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Impact of open channel blockers on the surface dynamics and organization of NMDA receptors

Alexandra Fernandes

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THÈSE PRÉSENTÉE
POUR OBTENIR LE GRADE DE
**DOCTEUR DE
L'UNIVERSITÉ DE BORDEAUX**

ÉCOLE DOCTORALE Sciences de la Vie et de la Santé
SPÉCIALITÉ Neurosciences

Par Alexandra FERNANDES

**Impact of open channel blockers on
the surface dynamics and organization
of NMDA receptors**

Sous la direction de Julien DUPUIS

Soutenue le 10 Novembre 2020

Membres du jury :

Mme SANS, Nathalie
M. DE KONINCK, Paul
Mme LÉVI, Sabine
Mme CARVALHO, Ana Luísa
M. DUPUIS, Julien

Directeur de recherche INSERM	Président
Professeur, University of Laval	Rapporteur
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Professeur, University of Coimbra	Examineur
Chargé de recherche INSERM	Directeur

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**Impact des bloqueurs de canal ouvert
sur la dynamique et l'organisation de
surface des récepteurs NMDA**

Sous la direction de Julien DUPUIS

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Insane in the membrane

Insane in the brain

- Cypress Hill, 1993

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Abstract

N-Methyl-D-Aspartate glutamate receptors (NMDAR) are key actors of excitatory synaptic transmission, synaptic plasticity and higher brain functions such as memory formation and learning. As a consequence, NMDAR dysfunctions are associated to pathological states and high investments have been made to develop modulators of NMDAR activity for clinical applications. While some NMDAR antagonists such as ketamine (anaesthetic, antidepressant) or memantine (prescribed as a treatment for Alzheimer's disease) have proven of great medical value, their clinical use is often limited by severe adverse effects (e.g. psychotic-like states induced by ketamine) and several questions regarding their action mode - including why some antagonists exhibit psychoactive properties when others do not - remain unanswered. Accumulating evidence suggests that beyond their channel function, physiological and pathological NMDAR signalling may involve non-canonical pathways independent from ion flux. Using a combination of epifluorescence, FRET-FLIM, biochemistry and single molecule localization microscopy approaches, we investigated the impact of competitive (D-AP5, CPP) and uncompetitive (MK-801, ketamine, memantine) NMDAR antagonists on the properties, redistribution and subsynaptic organization of surface NMDAR and their cytosolic partners in hippocampal neurons. We found that while all antagonists produce comparable inhibition of NMDAR ionotropic activity, exposure to the psychotomimetic blockers MK-801 and ketamine selectively triggers changes in the conformation of NMDAR. Interestingly, these conformational rearrangements were associated with a decreased surface diffusion and an increased residency time of receptors at synapses, suggesting MK-801 and ketamine binding possibly enhance NMDAR synaptic anchoring. Although drug exposure (1h) did not change the overall receptor abundance at excitatory synapses, super-resolution imaging revealed profound and antagonist-specific nanoscale reorganizations of synaptic NMDAR clusters, with exposure to the competitive antagonist D-AP5 causing a reduction in the size and an increase in the density of receptor nanodomains while inhibition by the uncompetitive psychotomimetic blockers MK-801 and ketamine triggered an enlargement of receptor nanodomains, and exposure to memantine prompted the fragmentation of these nanodomains. Moreover, we found that MK-801 and ketamine selectively enhanced the mobility of Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) within dendritic spines through an action mode that relies on the direct interaction between both partners, suggesting that drug-induced receptor redistributions may impact the intracellular dynamics and organization of downstream signalling partners of NMDAR. Altogether, our results provide evidence that besides inhibition of ion fluxes through the receptors, competitive and uncompetitive antagonists have a different impact on NMDAR surface dynamics and subsynaptic organization, and suggest that the psychoactive blockers MK-801 and ketamine may act on receptor function through non-canonical rearrangements in

the organization of NMDAR signalling complexes.

Key words: surface dynamics / nano-organization / NMDA

Résumé

Les récepteurs du glutamate N-méthyl-D-aspartate (RNMDA) sont des acteurs clés de la transmission synaptique excitatrice, de la plasticité synaptique et des fonctions cérébrales supérieures telles que la formation de la mémoire et l'apprentissage. En conséquence, les dysfonctionnements NMDAR sont associés à des maladies neuropsychiatriques sévères et des investissements importants ont été réalisés pour développer des modulateurs de l'activité NMDAR en vue d'applications cliniques. Si certains antagonistes des RNMDA (ex. : la kétamine comme anesthésique ou antidépresseur) se sont avérés d'une grande valeur médicale, leur utilisation clinique est souvent limitée par des effets indésirables graves. Plusieurs questions concernant leur mode d'action restent sans réponse. De nombreuses données suggèrent qu'au-delà de leur fonction de canal, la signalisation RNMDA physiologique et pathologique peuvent impliquer des voies non canoniques indépendantes du flux ionique. En utilisant une combinaison d'approches d'épifluorescence, de FRET-FLIM, de biochimie et de microscopie de localisation de molécule unique, nous avons étudié l'impact des antagonistes RNMDA compétitifs (D-AP5, CPP) et non-compétitifs (MK-801, kétamine, mémantine) sur les propriétés, la redistribution et l'organisation nanométrique des RNMDA de surface et de leurs partenaires cytosoliques dans les neurones d'hippocampe. Nous avons constaté que si tous les antagonistes produisent une inhibition comparable de l'activité ionotrope des récepteurs, l'exposition aux bloqueurs psychomimétiques MK-801 et kétamine déclenche sélectivement des changements de conformation des RNMDA. Ces réarrangements conformationnels sont associés à une diminution de la diffusion de surface et à une augmentation du temps de résidence des récepteurs aux synapses, suggérant que le MK-801 et la kétamine accroissent l'ancrage synaptique des RNMDA. Bien que l'exposition aux drogues (1h) ne modifie pas l'abondance globale des récepteurs aux synapses, l'imagerie de super-résolution révèle des réorganisations nanométriques profondes et antagoniste-spécifiques des clusters de RNMDA synaptiques, une exposition à l'antagoniste compétitif D-AP5 entraînant une réduction de la taille et une augmentation de la densité des nanodomains de récepteurs tandis que l'inhibition par les bloqueurs psychotomimétiques non compétitifs MK-801 et kétamine déclenche un élargissement des nanodomains récepteurs, et que l'exposition à la mémantine provoque la fragmentation de ces nanodomains. De plus, nous avons constaté que le MK-801 et la kétamine augmentent de manière sélective la mobilité de la protéine kinase Ca²⁺/calmoduline-dépendante (CaMKII) dans les épines dendritiques via un mode d'action qui repose sur l'interaction directe entre les deux partenaires, suggérant que les redistributions des récepteurs induites par les antagonistes pourraient avoir un impact sur la dynamique intracellulaire et l'organisation des partenaires de signalisation en aval des RNMDA. Dans l'ensemble, nos résultats montrent qu'en plus de l'inhibition des flux ioniques à

travers les récepteurs, les antagonistes compétitifs et non compétitifs ont un impact différent sur la dynamique de surface et l'organisation sous-synaptique des NMDAR, et suggèrent que les bloqueurs psychoactifs MK-801 et kétamine peuvent agir sur la fonction des récepteurs via des réarrangements non-canoniques de l'organisation des complexes de signalisation RNMDA.

Mots-clés : dynamique de surface / nano-organisation/ récepteurs NMDA

Résumé long

Les récepteurs du glutamate de type N-méthyl-D-aspartate (RNMDA) sont des acteurs clés de la transmission synaptique excitatrice, de la plasticité synaptique et des fonctions cérébrales supérieures telles que la formation de la mémoire et l'apprentissage. En conséquence, les dysfonctionnements des RNMDA sont associés à des états pathologiques. Une hypofonction des RNMDA a été associée à la schizophrénie, tandis que leur hyperfonction et l'excitotoxicité qui en résulte sont associées à des troubles neurodégénératifs tels que la maladie de Parkinson et la maladie d'Alzheimer. Des investissements importants ont été réalisés pour développer des modulateurs de l'activité RNMDA pour des applications cliniques. Il existe différents types d'antagonistes RNMDA: (i) des antagonistes compétitifs, tels que D-AP5 et son analogue CPP empêchent l'activation du récepteur en entrant en compétition avec l'agoniste du récepteur pour son site de liaison, (ii) des antagonistes non compétitifs ou bloqueurs de canal ouvert, tels que la dizocilpine (MK-801), la kétamine et la mémantine, qui bloquent physiquement le passage des ions à travers le récepteur en occupant son pore ionique. Alors que certains antagonistes du RNMDA tels que la kétamine (anesthésique, antidépresseur) ou la mémantine (prescrite comme traitement de la maladie d'Alzheimer) se sont avérés d'une grande valeur médicale, l'utilisation d'antagonistes des RNMDA comme thérapie est entravée en raison d'effets secondaires importants. Notamment, le MK-801 et la kétamine peuvent induire des états de type psychotique qui miment les symptômes caractéristiques de la schizophrénie. La mémantine, par contre, est cliniquement bien tolérée. Une accumulation d'éléments suggère que la signalisation des RNMDA physiologique et pathologique peut impliquer des voies non canoniques indépendantes du flux ionique. En effet, cette signalisation peut être déclenchée par des changements conformationnels RNMDA, induits par la liaison de l'antagoniste au complexe récepteur. Notre première question était de savoir si la liaison des antagonistes RNMDA aux domaines extracellulaires ou transmembranaires des récepteurs pouvait entraîner la transduction d'un signal moléculaire le long du récepteur et avoir un impact sur la signalisation intracellulaire post-synaptique. Nous

avons donc entrepris d'étudier les changements conformationnels des domaines intracellulaires du RNMDA. En utilisant la microscopie d'imagerie du temps de vie de fluorescence (FLIM), il est possible d'observer le transfert d'énergie par résonance Forster (FRET) entre deux fluorophores, ce qui permet d'estimer indirectement la distance entre eux. Nous avons utilisé des constructions portant des fluorophores fusionnés aux domaines carboxy-terminaux (CTD) de la sous-unité GluN1 des RNMDA et découvert que, bien que tous les antagonistes produisent une inhibition comparable de l'activité ionotrope des RNMDA, l'exposition aux bloqueurs psychotomimétiques MK-801 et kétamine déclenche sélectivement des changements dans la conformation de RNMDA. Nous avons étudié l'importance des interactions RNMDA avec les partenaires d'ancrage synaptique pour que ce changement conformationnel se produise. Pour cela, nous avons utilisé un peptide biomimétique compétitif composé des quinze derniers acides aminés de la sous-unité GluN2B des RNMDA, mimant une région des récepteurs importante pour l'interaction avec les protéines d'échafaudage de la densité post-synaptique. Nous avons constaté que la prévention des interactions au niveau de la sous-unité GluN2B CTD empêchait l'impact de la kétamine sur la conformation RNMDA. Cela pourrait signifier que 1) seuls les récepteurs préalablement ancrés aux protéines d'échafaudage peuvent subir un changement de conformation induit par la kétamine, 2) des interactions sont nécessaires pour stabiliser un changement de conformation se produisant au niveau du récepteur à la suite de l'application de kétamine, ou 3) la kétamine peut favoriser la survenue d'une interaction se produisant au niveau du CTD GluN2B entraînant le changement de conformation. En suivant les mouvements des RNMDA individuels à la surface des neurones à l'aide de techniques de suivi de particules uniques (SPT), nous avons constaté que ces réarrangements conformationnels étaient associés à une diffusion de surface réduite et à une augmentation du temps de résidence des récepteurs au niveau des synapses, suggérant que la liaison de MK-801 et de kétamine améliore l'ancrage synaptique des RNMDA. De manière surprenante, nous avons observé que l'injection intrapéritonéale aiguë de MK-801 à des rats adultes conduit à une diminution de l'abondance de RNMDA dans des préparations de synaptosomes corticaux, tandis que le CPP et la kétamine n'ont aucun impact.

Aucun des antagonistes n'a d'impact sur les niveaux d'expression synaptique de la protéine d'échafaudage PSD95 ou sur la co-immunoprécipitation RNMDA/PSD95 *in vivo*. Bien que l'exposition aux différents antagonistes du RNMDA *in vitro* n'ait pas modifié l'abondance globale des récepteurs au niveau des synapses excitatrices, la microscopie de reconstruction optique stochastique directe (dSTORM) révèle des réorganisations nanométriques profondes et antagoniste-spécifiques des clusters de RNMDA synaptiques, l'exposition à l'antagoniste compétitif D-AP5 entraînant une réduction de la taille et une augmentation de la densité des nanodomains récepteurs tandis que l'inhibition par les bloqueurs psychotomimétiques non compétitifs MK-801 et la kétamine déclenche un élargissement des nanodomains récepteurs, et l'exposition à la mémantine provoque l'augmentation du nombre de nanodomains par cluster. Comme les antagonistes du RNMDA diminuent également l'activité neuronale, nous avons également examiné l'effet de la TTX, un bloqueur de l'activité neuronale. Nous avons constaté la TTX réduit la surface et augmente la densité des nanodomains de RNMDA, de manière similaire à l'AP5, indiquant que les réorganisations provoquées par l'AP5 sont potentiellement dues à la suppression de l'activité neuronale. Le MK-801 et la kétamine ont l'effet inverse, élargissant la zone des nanodomains et entraînant une augmentation de la distance entre récepteurs. Nous avons également exploré l'impact de la kétamine à une concentration plus élevée pour vérifier si l'effet de cet antagoniste sur l'organisation nanométrique de surface RNMDA était le même, car chez l'homme différentes doses de kétamine induisent des états cliniques différents. A concentration élevée, la kétamine augmente la surface et diminue la densité des nanodomains de RNMDA de la même manière que le MK-801, ce qui pourrait provoquer un changement dans la signalisation post-synaptique. De plus, nous avons constaté que MK-801 et la kétamine augmentent de manière sélective la mobilité de la protéine kinase Ca^{2+} / calmoduline-dépendante (CaMKII) dans les épines dendritiques grâce à un mode d'action qui repose sur l'interaction directe entre les deux partenaires, suggérant que les redistributions des récepteurs induites par ces agents pharmacologiques peuvent avoir un impact sur la dynamique intracellulaire et l'organisation des partenaires de signalisation en aval du RNMDA. Une enquête plus approfondie sera

nécessaire afin de comprendre comment le MK-801 et la kétamine impactent l'activité de la protéine CaMKII. En résumé, en utilisant une combinaison d'approches d'épifluorescence, de FRET-FLIM, de biochimie et de microscopie de localisation de molécule unique sur neurones d'hippocampe, nous avons étudié l'impact des antagonistes compétitifs (D-AP5, CPP) et non compétitifs (MK-801, kétamine, mémantine) des RNMDA sur leur conformation, leur redistribution et leur organisation nanométrique de surface et celle de leurs partenaires cytosoliques. Dans l'ensemble, nos résultats montrent qu'en plus de l'inhibition des flux ioniques à travers les récepteurs, les antagonistes compétitifs et non compétitifs ont un impact différent sur la dynamique de surface et l'organisation sous-synaptique des RNMDA, et suggèrent que les bloqueurs psychoactifs MK-801 et la kétamine peuvent agir sur la fonction des récepteurs par des réarrangements non-canoniques de l'organisation des complexes de signalisation RNMDA. Le MK-801 et la kétamine semblent constituer un sous-type d'antagoniste RNMDA avec un potentiel psychotomimétique élevé qui présentent un impact spécifique sur la conformation intracellulaire des RNMDA, leur trafic de surface, leur organisation à l'échelle nanométrique et la mobilité cytosolique de leur partenaire de signalisation CaMKII dans les épines dendritiques. Plusieurs questions ouvertes par cette étude nécessiteront de futures investigations. Premièrement, la mobilité accrue de CaMKII se traduit-elle par son accumulation dans les épines dendritiques? Comment la translocation synaptique physiologique de la CaMKII suite à l'activation des RNMDA est-elle affectée par les différents antagonistes? Les antagonistes des RNMDA induisent-ils des altérations des interactions entre les RNMDA et les partenaires synaptiques? Le trafic de surface des RNMDA régit-il la nano-organisation des RNMDA? Il sera également nécessaire de déterminer dans quelle mesure ces altérations au niveau moléculaire pourraient contribuer aux changements d'activité neuronale et de comportement associés à ces agents pharmacologiques.

Conference Posters

Mechanistic insights on the action of NMDAR antagonists with distinct clinical properties

Fernandes A., Jézéquel J., Bouchet D., Durand P., Dupuis J., Groc L.

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Introduction

The glutamatergic synapse

The vertebrate central nervous system is made up of the spinal cord and the brain. In order to input, process, store, access, rework and output information, the brain performs intricate computations, which rely on the organization of its neuronal cells into a vast, fast, complex and reliable network of communications. Information flows between neurons through points of contact termed synapses. There are two types of synapses, electrical and chemical, of which the latter is distinctly predominant (Purves D, Augustine GJ, Fitzpatrick D, et al., 2001). Electrical synapses are gap junctions between two neurons, which ions cross by means of transcellular connexons. Information flow through electrical synapses is fast, but the signal cannot be amplified or modulated. Chemical synapses are characterized by the release of diffusible molecules, termed neurotransmitters, which can be sensed by specialized transmembrane receptors. At chemical synapses, information flow is regulated at many levels. The most prevalent type of chemical synapse is the glutamatergic synapse. Glutamatergic synapses result from the apposition of two specialized compartments: a presynaptic “emitting” terminal which releases the neurotransmitter glutamate upon depolarization, and a postsynaptic “receiving” terminal containing glutamate-sensitive receptors which convert glutamate binding into intracellular signalling in the cytosol of the postsynaptic neuron (Purves D, Augustine GJ, Fitzpatrick D, et al., 2001). Trans-synaptic adhesion proteins can ensure the proper alignment of presynaptic glutamate release sites with postsynaptic glutamate receptors and allow the formation of trans-synaptic nanocolumnar functional units, maximizing the efficiency of neurotransmissions (Tang *et al.*, 2016; reviewed in Biederer, Kaeser and Blanpied, 2017; Haas *et al.*, 2018). Glutamate receptors can be divided into two categories depending on their signalling modalities: ionotropic or metabotropic. Metabotropic glutamate receptors (mGluRs) are coupled to G proteins, which control the intracellular levels of second messengers. Their response is elicited seconds to minutes after receptor activation, triggering signalling pathways that result in increased neuronal excitability or neurotransmitter release (Pinheiro and Mulle, 2008; Niswender and Conn, 2010). Ionotropic glutamate receptors (iGluRs) are fast-acting receptors which, within milliseconds of receptor activation, allow the flow of cations into the neuron, thereby causing membrane depolarization. A sufficiently high level of depolarization results in the generation of an action potential at the axonal hillock, which triggers neurotransmitter release at axonal terminals and prompts further interneuronal communication (Purves D, Augustine GJ, Fitzpatrick D, et al., 2001). iGluRs are subdivided into three types: the *N*-Methyl-D-aspartic acid receptors (NMDAR), the kainate receptors (KAR)

and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) which are the main mediators of fast excitatory neurotransmission.

Excitatory postsynaptic terminals are commonly shaped as dendritic protrusions, termed dendritic spines. They contain a protein-rich zone adjacent to the postsynaptic membrane opposite of the presynaptic terminal, designated the postsynaptic density (PSD). The structure of the PSD (Figure1) is composed in layers. The first layer is that of transmembrane proteins **(1)**, which includes not only glutamate receptors, but also other ion channels and G-protein coupled receptors, tyrosine kinase receptors, and cellular adhesion proteins. At layer two, molecular scaffolds **(2)** interact with the intracellular portion of the transmembrane receptors. These scaffolds are organized in a mesh-like fashion, containing a layer of membrane-associated guanylate kinases (MAGUKs), of which the most abundant is the postsynaptic density protein 95 (PSD-95), oriented perpendicularly to the neuronal surface (Jeyifous *et al.*, 2016), linked through sublayers of scaffolds oriented parallelly to the membrane surface, which belong to the guanylate kinase-associated protein (GKAP) and SH3 and multiple ankyrin repeat domains (SHANK) protein families (Chen *et al.*, 2008; Feng and Zhang, 2009; Jeyifous *et al.*, 2016). This mesh of scaffolds is connected to elements of the cytoskeleton **(3)** (Sheng and Hoogenraad, 2007). The PSD also contains enzymes **(4)**, of which the most abundant is the holoenzyme Ca²⁺/calmodulin-dependent protein kinase 2 (CaMKII). These entities are mobile, and their localization and activation determine the outcome of molecular signalling cascades.

Chapter I – NMDA glutamate receptors (NMDAR)

A. The NMDAR: composition, expression, function and regulation

NMDA receptors have garnered much interest and have been extensively studied since their initial description in the 1980's (Watkins and Evans, 1981). They are glutamate-gated ion channels selectively permeant to sodium, potassium, and calcium. In particular, calcium influx through NMDAR is necessary for certain forms of synaptic plasticity and for learning and memory formation (Morris *et al.*, 1986). Additionally, NMDAR dysfunction is associated with multiple brain disorders (Zhou and Sheng, 2013a) (see Chapter III).

1. NMDAR composition

a. NMDAR subunits and genes

Seven different NMDAR subunits are encoded in the genome of mammals and can be divided in three families: the GluN1 subunit, four GluN2 subunits (GluN2[A-D]), and 2 GluN3 subunits (Cull-Candy and Leszkiewicz, 2004; Traynelis *et al.*, 2010; Paoletti, 2011), each of which is encoded by a different gene (Traynelis *et al.*, 2010). Table 1 summarizes the loci of these genes in the human and mouse genome, and the overall consequences of knocking-out NMDAR subunit-encoding genes (*GRIN*) in mice. Eight different isoforms of the GluN1 subunit are produced through alternative splicing of the *GRIN1* gene at exons 5 (N-terminal extracellular domain), 21 and 22 (C-terminal cytosolic domain): GluN1-[1-4]a and GluN1-[1-4]b, which extend the remarkable diversity of NMDAR (Figure 2a). Unlike GluN1-b isoforms, GluN1-a isoforms do not contain the 21-amino-acid stretch known as the N1 cassette, within the N-terminal extracellular domain of the receptor. As a result, incorporation of either GluN1-a or GluN1-b isoforms yields different three-dimensional structuration of the NMDAR complex, which results in different pharmacological properties and pH sensitivity (Regan *et al.*, 2018). Alternative splicing at exons 21 and 22 change the composition of the C-terminal cytoplasmic tail of GluN1 and affect NMDAR trafficking (Rumbaugh *et al.*, 2000; Horak and Wenthold, 2009; Vance, Hansen and Traynelis, 2012). Additionally, two splice variants of the GluN3A subunit have been reported, with no apparent functional relevance (Sun *et al.*, 1998).

Table 1: NMDAR subunit-encoding genes

NMDAR subunit	Gene	Human chromosome band	Number of amino acids	Mouse chromosome band	Consequences of gene KO in mouse
GluN1	<i>GRIN1</i>	9q34.3	938	2 A3.2	Neonatal death due to respiratory failure (Forrest <i>et al.</i> , 1994)
GluN2A	<i>GRIN2A</i>	16p13.2	1464	16 A1.16	Viable animals; deficits in spatial memory (Sakimura <i>et al.</i> , 1995)
GluN2B	<i>GRIN2B</i>	12p12	1484	6 G1.6	Neonatal death due to deficient suckling response (animals viable only though hand-feeding) (Kutsuwada <i>et al.</i> , 1996)
GluN2C	<i>GRIN2C</i>	17q25	1236	11 E2.11	Viable animals; deficits in sensorymotor gating (Kadotani <i>et al.</i> , 1996; Gupta <i>et al.</i> , 2016)
GluN2D	<i>GRIN2D</i>	19q13.33	1336	7 B3.7	Viable animals; hypolocomotion and reduction in spontaneous behavioural activity (Ikeda <i>et al.</i> , 1995; Shelkar <i>et al.</i> , 2019)
GluN3A	<i>GRIN3A</i>	9q31.1	1115	4 B1	Viable animals; impaired locomotor activity, increased sensitivity to inflammatory pain, enhanced recognition, spatial learning and memory functions (Das <i>et al.</i> , 1998; Mohamad <i>et al.</i> , 2013)
GluN3B	<i>GRIN3B</i>	19p13.3	1043	10 C1	Viable animals; impaired motor learning and impaired social behaviours (Niemann <i>et al.</i> , 2007; Lee <i>et al.</i> , 2018).

The first column indicates the name of the NMDAR subunits, as accorded by The International Union of Basic and Clinical Pharmacology (IUPHAR); the second column indicates the corresponding gene symbol, as accorded by the Human Genome Organisation (HUGO) Gene Nomenclature Committee at the European Bioinformatics Institute; the third column indicates the genomic localization of the gene in the human genome, and the fourth column indicates length of amino acids in the longest splice variant produced by expression of the human gene. Note the distinction in length between GluN1 and GluN2 subunits (also represented in Figure 2a). The fifth column indicates the localization of NMDAR subunit-encoding genes in the mouse genome, and the last column describes the effects of gene KO, reporting the viability of the KO, followed by either the cause of neonatal death or alterations in behaviour displayed by KO animals.

b. NMDAR topology

NMDAR are heterotetrameric structures composed of two obligatory GluN1 subunits and two GluN2 or GluN3 subunits, forming either diheteromers (two GluN1 and two GluN2 or GluN3 subunits of the same type) or triheteromers (two GluN1 and two different GluN2 or GluN3 subunits). Each NMDAR subunit can be divided into four structural and functional domains (Figure 2b). The extracellular N-terminal domain (NTD) is composed of two glomerular segments and contributes to proper NMDAR assembly at the endoplasmic reticulum, to extracellular protein-protein interactions and to allosteric modulation of the receptors (Traynelis *et al.*, 2010). The NTD is connected by a linker to the agonist-binding domain (ABD), which is composed by two discontinuous segments (S1 and S2). The ABD is where glutamate binds to GluN2 subunits, and where NMDAR co-agonists (glycine or d-serine) bind to GluN1 and GluN3 subunits. The transmembrane domain (TMD) is made of three transmembrane helices (M1, M3 and M4) plus one re-entering loop (M2), forming the ion pore of the receptor, where sodium, potassium and calcium ions flow, but magnesium ions become stuck. To date, it is unknown why NMDAR respond to calcium and magnesium so differently (Wollmuth, 2018). Two sites within transmembrane helices have been found to be key for calcium permeability: the Q/R/N site (N in NMDAR, Q/R in other iGluRs) at the M2 loop and the DRPEER motif at the M3 loop (Watanabe *et al.*, 2002; Wollmuth, 2018). The intracellular C-terminal domain (CTD) is made of dynamic amino acid stretches where protein-protein interactions between the NMDAR and intracellular partners occur (Paoletti, Bellone and Zhou, 2013). GluN1 subunit intracellular C-terminal tails are short relatively to those of GluN2 subunits (Figure 2a). These cytoplasmic domains are important for receptor assembly, membrane targeting, stabilization, post-translational modifications, trafficking, and targeting for degradation (Traynelis *et al.*, 2010).

In the last two decades, a series of crystallography studies considerably enlightened us on the structural features that underlie NMDAR functions. Early works on the extracellular domains of the receptor revealed that the GluN1/GluN2 subunit interface within the ABD controls NMDAR deactivation and allows coupling between NTD and channel gate (Furukawa *et al.*, 2005; Gielen *et al.*, 2008); that NTD/ABD inter-domain interfaces are crucial for GluN2 subunit-specific NMDAR functional properties and for the action of allosteric receptor modulators (Gielen *et al.*, 2008; Zhu *et al.*, 2013); and that GluN2 NTDs can assume specific conformations that are not found in other iGluR subunits (Karakas, Simorowski and Furukawa, 2009; Stroebel, Carvalho and Paoletti, 2011). Further studies also revealed that tetrameric NMDAR complexes are assembled in a 1-2-1-2 arrangement (Riou *et al.*, 2012), where the ABDs of NMDAR subunits are positioned under the NTDs of neighbouring subunits as illustrated by the first intact structures of full-length heterotetrameric NMDAR (Karakas and Furukawa, 2014; Lee *et*

al., 2014) (Figure 2c). This NTD/ABD subunit crossover creates possible GluN1/GluN2 subunit interactions, which may be the structural basis for the requirement of co-agonist binding for receptor activation (Hansen *et al.*, 2018).

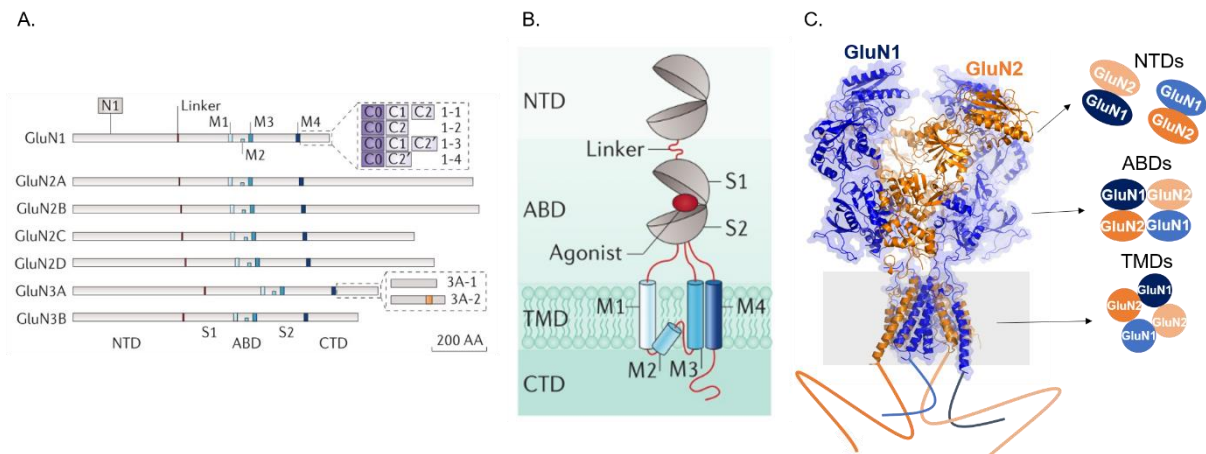


Figure 2. NMDAR topology. A. Representation of the linear amino acid sequence of NMDAR subunits and their isoforms. B. Organization of NMDAR subunits into different domains. C. 3D crystal structure of the NMDAR complex (left), and top view of the relative positions of each subunit (right). Adapted from (Paoletti, Bellone and Zhou, 2013; Karakas and Furukawa, 2014; Lee *et al.*, 2014; Hansen *et al.*, 2018)

c. NMDAR Activation

At hyperpolarized resting membrane potential, NMDAR are essentially closed due to the occupation of the ion pore by a magnesium ion. NMDAR activation can only occur when three conditions are gathered (Figure 3a,b): (i) binding of glutamate, its agonist, to the ABD of GluN2 subunits, (ii) binding of glycine or D-serine, its obligatory co-agonists, to the ABD of GluN1 subunits, and (iii) postsynaptic depolarization-elicited removal of the tonic blockade by magnesium ions (Kleckner and Dingledine, 1988). Since both neurotransmitter release and postsynaptic membrane depolarization are required for their activation, NMDAR act as molecular coincidence detectors of simultaneous pre- and postsynaptic activity, which, according to Hebbian theory, is the basis for the changes in synaptic strength underlying the learning process (Hebb, 1949). The first step of receptor activation is the binding of agonists and co-agonists to the cleft between the S1 and S2 segments of ABDs. All four binding sites need to be occupied for receptor activation. This leads to the approximation of S1 and S2, which separates the ABDs from one another. This movement creates tension in the linkers, resulting in the reorganization of the TMDs and ion pore opening (Paoletti, 2011).

d. NMDAR subunit composition determines NMDAR functional properties

Compared to other iGluRs, NMDAR have relatively slow gating kinetics and desensitization, and the highest affinity for glutamate (Traynelis *et al.*, 2010; Hansen *et al.*, 2018). NMDAR with different subunit compositions have distinct functional properties (Monyer *et al.*, 1992; Cull-Candy, Brickley and Farrant, 2001; Paoletti, Bellone and Zhou, 2013). To illustrate the role of distinct NMDAR subunits, this section refers to diheteromeric receptors unless otherwise stated. NMDAR-mediated excitatory postsynaptic currents (EPSCs) have remarkably different profiles depending on receptor composition (Figure 3c). The decay time constant for EPSCs of GluN2A is 40ms, of GluN2B and GluN2C is around 200-300ms, while of GluN2D is 2s (Vicini *et al.*, 1998). Additionally, NMDAR containing GluN1-a isoforms have a much slower decay time than those containing GluN1-b isoforms (Rumbaugh *et al.*, 2000) (Figure 3c). Receptors open probability can also vary greatly (up to 50 fold) depending on receptor subunit composition (0.5 for GluN2A, 0.1 for GluN2B, and 0.01 for GluN2C and GluN2D) (Wyllie, Béhé and Colquhoun, 1998; Chen, Luo and Raymond, 1999; Cull-Candy, Brickley and Farrant, 2001; Dravid, Prakash and Traynelis, 2008) (Figure 3d). To add to the complexity of NMDAR functional diversity, GluN2A and GluN2B have much higher conductance (around 1.35 fold), sensitivity to magnesium blockade (around 5.3 fold) and calcium permeability (around 1.6 fold) than GluN2C and GluN2D (Dingledine *et al.*, 1999; Paoletti, Bellone and Zhou, 2013)². Inversely, GluN2A have the lowest sensitivity to glutamate and glycine, followed by GluN2B, GluN2C, and GluN2D (Erreger *et al.*, 2007). Different NMDAR subtypes have distinct pharmacological modulators. Ions at the extracellular medium can act as endogenous NMDAR allosteric modulators, inhibiting NMDAR in a subunit-specific way. Protons preferentially inhibit GluN2B and GluN2D, while zinc is a highly specific inhibitor of GluN2A. Additionally, synthetic molecules have been developed to act as subunit-specific NMDAR allosteric inhibitors, such as ifenprodil and ifenprodil-derived molecules (e.g. Ro 25-6981) which have a high specificity for GluN2B (Paoletti, 2011).

Like GluN1 subunits, GluN3 subunits bind NMDAR co-agonists at their ABDs. GluN3 can therefore act as excitatory glycine receptors, as they do not bind glutamate but respond to NMDAR co-agonists (Pérez-Otaño, Larsen and Wesseling, 2016; Grand *et al.*, 2018). GluN3-containing diheteromers receptors have a very low calcium permeability and virtually no magnesium blockade. Ambient levels of glycine induce low amplitude and transient GluN3 currents (Sasaki *et al.*, 2002; Matsuda *et al.*, 2003; Pérez-Otaño, Larsen and Wesseling, 2016). CGP-78608, a recently developed GluN3A positive allosteric modulator, enhances and prolongs glycine-induced GluN3A currents (Grand *et al.*, 2018). Despite this example, there is

² Calculated based on values of maximum conductance, IC50[Mg²⁺], and *pCa/pCs*

a lack of pharmacological agents targeting these receptors. GluN3 are insensitive to competitive NMDAR antagonists since they do not possess glutamate binding sites, and to NMDAR open-channel blockers, likely due to particularities of their ion pore (Chatterton *et al.*, 2002; Pérez-Otaño, Larsen and Wesseling, 2016).

NMDAR can also combine different types of GluN2 and/or GluN3 subunits to form triheteromeric receptors (Chazot *et al.*, 1994; Dingledine *et al.*, 1999; Hatton and Paoletti, 2005; Mayer, 2006; Al-Hallaq *et al.*, 2007; Rauner and Köhr, 2011; Tovar, McGinley and Westbrook, 2013; Frank *et al.*, 2016). As an example, GluN1/2A/2B triheteromeric receptors have been estimated to represent from approximately one-third up to the majority of the total NMDAR population in the hippocampus of rats depending on the method used (Al-Hallaq *et al.*, 2007; Rauner and Köhr, 2011; Tovar, McGinley and Westbrook, 2013). Although still largely uncharacterized, triheteromeric receptors seem to display unique pharmacological properties. Unlike heterodimers, GluN1/GluN2A/GluN2B triheteromers show a high affinity for, but weak inhibition by, zinc and ifenprodil ($\approx 20\%$ maximal inhibition) (Hatton and Paoletti, 2005). They are globally more sensitive to GluN2A- than GluN2B-specific inhibitors and show kinetics similar to those of GluN2A receptors, raising the possibility that they might account for a significant fraction of what is usually considered as the functional contribution of GluN2A diheteromers (Hansen *et al.*, 2014; Cheriyan *et al.*, 2016; Sun, Hansen and Jahr, 2017). However, they retain some of the signalling properties of GluN2B-containing receptors (Sun, Hansen and Jahr, 2017). Triheteromeric receptors containing GluN2 and GluN3 subunits exhibit different properties than their GluN2-containing counterparts (Sanz-Clemente, Nicoll and Roche, 2013). GluN3-containing triheteromers have a dramatically low magnesium blockade and calcium permeability, and coexpression of GluN3A with GluN1 and GluN2A subunits causes a reduction in single-channel conductance and whole-cell currents compared to coexpression of GluN1 and GluN2A (Pérez-Otaño, Larsen and Wesseling, 2016; Grand *et al.*, 2018).

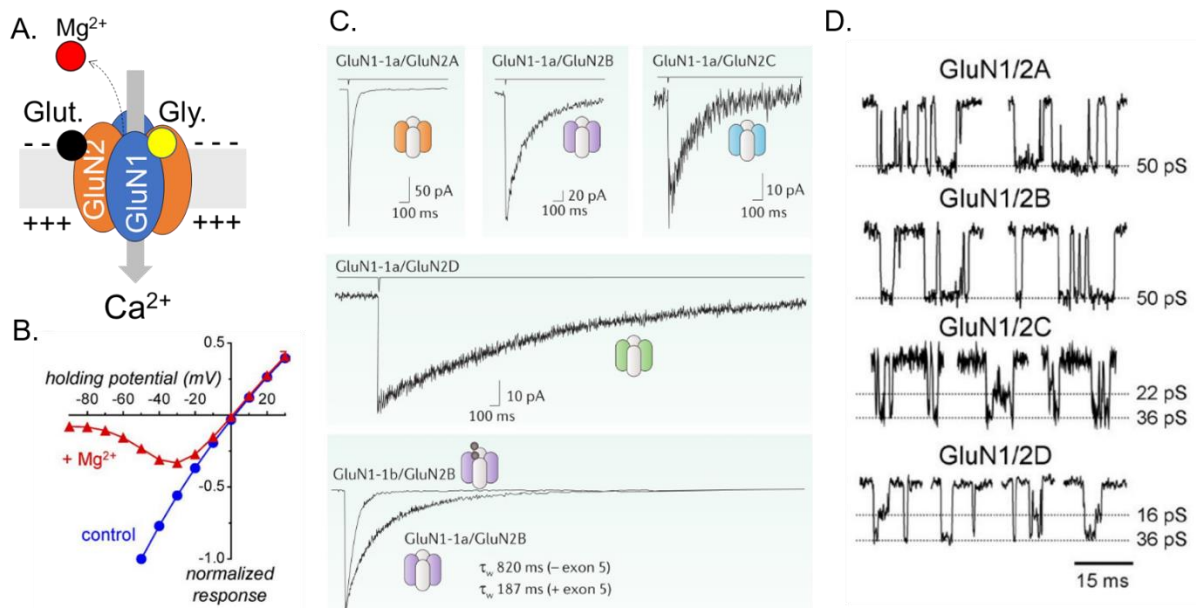


Figure 3. NMDAR functional properties. A) Illustration of the NMDAR activation mechanism. Note the binding of the receptor's agonist and co-agonist, and the membrane depolarization which removed the magnesium blockade, and allowed NMDAR-mediated ion flow. B) Voltage-dependence of NMDAR activation. Comparison between the membrane potential necessary to allow NMDAR-mediated ion flow in the presence and absence of magnesium blockade. C) Macroscopic currents generated by NMDAR with different subunit compositions (recombinant receptors, HEK293 cells). D) Single-channel records of NMDAR with different subunit compositions (recombinant receptors, *Xenopus* oocytes). Dotted lines indicate open states. Adapted from (Cull-Candy, Brickley and Farrant, 2001; Paoletti, Bellone and Zhou, 2013).

2. NMDAR assembly and transport to the cell membrane

NMDAR subunits are synthesized by ribosomes, assembled into tetrameric complexes at the endoplasmic reticulum (ER), processed by the Golgi apparatus, and undergo intracellular trafficking until finally reaching the neuronal surface. NMDAR early processing is regulated by the number of available subunits within the ER, the presence of ER retention and export signals, and posttranslational modifications, all of which contribute to a complex quality control system ensuring that only properly folded and assembled heterotetrameric receptors reach their final destination at the cell surface (Horak *et al.*, 2014; Lichnerova *et al.*, 2015).

GluN1 subunits are produced in excess, and only 40–50% of those generated reach the cell surface (Hall and Soderling, 1997). This is mostly due to the presence of two endoplasmic retention signals (KKK and RRR motifs) at the CTD of GluN1 subunits (Figure 4a). The association of GluN1 with other subunits at the ER masks these retention signals, and only properly assembled tetrameric NMDAR complexes can continue through the secretory pathway (McIlhinney *et al.*, 1998; Prybylowski and Wenthold, 2004; Horak *et al.*, 2014). The ER retention signals at the GluN1 CTD are located at the C1 amino acid stretch, which is determined by alternative splicing of exon 21. (Okabe, Miwa and Okado, 1999; Bradley *et al.*, 2006; Horak and Wenthold, 2009). The C1 cassette is present in GluN1-1 and GluN1-3 isoforms. Inversely, GluN1-3 and GluN1-4 isoforms comprise a C2' cassette determined by

alternative splicing of exon 22, which contains an ER export signal that promotes GluN1 export and compensates the retention action of the C1 cassette (Horak and Wenthold, 2009). Export from the ER is also favoured by post-translational modifications of the ER retention signals, such as PKC phosphorylation of ER retention signals at the C-terminal C1 cassette (Scott *et al.*, 2001) or *N*-glycosylation of the GluN1 subunit at two N-terminal asparagine residues (N203 and N368) (Lichnerova *et al.*, 2015).

Individual GluN2 and GluN3 subunits do not exit the ER without associating with GluN1 subunits (McIlhinney *et al.*, 1998; Pérez-Otaño *et al.*, 2001). GluN2A subunits contain a known ER retention signal (A2 amino acid stretch) at the ATD (Qiu *et al.*, 2009). GluN2B subunits contain an ER retention signal at an unknown location of the CTD, and an ER export signal (HLFY motif) at the M4 loop of the TMD (Hawkins *et al.*, 2004) (Figure 4a). GluN3B subunits contain ER retention signals at the CTD (Matsuda *et al.*, 2003). Finally, several sites at the M3 and M4 loops of NMDAR subunits are structural determinants for the ER trafficking of NMDAR (Horak *et al.*, 2014) (Figure 4a).

While passing through the ER, NMDAR associate with several other molecular partners, including MAGUKs (e.g. SAP102 and SAP97), postsynaptic adaptor proteins (e.g. CASK), components of the exocyst complex (e.g. Sec8), and motor proteins (e.g. microtubule-associated motor protein KIF17) (Sans *et al.*, 2003; Jeyifous *et al.*, 2009; L Bard and Groc, 2011) (Figure 4b). Once properly processed at the ER, these protein packets are inserted into vesicles and move on to the Golgi apparatus at the soma. NMDAR complexes associated with SAP102 and sec8 typically advance to the somatic Golgi apparatus. Then, by sourcing KIF17 associated with Lin-10, NMDAR within post-Golgi transport vesicles travel along dendrites through the microtubule network (Setou *et al.*, 2000). Receptors are subsequently deployed to the neuronal surface via exocytosis. Alternatively, NMDAR complexes associated with SAP97 and CASK have been found to exit the ER and move to dendritic Golgi outposts through non-canonical trafficking (Jeyifous *et al.*, 2009). Since SAP102 preferentially interacts with GluN2B while SAP97 preferentially interacts with GluN2A (Sans *et al.*, 2000; Jeyifous *et al.*, 2009), non-canonical trafficking becomes more important in mature neurons as expression levels of GluN2A increase during development (Zhang and Luo, 2013).

NMDAR can also be locally synthesized. At postsynaptic terminals, mRNAs can be read by polyribosomes and monosomes (Schuman, Dynes and Steward, 2006; Biever *et al.*, 2020). Nascent locally synthesized proteins can be processed at dendrites (Biever, Donlin-Asp and Schuman, 2019). The ER forms a network along dendrites with larger zones close to dendritic branching points and large spines (Cui-Wang *et al.*, 2012), and further processing can occur

at dendritic Golgi outposts or Golgi satellites (Golgi-related micro-organelles that are much smaller and more abundant than Golgi outposts) (Horton *et al.*, 2005; Mikhaylova *et al.*, 2016). GluN1 mRNA has been found at dendrites (Steward and Schuman, 2001), and local NMDAR synthesis is important for certain activity-dependent modulations in synaptic NMDAR content (Swanger *et al.*, 2013).

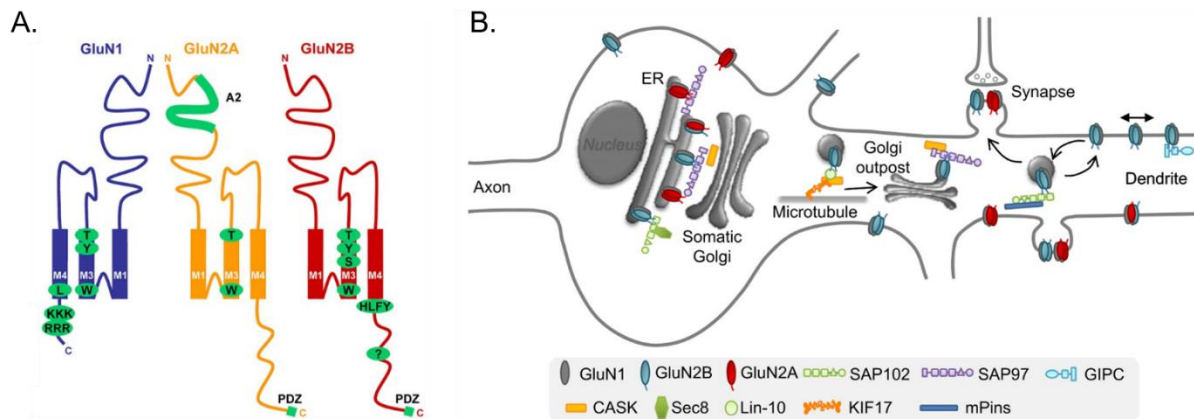


Figure 4. NMDAR assembly and transport to the cell membrane. A. Determinants of ER retention of NMDAR subunits. The scheme represents, in green, the locations of known ER retention or export signals of GluN1, GluN2A and GluN2B. B. NMDAR intracellular transport to the cell membrane. Note the NMDAR complex assembly and protein interactions occurring at the ER, the use of the molecular motor KIF17 for NMDAR intracellular transport, the presence of Golgi outposts, and the fusion of NMDAR-containing transport vesicles to the neuronal surface. Finally, NMDAR reach the synapse through lateral diffusion. Adapted from (L Bard and Groc, 2011; Horak *et al.*, 2014).

3. NMDAR localization

a. NMDAR distribution throughout the body

At the central nervous system (CNS), NMDAR are primarily expressed in neuronal cells, but can also be present at glial cells. Functional NMDAR can be found on astrocytes (Lalo *et al.*, 2006). Astrocytes respond to glutamate by increasing intracellular calcium concentration which triggers gliotransmitter release. However, the role of NMDAR in astrocytic calcium signalling is not clear (Skowrońska *et al.*, 2019). Oligodendrocyte lineage cells also express NMDAR, which may be involved in oligodendrocyte survival and in myelination (Salter and Fern, 2005; C. Li *et al.*, 2013), though this view is challenged by a study inducing the specific ablation of NMDAR in oligodendrocytes (de Biase *et al.*, 2011). Microglia express NMDAR *in vitro* (Kaindl *et al.*, 2012), yet their role and *in vivo* expression is still under debate. NMDAR expression has also been reported in other glial cell types, namely cerebellar radial glia, satellite glia, enteric glia, retinal Müller glia, and Schwann cells (Hogan-Cann and Anderson, 2016), and in cultured endothelial brain cells (Krizbai *et al.*, 1998; Sharp *et al.*, 2003).

While the vast majority of NMDAR are found at the CNS, NMDAR expression is present at the periphery across several organs, tissues and cell types (e.g. bone, skin, airways, the

cardiovascular system, kidney, pancreas, blood, testis, ileum, parathyroid gland, taste buds, and others), where they can be activated by glutamate, L-Homocysteic acid and quinolinic acid. Their functions at peripheral locations is reviewed in (Hogan-Cann and Anderson, 2016). The functions of peripheral NMDAR highlighted in this paragraph are particularly relevant in disease. NMDAR control smooth muscle contraction in lungs and airways, and they are involved in inflammation-associated airway hyperreactivity, a component of asthma and other respiratory diseases (Strapkova and Antosova, 2012; Anaparti *et al.*, 2015). NMDAR play a role in osteoblast differentiation and osteoclast survival and function, therefore impacting bone mineralization, bone matrix deposition, and bone resorption (Mentaverri *et al.*, 2003; Li *et al.*, 2011). Of interest, blocking NMDAR inhibits bone resorption, revealing a possible strategy for the treatment of osteoporosis (Itzstein *et al.*, 2000; Szczesniak *et al.*, 2005; Du *et al.*, 2017). In the kidney, NMDAR activation results in vasodilation of the glomerulus, which impacts renal blood flow, filtration, and reabsorption (Deng and Thomson, 2009; Dryer, 2015). Blocking NMDAR at proximal tubules can be of value to the treatment of kidney injury (Lin *et al.*, 2015). At the pancreas, islet β cells release insulin as a response to increased glucose concentration. There, NMDAR activation inhibits insulin release, creating a negative feedback loop that regulates insulin levels. NMDAR are a promising target for the treatment of diabetes (Marquard *et al.*, 2015).

As glutamate signalling is involved in several types of cancers (Stepulak *et al.*, 2014), NMDAR expression and activity promotes tumour cell proliferation and invasiveness (Hogan-Cann and Anderson, 2016; Robinson and Li, 2017; Bray, 2019; Q. Zeng *et al.*, 2019; Venkataramani *et al.*, 2019). Despite this knowledge, clinical trials aiming at a future therapy targeting NMDAR in cancer are still rather focused on pain management (www.clinicaltrials.gov). Ectopic expression of NMDAR in ovarian teratomas can also lead to the development of autoimmune antibodies against NMDAR and indirectly trigger anti-NMDAR encephalitis (Dalmau *et al.*, 2007), as discussed in more details in Chapter II.4.E.

b. NMDAR distribution throughout the brain

i. Regional brain distribution

NMDAR are present ubiquitously in the brain. The obligatory NMDAR subunit GluN1 is expressed across development, brain regions and cell types (Monyer *et al.*, 1994), and is particularly concentrated at the forebrain and cerebellum. Different GluN1 isoforms have distinct expression patterns. GluN1-a isoforms are expressed evenly across the brain, while GluN1-b isoforms are found mostly in sensorimotor cortex, neonatal lateral caudate, thalamus, hippocampus (CA3 region) and cerebellum (granule cells). The expression of GluN1-2

isoforms is the most widespread. GluN1-3 isoform expression is low and largely restricted to the cortex and hippocampus. GluN1-1 and GluN1-4 have an almost mutually exclusive expression pattern, as the former isoform is highly expressed in caudal structures and the latter in rostral structures (Laurie and Seeburg, 1994). While the pattern of GluN1 isoforms is mostly fixed from birth, the expression levels and patterns of GluN2 and GluN3 subunits is developmentally regulated. GluN2B and GluN2D are expressed prenatally. At birth, GluN2B expression is widespread, though GluN2B levels are highest at the forebrain, and GluN2D expression is mostly restricted to the midbrain. GluN2D levels start dropping after birth. GluN2B expression starts to decrease later in development, at around postnatal day (P) 7-10 in rat. In adulthood, the forebrain is the structure which retains the highest levels of GluN2B expression. The GluN2A subunit is postnatally expressed throughout the brain, and GluN2A levels gradually increase with time (Figure 5) (Watanabe *et al.*, 1992; Monyer *et al.*, 1994; Sheng *et al.*, 1994). GluN2A and GluN2B are the prevalent non-obligatory NMDAR subunits in higher brain structures (especially in the cortex and hippocampus) (Paoletti, Bellone and Zhou, 2013). The progressive alteration in NMDAR subunit composition (i.e. the GluN2B to GluN2A switch) prompts an adjustment in the threshold for synaptic plasticity, converting networks from a plastic/immature state to a stable/mature one (Bellone and Nicoll, 2007; Paoletti, Bellone and Zhou, 2013; Sanz-Clemente, Nicoll and Roche, 2013). Importantly, GluN2 subunits are also differentially expressed across cell types. For example, in the hippocampus, GluN2A- and GluN2B-containing receptors predominate in pyramidal neurons, while interneurons express GluN2C- and GluN2D-containing NMDAR (Monyer *et al.*, 1994). Post-natal GluN3A expression is widespread, and most relevant at the somatosensory cortex, hippocampus and visual cortex. GluN3A expression peaks at P8, after which it decreases until adulthood. GluN3B expression is only significant in adulthood, and is mostly restricted to the brainstem and motor neurons (Henson *et al.*, 2010; Pachernegg, Strutz-Seebohm and Hollmann, 2012; Pérez-Otaño, Larsen and Wesseling, 2016).

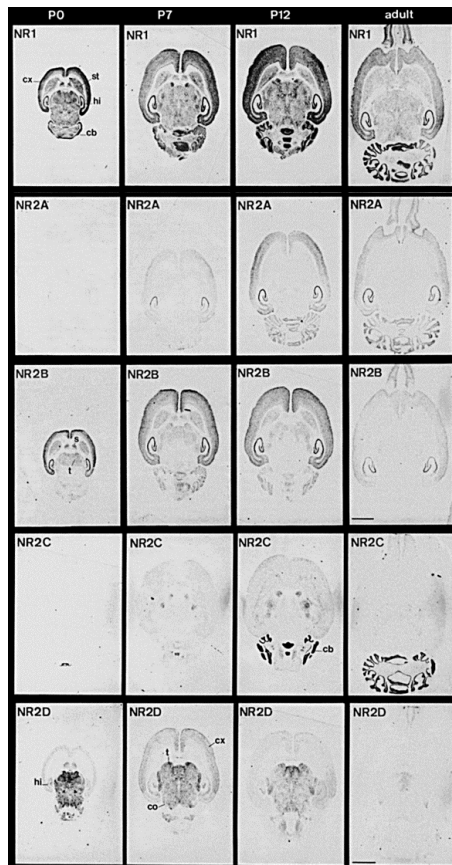


Figure 5. Expression pattern of NMDAR subunits throughout the brain across development. NMDAR are present all over the brain and across developmental stages, as demonstrated by the expression pattern of GluN1. GluN2A expression is negligible at P0, but steadily increases during development. Conversely, GluN2B expression is high in the immature brain, particularly at the forebrain, and decreases over time. GluN2C expression begins late in development and is mostly restricted to the cerebellum. GluN2D is highly expressed in midbrain structures initially, but its levels are considerably low in adulthood. In situ hybridization was used to identify mRNA encoding NMDAR subunits in axial rat brain sections of different ages. (P, post-natal day; NR1, GluN1; NR2A, GluN2A; NR2B, GluN2B; NR2C, GluN2C; NR2D, GluN2D; cx, cortex; st, striatum; hi, hippocampus; cb, cerebellum; t, thalamus; s, septum; co, colliculi). Scalebar, 3.4 mm. Adapted from (Monyer *et al.*, 1994).

ii. Presynaptic NMDAR

Evidence for NMDAR at presynaptic terminals first originated from electron microscopy studies. Presynaptic NMDAR have been imaged at the visual cortex (Aoki *et al.*, 1994; Larsen *et al.*, 2011); neocortex (DeBiasi *et al.*, 1996); cerebellar cortex (Charton *et al.*, 1999); somatosensory cortex (Brasier and Feldman, 2008), hippocampus (McGuinness *et al.*, 2010), and other structures (Banerjee *et al.*, 2016). The expression and composition of NMDAR at presynaptic terminals varies depending on brain regions and on the identity of the postsynaptic terminal (Bouvier *et al.*, 2018). Presynaptic NMDAR at immature neurons from the barrel cortex contain GluN2C and GluN2D subunits (Banerjee *et al.*, 2009), and those at immature neurons from the visual cortex are presumably GluN1/2B/3A triheteromeric receptors (Larsen *et al.*, 2011) (Figure 6). At mature stages, cerebellar presynaptic NMDAR contain GluN2A subunits (Bidoret *et al.*, 2009) while GluN2B- and GluN2C/D-containing receptors predominate in the cortex and the hippocampus, respectively (Woodhall *et al.*, 2001; Brasier and Feldman, 2008; Larsen *et al.*, 2011; Andrade-Talavera *et al.*, 2016).

