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Convergent evidence for impaired AKT1-GSK3β signaling in schizophrenia

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AKT-GSK3β signaling is a target of lithium and as such has been implicated in the pathogenesis of mood disorders. Here, we provide evidence that this signaling pathway also has a role in schizophrenia. Specifically, we present convergent evidence for a decrease in AKT1 protein levels and levels of phosphorylation of GSK3β at Ser9 in the peripheral lymphocytes and brains of individuals with schizophrenia; a significant association between schizophrenia and an *AKT1* haplotype associated with lower AKT1 protein levels; and a greater sensitivity to the sensorimotor gating–disruptive effect of amphetamine, conferred by AKT1 deficiency. Our findings support the proposal that alterations in AKT1-GSK3β signaling contribute to schizophrenia pathogenesis and identify *AKT1* as a potential schizophrenia susceptibility gene. Consistent with this proposal, we also show that haloperidol induces a stepwise increase in regulatory phosphorylation of AKT1 in the brains of treated mice that could compensate for an impaired function of this signaling pathway in schizophrenia.

Alterations in intracellular signaling pathways are important in treating complex neuropsychiatric disorders[1]. Signaling pathway components, including several protein kinases and phosphatases, are direct targets of some of the most effective medications for treating these disorders. For example, lithium, one of the most established treatments for bipolar disorder (BPD), is believed to exert its therapeutic effects by directly modulating the activity of inositol monophosphatase and of glycogen synthase kinase 3β (GSK3β)[1]. It is still unclear, however, whether abnormalities in signaling pathways are central to the pathophysiology of psychiatric illnesses, including schizophrenia. Nevertheless, genes regulating such signaling cascades, especially those affecting synaptic transmission and plasticity, are good candidate susceptibility genes.

RESULTS

Low levels of AKT1 in individuals with schizophrenia

It is well established that most of the central nervous system (CNS) protein kinases and phosphatases are involved in a wide variety of cellular functions and are expressed in diverse cell types including peripheral blood lymphocytes. We speculated that alterations in brain levels or activity of protein kinases and phosphatases may contribute to schizophrenia susceptibility in humans and that this might be observed in the peripheral tissues of individuals with schizophrenia. We examined the abundance of several kinases implicated in synaptic plasticity (PRKACA, AKT1, PRKC, MAP3K, GSK3β, PIK3CB and PIK3R2)[2–4]. We assessed changes in protein levels because they are more likely to be cell-autonomous and less likely to be influenced by the cellular environment when compared, for example, with rapid and reversible activity modifications through phosphorylation. Protein extracts from lymphocyte-derived cell lines from individuals with schizophrenia ($n = 28$) and unaffected controls ($n = 28$) were subjected to SDS-PAGE and immunoblot analysis. Levels of protein kinase AKT1 were 68% lower in individuals with schizophrenia than in controls (**Fig. 1a**, $P = 0.014$, Mann-Whitney test, corrected for eight tests). In contrast, we observed no differences in the levels of the other kinases tested.

This initial exploratory analysis may have been confounded by an imperfect match of cases and controls and the influence of Epstein–Barr virus (EBV) transformation on the levels of AKT1. Nonetheless, the specificity of our findings together with the fact that the same cellular pathway has been implicated in mood disorders[1] prompted us to follow up our initial observation using four complementary approaches. First, we attempted to verify a reduction in AKT1 levels in postmortem frontal cortex, one of the primary sites of disease pathology. Second, we examined whether the reduction in AKT1 levels was reflected by a reduction in substrate phosphorylation in both peripheral lymphocytes and postmortem frontal cortex. Third, we tested whether certain variants of the gene encoding AKT1 were preferentially transmitted in individuals with schizophrenia. Fourth, we used *Akt1*$^{-/-}$ mutant mice[5] to examine whether AKT1 deficiency affects gating of the startle reflex, which is impaired in individuals with schizophrenia[6].

In brain tissues acquired from a cohort of individuals with schizophrenia and controls, obtained from the MRC Brain Bank

