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Homozygous and heterozygous disruptions of *ANK3:* at the crossroads of neurodevelopmental and psychiatric disorders

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AnkyrinG, encoded by the *ANK3* gene, is involved in neuronal development and signaling. It has previously been implicated in bipolar disorder and schizophrenia by association studies. Most recently, *de novo* missense mutations in this gene were identified in autistic patients. However, the causative nature of these mutations remained controversial. Here, we report inactivating mutations in the Ankyrin 3 (*ANK3*) gene in patients with severe cognitive deficits. In a patient with a borderline intelligence, severe attention deficit hyperactivity disorder (ADHD), autism and sleeping problems, all isoforms of the *ANK3* gene, were disrupted by a balanced translocation. Furthermore, in a consanguineous family with moderate intellectual disability (ID), an ADHD-like phenotype and behavioral problems, we identified a homozygous truncating frameshift mutation in the longest isoform of the same gene, which represents the first reported familial mutation in the *ANK3* gene. The causality of *ANK3* mutations in the two families and the role of the gene in cognitive function were supported by memory defects in a *Drosophila* knockdown model. Thus we demonstrated that *ANK3* plays a role in intellectual functioning. In addition, our findings support the suggested association of *ANK3* with various neuropsychiatric disorders and illustrate the genetic and molecular relation between a wide range of neurodevelopmental disorders.

INTRODUCTION

Intellectual disability (ID), defined by an IQ < 70, is a cognitive disorder of major medical and socioeconomic importance. It is usually first diagnosed in infancy, childhood or adolescence and affects $\sim$1–3% of the population worldwide (1). At least half of the cases appear to be genetic in origin (2,3). Causative Mendelian mutations have been identified in more than 400 genes (4).

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However, the majority of ID genes has yet to be uncovered and most patients are still undiagnosed due to this extensive genetic heterogeneity (5). ID can manifest as the only clinical feature, but is often accompanied by a wide range of symptoms. These additional characteristics can be helpful in guiding the clinical diagnosis of a specific disorder. Particular common co-morbidities include neurological and psychiatric features, such as epilepsy, autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD) and other behavioral problems. For example, 40% of ID patients show features of ASD (6). Conversely, ID is seen in 50–85% of patients reported with ASD (6). ASD affecting 1 out of 150 live births is characterized by groups of symptoms and signs such as deficits in social interaction, communication difficulties and restricted stereotyped behavior (7). ADHD, with the core features of hyperactivity, inattention and impulsivity, is also frequently associated with ID. It is the most commonly diagnosed neuropsychiatric condition in children with a prevalence of 3–5% globally (8). Both ADHD and ASD show a high estimated heritability of 76% (9) and 90% (8), respectively. A well-known example of an ID disorder with autistic features and ADHD-like symptoms is the fragile X syndrome, the most common form of monogenic ID (10).

There is an increasing number of examples showing that the phenotypic overlaps between ID and other mental disorders is reflected by overlaps between the molecular underpinnings of these conditions. First, recent studies in twins suggest that common genetic factors underlie this co-morbidity (11). Secondly, copy number variations (CNVs) and genome-wide association studies (GWAS) have suggested chromosomal regions implicated in autism that are also susceptibility sites for ADHD (12–14). Finally, mutations and polymorphisms in several genes have been shown to cause variable neurodevelopmental and neuropsychiatric phenotypes, such as ID, schizophrenia, ASD and other neurological disorders (14–16).

Here, we report on the characterization of a balanced translocation leading to heterozygous disruption of the AnkyrinG gene (*ANK3* [MIM 600465]) in a patient with a cognitive deficit, ADHD and ASD. In an independent study, we identified a homozygous frameshift mutation in the same gene in a family with autosomal recessive ID, hypotonia, spasticity and sleep disturbances. *ANK3* has already been implicated in a number of mental disorders such as bipolar disorder, schizophrenia and autism but so far remained to be controversially discussed (17–24). Our data reveal the first familial case of *ANK3* and suggest that variants in *ANK3* can lead to various cognitive/psychiatric phenotypes. Finally, we use a *Drosophila* model to provide further experimental support for the role of *ANK3* in short-term memory, a core domain of cognition that is commonly affected in ID (25), autism (26) and ADHD (27).

RESULTS

Disruption of the ANK3 gene by a balanced translocation

In a patient referred to the genetic diagnostic department upon indication of behavioral problems including ADHD, ASD and cognitive problems, we identified a balanced translocation involving chromosome 2 and 10, using conventional karyotyping. Cognitive testing revealed a disharmonic cognitive profile with a verbal IQ of 67 and a performance IQ of 97 (see Materials and Methods for a detailed clinical description). After refining the translocation breakpoints to 2q11.2 and 10q21.2 using fluorescent *in situ* hybridization (FISH) and chromosome painting (Fig. 1A), CNV analysis was performed to exclude submicroscopic imbalances at the translocation breakpoints or elsewhere in the genome. At a practical resolution of 75 kb, no imbalances at the translocation breakpoints were detected using CNV-WebStore (28). No further pathogenic CNVs were found on genome-wide analysis. Specific amplicons were designed spanning the breakpoints on the flow sorted derivate chromosomes der(2) and der(10). These amplicons were sequenced and mapped against the human genome reference sequence to positions 95 427 597 and 61 875 979 on chromosomes 2 and 10, respectively (Fig. 1B).

