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The impact of antipsychotics on the trafficking of neurotransmitter receptors

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The impact of antipsychotics on the trafficking of neurotransmitter receptors

O impacto de antipsicóticos no tráfego de receptores de neurotransmissores

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requesitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Doutor Laurent Groc (Instituto Interdisciplinar de Neurociências, França) e da Professora Doutora Ana Luisa Carvalho (Universidade de Coimbra).

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Table of contents

List of Abbreviations i
Abstract v
Resumo vii
General Introduction
I. Schizophrenia 1
I.I Diagnosis and symptomatology 1
I.II Structural and cellular characterization 2
I.III Genetic and molecular characterization..5
I.III.I Neurodevelopment and schizophrenia..8
I.III.II Neurotransmission-associated signal transduction and schizophrenia…..9
I.IV Treatment for schizophrenia 12
II. Surface trafficking of NMDA receptors 15
II.I Neurotransmitter receptor trafficking: role in synaptic transmission............15
II.II The NMDAR: subunit composition, lateral diffusion and synaptic Plasticity…………………………………………………………………………16
II.III NMDAR surface diffusion and schizophrenia...20
Materials and Methods
I. Cell culture 23
II. Immunocytochemistry 23
III. Confocal laser scanning microscopy 23
IV. Single quantum dot tracking and surface diffusion 25
V. Statistical analysis 26
Results
I. Ketamine impairs the diffusion of NMDAR..27
II. Haloperidol does not affect NMDAR surface dissusion 29
III. Clozapine increases surface diffusion 31
IV. Clozapine rescues ketamine-elicited imapirments in NMDAR surface diffusion..33
V. Clozapine does not impact NMDAR surface expression and distribution....36

Discussion 39
Conclusions 45
Bibliography 47

Table Index

Table 1: Genetic risk factors for schizophrenia selected from information listed at the Online Mendelian Inheritance in Man database ……………………………..……………6

Figure Index

Figure 1: NMDA receptor hypofunction and the dopaminergic pathways associated with symptoms of schizophrenia ... .11

Figure 2: Pathways relevant for antipsychotic atypicality 14

Figure 3: Trafficking of ionotropic glutamate receptors at excitatory synapses 15

Figure 4: Topology of NMDA glutamate receptors 16

Figure 5: Mechanisms underlying long-term potentiation 18

Figure 6: Surface diffusion of NMDAR is essential for the expression of hippocampal synaptic plasticity 20

Figure 7: Surface diffusion of NMDAR is impaired in experimental models of psychotic disorders 21

Figure 8: Principle of confocal microscopy 24

Figure 9: Single particle tracking of NMDAR 25

Figure 10: Surface diffusion of NMDAR is impaired in a ketamine-based pharmacological model of schizophrenia 28

Figure 11: Haloperidol does not affect the surface diffusion of NMDAR 30

Figure 12: Clozapine increases the surface diffusion of NMDAR 32

Figure 13: Clozapine rescues the imapirments in NMDAR surface diffusion caused by ketamine 35

Figure 14: Clozapine does not impact NMDAR surface distribution 38

List of Abbreviations

In order of appearance:

NMDAR	N-methyl-D-aspartate Receptor
D1R	Dopamine Receptor 1
DSM-5	Fifth edition of the Diagnostic and Statistical Manual of Mental Disorders
PPI	Paired Pulse Inhibition
DLPFC	Dorsolateral Prefrontal Cortex
VTA	Ventral Tegmental Area
DA	Dopamine
LTP	Long Term Potentiation
CSF	Cerebrospinal Fluid
PV+	Expressing Parvalbumin
TNF-α	Tumor Necrosis Factor alpha
IL-1β and -6	Interleukin 1 beta and Interleukin 6
GWAS	Genome-wide Association Study
HLA	Human Leukocyte Antigen
ATP	Adenosine Triphosphate
OMIM	Online Mendelian Inheritance in Man
KO	Knock Out
KD	Knock Down
22q11.2 DS	22q11.2 Deletion Syndrome
COMT	Catechol-O-methyl-transferase
MTHFR	5,10-Methylenetetrahydrofolate Reductase
DISC1	Disrupted in Schizophrenia 1
ErbB4 and 2	Tyrosine Kinase Cell Surface Receptor HER4 and 2
Syn 2	Synapsin 2

GABRA1,P and 6	Gamma-Aminobutyric Acid receptor subunits 1, P and 6
DTNBP1	Dysbindin or Dystrobrevin Binding Protein 1
NRG1	Neuroregulin 1
PPP3CC	Calcineurin Gamma catalytic Subunit
DAO	D-amino Acid Oxidase
5HT2R	Serotonin Receptor 2A
DAOA	D-amino Acid Oxidase Activator
AKT1	RAC-alpha serine/threonine-protein kinase
MAM	Methylazoxymethanol Acetate
CNS	Central Nervous System
PET	Positron Emission Tomography
D2R and D3R	Dopamine Receptors 2 and 3
PCP	Phencyclidine
AP-5 or AP-V	2-amino-5-phosphonopentanoic Acid
LSD	Lysergic Acid diethylamide
EPS	Extrapyramidal Symptoms
Ach	Acetylcholine
M1R	Muscarinic receptor 1
5HT	Serotonin
5HT1AR	Serotonin Receptor 1A
α1R	Adenosine receptor 1
PSD	Post-synaptic Density
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptor
KAR	Kynurenic Acid receptor
PDZ	PSD95/Discs-large/ZO-1 Homology Domain
2A- or 2B-NMDAR	NMDAR containing the GluN2A or the GluN2B subunit

CaMKII	Calcium/Calmodulin-dependent Protein Kinase II
mGluR5	Metabotropic Glutamate Receptor 5
α7-nAchR	Nicotinic Acetylcholine Receptor α7
EPSP	Excitatory Post-synaptic Currents
LTD	Long Term Depression
LFS or HFS	Low or High Frequency Stimulation
ARA-C	Arabinofuranosyl Cytidine
PBS	Phosphate Buffered Saline
BSA	Bovine Serum Albumin
RT	Room Temperature
CLSM	Confocal Laser Scanning Microscopy
ROI	Region of Interest
FRAP	Fluorescence Recovery after Photobleaching
QD	Quantum Dot
SPT	Single Particle Tracking
SNR	Signal to Noise Ratio
D	Instantaneous Diffusion Coefficient
IQR	Interquartile range
Ket	Ketamine
Halo	Haloperidol
Cloza	Clozapine
MSD	Mean Square Displacement
SERT	Serotonin Transporter
NET	Norepinephrine Transporter
TTX	Tetrodotoxin

Abstract

Schizophrenia, a psychotic disorder that affects between 0,4 and 0,8 % of the population, is believed to result from an imbalance between glutamatergic and dopaminergic neurotransmission systems during neurodevelopment. In particular, alterations in glutamate N-methyl-D-aspartate (NMDA) and dopamine receptor signaling and trafficking are associated with the disease. As an example, NMDA receptor (NMDAR) antagonists can mimic in healthy individuals some of the symptoms associated with the disease, and alterations in NMDA receptor expression have been reported both in brain samples from patients and in experimental models of schizophrenia. However, the mechanisms underlying these impairments remain elusive. Long considered as immobile at the cell surface, neurotransmitter receptors are instead highly dynamic and diffuse laterally within the membrane plane. Surface diffusion recently emerged as a key regulator controlling the synaptic content in NMDAR to shape the strength of excitatory neurotransmissions. It also contributes to the dialogue between glutamatergic and dopaminergic pathways through dopamine D1 receptor (D1R)/NMDAR interaction-based surface redistributions of receptors. Moreover, changes in NMDAR surface diffusion have been reported in autoimmune neuropsychiatric disorders, suggesting that impaired receptor surface dynamics could contribute to psychosis. To address this challenging question, we assessed whether a psychotomimetic molecule, ketamine, acutely impacts NMDAR surface dynamics and distribution. We then explored if neuroleptics used to alleviate the symptoms of schizophrenia rescue ketamine-induced deficits in NMDAR surface trafficking. Our results show that NMDAR surface diffusion is decreased following acute application of ketamine, an effect which preferentially impacts extrasynaptic GluN2A subunit-containing NMDAR. Importantly, the atypical antipsychotic clozapine had the opposite action and increased the surface diffusion of GluN2A-NMDAR in perisynaptic and synaptic compartments, and of GluN2B-NMDAR at extrasynaptic sites, although it did not significantly affect the surface expression or synaptic localization of NMDAR as attested by immunostaining experiments. On the contrary, haloperidol, a typical first-generation neuroleptic, did not impact NMDAR surface dynamics. When combined with ketamine, clozapine successfully prevented the impairments in GluN2A-NMDAR surface diffusion elicited by the psychotomimetic molecule. Altogether, these results suggest that impairments in NMDAR surface trafficking could represent a new hallmark of psychotic disorders, and that one of the actions of atypical neuroleptics could be to restore NMDAR dynamics at the cell surface.

Keywords: schizophrenia, NMDAR, trafficking, lateral diffusion, ketamine, clozapine, haloperidol, antipsychotic, psychotomimetic

Resumo

Acredita-se que a esquizofrenia, uma doença caracterizada por psicose, que afeta entre 0,4 a 0,8 % da população, resulta de um desequilíbrio entre os sistemas glutamatérgicos e dopaminérgicos durante o desenvolvimento. Estão associadas a esta doença alterações nas vias sinalização e tráfego dos recetores de glutamato denominados recetores de N-metil-D-aspartato (NMDA) e dos recetores de dopamina. Por exemplo, antagonistas dos recetores NMDA (NMDAR) conseguem mimetizar em indivíduos saudáveis alguns dos sintomas associados a esta doença, e foram descritas alterações na expressão dos recetores NMDA tanto em cérebros de pacientes como em modelos experimentais de esquizofrenia. No entanto, os mecanismos subjacentes a estes danos são ainda pouco conhecidos. Embora tenham sido considerados como estando imóveis na superfície celular durante largas décadas, os recetores de neurotransmissores são ao invés altamente dinâmicos e difundem lateralmente dentro da membrana. A difusão superficial emergiu recentemente como um regulador-chave que controla o conteúdo sináptico de NMDAR de modo a moldar a força da neurotransmissão excitatória. Este mecanismo contribui também para o diálogo entre as vias glutamatérgicas e dopaminérgicas através da redistribuição de recetores, baseada na interação entre recetores de dopamina D1 (D1R) e NMDAR. Foram ainda descritas alterações na difusão superficial de NMDAR em doenças neuropsiquiátricas autoimunes, o que sugere que uma dinâmica superficial de recetores debilitada pode contribuir para a psicose. Para responder a esta intrigante questão, propusemo-nos a avaliar se uma molécula psicotomimética, a ketamina, tem um impacto agudo sobre a distribuição e/ou dinâmica superficial dos NMDAR. Exploramos ainda se os neurolépticos usados para aliviar os sintomas da esquizofrenia resgatam os danos no tráfego superficial dos NMDAR induzidos pela ketamina. Os nossos resultados demonstram que a difusão superficial dos NMDAR diminui após aplicação aguda de ketamine, um efeito com maior impacto sobre os NMDAR que contêm a subunidade GluN2A. Clozapine, um antipsicótico atípico, teve no entanto a ação contrária e aumentou a difusão superficial de GluN2A-NMDAR nos compartimentos perisináptico e sináptico, e de GluN2B-NMDAR em localizações extrasinápticas, apesar de não ter afetado significativamente a expressão superficial ou a localização sináptica de NMDAR, como demonstram as experiências de immunocitoquimica. Pelo contrário, haloperidol, um antipsicótico típico de primeira geração, não teve qualquer impacto sobre a dinâmica superficial dos NMDAR. Quando combinado com a ketamina, a clozapina impediu com sucesso os danos causados na difusão superficial dos GluN2A-NMDAR por esta molécula psicotomimética. Estes resultados sugerem que as alterações da difusão superficial dos NMDAR podem representar uma nova característica das doenças

psicóticas, e que uma das ações dos neurolépticos atípicos poderá ser o restauro da dinâmica dos NMDAR à superfície da célula.

Palavras-chave: NMDAR, tráfego, difusão lateral, ketamina, clozapina, haloperidol, antipsicótico, psicotomiméticos

General Introduction

General Introduction

I. Schizophrenia

I.I Diagnosis and symptomatology

Schizophrenia is a neuropsychiatric disorder that affects approximately 0.8% of the general population, and is considered as one of the most impactful mental illnesses today (Saha, Chant, Welham, & McGrath, 2005). Schizophrenics at the prodromal stage of the disease may already display an unwholesome psychological state, demonstrating affective dysregulations such as mania, anxiety, demoralization and impulsivity. At the onset of schizophrenia, typically during or shortly after adolescence, they begin to suffer psychotic outbreaks, often experiencing auditory hallucinations and falling into paranoid delusions. With time, their mental process deteriorates and many become unable to form a rational train of thought, resulting in disconnected, disordered or even incoherent speech. These distinctive signs of schizophrenia are categorized as 'positive symptoms', in the sense that they are an "addition" to reality. On the other side of the coin are the termed 'negative symptoms' of schizophrenia, which are minimally described in the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) as diminished emotional expression and/or avolition (decrease in the motivation to initiate and perform self-directed purposeful activities) (American Psychiatric Association, 2013). This is clearly a simplification with the intent of facilitating diagnosis. Negative symptoms are regarded in the literature in a broader sense as any symptom that is not a positive symptom, and include not only a general lack of motivation and a reduced range of emotions, but also the inability to extract pleasure from activities usually found enjoyable (anhedonia) and social withdrawal, rendering this disorder extremely debilitating for one's morale. It is estimated that death by suicide befalls 3-7% of schizophrenics. This high incidence of suicide has a major impact in the life expectancy of schizophrenics, which was determined to be 20 years shorter than that of an unaffected individual (Laursen, Nordentoft, & Mortensen, 2014). Therefore, it is imperative that treatment design for this illness is focused as much (or more) in the mitigation of these negative symptoms as in the riddance of positive ones. There is also a level of cognitive impairment associated with schizophrenia. Cognitive functions affected include memory, attention/concentration, problem solving, learning, executive function, processing speed, and social cognition (Kitchen, Rofail, Heron, & Sacco, 2012). Defective sensory gating and the resulting decline in selective attention are even basis for validation of animal models for schizophrenia, and the same behavioral tests, of which the most common is Paired Pulse Inhibition (PPI) assessment, have been successfully applied to humans and appear to reliably differentiate between healthy and schizophrenic individuals (Takahashi et al., 2011).

This contributes to the view of schizophrenia as a highly heterogeneous disorder, belonging within a spectrum, similarly to autism. Accordingly, the DSM-5 categorizes multiple syndromes as being within the schizophrenia spectrum (evidently including schizophrenia), and others as unrelated psychotic disorders (American Psychiatric Association, 2013). It is important to keep in mind just how different the cases of persons diagnosed with this same disorder can be. Regarding negative symptoms, cases exist of patients who do not demonstrate at all diminished emotional expression or avolition, and there is even more variability concerning cognitive impairments, as there are many cases of schizophrenics with a quite normal or even above average intelligence, so much that the presence or absence of a cognitive impairment is completely excluded from the diagnosis for this disorder (Regier, Kuhl, & Kupfer, 2013). Nevertheless, the link between cognitive impairments and schizophrenia has long been accredited, and is subject to much interest. Although it is not central for diagnosis, it is believed that most, if not all, schizophrenic patients suffer from a slight cognitive impairments, and that even while demonstrating a normal or superior intelligence they may not achieve their full intellectual potential (Stahl, 2013). Accordingly, all structural, cellular and molecular features of schizophrenia reviewed in the following subchapters would logically have an impact on cognitive function.

