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Increased expression of Kalirin-9 in the auditory cortex of schizophrenia subjects: Its role in dendritic pathology

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ABSTRACT

Reductions in dendritic arbor length and complexity are among the most consistently replicated changes in neuronal structure in post mortem studies of cerebral cortical samples from subjects with schizophrenia, however, the underlying molecular mechanisms have not been identified. This study is the first to identify an alteration in a regulatory protein which is known to promote both dendritic length and arborization in developing neurons, Kalirin-9. We found Kalirin-9 expression to be paradoxically increased in schizophrenia. We followed up this observation by overexpressing Kalirin-9 in mature primary neuronal cultures, causing reduced dendritic length and complexity. Kalirin-9 overexpression represents a potential mechanism for dendritic changes seen in schizophrenia.

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Introduction

Recent studies have identified several electrophysiological, morphological and cellular changes within the auditory cortex of schizophrenia subjects (Lewis and Sweet, 2009). Individuals with schizophrenia have reduced auditory cortex gray matter volume, already present at the first episode of psychosis (Hirayasu et al., 2000; Kasai et al., 2003; Salisbury et al., 2007). Related deficits in auditory sensory processing, evidenced by a reduced ability to discriminate pure tones, are also present in subjects with schizophrenia (Javitt et al., 2000; Leitman et al., 2010; Strous et al., 1995). Impaired tone discrimination is also correlated with reduced magnitude of Mismatch Negativity (MMN), an event-related potential arising from auditory cortex after auditory stimuli that deviate from a repetitive stimulus in one characteristic (e.g. pitch) (Javitt et al., 1994, 2000).Tone discrimination impairments correlate with core negative symptoms of this illness such as impairments in detecting spoken emotional tone, in phonologic processing and in reading attainment (Arnott et al., 2011; Javitt, 2009; Leitman et al., 2005, 2010).

Tone discrimination depends on the primary auditory cortex (AI), contained within Heschel's gyrus (HG), which sharpens the frequency representations present at lower levels of auditory processing (Liu et al., 2007; Oswald et al., 2006). Similarly tuned and reciprocally connected layer 3 pyramidal cells in AI excite each other, selectively amplifying the thalamocortical signal (Liu et al., 2007; Ojima et al., 1991; Wallace et al., 1991). MMN similarly reflects activity within layer 3 circuits of AI, arising after the initial thalamic volley, and is dependent on excitatory neurotransmission (Javitt et al., 1994). A previous study identified a 27% reduction in the density of spinophilin immunoreactive structures which serve as a marker of dendritic spines within deep layer 3 of AI in subjects with schizophrenia (Sweet et al., 2009). Dendritic spines are necessary components of excitatory glutamatergic signaling and spine density was correlated with the density of non-selectively labeled pre-synaptic axon boutons (Sweet et al., 2009). These findings establish dendritic pathology in the primary auditory cortex which likely contributes to the reduced auditory cortex gray matter volume in subjects with schizophrenia and to an impaired spread of activation within the layer 3 pyramidal cell networks of AI.

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While dendritic spines are responsible for receiving excitatory signals, the degree of connectivity as well as the integration and propagation of these excitatory signals are dependent on multiple dendritic structural elements. Such elements include the overall extent of the dendritic tree and its degree of aborization, both of which may be relevant to pathophysiological changes in schizophrenia. In fact, among the most consistently replicated findings in post mortem studies of schizophrenia subjects are decreases in dendritic arbor length and dendritic complexity (Black et al., 2004; Broadbelt et al., 2002; Glantz and Lewis, 2000; Kalus et al., 2000). Pyramidal cell activation is dependent on numerous electrophysiological events including excitatory post-synaptic potential signal attenuation as a function of distance from the soma and back propagation of action potentials (Larkum et al., 2001; Williams, 2004). Both of these properties may be affected by a decrease in dendritic length. Additionally, pyramidal cells have been noted to have precisely targeted apical and basal inputs from other layers and cortical regions (Petreanu et al., 2009). Selective alterations to these connections may contribute to the excitatory signal dependent deficits in AI of schizophrenia subjects through either a reduction or desynchronization of signals received from thalamocortical and corticocortical inputs, thereby disrupting auditory stimulus detection and refinement. To date no potential molecular mechanisms contributing to dendritic pathology in schizophrenia have been identified.

Within the complex molecular network that influences dendritic morphology and connectivity, converging lines of evidence support alteration of one key regulator, Kalirin, in the pathogenesis of schizophrenia. Kalirin is a GDP/GTP exchange factor which activates the Rho family of GTP binding proteins (Alam et al., 1997; Cerione and Zheng, 1996). Four major isoforms of Kalirin, generated through alternative splicing, are expressed in adult CNS (Johnson et al., 2000). A previous study noted a reduction in Kalirin mRNA in the dorsolateral prefrontal cortex in schizophrenia, using a probe sequence common to the Kalirn-7 and Kalirin-5 isoforms (Hill et al., 2006). Several rare missense mutations unique to regions of the Kalirin gene expressed only in the Kalirin-9 and Kalirin-12 splice variants have been found to be associated with schizophrenia (Kushima et al., 2010). While such rare mutations are unlikely to explain many cases of disease, they can identify pathways in which convergent alterations contribute to disease, for example as seen with rare cases of early onset Alzheimer Disease resulting from mutations in *APP* (Selkoe, 2005). Prior in vitro studies have demonstrated an interaction between the schizophrenia susceptibility molecules DISC1 and ERBB4 and Kalirin-7 via a sequence also shared with Kalirin-5, although whether other Kalirin isoforms may interact with these molecules through another mechanism was not tested (Cahill et al., 2011; Hayashi-Takagi et al., 2010).

