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Altered Serine/Threonine Kinase Activity in Schizophrenia

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Abstract

Converging evidence implicates alterations in multiple signaling pathways in the etiology of schizophrenia. Previously, these studies were limited to the analysis of one or a few phosphoprotein a time. Here, we use a novel kinase array platform to simultaneously investigate the convergence of multiple signaling cascades implicated in schizophrenia. This technology uses consensus peptide substrates to assess activity levels of a large number (>100) of serine/threonine protein kinases. 19 peptide substrates were differentially phosphorylated (>15% change) in the frontal cortex in schizophrenia. These peptide substrates were examined using Ingenuity Pathway Analysis to group them according to the functions and to identify processes most likely affected in schizophrenia. Pathway analysis placed 14 of the 19 peptides into cellular homeostatic pathways, 10 into pathways governing cytoskeletal organization, and 8 into pathways governing ion homeostasis. These data are the first to simultaneously investigate comprehensive changes in signaling cascades in a severe psychiatric disorder. The examination of kinase activity in signaling pathways may facilitate the identification of novel substrates for drug discovery and the development of safer and more effective pharmacological treatment for schizophrenia.

Keywords

kinase; postmortem; ion homeostasis; immune trafficking; phosphopeptides; synaptic remodeling

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1. Introduction

For decades the pharmacological treatment of severe mental illness has focused on modulation of neurotransmitter receptors. Compounds that activate or block receptors, transporters, or key enzymes are postulated to work by altering intracellular signaling cascades connected to these neurotransmitter systems, leading to symptom improvement via alterations in neuroplasticity (Beaulieu, 2012; Lewis, 2009; McCullumsmith et al., 2004). More recently, the discovery of signal integration molecules such as DARPP-32 highlights the significance of crosstalk between downstream targets of neurotransmitter receptors (Albert et al., 2002; Greengard et al., 1999; Kunii et al., 2011). However, the signal integration process is complex and includes multiple interconnected signaling pathways (Funk et al., 2012; Greengard, 2001). Due to this complexity, characterization of the phosphorylation state of one or a few signal integration molecules in a pathological substrate provides a markedly limited view of the underlying signaling millieu in a disease model or state.

A kinome represents the full complement of protein kinase activity in a cell or organism, while the term sub-kinome refers to a specific subset of these enzymes, such as serine/threonine kinases. High-throughput peptide array screening techniques for specific kinomes were recently developed to identify alterations in kinase activity in tumor cells (Arsenault et al., 2011; Sikkema et al., 2009; van Hattum et al., 2013). Kinome analyses have been instrumental in the identification of new targets for drug development and novel therapies for multiple cancer types (Engelman et al., 2005; Watts et al., 1995; Yuan et al., 2000). Here, we apply kinome array technology to investigate, for the first time, global patterns of kinase activity in frontal cortical tissue in schizophrenia.

The serine/threonine sub-kinome represents nearly 125 of the purported 500+ human kinases (Capra et al., 2006). Many of the serine/threonine kinases are involved in intracellular signaling pathways including protein kinase A (PKA), protein kinase C (PKC), mitogen-activated kinases (MAPKs), and inositol triphosphate-3 kinase (PI3K). These signaling cascades regulate fundamental cellular processes including cytoskeletal architecture, molecular transport, ion homeostasis, and protein synthesis (Dehghani et al., 2005). In the brain, many of these signaling cascades underlie cognitive processes such as attention, decision making, and learning, which are significantly disrupted in schizophrenia (Amadio et al., 2006; Yamauchi, 2005).

We used complimentary approaches to more fully elucidate putative abnormalities in signaling cascades in schizophrenia (Castillo et al., 2010; Funk et al., 2012; Moghaddam, 2003). We first applied Ingenuity Pathway Analysis (www.lngenuity.com) to available data from published studies measuring changes in phosphorylation states in postmortem schizophrenic brain. The IPA software algorithm combines the molecules into networks to maximize interconnectivity, calculates the most likely biological functions of the molecules, and compares the interconnectivity of molecules with a database of canonical pathways. Next, we used a novel kinome array to simultaneously interrogate resting-state kinase activity for 133 well-defined serine/threonine kinase targets in human postmortem brain from subjects with schizophrenia and matched control subjects.

2. Results

2.1 Integrated Pathway Analysis of previously published findings

The 12 studies examined (Table 1) a total of 23 proteins and phosphoproteins across five cortical brain regions including dorsolateral prefrontal cortex (DLPFC), anterior cingulate cortex (ACC), prefrontal cortex (PFC), frontal cortex (FC), and superior temporal gyrus (STG). 5 proteins were measured in more than one region. Expression levels were increased (9 proteins), decreased (10 proteins) or unchanged (4 proteins) in schizophrenia (Table 1). Not surprisingly, Ingenuity Pathway Analysis (www.ingenuity.com) identified cell-cell signaling and aspects of cellular survival, organization, morphology and development as the functional groupings best described by the dataset (p values from 5.83^{-3} to 8.94^{-14}) (Supplemental Table 1). This is consistent with the *a priori* hypotheses driving gene selection for these studies. IPA also categorized nervous system development and behavior as the top two systems most likely to be impacted by the described changes in kinase activity (p values between 5.88^{-3} and 8.94^{-14})(Supplemental Table 1).

2.2 Identification of Serine/Threonine Kinase Activity using PamChip

2.2 Quality Control Studies—We tested the feasibility of measuring kinase activity in human brain tissue lysates in a PamChip kinome array by comparing activity in fresh rat and postmortem human brain tissues. Using an overlay of all phosphorylated peptides we found similar kinase activity between rodent and postmortem human samples indicating that kinase activity was maintained in postmortem samples (Figure 1).

To assess the effects of postmortem interval (PMI) on cortical kinase activity, we measured kinase activity in samples with an increasing PMI. Rodent brain tissue was collected 0, 4, 8, or 12 hours after sacrifice and assayed for kinase activity. We found no significant differences in the kinase activity between 0, 4, 8, or 12 hours PMI, suggesting postmortem intervals of at least 12 hours did not impact kinase activity (Figure 2 and Supplemental Figure 1).

Non-specific binding to the array substrate was evaluated by eliminating ATP from the reaction mixture (Figure 3). Four peptide substrates showed high signal in the absence of ATP that did not increase further with the addition of ATP. These four substrates were eliminated from further analyses. Three additional peptide susbstrates were undetectable with ATP and were also excluded from our analyses, leaving 133 phosphopeptide sequences.

