Selective effects of typical antipsychotic drugs on SNAP-25 and synaptophysin in the hippocampal trisynaptic pathway

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Abstract

Recent studies indicate that levels of presynaptic proteins are altered in the post-mortem brain in schizophrenia. In particular, the hippocampus exhibits reduced levels of synaptophysin and the SNARE protein SNAP-25. The effects of treatment with antipsychotic drugs on levels of SNAP-25 in the hippocampus remains unknown. To determine the effects of typical antipsychotic drugs on levels of synaptophysin and SNAP-25 in the hippocampus, rats were treated with chlorpromazine, haloperidol or trifluoperazine for 21 d. Quantitative immunohistochemistry was used to measure immunoreactivity within the trisynaptic circuit of the hippocampus. Trifluoperazine decreased synaptophysin within the Schaffer collateral region of the radiatum lacunosum in CA1, while haloperidol and chlorpromazine increased SNAP-25 throughout the trisynaptic pathway of the hippocampus, with strongest effects in the mossy fibre region of CA3. These results indicate that presynaptic proteins represent a potential molecular substrate for the effects of antipsychotic drugs on hippocampal synaptic connectivity.

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Introduction

Schizophrenia is a complex psychiatric disorder, which probably arises from an elaborate, and as yet unknown, series of interactions between genetic and environmental factors (Lewis and Levitt, 2002). Accumulating evidence suggests that schizophrenia is largely a disorder of neural connectivity, in which the underlying pathophysiology of the condition is manifested at a synaptic level in the brain by abnormal ‘miswiring’ (Honer, 1999). Consistent with this position, an extensive body of research has demonstrated that the molecular machinery that regulates synaptic activity is altered in post-mortem brain tissue in schizophrenia (Sawada et al., 2002, 2005). In particular, the plasticity-associated soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein synaptosomal-associated protein-25 (SNAP-25) and the ubiquitous synaptic protein synaptophysin exhibit altered levels of protein or gene expression in different brain regions in this illness (Eastwood et al., 2001; Fatemi et al., 2001; Honer et al., 2002; Thompson et al., 2003; Young et al., 1998).

Levels of SNAP-25 and synaptophysin are altered throughout the brain in schizophrenia, and the hippocampus is highlighted as a region of special interest. The hippocampus plays a critical role in cognitive processing and memory formation, both of which are severely impaired in schizophrenia (Lewis, 2004). At a molecular level, we recently demonstrated that schizophrenia is associated with decreased levels in the hippocampus of the presynaptic proteins complexin I and II, and that a decrease in the complexin II:1 ratio is significantly associated with cognitive impairment (Sawada et al., 2005). However, rats treated with the typical antipsychotic drug haloperidol for 21 d do not exhibit altered levels of complexins in the hippocampus, suggesting that these presynaptic proteins are not important molecular targets for the drug in this brain region. The effects of this neuroleptic drug on alternate presynaptic proteins, such as SNAP-25, were not measured.
The purpose of the present study was, therefore, to determine whether antipsychotic drugs may exert their effects in part through altering levels of the presynaptic proteins SNAP-25 and synaptophysin in the hippocampus. The hippocampus exhibits a complex, multisympaptic circuitry that has been mapped precisely, rendering it amenable to analysis within the context of models of neural connectivity (Harrison, 2004). Furthermore, it has been proposed that the summed effect of the numerous molecular changes that occur in the different subregions of the hippocampus in schizophrenia may be to alter the functional circuitry of the macroarchitectural trisynaptic pathway (Benes, 1999). To test this hypothesis, animals were treated for 21 d with three different typical antipsychotics, and levels of SNAP-25 and synaptophysin were measured in the trisynaptic pathway of the hippocampus using quantitative immunohistochemistry.

Materials and methods

Twenty-four male Sprague–Dawley rats (250–275 g; Animal Care Centre, University of British Columbia, Vancouver) were maintained in a colony room at 20 °C (± 1 °C) on a 12 h light-dark cycle (lights on 07:00 hours) with unlimited access to water and food. One week after arrival, four randomly selected groups received single daily intraperitoneal injections of either haloperidol (1 mg/kg dissolved in 0.3% tartaric acid), chlorpromazine (10 mg/kg in distilled water), trifluoperazine (6 mg/kg in distilled water), or vehicle (0.3% tartaric acid) over 21 d (n = 5, trifluoperazine and vehicle; n = 6, haloperidol and chlorpromazine); drugs were dissolved in a volume of 1 ml/kg. Animals were injected between 10:00 and 12:00 hours. After the last injection, animals were deeply anaesthetized with chloral hydrate, transcardially perfused with ice-cooled 200 ml phosphate buffered saline (PBS) and 200 ml neutral buffered formalin (4% NBF), and sacrificed by decapitation. Brains were removed, fixed in NBF for 48 h at 4 °C, and stored in Tris-buffered saline (TBS)-azide (0.1% azide) at 4 °C until required. All experimental procedures were conducted in accordance with guidelines provided by the Canadian Council on Animal Care and the University of British Columbia Animal Care Committee.

