Copy Number Variation and Gene Expression of Brain Derived Neurotrophic Factor (BDNF) and Its Association with Pro and Anti Inflammatory Cytokines (IL 6, TNF α, and IL 10) in Awassi Sheep

Dhiaa Sabah Ibrahim [1*]

[1] Department of Biology, College of Education for Pure Sciences, University of Samarra, Iraq.

*Corresponding author's email: dyia.sabah@uosamarra.edu.iq

Email addresses of coauthors: dyia.sabah@uosamarra.edu.iq

Abstract

Copy number variation (CNV) in genes can lead to alteration in gene expression and might affect various biological processes. CNV in neurotrophin genes have been shown to be associated with differential expression of cytokines. The objective of this study was to evaluate effects of CNV in BDNF gene on cytokines regulation in Iraqi Awassi sheep. Blood samples were collected from 100 Iraqi Awassi sheep and analyzed for CNV status and expression levels of BDNF gene and serum concentrations of IL 6, TNF α and IL 10. Statistically significant differences between the groups with different CNV status in BDNF gene were found ($P < 0.05$). The results showed that sheep with deletion CNV had lower serum concentration of IL 6, TNF α and IL 10 compared with other CNV types (duplication and normal), also these sheep had lower expression level of BDNF. Sheep with duplication CNV had higher serum concentration of IL 6, TNF α and IL 10 than the sheep with normal CNV and had moderate expression of BDNF. Sheep with normal CNV status showed highest expression level of BDNF gene. Positive correlations between expression level of BDNF and serum concentration of IL6, TNF α and IL 10 indicated neurotrophic as well as immune regulatory effects of BDNF. This study provides evidence for direct effects of CNV on neuroimmune regulation mechanisms in sheep. Identification of CNV and their correspondent expression profiles in BDNF may provide molecular biomarkers for resiliency, immune homeostasis and productivity in sheep that will face severe environments

Keywords: BDNF, CNV, Cytokines, Neuroimmune regulation, Awassi sheep.

Introduction

Brain derived neurotrophic factor (BDNF), a member of the neurotrophin family, is responsible for survival, development, and function of neurons in both central nervous system (CNS) and peripheral nervous system (PNS). Recent studies in sheep have uncovered that BDNF also plays important roles in immune regulation and inflammatory control[1]. Neurotrophins such as BDNF have been found to regulate immunity and inflammatory response through expression in immune system cells and tissues where they participate in immunomodulation and maintenance of inflammatory homeostasis at least in part by modulation of oxidative stress[2]. This finding has been used as a foundation to further characterize neuroimmune interactions in ruminants and suggest neurotrophins as possible

ISSN 2072-3857

communicators between the nervous system and immune system [3]. Neuroimmune crosstalk has been identified as a crucial component to health, reaction to stress, and productive output of animals. In sheep, expression of neurotrophins can be regulated by pro and anti inflammatory cytokines[4]. In addition, BDNF can regulate cytokine secretion and immune cell function highlighting the possibility that changes to neurotrophic support can influence inflammatory regulation and physiology of small ruminants,Copy number variations (CNV) are deletions or duplicates of stretches of DNA that make up some of the most significant structural variants in the sheep genome [5]. CNV can cause changes in transcription levels of the genes in the duplicated or deleted region [6]. Analysis of the sheep genome have found that CNV are prevalent across the genome and can be associated with production traits, disease resistance, and adaptation to climate [7]. Copy number variations of genes involved in neurotrophic support or cytokine production could help to explain variation in inflammatory reactions and resilience,With this in mind, copy number variation provides an interesting mechanism to help uncover the genetics behind complex neuroimmune traits in sheep [8]. Pro and anti inflammatory cytokines work to regulate inflammation in ruminants. Interleukin 6 (IL 6) and tumor necrosis factor alpha (TNF α) are pro inflammatory cytokines that can be increased during both acute and chronic inflammation in sheep [9]. Both of these cytokines have been linked to disease, stress, and lower productivity in livestock species. IL-10 is an anti-inflammatory cytokine that aids in controlling the inflammatory response by limiting unnecessary immune cell activation and promoting anti oxidant protection and immune homeostasis [10]. An imbalance in pro and anti-inflammatory cytokines can have negative effects on animal health and productivity which can be exaggerated by stress from the environment or mismanagement[11].

