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Review

Mechanism of action of clozapine in the context of dopamine D_1-D_2 receptor hetero-dimerization – a working hypothesis

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Abstract:
The tight correlation between the clinical potency and the D_2R blocking action of antipsychotic medications suggests that dopamine hyperactivity plays a significant role in psychosis. Clozapine, one of the most effective antipsychotic drugs, has been shown to display moderate affinity for various neurotransmitter receptors, including the dopamine D_1 and D_2 receptors; however, the exact mechanism of action of clozapine has not yet been fully elucidated. Here, we describe our working hypothesis pointing to the role of dopamine D_1-D_2 receptor hetero-dimerization as a mechanism of action of clozapine. It has been widely assumed that D_1 and D_2 receptors are segregated to separate neuronal populations; however, other data suggest that D_1 and D_2 receptors are co-expressed by a moderate to substantial proportion of striatal neurons, as well as in the medial prefrontal cortex. Our recent studies indicate that concomitant stimulation of both D_1 and D_2 dopamine receptors induces an increase in their hetero-dimerization. In order to confirm the working hypothesis that clozapine influences D_1-D_2 receptor oligomerization, we employed fluorescence resonance energy transfer (FRET) technology, using fluorescently tagged dopamine receptors and fluorescence lifetime microscopy of intact living cells. The effect of clozapine on D_1R-D_2R hetero-oligomerization was strongly dependent on the drug concentration; the lower concentration, which resulted in binding to the high affinity sites, decreased the transfer efficiency, while the higher concentration of clozapine enhanced transfer efficiency. Further investigation confirmed the idea that high affinity binding sites exist when the receptor is coupled with G protein, and also that clozapine attenuates the hetero-oligomerization of a high affinity pool of dopamine D_1-D_2 receptors. The results discussed in the present study, showing the effect of clozapine on D_1-D_2 receptor hetero-oligomerization, together with the data pointing to the importance of receptors forming hetero-oligomers as a novel level for pharmacological intervention help to increase the understanding of the molecular mechanism of action of antipsychotic drugs.

Key words:
dopamine receptors, hetero-dimerization, FRET, clozapine, schizophrenia

Introduction

The idea that various neurotransmitter receptors form dimers or higher order oligomers has been well documented [49]. This phenomenon most likely provides an additional level of fine tuning for intercellular signaling. The data recently provided by Gonzalez-Maeso et al. [23] strongly suggest that receptor hetero-di-

merization plays an important role in the mechanism of action of psychotropic drugs, as well as in the etiopathogenesis of psychosis. These authors have shown that serotonin 5-HT_{2A} receptors form functional complexes with metabotropic GluR2 in brain cortex, which trigger unique cellular responses when targeted by hallucinogenic drugs. Convincing experimental data point to the importance of mutual regulation of these two receptors, the malfunction of which may be responsible for the altered cortical processes observed in schizophrenia.

Consequences of dopamine D_1-D_2 receptor interaction

Although it has been suggested that the role of dopamine may be only secondary to alterations in the glutamate system [50], schizophrenia is associated with increased dopamine releasability [9, 31]. The tight correlation between the clinical potency and the D_2R blocking action of the antipsychotic medications suggests that dopamine hyperactivity plays a significant role in psychosis. The pathophysiology of other psychological and cognitive abnormalities in schizophrenia remains unclear; however, dysregulation of dopamine signaling is one of the best explanations for the psychotic episodes [44].

Under the condition of increased dopamine releasability, both dopamine receptors are most likely stimulated. Our recent studies [17] indicate that such concomitant stimulation of both dopamine receptors induces an increase in their hetero-dimerization. It has been shown with the use of an *in vitro* model, however, that one might expect that a similar situation takes place in the membranes of neuronal cells co-expressing both receptors.