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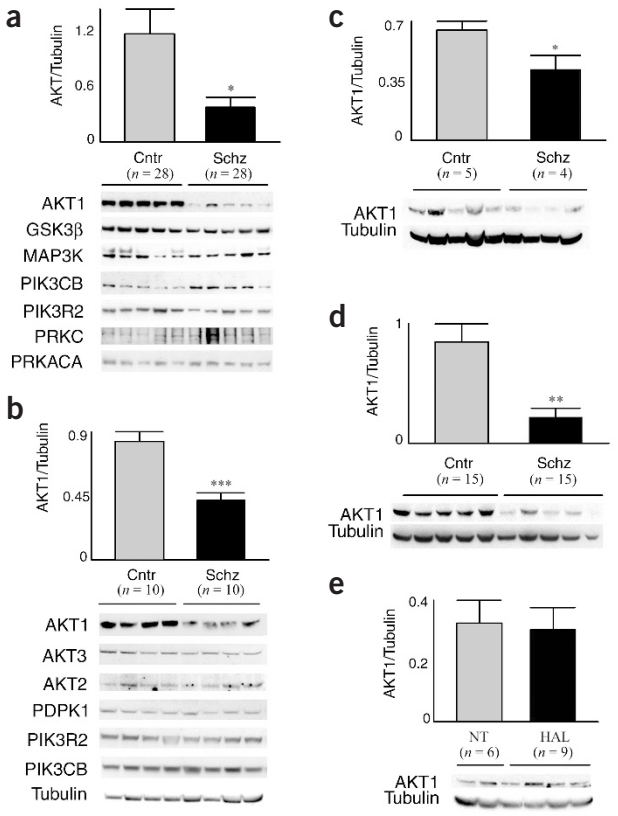

Figure 1 Lower AKT1 protein levels in the lymphocytes and frontal cortex of individuals with schizophrenia. (**a**) AKT1 protein levels in lymphocytes. Mean ± s.e.m. optical densities are shown, reflecting the normalized AKT1 levels in individuals with schizophrenia (Schz) and controls (Cntr). Total protein extract (30 g) was loaded in each lane. Representative immunoblots are shown, probed with antibodies to AKT1, GSK3β, MAP3K, PIK3CB, PIK3R2, PRKC and PRKACA, as well as with antibody to tubulin as a loading control. (**b**–**d**) AKT1 protein levels in frontal cortex (**b**,**d**) and hippocampus (**c**). Mean ± s.e.m. optical densities are shown, reflecting the normalized AKT1 levels in two independent brain tissue samples of individuals with schizophrenia (Schz) and controls (Cntr) from the MRC (**b**,**c**) and Stanley (**d**) Brain Banks. Total protein extract (100 g of MRC frontal cortex or 50 g of MRC hippocampus, Stanley) was loaded in each lane. Representative immunoblots are shown, probed with antibodies to AKT1, AKT3, AKT2, PDPK1, PIK3R2 and PIK3CB, as well as with antibody to tubulin as a loading control. (**e**) AKT1 protein levels in the frontal cortex of haloperidol-treated (HAL) mice and untreated control (vehicle only; NT) mice. Mean ± s.e.m. optical densities are shown, reflecting the normalized AKT1 levels in treated and untreated mice. Representative immunoblots are shown, probed with antibody to AKT1 as well as with antibody to tubulin as a loading control. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

(**Supplementary Table 1** online), AKT1 protein levels were indeed lower in the frontal cortex (**Fig. 1b**) and hippocampus (**Fig. 1c**; $P = 0.0001$ and $P = 0.04$, respectively, by unpaired two-tailed *t*-test) of individuals with schizophrenia compared with controls. AKT1 was specifically affected: similar analysis in the cortex did not detect any difference in expression of the closely related isoforms AKT2 and AKT3 (encoded by distinct genes) or of a number of other kinases acting upstream to AKT1 in the phosphatidylinositol-AKT signaling pathway in individuals with schizophrenia versus controls (**Fig. 1b**). In addition, Pearson correlation analysis found no relation between the AKT1 levels in the frontal cortex and age, gender or post-mortem interval (data not shown). We confirmed the difference in cortical AKT1 levels in brain tissues using an independent cohort of individuals with schizophrenia and controls obtained from the Stanley Consortium Brain Bank[7] (**Fig. 1d**; $P = 0.001$, unpaired two tailed *t*-test).

Consistent changes in protein levels in individuals with schizophrenia may reflect either a component of the disease process or a consequence of the pharmacological treatment of the disorder. To control for this latter confounding factor, we designed an *in vivo* chronic trial of haloperidol (one of the most commonly used antipsychotic medications in schizophrenia) in C57Bl/6 mice. Comparisons of haloperidol-treated and control mice did not detect any difference in total amount of AKT1 (**Fig. 1e**). Because an animal model may not accurately mirror the pharmacotherapy of schizophrenia, we cautiously interpreted these data to indicate that the changes in AKT1 protein levels we observed in individuals with schizophrenia were not a direct result of treatment with antipsychotic medication.

