Clozapine's multiple cellular mechanisms: what do we know after more than fifty years? A systematic review and critical assessment of translational mechanisms relevant for innovative strategies in treatment-resistant schizophrenia

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Abstract

Almost fifty years after its first introduction into clinical care, clozapine remains the only evidence-based pharmacological option for treatment-resistant schizophrenia (TRS), which affects approximately 30% of patients with schizophrenia.

Despite the long-time experience with clozapine, the specific mechanism of action (MOA) responsible for its superior efficacy among antipsychotics is still elusive, both at the receptor and intracellular signaling level. This systematic review is aimed at critically assessing the role and specific relevance of clozapine's multimodal actions, dissecting those mechanisms that under a translational perspective could shed light on molecular targets worth to be considered for further innovative antipsychotic development.

In vivo and *in vitro* preclinical findings, supported by innovative techniques and methods, together with pharmacogenomic and *in vivo* functional studies, point to multiple and possibly overlapping MOAs. To better explore this crucial issue, the specific affinity for 5-HT_2R, D1R, α_{2c}, and muscarinic receptors, the relatively low occupancy at dopamine D2R, the interaction with receptor dimers, as well as the potential confounder effects resulting in biased ligand action, and lastly, the role of the moiety responsible for lipophilic and alkaline features of clozapine are highlighted. Finally, the role of transcription and protein changes at the synaptic level, and the possibility that clozapine can directly impact synaptic architecture are addressed. Although clozapine's exact MOAs that contribute to its unique efficacy and some of its severe adverse effects have not been fully understood, relevant information can be gleaned from recent mechanistic understandings that may help design much needed additional therapeutic strategies for TRS.

Keywords: clozapine; treatment-resistant schizophrenia; psychosis; immediate early genes; postsynaptic density; antipsychotics.

Abbreviations:

TRS: treatment-resistant schizophrenia

EPS: extrapyramidal side effects

MOAs: mechanisms of action

PSD: post-synaptic density

PET: positron emission tomography

TD: tardive dyskinesia

DSP: dopamine supersensitivity psychosis

PFC: prefrontal cortex

Cav-1: Caveolin-1

PPI: Pre-pulse Inhibition

BRET: bioluminescence energy transfer

FRET: fluorescence resonance energy transfer techniques

PLA: Proximity Ligation Assay

DREADDs: designer receptors exclusively activated by designer drugs

CNO: clozapine N-oxide

mPFC: medial prefrontal cortex

SNAT: sodium-coupled neutral amino acid transporter

GAD: glutamic acid decarboxylase

FGF-2: fibroblast growth factor-2

IEGs: immediate early genes

BDNF: brain-derived neurotrophic factor

CREB: Cyclic adenosine monophosphate Response Element Binding protein

IFN-γ: interferon γ

at-RA: all-trans retinoic acid

GWA: genome-wide association study

COMT: catechol-O-methyltransferase

OXT: oxytocin gene

ITIH3: inter-Alpha-Trypsin Inhibitor Heavy Chain 3

HLA: human leukocyte antigen system

MRI: magnetic resonance imaging

SPECT: Single Photon Emission Computed Tomography

fMRI: functional Magnetic Resonance Imaging

OFC: orbitofrontal cortex

DTI: diffusion tensor imaging

FA: fractional anisotropy

MRS: Magnetic resonance spectroscopy

CIA: clozapine-induced idiosyncratic agranulocytosis

CIM: Clozapine-induced myocarditis

CIWG: Clozapine-induced weight gain

PLC: phospholipase C

PKC: protein kinase C

DARPP-32: dopamine- and cAMP-regulated phosphoprotein 32 kD

TRPC: transient receptor potential cation channel

ERK1/2: extracellular signal-regulated kinases 1/2

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1. Introduction

Antipsychotics are the cornerstone of the pharmacological treatment of schizophrenia (SCZ). However, approximately 30% of SCZ subjects respond poorly or do not respond at all to treatment with first- (typical) or second- (atypical) generation antipsychotics and are defined as treatment-resistant schizophrenia (TRS) patients (Kahn, et al., 2015; Kane & Correll, 2016). TRS is a severe condition associated with a greater clinical burden of symptoms, including positive, negative and cognitive (de Bartolomeis, Balletta, et al., 2013; Iasevoli, et al., 2016; K. M. Shannon, 2005), severely affecting individual functional capacity (Iasevoli, Balletta, Gilardi, Giordano, & de Bartolomeis, 2013; Iasevoli, et al., 2018). Moreover, a significant number of patients responsive to antipsychotics with high dopamine D2 receptor (D2R) affinity may experience neuromotor side effects that force them to interrupt the treatment. These patients require a different therapeutic strategy tackling both the psychotic symptoms and movement disorders induced by antipsychotics (Keepers & Casey, 1986; Pierre, 2005; Tonda & Guthrie, 1994). In these populations of patients, clozapine (a 5H-dibenzo[b,e][1,4]diazepine substituted by a chloro group at position 8 and a 4-methylpiperazin-1-yl group at position 11), the prototypical atypical antipsychotic, represents the current gold standard of treatment after more than fifty years from its first introduction into clinical care (Correll, et al., 2022; Fakra & Azorin, 2012; Meltzer, 2013).

Clozapine was first introduced into SCZ treatment in the 1970s (Wenthur & Lindsley, 2013) when the knowledge of SCZ pathophysiology was still scarce and the monoamine hypothesis was at its beginning. Even with the recognition of relevant adverse events, such as agranulocytosis (that forced withdrawal of clozapine from the therapeutic armamentarium for several years), myocarditis, constipation, weight gain and diabetes (Musil, Obermeier, Russ, & Hamerle, 2015; Shams & Müller, 2014), clozapine remains the only antipsychotic with a specific indication for TRS (de Leon, Ruan, Schoretsanitis, & De Las Cuevas, 2020; Meltzer, 2013). In fact, clozapine was found to be more effective than first-generation antipsychotics in many symptom domains (overall change in symptoms, and positive and negative symptoms) and induced fewer extrapyramidal side effects (EPS)

(Huhn, et al., 2019; Leucht, et al., 2009). However, clozapine's superior efficacy on negative cognitive symptoms domain is still debated, given the high variability of its effect on cognition (Torrisi, et al., 2020). The observed improvements in negative symptoms and neuropsychological test performance in subjects receiving clozapine (Buchanan, Holstein, & Breier, 1994; M. A. Lee, Thompson, & Meltzer, 1994; McGurk, 1999) may be mediated by the amelioration of positive symptoms, quality of life, and global functioning (Priyamvada, Ranjan, Jha, & Chaudhury, 2021; Verma, Grover, & Chakrabarti, 2021). Other data on the comparative efficacy have been sometimes conflicting (Samara, et al., 2016), either due to underdosing of clozapine, admixture of non-TRS subjects into randomized trials, and probably also due to the numerous antipsychotic treatments often preceding and delaying clozapine introduction, which may reduce its effectiveness (Czepielewski, et al., 2018; Nielsen, Nielsen, & Correll, 2012). However, clozapine appeared to be unique among antipsychotics in TRS patients, in early-onset schizophrenia, as well as for reduction of suicide risk (Meltzer, 2013; Schimmelmann, Schmidt, Carbon, & Correll, 2013; Taipale, Lähteenvuo, Tanskanen, Mittendorfer-Rutz, & Tiihonen, 2021). Additionally, although regulatory approval for these indications is lacking, clozapine has also demonstrated relevant efficacy for aggression/agitation, treatment-resistant bipolar disorder, impulsivity, and suicidality in conditions other than SCZ (Fornaro, et al., 2020; Frogley, Taylor, Dickens, & Picchioni, 2012; Meltzer, 1999b; Nielsen, Kane, & Correll, 2012; Rohde, Polcwiartek, Correll, & Nielsen, 2018; Spivak, et al., 1998).

Despite the long-lasting clinical experience with clozapine, its mechanisms of action (MOAs) remain yet to be unveiled (Nucifora, Mihaljevic, Lee, & Sawa, 2017), especially when compared to all other antipsychotics, both of first- and second-generation. It has been suggested that, paradoxically, the attempt to synthesize an antipsychotic agent similar to clozapine and with fewer side effects has in some way jeopardized the search for a truly innovative antipsychotic drug (Siskind, McCartney, Goldschlager, & Kisely, 2016; Tuunainen, Wahlbeck, & Gilbody, 2000), leading to several compounds that only partially mimic the action of clozapine without reaching comparable levels of efficacy.

However, unique and previously undiscovered MOAs of clozapine at the receptor and intracellular, as well as synaptic function and dendritic spine architecture level have started to emerge in preclinical *in vivo* and *in vitro* investigations, as well as in pharmacogenomic and functional studies in humans. Based on previous observations and given the lack of a recent overview on this topic, this review aimed at tackling the following issues:

1) If the antipsychotic MOA relies for most available antipsychotics on functional striatal D2R blockade of approximately 60-70%, how may clozapine work on the dopaminergic system blocking significantly less striatal D2Rs?

2) To what extent is clozapine's multi-receptor profile, beyond the D2R occupancy, responsible for its antipsychotic action and unique adverse events?

3) Which mechanisms downstream of the receptor level and which modulation of cell signaling and synaptic plasticity effects may lead to changes in neurotransmitter circuitries that are potentially relevant to clozapine's beneficial effects?

4) Could a better insight into clozapine's MOAs help discover innovative pharmaceutical targets in TRS?

Attempting to answer these questions to the degree currently possible, we reviewed the recent findings on clozapine's specific D2R binding and dissociation, the action on neurotransmitter vesicles at presynaptic terminals, the multimodal receptor action with specific regard to M1-M4 muscarinic and α_{2c} adrenoreceptors, the influence on intracellular signaling, as well as the impact on post-synaptic density (PSD) and dendritic spine architecture. We further accompanied the preclinical findings by *in vivo* imaging studies aimed at investigating clozapine's effects on brain structure and connectivity in SCZ patients. Finally, we reviewed discrete MOAs underlying severe adverse events of clozapine treatment, including pharmacogenomic data.

2. Search and selection strategy

A first comprehensive search in the PubMed database was conducted January 1st, 2021, which was updated on November 18th, 2021, before the final writing of the manuscript, for all studies containing data on clozapine's MOAs, without applying time limits. A search string combining "clozapine" with 30 terms related to pharmacodynamics, gene expression, and other molecular effects was used to identify relevant articles (see the Supplementary Text for more details and the combination of terms). Retrieved records and full texts were managed by using Endnote X. We included publications in peer-reviewed journals and written in English: i) reporting original data or reviewing the pharmacology of clozapine; and ii) evaluating the putative MOAs of clozapine *in vitro* or *in vivo*, both in animal models or humans. Case reports, case series, as well as articles describing behavioral clozapine effects without directly or indirectly addressing the action of clozapine at the mechanistic level (i.e., cellular-, molecular- neurotransmitter-, gene-related mechanisms) were excluded. Similarly, clinical trials not providing an explanatory model of clozapine's MOA were excluded. Furthermore, the reference lists of the full texts of the included records were screened for any possible relevant articles not primarily retrieved by the first search. The database search and cross-referencing yielded 4,898 records. Records were first assessed by title/abstracts by two blind reviewers (AB and LV), then relevant articles were selected for full-text assessment according to the above-mentioned eligibility criteria. Inconsistencies were resolved by consensus in a meeting with another researcher (FI); a final critical appraisal of relevant articles was performed by a second consensus meeting with two researchers (AdB and FI). Finally, 489 articles were included in the qualitative synthesis. The details of the methodological strategy are reported in the Prisma 2020 flow diagram (Fig. 1) and in Supplementary Text 1.

3. Clozapine's receptor profile and its action at neurotransmitter level

Despite more than five decades of research, the neurotransmitter conundrum of clozapine's MOAs, which are associated with its clinical superiority, is still not resolved. Multiple theories have been

proposed over time. The efficacy of clozapine in TRS and low propensity to lead to neuromotor adverse effects was originally attributed to its relatively low D2R striatal occupancy (Tauscher, et al., 2004). Later, its atypical characteristics were supposed to be related to its stronger affinity for D4Rs compared to D2Rs. Meltzer suggested that uniqueness of clozapine was due to a combination of the antagonism at D2Rs and serotonin 5-HT receptors (5-HTR) type 2A (5-HT_{2A}Rs), as well as its characteristic partial agonism at serotonin 5-HT_{1A}Rs (Z. Li, Prus, Dai, & Meltzer, 2009; Meltzer, 2012b). Nonetheless, this MOA appears to be distinctive of the entire class of second-generation antipsychotics. Kapur and Seeman, on the other hand, hypothesized that clozapine's effects were not due to a strong blockade of D4Rs, 5-HT_2Rs, or others, but due to its fast dissociation from D2Rs (Kapur & Seeman, 2001; Seeman, 2014). In fact, clozapine transiently occupies D2Rs, allowing endogenous dopamine to displace the loosely bound antipsychotic drug. On closer examination, the actual clozapine receptor binding profile is highly complex (Table 1), encompassing a multiple array of receptors, including adrenergic, histaminergic, and muscarinic receptors (Fig. 2). Furthermore, γ-aminobutyric acid (GABA) (O'Connor & O'Shea, 2015) and glutamate receptors have also been implicated in clozapine's MOA, supporting further theories on the unique clinical properties of clozapine in the treatment of TRS. In this section, we will consider in detail each receptor-mediated mechanism that has been proposed for clozapine's unique therapeutic effects.

3.1 Dopamine receptors

3.1.1 Clozapine and D2 dopamine receptors

Clozapine binds to all dopamine receptor subtypes. However, since the discovery of dopamine receptors, a significant differential action of clozapine at D2R compared to all other antipsychotics has been assumed on the basis of *in vitro*, *in vivo*, and human brain positron emission tomography (PET) studies using relatively specific radioligands, such as ^{11}C-raclopride, ^{11}C-N-methyl-spiperone, and ^{11}C-FLB 457 (Farde, et al., 1997).

Multiple clinical lines of evidence suggest a dual role for dopamine in the pathophysiology of SCZ, postulating the existence of cortical "hypodopaminergia" and subcortical "hyperdopaminergia", associated with the dysfunction of other neurotransmitter systems, mainly serotonergic and glutamatergic (Howes & Kapur, 2009; Moghaddam & Javitt, 2012). The presence of a hyperdopaminergic state is the theoretical rationale for the use of antipsychotics that block dopamine D2Rs, which is regarded as a prerequisite for antipsychotic action, but which is also linked to common side effects. For instance, blocking D2Rs in the mesocortical, nigrostriatal, and tuberoinfundibular pathways is believed to cause, respectively, neuroleptic-induced deficit syndrome or worsening of cognitive/negative symptoms and induction of dysphoria or depression, parkinsonism, dystonia, akathisia or tardive dyskinesia (TD) (collectively called extrapyramidal side effects, or EPS), and hyperprolactinemia with related sexual and reproductive dysfunction (Stępnicki, Kondej, & Kaczor, 2018). It has long been proposed that clozapine's "limbic selectivity", namely its ability to preferentially block mesolimbic rather than nigrostriatal D2Rs, may account for reduced EPS liability (Pilowsky, et al., 1997), although this hypothesis has later been questioned (Talvik, et al., 2001). Of interest, at therapeutic concentrations, clozapine's D2R occupancy is reported to be approximately 40-60% (Coward, 1992; Fakra & Azorin, 2012; Naheed & Green, 2001), and thus below the D2R occupancy threshold of most other antipsychotic agents, with the exception of quetiapine and lumateperone. Moreover, typical antipsychotics may display a prolonged occupancy of D2R in the human striatum, while clozapine only transiently occupies this site (Seeman, 2014). In fact, it has been hypothesized that the chemical structure of clozapine facilitates a relatively rapid dissociation from D2Rs, thus giving clozapine the unique capability of having an antipsychotic effect but no or low EPS liability, combined with the ability to avoid sustained hyperprolactinemia (Seeman, 2014). This mechanism has been fully described by Kapur and Seeman in the terms of a "fast dissociation"; the "fast-off" theory claims that atypical antipsychotics have low affinity for D2Rs, are weakly bound to, and rapidly dissociate from these receptors (Kapur & Seeman, 2001). Other major aspects involved in the development of side effects (i.e., EPS, hyperprolactinemia) seem to be related to the K_{on} and

K_{off} values at D2Rs. Sykes et al. have demonstrated that antipsychotic-induced hyperprolactinemia is strongly related to D2R K_{off}, while EPS depend on both D2R K_{off} and K_{on}, probably together with the contribution of other neuroreceptors (such as serotonergic ones) (Sykes, et al., 2017). On the other hand, the "fast-off hypothesis" has recently been revisited by Sahlholm and colleagues (Sahlholm, et al., 2016). In fact, these authors have shown that the K_{off} of typical antipsychotics may be often biased and underestimated due to the lipophilic nature of typical compounds, allowing for sequestration of the antipsychotic in the cell membrane or cell interior, which then facilitates subsequent rebinding to D2Rs (Sahlholm, et al., 2016). In this perspective, Sahlholm et al reported that clozapine's dissociation is only 6-fold faster in comparison to typical antipsychotics, such as haloperidol, instead of 100-fold faster as suggested in previous receptor binding kinetic assays (Sahlholm, et al., 2016). Therefore, factors other than D2R kinetic binding, probably involving a specific moiety of the compound, may participate in the specific clozapine dopaminergic action profile.

Furthermore, continuous dopamine blockade is known to induce D2R upregulation, and this effect has been potentially linked to antipsychotic tolerance, dopamine supersensitivity psychosis (DSP), and TD (Cornett, Novitch, Kaye, Kata, & Kaye, 2017). In this respect, clozapine's fast-off and its reduced ability to translocate D2Rs on the cell surface may help to avoid this harmful condition, being responsible, at least partially, for its effectiveness in treating TRS patients (Meltzer, 2012a, 2013; Moran-Gates, et al., 2006; Schrader, et al., 2019; Siskind, et al., 2016; Stevens, Denney, & Szot, 1997; Tarazi, Florijn, & Creese, 1997).

Selective and high-affinity D2R antagonism has been associated with poor cognitive performancedue to the disruption of the D2R-mediated signaling in the prefrontal cortex (PFC) (Mehta, Montgomery, Kitamura, & Grasby, 2008; Torrisi, et al., 2020; Watson, et al., 2012). In this respect, clozapine not only exerts a slight and transient blockade at prefrontal D2Rs, but can selectively augment the dopamine turnover in prefrontal regions sparing striatal areas, thus normalizing the dopaminergic transmission in brain regions relevant for cognitive functioning (Elsworth, Jentsch, Morrow, Redmond, & Roth, 2008). Moreover, clozapine's ability to stabilize phasic and basal dopamine

303 release in the amygdala of rats previously sensitized with amphetamine may contribute to improving
304 emotional cognitive processing in patients with schizophrenia (Kawano, et al., 2016).
305 It has also been demonstrated that D2R activation is relevant for the regulation of dopamine uptake
306 by the vesicular monoamine transporter-2 (VMAT-2) (Truong, Newman, Hanson, & Fleckenstein,
307 2004). In fact, long-term treatment with clozapine seems to induce an up-regulatory effect on
308 VMAT2, resulting in an augmented storage capacity of the presynaptic monoaminergic neurons. The
309 resultant greater monoamine availability may, in turn, account for clozapine's beneficial impact on
310 the pleomorphic symptomatology of SCZ, as well as its low propensity to cause EPS (Rehavi, Roz,
311 & Weizman, 2002).
312 Hence, the classical assumptions about the superior efficacy of clozapine encompass both its lower
313 D2R occupancy and the fast-off capacity, with a subsequent low likelihood of inducing D2R
314 upregulation. Since these features appear to be decisive for clozapine's "atypicality", further
315 explanation for its distinctive effectiveness in TRS may lie in the affinity for a broader array of
316 neuroreceptors.
317
318 ***3.1.2 Clozapine and non-D2 dopamine receptors***

319 Clozapine's affinity for the D4R subtype (Ki=39 nM) is 10-fold higher than for D2R (Ki=431 nM)
320 (R. A. Lahti, Evans, Stratman, & Figur, 1993). Since D4Rs seem to be involved in the cellular
321 mechanisms of hyperlocomotion (Ninan & Kulkarni, 1998; Wenthur & Lindsley, 2013), this peculiar
322 pharmacodynamic property may have interesting clinical implications. Furthermore, the regional
323 pattern of D4R distribution mainly involves prefrontal and temporolimbic structures, and spares the
324 basal ganglia. Therefore, compounds with preferential binding to D4Rs over D2Rs may selectively
325 reduce the dopaminergic tone in mesolimbic and mesocortical pathways without affecting the
326 nigrostriatal pathway and, hence, without producing motor side effects (Kulkarni & Ninan, 2000).
327 Since D4Rs are located on both pyramidal and GABAergic neurons in the cortex, hippocampus,

thalamus, globus pallidus, and substantia nigra (Mrzljak, et al., 1996), clozapine may modulate glutamatergic transmission via D4Rs, either directly or indirectly through GABAergic interneurons. However, despite these preclinical observations, selective D4R antagonists failed in clinical trials (Corrigan, Gallen, Bonura, & Merchant, 2004; Kramer, Last, Getson, & Reines, 1997; Lindsley & Hopkins, 2017), thereby questioning the hypothesis that antagonism at D4Rs may play a major role in clozapine's antipsychotic action. Recently, D4R antagonists have returned to the spotlight as a novel potential therapeutic strategy for treating central nervous system (CNS) diseases (i.e., addiction and L-DOPA-induced dyskinesias in Parkinson's Disease) and cancer (Bergman & Rheingold, 2015; Dolma, et al., 2016; Huot, et al., 2015; Ratna & Sastry, 2005; Schaeffer, Pilotto, & Berg, 2014).

According to the binding assays, clozapine displays also a higher affinity for D1Rs (Ki=189 nM) than D2Rs (Wenthur & Lindsley, 2013). PET findings suggest that clozapine's striatal D1R and D2R occupancy is nearly equivalent in humans (Tauscher, et al., 2004), with a D1R/D2R ratio of 0.88, which appears to be the highest among other antipsychotics, equaled only by asenapine (Huot, et al., 2015). A PET study by Chou and colleagues indicated that clozapine preferentially acts on D1Rs located in the frontal cortex rather than in striatum, assuming the regional selectivity at the basis of its peculiarity (Chou, Halldin, & Farde, 2006). However, whether clozapine behaves as a D1R agonist or antagonist is yet to be elucidated. Some reports indicated that clozapine behaves as a D1R agonist, which may potentially explain clozapine's efficacy on cognitive symptoms of SCZ (Ahlenius, 1999). In this perspective, it is worth emphasizing that SKF38393, a D1R agonist, may revert the behavioral sensitization, enhanced locomotor activity, and cognitive deficits induced by methamphetamine (Shuto, et al., 2006). Conversely, other authors suggested that clozapine may act as an inverse agonist or antagonist at D1Rs (Cai, Gurdal, Smith, Wang, & Friedman, 1999; Murray & Waddington, 1990). However, treatment with pure D1R antagonists failed to induce an antipsychotic response in patients suffering from SCZ (Karlsson, et al., 1995).

Of interest, the affinity of clozapine for D1R can vary depending on whether the receptor is expressed alone or concomitantly with D2R (Faron-Górecka, Górecki, Kuśmider, Wasylewski, & Dziedzicka-

Wasylewska, 2008). In fact, the clozapine affinity value for D1R has been found to increase when D2Rs are co-expressed, whereas D2R affinity remains unchanged (Faron-Górecka, et al., 2008). Moreover, low doses of clozapine have been found to dissolve D1R-D2R dimers, thereby reducing the intracellular calcium levels (Dziedzicka-Wasylewska, Faron-Górecka, Górecki, & Kuśemider, 2008; Faron-Górecka, et al., 2008). Since the formation and functional activation of D1R-D2R heterodimers have been found to be increased in the globus pallidus in SCZ (Hasbi, O'Dowd, & George, 2011; Perreault, et al., 2010), these findings could be relevant from a clinical point of view. The lack of suitable radioligands capable of differentiating the D1R from the D5R subtype has hindered research on clozapine's D5R receptor binding (Kilbourn, 2021).

