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Autism Associated Haplotype Affects the Regulation of the Homeobox Gene, *ENGRAILED 2*

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Abstract

Background—Association analysis identified the homeobox transcription factor, ENGRAILED 2 (EN2), as a possible Autism Spectrum Disorder (ASD) susceptibility gene (ASD [MIM 608636]; EN2 [MIM 131310]). The common alleles (underlined) of two intronic SNPs, rs1861972 (A/G) and rs1861973 (C/T), are over-transmitted to affected individuals both singly and as a haplotype in three separate datasets (518 families total, haplotype P=0.00000035). Methods: Further support that EN2 is a possible ASD susceptibility gene requires the identification of a risk allele, a DNA variant that is consistently associated with ASD but is also functional. To identify possible risk alleles, additional association analysis and LD mapping were performed. Candidate polymorphisms were then tested for functional differences by luciferase (luc) reporter transfections and Electrophoretic Mobility Shift Assays (EMSAs). Results: Association analysis of additional EN2 polymorphisms and LD mapping with Hapmap SNPs identified the rs1861972-rs1861973 haplotype as the most appropriate candidate to test for functional differences. Luc reporters for the two common rs1861972-rs1861973 haplotypes (A-C and G-T) were then transfected into human and rat cell lines as well as primary mouse neuronal cultures. In all cases the A-C haplotype resulted in a significant increase in luc levels (P < .005). EMSAs were then performed and nuclear factors bound specifically to the A and C alleles of both SNPs. Conclusions: These data indicate the AC haplotype is functional and together with the association and LD mapping results support EN2 as a likely ASD susceptibility gene and the A-C haplotype as a possible risk allele.

Keywords

Autism; ENGRAILED 2; risk allele

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INTRODUCTION

Autism Spectrum Disorder (ASD) is a polygenic disorder affecting CNS development ^{1,2}. Individuals with ASD display deficits in language, emotional reciprocity as well as increased repetitive behaviors and movements. Although strong evidence for a genetic contribution to ASD exists, few causative genetic defects have been implicated in the etiology of the disorder ³⁻⁹.

Our previous analysis has focused on EN2, an important regulator of CNS development¹⁰⁻¹³. EN2 maps to the distal portion of chromosome 7 (7q36.3) and is encoded by two exons and a single 3.5kb intron spanning 8.1kb of genomic DNA. EN2 association was tested previously in nuclear pedigrees obtained from the Autism Genetic Resource Exchange (AGRE) and the NIMH. The pedigrees have at least two siblings diagnosed with ASD and can also include unaffected siblings.

Two intronic EN2 SNPs, rs1861972 and rs1861973, are significantly associated with ASD individually and as a haplotype under both a narrow (autism) and broad (autism, Asperger's syndrome or Pervasive Developmental Delay-Not Otherwise Specified) phenotypic definition. The common alleles for both SNPs (A-rs1861972; C-rs1861973) are over-transmitted to affected individuals and under-represented in unaffected siblings. These results were observed in an original dataset of 167 families (AGRE I, rs1861972-narrow: P=.026, broad: P=.016; rs1861973- narrow: P=.008, broad: P=.012; rs1861972-rs1861973 haplotype, narrow: P=. 0009, broad: P=.0017)¹⁴. Association was then replicated in two separate datasets (AGRE II, 222 families, haplotype, narrow: P=.0048, broad: P=.0016 and NIMH, 129 families, haplotype, narrow: P=.0463, broad: P=.0431). When all three datasets were combined strong evidence for association was observed (518 families, haplotype, narrow: P=.00000065; broad: P=. 00000035)^{14,15}. Rs1861972 and rs1861973 display strong inter-marker LD with each other in these three datasets (D'=.903, r^2 =.767). In the combined three datasets the frequencies of the common A and C alleles for rs1861972 and rs1861973 both individually and as a haplotype are ~72% (rs1861972 A allele- 73%, rs1861973 C allele- 72%, A-C haplotype- 71%). Four other groups have reported some association for EN2 with autism in datasets of different ethnicities: a Northern French population ¹⁶, one of largely Western-Northern European descent ¹⁷, and two Chinese datasets ^{18,19}. However, polymorphic and allelic differences have been observed between these studies and our association data, suggesting that underlying causative genetic variant(s) may vary between datasets and ethnicities. Although many different rare and common variants are likely to contribute to ASD susceptibility, these data are consistent with EN2 being a likely ASD susceptibility gene. However further support for this possibility requires the isolation of a risk allele, an associated polymorphism that affects the expression or activity of EN2.