Each isoform appears to have unique temporal and spatial expression patterns as well as potentially unique functional roles in both development and continuing dendritic plasticity. Kalirin-9 and 12 are expressed through all developmental stages including adulthood, while Kalirin-7 is not expressed at birth with detectable expression first apparent at postnatal days 7 to 10 in rats and high expression levels in adulthood (Ma et al., 2003). When evaluated in immature primary cortical neuronal cultures, Kalirin-7 was found to be primarily expressed in dendritic spines, whereas Kalirin-9 was more uniformly distributed across dendritic processes and the cell soma and Kalirin-12 was largely isolated to the cell soma (Johnson et al., 2000).

Differences in the functional domains present in each Kalirin isoform also suggest different potential regulatory roles in development and plasticity. All four of the major Kalirin isoforms expressed in the CNS contain a GEF domain which activates Rac1 (Rabiner et al., 2005). Both Kalirin-9 and 12 contain a second GEF domain which activates Rho A (Rabiner et al., 2005). Rac1 is a regulator of dendritic remodeling, while RhoA is a regulator of dendritic growth (Li et al., 2000). Kalirin-7 and Kalirin-5 have a C terminus PDZ binding domain which both Kalirin-9 and Kalirin-12 lack. The PDZ domain interacts with PSD95 in the post synaptic density allowing targeting to dendritic spines (Penzes et al., 2001b).

Kalirin-7 has been evaluated in immature neuronal culture systems, where its underexpression results in decreases in spine emergence (Penzes et al., 2001b). In mature neuronal cultures, Kalirin-7 reductions lead to diminished spine persistence and reduced spine enlargement after long-term potentiation (Xie et al., 2007). These findings suggest that a reduction in Kalirin-7 may contribute to the reduction in dendritic spines noted in schizophrenia. In cultures of immature primary cortical neuron cultures, overexpression of Kalirin-9 results in the elongation of processes but not in the formation of additional processes (Penzes et al., 2001a). However, the role of Kalirin-9 in mature neurons is unknown. Given that overexpression of Kalirin-9 is known to increase dendritic length, its underexpression is a potential candidate contributing to the reduction in dendritic length in schizophrenia. The functions of Kalirin-5 and 12 for dendritic and spine development are less well characterized (Rabiner et al., 2005). We therefore evaluated Kalirin isoform expression in the primary auditory cortex in subjects with schizophrenia. Our objectives were to identify potential differences in Kalirin isoform protein expression in subjects with schizophrenia and to model any changes in protein expression in neuronal culture to decipher their possible structural implications.

Methods

Human subjects

A total of 26 subjects with a diagnosis of either schizophrenia or schizoaffective disorder were each matched to a control subject for sex, as closely as possible for age and post-mortem interval (PMI), and to the extent possible, for handedness (Supplemental Table 1). All brain specimens were obtained during autopsy at the Allegheny County Medical Examiner's Office after obtaining consent from the next of kin. An independent panel of experienced clinicians made consensus DSM-IV diagnoses using a previously described method (Glantz and Lewis, 2000) approved by the University of Pittsburgh Institutional Review Board. The right hemisphere was blocked coronally at 1–2 cm intervals and the resultant slabs snap frozen in 2-methyl butane on dry ice, and stored at −80 °C. Medications used at time of death were identified by the presence of an active prescription at the time of death or a positive toxicology screen at autopsy.

Sample preparation

Tissue slabs containing the superior temporal gyrus (STG) with an evident Heschl's Gyrus (HG) located medial to the planum temporal were identified and matched on rostral-caudal level within pairs (Sweet et al., 2003, 2004). From these slabs, the STG was removed as a single block (Supplemental Fig. 1). Gray matter was collected from HG by taking 40 μm sections, and frozen at -80 °C. Total protein was extracted using SDS extraction buffer (0.125 M Tris–HCl (pH 7), 2% SDS, and 10% glycerol) at 70 °C. Protein concentration was estimated using a bicinchoninic acid assay (BCA™ Protein Assay, Pierce). Pairs were run together, and assayed in triplicate. The final protein concentration utilized for each sample was the mean of the triplicate runs.

