Allele variants in functional microRNA target sites of the neurotrophin-3 receptor gene (*NTRK3*) as susceptibility factors for anxiety disorders

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ABSTRACT

Genetic and functional data indicate that variation in the expression of the neurotrophin-3 receptor gene (*NTRK3*) may have an impact on neuronal plasticity suggesting a role for *NTRK3* in the pathophysiology of anxiety disorders. MicroRNAs are post-transcriptional gene regulators that act by base pairing to specific sequences, usually at the 3'UTR of the target mRNA. Nucleotide variants at these sites might result in changes of gene expression contributing to disease susceptibility. We have investigated genetic variation in two different isoforms of *NTRK3* as candidate susceptibility factors for anxiety by re-sequencing their 3'UTRs in patients with panic disorder, obsessive-compulsive disorder and controls. We have found the C allele of rs28521337, located in a functional target site for miR-485-3p in the truncated isoform of *NTRK*3, to be significantly associated with the hoarding phenotype of obsessive-compulsive disorder. Furthermore, we have also identified two new rare allelic variants in the 3'UTR of *NTRK3*, ss102661458 and ss102661460, each present only in a chomosome of a patient with panic disorder. The ss102661458 variant is located in a functional target site for miR-765, and the ss102661460 in functional target sites for two microRNAs, miR-509 and miR-128, the latter being a brain-enriched microRNA involved in neuronal differentiation and synaptic processing. Interestingly, these two variants significantly alter the microRNA-mediated regulation of *NTRK3* and result in the recovery of gene expression. The reported data implicate microRNAs as key post-transcriptional regulators of *NTRK3* and provide a framework for allele-specific microRNA regulation of *NTRK3* in anxiety disorders.

Keywords: Functional allele variant, SNP, *NTRK3*, microRNA, posttranscriptional regulation, anxiety disorder, obsessive compulsive disorder, panic disorder, hoarding

INTRODUCTION

Anxiety disorders are nowadays considered a heterogeneous cluster of several related disorders that comprises panic disorder (PD) and obsessive compulsive disorder (OCD) among others [Association, 1994]. The defining features of PD are the recurrence of unexpected panic attacks characterized by brief episodes of intense terror and feelings of impending doom [Smoller and Tsuang, 1998]. Agoraphobia involves fear of panic attacks and is considered a common complication of PD. OCD, on the other hand, is characterized by obsessions (repetitive, intrusive thoughts or images that are realized as senseless) and/or compulsions (repetitive behaviours or mental acts that the person feels forced to do to reduce anxiety generated by obsessions). Approximately 18 to 42% of those suffering from OCD report hoarding obsessions, which is also called "pathological collecting". Despite the genetic influences on anxiety disorders are well established, the identification of the genes underlying the pathogenesis of these psychiatric diseases has proven to be extremely difficult. One of the limiting factors is the achievement of an accurate clinical diagnosis, together with an adjusted definition of its relationship with specific quantitative traits.

The search for factors predisposing to anxiety disorders has been classically focused on genes of the neurotransmitter system. However, molecules involved in neurodevelopment and neuronal plasticity such as neurotrophins and their receptors could also have an important role in the aetiology of these disorders [Gratacos, et al., 2007]. Besides their essential role in regulating the differentiation and proliferation of neuronal precursors, these molecules have also been involved in processes that underlie memory formation and cognitive attributes such as axonal guidance mediation [Teng and Hempstead, 2004]. In particular, the neurotrophin-3 receptor gene (*NTRK3*, MIM#191316) is highly expressed in the *locus coeruleus* [King, et al., 1999], a brain structure that has an important function in the fear/alarm modulatory circuitry [Sullivan, et al., 1999]. Furthermore, overexpression of a full length isoform of

NTRK3 has been shown to contribute to anxiety-like behaviour in *NTRK3* transgenic mice, whose anxious phenotype was reversed by the administration of benzodiazepines [Dierssen, et al., 2006; Sahun, et al., 2007]. Linkage and association studies suggest *NTRK3* as a candidate gene also for other psychiatric disorders such as eating disorders or major depression [Camp, et al., 2005; Holmans, et al., 2007; Verma, et al., 2008]. More concretely, a linkage disequilibrium (LD)-mapping based approach recently showed that the *NTRK3* locus may contribute to the genetic susceptibility to OCD [Alonso, et al., 2008]. The *NTRK3* gene encodes for at least four splice variants in humans [Huang and Reichardt, 2001], with two isoforms being more abundantly expressed, a full-length catalytic isoform (Refseq NM_001012338) and a truncated isoform (Refseq NM_001007156). The transcript coding for the truncated non-catalytic form lacks the last 6 exons at the 3' end, which contain the kinase domain. Instead of this 3' region, the *NTRK3* truncated isoform has two extra exons and a different 3'UTR. In mice, co-expression of both isoforms has been detected in several regions of the developing nervous system and in adults the two isoforms are present in many brain structures [Menn, et al., 1998]. Little is known about the function of the truncated isoform, however, transfection of the two isoforms into primary neurons has shown that different ratios of catalytic and non-catalytic receptors are associated with different axonal morphology [Ichinose and Snider, 2000]. Coordination on the expression of both isoforms has been suggested in a study that also shows the truncated isoform of *NTRK3* is regulated by at least two microRNAs (miRNAs) (miR-9 and miR-125) causing cell growth repression in a neuroblastoma cell line [Laneve, et al., 2007].

