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Recommended Citation

Jensen, Kevin P.; Covault, Jonathan; Conner, Tamlin S.; Kranzler, Henry R.; Tennen, Howard A.; and Furneaux, Henry M., "A Common Polymorphism in Serotonin Receptor 1B mRNA Moderates Regulation by miR-96 and Associates with Aggressive Human Behaviors" (2009). *Articles - Research*. Paper 69.
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Author Manuscript
Mol Psychiatry. Author manuscript; available in PMC 2011 August 26.

Published in final edited form as:
Mol Psychiatry. 2009 April ; 14(4): 381–389. doi:10.1038/mp.2008.15.

A common polymorphism in serotonin receptor 1B mRNA moderates regulation by miR-96 and associates with aggressive human behaviors

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Abstract

Non-coding regulatory elements can transduce the human genome's response to environmental stimuli. Thus, there is a possibility that variation in non-coding regulatory elements may underlie some of the diversity in human behavior. However, this idea has remained largely untested due to the difficulty in accurately identifying regulatory elements in the 98% of the human genome that does not encode protein. The recent recognition that small trans-acting RNAs anneal to mRNA and regulate gene expression provides a means to identify and test such variants. Here we show that microRNA-directed silencing of mRNA can be attenuated by a common human polymorphism. We have identified an element (A-element) within serotonin receptor 1B (*HTR1B*) mRNA that confers repression by miR-96. The repressive activity of this element is attenuated by a common human variant (G-element) that disrupts a nucleotide critical for its interaction with miR-96. Because deletion of the *HTR1B* gene leads to an aggressive phenotype in mice, we hypothesized an association between the A/G polymorphism and aggressive phenotypes in a sample of 359 college students. As predicted, individuals homozygous for the ancestral A-element reported more conduct-disorder behaviors than individuals with the G-element. Our studies suggest that such functional variants may be common and may help to refine the search for genes involved in complex behavioral disorders.

Keywords

microRNA; polymorphism; behavior; aggression; human

Introduction

Although there is evidence that human behavior is heritable, the molecular basis of the pronounced individual variability remains enigmatic. The comparison of human and chimpanzee genomes has suggested that the uniqueness of human behavior may not reside in the highly invariant protein coding sequences but in the much more variable and rapidly changing non-coding elements that regulate the timing and tissue specificity of gene expression (1, 2). Indeed, the polymorphic microsatellite in the promoter region of the gene

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encoding a vasopressin receptor has been shown to modulate gene expression and thereby predict socio-behavioral traits in voles (3). Thus, it is possible that variation in human non-coding elements may underlie some of the diversity in human behavior.

It is now recognized that human mRNAs are silenced by small trans-acting, non-coding RNAs called microRNAs (4-9). It is thought that microRNAs may regulate as many as 30% of all cellular mRNAs, and they have been found to play a critical role in virtually all cellular functions (10). The mRNA that is targeted by a given microRNA is determined by the nature and extent of the complementarity between the microRNA and the mRNA (11, 12). Thus, if polymorphisms occur in the microRNA target element, they may exert a singular effect upon gene expression with a concomitant change in phenotype. Indeed, a striking example of this has recently been provided by a sheep pedigree in which the acquisition of a single nucleotide variant was sufficient to enhance the microRNA-directed silencing of myostatin leading to exaggerated muscle mass (13). Recent studies on human microRNA target sequences suggest that a similar mechanism may underlie individual differences in human phenotypes (14-16). Therefore, we investigated whether commonly occurring human polymorphisms modulate microRNA-directed regulation of behavioral gene expression, and whether they associate with defined human phenotypes related to aggression.

In this study, we examined polymorphic elements in a number of behavioral genes and selected for further study only those elements that potentially modified microRNA annealing. From this work, we identified the A/G polymorphism within the gene encoding the serotonin receptor 1B (*HTR1B*) as the strongest modulator of gene expression, and we have confirmed that this polymorphism modifies the direct interaction with a microRNA, miR-96. We then tested the behavioral correlates of this SNP via secondary analysis of an existing behavioral data set of 359 college students. Given that *HTR1B* knock-out mice have an aggressive phenotype (17), we hypothesized that the A to G polymorphism would be related to a history of aggression-related behaviors measured in a conduct disorder history checklist. Consistent with our molecular research, we have found that individuals homozygous for the A-element report more aggression related conduct-disorder behaviors compared to individuals with one or two copies of the G-element.

Methods

Cell culture

HeLa cells were grown in Dulbecco's Modified Eagle Medium (D-MEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 100U/ml penicillin and 100ug/ml streptomycin. Cells were plated in either 12- or 24-well dishes.

Luciferase assay of microRNA response elements

The putative reference and variant elements were subcloned into the SacI and NheI sites in the 3′UTR of pIS-0 the modified pGL3 Control (Promega, Madison, WI) reporter plasmid devised by Lewis et al. (2003) (11). Reporter plasmids were transfected into HeLa cells (80% confluence) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The transfection was performed in OPTI-MEM (Invitrogen) which was removed after 4 hours and replaced with D-MEM containing 10% FBS. After 24 hours, reporter activity was determined by assaying the cell lysates for firefly luciferase activity according to the manufacture's protocol (Promega). Luciferase activity was determined by quantitative titration and was normalized using co-transfected β-galactosidase or renilla luciferase activities to control for variance in well-to-well transfection efficiency. Changes

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in luciferase activity were compared using the Dunnett T-test statistic using the SPSS v15 software package.

Exogenous expression of microRNA

Synthetic RNA duplexes were transfected into HeLa cells using Lipofectamine 2000 as described above. Commercially available miR-96 (Ambion, Austin, TX) was used for the experiment in Figure 3c. In the experiment in Figure 3d, RNA duplexes were generated by annealing miR-96 (5′P UUUGGCACUAGCACAUUUUUGC 3′) and miR-96[C] (5′ P UUCGGCACUAGCACAUUUUUGC 3′) guide strands to a common passenger strand (5′P AAAAAUGUGCUCGUGCCUAUAU 3′).

