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Effects of haloperidol and clozapine on synapse-related gene expression in specific brain regions of male rats

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Abstract

We investigated the effects of clozapine and haloperidol, drugs that are widely used in the treatment of schizophrenia, on gene expression in six cortical and subcortical brain regions of adult rats. Drug treatments started at postnatal day 85 and continued over a 12-week period. Ten animals received haloperidol (1 mg/kg bodyweight) and ten received clozapine (20 mg/kg bodyweight) orally each day. Ten control rats received no drugs. The ten genes selected for this study did not belong to the dopaminergic or serotoninergic systems, which are typically targeted by the two substances, but coded for proteins of the cytoskeleton and proteins belonging to the synaptic transmitter release machinery. Quantitative real-time PCR was performed in the prelimbic cortex, cingulate gyrus (CG1) and caudate putamen and in the hippocampal cornu ammonis 1 (CA1), cornu ammonis 3 (CA3) and dentate gyrus. Results show distinct patterns of gene expression under the influence of the two drugs, but also distinct gene regulations dependent on the brain regions. Haloperidol-medicated animals showed statistically significant downregulation of SNAP-25 in CA3 ($p=0.0134$) and upregulation of STX1A in CA1 ($p=0.0133$) compared to controls. Clozapine-treated animals showed significant downregulation of SNAP-25 in CG1 ($p=0.0013$). Our results clearly reveal that the drugs' effects are different between brain regions. These effects are possibly indirectly mediated through feedback mechanisms by proteins targeted by the drugs, but direct effects of haloperidol or clozapine on mechanisms of gene expression cannot be excluded.

Keywords Presynaptic proteins · Cytoskeletal proteins · BDNF · Gene expression · Schizophrenia

Introduction

Schizophrenia is a mental disorder characterized by pathological changes on both the morphological and molecular levels in various brain regions, especially in the frontal and temporal lobes [1] and in the hippocampus [2]. Although its aetiology remains enigmatic, it appears to be a developmental disorder. In line with this hypothesis, synaptic plasticity [3] and connectivity [4] and regulation of synaptic functions [5] seem to be disturbed in schizophrenia. On the molecular level, altered expression of genes involved in neurotransmission, neurodevelopment and presynaptic function has been reported in patients with schizophrenia [6–9].

The importance of cytoskeletal elements and additional structural elements in this context has probably been somewhat neglected. Collagen and laminin are the major components of the basal lamina and, therefore, part of the extracellular matrix. Changes in functionally relevant proteoglycans may influence interactions between the extracellular matrix and glial cells and contribute to the pathogenesis of schizophrenia [10]. Normal neurotransmitter release depends, for example, on dynactin (DCTN), a cytoskeletal protein that supports vesicle transport [11], and on complex interactions of presynaptic proteins such as synaptotagmin (SYT), syntaxin (STX), synaptophysin (SYP), synaptosomal-associated protein (SNAP-25) and vesicle-associated membrane protein (VAMP), which mediate the fusion and recycling of synaptic vesicles [5]. Moreover, synaptotagmins are necessary for calcium-dependent trafficking in neuronal synaptic

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vesicles, and their modified gene expression seems to be associated with the aetiology of schizophrenia [12]. Deficiencies of posttranslational phosphorylations in syntaxin, such as STX1, may have additional adverse effects during the progression of schizophrenia [13]. Apparently, SYP is not essential for neurotransmitter release [14], but mice lacking the protein show behavioural changes, such as increased exploratory behaviour, impaired object novelty recognition and reduced spatial learning [15]. Furthermore, SNAP-25, a key component in the molecular pathway of neurotransmitter release, is reduced in different brain areas of patients with schizophrenia [16, 17]. Finally, brain-derived neurotrophic factor (BDNF) affects many neurons of the central nervous system by mediating the survival of existing neurons and the growth and differentiation of new neurons and synapses and influencing synaptic efficiency and plasticity. Peripheral BDNF levels have been found decreased in patients with chronic schizophrenia [18, 19], but results are inconsistent [20, 21].