Awassi sheep are one of the largest sheep breeds in the middle eastern region and make up a large portion of the milk and meat produced in desert and near desert environments[12]. Awassi sheep are highly adaptive, can handle harsh climates and many different management styles, and have high genetic diversity. Awassi sheep will be used for this study[13].

Materials and Methods

Sample Collection

Blood samples were taken from jugular vein of one hundred Awassi sheep using disposable sterile medical syringes. Blood volume of 4.5 mL was collected per each animal. Sheep were obtained from Ruminant Research Station, Aqraquf, Abu Ghraib, Baghdad.The blood samples was adivided according to need volume for molecular assays and serum based biochemical analyses. Genomic DNA/RNA extraction: 1.5 mL blood samples from each animal were allocated into 2 mL EDTA tubes to avoid coagulation. Tubes were gently inverted for few times to mix EDTA completely with blood. Blood samples were transported into lab inside the cooled insulator boxes and kept frozen at

ISSN 2072-3857

–20 °C until further DNA and RNA extraction steps were performed. Biochemical based serum analysis: 3 mL blood samples were placed in serum separator tube (SST). Samples were left standing at room temperature until clot formed then centrifuged at 3000 rpm for 10 minutes to harvest serum layer. Serum samples were aliquoted into RNase and DNase free cryovials before being stored at –20 °C until analyzed.

Genomic DNA Extraction

Genomic DNA was isolated by Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instruction. All purification steps were performed under aseptic conditions using filter tips and RNase/DNase‑free disposable plastics to avoid nucleic acid degradation or cross‑contamination. The concentration and purity of extracted DNA was determined by NanoDrop spectrophotometer readings with an absorbance ratio A260/280. A260/280 values ranging from 1.8 to 2.0 indicated high‑quality DNA. Integrity of isolated DNA was validated by agarose gel electrophoresis, which confirmed high‑molecular‑weight bands.The DNA concentrations used for CNV assays were determined using Qubit dsDNA High‑Sensitivity fluorometric assay[14].

Total RNA Isolation

Total RNA extraction was performed using the ReliaPrep™ RNA Miniprep System (Promega), strictly adhering to RNase‑free workflows, including certified RNase‑free consumables. Purity was verified using NanoDrop spectrophotometry (A260/280 = 1.9–2.1). Structural integrity was evaluated using Bioanalyzer to obtain RNA Integrity Number (RIN) scores. RNA concentrations were confirmed using the Qubit RNA High‑Sensitivity assay. Extracted RNA was stored at −80°C until cDNA synthesis[15].

Primer Design and Sequences

Primer sets for CNV analysis of the BDNF gene, the CNV reference gene, BDNF mRNA expression, and the housekeeping gene GAPDH were designed using Ovis aries genomic and transcriptomic sequences retrieved from NCBI reference assemblies [16].Amplicon lengths were restricted to 70–200 bp. Primer specificity was validated using BLASTn. Primers were synthesized by Macrogen. Sequences: BDNF CNV F: CAATGAAGCAACAGCGAGCC, R: TTGGGCCCACTCCCTTCATTTC. CNV ref F: CCAACCCCTGTGCTCAGAGC, R: CGGGACCATCCACTCGTGA. BDNF expression F: CATTGGATGCCAGATTGTTTGAAT, R: CGAGACATCCAGGAATTGTTT. GAPDH F: GGGTGGAACCCATGGAAGTATAA, R: CCCTCCACGATGCCAAAGT.

qPCR‑BasedCNV Quantification

Copy‑number variation (CNV) analysis of the BDNF gene was conducted using SYBR Green–based quantitative PCR (qPCR) with Promega qPCR Master Mix in a 20‑µL reaction volume containing template DNA, primers, $MgCl_2$, and nuclease‑free water. Cycling: 95°C for 5 minutes, then 45 cycles of 95°C for 20 seconds, 63°C for 20 seconds, and 72°C for 20 seconds, followed by melt‑curve analysis. CNV

ISSN 2072-3857

values were calculated using the ΔΔCt method with the reference gene as calibrator. Samples were categorized into Loss (CN < 2), Normal (CN = 2), or Gain (CN > 2).

cDNA Synthesis and Gene Expression qPCR

Complementary DNA (cDNA) synthesis was performed using Promega reverse‑transcription kits in 20‑µL reactions containing RNA, MMLV‑RT enzyme, Oligo(T) primers, and reaction mix. Thermal program: 25°C for 5 min, 42°C for 60 min, 70°C for 15 min. Gene expression of BDNF was quantified using SYBR Green–based qPCR with GAPDH as reference. Cycling: 95°C for 5 min; 45 cycles of 95°C for 20 s, 57–60°C for 20 s, 72°C for 20 s; followed by melt‑curve analysis. Expression levels were calculated using the 2^-ΔΔCt method[17].

