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# Proteomic pathway analysis of the hippocampus in schizophrenia and bipolar affective disorder implicates 14-3-3 signaling, aryl hydrocarbon receptor signaling, and glucose metabolism: Potential roles in GABAergic interneuron pathology

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ABSTRACT

Neuropathological changes of the hippocampus have been associated with psychotic disorders such as schizophrenia and bipolar disorder. Recent work has particularly implicated hippocampal GABAergic interneurons in the pathophysiology of these diseases. However, the molecular mechanisms underlying structural and cellular hippocampal pathology remain poorly understood. We used data from comprehensive difference-in-gel electrophoresis (2-D DIGE) investigations of postmortem human hippocampus of people with schizophrenia and bipolar disorder, covering the acidic (isoelectric point (pI) between pH 4 and 7) and, separately, the basic (pI between pH 6 and 11) sub-proteome, for Ingenuity Pathway Analysis (IPA) of implicated protein networks and pathways. Comparing disease and control cases, we identified 58 unique differentially expressed proteins in schizophrenia, and 70 differentially expressed proteins in bipolar disorder, using mass spectrometry. IPA implicated, most prominently, 14-3-3 and aryl hydrocarbon receptor signaling in schizophrenia, and gluconeogenesis/glycolysis in bipolar disorder. Both disorders were characterized by alterations of proteins involved in the oxidative stress response, mitochondrial function, and protein-endocytosis, -trafficking, -degradation, and -ubiquitination. These findings are interpreted with a focus on GABAergic interneuron pathology in the hippocampus.



## 1. Introduction

The hippocampus, a temporal lobe structure crucially involved in memory formation and consolidation (Scoville and Milner, 1957; Squire, 2009), spatial memory and navigation (Moser et al., 2008), and the capacity to inhibit learned responses to stimuli (Gray and McNaughton, 2000), has been consistently implicated in schizophrenia and bipolar disorder (Harrison, 2004). Deficits of hippocampal connectivity and structural volumes have been demonstrated in schizophrenia and bipolar patients (Bilder et al., 1995; Glahn et al., 2008, 2010; Ng et al., 2009). While some findings are similar for both disorders (Haukvik et al., 2014; Mathew et al., 2014), differences are described in the overall extent and in the pre-morbid manifestation of hippocampal volume reductions (Fornito et al., 2009; Ellison-Wright and Bullmore, 2010; Fusar-Poli et al., 2011, 2012), which may relate to different underlying biological mechanisms (Bellivier et al., 2013).

At the histological and cellular level, several studies have found hippocampal neurons of compromised morphology and organization, as well as pre-synaptic and dendritic deficits, in schizophrenia (Harrison, 2004) and bipolar disorder (Konradi et al., 2011b). Amongst the neuron populations affected by these changes, GABAergic interneurons, and particularly those of the fast-spiking basket and chandelier type, have come into the focus in schizophrenia and bipolar disorder research, mainly because of solid evidence for widespread reductions in the concentration of proteins typically expressed by fast-spiking cells such as GAD67, somatostatin, and parvalbumin (Woo et al., 1997; Lewis et al., 2005) in several cortical regions (Hashimoto et al., 2008). In the hippocampus, an increase in $GABA_A$ receptor binding activity in people with schizophrenia was found to be predominantly located to non-pyramidal cells of the cornu ammonis areas 3 (CA 3) (Benes et al., 1996), and was hypothesized to represent a compensatory up-regulation of receptors in response to a loss or deficit of inhibitory GABAergic interneurons (Benes et al., 1989). Further, reduced numbers of non-pyramidal cells were found selectively in CA 2 of people with schizophrenia and bipolar disorder (Benes et al., 1998), and rodent

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models have demonstrated that these cells are almost certainly fast-spiking interneurons containing parvalbumin (Gisabella et al., 2009). Consistent with these findings, overall numbers of parvalbumin-positive cells are reduced in the hippocampus of people with schizophrenia and bipolar disorder (Konradi et al., 2011a, 2011b), and reductions in GAD67 immunoreactivity were detected in the stratum oriens of CA 2/3 in both diagnostic groups (Benes et al., 2007). Because fast-spiking GABAergic interneurons synapse onto the spike-initiating region of pyramidal cells, they are thought to possess key controlling properties within the overall firing patterns of brain networks (Lisman et al., 2008). In animal models, both inhibition of hippocampal interneurons and stimulation of hippocampal excitatory outputs were shown to induce increased dopamine release in the ventral tegmental area (VTA), which in turn is one of the hallmarks of psychosis pathophysiology (Moore et al., 2006; Lodge and Grace, 2007).

Proteomic studies of the brain can provide insights into the molecular mechanisms underlying psychotic disorders. In schizophrenia, systematic review and analysis of published studies indicate converging molecular pathways in various brain areas and across gray and white matter in schizophrenia, including glucose metabolism, mitochondrial function, and cytoskeletal and synaptic integrity (English et al., 2011). In bipolar disorder, no systematic review or meta-analysis of proteomic findings has been undertaken to date.

