Antipsychotics Alter the Protein Expression Levels of β-Catenin and GSK-3 in the Rat Medial Prefrontal Cortex and Striatum

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Background: *It has been demonstrated that schizophrenics have altered levels and/or phosphorylation states of several Wnt related proteins in the brain, including β-catenin and GSK-3, and may represent susceptibility loci for schizophrenia. The current study was conducted to assess the effects of antipsychotics on β-catenin and glycogen synthase kinase-3.*

Methods: *Western blotting and immunocytochemistry were employed to investigate the effects of antipsychotics on β-catenin and glycogen synthase kinase-3 following acute, subchronic and chronic drug administration. Specificity of the response was tested using additional drugs such as fluoxetine, amphetamine and valproic acid.*

Results: *Significant increases in the levels of β-catenin and glycogen synthase kinase-3 total protein were identified following administration of clozapine, haloperidol or risperidone. The phosphorylation state of GSK-3 was also increased but phosphorylated β-catenin levels were unaffected. Other drug compounds, with the exception of raclopride, had no effect on either GSK-3 or β-catenin protein levels or distribution.*

Conclusions: *Targeting of β-catenin and GSK-3 is a common feature of antipsychotics regardless of class and appears to be mediated by D_2 dopamine receptors. Therefore changes in β-catenin and GSK-3 may represent one of the mechanisms through which antipsychotics are able to exert behavioral changes.*

Key Words: Antipsychotics, Wnt, GSK-3, β-catenin, prefrontal cortex, striatum

Antipsychotics remain the only effective treatment for the symptoms of schizophrenia. Though antipsychotics are often classified as either typical or atypical and possess different receptor binding profiles, D_2 dopamine (DA) receptor blockade is an essential feature of both classes of drugs (Kapur and Mamo 2003). However, atypical antipsychotics are also strong serotonin receptor (5-HT) antagonists and the combined DA/5-HT action may be responsible for improved efficacy and reduced extrapyramidal symptoms (EPS) (Ichikawa and Meltzer 1999; Meltzer 1999). Current theories suggest that the blockade of receptors in the mesolimbic DA system is responsible for the ability of antipsychotics to alleviate psychosis whereas high levels of D_2 DA receptor saturation in the striatonigral system is thought to cause disabling side effects commonly referred to as EPS (Serretti et al 2004). Strong D_2 DA antagonists, such as typical antipsychotics, frequently induce EPS whereas atypical antipsychotics (weaker D_2 antagonists) are less likely to do so.

Despite a good understanding of the receptor binding profiles of antipsychotics, the cellular consequences of repeatedly blocking D_2 DA receptors remains unclear and traditional signaling pathways linked to DA receptors have not provided an easy or direct answer. Therefore other signaling pathways should be examined to ascertain their potential involvement in the antipsychotic response. Fortunately several insights have been provided recently by studies using post-mortem brain tissue obtained from schizophrenics and matched controls. One group of proteins that was identified as altered in schizophrenic brains belongs to the Wnt signal transduction pathway. Wnt is important in central nervous system (CNS) development (Cadigan and Nusse 1997; Miller and Moon 1996) and constituents of the pathway remain highly expressed in the adult brain. Wnt has also been associated with a number of CNS disorders including Alzheimer's disease and most recently schizophrenia (De Ferrari and Inestrosa 2000; Kozlovsky et al 2002).

The canonical Wnt signaling cascade is activated when a Wnt protein interacts with a Frizzled (Fz) receptor (Bhanot et al 1996). The interaction of Wnt with Fz leads to the phosphorylation of dishevelled (Dvl) (Noordermeer et al 1994). Dvl antagonizes the action of glycogen synthase kinase-3 (GSK-3) resulting in the cytoplasmic accumulation and translocation of β-catenin into the nucleus (van Leeuwen et al 1994). A phosphorylation regulatory site has been identified in GSK-3 (Ser 21/9 for GSK-3α and GSK-3β respectively) that inhibits the kinase activity of an otherwise constitutively active protein and may be involved in regulating cytoplasmic β-catenin levels (Doble and Woodgett 2003). Once in the nucleus, β-catenin binds to TCF/LEF (T-cell factor and lymphoid enhancer factor), forming a transcription factor complex that activates gene expression (Behrens et al 1996; Huber et al 1996).

