PAK1 Protein Expression in the Auditory Cortex of Schizophrenia Subjects

Anthony J. Deo1,2*, Isaac M. Goldszer1, Siyu Li3, James V. DiBitetto1, Ruth Henteleff1, Allan Sampson3, David A. Lewis1,4, Peter Penzes5,6, Robert A. Sweet1,7,8*

1 Translational Neuroscience Program, Department of Psychiatry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, United States of America, 2 Physician Scientist Training Program, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, United States of America, 3 Department of Statistics, University of Pittsburgh, Pittsburgh, Pennsylvania, United States of America, 4 Department of Neuroscience, University of Pittsburgh, Pittsburgh, Pennsylvania, United States of America, 5 Department of Physiology, Northwestern University Feinberg School of Medicine, Chicago, Illinois, United States of America, 6 Department of Psychiatry and Behavioral Sciences, Northwestern University Feinberg School of Medicine, Chicago, Illinois, United States of America, 7 Department of Neurology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, United States of America, 8 VISN 4 Mental Illness Research, Education and Clinical Center, VA Pittsburgh Healthcare System, Pittsburgh, Pennsylvania, United States of America

Abstract

Deficits in auditory processing are among the best documented endophenotypes in schizophrenia, possibly due to loss of excitatory synaptic connections. Dendritic spines, the principal post-synaptic target of excitatory projections, are reduced in schizophrenia. p21-activated kinase 1 (PAK1) regulates both the actin cytoskeleton and dendritic spine density, and is a downstream effector of both kalirin and CDC42, both of which have altered expression in schizophrenia. This study sought to determine if there is decreased auditory cortex PAK1 protein expression in schizophrenia through the use of quantitave western blots of 25 schizophrenia subjects and matched controls. There was no significant change in PAK1 level detected in the schizophrenia subjects in our cohort. PAK1 protein levels within subject pairs correlated positively with prior measures of total kalirin protein in the same pairs. PAK1 level also correlated with levels of a marker of dendritic spines, spinophilin. These latter two findings suggest that the lack of change in PAK1 level in schizophrenia is not due to limited sensitivity of our assay to detect meaningful differences in PAK1 protein expression. Future studies are needed to evaluate whether alterations in PAK1 phosphorylation states, or alterations in protein expression of other members of the PAK family, are present in schizophrenia.

Citation: Deo AJ, Goldszer IM, Li S, DiBitetto JV, Henteleff R, et al. (2013) PAK1 Protein Expression in the Auditory Cortex of Schizophrenia Subjects. PLoS ONE 8(4): e59458. doi:10.1371/journal.pone.0059458

Editor: Philipp J. Kahle, Hertie Institute for Clinical Brain Research and German Center for Neurodegenerative Diseases, Germany

Received May 31, 2012; Accepted February 18, 2013; Published April 22, 2013

This is an open-access article, free of all copyright, and may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose. The work is made available under the Creative Commons CC0 public domain dedication.

Funding: This work was supported by grants MH071533, MH084053 and MH071316 from the National Institutes of Mental Health. Anthony Deo was supported by a Clinical Research Fellow grant from the Doris Duke Charitable Foundation to the University of Pittsburgh School of Medicine. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Allan R. Sampson is a paid statistical consultant on design and analysis issues to Johnson & Johnson Pharmaceutical Research and Development. This does not alter his adherence to all the PLOS ONE policies on sharing data and materials.

* E-mail: sweetra@upmc.edu

* Current address: Department of Psychiatry, Columbia University, New York, New York, United States of America

Introduction

Recent studies have identified several electrophysiological, morphological and molecular changes in the auditory cortex of schizophrenia subjects. Individuals with schizophrenia have reduced auditory cortex gray matter volume [1;2] and deficits in auditory sensory processing. These deficits, evidenced by a reduced ability to discriminate pure tones, correlate with core negative symptoms of this illness such as impairments in detecting spoken emotional tone, in phonologic processing and in reading [3]. Impaired tone discrimination is also correlated with reduced magnitude of Mismatch Negativity (MMN), an event-related potential arising after auditory stimuli that deviate from a repetitive stimulus in one characteristic (e.g. pitch) [5;6].

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 [7;8]. Similarly tuned and reciprocally connected [9;10] layer 3 pyramidal cells in AI excite each other, selectively amplifying the thalamocortical signal [8]. MMN similarly reflects activity within layer 3 circuits of AI, arising after the initial thalamic volley, and is dependent on excitatory neurotransmission [11]. A previous study identified a 27% reduction in density of a marker of dendritic spines, post-synaptic components of excitatory glutamatergic signaling, within deep layer 3 of AI in subjects with schizophrenia [12]. Spine density was correlated with the density of non-selectively labeled pre-synaptic axon boutons [12;13]. These findings likely contribute 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. A reduction in dendritic spine density has also been observed in the dorsolateral prefrontal cortex in post-mortem studies of schizophrenia [14;15].

Dendritic spines remain plastic structures, with a proportion arising and retracting into adulthood [16–18]. A net elimination of spines will result from reduced spine emergence, persistence and/or increased spine retraction. Formation and maintenance of dendritic spines is dependent on the organization and stabilization...
of an actin cytoskeleton [19]. Hence, disturbances in molecular pathways known to regulate actin stabilization may contribute to abnormalities in dendritic spine number and morphology.

