



The Relationship Between BDNF Copy Number Variation and Humoral Immunity in Awassi Sheep: a Molecular–Immunological Study

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ABSTRACT

Humoral immunity (Secretory IgA (SIgA), IgM, IgG) and copy number variation (CNV) of BDNF gene and its expression were assessed in Awassi sheep blood samples (n = 40). CNV analysis were performed via qPCR ($\Delta\Delta C_t$ method), BDNF gene expression analysis through SYBR Green qPCR, and IgA, IgM and IgG ELISA were used to determine serum immunoglobulin levels. ANOVA followed by Duncan's test was used to compare differences among deletion (DEL), normal CNV (NOR) and duplication (DUP) groups and Pearson and Spearman correlations were used to determine linear and monotonic relationships. IgM and IgG serum levels were not significantly different among CNV groups and had no significant correlations with BDNF CNV coding respectively ($P > 0.05$). However, serum levels of IgA had a significant group effect ($P \leq 0.05$). Normal CNV group had higher levels of IgA compared to DEL and DUP groups. Similarly, BDNF gene expression was significantly higher in the NOR group compared to DEL and DUP groups showing a clear dose response relationship indicating gene dosage sensitivity and nonlinear response to CNV. Pearson correlation coefficient between IgA and CNV coding was not significant ($p > 0.05$), however Spearman correlation rho value approached significance showing a positive monotonic association ($\rho = 0.305$, $P = 0.056$). Both analyses suggest a relationship between IgA and CNV dosage that may become significant with increased sample size. Taken together our results indicate BDNF requires an optimal dosage range and BDNF-associated neuroimmune communication may contribute to mucosal immune response with IgA being the immunoglobulin most likely to reflect these changes.

Keywords: BDNF Gene, Copy Number Variation (CNV) , Humoral immunity, Gene Dosage Sensitivity , Awassi Sheep.

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INTRODUCTION

Brain-derived neurotrophic factor (BDNF) is among the most influential genetic elements known to maintain nervous system health. It supports neuronal viability and maintenance, synaptic plasticity, and functional neural network formation, all of which are critical components of behavior regulation, development, and stress response of an organism. Reduced expression of BDNF has been linked to neurologic malfunction, cognitive defects, and behavior disorders, whereas overexpression facilitates learning abilities, memory, and neuronal restoration. The sheep neuroimmune system, similar to other ruminants, is closely linked together, and BDNF directly regulates microglial cells in the central nervous system (CNS) [1]. Therefore, BDNF is capable of acting as a neuroimmune regulator that influences neuronal viability and activity through multiple signaling pathways. This mechanism may play a role in determining productive and physiological traits and feeding behavior of stressed sheep by reducing microglial damage while promoting neurorestoration, thereby allowing improved immunological and neurological compensation for stressful environmental factors [2].

Copy number variations (CNVs) are one of the most common types of structural variation observed in the genome. They account for deletions, insertions, and duplications of DNA segments that range anywhere between 1 kb to several megabases. CNVs are responsible for a significant amount of genetic variation between mammals, including sheep.

This variation has a much larger impact than single nucleotide polymorphisms (SNPs) because CNVs directly influence gene dosage, gene structure, and regulatory potential, which affects the expression levels of genes and traits they regulate [3]. Through comprehensive mapping of CNVs that span approximately 2–10% of the ovine genome, thousands of CNV regions (CNVRs) have been identified as regulators of growth, metabolism, fertility, and development of the body. Further assessment across breeds have identified upwards of 39,000 CNVs that are responsible for climatic adaptation and physiological attributes sculpted by geographical differences, defining breed-specific traits [4].

Studies have been performed targeting economically important traits such as weight gain, milk production, wool characteristics, and fertility rate; these studies have shown that CNVs alter expression levels of genes that relate to these traits. CNVs also play a role in reformatting gene regulatory networks. These changes cause transcriptional rewiring which subsequently affect proteins that are expressed [5]. In summary, CNVs are one of the building blocks of sheep genetics and gene-expression profiles. CNVs can be used in molecular selection programs, environmental-related studies, and programs working to enhance livestock productivity to help identify molecular differences between breeds.