Reduced phosphorylation of GSK3β at Ser9 in schizophrenic individuals

An increasing number of proteins have been proposed as substrates of AKT1, although evidence that many of them are phosphorylated by

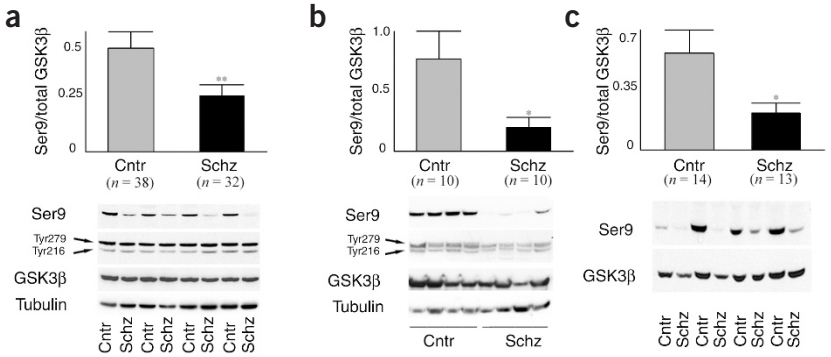

Figure 2 Reduced phosphorylation of GSK3β at Ser9 in the lymphocytes and frontal cortex of individuals with schizophrenia. (**a**) Phosphorylation of GSK3β in lymphocytes. Mean ± s.e.m. optical densities are shown, reflecting the normalized phosphorylation of GSK3β at Ser9 in individuals with schizophrenia (Schz) and controls (Cntr). Total protein extract (30 g) was loaded in each lane. Representative immunoblots are shown, probed with an antibody against total GSK3β, stripped and reprobed with antibodies to GSK3β phosphorylated at Ser9 and to GSK3β or GSK3α phosphorylated at Tyr216 or Tyr279, respectively, as well as with antibody to tubulin as a loading control. (**b**,**c**) Phosphorylation of GSK3β in the frontal cortex of individuals with schizophrenia. Mean ± s.e.m. optical densities are shown, reflecting the normalized phosphorylation of GSK3β at Ser9 in individuals with schizophrenia and controls from the MRC (**b**) and Stanley (**c**) cohorts. Total protein extract (100 g for MRC samples or 50 g for Stanley samples) was loaded in each lane. Representative immunoblots are shown, probed as in **a**. $^{*}P < 0.05$; $^{**}P < 0.01$.

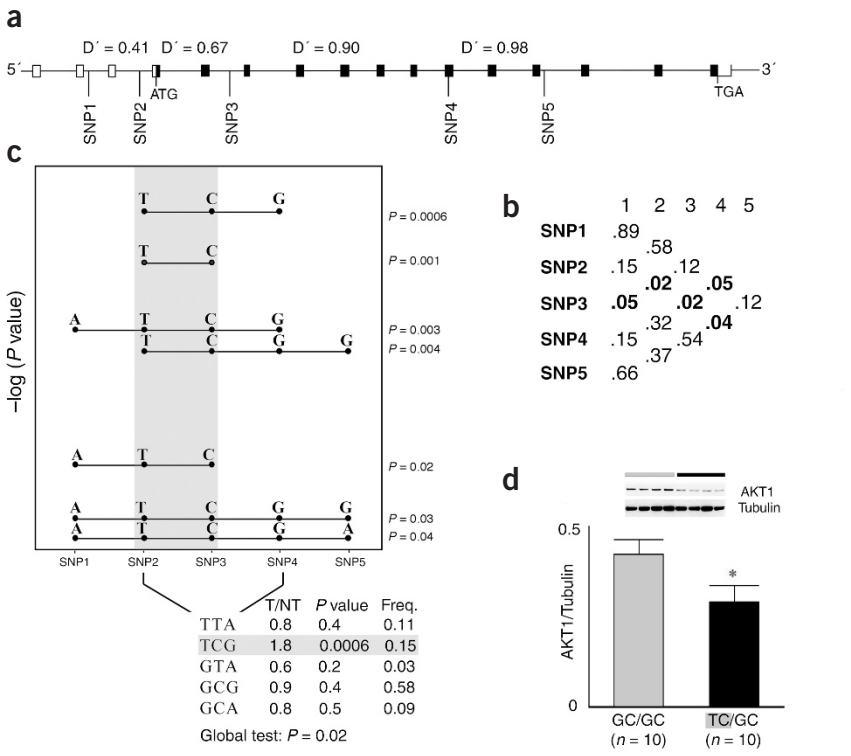

Figure 3 Preferential transmission to individuals with schizophrenia of an *AKT1* haplotype associated with lower AKT1 protein levels. (**a**) Genomic structure of the *AKT1* locus and polymorphic sites used. Exons and introns are not to scale; black blocks indicate coding sequences; white boxes indicate noncoding sequences. Pairwise linkage disequilibrium (D′; ref. 30) between the tested SNPs is also indicated. (**b**) Global *P* values from single-locus and multilocus (two to five) association analysis. Frequency data for individual SNPs were as follows (allele frequency): SNP1 (G = 0.09; A = 0.91), SNP2 (G = 0.71; T = 0.29), SNP3 (C = 0.83; T = 0.17), SNP4 (A = 0.25; G = 0.75), SNP5 (G = 0.44; A = 0.56). (**c**) Upper panel: Haplotypes showing significant transmission distortion at 1 degree of freedom. The two-SNP core haplotype is highlighted. Lower panel: Analysis of the transmission of the five SNP2/3/4 haplotypic variants and their corresponding frequencies in the parental chromosomes. (**d**) The *AKT1* core risk haplotype is associated with lower AKT1 protein levels. Mean ± s.e.m. optical densities are shown, reflecting the normalized AKT1 levels. Representative immunoblot is shown, probed with an antibody against total AKT1 and against tubulin as a loading control. Total protein extract (30 g) was loaded in each lane.