It has been widely assumed that D_1 and D_2 receptors are segregated to separate neuronal populations [21]; however, other data suggest that D_1 and D_2 receptors are co-expressed by a moderate to substantial proportion of striatal neurons [1, 48]. Earlier studies by Vincent et al. [52] have also shown that the laminar distribution of medial prefrontal cortex neurons expressing both D_1 and D_2 receptors was similar to that of the mesocortical dopamine afferents. This observation might be important given the role of the prefrontal cortex in the pathophysiology of schizophrenia, especially since this brain region has been commonly associated with deficits in the working memory of schizophrenic patients [22].

Cyclic AMP has long been recognized as a principal effector in dopamine-mediated signaling; however, as has been shown by Lee et al. [32] and Rashid et al. [41], concomitant activation of D_1 and D_2 receptors leads to the recruitment of a signaling pathway involving phospholipase C-mediated calcium mobilization, which is different from the intracellular responses observed after stimulation of either receptor alone. It has also recently been shown that two so-called dopamine receptor-interacting proteins, calcyon and neuronal calcium sensor-1 (NCS-1), are up-regulated in the dorsolateral prefrontal cortex of schizophrenic patients [4, 29]. Therefore, it may be assumed that the elevated level of these calcium binding proteins might result from an increased, concomitant stimulation of both dopamine receptors, which then tend to form hetero-dimers coupled to phospholipase C. Indeed, it has been found that dopamine is capable of inducing much stronger increases in phosphoinositide hydrolysis in the prefrontal cortex of patients with schizophrenia than in control tissue [25].

In our recent studies we have shown that clozapine is able to uncouple the D_1-D_2 receptor hetero-dimers [18]. This is especially interesting because clozapine, an atypical antipsychotic drug, seems to be superior to conventional antipsychotics in alleviating both positive and negative symptoms of schizophrenia [8, 26]. Also, in contrast to classic antipsychotics, clozapine does not produce extrapyramidal syndrome or elevate prolactin levels in plasma [5]. Despite numerous studies, the precise mechanism of action of clozapine is still not clear.

Intrinsic activity of clozapine

Clozapine has been shown to display moderate affinity for various neurotransmitter receptors [10, 45], including dopamine D_1 and D_2 receptors [51], but the issue of clozapine affinity for dopamine D_1 and D_2 receptors is still open. The consensus concerning this problem has not been reached, despite various experimental approaches employed so far [2, 3, 36, 38]. In functional tests, clozapine, similar to haloperidol, exhibited pure antagonistic properties [6, 36], although it has been shown that unlike classic antipsychotics chronic administration of clozapine does not increase

the density of striatal dopamine D_2 receptors [42]. This finding has led to the conclusion that clozapine is a weak D_2 receptor antagonist. Additionally, inverse agonism of atypical antipsychotics, including clozapine, at the D_2 receptor has been also postulated [2, 7, 53]. An interesting so-called "loose-binding" hypothesis recently put forward states that clozapine works by intermittently blocking D_2R [27]. Additionally, it has also been suggested by studies based on the mathematical modeling of receptor occupancy at the synapse that clozapine might block the single spikes of neurons at the D_2R but spare the spike bursts that mediate movement, cognition and affect [40].

In a number of *in vivo* assays, clozapine has some preferential although not selective action to antagonize D_1 receptor-mediated functions [37]. However, D_1 antagonism by itself has not been an effective antipsychotic principle; selective D_1 antagonists SCH 23390, SCH 39166 and NNC 01-0687 by themselves were ineffective as antipsychotics [38]. On the other hand, additional reports indicate that clozapine behaves as a D_1 agonist; clozapine-induced hypothermia in rats was fully antagonized by SCH 23390 or NNC 01-0687 [3, 39], which is especially interesting in the context of observations implicating D_1 receptor agonism in the prefrontal cortex in cognitive function [19]. The inverse agonism of clozapine towards the D_1 receptor has also been postulated [11].

