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ORIGINAL ARTICLE

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Association between temperament related traits and single nucleotide polymorphisms in the serotonin and oxytocin systems in Merino sheep

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Abstract

Animal temperament is defined as the consistent behavioral and physiological differences that are seen between individuals in response to the same stressor. Neurotransmitter systems, like serotonin and oxytocin in the central nervous system, underlie variation in behavioral traits in humans and other animals. Variations like single nucleotide polymorphisms (SNPs) in the genes for tryptophan 5-hydroxylase (*TPH2*), the serotonin transporter (*SLC6A4*), the serotonin receptor (*HTR2A*), and the oxytocin receptor (*OXTR*) are associated with behavioral phenotype in humans. Thus, the objective of this study was to identify SNPs in those genes and to test if those variations are associated with the temperament in Merino sheep. Using ewes from the University of Western Australia temperament flock, which has been selected on emotional reactivity for more than 20 generations, eight SNPs (rs107856757, rs107856818, rs107856856 and rs107857156 in *TPH2*, rs20917091 in *SLC6A4*, rs17196799 and rs17193181 in *HTR2A*, and rs17664565 in *OXTR*) were found to be distributed differently between calm and nervous sheep. These eight SNPs were then genotyped in 260 sheep from a flock that has never been selected on emotional reactivity, followed by the estimation of the behavioral traits of those 260 sheep using an arena test and an isolation box test. We found that several SNPs in *TPH2* (rs107856757, rs107856818, rs107856856 and rs107857156) were in strong linkage disequilibrium, and all were associated with behavioral phenotype in the non-selected sheep. Similarly, rs17196799 in *HTR2A* was also associated with the behavioral phenotype.

KEYWORDS

merino sheep, oxytocin, serotonin, single nucleotide polymorphisms, temperament

1 | INTRODUCTION

Temperament can be defined as the consistent behavioral and physiological differences that are observed between individuals in response to an eliciting event.[1] The differences are thought to reflect the individual's perception of the surrounding situation. Nonhuman animals, in a similar way as humans, evaluate a situation based on particular characteristics of the eliciting event such as its suddenness, familiarity, pleasantness, controllability, and predictability, and how the event accords or deviates from their expectations.[2] The

perception of a situation leads to an emotional state that is experienced by the individual. The emotion then elicits physiological and behavioral responses, and those responses have been used to quantify the temperament of individual animals.[2,3] Temperament is of more than academic interest because temperament can influence immunity, and production traits such as ovulation rate, growth performance, and meat quality.[4-6] In sheep, the phenotype and the genetic heritability of temperament traits have been assessed using behavioral tests like an arena test and an isolation box test.[7-9] Similarly, the temperament of bovines has been assessed for decades using behavioral tests such as the open field arena test, a flight speed test, an exit velocity test, and a crush score test.[4,10,11] However, the classic behavior tests, like the arena test, isolation box test, and flight speed test, are time and labor consuming, and difficult to carry out in young animals.[12] These limitations reduce the utility of temperament selection programs on farm, even if that selection can provide economic benefits.[13]

The development of sequencing techniques has led to the discovery of correlations between many single nucleotide polymorphisms (SNPs) and personality traits in humans (fear, impulsivity, aggression, and impulsivity).[14,15] Personality traits in humans are qualitatively equivalent to the traits that are associated with animal temperament. Recently, two SNPs that are associated with the temperament phenotype of sheep were derived from SNPs that had been described in humans.[16] An SNP in the gene encoding for the dopamine receptor 2 (SNP939),[16] and a variant of the gene that encodes the enzyme cytochrome P450 17α-hydroxylase/17,20-lyase (SNP628), an enzyme that is involved in the production of cortisol, were both associated with sheep temperament.[16] Moreover, SNPs in genes from the dopamine and serotonin pathway are associated with temperament traits in Charolais cows.[17] These findings not only indicate the importance of genes that code for elements of brain pathways that are important in temperament determination in cattle, but also hint that the SNPs in those genes might be associated with the temperament phenotype of animals in general.

Serotonin (5-HT) is a widely distributed neurotransmitter in the mammalian brain that has been reported to play an important role in psychological state.[18-21] In humans, several SNPs in the serotoninergic system have been associated with personality traits such as fear, impulsivity, and aggression.[15] Variations in the gene that codes for tryptophan 5-hydroxylase 2 (*TPH2*), the rate limiting enzyme in the synthesis of neuronal serotonin,[22] affect the serotonin concentration in the brain as well as behavior traits.[23,24] Two SNPs (rs4570625 and rs17110747) in *TPH2* have been associated with depressive disorder,[25] while, SNP 4570625 in *TPH2* has been associated with emotional dysregulation.[26] Other SNPs in *TPH2* (rs7305115, rs4290270, rs11178997 and rs13864923) have been related to emotional stability and suicidal behavior.[27-29] Further along the serotonin pathway, SNPs in the 5-HT transporter (*SLC6A4*, rs140701, rs3813034) and the 5-HT receptor (HTR2A, rs6313 and rs7322347) have been associated with anxiety-related traits, emotional dysregulation, and aggressive behavior in humans.[30-34] The serotonergic system is similar in other mammals and the results of pharmacological depletion of brain 5-HT in sheep suggests that the serotonin pathways is involved in affective state.[35] It is likely that genetic variations in the serotonin pathway in sheep will be associated with phenotypic differences in temperament.