miRNAs are a large class of small non-coding RNAs of about 19-25 nucleotides in length in their mature form that act as post-transcriptional regulators of gene expression by either mRNA degradation or translational repression [Filipowicz, et al., 2008]. Recent estimates indicate that miRNAs regulate approximately 30% of all protein-coding genes, building

complex regulatory networks that control almost every cellular process [Filipowicz, et al., 2008]. The recognition of target mRNAs is mediated by the complementarities between miRNAs and sequences located in the target mRNAs. The most critical region for target recognition is known as the seed region and consists of nucleotides 2-7 of the miRNA sequence [Brennecke, et al., 2005]. The identification of animal miRNA targets is a challenging assignment for both experimental and computational groups, with around 15 different computational miRNA target prediction programs developed since the publication of the first predictions [Lewis, et al., 2003; Rajewsky, 2006; Sethupathy, et al., 2006]. Increasing evidence indicates that genetic variation in regulatory regions could be a major contributor to phenotypic diversity in human populations [Feng, et al., 2008]. In fact, deregulation of the miRNA regulatory pathways has already been involved in human disorders such as cancer or fragile X syndrome [Gong, et al., 2005]. Furthermore, single nucleotide polymorphisms (SNPs) located within miRNA target sites have been demonstrated to affect the expression of the target gene and contribute to the susceptibility to common diseases. Abelson *et al*. were the first to associate a sequence variant in a miRNA target site with disease [Abelson, et al., 2005]. They reported a rare sequence variant disrupting a target site for miR-189 in the *SLITRK1* gene in two patients with Tourette's syndrome and in none of the controls tested. An altered interaction between this miRNA and the *SLITRK1* mRNA in the developing brain was suggested to contribute to this neuropsychiatric disorder. Since then, many reports have corroborated the effects of sequence variants in miRNA target sites in complex diseases and phenotypes, including asthma, hypertension and aggressiveness among others [Jensen, et al., 2008; Martin, et al., 2007; Sethupathy, et al., 2007; Tan, et al., 2007; Wang, et al., 2008]. Based on the recent discovery of miRNA-mediated regulation of *NTRK3,* we studied if genetic variation in miRNA target sites of *NTRK3* could be a predisposition factor for anxiety disorders in our population.

MATERIALS AND METHODS

Clinical Sample

OCD sample: between 2004 and 2006, 153 consecutive Spanish outpatients with OCD (85 males and 68 females, average age 33 (18-60), Supplementary Table1) , recruited from the OCD Clinic and Research Unit of Bellvitge Hospital (Barcelona, Catalonia) were included in the study. All patients met DSM-IV criteria for OCD [Association, 1994] and had had OCD symptoms for at least one year. Diagnosis was independently assigned by two senior psychiatrists using the Structured Clinical Interview for DSM-IV Axis I Disorders - Clinician Version (SCID-CV) [First MB, et al., 1997]. Exclusion criteria were: ages under 18 or over 65 years, mental retardation assessed by the WAIS-III, any other DSM-IV Axis I comorbid disorder except current or lifetime history of mood disorders and severe organic or neurological pathology, including Gilles de la Tourette or other chronic tic disorders. A clinician administered version of the Yale-Brown Obsessive-Compulsive Scale (Y-BOCS) [Goodman, et al., 1989] and the 21-item Hamilton Depression Rating Scale (HDRS) [Hamilton, 1960] were used to assess the severity of obsessive-compulsive and depressive symptoms, respectively. The Y-BOCS Symptom Checklist was employed to ascertain scores on the following symptom dimensions: contamination/cleaning, aggressive/checking, symmetry/ordering, sexual/religious and hoarding. So as to restrict the study to patients with clinically significant hoarding symptoms, 47 OCD patients (36 males and 11 females, average age 31 (18-52), Supplementary Table1), who gave affirmative answers on the Y-BOCS-SC items related to hoarding were considered hoarders only if they spent at least one hour a day on hoarding-related activities and reported at least moderate to severe distress and impairment secondary to hoarding.

PD sample: between 2001 and 2006, 212 consecutive adult Spanish outpatients with PD (55 males and 157 females, average age 36 (18-69), Supplementary Table1) recruited from the

Psychiatry outpatient unit in Hospital del Mar (Barcelona, Catalonia) were studied. The diagnosis of PD was independently assigned by two senior psychiatrists using the Structured Clinical Interview for DSM-IV Disorders - Clinician Version (SCID-CV). At the moment of the inclusion, 131 patients with PD also had agoraphobia. Exclusion criteria were: age under 18, organic brain syndromes, psychoactive abuse disorders (except nicotine abuse), any other DSM-IV Axis I comorbid disorder apart from other anxiety disorders and life prevalence of mood disorder and severe organic or neurological pathology including partial epilepsy. As some of the questionnaires were self-administered, illiterate patients also had to be excluded. A clinician administered version of the Panic and Agoraphobia scale [Bandelow, 1995], the Fear Survey Schedule (F100) of Wolpe [Arrindell, et al., 2003] and the 21-item HDRS [Hamilton, 1960] were used to assess the severity of panic and agoraphobia, fears and depressive symptoms, respectively.