Immunoglobulins such as IgA, IgM, and IgG are essential components of the humoral immune response observed in sheep. These proteins make up the terminal endpoints of B-cell proliferation once antigens have been successfully detected by the cell. Adaptive immunity is dependent on immunoglobulins to mount appropriate and suitable detection and eradication of pathogens. IgG, IgM, and IgA are important for immune homeostasis as well. Sheep possess five immunoglobulin classes; however, IgG, IgM, and IgA make up most of the antibodies involved with systemic and mucosal immunity. The sheep species express several isoforms of IgG and IgA that produce a variety of subclasses allowing for diverse antigen-specific responses to occur [6]. IgG accounts for the majority of antibodies circulating within the body and secondary tissue fluids. This immunoglobulin is responsible for long-term immunity. Sheep have been reported to have multiple IgG-encoding genes. IgG concentrations have been used to determine chronic infections and disease diagnostics such as toxoplasmosis [7]. Infected individuals were found to have decreased levels of IgG. IgM is the first antibody to be produced in response to a primary immune stimulus. It is very effective in complement activation and parasite/pathogen control. IgM concentrations have been shown to play a role in gastrointestinal parasite resistance, specifically *Haemonchus contortus* infections [8]. IgA is the most abundant immunoglobulin class that composes mucosal immunity. This antibody serves as the first line of defense along mucosal surfaces within the digestive tract and respiratory system. IgA concentrations are significantly higher within secretions than they are found in serum [9].

It has been well established that CNVs play a key role in genetic variation seen between sheep. CNVs can alter gene dosage allowing for upregulation or downregulation of gene expression to occur. Genome-wide CNV analyses have associated CNVs with neurological regulation and aspects of both innate and adaptive immunity. If a CNV was present on a gene involved with neural function, such as BDNF, it would be capable of modifying neuroimmune signaling by upregulating or downregulating inflammation-related genes. Studies have shown BDNF capable of suppressing inflammation by inhibiting microglial activity and downregulating pro-inflammatory cytokines such as IL-6 and TNF- α . Expression of BDNF is important for immune homeostasis as it helps prevent unwanted inflammatory reactions [10]. IgG, IgM, and IgA make up immunoglobulins which are used as indicators of humoral immunity. Moreover, these immunoglobulins contain structural characteristics that allow sheep to defend against a wide variety of microbial and parasitic pathogens. Secretory IgA (SIgA) is expressed abundantly in mucosal epithelia along the respiratory system and gastrointestinal tract. If CNVs were present on BDNF, they would have the potential to indirectly modify IgG, IgM, and IgA concentrations through regulation along the neuroimmune axis [11].

An association between CNVs and expression levels of immunoglobulins would be beneficial to molecular selection programs. Molecular markers that use CNVs could be applied to breeding programs in order to produce genetically superior sheep with improved disease resistance and adaptability to their surrounding environment.

The purpose of this study was to screen sheep for CNVs of BDNF and their relationship with immunoglobulin concentrations (IgG, IgM, and IgA). This study will determine if BDNF CNVs play a role in neuroimmune regulation, microglial activity, and humoral immune homeostasis during times of stress. It was also determined if BDNF CNVs had any measurable impact on productive, physiological, or behavioral traits. Finally, we aimed to determine if BDNF CNVs could be used as molecular markers to select for improved immunity, physiological environmental compensation, and health- or production-related traits in local sheep.

Materials And Method

Sample Collection

Blood samples were collected from jugular vein of 40 Awassi sheep using medically graded syringes, sterile needles (45 mL blood from each animal). Sheep were randomly selected from Ruminant Research Station herd located at

Akraquf, Abu Ghraib, Baghdad. Blood volume was equally divided for molecular assays requirements and serum based biochemical analyses.

1.5 mL blood aliquot from each animal was placed in EDTA tubes then were transported to the laboratory in cooled box provided by our facility. Samples were stored at -20°C until genomic DNA and RNA extraction was performed. Tubes containing EDTA were inverted several times to mix anticoagulant powder with blood samples thus preventing blood coagulation and preserving cell structure. For serum based biochemical analysis, blood samples (3 mL) were placed into SST and kept upright at room temperature for 30 minutes to allow blood clotting then centrifuged at 3000 rpm for 10 minutes. Serum layer was aspirated using transfer pipette and transferred into RNase and DNase free cryovials. Serum samples were stored at -20°C until analysis.

Genomic DNA Extraction

Genomic DNA was purified with Wizard® Genomic DNA Purification Kit (Promega, USA) according to manufacturer's instructions. All steps of genomic DNA purification were performed under aseptic condition using filtered pipette tips and RNase/DNase-free disposable products to avoid nucleic acid degradation or cross-contamination. The concentration and quality of purified DNA was determined by NanoDrop spectrophotometer using A260/280 ratios. DNA samples with $1.8 \leq \text{A260/280 ratios} \leq 2.0$ were accepted as high-quality genomic DNA and used for subsequent experiments. Additionally, the DNA integrity was examined by electrophoresis using 1% agarose gel, where high-molecular-weight bands of DNA were visualized. DNA concentrations used in CNV assays were determined by Qubit dsDNA High-Sensitivity fluorometric assay.

