Effects of TCB-2 and DOI on Serotonin (5HT2A) - Dopamine (D2) Heteroreceptor Complexes in the Hippocampus of the Rat Brain and Their Relevance for Schizophrenia

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ABSTRACT

As schizophrenia is a mental illness that affects tens of millions of people worldwide, it is of uttermost importance to find more effective treatments than are today available for this disease. In examining the complex of the 5-HT2A-D2 heteroreceptor and its presence in the pyramidal cell layer of the hippocampus with the use of the *In situ* Proximity Ligation Assay (PLA), an increase of the complex could be observed in the CA1 region of the hippocampus when induced with a hallucinogenic agonist, as opposed to the use of a regular agonist. This suggests that this area may be one of great importance to further investigate as it could prove to be an area from which certain schizophrenic symptoms derive.

INTRODUCTION

As one of the most prevalent mental disorders of our time that affects over 21 million people worldwide (1), schizophrenia has been the target of treatment for many years. Characterized by various positive, negative and cognitive symptoms, it has been proven that patients diagnosed with schizophrenia have a life expectancy around 15-20 years lower than the rest of the population (2). One major reason to the decreased life expectancy is the abnormally high rate of suicide among patients with schizophrenia, with the attempted suicide rate estimated to be 20-40% (3), and the successful suicide rate for people with new-onset schizophrenia being around 10-13% (4, 5) and the population of schizophrenia patients in total of 4.9% (4-6).

In recent years, allosteric receptor-receptor interactions (RRIs), with a focus on G-protein coupled receptor (GPCR) heteroreceptor complexes, have been targets for drug development to treat schizophrenia (7, 8). In this study the serotonin receptor (5-HT2AR) and the dopamine receptor (D2R) are investigated in their potential interactions in the pyramidal cell layer of the hippocampus to see if that could be an area worth further exploring in the search of new treatments for schizophrenia. Through studies of the patterns of regional cerebral blood flow (rCBF), it is known that patients with schizophrenia experience hyperactivity in the hippocampus, most probably due to excessive amounts of dopamine (9). It is already known that an increase of the D2R enhances the symptoms of schizophrenia (8, 9). Many of today's antipsychotics are therefore types of D2R antagonists. As for the serotonin receptor 5-HT2A, it is one of the many 5-HT subtypes (8). In combination with the D2R, hallucinogenic 5-HT2AR agonists, though not standard 5-HT2AR agonists, have been proven to act as enhancements to the former receptor protomer (8, 10). Another antagonist of the 5-HT2A receptors, however, is known to block this action (10). With the hallucinogenic 5-HT2A receptor agonists, the heteroreceptor complex 5-HT2A-D2 can increase D2R protomer recognition

and signaling, thus causing more severe symptoms of schizophrenia (11). In this study, we have examined how the hallucinogenic compound DOI, 2,5-dimethoxy-4-iodoamphetamine, interacts with the 5-HT2A-D2 heteroreceptor complex and compared that to the use of a non-hallucinogenic compound, TCB-2. Because of this, we could look at the idea of schizophrenia being triggered by the use of hallucinogenic agonists, and not regular agonists, along with how this change in the different studied areas of the brain. We also looked at if the occurrence of the 5-HT2A-D2 heteroreceptor complex had a homogenous distribution in the whole of the pyramidal cell layer in the hippocampus. Through this, an evaluation could be made of what the most valuable regions to further investigate to find new treatments for schizophrenia are. By finding a treatment that inhibits the D2Rs through the 5-HT2ARs, many of the side-effects of the first generation antipsychotics, which target only the D2Rs, and that can cause various side effects, such as parkinsonism and tardive dyskinesia, could be avoided (12).

MATERIALS AND METHODS

Animals. All experiments were performed using male Sprague-Dawley rats (SD) (Scanbur, Sweden). The animals were group-housed under standard laboratory conditions (20-22 °C, 50-60% humidity). Food and water were available *ad libitum.* All studies involving animals were performed in accordance with the Stockholm North Committee on Ethics of Animal Experimentation, the Swedish National Board for Laboratory Animal and European Communities Council Directive (2010/63/EU) guidelines for accommodation and care of Laboratory Animals.

Brain Tissue Samples and their Preparation. The animals were anesthetized by an intraperitoneal (i.p.) injection of pentobarbital (60 mg/ml, [0.1 ml/100 g]) followed by being perfused intracardially with 30-50 ml ice-cold 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS, pH 7.4) solution. The brains were collected and transferred into well-labeled glass vials filled with 4% PFA fixative solution for 6-12 h. The brains were subsequently placed in 10% and 30% sucrose (0.1 M PBS, pH 7.4) and incubated for 1 day (10% sucrose) at 4 °C with several sucrose buffer changes, until freezing the brain. The brains were frozen with isopentane and then sectioned (10-30 μm-thick) using a cryostat. The brain's slices were stored at -20 °C in Hoffman solution.