Enzyme Linked Immunosorbent Assay (ELISA) for Detection of IL-6, TNF-α, and IL-10

Quantification of cytokines IL-6, TNF-α, and IL-10 was conducted using commercially available ELISA kits (Chondrex Inc., USA) employing a sandwich-type Enzyme-Linked Immunosorbent Assay (ELISA), strictly following the manufacturer's instructions. Briefly, serum samples previously obtained from clotted blood and stored at −20 °C were thawed on ice and mixed gently prior to analysis to preserve cytokine stability.Microplate wells coated with monoclonal antibodies against IL 6, TNF α, or IL 10 were used for the determination of respective cytokines. Standards, controls, and twofold diluted serum samples were prepared at appropriate concentrations and 100 µL of each were allocated into individual wells. After incubation at room temperature the antigen–antibody reaction was allowed to proceed. The wells were then washed several times with PBST to remove unbound material. Each well received 100 µL of a cytokine-specific species appropriate HRP conjugated secondary antibody directed against ovine cytokines. Samples were again incubated at room temperature protected from light to enable a stable immunocomplex to form. The wells were washed extensively to remove any unbound conjugate and incubated with 100 µL of chromogenic substrate solution (TMB). The enzyme reaction produced a color change that was quantitated by optical density at 450 nm using a microplate reader after termination of the reaction with 1 N sulfuric acid. Serial dilutions of known concentrations of each cytokine were used to construct standard curves (supplied with the ELISA kits) and determine the serum concentrations of IL 6, TNF α, and IL 10. All determinations were performed in duplicates or triplicates and means used for statistical analysis to reduce intra assay variation. Negative and positive controls were run alongside all assays. Results were recorded and used for further statistical analysis [18].

Statistical Analysis

To assess the impact of BDNF CNV status (Deletion, Normal, Duplication) on raw values for BDNF gene expression and cytokine levels (IL 6, TNF α, IL 10), we conducted a one way analysis of variance (ANOVA; Type II sum of squares) with CNV group as fixed effect. If statistical significance was detected via ANOVA omnibus tests, we used Duncan's

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Multiple Range Test to further assess significance of differences between CNV groups (α = 0.05). Means are displayed as group means ± standard error (SEM) with significance letters denoting differences between CNV groups. Additionally, correlations between CNV values and BDNF gene expression and cytokine levels were performed. To determine the correlation between variables with normal distribution we used Pearson's correlation coefficient. In case of variables with non normal distribution Spearman's rank correlation coefficient was utilized. The IBM SPSS [19] Statistics correlation matrices and heatmaps visualize correlations and thus aid in interpretation of molecular–immunological interactions.

Result and Discussion

Result

Evaluation of BDNF Copy Number Variation on Cytokines and Gene Expression

There were significant differences ($P \leq 0.05$) due to the effect of CNV as shown by comparing fold changes in the transcriptional levels of BDNF gene between the three groups (Deletion, Duplication, Normal) for Immune cytokine levels&gene expression (Table 1). As shown in Table 1, levels of IL 10, TNF α and IL 6 were significantly lower in Deletion group as compared to other groups. These showed the minimum values and were statistically significant indicating a lowered immune activity in this group. Also fold changes of BDNF gene expression was significantly low in Deletion group indicating that loss of copy numbers lead to low neuro immune activity. Thus it can be suggested that CNV deletion acts as a repressor for immune and neuronal functions influenced by BDNF gene transcription.

Table (1) Impact of CNV Types in BDNF Gene on Cytokines Profiles and Gene Expression.

