Extended neuroleptic administration affects the NMDAR subunits immunoexpression in the rat diencephalon and neocortex

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Extended neuroleptic administration modulates NMDA-R subunit immunoexpression in the rat neocortex and diencephalon.

Abstract

Background: This study aimed to evaluate the effect of extended olanzapine, clozapine and haloperidol administration on NMDA-R subunit immunoexpression in the rat neocortex and diencephalon**.**

Methods: To explore NR1, NR2A and NR2B subunit protein expression,densytometric analysis of immunohistochemically stained brain slices was performed.

Results: Interestingly, all neuroleptics caused a downregulation of NMDA-R subunit expression in the thalamus but increased the level of NR1 in the hypothalamus. Olanzapine upregulated hypothalamic NR2A expression, while clozapine and haloperidol decreased hypothalamic levels.We observed no significant changes in NR2B immunoreactivity. None of the studied medications had significant influence on NMDA-R subunit expression in the neocortex.

Conclusions: Neuroleptic-induced reduction in the expression of thalamic NMDA-R subunits may play an important role in the regulation of glutamatergic transmission

disorders in cortico–striato–thalamo–cortical loop in schizophrenia. A decrease in NMDA signaling in this region after long-term neuroleptic administration may also cautiously explain the incomplete effectiveness of these drugs in the therapy of schizophrenia-related cognitive disturbances.

Key words: NMDA receptor, neuroleptics, glutamate, neocortex, diencephalon

Introduction

It has been established that during schizophrenia there are changes in NMDA receptor (NMDA-R) subunit composition in various regions of the brain [1-4, Paoletti and Neyton 2007]. However, current results are often inconsistent and our understanding of the influence of neuroleptics on NMDA-R expression remains incomplete. Nevertheless, on the basis of this data we can still try to create a hypothetical model of NMDA-R subunit expression changes in patients suffering from schizophrenia. In this model, NR1 subunit expression decreases in majority of brain structures of schizophrenia patients. Accordingly, these dysfunctions result from an NMDA-R dependent deficit in glutamatergic transmission in certain forebrain areas, such as the prefrontal cortex (PFC) and hippocampus [5-8]. The NMDA receptors indirectly regulate dopamine release in the brain cortex, suggesting that the dopaminergic hypofunction, typical in schizophrenia, is secondary to glutamatergic action [9]. Alternatively, dopaminergic receptors participate in the regulation of NMDA-R expression [10], and therefore, a final consensus on the sequence of signaling events underlying schizophrenia remains open. The ionotropic NMDA-R is

a heterotetramer, composed of one mandatory subunit NR1, and three different subunits: NR2, NR3 and NR4 [11,12]. At present, eight splice variants of subunit NR1, and at least four classes of the subunit NR2: A, B, C and D have been identified. In patients suffering from schizophrenia, NR1 and NR2A subunit expression decreases in dorso-lateral PFC [13, 14]. Transport of NR2B subunits is also disturbed, a lack of which can in turn impair NMDA-R function [15]. In frontal and occipital cortex there is an increase of NR2A subunit expression [16]. On the other hand, in the temporal cortex an increase of the number of NR2B subunits has been observed [17]. NR1 subunit expression is probably diminished in the thalamus [16] with simultaneous increase of the number of NR2B subunits in the dorso-medial area [18]. However, some studies have revealed no abnormalities in the thalamus [17]. In the hippocampus NR1 subunit expression decreases, while formation of NR2B subunits probably increases [19]. Moreover, the number of NMDA-Rs increases in putamen [20]. As the NR1 subunit is present in all NMDA-R, the results concerning putamen can be indirectly interpreted as an overall decrease in the number of NR1 subunits. Similarly, expression of NR1 subunit in substantia nigra is probably decreased in schizophrenia [16]. Described changes of NMDA-R subunit composition can result from disease pathogenesis or from the influence of neuroleptics. Indeed, antipsychotics can change NMDA-R subunit gene expression and lead to modifications of receptor function [21 We previously examined an effect of chronical treatment with haloperidol, clozapine and olanzapine on NMDA-R subunits expression in the rat hippocampus [22]. In the present article we aim to report and discuss the effect of these neuroleptics on NMDA receptor composition in the rat hypothalamus, thalamus and neocortex. It is especially worth mentioning that haloperidol is a classical neuroleptic with a wide spectrum of unfavorable dyskinetic and cardiac side effects (Leucht et al. 2013, Stracina et al. 2015), while olanzapine and clozapine are new generation atypical medications which are more suitable in drug resistant schizophrenia (Wahlbeck et al. 2000, Duggan et al 2003., Essali et al. 2009). In the context of this, comparing potential effects of these drugs on NMDA-R immunoexpression in various brain regions seems to be relatively novel and valid.