The control group consisted of 324 unrelated adult Spanish (187 males and 137 females, average age 39 (18-69), Supplementary Table1), who were recruited from a group of blood donors of Catalonia and that had not been psychiatrically screened. An additional control sample of 250 healthy individuals recruited in the hospital Vall d'Hebron also in Catalonia (74 males and 176 females, average age 47 (20-59)) was also screened for the presence of the identified rare allele variants.

Written informed consent was obtained from each individual after they were given a complete description of the study, which was approved by the hospital ethic committee. After obtaining written informed consent, DNA of patients who agreed to participate in the study was extracted from peripheral blood using standard procedures.

Genotyping for Population Admixture

To detect population admixture, we chose 48 anonymous unlinked SNPs derived from a panel of 52 Ancestral Informative Markers (AIM) reported to be polymorphic in European,

Asian and African populations [Sanchez, et al., 2006]. We used the computer program 'Structure' [Pritchard, et al., 2000] to identify clusters of genetically similar individuals. An admixture model with correlated frequencies was applied, using five putative K values (1 to 5). Analysis was performed both with and without prior population information. Only the SNPs in Hardy-Weinberg equilibrium (HWE), with a genotyping rate >90% and an r^2 <0.1, were retained for analysis from our data. In total, 37 SNPs out of 48 were used. No allelic differences were observed and the highest log likelihood scores were obtained when the number of populations was set to 1.

PCR and Direct Sequencing

Fragments ranging from 379 bp to 745 bp were amplified by polymerase chain reaction (PCR) in a final volume of 25 µl using standard protocols and the following primers: TF-1F (5'-CCGTCTCCACCTCTGCTTA-3'), TF-1R (5'-CAAAGGACGTCATGGCTTTTA-3'), TF-2F (5'-CATCTTGACCTTGAAATGCTG-3'), TF-2R (5'-CTCTCCCAGGATACCATGC-3'), TF-3F (5'-CTTCCCTCTCCTCAGTGGAT-3'), TF-3R (5'-CAGGAGGTTTTACCCATGAAC-3'), TF-4F (5'-TAAAGCCACACTTGGGAGGA-3'), TF-4R (5'-GTCCTCCTGTTCTCTGGTCA-3'), TF-5F (5'-TGGAACAGATTGTGAACACCT-3'), TF-5R (5'-CTCATCCTGCTCCCCATTG-3'), FL-F (5'-ATTTGCCAAACTGCCTTACAG-3'), FL-R (5'-AACTAATCCGGGAAGTTGTTG-3'). The sequencing reaction was performed using the ABI PRISM® BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and PCR products were purified with ExoSAP-IT (usb, Cleveland, OH, USA) following standard procedures. Sequence purification was performed with Millipore Montage seq96 (Millipore Corporation, Billerica, MA, USA). Sequencing was performed using an ABI PRISM® 3730*xl* DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The six new allelic variants

identified in this study have been confirmed in a second PCR product by pyrosequencing and have been submitted to the dbSNP database receiving an ss number (Table 1).

Genotyping

Genotyping for allelic ss102661458 and rs28521337 was done by Pyrosequencing (Biotage AB, Uppsala, Sweden) using a specific SNP assay for each variant. For ss102661458: forward 5'-CGGGAAAAAAGGAGCACAGTG-3', reverse biotinylated 5'-GCACCCTTTAACACCACCAGAT-3', and sequencing 5'- AAGGAGCACAGTGATGA-3' primers. For rs28521337 forward 5'-ACAAGCCCCCCACAGGAT-3', reverse biotinylated 5'-CATGGGGCCTCCATTGTA-3', and sequencing 5'-GGCAGCCTATGCTGA-3' primers. Sequence identification was performed with the PSQ SNP software (Biotage AB), and the percentage of mutation load was determined using the quantification function of the software. ss102661460 and ss102661463 were analyzed by direct sequencing using the forward primer 5'-AGAACTTGGAAAGCCTCATGT-3' and the reverse primer 5'-AACCCAATGGAATCAGCAT-3'.

Association Studies

HWE for all the biallelic SNP markers was confirmed using a chi-square test (1 degree of freedom). Association analyses were assessed by means of multivariate regression methods under dominant, recessive, and additive models controlling for sex and categorizing cases by clinical status. We estimated the crude odds ratio (OR) and 95% confidence intervals (95% CI). Bonferroni correction for multiple comparisons was applied taking into account 2 independent non-monomorphic SNPs and the use of four different genetic models. Since these four tests are not independent, a simulation study was carried out obtaining a factor of 2.5 as the effective number of tests performed when these four genetic models are used (data not shown). Using these criteria, the corrected level of significance was set to 0.01 (2 SNPs x 2.5 effective tests = 5 comparisons). These analyses were performed with the SNPassoc R

package [Gonzalez, et al., 2007]. Linkage disequilibrium (LD) between polymorphisms was evaluated with the Haploview software version 3.2 (http://www.broad.mit.edu/mpg/haploview/). For the haplotype estimations the haplo.stats R package was used.