2.3 Integrated Pathway Analysis of the serine/threonine kinome

Serine/threonine kinase activity was assayed in 12 subjects with schizophrenia and 12 age-, sex-, pH-, and PMI-matched comparison subjects (Table 2). Of the 133 peptide substrates, 19 showed changes in kinase activity larger than 15% (up or down) between schizophrenia and comparison groups (Figure 4 and Table 3). Seven of those had differences larger than 25%.

The Ingenuity algorithm identified 3 network functions for the dataset: 1) Cell movement, cell development and hematological development; 2) Connective tissue disorders, organismal injury and abnormalities, and cardiovascular disease; and 3) Cell signaling, nucleic acid metabolism and small molecule biochemistry. The top 3 functions within these networks were basic cell function and maintenance (16 peptides, p values from 7.09^{-11} to 5.85^{-3}), cell movement (13 peptides, p values from 8.97^{-8} to 6.23^{-3}), and molecular transport (10 peptides, p values from 1.16^{-7} to 5.85^{-3})(Table 4). Within these functions, 14/19 peptides relate to cell homeostasis ($p=7.09^{-11}$). Of those, 10/19 peptides relate to cytoskeletal organization ($p=5.46^{-7}$) and 7/19 relate to microtubule dynamics ($p=1.75^{-4}$) (Table 4). Eight of nineteen peptides in the dataset contribute to ion homeostasis ($p=2.05^{-7}$) with 7/19 relating to ion flux ($p=1.16^{-7}$), 6/19 relating specifically to Ca^{2+} flux ($p= 1.17^{-6}$) and 5 of those 6 most likely regulate Ca^{2+} influx ($p=2.67^{-6}$) and mobilization ($p=6.61^{-5}$) (Table 4).

Hematological development, hematopoeisis, lymphoid development and immune cell trafficking were the top four physiological systems identified by IPA as likely to be impacted based on this dataset (p values from 1.85^{-7} to 5.90^{-3})(Table 4).

3. Discussion

3.1 Historical IPA

There are a number of prevailing hypotheses for the etiology of schizophrenia. These include altered dopaminergic activity in the mesolimbic and mesocortical pathways (Laruelle et al., 1996; Laruelle et al., 1999; Meltzer and Stahl, 1976), glutamate and N-methyl-D-aspartate (NMDA) receptor signaling complex abnormalities (Hahn et al., 2006), prenatal exposure to pathogens and subsequent neuroinflammation (Bronson et al., 2011; Hahn et al., 2006; Vorhees et al., 2012), and developmental miscues resulting in defects in the wiring of neuronal circuits (Hoftman et al., 2013; Lewis et al., 1999; Marenco and Weinberger, 2000; Rapoport et al., 2005). Our IPA analyses of previously reported phosphoprotein irregularities in schizophrenia revealed that many of these proteins may be involved signaling pathways outside the specific pathway of interest that led to selection of the targets in the original studies (Supplemental Table 1). One of the pathways of interest in these studies is glutamate and N-methyl-D-aspartate (NMDA) receptor complex signaling. While NMDA signaling is critical for signal transduction and synaptic plasticity associated with cognitive processes (Coyle, 1996; Coyle et al., 2003; Tsai et al., 1995), many of the downstream targets of NMDA receptors function in other pathways as well. For example, ErbB4 and ERK function as signal integration molecules; PAK1, MLC, and Rap2, are involved in cytoskeletal architecture; and PACSIN and syntaxin assist with vesicle formation and docking (Funk et al., 2012; Hahn et al., 2006; Rubio et al., 2012; Swatton et al., 2004). Our analyses of the previously published findings support the hypothesis that altered kinase activity may impact other functional domains, including cellular morphology and organization, nervous system development, and cell survival, contributing to the underlying pathophysiology of this illness (Supplemental table 1). Inspired by these findings, we sought to replicate and expand these results using an unbiased kinome approach.

3.2 The schizophrenia kinome

A limitation of the previous phosphoprotein studies is that they were necessarily biased towards targets based primarily on strong pharmacological evidence implicating abnormalities of glutamate and dopamine neurotransmission in schizophrenia. D2 receptor antagonism has an antipsychotic effect, while NMDA receptor blockade with phencyclidine recapitulates the schizophrenia syndrome in humans (Greengard et al., 1999; McCullumsmith et al., 2004). Most of these studies tested well-developed hypotheses using Western blot analysis and phosphoprotein-specific antibodies, measuring one or a few targets at a time. Further, until recently proteomic approaches required large amounts of starting material, and had limited sensitivity for detecting phosphopeptides in complex biological samples. Finally, such Western blot and mass spectrometry approaches are only measuring the products of enzymatic reactions and not protein kinase activity. Thus, we sought to globally examine signaling cascades in schizophrenia by assessing kinase activity using an unbiased platform, the Pamgene kinome array, in subjects with schizophrenia.

Our kinome array studies yielded 19 peptides that were differentially phosphorylated (>15% change) in schizophrenia. These relatively modest changes in kinase activity raise the question of what magnitude of change in kinase activity has biological relevance. Subtle changes within and between converging signaling pathways may be more relevant to the etiology of disorders such as schizophrenia, which are highly heterogeneous, suggestive of profound disruption of cellular processes, and yet subtle with respect to morphological and structural changes within brain tissue. In vitro studies support this notion, as 1.11-1.25 fold changes in kinase activity stimulated cell proliferation and altered protein synthesis dynamics (Appuhamy et al., 2014; Cohen-Saidon et al., 2009; Gien et al., 2013). Additionally, small changes in kinase activity in ERK2, Wnt, and TGF-beta signaling had robust effects on cellular organization and survival (Goentoro and Kirschner, 2009; Goentoro et al., 2009; Haupt et al., 2012). Taken together, these data suggest that changes in kinase activity in the 15-25% range may significantly affect biological functions linked to altered signaling pathways.

