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RESEARCH ARTICLE

Recurrent deletions of *ULK4* in schizophrenia: a gene crucial for neuritogenesis and neuronal motility

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ABSTRACT

Although many pathogenic copy number variations (CNVs) are associated with neuropsychiatric diseases, few of them have been functionally characterised. Here we report multiple schizophrenia cases with CNV abnormalities specific to unc-51-like kinase 4 (*ULK4*), a serine/threonine kinase gene. Deletions spanning exons 21–34 of *ULK4* were present in 4 out of 3391 schizophrenia patients from the International Schizophrenia Consortium, but absent in 3181 controls. Deletions removing exons 33 and 34 of the large splice variant of *ULK4* also were enriched in Icelandic schizophrenia and bipolar patients compared with 98,022 controls ($P=0.0007$ for schizophrenia plus bipolar disorder). Combining the two cohorts gives a *P*-value less than 0.0001 for schizophrenia, or for schizophrenia plus bipolar disorder. The expression of ULK4 is neuron-specific and developmentally regulated. ULK4 modulates multiple signalling pathways that include ERK, p38, PKC and JNK, which are involved in stress responses and implicated in schizophrenia. Knockdown of *ULK4* disrupts the composition of microtubules and compromises neuritogenesis and cell motility. Targeted *Ulk4* deletion causes corpus callosum agenesis in mice. Our findings indicate that *ULK4* is a rare susceptibility gene for schizophrenia.

KEY WORDS: Neurite branching, Schizophrenia, serine/threonine kinase, ULK4

INTRODUCTION

Schizophrenia is a severe psychiatric disorder affecting about 1% of the world population. Although heritability is estimated between 60 and 80%, its genetic architecture remains elusive. A few rare, but highly penetrant, copy number variants (CNVs) account for ~2–3% of cases (International Schizophrenia Consortium, 2008; Stefansson et al., 2008; Walsh et al., 2008). However, very few specific susceptibility genes for schizophrenia, for example Neurexin1, have been implicated definitively by CNV analysis. In most cases CNVs are either too rare to obtain statistical evidence for association or, when recurrent, disrupt multiple genes. Association signals from common genetic variants in genome-wide association studies (GWAS) also are inconsistent, with only a small number showing genome-wide significant associations (Stefansson et al., 2008; O'Donovan et al., 2008; International Schizophrenia Consortium, 2009; Shi et al., 2011; Yue et al., 2011). There is now overwhelming evidence of abnormalities of neurodevelopment as well as of adult brain function in schizophrenia. A high proportion of CNVs associated with schizophrenia are involved in neurodevelopment in early life and neuronal integrity, connectivity, signalling and synaptic plasticity in the adult (Kirov et al., 2012). Clinical neuro-imaging studies have demonstrated disturbed neuronal connectivity in schizophrenia. Human post-mortem studies also revealed shorter and less branched dendrites (Black et al., 2004; Guidotti et al., 2000) and lower density of dendritic spines (Glantz and Lewis, 2000) in schizophrenic brains. Numerous alterations in nerve-terminal functions involving synaptic vesicle recycling, transmitter release and cytoskeletal dynamics are found in anterior prefrontal cortex and anterior cingulate cortex grey matter in schizophrenia.

Intriguingly, during a re-analysis of the CNV data from the International Schizophrenia Consortium (ISC) (International Schizophrenia Consortium, 2008), we discovered that unc-51-like kinase 4 (*ULK4*) was deleted in four schizophrenia patients but not in controls. We also found similar enrichment for *ULK4* in schizophrenia and bipolar Icelandic cases genotyped by deCODE Genetics, Iceland. ULK4 is one of five members of the unc-51-like serine/threonine kinase (STK) family that participates in a conserved pathway involving both endocytosis and axon growth (Ogura et al., 1994; Pelkmans et al., 2005; Tomoda et al., 2004). In *C. elegans*, a mutation in the *unc-51* gene results in stalled axon outgrowth, increased axon numbers (short and stunted) and abnormal accumulation of intracellular membranous structures (Ogura et al., 1994). In *Drosophila*, *Unc-51*-mediated membrane vesicle transport is pivotal for axonal and dendritic development. It aids targeted localization of guidance molecules and organelles that regulate the elongation and the compartmentalization of developing neurons and motor-cargo assembly (Mochizuki et al., 2011).

Here, we not only show that deletions of *ULK4* are associated with schizophrenia, but also demonstrate the importance of ULK4 in the regulation of neurite elongation, neurite branching and neuronal migration. We further show that ULK4 modulates the neuronal cytoskeleton and regulates multiple signalling pathways involving JNK, ERK and protein kinase C (PKC), all of which are well known for their roles in neurite elaboration and cell

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migration. In addition, *Ulk4*$^{-/-}$ newborn mice displayed defects in corpus callosum nerve fibres connecting the two hemispheres of the brain. Our findings demonstrate that ULK4 is crucial to brain development and a previously unidentified genetic risk factor in schizophrenia.

RESULTS

CNV analysis of serine/threonine kinases

Previously the ISC genotyped CNVs in 3391 schizophrenic patients and 3181 ancestrally matched controls. The CNV data were stratified against population on the basis of the genome-wide single nucleotide polymorphism (SNP) data, and there was no evidence of major population stratification within each site (International Schizophrenia Consortium, 2008). In this study, we re-analysed CNVs of the ISC datasets against a total of 420 genes with key words of 'serine/threonine kinase (*STK*)' on the UCSC (University of California Santa Cruz) database (hg18), and identified 124 patient CNVs (3.66%) and 75 control CNVs (2.36%) carrying *STK* genes (Table 1; $P=0.0014$, one-tailed Chi-squared test with Yates correction), showing ~1.6 times enrichment of *STK* CNVs in the schizophrenic population. A summary of the *STK* CNVs is shown in supplementary material Tables S1, S2.

Among the 124 *STK* CNVs in schizophrenia, *TSSK2* (11 cases/1 control, $P=0.0064$), *STK38L* (6 deletions in patients/1 duplication in control, $P=0.0765$) and *LRRK1* (3 cases/1 control, $P=0.3313$) were enriched in the disease. Five of the six *STK38L* CNVs at 12p11.23 involved two other genes (*ARNTL2* and *PPFIBP1*) and 12p11.23 CNVs were also associated with 1/708 Icelandic schizophrenia patients and 7/98,022 controls (supplementary material Fig. S1). The *PAK7* (6 cases, $P=0.0247$), *SGK196* (6 cases, $P=0.0247$), *ULK4* (4 cases, $P=0.0753$), *MKNK1* (3 cases, $P=0.1356$) and *MAST4* (3 cases, $P=0.1356$) CNVs were unique in the patient group. The *MKNK1*, *SGK196* and *TSSK2* genes were of less priority, as they were embedded in CNVs with many flanking genes. The *PAK7* and *MAST4* CNVs were involved in partial duplications. They were not chosen in this study because their mode of disease mechanism was uncertain, although loss of function was more likely.

We have focused on 3p22.1 deletions in this study because: (1) the 3p22.1 deletions are restricted to a single *STK* gene *ULK4* (supplementary material Table S1); (2) *ULK4* encodes a previously unknown kinase; and (3) all four patients are in Scotland. The four patients (three from Aberdeen and one in Edinburgh) did not share any other CNVs across the genome. The *ULK4* gene is located at chromosome 3p22.1 and the longest isoform with 1275 amino acids is encoded by 36 exons. The deletions in the four schizophrenic patients were intragenic, all spanning from intron 20 to 34 (Fig. 1A), which truncated the *ULK4* coding sequence in the 3′-half of the gene. The Aberdeen schizophrenia cohort has been independently genotyped by deCODE, which verified three *ULK4* CNVs in the same genomic regions by different platforms (supplementary material Fig. S2).