Luciferase Constructs for the 3'UTR of the human *NTRK3* truncated isoform

The 3'UTR of the truncated isoform of *NTRK3* was PCR-amplified from BAC CTD2508H23 using the forward primer (5'–ACACACTCTAGAAATAAGCCTTCCCGGACATT–3') and the Reverse primer (5'-ACACACTCTAGATGCAAAATTTCCAAATAAGAGG–3'). A PCR fragment of 2110 bp was purified and cloned into an *Xba*I site, located downstream of the Firefly luciferase gene in the pGL4.13 reporter plasmid (Promega Corporation, Madison, WI, USA). Mutant reporter plasmids (pGL4-v1-C, pGL4-v3-C, pGL4-rs-G) were generated using the wild-type pGL4-wt plasmid as a template and the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to manufacturers' instructions. Mutations were generated as described hereafter: PGL4-v1-C: a T>C mutation was introduced with primers (5'-CCCCTTCCCTCCA**G**TCATCACTGTGCTC-3') and (5'-GAGCACAGTGATGA**C**TGGAGGGAAGGGG-3'). PGL4-v3-C: a G>C mutation was introduced with primers (5'-CCATGAGAGGGGCTG**C**AGGAGGCCAAGTTTT-3') and (5'-AAAACTTGGCCTCCT**G**CAGCCCCTCTCATGG-3'). PGL4-rs-G: a C>G mutation was introduced with primers (5'-CAGCCTATGCTGACTT**G**ATACAATGGAGGCCC-3') and (5'-GGGCCTCCATTGTAT**C**AAGTCAGCATAGGCTG-3'). The authenticity, the inserts' orientation and the presence of the mutations were confirmed by direct sequencing.

Transfection and Luciferase Assay

Transfection of HeLa cells with small RNAs was optimized with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) using a fluorescein-labeled double-strand RNA oligomer.

Small RNAs that mimic endogenous mature miRNAs and the related negative controls (miRIDIAN^{TM} microRNA Mimics) were obtained from Dharmacon Inc. (Lafayette, CO, USA). HeLa cells were seeded at $13x10^4$ cells per well in 96-well plates and were cotransfected 24h after seeding with the Firefly reporter constructs described above (10 ng to 24 ng), the Renilla reporter plasmid pGL4.75 (3 ng), and the appropriate miRNA mimic at different concentrations (3.3 nM, 10 nM and 30 nM). 24h after transfection, cells were lysed and Firefly and Renilla luciferase activities were determined using the Dual-Glo^{TM} Luciferase Assay System (Promega Corporation). The relative reporter activity was obtained by normalization to the Renilla luciferase activity. Each experiment was done in triplicate and at least three independent experiments were performed for each tested miRNA. Data are reported as mean (of all experiments performed) ± s.e. Statistical significance was calculated using Student's t test.

Computational Methods

Genomic coordinates are according to the March 2006 human assembly release (*H. sapiens* hg18). Sequences were obtained from the University of California Santa Cruz (UCSC) Genome Browser (http://www.genome.ucsc.edu). Sequence analysis was performed using the 4peaks software (http://mekentosj.com/4peaks/) and Multalin (http://prodes.toulouse.inra.fr/multalin/multalin.html). Primers were designed using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi). Prediction of miRNA target sites was performed with the web-based versions of Targetscan (http://genes.mit.edu/tscan/targetscanS2005.html), PicTaR (http://pictar.bio.nyu.edu) and MiRANDA (http://microrna.sanger.ac.uk/targets; http://www.microrna.org). All miRNA predicted to regulate *NTRK3* by at least one of the programs were functionally tested. The analysis of illegitimate miRNA target sites was performed using miRanda (miRanda v1.0b microRNA Target Algorithm) and Patrocles (http://www.patrocles.org/) algorithms.

RESULTS

Resequencing of the 3'UTRs of *NTRK3* and Screening for Functional Allelic Variants

The short 3'UTR (178 bp) of the full-length isoform of *NTRK3* and the long 3'UTR (1981 bp) of the truncated isoform were re-sequenced in 30 patients with PD, 30 patients with OCD, and 50 control subjects to look for nucleotide changes that could disrupt miRNA target sites. The re-sequenced region included the last exon of each isoform as well as the 3'UTRs and an extended genomic region of more than 100bp; three different *in silico* algorithms (Miranda, TargetScan and PicTar) were used to predict all miRNA target sites at the 3' UTRs of *NTRK3*. While no allelic variants were identified in the 3'UTR of the full-length isoform, seven allelic variants located within the predicted target sites of 10 different miRNAs (5 conserved and 5 non-conserved miRNAS) were identified in the 3'UTR of the truncated isoform (Table 1, Figure 1). Three of these miRNAs are predicted to target *NTRK3* by at least two different programs (Table 1). Two of the seven identified variants (ss102661458 and rs28521337) fall within the predicted target sites of two and three different miRNAs, respectively (Figure 2). None of the identified allelic variants were predicted to create a novel illegitimate miRNA target site, according to either the Patrocles or miRanda prediction programs.

