

ORIGINAL ARTICLE

Association of *Per1* and *Npas2* with autistic disorder: support for the clock genes/social timing hypothesis

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Clock gene anomalies have been suggested as causative factors in autism. We screened eleven clock/clock-related genes in a predominantly high-functioning Autism Genetic Resource Exchange sample of strictly diagnosed autistic disorder progeny and their parents (110 trios) for association of clock gene variants with autistic disorder. We found significant association ($P < 0.05$) for two single-nucleotide polymorphisms in *per1* and two in *npas2*. Analysis of all possible combinations of two-marker haplotypes for each gene showed that in *npas2* 40 out of the 136 possible two-marker combinations were significant at the $P < 0.05$ level, with the best result between markers rs1811399 and rs2117714, $P = 0.001$. Haplotype analysis within *per1* gave a single significant result: a global $P = 0.027$ for the markers rs2253820–rs885747. No two-marker haplotype was significant in any of the other genes, despite the large number of tests performed. Our findings support the hypothesis that these epistatic clock genes may be involved in the etiology of autistic disorder. Problems in sleep, memory and timing are all characteristics of autistic disorder and aspects of sleep, memory and timing are each clock-gene-regulated in other species. We identify how our findings may be relevant to theories of autism that focus on the amygdala, cerebellum, memory and temporal deficits. We outline possible implications of these findings for developmental models of autism involving temporal synchrony/social timing.

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Introduction

This study aims to evaluate the hypothesis that clock genes are implicated in autistic disorder. This severe neurodevelopmental disorder is characterized by three areas of abnormality: impairment in communication (verbal and non-verbal) and reciprocal social interaction together with a markedly restricted repertoire of activities and interests, all in evidence before 3 years of age.¹ The primary focus of this paper is autistic disorder, as opposed to the more heterogeneous autistic spectrum disorders (ASDs). The prevalence of autistic disorder is 0.1–0.2%;² autistic disorder has a 60% concordance rate in monozygotic twins but no concordance is shown for autistic disorder in dizygotic twins.³ Such data suggest that there is a high degree of, but not complete, genetic control over the occurrence of autistic disorder; adverse inter-uterine or potentially protective effects may be involved in the manifestation of this disorder.⁴ The heritability of autistic disorder is best

explained by a model involving a number of genes at unlinked and some epistatic loci that together contribute to the phenotype.⁵ There is considerable comorbidity of mental retardation in autism (at least 75%⁶). Our study addressed sample heterogeneity by selecting for more intellectually able subjects that met strict diagnostic criteria for autistic disorder, thereby increasing experimental power.

Whole-genome screens and candidate gene studies for autism

There are significant whole-genome linkage findings for autism on 2q,⁷ 3q^{8,9} and 17q.⁹ 2q is highlighted as most likely harboring a locus for autistic disorder.^{7,10–12} However, the overall genome screen results are inconsistent, with a possible explanation being that most/all of these studies are underpowered to find genes of small effect set in the context of sample heterogeneity and diagnostic differences.

Candidate gene studies in autism have tested numerous genes, often on the basis of their proximity to LOD score peaks from whole-genome linkage studies. Such studies remain largely unreplicated, with the possible exceptions of GABRB3,^{13,14} *Engrailed2*^{15,16} and the serotonin transporter 5-HTT.^{17–20} The scope of candidate gene studies in autism is generally limited by the lack of credible supporting

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hypotheses. Our candidate gene study was prompted by Wimpory *et al.*'s²¹ hypothesis that autism reflects a disturbance of clock gene function, from a molecular level to the manifestation of autistic disorder as a psychological phenomenon.

Timing difficulties and autism

Timing difficulties have been proposed as being central to autism (e.g. Newson²²), with hypotheses encompassing circadian, communicative and/or neurological aspects of timing.^{21,23–29} For example, Boucher²⁵ suggests a core timing deficit presenting different manifestations by its effect on the elements of an integrated system of neural and physiological oscillators. Wimpory *et al.*²¹ hypothesize a causative, concurrent and developmental role³⁰ for timing deficit in autistic disorder and that this deficit is derived from pathological variations in the structure/function of clock/clock-related genes. Drawing on the above hypotheses, we conceive that timing deficit may manifest in autism as both temporally measured anomalies and apparently disparate symptoms (such as anomalous performance in tasks involving perceptual and cognitive coherence (see review, Happe and Frith³¹), relational memory (episodic^{32–35}) and diachronic thinking³⁶); each with some temporal and/or clock gene dependency.

Temporally measured anomalies in autism (including high-functioning autism and Asperger syndrome) range from circadian/sleep architecture (outlined below) to brain oscillations involved in neural binding,^{37,38} information processing,³⁹ attention^{40–42} including rapid attention-switching,^{29,43,44} and, motor coordination (from posture to eye-blink).^{45–48} They also extend to the reciprocity/temporal synchrony skills required for conversation.^{49–52} Objectively recorded atypical sleep architecture in young adults with high-functioning autism and Asperger syndrome⁵³ and in children with autistic disorder⁵⁴ shows association between sleep profiles and autistic symptomatology.^{53,54} Circadian hormone (melatonin) anomalies are found in autistic disorder^{55–57} as well as altered serotonin levels in autism.^{58,59} An altered circadian clock mechanism affecting normal sleep wake cycles could have an effect on the levels of these hormones that is additional to any other specific transport and reuptake effects.