In the deCODE dataset, we identified additional *ULK4* deletions, which removed exons 33 and 34 of the large splice variant (supplementary material Fig. S2). The deletions are present in 37 of 98,022 Icelandic controls, but significantly enriched in patients, with two in 708 Icelandic schizophrenia cases (odds ratio: OR=7.5, $P=0.01$), two in 1136 bipolar patients (OR=4.7, $P=0.05$) and one in 507 autism cases (OR=7.5, $P=0.2425$). Combined ISC and Icelandic datasets give a *P*-value less than 0.0001 for schizophrenia (OR=8.8) or for schizophrenia plus bipolar (OR=7.2).

ULK4 is an *unc-51*-like STK with five family members, ULK1–4 and STK36, in mammals. One additional genomic duplication involving the 5′-half of the *ULK2* gene was present in the patient group (Fig. 1C and supplementary material Table S1), and partial duplications of 3′-half of *ULK1* gene were found in two patients and one control of the ISC dataset (Fig. 1B and supplementary material Tables S1 and S2). One-tailed Chi-squared test with Yates' correction for seven *ULK* CNVs in 3391 patients and one CNV in 3,181 controls gives a *P*-value of 0.047, suggesting that the *ULK* gene family is associated with schizophrenia (Table 2).

The recurrent deletions of *ULK4* in schizophrenia and bipolar disorder patients highlight the potential importance of this kinase in psychosis. We therefore reviewed the recently published data by the Psychiatric GWAS Consortium (Psychiatric GWAS Consortium Bipolar Disorder Working Group, 2011; Major Depressive Disorder Working Group of the Psychiatric GWAS Consortium et al., 2012), and found no association with schizophrenia in this database. Association study of 3750 schizophrenia cases and 6468 controls of the Chinese Han population suggested a potential association with SNPs rs6599155 ($P=0.064$) and rs17062109 ($P=0.057$) in the *ULK4* gene. In addition, SNP analysis of the bipolar GWAS in the Psychiatric Genetics Consortium dataset showed a *P*-value of 0.0001 for rs17210774 in bipolar disorder, and a *P*-value of 0.001 for rs1722850 flanking the *ULK4* gene in major depression disorders. Collectively, these genetic data suggested strongly that *ULK4* is a rare, but high susceptibility risk factor for a range of psychiatric diseases including schizophrenia.

Postnatal switch of Ulk4 isoform

Multiple ULK4 splice variants, with similar N-termini but differing at the C-terminus, are predicted from mouse and human genomic sequences. To establish the biology of ULK4 function, we first examined ULK4 expression and regulation with an anti-ULK4 antibody. Western blotting revealed abundant expression of the ~105 kDa (Fig. 2A, arrowhead) and ~95 kDa (Fig. 2A, star) Ulk4 proteins in embryonic (E15.5 and E17.5) and early postnatal (P0 and P3) mouse brains. However, adult mouse brain expressed predominantly the large Ulk4 isoform of ~130 kDa (Fig. 2A, arrow), and it was the large isoform that was affected by genomic deletions in schizophrenic patients. These data suggest a postnatal switch of Ulk4 expression from the

Table 1. CNVs of *STK* genes are significantly enriched in schizophrenia patients recruited in a GWAS study by ISC

	STK gene deletion	*STK* gene duplication	Total *STK* CNV
Control ($n=3181$)	17	58	75 (2.36%)
Schizophrenia ($n=3391$)	34	90	124 (3.66%)
One-tailed chi-squared with Yates' correction, *P*			$\chi^2=8.995$ $P=0.0014$

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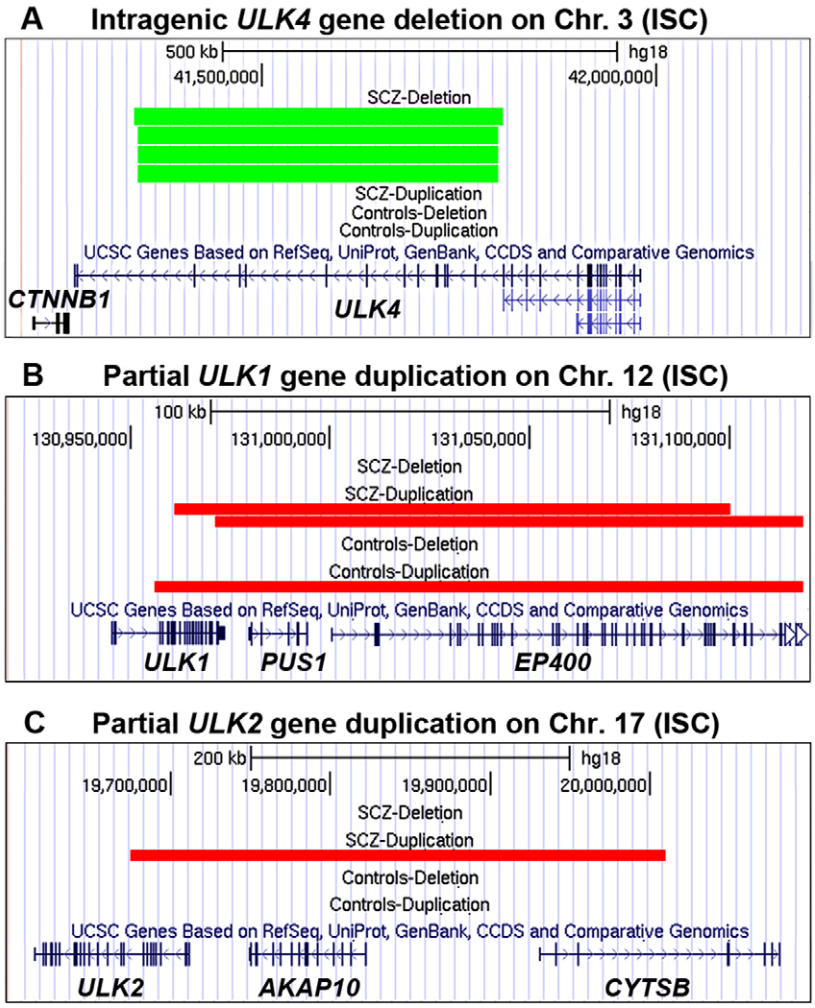

Fig. 1. Genetic association of *ULK4* gene family members with schizophrenia in the ISC dataset. (A). Schematic representation of an 800 kb chromosomal region, 3p22.1, which encodes *ULK4* and flanking *CTNNB1* genes. Four schizophrenia patients were identified to carry intragenic deletions spanning exons 20–34 of the *ULK4* gene. (B) Partial duplication of the *ULK1* gene was found in two patients and one control. (C) Partial duplication of the *ULK2* gene was found in one schizophrenia patient but not in controls.

small isoforms to the large isoform, and the requirement of the large isoform for postnatal brain function.

To investigate signalling pathways influencing ULK4 expression during neuronal differentiation, human neuroblastoma SH-SY5Y cells were treated with DMSO, 9-cis RA, all-trans retinoic acid (ATRA), dibutyryl cAMP (db cAMP), pituitary adenylate cyclase-activating peptide (PACAP) or nerve growth factor (NGF) for 2 days (Fig. 2C,D). The most abundant ULK4 species in SH-SY5Y cells was the ~95 kDa one, with weak expression of other isoforms of ~105 or ~130 kDa (Fig. 2C). With the existing reagents it was not possible to establish that all bands were ULK4-specific isoforms, but the ~95 kDa protein (star, Fig. 2C) was upregulated substantially by 9-cis RA and ATRA. This induction was time dependent and a significant increase was detected from 1 day onwards for ATRA ($P<0.05$), but not earlier (Fig. 2E,H), suggesting that *ULK4* could be a secondary RA-responsive gene, and the ~95 kDa isoform could be required during RA-mediated neuronal differentiation.