IPA analyses of our kinome array dataset identified cytoskeletal organization and Ca^{2+} flux as the cellular functions with the strongest associations with schizophrenia. While confirmatory studies are needed to determine how these disruptions impact the schizophrenic brain, roles for cytoskeletal elements and Ca^{2+} flux are well defined in the CNS. For example, actin and microtubule dynamics are critically important for spine and synapse remodeling (Sakakibara et al., 2013; Verbich et al., 2012), molecular transport (Kreft et al., 2009; Smith, 2004), localization of mitochondria, receptors and transmitters (Kreft et al., 2009; Schwarz, 2013; Verbich et al., 2012), as well as axonogenesis (Sakakibara et al., 2013). Dysregulation of cytoskeletal organization and Ca^{2+} flux may be consistent with an abnormal glia-neuron interface in the prefrontal cortex in schizophrenia (Gobel and Helmchen, 2007; Patrushev et al., 2013). Interestingly, nervous system tumor and glioma were the top cancer and nervous system disorders identified by the IPA analysis of the kinome array dataset, consistent with the notion that astroglial function is disrupted in schizophrenia. Finally, several postmortem studies have found altered neuron architecture, changes in levels of calcium-regulated actin-binding proteins, and in calcium signaling

molecules (English et al., 2011; Garey, 2010; Lewis et al., 2003; Martins-de-Souza et al., 2009), consistent with the identification of roles for cytoskeleton and calcium flux abnormalities in this illness.

Changes in interactions between neurons and glia (Halassa et al., 2007; Schafer et al., 2013) are consistent with converging hypotheses regarding the etiology of schizophrenia. Irregularities in T-cell development and cytokine expression in schizophrenia suggest inflammatory processes (Craddock et al., 2007; Fineberg and Ellman, 2013). In line with this, IPA placed the peptides identified in the kinome array into canonical FLT-3 and T-cell receptor signaling pathways (Figure 4). This is also consistent with a substantial body of literature demonstrating abnormalities in both adaptive and innate immunity in schizophrenia and other psychotic disorders (Beumer et al., 2012; Fineberg and Ellman, 2013; Lang et al., 2007). Additionally, multiple glial subtypes utilize “immune system” signaling pathways (Cornet et al., 2000; DeBoy et al., 2010; Meeuwsen et al., 2003). Astrocytes and microglia contribute to postnatal brain development through sculpting of circuits and synapses (Parpura et al., 1994; Parpura et al., 2012; Schafer et al., 2013; Verbich et al., 2012). The age of onset for schizophrenia in adolescence and early adulthood corresponds to the period of extensive pruning and remodeling of brain circuits (Paolicelli and Gross, 2011; Rapoport et al., 2005). Microglia are highly involved in postnatal synaptic pruning which requires localization of complement proteins at the synaptic membrane (Paolicelli et al., 2011; Schafer et al., 2012; Stephan et al., 2012).

Our kinome array IPA findings, specifically calcium flux, cytoskeletal organization and molecular transport, also link to glutamate signaling dysfunction in schizophrenia (Table 4) (Trudeau, 2004). Regulation of glutamate levels in the synapse is dynamic and complex, involving multiple glial and neuronal receptors, transporters and co-transporters (Bridges et al., 2012; Mattson, 2008; McCullumsmith et al., 2004; Rodriguez et al., 2013). Ion transport and calcium signaling are integral to glutamate regulation as Ca^{2+} stimulates pre-synaptic glutamate release whereas astrocytes may in turn use glutamate to regulate neuronal Ca^{2+} levels (Parpura et al., 1994). Reduced intracellular calcium levels in astrocytes diminished glutamate buffering in the synapse, increasing glutamate spillover to extrasynaptic microdomains (Tanaka et al., 2013). Extrasynaptic glutamate signaling contributes to neuronal synchronization, aspects of memory formation, microglial activation and oligodendricyte maturation (Gibbs et al., 2011; Goubard et al., 2011; Hassanpoor et al., 2012; Rodriguez et al., 2013).

Multiple lines of evidence support deficits in glutamate buffering in schizophrenia including altered glutamate synthesis, altered receptor trafficking, decreased expression of glutamate transporters, and decreased activity in enzymes of the glutamate-glutamine cycle (Shan et al., 2012; Shan et al., 2013). Chronic dysregulation of extrasynaptic glutamate may in turn lead to pathological remodeling of excitatory synapses and signaling pathways downstream of glutamate receptors (Shan et al., 2012). While our kinome IPA finding of calcium flux as an implicated biological system is consistent Swith this hypothesis, calcium mediated signaling events are linked to myriad neurotransmitter systems and signal integration molecules.

There are several putative limitations of our studies. The subjects in our study suffered from a lifetime of severe mental illness and treatment with (primarily) typical antipsychotics. Changes found in this postmortem substrate most likely do not reflect abnormalities in the brain from the onset of the illness; rather, they more likely reflect a lifetime of neuroplastic changes suggestive of a common pathophysiological lesion associated with the behavioral phenotype of the schizophrenia syndrome (McCullumsmith et al., 2014). We have addressed the limitation of PMI by matching our subjects (to the extent possible) for PMI, and performing a rodent PMI control study. While previous studies have shown that PMI may impact phosphoprotein stability (Li et al., 2003; Li et al., 2005; Oka et al., 2011), kinase activity appears to be preserved (Figure 2 and supplementary Figure 1). The majority of the peptide substrates examined here were stable up to 12 hours PMI. Kinase activity at a few peptide substrates, CHK2, CBL, and ELK1 among them, had higher variability across several postmortem intervals and may be more sensitive to innate genetic variability or external factors such as PMI. However, this is more likely to mask differences between groups and there was sufficient signal in our samples to warrant maintaining them in the analysis. In addition, we did not have sufficient numbers of schizophrenia subjects off of antipsychotic medications at the time of death to perform secondary analyses to probe for medication effects. Previous studies have found that antipsychotics regulate protein kinase signaling pathways via neurotrophic, monoaminergic, glutamatergic, and G-coupled protein receptors (Beaulieu et al., 2009; Hunsberger et al., 2009; Molteni et al., 2009). Antipsychotics differ in regional and temporal profiles in their actions on kinase activity and signaling pathways (Del'guidice and Beaulieu, 2008; Pereira et al., 2012). For example, in vivo administration of haloperidol in mice produced minimal effects on ERK phosphorylation in prefrontal cortex and more robust effects in striatum (Pereira et al., 2012). However, we cannot exclude a contribution of anti-psychotic treatment to the current findings. Finally, our kinome array studies used brain regions containing an admixture of different cell types and extracellular matrix. Such region level studies may mask changes in specific cell populations that may be differentially altered in this illness (McCullumsmith et al., 2014).