Tissue was prepared as described previously (Barr et al., 2003). Three coronal sections 3-μm thick were placed on individual slides. Sections were deparaffinized followed by incubation in TBS plus 0.2% Triton X-100 with 3% hydrogen peroxide for 30 min. Non-specific immunoreactivity was blocked by incubation in TBS-5% non-fat, powdered milk for 1 h at room temperature (RT). Primary antibodies (tissue culture supernatant SP12 and SP15) diluted 1:10 in TBS-milk were added and incubated overnight at 4 °C. Slides were washed in TBS (five times for 5 min) at RT. Biotinylated goat anti-mouse immunoglobulin G plus M (Jackson Immunolabs, West Grove, PA, USA) diluted 1:500 in TBS was added for 1 h at RT. Following a TBS wash (five times for 5 min), peroxidase-conjugated streptavidin (Jackson Immunolabs) was added in a dilution of 1:1000 in TBS for 30 min. After sections were washed in TBS (five times for 5 min), sections were incubated in 0.03% diaminobenzidine (Sigma, St. Louis, MO, USA), 0.015% hydrogen peroxide in 0.1 M Trizma-base (pH 7.4). The reaction was terminated after 8 min, the slides dried, dehydrated and then coverslipped using Permount. A common reference section was used as a positive control for synaptophysin and SNAP-25 staining for each run of immunohistochemistry. In addition, for a negative control, tissue culture medium conditioned by the parent, non-secreting myeloma cell line was substituted for the primary antibody on an additional section.

Images of the whole hippocampus were collected in triplicate blinded to drug treatment using a Sony CCD video camera, Nikon lens, and Northern Light constant source lightbox, with a single wavelength filter (Lee Chromalux Light Blue) centred around 450 nm and a custom series of monochrome slides for calibrating grey-scale intensities to OD values. Hippocampal regions of interest were demarcated using an atlas (Paxinos and Watson, 1986); all measurements were performed blind to treatment. Ammon’s horn subregions were defined by characteristics of the pyramidal cell layer including width and terminations of afferent projections, such as the mossy fibres (synaptic immunostained sections) or the Schaffer collateral regions. The CA1 and CA2 regions were combined as a single CA1+2 region. Regions were outlined using NIH Image version 1.61 and mean pixel intensity was calculated. For synaptophysin, white-matter values were subtracted from grey-matter image values on the same section. As SNAP-25 is present in axons to a small extent, this correction was not applied to SNAP-25 immunostaining; instead, the background value from the negative control section was subtracted from the image values.

For analysis of trisynaptic pathway regions of interest on immunostained sections, repeated-measures analysis of variance (ANOVA) was used for datasets obtained from CA1+2 (radiatum lacunosum), mossy fibres, and the molecular layer of the
dentate gyrus: drug treatment was a between-subjects factor while region of interest was a within-subjects repeated measure. When a significant interaction was indicated, Fisher’s post-hoc test was performed. Bonferroni corrections were applied for multiple comparisons, whereby statistical significance was defined as $p < 0.05/3$ for region of interest, or $p < 0.05/2$ for drug (to control for two antibodies).

**Results**

For measures of the whole hippocampus, slice-to-slice variability in staining intensity of sections from the same case was 2% for both synaptophysin and SNAP-25. Immunostaining yielded strong staining for both synaptophysin and SNAP-25 (Figure 1), whereby levels of both presynaptic proteins were clearly evinced throughout the hippocampal trisynaptic pathway.