Western blotting

This study consists of 26 matched pairs of control and schizophrenia subjects examined in 13 runs, with 2 pairs per run and each run consisting of 4 gels. Each gel contained two different pairs loaded using a total of 8 lanes so that the control member of each pair appears on 2 lanes and the schizophrenia member of each pair appears on 2 lanes, with members of a pair run in adjacent lanes. The experimenter was blind to diagnosis during experimentation and quantification of blots. Pilot studies were used to establish conditions providing for linear detection of all target proteins (Supplemental Fig. 2). Based on these studies, 25 μg

of protein was aliquoted in 1x LI-COR Protein Loading Buffer (Licor Inc. Lincoln, Nebraska, USA), loaded on 4–20% SDS-PAGE gradient gels (Thermo Scientific, Rockford, Illinois, USA), and separated for 2 h at room temperature in 1X SDS running buffer (Pierce 20X Tris Hepes SDS Buffer) at 75 V. Samples were then transferred to 0.45 μm nitrocellulose membrane (Licor) for human study samples [or for comparison with Kalirin KO mice to 0.45 μm PVDF (Millipore, Billerica, Massachusetts, USA)] in 1x Tris Glycine Blotting Buffer (Pierce) at 85 V for 50 min at 4 °C. Membranes were incubated for 1 h in Odyssey LiCor Blocking Buffer diluted 1:1 in 1x TBS. The membrane was incubated overnight in primary antibodies which recognized the spectrin domain of 4 Kalirin isoforms (5, 7, 9 and 12) (rabbit anti-Kalirin spectrin, Millipore # 07-122) diluted 1:500, mouse anti-β-tubulin (Millipore # 05-661) diluted 1:20,000, mouse anti-NR1 (BD Biosciences, #556308) diluted 1:1000, mouse anti-synaptophysin (Sigma, #S5768) diluted 1:3000, rabbit anti-spinophilin (Millipore, #AB5669) diluted 1:6000 in Pierce SuperBlock blocking buffer with 0.1% Tween 20 (Sigma-Aldrich, St. Louis, Missouri, USA). Membranes were then incubated in LiCor IRDye secondary antibodies (goat anti-rabbit 800 nm; goat anti-mouse 680 nm) 1:10,000 in Odyssey Licor Blocking Buffer diluted 1:1 with TBS (0.1% Tween 20 + 0.02% SDS). Blots were scanned while wet and bands detected using a Li-Cor Odyssey Infrared Scanner set at a resolution of 42 μm and the highest image quality.

Quantification of western blots

Images were quantified using MCID Core Version 7.0 (InterFocus Imaging Ltd., Linton, Cambridge, UK). The peak for each of the 4 isoforms of Kalirin and β-tubulin on the histogram were independently aligned to a single point for all lanes from all blots by translating each lane along the distance axis. Once aligned, a band definition encompassing the full range of each band was applied uniformly to all lanes from all blots in the study on the histogram for each protein (Supplemental Fig. 1e). The use of alternative band definitions confined more narrowly to the intensity peaks for each isoform gave highly correlated results (data not shown). The integrated intensity (mean intensity × number of pixels) was acquired for each protein.

Enrichment of PSD fractions in human tissue

PSD enrichment was based on a modification of methods described previously (Hahn et al., 2009; Phillips et al., 2001). 500–700 mg tissue was homogenized on ice in 1.5 ml 0.32 M sucrose, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 150 μl protease inhibitor (Sigma, #P8340) and 150 μl phosphatase inhibitor (Sigma, #P0044) with teflon (15 up and down strokes). The resulting homogenate was mixed in a solution containing (final) 1.25 M sucrose, 0.15 mM $MgCl_2$, 39 μM $CaCl_2$ and overlaid with 1 M sucrose for centrifugation at 100,000 × *g* for 3 h at 4 °C. Synaptosomes were harvested at the 1.25 M/1 M sucrose interface, incubated in 1% Triton X-100, 0.1 mM $CaCl_2$, 20 mM Tris, pH 6.0, shaken at 4 °C for 30 min and centrifuged at 40,000 × *g* for 30 min at 4 °C. The resulting pellet composed of synaptic junctions was resuspended in 20 mM tris buffer, 1% Tx-100 in 0.1 mM $CaCl_2$, pH 8.0 and homogenized with 10 strokes of a Teflon homogenizer. The homogenate was then incubated for 30 min at 4 °C and centrifuged at 40,000 × *g* at 4 °C for 30 min. The PSD pellet was resuspended in 150 μl 1% SDS for use in immunoblotting. The supernatant containing the presynaptic fraction was filtered using an Amicon Ultra-15, Ultracel-50 (Millipore, #UFC905024) at 5000 × *g*, precipitated with acetone, and resuspended in 1% SDS.

Statistical analyses

Assessment of diagnostic group effect

The Kalirin-5, Kalirin-7, Kalirin-9, and Kalirin-12 isoform expression levels were examined separately, expressed as the ratio of each isoform of Kal protein (Kal5, Kal7, Kal9, Kal12) level to the level of tubulin protein, a normalizing protein that does not differ between diagnostic groups ($t_{21.8} = -0.48$, $p = 0.63$). The response variable for each of these four analyses was the natural logarithm of the ratio, a standardly used transformation when doing linear modeling which is based on the normal distribution. For each subject, up to 8 measurements of this log ratio were made for each Kalirin isoform. Each analysis used two linear mixed models, in which subject and gel were treated as random effects due to the fact that the log ratio measurements are repeated within each subject and within each gel. The primary model treated diagnosis, pair nested in run, and run as fixed effects, while tissue storage time and brain pH were treated as covariates. The secondary model ignored subject pairings and instead replaced the fixed effect of pair nested in run by the covariates gender, age, and PMI with tissue storage time and brain pH as additional covariates. All tests were two-sided and conducted at the 0.0125 significance level to maintain an overall type 1 error rate of 0.05 for simultaneous testing of the four isoforms. The p-values for diagnostic group effect are based on the contrast of control effect minus schizophrenia effect. Age and gender effects were assessed by the secondary model, and their interactions with diagnosis were also assessed in a model identical to the secondary model only with the inclusion of the relevant interaction term.