AKT1 *in vivo* is still lacking[8]. GSK3β is one of the most established and thoroughly studied substrates of AKT1 (ref. 8). GSK3β is unusual in that it seems to be largely regulated by inhibition through AKT1-dependent phosphorylation at Ser9 (ref. 8). Analysis of protein extracts from lymphocytes did not show any differences in the levels of GSK3β (**Fig. 2a**). Similar analysis of frontal cortex lysates showed only a slight, insignificant decrease in protein levels (**Fig. 2b**). To quantify the relative level of phosphorylation of GSK3β at Ser9, we stripped the immunoblots prepared from lymphocyte and frontal cortex lysates and reprobed them with an antibody specific to GSK3β phosphorylated at Ser9. We used the ratio of Ser9 density to total protein density as a measure of phosphorylation of GSK3β at Ser9. This analysis showed that the relative phosphorylation at Ser9 was significantly lower in individuals with schizophrenia than in controls both in lymphocytes (48% lower, $P = 0.009$, Mann-Whitney test; **Fig. 2a**) and in frontal cortex from the MRC (73% lower, $P = 0.02$, Mann-Whitney test; **Fig. 2b**) and Stanley (62% lower, $P = 0.04$, Mann-Whitney Test; **Fig. 2c**) cohorts.

GSK3β is also regulated by an independent activation pathway through phosphorylation at Tyr216 in the kinase domain. In control experiments, we quantified the relative phosphorylation of GSK3β at Tyr216 by reprobing the same immunoblots with an antibody that recognizes a 46-kDa band corresponding to GSK3β phosphorylated at Tyr216 and a 51-kDa band corresponding to GSK3α phosphorylated at Tyr279. We observed no difference in the phosphorylation of GSK3β at Tyr216 between individuals with schizophrenia and controls in either lymphocytes (**Fig. 2a**) or frontal cortex samples from either the MRC (**Fig. 2b**) or the Stanley cohorts (data not shown). Thus, we observed a reproducible deficit in phosphorylation of GSK3β at Ser9 (but not at Tyr216) in the brains and peripheral lymphocytes of individuals with schizophrenia, consistent with the expectation from the observed reduction in AKT1 levels.

An *AKT1* haplotype associated with schizophrenia

The gene encoding AKT1 is located in the cytogenetic band 14q32, which is not included among the main susceptibility loci identified by linkage studies in families with schizophrenia. But genome-wide linkage scans for genetically heterogeneous disorders are unlikely to produce reliable signals when the contributing alleles are associated with moderate increase in risks or are present at low frequencies. Sequencing of the *AKT1* locus in nine individuals with schizophrenia, and searching of public variant databases, found no coding variants but identified several other polymorphisms. To examine whether variants of the gene are preferentially transmitted to individuals with schizophrenia, we typed five single-nucleotide polymorphisms (SNPs) at the *AKT1* locus (**Fig. 3a**) in 268 affected families. This sample included 210 proband-parent triads and 58 extended families, containing a total of 335 individuals affected with schizophrenia or schizoaffective disorder. This analysis identified a significant global transmission distortion of one SNP (SNP3, $P = 0.05$; **Fig. 3b**) and several multi-SNP haplotype systems (**Fig. 3b**). Further multilocus analysis (**Fig. 2c**) identified one three-SNP haplotype (SNP2/3/4, TCG) with a frequency of ~15% in parental chromosomes that was preferentially transmitted to probands with schizophrenia at a ratio of 1.8:1 and showed a highly significant association with the disease ($P = 0.0006$). This association remained significant ($P < 0.04$) even after overconservatively adjusting for the 55 comparisons involving individual SNP alleles and multi-SNP haplotypes (with frequencies higher that 3%) with the Bonferroni correction. Other multi-SNP haplotypes also showed significant association with the disease (**Fig. 3c**). They all share a common core that extends from SNP2 to SNP3 and includes the first two coding exons (**Fig. 3a**).

