Association Between Temperament Related Traits and SNPs in the Serotonin and Oxytocin Systems in Merino Sheep

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Abstract

Background: Animal temperament is defined as the consistent behavioural 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 the variation in temperament in humans. The variations like single nucleotide polymorphisms (SNPs) in the genes for tryptophan 5-hydroxylase (*TPH2*), the serotonin transporter (*SLC6A4*), the serotonin receptor (*HTR2A*), or the oxytocin receptor (*OXTR*) are associated with the behavior phenotypes in human. Thus, the objective of this study was to identify SNPs in *TPH2, SLC6A4, HTR2A* and *OXTR* and to test if those variations predict the temperament of Merino sheep.

Results: Using ewes from the UWA temperament flock, that 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 non-selected flock that has never been selected on emotion reactivity, followed by the estimation of the behavioural 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 behavioural phenotype in the non-selected sheep. Similarly, rs17196799 in *HTR2A* was also associated with the behavioural phenotype.

Conclusions: We thus conclude that, rs107856856 and rs17196799 could be used as gene markers for the temperament of Merino sheep, with allele C of rs107856856 and allele A of rs17196799 being associated with calm temperament.

Background

Animal temperament is often referred to as personality or a behavioural syndrome [1]. More specifically, animal temperament is the consistent behavioural and physiological differences that are observed between individuals in response to an eliciting event [2]. The variability in temperament is often described along dipoles such as shy to bold, sociable to aggressive, exploring to avoiding, or behavioural activity or inactivity [2]. That individual variability is thought to reflect the individual's perception of the surrounding situation. Non-human 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 [3]. The perception of a situation leads to an emotional state that is experienced by the individual. The emotion then elicits physiological and behavioural responses, and those responses have been used to qualify the temperament of individual animals [3, 4]. In sheep, the phenotype and the genetic heritability of temperament traits [5–7], have been assessed using behavioural tests. Temperament is of more than academic interest because temperament can influence production traits like ovulation rate [8]. Classic behaviour tests, like the arena test and the isolation box test, are time and labour consuming, and difficult to carry out in young animals [9]. These limitations reduce the utility of temperament selection programs on farm, even if that selection can provide economic benefits [10].

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) [11, 12]. 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 [13]. An SNP in the gene encoding for the dopamine receptor 2 (*DRD2*, SNP939) [13], and a variant of the gene that encodes the enzyme cytochrome P450 17α-hydroxylase/17,20-lyase (*CYP17*, SNP628), an enzyme that is involved in the production of cortisol, were both associated with sheep temperament [13]. Because the perception and evaluation of a situation occurs in the brain, and those SNPs are not in genes that encode for elements of brain pathways, additional SNPs might be associated with the temperament phenotype of sheep.

Serotonin (5-HT) is a widely distributed neurotransmitter in the mammalian brain that has been reported to play an important role in psychological state [14–17]. In humans, several SNPs in the serotoninergic system have been associated with

personality traits such as fear, impulsivity, and aggression [12]. Variations in the gene that codes for tryptophan 5-hydroxylase 2 (*TPH2*), the rate limiting enzyme in the synthesis of neuronal serotonin [18], affect the serotonin concentration in the brain as well as behaviour traits [19, 20]. Two SNPs (rs4570625 and rs17110747) in *TPH2* have been associated with depressive disorder [21], while, SNP 4570625 in *TPH2* has been associated with emotional dysregulation [22]. Other SNPs in *TPH2* (rs7305115, rs4290270, rs11178997 and rs13864923) have been related to emotional stability and suicidal behaviour [23–25]. 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 behaviour in humans [26–30]. The serotonergic system is similar in other mammals and the results of pharmacological depletion of brain 5HT in sheep suggests that the serotonin pathways is involved in affective state [31]. 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 an important regulator of social behaviours such as maternal and affiliative behaviour [32], recognition [33] and trust [34] in humans. Variations in the gene for the oxytocin receptor (*OXTR*) are associated with prosocial temperament (rs53576), reactivity to stressors (rs53576) and aggressive behaviours (rs6770632 and rs1042778) [35–37]. Polymorphisms in *OXTR* (rs53576, rs2254298 and rs2228485) have been associated with emotional loneliness [38]. In domesticated animals, oxytocin has been proposed as a marker of voluntary homo-specific and hetero-specific social contact [39]. 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 [40]. Since temperament in sheep has been defined by the response that a sheep shows during a social challenge, such as isolation [8], polymorphisms in the oxytocin pathway could be associated with phenotypic temperament in sheep.