In the absence of Wnt, cytoplasmic β-catenin levels are maintained at low levels through regulation by GSK-3, *adenomatous polyposis coli* (APC) and Axin (Hamada et al 1999; Ikeda et al 1998). The Axin-APC-GSK-3 complex promotes the phosphorylation of β-catenin by GSK-3 targeting it for destruction by the ubiquitin-proteasome pathway (Aberle et al 1997; Polakis 1997; Siegfried et al 1992). In the absence of β-catenin, TCF/LEF still binds to the TCF promoter element but fails to activate transcription (Brannon et al 1997; Riese et al 1997).

The purpose of the current study was to determine if antipsychotics cause alterations in the protein levels or distribution of β-catenin and GSK-3 in the medial prefrontal cortex (PFC) or striatum (Str). These two proteins were selected for characterization since β-catenin represents the key protein that transduces activation of the Wnt pathway into the nucleus while GSK-3 is

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the central regulator of cytoplasmic β-catenin levels (Novak and Dedhar 1999).

Methods and Materials

Animals

For all aspects of the study, adult female Sprague-Dawley rats 14+ weeks of age (Charles River, Quebec, Canada) were used. Female rats were selected over male rats because they maintained a more stable body weight and body mass index over the course of the study. Rats were housed in pairs with free access to food and water in a room scheduled on a 12-hour light/dark cycle. All efforts were made to minimize the number of animals used in the current study and to eliminate pain and suffering. Use of animals for the current study was reviewed and approved by the University of Western Ontario Animal Research Ethics Committee in accordance with the guidelines developed by the Canadian Council on Animal Care.

Drugs and Drug Paradigms

For the western blotting studies all rats received intramuscular or subcutaneous, injections of .25 mg/kg or 1.0 mg/kg haloperidol, .9 mg/kg or 2 mg/kg risperidone, 25 mg/kg clozapine or appropriate vehicles (n = 5 rats/treatment group) for 7 days (sub-chronic treatment) and sacrificed 2 hours post-injection. Two doses of haloperidol were selected for the study since a controversy has recently arisen concerning appropriate dosing in rats. One mg/kg is the dose that has often been used for antipsychotic studies but has been shown to induce catalepsy, a rat measure of EPS liability. A lower dose results in saturation of central DA receptors in the rat brain that more closely approximates what is observed in treated patients without significant signs of EPS and may not induce catalepsy in rats (Kapur et al 2000a; Wadenberg et al 2001). However, the receptor occupancy and behavioral studies were only conducted following acute drug administration and it is not clear if the results can be extrapolated to a subchronic or chronic injection paradigm. Therefore both doses of haloperidol were used in the study. Two doses of risperidone were also chosen, for similar reasons. The higher dose of risperidone (2 mg/kg) would be expected to break the threshold of atypicality and induce catalepsy whereas the lower dose (.9 mg/kg) is less likely to do so (Wadenberg et al 2001). Consistent with the behavioral observations, higher doses of risperidone induce immediate early genes in the lateral caudate-putamen (CPu) whereas the lower doses of risperidone do not (Robertson et al 1994). Finally, the dose of clozapine was selected based on a receptor occupancy study showing comparable D_2 DA receptor saturation between 25 mg/kg of clozapine and the lower doses of risperidone and haloperidol (Schotte et al 1993). Only a single dose of clozapine was selected since it is generally accepted that clozapine does not induce EPS in rats or humans and does not induce immediate early genes in the lateral CPu of rats (Robertson et al 1994).