Assessment of possible confound effects

The relationship of the natural logarithm of the tubulin normalized Kalirin expression ratios within each pair to each of the following confounds was also examined: schizoaffective diagnosis (yes/no), suicide as cause of death (yes/no), antipsychotic use at time of death (yes/no), history of substance use disorder (yes/no), age of onset, and duration of schizophrenia. Log tubulin normalized ratios for each Kalirin on each gel were obtained by averaging Kalirin and Tubulin expression levels of Schizophrenia subject and Control subject over the gel separately, getting two Tubulin normalized Kalirin values—[$Kalirin_{Schz}$/$Tubulin_{Schz}$] and [$Kalirin_{CNTL}$/$Tubulin_{CNTL}$], and then getting the log ratio of the two Tubulin normalized Kalirin values. In computing the log tubulin normalized ratios for a subject, if a Kalirin (or tubulin) measurement was missing on one lane, the corresponding tubulin (or Kalirin) measurement was also treated as missing on that lane. The analyses for each Kalirin isoform used linear mixed models to model the log tubulin normalized Kalirin ratio, where each of the confounds was treated as a fixed effect, and where gel and pair were treated as random effects. All tests were two-sided and conducted at the 0.05 significance level. No adjustments were made for multiple comparisons.

All analyses were implemented in SAS PROC MIXED (Version 9.2, SAS Institute Inc., Cary, NC).

In vitro studies

Cultured cortical neurons were prepared from Sprague–Dawley rat E18 embryos. Coverslips were coated with poly-D-lysine (0.2 mg/ml, Sigma), and neurons were plated onto these coverlips in feeding media with 5% fetal calf serum. After 1-hour, the media was changed to feeding media comprised of Neurobasal media with B27 (Invitrogen, Carlsbad, CA, USA) and 0.5 mM glutamine. 4 days later, 200 μM D,L-aminophosphonovalerate (D,L-APV, Ascent Scientific, Princeton, NJ, USA) was added to the media. Neurons were fixed in 4% formaldehyde and cold methanol at DIV25-28.

Neuronal transfections

Myc-tagged Kalirin-9 was co-expressed with GFP for 2-weeks. Neurons were DIV26 at the time of fixation. Neurons were fixed for 10-minutes in 3.7% formaldehyde followed by 10-minutes in cold methanol. Cells were immunostained using polyclonal anti-GFP and monoclonal anti-Myc primary antibodies. Secondary antibodies used were rabbit Alexa 488 and mouse Alexa 568.

For spine morphology analysis, confocal images of single- and double-stained neurons were obtained with a Zeiss LSM5 Pascal confocal microscope. Images of neurons were taken using the 63x oil-

immersion objective as a z-series of three to eight images, averaged four times, taken at 0.37 μm intervals, 1024×1024 pixel resolution at a scan speed of 2.51 μs/pixel. Two-dimensional maximum projection reconstructions and quantitative spine analysis of images were conducted using MetaMorph software (Universal Imaging). To assess dendrite length and complexity, neurons were imaged using a Zeiss Axioplan2 upright microscope. Images were taken with a 10× objective (NA = 0.17) and micrographs acquired using a Zeiss AxioCam MRm CCD camera. Dendrites were traced and measured using Image J utilizing the appropriate measurement correction factor for the Zeiss microscope. Sholl analysis was performed using Neuronstudio (Mount Sinai). The number of dendritic intersections was determined using 25 μm increments from the soma and plotted.

For the spine analysis 16 cells were analyzed for GFP alone and 11 cells were analyzed for GFP + Kalirin-9. For the dendrite analysis 12 cells were analyzed for GFP and 10 cells were analyzed for GFP + Kalirin-9.

Immunofluorescence staining

For endogenous Kalirin-9 and PSD-95 immunostaining, DIV 26 cultured cortical neurons were fixed for 10-minutes in 3.7% formaldehyde followed by 10-minutes in cold methanol. Neurons were immunostained with a polyclonal anti-Kalirin-9 primary antibody (Johns Hopkins University) and with a monoclonal PSD-95 primary antibody (Neuromab). Secondary antibodies used were rabbit Alexa 488 and mouse Alexa 568.

Results

Each of the 4 primary Kalirin isoforms was detected by western blotting in human postmortem auditory cortex tissue (Fig. 1a) and protein levels did not significantly differ with postmortem interval in mouse (Supplemental Fig. 3). Analysis of schizophrenia and control subjects revealed a significant effect of diagnosis for normalized Kalirin-9 expression ($t_{23.7} = -3.21$, $p = 0.003$) (Figs. 1b,c). Unexpectedly, there was an increase in Kalirin-9 expression in schizophrenia subjects relative to controls, present in 20/26 subject pairs and equating to an 11.3% increase in protein levels. This finding was surprising, as short term Kalirin-9 overexpression in immature rat cortical neurons increased dendritic length (Penzes et al., 2001a). Diagnosis was not significantly associated with the normalized expression of any of the other isoforms (Kalirin-5 $t_{25.4} = -0.56$, $p = 0.58$; Kalirin-7 $t_{25.4} = 0.04$, $p = 0.97$; Kalirin-12 $t_{23.8} = -0.78$, $p = 0.44$) (Fig. 1c). These results were unchanged when excluding the effects of pH and storage time from our models.

