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Distinct proteomic profiles in *post-mortem* pituitary glands from bipolar disorder and major depressive disorder patients

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ABSTRACT

Disturbances of the hypothalamic–pituitary–adrenal axis have been implicated in the pathophysiology of bipolar disorder (BD) and major depressive disorder (MDD). To examine this further, we carried out proteomic profiling of *post-mortem* pituitaries from 13 BD and 14 MDD patients, in comparison to 15 controls. Liquid chromatography–mass spectrometry (LC–MS^E) analysis showed that BD patients had significantly increased levels of the major pituitary hormones pro-opiomelanocortin (POMC) and galanin. BD patients also showed changes in proteins associated with gene transcription, stress response, lipid metabolism and growth signalling. In contrast, LC–MS^E profiling revealed that MDD patients had significantly decreased levels of the prohormone-converting enzyme carboxypeptidase E and follow-up enzymatic analysis showed decreased activity of prolyl-oligopeptidase convertase. This suggested that altered prohormone processing may occur in pituitaries of MDD patients. In addition, MDD patients had significant changes in proteins involved in intracellular transport and cytoskeletal signalling. Finally, we carried out selective reaction monitoring (SRM) mass spectrometry profiling for validation of protein changes in key biological pathways. This confirmed increased POMC levels in BD patients with no change in the levels of this prohormone in MDD. This study demonstrates that proteomic profiling analysis of the pituitary can lead to new insights into the pathophysiology of BD and MDD. Also, given that the pituitary directly releases a variety of bioactive molecules into the bloodstream, many of the proteins identified here could serve as focal points in the search for peripheral biomarkers in clinical or drug treatment studies of BD and MDD patients.

1. Introduction

Bipolar disorder (BD) and major depressive disorder (MDD) have a lifetime prevalence of 20% (Hirschfeld, 2012). Individuals with either of these disorders have decreased life expectancies due to suicide and increased comorbidities such as obesity, diabetes, dyslipidemia and cardiac disease (Ferrari et al., 2013; Hauser et al., 2013). Some of these co-morbidities can be side effects from psychiatric medications (Gazalle et al., 2007; McLaren and Marangell, 2004). Also, misdiagnosis or inappropriate treatment can have negative effects on health and result in a worse outcome for patients (Bowden, 2010). Recent studies have attempted to increase our understanding of the underlying pathophysiologies of BD and MDD through identification of disease-specific biomarkers (Alsaif et al., 2013; Schneider and Prvulovic, 2013). These studies have identified hypothalamic–pituitary–adrenal (HPA) axis perturbations in many patients with mood disorders (Banki et al., 1987; Grunze, 2011; Tsigos and Chrousos, 2002). The HPA axis is a component of the diffuse neuroendocrine system involved in regulation of vital homoeostatic systems throughout the body, including blood pressure, metabolism, inflammation and fluid balance. Effects on all of these processes have been implicated in BD and MDD. In addition, pituitary size is known to be enlarged in adolescents with BD and MDD, indicating that hormonal abnormalities may occur at early stages of disease (MacMaster et al.,

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2008). One of the most studied components of the HPA axis is regulation of the stress response via proteolytic conversion of pro-opiomelanocortin (POMC) to adrenocorticotropin (ACTH) in the anterior pituitary (Arlt and Stewart, 2005). Secretion of ACTH into the peripheral circulation stimulates release of cortisol from the adrenal cortex, which in turn can affect brain function (Frodl and O'Keane, 2013). Under normal circumstances, cortisol also acts on the hypothalamus and pituitary in a negative feedback loop to suppress production of ACTH.

Several other proteins produced by the pituitary have effects on the brain. For example, galanin regulates cognition, neuronal growth and neuronal protection (Mechenthaler, 2008). Growth hormone (somatotropin) has been implicated in several pathways including the sleep–wake cycle, brain development and neuronal repair (Arce et al., 2013). Prolactin is involved in diverse processes such as regulation of the immune response, neurogenesis and neuronal differentiation (Larsen and Grattan, 2012). Also, the production of prolactin is tightly regulated with that of neurotransmitters such as dopamine which can affect behaviour and mood (van den Pol, 2010). The pituitary also contains prohormone-converting enzymes such as carboxypeptidase E (CPE) and prolyl-oligopeptidase 1 (POP), which are involved in proteolytic maturation of pituitary hormones and other proproteins, leading to generation of a spectrum of bioactive peptides (Hook et al., 2009; Yoshimoto et al., 1981).

The aim of this study was to identify HPA axis biomarker candidates for BD and MDD using post-*mortem* pituitary tissue. Protein extracts were analysed using a combination of liquid chromatography mass spectrometry (LC–MS^E), selective reaction-monitoring (SRM) mass spectrometry and an enzyme assay to identify BD- and MDD-associated proteomic fingerprints. The analyses focussed on identification of secreted pituitary proteins as these could be translated into blood-based assays for future clinical studies.