Materials and methods

Adult (5 month old, 210-240 grams weight) male Sprague-Dawley rats from Medical University of Silesia Experimental Centre were housed at 22^0C with regular 12/12 light-dark cycle and access to standard Murigran chow and water *ad libitum*.

Four groups of animals (n=6) received either control vehicle, haloperidol (1 mg /kg/day), clozapine (20 mg/kg/day) or olanzapine (10mg/kg/day) by intraperitoneal injection for 4 weeks. Three hours after the last drug administration, rats were anaesthetized with isoflurane and their brains removed and fixed in 4% paraformaldehyde PBS (pH 7.2-7.4). 7 μm thick sections were cut on a microtome (Leica Microsystems, Germany) in the coronal plane (-3.30 to -3.80 from bregma, according to Paxinos & Watson's The Rat Brain in Stereotaxic Coordinates).

Sections were blocked with 5% goat serum and incubated with the following rabbit polyclonal primary antibodies against the NMDA receptor subunits; anti-NR1 (1:1500; AB17345), anti-NR2A (1:500; AB14596) and anti-NR2B (1:400; AB65875) were purchased from Abcam Company, Cambridge, UK.

Primary antibodies were followed by biotynylated goat anti-rabbit secondary antibody, and then visualized via avidin-biotin-horseradish peroxidase complex (Vectastain ABC kit, Vector Labs), and 3,3'-diaminobenzidine. Sections were mounted on gelatin-coated glass slides, dehydrated and coverslipped.

The same level of cortical, thalamic and hypothalamic sections were chosen from each slide, and three standarized areas were studied (Fig 1.). The relatively poorly investigated parietal cortex was examined. The hypothalamic frame contained ventro- and dorsomedial (VMH, DMH) as well as paraventricular nuclei (PVN) and lateral area (LH). In the thalamus we focused on the main laterodorsal and ventrobasal nuclei involved in the crucial sensory processing. The immunoreactivity of NR1, NR2A and NR2B, containing cells in the randomly selected regions, were measured as the integrated optical density (IOD) and a mean ± SEM was calculated. The data were collected and analyzed using Nikon optic systems and software Image ProPlus (Media Cybernetics, USA). Statistical analyses were performed using Kruskall-Wallis and HSD test. Differences were considered statistically significant at $p<0.05$.

Results

Following neuroleptic treatment, changes in rat NMDA-R subunit expression were greatly dependent on the area of rat brain analyzed. In the thalamus each neuroleptic treatment caused a decrease in expression of all three NMDA subunits examined, most notably the general subunit - NR1. Interestingly olanzapine and clozapine caused equivalent reductions in each NMDA subunit (Fig. 2B, Fig 3.). In the hypothalamus all examined neuroleptics increased NR1 expression and also, in the case of olanzapine administration, we observed increased NR2A expression (Fig. 2A and B). However, treatment with clozapine and haloperidol caused reduced NR2A subunit expression in the hypothalamus. No significant changes in NR2B expression were observed in this region (Fig. 2A and B.) In the cortex, drug treatment caused an increased trend in NR1 expression, but the result was not statistically significant. Moreover, none of the studied antipsychotics had significant influence on NR2A and NR2B expressions in the cortex.

Discussion

Collectively, these results suggest that the described neuroleptics inhibit the expression of NMDA receptors in the thalamus, which in turn can decrease the activity of the thalamic glutamatergic system. Alternatively, the NMDA-R subunit expression changes in the hypothalamus suggest an increase of glutamatergic circuit activity in this structure. In our analysis, we focused on the expression of the receptor subunits via protein quantification. Although, this may be a distinct limitation, we