I.II Structural and cellular characterization

Expectedly, the before-mentioned variability between patients extends to all levels, and it is only through studies with large cohorts and meta-analysis that one may find reliable information on what are the hallmarks of schizophrenia. Seeing as there are no large groups of treatment-naïve individuals, any study conducted longitudinally in humans does not realistically relay the course of this disorder, but is subject to the great confounder that is the effect of antipsychotic treatment. As such, only alterations that are verified at the prodromal phase of the illness, or at the instance of the first psychotic outbreak (first-episode) are truly not biased by the effects of these drugs. The most striking morphological changes observed in the brains of schizophrenics are the enlargement of the ventricles, the widening of sulci, and the loss of white and gray matter. This loss results in volume reduction of specific structures. At the temporal lobe, the insula, superior temporal gyrus, medial prefrontal temporal gyrus, amygdala and hippocampus show reduction. At the cortex, the dorsolateral prefrontal cortex (DLPFC) is the most affected (Honea, Crow, Passingham, & Mackay, 2005). The variation in size of these structures is small, commonly in the order of 3 to 5%, but nonetheless, well-established and present in first-episode cases (Honea et al., 2005). Pertinently, it has been reported in a meta-analysis that the administration of antipsychotics can cause further reductions in the volume of brain structures (Moncrieff & Leo, 2010). Among these, alterations to the hippocampus and the DLPFC are the most subject to study,

as these structures are associated to the cognitive symptoms of schizophrenia. A deficit in declarative memory tasks is attributed to hippocampal dysfunction, while a decrease of executive functions such as working memory and attention is attributed to underactivation of the DLPFC. It has been hypothesized that the hippocampus could even play a role in the positive symptoms of schizophrenia. In response to novelty, hippocampal activity indirectly leads to stimulation of dopaminergic neurons of the ventral tegmental area (VTA), which in turn project to the hippocampus and release dopamine (DA), resulting in an enhancement of long-term potentiation (LTP) of hippocampal synapses, and enables memory formation. In this functional loop, the action of the hippocampus is not only important for the formation of new memories, but also for the detection of novelty. It is postulated that the hippocampus serves as a "comparator" between external stimuli and previously acquired memories. Thus, an interesting theory to the nature of psychosis is that this comparison is lost, and external stimuli are mismatched to previous memories in confusing ways, resulting in an untrue perception of reality (J Lisman & Otmakhova, 2001; J. E. Lisman & Grace, 2005). One could also hypothesize that the lack of sensory gating in schizophrenia is a result of deeming every stimulus as novel. The link between hippocampal dysfunction and schizophrenia is further supported by reports of increased flow of cerebrospinal fluid (CSF) in this region, frequent co-occurrence of schizophrenia in temporal lobe epilepsy, and the manifestation of schizophrenia-like symptoms in animals with hippocampal lesions (Harrison, 2004). At a cellular level, reported alterations of the hippocampus include decreased neurogenesis and a disarray of mossy fiber layer cells, though these observations are not reproducible enough to be taken reliably (Harrison, 2004; Tamminga, Stan, & Wagner, 2010). Regarding the whole brain, there is a small, albeit consistent, decrease in neuronal size and neurite density associated to schizophrenia, and mixed results as to whether there is a general reduction in neuronal density or not (Bakhshi & Chance, 2015). However, a great number of more detailed studies steadily point to a decrease in the density of a specific neuronal type, parvalbumin-containing (PV+) GABAergic interneurons, particularly those found at the cortex and hippocampus (Gonzalez-Burgos & Lewis, 2012; Zhang & Reynolds, 2002). Currently, there is acceptance in the field that impairments in this particular neuronal type play a central role in the disorder. Their involvement is of utmost importance to understand the alterations of different neurotransmitter-associated pathways in schizophrenia [see sub-chapter I.III: genetic and molecular characterization]. Moreover, their role is essential for the generation of gamma oscillations required for high levels of cognitive control. Recent reviews that look into the role of oxidative stress and inflammation in schizophrenia were built on, among other insights, the fact that PV+ GABAergic interneurons are particularly sensitive to these insults (Feigenson, Kusnecov, & Silverstein, 2014; Hardingham & Do, 2016). Inflammation plays a part in virtually all psychiatric illnesses, from depression to Alzheimer's, and so, it is not

surprising to find it in schizophrenia. There is mild encephalitis, resulting from a pro-inflammatory environment in the brain, in first-episode cases of schizophrenia (Bechter, 2013). Accordingly, microglial cells have been found to be more active and in higher number in schizophrenics (Bernstein, Steiner, Guest, Dobrowolny, & Bogerts, 2015). Microglia, the macrophages of the brain, when active, are responsible for the release of pro-inflammatory cytokines such as tumor necrosis factor α (TNF- α) and interleukins 1β and 6 (IL-1β, IL-6). Aside from that, they influence many physiological processes other than immunity, including neurogenesis, synaptic transmission and synaptic pruning, processes that are most likely impaired in schizophrenia. Infections during pregnancy are a risk factor for schizophrenia, and so is the manifestation of autoimmune diseases (for example, psoriasis) (Vilain et al., 2013). Furthermore, genome-wide association studies (GWAS) indicate a strong relation between variations in genes of the Human Leukocyte Antigen (HLA) region and schizophrenia (Debnath, Cannon, & Venkatasubramanian, 2013; Ripke et al., 2014). Taking all this into account, it comes as no surprise that immunity is more and more at the center of schizophrenia research – with interesting questions currently being raised about a possible involvement of autoimmunity. With regard to astrocytes, there is no agreement as to whether their numbers are altered, but a series of studies report alterations in the expression of astrocytic enzymes, especially those related to the synthesis of glutamine, D-serine, adenosine triphosphate (ATP), and kynurenic acid, molecules important in astrocytic regulation of neurotransmission (Bernstein et al., 2015). Finally, the number of oligodendrocytes has been shown to be reduced, and myelin sheathing decreased, causing an observable reduction in white matter characteristic of schizophrenia (Bernstein et al., 2015). Oxidative stress and inflammation processes are also impacted by and impactful on glial cells, making it a complex matter to distinguish, among these features, which are causal or consequential of the underlying condition. As a final point, it has been well established that cytological marks of abnormal neural degeneration (such as gliosis, inclusion bodies, neurofibrillary tangles) are not more common in schizophrenics than in a healthy individual, nor is there a higher risk for Alzheimer's among schizophrenics, leading to the conclusion that schizophrenia is not a neurodegenerative condition (Bakhshi & Chance, 2015).

I.III Genetic and molecular characterization

Mental disorders in general do not have a Mendelian inheritance. The same applies to schizophrenia. However, there is a strong genetic component to it. Heritability is estimated at 0,81 and concordance between monozygotic twins is bordering on 50% (Cardno & Gottesman, 2000; Sullivan, Kendler, & Neale, 2003). Environmental risk factors are mainly (but not exclusively) related to prenatal insults towards the fetus, drug abuse, and traumatizing experiences during childhood, while genetic risk factors comprise allelic variants of over 100 genes (Ripke et al., 2014). To obtain a broad picture of the genetic risk factors deemed most important in schizophrenia, a table of the main ones featured at the Online Mendelian Inheritance in Man (OMIM) database was comprised **(Table 1)**. It is not only in genetic association studies that we find alterations in mechanisms linked with schizophrenia, but also in epigenetic, proteomic and functional studies. However, elaborating a comprehensive report of the literature on the subject would be an unfathomable task. Therefore, for the purpose of this thesis, the focus became attaining a comprehensive grasp on the field, and conveying, through a series of pertinent examples, the key conclusions. Historically, the most important gene to become associated with schizophrenia codes for DISC1, a protein that plays a key role in development, and is involved in the regulation of cell proliferation, differentiation, migration, neuronal axon and dendrite outgrowth. It was through a cross-generational study of a Scottish family with a history of mental perturbations that alterations in this gene were first linked to schizophrenia (Blackwood et al., 2001). One of the most employed animal models of schizophrenia based on genetic background is through the knock-out (KO) or knock-down/silencing (KD) of this gene. The second most common is through the deletion of the chromosomal region homologous to 22q11.2 in humans. It was found that individuals with 22q11.2 Deletion Syndrome (22q11.2DS) had a 30 times higher probability of developing schizophrenia (Bassett et al., 2003). In that region lies COMT, a gene which encodes the enzyme Catechol-O-methyl-transferase, responsible for the degradation of dopamine. The findings presented so far arose from opportune observations. In these cases, it happened that links from genes to schizophrenia were discovered through the study of one particular set of individuals for whom this disorder had an unusually high penetrance, and fortuitously resulted in the detection of solely one gene or chromosomal region of interest at fault. Only a small fraction of cases of schizophrenia can so easily be linked to a single genetic trait – a realistic depiction of genetic risk for schizophrenia is far more complex. GWAS became the largest contributors for the detection of genetic variants associated to risk for schizophrenia. These studies examine common genetic variants in different individuals to attest whether any variant is associated with a particular trait, and are

powerful tools in unveiling the whole picture of polygenic traits. Looking into the functions of the genes in Table 1 is informative on the underlying mechanisms affected in schizophrenia.

Table 1: *Genetic risk factors for schizophrenia selected from information listed at the Online Mendelian Inheritance in Man (OMIM) database, phenotype "SCHIZOPHRENIA; SCZD", ID #181500*

Location	Gene/Locus	MIM number	Variation	Gene function	Associated Mechanisms
1p36.22	**MTHFR** 5,10-Methylenetetrahydrofolate reductase	607093	Expression of mutated MTHFR with reduced enzymatic activity	Enzyme responsible for the conversion of folate to its active form – methylfolate, which is required to fulfill the high demand for methyl groups during the post translational methylations of the cytoskeleton in neural cells necessary for neural tube closure. Reduced activity from this enzyme also leads to elevated levels of homocysteine, which acts as an agonist of the NMDAR, intensifying phenomena of excitotoxicity.	Neuronal Development; Glutamatergic Signal Transduction
1q42.2	**DISC1** Disrupted in schizophrenia 1	605210	DISC1 is located at a DNA breakpoint, which causes a translocation that disrupts this gene and leads to a poorer function and/or underexpression	DISC1 is highly expressed during embryonic life, in the course of the development of the cerebral cortex. It regulates multiple aspects of embryonic and adult neurogenesis, neuronal migration and positioning. Its interactions with many pivotal partners influence key cellular proceedings, including apoptosis (AKT1), cAMP levels (phosphodiesterase-4B), microtubule-associated motor transport (dynein), and the proper functioning of the centrosome and cytoskeletal system.	Neuronal Development; Neuronal Migration; Apoptosis; Neurotransmitter Signal Transduction; Cell Division
2q34	**ErbB4** Tyrosine Kinase Cell Surface Receptor HER4	600543	Increased NRG1 and NRG3-induced activation of ERBB4	ErbB4 is a receptor for neuregulins, and is enriched in the postsynaptic density and associates with PSD95. Neuregulins and their associated receptors are essential for neuronal development and synaptic plasticity.	= NRG1
3p25.2	**SYN2** Synapsin 2	600755	Underexpression	Synaptic vesicle-associated protein essential for presynaptic activity, implicated in modulation of neurotransmitter release and in synaptogenesis.	Neurotransmitter Signal Transduction; Synaptogenesis

5q31-q35	**GABRA1**, **GABRP** and **GABRA6** $GABA_A$ receptor subunits	137160, 602769 and 137143	Lower expression and abnormal co-expression of these subunits	$GABA_A$ receptors are ligand-gated ion channels that respond to the chief inhibitory neurotransmitter in the mature vertebrate central nervous system.	GABAergic Signal Transduction
6p21-p22	**HLA region** region of chromosome 6 where mostly Human Leukocyte Antigen genes are present	All HLA genes' MIM identificat ion	Abnormal function or expression of HLAs (functional outcome unknown)	The HLA genes are the human versions of the immunity major histocompatibility complex (MHC) genes. The primary function of MHCs is to bind to peptide fragments derived from pathogens and display them on the cell surface for recognition by the appropriate T-cells. Class I MHC receptors are also involved in synaptic plasticity in the hippocampus and structural regression of synapses during development.	Immunity
6p22.3	**DTNBP1** or Dysbindin Dystrobrevin Binding Protein 1	607145	Underexpressio n	Dysbindin is a key component of biogenesis of lysosome-related organelles complex-1 (BLOC-1), which regulates the trafficking of proteins in the lysosomal pathway. In drosophila, dysbindin has been shown to be essential for neural plasticity.	Lysossomal Protein Degradation; Synaptic Plasticity
8p12	**NRG1** Neuregulin 1	142445	Altered expression of NRG1 isoforms	Neuregulins regulate the composition of neurotransmitter receptors (particularly of NMDARs) in maturing synapses in the brain. NRG signaling in the adult central nervous system may be responsible for modulation of synaptic plasticity. It is also a neuronal signal that promotes the proliferation and survival of the oligodendrocyte.	Neuronal Development; Synaptic Plasticity; Cell Survival
8p21.3	**PPP3CC** Calcineurin gamma catalytic subunit.	114107	Underexpressio n	Calcineurin acts as a Ca^{2+}-dependent modifier of phosphorylation status. Its activity plays a key role in the downstream regulation of dopaminergic signal transduction and in the induction of certain forms of NMDAR-dependent synaptic plasticity. Thus, calcineurin function could comprise a critical link between dopaminergic and glutamatergic signaling.	Dopaminergic and Glutamatergic Signal Transduction
12q24.11	**DAO** D-amino acid Oxidase	124050	Overactivation due to high levels of DAOA	Enzyme that catalyzes the oxidation of D-serine, a potent activator of N-methyl-D-aspartate (NMDA)-type glutamate receptor.	Glutamatergic Signal Transduction
13q14.2	**5HTR2A** serotonin 2A receptor	182135	Overexpression	Receptor for serotonin, a neurotransmitter that plays a role in physiological processes such as sleep, appetite, pain perception, hormone secretion, and sexual behavior. There seems to be a specific role for cortical HTR2A function in the modulation of conflict anxiety. Dopamine can act as a partial agonist for this receptor, and can also induce receptor internalization. HTR2A can also directly interact with mGluR2, to form functional complexes in brain cortex, which are targeted by hallucinogens.	Serotonergic Signal Transduction

13q33.2	**DAOA** D-amino acid Oxidase Activator	607408	Overexpression	Activator for the DAO enzyme.	=DAO
14q32.33	**AKT1** RAC-alpha serine/threonine -protein kinase	164730	Underexpressio n	This protein is fundamental for the transmission of stress-induced cellular responses, and protects the cells from undergoing apoptosis.	Stress Response; Cell survival
22q11.21	**COMT** Catechol-O-methyl-transferase	116790	Expression of a mutated COMT with reduced enzymatic activity	Enzyme involved in the metabolic degradation of catecholamines (e.g., dopamine, norepinephrine, epinephrin).	Dopaminergic Signal Transduction

The most represented processes are: neurodevelopment (DISC1, ErbB4, and NRG1), synaptic plasticity (SYN2, DTNBP1 and NRG 1) and neurotransmission-associated signal transduction, namely dopaminergic (COMT and PPP3CC), glutamatergic (MTHFR, PPP3CC, DAO and DAOA), GABAergic (GABRA1, GABRP and GABRA6), and serotonergic (5HTR2A).

I.III.I Neurodevelopment and schizophrenia

A main point of consensus today is that schizophrenia is a neurodevelopmental disorder. To begin with, the presence of affective dysregulations and structural alterations in first-episode cases indicates that these were present previously to the disease onset. Although cytoarchitectural abnormalities are not always found in schizophrenia, the most reported are cellular disarrays due to inadequate cellular migration during development. Additionally, obstetric complications are among the environmental risk factors for schizophrenia. Building on these premises, a neurodevelopmental animal model for schizophrenia was devised through the treatment of pregnant rat dams with methylazoxymethanol acetate (MAM), an anti-mitotic and anti-proliferative agent that methylates and specifically targets neuroblast proliferation in the central nervous system (CNS), to selectively affect brain development. MAM and DISC-1 KO models for schizophrenia mimic several pathological and behavioural alterations seen in schizophrenia (Jones, Watson, & Fone, 2011). This raises the question of why the age of onset for schizophrenia is typically in adolescence. During early puberty, there is an overproduction of axons and synapses, followed by rapid pruning in later adolescence (Crews, He, & Hodge, 2007). In schizophrenia, a number of genetic and environmental elements can build predisposition for instability of particularly sensitive neuronal circuits, which, when subjected to the added strain of (possibly excessive) synaptic pruning that occurs in adolescence, are pushed to a point of rupture, resulting in the incidence of psychotic symptoms. The evidence pointing to a role of immunity has been

addressed in the previous sub-chapter. It is now pertinent to mention that the immune system is very much involved in this process of selective pruning (Boulanger & Shatz, 2004). This is the termed progressive neurodevelopmental theory of schizophrenia, and it is presently the perspective that gathers the most support.

I.III.II Neurotransmission-associated signal transduction and schizophrenia

The involvements of dopaminergic and glutamatergic pathways are the most relevant for schizophrenia pathology. There are five dopaminergic pathways in the human brain: nigrostriatal, mesolimbic, mesocortial, tuberinfundibular, and thalamic. These are in general terms related to movement, reward, cognition, prolactin release, and sleep, respectively. Positron emission tomography (PET) can offer a mechanistic view of neurotransmission, e. g., by determination of dopamine receptor function and distribution, through binding of a receptor-type specific tracer and monitoring of tracer displacement by dopamine. This technique allowed the detection of increased dopamine receptor 1 (D1R) availability in patients with schizophrenia, (possibly as compensation for the sustained low levels of dopamine from the mesocortical dopaminergic pathway). Over- or underactivation of D1R is associated to cognitive impairment, as found in schizophrenia (Abi-Dargham et al., 2002). PET studies also reveal heightened presynaptic striatal dopaminergic function and elevated striatal dopamine receptor 2 and 3 (D2R and D3R) density in the brain of schizophrenics (Vyas, Patel, Nijran, Al-Nahhas, & Puri, 2010). After the observation that D2R blockers effectively prevent the positive symptoms of schizophrenia, excessive dopaminergic signaling in the mesolimbic pathway was assumed to be the cause for schizophrenics' positive symptoms. In fact, the first generation of antipsychotics was comprised entirely of substances that acted as D2R blockers, and this type of medication is still available as treatment for schizophrenia today. Amphetamine, a recreational drug that acts by increasing dopamine levels, induces psychotic outbreaks, also supporting this train of thought (S. H. Snyder, 1973). Thus, dopamine was coined "the wind of the psychotic fire'', and the dopaminergic hypothesis of schizophrenia emerged (Laruelle, Abi-Dargham, Gil, Kegeles, & Innis, 1999). Decreased dopaminergic signaling at the mesocortical pathway was later postulated to be the cause of negative, affective and cognitive symptoms (Jones et al., 2011). However insightful, this hypothesis does not account for signal convergence nor pathway interactions from other neurotransmitters. For instance, dopamine-based models of schizophrenia do not replicate the negative symptoms of schizophrenia. On the other hand, administrating glutamate N-methyl-D-aspartate receptor (NMDAR) non-competitive antagonists mimics the positive, negative and cognitive symptoms of schizophrenia in healthy individuals and worsen positive symptoms of schizophrenic patients, an observation which was at the basis of the glutamatergic hypothesis of schizophrenia (Krystal; Laurence

P. Karper, MD; John P. Seibyl, MD; Glenna K. Freeman; Richard Delaney & J. Douglas Bremner, MD; George R. Heninger, MD; Malcolm B. Bowers, Jr, MD; Dennis S. Charney, 1994). These compounds bind to the NMDAR at the ion-pore, and physically block the entry of positively-charged ions through the receptor. In particular, PCP and ketamine are non-competitive NMDAR antagonists used recreationally, and chronic users can be falsely diagnosed with schizophrenia. In high doses, competitive antagonists of the NMDAR such as AP-5, which bind to the receptor at the glutamate binding site, have a similar psychotomimetic effect - however, unlike PCP and ketamine, do not lead to addiction (Willetts, Balster, & Leander, 1990). When compared to the effects of other psychotomimetic drugs, such as amphetamines and lysergic acid diethylamide (LSD), the type of psychosis induced by non-competitive NMDAR antagonists is the most similar to those experienced by schizophrenics (Domino & Luby, 2012; Luby, Cohen, Rosenbaum, Gottlieb, & Kelley, 1959). Moreover, NMDAR antagonists effectively increase dopamine release in the limbic system (Aalto et al., 2005; Adams, Bradberry, & Moghaddam, 2002). Application of these same compounds to rats at an early phase of development is now common practice to engender valid pharmacological models of schizophrenia (Bubenkov-Valeov, Horek, Vrajov, & Hschl, 2008). The role of the glutamatergic system and particularly of the NMDA receptor in this ailment has complemented the previous representation of circuits' dysfunction in schizophrenia. A deficit of NMDAR expression and glutamatergic terminals in PV+-containing inhibitory neurons in the prefrontal cortex of schizophrenics was identified (Bitanihirwe, Lim, Kelley, Kaneko, & Woo, 2009). A current hypothesis is that, given that the triggering of cortical GABAergic interneurons is compromised due to decreased NMDAR function, downstream excitation of the dopaminergic mesolimbic pathway is exacerbated, as is the inhibition of the dopaminergic mesocortical pathway. The proposed pathway interactions are illustrated below **(Figure 1)**.

