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ve vědním oboru “Molekulárně-biologické a lékařské vědy”

Regulation of NMDA receptors and their pharmacological modulation

Komise pro obhajoby doktorských disertací v oboru “Biomedicína”

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Ústav experimentální medicíny AV ČR

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Contents

Summary

N-methyl-D-aspartate receptors (NMDARs) are pivotal in excitatory synaptic transmission and synaptic plasticity within the mammalian central nervous system (CNS). Their altered trafficking and functionality have been linked to a broad spectrum of psychiatric and neurological disorders, including Alzheimer's disease (AD), Huntington's disease, epilepsy, schizophrenia, and ischemia, with these alterations often attributed to pathogenic variants in the GluN subunits (Garcia-Recio et al., 2020; Hansen et al., 2021; Hu et al., 2016; Strehlow et al., 2019). The recent approval of esketamine has sparked a global surge in the research and development of modulators of NMDARs within scientific and industrial communities. Despite the availability of a comprehensive list of modulators of NMDARs, their application *in vivo* frequently fails to yield beneficial outcomes or is marred by significant adverse effects. A dual approach is critical to effectively address disorders linked to dysregulation of NMDARs: i.) elucidating the molecular mechanisms governing the regulation of NMDARs in mammalian cells, and ii.) creating innovative pharmacological compounds targeting both the standard and mutant forms of NMDARs. As the principal investigator at the Institute of Physiology CAS (IPHYS) and head of the Department of Neurochemistry in the Institute of Experimental Medicine CAS (IEM), my work encompassed directing various projects aimed at deciphering the molecular basis of regulation of NMDARs in terms of their early trafficking, surface mobility, and functional properties in mammalian cells under both normal and pathological conditions. Together with my collaborators, I also spearheaded an interdisciplinary initiative to design new pharmacological compounds that act at specific NMDAR subtypes through distinct mechanisms. This thesis consolidates over a decade of our research on NMDARs, conducted at both IPHYS and IEM, focusing on studies where I served as a (co-)corresponding author, showcasing our major scientific and technological breakthroughs. It begins with our findings on the regulation of NMDARs through membrane domains and extracellular regions, proceeds to explore the regulatory roles of *N*-glycans across various NMDAR subtypes, and includes a brief overview of our latest progress in tracking the surface mobility and localization of NMDARs using single-molecule imaging techniques. Finally, it details our persistent efforts to identify new pharmacological compounds targeting NMDARs, culminating in acquiring three domestic patents.

1. Introduction

References in blue refer to our publications used as a basis for this thesis.

N-methyl-D-aspartate receptors (NMDARs) represent a group within the ionotropic glutamate receptors, crucial for mediating excitatory neurotransmission in the mammalian central nervous system (CNS) (Hansen et al., 2017). The NMDAR family is encoded by seven distinct genes: one for the GluN1 subunit, four for the GluN2A to GluN2D subunits, and two for the GluN3A and GluN3B subunits (Hansen et al., 2021). Both glutamate and glycine activate traditional GluN1/GluN2 receptors, whereas the atypical GluN1/GluN3 receptors are solely responsive to glycine. The architecture of all GluN subunits consists of a uniform membrane topology that includes an extracellular amino-terminal domain (ATD), a ligand-binding domain (LBD) comprising S1 and S2 segments, a transmembrane domain (TMD), an intracellular C-terminal domain (CTD; FIG. 1). The TMD features three transmembrane-spanning helices (M1, M3, and M4) and a short membrane loop (M2) (Hansen et al., 2021; Wo and Oswald, 1995). The M2 loops, along with the M3 helices, construct the ion channel, with the M2 loops explicitly contributing to the channel's selectivity filter and serving as the Mg^{2+}-binding site for GluN1/GluN2 receptors (Kuner and Schoepfer, 1996; Zhang et al., 2021). Thus, at standard resting membrane potentials, extracellular Mg^{2+} blocks the ion channel of the GluN1/GluN2 receptor (Kuner and Schoepfer, 1996; Nowak et al., 1984). The dysregulation of NMDARs is known to significantly impact the development of numerous neuropsychiatric conditions, including Alzheimer's disease (AD), Huntington's disease, epilepsy, schizophrenia, and ischemia, with recent research linking many of these conditions to mutations within the coding sequences of GluN subunits (Hansen et al., 2017). Thus, a deep understanding of the regulatory mechanisms of NMDARs is crucial for creating effective treatment strategies. It is noteworthy that the expression profiles of the primary GluN2 subunits within the mammalian forebrain evolve throughout development; specifically, the GluN2B subunit is predominantly expressed at birth and subsequently declines, whereas the GluN2A subunit expression gradually rises postnatally. The GluN3A subunit, in contrast, is predominantly expressed during a critical window of intense synaptogenesis (Hansen et al., 2017). Our research addresses specific scientific questions regarding the regulatory mechanisms of diheteromeric GluN1/GluN2A, GluN1/GluN2B, GluN1/GluN2C, GluN1/GluN3A and GluN1/GluN3B receptors.

Our prior reviews and a book chapter highlighted that the population of NMDARs on the cell surface is initially dictated by the endoplasmic reticulum (ER) quality control system, which verifies the correct assembly of NMDARs before their release from the ER (Horak et al., 2021;

Horak et al., 2014a; Horak et al., 2014b). This screening detects specific ER retention and export signals within the various GluN subunit regions. A prevailing hypothesis posits that the ER quality control apparatus utilizes a common strategy to assess the correct ligand binding and functionality of NMDARs, similar to its approach with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and kainate receptors (Coleman et al., 2010; Coleman et al., 2009; Penn et al., 2008; Scholefield et al., 2019). Yet, evidence for this theory primarily stems from studies on a select number of mutant NMDARs (Kenny et al., 2009; She et al., 2012; Skrenkova et al., 2019), leaving the influence of other regions harboring specific ER retention and export signals on the ER processing of NMDARs unclear. Moreover, while the ER machinery robustly *N*-glycosylates the extracellular domains of the GluN subunits (Chazot et al., 1995; Everts et al., 1997; Huh and Wenthold, 1999; Kaniakova et al., 2016), the impact of these *N*-glycosylations on the sequential steps required for the ER processing of NMDARs is not fully understood. Additionally, it is well-accepted that NMDARs on the cell surface can be immobilized at the postsynaptic density (PSD) via lateral diffusion (Groc et al., 2004; Groc et al., 2006; Tovar and Westbrook, 2002). Nevertheless, questions remain regarding the suitability of previously used immunoglobulin G (IgG)-based probes for investigating the surface dynamics of NMDARs. Moreover, whether the surface mobility and localization of NMDARs containing GluN2A, GluN2B, and GluN3A subunits vary depending on the subtype is yet to be clarified using well-defined probes.

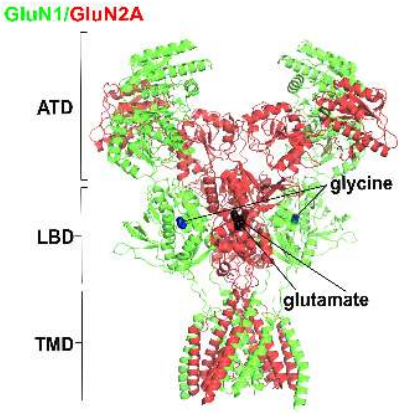

FIGURE 1. (A) The crystal structure of the GluN1/GluN2A heterotetramer (PDB code: 7eu7), including the glycine (blue) and glutamate (black) molecules. ATD stands for the amino-terminal domain; LBD stands for the ligand-binding domain; and TMD stands for the transmembrane domain (the C-terminal domain is not shown). The GluN1 subunit is shown in green, and the GluN2A subunit is shown in red.