Validation of miRNA Target Sites in the truncated isoform of *NTRK3*

Functional validation of the 10 predicted miRNA target sites containing the identified allelic variants was performed using a dual-luciferase assay in HeLa cells. A luciferase-reporter contruct containing the wild-type 3'UTR of the truncated isoform of *NTRK3* was co-transfected with the miRNAs mimics of interest. As shown in Figure 3, a statistically significant reduction of the luciferase activity was observed for five miRNAs (miR-128, miR-485-3p, miR-509, miR-625 and miR-765) when compared with two different control mimics. The down-regulation observed ranged from a moderate reduction of the luciferase

expression of approximately 17% in the case of miR-485-3p, up to a substantial reduction of more than 50% for at least two different miRNAs, miR-509 and miR-625. Since miRNAs have been reported to act synergistically, co-transfection experiments using different combinations of miRNAs were also performed, but the effect of co-transfected miRNAs was never higher than the effect observed when they were transfected separately (data not shown). We concluded that the truncated isoform of *NTRK3* is strongly regulated by at least five different miRNAs, some of them act on the same target site and are probably coordinated in a time and/or tissue specific fashion.

Association of Variants in *NTRK3* miRNA Target Sites with Anxiety Disorders

Case-control studies were performed for the four allelic variants located within functionally validated *NTRK3* miRNA target sites (ss102661458, ss102661460, ss102661463 and rs28521337, Table 1). Analyses of allele frequencies in 212 patients with PD (with and without agoraphobia), 153 patients with OCD (hoarding and non hoarding type) and in 324 controls showed that ss102661463 and rs28521337 are common variants (MAF >0.05). In contrast, ss102661458, which is located in the target sites of two different miRNAs (miR-128 and miR-509), and ss102661460, located within the miR-765 target site, are rare allele variants, each present in only one chromosome of a PD patient and in none of the 944 chromosomes analyzed from controls and OCD patients (Table 1). Furthermore, the presence of these two rare allele variants in another control sample was discarded by screening 500 additional chromosomes from healthy Catalan individuals. Interestingly, both PD patients are males presenting the agoraphobic phenotype.

Association analysis with the two common variants was performed under different models using logistic regression and adjusting for sex after verification of Hardy-Weinberg equilibrium in our population. LD analyses based on r^2 revealed a low correlation ($r^2<0.2$) between both SNPs in the affected individuals (either OCD or PD) as well as in the control

set. No significant association for any of the two variants was found for PD even when categorized by clinical status or after stratifying for the agoraphobia phenotype. In contrast, when assessing the OCD sample, a statistically significant association was found for rs28521337 under a recessive model (unadjusted p=0.020, OR= 0.55; 95% CI= 0.33-0.93; Table 2), although the significance was lost after Bonferroni correction (p<0.01, two independent SNPs and 2.5 effective tests). When the analysis was repeated taking into account symptom dimensions, we could detect an association with hoarding. Hoarding obsessions and compulsions were present in about 33% of the OCD patients, who also exhibited more severe OCD signs. As shown in Table 2, the association increased for the OCD hoarding sample under a recessive model (unadjusted p=0.010, OR=0.30; 95% CI= 0.10-0.86) and became significant after Bonferroni correction (p<0.005, two independent SNPs, two subphenotypes tested and 2.5 effective tests) under the log-additive model (unadjusted p=0.0048, OR=0.53; 95% CI= 0.33-0.84). The calculated odds ratios suggest that the C allele, with a frequency of 0.47 in the controls and 0.31 in the OCD hoarding sample, could have a moderate protective effect against OCD hoarding phenotype. Finally, haplotype analysis for ss102661463 and rs28521337 was also performed but no increase in the significance level was observed.

Functional Analysis of miRNA Targeting for rs28521337

We checked if rs28521337, located in the seed region of the functional miR-485-3p target site, was influencing the targeting of *NTRK3* by this miRNA. A construct containing the G allele for rs28521337 was generated by mutagenesis from the wild-type pGL4-wt construct (containing the C allele for rs28521337). These constructs were co-transfected with the miR-485-3p mimic and with two different control mimics and the resulting luciferase activity was normalized and compared between the two constructs, however, no significant recovery of the luciferase levels was observed for the G allele in comparison to the C allele (92% v.s.

85%, p=0.56, Figure 4a). Furthermore, we also tested if the G allele of this variant could improve the targeting by miRNAs miR-345 and miR-617, which were predicted to bind at this position but were proven not to be functional on the wild-type construct (Table 1 and Figure 3). Again, no significant changes in luciferase activity were observed between the constructs carrying the C or G alelles (Figure 4a) indicating that the rs28521337 variant is not interfering significantly with the binding of any of those 3 miRNAs. Since it could also be that HeLa cells might lack additional cofactors required for the release of *NTRK3* repression, we tried to perform the same luciferase experiment in the neuroblastoma cell line SH-SY5Y, unfortunately, we failed to optimize the triple transfection with the long plasmid containing the 3'UTR of the *NTRK3* truncated isoform.