Functionally, presynaptic NMDAR regulate spontaneous neurotransmitter release through activation of JNK2 in an ion flow-independent fashion, but also control the readily releasable

pool of neurotransmitter vesicles through an ion flow-dependent pathway involving RIM1a β (Abrahamsson *et al.*, 2017). Additionally, presynaptic NMDAR-mediated Ca²⁺ entry can play a role in synaptic plasticity through the activation of nitric oxide synthase (NOS) (Lev-Ram *et al.*, 1997) or calcineurin (Larsen *et al.*, 2014; Andrade-Talavera *et al.*, 2016). Early in development, GluN1/2B/3A triheteromers have been hypothesized to favour glutamate release and thereby mediate spike timing-dependent long-term depression (t-LTD) (Larsen *et al.*, 2011; Pérez-Otaño, Larsen and Wesseling, 2016). Indeed, the action of presynaptic NMDAR has long been associated to t-LTD, an assumption which stems from an initial study reporting that selectively blocking presynaptic NMDAR using an intracellular application of MK-801 prevents t-LTD induction at the somatosensory cortex (Rodríguez-Moreno and Paulsen, 2008). Congruently, presynaptic NMDAR ablation at the visual cortex prevents the induction of t-LTD (Larsen *et al.*, 2011). However, specific ablation of presynaptic or postsynaptic NMDAR at the somatosensory cortex indicates that t-LTD induction relies on postsynaptic NMDAR and points rather to a non-ionotropic signalling mechanism of postsynaptic receptors, as discussed in Chapter I.4.a. (Carter and Jahr, 2016). The validity of certain functional studies of presynaptic NMDAR is under debate (Bouvier *et al.*, 2018). For instance, the presence of presynaptic NMDAR was functionally confirmed by blocking postsynaptic NMDAR at layer 2 synapses of the entorhinal cortex using an intracellular application of MK-801, followed by recording miniature excitatory postsynaptic currents (mEPSCs, reflecting the quantal release of single presynaptic glutamate-containing vesicles) before and after blocking all NMDAR using AP5 (Berretta and Jones, 1996). AP5 decreased the frequency of mEPSCs, which was interpreted as evidence that glutamate release is being regulated presynaptically by NMDAR. This observation was extended to presynapses of the hippocampus and visual, entorhinal and somatosensory cortices (Sjöström, Turrigiano and Nelson, 2003; Mameli *et al.*, 2005; Yang, Woodhall and Jones, 2006; Li and Han, 2007; Corlew *et al.*, 2008). Some of the discrepancies in this field may be due to incorrect interpretations of pharmacological data. The intracellular application of NMDAR blockers has historically been used as a tool to probe for presynaptic NMDAR function (e.g. Berretta and Jones, 1996; Rodríguez-Moreno and Paulsen, 2008; McGuinness *et al.*, 2010). The validity of this approach is debatable, as intracellular application of MK-801 results in a ~30.000-fold decrease in drug affinity (W. Sun *et al.*, 2018). Moreover, in experiments with this design the functions attributed to presynaptic NMDAR may in fact be driven by postsynaptic NMDAR metabotropic signalling. Further research is needed to clarify the ionotropic and/or non-ionotropic functions of pre- and postsynaptic NMDAR.

iii. Synaptic and extrasynaptic NMDAR

The presence of NMDAR at extrasynaptic sites was first confirmed through immunohistochemistry (Aoki *et al.*, 1994; Siegel *et al.*, 1994). In mature hippocampal neurons,

20-50% of the NMDAR pool is extrasynaptic (Ivanov *et al.*, 2006; Harris and Pettit, 2007). Using electron microscopy to explore NMDAR distribution along neurons in greater detail, NMDAR could be found at axons, the cell body, the dendritic shaft, the neck of the dendritic spine and adjacent to the postsynaptic density (often referred to as perisynaptic area) (Petralia *et al.*, 2010). In immature neurons, NMDAR-mediated currents are highly sensitive to GluN2B-specific blockers, indicating that most synaptic NMDAR are GluN2B-containing receptors (Kew *et al.*, 1998). However, later in development (i.e. after the GluN2B / GluN2A switch), GluN2B-containing NMDAR are preferentially found at extrasynaptic locations while GluN2A-containing receptors become majority at synapses, even though there is still a significant portion of synaptic GluN2B-containing NMDAR that are mostly found at the periphery (Tovar and Westbrook, 1999, 2002; B. Li *et al.*, 2002; Groc *et al.*, 2004; L Groc *et al.*, 2006; Harris and Pettit, 2007; Shinohara *et al.*, 2008; L Bard and Groc, 2011) (Figure 6). GluN2D NMDAR are typically extrasynaptic, but can occasionally be found at synapses (Brothwell *et al.*, 2008; Harney, Jane and Anwyl, 2008). GluN3A-containing NMDAR are preferentially located at the perisynaptic region, while GluN3B-NMDAR receptors seem to be more tightly associated to the PSD, though both are much less prone to be found in the PSD compared to GluN2 subunit-containing receptors (Wee *et al.*, 2016) (Figure 6).

Extrasynaptic NMDAR activation is triggered by the presence of glutamate outside of the synaptic cleft, stemming from synaptic glutamate spill over (Clark and Cull-Candy, 2002; Giles E. Hardingham and Bading, 2010). Synaptic and extrasynaptic NMDAR are preferentially gated by different co-agonists, D-serine and glycine, respectively. Each co-agonist differentially impacts NMDAR surface trafficking and influences the distribution of NMDAR throughout synaptic and extrasynaptic areas (Papouin *et al.*, 2012; Ferreira *et al.*, 2017). The activation of extrasynaptic NMDAR is implicated in several brain diseases, as extrasynaptic / GluN2B-containing NMDAR can trigger cell-death associated signalling pathways (Giles E Hardingham and Bading, 2010; Parsons and Raymond, 2014).

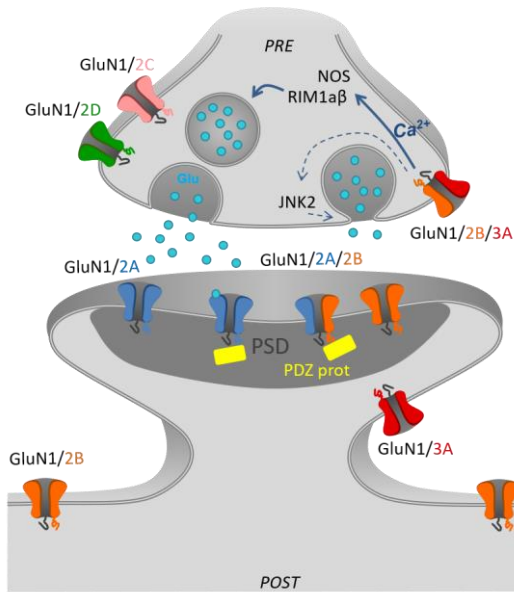


Figure 6. Representation of subtype-specific distribution of synaptic NMDAR. At the post-synapse, diheteromeric GluN1/2A, GluN1/2B and triheteromeric GluN1/2A/2B NMDAR are distributed within the postsynaptic density compartment, while GluN1/3A-NMDAR are located at the perisynaptic area and GluN1/2B- as well as GluN1/2D-NMDAR at extrasynaptic portions of the dendritic shaft. At the pre-synapse, diheteromeric GluN1/2C, GluN1/2D and triheteromeric GluN1/2B/3A receptors can be found. Presynaptic NMDAR can influence neurotransmitter release through ion flow-dependent (full arrow) and independent (dashed arrow) pathways. Metabotropic action of presynaptic NMDAR elicits spontaneous neurotransmitter release through JNK2. Ion flow through presynaptic NMDAR increases the readily releasable pool of neurotransmitter vesicles through RIM1aβ. This helps sustain high-frequency evoked neurotransmitter release. Calcium entry through presynaptic NMDAR may also influence presynaptic function through other signalling pathways (e.g. NOS).

c. NMDAR nanoscale organization

At mature cortical and hippocampal neurons, surface NMDAR form clusters with a diameter of around 400 nm (Benke *et al.*, 1993; Richmond *et al.*, 1996; Kellermayer *et al.*, 2018). The development of super resolution microscopy techniques allows us to study the distribution of NMDAR within those clusters, which would be impossible using classical diffraction-limited microscopy (van de Linde, Sauer and Heilemann, 2008; Zhuang, 2009; Liu, Lavis and Betzig, 2015; Sahl, Hell and Jakobs, 2017). Thanks to this technology, several membrane proteins have been found to contain within their clusters (i.e. regions of protein aggregation at the neuronal surface detected through diffraction-limited microscopy) one or more nanodomains (i.e. nanoscale regions within a cluster where the protein tends to concentrate) (Garcia-Parajo *et al.*, 2014).

Seeing past the diffraction-limit of light revealed a physical and functional co-organization between intracellular scaffolds and ionotropic receptors, both in excitatory postsynaptic terminals (Fukata *et al.*, 2013; MacGillavry *et al.*, 2013; Nair *et al.*, 2013; Broadhead *et al.*, 2016; Heine and Holcman, 2020) and in inhibitory ones (Specht *et al.*, 2013; Pennacchietti *et al.*, 2017). Presynaptic terminals also have a specific nanoscale organization (Ehmann *et al.*, 2014; Zhan *et al.*, 2014; Dudok *et al.*, 2015). The alignment of postsynaptic scaffolds to AMPAR and to presynaptic active zones forms functional nanocolumns that ensure a high efficiency of excitatory neurotransmission (Tang *et al.*, 2016; Haas *et al.*, 2018; Hruska *et al.*, 2018).

Surface NMDAR in hippocampal neurons are organized in clusters which contain nanodomains (MacGillavry *et al.*, 2013; Kellermayer *et al.*, 2018). This nanoscale organization

of NMDAR is subunit-dependent and developmentally regulated, and involves interactions between the C-terminal domains of GluN2 subunits and the PDZ domains of scaffolding proteins (Kellermayer *et al.*, 2018). Clusters of GluN2B subunit-containing NMDAR are smaller and have less nanodomains than those of GluN2A subunit-containing receptors. Interestingly, disrupting the nanoscale organization of GluN2A- or GluN2B-NMDAR by preventing NMDAR-MAGUK interactions in a subtype-specific manner does not affect basal NMDAR-mediated current amplitudes, suggesting either that this does not impair the ionotropic function of NMDAR or that compensatory mechanisms take place. However, releasing GluN2A-containing NMDAR from these anchors augments LTP, while releasing GluN2B-containing NMDAR prevents LTP induction (Kellermayer *et al.*, 2018). This suggests that besides ion flux, precise GluN2A- and GluN2B-NMDAR localization within the PSD and interactions with signalling partners have a significant impact on postsynaptic NMDAR-mediated signalling and plasticity (see Chapter I.4.a).

What could be the significance of postsynaptic NMDAR nanoscale organization? To date, there is no evidence that NMDAR form nanocolumns with the presynaptic glutamate release machinery. Unlike AMPAR, NMDAR have a high affinity for glutamate and slow gating kinetics (Traynelis *et al.*, 2010). Moreover, NMDAR activation does not rely solely on agonist binding but also requires the presence of co-agonists (i.e. glycine / D-serine). Therefore, having a perfect alignment of NMDAR to glutamate release sites would not necessarily have a significant impact on NMDAR ionotropic function. However, due to the slow binding rate of glutamate to NMDAR, simulations indicate that the activation probability of GluN2B-NMDAR decreases 65% if the receptors are located a mere 200nm away from the glutamate release site (Erreger *et al.*, 2005; Santucci and Raghavachari, 2008; Biederer, Kaeser and Blanpied, 2017). This is not the case for GluN2A-NMDAR, which have faster glutamate binding kinetics. Additionally, calcium entry through NMDAR may generate functionally relevant calcium nanodomains, as fine spatiotemporal control of calcium entry regulates several neuronal molecular events, such as presynaptic vesicle fusion at the active zone, the calcium dependent inactivation of L type calcium channels, and the activation of BK channels by NMDA receptors (Isaacson and Murphy, 2001; Liang *et al.*, 2003; Schneggenburger and Neher, 2005; Evans and Blackwell, 2015). Incidentally, NMDAR closely packed in a nanodomain may be more subject to autoinhibitory calcium-dependent inactivation (Iacobucci and Popescu, 2019). Moreover, the fact that proteins tend to spontaneously self-assemble into increasingly more complex structures opens the possibility that surface NMDAR nanodomains are strategically supported by large intracellular protein assemblies that keep key signalling partners in proximity to the receptors. This view is corroborated by the fact that NMDAR form protein complexes (~0.8MDa) and supercomplexes (~1.5MDa) at synapses during maturation (Frank

et al., 2016; reviewed in Frank and Grant, 2017). Protein nano-clustering may be a natural result of biochemical phenomena termed phase transitions. Phase transitions are self-forming protein-rich droplets which self-assemble within a hierarchy of different scales and govern the spatiotemporal organization of biochemical reactions. It is possible to assemble *in vitro* postsynaptic densities with the ability to clusterize receptors through phase transitions alone (Zeng *et al.*, 2018).

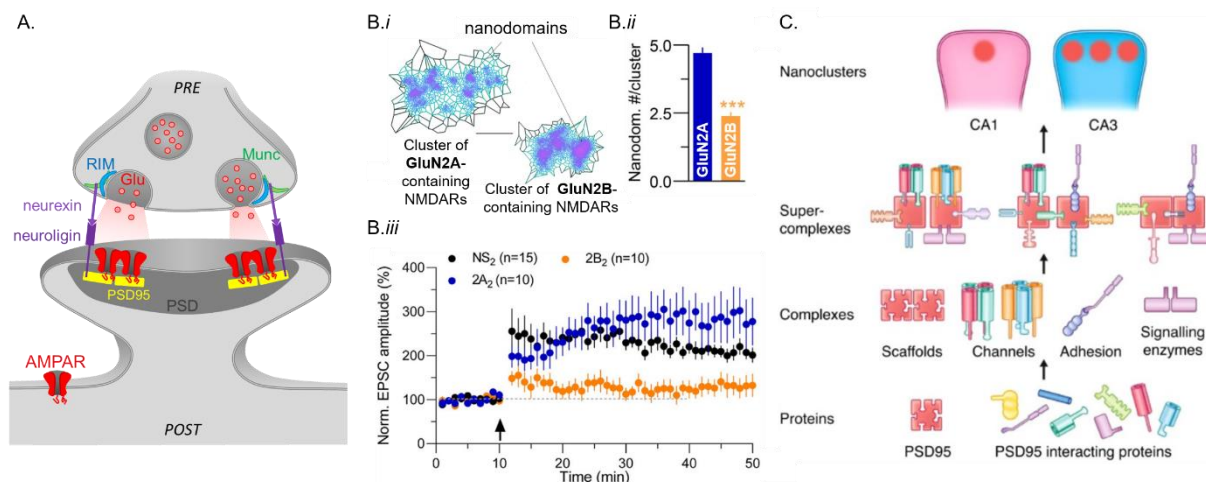


Figure 7. Nanoscale organization of receptors at glutamatergic synapses. A. iGluR nanocolumns. Glutamatergic synapses form functional nanocolumns through the co-alignment of active-zone machinery (RIM, MUNC), AMPAR, and scaffolds (PSD-95). This alignment is favoured by interactions with trans-synaptic partners (neurexin / neuroligin). **B. NMDAR nanoscale organization.** (i) Examples of clusters of surface NMDAR containing the GluN2A or the GluN2B subunit acquired from hippocampal neurons. Arrows point to NMDAR nanodomains. Polygons are generated by tessellation (scalebar, 100nm). (ii) GluN2A-NMDAR synaptic clusters show more nanodomains than GluN2B-NMDAR synaptic clusters. (iii) Normalized evoked EPSC amplitudes recorded from CA1 pyramidal neurons before and after application of an LTP conditioning protocol. Disrupting the organization of NMDAR using a competing peptide-based approach to break interactions with PDZ domain-containing proteins results in modulations of long-term plasticity. Black arrow, LTP induction. Bivalent peptides: NS₂, nonsense; 2A₂, GluN2A-targeting peptides; 2B₂, GluN2B-targeting peptides. Adapted from (Kellermayer *et al.*, 2018). **C. Protein self-assembly generates large and complex structures at postsynaptic terminals.** Postsynaptic proteins, including NMDAR, assemble into complexes and super complexes, and finally into nanoclusters. Represented is the relative difference in abundance of synaptic PSD-95 nanoclusters at two hippocampal regions (CA1 and CA3) (Broadhead *et al.*, 2016). Adapted from (Frank and Grant, 2017)

4. NMDAR function and regulation

a. NMDAR function: non-ionotropic dimensions

NMDAR have been categorized as ionotropic receptors forty years ago (Watkins and Evans, 1981) and their unique permeability to calcium has been found to be at the basis of their central role in Hebbian plasticity. However, several pieces of evidence suggest that considering NMDAR signalling as purely ionotropic is an incomplete view (Figures 8 and 9).

LTD

Since 1996, there have been accumulating indications for an additional non-ionotropic dimension to NMDAR function. Scanziani, Malenka and Nicoll were the first to report that heterosynaptic long-term depression (LTD) is not blocked by MK-801 nor intracellular calcium

chelation and is independent of mGluRs (Scanziani, Malenka and Nicoll, 1996). A more recent study replicated this effect, adding that LTD induction was not blocked by either MK-801 or 7CK, but was blocked by AP5. LTD induction mediated by non-ionotropic NMDAR function required basal levels of intracellular calcium and the activation of p38 MAPK. In addition, a protocol for inducing LTP resulted in the expression of LTD with receptors blocked by MK-801. (Nabavi *et al.*, 2013). These observations open the possibility that NMDAR can mediate plasticity in an ion-flow independent fashion. How can this happen? While NMDAR are blocked at the ion pore by open channel blockers (e.g. MK-801, magnesium) or at the co-agonist binding site with glycine site antagonists (e.g. 7CK), non-ionotropic signalling may still result from agonist binding, which can only be prevented by competitive NMDAR antagonists (e.g. AP5, CPP). Using a FRET-based approach to detect the relative distance between GluN1 CTDs, Dore, Aow and Malinow reported that agonist binding to NMDAR leads to a transient change in receptor conformation (Dore, Aow and Malinow, 2015). Agonist was applied while blocking the receptor at the ion pore with MK-801, at the glycine binding site with 7CK, or at the glutamate binding site with AP5. In all conditions except the latter, agonist binding caused GluN1 CTDs to transiently move away from one another. In a separate publication, the same authors reported that using intracellular antibodies which prevent the movement of GluN1 CTDs blocks non-ionotropic NMDAR-mediated induction of LTD. Additionally, agonist binding impacted NMDAR interactions with signalling enzymes relevant for synaptic plasticity processes, namely PP1 and CaMKII (Aow, Dore and Malinow, 2015). Hence, NMDAR non-ionotropic functions can arise from agonist-driven conformational changes to the receptor, thus impacting receptor interactions with signalling partners and processes of synaptic plasticity.

Importantly, there are studies opposing these findings, reporting that NMDAR-mediated LTD induction is not ion flow independent (Babiec *et al.*, 2014; Malenka 2012, personal communication). The disparities in these results may be due to differences in experimental designs and in developmental stages - more specifically, it is conjectured that relieving NMDAR blockade shortly after the LTD induction protocol prevents the expression of this form of plasticity, and that ion flow-independent NMDAR-mediated LTD is only found in earlier stages of development (Babiec *et al.*, 2014; Nabavi *et al.*, 2014).

Nevertheless, other forms of synaptic plasticity also seem to involve non-ionotropic contributions from NMDAR. Spike timing-dependent LTD (t-LTD) can occur while postsynaptic receptors are blocked by MK-801, implicating the involvement of presynaptic NMDAR, as described in Chapter 1.3.b.ii (Larsen *et al.*, 2011). However, in 2016, the observation that ablation of postsynaptic NMDAR, but not presynaptic NMDAR, prevents t-LTD induction indicated that this rather relies on the non-ionotropic function of postsynaptic NMDAR.

Corroborating this, the authors report that t-LTD induction was prevented by the NMDAR competitive antagonist CPP, but not by MK-801 or 7-CK (Carter and Jahr, 2016). Presynaptic NMDAR have also been reported to regulate spontaneous neurotransmitter release through an ion flow-independent mechanism. The frequency of spontaneous miniature EPSCs (mEPSCs) is decreased by AP5 and by the GluN2B-specific blocker Ro 25-6981, but not by the external application of MK-801. The presynaptic localization of receptors is assumed since the frequency of mEPSCs relates to presynaptic neurotransmitter release, and postsynaptic NMDAR in this study were not sensitive to Ro 25-6981. In addition, postsynaptic NMDAR were continuously blocked by intracellular application of MK-801 (Abrahamsson *et al.*, 2017). However, it is important to not generalize the ionotropic and the non-ionotropic functions of presynaptic NMDAR, since these can vary greatly according to the brain region being studied (Larsen *et al.*, 2011; Carter and Jahr, 2016). To note, besides LTD, long term synaptic depotentiation, or the reversal of synaptic strengthening that occurs after LTP induction, is yet another NMDAR-dependent form of synaptic plasticity that is blocked by the competitive NMDAR antagonist APV but not by MK-801 or high magnesium concentrations (Latif-Hernandez *et al.*, 2016).

Importantly, functional long-term depression or depotentiation of glutamatergic synapses is often associated with structural plasticity mechanisms (i.e. morphological changes to dendritic spines), which have also been reported to involve non-ionotropic NMDAR signalling. As an example, low-frequency stimulation used to induce LTD leads to spine shrinkage even when NMDAR are blocked by 7CK. Additionally, NMDAR blockade by 7CK or MK-801 also turns high frequency stimulation-induced spine enlargement into spine shrinkage, suggesting that NMDAR-dependent Ca^{2+} influx usually overcomes a non-ionotropic shrinkage signal to promote spine growth (Stein, Gray and Zito, 2015). Non-ionotropic NMDAR-dependent spine shrinkage requires nNOS, NOS1AP, p38 MAPK, MK2, cofilin and CaMKII activities (Nabavi *et al.*, 2013). Interestingly, non-ionotropic NMDAR-mediated signalling has also recently been proposed to participate in immune cell infiltration across the blood-brain barrier during inflammation. Pathological activation of GluN3A-containing receptors through a co-stimulation of endothelial cells by NMDAR agonists or co-agonists (NMDA, glycine) and by the serine protease tissue plasminogen activator (tPA) leads to the recruitment of the Rho / ROCK pathway, phosphorylation of myosin light chain and subsequent endothelial cell shrinkage which causes increased permeability of the barrier (Mehra *et al.*, 2020).

LTP

While blocked NMDAR can allow for synaptic depression and depotentiation, the expression of NMDAR-mediated LTP requires ionotropic activity (Scanziani, Malenka and Nicoll, 1996).

However, non-ionotropic aspects of NMDAR physiology may also contribute to the expression of LTP. In mature hippocampal neurons, LTP is inhibited by GluN2A-specific NMDAR antagonists, but not by GluN2B-specific NMDAR antagonists. However, knocking down GluN2B prevents the induction of LTP, which can be rescued by the expression of chimeras composed of GluN2A subunits without CTDs or with GluN2B CTDs, but not by chimeras with the opposite composition (GluN2A with GluN2B CTD) (Foster *et al.*, 2010). Therefore, NMDAR can have not only an ionotropic but also a structural role at synapses that is relevant for synaptic potentiation, one of which may be the co-trafficking and synaptic anchoring of molecular interactors (Barria and Malinow, 2002). As an example, quick GluN2B-containing NMDAR surface redistributions in immature hippocampal synapses support the early steps of LTP induction by promoting the recruitment and accumulation of CaMKII at dendritic spines. CaMKII recruitment and LTP are abolished either by antibody-based NMDAR immobilization or expression of a mutant GluN2B subunit unable not bind CaMKII, suggesting that NMDAR could carry signalling enzymes such as CaMKII as cargo (Dupuis *et al.*, 2014).

Self-regulation

Non-ionotropic actions of NMDAR can also play a role in the regulation of the receptor itself. For instance, the delivery of GluN2A-containing NMDAR into synapses requires agonist and co-agonist binding, but not ion flow (Barria and Malinow, 2002). Consecutive NMDAR stimulations lead to a progressive decline of the amplitude of NMDAR currents, corresponding to a use-dependent decrease in the number of functional NMDAR. The authors report that multiple applications of glutamate induce tyrosine dephosphorylation, which leads to NMDAR interaction with AP2 (a protein complex which internalizes cargo through clathrin-mediated endocytosis) independently of NMDAR-mediated ion influx (Vissel *et al.*, 2001). Co-agonist or agonist binding alone does not lead to NMDAR endocytosis. However, preemptive co-agonist application increases NMDAR-AP2 interactions and primes NMDAR for activity-dependent clathrin-mediated endocytosis (Nong *et al.*, 2003). Interestingly, using the same FRET approach as Dore, Aow and Malinow, Ferreira and colleagues showed that D-serine can lead to a conformational change in NMDAR, which is counteracted by agonist binding to the receptor. High levels of D-serine or an increase in D-serine/glycine ratio decreases GluN1/2B-NMDAR surface mobility and synaptic GluN1/2B-NMDAR content (Ferreira *et al.*, 2017). Of note, this may correspond to NMDAR ionotropic functions. Co-agonist binding has been reported to elicit non-ionotropic NMDAR functions in other contexts. Glycine potentiates AMPAR-mediated currents through GluN1/2A-NMDAR and ERK1/2 activation independently of NMDAR ionotropic function and of the activation of glycine receptors (Li *et al.*, 2016). Additionally, glycine reportedly enhances the activation of cell survival-promoting kinase Akt

via GluN2A-containing NMDAR in an ion flow-independent fashion. Accordingly, glycine acts as a neuroprotectant and decreases the infarct volume in rat middle cerebral artery occlusion (MCAO)³ (R. Hu *et al.*, 2016; J. Chen *et al.*, 2017).

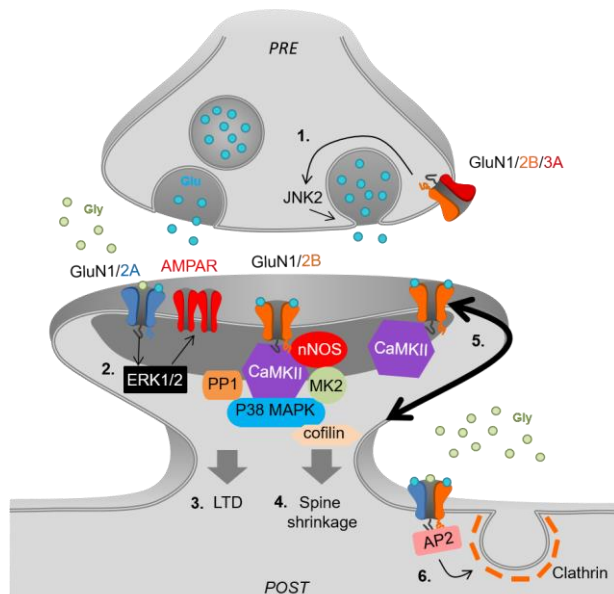


Figure 8. Non-ionotropic physiological functions of NMDAR. 1) GluN2B/3A-containing presynaptic NMDAR elicit spontaneous neurotransmitter release through JNK2 in an ion flux-independent fashion; 2) glycine binding to GluN2A-containing receptors increases synaptic AMPAR content through the recruitment of ERK1/2 signalling; 3) agonist binding to GluN2B-containing NMDAR triggers LTD through the recruitment of CaMKII, PP1, and the p38/MAPK signalling cascade; 4) agonist binding to GluN2B-containing NMDAR triggers spine shrinkage through nNOS, CaMKII, MK2, p38/MAPK and cofilin recruitment; 5) lateral diffusion-based surface redistributions of GluN2B-NMDAR allows the activity-dependent accumulation of CaMKII in dendritic spines to support LTP induction; 6) glycine binding favours interactions of GluN2A- and/or GluN2B-containing NMDAR with the AP2 complex, thus priming receptors for agonist binding-driven (i.e. use dependent) internalization.

Interactions with other neurotransmitter receptors

Non-ionotropic NMDAR functions can include regulation of other neurotransmitter receptors, for example through direct physical interactions occurring within heteroreceptor complexes. NMDAR can form complexes with several G-protein coupled neurotransmitter receptors (GPCRs), and through those impact metabotropic signalling. Heteroreceptor complexes containing NMDAR and dopamine receptors (DRs) are particularly interesting, as NMDAR and DR signalling often interrelate and impairments in their signalling pathways are relevant in psychiatric disorders (Wang, Wong and Liu, 2012). NMDAR activation leads to greater D1R surface expression and stabilization, directing the formation of NMDAR-D1R complexes at perisynaptic sites (Scott *et al.*, 2002; Fiorentini *et al.*, 2003; Pei *et al.*, 2004). This increase of D1R retention at dendritic spines is due to a change in the conformation of NMDAR, and is independent of its function as an ion pore (Scott *et al.*, 2006). There is also indication that NMDAR and D2R are able to unite at synapses (Liu *et al.*, 2006). However, an effect of NMDAR activation on D2R function or surface diffusion is yet to be confirmed. NMDAR and mGluR5a can physically interact either directly or indirectly (through PSD-95 and Homer1b/c), and their co-activation favours the transcription of immediate early genes, through a pathway involving ERK1/2, CREB and c-Fos (Yang *et al.*, 2004). The role of NMDAR in this co-activation effect is a result of agonist binding, since it is blocked by AP5 but not by MK-801 or magnesium, and constitutes a non-ionotropic NMDAR function. NMDAR can also interact with μ (Mu) opioid

³ MCAO is an animal model of cerebral ischemia-reperfusion injury, i.e. the damage caused when blood supply returns to cerebral tissue after a period of ischemia (restriction in blood supply) or lack of oxygen.

receptors (MORs). This interaction determines the effects of the MOR agonist morphine (Rodríguez-Múoz *et al.*, 2012). However, the effect of NMDA binding on MOR-NMDAR interactions is likely ion-flow dependent since it is blocked by both AP5 and MK-801. These aspects are discussed in more details at Chapter I.A.4.c.

Pathology

NMDAR are desirable clinical targets, particularly in neurological or neuropsychiatric conditions that involve excitotoxicity (neuronal damage caused by excessive excitatory neurotransmitter concentrations), such as ischemic brain stroke. NMDAR activity is essential for brain function, hence blocking NMDAR can be deleterious and bring about serious secondary effects (Krystal *et al.*, 1994). On the other hand, NMDAR antagonists reportedly act as antidepressants (Trullas and Skolnick, 1990). Notably, ketamine and its metabolites have recently emerged as fast-acting antidepressant molecules. However, it is unclear whether this effect is mediated by their impact on NMDAR (Zanos *et al.*, 2016, 2017; Suzuki *et al.*, 2017; Pham and Gardier, 2019) (for more details, see Chapter II.B.3). Instead of modulating NMDAR activity, it is possible to modulate the direct NMDAR interactions that elicit NMDAR-mediated signalling cascades underlying the mechanisms or symptoms of the disease. Peptides preventing GluN2 subunit interactions with PDZ domain-containing MAGUKS do not impair NMDAR ionotropic function, but affect NMDAR surface traffic, nanoscale organization and prevent LTP induction (Bard *et al.*, 2010; Kellermayer *et al.*, 2018)(see Chapter I.A.3.c). Therefore, GluN2 intracellular interactions can be important sites for the non-ionotropic/structural role of NMDAR at synapses in both physiological and pathological conditions. Importantly, these interactions regulate the number and composition of synaptic NMDAR. In levodopa-induced dyskinesia, the ratio of synaptic GluN2A-NMDAR to GluN2B-NMDAR at the striatum is increased (Gardoni *et al.*, 2006, 2012). Using interfering peptides which release GluN2B- or GluN2A-NMDAR from their synaptic anchors, this form of dyskinesia becomes more or less prevalent in levodopa-treated animals, respectively. Using the same interfering peptides prevents GluN2B-PSD95 association, uncouples NMDAR from nNOS and decreases NOS production, thus reducing neurotoxic signalling (Aarts *et al.*, 2002). These peptides protect cultured neurons from excitotoxicity, reduce focal ischemic brain damage in rats, and improve their neurological function. However, there are many different molecular cascades involving NMDAR in excitotoxicity and neuronal cell death (Aarts *et al.*, 2002; Soriano *et al.*, 2008; Weilingner *et al.*, 2016). Weilingner and colleagues report that excitotoxic blebbing of dendrites induced by application of high concentrations of NMDA is prevented by APV, but not by MK-801 and magnesium. The authors found that NMDA application favours the formation of a signalling complex made of NMDAR, Src kinase and Panx1. Using an interfering peptide to disrupt the NMDAR-Src-Panx1 complex was effective in reducing infarct

area caused by MCAO in rat (Weillinger *et al.*, 2016). Interestingly, this NMDAR-Src-Panx1 complex has a role in mediating presynaptic glutamate release. Panx1 blockade increases the levels of an endovanilloid, an agonist for the presynaptic TRPV1, and increases the frequency of mEPSCs. Blocking NMDAR with AP5 has the same effect. However, it isn't demonstrated that the NMDAR plays an important non-ionotropic role in this modulation of presynaptic activity (Bialecki *et al.*, 2020). Non-ionotropic NMDAR functions may also play a role in Alzheimer's disease, as studies report that A β -induced synaptic depression and synapse loss is blocked by AP5, but not MK-801 or 7CK⁴ (Kessels, Nabavi and Malinow, 2013; Tamburri *et al.*, 2013; Birnbaum *et al.*, 2015).

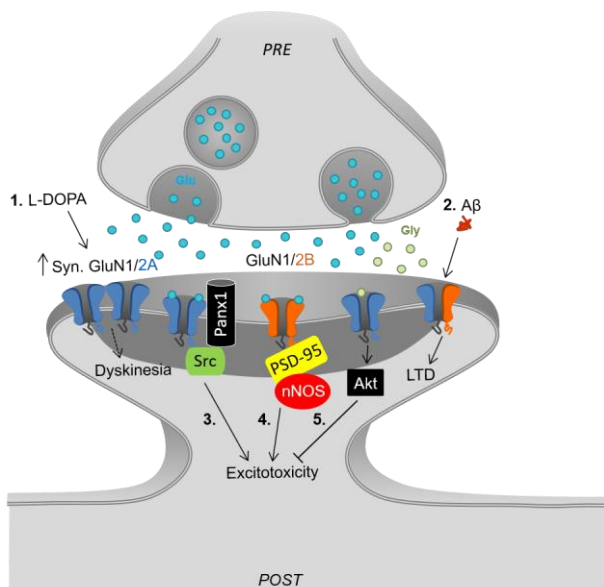


Figure 9. Role of non-ionotropic NMDAR functions in pathological brain conditions. 1) Levodopa-induced dyskinesia is associated with a high GluN2A/GluN2B ratio at striatal synapses, which can be rescued through modulations of NMDAR synaptic anchoring; 2) in Alzheimer's disease, A β induces LTD through non-ionotropic NMDAR signalling; 3, 4) eexcitotoxicity can result from excessive NMDAR agonist binding favouring the ion flux-independent formation of NMDAR-Src-Pannexin1 complexes (3) and is also promoted by the physical coupling of NMDAR to nNOS through the PSD-95 scaffolding protein (4); 5) glycine binding to GluN2A is protective against excitotoxic neuronal death as it enhances Akt activity.

b. Regulation through post-translational modifications

NMDAR expression and trafficking can be regulated through post-translational modifications (PTMs) (Lussier, Sanz-Clemente and Roche, 2015). These can be divided into two categories: modifications which involve the addition of a functional group to the receptor (phosphorylation, palmitoylation and glycosylation), and modifications which involve the covalent conjugation of the proteins ubiquitin or small ubiquitin-like modifier (SUMO) to the receptor (ubiquitination and SUMOylation).

Phosphorylation

The addition of phosphate groups, or phosphorylation, leads to a higher negative charge and hydrophobicity of the target protein, which favours interactions with membranes and other

⁴ Note: The effect of 7CK is not shown for synapse loss (Birnbaum *et al.*, 2015)

proteins. Phosphorylation is performed by kinases, while the removal of phosphate groups, or dephosphorylation, is performed by phosphatases. Phosphorylation of the NMDAR typically occurs at serine (S) and tyrosine (Y) residues. NMDAR are phosphorylated by a series of kinases, most notably CK2, CaMKII, Fyn/Src, Cdk5, PKA and PKC, most of which act at sites located within the C-terminal cytosolic tails of GluN1 and GluN2 subunits (Lussier, Sanz-Clemente and Roche, 2015) (Figure 10). PKA and PKC act on NMDAR early trafficking, by phosphorylating GluN1 S⁸⁹⁷ and S⁸⁹⁶, respectively, thus masking the RRR ER retention signal present at the C1 cassette and promoting NMDAR release from the ER (Scott *et al.*, 2001)(see Chapter I.A.2). Moreover, NMDAR phosphorylation, particularly at GluN2B subunits, is an important mechanism to regulate activity-dependent modulations of NMDAR surface levels. For instance, Cdk5 can phosphorylate GluN2B at S¹¹¹⁶ and favour activity-dependent decreases in levels of surface NMDAR (Plattner *et al.*, 2014). Additionally, phosphorylation of GluN2B on Y¹⁴⁷² by Fyn/Src kinases prevents activity-dependent GluN2B internalization, thus increasing NMDAR surface expression (Lavezzari *et al.*, 2003; Prybylowski *et al.*, 2005; Sanz-Clemente *et al.*, 2010). Fyn/Src kinases directly interact with MAGUKs, therefore primarily phosphorylating synaptic receptors. Conversely, GluN2B Y¹⁴⁷² can be dephosphorylated by STEP, a phosphatase which is mostly extrasynaptic since it becomes ubiquitinated and subsequently degraded after interacting with PSD-95 at synaptic sites (Chen *et al.*, 2012; Won *et al.*, 2016). STEP can also dephosphorylate and inactivate Fyn, additionally decreasing GluN2B Y¹⁴⁷² phosphorylation (Nguyen, Liu and Lombroso, 2002). Therefore, the surface distribution of NMDAR is also an important factor for modulations through phosphorylation. In fact, phosphorylation of the GluN2B PDZ domain at S¹⁴⁸⁰ by CK2 opposes the effects of Y¹⁴⁷² phosphorylation by releasing receptors from MAGUKs (and any associated Fyn/Src kinases), thus allowing NMDAR to diffuse away from synapses and become endocytosed (Hee *et al.*, 2004; Sanz-Clemente *et al.*, 2010). CK2 is a constitutively active kinase, but it mediates regulation of synaptic NMDAR number in an activity-dependent fashion by associating with activated CaMKII, forming a complex that favours the phosphorylation of GluN2B S¹⁴⁸⁰ and consequently the activity-dependent internalization of NMDAR (Sanz-Clemente *et al.*, 2013). At synapses, CK2-driven GluN2B S¹⁴⁸⁰ phosphorylation can be removed by PP1 (Chiu *et al.*, 2019), and CaMKII itself can phosphorylate GluN2B at S¹³⁰³, which disrupts GluN2B-CaMKII interactions (O'Leary *et al.*, 2011). At extrasynaptic sites, DAPK1 can phosphorylate the GluN2B subunit at S¹³⁰³ to regulate NMDAR channel conductance (Tu *et al.*, 2010). Other NMDAR subunits have also been found to be regulated through phosphorylation. For instance, GluN2A can be phosphorylated by Dyrk1a at S¹⁰⁴⁸, which impairs receptor internalization (Grau *et al.*, 2014). Finally, GluN3A can be phosphorylated at Y⁹⁷¹, promoting receptor internalization (Chowdhury *et al.*, 2013).

Palmitoylation

Palmitoylation consists on the addition of palmitic acid to cysteine (C) residues in a protein by palmitoyltransferases. This increases the hydrophobicity of the protein and favours interactions with membranes. The removal of palmitic acid, or depalmitoylation, is mediated by acyl-protein-thioesterases. There are two clusters of cysteines at the CTDs of GluN2A and GluN2B subunits which can be subject to palmitoylation, one close to the TMD (Cluster I) (GluN2A C⁸⁴⁸, C⁸⁵³ and C⁸⁷⁰; GluN2B C⁸⁴⁹, C⁸⁵⁴ and C⁸⁷¹), and one at the middle of the CTD (Cluster II) (GluN2A C¹²¹⁴, C¹²¹⁷, C¹²³⁶ and C¹²³⁹; GluN2B C¹²¹⁵, C¹²¹⁸, C¹²³⁹, C¹²⁴² and C¹²⁴⁵) (Hayashi, Thomas and Haganir, 2009; Thomas and Haganir, 2013; Naumenko and Ponimaskin, 2018) (Figure 10). Palmitoylation of Cluster I increases NMDAR surface expression, possibly via increased NMDAR-Fyn/Src interactions, while palmitoylation of Cluster II leads to NMDAR retention at the Golgi apparatus.

Glycosylation

Glycosylation is the addition of a polysaccharide to a protein through an enzymatic process. Most membrane proteins are glycosylated as they travel across the ER and Golgi apparatus (Aebi *et al.*, 2010; Moremen, Tiemeyer and Nairn, 2012). One of the most prevalent types of glycosylation is *N*-glycosylation, which links glycans to the nitrogen atom of asparagine (N) residues. The GluN1 subunit needs to be *N*-glycosylated at N²⁰³ and N³⁶⁸ in order to be released from the ER (Lichnerova *et al.*, 2015), as does the GluN3A subunit at N¹⁴⁵, N²⁶⁴ and N²⁷⁵ (Skrenkova *et al.*, 2018). Other sites of *N*-glycosylation are found across the NMDAR, and, although not much is known about their significance, computational simulations and experimental data indicate that *N*-glycosylation likely affects NMDAR structure, ionotropic function, and surface trafficking (Huh and Wenthold, 1999; Lichnerova *et al.*, 2015; Kaniakova *et al.*, 2016; Sinitskiy *et al.*, 2017; Skrenkova *et al.*, 2018).

Ubiquitination

Ubiquitination is the conjugation of a ubiquitin protein to lysine (K) residues of a target protein. Ubiquitin is added through the sequential action of E1 (activating), E2 (conjugating) and E3 (ubiquitin ligase) enzymes. Ubiquitination mostly labels proteins for degradation through the ubiquitin-proteasome system. However, ubiquitination has been found to be involved in protein quality control, membrane trafficking and internalization (Hicke and Dunn, 2003; Ciechanover and Iwai, 2004; DiAntonio and Hicke, 2004). The binding of E3 ubiquitin ligase Nedd4-1 to GluN2D CTD promotes receptor polyubiquitination, which leads to a reduction in NMDAR

currents, likely through increased receptor internalization and degradation (Gautam *et al.*, 2013). Conversely, the ubiquitin-specific protease USP6 reduces NMDAR ubiquitination and enhances NMDAR surface expression and formation of synaptic NMDAR clusters (F. Zeng *et al.*, 2019). Finally, the E3 ubiquitin ligase Mind bomb-2 ubiquitinates GluN2B at K¹⁴²⁶, which enhances NMDAR currents (Jurd *et al.*, 2008) (Figure 10). This ubiquitination is promoted by Fyn/Src phosphorylation of GluN2B. Interestingly, the UPS and NMDAR interact in unexpected ways, as GluN2B KO leads to decreased levels of proteasome subunits at the PSD, which decreases AMPAR endocytosis (Ferreira *et al.*, 2015). Enhancing UPS activity counteracts this effect of GluN2B KO.

SUMOylation

SUMOylation is the conjugation of a small ubiquitin-like modifier protein (SUMO) to lysine (K) residues of target proteins through the same enzymatic chain reaction as ubiquitination (Wilkinson and Henley, 2010). In mammals, four genes encode four SUMO isoforms, SUMO1-4. This PTM occurs mostly to nuclear proteins, although there is a multitude of membrane proteins that can become SUMOylated (Kamitani, Nguyen and Yeh, 1997; Wilkinson and Henley, 2010). SUMOylation of neuronal targets may result in altered conformation, changed protein interactions, and increased probability of target protein ubiquitination through direct interactions of SUMO with ubiquitin ligases (Henley, Craig and Wilkinson, 2014). Currently, there is no indication that NMDAR undergo SUMOylation. However, NMDAR-dependent chemical LTP induction is reported to be dependent on SUMOylation of neuronal targets (Jaafari *et al.*, 2013). Of note, in 2017, the detection of SUMO1-ylation of most neuronal targets previously described in the literature was refuted by Daniel and colleagues, showing that 1) in WT animals there is no shift on the molecular weight of most putative neuronal SUMOylation targets which would correspond to the addition of a SUMO1; 2) antibodies used to detect SUMO1 in previous studies stain preparations from SUMO1 KO animals; and 3) in a KI animal model expressing Ha-tagged SUMO1, immunoprecipitation experiments fail to detect SUMOylation of previously validated SUMO targets (Daniel *et al.*, 2017). Decreased SUMO1-ylation and compensation by SUMO2/3-ylation in this KI model is described (Tirard *et al.*, 2012). To date, there is no clear consensus on the validity of this refutation (Wilkinson *et al.*, 2017; Daniel *et al.*, 2018).

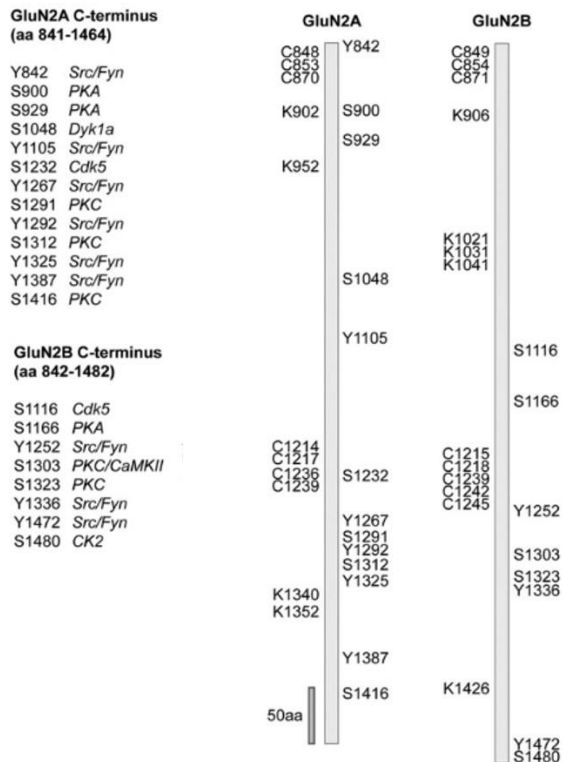


Figure 10. Post-translational modifications at the C-terminal domains (CTDs) of GluN2A and GluN2B subunits. On the left is a list of serine (S) and tyrosine (Y) residues at GluN2A and GluN2B CTDs and the kinases responsible for their phosphorylation. On the right is a representation of the stretch of amino acids corresponding to GluN2A and GluN2B CTDs indicating sites for phosphorylation (serine (S) and tyrosine (Y) residues), ubiquitination (lysine (K) residues), and palmitoylation (cysteine (C) residues). Adapted from (Lussier, Sanz-Clemente and Roche, 2015).

c. Regulation through protein-protein interactions

NMDAR interact with a multitude of molecular partners (Figure 11). Here we deliberately emphasize direct physical interactions which contribute to the regulation of NMDAR surface trafficking (see Chapter I.4.e).

NMDAR-DR interactions

D1-like dopamine receptors (D1Rs) can physically interact with NMDAR through two different sites within its intracellular carboxyl-terminal tail (CTD): the t2 segment (L387-L416), which can bind to the CTD of GluN1 and to CaMKII, and the t3 segment (S417-T446), which can bind to the CTD of GluN2A (Lee *et al.*, 2002). As described in Chapter I.4.a, NMDAR activation increases D1R surface levels and stabilization, directing the formation of NMDAR-D1R complexes at perisynaptic sites (Scott *et al.*, 2002; Fiorentini *et al.*, 2003; Pei *et al.*, 2004). Conversely, upon D1R activation, both NMDAR and D1Rs become more mobile. D1Rs decluster from perisynaptic sites, disperse in the membrane, and are often internalized, while NMDAR diffuse towards the synapse (Ladepêche *et al.*, 2013). D1R activation strengthens interactions through the t3 segment and weakens interactions through the t2 segment. D1R metabotropic activity thereby favours the assembly of D1R-GluN1/2A complexes and possibly NMDAR internalization, as the authors report a decrease in surface NMDAR (Lee *et al.*, 2002). Congruently, this reportedly leads to a reduction of NMDAR-mediated currents (Ladépêche,

Dupuis and Groc, 2014). At the same time, D1R activity leads to the uncoupling of D1R from GluN1. This allows for NMDAR interaction with CaMKII and induction of cell-survival signalling pathways and NMDAR-dependent LTP (Lee *et al.*, 2002; Nai *et al.*, 2010). The use of an interfering peptide that mimics the t2 segment and disrupts CamKII-GluN1 interaction results in serious reduction of LTP and a phenotype of impaired working memory (Nai *et al.*, 2010). This bidirectional interplay between surface D1Rs and NMDAR is conceptualized as a diffusion trap to stimulate synaptic plasticity. NMDAR activity creates a pool of NMDAR and D1R retained at perisynaptic sites. After D1R activation, perisynaptic NMDAR unbind from D1R and laterally diffuse towards synapses where they are free to interact with CaMKII and potentiate neurotransmission. The retention of D1Rs in the vicinity of NMDAR also potentiates the intracellular signalling pathways elicited by both these receptor types, which convey in the activation of CREB, a transcription factor necessary for the long-term effects of LTP on gene expression (Scott and Aperia, 2009; Ladepeche *et al.*, 2013).

Although the presence of D2R-NMDAR complexes hasn't been confirmed at the surface of neurons, D2Rs co-immunoprecipitate with NMDAR's 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 association and phosphorylation by CaMKII, and ultimately leading to the inhibition of NMDAR-mediated currents (Liu *et al.*, 2006).

NMDAR-mGluR interactions

NMDAR can also directly interact with group I metabotropic glutamate receptors mGluR5a and mGluR1a. mGluR5a intracellular carboxyl-terminal tail binds to GluN1/2B-NMDAR through an unknown site, and the direct physical interactions between these receptors leads to reciprocal constitutive inhibition (Perroy *et al.*, 2008). Coactivation of NMDAR and mGluR5 in neurons greatly enhances NMDAR currents, while mGlu5R activation in the absence of NMDAR stimulation lightly decreases NMDAR currents (Kotecha *et al.*, 2003). Furthermore, coactivation of NMDAR and mGluR5 synergistically increases phosphorylation of ERK1/2 (transcription factors participate in the immediate early gene response) in a way that is dependent on the crosstalk between NMDAR-associated synaptic adaptor protein PSD-95 and the mGluR5-linked adaptor protein Homer1b/c, but independent of NMDAR- or mGluR5-mediated calcium signalling (Yang *et al.*, 2004). NMDAR and mGluR5 coactivation also phosphorylates CREB and increases c-Fos expression.

NMDAR-MOR interactions

In neurons of the mesencephalic periaqueductal gray, NMDAR have also been reported to directly interact with μ (Mu) opioid receptors (MORs) through the MOR intracellular carboxyl-terminal tail and the GluN1 C1 amino acid stretch which is present in GluN1-1 and GluN1-3 isoforms (see Chapter I.A.1.b) (Rodríguez-Muñoz *et al.*, 2012). Disruption of MOR-NMDAR complexes leads to morphine tolerance and decreased morphine antinociceptive effect. MOR activation with morphine leads to PKC-mediated C1 phosphorylation and disruption of the MOR-NMDAR complex, while NMDAR activation with NMDA leads to PKA-mediated disruption of the MOR-NMDAR complex. Inhibition of PKC or PKA restores the MOR-NMDAR complex and restores the analgesic and antinociceptive effects of morphine, respectively.

NMDAR- α 7 nAChR interactions

Hippocampal GluN2A can interact with α 7 nicotinic acetylcholine receptors (α 7 nAChRs) through the intracellular loop 2 of α 7 nAChR and the CTD of GluN2A (Li *et al.*, 2012). Activation of α 7 nAChR increases the α 7nAChR–NMDAR interaction, upregulates NMDAR activity and favours LTP induction in cultured neurons (S. Li *et al.*, 2013). Disrupting α 7 nAChR–GluN2A interactions using an interfering peptide prevents this and decreases ERK activity, thereby impairing novel object recognition and blocking cue-induced reinstatement of nicotine seeking in animal models of nicotine relapse (Li *et al.*, 2012; S. Li *et al.*, 2013). Interestingly, α 7 nAChR–GluN2A complexes are decreased in the cortex from humans with Alzheimer's disease (Elnagar *et al.*, 2017). Activation of α 7 nAChR also indirectly regulates GluN2B traffic via Src-family tyrosine kinase (SFK), which may play a role on the impact that α 7 nAChR activity has on the severity of secondary effects caused by the volatile anaesthetic sevoflurane (Tang *et al.*, 2018). In fact, proteomics analysis indicates that there are likely many indirect pathways through which α 7 nAChR regulate NMDAR activity, additionally to physically interacting with the receptor, including the regulation of neurotransmitter levels (Zhang *et al.*, 2016). Additionally, as α 7 nAChRs at the neuronal surface are confined at perisynaptic areas, α 7 nAChRs have been proposed to act as a diffusion trap for NMDAR, similar to D1Rs (Bürli *et al.*, 2010; Ladepeche *et al.*, 2013).

NMDAR- EphB2R interactions

EphB2R is the tyrosine kinase receptor of EphB2. EphB2R is postsynaptic and EphB2 is presynaptic. As a result, the association of receptor and ligand creates a trans-synaptic column. NMDAR physically interact with EphB2R through the NTD of GluN1 (Dalva *et al.*, 2000; Washburn *et al.*, 2020). Ligand binding to EphB2R results in EphB2R Y⁵⁰⁴

phosphorylation, which promotes NMDAR-EphB2R interactions, and increases NMDAR stabilization at synapses (Dalva *et al.*, 2000; Nolt *et al.*, 2011; Hanamura *et al.*, 2017). This also favours NMDAR phosphorylation by Src and potentiates NMDAR-mediated calcium flow (Takasu *et al.*, 2002). As a consequence, knocking out EphB2R reduces NMDAR synaptic localization, NMDAR currents, and the amplitude of EPSCs after LTP (Henderson *et al.*, 2001). Interestingly, autoantibodies from patients suffering from anti-NMDAR encephalitis - a neurological disorder characterized by the production of pathogenic antibodies against NMDAR - have been shown to disrupt NMDAR-EphB2R interactions (Mikasova *et al.*, 2012), causing lateral redistribution of NMDAR from synapses and lateral redistribution at the neuronal surface (Mikasova *et al.*, 2012; Washburn *et al.*, 2020). Interestingly, though the autoantibodies from anti-NMDAR encephalitis patients impair an NMDAR-EphB2R interaction which relies on GluN1, i.e. the obligatory NMDAR subunit, patient antibodies have a distinct impact on GluN2A- and GluN2B-containing NMDAR (Mikasova *et al.*, 2012). While they increase the surface mobility of GluN2A-NMDAR, they have the opposite effect on GluN2B-NMDAR. This may reflect the subcellular localization of these receptor subtypes. The authors propose that, by breaking the synaptic NMDAR-EphB2 interactions, synaptic (mostly GluN2A-)NMDAR become less anchored and diffuse away from the postsynaptic terminals. At the same time, autoantibodies cross-link extrasynaptic (mostly GluN2B-)NMDAR, increasing NMDAR internalization. After prolonged exposure to autoantibodies from anti-NMDAR encephalitis patients, levels of surface NMDAR decline, causing NMDAR hypofunction and preventing LTP induction (Mikasova *et al.*, 2012).

NMDAR-NLG1 interactions

Neuroligins are cell adhesion proteins involved in synaptogenesis (Chih, Engelman and Scheiffele, 2005). While neuroligins (NLG) are present at postsynaptic terminals, their ligands neurexins (NRX) are expressed at presynaptic terminals and the binding of both partners forms trans-synaptic adhesions that help establish functional trans-synaptic nanocolumns (Haas *et al.*, 2018). NLG1 directly interacts with NMDAR through the NTD of GluN1 subunits and controls the synaptic abundance of receptors (Budreck *et al.*, 2013). Reciprocally, NMDAR activity modulates NLG1 function and surface expression and blocking NMDAR prevents the synaptogenic action of NLG1 (Chubykin *et al.*, 2007). NLG1 KO decreases NMDAR-mediated EPSCs and prevents NMDAR-dependent LTP (Chubykin *et al.*, 2007; Soler-Llavina *et al.*, 2011; Budreck *et al.*, 2013). Interestingly, NLG1 KO prevents the recovery of NMDAR EPSCs following washout of MK-801, which points towards a failure to introduce unblocked extrasynaptic NMDAR into the synapse, indicating an impairment in NMDAR surface trafficking (Budreck *et al.*, 2013).

NMDAR-Neto1 interactions

Neto1 is a postsynaptic CUB domain transmembrane protein most notable for acting as an auxiliary subunit to kainate receptors (Michishita *et al.*, 2003; Straub *et al.*, 2011). The first extracellular CUB domain of Neto1 can directly interact with GluN2A and GluN2B subunits (Ng *et al.*, 2009). Neto1 KO leads to a decreased synaptic GluN2A-NMDAR content, which is compensated by GluN2B-NMDAR. Although this results in basal NMDAR EPSCs with a normal amplitude, Neto1 KO prevents the induction of NMDAR-dependent LTP, and Neto1 KO animals have learning and memory impairments.