ULK4 is highly expressed in cortex and hippocampus

We next investigated *in vivo* neuroanatomical expression of Ulk4 by performing immunohistochemistry with antibody against Ulk4. We showed ubiquitous expression of Ulk4 in mouse brain, with high intensity in layers II/III and V of the cerebral

Table 2. CNVs of *ULK4* family members are significantly enriched (OR=6.77) in schizophrenia patients recruited in a GWAS study by ISC

	ULK1	*ULK2*	*ULK4*	Total *ULK* CNV
Control (n=3181)	1	0	0	1
Schizophrenia (n =3391)	2	1	4	7
One-tailed chi-squared with Yates' correction	0.3007	0.1664	0.0753	0.0466
One-tailed Fisher's exact test, P	0.5240	0.5160	0.0708	0.0426

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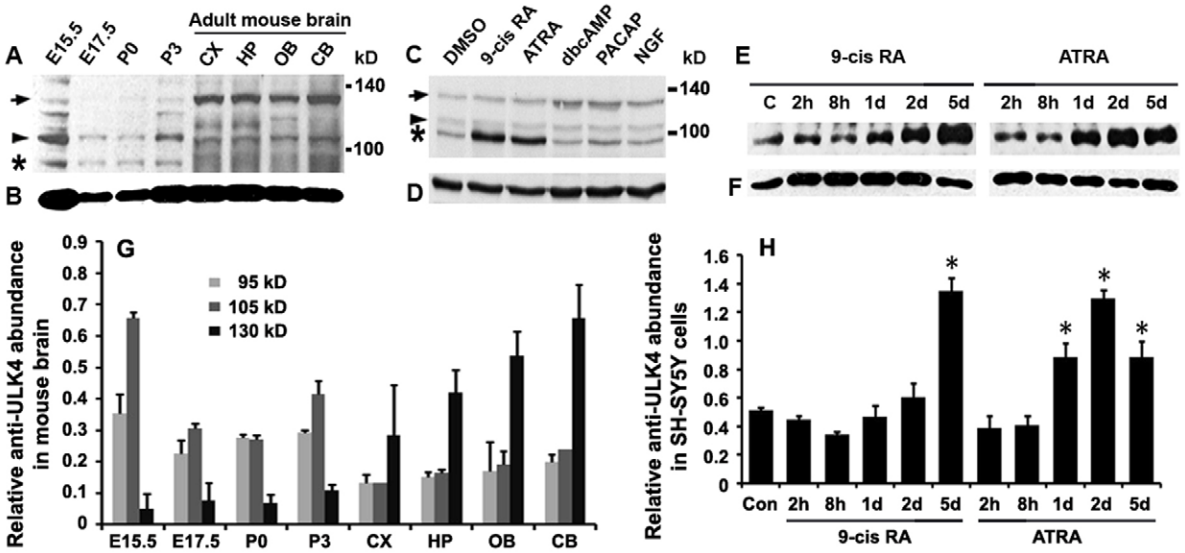

Fig. 2. Expression of ULK4 protein during brain development and neuronal differentiation. (A,B) Proteins were extracted from E15.5, E17.5, P0, P3 whole brain, and cortex (CX), hippocampus (HP), olfactory bulb (OB) and cerebellum (CB) of adult mouse brain, and immunoblotted with anti-ULK4 (A) or anti-α-tubulin (B), showing a switch of Ulk4 isoform expression from ~105 (arrowhead) and ~95 kDa (star) in embryos and early postnatal brain to ~130 (arrow) in adult brain. (C–F) Regulated expression of ULK4 during neuronal differentiation. SH-SY5Y cells (C,D) were treated with DMSO, 9-cis RA, all-trans retinoic acid (ATRA), dibutyryl cAMP (db cAMP), pituitary adenylate cyclase-activating peptide (PACAP) or nerve growth factor (NGF) for 2 days, and blotted with anti-ULK4 (C) or anti-α-tubulin (D). Note a substantial induction of ULK4 expression by 9-cis RA and ATRA RA in SH-SY5Y cells. (E,F) SH-SY5Y protein extracts from cells without (C, control) or with 9-cis RA or ATRA for 2 hours, 8 hours, 1 day, 2 days or 5 days, and blotted with anti-ULK4 (E) or anti-α-tubulin (F), showing time-dependent ULK4 upregulation by 9-cis RA and ATRA. (G) Relative expression of various Ulk4-reactive species in mouse brain. (H) Quantification of ULK4 relative to housekeeping protein. Values are means ± s.e.m.; *P<0.05.

cortex (Fig. 3A), piriform cortex (Fig. 3B), CA1-3 of hippocampus (Fig. 3D,G), dentate gyrus (Fig. 3H), ependymal cells lining the ventricles and choroid plexus (Fig. 3I), and in the thalamic reticular nucleus (Fig. 4G,H). There was no colocalization of Ulk4 with GFAP or 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase), specific markers for astrocytes (Fig. 4C) and oligodendrocytes (Fig. 4D), respectively. Double-labelling with anti-Ulk4 and anti-Sox10, a transcription factor specifically expressed in oligodendrocytes, further confirmed that Ulk4 was not present in Sox10-positive cells in the corpus callosum (Fig. 4M) or cortex (Fig. 4N). However, Ulk4 was co-expressed with NeuN (Fig. 4A,B) and GAD67 (Fig. 4E–H). Similar expression patterns were detected in post-mortem human brain sections (Fig. 4I–L). ULK4 expression was detected in human MAP2-positive (Fig. 4K) and GAD67-positive neurons (Fig. 4L), but not on GFAP-positive astrocytes of human cortex (data not shown) and hippocampus (Fig. 4I,J). The anti-ULK4 staining was specific, and expression of Ulk4 was almost undetected in Ki67-positive (red, supplementary material Fig. S3A) and BrdU-positive adult neural stem cells in the subventricular zone, or adjacent to the ependymal cells (green, supplementary material Fig. S3B). These data demonstrate that in adult brain anti-ULK4 immunoreactivity is mainly located in postmitotic neurons, including GABAergic neurons, which are commonly defective in schizophrenia.

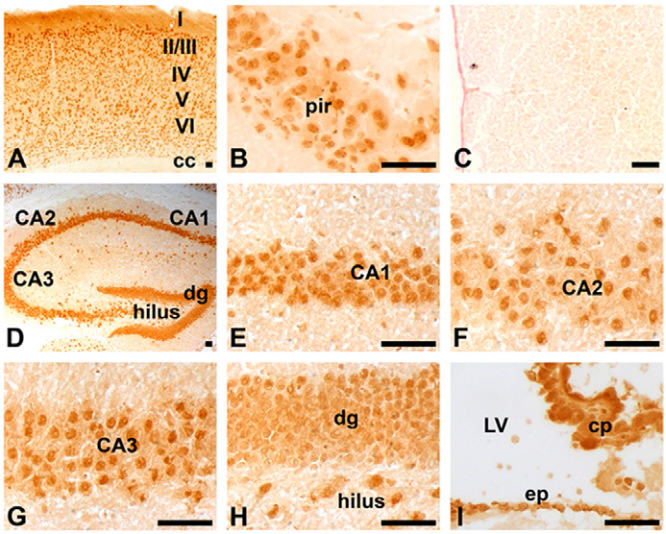

Fig. 3. Expression of Ulk4 in the mouse brain. (A–I) Brightfield staining of mouse brain sections with anti-Ulk4 antibody showing Ulk4 expression (in brown) in the cerebral cortex (A), piriform cortex (B), hippocampus (D–H), choroid plexus and ventricular ependymal cells (I), with a negative control in the absence of the primary antibody (C). I–VI, sublayers of cerebral cortex; CA1–3, subfields of the hippocampus; cc, corpus callosum; cp, choroid plexus; dg, dentate gyrus; ep, ependymal layer; LV, lateral ventricle; pir, piriform cortex. Scale bars: 50 μm.