3.3 Conclusions

In summary, we found modest but meaningful changes in kinase activity, that consistently plot with high significance to IP3K, MAPK/ERK, and PKC signaling pathways. Moreover, these data potentially integrate a number of hypotheses regarding the neuropathology of schizophrenia. There is strong evidence for lymphocyte irregularities in schizophrenia (Craddock et al., 2007; Drexhage et al., 2011) and maternal infection may be a risk factor for schizophrenia (Canetta and Brown, 2012). Immune system/autoimmune hypotheses in the etiology of schizophrenia include brain autoimmunity, inflammatory cytokines, and more recently, microglial dysfunction (Adams et al., 2012; Bayer et al., 1999; Fineberg and Ellman, 2013). In developmental hypotheses, genetic and epigenetic factors lead to mis-wiring of brain connections, either in utero or postnatally (Rapoport et al., 2005). However, we would argue that all of these theories converge on kinase activity and the regulation and coordination of key signaling cascades within different brain cell types (Perea and Araque, 2010; Santello et al., 2012). Future studies examining the kinome within individual cell types are critical to further define the nature of signaling pathway interactions in

schizophrenia. Comprehensive kinomic analyses, as we used here, may help shape new strategies for effective pharmacological treatment of devastating brain disorders such as schizophrenia.

4. Experimental Procedures

4.1 Literature Review

A PubMed search of “schizophrenia and postmortem and phosphorylation” produced 23 articles published between 2000 and 2012. These studies include profiles of protein and gene expression in human postmortem brain and targeted analysis of candidate proteins in various pathways. Of these, 12 measured expression of proteins and/or phospho-proteins in five brain regions (Amar et al., 2008; Carty et al., 2012; Castillo et al., 2010; Emamian et al., 2004; Funk et al., 2012; Hahn et al., 2006; Jones et al., 2002; Kozlovsky et al., 2001; Kunii et al., 2011; Rubio et al., 2012; Swatton et al., 2004)(Table 1). For re-analysis of the available data using IPA, each protein was considered of equal importance and a fold-change value of 1 was used when the exact fold change data were unavailable.

4.2 Tissue Collection

4.2.1 Human Tissue Samples—Fresh, frozen postmortem human anterior cingulate cortex for quality control studies was provided by the Alabama Brain Collection repository (Birmingham, AL). Fresh frozen postmortem anterior cingulate cortex for our schizophrenia studies was provided by the Mount Sinai NIH Brain and Tissue Repository (New York, NY) (Table 2). To control as much as possible for factors unrelated to schizophrenia that might impact kinase activity, schizophrenia and control samples were matched as pairs for age, sex, tissue pH and PMI (n=12 control to n=12 schizophrenia to make 12 schizophrenia-control pairs)(Table 2). Subjects were diagnosed with schizophrenia based on DSM-III-R criteria. Comparison subjects were selected using a formal blinded medical chart review instrument with no history of psychiatric or neurological disease. Exclusion criteria were a history of alcoholism, death by suicide, or coma for more than 6 hours before death. Next of kin consent was obtained for all subjects.

Approximately 500μg of tissue per subject was manually homogenized on ice for three minutes in Mammalian Protein Extraction Reagent buffer (ThermoScientific, Rockford, IL) with Halt™ phosphatase inhibitor cocktail (ThermoScientific, Rockford, IL) and Halt™ protease inhibitor cocktail (ThermoScientific, Rockford, IL). After homgenization tissue was stored at -80°C until use.

4.2.2 Rodent Tissue Samples—Rodent brain tissue for kinome analysis was collected from twelve adult, male, Sprague Dawley rats (225-275g; Harlan Laboratories, Houston, TX). The animals were housed in standard shoebox cages in a climate controlled vivarium on a 12:12 light cycle (lights on 06:00) with *ad libitum* access to food and water. All procedures involving animal subjects were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Alabama, Birmingham.

For the postmortem interval control studies, rats were sacrificed and stored at 4°C. Rat brains were extracted at 0, 4, 8, and 12 hours after sacrifice (n=3 per group). The frontal

cortex was dissected away from the rest of the brain and homogenized for three minutes on ice in Mammalian Protein Extraction Reagent buffer (ThermoScientific, Rockford, IL) with Halt™ phosphatase inhibitor cocktail (ThermoScientific, Rockford, IL) and Halt™ protease inhibitor cocktail (ThermoScientific, Rockford, IL). Tissue was stored at -80°C until use.

4.3 Serine-threonine kinase profiling using PamChip

Profiling of serine-threonine kinome (STK) activity was performed using the PamStationl2 microarray (PamGene International) and STK PamChips containing 144 consensus phosphopeptide sequences per well (4 of which are internal controls), immobilized on porous ceramic membranes. Each PamChip well was blocked with 2% bovine serum albumin (BSA) before 2μg of protein in the manufacturer's kinase buffer (PamGene), 157μM ATP, and FITC-labeled anti-phospho Ser/Thr antibodies (PamGene) were added in each well. The homogenized samples containing the active kinases and assay mix were pumped through the wells to facilitate interaction between kinases in the sample and specific peptide substrates immobilized on the chip. The degree of phosphorylation per well is measured in real time using Evolve (PamGene) kinetic image capture software. The software captures FITC labeled anti-phospho antibodies binding to each phosphorylated peptide substrate every 6 seconds for 90 minutes (Jarboe et al., 2012). Integrated spot intensities (slope of exposure brightness over multiple exposure times multiplied by 100) within the 99th percentile were then used to calculate a minimal positive shift using steady state as quality control and data were $\log^2$ transformed. These log transformed spot intensities are the "signal" for each peptide. Log transformation produces a more linear intensity distribution and allows fold-change comparisons of both increased and decreased kinase activity on the same scale (i.e. +1.5 and -1.5 represent equal magnitudes of change in opposite directions). The signal intensities for each peptide were analyzed using BioNavigator 5.2 Software (PamGene)(Jarboe et al., 2012).

A control array was run without the addition of ATP to identify non-specific binding of labeled antibody to the array substrate. Peptides with significant background signal in the -ATP condition (higher than the +ATP condition) were excluded from further analysis. Four peptide substrates were excluded based on these criteria. Peptide substrates for which signal was not detected in the +ATP condition were also excluded from analyses. Three additional peptides were excluded under this criterion, leaving a total of 133 peptide substrates.

