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Effects of typical (haloperidol) and atypical (risperidone) antipsychotic agents on protein expression in rat neural stem cells

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ABSTRACT

Neural stem cells (NSCs) play a crucial role in the development and maturation of the central nervous system. Recently studies suggest that antipsychotic drugs regulate the activities of NSCs. However, the molecular mechanisms underlying antipsychotic-induced changes of the activity of NSCs, particularly protein expression, are still unknown. We studied the growth and protein expression in haloperidol (HD) and risperidone (RS) treated rat NSCs. The treatment with RS (3 μM) or HD (3 μM) had no effect on morphology of NSCs after 24 h, but significantly promotes or inhibits the differentiation of NSCs after a 96 h of treatment. 2-DE based proteomics was performed at 24 h, a stage before phenotypic expression of NSCs. Gel image analysis revealed that 30 protein spots in HD- and 60 spots in RS-treated groups were differentially regulated in their expression compared to control group ($p < 0.05$; ANOVA). When these spots were compared between the two drug-treated groups, 23 spots overlapped leaving 7 HD-specific and 37 RS-specific spots. Of these 67 spots, 32 different proteins were identified. The majority of the differentially regulated proteins were classified into several functional groups, such as cytoskeletal, calcium regulating protein, metabolism, signal transduction and proteins related to oxidative stress. Our data shows that atypical RS expressed more proteins than typical HD, and these results might explain the molecular mechanisms underlying the different effects of both drugs on NSCs activities as described above. Identified proteins in this experiment may be useful in future studies of NSCs differentiation and/or understanding in molecular mechanisms of different neural diseases including schizophenia.

1. Introduction

Neural stem cells (NSCs) are self-renewable and can generate diverse types of neural tissue cells, such as neurons, astrocytes and oligodendrocytes, in the central nervous system (CNS) (McKay, 2001). NSCs play a pivotal role in the development and maturation of the CNS. In most brain regions, the generation of neurons is generally confined to a discrete developmental period.

In adult CNS, thousands of new NSCs are formed in the hippocampal dentate gyrus (Cameron and McKay, 2001) and in the subventricular zone of the forebrain (Petreanu and Alvarez-Buylla, 2002). Their self-renewal ability may contribute to tissue repair in some neurological diseases. Recent findings suggest that neurogenesis may be of importance for memory functioning as well as controlling mood (Shors, 2004, 2008). Furthermore, various chemicals, such as alcohol and toxins, and mental disorders, such as schizophrenia, have been shown to influence neurogenesis through changes in the activities of NSCs, leading to alterations of brain plasticity and cognitive functions (Madsen et al., 2000; Malberg et al., 2000; Manev et al., 2001; Czeh et al., 2001; Duman, 2004; Crews et al., 2004; Lepore and Maragakis, 2007; Donohoe et al., 2008).

Use of antipsychotic agents improves some aspects of clinical symptoms in schizophrenia. Typical antipsychotic agents, such as haloperidol (HD) are effective in reducing positive symptoms but not particularly useful against negative symptoms or cognitive deficits in schizophrenia. In contrast, atypical antipsychotic agents, such as risperidone (RS) have gained popularity over typical antipsychotics due to their efficacy in controlling both the positive and negative symptoms (Lin et al., 2006; Citrome et al., 2009; Konarzewska et al., 2009). Chemically, HD is a butyrophenone type of compound which is an antagonist at the dopamine receptors (DA_2), while the 2nd generation antipsychotic RS, has small to moderate affinity for serotonin receptors (5-HT_{2C}, 5-HT_{1A}, 5-HT_{1C}

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and 5-HT_{1D}) and high affinity for 5-HT_{2A}, DA_2, α_1 and α_2 adrenergic, histamine-H_1 receptors. Line of evidences suggested that better performance in administration of atypical agents against neuronal disorders is due the wide range of their mode of actions and activation of neurogenesis (Harvey et al., 2003; Kasper and Resinger, 2003; Purdon et al., 2000). Wakade et al. (2002) demonstrated that atypical neuroleptics (RS and olanzapine) increased neurogenesis in the subventricular zone compared to that of vehicle-treated control or HD-treated animals *in vivo*. A recent study using cultured embryonic NSCs, showed that olanzapine and RS increased the differentiation of NSCs into neurons compared to that of vehicle-treated controls; in contrast, HD caused a significant inhibition of this differentiation (Wakade et al., 2002; Ukai et al., 2004; Kurosawa et al., 2007). However, the molecular and cellular mechanisms by which the antipsychotic agents exert their specific effects on NSCs are not well understood.