In addition to the role that dopamine and serotonin play in the mammalian emotional response, oxytocin is important in social behaviors such as maternal and affiliative behavior,[36] recognition[37] and trust[38] in humans. Variations in the gene for the oxytocin receptor (*OXTR*) are associated with prosocial temperament (rs53576), reactivity to stressors (rs53576) and aggressive behaviors (rs6770632 and rs1042778).[39-41] Polymorphisms in *OXTR* (rs53576, rs2254298, and rs2228485) have been associated with emotional loneliness.[42] In domesticated animals, oxytocin has been proposed as a marker of voluntary homo-specific and hetero-specific social contact.[43] Importantly in sheep, oxytocinergic neurons are activated in several regions of the lamb brain when a known caregiver is present, suggesting that oxytocinergic pathways play a role in the response to positive social contact.[44] Since temperament in sheep has been defined by the response that a sheep shows during a social challenge, such as isolation,[5] polymorphisms in the oxytocin pathway could be associated with phenotypic temperament in sheep.

We have investigated the role of polymorphic variations in the central neurotransmitter systems described above in the expression of temperament in sheep. We first looked for SNPs in the genes for *TPH2*, *SLC6A4*, *HTR2*A, and *OXTR* in sheep and then investigated the association between those SNPs and the behavioral phenotype of temperament. The first objective was to identify SNPs that are associated with a hypo-(calm) and a hyper-(nervous) response to particular stressors in sheep. We first used sheep from the University of Western Australia temperament flock that has been selected for 20 generations on the basis of behavioral phenotypic responses to isolation and human presence.[45] Secondly, we tested whether the SNPs that we identify in the first part were associated with differences in behavioral reactivity to isolation and human contact in sheep that had never been selected for temperament.

We conducted the experimentation in two parts. To make the experimental protocol and results easier to follow, below we present the methods as Parts 1 and 2, followed by the results also as Parts 1 and 2.

2 | MATERIALS AND METHODS

2.1 | Part 1: The identification of SNPs that are associated with temperament

All experiments were carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th Edition, 2013) and were approved by the Animal Ethics Committee of The University of Western Australia, under approval number RA/3/100/1252.

2.1.1 | Animals

We used Merino sheep from the University of Western Australia (UWA) temperament flock that has been selected for more than

20 generations based on behavioral criteria related to emotional reactivity. The flock is kept at the UWA Farm Ridgefield, Pingelly, Western Australia. The temperament of the sheep in the flock was assessed within 2 weeks after weaning at around 16 weeks of age using two behavioral tests that are described in detail below under phenotyping of sheep temperament.[9] Sixty ewes from the "calm" line and 60 ewes from the "nervous" line, at 16 weeks of age, with a similar live weight (20 ± 1.6 kg) were used in the first study.

2.2 | Phenotyping of sheep temperament

2.2.1 | Behavioral tests

The temperament of each of the 120 sheep in part 1 was assessed using an open-field arena test and an isolation box test.[9,12,14]

Open-field arena test: Each sheep was introduced into a test arena (L: 7 m × W: 3.3 m) that was divided by lines on the floor into four sectors. A motionless human stood at the end of the arena that was opposite to the entrance, in front of a small external pen containing three sheep from the same flock as the tested sheep. The test places the sheep into a conflicting situation between approach to a human and access to its flock mates. Each sheep was in the arena test for 3 min. During that time, the locomotor activity was measured by counting the number of the sectors that the sheep crossed (Crosses). The number of bleats (Bleats) was also recorded. The results of Crosses plus Bleats were used to estimate the emotional response.

Isolation box test: Within 2 min after the completion of the arena test, each sheep was introduced into, and locked in, an enclosed wooden box (H: 1.5 m × L: 1.5 m × W: 0.75 m) and left for 1 min while deprived of visual contact with conspecifics.[9] The level of vibration of the box that was produced by the movement of sheep and the high pitch bleats were recorded by an apparatus (agitation meter) that was fixed to the outside of the box.[5] Prior to the test, the agitation meter was calibrated with an electric unit that produced three standardized levels of vibration.[5] The score recorded on the agitation meter reflects the temperament, with a higher score (known as the score on the isolation box test, IBT score) indicating a nervous phenotype.

An overall selection score was calculated for each animal by combining the results of the two tests.[12] The selection score was used to classify sheep as 'nervous' or 'calm' on the basis of the expression of high or low levels of physical activity and vocalization in response to those tests. Contrary to the "nervous" line, the "calm" line is less reactive to human presence and isolation.

2.2.2 | Blood sampling and isolation of genomic DNA

Whole blood from a jugular vein was sampled into a vacutainer tube that contained EDTA (Greiner Bio-One, Australia). Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (69,506, Qiagen, Hilden, Germany) following manufacturer instructions. The integrity of the DNA was assessed by agarose gel electrophoresis and the concentration of DNA was measured with a Nanodrop spectrophotometer (ThermoFisher, Scoresby, Australia).

2.2.3 | Amplification of the fragments in target genes

A total of 12 fragments from *TPH2*, *SLC6A4*, *HTR2A*, and *OXTR* were amplified by PCR. The primers of the amplified fragments (see electronic supplementary material, Table S1) were designed with Primer Express software 1.5 (Applied Biosystems) and synthesized by GeneWorks (Australia). The PCR reaction was performed using a 50 μl reaction mixture containing 0.2 mM deoxynucleotide triphosphates (dNTPs, Fisher Biotec, Australia), 2 mM $MgCl_2$ (Fisher Biotec, Australia), 2 U Taq DNA polymerase (Fisher Biotec, Australia), 0.3 μM forward and reverse PCR primer (GeneWorks, Australia), and 100 ng genomic DNA. The thermal cycling conditions consisted of a first denaturation step at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing for 30 s, and extension at 72°C for 15 s, with a final extension step at 72°C for 5 min. Finally, the amplification products were run in a 2% agarose gel that was stained with GoldView for a quality check.

