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The Analysis of Structural details of the NMDA receptor

Analýza strukturních detailů NMDA receptoru

Diploma thesis

Supervisor: RNDr. Aleš Balík, Ph.D.

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Prohlašuji, že jsem závěrečnou práci zpracovala samostatně pod vedením školitele RNDr. Aleše Balíka, Ph.D. a všechny použité informační zdroje a literaturu řádně citovala. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Most of all, thanks to Aleš.

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To all of them.

CONTENTS

1 LIST OF ABREVATION

AA	amino acid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid receptor
BSA	bovine serum albumin
CNS	central nervous system
CTD	C- terminal domain
H_2O T1	ultrapure water Type 1
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
eGFP	enhanced green fluorescent protein
FBS	foetal bovine serum
GFP	green fluorescent protein
GluA1-4	AMPAR subunits 1-4
GluN1-3	NMDAR subunits 1-3
HVL	hypervariable loop
iGluRs	ionotropic glutamate receptor
kDa	kilo Dalton
kPa	kilo Pascal
LBD	ligand-binding domain
L-Met	L-Methionine
NMDA	*N*-methyl-D-aspartate
NMDAR	*N*-methyl-D-aspartate receptor
NTD	N-terminal domain
PBS	phosphate buffer saline
PCR	polymerase chain reaction
photo-Met	photo-L-Methionine
RNA	ribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TMD	transmembrane domain

2 ABSTRACT

NMDA receptor is necessary for excitatory transmission in the central nervous system. Altered funtion of the NMDA receptors is associated with many neurodegenerative and neuropsychiatric diseases. All available crystal structures of the NMDAR meant great shift towards our understanding of details of the receptor and its function. Unfortunately, these up-to-date available structures present only certain functional states of receptors and also a few structural data are still missing. For complete comprehension of the process of activation and deactivation of NMDA receptors, we need to supplement the current information with more data. The aim of this thesis was to employ a combination of different approaches (computational modelling, cloning, biochemistry, protein expression and purification and mass spectrometry) to obtain new structural data, by which we would be able to fill in the gaps in current receptor models, especially at various functional states of the receptor.

Key words:

NMDA receptor, glutamate receptor, computational modelling, structure, cloning, protein expression

3 ABSTRAKT

NMDA receptor je nezbytnou součástí exitační transmise v centrální nervové soustavě. Pozměněná funkce NMDA receptorů je spojována s mnoha neurodegenerativními a neuropsychiatrickými chorobami. Vyřešené krystalové struktury NMDA receptorů znamenaly velký posun v pochopení detailů jejich funkce. Bohužel tyto v současnosti dostupné struktury představují jen některé funkční stavy receptoru a také několik strukturních informací stále chybí. Ke kompletnímu porozumění procesu aktivace a deaktivace NMDA receptorů je potřeba doplnit stávající informace dalšími studiemi. Cílem této práce bylo pomocí kombinace různých metod (počítačové modelování, klonování, biochemické metody, exprese a purifikace proteinu a strukturní hmotnostní spektrometrii) získat nová strukturní data, kterými bychom byli schopni doplnit mezery v současnosti dostupných modelech a strukturách receptoru, zejména pak receptoru v různých funkčních stavech.

Klíčová slova

NMDA receptor, glutamátový receptor, počítačové modelování, struktura, klonování, exprese proteinu

4 THEORETICAL PART

4.1 Introduction

Glutamatergic neurotransmission in the central nervous system (CNS) is mediated by glutamate-gated ion channels (iGluRs). They are present in all excitatory synapsis of the mammalian nervous system and underlie the processes of learning and memory. There are several types of iGluRs, and each of them forms ion channel. Activation of the receptor by specific ligand – L-glutamate (Curtis, Phillis and Watkins, 1959) causes intracellular influx of related cations (Na^+ and Ca^{2+}) conjugated with the potassium ions eflux. According to their pharmacology, there are four subtypes of iGluRs – kainate, NMDA, APMA and δ receptors (Niciu, Kelmendi and Sanacora, 2012).

NMDA receptors and AMPA receptors are responsible for the molecular mechanisms for synaptic plasticity. NMDA receptor is thought as major mechanism, responsible for forming synaptic plasticity. Opening of this receptor leads to the rise of intracellular Ca^{2+} concentration in postsynaptic neuron, an initial step in the long-term potentiation and long-term deprivation (Lynch, 2004; Luscher and Malenka, 2012). However, the overactivation, as well as lowered activity of NMDAR is associated with development of several neurodegenerative and neuropsychiatric disorders, such as the Alzheimer's disease or schizophrenia, due to the altered level of Ca^{2+} in neurons (Sf F. Traynelis *et al.*, 2010; Gupta, Hardingham and Chandran, 2013).

The presented thesis is focused mostly on NMDA receptor physiology. Detailed understanding of their structural features would lead to complete understanding of the receptor-gating process, therefore several *in silico* and biochemistry approaches leading to obtain novel information about NMDAR structure were designed. In the first part of my thesis, I am summarising theoretical knowledge about NMDA and AMPA receptor structures. Employing enlisted methods, I wanted to widen this current-state of knowledge with computational models as well as with experimental data. Up-to-date results are shown under with following discussion, where I am commenting on these data and suggesting possible explanations and I am proposing some optimizations for subsequent experiments.

4.2 Ionotropic glutamate receptors

Ionotropic glutamate receptors are excitation receptors spread across mammalian central nervous system (CNS) (see Fig. 1) (Sf F. Traynelis *et al.*, 2010), but also in several other tissues, such as kidneys (Anderson *et al.*, 2011; Lin *et al.*, 2015), osteoblasts (Genever *et al.*, 1999), taste buds (Chaudhari *et al.*, 1996; Lin and Kinnamon, 1999) etc.. Each of them is activated by its agonist – glutamate – the primary excitatory neurotransmitter in brain (Curtis, Phillis and Watkins, 1959; Meldrum, 2000). According to their pharmacological and structural features they can be divided into three main groups including: NMDA (N-methyl-D-aspartic acid), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and kainate receptors; and one minor group of δ receptors (Collingridge *et al.*, 2009; Matsuda *et al.*, 2010; Khan, 2017). They form transmembrane ligand-gated tetrameric bouquet-like shaped central ion channels and all subunits share similar architecture – extracellular amino(N)- terminal domain (NTD) and ligand-binding domain (LBD) (both in clamshell-like structure). NTD is connected to three polypeptide chains – linkers to the transmembrane domain (TMD) containing four α helixes (M1-M4) (Karakas, Regan and Furukawa, 2015) and intracellular C-terminal domain (CTD) (Stephen F. Traynelis *et al.*, 2010).

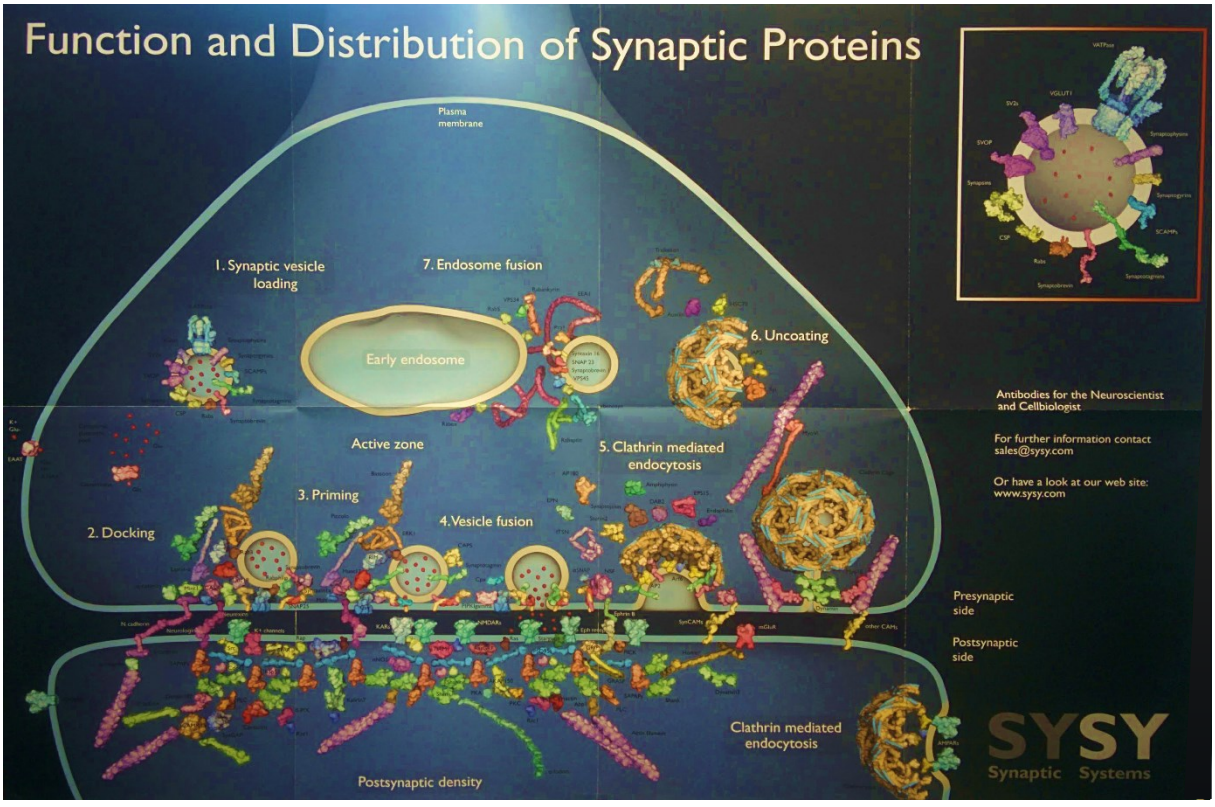


Fig. 1. Function and Distribution of Synaptc Proteins; adapted from Synaptic Systems (www.sysy.com/products)

AMPA and kainite receptors are be activated exclusively by glutamate. (Keinanen *et al.*, 1990; Werner *et al.*, 1991). NMDA receptors beside glutamate require for activation co-agonist glycine as well as membrane depolarization which leads to the release of magnesium block of the ion channel. (Mayer, Westbrook and Guthrie, 1984; Nowak *et al.*, 1984). This need of glutamate presence and membrane depolarization explain slower kinetics of activation/deactivation of NMDA receptors.

4.2.1 AMPA receptors

4.2.1.1 Structure of AMPA receptors

The first glutamate receptors were observed by electron microscopy in quaternary structure by Safferling *et al.*, 2001 and Tichelaar *et al.*, 2004. Even though the low resolution of these revealed structures (around 20 Å), it provided primary insights into outer receptor layout: internal 2-fold rotational symmetry, indicating receptor assembly dimer of dimers (Tichelaar *et al.*, 2004; Midgett and Madden, 2008). Consequent crystallographic studies reached resolution of 3.6 Å in membrane-spanning homotetrameric rat GluA2 receptor in complex with antagonist glutamate (see Fig. 2).

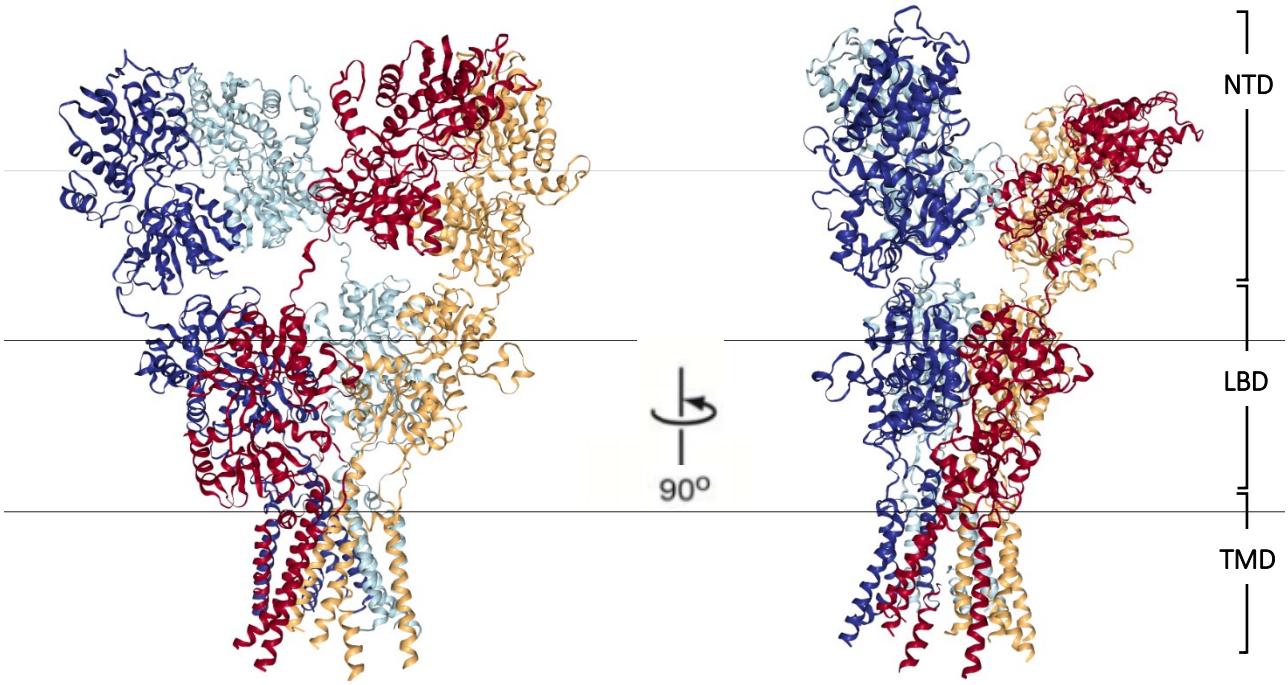


Fig. 2. Overall structure of homologous GluA2 AMPA receptor; PDB ID **3KG2**; adapted from (Sobolevsky, Rosconi and Gouaux, 2009).

According to this data, 2-fold symmetry perpendicular to the membrane, where NTD and LBD occupy dimers of dimers organization and ion channel domains are organized in 4-fold symmetry, was confirmed (Sobolevsky, Rosconi and Gouaux, 2009).

AMPA receptors assemble as a complex of four subunits at various stoichiometry, termed: GluA1, GluA2, GluA3 and GluA4 (Matsuda, Kamiya and Yuzaki, 2005; Sobolevsky, Rosconi and Gouaux, 2009). All these types can form homo- and heteromers as well and, as mentioned previously, assemble as dimers of dimers (Stephen F. Traynelis *et al.*, 2010). Formation of this conformation is apparently enabled through NTDs interaction that is crucial for initiation of oligomerization, as well as formation of the dimers and following tetramerization through LBDs and TMDs (Kuusinen *et al.*, 1999; Leuschner and Hoch, 1999; Ayalon and Stern-Bach, 2001; Mansour *et al.*, 2001; Ayalon *et al.*, 2005). The importance of initial dimerization via NTD is highlighted in studies proving that even isolated NTDs are able to create stable dimers in solution (Clayton *et al.*, 2009; Jin *et al.*, 2009; Kumar *et al.*, 2009; Rossmann *et al.*, 2011).

4.2.1.1.1 Receptor domains

4.2.1.1.1.1 N-terminal domain of the AMPA receptor

The NTD of GluA2 subunit is folded in clamshell-like structure (see Fig. 3). Similar structure, also occurs in NMDA receptor subunits (see 0). The clamshell is formed of two lobes – L1 (N-terminal) and L2 (C-terminal), linked together with three short loops. As many other molecular structures, NTD GluR2 subunit is post-translationally modified – by the N-linked glycosylation (Asn235/Asn349) and disuphide bond (Cys57/Cys309) (Jin *et al.*, 2009). NTD of GluA2 shares several structural similarities with mGluR-LBD – the six highly conserved residues, that are responsible for the mGluR-LBD-ligand interaction, are missing in GluA2-NTD. The AMPA receptor NTD is required for the assembly of the tetrameric receptor (Sf F. Traynelis *et al.*, 2010).

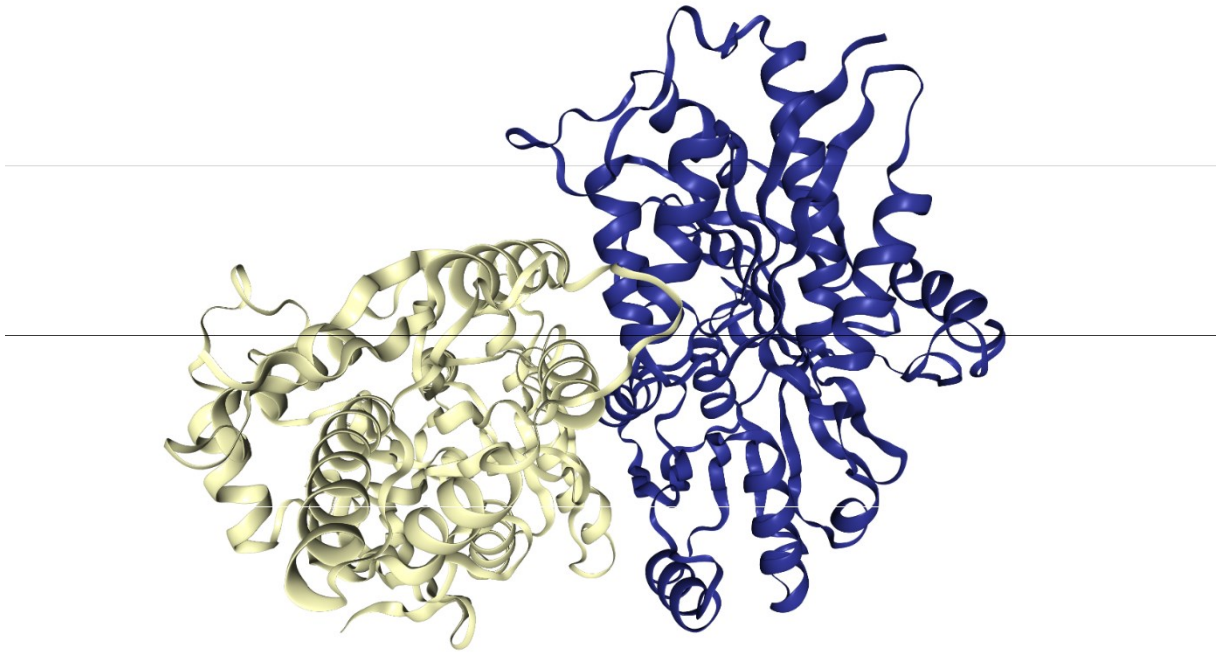


Fig 3. Structure of the hom 2-mer GluR2 NTD; PDB ID **3H5V**; adapted from (Clayton *et al.*, 2009).

The LBD is also conserved structure not only in AMPA receptors, but in NMDA receptors as well (see 4.2.2.1.2.1). Two polypeptidic chains, named S1 and S2 (Stern-Bach *et al.*, 1994) create conformation appearing like a clamshell (see Fig. 4). Agonists bind into the clamshell structure, between S1 and S2 AAAs proven in study (Stern-Bach *et al.*, 1994; Sun *et al.*, 2002). LBD is arranged in 2-fold axis symmetry. When ligand is bound the conformation of the clamshell changes, subsequently leading to the separation of the TMD via linkers and to the ion channel opening and influx of potassium into the cell (Sobolevsky, Rosconi and Gouaux, 2009).

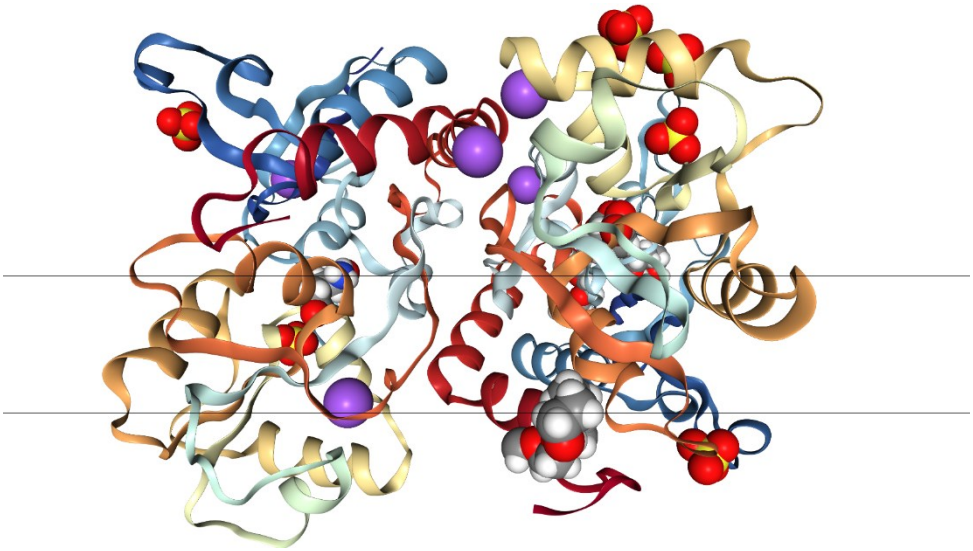


Fig 4. Crystal structure of the hom 2-mer GluR2 LTD in complex with glutamate; PDB ID **5NS9**; adapted from (Zhang *et al.*, 2017).

4.2.1.1.1.3 Transmembrane domain of the AMPA receptor

The TMD is forming the ion channel and presents the narrow 'base' of the AMPA receptor. TMD itself reminds 'Mayan temple' shape, that is ~42 Å broad(Sobolevsky, Rosconi and Gouaux, 2009)(Sobolevsky, Rosconi and Gouaux, 2009)(Sobolevsky, Rosconi and Gouaux, 2009)(Sobolevsky, Rosconi and Gouaux, 2009)(Sobolevsky, Rosconi and Gouaux, 2009). LBD passes onto the section of three transmembrane helices – M1, M3 and M4, forming the TMD, including central M2 loop forming the ion channel pore. S1 segment of the LBD is connected to the membrane helix M1, M3 and M4 helices are linked by two short polypeptidic chains to the S2 segment of the LBD. In the interface of extracellular domains and TMD a symmetry mismatch occurs. TMD is set out around 4-fold rotational symmetry, arranged as a tetramer, meanwhile extracellular domains NTD and LBD are set out around 2-fold symmetry, arranged as a dimer of dimers (Sobolevsky, Rosconi and Gouaux, 2009; Karakas, Regan and Furukawa, 2015).

4.2.1.1.1.4 C-terminal domain of the AMPA receptor

The CTD is generally the most variable (in sequence length or AA sequence) section of the receptor. Similarly to the NMDAR subunit CTD, GluA CTD mediates intracellular protein-protein interactions. For example with PSD93, PSD95 or cytoskeletal actinfilin (Sf F. Traynelis *et al.*, 2010).

4.2.2 NMDA receptors

NMDARs functional properties are distinct, compared to other iGluRs. The most overt difference is presented in their quite slow opening and consequential deactivation, that takes up to tens of milliseconds. As shown in several kinetic studies, the NMDA receptor function as ion channel also lays in intermediate transition states between closed and open state (Banke and Traynelis, 2003; Amico-Ruvio and Popescu, 2010). Aside from different time of activation/deactivation as mentioned before, there is also presence of voltage dependent Mg^{2+} block of ion channel in steady state potential (Mayer, Westbrook and Guthrie, 1984; Nowak *et al.*, 1984) or high permeability of Ca^{2+} ions (ten times higher than for Na^{+}) (Macdermott *et al.*,

1986). Over activation of NMDA receptors therefore can rise in excitotoxicity and further neural cell damage (Gupta, Hardingham and Chandran, 2013). NMDARs are involved in the process of learning and memory (Stephen F. Traynelis *et al.*, 2010). NMDA receptors are also implicated in pathophysiological disorders such as epilepsy, schizophrenia or Parkinson disease (Skolnick, Popik and Trullas, 2009; Hardingham and Bading, 2010; Hedegaard *et al.*, 2012).

4.2.2.1 Structure of NMDA receptors

NMDARs also assemble as a tetrameric complex of subunits (Laube, Kuhse and Betz, 1998), but unlike other glutamate receptors, NMDA receptors are exclusively heterotetrameric. There are three subtypes of NMDAR subunits: GluN1, GluN2 and GluN3 (Dingledine *et al.*, 1999) but overall more isoforms could be found. This is facilitated by alternative splicing of pertinent genes (Solnick and Lee, 1987; Goguel and Rosbash, 1993). RNA for GluN1 subunit can be processed up to eight isoforms: GluN1a-4a, GluN1b-4b due to alternative splicing of three exons 5 (A-terminal, cassette N1, marked a and b), 21 and 22 (both C-terminal, cassettes C1, C2, their combinations marked 1 to 4) (Hollmann *et al.*, 1993; Zukin and Bennett, 1995) and that underlines features of each isoform in connection to protein-protein intra- or extracellular interaction (Traynelis, Hartley and Heinemann, 1995; Horak and Wenthold, 2009). Contrary to that, GluN2 (A-D) subunit isoforms are encoded by four different genes. GluN2A/GluN2B and GluN2/GluN2D share similar primary structure and comparing NMDA receptor containing A/B to C/D shows fundamental differences in pharmacological properties, such as different sensitivity towards inhibitors or neurosteroids (Horak *et al.*, 2006). Similarly, GluN3 subunits are encoded by two genes: *GRIN3A* and *GRIN3B* (Ciabarra *et al.*, 1995; Andersson *et al.*, 2001).