Proteins are central to our understanding of normal cellular functioning and disease processes. Proteomics, a high throughput analysis of expressed proteins has gained increasing attention in the field of biological research (Kashem et al., 2008, 2007; Matsumoto et al., 2007; Clark et al., 2006; O'Brien et al., 2006). In order to elucidate the possible mechanisms underlying the changes induced by antipsychotic agents in the differentiation of NSCs, we investigated the effects of HD and RS on protein expression profiles in cultured rat embryonic NSCs culture, using two-dimensional gel electrophoresis (2-DE)-based proteomics.

2. Materials and methods

2.1. Preparation of neural stem cells culture

All animal experimental procedures were approved by the institutional animal care committee and conducted following the Sapporo Medical University Guidelines for the Care and Use of Laboratory Animals. NSCs were prepared from 13.5-day-old (E13.5) rat embryos. NSCs were cultured in a monolayer, as we described previously (Tateno et al., 2006). The telencephalon was dissected from the embryonic brain microscopically and trimmed in ice-cold Hank's balanced salt solution (HBSS; Invitrogen, CA, USA). The cells were dissociated by mechanical trituration with a glass pipette followed by filtration, and collected by centrifugation ($300 \times g$ for 5 min at 4 °C). The viable cells were determined by trypan blue (Invitrogen) exclusion. The cells were dispersed in neurobasal medium (NBM, Invitrogen)/2% B27 supplement (Invitrogen), 0.5 mM L-glutamine and 20 ng/ml recombinant human fibroblast growth factor 2 (FGF-2, PeproTech [London, UK]), and plated at a density of 3×10^6 cells per 100 mm dish. The dishes were coated with poly-L-ornithine (Sigma [St. Louis, MO, USA])/fibronectin (Invitrogen). After 4–6 days of incubation at 37 °C in a 5% CO_2 atmosphere, cultured cells were reseeded on either 100 mm dishes, 24-well microplates or glass bottom plates and differentiated by withdrawal of FGF-2. At the time of replating, most cells expressed nestin, an intermediate filament present in NSCs, and less than 5% expressed early neuronal marker tubulin β-III (Tuj-1), astrocytic marker glial fibrillary acidic protein (GFAP) or oligodendrocytic marker O4 (data not shown).

2.2. Drug treatment and sample collection

After replating, NSCs were treated with drugs by adding them to the medium. The vehicle (DMSO), antipsychotic 3 μM (final concentration in the medium) HD (Wako Tokyo, Japan) and 3 μM (final concentration in the medium) RS (generous gift from Janssen, L.P., NJ, USA) were treated for 96 h, respectively, until morphological observation with immunochemical assays. In this study, we used 3 μM risperidone and 3 μM haloperidol. The tested concentration of each antipsychotic used here were according to their previous reports (Aravagiri et al., 1998; Kornhuber et al., 1999; Sprague et al., 2004), and these concentrations did not show any toxic effects on cultured NSCs (Kurosawa et al., 2007). For proteomics sample collection, after 24 h incubation with drugs or vehicle, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested in 200 μl solubilizing buffer (7 M urea, 2 M thiourea, 1% C7bZO and 40 mM Tris–HCl, pH 10.4; Sigma). After 3 times sonication for 5 s, the samples were stored at −80 °C until protein extraction procedures. We selected 24 h treated cells for proteomics analysis because this time point initiate to start the expression of proteins that related to phenotypic expression, i.e. tissue differentiation.

2.3. Immunofluorescence

Cultured cells were fixed with 4% formalin neutral buffer solution for 30 min, followed by washing with PBS. The cells were than treated with 0.2% Triton-X 100 in PBS for 10 min, followed by washing with PBS. After incubation with 5% normal horse serum in PBS, cells were incubated with anti-microtubule-associated-protein 2 (MAP2, mouse, 1:1000; Sigma, St. Louis, MO, USA) for 30 min. The cells were further incubated in mouse Zenon IgG labeling reagent (1:200, Invitrogen) for 45 min and followed by washing with PBS.