We expect candidate risk alleles to be in strong LD with rs1861972 and rs1861973 and to display at least as significant association with ASD as the A-C haplotype under both diagnoses. Our prior re-sequencing, association, and LD mapping data identified the rs1861972rs1861973 A-C haplotype as a candidate for the EN2 risk allele. Previously 16 additional EN2 polymorphisms were typed in the AGRE I dataset. Only the intronic SNPs demonstrated high D' with rs1861972 and rs1861973, while one intronic SNP, rs2361688 (Minor Allele Frequency (MAF) = 27%), displayed high r^2 (rs1861972 = .730; rs1861973 = .807). Resequencing of the intron identified one new SNP with a MAF of ~1%. Association analysis for all 16 EN2 polymorphisms demonstrated that none of them were as strongly associated as rs1861972 or rs1861973 individually or as a haplotype. Rs2361688 and another intronic SNP (rs3824068) displayed minimal association but only under one diagnostic criterion (rs2361688: narrow P=.13, broad P=.04; rs3824068: narrow P=.04, broad P=.10)¹⁵. This analysis identified the rs1861972-rs1861973 haplotype as one possible candidate risk allele

but it was unknown whether additional polymorphisms were in strong r^2 with either associated SNP. We now address this possibility and provide additional genetic and molecular data implicating the *rs1861972-rs1861973* haplotype as a functional variant that may increase ASD risk.

MATERIALS AND METHODS

Hapmap

CEU Hapmap genotypes for *rs1861973* were obtained from the Hapmap consortium. All other genotypes were directly acquired from Hapmap (PhaseII, January 2007, NCBI: dbSNP b125). Haploview program (version 4.0) determined inter-marker LD relation between CEU Hapmap SNPs and *rs1861973*. For other datasets, LD data was directly downloaded from the Hapmap website.

Genotyping, association and LD analysis

Details concerning the genotyping, error checking, LD, and association analysis for eight *EN2* 3' polymorphisms typed as part of this analysis are available as Supplemental Information. The AGRE I, AGRE II, and NIMH datasets ¹⁵ were subdivided by ethnicity and *rs1861972* and *rs1861973* were analyzed for association in the White non-Hispanic subset (489 families, 2266 individuals, 790 individuals with narrow autism diagnosis, 938 individuals with broad ASD diagnosis). Three SNPs that displayed minimal association in the AGRE I dataset (*rs2361688, rs3824068, and rs12533271*) were also tested for association in the White non-Hispanic subset of AGRE I (154 families, 686 individuals, 241 individuals with autism narrow diagnosis, 298 individuals with broad ASD diagnosis).

Luciferase assays

Details concerning the generation of luc constructs are available as Supplemental Information. HEK293T cells were maintained in D-MEM supplemented with 10% FBS and 1% Penicillin/ Streptomycin. PC12 cells were maintained as above except with an additional 5% horse serum. Granule cells were isolated from P6 C57BL6 mice by standard protocols and maintained at 35°C under 5% CO₂ 5µg of pGL3 constructs and 300ng of phRL-null vector (Promega) were transfected by Amaxa electroporation into 5 million granule and PC12 cells. HEK293T cells were transfected-null vector using the lipofectamine 2000 system. 24 hours following transfection, cells were collected and lysed using a 1X Promega passive lysis buffer. Luciferase activities were measured using the VeritasTMMicroplate Luminometer where 85µl of Promega luciferase substrate (LARII) and 100µl of Promega Renilla luciferase substrate (Stop & Glow) were consecutively added to 35µl of cell lysates.