The hypothesis

Our study tests the hypothesis that there is association between strictly diagnosed autistic disorder and alterations in clock genes (specifically *per1*, *per2*, *per3*, *clock*, *npas2*, *arntl* (*bmal1*), *tim*, *cry1*, *cry2*, *dbp* and *ck1ε*; see Table 1). There is a high degree of functional and sequence similarity of canonical clock genes across widely different organisms^{60–64} and, in addition to affecting the circadian cycle, clock gene anomalies are specifically linked to sleep disorders (in humans^{65,66}), altered sleep architecture and contextual memory (in mice^{67,68}), and communicative timing and memory formation (in *Drosophila*^{69–72}):

Table 1 Candidate genes and their chromosomal locations

| <i>Gene</i> | <i>Chromosome location</i> |
|-------------------------------|----------------------------|
| <i>Arntl</i> (<i>bmal1</i>) | 11p15.2 |
| <i>Clock</i> | 4q12 |
| <i>Ck1ε</i> | 22q31.1 |
| <i>Cry1</i> | 12q23.3 |
| <i>Cry2</i> | 11p11.2 |
| <i>Dbp</i> | 19q13.33 |
| <i>Npas2</i> | 2q11.2 |
| <i>Per1</i> | 17p13.1 |
| <i>Per2</i> | 2q37.3 |
| <i>Per3</i> | 1p36.23 |
| <i>Tim</i> | 12q13.3 |

all are relevant to aspects of autism: sleep,^{53,54} memory^{32–35,73,74} and communicative timing^{50,52}).

Molecular genetic background: clock genes

In mammals, the core molecular clock model (see e.g. Looby and Loudon⁷⁵) comprises a suite of epistatic genes that operate as an integrated system of transcription/translation autoregulatory feedback loops. CLOCK and BMAL1 (ARNTL), the protein products of the genes *clock* and *bmal1* (*arntl*), positive elements of the system, heterodimerize and activate the transcription of the genes *per1*, *per2*, *per3*, *cry1*, *cry2*, *rev-erba* and other clock-controlled genes. The gene products of *per*, *cry* and *rev-erba*, the negative elements of the system, operate via a PER/CRY heterodimer that inhibits the activating effects of the CLOCK/BMAL1 heterodimer while REV-ERBα represses expression of *bmal1*. Protein turnover eventually releases the genes from repression and the cycle starts over. The time taken for the molecular migrations of proteins and mRNA, respectively, to and from the nucleus, together with the protein turnover time, broadly defines the ~24-h period of this oscillatory system.

To integrate this core clock mechanism into the life of the organism as a whole, genes associated with resetting the clock (*cry1*, *cry2*) serve in matching environmental time with biological time by resetting the clock in response to environmental cues such as day length.⁷⁶ *Ck1ε* is involved with regulating the clock by affecting the stability of the PER/CRY complex through phosphorylation⁷⁷ while output pathways transduce the clock's time signals into physiological response. It is this molecular clock that endows living organisms with the ability to maintain a state of appropriate physiological readiness that anticipates the environmental demands associated with a particular time of day or night. In addition to their role as clock elements, certain clock genes (e.g., *per1*, *per2* and *npas2*) appear to play roles in signaling pathways and in DNA repair.^{78–82}

Genetic studies also point toward a broader functionality for clock genes beyond their role in circadian rhythms. For example, *per* in *Drosophila melanogaster* is shown to regulate a short-period oscillator

involved with the fly's courtship song,⁷⁰ a primitive form of sonic communication. The male fly, as part of the mating ritual, produces a song by rhythmic beating of its wings. This song has several acoustic components, including pulse song where a series of rhythmic pulses are separated by inter-pulse intervals (ipi) of ~34-ms.

Kyriacou and Hall⁷⁰ studied song structure in *Drosophila* circadian *per* mutants⁸³ and showed a cyclic modulation of ipi-duration in the pulse song of *D. melanogaster*, the Kyriacou and Hall (K&H) cycle, of ~55 s is also under the control of *per*.^{69,70} The three circadian *per* mutants studied *per^s* (19 h: short period) *per^l* (28-h: long period) and *per⁰* (arrhythmic) showed K&H cycles that were also short, long and abolished respectively. Thus, *per*'s influence on the circadian cycle (~24 h) and on a short-period (~55 s) oscillatory function associated with the song appear to be qualitatively similar.⁷⁰ These observations show *per* operating not only in a circadian oscillator but also in modulating high-frequency oscillators controlling motor function associated with a communicative behavior.^{71,83,84} Kyriacou and Hall⁷⁰ also noted that where male flies interrupted their song, they restarted in phase with the initial portion of the song and that each fly's song may start at a different phase of the K&H cycle. This suggests that the *per*-determined K&H cycle is integral with a concurrent, short-interval timing process.

The clock genes *per1* and *npas2* also play key roles in memory formation. Experiments with *npas2* knockout mice show that the knockout mice performed statistically similarly to wild-type mice in a battery of behavioral tests apart from the test for cued and contextual fear. The results of this experiment suggest that *npas2-LacZ(-/-)* mice are deficient in complex emotional memory specifically, but not in non-emotional memory.⁶⁸ In *Drosophila*, *per1* plays a role in long-term memory formation, which is independent of the core circadian oscillator.⁷²

The candidate genes and their relevance to the hypothesis

Because of the epistatic nature of the core clock genes, we decided to screen all the canonical clock elements and also *npas2*, *dbp*, *tim* and *ck1ε* (see Table 1). Apart from including the core elements (*per1*, *per2*, *per3*, *arntl* (*bmal1*), *clock*, *cry1* and *cry2* – see Table 1) for their role in the central circadian oscillator, we also selected candidate genes on the basis of their non-circadian functions and/or association with syndromes implying possible links with autism. Thus, we included: *per1*, for its association with long-term memory formation⁷² and high-frequency oscillators involved with communicative timing in *Drosophila*;^{69–71} *per2*, for its implication in familial advanced sleep phase syndrome;⁶⁶ and, both *ck1ε* and *per3*, for their effect on sleep disorders⁸⁵ and association with delayed sleep phase syndrome.^{65,86}

