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# Functionality of promoter microsatellites of arginine vasopressin receptor 1A (AVPR1A): implications for autism

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## Abstract

**Background:** Arginine vasopressin (AVP) has been hypothesized to play a role in aetiology of autism based on a demonstrated involvement in the regulation of social behaviours. The arginine vasopressin receptor 1A gene (*AVPR1A*) is widely expressed in the brain and is considered to be a key receptor for regulation of social behaviour. Moreover, genetic variation at *AVPR1A* has been reported to be associated with autism. Evidence from non-human mammals implicates variation in the 5'-flanking region of *AVPR1A* in variable gene expression and social behaviour.

**Methods:** We examined four tagging single nucleotide polymorphisms (SNPs) (rs3803107, rs1042615, rs3741865, rs11174815) and three microsatellites (RS3, RS1 and AVR) at the *AVPR1A* gene for association in an autism cohort from Ireland. Two 5'-flanking region polymorphisms in the human *AVPR1A*, RS3 and RS1, were also tested for their effect on relative promoter activity.

**Results:** The short alleles of RS1 and the SNP rs11174815 show weak association with autism in the Irish population ( $P = 0.036$  and  $P = 0.008$ , respectively). Both RS1 and RS3 showed differences in relative promoter activity by length. Shorter repeat alleles of RS1 and RS3 decreased relative promoter activity in the human neuroblastoma cell line SH-SY5Y.

**Conclusions:** These aligning results can be interpreted as a functional route for this association, namely that shorter alleles of RS1 lead to decreased *AVPR1A* transcription, which may proffer increased susceptibility to the autism phenotype.

## Background

The neuropeptide arginine vasopressin (AVP) has been hypothesized to play a role in the aetiology of autism based on a demonstrated involvement in social bonding and in the regulation of a variety of socially relevant behaviours in animal models. Pharmacological experiments in rodents have shown a role for vasopressin in learning and memory, aggression and affiliative behaviours. Increases in AVP are associated with stressful or defensive circumstances and AVP is also involved in aggressive behaviours and pair bonding, mostly in males [1]. AVP regulates male social behaviour not just through higher expression in males but also in steroid-sensitive brain sexual dimorphisms in AVP neurons [1,2]. AVP may therefore influence

sexually dimorphic social behaviours in a range of species [3]. The role of sex hormones on AVP is of interest in the context of autism considering that the ratio of affected males with autism compared to affected females is highly skewed (4:1) [4]. Only one study has examined the relationship between AVP levels and autism; Boso *et al.* [5] found AVP levels to be higher in plasma from individuals with autism than controls ( $P = 0.02$ ).

AVP acts through binding to G-protein-coupled receptors. One of these receptors, arginine vasopressin receptor 1A (AVPR1A), is widely expressed in the brain. There is considerable evidence to implicate the *AVPR1A* gene in social behaviours. *AVPR1A*-knockout mouse models show impaired social recognition and decreased anxiety behaviours, while overexpression of *AVPR1A* in mice resulted in increased social memory [6,7]. Disruption in *AVPR1A* expression in the brain has been associated with major behavioural changes [8-11].

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In non-human mammals, the variable expression of *AVPR1A* is implicated in changes in social bonding, parental rearing behaviours and social recognition and memory. Hammock and Young [12] described behavioural differences in voles (genus *Microtus*) that was related back to the AVP neuropeptide. Prairie voles (*Microtus ochrogaster*) are socially monogamous through multiple breeding seasons with both the males and females contributing to rearing of the young. These behaviours are not typically seen within closely related species such as the montane vole (*Microtus montanus*) and meadow vole (*Microtus pennsylvanicus*). This phenomenon has been attributed to variable expression resulting from a microsatellite marker in the 5'-flanking region of the *AVPR1A* gene in *Microtus* [13,14].