Detailed sequence analysis showed an overlap of four nucleotides (ACCC) at the breakpoint between both translocation partners, which was only retained on der(2). In addition, a loss of five nucleotides (AATGG) from the reference sequence of chromosome 2 was detected (Fig. 1C). The combination of micro-homology with small insertions and deletions is characteristic of non-homologous end joining (NHEJ) following a double-stranded break (29). Both breakpoints were located within annotated transcripts. On chromosome 2, ankyrin repeat domain 20 family member 8P (*ANKRD20A8P)* is disrupted in its second to last intron. *ANKRD20A8P* is a pseudogene. The translocation breakpoint on chromosome 10 mapped to intron 30 of NM_020987.3 of the *ANK3* gene and interrupts all *ANK3* transcript variants (Fig. 2D).

Expression of the disrupted transcripts and other genes near the breakpoints was investigated using real-time PCR (RT-PCR) on RNA extracted from three independent lymphoblastoid cell cultures of the patient and eight cultures from healthy controls (Fig. 3). Two probes were designed for *ANK3*, located proximal and distal to the breakpoint. To exclude the position effect on genes previously associated with cognitive disorders, we included early growth response 2 (*EGR2* [MIM 129010]) and jumonji domain-containing protein 1C (*JMJD1C* [MIM 604503]) located, respectively, at 2.7 and 2.8 Mb from the breakpoint on chromosome 10 (30,31). Both *ANK3* probes detected significant underexpression of the gene (Mann–Whitney U, $P < 0.001$), whereas expression of the neighboring genes was unaffected ($P > 0.05$). Four randomly sampled genes located at a large distance on chromosome 2 {[ATPase family, AAA domain containing 2B (*ATAD2B*), NDC80 kinetochore complex component, homolog (*SPC25*)} and chromosome 10 [pitrilysin metallopeptidase 1 (*PITRM1*)], suppressor of clear, *Caenorhabditis elegans*, homolog of (*SHOC2* [MIM 602775])] did not show altered expression levels, suggesting no global consequences on gene expression. No expression of *ANKRD20A8P* was detected in the lymphoblasts of healthy controls or the patient. This clearly indicates the absence of an *ANK3-ANKRD20A8P* fusion transcript from der(2) in the patient lymphoblasts.

Identification of a homozygous ANK3 frameshift mutation

A Pakistani family, PKMR14 (Fig. 2A and B), was included from a parallel study on autosomal recessive ID upon

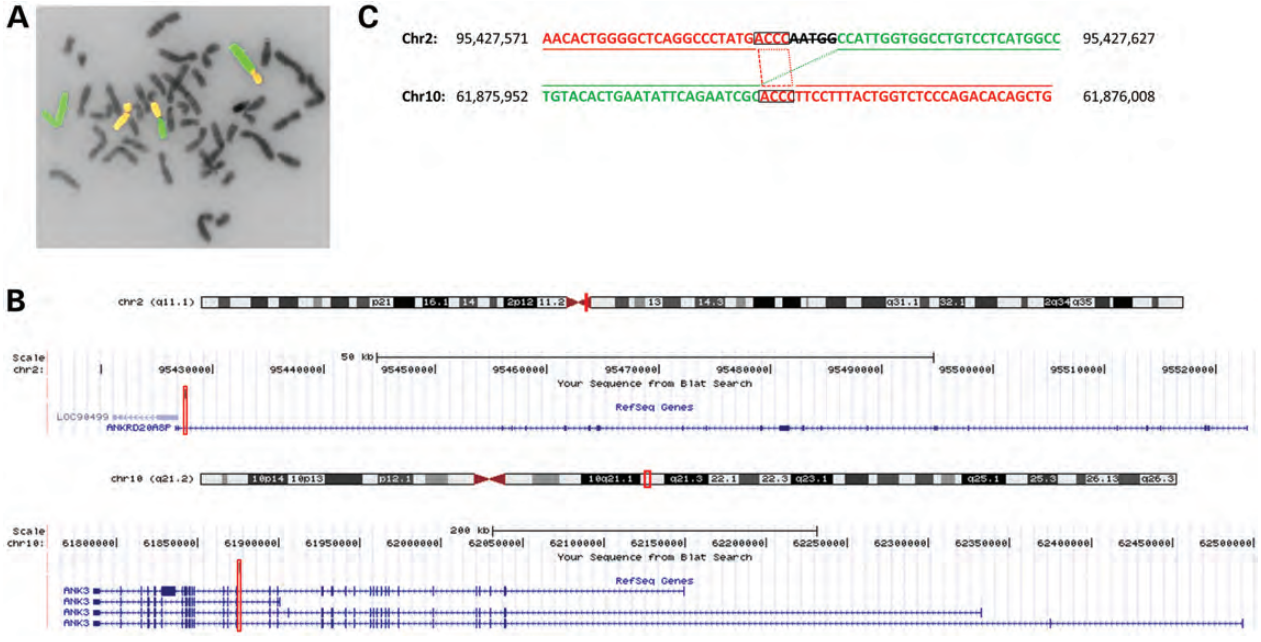

Figure 1. Molecular analysis of the balanced translocation. (**A**) Chromosome painting results confirm location of chromosome 2 (green) centromere to be located on derivate chromosome 2, with the attachment of q21–q26 of chromosome 10 (yellow). (**B**) Genomic context of translocation breakpoints (indicated by red boxes). Top: chromosome 2 breakpoint disrupts the *ANKRD20A8P* transcript. Bottom: chromosome 10 breakpoint disrupts all transcript variants of *ANK3*. (**C**) 2: Breakpoint sequences. Red: Sequence present on derivate chromosome 2. Green: Sequence present on derivate chromosome 10. Box: four nucleotides overlap in sequence between both chromosomes. Strikethrough: Sequence missing from both derivates. Possible recombination paths are indicated by dashed lines.