Of those proteins known to regulate actin stabilization, p21-activated kinase 1 (PAK1) is a plausible point of dysregulation contributing to a reduction in dendritic spines in schizophrenia, a premise supported by multiple lines of evidence. First, PAK1, a serine/threonine protein kinase, alters F-actin stabilization. PAK1 activates LIMK which phosphorylates cofilin, inactivating it so that it cannot depolymerize F-actin [20]. Second, overexpression of either dominant negative or kinase dead PAK1 constructs in hippocampal neuron cultures reduced spine density [21;22]. Dominant negative PAK1 also altered spine morphology [21]. Overexpression of wild type PAK1 or constitutively active PAK1 resulted in increased spine density [21;22]. In vivo models of reduced PAK1 expression also provide evidence of altered spine function and morphology, although less consistent evidence of reduced spine density are present. PAK1 knockout mice have been shown to have deficient long term-potentiation in hippocampal region CA1, with reduced F-actin in spines, although reductions in spine density were not present [23]. In contrast, PAK1/PAK3 double knockouts show a decrease in synaptic density and bidirectional synaptic plasticity [24], PAK1/PAK3 double knock-out was also associated with reduced numbers of spines with mature morphologies, although there was not a change in overall spine density due to increases in spines with immature morphologies [24]. Third, PAK1 is dually regulated by both kalirin (via Rac1) and CDC42. Kalirin has been shown to influence dendritic spine number and morphology [25;26], while CDC42 has been demonstrated to be involved with filopodia formation and actin stabilization [27]. mRNA for the kalirin-7 isoform and for CDC42 have been reported to be reduced in schizophrenia [28]. More recently we examined protein levels of kalirin isoforms in the auditory cortex of subjects with schizophrenia, finding increased levels of the kalirin-9 isoform, while the more abundant kalirin-5, kalirin-7, and kalirin-12 isoforms were unchanged [29].

We hypothesized that altered PAK1 protein expression may contribute to the reduction in dendritic spine density in the auditory cortex of schizophrenia subjects. We utilized quantitative western blotting to measure PAK1 levels in post mortem auditory cortex gray matter samples from 25 schizophrenia subjects and matched controls in whom we had previously measured kalirin isoform levels [28].

Results

PAK1 protein expression is not altered in schizophrenia

PAK1 protein level was not significantly different between the schizophrenia subjects and matched controls in our cohort ($t_{22.7} = -0.79$, $p = 0.44$). The distribution of PAK1 level was evenly distributed among schizophrenia and control subjects with 48% of schizophrenia subjects and 52% of control subjects having higher PAK1 levels (Figure 1 A–C).

There was no significant interaction between PMI group (High, Medium and Low) and diagnosis ($F_{2, 21} = 1.30$, $p = 0.29$). The 95% nominal confidence intervals for the diagnostic difference in the High, Medium and Low PMI groups were $(-0.19, 0.38)$, $(-0.18, 0.28)$ and $(-0.31, 0.11)$, indicating no effect of diagnosis in any of these subgroups.

We further examined whether PAK1 level (log scale) was selectively altered in additional subgroups of subjects (Figure 2). There was no difference in PAK1 levels between subject pairs when separated by sex ($t_{23} = -1.15$, $p = 0.26$), diagnosis of schizoaffective disorder ($t_{23} = -0.66$, $p = 0.52$), death by suicide ($t_{22.9} = 0.56$, $p = 0.58$), history of substance use disorder ($t_{22} = 0.49$, $p = 0.63$), or antipsychotic use at time of death ($t_{22.5} = 1.02$, $p = 0.32$). There was no significant correlation of the pairwise change in PAK1 levels with age of onset ($t_{23} = -0.17$, $p = 0.87$) or duration of schizophrenia ($t_{23} = 0.33$, $p = 0.74$).

We had previously determined relative protein levels of kalirin isoforms within auditory cortex gray matter from these subject pairs; the within subject-pair differences in log of PAK1 protein levels and log of total kalirin protein levels were positively correlated ($r = 0.55$, $p = 0.004$, Figure 1), although neither PAK1 nor total kalirin protein level (unpublished observation) significantly differed between schizophrenia and control subjects.

We similarly evaluated whether PAK1 protein levels were correlated with protein levels of spinophilin, a marker of dendritic spines [30;31] sensitive to changes in spine density [12;32]. Spinophilin protein levels were obtained in the same individuals. However, because of evidence for loss of spinophilin immunoreactivity when postmortem intervals were greater than 24 hours in an animal model (Figure S1) we restricted these analyses to the 20 pairs of subjects with observed postmortem intervals $\leq 24$ hours (Table 1). Spinophilin protein levels, like PAK1 levels, were unchanged in subjects with schizophrenia ($t_{18} = 0.58$, $p = 0.57$). Nevertheless, PAK1 protein levels and spinophilin protein levels were positively correlated among all subjects (Figure S2, $r = 0.37$, $p = 0.02$). A stronger correlation was seen for the within subject-pair differences in log PAK1 protein levels and differences in log spinophilin protein levels (Figure 1, $r = 0.61$, $p = 0.004$).

Post mortem interval does not affect PAK1 protein expression

Failure to detect differences between groups in postmortem studies can occur because the measured protein, although detectable, is present at such a reduced level in comparison to the in vivo state that identifying further reduction is not possible, i.e. a “floor effect”. We thus examined the effect of post mortem interval on PAK1 level in cortical grey matter in a mouse model (Figure 3). We found that the effects of post mortem interval on PAK1 level ($F_{7,24} = 0.35$, $p = 0.92$) were minimal in the PMI range utilized in this study (PMI $\leq 30$ hours). There was a small, but statistically significant effect of PMI on β tubulin level ($F_{7,24} = 9.56$, $p = 0.01$), however, this is readily accounted for by subject matching which includes PMI. In the human study there were no significant effects of PMI on PAK1 level ($F_{3,31} = 0.76$, $p = 0.39$), although we did detect the previously mentioned nominally significant interaction between PMI and diagnosis.

Discussion

The emergence and persistence of dendritic spines is likely regulated by numerous molecular pathways with complex interactions. Strong potential candidates contributing to dendritic spine abnormalities in schizophrenia will demonstrate regulatory effects on the actin cytoskeleton, dendritic spine number and spine morphology, as well as interactions with regulatory proteins whose expression are known to be altered in the disease state. While PAK1 fulfills all of these criteria and is stable in postmortem tissue, this study demonstrated that there is no change in PAK1 protein expression in whole grey matter extracted from the auditory cortex of schizophrenia subjects in our cohort.

