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Caspase-3 Activation in Rat Frontal Cortex Following Treatment with Typical and Atypical Antipsychotics

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In schizophrenia, studies indicate that apoptotic susceptibility in cortex may be increased. A role for apoptosis in schizophrenia could potentially contribute to post-mortem evidence of reduced cortical neuropil and neuroimaging studies showing progressive cortical gray matter loss. Interestingly, antipsychotic treatment has been associated with higher cortical levels of anti-apoptotic Bcl-2 protein in rat cortex and preliminary data has suggested a similar association in schizophrenia and bipolar disorder. To better understand the effects of antipsychotics on apoptotic regulation, rats were administered haloperidol, clozapine, quetiapine, or saline daily for 4 weeks. Multiple apoptotic markers, including Bcl-2, pro-apoptotic Bax, anti-apoptotic XIAP, and the downstream protease caspase-3 were measured in frontal cortex using Western blot. Caspase-3 activity, activated caspase-3-positive cell number, and DNA/histone fragmentation levels were also determined. Western blot showed that immunoreactivity of Bax and Bcl-2 bands were unchanged with treatment. However, mean density of the 19 kD activated caspase-3 band was 55% higher with haloperidol ($p<0.001$), 40% higher with clozapine ($p<0.05$), and 48% higher with quetiapine ($p<0.01$) compared to saline control. Specific activity of caspase-3 was also increased across all treatments ($p<0.0001$), while DNA fragmentation rates remained unchanged. These data suggest that sub-chronic antipsychotic treatment is associated with non-lethal caspase-3 activity. The findings do not support a prominent Bcl-2-mediated neuroprotective role for antipsychotics. Although the association between antipsychotic treatment and increased pro-apoptotic caspase-3 is intriguing, further study is needed to understand its potential effects.
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INTRODUCTION

Although the pathophysiology of schizophrenia remains obscure, both neuroimaging and post-mortem studies have identified small reductions in cortical gray matter (Selemon and Goldman-Rakic, 1999; Shenton *et al*, 2001). Furthermore, longitudinal imaging studies of prodromal (Pantelis *et al*, 2003), childhood-onset (Sporn *et al*, 2003), and first-episode psychosis (Cahn *et al*, 2002; Kasai *et al*, 2003) suggest that the reduction in cortical gray matter may be progressive, especially early in the course of illness. Although several post-mortem studies have found no evidence of cortical neuronal loss (Pakkenberg, 1993; Selemon *et al*, 1995; Akbarian *et al*, 1995), other studies indicate layer-specific reductions of interneuron (Benes *et al*, 1991, 2001) and glial cell density (Cotter *et al*, 2002; Stark *et al*, 2004) in several cortical areas. Taken together, these data have led to the hypothesis that apoptosis could contribute to the loss of cortical volume in schizophrenia (Margolis *et al*, 1994; Jarskog *et al*, 2005). Supporting evidence include altered levels of apoptotic regulatory proteins in the Bcl-2 family in post-mortem temporal cortex (Jarskog *et al*, 2004) and altered DNA fragmentation patterns in anterior cingulate cortex (Benes *et al*, 2003). Although the impact of antipsychotic medications on these apoptotic changes has been uncertain, one post-mortem study found a preliminary association between prior exposure to antipsychotics and higher Bcl-2 levels in schizophrenia and bipolar disorder (Jarskog *et al*, 2000). Also, investigators found higher Bcl-2 protein and mRNA levels in rat cortex following 1 month of olanzapine and clozapine treatment (Bai *et al*, 2004).

Apoptosis involves interactions among several protein families that regulate activation of proteolytic caspases.

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Although multiple pathways can induce apoptosis, the mitochondrial pathway has most frequently been implicated in central nervous system (CNS) apoptosis (Yuan and Yankner, 2000). In this pathway, the prototypical and best-studied interaction occurs between pro-apoptotic Bax and anti-apoptotic Bcl-2 in the mitochondrial membrane, and their relative ratio is an important determinant of whether cytochrome *c* is released in response to pro-apoptotic stimuli (Oltvai *et al*, 1993). If the Bax/Bcl-2 ratio is raised sufficiently, cytochrome *c* is released by mitochondria and leads, through a series of steps, to activation of caspase-3, the principal effector caspase in the CNS (Yuan and Yankner, 2000). Before activation, caspase-3 must be released from inhibition by members of the inhibitor-of-apoptosis (IAP) protein family (Lotocki and Keane, 2002). Activation of caspase-3 is a well-recognized marker of apoptosis (Krajewska *et al*, 1997).