In Situ Proximity Ligation Assay (PLA). This study utilised the well-established *in situ* Proximity Ligation Assay (13–16) to be able to detect the protein-protein interactions. Free-floating formalin-fixed brains sections (30 μm) at Bregma level (-3.6 mm) from acute treated (TCB2: DOI) Sprague-Dawley rats were employed using the following primary antibodies: mouse monoclonal anti-D2R (MABN53, 1:600, Millipore, Sweden) and rabbit monoclonal anti-5-HT2A (SAB4301791, 1 μg/ml, Sigma-Aldrich, Sweden). The PLA signal was visualized and quantified using a Leica TCS-SL confocal microscope (Leica) and the Duolink ImageTool software. Fixed free-floating rat brain sections (stored at -20 °C in Hoffman solution) were washed 3 times, for 5 minutes each, with PBS and quenched with 10 mM glycine buffer for 20 minutes at room temperature (RT). The sections were once again washed with PBS, 2x5 min at RT. With a permeabilization buffer (10% fetal bovine serum FBS and 0.3% Triton-X-100 or Tween 20 in Tris buffer saline (TBS), pH 7.4) for 30 minutes at RT. Again, the sections were washed twice, 5 min each, with PBS at RT and incubated with blocking buffer (0.2% BSA in PBS) for 15 min at RT. The brain sections were then incubated with the primary antibodies diluted in a suitable concentration in the blocking solution for 1-2 h at 37 °C or at 4 °C overnight. When done, the sections were

washed twice with the same procedure as earlier and the proximity probe mixture (1:7 in 0.2% BSA - 5% goat serum in PBS (0.5 µl PLA probe MINUS stock, 0.5 µl PLA probe PLUS stock in 40 µl)) was applied to the samples and incubated for 2 h at 37 °C in a pre-heated humidity chamber. The unbound proximity probes were removed by washing the slides twice, 5 min each, with PBS at RT under gentle agitation, and the sections were incubated with the hybridization-ligation solution(BSA (250 g/ml), T4 DNA ligase (final concentration, 0.05 U/µl), Tween 20 (0.05%), NaCl (250mM), adenosine 5´-triphosphate (1 mM), and the circularization connector oligonucleotides (125-250 nM)) and incubated in a humidity chamber at 37 °C for 1 h. The excess of connector oligonucleotides was removed by washing twice, 2 min each, with Wash Buffer A (Sigma-Aldrich; Duolink Buffer A (8.8 g of NaCl, 1.2 g of Tris base, and 0.5 ml of Tween 20 dissolved in 800 ml of high-purity water at pH 7.4)) at RT under gentle agitation, and the rolling circle amplification mixture was added to the slices and incubated in a humidity chamber for 90 min at 37 °C. The sections were then washed, 2x10 min, with Wash Buffer B (Sigma-Aldrich; Duolink Buffer B (5.84 g of NaCl, 4.24 g of Tris base, 26.0 g of Tris-HCl dissolved in 500 ml of high-purity water at pH 7.5)) at RT under gentle agitation. The free-floating sections were put on microscope slides and a drop of appropriate mounting medium (e.g., VectaShield or Dako) was applied. The coverslip was placed on the section and sealed with nail polish. The sections were protected against light and stored for several days at -20 °C before confocal microscope analysis.

Statistical Analysis. The number of samples (*n*) under each experimental condition is indicated in figure legends. Data from competition experiments were analysed by nonlinear regression analysis using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). Group differences after two ANOVAs and non-parametric student t-test were measured. A *P* value of 0.05 and lower was considered significant.