Evolution has tailored the functional properties of NMDAR subtypes to finely tune excitatory neurotransmission across different regions of the CNS during its development (Stroebel and Paoletti, 2021). This thesis explores how *N*-glycosylation influences the functional characteristics of these NMDAR surface subtypes. In the realm of the pharmacology of NMDARs, several key modulators stand out, such as memantine, ketamine, and dizocilpine (MK-801; FIG. 5), all of which are open-channel blockers of the GluN1/GluN2 receptors, operating within the ion

channel in a manner dependent of Mg^{2+}. Memantine has been approved for the treatment of AD (Robinson and Keating, 2006), ketamine serves both as an anesthetic and an antidepressant (Zanos et al., 2018), and MK-801 acts as a research tool to simulate schizophrenia symptoms (Rung et al., 2005). These compounds' distinct *in vivo* effects suggest variability in their interactions with NMDARs. Therefore, a comprehensive understanding of these compounds' inhibitory mechanisms is crucial for developing new drugs that overcome the limitations of existing therapies for disorders linked with dysregulation of NMDARs. A potential pharmacophore for advancement is 1,2,3,4-tetrahydro-9-aminoacridine (tacrine; THA; FIG. 5), initially approved for AD treatment, which exhibits mild inhibitory action at NMDARs; it was later withdrawn due to severe hepatotoxic side effects. Furthermore, the complexity of AD might benefit from a multi-target-directed ligand (MTDL) approach, which posits that a single molecule harboring multiple pharmacophores could simultaneously target NMDARs and other relevant systems. A particularly intriguing pharmacological target is the GluN1/GluN3A receptor, notable for its resistance to inhibition by Mg^{2+}, memantine, or MK-801, and for which specific, effective modulators are yet to be identified.

2. Aims

Our research endeavors aimed to elucidate the molecular mechanisms governing the regulation of NMDARs in mammalian cells across both standard and disease states to identify therapeutic avenues for CNS disorders stemming from dysregulation of NMDARs:

- How do the membrane domains and extracellular regions of specific NMDAR subtypes influence their early trafficking, functionality, and pharmacology?
- Can *N*-glycosylation differentially affect the early trafficking and functional properties of NMDAR subtypes?
- Are there any subtype-specific variations in the mobility and localization of NMDARs when examined using more miniature, nanobody-based probes?

Additionally, our goal was to innovate a new range of effective pharmacological agents targeting NMDARs, employing an MTDL approach and designing THA derivatives.

3. Methods

Our IPHYS and IEM team conducted all *in vitro* investigations related to NMDARs, spanning molecular biology, microscopy, biochemistry, and electrophysiology. Our methods are elaborated upon in our publications. Electrophysiological measurements in IPHYS were performed closely with Prof. Ladislav Vyklicky Jr. and his team. The application device for solutions was developed by Ing. Ivan Dittert, Ph.D.

Except for the specific case mentioned below, all work on compound design and synthesis, acetylcholinesterase assays, quantitative structure-activity relationship (QSAR) analysis, assessments of blood-brain barrier (BBB) permeability, monoamine oxidase A/B (MAO-A/B) inhibition, cytotoxicity evaluations on the SH-SY5Y cell line, measurements of dehydrogenase activity and glutathione levels, docking studies targeting the NMDARs, and other non-NMDAR pharmacological methods were primarily conducted under the guidance of Prof. Ondrej Soukup and Prof. Jan Korabecny at the Biomedical Research Center, University Hospital Hradec Kralove, alongside the named contributors and facilities.

Assays for β-secretase (BACE1) inhibition, thioflavin (ThT) fluorescence assays for evaluating the impact on Aβ1–40 amyloid fibril formation, two-electrode voltage-clamp recordings on nicotinic acetylcholine receptors (nAChRs), interactions with M1 muscarinic acetylcholine receptors (mAChRs), cytotoxicity assessments using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, and predictions of BBB penetration as published (Gazova et al., 2017), were executed by a cross-disciplinary team led by Prof. Kamil Kuca at the Biomedical Research Center, University Hospital Hradec Kralove; Department of Toxicology and Military Pharmacy, Faculty of Military Health Sciences, Hradec Kralove; and the National Institute of Mental Health, Klecany.

Dr. Karel Vales and his team at the National Institute of Mental Health, Klecany, implemented behavioral analyses and other *in vivo* examinations, including tests in open field environments, elevated plus maze experiments, NMDA-induced lesion studies in rats, pre-pulse inhibition of the acoustic startle reflex, and others.

Prof. Young Ho Suh's group at Seoul National University College of Medicine, South Korea, conducted biochemistry procedures with NMDARs, such as biotinylation assays in hippocampal neurons and the cloning and generation of lentiviruses.

Dr. Ronald S. Petralia conducted pre-embedding electron microscopy using immunoperoxidase techniques at the Advanced Imaging Core, National Institutes of Health, USA.

The preparation and characterization of the conjugated anti-green fluorescent protein (GFP) nanobodies were performed in collaboration with Dr. Evzen Boura's laboratory at the Institute of Organic Chemistry and Biochemistry, CAS.

Quantum dot (QD) trajectory analysis, statistical analysis, and data interpretation were performed in collaboration with Dr. Martin Zapotocky at IPHYS.

4. Results

The findings discussed herein represent the cumulative efforts of my team and our collaborators spanning over a decade. This section primarily focuses on research findings from studies where I have served as a (co-)corresponding author.

4.1. Regulation of NMDARs by their membrane domains and extracellular regions

Building upon our earlier reviews and a book chapter (Horak et al., 2021; Horak et al., 2014a; Horak et al., 2014b), we have documented how the early processing of NMDARs is influenced by ER retention and export signals, along with posttranslational modifications (Horak et al., 2014a; Horak et al., 2014b). In my first research endeavor upon returning from a postdoctoral fellowship at the National Institutes of Health (Bethesda, USA), our application of microscopic and electrophysiological methodologies revealed that the M3 domains in both GluN1 and GluN2 subunits contain essential amino acid residues that play a pivotal role in dictating the surface expression of NMDARs, likely through mechanisms affecting their early trafficking (Kaniakova et al., 2012a). Additionally, our investigations determined that these crucial residues are not imperative for subunit interaction or the assembly of functional NMDARs. In a follow-up study, we pinpointed a single amino acid residue in the M4 domain of the GluN1 subunit, L830, as a regulator of the early trafficking for NMDARs containing GluN2A and GluN2B subunits. Interestingly, this L830 residue does not influence the trafficking of the GluN1 subunit when expressed independently (Kaniakova et al., 2012b).

In our subsequent studies, we explored how three pathogenic variants (M641I, A645S, and Y647S) within the M3 domain of the GluN1 subunit influence the surface expression of NMDARs, sensitivity to agonists, susceptibility to Mg^{2+} blockage, and response to memantine inhibition (Kolcheva et al., 2021). Through microscopic analysis in human embryonic kidney 293 (HEK293) cells, we observed diminished surface levels of GluN1-M641I/GluN2A, GluN1-Y647S/GluN2A, and GluN1-Y647S/GluN2B receptors, while other variants, including those forming GluN1/GluN3A receptors, maintained normal levels of surface expression. Microscopical experiments using rat hippocampal neurons demonstrated that GluN1-M641I and GluN1-Y647S subunits showed reduced surface levels. Electrophysiological measurements revealed that GluN1-M641I/GluN2 and GluN1-A645S/GluN2 receptors expressed in HEK293 cells exhibited EC_{50} values for glutamate and glycine like those of the wild-type GluN1/GluN2 receptors; however, GluN1-Y647S/GluN2 receptors failed to generate glutamate-induced currents. Under 1 mM Mg^{2+} condition, GluN1-M641I/GluN2 receptors displayed decreased

IC_{50} values for memantine and exhibited slower dissociation rates, whereas GluN1-A645S/GluN2 receptors showed increased IC_{50} values for memantine and faster dissociation rates compared to the wild-type receptors. Corresponding with these electrophysiological findings, memantine provided the highest neuroprotection in hippocampal neurons expressing the GluN1-M641I subunits, compared with those expressing the wild-type GluN1 and GluN1-A645S subunits, in an NMDA-induced excitotoxicity model. These discoveries highlight how specific pathogenic variants in the M3 domain of the GluN1 subunit can distinctly affect the trafficking, function, and pharmacology of NMDARs.

Subsequently, we delved into the functional and pharmacological characterization of the GluN1-N650K, a variant linked to developmental delays and seizures (FIG. 2)(Kolcheva et al., 2023). Our microscopical examination in HEK293 cells revealed an elevation in surface levels of GluN1/GluN2A and GluN1/GluN2B receptors containing the GluN1-N650K variant; this increase in surface expression was mirrored in hippocampal neurons expressing the GluN1-N650K subunit. Electrophysiological measurements indicated that the GluN1-N650K variant enhances the sensitivity of GluN1/GluN2A receptors to glutamate and glycine, together with a reduction in receptor conductance and open probability. Notably, the GluN1-N650K variant precluded the detection of current responses of GluN1/GluN2B receptors induced by glutamate and glycine, but we observed the glycine-induced current responses of GluN1-N650K/GluN3A receptors. Under 1 mM Mg^{2+}, GluN1-N650K/GluN2A receptors exhibited a regular ketamine sensitivity and an enhanced memantine sensitivity. However, onset and offset rates for memantine and ketamine were slower than those observed with wild-type GluN1/GluN2A receptors. Crucially, hippocampal neurons expressing the GluN1-N650K variant demonstrated reduced excitotoxicity, with memantine offering notable neuroprotection. These findings illuminate the impact of the GluN1-N650K variant, significantly altering the functional and pharmacological properties of NMDARs.