This study has several strengths of that enhance confidence in the results. We evaluated 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
Figure 1. PAK1 Expression in schizophrenia. A) Detection of PAK1 in human gray matter extracts. Bands corresponding to the predicted molecular weights of PAK1 and Tubulin are readily detected in human gray matter extracts from subjects with schizophrenia (S) and matched control subjects (C). MW, Molecular Weight Markers. B) Comparison of PAK1 expression (normalized to tubulin expression) for the 25 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, points below the line indicate decreased expression in schizophrenia subjects. C) Mean (SD)
expression PAK1:tubulin in schizophrenia and control subjects. D) Correlation of within pair differences in log PAK1 expression with the corresponding measure of log total kalirin protein expression (r = 0.55, p = 0.004). E) Correlation of within pair differences in PAK1 expression with the corresponding measure of spinophilin protein expression (r = 0.61, p = 0.004).

do:10.1371/journal.pone.0059458.g001

fluorescence intensities. 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. Using this approach in these subjects, we had previously found an increased protein level of kalirin-9, indicating that our approach is sufficiently sensitive to detect protein changes.

Although there was no overall change in mean PAK1 protein levels in our subjects with schizophrenia, within pair differences in PAK1 levels were correlated with those of an upstream effector, total kalirin, and with levels of a dendritic spine marker, spinophilin. Detection of these correlated changes further suggests that our approach was sufficient to detect potential biologic effects. Similarly, evaluation of postmortem effects on PAK1 levels in a mouse model showed minimal impact for the intervals represented within our cohort, indicating that the failure to find a change in PAK1 was not likely an artifact of “floor effects” in the human postmortem cohort. One potential limitation of our study is the lack of evaluation of the potential influences of antipsychotic use on PAK1 levels in a model system, however, our analysis did not show an effect of antipsychotic use at the time of death in our human cohort.

Our finding of no change in PAK1 protein expression in the auditory cortex in combination with a recent study that found no change in PAK1 levels in the anterior cingulate cortex, but increased PAK1 levels in the dorsolateral prefrontal cortex, in subjects with schizophrenia, suggests there may be regional differences in disease-related PAK1 expression.[33] Alternatively, the significant correlation of within pair reductions in PAK1 and spinophilin raises the possibility that it is a subset of subjects with schizophrenia that have deficits in signaling via PAK1 to dendritic spines. Figure 4 summarizes the major PAK1 signaling pathway regulating actin dynamics and dendritic spines. PAK1 activity is regulated by the Rho GTPases, Rac1 and CDC42, which in their active GTP bound state bind to PAK1 dimers inducing a conformational change which removes the autoinhibitory domain from the kinase active site [34]. These conformational changes promote autophosphorylation at threonine 423 in the active site, which is required to maintain the uninhibited state and to promote full catalytic activity. In addition at least two other phosphorylation sites exist which promote and maintain catalytic activity regulated by numerous additional mediators (ie. Cdk-5, PDK1), thus creating a number of possible differentially phosphorylated, catalytically active states [34]. Prior studies have demonstrated enrichment of the phospho-threonine 423 PAK1 in the post-synaptic density of mouse cortical neurons [35;36]. It is plausible that while PAK1 protein expression is not changed, the fraction of PAK1 in its active phosphorylated state is altered. Measurement of levels of these specific phosphorylation states, through the use of phospho-specific antibodies, may provide more biologically relevant indications of dysregulation of this molecular cascade. However, phosphorylation states vary with postmortem time and conditions, making determination of individual phosphorylation states in post-mortem samples challenging [37]. Nevertheless, one recent study identified reduced levels of phospho-threonine 423 PAK1 in the dorsolateral prefrontal cortex and anterior cingulate cortex of subjects with schizophrenia [33].

Additional consideration must be given to the use of protein extracted from whole gray matter in this study, which does not allow for differentiation of PAK1 levels in individual layers or specific cellular compartments. Prior findings of altered kalirin protein levels and kalirin and Cdc42 mRNA expression in schizophrenia were not layer specific [28;29], leading us to evaluate whole gray matter. Nevertheless, it remains possible a selective decrease in PAK1 protein level within specific layers may contribute to spine loss. Such a specific change may not be detectable in the overall background of PAK1 protein expression and would require separate studies to detect changes confined to individual layers. Similarly, PAK1 levels may be selectively altered in the spine compartment, and may require measurement

Figure 2. Effect of subject variables on PAK1 expression in schizophrenia. Each point represents the mean within pair difference in log PAK1 expression. Horizontal bars are group means. No differences were significant.
doi:10.1371/journal.pone.0059458.g002

PAK1 in Schizophrenia
Table 1. Characteristics of control subjects and subjects with schizophrenia.

