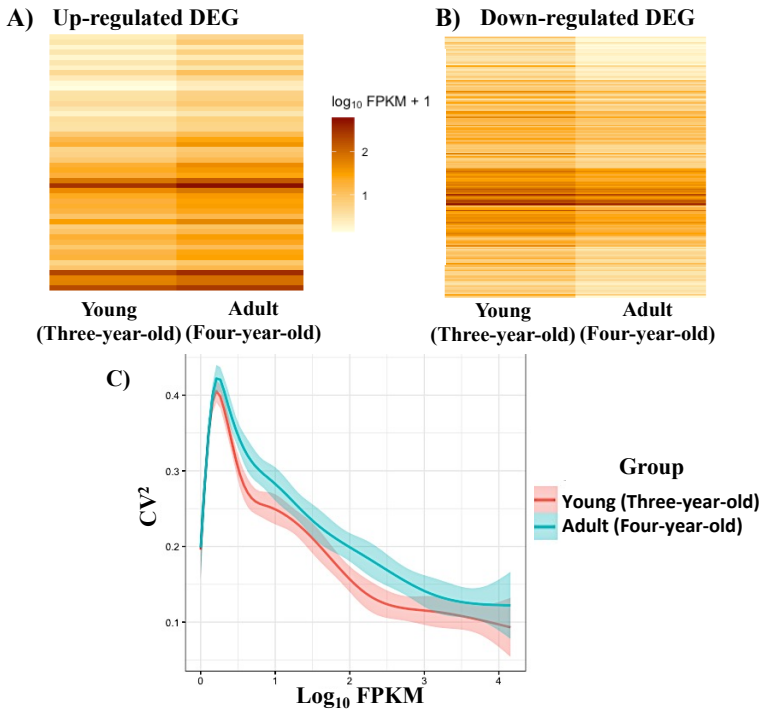
Figure 1. Heatmap of up-regulated (A) and down-regulated (B) differentially expressed genes (DEG) in the adult group. (C) CV-plot showing the biological variation of DEGs per group. The Y-axis shows the normalized coefficient of variation measurements (CV^2) between young and adult groups, and the X-axis correspond to the relative number of reads measured by fragments per kilobase of transcript per million mapped reads (FPKM).

Figure 2. Bar chart of up and down regulated DEGs in common with the cross-species comparative analysis (CSCA). Gene abundance is represented in fragments per kilobase of exon per million fragments mapped (FPKM) of the adult (4-year-old) and young (3-year-old) groups.

Figure 3. Regulator effects of the IPA package applied to the up-regulated DEG and diverse behavioral conditions (p-values < 10^{-5}). In the lower tier, the expected behavioral consequences of the up-regulated DEG changes are shown by considering the Ingenuity Knowledge Base. In orange color are marked those functions predicted to be activated and in blue those predicted to be suppressed.

Figure 4. Network analysis of the 29 DEGs in the adult group in common with the CSCA. Genes highlighted in color correspond to the DEGs in our study; in red the up-regulated and in green the down-regulated DEGs.

Figure 5. Network visualization of the four major upstream regulators of the DEGs in common with the CSCA. In orange, upstream regulators predicted to be activated; in red and green, genes whose expression increases or decreases in response to the activation of upstream regulation, respectively. The shapes of the nodes reflect the functional class of each gene product, as shown in the legend. The symbols marked in grey define functions. Solid and dashed lines between genes represent known direct and indirect interactions, respectively, with orange lines leading to activation and blue to inhibition.