identification of a homozygous truncating variant in *ANK3*, shared by three affected siblings from consanguineous parents. The affected siblings presented with moderate ID, hypotonia, spasticity and severe behavioral problems. Homozygosity mapping and CNV analysis were performed using SNP arrays (for details, see Materials and Methods section). No pathogenic CNVs were detected. Homozygosity mapping revealed a single homozygous region that was shared among the affected individuals of this family. This 20.4 Mb homozygous stretch is located on chromosome 10q21.1–22.1; chr10: 52 930 315–73 381 926 (hg19) and encompasses >80 genes (Fig. 2C). Subsequently, whole-exome sequencing was conducted for patient V:1. Stringent filtering resulted in 23 homozygous and 6 compound heterozygous possibly pathogenic variants (Supplementary Material, Table S1), out of which only a single homozygous variant was located in the identified homozygous region on 10q21.1–22.1 shared by the affected family members. The segregation of the homozygous variant was confirmed by Sanger sequencing (Fig. 2A). Furthermore, it was not detected in 268 ethnically matched chromosomes, dbSNP (135v), 200 Danish exomes (32), the 1000 genomes data set (33) and the Exome Sequencing Project [ESP, Seattle, WA (http://evs.gs.washington.edu/EVS/)]. This single base pair deletion (c.10995delC) results in a frame shift and leads to a premature stop codon in exon 42 of *ANK3*. Exon 42 is a large 7.8 kb exon which is unique to the largest isoform of *ANK3* (NM_020987.3) (Fig. 2D). The homozygous nucleotide deletion predicts a premature stop codon at position 3666 (p.Thr3666LeufsX2), which makes the mutant mRNA a target for nonsense-mediated mRNA decay (NMD) (34,35). Together, the genetic data indicate that the *ANK3* mutation is causative for the phenotype in family PKMR14. No cell lines were available to confirm the NMD and other functional impact of this homozygous frame shift mutation.

Disruption of the ANK3 homolog affects short-term memory in *Drosophila*

Common to ID, autism and ADHD are defects in cognitive processes such as memory. We therefore set out to provide independent indication for the role of ANK3 in cognition using an animal model. *Drosophila* is a powerful organism for research into the genetics of learning and memory and is an established model for cognitive disorders (36). The fly genome harbors two Ankyrin genes, *Ank* and *Ank2*. We studied *Drosophila Ank2*, the closest homolog of human *ANK2* and *ANK3*. Like human *ANK3*, but different from human *ANK2*, fly *Ank2* is expressed specifically in the nervous system (37,38) and has recently been demonstrated to be crucial for synaptic organization (39,40). Since *Ank2* mutants are lethal, we used inducible transgenic RNA interference (RNAi) to knockdown *Ank2* in a tissue-specific manner and analyze the effects on behavior. Pan-neuronal knockdown using the promoter line *UAS-dicer2*; *elav-Gal4* phenocopied the previously reported synaptic *Ank2* mutant phenotype at the larval neuromuscular junction. Synapses were small, show less synaptic boutons and were characterized by the absence of intrabouton space. Highly significant reduction in synapse area and length was found with *P*-values $4.1e^{-9}$ and 0.0025, respectively (Fig. 4A), confirming efficiency of the RNAi line. Using the *UAS-dicer2; 247-Gal4* driver, we next ablated *Ank2* in the mushroom bodies, the

