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Characterization of intronic SNP located in candidate genes influencing cattle temperament

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ABSTRACT - The objective of this study was to evaluate the effect of intronic single nucleotide polymorphisms (SNP) on temperament traits in a Brahman cattle population. The SNP located in *CACNG4*, *EXOC4*, *NRXN3*, and *SLC9A4* candidate genes were genotyped in 250 animals with temperament records of exit velocity, pen score, and temperament score. Rs3423464051:G>A in the *CACNG4* gene was associated with exit velocity and temperament score. An *in silico* analysis of the five intronic SNP showed that alternative alleles of *CACNG4*-rs3423464051, *EXOC4*-rs109393235, and *SLC9A4*-rs109722627 SNP could alter branch point sites during splicing, while a protein–protein interaction network analysis demonstrated a *GRIA2* gene-mediated interaction between *CACNG4* and *NRXN3*. The present results support previously reported evidence regarding bovine temperament-related candidate genes, particularly *CACNG4*, which is a confirmed candidate gene in need of more detailed analyses to reveal its role in temperament-related traits.

Keywords: beef cattle, behavior, molecular markers

1. Introduction

Temperament is a complex and economically relevant trait that reflects the welfare of an animal, directly affects production, and is closely related to genetic control (Haskell et al., 2014). Few studies have explored the characteristics of cattle temperament at the genomic level, due to the biological complexity and recording difficulty. Recent discoveries based on genome-wide association studies (GWAS) have revealed candidate genes implicated in cattle temperament (Lindholm-Perry et al., 2015; Valente et al., 2016; dos Santos et al., 2017; Chen et al., 2020).

Several previously identified genes that play a role in the central nervous system and excitatory synaptic transmission show differential expression during anxiety events related to cognitive impairment in humans and mice (Riley et al., 2016). These findings represent progress in defining the genetic architecture of cattle temperament and show that temperament regulation involves multiple genes. Knowledge of gene interactions influencing temperament and other traits is

limited, which hinders the elucidation of the group of genes that participate in regulating the trait of interest (Alvarenga et al., 2021).

Relevant evidence has recently been published, identifying similarities between bovine and human SNP related to temperament and involved in psychiatric and personality disorders through GWAS (Costilla et al., 2020; Paredes-Sánchez et al., 2020). These disorders may include neuroticism, schizophrenia, autism spectrum disorder, and developmental delay disorders related to the brain and cognition. Brahman cattle population studies identified 14 SNP with an effect on exit velocity in bovines (Paredes-Sánchez et al., 2020). From those, five SNP (rs3423464051, rs109393235, rs135982573, rs110864071, and rs109722627) were located on the intronic regions of the neurexin-3 (*NRXN3:NC_037337.1 BTA 10*), calcium voltage-gated channel auxiliary subunit gamma-4 (*CACNG4:NC_037346.1 BTA 19*), exocyst complex component-4 (*EXOC4:NC_037331.1 BTA 4*), and solute carrier family 9 member A4 (*SLC9A4: NC_037338.1 BTA 11*) genes (Paredes-Sánchez et al., 2020). The *CACNG4* and *SLC9A4* candidate genes have been previously implicated in human diseases (Guan et al., 2016; Calvete et al., 2021). Studies showed that members solute carrier gene family *SLC18A2*, *SLC9A9,* and *SLCO3A1* are associated with temperament traits (Garza-Brenner et al., 2017; Chen et al., 2020; Paredes-Sánchez et al., 2020). Furthermore, it was reported that SNP located in introns represented 41% of the variations associated with behavioral traits (Li et al., 2021). The relationship and concordance between these reports demonstrate the need to continue the efforts to characterize genes and their interactions to define the genetic architecture of cattle temperament (Garza-Brenner et al., 2017; Chen et al., 2020; Paredes-Sánchez et al., 2020; Li et al., 2021). Genetics contribute to differences in the expression of the phenotypes of complex traits, and population size can play an essential role in masking these differences. Focusing on individuals showing extreme expression of the trait of interest facilitated the discovery of associations (Paredes-Sánchez et al., 2020); however, the effect of variations and their distributions must be confirmed by studies in unselected populations (Petrakova et al., 2012).

Herein, we evaluated the effect of five previously reported intronic SNP (rs3423464051-*CACNG4*, rs109393235-*EXOC4*, rs135982573-*NRXN3*, rs110864071-*SLC9A4*, and rs109722627-*SLC9A4*) associated with temperament traits based on both *in silico* functional analysis and association analysis in a Brahman population.

2. Material and Methods

2.1. Ethical report

All practices complied with the Guide for the Care and Use of Agricultural Animals in Research and Teaching 2010 (AUP 2002–315).

2.2. Source of data

Data from 250 Brahman calves (126 males and 124 females) born in 2018 (n = 116; 56 males and 60 females), 2019 (n = 67; 37 males and 31 females), and 2020 (n = 66, 33 males and 33 females) were randomly selected and included in this study. The cattle management and temperament evaluation procedures were previously described (Schmidt et al., 2014). Briefly, the following phenotypic evaluations were performed at weaning. Exit velocity (EV), which is an objective test that measures the velocity of an animal traveling 1.83 m after receiving a stimulus, was measured with an infrared sensor (FarmerTek Inc., North Wylie, TX, USA) (Curley et al., 2006). Pen score (PS), which is a subjective measure based on the visual evaluation of animal behavior while confined to a pen in a group of five animals, was recorded on a scale from 1, indicating calm, to 5, indicating aggressive (Hammond et al., 1996). Finally, temperament score (TS) was calculated as the average of the EV and PS values: [TS = (EV + PS)/2] (Burdick et al., 2011).

Based on the reported EV records of the population, the animals were grouped into three temperament categories: calm, with a range of 0.16-1.82 m/s and 0.4-1.56 m/s; intermediate with a range of 1.83-3.12 m/s and 1.57-3.04 m/s; and temperamental with a range of 3.13-7.66 m/s and 3.05-10.83 m/s for females and males, respectively (Garza-Brenner et al., 2017; Paredes-Sánchez et al., 2020).