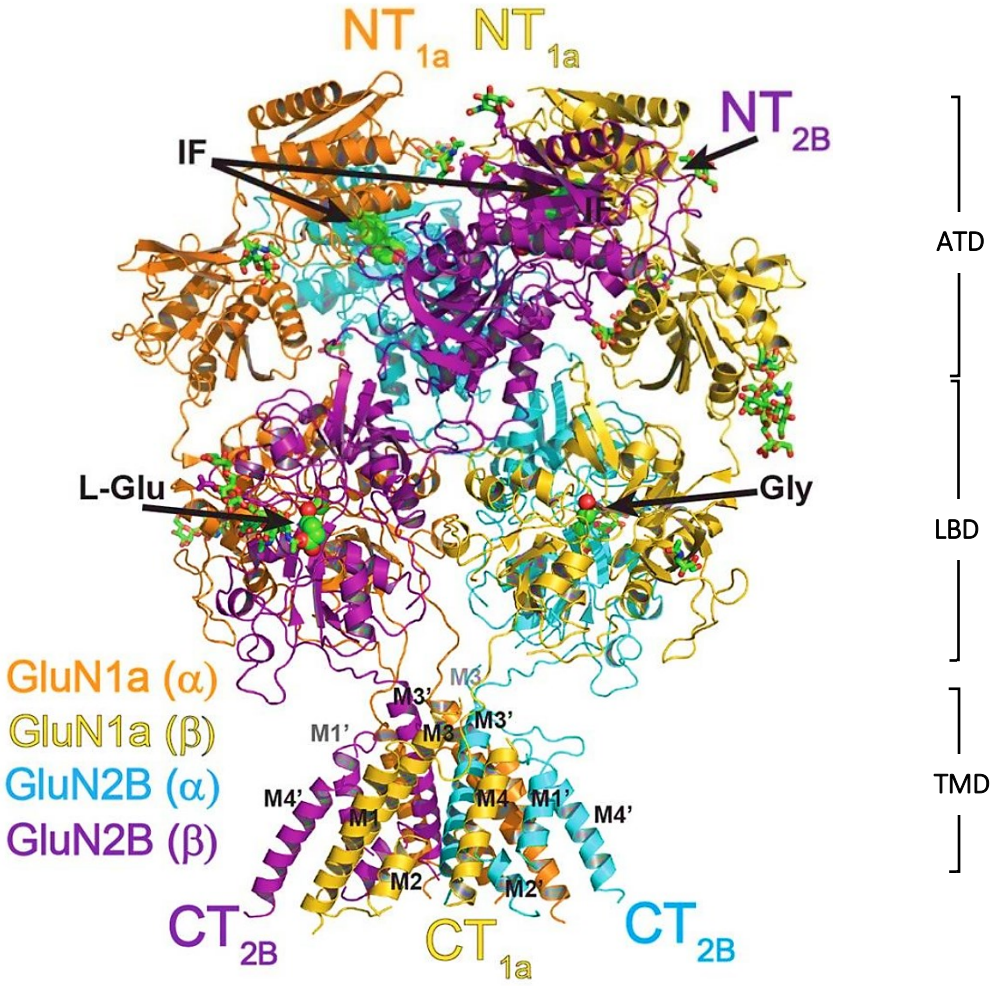


Fig. 5. Overall structure of GluN1a/GluN2B NMDA receptor; PDB ID **4PE5**;

„GluN1a and GluN2B subunits, labeled as GluN1a (α), GluN1a (β), GluN2B (α), GluN2B (β) are colored in orange, yellow, cyan and purple, respectively. The amino (NT) and carboxy (CT) termini are located on top and bottom, respectively. Ifenprodil (IF), located at the GluN1a/GluN2B ATD heterodimer interfaces, and agonists, glycine (Gly) and L-glutamate (L-Glu), lodged at the LBD clamshells, are shown in green spheres. *N*-glycosylation chains are shown in green sticks.“

Adapted from (Karakas, Simorowski and Furukawa, 2009).

Native NMDA receptor is composed of two obligatory GluN1 subunits combined in most cases with two GluN2 subunits –GluN2A and GluN2B (see Fig. 5). Spatial arrangement of subunits was formerly being thought as 1-1-2-2 (Schorge and Colquhoun, 2003), but recent crystal structure of NMDAR confirmed the arrangement as 1-2-1-2 (Karakas and Furukawa, 2014). GluN1 and GluN3 subunits include the binding site for glycine (Furukawa and Gouaux, 2003; Yao *et al.*, 2008), whereas GluN2 has the binding site for glutamate (Furukawa *et al.*, 2005). For complete activation of the NMDA receptor, several incidences must occur – the Mg^{2+} ion channel block must be eliminated from the gullet of the receptor by the membrane potential change and simultaneously is necessaire glycine and glutamate bind (Stephen F. Traynelis *et al.*, 2010).

4.2.2.1.1 Receptor domains

All NMDAR subunits share similar topology (from extracellular space – most distantly, cellular membrane, to intracellular): NTD, LBD connected to the TMD with linkers and CTD.

4.2.2.1.2 N-terminal domain of the NMDA receptor

NTD is the extracellular 'top' part of NMDAR subunits. It mediates important interaction between subunits and also contains subunit-specific binding sites for allosteric modulators, such as zinc ions or Ifenprodil (GluN2B-selective). NTD is capable to transduce conformational changes between two subunits as well as affects LBD (Lü *et al.*, 2017). Each NTD of NMDA receptor subunits share similar clamshell-like structure (likewise another extracellular domain – LBD).

NTD is assembled of two lobes R1 and R2 (see Fig. 6.), which are connected together via three well-structured loops (Karakas, Simorowski and Furukawa, 2009). In GluN1/GluN2B subunit composition, two R2 lobes of GluN2B subunits are situated proximally each to the other, whereas R2 lobes of GluN1 are rather distanced at the periphery of the receptor (Lee *et al.*, 2014).

In GluN2B, two types of post-translation modifications occur within the NTD – N-linked glycosylation and disulphide bonds are formed, that are i.e. between R1 and hypervariable loop (HVL). HVL is a peptide (15-20 AA long) that is conserved in other iGluRs and could take part in NMDAR subunit assembly (Karakas, Simorowski and Furukawa, 2009; Karakas and Furukawa, 2014).

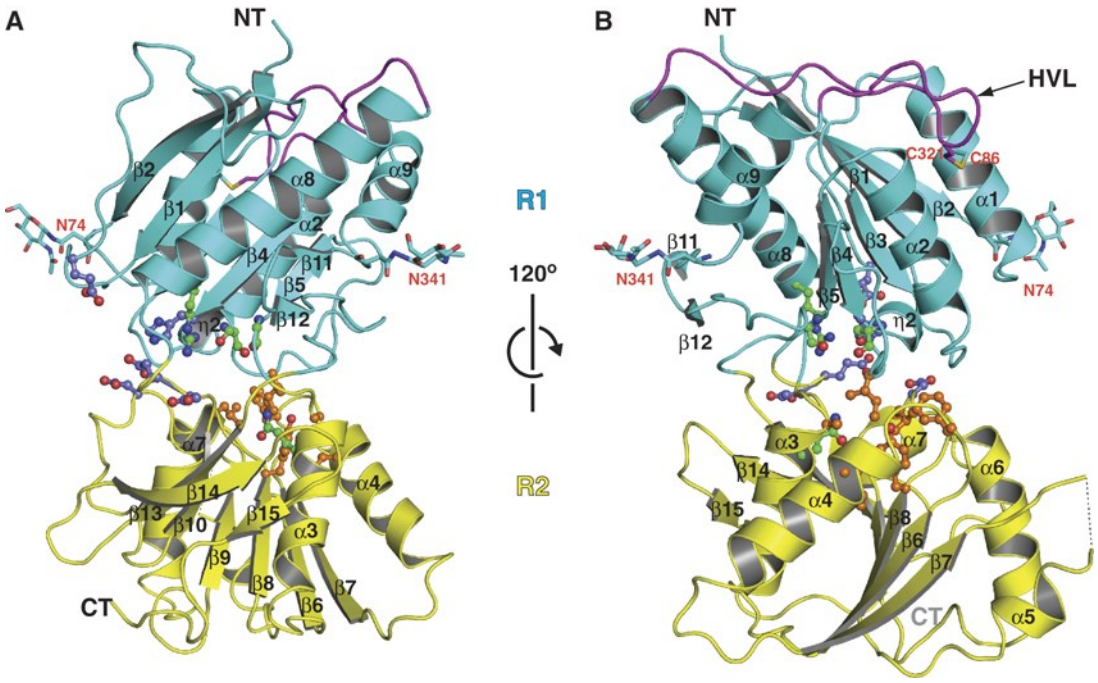


Fig. 6. Overall architecture of GluN2B NTD; R1 and R2 domains in complex with zinc. Hypervariable loop (HVL) is pointed out on the top of the R1 domain. Adapted from (Karakas, Simorowski and Furukawa, 2009).

4.2.2.1.2.1 Ligand-binding domain of the NMDA receptor

The second extracellular part of NMDAR subunits, that is tied together with NTD via variable linker, is LBD (see Fig. 7). The architecture of LBD is similar to NDT – it occupies clamshell-like structure and is formed of two polypeptide chains – S1 and S2. Both of them create a structure that reminds by its shape a half of the shell, where the S1 segment forms upper part of clamshell and S2 the lower part. The S1 lobe is connected via S1-M1 linker with the first TMD part – M1 helix, and S2 chain of the lower lobe, is situated between M3 and M4 helices of the TMD, where the connection is mediated by two linkers (M3-S2 and S2-M4) (Sf F. Traynelis *et al.*, 2010; Lee *et al.*, 2014; Cull-Candy, 2017). Clamshells between subunits are

situated in back-to-back fashion (Sf F. Traynelis *et al.*, 2010). Activation of the receptor is mediated through the conformational change of the LBD after the agonists are bound. LBD harbours binding site (pocket) for NMDAR agonist – glycine (GluN1, GluN3) and glutamate (GluN2A-D), (aspartate, D-serine). The binding pocket is located between S1 and S2 lobe and is able to create interactions with the α-carboxyl and α-amino groups of the agonist.

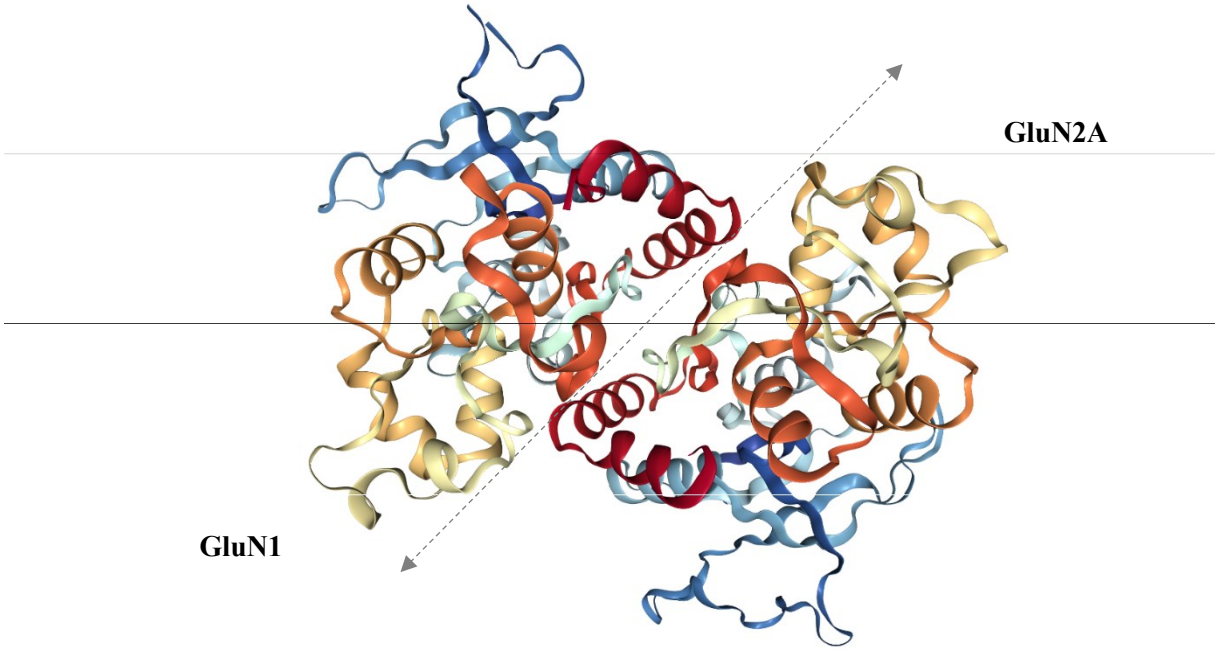


Fig 7. Structure of the human GluN1/GluN2A LBD; PDB ID **5H8H**; adapted from (Hackos *et al.*, 2016).

When the agonist is bound into the clamshell-like structure, it incites the LBD closure leading to conformational changes transferred via linkers into the TMD part and subsequent opening of the ion channel (Karakas and Furukawa, 2014). That triggers intracellular influx of Ca^{2+} and Na^{2+} ions.

4.2.2.1.2.2 LBD-TMD linkers of the NMDA receptor

Although several crystal structures of the NMDA receptor are available, the complete details of ion channel opening are still not well described (Karakas and Furukawa, 2014). The linkers (especially M3-S2 linker), connecting LBD to the TMD, are suggested to play a significant role in the process, mediating ion-channel opening. M3-S2 linkers differ in orientation within subunits GluN1 and GluN2, which also underlines the possible different role in the receptor

gating (Karakas and Furukawa, 2014; Lee *et al.*, 2014). Recent study moreover suggests, that the mode of the transduction of the signal from the LBD to the TMD is probably not fully dependent on the length of the M3-S2 linker, but the amino acid composition is important for ion channel opening. The proposed model indicates the interaction between the two M3-S2 linker residues (L657 in GluN1 and I655 in GluN2B), that take a place for temporal stabilization of the ion channel vestibule in its closed state (Ladislav *et al.*, 2018).

4.2.2.1.2.3 Transmembrane domain of the NMDA receptor

Together with LBD, TMD presents second fundamental structure for receptor function. Three transmembrane helices – M1, M3 and M4 and small re-entrant loop M2, form an ion channel in pseudo-fourfold symmetry, permeable for ions after activation of the receptor (Fig. 8). The topology of helices, according to available crystal structures of the NMDA receptors, shows M3 helices of NMDAR subunit TMDs closer to the central ion channel axis. Contrary, M1 and M4 helices are located at the periphery of the receptor (Karakas and Furukawa, 2014; Lee *et al.*, 2014). As mentioned above, M1 helix is connected via linker to the S1 polypeptide chain of the LBD and M3 helix to the S2 polypeptide chain of the LBD.

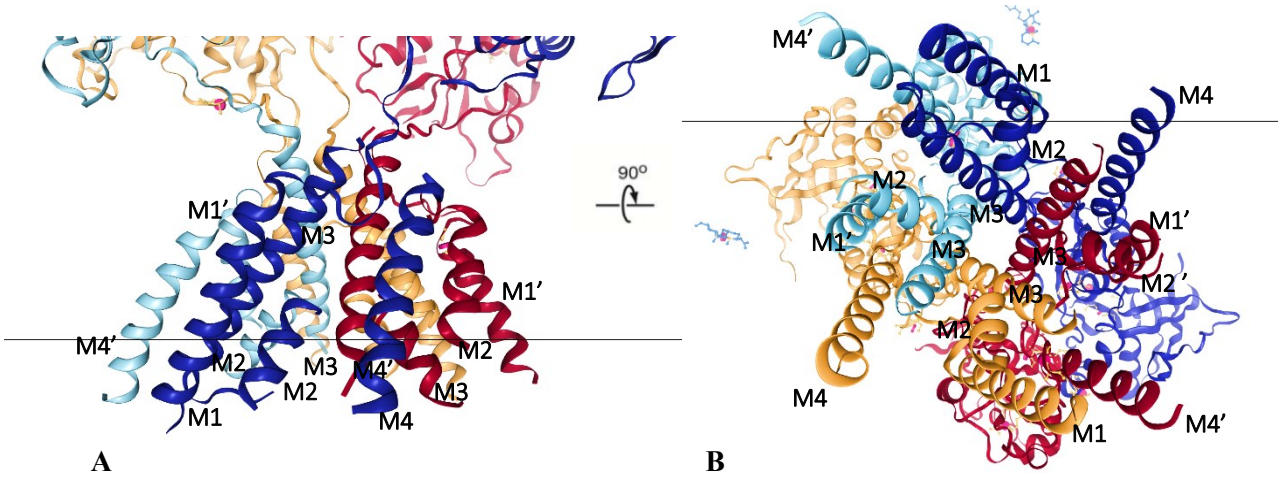


Fig. 8. Crystal Structure of GluN1a/GluN2B NMDA Receptor Ion Channel – Transmembrane helices neared; PDB ID **4PE5**; A – side view, B – bottom view; adapted from (Karakas and Furukawa, 2014).

4.2.2.1.2.4 C-terminal domain of the NMDA receptor

The CTD is the intracellular part of the NMDA receptor. Due to its variability and location in cytosol, any data indicating its structure does not exist. CTD varies in amino acid sequence as well as in length all across iGluRs subunits (Sf F. Traynelis *et al.*, 2010). CTD harbours short docking motifs, different phosphorylation sites and alternative splicing of CTD (C1/C2) of the GluN1 and GluN2B encodes also ER retention signals (Horak and Wenthold, 2009). Therefore, it operates within processes of membrane targeting of the protein, its stabilization, post-translation modifications, and targeting for degradation, as well as in interaction with intracellular proteins. Although, it does not directly participate in the receptor gating differences of GluN2A and GluN2B, GluN1 CTD alters deactivation kinetics of the receptor (Köhr and Seeburg, 1996; Steigerwald *et al.*, 2000; Vissel *et al.*, 2001; Maki *et al.*, 2012; Punnakkal, Jendritza and Köhr, 2012).

4.3 The details of the NMDA receptor structure

Due to their complexity, NMDA receptors were extensively studied using electrophysiological and immunochemical approaches (Moriyoshi *et al.*, 1991). One of the first data presenting the structure of NMDA receptors were deduced from their AA sequences (Mori and Mishina, 1995). Than many crystal structures of isolated LBDs or NRDs were published, including structures of agonist and antagonist bound in the LBD of GluN1 and GluN2A/2B subunits (Furukawa and Gouaux, 2003; Furukawa *et al.*, 2005; Karakas, Simorowski and Furukawa, 2009). Although, to obtain full-lengh receptor crystal structure was technically impossible until 2014, when the first crystal structure of the heterotetrameric GluN1/GluN2B NMDA receptor (*Rattus norvegicus* and *Xenopus laevis*) were obtained and published (Karakas and Furukawa, 2014; Lee *et al.*, 2014). Before, there existed only AMPAR (homomeric GluA2) crystal structures and the data from AMPAR were then used to model the full-lengh NMDAR (Paoletti and Neyton, 2007). The NMDAR crystal structure data provides us unprecedented details of intersubunit and interdomain interactions and their arrangement. However how great shift in understanding of the receptor structure this presented, substantial modifications (deletions, mutations, cys-bridges) within the receptor sequence were introduced to obtain the sufficiently high resolutions data, allowing to solve the crystal structure of NMDAR heteromeric complex (Karakas and Furukawa, 2014; Lee *et al.*, 2014). Due to this, many details of the NMDAR

structure may differ from the native GluN1/GluN2B NMDA receptor. Recent data from cryogenic electron microscopy (cryo-EM) of the triheteromeric NMDA receptor – GluN1/GluN2A/GluN2B (organism *Xenopus laevis*) provided more detailed insight into the triheteromeric NMDAR assembly and interactions between various subunits – especially the effect of two different GluN2 subunits on the whole receptor (Lü *et al.*, 2017).

5 AIMS OF THE THESIS

The aim of this master's thesis was to supplement our knowledge about NMDA receptor – its functional properties and structural dynamics. By the help of several approaches – from computational modelling to experimental data, obtain structure of NMDA receptor in different states and by comparison, draw on details of receptor gating. Specifically, three major aims were defined:

I. Collect the available NMDA receptor structural data and use molecular modelling to describe structural details mediating LBD-TMD function.

II. Establish suitable expression system of NMDA receptors/AMPA receptors and confirm presence of functional receptors.

III. Prepare samples of NMDA receptors/AMPA receptors at their different functional states to be subsequently analysed using mass spectrometry approach.

In order to achieve that, following experiments were designed:

1. NMDAR molecular modelling using available crystallographic data and, in homology model, analyse intramolecular interactions available for prospective chemical or UV crosslinking.
2. Clone and express two subunits GluN1 and GluN2B and as well as the tagged with 6xHis (GluN1) and 2xStrep (GluN2B) for protein purification.
3. With various biochemistry techniques and electrophysiology, confirm the presence of functional heteromeric NMDAR.
4. Expression of NMDAR subunits in larger amount and in connection with that, set in an appropriate expression system.
5. Using mass spectrometry, firstly confirm NMDAR subunits expression, and subsequently using crosslinking to obtain molecular structure data of the NMDA receptor subunits in different functional states and analyse it.
6. In parallel, design similar experiments for homomeric GluA2 subunit of AMPA receptor.

6 MATERIALS AND METHODS

6.1 Materials

6.1.1 Chemicals:

name	Empirical/linear formula	company
acetic acid	CH_3COOH	Lach:ner
agarose (TopVision)		ThermoFisher Scientific™
acrylamide	C_3H_5NO	MP Biomedicals
APS – ammonium persulfate	$(NH_4)_2S_2O_8$	Sigma-Aldrich (Merck)®
Blasticidin S	$C_{17}H_{26}N_8O_5 \cdot HCl$	InvivoGen
BSA – bovine serum albumin		Sigma-Aldrich (Merck)®
copper (II) sulfate	$CuSO_4$	Sigma-Aldrich (Merck)®
deionized water	dH_2O	
deoxynucleotides	dNTPs	Sigma-Aldrich (Merck)®
EDTA	$C_{10}H_{18}N_2O_8$	Sigma-Aldrich (Merck)®
ethanol	C_2H_6O	Lach:ner
FURA-2AM	$C_{44}H_{47}N_3O_{24}$	(Sigma-Aldrich (Merck)®)
glycerol	$C_3H_8O_3$	Lach:ner
glycine	NH_2CH_2COOH	Sigma-Aldrich (Merck)®
glucose	$C_6H_{12}O_6$	Sigma-Aldrich (Merck)®
H_2O T1 (Milli-Q®)		(Merck Millipore (Merck)®)
HEPES	$C_8H_{18}N_2O_4S$	Sigma-Aldrich (Merck)®
imidazole	$C_3H_4N_2$	Sigma-Aldrich (Merck)®
isopropanol	C_3H_8O	Lach:ner
magnesium chloride	$MgCl_2$	Sigma-Aldrich (Merck)®
magnesium sulphate	$MgSO_4$	AnalaR Normapur®
methanol	CH_3OH	Lach:ner
Midori Green		NIPPON Genetics Europe
milk (non-fat dry)		Bio-Rad
NMDA	$C_5H_9NO_4$	Sigma-Aldrich (Merck)®
PMSF – phenylmethylsulphonyl fluoride	$C_7H_7FO_2S$	Sigma-Aldrich (Merck)®

Ponceau S	$C_{22}H_{12}N_4Na_4O_{13}S_4$	(Merck Millipore (Merck) ®)
potassium chloride	KCl	AnalaR Normapur®
propidium iodide	$C_{27}H_{34}I_2N_4$	Sigma-Aldrich (Merck)®
QC Colloidal Coomasie Stain		Bio-Rad
Roti®-Load 1		CarlROTH
SDS – sodium dodecyl sulphate	$C_{12}H_{25}O_4S{\cdot}Na$	SERVA
sodium chloride	NaCl	PENTA
sodium dihydrogen phosphate dodecahydrate	$Na_2HPO_4 \cdot 12H_2O$	Lach:ner
sodium hydroxide	NaOH	Lach:ner
sodium phosphate monobasic dihydrate	$NaH_2PO_4 \cdot 2H_2O$	Lach:ner
TEMED – N,N,N',N'-tetramethylethylene-1,2-diamine	$(CH_3)_2NCH_2C_2N(CH_3)_2$	Sigma-Aldrich (Merck)®
TRIS – 2-Amino-2-(hydroxymethyl)propane-1,3-diol	$NH_2C(CH_2OH)_3$	MP Biomedicals /Sigma-Aldrich (Merck)®
Triton™ X-100		Sigma-Aldrich (Merck)®
Tryptone (BactoTryptone)		BD biosciences
Tween® 20		Sigma-Aldrich (Merck)®
Yeast extract (BactoYeast Technical)		BD biosciences

6.1.2 Bacterial strain

Escherichia coli XL-10 Gold; genotype: *endA1 glnV44 recA1 thi-1 gyrA96 relA1 lac The Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 tet^R F'[proAB lacl^{q}ZΔM15 Tn10(Tet^R Amy Cm^R)]*

Escherichia coli DH5α; genotype: *F$^-$ endA1 glnV44 thi-1 recA1 gyrA96 deoR nupG purB20 φ80d/acZΔM15 Δ(lacZYA-argF)U169, hsdR17 ($r_K^-m_K^+$), λ$^-$*

6.1.3 Cell lines

- HEK293T (ATCC® № CRL-3216™) – human embryonic kidney cell line
- HEK293S GnTI- (ATCC® № CRL-3022™) – human embryonic kidney cell line, transformed with adenovirus 5 DNASchneider's Drosophila Line 2 [D. Mel. (2), SL2] (ATCC® № CRL-1963™) – *Drosophila melanogaster* embryonal (20-24 h) cells

6.1.4 Culture media:

6.1.4.1 Bacterial strain media

LB (Luria-Bertani) medium: 1% tryptone, 0.5% yeast extract, 1% NaCl, dH_20

SOC medium: 2% tryptone, 0.5% yeast extract, 20 mM glucose, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$

Solid media were supplemented by 2% agarose.