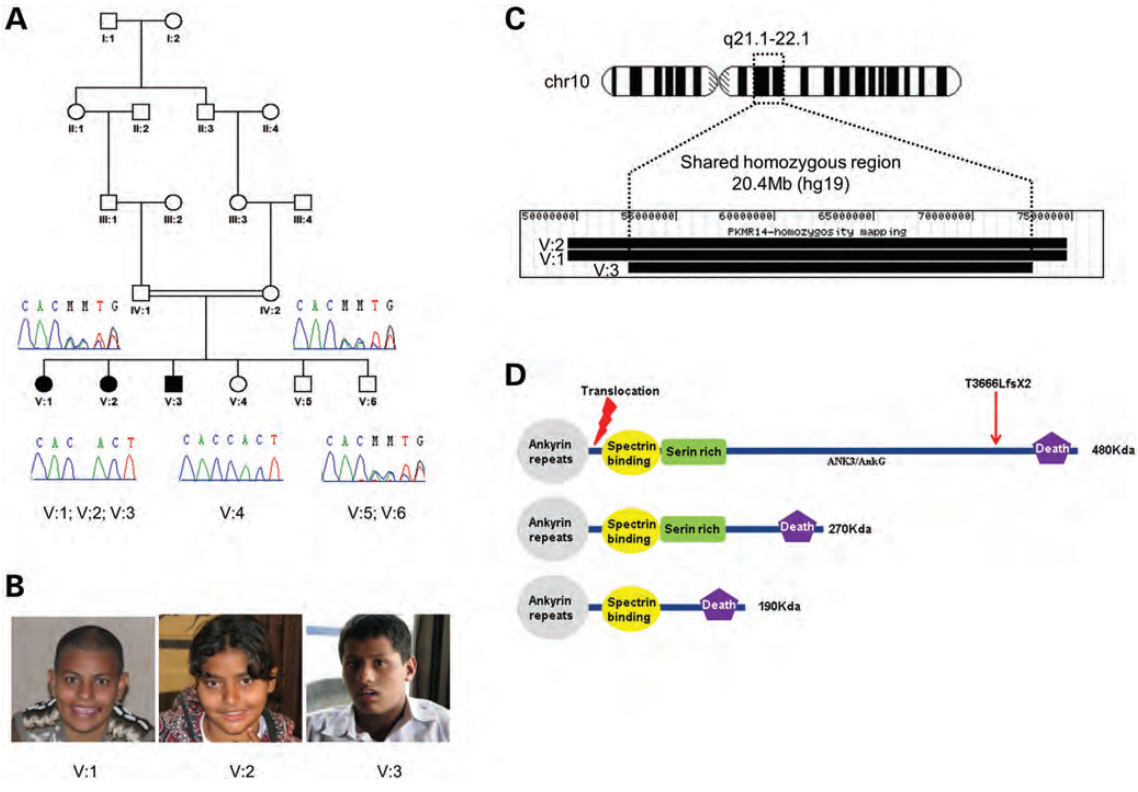

Figure 2. Identification of a homozygous frame shift mutation in a Pakistani family, PKMR14. (**A**) Pedigree structure of consanguineous Pakistani family PKMR14. Filled symbols indicate the affected members and the double horizontal lines demonstrate the consanguineous marriage. Chromatograms showing a complete segregation of g:61829644/c.10995del variant with phenotype are shown. (**B**) Photographs of patients V:1,V:2 and V:3, presenting no obvious features of facial dysmorphism. (**C**) Schematic representation of the 10q21.1–22.1 region that is homozygous in all three affected members (V:1,V:2,V:3) of family. The shared homozygous region among all three affected members was of 20.4 Mb (chr10: 52 930 315–73 381 926), which is indicated with blue lines. The genome assembly UCSC hg19 was used for the coordinates in the genetic map. (**D**) Ank3 protein structure with Ankyrin repeats domain (grey color), spectrin binding domain (yellow color), a serine rich domain (green color) and death domain (violet color). The position of the homozygous frame shift mutation is indicated as p.Thr3666LeufsX2. The position of the balanced translocation is indicated by the flash symbol.

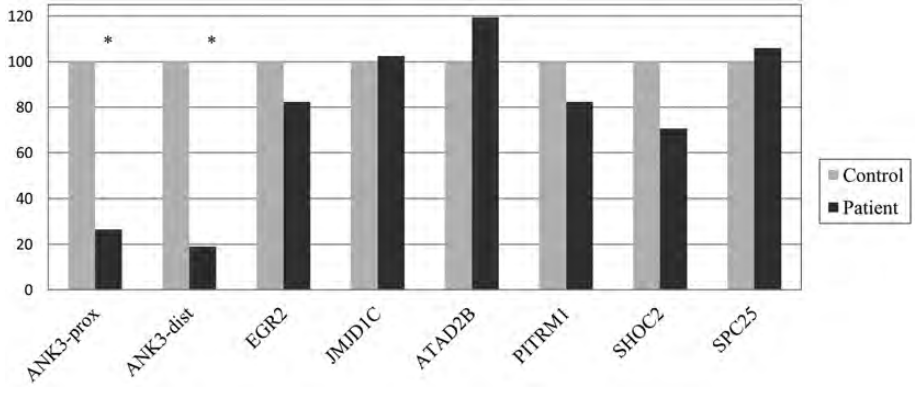

Figure 3. Expression analysis. Results are presented as the relative expression in patient lymphoblasts compared with healthy controls. Significant differences ($P < 0.001$) are indicated by asterisk.

learning and memory center of the fly brain, and assessed learning and memory in the courtship conditioning paradigm. We found that these mutants have normal learning but a significant reduction ($P < 0.05$, 10 000 bootstrap replicates) in short-term memory (Fig. 4B). This suggests that *Ank2* knockdown flies are affected in a specific aspect of cognition and do not show global neurological defects. To further support this conclusion, we quantitatively monitored *Drosophila* locomotion of individual flies over a period of several days. Both knockdown of *Ank2* in the mushroom body and pan-neuronal knockdown did not