ULK4 knockdown compromises neuritogenesis and impairs neurite branching

Depletion of ULK4 in SH-SY5Y cells by shRNA-mediated gene knockdown resulted in a significant reduction in ULK4 expression compared with control cells (P=0.005, n=3; Fig. 5H, top lane; Fig. 7; and supplementary material Fig. S5) Comparison of ULK4 knockdown and control cells revealed a substantial difference in neuritogenesis (Fig. 5A,G). On the first day of subculture, the control cells had much longer neurites (23.4±1.0 μm, n=115) than the *ULK4* knockdown cells (14.6±0.6 μm, n=118, P<0.01; Fig. 5A,D,G). On day 5 (Fig. 5C,F), the neurites of control cells had more than doubled

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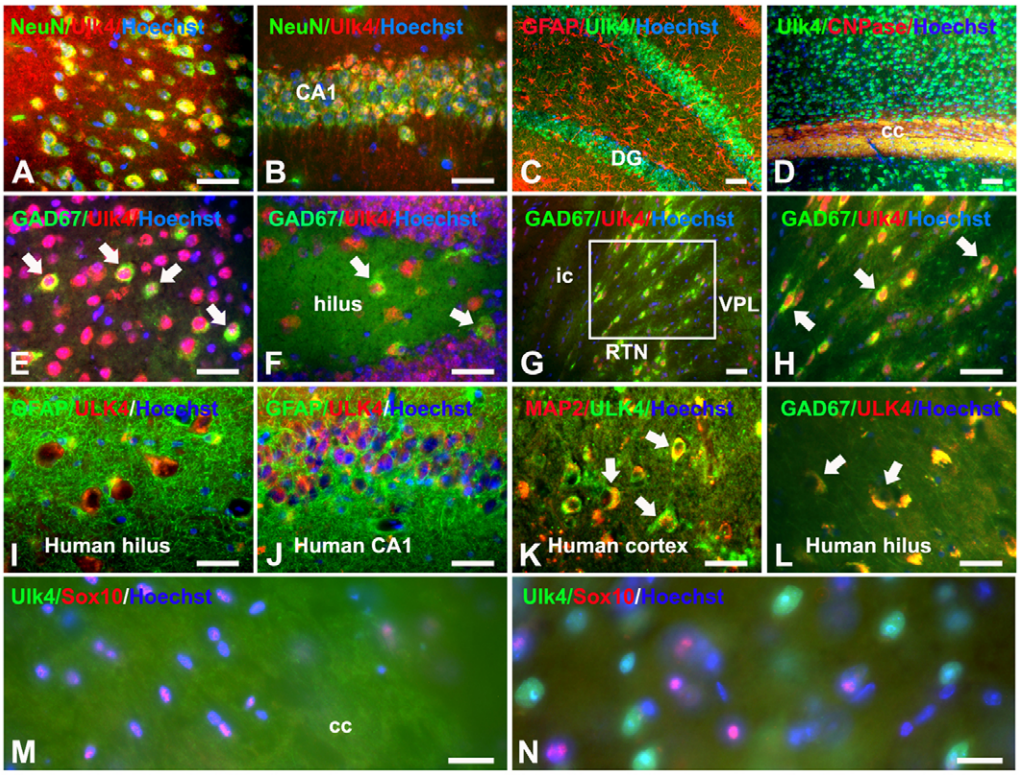

Fig. 4. Double immunofluorescent staining showing neuronal-specific ULK4 expression in mouse (A-H,M,N) and human (I–L) brain. Double immunofluorescent staining was carried out with antibodies against ULK4 and, NeuN (A,B), GFAP (C,I,J), CNPase (D), GAD67 (E–H,L) and MAP2 (K). Ulk4 was co-expressed with NeuN in mouse cortex (A) and mouse hippocampus (B), and also with MAP2 in human cortical neurons (K). No expression of Ulk4 was detected in GFAP-positive astrocytes in mouse hippocampus (C), human hippocampus (I,J) or CNPase-expressing mouse oligodendrocytes (D). However, ULK4 was colocalised with GAD67, a specific marker for GABAergic interneurons, in mouse cortex (E), mouse hippocampus (F), mouse reticular thalamic nucleus (G,H) and human brain (L). H is a higher magnification of boxed area in G, showing the double labelling of Ulk4 and GAD67 in mouse reticular thalamic nucleus. (M,N) Double immunostaining of corpus callosum (M) and layer V of the cortex (N) with anti-ULK4 (green), and anti-Sox10 (red), a specific marker for oligodendrocytes, showing no Ulk4 expression in oligodendrocytes. Arrows in E, F, H, K and L indicate typical double-labelled neurons. cc, corpus callosum; DG, dentate gyrus; ic, internal capsule; RTN, reticular thalamic nucleus; VPL, ventral posterolateral thalamic nucleus. Scale bars: 50 μm (A–L); 20 μm (M,N).

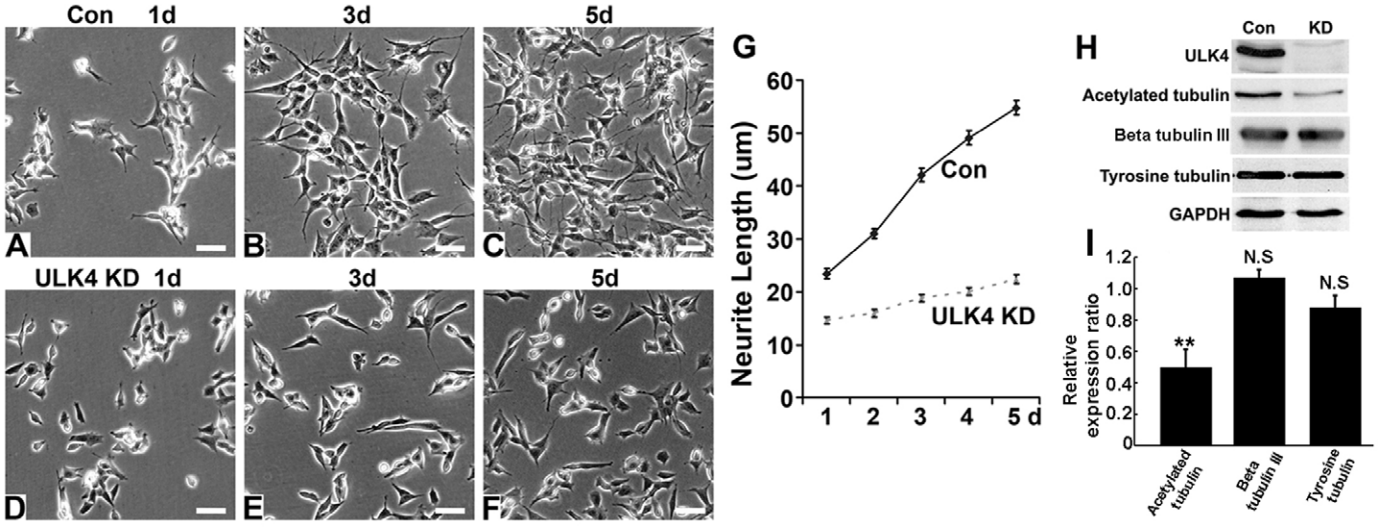

Fig. 5. *ULK4* knockdown compromises neurite elongation and branching. (A–F) Images of puromycin-resistant control (Con, A–C) or *ULK4* knockdown (KD; D–F) cells after subculture. (G) Quantification of neurite length in control (Con) and *ULK4* KD cells. Values are means ± s.e.m.; $P<0.01$; for control cells $n=115$ (day 1); 170 (day 2); 171 (day 3); 152 (day 4); 153 (day 5); for *ULK4* KD $n=118$ (day 1); 130 (day 2); 153 (day 3); 124 (day 4); 125 (day 5). (H,I) Protein extracts from control (Con) and *ULK4* KD cells were immunoblotted with the indicated antibodies. *ULK4* KD cells showed reduced expression of acetylated α-tubulin and un-altered expression of tyrosinated tubulin and β-tubulin III. $^{**}P<0.01$, $n=3$. N.S., not significant. Scale bars: 50 μm (A–F).

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in length (54.7±1.4 μm, n=153), whereas the knockdown cells showed reduced neurite extension (22.4±0.8 μm, n=125, P<0.01; Fig. 5G). F-actin staining strikingly revealed that the knockdown cells only had short, stout and rigid-looking neurite protrusions with considerably fewer secondary or higher order neurite branches (Fig. 6A,B,E,F), suggesting that ULK4 plays a crucial role in neuritogenesis.