Means ± SE				Number of observations	Copy Number Variation (CNV)
IL-10 ng/ml	TNF-α ng/ml	IL-6 ng/ml	*BDNF* Gene Expression(fold change)		
0.3245 ± 0.0551 c	0.3739 ± 0.0684 c	0.4605 ± 0.0915 c	1.0229 ± 0.0992 a	70	Deletion
1.013 ± 0.0755 a	1.4595 ± 0.0678 a	1.6695 ± 0.1385 a	1.1517 ± 0.084 b	15	Duplication
0.9985 ± 0.1676 b	1.2066 ± 0.2141 b	1.4355 ± 0.2736 b	1.6288 ± 0.2818 b	15	Normal
*	*	*	*	Significance level	

Different letters within the same column indicate significant differences among means at ($P \leq 0.05$) and NS = non-significant.

ISSN 2072-3857

Conversely, the Duplication group demonstrated a pronounced elevation in the three cytokines, recording the highest values with clear statistical significance. This outcome reflects a robust immune response that may be associated with hyper-inflammatory activity. However, the expression of the BDNF gene in this group was not the highest; rather, it remained at an intermediate level compared to the other groups. This suggests that duplication enhances immune activity more strongly than it augments neuronal activity. Such divergence highlights that increased copy number may amplify immune responsiveness, a phenomenon with dual implications: it may strengthen resistance to infection but simultaneously increase susceptibility to inflammatory disorders.

In contrast, the Normal group exhibited the highest level of BDNF gene expression, accompanied by moderate cytokine concentrations. This profile reflects a functional equilibrium between neurogenesis and immune responsiveness. The pattern indicates that the normal CNV state achieves optimal integration of neuronal and immune activities, consistent with the central role of BDNF in supporting synaptic plasticity and maintaining immune homeostasis. Collectively, these findings demonstrate that CNV in the BDNF gene directly influences both gene expression and cytokine levels: deletion suppresses neuro-immune activity, duplication enhances immune responsiveness, while the normal state represents a balanced physiological condition most favorable for functional stability.

Multivariate Correlation Analysis Between CNV, BDNF, and Cytokines

Pearson correlations were performed for the selected variables (CNV versus BDNF gene expression and cytokines immune markers IL 6, TNF α, and IL 10). Results are presented in Table (2). Correlations ranged from weak to moderate and are both significant and non-significant. The correlations revealed the interconnectedness of these variables contributing to the neuro immune paradigm tested within our sample. Weak correlations between CNV and BDNF gene expression ($r = 0.1289$) suggest that CNV is not predictive of this neurotrophin gene expression. As BDNF expression is not directly correlated with CNV there may be other factors such as epigenetic regulation or environment effecting BDNF expression. Moderate correlations were seen between CNV and immune cytokines IL 6 ($r = 0.528$), TNF α ($r = 0.5962$) and IL 10 ($r = 0.5194$). Together these results suggest CNV has a closer relationship to immune responsivity than to neuronal responsivity.

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Table (2) Pearson Correlation Matrix Between BDNF Copy Number Variation ,Cytokines and Gene Expression Traits.

IL-10 (ng/ml)	TNF-α (ng/ml)	IL-6 (ng/ml)	BDNF Expression	GeneCNV	Variable
0.5194	**0.5962**	**0.528**	0.1289	1.000	CNV
0.8258	**0.8051**	**0.8345**	1.000	0.1289	BDNF Expression
0.8917	**0.9144**	1.000	**0.8345**	**0.528**	IL-6 (ng/ml)
0.9090	1.000	**0.9144**	**0.8051**	**0.5962**	TNF-α (ng/ml)
1.000	**0.9090**	**0.8917**	**0.8258**	**0.5194**	IL-10 (ng/ml)

BDNF also showed very strong correlations with each cytokine measured. IL 6 (r = 0.8345), TNF α (r = 0.8051), and IL 10 (r = 0.8258). These strong correlations solidify BDNF's role as both a neurotrophic agent and as a modulator of immune activity. Higher expression of BDNF was associated with higher levels of both pro and anti inflammatory cytokines, which makes sense as BDNF plays roles in assisting synaptic plasticity as well as homeostasis of the immune system. Therefore, BDNF can be seen as the bridge linking nervous system to immune system as it plays roles in both physiologic and inflammatory responses.

There were also some very strong correlations between cytokines. IL 6 and TNF α (r = 0.9144), IL 6 and IL 10 (r = 0.8917), TNF α and IL 10 (r = 0.9090). These high numbers are to be expected as cytokines work in such a network like fashion. When one cytokine is turned on, others will follow. They all increased at the same time showing that they work together to create a response. You also see the pro-inflammatory cytokines (IL 6 and TNF α) being strongly correlated with an anti-inflammatory cytokine (IL 10). The strong correlations between the pro-inflammatory and anti-inflammatory cytokines show that there is a balance and that the body tries to regulate these cytokines to avoid over-inflammation and maintain homeostasis.