6.1.4.2 Cell line media:

Dulbecco's Modified Eagle's Medium – high glucose (Sigma-Aldrich (Merck)®)

EX-CELL® 293 Serum-Free Medium for HEK293 Cells (Sigma-Aldrich (Merck)®)

EX-CELL® 420 Serum-Free Medium for Insect Cells (Sigma-Aldrich (Merck)®)

Fetal Bovine Serum (Sigma-Aldrich (Merck)®)

HyClone™ SFX Insect™ Cell Culture Medium (GE Healthcare)

Opti-MEM® I Reduced Serum Medium (Gibco® by Life Technologies)

Schneider's Insect Medium (Sigma-Aldrich (Merck)®)

6.1.5 Antibiotics:

Antibiotic Antimycotic Solution (100x), Stabilized (Sigma-Aldrich (Merck)®)

Ampicillin (Gibco® by Life Technologies)

Ciprofloxacin (Sigma-Aldrich (Merck)®)

Gentamicin (Gibco® by Life Technologies)

Penicillin-Streptomycin (Sigma-Aldrich (Merck)®)

6.1.6 Solutions:

6.1.6.1 Solutions for SDS-PAGE and Western Blot:

SDS-PAGE – resolving gel buffer: 150 mM TRIS, 1.92 M glycine, 1% SDS; pH = 8.8 (25 °C)

SDS-PAGE – stacking gel buffer: 150 mM TRIS, 1.92 M glycine, 1% SDS; pH = 6.8 (25 °C)

Fixing solution: 40% ethanol, 10% acetic acid, dH_2O

Blocking solution: 0.5% milk in PBS-T (0.01%) / 0.2% BSA in PBS-T (0.01%)

Blotting buffer: 25 mM TRIS, 195 mM glycine, 1% SDS, 20% methanol

Running buffer: 25 mM TRIS, 195 mM glycine, 1% SDS

6.1.6.2 Solutions for sample preparation and agarose electrophoresis:

Lysis buffer: 150 mM NaCl, 20 mM HEPES, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, pH = 7.4 (25 °C))

Phosphate buffer saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH = 7.2 (25 °C)

TAE buffer for agarose electrophoresis: 40 mM TRIS, 20 mM acetic acid, 1 mM EDTA

6.1.6.3 Solutions for protein purification:

Buffer N: 50 mM Na_2HPO_4, 300 mM NaCl; 0.5% Tween 20; pH = 8.0 (25 °C)

Elution buffer: 50 mM Na_2HPO_4, 300 mM NaCl; 300 mM imidazole; 0.5% Tween 20; pH = 8.0 (25 °C)

Equilibration buffer: 50 mM Na_2HPO_4, 300 mM NaCl; 0.5% Tween 20; pH = 8.0 (25 °C)

6.1.7 Commercial kits

High-Speed Plasmid Mini Kit (PD 300) (Geneaid)

Geneaid™ Midi Plasmid Kit (Endotoxin Free) (Geneaid)

Gibson Assembly® Site-Directed Mutagenesis Kit (Synthetic Genomics®)

jetPEI® DNA transfection, HTS application (Polyplus transfection)

Monarch™ PCR & DNA Cleanup Kit (5 µg) (New England BioLabs®)

Monarch™ DNA Gel Extraction Kit (New England BioLabs®)

NucleoSpin® Gel and PCR Clean-up (Machery-Nagel)

SuperSignal™ West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific™)

6.1.8 Plasmids & vectors

Pcdna3_GluA2 – *Rattus norvegicus* AMPA receptor 2 flip and flop variants

pCDM8_GluN1 – expression plasmid carrying DNA sequence for *Rattus norvegicus* glutamate ionotropic receptor NMDA type subunit 1 (*GRIN1*), transcript variant 2

pCgNRp/CgACATp-nat_GluN2B – expression plasmid carrying DNA sequence for *Rattus norvegicus* glutamate ionotropic receptor NMDA type subunit 2B (*GRIN2B*)

pGem®-T Easy Vector (Promega)

pMTH BiP V5 His-B* (Invitrogen)

*edited MSC (multiple cloning site) sequence; go to supplementary

6.1.9 Primers for pMTH BiP V5 His-B* vector:

MT Forward: `CATCTCAGTGCAACTAAA` (Invitrogen)
BGH Reverse: `TAGAAGGCACAGTCGAGG` (Invitrogen)

6.1.10 Primers for standard restriction cloning:

6.1.10.1 Primers for *GRIN1*:

GluN1 Forward (5'-3'):

ACTAGTGCCCGCGCCGCCTGCGACCCCAAG

T_M = 71 °C; GC content: 83.33%

(*Spe*I restriction site underlined; yellow – overlapping)

GluN1 Reverse (5'-3'):

TTCGAACTGCTTCCTACGGGCATCC

T_M = 55 °C; GC content: 63.16%

(*BstB*I restriction site underlined; yellow – overlapping)

6.1.10.2 Final insert:

```
   1 actagtgccc gcgccgcctg cgaccccaag atcgtcaaca tcggcgcggt gctgagcacg
  61 cgcaagcatg aacagatgtt ccgcgaggca gtaaaccagg ccaataagcg acacggctct
 121 tggaagatac agctcaacgc cacttctgtc acccacaagc ccaacgccat acagatggcc
 181 ctgtcagtgt gtgaggacct catctctagc caggtctacg ctatcctagt tagccacccg
 241 cctactccca acgaccactt cactcccacc cctgtctcct acacagctgg cttctacaga
 301 atccctgtcc tgggactgac tacccgaatg tccatctact ctgacaagag tatccacctg
 361 agtttccttc gcacggtgcc gccctactcc caccagtcca gcgtctggtt tgagatgatg
 421 cgagtctaca actggaacca catcatcctg ctggtcagcg acgaccacga gggacgggca
 481 gcgcagaagc gcttggagac gttgctggag gaacgggagt ccaaggcaga gaaggtgctg
 541 cagtttgacc caggaaccaa gaatgtgacg gctctgctga tggaggcccg ggaactggag
 601 gcccgggtca tcatcctttc tgcaagcgag gacgacgctg ccacagtgta ccgcgcagcc
 661 gcaatgctga acatgacggg ctctgggtac gtgtggctgg tcggggaacg cgagatctct
 721 gggaacgccc tgcgctacgc tcctgatggc atcatcggac ttcagctcat caatggcaag
 781 aatgagtcag cccacatcag tgacgccgtg ggcgtggtgg cacaggcagt tcacgaactc
 841 ctagagaagg agaatatcac tgacccaccg cggggttgcg tgggcaacac caacatctgg
 901 aagacaggac cattgttcaa gagggtgctg atgtcttcta agtatgcgga cggagtgact
 961 ggccgtgtgg aattcaatga ggatggggac cggaagtttg ccaactatag tatcatgaac
1021 ctgcagaacc gcaagctggt gcaagtgggc atctacaatg gtacccatgt catcccaaat
1081 gacaggaaga tcatctggcc aggaggagag acagagaaac ctcgaggata ccagatgtcc
1141 accagactaa agatagtgac aatccaccaa gagcccttcg tgtacgtcaa gcccacaatg
1201 agtgatggga catgcaaaga ggagttcaca gtcaatggtg acccagtgaa gaaggtgatc
1261 tgtacggggc ctaatgacac gtccccaggc agcccacgcc acacagtgcc ccagtgctgc
1321 tatggcttct gcatagacct gctcatcaag ctggcgcgga ccatgaattt tacctatgag
1381 gtgcacctgg tggcagatgg caagtttggc acacaggagc gggtaaacaa cagcaacaaa
1441 aaggagtgga acggaatgat gggcgagcta ctcagtggcc aagcggacat gattgtggca
1501 ccactgacca tcaacaatga gcgtgcgcag tacatagagt tctccaagcc cttcaagtac
1561 cagggcctga ccattttggt caagaaggag attcccagga gcacactgga ctcatttatg
1621 cagccttttc agagcacact gtggttgcta gtaggactgt cagttcatgt ggtggctgtg
1681 atgctgtacc tgctggaccg cttcagtccc tttggccgat tcaaggtgaa cagtgaggag
1741 gaggaggaag atgcactgac cctgtcctct gccatgtggt tttcctgggg cgtcctgctc
1801 aactccggca ttggggaagg tgccccccgg agtttctctg cacgtatcct aggcatggtg
1861 tgggctggtt tcgccatgat catagtggct tcctacactg ccaacttggc agctttcctg
1921 gtgctggatc ggcctgagga gcgcatcacg ggcatcaatg accccaggct cagaaacccc
1981 tcagacaagt tcatctacgc aactgtaaag cagagctccg tggacatcta cttccggagg
2041 caggtggagt tgagtaccat gtaccggcac atggaaaaac acaattacga gagcgcagct
2101 gaggccatcc aggctgtgcg ggacaacaag ctgcacgcct ttatctggga ctcggccgtg
2161 ctggagtttg aggcttcaca gaagtgcgat ctggtgacca cgggtgagct gttcttccgc
2221 tcaggctttg gcatcggcat gcgcaaggac agcccctgga agcagaacgt ttccctgtcc
2281 atactcaagt cccatgagaa tggcttcatg gaagatctgg ataagacatg ggttcggtat
2341 caggaatgcg actcccgcag caatgctcct gcaaccctca cttttgagaa catggcaggg
2401 gtcttcatgc tggtggctgg aggcatcgta gctgggattt tcctcatttt cattgagatc
2461 gcctacaagc gacacaagga tgcccgtagg aagcagttcg aa
```

6.1.10.3 Primers for *GRIN2B*:

GluN2B Forward (5'-3'):

CTACTAGTCGTTCCCAAAAGAGCCCCCCCAGC

T_M = 64 °C; GC content: 66.67%

(*Spe*I restriction site underlined; yellow – overlapping)

GluN2B Reverse (5'-3'):

CCTCTAGAACCCATGAAGCAATGCCGG

T_M = 53 °C; GC content: 57.89%

(*Xba*I restriction site underlined; yellow – overlapping)

GluN2B Reverse II (5'-3')*:

CGTCCCTTCCAGCCATGTAG

T_M = 56 °C; GC content: 60%

*For GluN2B colony PCR MT Forward and GluN2B II Reverse primers were used.

6.1.10.4 Final insert:

```
  1 ctactagtcg ttcccaaaag agccccccca gcatcggcat cgctgtcatc ctcgtgggca
 61 cttcagacga agtggccata aaagacgccc acgagaaaga tgacttccat catctctcag
121 tagttccccg ggtggagctg gtagccatga acgaaactga cccaaagagc atcatcaccc
181 gtatctgcga tcttatgtct gaccggaaga tccaggggct ggtgttcgcg gatgacaccg
241 accaagaagc catcgctcag atcctcgact tcatttctgc tcagactctc acccccatcc
301 tgggcatcca tgggggctca tctatgataa tggcggataa ggatgagtcc tccatgttct
361 tccagtttgg cccgtctatc gaacagcaag cttccgtcat gctcaacatc atggaagaat
421 atgactggta catcttttcc atcgtcacca cctacttccc tggctaccag gactttgtga
481 acaagatccg cagtaccatc gagaacagct tcgtgggctg ggagctcgag gaagtcctcc
541 tgctagacat gtctctggac gatggcgact ctaagattca gaatcagctg aagaagctcc
601 aaagccccat cattctcctt tattgcacga aggaggaagc cacctacatt tttgaagtag
661 ctaactcagt tgggctgact ggctacggct acacgtggat tgtgccgagt ctggtggccg
721 gggatacgga cacggtgcct tcagagttcc ccacggggct tatctctgtg tcttatgatg
```

```
 781 aatgggacta tggccttcct gccagagtga gagatggaat tgccatcatc accactgctg
 841 cctcggacat gctgtccgaa cacagtttca tccctgagcc caagagcagt tgctacaaca
 901 cccacgagaa gaggatctac cagtctaaca tgttgaatag gtatctgatc aatgtcactt
 961 ttgaagggag aaacctgtcc ttcagcgaag atggctacca gatgcatccg aagctggtga
1021 taatccttct gaacaaggag aggaagtggg agagggtggg gaaatggaag gacaagtccc
1081 tgcagatgaa gtattatgtg tggcctcgga tgtgtcctga gactgaggag caagaggatg
1141 accatctgag cattgtcacc ttggaggagg cgccatttgt cattgtggaa agcgtggacc
1201 ctctcagtgg aacctgcatg aggaatacag tcccgtgcca gaagcgcatc atctctgaga
1261 ataaaacaga tgaggaacca ggctacatca aaaaatgctg caaggggttc tgtattgaca
1321 tccttaagaa aatttctaag tctgtgaagt tcacctatga cctttacctg gtgaccaatg
1381 gcaagcacgg gaagaagatt aatgggacct ggaatggcat gatcggtgag gtggtcatga
1441 agagggccta catggcagtg ggatcactaa ctatcaatga agaacggtca gaggtggttg
1501 acttctctgt acccttcata gaaactggca tcagtgtcat ggtatctcgc agcaatggga
1561 ctgtgtcacc ttctgccttc ttagagccat tcagcgctga cgtgtgggtg atgatgtttg
1621 tgatgctgct cattgtttct gcggtggctg tctttgtctt tgaatacttc agccctgtgg
1681 gttacaacag gtgcctagcc gatggcagag agccaggagg cccatctttc accatcggca
1741 aagcaatttg gttactctgg ggtctggtgt ttaacaactc cgtacctgtg cagaacccaa
1801 aggggaccac ctccaagatc atggtgtcag tgtgggcctt ctttgctgtc attttcctgg
1861 ccagctacac tgccaactta gcagccttca tgatccaaga ggagtatgtg gaccaggttt
1921 ctggcctgag tgacaagaag ttccagagac ctaatgactt ctcacccccc ttccgctttg
1981 ggactgtgcc caatggcagc acagagagga atatccgtaa taactatgca gaaatgcatg
2041 cctacatggg aaagttcaac caaaggggtg tagatgatgc attgctctcc ctgaaaacag
2101 ggaagcttga tgcattcatc tatgatgcag ctgtgctcaa ctacatggct ggaagggacg
2161 aaggctgcaa actggtgacc attggcagtg gcaaggtctt tgcttctacc ggctatggca
2221 ttgctatcca aaaggactcc gggtggaagc gccaggtgga cctggctatc ctgcagctgt
2281 ttggagatgg ggagatggaa gaactggaag ctctctggct cactggcatt tgccacaatg
2341 agaagaatga ggtgatgagc agccagctgg acatcgacaa tatggcaggt gtcttctata
2401 tgttgggggc agccatggcc ctcagcctca tcaccttcat ctgtgagcat ctgttctatt
2461 ggcagttccg gcattgcttc atgggtctta gagg
```

6.1.11 Primers for Gibson Assembly cloning method:

6.1.11.1 Primers for *GRIN2B* – 2xStrep tag:

GluN2B-2xStrep Forward (5'-3'):

GGCCTTTGTTGGCCTCTCGCTCGGGAGATCTTCCGCCTGGAGCCACCCGCAGTTCGAAAAGTCCGCCT

GGAGCCACCCGCAGTTCGAAAAGCGTTCCCAAAAGAGCCCCCCCAGC

T_M = 64 °C; GC content: 66.67%

GluN2B-2xStrep Reverse (5’-3’):

GTTTAAACTCAATGGTGATGGTGATGATGCTCGAGTCAACCCATGAAGCAATGCCGG

T_M = 61 °C; GC content: 56%

(yellow – overlapping, blue – Strep tag (2x))

6.1.11.2 Final insert:

```
   1 ggcctttgtt ggcctctcgc tcgggagatc ttccgcctgg agccacccgc agttcgaaaa
  61 gtggacgcca cccgcagttc gaaaagcgtt cccaaaagag ccccccccagc atcggcatcg
 121 ctgtcatcct cgtgggcact tcagacgaag tggccataaa agacgcccac gagaaagatg
 181 acttccatca tctctcagta gttccccggg tggagctggt agccatgaac gaaactgacc
 241 caaagagcat catcacccgt atctgcgatc ttatgtctga ccggaagatc caggggggtgg
 301 tgttcgcgga tgacaccgac caagaagcca tcgctcagat cctcgacttc atttctgctc
 361 agactctcac ccccatcctg ggcatccatg ggggctcatc tatgataatg gcggataagg
 421 atgagtcctc catgttcttc cagtttggcc cgtctatcga acagcaagct tccgtcatgc
 481 tcaacatcat ggaagaatat gactggtaca tcttttccat cgtcaccacc tacttccctg
 541 gctaccagga ctttgtgaac aagatccgca gtaccatcga gaacagcttc gtgggctggg
 601 agctcgagga agtcctcctg ctagacatgt ctctggacga tggcgactct aagattcaga
 661 atcagctgaa gaagctccaa agccccatca ttctccttta ttgcacgaag gaggaagcca
 721 cctacatttt tgaagtagct aactcagttg ggctgactgg ctacggctac acgtggattg
 781 tgccgagtct ggtggccggg gatacggaca cggtgccttc agagttcccc acggggctta
 841 tctctgtgtc ttatgatgaa tgggactatg gccttcctgc cagagtgaga gatggaattg
 901 ccatcatcac cactgctgcc tcggacatgc tgtccgaaca cagtttcatc cctgagccca
 961 agagcagttg ctacaacacc cacgagaaga ggatctacca gtctaacatg ttgaataggt
1021 atctgatcaa tgtcactttt gaagggagaa acctgtcctt cagcgaagat ggctaccaga
1081 tgcatccgaa gctggtgata atccttctga acaaggagag gaagtgggag agggtgggga
1141 aatggaagga caagtccctg cagatgaagt attatgtgtg gcctcggatg tgtcctgaga
1201 ctgaggagca agaggatgac catctgagca ttgtcacctt ggaggaggcg ccatttgtca
1261 ttgtggaaag cgtggaccct ctcagtggaa cctgcatgag gaatacagtc ccgtgccaga
1321 agcgcatcat ctctgagaat aaaacagatg aggaaccagg ctacatcaaa aaatgctgca
1381 aggggttctg tattgacatc cttaagaaaa tttctaagtc tgtgaagttc acctatgacc
1441 tttacctggt gaccaatggc aagcacggga agaagattaa tgggacctgg aatggcatga
1501 tcggtgaggt ggtcatgaag agggcctaca tggcagtggg atcactaact atcaatgaag
1561 aacggtcaga ggtggttgac ttctctgtac ccttcataga aactggcatc agtgtcatgg
1621 tatctcgcag caatgggact gtgtcacctt ctgccttctt agagccattc agcgctgacg
1681 tgtgggtgat gatgtttgtg atgctgctca ttgtttctgc ggtggctgtc tttgtctttg
1741 aatacttcag ccctgtgggt tacaacaggt gcctagccga tggcagagag ccaggaggcc
1801 catctttcac catcggcaaa gcaatttggt tactctgggg tctggtgttt aacaactccg
1861 tacctgtgca gaacccaaag gggaccacct ccaagatcat ggtgtcagtg tgggccttct
1921 ttgctgtcat tttcctggcc agctacactg ccaacttagc agccttcatg atccaagagg
1981 agtatgtgga ccaggtttct ggcctgagtg acaagaagtt ccagagacct aatgacttct
2041 caccccctttt ccgctttggg actgtgccca atggcagcac agagaggaat atccgtaata
2101 actatgcaga aatgcatgcc tacatgggaa agttcaacca aaggggtgta gatgatgcat
2161 tgctctccct gaaaacaggg aagcttgatg cattcatcta tgatgcagct gtgctcaact
2221 acatggctgg aagggacgaa ggctgcaaac tggtgaccat tggcagtggc aaggtctttg
2281 cttctaccgg ctatggcatt gctatccaaa aggactccgg gtggaagcgc caggtggacc
2341 tggctatcct gcagctgttt ggagatgggg agatggaaga actggaagct ctctggctca
2401 ctggcatttg ccacaatgag aagaatgagg tgatgagcag ccagctggac atcgacaata
2461 tggcaggtgt cttctatatg ttgggggcag ccatggccct cagcctcatc accttcatct
2521 gtgagcatct gttctattgg cagttccggc attgcttcat gggttgactc gagcatcatc
2581 accatcacca ttgagtttaa ca
```

6.1.12 Molecular weight markers for gel electrophoresis:

6.1.12.1 DNA agarose gel:

1 kb DNA Ladder (New England BioLabs®) – **I**

2-Log DNA Ladder (0.1-10 kb) (New England BioLabs®) – **II**

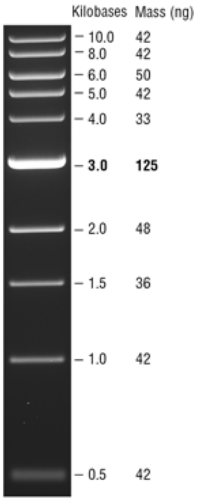
BenchMark™ Protein Ladder (Invitrogen) – **III**

6.1.12.2 SDS-PAGE:

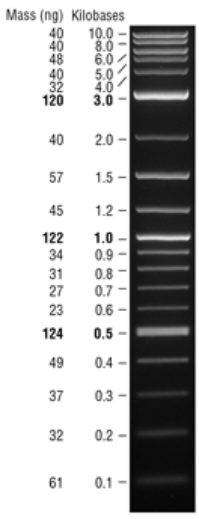
Spectra™ Multi Color Broad Range Protein Ladder (ThermoFisher Scientific™) – **IV**

Full Range Amersham ECL Rainbow™ Molecular Weight Marker (GE Healthcare) – **V**

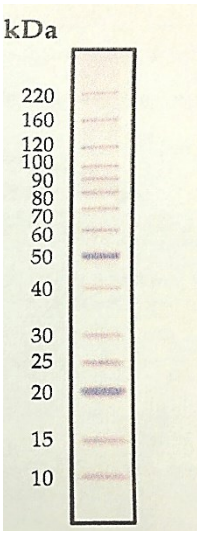
I



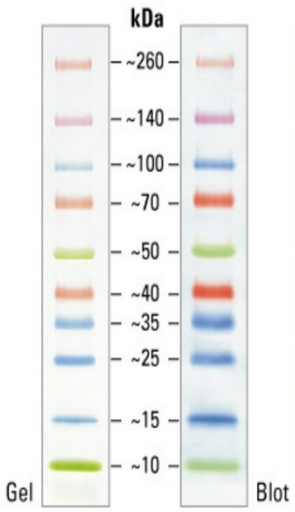
II



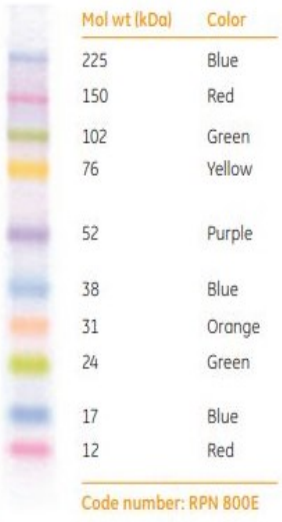
III



IV



V



6.1.13 Enzymes

TEMPase Hot Start DNA Polymerase (VWR™)

PfuUltra II Fusion HS DNA Polymerase (Agilent)

Phusion® High Fidelity DNA Polymerase (New England BioLabs®)

AmpONE Taq DNA polymerase (GeneAll)

*Dpn*I (New England BioLabs®)

*Not*I (10U/L) (ThermoFishér Scientific™)

*Spe*I-HF® (New England BioLabs®)

*Xba*I (New England BioLabs®)

T4 DNA Ligase (New England BioLabs®)

Benzonase® Nuclease (Sigma-Aldrich (Merck®))

6.1.14 Inhibitors

DL-2-Amino-5-phosphonopentanioc acid; D-AP5 (Tocris Bioscience)

$MgCl_2$ (Sigma-Aldrich (Merck®))

Ketamine hydrochloride (Tocris Bioscience)

cOmplete™, EDTA-free Protease Inhibitor Cocktail (Roche)

phenylmethylsulfonyl fluoride; PMSF (Sigma-Aldrich (Merck)®)

6.1.15 Antibodies

6.1.15.1 Primary antibodies

Anti-NMDA Receptor 1 (GluN1) (extracellular) Rabbit Polyclonal Antibody (Alomone labs)

NMDA Receptor1 (GluN1) (D65B7) Rabbit Monoclonal Ab (Cell Signaling Technology)

Anti-NR1 glutamate receptor, clone N308/48 (purified mouse monoclonal) (NeuroMab)

Anti-NR2B glutamate receptor, clone N59/36 (purified mouse monoclonal) (NeuroMab)

Anti-NMDA Receptor 2B (GluN2B) (extracellular) Rabbit Polyclonal Antibody (Alomone labs)

HRP Mouse Anti-6xHis (BD Pharmingen™)

6x-His Tag Mouse Monoclonal Antibody (HIS.H8) (ThermoFisher Scientific™)

Strep II Tag Mouse monoclonal antibody (Bioworld Technology, Inc.)