In humans, the 5' region of the gene contains four repeat elements; three promoter repeats ((GT)<sub>25</sub>, RS1, ((GATA)<sub>14</sub>) and RS3 ((CT)<sub>4</sub>TT(CT)<sub>8</sub>(GT)<sub>24</sub>)) and an intronic repeat (AVR ((GT)<sub>14</sub>(GA)<sub>13</sub>(A)<sub>8</sub>)) (see Figure 1) [15]. These polymorphisms, specifically RS1 and RS3 have been studied extensively in terms of their relationship to autism. The data reported thus far has produced inconsistent association with different alleles and markers implicated at this gene. In a sample of 115 autism spectrum disorder (ASD) proband-parent trios, Kim *et al.* [16] did not observe association for alleles of RS1 or RS3. In a smaller sample of 65 ASD trios, Wassink *et al.* [17] reported modest association with a shorter allele of the RS1 polymorphism being under-transmitted to probands with autism (max *P* = 0.008). This was not confirmed in a study of 128 individuals with ASD by Yirmiya *et al.* [18], where association was observed at the AVR polymorphism (max *P* = 0.003). More recently, Yang *et al.* [19] identified association at both RS1 and RS3 in a Korean ASD family-based study. However, the association at the RS1 markers was not consistent with previous findings, which may reflect population differences.

Various studies have linked different lengths of the *AVPR1A* polymorphisms to behavioural traits in non-clinical samples. Knafo *et al.* [20] reported that subjects with shorter length RS3 polymorphisms were found to

be less generous by the 'dictator game' indicating less altruistic behaviour [20]. Moreover, shorter variants in general were associated with lower levels of *AVPR1A* mRNA in postmortem hippocampus. Recent neuroimaging approaches have also indicated a link between gene expression levels and amygdala activation [21].

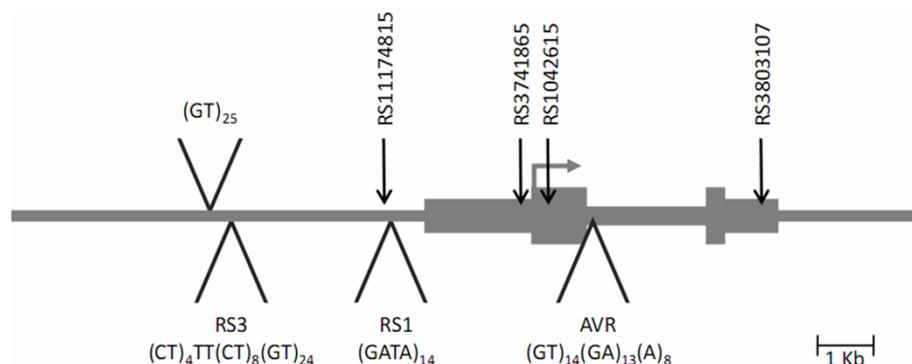
Recent studies have highlighted the role of rare *de novo* inherited copy number variants in the aetiology of autism [22]. However, only rare *de novo* and inherited variants are established as genetic risk factors, and these account for a small proportion of the total genetic risk [23]. Furthermore, in a recent paper from the International Schizophrenia Consortium, common genetic variants accounted for over 30% of the genetic risk for schizophrenia [24] and there is no reason to believe this will not be the case for autism as well. The genetic factors influencing the aetiology of autism are likely to be a combination of rare, private and more common variants. The action of which are not likely to be mutually exclusive.

Based on prior evidence suggesting a role for AVP in human and animal social cognition, and prior reports of genetic association between variants in vasopressin-related genes and autism, we hypothesized that variants in *AVPR1A* would be associated with autism in our Irish autism sample. We examined the variants RS1, RS3, AVR and four tagging single nucleotide polymorphisms (SNPs) across the gene locus, namely rs3803107, rs1042615, rs3741865 and rs11174815, for association with autism in the Irish autism trio collection. Furthermore, considering the evidence to support a role of 5'-flanking variation in the expression of *AVPR1A* in *Microtus*, we hypothesised that variation in the 5'-flanking region of the human *AVPR1A* gene at the microsatellites' RS3 and RS1 polymorphisms would influence the relative effects on promoter activity.