Antipsychotics only alleviate psychosis clinically in humans following repeated treatment and stabilization can take weeks or months. To determine if changes in β-catenin and GSK-3 followed a similar pattern, rats (n = 5/treatment group) were also injected with haloperidol (1 mg/kg) or risperidone (.9 mg/kg) acutely (single injection) or chronically for 28 days (28 daily injections) and sacrificed 2 hours post-injection. Only the PFC was examined following acute and chronic antipsychotic administration (and for most of the remaining portions of the study) since the results of the subchronic experiments suggested that the medial prefrontal cortex is commonly affected by all three drugs and both doses of haloperidol and risperidone whereas the changes in the Str are dependent on dose and/or drug class (i.e. typical versus atypical). In addition to the single daily bolus injection experiments, rats were also injected with a single dose of haloperidol decanoate (1 mg/kg/day), a slow continuous release formulation of haloperidol lasting 14-21 days, or sesame seed oil vehicle and sacrificed 14 days following injection. The haloperidol decanoate was used to ensure the results were not the consequence of delivery method or other factors such as sedation.

To examine receptor and drug specificity properties, rats (n = 5 rats/treatment group) were injected with raclopride (DA D_2/D_3 receptor antagonist, 3 mg/kg, 7 days), fluoxetine (selective serotonin reuptake inhibitor, 10 mg/kg, 9 days), ritanserin (serotonin 5-HT_{2a}, 5-HT_{1c} receptor antagonist, 1 mg/kg, 7 days), amphetamine (psychotomimetic, 2.5 and 5 mg/kg, 7 days), apomorphine (DA D_1/D_2 receptor agonist, .5 and 2.5 mg/kg, 7 days), quinpirole (DA D_2 receptor agonist, 2.5 mg/kg, 7 days), SKF-82958 (DA D_1/D_5 receptor agonist, 1 mg/kg, 7 days), valproic acid (mood stabilizer, 300 mg/kg, 10 days) or appropriate vehicles and sacrificed 2 or 4 hours following the final injection. The dose selected for each of the drugs was based on published work including behavioral studies involving pre-pulse inhibition (Geyer et al 2001). The length of treatment was chosen to match the subchronic antipsychotic injection paradigm (7 days) with the exception of valproic acid and fluoxetine (9 days). The treatment interval was extended for these two agents since several published studies used a slightly longer injection period. For example, a recent study examining the effects of lithium on GSK-3 used a nine day treatment interval for valproic acid (Gould et al 2004).

For the immunocytochemistry portion of the study, rats (n = 3/treatment) received intramuscular or subcutaneous injections of haloperidol (1 mg/kg), risperidone (.9 mg/kg or 2 mg/kg), amphetamine (5 mg/kg), apomorphine (2.5 mg/kg), quinpirole (2.5 mg/kg), valproic acid (300 mg/kg), ritanserin (1 mg/kg), fluoxetine (10 mg/kg), SKF-82958 (1 mg/kg) or vehicle daily for 7-10 days (as indicated above) and were killed 2 hours post-injection.

All drugs used throughout the study were obtained from Sigma-Aldrich (Mississauga, Ontario, Canada).

Western Blot

Following treatment the rats were decapitated, the brains rapidly removed and dissected to obtain the Str (CPu and caudal nucleus accumbens) and the PFC (medial prefrontal cortex plus anterior cingulate cortex). Tissue from individual rats was immediately homogenized on ice in ice-cold lysis buffer (137 mM NaCl, 20 mM Tris (pH 8.0), 1% NP-40, 10% glycerol and .1% sodium dodecyl sulfate) to which a protease inhibitor tablet (Roche, Laval, Quebec, Canada) was added using a dounce homogenizer. For the phosphorylation state portion of the study, Ser/Thr phosphatase inhibitors (Sigma) were included in the lysis buffer. The homogenized tissue was sonicated for 15 seconds, mixed with 5x loading buffer (125 mM Tris pH 6.8, 10% glycerol, 2% SDS, 2% 2-mercaptoethanol, .01% xylene cyanol and .01% bromophenol blue) and boiled for 5-7 minutes. The extracts were stored at -80°C until needed. Protein concentrations were determined using a bicinchoninic acid Protein Assay Kit (Pierce Chemical Co., Rockford, Illinois) and a μ-Quant plate reader.

For western blot analysis, 15-25 μg of protein from drug