The deficit in neurite formation may result from abnormal assembly of the cytoskeleton. Microtubules consist of α- and β-tubulin subunits, and acetylation of α-tubulin helps to stabilize them. We detected significantly less acetylated α-tubulin (P=0.009, n=3) in the knockdown cells (Fig. 5H,I; Fig. 6I,J; supplementary material Fig. S4A,B), whereas β-tubulin III expression was not altered (P=0.09, n=3; Fig. 5H,I; Fig. 6C,G; supplementary material Fig. S4C,D). Tubulin tyrosinylation is also involved in the assembly of microtubules and contributes to active neurite formation. Although there was no significant change in the quantity of tyrosinated tubulin (P=0.06, n=3; Fig. 5H,I), more diffuse distribution of tyrosinated tubulin was observed in the *ULK4* knockdown cells (Fig. 6D,H and supplementary material Fig. S4E,F). These data indicate that ULK4 is involved in remodelling of cytoskeletal components and in this way regulates neurite branching and elongation.

ULK4 knockdown reduces cell motility

Abnormal neuronal migration and faulty cell positioning could lead to disorganized brain development and hence a range of brain malfunctions including schizophrenia and autism. To examine the role of ULK4 in cell motility, we imaged the random motility of the *ULK4* knockdown cells and control SH-SY5Y cells and analysed their migration competence (Fig. 6K) (Lang et al., 2006). We found that control cells were constantly changing direction and moving with a mean rate of 48.1±1.8 μm/hour (T_t/T; see Materials and Methods) and 25.7±1.4 μm/hour (T_d/T; n=114 cells). However, the migration rate in *ULK4* knockdown cells was reduced significantly by 31% and 34%, respectively (T_t/T, 33.4±1.1 μm/h, T_d/T, 16.4±0.9 μm/h; n=140 cells, P<0.001, see supplementary material Movie 1). Therefore, ULK4 silencing significantly reduces cell motility *in vitro*.

ULK4 modulates ERK, p38 MAPK, PKC and JNK pathways

Nothing is known about ULK4 upstream signalling pathways or downstream substrates. This prompted us to examine major signalling pathways implicated in schizophrenia and neuronal function. Growth factors and cellular stress activate the MAPK family of signalling intermediates, known to play important roles in neuronal development. In *ULK4* knockdown cells, we observed a significant reduction in the phosphorylation of ERK1/2 (P=0.001, n=3) and p38 MAPKs (P=0.003, n=3). Upregulated activity of the stress-activated c-Jun N-terminal kinases (JNKs) is highly involved in neuritogenesis, including axon formation, polarization, extension, synaptic plasticity, and dendrite development during brain development or under a stress challenge (Coffey et al., 2000; Rosso et al., 2005; Oliva et al., 2006; Barnat et al., 2010; Qu, et al., 2013; Sun et al., 2013). In line with these reports, we observed consistently decreased expression of phosphorylated JNK in *ULK4* knockdown cells (P=0.008, n=3; Fig. 7). ELK1 is a major substrate of ERK/p38, and phosphorylated ELK1 is required for target gene activation. Consistently, we detected decreased phosphorylation of ELK1 in knockdown cells (P<0.001, n=3; Fig. 7). G-protein-coupled receptors often activate PKA and PKC. PKC mediates stress-related dendritic remodelling of cortical neurons and is implicated in bipolar and schizophrenic conditions. Strikingly, we found significantly increased phosphorylation of PKC in knockdown cells (P=0.02, n=3). *ULK4* knockdown, however, did not affect PKA, with no alteration of phosphorylated CREB (P=0.26, n=3; Fig. 7). These results indicate that multiple signalling pathways were affected by *ULK4* knockdown.

Defects of corpus callosum in *Ulk4*$^{-/-}$ mice

Although the non-specific shRNA had been widely used as control for knockdown experiments, it has its limitations. To examine the functional consequences of Ulk4 deficiency *in vivo*, we compared the neuroanatomy of the newborn littermates from *Ulk4*$^{+/-}$×*Ulk4*$^{+/-}$ mating. The brain sections were stained immunohistochemically with the neuronal marker Tuj1 (Fig. 8).

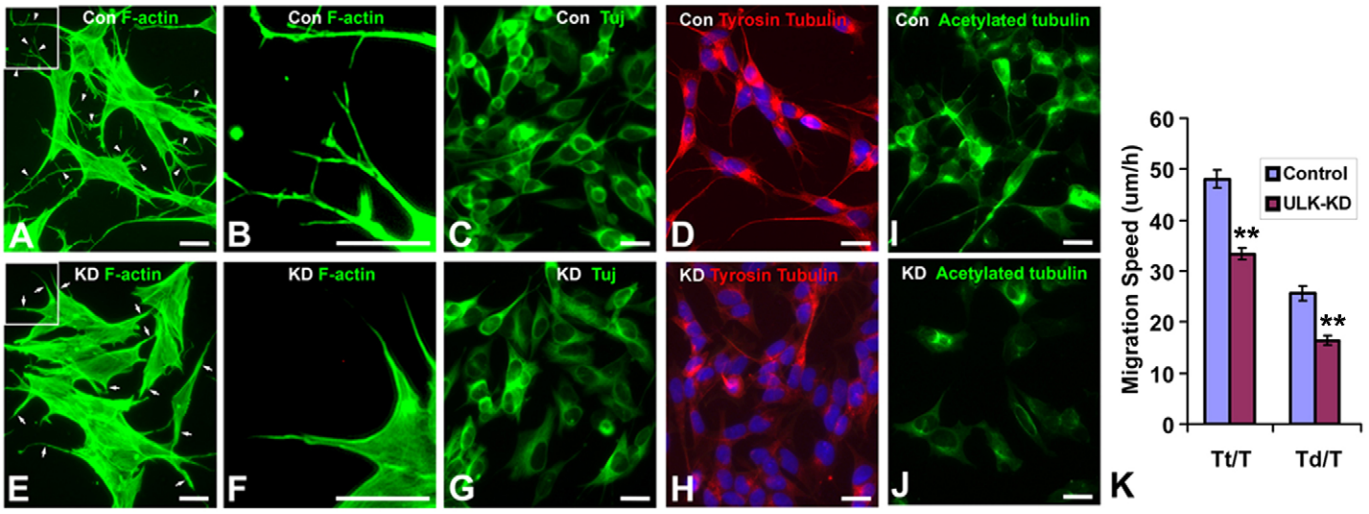

Fig. 6. *ULK4* knockdown impairs neuritogenesis and neuronal motility. (A–J) Puromycin-resistant control (Con) and *ULK4* KD cells were cultured for 2 days and stained for F-actin (A,B,E,F), β-tubulin III (Tuj, C,G), tyrosinated α-tubulin (D,H) and acetylated α-tubulin (I,J). B and F are magnified views of the boxed regions in A and E, respectively, showing defective neurite branching in KD cells (F). Arrowheads in A and arrows in E indicate the neurites of control and knockdown cells respectively. (K) Reduced migratory rates of ULK4 KD cells compared with puromycin-resistant control, indicating that ULK4 modulates random cell motility. Scale bars: 50 μm (A,C–E,G–J); 10 μm (B,F). **P<0.01.