We included *npas2* because it is a paralogue of *clock*, expressed in the brain,⁸⁷ and associated with complex (cued and contextual) memory in mice.⁶⁸

Subjects with autism also show impairment in complex memory;⁸⁸ contextualized episodic memory is specifically impaired even in high-functioning autism and Asperger syndrome.^{32–35,74} *Npas2* is also a transcriptional regulator of non-rapid eye movement sleep in mice;⁶⁷ this is relevant, given the altered sleep architecture in high-functioning autism, autistic disorder and Asperger syndrome.^{53,54} Finally, we included *dbp* for its role in the regulation of clock outputs such as sleep and locomotor activity in mice^{89,90} and timeless (*tim*) for its role in *Drosophila* circadian systems.⁹¹

Materials and methods

Subjects

In the interests of sample homogeneity, we selected a predominantly high-functioning sample where all subjects were strictly diagnosed with autistic disorder (detailed below). We obtained DNA first from 90 probands and all their parents (parent–offspring trios), of whom there were 65 male and 25 female subjects, from the Autism Genetic Resource Exchange (AGRE) (<http://www.agre.org/>). This is a publicly available database and central repository founded by the Cure Autism Now Foundation.⁹² In the second stage of the study, we obtained an additional 20 AGRE probands (14 males and six females) to form, together with first wave subjects, a larger sample in which we might attempt to replicate any positive results that reached a significance level of $P < 0.05$ in the first wave. The subjects were 91% Caucasian, 4% other and 5% unknown (see Table 2).

All probands analyzed in this study met strict diagnostic criteria for autistic disorder using both the Autism Diagnostic Interview-Revised (ADI-R⁹³) and the Autism Diagnostic Observation Schedule-Generic (ADOS-G⁹⁴), compatible with DSM-IV's definition of autistic disorder that includes high-functioning individuals.¹ We excluded those with borderline or 'not quite autism' (NQA) sometimes accepted as a research diagnosis.

We also used assessment measures available from AGRE, prioritizing inclusion of subjects on the basis of intelligence (83%) over language (6%), and language over behavioral (11%) measures. The assessment measures for these three fields were Raven Progressive Matrices (RPM⁹⁵), Peabody Picture Vocabulary Test (PPVT-III⁹⁶) and the Vineland Adaptive Behavior Scales (VABS⁹⁷) respectively.

Our criterion for accepting subjects tested on the RPM was a non-verbal intelligence quotient (IQ) of at least 51. In practice, they all had IQs of 58 or more (mean 95.4, s.d. 17.7, range 58–136, $n = 91$). We accepted additional subjects ($n = 7$) with IQ approximations, equivalent to our RPM selection criteria, derived from PPVT-III percentiles using Wechsler Percentile-IQ Correspondence Scale.⁹⁸ Remaining subjects ($n = 12$) had VABS percentile rankings (0.1 and above) equivalent to those of our PPVT-III selection criterion (see Table 3).

Table 2 Diagnostic criteria and demographics of the sample

| <i>Diagnostic measures and sample demographics</i> | <i>Total</i> | <i>1st stage</i> | <i>2nd stage</i> |
|--|------------------------|-----------------------|-----------------------|
| Autistic disorder (ADI-R) ^a | 100% (<i>N</i> = 110) | 100% (<i>n</i> = 90) | 100% (<i>n</i> = 20) |
| Autistic disorder (ADOS-G) ^b | 100% (<i>N</i> = 110) | 100% (<i>n</i> = 90) | 100% (<i>n</i> = 20) |
| Male | 72% (<i>n</i> = 79) | 72% (<i>n</i> = 65) | 70% (<i>n</i> = 14) |
| Female | 28% (<i>n</i> = 31) | 28% (<i>n</i> = 25) | 30% (<i>n</i> = 6) |
| Caucasian | 91% | 91% | 90% |
| Other race ^c | 4% | 3% ^d | 10% ^e |
| Unknown race ^b | 5% | 6% | 0% |

Abbreviations: ADI-R, Autism Diagnostic Interview Revised; ADOS-G, Autism Diagnostic Observation Schedule-Generic.

^aMet strict ADI-R criteria for autistic disorder.

^bMet strict ADOS-G criteria for autistic disorder (only subjects with consistent diagnoses were accepted; we rejected those for whom ADI-R or ADOS-G re-assessment had changed their diagnostic status to autistic disorder from another diagnosis that failed to meet our criteria (e.g., ASD; 'not quite autism' (NQA)/borderline autistic disorder etc.).

^cMixed race and Pacific Islander.

^dMixed race.

^e5% mixed race and 5% Pacific Islander (*n* = 1 + 1).

Table 3 Distribution of percentile ranking (PR) from ability/behavioral measures and corresponding intelligence levels

| <i>Assessment measure</i> | <i>Sample division</i> | <i>n</i> | <i>% Total sample</i> | <i>Mean PR (IQ)^a</i> | <i>s.d.</i> | <i>Range PR (IQ)^a</i> |
|-----------------------------------|------------------------|----------|-----------------------|---------------------------------|-------------|----------------------------------|
| Raven's Progressive Matrices | 1st wave | 72 | 66 | 42 (IQ 97.3) | 17.4 | 0.3–99 (IQ 58–136) |
| | 2nd wave | 19 | 17 | 21 (IQ 88.3) | 17.5 | 1–90 (IQ 63–119) |
| | 1st and 2nd wave | 91 | 83 | 37 (IQ 95.4) | 17.7 | 0.3–99 (IQ 58–136) |
| Peabody Picture Vocabulary Scales | 1st wave | 6 | 6 | 5.5 (IQ 77) | 9.7 | 0.1–25 (IQ 51–90) |
| | 2nd wave | 1 | 1 | 0.1 (IQ 51) | N/A | 0.1 (IQ 51) |
| | 1st and 2nd wave | 7 | 6 | 4.8 (IQ 75) | 9.1 | 0.1–25 (IQ 51–90) |
| Vineland Adaptive Behavior Scales | 1st wave only | 12 | 11 | 5.7 | 14.1 | 0.1–50 |

Abbreviation: N/A, not applicable.