Given the aforementioned association of hippocampal pathology with psychotic disorders, surprisingly few proteomic screens of the hippocampus in schizophrenia and bipolar disorder have been undertaken (Edgar et al., 1999). Our group recently undertook the first detailed proteomic study of the human hippocampus in schizophrenia and bipolar disorder for proteins with isoelectric points (pI) between pH 4 and pH 7, assessing the hippocampal subregions CA1, CA2/3, CA4, and DG separately (Focking et al., 2011a). We found a significant overlap between schizophrenia and bipolar disorder with regard to differentially expressed proteins. In both disorders, the most prominent protein changes were detected in CA 2/3. Pathway analysis particularly implicated cellular assembly and organization as well as Clathrin-Mediated Endocytosis (CME) in both disorders.

In order to complement these findings, we re-evaluated these results, using Ingenuity Pathway Analysis (IPA), in combination with novel data on differential protein expression relating to the basic sub-proteome in postmortem schizophrenia and bipolar hippocampus tissue, covering proteins with a pI between pH 6 and pH 11. This basic proteome contains many low abundance- and membrane bound proteins which cannot be identified using pH 4–7 approaches (McManus et al., 2010). We present the results of IPA, covering the combined acidic and basic pH ranges. The relevance of implicated pathways to GABAergic interneuron pathology in the hippocampus in schizophrenia and bipolar disorder is then discussed. The approach may help generate novel testable hypotheses for future research and drug development.

## 2. Methods

Proteomic screens were carried out separately for the pI pH ranges 4–7 and 6–11.

Methods and results of the pH 4–7 study, which included separate analyses for the hippocampal sub-regions CA1, CA2/3, CA4, and DG, have previously been published (Focking et al., 2011a).

For the present study, a screen of the sub-proteome with pI between pH-6 and 11 was added to previous results analyzing the whole hippocampus. For Ingenuity Pathway Analysis (IPA), proteins identified as differentially expressed in schizophrenia or bipolar disorders in either screen were considered.

### 2.1. Sample characteristics and preparation

All experiments were carried out on postmortem brain tissue donated by the Stanley Medical Research Institute's (SMRI) brain collection. The initial preparation of postmortem tissue was carried out by anatomist Maree J. Webster, PhD, Stanley Medical Research Institute, Maryland, USA. For the pH 4–7 study, a well-matched sub-sample of the Stanley Array Collection consisting of 20 cases with schizophrenia, 20 cases with bipolar disorder, and 20 healthy controls were selected, and hippocampi were prepared for proteomic analysis by laser-assisted dissection, as described previously (Focking et al., 2011a).

For the pH 6–11 experiments, a subsample of 27 cases of the Stanley Array collection (10 schizophrenia, 10 bipolar disorder, 7 controls, Supplementary Table 2), and hippocampi were prepared for further experiments as described in the Supplementary material.

For both studies, cases were matched as closely as possible for age, gender, postmortem interval (PMI), and brain pH (sTable 1 and sTable 2).

### 2.2. Separation of proteins by 2-dimensional difference-in-gel electrophoresis, digital image analysis, and statistical evaluation

For both proteomic screens (pH 4–7 and pH 6–11), samples were processed and separated by 2-dimensional difference-in-gel electrophoresis (2D-DIGE) as described previously (Behan et al., 2009; Pennington et al., 2008; English et al., 2009; Focking et al., 2011a). For the pH 6–11 study, sample homogenates were gradually introduced to the pH 6–11 immobilized pH gradient (IPG) strips (GE Healthcare) via a plastic cup placed over the anode (for details see Supplementary information).

### 2.3. Identification of differentially expressed proteins by mass spectrometry

For identification of differentially expressed proteins, mass spectrometry was used as previously described (Focking et al., 2011a), using an Agilent 6520 mass spectrometer coupled to a reverse phase HPLC column. For detailed descriptions of the experiments see Supplementary information.

### 2.4. Pathway analysis

Protein networks and canonical pathways associated with differentially expressed proteins in schizophrenia or bipolar disorder compared to controls were explored using QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity). For analysis, all proteins identified in any hippocampal subfield in either the pH 4–7 or the pH 6–11 experiment were entered into the software. IPA makes use of computational algorithms to identify local networks that are particularly enriched in original-omics datasets. Such local networks contain the most highly connected focus proteins, which, in turn, have specific interactions with other proteins in the network. Further, significant biological functions are determined using the right-tailed Fisher's exact test to compare the number of proteins that participate in a given function to the total number of occurrences of these proteins in all functional annotations. Additionally, multiple hypothesis correction is carried out based on the Benjamini–Hochberg (B–H) approach at 1% FDR threshold. Significance levels are expressed as the IPA p-value. IPA additionally identifies overrepresented Canonical Pathways in the entire dataset of differentially expressed proteins. Canonical pathways within the IPA knowledge library have a defined number of molecules, and an "IPA ratio" of specific dataset molecules to the total number of molecules in the pathway is reported. Additional weighting is given to functions or pathways containing the highest proportion of focus proteins (i.e. differentially expressed proteins from the dataset).

## 3. Results

### 3.1. Proteins differentially expressed in schizophrenia

Across all hippocampal subfields, 46 discrete proteins were identified as differentially expressed in schizophrenia compared to healthy

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