2.3. Genotyping

Ear notch samples were obtained from all animals at weaning. Samples were stored at −80 °C until DNA extraction. DNA extraction was performed using the commercial Genelute Mammalian Genomic DNA kit (Cat. G1N350, Sigma–Aldrich Co. LLC, St. Louis, Missouri, USA). Primers for the amplification of a gene region encompassing the rs3423464051-*CACNG4*, rs109393235-*EXOC4*, rs135982573-*NRXN3*, rs110864071-*SLC9A4*, and rs109722627-*SLC9A4* were designed in AmplifX 2.0.7 software. As part of Nextera® XT sequencing protocol (Part #15044223 Rev. B), primer design included an adapter sequence to each primer (forward: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG and reverse: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG). Table 1 shows the primer sequences and the expected PCR product sizes. Polymerase chain reaction (PCR) amplifications were done in 25 µL volumes on a Thermocycler DNA Engine TETRAD 2 Peltier thermal cycler (MJ Research, Inc., Waltham, MA, USA). The reaction mixtures contained 50 ng of genomic DNA, 1.5 mM MgCl2, 0.1 µM of each primer, 0.4 mM dNTPs, and 2.5 U of GoTaq® DNA Polymerase (Promega, Madison, WI, USA). A touchdown method was used, and the amplification profile included an initial denaturation step of 95 °C for 10 min, five three-step cycles of 45 s at 95 °C, an annealing step for 45 s that started at 65 °C but decreased by 2 °C during each cycle and 45 s at 72 °C, and 25 three-step cycles of 45 s each at 95 °C, 60 °C and 72 °C. The PCR products were verified in 2% agarose gel electrophoresis. Allelic discrimination of each SNP was achieved either by sequencing using the Nextera® XT DNA protocol in MINIseq Illumina equipment (Part #15044223 Rev. B) and by Restriction Fragment Length Polymorphism (PCR-RFLP). For the PCR-RFLP, amplicons were generated as in the previous description and were digested with the specific enzymes selected for allelic discrimination with Watcut software (Palmer, 2007), with digestion patterns verified *in silico* by NEBcutter (Vincze et al., 2003) (Table 2). The PCR products were digested using standard protocols under the conditions

Table 1 - Characteristics of candidate genes, SNP, and primers

Gene ID	Gene structure	SNP	POS	Primer	PCR size
CACNG4: NC_037346.1 BTA 19	51 Kbp Exon 4 Intron 3	rs3423464051	1: 63090595	F: GGTAGTGCCAGGGGGCC R: CACGTGGGGCTGTTGCTCCT	368 pb
EXOC4: NC_037331.1 BTA 4	806 Kbp Exon 18 Intron 17	rs109393235	7: 97051523	F: GGAGTACAGTGAAGAGAGAAAGA R: TTAACCTAAAGAGCATTCCACCA	368 pb
NRXN3: NC_037337.1 BTA 10	1.3 Mbp Exon 7 Intron 6	rs135982573	1: 90623799	F: TATATGTTCCTAAAATTTCTTCA R: TAATGGCAATGGCCTTTGC	368 pb
SLC9A4: NC_037338.1 BTA 11	56 Kbp Exon 12 Intron 11	rs110864071	2: 7257740	F: GGTCAGGGAGATCCCCTGGAG R: TATAGTTTCTTCATCTGTGAA	373 pb
		rs109722627	8: 7286313	F: TACCCAGGGTTCGAACCTGT R: CCTAATGCATTTTTAAAAACTGG	373 pb

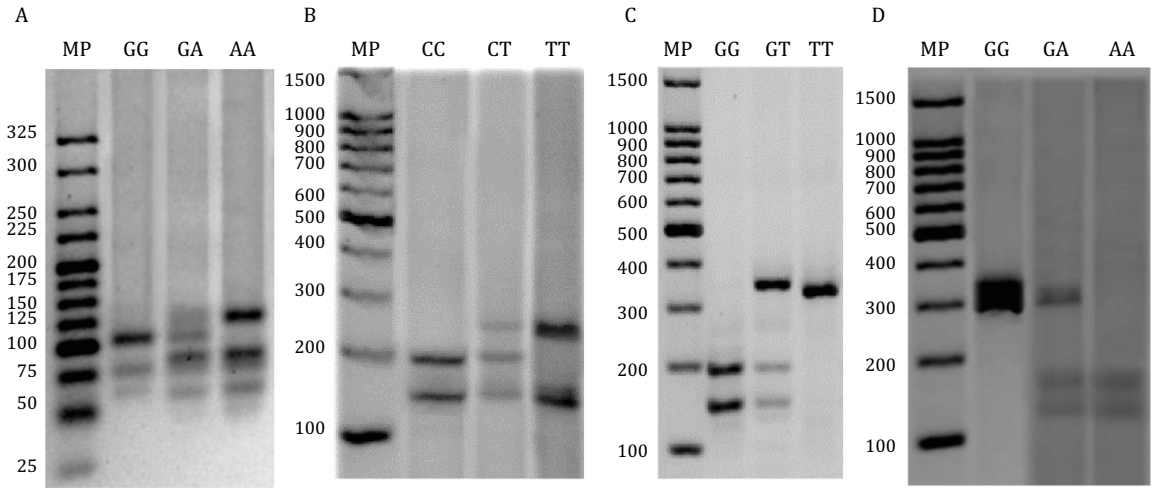
Gene ID includes gene abbreviation, reference sequence number, and *Bos taurus* autosome (BTA); gene structure - gene length and number of exons and introns; SNP - Ensembl database identifier; POS - intron locations after colon number of genomic position; primers: F - forward and R - reverse; PCR size - amplicon size in pair base (pb).

suggested by each enzyme provided and incubated at 37 °C during 5 h. The digested fragments were electrophoresed in 2.5% agarose gels stained with Syber Gold 1X and visualized on a transilluminator (Kodak Gel-Logic 112, Burlington, USA). Determination of genotype of each sample was achieved by visual analysis of the gels (Figure 1). A database with the genotypes was generated in the Excel tool of the Microsoft Office package. We used the database for computed allelic frequencies and Hardy-Weinberg equilibrium through the software Cervus 3.0.7 (Kalinowski et al., 2007).

Table 2 - Allelic discrimination of intronic SNP via RFLP analysis

Gene	SNP ID	Enzyme	Digestion pattern (bp)			
			A	C	G	T
CACNG4	rs3423464051	*Bs1*I	120, 77, 61, 55, 29, 26		104, 77, 61, 55, 29, 26, 16	
NRXN3	rs135982573	*Hyp*CH4V	182, 134, 52		316, 52	
SLC9A4	rs110864071	*Hinf*I		235, 138		190, 138, 45
	rs109722627	*Taq*I			189, 140, 44	329, 44

Gene - gene abbreviation; SNP ID - Ensembl database identifier; *CACNG4* - calcium voltage-gated channel auxiliary subunit gamma-4; *NRXN3* - neurexin-3; *SLC9A4* - solute carrier family 9 member A4; digestion pattern (bp) - digest pattern size in base pairs (bp).