We obtained qualitatively similar results (albeit at lower significance levels due to a decrease in statistical power) when we analyzed only the 210 proband-parent triads or when we reanalyzed the entire sample by including only one, randomly selected proband per

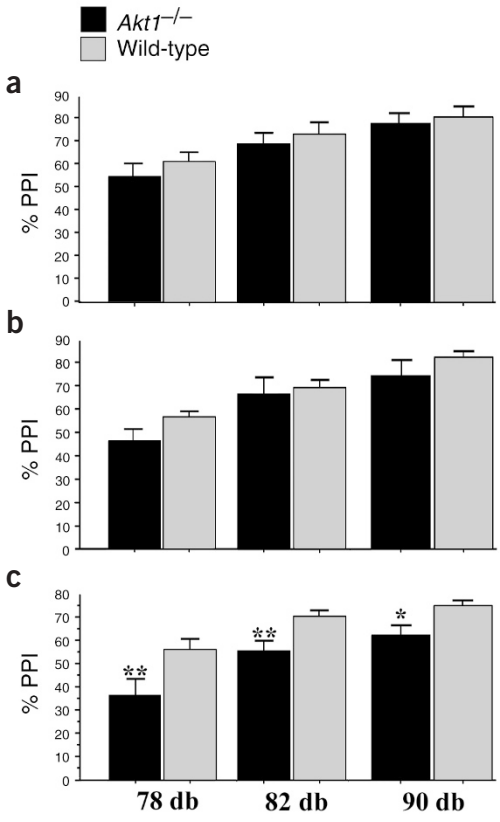

Figure 4 AKT1 deficiency confers increased sensitivity to the sensorimotor gating–disruptive effect of amphetamine. PPI analysis of *Akt1*$^{-/-}$ mice and wild-type littermate controls at baseline (vehicle; **a**) and in response to MK801 (**b**) or amphetamine (**c**). To increase the sensitivity of this approach we used the highest doses of amphetamine (4 mg kg^{-1}) and MK801 (0.3 mg kg^{-1}) that, in our pilot experiments, did not significantly affect PPI in wild-type littermates. *$P < 0.05$; **$P < 0.01$.

extended family ($P = 0.01$ and $P = 0.008$, respectively, for the core risk haplotype). We compared control lymphocyte-derived cell lines identified as unambiguous heterozygous carriers of the core two-SNP risk haplotype (TC) with cell lines homozygous with respect to the most common and neutral two-SNP variant (GC). Relative levels of AKT1 were consistently lower in cell lines with the genotype TC/GC versus those with the genotype GC/GC (**Fig. 3d**; $P = 0.03$, unpaired *t*-test), suggesting that the TC risk haplotype dictates impaired *AKT1* mRNA expression or processing, as compared with the most commonly occurring GC haplotype. Over-representation of this haplotype in individuals with schizophrenia may partially account for the observed difference in AKT1 levels in the brain and peripheral lymphocytes relative to controls, but additional *trans*-acting factors affecting *AKT1* gene expression or protein stability may also be involved. A previous genome scan in 22 extended families with BPD detected suggestive evidence for linkage on the distal portion of chromosome 14 in the vicinity of the *AKT1* locus (14q22–32; ref. 9), and a recent rank-based genome scan meta-analysis of 18 BPD genome scan data sets provided additional support for linkage in the combined data for the same locus[10]. Furthermore, preliminary analysis of transmission of SNP haplotypes from the *AKT1* gene showed weak evidence of association between specific *AKT1* haplotypes and BPD[11].

Greater sensitivity to sensorimotor gating disruption

Sensorimotor gating refers to the forebrain influences on the automatic startle reflex. An operational measure of sensorimotor gating is prepulse inhibition (PPI), the attenuation of a startle response by a weak prestimulus (prepulse) presented a short time (100 ms) before the startle stimulus. PPI in rodents has been used widely in studies addressing the neurobiology of impaired sensorimotor gating in schizophrenia[12]. Mice have robust PPI, which is impaired in a dose-dependent manner by treatment with nonselective dopamine receptor agonists (such as the dopamine releaser amphetamine) or more selective N-methyl-D-aspartate receptor antagonists (such as dizocilpine (MK-801))[12]. We recorded PPI from adult (2–6-month-old) *Akt1*$^{-/-}$ mice and wild-type littermates of both sexes as controls, both at baseline and in response to amphetamine and MK801. We used a combination of one startle level (120 dB) and three prepulse levels (78, 82 and 90 dB), and PPI was expressed as 100 – ((response to startle stimulus after prepulse / response to startle stimulus alone) 100). AKT1 deficiency did not cause a disruption of baseline PPI in mice that did not receive any treatment (**Fig. 4a**). Similarly, MK801 treatment at a dose (0.3 mg kg^{-1}) that did not affect PPI in wild-type mice did not cause a clear disruption of PPI in *Akt1*$^{-/-}$ mice (**Fig. 4b**). In contrast, treatment with amphetamine at a dose (4 mg kg^{-1}) that did not affect PPI in wild-type mice resulted in substantial reduction of PPI in *Akt1*$^{-/-}$ mice relative to identically treated wild-type controls at all three prepulse intensities (**Fig. 4c**). We also evaluated the amplitude of the startle response, which is affected by factors including the integrity and excitability of the responding neurons in subcortical auditory centers. We did not find any significant differences in the weight-corrected startle response amplitudes (data not shown). Although further dose-effect studies are warranted, our initial analysis indicates that AKT1 deficiency partially impairs the neuronal circuits that gate the startle reflex, as uncovered under challenge with amphetamine.