2.2.4 | Partial gene sequencing and identification of SNPs

The gene fragments from ten sheep in the calm line and ten in the nervous line were amplified. Each gene fragment was sequenced by Sanger sequencing at the Australian Genome Research Facility (Perth, Australia). SNPs were detected by comparing the sequence of gene fragments from each sheep with the software Sequencher (Version 5.4.6, Ann Arbor, America).

2.2.5 | Genotyping of the identified SNPs

For the remaining 100 sheep, an Agena Bioscience Mass ARRAY[46] was used to genotype the SNPs that had been identified with Sanger Sequencing. All of the DNA samples (10 ng/μl) were first added to 384 well PCR plates. The genotyping analysis was performed using an iPLEX Gold SNP genotyping kit (Agena, San Diego, US) in a MassArray platform (Agena) following the manufacture's protocols. Samples were firstly amplified from a 5 μl PCR mixture composed of PCR buffer (2 mM MgCl2, 500 μM dNTPs), 0.1 μM each of a forward and reverse primer (see electronic supplementary material, Table S2), 0.5 U Hotstar Tag enzyme, and 1 μl of the DNA sample. The PCR reaction was run as follows, 2 min at 95°C, 45 cycles of denaturation for 30 s at 95°C, 30 s at 56°C and 1 min at 72°C, with a final extension for 5 min at 72°C. To neutralize unincorporated dNTPs, the PCR products were incubated with 0.5 U shrimp alkaline phosphatase at 37°C for 40 min, followed by heating at 85°C for 5 min to inactivate the

enzyme. The purified PCR products were then mixed with iPLEX Gold extension reaction cocktail and extension primers to a final volume of 9 μl containing 0.222 units iPLEX buffer, 1-unit iPLEX termination mix, and 1-unit iPLEX enzyme. The iPLEX extension reaction was carried out under the following conditions, an initial denaturation step at 94°C for 30 s, followed by 40 cycles of a denaturation step at 94°C for 5 s, 5 cycles of annealing at 52°C for 5 s, extension at 80°C for 5 s, and a final extension step at 72°C for 3 min. After desalting the products using SpectroCLEAN resins following the manufacturer's protocol, the cleaned extension products were dispensed onto a 384 SpectroCHIP array using an RS1000 Nanodispenser, and finally, the array was introduced into a MassARRAY Compact mass spectrometer. Spectra were acquired using SpectroAcquire software, and data analysis, including automated allele calling, was done using MassARRAY Typer (version 4.0.5, Agena).

2.3 | Statistical analysis

The data for behavioral phenotype (Bleats, Crosses, and IBT score) were assessed for normality and homogeneity using a Shapiro–Wilk test and a Bartlett's test. A correlation between the behavioral phenotypes was done using a Pearson correlation test. The differences in behavioral phenotype between the sheep from the calm and the nervous line were analyzed using Kruskal-Wallis test, because the behavioral phenotypes were not homoscedastistic.

The data for Sanger sequencing (ten sheep from each temperament line) and Agena genotyping (fifty sheep from each temperament line) were collected together and firstly assessed for reliability using HWE, chisq function in R-studio. To further investigate the relationship between each SNP, and the relationship between SNPs and behavioral phenotypes, we first transformed the genotype into dosage data and an association analysis was performed using Spearman correlations between SNP dosage and the phenotype. The correlation for each SNPs was carried out using Spearman correlation. Differences were considered significant when $P < 0.05$.

2.4 | Part 2: The validation of SNPs that are associated with temperament

To validate the accuracy of predicting sheep temperament using the SNPs that were identified in Part 1, we tested the association between SNPs and behavioral phenotype in 14-month-old sheep (N = 260) from a commercial flock that had never been selected on emotional reactivity. This experiment was approved by the Animal Ethics Committee of The University of Western Australia with the same approval number RA/3/100/1252 as in part 1.

2.4.1 | Behavioral tests

The temperament of each of the 260 sheep in part 2 was assessed using the same behavioral tests as described in part 1.

2.4.2 | Genotyping of SNPs

A blood sample was obtained from each of the 260 sheep, and genomic DNA was extracted as described in part 1. An Agena Bioscience Mass ARRAY was used to genotype these 260 sheep for the temperament associated SNPs, following the steps described in part 1.

2.5 | Statistical analysis

2.5.1 | Correlation between the behavioral phenotypes was done using a Pearson correlation test

The frequencies of genotypes of each SNP were calibrated, and Hardy–Weinberg equilibrium was tested with HWE, Chisq function using R-studio. The differences in genotype frequency for each SNP among the sheep from calm line, nervous line and commercial flock were compared using Chi-square tests. To further investigate the association between the temperament related SNPs identified in part 1 and behavioral phenotype (Bleats, Crosses, and IBT score), the 260 sheep were grouped according to the genotype of the temperament associated SNPs. The data for behavioral phenotype was then assessed for normality and homogeneity, using a Shapiro–Wilk test and a Bartlett's test, respectively. The differences in behavioral phenotype between different genotype groups were analyzed using Kruskal-Wallis test, as all the behavioral phenotypes were not homoscedastistic. Differences were considered significant when $P < 0.05$.

3 | RESULTS

3.1 | Part 1: The identification of SNPs that are associated with temperament

3.1.1 | Phenotyping of sheep from the "Calm" and "Nervous" lines in the UWA temperament flock

The number of Bleats, Crosses, and the IBT score in the sheep from the UWA temperament flock were positively correlated with each other (see electronic supplementary material, Table S3). Moreover, the number of Bleats, Crosses, and the IBT score were significantly higher in sheep from the nervous line than in sheep from the calm line (Figure 1).