## Methods

### Sample description

A total of 177 families, consisting of an affected child and both parents were recruited through schools, parent



**Figure 1** Schematic of the arginine vasopressin receptor 1A gene (*AVPR1A*).

support groups and clinician referral. The male to female ratio was 4.68:1. Autism diagnoses were confirmed using Autism Diagnostic Interview - Revised (ADI-R) [25] and the Autism Diagnostic Observation Schedule - Generic (ADOS-G) [26]. IQ and Vineland Adaptive Behaviour Score (VABS) [27] were evaluated to establish a distribution of functioning within the sample. Individuals with known medical causes of autism (for example, tuberous sclerosis, extreme prematurity, congenital rubella) were excluded. Subjects met the ADI-R criteria for autism, and the ADOS-G criteria for autism or ASD. All individuals had an IQ >35 or a mental age (as defined by the VABS) >18 months. Ethical approval was obtained from the Health Services Executive Linn Dara Child and Adolescent Psychiatry Ethics Committee, Ireland. DNA was extracted from blood or buccal swabs using the phenol/chloroform extraction method.

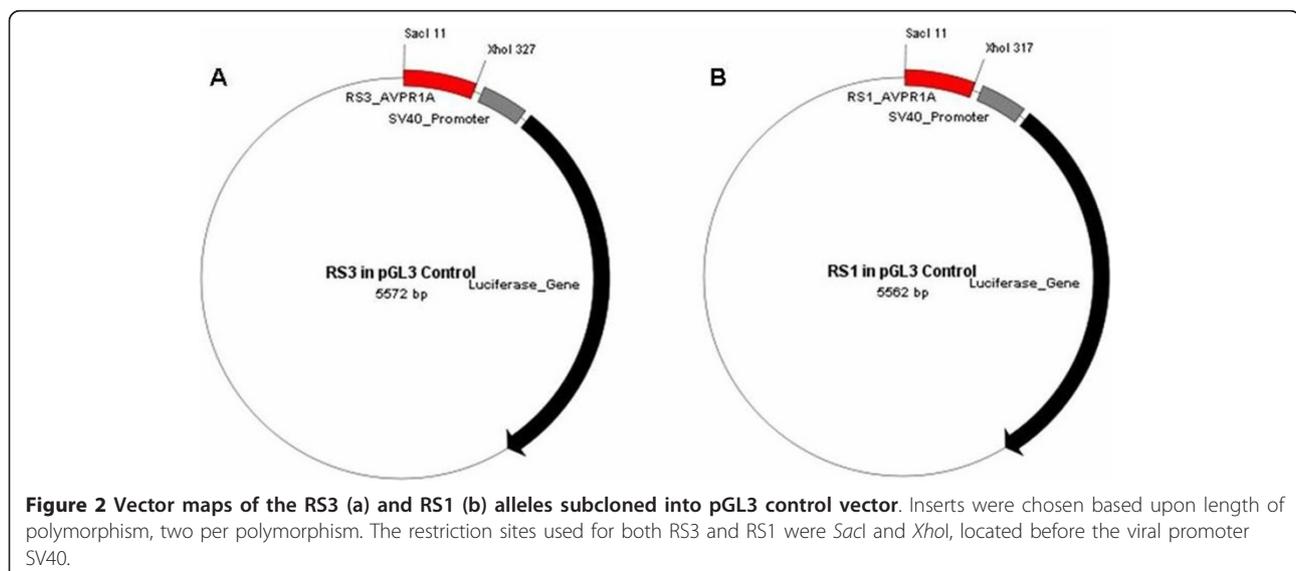
### Genotyping

Using the HapMap CEPH data, four SNPs in the *AVPR1A* locus were identified using the tagger function of Haploview [28]. SNPs were selected using pairwise tagging with an  $r^2$  threshold of 0.8 with an additional 2000 base pairs taken 5' and 3' of the gene transcript. SNP genotyping was performed by a commercial genotyping company, KBiosciences (<http://www.kbioscience.co.uk/>) using Kaspar assays. Three additional microsatellite polymorphisms (RS1, RS3, and AVR) at the *AVPR1A* gene locus were also genotyped. RS1: forward (fluorescent) 5'-AGG GAC TGG TTC TAC AAT CTG C-3'; reverse 5'-ACC TCT CAA GTT ATG TTG GTG G-3'. RS3: forward (fluorescent) 5'-CCT GTA GAG ATG TAA GTC CT-3'; reverse 5'-TCT GGA AGA GACT