MP - DNA marker ladder.
The figures show the PCR-RFLP results for each marker-genotype assignations.
A: For *CACNG4*-rs3423464051, the GG genotype is characterized by 104, 77, and 61 pb bands; AA genotype is distinguished for the 120 and 77-pb bands.
B: In the *SLC9A4*-rs110864071, the CC genotype displays two bands of 190 and 138-bp, while TT genotype has a differential 235 and 138-pb pattern.
C: The *SLC9A4*-rs109722627 GG genotype has two bands of 189 and 140 pb, while the TT genotype shows a main band of 329.
D: The GG genotype of *NRXN3*- rs135982573 shows a differential band of 316-pb and AA genotype, and has three bands of 182, 134, and 52-pb.
In all markers, the heterozygote carriers are characterized by a band pattern combining both homozygotes.

Figure 1 - Allelic discrimination of intronic SNP.

2.4. Statistical analysis

To determine the effect of loci on the studied temperament characteristics (EV, PS, and TS), a mixed model was fitted as follows:

$$Y_{ijklm} = \mu + Si_i + Sx_j + Y_k + \beta_1 X_l + \beta_2 X_l^2 + G_m + \varepsilon_{ijklm}$$

in which Y_{ijklm} was the random variable (EV, PS, TS), μ was the overall mean, Si_i was the random effect of the sire (i = 11), Sx_j was the fixed effect of animals' sex (j = male, female), Y_k was the fixed effects of animals' year of birth (k = 2018, 2019, 2020), $\beta_1 X_l + \beta_2 X_l^2$ was the linear and quadratic effect of age of dam (l = 2022-2019), G_m was the fixed effect of genotypes in assessed loci (m = 0, 1, 2), and ε_{ijklm} was de random residual error. Least square means were estimated of each trait and compared using a Bonferroni adjustment. All analyses were performed using SAS software (Statistical Analysis System, SAS OnDemand for Academics).

2.5. Functional analysis of intronic SNP

2.5.1. Prediction of the effect of intronic SNP on splicing sites

To perform an *in silico* analysis of the effects of previously associated intronic SNP (Paredes-Sánchez et al., 2020), a search for splicing sites was carried out in the region where the SNP were located. Briefly, for the generation of mature mRNA, exons need be identified and joined together in a precise process that requires the coordinated activity of small nuclear RNA. The ESEfinder 3.0 (Cartegni et al., 2003; Smith et al., 2006) tool enabled the identification of splicing site motifs. This tool analyzes 5',3' splice sites and branch sites to reveal motifs needed to correct splicing. A pair of input sequences (500 bp flanking the position of the SNP) that contained the alleles to be evaluated were identified, and the existence of a splicing site was determined based on the default threshold values (5 'donor = 6.67, 3 'acceptor = 6.632 and branch site = 0) suggested for the platform.

2.5.2. Interaction networks

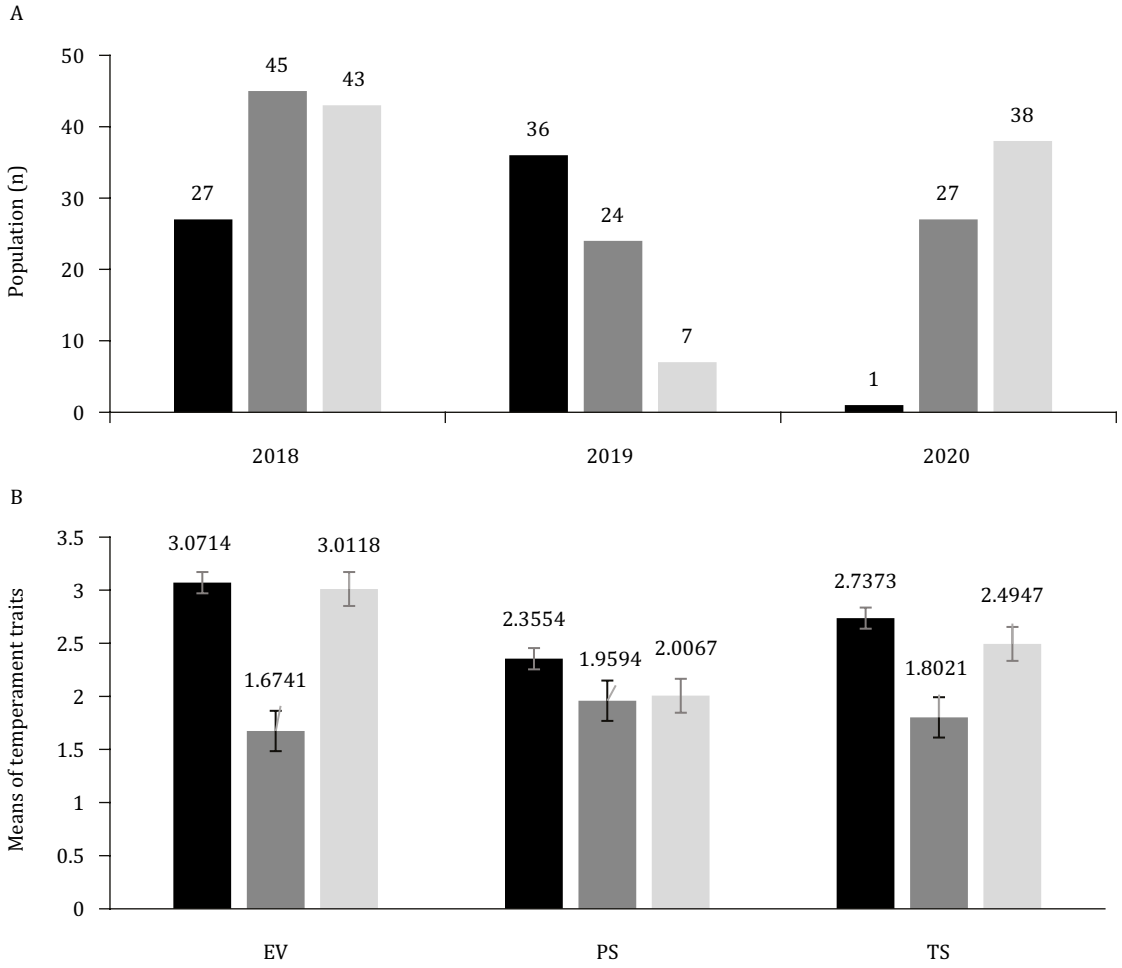
To evaluate the relationships between the studied genes and thus verify whether they act together in the control of bovine temperament, the protein–protein interactions of the *CACNG4*, *EXOC4*, *NRXN3*, and *SLC9A4* genes were considered based on the STRING database (Szklarczyk et al., 2015), displaying a reference of the interactions reported for homologous proteins in humans. The references used by the STRING database were known interactions based on curated databases and experimental determination; predicted interactions based on the gene neighborhood, gene fusions, and gene co-occurrence; and other interactions based on text mining, co-expression, and protein homology. Information that was in accord with the STRING interaction database was then employed to produce an individual score and a combined score.