In order to better understand the potential relationship between antipsychotic treatment and apoptotic regulation, rats were treated with typical and atypical antipsychotics daily for 4 weeks and the effect on upstream and downstream apoptotic proteins by Western blot (Bax, Bcl-2, caspase –3, and X-linked IAP (XIAP)), caspase-3 activity, caspase-3-immunopositive cell number, and DNA fragmentation in frontal cortex was measured. It was hypothesized that antipsychotic treatment would increase Bcl-2 immunoreactivity, reduce Bax/Bcl-2 ratio, and that clozapine and quetiapine would produce differentially greater effects than haloperidol on these measures. Furthermore, given the anti-apoptotic effects of Bcl-2, it was expected that caspase-3 levels, activity, and cell number would not be altered from baseline.

MATERIALS AND METHODS

Animals and Tissue Preparation

This study was approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. Singly housed, male Sprague–Dawley rats (150–200 g, Charles River) received daily i.p. injections of haloperidol 1 mg/kg/day ($n=12$), clozapine 10 mg/kg/day ($n=13$), quetiapine 10 mg/kg/day ($n=12$), or saline 0.9% ($n=12$) for 4 weeks. These doses are consistent with previous studies using *in vivo* rat models of neurochemical measures of cellular viability (Xu *et al*, 2002; Jarskog *et al*, 2004; Bai *et al*, 2004; Parikh *et al*, 2004; Fumagalli *et al*, 2004). One hour after the final dose, rats were killed and brains were removed and hemisected. Anterior right medial frontal cortex was dissected out and frozen on dry ice. Left hemicortex was frozen whole and 15 μm coronal sections were cut using a cryostat (Bright Instruments, Huntingdon, UK) through the frontal cortex. All tissue was kept frozen at −80°C until use.

Homogenization

Right medial cortex was placed in 10 volumes (w/v) of 50 mM Tris-HCl buffer (pH 7.4) with 0.6 M NaCl, 0.2% Triton X-100, 0.5% BSA, 1 mM benzamidine, 0.1 mM benzethonium chloride, and 0.1 mM PMSF. Samples were homogenized on ice for 30 s (PowerGen 125, Fisher Scientific, Pittsburgh, PA) and sonicated for 10 s at 10 mV (Sonic Dismembrator 60, Fisher Scientific). The homogenates were centrifuged for 15 min at 15 000 *g* and at 4°C. Supernatants were assayed for total protein by the bicinchoninic acid method (Micro BCA Protein Assay Kit, Pierce Chemical, Rockford, IL). Chemicals were obtained from Sigma (St Louis).

Semi-Quantitative Western Blots

Samples were resolved on 8 (XIAP), 12 (Bcl-2, procaspase-3), 14 (Bax), and 16% (activated caspase-3) 10-well Tris-glycine polyacrylamide mini-gels using established methods (Jarskog *et al*, 2000). Briefly, equal amounts of total protein (10 μg for Bax, 30 μg for Bcl-2, 20 μg for procaspase-3, 100 μg for activated caspase-3, 25 μg for XIAP) were boiled for 5 min in Tris-glycine SDS sample buffer and applied to gels in triplicate. Two samples from each treatment arm, a low-range molecular weight ladder and a pooled sample were applied to each gel. Thus, for each protein, 18 gels were used to run each sample in triplicate. Band positions were verified with controls as follows: HeLa cell lysate for Bax and XIAP, Jurkat cell lysate for Bcl-2 and procaspase-3, and a recombinant human caspase-3 fragment for activated caspase-3 that migrated to 17 kDa (Pharmingen). Separated proteins were electrophoretically transferred to PVDF membranes (Immobilon-P, Millipore, Billerica, MA) at 25 V for 90 min and complete transfer was ascertained with Ponceau S stain. Membranes were blocked with 5% (w/v) nonfat dry milk (Carnation brand) in 0.1% Tween in Tris-buffered saline (TBST) for 60 min at 25°C and then for 90 min at 25°C with primary antibodies as follows: monoclonal anti-human Bcl-2 (1 : 350, Pharmingen), polyclonal anti-rabbit Bax (N-20, 1 : 1500, Biotechnology, Santa Cruz, CA), polyclonal anti-rabbit procaspase-3 (H-277, 1 : 200, Santa Cruz), and monoclonal anti-human cleaved caspase-3 (IMG-144, 1 : 200, Imgenex); incubation with polyclonal anti-rabbit XIAP antibody (1 : 1000, Santa Cruz) was performed overnight at 4°C. Membranes were then washed and incubated for 90 min at 25°C with appropriate (anti-mouse or anti-rabbit) horseradish peroxidase-labeled secondary antibodies (1 : 3000, Amersham Pharmacia, Piscataway, NJ), diluted in 5% milk TBST. Membranes were developed using chemiluminescence (ECL, Amersham) and protein bands were detected on radiographic film (Hyperfilm ECL, Amersham). Optical densitometry was performed using the Bioquant system and image analysis using Adobe Photoshop 7.0. Band densities were normalized to the pooled sample that was applied to each gel to permit inter-gel comparisons. Finally, a standard curve of increasing protein amounts of pooled sample was immunoblotted for each protein to ascertain that all band densities fell within the linear portion of the densitometric curve. This linear relationship between increasing total protein levels and increasing immunodensity of the respective bands allows for comparisons of relative differences in protein quantity across samples.