Given the role that variations in genes that play a role in these central neurotransmitter systems have in emotional responses, we have investigated the role of polymorphic variations in these systems in the expression of temperament in sheep. We investigated the presence of SNPs in the genes for *TPH2*, *SLC6A4*, *HTR2*A, and *OXTR* in sheep and the impacts of those variations on the behavioural 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 behavioural phenotypic responses to isolation and human presence [41]. Secondly, we tested whether the SNPs that we identify in the first part were associated with differences in behavioural reactivity to isolation and human contact in sheep that had never been selected for temperament.

Methods

Part 1: The identification of SNPs that are associated with the “calm” and “nervous” temperament lines in the UWA temperament flock

Animals

We used Merino sheep from the UWA temperament flock that has been selected for more than 20 generations based on behavioural 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 two weeks after weaning at around 16 weeks of age using two behavioural tests that are described in detail below under phenotyping of sheep temperament [6]. An overall selection score was calculated for each animal by combining the results of two tests [9]. 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 vocalisation in response to specific stressors. Contrary to the “nervous” line, the “calm” line is less reactive to human presence and isolation. 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.

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 (69506, 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).

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 (Table 1) 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℃ 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.

Table 1
Primers used for the PCR amplification of different fragments in *TPH2, SLC6A4, HTR2A* and *OXTR* gene.

Gene name	ID number	Fragment	Sequence (5'to3')	Product size, bp	Annealing temperature, °C
TPH2	XM_015094535.2	Intron 1	F: GGAAGCTAATGCCACTCACC	527	59
			R: AGCTGAATGGTGACCAGAGC		
		Exon 2	F: CAGCGCAAAAACAAAAACC	494	60
			R: CAACACACCAGCATGATTCC		
		Intron 2	F: TTGAAGTGCACGTTTTGTGG	464	60
			R: CCTCTCCTCAGCAGTCAACC		
SLC6A4	AF246893.1	Exon 1	F: ACAGGCCCACACAGACTAGC	543	60
			R: GTCAGCATCACCTCCTAGCC		
		Exon 2	F: CGGACTTAGCCACACATACG	517	59
			R: GACAGGGTGGCTTCATGG		
		Exon 8	F: TTAAACCGGTCCCTTAGTGC	544	59
			R: TCCCAGGATGTCAGGATAGC		
		Exon 12	F: TGCTATACACCAGGCTGTGC	505	60
			R: TTCACTTTCTGCGGTATCAGG		
HTR2A	XM_012124453.2	Exon 1	F: GGAAGCTAATGCCACTCACC	506	60
			R: AGCTGAATGGTGACCAGAGC		
		Exon 2	F: CAGCGCAAAAACAAAAACC	530	59
			R: CAACACACCAGCATGATTCC		
		Exon 3	F: TTGAAGTGCACGTTTTGTGG	490	61
			R: CCTCTCCTCAGCAGTCAACC		
OXTR	NM_001009752.1	Exon 1	F: CATCACGTTCCGTTTCTACG	527	59
			R: CATCTGCACGAAAAAGAATGG		

Gene name	ID number	Fragment	Sequence (5'to3')	Product size, bp	Annealing temperature, °C
		Exon 3	F: GAAGCATGTGGTAGGGAAGC	541	60
			R: TGTACACAGCCACCAAGAGC		

Partial gene sequencing

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) for the detection of SNPs.

Part 2: The association between temperament associated SNPs and temperament traits related to the stress response in non-selected sheep

To validate the accuracy of predicting sheep temperament using the SNPs that were identified in Part 1, we tested the association between SNPs and behaviour phenotype in 14 month-old sheep (N = 260) from a commercial flock that had never been selected on emotional reactivity.