Little is known about the influence of haloperidol and clozapine on synaptic gene expression. Studies have shown that haloperidol reinforces postsynaptic gene expression throughout the striatum and has a greater impact on lateral subregions implicated in motor performances [22]. A comparison of clozapine and haloperidol [23] revealed that clozapine specifically regulates transcripts related to presynaptic proteins and that gene expression of BDNF is changed by clozapine but not by haloperidol. Genes encoding presynaptic proteins that are involved in vesicle transport, synaptic docking and the regulation of synaptic activity have been investigated, but independently of each other. Only a few studies have simultaneously examined the impact of FGAs or SGAs on the expression of multiple components of synaptic function. Animals treated with haloperidol had increased cortical SNAP-25 levels compared to non-treated controls [24]. In contrast, in CPU, haloperidol or clozapine showed no effects on SNAP-25A or SNAP-25B protein. In the hippocampus, haloperidol increased SNAP-25 immunoreactivity with strongest effects in CA3 [25]. These results speak for differential effects of antipsychotics in specific brain regions, but comparative studies and information on the gene expression level are lacking.

Thus, studies to date do not provide a great deal of insight into possible mechanisms by which gene expression can be changed, e.g. by feedback activities of the proteins affected by the drugs. Therefore, the present study investigated RNA transcription. We decided to focus on disease-related genes identified in our previous investigations in the post-mortem superior temporal cortex of schizophrenia patients [7] with the aim to broaden the understanding of drug pleiotropy. For this reason, we examined the expression of genes coding for BDNF, collagen type IV (COL1A1) and laminin subunit gamma-3 (LAMC3) and of the genes DCTN6, SNAP-25,

STX1a, STX12, SYP, SYT6 and VAMP2. We chose to study these genes to gain further insight into the altered neurotransmission of their products under the influence of clozapine and haloperidol.

Moreover, the likelihood has largely been neglected that the multiple brain regions affected by schizophrenia, such as the prefrontal cortex, cingulate cortex, striatum and limbic structures (e.g. the hippocampus), may be targeted simultaneously by clozapine and haloperidol. Therefore, the study also aimed to identify drug actions specific for a number of important brain regions known to be involved in the pathophysiology of schizophrenia, i.e. the prelimbic cortex (PrLC), cingulate gyrus (CG1) and caudate putamen (CPU) and the *cornu ammonis 1* (CA1), *cornu ammonis 3* (CA3) and dentate gyrus (DG) of the hippocampus. We studied a variety of brain regions because the up- or downregulation of a particular gene can differ between regions.

Experimental procedures

Animals

All experiments were carried out in accordance with the laws of the local authorities for animal experimentation and approved by the Landesamt für Natur, Umwelt- und Verbraucherschutz NRW, Recklinghausen (Reference number 9.93.2.10.34.07.227). On postnatal day (PD) 21, 30 healthy male pups of Sprague Dawley rats (Taconic, Denmark) were removed from their mothers and maintained on a 12:12 light/dark cycle (lights off at 8 p.m.) at a temperature of 21 °C and 60% humidity until PD 169.

Treatment groups

The animals were fed as described previously [26]. To avoid effects of estrous cycle, we studied only male rats. To exclude any influence of puberty, drug treatments were started at PD 85 and given daily for 12 weeks as chronic medication. The rats were individually housed and their weights determined twice a week (Monday and Thursday) and averaged for a week. In accordance with the studies of Minet-Ringuet et al. [27, 28] and Kapur et al. [29], ten males received 1 mg/kg body weight (BW) haloperidol (Haloneurol®, Hexal, Germany) corresponding to an effective average dose rate of 0.8 ± 0.03 mg/kg BW. Ten males received 20 mg/kg BW clozapine (Leponex®, Novartis, Germany), corresponding to 18.5 ± 0.26 mg/kg BW per day. The drugs were mixed homogenously in a measured quantity of ground pellets, which had the following composition: 19.0% crude protein, 6.0% crude fibre, 7.5% crude ash and 4.0% crude fat, with an additional 15% fat in the dry matter (Altromin

Spezialfutter GmbH, Germany). The control group received only ground pellets.

The mean of food conversion ratio, food conversion efficiency, growth rate, body weight gain, food intake related to 1 kg body weight (relative food intake) and locomotor activity by voluntary wheel running were published previously [26]. Briefly, food conversion and growth rate are unchanged within the groups. Male clozapine- or haloperidol-medicated animals showed significantly decreased weight gain in comparison with controls. Male haloperidol-medicated animals ate significantly less than the control and clozapine-medicated group.