Caspase-3 Immunoprecipitation (IP)

Homogenates within each treatment arm were equally pooled to produce one pooled sample per treatment arm.

Samples were precleared for 30 min at 4°C by addition of protein A-agarose beads (Santa Cruz). Samples were centrifuged and supernatants were transferred to new tubes, where monoclonal rabbit cleaved caspase-3 antibody (1 : 100, Asp 175, Cell Signaling, Beverly, MA) was mixed with 100 μg of precleared protein and incubated for 24 h at 4°C with agitation. Protein A-agarose was added and incubated with agitation for 2 h at 4°C. Samples were centrifuged for 5 min at 2500 r.p.m. and at 4°C. The pellet was washed four times by centrifugation and resuspension in cold IP buffer (50 mM Tris pH7.5, 150 mM NaCl, 0.1%Triton X-100), and washed with 50 mM Tris buffer pH 7.5. The bound cleaved caspase-3 and its complexes were eluted from immunoaffinity beads by resuspension in 60 μl Tris-glycine SDS sample buffer. Normal rabbit IgG antibody (1 : 400, Santa Cruz) diluted in sterile PBS was used for control IP. Samples were boiled for 5 min before immunoblotting for XIAP using methods as described above except that the primary XIAP antibody dilution was 1 : 2000.

Caspase-3 Activity Assay

Caspase-3-like activity was measured spectrophotometrically using the caspase-3 colorimetric tetrapeptide substrate N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (DEVD-pNa) in cortical homogenates using a 96-well microplate kit (Caspase-3 Cellular Activity Assay Kit, Calbiochem, San Diego, CA) according to the manufacturer's protocol. Briefly, 45 μl of sample and 45 μl assay buffer (100 mM NaCl, 50 mM HEPES, 10 mM DTT, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, pH7.4) were added to wells and the microplate was equilibrated at 37°C. The reaction was initiated by adding 10 μl of DEVD-pNA substrate (200 μM final concentration). Absorbance was recorded at 10 min intervals for 2 h at 405 nm using a microplate reader (Vmax, Molecular Devices, Sunnyvale, CA). All samples were run in duplicate and purified activated caspase-3 was run on each microplate as a positive control. In this assay, the initial substrate concentration is saturating, leading to a linear absorbance *vs* time relationship until the substrate begins to deplete, after which the absorbance gradually reaches a plateau. Specific activity of DEVD-pNa cleavage (pmol pNa/min/μg total protein) for each sample was calculated by measuring the slope of the linear portion of the absorbance *vs* time graph, as follows: specific activity = activity (slope(ΔA_{sample}/min) × (50 μM/$A_{405(100\,\mu l\ of\ 50\,\mu M\ pNa]}$) × 100 μl (assay volume))/μg total protein.

Activated Caspase-3 Immunohistochemistry

Sections were fixed in cold 100% acetone for 10 min, washed in PBS for 5 min, then treated with 0.3% hydrogen peroxide in 100% methanol to remove endogenous peroxidases. Slides were hydrated through an ethanol gradient, then permeabilized and blocked in 5% goat serum in 0.3%Triton X-100 (PBS-T) for 1 h. Subsequently, slides were incubated for 24 h at 4°C with rabbit polyclonal cleaved caspase-3 antibody (1 : 200, Cell Signaling) in blocking solution, washed, and incubated for 1 h at 25°C with a secondary goat anti-rabbit biotinylated IgG antibody (1 : 200, Vector Labs) in blocking solution. Sections were immunostained using the ABC method (Vectastain Elite Kit, Vector Labs, Burlingame, CA) and diaminobenzidine (DAB), counterstained with toluidine blue, dehydrated in an ethanol gradient, immersed in xylene and coverslipped. The specificity of the immunoreaction was assessed by performing all steps except leaving out the primary antibody in which case no immunolabeling was observed.