NMDAR-CaMKII interactions

CaMKII is a large holoenzyme which plays a central role in synaptic plasticity (Silva *et al.*, 1992; Stevens, Tonegawa and Wang, 1994; Coultrap *et al.*, 2014). At basal conditions, CaMKII is constitutively autoinhibited. After LTP-inducing stimulation, calcium entry through the NMDAR activates calcium-calmodulin, which binds to and disinhibits CaMKII, leading to CaMKII autophosphorylation at the threonine (T) residue at position 286 (T²⁸⁶). Activated CaMKII is then translocated to the postsynaptic terminal. CaMKII can physically interact the CTD of GluN1, GluN2A and GluN2B subunits (Gardoni *et al.*, 1999; Leonard *et al.*, 1999; Strack, McNeill and Colbran, 2000; Bayer *et al.*, 2001, 2006; Lisman, Yasuda and Raghavachari, 2012), though association to GluN2B is stronger than to other NMDAR subunits (Strack and Colbran, 1998; Leonard *et al.*, 1999). This kinase can bind to GluN2B at two sites, one near a serine (S) residue at position 1303 (S¹³⁰³), the other between amino acids 839-1120. Binding to the latter requires that CaMKII be active and that T²⁸⁶ be phosphorylated (Bayer *et al.*, 2001). At the same time, active CaMKII can become autophosphorylated at T^{305/6} and phosphorylate GluN2B at S¹³⁰³, both factors which would disrupt GluN2B-CaMKII interactions (O'Leary *et al.*, 2011). However, analysis of the kinetic properties of CaMKII autophosphorylations and of ATP binding to CaMKII indicates that positive regulation of GluN2B-CaMKII interactions by ATP binding and T²⁸⁶ autophosphorylation occurs faster than negative modulation by GluN2B S¹³⁰³ phosphorylation and CaMKII T^{305/6} phosphorylation, resulting in the upholding of the GluN2B-CaMKII interaction. This interaction locks CaMKII in an active state for over 30 minutes (Bayer *et al.*, 2006). This way, autophosphorylated CaMKII sustains its activity even after the transient NMDAR-mediated increase in calcium concentration has passed, allowing for long-term modifications in synaptic strength (Lisman, Yasuda and Raghavachari, 2012). CaMKII is less mobile within spines, and becomes even less diffusive after NMDAR activation (Lu *et al.*, 2014). CaMKII binding to GluN2B is necessary for the expression of LTP (Barria and Malinow, 2005; Dupuis *et al.*, 2014; Incontro *et al.*, 2018). Interestingly, NMDAR-CaMKII interactions affect the trafficking of both molecular partners

(Dupuis *et al.*, 2014). Dupuis and colleagues report that in immature neurons the translocation of CaMKII into synapses after LTP-inducing stimulation is dependent on GluN2B-CaMKII association and NMDAR surface redistributions. The authors report an increased GluN2B surface diffusion after LTP induction which is dependent on CaMKII and CK2 activity, and show that NMDAR surface immobilization or disruption of CaMKII interaction both prevent the intracellular relocalization of CaMKII to dendritic spines and the expression of LTP. Interestingly, CaMKII autophosphorylation also occurs following LTD-inducing stimulation, but no interaction with GluN2B has been reported in this case (Barria *et al.*, 1997; Coultrap *et al.*, 2014). LTD induction leads to activation of calcineurin, which subsequently activates DAPK1. DAPK1 then binds to GluN2B, masking the CaMKII binding site, and phosphorylates GluN2B at S¹³⁰³, preventing GluN2B-CaMKII interactions (Strack, McNeill and Colbran, 2000; Tu *et al.*, 2010; O'Leary *et al.*, 2011). DAPK1-mediated suppression of CaMKII/GluN2B binding is required for LTD (Goodell *et al.*, 2017). Hence, modulation of GluN2B-CaMKII interactions is central in establishing the direction of synaptic plasticity.

NMDAR-MAGUK interactions

Membrane-associated guanylate kinases (MAGUKs) are molecular scaffolds containing PDZ, SH3 and GUK domains. Unlike what the name suggests, MAGUKs have no enzymatic activity. The MAGUK protein super-family is composed by SAP-102, PSD-95, SAP-97, PSD-93, and DLG5. The two first PDZ domains of MAGUKs bind to the PDZ-binding motifs (xSxV) which constitute the four last amino acids of the CTDs of GluN2 subunits (L Bard and Groc, 2011). MAGUKs can also interact with NMDAR through other stretches of GluN2 CTDs. For example, PSD-95 can interact with GluN2A amino acids at positions 1382–1420 and GluN2B amino acids at positions 1086–1157, which correspond to putative SH3-binding domains (Cousins, Kenny and Stephenson, 2009). Additionally, certain splice variants of SAP-102 contain a GluN2B-specific binding site, which depends on two aspartic acid (D) residues at the GluN2B CTD (D¹³⁹¹ and D¹³⁹²) (Chen *et al.*, 2011, 2012). This may confer some NMDAR subunit specificity to NMDAR-MAGUK interactions. In fact, it has been reported that different GluN2 subunits preferentially interact with specific MAGUKs (namely, that GluN2A preferentially binds to the mostly synaptic MAGUK PSD-95, while GluN2B preferentially binds to the mostly peri- and extrasynaptic MAGUK SAP-102) (Sans *et al.*, 2000; van Zundert, Yoshii and Constantine-Paton, 2004; Groc, Bard and Choquet, 2009; Zhang and Diamond, 2009; Bard Groc, L., 2011, but see Al-Hallaq *et al.*, 2007). This may be an important factor conferring a differential localization to NMDAR according to their subunit composition (see Chapter I.A.3.b.iii). Additionally, NMDAR can interact with other spatially segregated scaffolds outside of the MAGUK super-family, such as the PDZ domain-containing scaffold GIPC which is mainly

extrasynaptic (Yi *et al.*, 2007).

NMDAR-MAGUK interactions are essential for early NMDAR processing and traffic into synapses (Setou *et al.*, 2000; Sans *et al.*, 2003; Jeyifous *et al.*, 2009; L Bard and Groc, 2011) (see Chapter I.A.2). Furthermore, MAGUKs play a central role in the anchoring of glutamate receptors to the PSD (Elias and Nicoll, 2007; Bard *et al.*, 2010; L Bard and Groc, 2011). When compared to wild type GluN2A or GluN2B subunits, truncated GluN2A or GluN2B subunits lacking the last six amino acids of their CTD are barely introduced into synapses (Barria and Malinow, 2002). While overexpression of wild type GluN2B leads to an increase in NMDAR EPSCs, overexpression of GluN2B subunits incapable of interacting with PDZ domains does not, indicating that these receptors are not inserted or do not become anchored to the synapse (Prybylowski *et al.*, 2002). Whether the insertion of GluN2A into synapses is similarly controlled by PDZ binding domains or not is still controversial (Barria and Malinow, 2002; Prybylowski *et al.*, 2005; Lucie Bard and Groc, 2011).

Cell-permeant peptides containing the same amino acid sequence as the last 15-9 residues of GluN2 CTDs (TAT-2A, TAT-2B) selectively prevent the interactions between GluN2A and GluN2B subunits (despite the high sequence homology of GluN2 CTDs) and their synaptic anchors through competition for NMDAR binding sites at MAGUKs (Aarts *et al.*, 2002; Aarts and Tymianski, 2003; Lim *et al.*, 2003; Gardoni *et al.*, 2006, 2009; Bard *et al.*, 2010). . As the last 6 amino acids of GluN2A and GluN2B CTDs are identical, the NMDAR subunit specificity attained by the aforementioned competing peptides is presumably conferred by the residues preceding them (L Bard and Groc, 2011). Of note, as it is likely that the CTDs of two GluN2 subunits belonging to the same NMDAR occupy two adjacent PDZ domains of one MAGUK, competing peptides which contain two copies of GluN2 CTDs have been designed (Bard *et al.*, 2010). These divalent peptides are more efficient at disrupting NMDAR-MAGUK interactions than classical monomeric ones.

TAT-2A releases GluN2A-containing NMDAR from their synaptic anchors, consequently increasing their surface mobility and disrupting their nanoscale organization (Bard *et al.*, 2010; Kellermayer *et al.*, 2018). Conversely, TAT-2B has the same effect on GluN2B-containing receptors. Ultimately, TAT-2A partially depletes GluN2A-NMDAR from synapses, which results in a decreased GluN2A synaptic content. Importantly, these competing peptides do not impact the overall amplitude of NMDAR-mediated EPSCs, although they alter the relative contribution of NMDAR subtypes (namely, GluN2A-containing NMDAR and GluN2B-containing NMDAR). This likely results from the fact that the removal of an NMDAR subtype from the synapse is compensated by the introduction of another (e.g. when TAT-2A decreases GluN2A synaptic content the amount of GluN2B at synapses rises). Although it is unclear whether TAT-2B

actually decreases the synaptic content of GluN2B-NMDAR, it does decrease the contribution of GluN2B-NMDAR to NMDAR EPSCs, which may be due to receptor displacement rather than exclusion from the synapse, given the high GluN2B sensitivity to distance from glutamate release sites (Aarts *et al.*, 2002; Erreger *et al.*, 2005; Gardoni *et al.*, 2006, 2009; Santucci and Raghavachari, 2008; Biederer, Kaeser and Blanpied, 2017; Kellermayer *et al.*, 2018) (see Chapter I.A.3.c). Despite not affecting the amplitude of basal NMDAR EPSCs, these manipulations of NMDAR-MAGUK interactions bidirectionally impact LTP (Gardoni *et al.*, 2009; Kellermayer *et al.*, 2018). Decreasing the GluN2A/GluN2B ratio at synapses using TAT-2A boosts LTP, while increasing it with TAT-2B prevents LTP induction, suggesting that both receptor subtypes serve different roles in synaptic adaptation (Kellermayer *et al.*, 2018).

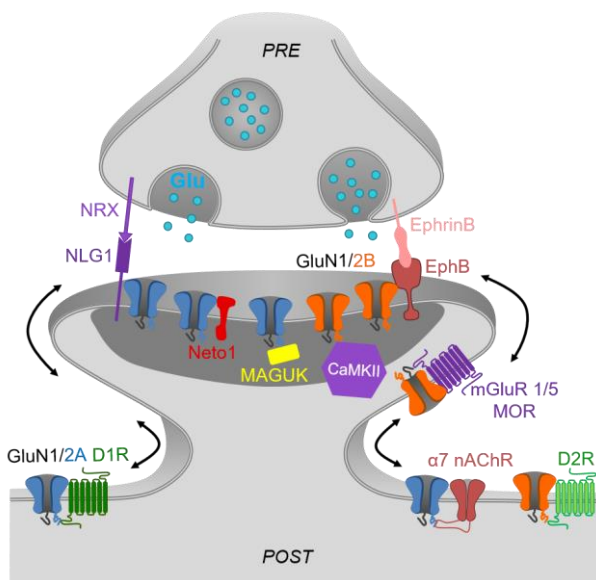


Figure 11. Direct interactions impact the surface trafficking and synaptic anchoring of NMDAR. Surface NMDAR interact with several partners, including NLG1, Neto1 and EphB2R at synaptic sites, D1R, D2R, mGluRs, MOR and $\alpha 7$ nAChRs at perisynaptic and extrasynaptic sites. Synaptic NMDAR interact with cytosolic MAGUKs which help stabilize them at the postsynaptic density. They also bind to CaMKII to promote synaptic plasticity.

d. Regulation through endocytosis/exocytosis cycling

i. NMDAR endocytosis

Endocytosis is the active process through which membrane proteins become internalised. In neurons, specific endocytic zones neighbouring postsynaptic terminals suggest that NMDAR first exit synapses through lateral diffusion before being trapped and internalized at extrasynaptic sites (Blanpied, Scott and Ehlers, 2002; Petralia, Wang and Wenthold, 2003; Rácz *et al.*, 2004). There, clathrin coats assemble to create a budding of the neuronal membrane. A patch of neuronal membrane thus becomes a clathrin-coated intracellular vesicle, consequently internalising all membrane-bound proteins. The $\mu 2$ subunit of the AP2 protein complex, which is a part of the clathrin-mediated endocytosis machinery, interacts with

both GluN2A and GluN2B subunits, although only the interaction with GluN2B, which occurs through an internalization motif (YEKL) at the CTD, can induce clathrin-mediated endocytosis (Slepnev and De Camilli, 2000; Roche *et al.*, 2001; Vissel *et al.*, 2001; Lavezzari *et al.*, 2003, 2004). NMDAR-PSD95 association opposes the effect of NMDAR interactions with AP2, decreasing NMDAR internalization and increasing NMDAR surface levels (Roche *et al.*, 2001). NMDAR-AP2 interactions are enhanced by co-agonist binding to the NMDAR, thus priming receptors for internalization (Nong *et al.*, 2003). High NMDAR activation leads to NMDAR internalization. This relies on NMDAR-AP2 interactions and depends on NMDAR agonist binding but is ion-flow independent (Vissel *et al.*, 2001; Prybylowski *et al.*, 2005) (see Chapter I.A.4.a). NMDAR internalization is also highly regulated by post-translational modifications (see Chapter I.A.4.b)

ii. NMDAR exocytosis

Exocytosis is the active process through which proteins are integrated into the plasma membrane. NMDAR exocytosis at neurons can occur via the SNARE complex at extrasynaptic sites (Gu and Huganir, 2016). Again, this suggests that NMDAR laterally diffuse from their site of deployment in order to enter synapses. NMDAR are exocytosed in a constitutive fashion. Constitutive NMDAR exocytosis is regulated through NMDAR interactions with Rab proteins (Gu and Huganir, 2016). At the same time, there are processes of activity-dependent NMDAR exocytosis. PKC activity, which is controlled by intracellular calcium levels and necessary for the expression of LTP, upregulates SNARE-dependent delivery of functional NMDAR to the neuronal surface (Tanaka and Nishizuka, 1994; Lan, Skeberdis, Jover, Grooms, *et al.*, 2001). Additionally, the activation of other receptors, such as group I mGluR, dopamine receptors, and TNF α , promotes the insertion of NMDAR to the neuronal surface (Dunah and Standaert, 2001; Lan, Skeberdis, Jover, Zheng, *et al.*, 2001; Wheeler *et al.*, 2009). NMDAR membrane integration is also controlled by NMDAR post-translational modifications (see Chapter I.A.4.b).

e. Regulation through lateral diffusion

Long considered to be fixed in the neuronal surface, neurotransmitter receptors (NTRs) are now known to be constantly redistributed through surface diffusion along the plasma membrane, as revealed by fluorescence imaging. Two main strategies have been developed to explore redistributions of surface proteins, i.e. ensemble approaches such as electrophysiology or fluorescence recovery after photobleaching (FRAP), allowing to monitor the relocalization of surface receptors in bulk, and single molecule imaging approaches where traceable probes bound to individual receptors can be tracked with great spatiotemporal precision (Groc, Bard and Choquet, 2009; Dupuis and Groc, 2020).

FRAP is a live fluorescence microscopy technique where the diffusion of fluorescently labelled proteins into a photobleached portion of the cell is monitored. Fluorescence recovery to the photobleached area occurs due to protein trafficking. In order to label receptors at the neuronal surface, neurons can be elicited to express recombinant receptors that are fused to a super-ecliptic pHluorin (SEP), a pH-sensitive molecule that becomes fluorescent only when in contact with the neutral pH of the extracellular medium (Miesenböck, De Angelis and Rothman, 1998). Another strategy, for example, would be to irreversibly bind membrane-impermeable fluorescent markers to the receptors (Groc and Choquet, 2008). Historically, FRAP experiments have been elemental to study receptor surface trafficking. In fact, the first records of NTR lateral diffusion come from FRAP studies establishing that acetylcholine receptors are highly mobile at the muscle membrane, but become confined and accumulate at neuromuscular junctions (Axelrod *et al.*, 1976; Young and Poo, 1983). This “diffusion trap” mechanism is presumed to play an important role during muscle innervation.

Another historically relevant ensemble approach is the electrophysiological recording of ionotropic receptor-mediated currents coupled with the irreversible blockade of synaptic receptors. Fast current recovery is an indication that unblocked receptors are laterally diffusing into synapses (Tovar and Westbrook, 2002; Adesnik, Nicoll and England, 2005; Thomas *et al.*, 2005). In the first experiment of the sort, MK-801 and ketamine were used to irreversibly block NMDAR in autaptic synapses *in vitro* (Tovar and Westbrook, 2002). The timescale of NMDAR current recovery could not be accounted for by endo- and exocytosis or antagonist unbinding. Additionally, blockade of all surface NMDAR by co-application of MK-801 with NMDA prevented NMDAR-mediated current recovery, indicating that lateral diffusion of extrasynaptic NMDAR into the synapse was responsible for this phenomenon. There have been attempts of replicating this effect in more complex hippocampal preparations, with mixed results. While Harris and Pettit could not find NMDAR current recovery post-MK-801 washout in acute hippocampal slices, McQuate and Barria recently reported that this occurs in CA1 neurons at organotypic hippocampal preparations (Harris and Pettit, 2007; McQuate and Barria, 2020). While the electrophysiological approach is informative on a functional level, it seems to be sensitive to differences in stimulation protocols and biological preparations (Tovar and Westbrook, 2002; Harris and Pettit, 2007; McQuate and Barria, 2020). Additionally, the receptor antagonists used as tools may themselves affect receptor mobility.

Both ensemble approaches only allow for the extrapolation of data on the averaged lateral diffusion of receptor subpopulations, without providing detailed information on the surface behaviour of single NTRs. Single molecule imaging approaches were developed to overcome this limitation. These approaches consist on the tracking of individual receptors using latex

beads, organic dyes, gold particles or quantum dots (QDs) immunologically associated to extracellular epitopes of receptors. It is then possible to track these probes in real-time with great precision. To allow for the high-resolution spatial reconstruction of single receptor trajectories, single molecule imaging techniques resort to sparse receptor labelling with photostable probes (single particle tracking, SPT) or with photobleachable probes (universal Point Accumulation for Imaging in Nanoscale Topography, uPAINT); if not to continual stochastic activation of small subsets of receptor-bound fluorophores (photo-activated localization microscopy, PALM) (Dupuis and Groc, 2020).

Through the combination of all previously mentioned approaches, NTRs have been found to be mobile at the surface of neurons with distinct lateral diffusion profiles (e.g. GABA receptors; glycine receptors; glutamate receptors, dopamine receptors, acetylcholine receptors) (Borgdorff and Choquet, 2002; Sergé *et al.*, 2002; Dahan *et al.*, 2003; Jacob *et al.*, 2005; Laurent Groc *et al.*, 2006; Scott *et al.*, 2006; Bürli *et al.*, 2010). Today, it is assumed that all NTRs are mobile at the neuronal surface, and can travel across membrane compartments (synaptic, perisynaptic, extrasynaptic) (Choquet and Triller, 2013). Knowing that synaptic and extrasynaptic receptors are interchangeable, lateral diffusion can be appreciated as means of quickly altering the receptor composition and therefore functional output of a synapse. This dynamic behaviour within the membrane was 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; Groc and Choquet, 2020).

The surface expression of NMDAR is developmentally controlled and modulated by synaptic activity and sensory experience (Bellone and Nicoll, 2007; Paoletti, Bellone and Zhou, 2013; Sanz-Clemente, Nicoll and Roche, 2013). While the overall number and composition of NMDAR at the neuronal surface is regulated through cycles of exocytosis and endocytosis, their spatial organization is finely modulated through surface trafficking (Groc, Bard and Choquet, 2009; Bard *et al.*, 2010; L Bard and Groc, 2011; Groc and Choquet, 2020). This highly impacts NMDAR function as, depending on their location, NMDAR contribute either to synaptic transmission, protein synthesis-associated signalling pathways, cell survival or apoptosis (Cull-Candy and Leszkiewicz, 2004; Lucie Bard and Groc, 2011). Moreover, the relative content of NMDAR subtypes at a synapse (notably GluN2A and GluN2B-NMDAR) may constitute a metaplasticity mechanism, as it impacts the plastic range of synapses (Dupuis *et al.*, 2014; Kellermayer *et al.*, 2018). Importantly, dysregulating NMDAR surface trafficking leads to significant impairments in synaptic plasticity and cognitive deficits (Mikasova *et al.*, 2012; Dupuis *et al.*, 2014; Potier *et al.*, 2015; Kellermayer *et al.*, 2018). Therefore, it is crucial

to understand the physiological function and regulation of NMDAR lateral diffusion.

- i. Activity-dependent changes in NMDAR surface trafficking: developmental switch, synapse maturation and synaptic plasticity

Synaptic NMDAR composition changes during development, from mainly GluN2B-containing NMDAR to GluN2A-containing NMDAR during the second postnatal week (Monyer *et al.*, 1994). Progressive synaptic insertion of functional GluN2A-NMDAR is dependent on synaptic activity (Barria and Malinow, 2002). Hence, while immature synapses initially contain only GluN2B-NMDAR, spontaneous synaptic activity and changes in GluN2 expression levels drive the synaptic incorporation of GluN2A-containing NMDAR during development. Consistent with this GluN2B to GluN2A switch, NMDAR EPSCs from mature synapses have faster kinetics and are much less sensitive to the GluN2B-specific NMDAR antagonist ifenprodil (Bellone and Nicoll, 2007; Rauner and Köhr, 2011). The precise mechanisms through which the GluN2B to GluN2A switch occurs are not yet fully understood. However, modulations of NMDAR surface trafficking are likely to play a role in this.

The diffusion properties of NMDAR depend on their subunit composition. By tracking the movements of individual NMDAR at the neuronal surface, Groc and colleagues found that GluN2A-containing NMDAR are less mobile, show a longer synaptic dwell time than GluN2B-NMDAR, and are generally concentrated within synapses while GluN2B-NMDAR are rather at the periphery (Laurent Groc *et al.*, 2006). The authors reported that GluN2B-containing NMDAR are much more mobile in mature neurons compared to immature neurons, while the opposite is true for GluN2A-containing NMDAR. Decreased NMDAR surface diffusion was associated with an increase in the time spent within synapses. Therefore, the developmental switch in synaptic NMDAR composition may depend on the surface stabilization and synaptic retention of specific NMDAR subtypes.

The developmental switch from GluN2B- to GluN2A-NMDAR is impelled by sensory experience, and is associated with the refinement of cortical networks (Philpot *et al.*, 2001; van Zundert, Yoshii and Constantine-Paton, 2004). Visual experience modulates the synaptic GluN2A/GluN2B ratio at the visual cortex (Philpot *et al.*, 2001). In dark-reared animals, this ratio is decreased, as implied by the higher sensitivity to ifenprodil and slower decay kinetics of NMDAR EPSCs. The experience-driven modulation of the GluN2A/GluN2B ratio is dynamic, as putting light-reared animals in the dark reduces their GluN2A/GluN2B ratio over time, until their NMDAR EPSCs display the same properties as those of dark-reared animals. Synaptic plasticity at immature hippocampal preparations also leads to bidirectional adjustments of synaptic GluN2A/GluN2B ratio. LTP induction in hippocampal slices from young animals lead

to a rapid (within 5 minutes) increase in this ratio, while subsequent synaptic depotentiation decrease it (Bellone and Nicoll, 2007). The timescale of this adjustment is inconsistent with regulation through exo- and endocytosis. Fast changes in synaptic NMDAR composition are therefore likely to occur via NMDAR surface trafficking. Indeed, Dupuis and colleagues reported that this remodeling involves a transient increase in the lateral diffusion of GluN2B-NMDAR which favours the accumulation of CaMKII within dendritic spines through their direct interaction. Preventing either the physical interaction between CaMKII and GluN2B subunits, or the ability of GluN2B-NMDAR 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). Additionally, decreasing the GluN2A/GluN2B ratio by destabilizing GluN2A-NMDAR from synapses using competing peptides boosts LTP, while increasing the GluN2A/GluN2B ratio by eliciting GluN2B-NMDAR lateral redistribution prevents LTP induction (Kellermayer *et al.*, 2018). Surface trafficking of NMDAR is therefore an important regulator of synaptic plasticity and is necessary for LTP induction. Supporting this, the administration of cross-linking anti-NMDAR antibodies into the Cornu Ammonis (CA) 1 hippocampal region of anesthetized mice does not alter basal NMDAR-mediated transmission but indeed prevents CA1 LTP induction, as demonstrated through *in vivo* electrophysiological recordings of CA1 field EPSCs induced by contralateral CA3 fibres stimulation (Potier *et al.*, 2015). The authors also report that infusing the dorsal hippocampus of animals with NMDAR cross-linking antibodies before fear conditioning results in impaired acquisition and retention (24-26 hours) of contextual and temporal fear association memories in mice. Cross-linking receptors at the dentate gyrus had no such effect. Restricting antibody infusion to the dorsal CA1 selectively prevented the retention of temporal associative fear memory in that task. However, this manipulation did not impact the performance of animals on the object location task, a hippocampus-dependent task that does not require any temporal association. Therefore, region-specific manipulations of NMDAR surface trafficking can impact particular cognitive functions. These deleterious effects of blocking NMDAR surface diffusion allude to the possible consequences of NMDAR surface trafficking dysregulations.

ii. Regulators of NMDAR surface trafficking

NMDAR surface trafficking is developmentally regulated and dependent on NMDAR subunit composition. Given the importance of quickly and finely adjusting NMDAR synaptic content and surface distribution for neuronal functions, NMDAR surface diffusion is likely a highly controlled form of trafficking, of which the mechanisms of regulation have begun to emerge in the last decade.

Stabilization through direct physical interactions

Several membrane and intracellular proteins regulate NMDAR surface trafficking through direct interactions. The stabilization of diffusive receptors within a specific surface compartment depends highly on the receptors' affinity to locally available molecular partners which physically interact with them and peg them in place. For example, synaptic retention of receptors is largely owed to their binding to intracellular scaffolds enriched at the PSD, such as MAGUKs (Bard *et al.*, 2010; L Bard and Groc, 2011). Trans-synaptic columns created by the binding of pre- and postsynaptic elements, such as EphB2R-EphB2, also play a relevant role in the synaptic anchoring of NMDAR (Dalva *et al.*, 2000; Mikasova *et al.*, 2012). Even other neurotransmitter receptors are capable of stabilizing surface NMDAR, as is the case of D1R (Ladepêche *et al.*, 2013). Indeed, D1R activation regulates NMDAR-D1R interactions, rendering NMDAR surface trafficking sensitive to dopaminergic neurotransmission. For more details on these interactions, see Chapter I.A.4.c.

Post-translational modifications

Nevertheless, the impact of an interactor on NMDAR surface trafficking may not be restricted to diffusional confinement. For example, CaMKII can influence NMDAR diffusion through its kinase activity (Dupuis *et al.*, 2014). It has been reported that during LTP, there is an increase in GluN2B surface trafficking which is dependent on GluN2B-CaMKII interactions (see Chapter I.A.4.c. and Chapter I.A.4.e.i). This effect is prevented by inactivation of CaMKII or CK2, but not PKA or PKC. Interestingly, CaMKII inhibition also significantly decreased GluN2B lateral diffusion in basal conditions, resulting in a considerable impairment of GluN2B-NMDAR mobility. However, inhibition of CK2 does not impact basal GluN2B-NMDAR lateral diffusion. Therefore, NMDAR surface trafficking is modulated through post-translational modifications such as specific phosphorylation states.

Extracellular matrix proteins

NMDAR can be regulated by extracellular matrix proteins. For instance, reelin is a secreted glycoprotein which impacts GluN2B surface trafficking (Groc *et al.*, 2007). The expression pattern of reelin is developmentally regulated, and during maturation a synaptic enrichment of reelin occurs concomitantly with the decrease of synaptic GluN2B levels. Reelin overexpression increases GluN2B surface diffusion, decreasing GluN2B synaptic levels and thus decreasing the contribution of GluN2B-NMDAR for synaptic transmission, in a mechanism that is dependent on the activity of integrin- β 1. Conversely, reelin inhibition decreases GluN2B surface diffusion. Matrix metalloproteins (MMPs) are endopeptidases which cleave the

extracellular matrix. MMP-9 increases NMDAR surface mobility, also in an integrin- β 1 dependent fashion (Michaluk *et al.*, 2009). Inactivation of MMP-9 and integrin- β 1 prevents this effect.

Diffusible molecules

Additionally, receptor surface trafficking can be modulated by diffusible molecules at the extracellular space. Hormones, for instance, can have an impact on NMDAR lateral diffusion. The sex hormone 17 β -estradiol E2 (E2) is a strong physiological synaptic potentiator, which greatly affects cognitive functions (Smith, Vedder and McMahon, 2009; Luine and Frankfurt, 2020). Potier and colleagues observed that acute E2 application to cultured neurons stabilized synaptic GluN2B-containing NMDAR (Potier *et al.*, 2015). The authors reported that blocking NMDAR lateral diffusion using an antibody cross-linking protocol precludes E2-induced increase in spine density *in vitro* and E2-induced synaptic potentiation and enhancement of temporal associative memory *in vivo*. Stress hormones, such as corticosteroids, also modulate NMDAR surface trafficking (Mikasova *et al.*, 2017). Corticosterone anchors GluN2B-NMDAR within synapses. Interestingly, cross-linking of NMDAR prevents corticosterone-induced increases in synaptic AMPAR content.

Molecules which modulate NMDAR activity, such as NMDAR co-agonists, can regulate NMDAR surface trafficking. NMDAR co-agonists, glycine and D-serine, are spatially segregated (Henneberger *et al.*, 2013). D-serine concentrations are higher at the synaptic cleft, where most NMDAR contain GluN2A, while glycine concentrations are higher outside, where most NMDAR contain GluN2B. Glycine selectively decreases GluN2A-NMDAR surface diffusion while D-serine selectively decreases GluN2B-NMDAR surface diffusion. The authors propose that this helps maintain NMDAR subtypes restricted to their membrane compartment. Importantly, there is a developmental switch on the co-agonist which gates synaptic NMDAR from glycine to D-serine during development, closely paralleling the GluN2B to GluN2A switch on synaptic NMDAR composition (Bellone and Nicoll, 2007; Le Bail *et al.*, 2015; Ferreira *et al.*, 2017). These observations suggest that co-agonists steer the spatial segregation of NMDAR subtypes through modulations of NMDAR surface trafficking. Interestingly, D-serine application leads to a conformational change at the intracellular portion of NMDAR (Ferreira *et al.*, 2017). Furthermore, D-serine decreases GluN2B interactions with PSD-95 but not SAP-102, and leads to a decrease of GluN2B synaptic content, an effect which is occluded by TAT-2B. Therefore, NMDAR-MAGUK interactions play a role in the effect of NMDAR co-agonists.

Diffusible modulators of NMDAR surface trafficking can also emerge in pathological contexts. Importantly, autoantibodies from patients suffering anti-NMDAR encephalitis (an autoimmune

brain disorder characterized by severe psychotic episodes) bind to GluN1 NTDs and impair NMDAR surface trafficking, thereby preventing hippocampal LTP, which could explain the cognitive deficits observed in these patients (Mikasova *et al.*, 2012). Thus, impairments in NMDAR diffusion within the plane of the plasma membrane can be associated with neuropsychiatric conditions.

Chapter II – NMDAR dysfunction in pathology

NMDAR are ubiquitous in the brain and play a central role in neurodevelopment, synaptic plasticity, and maintenance of essential brain functions. Unsurprisingly, abnormal NMDAR hypo- and hyperfunction are associated with several pathologies (Zhou and Sheng, 2013b).

A. NMDAR hyperfunction in neurological disorders

NMDAR hyperactivation has been shown to cause excitotoxicity, which is one of the main ways through which NMDAR dysfunction contributes to brain disorders. Excitotoxicity is the cellular damage or death caused by excessive excitatory signalling. This can occur, for instance, when a damaged cell releases all its intracellular glutamate content into the extracellular space (Mehta *et al.*, 2013). An overabundance of glutamate can also result from impaired glutamate uptake or even reverse glutamate uptake by astrocytic glutamate transporters, as is the case in ischaemic brain injury (Rossi, Oshima and Attwell, 2000; Sattler and Rothstein, 2006). Increased glutamate levels correlate with mitochondrial damage and oxidative stress, though since these effects influence one another, it is difficult to determine which of them firstly triggers neurotoxicity in pathological conditions (Armada-Moreira *et al.*, 2020).

Glutamate-induced neurotoxicity was first serendipitously observed in retinal neurons of mice in 1957, in a study investigating the therapeutic value of glutamate for the treatment of a hereditary form of retinal dystrophy. The term excitotoxicity was later coined in 1969, and it has since been reported to be dependent on Ca^{2+} influx, mainly through NMDAR (Lucas and Newhouse, 1957; Olney, 1969; Choi, 1987; Tymianski *et al.*, 1993). NMDAR activation can either lead to neuroprotection or neurotoxicity. Initially it was supposed that NMDAR overactivation during excitotoxicity lead to intracellular calcium overload, causing the activation of calcium-dependent kinases which trigger signalling pathways resulting in neuronal damage or death (Dong, Wang and Qin, 2009). However, studies show that what determines whether NMDAR activation results in excitotoxicity or not is not the concentration of intracellular calcium, but receptor surface localization (Hardingham, Fukunaga and Bading, 2002; von Engelhardt *et al.*, 2007; Giles E Hardingham and Bading, 2010; Zhou *et al.*, 2013). Activation of synaptic NMDAR is associated with neuronal survival, while activation of extrasynaptic NMDAR is associated with neuronal death through opposing signalling pathways. Of note, there is also a possible ion-flow independent role of NMDAR in excitotoxicity (Weilinger *et al.*, 2016) (see Chapter I.A.4.a). The following paragraphs summarise the role of prominent players involved in NMDAR excitotoxic signalling (Giles E Hardingham and Bading, 2010) (Figure 12):

Synaptic NMDAR activity indirectly activates ERK1/2 (Ivanov *et al.*, 2006), which in turn phosphorylates and promotes the activity of the transcription factor CREB (Mayr and Montminy, 2001), resulting in the transcription of pro-survival genes (Giles E Hardingham and Bading, 2010). Active ERK1/2 also prevents Jacob-driven cell death signalling (Karpova *et al.*, 2013). Synaptic NMDAR activation additionally activates Akt through PI3K, which dephosphorylates and promotes the nuclear export of the pro-apoptotic/pro-death transcription factor FOXO1/FOXO3 (Papadia *et al.*, 2008; Dick and Bading, 2010).

Calcium entry through extrasynaptic NMDAR results in calpain-mediated cleavage of STEP (Xu *et al.*, 2009). Cleaved STEP activates p38, which results in the transcription of pro-apoptotic factors. Activation of extrasynaptic NMDAR also directly opposes synaptic NMDAR signalling, since it triggers the dephosphorylation and inactivation of ERK1/2 (Ivanov *et al.*, 2006) and CREB (Hardingham and Bading, 2002; Hardingham, Fukunaga and Bading, 2002) and favours the transcription of pro-death genes by Jacob (Dieterich *et al.*, 2008) and FOXO1/FOXO3 (Dick and Bading, 2010).

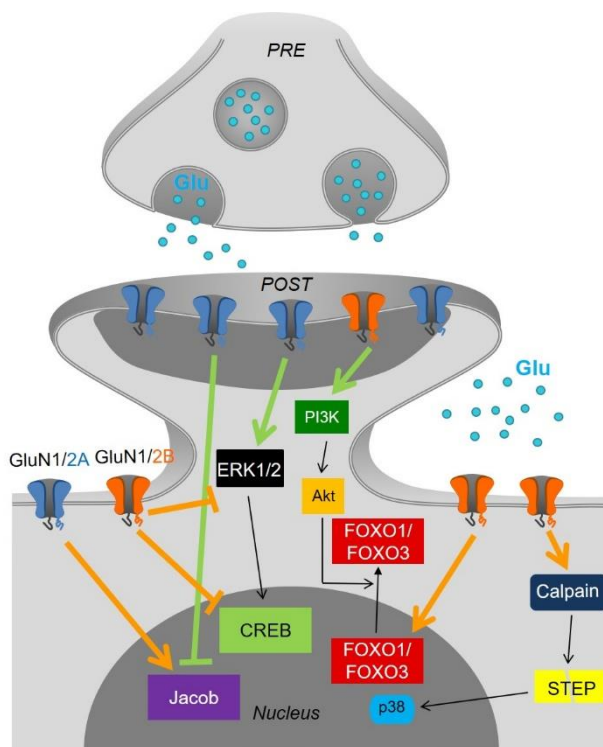


Figure 12. Synaptic and extrasynaptic NMDAR signalling. Extrasynaptic NMDAR activation (orange arrows) favours the transcription of cell death genes by regulation of Jacob and activation and nuclear translocation of FOXO1/FOXO3. Additionally, extrasynaptic NMDAR activity triggers calpain-mediated STEP cleavage, leading to the activation of p38 and pro-death signalling. Synaptic NMDAR activation (green arrows) activates ERK1/2 and favours CREB-mediated transcription of cell survival genes. Extrasynaptic NMDAR activity counteracts this, by preventing ERK1/2 and CREB activation. Finally, synaptic NMDAR activity also counteracts extrasynaptic NMDAR signalling by promoting the dephosphorylation and nuclear export of FOXO1/FOXO3, through Akt and PI3K activity.

Excitotoxicity is a component of several neurological disorders, such as ischaemic stroke and epilepsy, as well as neurodegenerative diseases, such as Parkinson's disease, Huntington's disease and Alzheimer's disease.

1. Parkinson's and Huntington's diseases

Parkinson's disease (PD) is a neurodegenerative disorder that presents a great deterioration of motor functions. PD is characterized by the loss of dopaminergic neurons from the substantia nigra pars compacta (SNc), which reduces dopaminergic drive onto the striatum, a key structure controlling voluntary movement execution (Kalia and Lang, 2015). Parkinsonian syndrome, i.e. the set of motor impairments arising in PD, is characterized by tremors, bradykinesia, limb rigidity and gait and balance problems (Sveinbjornsdottir, 2016). Dementia and depression are common in late phases of the disease. To model PD in animals, MPTP or 6-OHDA injection into the median forebrain bundle is a commonly used method to selectively kill SNc dopaminergic neurons, resulting in dopamine-denervation of the striatum (Blum *et al.*, 2001). It has been proposed that SNc dopaminergic neurons are more susceptible to excitotoxic insults due to high metabolic demands (Greenamyre and Hastings, 2004). NMDAR antagonists have indeed been reported to act as neuroprotectants in PD animal models (Greenamyre and O'brien, 1991; Ferro *et al.*, 2007; Majláth and Vécsei, 2014; Vanle *et al.*, 2018). Besides this, NMDAR antagonists also have the potential to alleviate PD non-motor symptoms (Vanle *et al.*, 2018). 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 and Calabresi, 2012), suggesting that an abnormal redistribution of NMDAR occurs during the emergence of the pathology. Indeed, in striata lacking dopaminergic innervation, synaptic PSD95, GluN1 and GluN2B levels are reduced, as well as GluN2B association with SAP102 and SAP97 (Picconi *et al.*, 2004; Bagetta *et al.*, 2010; Johnson, Conn and Niswender, 2012). Additionally, CaMKII autophosphorylation and CaMKII-mediated NMDAR phosphorylation is increased in a PD animal model, and both L-DOPA and intrastriatal CaMKII inhibition rescued deficits in motor skills and in NMDAR-dependent LTP (Picconi *et al.*, 2004). Levodopa (L-DOPA) administration is used as means of achieving a generalized increase in brain dopamine concentrations, and is the most efficient therapy for PD. However, L-DOPA treatment often induces dyskinesia through mechanisms which are still unclear (Angela Cenci, 2014). In 6-OHDA-treated animals presenting L-DOPA-induced dyskinesia (LID), the GluN2A-/GluN2B-NMDAR synaptic ratio is even more increased than animals which don't present this side effect (Gardoni *et al.*, 2006, 2012), and lowering the GluN2A-/GluN2B-NMDAR ratio with using biomimetic peptides (TAT-2A) reduces the prevalence of LID, while increasing it has the opposite effect. Therefore, the surface distribution of NMDAR is a determinant factor for the result of long-term dopamine therapy in PD.

Huntington's disease (HD) is also a neurodegenerative disorder that is characterized by a loss

of movement control. HD is caused by an autosomal dominant mutation in the gene coding for the Huntingtin protein, which is expressed by all cells, and, in neurons, plays an important part in mediating intracellular signalling cascades, transport of vesicles along neurites and synaptic neurotransmission (Cattaneo, Zuccato and Tartari, 2005). This mutation leads to an excessive number of glutamine residues at the polyglutamine tract of the Huntingtin protein, which causes it to misfold and form aggregates (Dayalu and Albin, 2015). Mutant huntingtin (mHTT) primarily affects medium spiny neurons, which make up more than 95% of all neurons in the striatum (Yager *et al.*, 2015). At the prodromal stage of HD, patients may exhibit altered personality and slight cognitive and motor deficits (Dayalu and Albin, 2015). HD patients eventually begin displaying uncontrolled and uncoordinated movements, in what is termed Huntington's chorea. As the disease progresses, cognitive abilities deteriorate and motor symptoms intensify, leading to rigidity and abnormal posturing. Several lines of research indicate that HD involves NMDAR-dependent excitotoxicity processes (Fan and Raymond, 2007; Carvajal, Mattison and Cerpa, 2016). In the striatum of animals expressing mHTT, NMDAR subunits GluN1, GluN2A and GluN2B are enriched at extrasynaptic sites, and extrasynaptic NMDAR activity is augmented, while CREB activation is accordingly decreased (Milnerwood *et al.*, 2010). In fact, the use of low concentrations of memantine to, in theory, specifically block extrasynaptic NMDAR rescues CREB signalling, and attenuates striatal atrophy and motor deficits in animal models of HD (Cummings *et al.*, 2007; Okamoto *et al.*, 2009; Milnerwood *et al.*, 2010). Furthermore, synaptic NMDAR activity induces mHTT inclusion formation, a neuroprotective strategy in HD (Arrasate *et al.*, 2004; Okamoto *et al.*, 2009). Conversely, activation of extrasynaptic NMDAR promotes neuronal death in neurons expressing mHTT not only by inhibiting CREB, but also by activating Rhes, which promotes the disaggregation of mHTT (Okamoto *et al.*, 2009).

2. Alzheimer's disease

Alzheimer's disease (AD) is a progressive form of dementia associated with ageing. At the prodromal phase of the disease, those affected by it display mild cognitive impairments, and, as the disease progresses, learning and memory functions deteriorate until patients become fully dependent on caregivers (Förstl and Kurz, 1999). At late phases of the disease, AD leads to behavioural and neuropsychiatric changes and complete loss of speech and mobility. AD is characterized by the abnormal build-up of amyloid beta (A β) peptide and hyperphosphorylated tau protein in the brain. While the heritability of AD is estimated at 49-79%, familial forms of AD caused by autosomal dominant mutations constitute only around 0.1% of total cases (Blennow, de Leon and Zetterberg, 2006; Wilson *et al.*, 2011). Several studies report decreased function and expression levels of glutamate transporters (particularly vesicular

glutamate transporter (VGluT) and excitatory amino acid transporter 2 (EEAT2)) in human AD samples (Masliah *et al.*, 1996; Li *et al.*, 1997; Kirvell, Esiri and Francis, 2006; Scott *et al.*, 2011; Wang and Reddy, 2017). As a consequence, impaired glutamate uptake/recycling mechanisms increase glutamate availability and contribute to excitotoxicity in AD, possibly through the action of pathological forms of amyloid β ($A\beta$) which induce astrocytic glutamate release and activate extrasynaptic NMDAR (Arias, Arrieta and Tapia, 1995; Parpura-Gill, Beitz and Uemura, 1997; Fernández-Tomé *et al.*, 2004; Wang and Reddy, 2017). Therefore, there is a rationale for the therapeutic use of NMDAR antagonists in AD. As such, the NMDAR antagonist memantine has been approved and prescribed as AD therapy. A recent meta-analysis revealed that memantine indeed slightly improves cognition in moderate to severe AD (Mcshane *et al.*, 2019). Moreover, Talantova and colleagues report that $A\beta$ induces astrocytic glutamate release and leads to extrasynaptic NMDAR activation and synapse loss, which is prevented by memantine (Talantova *et al.*, 2013). Consistently, a multitude of studies report $A\beta$ -induced rise in NMDAR currents which can either be mitigated or fully prevented by NMDAR antagonists (Le *et al.*, 1995; Kamenetz *et al.*, 2003; Ye *et al.*, 2004; Domingues *et al.*, 2007; Kawamoto *et al.*, 2008; Alberdi *et al.*, 2010; Texidó *et al.*, 2011; Ferreira *et al.*, 2012; Wang and Reddy, 2017). This is associated with early-stage AD, and is postulated to ultimately lead to NMDAR desensitization and internalization (Palop and Mucke, 2010; Liu *et al.*, 2019). Supporting this, Snyder and colleagues report that $A\beta$ peptides promote dephosphorylation of GluN2B at Y¹⁴⁷² and subsequent endocytosis, leading to a reduction of NMDAR currents (Snyder *et al.*, 2005). $A\beta$ additionally contributes to synaptic dysfunction by downregulating PSD-95 and synaptophysin, an effect which is also prevented by the NMDAR antagonists (Liu *et al.*, 2010; Röncke *et al.*, 2011). Interestingly, preventing ligand binding to the NMDAR - without necessarily blocking the receptor - precludes $A\beta$ -induced synaptic depression and synapse loss, indicating a possible non-ionotropic role of the NMDAR in AD (Birnbbaum *et al.*, 2015; Stein, Gray and Zito, 2015) (see Chapter I.A.4.a).

3. Epilepsy

Epilepsy is a neurological condition characterized by recurring seizures. Seizures present as an absence of awareness, conscience or movement control (such as uncontrolled shaking), caused by abnormally excessive or synchronous neuronal activity (Figure 13). In epilepsy, many factors, which can be genetic, structural (e.g. stroke, traumatic brain injury), infectious, metabolic, immune or (in around 50% of the cases) unknown, increase susceptibility to seizures (WHO, 2017). The process of developing epilepsy as a result from a primary insult, such as brain injury, is termed epileptogenesis. Seizure generation in patients with epilepsy is termed ictogenesis. Ictogenesis stems from dysfunctional brain circuitry, which creates

feedback loops that cause neurons to fire intensely and in tandem. The mechanisms of ictogenesis can be examined at different levels: the level of ion gradients across the plasma membrane, the cellular level, and the circuits level. Ionic imbalance caused by alterations in ion pumps and channels, including ionotropic neurotransmitter receptors, can depolarize the neuronal membrane, resulting in neuronal hyperexcitability (Meisler *et al.*, 2001). Increased glutamatergic drive or decreased GABAergic drive can result in circuit hyperexcitability through overexcitation or disinhibition, respectively (Cobb *et al.*, 1995)(Figure 13). Epilepsy is thus considered to stem from an excitation/inhibition imbalance. Finally, circuits can adapt to seizure-associated activity through axonal sprouting and synaptic plasticity, further sustaining seizure generation (Sutula and Dudek, 2007)(Figure 13). Conversely, seizures may also lead to neuronal loss (Thom, 2014)(Figure 13). These adaptations may enable the self-perpetuation of seizure activity, resulting in prolonged seizures. A seizure that lasts longer than 5 minutes is termed status epilepticus (SE) and constitutes a medical emergency.

NMDAR mutations can be found in cases of childhood-onset epilepsy (see also Chapter II.B.1), and NMDAR hyperfunction underlies some forms of monogenic epilepsy, in what is described as NMDA-pathy (Burnashev and Szepietowski, 2015; Gataullina *et al.*, 2019). However, the role of NMDAR in epilepsy is not clear-cut. Mutations of NMDAR resulting in NMDAR gain- or loss-of-function have both been found to be associated with epilepsy (Xu and Luo, 2018). Moreover, cerebrospinal fluid from patients with epilepsy have high levels of glutamate (Stover *et al.*, 1997), and increased glutamate tone is associated with seizure generation, which would point to excitotoxicity in ictogenesis (Stover *et al.*, 1997; Davis *et al.*, 2015; Çavuş *et al.*, 2016; Hanada, 2020). Application of NMDA induces acute seizures without triggering epileptogenesis (Velíšek *et al.*, 2007). While the implication of NMDAR in epilepsy is still under scrutiny, NMDAR antagonists which block the receptor at the glutamate binding site or at the ion pore act as anticonvulsants and delay epileptogenesis in animal models of epilepsy (Löscher, Nolting and Hönack, 1988; Bertram and Lothman, 1990; Löscher and Brandt, 2010; Ghasemi and Schachter, 2011; Hanada, 2020). An NMDAR glycine site antagonist and two partial NMDAR agonists also show anticonvulsive properties (Rundfeldt, Wlaż and Löscher, 1994).

NMDAR play a role in the aetiology of status epilepticus. The subunit composition of neurotransmitter receptors is altered in response to SE, resulting in a profile that resembles immature developmental stages (increased ratio of non- $\alpha 1/\alpha 1$ in GABAA receptors, GluA1/GluA2 in AMPA receptors, and decreased GluN2A/GluN2B ratio in NMDA receptors) (Loddenkemper *et al.*, 2014). While NMDAR antagonists amantadine and ketamine themselves do not ameliorate SE in animal models (Martin and Kapur, 2008; H. and C., 2018;

Mohammad *et al.*, 2019), ketamine has a synergistic effect with benzodiazepines in treating SE (Martin and Kapur, 2008; Niquet *et al.*, 2017). Furthermore, due to several clinical case reports, a medical consensus has recently been reached that ketamine could be used as a last resort treatment for super-refractory SE (Gomes *et al.*, 2018; Kapur, 2018). Finally, around 80% of patients suffering from anti-NMDAR encephalitis develop seizures through unknown mechanisms, which could constitute a form of autoimmune epilepsy resulting from alterations to NMDAR functions (Dalmau *et al.*, 2007, 2008; Liu *et al.*, 2017; Dalmau and Graus, 2018).

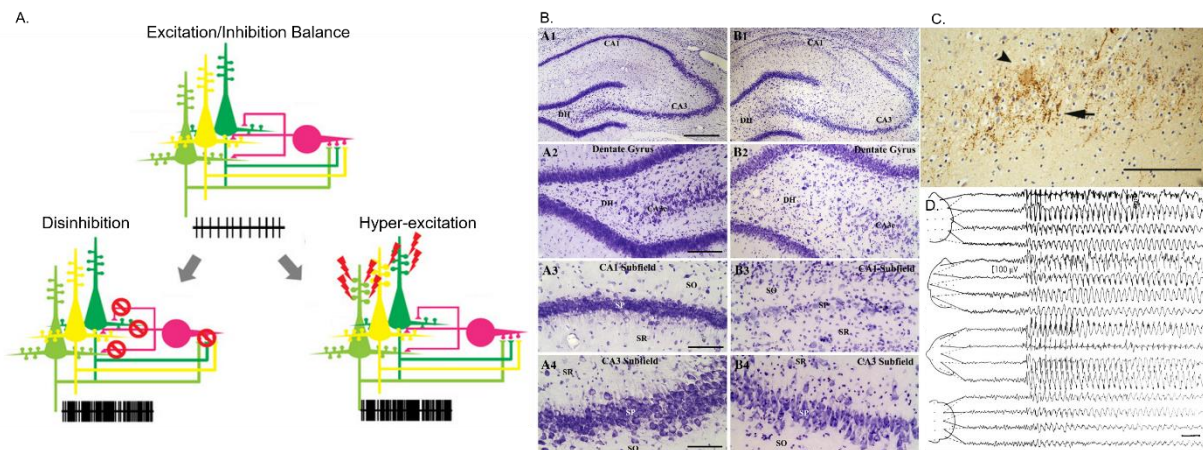


Figure 13. Neurological features of epilepsy. A. Physiological and pathological N-methyl-D-aspartate (NMDA) receptor function in epilepsy. Top: Physiological interaction between excitatory and inhibitory neurons. Lower left: Excitatory input to the inhibitory neuron is diminished by hypofunction of NMDA receptors; silencing of inhibitory interneurons results in an increase in the firing of excitatory neuron. Lower right: NMDAR hyperfunction could enhance neuronal excitation causing hyper-excitation of excitatory neurons. Green and yellow: excitatory/glutamatergic neurons, purple: inhibitory/GABAergic neurons, Black traces, indication of neuronal firing rate (Hanada, 2020). B. Cytoarchitecture of the hippocampus in an intact control rat (A1) and a chronically epileptic rat (B1). A2–4 and B2–4 are micrographs of hippocampal dentate gyrus (2), CA1 (3) and CA3 (4) regions. Note the considerable neuronal loss in the hippocampus of the epileptic rat. Scale bar panels (1)=500 μm , (2)=100 μm ; (3 and 4)=50 μm (Rao *et al.*, 2007). C. Complex parvalbumin terminals (brown) are seen surrounding somas (nucleus in blue) in the dentate gyrus granule cell layer in hippocampal sclerosis associated with medial temporal lobe epilepsy, indicative of maladaptive neuronal plasticity. Scale bar=75 μm (Thom, 2014); D. EEG recording of an absence seizure, which is characterized by brief lapses of consciousness. Note the increased and synchronous electrical brain activity. Left: electrode placement. Scale bar =1 second (Smith, 2005).

4. Ischaemic Stroke

Acute brain injury (i.e. traumatic brain injury or cerebrovascular injury) constitutes an excitotoxic insult. In traumatic brain injury (TBI), a primary lesion is caused by external force, causing secondary inflammation, oxidative stress and excitotoxicity (Davis, 2000). In cerebrovascular injury (stroke), internal bleeding (haemorrhagic stroke) or lack of blood supply (ischaemic stroke) leads to metabolic imbalances resulting in cell death through those same processes (Deb, Sharma and Hassan, 2010). Around 80% of strokes are ischaemic (Della-Morte *et al.*, 2012). In ischaemic stroke (IS), lack of oxygen and glucose supply leads to energetic deficits, resulting in the depletion of ATP. Without ATP, active transporters are unable to maintain transmembrane ion gradients, and astrocytic glutamate transporters start to reverse glutamate uptake. Neurotoxic glutamate levels in ischaemic stroke are mainly caused by reverse uptake by astrocytes (Rossi, Oshima and Attwell, 2000). In around 50-70% of IS cases, blood flow into the damaged area is spontaneously restored (Baird *et al.*, 1994).

This substantially intensifies oxidative stress, causing a secondary reperfusion injury (Warach and Latour, 2004; Lin, Wang and Yu, 2016).

NMDAR are central to IS pathological processes (Simon *et al.*, 1984; Lipton, 2006; Y. Sun *et al.*, 2018). However, NMDAR antagonists have failed to pass clinical trials to improve IS outcomes, with several likely explanations (Albers *et al.*, 1995, 2001; Davis *et al.*, 2000; Sacco *et al.*, 2001; Ikonomidou and Turski, 2002; Saver *et al.*, 2015; Rajah and Ding, 2017). First, NMDAR antagonists produce serious secondary effects, which severely restrict their clinical use (Krystal *et al.*, 1994). Secondly, NMDAR activity not only mediates neurotoxic, but also neuroprotective signalling, which may be necessary for recovery after IS. In the future, will it become possible to reduce NMDAR-mediated excitotoxicity without blocking NMDAR ionotropic functions? Interestingly, the NMDAR co-agonist glycine acts as a neuroprotectant and decreases the volume of infarct caused by middle cerebral artery occlusion (MCAO) - a common strategy to induce focal cerebral ischemic and ischemic-reperfusion injury in order to model IS - through modulations of ion flow-independent NMDAR signalling (See Chapter I.A.4.a) (Li *et al.*, 2016; R. Hu *et al.*, 2016; J. Chen *et al.*, 2017). Thus, development of innovative IS treatments may involve targeting non-ionotropic NMDAR functions, such as receptor interactions with neuronal death-promoting signalling partners. For instance, nNOS controls the production of nitric oxide (NO) which mediates oxidative stress. This enzyme is activated by calcium, and is abnormally relocated near calcium-permeable NMDAR ion pores in IS through the assembly of a GluN2B-PSD95-nNOS complexes (Sattler *et al.*, 1999; Girouard *et al.*, 2009; Zhou *et al.*, 2010). Both releasing GluN2B CTDs from MAGUKs (including PSD-95) and releasing nNOS from PSD-95 have been shown to prevent NMDAR-mediated excitotoxicity and ameliorate focal cerebral ischemic damage following MCAO without affecting basal NMDAR activity (Aarts *et al.*, 2002; Zhou *et al.*, 2010). Additionally, IS promotes the interaction between GluN2B CTD and death-associated protein kinase 1 (DAPK1) and thereby enhances NMDAR conductance (Tu *et al.*, 2010). Disrupting this interaction protects neurons against excitotoxicity and significantly reduces the volume of MCAO-induced infarction. NMDAR interactions involved in IS are now being heavily explored. Other interactions at the GluN2B CTD, namely with CaMKII and AP2, have been found to be necessary for neurotoxicity by oxygen and glucose deprivation (OGD) *in vitro* (Vieira *et al.*, 2016). Additionally, a recent study explored the role of an unconventional NMDAR partner in IS by reporting that interactions between NMDAR and $\alpha 2\delta$ -1 - a subunit of voltage gated calcium channels (VGCCs) - are enhanced by OGD and are essential for ischemia-induced NMDAR hyperactivity and neurological deficits (Luo *et al.*, 2018).