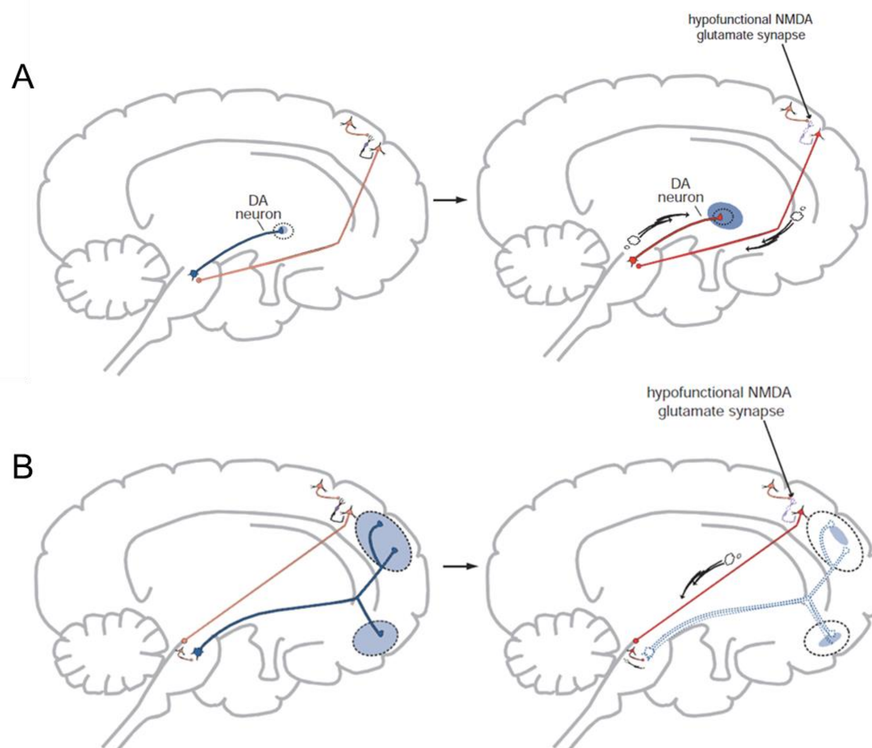

Figure 1: *NMDA receptor hypofunction and the dopaminergic pathways associated with symptoms of schizophrenia* ***A.*** *Cortical glutamate projections (represented by long pyramidal neuron, orange color indicates normal function, red color indicates hyperfunction) communicate with the mesolimbic dopamine pathway (represented by DA neuron, blue color indicates normal function, red color indicates hyperfunction) in the VTA (at the brainstem) to regulate dopamine release in the nucleus accumbens (dotted area at the end of mesolimbic DA neuron, blue color indicates dopamine release). If NMDA receptors on cortical GABA interneurons (small neuron at the cortex, purple color indicates normal function, dotted line indicates hypofunction) are hypoactive, then the cortical brainstem glutamatergic pathway to the VTA will be overactivated, leading to excessive release of glutamate in the VTA. This will result in overstimulation of the mesolimbic dopamine pathway and thus excessive dopamine release in the nucleus accumbens. This is the theoretical biological basis for the mesolimbic dopamine hyperactivity thought to be associated with the positive symptoms of psychosis.* ***B.*** *The cortical brainstem glutamatergic pathway (represented by long pyramidal neuron, orange color indicates normal function, red color indicates hyperfunction) communicates with the mesocortical dopamine pathway (represented by long ramified neuron, blue color indicates normal function, dotted line indicates hypofunction) in the VTA via pyramidal interneurons (small neuron at the brainstem, orange color indicates normal function, red color indicates hyperfunction), thus regulating dopamine release in the prefrontal cortex. If NMDA receptors on cortical GABA interneurons are hypoactive, then the cortical brainstem pathway to the VTA will be overactivated, leading to excessive release of glutamate in the VTA. This will result in excessive stimulation of brainstem pyramidal neurons, which in turn leads to inhibition of mesocortical dopamine neurons. This reduces dopamine release in the prefrontal cortex (dotted areas at the end of mesocortical DA neuron, blue color indicates dopamine release) and is the theoretical biological basis for the negative symptoms of schizophrenia. Ilustrations adapted from* (Stahl, 2013).

By decreasing NMDAR numbers selectively at cortical and hippocampal interneurons, a mouse model was created that mimics the phenotype of schizophrenia, including the post adolescent onset (Belforte et al., 2010). The termed NMDA hypofunction hypothesis attributes the genesis of schizophrenia to an imbalance between glutamatergic, GABAergic and dopaminergic neurotransmission systems and is nowadays the most accredited theory of schizophrenia. This is not in conflict with the previously mentioned progressive neurodevelopmental theory of schizophrenia, as NMDAR hypofunction can lead to, or occur due to, alterations in development.

I.IV Treatment for schizophrenia

As mentioned previously, first generation antipsychotics (also known as typical antipsychotics or neuroleptics) act as D2R blockers. Since these drugs do not target the mesolimbic dopaminergic pathway specifically, their use has severe consequences. The blockade of D2Rs at the mesolimbic system leads not only to the eradication of positive symptoms, but of most dopaminergic signaling at the nucleus accumbens, a structure considered to be the “pleasure center” of the brain (Adinoff, 2004). As such, typical antipsychotics induce neurolepsis, a quiescence state of reduced responsiveness and indifference to surroundings. Moreover, antagonism of dopamine receptors at the tuberoinfundibular pathway can cause hyperprolactinemia. The most serious secondary effects of typical antipsychotics comes from the excessive blockade of D2Rs (more specifically, the blockade of over 80% of these receptors (Farde et al., 1992; Kapur, Zipursky, Jones, Remington, & Houle, 2000) at the nigrostriatal pathway, resulting in movement-related extrapyramidal side effects (EPS), which may even become irreversible. This is partly due to the disablement of nigrostriatal dopamine neurons’ inhibitory effect on striatal cholinergic interneurons. The augmented acetylcholine (Ach) release at the striatum results in enhanced excitation of neurons innervating the motor cortex. To mitigate this, typical neuroleptics are commonly muscarinic 1(M1) cholinergic receptor antagonists. Haloperidol is a typical first-generation antipsychotic that acts as a D2R and M1R antagonist. This drug is still widely used today as one of the fastest-acting treatments for positive symptoms of schizophrenia. The serendipitous discovery of the actions of clozapine, the first atypical neuroleptic to be discovered, triggered the dawn of a new age in treatment for schizophrenia. Clozapine was the first antipsychotic virtually devoid of EPS. It acts on positive and negative symptoms of schizophrenia, and it is the most efficient medication for patients refractory to treatment. On the other hand, secondary effects of clozapine include agranulocytosis, myocarditis, weight gain and diabetes. Most antipsychotics synthesized since its discovery have been an attempt to isolate the mechanisms that grant clozapine its superiority from those causing its nefarious side effects. These new drugs are second-generation or atypical antipsychotics. An antipsychotic is generally considered as atypical when patients treated with it lack or have very low incidence of EPS. The ability to ameliorate negative symptoms and to take effect on previously treatment-resistant schizophrenics are also considered as features of atypicality, though not all second generation antipsychotics exhibit these qualities. Although clozapine’s receptor binding profile has been thoroughly explored, there is no clear interpretation of it, and no consensus as to what is its mechanism of atypicality. The pharmacological properties that have received the most attention are the involvement of clozapine with serotonin receptors. 5-hydroxytryptamine (5HT or serotonin) is

a monoamine neurotransmitter that, like dopamine, acts as a neuromodulator, meaning that its action is neither exclusively excitatory nor inhibitory to neurotransmission. Serotonin receptors are G-protein-coupled metabotropic receptors. 5HT 2A receptors (5HT2ARs) are linked to G_q proteins, and are exclusively post-synaptic. 5HT 1A receptors (5HT1ARs), which are linked to G_i proteins, can be found both pre- and post-synaptically. The role of serotonin receptors in schizophrenia treatment is associated to the nigrostriatal dopaminergic pathway. 5HT2ARs are present at deep layer cortical glutamatergic neurons projecting to the brainstem, where their activation facilitates glutamate release. Glutamate will act on GABAergic interneurons at the brainstem, exacerbating inhibition on dopaminergic neurons, specifically those of the nigrostriatal pathway. 5HT1ARs are present at the same neurons, and their stimulation leads to an inhibition of glutamate release to the brainstem **(Figure 2)** (Stahl, 2013). The outcome of 5HT2AR activation has the same direction as the dopaminergic imbalances brought upon by the use of D2R blockers: there is less effect of dopamine in the striatum, while activation of 5HT1Rs has the opposite outcome. Although both 5HT2AR antagonism and 5HT1A agonism are properties of clozapine which decrease the incidence of EPS by inducing dopamine release in the striatum, dopamine receptor blockade is nevertheless necessary to counteract the hyperdopaminergic state of the mesolimbic pathway, and confer the drug an antipsychotic action (Stahl, 2013). As a parallel to the role of the 5HT2AR, clozapine is also an antagonist of the alpha-1 (α1) adrenergic receptor, a receptor equally present at the before-mentioned deep layer glutamatergic neurons **(Figure 2)**, which, in response to noradrenaline, equally elicits glutamate release (Stahl, 2013). Clozapine acts both as a 5HT2A and an α1R antagonist, as well as a 5HT1A agonist. It was proposed that the unique binding profile of clozapine to receptor subtypes present in convenient brain reagions aligned with a mitigation of EPS mainly by increasing dopamine relase in the striatum. Clozapine is a D2R antagonist, but the increased levels of dopamine at the striatum would displace clozapine from the receptors and lower D2R ocupancy from 80% to closer to 60%

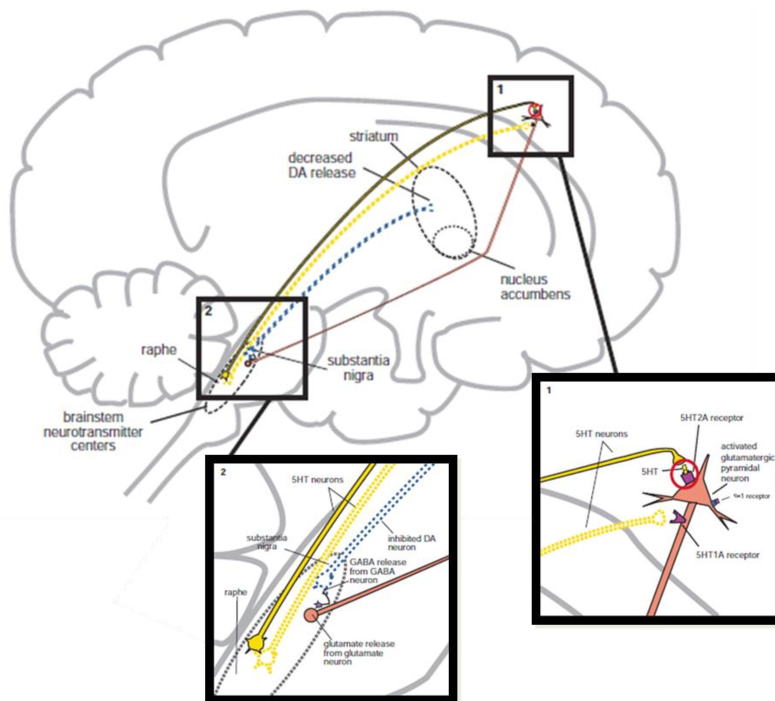

***Figure 2**: Pathways relevant for antipsychotic atypicality. Serotonin projections (yellow) from the raphe nucleus **(2)** to the cortex **(1)** make connections with glutamatergic pyramidal neurons (orange). Serotonin released in the cortex binds to 5HT2A receptors on glutamatergic pyramidal neurons, causing activation of those neurons. This leads to glutamate release in the brainstem, which in turn stimulates GABA release. GABA binds to dopaminergic neurons projecting from the substantia nigra to the striatum, inhibiting dopamine release at the nigrostriatal pathway (blue dotted line). Serotonin released also binds to 5HT1A receptors, which causes inhibition of the glutamatergic neuron. As a result, there is no inhibition of dopamine release from the substantia nigra into the striatum. Thus, cortical 5HT1A receptor stimulation is functionally analogous to cortical 5HT2A receptor blockade, in that both lead to increased dopa mine release in the striatum. Cortical α1 receptor stimulation is functionally analogous to cortical 5HT2A receptor stimulation, as norepinephrine binding to α1 receptors on the cortical glutamate neuron causes glutamate release in the brainstem, which in turn causes GABA release in the substantia nigra, inhibiting dopaminergic neurons and therefore decreasing dopamine release into the striatum. Ilustrations adapted from* (Stahl, 2013)

(Kessler et al., 2006). One other hypothesis of is that clozapine acts mainly as a D2R antagonist, but due to its high dissociation constant, it is so quick to leave the D2R that exaggerated receptor blockade is prevented (Seeman, 2014). It is important to keep in mind how rich the pharmacological profile of this drug is, and how complex interactions between different neurotransmitter pathways can be. Other theories and central roles for other receptor types have been proposed, but have not gathered enough interest to be as thoroughly pursued (Bymaster et al., 2003; Quik, Perez, & Grady, 2011; Svensson, 2003; Wong & Van Tol, 2003). Moreover, there are functions of clozapine that are not directly tied to its receptor binding profile. For example, clozapine modulates glutamatergic transmission by inducing astrocytic release of D-serine, and not by directly interacting with glutamatergic receptors (Tanahashi, Yamamura, Nakagawa, Motomura, & Okada, 2012). In conclusion, a comprehensive grasp on the complete pharmacological actions of this drug remains elusive.

II. Surface trafficking of NMDA receptors

II.I Neurotransmitter receptor trafficking: role in synaptic transmission

Information storage in the brain requires long-term changes in the strength of neurotransmissions through synaptic plasticity. These adaptive processes may involve modifications in the amount of neurotransmitter released by the pre-synapse, but also additions or retractions of neurotransmitter receptors located in the postsynaptic element. This regulation of the number of synaptic receptors has long been exclusively attributed to exo- and endocytosis events. However, the recent development of single molecule tracking techniques has revealed that receptors diffuse laterally in and out of synaptic sites within the membrane plane and are dynamically redistributed in order to ensure a fine control of their number and composition within synapses (Choquet & Triller, 2013) Receptors are thus in a dynamic equilibrium between intracellular, synaptic and extrasynaptic compartments through a combination of exo-/endocytosis phenomenon controlling the amount of receptors at the surface, and lateral diffusion favoring the entry and exit of receptors from synapses (**Figure 3**).

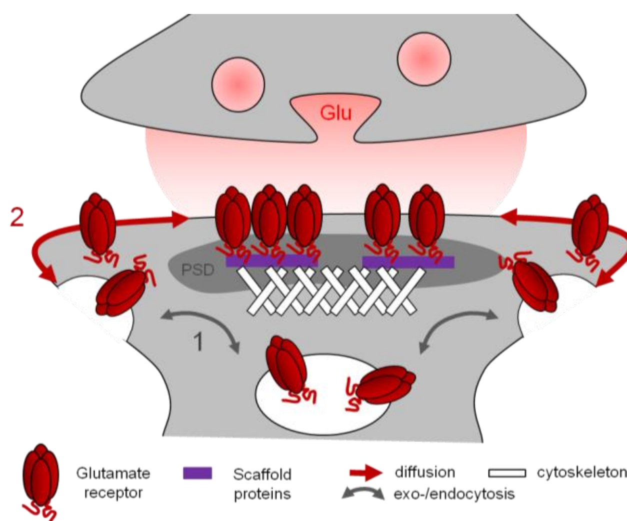

***Figure 3**: Trafficking of ionotropic glutamate receptors at excitatory synapses. **(1)** Perisynaptic exo-/endocytosis processes regulate the amount of receptors expressed at the plasma membrane within the timescale of a minute. Lateral diffusion **(2)** then enables the entry and exit of receptors in and out of synapses within tens of milliseconds. Lateral diffusion of receptors is regulated within synapses by their interaction with the scaffolding proteins (violet bars) of the postsynaptic density (PSD, dark grey). These scaffolding proteins in turn interact with cytoplasmic partners including the actin cytoskeleton (white bars).*

Knowing that synaptic and extrasynaptic receptors are interchangeable, lateral diffusion can be appreciated as means of quickly altering the receptor composition and therefore functional outcome of a synapse. Indeed, this dynamic behavior within the membrane was recently shown to enable the rapid exchange of desensitized synaptic glutamate receptors with naïve perisynaptic ones and thus to be a critical contributor to the fidelity of excitatory synaptic transmission (Heine et al., 2008) Thus, lateral diffusion of neurotransmitter receptors at the surface of neurons is now considered as a key regulation mechanism of

synaptic physiology. The stabilization of diffusive receptors within specific surface compartment depends on the receptors' affinity to locally available molecular partners that physically interact with them and peg them in place. For example, synaptic retention of receptors can be explained by their binding to intracellular interactors enriched at the post-synaptic density (PSD). Receptors tether at the cytoskeleton through connections to scaffolding proteins, which ensures their stabilization at synaptic sites. Regulation of their lateral diffusion also involves allosteric changes or modifications in the trafficking of either the receptors or their interactors.