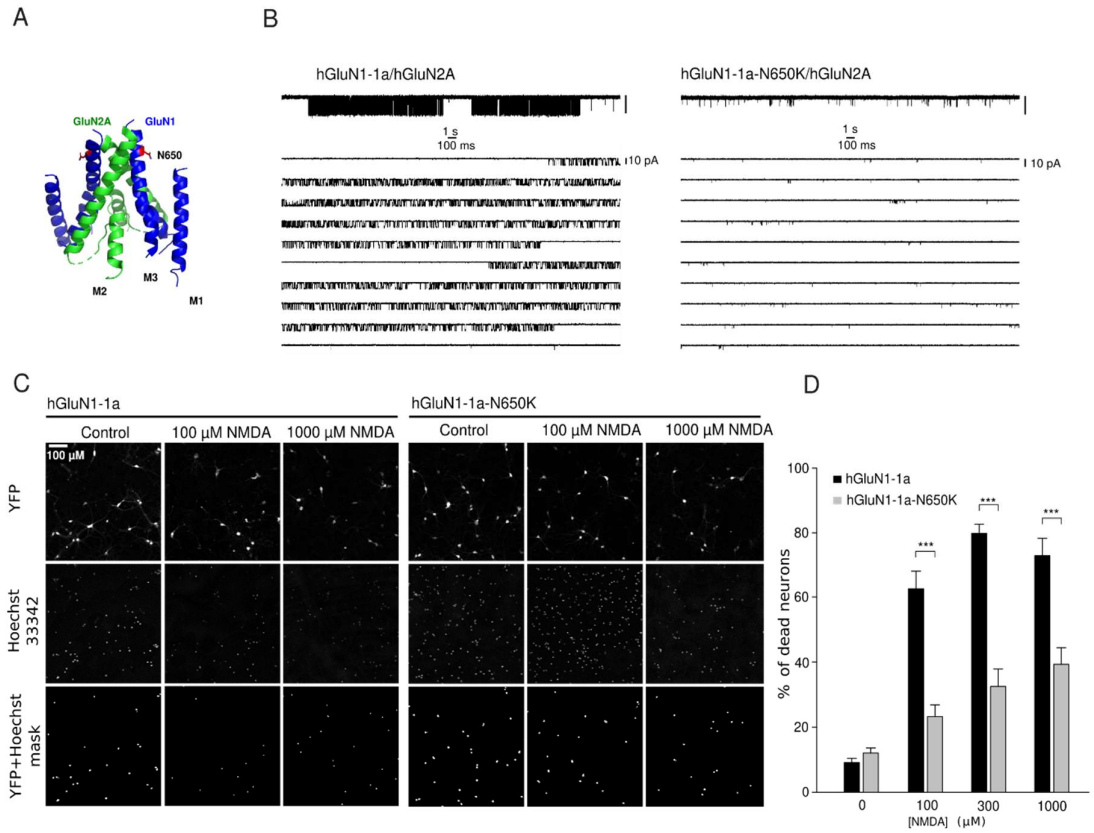

FIGURE 2. The pathogenic GluN1-N650K variant regulates open probability of NMDARs and reduces NMDA-induced excitotoxicity. A. Structural model of the M1, M2, and M3 domains in the GluN1/GluN2A heterotetramer; the residue studied here is indicated in red; adapted from our study (Kolcheva et al., 2023). B. Single-channel recordings from HEK293 cells expressing either human version of the GluN1/GluN2A (hGluN1/hGluN2A; left) or hGluN1-N650K/hGluN2A (right) receptors C. Representative images of yellow fluorescent protein (YFP) and Hoechst 33342 fluorescence in hippocampal neurons expressing YFP- hGluN1-1a or YFP-hGluN1-1a-1a-N650K subunits. D. Summary of the percentage of dead neurons expressing the indicated YFP-hGluN1-1a subunits, treated with the indicated concentrations of NMDA.

Our next investigation explored the characteristics of rare NMDAR subtypes, particularly those incorporating GluN2C and GluN3A subunits. By applying microscopical techniques to mammalian cell lines and cultured cerebellar granule cells, we discovered that NMDARs containing GluN2C subunits exhibit lower surface expression levels in comparison to NMDARs containing GluN2A or GluN2B subunits (Lichnerova et al., 2014). Additionally, we pinpointed three crucial segments within the ATD, M3 domain, and CTD regions of the GluN2C subunits essential for the early trafficking of NMDARs. Our findings indicate that the structural integrity of the membrane domains critically regulates the early trafficking of NMDARs, likely working in tandem with other ER retention and export signals located in the extracellular and intracellular domains of GluN subunits.

Pursuing the hypothesis that ligand binding influences the release of NMDARs from the ER, our study focused on analyzing the impact of mutations within the glycine-binding sites of GluN1 and GluN3A subunits on the surface delivery and functionality of the NMDARs (Skrenkova et al., 2019). Through microscopical and electrophysiological experiments in HEK293 cells and rat hippocampal neurons, we found a correlation between the structural changes of the glycine-binding sites in GluN1 and GluN3A subunits and the early trafficking of GluN1/GluN3A receptors. We also identified a pathogenic variant within the glycine-binding site of the GluN3A subunit that notably diminishes the surface expression of NMDARs, underscoring the critical role of these sites in regulating GluN3A-containing NMDARs.

In a further investigation, we employed *in silico* modeling alongside microscopy, biochemistry, and electrophysiology in HEK293 cells and hippocampal neurons to explore the consequences of the pathogenic GluN1-S688Y variant on trafficking and function of NMDARs (Skrenkova et al., 2020). This variant decreased the surface number of GluN3A-containing NMDARs without impacting the trafficking of GluN2-containing NMDARs. Additionally, the GluN1-S688Y variant elevated the EC_{50} values for glycine and D-serine at GluN1/GluN2A and GluN1/GluN2B receptors and induced slower desensitization rates in GluN1/GluN3A receptors. Notably, this variant also reduced Ca^{2+} influx through NMDARs and diminished NMDA-triggered excitotoxicity in cultured hippocampal neurons. These results highlight how an individual missense pathogenic variant in the glycine-binding site can dramatically influence the regulation of NMDARs.

4.2. Regulation of NMDARs by their *N*-glycosylation

We investigated the role of extracellularly attached *N*-glycans to various NMDAR subtypes (FIG. 3). Through biochemical assays, confocal and electron microscopy, electrophysiological analyses, and employing a lentivirus-based molecular replacement approach, we determined that NMDARs are permitted to exit the ER and reach the cell surface only if two specific asparagine residues in the GluN1 subunit (N203 and N368) undergo *N*-glycosylation (Lichnerova et al., 2015). While *N*-glycosylation also occurs on GluN2A and GluN2B subunits, these modifications were not critical for the surface expression of NMDARs. Our studies furthermore revealed that removing *N*-glycans from the native NMDARs (e.i., GluN1/GluN2 subtypes) in cultured cerebellar granule cells only slightly affected their functional properties. These findings introduce a previously unrecognized mechanism through which neurons may regulate the surface number of NMDARs.