Total RNA Isolation

Total RNA was extracted with ReliaPrep™ RNA Miniprep System (Promega), using RNase-free protocols including certified RNase-free consumables. RNA purity was assessed by NanoDrop spectrophotometry ($\text{A260/280} = 1.9\text{--}2.1$). RNA structure was determined using Bioanalyzer to assess RNA Integrity Number (RIN) scores. RNA concentrations were validated using Qubit RNA High-Sensitivity assay. All RNA samples were stored at -80°C prior to cDNA synthesis.

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 [12]. 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: GGGTGGAAACCCATGGAAGTATAA, R: CCCTCCACGATGCCAAAGT.

qPCR-Based CNV Quantification

Copy-number variation (CNV) of the BDNF gene was measured by SYBR Green-based quantitative PCR (qPCR) performed using Promega qPCR Master Mix in a $20\text{ }\mu\text{L}$ reaction volume. The reaction included template DNA, primers, MgCl_2 , and nuclease-free water. Cycling parameters were as follows: 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. The qPCR products were subjected to melt-curve analysis. Relative CNV values were determined by using the $\Delta\Delta\text{Ct}$ method with reference gene as calibrator. Samples were classified as Loss ($\text{CN} < 2$), Normal ($\text{CN} = 2$), or Gain ($\text{CN} > 2$).

cDNA Synthesis and Gene Expression qPCR

Reverse-transcription reactions to synthesize cDNA were performed in $20\text{ }\mu\text{L}$ reactions using Promega reverse-transcription kits with MMLV-RT enzyme and Oligo(T) primers. Thermal cycling conditions were: 25°C for 5 min, 42°C for 60 min and 70°C for 15 min. Gene expression levels of BDNF were quantified by SYBR Green-based qPCR using GAPDH as reference gene. Thermal cycling conditions were: 95°C for 5 min; followed by 45 cycles of: 95°C for 20 s, $57\text{--}60^{\circ}\text{C}$ for 20 s, 72°C for 20 s; followed by melt-curve analysis. Expression levels were determined using the $2^{-\Delta\Delta\text{Ct}}$ method.

Assessment of IgG, IgM, and IgA Levels Through an Immunoenzymatic Detection Method

Measurement of total serum IgG, IgM, and IgA was determined by sandwich type Enzyme Linked Immunosorbent Assay (ELISA). Kits for ELISA analysis of IgG, IgM, and IgA were purchased from Bethyl Laboratories Inc. (Montgomery, TX). Serum samples that were stored at -20°C as described above were thawed on ice and vortexed briefly before analysis. Microplate wells were coated with monoclonal antibodies directed against sheep IgG, IgM, or IgA (Rocky Mountain Diagnostics Inc., Westminster, CO). Diluted serum samples (standard curve), calibrators and controls were dispensed into the wells and incubated for 2 hours at room temperature to permit antigen-antibody

complex formation. Wells were washed with phosphate buffered saline Tween 20 (PBST) buffer, and horseradish peroxidase (HRP)-linked secondary antibody specific for sheep immunoglobulins was added and incubated for 1 hour. Wells were washed again to remove unbound conjugate and the substrate solution was added to each well. The amount of bound antibody in the microplate wells was determined by measuring the optical density (OD) produced by an enzyme-substrate reaction. The amount of OD was directly proportional to the amount of antibody bound to the well. Known amounts of purified sheep IgG, IgM, and IgA used to create a standard curve were supplied with each ELISA kit. OD readings were converted to concentrations using these standard curves. Total IgG, IgM, and IgA measurements were performed in duplicate or triplicate to minimize intra assay variability. Negative and positive controls were run with each assay to confirm assay reproducibility. Final concentrations were calculated and used for analysis.

Statistical Analysis

Statistical analyses were conducted to evaluate the effect of *BDNF* CNV status (Deletion, Normal, Duplication) on four dependent variables: *BDNF* gene expression, IgA, IgG, and IgM concentrations. A one-way Analysis of Variance (ANOVA; Type II Sum of Squares) was employed with CNV category as a fixed factor. Post-hoc comparisons among CNV groups were performed using Duncan's Multiple Range Test at a significance threshold of $\alpha = 0.05$. Results are presented in SPSS-style ANOVA tables followed by Duncan grouping tables and corresponding group means.