3.1.2 | Identification of SNPs in sheep from the UWA temperament flock

We identified 12 SNPs in different segments of the four target genes in sheep from the UWA temperament flock (Table 1). Four SNPs were identified in the *TPH2* gene, one T > C transition at position 107,856,757, as well as G > A transitions in exon 1 (position 20,933,178), exon 2 (position 20,930,506), and exon 12 (position

20,917,901). Three G > A transitions were found in *SLC6A4*, one in each of exon 1 (position 20,933,178), exon 2 (position 20,930,506) and exon 12 (position 20,917,901). Four SNPs were identified in the fragments of *HTR2A*, two G > A transitions at position 17,196,799 and 17,196,697 in exon 1, one C > T transition at position 17,193,313, and one G > A transition at position 17,193,181 in exon 2. In the *OXTR* gene, one C > T transition was identified at position 17,664,565 in exon 3.

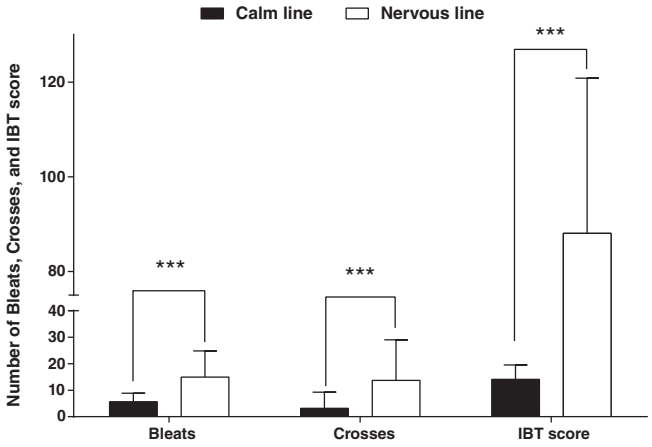

FIGURE 1 Results of arena test and isolation box test of sheep selected from the UWA temperament flock. *** means $P < 0.001$. UWA, University of Western Australia

The four SNPs in *TPH2* (rs107856757, rs107856818, rs107856856 and rs107857156) were in very strong linkage disequilibrium, and the 3 SNPs in *HTR2A* (rs17196697, rs17193313 and rs17193181) were also in strong linkage disequilibrium (Table 2).

3.2 | Association between the identified SNPs and temperament phenotype in the UWA temperament flock

There were significant associations between temperament and the four SNPs in *TPH2* (rs107856757, rs107856816, rs107856856 and rs107857156), the two SNPs in *HTR2A* (rs17196799 and rs17193181), the single SNP in *SLC6A4* (rs201917901), and the one in *OXTR* (rs17664565) (Table 3). The SNPs in *TPH2* explained 28% of the total variance in temperament. The distribution of the SNPs in *SLC6A4* (rs20933178 and rs20930506) and *HTR2A* (rs17196697 and rs17193313) were not different between the sheep of the "calm" and the "nervous" lines.

TABLE 1 Localization of SNPs in *TPH2*, *SLC6A4*, *HTR2A* and *OXTR* genes in the UWA temperament flock

CHR	Gene name	Location	SNP	Minor allele	MAF	Nucleotide Sequence
3	*TPH2*	Intron 2	rs107856757	C	0.39	CAAAGAGACCAAACT**T**
						CAAAGAGACCAAACT**C**
		Exon 2	rs107856816	T	0.39	GATAAAAAAGG**C**
						GATAAAAAAGG**T**
		Exon 2	rs107856856	A	0.39	ACACGGCTACCGAGAGC**G**
						ACACGGCTACCGAGAGC**A**
		Intron 1	rs107857156	A	0.39	TAATACTTTGGTGTGT**G**
						TAATACTTTGGTGTGT**A**
11	*SLC6A4*	Exon 1	rs20933178	A	0.49	GGGTACTCGGCGGTTCC**G**
						GGGTACTCGGCGGTTCC**A**
		Exon 2	rs20930506	A	0.02	GCCATTTTTGGGGGGATCCC**G**
						GCCATTTTTGGGGGGATCCC**A**
		Exon 12	rs20917901	A	0.20	TTCATCATCTGCAGTTTTTTGAT**G**
						TTCATCATCTGCAGTTTTTTGAT**A**
10	*HTR2A*	Exon 1	rs17196799	A	0.06	CTTCTTTGAGCTCAACTAC**G**
						CTTCTTTGAGCTCAACTAC**A**
		Exon 1	rs17196697	A	0.28	TAACTGGACCGTGGACTC**G**
						TAACTGGACCGTGGACTC**A**
		Exon 2	rs17193313	T	0.41	GGCCTCTGCCCAGCAAGCTCTG**T**
						GGCCTCTGCCCAGCAAGCTCTG**C**
		Exon 2	rs17193181	A	0.38	TCAACTCCAGAACTAAGGC**G**
						TCAACTCCAGAACTAAGGC**A**
19	*OXTR*	Exon 3	rs17664565	T	0.27	ATTCGTACACCTTTGTCCTGAG**T**
						ATTCGTACACCTTTGTCCTGAG**C**

Abbreviations: CHR, Chromosome; MAF, Minor allele frequency.