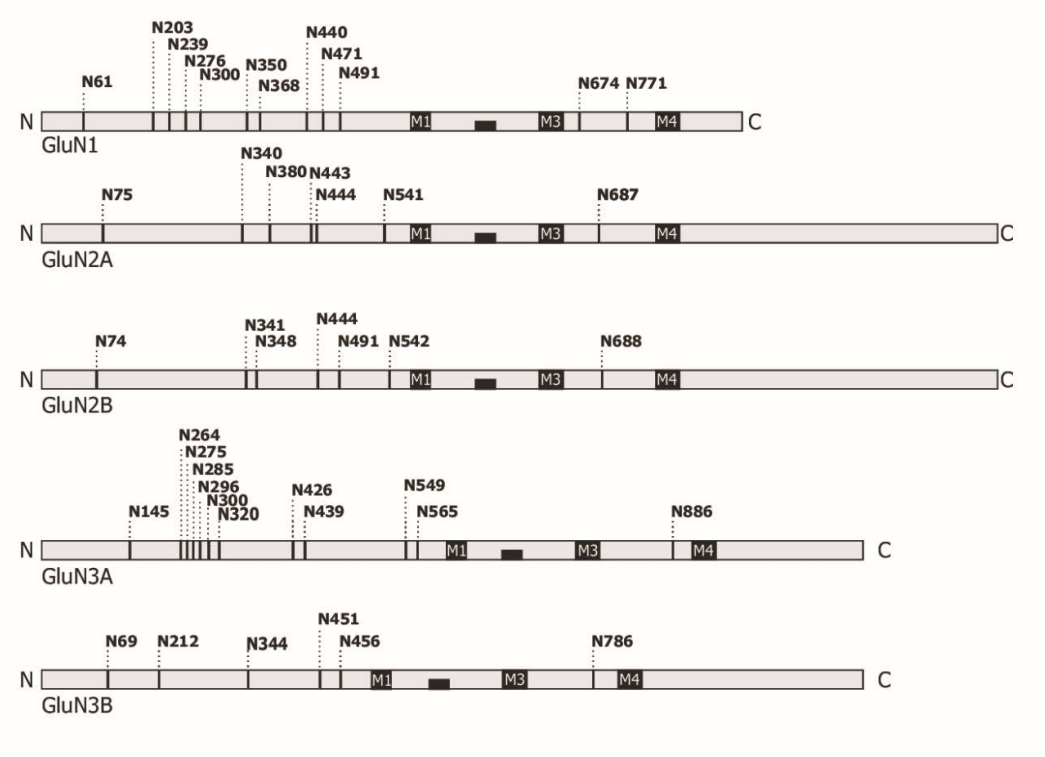

FIGURE 3. Schematic diagram showing the approximate locations of the predicted N-*glycosylation consensus sites (N-X-S/T) in the GluN subunits. The four membrane domains (M1 through M4) are indicated; adapted from our review (Horak et al., 2021).*

We analyzed the specific *N*-glycan profiles attached to endogenous NMDARs in cerebellar tissue (Kaniakova et al., 2016). Through biochemical techniques, we confirmed the extensive *N*-glycosylation of native NMDARs and noted distinct differences in endoglycosidase H sensitivity between GluN1 and GluN2B subunits. A comprehensive assessment using a wide range of lectins was conducted to decode the glycan composition of NMDARs in cerebellar tissue and cultured cerebellar granule cells. This approach allowed us to identify 23 lectins binding to GluN1 and GluN2B subunits. Further electrophysiological studies revealed that treatment of cerebellar granule cells with three selected lectins—Concanavalin A (ConA), Wheat Germ Agglutinin (WGA), and Aleuria Aurantia Lectin (AAL)—modifies the functional characteristics of NMDARs, an effect reversed by deglycosylation with peptide-N-glycosidase F. Similar alterations in functional properties of NMDARs were observed in HEK293 cells expressing recombinant GluN1/GluN2B receptors. Analysis of mutant GluN1/GluN2B receptors in HEK293 cells identified that 11 out of 12 anticipated *N*-glycosylation sites on the GluN1 subunit and all 7 sites on the GluN2B subunit were utilized for *N*-glycan attachment. These observations advanced our understanding of the regulatory influence of *N*-glycosylation on functional properties of NMDARs.

Given the paucity of data on the influence of *N*-glycosylation on GluN3A-containing NMDARs, we extended our methodologies to mutated GluN1 and GluN3A subunits (Skrenkova et al., 2018). Our findings indicated that two asparagine sites (N203 and N368) in the GluN1 subunit and three sites (N145, N264, and N275) in the GluN3A subunit are essential for the surface expression of the GluN3A-containing NMDARs. We also discovered through deglycosylation and lectin analyses that the GluN3A subunits bear complex *N*-glycan modifications, including hybrid/complex types. Furthermore, our research, whether utilizing a series of inhibitors or analyzing human fibroblasts from patients with congenital disorders of glycosylation (CDG), showed that *N*-glycan remodeling is unnecessary for surface expression of GluN3A-containing NMDARs. These insights contribute valuable knowledge to the regulatory mechanisms controlling the surface delivery of GluN3A-containing NMDARs through *N*-glycosylation.

In our subsequent studies, we employed electrophysiological techniques to investigate how specific *N*-glycans and their interactions with selected lectins influence the functional properties of GluN1/GluN3A and GluN1/GluN3B receptors in HEK293 cells (Hemelikova et al., 2019). Our analysis revealed that eliminating potential *N*-glycosylation sites impacts the functionality of GluN1/GluN3B receptors, while GluN1/GluN3A receptors remain unaffected. Additionally, we observed that the activity of both GluN1/GluN3A and GluN1/GluN3B receptors can be modified through the application of various lectins, such as ConA, WGA, and AAL, showing that these effects are primarily due to a decrease in GluN1 subunit-mediated desensitization. Furthermore, our experiments indicated that the influence of lectins is specific to non-activated GluN1/GluN3 receptors and does not persist in the continuous presence of glycine. These results further highlight the distinctive regulatory mechanisms and functional properties of unconventional GluN1/GluN3 receptors compared to the conventional GluN1/GluN2 receptors.

4.3. Surface mobility and localization of NMDARs

In an earlier study, we discovered that the surface mobility of GluN3A-containing NMDARs in hippocampal neurons increases with the treatment of 1-deoxymannojirimycin (DMM), an inhibitor of hybrid/complex *N*-glycan formation and decreases upon exposure to specific lectins (Skrenkova et al., 2018). However, this investigation used relatively bulky rabbit anti-GFP antibodies and secondary antibodies linked to QD605 (antiGFP-QD605) to visualize NMDARs. In our subsequent research, we have employed novel anti-GFP nanobodies tethered to the smallest available QD525 or a slightly larger, thus more luminous, QD605,

designated as nanoGFP-QD525 and nanoGFP-QD605, respectively (Fig. 4)(Kortus et al., 2023). By targeting the YFP-GluN1 subunit in rat hippocampal neurons, we evaluated these nanoprobes against the previously used antiGFP-QD605. Introducing nanoGFP-based probes resulted in a faster lateral diffusion of the NMDARs, evidenced by a substantial increase in the median diffusion coefficient (*D*) values. Furthermore, when demarcating synaptic regions with thresholded tdTomato-Homer1c signals, we observed a significant elevation in the *D* values of the nanobody-tagged NMDARs, particularly at distances greater than 100 nm from the synaptic edge. Conversely, the *D* values for the antiGFP-QD605 showed no variation within a distance of up to 400 nm. Utilizing the nanoGFP-QD605 in hippocampal neurons expressing GFP-GluN2A, GFP-GluN2B, or GFP-GluN3A subunits, we identified differences in the synaptic localization of NMDAR subunits, including variations in their *D* values, duration of synaptic residence, and the rate of movement between synaptic and extrasynaptic regions. Additionally, we verified the effectiveness of the nanoGFP-QD605 for discerning the distribution patterns of synaptic NMDARs, comparing these results against findings obtained with nanoGFPs linked to organic dyes through techniques like universal point accumulation imaging in nanoscale topography and direct stochastic optical reconstruction microscopy.