Results of the ANOVA for SNAP-25 OD values throughout the trisynaptic pathway indicated that there was a significant main effect of drug treatment ($F = 4.90$, d.f. = 3, 18, $p = 0.012$). There was no significant main effect of trisynaptic pathway subregion, nor was there a significant interaction of drug × subregion. Further analysis of the data with post-hoc tests (Table 1) indicated that the drug effect was predominantly due to increased levels of SNAP-25 in the chlorpromazine- and haloperidol-treated rats (Figure 2), compared to trifluoperazine- and vehicle-treated animals. The main effect of drug treatment was initially significant in all three trisynaptic pathway subregions, but significance was lost for the dentate gyrus following application of the Bonferroni correction. Additional analysis of drug effects in the two significantly different subregions, i.e. the mossy fibre projections and Schaffer collateral regions, demonstrated that levels of SNAP-25 were significantly greater ($p < 0.05$) compared to vehicle animals for chlorpromazine-treated rats in the mossy fibre and Schaffer collateral regions, while haloperidol-treated rats displayed greater levels in the mossy fibre region. Following application of the Bonferroni correction, the significance for chlorpromazine-treated rats was lost in both hippocampal subregions, whereas the increased levels of SNAP-25 in the mossy fibre region of haloperidol-treated rats remained strongly significant.
Analysis of synaptophysin data indicated that there was a significant main effect of drug treatment ($F = 3.43$, d.f. $= 3, 18$, $p = 0.039$); however, following application of the Bonferroni correction, the significance of this effect was lost. The ANOVA indicated that there was also a significant main effect of trisynaptic pathway subregion ($F = 22.85$, d.f. $= 2, 18$, $p < 0.0001$) that remained highly significant after application of the Bonferroni correction. There was no significant drug x subregion interaction. Post-hoc tests (Table 1) revealed that levels of synaptophysin tended to be greater in haloperidol- and chlorpromazine-treated rats than vehicle animals, while levels of synaptophysin were lower in trifluoperazine-treated rats compared to vehicle animals ($p = 0.022$) but this remained non-significant after the Bonferroni correction. Interestingly, the significant main effect of the hippocampal subregion on levels of synaptophysin revealed that levels of this presynaptic protein were greater in the Schaffer collateral region of the radiatum lacunosum of CA1 than in other subregions.

### Discussion

The results of the present experiment demonstrate that levels of the presynaptic proteins SNAP-25 and synaptophysin are altered in a drug- and region-specific manner in the trisynaptic pathway, following administration of three different antipsychotic drugs for 21 d. Chlorpromazine-treated rats exhibited increased levels of SNAP-25 in the mossy fibre and Schaffer collateral regions while haloperidol-treated rats displayed increased levels in the mossy fibre region; these effects only remained significant for the haloperidol-treated rats following application of Bonferroni correction for multiple comparisons. There was also evidence that levels of synaptophysin were decreased in trifluoperazine-treated rats, although the significance of this effect also disappeared following the Bonferroni correction.

The results of the present study are in general agreement with previous reports. Prior experiments with similar doses of haloperidol have observed either no effect or non-significant increases in levels of hippocampal synaptophysin mRNA and protein (Eastwood et al., 1995, 1997, 2000; Vawter et al., 2002), while chlorpromazine had no significant effect on hippocampal levels of synaptophysin (Eastwood et al., 2000). Our observation that trifluoperazine decreased levels of synaptophysin within the Schaffer collateral region represents the first report of the effects of this drug on levels of presynaptic proteins, and provides an important example that drugs of a similar class, such as neuroleptics, may produce dissimilar effects on hippocampal connectivity. The doses of the drugs used in the present study were chosen to be as similar as possible. Both haloperidol and chlorpromazine, at the current doses, display $\sim 80\%$ dopamine D$_2$ receptor occupancy in vivo in the rat (Crocker and Hemsley, 2001; Turrone et al., 2003), which...
corresponds closely with clinical efficacy in humans. While we are unaware of pre-clinical studies reporting in-vivo D2 occupancy with trifluoperazine, this compound exhibits a low nanomolar affinity for the D2 receptor (McMillen, 1985), and thus, should exert similar physiological actions as the other neuroleptics.