In addition to group-based comparisons, correlation analyses were conducted to investigate the relationships among *BDNF* CNV values, *BDNF* gene expression levels, and the measured immunoglobulins (IgA, IgG, IgM). Pearson's correlation coefficient was used for normally distributed variables, whereas Spearman's rank correlation coefficient was applied for non-normal distributions or variables exhibiting ordinal characteristics. Correlation matrices were generated based on the previously provided datasets, and diagonal values were used to construct the graphical correlation figures already produced. These visualizations supported the interpretation of the direction and strength of associations between molecular (CNV and gene expression) and immunological parameters.

All statistical analyses and visual outputs—including ANOVA tables, Duncan's post-hoc groupings, correlation matrices, and correlation heatmap figures—were performed and interpreted in accordance with standard biometrical procedures.

Results

Evaluation of *BDNF* Copy Number Variation on Immune Parameters and Gene Expression

Effect of *BDNF* CNV on serum immunoglobulin levels and *BDNF* mRNA expression results show significantly different means of studied traits Table (1). Serum levels of IgM and IgG antibodies were not significantly affected by *BDNF* CNV as indicated by Normal, Deletion, and Duplication groups sharing superscript letter a and having NS P-value. This indicates that *BDNF* copy number changes have no effect on primary and secondary immune responses. Serum IgA level was significantly affected by *BDNF* CNV ($P \leq 0.05$). Sheep with Normal copy number had significantly greater IgA than those with Deletion and Duplication copies. *BDNF* copy number deletion or duplication have a negative effect on serum IgA levels Table (1). Change in *BDNF* copy number also affected *BDNF* mRNA expression. Both Deletion and Duplication groups had significantly lower mRNA expression than Normal copies ($P \leq 0.05$). Since changes on either side of normal resulted in down regulation of *BDNF* expression, this suggests *BDNF* gene expression is optimal at one copy number and may be prone to instability as with other neurotrophins when copy number is altered.

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

Copy Number Variation (CNV)	Number of observations	Means $\pm$ SE			
		Gene Expression	IgA mg/ml	IgG mg/ml	IgM mg/ml
Normal	3	3.1836 $\pm$ 1.0740 a	0.4908 $\pm$ 0.1456 a	22.2720 $\pm$ 1.589 a	1.2342 $\pm$ 0.1618 a
Deletion	32	0.9666 $\pm$ 0.2163 b	0.1877 $\pm$ 0.0294 b	19.2581 $\pm$ 0.937 a	1.1235 $\pm$ 0.1124 a
Duplication	5	0.8674 $\pm$ 0.1667 b	0.1768 $\pm$ 0.0226 b	20.1121 $\pm$ 2.426 a	0.6174 $\pm$ 0.1696 a
Significance level		*	*	NS	NS

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

The analysis of the effect of copy number variation (CNV) in the *BDNF* gene on immune variables and gene expression in sheep revealed no significant linear association between CNV coding (Deletion = 1, Normal = 2, Duplication = 3) and the expression levels of the immunoglobulins IgG and IgM. This finding was supported by Pearson correlation coefficients, which were weak and statistically non-significant ($p > 0.05$) across all examined variables. Similarly, Spearman's rank correlation coefficients—more appropriate given the ordinal nature of the CNV variable—showed no significant monotonic relationship with these immune parameters. These results indicate that variation in *BDNF* gene copy number is not directly or consistently associated with gene expression or with IgG and IgM levels in the studied sample (Table 2).

Table (2) Correlation Matrix Between *BDNF* Copy Number Variation and Key Immunological and Gene Expression Traits.

Category	Variable	GeneEx	IgG	IgM	IgA
Normal	GeneEx	1.0	0.224	1.0	1.0
Normal	IgG	0.224	1.0	0.224	0.224
Normal	IgM	1.0	0.224	1.0	1.0
Normal	IgA	1.0	0.224	1.0	1.0
Deletion	GeneEx	1.0	0.519	0.305	0.996
Deletion	IgG	0.519	1.0	0.292	0.552
Deletion	IgM	0.305	0.292	1.0	0.33
Deletion	IgA	0.996	0.552	0.33	1.0
Duplication	GeneEx	1.0	0.208	0.214	1.0
Duplication	IgG	0.208	1.0	0.165	0.208
Duplication	IgM	0.214	0.165	1.0	0.214
Duplication	IgA	1.0	0.208	0.214	1.0

The IgA variable had a different profile, however. While Pearson's correlation coefficient was not significant ($p = 0.508$), Spearman's rho did show a positive correlation nearing significance ($\rho = 0.305$, $p = 0.056$). While not reaching significance, there was a clear monotonic trend that likely would with increased sample size (Table 3). This finding is also supported by examining the differences across CNV groups, which did show statistically significant differences in IgA expression. The Normal CNV group had the highest IgA levels while both deletion and duplication groups had significantly lower levels.