Activated Caspase-3 Cell Counting

Activated caspase-3 immunopositive cell density was assessed in left medial frontal cortex in the corresponding region of right medial frontal cortex that was homogenized for Western blotting. The starting point for cell counting was the anterior aspect of corpus callosum. Cells were counted within a 90° sector formed by two perpendicular lines drawn from the superior tip of the corpus callosum, one drawn vertically to the superior cortical surface and one drawn horizontally to the medial cortical surface. Systematic random sampling of every 30th section among the first 100 sections was performed, starting with a randomly selected section among the first 10 sections that contained corpus callosum. Counting was performed blind to condition. Three sections were counted per brain using the StereoInvestigator system (MicroBrightField, Inc., Williston, VT) using a 250 μm counting frame. Approximately, 300 caspase-3 immunopositive cells were counted per brain.

Activated Caspase-3 Immunodensity

Densitometric measurements of immunhistochemically stained cells in each section were quantified by capturing an image with a CCD camera connected to an Olympus microscope and a Bioquant Image Analysis system. For each section, a digital image was captured at × 2 under the same illumination and camera settings, and a region of interest (ROI) was chosen at a fixed rectangular size (width: 500 μm; height: 2200 μm) placed within the same region used for cell counting adjacent to the corpus callosum. The measurement threshold used for each section was set at a level that excluded background and included only the dark pixels of the immunoreaction product, which indicated the varying levels of caspase-3 staining. Density was measured in relative grayscale units (0–255) with 0 being darkest and 255 lightest. Semi-automatic multiple average density measurements occurred in the ROI at × 20 magnification on slightly overlapping fields of view to prevent redundancy, and generated numeric labels in order to keep a numeric count of stained cells.

Cell Death Detection ELISA

An ELISA assay kit for DNA/histone nucleosomes was used to quantify the rate of apoptosis across homogenized samples using the manufacturer's recommended methods (Cell Death Detection ELISA plus, Roche). This assay is analogous to measuring apoptosis-induced DNA laddering by Southern blot. The method is based on quantifying mono- and oligonucleosomes generated by apoptosis via endonuclease-mediated cleavage of chromatin into 180-base pair (bp) nucleosomes and multiples thereof. Nucleosomes

are units of chromatin formed by a histone octamer wrapped by 146 bp DNA. In this assay, 20 µl homogenized samples (80 µg total protein/sample) were diluted with 80 µl immunoreagent containing monoclonal mouse anti-histone (H1, H2A, H2B, H3, and H4) biotin and anti-DNA (single and double-stranded)-peroxidase antibodies and were placed in 96-well streptavidin-coated microplates. All samples were run in triplicate. A positive control using DNA-histone complex was run on each plate. Samples were shaken (300 r.p.m.) for 2 h at 25°C and unbound components were removed by rinsing with incubation buffer. A measure of 100 µl ABTS substrate was added to each well, the plate was incubated at 25°C while shaking (250 r.p.m.) and retained peroxidase was measured spectrophotometrically at 405 nm using a microplate reader (Vmax, Molecular Devices).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism Version 4.01 (GraphPad Software, San Diego, CA). Outcome variables were compared by one-way analysis of variance (ANOVA) across all diagnostic groups with significance set at $p<0.05$. For significant changes by ANOVA, *post hoc* Dunnett's multiple comparison tests were also performed with two-tailed p-values considered significant at $p<0.05$.

RESULTS

Bax and Bcl-2 Immunoblots

Using semi-quantitative Western blots, immunoreactivity of Bcl-2 bands in rat frontal cortex did not show an effect of antipsychotic treatment by ANOVA ($F=0.69$, $df=3, 48$, $p=0.563$) (see Table 1). Although immunoreactivity of Bax bands showed a trend toward a medication effect ($F=2.70$, $df=3, 48$, $p=0.057$), *post hoc* analysis revealed no changes in Bax between the treatment arms (see Table 1). The Bax/Bcl-2 ratio has been identified as an important determinant of apoptotic vulnerability and neurons with high Bax/Bcl-2 ratio are more susceptible to undergo apoptosis (Oltvai *et al*, 1993; Vekrellis *et al*, 1997). The Bax/Bcl-2 ratio was derived by normalizing the optical density (OD) of Bax and Bcl-2 bands for each sample to the mean OD of the saline control group, respectively. Bax/Bcl-2 quotients of the normalized Bax and Bcl-2 values were calculated for each sample. This analysis did not show evidence of a medication effect on Bax/Bcl-2 ratios by ANOVA ($F=0.994$, $df=3, 48$, $p=0.405$) (see Table 1).