decided not to analyze expression at the mRNA level, given previous studies [23] and since changes at the mRNA level are rather difficult to interpret without specific information related to translation of receptor molecules [14]. For this reason, in the present experiment, we have emphasized a necessity of immunohistochemical assessment of the expression of NMDA receptor protein subunits. Neuroleptic-induced reduction of the expression of thalamic NMDA-R subunits has so far not been described in the literature. This effect may play an important role in the regulation of glutamatergic transmission disorders in cortico–striato–thalamo–cortical loop in the schizophrenia. Decreased expression of the NMDA-R subunits in the thalamus after neuroleptic administration may correspond with our previous results reporting that all studied drugs decreased the number of NMDA receptor molecules in the CA1 and CA2 hippocampal areas [22]. Interestingly, treatment with a potent NMDA-R antagonist phencyclidine (PCP) markedly increased c-fos expression in the rat centromedial and mediodorsal thalamic nuclei as well as in the amygdala (Celada et al. 2013). Clozapine administration completely reversed the elevation in firing rate produced by PCP in thalamic and PFC glutamatergic neurons (Kargieman et al., 2007; Santana et al., 2011). Furthermore, some behavioural alterations after NMDA-R blockers administration in rats are completely abolished by neuroleptics (Carlsson and Carlsson, 1989; Geyer et al., 2001). However, the neuronal pathways involved in these effects remains so far poorly investigated. Our results suggest cautiously, that the effect of neuroleptics might be based on the decrease of glutamatergic activity in the thalamic neurons. This downregulation may be particularly important for understanding the influence of antipsychotics on the cognitive symptoms of schizophrenia. For instance, clozapine has been shown to improve spatial memory in some animal models. The effects of this neuroleptic on delayed spatial alternation deficits in rats with NMDA-induced excitotoxic hippocampal damage was tested. The results obtained suggest that long-term, but not acute, drug administration enables animals with hippocampal dysfunction to develop new spatial learning (Bardgett et al. 2006). It may have potential implications for the treatment of cognitive deficits in schizophrenic patients.

According to the glutamatergic model of schizophrenia, dysfunction of NMDA receptors causes a dysfunctional hyperactivity of glutamatergic pyramidal neurons of the cerebral cortex and an increased glutamatergic transmission [24, 25].

Neuroleptics could potentially limit this hyperactivity of the glutamatergic system by reducing the number of receptors. The increase of NRI expression in the hypothalamus may be related to the blockade of D2 receptors by neuroleptics. Dopaminergic D2 and NMDA receptors remain in a functional antagonistic relationship [26]. Chronic inhibition of D2 receptors by neuroleptics and their upregulation leads to the decreased expression of the NMDA-R [27]. Moreover, a decrease of NR2A subunit expression causes an increase in the proportion of NR2B regulatory subunits, which can also lead to increased activity of the NMDA receptor. [15]. In summary, we found that all of the examined neuroleptics increased glutamatergic activity in the rat hypothalamus. Results from this brain region are in line with results obtained by Riva *et al.* [28], who have demonstrated that a 3 week-long haloperidol and clozapine administration decreased the expression of NR2A subunits in the hypothalamus. It should not be excluded that the effect observed may play an alternative role in the central mechanism of neuroleptic-dependent weight-gain.

The observed trend for an increase in NR1 subunit expression in the sensory cortex is consistent with the findings of Ułas *et al.* [29] who have seen an increase in NR1 expression in the rat parietal cortex after 3 weeks of haloperidol administration. The results of various independent studies have revealed that changes in the composition of NMDA subunits can be specific for particular cortex areas. In some experiments it was observed, that after haloperidol treatment there was a slight increase of NR1 expression in the rat temporal cortex. In the studies performed by Hanaoka *et al.* [30] 2 week-long administration of haloperidol and clozapine leads to a decrease of NR1 and NR2B subunit protein level in the frontal cortex. On the other hand, clozapine administered for 6 months decreases the number of NR1 subunits in the dorsolateral part of the PFC. These above-mentioned neuroleptics cause a decrease of NR2A subunits expression in the rat PFC [31]. Presented changes in NMDA-R subunit expression in different areas of the sensory cortex suggest the regional specificity of the influence of neuroleptics on the NMDA receptor composition. Special attention should be given to the comparison of obtained results with changes of NMDA receptor subunit expression in patients suffering from schizophrenia. Human studies have revealed an increased expression of NR2B subunits in the hippocampus, thalamus and in some parts of the cerebral cortex. This effect is opposite to the one