BDNF transcription was also seen to decrease significantly in deletion and duplication groups when compared to controls. This shows there to be clear dosage sensitivity with this gene. Rather than upregulating or buffering expression with additional or lost copy number, as one might expect from housekeeping genes, both increases and decreases to copy number hindered expression. This is typical of many neuroregulatory genes which have tight transcriptional control.

Taken together, it would appear that CNV variation in *BDNF* is not correlated with many standard immune markers (IgG, IgM), but that IgA is likely the immunologic readout most sensitive to genetic fluctuation. This is true by observational standards (a monotonic trend nearing significance), as well as by confirmed differences across CNV groups. The gene itself shows a typical expression pattern for transcripts that are highly dosage sensitive.

Table (3) Correlation Matrix Between *BDNF* Copy Number Variation and Immunoglobulin Profiles and Gene Expression.

	CNV	Gene Expression	IgG	IgM	IgA
CNV	1.000	0.281	0.136	-0.116	0.305
Gene Expression	0.281	1.000			
IgG	0.136		1.000		
IgM	-0.116			1.000	
IgA	0.305				1.000

Discussion

This study provides an integrated analysis of the relationship between copy number variation (CNV) in the *BDNF* gene, its expression levels, and humoral immune markers (IgA, IgM, IgG) in the investigated sheep cohort. The patterns observed in Figures 1 and 2 indicate a complex, dose-sensitive regulatory architecture that goes beyond simple linear expectations of duplication/deletion, with effects extending to the neuroimmune axis—particularly the mucosal milieu selectively reflected by IgA.

The Impact of CNV on *BDNF* Gene Expression: An Optimal-Dosage Window and Disruption of Regulatory Pathways

Figure 1 shows that the highest *BDNF* expression was detected in the normal CNV group (two copies), whereas expression decreased in both deletion and duplication groups, clearly indicating a nonlinear, dose-sensitive response. Notably, duplication did not lead to a net increase in expression. This pattern aligns with recent reviews highlighting *BDNF*'s multiple promoters, diverse isoforms, and heterogeneous 5'UTR structures, which together enable finely tuned spatiotemporal expression control and make the net output an emergent property of layered regulation—transcriptional, post-transcriptional, translational, and activity- and calcium-dependent regulated secretion [13]. Specifically, canonical references emphasize that *BDNF* harbors numerous transcripts driven by independent promoters, with regulatory inputs from CREB, ATF2, EGR, and others under neuronal depolarization and TrkB signaling. Such architecture can shift promoter and 5'-exon usage when gene dosage is altered, thereby changing translational efficiency and yielding a measured net decrease in expression despite increased copy number [14].

Our findings further indicate that *BDNF* secretion dynamics follow a regulated secretory pathway via the Golgi apparatus and secretory granules, with sorting proteins such as sortilin and CPE. Any dosage pressure or impairment in sorting/vesicular trafficking can introduce a bottleneck that reduces the measurable bioavailability of the protein—a pattern consistent with the reduced expression observed in the duplication group in Figure 1. Moreover, the proBDNF/mature BDNF balance is pivotal: proBDNF engages the p75NTR–sortilin complex, promoting apoptotic/inflammatory cascades, whereas mature BDNF activates TrkB with distinct plasticity outcomes. Competition between these forms can translate into diminished functional output when copy number increases or decreases, due to shifts in proteolytic processing and endocytosis.

This result is in line with [15], which notes that dosage–expression relationships in CNV are not invariably one-to-one; evolutionary and functional evidence points to dosage compensation and inverse dosage effects across regulatory networks, resulting in selective patterns whereby deletion and duplication can exert opposing impacts on distinct functions.

The present study also recognizes that CNV is common in immune-related gene families (e.g., FCGR and KIR) and modulates response amplitudes and immune thresholds among individuals, with examples such as CNV in FCGR3B/3A influencing therapeutic and inflammatory responsiveness. This underscores how CNV can create a functionally broadened response space that is not necessarily linear. While *BDNF* is not an immune gene family member per se, our findings converge conceptually: both deletion and duplication can compromise the optimal functional balance.