Splicing RTPCR

HEK293T cells were transfected as described above with TATA-Luc-Intron A-C and G-T constructs. 24 hours after transfection, RNA was isolated and cDNA was generated. Primer sequences and RT-PCR conditions are available as Supplemental Information. The expected RT-PCR products are 1758bp and 342bp using the F1/R and F2/R primers respectively. Cerebellar post-mortem samples (lobule 6) were obtained from The Harvard Brain Tissue Resource Center. The *rs1861972* and *rs1861973* genotype was determined as described previously ¹⁵. Total RNA was isolated from two affected (1 A-C/G-T, 1 G-T/G-T) and two psychiatrically normal (1 A-C/G-T, 1 G-T/GT) individuals by standard RNA purification procedure using RNA*later*-ICE (Ambion) and mirVana PARIS kit (Ambion). cDNA was generated and RT-PCR was performed (see Supplemental Information). The predicted size of the amplicon indicative of correct splicing is 134bp.

qRT-PCR

HEK293T cells were transfected with TATA-Luc, TATA-Luc-Intron A-C, and G-T constructs as described above. Primers for qPCR were designed using the Primer Express[®] software version 2.0 and available as Supplemental Information. qPCR was performed by adding 20µM of each primer, 12.5µl of 2x SYBR® Green and 2.5µl of cDNA using the ABI PRISM® 7000HT Sequence Detection System.

Electrophoretic mobility shift assays

Nuclear extracts prepared from P6 mouse granule neurons cultured for 24 hours were isolated using Panomics nuclear extraction kit (AY2002). Biotin-labeled, sense and anti-sense 21bp probes were designed such that 10bp of sequence both 5' and 3' flanked the polymorphic alleles of *rs1861972* and *rs1861973* (see Supplemental Information). Using the Panomics EMSA kit (AY1000),100ng of nuclear extracts was incubated with 1µg of Poly d(I-C) for 5 minutes at room temperature. 2µl of 5x binding buffer and 10ng of biotin labeled probes were then added to a final volume of 10µl and incubated for 30 minutes at 20°. For competition assays, 100 to 80 fold molar excess of competitors was added to the mixture prior to the 30 minutes incubation. The protein/DNA complex was separated on a non-denaturing 6% acrylamide gel in 0.5X Trisborate-EDTA (TBE) buffer and wet-transferred onto a Biodyne Nylon membrane (PALL) which was exposed to a HyBlotCLTM Autoradiography film (Denville Scientific Inc) for chemiluminescence detection.

RESULTS

Association and LD mapping analysis

Candidate risk alleles responsible for rs1861972-rs1861973 ASD association are anticipated to fulfill the following three criteria: i) display high inter-marker r^2 with rs1861972 and rs1861973, ii) exhibit at least as strong association as the rs1861972-rs1861973 haplotype under both narrow and broad diagnostic definitions, and iii) demonstrate a functional difference between alleles.

Because the region immediately 3' of *EN2* was not densely analyzed in our previous study, eight additional polymorphisms were typed in AGRE I. None of these polymorphisms displayed pairwise r^2 values exceeding 0.05 with *rs1861972* or *rs1861973* (Supplemental Table 1). In addition, one SNP (*rs12533271*) was marginally associated with ASD but only under the broad diagnosis (Supplemental Table 2).

To extend our LD map, we then examined publicly available Hapmap data, which was typed for rs1861973 but not rs1861972. To validate the applicability of the HapMap data to the AGRE and NIMH samples, the following was performed. First, r^2 and D' values were first determined for 5 SNPs (rs1861973, rs6460013, rs3824067, rs3808331 and rs1861958) typed in both the Hapmap and our ASD datasets. Because 70.3% of the AGRE datasets tested for association were of Northern/Western European descent, the CEU inter-marker LD values were evaluated first. Very similar r^2 and D' values were observed in both datasets (Supplemental Table 3). Second, the three ASD datasets tested previously for association (518 families) were then subdivided by ethnicity and 489 White non-Hispanic families were selected for analysis. Individual and haplotype association for rs1861972 and rs1861973 association was very similar between the White non-Hispanic subset and our previously reported results (Supplemental Tables 4 and 5). These studies validate using Hapmap CEU data to identify additional candidate risk alleles.

The Hapmap pairwise r^2 values with *rs1861973* were then ascertained in the CEU dataset for 3120 SNPs within 2 Mb of *EN2* (~1 SNP/641bp; ~66% of validated Ensembl SNPs). This

region was selected because it is likely to include most of the important cis-regulatory elements for *EN2* expression $^{20-22}$. We found that all Hapmap SNPs within the 2 Mb region were in weak r² with *rs1861973* (<.370)(Fig 1). In addition, little difference in inter-marker r² values with *rs1861972* and *rs1861973* was noted in the White non-Hispanic subset (Fig 1, Supplemental Fig 1) or the other Hapmap datasets (Supplemental Table 6).