3. Results

3.1. Temperament assessment

Regarding the temperament distribution of the population, the frequency of intermediate animals was highest, followed by temperamental and calm animals. The distribution of animals in the three groups differed depending on the year of birth (Figure 2A). Animals born in 2018 presented higher frequencies of the intermediate and temperamental phenotypes than the calm phenotypes, while in the 2019 population, the calm phenotype showed the highest frequency. The temperamental phenotype was greater in those animals born in 2020.

The results of our mixed model-based population analysis allowed us to identify differences in the mean results of the temperament tests according to year of birth. Calves born in 2019 presented lower mean EV and TS values (1.6741 and 1.8021) than those born in 2018 and 2020, which presented higher mean values (Figure 2B), whereas no differences by year were found between PS means.



A: Distribution of animals segregated into three temperament classifications and year of birth (black: calm; dark gray: intermediate; light gray: temperamental).
B: Mean values of exit velocity (EV), pen score (PS), and temperament score (TS) by year of birth (black: 2018, dark gray: 2019, and light gray: 2020).

Figure 2 - Population and temperament values by year of birth.

3.2. Effect of SNP on temperament traits

Four of the five evaluated SNP were determined to be polymorphic (*CACNG4*-rs3423464051, *NRXN3*-rs135982573, *SLC9A4*-rs110864071, and rs109722627) in the population (minor allele frequency > 10%). Based on the allelic frequency (Table 3), the *EXOC4* SNP was excluded from further evaluation.

Table 3 - Allelic frequencies of candidate genes for temperamental traits in the Brahman cattle population

Gene	SNP ID	N	HW	Allele			
				A	T	C	G
CACNG4	rs3423464051	241	NS	0.6245			0.3755
EXOC4	rs109393235	95	NS	0.9632			0.0368
NRXN3	rs135982573	208	NS	0.3269			0.6731
SLC9A4	rs110864071	248	NS		0.7278	0.2722	
	rs109722627	248	***		0.4315		0.5685

Gene - gene abbreviation; SNP ID - Ensembl database identifier; N - number of samples; HW - Hardy-Weinberg equilibrium; NS - non-significant.
*** Significant at the 0.1% level.

Only rs3423464051:G>A of the *CACNG4* gene was significantly associated with EV and TS (P<0.05; Table 4). For EV and TS, both homozygous genotypes were different (P = 0.0223 and P = 0.0211) from the heterozygote genotype and had a higher mean EV than heterozygote, ranging from 0.51-0.54 m/s for AA and GG; in the TS test, both genotypes also presented values higher than the heterozygous range of 0.36-0.46 for AA and GG.

Table 4 - Least-square means of the effect of assessed SNP on the temperament traits of Brahman cattle, exit velocity (EV), pen score (PS), and temperament score (TS) in the confirmation population

Gene	SNP ID	N	Genotype	EV	PS	TS
CACNG4	rs3423464051			(0.0223)	(0.1919)	(0.0211)
		33	GG	2.959±0.255a	2.386±0.243	2.650±0.220a
		115	AG	2.419±0.166b	1.995±0.187	2.186±0.169b
		93	AA	2.933±0.172a	2.1906±0.190	2.545±0.171a
SLC9A4	rs110864071			(0.2446)	(0.6771)	(0.3799)
		130	TT	2.560±0.162	2.133±0.177	2.336±0.159
		101	TC	2.788±0.170	2.100±0.181	2.435±0.163
		17	CC	2.982±0.328	2.336±0.291	2.648±0.263
SLC9A4	rs109722627			(0.3173)	(0.8153)	(0.5265)
		57	GG	2.604±0.205	2.202±0.206	2.395±0.187
		168	GT	2.655±0.156	2.101±0.175	2.366±0.159
		23	TT	3.054±0.286	2.146±0.260	2.600±0.236
NRXN3	rs135982573			(0.7698)	(0.9788)	(0.9042)
		66	GG	2.944±0.188	2.201±0.200	2.573±0.193
		72	GA	3.004±0.180	2.186±0.199	2.553±0.193
		25	AA	3.174±0.278	2.237±0.257	2.653±0.243

Gene - gene abbreviation; SNP ID - Ensembl database identifier; N - number of samples.
P-values are in parentheses.
a,b - Means with different letters for each trait and SNP are significantly different (P<0.05).

3.3. Prediction of the effect of SNP on splicing sites

Because of the locations of the SNP in the introns of genes, the SNP were analyzed to determine the effect of each alternative allele in the splicing process (Table 5). The allelic variants in *CACNG4, EXOC4*, and *SLC9A4* caused a change in the branch point (BS) recognition site, although the SNP in the *NRXN3* gene did not. According to the prediction matrix, the *CACNG4*-rs3423464051, *EXOC4*-rs109393235, and *SLC9A4*-rs109722627 SNP had an effect on BS due to a change in the alternative allele.