TABLE 2 Correlation between the SNPs that were identified in sheep from the UWA temperament flock

SNPs[a]	1	2	3	4	5	6	7	8	9	10	11	12
1 rs107856757		**−1.00**	**1.00**	**1.00**	0.02	0.07	−0.25	−0.37	0.17	0.05	−0.05	0.31
2 rs107856816	$<10^{-4}$		**−1.00**	**−1.00**	−0.02	−0.07	0.25	0.38	−0.17	−0.05	0.05	−0.31
3 rs107856856	$<10^{-4}$	$<10^{-4}$		**1.00**	0.02	0.07	−0.25	−0.38	0.17	0.05	−0.05	0.31
4 rs107857156	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$		0.02	0.07	−0.24	−0.37	0.18	0.05	−0.04	0.31
5 rs20933178	0.82	0.82	0.82	0.82		−0.17	−0.37	0.12	0.02	−0.02	0.06	0.02
6 rs20930506	0.47	0.47	0.47	0.47	0.08		0.00	−0.08	−0.13	0.12	−0.17	0.18
7 rs20917901	0.01	0.01	0.01	0.01	$<10^{-4}$	0.97		0.19	0.09	−0.11	0.13	−0.19
8 rs17196799	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$	$<10^{-4}$	0.23	0.43	0.05		−0.18	0.20*	−0.16	−0.22
9 rs17196697	0.07	0.07	0.07	0.07	0.84	0.18	0.36	0.07		**−0.79**	**0.81**	−0.03
10 rs17193313	0.63	0.63	0.63	0.63	0.87	0.23	0.28	0.04	$<10^{-4}$		**−1.00**	0.00
11 rs17193181	0.62	0.62	0.62	0.65	0.54	0.07	0.18	0.11	$<10^{-4}$	$<10^{-4}$		−0.08
12 rs17664565	0.0009	0.0009	0.0009	0.001	0.82	0.06	0.04	0.02	0.79	0.98	0.40	

Note: R-values^2 > 0.500 are shown in boldface.

[a]Upper diagonal: r-values for pair correlation analysis; Lower diagonal: *P*-values for pair correlation analysis.

TABLE 3 Association between the identified SNPs and temperament phenotypes in sheep from the UWA temperament flock

Gene name	SNP	Effect size (r)	*P*-value
TPH2	rs107856757	0.53	$\mathbf{3.81 \times 10^{-09}}$
TPH2	rs107856816	−0.53	$\mathbf{3.05 \times 10^{-09}}$
TPH2	rs107856856	0.53	$\mathbf{3.05 \times 10^{-09}}$
TPH2	rs107857156	0.53	$\mathbf{3.05 \times 10^{-09}}$
SLC6A4	rs20933178	−0.09	0.34
SLC6A4	rs20930506	0.13	0.17
SLC6A4	rs20917901	−0.38	$\mathbf{3.73 \times 10^{-05}}$
HTR2A	rs17196799	−0.29	$\mathbf{2.00 \times 10^{-3}}$
HTR2A	rs17196697	−0.03	0.75
HTR2A	rs17193313	0.12	0.23
HTR2A	rs17193181	−0.21	**0.03**
OXTR	rs17664565	0.38	$\mathbf{5.05 \times 10^{-5}}$

Abbreviation: SNPs, single nucleotide polymorphisms.

3.3 | Part 2: The validation of SNPs that are associated with temperament

The number of Bleats, Crosses, and the IBT score were positively correlated with each other in the sheep from the commercial flock (see electronic supplementary material, Table S4).

The association between behavioral phenotype and the genotype of temperament related SNPs are shown in Table 4. There were main effects of SNP rs107856856 which is in the gene encoding for tryptophan 5-hydroxylase. The sheep carrying genotype (G/G) had more Crosses and a higher IBT score than the sheep carrying genotype (A/G). Conversely, for SNP rs17193181, which is in the gene encoding for the 5-HT receptor, the sheep carrying genotype (G/G) had a lower IBT score than the sheep carrying genotype (A/G). The other temperament associated SNPs (rs20917901, rs17196799, and rs17664565) were not associated with any of the three measures of behavioral phenotype in the nonselected sheep.

4 | DISCUSSION

The present study shows, for the first time, an association between polymorphisms in brain serotoninergic pathways and phenotypic markers of temperament, or emotional reactivity, in Merino sheep. In the serotonin pathway, four genes that encode for a production enzyme (*TPH2*), a transporter (*SLC6A4*), and a receptor (*HTR2A*), and a receptor in the oxytocin pathway (*OXTR*) were selected for identification of SNPs based on the literature in other species. We found twelve SNPs in these four genes, with eight of those SNPs being associated with calm or nervous temperament in the UWA temperament flock. These results suggested that the serotonin pathway, at the level of synthesis, transport, and potentially sensitivity to 5-HT, and the oxytocin pathway at the level of sensitivity to oxytocin, are involved in the expression of emotional state in sheep, similar to what has been

TABLE 4 Association between temperament related SNPs and bleats, crosses, and IBT score in the commercial flock