Haloperidol effects on phosphorylation of AKT1

Stimulation of the catalytic activity of AKT results from phosphorylation at two regulatory residues: Thr308 and Ser473. Phosphorylation at Thr308, catalyzed by PDPK1, is necessary and sufficient to activate AKT1; this activity is augmented by phosphorylation at Ser473 through an uncharacterized kinase[13]. Several lines of evidence suggest that phosphorylation of AKT and GSK3β is a principal target of lithium and sodium valproate, two therapeutic agents for BPD. Lithium increases phosphorylation of AKT at Ser473 in cultures of cerebellar granule cells[14] and increases phosphorylation of GSK3β at Ser9 in the cerebral cortex and hippocampus of chronically treated mice[15]. Sodium valproate caused a gradual but relatively large increase in phosphorylation of AKT at Ser473, as well as a more modest increase in the inhibition-associated phosphorylation of GSK3β at Ser9 in cultures of human neuroblastoma cells[15].

The effect of antipsychotic medications on AKT-GSK3β signaling *in vivo* has not been addressed. Although haloperidol treatment does not affect AKT1 protein levels *in vivo* (**Figs. 1e** and **5a**), we examined whether it can affect enzymatic activity by modulating regulatory phosphorylation levels. Our analysis showed a stepwise haloperidol-dependent increase in phosphorylation of total AKT. In the acute paradigm, haloperidol caused an increase in phosphorylation at Thr308 and did not affect phosphorylation at Ser473 (**Fig. 5a**). In the chronic paradigm, haloperidol caused an increase in phosphorylation at both Thr308 and Ser473 (**Fig. 5a**), and this increase in phosphorylation was reflected in increased phosphorylation of GSK3β at Ser9 (**Fig. 5b**).

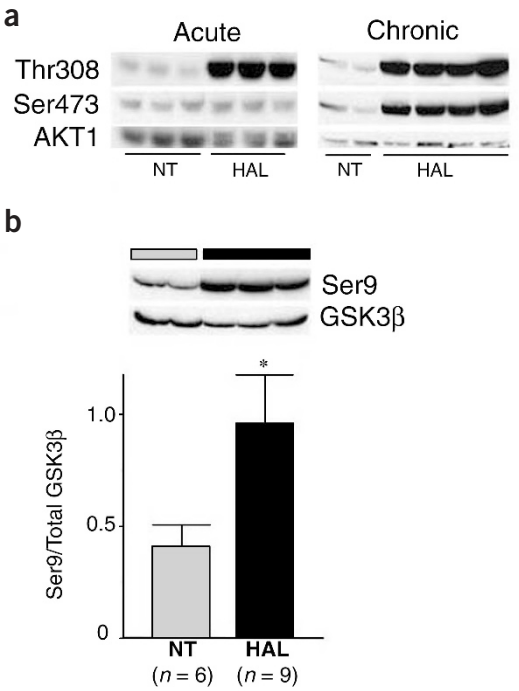

Figure 5 Haloperidol effects on phosphorylation of AKT1. (**a**) Acute haloperidol treatment increases phosphorylation of AKT at Thr308. Chronic haloperidol treatment increases phosphorylation of AKT at both Thr308 and Ser473. Representative immunoblots are shown probed with antibodies to AKT1, AKT phosphorylated at Thr308 and Ser473. HAL, haloperidol-treated mice; NT, untreated mice. (**b**) Chronic haloperidol treatment induces increase in phosphorylation of GSK3β at Ser9. Mean ± s.e.m. optical densities are shown, reflecting the normalized phosphorylation of GSK3β at Ser9 in haloperidol-treated (HAL) and untreated (NT) mice. The same chronic treatment immunoblot shown in **a** was reprobed with antibodies against total GSK3β and against GSK3β phosphorylated at Ser9. $^*P < 0.05$.

Because both antibodies that recognize phosphorylated AKT1 recognize all three isoforms, it is not clear at this point whether haloperidol affects the phosphorylation of one or more isoforms. More detailed analysis with the use of mice singly deficient for each isoform, as well as double and triple combinations, will be necessary to address the specificity of the haloperidol effect. Nevertheless, our data suggest that antipsychotic medications could compensate for the difference in total AKT1 levels by increasing regulatory phosphorylation levels of AKT, supporting a role for AKT1-GSK3β signaling in schizophrenia pathogenesis and uncovering a previously unknown aspect of the haloperidol mechanism of action. The fact that some of the most commonly used medications in the treatment of schizophrenia and BPD modulate the AKT-GSK3β signaling pathway and the observation that *AKT1* variants may contribute to the susceptibility to both schizophrenia and BPD are in agreement with observations that these two disorders share many genetic and biochemical abnormalities[16].