Human sample

Secondary analyses were conducted on a sample of 359 (167 men; 192 women) non-Hispanic Caucasian subjects from a mid-sized state university. This sample reflects a subset of the 574 participants in a recently completed multi-year study who provided DNA samples (n = 416), were Caucasian (to reduce risk of population stratification; n = 361), and completed the first-year assessment battery, which included a conduct-disorder behavior checklist (n = 359). This sample included individuals analyzed elsewhere (18) for unrelated hypotheses. The average age of sample participants was 18.6 ± 0.9 yrs with 61% percent freshmen, 32% sophomores, and 7% upper classmen at study entry. All participants gave written informed consent, which was conducted under guidelines approved by the Institutional Review Board at the University of Connecticut. In their first year, participants completed the assessment battery approximately one month following the start of either the fall or spring semester by logging onto a secure website. The assessment included demographic and several personality and health questionnaires, including one questionnaire expressly relevant to aggressive behaviors—a 9-item inventory of conduct-disorder related behaviors. Participants answered Yes/No to the following questions that covered a range of aggressive or manipulative behaviors: (1) Have you been expelled or suspended from school 3 or more times? (2) Do you frequently tell a lot of lies? (3) Have you stolen money or other things from family members or friends 3 or more times? (4) Have you started fights with persons other than brothers and sisters 3 or more times? (5) Have you ever damaged someone's property on purpose? (6) Have you ever broken into someone's car or house or any place else (not because you have been locked out)? (7) Have you ever deliberately started fires you were not supposed to? (8) Have you ever taken property or money from someone by threatening them or using force? (9) Were you ever mean or cruel to animals, including hurting them on purpose? These items were derived from the 15 item list of conduct disorder behaviors specified in the Diagnostic and Statistical Manual of Mental Disorders, 4th ed. (American Psychiatric Association, 1994). The nine items used in the baseline questionnaire were those that we anticipated would be relatively more prevalent in a college student population.

Genotype analysis of human samples

DNA was extracted from Scope™ (Procter and Gamble, Cincinnati, OH) mouthwash-stabilized samples using a commercial DNA isolation protocol (PureGene, Gentra Systems, Minneapolis, MN). The *HTR1B* 3′UTR polymorphism (rs13212041) was genotyped using a PCR-based TaqMan allelic discrimination assay. A 10μl PCR reaction was performed in 1× ABI TaqMan Universal master mix (ABI-Applied Biosystems Inc., Foster City, CA) containing 100nM of each TaqMan MGB probe, FAM labeled *HTR1B*[C] 5′ TGCAGACTTCGGC, and VIC labeled *HTR1B*[T] 5′ TGCAGACTTTGGC, and 600nM of each primer, Forward Primer- 5′ AAAGTGACAGGTACATGAAATTAAGAGAA, reverse primer-5′ CACAACTCTAACAAACAAACCATTATGTG, Polymerase chain reaction was performed with the following cycling parameters, 95°C for 10 minutes, followed by 40

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cycles of 95°C for 15 seconds, followed by 60°C for 60 seconds. End point FAM and VIC fluorescence levels were captured using an ABI 7500 Sequence Detection System and genotype calls were made based on the level of fluorescence signal. The genotyping assay was repeated for 14% percent of samples and there was no discrepancy in genotype calls

Results

Candidate behavioral genes have polymorphisms in putative microRNA target sites

First, we arbitrarily selected a list of 22 mRNAs that were previously implicated in the regulation of human behavior. Using the Miranda, TargetScan, PicTar and RNAHybrid prediction algorithms (11, 19-21), we established that the vast majority of these mRNAs (20/22) contained at least one putative microRNA target site (Figure 1a). Importantly, we found that half (11/20) of these mRNAs harbored polymorphisms within elements predicted to be targeted by microRNAs. Interestingly, these polymorphisms occurred more frequently at nucleotides complementary to the critical seed sequences of the putative microRNA, suggesting that they might be of functional significance (Figure 1b).

Polymorphic elements from the 3′UTR of candidate behavioral genes regulate the expression of a luciferase reporter

To test whether any of these polymorphisms modulated the regulation directed by microRNAs, we utilized the luciferase reporter assay devised by Lewis et al (2003) (11). In this assay, the putative element is inserted into the 3′UTR of luciferase mRNA and its repressive activity is measured by the inhibition of luciferase activity compared to a parental luciferase reporter plasmid that lacks an inserted test element. In our experiments, we used HeLa cells, since the concentration of a given microRNA can be readily increased by transfection of the appropriate precursor duplex.

We selected putative elements from three candidate genes for further study: the cannabinoid receptor 1 (*CNR1*), serotonin receptor 1B (*HTR1B*), and serotonin transporter (*SLC6A4*) mRNAs. This selection was based on the minor allele frequencies at each of these loci, the likely effect upon microRNA regulation as surmised by the position of the variant nucleotide and our ability to test for possible associations with human behavioral phenotypes. With the exception of the *HTR1B* G-element, all the microRNA response elements in the three genes were found to repress luciferase expression ($p<0.001$ compared with the parental construct), indicating that they could mediate repression by microRNAs (Figure 2). The serotonin transporter (*SLC6A4*) element had the least effect on repressing luciferase activity, and the U to G change had no discernable effect on its ability to repress gene expression (38% vs. 37% repression, $p=0.88$). Thus, we concluded that either this element represses expression by a microRNA–independent mechanism or that HeLa cells might lack additional cofactors required for the relief of repression. In contrast, the G to U change in the cannabinoid receptor (*CNR1*) element did diminish its repressive activity by a moderate extent (76% vs. 65% repression, $p=0.056$), and the A to G change in the serotonin receptor 1B (*HTR1B*) element markedly attenuated its repressive effect (64% vs. 0%, $p=0.001$). Thus, we conclude that some common variants in microRNA target sites can modulate the repression of gene expression and most likely do so by altering the interaction between mRNA and a microRNA.