On PD 169 (week 25), 12 h after food had been removed, the animals were anaesthetized by pentobarbital (Narcoren, Merial, Germany). Before brain dissection, blood was collected by aorta puncture in s-monovettes for serum preparation and s-monovettes for haematological testing (Sarstedt, Germany) with following centrifugation at 3000*g* for 10 min.

Determination of blood levels of antipsychotics

Serum levels of clozapine, *N*-desmethylclozapine and haloperidol were quantified by HPLC in the biochemical laboratory of the LVR Klinikum Düsseldorf using equipment from Dionex with the 580 pump and the GINA50 autosampler. For detection, the LC spectrometer Lambda Max 481 (Waters) was used. Drugs were extracted from the blood samples with ethyl acetate by rigorous vortexing for 30 min at room temperature. Prior to extraction, step LY170222 (Lilly) and chlorinated haloperidol analogue (Sigma–Aldrich) were added as internal standards for the quantification of clozapine as well as *N*-desmethylclozapine and for haloperidol, respectively. After ethyl acetate extraction, samples were centrifuged at 2500×*g* for 15 min, the supernatants were collected and evaporated to dryness with a speed vac (SC110A). Residues were dissolved in mobile phase [ammonium acetate (pH 4.5) with 30% acetonitrile]. Separation was performed with a Hypersil CPS (MZ, Germany) combined with Phenomenex security guard cartridges at 36 °C. The flow rate was adjusted to 1 mL/min and the adsorbance was measured at 254 nm. Drug quantification was performed with the Chromeleon software (Dionex) [26].

Tissue preparation

The brains were removed and snap frozen at −80 °C by 2-methylbutane, which was maintained at this temperature by liquid nitrogen. The temperature of 2-methylbutane was monitored by a thermometer and adjusted by removing it from the liquid nitrogen, if required. The frozen brains were then stored at −80 °C. Slices of 60 µm thickness were cut on a cryostat (CM3000, Jung, Germany), and the regions of interest (PrLC, CG1, CPU, CA1, CA3 and DG) were excised by sterile biopsy

punches from the right hemispheres, according to the brain atlas of Paxinos and Watson [30]. Biopsies were made with a 1.0-mm or 1.5-mm plunger (kai Europe GmbH, Germany), depending on the size of the region.

Quantitative real-time PCR (qRT-PCR)

Tissue punches were homogenized in Trizol (Life Technologies, Germany), and RNA was purified and concentrated by RNeasy micro kit (Qiagen, Germany) according to the manufacturer's instructions. The quality of RNA was evaluated by NanoDrop measurements and by the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, USA). Only samples with RIN values > 7.8 were used for further examinations in qRT-PCR. qRT-PCR was performed on a 7900HT Fast Real-Time PCR machine (Life Technologies, Germany) using Power SYBR Green PCR Master Mix (Life Technologies, Germany). All samples were run in duplicates. Primer pairs used for the respective genes and for housekeeping genes are listed in Table 1. ACTB, GAPDH, RPL6 and RPL27 were used as housekeeping genes to normalize Ct values (i.e. cycle number when a defined fluorescence threshold was passed) of genes of interest.

Applied Biosystems' SDS 2.2.2 software was used to analyze the SYBR green fluorescence intensity and to calculate the Ct values. Reactions were normalized by subtracting the geometric average of the Ct values of the endogenous controls from the Ct values of the candidate genes ($\Delta\Delta$Ct).

Statistical analysis

Statistical testing was performed with R statistical software. The data were normally distributed using Shapiro Wilk Test. Therefore, significant gene expression was determined by analysis of variance and Tukey's Honestly Significant Difference (HSD) test on the basis of the difference between the geometric average of the housekeeping genes and gene of interest. Gene expression was normalized to the average value of the control samples for graphical presentation (distribution changes in comparison with control group). p values ≤ 0.05 were considered as statistically significant. Bonferroni adjustments of the type I error probability were not applied, since such adjustments would significantly decrease the power to detect existing mean differences. Because of the explorative study design, the findings presented here are not conclusive for a causal relationship.