Recent PET studies have shown that D_1 receptor occupancy with clozapine in the primate brain was higher in the frontal cortex than in the striatum after administration of clinically relevant doses of the drug [13]. A regional selectivity of clozapine action has already been suggested, based on the pharmacological studies showing down-regulation of D_1-like dopamine receptors in the frontal cortex but not in the striatum after chronic administration of the drug [33, 34]. Another interpretation of these differences was that endogenous dopamine might have competed with radioligand binding. Similar arguments are being frequently provided for the interpretation of the binding of antipsychotic drugs, including clozapine, to dopamine D_2 receptors [14, 43, 46].

In vitro studies

Because of the problem of endogenous dopamine interference with specific ligand binding, we find cell model systems, which are devoid of endogenous dopamine, to be particularly useful for studying the affinity of compounds for dopamine receptors. The results recently obtained with the use of an *in vitro* model system, HEK 293 cells transiently transfected with plasmids encoding dopamine receptor proteins [18], indicated that there are two binding sites for clozapine at both dopamine receptors studied (in contrast to haloperidol, for which a single binding site has been detected, both at the D_1 as well as the D_2 dopamine receptors). The most intriguing results provided by these binding studies revealed that significantly higher D_1R binding-site affinity for clozapine could be observed upon concomitant expression of that receptor together with dopamine D_2R, while co-expression of both receptors did not affect the affinity of clozapine for D_2R.

Since dopamine D_1R displays differential sensitivity to clozapine depending on whether it is present alone in the cell membrane or together with another receptor (D_2R), one can assume that dopamine D_1-D_2 receptor hetero-oligomerization most likely plays a role in the observed phenomenon. Additionally, it has recently been shown by us as well as by others [17, 47] that these two dopamine receptors can indeed form hetero-oligomers constitutively, and this process is enhanced by specific agonists [17]. Physical contact of dopamine receptors seems to influence their affinity for ligands.

$D_2^{Ser311Cys}$ receptor mutant

Interesting results were also obtained from binding studies using a D_2R mutant, $D_2^{Ser311Cys}$. While there is some evidence that naturally occurring polymorphisms in dopamine receptor genes [20] can affect susceptibility to schizophrenia and an individual patients' response to therapeutic agents [28, 30, 54], relatively little work has been devoted to the study of the pharmacological function of these polymorphisms. The *Ser311Cys* mutation was shown to decrease the inhibition of cAMP synthesis [15], and examination of the affinity of clozapine for $D_2^{Ser311Cys}$ revealed that only a single low affinity binding site was detected when this receptor was expressed alone. However, upon co-expression of $D_2^{Ser311Cys}$ with D_1R, two binding sites for clozapine, a low and high affin-

ity site, were observed. This indicates some kind of allosteric modification, most likely exerted by D_1R on the $D_2^{Ser311Cys}$ receptor variant. Since $D_2^{Ser311Cys}$ does not show the high affinity binding site for clozapine by itself, according to the ternary complex theory this indicates that the mutant is uncoupled from the G protein. Co-expression with D_1R brings the high affinity site back; therefore, it may be concluded that the $D_2^{Ser311Cys}$-G protein ternary complex is restored by that interaction. Alteration of the K_i value for the high affinity clozapine binding site at D_1R was also observed when D_1R was co-expressed with $D_2^{Ser311Cys}$ as well as with D_2R. Therefore, one might conclude that allosteric modification works also in another direction; the presence of D_2R (and its genetic variant) modifies the affinity with which the D_1R recognizes clozapine.

It has been widely assumed that the high affinity state of the receptor exists when the receptor is associated with G protein and the agonist binds to this high-affinity state to form a ternary complex [16], while the low affinity state occurs when the G protein is not associated with the receptor. Chidiac et al. [12] have proposed an alternative model attempting to explain the high-affinity state of the receptor – the so called "cooperativity model", which proposes that the receptor cooperates with other receptors to form a dimer, or a larger oligomer. The receptor is in the high-affinity state when it is vacant (unoccupied by the agonists). When the agonist binds to the vacant receptor, the occupied receptor interacts or "cooperates" with the other receptors in such a way that the affinity of the other receptors for the agonist is markedly reduced [12]. According to this model, the reduced affinity for the agonist is a result of a "negative cooperativity" between the receptors, and corresponds to the receptor's low affinity state. With the results previously obtained [18] we propose that the "cooperativity" does not have to be "negative", and instead involves high affinity states of receptors. That means that for allosteric interactions, not only are the receptor protein partners necessary, the G proteins must be present as well.