| Pair | Control | Schizophrenia |
|------|---------|---------------|
|      | PMI     | Storage       | Diagnosis       | pH | Hand | PMI     | Storage       | Diagnosis       | pH | Hand |
|      | (hrs)   | Time (mos)    | COD             |    |     | (hrs)   | Time (mos)    | COD             |    |     |
|      | [Obs]   |              | None            | R  | O   |      |              | Paranoic schizophrenia; ADR | R  | BO  |
| 1*   | 1326 M/W/58 | 16.4 [16.4] | ASCVD           | None | 6.7 | R | O  | 11.1 [11.1] | Trauma | Paranoic schizophrenia; ADR | 6.4 | R  | BO  |
| 2*   | 1247 F/W/58 | 22.7 [22.7] | ASCVD           | None | 6.4 | R | O  | 22.9 [20.2] | ASCVD | Undifferentiated schizophrenia; ADR | 6.3 | R  | OP  |
| 3    | 1086 M/W/51 | 24.2 [23.8] | ASCVD           | None | 6.8 | R | N  | 27.1 [27.1] | ASCVD | Disorganized schizophrenia; OAR | 6.7 | R  | N   |
| 4    | 10005 M/W/42 | 23.5 [23.5] | Trauma         | None | 6.7 | R | O  | 27.4 [24.8] | Hanging | Undifferentiated schizophrenia | 6.4 | R  | P   |
| 5    | 1255 M/B/37 | 22.0 [21.8] | Pulmonary embolism | None | 5.9 | R | O  | 28.8 [28.8] | Salicylate overdose | Paranoic schizophrenia; ADR; OAC | 6.6 | R  | CDP |
| 6*   | 1480 M/W/62 | 20.1 [19.8] | ASCVD           | Reading D/O | 6.7 | R | O  | 22.7 [22.7] | Asphyxiation | Undifferentiated schizophrenia; ADR | 7.1 | R  | DP  |
| 7*   | 1119 M/W/57 | 20.4 [20.4] | ASCVD           | None | 6.8 | R | N  | 22.9 [18.8] | ASCVD | Disorganized schizophrenia; ADR | 6.4 | R  | OP  |
| 8*   | 1317 M/W/56 | 22.9 [22.9] | ASCVD           | None | 6.5 | L | O  | 23.2 [23.2] | Cardio-myoopathy | Schizoaffective disorder; ODC | 6.4 | U  | COP |
| 9*   | 1307 M/B/32 | 4.8 [4.8]    | ASCVD           | None | 6.7 | R | N  | 6.0 [6.0]    | ASCVD | Paranoic schizophrenia | 6.1 | L  | O   |
| 10*  | 1067 M/W/49 | 6.5 [6.5]    | ASCVD           | None | 6.6 | R | O  | 7.8 [7.8]    | Pneumonia | Undifferentiated schizophrenia | 6.5 | L  | DOP |
| 11*  | 1196 F/W/36 | 14.5 [14.5]  | Asphyxiation   | None | 6.4 | R | O  | 20.1 [20.1] | Sudden unexpected death | Schizoaffective disorder | 6.3 | L  | DOP |
| 12*  | 1099 F/W/24 | 9.1 [9.1]    | Cardio-myoopathy | None | 6.5 | R | O  | 20.1 [20.1] | Drowning | Disorganized schizophrenia | 6.7 | R  | BDP |
| 13*  | 806 M/W/57  | 24.0 [24.0]  | Pulmonary embolism | None | 6.9 | R | O  | 28.1 [12.9] | Intestinal hemorrhage | Paranoic schizophrenia; ADC | 6.9 | R  | DOP |
| 14*  | 739 M/W/40  | 15.8 [15.8]  | ASCVD           | None | 6.9 | R | N  | 21.5 [21.5] | Combined drug overdose | Undifferentiated schizophrenia; ADR; OAC | 6.5 | R  | DOP |
| 15   | 822 M/B/28  | 25.3 [25.3]  | ASCVD           | None | 7.0 | L | N  | 19.2 [19.2] | Gunshot | Schizoaffective disorder; ODC | 6.7 | L  | OP  |
| 16*  | 727 M/B/19  | 7.0 [7.0]    | Trauma         | None | 7.2 | R | N  | 12.0 [12.0] | Salicylate overdose | Schizoaffective disorder; ADC; ADR | 6.8 | L  | BC  |
| 17*  | 659 M/O/46  | 22.3 [22.3]  | Peritonitis     | None | 6.9 | R | N  | 15.7 [15.7] | ASCVD | Disorganized schizophrenia; ADR; OAR | 6.2 | R  | COP |
| 18*  | 852 M/W/54  | 8.0 [4.8]    | Cardiac Tamponade | None | 6.8 | R | N  | 15.7 [15.7] | ASCVD | Disorganized schizophrenia; ADR; OAR | 6.2 | R  | OP  |
| 19*  | 685 M/W/56  | 14.5 [13.4]  | Hypoplastic coronary artery | None | 6.6 | R | O  | 7.9 [7.9]    | ASCVD | Schizoaffective disorder | 6.2 | R  | P   |
Table 1. Cont.

| Control | Schizophrenia |
|---------|---------------|
| Pair    | Case | S/R/A | PMI (hrs) [Obs] | Storage Time (mos) | COD | Diagnosis | pH | Hand | Meds ATOD | Pair | Case | S/R/A | PMI (hrs) [Obs] | Storage Time (mos) | COD | Diagnosis | pH | Hand | Meds ATOD |
| 20*     | 686   | F/W/52 | 22.6 [20.4]  | 167 | ASCVD | None | 7.0 | R | O | 802 | F/W/63 | 29.0 [19.9]  | 146 | Right ventricular dysplasia | 6.4 | M | COP |
| 21*     | 1092  | F/B/40 | 16.6 [16.6]  | 95  | Mitral valve prolapse | None | 6.8 | R | O | 1010 | F/B/44 | 18.7 [18.7]  | 108 | Sudden unexpected death | 6.4 | L | CDP |
| 22*     | 1488  | M/B/39 | 21.5 [21.5]  | 33  | Pulmonary embolism | None | 6.4 | R | N | 1222 | M/W/32 | 30.8 [18.6]  | 79  | Combined drug overdose | 6.4 | R | DP |
| 23*     | 1047  | M/W/43 | 13.8 [13.8]  | 101 | ASCVD | None | 6.6 | R | O | 933  | M/W/44 | 8.3 [8.3]   | 119 | Myocarditis | 6.4 | R | CDOP |
| 24*     | 700   | M/W/42 | 26.1 [26.1]  | 164 | ASCVD | None | 7.0 | R | N | 625  | M/W/49 | 23.5 [23.5]  | 174 | Disorganized schizophrenia | 7.3 | R | DOP |
| 25*     | 818   | F/W/67 | 23.5 [23.5]  | 144 | Anaphylactic reaction | None | 7.1 | R | O | 917  | F/W/71 | 23.8 [23.1]  | 123 | Undifferentiated schizophrenia | 6.8 | U | OP |