Affinity for D3Rs is low for clozapine (Ki=646 nM) (Wenthur & Lindsley, 2013), being higher for norclozapine, its main metabolite, also known as N-desmethyl-clozapine (Fig. 3) (Maggio & Millan, 2010; Scarselli, et al., 2001). D3R occupancy may be implicated in antipsychotic effects and cognitive improvements (Leriche, Schwartz, & Sokoloff, 2003; Scharfetter, et al., 1999; X. Sun, et al., 2016) by enhancing the release of acetylcholine in the PFC (Nakajima, et al., 2013). An epistatic interaction between genes encoding D3R and dysbindin, a top candidate gene in schizophrenia, has been reported: genetic disruption of dysbindin may affect the intracellular trafficking of D2-like receptors, including D3R. Of interest, the concomitant reduction in dysbindin and D3R activity resulted in pro-cognitive effects in humans and mice (Leggio, et al., 2021), emphasizing the role of regional D2R/D3R balance and D3R antagonism in improving cognitive symptoms.

It has been reported that clozapine is responsible for D3R upregulation in rat brain after acute administration (Buckland, O'Donovan, & McGuffin, 1993), an effect shared with haloperidol. However, the clinical relevance of D3R blockade by clozapine remains unclear (Malhotra, et al., 1998), given that several preclinical studies point to negligible D3R occupancy of only 33-35%, with a D2R/D3R selectivity ratio of 2.82 ± 2.01 (Girgis, et al., 2011; McCormick, Wilson, Wilson, & Remington, 2013). Moreover, a PET study in baboons reported an even higher D2R/D3R selectivity

ratio, which reached 5.25 (Girgis, et al., 2011). Therefore, despite representing a promising target, the D3R blockade may limitedly contribute to clozapine pharmacodynamics.

In summary, considering the overall effects of clozapine's action on non-D2R, the most relevant finding is that clozapine, compared to other antipsychotics, has an almost equal affinity for D1R and D2R, while the biological roles of D3R and D4R antagonism appear to have only limited relevance for clozapine's clinical efficacy. The therapeutic potential of these receptors as a target could be better clarified when molecules with optimal D3R/D2R and D4R/D2R ratios will be developed and tested.

3.1.3 In vivo imaging of dopamine receptors

To investigate the extent and localization of the D2R blockade by clozapine, *in vivo* in the human brain, PET studies have been strongly instrumental and have shown that clozapine's D2R occupancy is low compared to typical antipsychotics (Farde, Nordström, Nyberg, Halldin, & Sedvall, 1994; Nordström, et al., 1995). In a PET study, Nordstrom and colleagues analyzed scans from seventeen patients treated with clozapine (125-600 mg/day) using three D2R-selective radioligands: ^{11}C-SCH23390, ^{11}C-raclopride and ^{11}C-N-methyl-spiperone. The occupancy of D2R was lower (20%-67%) than reported in patients treated with other antipsychotics (70%-90%), whereas occupancy at D1R and 5-HT$_2$R was significantly higher (Nordström, et al., 1995). A higher dopamine D2R occupancy can be transiently reached with very high doses of clozapine (Nyberg, Chou, & Halldin, 2002; Suhara, et al., 2002; Takano, et al., 2006). It is not certain whether there is a correlation between D2R occupancy and clinical improvement in treated patients, but there is probably not a critical degree of D2R occupancy required to obtain an antipsychotic effect with clozapine (Pickar, et al., 1996; Tauscher, et al., 1999) as is the case for other antipsychotics. On the other hand, the characteristic low striatal D2R occupancy may explain why EPS occur rarely in patients treated with clozapine (Scherer, et al., 1994; Tauscher, Küfferle, Asenbaum, Tauscher-Wisniewski, & Kasper, 2002).

Regarding the preferential occupancy of D2Rs by clozapine in different brain regions (cortical and striatal), imaging studies have produced conflicting results (Kessler, et al., 2006). For instance, Farde et al., as well as Talvik et al., concluded that clozapine did not show a regional pattern of D2R occupancy (Farde, et al., 1997; Talvik, et al., 2001). On the other hand, Xiberas et al. demonstrated that clozapine and other atypical antipsychotics act in a region-specific manner (Xiberas, et al., 2001). In particular, cortical D2Rs appear to be a common target of both typical and atypical antipsychotics, while basal ganglia receptors are primarily occupied by typical agents (Xiberas, et al., 2001). It has been argued that the discrepancy found in the literature about the regional selectivity of clozapine may partially be explained by the underestimation of the calculated drug occupancy values for antipsychotics with poor D2R occupancy, such as clozapine, which may give the impression of extrastriatal selectivity (Olsson & Farde, 2001).

Taken together, these reports indicate that clozapine has lower selectivity for D2Rs than D1Rs and 5-HT_{2A}Rs *in vivo*, which may contribute to its unique neurobiological and clinical features. In contrast, to date, there is little evidence to support the view that part of the action of clozapine may depend on extrastriatal D2R regional selectivity.

3.1.4 Clozapine activity at presynaptic dopaminergic terminals

While adequate central D2R blockade is generally achieved within a few hours after antipsychotic administration, a noticeable antipsychotic effect appears after days or weeks of treatment (Takano, et al., 2004). The delayed onset of symptom improvement questioned the paradigm of post-synaptic dopamine D2R blockade as the primary way in which antipsychotics may act, paving the way for the hypothesis that a clozapine non-obvious MOA could lie more in the pre-synaptic than in the post-synaptic dopaminergic terminal.

In line with this hypothesis, it has been demonstrated by use of the fluorescent reporter LysoTracker Red, which mimics the drug behavior, that weak-base antipsychotics (i.e., agents that do not

completely dissociate into their constituent ions when dissolved in solutions) may progressively accumulate in endosomes and synaptic vesicles at presynaptic dopaminergic nerve terminals, as a result of vesicular delivery of the drug (Morton & Cousin, 2012; Tischbirek, et al., 2012). Thus, chronic treatment would generate an intracellular reservoir of the drug, which is available for release during synaptic activity (Tischbirek, et al., 2012). The hypothesis formulated by Tischbirek et al. is that antipsychotics are co-released from vesicles along with endogenous dopamine, resulting in the inhibition of presynaptic voltage-gated sodium channels, exerting in turn an overall auto-inhibitory effect on dopamine release. According to this intriguing theory, voltage-gated sodium channels may represent the primary presynaptic target of antipsychotic action. Moreover, clozapine may be particularly suitable for this purpose, due to its alkaline and lipophilic properties allowing for its intravesicular accumulation.

Another presynaptic mode of action covered by antipsychotics may lie in the formation of a "reserve" of unblocked D2 presynaptic autoreceptors, available for binding to endogenous dopamine. In such case, extracellular endogenous dopamine could behave, perhaps counterintuitively, as an antipsychotic itself, by binding to this D2 inhibitory autoreceptor reserve, resulting in a reduction of presynaptic synthesis and release of dopamine (Amato, Vernon, & Papaleo, 2018). Nonetheless, the initial increase in synaptic dopamine availability after antipsychotic exposure appears to decline over time, and the decreased dopamine levels detectable during chronic treatment have been associated with loss of antipsychotic efficacy (Amato, et al., 2020). Therefore, reasons for drug tolerance or treatment failure should be sought in reduced dopamine levels at dopaminergic synapses, and the subsequent loss of stimulation of the D2R presynaptic reserve. It has been supposed that restoring the initial levels of synaptic dopamine may reinstate the antipsychotic efficacy in long-term treatment, and a viable therapeutic option would be the blocking of Dopamine transporter (DAT) as an augmentation strategy (Amato, et al., 2020). Very curiously, clozapine exhibits a moderate affinity for DAT (Miyamoto, Duncan, Marx, & Lieberman, 2005), and genetic variants in DAT gene have

been reported among clozapine-resistant patients (Xu, et al., 2010), suggesting that DAT-antagonism may be a crucial target for clozapine to express its considerable therapeutic potential.

In sum, this evidence challenges the traditional view of postsynaptic receptor blockade as the main dopaminergic mechanism exerted by antipsychotics, pointing to other unexpected indirect actions at the presynaptic dopaminergic nerve terminal, either by inhibiting voltage-gated sodium channels or via indirect stimulation of the D2 autoreceptor reserve. These considerations are helpful to achieve an in-depth understanding of the complex plastic changes induced by antipsychotics within synapses, but also shed light on DAT blockade as a novel putative target for treating TRS.

3.1.5 Clozapine, dopamine supersensitivity psychosis, and tardive dyskinesia

DSP was first conceptualized in the 1970s, following the observation of: 1) the sudden worsening of psychotic symptoms after drug discontinuation/reduction/switch; 2) tolerance to previous treatments and the need for higher doses to control relapse episodes; and 3) the occurrence of TD, a disorder characterized by abnormal and continuous involuntary movements of the tongue, neck, facial muscles, truncal musculature, and limbs (Chouinard & Jones, 1980; Chouinard, Jones, & Annable, 1978; Vasan & Padhy, 2021). It is known that polypharmacy and high doses of antipsychotics, especially of high potency D2R-blockers, are major determinants in developing TD (Solmi, Pigato, Kane, & Correll, 2018) which has an estimated prevalence of approximately 20% in individuals receiving antipsychotics (Carbon, Hsieh, Kane, & Correll, 2017; Chouinard & Chouinard, 2008; Stegmayer, Walther, & van Harten, 2018), and which remains an issue even with atypical antipsychotics (Carbon, Kane, Leucht, & Correll, 2018). Davis and Rosenberg hypothesized that DSP was the limbic equivalent of TD, because of a similar underlying mechanism (Davis & Rosenberg, 1979). There is no consensus on the etiology of DSP and TD, but it has been proposed that long-term blockade of D2R in the brain's mesolimbic system might lead to receptor upregulation, an increase in D2R density, and/or shifting from a "low-affinity" to a "high affinity" state (Iyo, et al., 2013;

Tenback & van Harten, 2011), resulting in a subsequent "dopamine supersensitivity" condition responsible for the dyskinetic movements. Long-term administration of typical and atypical antipsychotics generally increases D2R binding and density in the striatum (Köhler, Schröder, Augustin, & Sabel, 1994; Samaha, Seeman, Stewart, Rajabi, & Kapur, 2007; Silvestri, et al., 2000). Although clozapine's MOAs remains still unclear, its superior effectiveness compared to all other antipsychotics may encompass the ability to correct, or at least mitigate, the dopamine supersensitivity state (D. D. Kim, Barr, Honer, & Procyshyn, 2018). Not surprisingly, clozapine seems to be a viable therapeutic option for DSP, and if TD develops, a stepwise reduction of the offending agent and the switch to clozapine are part of the recommended treatment for TD (Ricciardi, et al., 2019). In fact, Schrader and colleagues proposed that almost all known antipsychotics, except for clozapine and partial D2 agonists, such as aripiprazole, act as pharmacological chaperones at D2R sites, inducing receptor translocation to the cell surface (Schrader, et al., 2019). On the contrary, clozapine displays low efficacy in behaving as a chaperone, and the ratio between the D2Rs expressed on the surface and the total cellular amount is the lowest after clozapine exposure compared to other antipsychotics, resulting in a reduced D2R upregulation (Schrader, et al., 2019) (Fig. 4). Against this background, it has been suggested that clozapine, due to its lower D2R occupancy, its rapid dissociation from the D2Rs, combined with the reduced ability to translocate D2Rs to the cell surface and the favorable 5-HT_{2A}R/D2R ratio, has the lowest potential among antipsychotics to sensitize D2R and cause DSP and TD (Nordström, et al., 1995; Schrader, et al., 2019; Seeman, 2011; Vasan & Padhy, 2021).

Moreover, the occurrence of neuroleptic-induced DSP has been associated also with persistent changes in serotonin receptor pattern of expression (Charron, Hage, Servonnet, & Samaha, 2015). A novel striking theory pointing to a crucial role for 5-HT_6R for the development of TD has recently been proposed (Aldrin-Kirk, et al., 2016). To date, selective activation of the 5-HT_6R in transplanted dopaminergic neurons has proved to be responsible for excessive dopamine release and subsequent 'graft-induced dyskinesia', a challenging side effect of dopaminergic neuron transplantation in

Parkinson's disease. Therefore, a mechanistic link between this specific serotonin receptor subtype and dyskinesia has been established (Aldrin-Kirk, et al., 2016). Hence, the binding potency of clozapine for 5-HT_6Rs (Ki=7 nM) (Wenthur & Lindsley, 2013) and its antagonist properties at this site, may partially account for this distinctive safer profile with respect to motor side effects compared to all other antipsychotics.

Another putative mechanism implicated in the development of DSP may be related to the oxidative stress resulting from free radicals generated by dopamine metabolism. In fact, catecholamine metabolism is a direct source of reactive oxygen species, and dopaminergic neurons in the substantia nigra and basal ganglia are particularly vulnerable to alterations of cellular redox homeostasis (Meiser, Weindl, & Hiller, 2013), which may represent one important biological underpinning of TD. Oxidative stress, as measured by lipid peroxidation, was found elevated in TRS patients compared to antipsychotic-responsive patients, but its relevance for DSP has not yet been investigated (Medina-Hernández, et al., 2007). Although with conflicting evidence (Elmorsy, Al-Ghafari, Aggour, Khan, & Amer, 2017b; Elmorsy, et al., 2017a; Elmorsy, Elzalabany, Elsheikha, & Smith, 2014; Elmorsy & Smith, 2015), clozapine has shown the ability to counter oxidative stress (Sadowska-Bartosz, et al., 2016; Sommer, et al., 2018), protecting cells against DNA damage (Topak, Ozdel, Dodurga, & Secme, 2018), mitochondrial dysfunction (Tran, et al., 2018), death induced by oxygen radicals (Magliaro & Saldanha, 2009), as well as to correct cortico-striatal redox disturbances in SCZ (Möller, et al., 2013). These preclinical observations suggest that antioxidant properties of clozapine may contribute to its peculiar effectiveness in treating re-emergent unresponsive psychotic symptoms in the event of DSP.

3.2 Serotonin receptors, new and old findings on clozapine's MOAs: the heterodimer connection

Clozapine has relevant affinity also for serotonergic receptors. The main 5-HT receptors implicated in the action of clozapine include the following: 5-HT_{1A}R, 5-HT_{2A}R, 5-HT_{2C}R, 5-HT_3R, 5-HT_6R, and 5-HT_7R (Meltzer, 1991) (Fig. 2).

Clozapine acts as a partial agonist of 5-HT_{1A}Rs, exhibiting moderate affinity (Ki=105 nM), whereas norclozapine displays a greater affinity at this site (Ki=14 nM) (Fig. 3) (Newman-Tancredi, Chaput, Verriele, & Millan, 1996; Newman-Tancredi, et al., 1998; Odagaki & Toyoshima, 2007; Wenthur & Lindsley, 2013). Extensive evidence indicates that 5-HT_{1A}R partial agonists have effects partially shared by 5-HT_{2A}R antagonists in several biological systems (Darmani, Martin, Pandey, & Glennon, 1990). The 5-HT_{1A}R has long been implicated in the etiopathogenesis and therapy of anxiety and depressive disorders (Blier & Ward, 2003; Feighner & Boyer, 1989; Pucadyil, Kalipatnapu, & Chattopadhyay, 2005). Clozapine has been shown to upregulate these receptors in PFC and hippocampus of adult rats (Choi, Gardner, & Tarazi, 2017). However, the translational meaning of 5-HT_{1A}R modulation in SCZ does not appear clear enough. 5-HT_{1A}R agonism has been suggested to contribute to the atypical antipsychotic drug profile (Protais, Chagraoui, Arbaoui, & Mocaër, 1994), helping to reduce movement disorders (Naidu & Kulkarni, 2001; Zazpe, et al., 2006) and improve cognitive and affective symptoms (Meltzer & Sumiyoshi, 2008; Schreiber & Newman-Tancredi, 2014). However, it should not be neglected that clozapine also reverts MK-801-induced hyperactivity in 5-HT_{1A}R receptor knock-out rodents, leading to the conclusion that this target is not indispensable for an antipsychotic effect (Newman-Tancredi, 2010; Scorza, Castañé, Bortolozzi, & Artigas, 2010). Therefore, although the action on 5-HT_{1A}Rs does not explain the antipsychotic potential of clozapine, it may contribute at least in part to its beneficial clinical effects including the purported amelioration of affective symptoms and cognitive impairment.

A mechanism that has been considered pivotal for atypicality of clozapine is the antagonism at 5-HT_{2A}R, which has been associated with preclinical antipsychotic properties (M. Li, Sun, & Mead, 2012) first of all locomotor suppressing effects (Maroteaux, et al., 2017; McOmish, Lira, Hanks, & Gingrich, 2012; Newman-Tancredi, et al., 1996). Higher affinity for 5-HT_{2A}Rs (Ki=13 nM) compared

554 to D2Rs, namely a high 5-HT_{2A}R/D2R ratio, is considered one of the best predictors of "atypicality",
555 which has been initially linked to the low liability to induce EPS (Newman-Tancredi, et al., 1996;
556 Schmidt, Sorensen, Kehne, Carr, & Palfreyman, 1995; Wenthur & Lindsley, 2013). Other atypical
557 antipsychotics, such as risperidone and olanzapine, show greater 5-HT_{2A}R than D2R occupancy, but
558 their 5-HT_{2A}R/D2R ratio is lower than that of clozapine (Kapur, Zipursky, & Remington, 1999).
559 Small doses of clozapine can induce very high 5-HT_2R occupancy in the frontal cortex of treated
560 animals (Nordström, Farde, & Halldin, 1993; Sumiyoshi, et al., 1993). Therefore, the high affinity
561 for 5-HT_{2A}Rs appears to be a pivotal MOA for clozapine, as well as other atypical antipsychotics.
562 Notably, Caveolin-1 (Cav-1), a scaffolding protein that interacts with 5-HT_{2A}Rs and participates in
563 both inverse agonist and agonist actions at this site, has been reported to modulate the antipsychotic
564 efficacy of clozapine and olanzapine (Allen, Yadav, Setola, Farrell, & Roth, 2011; A. W. Cohen,
565 Hnasko, Schubert, & Lisanti, 2004). Rare structural variants of the Cav-1 gene have been associated
566 with SCZ in human genetic studies (Walsh, et al., 2008). In preclinical paradigms, the knock-out of
567 Cav-1 attenuated the ability of clozapine and olanzapine to normalize the Prepulse Inhibition (PPI)
568 (a measure of sensorimotor gating found reduced in SCZ) and hyperlocomotion in the phencyclidine
569 (PCP) animal model of psychosis (Allen, et al., 2011). Therefore, Cav-1 may represent a key element
570 mediating the biological effect of clozapine and olanzapine via 5-HT_{2A}Rs, and genetic disruption of
571 this molecule may underlie poor response to at least these agents. The 5-HT_{1A}R may form 5-HT_{1A}R/5-
572 HT_{2A}R and D2R/5-HT_{1A}R heterodimers, which activate a downstream signaling pathway distinct
573 from those of monomers, through a mechanism known as heterodimer-directed signal specificity
574 (Łukasiewicz, Błasiak, Szafran-Pilch, & Dziedzicka-Wasylewska, 2016). It has been demonstrated
575 that clozapine may increase the levels of these heterodimers in the PFC of mice, in contrast with
576 haloperidol that decreases their formation (Szlachta, et al., 2018). Using *in vitro* bioluminescence
577 energy transfer (BRET), fluorescence resonance energy transfer (FRET) techniques, and *in vivo*
578 Proximity Ligation Assay (PLA), dimerization of 5-HT_2Rs with D2Rs has been observed in rat
579 striatum (Borroto-Escuela, et al., 2014; Borroto-Escuela, et al., 2010; Lukasiewicz, et al., 2010).

Moreover, stimulation of 5-HT_{2A}R/D2R dimers with D2R agonists could be counteracted or inhibited by co-administration of 5-HT_{2A}-agonists, probably due to a 5-HT_{2A}R-mediated trans-inhibition of D2Rs, resulting in an enhanced G_q signaling over $G_{i/o}$ signaling (Borroto-Escuela, et al., 2010). Interestingly, an *in vitro* study has found that the 5-HT_{2A}R mutant H452Y, which is associated in humans with clozapine resistance, has a lower dimerization capacity with D2R compared to the wild-type (Łukasiewicz, Faron-Górecka, Kędracka-Krok, & Dziedzicka-Wasylewska, 2011). Taken together, these observations suggest that 5-HT_{2A}R/D2R heterodimers may be an example of asymmetrical and ligand-dependent cross-regulation that allows clozapine to exert its therapeutic effect (Maroteaux, Béchade, & Roumier, 2019). 5-HT_{2A}Rs can also form dimers with the metabotropic glutamate receptor 2 (mGluR2), as illustrated by *in vitro* and *in vivo* studies (González-Maeso, et al., 2008; Moreno, et al., 2012). As an effect of 5-HT_{2A}R/mGluR2 heterodimerization, G_i signaling downstream of mGluR2 is potentiated, while the G_q signaling from 5-HT_{2A}Rs is inhibited (Fribourg, et al., 2011). Clozapine, as opposed to hallucinogenic drugs, seems to be able to restore the correct balance between the G_i and G_q signaling pathways (Fribourg, et al., 2011). Although the functional consequences are not known in detail and their clinical relevance is still largely questioned, clozapine's ability to modulate these heterodimers should be considered as a putative additional mechanism for its unique antipsychotic property, and future investigations on heterodimers are clearly warranted.

Clozapine also binds with high affinity to 5-HT_{2C}Rs (Ki=29 nM), behaving as an inverse agonist at this site (Navailles, De Deurwaerdère, & Spampinato, 2006; Wenthur & Lindsley, 2013). Due to its action on 5-HT_{2C}Rs, clozapine is expected to increase dopamine and norepinephrine release in the PFC (Meltzer, 1999a), putatively responsible for antidepressant and pro-cognitive actions. Nonetheless, blockade of 5-HT_{2C}R receptors may also account for weight gain and metabolic disturbances associated with clozapine use (Montastruc, et al., 2015). Since the action on 5-HT_{2C}Rs is shared by multiple antipsychotic and antidepressant medications, it is unlikely that this mechanism may explain *per se* the unique clinical efficacy of clozapine.

Multiple behavioral, neurochemical, and electrophysiological investigations indicate that 5-HT_3Rs are implicated in the modulation of dopaminergic activity in mesolimbic and nigrostriatal pathways (Barnes & Sharp, 1999; Mylecharane, 1996; R. Y. Wang, Ashby, Edwards, & Zhang, 1994). This is the reason why the 5-HT_3R antagonists at could mimic the effects of antipsychotic drugs. Clozapine's moderate antagonism at 5-HT_3Rs (Ki= 241nM) may therefore possibly contribute to its antipsychotic effect (Hermann, et al., 1996; Rammes, et al., 2009; Wenthur & Lindsley, 2013).

More recently, a potential role of 5-HT_6Rs in the MOA of clozapine has been suggested. In fact, clozapine is a 5-HT_6R antagonist (Ki=17 nM) (Wenthur & Lindsley, 2013), and the antagonism at this receptor is believed to increase dopamine levels in the medial PFC (mPFC) (Lacroix, Dawson, Hagan, & Heidbreder, 2004) and hippocampus (Z. Li, Huang, Prus, Dai, & Meltzer, 2007). However, biological and clinical effects of antagonism at 5-HT_6Rs remain to be elucidated (Dawson, Nguyen, & Li, 2003), and a clue comes from the observation that administration of 5-HT_6R antagonists mitigates the pro-psychotic effects of MK-801 and PCP in animal models of SCZ (de Bruin, et al., 2013; Rodefer, Nguyen, Karlsson, & Arnt, 2008).

Similarly, clozapine has also a relevant affinity for 5-HT_7Rs (Ki=18 nM) (Wenthur & Lindsley, 2013) and regulates the internalization and subsequent degradation of this receptor (Andressen, et al., 2015). The role of 5-HT_7R blockade is still equivocal, but a selective 5-HT_7R receptor antagonist, SB-269970, has proved to be effective in ameliorating ketamine-induced attentional deficits and cognitive inflexibility (Nikiforuk, et al., 2013).