The common A to G polymorphism in the serotonin receptor 1B mRNA moderates the regulation of miR-96

In the rest of our studies here, we focused on the element within the *HTR1B* mRNA. The structure of the *HTR1B* element and its putative complementarity to miR-96 are shown in Figure 3. The human element with the A residue is likely the ancestral version since it is

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conserved from cow to chimpanzee (Figure 3a). The polymorphism (A/G) in the mRNA corresponds to the nucleotide that would anneal to the third nucleotide of miR-96 and changes an A:U pairing to a weaker G:U pair (Figure 3b). Henceforth, we will describe the ancestral element as the A-element and the unresponsive element as the G-element. To further confirm the role of miR-96, we increased its concentration in HeLa cells with the expectation that this would increase the suppressive effect of the ancestral A-element, with comparatively little effect on the G-element. Indeed, upon transfection of miR-96, the suppression exerted by the A-element increased significantly (p=0.006 and 0.004 for 1 and 2 nM respectively compared with no added miR-96), while there was little effect on the G-element (p= 0.46 and 0.34 for 1 and 2 nM miR-96) (Figure 3c). Thus, we concluded that miR-96 silences mRNAs containing the A-element. Although unlikely, it remained possible that miR-96 exerted its effect indirectly by modulating another mRNA whose protein product somehow differentially influenced the repressive effect of the A-element. It is also important to validate that the G-element luciferase construct can be repressed. Accordingly, we designed a rescue microRNA, miR-96 [C], in which the third nucleotide was changed from uracil to cytosine. The introduction of 2nM miR-96[C] had no effect on the repressor activity of the A-element (p=0.34), but it markedly increased the suppressive activity of the G-element (p= 0.014) (Figure 3d). Thus, we concluded that this element in *HTR1B* mRNA mediates suppression by miR-96 and that this suppression may be attenuated by a common human variant.

The common polymorphism in the serotonin receptor 1B mRNA is associated with aggressive behavior

Although the precise contribution of the *HTR1B* protein to serotonergic circuits is not yet well understood, mice lacking this receptor are more aggressive than their littermates (17). Therefore, we postulated that human A-element homozygotes, harboring an increased potential for the suppression of *HTR1B* expression, might exhibit increased aggression-related behavior. To test this hypothesis, we examined the association between genotype and an available measure of behavior related to aggression, i.e., conduct-disorder behaviors. As predicted by the HapMap dataset (22), the majority of this Caucasian population were A-element homozygotes (Table 1; AA = 231; AG = 113; GG = 15; HWE X^2 = .06, p = .80). In contrast, the prevalence of G-element homozygotes was low (4%). Other populations show a far lower prevalence of the ancestral A-element. For example, only 20% of YRI (Yoruba in Ibadan, Nigeria) are homozygous with respect to the ancestral A-element and, even more remarkably, 33% are homozygous with respect to the G-element. The expected low prevalence of G-element homozygotes precluded their independent analysis and thus we compared A-element homozygotes to G-element carriers (Table 2). As predicted, A-element homozygotes reported a history of more conduct-disorder related behaviors (mean = 1.17) than did the G-element carriers (mean = 0.81). The difference between the A-element homozygotes and the G-element carriers was statistically significant but reflected a small effect size (d = 0.28) (23), with the genotype accounting for 2.3% of the variance in conduct disorder outcomes. Patterns were observed for both men and women, as shown in Figure 4. Importantly, we observed a marked specificity in the reported conduct-disorder behaviors. For example, there was no discernable association between the homozygous A-element genotype and self-reported propensity to lie or to break and enter. On the other hand, the A-element homozygotes were much more likely to report having started fires and damaged property. Similar trends were also noted for their reports of having stolen and started fights. Thus, these data provide preliminary evidence linking the A-element allele to increased aggression among young adults.

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Discussion

Our studies are a direct demonstration that common human polymorphisms can attenuate microRNA-mediated repression of gene expression by conserved elements in mRNA. Importantly, our results corroborate recent studies suggesting that microRNA target variants may be determinants of a range of human phenotypes including cardiovascular disease risk (24, 25) and response to chemotherapy (26). Specifically in terms of behavioral phenotypes, our study supports an earlier finding that a rare variant enhances microRNA-directed suppression of *SLITKR* mRNA and may underlie certain brain developmental defects associated with Tourette's Syndrome (27).

Although the current studies indicate that such microRNA-directed repression of *HTR1B* may be an important facet in the development of aggressive behaviors, additional research is warranted given the limited phenotype tested. The percentage of variance in conduct disorder outcomes accounted for by the *HTR1B* polymorphism was small but comparable to that seen in other single gene association studies, accounting for ≈2% of the variance. In this regard, we have not fully categorized the potential behavior effects manifested by A-element homozygotes. Indeed the absolute role of the *HTR1B* gene in behavior has been difficult to ascertain, and research on *HTR1B* has yielded conflicting results in terms of its pharmacology and genetics. Genetic studies have shown an association between variation in the *HTR1B* gene and a number of phenotypes including ADHD(28-30), obsessive compulsive disorder (31) antisocial alcoholism(32), substance dependence and major depression(33). However many of the association findings have been inconsistent(34-36), and this may be due to an incomplete awareness of the extent of functional variation within the *HTR1B* gene. Another difficulty in determining the contribution of the *HTR1B* gene to behavior is the lack of an appropriate behavioral measure. For example it is possible that A-element homozygotes might score more highly in paradigms designed to test aggression-related behaviors more directly (e.g., through more sensitive self-reports or through observational paradigms). Additional evidence from the sample of college students studied here supports this assertion (Conner, Jensen, Tennen, Covault, Furneaux, & Kranzler, under review). In that report we provide evidence of an association between the HTR1B A/G polymorphism and daily self-reports of angry and hostile mood obtained across a four-year longitudinal assessment. Interestingly, patterns were strongest among the young men and not observed in women (see Conner et al. for possible explanations). Although these additional findings paint a more complex phenotypic picture, together, they support a link between the microRNA binding site polymorphism and aggression related phenotypes that is consistent with the animal literature. It should be noted that this is the same population studied by Covault et al. (18), for an unrelated hypothesis, and that the self-reported ethnicity was found to be consistent with the genotype of an ancestry informative marker described by Lautenberger et al (37). Although we believe that this population is homogeneous, it should be acknowledged that it is possible that our results are confounded by population stratification, which could contribute to type 1 error.