II.II The NMDAR: subunit composition, lateral diffusion and synaptic plasticity

NMDA receptors are heterotetrameric ionotropic glutamate receptors composed of two obligatory GluN1 subunits associated with any combination of GluN2A, GluN2B, GluN2C, GluN2D, GluN3A or GluN3B subunits. When bound to their endogenous agonist, glutamate, and either one of their endogenous co-agonists, glycine or D-serine, NMDARs are permeable to Ca^{2+} , but only when Na^{+}/K^{+} entries through ionotropic glutamate receptors α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPARs) and kainate receptors (KARs) generate enough membrane depolarization to displace Mg^{2+} ions which block the ion pore at resting potentials. Each subunit is composed of an extracellular N-terminal domain, three transmembrane segments and an intracellular C-terminal tail which in GluN2 subunits harbors several phosphorylation sites and an interaction motif for PDZ domain-containing scaffolding proteins of the post-synaptic density ((Bard & Groc, 2011); **Figure 4**).

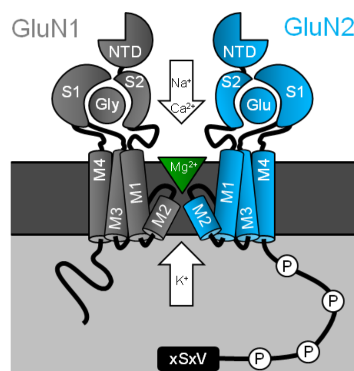

***Figure 4**: Topology of NMDA glutamate receptors. The receptor complex results from the heterotetrameric combination of two GluN1 subunits with two GluN2 and/or GluN3 subunits. Each subunit features three membrane-spanning segments (M1, M3, M4), a re-entering loop (M2) involved in the formation of the cation-selective ion pore, an extracellular N-terminal domain (NTD), two extracellular loops (S1/S2) forming the ligand binding site (glutamate, glycine or D-serine depending on the nature of the subunit), and a large intracellular C-terminal tail. The C-terminal tail of GluN2 subunits presents several phosphorylation sites (P) as well as four terminal amino acids forming the xSxV interaction motif for PDZ domain-containing scaffolding proteins of the post-synaptic density.*
Adapted from (Bard & Groc, 2011).

GluN2A and GluN2B–containing NMDAR subtypes are the most highly expressed in the human brain. In immature neurons (up until the second post-natal week of development in rodents), NMDARs containing the GluN2B subunit (2B-NMDARs) are the predominant subtype mediating synaptic transmission. During neurodevelopment, a 2B/2A switch occurs in synaptic receptor composition. The functional properties of NMDAR (i.e. glutamate affinity,

rectification, unitary conductance, open probability, kinetics) as well as their intracellular trafficking, membrane expression and surface trafficking depend on the nature of GluN2 subunits. For instance, 2A-NMDARs possess the highest open channel probability, 3-5 higher than 2B-NMDARs, and the fastest glutamate deactivation kinetics (Paoletti, Bellone, & Zhou, 2013). NMDARs are then, to some extent, segregated to different compartments according to their constituting subunits, as 2A-NMDARs become enriched in synapses, and 2B-NMDARs become the most present subtype at extrasynaptic sites (Sheng, Cummings, Roldan, Jan, & Jan, 1994) (Bellone & Nicoll, 2007). Depending on their location at the surface of neurons, NMDARs contribute either to synaptic transmission, protein synthesis-associated signaling pathways, cell survival or apoptosis (Bard & Groc, 2011; Cull-Candy & Leszkiewicz, 2004). Their surface trafficking thus requires a fine regulation. The synaptic retention of NMDAR relies on intracellular interactions with scaffolding proteins of the PSD (e.g. PSD-95) regulated through phosphorylation by kinases (e.g. CaMKII) (Bard & Groc, 2011; Bard et al., 2010). It also involves modulations by NMDAR co-agonists (glycine/D-serine) interactions with transmembrane and extracellular partners such as Ephrin B2 receptors, adhesion molecules, extracellular matrix proteins and neurotransmitter receptors such as D1 dopamine receptors (Dalva et al., 2000; Ladépêche, Dupuis, & Groc, 2013; Michaluk et al., 2009) It is very likely that other receptors which physically interact with NMDARs at the membrane surface (namely D2R, mGluR5 and α7-nAchR) also affect their surface diffusion, although this is yet to be demonstrated (Ladépêche et al., 2013).

The diffusion properties of NMDAR depend on their subunit composition. Indeed, GluN2A-containing NMDAR are less mobile and show a longer synaptic dwell time than GluN2B-NMDAR, and are generally concentrated within synapses while GluN2B-NMDAR are rather at the periphery (Groc et al., 2006). It is however important to keep in mind that the adult brain contains not only purely diheteromeric 2A- or 2B-NMDARs, but also a substantial portion of triheteromeric 2A/2B-NMDARs (Chazot & Stephenson, 1997; Rauner & Köhr, 2011). While their respective contributions are still a matter of debate, 2A- and/or 2B-containing NMDAR are central actors of neurodevelopment and synaptic plasticity processes.

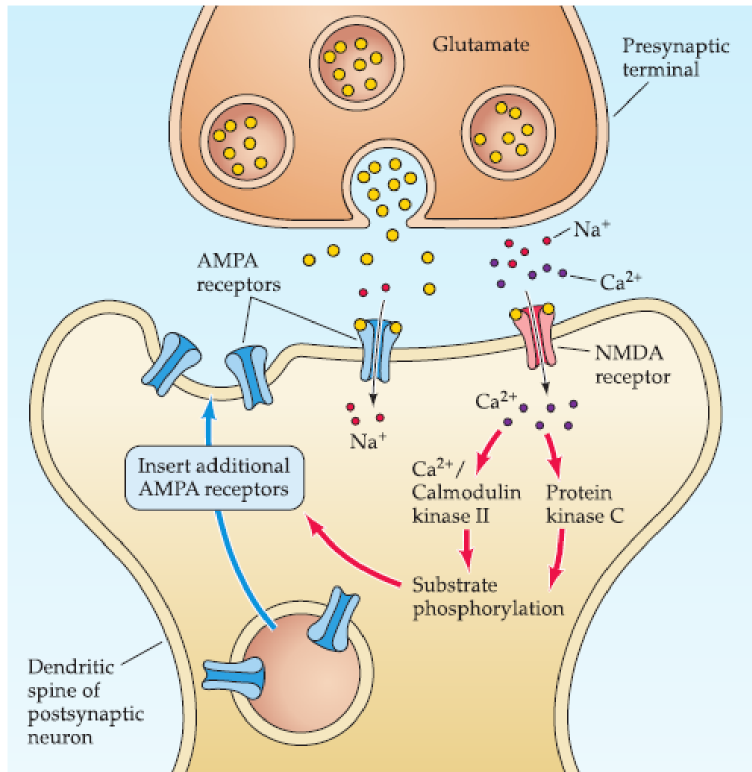

Figure 5: *Mechanisms underlying long-term potentiation. In the presence of glutamate, the NMDAR acts as an ion-pore only if the postsynaptic membrane potential is sufficiently depolarized. Conditions that induce LTP, such as high-frequency stimulation, will cause prolonged glutamate release and AMPAR activation, resulting in the depolarization of the postsynaptic membrane potential, allowing Ca^{2+} to enter through NMDARs. The increase in Ca^{2+} concentration within dendritic spines activates postsynaptic protein kinases. These kinases act in complex pathways to insert new AMPA receptors to the synapse, thereby increasing the sensitivity to glutamate. The induction of LTP seems to rely on the activity of NMDARs, while maintenance of LTP is due to AMPAR insertion into the postsynaptic membrane. Illustration from* (Purves D, Augustine GJ, Fitzpatrick D, et al., 2001).

Repeated synaptic stimulations induce adaptations that alter the amplitude of excitatory post-synaptic potentials (EPSPs) over time scales of 30 minutes or longer: long-term potentiation (LTP) and long-term depression (LTD). This phenomena is input-specific (meaning that if only one synapse is stimulated, only that synapse will undergo potentiation or depression), and associative (if one synapse is stimulated while a neighboring synapse has a weak activity, both undergo potentiation or depression), the same way that knowledge results from long-lasting storage of specific information, and perceptual associations are made between concomitant stimuli. Because of these characteristics, synaptic plasticity is considered to be the substrate for learning (Purves D, Augustine GJ, Fitzpatrick D, et al., 2001). LTD and LTP are elicited through high- and low-frequency synaptic stimulations (LFS and HFS), respectively, and the direction of plasticity depends directly on the size of the NMDAR-mediated calcium influx. In order to elicit synaptic plasticity, there needs to be Hebbian activity, meaning a simultaneous activation of both the pre- and post-synaptic terminals (Hebb, 1949). The NMDAR acts as a coincidence detector, as its activation requires pre-synaptic glutamate release and alterations in the post-synaptic membrane potential. HFS leads to a great influx of extracellular Ca^{2+} through NMDARs, resulting in the recruitment and activation of Ca^{2+}-dependent kinases, of which the most relevant is the calcium-calmoduline kinase II (CaMKII). The accredited mechanism for LTP induction is that intracellular kinase activation leads to substrate phosphorylation, and ultimately to the deployment of intracellular AMPAR reservoirs to the synapse (Purves D, Augustine GJ, Fitzpatrick D, et al., 2001)

(Figure 5). CaMKII phosphorylates stargazin at the PSD, which stabilizes AMPARs at the synapse (Opazo et al., 2010). On the other hand, in the induction of LTD, low Ca^{2+} influx elicited by LFS evokes the recruitment of intracellular phosphatases, favoring the internalization of AMPAR (John Lisman, Schulman, & Cline, 2002). The contributions of 2A- and 2B-NMDARs are not the same for both types of plasticity. LTD is unaffected by GluN2A antagonists, but prevented by GluN2B antagonism. This is true even after blockade of all synaptic NMDARs, indicating that both receptor subtype and localization are determinants of synaptic plasticity (Collingridge, Isaac, & Wang, 2004). When it comes to synaptic potentiation, GluN2B subunit antagonism is only able to prevent LTP induction in immature neurons. As the 2B/2A switch takes place, it becomes possible to prevent LTP by use of GluN2A, but not GluN2B, antagonists. In spite of this, silencing protein expression of the GluN2B subunit is impeditive for potentiation at all developmental stages. Foster and colleagues (2010) pinpointed this specifically to the absence of the C-terminal cytoplasmic tail of the GluN2B subunit, where lie binding sites between NMDARs and CaMKII (Bayer, De Koninck, Leonard, Hell, & Schulman, 2001; Leonard, Lim, Hemsworth, Horne, & Hell, 1999). Altogether, it appears that synaptic adaptations necessitate the presence of both GluN2A- and GluN2B-NMDAR at synapses, and that its intensity and direction depend on the GluN2A/GluN2B balance (Yashiro & Philpot, 2008). Moreover, this balance evolves during synaptic plasticity. Indeed, LTP induction at hippocampal synapses between Schaffer collaterals and CA1 PNs is associated with a decrease in the contribution of GluN2B-NMDAR paralleled by a synaptic enrichment in GluN2A-NMDAR (Bellone & Nicoll, 2007). The Groc laboratory recently demonstrated that this remodeling involves a transient increase in the lateral diffusion of GluN2B-NMDAR which favors the accumulation of CaMKII within dendritic spines through their direct interaction **(Figure 6)**. Preventing either the physical interaction between CaMKII and GluN2B subunits, or the ability of 2B-NMDARs to laterally diffuse, resulted in the same outcome - CaMKII recruitment to the synapse was decreased, and LTP did not take place. Because of this, NMDAR diffusion is postulated to be the driving force for CaMKII relocation (Dupuis et al., 2014). Lateral diffusion of NMDARs is then necessary for LTP induction. *In vivo* electrophysiological recordings in the hippocampus of anesthetized mice confirmed this, as HFS did not induce LTP in mice in which NMDAR diffusion had been deliberately blocked (Potier et al., 2015). Importantly, auto-antibodies from patients suffering anti-NMDAR encephalitis - an autoimmune brain disorder characterized by severe psychotic episodes – also immobilize NMDAR and thereby prevent hippocampal LTP, which could explain the

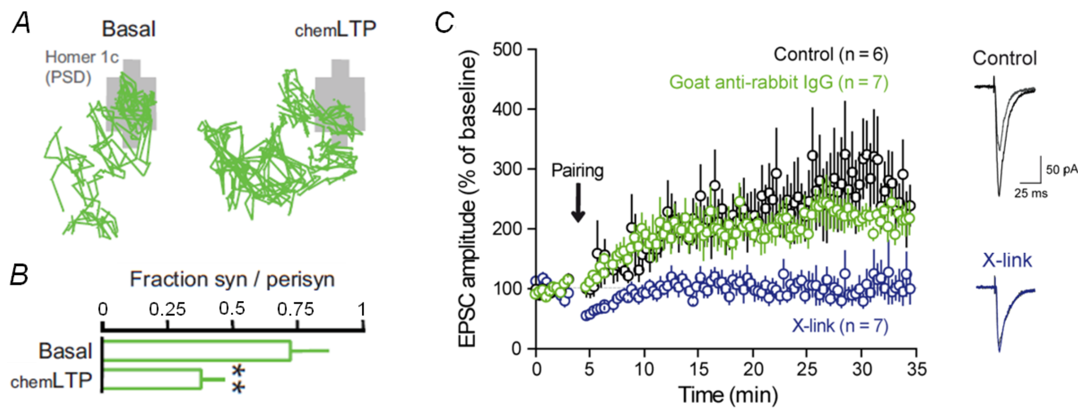

***Figure 6**: Surface diffusion of NMDAR is essential for the expression of hippocampal synaptic plasticity. (A) Surface trajectories of single GluN2B-NMDAR (20 Hz acquisition, 30 s duration) labelled with quantum dots (QD), before (basal) and after chemical LTP (chemLTP). LTP induction triggers an increase in the diffusion of GluN2B-NMDAR (A) which in turn favors their redistribution towards extrasynaptic areas (B; **P<0.01). (C) Patch-clamp recordings on CA1 pyramidal neurons from acute hippocampal brain slices. Impairing GluN2B-NMDAR diffusion through antibody-based receptor cross-linking prevents redistribution and thereby occludes LTP. Insets: excitatory postsynaptic currents (EPSCs) before and after LTP induction in control and cross-link (X-link) conditions. Adapted from Dupuis et al., 2014.*

cognitive deficits observed in these patients. Thus, NMDAR diffusion within the plane of the plasma membrane is a novel regulatory level of synaptic activity, and impairments in this regulation can be associated with neuropsychiatric conditions.

II.III NMDAR surface diffusion and Schizophrenia

Impairments in NMDAR trafficking and function are involved in the onset and/or the expression of severe neurological and psychiatric disorders. For example, Alzheimer's disease is associated with an excessive internalization of GluN2B-NMDAR induced by β-amyloïd peptides (E. M. Snyder et al., 2005). Huntington's disease is also considered as involving NMDAR-dependent excitotoxicity processes (Fan & Raymond, 2007). Moreover, the striatal dopamine depletion characteristic of Parkinson's disease is associated with an increased GluN2A-/GluN2B-NMDAR synaptic ratio and consequent impairments in cortico-striatal plasticity which are directly linked to the expression of motor symptoms (Picconi, Piccoli, & Calabresi, 2012), suggesting that an abnormal redistribution of NMDAR occurs during the emergence of the pathology. Impairments in NMDAR signaling and in the glutamate-dopamine functional interplay also contribute to the onset of psychiatric disorders such as schizophrenia (Carlsson et al., 2001; Lewis & Levitt, 2002). As an example, post-mortem brain samples from patients with schizophrenia show abnormally low levels of NMDAR surface expression (Catts, Lai, Weickert, Weickert, & Catts, 2015). More recently, individuals with autoimmune anti-NMDAR encephalitis, a recently characterized neuropsychiatric disorder with initial symptoms mimicking those of schizophrenia, were found to produce antibodies that acutely impair the surface expression and diffusion of NMDARs without affecting their functions, apparently by disrupting their interaction with (Ephrin B2) EphB2 (Mikasova et al., 2012). After treatment involving immunosuppression, elimination of

the circulating anti-NMDAR antibodies, and temporary IgG replacement (passive immunity), patients typically recover completely. These observations establish a new and unexpected link between NMDAR surface diffusion deficits and psychotic disorders. Excitingly, additional unpublished data from the laboratory show that surface diffusion of NMDAR is impaired at specific time windows of development in primary cultures of rat hippocampal neurons, both in a genetic (DISC1 knock-down) and a neurodevelopmental model (methylazoxymethanol acetate exposure during gestation) of schizophrenia (**Figure 7**; Espana A.et al., in preparation).