Finally, the three other SNPs (*rs2361688*, *rs3824068*, and *rs12533271*) demonstrating minimal association in the AGRE I dataset were analyzed in the White non-Hispanic subset (n=154). *Rs2361688* is not associated under either diagnostic definition while *rs3824068* and *rs12533271* display minimal association only under one diagnostic criterion (Supplemental Table 7).

Thus only rs1861972 and rs1861973 fulfill the first two criteria for an ASD risk allele responsible for our previously reported EN2 association. One, they are in high r^2 with each other, and two both SNPs display consistent association with ASD under both diagnostic criteria. For these reasons we first decided to test the possible functionality of rs1861972 and rs1861973.

Luciferase assays

To investigate whether a functional difference could be observed between the two common rs1861972-rs1861973 haplotypes (A-C and G-T), luciferase (luc) assays were performed. Luc assays measure quanta of light and due to their reproducibility and sensitivity are commonly used to test functional activity of cis-regulatory sequences. Since the activity of cis-regulatory elements can be affected by position and functional variants associated with common disorders often have subtle effects on gene regulation $^{23-29}$, we designed the luc constructs to approximate the endogenous locus. The intron was cloned 3' of the promoter and the luc protein coding sequence but 5' of the SV40 poly-adenylation site so that the intron would be transcribed and spliced as the endogenous gene. Two promoters were used: the SV40 minimal promoter or the EN2 promoter (-1 to -5500) that is evolutionarily conserved from humans to rodents. These constructs were transiently transfected into three different cell types: a human nonneuronal cell line (HEK293T), a rat neuronal cell line (PC12) and primary cultures of mouse post-natal day 6 (P6) cerebellar granule neurons. Immunohistochemistry and *in situ* analysis have established that En2 is expressed abundantly in P6 post-mitotic granule neurons 13,30. Our RTPCR experiments demonstrated that En2 transcripts are detected in P6 primary granule cell cultures and HEK293T cells but not PC12 cells (Supplemental Fig 2). In all three cell types and for both promoters, the A-C haplotype resulted in a significant increase in luc levels compared to the G-T haplotype (Fig 2).

We also transfected the SV40 minimal promoter intronic constructs into HEK-293T cells and measured *luc* mRNA levels by q-RTPCR. A similar difference in normalized *luc* RNA levels was observed between haplotypes (Supplementary Fig 3). These results demonstrate a consistent functional difference between the A-C and G-T haplotypes.

Splicing assays

Because the intron is transcribed, we also investigated whether the A-C haplotype affects splicing. For the above A-C and G-T constructs the intron also included the splice acceptor and donor sequences of each *EN2* exon so that potential splicing effects of the haplotype could be investigated. The SV40 minimal promoter intronic constructs were then transfected into HEK-293T cells and RTPCR experiments with multiple primer sets to *luc* and the SV40 polyA sequence were performed (Supplemental Fig 4A). Appropriate cycling conditions were used to amplify the intron if it was present in the cDNA. Only amplicons of the correctly spliced transcripts were observed, indicating that neither haplotype resulted in cryptic splicing

(Supplemental Fig 4B, C). This was confirmed by performing RTPCR for *EN2* on cerebellar post-mortem samples with and without the risk allele (Supplemental Fig 4D).

Electrophoretic Mobility Shift Assay (EMSA) analysis

To investigate whether the associated SNPs affect the binding of DNA proteins, EMSAs were conducted. Nuclear extracts from P6 post-mitotic cerebellar granule cells were isolated and incubated with labeled oligonucleotides containing either allele of *rs1861972* and *rs1861973*. For *rs1861972*, we detected two bands that specifically interacted with the common A allele but not the rare G allele (Fig 3A). These protein-DNA complexes were consistently observed in all nuclear extract preps (n=4). A third complex that interacted with both alleles was also detected but its presence was more variable between extracts (Fig 3A, B).

Similar results were observed for *rs1861973*. Two specific DNA-protein complexes were consistently detected for the common C allele but not the rare T allele while one shifted band was observed for both alleles in some extracts (Fig 3A, B). All *rs1861972* and *rs1861973* DNA-protein complexes were competed with 100 molar excess of unlabelled oligonucleotide. These data demonstrate the specific binding of factors to the common alleles of both SNPs, which are over-transmitted to individuals with ASD.