obtained in animal studies, where an inhibition of NR2B subunit expression was observed after exposure to neuroleptics. The data from experiments on humans suggests that an increase of NR2B subunits expression is related to the pathogenesis of schizophrenia, rather than with exact effects of neuroleptics used in the therapy. It may indicate the importance of the modulating influence of neuroleptics on the NMDA receptor composition and thereby on their activity. An inhibitory effect of neuroleptics on NMDA-R expression as well as the drug-induced reduction of thalamic glutamatergic activity could be important elements of the mechanism of neuroleptic anti-schizophrenic activities. A decreased NR1 phosphorylation was also found in some other brain regions in schizophrenia patients [32]. Mice with mutation in the NR1 molecule exhibit an impairment in social behaviour [16]. However, it seems that the specific activity of the NMDA-R has mostly been determined via the analysis of the subunit NR2 [6,33]. Varied expression of the alternative subunit classes could determine a level of receptor sensitivity to different antipsychotic actions. Haloperidol, clozapine and olanzapine, are dopaminergic receptor antagonists and despite their lack of affinity to the NMDA-R, present a significant increase in glutamatergic transmission [14]. Some studies suggested that the blockage of the dopamine receptors D_2 and D_4 by haloperidol, cause an increase in phosphorylation of the NR1 subunit via protein kinase, leading to sensitization of the glutamate receptor [28]. However, alternative studies, demonstrate no influence of haloperidol on NMDA-R activity in the rat PFC [30]. Conversely, it has also been reported that atypical antipsychotic drugs enhance glutamatergic activity in the PFC [31]. In turn, clozapine, may also indirectly influence the activity of the glutamatergic system, by inhibiting glutamate reuptake, through a decreased expression of the glycine transporters, EAAT 3 and EAAT 2, in neurons and astrocytes. Perhaps, the observed dynamic changes in composition of NMDA-R subunits could explain differences in the spectrum of cognitive process modification in schizophrenic patients, treated with classical and atypical antipsychotic drugs [26]. A dysfunction of PFC has been associated with the augmented response to stress observed in schizophrenic patients. Interestingly, a recent report suggests that the hypofunction of prefrontal NMDA-Rs does not affect the sensitivity to acute stress of dopamine and noradrenaline projections to amygdala but impairs the acquisition of aversive memory (Del Arco et al. 2015). On the other hand, it is known that schizophrenic patients often show abnormal affective multisensory integration and an emotion

recognition impairment (Van den Stock et al. 2011). We can not exclude that these disturbances may be associated with some deficits in glutamatergic signaling in the limbic structures.

It has been also proposed that NMDA-Rs deficits in the prefronto-thalamo-cerebellar circuit may play a role in the pathogenesis of schizophrenia (Andreasen et al. 1999, Yeganeh-Doost et al. 2011). A study by Schmitt et al. (2010) reported that clozapine may be superior to haloperidol in restoring a deficit in NMDA-R subunit NR2C expression in the cerebellum of rats chronically treated with these neuroleptics. Noteworthy, there was no significant difference in the gene expression of NR1, NR2A, NR2B, NR2C and NR2D subunits or NMDA receptor binding between control animals and groups receiving drug treatment. In the current study we have investigated whether chronic administration of haloperidol, clozapine and olanzapine affects the immunoexpression of the NR1, NR2A and NR2B subunits in the rat hypothalamus, thalamus and PFC. We postulate that the possible changes in NMDA-R expression among the examined neuroleptics may contribute to their different pharmacological effects, especially in the context of cognitive dysfunction therapy.

Conclusions

Our results support a hypothesis that the neuroleptic-dependent changes in expression of NMDA receptor subunits in the rat thalamus, may theoretically and cautiously explain the incomplete effectiveness of these medications in the therapy of cognitive dysfunctions in schizophrenia. On the other hand, the increase of hypothalamic glutamatergic activity may be related to common neuroleptic side effects such as body mass increase.

Conflict of interest

Authors declare no conflict of interest

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Fig 1. Standarized frames used for the integrated optical density (IOD) measurement in the examined brain regions after immunohistochemical procedure. Nissl staining. Scale bar: 500 μm.

Fig 2. Representative expression of NR1, NR2A and NR2B in the hypothalamus of control and indicated neuroleptic treated animals. The immunopositive perikarya and dendrites of multipolar neurons are visible (A). Scale bars: 100 μm. Changes in the expression of NR1, NR2A and NR2B subunits in rat hypothalamus and thalamus (B.). Results are based on the mean values of integrated optical density (IOD), and expressed as percentages of control (100%) for n=6 rats. OLZ – olanzapine, KLO – clozapine, HAL – haloperidol. *p<0,05, **p<0,01.

Fig 3. The NMDA-Rs expressing cells in the thalamus. Immunopositive perikarya and processes of multipolar neurons are visible. Scale bars: 100 μm