In conclusion, CNV appears to be a master control with only moderate influence over immune activity. BDNF strongly connects the nervous system to the immune system. The cytokines themselves create a very integrated system showing that the immune system of sheep act as one system rather than individual components. These results show that BDNF with variations in copy number can indirectly influence immunity by changing gene expression, and that cytokines work as a team when trying to adapt to change.

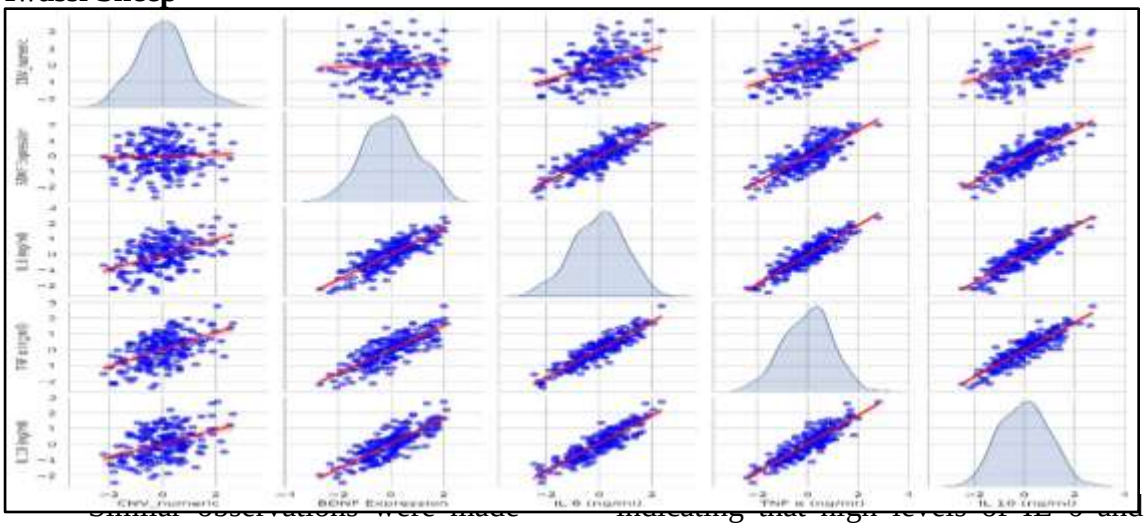
Discussion

Molecular Insights into CNV–BDNF and Cytokine Dynamics in Sheep

The findings in this study revealed that CNV of BDNF gene expression in Iraqi Awassi sheep had strong linkages with the levels of pivotal

cytokines (IL 6, TNF α, IL 10). As shown in figure (1) positive correlation between CNV and BDNF with all cytokines except (TNF α) showed obvious linear directions which confirmed the proposed theory of existence mutual regulatory relationships among studied parameters. The elevation of IL 6 and TNF α along with increased CNV & BDNF expression may be suggestive of induction of regulated inflammatory mechanisms while the raise of IL 10 may reflect anti-inflammatory processes. All together these findings represents a counter balance between inflammatory immune mechanisms indicating that Awassi sheep may have immune hardy capacities that allow them to withstand the effects of environmental and climatic changes in Iraq and this might be attributed to their ancestral resistant origin.

Figure (1) Correlation Matrix of CNV, BDNF Expression, and Cytokine Levels in Awassi Sheep



Similar observations were made in Iranian sheep population, in which CNVs were enriched for adaptive traits like tail fat deposition and immune traits, where CNVs identified in thin tail sheep breeds were involved in regulation of immunity and oxidative processes. Another recent review also showed that CNV were associated with growth, reproduction, disease, and adaptation to environment traits in ruminants[20].

In sheep model, experiments showed that supplementation of BDNF to oocyte cultures promoted maturation rate and decreased oxidative stress demonstrating the effect of BDNF in cell growth regulation and immune defense mechanism. There are also reports indicating that high levels of IL 6 and TNF α lowered the concentration of BDNF in sheep[21].