TAG ATG G-3'. AVR: forward (fluorescent) 5'-ATC CCA TGT CCG TCT GGA C-3'; reverse 5'-AGT GTT CCT CCA AGG TGC G-3' [15,18]. Each reaction contained 0.5 mM primer. The sample was initially heated for 10 min at 95°C followed by 30 cycles of 95°C (30 s), 55°C (30 s), 72°C (40 s) and a final extension step of 72°C for 10 min. An ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) was used to determine the length of each PCR product. Samples were analysed using Genemapper v2.7 (Applied Biosystems).

### Construction of the reporter vectors

DNA samples of individuals homozygous for the RS3 variants (allele 325 and allele 339) and RS1 (allele 306 and 322) were selected from CEPH HapMap individuals. PCR amplification of a DNA fragment containing allele 325 and allele 339 of RS3 were amplified using primers: forward 5'-CCT GTA GAG ATG TAA GTG CT-3'; reverse 5' CTC GAG TCT GGA AGA GAC TTA GAT GG-3'. For RS1, allele 306 and allele 322 were amplified using primers: forward 5'-AGG GAC TGG TTC TAC AAT CTG C-3'; reverse 5'-CTC GAG ACC TCT CAA GTT ATG TTG GTG G-3'. Amplified sequences were cloned into the pGEM-T vector (Promega, Southampton, UK). The RS3 and RS1 alleles were subcloned into the *SacI* and *XhoI* site in the pGL3 control vector (Promega). The amplified promoter fragments do not contain the core promoter elements of *AVPR1A*. Therefore, the pGL3 control vector as opposed to the pGL3 Basic vector was used to examine the modulating influence of these elements as 5'-flanking polymorphism through an SV40 promoter. Vector maps of the tested constructs are shown in Figure 2. Sequence analysis using a BigDye



Terminator v3.1 Cycle Sequencing Kit (ABI PRISM, Applied Biosystems) was performed to confirm the fidelity and orientation of all inserts.

#### Cell culture and transfection

The human neuroblastoma cell line SH-SY5Y, were maintained in RPMI 1640 containing 10% foetal bovine serum (FBS) at 37°C and 5% CO<sub>2</sub>. Transfections were performed in triplicate a minimum of three times using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

#### Luciferase assay

Luciferase assays were performed using the dual-glow luciferase system (Promega) according to the manufacturers' instructions. Luminescence was measured on a Victor plate reader (PerkinElmer, Boston, MA, USA). Transfection efficiency was corrected for by normalising data relative to the cotransfected renilla luciferase pRL-cytomegalovirus (CMV) control vector (Promega). For the RS3 and RS1 constructs, 100 ng of test constructs were cotransfected with 500 ng pGL3 control vector. Expression was reported as relative to the pGL3 control vector.

#### Statistical analyses

Transmission disequilibrium test (TDT) for the three microsatellites from *AVPR1A* were analysed in UNPHASED [29]. Each allele is tested independently for transmission disequilibrium. Research previously undertaken in voles demonstrated the repeat length of the polymorphism modified gene expression, transcriptional pattern in the brain and social behaviours [14]. Therefore, we decided to test for association between the length of polymorphic repeat and autism. Alleles were grouped in 'long' alleles and 'short' alleles for RS1, RS3 and AVR polymorphisms. Nomenclature of alleles for RS1 and RS3 can be found in Yirmiya *et al.* [18]. For RS3, 'long' refers to allele 327 and all repeats longer than 327 and 'short' refers to allele 325 and all repeats shorter than allele 325 consistent with the previously published study of Knafo *et al.* [20]. For RS1, 'long' consists of allele 314 and all repeats larger than allele 314 and 'short' consists of allele 310 and all repeats shorter than allele 310. For AVR, 'short' includes allele 213 and all repeats shorter than allele 213, while 'long' includes allele 215 and all repeats larger than allele 215. A similar procedure has been applied to other complex polymorphism, such as the nitric oxide synthase 1 (NOS1) ex1f- variable number tandem repeat (VNTR) [30-33].