Functional Analysis of miRNA Targeting for the *NTRK3* Rare Allele Variants

We tested if the two rare variants (ss102661458 and ss102661460), identified in heterozygosity in one PD patient each, had a similar effect on the regulation of *NTRK3*. Two more constructs containing the corresponding alleles were created: pGL4-v1-C (C allele for ss102661458) and pGL4-v3-C (C allele for ss102661460). For the first allelic variant, which is located in the target site of two different miRNAs (miR-128 and miR-509, Figure 2), an increase in luciferase activity was observed for the two miRNAs; with miR-128, a moderate but significant recovery of luciferase expression was observed (approximately 10% of recovery, Figure 4b). Interestingly, in the case of miR-509, the luciferase expression increased by about 18% when compared to the wild-type (Figure 4b), the difference being highly significant ($p<0.005$) at the three different mimic concentrations tested (3.3 nM, 10 nM and 30 nM). Similarly, in the case of ss102661460, located on the seed region of miR-765 (Figure 2), a significant recovery of luciferase activity (approximately 15%, $p<0.005$) was observed for the mutant C allele (Figure 4c). The nucleotide at the ss102661458 position

would anneal to the fourth nucleotide in the seed region of miR-509 (Figure 2) and it represents a change from an A·U pairing to a weaker G·U wobble at the RNA level. In the case of ss102661460, a strong G·C pairing is changed to a G·G mismatch in the binding site corresponding to the second nucleotide on the seed region of miR-765. These results indicate that these three miRNAs bind the 3'UTR of the truncated *NTRK3* causing a reduction of the luciferase activity between 30% and 65%. In addtion, the rare alleles of the two variants identified in PD patients create a mismatch in the respective miRNA target sites (miR-128, miR-509 and miR-765, Figure 2) that affect the proper miRNA binding and result in a significant recovery of the luciferase expression.

DISCUSSION

Polymorphisms at miRNA target sites may be determinant of a range of human traits related to disease such as hypertension, asthma or Parkinson's disease [Martin, et al., 2007; Sethupathy, et al., 2007; Tan, et al., 2007; Wang, et al., 2008]. In terms of psychiatric phenotypes, variants at the 3'UTR of the *SLITRK1* and the *HTR1B* (serotonin receptor 1B) genes have been associated with Tourette syndrome and with aggressive human behaviour, respectively [Abelson, et al., 2005; Jensen, et al., 2008]. These findings demonstrate that changes in regulatory elements that lead to minor variations in the dosage of proteins involved in neuronal pathways may play an important role in fine-tuning the activity of these proteins, contributing to the susceptibility to psychiatric disorders. This could be the case for *NTRK3* that has recently been associated to anxiety-like behaviour in a mouse model overexpressing the full length of *NTRK3* [Dierssen, et al., 2006]. Furthermore, post-transcriptional regulation of the truncated isoform of *NTRK3* by two miRNAs (miR-9 and miR-125) has recently been demonstrated [Laneve, et al., 2007]. In the present study we have tested 10 miRNAs predicted as putative regulators of the truncated isoform of *NTRK3* and found that 5 of them are functional. Two of the miRNAs (miR-509 and miR-625) caused a striking reduction of more than 50% in luciferase activity indicating that *NTRK3* is strongly repressed by these miRNAs. Post-transcriptional regulation by miRNAs may thus represent an important mechanism through which the different isoforms of *NTRK3* are precisely regulated, stressing the importance of the study of the genetic variability at 3'UTRs of *NTRK3*. Regulatory regions at 3'UTRs have been poorly studied in the past and information about their genetic variation is almost non-existent. This definitely applies to the truncated isoform of *NTRK3*, for which very little is known and it has only recently been demonstrated to activate a specific signalling pathway [Esteban, et al., 2006]. The lack of SNPs in the 3'UTRs of *NTRK3* in public databases could be explained by a stronger action of negative