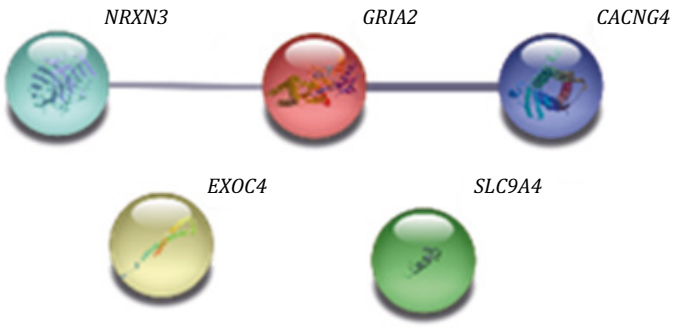
3.4. Protein–protein interaction analysis

The protein–protein interaction analysis showed that the tested proteins did not interact directly (Figure 3). However, *CACNG4* and *NRXN3* were connected through an interaction with the *GRIA2* gene (glutamate receptor 2). This interaction was supported by functional evidence from co-expression, experimental or biochemical data, and data mining in the STRING database, where the combined scores representing evidence of interactions with *GRIA2* were 0.862 for *CACNG4* and 0.578 for *NRXN3*.

Table 5 - Sites and prediction values of the effect of intronic SNP on the splicing mechanism

Gene	SNP ID	Allele	Motif	Position	Site	Score
CACNG4	rs3423464051	G	Branch Site	500	GGGTTAT	0.4412
		A	Branch Site	-	-	-
EXOC4	rs109393235	A	Branch Site	500	CTCTCAT	5.9828
		G	Branch Site	-	-	-
SLC9A4	rs110864071	T	Branch Site	506	ATCTCAT	4.0119
		C	Branch Site	506	ACCTCAT	4.6018
	rs109722627	G	Branch Site	503	ACTCGAA	0.1398
		T	Branch Site	-	-	-
		G	Branch Site	504	CTCGAAC	0.9333
		T	Branch Site	504	CTCTAAC	6.7452

Gene - gene abbreviation; SNP ID - Ensembl database identifier; motif - splicing motif site; site: sequence heptamer of recognition.



Candidate genes linked by a line have a prediction based on physical or functional interaction. The *GRIA2* gene mediates the interaction between the *NRXN3* and *CACNG4* genes. However, the *EXOC4* and *SLC9A4* genes do not exhibit direct interactions with each other or with other genes.

Figure 3 - Protein–protein interactions of temperament-related candidate genes.

4. Discussion

4.1. SNP effects on temperament: Role of the *CACNG4* gene in bovine temperament

Polymorphisms in four genes previously associated with temperament traits in beef cattle were assessed, and rs3423464051 of the *CACNG4* gene was confirmed to be associated with EV and TS in a Brahman cattle population. Previous studies of this SNP showed that genotype AA was significantly different from genotypes GG and AG (Paredes-Sánchez et al., 2020), and the authors ascertained a genotypic effect reflected by the AA genotype with a higher mean EV value than the heterozygous AG and homozygous GG genotypes. Here, we were unable to differentiate the effect of each homozygous genotype. It is important to increase the investigated sample number to confirm whether rs3423464051 may be useful for identifying temperamental animals.

The functions of most genes harboring markers associated with cattle temperament and their roles in determining this trait are still unclear. To elucidate these functions, the study of temperament relies on gene orthology, since neural disorders and addictions present similarities with temperament regarding the genes involved in these phenotypes. Bioinformatic approaches have been used as an essential tool facilitating the analysis of complex traits in different organisms. These efforts focus on the identification of a candidate gene and the characterization of potential mutations affecting the trait (Zhang et al., 2017). Here, we applied two bioinformatic approaches to obtain evidence about the roles of the studied intronic SNP. Specifically, the confirmed candidate gene *CACNG4* (encoding

the gamma-4 subunit) identified in cattle has been reported to be involved in the MAP kinase and oxytocin signaling pathways and in contraction function and heart muscle problems, as reported in the KEGG pathway database (Kanehisa and Sato, 2020).

A specific function of this gene in representative cattle temperament pathways is still unknown. However, *CACNG4* plays an integral role in calcium channels, allowing the entry of Ca^{2+} into the cell, which in turn uses it as a secondary messenger in the functions and differentiation of nerve cells. The associations of *CACNG4*, thus, indicate that it is related to information transmission activities and the formation of nerve cells (Yin et al., 2016).

CACNG4 presents similar characteristics among human, mouse, and bovine models (Kious et al., 2002). Evidence indicates that gamma 2-8 subunits are not found in neuronal or cardiac calcium complexes but act as modifying proteins of the AMPA glutamate receptor (Heyes et al., 2015). The modifying activity of *CACNG4* toward the AMPA receptor is an important finding in temperament research, and it is essential to investigate whether *CACNG4* modification acts in a general way or is specific to AMPA receptor subunits.

Our *in silico* protein–protein analysis included modifying proteins of AMPA receptors. The interaction between *CACNG4* and *NRXN3* is mediated by glutamate receptor 2 (*GRIA2*, AMPA subunit), which acts as a ligand in channels of the central nervous system and plays an essential role in excitatory synaptic transmission according to the STRING database. *GRIA2* has been previously associated with temperament (Lindholm-Perry et al., 2015), and subsequently identified an interaction between *NRXN3* and *GRIA2* (Paredes-Sánchez et al., 2020). Reports in humans have shown that the *GRIA2* gene works in conjunction with AMPA-type glutamate receptors. One of its primary activities is to connect excitatory transmissions underlying perception, cognition, and movement. Its function requires auxiliary proteins such as calcium channel subunits (*CACNG4*). It participates in multiple activities in the brain; its expression has been identified in subcortical regions (Shen and Limon, 2021) and its dysregulation has been associated with abnormal behavior. Some gamma calcium channel subunits, such as *CACNG2* and *CACNG4,* show similarities in the binding sites between AMPA receptor subunits and PDZ proteins (proteins with AMPA subunit interaction domains), which suggests a physical association with AMPA receptors and PDZ proteins during synapse formation (Kious et al., 2002). The AMPA receptor must contain at least one *GRIA2* subunit, which strengthens the interaction domain (Tomita et al., 2001). In contrast to *CACNG4*, acting in the subcortex of the brain, both *GRIA2* and *NRXN3* act in the hippocampus. Specifically, the *NRXN3* protein is necessary for the control of postsynaptic AMPA expression and the formation of the postsynaptic density protein complex (Bayés et al., 2011; Aoto et al., 2015). The *NRXN3* gene has been associated with human neurodevelopmental disorders and with a locus related to characteristics of empathy or fear in mice (Di Gregorio et al., 2017; Keum et al., 2018). In general, the pathways involving the *CACNG4*, *GRIA2*, and *NRXN3* genes include postsynaptic activities, protein aggregation, and functions within complexes formed at synapses, and variants in these genes can disturb these complexes, with proportionate changes in signal transmission to the environment.