B. NMDAR hypofunction in neuropsychiatric disorders

1. Autism and Intellectual disability

Alterations in NMDAR function are also associated to neurodevelopmental disorders, such as autism and intellectual disability. Autism is characterised by deficits in social interaction, communication and repetitive and restricted patterns of behaviour, interests or activities, which present within the first years of infancy (First, 2013; Lai, Lombardo and Baron-Cohen, 2014; Lord *et al.*, 2018). Due to high heterogeneity in symptom presentation and severity, autism is considered as a spectrum of disorders. The aetiology of autism spectrum disorders (ASDs, also known as pervasive developmental disorders) has a strong genetic component. Indeed, 74–93% of ASD risk is heritable, although monogenic ASDs are rare (only around 5% of ASD cases) (Sztainberg and Zoghbi, 2016; Tick *et al.*, 2016). A high proportion of the genetic mutations or deletions associated with ASD affect genes encoding for synaptic proteins and cause impairments in synaptic structure and function, which is why ASDs are considered as synaptopathies (Bagni and Zukin, 2019). These include genes encoding for the cell adhesion proteins neuroligins, postsynaptic density proteins SHANKs, the actin skeleton adaptor protein IRSp53, and the transcription repressor MeCP2 (mutations in MeCP2 cause Rett syndrome, which is within the autism spectrum) (Lee, Choi and Kim, 2015). In general, ASDs are associated with a low density of glutamatergic spines and with spine morphology indicative of an immature state, although this is highly dependent on the brain region and animal model examined (Martínez-Cerdeño, 2017). Impairments in NMDAR function have been reported in several models of ASD (Lee, Choi and Kim, 2015). Particularly, ASD models involving neuroligin-1 KO or Shank 2 deletions are associated with NMDAR hypofunction, while IRSp53 KO is associated with NMDAR hyperfunction, and MeCP2 KO has been reported to accelerate the GluN2B to GluN2A developmental switch (Lee, Choi and Kim, 2015; Katz, Menniti and Mather, 2016). NMDAR modulators have been found to ameliorate autism-like behavioural impairments according to the NMDAR dysfunction at hand. Namely, D-cycloserine has been reported to rescue grooming or sociability in animals with neuroligin-1 KO and Shank 2 deletions (exons 6 and 7) respectively, while memantine improves sociability in IRSp53 KO animals and ketamine can rescue Rett syndrome phenotype in MeCP2 KO animals (Lee, Choi and Kim, 2015; Patrizi *et al.*, 2016). At the same time, the NMDAR co-agonist D-cycloserine and the NMDAR antagonists memantine and amantadine have all been reported to ameliorate symptoms of patients with ASDs (Posey *et al.*, 2004; Hosenbocus and Chahal, 2013a, 2013b; Urbano *et al.*, 2014; Lee, Choi and Kim, 2015).

Intellectual disability (ID), previously termed mental retardation, is a frequent co-morbidity of ASDs. ID is defined by an intellectual quotient under 70 plus deficits in adaptive functioning

that affect daily life (First, 2013). About a quarter to half of ID have a genetic cause (Srouf and Shevell, 2014). For instance, ID is a feature of Rett syndrome. Individuals affected by genetic syndromes causing ID also often present autism. Animal models of ID include mutation or deletion of the RNA-binding protein FMRP (mutations in FMRP can cause Fragile X syndrome) or of the synaptic Ras GTPase-activating protein SYNGAP1 (mutations in SYNGAP1 cause SYNGAP1-associated intellectual disability) (Verma *et al.*, 2019). Like ASD, ID can be associated with synaptic alterations. For example, fragile X syndrome is associated with a high spine density comprised mostly of immature filopodia-like spines, while Down syndrome is associated with a low density of large spines (Levenga and Willemsen, 2012). At the molecular level, Fragile X syndrome is associated with NMDAR hypofunction, reduced levels of NMDAR subunits, impaired surface trafficking and synaptic function of mGluR5 and NMDAR, high AMPAR/NMDAR currents ratio, and impairments in NMDAR-mediated synaptic plasticity at the dentate gyrus (Bostrom *et al.*, 2015; Yau *et al.*, 2016, 2019; Aloisi *et al.*, 2017). Synaptic plasticity impairments can be rescued by glycine or D-serine application, or ameliorated by selective inhibition of GluN2A-NMDAR (Bostrom *et al.*, 2015; Lundbye, Toft and Banke, 2018). SYNGAP1 mutations are linked to low AMPAR/NMDAR currents ratio (Clement *et al.*, 2012; Verma *et al.*, 2019). SynGAP binds to PSD-95 and is part of the NMDAR signalosome, coupling NMDAR-mediated calcium entry to MAPK activation, ultimately impacting ERK activity (Kim *et al.*, 1998; Iida *et al.*, 2001; Kennedy *et al.*, 2005). Therefore, altered NMDAR signalling is a feature of SYNGAP1-associated intellectual disability.

NMDAR dysfunction associated with neurodevelopmental disorders can arise directly from mutations in NMDAR. Mutations to NMDAR subunits are highly associated with autism, childhood-onset epilepsy and ID, with a high co-morbidity of this triad of neurodevelopmental impairments (Zafeiriou, Ververi and Vargiami, 2007; Burnashev and Szepetowski, 2015; Amin, Moody and Wollmuth, 2020)(Figure 14). Genetic syndromes caused by GRIN2B (GluN2B-encoding gene) mutations induce developmental delay and ID (Platzer and Lemke, 1993). Epilepsy, ASD and muscle tone anomalies are also common in GRIN2B-related neurodevelopmental disorders. GRIN1 mutations leading to loss-of-function result in severe ID, movement disorder, and cortical visual impairment (Lemke *et al.*, 2016). GRIN2A mutations have been highly associated with epilepsy and aphasia⁵, while being more weakly associated with developmental delay, ID and ASD than GRIN2B mutations (Endele *et al.*, 2010; Burnashev and Szepetowski, 2015; Myers *et al.*, 2019; Strehlow *et al.*, 2019). However, the effect of these mutations on NMDAR function is variable, and often loss-of-function and gain-of-function mutations are associated with the same clinical phenotype (Burnashev and

⁵ Aphasia is an impairment of language skills due to damage to specific brain regions

Szepetowski, 2015; Xu and Luo, 2018). In *GRIN2A*, mutations in TMD are commonly gain-of-function and associated with more severe phenotypes (Strehlow *et al.*, 2019). Mutations can also affect NMDAR trafficking, as is the case of GluN1^{G620R}, a *de novo* mutation found in individuals with developmental delay and ID which significantly decreases GluN1^{G620R}-GluN2B-NMDAR surface expression (W. Chen *et al.*, 2017). The efficiency of NMDAR antagonists on the treatment of neurodevelopmental disorders originating from *GRIN* mutations depends on how these mutations affect NMDAR structure and function. For instance, Fedele and colleagues report that two GluN2B mutations associated with West syndrome⁶, GluN2B^{V618G} and GluN2B^{N615I}, result in loss of magnesium NMDAR blockade (Fedele *et al.*, 2018). However, due to the structural rearrangements of the ion pore caused by these mutations, memantine has reduced binding to GluN1–GluN2B^{V618G}-NMDAR and increased binding to GluN2B^{N615I}-NMDAR.

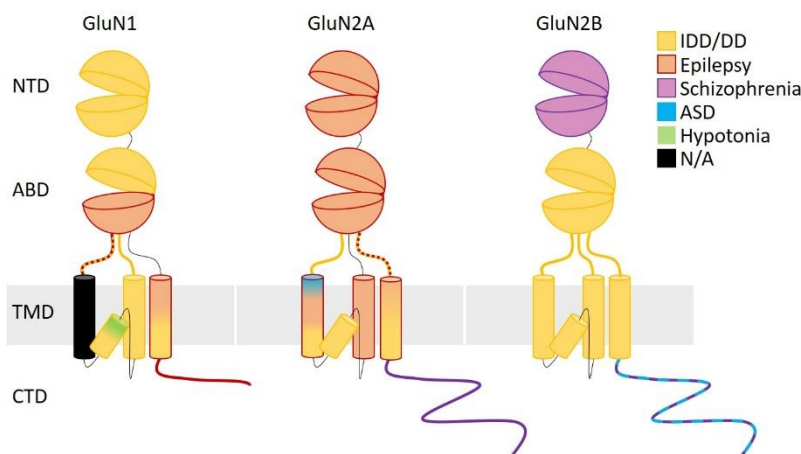


Figure 14. NMDAR mutations in neurodevelopmental disorders. Main phenotypes associated with mutations in the different domains of NMDAR subunits, as reviewed by Amin, Moody and Wollmuth (Amin, Moody and Wollmuth, 2020). Double- and triple-coloured structures indicate the same prevalence of different phenotypes. Note the high occurrence of intellectual disability, epilepsy, schizophrenia and autism spectrum disorders associated with NMDAR mutations. IDD/DD, intellectual disability and developmental delay; ASD, autism spectrum disorders; N/A, non-applicable.

In conclusion, neurodevelopmental disorders can associate with NMDAR hypo- and hyperfunction. This and possible structural alterations caused by mutations in NMDAR subunits determine which pharmacological tools could best modulate and normalize NMDAR function in these disorders.

2. Depression

Major depressive disorder (MDD) is a common mental disorder affecting around 2% of the world's population (James *et al.*, 2018). MDD is characterized as a low mood and/or loss of interest or pleasure in activities for at least two weeks (First, 2013). Cognitive impairments, though not central for diagnosis, are also a feature of depression (Rock *et al.*, 2014). Risk factors for MDD include childhood trauma, stress, and family history of depression (Hammen,

⁶ West syndrome is an epileptic disorder characterized by infantile spasms and developmental regression, associated with a distinctive electroencephalography pattern in the period between seizures.

2018). The heritability of MDD is around 40%, and genetic risk for MDD reflects the cumulative effects of many low-penetrance genetic variants (Sullivan, Neale and Kendler, 2000; Sullivan *et al.*, 2013). By tracking the recovery of untreated patients, the median time for a depressive episode in MDD was found to be three months (Posternak *et al.*, 2006). However, for 15% of untreated patients, this took over a year. Depression increases the risk of death by suicide by about 15%, and is estimated to be responsible for around 60% of deaths by suicide; which is a projected number of around 500 000 globally each year (Kessler and Bromet, 2013; Turecki and Brent, 2016; Ng, How and Ng, 2017). As 30-40% of MDD cases are treatment-resistant, it is imperative to understand the pathological mechanisms occurring in MDD and design therapies which allow to treat depressive states and combat suicidal ideation (Rush *et al.*, 2006; McIntyre *et al.*, 2014).

Several biochemical and neurological alterations occur in depression. From a neuroanatomical point of view, MDD is associated with decreased volume of the hippocampus, amygdala and cingulate cortex, and altered functional connectivity between those structures (Sheline *et al.*, 1996, 2013; Vakili *et al.*, 2000; Bell-McGinty *et al.*, 2002; Koolschijn *et al.*, 2009; Bora *et al.*, 2012; De Kwaasteniet *et al.*, 2013; Zhao *et al.*, 2017; Gray *et al.*, 2020). Additionally, dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis regulating glucocorticoid⁷ production leads to elevated cortisol levels in depression. This increased cortisol production in response to stress can induce neuronal death, inhibit neurogenesis, and thus play a role in decreasing hippocampal volume (Sapolsky, 1996, 2000; Sapolsky, Romero and Munck, 2000; Pariante, 2003; Abrous, Koehl and Le Moal, 2005). Several lines of evidence also point to the role of pro-inflammatory cytokine processes in depression, including the effectiveness of anti-inflammatory treatment in ameliorating symptoms of depression (Elenkov *et al.*, 2005; Köhler *et al.*, 2014). Most importantly, depression was historically associated with decreased monoaminergic neurotransmission, due to the serendipitous observation that drugs which increase monoamine levels act as antidepressants (Krishnan and Nestler, 2008). However, while certain antidepressants have an immediate impact on monoamine levels (via blocking monoamine uptake or preventing monoamine degradation), their antidepressant action takes weeks to occur (David *et al.*, 2003). Furthermore, acutely decreasing monoamine levels in healthy individuals does not affect their mood (Ruhé, Mason and Schene, 2007). Therefore, a deficit in monoaminergic transmission does not explain all aspects of depression.

The most commonly used animal models for depression are based on acute or chronic stress

⁷ Glucocorticoids (colloquially dubbed stress hormones) are steroid hormones that are produced by the adrenal gland. In a physiological context, glucocorticoids have anti-inflammatory and immunosuppressive actions. However, exaggerated glucocorticoid levels can lead to cell death (Pariante, 2003). The most common glucocorticoid in humans is cortisol, and in rats and mice is corticosterone.

exposure, though animal models for depression can also be attained through exogenous administration of glucocorticoids, selective breeding of animals with a depressive phenotype, or manipulations of genes controlling monoamine levels (Wang *et al.*, 2017). Stress leads to dendritic atrophy, decreased spine number, and impairments in synaptic plasticity (Gorman and Docherty, 2010). The actions of antidepressants targeting monoaminergic systems were later associated with increased neurogenesis and neurotrophic signalling, namely increased BDNF levels at limbic structures (Duman and Monteggia, 2006; Warner-Schmidt and Duman, 2006; Krishnan and Nestler, 2008; Racagni and Popoli, 2008; David *et al.*, 2009). This contributed to the conception of a neuroplasticity-based theory of depression (Krishnan and Nestler, 2008). Stress also highly impacts iGluR expression and trafficking. The stress hormone corticosterone increases AMPAR lateral diffusion and potentiates synaptic AMPAR content, blocking further synaptic potentiation (Groc, Choquet and Chaouloff, 2008). Corticosterone also increases NMDAR currents and trapping of GluN2B-NMDAR at synapses, and lateral diffusion-based NMDAR surface redistribution is necessary for corticosterone-induced increases in synaptic AMPAR content (Mikasova *et al.*, 2017). While acute stress potentiates glutamatergic synapses and transiently increases the surface expression of AMPAR and NMDAR *in vivo* (which is reflected by the previously described effects of corticosterone *in vitro*), chronic stress decreases AMPAR and NMDAR levels by enhancing their degradation via the UPS (Gourley *et al.*, 2009; Yuen *et al.*, 2009, 2012). Therefore, glutamatergic neurotransmission is modulated by stress, which contributes to the pathogenesis of depression.

As such, targeting glutamatergic signalling proved to be an efficient strategy to relieve the symptoms of depression. As an example, modulators NMDAR such as D-cycloserine can act as efficient antidepressants (Newport *et al.*, 2015; Hashimoto, 2019). Additionally, it has been reported since 1990 that NMDAR antagonists have antidepressant properties, and can rescue stress-induced impairments in synaptic plasticity (Trullas and Skolnick, 1990). The most effective antidepressant out of the NMDAR antagonists is ketamine (Newport *et al.*, 2015; Kishimoto *et al.*, 2016). A single low (subanaesthetic) dose of ketamine has been found to have a fast antidepressant effect (Berman *et al.*, 2000; Diazgranados *et al.*, 2010). Ketamine can increase ambient glutamate concentrations, stimulate synaptogenesis in the medial prefrontal cortex in an AMPAR-dependent fashion, and reverse stress-induced behavioural impairments and deficits in glutamatergic neurotransmission (Moghaddam *et al.*, 1997; Li *et al.*, 2010). The mechanisms underlying this are currently being extensively studied (Hashimoto, 2019; Pham and Gardier, 2019). One hypothesis to explain these effects is that ketamine at these doses specifically blocks NMDAR at GABAergic interneurons, thus disinhibiting cortical glutamatergic neurotransmission (Miller, Moran and Hall, 2016). In fact,

ketamine impacts not only glutamatergic and GABAergic, but also serotonergic neurotransmission, as it increases extracellular 5-HT levels at the medial prefrontal cortex through an unknown AMPAR-dependent mechanism (Cryan, Markou and Lucki, 2002; Pham and Gardier, 2019). Another possibility is that the antidepressant actions of ketamine are due specifically to blockade of NMDAR-dependent bursting activity in the lateral habenula (LHb) (Yang *et al.*, 2018). Yang and colleagues report that increased bursting at the LHb induces a depressive phenotype, and that blocking LHb NMDAR either with ketamine or AP5 had an antidepressant effect. Additionally, the antidepressant actions of ketamine may not be entirely caused by the drug *per se*, but also by ketamine-derived metabolites. Zanos and colleagues reported that the ketamine metabolite (2R,6R)-HNK contributes to ketamine-induced antidepressant effects in an AMPAR-dependent, but NMDAR-independent fashion (Zanos *et al.*, 2016, 2019). At the molecular level, the antidepressant effects of ketamine have been inferred to occur through mTORC, BDNF, VEGF, GSK-3, P11, HCN1, AMPAR, opioid receptors, several micro RNAs and even the gut microbiota (for review see Hashimoto, 2019).

Although the mechanisms supporting the antidepressant action of NMDAR antagonists remain elusive, a recent meta-analysis on available therapies for treatment-resistant depression revealed that strategies targeting the NMDAR are the most successful (Strawbridge *et al.*, 2019), and the American Food and Drug Administration (FDA) recently approved the use of an (S)-ketamine nasal spray as therapy for treatment-resistant depression. However, as the antidepressant benefits of ketamine typically occur along with cognitive and psychotomimetic adverse effects (Farber, 2019), understanding and curtailing the unwanted actions of NMDAR antagonists will be an important step for their implementation as a standard therapy for treatment-resistant MDD.

3. Autoimmune brain disorders

Autoimmune disorders are defined by the targeting of endogenous epitopes by pathogenic autoantibodies. An autoantibody is considered as pathogenic if the following conditions are met: 1) the autoantibody is present during presentation of the symptoms; 2) the autoantibody targets a cell surface protein; 3) autoantibody transfer to healthy individuals or animals induces the symptoms of the autoimmune disease; 4) elimination of the autoantibody ameliorates the symptoms of the disease or prevents disease progression (Rose and Bona, 1993). These criteria are met by anti-NMDAR autoantibodies in anti-NMDAR encephalitis. The production of pathogenic anti-NMDAR autoantibodies (NMDAR-Abs) is commonly triggered by tumours, typically ovarian teratomas (Dalmau *et al.*, 2019). Other factors, such as herpes simplex encephalitis, can elicit anti-NMDAR-Abs production. However, every so often, there is no known cause for NMDAR-Ab production. During the prodromal phase of anti-NMDAR

encephalitis, symptoms resemble those of a common viral infection (Figure 15.A). In one week of time, psychiatric symptoms arise, such as delusions, hallucinations, mania, catatonia, disorganized thoughts and speech alterations, often accompanied by memory impairments and seizures. Neurological complications generally emerge a couple of weeks later, including dysautonomia and abnormal movements. In 5% of cases, patients with anti-NMDAR encephalitis present demyelination, and there are cases of comorbidity of anti-NMDAR encephalitis with other demyelinating autoimmune disorders, such as multiple sclerosis and neuromyelitis optica spectrum disorder⁸ (Kruer *et al.*, 2010; Uzawa *et al.*, 2012; Titulaer *et al.*, 2014; Alam *et al.*, 2015; Fleischmann *et al.*, 2015; Luo *et al.*, 2016). The severity of symptoms worsens for weeks to months as the disease progresses until the patient becomes comatose. Immunotherapy and, if needed, tumour removal, effectively treats around 80% of patients with anti-NMDAR encephalitis (Titulaer, Kayser and Dalmau, 2013). However, most patients will retain long lasting cognitive impairments after the incidence of the disease (Finke *et al.*, 2012).

NMDAR-Abs epitope recognition is only possible when NMDAR subunits form a receptor complex (Gleichman *et al.*, 2012). NMDAR-Abs target the GluN1 subunit in a way that is dependent on the asparagine and glycine residues at positions 368 and 369 (N³⁶⁸,G³⁶⁹), regardless of glycosylation state, located at the lower lobe of the extracellular NTD (Gleichman *et al.*, 2012). While GluN1 is ubiquitously distributed in the brain, there is an striking and unexplained preference of NMDAR-Abs for binding to the hippocampus (Dalmau *et al.*, 2007, 2008)(Figure 15). This may contribute to the symptomatology of anti-NMDAR encephalitis, namely the cognitive deficits. NMDAR-Abs preference for the hippocampus has been hypothesized to originate from a preference for a specific NMDAR subunit composition. However, this was shown not to be true for GluN1 coupling with any individual GluN2 or GluN3 subunit (Dalmau *et al.*, 2007; Gleichman *et al.*, 2012). There is still the possibility that NMDAR-Abs have a higher affinity for GluN1/2A/2B triheteromeric receptors, which are prevalent at the hippocampus (Tovar, McGinley and Westbrook, 2013). NMDAR-Abs from patients with anti-NMDAR encephalitis lead to NMDAR hypofunction. However, this is not a direct effect, as NMDAR-Abs do not act as receptor antagonists (Mikasova *et al.*, 2012). Instead, NMDAR-Abs acutely disrupt NMDAR-EphB2R interactions, which impacts receptor surface trafficking and distribution. The surface diffusion of GluN2A-NMDAR, which are predominantly synaptic, is acutely increased by NMDAR-Abs, while the opposite is true for GluN2B-NMDAR, which are predominantly extrasynaptic (Mikasova *et al.*, 2012). Interestingly, cerebrospinal fluid (CSF) of anti-NMDAR encephalitis patients (hence, containing NMDAR-Abs) suppresses the global activity of *in vitro* neuronal networks within 15 minutes (Jantzen *et al.*, 2013). The swiftness of

⁸ Neuromyelitis optica is an autoimmune disorder characterized by the production of autoantibodies against the astrocytic water channel aquaporin-4.

this effect is consistent with alterations in surface trafficking. By releasing NMDAR from EphB2R, NMDAR-Abs increase the mobility of synaptic receptors. Using superresolution microscopy, Ladépêche and colleagues reported that in the 2 hours following NMDAR-Abs binding, NMDAR nanodomains increase in size and receptor density, later decreasing back to their original features (Ladépêche *et al.*, 2018). Therefore, disrupting NMDAR-EphB2R interactions releases EphB2R-bound synaptic receptors, but increases overall receptor content due to antibody cross-linking. Eventually, receptor cross-linking leads to internalization through clathrin-mediated endocytosis at extrasynaptic sites, and degradation through the endolysosomal pathway (Hughes *et al.*, 2010; Mikasova *et al.*, 2012; Moscato *et al.*, 2014)(Figure 17). Receptor internalization triggered by NMDAR-Abs can occur in the presence of AP5, suggesting that this effect is independent of NMDAR ionotropic function (Moscato *et al.*, 2014). This results in a decline in the number of NMDAR clusters, particularly extrasynaptic ones (Ladépêche *et al.*, 2018). Through these alterations in surface trafficking, NMDAR-Abs reduce NMDAR currents, decrease surface receptor levels in a titre-dependent fashion, and prevent NMDAR-dependent LTP induction (Dalmau *et al.*, 2008; Hughes *et al.*, 2010; Mikasova *et al.*, 2012; Zhang *et al.*, 2012; Dupuis *et al.*, 2014; Moscato *et al.*, 2014; Würdemann *et al.*, 2016). EphB2, the EphB2R ligand, increases synaptic NMDAR clustering (Dalva *et al.*, 2000). Application of EphB2 counteracts NMDAR-Ab-mediated impairments in NMDAR surface trafficking, nanoscale organization and surface expression levels (Dalva *et al.*, 2000; Mikasova *et al.*, 2012; Ladépêche *et al.*, 2018). *In vivo* studies confirm that NMDAR-Abs from patients downregulate NMDAR levels and induce cognitive and behavioural impairments in animals, which can be reversed by EphB2 application (Hughes *et al.*, 2010; Mikasova *et al.*, 2012; Planagumà *et al.*, 2015, 2016). Most studies aiming at characterizing the pathogenic action of NMDAR-Abs have been based on *in vitro* or *in vivo* models of exposure to patient CSF or purified IgGs. Therefore, there is the possibility that components of patients CSF or IgGs other than NMDAR-Abs are producing the before-mentioned impairments. To confirm the effects of NMDAR-Abs, Kreye and colleagues isolated memory B cells and antibody secreting cells of patients to produce monoclonal NMDAR-Abs, which also decrease NMDAR surface levels and NMDAR-mediated currents (Kreye *et al.*, 2016).

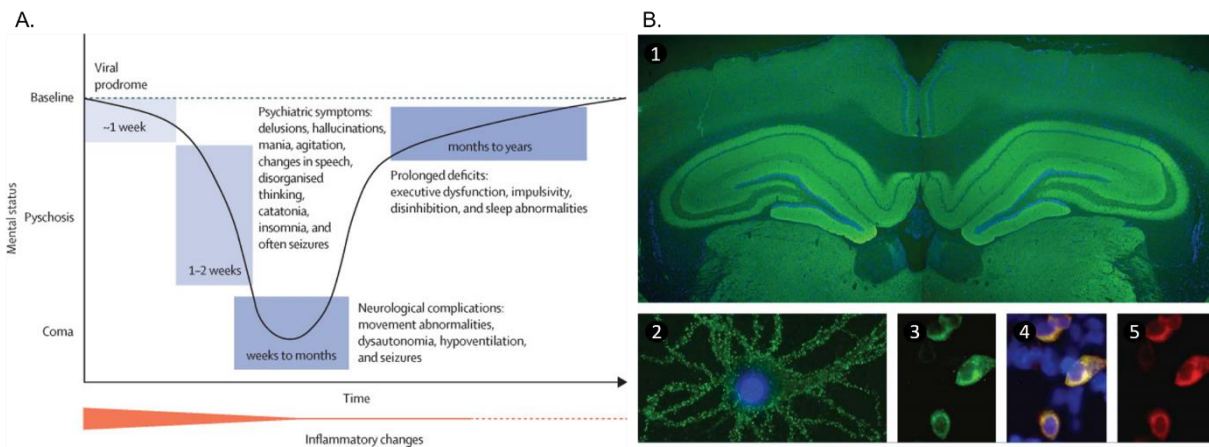


Figure 15. Anti-NMDAR encephalitis. A. Symptom progression in anti-NMDAR encephalitis (Dalmau *et al.*, 2019). B. Antibody reactivity of sera and CSF from patients shows that (1) NMDAR-Abs preferentially target the hippocampus, (2) NMDAR-Abs target a protein at the neuronal surface, and (3-5) NMDAR-Abs react to NMDAR-expressing HEK239 cells; green, NMDAR-Abs reactivity; blue, nucleus; red, GluN2B staining; yellow, overlap of red and green signals (Dalmau *et al.*, 2008).

The presence of anti-NMDAR autoantibodies can also be found in other disorders with psychotic features. Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder with indeterminate causes, sometimes associated with the presentation of psychiatric symptoms such as mood alterations and psychosis (it is then termed neuropsychiatric SLE, NSLE). Estimates of the prevalence of neuropsychiatric symptoms in SLE range from 14% to 75% (Jones *et al.*, 2005). The presence of autoantibodies against brain targets is more prevalent in NSLE than SLE (Ho *et al.*, 2016). Clinical studies indicate that 40–50% of SLE patients present anti-dsDNA antibodies which cross-react with GluN2A and GluN2B subunits (anti-dsDNA/GluN2 antibodies) (Omdal *et al.*, 2005; Hanly, Robichaud and Fisk, 2006; Lapteva *et al.*, 2006; Yoshio *et al.*, 2006; Steup-Beekman *et al.*, 2007; Fragoso-Loyo *et al.*, 2008). Unlike NMDAR-Abs from encephalitis patients, application of anti-dsDNA/GluN2 antibodies to acute hippocampal slices results in amplified NMDAR currents at low titres, and promotion of excitotoxicity at high titres (Faust *et al.*, 2010). Application of these antibodies directly to the brains of live animals induces impairments in cognition and behaviour (Huerta *et al.*, 2006; Kowal *et al.*, 2006; Lee *et al.*, 2009). Other neuropsychiatric disorders have often been associated with anti-NMDAR Abs detection, such as PD (Dahm *et al.*, 2014), dementia (Busse *et al.*, 2014), autism (Creten *et al.*, 2011; Scott *et al.*, 2014; Hacoen *et al.*, 2016), bipolar disorder (Eaton *et al.*, 2010; Dickerson *et al.*, 2012; Sidhom *et al.*, 2012; Hammer *et al.*, 2014; Pearlman and Najjar, 2014), MDD (Pearlman and Najjar, 2014), and, most notably, the psychotic disorder schizophrenia (Ezeoke *et al.*, 2013; Pearlman and Najjar, 2014; Pollak *et al.*, 2014; Jézéquel *et al.*, 2018; Tong *et al.*, 2019).

4. Schizophrenia

Schizophrenia is a neuropsychiatric disorder characterized by an altered perception of reality, which affects approximately 0.8% of the general population (Saha *et al.*, 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, affected individuals begin to suffer psychotic outbreaks, often experiencing auditory hallucinations and falling into paranoid delusions. With time, mental processes deteriorate, resulting in disconnected, disordered or even incoherent thought and speech patterns. These distinctive signs of schizophrenia are categorized as 'positive symptoms', in the sense that they are an "addition" to reality. The 'negative symptoms' of schizophrenia include blunted affect, avolition, anhedonia and social withdrawal. Although it is not central for diagnosis, cognitive impairments are a feature of schizophrenia (Stahl, 2013). Other cognitive processes affected include memory, learning, processing speed, and social cognition (Kitchen *et al.*, 2012). Symptom presentation in schizophrenia is highly heterogeneous, therefore, schizophrenia is considered as a spectrum, similarly to autism. The life expectancy of schizophrenics, which is influenced by a high incidence (3-7%) of suicide, is 20 years shorter than that of an unaffected individual (Laursen, Nordentoft and Mortensen, 2014).

Schizophrenia heritability is estimated at 81%, and concordance between monozygotic twins is bordering on 50% (Cardno and Gottesman, 2000; Sullivan, Kendler and Neale, 2003). Environmental risk factors for schizophrenia include prenatal insults, perinatal viral infection and childhood trauma, while genetic risk factors comprise allelic variants of over 100 genes (Ripke, Neale, Corvin, James T. R. Walters, *et al.*, 2014). Of note, schizophrenia has been linked to variants in genes involved in immunity (HLA), neurodevelopment (DISC1, ERBB4, and NRG1), synaptic plasticity (PPP3CC, SYN2, DTNBP1), and dopaminergic (COMT), glutamatergic (DAO and DAOA), GABAergic (GABRA1, GABRP and GABRA6), and serotonergic (5HTR2A) neurotransmission (Allen *et al.*, 2008; Debnath, Cannon and Venkatasubramanian, 2013; Ripke, Neale, Corvin, James T. R. Walters, *et al.*, 2014). Moreover, mutations of NMDAR subunits, particularly at the CTDs, are associated with schizophrenia (Tarabeux *et al.*, 2011; C. Hu *et al.*, 2016; Hardingham and Do, 2016; Myers *et al.*, 2019; Amin, Moody and Wollmuth, 2020).

The brains of schizophrenics undergo structural alterations, namely enlargement of the ventricles, widening of sulci, loss of white and gray matter, and reduction in the volume of several structures (specifically, the insula, superior temporal gyrus, medial prefrontal temporal gyrus, amygdala, hippocampus and dorsolateral prefrontal cortex) which generally precedes

the onset of the first psychotic episode (Honea *et al.*, 2005). Deficits in hippocampal-dependent cognitive functions, high comorbidity of schizophrenia and temporal lobe epilepsy, and the manifestation of schizophrenia-like cognitive and behavioural deficits in animals with hippocampal lesions indicate that hippocampal dysfunction plays a major role in schizophrenia (Harrison, 2004). Disorganization of hippocampal mossy fibre layer cells in the brains of schizophrenics has also been reported (Harrison, 2004; Tamminga, Stan and Wagner, 2010). Additionally, one of the features of this illness is a decrease in the number of cortical and hippocampal parvalbumin-containing (PV+) GABAergic interneurons (Zhang and Reynolds, 2002; Gonzalez-Burgos and Lewis, 2012), which are essential for the generation of gamma oscillations required for high levels of cognitive control, and are particularly sensitive to insults such as oxidative stress and inflammation (Feigenson, Kusnecov and Silverstein, 2014; Hardingham and Do, 2016). Given the central role of these interneurons in schizophrenia pathophysiology, an imbalance in excitatory and inhibitory neurotransmission is a possible feature of this disorder. In terms of cellular morphology, a decrease in neuronal size, neurite density, and glutamatergic spine density are associated to schizophrenia (Bakhshi and Chance, 2015; Van Berlekom *et al.*, 2020). Microglia are likely to be involved in spine density decline in schizophrenia, as they play a role in the developmental pruning of synapses, and Sellgren and colleagues have recently reported that microglia derived from schizophrenic patients carry out exacerbated synaptic pruning *in vitro* (Sellgren *et al.*, 2019). Microglial cells have also been found to be more active and in higher number in schizophrenics, consistent with reports of a pro-inflammatory environment in the brain of first-episode cases of schizophrenia (Bechter, 2013; Bernstein *et al.*, 2015).

The discovery that first-generation antipsychotics effectively prevent the positive symptoms of schizophrenia by blocking D2 dopamine receptors led to the early hypothesis that excessive dopaminergic signalling was the cause for the disease. This hypothesis was further supported by the psychotogenic action of amphetamines - which increase dopamine levels and induce psychotic outbreaks (Snyder, 1973) - and by positron emission tomography (PET) functional imaging studies which revealed alterations to the expression, activity and availability of dopamine receptors associated with cognitive impairments in patients with schizophrenia (Abi-Dargham *et al.*, 2002; Vyas *et al.*, 2010). Thus, dopamine was coined “the wind of the psychotic fire” (Laruelle *et al.*, 1999). However, the dopaminergic hypothesis does not account for all aspects of schizophrenia pathophysiology. For instance, dopamine-based models of schizophrenia do not replicate the negative symptoms of schizophrenia, and classical dopaminergic antipsychotics do not treat these symptoms well. On the other hand, several pieces of evidence support a contribution of NMDAR dysregulations in the aetiology of schizophrenia at the basis of a glutamatergic hypothesis for the disease (Figure 16): (1)

administering non-competitive NMDAR antagonists (PCP, MK-801, Ketamine) mimics the positive, negative and cognitive symptoms of schizophrenia in healthy individuals (Krystal *et al.*, 1994); (2) genetic and epigenetic changes in genes coding NMDAR subunits or proteins involved in NMDAR signalling are associated with schizophrenia (Blackwood *et al.*, 2001; Kantrowitz and Javitt, 2010; Zoghbi and Bear, 2012; Ripke, Neale, Corvin, James T.R. Walters, *et al.*, 2014; Burnashev and Szepietowski, 2015; Volk *et al.*, 2015; C. Hu *et al.*, 2016; Lemke *et al.*, 2016; Snyder and Gao, 2020); (3) post-mortem brain samples from patients with schizophrenia show abnormally low levels of NMDAR surface expression (Catts *et al.*, 2015), particularly at glutamatergic terminals in PV+ interneurons (Bitanhirwe *et al.*, 2009), which can be emulated to produce animal models of schizophrenia (GluN1 KD model of schizophrenia: Mohn *et al.*, 1999; Ramsey, 2009; Jones, Watson and Fone, 2011; NMDAR ablation at interneurons model of schizophrenia: Belforte *et al.*, 2010; but see Bygrave *et al.*, 2016); (4) alterations in the expression of astrocytic enzymes related to the synthesis of the NMDAR co-agonist D-serine and the endogenous NMDAR and $\alpha 7$ -nAChR antagonist kynurenic acid are associated with the disease (Kantrowitz and Javitt, 2010; Bernstein *et al.*, 2015). 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 (Luby *et al.*, 1959; Domino and Luby, 2012). Moreover, NMDAR antagonists effectively increase dopaminergic neurotransmission in the limbic system, suggesting that dopaminergic hyperfunction could be a consequence of NMDAR hypofunction (Adams, Bradberry and Moghaddam, 2002; Aalto *et al.*, 2005). Application of these same compounds to animals is now common practice to engender valid pharmacological models of this disease (Bubenkov-Valeov, Horek, Vrajov, & Hschi, 2008). Therefore, NMDAR hypofunction is central for schizophrenia aetiology.

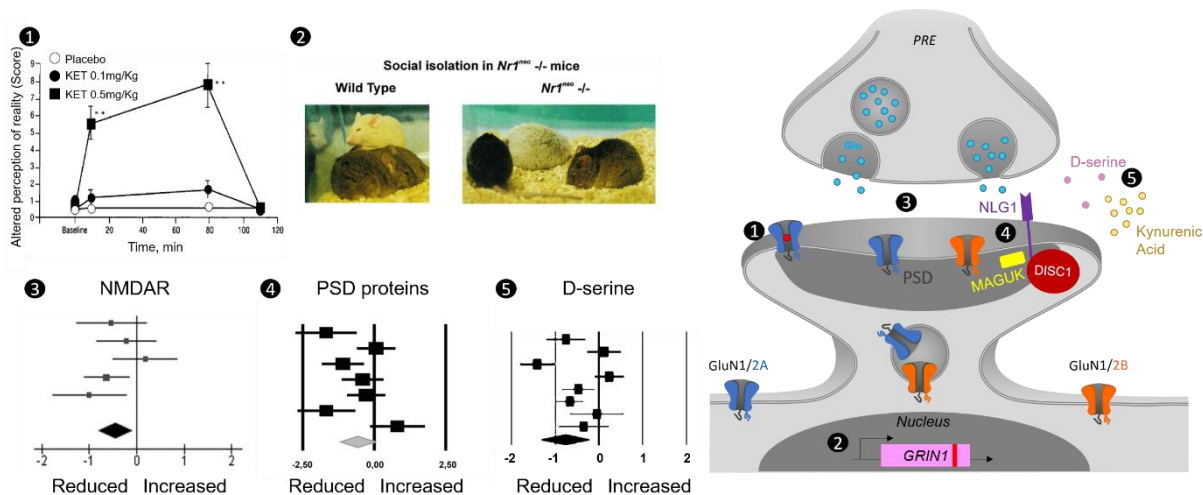


Figure 16. The glutamatergic hypothesis of schizophrenia. The theory that NMDAR hypofunction is at the origin of schizophrenia is supported by several arguments: (1) NMDAR blockers such as Ketamine, PCP and MK-801 mimic in healthy individuals the symptoms of schizophrenia. Graph indicates altered perception of reality in response to a high dose of ketamine (Krystal *et al.*, 1994); (2) Not only are mutations in genes encoding NMDAR subunits in humans associated with schizophrenia, but GluN1 KD results in a schizophrenia-like behavioural phenotype in animals. Example shows social isolation in GluN1 KD rats compared to controls (Mohn *et al.*, 1999); (3) NMDAR expression is decreased in *post-mortem* samples from patients with schizophrenia (Catts *et al.*, 2015); (4) NMDAR interactors are affected by the disorder. MAGUK levels are decreased in schizophrenia (Van Berlekom *et al.*, 2020); (5) Levels of endogenous NMDAR modulators, namely NMDAR co-agonists and the endogenous NMDAR antagonist kynurenic acid, are altered in schizophrenia. For example, D-serine levels are lower in patients with schizophrenia than healthy individuals (Cho *et al.*, 2016).

a. NMDAR trafficking impairments in schizophrenia

The NMDAR hypofunction typically associated with schizophrenia can result from deficits at different levels of NMDAR regulation, including NMDAR trafficking.

i. Intracellular trafficking impairments

In physiological conditions, phosphorylation of GluN1 at S⁸⁹⁷ masks an ER retention signal and promotes NMDAR release from the ER (Scott *et al.*, 2001). Emamian and colleagues found this phosphorylation-based export from the ER to be decreased in brain tissue from schizophrenics (Emamian, Karayiorgou and Gogos, 2004). GluN2A tyrosine phosphorylation has also been found to be decreased in *post-mortem* samples from schizophrenic patients (Hahn *et al.*, 2006; Banerjee *et al.*, 2015). NMDAR dephosphorylation is, in general, associated with receptor internalization. STEP61 dephosphorylates GluN2B-NMDAR and decreases receptor surface expression. Levels of this phosphatase have been found to be increased in cortical samples from schizophrenics (Carty *et al.*, 2012). Neuregulin-1 (NRG1) is a presynaptic adhesion protein which can bind to the postsynaptic tyrosine kinase receptor ErbB4. NRG1-ErbB4 interactions lead to ErbB4-mediated GluN2A dephosphorylation and favour GluN2A-NMDAR internalization. Both NRG1 and ErbB4 are overexpressed in schizophrenia, which contributes to decreased NMDAR surface levels (Hahn *et al.*, 2006;

Geddes, Huang and Newell, 2011). Thus, pathological impairments to NMDAR post-translational modifications could cause intracellular trafficking impairments associated with schizophrenia. Additionally, direct protein interactions can modulate NMDAR intracellular trafficking. After studying a large Scottish family with translocation of the DISC1 gene and several psychiatric problems for four generations, DISC1 mutations were associated with an unusually high prevalence of schizophrenia (Blackwood *et al.*, 2001). DISC1 is a scaffold protein that is also involved in intracellular transport, and can directly and indirectly interact with the NMDAR (Yerabham *et al.*, 2013; Malavasi *et al.*, 2018). DISC1 translocation increases NMDAR surface expression and synaptic localization (Malavasi *et al.*, 2018).

ii. Surface trafficking impairments

NMDAR surface trafficking is an important level of receptor regulation. Several molecular partners which directly interact with the NMDAR and regulate its surface trafficking are underexpressed (e.g. reelin, PSD-95, mGluR1) or overexpressed (e.g. D1R, D2R) in schizophrenia (Abi-Dargham *et al.*, 2002; Toro and Deakin, 2005; Kristiansen *et al.*, 2006; Funk *et al.*, 2009; Vyas *et al.*, 2010; Berretta, 2012; Catts *et al.*, 2015; Matosin *et al.*, 2016). DAAO - the astrocytic enzyme which metabolizes the NMDAR co-agonist D-serine that regulates NMDAR surface trafficking (Papouin *et al.*, 2012; Ferreira *et al.*, 2017) - has been found to be more active in samples from schizophrenic patients (Verrall *et al.*, 2010), and D-serine levels are decreased in schizophrenics CSF (Cho *et al.*, 2016). In fact, mutations in DAAO are highly associated with schizophrenia, and DAAO inactivation or D-serine application have been found to be ameliorate behavioural deficits in animal models of this disorder (Verrall *et al.*, 2010). In fact, combining D-serine with antipsychotics is more efficient than antipsychotic treatment alone (Cho *et al.*, 2016). Most importantly, a significant proportion (Jézéquel and colleagues report a value of 18.7%) of patients with schizophrenia produces NMDAR-Abs (Jézéquel, Johansson, *et al.*, 2017), and higher levels of NMDAR-Abs in first-episode schizophrenia are linked to more severe cognitive, positive and negative symptoms (Tong *et al.*, 2019).

Anti-NMDAR autoantibodies: a link between surface trafficking alterations and psychosis?

NMDAR-Abs from schizophrenia patients have been shown to increase GluN2A-NMDAR and EphB2R surface trafficking and expression and to prevent plasticity induction without directly impacting NMDAR ionotropic function, similarly to NMDAR-Abs from anti-NMDAR encephalitis (Jézéquel, Johansson, *et al.*, 2017)(Figure 17). However, NMDAR-Abs from the two disorders do not compete for the same epitope. Therefore, while it is possible that, given the overlap in symptomatology of both disorders, schizophrenics which are seropositive for NMDAR-Abs

could be misdiagnosed cases of anti-NMDAR encephalitis, it is also possible that this is a distinct autoimmune psychotic disorder induced by anti-NMDAR autoantibodies. Importantly, anti-NMDAR autoantibody detection is highly dependent on the method employed, and perhaps also on the stage of disease progression and the presence or absence of ongoing treatment (Jézéquel, Rogemond, *et al.*, 2017). Of note, a small portion (Jézéquel and colleagues report a value of 2.9%) of healthy individuals also produce (presumably non-pathogenic) anti-NMDAR Abs, and the clinical relevance of these anti-NMDAR antibodies is now under investigation (Jézéquel, Johansson, *et al.*, 2017; Hara *et al.*, 2018). Some studies point to differential impact of anti-NMDAR antibodies from healthy individuals and from patients with anti-NMDAR encephalitis or schizophrenia (Jézéquel, Johansson, *et al.*, 2017), while others report that anti-NMDAR Abs from healthy individuals and from patients presenting anti-NMDAR encephalitis or schizophrenia induce NMDAR internalization, suggesting that all naturally occurring anti-NMDAR Abs are potentially pathogenic (Castillo-Gómez *et al.*, 2017). However, their pathogenic action requires that they reach the brain via a yet elusive pathological increase in blood-brain barrier permeability (Pan *et al.*, 2019). Defining whether all autoantibodies directed against NMDAR are pathogenic will require further molecular characterization.

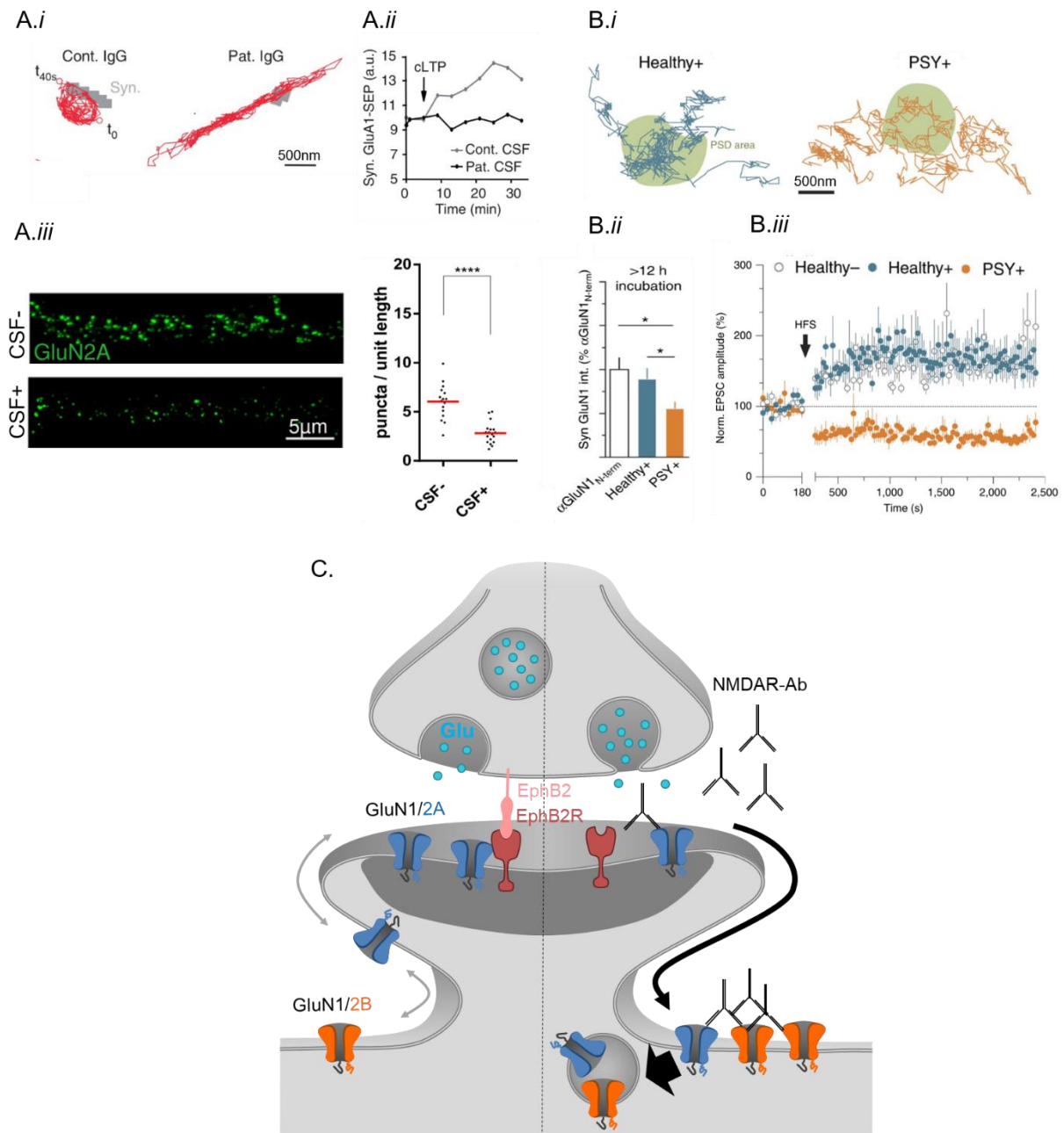


Figure 17. Anti-NMDAR autoantibodies in psychotic disorders. A. Effects of NMDAR-Abs from patients with anti-NMDAR encephalitis; *i.* patients IgGs increase GluN2A-NMDAR diffusion compared to IgGs from controls, as represented by the NMDAR trajectories in red (Mikasova *et al.*, 2012); *ii.* patients CSF prevent the induction of LTP. Note the lack of synaptic accumulation of AMPAR (GluA1-SEP) in neurons treated with patients CSF after a chemical LTP induction protocol (Mikasova *et al.*, 2012); *iii.* patients CSF decrease surface NMDAR levels. Note the drastically decreased density of super resolved GluN2A puncta following 24h treatment of cultured neurons with patient CSF (Ladépêche *et al.*, 2018). B. Effects of NMDAR-Abs from patients with schizophrenia; *i.* patients IgGs (PSY+) increase GluN2A-NMDAR diffusion compared to IgGs from healthy individuals seronegative for anti-NMDAR antibodies (Healthy+) (Jézéquel, Johansson, *et al.*, 2017); *ii.* PSY+ IgGs decrease the intensity of synaptic NMDAR clusters compared to control IgGs and IgGs from Healthy+; *iii.* Patients IgGs prevents LTP induction. Human IgGs were administered to animals via intrahippocampal stereotaxic injection. Unlike IgGs from Healthy+ or healthy individuals seronegative for anti-NMDAR antibodies (Healthy-), PSY+ IgGs prevented LTP induction in acute hippocampal slices (Jézéquel, Johansson, *et al.*, 2017). C. Surface trafficking impairments caused by NMDAR-Abs. NMDAR-Ab binding acutely disrupts NMDAR-EphB2R interactions, which causes synaptic NMDAR to become more mobile. NMDAR-Abs cross-link surface receptors, which immobilizes them, and favours receptor internalization at extrasynaptic sites.

Chapter III – NMDAR antagonists

The term antagonist derives from the Greek word ἀνταγωνιστής (antagonistes), meaning opponent, competitor, rival. In neuropharmacology, a receptor antagonist is a molecule which, by binding to a receptor, blocks or inhibits its function. Endogenous NMDAR antagonists include magnesium and zinc ions, L-phenylalanine, and kynurenic acid. The binding of NMDAR antagonists to their target is the first step that underlies both the beneficial and the detrimental effects of these drugs. NMDAR antagonists can be classified into different types according to their binding sites at the receptor (Figure 18).

A. Types of NMDAR antagonists

Competitive antagonists act on receptors by binding to the same site as their agonist (i.e. glutamate in the case of NMDAR) without producing activation, thus preventing the action of the agonist. Competitive NMDAR antagonists, such as (2R)-amino-5-phosphonovaleric acid (AP5) and 3-((R)-2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), compete with glutamate for its binding site at the ABD of GluN2 subunits. NMDAR antagonists can also act through competition with NMDAR co-agonists, i.e. glycine and D-serine. Glycine site NMDAR antagonists, such as kynurenic acid and 7-chlorokynurenic acid (7-CK), compete with glycine and D-serine for their binding site at the ABD of GluN1 subunits, thus effectively preventing receptor activation. Antagonists may also physically obstruct the passage of ions through the ion channel. For instance, MK-801, PCP, ketamine, memantine, and magnesium, penetrate the NMDAR and bind within the ion pore. The binding of these antagonists requires ion pore opening associated to receptor activation (MacDonald, Miljkovic and Pennefather, 1987; Huettner and Bean, 1988; MacDonald *et al.*, 1991). Hence, they can be classified as open channel blockers (OCB) or use-dependent antagonists. The term uncompetitive antagonist is also applied, defined in opposition to non-competitive antagonism, since the binding of these antagonists is not independent from the action of the agonist (Kornhuber and Bormann, 1993; Lipton, 2004). Finally, allosteric modulators are drugs which bind to the receptor outside of agonist (or co-agonist) binding sites and that do not change the properties of the ion pore, but alter the transition rates between receptor conformational states. The actions of NMDAR positive and negative allosteric modulators (PAMs and NAMs, respectively) are reviewed in (Zhu and Paoletti, 2015; Burnell *et al.*, 2019). Certain NAMs can be used to selectively inhibit receptors with a specific subunit composition (Figure 18).

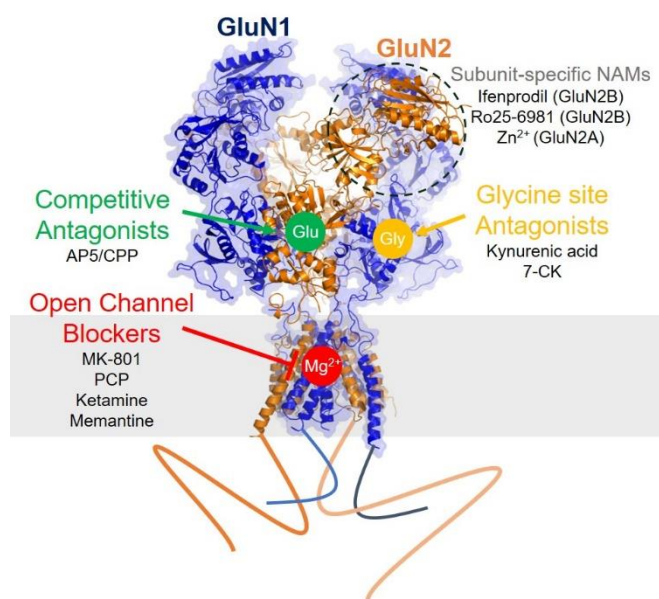


Figure 18. Types of NMDAR antagonists. The subunit-specific NAMs listed as examples here (ifenprodil and Ro25-6981 specifically block GluN2B-NMDAR, and zinc at certain concentrations is selective for GluN2A-NMDAR) bind at the NTDs of the NMDAR. Competitive antagonists, such as AP5 and CPP, compete with NMDAR agonists for their binding site. Glycine site antagonists, such as kynurenic acid and 7-CK, compete with NMDAR co-agonists for their binding site. Open channel blockers, such as MK-801, PCP, ketamine and memantine, obstruct the receptor ion pore, similarly to magnesium.

B. Introduction to the antagonists used in our study

i. Competitive antagonists: AP5 and CPP

By lengthening the carbon backbone of glutamate, it is possible to synthesize competitive NMDAR antagonists. Aminoadipate and aminosuberic acid (specifically isomers with the α -carbon in D- (*R*-) form) are examples of this, and were among the first NMDAR antagonists to ever be produced (Davies and Watkins, 1979; Watkins, 1981; Monaghan and Jane, 2008) (Figure 20). If the carboxyl group in these compounds is replaced by a phosphono group, their antagonism potency increases. The phosphonic acid analog of D- α -Aminoadipate is D-AP5⁹ (or D-APV), and was synthesized in 1981 by Jeff Watkins (Davies *et al.*, 1981; Watkins, 1981). Historically, AP5 was of utmost importance, as it was used to first demonstrate the NMDAR-dependency of hippocampal synaptic plasticity essential for learning and memory formation (Collingridge, Kehl and McLennan, 1983; Morris *et al.*, 1986). The phosphonic acid analog of D- α -Aminosuberic acid is D-AP7¹⁰ (Ferkins, Collins and Stone, 1982). Incorporating the backbone of D-AP5 or D-AP7 into a piperazine ring further increases antagonism potency (Monaghan and Jane, 2008). D-CPP was synthesized as a piperazine ring analog of D-AP7. D-CPP can cross the blood-brain barrier and is better suited for *in vivo* applications than D-AP5 and D-AP7. Adding a double bond to the carbon chain creates the slightly more potent NMDAR competitive antagonist, D-CPP-ene¹¹ (Lowe *et al.*, 1994). Clinical use of competitive NMDAR antagonists has been cast off due to serious adverse effects such as confusion, ataxia, sedation, and acute paranoia (Kristensen, Svensson and Gordh, 1992; Chadwick *et al.*,

⁹ D-AP5; IUPAC name, (2*R*)-2-amino-5-phosphonopentanoate; molecular formula, C₅H₁₂NO₅P; PubChem CID, 135342

¹⁰ D-AP7; IUPAC name, (2*R*)-2-amino-7-phosphonopentanoate; molecular formula, C₇H₁₆NO₅P; PubChem CID, 1617430

¹¹ D-CPP-ene; IUPAC name, 3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid; molecular formula, C₈H₁₅N₂O₅P; PubChem CID, 6437356

1993; Muir and Lees, 1995; Yenari et al., 1998; Mion and Villevieille, 2013).