selection on these regions in comparison to other non-coding regions. In fact, a recent work has reported the existence of negative selection in conserved miRNA target sites at 3'UTRs where the SNP density is lower (0.5 SNPs/kb) than in their flanking control sites (0.73 SNPs/kb) [Chen and Rajewsky, 2006]. Polymorphisms in miRNA binding sites are thus likely to be deleterious and are arise as good candidates to contribute to human disease. After re-sequencing the 3'UTRs of *NTRK3* in patients and controls, we found 7 allele variants from which 4 are located in validated miRNA target sites, two of them being conserved target sites for miR-765 and miR-128, and could be functional and influence genetic susceptibility to disease (Table 1). Five of the identified allelic variants are common SNPs (MAF >0.05) but the other two are rare variants that altered the regulation of *NTRK3* by three different miRNAs (miR-128, miR-509 and miR-765), each of the variants found in only one male patient with PD and agoraphobia. Similarly, in a recent report, a rare allelic variant in the 3'UTR of *SLITRK1* affecting the binding of miR-189 was only identified in two patients with Tourette syndrome and in none of the controls tested [Abelson, et al., 2005]. It was argued that these results might be confounded because the screening for new causative allelic variants was carried out only in cases [Keen-Kim, et al., 2006]. In order to exclude a possible ascertainment bias due to population stratification, we performed re-sequencing of both, patients and controls, and population homogeneity was assessed. Regardless of the genetic mechanism, the phenotype associated to the allele-specific targeting found for *NTRK3* will depend upon the expression and activity of these miRNAs. Although little is known about the factors that regulate the function and expression of most of these miRNAs, some of them are known to be expressed in the brain and could have important roles in the central nervous system. In fact, miR-485-3p is a brain enriched miRNA that is expressed in the adult hippocampus and cerebellum [Betel, et al., 2008], miR-765 is located in the intron of *ARHGEF11*, a gene highly expressed in brain that has been described to have a role as

modulator in glutamatergic synapses [Jackson, et al., 2001]. More interestingly, miR-128 has been implicated in the differentiation of neurons [Smirnova, et al., 2005] and, very recently, in the inhibition of the pre-synaptic protein SNAP25 [Eletto, et al., 2008] fact that strengthens the importance of the miR-128 mediated regulation of *NTRK3* in the context of synaptic plasticity. In the case of miR-509, a miRNA that shares the target site of miR-128, its expression has been observed to be restricted to testis [Betel, et al., 2008], that could suggest tissue dependent regulation of *NTRK3* at this site.

Two general models of the genetics of complex diseases have been proposed. The common disease–common variant model and an alternative model that states that susceptibility to common diseases is the result of multiple rare alleles [Pritchard, 2001], which could be the case for PD. Even though the contribution to heterozygosity of these rare allele variants is very low, their putative role in disease cannot be ruled out as emphasized by the lack of agreement among recently published results on whole genome association studies, which point out that susceptibility alleles are likely to be modest in effect size and require large samples for detection [Sklar, et al., 2008]. Furthermore, the lack of replications in recent association studies in complex disorders, and specially in the case of psychiatric traits, remarks the importance of performing functional studies rather than replicating in other cohorts, in which the effect of distinct genetic backgrounds and the use of different diagnostic criteria often lead to false negative replications [Burmeister, et al., 2008]. The complex modes of inheritance observed in PD [Smoller and Tsuang, 1998] could lead to the conclusion that multiple genes of small effect, as well as accumulation of rare variants like those found in this study, may interact with each other and/or with non-genetic factors, and produce vulnerability to the disorder. However, in order to demonstrate the rare variant hypothesis in PD, a larger sample of cases and controls should be analyzed to reach statistical significance, which is out of the scope of this study. In fact, *NTRK3* seems to be precisely

regulated and its dosage imbalance may have pathological consequences. The neurotrophic support is critical for neuronal systems and its reduction may cause developmental alterations of the fear circuitry or impair neural adaptation to stress [Gratacos, et al., 2007]. Data from a transgenic mouse model overexpressing the full-length isoform of *NTRK3* supports this hypothesis [Dierssen, et al., 2006] as these mice present anxiety-like behaviour and show increased cellularity in the LC and other catecholaminergic nuclei. Neurotrophin-3 has trophic effects on LC neurons and mediates the effect of stress and of some antidepressants on LC function and plasticity [Smith, et al., 1995].

In contrast to the rare variant hypothesis for PD, association with a common SNP (rs28521337) was found in OCD. The association was specific for the hoarding sub-clinical type, suggesting a different pattern of genetic inheritance for this group of patients, which would be in agreement with recent reports that indicate that hoarding syndrome may constitute a neurobiologically and etiologically distinct variant of OCD [Miguel, et al., 2005; Samuels, et al., 2007] being highly heritable as a quantitative trait [Mathews, et al., 2007]. The contribution of rs28521337 to disease remains unclear and, although the variant is located in the seed region of a validated target site for miR-485-3p, it does not significantly change the affinity and efficiency of this miRNA. Albeit it is possible that rs28521337 is only in LD with the real cause of the disorder, we cannot exclude that it could alter the expression of *NTRK3* in a miRNA independent mechanism or that HeLa cells might lack the additional cofactors required for the release of miRNA mediated repression of *NTRK3*. In this sense, it would be interesting to analyze the functional consequences of this SNP in a more biologically relevant context as we tried using SH-SY5Y neuroblastoma cell line. However, the high specificity of the expression of the truncated isoform of *NTRK3* makes this approach complicated. Furthermore, the possibility that other still unknown miRNAs could be regulating *NTRK3* at the site where the rs28521337 SNP is located should also be

considered. More comprehensive work on 3'UTRs sequencing in human individuals, as well as deep sequencing of miRNAs at the RNA and DNA level, are likely to increase our knowledge and catalogue of functional variants and new miRNAs associated with this disorder.