With respect to the link between this SNP and aggression, it is important to note that the aggressive phenotype exerted by the A-element will depend upon the expression and activity of miR-96. It is known that miR-96 is expressed in brain (38, 39), but as is the case with virtually all microRNAs, the factors that regulate its expression and activity are not known. It will be interesting to see if factors (for example, steroids) that influence aggressive behavior may do so by modulating the level or activity of miR-96. In any event, our observations suggest the possibility that aggressive behavior might be modifiable by administration of inhibitors of miR-96.

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We are intrigued that the ancestral A-element is less common among Yorubans, the model ancestral population (40) compared with Caucasian or Asian populations. These contrasting patterns suggest that this regulatory element has been subjected to unknown but profound selective pressures. Although it is possible that the divergent frequencies of this element are a result of a neutral process such as genetic drift, perhaps it evolved quickly in response to environments in which hostile behavior is disadvantageous. In light of this, it will be interesting if a study of closely-related African populations reveals the nature of the responsible evolutionary force, as has occurred in the case of lactose intolerance (41).

Our initial screen, albeit on a sample of only 22 genes, suggests that modulation of microRNA regulation by common variants in mRNA sequences may be widespread in the human genome. Indeed, more comprehensive sequencing of individual humans and deep sequence cloning of new microRNAs are likely to substantially increase our catalog of such functional variants. In addition, with the discovery of new small RNA classes with purported roles in DNA methylation and transcriptional silencing, a similar strategy may readily identify new functional variants in the 5′ untranslated region and segments of genes that are proximal to the promoter region (42). In sum, we hope the current studies prompt an enthusiastic search for new classes of functional polymorphisms that reside in RNA regulatory elements and an informed evaluation of their contribution to the human phenotype. Of specific interest in the present report is the apparent role of miR-96 in regulating aggressive behaviors among young adults. Given the important clinical significance of such behaviors (43), a clearer understanding of their etiologies and the potential for intervening to prevent such behaviors have important clinical implications.

Acknowledgments

This study was supported by NIH grants P50 AA03510, M01 RR06192 (University of Connecticut GCRC), K24 AA13736, T32 AA07290, and DA 022226. We gratefully acknowledge the assistance of Nicholas Maltby for web programming and the technical assistance of Linda Burian.

References

1. King MC, Wilson AC. Evolution at *two* levels in humans and chimpanzees. Science. 1975 Apr 11; 188(4184):107–116. [PubMed: 1090005]
2. Hellmann I, Zollner S, Enard W, Ebersberger I, Nickel B, Paabo S. Selection on human genes as revealed by comparisons to chimpanzee cDNA. Genome Res. 2003 May; 13(5):831–837. [PubMed: 12727903]
3. Hammock EA, Young LJ. Microsatellite instability generates diversity in brain and sociobehavioral traits. Science. 2005 Jun 10; 308(5728):1630–1634. [PubMed: 15947188]
4. Lee RC, Ambros V. An extensive class of small RNAs in Caenorhabditis elegans. Science. 2001 Oct 26; 294(5543):862–864. [PubMed: 11679672]
5. Lau NC, Lim LP, Weinstein EG, Bartel DP. An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science. 2001 Oct 26; 294(5543):858–862. [PubMed: 11679671]
6. Hutvagner G, Zamore PD. A microRNA in a multiple-turnover RNAi enzyme complex. Science. 2002 Sep 20; 297(5589):2056–2060. [PubMed: 12154197]
7. Meister G, Landthaler M, Patkaniowska A, Dorsett Y, Teng G, Tuschl T. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol Cell. 2004 Jul 23; 15(2):185–197. [PubMed: 15260970]
8. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. Science. 2001 Oct 26; 294(5543):853–858. [PubMed: 11679670]
9. Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, et al. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature. 2000 Nov 2; 408(6808):86–89. [PubMed: 11081512]