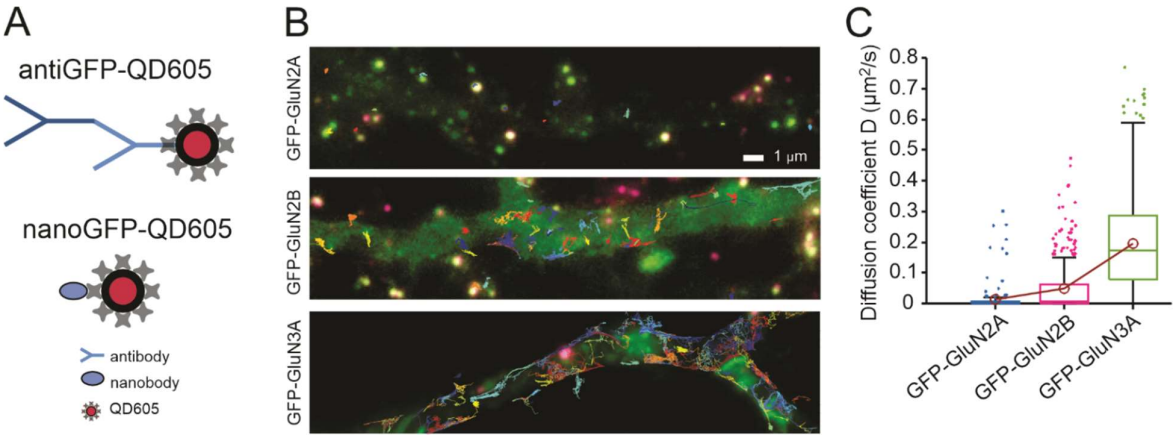

FIGURE 4. Subunit-dependent surface mobility and localization of NMDARs in hippocampal neurons measured using nanobody probe. A) Schematic diagram depicting QD-based probes. The antiGFP-QD605 probe contains a large IgG antibody and another IgG antibody conjugated to QD605. The nanoGFP-QD605 probe contains a small nanobody directly conjugated to QD605. B) Representative surface trajectories of GFP-GluN2A-containing, GFP-GluN2B-containing, or GFP-GluN3A-containing NMDARs (green) in hippocampal neurons expressing synaptic marker Homer (red) tracked using nanoGFP-QD605 (colored trajectories). C) Graph summarizing the D *values for nanoGFP-QD605 trajectories, mobility of NMDAR subunits is as follows: GFP-GluN2A < GFP-GluN2B < GFP-GluN3A. Adapted from our study (Kortus et al., 2023).*

4.4. Novel pharmacological compounds acting at NMDARs

AD is a challenging and progressively worsening neurodegenerative condition for which effective treatments are still not available. At present, AD treatment is mainly limited to symptomatic relief through cholinesterase inhibitors and antagonists of NMDARs (FIG. 5). Our initial research embraced the MTDL approach, focusing on developing 7-methoxytacrine (7-MEOTA)-memantine heterodimers (Gazova et al., 2017). Employing a range of spectroscopic, microscopic, and cell culture techniques, we systematically explored how these heterodimers affect BACE1 activity, interfere with Aβ peptide amyloid fibril formation (a key aspect of the amyloid hypothesis), and interact with mAChRs and nAChRs (addressing the cholinergic hypothesis), as well as NMDARs (pertaining to the glutamatergic hypothesis). The findings revealed that this new series of compounds exhibits a broad-spectrum impact, underscoring their potential to mitigate the neurodegenerative progression associated with AD.

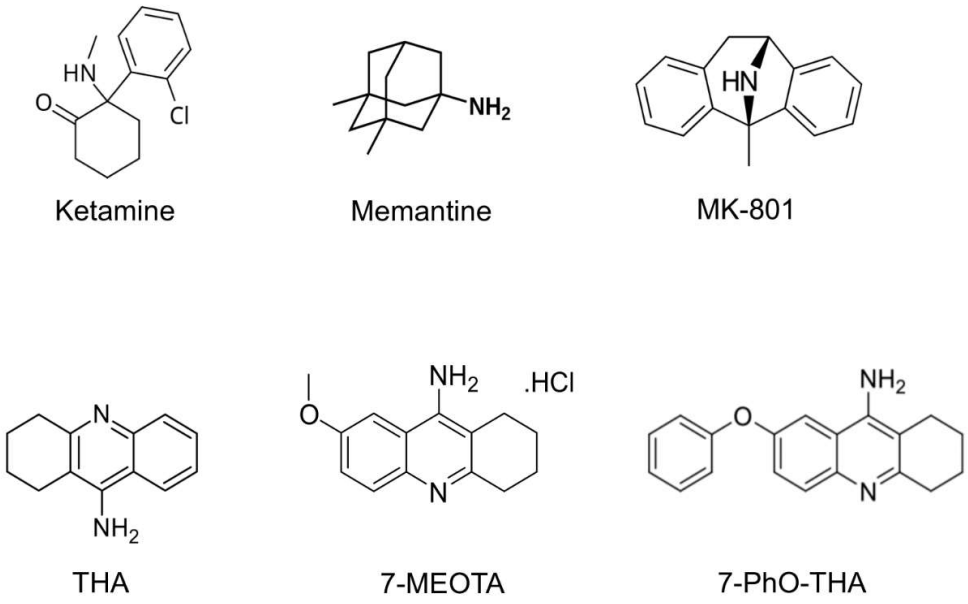

FIGURE 5. Chemical structures of the studied pharmacological modulators of NMDARs. See text for more details.

Advancing with our MTDL approach, we ventured into organic chemistry to synthesize a hybrid molecule combining 6-chlorotacrine (6-Cl-THA) and memantine (Kaniakova et al., 2019). This innovative hybrid demonstrated the acetylcholinesterase (AChE) inhibitory potency of its progenitor, 6-Cl-THA, and exhibited NMDAR-blocking activity akin to memantine. It also showed promise for BBB penetration through passive diffusion. Crucially, *in vivo* studies indicated that this new 6-Cl-THA-memantine hybrid offered a superior

neuroprotective effect compared to memantine alone, indicating a significant advancement in the search for effective AD treatments.

We embarked on the creation, synthesis, and biological assessment of 15 new fluoren-9-amine derivatives (Konecny et al., 2020). The biological evaluation focused on their ability to inhibit cholinesterases, antagonize NMDARs, and their cytotoxicity impact on the CHO-K1 cell line. The findings indicated a preference for butyrylcholinesterase (BChE) inhibition, antagonistic effects at NMDARs, and a benign cytotoxicity profile. Furthermore, we ventured into the development and biological evaluation of 24 new *N*-methylpropargylamino-quinazoline derivatives (Svobodova et al., 2023). These compounds underwent initial *in silico* analysis to predict their oral and CNS bioavailability and were experimentally tested for their inhibitory effects on cholinesterases and MAO-A/B, NMDAR antagonism, effects on dehydrogenase activity, and glutathione levels. Their cytotoxicity was also assessed on undifferentiated and differentiated neuroblastoma SH-SY5Y cell lines. Compound II-6h emerged as a standout, exhibiting selective MAO-B inhibition, NMDAR antagonism, a favorable cytotoxicity profile, and BBB permeability potential. This MTDL approach introduces a groundbreaking methodology for the rational discovery of drugs, significantly advancing our pursuit of novel treatments for AD.

We also recently evaluated the potential of transforming an amiridine-based drug, known for its cholinesterase inhibitory action, into a pioneering category of MTDLs. By employing a linking strategy, amiridine was combined with components such as memantine/adamantylamine, trolox, and substituted benzothiazole groups, creating innovative MTDLs that possess additional properties, including affinity for NMDARs, antioxidant capabilities, and anti-amyloid effects. Additionally, the leading amiridine-derived molecule, compound 5d, underwent *in silico* analysis to compare its butyrylcholinesterase binding affinity with that of a closely related structural variant, 5b. This investigation contributes to the advancement of new amiridine-based medications by expanding their therapeutic targets from solely cholinesterase inhibition to encompassing MTDLs, potentially enhancing AD treatment options (Mezeiova et al., 2024).

From our research, THA derivatives that simultaneously modulate cholinesterase and NMDARs emerge as a promising direction for developing treatments for cognitive impairments, such as those seen in AD. Our review focused on THA and its derivatives' impact on NMDARs, examining their action mechanism and relevance to AD therapy (Horak et al., 2017). Although antagonistic effects of THA on NMDARs are documented *in vitro*, these effects require high compound concentrations. Consequently, we explored the inhibitory

potency of THA compared to 7-MEOTA, the latter being less toxic and exhibiting encouraging outcomes in patients suffering from tardive dyskinesia (Kaniakova et al., 2018). Our findings indicated that THA and 7-MEOTA act as "foot-in-the-door" open-channel blockers on GluN1/GluN2 receptors, with 7-MEOTA demonstrating greater potency albeit slower kinetics than THA. The IC_{50} values for THA and 7-MEOTA showed a sequence of GluN1/GluN2A < GluN1/GluN2B < GluN1/GluN2C = GluN1/GluN2D in terms of potency. Moreover, 7-MEOTA uniquely blocked GluN1/GluN3 receptors as a "foot-in-the-door" mechanism, a property not observed with memantine. Additionally, 7-MEOTA offered superior neuroprotection compared to THA and memantine in rat models of NMDA-induced hippocampal lesions. Intraperitoneal administration of 7-MEOTA also mitigated MK-801-induced hyperlocomotion and deficits in pre-pulse inhibition in rats. Based on these outcomes, 7-MEOTA emerges as a viable candidate for addressing disorders linked to dysfunction of NMDARs.