Bioinformatic analysis (Transcription Element Search Software-TESS)³¹ supports our EMSA results. The common A allele of *rs1861972* (underlined) is situated in a canonical CCA<u>A</u>T binding site recognized by three transcription factor families (NF1, NFY and C/EBP) composed of multiple genes. The rare G allele (CCAGT) replaces one of the obligatory A nucleotides required for transcription factor recognition, which is predicted to completely disrupt binding of all three transcription factor families (Fig 4, Supplemental Table 8). For *rs1861973*, the sequence containing the common C allele is situated in overlapping consensus sites for the Sp1 and Ets family of transcription factors (Fig 4). Similar to *rs1861972*, the rare T allele of *rs1861973* replaces a cytosine, which is required for the sequence-specific DNA binding of Sp1 and Ets family members. Transcription factors are also predicted to bind equally well to both alleles of *rs1861972* and *rs1861973*, consistent with the common shifted complexes observed in some extract preps.

We further investigated the specificity of binding by performing additional competitions. Oligonucleotides mutated for either the CCAAT sequence for *rs1861972* or the overlapping Sp1/Ets binding site for *rs1861973* did not compete in our EMSAs (Fig 4B). Finally, oligonucleotides containing the rare alleles for *rs1861972* (G allele) and *rs1861973* (T allele) also did not compete as well as equimolar amounts of the associated alleles (Fig 4B). These studies are consistent with *rs1861972* and *rs1861973* affecting the binding of nuclear factors.

DISCUSSION

Our previous data demonstrated that the rs1861972-rs1861973 A-C haplotype is consistently associated with ASD in three separate datasets. LD mapping, association analysis and resequencing identified the rs1861972-rs1861973 haplotype as a possible risk allele. It was equally possible the associated SNPs were in strong LD with a risk variant mapping at a distance from EN2. In addition, no functional difference between the rs1816972-rs1861973 A-C and G-T haplotypes had yet been demonstrated ^{14,15}. We have now extended the LD map and none of the new markers display high inter-marker r^2 with rs1861973. These data are consistent with the shorter LD spans typically observed in telomeric positions ³² but it remains formally possible that rs1861972 and rs1861973 are in high r^2 with other polymorphisms not typed in our analysis and these unidentified variants may also contribute to a functional difference. Nevertheless our LD mapping and association results identified the rs1861972-rs1861973 haplotype as the best candidate for functional experiments. Our luc assays demonstrate a

consistent increase in levels for the A-C haplotype in three cell types using two different promoters. The specific binding of nuclear factors to the A and C alleles support this functional difference. In summary only *rs1861972* and *rs1861973* currently fulfill all three criteria of a risk allele responsible for our reported *EN2* association: i) these SNPs are consistently associated with ASD under a narrow (autism) and broad (ASD) diagnostic criteria both individually and as a haplotype, ii) *rs1861972* and *rs1861973* are in high inter-marker r^2 with each other, and iii) a functional difference between alleles has been observed. Together these data support *EN2* as a likely ASD susceptibility gene and the A-C haplotype as a possible risk allele.

Rs2361688 is the only tested polymorphism, which is in high but not perfect r² with both rs1861972 and rs1861973 and displays minimal association with ASD. These results could be explained in two ways. One, rs2361688 is a SNP that segregates frequently with rs1861972 and rs1861973 but individually is not functional. The difference in association for rs2361688 versus rs1861972 and rs1861973 is consistent with this possibility (rs2361688: narrow P=.128; broad P=.040; rs1861972: narrow P=.026, broad P=.016; rs1861973: narrow P=.008, broad P=.012). Alternatively, rs2361688 may function in concert with the A-C haplotype. However if this were the case, the common rs2361688-rs1861972-rs1861973 haplotype (G-A-C) would be expected to display more significant association than the A-C haplotype, which is not observed (G-A-C: narrow P=.009, broad P=.004; A-C: narrow P=.002, broad P=.004). Thus our current data suggests that rs2361688 is non-functional but segregates with the functional rs1861972-rs1861973 haplotype. Nevertheless to further investigate the possible involvement of rs2361688, additional association analysis in the AGRE II and NIMH datasets in the AGRE II and NIMH datasets are ongoing. If positive results are obtained, then functional experiments can be performed. Finally several other EN2 polymorphisms that are not in high r² with rs1861972 or rs1861973 also exhibit minimal association in our study and other published reports. These data suggest the possible presence of additional EN2 risk alleles. Future association, LD mapping and functional experiments will test this possibility.