The $D_2^{Ser311Cys}$ receptor mutant illustrates this thesis the best. Co-expression of that receptor variant with D_1R not only induced changes in the affinity of D_1R for clozapine (however, less pronounced than the changes induced by D_2R), upon co-expression the high affinity pool of $D_2^{Ser311Cys}$ also started to become apparent. Therefore, one may conclude that the affinity state of a given receptor is finely tuned; namely, it depends not only on coupling with the G protein but also on the other interacting protein partners.

Additionally, the observations of the altered affinity of clozapine for the $D_2^{Ser311Cys}$ receptor variant and its influence on the affinity of D_1R while these two receptors are co-expressed might have a great impact on understanding the links between this genetic polymorphism, the vulnerability to schizophrenia and the differential sensitivity for the applied therapy.

Clozapine and D_1-D_2 receptor oligomerization

In order to confirm the working hypothesis that clozapine indeed influences D_1-D_2 receptor oligomerization, we employed fluorescence resonance energy transfer (FRET) technology, which used fluorescently tagged dopamine receptors combined with fluorescence lifetime microscopy of living cells [35]. From the results obtained, it may be concluded that clozapine does affect the formation of hetero-oligomers but only initially when present for 30 min in the incubation medium, and ceases to affect this process after a longer time (120 min). Moreover, the effect of clozapine on D_1R-D_2R hetero-oligomerization was strongly dependent on the drug concentration; the lower concentration, which resulted in binding to the high affinity sites, decreased the transfer efficiency, while the higher concentration of clozapine enhanced transfer efficiency.

On the other hand, when a low concentration of clozapine was added in the presence of Gpp(NH)p (a non-hydrolyzable GTP analog), which should mean that the high affinity binding sites were no longer present, the degree of D_1-D_2 receptor hetero-oligomerization significantly increased. In contrast, when clozapine was present without Gpp(NH)p hetero-oligomerization was not different from the control. These results further confirm the idea that high affinity binding sites exist when the receptor is coupled with G protein, and that clozapine attenuates the hetero-oligomerization of a high affinity pool of dopamine D_1-D_2 receptors. A similar conclusion can be drawn from the data obtained with the higher concen-

tration of clozapine, which "sees" both the low and the high affinity binding sites.

In light of the obtained data, we propose that clozapine is capable of producing opposite effects on G protein-coupled receptors depending on whether or not they are coupled to their given G proteins. Such a conclusion has a fundamental significance, since therapeutic doses of clozapine are not sufficient to saturate the low affinity binding sites; therefore, the compound acts at high affinity binding sites. On the basis of the results obtained with the use of FRET measurements, one may assume that clozapine decreases the degree of hetero-oligomerization of dopamine receptors. Since concomitant agonist stimulation of both receptors enhances the degree of D_1-D_2 hetero-dimerization [17], one may assume that excessive dopamine release, which has been postulated in schizophrenia, exerts a similar effect.

Concluding remarks

Despite its well-recognized superiority over other antipsychotics, clozapine is an imperfect drug. The search for safer alternatives to clozapine that would not cause agranulocytosis, seizures, obesity or diabetes [24, 55] is currently one of the most important topics in the field of schizophrenia research. We strongly believe that the results discussed in the present study showing the effect of clozapine on D_1-D_2 receptor hetero-oligomerization, together with the data pointing to the importance of receptors forming hetero-oligomers as a novel level for pharmacological intervention, are an important contribution to the field, and will help to increase the understanding of the molecular mechanism of action of antipsychotic drugs.

Future studies dedicated to finding compounds targeting the fine tuning of D_1-D_2 receptor hetero-oligomerization are therefore necessary to develop new potential drugs as efficacious as clozapine, but devoid of its serious side effects.

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