RESULTS

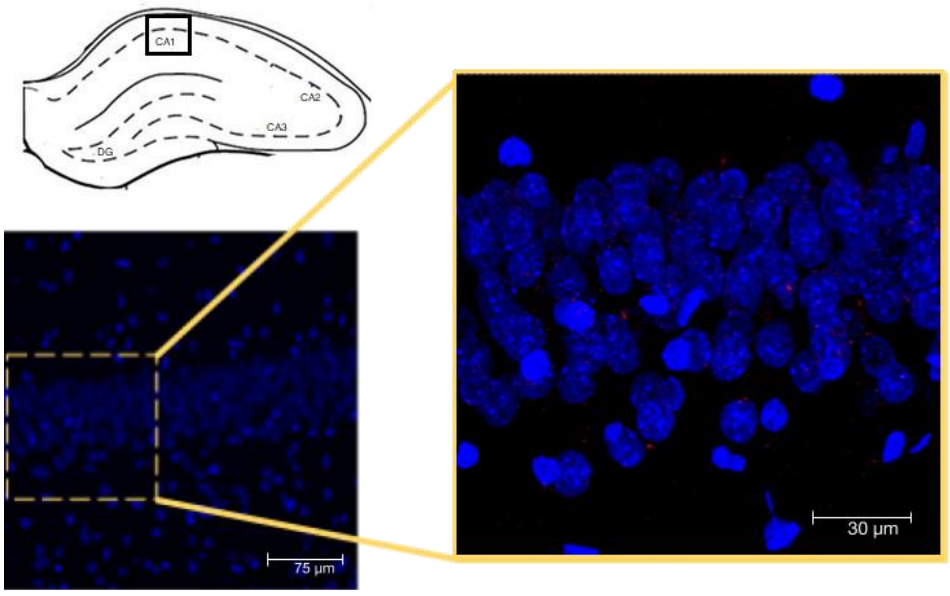


Figure 1.
A representative sample showing the positive *In situ* PLA in the in the *comus amonis 1* (CA1) region of the pyramidal cell layer in the hippocampus of the TCB-2 treated SD rats. In the photo, the nuclei of the neurons are colored in blue whereas the heteroreceptor complexes of the 5-HT2A-D2 reeptor are in shown in red.

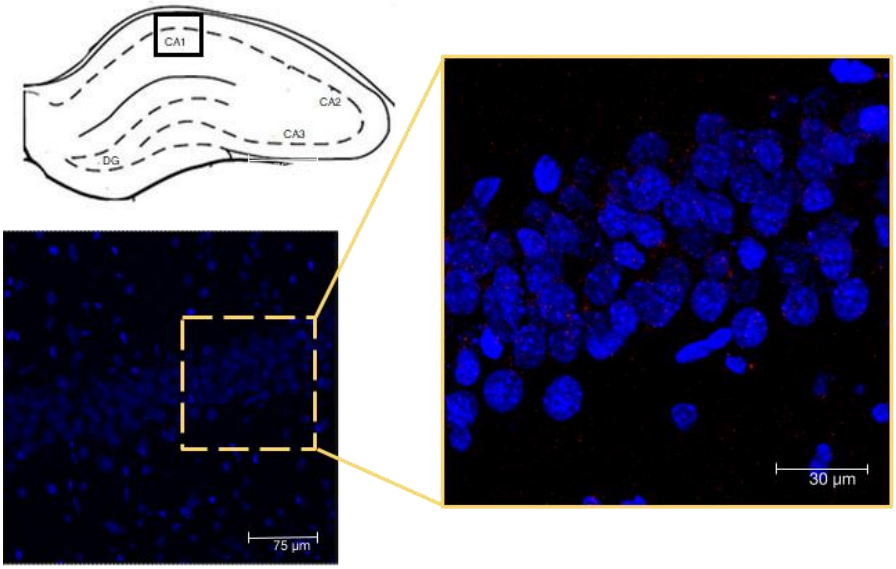
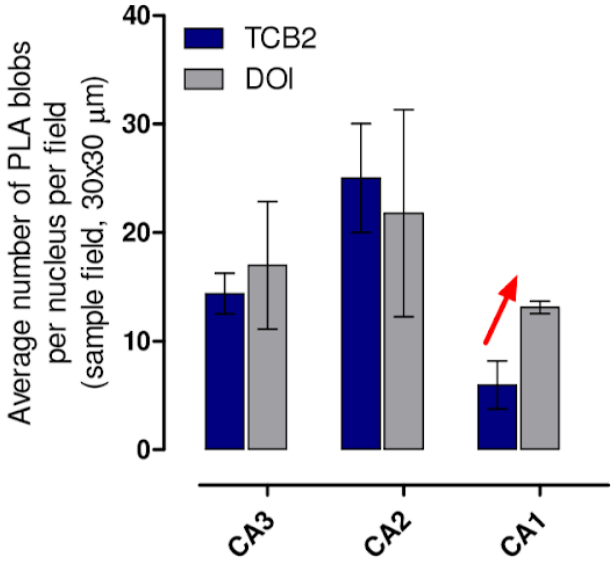


Figure 2.
A representative sample showing the positive *In situ* PLA 5-HT2A-D2 heteroreceptor complex in the *comus amonis 1* (CA1) region of the pyramidal cell layer in the hippocampus of the DOI treated SD rats. The photo shows the nuclei of the neurons in blue and the heteroreceptor complexes of the 5-HT2A-D2 reeptor in red.

Figure 3.
An illustration showing the average number of PLA blobs per nucleus per field in the *comus amonis 1, 2 and 3* (CA1, CA2 and CA3) in the pyramidal cell layer of the hippocampus of the SD rats that were TCB-2 or DOI treated, respectively. Here, non-conclusive results can be seen in the CA3 and the CA2 regions as the deviations overlap for the greatest part. For the CA1 region, however, a clear trend in the increase of the PLA blobs could be seen in the DOI treated brains in the CA1 region. The areas measured were those for which *figure 1* and *figure 2* stand as representatives. Though not of statistically significant values, the number of average PLA blobs in the CA1 region indicates that this is an area in which the heteroreceptor complex of the 5-HT2A-D2 receptor interacts more with the hallucinogenic agonist compared to that of the standard agonist, as opposed to the CA2 region or the CA3 region.