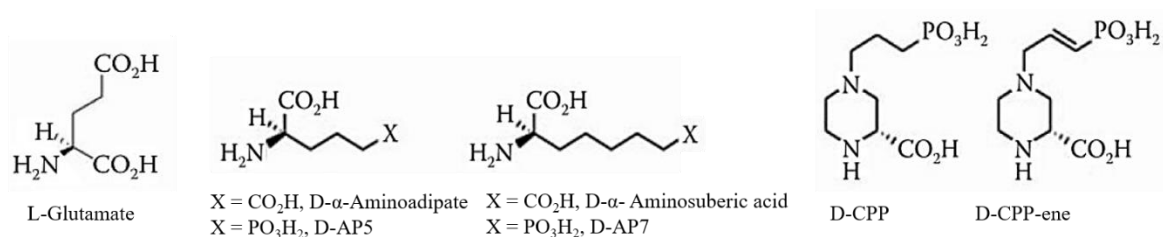


Figure 19. Structure of glutamate and NMDAR competitive antagonists. From left to right: L-Glutamate; if X represents a carboxyl group, D- α -Amino adipate, if X represents a phosphono group, D-AP5; if X represents a carboxyl group, D- α -aminosuberic acid, if X represents a phosphono group, D-AP7; D-CPP; D-CPP-ene (Monaghan and Jane, 2008).

ii. Uncompetitive antagonists: MK-801, ketamine and memantine

Phencyclidine (PCP¹²), the first synthetic uncompetitive NMDAR antagonist, was produced in the 1950s by Parke-Davies industries to be used as an anesthetic (Johnstone, Evans and Baigel, 1959; Mion and Villevieille, 2013). However, it was quickly observed that this cyclohexamine compound was hallucinogenic and induced psychotic-like states (Luby *et al.*, 1959; Bakker and Amini, 1961). Production stopped as PCP was illicitly used for recreational purposes, becoming a drug of abuse. Later on, the cyclohexanone ketamine¹³ was developed based on the backbone of PCP (Figure 20), and it is still being used safely in human and veterinary medicine as an anesthetic presenting low risk for respiratory and cardiovascular complications, despite also displaying psychotomimetic adverse effects.

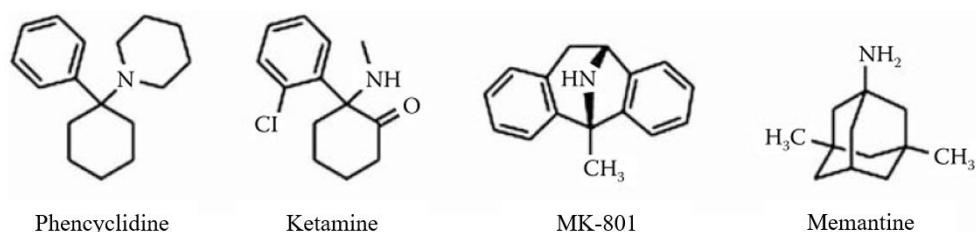


Figure 20. Structure of NMDAR OCB. From left to right: PCP; ketamine; MK-801; memantine (Monaghan and Jane, 2008).

Like PCP, ketamine is used recreationally, and chronic PCP and ketamine users can be falsely diagnosed with schizophrenia (Krystal *et al.*, 1994; Jentsch and Roth, 1999; Cheng *et al.*, 2018). It was only much later, in the 1980s, that PCP and ketamine were found to act as NMDAR antagonists (Lodge and Anis, 1982; Anis *et al.*, 1983; Martin and Lodge, 1985). Around the same time, dizocilpine (MK-801¹⁴), known to be a powerful anticonvulsant, was discovered to be an extremely potent NMDAR antagonist (Clineschmidt, Martin and Bunting,

¹² PCP; IUPAC name, 1-(1-phenylcyclohexyl)piperidine;hydrochloride; molecular formula, C₁₇H₂₆ClN; PubChem CID, 9795678

¹³ Ketamine; IUPAC name, 2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one; molecular formula, C₁₃H₁₆ClNO; PubChem CID, 3821

¹⁴ MK-801; IUPAC name, (1S,9R)-1-methyl-16-azatetracyclo[7.6.1.0^{2,7}.0^{10,15}]hexadeca-2,4,6,10,12,14-hexaene; molecular formula, C₁₆H₁₅N; PubChem CID, 180081

1982; Wong *et al.*, 1986). These substances were soon after reported to act as use-dependent antagonists or open channel blockers of the NMDAR (Kemp, Foster and Wong, 1987; MacDonald, Miljkovic and Pennefather, 1987; Huettner and Bean, 1988; MacDonald *et al.*, 1991; Kornhuber and Bormann, 1993; Lipton, 2004). Clinical use of PCP, MK-801, and ketamine was stifled as these drugs were shown to induce psychotomimetic effects and severe neurotoxicity in animals (Figure 21) (Olney, Labruyere and Price, 1989; Krystal *et al.*, 1994; Neill *et al.*, 2010). Later, competitive antagonists at high doses were also found to produce these lesions (Olney *et al.*, 1991). More recently, ketamine has been found to have important antidepressant properties at sub-anaesthetic doses through indeterminate mechanisms, and, despite the possible side effects, approval for the commercialization of (S)-ketamine for use in cases of treatment-resistant depression was given in 2019 (Berman *et al.*, 2000; Autry *et al.*, 2011; Fond *et al.*, 2014; Pham and Gardier, 2019).

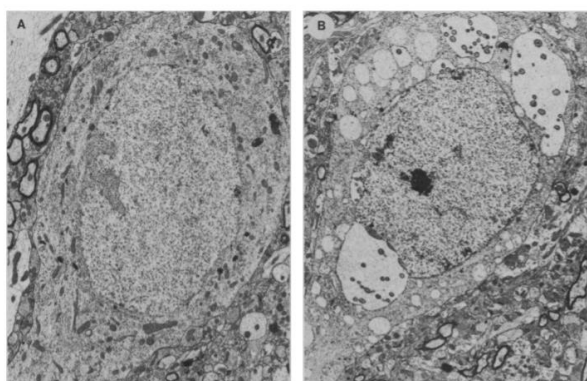


Figure 21. Olney's lesions - brain histology changes in rats caused by high doses of NMDAR antagonists. Electron micrograph depicting a large posterior cingulate cortical neuron from the brain of (A) a normal untreated rat and (B) a rat treated with PCP 4 hours earlier. A. The cytoplasm of the neuron from the control animal contains normal-appearing mitochondria, and there are no vacuoles. B. The cytoplasm of the neuron from the PCP-treated animal contains few normal mitochondria and many vacuoles, some of which contain multiple small, round structures that appear to be remnants of mitochondria. The neuropil surrounding this neuron is well preserved, and there are many normal-appearing mitochondria in the neuropil components. The same effects were seen in rats treated with MK-801 and ketamine at very high doses. Magnification: 7000x (Olney, Labruyere and Price, 1989).

In the 1970s, the aminoadamantane compound amantadine¹⁵, while being employed as treatment for influenza, was serendipitously found to improve symptoms of Parkinson's disease (Hubsher, Haider and Okun, 2012). The amantadine derivative memantine¹⁶ was produced by Eli Lilly and Company in 1968 as an unsuccessful anti-diabetic agent, and was only found to act as an NMDAR open channel blocker in 1989 (Bormann, 1989). Observations of anti-cataleptic effects further cemented the classification of memantine as an anti-parkinsonian agent, and as treatment for neurodegenerative disorders in general (Danysz *et al.*, 1997). Memantine has consistently been shown to slow cognitive decline in Alzheimer's disease (Liu *et al.*, 2019). Unlike ketamine, memantine is very well tolerated and appears to have no abuse potential (Johnson and Kotermanski, 2006; Parsons, Stöffler and Danysz, 2007; Parsons, Rammes and Danysz, 2008).

¹⁵ Amantadine; IUPAC name, adamantan-1-amine; molecular formula, C₁₀H₁₇N; PubChem CID, 2130

¹⁶ Memantine; IUPAC name, 3,5-dimethyladamantan-1-amine; molecular formula, C₁₂H₂₁N; PubChem CID, 4051

C. Pharmacology and structural basis for the action of NMDAR antagonists

The potency of an antagonist and its affinity for the receptor can be ascertained through the calculation of the half maximal inhibitory concentration (IC_{50})¹⁷ and inhibition constant (K_i)¹⁸ of these drugs. These parameters can be determined through dose-response studies of inhibition of agonist-driven response or through binding competition assays with receptor radioligands (e.g. [3H]MK-801 for NMDAR). IC_{50} and K_i values for the antagonists in this study can be found in Table 2.

Table 2: Pharmacological properties of NMDAR antagonists.

	IC_{50}	K_i	% trapping	T_{on} (s)	T_{off} (s)
D-AP5	3.7 μ M ¹	1.93 μ M ²	-	-	-
D-CPP-ene	0.64 μ M ¹	0.23 μ M ²	-	-	-
(+)-MK-801	4.1 nM ³	2.5 nM ³	100% ⁴	8.1 ⁴	92 ⁴
Ketamine	508.5 nM ³	323.9 nM ³	86% ⁵	5.2 ⁵	10.5 ⁵
Memantine	594.2 nM ³	378.4 nM ³	70% ⁵	3.5 ⁵	9.8 ⁵

¹inhibition of neuronal depolarization in cortical wedges cut from slices of rat cingulate cortex was detected across a grease seal barrier placed near the junction between grey and white matter, in magnesium-free aCSF containing TTX. Stimulus: NMDA 40 μ M. (Lodge *et al.*, 1988) ² mouse hippocampal neurons in culture, whole-cell voltage clamp, holding potential of -60mV in magnesium-free aCSF containing TTX, glycine, and bicuculine. Stimulus: NMDA 10 μ M. (Benveniste and Mayer, 1991) ³ IC_{50} and K_i values for displacement of [3H]MK-801 in rat forebrain homogenate (Wallach *et al.*, 2016) ⁴ rat visual cortex neurons in culture, whole-cell voltage clamp, holding potential of -70mV in aCSF containing zero magnesium, TTX, glycine, and bicuculine. Stimulus: NMDA 30 μ M. (Huettner and Bean, 1988) ⁵ rat cortical neurons in culture, whole-cell voltage clamp, holding potential of -60mV in aCSF containing zero magnesium, TTX, glycine, and strychnine. Stimulus: NMDA 10 μ M. (Mealing *et al.*, 1999).

For OCB, a percentage of receptor blockade due to antagonist trapping to the ion pore is presented. This was determined by double pulse protocols (Figure 22) which reveal the degree of EPSC inhibition produced by the molecules that remain “trapped” inside the NMDAR after drug washout (Mealing *et al.*, 1999). The time required for the development and relief of blockade (T_{on} and T_{off} , respectively) was also calculated. Values for (+)-MK-801 are shown since, though MK-801 is a mixture of stereoisomers, most MK-801 molecules are protonated at a physiological pH (Huettner and Bean, 1988; Dravid *et al.*, 2007).

¹⁷ IC_{50} : concentration of antagonist that produces half of the maximal blockade possible.

¹⁸ K_i : equilibrium constant between the rates of antagonist binding and unbinding from the receptor.

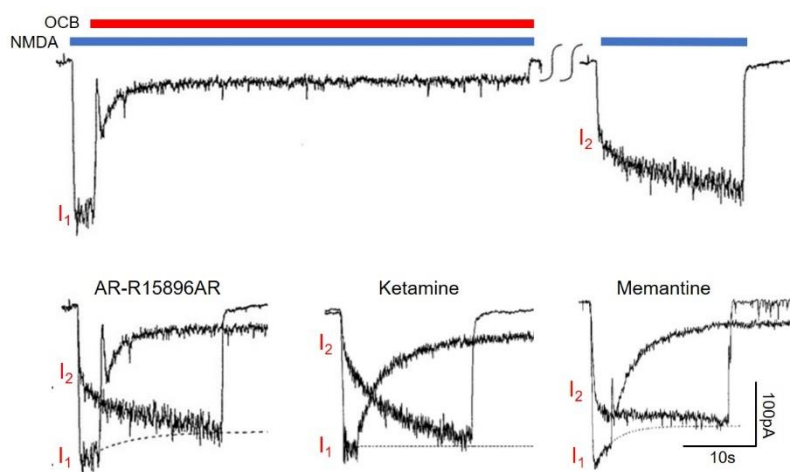


Figure 22. Determining OCB trapping. Top: Example of a double pulse protocol to determine the trapping blockade of OCB. Cortical neurons in culture were patched in whole-cell voltage clamp mode, at a holding potential of -60mV or -70mV, in artificial CSF solution containing zero magnesium, TTX, glycine, and either bicuculline or strychnine. The application of NMDA (blue) induces an inward current (black line) indicating membrane depolarization. Application of an OCB (red) (example shown, AR-R15896AR¹⁹) gradually blocks receptors. Following drug washout, a second application of NMDA reveals residual EPSC inhibition due to drug trapping inside the receptor. Percentage of blockade due to

drug trapping is calculated as: % trapping = $[(I_1 - I_2)/I_1] \times 100$ (Huettner and Bean, 1988; Mealing *et al.*, 1999; Bolshakov *et al.*, 2003). Bottom: comparison of trapping blockade by different OCB: AR-R15896AR (same trace as top), ketamine and memantine (Mealing *et al.*, 1999).

The IC₅₀ values of the antagonists in this study for diheteromeric NMDAR with different subunit compositions are presented in Table 3. Subunit specificity is generally considered to be achieved when the IC₅₀ of an antagonist for an NMDAR subtype is over 50 fold lower than for others (Ogden and Traynelis, 2011). According to this criterion, none of the antagonists in this study exhibit a clear subunit preference.

Table 3: IC₅₀ values of NMDAR antagonists for the different diheteromeric receptor subtypes.

	GluN1/2A	GluN1/2B	GluN1/2C	GluN1/2D
D-AP5	0.3 μM ¹	0.5 μM ¹	1.6 μM ¹	3.7 μM ¹
D-CPP-ene	0.11 μM ¹	0.14 μM ¹	1.5 μM ¹	1.8 μM ¹
(+)-MK-801	0.015 μM ²	0.009 μM ²	0.024 μM ²	0.038 μM ²
Ketamine	0.33 μM ³	0.31 μM ³	0.51 μM ³	0.83 μM ³
Ketamine in Mg ²⁺ 1mM	5.4 μM ³	5.08 μM ³	1.2 μM ³	2.9 μM ³
Memantine	0.80 μM ³	0.57 μM ³	0.52 μM ³	0.54 μM ³
Memantine in Mg ²⁺ 1mM	13 μM ³	10 μM ³	1.6 μM ³	1.8 μM ³

¹IC₅₀ values for displacement of [³H]Glutamate in recombinant NMDAR expressed in *Xenopus* oocytes (Buller and Monaghan, 1997) ²two-electrode voltage-clamp recordings of *Xenopus* oocytes expressing recombinant NMDAR, holding potential of -40mV in magnesium-free buffer solution. Stimulus: glutamate 50μM with glycine 30μM. (Dravid *et al.*, 2007) ³HEK293T cells expressing recombinant NMDAR, holding potential -66mV in buffer solution containing 0 or 1 mM magnesium. Stimulus: glutamate 1mM with glycine 100μM. (Kotermanski and Johnson, 2009)

Studies on the functional properties of triheteromeric NMDAR have so far mainly focused on the activation and deactivation kinetics, magnesium blockade and sensitivity to subunit-specific

¹⁹ AR-R15896AR; IUPAC name, (1S)-1-phenyl-2-pyridin-2-ylethanamine; molecular formula, C₁₃H₁₄N₂; PubChem CID, 9794203

modulators (McClymont, Harris and Mellor, 2012; Tovar, McGinley and Westbrook, 2013; Hansen *et al.*, 2014; Cheriyan *et al.*, 2016; Yi *et al.*, 2018, 2019). The few studies in which the sensitivity of triheteromeric NMDAR to subunit-independent antagonists were studied are summarized in Table 4.

Table 4: IC₅₀ values of NMDAR antagonists for different triheteromeric receptor subtypes.

	GluN1/2B/2D	GluN1/2A/3A	GluN1/2A/3B
(+)-MK-801		0.49 μM^2	3.56 μM^2
Ketamine	1.1 μM (5.7 μM in Mg^{2+} 1mM) ¹		
Memantine	0.6 μM (2.6 μM in Mg^{2+} 1mM) ¹	15.89 μM^2	18.23 μM^2

¹two-electrode voltage-clamp recordings of *Xenopus* oocytes expressing recombinant NMDAR, holding potential of -60mV in buffer solution containing 0 or 1 mM magnesium. Stimulus: glutamate 300 μM with glycine 100 μM . (Yi *et al.*, 2019) ²two-electrode voltage-clamp recordings of *Xenopus* oocytes expressing recombinant NMDAR, holding potential of -50mV in magnesium-free buffer solution. Stimulus: NMDA 100 μM with glycine 10 μM . (McClymont, Harris and Mellor, 2012)

i. Competitive antagonists

Given that the binding site for competitive antagonists is at the GluN2 subunit, a certain level of subunit-specificity might be expected of competitive antagonists. However, despite a general slight preference for GluN2A-NMDAR, followed by GluN2B-, GluN2C- and GluN2D-NMDAR (in this order), competitive NMDAR antagonists are not subunit-selective (Buller and Monaghan, 1997; Erreger *et al.*, 2007; Andaloro VJ, et al., *Pharmacology of NMDAR subtypes*, in Soc. Neurosci. Abstr. 1996;60:4.) (Table 3). Information on off-target effects of these competitive antagonists is limited, but D-AP5 and D-CPP-ene at 10 μM concentration did not pass a primary screen for the displacement of [3H]Kainate and [3H]AMPA (Whitten *et al.*, 1990). D-CPP-ene is also reported to have no affinity for acetylcholine, serotonin, dopamine and GABA receptors (Lowe *et al.*, 1994). From a structural point of view, Jespersen and colleagues recently resolved the D-AP5 binding site at the GluN2A ABD and reported conformational changes that arise from D-AP5 binding (Jespersen *et al.*, 2014) (Figure 23). D-AP5 interacts with virtually the same residues as glutamate, but its phosphono group additionally interacts with residues at positions 685-691 (excluding 687) as well as a tyrosine residue at position 730 (Y⁷³⁰). Y⁷³⁰ is only found in NMDAR GluN2 subunits and replacing it by a phenylalanine leads to a 5-fold decrease in receptor sensitivity to D-AP5. The bulky phosphono group separates the bottom lobe of GluN2A ABD from the top lobe, and impacts LBD-TMD linkers, decreasing inter-linker distance in the receptor complex. This may be the basis for D-AP5-induced NMDAR inhibition (Talukder and Wollmuth, 2011; Jespersen *et al.*, 2014). A study using GluN2B-NMDAR showed that there is conformational heterogeneity in receptors bound to both D-AP5 and the glycine site antagonist DCKA (Zhu *et al.*, 2016), indicating that receptor conformation is dynamic even while the receptor is inhibited. This study

reports that D-AP5 leads to a twisting of GluN2B ABDs and an increase in distance between ABDs, from ABDs to NTDs, and between GluN2B NTDs.

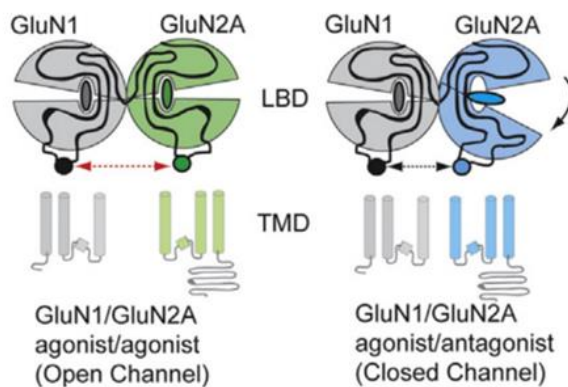


Figure 23. Impact of competitive NMDAR antagonists on receptor structure. Schematic representation of different NMDAR structural domains to show the competitive antagonist-induced domain opening of GluN2 LBDs. This leads to a decrease in the interlinker distance (arrows between two spheres), thereby causing the transmembrane ion channel to close (Jespersen *et al.*, 2014).

ii. Uncompetitive antagonists

Several factors determine channel blockade by uncompetitive antagonists (Huettnner and Bean, 1988; Blanpied *et al.*, 1997; Parsons, Stöffler and Danysz, 2007; Parsons, Gilling and Jatzke, 2008a). OCB are use-dependent antagonists, meaning that NMDAR activation is required to allow ion channel blockade. There is competition between magnesium and OCB for occupancy of the NMDAR ion pore. The binding of OCB, which are all positively charged, is highly voltage dependent: while entrance/stabilization of OCB into the pore is facilitated by membrane hyperpolarization, the escape of OCB from the ion pore is favoured by membrane depolarization. To note, the size of OCB does not correlate with their blocking potency (Bolshakov *et al.*, 2003). Uncompetitive antagonists used in this study exhibit different levels of trapping to the ion pore (MK-801 \ll ketamine $<$ memantine; Table 1), with MK-801 producing a practically irreversible channel blockade. Unlike MK-801, ketamine and memantine can unbind from the ion pore, leading to a weaker inhibition of NMDAR currents after a second stimulation, a phenomenon that is termed partial trapping. Importantly, the off-rate of OCB is not correlated with their lipophilicity and OCB do not escape through the cell membrane (Mealing *et al.*, 2001). Instead, relief of drug trapping occurs through OCB unbinding and exiting into the extracellular compartment.

In 2018, Song and colleagues resolved the MK-801 and memantine binding sites within the ion pore of GluN1/2B NMDAR at the atomic level (Song *et al.*, 2018) (Figure 24). In order to crystallize the NMDAR complex, the authors deleted the GluN NTDs and added a thermostabilizing mutation (G610R) within the TMD, which increased MK-801 dissociation by ~ 15 fold. Despite this, experimental data agreed with structure simulations for the interactions which mediate MK-801 and memantine association to the NMDAR ion channel. After passing

the bundle-crossing region of the NMDAR (corresponding roughly to residues at positions 645-655 in the M3 helix of the GluN2B subunit), MK-801 binds to the ion pore. The two aromatic rings of MK-801 become lodged at a relatively superficial location within the ion pore, next to the V642 residues of the M3 helix of the GluN1 subunit, while the methyl (CH₃) substituent lodges next to the L640 residues of the M3 helix of the GluN2B subunit. Accordingly, mutations affecting these residues impair channel blockade by MK-801 (Kashiwagi *et al.*, 2002). At the pore constriction of the receptor lie the “N-site asparagines”, N614 (GluN1) and N612 (GluN2B), which are at the most inner point of the M2 coil recesses within the ion channel. The GluN2B “N+1” N613 residue is located deeper than GluN2B N612 and aligns with the N-site asparagine N614 of GluN1. The amine (NH or NH₂) group of both MK-801 and memantine form a hydrogen bond with N-site asparagines, regardless of voltage (Song *et al.*, 2018). However, applying voltage drives the OCB deeper into the ion pore, where they form hydrogen bonds with the N613 residue GluN2B residue. Therefore, the presence of the “N+1” asparagine residue leads to voltage dependency of MK-801 and memantine binding. The binding of the drugs is then predicted to be followed by a hydrophobic collapse of residues at bundle-crossing region around the blocker, thus closing the receptor (Figure 23).

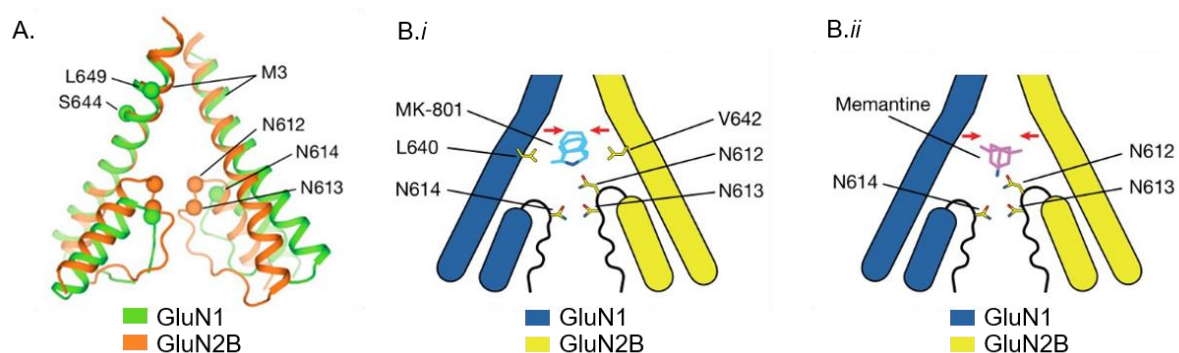


Figure 24. Impact of uncompetitive NMDAR antagonists on receptor structure. A. Representation of NMDAR TMDs with relevant amino acid residues. B. Schematic representations of the binding of *i.* MK-801 and *ii.* memantine. Both OCB interact with key asparagine residues and induce channel closure at the bundle-crossing region, therefore blocking the receptor (red arrows) (Song *et al.*, 2018).

Although OCB act as use-dependent blockers, memantine, in particular, can also bind to and inhibit the NMDAR in a use-independent-way (Blanpied *et al.*, 1997; Sobolevsky and Koshelev, 1998; Sobolevsky, Koshelev and Khodorov, 1998; Chen and Lipton, 2005; Kotermanski, Wood and Johnson, 2009). This was observed using a modified double-pulse protocol, whereby applying memantine without stimulation and then quickly washing it away resulted in NMDAR current inhibition (Blanpied *et al.*, 1997; Kotermanski, Wood and Johnson, 2009)(Figure 25). Ketamine does not produce use-independent inhibition, as application and subsequent removal of ketamine without stimulation does not impact NMDAR currents (Figure 25). This characteristic of memantine is termed second-site inhibition, as it is proposed that memantine can bind weakly to closed receptors through a secondary site, located more superficially than

the ion pore (Kotermanski, Wood and Johnson, 2009). Long periods of washing during standard double-pulse protocols (Figure 24) decrease memantine inhibition to the second stimulation, indicating that washing leads to the unbinding of memantine from this superficial site (Blanpied *et al.*, 1997; Kotermanski, Wood and Johnson, 2009).

Kotermanski and colleagues noted that ketamine also exhibits a washing time-dependent disinhibition, albeit to a nearly negligible degree (Kotermanski, Wood and Johnson, 2009). Unlike ketamine, memantine dissociation from the ion pore is concentration-dependent, with higher initial concentrations producing a slower removal of trapped molecules in double-pulse protocols (Glasgow, Wilcox and Johnson, 2018). This is thought to be the influence of second-site inhibition, as dissociation kinetics from the superficial site are proposed to be slower than unbinding from the deep site (Kotermanski, Wood and Johnson, 2009). Memantine interaction with the superficial site is suggested to compete with occupation of the “deep site”, where the binding of magnesium occurs (Figure 25). Consistent with this, the presence of magnesium during the first stimulation in a modified double-pulse protocol prevents NMDAR inhibition induced by memantine application without stimulation (Glasgow, Wilcox and Johnson, 2018). This is only true for NMDAR subtypes which have a high sensitivity to magnesium (GluN2A and GluN2B, not GluN2C and GluN2D) (Paoletti, Bellone and Zhou, 2013; Glasgow, Wilcox and Johnson, 2018). However, Glasgow and colleagues propose that the binding of memantine at the superficial site is not sufficient to inhibit the receptor, but can only cause inhibition by transposing to the deep site. This is due to two observations: 1) mutating the deep site prevents second-site inhibition, and 2) memantine can bind to the superficial site of the receptor, but not the deep site, when the membrane is depolarized, but only produces second-site inhibition at hyperpolarizing potentials, when the channel is open and the deep site is available (Glasgow, Wilcox and Johnson, 2018).

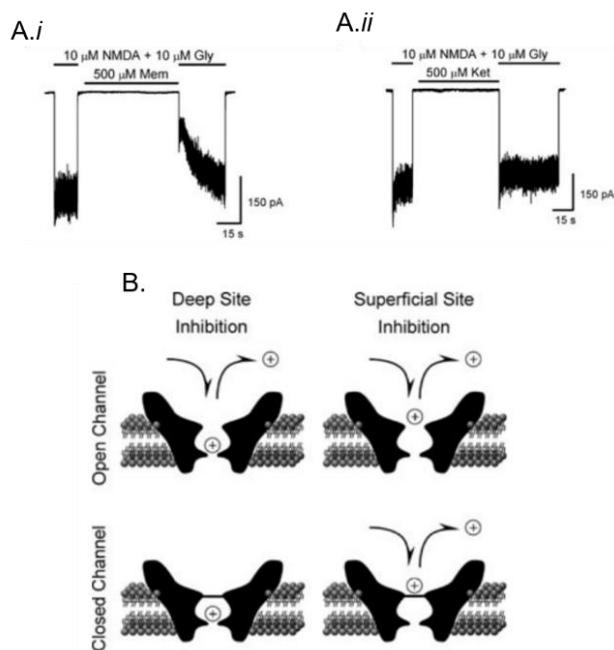


Figure 25. Use-independent binding of memantine to a superficial site at the NMDAR. A. Modified double-pulse protocol used to unveil second-site inhibition. Whole-cell patch-clamp recordings at a holding potential of -66mV were performed on HEK293T cells expressing GluN2A-containing NMDAR. Co-application of NMDA with glycine lead to an inward current congruent with NMDAR activation. The OCB is applied in the absence of stimulus and is quickly washed away before a second stimulation. i, Memantine can bind to a superficial site at the NMDAR even though it is closed and inhibit NMDAR currents triggered by the second stimulation. ii, Ketamine cannot bind to the closed receptor and therefore does not inhibit NMDAR currents triggered by the second stimulation. B. Kotermanski and colleagues hypothesized that memantine can bind at two inhibitory binding sites of the NMDAR that cannot be occupied simultaneously. Memantine (+) can only be trapped by channel closure (bottom left) when occupying the deep trapping site. Memantine binding and unbinding at the deep site requires that the channel be open and is strongly voltage dependent. Binding and unbinding of memantine at the superficial site (right) may occur whether the channels are open or not. Memantine, but not ketamine, can bind at the superficial site, which is located outside the “trapping gate” (horizontal line at channel entrance in lower panels). Memantine can unbind from the superficial site of closed receptors (bottom right) (Kotermanski, Wood and Johnson, 2009).

This assumes a fast displacement of memantine from one site to another, which Glasgow and colleagues report happens immediately in GluN2A receptors, but not in GluN2D receptors (Glasgow, Wilcox and Johnson, 2018). Memantine is not the only OCB that has been posited to bind to two sites at the NMDAR. Using single channel currents recordings, Orser and colleagues report that ketamine not only reduces the mean open time of NMDAR, but also decreases the frequency of channel opening (Orser, Pennefather and MacDonald, 1997). If applied outside of a cell-attached patch, ketamine reaches and decreases the frequency of opening of receptors within the patch. The authors propose that ketamine reduces channel mean open time by blocking the open ion pore and impacts the frequency of channel opening by crossing the membrane and reaching an allosteric site at the intracellular portion of the receptor. Interestingly, while intracellular application of MK-801 can lead to receptor blockade (although with much lower potency), intracellular application of memantine cannot (Berretta and Jones, 1996; Parsons, Gilling and Jatzke, 2008b; W. Sun *et al.*, 2018).

The high clinical tolerability of memantine has been largely attributed to its fast unbinding kinetics, which creates a steady-state NMDAR inhibition that allows basal NMDAR activity but limits pathological NMDAR hyperactivity (Parsons, Stöffler and Danysz, 2007). However, the kinetics of binding and unbinding from the receptor are not very different between memantine and ketamine (Table 2). Memantine has also been thought to preferentially block extrasynaptic over synaptic NMDAR (Léveillé *et al.*, 2008; Xia *et al.*, 2010; Wu and Johnson, 2015). Physiologically, extrasynaptic NMDAR are continuously activated by low levels of ambient glutamate caused by the spill over of synaptic glutamate release, and generate a tonic NMDAR

current (Sah, Hestrin and Nicoll, 1989). To distinguish between synaptic and extrasynaptic NMDAR populations, electrical stimulation is usually used to evoke synaptic currents, elevating glutamate levels at synaptic sites for only ~1-2 milliseconds (Clements *et al.*, 1992). Bath application of NMDAR agonists is then employed to open extrasynaptic receptors, leading to prolonged NMDAR activation, as would be the case in physiological conditions (in the scale of minutes or longer). Using these methods, Xia and colleagues inferred that memantine inhibited extrasynaptic receptors with a twofold higher potency than synaptic ones (Xia *et al.*, 2010). More recently, Glasgow and colleagues established that memantine inhibition increases with increasing duration of NMDAR activation, irrespective of subcellular localization (Glasgow *et al.*, 2017). Long periods of NMDAR stimulation result in receptor desensitization through different mechanisms (Dingledine *et al.*, 1999; Traynelis *et al.*, 2010). Glasgow and colleagues report that memantine stabilizes NMDAR specifically in a calcium-dependent desensitized state, thus reducing the rate of NMDAR recovery from desensitization (Glasgow *et al.*, 2017) (Figure 26).

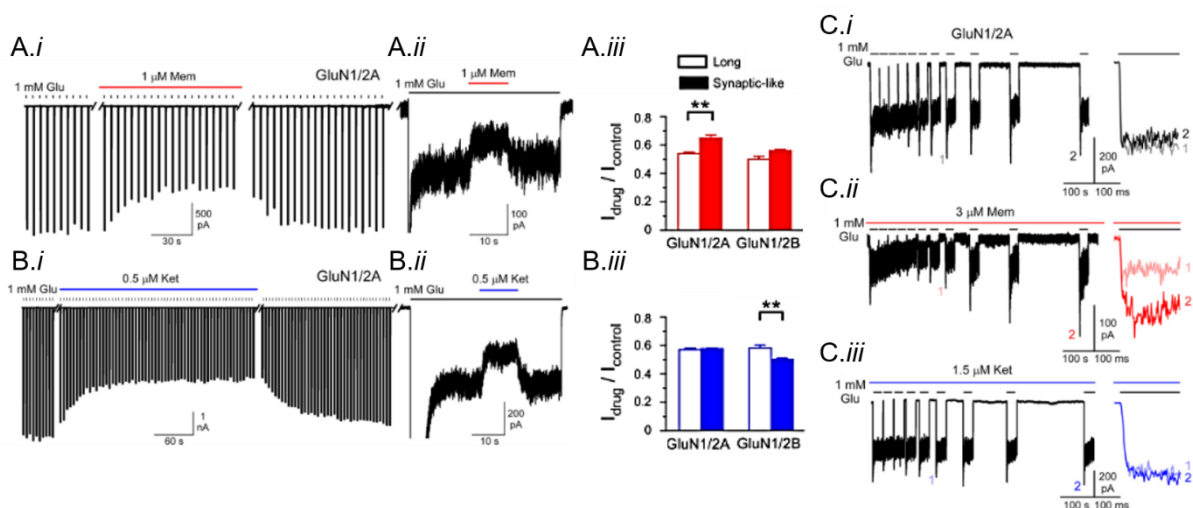


Figure 26. Memantine reduces the rate of GluN2A-NMDAR recovery from desensitization. A and B, effect of memantine and ketamine on NMDAR-mediated currents acquired through whole-cell recordings from tsA201 cells expressing GluN2A-containing NMDAR held at -65 mV in a setup that allows fast solution exchange, *i.* example of fast, synaptic-like stimulation (1 mM glutamate, ~2.5 ms, 0.2 Hz) of NMDAR to achieve steady-state NMDAR inhibition with memantine and ketamine while allowing receptor deactivation and recovery from desensitization between glutamate applications; *ii.* example of long (over 45s) agonist application followed by drug application to study drug binding to receptors in desensitized states; *iii.* impact of memantine and ketamine on GluN2A or GluN2B-NMDAR currents after each stimulation protocol C, 1 mM glutamate was applied for 30s to GluN1/2A-expressing tsA201 cells held at -65mV to allow receptors to reach a steady-state level of activation, and washed in intervals ranging from 0.2s to 200s before re-application, in order to study the time course of receptor recovery from desensitization in (*i*) control conditions and in the presence of (*ii*) memantine or (*iii*) ketamine. Note that memantine slows recovery from desensitization (Glasgow *et al.*, 2017).

Accordingly, inhibition of NMDAR currents by memantine increases as a function of intracellular calcium concentration. The authors also report that, in a heterologous expression system, memantine inhibits GluN2A-containing NMDAR more intensely after long-term receptor activation than after short-term receptor activation, while ketamine inhibits GluN2B-containing NMDAR more intensely after short-term receptor activation through unknown

mechanisms (Figure 26). Memantine had no effect on GluN2B-containing NMDAR, likely since this receptor subtype is less subject to calcium-dependent desensitization than GluN2A-NMDAR (Dingledine *et al.*, 1999; Traynelis *et al.*, 2010; Sibarov and Antonov, 2018). GluN2C- and GluN2D-NMDAR practically do not undergo calcium-dependent desensitization. Uncompetitive NMDAR antagonists typically do not distinguish between NMDAR subunits (Yamakura *et al.*, 1993; Dravid *et al.*, 2007) (Table 2). However, physiological concentrations of magnesium (~1 mM) substantially increase the IC₅₀, modify the voltage dependence, and alter the NMDAR subtype-selectivity of both memantine and ketamine (Table 2). Kotermanski and colleagues report that 1 mM of magnesium decreased memantine and ketamine inhibition of GluN2A and GluN2B receptors ~16-fold, while only decreasing OCB-driven inhibition of GluN2C and GluN2D receptors ~3-fold, making ketamine and memantine ~5 or ~8 fold more potent for inhibition of GluN2C/D- than GluN2A/B-containing receptors, respectively (Kotermanski and Johnson, 2009). Ketamine and memantine inhibit triheteromeric GluN1/2B/2D NMDAR with an IC₅₀ similar to that of other NMDAR subtypes. 1mM magnesium decreases the inhibition caused by ketamine and memantine over triheteromeric GluN1/2B/2D receptors ~5 or ~4 fold, respectively (Yi *et al.*, 2019) (Table 3). GluN3-containing diheteromeric NMDAR do not show significant sensitivity to blockade by magnesium nor by OCB (Chatterton *et al.*, 2002; Smothers and Woodward, 2007). MK-801 and memantine are substantially less effective at inhibiting GluN3-containing triheteromeric receptors than any GluN2-containing diheteromeric receptor subtype (McClymont, Harris and Mellor, 2012) (Table 3). Importantly, the impact of OCB is not limited to the NMDAR. Information on MK-801 off-target effects is restricted to the knowledge that MK-801 does not impact GABARs and its IC₅₀ for the displacement of an nAChR radioligand is 1.9 μM (Halliwell, Peters and Lambert, 1989; Arias, Mccardy and Blanton, 2001). Memantine however, can act as an antagonist for serotonin, acetylcholine, and dopamine receptors (Table 5). Ketamine also has a complex pharmacology and can interact with many targets other than the NMDAR, including opioid, serotonin, acetylcholine, and dopamine receptors (Table 6).

Table 5: IC₅₀ values for off-target actions of Memantine

Target	Action	IC ₅₀ Value (μM)
NMDAR	antagonist	2.3
5-HT3A	antagonist	2.29
α7 nAChR	antagonist	0.34 (rat)
AChR	antagonist	5
D2R	antagonist	At doses of 0.2-2μM, inhibited the D2R-dependent release of prolactin from isolated rat anterior pituitary cells in primary culture

Based on BindingDB accession DB01043. IC₅₀ value estimated for human receptors, unless otherwise stated.

Table 6: Ki values for off-target actions of ketamine

Target	Action	Ki Value (μM)
NMDAR	Antagonist	0.25–0.66
$\mu\text{OR}2$	Antagonist	12.1
$M_1\text{R}$	Antagonist	45
$\alpha 3\beta 4\text{-nAChR}$	Antagonist	IC_{50} : 3.1 μM (rat)
$M_3\text{R}$	Antagonist	246
$M_2\text{R}$	Antagonist	294
5-HT ₃	Antagonist	420
5-HT _{2A}	Unknown	>10
D2R	Agonist	0.05–0.5
SERT	Inhibitor	>10
κOR	Agonist	23.1–60.0
μOR	Agonist	28.1–272
δOR	Agonist	205–286
$\sigma 2\text{R}$	Agonist	26.3 (rat)
$\sigma 1\text{R}$	Agonist	66–140 (rat)
GABA _A R	Agonist	EC_{50} : 600–1800
NET	Inhibitor	66.8
ChE	Inhibitor	494

Based on (Scheller *et al.*, 1996; Moaddel *et al.*, 2013; Roth and Driscoll, 2013; Fröhlich and Van Horn, 2014; Mathew and Zarate, 2016; Zanos *et al.*, 2018) and BindingDB accession DB01221. Ki value estimated for human receptors, unless otherwise stated.

There is a possibility that the dissimilarities between the behavioural impact of ketamine and memantine are related to off-target effects, or even to the effects of their metabolites. Ketamine is a racemic mixture of (*R*)-ketamine and (*S*)-ketamine. (*R*)-ketamine is a more potent antidepressant and induces less psychotomimetic side effects than (*S*)-ketamine (Mathisen *et al.*, 1995; Chang *et al.*, 2019).

D. Behavioural impact and clinical interest of NMDAR antagonists

NMDAR antagonists have a series of clinically relevant properties, including analgesic, antidepressant, anti-convulsant, psychotomimetic and anesthetic effects. The impact of a single administration of these antagonists at different doses on animal behaviour is summarized in Table 7. In order to compare dose-effect relations, Table 7 reports almost exclusively studies using a single intraperitoneal drug administration (i.p.) on rats. Behavioural effects of D-AP5 were not included, as this compound does not easily permeate the blood-brain barrier. The doses at which NMDAR antagonist-induced neurotoxicity was observed are also included.

Table 7: Behavioural impact of NMDAR antagonists on animals

	Dose	Observation	Reference
CPP	0.5; 1; 5 mg/kg (s.c.)	no disruption of PPI	(Bakshi <i>et al.</i> , 1999)
	10 mg/kg	anti-convulsant	(Lowe <i>et al.</i> , 1994)
	10-20 mg/kg	increase in locomotion, ataxia, decreased muscle tone	(Lowe <i>et al.</i> , 1994)
	10,30 mg/kg	deficits in spatial memory processing, assessed by increased rate of errors in radial arm maze	(Ward, Mason and Abraham, 1990)
	30 mg/kg	hyperexcitability to environmental stimuli, ataxia and motor incoordination, stereotyped behaviour	(Ward, Mason and Abraham, 1990)
	50 mg/kg (i.v.)	presence of Olney's lesions (neuronal vacuolization in the cingulate gyrus and retrosplenial cortex)	(Olney <i>et al.</i> , 1991)
	100 mg/kg	absence of Olney's lesions (neuronal vacuolization in the cingulate gyrus and retrosplenial cortex)	(Hargreaves <i>et al.</i> , 1993)
	138 mg/kg	ataxia, no anaesthesia	(Kelland <i>et al.</i> , 1993)
	0.05; 0.1 mg/kg	animal does not appear intoxicated	(Wozniak <i>et al.</i> , 1990)
	>0.1 mg/kg	hyperlocomotion	(Eyjolfsson <i>et al.</i> , 2006)
MK-801	0.1,0.2 mg/kg	anti-cataleptic against haloperidol induced catalepsy	(W. Danysz <i>et al.</i> , 1994)
	0.2 mg/kg	animal appears grossly intoxicated	(Wozniak <i>et al.</i> , 1990)
	0.1; 0.2; 0.5 mg/kg	deficits in spatial memory processing, assessed by increased rate of errors radial arm maze	(Ward, Mason and Abraham, 1990; Wozniak <i>et al.</i> , 1990)
	0.2 mg/kg	ataxia, stereotypy	(Wojciech Danysz <i>et al.</i> , 1994)
	0.2, 0.4 mg/kg	deficits in PPI	(Wędzony, Gołembowska and Zazula, 1994)
	0.3-1 mg/kg	deficits in PPI	(Mansbach and Geyer, 1989)
	1 mg/kg	lethargy, ataxia, slowed movements, occasional myoclonic jerks	(Chen <i>et al.</i> , 1998)
	1 mg/kg	learning impairment, failure to learn the position of a hidden platform in the morris water maze	(Chen <i>et al.</i> , 1998)
	1 mg/kg	hyperexcitability to environmental stimuli, ataxia and motor incoordination, stereotyped behaviour	(Ward, Mason and Abraham, 1990)
	5; 10 mg/kg (s.c.)	presence of Olney's lesions (neuronal vacuolation in the cingulate gyrus and retrosplenial cortex)	(Olney, Labruyere and Price, 1989)
Ketamine	18.4 mg/kg	respiratory distress	(Kelland <i>et al.</i> , 1993)
	18.4 mg/kg (i.v.)	anaesthesia	(Kelland <i>et al.</i> , 1993)
	10 mg/kg	antidepressant	(Garcia <i>et al.</i> , 2008)
	10 mg/kg	impairment of PPI	(Mansbach and Geyer, 1991)
	10; 20 mg/kg (s.c.)	absence of Olney's lesions (neuronal vacuolation in the cingulate gyrus and retrosplenial cortex)	(Olney, Labruyere and Price, 1989)
	>30 mg/kg	decreased activity	(Becker <i>et al.</i> , 2003)
	30 mg/kg	antidepressant	(Yang <i>et al.</i> , 2018)
	30 mg/kg	impairment of spatial short-term memory	(Moghaddam <i>et al.</i> , 1997)
	30 mg/kg	learning impairment, failure to learn assessed in the morris water maze	(Duan <i>et al.</i> , 2013)
	30 mg/kg	disruption of PPI	(Duan <i>et al.</i> , 2013)
Memantine	30 mg/kg	impairment of latent inhibition	(Razoux, Garcia and Léna, 2007)
	40 mg/kg (s.c.)	presence of Olney's lesions (neuronal vacuolation in the cingulate gyrus and retrosplenial cortex)	(Olney, Labruyere and Price, 1989)
	60 mg/kg	stereotypy	(Wojciech Danysz <i>et al.</i> , 1994)
	100 mg/kg	ataxia, hyperlocomotion	(Wojciech Danysz <i>et al.</i> , 1994)
	150 mg/kg	anaesthesia	(Kelland <i>et al.</i> , 1993)
	1; 3 mg/kg (s.c.)	no disruption of PPI	(Wiley <i>et al.</i> , 2003)
	5-7.5 mg/kg	cognitive enhancer	(Parsons, Rammes and Danysz, 2008)
	9.7 mg/kg	anti-convulsant	(Parsons, Rammes and Danysz, 2008)
	5-10 mg/kg	anti-cataleptic against haloperidol induced catalepsy	(W. Danysz <i>et al.</i> , 1994)
	5; 10 mg/kg	decreased spatial memory retention in rotating hole-board task; mild locomotor impairment, latency to initiate movement, decreased beam crossing performance	(Creeley <i>et al.</i> , 2006)
Memantine	10 mg/kg	muscle relaxant, locomotor impairment	(W. Danysz <i>et al.</i> , 1994)
	>10-60 mg/kg	hyperlocomotion	(Wojciech Danysz <i>et al.</i> , 1994)
	10; 20 mg/kg	locomotor impairments, increased ambulation, rearing and latency to initiate movement, severely impaired beam crossing performance, latency to turn around and climb in a 90° inclined wire mesh grid	(Creeley <i>et al.</i> , 2006)
	10;17 mg/kg (s.c.)	disruption of PPI	(Wiley <i>et al.</i> , 2003)
	20 mg/kg	disruption of PPI	(Swerdlow <i>et al.</i> , 2009)
	20 mg/kg	absence of Olney's lesions (neuronal vacuolation in the cingulate gyrus and retrosplenial cortex)	(Chen <i>et al.</i> , 1998)

20 mg/kg	no learning impairment, as assessed using the morris water maze	(Chen <i>et al.</i> , 1998)
30 mg/kg	ataxia	(Wojciech Danysz <i>et al.</i> , 1994)
50 mg/kg	presence of Olney's lesions (neuronal vacuolization in the cingulate gyrus and retrosplenial cortex)	(Creeley <i>et al.</i> , 2008)

All observations performed on rats after acute drug administration. Route of administration is intraperitoneal injection, unless otherwise stated: s.c., subcutaneous injection; i.v., intravenous administration. PPI, prepulse inhibition

Competitive vs uncompetitive antagonists

Competitive and uncompetitive antagonists share certain important preclinical features. For instance, both competitive and uncompetitive NMDAR antagonists act as anticonvulsants (Croucher, Collins and Meldrum, 1982; Wilmot, 1989). OCB, in particular, additionally show anti-cataleptic and analgesic properties (W. Danysz *et al.*, 1994; Pud *et al.*, 1998; Amin and Sturrock, 2003; Eisenberg *et al.*, 2007; Collins *et al.*, 2010; Mion and Villevieille, 2013; Pickering and Morel, 2018). At high doses, both competitive and uncompetitive antagonists can produce locomotor impairments, characterized by hyperlocomotion and hyperexcitability paired with loss of muscle tone, ataxia and stereotypy (Kelland *et al.*, 1993; Wojciech Danysz *et al.*, 1994; Creeley *et al.*, 2006; Eyjolfsson *et al.*, 2006). At even higher doses, NMDAR antagonists cause sedation and, particularly uncompetitive antagonists, induce anaesthesia (Kelland *et al.*, 1993). Both competitive and uncompetitive NMDAR antagonists potentially trigger the appearance of Olney's lesions in rats, however, the presence of these lesions in humans is more controversial. Thus far the presence of OCB-induced histopathological changes has only been reported in persons suffering from ketamine addiction (Kornhuber *et al.*, 1999; Jansen, 2004; Wang *et al.*, 2013). Also, unsurprisingly given the importance of NMDAR for learning and memory formation, NMDAR antagonists generate cognitive impairments, notably deficits in spatial memory, as assessed in tasks such as the radial arm maze and morris water maze (Ward, Mason and Abraham, 1990; Moghaddam *et al.*, 1997; Chen *et al.*, 1998; Creeley *et al.*, 2006; Duan *et al.*, 2013). Memantine, however, seems to induce only mild cognitive impairment, with some reports even suggesting that it acts as a cognitive enhancer (Parsons, Rammes and Danysz, 2008). NMDAR antagonists also affect sensory motor gating (Mansbach and Geyer, 1989). Sensory motor gating is our ability to unconsciously filter out irrelevant environmental stimuli, This can be assessed through a test of prepulse inhibition (PPI), which measures the decrease in startle response to a high intensity stimulus if the same stimulus is presented at a lower intensity shortly beforehand. Deficits in PPI are associated with schizophrenia, as both schizophrenic patients and animal models of the disorder consistently show impairments in sensory motor gating (Jones, Watson and Fone, 2011). MK-801, PCP and ketamine have been used to engender models of schizophrenia with phenomenological validity and suitability for research on antipsychotic treatments (Bubeníková-Valešová *et al.*, 2008). These psychotomimetics induce schizophrenia-like

manifestations, including deficits in PPI, in humans and animals. Interneurons have been found to be highly affected in schizophrenia and in NMDAR antagonist-based models of schizophrenia (Cochran *et al.*, 2003; Keilhoff *et al.*, 2004; Rujescu *et al.*, 2006; Behrens *et al.*, 2007; Morrow, Elsworth and Roth, 2007; Bitanirwe *et al.*, 2009; Gonzalez-Burgos and Lewis, 2012; Koh *et al.*, 2016). As use-dependent antagonists, OCB are more potent for neuronal types which have high activity rates (Homayoun and Moghaddam, 2007; Su *et al.*, 2019). Supporting this claim, acute systemic administration of uncompetitive NMDAR antagonists results in hyperactivity of cortical pyramidal neurons (Q. Li *et al.*, 2002; Suzuki *et al.*, 2002; Jackson, Homayoun and Moghaddam, 2004; Homayoun and Moghaddam, 2007; Povysheva and Johnson, 2016; Ali *et al.*, 2020), and preferential inhibition of hippocampal interneurons (Ling and Benardo, 1995; Grunze *et al.*, 1996). This could relate to the psychotomimetic effects of certain OCB. Are competitive antagonists also psychotomimetic? The competitive antagonists SDZ 220-581²⁰ and SDZ EAB-515²¹, for instance, elicit deficits in PPI (Bakshi *et al.*, 1999). On the other hand, the competitive antagonists CGP-37849²², NPC-12626²³ and CGS-19755²⁴ do not affect PPI, even at high doses (20, 3-30, and 1-10 mg/kg i.p. in rat, respectively) (Mansbach, 1991; Wędzony, Gołembiowska and Zazula, 1994). Hyperlocomotion, which is also associated to psychotomimetic potential, is more consistently induced by competitive antagonists than deficits in PPI (Bubeníková-Valešová *et al.*, 2008). As the impact of competitive and uncompetitive antagonists on monoaminergic systems is not the same, CPP- or MK-801-induced hyperlocomotion may arise from distinct pathways of the basal ganglia. In mice, CPP does not impact nigrostriatal dopamine levels, while MK-801 increases it (Svensson, Pileblad and Carlsson, 1991; Svensson, Carlsson and Carlsson, 1992). It is possible that uncompetitive antagonists mostly affect the tonically active indirect basal ganglia pathway, thus disinhibiting striatal dopamine release, while competitive antagonists impact both direct and indirect pathways, ultimately moderating the dopaminergic tone (Rao, Cler, *et al.*, 1991; Rao, Contreras, *et al.*, 1991; Svensson, Pileblad and Carlsson, 1991; Svensson, Carlsson and Carlsson, 1992). Hence, induction of dopamine-driven hyperlocomotion by competitive antagonists is less likely. In conclusion, at very high doses, competitive antagonists can induce some psychotomimetic effects in rodents, although given their low psychotomimetic potential they are not considered psychotomimetic drugs in the same way that the open channel blockers PCP, MK-801 and ketamine are (Kornhuber and Weller, 1997). However, in

²⁰SDZ 220-581; IUPAC name, (2S)-2-amino-3-[3-(2-chlorophenyl)-5-(phosphonomethyl)phenyl]propanoic acid; molecular formula, C₁₆H₁₇ClNO₅P; PubChem CID, 128019

²¹SDZ EAB-515; IUPAC name, (2S)-2-amino-3-[3-phenyl-5-(phosphonomethyl)phenyl]propanoic acid; molecular formula, C₁₆H₁₈NO₅P; PubChem CID, 159489

²²CGP-37849; IUPAC name, (E,2R)-2-amino-4-methyl-5-phosphonopent-3-enoic acid; molecular formula, C₆H₁₂NO₅P; PubChem CID, 6604869

²³NPC-12626; IUPAC name, 2-amino-3-[2-(2-phosphonoethyl)cyclohexyl]propanoic acid; molecular formula, C₁₁H₂₂NO₅P; PubChem CID, 108099

²⁴CGS-19755; IUPAC name, (2S,4R)-4-(phosphonomethyl)piperidine-2-carboxylic acid; molecular formula, C₇H₁₄NO₅P; PubChem CID, 68736

humans, secondary effects of competitive antagonist administration include anxiety, confusion, altered sensory perception, ataxia, visual distortion, sedation, nightmares, acute paranoid psychosis, and hallucinations, which resemble the psychodysleptic effects of PCP and ketamine (Kristensen, Svensson and Gordh, 1992; Chadwick et al., 1993; Muir and Lees, 1995; Yenari et al., 1998; Mion and Villevielle, 2013). To further complicate the picture, Swerdlow and colleagues report that memantine can cause deficits in PPI in rats, but not in humans (Swerdlow *et al.*, 2009). Overall, memantine is not considered to be a psychotomimetic drug. A study designed to assess whether the experience of these NMDAR antagonists are of similar quality in humans would be ethically questionable. Instead, drug discrimination studies can be used to indirectly compare the subjective experience of psychoactive drugs in animals (Figure 27). Animals exhaustively trained to pull on a given lever in response to PCP or vehicle readily pull the “PCP” lever when administered MK-801, indicating they are unable to discriminate between the two OCB (Willetts, Balster and Leander, 1990). This is an indication that the experience of having been administered either substance is similar to the animals. Additionally, if the drug being substituted is a substance of abuse, the abuse potential of the substituting compounds is also being tested. PCP is not substituted by CPP (Figure 26). Additionally, while MK-801 and ketamine substitute PCP, memantine only does so at doses that decrease the response rate of the animal, leaving an open question on whether these results are a consequence of the muscle relaxant properties of this OCB (Parsons, Rammes and Danysz, 2008; Swedberg, Ellgren and Raboisson, 2014).

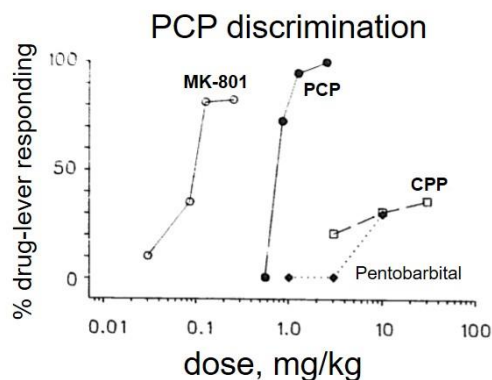


Figure 27. Drug discrimination between PCP and MK-801 or CPP. Animals learned to respond to the administration of either vehicle or 1,25 mg/kg PCP by pulling the corresponding lever during 30-40 daily training sessions. Animals were later administered varying doses of the psychotomimetic OCB MK-801, the training psychotomimetic OCB PCP, the competitive NMDAR antagonist CPP or the hypnotic sedative pentobarbital. Note that animals hardly discriminate between PCP and MK-801, but not CPP, even at very high doses (Willetts, Balster and Leander, 1990).

Since ketamine has been extensively used for clinical practices, the subjective and objective effects of this drug in humans is very well characterized. A summary of the effects of different plasma levels of ketamine in humans is presented in Table 8 (each value is associated to its corresponding concentration in micromolar).