			Behavioral phenotype		
Gene name	**SNP**	**Genotype**	**Bleats**	**Crosses**	**IBT score**
TPH2	rs107856856[a]	A/A	8.0 ± 8.16	7.5 ± 5.20[c]	55.3 ± 91.26[d]
		A/G	3.0 ± 4.90	5.3 ± 3.37[b]	45.9 ± 41.90[b]
		G/G	3.9 ± 5.87	6.6 ± 3.81[a]	65.0 ± 43.14[a]
SLC6A4	rs20917901	A/A	5.6 ± 9.32	6.6 ± 4.10	66.6 ± 35.30
		A/G	3.8 ± 5.20	6.7 ± 4.12	60.5 ± 44.50
		G/G	3.6 ± 5.79	6.2 ± 3.63	59.9 ± 44.63
HTR2A	rs17196799	A/A	2.0 ± 1.41	7.5 ± 0.71	18.5 ± 16.26
		A/G	4.8 ± 5.90	6.2 ± 3.98	64.0 ± 37.81
		G/G	3.6 ± 5.70	6.3 ± 3.76	60.1 ± 45.04
	rs17193181[b]	A/A	1.0 ± 1.73	4.0 ± 1.00	35.7 ± 30.53[c]
		A/G	3.9 ± 5.01	5.8 ± 2.39	70.1 ± 37.65[a]
		G/G	3.8 ± 5.93	6.5 ± 4.02	57.6 ± 45.26[b]
OXTR	rs17664565	C/C	4.0 ± 5.84	6.4 ± 3.65	59.2 ± 43.55
		C/T	3.4 ± 5.57	6.1 ± 4.05	63.3 ± 46.69
		T/T	1.7 ± 2.43	7.3 ± 3.04	48.9 ± 37.61
P-value					
TPH2 rs107856856[a]			0.19	0.03	<0.001
SLC6A4 rs20917901			0.80	0.65	0.74
HTR2A rs17196799			0.30	0.67	0.17
HTR2A rs17193181[a]			0.19	0.52	0.02
OXTR rs17664565			0.60	0.22	0.67

[a]rs107856856 was selected as the nonindependent marker for the four SNPs that were completely correlated in the *TPH2* gene (rs107856856, rs107856757, rs107856816 and rs107857156).
[b]rs17193181 was selected as the nonindependent marker for the SNPs (rs17193181, rs17196697 and rs17193313) in the *HTR2A* gene.
[c]Different lowercase superscripts in the same column indicate difference at $P < 0.05$.
[d]Different uppercase superscripts in the same column indicate difference at $P < 0.001$.
Abbreviation: SNPs, single nucleotide polymorphisms.

reported in humans, laboratory animals, and domesticated bovines. However, only the SNPs in *TPH2* and *HTR2A* were associated with temperament phenotype, as assessed by the arena test and the isolation box test, in sheep from a flock that had never been selected on behavioral phenotype. The lack of association between the SNPs in *SLC6A4* and *OXTR* and temperament phenotype in the nonselected sheep, while they were associated with temperament in the selected lines, could be a result of differences that have emerged over the 20 years of temperament selection. The proportion of the different genotypes of most SNPs were significantly different between the sheep from the calm line and nervous line in the UWA temperament flock, and the commercial flock (Figure 2).

Our results support the suggestion that SNPs in *TPH2* play an important role in serotonin synthesis and impact on the response to stress and the development of animal temperament.[47] In the present study, the missense variant (rs107856856) in exon 2 of *TPH2* explained 28.1% of the total variance in measures of temperament in the UWA temperament flock. That association suggests that functional changes in the gene that codes for an enzyme involved in serotonin production underpins some of the variance in the temperament differences. In humans and other animal species, missense SNPs in *TPH2* have been associated with variance in the occurrence of psychological disorders or behaviors, suggesting that the activity of tryptophan 5-hydroxylase plays a role in behavior (Table 5).[47-51] The results observed in other species strongly suggest that the mutations we observed in sheep could also modify the production of serotonin. Unfortunately, for technical reasons, the measurement of the concentration of serotonin in the brain was not possible in the present study.

In contrast to rs107856856, another SNP (rs107856818) in the coding region of *TPH2* that was associated with temperament differences in the UWA temperament flock, is a synonymous SNP with a change in base-pair but no change in amino acid (a glycine in both cases). The synonymous SNPs should be functionally neutral, and in humans, no association has been reported between synonymous SNPs in *TPH2* and phenotype (Table 5).[52,53] We suggest that the association between the synonymous variant (rs107856818) and temperament might be explained by its strong linkage with the missense variant (rs107856856).

In our sheep, the four SNPs in *TPH2* (rs107856856, rs107856818, rs107857156, and rs107856757) were in a very strong