An interesting characteristic of intronic SNP is their potential to exert regulatory effects. Intronic SNP have been associated with the splicing and alteration of recognition sites for spliceosome binding (Kol et al., 2005). Due to the gene locations of all intronic SNP analyzed in this report, their possible roles in splicing were evaluated. Three of these five SNP exhibited one allelic variant with the potential to alter branch point recognition. Although the splicing machinery is highly conserved in mammals, branch point sequences are highly variable, and this variability may be a mechanism allowing the alternative splicing of a gene (Ast, 2004). It has been reported that the presence of a SNP near a branch point site that does not interrupt splicing recognition can lead to a change in splicing efficiency and, consequently, increased or decreased expression at the mRNA and protein levels (Zhang et al., 2018; Mucaki et al., 2020).

Based on the characterization and prediction of intronic branch points in the bovine genome, it has been suggested that branch points in bovine introns occur within degenerate heptamers

with a consensus sequence of "nnyTrAy", indicating conserved thymine and adenine residues in the branch point sequence (Kadri et al., 2021). Therefore, the conserved sequence can change in the presence of an SNP allele that interferes with the recognition of the sequence by the spliceosome. Branch points analysis in human model indicate a strong preference toward adenine (Kadri et al., 2021). Simultaneously, the *EXOC4* and *CACNG4* markers harbor the A allele, where we found that allelic change in the preferential residues of the heptamer causes the loss of the recognition site. In human diseases, it is common to find structural variations associated with a pathological condition or that contribute to it (Kozyrev et al., 2008). The SNP located at a branch point accompanied by a nonsynonymous change with strong linkage disequilibrium caused variation in gene expression and protein localization by inducing splicing and modifying the length of the protein (Martínez-Bueno et al., 2018). This was experimentally confirmed through the construction of a minigene with allelic variants at intronic and exonic positions corresponding to the associated SNP (Kozyrev et al., 2012).

5. Conclusions

The genotype-phenotype association analysis showed the effect of *CACNG4* gene polymorphism on exit velocity and temperament score. *In silico* analysis allowed us to infer that *CACNG4* and *NRXN3* are regulated by the *GRIA2* gene, previously associated with bovine temperament. Likewise, intronic allelic variants can alter branch points, which are essential sites for spliceosome recognition.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: G. Ruiz-De-La-Cruz, A.M. Sifuentes-Rincón, F.A. Paredes-Sánchez and R.D. Randel. Data curation: T.H. Welsh Jr. and R.D. Randel. Formal analysis: G. Ruiz-De-La-Cruz, A.M. Sifuentes-Rincón, F.A. Paredes-Sánchez and E. Casas. Funding acquisition: A.M. Sifuentes-Rincón. Investigation: G. Ruiz-De-La-Cruz, E. Casas and R.D. Randel. Methodology: F.A. Paredes-Sánchez, G.M. Parra-Bracamonte, T.H. Welsh Jr., D.G. Riley and G. Perry. Project administration: A.M. Sifuentes-Rincón. Writing – original draft: G. Ruiz-De-La-Cruz and A.M. Sifuentes-Rincón. Writing – review & editing: F.A. Paredes-Sánchez, G.M. Parra-Bracamonte, E. Casas, T.H. Welsh Jr., D.G. Riley, G. Perry and R.D. Randel.

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References

Alvarenga, A. B.; Oliveira, H. R.; Chen, S. Y.; Miller, S. P.; Marchant-Forde, J. N.; Grigoletto, L. and Brito, L. F. 2021. A systematic review of genomic regions and candidate genes underlying behavioral traits in farmed mammals and their link with human disorders. Animals 11:715. https://doi.org/10.3390/ani11030715

Aoto, J.; Földy, C.; Ilcus, S. M. C.; Tabuchi, K. and Südhof, T. C. 2015. Distinct circuit-dependent functions of presynaptic neurexin-3 at GABAergic and glutamatergic synapses. Nature Neuroscience 18:997-1007. https://doi.org/10.1038/nn.4037

Ast, G. 2004. How did alternative splicing evolve? Nature Reviews Genetics 5:773-782. https://doi.org/10.1038/nrg1451

Bayés, À.; van de Lagemaat, L. N.; Collins, M. O.; Croning, M. D. R.; Whittle, I. R.; Choudhary, J. S. and Grant, S. G. N. 2011. Characterization of the proteome, diseases and evolution of the human postsynaptic density. Nature Neuroscience 14:19-21. https://doi.org/10.1038/nn.2719

Burdick, N. C.; Agado, B.; White, J. C.; Matheney, K. J.; Neuendorff, D. A.; Riley, D. G.; Vann, R. C.; Welsh Jr., T. H. and Randel, R. D. 2011. Technical note: Evolution of exit velocity in suckling Brahman calves. Journal of Animal Science 89:233-236. https://doi.org/10.2527/jas.2010-2973

Calvete, O.; Reyes, J.; Valdés-Socin, H.; Martin, P.; Marazuela, M.; Barroso, A.; Escalada, J.; Castells, A.; Torres-Ruiz, R.; Rodríguez-Perales, S.; Currás-Freixes, M. and Benítez, J. 2021. Alterations in SLC4A2, SLC26A7 and SLC26A9 drive acid–base imbalance in gastric neuroendocrine tumors and uncover a novel mechanism for a co-occurring polyautoimmune scenario. Cells 10:3500. https://doi.org/10.3390/cells10123500

Cartegni, L.; Wang, J.; Zhu, Z.; Zhang, M. Q. and Krainer, A. R. 2003. ESEfinder: a web resource to identify exonic splicing enhancers. Nucleic Acids Research 31:3568-3571. https://doi.org/10.1093/nar/gkg616

Chen, Q.; Zhang, F.; Qu, K.; Hanif, Q.; Shen, J.; Jia, P.; Ning, Q.; Zhan, J.; Zhang, J.; Chen, N.; Chen, H.; Huang, B. and Lei, C. 2020. Genome-wide association study identifies genomic loci associated with flight reaction in cattle. Journal of Animal Breeding and Genetics 137:477-485. https://doi.org/10.1111/jbg.12461

Costilla, R.; Kemper, K. E.; Byrne, E. M.; Porto-Neto, L. R.; Carvalheiro, R.; Purfield, D. C.; Doyle, J. L.; Berry, D. P.; Moore, S. S.; Wray, N. R. and Hayes, B. J. 2020. Genetic control of temperament traits across species: association of autism spectrum disorder risk genes with cattle temperament. Genetics Selection Evolution 52:51. https://doi.org/10.1186/s12711-020-00569-z