Hardy-Weinberg equilibrium and TDT analyses for SNP markers were performed using PLINK [34]. TDT for dichotomised microsatellites (long versus short) was also performed using PLINK. No Mendelian incompatibilities

were detected and all variants were found to be in Hardy-Weinberg equilibrium ( $P > 0.01$ ) in the parents. Permutation procedures were performed in PLINK using the mperm function ( $n = 1000$ ). Haplotypes were defined using Gabriel's confidence intervals and tested for association using permutation in Haploview ( $n = 1000$ ) [28,35].

The statistical significance of differences in relative gene reporter activity was examined using two-tailed Student's *t* test in Stata v. 10 (StataCorp, College Station, TX, USA).

#### Power calculation

In the Irish autism sample ( $n = 177$ ) at minor allele frequencies of 0.20, there was greater than 80% power to observe an effect of odds ratio (OR) approximately 1.35 at an uncorrected  $P = 0.05$  level. This calculation considers a modest effect size and also no linkage disequilibrium between the test and causative marker.

## Results

#### Genetic association analyses

Of the seven markers (four SNPs and three microsatellite markers), only one showed a significant association with autism in the Irish autism sample (rs11174815, nominal  $P = 0.008$ ). Dichotomisation of the RS1 allele revealed a weak association of the RS1 short allele and autism (nominal  $P = 0.036$ ; odds ratio (OR) 1.44; 95% confidence interval 1.02 to 2.04) (Tables 1 and 2). No significant haplotype associations were observed (data not shown).

#### Gene reporter assays for the RS1 and RS3 polymorphism of AVPR1A

Differential activity was observed for alleles of both the RS1 and RS3 variants when expressed as a luciferase construct in the pGL3 control vector. In SH-SY5Y cells, the long repeat of the RS1 polymorphism showed 2.7 times higher relative activity compared to the shorter length repeat ( $P = 0.0005$ , Student's *t* test) (Figure 3). Similarly, for the long repeat of the RS3 variant was 1.4 times more active than the short-repeat allele ( $P = 0.0081$ , Student's *t* test) (Figure 3).

## Discussion

In the current work we present the results of genetic investigations of the *AVPR1A* gene in an Irish autism sample. We followed association analysis with investigations to examine the role of promoter region polymorphisms, RS1 and RS3, on relative promoter activity.

Overall, we observed a single association with *AVPR1A* in the Irish autism trio collection (rs11174815, corrected  $P = 0.01$ ). Due to the very low minor allele frequency (minor allele frequency (MAF) = 0.015) and lack of observations for transmissions of the A allele, it

**Table 1 Genotype association results using UNPHASED for multiallelic markers and PLINK for dichotomous markers**

Marker	Allele	Frequency	T	NT	$\chi^2$	P value
RS3	305	0.001	0	1	1.00	0.32
	311	0.005	2	1	0.33	0.56
	319	0.001	0	1	1.00	0.32
	321	0.008	3	1	1.00	0.32
	323	0.058	19	16	0.26	0.61
	325	0.102	31	24	0.89	0.35
	327	0.214	45	36	1.00	0.32
	329	0.210	43	54	1.25	0.26
	331	0.099	25	26	0.02	0.89
	333	0.167	44	42	0.05	0.83
	335	0.026	6	11	1.47	0.23
	337	0.014	4	2	0.67	0.41
	339	0.059	14	20	1.06	0.30
	341	0.015	4	5	0.11	0.74
	343	0.017	5	6	0.09	0.76
	345	0.004	2	1	0.33	0.56
	Short	0.390	86	65	2.92	0.09
RS11174815	<b>A</b>	<b>0.015</b>	<b>0</b>	<b>7</b>	<b>7.00</b>	<b>0.008<sup>a</sup></b>
RS1	302	0.004	0	3	3.00	0.08
	306	0.161	39	31	0.91	0.34
	310	0.448	67	52	1.89	0.17
	314	0.193	33	46	2.14	0.14
	318	0.079	20	26	0.78	0.38
	322	0.096	24	30	0.67	0.41
	330	0.014	5	1	2.67	0.10
	334	0.005	2	1	0.33	0.56
	<b>Short</b>	<b>0.518</b>	<b>78</b>	<b>54</b>	<b>4.36</b>	<b>0.036<sup>b</sup></b>
RS3741865	T	0.008	2	1	0.33	0.56
RS1042615	T	0.436	55	58	0.08	0.78
AVR	204	0.002	1	0	1.00	0.32
	208	0.010	2	0	2.00	0.16
	210	0.108	16	21	0.68	0.41
	212	0.272	43	41	0.05	0.83
	214	0.461	51	48	0.09	0.76
	216	0.040	10	10	0.00	1.00
	218	0.006	0	1	1.00	0.32
	220	0.064	10	11	0.05	0.83
	222	0.001	0	1	1.00	0.32
	Short	0.411	79	79	0.00	1.00
RS3803107	T	0.178	32	30	0.06	0.79