Our findings indicated that 7-MEOTA exhibits a marginally higher potency at GluN1/GluN2A receptors than at GluN1/GluN2B receptors. In our recent work, we explored a series of 30 new THA derivatives to analyze the structure-activity relationship, noting varied blocking efficacies across different compounds and NMDAR subtypes (Gorecki et al., 2021). Specifically, certain compounds (4 and 5) demonstrated potent inhibition of both GluN1/GluN2A and GluN1/GluN2B receptors, while others (7 and 23) were more effective against GluN1/GluN2B, or GluN1/GluN2A receptors (21 and 28), respectively. QSAR analysis provided a predictive model for the inhibitory effect at GluN1/Glu2B receptors at -60 mV (IC_{50} values) and the relative inhibition of GluN1/Glu2A receptors at +40 mV, which could facilitate ligand-based virtual screenings for new inhibitors of NMDARs. Unlike MK-801, our novel compounds did not induce hyperlocomotion in open-field tests or disrupted pre-pulse inhibition of the startle reflex in rats, indicating a lower risk of psychotomimetic side effects. This suggests that THA derivatives hold promise as inhibitors of NMDARs with subtype specificity and minimal behavioral adverse effects.

In response to the critical role of GluN2B-containing NMDARs in neuronal excitotoxic damage, we developed a dual-function compound, 7-phenoxytacrine (7-PhO-THA; FIG. 5), and assessed its neuropsychopharmacological properties and potential therapeutic application (Kaniakova et al., 2021). Our experiments confirmed the dual efficacy of 7-PhO-THA in inhibiting AChE and NMDARs. Moreover, 7-PhO-THA was found to selectively inhibit GluN1/GluN2B receptors through an ifenprodil-binding site, alongside a voltage-dependent inhibition of both GluN1/GluN2A and GluN1/GluN2B receptors. NMDA-induced

hippocampal lesion studies validated its strong neuroprotective capabilities. Behavioral tests also revealed a cholinergic effect, particularly in reducing hyperlocomotion, without inducing schizophrenia-like side effects. The CNS penetration and favorable behavioral profile of 7-PhO-THA underscore its potential for further therapeutic research.

Building on our initial discovery of 7-PhO-THA, we expanded our investigation to include 30 new derivatives, prioritizing reduced hepatotoxicity, effective AChE activity, and specific antagonism at the GluN1/GluN2B receptors (Misiachna et al., 2024). Through a rigorous selection process by *in vitro* approaches, two derivatives, I-52 and II-52, emerged as promising inhibitors of GluN1/GluN2B receptors due to their affinity for the ifenprodil-binding site and thus subsequently advanced to *in vivo* pharmacodynamic evaluation. Among these, compound I-52, with its negligible AChE binding, was recognized as the leading candidate based on its positive outcomes in behavioral tests, including open-field and pre-pulse inhibition assessments, as well as in scopolamine-induced behavioral paradigms and NMDA-induced hippocampal lesion studies. The findings suggest that I-52 demonstrates low toxicity and reduced hepatotoxicity, a notable concern with THA-derived compounds.

5. Conclusions

- The structural components of the GluN subunits, including their membrane domains and extracellular regions, are crucial for directing the early trafficking, influencing the functional properties, and determining the pharmacological properties of NMDARs. This assertion is supported by our research into pathogenic variants within the GluN1 subunit, which revealed significant impacts on regulating NMDARs that often vary according to the receptor subtype.
- *N*-glycosylation of the GluN1 subunit is essential in the early trafficking of NMDARs. While *N*-glycosylation is not pivotal for the functional properties of NMDARs, the application of lectins significantly influences the desensitization of the GluN1/GluN3 receptors.
- Nanobody-based imaging tools are more effective than traditional antibodies for investigating the surface dynamics and distribution of NMDARs. Notably, NMDARs containing the GluN3A subunits exhibit increased mobility and a reduced tendency for synaptic localization compared to those incorporating GluN2A and GluN2B subunits.
- The MTDL approach is promising for developing new pharmacological agents targeting also NMDARs. Additionally, derivatives of THA display considerable potential for selectively modulating distinct NMDAR subtypes through specific inhibitory mechanisms.

6. Discussion and future directions

6.1. Regulation of NMDARs by their membrane domains and extracellular regions

The discovery of critical amino acid residues within the M3 domains of both GluN1 (W636 and Y647/T648) and GluN2B (W635 and S645/Y646/T647) subunits, along with the M4 domain (L830) of GluN1 subunits that influence the trafficking of NMDARs, presumably at the level of ER processing, aligns with our earlier findings, which highlighted the importance of the M3 and M4 domains in the GluN1 subunit and the M3 domains in the GluN2A-B subunits for release of the NMDARs from the ER (Horak et al., 2008). Further investigations revealed that the GluN1-M641I variant diminishes the surface number of GluN1/GluN2A receptors without affecting GluN1/GluN2B and GluN1/GluN3A receptors. Conversely, the GluN1-A645S variant showed no impact on the surface delivery of any NMDAR subtype examined. However, the GluN1-Y647S variant led to decreased surface expression of GluN1/GluN2A and GluN1/GluN2B receptors but not GluN1/GluN3A receptors, aligning with our prior observations involving GluN1-Y647A/GluN2 receptors (Horak et al., 2008; Kaniakova et al., 2012a). Additionally, the GluN1-N650K variant selectively influenced the surface expression of NMDAR subtypes, slightly enhancing the expression of GluN1/GluN2A and GluN1/GluN2B receptors, but not affecting GluN1/GluN3A receptors. The observation that mutations in GluN1 subunits do not alter surface trafficking of the GluN3A-containing NMDARs could be attributed to structural differences in the membrane domains of GluN1/GluN2 and GluN1/GluN3 receptors (Wada et al., 2006).

The pursuit of therapies targeting NMDARs is experiencing a resurgence in treating various genetic and autoimmune disorders (Hansen et al., 2021). Considering that memantine levels in the plasma and cerebrospinal fluid of patients are reported to be between 0.5-1 μM (Kornhuber and Quack, 1995; Parsons et al., 2007; Quack et al., 1995), memantine emerges as a viable therapeutic avenue for individuals harboring specific pathogenic variants in GluN subunits. For instance, memantine has effectively reduced seizure frequency in patients with variants in the M3 domain of the GluN2D subunit (Li et al., 2016) and improved social impairments in pervasive developmental disorders (Erickson et al., 2007). Our research indicates that the pathogenic GluN1-M641I and GluN1-A645S variants, linked to intellectual disability, seizures, and movement disorders (Kolcheva et al., 2021; Lemke et al., 2016), respond differently to memantine. Likewise, the GluN1-N650K variant, associated with movement disorders, developmental delays, severe intellectual disability, and childhood seizures (Lemke et al., 2016; Ohba et al., 2015), alters the inhibitory kinetics of memantine and ketamine at GluN1/GluN2A receptors. The potential benefits of memantine and ketamine treatment for patients with these GluN1 variants remain speculative.

Consequently, *in vivo* studies (e.g., by using knock-in mouse models) are essential to explore the functional consequences of these GluN1 pathogenic variants, particularly regarding excitatory synaptic transmission and synaptic plasticity. Future investigations should also consider whether inhibiting (e.g., via memantine or ketamine) or enhancing (e.g., through positive allosteric modulators) the activity of NMDARs with pathogenic variants could be therapeutically advantageous.

We have pinpointed three critical areas within the GluN2C subunit—located within the M3 domains and the ATD and CTD regions—that are crucial for the surface expression of GluN1/GluN2C receptors. This underscores a distinctive regulatory mechanism of surface expression for NMDARs among glutamate ionotropic receptors, where the extensive N- and C-termini of GluN subunits likely play a significant role in the early trafficking stages, possibly through interactions with various proteins *en route* to the cell surface. Multiple trafficking signals within the NMDARs might implement rigorous control during ER processing, ensuring the transport of only correctly assembled heterotetramers of NMDARs. Moreover, these diverse regulatory strategies may be selectively activated under specific conditions, such as during synaptic plasticity or neuronal development.