These conclusions are concordant with [16], which reported that deletion/duplication can have bidirectional effects on functional outputs, and that dose sensitivity spans multiple pathways and functions—supporting the hypothesis that an optimal dosage window yields the best functional performance. This aligns well with our results as illustrated in Figure 1.

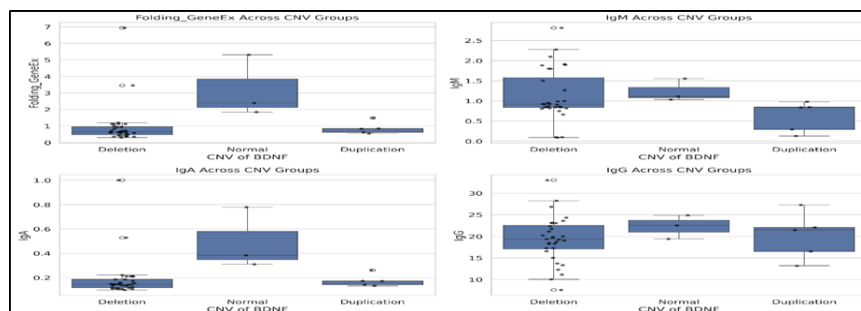


Figure 1. Comparative Analysis of Gene Expression and Immunoglobulin Levels Across BDNF CNV Groups.

The findings of this study suggest that the reduced expression observed in the duplication group may be explained by position-effect influences and chromatin context, whereby additional gene copies may be placed within a transcriptionally repressive environment, leading to diminished transcriptional output. This reduction may be further compounded by potential bottlenecks within the regulated secretory pathway, which relies on sortilin, CPE, and fine-tuned motor-protein-mediated transport (kinesin/dynactin). Such disturbances can lower the bioavailability of BDNF at its target sites. In addition, a shift in the proBDNF/mature BDNF balance toward the less functional—or even functionally opposing—form acting through the p75NTR–sortilin complex may further contribute to the reduced overall biological output.

All these mechanisms are well documented in specialized cellular, molecular, and behavioral reviews, and align

closely with the findings of studies [17, 18, 19], which report that the regulated secretion dynamics of BDNF operate within an inherently dose-sensitive pathway. This pathway relies heavily on Ca^{2+} -dependent and activity-dependent triggers, such that an unbalanced increase in gene copies may disrupt the kinetics of sorting and release rather than enhance them. This interpretation is consistent with the observation that BDNF expression reaches its peak at the optimal dosage state ($\text{CNV} = 2$), as demonstrated in the present study.

Multivariate Correlation Analysis Between CNV, BDNF, and Humoral Immunity

Figure (2) depicts the bi variate and multivariate correlation heatmap of CNV status vs. BDNF expression vs. observed immunoglobulins. Notice that correlation between CNV variation and BDNF expression is much weaker linearly-trended—unsurprisingly as the biological effect is non linear (both deletion and duplication show lower than normal expression). Any U shape curve will reduce the linear correlations coefficient despite obvious differences between groups.

Notice also Figure (2) indicates a stronger and more selective relationship between BDNF expression and IgA versus IgG and IgM levels. This sort of signature is what would be expected if there is indeed neuro immune mucosal tuning occurring where BDNF directly or indirectly through microglia/cytokine effects tunes the mucosal immune response. If this is the case then IgA would be the most sensitive humoral reflection of these neuro immune effects. This correlates well with findings from [20] where it was shown that BDNF suppressed microglial inflammatory responses and helped reshape inflammation through STAT3 and TRAF6 signaling-how neural circuits and immune responses are intimately connected within tissue.

IgG and IgM on the other hand are humoral markers that are far more systemic in nature, and you would usually need an immune challenge (ie. infection/vaccination) or longitudinal design/larger N to observe statistical differences at the group level (thus the minimal differences you see in Figure (1)). The correlation coefficients should also be lower as shown in Figure (2). Lastly reviews in mucosal immunology continually stress that IgA (unlike IgG and IgM) is regulated by MALT networks and shows increased sensitivity to endogenous neuro immune influences.