N (%), or Mean (SD)

M: 19 (76.4); Mean: 45.8 (12.3)

665: Min (12.9) Max (43.2) Avg (28.1) **One Pair Not Shown: HU 1201 (C) and 1189 (S) 802: Min (19.9) Max (37.9) Avg (29) 1222: Min (18.6) Max (43) Avg (30.8)

All subject pairs shown were included in analyses of PAK1 and kalirin protein levels. Subject pairs also analyzed for spinophilin protein levels (Observed PMI=24 hours) are indicated by an asterisk. Control and schizophrenia subjects significantly differ in pH and handedness, but not in age, sex, PMI, and tissue storage time. S/R/A, Sex/Race/Age; PMI, postmortem interval (calculated as lag from midpoint of time last seen alive – time discovered dead to time of brain fixation); Observed is calculated as time discovered dead to time of brain fixation); COD, cause of death; Hand, handedness; Meds ATOD, medications used at time of death; ADC, Alcohol Dependence, current at time of death; ADR, Alcohol Dependence, in remission at time of death; AAR, Alcohol Abuse, current at time of death; AAC, Alcohol Abuse, in remission at time of death; ODC, Other Substance Dependence, current at time of death; ODR, Other Substance Dependence, in remission at time of death; OAC, Other Substance Abuse, current at time of death; OAR, Other Substance Abuse, in remission at time of death; L, Left; M, Mixed; R, Right; U, Unknown; B, Benzodiazepines; C, Anticonvulsants; D, Antidepressants; L, Lithium; N, No medications; O, Other medication(s); P, Antipsychotic; U, Unknown.

doi:10.1371/journal.pone.0059458.t001
specifically in this compartment. Finally, other members of the PAK family may be relevant to spine density. For instance, a rare chromosomal deletion including the \( \text{PAK2} \) gene has recently been associated with schizophrenia [38]. Also of note, mutations in the \( \text{PAK3} \) gene, discovered to be associated with nonsyndromic mental retardation, have been noted to alter spine number through a CDC42 mediated pathway [39]. Recent data indicate that PAK1 protein and PAK3 protein colocalize within dendritic spines where they heterodimerize, regulating PAK3 signaling. Of interest, this dimerization is inhibited by mutations in PAK3 that lead to mental retardation, suggesting that PAK1 levels may contribute to spine reductions through this mechanism [40]. Thus, while we found no change in overall PAK1 protein expression in schizophrenia, further exploration of possible changes in both biochemical properties and compartmental localization of members of the PAK family of proteins will be required to fully evaluate whether they play a role in dendritic pathology in schizophrenia.

Materials and Methods

Ethics Statement

All brain specimens were obtained during autopsy after obtaining a witnessed verbal consent from the next of kin, using a procedure reviewed and approved by the University of Pittsburgh Committee for Oversight of Research and Clinical Training Involving Decedents. Verbal consent was audiotaped.
PAK1 in Schizophrenia

Figure 4. PAK1 Signaling Pathway. PAK1 resides in inactive homo-
or heterodimers. Binding of the Rho-GTPase, RAC1 (or CDC42) causes
dissociation of the dimers and activation of PAK1. PAK1 activates LIMK1,
which inhibits cofilin-mediated f-actin depolymerization. PAK1 may be
subject to activation by non-GTPase mechanisms, and can activate
other effector pathways, some of which (e.g. MLCK and MLC) may also
impact spine dynamics. Blue indicates promotion of dendritic spine
perseverence, red indicates promotion of spine elimination.

doi:10.1371/journal.pone.0059458.g004

PAK1 in Schizophrenia

Figure 4. PAK1 Signaling Pathway. PAK1 resides in inactive homo-
or heterodimers. Binding of the Rho-GTPase, RAC1 (or CDC42) causes
dissociation of the dimers and activation of PAK1. PAK1 activates LIMK1,
which inhibits cofilin-mediated f-actin depolymerization. PAK1 may be
subject to activation by non-GTPase mechanisms, and can activate
other effector pathways, some of which (e.g. MLCK and MLC) may also
impact spine dynamics. Blue indicates promotion of dendritic spine
perseverence, red indicates promotion of spine elimination.

doi:10.1371/journal.pone.0059458.g004

and a written document summarizing the consent process was
generated and signed by the individual obtaining consent and an
independent witness. Family members of decedents provided written
consent to participate in a postmortem research diagnostic
interview as approved by the University of Pittsburgh Institutional
Animal Care and Use Committee.

Human Subjects

A total of 25 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 (Table 1). All brain
specimens were obtained during autopsy at the Allegheny County
Medical Examiner’s Office after obtaining consent from the next
member of that pair appears on 2 adjacent lanes. The
experimenters were blind to diagnosis during experimentation and
quantification of blots. Pilot studies were used to establish
conditions providing for linear detection of all target proteins
(Figure 5 E). Based on these studies, 20 mg of protein was
aliquoted in 1 × 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 hours at room temperature in 1 × SDS running buffer (Pierce
20 × Tris Hepes SDS Buffer) at 75 V. Samples were then
transferred to 0.45 μm PVDF [Millipore, Billerica, Massachusetts,
USA] in 1 × Tris Glycine Blotting Buffer (Pierce) at 85 V for 50
minutes at 4°C. Membranes were incubated for 1 hour in Odyssey
LiCor Blocking Buffer diluted 1:1 in 1 × TBS. The membrane was
incubated overnight in PAK1 primary antibody (rabbit anti-
PAK1, Invitrogen, 71–9300, Camarillo, Ca 93012) diluted 1:1000
and mouse anti-β tubulin (Millipore # 05–661) diluted 1:60,000, in
Pierce SuperBlock blocking buffer with 0.1% Tween 20 (Sigma-
Aldrich, St. Louis, Missouri, USA). The anti-PAK1 antibody did
not detect PAK2 or PAK3 (Figure 5 F). 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 dry and bands detected using a Li-Cor
Odyssey Infrared Scanner set at a resolution of 42 mm and the
highest image quality. Procedures for spinophilin (detected using
rabbit anti-spinophilin, Millipore # AB5669, Millipore, Billerica,
Massachusetts, USA) diluted 1:2000 were identical, with the
exception that 10 μg of protein was loaded based on pilot studies
to determine conditions providing for linear detection of
spinophilin (Figure S1).