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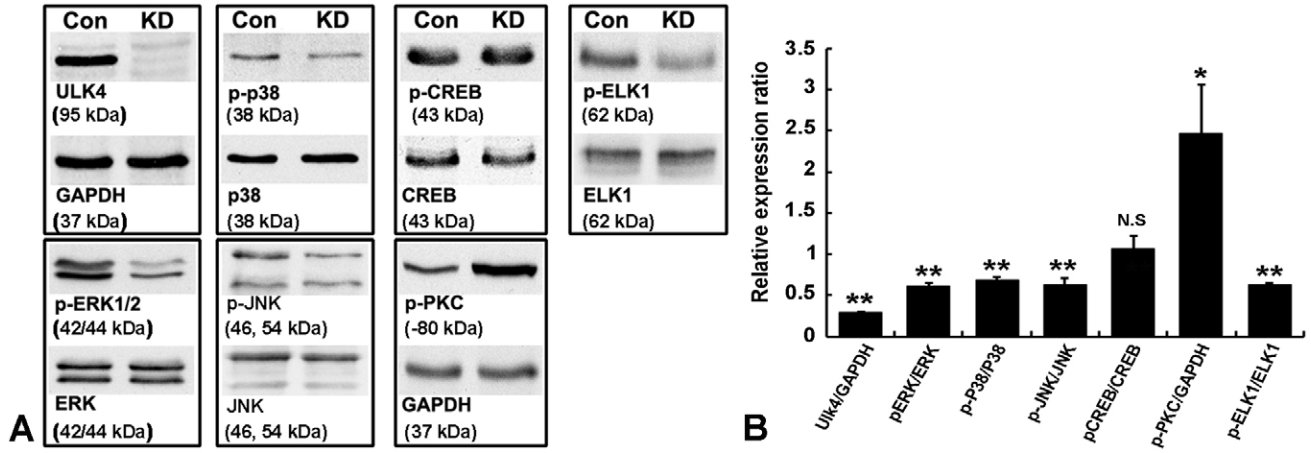

Fig. 7. ULK4 modulates multiple signalling pathways. (A) Equal amounts of protein from control (Con) and *ULK4* KD cells were blotted with the antibodies specified. ULK4 KD cells showed effective knockdown, increased expression of phosphorylated (p-) PKC and reduced levels of p-ERK1/2, p-p38, p-ELK1 and p-JNK. The expression of p-CREB was not altered. (B) Protein expression in KD cells was normalised to controls. Data are averages of three independent experiments ± s.d. *$P<0.05$, **$P<0.01$, $n=3$. N.S., not significant.

Ulk4$^{-/-}$ mice were found to have partial agenesis of the corpus callosum. The corpus callosum consists of nerve fibres projecting from cortical neurons to communicate between the two hemispheres. At post-natal day 0, high magnification views of the brain sections revealed a dense layer of nerve fibres crossing the midline in the wild-type newborn mice (Fig. 8A,C,E), whereas in the *Ulk4*$^{-/-}$ brain, the corpus callosum had many areas lacking nerve fibres (Fig. 8B,D,F). Consistent with neuritogenesis defects in knockdown neuroblastoma cells, the apparent agenesis of corpus callosum in *Ulk4*$^{-/-}$ mice might result, in part, from short cortical projections and reduced branching during mouse brain development.

DISCUSSION

We have identified by recurrent CNVs that the *ULK4* gene is associated significantly with schizophrenia and other major psychiatric disorders in two large independent cohorts (ISC and deCODE). Although concerns may be raised that different datasets analysed platforms of different resolution, we find this does not appear to affect the *ULK4* gene in this study. For example, three of the four *ULK4* large deletion samples have been independently genotyped by ISC (Fig. 1A) and deCODE (supplementary material Fig. S2), and both datasets mapped the deletions to reproducible regions in these patients. In addition, in the Icelandic control population, deletions in both 3′ and 5′ regions of the *ULK4* gene as well as in the neighbouring *TRAK1* gene were detected (supplementary material Fig. S2). Therefore, different regions of the *ULK4* gene are well covered by the deCODE platforms.

In both datasets the disruptions were localized to the 3′-half of the *ULK4* gene (albeit different regions) and affected the large ULK4 transcript, which is predicted to encode a STK domain at its N-terminus, with two ARM-like protein–protein interaction domains in the middle and at the C-terminus. We were unable to examine the transcripts in any of the CNV cases. Given that all the deletion boundaries are intronic, it is more likely that haplo-insufficiency through nonsense-mediated decay is contributing to increased risk of major mental illness, although a dominant-negative or gain-of-function mechanism cannot be ruled out completely. As there is no evidence of a single origin of founder mutation in either population, this highlights again presumed heterogeneity across populations in vulnerability to specific individual mutational events. The data on SNP allelic associations are less compelling and need to be treated with caution. Although no significant association signals were observed with schizophrenia in Caucasian populations, and only marginal associations with schizophrenia with two SNPs (rs6599155, $P=0.064$ and rs17062109, $P=0.057$) in a large Chinese case control cohort, SNP rs17210774 of *ULK4* was highly associated with bipolar disorder in Caucasians, and another SNP (rs1722850), close to but downstream of *ULK4*, was associated with major depressive disorders. These results strongly suggest that *ULK4* can be a rare genetic risk factor of a range of psychiatric disorders.

On this basis, we decided to characterize some of the functional neurobiology of ULK4. We have shown, for the first time, that an appropriate level of ULK4 expression is fundamental to neuritogenesis, neurite branching, neuronal motility and consequently brain development. The genesis of the corpus callosum was compromised in the *Ulk4*$^{-/-}$ newborn mice. Furthermore, we demonstrated that ULK4 is modulated by several signalling molecules including RA, and in turn, regulates multiple signalling pathways involved in the control of the neuronal cytoskeleton frequently implicated in schizophrenia neuropathology.

Although five members of unc-51 like kinases (ULK1–4 and STK36) have been documented in mammals, little information is available about ULK4. Recent GWAS studies suggest it is a risk locus for multiple myeloma (Broderick et al., 2011) and inter-individual diastolic blood pressure variation (Levy et al., 2009). Null mice with targeted deletion of *Ulk4* were recently reported to develop congenital hydrocephalus, and their respiratory epithelia and ependymal cells had shorter cilia than normal, indicating ciliopathies (Vogel et al., 2012). Intriguingly, many imaging studies have suggested that schizophrenia patients have increased global or regional cerebrospinal fluid (Ananth et al., 2002; Bose et al., 2009; Hulshoff Pol et al., 2002). We have shown strong expression of Ulk4 in the choroid plexus and ependymal cells, which hints at the possible modulation by Ulk4 of the production and directed flow of cerebrospinal fluid. Remarkably, *Stk36*,

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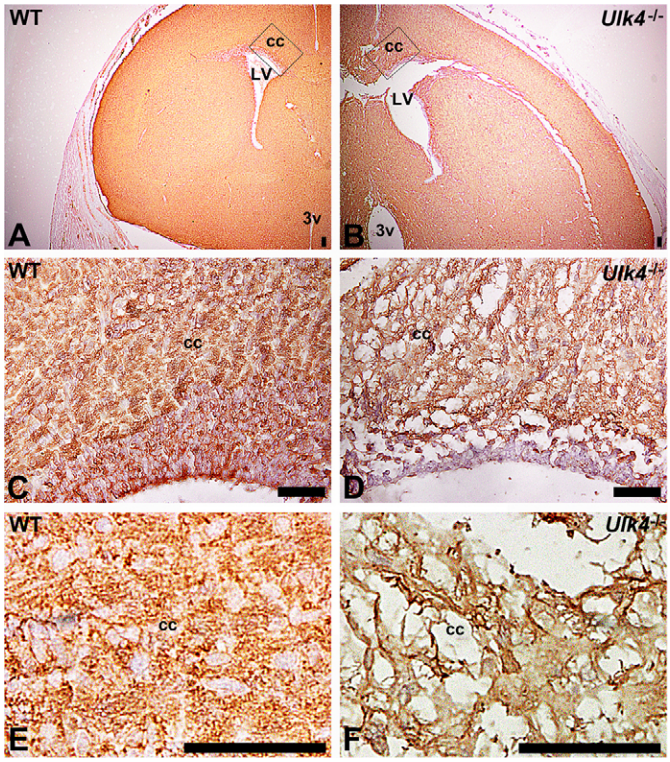

Fig. 8. *Ulk4*$^{-/-}$ mice exhibited defects in integrity of the corpus callosum . (A–F) β-tubulin III (Tuj1) immunostaining of P0 wild-type (WT; A,C,E) and *Ulk4*$^{-/-}$ (B,D,F) mouse brains. C and D are magnified (40×) views of the region of the corpus callosum (cc) indicated by the black boxes in A and B, showing dense and directional nerve fibres in the cc of WT mice (C), but in *Ulk4*$^{-/-}$ mice the structure is abnormal, with loose, fractured and collapsed fibres (D). E and F show even higher magnification (60×) of the cc with extensive and clear space between fibres in the *Ulk4*$^{-/-}$ mice. Scale bars: 50 μm.

another Ulk family member, is also associated with hydrocephalus (Merchant et al., 2005). This evidence indicates that ULK4 plays important but largely neglected roles in brain development and tissue homeostasis.