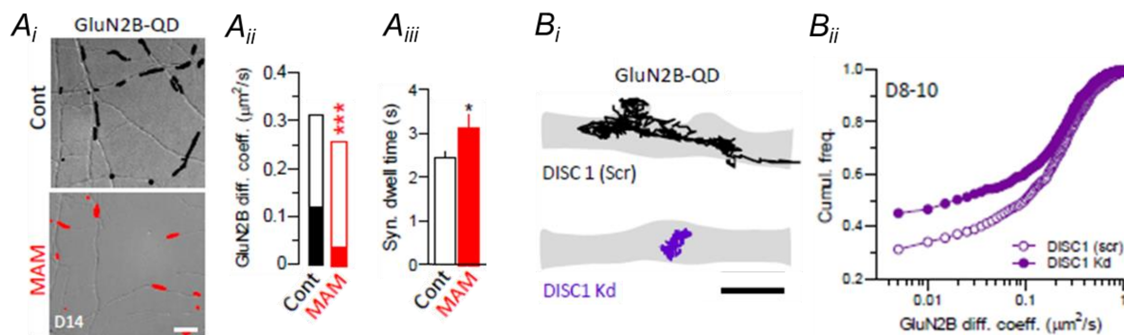

Figure 7: *Surface diffusion of NMDAR is impaired in experimental models of psychotic disorders. (A_i) Surface trajectories of single GluN2B-NMDAR labelled with quantum dots (GluN2B-QD; 20 Hz acquisition, 30 s duration) in hippocampal neuronal cultures from control (Cont, black) and MAM-treated rats (MAM, red; scale bar: 5 μm).The surface diffusion of GluN2B-NMDAR is reduced in cultures from MAM-treated rats, while their synaptic dwell time is increased ($A_{ii\text{-}iii}$; ***P<0.001, *P<0.05). (B_i) Representative trajectories of QD-labelled GluN2B-NMDAR on cultured hippocampal neurons transfected with a scramble siRNA (Scr, black) or knocked down for DISC-1 (Kd, purple). (B_{ii}) Cumulative distributions of the instantaneous diffusion coefficients of GluN2B-NMDAR in control (Scr, white dots) and DISC-1 knock-down conditions (Kd, purple dots). The leftward shift in the distribution and higher initial point reveal a decrease in the diffusion and higher fraction of immobile receptors when DISC-1 is knocked down.*

Based on these observations, we hypothesized that impairments in NMDAR surface trafficking could represent a new hallmark of the disease, and that rescuing these deficits might contribute to alleviate the symptoms of schizophrenia. To address this question, we explored the impact of acute exposure to a psychotomimetic molecule and neuroleptic agents on NMDAR trafficking. Combining single-particle tracking and immunocytochemistry, we assessed here whether ketamine – a non-competitive NMDAR antagonist with psychotomimetic properties – affects the surface diffusion and distribution of NMDAR, and if these ketamine-elicited impairments can be rescued by classical (haloperidol) or atypical (clozapine) neuroleptics.

Material and methods

Materials and Methods

I. Cell culture

Cultures of hippocampal neurons were prepared from embryonic day 18 Sprague-Dawley rats of either sex following a previously described method (Mikasova et al., 2012). Briefly, cells were plated at a density of 200×10^3 to 300×10^3 cells per dish on poly-lysine-pre-coated coverslips. Coverslips were maintained in a 3% horse serum-containing neurobasal medium supplemented with SM1 and kept at 37°C in 5% CO_2. This medium was replaced after 4-5 days in vitro (div) by a serum-free neurobasal medium supplemented with SM1 and kept as previously indicated. When needed, cytosine d-D-arabinofuranoside (commonly known as Ara-C), an inhibitor of DNA synthesis, was added at a concentration of 2 μM at div 7 to prevent excessive glial proliferation. Cultures were kept for 14-15 div for single particle tracking experiments and 17-18 div for immunocytochemistry.

II. Immunocytochemistry

Surface GluN2A and GluN2B were specifically stained using a monoclonal anti-GluN2A or 2B subunit rabbit polyclonal antibody (1:200 or 1:100 respectively) for 10 minutes on live neurons incubated at 37°C in culture medium with added bovine serum albumin (BSA) at 1%. Briefly, neurons were fixed with 4% paraformaldehyde, washed, and kept on phosphate-buffered saline (PBS) with 5-10% BSA for blocking of nonspecific antibody binding sites. Secondary anti-rabbit Alexa 488 antibodies (1:500) were added to act as fluorophore labels of surface 2A- or 2B-NMDARs. To label synaptic sites, neurons then were permeabilized by using 0.3% Triton X-100, kept on phosphate-buffered saline (PBS) with 5-10% BSA for blocking of nonspecific intracellular antibody binding sites, and incubated with a mouse polyclonal anti- antibody (1:500) directed against Homer, a protein of the post-synaptic density, followed by secondary incubation with anti-mouse Alexa 594 antibodies (1:500). All steps following fixation were intercalated by washes with PBS and performed at room temperature (RT).

III. Confocal laser scanning microscopy

The basis of all fluorescence microscopy is that light of a specific wavelength is absorbed by fluorophores, causing them to emit light of longer wavelengths. The excitatory fluorescence wavelengths are sorted from white light through the use of an excitation filter, and travel to a dichroic beamsplitter, reflective for excitation wavelengths and transmissive for emitted wavelengths, to be directed to the sample. Fluorophores then emit light at the emission

wavelength, which travels through the beamsplitter to the light detector. Confocal laser scanning microscopy (CLSM) is an optical imaging technique for increasing the resolution and contrast of classical epifluorescence microscopy. The maximal resolution of this technique is of 180-250 nanometers in the x,y plane (Schermelleh, Heintzmann, & Leonhardt, 2010). In CLSM, the light source for excitatory fluorescence is a powerful laser, allowing for fluorescence emission with high intensity. The improvement of resolution is achieved through the addition of a pinhole close to the light detector (**Figure 8**). Light from fluorophores that are not at the focal plane is dispersed, and does not align with the aperture of the pinhole.

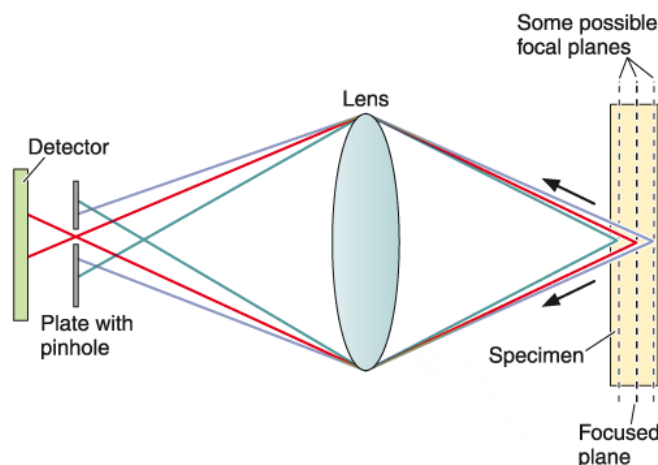

***Figure 8**: Principle of confocal microscopy: The presence of a pinhole prevents out-of-focus light (blue and green lines) derived from different focal planes of the sample (light yellow) to enter the light detector (light green). Image from* (Junqueira & Carneiro, 2005)

A CLSM microscope equipped with a 63X oil-immersion objective was used. Images of separate focal planes were attained for all fields of acquisition, each containing one cell for analysis, by means of the Leica LAS-AF software. Maximum projections were compiled resourcing to the BioFormats plugin of FIJI image analysis software. Fluorescence analysis was performed blind, using a custom-made macro operating with tools provided by FIJI or its predecessor, ImageJ. Homer and NMDAR clusters were identified by selecting regions of interest (ROIs) corresponding to dendrites of stained neurons, and manually defining the threshold between signal and background. Cluster number, area, and fluorescence intensity was then measured for each cluster, and their sums calculated for each cell and divided by the maximal distance of the ROI, an estimation of dendritic length. Quantification of surface NMDAR staining within individual synapses was achieved using Homer staining as a mask filter to isolate surface GluN2A or 2B subunit staining in individual Homer clusters. For the colocalization measurement, the number of synaptic NMDAR clusters was compared to the total number of NMDAR clusters in each cell.

IV. Single quantum dot tracking and surface diffusion

Ensemble imaging approaches such as fluorescence recovery after photobleaching (FRAP) do not allow access to individual molecule dynamics. To overcome this limitation, we favored a single particle tracking-based approach where traceable probes bound to antibodies targeting extracellular epitopes are used to investigate the behavior of surface proteins of interest. The most commonly used nanoparticles to perform single molecule are semiconductor nano-crystals called Quantum Dots (QD) which can be composed of various elements, such as a core of CdSe beneath a shell of ZnS. Their small size (5-10 nm), large Stokes shift, narrow emission spectrum, brightness (an order of magnitude brighter than organic dyes) and photostability, have allowed extensive use over recent years to label a large variety of biomolecules with nanometric precision (30-40 nm resolution), in particular in the field of neuroscience where QD helped disclose unexpected mechanisms regulating synaptic transmission (**Figure 9**).

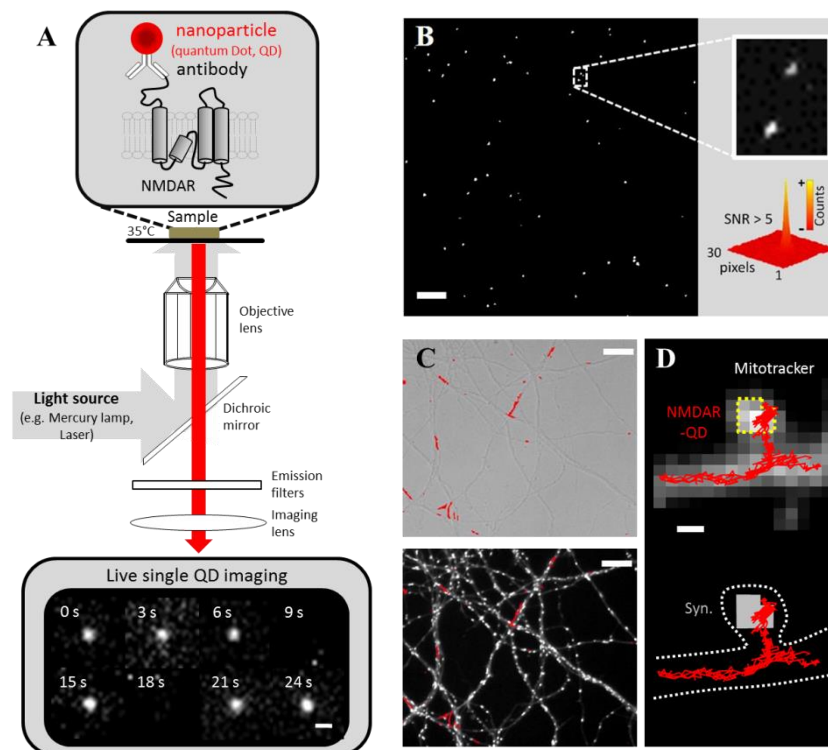

***Figure 9**: Single-particle tracking of NMDAR. (A) Schematic microscopy setting to perform SPT in live brain cells. Nanoparticles (quantum dots, QD) are coupled with polyclonal rabbit antibodies directed against extracellular epitopes of NMDAR. (scale bar: 500 nm). (B) Detection of NMDAR-QD. The brightness and photostability of QD allow prolonged recordings with high signal-to-noise ratio (SNR; scale bar: 5 µm). (C) Trajectories of QD-labeled NMDAR on the dendrites of hippocampal cultured neurons (20 Hz acquisition, 30 s duration; scale bars: 5 µm). Lower panel, Mitotracker staining of synaptic areas. (D) Representative NMDAR trajectory (NMDAR-QD) on a dendrite of a hippocampal neuron. A postsynaptic density labelled with Mitotracker Green is outlined in yellow on the upper picture and shown in grey (syn.) in the lower (white dotted line: shape of the dendrite; scale bar: 500 nm).*

As previously described (Groc et al., 2006), coverslips were first incubated for 10 min with polyclonal rabbit antibodies against GluN2A (1:200 dilution; Alomone Labs; epitope

corresponding to residues 41–53 of GluN2A subunit) and GluN2B subunits (1:200 dilution; Alomone Labs; epitope corresponding to residues 323–337 of GluN2B subunit), followed by quantum dots (QD) 655 goat F(ab')2 anti-rabbit antibodies (1:10000; 10 min incubation). Synaptic localization was achieved by labeling mitochondria with MitoTracker Green (1:50000; 30 s exposure), and the perisynaptic region was established as a 500 nm annulus around the perceived synapse. Imaging sessions were performed on a Nikon Eclipse Ti inverted microscope equipped with a 63X oil-immersion objective. Signals were detected with and EMCCD camera (Evolve, Photometrics) at an acquisition rate of 20 Hz with up to 500 frames, and processed with Metamorph software (Molecular Devices). To determine the distribution and synaptic fraction of single QD complexes, frame stacks were obtained and on each frame the receptor-particle complexes were precisely located in synaptic, perisynaptic, and extrasynaptic compartments. Then, those locations were projected on a single image, providing a high-resolution distribution of receptor-QD complexes. The two-dimensional trajectories of single molecules in the plane of focus were constructed by correlation analysis between consecutive images by using a Vogel algorithm. The instantaneous diffusion coefficient "D" was calculated for each trajectory, from linear fits of the first four points of the mean square displacement versus time function using $MSD(t) = \langle r^2 \rangle (t) = 4Dt$. Single-particle detection and synaptic staining identification was performed under Metamorph environment. Trajectories were reconnected with Matlab software (Mathworks) and extraction of receptor diffusion parameters was conducted with a custom-designed software (MSDTurbo).

V. Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Normally distributed data sets were tested by Student's unpaired t-test for two independent groups. Non-Gaussian distribution datasets were tested by Mann-Whitney U test. Multiple-comparisons were performed by One-way ANOVA using Dunnett's *post hoc* multiple comparison test. Indications of significances correspond to p values <0.05(*), $p<0.01$(**), and $p<0.001$(***).

Results

Results

I. Ketamine impairs the surface diffusion of NMDAR

Rapid NMDAR surface redistributions were recently shown to be a critical step in synaptic plasticity processes (Dupuis et al., 2014; Ladépêche et al., 2013; Potier et al., 2015), and impairments in these redistribution processes appear to occur in experimental models of neuropsychiatric diseases (**Figure 9**;(Mikasova et al., 2012); Espana et al., in preparation). To determine if alterations in NMDAR surface dynamics could be a hallmark of psychotic disorders, we first assessed whether ketamine, a non-competitive psychotomimetic NMDAR antagonist which induces psychotic episodes in healthy individuals, impacts the diffusion and distribution of NMDAR at the surface of cultured hippocampal neurons. Using single-particle tracking methods, we imaged the dynamics of GluN2A and GluN2B subunit-containing NMDAR before and after acute application of ketamine (1 µM). Tracking of single 2A- and 2B-NMDAR revealed that ketamine downregulates the surface diffusion of both receptor subtypes in the extrasynaptic compartment (**Figure 10**; median ± interquartile range [IQR]; extrasynaptic GluN2A-NMDAR, Before Ket: 0.1454±0.04578-0.2428, n = 838 trajectories; After Ket: 0.1274±0.216-0.2286, n = 1832; Before Ket vs After Ket: ** p<0.01; extrasynaptic GluN2B-NMDAR, Before Ket: 0.1439±0.0477-0.2398, n = 2007; After Ket: 0.1336±0.03595-0.2283, n = 4077; Before Ket vs After Ket: ** p<0.01). Consistently, the leftward shift and higher initial point in the cumulative distributions of extrasynaptic diffusion coefficients shows a decrease in the mobility and higher fraction of immobile receptors (membrane diffusion < 0.005 $\mu m^2/s$) after exposure to ketamine, in particular when considering 2A-NMDAR (**Figure 10 A_{ii}**). To note, perisynaptic and synaptic 2A- and 2B-NMDAR were not affected by ketamine (**Figure 10;** median ± IQR; perisynaptic GluN2A-NMDAR, Before Ket: 0.0963±0.03668-0.1957, n = 330 trajectories; After Ket: 0.975± 0.0304-0.1920, n = 735; Before Ket vs After Ket: p>0.05; synaptic GluN2A-NMDAR, Before Ket: 0.06945±0.03573-0.1797, n = 34; After Ket: 0.05445±0.01585-0.1401, n = 76, Before Ket vs After Ket: p>0.05; perisynaptic GluN2B-NMDAR: Before Ket: 0.1032±0.04415-0.1845, n = 1004 trajectories; After Ket: 0.0975± 0.03712-0.1852, n = 2173, Before Ket vs After Ket: p>0.05; Synaptic GluN2B-NMDAR: Before Ket: 0.04514±0.01735-0.1323, n = 129; After Ket: 0.0528±0.01895-0.1201, n = 260, Before Ket vs After Ket: p>0.05). Thus, acute exposure to ketamine results in an overall decrease of NMDAR surface dynamics on primary cultures of hippocampal neurons, most notably in extrasynaptic compartments, suggesting that NMDAR diffusion impairments also occur in this pharmacological model of schizophrenia.