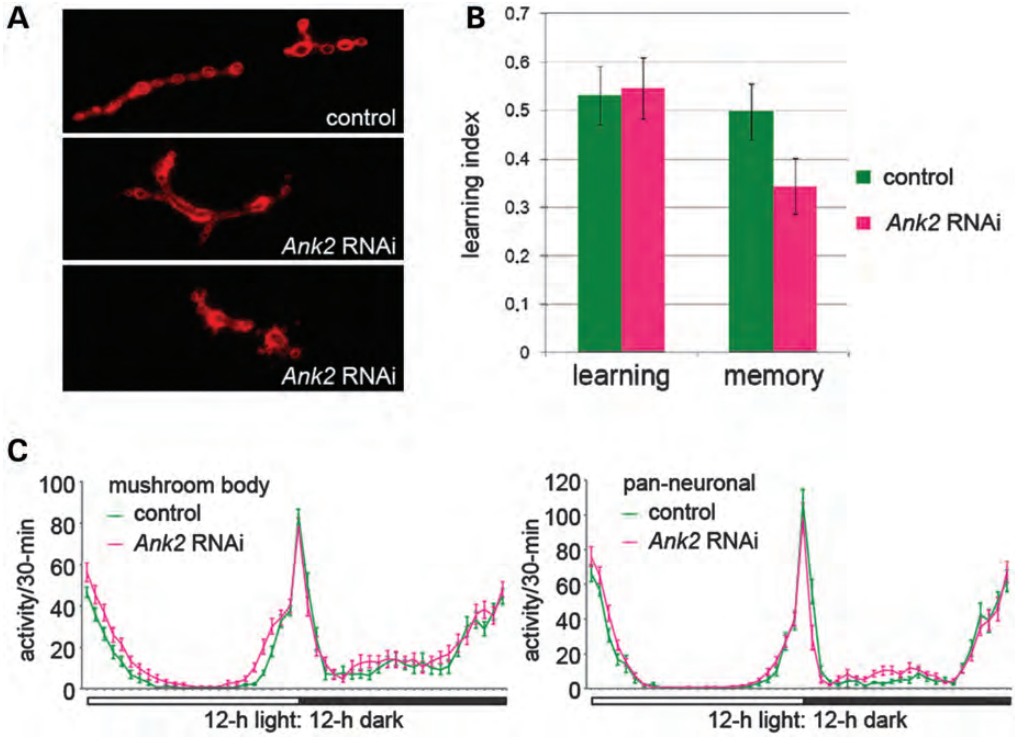

Figure 4. Analysis of synapse morphology, learning, memory and locomotion in *Drosophila* knockdown (RNAi) models of the Ank3 homolog, Ank2. (**A**) Synapse morphology at the larval neuromuscular junction (NMJ), revealed by anti-DLG1 labeling. Knockdown of Ank2 (*Ank2 RNAi*) using vdrc UAS-RNAi line 207 238 and the pan-neuronal driver elav-Gal4 phenocopies the reported mutant synapse phenotype. Eighteen NMJs of the *Ank2* knockdown condition were compared with 384 NMJs from control lines. Representative images are shown. (**B**) Learning and memory in *Ank2* mushroom-body specific knockdown flies versus their genetic background control in the courtship conditioning paradigm. Loss of *Ank2* causes a statistically significant reduction in courtship-based short-term memory. Error bars indicate SEM of the average learning index from four individual testing days ($P < 0.05$, 10 000 bootstrap replicates). (**C**) Activity profiling of mushroom body-specific and pan-neuronal *Ank2* knockdown models shows normal locomotion in both conditions. Average 30 min bin activity is calculated for 24-h with a 12-h light: 12-h dark cycle. Error bars indicate 95% confidence intervals.

affect locomotion, demonstrating that overall fitness of these knockdown conditions is not affected (Fig. 4C). Our results suggest that *Drosophila Ank2* is crucial for memory function.

DISCUSSION

ANK3 has previously been suggested to play a role in a number of psychiatric and cognitive disorders. Multiple independent GWAS strongly indicate that *ANK3* is associated with bipolar disorder (17–19,41). However, other studies failed to replicate these findings (21,22). In addition, SNPs in *ANK3* have been associated with late-onset Alzheimer's disease (42) and schizophrenia (23,24). This was supported by a joint GWAS of the Bipolar Disorder and Schizophrenia working groups of the Psychiatric GWAS Consortium that showed genome-wide significant associations with bipolar disorder and schizophrenia for an SNP in the 5′ UTR region of *ANK3* (rs10994359) (43). Further studies indicated that the *ANK3* risk variant for bipolar disorder has a significant impact on sustained attention (44,45). Sustained attention has been studied extensively in the context of both ASD and ADHD and is affected in both disorders (46,47). Moreover, the *ANK3* rs9804190 C allele was significantly associated with decreased *ANK3* mRNA levels in the superior temporal gyrus of schizophrenia patients (48). Here, we show that inactivating mutations in the *ANK3* gene result in a severe cognitive deficit. A heterozygous disruption of all isoforms of the *ANK3* gene results in a cognitive deficit, sleeping problems, autism, severe ADHD and aggressive behavior. The first reported familial *ANK3* mutation, a homozygous frameshift mutation affecting the longest isoform of the same gene, causes recessive ID, epilepsy, sleeping disorder, hypotonia, spasticity, hyperactivity and aggressive behavior. A causative role of *ANK3* in several of these cognitive functions and features is further supported by decreased short-term memory performance in a *Drosophila* model.

The AnkyrinG protein encoded by *ANK3* is located mainly at the nodes of Ranvier and the axon initial segment (AIS), two sub-compartments of neurons responsible for action potential generation (49). AnkyrinG consists of four domains, a globular head domain containing membrane-binding ankyrin repeats, a spectrin cytoskeleton-binding domain, a serine-rich domain and an extended tail domain containing a death domain (Fig. 2D). There are multiple isoforms of AnkyrinG, three of which are brain specific (190, 480 and 270 kDa). The 480 and 270 kDa isoforms harbor a unique large tail domain encoded by the exon 42 (for the 270 kDa only part of this exon), containing a serine-rich *O*-GlcNAc glycosylated part necessary to restrict these two larger isoforms to the AIS and nodes of Ranvier (50). AnkyrinG is recruited together with voltage-dependent sodium channels at the nodes of Ranvier (51), where AnkyrinG stabilizes the Na^+ channels by cross-linking

them to the spectrin–actin cytoskeleton proteins, thus ensuring proper action potential propagation (52). The involvement of ankyrinG in the composition of the AIS also implies an involvement in action potential generation by ion channels located at the diffusion barrier. In addition, it was shown that AnkyrinG is required to maintain the axo-dendritic cell polarity (53). Cerebellar depletion of the 270/480 kDa ankyrinG isoforms leads to axonal protrusions that resemble dendritic spines, containing post-synaptic densities and typical post-synaptic proteins such as SHANK2 [MIM 603290]. These axonal spines were contacted by pre-synaptic glutamatergic boutons and lack typical ultrastructural features of AIS, such as cytoplasmic bundles of microtubules (53). Interestingly, the identified homozygous frameshift mutation is predicted to specifically affect the synthesis of the 270/480 kDa isoforms, which may similarly result in disruption of the axo-dendritic polarity in patients carrying this mutation. The breakpoint in the translocation carrier inactivates these isoforms as well as all other isoforms of AnkyrinG. Surprisingly, although only a single copy of the *ANK3* gene is interrupted by the translocation, we measured a residual expression of this gene of only 25% in lymphoblastoid cell lines. This reduced expression might explain why the balanced translocation carrier is affected, whereas heterozygous mutation carriers appear to be unaffected. The significant reduction beyond the expected 50% may reflect the complex allele-specific regulation of *ANK3* transcription, as demonstrated recently (54). The complex transcriptional regulation may also account for the observations in a patient with a balanced translocation disrupting both forkhead box P1 (*FOXP1* [MIM 605515]) on chromosome 3p13 and *ANK3* on chromosome 10q21.2 (14). In this patient, *ANK3* expression is unaffected by the translocation and the neurodevelopmental abnormalities were postulated to be the result of the inactivation of the *FOXP1* gene. *De novo* chromosomal aberrations leading to reduced *ANK3* expression have been associated previously with an ASD phenotype and/or ADHD-like problems and/or developmental delay (55) (DECIPHER UPP001479 and AR256229, personal communication with Dr Vogel, Aarhus University Hospital). In addition, very recently Bi *et al.* (56) reported two *de novo* ANK3 missense mutations in the same exon as our homozygous frameshift mutation in 4 out of 47 ASD patients and one recurrent *de novo* missense mutation in exon 41 (56). Finally, Sanders *et al.* (57) also reported a *de novo* missense mutation in exon 42 of *ANK3* in a patient with ASD.