In summary, serotonergic receptors represent a relevant target for clozapine's MOA both for efficacy and reduced EPS liability (Tarsy, Baldessarini, & Tarazi, 2002). The receptor affinity ratio between D2Rs and 5-HT_{2A}Rs has been proposed as a key mechanism for the atypicality of clozapine, paving the way for other compounds sharing similar molecular properties. The formation of heterodimers opens a new chapter on the effects of antipsychotics with respect to the asymmetrical and ligand-dependent cross-regulation of multiple post-receptor signaling pathways. Nonetheless, the action on

631 other serotonin receptors, such as 5-HT_{1A}R, 5-HT_3R, 5-HT_6R, and 5-HT_7R, may be beneficial,
632 potentially, in treating negative symptoms of SCZ.
633
634 ***3.3 Acetylcholine receptors***

635 Clozapine significantly acts upon acetylcholine neurotransmission, through muscarinic receptors (R.
636 J. Miller & Hiley, 1974) (Fig. 2). Obara et al. described the strong anticholinergic potential of
637 clozapine in the cortex of mice, mediated by its binding to muscarinic receptors, which counteract
638 the clinical effects of cholinesterase inhibitors (Sadasiva, et al., 2019). Subjects with SCZ show a
639 selective reduction in the expression of muscarinic receptors (in particular M1 receptors) in the CNS
640 (Crook, Dean, Pavey, & Copolov, 1999; Crook, Tomaskovic-Crook, Copolov, & Dean, 2000, 2001;
641 Dean, Thomas, Lai, Chen, & Scarr, 2015) that could be responsible for cognitive impairment.
642 Therefore, reversing M1 expression could be a potential therapeutic target for antipsychotics
643 (Carruthers, Gurvich, & Rossell, 2015; Malkoff, Weizman, Gozes, & Rehavi, 2008; Meltzer, 2015).
644 The supposed efficacy for cognition with clozapine treatment seems to be at odds with clozapine's
645 antagonism at M1 (Ki= 4 nM), M3, and M5 receptors. There is some evidence, however, that the M1-
646 mediated cognitive improvement could be rather exerted by the agonism of the active metabolite
647 norclozapine, which behaves as a positive allosteric modulator at M1 muscarinic receptors (Ki=68
648 nM) (Fig. 3) (Chew, et al., 2008; Weiner, et al., 2004; Wenthur & Lindsley, 2013; Yohn & Conn,
649 2018). Of interest, positive allosteric modulation at M1 receptors can potentiate hippocampal N-
650 methyl-D-aspartate receptor (NMDAR) currents (Sur, et al., 2003). Furthermore, it has been
651 demonstrated that the administration of a full M1 agonist improved long-term depression, cognitive
652 functions, and social skills in mouse models of SCZ (Ghoshal, et al., 2016). Accordingly, a low
653 plasma clozapine/norclozapine ratio could enhance attention/vigilance, working memory, and social
654 cognition in patients with SCZ (Park, Kim, & Kim, 2020). In summary, norclozapine may therefore

contribute to clozapine's clinical effects by modulating both muscarinic and glutamatergic neurotransmission (Heusler, Bruins Slot, Tourette, Tardif, & Cussac, 2011; Islam, et al., 2021).

Clozapine is also a potent antagonist at M3 receptors (Ki=25 nM) (Wenthur & Lindsley, 2013). The role of these receptors in the pathophysiology of SCZ is unclear, but they are probably involved in the development of adverse effects, in particular of second-generation antipsychotic-induced type 2 diabetes (Weston-Green, Huang, & Deng, 2013). In fact, M3 is responsible for insulin release from the pancreatic β cells via G_q protein signaling (Ruiz de Azua, Gautam, Guettier, & Wess, 2011), and through a G protein-independent mechanism (via arrestin and PKD1 signaling, during the enteric digestive phase). The M3 receptor is also involved in the central regulation of insulin release in the hypothalamus and brainstem, affecting insulin levels through the parasympathetic vagal innervation of the pancreas (Weston-Green, et al., 2013). Therefore, M3 antagonism may be mostly implicated in adverse effects rather than the therapeutic efficacy of clozapine.

A preclinical study has demonstrated the involvement of M4 receptors in cognitive functioning (Galloway, Lebois, Shagarabi, Hernandez, & Manns, 2014) and in the prevention of hyperexcitability in midbrain dopamine neurons (Tzavara, et al., 2004). Clozapine acts as a M4 receptor antagonist (Ki=29 nM) in the rat striatum (Olianas, Maullu, & Onali, 1997; Wenthur & Lindsley, 2013), while its active metabolite norclozapine behaves as a M4 receptor agonist (Ki=170 nM) in the human neocortex (Gigout, Wierschke, Dehnicke, & Deisz, 2015; Wenthur & Lindsley, 2013), possibly contributing to sialorrhea (Zorn, Jones, Ward, & Liston, 1994). These findings are in contrast with early studies that traditionally recognized clozapine only as a muscarinic receptor antagonist. The M1-M4 receptor agonism appears to be an interesting pharmacological effect for antipsychotics since dopamine-acetylcholine balance is relevant to the expression of SCZ symptoms (H. E. Shannon, et al., 1999; H. E. Shannon, et al., 2000; Thomsen, Wess, Fulton, Fink-Jensen, & Caine, 2010). In support of this hypothesis, xanomeline, a selective agonist at M1 and M4 receptors, has been found to significantly reduce positive and negative symptoms, as measured by positive and negative syndrome scale (PANSS) in a recent double-blind randomized multicenter phase II trial (Dean &

Scarr, 2020). Nevertheless, patients in this trial were also acutely exacerbated, and the efficacy of M1/M4 muscarinic agonism for improving negative symptoms independent of improved secondary negative symptoms (Correll and Schooler 2020), i.e., in patients with predominant negative symptoms, remain to be examined.

Notably, the interaction between clozapine and M3/M4 receptors has proven useful for a new class of chemogenetically-engineered proteins, the designer receptors exclusively activated by designer drugs (DREADDs), respectively hM3Dq and hM4Di. These receptors are not activated by acetylcholine or other endogenous neurotransmitters, but exclusively by clozapine or clozapine N-oxide (CNO), an inert and inactive clozapine metabolite (Armbruster, Li, Pausch, Herlitze, & Roth, 2007). Several findings demonstrated that CNO does not cross the blood–brain barrier (BBB) and the observed effects are probably related to its back-conversion into clozapine and subsequent transport of clozapine into the brain (X. Chen, et al., 2015; Gomez, et al., 2017; Manvich, et al., 2018; Schotte, Janssen, Megens, & Leysen, 1993). This technological advance may also provide additional data on the neurobiology of clozapine.

Clozapine acts as an antagonist also at M5 receptors, although the neurobiology of this interaction has been limitedly studied, and clinical effects have not yet been fully elucidated (Zorn, et al., 1994). Of interest, single nucleotide polymorphisms (SNPs) in the M5 gene have been associated with the susceptibility to SCZ (De Luca, et al., 2004). In agreement, Thomsen et al. demonstrated the occurrence of hyperactivity and an impairment in PPI in mice with constitutive deletion of the M5 gene (Thomsen, et al., 2007). Clozapine was able to ameliorate these deficits in mice, indirectly implying that the M5 receptor subtype is not indispensable for its antipsychotic action. Rather, the absence of functional M5 receptors appears to confer increased sensitivity to clozapine (Thomsen, et al., 2007).

One of the most intriguing features of clozapine receptor profile is the action on α7 nicotinic receptors (Martin, Kem, & Freedman, 2004; Singhal, Zhang, Morales, & Oz, 2007). Reduced levels of α7 nicotinic receptors have been reported in the hippocampus of subjects affected by SCZ, and are

associated with impaired auditory gating (Lloyd & Williams, 2000). Noteworthy, clozapine has been found to normalize in a dose-dependent manner the auditory gating in rats precisely via α7 nicotinic receptors (Simosky, Stevens, Adler, & Freedman, 2003). Whereas subchronic administration of MK-801 has been found to reduce protein and gene expression of α7 nicotinic receptors in the hippocampus, clozapine treatment restored α7 expression and reversed cognitive deficits in male rats (Unal, Sirvanci, & Aricioglu, 2021).

Although typical antipsychotics are associated with cigarette smoking in patients with SCZ, clozapine appears to decrease nicotine use (George, Sernyak, Ziedonis, & Woods, 1995; J. McEvoy, et al., 1995a; J. P. McEvoy, Freudenreich, Levin, & Rose, 1995b; B. J. Wu, Chen, & Lee, 2013), probably due to its action at nicotinic receptors reducing the need to self-medicate with cigarette smoking. In the light of these findings, α7 nicotinic receptors attracted a lot of attention, and positive allosteric modulators at this site have recently been developed as an add-on strategy to mitigate cognitive symptoms of SCZ (Simosky, Stevens, & Freedman, 2002; Unal, Bekci, Cumaoglu, Yerer, & Aricioglu, 2020; Unal, et al., 2021).

The central and peripheral antimuscarinic affinity of antipsychotics is believed to be responsible for side effects, such as dizziness, drowsiness, confusion, blurred vision, and others (Lavrador, et al., 2021; J. A. Lieberman, 3rd, 2004). Moreover, muscarinic receptors are involved in the development of constipation and decreased peristalsis, which may worsen as a result of the antiadrenergic properties of clozapine, thus reducing intestinal perfusion and conferring the risk of intestinal ischemia (Palmer, McLean, Ellis, & Harrison-Woolrych, 2008). Beyond the agonism exerted by norclozapine at M1/M4, positive synergistic interaction with the vasoactive intestinal peptide (VIP) may also explain the unexpected propensity of clozapine to produce sialorrhea (Ekström, Godoy, Loy, & Riva, 2014; S. Ishikawa, et al., 2020).

Taken together, these observations point to the cholinergic receptors as candidate targets accounting for the unique superior efficacy of clozapine. In particular, muscarinic agonism of norclozapine at M1 and M4 receptor, as well as agonism of clozapine at α7 receptor, could exert beneficial effects on

cognition and psychotic symptoms. As confirmed by the positive results obtained with xanomeline combined with the peripheral anticholinergic trospium in a recent phase II clinical trial, M1/M4 muscarinic acetylcholine receptors may represent strategic targets for the development of novel antipsychotics with a mechanism unrelated to postsynaptic dopamine receptor occupancy.

3.4 Norepinephrine receptors

Clozapine has high affinity for α_{1A} (Ki=1.6 nM) and α_{1B} (Ki=7 nM) receptors and has a low-to-moderate affinity for α_{2C} (Ki=142 nM) receptors (Wenthur & Lindsley, 2013). Clozapine acts as an α_1 antagonist, a mechanism that contributes to the regulation of the firing of mesolimbic dopaminergic neurons, allowing positive symptom control (Svensson, 2003). The specific α_1 antagonist prazosin has been found to ameliorate the performance of rats pretreated with MK-801 in the active place avoidance task, a behavioral test that assesses spatial navigation and learning (Stuchlík, Petrásek, & Vales, 2009), suggesting a beneficial effect of α_1 receptor blockade on cognitive symptoms. On the other hand, α_1 antagonism could be responsible for severe hypotension when starting clozapine at too high a dose (Nourian, et al., 2008; E. Y. Yuen, Zhong, & Yan, 2010).

Moreover, clozapine's antagonism at α_2 receptors has been hypothesized to contribute to its clinical profile (Aringhieri, et al., 2018; Larrauri & Levin, 2012; Semenova & Markou, 2010), in particular to its antidepressant characteristics, which could underlie the effect of this compound in preventing suicide (Meltzer, et al., 2003). The molecular basis of this effect on mood that could be relevant for negative symptoms may lie in the fact that α_2 antagonists modulate the firing of dopamine neurons in the ventral tegmental area (VTA), thus inducing a net increase in dopamine in the PFC (Svensson, 2003). In particular, the antagonism at α_{2C} subtype has been related to antidepressant, antipsychotic, and procognitive effects in preclinical studies (Sallinen, et al., 2007; Sallinen, et al., 2013; Uys, et al., 2016). A recent line of research pointed out that α_{2C}, instead of α_{2A}, receptors are mainly involved in the regulation of GABA release in the striatum, playing an inhibitory role on GABA neurons

projecting to cortical pyramidal neurons. In this perspective, it follows that α_{2C} receptor antagonists may be beneficial in mitigating the "interneuronopathy" associated with SCZ (M. M. Uys, M. Shahid, & B. H. Harvey, 2017). In fact, α_{2C} receptor-selective antagonists have been found to ameliorate cognitive deficits and PCP-induced social interaction impairment (Dutra, Andreazza, Andreatini, Tufik, & Vital, 2002; Franowicz, et al., 2002; Ramos & Arnsten, 2007). Considering this evidence, we should emphasize that clozapine exhibits a selectivity for the α_{2C} receptor subtype, displaying a high α_{2C}/α_{2A} ratio, as well as one of the highest α_{2C}/D2R ratios among antipsychotics, which is believed to underlie clozapine's potential ability to mitigate negative symptoms (Kalkman & Loetscher, 2003; Savolainen, Ihalainen, Jalkanen, & Forsberg, 2019).

Therefore, available data may suggest that clozapine's α_1 receptor antagonism may affect positive symptoms by mitigating limbic hyperdopaminergia, whereas α_2 receptor blockade may be implicated in the decrease of negative symptoms by augmenting prefrontal dopaminergic activity, although independence of negative symptom improvement with clozapine from positive symptom improvement and lower risk for Parkinsonian adverse effects, both of which can ameliorate secondary negative symptoms (Correll and Schooler 2020) requires further clarification.

On the other hand, clozapine displays very low affinity to β_1 and β_2 receptors (Ki> 10000 nM). Thus, β_1 and β_2 receptors do not appear to be directly involved in clozapine's MOAs (Wenthur & Lindsley, 2013).

In summary, although the significance of the adrenoreceptor blocking properties of antipsychotics for treating SCZ still requires further investigations, the prominent action of clozapine at α_1 and α_2 receptors may globally stabilize the dopaminergic system and, at least partially, explain its clinical effectiveness, despite lower D2R occupancy.

3.5 Histamine receptors

Clozapine is a potent H1 receptor antagonist, showing high affinity (Ki=2 nM) (Sato, et al., 2015; Wenthur & Lindsley, 2013) at this site. This interaction is held responsible for several side effects of clozapine, including weight gain, sedation, orthostatic hypotension, and hypersalivation (Cardozo, et al., 2017; Fang, et al., 2016; S. F. Kim, Huang, Snowman, Teuscher, & Snyder, 2007; Kroeze, et al., 2003; Solismaa, et al., 2017). Sedation may, in turn, increase the risk of pneumonia (up to 20 times higher for clozapine compared to other antipsychotics) (Kuo, et al., 2013; Schoretsanitis, et al., 2021). In addition, H1 receptor antagonism may contribute to the risk of cerebral ischemia, as highlighted by a case-crossover study conducted in a cohort of SCZ patients exposed to different antipsychotics (W. Y. Chen, et al., 2019). On the other hand, Roegge et al. demonstrated that H1 receptor blockade is implicated in the improvement of sensorimotor gating and memory functions, as shown by results in PPI and radial-arm maze choice accuracy (RAM) tests in rats (Roegge, Perraut, Hao, & Levin, 2007), suggesting that the ability of clozapine to target the H1 receptor may be relevant for its antipsychotic action.

Clozapine H4 receptor agonism appears to be related to serious side effects, such as agranulocytosis (Goto, et al., 2016), while antagonism at H3 receptors may contribute to its overall clinical efficacy (Ito, 2009; Kathmann, Schlicker, & Göthert, 1994; Mahmood, Akhtar, Jahan, & Goswami, 2016; Rodrigues, Jansen, Leurs, Timmerman, & Prell, 1995). Probably, both H3 and H4 receptors could have a role in mediating complex interactions between multiple neurotransmitter systems involved in the regulation of appetite, satiety and food intake, thus explaining some of clozapine's cardiometabolic side effects, including body weight gain (Deng, Weston-Green, & Huang, 2010; Humbert-Claude, Davenas, Gbahou, Vincent, & Arrang, 2012).

Overall, the action on histaminergic receptors contributes only to a limited extent to the unique clinical efficacy profile of clozapine, while being substantially involved in multiple side effects of this agent.

3.6 Glutamate receptors and glycine transporter

Glutamate is the predominant excitatory neurotransmitter in the human CNS. Despite not having a direct action on ionotropic glutamate receptors, a role of clozapine in the modulation of NMDAR currents has been hypothesized. Clozapine could have intrinsic agonist or partial agonist activity at the glycine binding site (Glycine B-site) of NMDARs (Arvanov, Liang, Schwartz, Grossman, & Wang, 1997; Heresco-Levy, 2000; Kargieman, Santana, Mengod, Celada, & Artigas, 2007; Ninan, Jardemark, & Wang, 2003), but this hypothesis has not yet been experimentally confirmed (Schwieler, Linderholm, Nilsson-Todd, Erhardt, & Engberg, 2008). Furthermore, norclozapine dose-dependently potentiates NMDAR currents, as shown by an electrophysiology study in hippocampal slices (Sur, et al., 2003). Tanahashi et al. demonstrated that clozapine is responsible for an increase in glial D-serine and L-Glutamate, which act as NMDAR activators, in mPFC of rats (Tanahashi, Yamamura, Nakagawa, Motomura, & Okada, 2012). Williams et al. provided further evidence on glycine and clozapine interactions, showing that the glycine transporter 1 (GlyT1) in glial cells can be inhibited by clozapine (resulting in a net increase in glycine levels in the synaptic cleft) (Williams, Mallorga, Conn, Pettibone, & Sur, 2004). Moreover, clozapine increased glycine levels also by inhibiting sodium-coupled neutral amino acid transporter 1 (SNAT1), and eventually sodium-coupled neutral amino acid transporter 2 site (SNAT2) on neuronal cells (Javitt, et al., 2004; Schwieler, Engberg, & Erhardt, 2004). Since glycine is a known NMDAR co-agonist, clozapine-induced glycine increase in the synaptic cleft can enhance, in turn, NMDAR activation (de Bartolomeis, et al., 2020). This pro-glutamatergic effect may mitigate NMDAR hypofunction, which has been hypothesized as a key pathophysiological alteration in SCZ (Olney, Newcomer, & Farber, 1999). Clozapine's ability to modulate glutamatergic firing and mitigate NMDAR hypofunction may be relevant in the treatment of SCZ symptoms, as confirmed by the positive results in clinical trials obtained by sodium benzoate, an inhibitor of D-amino-oxidase (the enzyme that metabolizes D-amino acids) that enhances NMDAR function (Lin, et al., 2018).

Clozapine has been associated with changes in cortical and striatal NMDARs, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), and Kainate subunit composition in both

preclinical and postmortem studies (Hanaoka, et al., 2003; Healy & Meador-Woodruff, 1997). Although clinical implications of this subunit shift in ionotropic receptor subunits is not entirely clear, it may take part in clozapine's toxic as well as therapeutic effects.

Furthermore, clozapine has been shown to act on the inhibitory presynaptic metabotropic glutamate receptor 3 (mGluR3), thus preventing the hyperactivation of glutamatergic transmission between the mediodorsal thalamic nucleus (MDTN) and mPFC induced by administration of MK-801(Fukuyama, Kato, Murata, Shiroyama, & Okada, 2019).

On the other hand, clozapine's activity on glutamate transmission may also account for dose-dependent adverse events, such as convulsions, as suggested by Fukuyama et al. In fact, toxic concentrations of clozapine may activate astroglial hemichannels, which are crucial for both astrocyte-astrocyte communication, and neuron-astrocyte cross-talk (Orellana & Stehberg, 2014). Hemichannel activation could be responsible for a strong release of L-glutamate, resulting in a hyperglutamatergic state, and subsequent clozapine-induced seizures (Fukuyama, et al., 2019).

In summary, clozapine may affect the glutamatergic system in different ways, acting on several glutamatergic targets, namely receptors and transporters. This molecular action may be unique to clozapine among antipsychotics and may represent one of the key mechanisms for its unique clinical profile. Clozapine's action on glutamate release and regulation emphasizes glutamatergic targets in the development of novel antipsychotics, especially in the field of TRS, in which the other available antipsychotics are ineffective.

3.7 GABA receptors

Gamma-aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in the human CNS. GABAergic neurons play a crucial role in the maturation of neural circuitry during postnatal development and appear to contribute to the pathophysiology of psychiatric disorders, including SCZ (Benes, McSparren, Bird, SanGiovanni, & Vincent, 1991; Ghose, Winter, McCarson, Tamminga, &

Enna, 2011; Sands, Reisman, & Enna, 2004). In fact, several alterations in GABA neurotransmission have been detected in SCZ patients, including: i) morphological changes in cortical and hippocampal GABA interneurons (Benes, et al., 1991); ii) significant reduction in dorsal PFC expression of glutamic acid decarboxylase (GAD67), the GABA-synthesizing enzyme (Akbarian, et al., 1995); iii) marked reduction of GABA levels in cerebrospinal fluid in early-stage SCZ, positively correlated with the severity of symptoms (Orhan, et al., 2018); and iv) a reduction in $GABA_B$ receptor protein levels in the lateral cerebellum and other brain regions (M. Ishikawa, Mizukami, Iwakiri, & Asada, 2005; Mizukami, et al., 2000).

Clozapine exerts a pharmacological effect on the GABAergic system (O'Connor & O'Shea, 2015). In the beginning, clozapine was considered a $GABA_B$ positive allosteric modulator facilitating the binding of GABA (Y. Wu, et al., 2011). It has only recently emerged that clozapine may directly bind to the $GABA_B$ receptor at the GABA binding site (Nair, McKinnon, Miners, & Bastiampillai, 2020). This ability appeared quite attractive, also in the light of genetic findings supporting a significant association between variation in GABA-related genes (such as GAD1 and GABBR2) and TRS (Miyazawa, et al., 2022). However, it remains to be elucidated whether clozapine acts as an agonist or partial agonist at this site (Nair, et al., 2020).

Patients affected by SCZ generally have an impaired ability to filter extraneous sensory information, which is likely responsible for misattribution of salience to environmental stimuli, which has been related to an impaired firing of $GABA_B$ receptors (Adler, et al., 2004; Freedman, et al., 2000). Interestingly, unlike other antipsychotics, clozapine resulted in significant amelioration of signal-to-noise discrimination in SCZ patients (Daskalakis & George, 2009), probably by potentiating $GABA_B$-mediated inhibitory transmission. In this regard, an increase in the cortical silent period, an electrophysiological parameter positively correlated with GABA function, has been observed in TRS patients receiving clozapine compared to other antipsychotics. The underlying mechanism seems to involve an enhancement in $GABA_B$ neurotransmission (Miyazawa, et al., 2021).