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10. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 2005 Jan 14; 120(1):15–20. [PubMed: 15652477]
11. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. Cell. 2003 Dec 26; 115(7):787–798. [PubMed: 14697198]
12. Doench JG, Sharp PA. Specificity of microRNA target selection in translational repression. Genes Dev. 2004 Mar 1; 18(5):504–511. [PubMed: 15014042]
13. Clop A, Marcq F, Takeda H, Pirottin D, Tordoir X, Bibe B, et al. A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. Nat Genet. 2006 Jul; 38(7):813–818. [PubMed: 16751773]
14. Chen K, Rajewsky N. Natural selection on human microRNA binding sites inferred from SNP data. Nat Genet. 2006 Dec; 38(12):1452–1456. [PubMed: 17072316]
15. Saunders MA, Liang H, Li WH. Human polymorphism at microRNAs and microRNA target sites. Proc Natl Acad Sci U S A. 2007 Feb 27; 104(9):3300–3305. [PubMed: 17360642]
16. Adams BD, Furneaux H, White BA. The micro-ribonucleic acid (miRNA) miR-206 targets the human estrogen receptor-alpha (ERalpha) and represses ERalpha messenger RNA and protein expression in breast cancer cell lines. Mol Endocrinol. 2007 May; 21(5):1132–1147. [PubMed: 17312270]
17. Saudou F, Amara DA, Dierich A, LeMeur M, Ramboz S, Segu L, et al. Enhanced aggressive behavior in mice lacking 5-HT1B receptor. Science. 1994 Sep 23; 265(5180):1875–1878. [PubMed: 8091214]
18. Covault J, Tennen H, Armeli S, Conner TS, Herman AI, Cillessen AH. Interactive Effects of the Serotonin Transporter 5-HTTLPR Polymorphism and Stressful Life Events on College Student Drinking and Drug Use. Biol Psychiatry. 2006 Aug 18.
19. John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS. Human MicroRNA targets. PLoS Biol. 2004 Nov.2(11):e363. [PubMed: 15502875]
20. Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, et al. Combinatorial microRNA target predictions. Nat Genet. 2005 May; 37(5):495–500. [PubMed: 15806104]
21. Rehmsmeier M, Steffen P, Hochsmann M, Giegerich R. Fast and effective prediction of microRNA/target duplexes. Rna. 2004 Oct; 10(10):1507–1517. [PubMed: 15383676]
22. A haplotype map of the human genome. Nature. 2005 Oct 27; 437(7063):1299–1320. [PubMed: 16255080]
23. Cohen, J. Statistical power analysis for the behavioral sciences. 2nd. Hillsdale; NJ: 1988.
24. Sethupathy P, Borel C, Gagnebin M, Grant GR, Deutsch S, Elton TS, et al. Human microRNA-155 on chromosome 21 differentially interacts with its polymorphic target in the AGTR1 3′ untranslated region: a mechanism for functional single-nucleotide polymorphisms related to phenotypes. Am J Hum Genet. 2007 Aug; 81(2):405–413. [PubMed: 17668390]
25. Martin MM, Buckenberger JA, Jiang J, Malana GE, Nuovo GJ, Chotani M, et al. The Human Angiotensin II Type 1 Receptor +1166 A/C Polymorphism Attenuates MicroRNA-155 Binding. J Biol Chem. 2007 Aug 17; 282(33):24262–24269. [PubMed: 17588946]
26. Mishra PJ, Humeniuk R, Longo-Sorbello GS, Banerjee D, Bertino JR. A miR-24 microRNA binding-site polymorphism in dihydrofolate reductase gene leads to methotrexate resistance. Proc Natl Acad Sci U S A. 2007 Aug 14; 104(33):13513–13518. [PubMed: 17686970]
27. Abelson JF, Kwan KY, O'Roak BJ, Baek DY, Stillman AA, Morgan TM, et al. Sequence variants in SLITRK1 are associated with Tourette's syndrome. Science. 2005 Oct 14; 310(5746):317–320. [PubMed: 16224024]
28. Quist JF, Barr CL, Schachar R, Roberts W, Malone M, Tannock R, et al. The serotonin 5-HT1B receptor gene and attention deficit hyperactivity disorder. Mol Psychiatry. 2003 Jan; 8(1):98–102. [PubMed: 12556913]
29. Hawi Z, Dring M, Kirley A, Foley D, Kent L, Craddock N, et al. Serotonergic system and attention deficit hyperactivity disorder (ADHD): a potential susceptibility locus at the 5-HT(1B) receptor gene in 273 nuclear families from a multi-centre sample. Mol Psychiatry. 2002; 7(7):718–725. [PubMed: 12192616]

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30. Smoller JW, Biederman J, Arbeitman L, Doyle AE, Fagerness J, Perlis RH, et al. Association between the 5HT1B receptor gene (HTR1B) and the inattentive subtype of ADHD. Biol Psychiatry. 2006 Mar 1; 59(5):460–467. [PubMed: 16197923]
31. Mundo E, Richter MA, Zai G, Sam F, McBride J, Macciardi F, et al. 5HT1Dbeta Receptor gene implicated in the pathogenesis of Obsessive-Compulsive Disorder: further evidence from a family-based association study. Mol Psychiatry. 2002; 7(7):805–809. [PubMed: 12192628]
32. Lappalainen J, Long JC, Eggert M, Ozaki N, Robin RW, Brown GL, et al. Linkage of antisocial alcoholism to the serotonin 5-HT1B receptor gene in 2 populations. Arch Gen Psychiatry. 1998 Nov; 55(11):989–994. [PubMed: 9819067]
33. Huang YY, Oquendo MA, Friedman JM, Greenhill LL, Brodsky B, Malone KM, et al. Substance abuse disorder and major depression are associated with the human 5-HT1B receptor gene (HTR1B) G861C polymorphism. Neuropsychopharmacology. 2003 Jan; 28(1):163–169. [PubMed: 12496953]
34. Ickowicz A, Feng Y, Wigg K, Quist J, Pathare T, Roberts W, et al. The serotonin receptor HTR1B: gene polymorphisms in attention deficit hyperactivity disorder. Am J Med Genet B Neuropsychiatr Genet. 2007 Jan 5; 144(1):121–125. [PubMed: 16958036]
35. Kranzler HR, Hernandez-Avila CA, Gelernter J. Polymorphism of the 5-HT1B receptor gene (HTR1B): strong within-locus linkage disequilibrium without association to antisocial substance dependence. Neuropsychopharmacology. 2002 Jan; 26(1):115–122. [PubMed: 11751038]
36. Proudnikov D, LaForge KS, Hofflich H, Levenstien M, Gordon D, Barral S, et al. Association analysis of polymorphisms in serotonin 1B receptor (HTR1B) gene with heroin addiction: a comparison of molecular and statistically estimated haplotypes. Pharmacogenet Genomics. 2006 Jan; 16(1):25–36. [PubMed: 16344719]
37. Lautenberger JA, Stephens JC, O'Brien SJ, Smith MW. Significant admixture linkage disequilibrium across 30 cM around the FY locus in African Americans. Am J Hum Genet. 2000 Mar; 66(3):969–978. [PubMed: 10712211]
38. Barad O, Meiri E, Avniel A, Aharonov R, Barzilai A, Bentwich I, et al. MicroRNA expression detected by oligonucleotide microarrays: system establishment and expression profiling in human tissues. Genome Res. 2004 Dec; 14(12):2486–2494. [PubMed: 15574827]
39. Sempere LF, Freemantle S, Pitha-Rowe I, Moss E, Dmitrovsky E, Ambros V. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. Genome Biol. 2004; 5(3):R13. [PubMed: 15003116]
40. Voight BF, Kudaravalli S, Wen X, Pritchard JK. A map of recent positive selection in the human genome. PLoS biology. 2006 Mar.4(3):e72. [PubMed: 16494531]
41. Tishkoff SA, Reed FA, Ranciaro A, Voight BF, Babbitt CC, Silverman JS, et al. Convergent adaptation of human lactase persistence in Africa and Europe. Nat Genet. 2007 Jan; 39(1):31–40. [PubMed: 17159977]
42. Martianov I, Ramadass A, Serra Barros A, Chow N, Akoulitchev A. Repression of the human dihydrofolate reductase gene by a non-coding interfering transcript. Nature. 2007 Feb 8; 445(7128):666–670. [PubMed: 17237763]
43. Loeber R, Burke JD, Lahey BB, Winters A, Zera M. Oppositional defiant and conduct disorder: a review of the past 10 years, part I. J Am Acad Child Adolesc Psychiatry. 2000 Dec; 39(12):1468–1484. [PubMed: 11128323]