4.4 Data Analysis

Signal intensity of phosphopeptides at increasing postmortem intervals were analyzed individually using ANOVA (Graphpad Prism). Data is expressed as mean +/- standard error of the mean. Signal intensity data for each peptide (excluding the 4 control peptide substrates, 3 peptide substrates for whom kinase activity could not be detected and 4 peptides with non-specific activity) for all 24 subjects were tested for outliers (+/- 2.5 standard deviations from the mean of the entire group of 12 schizophrenia or control samples). When an outlier was identified in either member of the Schizophrenia-Comparison pair, the pair was eliminated from analysis for that probe sequence. Comparisons for each peptide substrate were made in at least 10-12 pairs (n=20-24 subjects). The mean of the Schizophrenia-Comparison ratio was used to calculate fold-

change for each peptide. Peptides with a fold change +/- 1.15 were considered significant for further analysis.

IPA uses a knowledge base, compiled from published findings and gene and protein databases to assign genes and proteins of interest to functionally related groups and categories. IPA utilizes Fisher's exact test to determine the probability (with associated p values) that a peptides or group of peptides from the data set, acting within a particular pathway or function, would occur by chance (Choe et al., 2007; English et al., 2011; Yang et al., 2010). Results are typically presented in three levels starting with broad categories of 1) Molecular and Cellular Functions, 2) Physiology and Developmental Functions, and 3) Diseases and Disorders. Within these broad categories, the analyses provide more refined functional domains (i.e. inflammatory response, cell or tissue morphology, or cellular movement) with a range of p-values. These p-values correspond to specific disorders, processes and functions within the domain that are enriched for peptides in the dataset.

Pathway analysis of published findings and the results of the kinome array were analyzed independently using Ingenuity Pathway Analyses (IPA). Functions and pathways with assigned p-values less than .05 and containing more than 3 peptides with a +/-1.15 fold change were considered most relevant to changes associated with schizophrenia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We use kinome array technology to examine global patterns of kinase activity in schizophrenia.
- The kinome array differentially detected kinase activity in anterior cingulate in schizophrenia.
- Ca^{2+} and cytoskeletal dynamics were pathways most affected in schizophrenia.

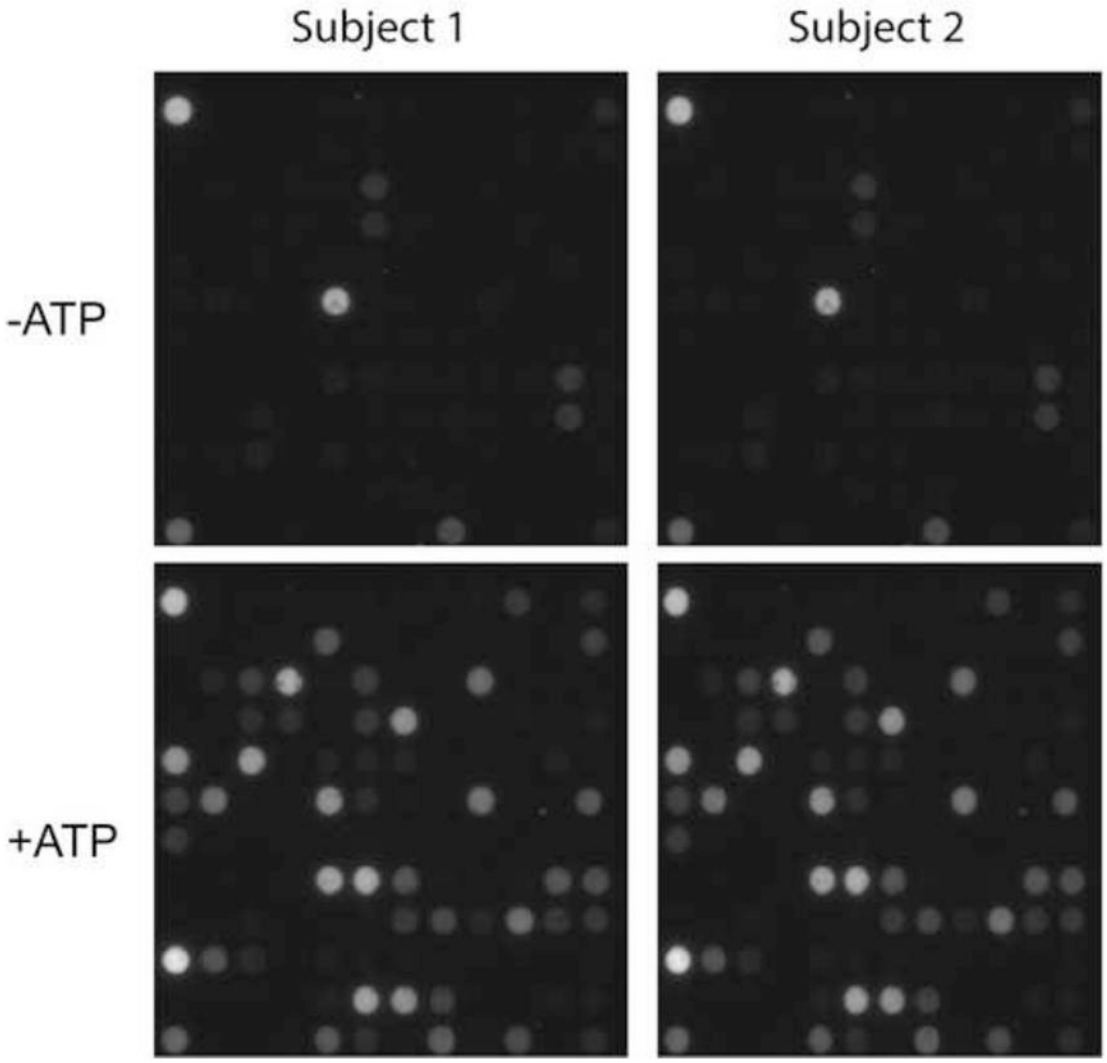

Figure 1.

Detection of kinase activity in human postmortem brain. The increase in signal intensity of all peptide substrates during PamChip data collection was similar for fresh rodent and human postmortem brain.