In conclusion, we show that the truncated isoform of *NTRK3* is regulated by at least five different miRNAs and we find an association of a common SNP in *NTRK3* with OCD hoarding type. Several authors have reported association of *NTRK3* with other psychiatric disorders, such as mood and eating disorders [Feng, et al., 2008; Mercader, et al., 2008; Verma, et al., 2008]. These reports are based on the study of LD and do not focus on the investigation of functional DNA variants that could explain the contribution of *NTRK3* to the phenotypes. These approaches could underestimate the contribution of a gene to a phenotype in the case of functional allele variants with low frequency, as reported here. Very recently, re-sequencing of *NTRK3* was performed in patients with major depression, the study focused on coding and other conserved regions, but no association was found with the 3'UTR of the gene and no functional variants were identified [Verma, et al., 2008]. Here, we show that two rare variants identified in patients with PD affect the miRNA-mediated regulation of *NTRK3*, leading to overexpression compared to the wild type allele. Regardless variants in *NTRK3* may not largely contribute to PD, the discovery of *NTRK3* 3'UTR variants affecting miRNA targeting reveals a functional mechanism by which non-coding polymorphisms may underlie the aetiology of PD, a disorder to which many different loci with small but cumulative effects are likely to be involved.

ACKNOWLEDGEMENTS

This work was supported by the “Instituto Carlos III and Fondo de Investigaciones Sanitarias” [CIBER-CB06/02/0058, CIBER-SAM, FIS/ISCIII:P1052565, ISCIII:GO3/184], the “Fundació la Marató-TV3” [014331], the “Departament d’Universitats Innovació i Empresa, Generalitat de Catalunya” [2005SGR00008] and the European Union Sixth Framework Programme Integrated Project SIROCCO [Grant LSHG-CT-2006-037900]. The Spanish National Genotyping Center (CeGen) is supported by “Genoma España” and the “Ministerio de Educación y Ciencia” (Spanish Government). M.M is a recipient of a FIS fellowship [FI05/0006]. Y.E was supported by the “Ramón y Cajal” Program [Spanish Ministry of Science and Education]. We would like to thank J.M. Mercader for his help in technical issues and suggestions as well as B. Cormand, A. Macaya, R. Corominas and E. Cuenca (Hospital Universitari Vall d'Hebron, Barcelona) for kindly proving us with control samples.

LEGENDS TO FIGURES

Figure 1: Schematic view of the full length and truncated isoforms of *NTRK3*. Allelic variants on the 3'UTRs are depicted by triangles. For those allelic variants located within predicted miRNA target sites the name of the respective miRNA is shown. Allelic variants located within the seed region of a predicted miRNA target site are also indicated (^{s}).

Figure 2: Sequence alignments for the four allelic variants identified in functional miRNA target sites of the truncated isoform of *NTRK3*, showing the predicted binding to the corresponding miRNAs. For each variant both alleles are shown. Allelic variants located within a functional miRNA target site are depicted together with other predicted miRNA targets they might be affecting. The position of the allelic variant is indicated in bold and the seed region of the miRNAs is underlined: a, predicted target sites for miRNAs miR-128 and miR-509 are shown for ss102661458; b, predicted target site for miR-765 is shown for ss102661460; c, predicted target site for miR-625 is shown for ss102661463; d, predicted target sites for miR-485-3p, miR-345 and miR-617 are shown for rs28521337. Note: the sequences shown here correspond to the *NTRK3* RNA, which is transcribed from the antisense (-) DNA strand.

Figure 3: Results of the luciferase-reporter assay, which was used to test the interaction between different miRNAs and the truncated isoform of *NTRK3* in HeLa cells. Ratios of the Firefly and Renilla luciferase luminescence are presented after normalization to the empty plasmid pGL4.13 and to the mean of two different mimic controls. Each experiment was done in triplicate and at least three independent experiments were performed for each miRNA tested, at a concentration of 10 nM. Data reported here are the means ± s.e of all experiments performed. Significant associations ($p<0.01$, Student's t test) are indicated with an asterisk. Light-colour bars correspond to those miRNAs found to significantly reduce the luciferase expression.

Figure 4: Effect of miRNA overexpression on three different allelic variants at the 3'UTR of the truncated isoform of *NTRK3*: a, ratios of the firefly and renilla luciferase activity for the C (PGL4-wt) and the G allele (PGL4-rs-G) of the rs28521337 SNP when transfected with two control mimics and miR-485-3p, miR-345 and miR-617 at 10 nM. b, results for the T (PGL4-wt) and the C allele (PGL4-v1-C) of ss102661458 when transfected with miR-128 and miR-509 at 3 different concentrations, showing a significant increase in the luciferase activity for the C allele. c, results for the G (PGL4-wt) and the C allele (PGL4-v3-C) of ss102661460 when transfected miR-765 at 3 different concentrations, showing a significant increase in luciferase activity for the C allele. Each experiment was normalized to the empty plasmid pGL4.13 and to the mean of the two controls. Each experiment was done in triplicate and at least three independent experiments were performed. Data reported here are the means ± s.e of all experiments performed. Significant associations ($p<0.01$, Student's t test) are marked with an asterisk.

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