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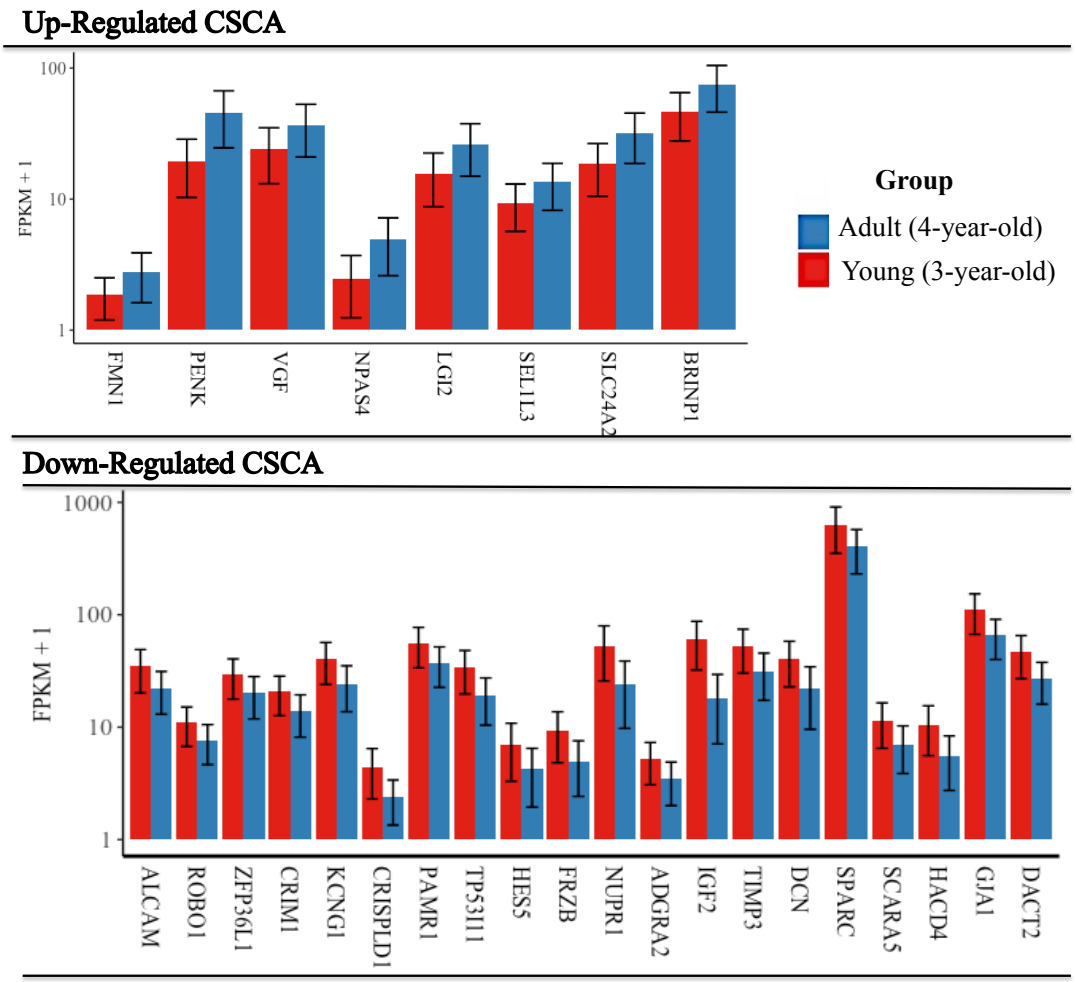

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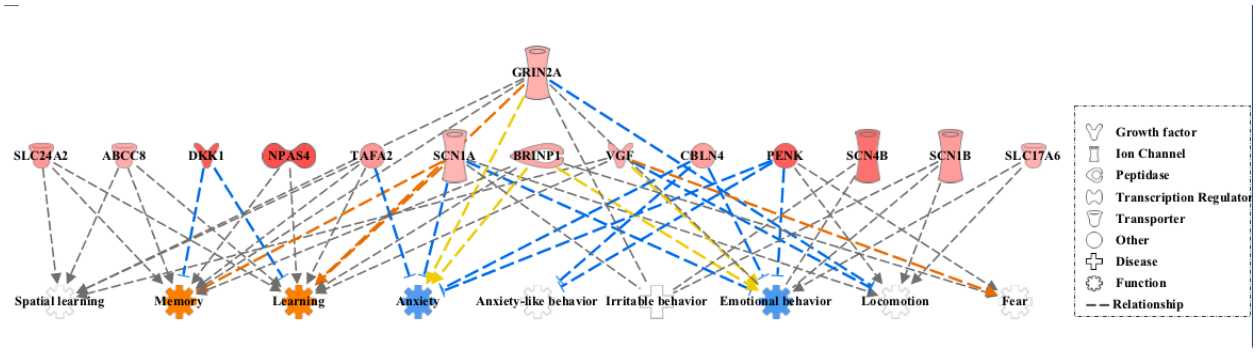