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 SNPs that were identified in Part 1 [42]. 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 (Table 2), 0.5 U Hotstar Tag enzyme, and 1 μL of the DNA sample. The PCR reaction was run as follows, 2 min at 95℃, 45 cycles of denaturation for 30 s at 95℃, 30 s at 56℃ and 1 min at 72℃, with a final extension for 5 min at 72℃. To neutralize unincorporated dNTPs, the PCR products were incubated with 0.5 U shrimp alkaline phosphatase at 37℃ for 40 min, followed by heating at 85℃ 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℃ for 30 s, followed by 40 cycles of a denaturation step at 94℃ for 5 s, 5 cycles of annealing at 52℃ for 5 s, extension at 80℃ for 5 s, and a final extension step at 72℃ 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).

Table 2
Primers used for the genotyping of SNPs of the genes *TPH2, SLC6A4, HTR2A* and *OXTR*.

Gene name	SNP	Primer	Sequence (5' to 3')
TPH2	rs107856757	Forward	ACGTTGGATGAGTATCACTGAATCCGTTTC
		Reverse	ACGTTGGATGCAGACCAACCCCTCTTATAG
		Extension	GCTTCAAAGAGACCAAACT
	rs107856816	Forward	ACGTTGGATGTCAAGGAGAATACAACCGCC
		Reverse	ACGTTGGATGGAGAACTCTGGAATCCTAAC
		Extension	TTGCTGCTTCCCTTGTT
	rs107856856	Forward	ACGTTGGATGTCAAGGAGAATACAACCGCC
		Reverse	ACGTTGGATGGAGAACTCTGGAATCCTAAC
		Extension	AGGAGAATACAACCGCCGTCTTGC
	rs107857156	Forward	ACGTTGGATGTCAAGGAGAATACAACCGCC
		Reverse	ACGTTGGATGGAGAACTCTGGAATCCTAAC
		Extension	TAGCTTCAAGAAGGTAGAAA
SLC6A4	rs20933178	Forward	ACGTTGGATGAGTCTGGCCAGATCTCCAAC
		Reverse	ACGTTGGATGATGGAGTGCTGAGTGTCGTC
		Extension	ACAACGGGTACTCGGCGGTTCC
	rs20930506	Forward	ACGTTGGATGCATCCATTTCGGTGGTACTG
		Reverse	ACGTTGGATGCTATCATGGCCATTTTTGGG
		Extension	GAGCTCCATGTAGAAGAG
	rs20917901	Forward	ACGTTGGATGGTCTCTGTGTGGGTGTTGTG
		Reverse	ACGTTGGATGTACCGGAAAAGTCGTAGCTG
		Extension	AGTTCATCATCTGCAGTTTTTTGAT
HTR2A	rs17196799	Forward	ACGTTGGATGTCACAGGAAAGGTTGGTTCG
		Reverse	ACGTTGGATGGAAGAAAACACTTCTTTGAGC
		Extension	AATGTAATTGCATTAAGGAGTT
	rs17196697	Forward	ACGTTGGATGTCACAGGAAAGGTTGGTTCG
		Reverse	ACGTTGGATGGAAGAAAACACTTCTTTGAGC
		Extension	TAAAGGTTGGTTCGATTTTC
	rs17193313	Forward	ACGTTGGATGCCACTTACCCACTGATATCG
		Reverse	ACGTTGGATGGGTGGCCTCTGCCCAGCAA
		Extension	CCATCCAGGTAAATCCAAACAGC
	rs17193181	Forward	ACGTTGGATGGGTGGCCTCTGCCCAGCAA
		Reverse	ACGTTGGATGCCACTTACCCACTGATATCG

Gene name	SNP	Primer	Sequence (5' to 3')
		Extension	AACTCCAGAACTAAGGC
OXTR	rs17664565	Forward	ACGTTGGATGAGACGAGCGTCAGCAAAAAG
		Reverse	ACGTTGGATGATCAGTCACACCGTGGATGG
		Extension	CGTACACCTTTGTCCTGAG

Phenotyping of sheep temperament

The temperament of each of the 260 sheep in Part 2 was assessed using an open-field arena test and an isolation box test [6, 9, 11].

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 three min. During that time, the locomotor activity was measured by counting the number of the sectors that the sheep crossed (Cross). The number of bleats (Bleats) was also recorded. The results of Cross plus Bleats was used to estimate the emotional response.

Isolation box test: Within two minutes 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 one min while deprived of visual contact with conspecifics [6]. 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 [8]. Prior to the test, the agitation meter was calibrated with an electric unit that produced three standardised levels of vibration [8]. The score recorded on the agitation meter reflects the temperament, with a higher score (known as the score on the isolation box test, IBT) indicating a nervous phenotype.