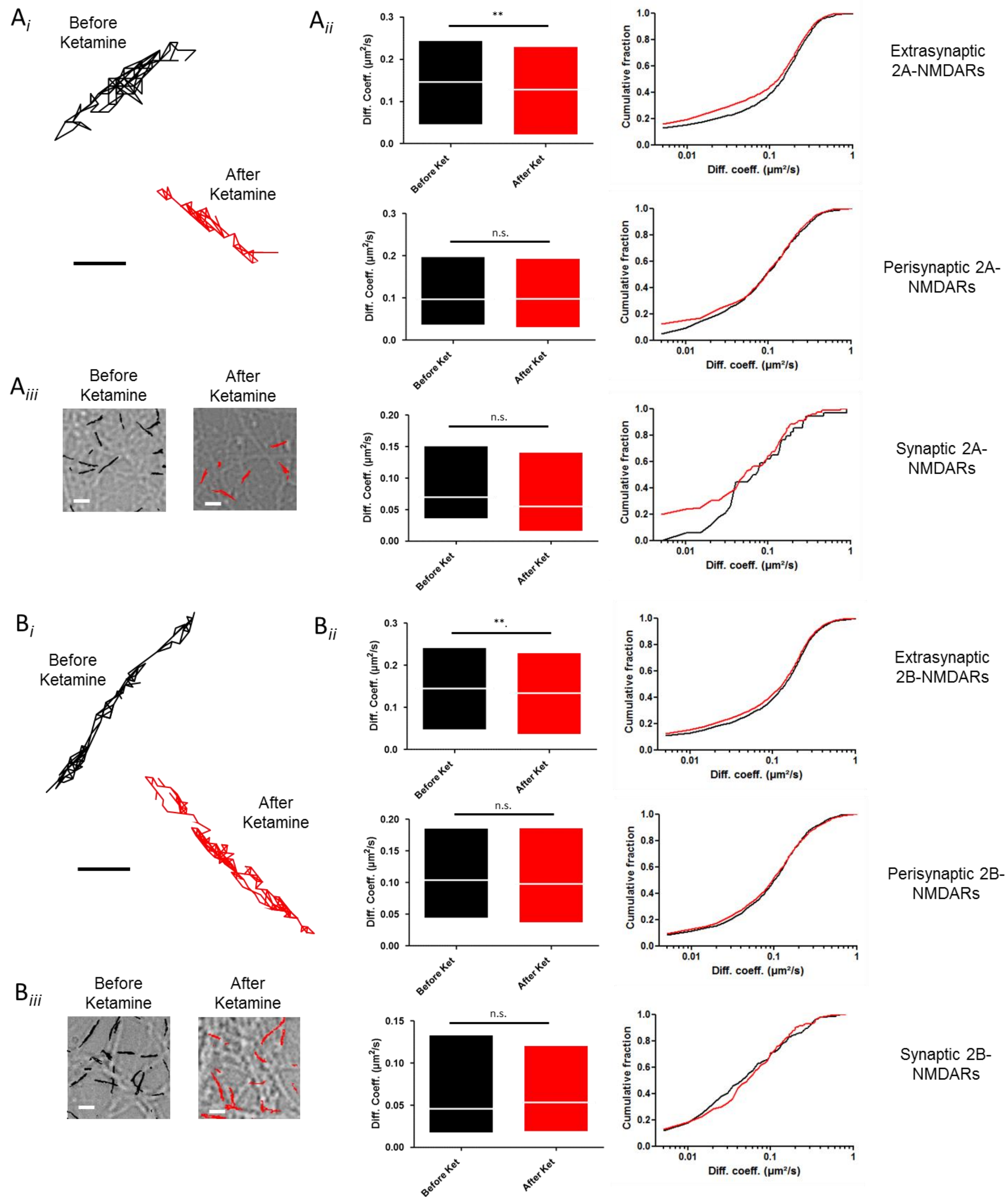

Figure 10: *Surface diffusion of NMDAR is impaired in a ketamine-based pharmacological model of schizophrenia. (A$_i$) Representative trajectories (25-s duration, 20-Hz acquisition, 0-10 min after acute drug application) of surface QD-conjugated 2A-NMDARs from control (Before Ketamine, black) and ketamine-treated neurons (After Ketamine, red; scale bar: 1 µm) (A$_{ii}$) Left: Comparison of the instantaneous membrane diffusion coefficients of extrasynaptic (E), perisynaptic (P) and synaptic (S) 2A-NMDARs in control (Before Ket, black) and ketamine conditions (After Ket, red) Floating bars central lines indicate median and boxes above and below are interquartile range (IQR). Right: Cumulative distributions of the instantaneous diffusion coefficients of extrasynaptic, perisynaptic and synaptic 2A-NMDARs in different experimental conditions (Before Ketamine, black dots; After Ketamine, red dots). (A$_{iii}$) Surface trajectories of single 2A-NMDARs labelled with quantum dots (25-s duration, 20-Hz acquisition) in control (Before Ketamine, black) and ketamine-treated (After Ketamine, red; scale bar: 4 µm) neurons from hippocampal neuronal cultures (n = 4 cultures).(B$_i$) Representative trajectories (25-s duration, 20-Hz acquisition, 0-10 min after acute drug application) of surface QD-conjugated 2B-NMDARs from control (Before Ketamine, black) and ketamine-treated neurons (After Ketamine, red; scale bar: 1 µm) (B$_{ii}$) Left: Comparison of the instantaneous membrane diffusion coefficients of extrasynaptic, perisynaptic and synaptic 2B-NMDARs in control (Before Ket, black) and ketamine conditions (After Ket, red). Right: Cumulative distributions of the instantaneous diffusion coefficients of extrasynaptic, perisynaptic and synaptic 2B-NMDARs in different experimental conditions (Before Ketamine, black dots; After Ketamine, red dots). (B$_{iii}$) Surface trajectories of single 2B-NMDARs labelled with quantum dots (25-s duration, 20-Hz acquisition) in control (Before Ketamine, black) and ketamine-treated neurons (After Ketamine, red; scale bar: 4 µm) from hippocampal neuronal cultures (n = 6 cultures).*

II. Haloperidol does not affect NMDAR surface diffusion

Because changes in NMDAR surface dynamics may thus be associated with the switch between healthy and psychotic states, we then tested whether neuroleptic drugs used in the clinics to alleviate psychotic symptoms could also exert an influence on receptor lateral diffusion. To this aim, we examined in parallel the actions of a classical first-generation antipsychotic, haloperidol, and an atypical second-generation neuroleptic agent, clozapine. A 1 hour pre-incubation with haloperidol (1 μM) did not alter the overall surface trafficking properties of 2A- and 2B-NMDARs, either regarding instantaneous diffusion coefficients, cumulative distributions of diffusion speeds and mean squared displacements versus time (i.e. surface explored as a function of time; **Figure 11 A_{ii} & B_{ii}**). While this observation applied to all surface compartments for 2A-NMDAR (**Figure 11;** median ± IQR; extrasynaptic GluN2A-NMDAR, PBS: 0.1417±0.05321-0.2463, n = 5220 trajectories; Halo: 0.1394±0.05038-0.2516, n = 4226; PBS vs Halo: p>0.05; perisynaptic GluN2A-NMDAR, PBS: 0.1006±0.03691-0.1950, n = 5521; Halo: 0.09971±0.03796-0.1971, n = 4501; PBS vs Halo: p>0.05; synaptic GluN2A-NMDAR, PBS: 0.06956±0.02442-0.9140, n = 1071; Halo: 0.07871±0.02529-0.1601, n = 939; PBS vs Halo: p>0.05) , one should note though that membrane diffusion coefficients of perisynaptic 2B-NMDARs were significantly increased after exposure to haloperidol (**Figure 11;** median ± IQR; extrasynaptic GluN2B-NMDAR, PBS: 0.1515±0.06314-0.2611, n = 4862 trajectories; Halo: 0.1586±0.06690-0.2664, n = 3679; PBS vs Halo: p>0.05; perisynaptic GluN2B-NMDAR, PBS: 0.1077±0.03973-0.2059, n = 4695; Halo: 0.1127±0.04615-0.2151, n = 3978; PBS vs Halo: * p<0.05; synaptic GluN2B-NMDAR, PBS: 0.07740±0.02581, n = 854; Halo: 0.08176±0.02882-0.1655, n = 868; PBS vs Halo: p>0.05) but this effect was not confirmed when considering cumulative distributions of diffusion coefficient values or surface explored by receptors over time (**Figure 11 B_{ii}**).

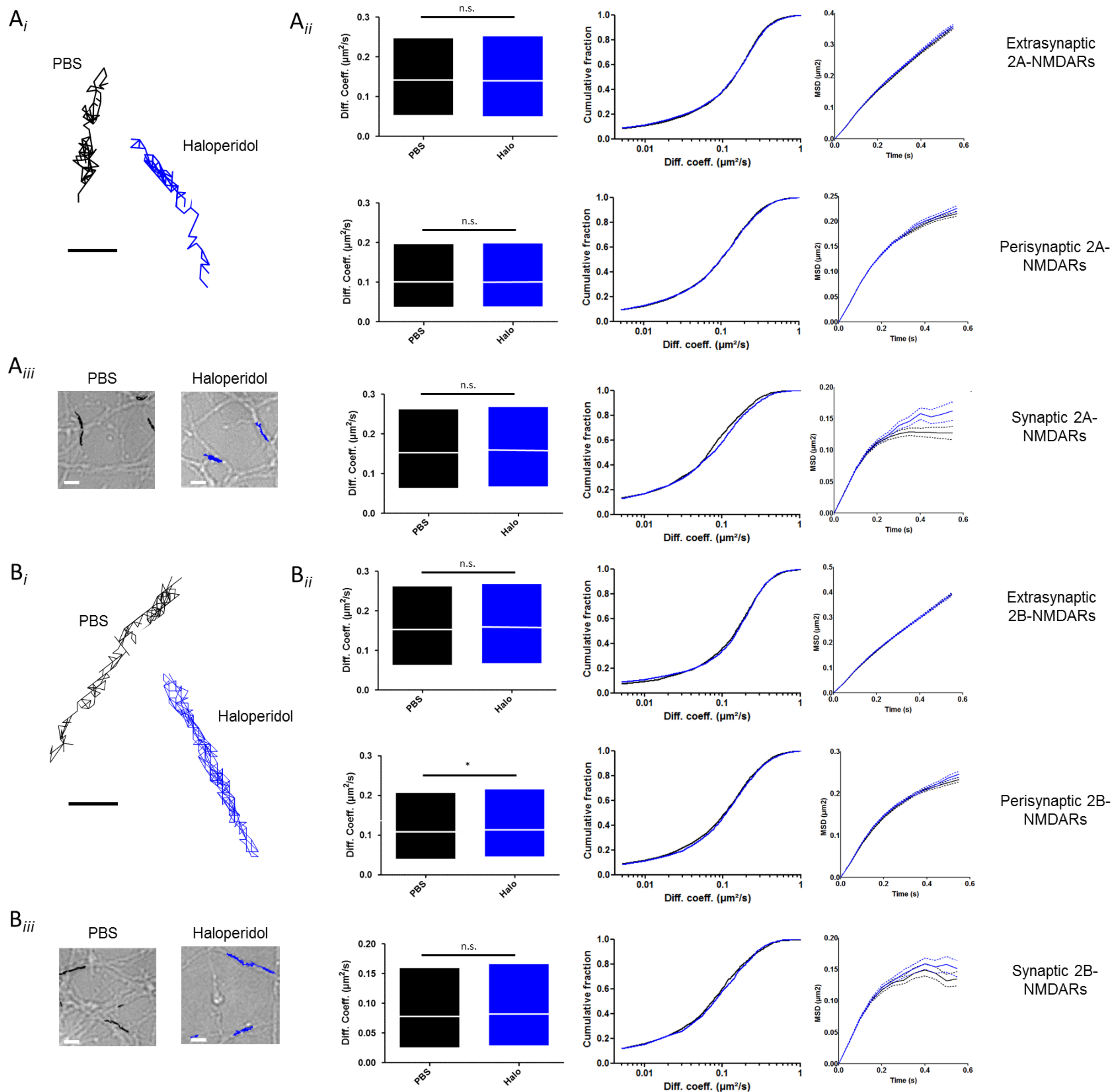

Figure 11: *Haloperidol does not affect the surface diffusion of NMDAR. (A_i) Representative trajectories (25-s duration, 20-Hz acquisition, 1h after drug application) of surface QD-conjugated 2A-NMDARs from control (PBS, black) and haloperidol-treated neurons (Haloperidol, blue; scale bar: 1 µm) (A_{ii}) Left: Comparison of the instantaneous membrane diffusion coefficients of extrasynaptic, perisynaptic and synaptic 2A-NMDARs in control (PBS, black) and haloperidol conditions (Halo, blue); Center: Cumulative distributions of the instantaneous diffusion coefficients of extrasynaptic, perisynaptic and synaptic 2A-NMDARs in different experimental conditions (PBS, black dots; Haloperidol, blue dots) Right: Mean square displacement (MSD) of 2A-NMDARs in the different surface compartments of neurons in control conditions (PBS, black) and treated with the antipsychotic agent (Haloperidol, blue) Full lines represent the average MSD, while dotted lines represent the mean ± s.e.m. (A_{iii}) Surface trajectories of single 2A-NMDARs labelled with quantum dots (25-s duration, 20-Hz acquisition) in control (PBS, black) and haloperidol-treated neurons (Haloperidol, blue; scale bar: 4 µm) from hippocampal neuronal cultures (n = 5 cultures). (B_i) Representative trajectories (25-s duration, 20-Hz acquisition, 1h after drug application) of surface QD-conjugated 2B-NMDARs from control (PBS, black) and haloperidol-treated neurons (Haloperidol, blue; scale bar: 1 µm) (B_{ii}) Left: Comparison of the instantaneous membrane diffusion coefficients of extrasynaptic, perisynaptic and synaptic 2B-NMDARs in control (PBS, black) and haloperidol conditions (Halo, blue); Center: Cumulative distributions of the instantaneous diffusion coefficients of extrasynaptic, perisynaptic and synaptic 2B-NMDARs in different experimental conditions (PBS, black dots; Haloperidol, blue dots) Right: Mean square displacement (MSD) of 2B-NMDARs in the different surface compartments of neurons in control conditions (PBS, black) and treated with the antipsychotic agent (Haloperidol, blue) Full lines represent the average MSD, while dotted lines represent the mean ± s.e.m. (B_{iii}) Surface trajectories of single 2B-NMDARs labelled with quantum dots (25-s duration, 20-Hz acquisition) in control (PBS, black) and haloperidol-treated neurons (Haloperidol, blue; scale bar: 4 µm) from hippocampal neuronal cultures (n = 5 cultures).*

III. Clozapine increases NMDAR surface diffusion

Interestingly, 1 hour exposure to a concentration of clozapine matching the one observed in patient sera (1 μM) increased 2A-NMDAR diffusion in perisynaptic and synaptic compartments (**Figure 12;** median ± IQR; extrasynaptic GluN2A-NMDAR, PBS: 0.147991±0.0502116-0.264757, n = 5902 trajectories; Cloza: 0.152165±0.0508012-0.271081, n = 4820; PBS vs Cloza: $p>0.05$; perisynaptic GluN2A-NMDAR, PBS: 0.0956966±0.032132-0.193584, n = 6742; Cloza: 0.101732±0.038-0.210962, n = 5252; PBS vs Cloza: ** $p<0.01$; synaptic GluN2A-NMDAR, PBS: 0.0739811±0.0269784-0.162663, n = 1874; Cloza: 0.0916624±0.032824-0.186451, n = 1388; PBS vs Cloza: ** $p<0.01$), as well as the diffusion of extrasynaptic 2B-NMDAR (**Figure 12;** median ± IQR; extrasynaptic GluN2B-NMDAR, PBS: 0.154938±0.0586542-0.267871, n= 4454 trajectories; Cloza: 0.170152±0.064025-0.288081, n = 3714; PBS vs Cloza: ** $p<0.01$; perisynaptic GluN2B-NMDAR, PBS: 0.111209±0.0448012-0.214751, n = 4996; Cloza: 0.1139±0.044194-0.223439, n = 4074; PBS vs Cloza: $p>0.05$; synaptic GluN2B-NMDAR, PBS: 0.0929845±0.0350653-0.171509, n = 1190; Cloza: 0.0816806±0.0277471-0.172572, n = 950; PBS vs Cloza: $p>0.05$). This was confirmed by rightward shifts in the cumulative distributions of peri/synaptic 2A-NMDAR and 2B-NMDAR, respectively, as well as an increase in their respective surface explored over time (**Figure 12 A_{ii} & 12 B_{ii}**). Thus, while haloperidol did not affect NMDAR surface dynamics in our experimental paradigm, clozapine seems to increase the diffusion and exploration capacities of NMDAR at the surface of cultured hippocampal neurons.