Quantification of Western Blots

Images were quantified using MCID Core Version 7.0
(InterFocus Imaging Ltd., Linton, Cambridge, UK). The peaks for
PAK1, spinophilin, 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 (Figure 5 D). The integrated intensity
(mean intensity × mm²) and maximum intensity was acquired for
each protein.

Statistical Analyses

The response variable for the analysis was the natural logarithm
of the ratio of PAK1 protein integrated intensity to the integrated

Total protein was extracted using SDS extraction buffer (0.125 M
Tris-HCl (pH 7), 2% SDS, and 10% glycerol) at 70°C. Protein
centrifugation was estimated using a bicotinic acid assay (BCA™
Protein Assay Pierce # 23225). 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 25 matched pairs of control and
schizophrenia subjects examined in 13 runs, with 2 pairs per run
for 12 runs and 1 pair for 1 run with each run consisting of 4 gels.
For the 12 runs with 2 pairs in each run, 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. For the run with 1 pair, each gel was loaded
using a total of 4 lanes so that the control and schizophrenia
member of that pair appears on 2 adjacent lanes. The
experimenters were blind to diagnosis during experimentation and
quantification of blots. Pilot studies were used to establish
conditions providing for linear detection of all target proteins
(Figure 5 E). Based on these studies, 20 mg of protein was
aliquoted in 1 × 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 hours at room temperature in 1 × SDS running buffer (Pierce
20 × Tris Hepes SDS Buffer) at 75 V. Samples were then
transferred to 0.45 μm PVDF [Millipore, Billerica, Massachusetts,
USA] in 1 × Tris Glycine Blotting Buffer (Pierce) at 85 V for 50
minutes at 4°C. Membranes were incubated for 1 hour in Odyssey
LiCor Blocking Buffer diluted 1:1 in 1 × TBS. The membrane was
incubated overnight in PAK1 primary antibody (rabbit anti-
PAK1, Invitrogen, 71–9300, Camarillo, Ca 93012) diluted 1:1000
and mouse anti-β tubulin (Millipore # 05–661) diluted 1:60,000, in
Pierce SuperBlock blocking buffer with 0.1% Tween 20 (Sigma-
Aldrich, St. Louis, Missouri, USA). The anti-PAK1 antibody did
not detect PAK2 or PAK3 (Figure 5 F). 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 dry and bands detected using a Li-Cor
Odyssey Infrared Scanner set at a resolution of 42 mm and the
highest image quality. Procedures for spinophilin (detected using
rabbit anti-spinophilin, Millipore # AB5669, Millipore, Billerica,
Massachusetts, USA) diluted 1:2000 were identical, with the
exception that 10 μg of protein was loaded based on pilot studies
to determine conditions providing for linear detection of
spinophilin (Figure S1).

Quantification of Western Blots

Images were quantified using MCID Core Version 7.0
(InterFocus Imaging Ltd., Linton, Cambridge, UK). The peaks for
PAK1, spinophilin, 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 (Figure 5 D). The integrated intensity
(mean intensity × mm²) and maximum intensity was acquired for
each protein.

Statistical Analyses

The response variable for the analysis was the natural logarithm
of the ratio of PAK1 protein integrated intensity to the integrated
intensity of tubulin, a normalizing protein that does not differ between diagnostic groups ($t_{22.5} = 1.16$, $p = 0.26$). For each subject, up to 8 measurements of this log ratio were made after excluding any measurements with gel artifacts. 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. [For the primary model, run*diagnosis interaction was not significant (F12, 10 = 0.31, p = 0.97), thus not included in the model. For the secondary model, there were no significant interactions between diagnosis and age (F3, 38.4 = 0.06, P = 0.80) or diagnosis and sex (F1, 30.3 = 0.86, P = 0.36), thus neither of them was included in the model]. We did however identify a marginally significant interaction between diagnosis and PMI (F1, 30 = 3.31, P = 0.079). To further evaluate this we categorized the PMIs into Low (<14), Medium (14–23) and High (>23) and then examined the PMI*diagnosis interaction in the secondary model, now treating PMI as a categorical variable, and estimated the diagnostic effects in the three PMI groups.

To guard against bias in our band definitions, we also evaluated an alternate response variable, the natural logarithm of the ratio of the maximum intensities of PAK1 and tubulin. Similar primary and secondary analyses were done for this alternate variable and provided results consistent with the analysis utilizing the integrated intensity level so are not reported further.

The relationship of the natural logarithm of the tubulin normalized PAK1 ratios within each pair to each of the following confounds was also examined: schizophrenia diagnosis (yes/no), suicide as cause of death (yes/no), antipsychotic use at time of suicide (yes/no), history of substance use disorder (yes/no), age of onset, and duration of schizophrenia. Log tubulin normalized ratios for PAK1 on each gel were obtained by averaging PAK1 and Tubulin levels of Schizophrenia subject and Control subject over the gel separately, getting two Tubulin normalized PAK1 values, [PAK1 schizophrenia/Tubulin schizophrenia] and [PAK1 control/Tubulin control], and then getting the log ratio of the two Tubulin normalized PAK1 values. In computing the log tubulin normalized ratios for a subject, if a PAK1 (or tubulin) measurement was missing on one lane, the corresponding tubulin (or PAK1) measurement was also treated as missing on that lane. The analyses used linear mixed models to model the log tubulin normalized PAK1 ratio, where each of the confounds was treated as a fixed effect, and where gel and pair were treated as random effects in order to account for the four gels per pair and the two pairs per gel.