Statistical analysis

The results of the Sanger sequencing and the Agena Bioscience Mass ARRAY test were analysed using the software Sequencher (Version 5.4.6, Ann Arbor, America) and the MassARRAY Typer (version 4.0.5, Agena), respectively. The reliability of the data was first analysed using Hardy-Weinberg equilibrium. To further investigate the relationship between each SNP and behavioural phenotype, we first transformed the genotype into dosage data and an association analysis was performed by calculating Spearman correlations between SNP dosage and the phenotype. The correlation between different SNPs was carried out using Spearman correlation.

Results

Part 1: Identification of temperament related SNPs that are associated with “calm” and “nervous” temperament in the UWA temperament flock

Phenotyping of sheep from the “Calm” and “Nervous” lines in the UWA temperament flock

The number of Bleats, Cross, and the IBT score were significantly higher in sheep from the nervous line than in sheep from the calm line (Fig. 1).

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 3). Four SNPs were identified in the *TPH2* gene, one T > C mutation at position 107856757, as well as G > A mutations in exon 1 (position 20933178), exon 2 (position 20930506), and exon 12 (position 20917901). Three G > A mutations were found in *SLC6A4*, one in each of exon 1 (position 20933178), exon 2 (position 20930506) and exon 12 (position 20917901). Four SNPs were identified in the fragments of *HTR2A*, two G > A mutations at position 17196799 and 17196697 in exon 1, one C > T mutation at position 17193313, and one G > A mutation at position 17193181 in exon 2. In the *OXTR* gene, one C > T mutation was identified at position 17664565 in exon 3.

Table 3
Localisation of SNPs in *TPH2, SLC6A4, HTR2A* and *OXTR* gene in the UWA temperament flock.

CHR	Gene name	Location	SNP	Minor allele	MAF	Nucleotide Sequence
3	*TPH2*	Intron 2	rs107856757	C	0.39	TAATACTTTGGTGTGT**G**
						TAATACTTTGGTGTGT**A**
		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	CAAAGAGACCAAACT**T**
						CAAAGAGACCAAACT**C**
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**

CHR: Chromosome; MAF: Minor allele frequency

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 4).

Table 4

Correlation between the different SNPs that were identified in sheep from the UWA temperament flock[a].

SNPs	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	0.010	0.010	0.010	0.011	$< 10^{-4}$	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	0.046		-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	

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

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

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 5). 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 5
Association between the identified SNPs and temperament phenotypes of sheep from the UWA temperament flock.

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

Part 2: The association between the SNPs identified in part 1 and temperament traits in a commercial flock

In the gene encoding for tryptophan 5-hydroxylase, SNP rs107856856 was associated with the phenotypic measurements of Cross and IBT, but was not associated with Bleats (Table 6). In the gene encoding for 5-HT receptor, SNP rs17193181 showed only a trend for association with IBT, and no association with Bleats or Cross. The other temperament associated SNPs (rs20917901, rs17196799 and rs17664565) were not associated with any of the three measures of temperament phenotype (Table 6) in the non-selected sheep.

Table 6
Association between the temperament related SNPs and bleats, cross and IBT in the commercial flock.

Gene name	SNP	Bleats		Cross		IBT	
		Effect size (r)	*P*-value	Effect size (r)	*P*-value	Effect size (r)	*P*-value
TPH2	rs107856856[a]	0.04	0.48	0.15	**0.02**	0.25	**3.98 × 10 − 5**
SLC6A4	rs20917901	-0.04	0.50	-0.06	0.37	-0.01	0.86
HTR2A	rs17196799	-0.10	0.12	0.00	0.97	-0.02	0.79
HTR2A	rs17193181[b]	-0.08	0.21	0.03	0.59	-0.15	**0.02**
OXTR	rs17664565	-0.06	0.31	-0.05	0.40	0.01	0.81

[a]rs107856856 was selected as the non-independent marker for the four SNPs that were completely correlated in the *TPH2* gene (rs107856856, rs107856757, rs107856816 and rs107857156).

[b]rs17193181 was selected as the non-independent marker for the SNPs (rs17193181, rs17196697 and rs17193313) in the *HTR2A* gene.