Additional analyses revealed associations of age with normalized expression of all isoforms, with decreasing protein expression with increasing age (Fig. 2): Kalirin-5 ($t_{34} = -4.77$, $p < 0.001$), Kalirin-7 ($t_{32} = -3.16$, $p = 0.003$), Kalirin-9 ($t_{35.1} = -5.04$, $p < 0.001$), and Kalirin-12 ($t_{35.2} = -6.75$, $p < 0.001$). Males had significantly lower expression levels, relative to females for Kalirin-7 ($t_{27.1} = -2.28$, $p = 0.03$) and Kalirin-9 ($t_{29.2} = -2.28$, $p = 0.03$). There were no significant interactions between diagnosis and age or diagnosis and sex for any of the normalized isoforms (all $p > 0.1$). The magnitude of increase in Kalirin-9 was not significantly different between subject pairs including a subject with schizophrenia and subject pairs including a subject with schizoaffective disorder ($t_{23.8} = 0.03$, $p = 0.978$). There were no significant effects of several possible confounds including death by suicide, history of substance use disorder, or antipsychotic use at time of death on expression of Kalirin-9 and the other isoforms (all $p > 0.1$). Additionally, there was no significant correlation of the ratios (schizophrenia subject to its control subject) of normalized expressions of all isoforms with age of onset or duration of schizophrenia.

Since prior studies of Kalirin-9 compartmental localization and overexpression had been limited to short-term studies of immature primary neuronal cultures (Johnson et al., 2000; Penzes et al., 2001a), we evaluated the distribution of Kalirin-9 in mature primary cortical pyramidal neuron cultures and the long-term effects of Kalirin-9 overexpression to model the impact in the adult. Endogenous Kalirin-9 was strongly expressed in cortical pyramidal neuronal dendrites and dendritic

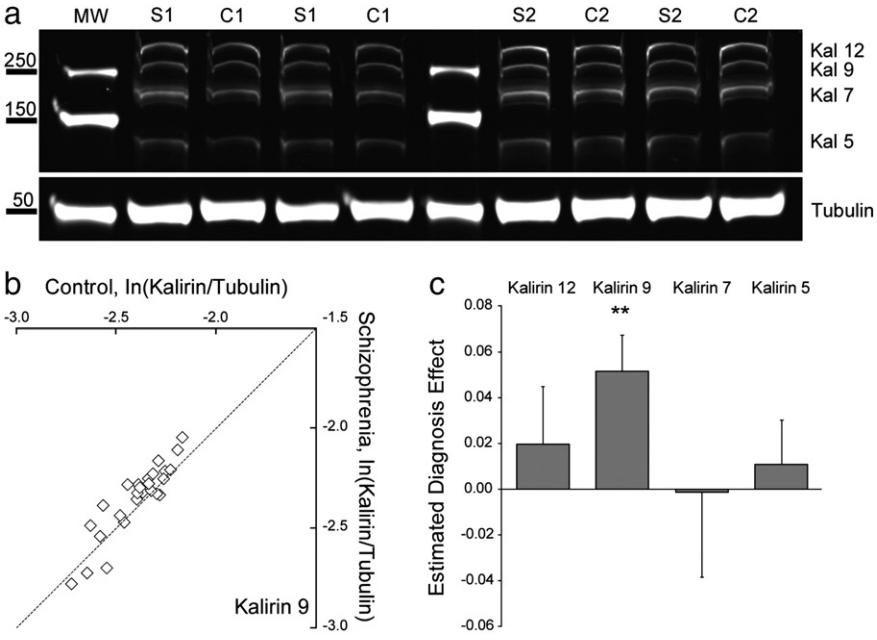

Fig. 1. a) An example blot from the comparison of subjects with schizophrenia (S) and Control (C) subjects. S1/C1 refers to Pair 2 in Supplemental Table 2. S2/C2 refers to Pair 4. b) Comparison of Kalirin-9 expression (normalized to tubulin expression) for the 26 pairs of subjects. Each point represents a pair of subjects. The diagonal reference line represents a control:schizophrenia ratio of one. Points above the line indicate increased expression in schizophrenia subjects. The increase in Kalirin-9 expression in schizophrenia subjects was significant ($p = 0.0038$). c) Adjusted mean pairwise difference in Kalirin isoform expression (normalized to tubulin expression). Error bars represent adjusted standard errors. ** $p = 0.0038$.