We examined the correlation of the pairwise differences (the differences of averaged log tubulin normalized ratios between Schizophrenia subject and Control subject within a pair where the average is over the four gels) for PAK1 with similarly calculated pairwise difference for total kalirin protein levels (as all 4 kalirin isoforms present in human cortex have Rac1 guanine nucleotide exchange factor activity and thus are likely to be upstream of PAK1 [44]). The log tubulin normalized ratios were computed for spinophilin using the same method. Furthermore, we evaluated the correlation of the pairwise difference in PAK1 values with the pairwise difference in spinophilin values, and also the correlation between corresponding PAK1 and spinophilin values among all subjects. All tests were two-sided and conducted at the 0.05 significance level. The p-values for diagnostic group effect are based on the contrast of control effect minus schizophrenia effect. All analyses were implemented in SAS PROC MIXED (Version 9.2, SAS Institute Inc., Cary, NC).

**Figures**

Figure S1 Spinophilin Western Blot Characterization and Validation. A) Spinophilin immunoactivity in human, wild type mouse (+/+) and spinophilin knockout mouse (−/−) demonstrate specificity of spinophilin detection. B) Spinophilin optical density as a function of micrograms of protein loaded per lane. The mean (SD) of a eight repeated assays of a human subject is shown. It can be seen that the protein loading used in the comparison of schizophrenia and control subjects (10 μg) sits within the linear detection range. C) Optical densities for spinophilin in mice (N=2) in which the interval from sacrifice to brain harvesting was experimentally varied between 0 and 48 hours. Mean (SEM) value at each time point is shown. Time points sharing the same superscript letter differ significantly. D) Detection of spinophilin in auditory cortex gray matter extracts from two subject pairs. S, subjects with schizophrenia, C, matched control subjects.

**Acknowledgments**

Allan R. Sampson is a statistical consultant to Johnson & Johnson Pharmaceutical Research and Development. The authors thank Josephine Anaf-Adjei for statistical assistance.

**Author Contributions**

Conceived and designed the experiments: AJD RAS JVD. Performed the experiments: AJD IG RH JVD. Analyzed the data: SL AS. Contributed reagents/materials/analysis tools: DAL PP RAS. Wrote the paper: AJD SL AS DAL PP RAS.
References