Anti-GluA1/GluR1 glutamate receptor, clone N355/1 (purified mouse monoclonal) (NeuroMab)

Anti-GluR2 ionotropic glutamate receptor, clone L21/32 (purified mouse monoclonal) (NeuroMab)

eGFP Tag Monoclonal Antibody (F56-6A1.2.3) Mouse Monoclonal (ThermoFisher Scientific™)

ß3-Tubulin (D71G9) XP® Rabbit Monoclonal Ab (Cell Signaling Technology)

6.1.15.2 Secondary antibodies

Goat Anti-Mouse IgG & IgM Antibody, HRP Horseradish Peroxidase (HRP) conjugated, affinity purified (Millipore (Merck))

Goat Anti-Rabbit IgG Antibody, F(ab')2 HRP Horseradish Peroxidase (HRP) conjugated affinity purified (Millipore (Merck))

6.2 Methods

6.2.1 DNA related work

6.2.1.1 Plasmid isolation

6.2.1.1.1 Plasmid Mini prep isolation

Transformed bacteria cells (grown over-night, see 6.2.2.1.1) in suspension were centrifuged (10 min, 3000 rcf, RT). For the plasmid isolation High-speed Plasmid Mini Kit (Geneaid™) was used according to manufacturers' instructions. Briefly, the harvested cell pellet was resuspended in 200 µl of PD1 Buffer thoroughly. Cell lysis was performed by adding of 200 µl of PD2 Buffer and by subsequent incubation at RT for 2 minutes. 300 µl of PD3 Buffer neutralized lysis and samples were spun down (16 000 rcf, 3 min, RT). Supernatant was transferred on the PD Columns and centrifuged (16 000 rcg, 30 sec, RT). Due to upcoming sequencing analysis, recommended 400 µl of W1 Buffer was added on the PD Columns and spun down (16 000 rcf, 30 sec, RT). Columns were washed by 600 µl of Wash Buffer. DNA was finally eluted by 50 µl of preheated (50 °C) Elution Buffer. DNA concentration was analysed on the NanoDrop™ (ThermoFisher Scientific™) and stored at -20 °C.

6.2.1.1.2 Plasmid Midi prep isolation

Transformed bacteria cells grown over-night (see 6.2.2.1.1), grown up to OD_{600} = 4 (100 ml), were centrifuged (15 minutes, 3000 rcf, RT) and supernatant was discarded. For endotoxin free plasmid isolation Midi Plasmid Kit (Geneaid™) was used according to manufacturers' instructions. Plasmid Midi Columns were equilibrated by 5 ml of PEQ Buffer and harvested cell pellet was resuspended by mixture of 4 ml PM1 Buffer with RNase A + 40 µl of TrueBlue Lysis Buffer. Right after, 4 ml of PM2 Buffer was added to the solution, gently mixed and let stand at RT for 2 minutes. Neutralization of the lysis was performed by adding 4 ml of PM3 Buffer, mixed and centrifuged (3000 rcf, 20 min, RT). Endotoxin removal step followed, mixing of 1.2 ml PER Buffer with supernatant and incubation on ice for 30 minutes. Whole solution was transferred on equilibrated Plasmid Midi Columns and let to flow through. Columns were washed with 12 ml of PW Buffer. DNA was eluted in clean tube by 8 ml of PEL Buffer and precipitated by adding of 0.75 volumes of isopropanol (Lach:ner), and pelleted by centrifugation (3000 rcf, 5 minutes, 4 °C). Air-dried pellet was resuspended in 500 µl of dH_20 and stored at -20 °C.

6.2.1.2 DNA purification

DNA clean up step was performed by using two types of kits – Monarch® PCR and DNA Cleanup Kit (5 µg) (New England BioLabs®) or NucleoSpin® Gel and PCR Clean-up for DNA, RNA, and protein purification (Marchery-Nagel). Cleaning up performed with Monarch Kit for plasmid or insert according to manufacturers' instructions. Samples were diluted in DNA Cleanup Binding Buffer in ratio of Binding Buffer : Sample; 2 : 1 and loaded onto column. DNA was washed with 200 µl of DNA Wash Buffer and eluted with preheated (50 °C) 20 µl of DNA Elution Buffer. After purification, DNA fragments were stored at -20 °C. Similar workflow was performed according to manufacturers' instruction with NucleoSpin® Gel and PCR Clean-up (Marchery-Nagel).

6.2.1.3 Molecular cloning

Plasmids, encoding *GRIN1* or *GRIN2B* sequence formerly used for HEK293T cell transfection were used for preparation of insert using polymerase chain reaction. Desired restriction sites, as well as 6xHis (for GluN1) or 2xStrep (for GluN2B) tags, were implemented via primers or through a first-step cloning to pGEM®-T Easy vector (Promega Corporation). Inserted restriction sites were further used with corresponding restriction endonucleases. Concentrations of all components, including restriction enzymes, were selected in compliance with manufacturer's instructions. Alternatively, Gibson Assembly protocol was done in order to heighten efficacy of long insert (≥3000 kbp) cloning.

6.2.1.4 Restriction

6.2.1.4.1 *GRIN1*-pMT BiP V5-His* vector cloning

6.2.1.4.1.1 *GRIN1*-pGEM®-T Easy vector cloning

GluN1 receptor subunit was initially cloned into pGEM®-T Easy vector (Promega Corporation) for the purpose to insert appropriate restriction sites for following restriction cloning. Sequence of GluN1 subunit gene was multiplied via PCR under following conditions:

5X PCR Buffer ($MgCl_2$)	–	10 µl
dNTPs (10 mM)	–	1 µl
primers FW + REV (10 µM)	–	2.5 µl
plasmid	–	1 µg
polymerase*	–	0.5 µl
H_2O T1	–	up to 50 µl

*The PCR amplification efficiency was optimized by compariosn of yield of three different polymerases:

- AmpONE Taq DNA polymerase (GeneAll)
- PfuUltra II Fusion HS DNA Polymerase (Agilent)
- Phusion® High Fidelity DNA Polymerase (New England BioLabs®)

PCR ran at following parameters (for *GRIN1*):

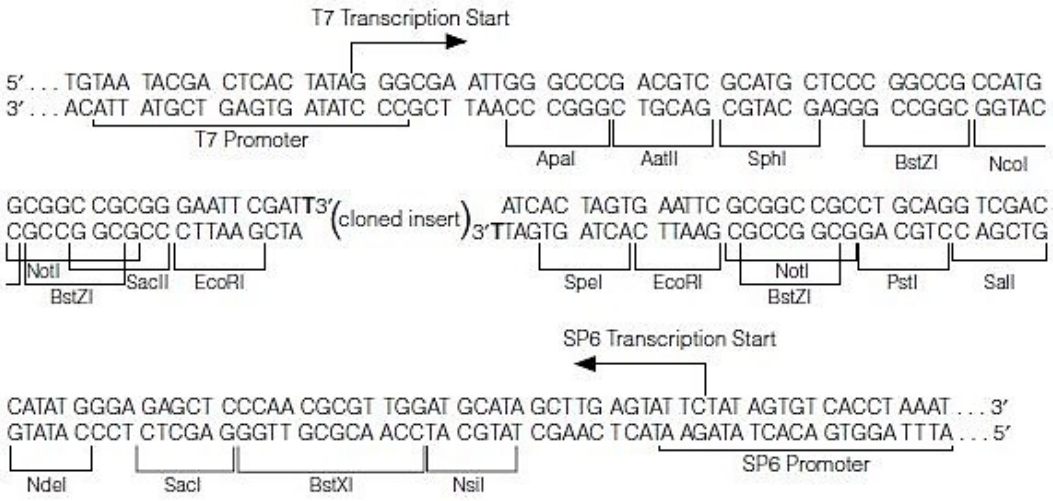
98 °C	–	30 sec	
98 °C	–	10 sec	35x
63 °C	–	30 sec	
72 °C	–	3 min	
72 °C	–	10 min	
4 °C	–	∞	

Subsequently an aliquotes of PCR results were analysed on an agarose gel (1.5%) and, along with the protocol (Promega Corporation), ligation into pGEM®-T Easy vector was done by setting ligation conditions:

2X Rapid Ligation Buffer, T4 DNA Ligase	–	5 µl
pGEM®-T Easy vector (50 ng/µl)	–	1 µl
PCR product (GluN1 subunit sequence)*	–	150 ng
T4 DNA Ligase (3 Weiss units/µl)	–	1 µl
nuclease-free water to final volume of	–	10 µl

*molar ratio of pGEM®-T Easy vector : insert was 1 : 3; required concentration of insert DNA was estimated according to recommended equation:

$$\frac{ng\ of\ vector \cdot kb\ size\ of\ insert}{kb\ size\ of\ vector} \cdot \frac{3}{1} = ng\ of\ insert$$



pGEM®-T Easy vector multiple cloning sequence (Promega Corporation)

Reaction mixture was set up and incubated at RT for one hour, followed by transformation of competent *E. coli* XL-10 Gold with product of ligation reaction (see 6.2.2.1.1).

Selection of positively transformed cells was facilitated by LB/ampicillin/IPTG/X-Gal plates, where successfully transformed colonies stayed white due to the property of the vector to interrupt the coding sequence of ß-galactosidase. False positive colonies (plasmids without insert) grown on selective plates turned blue. White colonies were selected and grown over-night in 10 ml LB/ampicillin media. Next day Mini prep was performed and *GRIN1*-pGEM®-T Easy vector plasmid was eluted and used for further cloning by specific sites restriction.

6.2.1.4.1.2 *GRIN1*-pMT BiP V5-His* vector cloning

One restriction enzyme creating sticky ends in plasmid as well as in *GRIN1*-pGEM®-T Easy vector plasmid was selected – *Not*I. Restriction conditions were set up:

Restriction mixture:

restriction endonuclease (*Not*I)	–	0.5 µl
CutSmart® Buffer (New England BioLabs®) (10x)	–	2 µl
pGEM®-T Easy vector/GluN1 PCR product	–	1 µg
H_2O T1	–	up to 20 µl

Restriction mixture was incubated over-night at 37 °C. Products were cleaned up via agarose gel (1.5%) electrophoresis and gel extracted (see 6.2.1.6). Ligation followed. DNA concentration was analysed on NanoDrop™ (ThermoFisher Scientific™) and sticky-end ligation conditions were set up:

T4 DNA Ligase Buffer (10X)	–	2 µl
Vector DNA (pMT BiP V5-His*)	–	50 ng
Insert DNA (*GRIN1*)	–	50 ng
T4 DNA Ligase (5U/L)	–	1 µl
Nuclease-free water	–	up to 20 µl

*vector : insert ratio – 1 : 1

Ligation run at 16 °C for 16 hours. Morning after, transformation of competent cells with ligation reaction was performed (see 6.2.2.1.1). Next day, colonies were selected, diluted in 15 µl of H_20 T1 and colony PCR was set up:

10X Key Buffer (VWR^{TM})	–	5 µl
$MgCl_2$ (15mM)	–	2 µl
NTPs (10 mM)	–	1 µl
primers FW + REV (10 µM)*	–	1 µl
colony diluted in H_2O T1	–	5 µl
TEMPase Hot Start Polymerase (5U/µl)	–	0.2 µl
H_2O T1	–	up to 50 µl

*MT Forward + BGH Reverse primers

Colony PCR ran at following conditions:

95 °C	–	15 min	
95 °C	–	30 sec	50x
60 °C	–	30 sec	
72 °C	–	3 min	
72 °C	–	5 min	
4 °C	–	∞	

Expected colony PCR products (2836 bp) were visualised on agarose gel and positive colonies were further grown for DNA Mini or Midi prep isolation (see 6.2.1.1). Obtained plasmids were sequenced and stored at -20 °C.

6.2.1.4.2 *GRIN2B*-pMT BiP V5-His* vector cloning

Gene for GluN2B receptor subunit was cloned directly in pMT BiP V5-His* vector using GluN2 PCR product with inserted restriction sites *Spe*I and *Xba*I. GluN2B sequence was multiplied with PCR under following conditions:

5X Buffer	–	10 µl
dNTPs (10 mM)	–	1 µl
primers FW + REV (10 µM)	–	2.5 µl
GluN2B plasmid	–	1 µg
polymerase*	–	0.5 µl
H_2O T1	–	up to 50 µl

*The PCR amplification efficiency was optimized by comparison of yield of three different polymerases:

- AmpONE Taq DNA polymerase (GeneAll)
- PfuUltra II Fusion HS DNA Polymerase (Agilent)
- Phusion® High Fidelity DNA Polymerase (New England BioLabs®)

PCR ran at following conditions (for *GRIN2B*):

98 °C	–	30 sec	
98 °C	–	10 sec	35x
60 °C	–	30 sec	
72 °C	–	3 min	
72 °C	–	10 min	
4 °C	–	∞	

Aliquot of PCR product was analysed on an agarose gel (1.5%) and restriction reaction was set up.

Restriction mixture:

restriction endonuclease (*Spe*I/*Xba*I)	–	0.5 µl
CutSmart® Buffer (New England BioLabs®) (10x)	–	2 µl
pMT BiP V5-His*/PCR product (GluN2B)	–	1 µg
H_2O T1	–	up to 20 µl

Restriction was performed over-night at 37 °C and following morning, restricted DNA was visualised on agarose gel (1.5%) and extracted from the gel (see 6.2.1.56.2.1.6). DNA concentration was measured on NanoDrop™ (ThermoFisher Scientific™). Right after, ligation with T4 DNA Ligase (New England BioLabs®) followed up under ligation conditions as follow:

T4 DNA Ligase Buffer (10X)	–	2 µl
Vector DNA (pMT BiP V5-His*)	–	50 ng
Insert DNA (*GRIN2B*)	–	50 ng
T4 DNA Ligase (5U/L)	–	1 µl
Nuclease-free water	–	up to 20 µl

*vector : insert ratio – 1 : 1

Ligation ran at 16 °C for 16 hours and after ligation ended, competent cells Xl-Blue were transformed (see 6.2.2.1.1). Colonies were picked up randomly, diluted in 15 µl of H_20 T1 and colony PCR was set up:

10X Key Buffer (VWR™)	–	5 µl
$MgCl_2$ (15mM)	–	2 µl
NTPs (10 mM)	–	1 µl
primers FW + REV (10 µM)*	–	1 µl
colony dilution	–	5 µl
TEMPase Hot Start Polymerase (5U/µl)	–	0.2 µl
H_2O T1	–	up to 50 µl

*MT Forward + BGH Reverse primers

Colony PCR ran at following program:

95 °C	–	15 min	
95 °C	–	30 sec	50x
60 °C	–	30 sec	
72 °C	–	3 min	
72 °C	–	5 min	
4 °C	–	∞	

Expected PCR products (2726 bp) were visualised with agarose gel electrophoresis and positive colonies were further grown for DNA Mini or Midi prep (see 6.2.1.1). Purified plasmids were sequenced and stored at -20 °C.

6.2.1.4.3 *GRIN2B*-2xStrep tag-pMT BiP V5-His*- vector cloning

6.2.1.4.3.1 Gibson Assembly® (Isothermal Assembly)

6.2.1.4.3.1.1 DNA preparation for Gibson Assembly®

GRIN2B-2xStrep tag insert was firstly amplified with PCR under following conditions:

5X Buffer	–	10 µl
dNTPs (10 mM)	–	1 µl
primers FW + REV (10 µM)	–	2.5 µl
GluN2B plasmid	–	1 µg
polymerase*	–	0.5 µl
H_2O T1	–	up to 50 µl

*The PCR amplification efficiency was optimized by compariosn of yield of three different polymerases:

- AmpONE Taq DNA polymerase (GeneAll)
- PfuUltra II Fusion HS DNA Polymerase (Agilent)
- Phusion® High Fidelity DNA Polymerase (New England BioLabs®)

PCR ran at following program (for *GRIN2B*-2xStrep tag):

98 °C	–	30 sec	
98 °C	–	10 sec	35x
62 °C	–	30 sec	
72 °C	–	3 min	
72 °C	–	10 min	
4 °C	–	∞	

PCR products were treated with *Dpn*I (New England BioLabs®) and separated on agarose gel (1.5%) electrophoresis and extracted from the gel (see 6.2.1.6). Vector – linearized and purified pMT BiP V5-His* plasmid was obtained from Jiří Zahradník (IBT). According to recommendation of the protocol, overlapping regions (insert/vector) were designed as 31 (5') and 32 (3') nucleotides long (Fig. 9). Along with the protocol (Syntetic Genomics®), Gibson Assembly® (Gibson *et al.*, 2009) Single Site Mutation (< 5 kbp) reaction was set up.

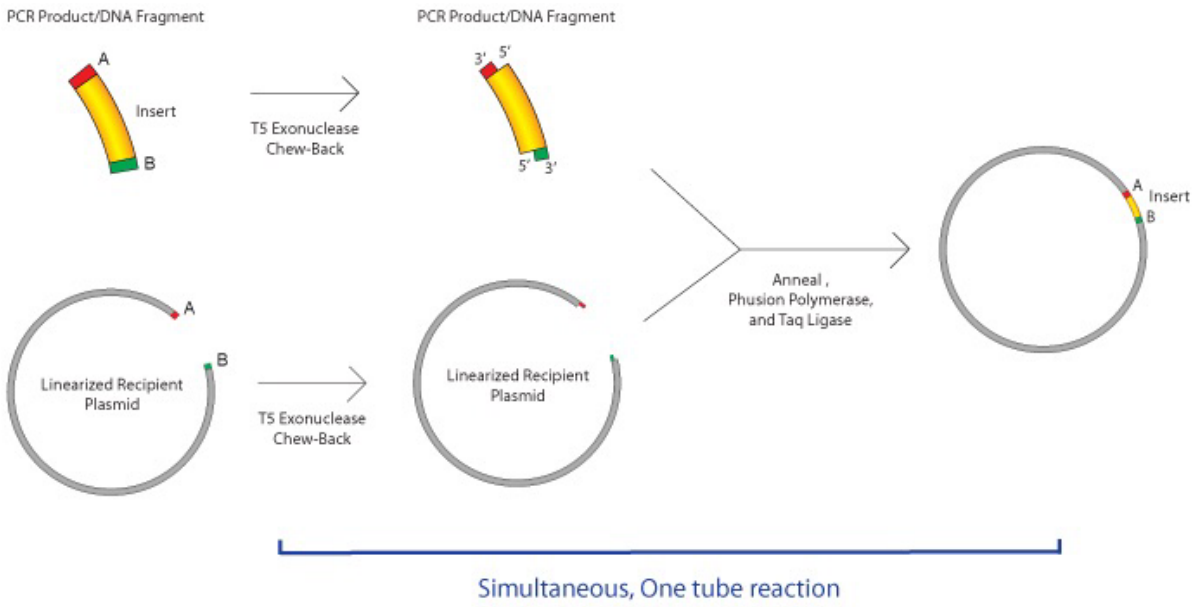


Fig. 9. Gibson assembly® illustration, adopted from: Moser and Matsen, n.d.

According to manufacturers' instructions insert and vector concentration was 25 ng and final volume was brought up to 5 µl, where insert : vector ratio was 1 : 1. Thawed GA SDM Assembly Mix A (2X) was mixed by Vortex device and 5 µl were added to 5 µl of DNA fragments mixture. Reaction was mixed, briefly centrifuged, and tubes were placed into the thermocycler. Gibson Assembly® Reaction of Mutagenesis Fragments program was set (1 cycle):

3' end Chew Back	–	37 °C for 5 min
Inactivation	–	75 °C for 20 min
Slowly Cool	–	0.1 °C/sec to 60 °C
Anneal	–	60 °C for 30 min
Slowly Cool	–	0.1 °C/sec to 4 °C

GA SDM Assembly Mix B (2X) was thawed on ice and 10 µl was added to the completed reaction. Samples were mixed and incubated under following conditions:

Repair cycle (1) – 45 °C for 15 min

Obtained plasmids were used for transformation of competent cell (see 6.2.2.1.1). Next day obtained colonies were picked up, diluted in 15 µl of H_20 T1 and colony PCR was set up:

10X Key Buffer (VWR™)	–	5 µl
$MgCl_2$ (15mM)	–	2 µl
NTPs (10 mM)	–	1 µl
primers FW + REV (10 µM)*	–	1 µl
colony diluted in H_2O T1	–	5 µl
TEMPase Hot Start Polymerase (5U/µl)	–	0.2 µl
H_2O T1	–	up to 50 µl

*MT Forward + BGH Reverse primers

Colony PCR ran at following program:

95 °C	–	15 min	
95 °C	–	30 sec	50x
60 °C	–	30 sec	
72 °C	–	3 min	
72 °C	–	5 min	
4 °C	–	∞	

Amplified DNA fragments (2697 bp) were visualised with agarose gel electrophoresis and positive colonies were further grown for DNA Mini or Midi prep isolation (see 6.2.1.1). Obtained plasmids were sequenced and stored at -20 °C.

6.2.1.5 Agarose gel electrophoresis

For DNA separation, 1.5% agarose gel was prepared by dissolving 1 g of agarose in 70 ml TAE buffer (heated up in microwave oven). After cooling the solution down to circa 50 °C, 3 µl of DNA/RNA stain Midori Green (NIPPON Genetics Europe) was added. Solution was mixed thoroughly and poured into prepared electrophoresis casting tray. When gel was solidified, tray was transferred into electrophoresis apparatus and poured over with TAE buffer. Samples, previously mixed with sample buffer (6x), were applied into wells with one separately for DNA marker. Power pack was set up to electric field intensity of 5V/cm and turned on. Electrophoresis was ended when reference dye – bromophenol blue, reached bottom end of the gel. Separated DNA samples were visualized on imaging system (Fusion Solo S, Vilber Lourmat).

6.2.1.6 DNA extraction from agarose gel

When the electrophoresis ended, gels were transferred and observed under UV light (Benchtop 2UV™ Transilluminator, UVP). Required bands of DNA were excised (circa 300 µg) and transferred into microtubes. According to manufacturers' instructions (Geneaid™), each gel slice was than mixed thoroughly with 500 µl of DF Buffer and incubated at 55 °C until complete sample dissolvement. Mixture was put on filter columns and spun down (16000 rcf, 30 sec, RT). Column was casted with 400 µl of W1 Buffer centrifuged (16 000 rcf, 30 sec, RT) and washed with 600 µl of Wash Buffer containing ethanol (Lach:ner). DNA fragments were eluted with 30 µl of preheated (60 °C) Elution Buffer (Geneaid™) and stored for further usage at -20 °C. Alternatively, some DNA fragments were extracted with Monarch DNA Gel Extraction Kit (New England BioLabs®). After DNA containing gel dissection, slice was melted in 4 volumes of Gel Dissolving Buffer and incubated in 50 °C until total sample dissolvement. Sample was loaded onto the columns and spun down (16 000 rcf, 1 min, RT). DNA was washed by 200 µl of DNA Wash Buffer containing 4 volumes of ethanol (Lach:ner). DNA was extracted by 20 µl of preheated (50 °C) DNA Elution Buffer and stored at -20 °C.

6.2.1.7 Sequencing

Mix2 Seq Kit OVERNIGHT service (Eurofins Genomics) was used for sequencing. Sequencing reaction mix was of total volume 17 µl, 2 µl (10 pmol/µl) of forward primer was added with 15 µl of purified DNA (min. 50 ng/µl). Samples were sent out via Eurofins DropBox and sequenced over-night.

6.2.2 Cells related work

6.2.2.1 Bacterial cells work

6.2.2.1.1 Transformation of competent bacteria

10 µl of reaction or diluted DNA was added to 100 µl of Xl-Blue cell suspension (thawed on ice) and left on ice for 20 minutes. Then heat shock was performed – 1 minute in 42 °C water bath and tubes were cooled down on ice for 2 minutes. Right after 120 µl of preheated SOC media were added and whole mixture was incubated in shaking incubator (37 °C, 250 rpm) for

at least 40 minutes. Transformed cells were plated on agar plates containing selective antibiotic – ampicillin. Plates were incubated over-night in incubator at 37 °C. Following day a colony PCR was performed (see further) and positive colonies were picked up and inoculated into tube containing 10 ml LB media with ampicillin for DNA Mini prep, or into over 100 ml of LB media with ampicillin for DNA Midi prep. Bacteria were cultivated in shaking incubator (37 °C, 250 rpm) for 16 hours or until they reached optimal optical density for Midi prep (measured on NanoDrop™ ONE^{C} Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific™)).

6.2.2.1.2 Bacterial glycerol-stock preparation

At the end of cultivation of positively transformed bacterial cells in LB medium, 400 µl of bacteria were mixed with 200 µl of 100% glycerol. Suspension was frozen immediately and stored at -80 °C.

6.2.2.2 Thawing frozen HEK293T/HEK293S GnTI- cells

Cryovials were taken from liquid nitrogen and quickly transferred in 37 °C preheated water bath for about one minute until small piece of ice remained. Cryovials were washed in 70% ethanol and suspension was drop-like transferred into a sterile centrifuge tube containing 2 ml of preheated media in flow box. Tubes were centrifuged (300 rcf, 5 min, RT), media was removed and cells were resuspended in fresh media (Opti-MEM® I Reduced Serum Medium with 5% FBS (Gibco® by Life Technologies) for HEK293T and DMEM (Sigma-Aldrich (Merck)®) + 10% FBS for HEK293S GnTI- cells). According to the cell density, cells were grown in appropriate culture tissue vessel in 37 °C, 5% CO_2.

6.2.2.3 Thawing frozen Schneider's Drosophila Line 2 cells

Cryovials containing Schneider's Drosophila Line 2 cells were taken from liquid nitrogen and quickly transferred in 37 °C preheated water bath for about one minute until small piece of ice remained. Cryovials were washed in 70% ethanol and suspension was drop-like transferred into

a sterile centrifuge tube containing 2 ml of preheated media (25 °C, EX-CELL® 420 Serum-Free Medium for Insect Cells (Sigma-Aldrich (Merck)®) in flow box. Tubes were centrifuged (800 rcf, 5 min, RT), media was removed and cells were resuspended in fresh media enriched by 10% FBS and antibiotics (Antibiotic Antimycotic Solution (100x), Stabilized (Sigma-Aldrich (Merck)®); Ciprofloxacin (Sigma-Aldrich (Merck)®)). Cells were grown in appropriate culture tissue flasks at 27 °C. Next day, cells were gently resuspended and transferred in centrifuge tube. Suspension was spun down (800 rcf, 5 min, RT), former media was removed and cells were resuspended in fresh media containing 10% FBS and antibiotics. Schneider's Drosophila Line 2 cells were further grown at 27 °C.

6.2.2.4 Coating of cell culture plastics

All plastics for cell cultivation were treated with poly-L-lysine (10 µg/ml) and incubated in thermostat (37 °C) for one hour. Used solution was aspirated and surface was briefly washed with sterile water and stored at 4 °C.