^aRPM non-verbal IQ estimations, together with PPVT-III, and VABS-composite percentile rankings, were obtained from AGRE. For comparison purposes in this table, Wechsler Percentile-IQ Correspondence Scale was employed to give percentiles and IQ approximations (for RPM and PPVT-III, respectively).

On the Wechsler Percentile-IQ Correspondence Scale a percentile rank of 2 corresponds to an IQ range of 68–70 inclusive and we therefore used an IQ cutoff of 68 (or percentile ranking of 2) to define 'high-functioning' autism (cf. Mottron⁹⁹). Our total sample was 85% high functioning, with remaining subjects ensuring appropriate sample size. We thereby derived the most able autistic disorder sample available from AGRE at the time of subject selection.

Possible non-idiopathic autistic disorder cases were excluded, as were probands who had any known additional major medical or neurological condition that might have been perceived to cause secondary autism. Similarly, cases with a previous or concurrent alternative diagnosis (e.g. ASD on ADI-R and/or schizophrenia) were excluded, together with those with identified chromosomal aberrations.

Screening the candidate genes

Candidate clock genes were systematically screened for indirect association with autism by genotyping

single-nucleotide polymorphisms (SNPs) covering the genes. We aimed at an average spacing of one common SNP at every 3–5 kb, but this was not always possible, or practical. For example, some of the genes were too long to allow such a dense coverage within our budget (for example, for *npas2* we tried to cover some 160 kb), and in some cases there were long gaps with no validated common SNPs at the time we performed the work. In addition, for some SNPs our genotyping assay could not be designed or failed. These reasons prevented us from providing a uniform coverage of each gene. SNPs were chosen from the publicly available databases HAPMAP (www.hapmap.org/), NCBI (www.ncbi.nlm.nih.gov/), CHIP Bioinformatics tools (snpper.chip.org/).¹⁰⁰ Collaboration with the team of Professor Vishwajit Nimgaonkar, who screened these genes in a study of bipolar affective disorder,¹⁰¹ provided details on additional SNPs that they had identified. During the course of our study a number of these SNPs became available in the public databases.

Genes where only a few or no SNPs were available were screened for mutations in the promoter, all exons and 3' UTR using denaturing high performance liquid chromatography (dHPLC) analysis on a WAVE dHPLC system (Transgenomics Inc., Cambridge, MA, USA). In addition, we screened the genes that showed evidence for association after individual genotyping of the first stage of our sample. For mutation screening, we used 16 autistic probands from our sample. Temperature and solution gradients for individual fragments for dHPLC were calculated using the Stanford Genome Technology Center DHPLC Melt Program freely available on the web (<http://insertion.stanford.edu/melt.html>).

In addition to screening the promoter regions of these genes, we also screened predicted regulatory regions of the gene using the Cister program (<http://zlab.bu.edu/~mfrith/cister.shtml>). This freely available automated resource explores clusters of cis-elements in DNA sequences and such regions were also included for screening. On average, we covered genes for screening with ~20–25 DNA fragments of 400–500 bp length each. The genes that we screened for mutations were *clock*, *per1* and *npas2*. Fragments showing possible mutations via dHPLC were sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 3.1 on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, CA, USA).

Individual genotyping was carried out using the Amplifluor SNPs Genotyping Systems (Serologicals Corporation, Norcross, GA, USA),¹⁰² which is a one-step PCR-based reaction using allele-specific primers. All forward and reverse primers for the Amplifluor reaction were designed using the automated primer design software, Amplifluor AssayArchitect, freely available through the Serologicals Corporation website (www.assayarchitect.com). Amplifluor reactions were performed in a 5 µl volume in 96-well black propylene plates (ABgene, Epsom, UK) with 15 ng of dried genomic DNA. Data were analyzed using an Analyst AD fluorescence multiwell plate reader (LJL Biosystems, CA, USA). Every assay designed was first tested on one 96-well plate of DNA samples and only SNPs that gave satisfactory clusters and no non-mendelizations were genotyped in the full sample of trios. On average, some 30% of all SNPs we attempted to genotype failed and were therefore discarded; we present results only on the SNPs that produced good traces.

Statistics

For statistical analysis of genotyping results we used the transmission disequilibrium test (TDT¹⁰³) that examines the transmission of alleles from heterozygous parents and is thus a robust test against population stratification. Haplotype analysis was performed with the program TDTPHASE.¹⁰⁴

Results

All results from individual genotyping are presented in Table 4. The table also shows the genomic

positions of SNPs according to the Golden Path database (<http://www.genome.ucsc.edu/>) (Build 35, May 2004). Initially, for the sample of 90 trios, we obtained significant evidence for association for two of the genes studied. In *per1* two intronic SNPs gave significant results: rs885747 with a $P=0.014$ and rs6416892 with $P=0.035$. Re-sequencing the gene did not identify any novel polymorphisms in the 16 individuals we genotyped, although we found a number of SNPs already available in public databases. All six SNPs genotyped in the first wave were genotyped in the additional 20 trios. The two significant SNPs remained significant: $P=0.047$ and 0.042 , respectively.