884 Otherwise, in a preclinical study, Barbaccia et al. have shown that clozapine increases brain
885 concentrations of positive modulators at $GABA_A$ receptors, namely allopregnanolone and
886 allotetrahydrodeoxycorticosterone (Barbaccia, et al., 2001; Gee, McCauley, & Lan, 1995; Lambert,
887 Belelli, Hill-Venning, & Peters, 1995). Clozapine has also been reported to upregulate the GABA
888 transporter (VGAT) in the rat frontal cortex (Bragina, Melone, Fattorini, & Conti, 2007), which is
889 crucial for GABAergic function and contributes to the transmitter storage and release at GABAergic
890 synapses (De Gois, et al., 2005; Wojcik, et al., 2006).
891 In a recent study investigating the GABAergic system gene expression profile in patients with SCZ
892 receiving clozapine, GAD1, GAD67, GAD25 messenger ribonucleic acids (mRNAs) were found
893 significantly higher in peripheral blood lymphocytes (Sershen, et al., 2021). Upregulation of GABA
894 pathway genes may suggest an overall improvement in GABAergic function, probably mediating
895 clinical response in SCZ patients on clozapine treatment (Sershen, et al., 2021).
896 In summary, GABA dysfunctions in the PFC and hippocampus may represent crucial features of the
897 pathophysiology of TRS, and it has been argued that $GABA_B$ receptors may be a molecular target for
898 the action of clozapine. Unfortunately, no other antipsychotic agents specifically targeting the
899 GABAergic system are currently available, and further studies on the detailed binding mechanism,
900 the identification of the binding site, and the biological effects of clozapine at $GABA_B$ receptors have
901 the potential to provide a novel platform for designing novel psychopharmacological interventions
902 for TRS.
903

904 ***3.8 Sigma receptors***

905 Since their discovery, sigma binding sites have been the subject of investigations aimed at exploring
906 their functional role and their relevance for antipsychotic activity (Karbon & Enna, 1991). The
907 affinity of clozapine for σ receptors is very low (Ki>10000 nM) (Wenthur & Lindsley, 2013), but it
908 is conceivable that clozapine may indirectly exert a clinically relevant action on σ-related

transmission (Navarro, et al., 2010). The σ1 receptor is involved in many biological functions: it modulates Ca^{2+} signaling via inositol trisphosphate (IP3) (Hayashi & Su, 2007), activates ryanodine receptor (RyR) (Tagashira, Bhuiyan, & Fukunaga, 2013), and binds to many channels and receptors, such as voltage-gated K^+, Na^+, and Ca^{2+} channels, NMDAR, Rac-1 guanosine triphosphate hydrolase (GTPase), and, finally, D1Rs and D2Rs (Johannessen, et al., 2009; Kourrich, et al., 2013; Natsvlishvili, Goguadze, Zhuravliova, & Mikeladze, 2015; Navarro, et al., 2010; Navarro, et al., 2013; Pabba & Sibille, 2015; Tchedre, et al., 2008). Ovalle et al. demonstrated that the σ_1 receptor ligand E-5842 modulates the expression of fibroblast growth factor-2 (FGF-2) in the rat brain, which is also increased during learning process (Gómez-Pinilla, So, & Kesslak, 1998; Ovalle, Zamanillo, Andreu, Farré, & Guitart, 2001). Of interest, an increase in FGF-2 levels has been detected after chronic administration of clozapine (but not with other atypical antipsychotics) in the rat striatum. Based on these findings, we can conclude that the induction of FGF-2 is unique to clozapine among other antipsychotics and may possibly depend on interactions with the σ1 receptor downstream pathway. Since FGF-2 exhibits also a trophic and protective activity on dopaminergic neurons, allowing for their survival in Parkinson's disease models, clozapine's ability to induce FGF-2 may explain its low likelihood of causing clinically discernible EPS (Riva, Molteni, Tascedda, Massironi, & Racagni, 1999). In summary, although there is no evidence that clozapine binds to σ receptors *per se*, the downstream activated signaling pathways seem to converge, suggesting a potential interplay between physiological systems activated by sigma ligands and clozapine.

4. The lipophilic structure of clozapine and its role in clinical efficacy

Clozapine is a highly lipophilic drug with an experimentally derived distribution coefficient $LogD_{(pH\ 7.4)}$ of 2.754, a value allowing for its passive diffusion across the BBB, which is regarded as optimal to be readily and equally distributed into the CNS (Härtter, et al., 2003; van de Waterbeemd, Camenisch, Folkers, Chretien, & Raevsky, 1998). Furthermore, according to the "Overton rule" (Al-

Awqati, 1999), its lipophilicity may represent the driving force for cellular uptake through the plasma membrane, although there is also the possibility that its uptake may be a carrier-mediated process (Dickens, et al., 2018). Clozapine is a dibenzodiazepine derivative with a piperazinyl side chain that is rapidly absorbed orally with a bioavailability of 0.27 (Jann, 1991). Although clozapine is not likely to be accumulated or sequestered in the cell membrane in comparison to other lipophilic typical antipsychotics (Härtter, et al., 2003), as mentioned above, its lipophilic nature may at least influence the ability to cross the BBB (Fig. 5). Several studies have underlined a relevant structure-activity relationship, as well as the influence of plasma triglyceride and lipoproteins levels on clozapine's efficacy (Pande, Procyshyn, Nazerali, Attwood, & Chow, 2002; Procyshyn, Honer, & Barr, 2009). An *in vitro* study showed that clozapine is redistributed, depending on plasma triglyceride levels, from the lipoprotein-deficient fraction to the low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) fraction (Procyshyn, Kennedy, Marriage, & Wasan, 2001). Given the lipophilic nature of lipoproteins, this property could affect the pharmacokinetics of clozapine increasing its ability to cross the BBB (Procyshyn, et al., 2009; Procyshyn, et al., 2001). Moreover, clozapine's combination with lipoproteins may result in a kind of "physiological depot" from which the medication may be released in a protracted way (Fig. 5) (Yamamoto, et al., 2017). These preclinical observations seem to be confirmed by the higher efficacy of clozapine in patients with high plasma triglyceride levels (Dursun, Szemis, Andrews, & Reveley, 1999). For instance, several studies reported a significant association between increases in triglyceride levels and improvements in SCZ symptoms in patients treated with clozapine (Atmaca, Kuloglu, Tezcan, & Ustundag, 2003; Lally, et al., 2013; Procyshyn, et al., 2007). The association between serum lipids and the antipsychotic response appears to be more consistent during treatment with clozapine rather than with other antipsychotics (D. D. Kim, Barr, Fredrikson, Honer, & Procyshyn, 2019).

Interestingly, clozapine has recently been inserted into solid lipid nanoparticles (SLNs) to improve the oral bioavailability of the compound. In this study, Manjunath and Venkateswarlu demonstrated

a 2-4-fold higher bioavailability and a better tissue distribution of clozapine incorporated in SLNs compared to the suspension (Manjunath & Venkateswarlu, 2005).

In summary, the lipophilic properties of clozapine and its combination with lipoproteins, which allows it to act as an intracellular depot and be released in a prolonged manner, may potentially contribute to its unique effectiveness.

5. Clozapine's intracellular mechanisms of action

One of the most replicated findings of clozapine's intracellular effects is the differential activation of transcription factors belonging to the class of immediate early genes (IEGs), including *c-Fos, Arc, Zif268,* and *Homer1a*, as well as long-acting early genes (e.g., *DeltaFosB*) in comparison to other antipsychotics, which has led to the characterization of its unique "molecular fingerprint" (Hiroi & Graybiel, 1996; Polese, de Serpis, Ambesi-Impiombato, Muscettola, & de Bartolomeis, 2002). In fact, clozapine has been shown to activate IEGs, such *c-Fos* and *Zif-268* preferentially in the PFC and accumbens, whereas the prototypical typical antipsychotic drug haloperidol and other potent D2R antagonists induce the activation of IEGs predominantly in the striatum (de Bartolomeis, et al., 2017). Furthermore, when administered subchronically or chronically, clozapine, unlike haloperidol, is capable of inducing DeltaFosB (Robertson, et al., 2004).

The "early" description of clozapine's impact on IEGs has been more recently been re-examined by innovative techniques of cellular biology unveiling novel and unsuspected targets of clozapine. For instance, in transgenic "FosTRAP" mice, a fluorescent reporter marks the cells responsive to antipsychotic administration. With this technique, acute administration of clozapine has been shown to induce *c-Fos* in cortical regions and ependymal cells. In particular, ependymal cells seem to be highly sensitive to clozapine, even in the absence of 5-HT_{2A}R (Joshi & Panicker, 2018). However, the exact meaning of these findings for clozapine's MOAs that help improve TRS are unclear, soliciting further investigation of clozapine's intracellular signaling.

5.1 Neuroprotective actions

Emerging findings at the cellular level have highlighted that clozapine may act through non-canonical biological mechanisms, different at least in part from other antipsychotics, involving various protein kinases resulting in a number of potentially relevant neuroprotective effects: 1) increase in hippocampal neurogenesis (Halim, Weickert, McClintock, Weinberger, & Lipska, 2004); 2) prevention of apoptosis, proteolytic degradation, and DNA fragmentation in neuronal cells that promote cortical atrophy (Abekawa, Ito, Nakagawa, Nakato, & Koyama, 2011; Bai, Zhang, & Li, 2004; Lundberg, et al., 2020; Qing, Xu, Wei, Gibson, & Li, 2003); 3) mitigation of the neuroinflammatory response (L. K. Green, et al., 2017) and inhibition of microglia activation (Jiang, et al., 2016); 4) regulation of protein degradation (J. H. Jeon, et al., 2021) in order to achieve an optimal homeostatic balance and remove misfolded proteins (Chong, et al., 2004; S. H. Kim, et al., 2018); and 5) release of neurotrophins, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), supporting survival, differentiation, and connectivity functions, as well as preventing metabolic or excitotoxic injuries in neurons (Bai, Chlan-Fourney, Bowen, Keegan, & Li, 2003; Ghosh, Carnahan, & Greenberg, 1994; Parikh, Khan, Terry, & Mahadik, 2004; Shao, Dyck, Wang, & Li, 2006; Turner, Rembach, Spark, Lopes, & Cheema, 2003).

It has been recently observed in a transcriptome analysis in human-induced excitatory neurons that clozapine massively affects the expression of genes involved in cholesterol metabolism and biosynthesis. Exposure to clozapine leads to a similar upregulation of lipogenesis-related genes also in glial-like cells (Fernø, et al., 2005). Of interest, cholesterol is a major component of neuronal membrane and myelin, influencing the activity of many membrane-bound proteins including ion channels, transporters, and receptors, which are the primary elements in multiple signaling pathways (Pfrieger, 2003). Overall, these results suggest that clozapine may have some neuroprotective actions

in regulating myelin formation, membrane fluidity, and composition in neurons as well as in glial cells, which may relate to its unique efficacy profile.

5.1.2. Action upon growth factors: brain-derived neurotrophic factor

BDNF is believed to influence the survival of dopaminergic, cholinergic, and serotonergic neurons, being involved in the synaptic plasticity regulation and counterbalance of maladaptive plastic changes within brain circuitries (Ninan, 2014), which are linked to the cognitive dysfunction of SCZ. A significant decrease of BDNF has been reported in the hippocampus of postmortem brains tissue from patients affected by SCZ (Durany, et al., 2001). Chronic treatment with typical antipsychotics results in decreased expression of BDNF (Bai, et al., 2003; Xiu, et al., 2009), while dopamine agonists (i.e., levodopa) and 5-HT_{2A}R antagonists (i.e., ketanserin) are known to be capable of upregulating BDNF (Okazawa, Murata, Watanabe, Kamei, & Kanazawa, 1992; Vaidya, Marek, Aghajanian, & Duman, 1997). Unlike first-generation antipsychotics, it has been argued that clozapine, due to its fast-off properties at D2Rs and strong 5-HT_{2A}R antagonism, may restore BDNF signaling by increasing its expression in several brain regions (Pedrini, et al., 2011). BDNF and its receptor TrkB are among the target genes of the transcription factor cyclic cyclic adenosine monophosphate response element binding protein (CREB) (Nibuya, Nestler, & Duman, 1996), and the entire BDNF-CREB signaling pathway seems to be implicated in clozapine response. In fact, chronic administration of clozapine may counterbalance the dysregulation in the BDNF/TrkB signaling in animal models of depression and increase CREB mRNA expression in frontal cortex and hippocampus of rodents (Einoch, et al., 2017; Yang, et al., 2020). Since CREB is responsible, in turn, for the induction of NGF, clozapine's ability to upregulate CREB signaling may support the hypothesis that clozapine may take part in the processes of neuronal differentiation and neurite outgrowth (S. Jeon, Kim, Chung, & Kim, 2015).

5.1.3 Clozapine and CREB dependent signaling: the Akt and GSK connection

Signaling cascades converging on CREB and targeted by clozapine involve two major upstream kinases, Akt and its substrate glycogen synthase kinase 3 (GSK-3β), which is phosphorylated and then inhibited by Akt. GSK3 phosphorylates around 40 different substrates including Activator protein 1 (AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), Heat shock factor 1 (HSF-1), CREB, p53, β-catenin, which are gene expression regulators influencing survival, cell structure, and spine shape remodeling (Zeng, et al., 2017). Individuals with SCZ usually exhibit lower levels of Akt and reduced phosphorylation of GSK-3β in brain and peripheral lymphocytes (Olianas, Dedoni, Ambu, & Onali, 2009) compared to healthy subjects. Noteworthy, clozapine is capable of activating Akt (Alimohamad, Rajakumar, Seah, & Rushlow, 2005a; Alimohamad, Sutton, Mouyal, Rajakumar, & Rushlow, 2005b; Kozlovsky, Amar, Belmaker, & Agam, 2006; Xi, et al., 2011) and enhancing the inhibitory phosphorylation of GSK-3β in PFC, striatum, and ventral midbrain (M. R. Ahmed, Gurevich, Dalby, Benovic, & Gurevich, 2008; Kenakin, 2012; Takaki, et al., 2018). Therefore, clozapine's effect on the GSK-3β cascade may be crucial in regulating dendritic spine density and morphology(Samuels, Saitta, & Landreth, 2009), thereby contributing to those changes in synapse conductance, which are at the basis for long-term synaptic plasticity underlying learning and memory functions. Although this molecular mechanism may be extremely promising for deepening our understanding of clozapine action, many other antipsychotics have been reported to exert an action on the Akt-GSK3 pathway, albeit with multiple regional and molecular differences. Hence, the exact relevance of action on the Akt-GSK3 pathway for antipsychotic efficacy remains to be further elucidated.

5.1.4 Clozapine and extracellular signal-regulated kinase (ERK) signaling

Among other post-receptor MOAs, clozapine has been found to affect the function of ERK, a kinase belonging to the mitogen-activated protein (MAP) kinase family. Several studies have investigated the differential ability of antipsychotics in modulating ERK activity, pointing to a superiority of

clozapine over other tested agents in augmenting ERK signaling (Pereira, Zhang, Malcolm, & Sundram, 2013). A study by Aringhieri et al., reported that ERK1/2 phosphorylation increased up to four-fold from baseline after clozapine exposure in HeLa cells [347]. The same authors have proposed that clozapine-induced ERK1/2 activation may be mediated by 5-HT_{2A}R agonism, and that clozapine could then behave as an agonist at this site, despite being widely recognized as a 5-HT_{2A}R antagonist. This paradoxical activity would be explained by a mechanism known as "biased agonism", through which clozapine might act either as agonist or antagonist at 5-HT_{2A}Rs, selectively recruiting specific effectors such as β-arrestin or others and activating intracellular pathways that are independent of G proteins [355]. Hence, the higher efficacy of clozapine in the activation of ERK1/2 [356], which is relevant for neuronal connectivity, synaptogenesis, and plasticity [347, 357], might account for some differential therapeutic effects of clozapine versus other antipsychotics.

5.2 Clozapine's putative antiproliferative action

Clozapine has been proposed to have putative antineoplastic/antimitotic features. In fact, clozapine's agonism at the H4 receptor has recently attracted increasing interest as a potential adjuvant anticancer target for the treatment of human breast cancer and metastatic melanoma (Martinel Lamas, et al., 2013; Massari, et al., 2013; Massari, et al., 2017). Furthermore, clozapine has been found to decrease, in a dose-dependent manner, the growth/survival rates in cultures of cancer cells, via direct inhibition of ErbB kinases (Kobayashi, et al., 2019). Interestingly, impairments in the epidermal growth factor (EGF)/ErbB system have been largely associated with SCZ (Shamir, et al., 2012), and ligands for ErbB1 and ErbB4 are known to induce behavioral deficits, such as impaired sensorimotor gating, reduced mismatch negativity, amphetamine-induced dopamine hypersensitivity, and reduced social drive (Futamura, et al., 2003; Jodo, et al., 2019; Mizuno, et al., 2007; N. Tsuda, et al., 2008). Therefore, the kinase-inhibitory activity impacting non-canonical pharmacological pathways, may

account for clozapine's unique antipsychotic effects. On the other hand, these mechanisms may explain also some serious adverse effects shared with other chemotherapy agents, such as myocarditis and agranulocytosis (Dang, et al., 2016).

5.3 Clozapine putative anti-inflammatory action

Among theories of SCZ pathophysiology, the vulnerability-stress-inflammation model and the genetic microvascular-inflammatory disease model have been conceptualized in recent years (Hanson & Gottesman, 2005; N. Müller, 2018), also in light of the evidence of pro-inflammatory cytokine dysregulation, abnormal microglia activation, and the potential advantage of certain anti-inflammatory medications found in SCZ (Rothermundt, Arolt, & Bayer, 2001). Several studies have proposed the role of clozapine as an anti-inflammatory and immunomodulatory drug within the CNS (Al-Amin, Nasir Uddin, & Mahmud Reza, 2013; Leykin, Mayer, & Shinitzky, 1997; Maes, et al., 1997; Ribeiro, et al., 2019; Sugino, Futamura, Mitsumoto, Maeda, & Marunaka, 2009). In fact, by preferentially activating the anti-inflammatory docosahexaenoic acid (DHA) cascade over the arachidonic acid (AA) cascade (H. W. Kim, Cheon, Modi, Rapoport, & Rao, 2012), and inhibiting Ca^{2+}/CaM/Akt-mediated nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) activation (Seol, Kuo, & Kim, 2004), clozapine treatment exerts a net anti-inflammatory action. Likewise, clozapine prevents degranulation of mast cells, immune cells resident in the CNS that orchestrate inflammatory processes (Szuster-Ciesielska, Słotwińska, Stachura, Marmurowska-Michałowska, & Kandefer-Szerszeń, 2004). Moreover, clozapine treatment can affect levels of cytokines, which are relevant humoral mediators of the immune response. For instance, clozapine enhances the production of anti-inflammatory cytokines such as interleukin (IL)-10, IL-1 receptor antagonist (IL-1RA), leukemia inhibitory factor receptor (LIF-R) (Maes, et al., 2002; Song, Lin, Kenis, Bosmans, & Maes, 2000; Sugino, et al., 2009; Szuster-Ciesielska, et al., 2004), IL-4 (Himmerich, et al., 2011), and reduces the levels of pro-inflammatory IL-8 (Möller, et al., 2013) in

cerebrospinal fluid. Moreover, clozapine significantly suppresses interferon (IFN)-γ production in peripheral blood mononuclear cells by inhibiting the Th1 cell-differentiation processes (M. L. Chen, et al., 2012).

Recently, clozapine has been tested in animal models of multiple sclerosis, revealing its ability to reduce infiltration of peripheral immune cells (monocytes, neutrophils, and T cells) into the CNS (Robichon, Patel, Connor, & La Flamme, 2020). In fact, clozapine seems to directly target resident microglia and macrophages (Okazaki, et al., 2021), reducing the release of chemokines CCL2 and CCL5 in the brain and spinal cord, subsequently resulting in impaired migration of immune cells, and an overall mitigation of the disease severity (Robichon, et al., 2020). Clozapine has also been found to enhance the rate of functional recovery in an animal model of demyelination, being capable of modulating cellular events surrounding demyelination and remyelination by reducing astrocyte and microglial activation (Templeton, Kivell, McCaughey-Chapman, Connor, & La Flamme, 2019), as well as inducing glial cell lipogenesis (Fernø, et al., 2005; Fernø, Skrede, Vik-Mo, Håvik, & Steen, 2006). Additionally, clozapine is known to correct tryptophan metabolism in the rat model of SCZ, by diverting away from the production of neurotoxic metabolites such as quinolinic acid (Möller, Du Preez, Emsley, & Harvey, 2012).

In summary, the role of anti-inflammatory effect in clozapine's MOAs is still overlooked, and further studies are warranted especially in light of the increasing recent evidence of a potential involvement of inflammation and immune dysregulation in SCZ and in TRS (Chauhan, Kaur, Prasad, & Singh, 2021; Leboyer, et al., 2021; Mohd Asyraf, Nour El Huda, Hanisah, Norsidah, & Norlelawati, 2022).

5.4 Nuclear receptors targeted by clozapine: focus on retinoid receptors

Retinol regulates many dynamic processes involved in the formation, stabilization, and pruning of synapses (Cao, et al., 2020; L. Chen, Lau, & Sarti, 2014; Hsu, Li, Wu, Südhof, & Chen, 2019; Zhong, Chen, Park, Südhof, & Chen, 2018). In fact, whereas in the early stages of life the synapse formation

prevails, during adolescence the pruning of little used synapses is a key process underlying physiological adaptations allowing for correct neurodevelopment (Batool, et al., 2019). Synapse pruning appears to be accelerated in SCZ (Hall, Trent, Thomas, O'Donovan, & Owen, 2015), and several authors suggest, among other mechanisms, a disturbed retinoid signaling at the basis of this alteration (J. Feng, et al., 2005; Reay, et al., 2020; Wan, et al., 2006). Furthermore, it is noteworthy that D2Rs, the main pharmacological target of antipsychotics, are under transcriptional control of retinoids (Samad, Krezel, Chambon, & Borrelli, 1997). Clozapine and norclozapine have been found to inhibit the catabolism of *all-trans* retinoic acid (at-RA), an active metabolite of Vitamin A, both in clinical and preclinical models (Regen, et al., 2021). It has been demonstrated that patients affected by SCZ have lower serum levels of at-RA and retinol compared to healthy subjects. Of interest, clozapine, unlike other antipsychotics, has been found to increase at-RA levels in SCZ patients (Regen, et al., 2021). Clozapine appears to also be responsible for an increase in transcription of transthyretin, a retinol carrier protein, in the cortex of rodents (M. L. Chen & Chen, 2007).

In light of the "retinoid hypothesis of SCZ", the ability of clozapine to considerably impact retinoid signaling may disclose a novel approach to treat psychotic symptoms (Goodman, 1998). As an example, clinical trials investigating the effects of bexarotene, a synthetic retinoid agonist, as adjuvant treatment for SCZ, provided promising results on positive symptoms (Lerner, et al., 2008; Lerner, et al., 2013).

Despite the preliminary nature of these findings, clozapine's capability of targeting retinoid receptors may shed light on a novel potential antipsychotic MOA worth to be explored (Lerner, McCaffery, & Ritsner, 2016). Moreover, considering the role of retinoids in regulating D2R expression, it is tempting to consider clozapine's action on retinoid receptors as a potential mechanism that further contributes to the overall beneficial effect on dopamine function.

6. Clozapine's effect on synaptic plasticity and post-synaptic density proteins

The pathophysiology of SCZ has been related to altered connectivity within and between multiple brain regions, which may reflect, in turn, abnormalities in dendritic spines, small protrusions from dendritic shafts crucial in receiving synaptic inputs and recognized as the locus of synaptic plasticity events (Konrad & Winterer, 2008). Because of the onset of SCZ during adolescence or early adulthood, three hypotheses are plausible: reduced dendritic spine density in SCZ could result from either a failure to elaborate normal numbers of dendritic spines in early development, or from more rapid elimination of dendritic spines during adolescence, or a combination of both mechanisms together may contribute to the pathophysiology of SCZ (Feinberg, 1982; Keshavan, Anderson, & Pettegrew, 1994; McGlashan & Hoffman, 2000; Moyer, et al., 2016). Preclinical findings demonstrated that clozapine administration results in synaptic plasticity augmentation in the hippocampal-mPFC pathway, probably via a D1R-dependent mechanism (Matsumoto, et al., 2008). Moreover, clozapine is also able to upregulate Neuregulin-1 in rat mPFC, a trophic factor that contains an epidermal growth factor domain implicated in neurodevelopment and synaptic plasticity (Chana, et al., 2009). The role of clozapine in neuroplasticity is also confirmed by its ability to regulate calcium homeostasis and cytoskeleton rearrangements via the Rho/Cdc42 signal pathway in neuronal and glial cells (M. L. Chen, Tsai, Lee, & Lin, 2016; Kedracka-Krok, et al., 2015; Kedracka-Krok, et al., 2016). Clozapine's effects on synaptic plasticity are reflected by changes in PSD composition. In fact, the PSD is a specialized ultrastructure that is detected by electron microscopy as a thickness of glutamatergic synapses, which is believed to act as a molecular switchboard for multiple intracellular signaling. The PSD is constituted by different orders of layered molecules including receptors (i.e., NMDAR, AMPAR, mGluRs type I), scaffolding proteins (i.e., PSD-95, Shanks, Homers, Arc), cytoskeleton proteins (i.e., tubulin, actin, α-internexin), and enzymes (Suzuki, Kametani, Guo, & Li, 2018). PSD proteins have been reported to be involved in the pathophysiology of psychosis by genome-wide association study (GWAS) analyses conducted on 60,000 participants from the Psychiatric Genomics Consortium (N. a. P. A. S. o. P. G. Consortium, 2015). In fact, the risk variants associated with SCZ were found to aggregate especially in the biological pathway of the

PSD and /or in those regulating immune response and epigenetic mechanisms, as already suggested by exome sequencing and array comparative genomic hybridization (array-CGH) studies (Fromer, et al., 2014; Kirov, et al., 2012; Ting, Peça, & Feng, 2012). PSD rearrangements have been demonstrated in preclinical studies to be responsive to antipsychotics administration, alone (de Bartolomeis, et al., 2019; de Bartolomeis, et al., 2015; de Bartolomeis, Latte, Tomasetti, & Iasevoli, 2014; de Bartolomeis, Sarappa, et al., 2013; Iasevoli, et al., 2014; Iasevoli, Tomasetti, & de Bartolomeis, 2013) or in combination with other psychotropic agents (Buonaguro, et al., 2017; Dell'aversano, Tomasetti, Iasevoli, & de Bartolomeis, 2009; Tomasetti, Dell'Aversano, Iasevoli, Marmo, & de Bartolomeis, 2011).