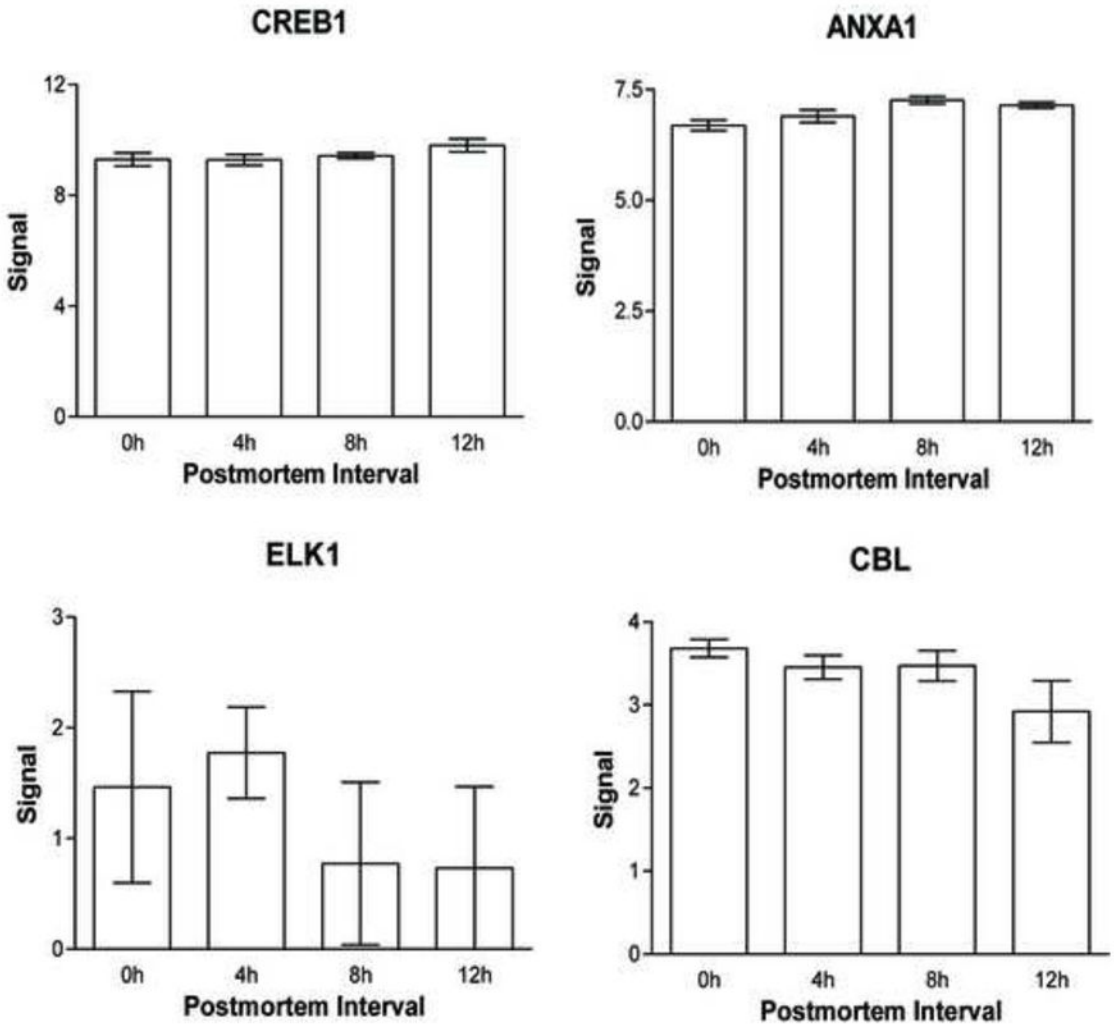

Figure 2.

Signal intensities for representative rodent peptide substrates over increasing postmortem intervals. Phosphorylation of peptide substrates did not significantly change between 0 and 12 hours postmortem (n=3).

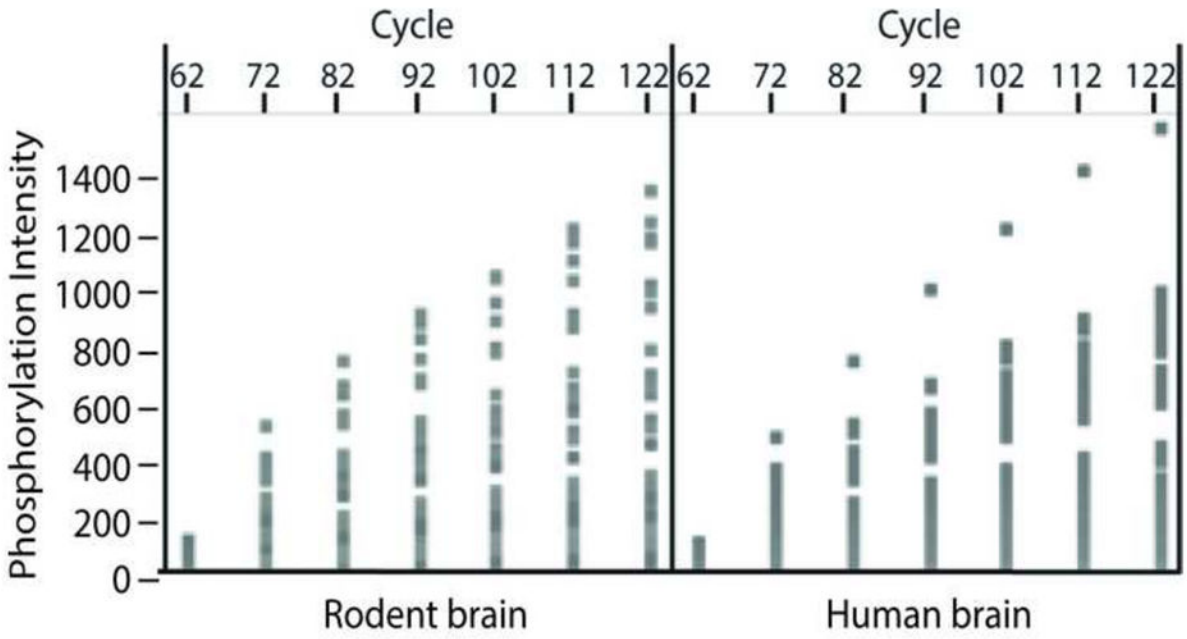

Figure 3.

Non-specific kinase signal from postmortem human tissue. High background signal that did not increase with the addition of ATP to the reaction was detected in four substrates at positions (5,6), (8,12), (11,8), and (11,9).