The associations between temperament phenotype and genotypes rs107856856 and rs17193181 are shown in Fig. 2. Three genotypes (CC, CT and TT) were identified in rs107856856, and the genotype TT was associated with higher scores for both Cross and IBT. Furthermore, three genotypes (AA, AG and GG) were found in rs17193181, and the genotype GG was associated a higher score in the IBT.

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 oxtocin pathway at the level of sensitivity to oxytocin, are involved in the expression of emotional state in sheep, similar to what has been reported in humans and laboratory animals. However, only the SNPs in *TPH2* and *HTR2A* were associated with temperament phenotype, as assessed by the arena test and the isolation box tests, in sheep from a flock that had never been selected on behavioural phenotype. The lack of association between the SNPs in *SLC6A4* and *OXTR* and temperament phenotype in the non-selected 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.

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 [43]. In the present study, the SNP 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. The missense variant (rs107856856) in the coding region causes an amino acid change from glycine (GGC) to serine (AGC). While the functionality of rs107856856 has not yet been demonstrated in sheep, alteration of the activity of tryptophan 5-hydroxylase by polymorphism has been reported. In humans with unipolar major depression, the SNP G1463A in the coding region of *TPH2* leads to the replacement of an arginine with a histidine, resulting in an 80% loss of function in the activity of tryptophan 5-hydroxylase [43]. Similarly, in chimpanzees, SNP Q468R, which replaces a glutamine with an arginine at the 1404th position of tryptophan 5-hydroxylase, changes the activity of the enzyme [44], and is associated with neuroticism traits [45] and is related to depression and aggressive behaviours [46]. In mice, a similar missense variant to SNP Q468R, SNP C1473G in exon 11, modifies the activity of tryptophan 5-hydroxylase and thereby the production of 5-HT and is linked to aggressive behaviour [47]. 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). Synonymous SNPs are functionally neutral [48] and, in humans, no association has been reported between synonymous SNPs in *TPH2* and specific phenotypes [49]. However, another study in humans identified 10 SNPs in *TPH2*, most were in strong linkage disequilibrium and associated with the major depression [50]. We suggest that the association between the synonymous variant (rs107856818) and temperament might be explained by its strong linkage with the missence variant (rs107856856).

In our sheep, the four SNPs in *TPH2* (rs107856856, rs107856818, rs107857156, and rs107856757) were in a very strong 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 [50]. 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 non-selected 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 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 non-coding regions can also play an important role by affecting gene expression or by altering biological function directly [51]. For example, intronic SNPs can modify the function of the related protein by affecting splicing and expression [52]. In humans, the activity of the amygdala (a central structure in behavioural mediation) *in vitro* has been shown to be altered by SNP G844T [11], a SNP that is located in a non-coding region of the *TPH2* gene. *In vivo*, SNP rs4570625 in the promoter region of *TPH2* alters the response of the amygdala and cortical regions to affective stimuli [53] and is associated with depression, anxiety, and the personality traits of emotional dysregulation [22, 54, 55]. Those effects may be due to the changes in the mRNA expression of *TPH2* that can be caused by rs4570625, an hypothesis that was supported by an *in vitro* study [56]. Conversely, in humans, some other SNPs (rs1386494) in introns of *TPH2* show no association with personality traits that are related to depression [57]. 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 that are in introns (rs107857156 and rs107856757) and temperament could arise from their strong linkage to the SNP (rs107856856) that is in the coding region.

In the gene that codes for the serotonin transporter (*SLC6A4*), three SNPs (rs20933178, rs20930506 and rs20917091) were identified in sheep from the UWA temperament flock, but only rs20917091 showed an association with temperament. In other species, *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 [58]. In humans, several SNPs in *SLC6A4* (rs25531, rs25532, I425V) are related to temperament traits and mental disease [59, 60]. 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. In humans, a missense mutation in position 255 of the protein sequence (L255M; leucine to methionine) has been linked to severe depression [61]. Another missense mutation (I425V; valine to isoleucine) is associated with a complex neuropsychiatric phenotype [62], presumably by affecting the activity of the serotonin transporter [63].