In *npas2* we found one significantly associated SNP in the first wave of genotyping: rs1811399 at $P=0.009$ level. *Npas2* was re-sequenced in an attempt to identify markers that were more strongly associated, or have a putative functional significance. We identified several new SNPs and genotyped them individually: a C/T polymorphism in intron 3 (called NPAS2_X3_C_T in Table 4), a G/A polymorphism in intron 7 (NPAS2_IN7_G_A), a G/A polymorphism in exon 8 (NPAS2_X8_G_A), an A/G polymorphism in intron 11 (NPAS2_X11_A_G), an A/T polymorphism in intron 12 (NPAS2_X12_A_T) and a C/T polymorphism in exon 15 of the gene (NPAS2_X15_C_T). These, as well as all the markers typed in the first wave, were genotyped in the complete set of 110 trios (a total of 17 SNPs). As shown in Table 4, only one of the new markers reached statistical significance in the full set of trios: NPAS2_X3_C_T with a P -value of 0.028, whereas the originally significant marker, rs1811399, remained significant at $P=0.018$.

Haplotype analysis

We performed analysis of all possible combinations of two-marker haplotypes for each gene (see Figure 1). In *npas2*, 40 out of the 136 possible two-marker haplotype combinations were significant at the $P<0.05$ level, with the best result between markers rs1811399 and rs2117714, $P=0.001$. If we perform a Bonferroni correction for 136 tests, this result would lose its significance; however, this correction is overconservative due to the high number of SNPs that are in linkage disequilibrium in *npas2* (Figure 1).

Within *per1* there was a single significant result: a global $P=0.027$ for the haplotype analysis of markers rs2253820–rs885747. No two-marker haplotype was significant in any of the other genes, despite the high number of tests performed.

Discussion

We hypothesized that clock genes are implicated in autistic disorder.²¹ In this candidate gene study, we found significant associations in two of the genes studied: *per1* and *npas2*, with two SNPs in each gene reaching conventional levels of statistical significance. In addition, a high proportion of all possible haplotypes in *npas2* was also significant. It is difficult