Curley Jr., K. O.; Paschal, J. C.; Welsh Jr., T. H. and Randel, R. D. 2006. Technical note: Exit velocity as a measure of cattle temperament is repeatable and associated with serum concentration of cortisol in Brahman bulls. Journal of Animal Science 84:3100-3103. https://doi.org/10.2527/jas.2006-055

Di Gregorio, E.; Riberi, E.; Belligni, E. F.; Biamino, E.; Spielmann, M.; Ala, U.; Calcia, A.; Bagnasco, I.; Carli, D.; Gai, G.; Giordano, M.; Guala, A.; Keller, R.; Mandrile, G.; Arduino, C.; Maffè, A.; Naretto, V. G.; Sirchia, F.; Sorasio, L.; Ungari, S.; Zonta, A.; Zacchetti, G.; Talarico, F.; Pappi, P.; Cavalieri, S.; Giorgio, E.; Mancini, C.; Ferrero, M.; Brussino, A.; Savin, E.; Gandione, M.; Pelle, A.; Giachino, D. F.; De Marchi, M.; Restagno, G.; Provero, P.; Silengo, M. C.; Grosso, E.; Buxbaum, J. D.; Pasini, B.; De Rubeis, S.; Brusco A. and Ferrero, G. B. 2017. Copy number variants analysis in a cohort of isolated and syndromic developmental delay/intellectual disability reveals novel genomic disorders, position effects and candidate disease genes. Clinical Genetics 92:415-422. https://doi.org/10.1111/cge.13009

dos Santos, F. C.; Peixoto, M. G. C. D.; Fonseca, P. A. S.; Pires, M. F. A.; Ventura, R. V.; Rosse, I. C.; Bruneli, F. A. T.; Machado, M. A. and Carvalho, M. R. S. 2017. Identification of candidate genes for reactivity in Guzerat (*Bos indicus*) cattle: A genome-wide association study. PLoS One 12:e0169163. https://doi.org/10.1371/journal.pone.0169163

Garza-Brenner, E.; Sifuentes-Rincón, A. M.; Randel, R. D.; Paredes-Sánchez, F. A.; Parra-Bracamonte, G. M.; Arellano Vera, W.; Rodríguez Almeida F. A. and Segura Cabrera, A. 2017. Association of SNPs in dopamine and serotonin pathway genes and their interacting genes with temperament traits in Charolais cows. Journal of Applied Genetics 58:363-371. https://doi.org/10.1007/s13353-016-0383-0

Guan, F.; Zhang, T.; Liu, X.; Han, W.; Lin, H.; Li, L.; Chen, G. and Li, T. 2016. Evaluation of voltage-dependent calcium channel γ gene families identified several novel potential susceptible genes to schizophrenia. Scientific Reports 6:24914. https://doi.org/10.1038/srep24914

Hammond, A. C.; Olson, T. A.; Chase Jr., C. C.; Bowers, E. J.; Randel, R. D.; Murphy, C. N.; Vogt, D. W. and Tewolde, A. 1996. Heat tolerance in two tropically adapted *Bos taurus* breeds, Senepol and Romosinuano, compared with Brahman, Angus, and Hereford cattle in Florida. Journal of Animal Science 74:295-303. https://doi.org/10.2527/1996.742295x

Haskell, M. J.; Simm, G. and Turner, S. P. 2014. Genetic selection for temperament traits in dairy and beef cattle. Frontiers in Genetics 5:368. https://doi.org/10.3389/fgene.2014.00368

Heyes, S.; Pratt, W. S.; Rees, E.; Dahimene, S.; Ferron, L.; Owen, M. J. and Dolphin, A. C. 2015. Genetic disruption of voltage-gated calcium channels in psychiatric and neurological disorders. Progress in Neurobiology 134:36-54. https://doi.org/10.1016/j.pneurobio.2015.09.002

Kadri, N. K.; Mapel, X. M. and Pausch, H. 2021. The intronic branch point sequence is under strong evolutionary constraint in the bovine and human genome. Communications Biology 4:1206. https://doi.org/10.1038/s42003-021-02725-7

Kalinowski, S. T.; Taper, M. L. and Marshall, T. C. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Molecular Ecology 16:1099-1106. https://doi.org/10.1111/j.1365-294x.2007.03089.x

Kanehisa, M. and Sato, Y. 2020. KEGG Mapper for inferring cellular functions from protein sequences. Protein Science 29:28-35. https://doi.org/10.1002/pro.3711

Keum, S.; Kim, A.; Shin, J. J.; Kim, J. H.; Park, J. and Shin, H. S. 2018. A missense variant at the *Nrxn3* locus enhances empathy fear in the mouse. Neuron 98:588-601. https://doi.org/10.1016/j.neuron.2018.03.041

Kious, B. M.; Baker, C. V. H.; Bronner-Fraser, M. and Knecht, A. K. 2002. Identification and characterization of a calcium channel γ subunit expressed in differentiating neurons and myoblasts. Developmental Biology 243:249-259. https://doi.org/10.1006/dbio.2001.0570

Kol, G.; Lev-Maor, G. and Ast, G. 2005. Human-mouse comparative analysis reveals that branch-site plasticity contributes to splicing regulation. Human Molecular Genetics 14:1559-1568. https://doi.org/10.1093/hmg/ddi164

Kozyrev, S. V.; Abelson, A. K.; Wojcik, J.; Zaghlool, A.; Reddy, M. V. P. L.; Sanchez, E.; Gunnarsson, I.; Svenungsson, E.; Sturfelt, G.; Jönsen, A.; Truedsson, L.; Pons-Estel, B. A.; Witte, T.; D'Alfonso, S.; Barizzone, N.; Danieli, M. G.; Gutierrez, C.; Suarez, A.; Junker, P.; Laustrup, H.; González-Escribano, M. F.; Martin, J.; Abderrahim, H. and Alarcón-Riquelme, M. E. 2008. Functional variants in the B-cell gene *BANK1* are associated with systemic lupus erythematosus. Nature Genetics 40:211-216. https://doi.org/10.1038/ng.79