Caspase-3 Immunoblots

Immunoreactivity of procaspase-3 bands did not show an effect of antipsychotic treatment by ANOVA ($F=0.32$, $df=3, 48$, $p=0.810$) (see Table 1). However, antipsychotic medications produced a robust effect on the immunoreactivity of the 19 kD fragment of activated caspase-3 by ANOVA ($F=7.43$, $df=3, 48$, $p=0.0004$) (see Figure 1). Activated caspase-3 fragments are seen as multiple cleaved bands migrating in the 17–20 kD range and a smaller band ~12–14 kD can sometimes be visualized (Faleiro *et al*, 1997). In the current study, a 19 kD band was most abundant and clearly visualized across all samples while 17 and 20 kD bands were very faint and no 12–14 kD bands

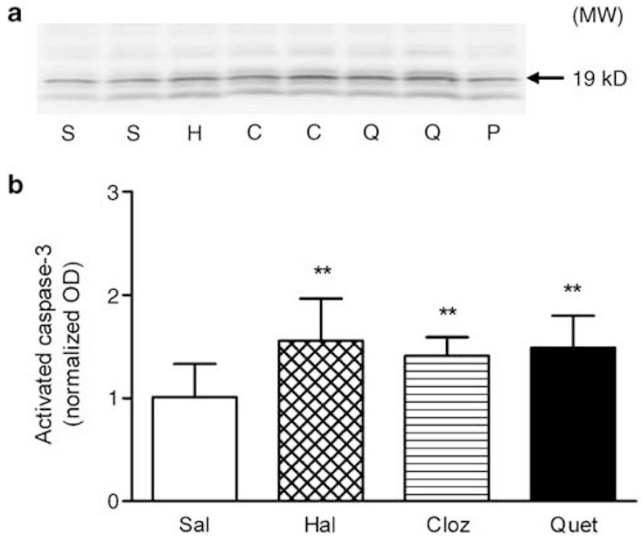

Figure 1 (a) Representative Western blot of activated caspase-3 in rat frontal cortex from animals treated daily for 4 weeks with i.p. saline (S) 0.9%, haloperidol (H) 1 mg/kg, clozapine (C) 10 mg/kg, and quetiapine (Q) 10 mg/kg. Pooled sample (P) was included to allow for inter-gel comparisons. Of the large fragments of activated caspase-3, only the 19 kD molecular weight band was visualized in all samples. (b) A significant treatment effect emerged for activated caspase-3 levels by ANOVA. Compared to saline control, mean activated caspase-3 levels were 55% higher with haloperidol (**$p<0.01$), 40% higher with clozapine (**$p<0.01$), and 47% higher with quetiapine (**$p<0.01$) using *post hoc* Dunnett's multiple comparison tests.

Table 1 Immunoreactivity of Apoptotic Protein Bands by Semi-Quantitative Western Blot

Treatment	Bax	Bcl-2	Bax/Bcl-2	Procaspase-3	Act. caspase-3	XIAP
Saline	1.41 ± 0.27	1.20 ± 0.30	1.06 ± 0.34	1.11 ± 0.29	1.01 ± 0.32	1.38 ± 0.39
Haloperidol	1.30 ± 0.26	1.10 ± 0.20	1.05 ± 0.32	1.10 ± 0.24	1.56 ± 0.41**	1.47 ± 0.30
Clozapine	1.53 ± 0.25	1.18 ± 0.23	1.13 ± 0.28	1.03 ± 0.35	1.41 ± 0.18**	1.40 ± 0.29
Quetiapine	1.25 ± 0.27	1.26 ± 0.35	0.91 ± 0.35	1.01 ± 0.35	1.49 ± 0.31**	1.33 ± 0.34

Data represents normalized optical density measurements (mean ± SD) of immunoreactive bands representing Bax, Bcl-2, Bax/Bcl-2 ratio, procaspase-3, activated (act.) caspase-3 (19 kD fragment) and XIAP protein in rat frontal cortex following 4 weeks of daily treatment with haloperidol, clozapine, quetiapine, or saline. **$p<0.01$ by *post hoc* Dunnett's multiple comparison test.

were seen. Compared to saline control samples, the mean immunoreactivity of activated caspase-3 (19 kD fragment) bands were 55% higher with haloperidol ($p<0.01$), 40% higher with clozapine ($p<0.01$), and 47% higher with quetiapine ($p<0.01$) using a *post hoc* Dunnett's multiple comparison test (see Table 1).

XIAP Immunoblots

Immunoreactivity of XIAP protein in frontal cortical homogenates showed no effect of antipsychotic treatment by ANOVA ($F=0.391$, $df=3, 47$, $p=0.760$) (see Table 1). Similarly, there was no effect on XIAP bound to activated caspase-3 IP following treatment with haloperidol (0.88 ± 0.09), clozapine (1.07 ± 0.23), and quetiapine (1.18 ± 0.53) compared to saline (1.0) by ANOVA ($F=0.586$, $df=3, 11$, $p=0.641$). Note that in the IP experiment, samples were pooled from each treatment arm and results were normalized to the saline control arm.