Common functional variants reported to increase risk for other diseases typically affect the regulation of the associated gene ^{24-29,33}. The significant increase in luc levels for the A-C haplotype is consistent with these published results and can be explained by two possible molecular mechanisms. One, since the intron is transcribed and spliced in our constructs, the functional difference could be due to the haplotypes affecting splicing efficiency or stability of nuclear pre-mRNA. This would reduce the amount of luc protein and be consistent with the functional effects of intronic SNPs for other common disorders²³. Two, the rs1861972rs1861973 haplotype could regulate transcription initiation. This possibility is supported by our EMSA data and the bioinformatics indicating that both associated alleles are situated in well-defined consensus transcription factor binding sequences. It is also well established that these transcription factors can function at a distance and in a position independent manner. Published reports for other intronic risk alleles are consistent with this idea ^{24,33,34}. Finally, current bioinformatic data does not support another transcript or miRNA mapping to the EN2 intron and contributing to the functional difference between alleles (genome.ucsc.edu). Regardless of the molecular mechanism, our *in vitro* results indicate that the rs1861972rs1861973 haplotype is functional and suggest the A-C haplotype will affect EN2 levels in vivo.

A large number of transcription factors are predicted to bind to the A and C alleles of *rs1861972* and *rs1861973*. The A allele of *rs1861972* is situated in a CCAAT box which is a consensus binding site for the C/EBP, NF1 and NFY transcription factor family of proteins. Each of these protein families is comprised of multiple genes (*NFIA*, *B*, *C* and *X*; *C/EBPA*, *B*, *D*, *E*, *G* and *Z*; *NFYA*, *B* and *C*). In addition each NFI and NFY gene also generates multiple protein isoforms through alternative splicing and processing ³⁵. Approximately 40 different

transcription factors could then bind to the rs1861972 A allele. For rs1861973, a similar large number of proteins are predicted to bind to the rs1861973 C allele, nine Sp1 members and ~25 Ets factors ³⁶⁻³⁸. Previous *in situ* studies have demonstrated that a large percentage of these genes are widely expressed in the developing and adult brain including neuronal cell types that transcribe *EN2* such as post-mitotic granule cells ³⁰. Microarray analysis has also determined that these putative transcription factors are expressed in HEK-293 and PC12 cells used in our transfection analysis (Gene Expression Omnibus). Interestingly, these transcription factor family members can function as either activators or repressors ^{35,39-41}. Because *EN2* is expressed in a variety of different developmental cell types, the magnitude and direction of the haplotype functional effect could vary between cells depending upon the expression of these various transcription factor isoforms. Alternatively, it is possible that other unidentified factors could be responsible for the observed protein-DNA complexes. Future experiments will be directed at identifying the nuclear proteins that bind to rs1861972 and rs1861973 using a variety of adult and developmental cell types, in which the haploype has been shown to be functional.

To investigate whether the *rs1861972-rs1861973* haplotype affects *EN2* levels *in vivo*, both post-mortem analysis and mouse models will be employed. Post-mortem cerebellar samples are currently being obtained to investigate whether affection status and/or haplotype are correlated with altered EN2 mRNA and protein levels. Transgenic mice have been created for both haplotypes where *EN2* cis-regulatory sequences drive the expression of a fluorescent reporter. These mice will allow us to determine the potential regulatory effects of the haplotype in the developing and adult CNS. Knock-in mice are also being generated where the mouse locus is being replaced with either human haplotype. These knock-in mice will provide an important resource for determining potential phenotypic effects caused by altered *EN2* levels. These ongoing *in vivo* studies will extend our current *in vitro* analysis and provide information regarding when, where, and how the haplotype is functional.