Clozapine differentially affects the gene expression of post-synaptic proteins compared to other antipsychotics (Iasevoli, et al., 2011; Polese, et al., 2002; Purkayastha, et al., 2012; Takaki, et al., 2018). One of the most striking differences occurring in PSD after antipsychotic administration is the pattern of Homer1a expression, which is induced mainly in prefrontal and cortical regions after acute clozapine exposure, while being expressed primarily in the striatal regions after potent D2R blockers such as haloperidol or ziprasidone (Barone, et al., 2021; Iasevoli, et al., 2011). Since Homer1 proteins have been reported to be involved in the shaping and maintenance of dendritic spines (Sala, et al., 2003), the differential action of clozapine compared to other antipsychotics may be crucial for synaptic plasticity.

Clozapine also significantly increased Shank1 expression in primary and secondary dendritic spines of rat hippocampal neurons, whereas haloperidol, conversely, induced a reduction. Even if it is premature to draw conclusions from these preclinical findings, it is intriguing to speculate that a putative alteration of Shank1 proteins can be counterbalanced by clozapine treatment (Lennertz, et al., 2012).

Furthermore, clozapine has been found to directly bind to α and β tubulin heterodimers, preventing their polymerization to microtubules in HeLa cells (Hino, et al., 2021). Since disturbances in microtubule networks have been observed in schizophrenia, including increased ratio of polymerized

tubulin responsible for reduced dynamic properties of microtubules (Benítez-King, et al., 2016), clozapine may restore the dynamic instability of cytoskeletal structures.

However, clozapine's peculiar ability to induce the expression of immediate early genes involved in PSD composition recruiting the cortical structures may parallel its meaningful effects on restoring the synaptic plasticity processes underlying cognitive functioning.

7. Potential predictors of response to clozapine: current insight on pharmacogenomic and pharmacokinetic-related studies

Although clozapine is currently considered the only therapy for TRS, approximately 30-60% of TRS do not fully respond even to clozapine, and such patients are termed "clozapine-resistant", "super-refractory" or "ultra-resistant" (Siskind, Siskind, & Kisely, 2017; D. M. Taylor & Duncan-McConnell, 2000). Pharmacogenomic studies over the last 30 years have attempted to address challenges related to the prediction of both resistance and favorable response to clozapine, for the purpose of a timelier introduction of this antipsychotic in patients where it is expected to be effective. The initial research on genetic determinants of response to clozapine was mainly focused on candidate genes, chosen *a priori* based on the putative MOAs of clozapine. Thus, several authors have investigated the polymorphisms, functional or not, of dopamine and serotonin receptors as well as transporter genes. However, most of the statistically significant genetic associations reported with clozapine response have not been confirmed by replication analyses.

Given the action of clozapine on D4Rs, the first gene that has been investigated was the DRD4 gene (Shaikh, et al., 1993), although with negative results. In fact, several studies failed to find a significant association between clozapine response and repeat length variations or other polymorphisms of the DRD4 gene (Hong, Lee, Sim, & Hwu, 1997; Hwang, et al., 2012; Kaiser, et al., 2000; Kohn, et al., 1997; Rao, et al., 1994; Rietschel, et al., 1996; Shaikh, et al., 1993; Shaikh, et al., 1995; A. L. Zhao, et al., 2005). Patients carrying the Ser9Gly polymorphism in the DRD3 gene (Barlas, et al., 2009;

Jönsson, et al., 2003; Malhotra, et al., 1998; Schaeffer, et al., 2014; Shaikh, et al., 1996; Szekeres, et al., 2004) appeared to exhibit a poorer response to clozapine compared to non-carrier subjects. Nonetheless, a meta-analysis by Hwang et al. pointed to a lack of association between the DRD3 Ser allele and response to clozapine (Hwang, et al., 2010).

Several studies have explored the role of polymorphisms in the DRD1, DRD2, DRD4, and DAT genes (S. W. G. o. t. P. G. Consortium, 2014; Lencz, et al., 2006; Xu, et al., 2010), reporting a significant association of few variants with poor response to clozapine (J. P. Zhang, Lencz, & Malhotra, 2010). Exploratory studies by Hwang et al. (Hwang, et al., 2005; Hwang, et al., 2006) showed that some DR2 polymorphisms may explain the interindividual variability of clozapine response in African-American patients with SCZ. Of interest, Potkin et al. (Potkin, et al., 2003) found significant regional brain metabolic changes associated with DRD1 gene variants, that parallel substantial clinical improvements during clozapine treatment. However, evidence supporting the role of genetic variations of the D1R is conflicting (de Matos, Santana, & Souza, 2015; Hwang, et al., 2007).

Bosia et al. investigated the impact of polymorphisms of catechol-O-methyltransferase (COMT) and 5-HT_{1A}R genes on the clinical response, showing that clozapine-treated patients carrying COMT Val/Val and 5-HT_{1A}R G/G genotypes achieved greater improvement in the negative symptom domain (Bosia, et al., 2015). Another gene-gene interaction analysis showed that patients with the COMT Val/Met or Met/Met genotype, with a concomitant DRD4 polymorphism, had a significantly better clinical response to clozapine compared to non-carriers (Rajagopal, Rajkumar, Jacob, & Jacob, 2018). Several genetic variants in 5-HT_{2A}R, 5-HT_{2C}R, 5-HT_{6}R and serotonin transporter (SERT) genes have been described as being associated with clozapine's superior efficacy (M. Arranz, et al., 1995b; M. J. Arranz, et al., 2000b; M. J. Arranz, et al., 1996; M. J. Arranz, et al., 1998b; Harvey, et al., 2003; Kohlrausch, et al., 2010; Masellis, et al., 1998; Yu, et al., 1999). However, associations lacked consistent replications (C. H. Chen, et al., 1997; Lin, et al., 1999; Malhotra, Goldman, Ozaki, Breier, et al., 1996; Malhotra, Goldman, Ozaki, Rooney, et al., 1996; Masellis, et al., 2001; Masellis, et al.,

1995; Nöthen, et al., 1995; Rietschel, et al., 1996; Shinkai, et al., 1998; Sodhi, et al., 1995; Verga, Macciardi, Cohen, Pedrini, & Smeraldi, 1997). Lack of association has been reported with variants of 5-HT_{3B}R genes (Gutiérrez, et al., 2002). Discrepancies have also been observed with respect to the polymorphisms within the 5-HT_{3A}R gene and clozapine response (Souza, de Luca, Meltzer, Lieberman, & Kennedy, 2010a), which may vary consistently depending on the definition of the "clinical response" outcome (Rajkumar, et al., 2012).

Negative findings have been reported from pharmacogenetic studies investigating genes encoding adrenergic and histaminergic receptors (Bolonna, et al., 2000; Mancama, et al., 2002). Although controversial, a positive association between TNF-α and Neurexin1 polymorphisms and clozapine response has been proposed (Souza, Meltzer, Lieberman, Le Foll, & Kennedy, 2010; Tsai, Hong, Yu, Lin, & Liu, 2003; G. Zai, et al., 2006). Although the role of BDNF variants in modulating clozapine response is still uncertain, several SNPs have been found to be associated with clozapine resistance (Hong, Yu, Lin, & Tsai, 2003; J. P. Zhang, et al., 2013). Moreover, genetic variability in genes encoding other trophic factors, such as neurotrophic receptor tyrosine kinase 2 and glial-derived neurotrophic factors may also underlie differences in individual clinical response to clozapine (Mitjans, et al., 2015; Souza, Romano-Silva, et al., 2010).

Concerning second messenger proteins, there is suggestive evidence that the C825T polymorphism of the G-protein beta3 subunit gene (GNB3) could influence the response to antipsychotics, including clozapine (Kohlrausch, et al., 2008; D. J. Müller, et al., 2005). The relevance of variations in ABCB1 gene has been supported by several studies (S. T. Lee, et al., 2012; M, et al., 2020).

The rs2740204 polymorphism in the oxytocin (OXT) gene has been found to be significantly associated with clozapine response (Souza, de Luca, Meltzer, Lieberman, & Kennedy, 2010b), a finding set in the context of mounting evidence implicating the oxytocin signaling pathway in SCZ. Moreover, the rs2535629 of the Inter-Alpha-Trypsin Inhibitor Heavy Chain 3 (ITIH3) gene has been associated with improvements in negative symptoms after six months of clozapine treatment (Brandl, et al., 2016).

Given the role of the glutamatergic system in SCZ and its possible involvement in clozapine's MOAs, several studies investigated the effects of genetic variation within the NMDAR subunits (GRIN1, GRIN2A, and GRIN2B) genes in clozapine response (Hwang, et al., 2011; D. L. Taylor, et al., 2016), albeit reporting negative results.

There is limited evidence supporting the role of genetic variations within the human leukocyte antigen system (HLA) chromosomal area and clozapine response. In fact, Lahdelma et al. showed that the HLA-A1 allele was significantly associated with response to clozapine (Lahdelma, et al., 1998; Lahdelma, et al., 2001).

Indeed, as well as for many other traits of psychiatric disorders, clozapine response is likely to be explained by the cumulative effect of the alleles of many polymorphisms associated with different degrees of magnitude. It is not surprising that none of these single polymorphisms have been able to predict clozapine response alone, and models of a cumulative combination of genetic variants in several neurotransmitter pathways should be investigated.

A seminal approach in this direction has been made by Arranz et al. (2000a), who conducted association studies in multiple neurotransmitter-receptor related genes, showing that a combination of six SNPs resulted in 76.7% success in the prediction of clozapine response, although these findings have not been replicated by further studies (Schumacher, Schulze, Wienker, Rietschel, & Nöthen, 2000). Clearly, large-scale genome-wide investigations can lead to the identification of candidates that need to be tested for replication in smaller samples to verify their clinical utility. However, despite the need to explore the determinants of clozapine response at a genomic level, there is still a paucity of evidence from GWAS.

Poor response to clozapine may be also accounted for by extensive or ultra-rapid metabolism (Eap, et al., 2004), particularly in those patients who do not reach the minimum threshold of clozapine plasma levels of 350 ng/L, as well as those who early develop a toxic rise in plasma concentrations, such as approximately 10% of Asian population which may fall into the poor metabolizer category (Ruan, et al., 2019). Polymorphisms that affect the activity of cytochrome P450 isoenzymes may

strongly influence the pharmacological response to clozapine, by interfering with elimination and biotransformation processes. For instance, homozygosity for CYP1A2*1F polymorphism, the most common variant of the enzyme primarily involved in clozapine metabolism, has been associated with a 2.4-fold reduction in treatment response in a European sample (Balibey, et al., 2011). This genotype seems to be observed more frequently in ultra-resistant SCZ patients and ultra-rapid metabolizers with low-plasma clozapine levels while receiving an adequate dose of the drug, suggesting that CYP1A2*1F may act as a moderator of clinical response (Balibey, et al., 2011). Since CYP1A2 activity is a main determinant for clozapine clearance, it follows that the time course of plasma levels of clozapine and its major metabolites can strongly affect the clinical response. A retrospective pharmacokinetic study showed that clozapine non-responders usually exhibit clozapine plasma levels below the value of 260 ng/L and higher levels of N-desmethylclozapine, using N-demethylation as the preferred metabolic route (Fabrazzo, et al., 2002). In this regard, it should be underlined that among conditions responsible for clinically significant interactions there is the well-known influence of smoking on clozapine plasma levels, due to smoking-induced CYP1A2 liver enzyme activity (de Leon, 2004; Y. Tsuda, Saruwatari, & Yasui-Furukori, 2014). For the same reason, in the case of smoking cessation, a dosage adjustment is usually required to avoid clozapine toxicity (Kocar, Freudenmann, Spitzer, & Graf, 2018; Schaffer, Yoon, & Zadezensky, 2009). Moreover, it has been reported that smoking TRS patients had significantly higher scores than non-smokers in the PANSS score and performed worse in problem-solving cognitive tasks (Iasevoli, Balletta, et al., 2013), probably due to the ability of nicotine to increase dopamine release in the mesocortical and mesolimbic pathways. In summary, monitoring clozapine plasma levels is currently essential to identify potential pharmacokinetic implications of suboptimal clozapine responses, whereas genetic and other biomarker information might in the future help to predict pharmacodynamic efficacy of clozapine treatment.

8. Structural and functional neuroimaging findings associated with clozapine treatment

Clozapine has been suggested to trigger remodelling effects on cortical and subcortical brain architecture, as demonstrated by clozapine-specific changes in functional and non-functional neuroimaging findings. Indeed, clozapine has been reported to affect cortical thickness, grey and white matter volume, metabolic activity of discrete brain regions as well as interregional connectivity. Although the above-mentioned effects have been interpreted as neuroprotective, longitudinal magnetic resonance imaging (MRI) studies showed that clozapine induces a wide range of anatomical alterations: volume reductions in the caudate nucleus (Chakos, Lieberman, Alvir, Bilder, & Ashtari, 1995; Frazier, et al., 1996; Scheepers, de Wied, et al., 2001; Scheepers, Gispen de Wied, Hulshoff Pol, & Kahn, 2001), thalamus, hippocampus, and putamen, as well as the enlargement of lateral ventricles (Tronchin, et al., 2020), a decrease in cortical thickness (Mattai, et al., 2010; Molina, Taboada, Aragüés, Hernández, & Sanz-Fuentenebro, 2014), and gray matter loss (N. Liu, et al., 2020). Noteworthy, clozapine-related cortical thinning and subcortical volume reduction could even predict, according to a few studies, better clinical outcomes and improvements in cognitive symptoms (Molina, et al., 2014; Tronchin, et al., 2020) suggesting that these apparently structural derangements may represent an adaptive process rather than a harmful effect (de Bartolomeis, Barone, Begni, & Riva, 2022).

In contrast with other reports by structural MRI, Molina et colleagues detected a net increase in grey matter volume of frontal, parietal, and occipital lobes after chronic clozapine treatment, together with a reduction in the white matter volume of parietal and occipital lobes (Molina, Reig, et al., 2005), suggesting the co-occurrence of grey matter increase and white matter decrease, and a potential complementarity of these structural changes.

Moreover, PET and Single Photon Emission Computed Tomography (SPECT) studies revealed that clozapine increased perfusion in basal ganglia (Buchsbaum, et al., 1992; A. C. Lahti, Holcomb, Weiler, Medoff, & Tamminga, 2003), thalamus, temporal, and occipital regions while reducing

limbic as well as prefrontal metabolism (Machielsen, Veltman, van den Brink, & de Haan, 2018; Mier, et al., 2019; Potvin, et al., 2015; Remijnse, et al., 2006; Schirmbeck, et al., 2015). Although the correction of limbic hyperactivity is consistent with theoretical expectations, the reduction in prefrontal activity should be hopefully alleviated rather than induced by antipsychotics (Molina, Gispert, et al., 2005; Molina, Sanz, Sarramea, & Palomo, 2007; J. Zhao, He, Liu, & Yang, 2006). On the other hand, Ertugrul et al. detected an increase in the right frontal (superior and medial)/caudate perfusion ratio in patients receiving clozapine, associated with improvement in cognitive domains, suggesting that clozapine may relatively decrease striatal perfusion in favour of perfusion in the frontal lobes (Ertugrul, et al., 2009). In line with these findings, Lahti and colleagues reported the activation of the anterior cingulate and dorsolateral frontal cortex after exposure to clozapine, which was not detectable with other antipsychotics (A. C. Lahti, et al., 2003). However, these results have not been replicated, whereas the frequently reported reduction in frontal activity has been explained by the high clozapine D1R/D2R affinity ratio and the extensive expression of D1Rs over D2Rs in the PFC. In fact, in contrast to D2Rs, D1Rs are a G_s-coupled receptors that stimulate adenylyl cyclase, and their blockade may perhaps contribute to frontal hypometabolism (R. M. Cohen, et al., 1997). On the other hand, it should be noted that, despite its high affinity for D1Rs, it has not yet been clarified whether clozapine acts as an antagonist or rather as an agonist at this site, and the hypofrontalism may be primarily related to the TRS condition.

Whole-brain functional Magnetic Resonance Imaging (fMRI) in TRS patients stabilized on clozapine displayed hyperactivation of the dorsomedial PFC in response to neutral emotional stimuli, compared to non-TRS and healthy subjects (Potvin, et al., 2015). However, it is possible that these findings, suggesting an abnormal salience assignment to irrelevant stimuli, are the effect of TRS, rather than the direct effect of clozapine use. fMRI studies have highlighted other clozapine effects. For instance, it has been proposed that clozapine's ability to weakly increase orbitofrontal cortex (OFC) activation during attentional tasks and reduce left amygdala activation in response to emotional stimuli, may be related to its "pro-obsessive" effect, namely the likelihood of inducing or worsening obsessive-

compulsive symptoms in SCZ patients (Mier, et al., 2019; Remijnse, et al., 2006; Schirmbeck, et al., 2015). Moreover, a recent fMRI study disclosed the role of clozapine in mitigating craving for drugs and cue reactivity for cannabis-related stimuli in patients with SCZ and comorbid cannabis use disorder, exerting a greater decrease in amygdala activation compared to patients receiving risperidone (Machielsen, et al., 2018).

Furthermore, a diffusion tensor imaging (DTI) study, evaluating structural connectivity in the white matter of patients receiving antipsychotics, showed that 12-week clozapine treatment increased a parameter known as "fractional anisotropy" (FA), a proxy measure of white matter integrity, in widespread brain regions (Ozcelik-Eroglu, et al., 2014). In addition, this neuroimaging finding positively correlated with improvement in semantic fluency, leading to the conclusion that clozapine may ameliorate cognitive functions by reversing discrete microstructural connectivity alterations observed in SCZ (Ozcelik-Eroglu, et al., 2014). A cross-sectional study supported these results, showing that patients treated with clozapine for five years exhibited increased FA in the anterior region of corpus callosum compared to patients who had never been treated with antipsychotics (Tao, et al., 2021) On the other hand, a recent 6-month longitudinal DTI study questioned the hypothesis that clozapine may act by restoring white matter integrity: a significant reduction in the FA of corpus callosum and corona radiata was reported by Tronchin and collaborators (Tronchin, et al., 2021), pointing to progressive white matter abnormalities in TRS patients apparently unaffected by clozapine treatment (Matrone, et al., 2022), although it is unclear if the progression would have been even worse without clozapine treatment.

Magnetic resonance spectroscopy (MRS) studies have helped to understand previously unnoticeable clozapine effects. Riehmann et al. showed that clozapine treatment decreased the intracellular pH value in the right frontal lobes, probably via the α2 receptor-mediated inhibition of protein kinase C (Riehemann, Hübner, Smesny, Volz, & Sauer, 2002). Although the clinical meaning of this result is not entirely clear, clozapine may affect the control of the pH value that is crucial to ensure biochemical cell functions. Of interest, McLoughlin and colleagues detected increases in lactate

levels associated with clozapine exposure, suggesting the ability of clozapine to affect cerebral energy metabolism and potentially lead to a glycolytic shift (McLoughlin, et al., 2009), which may be consistent with the acidifying effects observed by Riehemann et al. (Riehemann, et al., 2002). Moreover, MRS studies provided details related to the major brain metabolites and their variations as a consequence of clozapine treatment. In this perspective, several MRS studies failed to identify significant differences in N-acetylaspartate (NAA) levels, which are assumed to be a neuronal function index, after clozapine treatment (Lindquist, Dunn, & Cecil, 2011; Szulc, et al., 2007). Furthermore, preclinical evidence suggested that clozapine might reduce glutamate levels in the PFC and hippocampus (McLoughlin, et al., 2009), which is opposite to the changes that have been detected in TRS patients. McQueen and colleagues observed a reduction in glutamate content in the caudate of individuals suffering from TRS after 12-week clozapine treatment, which was positively correlated with improvements in psychotic symptomatology (McQueen, et al., 2021). The persistently high glutamate content in the anterior cingulate cortex has been suggested to represent a stable neurobiological trait of resistance to antipsychotics, including clozapine (Matrone, et al., 2022).

In other words, clozapine may play an anti-glutamatergic role, by mitigating the disinhibition of pyramidal neurons, preventing glutamate-mediated excitotoxicity, which may otherwise result in cell damage and cortical thinning (Shah, et al., 2020; Snyder & Gao, 2013).

In summary, few relevant conclusions can be drawn: i) structural MRI showed a complex array of brain rearrangements, with substantial differences from one region to another and with contrasting effects on white and gray matter volumes, partially explaining the inconsistency in reports of volumetric changes after clozapine exposure; ii) it is not entirely clear what impact clozapine has on metabolic activity in frontal cortical regions, since reduced prefrontal metabolism is frequently reported in brain PET scans of TRS subjects; iii) clozapine treatment has been associated with a wide array of neuroprotective effects, as confirmed by disparate neuroimaging techniques, such as improving white matter integrity, decreasing craving-related amygdala activation, and reducing glutamate concentrations in PFC, thus potentially preventing glutamate-induced excitotoxicity.

9. Putative mechanisms of clozapine-related side effects

Although data support superior efficacy of clozapine in comparison to other antipsychotic drugs for TRS (Essali, Al-Haj Haasan, Li, & Rathbone, 2009; Glick, et al., 2011; J. P. McEvoy, et al., 2006), clozapine treatment is often delayed and limited by its potentially serious adverse effects, with up to 17% of patients being forced to discontinue treatment (Grohmann, Rüther, Sassim, & Schmidt, 1989). Clozapine's most relevant side effects include sedation, hypotension, reduction in seizure threshold (Fitzsimons, Berk, Lambert, Bourin, & Dodd, 2005), weight gain and metabolic abnormalities (Newcomer, 2005), constipation, pneumonia, myocarditis (Layland, Liew, & Prior, 2009), and rare but potentially life-threatening agranulocytosis (Honigfeld, Arellano, Sethi, Bianchini, & Schein, 1998). Most of the clozapine-induced side effects are assumed to derive from its unique pharmacological action, as described in the next paragraphs.

9.1 Agranulocytosis, blood dyscrasias, and immune system dysfunctions

Clozapine-induced neutropenia (1.500-500/mmc granulocytes) and, especially, agranulocytosis (neutrophils < 500/mmc) are rare but very severe adverse effects, constraining the use of this medication. In fact, a systematic literature review of case reports suggested the possibility of clozapine rechallenge in patients developing neutropenia, but not agranulocytosis (Manu, Lapitskaya, Shaikh, & Nielsen, 2018; Manu, Sarpal, Muir, Kane, & Correll, 2012; Nielsen, Correll, Manu, & Kane, 2013).