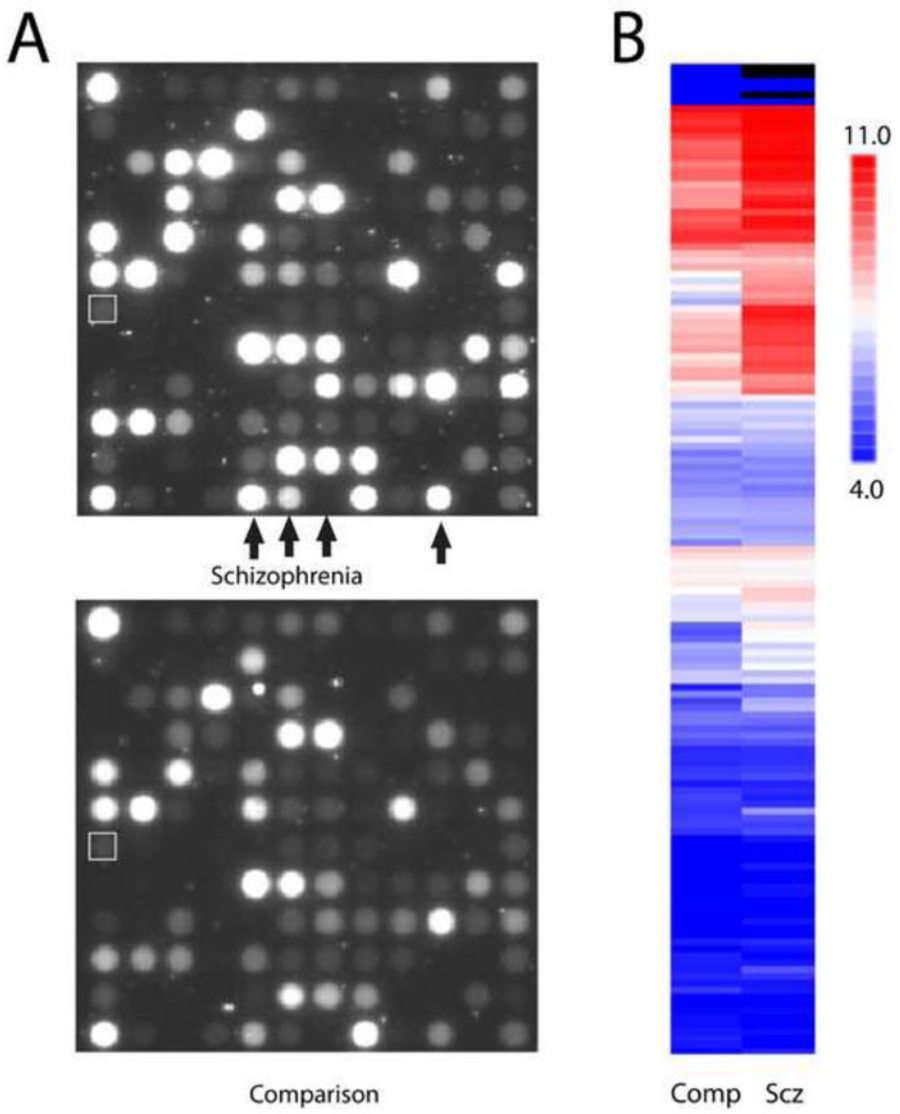

Figure 4.

Substrate phosphorylation differs between schizophrenia and comparison samples. A) Representative serine-threonine kinase activity profiles from schizophrenia (top) and comparison (bottom) samples. The arrows highlight individual substrates that are differentially phosphorylated between this schizophrenia-comparison pair. B) Heat map comparison of peptide phosphorylation depicted in panel A.

Table 1

Summary published studies of phosphoproteins and protein kinases in schizophrenia

Findings	Brain Region	Brain Bank	Samples	Level of Evidence	Reference
STEP61 ↑	ACC, DLPFC	Stanley Consortium, Mount Sinai Brain Bank	12 scz and 12 ctrl, 14 scz and 14 ctrl	Western	(Carty et al, 2012)
PAKl ↓, MLC ↑(ACC) ↔ (DLPFC), Cofilin 2 ↔	ACC, DLPFC	Mount Sinai Brain Bank	36 scz and 33 ctrl, 35 scz and 29 ctrl	Western	(Rubio et al, 2012)
DARPP-32 ↓ (II-V), phosphoDARPP-32 ↑ (V)	DLPFC (layers II-V)	Fukushima Brain Bank	9 scz and 9 ctrl	Immunohistochemistry	(Kunii et al, 2011a)
phosphoDARPP-32 ↑	STG	Fukushima Brain Bank	11 scz and 11 ctrl	Immunohistochemistry	(Kunii et al, 2011b)
Casein Kinase II ↓ Syntaxin 1 ↓	BA10	Dallas Brain Collection	15 scz and 15 ctrl	Western	(Castillo et al, 2010)
MAP2 ↓	BA9, BA32 (layers III and V)	Harvard Brain Tissue Resource Center	7 scz and 7 ctrl	Immunocytochemistry	(Jones et al, 2002)
ERK1/2 ↑, phosphoERK ↔	PFC	Tokyo Metropolitan Matsuzawa Hospital	8 scz and 5 ctrl	Western	(Swatton et al, 2004)
GSK3Beta ↓	FC	Rebecca L. Cooper Brain Bank	15 scz and 15 ctrl	Western	(Amar et al, 2008)
phosphoCREB ↓	FC	Stanley Consortium	15 scz and 15 ctrl	Western	(Kozlovsky, et al 2001)
Rap2 ↓, phosphoJNK1 ↓, phosphoJNK2 ↓, phosphoPSD95 ↓ (ACC) ↑(DLPFC), Rack1 ↑, Fyn ↑, Cdk5 ↑	ACC, DLPFC	Mount Sinai Brain Bank	36 scz and 33 ctrl, 35 scz and 31 ctrl	Western	(Funk etal, 2012)
phosphoGluN2B ↑	FC	Medical Research Council Brain Bank, Stanley Consortium	10 scz and 10 ctrl, 10 scz and 10 ctrl	Western	(Emamian et al, 2004)
NRG1 ↔, ErbB4 ↔	BA9, BA10, BA46	Schizophrenia Research Center (University of Pennsylvania)	14 scz and 14 ctrl	Western	(Hahn et al, 2006)

Abbreviations: Anterior Cingulate Cortex (ACC), Dorsolateral Prefrontal Cortex (DLPFC), Superior Temporal Gyrus (STG), Brodmann Area (BA), Prefrontal Cortex (PFC), Frontal Cortex (FC), Schizophrenia (scz), Control (ctrl), Increased (↑), Decreased (↓), Unchanged (↔).