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Gene	Predicted Target of a MicroRNA	Polymorphism in predicted microRNA target
Brain Derived Neutrophic Factor *(BDNF)*	Yes	rs1048241
Cannabinoid Receptor 1 *(CNR1)*	Yes	rs16880260
Catechol -*O*- methyl transferase *(COMT)*	Yes	rs9332380
Dopamine Receptor 2 *(DRD2)*	Yes	rs6274,rs1130354
muOpiod Receptor *(OPRM1)*	Yes	rs10543127
Nicotinic Acetylcholine Receptor Beta-2 *(CHRNB2)*	Yes	rs2072660
Notch 3	Yes	rs1804976
Reticulon 4 *(RTN4)*	Yes	rs7573286
Serotonin Receptor 1B *(HTR1B)*	Yes	rs13212041
Serotonin Receptor 2A *(HTR2A)*	Yes	rs17069218
Serotonin Receptor 2C *(HTR2C)*	Yes	rs1801412
Serotonin Transporter *(SLC6A4)*	Yes	rs1042173
Dopamine Receptor 3 *(DRD3)*	Yes	none detected
Dopamine Receptor 4 *(DRD4)*	Yes	none detected
GABA Receptor Alpha-2 Subunit *(Gabra2)*	Yes	none detected
Muscarinic Acetylcholine Receptor 2 *(CHRM2)*	Yes	none detected
Nicotinic Acetylcholine Receptor Alpha 7 *(CHRNA7)*	Yes	none detected
Serotonin Receptor 3A *(HTR3A)*	Yes	none detected
Tryptophan Hydroxylase 1 *(TPH1)*	Yes	none detected
Tryptophan Hydroxylase 2 *(TPH2)*	Yes	none detected
Alcohol Dehydrogenase 2 *(ADH2)*	No	none detected
Monoamine Oxidase A *(MAOA)*	No	none detected

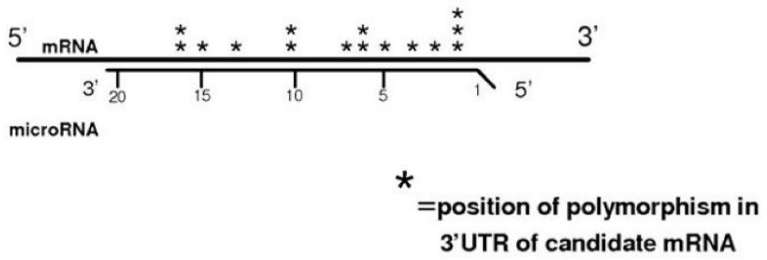

Figure 1. Polymorphisms frequently occur in the putative microRNA response elements in behavioral genes

A. In our survey of 22 behavioral genes, we found that the majority have a predicted microRNA binding site in their mRNAs. Among these genes, 11 had a polymorphism that occurred in the microRNA binding element. **B**. The polymorphisms in predicted microRNA binding elements cluster at the 3′ end of the putative response element. The asterisk represents the position of the polymorphism in the putative microRNA:mRNA duplex; a total of 15 polymorphisms that potentially affect microRNA binding sites are represented. One microRNA binding site has two polymorphisms, and one polymorphism occurs in the predicted binding site of two microRNAs.