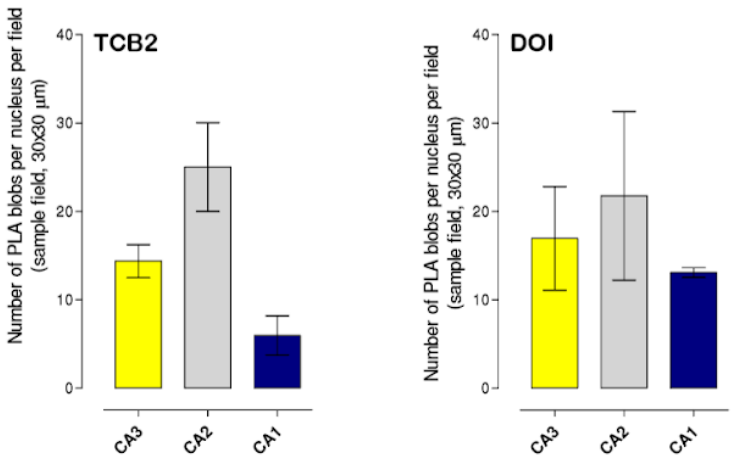


Figure 4 and 5.
A comparison of the number of PLA blobs per nucleus per field in the *comus amonis 1, 2 and 3* (CA1, CA2 and CA3) in the pyramidal cell layer of the hippocampus of the SD rats that were TCB-2 or DOI treated respectively. An higher quantity of the positive PLA blobs could be observed in both the TCB-2 treated and the DOI treated brains, however, in the TCB-2 treated brains, there is a larger distinction between the different examined areas than can be seen in the DOI treated brains.

As the study involved only 2 hallucinogenic treated and 2 control SD rats, no statistically significant results could be drawn from the collected data in the experiments. When looking at the *comus amonis 1* (CA1) region of the hippocampus, however, what was seen was a trend of an increase of the 5-HT2A-D2 heteroreceptor complex in the DOI treated SD rats as compared to that of the TCB-2 treated SD rats (figure 1 and 2). This suggested the DOI induced increase of the 5-HT2A-D2 heteroreceptor pyramidal cell layer of the hippocampus.

The frequency of the receptor complexes was compared and showed that the 5-HT2A-D2 heteroreceptor complexes were not homogeneously distributed along the pyramidal cell layer of the hippocampus in the rat brain. Both in the TCB-2 treated and the DOI treated brains, the complexes were of more prominent occurrence in the CA2 region. Also, in the TCB-2 treated brains, the CA1 region inhibited a considerably lower content of positive 5-HT2A-D2 heteroreceptor complexes than what could be observed in the other regions of the same samples.

DISCUSSION

The DOI treated rats showed an increased expression of the 5-HT2A-D2 heteroreceptor complexes over the non-treated. This trend, however, could solely be observed in the CA1 region of the pyramidal cell layer of the hippocampus in the rat brain. It can therefore be assumed that this is an area that can be a potential target for future drug development.

The results could be seen as inconclusive for the majority of the studied areas, though, as in both the CA2 and CA3 regions, the expression of a difference between the agonists could not be recognized. This can, nonetheless, be seen as an indication of the CA1 region, where the expression of interaction of the target receptor complex and the DOI was much higher than that of the TCB-2, being an area of the hippocampus more sensitive to the change of hallucinogenic vs. regular agonists. This, in turn, can lead to the symptoms of schizophrenia, and could therefore be a more promising area for further research.

The change in the distribution of the 5-HT2A-D2 heteroreceptor complex could also be observed, both when examining the hallucinogenic treated and the control brains. The CA2 region was a region particularly dense in the examined complexes as both the DOI and the TCB-2 showed the highest number of PLA blobs per nucleus per field in this area. In the DOI treated brains, however, the heteroreceptor complexes seem to be more levelled than in the TCB-2 treated. This may indicate a stronger interaction between the different regions when induced with a hallucinogenic agonist compared to that of a normal agonist.

More research is needed, however, as schizophrenia is a complex disease that does not solely affecting one area of the brain and is caused by multiple receptor complex dysfunctions, though mainly different subtypes of serotonin receptors and dopamine receptors. Schizophrenia is a disease that affects tens of millions of people all around the world, and harvests the lives of many, and as such, the idea of hallucinogenic agonists reacting more frequently, and with a higher affinity, than regular agonists with the 5-HT2A-D2 heteroreceptor complex in the CA1 region of the hippocampus show great potential for further studies in developing more effective treatments than is today available for this mental illness.

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