It thus appears that *ANK3* mutations and polymorphisms are associated with a wide spectrum of mental disorders presenting a continuum of mild-to-severe neuronal and cognitive manifestations. The severe end of this spectrum is represented by inactivating mutations affecting the neuronal isoforms of AnkyrinG. Several *de novo* missense mutations and structural variants have been associated with ASD so far. However, given the isolated occurrence of the respective patients it is not known whether these represent monogenic traits or whether these variants are contributory to the phenotype. Indeed, oligogenic inheritance is suspected in some cases because of the normal *ANK3* expression in a patient with a translocation disrupting *ANK3* and another established ID gene (*FOXP1*) (14). Finally, at the least severe end of the spectrum are *ANK3* polymorphisms which confer an increased risk to psychiatric conditions such as bipolar disorder, autism and dementia. Although these disorders are traditionally considered as distinct diseases, they share several behavioral and cognitive characteristics, together with a convergence on processes involving the development and regulation of neuronal signal transduction (58,59). Thus, our findings suggest that ID genes are excellent candidates for studying the etiology of genetically more complex disorders, for which the molecular underpinnings are usually poorly understood.

Mutations and variants in several other genes implicated in a comparably wide spectrum of mental disorders have been described (4). These included genes such as glutamate receptor, ionotropic, *N*-methyl-D-aspartate, subunit 2b (*GRIN2B* [MIM 138252]), *SHANK2*, contactin-associated protein-like 2 (*CNTNAP2* [MIM 604569]), neurexin 1 (*NRXN1* [MIM 600565]), *transcription factor 4* (*TCF4* [MIM 602272]), euchromatic histone methyltransferase 1 (*EHMT1* [MIM 607001]) and *FOXP1* (11,13,48–58). An intriguing finding emerging from these studies is that clusters of affected genes involved in networks of neuronal motility, synaptic development and axonal guidance, and genes encoding transcription factors and epigenetic regulators are significantly enriched in these disorders (4,60,61). The *ANK3* variants reported here fit well in this 'common pathways' model, as AnkyrinG is implicated in several of the core processes implicated in cognitive disorders, especially axonal guidance, transmission of nerve impulses and maintenance of cell polarity. Furthermore, the conditional ablation of *Drosophila Ank2* (the fly homolog of *ANK3*) recapitulated synaptic defects previously identified in *Ank2* mutant condition, and allowed us to implicate this essential gene in short-term memory. Defects in short-term memory have been reported in ID (although many conditions are too severe to conduct quantitative tests) and ID models, but also in autism and ADHD (25–27).

We hypothesize that there will be many more genes for which genotype–phenotype associations await discovery in mental disorders. Given that the first and most compelling evidence for a role of these genes in mental disorders was typically obtained by studying monogenic mutations in ID individuals with or without associated (neural) deficits, we believe that targeted analysis of ID genes in psychiatric disorders may reveal such genotype–phenotype associations. Moreover, in many cases such approach will immediately provide a biological correlate for a complex psychiatric disorder, which is often not the case in hypothesis-free GWAS.

MATERIALS AND METHODS

Case report of balanced translocation patient

The proband was the second child of a healthy mother and first child of the father. The parents are non-consanguineous. He was born prematurely at 32 weeks with a birth weight of 2 kg and length of 44 cm (both on the 50th centile). There is no record of neonatal problems. Gross motor development was initially normal as he sat unsupported at 7 months and crawled at 8 months. He walked unsupported at the age of 18 months and cycled when he was 6 years. Language development was delayed and speech therapy was initiated when he was 2.5 years old. As from nursery class, behavioral problems were noted: he was constantly fidgety, hyperactive, attention-

seeking and exhibited aggressive behavior towards his classmates for no obvious reasons. At 5 years of age, the diagnosis of ADHD was suspected and a year later formal cognitive testing by WPPSI-R showed borderline intelligence with a verbal IQ of 67, a performance IQ of 97 and a total IQ of 76. He was orientated into a special needs school and Ritalin treatment was initiated. On clinical examination at the age of 10 years, both weight and height were on the 25th centile and head circumference was on the 3rd centile. His facial appearance was somewhat peculiar as he had mild upslant of the palpebral fissures, a broad nose and flat philtrum. He had two sacral dimples and on abdomen and back several patches of depigmented skin were present as seen in vitiligo. Fragile X testing was negative.

Extensive neuropsychological testing was performed at the age of 14 years. The ADI-R (Autism Diagnostic Interview-Revised) from mother and half-sister showed scores above the cut-off for autism in the patient. Questioning on social interaction revealed that he was a loner and he did not have any friends. There was poor quality of social interaction and hardly any facial expression. Further assessment of his autism was obtained through an ADOS-G (Autism Diagnostic Observation Schedule). Scores on communication and social interaction were well above the cut-off for autism. The score regarding repetitive behavior and imagination was on the cut-off. Additional evaluation of language fundamentals by a speech therapist revealed poor expressive and receptive language equivalent to that of a 6-year-old.