EN2 is a homeobox transcription factor that regulates gene expression during embryonic and post-natal CNS development and continues to be expressed in a subset of differentiated neurons in the adult. Mutational analysis using model organisms has demonstrated that *En2* is necessary for the development of the cerebellum, ventral neurons of the serotonin, norepinephrine and dopamine neurotransmitter systems as well as the proper topographic mapping of retinal axons onto the tectum $^{10,13,42-46}$. Various anatomical, neurochemical and eye tracking studies have implicated these structures and neurotransmitter systems in the etiology of autism 1,47 . Thus altered levels of *EN2* may affect these or other developmental systems, which will be investigated in our *rs1861972-rs1861973* knock-in mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

ENGRAILED 2 LD map. Inter-marker r^2 values for *rs1861973* are shown. The map includes 26 *EN2* polymorphisms typed in the AGRE I dataset (167 families not subset on ethnicity) plus 3120 Hapmap SNPs within 2Mb of *EN2* (+1Mb 5', -1Mb 3') typed in the CEU dataset. Only *rs1861972* and *rs2361688* display high r^2 values (>.75) with *rs1861973*. However, *rs2361688* is not consistently associated with ASD ¹⁵, identifying *rs1861972* and *rs1861973* as the most appropriate candidates to test for functional allelic differences.



Figure 2.

Functional difference between *rs1861972-rs1861973* A-C and G-T haplotypes. (a) The functional difference between the A-C and G-T intronic haplotypes was investigated by generating the diagrammed *luc* reporter constructs: T-SV40 minimal promoter 5' of luc without *EN2* intron, P-*EN2* promoter (-1 to -5735) 5' of luc without *EN2* intron, AC-*EN2* intron with *rs1861972-rs1861973* A-C haplotype cloned 3' of luciferase but 5' of the SV40 polyadenylation signal to approximate the endogenous locus, GT-*EN2* intron with *rs1861972-rs1861973* G-T haplotype cloned 3' of luciferase but 5' of the SV40 polyadenylative light units of luciferase normalized to *Renilla reniformis* and expressed as percent of control, pgl3 promoter vector (T), is shown for the SV40 minimal promoter constructs transiently transfected

into (b) P6 cerebellar granule neurons (n=6), (c) PC12 cells (n=6) and (d) HEK293T cells (n=6). (e-g) Normalized relative light units of luciferase for luc *EN2* promoter constructs expressed as percent of control (P) is shown for (e) P6 cerebellar granule neurons (n=7), (f) PC12 cells (n=6) and (g) HEK293T cells (n=6). * P<.005, ** P<.001, *** P < .00001, two tailed paired Student's T test



Figure 3.

Differential binding of nuclear proteins to *rs1861972* and *rs1861973* associated alleles. (A) To investigate whether the associated SNPs affect the binding of nuclear proteins, EMSAs were conducted with biotinylated 20-mer oligonucleotides and nuclear extract isolated from P6 mouse cerebellar granule cells. Extract was incubated with oligonucleotides specific to each allele, separated on a denaturing acrylamide gel, transferred to a membrane and detected by chemiluminescence. Several protein-DNA complexes were observed for both SNPs. Specificity was determined by competing with 100 molar excess of unlabelled oligonucleotide. Protein-DNA complexes specific to the associated *rs1861972* A allele or *rs1861973* C allele were observed (arrows) that were not detected for the corresponding *rs1861972* G allele or

rs1861973 T allele biotinylated oligonucleotides. In addition, protein-DNA complexes common to both alleles for *rs1861972* or *rs1861973* were observed (arrowheads). Abbreviations: 972-A: 20-mer oligonucleotide specific to the *rs1861972* A allele, 972-G: 20-mer oligonucleotide specific to the *rs1861973* C allele, 973-T and T: 20-mer oligonucleotide specific to the *rs1861973* T allele, + or -: presence or absence respectively of extract or 100 molar excess of unlabelled oligonucleotide. (B) To examine allele-specific binding of nuclear proteins to *rs1861972* (left) and *rs1861973* (right), additional competitions were performed. 80 molar excess of 3 different unlabelled oligonucleotides were each added individually to the probe and nuclear extract: oligonucleotides predicted to disrupt NF1, NFY, C/EBP binding to the A allele of *rs1861972* or Sp1 and Ets binding to the C allele of *rs1861873*, and oligonucleotides for the non-associated G (972-G) and T (973-T) alleles. The sequence for each oligonucleotide is shown. Abbreviation: - absence of competitor



Figure 4.

Conservation of transcription factor binding sites for associated and non-associated alleles of *rs1861972* and *rs1861973*. The 20 bp sequence encompassing *rs1861982* and *rs1861973* and used as probes in our EMSAs is depicted. Conserved transcription factor sites are underlined with the polymorphic allele for each SNP designated with an asterisk.