T = transmitted, NT = non-transmitted.

Associations at the suggestive  $\alpha$  level 0.05 are shown in bold text.

<sup>a</sup>Empirical  $P = 0.01$ . <sup>b</sup>Empirical  $P = 0.08$ .

**Table 2 Multimarker transmission disequilibrium test (TDT) for the microsatellite markers RS3, RS1 and AVR reporting the likelihood ratio statistic (LRS), degrees of freedom (df) and global P value from UNPHASED**

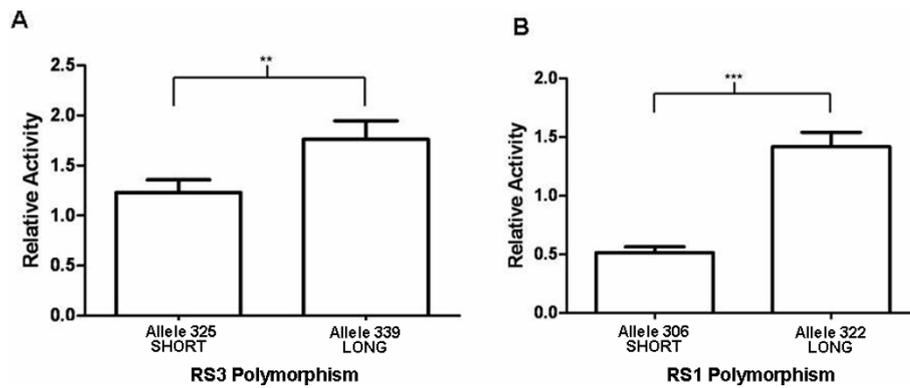
Marker	LRS	df	P value
RS3	10.76	15	0.769
RS1	12.22	7	0.094
AVR	8.085	8	0.425

is difficult to conclude the relationship between this marker and autism in the Irish autism sample from our study. Furthermore, a weak association signal was seen between the dichotomised RS1 polymorphism and individuals with autism in the Irish sample. The short alleles were overtransmitted in individuals with autism.

We performed an experimental examination of the effect of allele length of the RS1 and RS3 polymorphism on relative promoter activity in the luciferase gene reporter assay. For the RS1 variant we observed the shorter allele to show lower relative promoter activity. Previous studies have reported shorter RS1 polymorphisms show more activity in the left amygdala in a non-clinical sample [36]. Excessive amygdala activation during social interaction has been reported to be associated with increase anxiety and eventually social withdrawal [37]. These aligning results suggest shorter alleles of RS1 result in lower relative promoter activity and possibly reduced transcription of *AVPR1A*, increasing amygdala activity leading to social withdrawal.

Shorter alleles of the RS3 polymorphism also lead to lower relative promoter activity in the luciferase gene reporter assay. While the RS3 polymorphism was not shown to be associated with autism in the Irish autism sample, it has been shown previously to be associated with individuals with autism [17,18,38]. Knafo *et al.* [20] reported individuals with shorter variants of the RS3 polymorphism have lower hippocampal *AVPR1A* mRNA and shorter alleles lead to less prosocial behaviours than longer alleles. Potentially, shorter alleles of the RS3 polymorphism, which show lower relative promoter activity, lead to reduced levels of *AVPR1A* mRNA and lower levels of *AVPR1A* in cells. Decreased levels of *AVPR1A* potentially could result in less altruistic behaviours (Table 3).