Defective neuritogenesis is the most consistent neuroanatomical finding in post-mortem studies of schizophrenia (Black et al., 2004; Guidotti et al., 2000; Glantz and Lewis, 2000) and in functional studies using animal models (Shen et al., 2008; Ozeki et al., 2003; Bellon, 2007). Recently, two studies using induced pluripotent stem cell techniques showed that neurons derived from fibroblasts of human schizophrenia patients had reduced neurite numbers and neuronal connectivity (Brennand et al., 2011; Chiang et al., 2011). In our study, knockdown of *ULK4* greatly decreased neuritogenesis. Intriguingly, *ULK4* knockdown cells have less acetylated α-tubulin. This is important because α-tubulin is acetylated in the polymeric form and deacetylation is closely coupled to microtubular depolymerisation. Acetylation of assembled α-tubulin increases the stability of microtubules, which in turn contributes to active growth cone initiation and neurite elongation (Black et al., 1989). Reduced acetylation decreases dendrite length and/or branching and impairs radial migration of cortical neurons, whereas pharmacological restoration of α-tubulin acetylation rescues these deficits (Creppe et al., 2009). We suspect that areas of disassembled α-tubulin might underlie the defective neurite elaboration in the *ULK4* knockdown cells.

PKC has long been regarded as underpinning neuritogenesis and neuronal connectivity. Overactivity of PKC leads to marked retraction of dendrites and loss of spine density in the prefrontal cortex and hippocampus and further disrupts working memory, spatial learning and memory performance (Hains et al., 2009; Wood et al., 2004; Birnbaum et al., 2004). In addition, genetic alteration leading to overactivated PKC signalling has been reported in bipolar disorder (Baum et al., 2008) and schizophrenia (Mirnics et al., 2001). Lithium treatment reduces PKC activation in bipolar manic patients (Hahn et al., 2005), restores stress-induced dendritic retraction in rats (Wood et al., 2004), and increases brain grey matter in human patients (Moore et al., 2000). The CNVs of the *PKC* genes appeared to be enriched in the ISC schizophrenia cohort, with one *PRKCG* duplication and two *PRKCZ* duplications in patients, and only one *PRKCG* duplication in controls. We show that *ULK4* knockdown leads to a significant increase of PKC activity, which suggests that appropriate levels of ULK4 are required to maintain normal PKC activity.

Extracellular signal-regulated kinase (ERK1/2) also facilitates neurite outgrowth (Miñano et al., 2008; Desbarats et al., 2003). Recently, a compartmentalized ERK activation–deactivation switch was reported to govern neurite growth and retraction (Wang et al., 2011). Alteration of the MAPK pathway has also been implicated in schizophrenia (Walsh et al., 2008). In addition, phencyclidine abuse decreases ERK activity and reduces synaptic connectivity (Adachi et al., 2012), indicating important roles of ERK1/2 in the regulation of cytoskeletal dynamics and synaptic plasticity. In agreement with these reports, we show that ULK4 regulates the MAPK pathway and that ULK4 silencing reduces activation of ERK1/2 and p38, and produces defective neuritogenesis.

Mammalian JNKs consist of JNK1–3 with more than 10 splice variants encoded by *MAPK8–10* genes. They are strongly expressed in the brain and mostly studied in the context of cellular stress and apoptosis. Recently, compelling evidence indicates that they also govern the process of neurite outgrowth, branching and elongation. Depletion or pharmacological inhibition of JNKs substantially delayed neuritogenesis, neurite elongation (Coffey et al., 2000; Barnat, et al., 2010) and axonal guidance (Qu et al., 2013). *Jnk1*$^{-/-}$ mice exhibit disrupted anterior commissure tract formation and abnormal axonal microtubule integrity (Chang et al., 2003), as well as altered dendritic architecture (Björkblom et al., 2005). In addition, JNK-interacting protein 3 also promotes axon elongation in a JNK-dependent manner through facilitating actin polymerization (Sun et al., 2013). In the present study, the CNV frequency of *MAPK8–10* was comparable in the ISC cases and controls, with one *MAPK8* duplication plus one *MAPK9* partial duplication in the ISC cases, and one *MAPK9* plus one *MAPK10* partial duplication in the controls. However, ULK4 depletion significantly downregulated the expression of phosphorylated JNK. This suggests that ULK4 may help to maintain cytoskeletal integrity and microtubule assembly through regulation of the JNK pathway. We propose that a decrease in JNK activation impairs the formation of dendrite- and axon-like structures in the knockdown cells. In addition, consistent with observations of white matter reduction (including corpus callosum agenesis) and decreased dendritic arborisation in schizophrenia patients (Bose et al., 2009; Douaud et al., 2007; Fornito et al., 2009; Francis et al., 2011), we found evidence that the genesis of corpus callosum was compromised in the *Ulk4*$^{-/-}$ newborn mice.

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Together our data show that ULK4 plays important roles in regulating stress-related pathways including those involving PKC, ERK, p38 MAPK and JNK.

Although this study has primarily focused on the *ULK4* gene, the systematic analysis of the CNVs in the ISC data in this study has identified 34 genomic deletions (1%) and 91 (2.7%) duplications in 3391 schizophrenic cases that harbour *STK* genes. The STK CNV is 1.6-fold enriched in patients compared with 17 (0.5%) deletions and 58 (1.8%) duplications in 3,181 controls. Although these disease-specific CNVs are rare and not sufficient to conclude inter-relationships of the kinase pathways, we suggest that some of them may be worth following up, including the MAP kinase cascade (MINK1, MAPK8, MKNK1), PKC signalling (PRKCZ, PRKCG, PKN2), stress (MAPKAPK3, STK39, EIF2AK1, SMG1) and hormonal (ADRBK1, ADRBK2, HUNK) response, cytoskeleton remodelling (PAK7/LIMK1/RIOK3), cell proliferation and apoptosis (MAPK6, PKMYT1, TAOK2, CDK14, PAK2, SIK1, RPS6KB2, NRBP2), and neuronal differentiation, maturation and neurotransmission (STK38L, LRRK1, MYLK4 and ULK4). GABAergic neurons are commonly defective in schizophrenia. ULK4 is expressed in GABAergic neurons, and it is now appropriate to investigate whether Ulk4 deficiency affects birth and/or function of GABAergic neurons.

In summary, we have identified recurrent rare deletions of the *ULK4* gene in patients with schizophrenia and other psychiatric disorders. Our data show that ULK4 may be involved in fundamental neurobiological and molecular processes implicated in the pathogenesis of multiple neurodevelopmental disorders and that ULK4 deficiency leads to defects in neuritogenesis *in vitro* and *in vivo*.

MATERIALS AND METHODS

Ethics statement

The study was approved by the Multi-Centre Research Ethics Committee for Scotland and patients gave written informed consent for the collection of DNA samples for use in genetic studies. All the mouse work and molecular studies have been approved by the ethical committees of the Universities of Aberdeen and of Edinburgh.