The present data also indicate that the typical antipsychotic drugs haloperidol and chlorpromazine tend to increase levels of SNAP-25 throughout the hippocampal trisynaptic pathway, with greatest effects in the mossy fibre and Schaffer collateral regions. These data are the first to describe the effects of treatment with antipsychotic drugs on levels of SNAP-25 within the hippocampus in rodents. One previous study failed to observe an effect of chronic treatment with haloperidol on SNAP-25 mRNA within other brain regions (Nakahara et al., 1998), including dopamine-relevant regions such as the prefrontal cortex, nucleus accumbens, striatum, substantia nigra and ventral tegmental area. However, the multisynaptic structure of the hippocampus, and its consequent cytoarchitectural complexity, allows for the measurement of subtle and regionalized changes. These defined alterations are evident in the post-mortem hippocampus of schizophrenia and other psychiatric groups. For example, we have demonstrated previously that schizophrenia is associated with significant reductions in SNAP-25 immunoreactivity within the perforant path termination zones in the molecular layers of CA1 and CA2, as well as the outer molecular layer of the dentate gyrus, although no significant changes were observed in the presubiculum or CA3 region (Young et al., 1998). Similar, regionalized changes were observed by Fatemi and colleagues (2001) who noted general reductions in hippocampal SNAP-25 immunoreactivity and significant decreases within the stratum granulosum. Additionally, reduced levels of SNAP-25 have also been observed in schizophrenia when entire hippocampal homogenates were quantified using immunoblotting (Thompson et al., 2003). The mechanisms underlying decreased SNAP-25 in trifluoperazine-treated rats remains unknown, as doses of the three neuroleptics were chosen to be approximately equivalent, and no differences in behaviour were observed between treatment groups (Barr, A. M., personal observation). Future studies should address this issue with more complete dose–response evaluations.

In the present study, the significant increase in SNAP-25 that occurred following treatment with haloperidol was localized to the mossy fibre region of CA3. This region has not been shown to exhibit significantly reduced levels of SNAP-25 in schizophrenia, although, interestingly, it has been identified as an especially vulnerable region of the hippocampus to the effects of chronic stress (Galea et al., 1997). The dentate gyrus and CA1/Schaffer collateral regions in rats also exhibited a strong trend for increased SNAP-25 immunoreactivity, both of which regions were shown previously to exhibit reduced SNAP-25 in schizophrenia (Young et al., 1998). This observation corresponds closely with clinical efficacy in humans.
Further studies, using a wider range of drug doses of typical antipsychotics, affirming a role for elevated levels of SNAP-25 as a substrate for the action of antipsychotic drugs. This protein appears to be relatively enriched in subsets of synapses, with reports of preferential co-localization in glutamatergic terminals, and relative exclusion from interneuron GABAergic terminals (Frassoni et al., 2005). Partial loss of the SNAP-25 gene, as evident in the Coloboma heterozygous mutant mouse, is associated with hyperactivity and loss of synaptic plasticity, measured by reduced hippocampal long-term potentiation (Steffensen et al., 1996). Of interest, the behavioural deficits in Coloboma mice are proposed to reflect increases in dopaminergic neurotransmission, and it is therefore intriguing that the selective dopamine D2 receptor antagonists used in the present study increase levels of SNAP-25. Furthermore, allelic variants and haplotypes in the SNAP-25 gene have recently been linked to attention deficit hyperactivity disorder (Barr et al., 2000). Certain symptoms of this disorder, including hyperactivity and aggressiveness, may be treated effectively by low doses of both haloperidol and chlorpromazine (Bond, 1987), and it is also interesting to note that a recent study reported that specific SNAP-25 polymorphisms were significantly associated with clinical response to antipsychotic drugs (Muller et al., 2005).

In our previous study we reported no effects of treatment of rats with haloperidol on levels of complexin I, complexin II or the ratio of these proteins in a subregional analysis of the hippocampal formation. We re-analysed this data focusing on the trisynaptic pathway, using a similar statistical strategy as in the present paper. No effects of haloperidol on trisynaptic pathway levels of complexin I, complexin II or the ratio of these proteins were observed. These observations, along with the minimal effect of antipsychotic treatment on synaptophysin reported here, support the possibility that there may be SNAP-25-specific effects of antipsychotic drug treatments. This protein appears to be relatively enriched in subsets of synapses, with reports of preferential co-localization in glutamatergic terminals, and relative exclusion from interneuron GABAergic terminals (Frassoni et al., 2005). However, the tendency for the neuroleptic trifluoperazine to decrease trisynaptic pathway levels of SNAP-25 raises a note of caution in globally affirming a role for elevated levels of SNAP-25 as a general therapeutic effect of typical antipsychotics. Further studies, using a wider range of drug doses of both typical and atypical antipsychotic drugs, as well as valid animal models of schizophrenia (Barr et al., 2004a,b), are required to determine the relevance of pharmacotherapy-induced increases in SNAP-25 as a substrate for the action of antipsychotic drugs.

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Statement of Interest

None.

References