In a recent study, the human RS1 polymorphism showed no differences in COS7 cells [39]. Furthermore, Hong *et al.* [39] repeated the experiment in the PC12 cell line with no difference shown; however expression levels were extremely low indicating a potential weak promoter in *AVPR1A*. The difference in results between this study and ours is not surprising given recent



**Figure 3** Relative activity of the RS3 (a) and RS1 (b) polymorphisms constructs in the neuronal SH-SY5Y cell line. (a) Longer length repeat of the RS3 polymorphism of the arginine vasopressin receptor 1A gene (*AVPR1A*) showed higher relative activity compared to the shorter length repeat of RS3 in SH-SY5Y cell lines ( $P = 0.0081$ , Student's  $t$  test). (b) Longer length repeat of the RS1 polymorphism of *AVPR1A* showed higher relative activity compared to the shorter length repeat ( $P = 0.0005$ , Student's  $t$  test). \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . The graph shows the mean of the data and standard error of the mean.

**Table 3** Results from previous studies on the arginine vasopressin receptor 1A gene (*AVPR1A*) RS3 polymorphism demonstrating the functionality of this region

<i>AVPR1A</i> RS3 repeat length classification	Postmortem hippocampal mRNA levels [20]	Prepulse inhibition [45]	Dictator game and real money allocations [20]	Amygdala activation to face recognition [21]	Relative transcriptional activity (current study)
Short	Down	Down	Down	Down	Down
Long	Up	Up (males)	Up	Up	Up

Up/down indicate the direction of effect. Adapted from Levin *et al.* [45].

evidence that *cis*-acting variation differs between tissue and cell types [40-44]. Our study examines the enhancer effects of RS1 and RS3 in an appropriate and relevant cell type for consideration in the aetiology of autism, a neurological disorder. In the study undertaken here, RS1 and RS3 are examined as enhancers modulating a viral promoter SV40 instead of its own natural promoter. Gene reporter systems do not take into account the genomic or chromatin context of the elements tested, however it is encouraging that our data agree with post-mortem mRNA levels as shown in Knafo *et al.* [20], although additional studies are warranted. While the genomic environment tested was an artificial construction without the use of the endogenous promoter, these data do indicate that the repeat elements influence the relative activity of a core promoter in neuronal cells.

### Conclusions

The results from both RS3 and RS1 luciferase gene reporter assays are intriguing, with potentially shorter variants of both RS3 and RS1 leading to decreases in *AVPR1A* mRNA. Our results align with the study undertaken on the *Microtus* microsatellites, which showed decreased microsatellite length contributes to decreased sociability [13]. This suggests a potential conservation of function across species highlighting the

importance of these polymorphisms. However, the functionality of both polymorphisms in our study complicates any conclusions. A limitation of the gene reporter approach is the removal of additional regulatory mechanisms, which may affect the DNA region of interest, and in these particular polymorphisms, how these polymorphisms may interact with each other in the regulation of *AVPR1A* transcription. Our haplotype analysis between the dichotomised alleles of RS1 and RS3 showed no association in the Irish sample. Rigorous meta-analysis is currently not possible due to the use of different genotyping methodologies [45]. We have used the same genotyping primers as the Knafo *et al.* [20], Meyer-Lindenberg *et al.* [21] and Levin *et al.* [45] allowing us to draw conclusions easily across these studies. Future work examining the regulatory relationship between these two polymorphisms will be essential for better understanding of how they influence *in vivo* transcription of *AVPR1A* and ultimately how they potentially play a role in the aetiology of autism.

### Acknowledgements

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#### Authors' contributions

KET and RJLA wrote the manuscript. KET performed the laboratory work and data analysis undertaken in this manuscript. KET, MJH and RJLA conceived the experiment. MJH and RJLA supervised design, laboratory methods, and data analysis. MJH, LEC, MG and LG aided in manuscript preparation. MG, LG and RJLA were involved in the supervision and conception of the experiment.

#### Competing interests

The authors declare that they have no competing interests.

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