Table 4 Association analysis for selected markers in the candidate genes

| <i>Gene/marker</i> | <i>Coordinate (bp)</i> | <i>Distance from previous marker (bp)</i> | <i>Number of trios typed</i> | <i>Allele</i> | <i>Frequency in parents</i> | <i>Frequency in children</i> | <i>Transmitted</i> | <i>Non-transmitted</i> | <i>P-value</i> |
|--------------------|------------------------|---|------------------------------|---------------|-----------------------------|------------------------------|--------------------|------------------------|----------------|
| ARNTL_rs2279287 | 13255061 | | 87 | A > G | 0.305 | 0.328 | 35 | 27 | 0.31 |
| ARNTL_rs2279285 | 13255263 | 202 | 32 | T > G | 0.703 | 0.688 | 8 | 10 | 0.63 |
| ARNTL_rs1982350 | 13306707 | 51444 | 88 | A > G | 0.401 | 0.403 | 38 | 37 | 0.91 |
| ARNTL_rs3789327 | 13341892 | 35185 | 85 | A > G | 0.556 | 0.559 | 37 | 36 | 0.91 |
| ARNTL_rs3816358 | 13348048 | 6156 | 86 | A > C | 0.111 | 0.116 | 15 | 13 | 0.71 |
| ARNTL_rs2278749 | 13354454 | 6406 | 86 | T > C | 0.201 | 0.174 | 20 | 29 | 0.2 |
| ARNTL_rs2290035 | 13364347 | 9893 | 87 | A > T | 0.489 | 0.454 | 33 | 45 | 0.17 |
| CLOCK_SNP4_G_A | 56134430 | | 88 | G > A | 0.949 | 0.949 | 9 | 9 | 1 |
| CLOCK_rs1801260 | 56142297 | 7867 | 88 | T > C | 0.770 | 0.778 | 34 | 31 | 0.71 |
| CLOCK_rs6811520 | 56156106 | 13809 | 85 | A > G | 0.309 | 0.282 | 27 | 36 | 0.26 |
| CLOCK_rs2272073 | 56217272 | 61166 | 85 | G > A | 0.609 | 0.565 | 33 | 48 | 0.1 |
| Cry1_3741890 | 105889492 | | 31 | G > T | 0.436 | 0.436 | 14 | 14 | 1 |
| Cry1_rs1921126 | 105914072 | 24580 | 84 | A > G | 0.545 | 0.560 | 45 | 40 | 0.59 |
| Cry1_rs1861591 | 105919394 | 5322 | 83 | G > A | 0.542 | 0.566 | 45 | 37 | 0.38 |
| Cry1_rs1420399 | 105919649 | 255 | 62 | C > T | 0.419 | 0.403 | 24 | 32 | 0.28 |
| Cry1_rs2078074 | 105939273 | 19624 | 85 | A > G | 0.562 | 0.582 | 48 | 41 | 0.46 |
| CRY2_rs7121775 | 45820899 | | 31 | C > T | 0.2823 | 0.2742 | 12 | 13 | 0.84 |
| CRY2_3_A_G | 45823734 | 2835 | 87 | A > G | 0.287 | 0.293 | 35 | 33 | 0.81 |
| CRY2_rs2292912 | 45834264 | 10530 | 87 | C > G | 0.2011 | 0.1897 | 26 | 30 | 0.59 |
| CRY2_rs1401417 | 45836686 | 2422 | 31 | C > G | 0.7016 | 0.6935 | 11 | 12 | 0.84 |
| CRY2_rs12364060 | 45846351 | 9665 | 29 | C > T | 0.724 | 0.724 | 11 | 11 | 1 |
| CRY2_rs7933420 | 45853373 | 7022 | 87 | T > A | 0.5374 | 0.5517 | 45 | 40 | 0.59 |
| CRY2_rs877412 | 45854227 | 854 | 84 | A > C | 0.5863 | 0.5893 | 31 | 30 | 0.9 |
| CRY2_rs3824872 | 45862181 | 7954 | 32 | C > A | 0.7891 | 0.7656 | 9 | 12 | 0.5 |
| CKI_rs2075983 | 37015198 | | 90 | T > C | 0.2611 | 0.2889 | 39 | 29 | 0.22 |
| CKI_rs2075984 | 37015389 | 191 | 106 | G > T | 0.5212 | 0.5283 | 49 | 42 | 0.46 |
| CKI_rs1005473 | 37018873 | 3484 | 108 | T > G | 0.9444 | 0.9352 | 10 | 14 | 0.41 |
| CKI_rs1534891 | 37019599 | 726 | 108 | G > A | 0.8727 | 0.8519 | 19 | 28 | 0.19 |
| DBP_rs3745734 | 53826200 | | 90 | C > G | 0.9333 | 0.9222 | 12 | 12 | 1 |
| DBP_rs550455 | 53828327 | 2127 | 87 | A > G | 0.7098 | 0.7184 | 35 | 34 | 0.9 |
| DBP_rs386551 | 53830098 | 1771 | 88 | G > C | 0.7045 | 0.7159 | 35 | 33 | 0.81 |
| DBP_rs3848543 | 53833344 | 3246 | 88 | G > A | 0.8693 | 0.8523 | 17 | 21 | 0.51 |
| NPAS2_rs3849381 | 100910337 | | 107 | C > T | 0.2734 | 0.2944 | 48 | 39 | 0.34 |
| NPAS2_rs1811399 | 100937532 | 27195 | 109 | C > A | 0.2294 | 0.1789 | 32 | 54 | 0.018 |
| NPAS2_rs2117714 | 100980295 | 42763 | 106 | A > G | 0.7005 | 0.7358 | 48 | 33 | 0.1 |
| NPAS2_rs356651 | 101000316 | 20021 | 106 | C > T | 0.1439 | 0.1321 | 25 | 30 | 0.5 |
| NPAS2_X3_C_T | 101000390 | 74 | 110 | T > C | 0.1886 | 0.2318 | 47 | 28 | 0.028 |
| NPAS2_rs895521 | 101010613 | 10223 | 108 | C > T | 0.8333 | 0.8519 | 37 | 29 | 0.32 |
| NPAS2_rs13017718 | 101012554 | 1941 | 108 | T > C | 0.0718 | 0.0694 | 14 | 15 | 0.85 |
| NPAS2_rs895520 | 101036455 | 23901 | 105 | A > G | 0.4167 | 0.3952 | 37 | 46 | 0.32 |
| NPAS2_INT7_G_A | 101038851 | 2396 | 108 | A > G | 0.0856 | 0.088 | 18 | 17 | 0.87 |
| NPAS2_X8_G_A | 101039093 | 242 | 108 | G > A | 0.831 | 0.8565 | 32 | 21 | 0.13 |
| NPAS2_X11_A_G | 101043469 | 4376 | 108 | A > G | 0.8588 | 0.875 | 30 | 23 | 0.34 |
| NPAS2_rs1562313 | 101045973 | 2504 | 109 | C > T | 0.8211 | 0.8028 | 27 | 35 | 0.31 |
| NPAS2_X12_A_T | 101046160 | 187 | 108 | A > T | 0.8542 | 0.875 | 32 | 23 | 0.22 |
| NPAS2_rs2305160 | 101049822 | 3662 | 108 | G > A | 0.6829 | 0.6435 | 38 | 55 | 0.08 |
| NPAS2_rs2305159 | 101049961 | 139 | 108 | A > C | 0.6898 | 0.6898 | 45 | 45 | 1 |
| NPAS2_X15_C_T | 101052709 | 2748 | 108 | C > T | 0.8264 | 0.8519 | 33 | 22 | 0.14 |
| NPAS2_rs2305158 | 101070569 | 17860 | 110 | A > G | 0.2341 | 0.2318 | 40 | 41 | 0.91 |
| Per1_rs2289591 | 7988735 | | 106 | T > G | 0.2264 | 0.1887 | 29 | 45 | 0.063 |
| Per1_rs2253820 | 7988894 | 159 | 108 | A > G | 0.2269 | 0.2315 | 30 | 24 | 0.41 |
| Per1_rs885747 | 7991462 | 2568 | 107 | C > G | 0.6262 | 0.5841 | 32 | 50 | 0.047 |
| Per1_rs3027178 | 7993810 | 2348 | 110 | A > C | 0.6864 | 0.7273 | 55 | 41 | 0.15 |
| Per1_rs6416892 | 7983585 | 75 | 107 | C > A | 0.3364 | 0.3879 | 48 | 30 | 0.042 |
| Per1_rs2585398 | 7995585 | 1700 | 104 | G > T | 0.5048 | 0.4663 | 39 | 51 | 0.21 |
| Per2_In1_C_T | 238926115 | | 87 | C > T | 0.954 | 0.954 | 7 | 7 | 1 |
| Per2_rs2304669 | 238947663 | 21548 | 32 | G > A | 0.1719 | 0.125 | 6 | 12 | 0.16 |
| Per2_In17_G_T | 238947792 | 129 | 90 | G > T | 0.9806 | 0.9889 | 5 | 2 | 0.26 |