1. McCarley RW, Whibley OG, Frumin M, Hirayasu Y, Levitin JJ, et al. (1999) MRI anatomy of schizophrenia, Biol Psychiatry, 45: 1099–1119.
2. Honea R, Crow TJ, Pasingham D, Mackay CE (2005) Regional deficits in brain volume in schizophrenia: a meta-analysis of voxel-based morphometry studies, Am J Psychiatry, 162: 2223–2245.
3. Asprone M, Saccante E, Zivic I, Javitt DC, Revheim N (2008) Sounding it out: auditory processing deficits and reading impairment in schizophrenia. Biological Psychiatry 63: 2465–2470.
4. Leitman DI, Faye J, Butler PD, Saperstein A, Revheim N, et al. (2005) Sensory contributions to impaired prosodic processing in schizophrenia, Biol Psychiatry, 58: 56–61.
5. Javitt DC, Shelley AM, Ritter W (2000) Associated deficits in mismatch negativity generation and tone matching in schizophrenia, Clin Neurophysiol, 111: 1734–1737.
6. Javitt DC, Stiops RD, Grochoski S, Ritter W, Cowan N (1997) Impaired precision, but normal retention, of auditory sensory “echoes” memory information in schizophrenia. Journal of Abnormal Psychology, 106: 315–320.
7. Oswald AM, Schiffl ML, Reyes AD (2006) Synaptic mechanisms underlying auditory processing. Curr Opin. Neurobiol, 16: 371–376.
8. Liu BH, Wu G, Arbuckle R, Tao HW, Zhang LJ (2007) Defining cortical frequency tuning with recurrent excitatory circuitry. Nat, Neurosci., 10: 1594–1600.
9. Ojima H, Honda CN, Jones EG (1991) Patterns of axon collateralization of identified supragranular pyramidal neurons in the cat auditory cortex, Cereb. Cortex, 1: 80–94.
10. Wallace MN, Kittes LM, Jones EG (1991) Intrinsically inter- and intralaminar connections and their relationship to the tonotopic map in cat primary auditory cortex, Exp. Brain Res., 86: 527–544.
11. Javitt DC, Steinschneider M, Schroeder CE, Vaughan HG Jr, Arezzo JC (1994) Detection of stimulus deviance within primary auditory auditory cortex: intracortical mechanisms of mismatch negativity (MMN) generation, Brain Res., 667: 192–200.
12. Sweet RA, Henteleff RA, Zhang W, Sampson AR, Lewis DA (2009) Reduced dendritic spine density in auditory cortex of subjects with schizophrenia, Neuropsychopharmacology, 34: 374–389.
13. Sweet RA, Bergen SE, Sun Z, Marcius M, Sampson AR, et al. (2007) Anatomical evidence of impaired feedforward auditory processing in schizophrenia, Biol Psychiatry, 61: 854–864.
14. Glantz LA, Lewis DA (2000) Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia, Arch Gen. Psychiatry, 57: 65–73.
15. Garey LJ, Ojima H, Honda CN, Jones EG (1991) Patterns of axon collateralization of identified supragranular pyramidal neurons in the cat auditory cortex, Cereb. Cortex, 1: 80–94.
16. Aspromonte J, Saccente E, Ziwich R, Javitt DC, Revheim N (2008) Sounding it out: auditory processing deficits and reading impairment in schizophrenia, Biological Psychiatry 63: 2465–2470.
17. Hayashi K, Ohshima T, Hashimoto M, Mikoshiba K (2007) Pak1 regulates bidirectional plasticity, Nat. Neurosci., 7: 1104–1112.
18. Zuo Y, Lin A, Chang P, Gan WB (2005) Development of long-term dendritic spine density on cerebral cortical pyramidal neurons in schizophrenia, Arch. Gen. Psychiatry, 62: 656–664.
19. Okamoto K, Nagai T, Miyawaki A, Hayashi Y (2004) Rapid and persistent dendritic spine alterations in the prefrontal cortex of subjects with schizophrenia, Mol Psychiatry, 9: 537–546.
20. Edwards DC, Sanders LC, Bokoch GM, Gill GN (1999) Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics, Nat Cell Biol, 1: 253–258.
21. Hayashi K, Ohshima T, Hashimoto M, Mikoshiba K (2007) Pak1 regulates dendritic branching and spine formation, Dev Neurobiol, 67: 655–660.
22. Zhang H, Webb DJ, Assussen H, Niu S, Horwitz AF (2005) A GGT1/PIX/Pak signaling module regulates spine morphogenesis and synapse formation through MLCK, J Neurosci, 25: 3379–3386.
23. Asrar S, Meng Y, Zhou Z, Todorovski Z, Huang WW, et al. (2009) Regulation of hippocampal long-term potentiation by p21-activated protein kinase 1 (Pak1). Neuropharmacology, 56: 73–89.
24. Huang W, Zhou Z, Asrar S, Henkelman M, Xie W, et al. (2011) P21-Activated kinases 1 and 3 control brain size through coordinating neuronal complexity and synaptic properties, Mol Cell Biol, 31: 383–393.
25. Prenzes P, Johnson RC, Settler R, Zhang X, Huganir RL, et al. (2003) The neuronal Rho-GEF Kalirin-7 interacts with PDZ domain-containing proteins and regulates dendritic morphogenesis, Neuron, 29: 229–242.
26. Xie Z, Srivastava DP, Photowala H, Kai I, Cahill ME, et al. (2007) Kalirin-7 controls activity-dependent structural and functional plasticity of dendritic spines, Neuron, 56: 640–656.
27. Bishop AL, Hall A (2000) Rho GTPases and their effector proteins, Biochem, J, 340 Pt 2: 241–255.
28. Hill JJ, Hashimoto T, Lewis DA (2006) Molecular mechanisms contributing to dendritic spine alterations in the prefrontal cortex of subjects with schizophrenia, Mol Psychiatry, 11: 557–566.
29. Deo AJ, Cahill ME, Li S, Goldzer I, Henteleff R, et al. (2011) Increased expression of Kalirin-9 in the auditory cortex of schizophrenia subjects: its role in dendritic pathology, Neurobiol Dis.
30. Allen PB, Ouniet CC, Greenberg P (1997) Spinothin, a novel protein phosphatase 1 binding protein localized to dendritic spines, Proc. Natl. Acad. Sci. U S A, 94: 9556–9561.
31. Muly EC, Smith Y, Allen P, Greenberg P (2004) Subcellular distribution of spinothin immunolabeling in primate prefrontal cortex: localization to and within dendritic spines, J Comp Neurol, 469: 185–197.
32. Tang Y, Janssen WG, Hao J, Roberts JA, McKay H, et al. (2004) Estrogen replacement increases spinothin-immunoreactive spine number in the prefrontal cortex of female rhesus monkeys, Cereb. Cortex, 14: 215–223.
33. Rubio MD, Haroutunian V, Meador-Woodruff JH (2012) Abnormalities of the Dna/Ras-related C3 botulinum toxin substrate 1/p21-activated kinase 1 pathway drive myosin light chain phosphorylation in frontal cortex in schizophrenia, Biol Psychiatry, 71: 906–914.
34. Bokoch GM (2003) Biology of the p21-activated kinases, Annu Rev Biochem., 72: 743–781.
35. Hayashi ML, Choi SY, Rao BS, Jung HY, Lee HK, et al. (2004) Altered cortical synaptic morphology and impaired memory consolidation in forebrain-specific dominant-negative Pak transgenic mice, Neurosci, 42: 773–787.
36. Hayashi K, Ohshima T, Mikoshiba K (2002) Pak1 is involved in dendrite initiation as a downstream effector of Rac1 in cortical neurons, Mol Cell Neurosci, 20: 579–594.
37. Oka T, Tagawa K, Ito H, Okazawa H (2011) Dynamic changes of the phosphoproteome in postmortem mouse brains, PLoS One, 6: e21405.
38. Mulle JG, Dodd AF, McGrath JA, Wolney PIS, Mitchell AA, et al. (2010) Microdeletions of 3q29 confer high risk for schizophrenia, Am J Hum Genet., 87: 229–236.
39. Kreis P, Thervenot E, Rousseau V, Boda B, Muller D, et al. (2007) The p21-activated kinase 3 implicated in mental retardation regulates spine morphogenesis through a Cdc42-dependent pathway, J Biol Chem., 282: 21497–21506.
40. Combeau G, Kreis P, Donnenhini F, Amar M, Fossier F, et al. (2012) The p21-activated kinase Pak1 forms heterodimers with Pak1 in brain implementing trans-regulation of Pak3 activity, J Biol Chem., 287: 30084–30096.
41. Asrar RA, Weipert NA, Sampson AR, Lewis DA (2003) Reduced pyramidal cell somal volume in auditory association cortex of subjects with schizophrenia, Neuropsychopharmacology, 28: 599–609.
42. Sweet RA, Bergen SE, Sun Z, Sampson AR, Lewis DA (2003) Reduced pyramidal cell somal volume in auditory association cortex of subjects with schizophrenia, Neuropsychopharmacology, 28: 599–609.
43. Sweet RA, Bergen SE, Sun Z, Sampson AR, Lewis DA (2003) Reduced pyramidal cell somal volume in auditory association cortex of subjects with schizophrenia, Neuropsychopharmacology, 28: 599–609.