Table 8: Effects of different ketamine plasma concentrations in humans

Plasma concentration		Response
ng/mL	µM	
>50 ng/ml	>0.2	Minor psychodysleptic effects (severity increases with dose)
70 ng/ml	0.29	Deficits in memorization
70-160 ng/ml	0.29-0.67	Analgesia
75-185 ng/ml	0.32-0.78	Antidepressant effects
100 ng/ml	0.42	Mild psychodysleptic effects, feeling “high”
120 ng/ml	0.5	Psychotomimetic effects in subjects with schizophrenia
100–250 ng/ml	0.42–1.1	Psychotomimetic effects in healthy subjects
200 ng/ml	0.88	Nystagmus (involuntary rapid eye movement)
342 ng/ml †	1.43	Hypnosis
350 ng/ml	1.5	Cognition and memory impairment
360-630 ng/ml †	1.5-2.63	Narcosis (absence of verbal response)
500 ng/ml	2	Severe psychodysleptic effects, anxiety, paranoia
594 ng/ml †	2.48	Anaesthesia (absence of response to the nociceptive stimulus)
1200–2400 ng/ml	5-9.3	Dissociative anaesthesia
600–1100 ng/ml	2.7-4.7	Awakening from anaesthesia

adapted from (Mion and Villeveille, 2013; Zanos *et al.*, 2018). † plasma concentration values were extrapolated from the i.v. dosage using the pharmacokinetic study of (Grant *et al.*, 1983).

Ketamine can induce a myriad of effects according to the dose. Ketamine is psychoactive even at low levels (Mion and Villeveille, 2013). The antidepressant properties of this uncompetitive antagonist are induced at the threshold levels for the emergence of psychodysleptic side effects. Disinhibition of pyramidal cells via decreased output of fast-spiking GABAergic interneurons has been proposed as a mechanism that triggers ketamine-induced antidepressant response (Gerhard *et al.*, 2020). The psychodysleptic effects of ketamine increase linearly with plasma concentrations in humans, and at the doses inducing antidepressant effects, these tend to be mild (Bowdle *et al.*, 1998). Cognition can be impaired by ketamine at low levels as well. Patients suffering from schizophrenia reported that ketamine intensified pre-existing positive symptoms, indicating an overlap between the effects of the drug and symptoms of the disorder (Lahti *et al.*, 2001). Ketamine worsens the mental state of patients and affects their mental state more than healthy individuals at similar doses (Lahti *et al.*, 2001). At higher dosages, ketamine induces anaesthesia. As the plasma levels of ketamine decrease along the course of anaesthesia, patients awake. Since ketamine has been widely used as an aesthetic, the psychodysleptic effects which accompany awakening from ketamine-

induced anaesthesia were termed emergence phenomena. Ketamine is metabolized by the cytochrome P450 system into a series of metabolites (Figure 28; for a review on the pharmacology of ketamine metabolites see (Zanos *et al.*, 2018). Zanos and colleagues reported that the ketamine metabolite 6-hydroxy-norketamine contributes to ketamine-induced antidepressant effects in an AMPAR-dependent, but NMDAR-independent fashion (Zanos *et al.*, 2016, 2019). Potentiation of AMPAR-mediated responses have been associated to antidepressant qualities of ketamine (Moghaddam *et al.*, 1997; Li *et al.*, 2010). Accordingly, in hippocampal slices, ketamine application for 30 minutes increases the slope of field EPSCs (Autry *et al.*, 2011).

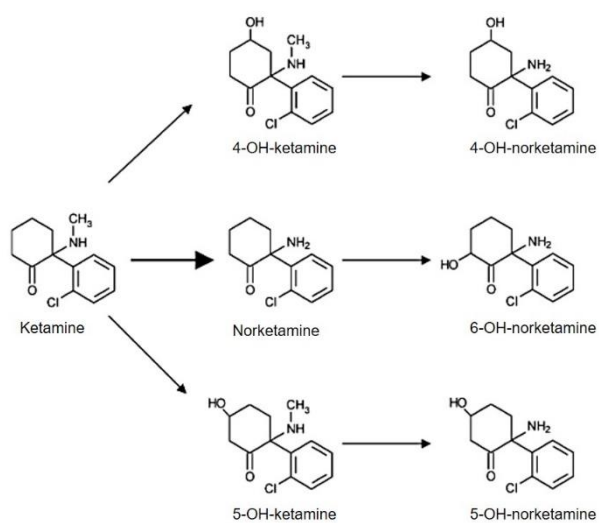


Figure 28. Main pathways of ketamine metabolism. Ketamine is metabolized mainly to Norketamine (80%), itself secondarily transformed into hydroxyl-norketamine (OH-norketamine) (15%), mainly 6-hydroxy-norketamine. Through an accessory pathway, ketamine transforms directly into hydroxy-ketamine (5%) (Mion and Villeveille, 2013).

In humans, memantine used for the treatment of Alzheimer's disease reaches steady-state plasma concentrations of 70-150 ng/ml (0.5-1 μ M), and commonly induces side effects such as dizziness, drowsiness and headache (Datapharm Ltd, 2020). One in 100-1000 users report hallucinations. This side effect occurs mainly patients with severe Alzheimer's disease. There have been isolated cases of psychotic reactions reported in post drug-marketing experience. Unlike PCP and ketamine, memantine is not a substance of abuse. Memantine also lacks antidepressant qualities both in animals and in humans (Zarate, Singh, Quiroz, *et al.*, 2006; Gideons, Kavalali and Monteggia, 2014). As previously mentioned, ketamine and memantine have a similar pharmacological profile, although more differences between the two OCB have been uncovered in the last decade (Glasgow *et al.*, 2017; Glasgow, Wilcox and Johnson, 2018). Despite this, the dissimilarities in the effects of the two OCB are still surprising.

As the clinical effects of NMDAR antagonists are highly heterogeneous, despite their comparable impact on receptor ionotropic function, we intend to study the non-canonical

effects of these drugs over their target. The function of NMDAR may go beyond their ionotropic role, since the conformation and interactions of this receptor are dynamic even in the absence of ion flow. The goal of my thesis is to characterize the effects of different NMDAR antagonists on unexplored aspects of NMDAR physiology.

Objectives of the thesis

N-Methyl-D-Aspartate glutamate receptors (NMDAR) are key actors of excitatory synaptic transmission, synaptic plasticity and higher brain functions such as memory formation and learning. As NMDAR dysfunctions are associated to pathological states, there is a high investment in the development of modulators of ionotropic NMDAR activity for clinical use. However, the outcome of this strategy is not clear-cut, as, for instance, certain uncompetitive NMDAR antagonists (e.g. MK-801, ketamine) induce psychotic-like episodes in humans and animals while other NMDAR blockers (e.g. AP5, memantine) do not elicit comparable behavioural deficits. Recently, studies have shown that NMDAR function may go beyond their role as an ion channel, as NMDAR conformation and interactions are dynamic even in the absence of ion flow. Different NMDAR antagonists can elicit very diverse clinical effects despite acting on the same receptor. The goal of this study is to explore the impact of these drugs on previously unexplored/overlooked aspects of NMDAR physiology.

We first confirm that all antagonists being used efficiently block NMDAR. To do this, we co-apply the drugs with an agonist in order to allow OCB access to the ion pore. Once this step is complete, the first objective of the thesis is to characterize the impact of NMDAR antagonists on NMDAR physiology. We aim to answer the following questions:

NMDAR conformational changes have been reported to be important for non-ionotropic NMDAR function. How do NMDAR antagonists impact NMDAR conformation? A follow-up to this point would be: what are the molecular mechanisms supporting a possible NMDAR antagonist-induced conformational change? Would it occur directly due to occupancy of the drug binding site, or would it rely on protein-protein interactions?

The mobility of surface NMDAR has been shown to be impaired in neuropsychiatric conditions associated with psychosis. What is the impact of NMDAR antagonists, particularly psychotomimetic ones, on NMDAR surface trafficking?

As the acute effects of NMDAR antagonists occur at the minutes to hours timescale, we asked ourselves if in that period the amount of NMDAR is modulated through homeostatic mechanisms. Do NMDAR antagonists affect synaptic NMDAR levels?

The nanoscale organization of NMDAR is regulated by NMDAR subunit composition, developmental stage, and interactions with intracellular scaffolds. NMDAR nanoscale organization may have an impact on NMDAR-mediated signalling, as changing it with biomimetic peptides was found to have an impact on synaptic plasticity. How do the different

NMDAR antagonists impact NMDAR nanoscale organization?

Finally, we aim to understand how NMDAR antagonist-induced modulations of NMDAR physiology may impact synaptic signalling. One of the most abundant postsynaptic NMDAR interactors is CaMKII, and CaMKII mobility is impacted by NMDAR activity and direct interactions with the receptor. Do the different NMDAR blockers have an impact on CaMKII transport?

Materials and Methods

A. *In vitro* assays

1. Cell culture

Primary cultures of hippocampal neurons were prepared from embryonic day 18 Sprague Dawley (SD) rat embryos as described by Bard and colleagues (Bard *et al.*, 2010). Cerebral tissue was immersed in Hank's Balanced Salt Solution (HBSS) (KCl 5.33 mM, KH₂PO₄ 0.44 mM, NaHCO₃ 4.16 mM, NaCl 137.93 mM, Na₂HPO₄ 0.33 mM, D-Glucose 5.55 mM) (ThermoFisher Scientific, Waltham, MA, USA, ref. N°14175-053) for dissection. Hippocampi were incubated with 0.05% trypsin-EDTA (1x, 15 min., 37°C) (ThermoFisher Scientific, Waltham, MA, USA, ref. N° 25300-054) and rinsed before undergoing mechanical dissociation. Dissociated neurons were then plated at a density of 2.75 to 3.25 x 10⁵ neurons/mL in 60 mm Petri dishes containing 18 mm glass coverslips pre-coated with poly-L-lysine (Sigma-Aldrich, Saint-Louis, MO, USA, ref. N° P26361G). Neurons were maintained at 37°C and 5% CO₂ for up to 21 days. 1.5 to 3% of Horse Serum (ThermoFisher Scientific, Waltham, MA, USA, ref. N° 26050-088) was present in the culture medium until *day in vitro (div)* 4-7. Neurons were cultured in Neurobasal™ or Neurobasal™ Plus medium (ThermoFisher Scientific, Waltham, MA, USA, ref. N° 12348-017 or A3582901) supplemented with NeuroCult™ SM1 (STEMCELL technologies, Vancouver, BC, Canada, CAT#05711). Progressively, Neurobasal was partially replaced with equally supplemented BrainPhys™ medium (STEMCELL technologies, Vancouver, BC, Canada, CAT#05790). For live imaging experiments, neurons were transfected at *div* 10 using the calcium-phosphate co-precipitation method (Jiang and Chen, 2006). Precipitates containing 1-1.5 mg plasmidic DNA (GFP, GCaMP6f, Homer-DsRed, Homer-GFP, GluN1-GFP, GluN1-mCherry, GluN1-flag, GluN2B-flag, GFP-αCaMKII, GFP-αCaMKII^{I205K}) were prepared using the following solutions: TE (1 mM Tris-HCl pH 7.3, 1 mM EDTA), CaCl₂ (2.5 M CaCl₂ in 10 mM HEPES, pH 7.2), 2X HEPES-buffered saline (HEBS; 12 mM dextrose, 50 mM HEPES, 10 mM KCl, 280 mM NaCl and 1.5 mM Na₂HPO₄-2H₂O, pH 7.2). Coverslips were transferred to 12-well plates containing 250 μL/well of conditioned culture medium supplemented with 2 mM kynurenic acid (Sigma-Aldrich, Saint-Louis, MO, USA, ref. N°K3375), and 50 μL of DNA precipitate solution was added to each well. Cells were incubated for 1 h at 37°C, then washed with unsupplemented Neurobasal medium containing 2 mM kynurenic acid and moved back to their original culture dishes. Plasmid DNA was expressed for a minimum of 2 days before experiments.

2. Drugs

TTX (ref. N° 1069), D-AP5 (ref. N° 0106), (+)-MK-801 maleate (ref. N° 0924) and memantine (ref. N° 10A/189732) were purchased from Tocris Bioscience (Bio-techne, Minneapolis, MN, USA), NMDA from Sigma-Aldrich (Sigma-Aldrich Saint-Louis, MO, USA, ref. N° M3262), and ketamine from Virbac (Virbac, Carros, France, ref. N° 03597132111010). Unless otherwise stated, drugs were used for *in vitro* assays at the following concentrations: NMDA 5 μ M, TTX 20 μ M, D-AP5 50 μ M, MK-801 20 μ M, ketamine 1 μ M, memantine 1 μ M.

3. Calcium imaging

Live neurons at *div* 15-19 expressing GCaMP6f and Homer-DsRed were transferred to an imaging chamber filled with a Tyrode solution containing (in mM): 110 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂, 25 HEPES, 15 D-glucose. Three time-lapse movies (3000 frames, 20 Hz frame rate) were successively recorded on a widefield Nikon eclipse Ti microscope (Nikon France S.A.S., Champigny-sur-Marne, France) equipped with a Plan Apo \square 60X oil immersion objective (NA 1.40) using a mercury lamp, appropriate excitation/emission filters and an Evolve EMCCD camera (Teledyne Photometrics, Tucson, AZ, USA). Cells were imaged before (baseline) and after being exposed to NMDA combined with either D-AP5, MK-801, ketamine (1 μ M or 100 μ M) or memantine for 5 minutes. D-AP5 was then added in the imaging chamber for 5 minutes to obtain a baseline recording free of NMDAR-dependent calcium transients. Dendritic spines were visually identified using Homer-DsRed signal to avoid bias towards more active spines, and average fluorescence (F) values for each spine were recorded over time. Time-lapse movies were concatenated and realigned in ImageJ (PoorMan3DReg plugin, Michael Liebling, and Template Matching plugin, Qingzong Tseng). Fluorescence from calcium transients vs. time was measured within individual ROIs manually defined by the experimenter (ImageJ, NIH). All pixels within each ROI were averaged to give a single value time course associated to the ROI. Mean normalized fluorescence ($\Delta F/F$) was calculated by subtracting each value with the mean of the previous 5 s values lower than P50 (μ) and dividing the result by μ to obtain $\Delta F/F$. Positive calcium transients were identified following a two-step procedure: initially, $\Delta F/F$ traces were smoothed by convoluting the raw signal with a 10 s squared kernel. True positives (with minimal intervals of 1 s between transients) were then defined on an automated basis using custom-written MATLAB (MathWorks, Natick, MA, USA) routines where the threshold was set at 5 times the standard deviation of the corresponding D-AP5 average trace.

4. Fluorescence Lifetime Imaging Microscopy - Förster Resonance Energy Transfer (FLIM-FRET)

Neurons were transfected to express C-terminally-tagged GluN1-GFP, GluN1-mCherry (Aow, Dore and Malinow, 2015; Dore, Aow and Malinow, 2015) (gift from Paul de Koninck) together with N-terminally-tagged GluN2B-flag (gift from Robert Wenthold) as described by Ferreira and colleagues (Ferreira *et al.*, 2017). FLIM-FRET experiments were performed at *div* 12-14. Live neurons were transferred to an imaging chamber filled with a Tyrode solution containing (in mM): 110 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂, 25 HEPES, 15 D-glucose. Acquisitions were performed on a Leica DMI6000B microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Yokogawa CSU-X1 spinning-disk system (Yokogawa Electric Corporation, Tokyo, Japan), a motorized stage controlled with MetaMorph software (Molecular Devices, San Jose, CA, USA) and a FLIM attachment (Lambert Instruments BV, Groningen, The Netherlands), using a Plan Apo 100X oil immersion objective (NA 1.4-0.7 iris). Epifluorescence microscopy was used to identify and record the positions of GluN1-GFP expressing neurons. GFP lifetime was recorded using a Li2CAM CCD camera (Lambert Instruments BV, Groningen, The Netherlands). A modulated 478 nm LED (100 mA LED DC, 2V LED AC, frequency modulation of 36 MHz) was used as light source to measure FLIM-FRET by frequency domain. Lifetimes were calibrated using a solution of erythrosin B (1 mg/ml) that was set to 0.086 ns as a reference (30ms exposure time). GluN1-GFP lifetime was determined from the fluorescence phase-shift between the sample (250ms exposure time) and the reference from a set of 12 phase settings using LI-FLIM software (Lambert Instruments BV, Groningen, The Netherlands). GFP lifetime was acquired in user-defined regions manually selected using ImageJ (NIH) based on the presence of GluN1-GFP clusters (blind to the FLIM image), before and after application of NMDA plus D-AP5, MK-801, ketamine or memantine to the imaging chamber for 5 minutes. As per (Dore, Aow and Malinow, 2015), clusters with the highest 5% initial GFP lifetime values were excluded from analysis due to disproportionate time-dependent decay in GFP lifetime. To determine the importance of interactions between C-terminal amino-acids of GluN2B and PDZ domain-containing cytosolic proteins on ketamine-induced alterations of NMDAR conformation, live neurons were pre-incubated for 60 minutes either with a nonsense (TAT-NS; YGRKKRRQRRRGSEVILDQPVIKPLIPALSVKKEEA, 10 μ M) (CASLO ApS, Kongens Lyngby, Denmark, ref N° P041012-03-01) (Ladepêche *et al.*, 2013) or a biomimetic peptide (TAT-2B; YGRKKRRQRRRNGHVYEKLSIESDV, 10 μ M) (CASLO ApS, Kongens Lyngby, Denmark, ref N° P051015-01-02) which selectively competes with GluN2B for binding to PDZ domains (Bard *et al.*, 2010). GFP lifetime was acquired in the presence of the peptide before and after application of NMDA plus ketamine to the imaging chamber for 5 minutes. Calculation of the effect of NMDAR antagonists on FRET efficiency and on the

presumed distance between GluN1 C-terminal tails was based on (Lakowicz, 2006; Lam *et al.*, 2012; Dore, Aow and Malinow, 2015) FRET efficiency was calculated as $\mathbf{EFRET} = 1 - \text{TDA}/\text{TD}$, and distance between fluorophores as $r = R_0 \times [(1/\text{EFRET}) - 1]^{1/6}$, where EFRET = FRET efficiency; TDA = lifetime of the donor in the presence of the acceptor (in picoseconds); TD = average lifetime of the donor alone (in picoseconds); r = presumed distance separating the fluorophores (in nm); R₀ = Förster radius for the GFP and mCherry FRET pair (5.4 nm).

5. Single particle tracking (SPT)

QD labelling and microscopy were performed as previously described by Mikasova and colleagues (Mikasova *et al.*, 2012). Neurons at *div* 9-11 were treated with D-AP5, MK-801 or ketamine for 60 minutes. Live neurons were then incubated with polyclonal anti-GluN1 rabbit antibodies (Alomone Labs, Jerusalem, Israel, ref. N°AGC-001, 1/200, 10 min, 37°C, 5% CO₂), then washed and incubated with Quantum dot (QD) 655-conjugated F(ab')₂-Goat anti-Rabbit IgG (H+L) (ThermoFisher Scientific, Waltham, MA, USA, ref. N°Q11422MP, 1/1000010 min, 37°C, 5% CO₂) secondary antibodies. All incubations were done in pre-heated Tyrode solution (composed of, in mM: 105 NaCl, 5 KCl, 2 MgCl₂, 2 CaCl₂, 12 D-glucose, 25 HEPES, pH 7.4) supplemented with 1% BSA (Sigma-Aldrich, Saint-Louis, MO, USA, ref. N°A3059) to avoid non-specific binding. MitoTracker™ Green FM (ThermoFisher Scientific, Waltham, MA, USA, ref. N°M7514, 1/2000) was used as an endogenous synaptic marker. QDs were detected on a Nikon Eclipse Ti microscope (Nikon France S.A.S., Champigny-sur-Marne, France) equipped with a Plan Apo 60X oil immersion objective (NA 1.4) using a mercury lamp, appropriate excitation/emission filters and an Evolve EM-CCD camera (Teledyne Photometrics, Tucson, AZ, USA). Images were obtained with an acquisition time of 50ms (20 Hz frame rate) with up to 500 consecutive frames. QDs were followed on randomly-selected dendritic regions for up to 20 min. Images were processed with the MetaMorph software (Molecular Devices, San Jose, CA, USA). Two-dimensional trajectories of single molecules were constructed by correlation analysis using a Vogel algorithm. The instantaneous diffusion coefficient (D) was calculated for each trajectory from linear fits of the first 4 points of the mean square displacement versus time function using $\text{MSD}(t) = \langle r^2 \rangle(t) = 4Dt$.

6. Immunocytochemistry

Live cultured neurons at *div* 12-14 expressing recombinant Homer-GFP and GluN1-flag were exposed to TTX, D-AP5, MK-801, ketamine 1 μM or 50 μM or memantine for 60 minutes. Surface exogenous GluN1-flag-containing receptors were immunostained live in the presence of these drugs using a mouse monoclonal anti-flag antibody (Sigma-Aldrich, Saint-Louis, MO, USA, ref. N° F1804, 1/500, 10 min, 37°C, 5% CO₂). Neurons were then fixed in 4% PFA for

15 min at room temperature (RT). Fixed samples were carefully washed and immersed in a PBS 1X-NH₄Cl 50mM quenching solution for 10 minutes. Samples were subsequently labeled for 1h (RT) with an anti-mouse Alexa 647-conjugated secondary antibody (ThermoFisher Scientific, Waltham, MA, USA, ref. N° A31571, 1/500) in a PBS 1X -BSA 1% blocking solution. Coverslips were carefully washed again and mounted onto glass slides with Mowiol mounting medium (composed of: Mowiol 4-88 9.6% (w/v) (Sigma-Aldrich, Saint-Louis, MO, USA, ref. N° 475904, Glycerol 24% (w/v) (Sigma-Aldrich, Saint-Louis, MO, USA, ref. N°G5516), and Tris-Cl (0.2 M, pH 8.5) 0.1 M (Sigma-Aldrich, Saint-Louis, MO, USA, CAT#15,456-3)). Acquisitions were performed using a Yokogawa CSU-X1 spinning-disk system (Yokogawa Electric Corporation, Tokyo, Japan) in a Leica DMI6000B microscope (Leica Microsystems, Wetzlar, Germany). Samples were excited using a diode-pumped solid-state 491 laser (200 mW, 8.5-10% power, 100-200ms exposure time) and a 642 laser diode (100 mW, 7-7.5% power, 500ms exposure time). Samples were acquired using a Plan Apo 63X oil immersion objective (NA 1.4-0.6 iris), the appropriate excitation/emission filters and an Evolve EMCCD camera (Teledyne Photometrics, Tucson, AZ, USA). 1 out of a total of 4 experiments was performed in a system of the same kind, using a coolSNAP HQ2 CCD camera (Teledyne Photometrics, Tucson, AZ, USA), a Plan Apo 100X oil immersion objective (NA 1.4-0.7 iris), and diode-pumped solid-state 491 (100 mW, 30% power, 700ms exposure time) and 642 (50mW, 50% power, 800ms exposure time) lasers. Quantification analysis was performed on one user-defined dendrite per cell using ImageJ (NIH). Clusters were identified using a pixel intensity threshold based on image background fluorescence for each experiment.

7. Direct Stochastic Optical Reconstruction Microscopy (dSTORM)

Live cultured neurons at *div* 14-17 expressing Homer-GFP and GluN1-flag were exposed to TTX, D-AP5, MK-801, ketamine 1 μ M or 100 μ M or memantine for 60 minutes. In the presence of these drugs, live neurons were quickly incubated with blocking agents (HEPES 10 mM, BSA 1%; 5 min, 37°C), and labeled using a mouse monoclonal anti-flag antibody (Sigma-Aldrich, Saint-Louis, MO, USA, ref. N° F1804, 1/500, 10 min, 37°C). Samples were fixed with 4% PFA (15 min, RT) and then carefully washed in a quenching solution (PBS-1X, NH₄Cl 50 mM). Unspecific antibody binding sites were masked using a blocking solution (1.5% BSA, 0.1% fish gel, 0.1% Triton-100X; 45 min, RT). Samples were labeled with an anti-mouse Alexa 647-conjugated secondary antibody (ThermoFisher Scientific, Waltham, MA, USA, ref. N° A31571, 1/500; 1h, RT). Coverslips were carefully washed and stored in PBS-1X at 4°C until imaging. Multicolor fluorescent TetraSpeck™ microbeads were added to the samples before image acquisition (ThermoFisher Scientific, Waltham, MA, USA, ref. N° T7279; 1/500; 10 min, RT). Imaging sessions were performed on a Nikon Eclipse Ti microscope (Nikon France S.A.S.,

Champigny-sur-Marne, France) equipped with a Perfect Focus System (PFS), an azimuthal llas² TIRF arm and scanner system (Gataca Systems, Massy, France), a Ti-S-ER motorized stage controlled by MetaMorph software (Molecular Devices, San Jose, CA, USA), an Apo TIRF 100 X oil-immersion objective (NA 1.49) and an Evolve EMCCD camera (Teledyne Photometrics, Tucson, AZ, USA) with a final pixel size of 160 nm. Alexa 647 fluorophores were converted into the dark state using a 642 nm fibre laser at maximum power (1000 mW), and a stable optimized rate of stochastically-activated molecules per frame was achieved by controlling the power of a diode-pumped solid-state 405 nm laser (100 mW) while fixing the 642 nm laser power to around 30% of maximum. Samples were illuminated in TIRF mode and images were obtained with an exposure time of 20 ms (50 Hz frame rate) up to 80,000 consecutive frames. Imaging was carried out at room temperature in a closed Ludin chamber (Life Imaging Services, Switzerland) using a pH-adjusted extracellular solution containing oxygen scavengers and reducing agents (Heilemann *et al.*, 2008; van de Linde, Sauer and Heilemann, 2008). Single-molecule localization and reconstruction was performed online with automatic feedback control of the lasers using the WaveTracer module, enabling optimal single-molecule density during the acquisition (Kechkar *et al.*, 2013). The acquisition and localization sequences were driven by MetaMorph software in streaming mode using a region of interest of 256x256 pixels. Super-resolution images were reconstructed with the PALMTracer software plugin for MetaMorph using a Gaussian fit (xy sigma) to determine the centroid-coordinates of a single molecule and lateral drift correction was achieved using the positions of the photostable TetraSpeckTM beads. SR-Tesseler software (Levet *et al.*, 2015) was used to quantify protein clustering from the detected fluorophore coordinates. This method uses a Voronoi diagram to decompose a super-resolution image into polygons of various sizes, which are drawn by equally dividing the distances between all adjacent detections. From those polygons, several parameters can be extracted, such as the first-rank density σ_i^1 of a detected molecule i . Automatic segmentation of clusters was performed by selecting sets of detections having a density σ_i^1 higher than $2\sigma^d$, with σ^d being the average density of a user-defined region (containing one dendrite). All selected neighboring molecules were merged and only clusters having a minimum area of 1.25 px² (minimum area of 180 nm² based on the size of GluN1 clusters in epifluorescence) and a minimum number of localizations of 5, as previously defined by Kellermayer and colleagues (Kellermayer *et al.*, 2018) were considered. For each cluster j , automatic segmentation of the nanodomains was achieved by applying $\sigma_{(i,j)}^1 > 1\sigma_j^o$, with σ_j^o the average density of the cluster j and $\sigma_{(i,j)}^1$ the density of its i^{th} molecule. As for clusters, all selected neighboring molecules were merged and only nanodomains having a minimum area of 0.00625 px² (minimum area of 12.65 nm² based on the size of an NMDAR as defined by (Patriarchi, Buonarati and Hell, 2018) and a minimum number of localizations of 25 based on the number of times a single emitter is expected to blink during the total length of an acquisition

(Kellermayer *et al.*, 2018) were considered. Size parameters of both the clusters and the nanodomains were extracted by principal component analysis. Local detection densities were calculated as the number of localizations divided by the respective area of the cluster or nanodomain. Synaptic NMDAR clusters were identified manually by superimposing an epifluorescence image of Homer-GFP to a super-resolved image of GluN1-flag clusters.

8. Glutamatergic spine counting

GFP-expressing neurons at *div* 13-14 were exposed to TTX 1 μ M, D-AP5, MK-801, ketamine or memantine for 1h, and subsequently fixed in 4% PFA (15 min, RT). Coverslips were carefully washed and mounted onto glass slides. Image acquisition was performed on a confocal spinning-disk system (Yokogawa CSU-X1, Leica DMI6000B microscope) with an Evolve EM-CDD camera (Teledyne Photometrics, Tucson, AZ, USA), using a Plan Apo 100x oil immersion objective (NA 1.4-0.7 iris). Samples were excited using a diode-pumped solid-state 491 laser (100mW, 3-7% power, 300ms exposure time, Binning: 2) and imaged with the appropriate excitation/emission filters. Dendritic spines were manually identified and labeled using ImageJ (NIH).

9. Fluorescence recovery after photobleaching (FRAP)

GFP- α CaMKII or GFP- α CaMKII^{I205K} expressing neurons at *div* 12-14 were exposed to TTX, D-AP5, MK-801, ketamine or memantine for 1h, and subsequently imaged on an inverted confocal Leica DMI6000B microscope (Leica Microsystems, Wetzlar, Germany) with a Yokogawa CSU-X1 spinning-disk system (Yokogawa Electric Corporation, Tokyo, Japan). Acquisitions were performed using a Plan Apo 63X oil immersion objective (NA 1.4) and a Prime 95B camera (Teledyne Photometrics, Tucson, AZ, USA). A 488 nm laser (400 mW power) at 50% intensity was used to photobleach locally. Recovery from photobleaching was monitored by three consecutive acquisition periods at 2, 0.5, and 0.1 Hz acquisition rates, respectively, using the appropriate excitation/emission filters. Clusters were imaged over a period of 180 seconds. Fluorescence intensity was measured using MetaMorph software (Molecular Devices, San Jose, CA, USA) and corrected for acquisitional photobleaching and background noise using homemade plugins in ImageJ (NIH). Image analysis was performed with ImageJ (NIH).

B. *In vivo* assays

10. Animals

All animal experimentation was approved by the ethical committee of the University of

Bordeaux and performed in accordance to University of Bordeaux guidelines and regulations. Adult (P60) male SD rats (Janvier) were housed at an on-campus conventional animal facility for experimentation. Experimental conditions were assigned randomly.

11. Biochemistry

a. Synaptosome preparation

P60 SD rats were injected intraperitoneally with either saline solution; (R,S)-CPP 10 mg/kg (Tocris Bioscience/Bio-technie, Minneapolis, MN, USA, ref. N° 0173); ketamine 100 mg/kg or MK-801 5mg/Kg. Animals were anaesthetized with 5% isoflurane and decapitated with a guillotine 1h post-injection. Cortices were dissected in artificial cerebrospinal fluid (containing in mM: 125 NaCl, 2.5 KCl, 2 MgSO₄, 1.25 KH₂PO₄, 26 NaHCO₃, 10 glucose, 4 sucrose, 2.5 CaCl₂; pH 7.3-7.4) and snap frozen in liquid nitrogen. For subcellular fragmentation, tissue was mechanically dissociated and homogenized with a Teflon glass potter in TPS (0.32 M sucrose, 4 mM HEPES pH 7.4), and a protease inhibitor cocktail (Sigma-Aldrich, Saint-Louis, MO, USA, ref. N° 539134; 1/1000). This homogenate was centrifuged (1000 g, 8 min, 4°C). The resulting supernatant fraction (S1) was collected and centrifuged (12500 g, 13 min, 4°C). The pellet resulting from this centrifugation (membrane fraction, P2) was resuspended in TPS. A sample of membrane fraction was collected, and the remaining volume was carefully deposited over a two-tier sucrose gradient (0.8 M sucrose, 4 mM HEPES pH 7.4; and 1.2 M sucrose, 4 mM HEPES pH 7.4) for ultracentrifugation (50000 g, 70 min, 4°C). Synaptosome-enriched fraction (containing synapses, synaptic plasma membranes and synaptic vesicles) was carefully collected from the resulting stratified biological material and snap frozen in liquid nitrogen. Samples were stored at -80°C prior to biochemical analysis. Protein quantification was performed using the Pierce BCA protein assay kit (ThermoFisher Scientific, Waltham, MA, USA, ref. N° 23225) and a POLARstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). 1 µg of each sample was diluted in 1 part water and 1.3 parts Tris-Glycine SDS Sample Buffer (63 mM Tris HCl, 10% Glycerol, 2% SDS, 0.0025% Bromophenol Blue, pH 6.8 and 5% β-mercaptanol blue). Samples were heated (5 min, 95°C) before being analyzed.

b. GluN1 signal detection using Wes™ technology

GluN1 quantification was performed using the Wes™ protein simple technology (Protein simple, Bio-technie, Minneapolis, MN, USA). 3 microliters of each sample (0,1 mg/ml) were analyzed using a WES-standard pack 12-230 kDa (Protein simple, Bio-technie, Minneapolis, MN, USA ref N° SM-W004). Total protein detection was performed with a WES-total protein pack (Protein simple, Bio-technie, Minneapolis, MN, USA, ref N° DM-TP01), GluN1 detection was performed with a mouse monoclonal anti-GluN1 antibody (Merck Millipore, Burlington,

MA, USA, ref N° Mab363) and an Anti-Mouse Detection Module for Wes (Protein simple, Biotechne, Minneapolis, MN, USA ref N° DM-002). The signal was normalized on total protein detection. Values were then reported to the mean of saline animals.

c. PSD95-GluN2 co-immunoprecipitation

Dynabeads Protein A (Invitrogen, Carlsbad, California, USA, CAT#10001D) were prepared following the manufacturer's recommendations. In brief, beads were incubated for 30min at 4°C under rotation with a mouse anti-PSD95 (ThermoFisher Scientific, Waltham, MA, USA, ref. N° MA1-046). The antibody-bead mixtures were washed with blocking buffer (PBS-0.5% BSA, pH 7.4), 1 h at 37°C under constant agitation and with washing buffer (PBS-0.1% BSA, pH 7.4). Synaptosomes (50µg) were then added and rotated overnight at 4°C. Supernatant was removed and saved, and immunoprecipitates were washed three times in lysis buffer. SDS-PAGE buffer was added to the washed immunoprecipitates, which then were resolved on 7.5% Mini-PROTEAN TGX Precast SDS-polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA, ref. N° 456 1023). Efficiency of the immunoprecipitation was determined by examining the eluted fractions obtained from the procedure on images obtained from a Chemidoc apparatus (Bio-Rad Laboratories, Hercules, CA, USA) (see western blots section). Band density values for coimmunoprecipitated GluN2A and GluN2B were normalized to immunoprecipitated PSD95.

d. Signal detection using standard Western Blot techniques

For standard western blot protein detection, samples were loaded onto a Mini-PROTEAN TGX Precast Protein gradient gel 4-15% (Bio-Rad Laboratories, Hercules, CA, USA, ref. N° 456 1096). Each gel was also loaded with a PAGERuler Prestained plus protein ladder (ThermoFisher Scientific, Waltham, MA, USA, ref N° 26619) and a positive control sample. Gels were immersed in TGS solution (25 mM Tris, 192 mM glycine and 0.1% SDS) and SDS-PAGE protein separation occurred by electrophoresis (200 V, 400 mA, 40 min). Proteins were transferred to a nitrocellulose membrane in transfer buffer (25 mM Tris Base, 195 mM glycine, and 20% (v/v) ethanol) by electrophoresis (100 V, 400 mA, 60-80 min). Membranes were blocked with TBST (Tris-saline - 0.05% tween 20) with added 5% milk (60 min, RT), and carefully washed with TBST. Membranes were incubated (120 min, RT; or overnight, 4°C) with mouse primary antibodies against PSD95 (ThermoFisher Scientific, Waltham, MA, USA, ref. N° MA1-046, 1/1000), Synaptophysin (Synaptic Systems GmbH, Göttingen, Germany, ref. N° 101011, 1/5000) and Actin (Sigma-Aldrich, Saint-Louis, MO, USA, ref. N° A5316, 1/5000), then carefully washed and incubated (40 min, RT) with donkey anti-mouse IgG (H+L) HRP secondary antibodies (Jackson ImmunoResearch, ref. N° 715-035-150; 1/1000) diluted in

TBST 0.5% milk. SuperSignal™ West Femto Maximum Sensitivity Substrate detection (ThermoFisher Scientific, Waltham, MA, USA, ref. N° 34095) and a ChemiDoc system (Bio-Rad Laboratories, Hercules, CA, USA) were used to reveal the protein bands, and band intensity was analyzed using Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA). Band density values for PSD95 were normalized to synaptophysin or actin.

C. Statistical analysis

All statistical analysis was performed using GraphPad Prism (GraphPad Software Corporation, San Diego, CA, USA). A D'Agostino and Pearson omnibus normality test was applied to determine the normality of the data. For normally distributed data, the following parametric tests were applied: for unpaired data, Student t-test; for paired data, Paired t-test test; for unmatched grouped data, One-way ANOVA followed by Tukey's multiple comparison test. For data that did not follow a normal distribution, the following non-parametric tests were applied: for unpaired data, Mann-Whitney test; for paired data, Wilcoxon matched-pairs signed rank test; for unmatched grouped data, Kruskal-Wallis test followed by Dunn's multiple comparison test. Statistically significant differences between conditions are represented as asterisks ($p > 0.05$, $*p > 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$).

Contribution of the candidate

I directly contributed to all experiments presented in this manuscript apart from SPT and FRAP. For biochemistry experiments, I contributed only to the preparation of synaptosomes.

Results

NMDAR antagonists selectively impact receptor conformation in a subtype-dependent manner

Recent studies demonstrated that agonist binding to the extracellular domains of NMDAR can trigger conformational rearrangements within cytosolic domains of the receptors and transmit information into the cell in the absence of ion flow (Vissel *et al.*, 2001; Nabavi *et al.*, 2013; Aow, Dore and Malinow, 2015; Dore, Aow and Malinow, 2015; Stein, Gray and Zito, 2015; Carter and Jahr, 2016; Weilingner *et al.*, 2016; Stein *et al.*, 2020). To explore whether antagonists may also trigger ion flux-independent changes to the physiology of NMDAR, we compared the actions of competitive (D-AP5) and uncompetitive (MK-801, ketamine, memantine) receptor blockers. We first ensured that all drugs efficiently inhibited NMDAR by performing calcium imaging experiments in dissociated hippocampal neurons. All drugs were applied in combination with the agonist NMDA (5 μ M) to allow the action of uncompetitive open channel blockers (OCB), which require channel aperture to reach and block the ion pore. Using GCamp6f as a fluorescent calcium indicator, we observed that D-AP5 (50 μ M), MK-801 (20 μ M), ketamine (1 μ M and 100 μ M) and memantine (1 μ M) all lead to a significant inhibition of spontaneous NMDAR-mediated calcium events in dendritic spines (average inhibition: D-AP5, 80.48%; MK-801, 99.99%; ketamine 1 μ M, 65.33%; ketamine 100 μ M, 88.4%; memantine, 94.39%; Figures 1, S1 and S7). In order to monitor conformational changes in the cytosolic domain of NMDAR upon antagonist binding, we then co-expressed C-terminally-tagged GluN1-GFP and GluN1-mCherry subunits together with GluN2B-flag (1:3:2 ratio) to favour the formation and synaptic delivery of recombinant receptor complexes, and used Fluorescence Lifetime Imaging Microscopy (FLIM) to measure the Förster Resonance Energy Transfer (FRET) between GluN1-GFP (donor) and GluN1-mCherry (acceptor) as a readout of the proximity between C-terminal ends of GluN1 subunits (Figure 2A), as previously described (Dore, Aow and Malinow, 2015; Ferreira *et al.*, 2017). Fluorescence lifetime images were collected and analyzed from manually selected GluN1-GFP clusters on dendritic spines (Figure 2B). As a negative control, GluN1-GFP was expressed alone to set basal levels of GFP lifetime in the absence of FRET (Figure S2). Of note, none of the drugs in this study impaired the ability of GluN1-GFP and GluN1-mCherry to express FRET (Figure S2), nor did they affect the lifetime of GluN1-GFP alone (Figure S3). When GluN1-GFP (donor) and GluN1-mCherry (acceptor) were co-expressed, FRET efficiency-based calculations yielded an estimation of the distance between fluorophores of (mean \pm SEM) $7,9 \pm 0,1$ nm (Table 1), as previously observed by Dore and colleagues (Dore, Aow and Malinow, 2015), which is substantially smaller than the average distance between synaptic NMDAR (Santucci and Raghavachari,

2008), indicating that FRET indeed results from intra-receptor rather than inter-receptor interactions. Application of NMDA (5 μ M, 5 min) alone did not affect FRET efficiency between C-terminally-located fluorophores (Figure 2B,C), unlike previously reported (Dore et al., PNAS 2015; Ferreira et al., eLife 2017). Instead, 5 min co-application of NMDA with the OCB MK-801 (20 μ M) or ketamine (1 μ M) resulted in a significant decrease in GFP fluorescence lifetime (Figure 2B-D). FRET efficiency-based calculations suggest that exposure to MK-801 and ketamine triggered a shortening of the GFP / mCherry distance by 0.20 nm and 0.12 nm, respectively (Table 1). One important caveat to these calculations is that decreases in FRET efficiency may alternatively be due to a change in fluorophore orientation. Importantly, co-exposure to NMDA and memantine (1 μ M) or the competitive antagonist D-AP5 did not affect GFP fluorescence lifetime. Altogether, while all competitive and uncompetitive NMDAR antagonists produce channel closure and although OCB are considered equivalent in terms of binding site and mechanism of ion pore obstruction, our results suggest that MK-801 and ketamine selectively affect NMDAR cytosolic domain conformation, while D-AP5 and memantine do not.

Open channel blockers decrease synaptic NMDAR mobility

NMDAR are mobile at the cell surface and exchange between synaptic and extrasynaptic compartments through lateral diffusion within the membrane plane (Tovar and Westbrook, 2002; Groc *et al.*, 2004 & 2006). They get anchored at synapses through interactions with trans-synaptic adhesion molecules and scaffolding proteins of the postsynaptic density, to which they bind through C-terminal cytosolic residues (Tovar and Westbrook, 2002; Bard *et al.*, 2010). We used single particle tracking methods to monitor NMDAR trafficking at the surface of spontaneously active dissociated hippocampal neurons (Figure S1) exposed to either buffer or antagonists, hypothesizing that drug-elicited changes in C-terminal conformation could possibly translate into modifications of NMDAR stabilization within synaptic areas. Synapses were labeled with an active mitochondria marker (Mitotracker, rhodamine derivative) and individual NMDAR were tracked using quantum dots (QD) functionalized with anti-GluN1 antibodies (Figure 3A,B). While preventing receptor-mediated ion flow with D-AP5 did not affect the diffusion properties of receptors at synapses, single-particle tracking sessions revealed that the uncompetitive OCB ketamine and MK-801 decrease lateral diffusion of synaptic NMDAR, suggesting that these blockers favour receptor stabilization at synaptic sites (Figure 3C). Consistently, the fraction of mobile synaptic receptors decreased significantly after exposure to MK-801 and ketamine, and the synaptic residency time of NMDAR increased accordingly (Figure 3D,E), while D-AP5 had no effect on these parameters. Together, our data shows that besides blocking the channel function of receptors, MK-801 and ketamine

selectively restrain the diffusion of NMDAR and favour their anchoring at synapses.

NMDAR blockade (1h) does not affect synaptic receptor abundance *in vitro*

Long-term NMDAR blockade increases synaptic levels of the receptor through homeostatic mechanisms (Williams, Dichter and Molinoff, 1992). Our results suggest that OCB increase the stabilization of synaptic NMDAR, which could lead to synaptic accumulation of receptors and changes in NMDAR-mediated signalling. To address this question, we first immunostained surface NMDAR in cultured neurons to assess whether exposure to D-AP5, MK-801, ketamine or memantine impacted synaptic receptor cluster area and intensity, using the recombinant scaffolding protein Homer-dsRed as a synaptic marker (Figure 4A,B). As NMDAR inhibition results in depression of neuronal firing, exposure to TTX was used as a control to ensure that the effects observed resulted from drug-elicited changes in receptor properties rather than modifications in network activity. We observed that a 1h treatment of cultured neurons with D-AP5 (50 μ M), MK-801 (20 μ M), ketamine (1 μ M and 50 μ M), memantine (1 μ M) or TTX (20 μ M) does not alter the area or intensity of synaptic NMDAR clusters (Figures 4C, S7 and S8). The total pool of surface NMDAR clusters is also not affected by the drugs (Figure S4). Additionally, a 1h-long inhibition of NMDAR by these drugs does not affect the number of synapses (with the exception of ketamine 50 μ M), as assessed using the linear density of Homer clusters (Figure S4, S8 and S9) and the number of visually-identified dendritic spines as a readout (Figure S5). Together, these *in vitro* results suggest that a 1h exposure to either competitive or uncompetitive NMDAR antagonists does not trigger major reorganizations in synapse numbers and receptor synaptic content.

To investigate the impact of antagonists on NMDAR synaptic levels *in vivo*, we injected saline solution (control), MK-801 (5 mg/kg) or ketamine (100 mg/kg) intraperitoneally to rats and prepared cortical synaptosomes from brain tissue collected 1h post-injection (Figure 5A). The competitive antagonist CPP (10 mg/kg) was used instead of D-AP5, which displays poor blood-brain barrier penetration. Quantitative immunoblot analysis revealed that GluN1 levels in membrane fraction were not affected by the antagonists (Figure 5B*i,Ci*). Exposure to CPP and ketamine did not affect GluN1 levels in cortical synaptosomes either (Figure 5B*ii,Cii*). However, GluN1 levels in cortical synaptosomes were decreased by 19% following exposure to MK-801 when compared to saline, although no significant difference was observed when comparing MK-801, CPP and ketamine conditions (Figure 5B*ii,Cii*). This apparent discrepancy between *in vitro* and *in vivo* experiments regarding MK-801 could reflect a structure-specific effect or *in vivo* pharmacokinetic specificities. Importantly, NMDAR antagonists did not affect the synaptic levels of the PDZ domain-containing protein PSD-95, suggesting that short exposure to antagonists does not affect the postsynaptic density content in scaffolding proteins (Figure

5C,D). Additionally, co-immunoprecipitation analysis showed that inhibiting NMDAR with either CPP or MK-801 did not affect interactions between PSD-95 and synaptic GluN2A- or GluN2B-NMDAR subunits (Figure 5F,G). Therefore, drug-elicited changes in receptor conformation and surface dynamics do not seem to result from alterations in interactions with the PDZ domain-containing scaffold PSD-95, nor to produce major adjustments in NMDAR synaptic abundance.

NMDAR antagonists elicit drug-specific nanoscale reorganizations of postsynaptic NMDAR clusters

Over the past decade, the development of super-resolution imaging approaches revealed that the nanoscale organization of pre- and postsynaptic molecular actors contributes to shape the efficacy of glutamatergic synaptic transmissions (MacGillavry et al., 2013; Nair et al., 2013; Tang et al., 2016; Hruska et al., 2015 and 2018; Kellermayer et al., 2018; Haas et al., 2018; Goncalves et al., 2020; Ferreira et al., 2020). Stochastic optical reconstruction microscopy (STORM) at hippocampal synapses uncovered that NMDAR are not randomly distributed within postsynaptic terminals but form clusters of ~400 nm diameter on average, which harbor between one and three zones of receptor accumulation (~70 nm diameter on average) termed nanodomains (MacGillavry et al., 2013; Kellermayer et al., 2018; Ferreira et al., 2020). The organization of these nanodomains is dynamically regulated through interactions with cytosolic proteins and tunes NMDAR signalling and plasticity (Santucci and Raghavachari, 2008; Tang et al., 2016; Kellermayer et al., 2018). As diffraction-limited immunofluorescence acquisitions did not show major changes in the overall amount of NMDAR at synapses following exposure to antagonists (Figure 4), we used super-resolution imaging to investigate whether drug-elicited modulations of receptor conformation and surface diffusion would translate into variations in their subsynaptic organization (Figure 6A,B). As previously reported, glutamatergic synapses were found to harbor one NMDAR cluster on average, containing between one up to five nanodomains of receptors (Figure S6). Interestingly, blocking NMDAR with D-AP5 led to a decrease in nanodomain area and to an increase in nanodomain density, paralleled by a decrease in cluster area and an increase in cluster density, illustrating a profound reorganization of NMDAR at the nanoscale (Figures 6C and S6). Conversely, exposure to the OCB MK-801 and ketamine resulted in an increase in nanodomain area that was associated with a decrease in nanodomain density in the case of MK-801, but none of them affected either cluster area or cluster density, suggesting an opposite action at the nanoscale compared to D-AP5 (Figure 6C and S6). Higher concentrations of ketamine (100 μ M) had a similar action and yielded an increase in nanodomain area and a decrease in nanodomain density (Figure S7), together with an increase in synaptic NMDAR cluster area and unchanged cluster density. Additionally, ketamine at 100 μ M increased the number of

nanodomains per cluster (Figure S9). Importantly, the OCB memantine did not affect nanodomain area and density, nor did it impact NMDAR cluster area, although it increased the median number of nanodomains per cluster, suggesting that within the class of OCB, MK-801 and ketamine share the ability to trigger specific rearrangements in the subsynaptic organization of receptors (Figures 6C and S6). Interestingly, blocking neuronal firing with TTX (20 μ M) yielded similar rearrangements to those observed after exposure to D-AP5, i.e. a decrease in cluster area and increase in cluster and nanodomain density (Figures 6C and S6). Thus, complete blockade of NMDAR activity either by means of receptor or neuronal inhibition elicit comparable receptor reorganizations at the nanoscale that differ profoundly from those originating from exposure to memantine, MK-801 and ketamine. Taken together, these results suggest that the subsynaptic organization of NMDAR depends on neuronal activity and is impacted by antagonist binding in a subtype-specific manner, with the psychotomimetic OCB MK-801 and ketamine sharing a peculiar influence on receptor nanoscopic distribution at the postsynaptic density, possibly resulting from changes in signalling or interactions with scaffolding partners.

Direct interactions with PDZ domain-containing proteins and CaMKII contribute to the action of MK-801 and ketamine on NMDAR

We explored the mechanisms underlying the action of MK-801 and ketamine on NMDAR, and investigated its consequences on downstream signalling partners of the receptors. Interactions between the C-terminal domains of GluN2-NMDAR subunits and PDZ domain-containing scaffolds at the postsynaptic density regulate NMDAR synaptic anchoring in a ligand binding-dependent manner (Bard *et al.*, 2010). In order to assess the involvement of these interactions in OCB-driven receptor rearrangements, we went back to FLIM-FRET experiments using the protocol described above while disrupting the cytosolic association between GluN2B subunits and PDZ domain-containing scaffolds using a cell-permeant biomimetic peptide (TAT-2B) which competes with receptors for the binding to PDZ domains (Bard 2010). As a control, we first checked that a 1h pretreatment with either TAT-2B (10 μ M) or a non-sense peptide (TAT-NS; 10 μ M) would not harm FRET efficiency in GluN1-GFP, GluN2B-flag and GluN1-mCherry co-expressing neurons, and that co-application of ketamine (1 μ M) and NMDA (5 μ M) would not affect GFP fluorescence lifetime in the absence of an acceptor fluorophore (Figures 7A, S10, and S11). Co-application of ketamine and NMDA led to an increase in FRET efficiency in cells treated with the non-sense peptide, consistent with our previous findings. However, pretreatment with TAT-2B prevented this effect, revealing that interactions between GluN2B and PDZ domain-containing scaffolds are necessary for ketamine-driven conformational changes (Figure 7B,C). As PSD-95 appears as an unlikely candidate from *in vivo* biochemistry

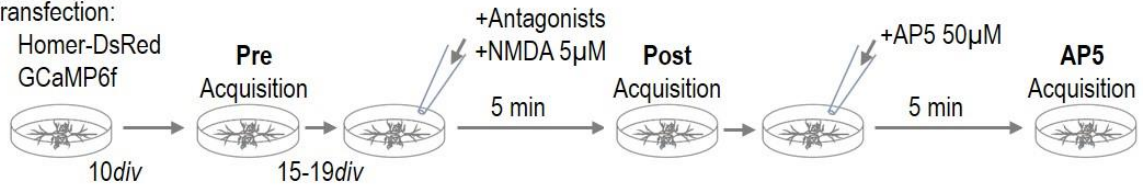
experiments, the respective contributions of other members of the MAGUK family will have to be dissected. Identifying whether ketamine binding enhances the affinity of NMDAR for these proteins will require further exploration.

Importantly, blocked receptors can still transmit information upon ligand binding, and non-ionic NMDAR signalling likely relies on receptor interactions with molecular partners within the PSD (Weilinger *et al.*, 2016). One of the most powerful regulators and downstream signalling targets of NMDAR is Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), which translocates and stabilizes at dendritic spines upon receptor activation through high affinity receptor binding, and contributes to its signalling both in ion flux-dependent and -independent manners to support the expression of synaptic plasticity and the formation of memory (Silva *et al.*, 1992; Stevens, Tonegawa and Wang, 1994; Lisman, Schulman and Cline, 2002; Coultrap *et al.*, 2014). An interesting feature of the activity-elicited recruitment of CaMKII to dendritic spines is that it is prevented by the NMDAR competitive antagonist D-AP5, by the disruption of GluN2B-CaMKII complexes or by alterations to NMDAR surface redistributions, indicative of a strong dependence upon functional and physical interactions with NMDAR (Morris *et al.*, 1986; Shen and Meyer, 1999; Bayer *et al.*, 2006; Dupuis *et al.*, 2014). To investigate whether OCB-driven changes receptor synaptic organization and diffusion may result in modifications of CaMKII intracellular dynamics, we performed FRAP experiments to assess the redistributions of α CaMKII within dendritic spines after a 1h incubation with either culture medium (control), D-AP5 (50 μ M), MK-801 (20 μ M), ketamine (1 μ M), memantine (1 μ M) or TTX (20 μ M) (Figure 8A). We observed that MK-801 and ketamine selectively increase the basal mobile fraction of α CaMKII, unlike D-AP5, memantine, and TTX (Figure 8B,C). To understand whether this effect was dependent on NMDAR-CaMKII interactions, we repeated this experiment while expressing a mutant of α CaMKII (lysine substitution by isoleucine at position 205, I205K) that is deficient for binding with GluN2B (Bayer *et al.*, 2001, 2006; Hudmon *et al.*, 2005). We found that MK-801 and ketamine do not affect the intracellular dynamics of α CaMKII^{I205K} within dendritic spines (Figure 8D,E). These results suggest that MK-801 and ketamine cause a reduction in CaMKII binding to the NMDAR, effectively increasing its mobility inside spines. Collectively, these findings suggest that by affecting receptor conformation and/or organization, the psychotomimetic OCB MK-801 and ketamine may change the distribution and activity of cytosolic NMDAR signalling partners in an ion flow-independent fashion via direct interactions (Figure 9).

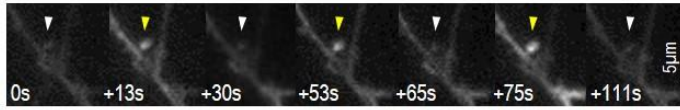
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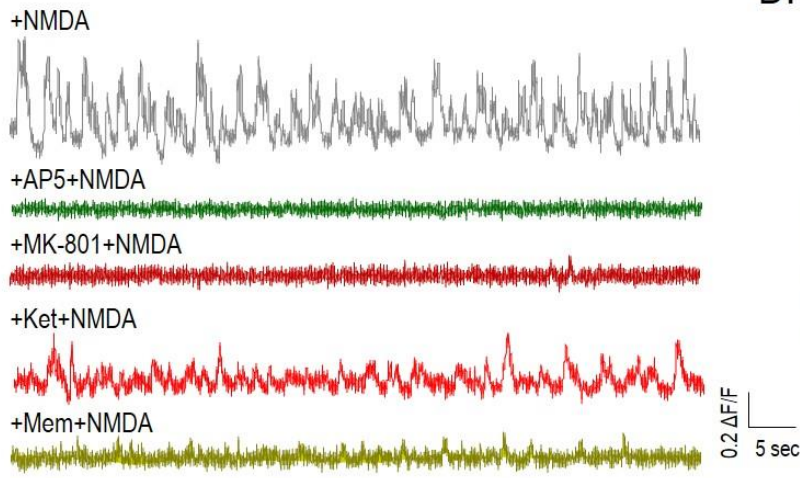
- Homer-DsRed
- GCaMP6f



B.



C.



D.