Table 4 Continued

| Gene/marker | Coordinate (bp) | Distance from previous marker (bp) | Number of trios typed | Allele | Frequency in parents | Frequency in children | Transmitted | Non-transmitted | P-value |
|----------------|-----------------|------------------------------------|-----------------------|--------|----------------------|-----------------------|-------------|-----------------|---------|
| Per2_Ex19F_L | 238951288 | 3496 | 90 | C>T | 0.025 | 0.0111 | 2 | 7 | 0.1 |
| Per2_Ex23C_T | 238959378 | 8090 | 87 | T>C | 0.1408 | 0.1322 | 17 | 20 | 0.62 |
| Per3_rs228729 | 7779961 | | 87 | C>T | 0.6839 | 0.6379 | 28 | 42 | 0.094 |
| Per3_rs228688 | 7813396 | 33435 | 24 | T>G | 0.3854 | 0.375 | 12 | 13 | 0.84 |
| Per3-21-T/G | 7814949 | 1553 | 86 | G>T | 0.1512 | 0.1395 | 18 | 22 | 0.53 |
| Per3_rs2859387 | 7821514 | 6565 | 85 | A>G | 0.45 | 0.4059 | 34 | 47 | 0.15 |
| Per3_rs228665 | 7825222 | 3708 | 84 | C>G | 0.3065 | 0.3333 | 37 | 30 | 0.39 |
| Per3-27-C/T | 7831399 | 6177 | 24 | C>T | 0.1667 | 0.1875 | 8 | 6 | 0.59 |
| Per3_rs4908699 | 7839540 | 8141 | 85 | A>G | 0.1412 | 0.1176 | 18 | 26 | 0.23 |
| TIME_rs2291739 | 55100920 | | 88 | C>T | 0.4489 | 0.4659 | 50 | 44 | 0.54 |
| TIME_rs2291738 | 55101548 | 628 | 86 | G>A | 0.5174 | 0.5 | 43 | 49 | 0.53 |
| TIME_rs774047 | 55102189 | 641 | 85 | G>A | 0.5471 | 0.5412 | 44 | 46 | 0.83 |
| TIME_rs774033 | 55111578 | 9389 | 88 | G>A | 0.5426 | 0.5284 | 44 | 49 | 0.6 |
| TIME_rs2279665 | 55113961 | 2383 | 84 | G>C | 0.5387 | 0.5119 | 40 | 49 | 0.34 |

P-values <0.05 are shown in bold type.

| | (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) | (9) | (10) | (11) | (12) | (13) | (14) | (15) | (16) | (17) |
|--------------------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| rs3849381 (1) | | 0.90 | 0.89 | 0.18 | 0.72 | 0.31 | 0.72 | 0.21 | 0.36 | 0.18 | 0.62 | 0.14 | 0.63 | 0.07 | 0.03 | 0.16 | 0.37 |
| rs1811399 (2) | 0.08 | | 0.64 | 0.30 | 0.79 | 0.22 | 0.48 | 0.41 | 0.01 | 0.01 | 0.14 | 0.13 | 0.13 | 0.08 | 0.05 | 0.02 | 0.04 |
| rs2117714 (3) | 0.11 | 0.04 | | 0.86 | 1.00 | 0.75 | 0.17 | 0.05 | 0.19 | 0.34 | 0.25 | 0.11 | 0.22 | 0.26 | 0.06 | 0.24 | 0.20 |
| rs356651 (4) | 0.02 | 0.05 | 0.05 | | 1.00 | 0.78 | 0.23 | 0.45 | 0.50 | 0.37 | 0.40 | 0.63 | 0.42 | 0.22 | 0.60 | 0.37 | 0.50 |
| NPAS2_X3_C_T (5) | 0.04 | 0.04 | 0.09 | 0.03 | | 1.00 | 0.27 | 0.11 | 0.47 | 0.02 | 0.26 | 0.44 | 0.26 | 0.08 | 0.50 | 0.01 | 0.00 |
| rs895521 (6) | 0.05 | 0.03 | 0.04 | 0.52 | 0.04 | | 0.74 | 0.29 | 0.17 | 0.30 | 0.23 | 0.03 | 0.26 | 0.21 | 0.11 | 0.30 | 0.38 |
| rs13017718 (7) | 0.01 | 0.00 | 0.01 | 0.00 | 0.03 | 0.01 | | 0.88 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.76 | 1.00 | 0.80 | 0.56 |
| rs895520(8) | 0.02 | 0.03 | 0.00 | 0.05 | 0.00 | 0.02 | 0.09 | | 1.00 | 1.00 | 1.00 | 0.32 | 1.00 | 0.98 | 0.64 | 0.97 | 0.03 |
| NPAS2_INT7_G_A (9) | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.01 | 0.07 | | 1.00 | 0.96 | 1.00 | 0.96 | 0.88 | 0.95 | 1.00 | 0.10 |
| NPAS2_X8_G_A (10) | 0.02 | 0.00 | 0.01 | 0.12 | 0.00 | 0.09 | 0.01 | 0.29 | 0.02 | | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 0.00 |
| NPAS2_X11_A_G (11) | 0.02 | 0.01 | 0.02 | 0.00 | 0.00 | 0.00 | 0.01 | 0.12 | 0.53 | 0.03 | | 0.53 | 1.00 | 1.00 | 0.97 | 1.00 | 0.03 |
| rs1562313 (12) | 0.01 | 0.00 | 0.00 | 0.01 | 0.01 | 0.00 | 0.02 | 0.01 | 0.02 | 0.04 | 0.01 | | 0.54 | 1.00 | 1.00 | 1.00 | 0.20 |
| NPAS2_X12_A_T (13) | 0.02 | 0.01 | 0.02 | 0.00 | 0.00 | 0.00 | 0.01 | 0.13 | 0.50 | 0.03 | 0.96 | 0.01 | | 1.00 | 0.97 | 1.00 | 0.02 |
| rs2305160 (14) | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.02 | 0.29 | 0.03 | 0.09 | 0.06 | 0.09 | 0.07 | | 1.00 | 1.00 | 0.02 |
| rs2305159 (15) | 0.00 | 0.00 | 0.00 | 0.02 | 0.02 | 0.00 | 0.03 | 0.13 | 0.20 | 0.08 | 0.36 | 0.48 | 0.38 | 0.20 | | 1.00 | 0.12 |
| NPAS2_X15_C_T (16) | 0.02 | 0.00 | 0.00 | 0.11 | 0.00 | 0.08 | 0.01 | 0.29 | 0.02 | 0.96 | 0.03 | 0.04 | 0.03 | 0.09 | 0.09 | | 0.01 |
| rs2305158 (17) | 0.02 | 0.00 | 0.03 | 0.01 | 0.00 | 0.01 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | |

Figure 1 Linkage disequilibrium within the NPAS2 gene. Above the diagonal: D' , below the diagonal: r^2 . Values above 0.5 are highlighted.

to interpret the real significance of these results. On the one hand, if we perform a correction for the multiple testing of all SNPs analyzed in this study,

none of the results will remain significant. On the other hand, our sample size is too small to allow us to identify markers that would withstand correction for

multiple testing, unless they conferred huge effects. This is unlikely to be the case in a disorder of complex inheritance such as autism where current models predict several interacting genes of small effect, each contributing to the phenotype of autistic disorder.⁵ In this context, we note that the NPAS2/ARNTL (BMAL1) heterodimer is an activator of *per1*;⁸⁷ it follows that genetic variation affecting the function of NPAS2 could be additive to genetic variation affecting the function of PER1.

We used all available cases of autistic disorder in the AGRE database that satisfied our strict selection criteria and we were therefore unable to consider replicating our initial results in a large sample. We placed considerable emphasis on subject diagnosis and intelligence in order to select a very homogeneous sample; restricting the phenotype in this way also increased the experimental power over a limited sample size. We satisfied the strictest diagnostic criteria for autistic disorder using ADOS-G and ADI-R for each case. By selecting for more able subjects, we derived a sample that was predominantly (85%) high functioning and unusually homogeneous in this field of research to date. To confirm or refute our results, future studies would need to collect other samples that have a similar composition.

The expression patterns of *npas2*^{68,105} and *per1*,¹⁰⁶ when considered together, cover brain areas found to be altered in individuals with autism: the cerebellum, forebrain and limbic system including the hippocampus and amygdala.^{107–110} Non-circadian expression of *per1* occurs in the cerebrum, hypothalamus and cerebellum (granular layer), while circadian *per1* expression is found in the SCN hypothalamus and Purkinje cells.¹⁰⁶

The implication of *npas2* in complex emotional memory,⁶⁸ and our results associating *npas2* with autistic disorder, lend tentative support to findings that the thalamo-cortico-amygdalo pathway (associated with complex emotional memory^{111–113}) is dysfunctional in autism.^{109,114,115} This may be relevant for related neurological and psychological theories that focus on episodic/contextual memory dysfunction and the hippocampus/limbic system^{34,116} and/or the amygdala.^{117–119}

Evidence of altered sleep architecture in autism is also supported by our positive result for *npas2*, as *npas2* is a transcriptional regulator of non-rapid eye movement sleep in mice.⁶⁷ Several of the sleep features attributable to *npas2* knockout in mice are recognized in high-functioning autism and Asperger syndrome; longer sleep latency, prolonged waking after sleep onset and reduced non-rapid-eye-movement sleep.⁵³ Franken *et al.*⁶⁷ also show that the effect of *npas2* knock-out on sleep is modulated by the sex of the animal (with males being more adversely affected than females), an observation that may have relevance to autism, given the male to female ratio of 3.8:1.⁶

The role of *per1* in modulating high-frequency oscillators concerned with communicative timing,^{69–71}

together with our findings of association of *per1* with autistic disorder, strengthens the notion that temporal deficits are quintessential to autistic disorder.^{21–26,28,29,39} Purkinje neurons are important for learning appropriate timing^{120,121} and their abnormally low number in the cerebella of autistic subjects^{115,122} is considered a keystone biological observation implicating cerebellar dysfunction.^{123,124} In this context, reports of a *per1* interacting protein (PIPS) in rat that co-translocates with *per1* into the nucleus¹²⁵ and which is further shown to be required for neuronal growth factor-mediated neuronal survival in P12 cells,¹²⁶ tentatively suggests a role for *per1* in the lack of Purkinje neurons of the cerebellum in autism. We are currently engaged in an investigation of PIPs as well as further analysis of *hper1* and *hper2*.

The gene *engrailed2* (*en2*) functions in the development of the cerebellum¹²⁷ and has been shown to be associated with autistic spectrum disorders.^{15,16} A risk allele that interacts with *en2* variants to perturb the normal spatial/temporal expression of *en2* could alter normal brain development, a point that has resonance with *per1*'s role in the cell division cycle¹²⁸ and dopamine D2 receptor-mediated signaling.¹²⁹

The timing, contextual/memory deficits and other implications suggested by the association of *per1* and *npas2* with autistic disorder may manifest concurrently and/or developmentally, for example, through infant–adult interaction that is timing-dependent.^{130–133} Neonatal physiological oscillations (sleep–wake cyclicality and cardiac vagal tone) predict mother–infant synchrony at 3 months.¹³⁴ In turn, the temporal organization of 3-month-old infants' social attention predicts mother–infant synchrony at 9 months.¹³⁵ Mother–infant synchrony itself predicts wide-ranging later developments.^{135–137} Temporal synchrony/reciprocity difficulties of autism^{49–51} have also been recorded in autistic infancy,^{52,138,139} where they may serve as a functional deficit contributing to the developmental cascade of autistic disorder.^{21,22,44,140,141}

Problems in timing, memory and sleep are all characteristics of autistic disorder and aspects of timing, memory and sleep are each clock-gene-regulated in other species. The association of clock genes with autistic disorder suggests a role for these genes in autism. It also encourages replication of our study and the collection of further data, particularly on timing, sleep and emotional complex/contextual memory, in high-functioning autistic disorder.

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