6.2.2.5 Cultivation of HEK293T and HEK293S GnTI- cells

When cell density reached about 80%, splitting of cells was performed. Cells were washed by 1xPBS and gently detached from the tissue culture flask using trypsin treatment (15 minutes, 37 °C, 5% CO_2) and cell suspension was then transferred to centrifuge tube. Cells were spun down (300 rcf, 5 min, RT), trypsin containing solution was removed by gentle aspiration and cells were resuspended in new media containing 10% FBS and antibiotics: Antibiotic Antimycotic Solution (100x), Stabilized (Sigma-Aldrich (Merck)®), gentamicin. HEK293T were grown in Opti-MEM® I Reduced Serum Medium with 5% FBS (Gibco® by Life Technologies) and HEK293S GnTI- cells were grown in Dulbecco's Modified Eagle's Medium – high glucose enriched by 10% FBS (Sigma-Aldrich (Merck)®).

6.2.2.6 Cultivation of Schneider's Drosophila Line 2 cells

Schneider's Drosophila Line 2 cells are non-adherent/loosely adherent cells grown in suspension. Due to this fact, only gentle resuspension was performed without trypsinization. Late-exponential phase cells (3 to 4 days) were harvested (800 rcf, 5 min, RT) and subsequently resuspended in fresh media containing antibiotics (Antibiotic Antimycotic Solution (100x), Stabilized (Sigma-Aldrich (Merck)®; Ciprofloxacin (Sigma-Aldrich (Merck)®)) with an appropriate dilution factor (1 : 10 / 1 : 5).

6.2.2.7 Cell count and cell viability

For cell count, 5 µl of cell suspension was taken from cultivation flask and dyed with 5 µl trypan blue Appropriate amount of mixture was applied in Bürker's cell counting chamber and cells within 12 different squares (0.2 mm) were counted. Average number of cells in 1/250 mm^3 volume was then multiplied into 1 ml media volume (x 2.5 x 10^5). Thanks to the ability of the dye to penetrate death cell membrane, viable cells were observed as unstained and dead cells underwent whole cell staining.

6.2.2.8 Cryo-preservation of cell lines

For cryo-preservation of cell lines, freezing medium was freshly prepared by adjusting Dulbecco's Modified Eagle's Medium – high glucose (Sigma-Aldrich (Merck)®) with 10% FBS and 10% DMSO as cryoprotective agent. Adherent cells – HEK293T/HEK293S GnTI- were firstly detached from the tissue culture vessel (15 minutes of trypsin treatment; 37 °C, 5% CO_2) and spun down (300 rcf, 5 min, RT). Schneider's Drosophila Line 2 cells were gently collected into falcon and spun down as well (800 rcf, 5 min, RT). Then rest of the media was removed and cells were resuspended in freezing media. Suspensions were dispensed into cryogenic storage vials. To ensure lowering the temperature of 1 °C per minute, cryovials were placed in chamber containing isopropanol or Thermo Scientific™ Mr. Frosty™ Freezing Container and stored at -80 °C over-night. Next day were cryovials transferred to liquid nitrogen and stored there for long-term period.

6.2.2.9 Transfection

Transfection of both HEK293T/HEK293S GnTI- as well as Schneider's Drosophila Line 2 cells was performed with jetPEI® DNA Transfection Reagent (Polyplus transfection®) which is suitable for cells grown in suspension as well as for adherent cells.

6.2.2.9.1 Transient transfection of adherent cells – HEK293T, HEK293S GnTI-

Cells marked for transfection were seeded the day before transfection to reach optimal 50-70% confluency on the day of transfection. Medium containing FBS (Sigma-Aldrich (Merck)®) was changed in the morning before transfection. In compliance with the manufacturers' instructions (Polyplus transfection®), transfection complexes should be stable no matter the presence of serum. According to number of cells plated for transfection, required amount of DNA (plasmids with NMDAR subunits genes/AMPAR subunit gene and eGFP gene encoding plasmid) was diluted in 150 mM NaCl. Separately required amount of jetPEI® (2 µl of jetPEI® per µg of DNA) was also diluted in 150 mM NaCl. jetPEI® solution was added to the DNA solution, mixed by vortex device and incubated for 30 minutes at RT for complex formation. Final mixture was drop-wisely added to cell culture where NMDA receptor inhibitors were added – D-AP5, $MgCl_2$ and ketamine. Cells were kept in the incubator till the next day (37 °C, 5% CO_2) when protein expression assay was performed.

6.2.2.9.2 Transient and stable transfection of cells grown in suspension – Schneider's Drosophila Line 2

500 µl of Schneider's Drosophila Line 2 cells (4.5 x10^7 cell/ml) grown in stock suspension (27 °C) were taken and diluted in fresh media (Sigma-Aldrich (Merck)®) in binary row into sterile 24 well plate. After one-hour incubation in thermostat, well containing one layer of cells (≥ 90% confluency) were selected for transformation. Per well, 2 µg of DNA (NMDAR subunits/AMPAR subunit gene encoding plasmid + eGFP gene encoding plasmid) were diluted in 150 mM NaCl. 4 µl of jetPEI® were diluted in 150 mM NaCl and solution was added to the DNA solution. Mixture was incubated for 30 minutes, RT for complex establishment. jetPEI®/DNA solution was drop-wisely added onto the cells and NMDA receptor inhibitors

(D-AP5, $MgCl_2$, ketamine) were added. Plate was returned to the incubator (27 °C). Following day, media with inhibitors was changed and cells were grown for another 24 hours. At least two days after transfection, gene expression was induced by adding sterile $CuSO_4$ (0.7 mM, Sigma-Aldrich (Merck)®) to the media. Cells were returned into thermostat (27 °C). Next day, protein expression assay was performed. A part of transfected Schneider's Drosophila Line 2 cells was also exposed to selective antibiotics – Blasticidin S (InvivoGen). Successfully transfected cells survived selection and were continuously grown at same conditions with media enriched by Blasticidine S.

6.2.2.10 Cytotoxicity Test

A part of Schneider's Drosophila Line 2 transfected cells was used for cytotoxicity test. Cells were washed with 1xPBS and cell media (without NMDAR inhibitors) was changed including PI dye and cells were transferred in 6 well plates and incubated for 15 minutes (27 °C). Pictures documenting background situation were acquired (DMi8 microscope (Leica)). Then, NMDA receptor agonists were added directly to the media: NMDA (1mM) (Sigma-Aldrich (Merck)®) + glycine (10 µM) (Sigma-Aldrich (Merck)®) and cells were returned to the incubator (27 °C). After one hour an aliquot of cell suspension was taken and death cells stained with Propidium iodide (Sigma-Aldrich (Merck)®) (50 µg/ml) were observed. Pictures were acquired (DMi8 microscope (Leica)), where cells expressing any NMDA receptor subunit were visible due to the GFP co-expression and death cells in red spectra. Cells with overlap of both channels were considered as functional NMDAR expressing cells that respond to high glutamate concentration by increased intracellular level of Ca^{2+} ions leading to apoptosis. This observation was twice repeated in one-hour intervals.

6.2.2.11 Fluorescence-activated cell sorting (FACS)

Sorting of NMDAR-carrying plasmid cells co-transfected with GFP as positive control was done by Galina Kislik from Imaging Methods Core Facility (IMCF Biocev). Fluorescence activated cell sorter – FACSAria™ Fusion (BD Biosciences) with configurable temperature, placed in Class II Type A2 biosafety cabinet was used. Cells were collected in microtubes and used for further cultivation or SDS-PAGE.

6.2.2.12 Calcium imaging

Ca^{2+} imaging was performed by Dr. Jan Krůšek (Institute of Physiology). Cells assigned to Ca^{2+} imaging were plated on the glass coverslips the day before Ca^{2+} imaging assay. Glutamate (1mM) + Glycine (100 mM) were applied on the cells in the Mg^{2+}-free media (0.5 mM Ca^{2+}) at times: $t_1 = 0.5$ h, $t_2 = t_0 + 2.5$ h; followed by application of Glutamate (1 mM) + Glycine (100 mM) in the Mg^{2+}-containing media (0.5 mM Ca^{2+}) at times: $t_3 = t_0 + 4.5$ h, $t_4 = t_0 + 6.5$ h. Changes in Ca^{2+} concentration were measured using indicator Fura-2 (1 μM; Fura-2AM (Sigma-Aldrich (Merck)®) added in cell culture 1 hour staining, RT, before the measurement. Excitation of cells leading to emission of two different wave lengths at 340 nm (300 ms) and 380 nm (100 ms) was observed (microscope Olympus IX81 system CellR (Olympus), excitation Polychrome V (Till Photonics), camera Hamamatsu Orca ER) and recorded in presence or absence of glycine and glutamate, and ratio of 340/380 nm indicated intracellular Ca^{2+} concentration. Analogously to the cytotoxicity test, increased Ca^{2+} concentration level indicated on cells expressing functional NMDA receptors.

6.2.2.13 Electrophysiology

Electrophysiological measurement in voltage clamp configuration was performed by Marek Ladislav (Institute of Physiology). Axon Patch-Clamp Amplifier Axopatch 200A (Molecular Devices) was used for current recording. The outcoming signal was filtered by 8-Pole Bessel Low Pass Filter (LPF-8) at 2 kHz for whole-cell recording. Signal was amplified by Digidata 1440A Digitizer (Molecular Devices) and obtained data were sampled in pClamp 10 software (Molecular Devices). Inverted microscope Olympus CKX41 placed into Faraday cage was gripped to anti-vibration table TMC (Ametek®, TMC™). Electrophysiological measurement was performed with argent-chloride electrode at RT. Final data were analysed in Clampfit 10 software (Molecular Devices).

6.2.2.14 Protein sample harvesting

6.2.2.14.1 HEK293T and HEK293S GnTI- cells

When cells reached maximum confluence, the media was removed and plates were transferred on ice. According to the well/plate size appropriate amount of lysis buffer was added together with Protease Inhibitor Cocktail (Roche) and PMSF. Plates were incubated on ice for 45 minutes. Lysate was then transferred into microtube and briefly spun down (13 000 rcf, 3 min., 4 °C). Supernatant (membrane fraction) containing NMDA receptors was used for further SDS-PAGE or stor ed at -80 °C.

6.2.2.14.2 Schneider's Drosophila Line 2

Cells were grown in suspension, harvested into 15 ml tube and centrifuged (800 rcf, 5 min., RT). The media was removed and cells were washed with 1xPBS and centrifuged again at same conditions. Cells were subsequently moved into microtubes on ice and lysed with lysis buffer supplemented by Protease Inhibitor Cocktail (Roche) and PMSF for 15 minutes. Lysate was centrifuged (13 000 rcf, 3 min., 4 °C) and supernatant was applied on SDS-PAGE or stored at -80 °C.

6.2.3 Protein related work

6.2.3.1 Ni-NTA column NMDAR subunit purification

Purification of 6xHis-tagged GluN1 subunit was done by Jiří Zahradník (IBT). HEK293S GnTI- cells co-transfected with 6xHis-tagged GluN1 subunit and wild-type GluN2B subunit, were harvested and centrifuged (1000 rcf, 10 min, RT). Supernatant was removed and pellet was resuspended in 5 ml of Buffer N. Cells were lysed by freeze/thaw steps (-20 °C, 3x) and following sonication 15W with Misonix Sonicator 3000 Ultrasonic Cell Disruptor with Temperature Control (MISONIX, Inc.) (2.5 A; 20 s pulse/40 s off; total time 10 min). To remove cell debris and coarse impurities, lysate was centrifuged (9000 rcf, 15 min, 4 °C). Benzonase® Nuclease (Sigma-Aldrich (Merck®)) (100 U) was added to lower viscosity and samples were further incubated for 20 min at 4 °C. For purification, NGC™ FPLC instrument (Bio-Rad) and 1 ml HisTrap HP (Ni-NTA column) (GE Healthcare Life Sciences) was used.

Column was equilibrated by 5 volumes of Equilibration buffer (flow rate 1 ml/min). After the sample application, unbounded fraction was washed with additional 5 ml of Equilibration buffer. Elution of fraction was done with 6 ml of Elution buffer and collected samples were further analysed on SDS-PAGE, mass spectrometer or stored at -20 °C.

6.2.3.2 SDS-PAGE (polyacrylamide gel electrophoresis)

Polyacrylamide gel electrophoresis exploits detergent – sodium dodecyl sulfate (SDS), which denatures proteins and induce them negative charge. Afterwards all proteins can be separated according to their size in polyacrylamide gel (Laemmli, 1970).

6.2.3.3 Gel preparation and electrophoresis

Gels were prepared in Mini-PROTEAN® Tetra Cell (Bio-Rad) set. Stands for polyacrylamide gel preparation were set and controlled for leakage with dH_2O. Then glasses were drought, and liquid 10-12% polyacrylamide gel was poured between two glasses. After approximately 40 minutes gel became firm, water was removed and 5% polyacrylamide gel was applied to the top, forming the stacking gel. Comb marking off wells was put in and gel was left to polymerase at room temperature for at least 15 minutes. Samples were mixed with 4x Laemmli buffer and parboiled at 95 °C for 10 minutes. Meanwhile gel became solid, comb was removed and wells were washed out with running buffer. Maximum sample volume was applied onto wells. Electrophoresis ran at voltage 8 V/cm for 20 minutes and more ~2 hours at 10 V/cm until blue colour reached the end of the gel.

6.2.3.3.1 Resolving gel (10%; 20 ml):

- H_20 – 9.7 ml
- 40% acrylamide – 5 ml
- 1.5 M TRIS buffer (pH = 8.8 (25 °C)) – 5 ml
- 10% SDS – 200 µl
- 10% APS – 100 µl
- TEMED – 10 µl

6.2.3.3.2 Stacking gel (4%; 5 ml):

- H_20 – 3.2 ml
- 40% acrylamide – 0.5 ml
- 0.5 M TRIS buffer (pH = 6.8 (25 °C)) – 1.25 ml
- 10% SDS – 50 µl
- 10% APS – 50 µl
- TEMED – 10 µl

6.2.3.4 SDS-PAGE gel staining

When electrophoresis ended, gels were fixed in Fixing solution for 15 minutes and stained with QC Colloidal Coomassie Stain (Bio-Rad) (over-night, RT). Excess stain was eluted with a deionized water for time sufficient to visualise bands of proteins.

6.2.3.5 Western blot

Wet western blot transfer followed after SDS-PAGE ended. Gel was taken out of the apparatus and left for 30 minutes at room temperature in blotting buffer to let the running buffer dilute. Meanwhile blotting sandwich was prepared – filtrate papers were cut, nitrocellulose membrane (Amersham™ Protran™ 0.2 µm NC (GE Healthcare)) was cut and incubated in blotting buffer. Blotting sandwich was arranged: outer sponge, filtrate papers, SDS-PAGE gel, nitrocellulose membrane, filtrate papers and another outer sponge. Each layer was soaked in blotting buffer thoroughly and put down carefully in order to prevent air bubbles. Blotting sandwich was placed in the apparatus for western blotting, poured over with blotting buffer and magnetic stirrer was added. Blotting was performed over-night for 15-16 hours at room temperature at constant current 30 mA. At the end sandwich was decomposed and nitrocellulose membrane with transferred proteins was ready for immunodetection.

6.2.3.6 Ponceau S membrane staining

Before blocking was done, control of protein transfer to membrane was performed by reversible Ponceau S dye (Merck®). Ponceau S solvent stock was prepared: 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid) and nitrocellulose membrane was incubated in this solution for an hour, RT. Destaining was mediated with dH_2O until the background was clean. Complete wash out was done in case of following immunodetection.

6.2.3.7 Immunodetection

Right after transfer of proteins to nitrocellulose membrane, blocking of residual binding sites on membrane was performed. In dependence on required antibody, different blocking solution was used: 5% non-fat milk in PBST or 0.2-0.3% BSA in PBST. Membrane was incubated in blocking solution at room temperature for 1 hour. Three times 10 minutes washing steps (with PBST) followed and membrane was incubated in desired primary antibody over-night at laboratory rocker, 4°C. After, membrane was washed 3 times 10 minutes in PBST and incubated with secondary antibody (PBST) at rocker for an hour at room temperature.

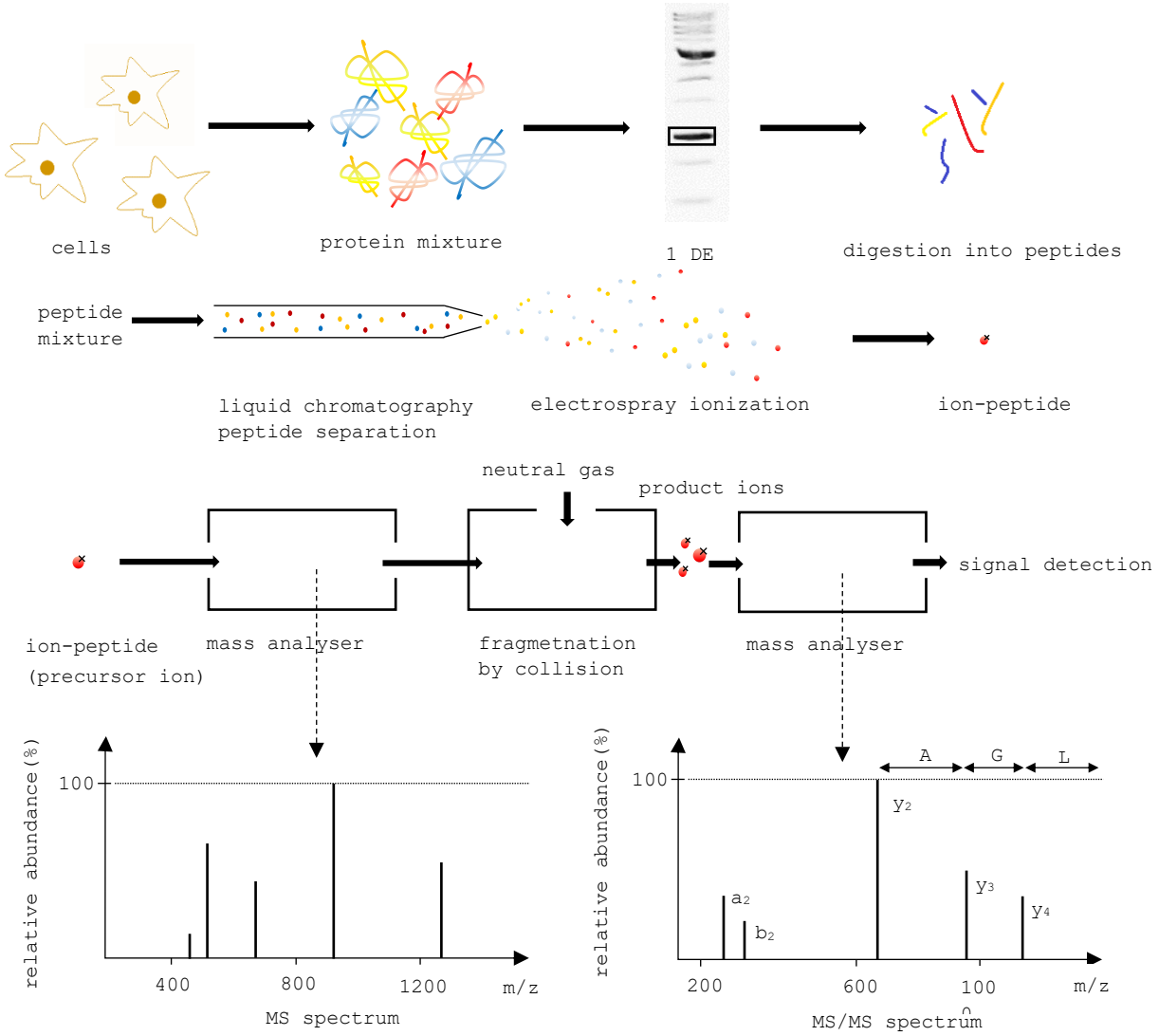
Development of the membrane was performed with Chemiluminescent Substrate (ThermoFisher Scientific™) according to manufacturers' instructions and was visualized on imaging system (Fusion Solo S, Vilber Lourmat).

6.2.3.8 Mass spectrometry

Major mass spectrometry measurement was done at Centre of Molecular Structure (Biocev). Bruker Daltonics 15T-Solarix XR FT-ICR mass spectrometer, with ESI and MALDI ion sources were used. It enabled ultra-high resolution to determination – peptide mass fingerprinting (confirmed in sequence) and characterization of NMDA receptors from SDS-PAGE by using MALDI as well as protein structural changes. For separation of protein complexes, Agilent Technologies 1200 HPLC system was used (coupled to the 15T-SolariX XR mass spectrometer). Recorded data were analysed by Dr. Petr Pompach.

Another mass spectrometry measurement was done at OMICS Proteomics (Biocev) division by Karel Harant. For this analysis Thermo Orbitrap Fusion with nano LC mass spectrometer was used.

Mass spectrometry workflow:

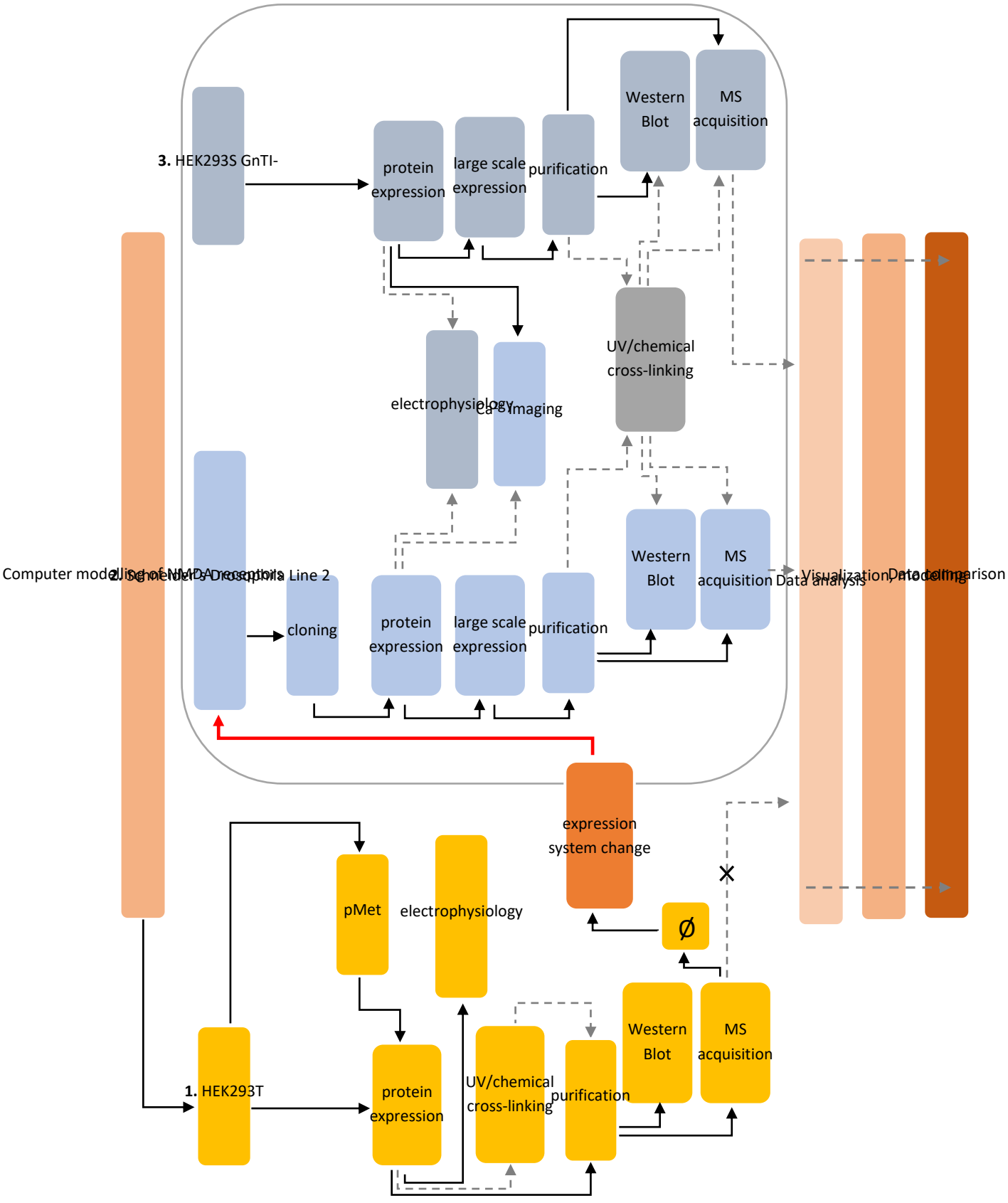


6.2.3.9 Sterilization of laboratory supplies and solutions

All plastics – tips, tubes etc. were sterilized at 121 °C, 97.4 kPa, 15 minutes in autoclave (Tuttnauer). Solutions were sterilized at 121 °C, 97.2 kPa, 20 minutes in autoclave (Tuttnauer) or filtered through 0.22 µm (Whatman™, GE Healthcare Life Sciences). Rest of laboratory supplies was sterilized in Biocev: Media preparation and washing units.