CNV analyses

CNV data in the ISC dataset of 3391 schizophrenia patients and 3181 ancestrally matched controls were analysed for 255 serine/threonine genes. Recruitment and assessment of cases and controls and the methods of CNV analysis have been described before (International Schizophrenia Consortium, 2008). Four CNV deletions in *ULK4* were present in Scottish patients. Diagnoses of schizophrenia according to DSM4 criteria were reached by consensus between two trained psychiatrists. *P*-values were calculated by one tailed chi-squared tests with Yates' correction.

deCODE has genotyped 100,373 Icelandic subjects using microarrays from Illumina (Human317K and larger). This includes 708 schizophrenia patients (2 deletion carriers), 1,136 bipolar patients (2 deletion carriers), 507 autistic individuals (1 deletion carrier) and 98,022 controls (37 deletion carriers), which were described previously (Stefansson et al., 2008; Steinberg et al., 2014; Vassos et al., 2012; Rujescu et al., 2009; Weiss et al., 2008). CNV analysis of the *ULK4* region was performed by interrogation of chip data spanning exons of *ULK4*. *P*-values were calculated by one tailed chi-squared tests with Yates' correction.

SNPs, typed using Affymetrix 500 or 2M chip, mapping between the above coordinates were examined for allelic association to schizophrenia and bipolar disorder (Psychiatric GWAS Consortium Bipolar Disorder Working Group, 2011) and major depression disorder (Major Depressive Disorder Working Group of the Psychiatric GWAS Consortium, 2012) in the Psychiatric GWAS Consortium dataset and a large case control schizophrenia cohort of Han Chinese from Shanghai Jiaotong University (Shi et al., 2011).

Immunohistochemistry and immunoblotting

Immunohistochemistry was carried out on human brain sections without clinical signs of neuropsychiatric disease (a gift from Prof. T. Harkany, University of Aberdeen) and mouse snap-frozen coronal brain sections. For immunoblotting, proteins were extracted with RIPA buffer containing complete protease and phosphatase inhibitors (Roche). Equal amounts of protein were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked and incubated with primary antibodies overnight at 4°C, incubated with HRP-conjugated secondary antibody (Invitrogen) and developed using an enhanced chemiluminescence kit (Millipore).

The following primary antibodies were used in these experiments: rabbit anti-ULK4 (NBP1-20229, Novus Biologicals), mouse anti-BrdU (BD biosciences), rat anti-Ki67 (DAKO), mouse anti-GFAP and mouse anti-CNPase (Abcam), mouse anti-α-acetylated tubulin, rabbit anti-β-tubulin III, mouse anti-tyrosine tubulin, mouse anti-β-actin and mouse anti-MAP2 (Sigma-Aldrich). Mouse anti-NeuN, mouse anti-GAD67, rabbit anti-phospho-CREB (ser133) (Millipore). Mouse anti-GAPDH, ELK1 and phospho-ELK1 (Santa Cruz, USA); mouse anti-phospho-ERK1/2, rabbit anti-ERK1/2, rabbit anti-phospho-p38, rabbit anti-p38, rabbit anti-CREB, rabbit anti-phospho-PKC, rabbit anti-phospho-JNK and rabbit anti-JNK (Cell Signaling Technology). Anti-Sox10 was kindly provided by Prof. Michael Wegner, University Medical Center Hamburg-Eppendorf, Hamburg, Germany.

Horseradish-peroxidase-conjugated-secondary antibodies for western blotting were purchased from Sigma. Fluorescent secondary antibodies for immunofluorescence staining were Alexa Fluor 488 or 594 conjugated (Molecular Probes). Secondary antibodies and the developing kits for DAB staining were from Sigma and Vector. F-actin was labelled with Alexa-Fluor-488–phalloidin (Molecular Probes) according to the manufacturer's instructions.

Cell culture

SH-SY5Y cells were maintained in DMEM:F12 with 10% foetal bovine serum. To examine regulation of ULK4 expression, cells were grown in reduced serum medium (0.2% FBS) for 24 hours and then treated with DMSO, or 25 μM ATRA, 25 μM 9-cis RA, 1 mM db cAMP, 100 nM PACAP or 100 ng/ml NGF, respectively.

ULK4 knockdown

ULK4 silencing was achieved by expression of a lentiviral shRNA against exon 1 of *ULK4* (Sigma, TRCN0000002205; 5′-CCGGCGACG-GAAGGGAACAATCAATCTCGAGATTGATTGTTCCCTTCCGTCG-TTTTT-3′). A vector containing a non-specific sequence (Sigma, SHC002; 5′CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGG-TGCTCTTCATCTTGTTGTTTTT-3′) for any human gene was chosen as control. Detailed procedures have been described previously (Martin-Granados et al., 2008). Three batches of puromycin-resistant stable cell lines were generated by two researchers with three independent batches of virus expressing ULK4 or control shRNAs. The knockdown efficiency was analysed statistically on three independent batches of cell lines and shown as averages of three independent experiments ± standard deviation (s.d.; $P<0.05$ and $P<0.01$were considered significant).

Neurite outgrowth assay

ULK4 knockdown and control cells were plated in 24-well dishes. Medium was regularly replenished and random images were taken daily for 5 days using an Axiovert 40CFL microscope (Leica) with a 20× objective lens. The length of neurites was measured and analysed statistically as previously (Shen et al., 2008). $P<0.05$ was considered significant.

Cell migration

ULK4 knockdown and control cells were seeded at a low density (5×10^4) onto six-well plates 16–20 hours before migration assay. Cells were cultured in CO_2-independent medium (Invitrogen) in a temperature-controlled chamber. Images were recorded every 15 minutes for 2 hours (5 hours for supplementary material Movie 1) and analysed using

MetaMorph software (Universal Imaging Corp). Migration rate was presented with trajectory speed (T_t/T) – the total length of the migration trajectory of a cell (T_t) divided by the given period of time (T), and displacement speed (T_d/T) – the straight-line distance between the start and end positions of a cell (T_d) divided by the time (T). All experiments were independently performed in triplicate.

Ulk4 knockout mice

Ulk4$^{+/-}$ mice on a C57BL/6N strain background were purchased from KOMP Repository (knockout mouse project, USA). All experimental procedures were conducted under a licence approved by the Irish Department of Health and Children in accordance with Cruelty to Animals Act of 1876 and under a certificate approved by the Animal Care and Research Ethics Committee (ACREC) of the National University of Ireland Galway, Galway, Ireland. *Ulk4*$^{-/-}$, *Ulk4*$^{+/-}$ and wild-type littermates were obtained from *Ulk4*$^{+/-}$×*Ulk4*$^{+/-}$ matings. Mice were genotyped by PCR of genomic DNA with two pairs of PCR primers: Ulk4EndE7For (5′-TAACTTGCTGGACGGATTGCTG-3′) and with Ulk4EndIn7Rev (5′-TGATCTGTAATCGCAGTGCAGG-3′) amplifying a 271 bp DNA fragment from the wild-type allele, and Ulk4KOMPKOFor (5′-GAGATGGCGCAACGCAATTAATG-3′) and Ulk4KOMPKORev (5′-CTGAGGAGACAATGTAACCAGC-3′) to produce a 621 bp DNA fragment from the knockout allele. Newborn mice were humanely killed with an over-dose of sodium pentobarbitone and P0 brains were immersed overnight in PBS containing 4% paraformaldehyde at 4℃. They were then embedded in paraffin and sectioned at 10 µm. Brain sections were immunohistochemically processed with the primary mouse anti-βIII tubulin (Tuj1; 1:1000, Promega) and biotinylated goat anti-mouse secondary (KPL) antibodies. Then they were developed with the Vectastain Elite ABC system (Vector). Images were captured using an Olympus BX51 upright, fluorescence microscope.

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Competing interests

The authors declare no competing interests.

Author contributions

S.S. and C.D.M. conceived of the experiments; B.L., I.H., M.L., D.S.C., C.D.M. and S.S. wrote the manuscript; B.L., J.P., I.H., M.L., C.M., T.J.R. performed experiments; B.L., I.H., J.P., G.D.G., Z.L.G., W.D.L., Y.Y.S., G.H., L.H., H.S., D.S.C., D.H.B., S.S. analyzed data and provide manuscript suggestions.

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Supplementary material

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.137604/-/DC1

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