Our results support the correlation between CNV, BDNF expression and cytokine regulation in Awassi sheep population. Although Several studies indicate the connection between CNVs and cytokine genes expression, these reports were insufficient to establish a strong relationship. Therefore, our findings add information on the genomic–immune axis in sheep population and can be utilized as molecular markers in selection programs to gain healthy animals that can tolerate harsh environmental conditions.

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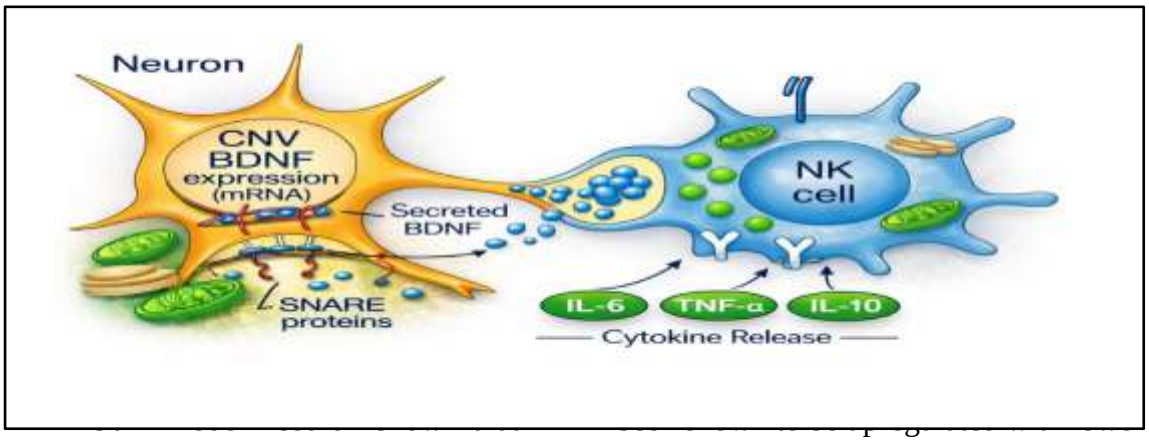
Association of CNV in BDNF Gene Expression with Cytokine Regulation

Figure (2) depicts one possible mechanism of molecular communication between neurons and NK cells that uses a BDNF gene expression dependent pathway affected by CNV. A neuron creates BDNF mRNA followed by secretion of the protein through the SNARE complex, a protein complex that controls exocytosis. Secreted BDNF is able to travel to NK cells causing them to secrete important cytokines including IL 6, TNF α, and IL 10.

Figure (2) Neuronal CNV–BDNF Pathway Driving Cytokine Release in NK Cells



CNVs effect through BDNF is not neuronal specific but rather coordinates immune activity. In our model BDNF secretion induced NK cells to secrete both pro-inflammatory cytokines (IL 6, TNF α) and an anti-inflammatory cytokine (IL 10), indicating pro- and anti-inflammatory response reciprocation. This could explain the positive statistical correlation seen between CNV, BDNF and most cytokines measured[22].

Although studies have shown correlations between CNVs and adaptive characteristics such as growth, immunity and disease-resistance in sheep and other ruminants there has not been a candidate gene identified until now. In other studies analyzing BDNF expression it has been shown to have positive influences on cell activity and decreasing effects on oxidative stress[23]. IL 6 and TNF α have also

expression of BDNF[24].

Our results conclude that BDNF regulated by CNV is involved not only in neurological but also immune responses in sheep. This unique pathway may serve as a factor to the response to stress Awassi sheep face showing their merit as a 'calm' genotype and in understanding the genomic contributions to immune homeostasis in livestock.

Conclusion

In this investigation, we showed that BDNF CNV variation affects BDNF expression and cytokine regulation in Awassi sheep. The deletion CNV genotype was associated with reduced immune-neurotrophic activity, duplication increased immune activity but could not induce the highest expression of BDNF, whereas the wild-type genotype could mediate the optimal level of neuronal and immune system activity. Furthermore, expression levels of BDNF showed significant correlation

ISSN 2072-3857

with cytokine production suggesting BDNF as a key regulator between immune system and nervous system activities. Overall, we concluded that CNV could be used as a molecular marker of adaptation linked with immune homeostasis in Awassi sheep and BDNF as a marker of resilience in sheep. Our findings will contribute to selection criteria for livestock breeding programs to keep the genomic stable in BDNF genes to facilitate better adaptation and productivity associated traits along with disease resistance when raised in stressful conditions.

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