7 RESULTS

7.1 Workflow diagram

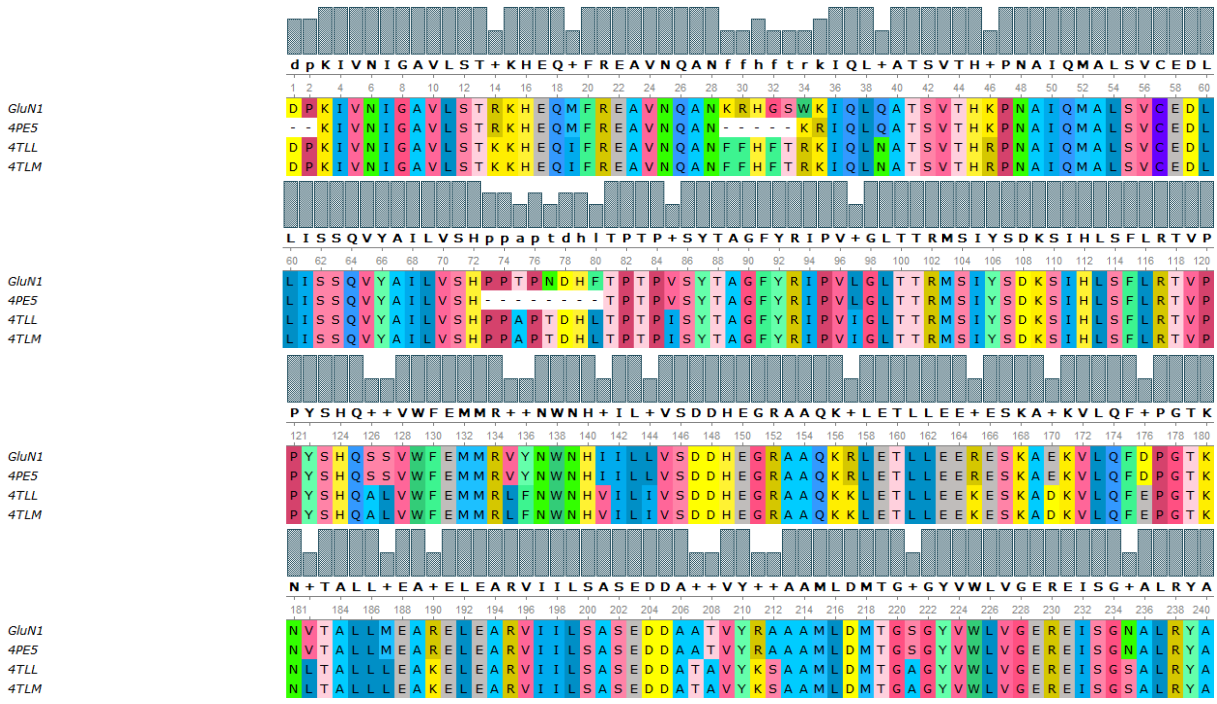


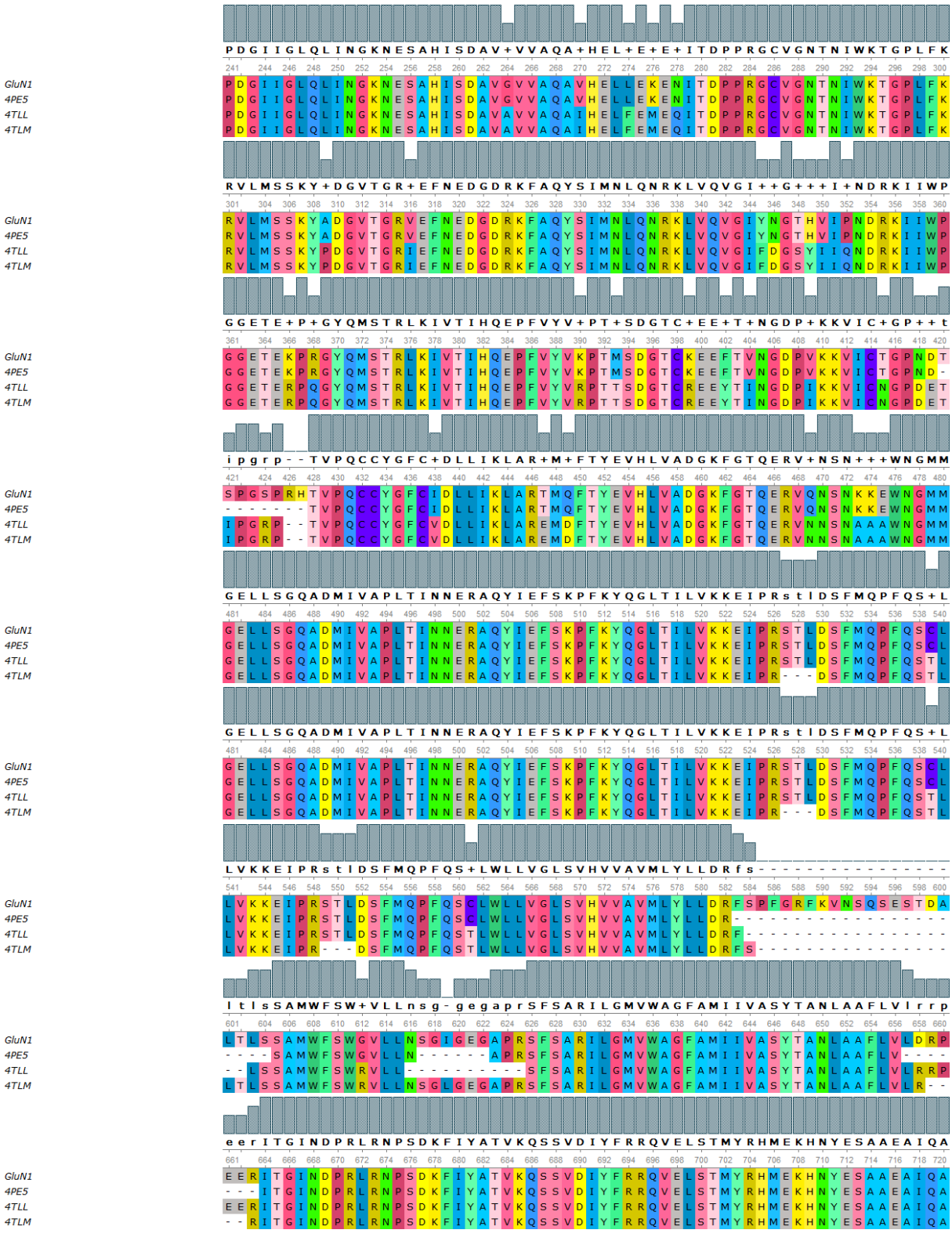
7.2 Computational modelling

The computations were performed with great help from Dr. Jiří Černý (IBT). The homology model construction was performed using toolbox of Modeller 9v14. Three recently available crystal structure templates were selected for homologue protein model construction: PDB IDs: 4PE5, 4TLL and 4TLM (Karakas and Furukawa, 2014; Lee *et al.*, 2014). All of these three structures feature of several sequence modifications (compared to the GluN1/GluN2B subunits of *Rattus norvegicus* – go to supplementary for complete GluN1 and GluN2B FASTA sequences), such as deletions, substitutions and others in order to stabilize inter-subunits interactions (NTD-NTD or TMD-TMD) (see 7.2.1). Therefore, obtained data more-likely display receptors with bounded ligands in closed state. Missing residues within templates were found after alignment of homologous sequences using MUSCLE and visualised with Unipro UGENE program.

7.2.1 4PE5, 4TLL and 4TLM and the NMDAR model (GluN1 and GluN2B) FASTA sequences alignment

- GluN1 NMDAR model alignment




GluN1
4PE5
4TLL
4TLM

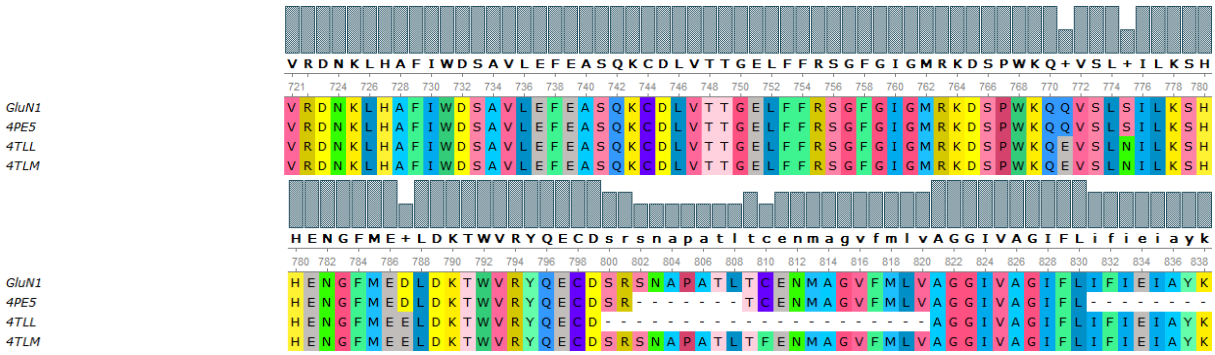
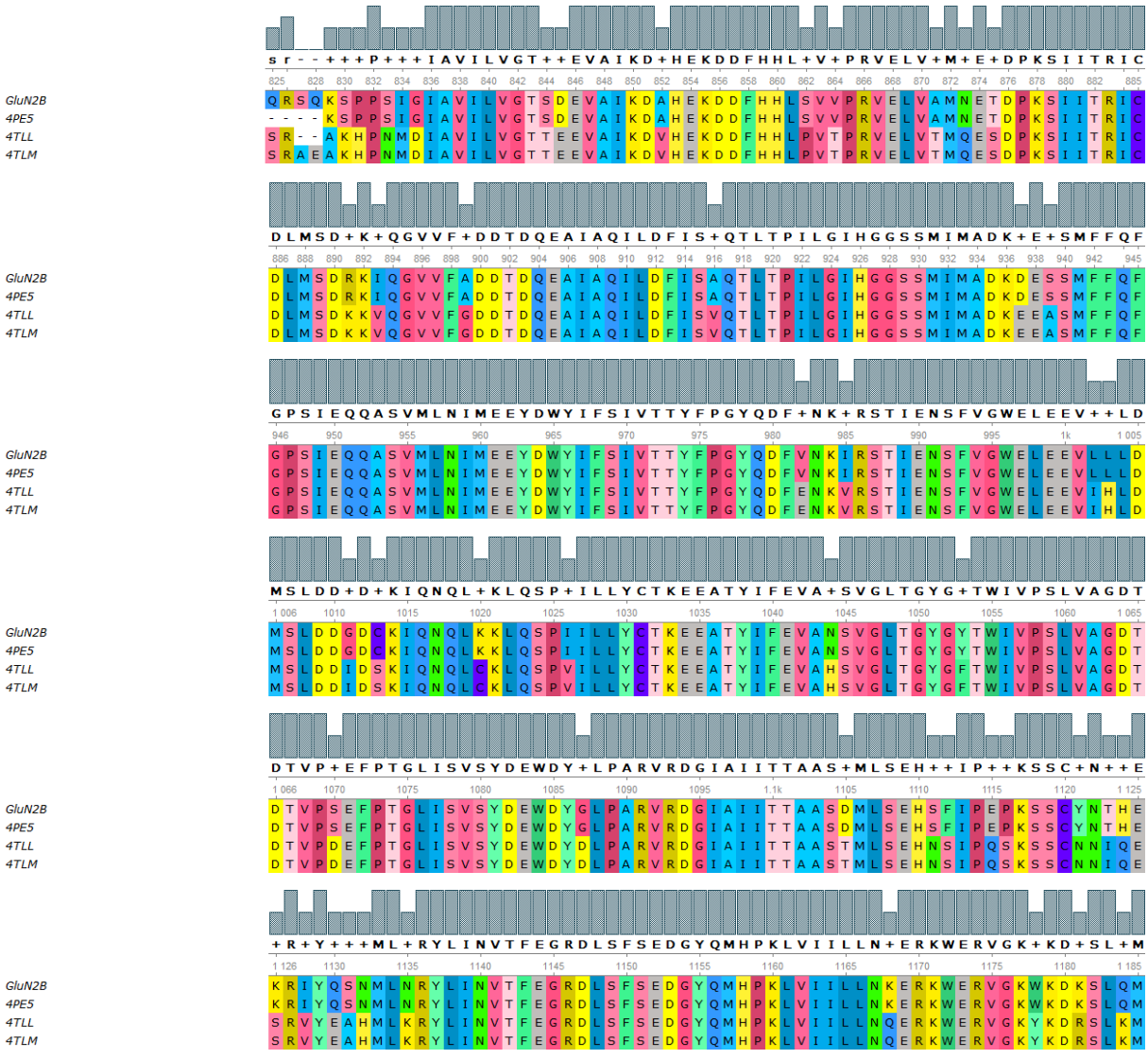


Fig. 10. **FASTA sequences alignment**; PDB ID: 4PE5, 4TLL and 4TLM sequences aligned with GluN1 NMDAR model subunit.

- GluN2B NMDAR model alignment




GluN2B
4PE5
4TLL
4TLM


GluN2B
4PE5
4TLL
4TLM


GluN2B
4PE5
4TLL
4TLM

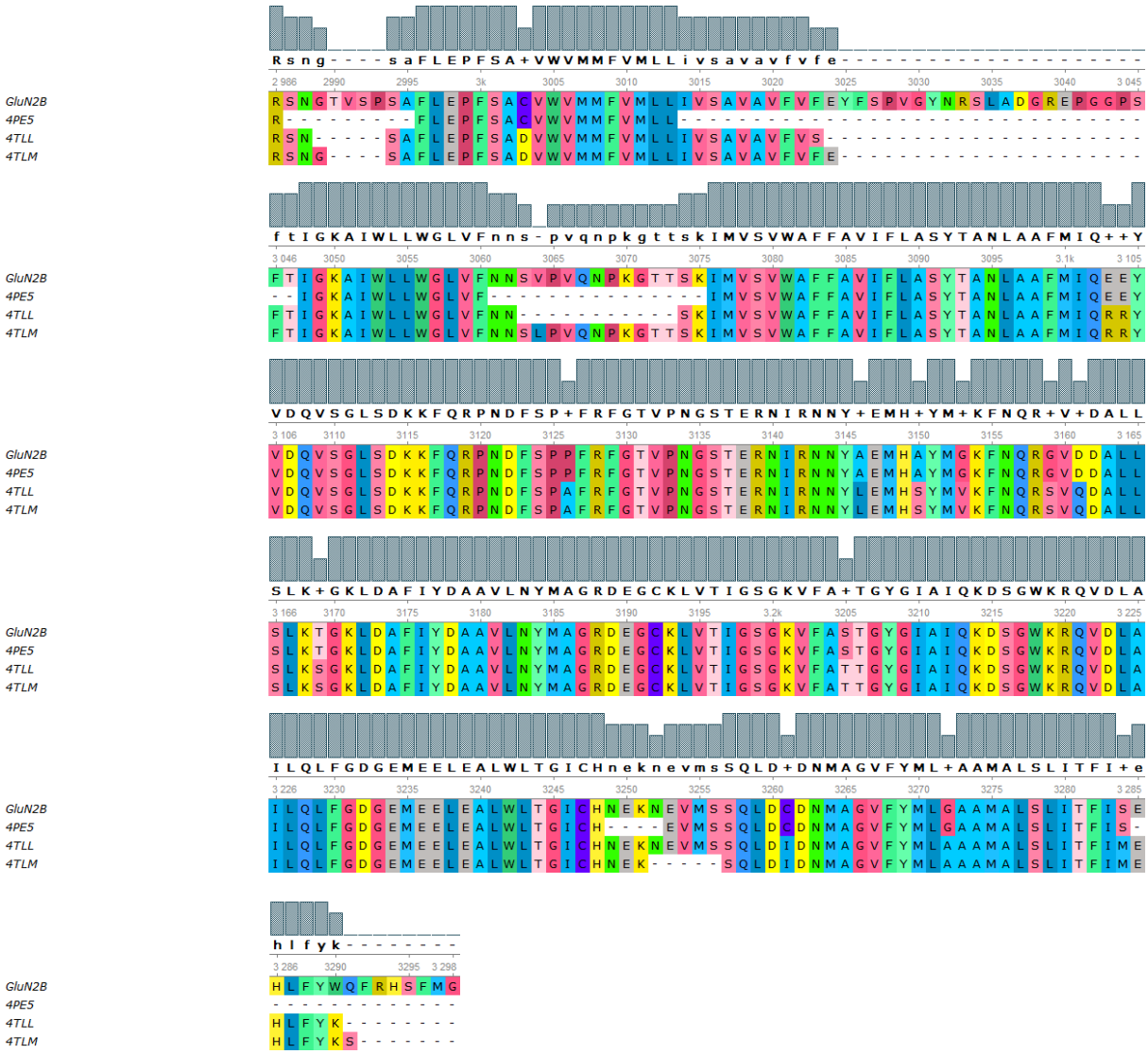


Fig. 11. **FASTA sequences alignment**; PDB ID: 4PE5, 4TLL and 4TLM sequences aligned with GluN2B NMDAR model subunit.

Comparison of the three available structures was performed in sequence alignment (Fig. 10-11) and 4PE5/4TLL/4TLM structures alignment (Fig. 12). Due to the high flexibility of certain parts of the NMDAR, there are several sub-domains (dashed line) within the receptor (linkers between LBD and TMD), that are still unrevealed (Fig. 13). We have constructed the above-mentioned complete homology atomic model of GluN1/GluN2B receptor (FASTA sequences alignment – NMDA_model), using the toolbox of Modeller 9v14 based on the three NMDAR crystal structure templates. The missing residues in the template structures were modelled using the "automodel" function of MODELLER, including the symmetry restrains for the C_α atoms of corresponding pairs of subunits. Final NMDA receptor model was created (Fig. 14).

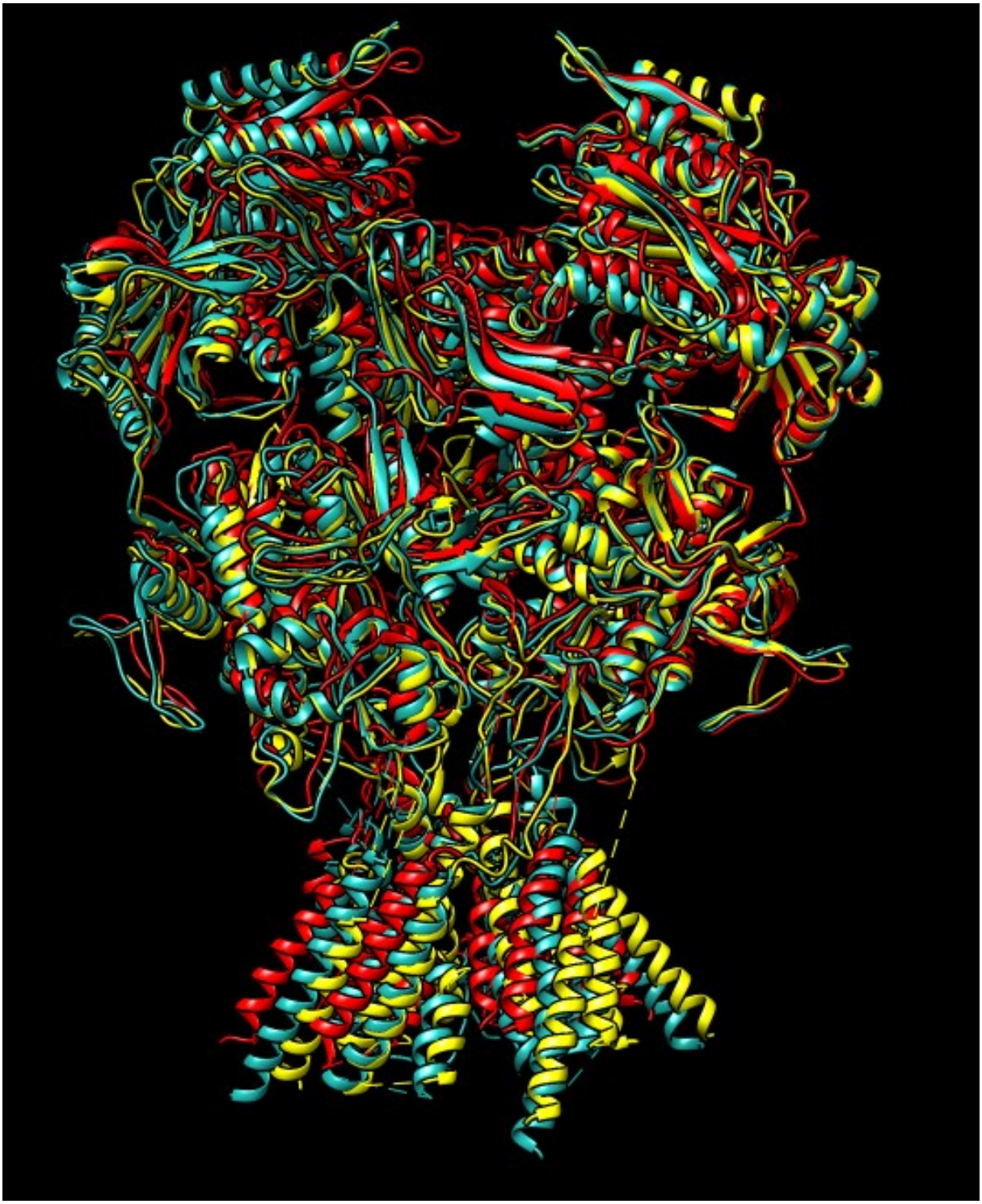


Fig. 12. **Three available structrures of whole NMDA receptor – aligned**; within the LBD-TMD part, several residues are missing, due to the flexibility of linkers.

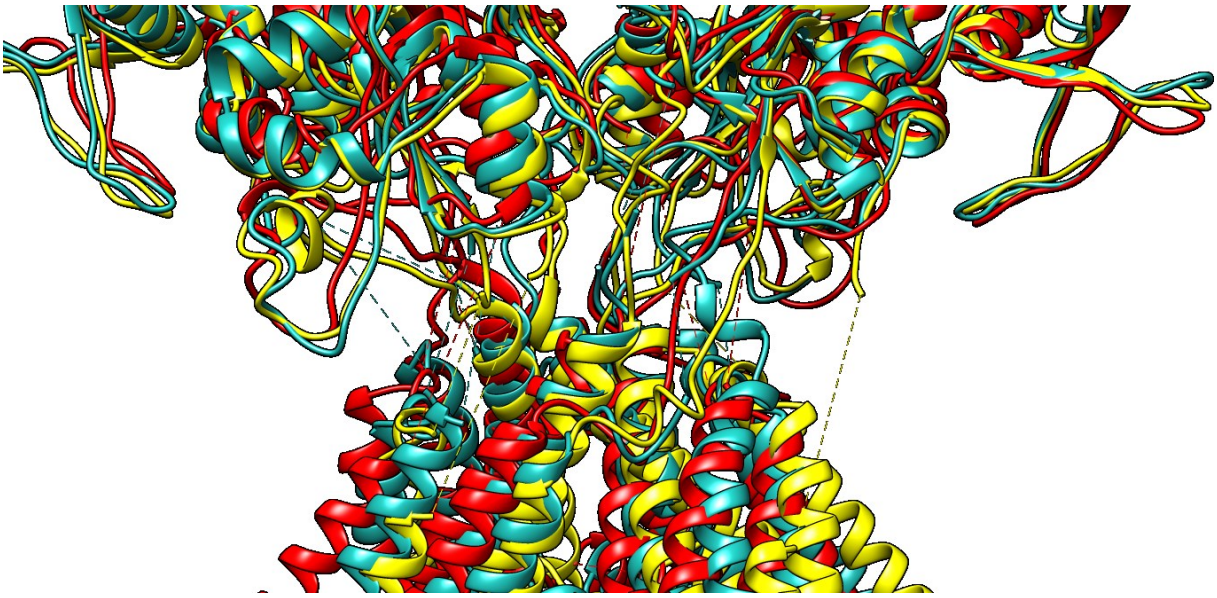


Fig. 13. **Close up insight onto the linker region**; missing parts of the NMDAR structure shown by dashed line.

The structure of NMDA receptor molecular model was also analysed in terms of future crosslinking assay (UV and chemical). For UV-crosslinking method, L-Methionines suitable for this assay (in the meaning of the distance and positions of atoms) within the subunits were selected and visualised within the NMDAR model (Fig. 19).

For chemical crosslinking, two approaches were considered – the "zero lengh" crosslinker 1-ethly-3-[3-dimethylaminopropyl]carbodiimide (EDC), which couples carboxyl groups with primary amines within 12 Å length; and disuccinimidyl suberate and disuccinimidyl glutarate (DSS and DSG), where both of them couple two amino groups within 20 Å and 24 Å length. To inspect the availability of residues for the chemical crosslinking method, analysis of lysine (for DSS and DSG) and aspartates, glutamates respectively (for EDC) within the NMDAR model was performed. AA residues were selected, and their position within the NMDAR model was inspected. Only those localized at a position available for the chemical crosslinker, and at required distance from each other, were chosen (Fig. 20).