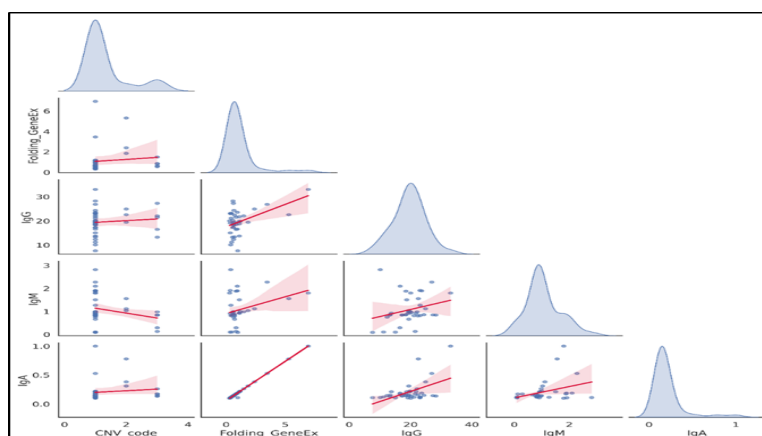


Figure 2. Multivariate Correlation Structure Among CNV C, Gene Expression, and Immunoglobulin Profiles.

Supporting the notion that peripheral BDNF measurement should not be construed as a direct readout of CNS expression, our findings here can also be rationalized when considering that reference-based comparisons to tissue level maps indicate unequivocal enrichment of BDNF expression to brain, with low, but measurable expression to non neural tissues. Gene expression patterns from GTEx and HPA highlight conserved expression dynamics both developmentally and across tissues including the receptors TrkB and p75NTR. Thus, peripheral blood measurements of BDNF expression are likely an integrated systemic output, influenced by but not exclusively representative of central expression and should therefore be expected to be highly sensitive to dysregulation by neuroendocrine-immune mediators as well as environmental stress. This provides mechanistic basis for the observed sensitivity of the results to gene dosage.

This hypothesis is supported by data demonstrating central-peripheral reciprocal regulation of BDNF through the vagus nerve as well as peripheral afferent signaling [21], and findings that targeted disruptions of promoter IV in mice results in modified BDNF concentrations across several peripheral tissues (muscle, liver, intestine, thymus/serum) indicative of multi organ compensatory systemic control [22]. Together these findings suggest that peripheral serum measurements of BDNF are likely reflective of a networked regulatory schema rather than simple correlative expression or transcriptional readout of abundance to any specific tissue.

IgA is considered the primary mediator of intestinal and pulmonary mucosal immunity. Produced locally or by immune cells primed in peripheral lymphoid tissues (as reviewed by IgA functions to initiate immune exclusion, establish a function mucous barrier and regulate microbiome homeostasis). Mediated by B cells residing primarily in MALT structures IgA is locally produced and shuttled across epithelial barriers via pIgR where it establishes as secretory IgA (SIgA) with exceptional binding efficiency at mucosal surfaces. Thus reviews on IgA structure/secretion have also consistently described IgA as the immunoglobulin isotype most sensitive to mucosal environmental signals, such as neural immune signals that regulate epithelial immunity, modulate microglia and govern enteric nerves [22].

Considering the mucosal immune system is the largest standing functional immune organ in ruminants and IgA (along with antimicrobial peptides and mucus) serves as the critical exclusion barrier preventing microbial penetration across epithelial surfaces, with IgA immune cells trafficked systemically via common mucosal immune system pathways. One may therefore conclude that heightened sensitivity of IgA to BDNF dosage imbalance (Figures (1) and (2)) may indicate that changes in gene dosage more drastically influences regulation of this mucosal immunological “code”, compared to circulating systemic markers like IgG and IgM in a cross sectional study design lacking a specific immune stimulus.

In particular, sheep in this flock experience recurrent nutritional and heat stress. We know glucocorticoids influence BDNF transcription and synaptic plasticity relationships and that BDNF can at least partially buffer against chronic stress. Thus, imbalance of gene dosage could induce greater fluctuations of neuro immune driven inflammatory dynamics with the largest measurable effects present in mucosal markers such as IgA (Figures (1) and (2)) while IgG and IgM appear less sensitive in a system level design without targeted immune stimulation.

While there are no studies to reference directly for Awassi or other indigenous Iraqi sheep breeds, basic biological concordance between stress and BDNF gene expression supports the logic of this interpretation.