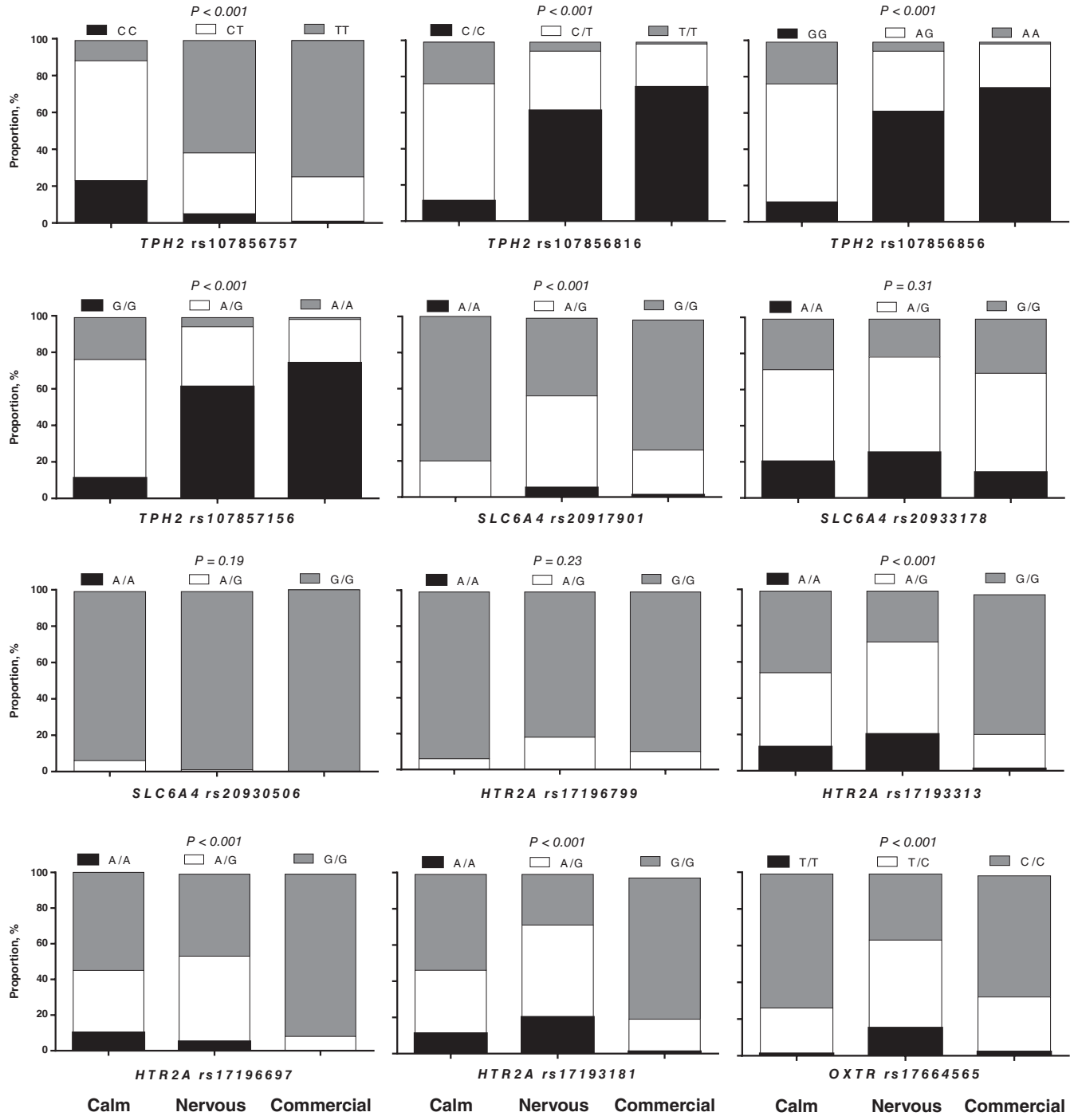

FIGURE 2 Frequency distribution of SNPs in the *TPH2*, *SLC6A4*, *HTR2A*, and *OXTR* genes in sheep from the calm line (Calm) the nervous line (Nervous) of the UWA temperament flock, and from nonselected sheep in the commercial flock (Commercial). UWA, University of Western Australia

linkage disequilibrium, similar to what has been described in eight out of the ten SNPs that span between exons 5 and 7 of the human *TPH2* gene.[54] The presence of allelic associations in the temperament flock is unlikely to be the result of the long lasting phenotypic selection that has been imposed on the flock (20 years), because the same linkage disequilibrium for the same four SNPs was also present in the commercial flock that has never had a selection pressure for temperament imposed on it. However, it will be difficult to ascertain the impact of these associations of SNPs within a gene on the functionality of tryptophan 5-hydroxylase in vivo since the four mutations do not appear independently of each other, even in the nonselected sheep.

The other two SNPs in *TPH2* that we identified (rs107857156 in intron 1 and rs107856757 in intron 2) were also associated with temperament in sheep from the UWA temperament flock. While it is well

TABLE 5 The association between behavioral phenotypes and SNPs in the serotonin system

Genes	SNPs	Location and types	Function	Species	Associated behavioral phenotypes	Ref.
TPH2	G1463A	Coding region, missense	Regulate tryptophan 5-hydroxylase activity	Humans	Unipolar major depression	47
TPH2	C1473G	Coding region, missense	Regulate tryptophan 5-hydroxylase activity	Mice	Aggressive behaviors	48
TPH2	Q468R	Coding region, missense	Regulate tryptophan 5-hydroxylase activity	Chimpanzees	Neuroticism traits, depression, and aggressive behaviors	49–51
TPH2	rs7305115	Coding region, synonymous	None	Humans	None	53
TPH2	rs2887148	Coding region, synonymous	None	Humans	None	53
TPH2	rs4290270	Coding region, synonymous	None	Humans	None	53
TPH2	rs4570625	Promoter region	Regulate the mRNA expression of TPH2, and response of the amygdala and cortical regions to affective stimuli	Humans	Depression, anxiety, and the personality traits of emotional dysregulation	14,26,56–59
TPH2	rs1386494	Intronic region	None	Humans	None	60
SLC6A4	L255M	Coding region, missense		Humans	Serve depression	63
SLC6A4	I425V	Coding region, missense		Humans	A complex neuropsychiatric phenotype	64
SLC6A4	I425V	Coding region, missense	Affect the activity of 5-HT transporter	Human cervical epithelioid carcinoma and COS-7 cells		62
HTR2A	rs43696138	Coding region, synonymous		Cows	Temperament traits	17
HTR2A	rs6313	Coding region, synonymous		Humans	Schizophrenia, mood disorders, and anxiety	65–67
HTR2A	rs6313	Coding region, synonymous		Humans	None	68

Abbreviation: SNPs, single nucleotide polymorphisms.

established that changes to the coding region of a gene can impact on the activity of the resulting protein because those changes alter the amino acid sequence of that protein, mutations in noncoding regions can also play an important role by affecting gene expression or by altering biological function directly.[55] For example, SNP rs4570625 in the noncoding region of *TPH2* affects the mRNA expression of *TPH2*, the responses of the amygdala (a central structure in behavioral mediation) and cortical regions to affective stimuli, and the subsequent behavioral phenotypes (Table 5).[14,26,56-59] Conversely, in humans, the other SNP rs1386494, in introns of *TPH2*, shows no association with personality traits that are related to depression (Table 5).[60] Given the findings in human *TPH2*, the two mutations that we have identified in the introns of *TPH2* in sheep might not have any functional significance in the expression of temperament. The significant association between the SNPs (rs107857156 and rs107856757) and temperament could arise from their strong linkage to the SNP (rs107856856) that is in the coding region.