Table 1 Primers and housekeeping genes used in quantitative real-time PCR

Gene	Primer forward (5′–3′)	Primer reverse (5′–3′)
BDNF	GCGGCAGATAAAAAGACTGC	GCAGCCTTCCTTCGTGTAAC
COL1A1	CCAGGATTCCAAGGTCAGAA	CCCTGGTTCTCCTTTGATGA
LAMC3	CACATGGATCCTTGCATCAC	TCCAAGTTGTGCTTGTCAGC
DCTN6	TCATTATCGGCGAAGGAAAC	TCCTGCCTACGTACGCTTTT
SNAP25, var. 1–3	CTGGAGGAGATGCAGAGGAG	GATTGGTCCATCCCTTCCT
STX1A	GCCCTCAGTGAGATCGAGAC	CACGTAGTCCACAGCGTGTT
STX12	GCAGGACTCAAGCAAACTCC	TAGGGGCAAGGACCCTAACT
SYP	CAGTGGGTCTTTGCCATCTT	ATCTTGGTAGTGCCCCCTTT
SYT6	CCTATGAGGAGCTGGCTGAC	TTGCTTTGAGATTGCGACAC
VAMP2/1	ATGTGGACAAGGTCCTGGAG	CTTGGCTGCACTTGTTTCAA
Housekeeping genes		
GAPDH	CTCATGACCACAGTCCATGC	TTCAGCTCTGGGATGACCTT
ACTB	GTCGTACCACTGGCATTGTG	TCTCAGCTGTGGTGGTGAAG
RPL6	AAGTTTGTCATCGCCACCTC	GCTTTCTGATCAGCCTTTCG
RPL27	GAATTGACCGCTATCCCAGA	CAGTGCTGGGTCTCTGAACA

Results

Drug concentrations in blood

Drug concentrations in blood were reported as mean + standard error (SEM): 29.4 ± 8.4 ng/ml for haloperidol, $80.4 + 5.6$ ng/ml for clozapine and 69.8 ± 5.5 ng/ml for *N*-desmethylclozapine. These values are within the therapeutic ranges used in humans and confirm that adequately high blood levels of the drugs were achieved [26].

Effects of haloperidol and clozapine on gene regulation

In *cornu ammonis 1* (CA1), haloperidol significantly upregulated STX1A [ANOVA: $F(2, 14) = 10.16$, $p = 0.0036$, Tukey-HSD$_{\text{haloperidol vs control}}$: $p = 0.0133$]. There was no significant difference after clozapine treatment (Fig. 1).

In *cornu ammonis 3* (CA3) haloperidol significantly downregulated the expression of SNAP25 [ANOVA: $F(2, 13) = 7.51$, $p = 0.0103$, Tukey-HSD$_{\text{haloperidol vs control}}$: $p = 0.0134$], while clozapine treatment did not affect the expression of any of the studied genes significantly (Fig. 2).

In the cingulate gyrus (CG1) clozapine significantly downregulated SNAP25 expression [ANOVA: $F(2, 14) = 3.79$, $p = 0.0004$, Tukey-HSD$_{\text{clozapine vs control}}$:

Fig. 1 Haloperidol- vs clozapine-mediated gene regulation in the *cornu ammonis 1* (CA1). * = 0.05, ** = 0.01. The results of the clozapine- and haloperidol-medicated groups are normalized to the controls (uncorrected *p*, Tukey's Honestly Significant Difference test)
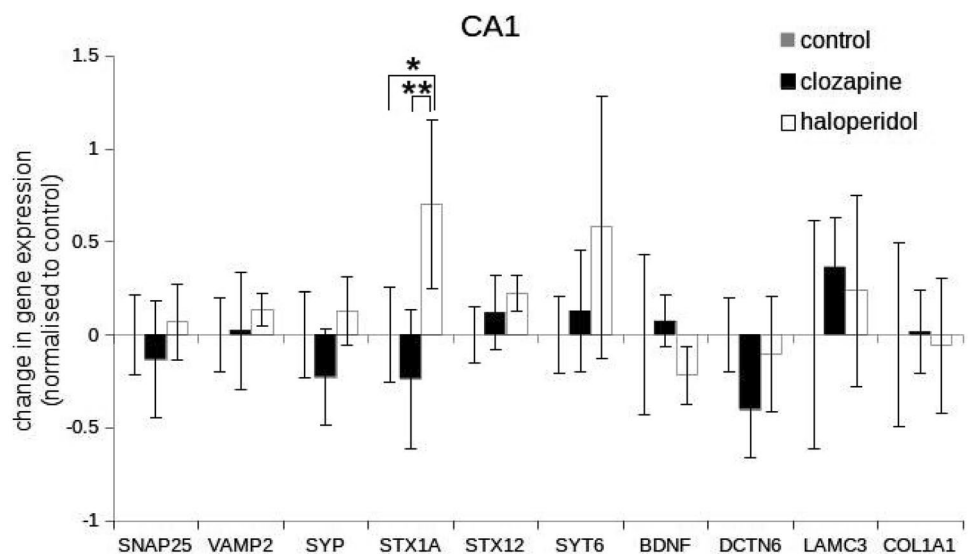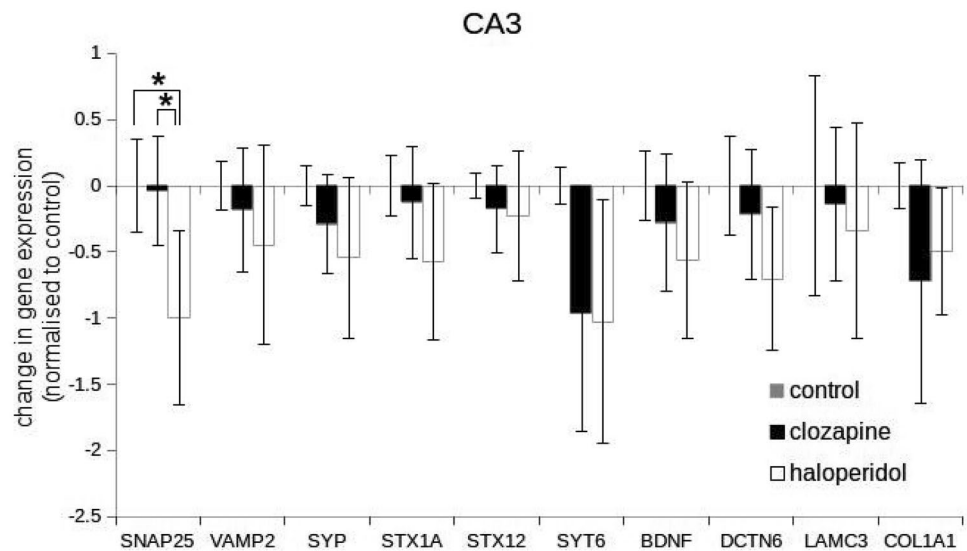
Fig. 2 Haloperidol- vs clozapine-mediated gene regulation in the *cornu ammonis 3* (CA3). *=0.05. The results of the clozapine- and haloperidol-medicated groups are normalized to the controls (uncorrected *p*, Tukey's Honestly Significant Difference test)

$p = 0.0013$] (Fig. 3). From ANOVA, we received a significant gene expression effect for SYP [$F(2,14) = 6.68$, $p = 0.0242$], but following Tukeys HSD tests showed only a significant difference between the haloperidol and the clozapine group ($p = 0.0267$), while haloperidol vs controls was only upregulated on trend level (Tukey-HSD$_{haloperidol \ vs \ control}$: $p = 0.0838$).
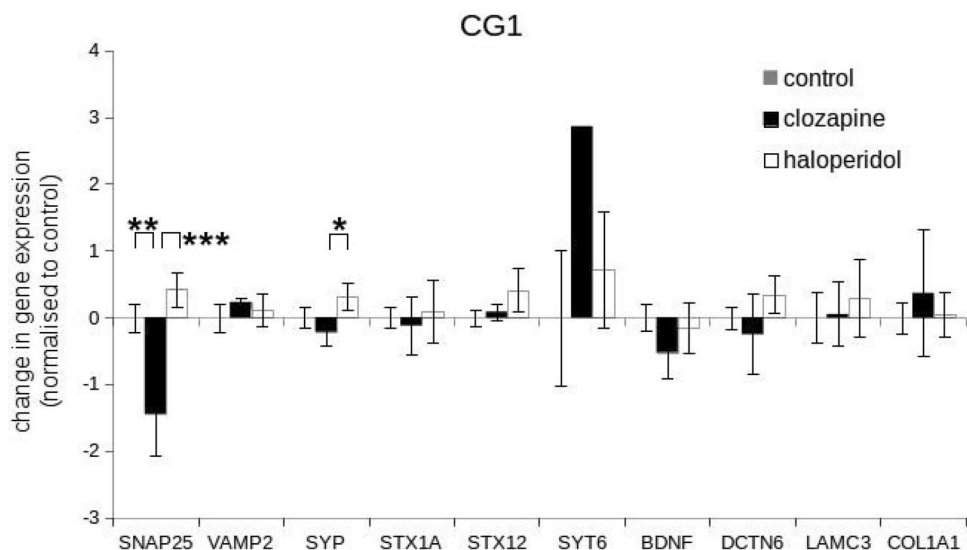
In the dentate gyrus (DG), in the caudate–putamen complex (CPU) and in the prelimbic cortex (PrLC), neither haloperidol nor clozapine affected the expression of any of the studied genes significantly (Supplement Figs. 1–3).