Regarding GluN3A-containing NMDARs, our observations indicate that alterations in the glycine-binding sites of GluN1 and GluN3A subunits critically influence the early trafficking of these receptors, including those incorporating endogenous GluN subunits in hippocampal neurons. The exact mechanisms by which mutations in these glycine-binding sites affect the surface expression of GluN3A-containing NMDARs remain elusive, though they appear to share similarities with those identified for GluN1/GluN2 receptors and AMPARs (Kenny et al., 2009; Penn et al., 2008; She et al., 2012). Our latest findings, highlighting the significant impact of the GluN1-S688Y variant within the glycine-binding site on reducing the surface expression of GluN3A-containing NMDARs, align with our hypothesis that glycine sensitivity plays a pivotal role in regulating their trafficking to the cell surface (Skrenkova et al., 2019).

Conversely, our observation that the GluN1-S688Y variant does not impact the surface expression of GluN1/GluN2A or GluN1/GluN2B receptors contrasts with previous findings indicating that the GluN1-D732A mutation reduces both trafficking and glycine potency of GluN1/GluN2A receptors (Hawkins et al., 2004). This discrepancy underscores the importance of further investigations to unravel the complexities of subtype-specific variations in the processing of NMDARs at early stages. It is posited that glutamate concentrations within the ER are sufficiently high, in the millimolar range (Berger et al., 1977; Meeker et al., 1989), to fully occupy the glutamate-binding sites of newly synthesized NMDARs. While the exact glycine levels in the

ER remain uncertain, considering glycine's involvement in numerous metabolic functions (Wang et al., 2013), it is plausible that it could at least partially occupy glycine-binding sites on newly formed NMDARs. Theoretically, neurons may modulate the number of surface NMDARs by adjusting the synthesis of agonists like glycine and D-serine or naturally occurring competitive antagonists such as kynurenic acid (Moroni et al., 1988; Traynelis et al., 2010). Future research should include identifying new pharmacological compounds capable of influencing the surface expression of NMDARs, offering potential therapeutic avenues for treating specific diseases.

6.2. Regulation of NMDARs by their *N*-glycosylation

Our research uncovered a distinctive molecular mechanism reliant on two *N*-glycosylation sites within the GluN1 subunit—absent in GluN2A and GluN2B—critical for releasing NMDARs from the ER. Utilizing various cell models, including CV-1 (simian) in Origin, and carrying the SV40 genetic material (COS-7) and HEK293 cells, as well as cultured hippocampal neurons and cerebellar granule cells, our studies corroborate the notion that ER quality control systems, ubiquitous in mammalian cells, assess the presence of *N*-glycans at two pivotal asparagine sites on the GluN1 subunit. Notably, our investigations suggest that the trafficking of GluN1/GluN3A receptors relies more on the *N*-glycosylation state of the GluN1 subunit than that of the GluN3A subunit. The observation that *N*-glycan remodeling is not essential for the cell surface expression of GluN3A-containing NMDARs aligns with findings from Hanus *et al.*, who documented similar mechanisms across a range of neuronal membrane proteins (Hanus et al., 2016).

Our research demonstrated that removing *N*-glycans from endogenous GluN1/GluN2 receptors modifies their potency for NMDA, aligning with findings from studies on *Xenopus* oocytes (Everts et al., 1997). Moreover, the complete elimination of accessible *N*-glycans from NMDARs did not influence their open probability or desensitization characteristics. Similarly, mutations of *N*-glycosylation sites on GluN1 and GluN3A subunits did not alter the desensitization kinetics of GluN1/GluN3A receptors, supporting our earlier work (Skrenkova et al., 2018). This suggests that *N*-glycans may fine-tune the functional properties of specific NMDARs during excitatory synaptic transmission (Traynelis et al., 2010). The functional impact of lectins on GluN1/GluN2 receptors is minimal (Kaniakova et al., 2016; Mayer and Vyklicky, 1989), echoing a prior observation that desensitization of AMPARs is diminished by ConA (Mayer and Vyklicky, 1989) and another study showing lectins' impact on desensitization of AMPARs and kainate receptors in *Xenopus* oocytes (Thalhammer et al., 2002). Our findings also highlighted a strong interaction between GluN3A-containing NMDARs and various lectins, including ConA, WGA,

and AAL (Skrenkova et al., 2018), which bind to mannose, hybrid, and complex *N*-glycan forms, and mitigate desensitization in both GluN1/GluN3A and GluN1/GluN3B receptors.

Future investigations should explore if *N*-glycan-interacting proteins might influence the functional properties of endogenous GluN3-containing NMDARs in both standard and diseased states, drawing parallels with galectin modulation of AMPARs and kainate receptors (Copits et al., 2014). Additionally, there is a need to catalog the full spectrum of *N*-glycans on NMDARs under physiological conditions and identify changes under disease states, as has been done for AMPARs in schizophrenia cases (Tucholski et al., 2013). Exploring the use of lectins for NMDAR-targeted pharmacological interventions may also hold promise. Given that CDG frequently manifests with CNS symptoms, including cognitive deficits, seizures, and epilepsy (Freeze, 2006; Moremen et al., 2012), understanding *N*-glycosylation's role in CNS function has significant clinical implications. The scarcity of human brain samples from CDG patients underscores the value of innovative methods for transforming human fibroblasts into neuronal cells (Vierbuchen et al., 2010), offering new avenues for studying how glycosylation impairments affect the regulation of NMDARs.

6.3. Surface mobility and localization of NMDARs

Our studies revealed that the mobility of GluN3A-containing NMDARs is enhanced upon the removal of hybrid and complex *N*-glycans, aligning with findings that the mobility of AMPARs is modulated by the enzymatic stripping of the extracellular matrix (Frischknecht et al., 2009). Notably, the *D* values of GluN3A-containing NMDARs profoundly decreased in the presence of lectins, suggesting that lectins might indirectly influence the mobility of GluN3A-containing NMDARs through interactions with other glycosylated proteins on the neuronal surface. Considering the diverse array of lectins present in the mammalian CNS, such as sialic acid–binding Ig-like lectins and galactose-binding lectins (Macauley et al., 2014), it is plausible these molecules could modulate the mobility of NMDARs in a physiological context.

Recent investigations assessing synaptic access for AMPARs labeled with QDs of varying sizes and fluorescent dyes indicated a marked reduction in the proportion of receptors within the synaptic cleft for larger probes (Le et al., 2020; Lee et al., 2017). Our analysis of QD trajectories in neurons expressing the YFP-GluN1-1a subunit showed no significant disparity in synaptic cleft access between nanoGFP-QD-based probes and the traditional antiGFP-QD605. However, the nanoGFP-QD-based probes exhibited notably higher *D* values for both synaptic and extrasynaptic receptor pools, with a more pronounced increase in *D* values moving away from the synaptic edge. The *D* values obtained with nanoGFP-QD-based probes closely mirrored those for the YFP-

GluN1-1a subunit labeled with nanoGFP-ATTO647N probe, where a smaller fluorescent dye was used instead of a QD. Owing to the superior localization precision of the nanoGFP-QD605 over the nanoGFP-QD525, we selected the nanoGFP-QD605 for our comprehensive analysis of the surface mobility of NMDARs.

The *D* values we obtained for the extrasynaptic QD trajectories of NMDARs incorporating GFP-tagged GluN2 subunits (with GFP-GluN2A < GFP-GluN2B) align with prior research utilizing conventional IgG antibodies (Groc et al., 2006). Upon accurately delineating synaptic regions, we observed no distinction in *D* values between synaptic GFP-GluN2A- and GFP-GluN2B-containing NMDARs, highlighting the importance of precise synaptic region identification for accurate QD trajectory analysis of NMDARs. Our examination of QD trajectories of GFP-GluN3A-containing NMDARs underscored that these receptors exhibit i) the shortest synaptic dwelling time and ii) the most rapid rate of movement between synaptic and extrasynaptic regions, aligning with the observed tendency of GluN3A-containing NMDARs to localize in perisynaptic and extrasynaptic areas (Perez-Otano et al., 2006). This observation could hold significant implications in a physiological context, such as facilitating swift shifts in the synaptic composition of NMDARs during synaptic development (Crawley et al., 2022; Holtmaat et al., 2005; Kehoe et al., 2014; Rakic et al., 1986). Although synaptic regions were identified using 2D imaging, which is considered reliable when analyzing a broad set of synapses under the assumption of random synaptic orientation (Ferreira et al., 2017; Groc et al., 2006), variations in synaptic regions and dendritic spine structure could influence our results (Kehoe et al., 2014; Roberts et al., 2009). Consequently, subsequent research should integrate detailed structural insights regarding dendritic spine morphology in live neuron imaging. Our uncertainty analysis revealed that the nanoGFP-QD605 boasts remarkable localization precision (~6 nm) and an essentially unlimited operational lifespan, suggesting that our experimental approach is well-suited for exploring the impact of endogenous ligands, pharmacological modulators like ketamine and memantine (Song et al., 2018; Zhang et al., 2021), and pathogenic GluN subunit variants on the specific surface mobility and localization of NMDARs.