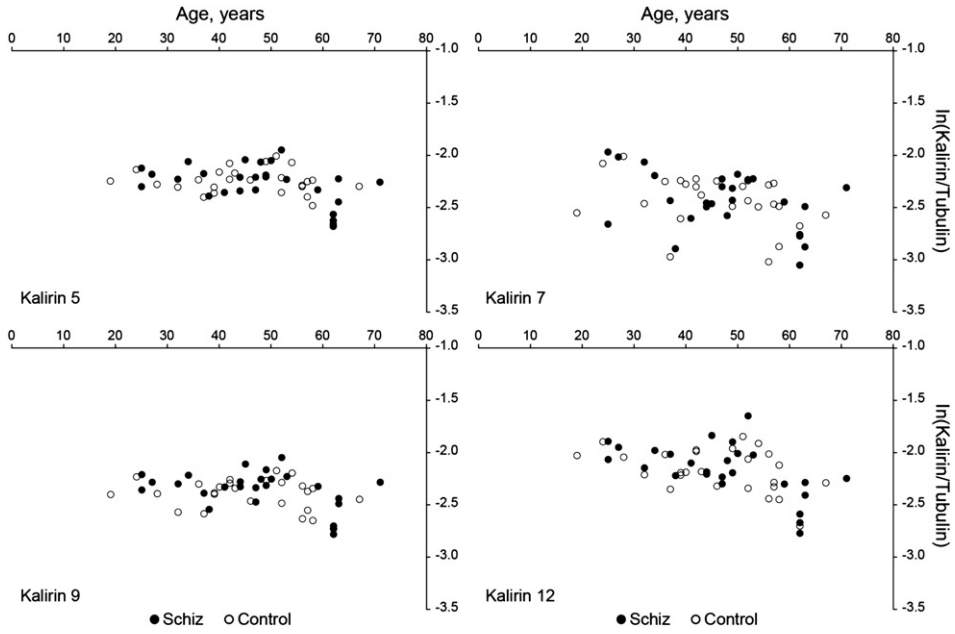

Fig. 2. The effect of age on Kalirin isoform expression (normalized to tubulin expression) in Schizophrenia and Control subjects. Increasing age was associated with significantly lower expression of all isoforms: Kalirin-5 ($t_{34} = -4.77$, $p<0.001$), Kalirin-7 ($t_{32} = -3.16$, $p = 0.003$), Kalirin-9 ($t_{35.1} = -5.04$, $p<0.001$), and Kalirin-12 ($t_{35.2} = -6.75$, $p<0.001$). There were no significant interactions between diagnosis and age for any of the isoforms (all $p>0.1$).

spines, with colocalization between Kalirin-9 and PSD-95 (Fig. 3a). We confirmed the presence of Kalirin-9 in the spine compartment in humans using a PSD enriched isolate from the cerebral cortex of human subjects (Fig. 4).

Surprisingly, after 2 weeks of Kalirin-9 overexpression, a statistically significant reduction in total dendritic length was detected (Fig. 3b). Sholl analysis further revealed a reduction in dendrite complexity in both the apical and basal trees. Both the reductions in dendritic length and complexity parallel changes found in post mortem studies of schizophrenia (Black et al., 2004; Broadbelt et al., 2002; Glantz and Lewis, 2000; Kalus et al., 2000). These effects were not present when Kalirin-9 was overexpressed for only 3 days (Supplemental Fig. 4). A small, but statistically significant increase in spine density was also detected, with no significant change in spine area. Total apical spine number (spine density per μm × total apical dendrite length) did not reveal differences between GFP alone and GFP with Kalirin-9.

Discussion

A notable strength of the current study is the use of a reverse translational approach to reveal several novel and unanticipated findings. Kalirin-9 expression was increased in subjects with schizophrenia and sustained overexpression of Kalirin-9 in a neuronal cell culture model caused detrimental effects on dendrites. These latter findings correspond well with structural changes detected previously in post mortem studies of schizophrenia subjects, in whom reductions in dendritic arborization and dendrite length have been reported in multiple cortical regions (Black et al., 2004; Broadbelt et al., 2002; Glantz and Lewis, 2000; Kalus et al., 2000). Also of note was the discovery that Kalirin-9 is localized to the post-synaptic density.

Finding increased expression of Kalirin-9 in schizophrenia subjects assessed after long-term illness leaves open the question of whether Kalirin-9 overexpression is a contributor to disease or a consequence of the illness. The in vitro findings of this study suggest that upregulation of Kalirin-9 contributes to primary dendritic pathology establishing a plausible disease pathway. This conclusion must be tempered by the limitations of this model system. For example, we cannot exclude the possibility that acute Kalirin-9 overexpression may alter endogenous expression of other Kalirin isoforms. It should also be noted that our use of 4 week old primary neuronal cultures, chosen to be reflective of adolescent or early adult neurodevelopment, does not model the effects of altered Kalirin-9 expression through the dynamic neurodevelopmental process. This limitation may be relevant, as Kalirin-9 expression leads to process elongation in immature neuronal cultures, but reduced dendritic complexity in mature neurons (via a maturation dependent mechanism which is not known), leaving open the question of the net effect of these opposing tendencies over the full neurodevelopmental timeline.

Nevertheless, the association of mutations in Kalirin with schizophrenia risk in some subjects would also support the interpretation that alterations in Kalirin-9 can contribute to the primary pathogenesis of schizophrenia and do not reflect a change secondary to the disease state (Kushima et al., 2010). While it is not known whether any of these mutations may affect Kalirin protein expression, the most common mutation, P2255T, results in a substitution of a threonine for a proline that is predicted to create several additional phosphorylation sites in close proximity to the RhoA GEF domain unique to both Kalirin-9 and 12 (Kushima et al., 2010). Such a change would likely effect net Kalirin-9 activity as Kalirin activity has been demonstrated to be regulated through phosphorylation (Rabiner et al., 2005). Several of the current findings also argue against increased Kalirin-9 expression emerging solely as a consequence of disease. Kalirin-9 expression was not correlated with illness duration or several possible confounds of chronic schizophrenia including death by suicide, substance use, or antipsychotic use at time of death in our subjects.

