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THE EFFECTS OF NEUROPSYCHIATRIC DRUGS ON GLYCOGEN SYNTHASE KINASE-3 SIGNALING

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Abstract—Glycogen synthase kinase-3 (GSK-3) has been implicated in the action of antipsychotics, mood stabilizers, and antidepressants. Given that only antipsychotics are able to alleviate the positive symptoms of schizophrenia, the regulation of GSK-3 by antipsychotics would be expected to differ from other neuropsychiatric drugs if GSK-3 is involved in the alleviation of psychosis. Consequently, the current study examined the effects of antipsychotics (haloperidol and clozapine), mood stabilizers (lithium and valproic acid), and antidepressants (imipramine and fluoxetine) on GSK-3, as well as Akt and Wnt in the prefrontal cortex and striatum. Western blotting and co-immunoprecipitation experiments showed that only antipsychotic treatment increased Dvl-3, GSK-3, and β-catenin levels and enhanced the association of GSK-3 at the dopamine D2 receptor (D_2DR) complex in the rat prefrontal cortex. In the striatum, haloperidol had the same effect on Wnt signaling as observed in the prefrontal cortex, whereas clozapine did not affect Dvl-3, GSK-3 or β-catenin levels. All three classes of drugs were able to activate Akt signaling as shown by the increased phosphorylated Akt and phosphorylated GSK-3 protein levels in the prefrontal cortex and/or striatum. In conclusion, regulation of the Wnt pathway is specific to antipsychotics, whereas antipsychotics, mood stabilizers, and antidepressants all affect Akt.

Key words: antipsychotic, mood stabilizer, antidepressant, GSK-3, signal transduction, Akt.

Glycogen synthase kinase-3 (GSK-3) is a constitutively active protein kinase that has been implicated in schizophrenia and antipsychotic drug action. Decreases in total and phosphorylated GSK-3β protein levels and a reduction in GSK-3β mRNA levels have been reported in the brain of schizophrenia patients (Kozlovsky et al., 2000, 2004). GSK-3 protein levels and/or phosphorylation state were also found to be decreased in several animal models of schizophrenia including the amphetamine model, ventral hippocampal lesion model, and the dopamine transporter knockout mice (Nadri et al., 2003; Beaulieu et al., 2004; Alimohamad et al., 2005b). Furthermore, hyperlocomotor activity in dopamine transporter knockout mice was reduced to wild-type levels following treatment with inhibitors of GSK-3 (Beaulieu et al., 2004). Antipsychotics, such as haloperidol, clozapine, and risperidone, in contrast, have been shown to increase total and phosphorylated GSK-3α/β (Ser21/9) protein levels in the prefrontal cortex (PFC) and/or striatum (STR) of rats following repeated administration (Alimohamad et al., 2005a; Sutton et al., 2007). Consequently, it has been suggested that GSK-3 may play a role in the manifestation and amelioration of some of the symptoms of schizophrenia (Freyberg et al., 2010). However, mood stabilizers and antidepressants have also been shown to regulate the phosphorylation state of GSK-3 *in vivo* (Klein and Melton, 1996; Li et al., 2004; Kozlovsky et al., 2006; Roh et al., 2005). Antipsychotics, mood stabilizers, and antidepressants all have different clinical profiles, and only antipsychotics alleviate the positive symptoms of schizophrenia. Consequently, it has been suggested that there may be something unique in the way GSK-3 is affected by antipsychotics compared with drugs used to treat other neuropsychiatric disorders (Ming and Song, 2009).

GSK-3 function is regulated by several mechanisms, including phosphorylation, association of GSK-3 with specific protein complexes (i.e. β-catenin phosphorylation complex), and sub-cellular localization (Frame and Cohen, 2001; Jope and Johnson, 2004). Several signaling cascades are responsible for controlling the characteristics of GSK-3 but the Akt and Wnt pathways are of particular interest given that they have both been linked to schizophrenia and antipsychotic drug action (Emamian et al., 2004; Alimohamad et al., 2005a,b). Akt and the Wnt pathway negatively regulate GSK-3, although via different mechanisms. Akt reduces GSK-3 kinase activity through phosphorylation of both isoforms of GSK-3 (pGSK-3α/β) (Cross et al., 1995). The Wnt pathway regulates GSK-3 through its association with the β-catenin phosphorylation complex. GSK-3 associates with several proteins, including Axin and adenomatous polyposis coli forming a β-catenin phosphorylation complex that facilitates the phosphorylation and eventual degradation of the transcription factor, β-catenin. Activation of the Wnt pathway leads to the phosphorylation and/or increase in protein levels of the signal transducer protein Dishevelled (Dvl). Dvl negatively regulates GSK-3 by interfering with the β-catenin phosphorylation complex preventing the phosphorylation of β-catenin. The resultant accumulation of β-catenin in the cytoplasm followed by its translocation to the nucleus