The mother reported an unusual sleep disturbance. The boy wakes up in the middle of the night, several times a week, gets dressed and starts his daily activities.

Ethics statement

The study was approved by the Institutional Review Board (IRB) of the Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore, Pakistan, and the local Ethics Committee of the Radboud University Medical Centre, Nijmegen, The Netherlands.

Family report of PKMR14

A consanguineous Pakistani family PKMR14 was ascertained from the southern part of Punjab, Pakistan. This family has three affected individuals: females V:1, V:2 and male V:3 (Fig. 2A). At counseling, they were 25, 22 and 18 years of age, respectively. The birth of all three affected individuals was uneventful. At the age of 12, individual V:3 developed epilepsy with tonic clonic seizures of severe intensity. The two affected females V:1 and V:2 did not show epilepsy. All of the affected members faced sleep disturbances before 10 years of age. Individual V:3 was treated with sleeping pills for 5–7 years. Nowadays, all affected members sleep at normal times, but they regularly fall asleep during daytime. Unaffected individuals of the family did not show any sleeping disturbances. All patients are very aggressive, hyperactive and beat others when they are in anger. Grinding of teeth is common to them especially in aggressive outbursts. Breathing and heart beat patterns were found normal in the affected members. They presented with moderate ID (IQ level < 50), hypotonia and spasticity (Table 1). Clinical evaluation did not reveal any dysmorphic features in the patients of this family (Fig. 2B). Computed tomography (CT) scan of the two affected individuals (V:2,V:3) was performed at Multan MRI Center, Multan, Pakistan. The CT scan did not reveal any structural brain abnormalities.

Table 1. Clinical features of PKMR14 patients

Patient	V:1	V:2	V:3
Age	25 Years	22 Years	18 Years
Gender	F	F	M
ID level	Moderate (IQ < 50)	Moderate (IQ < 50)	Moderate (IQ < 50)
Speech delay	+	+	+
Hypotonia	+	+	+
Spasticity	+	+	+
Hyperactivity	+	+	+
Epilepsy	−	−	+
Aggressiveness	+	+	+
Sleeping disorder	+	+	+

Chromosomal analysis and molecular characterization

Metaphase spreads were prepared from lymphocyte cultures using standard procedures. FISH analysis was performed as described before (62). Chromosomes were flow sorted as described elsewhere (63). Sorted material was amplified using an REPLI-g mini kit following standard protocol (Qiagen, Hilden, Germany). PCR primers were designed using Primer3 (64), and checked for unfavorable dimerization with UNAfold (64,65). PCRs were performed using GOtaq polymerase (Promega, Madison, WI, USA) following standard protocol. Sequencing was performed as described subsequently.

Real-time PCR

mRNA expression was examined by an optimized three-step real-time quantitative PCR assay following the protocol described before (66,67). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH* [MIM 138400]), hypoxanthine guanine phosphoribosyltransferase 1 (*HPRT* [MIM 308000]) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta isoform (*YWHAZ* [MIM 601288]) were selected as reference genes. qPCR primers were designed using an in-house automated pipeline (http://medgen.ua.ac.be/~gvandeweyer/index.php?page=spanprimers), conforming to requirements of intron-spanning location, no SNP content, no dimer formation at the 3′ end of the primers and low amplicon folding, with no folding in primer-binding sites. Primer sequences are available on request.

Homozygosity mapping and CNV analysis

Homozygosity mapping in individuals IV:1, IV:2, V:1, V:3, V:4, V:5 and V6 of the PKMR14 family was performed by using the Illumina 6k SNP microarray platform. One of the affected individuals (V:2) was genotyped on a higher

resolution platform, an Affymetrix 250K SNP array, to investigate CNVs and also to confirm the homozygosity data. The resolution of Illumina 6K array is high enough to identify the large regions of homozygosity that are expected in consanguineous populations such as Pakistan. For CNV analysis, the Affymetrix 250K SNP array data were analyzed using Copy Number Analyzer for GeneChip (68). The Affymetrix Genotyping Console (version 2.0) and Illumina BeadStudio genotyping module software (Illumina, Inc., San Diego, CA, USA) were used to generate the genotype calls. The regions of homozygosity of at least 1 Mb were observed by visual inspection. CNV analysis for the translocation carrier was performed using the Illumina HumanCNV370-Duo BeadChip (Illumina Inc.,{location already mentioned}) according to standard protocol. The chip was scanned on the BeadStation and image data were normalized using BeadStudio (3.1.3.0) and Illumina's genotyping module (v3.3.4). Copy number analysis was performed using CNV-WebStore at a practical resolution of 75 kb (28). All data were mapped using Human Genome Build hg19.

Whole-exome sequencing

Exome sequencing of patient V:1 was carried out by capturing the exome using the SureSelect Human All Exon 50 Mb kit (Agilent, Santa Clara, CA, USA) followed by multiplexed analysis on a SOLiD 4 System sequencing slide (Life Technologies, Carlsbad, CA, USA). Color space reads were mapped to the hg19 reference genome with the SOLiD BioScope version 1.3 software, which utilizes an iterative mapping approach. A total of 67% of bases (3.75 Gb) came from the targeted exome, resulting in an average target coverage of 43-fold, and 73% of targeted exons were covered more than 10 times. Single-nucleotide variants were subsequently called by the Di-Bayes algorithm with high call stringency. Called variants were combined and annotated with a custom analysis pipeline (69,70). A total of 19 554 variants were identified. Of these, 9427 variants were located in the exonic sequence or located in canonical splice sites, defined as the dinucleotides up- and downstream of exonic sequence. For this exome, the transitions/transversions (Ti/Tv) ratio (genotype quality control parameter) of 2.64 was obtained. We then applied a filtering scheme excluding variants observed in dbSNP (v132) and in our in-house database, containing data on 177 exomes (consisting of healthy individuals and patients from other rare disorders). This filtering step reduced the number of variants to a total of 548, of which 381 were predicted to lead to non-synonymous changes and/or altered transcripts. Assuming a recessive pattern of inheritance, 41 homozygous (with $>70\%$ variant reads) and 7 compound heterozygous ($20-80\%$ variant reads) variants were retained. As a next filtering step based on the quality of the raw sequence reads, we applied a Binary Sequence Alignment/Map format (BAM file) inspection to verify the presence and the minimal percentage (20% for heterozygous and 70% for homozygous variants) of the variant reads. As the number of variant reads in the automatically computed output file often contradict with the actual number of variant reads present in BAM files. This filtering step resulted in 23 possible homozygous and 6 compound heterozygous variants (Supplementary Material, Table S1).