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 humans, SNPs in *HTR2A* are associated with depression, obsessive compulsive disorder, schizophrenia [28, 64], and a predisposition to aggressive traits [64]. 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 [65]. In humans, SNP rs6313, a synonymous mutation in coding region of *HTR2A*, is associated with schizophrenia, mood disorders, and anxiety [66–68]. In contrast, other studies have shown no associations between rs6313 and psychiatric traits [69]. It has been suggested that these conflicting results in humans could be partly explained by non-identified variants, or different sample sizes between studies, laboratory techniques, or ethnic heterogeneity [70]. 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 non-independent marker for rs107856757, rs107856818, and rs107857156) in the *THP2* gene and rs17196799 in *HTR2A* had predictive power in the non-selected 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 behaviour or ovulation rate, traits that have been associated with temperament [8, 71]. In silver foxes that have been selected for contact-seeking behaviour with humans (tame / aggressive) [72], as well as differences in the gene expression and activity of key enzymes (tryptophan 5-hydroxylase, monoamine oxidase, and 5-HTT) in neurotransmitter systems [73], the allele frequency of other exonic SNPs, together with the expression of related genes, changed with the selection for tameness [74].

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 scoring lower for both Cross and IBT score in the non-selected flock, and sheep with allele A of rs17196799 in *HTR2A* scoring a lower IBT. 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 non-selected 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 behaviours such as parental care, pair-bonding, and social aggression in non-human 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 [75] and with autism spectrum disorder and loneliness in humans [38, 76]. 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, Cross and IBT) in the non-selected 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 behaviour, and bonding behaviour between the ewe and lamb [41].

Conclusions

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. Amongst the eight SNPs that associated significantly with temperament in the UWA temperament flock, only rs107856856 in *TPH2* (non-independent 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 non-selected 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.

Abbreviations

Bleats
the number of bleats in arena test in the 3 min arena test; Cross:the number of the sectors that the sheep crossed in the 3 min arena test; CHR:chromosome; CYP17:enzyme cytochrome P450 17α-hydroxylase/17,20-lyase; DRD2:dopamine receptor 2; HTR2A:serotonin receptor 2A; 5-HT:serotonin; IBT:the score recorded on the agitation meter in the isolation box test; OXTR:oxytocin receptor; SLC6A4:serotonin transporter; SNPs:single nucleotide polymorphisms; TPH2:tryptophan 5-hydroxylase 2; UWA:The University of Western Australia; MAF:minor allele frequency.

Declarations

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Availability of data and materials

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

Authors' contributions

Conceived and designed the experiments: LD, SKM, MW and DB. Performened the experiments: LD and DB. Analyzed the data: LD, JR and LC. Wrote and revised the paper: LD, DB and SKM.

Ethics approval

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.

Competing interests

The authors declare that they have no competing interests.

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Figures

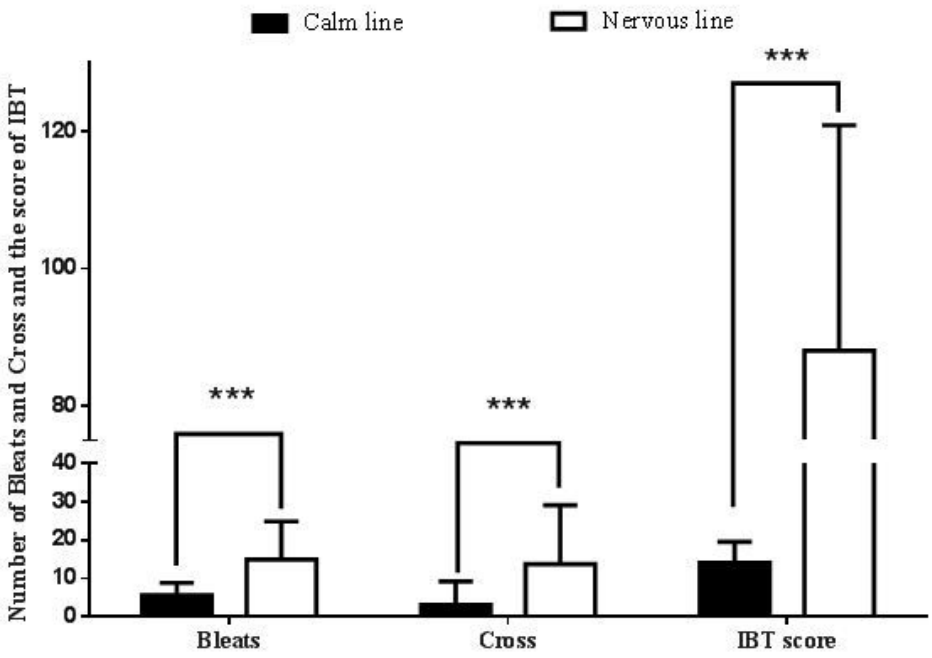

Figure 1

Results of arena test and isolation box test of sheep selected from UWA temperament flock. *** means P < 0.001.