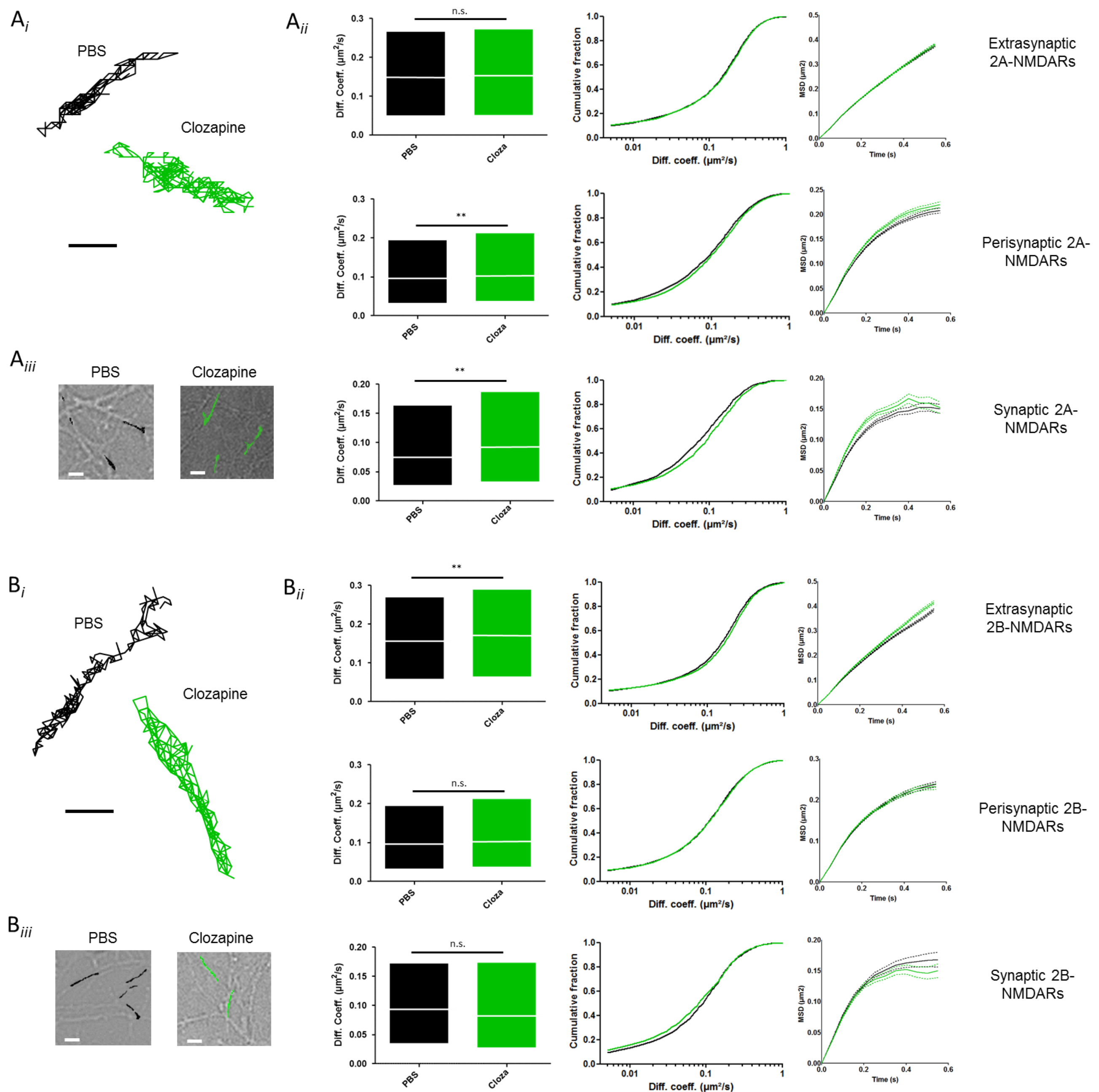

Figure 12: *Clozapine increases the surface diffusion of NMDAR. (A$_i$) Representative trajectories (25-s duration, 20-Hz acquisition, 1h after drug application) of surface QD-conjugated 2A-NMDARs from control (PBS, black) and clozapine-treated neurons (Clozapine, green; scale bar: 1 µm) (A$_{ii}$) Left: Comparison of the instantaneous membrane diffusion coefficients of extrasynaptic, perisynaptic and synaptic 2A-NMDARs in control (PBS, black) and clozapine conditions (Cloza, green); Center: Cumulative distributions of the instantaneous diffusion coefficients of extrasynaptic, perisynaptic and synaptic 2A-NMDARs in different experimental conditions (PBS, black dots; Clozapine, green dots) Right: Mean square displacement of 2A-NMDARs in the different surface compartments of neurons in control conditions (PBS, black) and treated with the antipsychotic agent (Clozapine, green). Full lines represent the average MSD, while dotted lines represent the mean ± s.e.m. (A$_{iii}$) Surface trajectories of single 2A-NMDARs labelled with quantum dots (25-s duration, 20-Hz acquisition) in control (PBS, black) and clozapine-treated neurons (Clozapine, green; scale bar: 4 µm) from hippocampal neuronal cultures (n = 7 cultures). (B$_i$) Representative trajectories (25-s duration, 20-Hz acquisition, 1h after drug application) of surface QD-conjugated 2B-NMDARs from control (PBS, black) and clozapine-treated neurons (Clozapine, green; scale bar: 1 µm) (B$_{ii}$) Left: Comparison of the instantaneous membrane diffusion coefficients of extrasynaptic, perisynaptic and synaptic 2B-NMDARs in control (PBS, black) and clozapine conditions (Cloza, green); Center: Cumulative distributions of the instantaneous diffusion coefficients of extrasynaptic, perisynaptic and synaptic 2B-NMDARs in different experimental conditions (PBS, black dots; Clozapine, green dots) Right: Mean square displacement of 2B-NMDARs in the different surface compartments of neurons in control conditions (PBS, black) and treated with the antipsychotic agent (Clozapine, green) Full lines represent the average MSD, while dotted lines represent the mean ± s.e.m. (B$_{iii}$) Surface trajectories of single 2B-NMDARs labelled with quantum dots (25-s duration, 20-Hz acquisition) in control (PBS, black) and clozapine-treated neurons (Clozapine, green; scale bar: 4 µm) from hippocampal neuronal cultures (n = 7 cultures).*

IV. Clozapine rescues ketamine-elicited impairments in NMDAR surface diffusion

Since ketamine slows down the lateral diffusion of NMDAR whilst clozapine affects NMDAR mobility in the opposite direction, we hypothesized that clozapine might favor the rescue of ketamine-elicited impairments in NMDAR diffusion. To explore this, we compared the effects of acute ketamine application on NMDAR dynamics at the surface of hippocampal neurons with or without a 1h pre-incubation with clozapine. For a more detailed analysis of the kinetics of ketamine action, and in order to control for receptor diffusional rundown associated with the length of acquisition time, we built time-lapsed representations of instantaneous diffusion coefficients and included a control condition where PBS replaced ketamine. Variability associated with the properties of individual cells was abated by normalizing time-lapsed traces to the initial acquisition timepoint, before any substance was applied. Our results show, consistently with previous observations, that diffusion of 2A-NMDAR is significantly decreased after 5-10 min of application of ketamine (**Figure 13;** median ± IQR; total GluN2A-NMDAR, PBS: 0.7622±0.2234-1.368, n = 5755 trajectories; Ket: 0.7195±0.1560, n = 2643; PBS vs Ket: * p<0.05). When compared to neurons exposed to antipsychotic pre-treatment, 2A-NMDAR diffusion following ketamine application is lower (**Figure 13;** median ± IQR; total GluN2A-NMDAR, Ket: 0.7195±0.1560, n = 2643 trajectories; Ket+Cloza: 0.7819±0.2468-1416, n = 2869; Ket vs Ket+Cloza: ** p<0.01), while there is no statistically significant difference between PBS and Ketamine+Clozapine conditions lower (**Figure 13;** median ± IQR; total GluN2A-NMDAR, PBS: 0.7622±0.2234-1.368, n = 5755 trajectories; Ket+Cloza: 0.7819±0.2468-1416, n = 2869; PBS vs Ket+Cloza: p>0.05), indicating that clozapine successfully prevents the effect of ketamine on 2A-NMDAR diffusion. This is verified for extrasynaptic 2A-NMDAR (**Figure 13;** median ± IQR; extrasynaptic GluN2A-NMDAR: PBS: 0.8043±0.2269-1.340, n = 4037 trajectories; Ket: 0.7590±0.1282-1.366, n = 1832; Ket+Cloza: 0.8261±0.2567, n = 1952; PBS vs Ket: * p<0.05; Ket vs Ket+Cloza: ** p<0.01; PBS vs Ket+Cloza: p>0.05), but not for receptors in perisynaptic and synaptic compartments, where neither ketamine, nor clozapine and ketamine combined seem to take effect (**Figure 13;** median ± IQR; Perisynaptic + Synaptic GluN2A-NMDAR, PBS: 0.6878±0.2371-1.369, n = 1514 trajectories; Ket: 0.6920±0.2204-1.375, n = 811; Ket+Cloza: 0.6541±0.2504-1.400, n = 917; PBS vs Ket: p>0.05; Ket vs Ket+Cloza: p>0.05; PBS vs Ket+Cloza: p>0.05). Viewing all surface compartments as a whole, ketamine as well as clozapine and ketamine combined do not significantly affect the diffusion of 2B-NMDARs (**Figure 13;** median ± IQR; total GluN2B-NMDAR, PBS: 0.7736±0.2158-1.420, n = 5366 trajectories; Ket: 0.7390±0.2371-1.357, n = 4699; Ket+Cloza: 0.7023±0.1902-1.404, n = 3882; PBS vs Ket: p>0.05; Ket vs Ket+Cloza: p>0.05; PBS vs Ket+Cloza: p>0.05). However, detailed analysis of receptor behavior in the different surface compartments reveals that the diffusion of 2B-NMDARs is decreased in the extrasynaptic

compartment 5-10 min after application of ketamine (**Figure 13;** median ± IQR; extrasynaptic GluN2B-NMDAR, PBS: 0.8703±0.2469-1.472, n = 3573 trajectories; Ket: 0.7743±0.2220-1.336, n = 3077; PBS vs Ket: *** p<0.001) and increased in perisynaptic and synaptic compartments (**Figure 13;** median ± IQR; perisynaptic + synaptic GluN2B-NMDAR, PBS: 0.5882±0.1835-1.256, n = 1793 trajectories; Ket: 0.7079±0.2774-1.315, n = 1622; PBS vs Ket: *** p<0.001). Although there is no statistically significant difference between ketamine and ketamine + clozapine conditions (**Figure 13;** median ± IQR; extrasynaptic GluN2B-NMDAR, Ket: 0.7743±0.2220-1.336, n = 3077 trajectories; Ket+Cloza: 0.7632±0.1822-1.389, n = 2534; Ket vs Ket+Cloza: p>0.05; perisynaptic + synaptic GluN2B-NMDAR, Ket: 0.7079±0.2774-1.315, n = 1622; Ket+Cloza: 0.6644±0.2108-1.296, n = 1348; Ketvs Ket+Cloza: p>0.05), pre-treatment with clozapine is counteracting the effects of ketamine for receptors at perisynaptic and synaptic sites, as there is a sound difference between PBS and Ketamine+Clozapine conditions for extrasynaptic 2B-NMDARs (**Figure 13;** median ± IQR; extrasynaptic GluN2B-NMDAR, PBS: 0.8703±0.2469-1.472, n = 3573 trajectories; Ket+Cloza: 0.7632±0.1822-1.389, n = 2534; PBS vs Ket+Cloza *** p<0.01), but not for perisynaptic and synaptic ones (**Figure 13;** median ± IQR; perisynaptic + synaptic GluN2B-NMDAR, PBS: 0.5882±0.1835-1.256, n = 1793 trajectories; Ket+Cloza: 0.6644±0.2108-1.296, n = 1348; PBS vs Ket+Cloza: p>0.05). Altogether, our results show that while ketamine slows down the surface dynamics of both 2A- and 2B-NMDAR, clozapine compensates mainly for ketamine-induced impairments in 2A-NMDAR diffusion.

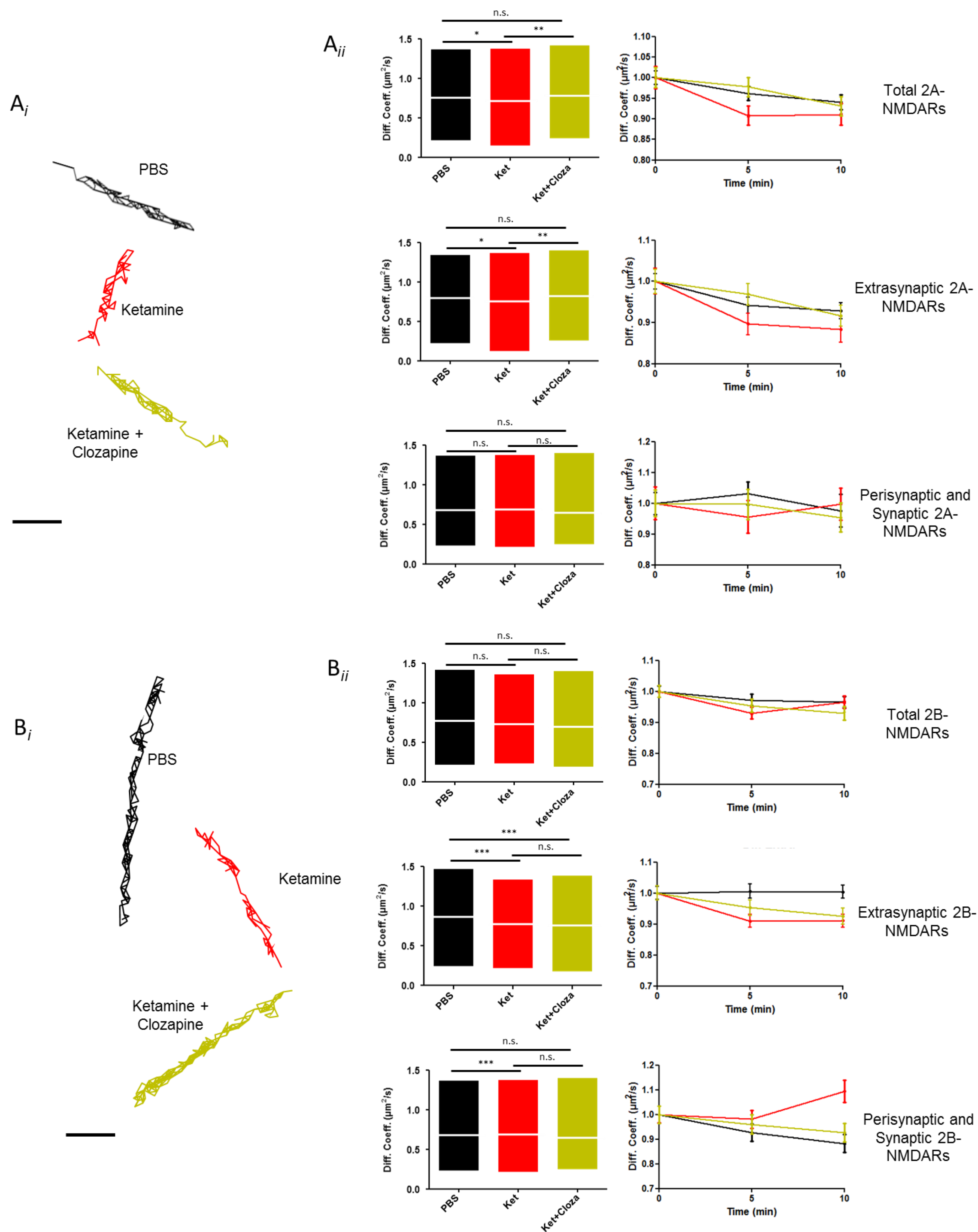

Figure 13: *Clozapine rescues the impairments in NMDAR diffusion caused by ketamine. (A_i) Representative trajectories (25-s duration, 20-Hz acquisition, 1h after PBS or antipsychotic application, 0-10 min after acute PBS or psychomimetic application) of surface QD-conjugated 2A-NMDARs from control conditions (PBS, black), after acute ketamine application (Ketamine, red) and clozapine-pretreated neurons after acute ketamine application (Ketamine+Clozapine, golden; scale bar: 1 µm) (A_{ii}) Left: Comparison of the instantaneous membrane diffusion coefficients of total (T), extrasynaptic (E), and perisynaptic+synaptic (P+S) 2A-NMDARs in all experimental conditions (PBS, black; Ket, red; Ket+Cloza, golden). Instantaneous diffusion coefficients were normalized to the initial timepoint in each of the experimental conditions; Right: Timelapse representation of mean instantaneous membrane diffusion coefficients ± s.e.m. normalized to values of initial timepoint of acquisition of each experimental condition (PBS, black; Ketamine, red; Ketamine+Clozapine, golden) (n=4 cultures). (B_i) Representative trajectories (25-s duration, 20-Hz acquisition, 1h after PBS or antipsychotic application, 0-10 min after acute PBS or psychomimetic application) of surface QD-conjugated 2B-NMDARs from control conditions (PBS, black), after acute ketamine application (Ketamine, red) and clozapine-pretreated neurons after acute ketamine application (Ketamine+Clozapine, golden; scale bar: 1 µm) (B_{ii}) Left: Comparison of the instantaneous membrane diffusion coefficients of total (T), extrasynaptic (E), and perisynaptic+synaptic (P+S) 2B-NMDARs in all experimental conditions (PBS, black; Ket, red; Ket+Cloza, golden). Instantaneous diffusion coefficients were normalized to the initial timepoint in each of the experimental conditions; Right: Timelapse representation of mean instantaneous membrane diffusion coefficients ± s.e.m. normalized to values of initial timepoint of acquisition of each experimental condition (PBS, black; Ketamine, red; Ketamine+Clozapine, golden) (n=6 cultures).*

V. Clozapine does not impact NMDAR surface expression and distribution

Changes in NMDAR lateral diffusion are associated with quick remodelling in their distribution at the surface of neurons which impact profoundly the activity of excitatory synapses (Ladepeche et al., 2013; Dupuis et al., 2014). Thus, we next explored if changes in NMDAR dynamics induced by acute exposure to neuroleptics and psychotomimetics translate into modifications of NMDAR distribution among surface compartments of hippocampal neurons. When focusing on the effects of clozapine, review of studies quantifying NMDAR mRNA and protein expression show contradictory results (Arvanov, Liang, Schwartz, Grossman, & Wang, 1997; Fitzgerald, Deutch, Gasic, Heinemann, & Nestler, 1995; Hanaoka et al., 2003; Meshul, Bunker, Mason, Allen, & Janowsky, 1996; Oretti, Spurlock, Buckland, & McGuffin, 1994). In order to clarify this conundrum, we employed immunocytochemistry to study the effects of 1h pre-incubation with this antipsychotic on 2A- and 2B-NMDAR surface expression in cultured hippocampal neurons. We labeled surface NMDAR exclusively by applying a live staining protocol, while labeling synaptic sites by staining of Homer, a postsynaptic density protein, after fixation and permeabilization of the cells. Our results show that treatment with clozapine does not affect the expression of 2A- or 2B-NMDARs nor of Homer (**Figure 14**). Moreover, we report no alteration in protein cluster number nor area, whether it be for Homer, NMDAR, or specifically synaptic NMDARs (**Figure 14,** mean ± s.e.m.; Homer Cluster Area, PBS: 1±0.07485; Cloza: 0.8919±0.04601; PBS vs Cloza: p>0.05; Homer Cluster Number, PBS: 1±0.06313; Cloza: 0.9074±0.05350; PBS vs Cloza: p>0.05; 2A-NMDAR Cluster Area, PBS: 1±0.06257; Cloza: 0.9483±0.05460; PBS vs Cloza: p>0.05; 2A-NMDAR Cluster Number, PBS: 1±0.06149; Cloza: 0.9266±0.05354; PBS vs Cloza: p>0.05; synaptic 2A-NMDAR Cluster Area, PBS: 1±0.09522; Cloza: 0.9107±0.06985; PBS vs Cloza: p>0.05; synaptic 2A-NMDAR Cluster Number, PBS: 1±0.06292; Cloza: 1±0.05896; PBS vs Cloza; p>0.05; Homer Cluster Area, PBS: 1±0.09178; Cloza: 0.9190±0.05836; PBS vs Cloza p>0.05; Homer Cluster Number, PBS: 1±0.1085; Cloza: 0.8549±0.06021; PBS vs Cloza: p>0.05; 2B- NMDAR Cluster Area, PBS: 1±0.08556; Cloza: 0.9141±0.07755; PBS vs Cloza: p>0.05; 2B- NMDAR Cluster Number, PBS: 1±0.06405; Cloza: 0.9890±0.06559; PBS vs Cloza: p>0.05; Synaptic 2B-NMDAR Cluster Area, PBS: 1±0.1052; Cloza: 0.9247±0.08440; PBS vs Cloza: p>0.05; Synaptic 2B-NMDAR Cluster Number, PBS: 1±0.07081; Cloza: 0.9916±0.06734; PBS vs Cloza: p>0.05). Likewise, clozapine does not alter the percentage of NMDAR that co-localizate with Homer (**Figure 14,** mean ± s.e.m.; co-localization, PBS: 1±0.04223; Cloza:1.048±0.03537; PBS vs Cloza: p>0.05; co-localization, PBS: 1±0.02718; Cloza: 1.014±0.02973; PBS vs Cloza: p>0.05**)**. The same is verified for both receptor subtypes at study. While these results suggest that clozapine itself does not spontaneously affect the surface distribution of NMDAR, further experiments will be required to address whether this

distribution is impaired by psychotomimetics (ex: ketamine), and if clozapine is then able to compensate for these ketamine-induced deficits as it does for impairments in receptor diffusion.