The discussion about clozapine-induced idiosyncratic agranulocytosis (CIA) is still open and genetic, toxic, or immunological mechanisms are thought to be involved (Frimat, et al., 1997; Palmblad, Papadaki, & Eliopoulos, 2001). Gerson et al. suggested the possibility of direct toxic action of norclozapine (Gerson, Arce, & Meltzer, 1994), while other authors showed that an oxidized metabolic intermediate of clozapine may play a role in this adverse event by increasing Fas ligand expression

with subsequent apoptosis in polymorphonuclear leukocytes (Husain, et al., 2006). As a result of clozapine oxidation by myeloperoxidase to a chemically reactive nitrenium ion (Iverson, Kautiainen, Ip, & Uetrecht, 2010; Maggs, Williams, Pirmohamed, & Park, 1995; Mosyagin, Dettling, Roots, Mueller-Oerlinghausen, & Cascorbi, 2004; Pirmohamed, Williams, Madden, Templeton, & Park, 1995), the agent may play direct toxic effects on both bone marrow cells and leukocytes. In fact, the nitrenium ion may promote granulocyte apoptosis or lead to membrane alterations, thus triggering an immune-mediated reaction (Uetrecht, 1992). In this respect, the association of an antioxidant drug such as N-acetylcysteine has recently been proposed in clozapine-treated patients to address safety concerns related to clozapine-induced oxidative stress and to encourage clozapine use when indicated (Chrétien, et al., 2021; Polydoro, et al., 2004; Reinke, et al., 2004). Although recognized as an idiosyncratic effect depending on the balance between clozapine bioactivation and detoxification (Rattay & Benndorf, 2021), it has also been proposed that the toxicity on bone marrow stromal cells may be dose- and titration speed-related (Pereira & Dean, 2006).

A reduction in gene expression of dihydronicotinamide riboside quinone oxidoreductase 2 (NQO2), which is involved in the detoxification of clozapine, may take part in the development of this adverse event (Ostrousky, et al., 2003). An elevation of soluble IL-2 receptor (SIL-2R) during treatment with clozapine would also imply the involvement of this cytokine (Maes, Meltzer, & Bosmans, 1994; Pollmächer, Hinze-Selch, Mullington, & Holsboer, 1995) and the immune system. Moreover, as mentioned before, clozapine's potential antiproliferative action carried out by targeting ErbB kinase, could help to understand this serious adverse effect shared with other anti-cancer medications (Kobayashi, et al., 2019).

The best evidence for genetic variants in components of the immune system, underlying an increased susceptibility to CIA, points to genes belonging to the pathways of nicotinamide adenine dinucleotide (NAD^+), glyoxylate, dicarboxylate, and drug metabolism (Platanić Arizanović, et al., 2021), as well as HLA. CIA has been associated with HLA-B38, DR4, DQW3 (J. A. Lieberman, et al., 1990), HLA-Cw*7, DQB *0502, DRB1 *0101, and DRB3 *0202 (Dettling, Cascorbi, Roots, & Mueller-

Oerlinghausen, 2001). A recent GWAS and exome-sequencing analysis emphasized the role of two independent loci: HLA-B and HLA-DQB1 (Goldstein, et al., 2014), opening the way to the clinical application of screening tests. As shown by the analysis of case-control studies, SNPs in HLA-DQB1 (6672G>C or "REC 21G"), may confer a 16.9-fold increase in the risk of developing CIA (Athanasiou, et al., 2011). Although these data have been sufficiently replicated, most of the patients who develop CIA are not carrier of risk-alleles, thus, further causative mechanisms still need to be explored.

Although clozapine treatment causes agranulocytosis in only very few cases, it may also display relevant effects in the opposite direction on neutrophil counts and bone marrow. In fact, clozapine may increase granulopoiesis as well as induce the release of neutrophils from the bone marrow into the circulation, leading to transient neutrophilia, particularly at the initial stage of the therapy (Lobach & Uetrecht, 2014). Furthermore, clozapine has been found to affect platelet differentiation, aggregation, and increase clot formation time, presumably via inhibition of the pathway downstream of the purinergic receptors P2Y1 and P2Y12, thereby determining a potential risk of hemorrhaging in patients on chronic clozapine treatment (Dietrich-Muszalska, Rabe-Jabłońska, & Olas, 2010; C. C. Wu, et al., 2016). It is still unknown whether other hematological or cardiovascular side effects induced by clozapine may be related to its ability to decrease platelet aggregability, and further studies are required. In contrast to this line of research, preclinical evidence pointed out that antagonism at 5-HT_{2A}Rs, and the subsequent activation of MAPK and fibrinogen may play a role in clozapine-induced venous thromboembolism (Axelsson, Hägg, Eriksson, Lindahl, & Whiss, 2007; Gligorijević, et al., 2020).

Clozapine appears also to be associated with abnormal humoral immunity, and a novel striking association was observed between the use of clozapine and secondary antibody deficiency, which accounts for the elevated risk of respiratory infection/sepsis during clozapine treatment (Ponsford, Pecoraro, & Jolles, 2019), with the risk of pneumonia being potentially increased through the unique clozapine side effect of sialorrhea. A cross-sectional case-control study has shown recently that

patients receiving clozapine had a substantial reduction in serum immunoglobulins (IgG, IgA, and IgM) compared to the clozapine-naïve group, and that the risk of hypogammaglobulinemia increased over time, being positively correlated with the duration of clozapine treatment (Ponsford, et al., 2019).

9.2 Clozapine-induced Myocarditis

Clozapine therapy may be associated with potentially fatal myocarditis and cardiomyopathy, even in physically healthy young adults with SCZ (Kilian, Kerr, Lawrence, & Celermajer, 1999). Clozapine-induced myocarditis (CIM) has an incidence of approximately 3% among treated patients and typically occurs within 3-4 weeks after starting clozapine (Ronaldson, Fitzgerald, & McNeil, 2015). Clinical and demographic risk factors may be rapid dose titration, concomitant use of sodium valproate, older patient age, a comorbid neurodevelopmental disorder (Iasevoli, Barone, Buonaguro, Vellucci, & de Bartolomeis, 2020; Ronaldson, et al., 2012). Although the exact mechanism underlying CIM is still unclear, it has been hypothesized that clozapine induces an IgE-mediated hypersensitivity reaction, as supported by the presence of myocardial eosinophilic infiltrates in biopsy tissues (Ronaldson, et al., 2010) and hypereosinophilia (circulating blood eosinophil count above 1.500/μL), occurring after 2-3 weeks of treatment (J. F. Wang, et al., 2008). CIM has also been associated with an increased release of inflammatory cytokines (Basel A. Abdel-Wahab, Abdalla, & El-khawanki, 2014a; Haack, et al., 2003) and redox imbalance (Nikolić-Kokić, et al., 2018). In fact, the histopathology of clozapine-treated mice showed a prominent myocardial inflammation, which is positively correlated with clozapine doses, TNF-α levels, and eosinophilia (Elman, et al., 1999). Other immunohistochemical findings include the increase in cardiac levels of TNF-α, nitric oxide, myeloperoxidase, caspase-3, NF-κB, p65, 8-OHdG (a marker of DNA damage) (B. A. Abdel-Wahab & Metwally, 2014b, 2015), and MDA (a lipid peroxidation marker) (F. Zhang, et al., 2021). A study investigating the cardiotoxic effects of clozapine on zebrafish embryos reported reduced activity of

antioxidant enzymes, such as catalase and superoxide dismutase after acute clozapine exposure, suggesting a clozapine-induced impairment in the ability to scavenge free radicals (F. Zhang, et al., 2021).

Moreover, clozapine treatment has been also associated with increased levels of norepinephrine and epinephrine (Brown, et al., 1997; Fleischhaker, Schulz, & Remschmidt, 1998; A. I. Green, et al., 1993; Krentz, Mikhail, Cantrell, & Hill, 2001), and these findings are of great relevance given that hypercatecholaminergia has been associated with myocarditis in both animals and patients (J. F. Wang, et al., 2008). In addition, Arzuk et al. demonstrated that cardiac mitochondria may be primarily targeted by clozapine cardiotoxicity. In fact, clozapine is largely distributed in the heart and may be converted into reactive metabolites in cardiac mitochondria, leading to a reduction of oxygen consumption rate (Arzuk, Karakuş, & Orhan, 2021).

As mentioned above, clozapine blockade of the cardiac Neuregulin-1/Erb2 might also be implicated in CIM (Dang, et al., 2016). Of interest, clozapine has been found to induce Connexin43 translocation at the plasmatic membrane and activate the Connexin43-associated channel in myocardial cells. Since this protein is upregulated in the early stages of cardiomyopathies, it has been proposed as one potentially responsible mechanism for CIM (Fukuyama, Okubo, Murata, Shiroyama, & Okada, 2020). In addition, both clozapine and olanzapine treatment have been found to disrupt spliceosome signaling, leading to multiple alternative splicing events in mouse hearts resulting in the dysregulation of molecular pathways underlying cardiac remodeling (J. Wang, et al., 2021).

A GWAS identified novel SNPs associated with a significantly increased risk of developing CIM, including a polymorphism located within the intron 2 of the GNA15 gene (Lacaze, et al., 2020), coding for a $G_{a/q}$ protein and found in some GPCRs, such as adrenergic, endothelin, and angiotensin II receptors, which is involved in the regulation of cardiac output and hemodynamic parameters (Ronaldson, et al., 2015). Moreover, the HLA class I allele HLA-C*07:01, already associated with CIA, has also been found to confer an increased risk of CIM. A polygenic risk score generated through the weighted contribution of 96 different SNPs revealed the strong genetic liability underlying CIM,

being able to explain about 66% of the susceptibility to develop this potentially fatal side effect (Lacaze, et al., 2020).

9.3 Weight gain and metabolic disorders

Clozapine is associated with a high risk of metabolic syndrome and one of the largest increases in weight gain among antipsychotics, including in children and adolescents (Allison, et al., 1999; Maayan & Correll, 2011; Vancampfort, et al., 2015). Clozapine-induced weight gain (CIWG) contributes to non-adherence to treatment and to the risk of developing dyslipidemia, diabetes mellitus type II, cardiovascular diseases, and related medical conditions (Leung, Barr, Procyshyn, Honer, & Pang, 2012; Masuda, Misawa, Takase, Kane, & Correll, 2019; Nielsen, Skadhede, & Correll, 2010; Umbricht, Pollack, & Kane, 1994). Many mechanisms have been hypothesized to be associated with weight gain: alterations in neurotransmitter and neuroendocrine system, dysregulation of neuropeptides, such as leptin, ghrelin, and neuropeptide Y (NPY). In particular, preclinical studies have shown that CIWG may be mediated by clozapine-induced reduction of NPY mRNA in striatum, anterior cingulate cortex, and accumbens (X. F. Huang, Deng, & Zavitsanou, 2006; Kirk, Cahir, & Reynolds, 2006; Palmiter, Erickson, Hollopeter, Baraban, & Schwartz, 1998). The histaminergic system is implicated in the regulation of food intake and energy (Yoshimatsu, 2006). H1 receptor antagonism results in increased food intake (Han, Deng, Burne, Newell, & Huang, 2008; Sakata, Yoshimatsu, & Kurokawa, 1997) and impairment in insulin action and energy sensing (Kowalchuk, Kanagasundaram, Belsham, & Hahn, 2019). Several studies found a correlation between antipsychotic H1 receptor affinity and weight gain (Kroeze, et al., 2003; Matsui-Sakata, Ohtani, & Sawada, 2005). Clozapine and olanzapine, for instance, activate the AMP-protein kinase (AMPK) via H1 receptor blockade, leading to an increase in food intake (Minokoshi, et al., 2004). Furthermore, clozapine could induce weight gain due to its antagonism on H3 receptors located on noradrenergic and cholinergic neurons (Schlicker & Marr, 1996), in turn increasing norepinephrine

and acetylcholine neurotransmitters that can act as orexigens (Kurose & Terashima, 1999). While histaminergic antagonism may account for weight gain, M3 and 5-HT_{2A} receptor antagonism are believed to be responsible for the diabetogenic side effects (Joshi, Singh, & Panicker, 2019; X. Liu, et al., 2017; Weston-Green, et al., 2013). In fact, the blockade of M3 may inhibit the acetylcholine pathway controlling insulin secretion (Sacks, et al., 2018). It should be noted that the ganglionic blocker mecamylamine, a non-selective, non-competitive antagonist of the nicotinic receptor, as well as β_1 and β_2 antagonists, may effectively mitigate clozapine-induced insulin resistance, suggesting that peripheral catecholamines may also play a role in the development of glucometabolic adverse events (J. W. Y. Yuen, et al., 2021).

Furthermore, blockade of hypothalamic 5-HT_{2C}Rs, mediating appetite regulation and satiety response, is likely to contribute to CIWG and metabolic disturbances (Montastruc, et al., 2015; Reynolds, Hill, & Kirk, 2006). CIWG has been investigated in relationship to genetic risk factors [(J. P. Zhang, et al., 2016). For example, CIWG has been related to the 759C/T polymorphism in the promoter region of the 5-HT_{2C}R gene (D. D. Miller, Ellingrod, Holman, Buckley, & Arndt, 2005; Reynolds, Zhang, & Zhang, 2003; Tsai, Hong, Yu, & Lin, 2002), and the rate of homozygous carriers among obese subjects receiving clozapine has been found to be significantly higher compared to non-obese subjects treated with clozapine (Gunes, Melkersson, Scordo, & Dahl, 2009). Noteworthy, clozapine showed the ability to increase motivation for food, independently of any concomitant experience of satiety, in preclinical studies. In fact, even rats with ad libitum access to food displayed a significant increase in motivation to work for food after clozapine exposure (Abela, Ji, Li, Lê, & Fletcher, 2020). This effect, stable over repeated testing, cannot be explained by clozapine antagonism at a single receptor, but seems to be related to a combined action at multiple binding sites (Abela, et al., 2020). Inflammatory cytokines have also been implicated in weight gain (Contreras-Shannon, et al., 2013; C. Zhang, Zhang, Cai, Chen, & Song, 2017; Zimmermann, Kraus, Himmerich, Schuld, & Pollmächer, 2003), and CIWG has been associated with increased levels of TNF-α, IL-1β, IL-6, soluble tumor necrosis factor receptors 1 and 2 (sTNFR-1 and sTNFR-2) (Brömel, et al., 1998),

as well as plasma leptin (Hägg, Söderberg, Ahrén, Olsson, & Mjörndal, 2001; Kluge, et al., 2009; Kraus, et al., 1999), yet, these alterations are likely consequences rather than mechanisms of CIWG. Of interest, clozapine may promote the differentiation of pre-adipocytes and the morphological changes accompanying the upregulation of the mature adipocyte markers (Cottingham, Patrick, Richards, & Blackburn, 2020). Clozapine administration may also upregulate major brown and beige adipocyte marker gene (UCP1), perhaps via inhibiting 5-HTRs, modifying the differentiation program of human adipocyte progenitor cells (Kristóf, et al., 2016), and inducing a developmental shift from white to beige adipocytes. Clozapine-induced beige cells display smaller lipid droplets, higher levels of Ucp1 protein, increased oxygen consumption, but exhibit lower sensitivity to anti-obesity cues, contributing to CIWG (Kristóf, et al., 2016).

Since a GWAS found the $GABA_A$ receptor subunit α2 (GABRA2) among the candidate genes responsible for CIWG, the involvement of the GABA pathway in the development of CIWG has been hypothesized (C. C. Zai, et al., 2015). GABRA2 is a target gene of rno-miR-200a-3p, a microRNA (miRNA) found to be modulated by clozapine administration in an animal model of SCZ (W. Huang, et al., 2021). These data make rno-miR-200a-3p a promising therapeutic target for the management of metabolic comorbidities in TRS patients (W. Huang, et al., 2021).

To what degree metabolic disturbances during antipsychotic treatment are solely the consequence of the weight gain has long been debated, but current evidence suggests that at least some antipsychotics, including clozapine, may directly increase insulin resistance and fasting triglyceride levels, even in the absence of weight gain (De Hert, Detraux, van Winkel, Yu, & Correll, 2011; Stahl, Mignon, & Meyer, 2009). Of interest, an *in vitro* study showed that clozapine may induce mitochondrial dysfunctions in cultured beta-cells, namely an increase in mitochondrial membrane fluidity and polyunsaturated fatty acid content, resulting in beta-cell apoptosis and suppression of cell proliferation (Elmorsy, et al., 2021; C. H. Huang, et al., 2012). On the other hand, chronic clozapine treatment has been found to induce noticeable histopathological abnormalities in pancreatic islets of rats, including beta-cell hyperplasia, irregularities in morphology, sprouting of new islets from pre-

existing ones (Abdelrahim, 2013). Possibly, both effects underlie the diabetogenic adverse events induced by clozapine.

Liu and colleagues showed that chronic exposure to olanzapine and clozapine led to deranged Akt signaling and GSK3β phosphorylation in liver tissues of female rats, resulting in reduced insulin responsiveness and thereby in glucose intolerance (Aoki, et al., 2012; El-Seweidy, Sadik, Malek, & Amin, 2014; X. Liu, et al., 2017). Compared to ziprasidone and sertindole, 4-week clozapine treatment was found to induce more severe alterations in liver histopathology as well as in antioxidant defense enzyme activity (Platanić Arizanović, et al., 2021).

Clozapine treatment significantly increased the protein levels of several nuclear transcription factors, including sterol regulatory element-binding protein 1c and 2 (SREBP-1c and SREBP-2), resulting in an up-regulation of hepatic H1 receptors, and carbohydrate-responsive element-binding protein (ChREBP), a glucose sensor that activates *de novo* lipogenesis in the liver (X. Liu, et al., 2017). Since clozapine-treated female rats displayed all these metabolic abnormalities without changes in body weight, it can be suggested that impaired glucose-lipid homeostasis induced by clozapine may be, at least partly, independent of adiposity.

In summary, many receptors targeted by clozapine appear to be implicated in metabolic disturbances, including H1, H3, 5-HT_{2A}R, 5-HT_{2C}R, M3, and $GABA_A$ receptors, and other possible mechanisms involved in weight gain and diabetogenic side effects may be related to inflammatory processes, mitochondrial dysfunction, and oxidative stress.

9.4 Reduced seizure threshold

Seizures are a dose-dependent side effect that may occur during the initiation phase of clozapine treatment. Nonetheless, even low-dose clozapine may cause minor electroencephalographic (EEG) abnormalities (Varma, Bishara, Besag, & Taylor, 2011). The exact mechanism through which clozapine lowers the threshold for generalized seizures (Hedges, Jeppson, & Whitehead, 2003) still remains unclear.

A polymorphism at the CYP1A2 gene has been significantly associated with reduced seizure threshold induced by clozapine (Bolla, et al., 2011). This SNP has been associated with lowered CYP1A2 mRNA expression in circulating lymphocytes, which may predispose to clozapine intolerance (Ferrari, et al., 2012). Another putative mechanism may derive from the non-competitive antagonist action of clozapine at the glycine receptor (GlyR) (Kohlrausch, et al., 2013). In fact, since GlyR function may partially overlap with those exerted by $GABA_A$, clozapine antagonism at inhibitory GlyRs may result in seizure-like activity (Y. Liu, et al., 2009; Lozovaya, Yatsenko, Beketov, Tsintsadze, & Burnashev, 2005). Clozapine-induced EEG abnormalities include a generalized slowing, especially involving theta and delta waves, as well as spikes and sharp activity (Malow, et al., 1994; Schuld, et al., 2000; Treves & Neufeld, 1996; Welch, Manschreck, & Redmond, 1994). A positive correlation has been observed between clozapine plasma levels and the occurrence of EEG alterations (Varma, et al., 2011). Furthermore, overall sleep architecture and cortical sleep EEG generation mechanisms appear to be affected by exposure to clozapine (Tsekou, et al., 2015).

On the other hand, clozapine is also capable of normalizing dysfunctional high gamma oscillations in a dose-dependent manner, either in chronic or acute paradigms (Ahnaou, Huysmans, Van de Casteele, & Drinkenburg, 2017; Anderson, Pinault, O'Brien, & Jones, 2014; Jones, et al., 2012; Lladó-Pelfort, et al., 2016; Olszewski, Piasecka, Goda, Kasicki, & Hunt, 2013; Rebollo, Perez-Zabalza, Ruiz-Mejias, Perez-Mendez, & Sanchez-Vives, 2018). In fact, synchronized gamma oscillations are critical for cognitive functioning and efficient brain connectivity, which are altered in SCZ. Clozapine has been found to affect gamma frequencies, without impacting other bands, in the hippocampus and PFC of mice (D. Sun, et al., 2021). Clozapine ability to normalize disturbed gamma activity may involve its action at D4R (Andersson, Johnston, & Fisahn, 2012), 5-HT_3R, and D3R receptors (Schulz, et al., 2012).

In summary, reduced seizure threshold by clozapine may derive from multiple unique pharmacodynamic and pharmacokinetic features, including GlyR antagonism, or polymorphisms of the CYP1A2 gene. However, the action of clozapine on the regulation of gamma oscillation

rhythmicity may reflect its beneficial effect on interneuronal synchronization and, therefore, on cognitive symptoms.

10. Discussion

Clozapine remains one the most effective antipsychotic drugs after almost 50 years after its first introduction in therapy, and the only antipsychotic with the specific indication for TRS. Even though the precise MOAs of clozapine remain unveiled, preclinical and clinical findings have highlighted multiple and convergent mechanisms at multiple receptor and intracellular levels, which are putatively responsible for the efficacy of clozapine and worth to be further explored for the search of new antipsychotic targets as well as for the design of innovative compounds.

Among multiple mechanisms, the action at the dopamine system pointing to low affinity and fast-off antagonism at D2Rs, and a relatively high affinity for D1Rs and D4Rs compared to other antipsychotics, remains pivotal.

The recent finding of clozapine's particular lipophilicity influencing its pharmacokinetics, and the loose binding at dopamine D2Rs are key features that should be considered for designing potential novel antipsychotics. Another issue related to the structure of clozapine is the recent observation that this drug by virtue of its alkaline moiety can build up preferentially in presynaptic vesicles, exerting an auto-inhibitory effect when released, with the net result of reducing dopamine release (Amato, et al., 2018). This, again, is an appealing mechanism for the development of new antipsychotics especially because it may tackle the "core" or at least one of the major mechanisms of the dopamine dysfunction in psychosis, which has been related to the aberrant neurotransmitter release at the presynaptic level (Amato, et al., 2020; Amato, Kruyer, Samaha, & Heinz, 2019).

Considering the action of clozapine at the postsynaptic level, one of the most innovative findings is the discovery that clozapine interacts with receptor dimers and that it may sequester in the cytoplasm

D2Rs during their recycling, impacting D2R trafficking and localization on the membrane surface, thus potentially preventing D2R upregulation (Schrader, et al., 2019).

Among the hypotheses regarding the uniqueness of clozapine, the binding to muscarinic receptors have for a long time attracted attention, in particular with regard to the norclozapine agonist action at M1 and M4 sites (differently from clozapine that exerts antagonist muscarinic action) (Chew, et al., 2008; Gigout, et al., 2015; Weiner, et al., 2004; Wenthur & Lindsley, 2013; Yohn & Conn, 2018), positioning these receptors as a relevant target for treating psychotic and cognitive symptoms of SCZ. This hypothesis has recently received support from the positive results obtained in a phase II clinical trial with xanomeline, a M1-M4 agonist, combined with the peripheral anticholinergic trospium (Dean & Scarr, 2020).

Clozapine's multimodal effect on glutamate regulation have been considered as one of its most distinctive features compared to other antipsychotics and support the involvement of the glutamate system in the pathophysiology of TRS. Modulation of the glutamatergic system as a therapeutic target in the treatment of TRS received support by the positive results in the phase two clinical trial obtained by sodium benzoate (Lin, et al., 2018), which increases glutamate NMDAR functions by acting as an inhibitor of the d-amino acid oxidase.

An additional striking feature of clozapine is its differential impact on synaptic plasticity and dendritic spine architecture compared to other antipsychotics, as demonstrated by *in vivo* and *in vitro* preclinical studies (Asenjo Lobos, et al., 2010). This observation, however, still lacks a clear counterpart in human studies, although human MRI studies have shown that exposure to clozapine may affect cortical thickness.