DISCUSSION

Our findings support the proposal that alterations in AKT1-GSK3β signaling contribute to schizophrenia pathogenesis and identify *AKT1* as a potential schizophrenia susceptibility gene. At this point we cannot exclude a contribution from the genes *AKT2* and *AKT3*. Genetic analysis of these genes, and of additional genes involved in the AKT1-GSK3β signaling cascade, in large family samples will be of considerable interest. AKT and GSK3β have emerged as the focal point for many signal-transduction pathways, regulating multiple cellular processes including transcription, apoptosis, endoplasmic reticulum stress response and cell proliferation[17,18]. In the developing CNS, in addition to the functions mentioned above, AKT-GSK3β signaling is required for axon elongation and branching[19] and has a role in the reelin-dependent migration of cortical neurons[20]. Decreases in the levels of reelin have been consistently described in brains of individuals with schizophrenia[21].

During development, AKT is also a crucial mediator of the neuronal survival-promoting effect of *NRG1* (a candidate schizophrenia susceptibility gene)[22]. In the adult CNS, AKT1 may have a crucial role in normal synaptic plasticity and memory formation. For example, AKT phosphorylates GABRA, the principal receptors mediating fast inhibitory synaptic transmission in the mammalian brain, thereby facilitating their transfer to the plasma membrane[23]. AKT also modulates dendritic spine protein expression[24]. Correlative evidence suggests that AKT1 may have a crucial role in memory formation. Specifically, increased phosphorylation of AKT was observed in the amygdala after long-term potentiation and fear-conditioning training[4]. In addition, brain-derived neurotrophic factor–dependent phosphorylation of AKT1 in the hippocampus increases in parallel with spatial reference and working memory formation[25]. The possibility of AKT1 involvement in working memory formation is of interest because a large number of studies have shown that individuals with schizophrenia have performance deficits on a wide range of working memory tasks.

One additional possibility is that GSK3β signaling is required for gene expression mediated by NFATC (nuclear factor of activated T cells, cytoplasmic). In hippocampal neurons NFATC rapidly translocates from cytoplasm to nucleus and activates a transcriptional response to synaptic activation. The translocation is crucially dependent on the calcium-regulated phosphatase calcineurin, which stimulates nuclear import[26]. Hyperactive GSK3β can phosphorylate NFATC, promoting its export from the nucleus and thus antagonizing calcineurin and NFATC–dependent transcription. Human genetic studies, as well as the behavioral analysis of an animal model, have provided evidence for an involvement of calcineurin signaling in schizophrenia susceptibility[27,28]. It is therefore conceivable that impairment in both AKT1-GSK3β and calcineurin signaling could be contributing factors in schizophrenia pathogenesis through a synergistic inhibition of NFATC-mediated gene expression that could have profound effects on neuronal function and synaptic plasticity.

There are several possible mechanisms by which altered AKT1 function could influence the formation and function of neuronal circuits whose integrity is necessary to prevent progression to psychosis. This influence may be additive, perhaps due to small impairment of several processes, or may be restricted to a small number of key processes that are particularly AKT1 dosage–dependent. In any case, our data is consistent with a model in which impairment of AKT1-GSK3β signaling increases the liability of these neuronal circuits to additional genetic or environmental insults that ultimately lead to the disease.

METHODS

Cell cultures, protein extraction and immunoblot analysis. We isolated lymphocytes from peripheral blood obtained from individuals with schizophrenia we recruited from the US (of northern European origin) and transformed them by EBV with standard techniques. We obtained EBV-transformed lymphocytes from controls from the Coriell Repository's collection of Utah and French CEPH families. In addition to being matched broadly for race and ethnicity, samples were also sex-matched. We cultured lymphocytes in RPMI media (Gibco-BRL) supplemented with 10% fetal bovine serum (Sigma), penicillin-streptomycin and L-glutamine (Gibco-