Several lines of prior evidence have linked the Kalirin-7 isoform to schizophrenia. This includes evidence for reduced expression of Kalirin mRNA in prefrontal cortex of subjects with schizophrenia detected using a probe that identifies the Kalirin-5 and Kalirin-7 isoforms (Hill et al., 2006) and evidence from model systems that Kalirin-7 interacts with DISC1 and ERBB4 (Cahill et al., 2011; Hayashi-Takagi et al., 2010). Additionally, strong evidence from studies in model systems have indicated that reduced expression of Kalirin-7 is deleterious to dendritic spines (Ma et al., 2003; Penzes et al., 2000, 2001b, 2003; Xie et al., 2007). It should be noted that our findings do not preclude a role for Kalirin-7 in schizophrenia. The possibility remains that a reduction in Kalirin-7 protein in schizophrenia is present in brain regions other than auditory cortex (e.g. prefrontal cortex). Additionally, the

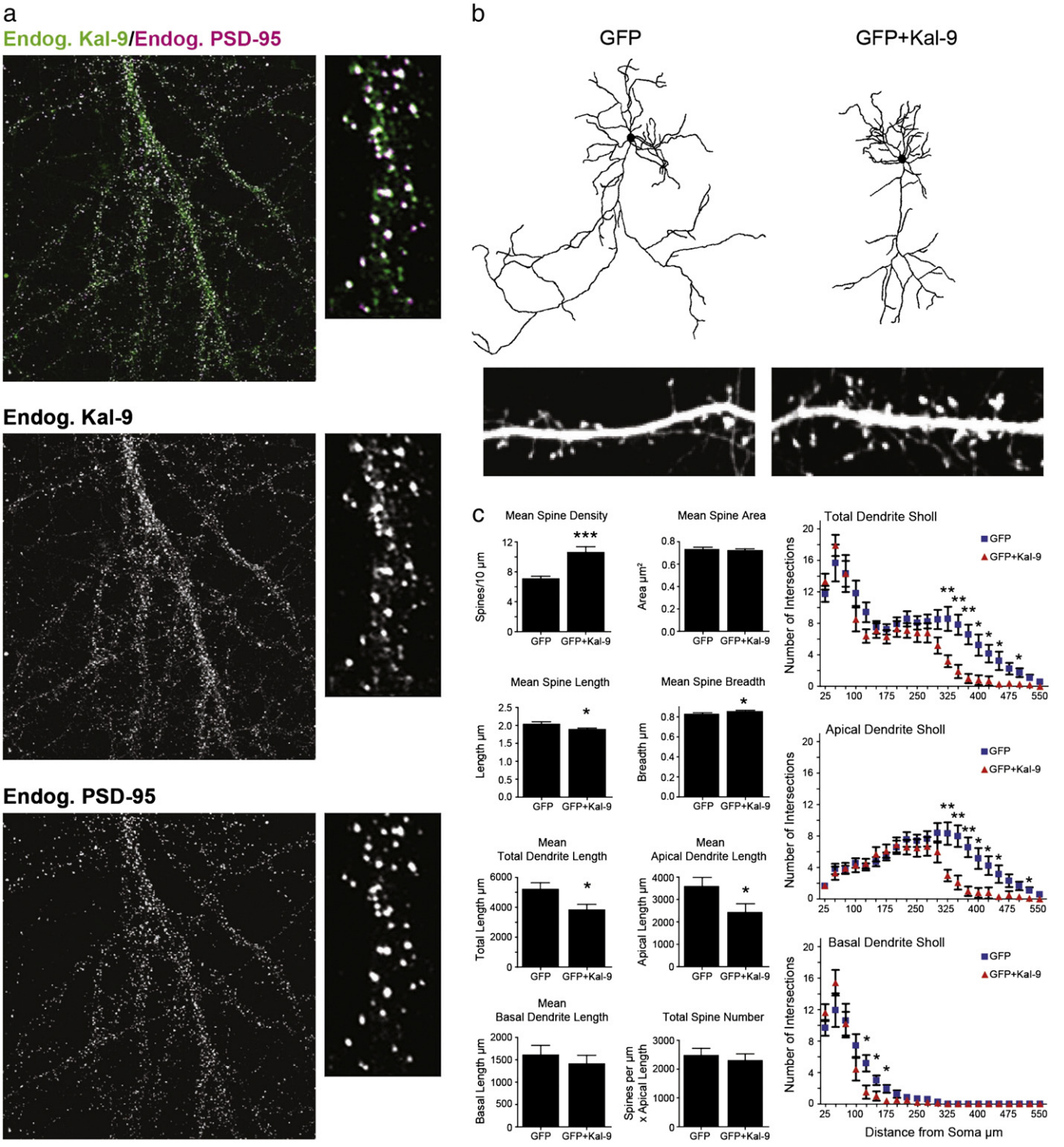

Fig. 3. a) DIV26 cultured cortical neurons were immunostained for endogenous Kalirin-9 and endogenous PSD-95. Left images show the dendritic tree of a representative neuron. The images to the right show a high magnification image of spines protruding from the dendritic shaft. The Kalirin-9 and PSD-95 channels are shown separately, and together, to reveal colocalization. Kalirin-9 colocalized with PSD-95 in spines. b) DIV26 cultured cortical neurons overexpressing either GFP alone or in combination with 2-week Kalirin-9 overexpression. Representative images of dendrite tracings and spine images are shown. c) Quantification of the effects of 2-week Kalirin-9 overexpression on spine density and dendrite morphology. Kalirin-9 overexpression resulted in a significant increase in spine density ($p<0.001$) with no effect on spine area. Kalirin-9 overexpression reduced total dendritic length and reduced apical dendritic length ($p<0.05$). Basal dendrite length was not affected. Sholl analysis revealed a reduction in the complexity of the total dendritic tree with reduced complexity evident in both basal and apical dendrites. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