Caspase-3 Activity

Specific activity of DEVD-pNA cleavage (caspase-3-like activity) demonstrated a significant treatment effect ($F=9.15$, $df=3, 47$, $p<0.0001$) (see Figure 2). A *post hoc* Dunnett's test showed that specific activity of caspase-3 was 30% higher in the haloperidol arm (0.086 ± 0.015 pmol pNa/min/μg total protein, $p<0.01$), 46% higher in the clozapine arm (0.0970 ± 0.020 pmol pNa/min/μg total protein, $p<0.01$), and 39% higher in the quetiapine arm (0.093 ± 0.014 pmol pNa/min/μg total protein, $p<0.01$) when compared to the control arm (0.066 ± 0.012 pmol pNa/min/μg total protein).

Activated Caspase-3 Immunohistochemistry

Activated caspase-3 immunopositive cells were found in all areas of medial frontal cortex in both saline and antipsychotic-treated animals (see Figure 3a). No effect emerged for activated caspase-3 cell density with haloperidol (1.11 ± 0.34 normalized cell density), clozapine (1.01 ± 0.36) and quetiapine (0.95 ± 0.21) compared to saline (1.00 ± 0.21) by ANOVA ($F=1.87$, $df=3, 140$, $p=0.138$) (see Figure 3b). Similarly, there was no effect on activated caspase-3 immunodensity per cell with haloperidol (35.75 ± 15.50 grayscale units), clozapine (33.04 ± 10.98), and quetiapine (36.86 ± 12.11) compared to saline (33.79 ± 10.02) by ANOVA ($F=0.244$, $df=3, 48$, $p=0.865$).

DNA Fragmentation

In this ELISA-based assay, histone-associated DNA fragment levels were measured. Apoptosis-induced histone/DNA fragments are analogous to DNA laddering that is visualized with Southern blotting. Medial frontal cortex did not demonstrate an effect of antipsychotic treatment on histone-associated DNA fragmentation with haloperidol (0.96 ± 0.16 absorption units (au)), clozapine (0.97 ± 0.13 au), and quetiapine (1.06 ± 0.14 au) compared to saline-treated animals (1.06 ± 0.13 au) by ANOVA ($F=1.93$, $df=3, 48$, $p=0.139$).

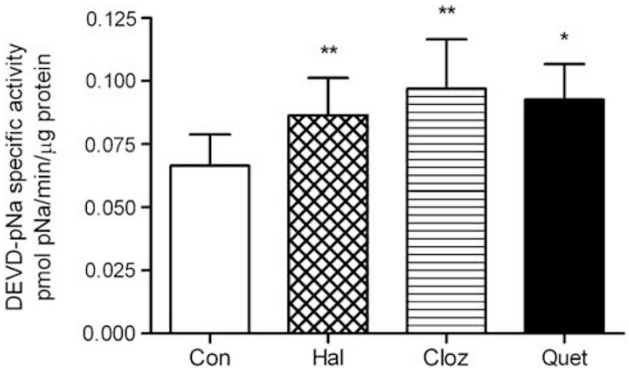

Figure 2 A significant effect of antipsychotic treatment was demonstrated on caspase-3-like activity measuring DEVD-pNA (Asp-Glu-Val-Asp-p-nitroanilide) cleavage, by ANOVA ($F=9.15$, $df=3, 47$, $p<0.0001$). Compared to control, *post hoc* Dunnett's multiple comparison tests showed caspase-3-like specific activity was 29.9% higher in the haloperidol arm (**$p<0.01$), 45.9% higher in the clozapine arm (**$p<0.01$), and 39.4% higher in the quetiapine arm (*$p<0.05$).