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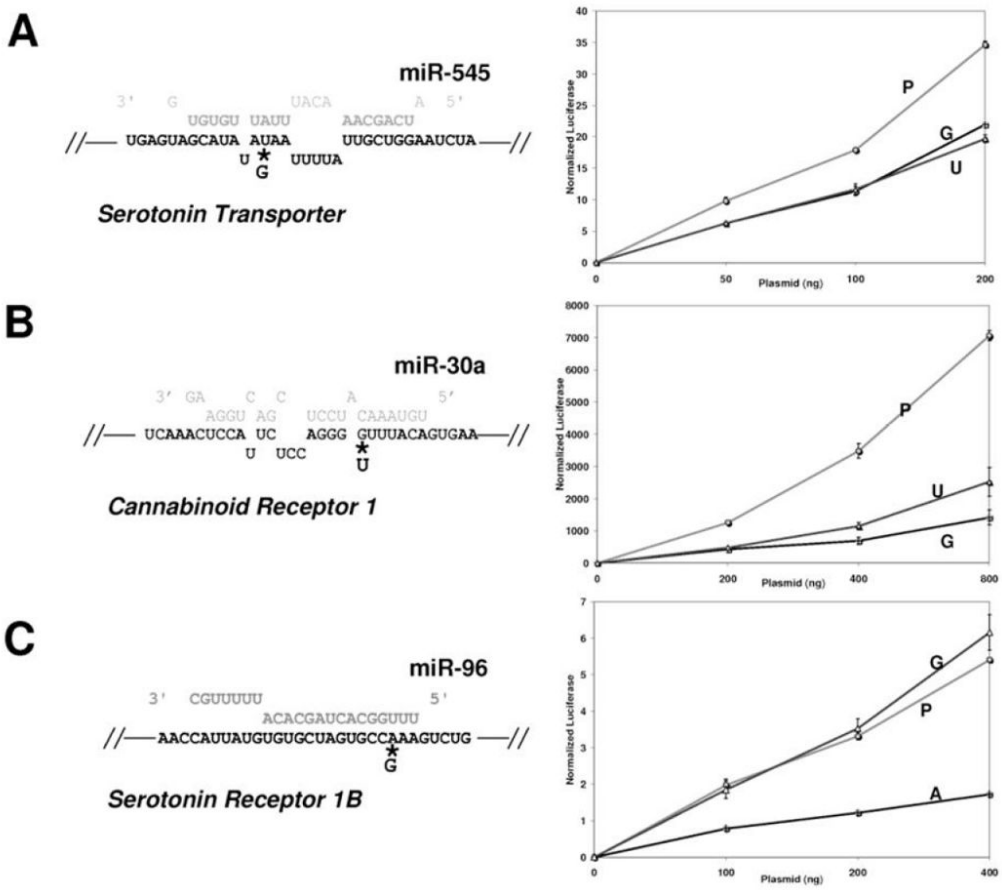

Figure 2. Polymorphisms can attenuate the repressive effect of the microRNA response elements

The position of the polymorphism in the putative element within the serotonin transporter mRNA (A), cannabinoid receptor 1 mRNA (B), and the serotonin receptor 1B mRNA (C) are marked by an asterisk (*). The putative elements were subcloned into the 3′UTR of the pIS-0 luciferase reporter plasmid. The parental (P), variant, and reference plasmids were transfected into HeLa cells and assayed for luciferase activity. Luciferase expression was normalized for transfection efficiency by co-transfecting a renilla luciferase plasmid (panels A and C) or a beta galactosidase plasmid (panel B). The reference (U) and variant (G) element from the serotonin transporter mRNA both repress luciferase expression to a similiar extent. The reference element (G) from the cannabinoid receptor 1 strongly repressed expression and this repression was moderately attenuated by the polymorphism (U). The reference element (A) from the serotonin receptor 1B mRNA strongly repressed expression and was completely attenuated by the polymorphism (G). Error bars represent the standard deviation of two measurements of luciferase activity. The average luciferase expression of all elements (except the *HTR1B* G-element) was significantly different than the parental plasmid ($p<0.001$). The difference in luciferase expression between the polymorphic elements was significant only for the *HTR1B* A versus G element ($p=0.001$).

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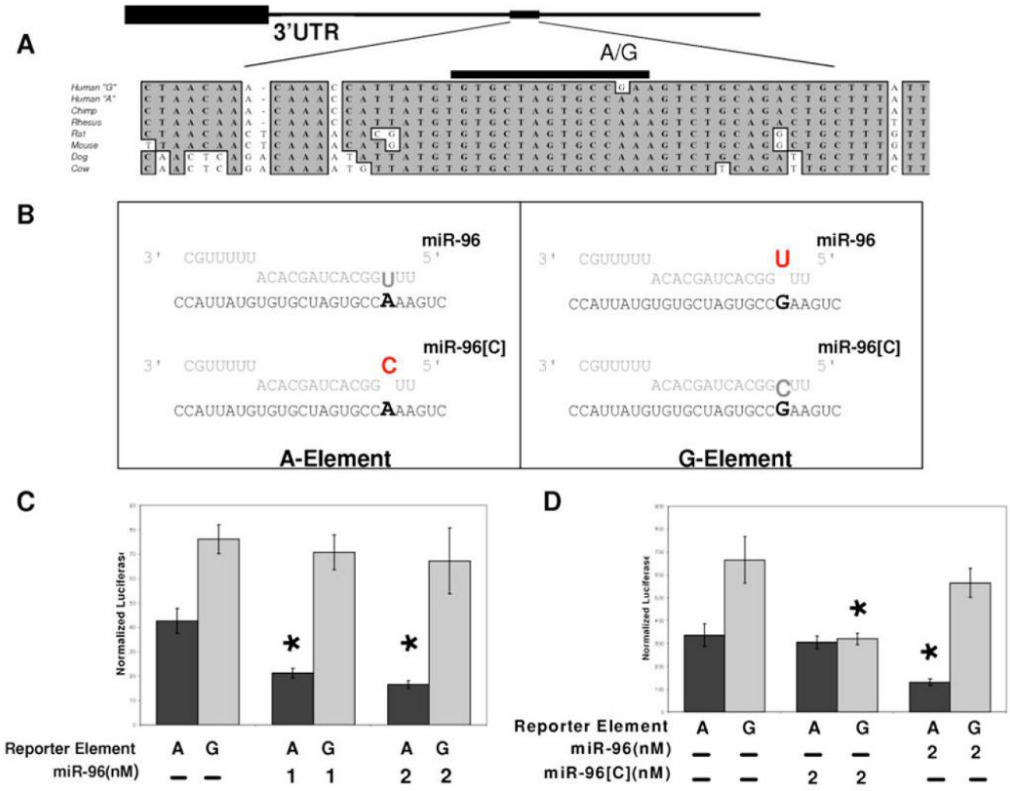

Figure 3. The A-element in the serotonin receptor 1B mRNA is repressed by miR-96

A. The region surrounding the *HTR1B* A/G SNP is highly conserved in several mammalian species, and in all (except humans) it encodes the A-variant. The segment of miR-96 that is predicted to anneal to the A-element is shown by the gray bar. **B**. The predicted annealing of the wild-type (miR-96) and the compensatory mutant (miR-96[C]) microRNAs to the A- and G-elements. **C**. HeLa cells were transfected with the indicated reporter plasmids and microRNAs. Introduction of exogenous miR-96 increases the repressive activity of the A-element but has little effect on the G-element. **D**. Introduction of the compensatory mutant (miR-96[C]) has no effect on the A-element but significantly increases the repressive activity of the G-element. Error bars represent the standard deviation of two measurements of luciferase activity. The asterisk (*) represents $p<0.01$ for panel C and $p<0.05$ for panel D, for the change in luciferase activity of either the A-element or G-element plasmid after the addition of microRNA.