Sanger sequencing in PKMR14

Sanger sequencing was used to confirm the candidate variant identified on chromosome 10 at position 61829644 (hg19). Primers for the amplification of part of the DNA encompassing the candidate variant were designed by using the Primer3 program (64). A 454 bp fragment carrying the candidate mutation was amplified by using primers 5′-TAACCGAGGGG ATGATGAAG-3′ (forward) and 5′-ACTGGATGTGCCT TCCTGAC-3′ (reverse). PCR amplification was carried out on 50 ng of genomic DNA with Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). A Nucleofast 96 PCR plate (Clontech Lab, Mountain View, CA, USA) was used to purify the PCR amplicons, according to the manufacturer's protocol. Sequence analysis was performed with the ABI PRISM Big Dye Terminator Cycle Sequencing V3.1 ready Reaction Kit and the ABI PRISM 3730 DNA Analyzer (Applera Corp, Foster City, CA, USA).

Drosophila stocks and breeding

Flies were raised on standard medium (cornmeal/sugar/yeast) at 25°C for the courtship conditioning paradigm, and at 28°C for evaluation of synapse morphology and activity monitoring. They were kept at 60% humidity on a 12 h light:dark cycle. RNA interference was induced in a tissue-specific manner with the UAS-GAL4 system (71). The *Ank2* RNAi line and its genetic background control were obtained from the Vienna *Drosophila* Research Center (vdrc stocks 107 238 and 60 100, respectively). The pan-neuronal driver *w; UAS-dicer2; elav-Gal4* was assembled in-house using *w; UAS-dicer2* (vdrc stock 60008) and *w; elav-Gal4* stocks (Bloomington *Drosophila* stock center, Indiana University, stock 8760). The mushroom body specific driver w^{+}*,UAS-dicer;247-Gal4* was a gift from K. Keleman (IMP Vienna).

Drosophila synapse morphology

Type 1b neuromuscular junctions (NMJs) at muscle 4 of *w/Y; UAS-dicer2/UAS-Ank2 RNAi; elav-Gal4/+* (UAS-Ank2 RNAi) and *w/Y; UAS-dicer2; elav-Gal4/+* (control) male wandering L3 larvae were analyzed after dissection, a 30 min fixation in 3.7% PFA and immunolabeling with an anti-Discs large 1 antibody (supernatant, 1:25) (Developmental Studies Hybridoma Bank, University of Iowa). NMJ pictures were obtained using a Leica automated brightfield multi-color epifluorescent microscope. Images were automatically processed and measured by an advanced self-made ImageJ/Fiji macro.

Drosophila learning and memory

Courtship conditioning (72) was performed as previously described (73) using males of the genotypes w^{+}*,UAS-dicer/Y;247-Gal4/UAS-Ank2 RNAi;+/+* (UAS-Ank2 RNAi) and w^{+}*,UAS-dicer/Y;247-Gal4/+;+/+* (control). Males were collected at eclosion and kept in isolation until 4 days of age. They were then trained by pairing with a single premated female for 5 h. After training, males were transferred to a 1 cm diameter chamber and filmed for 10 min in the presence of a new premated female, either immediately after training

(learning) or after 1 h (short-term memory). Courtship behavior of male flies toward females was quantified using Actual FlyTrack software (Actual Analytics Ltd). The mean Courtship Index (CI, the percentage of time spent on courtship during a 10 min interval) of trained males and of socially naïve males was used to calculate the Learning Index (LI), which is defined as the percent reduction in mean courtship activity in trained males compared with naïve males; $LI = (CI_{naive} - CI_{trained})/CI_{naive}$. For all conditions, we analyzed between 66 and 137 male–female pairs over the course of 7 days. For non-parametric statistical comparison of learning indexes between *Ank2* knockdown and control flies, we used a custom SAS (SAS Institute, Inc.) script to perform bootstrapping as described (74). Briefly, CI values were randomly sampled with replacement to generate 10 000 hypothetical LIs, which were used to determine the 95% confidence interval of the difference between LI(control) and LI(knockdown). This analysis revealed a significant difference between LI(control) and LI(knockdown) ($P < 0.05$).

Drosophila activity monitoring

Locomotor activity of individual 3- to 5-day-old male flies was recorded with the *Drosophila* Activity Monitor (DAM) system (Trikinetics, Waltham, MA, USA). Activity of $\geq$21 flies per genotype was recorded over 4 days on a 12 h light:dark cycle. The 30 min bin activity measurements for individual flies were averaged into 1 day, after which the average activity of a genotype was calculated with 95% confidence intervals. Genotypes were as indicated in synapse morphology and learning and memory sections discussed earlier.

WEB RESOURCES

The URLs for data presented herein are as follows:

1000 Genomes project, http://www.1000genomes.org/

IGV browser, http://www.broadinstitute.org/igv

Online Mendelian Inheritance in Man, http://www.omim.org/

UCSC Genome Browser, http://genome.ucsc.edu/

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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