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Abbreviations: co-IPs, co-immunoprecipitations; D_2DR, dopamine D2 receptor; D_3DR, dopamine D3 receptor; Dvl, dishevelled; EPS, extrapyramidal side effects; GSK-3, glycogen synthase kinase-3; Li, lithium; pAkt, phosphorylated Akt; PFC, prefrontal cortex; pGSK-3, phosphorylated GSK-3; STR, striatum.

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leads to modifications in mediated gene transcription (Novak and Dedhar, 1999).

Previous studies have shown that repeated treatment with the antipsychotics haloperidol and/or clozapine target Akt and Wnt pathway proteins in the PFC/frontal cortex (Emamian et al., 2004; Alimohamad et al., 2005a; Sutton et al., 2007). However, given that other neuropsychiatric drugs may regulate GSK-3 signaling, the current study investigated whether the mood stabilizers, lithium and valproic acid and/or the antidepressants, fluoxetine and imipramine mimic the effects of antipsychotics on Akt and Wnt signaling in the PFC and STR or if there are key differences in the response of these drugs.

EXPERIMENTAL PROCEDURES

Drug paradigm

Adult male Sprague–Dawley rats 14+ weeks of age (Charles River, Quebec, Canada) were housed in pairs in a 12-h light/dark cycle with free access to food and water. Rats were injected once daily with haloperidol (0.5 mg/kg, i.m.), clozapine (25 mg/kg, s.c.), or appropriate vehicle for 14 days (n=8 rats/treatment). The doses of haloperidol and clozapine were based on a previous study showing changes in the proteins of interest in the PFC and/or STR (Alimohamad et al., 2005a). Rats were also given a bolus injection of haloperidol decanoate (1 mg/kg/d), a continuous release form of haloperidol or sesame seed oil (vehicle) and sacrificed after 14 days to address a question that arose with Akt Ser473 during the course of the study (n=4 rats/treatment). To examine drug specificity, rats were injected with fluoxetine (10 mg/kg, s.c., Sigma-Aldrich), imipramine (10 mg/kg, s.c., Sigma-Aldrich), or appropriate vehicle for 14 days (n=8 rats/treatment). Fluoxetine and imipramine were chosen since they represent two different classes of antidepressants, and because they were found to alter phosphorylated GSK-3 levels following acute treatment (Li et al., 2004). In addition, rats were treated with the mood stabilizers, lithium or valproic acid via their chow or drinking water (n=8 rats/treatment). Rats were fed chow containing 2.0 g/kg LiCl or chow lacking LiCl for 30 days *ad libitum*. Valproic acid (12 g/L, Sigma Aldrich) was mixed in the rats' drinking water and supplemented with saccharin (300 mg/L, Sigma Aldrich) for palatability, whereas the control rats received drinking water supplemented with saccharin alone for 30 days. The doses and delivery method for lithium and valproic acid were selected based on previous studies showing they achieve clinically relevant serum concentrations in Sprague–Dawley rats (Bersudsky et al., 1997; Shaldubina et al., 2002). The PFC and STR of rats were isolated 2 h following final treatment of haloperidol, clozapine, fluoxetine, and imipramine, whereas rats treated with haloperidol decanoate, lithium, and valproic acid were sacrificed at the same time of day as the animals that received drugs via systemic injections. Every effort was made to reduce the number of rats used in the experiments and to minimize pain and suffering. All experiments were reviewed and approved by the University of Western Ontario Animal research ethics committee in compliance with Canadian Council on Animal Care.