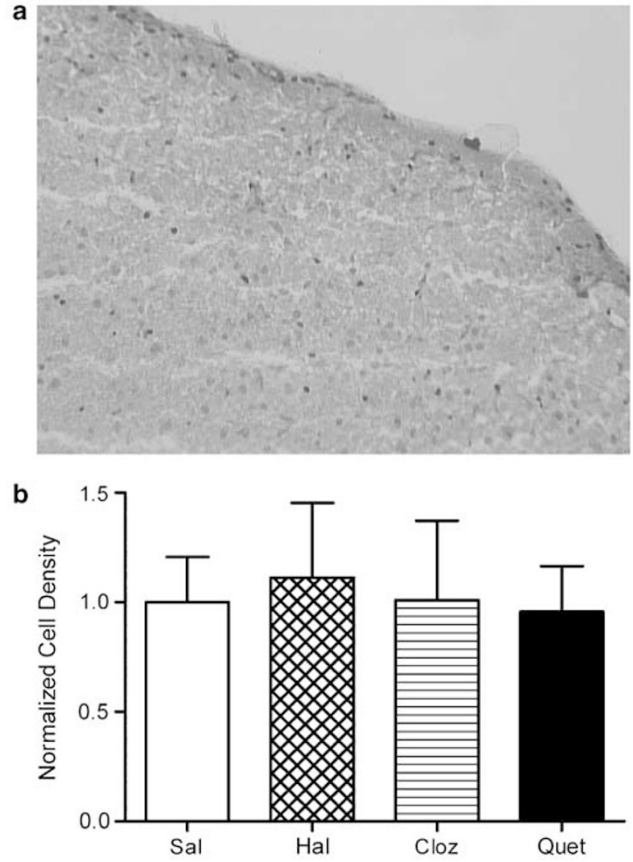

Figure 3 (a) Representative section of activated caspase-3 immunohistochemistry at × 20 following clozapine treatment. Caspase-3-positive cells are seen throughout the cortical layers. (b) Mean activated caspase-3-immunopositive cell density was not altered between saline control, haloperidol, clozapine, and quetiapine treatment arms.

DISCUSSION

As Bcl-2 has been established as a potent neuroprotective (Oltvai *et al*, 1993; Zhong *et al*, 1993) and neurotrophic protein (Oh *et al*, 1996; Chen *et al*, 1997), the potential for

antipsychotic-mediated Bcl-2 upregulation was examined. The particular interest in antipsychotic effects on Bcl-2 stemmed from the observation that Bcl-2 was reduced in temporal cortex in schizophrenia, along with a preliminary association between higher cortical Bcl-2 levels in those patients previously treated with antipsychotics when compared with treatment-naive patients (Jarskog *et al*, 2000). Contrary to our hypothesis, immunodensity of Bcl-2 bands in rat cortex was not increased after 4 weeks of haloperidol, clozapine, or quetiapine treatment when compared to vehicle-control treated animals. Likewise, pro-apoptotic Bax immunodensity and Bax/Bcl-2 ratio were unchanged with antipsychotic treatment. This is in contrast to a study that found 4 weeks of clozapine and olanzapine treatment produced 30–50% higher Bcl-2 mRNA and protein in rat frontal cortex and hippocampus (Bai *et al*, 2004). The main differences between these two studies were that a different rat strain was used by Bai *et al* (Wistar) as compared to the current study (Sprague–Dawley) and that only clozapine was used in common between the two studies. This could suggest strain-dependent differences in apoptotic signaling cascades or relate to pharmacokinetic differences. Based on the absence of an antipsychotic effect on Bcl-2 immunoreactivity in the current study, two main conclusions can be drawn. First, the current data do not support a prominent Bcl-2-mediated neuroprotective effect of typical or atypical antipsychotics in frontal cortex. Second, these findings strengthen the conclusion that low Bcl-2 levels in schizophrenia relate to pathophysiology and do not represent a confounding effect of treatment.

Although Bax and Bcl-2 were unchanged following treatment, typical, and atypical antipsychotic medications unexpectedly increased immunoreactivity and activity of activated caspase-3 in rat cortex. Caspase proteins are cysteine-dependent proteases that serve to cleave specific structural and functional cellular substrates during apoptosis. Caspase-3 represents the predominant 'effector' caspase in the CNS, both in normal neurodevelopment and in neuropathological states (Yuan and Yankner, 2000). Western blotting demonstrated a 40–55% increase in immunoreactivity of activated caspase-3 bands following 1 month of daily haloperidol, clozapine, or quetiapine treatment. Moreover, a previous study found that activated caspase-3 in rat cortex was not altered following 1 week of daily haloperidol treatment (Jarskog *et al*, 2004), suggesting that the increase in caspase-3 is a delayed effect, occurring only after several weeks of treatment. Consistent with elevated immunoreactivity of activated caspase-3 bands by Western blot, caspase-3-like specific activity was increased by 30–46% across the antipsychotic treatments. The current data represent the first *in vivo* evidence that both typical and atypical antipsychotics can activate caspase-3, a key downstream apoptotic protease that is extensively implicated in normal and pathological functions in the CNS.

To investigate the impact of increased caspase-3 levels on cortical cell survival, relative levels of DNA fragmentation were assessed. DNA fragmentation is recognized as a hallmark of apoptotic activity (Arends *et al*, 1990), and occurs as a consequence of caspase-3-mediated cleavage of DNA fragmentation factor (DFF/CAD) and other key cellular substrates (Yuan *et al*, 2003). Owing to its association with DNA fragmentation, the presence of activated caspase-3 has been identified as a marker for active apoptosis (Krajewska *et al*, 1997). However, in the current study, caspase-3 activation was not associated with increased rates of DNA fragmentation, indicating that antipsychotic treatment did not induce apoptosis. Taken together, these data indicate that sub-chronic antipsychotic treatment in a rat model is associated with non-lethal caspase-3 activity.