Conclusion

Here we have shown that BDNF CNV has dose dependent, nonlinear effects on BDNF expression in sheep and that both deletion and duplication alleles are associated with decreased expression compared to the wildtype two copy genotype. Although humoral correlates (IgG, IgM) were unaffected by CNV state, we did observe differences among IgA levels of the three CNV groups and a trend toward monotonic effect of CNV coding. This makes IgA the strongest immunological correlate of BDNF-mediated neuroimmune effects measured here. These data suggest that there is a 'sweet spot' for BDNF expression that agrees with previous work on strict regulation of neurotrophins, as well as potential position/chromatin and secretory pathway buffering under variable dosage. From an analytical standpoint, we found that ANOVA/Duncan testing coupled with Pearson/Spearman correlation failed to illuminate biological effects here, indicating that nonlinear modeling with larger samples and specific immune challenges are required to definitively parse out subtle differences. From a practical perspective, BDNF CNV may be useful as a resilience-related trait for selection in a context that involves mucosal immune response. Absolute copy number determination (i.e. ddPCR), longitudinal sampling, and expanded neuroimmune profiling (cytokines, SIgA, stress hormones, etc.) in future studies may help strengthen the connections between gene dosage and mucosal immunity via neuroimmune modulation.

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Data available upon request.

Conflicts of Interest:

The authors declare no conflict of interest.

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Conflict Of Interest

The authors declare no conflicts of interest associated with this manuscript.

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العلاقة بين تباين عدد نسخ جين BDNF والمناعة الخلوية في الأغنام العواسية: دراسة جزيئية-مناعية

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الخلاصة

تم تقييم المناعة الخلوية - والمتمثلة في الغلوبولينات المناعية *IgA*: الإفرافية (*SIgA*)، و *IgM*، و *IgG* - إضافةً إلى تباين عدد النسخ (*CNV*) لجين *BDNF* ومستويات التعبير الجيني له في عينات دم الأغنام العواسية. ($n = 40$) أجري تحليل تباين عدد النسخ باستخدام تفاعل البلمرة المتسلسل الكمي (*qPCR*) وفق طريقة *1ΔCt*، بينما جرى تحليل التعبير الجيني لجين *BDNF* باستخدام *qPCR* بتقنية *SYBR Green*، كما استُخدمت فحوصات *ELISA* لتحديد مستويات *IgM* و *IgA* و *IgG* في مصل الدم. استُخدم تحليل التباين (*ANOVA*) متبوعاً باختبار دنكن (*Duncan*) للمقارنة بين مجموعات الحذف (*DEL*)، والعدد الطبيعي للنسخ (*NOR*)، ومجموعة الازدواج (*DUP*)، كما استُخدمت معاملات ارتباط بيرسون وسبيرمان لتحديد العلاقات الخطية والرتبية. لم تُظهر مستويات *IgM* و *IgG* في المصل فروقاً معنوية بين مجموعات *CNV*، كما لم تُظهر ارتباطاً معنوياً مع ترميز *CNV* لجين *BDNF* ($P > 0.05$) ومع ذلك، أظهرت مستويات *IgA* في المصل تأثيراً معنوياً حسب المجموعات ($P \leq 0.05$)، إذ سجلت مجموعة العدد الطبيعي للنسخ (*NOR*) مستويات أعلى من *IgA* مقارنةً بمجموعتي *DEL* و *DUP*. وبالمثل، كان التعبير الجيني لجين *BDNF* أعلى بشكل معنوي في مجموعة *NOR* مقارنةً بمجموعتي *DEL* و *DUP*، ما يُظهر علاقة واضحة تعتمد على الجرعة الجينية تدل على حساسية التعبير الجيني لكمية النسخ وعدم خطية الاستجابة تجاه *CNV*. لم يكن معامل ارتباط بيرسون بين *IgA* وترميز *CNV* معنوياً ($P > 0.05$)، إلا أن قيمة معامل سبيرمان اقتربت من الدلالة الإحصائية مشيرةً إلى ارتباط رتبي موجب ($\rho = 0.305$)، ($P = 0.056$) وتشير التحليلات مجتمعةً إلى وجود علاقة بين *IgA* وجرعة *CNV* قد تصبح معنوية عند زيادة حجم العينة. تدل نتائجنا بالمجمل على أن جين *BDNF* يحتاج إلى نطاق جرعة أمثل للحفاظ على وظيفته، وأن التواصل العصبي-المناعي المرتبط بـ *BDNF* قد يسهم في الاستجابة المناعية المخاطية، مع كون *IgA* هو الغلوبولين المناعي الأكثر احتمالاً للتأثر بهذه التغيرات.

الكلمات المفتاحية: جين *BDNF*، تباين عدد النسخ (*CNV*)، المناعة الخلوية، حساسية الجرعة الجينية، الأغنام العواسية.