A reduction in cortical thickness, a volumetric reduction in white matter of discrete regions, and a worsened metabolic activity in frontal areas have been reported in patients receiving clozapine (M. Ahmed, et al., 2015; Itahashi, et al., 2021). On the other hand, clozapine has been found to improve white matter integrity, increase grey matter volume in specific regions (Ozcelik-Eroglu, et al., 2014). Therefore, a variety of multiple functional and structural changes should be considered to

clarify the overall effect of clozapine, and to what degree at first sight potentially harmful volumetric remodelling may participate in an adaptive and functionally beneficial global reorganization of brain architecture.

In conclusion, the most recent discoveries on the molecular underpinnings of *in vitro* and *in vivo* clozapine effects have allowed to better understand the multimodal MOA of this unique compound, and have highlighted potential novel molecular targets that might be fruitfully tested further for the development of innovative antipsychotics treatments. Finally, the unique history of clozapine and its true atypicality indicate the need for innovative strategies beyond the present already available agents, including, paradoxically, clozapine. Thus, an in-depth understanding of the MOAs of clozapine should be harvested to replace or extend upon clozapine, which is hampered by many potentially severe adverse effects that are hopefully not be directly mechanistically related to its superior antipsychotic efficacy for TRS. It is hoped that soon additional treatment options for TRS will be developed and available, which is much needed for improving the outcome of some of the most severely ill patients with SCZ (Kane, et al., 2019; Potkin, et al., 2020).

Conflicts of Interest:

Dr. de Bartolomeis has received unrestricted research funding from Astra Zeneca, Janssen-Cilag, Bristol-Myers Squibb, Otsuka, Sanofi and Lundbeck. The funding was made available to the Department of Neuroscience, University of Naples Federico II. He received honoraria for advisory board activity from Janssen-Cilag Italy, Eli Lilly, Bristol-Myers Squibb. Astra-Zeneca, Takeda, Recordati, Mylan, Trivia, Chiesi, Otsuka, Lundbeck. He received honoraria as speaker at non-educational activities sponsored by Astra-Zeneca Italia, Janssen-Cilag Italy, Eli Lilly, Bristol-Myers Squibb. Astra-Zeneca, Takeda, Recordati, Mylan, Trivia, Chiesi, Otsuka, Lundbeck.

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All other authors declare that, except for income received from their primary employer, no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional service, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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Legend to Figures and Tables embedded in text

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CLOZAPINE						
Target	**Ki (nM)**	**Agonist / antagonist / partial agonist**	**Molecular effects**	**Preclinical observations**	**Clinical effects**	**References**
D1R	189	Unknow	$G_{\alpha s/olf}$ pathway: from PKA to CREB, glutamate receptors, GABA receptors, ion channels, DARPP-32 G protein independent pathway: activation of sodium channels, Na^+/K^+ ATPase, or transactivation of BDNF receptors	Epigenetic modification in PFC of rats Enhancing of cognitive performances as well as attenuation of stimulant-induced cognitive deficits, sensitization and seeking behavior in rats	D1R/D2R ratio may be responsible for clozapine's unique effectiveness in TRS patients	(Aoyama, et al., 2014; Baracskay, Haroutunian, & Meador-Woodruff, 2006; Wenthur & Lindsley, 2013)

D2R	431	Antagonist	D_2-like receptors are associated with the alpha subunit of inhibitory G protein and G_q protein that modulates the activity of PLC-β Fast-off on this receptor and 5-HT_{2A} antagonist could be responsible for BDNF brain expression restore	Upregulated in dopamine supersensitivity	Antipsychotic effect; Favorable D2R/5-HT_{2A} ratio and consequent reduced incidence of EPS and hyperprolactinemia	(Kebabian & Greengard, 1971; Okazawa, et al., 1992; Seeman, 2014; Stevens, et al., 1997; Vaidya, et al., 1997; Wenthur & Lindsley, 2013)

D3R	646	Antagonist	D_2-like receptors are associated with the alpha subunit of inhibitory G protein and G_q protein that modulates the activity of PLC-β	Upregulation of D3R after acute administration D3R occupancy of approximately 33-35%	Tolerance Normalization of high gamma oscillation	(Andersson, et al., 2012; M. Feng, Gao, Sui, & Li, 2015; Girgis, et al., 2011; Kebabian & Greengard, 1971; McCormick, et al., 2013; Wenthur & Lindsley, 2013)

D4R	39	Antagonist	D_2-like receptors are associated with the alpha subunit of inhibitory G protein and G_q protein that modulates the activity of PLC-β	Cellular mechanisms of hyperlocomotion	Normalization of high gamma oscillation	(Andersson, et al., 2012; Ninan & Kulkarni, 1998; Wenthur & Lindsley, 2013)
5-HT_{1A}R	105 Norclozapine shows higher affinity for this receptor	Partial agonist	Activation of inhibitory G protein with inhibition of AC	Increase in PFC dopamine release in rats	Atypical antipsychotic drug profile; Amelioration in psychotic and cognitive symptoms	(Meltzer & Sumiyoshi, 2008; Protais, et al., 1994; Rollema, Lu, Schmidt, & Zorn, 1997;

	(Ki= 14 nM)					Wedzony, Maćkowiak, Fijał, & Gołembiowska, 1996; Weinberger & Lipska, 1995; Wenthur & Lindsley, 2013)
5-$HT_{2A}R$	13	Inverse agonist	Activation of G_q protein an than activation of PLC/PKC and Rho proteins Activation of Akt that, through the inhibition of GSK3, actives CREB Increase in BDNF Activation of ERK 1/2	Very high 5-HT_2R occupancy in the frontal cortex of rats; increase in the release of dopamine in rats PFC	Atypical antipsychotic drug profile; Amelioration in negative symptoms	(Ichikawa, et al., 2001; McGrew, Price, Hackler, Chang, &

						Sanders-Bush, 2004; Sah, Seasholtz, Sagi, & Brown, 2000; Sullivan, Clarke, & Berg, 2015; Sumiyoshi, et al., 1993; Vaidya, et al., 1997; Wenthur & Lindsley, 2013)

5-$HT_{2C}R$	29	Inverse agonist	Activation of G_q protein an than activation of PLC/PKC and Rho proteins	Increase the release of dopamine in nucleus accumbens and striatum of rats	Atypical antipsychotic drug profile; Amelioration in negative symptoms Weight gain and metabolic disturbances	(Ichikawa, et al., 2001; Montastruc, et al., 2015; Sullivan, et al., 2015; Wallace, Zai, Brandl, & Müller, 2011; Wenthur & Lindsley, 2013)
5-HT_3R	241	Antagonist	-	Modulation of dopaminergic activity in mesolimbic and nigrostriatal pathways	Antipsychotic effect Slow gastrointestinal transit time	(Andersson, et al., 2012; Barnes & Sharp,

					Normalization of high gamma oscillation	1999; Mylecharane, 1996; Palmer, et al., 2008; Wenthur & Lindsley, 2013)
5-HT$_6$R	17	Antagonist	Activation of G_q protein an than activation of PLC/PKC and Rho proteins	Increase dopamine levels in the medial PFC and hippocampus in rats 5-HT$_6$R antagonism reduces the effects of MK-801 and PCP in an animal model of SCZ	Reduction in long-term complication such us tardive dyskinesia	(de Bruin, et al., 2013; Lacroix, et al., 2004; Z. Li, et al., 2007; Wenthur & Lindsley, 2013)

5-HT$_7$R	18	Antagonist	Activation of G_q protein an than activation of PLC/PKC and Rho proteins	5-HT$_7$R receptor antagonism has proved to be effective in ameliorating ketamine-induced attentional deficits and cognitive inflexibility Regulates of receptor internalization and subsequent degradation	It could be involved in improvement in cognitive and negative symptoms of SCZ	(Andressen, et al., 2015; Nikiforuk, et al., 2013; Wenthur & Lindsley, 2013)
M1	14	Antagonist	G_q resulting in activation of PLC and PLD	-	Dizziness, drowsiness, confusion, blurred vision, constipation;	(Caulfield, 1993; Chew, et al., 2008; Ghoshal, et al., 2016; J. A. Lieberman, 3rd, 2004;
	68	Norclozapine behaves as an agonist		M1 agonism could potentiate hippocampal NMDA receptor currents improve the LTD, cognitive function and social skills in mouse models of SCZ	Sialorrhea Amelioration of cognitive symptoms	

						Rümenapp, et al., 2001; Weiner, et al., 2004; Wenthur & Lindsley, 2013)
M3	25	Antagonist	G_q resulting in activation of PLC and PLD G protein-independent mechanism (via arrestin and PKD1 signaling)	-	Second Generation Antipsychotic-Induced Type 2 Diabetes that involved also 5-HT_{2A}R antagonism and D2R/D3R antagonism	(Caulfield, 1993; Hahn, et al., 2011; Ruiz de Azua, et al., 2011; Rümenapp, et al., 2001; Wenthur & Lindsley,

						2013; Weston-Green, et al., 2013)
M4	29	Antagonist	Gi/o resulting in inhibition of AC	It seems to be implicated in cognitive function in animal model	The role of M4 is unknown, but dopamine-acetylcholine balance seems to be relevant to the expression of SCZ symptoms Sialorrhea maybe responsible for major risk of pneumonia	(Caulfield, 1993; Galloway, et al., 2014; Gigout, et al., 2015; Rümenapp, et al., 2001; Wenthur & Lindsley, 2013)

		Norclozapine behaves as an agonist	-	-	Sialorrhea for synergistic interaction with vasoactive intestinal peptide (VIP)	(S. Ishikawa, et al., 2020; Weiner, et al., 2004)
M5	94	Antagonist	G_q resulting in activation of PLC and PLD	Clozapine was able to ameliorate PPI in KO $M5^{-/-}$ mice indirectly implying that the M5 receptor subtype is not indispensable for its antipsychotic action	-	(Caulfield, 1993; De Luca, et al., 2004; Rümenapp, et al., 2001; Thomsen, et al., 2007; Wenthur & Lindsley, 2013)
α_{1A}	1.6	Antagonism				

α_{1B}	7		G_q and consequent activation of PLC, β arrestin, MAPK, Rho protein and TRPC	Restoring the correct firing of dopaminergic mesolimbic neurons	Amelioration in positive symptoms; Orthostatic hypotension Reduction of perfusion responsible for major risk of intestinal ischemia	(Michelsen & Meyer, 2007; Svensson, 2003; Wenthur & Lindsley, 2013; West, Rowbotham, Xiong, & Kenedi, 2017)
α_{2A}	-	Antagonist	$G_{i/o}$ resulting in inhibition of AC and voltage sensitive calcium channels, and in an activation of potassium channels	It modulates firing of dopamine neurons in (VTA) and seems to be relevant to increase dopamine in PFC	Anti-depressive characteristics that could underlie the effect of this compound in preventing suicide	(Aringhieri, et al., 2018; Meltzer, et al., 2003; Svensson,
α_{2B}	-					

						2003; Wenthur & Lindsley, 2013)
α 2C	142	Antagonist	Amelioration of PCP-induced social interaction impairment	Precognitive effects It mitigates the GABA interneuronopathy associated with SCZ	Antidepressant, antipsychotic effects	(Madeleine Monique Uys, Mohammed Shahid, & Brian Herbert Harvey, 2017); (Franowicz, et al., 2002)

H1	2	Antagonist	$G_{q/11}$ with activation of PLC and increase in intracellular levels of Ca^{2+}, increases in NO levels, activates phospholipase A2 and NO-dependent guanyl-cyclases resulting in an increase in several transcription factors, in particular in NF-κB	Improvement in sensorimotor plasticity and memory function in rats; Increase in food intake in rats	Weight gain, orthostatic hypotension hypersalivation sedation	(Jacoby, Bouhelal, Gerspacher, & Seuwen, 2006; Prast & Philippu, 2001; Richelson, 1978; Roegge, et al., 2007; Sakata, et al., 1997; Snider, McKinney, Forray, & Richelson,

						1984; Wenthur & Lindsley, 2013)
H3	236±87	Antagonism	Gi protein resulting in a decrease of cAMP, activation of MAPK, Akt, and PLC	Reduction in MK-801-induced locomotor hyperactivity Weight loss in obese rats	Cognitive improvements in SCZ hyperphagia and body weight gain	(Hancock, Bush, Jacobson, Faghih, & Esbenshade, 2004; Ito, 2009; Mahmood, et al., 2016; Panula, et al., 2015; Rodrigues, et al., 1995)

H4	-	Agonism	Gi, resulting in inhibition of AC, ERK, PI3K, and p38 and the transcription factor activating protein-1	Agranulocytosis	-	(Goto, et al., 2016; Gutzmer, et al., 2005; Nakamura, Itadani, Hidaka, Ohta, & Tanaka, 2000);
σ_1	>10000 nM	Probably indirect effect	Modulation of Ca^{2+} signaling via IP3, activation of RyR, and binding of a lot of channels and receptors, such as voltage-gated K^+, Na^+, and Ca^{2+} channels, NMDAR, Rac-1 GTPase, and finally D1R and D2R	Increase in fibroblast growth factor-2 in the rat brain after chronic clozapine administration may be related to this receptor	Improvement in tardive dyskinesia and parkinsonism	(Gómez-Pinilla, et al., 1998; Hayashi & Su, 2007; Navarro, et al., 2010;

						Ovalle, et al., 2001; Riva, et al., 1999; Tagashira, et al., 2013; Wenthur & Lindsley, 2013) (Johannesse n, et al., 2009; Kourrich, et al., 2013; Natsvlishvil i, et al., 2015;

						Navarro, et al., 2013; Pabba & Sibille, 2015; Tchedre, et al., 2008)
NMDAR	-	Intrinsic agonist or partial agonist at Glycine B-site (this hypothesis is yet to be confirmed	-	Increase in L-serine and L-Glutamate in medial PFC of rat;	Antipsychotic effects	(Tanahashi, et al., 2012)

		experimentally)				
GlyT	-	Antagonist	-	Increase in glycine levels at synaptic cleft thereby potentiating NMDAR signaling	Antipsychotic effects	(Williams, et al., 2004)
SNAT1 / SNAT2	-	Antagonist	-	Increase in glycine levels in neuronal cells	-	(Javitt, et al., 2004; Schwieler, et al., 2004)
GABA B	-	Agonist/partial agonist	G_i protein resulting in a decrease of voltage-gated Ca^{2+} channels, as well as the opening of G protein-coupled inward rectifying potassium channels	-	Improvement in ability to filter extraneous sensory information	(Daskalakis & George, 2009; Franek, et al., 1999; Mannoury la Cour, Herbelles,

						Pasteau, de Nanteuil, & Millan, 2008)

Table 1. Clozapine receptors binding: molecular and clinical effects. Ki (inhibitory constant); D1R (dopamine receptor 1); D2R (dopamine receptor 2); D3R (dopamine receptor 3); D4R (dopamine receptor 4); 5-HT_{1A}R (serotonin receptor 1A); 5-HT_{2A}R (serotonin receptor 2A); 5-HT_{2B}R (serotonin receptor 2B); 5-HT_{2c}R (serotonin receptor 2C); 5-HT_{3}R (serotonin receptor 3); 5-HT_{6}R (serotonin receptor 6); 5-HT_{7}R (serotonin receptor 7); M1 (muscarinic receptor 1); M3 (muscarinic receptor 3); M4 (muscarinic receptor 4); M5 (muscarinic receptor 5); α_{1A} (adrenergic receptor α 1A); α_{1B} (adrenergic receptor α 1B); α_{2A} (adrenergic receptor α 2A); α_{2B} (adrenergic receptor α 2B); α_{2c} (adrenergic receptor α 2C); H1 (histaminergic receptor 1); H3 (histaminergic receptor 3); H4 (histaminergic receptor 4); σ1 (sigma receptor 1); NMDAR (N-methyl-D-aspartate receptors); GlyT (glycine transporter); SNAT 1/2 (sodium-coupled neutral amino acid transporters 1/2); $GABA_B$ (γ-aminobutyric acid type B receptor); NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells); DARPP-32 (Dopamine- and cAMP-regulated phosphoprotein 32 kD); PLC (Phospholipase C); PKC (Protein kinase C); PLD (Phospholipase D); BDNF (Brain-derived neurotrophic factor); AC (Adenylyl cyclase); PFC (Prefrontal cortex); PPI (Prepulse inhibition); VTA (Ventral tegmental area); KO (Knock-out); IP3 (Inositol triphosphate); RyR (Ryanodine receptor); MAPK (Mitogen-activated protein kinase); NO (Nitric oxide); PI3K (Phosphoinositide 3-kinase); PKD1 (Polycystin 1 transient receptor potential channel interacting); TRPC (Transient receptor potential cation channel).

Clozapine intracellular mechanisms of action						
	Molecular targets	**Signaling cascades**	**Intracellular MOA**	**Biological role**	**Preclinical and clinical effects**	**References**
Neuroprotective action	BDNF- CREB	Akt-GSK3	Increase maybe mediated by fast-off D2R and the strong $5HT_{2A}R$ antagonism	Survival of dopaminergic, cholinergic and serotonergic neurons; synaptic plasticity regulation.	Improvements in cognitive dysfunctions	(Alimohamad, et al., 2005b; Kozlovsky, et al., 2006; Ninan, 2014; Pedrini, et al., 2011; Takaki, et al., 2018)
	ERK 1/2	β-arrestin	Activation maybe mediated by "biased agonism" on 5-HT2A	Connectivity, synaptogenesis, and plasticity.	-	(M. R. Ahmed, et al., 2008; Aringhieri, et al., 2017; Kenakin, 2012; Samuels, et al., 2009)

Antiproliferative action	ErbB kinases	ErbB1 and ErbB4	Direct inhibition	Reduced growth/survival rates in cultures of cancer cells.	Improvement in PPI, mismatch negativity, amphetamine-induced sensitization, social drive.	(Kobayashi, et al., 2019) (Futamura, et al., 2003; Jodo, et al., 2019; Mizuno, et al., 2007; Shamir, et al., 2012; N. Tsuda, et al., 2008)
	H4	-	-	-	Tested as adjuvant for anti-cancer therapy	(Martinel Lamas, et al., 2013; Massari, et al., 2013; Massari, et al., 2017)
Anti-inflammatory action	NF-κB	Ca^{2+}/CaM/Akt	Inhibition	Inhibition of proinflammatory signals;	-	(Lutz-Bucher, Boudjada,

				prevention of degranulation of mast cells		Heisler, Pelletier, & Koch, 1988)
	Anti-inflammatory cytokines (IL-10, IL1RA, leukemia inhibitory factor receptor, IL-4	Different cascade	Increase	Inhibition of proinflammatory signals.	-	(Himmerich, et al., 2011; Maes, et al., 2002; Song, et al., 2000; Sugino, et al., 2009; Szuster-Ciesielska, et al., 2004)
	IFN-γ	-	Suppression		Induction of Th1 cell differentiation	(M. L. Chen, et al., 2012)
	Docosahexaenoic acid	-	Activation		-	(H. W. Kim, et al., 2012)

Neurodevelopmental action	At-RA	-	Increase	Formation, stabilization, and the pruning of the synapse	Tested as adjuvant treatment for positive symptoms	(M. L. Chen & Chen, 2007; Goodman, 1998; Regen, et al., 2021)
	Transthyretin	-	Increase			(Goodman, 1998)

Table 2. Clozapine intracellular mechanism of action, focus on neuroprotective, antiproliferative, anti-inflammatory, and neurodevelopmental actions. BDNF (Brain-derived neurotrophic factor); CREB (Cyclic AMP response element binding protein); GSK3 (Glycogen synthase kinase 3); ERK1/2 (Extracellular signal-regulated kinases 1/2); H4 (Histamine receptor 4); NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells); IFN-γ (Interferon-γ); At-RA (All-trans retinoic acid); Ca^{2+}/CaM/AKT (Ca^{2+}/calmodulin/Akt); IL-10 (Interleukin 10); IL-4 (Interleukin 4); IL1RA (Interleukin 1 receptor antagonist).

POTENTIAL GENETIC PREDICTORS OF RESPONSE TO CLOZAPINE			
Gene	**Genotype**	**Response**	**Reference**
DRD1	rs4532	Poor response	(Hwang, et al., 2007)
DRD3	Ser9Gly polymorphism	Poor response	(Jönsson, et al., 2003; Shaikh, et al., 1996)
DRD2	-141C Ins/Del polymorphism	Poor response	(J. P. Zhang, et al., 2010)
	rs2514218	Good response	(E. Huang, et al., 2016)
DAT	rs2975226	Good response	(Xu, et al., 2010)
COMT	Val/Val	Good response	(Bosia, et al., 2015)
	Val/Met		(Rajagopal, et al., 2018)
	Met/Met		
5-HT$_{1A}$R	G/G	Good response	(Bosia, et al., 2015)
TNF-α	G-308A	Good response	(G. Zai, et al., 2006)
NRXN1	rs1045881	Good response	(Lett, et al., 2011)
BDNF	rs10501087	Treatment resistance	(J. P. Zhang, et al., 2013)

NTRK2	rs1778929	Good response	(Mitjans, et al., 2015)
FKBP5	rs1360780	Poor response	
GNB3	C825T	Good response	(Kohlrausch, et al., 2008; D. J. Müller, et al., 2005)
DTNBP1	diplotype ACCCTC/GTTGCC genotypes T/T+T/C allele T of marker rs742105	Good response	(Zuo, et al., 2009)
GDNF receptor family genes	T-G-G rs1128397-rs13250096-rs4567028 haplotype	Good response	(Souza, Romano-Silva, et al., 2010)
OXT	rs2740204 polymorphisms	Good response	(Souza, de Luca, et al., 2010b)
ITIH3	rs2535629	Good response	(Brandl, et al., 2016)
HLA-A1		Good response	(Lahdelma, et al., 1998; Lahdelma, et al., 2001)
	22q11.2	Good response	(Butcher, et al., 2015)
ABCB1	rs7787082 rs10248420 rs7787082 G	Poor response	(S. T. Lee, et al., 2012; M, et al., 2020)

	rs10248420 A		

Table 3. Potential genetic predictors of response to clozapine. DRD1 (Dopamine Receptor 1 gene); DRD2 (Dopamine Receptor 2 gene); DRD3 (Dopamine Receptor 3 gene); DAT (Dopamine Transporter gene); COMT (Catechol-O-methyltransferase gene); 5-HT$_{1A}$R (Serotonin Receptor 1A gene); TNF-α (Tumor Necrosis Factor-α gene); NRXN1(Neurexin 1 gene); BDNF (Brain-Derived Neurotrophic Factor gene), NTRK2 (Neurotrophic Receptor Tyrosine Kinase 2 gene); FKBP5 (FKBP Prolyl Isomerase 5 gene); GNB3 (G Protein Subunit Beta 3 gene); DTNBP1 (Dystrobrevin Binding Protein 1); GDNF (Glial Cell Line-derived Neurotrophic Factor gene); OXT (Oxytocin/Neurophysin I Prepropeptide gene); ITIH3 (Inter-Alpha-Trypsin Inhibitor Heavy Chain 3 gene); HLA-A (Human leukocyte antigen-A gene); ABCB1 (ATP Binding Cassette Subfamily B Member 1 gene).