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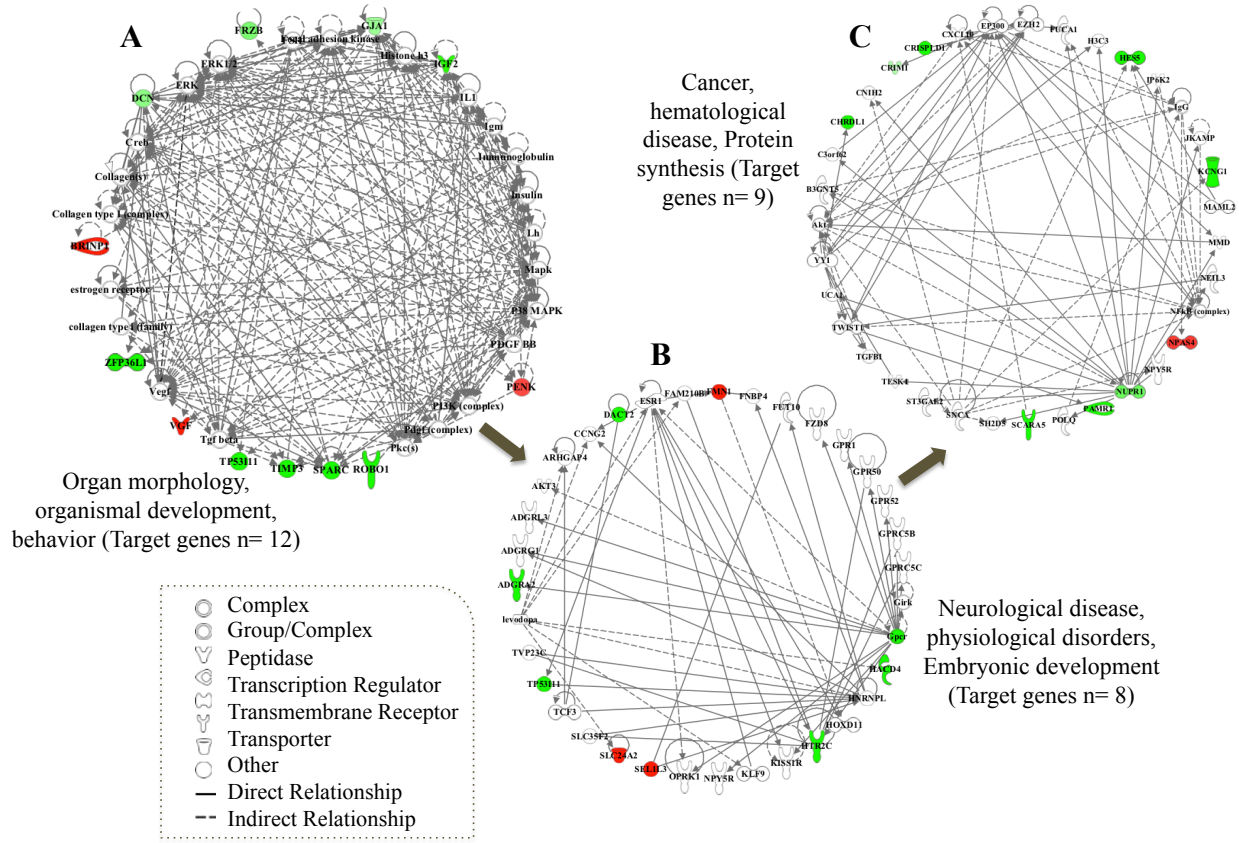

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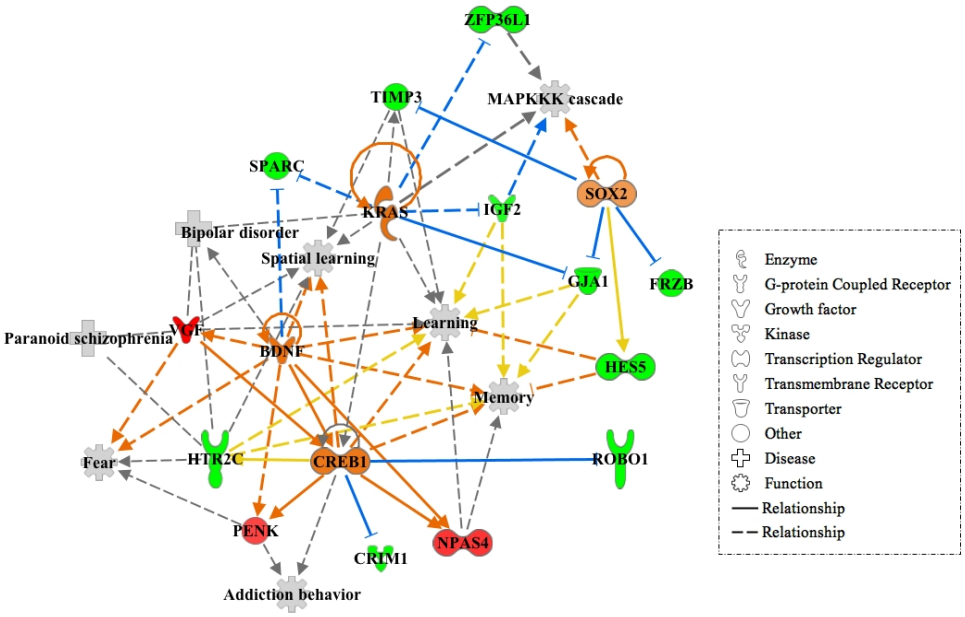

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