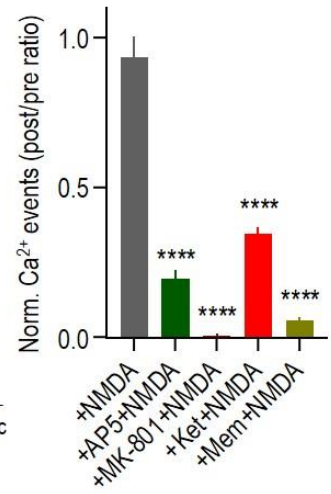
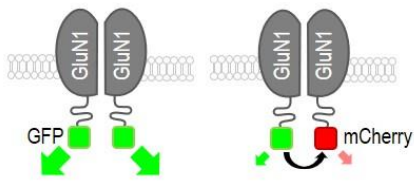


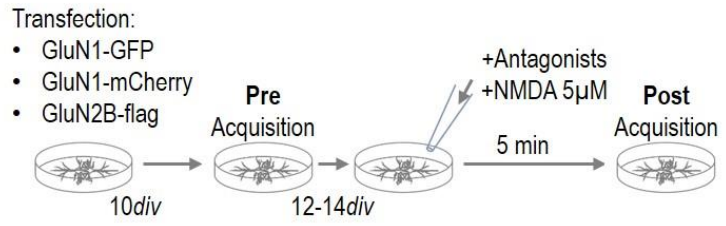
Figure 1 – NMDAR antagonists yield comparable inhibition of calcium transients in cultured hippocampal neurons

- (A) Schematic representation of the experimental design. Neurons were transfected with a fluorescent synaptic marker (Homer1c-DsRed) and a fluorescent calcium indicator (GCaMP6f). Calcium transients were recorded from individual spines of live neurons at basal state and after a 5 min co-application of NMDAR antagonists and NMDA (5 μ M). A final application of AP5 (50 μ M) for 5 min blocked all NMDAR-dependent activity. A calcium event was determined as a transient signal which surpassed the baseline of the recording by 5 standard deviations of the “AP5” acquisition.
- (B) Example of spontaneous calcium activity (70 s, 20 Hz acquisition rate) in a dendritic spine over time (white arrow, inactive spine, yellow arrow, calcium event).
- (C) Representative example traces of NMDAR-mediated calcium transients in single spines ($\Delta F/F$) after incubation with NMDA alone or in combination with the antagonists.
- (D) Normalized frequency of NMDAR-mediated calcium transients (ratio of calcium transient frequency before and after application of antagonists + NMDA). Data are expressed as mean \pm SEM (+NMDA, Nf = 0.9361 ± 0.0709 , n = 189 spines, N = 5 cells; +AP5+NMDA, Nf = 0.1952 ± 0.0294 , n = 97 spines, N = 4 cells; +MK-801+NMDA, Nf = 0.0076 ± 0.0057 , n = 79 spines, N = 3 cells; +Ket+NMDA, Nf = 0.3467 ± 0.0211 , n = 149 spines, N = 7 cells; +Mem+NMDA = 0.0561 ± 0.0121 , n = 85 spines, N = 3 cells; one-way ANOVA followed by Tukey's multiple comparisons test, ****p<0.0001).

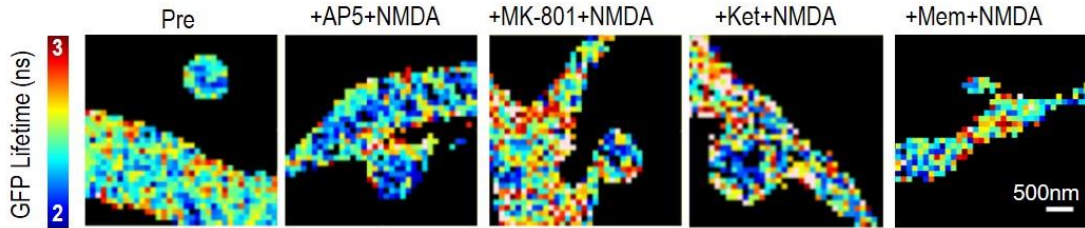
A.i



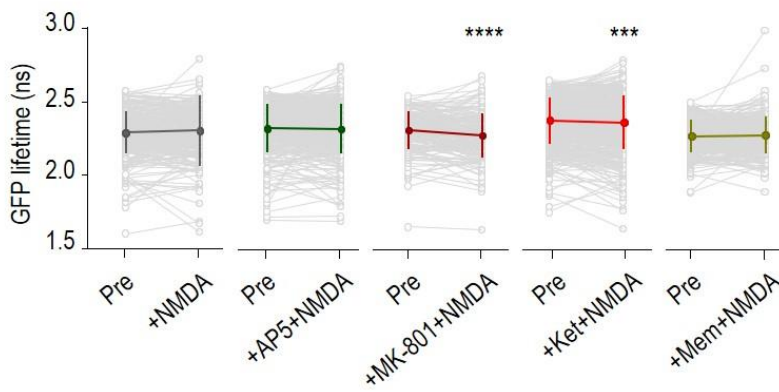
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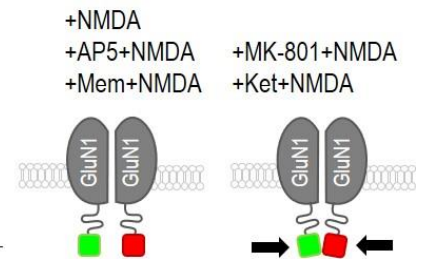


Figure 2 – Ketamine and MK-801 binding induces conformational changes in NMDAR cytoplasmic domains

(A) Schematic representation of the experimental design. (i) Principle of intra-receptor FRET experiments. Hippocampal neurons were transfected either with a recombinant GluN1 subunit fused with GFP at its C-terminal end alone (donor fluorophore only, left panel) or in combination with another recombinant version of GluN1 fused with mCherry at its C-terminal end (donor + acceptor fluorophores, right panel). All constructs were co-transfected along with GluN2B-flag to promote their surface expression. When both GluN1-GFP and GluN1-mCherry are co-expressed within a receptor, the donor fluorophore (GFP) transfers fluorescence resonance energy (black arrow) to the acceptor fluorophore (mCherry), causing excitation of the acceptor fluorophore and a subsequent decrease in the fluorescence lifetime of the donor fluorophore (green arrows). (ii) GFP fluorescence lifetime was acquired using Fluorescence Lifetime Imaging Microscopy before and after a 5 min application of NMDAR antagonists with NMDA (5 μ M).

(B) Representative images of the GFP lifetime in dendritic segments after the co-application of NMDAR antagonists with NMDA.

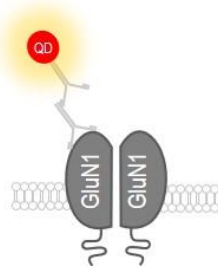
(C) GFP lifetime per GluN1-GFP cluster before and after acute NMDAR antagonist co-application with NMDA (Mean \pm SD GFP lifetime; Pre = 2.287 \pm 0.1440 ns, +NMDA = 2.301 \pm 0.2390 ns, n = 337 clusters, N = 36 cells; Pre = 2.316 \pm 0.1629 ns, +AP5+NMDA = 2.315 \pm 0.1687 ns, n = 373 clusters, N = 33 cells; Pre = 2.304 \pm 0.1269 ns, +MK-801+NMDA = 2.271 \pm 0.1462 ns, n = 183 clusters, N = 16 cells; Pre = 2.372 \pm 0.1561 ns, +Ket+NMDA = 2.358 \pm 0.1774 ns, n = 480 clusters, N = 44 cells; Pre = 2.266 \pm 0.1112 ns, +Mem+NMDA = 2.273 \pm 0.1208 ns, n = 285 clusters, N = 72 cells; Wilcoxon test, ***p<0.001, ****p<0.0001).

(D) Schematic representation of the effect of NMDAR antagonists.

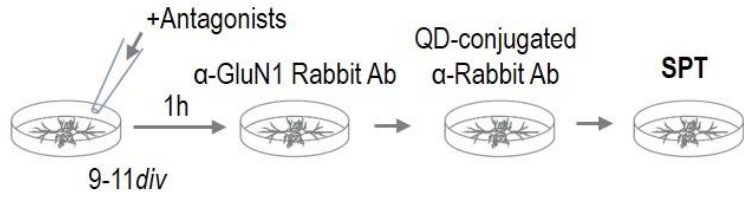
Table 1: Calculation of the effect of NMDAR antagonists on FRET efficiency and on the distance between GluN1 C-terminal tails

	<u>TD(ps)</u>	<u>TDA(ps)</u>	<u>EFRET</u>	<u>EFRET (%)</u>	<u>[(1/EFRET)-1]</u>	<u>[(1/EFRET)-1]^{1/6}</u>	<u>r(nm)</u>	<u>Pre/post difference (nm)</u>
Pre NMDA	2521	2287	0,093	9,282	9,774	1,462	7,896	
Post NMDA	2526	2301	0,089	8,907	10,227	1,473	7,956	0,060
Pre AP5+NMDA	2609	2316	0,112	11,230	7,904	1,411	7,621	
Post AP5+NMDA	2602	2315	0,110	11,030	8,066	1,416	7,647	0,026
Pre MK-801+NMDA	2553	2304	0,098	9,753	9,253	1,449	7,824	
Post MK-801+NMDA	2558	2271	0,112	11,220	7,913	1,412	7,623	-0,201
Pre Ket+NMDA	2596	2372	0,086	8,629	10,589	1,482	8,002	
Post Ket+NMDA	2601	2358	0,093	9,343	9,704	1,460	7,886	-0,116
Pre Mem+NMDA	2449	2266	0,075	7,472	12,383	1,521	8,214	
Post Mem+NMDA	2452	2273	0,073	7,300	12,698	1,527	8,248	0,035
	<u>TD(ps)</u>	<u>TDA(ps)</u>	<u>EFRET</u>	<u>EFRET (%)</u>	<u>[(1/EFRET)-1]</u>	<u>[(1/EFRET)-1]^{1/6}</u>	<u>r(nm)</u>	<u>Pre/post difference (nm)</u>
TAT-NS	2460	2279	0,074	7,358	12,591	1,525	8,236	
TAT-NS Ket+NMDA	2470	2264	0,083	8,340	10,990	1,491	8,052	-0,185
TAT-2B	2484	2245	0,096	9,622	9,393	1,453	7,844	
TAT-2B Ket+NMDA	2477	2252	0,091	9,084	10,009	1,468	7,927	0,083

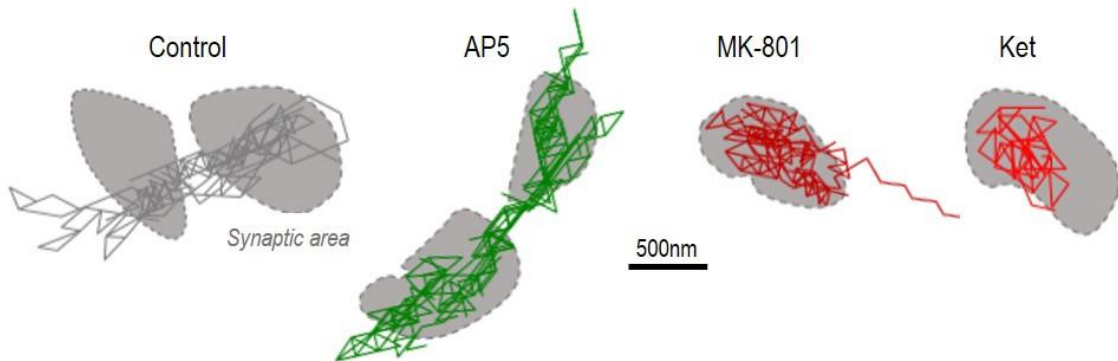
A.i



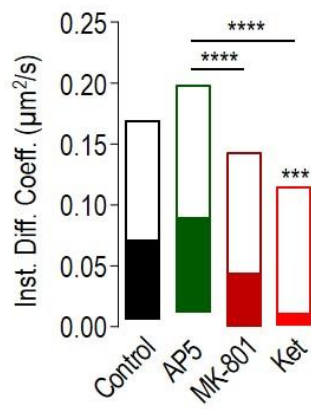
A.ii



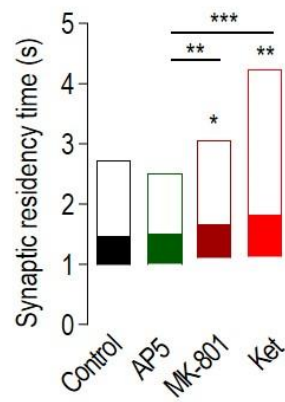
B.



C.



D.



E.

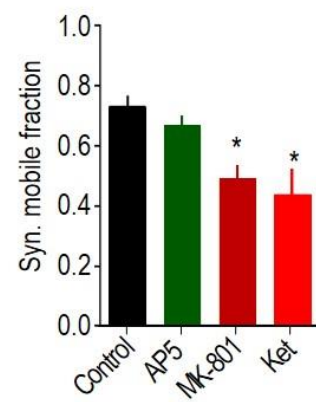


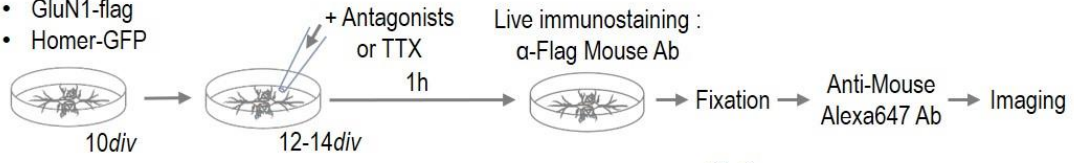
Figure 3 – NMDAR open-channel blockers change NMDAR surface trafficking at synapses

- (A) Schematic representation of the experimental design. (i) Schematic representation of a QD-labeled NMDAR. (ii) After a 1h treatment with NMDAR antagonists, live neurons were sequentially incubated with primary antibodies against an N-terminal epitope of the GluN1 subunit and with QD-conjugated secondary antibodies to track the surface movements of individual NMDAR.
- (B) Representative trajectories (25 s, 20 Hz acquisition rate) of synaptic NMDAR in the control condition (grey) or after 1h treatment with D-AP5 (50 μM , green), MK-801 (20 μM , wine) or ketamine (1 μM , red). Grey dotted areas, postsynaptic density.
- (C) Instantaneous diffusion coefficients of synaptic receptors. Data are expressed as median \pm 25%-75% IQR (Control, $D = 0.0698 \pm 0.0057\text{-}0.1685 \mu\text{m}^2/\text{s}$, $n = 341$ trajectories, $N = 14$ cells; AP5, $D = 0.0688 \pm 0.0106\text{-}0.1970 \mu\text{m}^2/\text{s}$, $n = 853$ trajectories, $N = 27$ cells; MK-801, $D = 0.0425 \pm 0.0006\text{-}0.1428 \mu\text{m}^2/\text{s}$, $n = 540$ trajectories, $N = 24$ cells; Ket, $D = 0.010 \pm 0.0003\text{-}0.1130 \mu\text{m}^2/\text{s}$, $n = 226$ trajectories, $N = 14$ cells; Kruskal-Wallis followed by Dunn's multiple comparison test, *** $p < 0.001$, **** $p < 0.0001$).
- (D) NMDAR synaptic residency time. Data are expressed as median \pm 25%-75% IQR (Control, $R_t = 1.45 \pm 1.00\text{-}2.70$ s, $n = 341$ trajectories, $N = 14$ cells; AP5, $R_t = 1.50 \pm 1.00\text{-}2.50$ s, $n = 835$ trajectories, $N = 27$ cells; MK-801, $R_t = 1.65 \pm 1.10\text{-}3.05$ s, $n = 540$ trajectories, $N = 24$ cells; Ket, $R_t = 1.80 \pm 1.14\text{-}4.20$ s, $n = 226$ trajectories, $N = 14$ cells; Kruskal-Wallis followed by Dunn's multiple comparison test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).
- (E) Synaptic NMDAR mobile fraction. Data are expressed as mean \pm SEM (Control, $M_f = 0.733 \pm 0.032$, $n = 341$ trajectories, $N = 14$ cells; AP5, $M_f = 0.663 \pm 0.036$, $n = 835$ trajectories, $N = 27$ cells; MK-801, $M_f = 0.489 \pm 0.047$, $n = 540$ trajectories, $N = 24$ cells; Ket, $M_f = 0.44 \pm 0.082$, $n = 226$ trajectories, $N = 14$ cells; one-way ANOVA followed by Tukey's multiple comparison test, * $p < 0.05$).

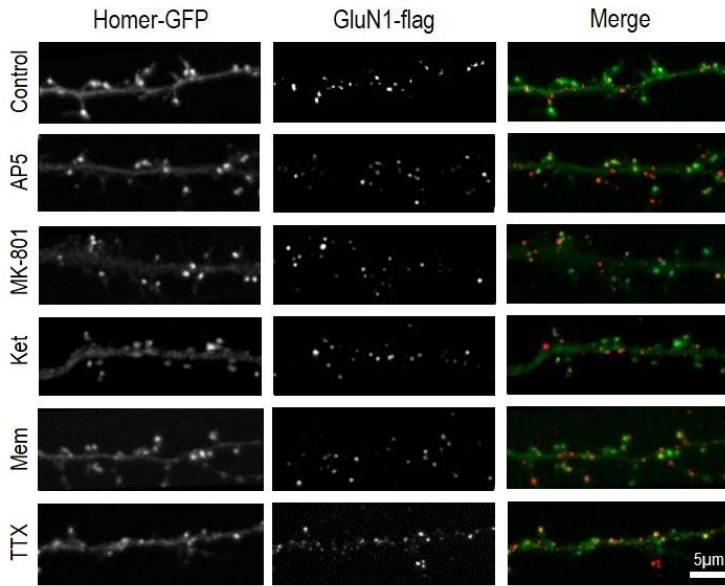
A.

Transfection

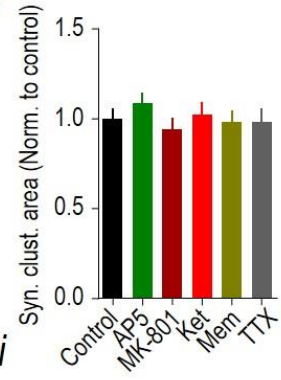
- GluN1-flag
- Homer-GFP



B.



C.i



C.ii

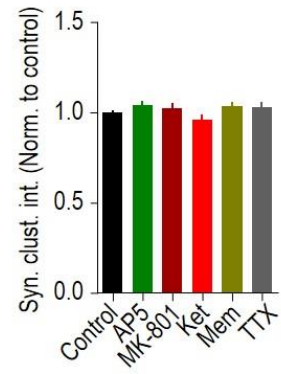


Figure 4 – Inhibition of NMDAR does not impact NMDAR surface expression

- (A) Schematic representation of the experimental design. Neurons were elicited to express GluN1-flag and Homer-GFP. After a 1h treatment with TTX or NMDAR antagonists, live neurons were incubated with primary antibodies against flag, then fixed and stained with secondary antibodies.
- (B) Representative dendritic segments of hippocampal neurons immunostained for GluN1-flag-containing NMDAR (red) and Homer-GFP (green) after exposure to buffer (control), AP5, MK-801, ketamine, memantine, or TTX.
- (C) (i) Synaptic NMDAR cluster area (normalized to control). Data are expressed as mean \pm SEM (Control, $Ca = 1 \pm 0.057$, $N = 50$ cells; AP5, $Ca = 1.085 \pm 0.057$, $N = 40$ cells; MK-801, $Ca = 0.938 \pm 0.066$, $N = 32$ cells; Ket, $Ca = 1.019 \pm 0.074$, $N = 38$ cells; Mem, $Ca = 0.979 \pm 0.064$, $N = 43$ cells; TTX, $Ca = 0.983 \pm 0.070$, $N = 34$ cells; one-way ANOVA followed by Tukey's multiple comparison test, $p > 0.05$). (ii) Synaptic NMDAR cluster intensity (normalized to control). Data are expressed as mean \pm SEM (Control, $Ci = 1 \pm 0.015$, $N = 50$ cells; AP5, $Ci = 1.041 \pm 0.022$, $N = 40$ cells; MK-801, $Ci = 1.024 \pm 0.032$, $N = 32$ cells; Ket, $Ci = 0.960 \pm 0.028$, $N = 38$ cells; Mem, $Ci = 1.036 \pm 0.024$, $N = 43$ cells; TTX, $Ci = 1.030 \pm 0.027$, $N = 34$ cells; one-way ANOVA followed by Tukey's multiple comparison test, $p > 0.05$).

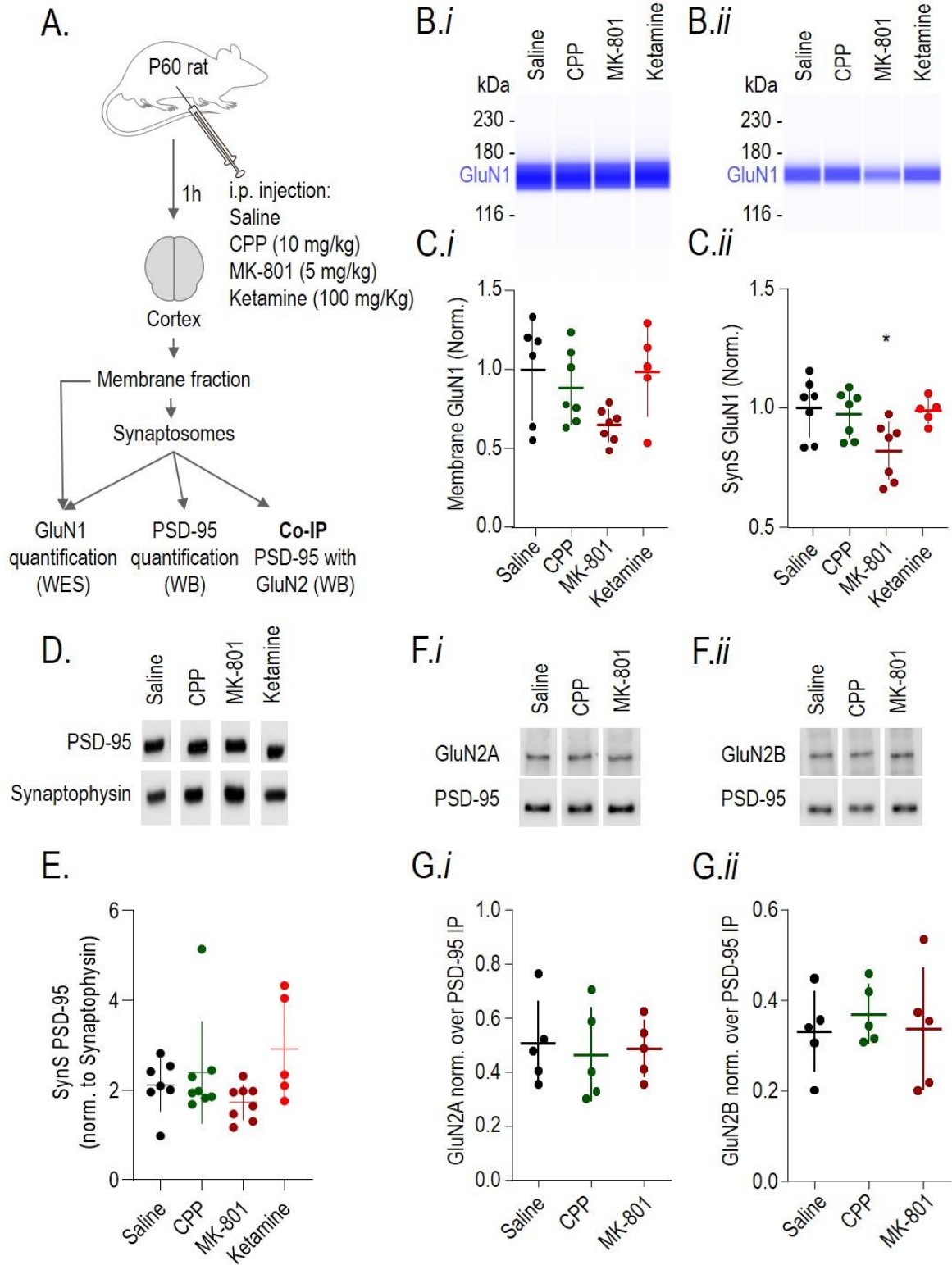


Figure 5 – MK-801 administration (1h) decreases synaptic NMDAR content *in vivo* without affecting interactions between GluN2-NMDAR and PSD-95

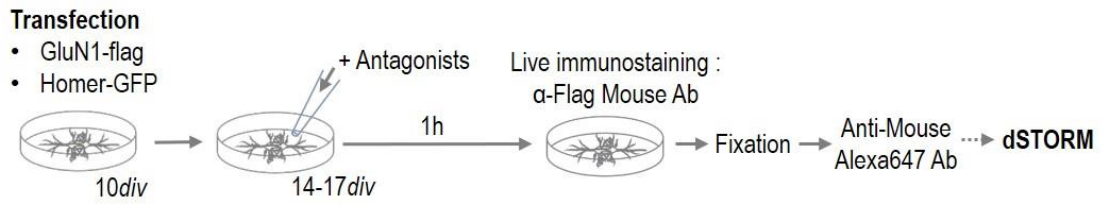
- (A) Schematic representation of the experimental design. Saline, CPP (10 mg/kg), MK-801 (5 mg/kg) or ketamine (100 mg/kg) were administered intraperitoneally to P60 Sprague-Dawley rats. 1h after injections, animals were sacrificed and whole cortices were removed for synaptosome preparation. GluN1 quantification was performed in cortical synaptosomes and membrane fraction. PSD-95 quantification and co-immunoprecipitation of PSD-95 and GluN2 subunits were performed in synaptosomes.
- (B) Representative immunoblots created through WES™ technology showing the expression of the GluN1 NMDAR subunit in (i) membrane-enriched fraction or (ii) synapse-enriched fraction of cortical samples of P60 rats injected with saline or the NMDAR antagonists CPP (10 mg/kg), MK-801 (5 mg/kg), or ketamine (100 mg/kg).
- (C) (i) Quantification of GluN1 in membrane fraction (normalized first to total protein content and then to the average value of control (saline injection) samples in each run). Data are represented as mean ± SD (Saline, GluN1 = 1 ± 0.3252, n = 6 animals; CPP, GluN1 = 0.8860 ± 0.2340, n = 7 animals; MK-801, GluN1 = 0.6462 ± 0.1056, n = 7 animals; Ketamine, GluN1 = 0.9879 ± 0.2858, n = 5 animals; one-way ANOVA followed by Tukey's multiple comparisons test, p>0.05). (ii) Quantification of GluN1 subunit content in cortical synapse-enriched fractions (synaptosomes, SynS) (normalized first to total protein content and then to the average value of control (saline injection) samples in each run). Data are expressed as mean ± SD (Saline, GluN1 = 1 ± 0.1239, n = 7 animals; CPP, GluN1 = 0.9727 ± 0.09846, n = 7 animals; MK-801, GluN1 = 0.8202 ± 0.1233, n = 7 animals; Ket, GluN1 = 0.9879 ± 0.05426, n = 5 animals; one-way ANOVA followed by Tukey's multiple comparison test, *p<0.05).
- (D) Representative immunoblots achieved through classical Western Blot protein detection showing the expression of PSD-95 and Synaptophysin in cortical synaptosomes of P60 rats injected with saline or the NMDAR antagonists CPP (10 mg/kg), MK-801 (5 mg/kg), or ketamine (100 mg/kg).
- (E) Quantification of PSD-95 in synapse-enriched fraction (synaptosomes, SynS)(normalized to Synaptophysin). Data are expressed as mean ± SD (Saline, PSD-95 = 2.117 ± 0.5911,

n = 7 animals; CPP, PSD-95 = 2.397 ± 1.138 , n = 8 animals; MK-801, PSD-95 = 1.729 ± 0.3943 , n = 8 animals; Ketamine, PSD-95 = 2.918 ± 1.186 , n = 5 animals; one-way ANOVA followed by Tukey's multiple comparisons test, $p > 0.05$).

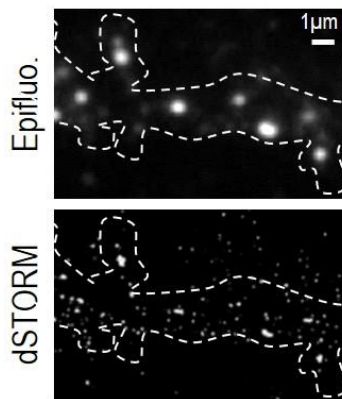
(F) Representative immunoblots showing co-immunoprecipitation of the (i) GluN2A or (ii) GluN2B NMDAR subunits (~170 kDa) with PSD-95 (~95 kDa) in cortical synaptosomes from P60 rats injected with saline, CPP (10 mg/kg) or MK-801 (5 mg/kg).

(G) Quantification of GluN2-NMDAR/PSD-95 co-immunoprecipitation in cortical synapse-enriched fractions. (i) Quantification of PSD-95 / GluN2A-NMDAR co-immunoprecipitation. Data are represented as mean \pm SD (Saline, GluN2A = 0.5049 ± 0.1589 , n = 5 animals; CPP, GluN2A = 0.4644 ± 0.1754 , n = 5 animals; MK-801, GluN2A = 0.4847 ± 0.1065 , n = 5 animals; one-way ANOVA followed by Tukey's multiple comparisons test, $p > 0.05$) (ii) Quantification of PSD-95 / GluN2B-NMDAR co-immunoprecipitation. Data are represented as median \pm SD (Saline, GluN2B = 0.3307 ± 0.0892 , n = 5 animals; CPP, GluN2B = 0.3692 ± 0.0665 , n = 5 animals; MK-801, GluN2B = 0.3371 ± 0.1358 , n = 5 animals; one-way ANOVA followed by Tukey's multiple comparisons test, $p > 0.05$).

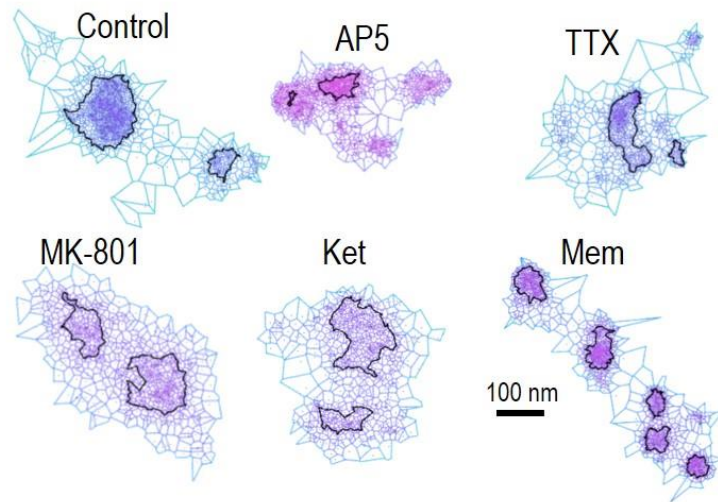
A.



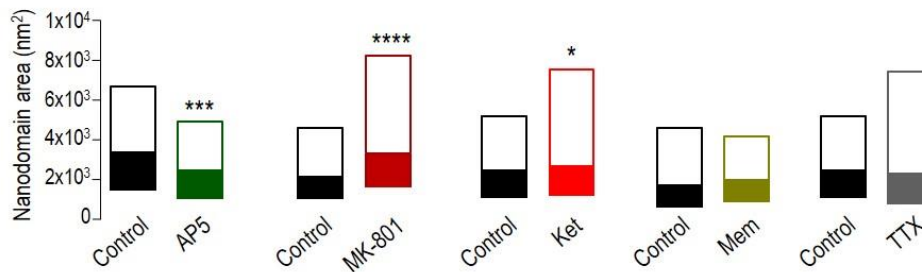
B.i



B.ii



C.i



C.ii

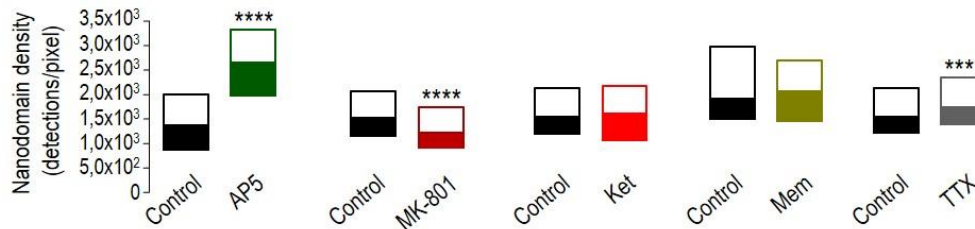


Figure 6 – Ketamine and MK-801 promote a nanoscale reorganization of postsynaptic NMDAR clusters

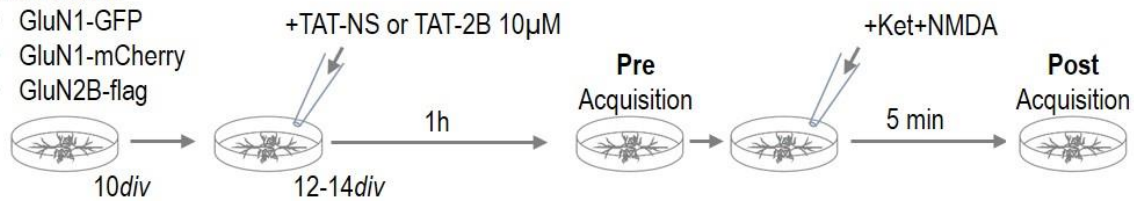
- (A) Schematic representation of the experimental design. Neurons were elicited to express Homer1c-GFP and GluN1-flag. After a 1h treatment with TTX or NMDAR antagonists, live neurons were incubated with primary antibodies against flag, then fixed and stained with secondary antibodies. Fixed samples were imaged using direct Stochastic Optical Reconstruction Microscopy.
- (B) (i) Epifluorescence (upper panel) and dSTORM (lower panel) images of a dendritic segment with GluN1-flag staining. (ii) Examples of super-resolved postsynaptic NMDAR clusters from each experimental condition. Grey points indicate fluorophore detections. Polygons around detections were generated through tessellation. Black outlines indicate intra-cluster receptor nanodomains.
- (C) (i) Nanodomain area. Data are expressed as median \pm 25%-75% IQR (Control, Na = 3359 \pm 1411-6676 nm², n = 333 nanodomains, N = 7 cells; AP5, Na = 2421 \pm 989.8-4902 nm², n = 222 nanodomains, N = 7 cells; Control, Na = 2275 \pm 1138-4653 nm², n = 305 nanodomains, N = 9 cells; MK-801 = 3433 \pm 1733-8232 nm², n = 327 nanodomains, N = 6 cells; Control, Na = 2583 \pm 1214-5246 nm², n = 228 nanodomains, N = 8 cells; Ket, Na = 2804 \pm 1353-7564 nm², n = 522 nanodomains, N = 8 cells; Control, Na = 1875 \pm 793.1-4713 nm², n = 149 nanodomains, N = 8 cells; Mem, Na = 2113 \pm 1010-4272 nm², n = 574 nanodomains, N = 7 cells; Control, Na = 2583 \pm 1214-5246 nm², n = 228 nanodomains, N = 8 cells; TTX, Na = 2434 \pm 904-7481 nm², n = 330 nanodomains, N = 10 cells; Mann-Whitney test, *p<0.05, ***p<0.0005, ****p<0.0001). (ii) Nanodomain density. Data are expressed as median \pm 25%-75% IQR (Control, Nd = 1365 \pm 866.2-1989 detections/pixel, n = 333 nanodomains, N = 7 cells; AP5, Nd = 2646 \pm 1952-3312 detections/pixel, n = 222 nanodomains, N = 7 cells; Control, Nd = 1502 \pm 1113-2061 detections/pixel, n = 305 nanodomains, N = 9 cells; MK-801, Nd = 1215 \pm 879.6-1739 detections/pixel, n = 327 nanodomains, N = 6 cells; Control, Nd = 1531 \pm 1189-2116 detections/pixel, n = 228 nanodomains, N = 8 cells; Ket, Nd = 1608 \pm 1028-2163 detections/pixel, n = 522 nanodomains; N = 8 cells; Control, Nd = 1916 \pm 1484-2966 detections/pixel, n = 149 nanodomains, N = 8 cells; Mem, Nd = 2063 \pm 1425-2697 detections/pixel, n = 574 nanodomains, N = 7 cells; Control, Nd = 1531 \pm 1189-2116 detections/pixel, n = 228

nanodomains, N = 8 cells; TTX, Nd = 1712 ± 1381 -2323 detections/pixel, n = 330 nanodomains, N = 10 cells; Mann-Whitney test, ****p<0.0001).

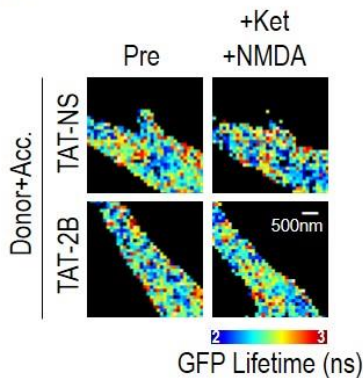
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Transfection:

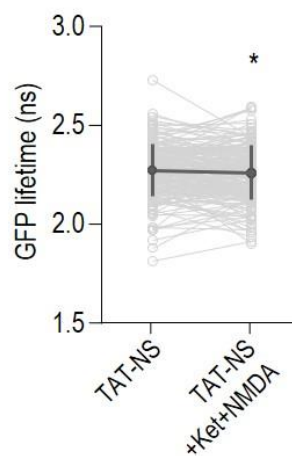
- GluN1-GFP
- GluN1-mCherry
- GluN2B-flag



B.



C.i



C.ii

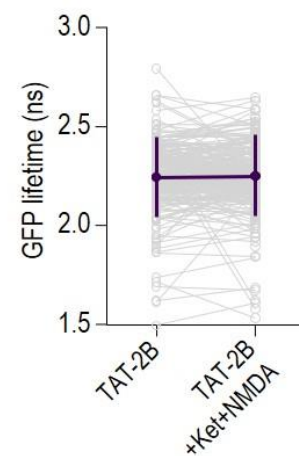


Figure 7 – Interactions between GluN2B and PDZ domain proteins are necessary for ketamine-induced conformational changes

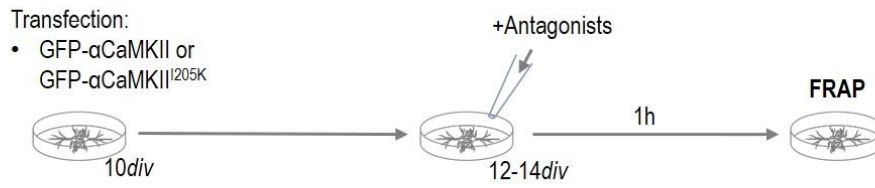
(A) Schematic representation of the experimental design. Neurons were transfected with GluN1-GFP, GluN1-mCherry, and GluN2B-flag. After a 1h incubation with either TAT-NS or TAT-2B, GFP fluorescence lifetime was acquired using Fluorescence Lifetime Imaging Microscopy before and after a 5 min application of NMDAR antagonists with NMDA (5 µM).

(B) Representative images of the GFP lifetime in dendritic segments neurons treated with either TAT-NS or TAT-2B before and after co-application of ketamine and NMDA 5µM.

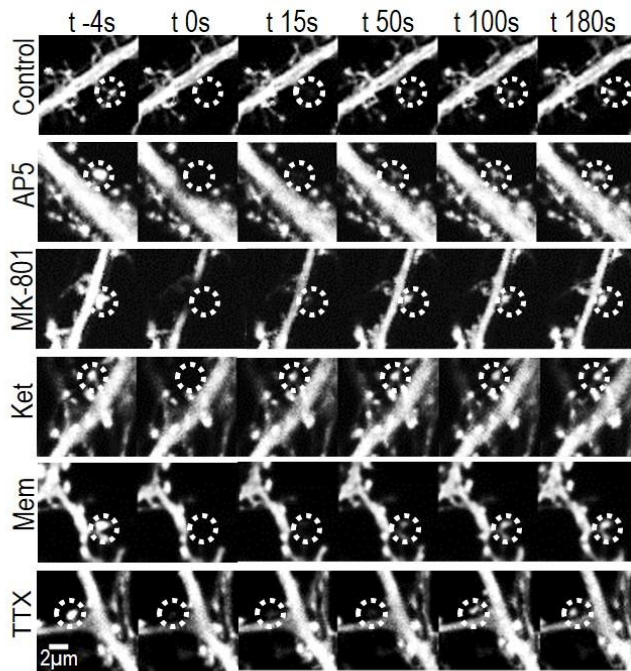
(C) (i) Impact of Ketamine + NMDA application on GFP lifetime per GluN1-GFP cluster after pre-treatment with TAT-NS (Mean ± SD GFP lifetime; TAT-NS = 2.279 ± 0.1330 ns, TAT-NS + Ket + NMDA = 2.264 ± 0.1374 ns, n = 195 clusters, N = 81 cells; Wilcoxon test, *p<0.05). (ii) Impact of Ketamine + NMDA application on GFP lifetime per GluN1-GFP

cluster after pre-treatment with TAT-2B (Mean \pm SD GFP lifetime; TAT-2B = 2.245 ± 0.2043 ns, TAT-2B + Ket + NMDA = 2.252 ± 0.2056 ns, n = 234 clusters, N = 88 cells; Wilcoxon test, $p > 0.05$).

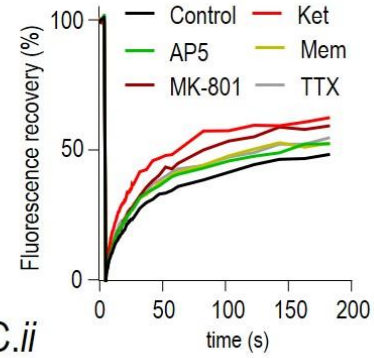
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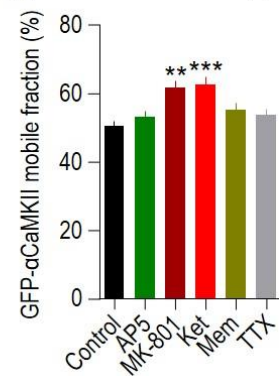
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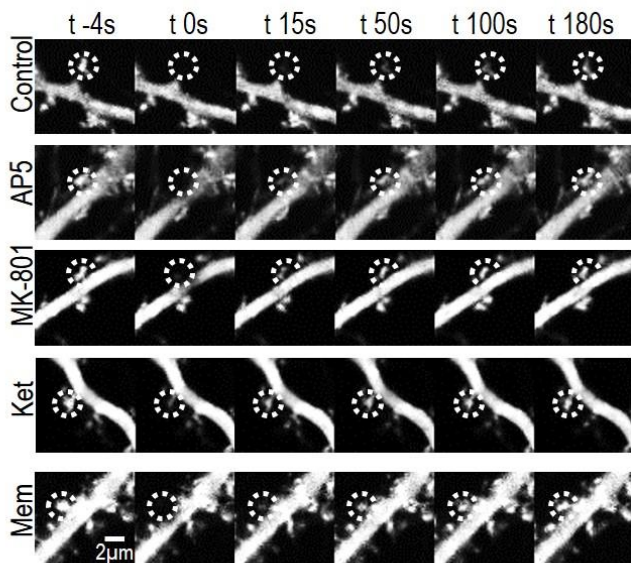
C.i



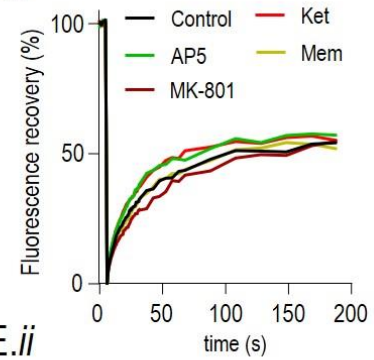
C.ii



D.



E.i



E.ii

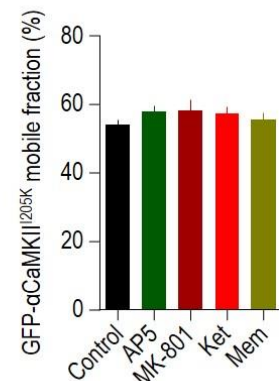


Figure 8 – Ketamine and MK-801 promote CaMKII spine mobility through direct interactions with NMDAR

- (A) Schematic representation of the experimental design. Neurons were transfected to express GFP- α CaMKII. After a 1h treatment with NMDAR antagonists or TTX, the intracellular dynamics of GFP- α CaMKII into dendritic spines were imaged in live neurons using fluorescence recovery after photobleaching (FRAP).
- (B) Representative images of GFP- α CaMKII fluorescence in each experimental condition before (t-4s), immediately after (t0s), and 15, 50, 100, and 180 seconds after dendritic spine photobleaching (dotted circle, photobleached area).
- (C) (i) Fluorescence recovery of the photobleached area over time. (ii) GFP- α CaMKII mobile fraction. Data are expressed as mean \pm SEM (Control, Mf = 50.32 ± 1.64 %, n = 100 spines, N = 11 cells; AP5, Mf = 53.23 ± 1.83 %, n = 87 spines, N = 8 cells; MK-801, Mf = 61.59 ± 2.16 %, n = 85 spines, N = 8 cells; Ket, Mf = 62.56 ± 2.47 %, n = 85 spines, N = 7 cells; Mem, Mf = 55.11 ± 2.08 %, n = 116 spines, N = 7 cells; TTX, Mf = 53.56 ± 1.915 %, n = 108 spines, N = 8 cells; one-way ANOVA followed by Tukey's multiple comparisons test, **p<0.01, ***p<0.001).
- (D) Representative images of GFP- α CaMKII^{I205K} fluorescence in each experimental condition before (t-4s), immediately after (t0s) and 15, 50, 100, and 180 seconds after (t50s) photobleaching (dotted circle, photobleached area).
- (E) (i) Fluorescence recovery of the photobleached area over time. (ii) GFP- α CaMKII^{I205K} mobile fraction. Data are expressed as mean \pm SEM (Control, Mf = 54.09 ± 1.53 %, n = 84 spines, N = 10 cells; AP5, Mf = 57.89 ± 1.88 %, n = 63 spines, N = 8 cells; MK-801, Mf = 58.14 ± 3.39 %, n = 66 spines, N = 6 cells; Ket, Mf = 57.28 ± 2.02 %, n = 69 spines, N = 8 cells; Mem, Mf = 55.53 ± 2.15 %, n = 74 spines, N = 7 cells; one-way ANOVA followed by Tukey's multiple comparisons test, p>0.05).

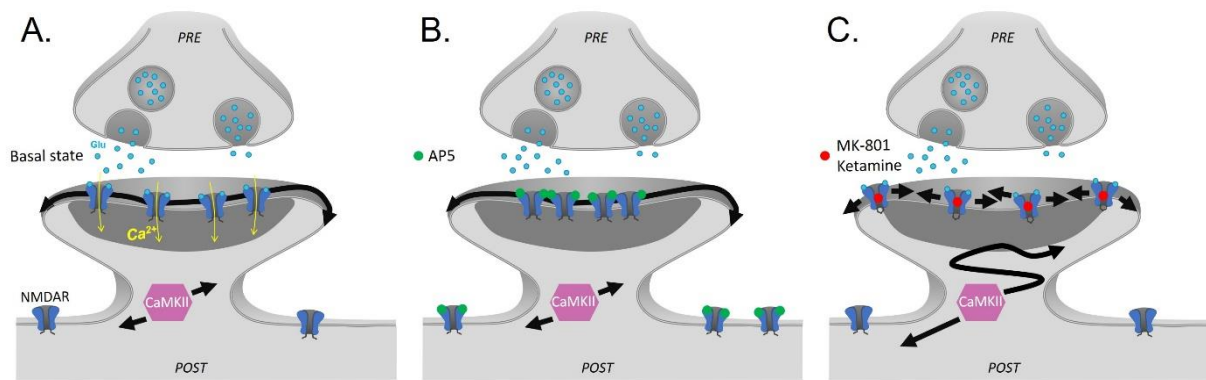


Figure 9 – Schematic representation of the main results

- (A) In basal state, synaptic NMDA receptors (blue and grey structures, GluN1 carboxy-terminal tails are represented) allow for the influx of calcium (yellow arrows) upon the binding of glutamate (blue circles). NMDAR are mobile at the neuronal surface and CaMKII (purple hexagon) is mobile at the cytoplasm (black arrows).
- (B) The competitive antagonist AP5 (green circles) binds to the glutamate site and prevents receptor activation, leading to a huddling of surface receptors without affecting NMDAR surface trafficking or CaMKII mobility.
- (C) The uncompetitive antagonists MK-801 and ketamine bind to NMDAR which had been previously activated. The binding of these drugs to the ion pore of the receptor leads to a change in NMDAR conformation that results in the approximation of GluN1 carboxy-terminal tails, a decrease in the surface mobility of NMDAR, a scattering of surface receptors, and an increase in mobility of synaptic CaMKII.

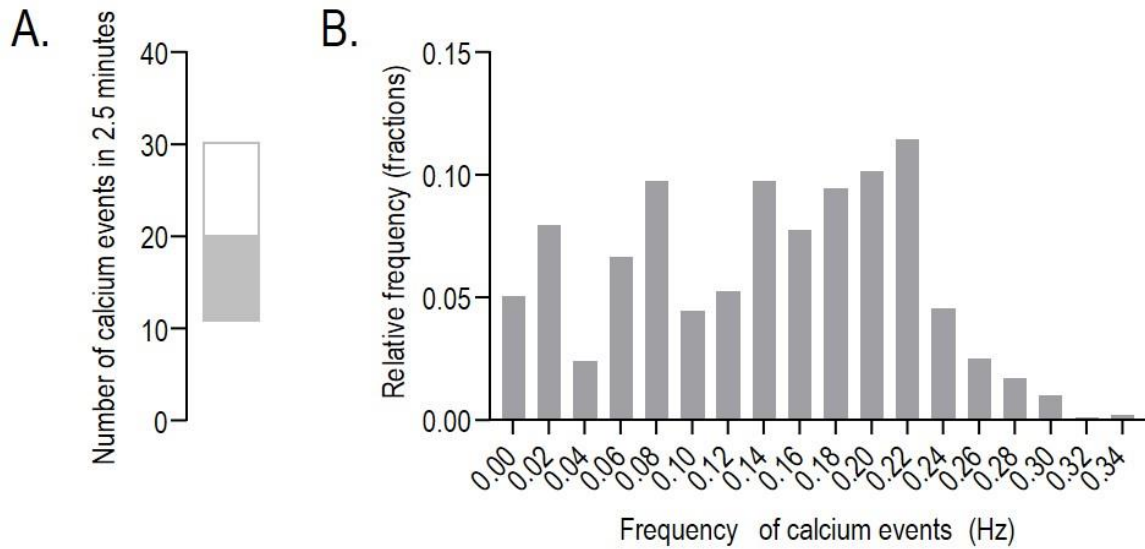


Figure S1 – Spontaneous activity in cultured hippocampal neurons

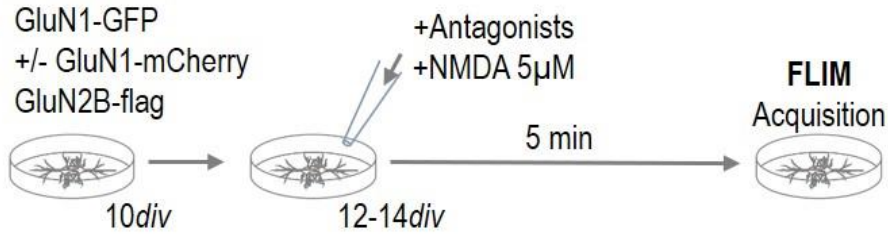
(A) Number of calcium events at basal state in individual dendritic spines over the length of an acquisition (2.5 minutes). Data are expressed as median \pm 25-75% IQR (median number of events = $20 \pm 11-30$, $n = 888$ spines, $N = 33$ cells).

(B) Relative distribution of calcium event frequency in individual dendritic spines. Data are expressed as median \pm 25-75% IQR (median frequency = $0.1467 \pm 0.0733-0.2067$ Hz, $n = 888$ spines, $N = 33$ cells).

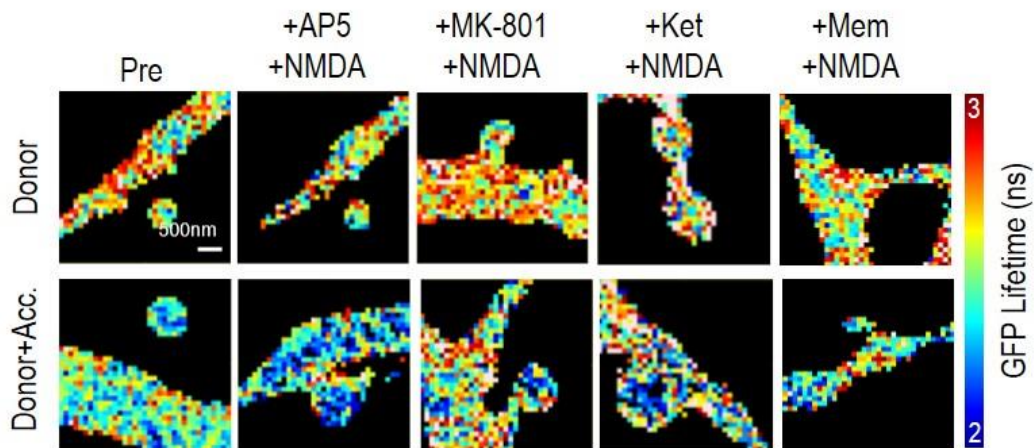
A.

Transfection:

- GluN1-GFP
- +/- GluN1-mCherry
- GluN2B-flag



B.



C.

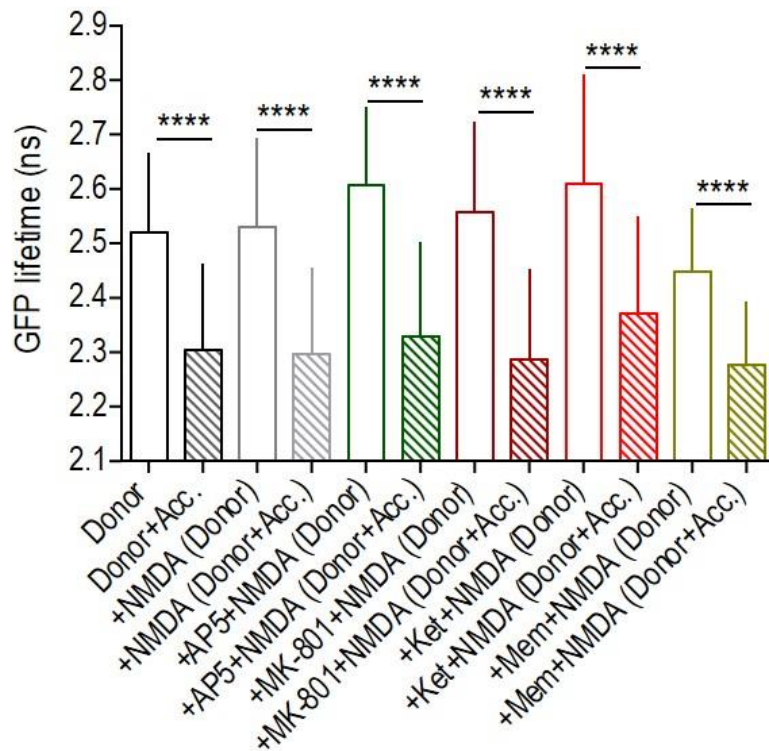


Figure S2 – NMDAR antagonists do not prevent FRET between GluN1-GFP and GluN1-mCherry

- (A) Schematic representation of the experimental design. Neurons were transfected with GluN2B-flag and GluN1-GFP, either with or without co-transfection with GluN1-mCherry. GFP fluorescence lifetime was acquired using Fluorescence Lifetime Imaging Microscopy after a 5 min application of NMDAR antagonists with NMDA 5 μ M.
- (B) Representative images of the GFP lifetime in dendritic segments of cells transfected with either GluN1-GFP alone (Donor, upper panels) or GluN1-GFP with GluN1-mCherry (Donor+Acc., lower panels) after co-application of NMDAR antagonists with NMDA.
- (C) GFP lifetime per GluN1-GFP cluster after exposure to NMDAR antagonists of cells transfected with either GluN1-GFP alone (Donor) or GluN1-GFP with GluN1-mCherry (Donor + Acc.). Data are expressed as mean \pm SD (Donor, GI = 2.521 \pm 0.1456 ns, n = 428 clusters, N = 36 cells; Donor + Acc., GI = 2.287 \pm 0.1440 ns, n = 337 clusters, N = 36 cells; +NMDA(Donor) = 2.526 \pm 0.1627 ns, n = 428 clusters, N = 36 cells; +NMDA(Donor+Acc.), GI = 2.301 \pm 0.2390 ns, n = 337 clusters, N = 36 cells; +AP5+NMDA(Donor), GI = 2.602 \pm 0.1449 ns, n = 488 clusters, N = 28 cells; +AP5+NMDA(Donor+Acc.), GI = 2.315 \pm 0.1687 ns, n = 373 clusters, N = 33 cells; +MK-801+NMDA(Donor), GI = 2.558 \pm 0.1599 ns, n = 389 clusters, N = 17 cells; +MK-801+NMDA(Donor+Acc.), GI = 2.271 \pm 0.1462 ns, n = 183 clusters, N = 16 cells; +Ket+NMDA(Donor), GI = 2.601 \pm 0.2053 ns, n = 310 clusters, N = 43 cells; +Ket+NMDA(Donor+Acc.), GI = 2.358 \pm 0.1774 ns, n = 480 clusters, N = 44 cells; +Mem+NMDA(Donor), GI = 2.452 \pm 0.1159 ns, n = 207 clusters, N = 53 cells; +Mem+NMDA(Donor+Acc.), GI = 2.273 \pm 0.1208 ns, n = 285 clusters, N = 72 cells; Kruskal-Wallis followed by Dunn's multiple comparison test, ****p<0.0001).

A.