BRL). To restrict confounding effects from the EBV transformation, we matched cultures closely for growth rate (three slow-growing cultures were discarded). We precisely dissected frozen rostro-caudally matched blocks obtained from MRC Brain Bank (London, UK) under a dissection microscope or obtained frozen sections from the Stanley Consortium Brain Bank and then homogenized and lysed them. Lymphocytes (~50 10^6), frontal cortex blocks (0.1–0.2 g) or frozen sections were homogenized in ice-cold lysate buffer (0.25 M Tris, pH 7.5) containing protease inhibitors (Protease Inhibitor Cocktail tablets, Boehringer Mannheim) and phosphatase inhibitors (Phosphatase Inhibitor Cocktails I & II, Sigma) and lysed through three cycles of freezing (in liquid nitrogen) and thaw (in 37 °C water bath). We measured protein concentration by Bio-Rad's protein assay and spectrometry at 595 nm. Equal amounts of total protein were loaded onto 4–12% gradient Bis-Tris gels, separated with the NuPAGE system (Invitrogen) and transferred onto nitrocellulose membrane. We probed the membrane with primary and secondary antibodies and detected signals by chemiluminescence followed by autoradiography. We used antibodies to AKT1 (Upstate Biotechnology, 1:1,000), AKT2 (Alpha Diagnostic International, 1:1,000), AKT3 (Upstate Biotechnology, 1:500), GSK3β (Transduction Laboratories, 1:2,000), GSK3β phosphorylated at Ser9 (Cell Signaling, 1:1,000), GSK3β phosphorylated at Tyr216 (Upstate Biotechnology, 1:1,000), MAP3K (Zymed, anti-MAP kinase (ERK1+ERK2), 1:1,000), PIK3R2 (Upstate Biotechnology, anti-p85, 1:1,000), PIK3CB (Upstate Biotechnology, anti-p110, 1:2,000), PDPK1 (Calbiochem, anti-PDK-1, 1:1,000), PRKACA (U.S. Biological, anti-PKA, 1:1,000), PRKC (Sigma, anti-PKC, 1:1,000) and AKT phosphorylated at Thr308 (Upstate Biotechnology, 1:1,000) and Ser473 (Cell Signaling, 1:1,000), as well as an antibody to tubulin (Sigma, 1:5,000), which was used as a loading control.

Human samples. The schizophrenia family sample included 210 proband-parent triads collected by us from the US (of northern European origin) and 58 extended families with multiple affected individuals (also of northern European origin) obtained from the NIMH Genetics Initiative. A total of 335 individuals met DSM-IV criteria for schizophrenia or schizoaffective disorder. All participants provided written informed consent before participation in the study. The protocol and the consent forms were approved by the Rockefeller University Institutional Review Board.

PCR primers and conditions. Primer sequences are available on request. After PCR amplification, we digested DNA samples with *Bsa*I (5 U) for SNP1, *Xcm*I (5 U) for SNP2, *Hae*III (3 U) for SNP3, *Afl*III (5 U) for SNP4 and *Pvu*II (3 U) for SNP5.

Haloperidol trial. We used male and female C57Bl/6 mice. Mice were housed in groups of four per cage on a 12 h light-dark cycle. Haloperidol (Sigma) was dissolved in 0.5 ml of 0.1 N HCl and brought up to the final concentration in 0.9% saline. All mice received the same number and volume (100 l) of injections, haloperidol (1 mg per kg body weight) or vehicle intraperitoneally. In the acute paradigm, each mouse received two injections, 20 min apart, and was killed 2 h later. In the chronic paradigm, mice received haloperidol (1 mg per kg body weight) or vehicle for 12 d and were killed 2 h after the last injection. All mouse experiments were approved by the Rockefeller University Institutional Animal Care and Use Committee.

Statistical analysis. We compared means and standard deviations of total protein or phosphorylation levels with the use of the unpaired two-tailed *t*-test or the Mann-Whitney test when appropriate. We carried out single-locus and multilocus haplotype association analysis using the program PDTPHASE v2.3 (ref. 29), a test of linkage disequilibrium in general pedigrees. PDTPHASE v2.3 includes extensions to deal with haplotypes and missing data of the program PDT. Maximum-likelihood gametic frequencies under the null were calculated with an EM algorithm. In all analyses, we retained only the haplotypes with a frequency >3% to avoid the application of statistics to a small number of chromosomes.

Assay for PPI of the acoustic startle response. We generated *Akt1*$^{-/-}$ mice as described[5] and back-crossed the mutation onto the C57Bl/6 genetic background. Loss of expression of AKT1 results in partial lethality probably during the early neonatal period[5]. But the surviving *Akt1*$^{-/-}$ pups continue to grow into adulthood and are fertile but weigh less than their wild-type littermates (regardless of sex). Adult *Akt1*$^{-/-}$ and wild-type littermate mice were housed individually for 2 weeks before testing. Testing was carried out in a SR-Lab system (San Diego Instruments). Response amplitude was calculated as the maximum response level occurring during the 100-ms recording. Because mice can, in principle, habituate to the prepulse as well as to the startle stimulus, we kept the number of trials to a minimum. Immediately after placement in the chamber, the mice were given a 5-min acclimation period, during which background noise (67 dB) was continually present, and then received ten sets of the following five trial types distributed pseudorandomly and separated by an average of 15-s intertrial intervals. Trial 1: 40 ms, 120-dB noise burst alone; trials 2–4: 120-dB startle stimuli preceded (by 100 ms) by a 20-ms, 78-dB, 82-dB or 90-dB noise burst (prepulse); trial 5: no stimulus, background noise alone (67 dB). Data were analyzed with ANOVA with repeated measures. We included 10 *Akt1*$^{-/-}$ and 11 wild-type littermates in each analysis.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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