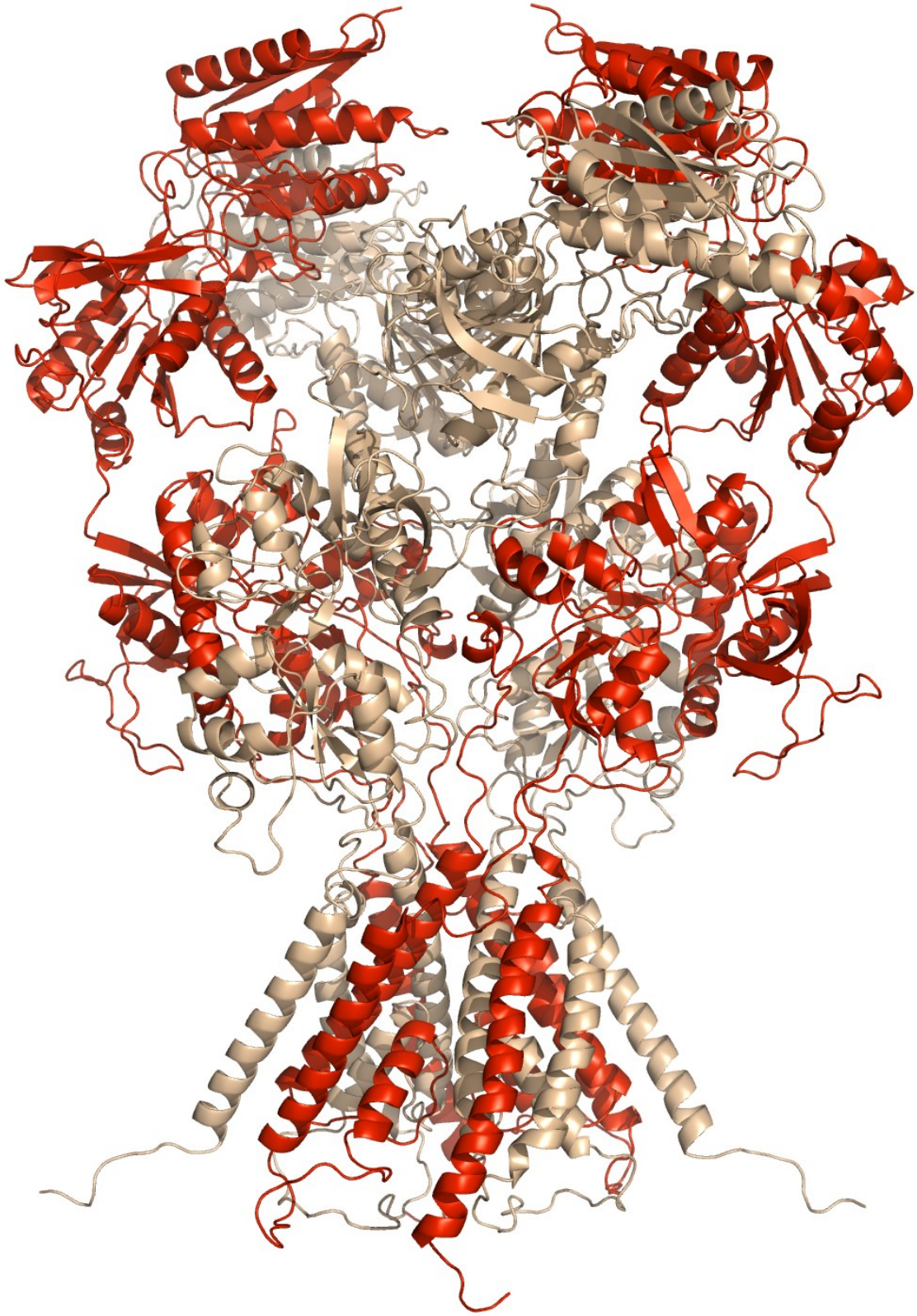


Fig. 14. **The model of the NMDA receptor**, constructed of three available template structures: 4PE5, 4TLL and 4TLM; missing residues were supplemented from *GRIN1*, *GRIN2B* (*Rattus norvegicus*) FASTA sequences.

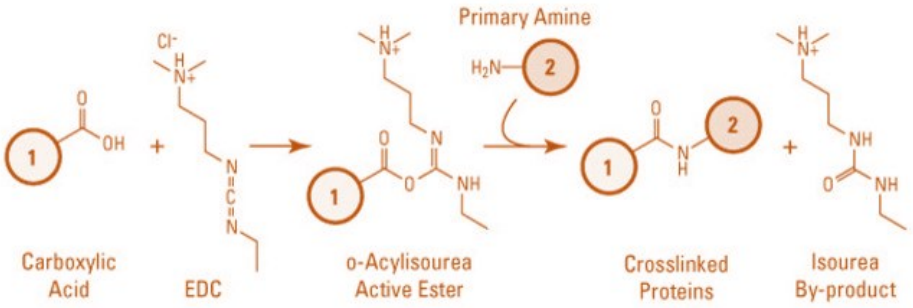


Fig. 15. **EDC crosslinking reaction scheme**; adopted from ThermoFisher Scientific™ (https://www.thermofisher.com/order/catalog/product/22980)

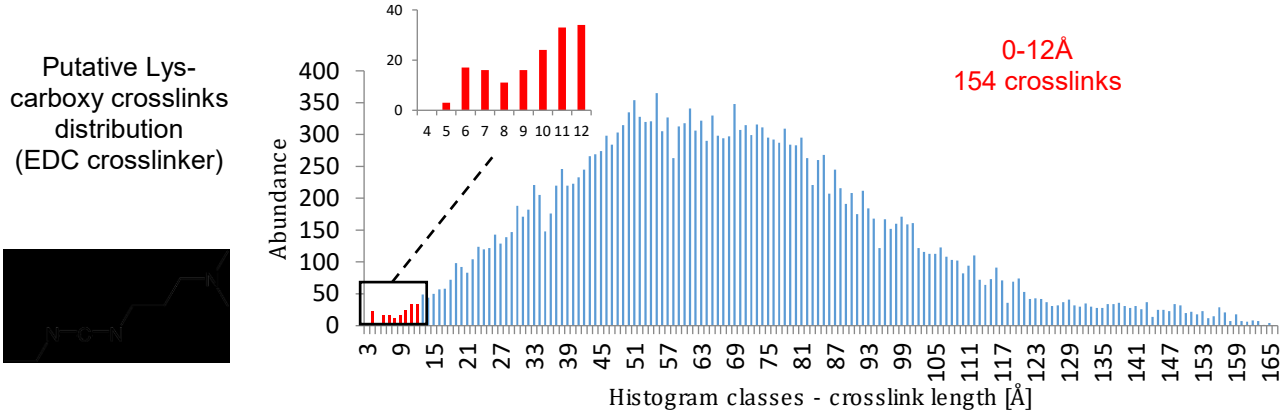


Fig. 16. **Lysine-carboxy crosslink distribution for EDC crosslinking**

The EDC crosslinker is able to activate carboxyl groups to conjugate primary amines (within lengh 12 Å) (Fig. 15). The DSS and DSG crosslinker react with amino groups . The DSS reacts via ester group with primary amines – lysine (K) and forms stable amide bonds. DSG is simple homobifunctional crosslinker (Fig. 17) and, as well as DSS, carries NHS (N-hydroxysuccinimide) ester group.

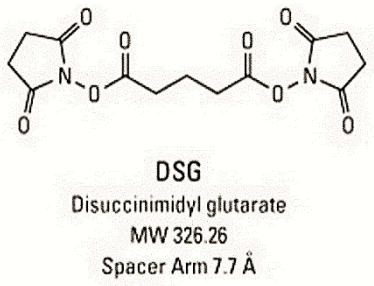


Fig. 17. **DSG molecule**; adopted from ThermoFisher Scientific™ (https://www.thermofisher.com/order/catalog/product/20593)

The distribution of thought residues is shown in histograms (Fig. 16, 18). Due to the flexibility of the NMDAR parts (linkers connecting LBD and TMD), it is possible for more residues to form crosslinkage. According to these data, we shifted the experiments *in vitro*.

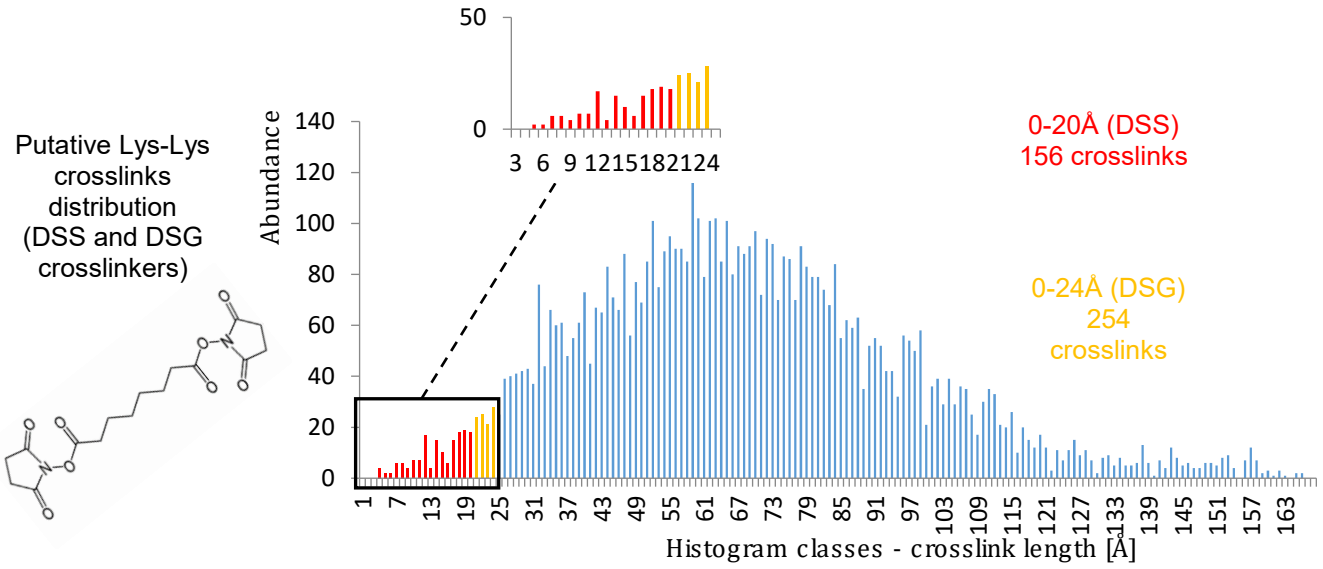


Fig. 18. **Lysine-lysine crosslink distribution for DSS and DSG crosslinking**

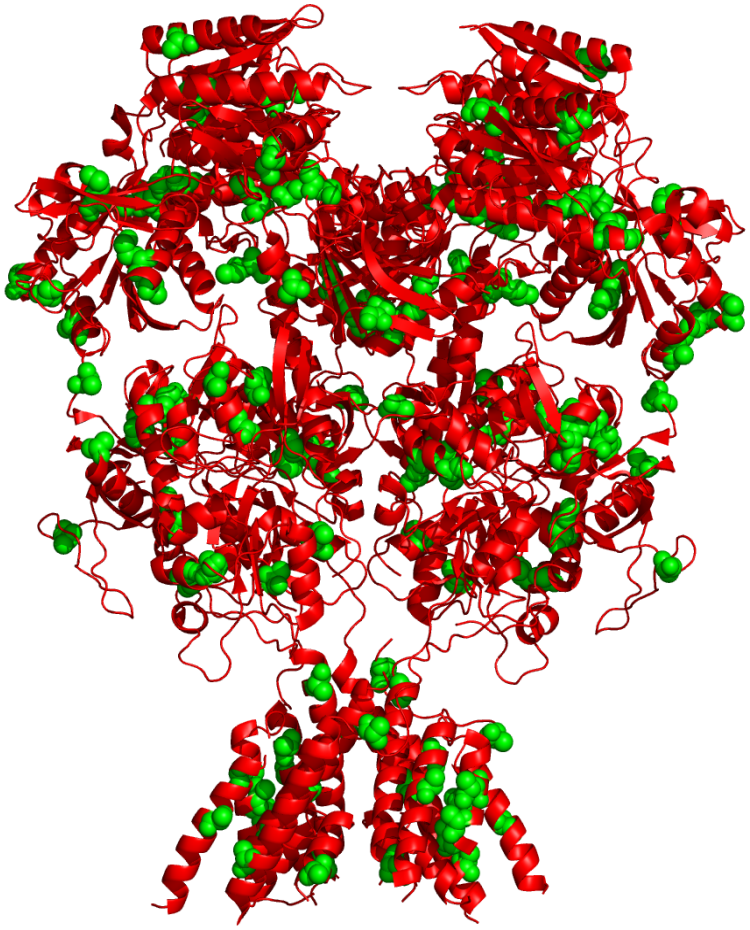


Fig. 19. **Model of the NMDA receptor**; L-Methionine residues disposable for the UV-crosslinking highlighted (green).

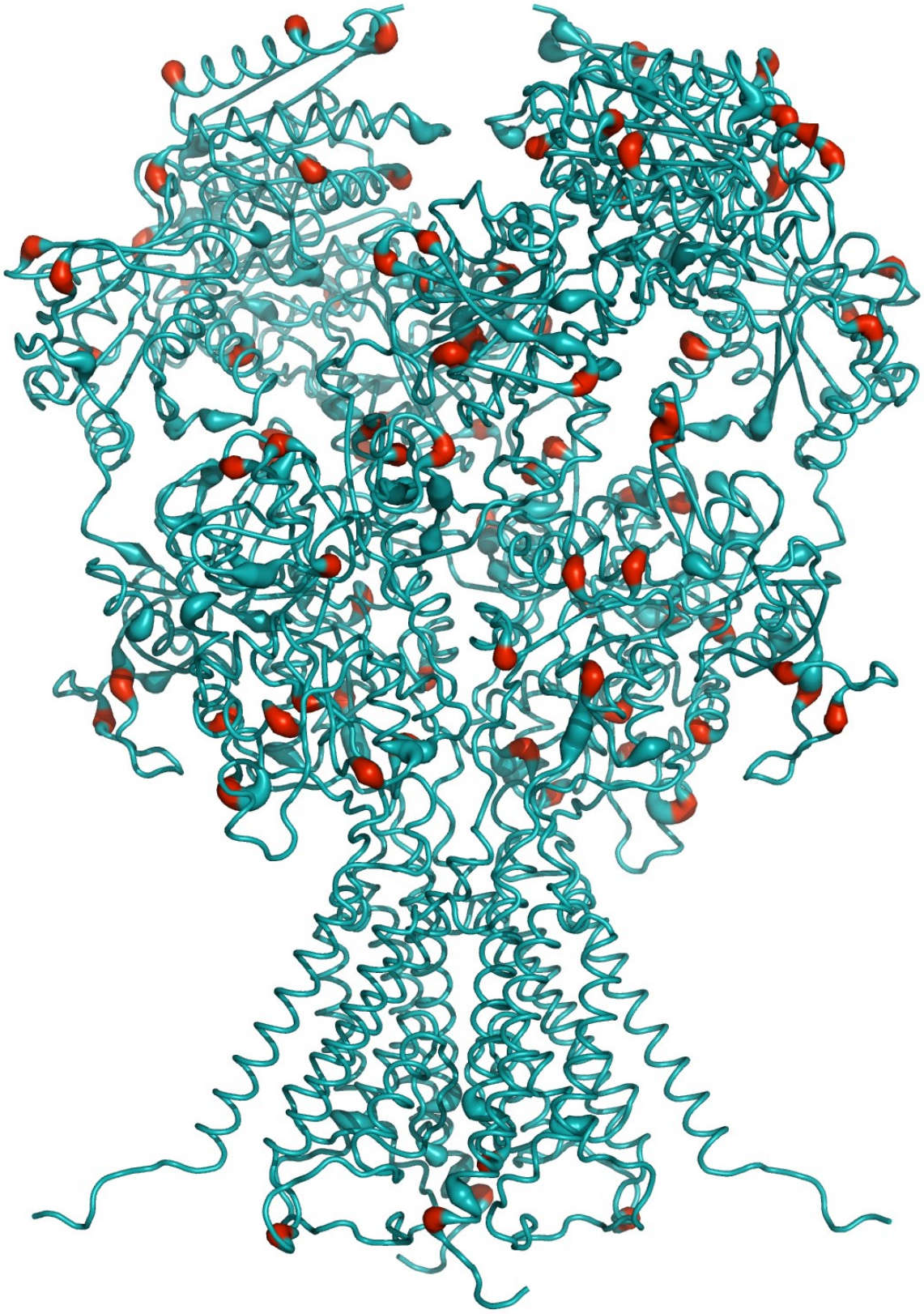


Fig. 20. **Model of the NMDA receptor**; lysine residues disposable for the chemical crosslinking highlighted (red).

7.3 NMDAR expression in HEK293T cells

7.3.1 Verification of the expression of functional NMDAR in HEK293 cells

In the primary experiments, adherent cells were co-transfected with pCDM8_GluN1 6xHis tag and pcDNA3_GluN2B Strep tag expression plasmids, encoding DNA sequences for *Rattus norvegicus* NMDA receptor. Both tags in the constructs were sub-cloned using site direct mutagenesis by Dr. Aleš Balík before my work on the project has started. The verification of the successful expression of GluN1/GluN2B tagged constructs assembled as functional NMDA receptors was performed using electrophysiology measurement in whole cell recording. Identification of NMDA receptor subunits in cell lysates was further confirmed by immunochemistry and mass spectrometry analysis.

7.3.1.1 NMDAR current recording in the HEK293T cells

Agonists (glycine and glutamate) were co-applied to the cells in the voltage clamp configuration. The elicited whole cell current responses from the cells expressing tagged subunits (Fig. 21 and 22) corresponded to the control response of the wild-type GluN1/GluN2B receptor (comparing amplitude and low level of desensitization).

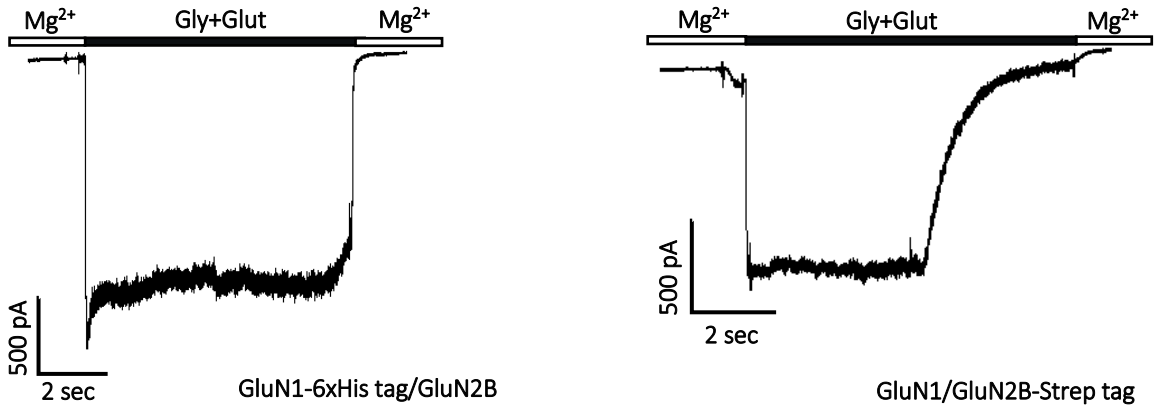
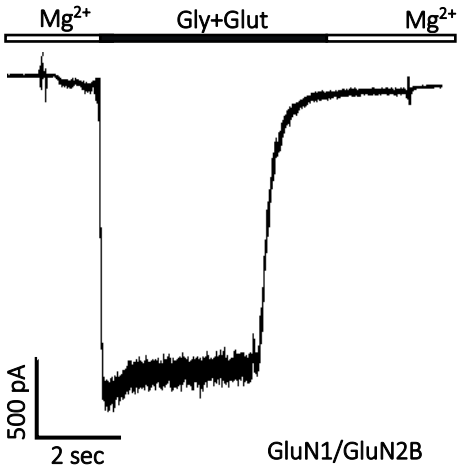


Fig. 21. The current traces recorded in whole cell configuration from cells expressing GluN1-6xHis/ GluN2B (left), GluN1/GluN2B-Strep (right).

Fig. 22. The current traces recorded in whole cell configuration from cells expressing GluN1/GluN2B (right).



7.3.1.2 NMDAR current recording in the HEK293T cells grown in media enriched with photo-L-Methionine

For planned UV-crosslinking experiment, the L-Methionine residues in the expressed subunits are substituted by photo-L-Methionine. To ensure, that this amino-acid substitution would not alter the function (thus the basic structure) of the receptor, cells were grown in media without L-Methionine, substituted by photo-L-Methionine. Identically to the previous experiment, the whole cell recording was performed, showing agonist-evoked currents (see Fig. 23), corresponding to currents elicited in the sister cells grown in media with L-Methionine (not shown). This and the previous recording were performed by Dr. Aleš Balík and Marek Ladislav, using transfected cell cultures I prepared.

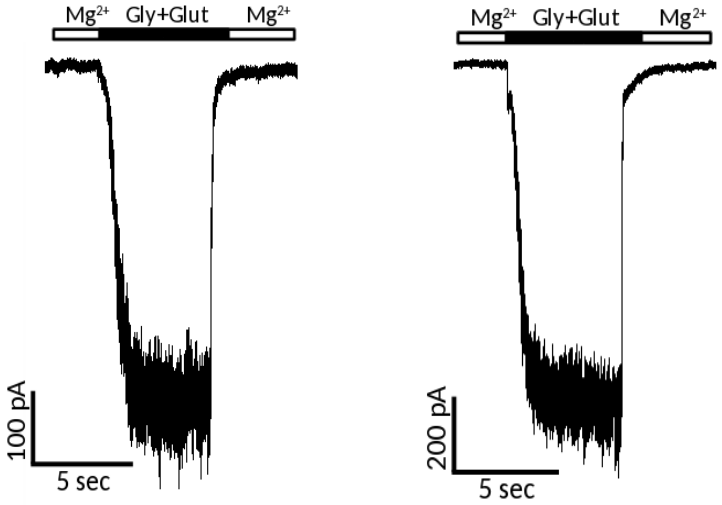


Fig. 23. The current traces recorded in whole cell configuration from cells expressing GluN1-6xHis/ GluN2B (left) and GluN1/GluN2B-Strep (right), grown in media containing photo-Met.

7.3.2 Immunodetection of NMDARs

Tagged NMDAR subunits were also analysed using Western blot technique. Cells grown in small scale (12 well plate) were transfected with tagged NMDAR subunits in combination of correspondning GluN1/GluN2B NMDAR subunits, harvested two days after transfection, lysed and crude membrane fractions were analysed on Western blot. Specific primary antibodies – anti-GluN1, anti-GluN2B, and anti-His were used in order to obtain signal from GluN1/GluN2B/His tag epitopes (Fig. 24).

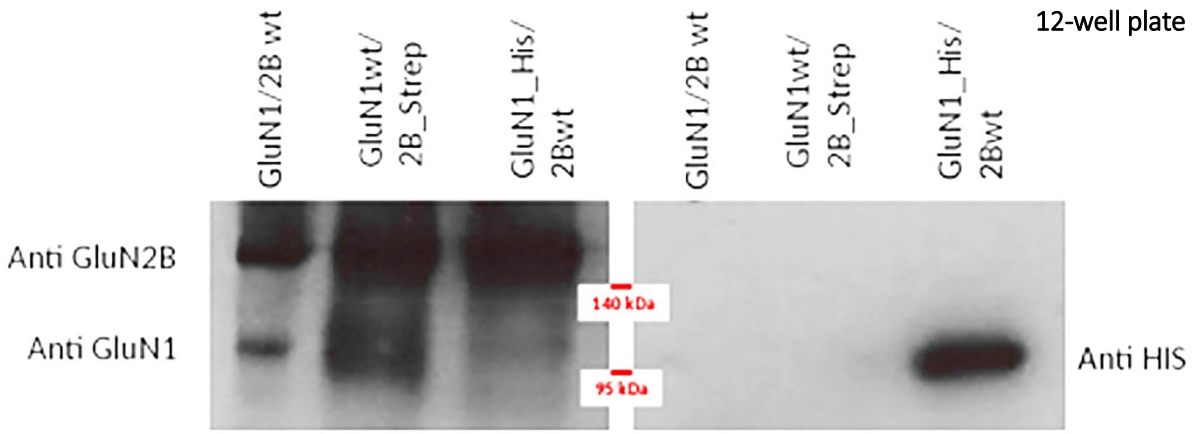


Fig. 24. Western blot of HEK293T cell lysates – expressed combinations of GluN1/GluN2B variants; anti-NR1 glutamate receptor antibody (NeuroMab), anti-NR2B glutamate receptor antibody (NeuroMab), 6x-His Tag Mouse Monoclonal antibody (ThermoFisher Scientific™).

7.3.3 Purification of NMDAR subunits

Subsequently, cells expressing 6xHis-tagged GluN1 subunit in combination with GluN2B were grown in larger scale (10 cm dish, standard or photo-Met supplemented culture medium) in order to be purified. Two days after transfection cells were harvested and crude membrane franctions prepared. We used Ni-NTA affinity chromatography (Fig. 25 and 26) (see 6.2.3.1) for 6xHis- tagged GluN1 subunit purification; obtained fractions were analysed on Western blot as well (Fig. 27).

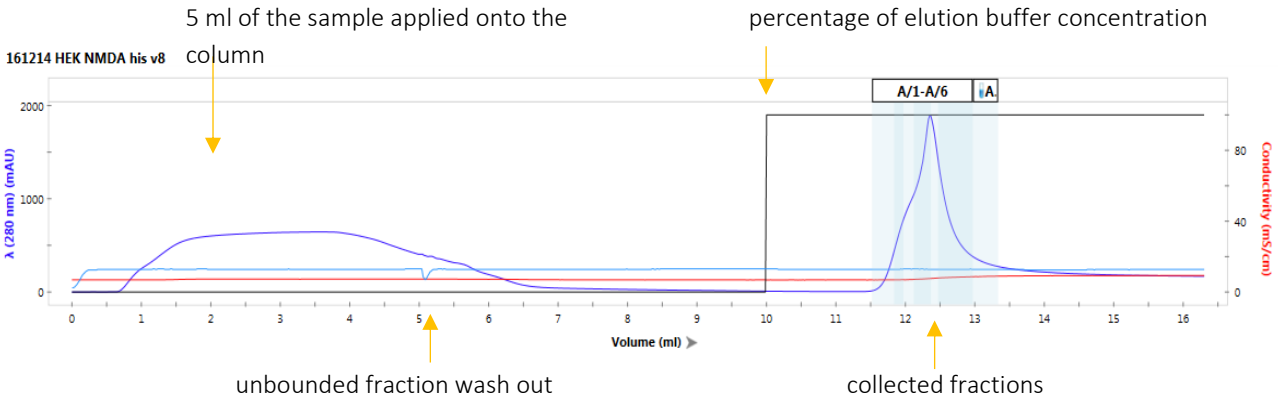


Fig. 25. The report of Ni-NTA purification profile of the GluN1-6xHis protein variant.