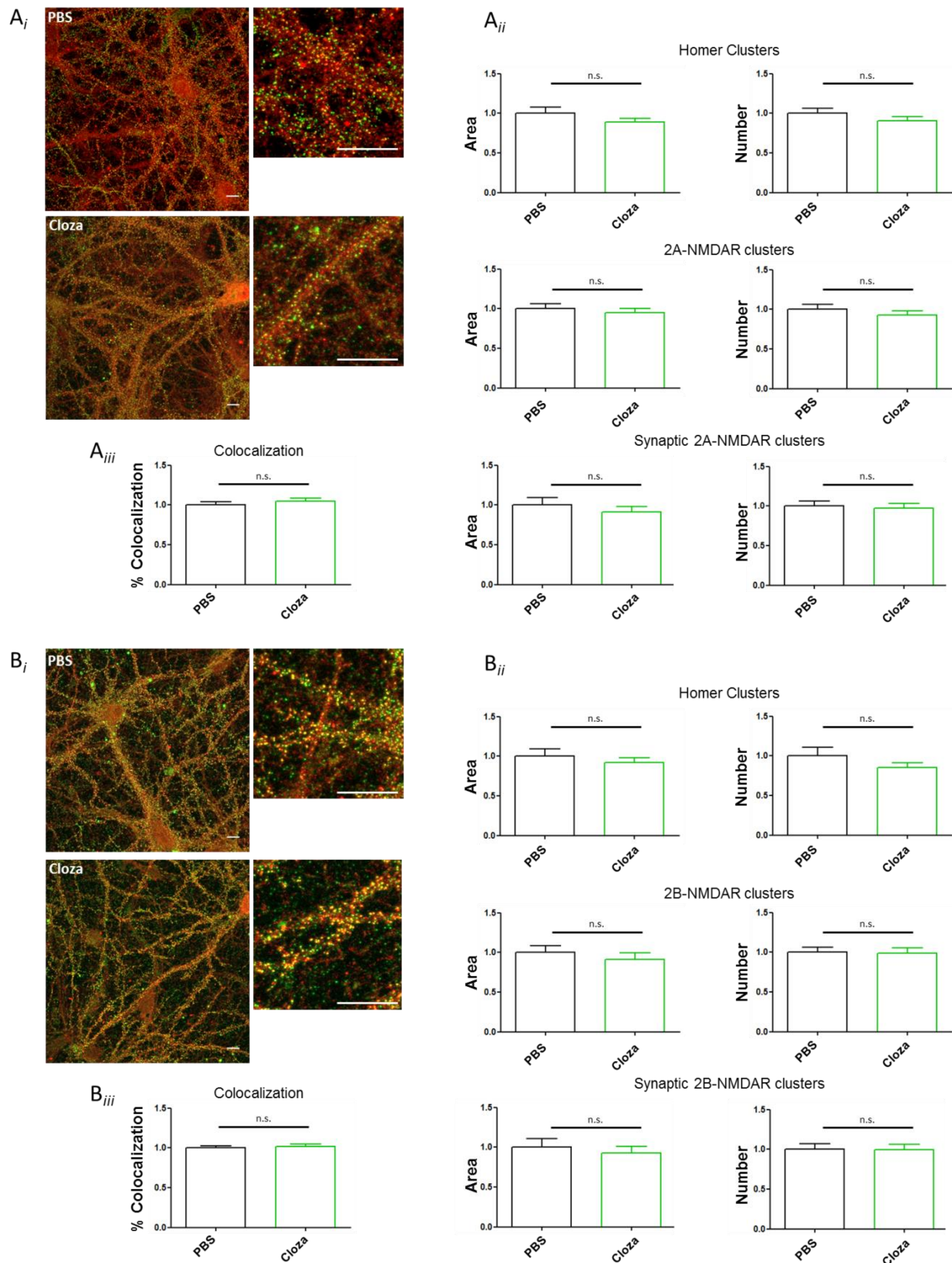

Figure 14: *Clozapine does not impact NMDAR surface distribution. (A_i) Representative images of surface 2A-NMDAR (green) and postsynaptic density protein Homer (red) immunostaining in hippocampal neurons in control and clozapine conditions (1h after drug application, scale bar: 10 µm) (n=4 cultures) (A_{ii}) Comparison between cluster area and number of 2A-NMDAR, Homer and synaptic 2A-NMDAR clusters, divided by the ferret of each ROI, of neurons in control conditions (PBS, black, n = 33 neurons) or subjected to antipsychotic treatment (Cloza, green, n = 38 neurons). Bars represent mean ± s.e.m. Results were normalized to control. (A_{iii}) Percentage of co-localization between 2A-NMDARs and Homer clusters in both experimental conditions (PBS, black; Cloza, green). Bars represent mean ± s.e.m. Results were normalized to control. (B_i) Representative images of surface 2B-NMDAR (green) and postsynaptic density protein Homer (red) immunostaining in hippocampal neurons in control and clozapine conditions (1h after drug application, scale bar: 10 µm) (n=4 cultures) (B_{ii}) Comparison between cluster area and number of 2B-NMDAR, Homer and synaptic 2B-NMDAR clusters, divided by the ferret of each ROI, of neurons in control conditions (PBS, black, n = 39 neurons) or subjected to antipsychotic treatment (Cloza, green, n = 45 neurons). Bars represent mean ± s.e.m. Results were normalized to control (B_{iii}) Percentage of co-localization between 2B-NMDARs and Homer clusters in both experimental conditions (PBS, black; Cloza, green). Bars represent mean ± s.e.m. Results were normalized to control.*

Discussion

Discussion

Using a high-resolution single particle tracking approach, we explored whether molecules used to induce or alleviate psychotic states impact the trafficking of glutamate NMDA receptors on hippocampal neurons. We here demonstrate that the psychotomimetic ketamine and the second-generation antipsychotic clozapine affect the surface diffusion of NMDAR, and, most remarkably, that clozapine is able to restore NMDAR diffusion impairments caused by ketamine. Combined with previous observations from autoimmune, genetic and neurodevelopmental models of psychosis, these results suggest that impairments in NMDAR surface trafficking could be a hallmark of psychotic disorders, and that one of the actions of atypical antipsychotics could be to restore proper NMDAR surface trafficking.

Indeed, the results of ketamine application align with former observations from the MAM and DISC-1 knock-down models of schizophrenia where the diffusion of GluN2B-containing NMDAR was found to be transiently diminished during development (Espana et al., in preparation). We report here that NMDAR diffusion is decreased as soon as 5 minutes following acute application of ketamine at 1 µM concentration. Though statistical significance was attained only when comparing the diffusion between receptors at extrasynaptic sites, this can be accounted to the number of trajectories recorded at the extracellular compartment, the largest of all compartments, which would confer statistical significance even to a small effect. We infer that a global look at the effects of ketamine points to a general decrease in NMDAR diffusion, and that this effect is particularly impactful on NMDARs with the GluN2A subunit. When making an argument on the dissimilarities between the previously obtained results where GluN2B-NMDAR seem to be more affected and these new findings where GluN2A-NMDAR diffusion is predominantly impaired, one must take into consideration the differences between experimental paradigms. At the age at which an effect on 2B-NMDARs had been previously observed (9-10 div), GluN2B is the main NMDAR non-obligatory subunit expressed, and 2B-NMDARs govern synaptic transmission. On the other hand, our experiments were performed after 14 days *in vitro*, a developmental stage at which the GluN2A/GluN2B switch has occurred and when GluN2A-NMDAR become the majority, which could account for this apparent discrepancy.

Interestingly, recent literature focuses on the role of ketamine not only as an antagonist of glutamatergic neurotransmission but also as an enhancer of neuronal potentiation. NMDAR antagonism is responsible for the anesthetic, amnesic, dissociative, and hallucinogenic, but not the antidepressant effects of ketamine. Owing to the study of the antidepressant effects of ketamine, there is evidence for upregulation of AMPA receptor expression following

ketamine administration (Kavalali & Monteggia, 2012). In a recent paper, the antidepressant property of ketamine was proposed to be caused by a particular metabolite, (2R,6R)-hydroxynorketamine, which promotes sustained activation of the AMPA receptor (Zanos et al., 2016). This seems counterintuitive to the notion that ketamine is capable of inducing NMDAR hypofunction and thereby weakening neuronal activity, namely that of the PV+ GABAergic interneurons deemed central in schizophrenia. Regarding this point, it must be kept in mind that CSF concentrations at which ketamine induces psychosis are much higher, and much closer to the concentration used in our study, than the ones at which ketamine exerts its antidepressant effect (WHO, 2002). Ketamine is, first and foremost, an NMDAR antagonist, and, when blockade of NMDAR is robust enough, the effect is unquestionably unfavorable for synaptic plasticity. The output of ketamine application is a decrease in glutamate sensitivity as well as in NMDAR diffusion. An interesting question will be to further investigate if other non-competitive antagonists of NMDAR, such as phencyclidine (PCP), and competitive ones, such as aminophosphovalerate (APV), also produce similar outcomes on NMDAR surface trafficking.

Also, further efforts will have to be devoted to understand why typical (haloperidol) and atypical (clozapine) neuroleptics produce different effects on NMDAR diffusion. While the results of exposure to haloperidol seem unimposing, there is a small but sound effect on the diffusion of 2B-NMDARs at perisynaptic sites. It is possible that this is the site of physical interactions between D2R and NMDAR. Studies on the communication between dopamine receptors and glutamate NMDA receptors have consigned the neuronal membrane as the stage for direct surface interaction of D1, D2 and NMDA receptor types (Liu et al., 2006; Scott & Aperia, 2009). Although the presence of D2R-NMDAR complexes has not been confirmed at the surface of neurons, D2R co-immunoprecipitate with NMDAR GluN2B subunit and PSD proteins, indicating that these are able to unite at synapses. D2R activation leads to a strengthening of its bond to GluN2B, preventing this subunit's phosphorylation by CaMKII, and ultimately leading to the inhibition of NMDAR-mediated currents (Scott & Aperia, 2009). However, an effect of D2R activation on NMDAR or D2R surface diffusion is yet to be confirmed. As mentioned before, haloperidol acts primarily as a dopamine D2 receptor antagonist. As the first to take note on the outcome of the effect of D2R blockade on NMDAR surface dynamics, we found that haloperidol does not seem to have much impact on NMDAR diffusion in cultured hippocampal neurons. However, we must keep in mind that haloperidol is a "dirty" drug, meaning it plays more functions other than what is considered its central active principle. Indeed, aside from antagonism of D2Rs, haloperidol at high doses impacts, in order of magnitude, sigma, serotonin, acetylcholine, adenosine and even GluN2B-containing NMDA receptors (Cobos, Pozo, & Baeyens, 2007; Colabufo et al., 2004;

Ilyin, Whittemore, Guastella, Weber, & Woodward, 1996; Kroeze et al., 2003). To observe differences on the effects of clozapine and haloperidol was not unexpected. We previously mentioned how the central feature of haloperidol conferring its antipsychotic actions is the blockade of D2Rs, while the currently most accepted view on the efficiency of clozapine is that it is due to a combination of its effects on dopamine and serotonin receptors. While haloperidol has, as referred in the previous paragraph, many actions that seem secondary to its key feature, the picture for clozapine is all the more dense, given that this is one of the most complex compounds existing today, with affinity (in order of magnitude) for histamine, adenosine, serotonin, acetylcholine, dopamine and opioid receptors, and the serotonin and norepinephrine transporters SERT and NET (Meltzer, 1994; Zhao & Sun, 2008). Moreover, clozapine induces astrocytic release of D-serine (Tanahashi et al., 2012). This NMDAR co-agonist is known to impact NMDAR diffusion, specifically by decreasing the diffusion of 2B-NMDARs (Papouin et al., 2012). What we have observed, however, is a general upsurge in NMDAR diffusion. Given that clozapine acts through many diverse and distinct pathways, to an extent that a detailed examination would take a great deal of time to conclude, it is most likely that D-serine release is not the major contributor to the effect of this antipsychotic on NMDAR surface dynamics.

Immunocytochemistry experiments were conducted to study the effect of clozapine on NMDAR surface expression, and surface NMDAR distribution. Were there an effect of this antipsychotic on NMDAR expression, or had exposure to this drug led to a shift or enrichment of an NMDAR subtype at the synapse, one would infer a functional significance to the results. However, surface expression and distribution of NMDARs remained persistently unaffected by the actions of clozapine. We propose that treatment with clozapine is inconsequential in a healthy system, whereas, in face of impairments typically associated with schizophrenia, clozapine acts to restore normal function, impacting, among other mechanisms over and under the cell surface interface, the lateral diffusion of the NMDA receptor. It is possible that what the observed effect of clozapine on NMDAR diffusion is the result of its binding to an array of distinct receptor types, or even neurotransmitter transporters. To propose that haloperidol is impacting receptor types other than D2R (though it is pertinent to mention that at our working concentration, there is not an important effect of haloperidol on NMDAR ionotropic function), would also be reasonable. Besides this, the psychomimetic ketamine is known to inhibit the reuptake of serotonin, dopamine, and norepinephrine (Quibell, Prommer, Mihalyo, Twycross, & Wilcock, 2011). It is important that we keep this in mind, as many neurotransmitter systems associated with the actions of these drugs would be meaningful for hippocampal neurons under physiological conditions; however, the presence of neurotransmitters other than glutamate in our cultures is negligible,

given that their release to the hippocampus is dependent on long-range projections from other cerebral nuclei. All neuronal machinery is nevertheless available, including all types of neurotransmitter receptors, meaning that physical interactions between drugs and these, as well as between these and NMDAR, are feasible.

Another crucial point is that the level of neuronal activity is reduced and more subject to variability in cultures. Therefore, to substantiate our observations, we must explore to what extent the actions of ketamine, clozapine and haloperidol treatments are activity-dependent. As an NMDAR antagonist, the effect of ketamine is most certainly activity-dependent. However, ketamine is able to bind to NMDARs at an allosteric site, which might induce adjustments in the conformation of the receptors, and affect lateral diffusion regardless of neuronal transmission (Hirota & Lambert, 2011). Whether the actions of clozapine and haloperidol on NMDAR surface diffusion are activity-dependent is even more of an enigmatic question. To study this, one might block neuronal activation resorting to tetrodotoxin (TTX), and observe whether treatment-induced adjustments in NMDAR diffusion are a match to what we have so far reported.

Finally, we have hereby described how impairments in diffusion brought upon by exposure to ketamine are prevented by clozapine. This is true for 2A-NMDARs, but observations regarding 2B-NMDARs are not so clear-cut. It seems that clozapine in combination with ketamine did not affect 2B-NMDAR diffusion at extrasynaptic sites. This seems contrary to data reported on the effects of clozapine alone, however, we must keep in mind how different these experimental conditions are, and how the impact of ketamine on this receptor subtype may be stronger than the counteraction of clozapine. Much would be gained by looking into the surface expression and distribution of NMDAR to better assess the impact of ketamine, and the restorative actions of clozapine on this psychotomimetic model.

We propose that future insight into the topic of clozapine action mechanisms be functionally oriented, as to better understand the role of these on NMDAR currents. It has been reported that both clozapine and lusaridone, a different second-generation antipsychotic, both increment NMDAR currents and rescue the impairments produced by PCP. Remarkably, a serotonin 5HT7R antagonist produced the same effect (Yuen et al., 2012). It would be interesting to find such a functional correlate to our data on NMDAR surface dynamics, and, separately test, both at the functional and at the surface lateral distribution level, the main action mechanisms associated with this antipsychotic.

Moreover, surface dynamics of other receptor types may be at play, directly or secondarily affecting NMDAR movement and function. The possible role of diffusion of dopamine receptors in schizophrenia has yet to be investigated, despite the acknowledgement that

alterations in NMDAR and dopamine receptor signaling and trafficking are associated with this illness, and that the dialogue between glutamatergic and dopaminergic pathways is not restricted to intracellular signaling convergence.

Conclusions

Conclusions

Schizophrenia is closely associated with deficits in NMDAR signaling. However, the mechanisms leading to this pathological hypofunction remain poorly understood. Here, we report that the surface dynamics of the NMDAR are deviant in a pharmacological model of schizophrenia (acute ketamine exposure). Our data corroborate observations resulting from neurodevelopmental and genetic models of this illness where similar impairments in NMDAR lateral diffusion were revealed, and stress the importance of neurotransmitter receptor cell surface dynamics as a reporter of pathological states in psychotic disorders. We also show that atypical (but not typical) neuroleptics used to alleviate the symptoms of schizophrenia can also influence NMDA receptor-mediated signaling by acting through modulations of receptor surface distribution: while the typical antipsychotic haloperidol did not affect NMDAR surface dynamics, clozapine increased the diffusion of NMDAR, although not to the extent of remodeling NMDAR surface expression and distribution in our experimental paradigm. Importantly, combining the actions of ketamine and clozapine revealed that the antipsychotic is able to partially rescue impairments in NMDAR surface motility caused by the psychomimetic non-competitive NMDAR antagonist. We see this as a hint that atypical antipsychotics may act partly by restoring the proper surface dynamics of neurotransmitter receptors.

We conclude that these interesting findings are basis for more research into the effects of pharmacological modulators which trigger, mimic or temper neuropsychiatric conditions upon the dynamics of neurotransmitter receptors across the surface of the cell, which could represent an unexpected therapeutical target.

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