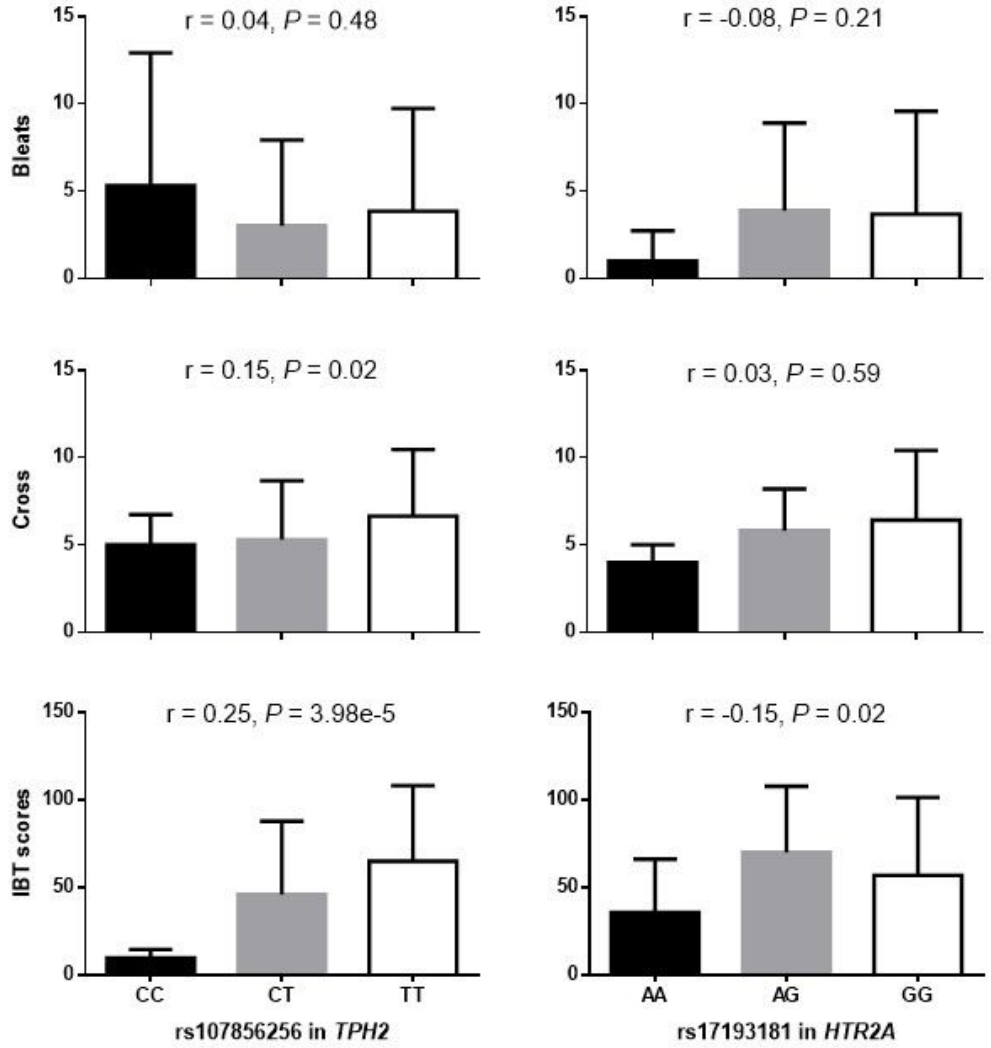

Figure 2

Results of arena test and isolation box test in sheep from the commercial flock. The left panels compare the three possible genotypes of rs107856256 in TPH2, and the right panels compare the genotypes of rs17193181 in HTR2A.