Discussion

Selection of genes of interest

To reveal multi-target effects of the drugs used here, we selected the genes to be studied on the basis of the literature, because of their involvement in synaptic processes which are hypothesized to play a role in schizophrenia [31]. In our previous hypothesis-free post-mortem studies in the temporal cortex of schizophrenia patients, we observed a downregulation of SYT6 and STX12 [7] and another post-mortem study showed that SNAP-25 immunoreactivity was decreased in hippocampal subfields and SYP was increased in the DG of schizophrenia patients [16]. Because all patients in these studies had been treated with antipsychotics for decades, in the present study we aimed to investigate the influence of

Fig. 3 Haloperidol- vs clozapine-mediated gene regulation in the cingulate gyrus (CG). *=0.05, **=0.01, ***=0.001. The results of the clozapine- and haloperidol-medicated groups are normalized to the controls (uncorrected *p*, Tukey's Honestly Significant Difference test)

long-term haloperidol or clozapine treatment on multi-target gene expression of synapse-related genes.

Single effects of the two drugs on expression of genes of interest

Genes of the neurotransmitter release machinery (SNAP-25, STX1A, STX12, SYP, SYT6, VAMP2)

Expression and regulation of these genes is not specific for any neurotransmitter, including dopamine and serotonin. Consequently, any effect of the two drugs may be a priori an effect on the release of any possible neurotransmitter.

Except for SYP, which encodes for synaptophysin, all the other genes encode proteins of the SNARE complexes, which mediate vesicle fusion and exocytosis of synaptic vesicles. These complexes include synaptotagmins, which are located at membranes of the presynaptic axon terminal and act as calcium sensors in the regulation of neurotransmitter release, and synaptobrevin, which mediates Ca^{2+}-mediated fusion of synaptic vesicles [32]. Various genes involved in calcium regulation appear to be regulated by antipsychotics [33].

The present results reveal prominent effects of haloperidol on expression of STX1A (in CA1) and SNAP-25 (in CA3). In general, the effects of haloperidol appear to be stronger than those of clozapine. CA3 is the mossy fibre region and connects the hippocampus to CA1, for example by glutamatergic Schaffer collaterals. In this region, expression of SNAP-25 was downregulated by haloperidol treatment. This is in contrast to findings of increased SNAP-25 expression on the protein level in CA3 after haloperidol treatment [34]. Therefore, it cannot be concluded that downregulation of genes of the synaptic transmitter release machinery are translated into changes at the protein level. However, any expression changes of presynaptic genes could increase the probability of vesicle fusion and neurotransmitter release [35].

Components of the extracellular matrix (collagen IV [COL1A1] and laminin [LAMC3]) and dynactin6 (DCTN6)

The products of these genes serve more basic functions of cell metabolism than any of the other selected genes. As parts of the cytoskeleton, collagens and laminin are the least mobile molecules of the cell. They form supramolecular networks that influence cell adhesion, migration and differentiation [36], have an organizing function in the developing nervous system [37] and are involved in neuronal migration, neurite outgrowth and synaptic function [38]. Moreover, COL1A1 ensures the precise maturation and function of synapses and its disturbed expression leads to severe disorders. Of interest in this context is that downregulation

of collagens has been observed in schizophrenia [7]. However, a specific involvement in the storage and release of neurotransmitters is questionable. Therefore, it comes as no surprise that neither haloperidol nor clozapine showed any appreciable effects on the expression of these genes in the adult brain. In contrast, dynactin 6 is an essential part of the dynactin–dynein complex, a highly active protein association responsible for microtubule-based movements of vesicles and organelles, activities that are required during all periods of life [39]. Moreover, interactions were found between the schizophrenia susceptibility gene dysbindin and the dynactin complex in murine striatum, suggesting that impairment of the vesicle life cycle may be a pathogenic mechanism in schizophrenia [40]. Possibly, these features make dynactin 6 more amenable to actions of haloperidol (in CA3) and clozapine (in CA1).