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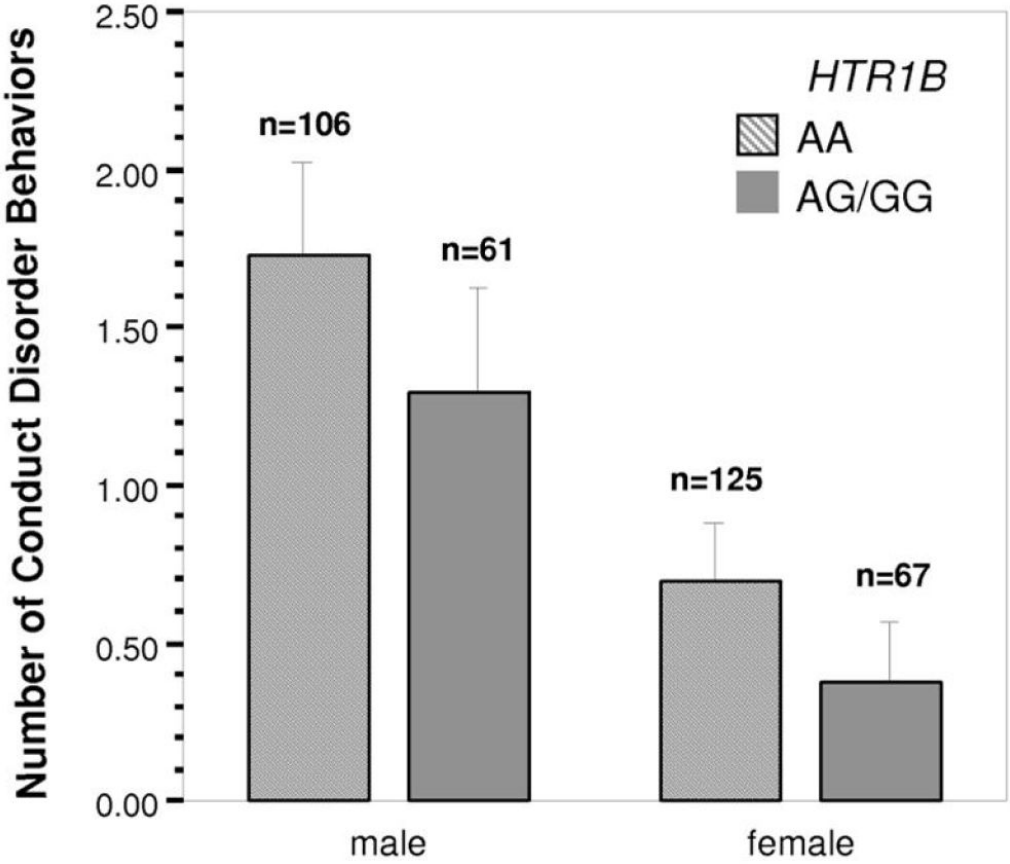

Figure 4. *HTR1B* genotype differences with conduct-disorder behavior were observed for men and women

The mean number of conduct disorder items (with 95% CI) endorsed by men and women was higher for AA individuals, as compared to G carrier individuals.

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Table 1

The frequency of the polymorphism in the serotonin receptor 1B microRNA target element is remarkably divergent in HapMap populations.

HapMap Population	G	A	% G Homozygous
Han Chinese in Beijing, China(**CHB**)	0.22	0.78	0 (0/45)
CEPH (Utah residents with ancestry from northern and western Europe)(**CEU**)	0.16	0.84	2 (1/60)
Japanese in Tokyo, Japan(**JPT**)	0.26	0.74	7 (3/45)
Yoruba in Ibadan, Nigeria(**YRI**)	0.57	0.43	33 (20/59)

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Table 2

A-element homozygote subjects endorsed more conduct-disorder items.

Conduct Disorder Items	AA (n = 231)	G Carriers (n = 128)	*t*	*P*
***M* (*SD*)**	**1.17 (1.38)**	**.81 (1.16)**	**2.61**	**0.005**
Min* - *Max	**0 - 6**	**0 - 5**		
	N (%)	**N (%)**	X^2	***P***
Started Fires	39 (16.9%)	10 (7.8%)	5.75	0.008
Damaged Property	79 (34.2%)	31 (24.2%)	3.86	0.025
Stealing	29 (12.6%)	9 (7.0%)	2.70	0.051
Started Fights	54 (23.4%)	21 (16.4%)	2.32	0.064
School Expulsion	2 (0.9%)	0 (0.0%)	1.11	0.146
Cruelty to Animals	28 (12.1%)	11 (8.6%)	1.06	0.152
Breaking and Entering	16 (6.9%)	7 (5.5%)	0.30	0.291
Lying Frequently	20 (8.7%)	13 (10.2%)	0.24	0.311
Took Property by Force	3 (1.3%)	2 (1.6%)	0.04	0.421

Note. *M* (*SD*) *Min* - *Max* = mean, standard deviation, and minimum/maximum number of total items endorsed. *N (%)* = number and percentage of individuals in each genotype group endorsing that conduct disorder item; *t* = t-test coefficient (computed with unequal variances) comparing mean differences in the number of items endorsed between AA and G element carriers; X^2 = Chi-Square coefficient comparing differences in percentages between AA and G element carriers (AG, GG); *p* = chance probability value based on one-sided test.