The gene *SLC6A4* plays an important role in maintaining the 5-HT pool that is available for subsequent release, and is associated with anxiety, depression, and aggression in humans.[61] We identified three SNPs (rs20933178, rs20930506 and rs20917091) *in SLC6A4* in sheep from the UWA temperament flock, but only rs20917091 showed an association with temperament. The lack of association between the other two SNPs (rs20933178 or rs20930506) and temperament in sheep is not surprising because both are synonymous mutations, with all versions coding for proline. In contrast, rs20917091 causes a change from methionine (ATG) to isoleucine (ATA), thus potentially resulting in a change in the structure and function of the serotonin transporter. Similarly, the missense SNPs (I425V and L255M) are associated with severe depression and a complex neuropsychiatric

phenotype in humans, which is presumably due to the effect of the missense SNPs on the activity of 5-HT transporter (Table 5).[62-64]

While we have identified clear associations between temperament and SNPs in enzymes that facilitate serotonin production and transport, the role of SNPs in the serotonin receptor are less clear. In the present study, we identified four SNPs in *HTR2A*: rs17196799 (exon 1), rs17196697 (exon 1), rs17193313 (exon 2) and rs17193181 (exon 2). All four SNPs were synonymous mutations that might be considered inconsequential for the protein structure and function. However, in the UWA temperament flock, temperament traits were associated with rs17196799 (both alleles coding for threonine) and rs17193181 (both alleles coding for alanine). Similarly, the synonymous mutation rs43696138 in exon 3 of *HTR2A* has been associated with temperament traits in Charolais cows (Table 5).[17] In humans, SNP rs6313, a synonymous mutation in coding region of *HTR2A*, is associated with schizophrenia, mood disorders, and anxiety.[65-67] In contrast, other studies have shown no associations between rs6313 and psychiatric traits.[68] It has been suggested that these conflicting results in humans could be partly explained by nonidentified variants, or different sample sizes between studies, laboratory techniques, or ethnic heterogeneity.[69] In the present study, the association between the synonymous mutations (rs17196799 and rs17193181) and temperament in sheep could be due to other SNPs in *HTR2A* that are as yet unidentified.

Of the SNPs that were associated with temperament in the UWA temperament flock, only rs107856856 (as a nonindependent marker for rs107856757, rs107856818, and rs107857156) in the *THP2* gene and rs17196799 in *HTR2A* had predictive power in the nonselected sheep. The lack of predictive power of the other SNPs that were different between the selected lines could be due to a long-term effect of the selection on temperament on mutations in other genes that encode for traits such as maternal behavior or ovulation rate, traits that have been associated with temperament.[5,70] In silver foxes that have been selected for contact-seeking behavior with humans (tame / aggressive),[71] as well as differences in the gene expression and activity of key enzymes (tryptophan 5-hydroxylase, monoamine oxidase, and 5-HTT) in neurotransmitter systems,[72] the allele frequency of other exonic SNPs, together with the expression of related genes, changed with the selection for tameness.[73]

The number of Crosses during the arena test, and the IBT score, both were associated with rs107856856 in *TPH2*, with the sheep with allele C being lower for both Crosses and IBT score in the nonselected flock, and sheep with allele A of rs17196799 in *HTR2A* having a lower IBT score. The other SNPs that were associated with temperament in the UWA temperament flock, rs20917091 and rs17193181, did not associate with the temperament phenotype in the nonselected commercial flock. One possible explanation for the apparent contradiction is that there might be some unidentified functional variants that are in strong linkage disequilibrium with rs20917091 and rs17193181, that contribute to the association with temperament in the UWA temperament flock.

In addition to the SNPs that we identified in the serotonin pathway, the *OXTR* gene that codes for the oxytocin receptor was sequenced. The oxytocin receptor is distributed in various brain regions and is associated with social behaviors such as parental care, pair-bonding, and social aggression in nonhuman mammals. We identified a synonymous mutation in *OXTR* (rs17664565), with both versions encoding for serine. Synonymous mutations in *OXTR* have been associated with temperament traits in cats[74] and with autism spectrum disorder and loneliness in humans.[42,75] While the synonymous mutation in *OXTR* (rs17664565) was associated with temperament differences in the UWA temperament flock, it was not associated with temperament phenotype (Bleats, Crosses and IBT score) in the nonselected commercial flock. It is possible that different genes (that code for the synthesis, transport, or reception) and different neurotransmitters (serotonin and oxytocin), where these SNPs were identified, work together as part of a system, but also play specific roles in other traits that have been associated with temperament phenotype, like sociability, maternal behavior, and bonding behavior between the ewe and lamb.[45]

The present study shows, for the first time, associations between SNPs in the serotonin and oxytocin pathways and phenotypic traits of temperament in Merino sheep. Among the eight SNPs that associated significantly with temperament in the UWA temperament flock, only rs107856856 in *TPH2* (nonindependent marker for the SNPs in linkage disequilibrium) and rs17196799 in *HTR2A* were predictors of temperament traits that are related to the response to stress in a nonselected flock. Our results suggest that serotonin pathways are involved in the expression of emotional state in sheep, as has been proposed in humans and laboratory animals.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

DATA AVAILABILITY STATEMENT The data analyzed during the current study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

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