Neuroimaging Techniques	Medication	Duration	Region assessed	Findings	References
MRI	CLZ *vs* Typical and previous typical APs	54.6 weeks	Caudate nucleus	Decreased caudate nucleus volume	(Chakos, et al., 1995)
	CLZ *vs* previous typical APs	2 years	Basal ganglia Lateral ventricles	Decreased caudate nucleus volume	(Frazier, et al., 1996)
	CLZ *vs* Previous typical APs	24 weeks	Caudate nucleus	Decreased caudate nucleus volume	(Scheepers, Gispen de Wied, et al., 2001)
	CLZ *vs* Previous typical APs	52 weeks	Caudate nucleus	Decreased right caudate nucleus volume	(Scheepers, Gispen de Wied, et al., 2001)
	CLZ *vs* RSP and Previous typical APs	26 months	Frontal cortex Parietal cortex Temporal cortex Occipital cortex	Increased GM volume in frontal, parietal, and occipital cortex; decreased WM volume in frontal, parietal, and occipital cortex	(Molina, Reig, et al., 2005)

	CLZ *vs* Typical AP Atypical AP Previous typical/ atypical AP	5 years	Left frontal gyrus	Attenuated loss of GM density in superior left frontal gyrus	(van Haren, et al., 2007)
	CLZ *vs* OLA	6 years	Cortex	Thinning of small circumscribed area in the right prefrontal cortex	(Mattai, et al., 2010)
	CLZ	6-9 months	GM	Reductions in GM volume in the right and left medial prefrontal cortex and in the periventricular area	(M. Ahmed, et al., 2015)
	CLZ	6 months	Cortex Basal ganglia Thalamus Hippocampus	Decreased caudate nucleus, thalamus, hippocampus, and putamen; enlargement of lateral ventricles	(Tronchin, et al., 2020)
fMRI	CLZ	Single scan during motor task	Sensorimotor cortex	Decreased activation in comparison with controls	(Wenz, et al., 1994)

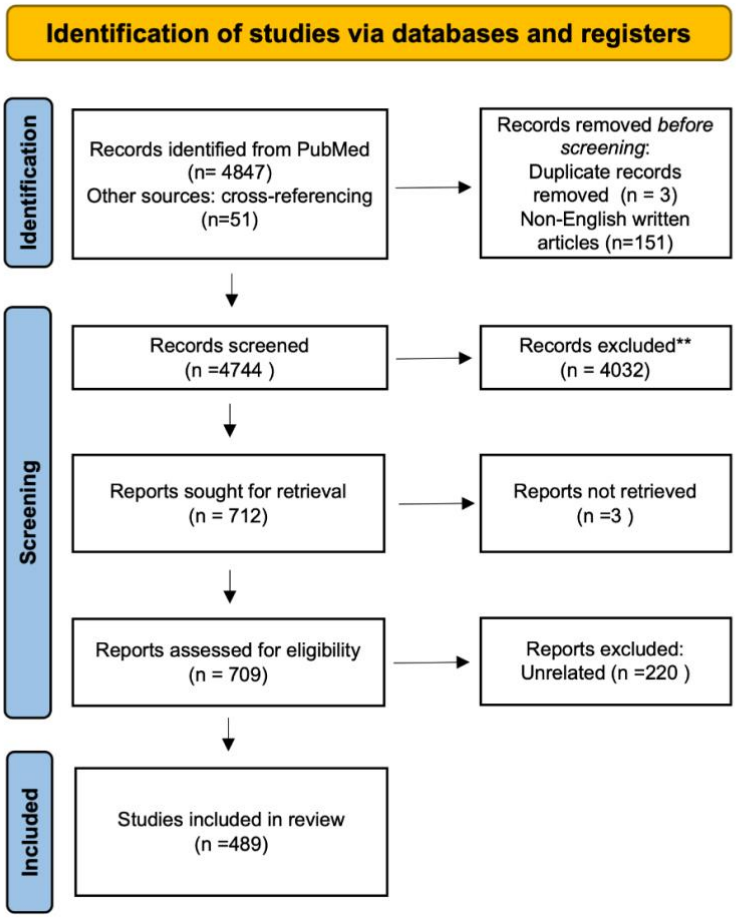
	CLZ	Single scan	Orbitofrontal cortex	Activation during attentional tasks	(Schirmbeck, et al., 2015)
			Left amygdala	Reduction in response to emotional stimuli	(Mier, et al., 2019)
	CLZ	Single scan	Amygdala	Decrease in activation during cannabis-related images	(Machielsen, et al., 2018)
SPECT	CLZ *vs* Typical AP and Previous typical AP	26 weeks	Thalamus Basal ganglia Frontal cortex	Decreased perfusion in thalamus, basal ganglia, superior left DLPFC and anterior PFC	(Rodríguez, et al., 1997)
	CLZ *vs* Risperidone and Previous typical/ atypical AP	8 weeks	Brain	Increased posterior temporal, occipital and brainstem Decreased perfusion in posterior cingulate and hippocampus Increased perfusion of medial occipital cortex and head of the caudate	(Molina, et al., 2008)
	CLZ *vs* Previous typical/ atypical AP	8 weeks	Frontal lobe Parietal lobe Temporal lobe Occipital lobes Caudate	Increased perfusion in left and right frontal cortex and caudate	(Ertugrul, et al., 2009)

			Thalamus Cerebellum		
PET	CLZ *vs* TTX	28-49 day	Basal ganglia	Increased metabolism in basal ganglia	(Buchsbaum, et al., 1992)
	CLZ *vs* FLZ	Single scan	PFC Occipital cortex Temporal lateral cortex Limbic cortex Subcortex	Decreased metabolism in the PFC Increased metabolism Of limbic, parietal, and occipital cortices	(R. M. Cohen, et al., 1997)
	CLZ *vs* FLZ	Single scan	Superior and inferior PFC Occipital cortex Parietal cortex Temporal lateral cortex	Increased metabolism in occipital and parietal *vs* controls and in temporal medial cortex Decreased perfusion in superior and inferior PFC	(R. M. Cohen, Nordahl, Semple, & Pickar, 1999)
	CLZ *vs* Haloperidol and other typical APS	35 weeks	Cerebral activation pattern	Increased metabolism in right ventral striatum, left caudate, and left DLPFC *vs* controls and in in anterior cingulate, medial frontal cortex, DLPFC, and occipital cortex *vs* haloperidol	(A. C. Lahti, et al., 2003)

				Decreased metabolism in left hippocampus and VLPFC *vs* controls and in ventral striatum, putamen, right VLPFC *vs* haloperidol	
	CLZ *vs* Haloperidol and other typical APS	6 months	Brain activation pattern	Decreased metabolism in DLPFC, medial prefrontal, left inferior medial temporal cortex, and basal ganglia *vs* haloperidol Increased metabolism in in occipital *vs* haloperidol	(Molina, Gispert, et al., 2005)
	CLZ *vs* Previous typical and atypical AP Neuroleptic-naive	Single scan	Brain activation pattern	Decreased metabolism in dorsolateral cortex, orbitofrontal, insular, and anterior cingulate *vs* controls and neuroleptic-naive	(Molina, et al., 2007)
MRS	CLZ	Single evaluation	Right frontal lobes	Decrease in intracellular pH value	(McLoughlin, et al., 2009; Riehemann, et al., 2002)

	CLZ	Single evaluation	Cingulate cortex	Increased glutamate levels in anterior cingulate cortex	(Matrone, et al., 2022)
DTI	CLZ	Single evaluation	WM	Low fractional anisotropy in specific WM tracts	(Matrone, et al., 2022)
	CLZ	12 weeks	WM	Increased fractional anisotropy in 6 brain regions	(Ozcelik-Eroglu, et al., 2014)

Table 4. Morphological and functional effects of clozapine in different brain regions based on neuroimaging studies. CLZ (Clozapine); AP (Antipsychotic); OLA (Olanzapine); FLZ (Fluphenazine); TTX (Thiothixene); MRI (Magnetic resonance imaging); fMRI (Functional magnetic resonance imaging); SPECT (Single photon emission computed tomography); PET (Positron emission tomography); MRS (Magnetic resonance spectroscopy); DTI (Diffusion tensor imaging); GM (Gray matter); WM (White matter); PFC (Prefrontal cortex); DLPFC (Dorsolateral prefrontal cortex); VLPFC (Ventrolateral prefrontal cortex).



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3971 *Fig. 1. The Prisma flow-diagram maps out the number of records identified, included and excluded.*

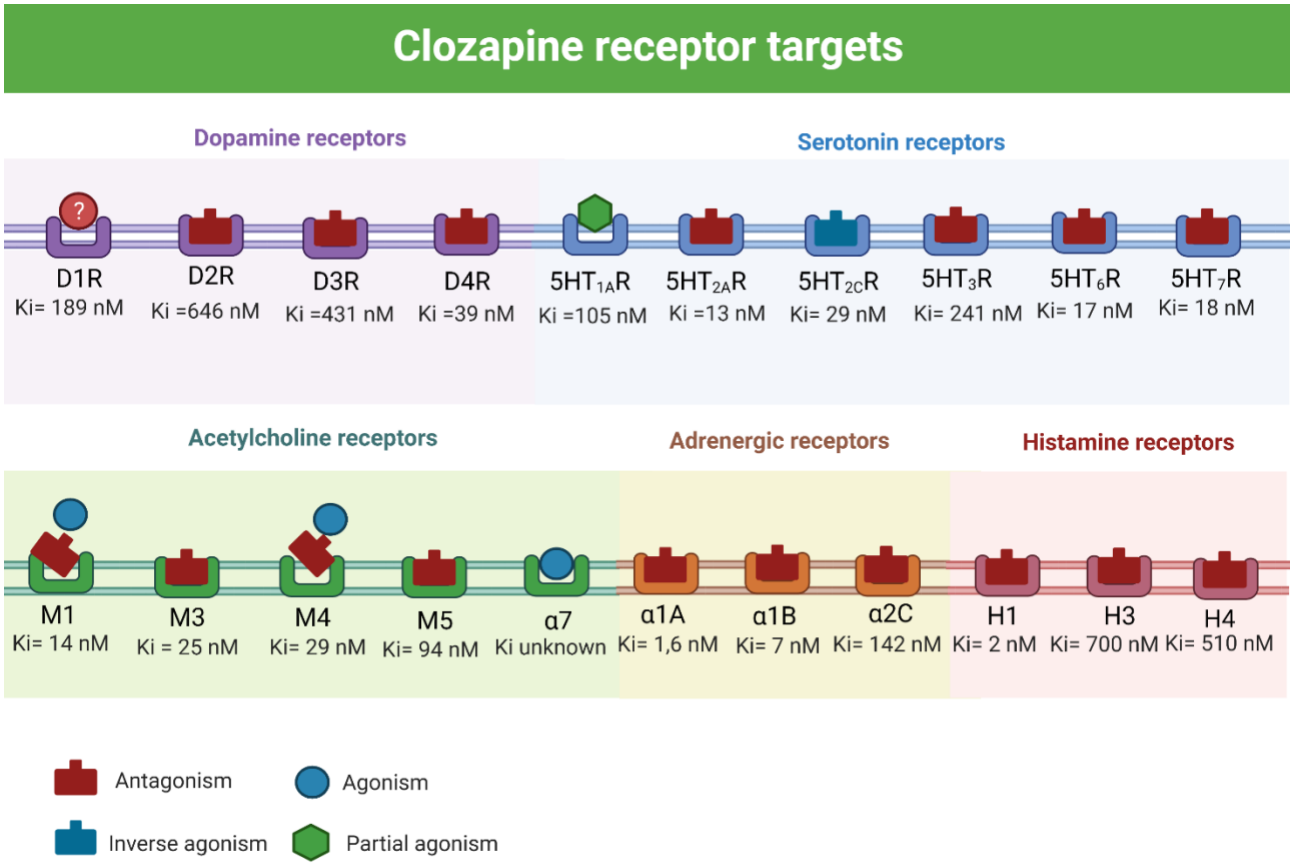

Fig. 2. Clozapine multireceptor profile encompassing a multiple array of receptors. D1R (dopamine receptor 1); D2R (dopamine receptor 2); D3R (dopamine receptor 3); D4R (dopamine receptor 4); 5-HT_{1A}R (serotonin receptor 1A); 5-HT_{2A}R (serotonin receptor 2A); 5-HT_{2C}R (serotonin receptor 2C); 5-HT_3R (serotonin receptor 3); 5-HT_6R (serotonin receptor 6); 5-HT_7R (serotonin receptor 7); M1 (muscarinic receptor 1); M3 (muscarinic receptor 3); M4 (muscarinic receptor 4); M5 (muscarinic receptor 5); α_7 (nicotinic receptor α7); α_{1A} (adrenergic receptor α1A); α_{1B} (adrenergic receptor α1B); α_{2c} (adrenergic receptor α_{2C}); H1 (histaminergic receptor 1); H3 (histaminergic receptor 3); H4 (histaminergic receptor 4).

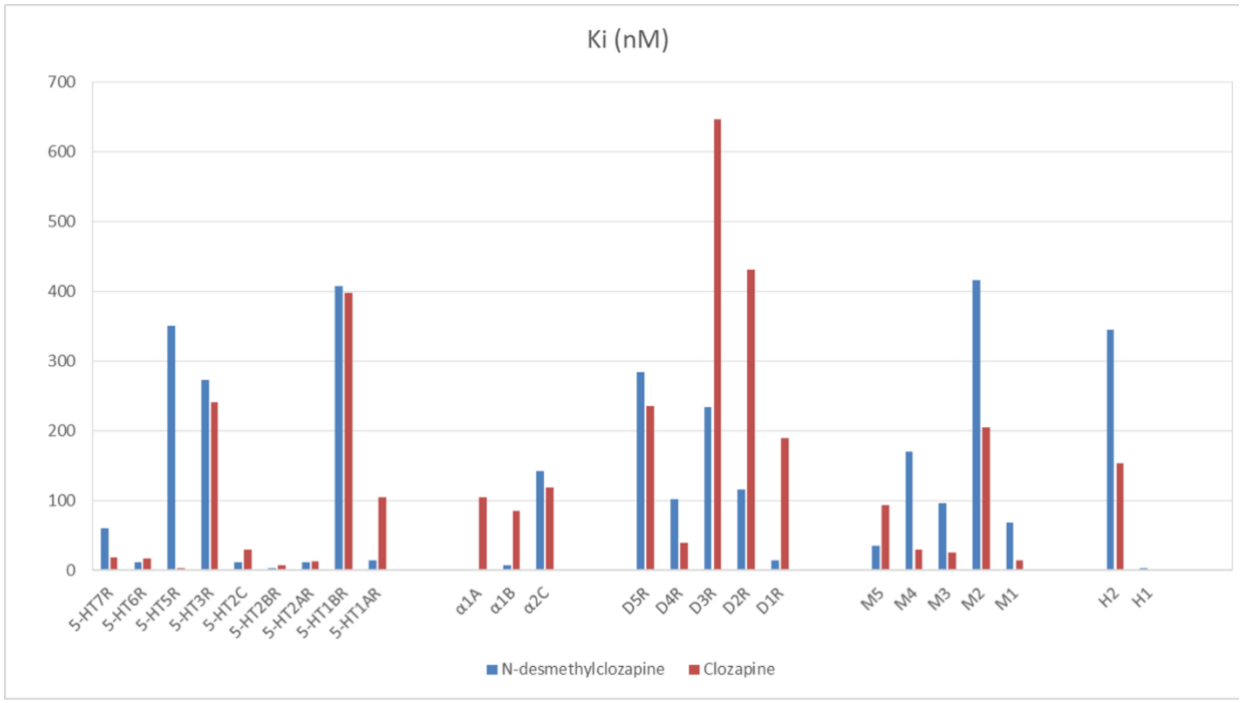

Fig. 3 Receptor profile signature of clozapine and norclozapine. K_i values as determined by the NIMH Psychoactive Drug Screening Program (available at https://pdsp.unc.edu/pdspweb/). D1R (dopamine receptor 1); D2R (dopamine receptor 2); D3R (dopamine receptor 3); D4R (dopamine receptor 4); D5R (dopamine receptor 5); 5-HT_{1A}R (serotonin receptor 1A); 5-HT_{1B}R (serotonin receptor 1B); 5-HT_{2A}R (serotonin receptor 2A); 5-HT_{2B}R (serotonin receptor 2B); 5-HT_{2C}R (serotonin receptor 2C); 5-HT_3R (serotonin receptor 3); 5-HT_5R (serotonin receptor 5); 5-HT_6R (serotonin receptor 6); 5-HT_7R (serotonin receptor 7); M1 (muscarinic receptor 1); M2 (muscarinic receptor 2); M3 (muscarinic receptor 3); M4 (muscarinic receptor 4); M5 (muscarinic receptor 5); α_{1A} (adrenergic receptor α1A); α_{1B} (adrenergic receptor α1B); α_{2c} (adrenergic receptor α_{2C}); H1 (histaminergic receptor 1); H2 (histaminergic receptor 2).

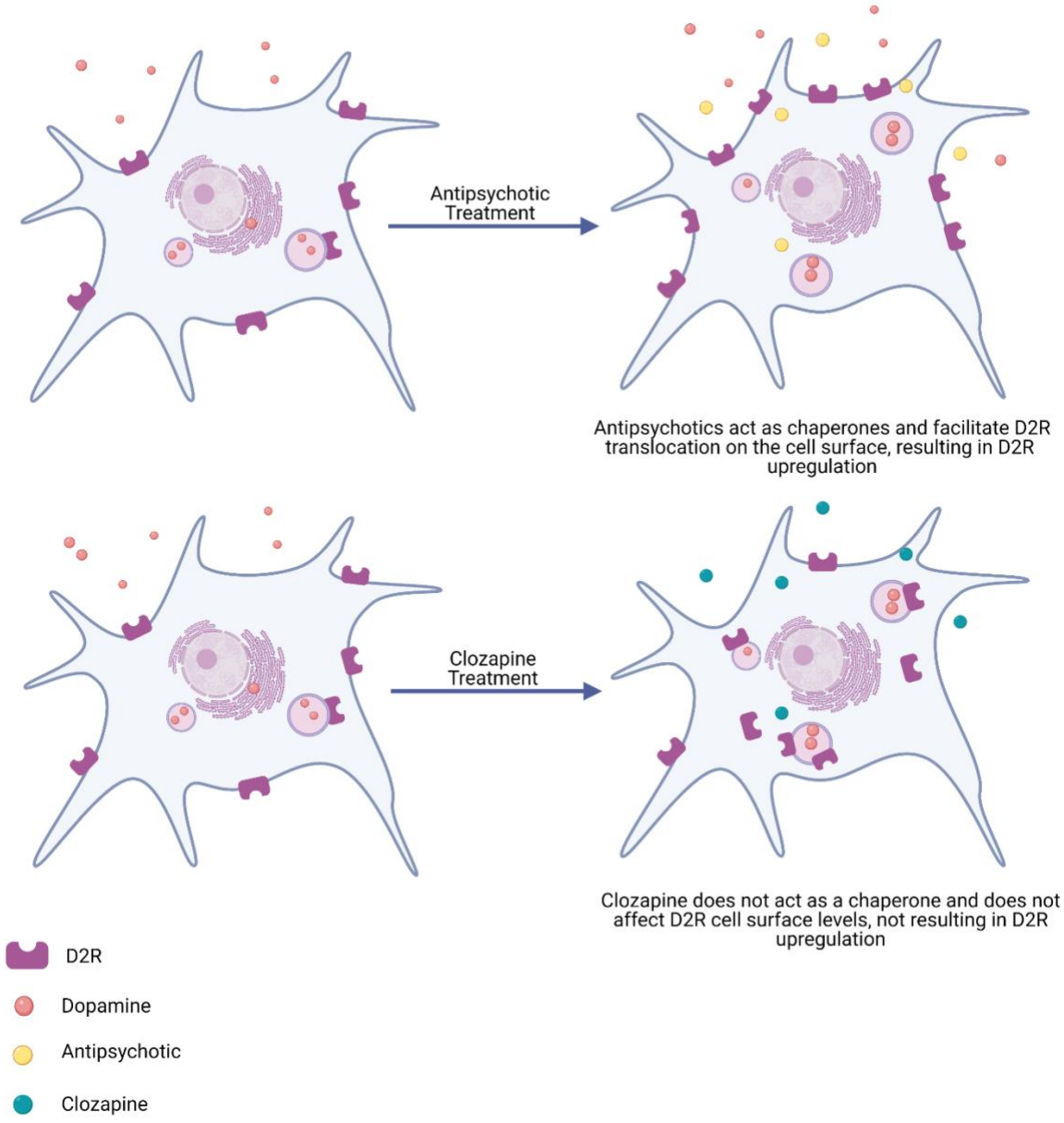

Fig. 4. Other antipsychotics act as pharmacological chaperones facilitating D2R translocation on cell surface and upregulation. Clozapine shows low activity as a chaperone for D2R in vitro. Therefore, D2Rs do not translocate easily to the surface of cell membrane and D2R upregulation is partially prevented.

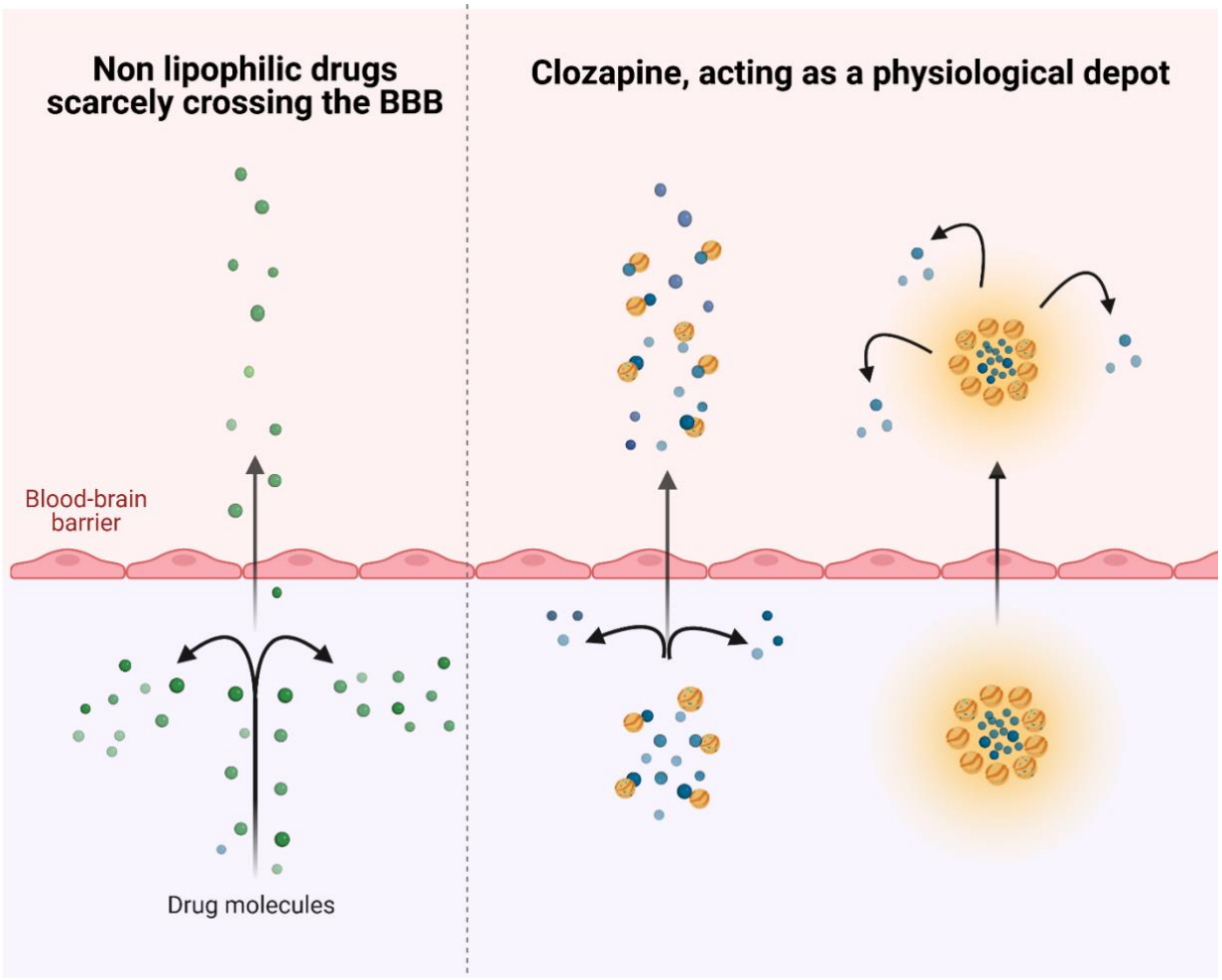

Fig. 5. Due to its lipophilic nature, clozapine readily passes the blood-brain barrier in comparison to other scarcely lipophilic antipsychotic compounds. Moreover, the combination of clozapine with low-density lipoprotein and very-low-density lipoprotein may explain its ability to act as a "physiological depot". BBB= Blood brain barrier.