2.4. Protein extraction

To identify protein expression in relation to NSC differentiation, we have selected 24 h drug-treated cells. Protein extractions and gel analysis were conducted in the Department of Pathology, University of Sydney, according to the method previously described by our research groups (Kashem et al., 2008, 2007; Iwazaki et al., 2007; Clark et al., 2006; O'Brien et al., 2006). In brief, the collected samples (from Japan) were thawed and pelleted at $16{,}000 \times g$ for 20 min at 15 °C. The supernatant was reduced and alkylated in 5 mM tributyl phosphine and 10 mM acrylamide monomer at room temperature for 2 h. The reaction was quenched using dithiothreitol (DTT) to 10 mM. Acetone and citric acid (20 mg) were used to induce precipitation for 5 min and then centrifuged at $2500 \times g$ for 15 min at 15 °C. The pellet was air dried and resuspended in 1 ml of buffer consisting of 7 M urea, 2 M thiourea and 1% C7bZO. The final extract was aliquot and stored at −80 °C before isoelectric focusing.

2.5. Two-dimensional gel electrophoresis

Protein concentration was determined following the procedure described by Bradford (1976) using human brain protein as a standard. Immobilised pH gradient strips (11 cm, pH 4–7) were re-hydrated with samples [run in duplicate of each replications ($n = 7$)] containing 400 μg of protein for 6 h at room temperature. The re-hydrated strips were focused for a total of 120 kVh. IPG strips were equilibrated using equilibration buffer followed by loading onto SDS-PAGE gels (8–16%, 10 cm × 15 cm) for second dimension (50 mA/gel, 25 °C for 110 min). The gel was fixed in a solution consisting of 25% (v/v) methanol and 10% (w/v) acetic acid and stained using colloidal Coomassie Blue.

2.6. Image analysis

The resultant 42 gels (duplicate runs for each sample; seven replications of samples per group) were scanned using a transmissive, flatbed scanner (UMAX) and the images were analysed using Phoretix 2D Expression software (nonlinear). To assist comparison and reduce group-to-group variation, averaged gels were created for each drug-treated/control group and averaging parameters were set at 70%. To verify the expression differences (spot volume) between control and drug-treated groups, one-way analysis of variance (ANOVA, $p < 0.05$) was performed. The false discovery rate (FDR) was calculated according to the method previously described by Storey (2002) based on all p values of HD and RS groups. Spots of interest were recovered for identification by mass spectrometry.

2.7. Mass spectrometry (MS) and database searching

Protein spots of interest were excised and de-stained using 25 mM NH_4HCO_3/50% (v/v) acetonitrile (ACN) for 3 × 15 min at 37 °C. The gel portions were then dehydrated using 100% ACN. Each gel piece was incubated with 12.5 ng/ml trypsin (Roche, sequencing grade) buffer (25 mM NH_4HCO_3/0.1% trifluroacetic acid (TFA) for 45 min at 4 °C and incubated at 37 °C overnight. The peptide mixtures were purified from the supernatant using C-18 purification tips (Eppendorf). The sample was eluted from the purification tip onto a MALDI sample plate with 3 μl of matrix (5 mg/ml solution of α-cyano-4-hydroxycinnamic acid in 70% (v/v) ACN/0.1% (v/v) TFA) and allowed to air dry. Samples were then analysed using Qstar XL Excll Hybride MS system (AB Applied Biosystems) in positive reflector mode, with delayed extraction.

The spectra obtained from MALDI-TOF were searched against the Swissport protein databases using the MASCOT search engine (http://www.matrixscience.com/). Positive protein identification was performed based on a MOWSE score (>54, rattus database) with matched p*I* and MW values (estimated from 2D gels) and sequence coverage.

2.8. Western blot analysis

Proteins (40 μg) of neuroleptic-treated samples were separated by SDS-polyacrylamide gel electrophoresis (10%, 1 mm gels) and followed by transferring proteins to PVDF membranes according to the protocol described by proteome IQ blotting kit (Proteome Systems Ltd.). The membranes were then blocked using the protocol previously described by Kashem et al. (2000). The primary antibodies (monoclonal anti-rabbit tryptophan hydroxylase (dilution 1:1000) (Sigma–Aldrich) and mouse neurofilament light chain (68 kDa) (Zymed laboratory) were incubated according to the manufacturers' instructions. Secondary antibodies were added according to the manufacturer's instructions (anti-mouse IgG). The protein bands were visualized using ECL system (GE Healthcare).