6.4. Novel pharmacological compounds acting at NMDARs

Despite extensive research on AD, the market has seen only a limited number of drugs that offer symptomatic relief. Our study supports the concept that developing MTDL is a promising strategy for creating viable preclinical candidates for AD treatment. Nonetheless, challenges such as reduced efficacy, insufficient BBB penetration, and toxicity remain with newly developed compounds acting at NMDARs. Given the comprehensive nature of pharmacological evaluation

for novel compound inhibitory activities, we primarily concentrated on crafting and analyzing novel THA derivatives. Our work with recombinant GluN1/GluN2 receptors highlighted that THA and 7-MEOTA preferentially target GluN1/GluN2A receptors, with 7-MEOTA being approximately three times as potent as THA. Our findings that THA and 7-MEOTA act as "foot-in-the-door" open-channel blockers align with earlier research identifying THA as a reversible blocker for native NMDARs (Costa and Albuquerque, 1994; Vorobjev and Sharonova, 1994). Remarkably, 7-MEOTA also serves as a "foot-in-the-door" blocker for GluN1/GluN3A and GluN1/GluN3B receptors, a novel observation given past evidence suggesting these receptors are not affected by traditional blockers of GluN1/GluN2 receptors (Chatterton et al., 2002; Henson et al., 2010; Kehoe et al., 2013; Low and Wee, 2010; Pachernegg et al., 2012; Perez-Otano et al., 2016). This suggests that 7-MEOTA's unique structure interact within the ion channel pores of both GluN1/GluN2 and GluN1/GluN3 receptors. Intriguingly, human volunteers tolerated 7-MEOTA well at doses of 2 mg/kg and 7 mg/kg per day, with no adverse effects on cognitive functions observed. Furthermore, 7-MEOTA demonstrated efficacy in oral administration to patients with tardive dyskinesia resulting from prolonged neuroleptic use (Patocka et al., 2008). Consistent with its tolerability, we noted that 7-MEOTA did not affect rat behavior in open-field tests, unlike MK-801 (Martin et al., 1997; Stuchlik et al., 2004; van der Staay et al., 2011). Therefore, our results suggest 7-MEOTA does not have the notable behavioral side effects associated with many antagonists of NMDARs, highlighting its therapeutic potential for conditions involving dysfunction of NMDARs.

We proceeded to develop THA derivatives capable of either non-selectively blocking GluN1/GluN2A and GluN1/GluN2B receptors or exhibiting enhanced inhibitory action towards either GluN1/GluN2A or GluN1/GluN2B receptors. Through QSAR analysis, we established a meaningful correlation with our experimental data, laying the groundwork for future ligand-based virtual screenings to identify promising ligands of NMDARs. Subsequently, we explored the characteristics of 7-PhO-THA, which effectively targets GluN1/GluN2B receptors through the extracellular ifenprodil-binding site. Intriguingly, the *in vitro* inhibitory effectiveness of 7-PhO-THA, as reflected in its IC_{50} values against both NMDARs and AChE, falls within a comparable range, underscoring its "multitarget" potential (Van der Schyf, 2011). Given the link between overstimulation of GluN1/GluN2B receptors and excitotoxicity-induced neurodegeneration (Hardingham and Do, 2016; Vieira et al., 2016; Vizi et al., 2013; Zhou et al., 2013), 7-PhO-THA demonstrated efficacy in protecting brain tissue adjacent to the site of NMDA application. Together, compounds such as THA, 7-MEOTA, and 7-PhO-THA show promise for the treatment of CNS disorders characterized by neurodegeneration, including AD. Future research should

assess their long-term effectiveness in AD animal models and delve into the underlying mechanisms of THA-associated toxicity, particularly hepatotoxicity.

7. References

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8. List of my publications as a basis of this thesis

The impact factor (IF) is from each scientific study's publication year and for 2023.

[number of citations including self-citations] according to Web of Science as of 12.8.2024.

A) Regulation of NMDARs by their membrane domains and extracellular regions

Horak M*, Barackova P, Langore E, Netolicky J, Rivas-Ramirez P, Rehakova K (2021) The Extracellular Domains of GluN Subunits Play an Essential Role in Processing NMDA Receptors in the ER. Front Neurosci 15:603715. **IF_{2021}: 5.2; IF_{2023}: 3.2 [4**]

Horak M, Seabold GK, Petralia RS (2014) Trafficking of glutamate receptors and associated proteins in synaptic plasticity. In: The Synapse: Structure and Function (Pickel V, Segal M, eds). New York: Elsevier. Chapter 8: 221-279.

Horak M*, Petralia RS, Kaniakova M, Sans N* (2014) ER to synapse trafficking of NMDA receptors. Front Cell Neurosci 8:394. **IF_{2014}: 4.3; IF_{2023}: 4.2 [59**]

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Kaniakova M, Lichnerova K, Vyklicky L, **Horak M*** (2012) Single amino acid residue in the M4 domain of GluN1 subunit regulates the surface delivery of NMDA receptors. J Neurochem. 123(3):385-95. **IF_{2012}: 4.0; IF_{2023}: 4.2 [13**]

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Skrenkova K, Hemelikova K, Kolcheva M, Kortus S, Kaniakova M, Krausova B, **Horak M*** (2019) Structural features in the glycine-binding sites of the GluN1 and GluN3A subunits regulate the surface delivery of NMDA receptors. Sci Rep 9:12303. **IF_{2019}: 4.0; IF_{2023}: 3.8 [19**]

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Krausova BH, Kucera T, Korabecny J, Suh YH*, **Horak M*** (2020) The pathogenic S688Y mutation in the ligand-binding domain of the GluN1 subunit regulates the properties of NMDA receptors. Sci Rep 10:18576. **IF$_{2020}$: 4.4; IF$_{2023}$: 3.8 [11**]

B) Regulation of NMDARs by their *N*-glycosylation

Lichnerova K, Kaniakova M, Park SP, Skrenkova K, Wang YX, Petralia RS, Suh YH*, **Horak M*** (2015) Two N-glycosylation Sites in the GluN1 Subunit Are Essential for Releasing N-methyl-d-aspartate (NMDA) Receptors from the Endoplasmic Reticulum. J Biol Chem 290:18379-18390. **IF$_{2015}$: 4.3; IF$_{2023}$: 4.0 [40**]

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C) Surface mobility and localization of NMDARs

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D) Novel pharmacological compounds acting at NMDARs

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9. Abbreviations

AD: Alzheimer's disease

Aβ: Amyloid beta

ATD: amino-terminal domain

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

AAL: Aleuria Aurantia Lectin

BACE: β-secretase

BBB: blood-brain barrier

BChE: butyrylcholinesterase

CAS: Czech Academy of Sciences

CDG: congenital disorders of glycosylation

6-Cl-THA: 6-chlorotacrine

CNS: central nervous system

ConA: Concanavalin A

COS: CV-1 (simian) in Origin, and carrying the SV40 genetic material

CTD: C-terminal domain

D: diffusion coefficient

DMM: 1-deoxymannojirimycin

EC_{50}: half maximal effective concentration

ER: endoplasmic reticulum

GFP: green fluorescent protein

HEK293: human embryonic kidney 293 (HEK293)

IC_{50}: half maximal inhibitory concentration

IEM: Institute of Experimental Medicine CAS

IgG: immunoglobulin G

IPHYS: Institute of Physiology CAS

LBD: ligand-binding domain

MAO: monoamine oxidase

7-MEOTA: 7-methoxytacrine

MK-801: dizocilpine

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

mAChR: muscarinic acetylcholine receptor

MTDL: multi-target-directed ligand

nAChR: nicotinic acetylcholine receptor

NMDAR: *N*-methyl-D-aspartate receptor

7-PhO-THA: 7-phenoxytacrine

PSD: postsynaptic density

QSAR: quantitative structure-activity relationship

QD: quantum dot

THA: 1,2,3,4-tetrahydro-9-aminoacridine (tacrine)

ThT: thioflavin

TMD: transmembrane domain

YFP: yellow fluorescent protein

WGA: Wheat Germ Agglutinin

The English language corrections were made using ChatGPT 4.0 and Grammarly.