2. Materials and methods

2.1. Samples

Whole *post-mortem* pituitaries from 15 control, 13 BD and 14 MDD subjects were provided by the Stanley Medical Research Institute (Bethesda, MD, USA). These were matched for variables including age at death, gender, *post-mortem* brain interval (PMI), duration of storage, onset age, duration of illness, death by suicide and history of substance abuse (Table 1). The control subjects had no history of psychiatric or neurological disorders. Tissue had been collected *post-mortem* with informed consent by a first degree relative in compliance with the Declaration of Helsinki. The protocol was reviewed and accepted by the Uniformed Services University of Health Sciences institutional review board and samples were de-identified and personal information anonymised. Local ethical approval for use of this tissue was granted by the Cambridgeshire Local Research Ethics Committee.

2.2. Sample preparation

Biochemical reagents were from Sigma–Aldrich (Poole, UK), unless specified otherwise. Whole pituitaries were ground on dry ice using a pestle and mortar, and stored at −80 °C. Approximately 50 mg powdered tissue was homogenised using a Branson Sonifier 150 (Thistle Scientific; Glasgow, UK) for 10 s in 250 μL ice-cold 30 mM Tris-HCl (pH 7.4), containing a 1:200 dilution of protease inhibitor cocktail (Merck Calbiochem; Nottingham, UK). Samples were centrifuged at 16,000 g for 30 min at 4 °C and supernatants were transferred into fresh tubes and the pellets re-extracted in Tris-HCl. The supernatants were combined with those from the first extraction, generating the soluble fraction. The pellets were sonicated in 50 μL of 8 M urea, vortexed and centrifuged as above. The final supernatants were transferred into new tubes, producing the insoluble fraction. A 5 μL aliquot of each sample was combined to create 4 quality control samples for each fraction, for use in assessing variation of the extraction procedure. Protein concentrations were estimated using the DC Protein Assay Kit (Bio-Rad; Hercules, CA, USA).

Approximately 20 μL of each sample (100 μg) was made to 100 μL with 50 mM ammonium bicarbonate. Dithiothreitol was added to a final concentration of 5 mM, followed by incubation at 60 °C for 30 min to reduce protein disulfide bonds. Next, iodoacetamide was added to a final 10 mM concentration, with incubation at room temperature in the dark for 30 min to alkylate reduced sulfhydryl groups. Porcine trypsin (Promega; Madison, WI, USA) was added at a 1:50 ratio, followed by incubation overnight at 37 °C for limited proteolysis of proteins. Reactions were stopped after 16 h by addition of 1.67 μL of 10 M HCl. The samples were stored at −80 °C prior to analysis.

2.3. LC–MS^E analysis

LC–MS^E profiling was performed using a quadrupole time-of-flight (QTOF)-Premier™ mass spectrometer (Waters Corporation; Wythenshawe, UK) (Krishnamurthy et al., 2013). Samples (0.6 μL) were made to 5 μL in buffer A (H_2O + 0.1% formic acid) and analysed in duplicate. Desalting was performed with 100% buffer A for 2 min, using a 5 μm particle size 0.18 × 20.0 mm C18 trapping column (Waters Corporation). Peptides were separated using a 1.7 μm particle size 0.075 × 200 mm C18 BEH nano-column (Waters Corporation) at 0.3 μL/min. The gradient had an initial concentration of 97% A/3% B (acetonitrile + 0.1% formic acid), followed by successive linear increases to 30% B over 90 min, 90% B over 25 min and 97% B in 5 min. The concentration of buffer B was kept constant at 97% for 10 min and then returned to 3% B over 1 min.

Table 1
Demographic details of *post-mortem* pituitary samples from control, bipolar disorder (BD) and major depressive disorder (MDD) individuals. For continuous variables, the mean/median (standard deviation) is presented.

	BD	MDD	CT	*P*-value
Age of death	41.50/42.50 (11.63)	46.38/46.00 (9.43)	48.07/52.00 (10.67)	0.337^K
PH	6.19/6.30 (0.26)	6.17/6.20 (0.20)	6.27/6.30 (0.24)	0.530^K
PMI	35.00/28.50 (16.98)	28.15/28 (11.42)	23.73/26.00 (9.95)	0.243^K
Duration of storage (days)*	615.30/630.50 (191.63)	465.60/512.00 (299.04)	338.30/286.00 (234.32)	0.033^K
Age of onset (years)	19.67/18.50 (8.02)	33.85/32.00 (12.44)	–	0.003^W
Duration of illness (years)	20.92/22.00 (9.95)	12.62/11.00 (11.36)	–	0.026^W
Gender (male/female)	5/7	6/7	6/9	0.945^C
Death by suicide (yes/no)	9/3	6/7	0/15	0.0002^C
Substance (never/current/past)	5/5/2	9/3/1	12/1/2	0.230^C
Brain side (right/left)	7/5	6/7	7/8	0.788^C

*Storage time as of April 1, 1997; K: Kruskal Wallis test; W: Wilcoxon rank sum test; C: Chi-squared test.