effect of Kalirin-7 on spines is regulated by phosphorylation (Kushima et al., 2010) and protein–protein interactions (Hayashi-Takagi et al., 2010), either of which might be altered in disease independently of any changes in total protein expression. Although we did not see any relationship of disease duration with Kalirin-7 expression, we did find age dependent decreases in expression of all isoforms, including Kalirin-7. Thus it is possible that Kalirin-7 plays a larger role in the development of auditory cortex spine deficits in schizophrenia at other stages of the disease process than sampled in this study. For example, gray matter volume reductions appear to be developing in the auditory cortex of

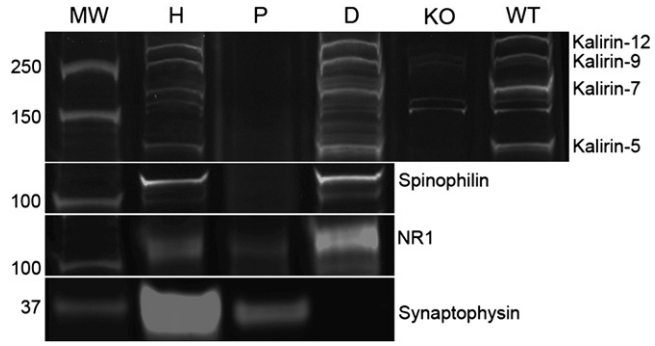

Fig. 4. Example western blot of Kalirin isoforms in post-synaptic density (PSD) enriched fraction from human cortical gray matter from a 53-year-old female subject with a PMI of 13 h. 15 μg of protein from human samples and 10 μg of protein from mouse (Kal KO and WT) were loaded. Spinophilin and n-methyl-D-aspartate receptor 1 (NR1) are postsynaptic markers while synaptophysin indicates a presynaptic marker. H = total protein extracted from a cortical gray matter homogenate from the same human subject, P = presynaptic fraction, D = PSD, KO = total protein extracted from brain homogenate from a Kalirin knock-out mouse, WT = total protein extracted from brain homogenate from a wild-type mouse. A previously described (Penzes et al., 2000) non-specific band with an apparent MW of ~150 can be detected in the KO.

subjects with schizophrenia in the time period surrounding the first episode of illness in adolescence and early adulthood (Salisbury et al., 2007; Takahashi et al., 2009). Finally, when considering spine pathology it is important to note that Kalirin-7 is just one piece of multiple pathways that converge on common downstream effectors to regulate actin dynamics and spine plasticity (Calabrese et al., 2006; Matsuzaki, 2007; Negishi and Katoh, 2005).

The results of this study should be considered cautiously pending replication. However, several strengths of this study enhance confidence in these findings. Such strengths include the use of a moderately large human tissue cohort, with sudden causes of death and indices of excellent tissue preservation. Initial studies established the linearity of our assay conditions allowing interpretation of the relative change in fluorescence intensities. We similarly established the absence of significant postmortem effects on isoform expression for the intervals represented within our cohort in a mouse model. Subjects with schizophrenia and matched control subjects were processed in pairs through all stages of the assay, including tissue harvesting, protein extraction and measurement, and western blotting. Since gel effects in western blotting can be substantial, this latter design element in which pairs of subjects were present in adjacent lanes, with replicates of each pair both within and across gels, was essential to reduce variability in our diagnostic comparison. Moreover, we used analytic models that explicitly addressed these nested repeated comparisons, enhancing the precision of our estimate of the diagnosis effect.

Further exploration of the role of Kalirin-9 in the pathogenesis of schizophrenia will require a detailed analysis of both upstream and downstream regulatory networks specific to this isoform. Kalirin-9 colocalization with PSD-95 at excitatory synapses suggest that it may be subject to regulation by synaptic signaling processes, as previously demonstrated for the Kalirin-7 isoform (Hayashi-Takagi et al., 2010; Penzes et al., 2003; Xie et al., 2007). Conversely, in order to elucidate the downstream regulatory cascade which may induce the deleterious structural changes demonstrated in Kalirin-9 overexpression, consideration of individual functional domains is likely to be informative. Of particular interest are the two GEF domains within Kalirin-9 that activate RhoA and Rac1, effectors with complementary actions on dendritic complexity. Increased expression of RhoA has been shown to decrease dendritic length whereas increased Rac1 expression has been demonstrated to reduce the number of dendritic branch additions and dendritic complexity (Li et al., 2000). These studies suggest that Kalirin-9 overexpression in mature neurons reduces dendritic length through increased RhoA activity and reduces overall dendritic complexity through increased Rac1 activity. Future development and evaluation of interventions to prevent, or reverse, dendritic impairments in disease may benefit from targeting these pathways in combination.

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