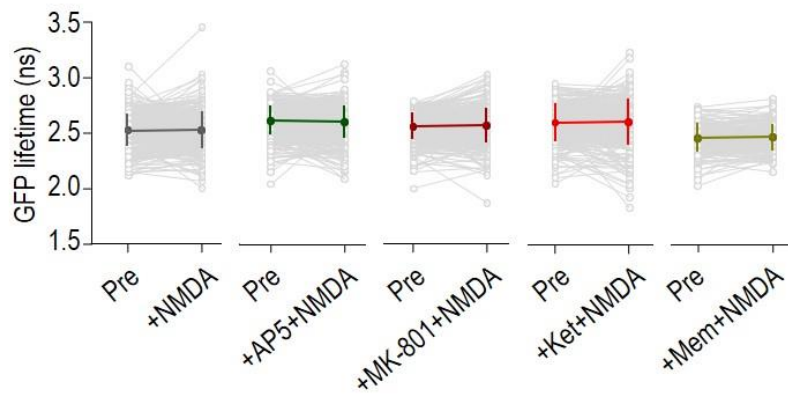
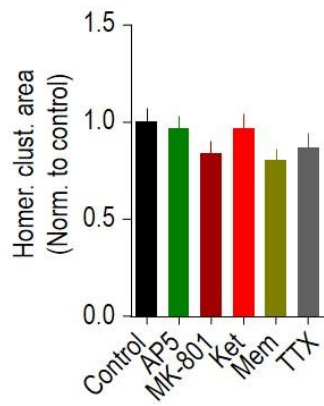


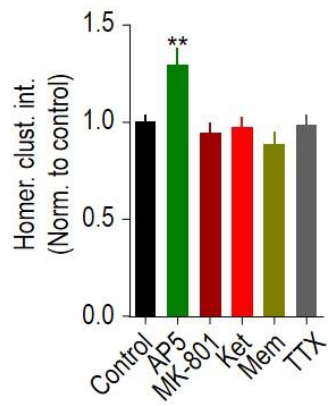
Figure S3 – NMDAR antagonists do not impact the fluorescence lifetime of the donor fluorophore

(A) Impact of NMDAR antagonists + NMDA 5 μ M application on GFP lifetime per GluN1-GFP cluster in cells expressing only GluN1-GFP and GluN2B-flag (Mean \pm SD GFP lifetime; Pre = 2.521 \pm 0.1456 ns, +NMDA = 2.526 \pm 0.1627 ns, n = 428 clusters, N = 36 cells; Pre = 2.609 \pm 0.1299 ns, +AP5+NMDA = 2.602 \pm 0.1449 ns, n = 488 clusters, N = 28 cells; Pre = 2.553 \pm 0.1196 ns, +MK-801+NMDA = 2.558 \pm 0.1599 ns, n = 389 clusters, N = 17 cells; Pre = 2.596 \pm 0.1671, +Ket+NMDA = 2.601 \pm 0.2053 ns, n = 310 clusters, N = 43 cells; Pre = 2.449 \pm 0.1256 ns, +Mem+NMDA = 2.452 \pm 0.1159 ns, n = 207 clusters, N = 53 cells; Wilcoxon test, p>0.05).

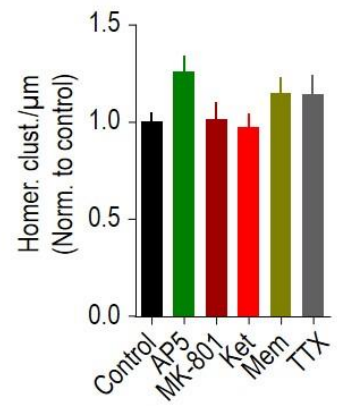
A.i



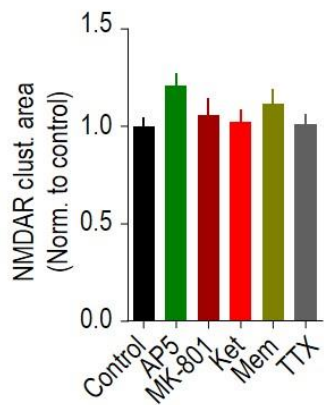
A.ii



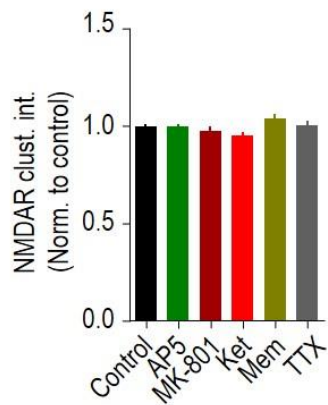
A.iii



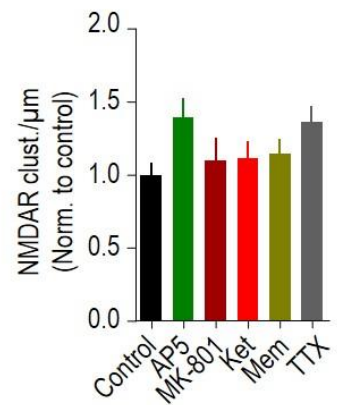
B.i



B.ii



B.iii



C.

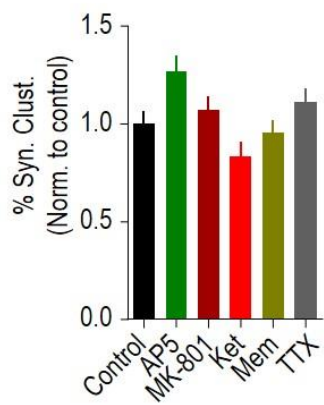


Figure S4 – Exposure to NMDAR antagonists and TTX (1h) does not impact the expression and distribution of surface NMDAR

(A) (i) Homer cluster area (normalized to control). Data are expressed as mean \pm SEM (Control, Ca = 1 ± 0.0714 , n = 50 cells; AP5, Ca = 0.9683 ± 0.0611 , n = 40 cells; MK-801, Ca = 0.8370 ± 0.0641 , n = 32 cells; Ket, Ca = 0.9670 ± 0.0765 , n = 38 cells; Mem, Ca = 0.8048 ± 0.0546 , n = 43 cells; TTX, Ca = 0.8696 ± 0.0724 , n = 34 cells; one-way ANOVA followed by Tukey's multiple comparison test, $p > 0.05$). (ii) Homer cluster intensity (normalized to control). Data are expressed as mean \pm SEM (Control, Ci = 1 ± 0.0393 , n = 50 cells; AP5, Ci = 1.293 ± 0.0885 , n = 40 cells; MK-801, Ci = 0.9437 ± 0.0522 , n = 32 cells; Ket, Ci = 0.9740 ± 0.0478 , n = 38 cells; Mem, Ci = 0.8857 ± 0.0636 , n = 43 cells; TTX, Ci = 0.8696 ± 0.0724 , n = 34 cells; one-way ANOVA followed by Tukey's multiple comparison test, $**p < 0.005$). (iii) Homer cluster number/ μm (normalized to control). Data are expressed as mean \pm SEM (Control, Cd = 1 ± 0.0484 , n = 50 cells; AP5, Cd = 1.258 ± 0.0814 , n = 40 cells; MK-801 = 1.014 ± 0.0848 , n = 32 cells; Ket, Cd = 0.9746 ± 0.0665 , n = 38 cells; Mem, Cd = 1.146 ± 0.0817 , n = 43 cells; TTX, Cd = 1.142 ± 0.1010 , n = 34 cells; one-way ANOVA followed by Tukey's multiple comparison test, $p > 0.05$).

(B) (i) NMDAR cluster area (normalized to control). Data are expressed as mean \pm SEM (Control, Ca = 1 ± 0.0437 , n = 55 cells; AP5, Ca = 1.2080 ± 0.0616 , n = 40 cells; MK-801, Ca = 1.057 ± 0.0848 , n = 35 cells; Ket, Ca = 1.0230 ± 0.0635 , n = 40 cells; Mem, Ca = 1.1180 ± 0.0717 , n = 47 cells; TTX = 1.0120 ± 0.0530 , n = 38 cells; one-way ANOVA followed by Tukey's multiple comparison test, $p > 0.05$). (ii) NMDAR cluster intensity (normalized to control). Data are expressed as mean \pm SEM (Control, Ci = 1 ± 0.0087 , n = 55 cells; AP5, Ci = 0.9961 ± 0.0162 , n = 40 cells; MK-801, Ci = 0.9772 ± 0.0201 , n = 35 cells; Ket, Ci = 0.9523 ± 0.0175 , n = 40 cells; Mem, Ci = 1.0400 ± 0.0215 , n = 47 cells; TTX, Ci = 1.0050 ± 0.0218 , n = 38 cells; one-way ANOVA followed by Tukey's multiple comparison test, $p > 0.05$). (iii) NMDAR cluster number/ μm (normalized to control). Data are expressed as mean \pm SEM (Control, Cd = 1 ± 0.0830 , n = 55 cells; AP5, Cd = 1.3950 ± 0.1312 , n = 40 cells; MK-801, Cd = 1.0990 ± 0.1539 , n = 35 cells; Ket, Cd = 1.1100 ± 0.1188 , n = 40 cells; Mem, Cd = 1.1410 ± 0.1040 , n = 47 cells; TTX, Cd = 1.3600 ± 0.1154 , n = 38 cells; one-way ANOVA followed by Tukey's multiple comparison test, $p > 0.05$).

(C) Percentage of synaptic NMDAR clusters (normalized to control). Data are expressed as mean \pm SEM (Control, Sc = 1 ± 0.0642 , n = 50 cells; AP5, Sc = 1.2680 ± 0.0816 , n = 40 cells; MK-801 = 1.0700 ± 0.0675 , n = 32 cells; Ket, Sc = 0.8315 ± 0.0786 , n = 38 cells; Mem, Sc = 0.9505 ± 0.0684 , n = 43 cells; TTX, Sc = 1.1130 ± 0.0690 , n = 34 cells; one-way ANOVA followed by Tukey's multiple comparison test, $p > 0.05$).

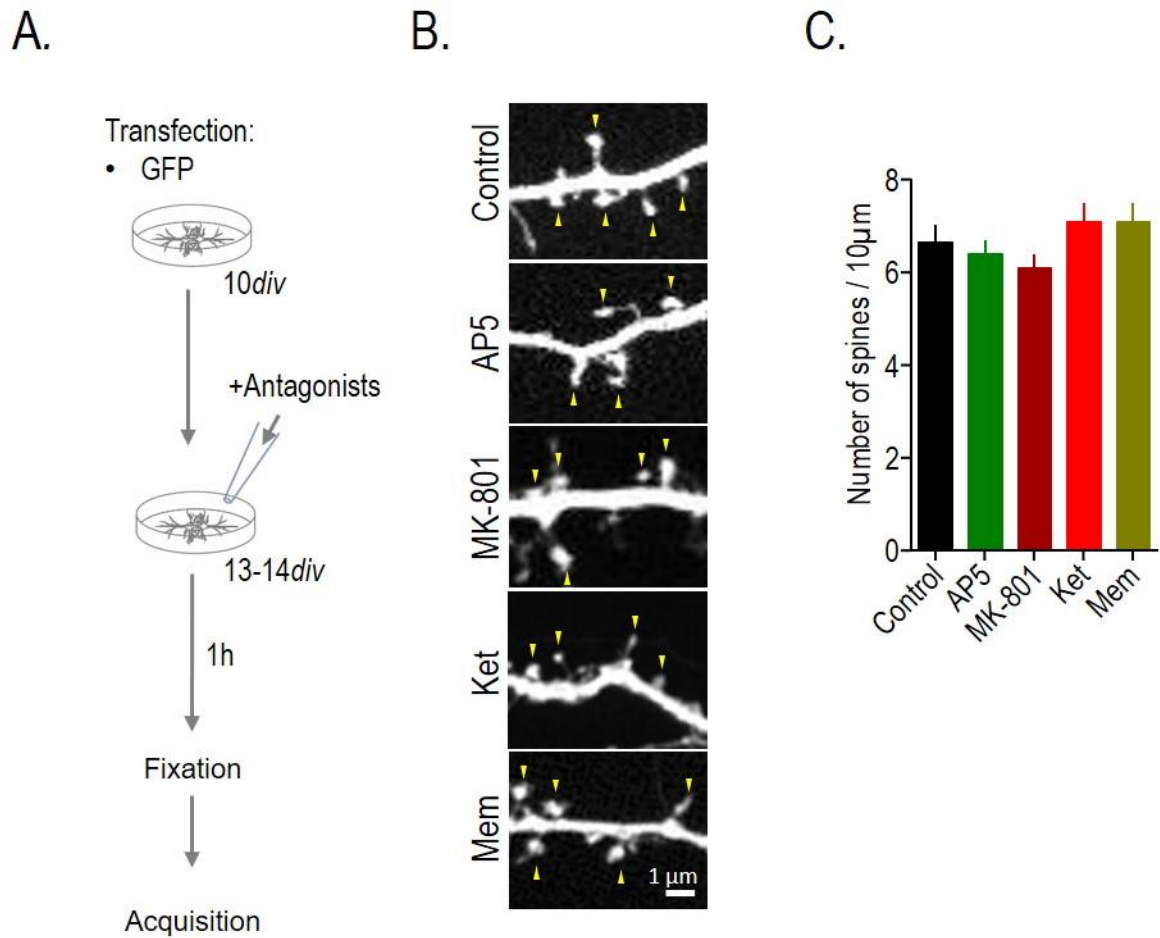


Figure S5 – Exposure to NMDAR antagonists (1h) does not alter the number of dendritic spines

(A) Schematic representation of the experimental design. Neurons were transfected to express GFP. After a 1h treatment with NMDAR antagonists, neurons were fixed and dendritic spines were visually identified.

(B) Representative dendritic stretches from neurons in each experimental condition. Yellow arrows indicate visually identified dendritic spines.

(C) Number of dendritic spines per 10 μ m. Data are expressed as mean \pm SEM (Control, $n = 6.6360 \pm 0.3785$ spines, $n = 63$ dendrites, $N = 30$ cells; AP5, $n = 6.3910 \pm 0.3131$ spines, $n = 52$ dendrites, $N = 28$ cells; MK-801, $n = 6.0860 \pm 0.3200$ spines, $n = 50$ dendrites, $N = 25$ cells; Ket, $n = 7.0840 \pm 0.4123$ spines, $n = 60$ dendrites, $N = 30$ cells; Mem, $n = 7.0730 \pm 0.4163$ spines, $n = 60$ dendrites, $N = 30$ cells; one-way ANOVA followed by Tukey's multiple comparisons test, $p > 0.05$).

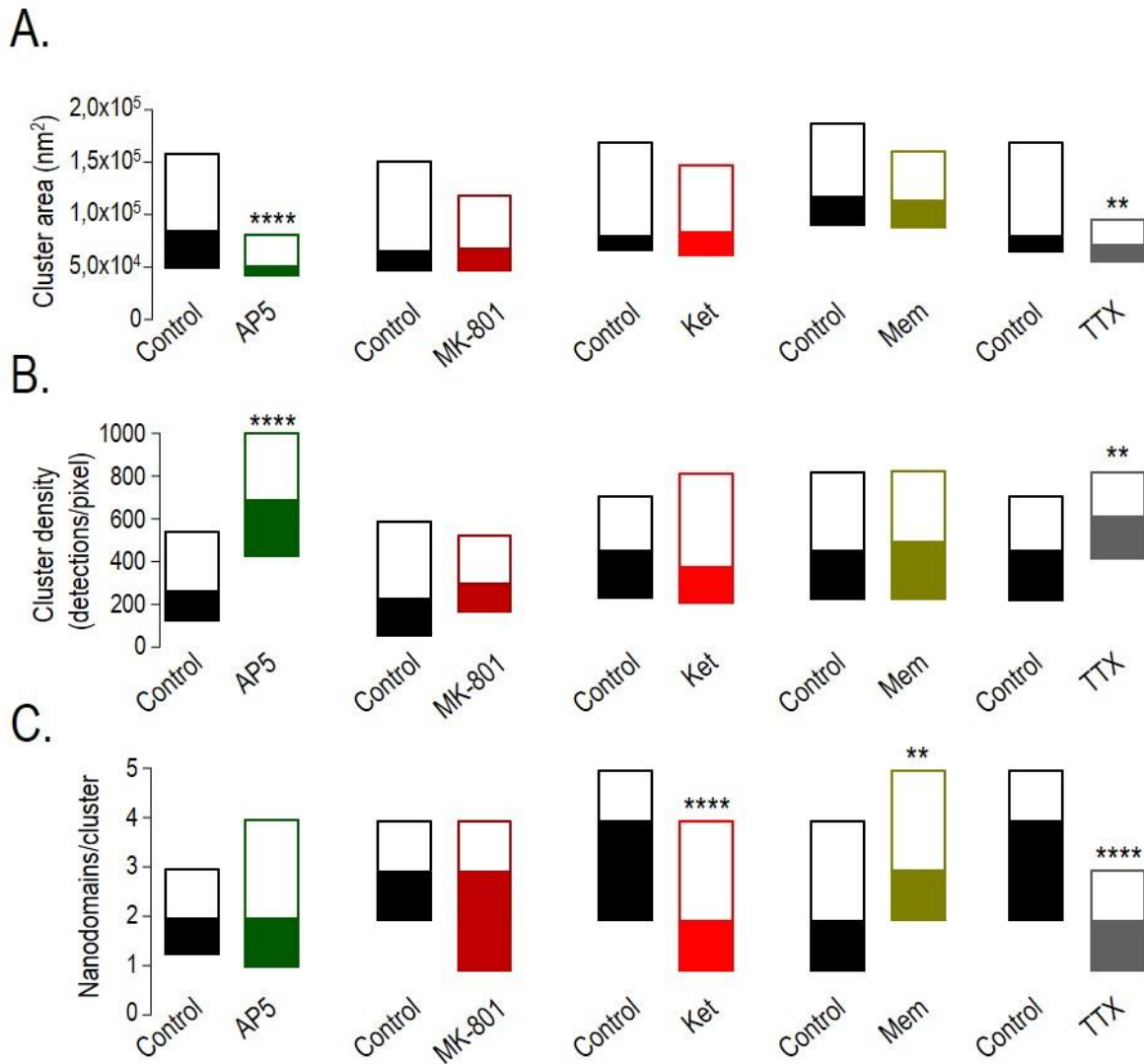


Figure S6 – Exposure to NMDAR antagonists and TTX (1h) affects the nanoscale organization of NMDAR clusters

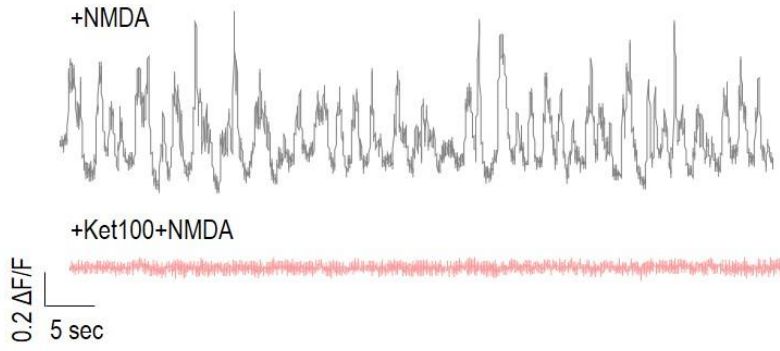
(A) Cluster area. Data expressed as median \pm 25%-75% IQR (Control, Ca = 82905 \pm 48966-156313 nm², n = 124 clusters, N = 7 cells; AP5, Ca = 49876 \pm 39901-79533 nm², n = 79 clusters, N = 7 cells; Control, Ca = 63964 \pm 43913-148871 nm², n = 102 clusters, N = 9 cells; MK-801, Ca = 65741 \pm 44489-117736 nm², n = 113 clusters, N = 6 cells; Control, Ca = 59497 \pm 47060-125786 nm², n = 57 clusters, N = 8 cells; Ket, Ca = 61820 \pm 44114-109415 nm², n = 179 clusters, N = 8 cells; Control, Ca = 57796 \pm 43961-92888 nm², n = 59 clusters, N = 8 cells; Mem, Ca = 56332 \pm 43180-79739 nm², n = 164 clusters, N = 7 cells; Control, Ca = 59497 \pm 47060-125786 nm², n = 57 clusters, N = 8 cells; TTX, Ca =

52563 ± 40320-70432 nm², n = 139 clusters, N = 10 cells; Mann-Whitney test, **p<0.005, ****p<0.0001).

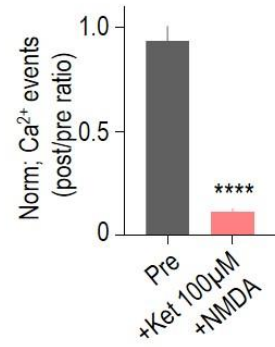
(B) Cluster density. Data expressed as median ± 25%-75% IQR (Control, Cd = 261.7 ± 117.8-538.1 detections/pixel, n = 124 clusters, N = 7 cells; AP5, Cd = 688.0 ± 417.5-995.9 detections/pixel, n = 79 clusters, N = 7 cells; Control, Cd = 271.8 ± 103.2-615.3 detections/pixel, n = 102 clusters, N = 9 cells; MK-801, Cd = 341.9 ± 212.8-553.3 detections/pixel, n = 113 clusters, N = 6 cells; Control, Cd = 480.2 ± 269.5-724.6 detections/pixel, n = 57 clusters, N = 8 cells; Ket, Cd = 411.7 ± 245.2-825.6 detections/pixel, n = 179 clusters, N = 8 cells; Control, Cd = 481.0 ± 259.0-829.8 detections/pixel, n = 59 clusters, N = 8 cells; Mem, Cd = 525.4 ± 263.8-836.6 detections/pixel, n = 164 clusters, N = 7 cells; Control, Cd = 480.2 ± 269.5-724.6 detections/pixel, n = 57 clusters, N = 8 cells; TTX, Cd = 636.1 ± 441.9-834.9 detections/pixel, n = 139 clusters, N = 10 cells; Mann-Whitney test, **p<0.005, ****p<0.0001).

(C) Number of nanodomains per cluster. Data are expressed as median ± 25%-75% IQR (Control, Nb = 2 ± 1.25-3 nanodomains/cluster, n = 124 clusters, N = 7 cells; AP5, Nb = 2 ± 1-4 nanodomains/cluster, n = 79 clusters, N = 7 cells; Control, Nb = 3 ± 2-4 nanodomains/cluster, n = 102 clusters, N = 9 cells; MK-801, Nb = 3 ± 1-4 nanodomains/cluster, n = 113 clusters, N = 6 cells; Control, Nb = 4 ± 2-5 nanodomains/cluster, n = 57 clusters, N = 8 cells; Ket, Nb = 2 ± 1-4 nanodomains/cluster, n = 179 clusters, N = 8 cells; Control, Nb = 2 ± 1-4 nanodomains/cluster, n = 59 clusters, N = 8 cells; Mem, Nb = 3 ± 2-5 nanodomains/cluster, n = 164 clusters, N = 7 cells; Control, Nb = 4 ± 2-5 nanodomains/cluster, n = 57 clusters, N = 8 cells; TTX, Nb = 2 ± 1-3 nanodomains/cluster, n = 139 clusters, N = 10 cells; Mann-Whitney test, **p<0.005, ****p<0.0001).

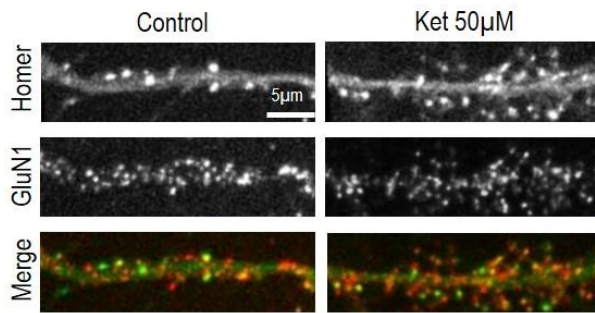
A.



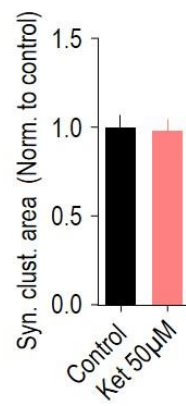
B.



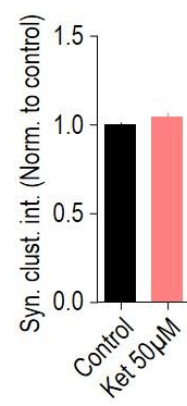
C.



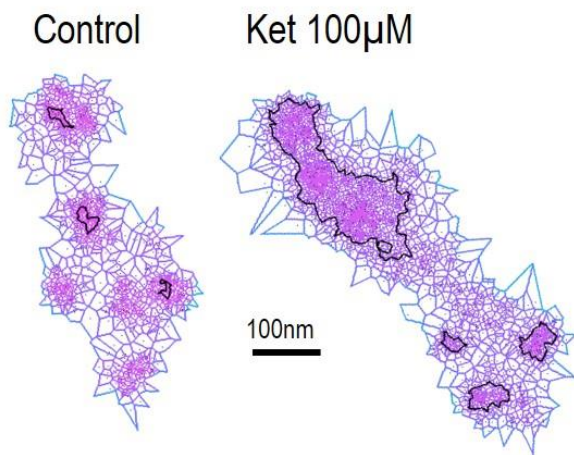
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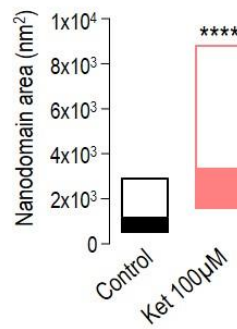
D.ii



E.



F.i



F.ii

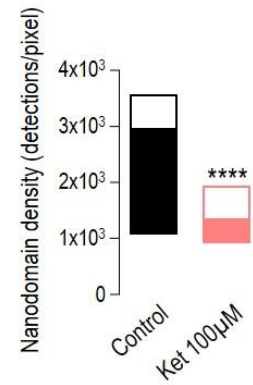
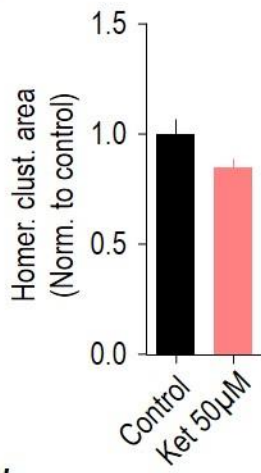


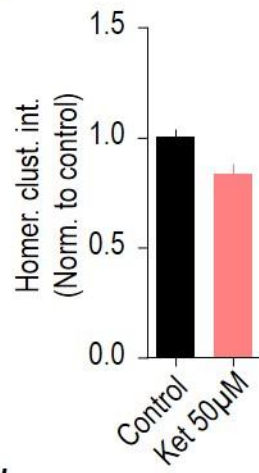
Figure S7 – Increasing doses of ketamine have a comparable impact on NMDAR function and organization

- (A) Representative example traces of NMDAR-mediated calcium transients in single spines ($\Delta F/F$) after incubation with NMDA alone (5 μM) or in combination with ketamine (100 μM).
- (B) (i) Normalized frequency of spontaneous NMDAR-mediated calcium transients (ratio of calcium transient frequency before and after application of ketamine (100 μM) and NMDA (5 μM)). Data are expressed as mean \pm SEM. (+NMDA, $N_f = 0.9361 \pm 0.0709$, $n = 189$ spines, $N = 5$ cells; +Ket100+NMDA, $N_f = 0.1160 \pm 0.0125$, $n = 132$ spines, $N = 5$ cells, Student's t-test, **** $p < 0.0001$).
- (C) Representative dendritic segments of control neurons or neurons treated with ketamine (50 μM) for 1h.
- (D) (i) Synaptic NMDAR cluster area (normalized to control). Data are expressed as mean \pm SEM (Control, $C_a = 1 \pm 0.0570$, $n = 50$ neurons; Ket 50 μM , $C_a = 0.9767 \pm 0.0682$, $n = 49$ neurons; Student's t-test, $p > 0.05$). (ii) Synaptic NMDAR cluster intensity (normalized to control). Data are expressed as mean \pm SEM (Control, $C_i = 1 \pm 0.0151$, $n = 50$ neurons; Ket 50 μM , $C_i = 1.241 \pm 0.0963$, $n = 49$; Student's t-test, $p > 0.05$).
- (E) Examples of super-resolved postsynaptic NMDAR clusters from control cells or cells treated with ketamine (100 μM) for 1h. Grey points indicate fluorophore detections. Polygons around detections were generated through tessellation. Black outlines indicate intra-cluster receptor nanodomains.
- (F) (i) Nanodomain area. Data expressed as median \pm 25%-75% IQR (Control, $N_a = 1112 \pm 496.4\text{-}2889 \text{ nm}^2$, $n = 97$ nanodomains, $N = 7$ cells; Ket 100 μM , $N_a = 3298 \pm 1525\text{-}8785$, $n = 312$ nanodomains, $N = 7$ cells; Mann-Whitney test, **** $p < 0.0001$). (ii) Nanodomain density. Data expressed as median \pm 25%-75% IQR (Control, $N_d = 2930 \pm 1103\text{-}3533$ detections/pixel, $n = 97$ nanodomains, $N = 7$ cells; Ket 100 μM , $N_d = 1340 \pm 932.8\text{-}1910$ detections/pixel, $n = 312$ nanodomains, $N = 7$ cells; Mann-Whitney test, **** $p < 0.0001$).

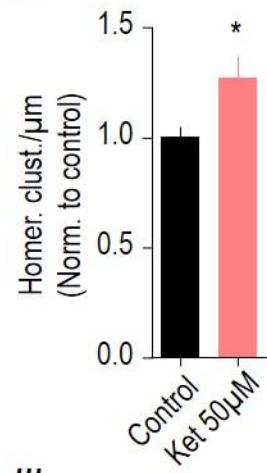
A.i



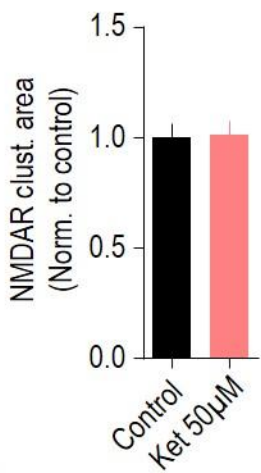
A.ii



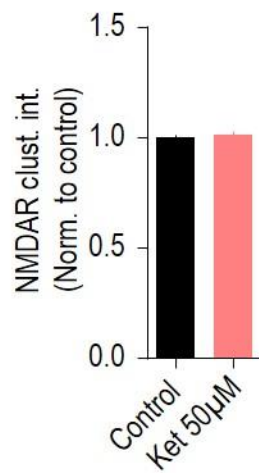
A.iii



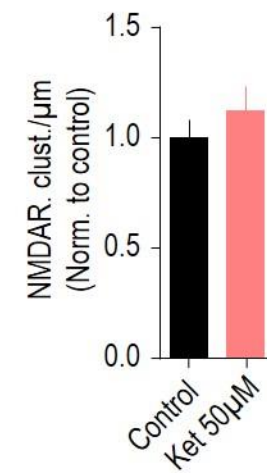
B.i



B.ii



B.iii



C.

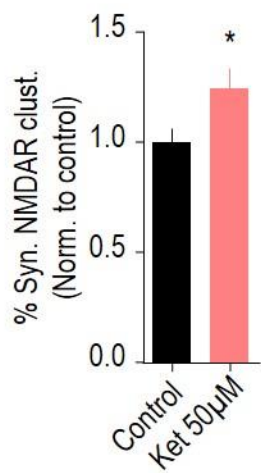


Figure S8 – High doses of ketamine increase the proportion of synaptic NMDAR

- (A) i) Homer cluster area (normalized to control). Data are expressed as mean \pm SEM (Control, $Ca = 1 \pm 0.0714$, $n = 50$ neurons; Ket 50 μ M, $Ca = 0.8516 \pm 0.0412$, $n = 49$ neurons; Student's t-test, $p > 0.05$). (ii) Homer cluster intensity (normalized to control). Data expressed as mean \pm SEM (Control, $Ci = 1 \pm 0.0393$, $n = 50$ neurons; Ket 50 μ M, $Ca = 0.8323 \pm 0.0466$, $n = 49$ neurons; Student's t-test, $p > 0.05$). (iii) Homer cluster number/ μ m (normalized to control). Data expressed as mean \pm SEM (Control, $Cd = 1 \pm 0.0484$, $n = 50$ neurons; Ket 50 μ M, $Cd = 1.2700 \pm 0.0985$, $n = 49$ neurons; Student's t-test, $p > 0.05$).
- (B) NMDAR cluster area (normalized to control). Data expressed mean \pm SEM (Control, $Ca = 1 \pm 0.0437$, $n = 55$ neurons; Ket 50 μ M, $Ca = 1.0130 \pm 0.0598$, $n = 52$ neurons; Student's t-test, $p > 0.05$). (ii) NMDAR cluster intensity (normalized to control). Data expressed as mean \pm SEM (Control, $Ci = 1 \pm 0.0087$, $n = 55$ neurons; Ket 50 μ M, $Ci = 1.0120 \pm 0.0177$, $n = 52$ neurons; Student's t-test, $p > 0.05$). (iii) NMDAR cluster number/ μ m (normalized to control). Data expressed as mean \pm SEM (Control, $Cd = 1 \pm 0.0830$, $n = 55$ neurons; Ket 50 μ M, $Cd = 1.1230 \pm 0.1082$, $n = 52$ neurons; Student's t-test, $p > 0.05$).
- (C) % of Synaptic NMDAR (normalized to control). Data expressed mean \pm SEM (Control, $Sc = 1 \pm 0.0642$, $n = 50$ neurons; Ket 50 μ M, $Sc = 1.2410 \pm 0.0963$, $n = 49$ neurons; Student's t-test, $*p < 0.05$).

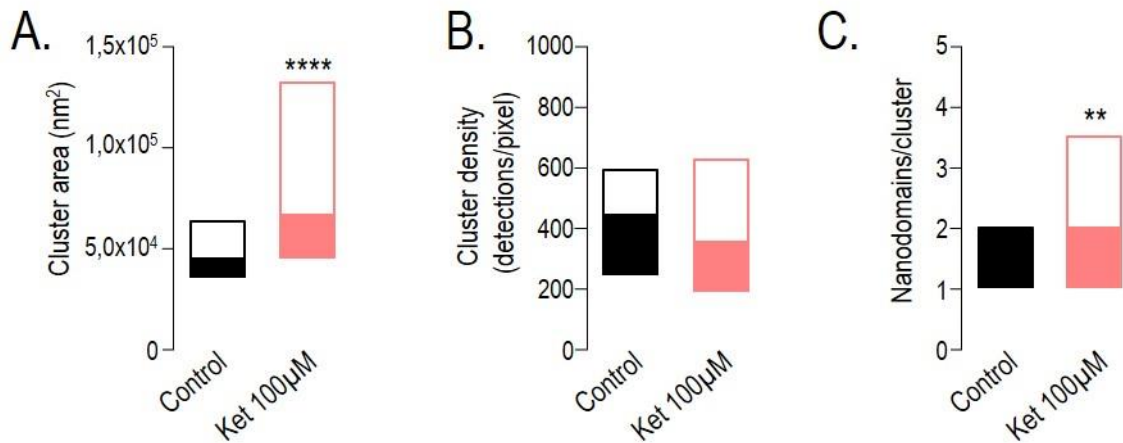


Figure S9 – High doses of ketamine increase the area of NMDAR clusters and the number of NMDAR nanodomains per cluster

(A) Cluster area. Data expressed as median \pm 25%-75% IQR (Control, Ca = 44788 \pm 35500-63640 nm², n = 58 clusters, N = 7 cells; Ket 100 μ M, Ca = 66767 \pm 44862-131657 nm², n = 129 clusters, N = 7 cells; Mann-Whitney test, ****p<0.0001).

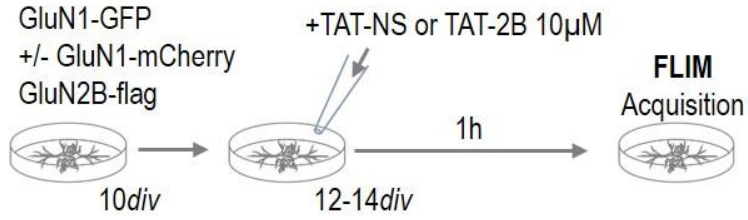
(B) Cluster density. Data expressed as median \pm 25%-75% IQR (Control, Cd = 444.8 \pm 246.3-589.3, n = 58 clusters, N = 7 cells; Ket 100 μ M, Cd = 355.4 \pm 189.4-623, n = 129 clusters, N = 7 cells; Mann-Whitney test).

(C) Number of nanodomains per cluster. Data expressed as median \pm 25%-75% IQR (Control, Nb = 2 \pm 1-2 nanodomains/cluster, n = 58 clusters, N = 7 cells; Ket 100 μ M, Nb = 2 \pm 1-3.5 nanodomains/cluster, n = 129 clusters, N = 7 cells; Mann-Whitney test, **p<0.005).

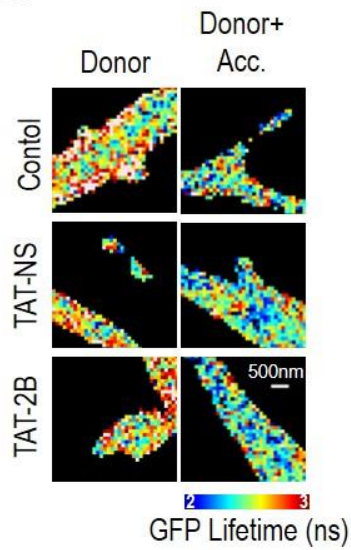
A.

Transfection:

- GluN1-GFP
- +/- GluN1-mCherry
- GluN2B-flag



B.



C.

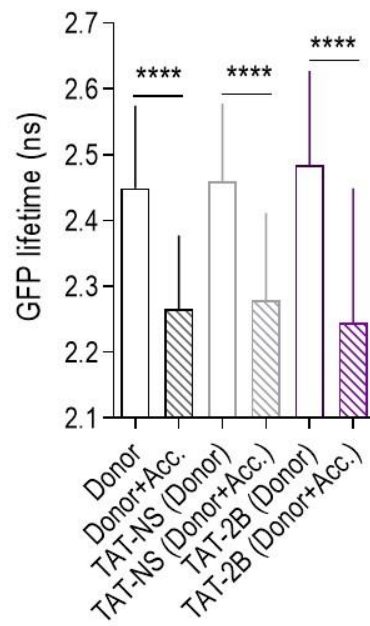


Figure S10 – Exposure to TAT-conjugated peptides does not prevent FRET between GluN1-GFP and GluN1-mCherry

- (A) Schematic representation of the experimental design. Neurons were transfected with GluN1-GFP and GluN2B-flag, and either with or without GluN1-mCherry. After a 1h incubation with either TAT-NS or TAT-2B, GFP fluorescence lifetime was acquired using Fluorescence Lifetime Imaging Microscopy.
- (B) Representative images of GFP lifetime in dendritic segments of neurons transfected with either GluN1-GFP alone (Donor, left) or GluN1-GFP and GluN1-mCherry (Donor+Acc., right) treated with either buffer (Control), TAT-NS or TAT-2B.
- (C) GFP lifetime per GluN1-GFP cluster after 1h exposure to TAT peptides in cells transfected with either GluN1-GFP alone (Donor) or GluN1-GFP and GluN1-mCherry (Donor+Acc.). Data are expressed as mean \pm SD (Donor, GI = 2.449 ± 0.1256 ns, n = 207 clusters, N = 53 cells; Donor+Acc., GI = 2.266 ± 0.1112 ns, n = 285 clusters, N = 72 cells; TAT-NS(Donor), GI = 2.460 ± 0.1183 ns, n = 318 clusters, N = 83 cells; TAT-NS(Donor+Acc.), GI = 2.279 ± 0.1330 ns, n = 295 clusters, N = 81 cells; TAT-2B(Donor), GI = 2.484 ± 0.1431 ns, n = 327 clusters, N = 77 cells; TAT-2B(Donor+Acc), GI = 2.245 ± 0.2043 ns, n = 234 clusters, N = 88 cells; Kruskal-Wallis followed by Dunn's multiple comparison test, ****p<0.0001).

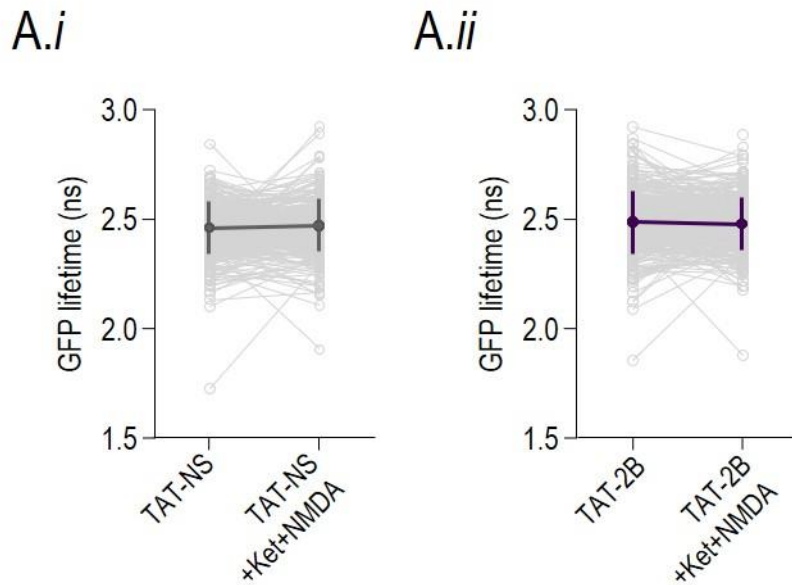


Figure S11 – Ketamine application after pre-treatment with biomimetic peptides does not affect the fluorescence lifetime of the donor fluorophore

(A) (i) Impact of Ketamine + NMDA 5 μ M application on GFP lifetime per GluN1-GFP cluster after pre-treatment with TAT-NS in cells expressing only GluN1-GFP and GluN2B-flag (Mean \pm SD GFP lifetime; TAT-NS = 2.460 \pm 0.1183 ns, TAT-NS+Ket+NMDA = 2.470 \pm 0.1203 ns, n = 318 clusters, N = 83 cells; Wilcoxon test, p>0.05). (ii) Impact of Ketamine + NMDA 5 μ M application on GFP lifetime per GluN1-GFP cluster after pre-treatment with TAT-2B in cells expressing only GluN1-GFP and GluN2B-flag (Mean \pm SD GFP lifetime; TAT-2B = 2.484 \pm 0.1431 ns, TAT-2B+Ket+NMDA = 2.477 \pm 0.1230 ns, n = 327 clusters,

Discussion and Perspectives

Using a combination of epifluorescence, FRET-FLIM, single particle tracking, single molecule localization microscopy and biochemistry approaches, we investigated the impact of competitive (D-AP5, CPP) and uncompetitive (MK-801, ketamine, memantine) NMDAR antagonists on the properties, redistribution and subsynaptic organization of surface NMDAR and their cytosolic partners in hippocampal neurons. We found that while all antagonists produced comparable inhibition of NMDAR ionotropic activity, exposure to the psychotomimetic NMDAR uncompetitive channel blockers MK-801 and ketamine elicited noteworthy changes in the conformation, surface trafficking and organization of NMDAR. Although drug exposure for one hour did not change the overall receptor abundance at excitatory synapses, single molecule imaging revealed that MK-801 and ketamine enhanced the trapping and triggered nanoscale reorganizations of synaptic receptor clusters possibly associated with rearrangements in NMDAR-mediated signalling. Indeed, we found that MK-801 and ketamine favoured the redistribution of CaMKII within dendritic spines through a direct interaction, suggesting that drug-induced receptor redistributions may impact the intracellular dynamics and organization of downstream signalling partners of NMDAR. Altogether, our results provide evidence that competitive and uncompetitive antagonists have a different impact on NMDAR surface dynamics and subsynaptic organization. They also suggest that besides inhibition of ion flux through the receptors, the psychoactive blockers MK-801 and ketamine may act on receptor function and behaviour through non-canonical rearrangements in the organization of NMDAR signalling complexes.

NMDAR antagonists display different behavioural outcomes depending on the dose administered (see Table 6). While the saturating concentrations of AP5 (50 μM) and MK-801 (20 μM) used here are well above their IC_{50} values, the doses of ketamine (1 μM) and memantine (1 μM) were chosen to match clinically-relevant concentrations while efficiently inhibiting NMDAR-dependent synaptic calcium transients. Studies determining the levels of ketamine in human CSF are lacking, but research in animals reveals that ketamine readily crosses the blood-brain barrier and accumulates in brain tissue (Can *et al.*, 2016). Yang and colleagues reported that a 30 mg/kg i.p. injection in rats yielded a 10 μM ketamine concentration in the hippocampus, a dose which produced both psychotomimetic and antidepressant actions (Duan *et al.*, 2013). Ketamine is also frequently administered at 10 mg/kg i.p. in rodent studies investigating its antidepressant action, although what this corresponds to in terms of CSF concentrations is unknown (Garcia *et al.*, 2008; Maeng *et al.*, 2008; Li *et al.*, 2010). In humans, a 40-min intravenous (i.v.) infusion of 0.5 mg/kg ketamine is typically exercised for experimental off-label use of ketamine as an antidepressant and results

in a 1 μM (~200 ng/mL) plasma concentration, a dose which robustly relieves treatment-resistant depression but also induces a state of dissociation and psychedelic experiences similar to the symptoms of schizophrenia (Zarate, Singh, Carlson, *et al.*, 2006; Singh *et al.*, 2016; Sanacora *et al.*, 2017; Phillips *et al.*, 2019; Fava *et al.*, 2020). Importantly, these behavioural outcomes may be mediated by different ketamine metabolites. Indeed, ketamine is highly metabolized into several derivatives by the cytochrome P450 liver enzymes CYP2B6 and CYP3A4 (Zanos *et al.*, 2018). In a study using 40-min i.v. infusion of 0.5 mg/kg of ketamine on treatment-resistant depression, peak plasma concentrations registered were of 0.86 μM for ketamine, but also 0.33 μM for its metabolite norketamine, 0.06 μM for dehydronorketamine and 0.097 μM for 6-hydroxy-norketamine (Zarate *et al.*, 2012; Zanos *et al.*, 2018). Ketamine metabolites were not tested here and more research will be necessary to understand how these affect NMDAR physiology, either directly or indirectly, especially if they are indeed responsible for the antidepressant actions of ketamine. Memantine hydrochloride is commonly used as therapy for Alzheimer's disease (AD). In rodent experimental models, a single 1.22 mg/kg subcutaneous (s.c.) administration of memantine results in a maximal concentration of 0.98 μM in the plasma and 10.62 μM in the brain, which drops to 1 μM after 8h (Beconi *et al.*, 2012). In humans, a single oral dose of 20 mg (recommended daily dose for AD treatment) results in a maximum plasma concentration of 0.1-0.2 μM (Kornhuber *et al.*, 2007). After 11 days of daily treatment, a steady-state plasma concentration of 0.5-1 μM is attained, which results in a CSF concentration of around 50% of the plasma concentrations (Kornhuber and Quack, 1995; Valis *et al.*, 2019). However, interindividual variability in steady state plasma concentrations and also in CSF/plasma ratio is high, and there is no indication if higher or lower plasma or CSF levels of memantine correlate with higher treatment efficiency (Kornhuber *et al.*, 2007; Valis *et al.*, 2019). Of note, unlike ketamine, 75–90% of memantine is excreted unmetabolized, and memantine metabolites are reportedly inactive (Kornhuber *et al.*, 2007).

Defining the neuroanatomical basis for the action of clinically relevant NMDAR antagonists is a central question. Cell types and brain regions with higher NMDAR content and more intense activity are more likely to be more impacted by OCB. As an example, glucose consumption in the medium prefrontal cortex and in the stratum lacunosum moleculare of the hippocampus was found to be highly increased by MK-801 and ketamine but not by competitive antagonists (Clow, Lee and Hammer, 1991; Miyamoto *et al.*, 2000). More recently, it has been proposed that robust oscillations in neuronal activity in the retrosplenial cortex generated by altered communication between cortical and subcortical regions are responsible for the dissociative state produced by MK-801, ketamine and PCP (Vesuna *et al.*, 2020). Over the past two decades, intense efforts have been made to understand the basis for the antidepressant properties of ketamine, of which administration of a single, sub-anesthetic dose relieves

treatment-resistant depression within a few hours (Berman *et al.*, 2000; Duman *et al.*, 2016). It was initially assumed that the antidepressant properties of ketamine resulted from NMDAR blockade. The following mechanisms have been proposed: (i) preferential blockade of NMDAR on interneurons promoting disinhibition of pyramidal cells (Li *et al.*, 2010; Widman and McMahon, 2018; Ali *et al.*, 2020; Gerhard *et al.*, 2020), and (ii) brief inhibition of synaptic (or extrasynaptic) NMDAR on pyramidal cells leading to eukaryotic elongation factor 2 (eEF2)-elicited intracellular cascades (Autry *et al.*, 2011). Both of these hypotheses involve the release of brain-derived neurotrophic factor (BDNF) and subsequent activation of postsynaptic TrkB receptors to recruit Akt/mTOR and ERK signalling pathways supporting protein synthesis and plasticity (Duman *et al.*, 2016; Gould, Zarate and Thompson, 2019). Inhibition of NMDAR-dependent bursts in neuronal networks associated with abnormal negative valence in the lateral habenula has also been proposed to support the antidepressant action of ketamine (Yang *et al.*, 2018). Subanaesthetic doses of ketamine also promote functional recovery of visual acuity defects by inhibiting NMDAR located on interneurons, which results in sustained cortical disinhibition (Grieco *et al.*, 2020). The following evidence suggests that the antidepressant action of ketamine could be independent of NMDAR inhibition or involve additional mechanisms: (i) (*R*)-ketamine is a more potent antidepressant than (*S*)-ketamine, despite being a weaker NMDAR antagonist (Li *et al.*, 2010), (ii) other classical NMDAR antagonists do not exhibit antidepressant properties, and (iii) the ketamine metabolites (*2R,6R*)- and (*2S,6S*)-hydroxynorketamine (HNK) have been proposed to retain antidepressant efficacy and trigger neural plasticity rather than ketamine itself (Zanos *et al.*, 2016). While these questions were not addressed here, assessing (i) whether antagonists and/or their derivatives preferentially cause receptor rearrangements in principal cells or interneurons (ii) and defining if these rearrangements are restricted to specific brain areas will be of primary importance to understand their action mode.

Expanding our observations at the cellular and molecular level will also be an essential step to further characterize the mechanisms underlying the properties of clinically relevant NMDAR antagonists. Although studies on homeostatic upregulation of NMDAR *in vitro* show that increased surface levels of synaptic NMDAR can be detected through classical immunocytochemistry experiments following a prolonged blockade of NMDAR by both competitive or uncompetitive antagonists (Rao and Craig, 1997; Crump, Dillman and Craig, 2001; Pérez-Otaño and Ehlers, 2005), a sixty minutes application of NMDAR antagonists did not impact the synaptic levels of surface NMDAR in our conditions. While one may hypothesize that we may not be saturating every available receptor during an hour-long incubation with antagonists at physiological magnesium concentrations (1 mM), where the IC₅₀ of ketamine and memantine is above 1 μM for all diheteromeric NMDAR subtypes, our SPT results (namely

that ketamine triggers a decrease in synaptic NMDAR surface mobility) were the same whether the antagonists were applied for five minutes with NMDA or for one hour in culture medium (data not shown), suggesting that receptors are efficiently targeted by the drugs in this experimental configuration and that one hour is too short of an exposure to allow profound modulations in receptor numbers.

Several interaction-based mechanisms contribute to the regulation of NMDAR surface dynamics, synaptic retention and organization which can be subdivided in two main categories: i) N-terminal interactions with extracellular and transmembrane partners, and ii) C-terminal interactions with PDZ domain-containing cytosolic scaffolding proteins of the MAGUK family (Elias and Nicoll 2007; Bard and Groc 2011; Ladépêche *et al.*, 2013). Our data suggests that the enhanced trapping and reorganization of NMDAR at synapses elicited by uncompetitive channel blockers may involve modulations of their interactions with anchoring partners. Based on FRET-FLIM data showing that - unlike D-AP5 and memantine - MK-801 and ketamine selectively change the conformation of NMDAR and trigger an approximation of GluN1 C-terminal domains of ~0.2 nm, we hypothesize that the affinity for cytosolic partners may vary due to the binding of these drugs, as previously reported for MK-801 which was shown to promote the association of NMDAR and PSD-95 and to prevent receptor activation-induced disruption of this interaction (Doré *et al.*, 2014). To note, we did not observe conformational changes following activation of the receptors by NMDA alone, unlike previously reported (Doré *et al.*, 2014; Dore, Aow and Malinow, 2015; Ferreira *et al.*, 2017). Although we did not observe changes in GluN2A/PSD-95 and GluN2B/PSD-95 co-immunoprecipitation in *ex vivo* brain samples of animals injected with MK-801, disrupting GluN2 CTD interactions with a competing peptide carrying the final 15 amino acids of the GluN2B CTD prevented the conformational change induced by ketamine, an intriguing result that could support several interpretations. Either (i) only scaffold-bound receptors may undergo ketamine-elicited conformational change; (ii) GluN2B CTD interactions with scaffolding protein are necessary to stabilize ketamine-induced conformational changes; (iii) drug-induced conformational rearrangements proceed from post-translational modifications and biomimetic peptides compete with GluN2B CTDs for enzyme-driven modifications, as the final 15 amino acids of GluN2B are the targets of kinases and phosphatases, notably Fyn/Src, PP1 and CK2 (Traynelis *et al.*, 2010); or (iv) ketamine binding favours an interaction occurring at the GluN2B CTD which is responsible for the change in conformation. Thus, the OCB-induced changes in NMDAR surface mobility may rely on an increase in the affinity for PDZ domain-containing proteins of the postsynaptic density, and further FRET-FLIM experiments will be required to address this question (Doré *et al.*, 2014). Besides, single molecule imaging-based approaches will have to be implemented to further explore whether variations in the interaction with transmembrane actors (typically,

trans-synaptic adhesion molecules such as Ephrin B2 receptors or Neuroligin) may also be involved in the action of uncompetitive antagonists.

Importantly, we documented here that NMDAR antagonists elicit profound changes in the nanoscale organization of NMDAR at excitatory synapses. What could be the functional outcome of these reorganizations in terms of NMDAR-mediated synaptic signalling? Physical proximity between NMDAR increases calcium-dependent receptor desensitization (Iacobucci and Popescu, 2019), suggesting that drug-induced enhancement in NMDAR cluster and nanodomains density could favour calcium-induced desensitization following receptor activation (Glasgow *et al.*, 2017). Rearrangements in NMDAR subsynaptic organization may change the activity of downstream associated intracellular signalling nanodomains within dendritic spines (Tang *et al.*, 2016, Haas *et al.*, 2018; Hruska *et al.*, 2018), which may form as a result of diffusional confinement of secondary messengers such as calcium and cAMP or due to the self-assembly of nanoscale interacting protein hubs through phase transitions (Blackstone and Sheng, 2002; Frank and Grant, 2017; Bock *et al.*, 2020; Zhang *et al.*, 2020). Interestingly, selectively modulating the nanoscale distribution of GluN2A- or GluN2B-NMDAR at synapses using biomimetic peptides to disrupt interactions with scaffolding proteins does not affect the amplitude of NMDAR-mediated currents but results in major modulations of NMDAR-dependent signalling and plasticity (Bard *et al.*, 2010; Kellermayer *et al.*, 2018). While the mechanisms underlying these reorganization-based modulations of receptor signalling are still elusive, the close physical and functional interplay between NMDAR and CaMKII - through which both partners reciprocally influence the activity and distribution of one-another - appears as one of the downstream actors that may be affected by exposure to receptor antagonists and requires careful examination. NMDAR activity, redistributions and organization influence the dendritic recruitment and organization of CaMKII (Dupuis *et al.*, 2014; Ferreira *et al.*, 2020). In return, CaMKII activity and physical association was recently shown to regulate the nanoscale organization of NMDAR (Ferreira *et al.*, 2020). We found that exposure to either MK-801 or ketamine - but not to AP5 or memantine - promotes the cytosolic trafficking CaMKII at synapses, suggesting that drug-induced receptor rearrangement (and not inhibition) could potentially change the organization of NMDAR signalling complexes. This result opens several questions that will have to be addressed through further work. First, do MK-801- and ketamine-elicited CaMKII trafficking result in an evolution of its content and organization within dendritic spines? Also, is this redistribution process paralleled by changes in the activity of CaMKII? While the first point may be tackled using a combination of live CaMKII-GFP fluorescence imaging and STORM in dendritic spines (see Ferreira *et al.*, 2020), FRET-based fluorescent reporters can be used to monitor CaMKII activity and follow changes in the nanoscale localization of this activity following drug binding to NMDAR (Bock *et al.*, 2020; Zhang *et al.*,

2020). Besides investigations at the molecular level, assessing the correlation between MK-801- and ketamine-elicited changes in NMDAR signalling complexes and the psychotomimetic properties of these drugs through biomimetic peptide- or antibody-based modulations combined with *in vivo* recordings of neuronal activity and behavioural approaches may shed new lights on the molecular mechanisms underlying the psychogenic action of these antagonists.

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