Western blotting

The PFC and STR were selected for the current study because both regions have been implicated in neuropsychiatric disorders and have been investigated following neuropsychiatric drug treatment. Following treatment, the rats were decapitated, and the STR (CPu and caudal nucleus accumbens) and PFC (medial prefrontal cortex plus anterior cingulate cortex) were immediately dissected. Tissue from individual rats was homogenized using a dounce homogenizer on ice in ice-cold lysis buffer (137 mM NaCl, 20 mM Tris (pH 8.0), 1% NP-40, 10% glycerol, and 0.1% sodium dodecyl sulfate) to which a protease inhibitor tablet (Roche) and phosphatase inhibitors (Sigma) were added. The homogenized tissue was sonicated (15 s), mixed with 5× loading buffer (125 mM Tris (pH 6.8), 10% glycerol, 2% SDS, 2% 2-mercaptoethanol, 0.01% Bromophenol Blue), and boiled for 5 min. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce) and a μ-Quant plate reader.

For western blots, 20–40 μg of protein was resolved on a polyacrylamide gel using a mini-protean III system (Bio-Rad) and then transferred to nitrocellulose membrane. To detect the proteins of interest, the membranes were blocked in 5% non-fat dry milk dissolved in Tris buffered saline (TBS)+0.05% Tween 20 (TBST) for 1 h. The blocking serum was replaced with fresh milk solution containing antibodies to the protein of interest for 1 h at room temperature or overnight at 4 °C. Following three 10-min washes in TBST, appropriate horseradish peroxide-conjugated in milk solution was added to the membrane for 1 h and then washed with TBST (three washes) and then with TBS. To visualize the proteins of interest, chemiluminsence (Pierce) and X-ray film (Kodak X-Omat LS) were used. Membranes were stripped (Restore plus western blotting stripping buffer, Thermo Scientific) according to the manufactures' instructions and re-probed for α-tubulin. For quantification densitometry values were obtained from X-ray film using a gray scale calibrated scanner (Epson) and Kodak 1-D Molecular Imaging software. Densitometry values were corrected for α-tubulin and expressed as a percentage of control. Data were analyzed using Student's t-test comparing each treatment with its specific vehicle control. The source and dilution of the antibodies used in the study were as follows; Dvl-1 (Santa Cruz Biotechnology, 1:100), Dvl-2 (Chemicon, 1:1000), Dvl-3 (Santa Cruz Biotechnology, 1:100), GSK-3 (Santa Cruz Biotechnology, 1:300), phospho-GSK-3 (Cell Signaling Technology, 1:1000), β-catenin (Sigma-Aldrich, 1:20000), Akt (Cell Signaling Technology, 1:1250), phospho-Akt Ser473 (Cell Signaling Technology, 1:1000), phospho-Akt Thr308 (Upstate, 1:1000), and α-tubulin (Sigma-Aldrich, 1:120000).

Co-immunoprecipitations

Protein was isolated from the PFC of haloperidol, clozapine, lithium and valproic acid treated rats along with their appropriate vehicle controls using a non-denaturing lysis buffer containing protease (Complete Mini, Roche Diagnostic) and phosphatase inhibitors (cocktail Set 2, Sigma-Adrich; cocktail Set IV, Calbiochem). Co-immunoprecipitations (co-IPs) were performed with the ExactaCruz system (Santa Cruz Biotechnology) according to the manufacturer's instructions using 500 μg of protein and antibodies specific for the D_2DR (3 μg/IP, Santa Cruz Biotechnology, rabbit and goat polyclonal) as outlined previously (Sutton et al., 2007). Co-IP samples and lysate used in the co-IPs were run on western blots and probed for Akt, GSK-3, Dvl-3, or the D_2DR. The blots were quantified using the densitometry values as described previously.

RESULTS

Differential effects of haloperidol and clozapine in the PFC and STR

The current study used the typical antipsychotic, haloperidol and the atypical antipsychotic, clozapine to examine changes in Akt and Wnt pathway proteins in the PFC and the STR. In the PFC, repeated haloperidol or clozapine injections increased the protein levels of Dvl-3, GSK-3α/β, pGSK-3α/β, and β-catenin but not Dvl-1 or Dvl-2 (Fig. 1a, b) consistent with previous results (Sutton et al., 2007). Increases in pAkt Thr308 but not pAkt Ser473 or total Akt were also observed (Fig. 1c, d). A previous study that examined pAkt in the cortex of mice following repeated