Table 2

Subject demographics for schizophrenia and comparison subjects

Pair	Subject	Sex/Age, y	pH	PMI, m	Medication
1	Control	M/59	6.63	1225	No
	Schizophrenia	M/57	6.10	1220	Yes
2	Control	F/80	6.20	285	No
	Schizophrenia	F/80	5.80	415	No
3	Control	M/65	6.82	230	No
	Schizophrenia	M/63	5.90	372	Yes
4	Control	F/84	6.21	1110	No
	Schizophrenia	F/86	5.80	1092	Yes
5	Control	F/66	6.51	960	No
	Schizophrenia	F/69	6.20	820	Yes
6	Control	M/66	6.58	454	No
	Schizophrenia	M/66	6.50	725	Yes
7	Control	F/88	6.40	305	No
	Schizophrenia	F/90	5.97	465	Yes
8	Control	M/72	6.60	720	No
	Schizophrenia	M/73	6.50	475	Yes
9	Control	F/74	6.00	180	No
	Schizophrenia	F/74	6.30	417	Yes
10	Control	M/95	6.53	245	No
	Schizophrenia	M/97	6.50	555	Yes
11	Control	M/69	6.30	255	No
	Schizophrenia	M/68	6.27	534	Yes
12	Control	F/78	6.19	600	No
	Schizophrenia	F/77	6.01	583	Yes

Abbreviations: Female (F), Male (M), years (y), minutes (m), Postmortem Interval (PMI). Medication status of “no” means off anti-psychotic medications six weeks or longer before time of death. Medication status of “yes” means administered anti-psychotic medication within six weeks of time of death.

Table 3

Substrate peptides differentially phosphorylated between schizophrenia and comparison subjects in the kinome array.

Increased Signal	Uniprot Accession	Abbreviation	Fold Change	Upstream kinase pathway
G-protein signaling modulator 2	P81274	GPSM2	1.329	PKC
Phosphorylase b kinase alpha M subunit	P46020	PHKA1	1.295	PIM1; PKA
Cystic fibrosis transmembrane conductance regulator	P13569	CFTR	1.263	PIM1
β-2 adrenergic receptor	P07550	ADRB2	1.250	MSK; CAMKIV; PKA
cAMP-responsive element-binding protein	P16220	CREB1	1.221	PIM1; MSK; PKC
Macrophage colony-stimulating factor 1 receptor	P07333	CSF1R	1.217	PIM1
Annexin 1	P04083	ANXA1	1.204	NPR1; PKG2
Vasodilator-stimulated phosphoprotein	P50552	VASP	1.192	MSK; PKG1
Erythrocyte protein 4.2	P16452	EPB42	1.185	PIM1; PKC
Protein tyrosine kinase 6	Q13882	PTK6	1.180	PKC; AKT; PIM3
G-protein coupled receptor 6	P46095	GPR6	1.164	p38 MAPK; ERK1
Phospholemman ion transport regulator	O00168	FXYD1	1.156	PKC; PIM1
Proto-oncogene c-Rel	Q04864	REL	1.152	PIM1; PKG
Vitronectin adhesion molecule	P04004	VTN	1.152	PKA; PKC; RSK
Decreased Signal	Uniprot Accession	Abbreviation	Fold Change	Upstream Kinase Pathway
Ets-domain containing protein	P19419	ELK1	-1.446	ERK1/2; p38; JNK
Myelin basic protein	P02686	MBP	-1.300	mTOR; JNK; ERK1/2
E3 ubiquitin ligase	P22681	CBL	-1.277	CHEK1; PKC; JNK; GSK3B
Serine/threonine protein kinase 2	O96017	CHEK2	-1.242	PIM1; CHEK2; MEK; PDK1; JNK
Tyrosine protein kinase	P43403	ZAP70	-1.173	mTOR; CDK4

14 peptides exhibited a 1.15-fold or more increase in kinase activity while 5 exhibited a 1.15-fold or more decrease in phosphorylation.

Table 4

Ingenuity Pathway Analysis of the schizophrenia kinome

Molecular and Cellular Functions	**IPA p-value**	**# Molecules**	**Function Annotation (# molecules)**
Cellular Function and Maintenance	7.09E-11–5.85E-3	16	Cellular homeostasis (14); Cytoskeletal organization (10); Microtubule dynamics (7); Ion homeostasis (8); Ca^{2+} flux (6); T cell development (7)
Cellular Movement	8.97E-8–6.23E-3	13	Leukocyte migration (9); Migration of cells (11); invasion of cells (7); cell movement of myeloid cells (6)
Molecular Transport	1.16E-9–5.85E-3	10	Ion flux (7); Flux of Ca^{2+} (6); influx of Ca^{2+} (5); mobilization of Ca^{2+} (5); uptake of D-glucose (4)
Cell Development	1.85E-7–4.92E-3	14	Development of leukocytes (8); Proliferation of blood cells (9); Differentiation of cells (12)
Cellular Growth and Proliferation	3.03E-7–6.03E-3	15	Proliferation of blood cells (9); Proliferation of monocytes (8); Proliferation of T lymphocytes (7)
Physiological Systems and Development	IPA p-value	# Molecules	Physiological System Annotation
Hematological System and Development	1.85E-7-5.9E-3	14	Leukocyte development (8); T-cell development (7); proliferation of monocytes (8)
Hematopoeisis	1.85E-7-5.85E-3	10	Leukocyte development (8); T-cell development (7); proliferation of monocytes (8)
Lymphoid Tissue Structure and Development	1.85E-7-4.23E-3	11	Leukocyte development (8); T-cell development (7); Differentiation of T lymphocytes (5)
Immune Cell Trafficking	5.35E-7-5.9E-3	10	Leukocyte migration (9); Cell movement of leukocytes (8) Cell movement of myeloid cells (6)
Top Canonical Pathways	IPA p-value		Downstream Pathways
FLT3 Signaling in Hematopoietic Progenitor Cells	5.11E-5		PI3K-Akt; Ras-MEK-ERK; JAK-STAT
Glucocorticoid Receptor Signaling	1.15E-4		PI3K-Akt; Ras-MEK-ERK; GSK3β; NFκB; SGK
T Cell Receptor Signaling	1.28E-4		PI3K-Akt-NFκB; Ras-MEK-ERK; p38; PKCθ-JNK
Gαs signaling	1.87E-4		PKA; Ras-MEK-MAPK
PI3K signaling in B Lymphocytes	2.8E-4		AKT-GSK3β; AKT-NFκB

Molecular and cellular functions, physiological and developmental functions, and canonical pathways determined by IPA to correspond to the differentially activated kinases in the kinome array data set.