BDNF

Ample literature has reported on the role of BDNF in schizophrenia. Decreased BDNF concentrations have been reported in cortical and hippocampal areas of post-mortem brains from schizophrenia patients [41]. We included the gene in the present study because influences of higher doses of clozapine and haloperidol have been shown on BDNF in hippocampal regions after chronic but not acute administration [42]. Because none of our findings were significant, we can only speculate that the influence of antipsychotic treatment on BDNF expression depends on the brain region and type of antipsychotic.

In the present study, the effects of clozapine and haloperidol on the genes studied were not necessarily in the same direction. For instance, in CA1 STX1A was upregulated by haloperidol but non-significantly downregulated by clozapine. In CG1 haloperidol upregulated non-significantly SNAP-25 and SYP, whereas clozapine downregulated SNAP-25 significantly. Moreover, clozapine appears to influence fewer genes than haloperidol (cf. Supplement Figs. 4 and 5). This finding, however, is not entirely in line with the literature, which indicates that drugs addressing multiple targets often exert subtler effects on each target but have fewer unwanted adverse effects. For instance, clozapine is known for its absence of debilitating extrapyramidal side effects. Similarly, antidepressants with complex modes of action have been shown to have superior clinical effects to single-action antidepressants [43]. Unfortunately, in studies these effects may not reach statistical significance, as may also have been the case in the present study.

As a limitation, the sample size was small and the present study investigated only ten molecular targets. Therefore, correction for multiple comparisons was not applied. It would be important to extend the number of targets considerably to obtain more insight into the multiple effects of these two

interesting compounds. Moreover, the results show different effects of clozapine or haloperidol medication on the examined genes in the regions of rat brain selected for this investigation. For example, marked effects were observed in CA3, which was the only region of the hippocampus where all genes were downregulated by clozapine or haloperidol. And equally interesting are the brain regions CPU and PrLC, where levels of all genes remained close to those of controls, with particularly small standard deviations in the CPU for all genes except BDNF and LAMC3 (bold horizontal lines at control levels in Supplement Figs. 4 and 5). However, we have to keep in mind that changes in mRNA levels might not always translate into comparable changes in protein levels [35]. Furthermore, rats were fed orally with antipsychotics during single-housing, which, on the one hand, may represent a stress factor for the animals. On the other hand, stress and pain caused by repeated i.p. injections have been avoided in our chronic treatment experiment. Moreover, future studies should show if similar changes are observable not only in healthy rats but also in rat schizophrenia models with associated schizophrenia-like neurotransmitter imbalances.

The differential expression of the genes examined in this study in the brain regions of interest gives rise to speculations about differential responses of the same molecular targets to haloperidol or clozapine, depending on the environment, and also about secondary effects, i.e. that drug effects occurring in one region may trigger changes of gene expression in another region (effects of neuronal projections). For example, regulation of SNARE mRNA expression occurs predominantly in the cell body, which may be located in a different brain region to the synapse. Thus, the effects of haloperidol could lead us to distinguish some interregional connectivity patterns of gene regulation specific for this drug, as outlined in Supplement Fig. 4 (for the proteins of the synaptic release machinery only). Thus, Figs. 4 and 5 (Supplement) are meant to give an idea (in 3D) of region-specific drug effects on the expression of the ten genes studied here. Clearly, the haloperidol-specific pattern of gene expression is distinct from the clozapine-specific one (Supplement Figs. 4 and 5).

Summary

Although it is impossible to distinguish between direct and indirect effects, the results clearly document that the expression of the genes of interest is differentially regulated by haloperidol and clozapine. Therefore, we could extend results from recent studies showing altered immunoreactivity of SNAP-25 in single brain regions. In the next step, effects of antipsychotic treatment in animal models of schizophrenia should be investigated [44] to elucidate relevance to the disease.

Compliance with ethical standards

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