Kozyrev, S. V.; Bernal-Quirós, M.; Alarcón-Riquelme, M. E. and Castillejo-López, C. 2012. The dual effect of the lupus-associated polymorphism rs10516487 on *BANK1* gene expression and protein localization. Genes & Immunity 13:129-138. https://doi.org/10.1038/gene.2011.62

Li, H. D.; Funk, C. C.; McFarland, K.; Dammer, E. B.; Allen, M.; Carrasquillo, M. M.; Levites, Y.; Chakrabarty, P.; Burgess, J. D.; Wang, X.; Dickson, D.; Seyfried, N. T.; Duong, D. M.; Lah, J. J.; Younkin, S. G.; Levey, A. I.; Omenn, G. S.; Ertekin-Taner, N.; Golde, T. E. and Price, N. D. 2021. Integrative functional genomic analysis of intron retention in human and mouse brain with Alzheimer's disease. Alzheimer's & Dementia 17:984-1004. https://doi.org/10.1002/alz.12254

Lindholm-Perry, A. K.; Kuehn, L. A.; Freetly, H. C. and Snelling, W. M. 2015. Genetic markers that influence feed efficiency phenotypes also affect cattle temperament as measured by flight speed. Animal Genetics 46:60-64. https://doi.org/10.1111/age.12244

Martínez-Bueno, M.; Oparina, N.; Dozmorov, M. G.; Marion, M. C.; Comeau, M. E.; Gilkeson, G.; Kamen, D.; Weisman, M.; Salmon, J.; McCune, J. W.; Harley, J. B.; Kimberly, R.; James, J. A.; Merrill, J.; Montgomery, C.; Langefeld, C. D. and Alarcón-Riquelme, M. E. 2018. Trans-ethnic mapping of *BANK1* identifies two independent SLE-risk linkage groups enriched for co-transcriptional splicing marks. International Journal of Molecular Sciences 19:2331. https://doi.org/10.3390/ijms19082331

Mucaki, E. J.; Shirley, B. C. and Rogan, P. K. 2020. Expression changes confirm genomic variants predicted to result in allele-specific, alternative mRNA splicing. Frontiers in Genetics 11:109. https://doi.org/10.3389/fgene.2020.00109

Palmer, M. 2007. WatCut: An on-line tool for restriction analysis, silent mutation scanning, and SNP-RFLP analysis. University of Waterloo, Ontario, Canada.

Paredes-Sánchez, F. A.; Sifuentes-Rincón, A. M.; Casas, E.; Arellano-Vera, W.; Parra-Bracamonte, G. M.; Riley, D. G.; Welsh Jr., T. H. and Randel, R. D. 2020. Novel genes involved in the genetic architecture of temperament in Brahman cattle. PloS One 15:e0237825. https://doi.org/10.1371/journal.pone.0237825

Petrakova, L.; Kerzienė, S. and Razmaitė, V. 2012. Contribution of different breeds to Lithuanian red cattle using pedigree information with only a fraction of the population analyzed. Veterinarija ir Zootechnika 57:62-66.

Riley, D. G.; Gill, C. A.; Boldt, C. R.; Funkhouser, R. R.; Herring, A. D.; Riggs, P. K.; Sawyer, J. E.; Lunt, D. K. and Sanders, J. O. 2016. Crossbred *Bos indicus* steer temperament as yearlings and whole genome association of steer temperament as yearlings and calf temperament post-weaning. Journal of Animal Science 94:1408-1414. https://doi.org/10.2527/jas.2015-0041

Schmidt, S. E.; Neuendorff, D. A.; Riley, D. G.; Vann, R. C.; Willard, S. T.; Welsh Jr., T. H. and Randel, R. D. 2014. Genetic parameters of three methods of temperament evaluation of Brahman calves. Journal of Animal Science 92:3082-3087. https://doi.org/10.2527/jas.2013-7494

Shen, K. and Limon, A. 2021. Transcriptomic expression of AMPA receptor subunits and their auxiliary proteins in the human brain. Neuroscience Letters 755:135938. https://doi.org/10.1016/j.neulet.2021.135938

Smith, P. J.; Zhang, C.; Wang, J.; Chew, S. L.; Zhang, M. Q. and Krainer, A. R. 2006. An increased specificity score matrix for the prediction of SF2/ASF-specific exonic splicing enhancers. Human Molecular Genetics 15:2490-2508. https://doi.org/10.1093/hmg/ddl171

Szklarczyk, D.; Franceschini, A.; Wyder, S.; Forslund, K.; Heller, D.; Huerta-Cepas, J.; Simonovic, M.; Roth, A.; Santos, A.; Tsafou, K. P.; Kuhn, M.; Bork, P.; Jensen, L. J. and von Mering, C. 2015. STRING v10: protein–protein interaction networks, integrated over the tree of life. Nucleic Acids Research 43:D447-D452. https://doi.org/10.1093/nar/gku1003

Tomita, S.; Nicoll, R. A. and Bredt, D. S. 2001. PDZ protein interactions regulating glutamate receptor function and plasticity. Journal of Cell Biology 153:F19-F24. https://doi.org/10.1083/jcb.153.5.F19

Valente, T. S.; Baldi, F.; Sant'Anna, A. C.; Albuquerque, L. G. and Paranhos da Costa, M. J. R. 2016. Genome-wide association study between single nucleotide polymorphisms and flight speed in Nellore cattle. PLoS One 11:e0156956. https://doi.org/10.1371/journal.pone.0156956

Vincze, T.; Posfai, J. and Roberts, R. J. 2003. NEBcutter: a program to cleave DNA with restriction enzymes. Nucleic Acids Research 31:3688-3691. https://doi.org/10.1093/nar/gkg526

Yin, K.; Baillie, G. J. and Vetter, I. 2016. Neuronal cell lines as model dorsal root ganglion neurons: a transcriptomic comparison. Molecular Pain 12:1744806916646111. https://doi.org/10.1177/1744806916646111

Zhang, Q.; Fan, X.; Wang, Y.; Sun, M. A.; Shao, J. and Guo, D. 2017. BPP: a sequence-based algorithm for branch point prediction. Bioinformatics 33:3166-3172. https://doi.org/10.1093/bioinformatics/btx401

Zhang, X.; Wu, Y.; Cai, F.; Liu, S.; Bromley-Brits, K.; Xia, K. and Song, W. 2018. A novel Alzheimer-associated SNP in *Tmp21* increases amyloidogenesis. Molecular Neurobiology 55:1862-1870. https://doi.org/10.1007/s12035-017-0459-9