Consistent with the DNA fragmentation data, activated caspase-3 immunopositive cell number in frontal cortex was unaffected by treatment. This suggested that higher activated caspase-3 immunoreactivity may instead have increased on a per cell basis. By tracing caspase-3-immunopositive cell bodies, immunoreactivity was numerically higher by 6% in the haloperidol group and 9% in the quetiapine group compared to saline control; however, this effect was not significant. Variability associated with immunohistochemical assessments could account for the failure to detect an increase in caspase-3 immunodensity per cell.

XIAP, a member of the IAPs, is a critical downstream inhibitor of apoptosis in the CNS that binds directly to active caspase-3 (Lotocki and Keane, 2002). As higher XIAP levels could potentially account for the absence of DNA fragmentation in the face of higher activated caspase-3 levels, XIAP levels were measured to rule out a potential effect of antipsychotics on XIAP. As demonstrated, total cortical XIAP levels did not differ between antipsychotic- and saline-treated animals. Furthermore, XIAP bound to immunoprecipitated activated caspase-3 was also unchanged across treatment groups. These data do not implicate XIAP in antipsychotic-mediated effects on caspase-3 activity.

The clinical relevance of increased caspase-3 activity following 1 month of antipsychotic treatment remains uncertain. One possibility is that caspase-3 activity could contribute to the evidence for progressive cortical gray matter loss in the early stages of schizophrenia (Gur *et al*, 1998; Cahn *et al*, 2002; Kasai *et al*, 2003; Lieberman *et al*, 2005). Yet, in human post-mortem studies, activated caspase-3 levels are slightly reduced in temporal cortex in schizophrenia (Jarskog *et al*, 2004), and the rate of neuronal DNA fragmentation is also reduced in anterior cingulate cortex in schizophrenia compared to control subjects (Benes *et al*, 2003). These differences in apoptotic marker profiles between schizophrenia cortex and the current antipsychotic-treated animal study suggest that the apoptotic deficits in schizophrenia are more likely to be pathophysiological in nature rather than an effect of treatment. Nevertheless, further study is needed to assess whether increased caspase-3 activity in response to antipsychotic treatment may be time-limited and whether such activity could impact synaptic viability, as has been suggested by evidence of caspase-3-mediated synaptically localized apoptotic activity (Mattson *et al*, 1998; Gylys *et al*, 2002).

A limitation of the current study is the uncertainty over the comparability and clinical relevance of the antipsychotic doses tested. The argument that antipsychotic dosing in rodent models often lacks relevance to the clinical condition is primarily based on studies of equivalent D_2 receptor occupancy. One study found that clinically relevant D_2

occupancy for single doses of quetiapine and clozapine are comparable to those used in the current study (Kapur *et al*, 2003), while for haloperidol, reports of comparable occupancy ranges from doses of 0.06 mg/kg (Kapur *et al*, 2003) to 0.6 mg/kg (Schotte *et al*, 1996). These studies suggest that our haloperidol dose was somewhat high. However, haloperidol plasma levels for long-term dosing at 1 mg/kg/day in rats is typically around 10 ng/ml (Gao *et al*, 1997; Andersson *et al*, 2002) and 10 ng/ml is also the plasma level that has associated with better clinical response in one of the few double-blind randomized studies that targeted specific haloperidol plasma levels—2 ng/ml *vs* 10 ng/ml—in acutely ill schizophrenic patients (Volavka *et al*, 1995). By this measure, our haloperidol dose was not unreasonably high.

In conclusion, sub-chronic treatment with haloperidol, clozapine, and quetiapine was not associated with increased Bcl-2 immunoreactivity in rat frontal cortex. This finding does not support a Bcl-2-mediated neuroprotective effect of antipsychotics in frontal cortex. However, antipsychotic treatment was associated with increased levels and activity of caspase-3, a key downstream apoptotic protease that is extensively involved in CNS development and disease. The increase in caspase-3 activity occurred without increased DNA fragmentation, indicating that this activity was probably non-lethal. Given several lines of evidence that apoptotic activity is downregulated in post-mortem cortex in the chronic stages of schizophrenia, this study indicates that the neuropathology of schizophrenia is not simply the result of the effects of antipsychotic medications on apoptotic signaling. Although speculative, it remains possible that caspase-3 activity could contribute to the evidence for progressive neurostructural changes seen in the early stages of schizophrenia. Further studies are needed to understand the relationship between antipsychotic treatment and apoptotic regulation and the potential clinical impact of this interaction.

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