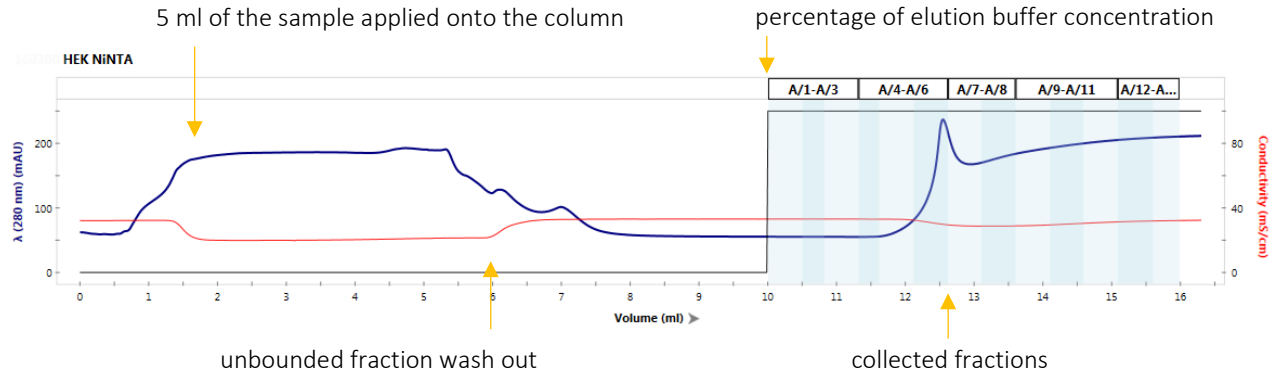


Fig. 26. The report of Ni-NTA purification profile of the all-photo-Met GluN1-6xHis protein variant.

According to the report of the Ni-NTA purification (Fig. 24), three collected fractions with highest signal, flow through sample (negative control) and brain lysates (positive control) were analysed on Western blot, using specific anti-GluN1 receptor antibody (Fig. 27).

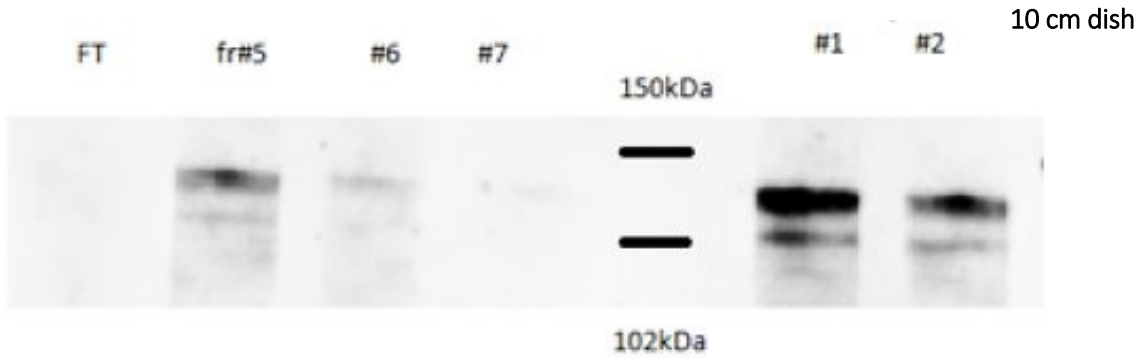


Fig. 27. **Western blot of collected fractions from Ni-NTA affinity chromatography purification of HEK293T cell lysate expressing combinations of GluN1-6xHis tag/GluN2B**. FT – flow throught; fr#5 – #7 – collected fractions 5 – 7; #1 – *Rattus norvegicus* hippocampus lysate; #2 – *Rattus norvegicus* cortex lysate; anti-GluN1 receptor antibody (Neuromab).

7.3.4 Mass spectrometry

The promising results from previous analysis (above) led us to go forward to the pilot mass spectrometry analysis. Two aims were settled: (1.) to identify NMDAR subunit (GluN1 or GluN2B) in prepared protein samples, and further (2.) to obtain the first data for NMDAR subunit expressed with L-Methionine and subunits expressed in medium supplemented with photo-Met to be compared. Therefore, it would be possible for us to detect possible changes, that this substition may be responsible for. Subsequent experimental step would include UV-crosslinking of photo.Met containing NMDAR. Two different mass spectrometry approaches were used. The first one (MS I) – the high resolution Bruker Daltonics 15T-Solarix XR – Fourier transform-ion cyclotron resonance mass spectrometer (FT-ICR) operating in the survey scan (CMS, Biocev), detects peptides in super resolution and accuracy (bellow 1 ppm – parts per-million; mass error unit) intended for structural data acquisition of purified samples. The second one (MS II) – Thermo Orbirap Fusion with nano LC mass spectrometer (OMICS Proteomics; Biocev) detecting wide dynamic range and ultrahigh mass recolution (sub-1-ppm), was used for basic protein identification from the whole cell extract.

MS I

Despite of promising results from electrophysiology and immunochemistry, analysis via Bruker Solarix XR mass spectrometer (Dr. Petr Pompach, Institute of Microbiology, Biocev) was not able to detect subunit GluN1 of NMDA receptors (go to supplementary for complete MS I record). Several proteins were detected at corresponding size, and all of them were identified as Poly (ADP-ribose) polymerase, LONP1 – Lon protease mitochondrial homolog and Serum albumin; but no trace of NMDAR subunits was found.

MS II

With the respect to negative results from MS I, crude protein samples, from cells expressing NMDARs, were consequently analysed with Thermo Orbitrap Fusion mass spectrometer. This protein identification was performed by Karel Harant (Faculty of Science, Charles University, Biocev). This wide range mass spectrometry identified almost 6000 proteins/protein fragments

(go to supplementary for MS II record). Of those, GFP at molecular weight 28.1 kDa and GluN2B subunit at molecular weight 166 kDa were positively identified. However, the amount of GluN1 subunit was probably under detectable limit. From our previous experience, both GluN1 and GluN2B subunits were certainly detected in our mouse brain samples, employing this mass spectrometry technique.

7.4 Expression system of Schneider's Drosophila Line 2

7.4.1 NMDAR subunits cloning into pMT BiP V5-His* vector

Based on previous results, that were obtained from mass spectrometry analysis, we have decided for the change of the expression systém similar to this, that other laboratories used for large-scale protein production, to set up crystallographic experiments. For successful mass spectrometry and following comparing analysis, we needed reasonable amount (up to mg) of protein. Schneider's Drosophila Line 2 cells were chosen as the new expression system due to advatages (Adriaan de Jongh, Salgueiro and Dyring, 2013), listed below:

- fast and robust protein production
- produce proteins in native conformation
- cell densities up to 70 million cells/ml without aggregation or toxic metabolite issues
- suitable for consitiutive and inducible expression
- low risk of human viral contaminants
- optimizable culture conditions

For transfection of Schneider's Drosophila Line 2 cells, cloning of *GRIN1* and *GRI2B* sequences into compatible vector pMT BiP V5-His* (vector-His tag is encoded beyond the stop codon) had to be done. NMDAR subunits (GluN1, GluN2B and GluN2B-2xStrep tag) were designed with truncated CTD. Sequence encoding CTD, would prolong length of DNA needed to be cloned, it does not affect ion channel opening of the receptor and we also considered a technical difficulty to analyse any structure of this intracellular part of the receptor (Krupp *et al.*, 1998; Sf F. Traynelis *et al.*, 2010).

7.4.1.1 GluN1 subunit sub-cloning

For NMDA receptors expression in Schneider's Drosophila Line 2 cells, GluN1 subunit of NMDA receptor had to be cloned in compatible vector: pMT BiP V5-His*. Due to few difficulties, including unsuccessful cloning with Gibson Assembly® and restriction cloning, cDNA for *GRIN1* was firstly amplified (Fig. 28) and cloned into pGem®-T Easy Vector (Promega). One positively *GRIN1*-pGem®-T Easy Vector transformed *E. coli* colony was selected (see Fig. 29) and pure plasmid was obtained.

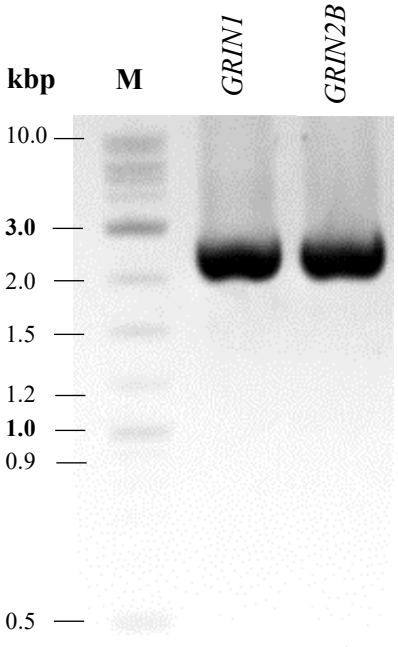


Fig. 28. **PCR products**; *GRIN1* and *GRIN2B* at size around 2.5 kbp.

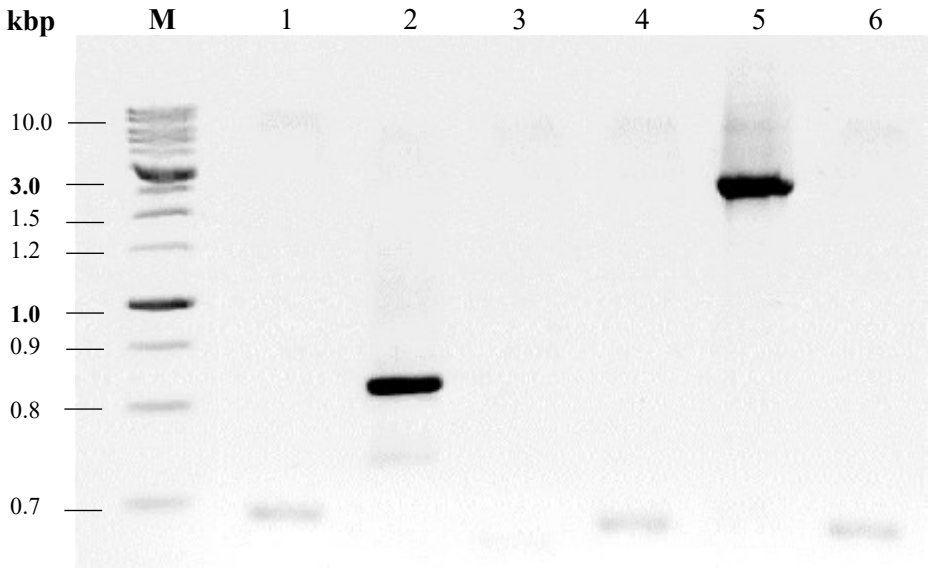


Fig. 29. **Colony PCR**; 5 – positively *GRIN1*-pGem®-T Easy Vector transformed colony.

pGem®-T Easy Vector adds additional restriction sites, which were subsequently used for restriction cloning into recquired pMT BiP V5-His* vector.

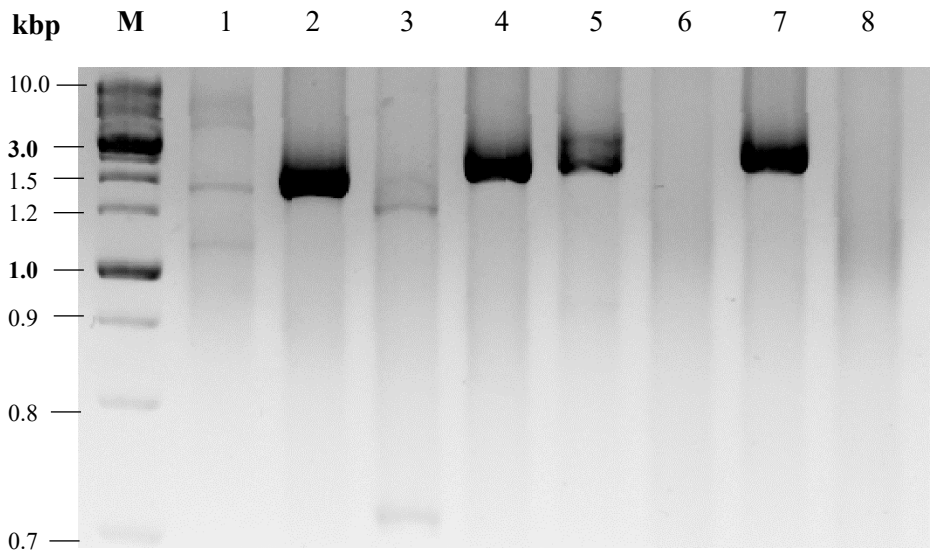


Fig. 30. **Colony PCR**; *GRIN1*-pMT BiP V5-His*; transformed colonies sent for sequencing at size around 3.0 kbp (2, 4, 5 and 7).

The colony N° 7 (Fig. 30) was sequencely confirmed as colony containing *GRIN1* gene, sub-cloned into pMT BiP V5-His* (see Fig. 31 for final construct).

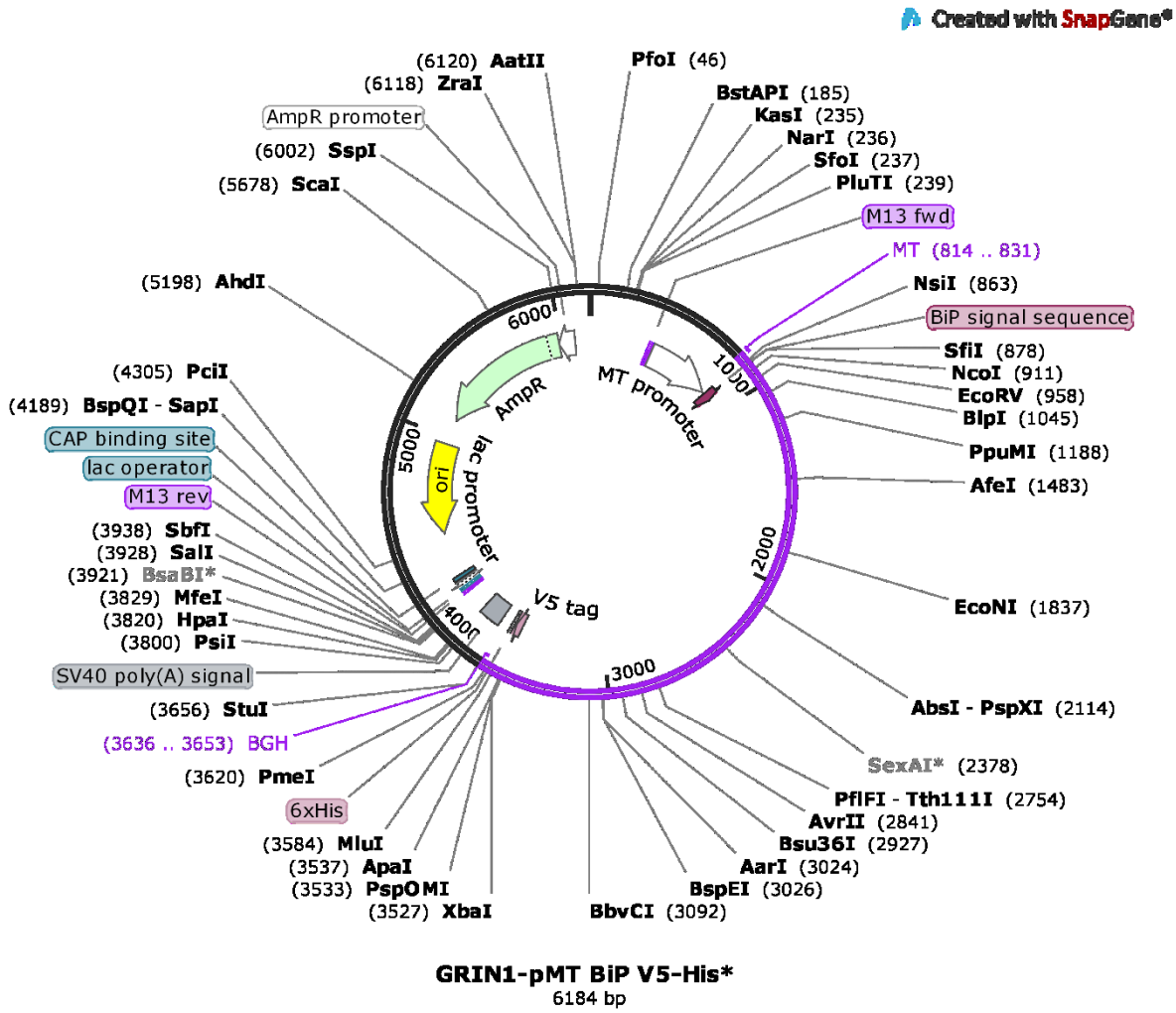


Fig. 31. **Map of *GRIN1*-pMT BiP V5-His* plasmid**; MT Forward and BGH Reverse primers highlighted (violet).

7.4.1.2 GluN2B sub-cloning

Identically to the GluN1 sub-cloning, GluN2B subunit sequence further used as inserts was amplified with PCR and cleaned up with agarose gel electrophoresis (Fig. 32). PCR primers contained sequences for restriction enzymes used in subsequent cloning.

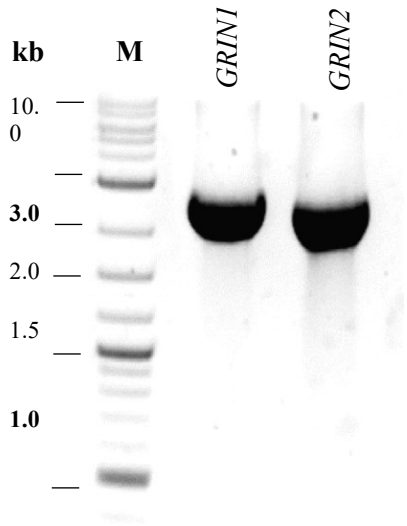


Fig. 32. **PCR products**; *GRIN1* and *GRIN2B* at size around 2.5 kbp.

GluN2B insert was digested with *Spe*I and *BstB*I restriction enzymes, as well as pMT BiP V5-His* vector, ligated, and competent *E. coli* cells were transformed. Selected colonies were used for colony PCR to detect positive clones containing *GRIN2B*-pMT BiP V5-His* (Fig. 33).

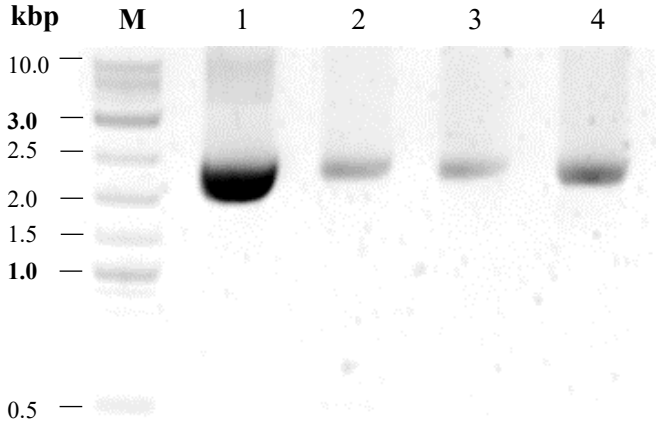


Fig. 33. **Colony PCR**; *GRIN2B*-pMT BiP V5-His*; transformed colonies sent for sequencing at size around 2.3 kbp (1, 4).

The clone N° 1 was confirmed by sequencing as positive, containing *GRIN2B*-pMT BiP V5-His* plasmid (Fig. 34).

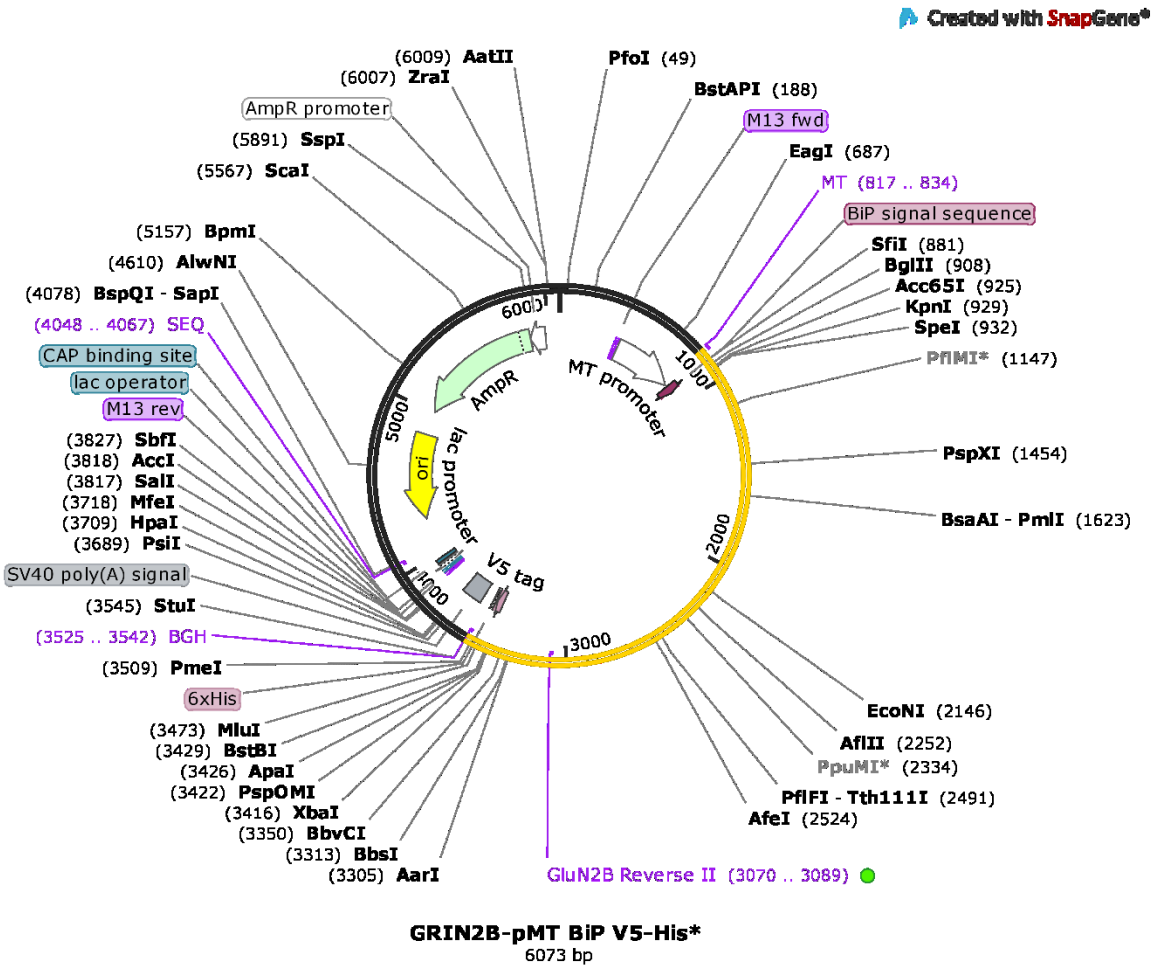


Fig. 34. **Map of *GRIN2B*-pMT BiP V5-His* plasmid**; MT Forward, BGH Reverse primers highlighted (violet) and GluN2B Reverse II primer (green point) highlighted.

7.4.1.3 GluN2B-2xStrep tag cloning

For specific GluN2B subunit purification via liquid chromatography, 2xStrep tag was added using forward primer with 2xStrep tag sequence (see 6.1.11.1). After preparation of the insert in adequate concentration (200 ng/µl) (Fig. 35), gene for GluN2B subunit with 2xStrep tag was cloned into pMT BiP V5-His* plasmid using Gibson Assembly® method.

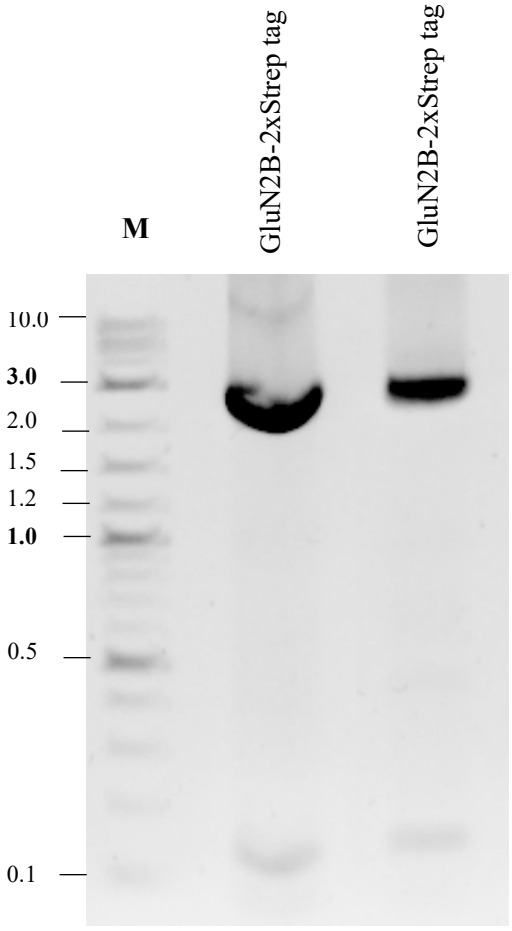


Fig. 35**. PCR products**; *GRIN2B*-2xStrep tag at size around 2.5 kbp

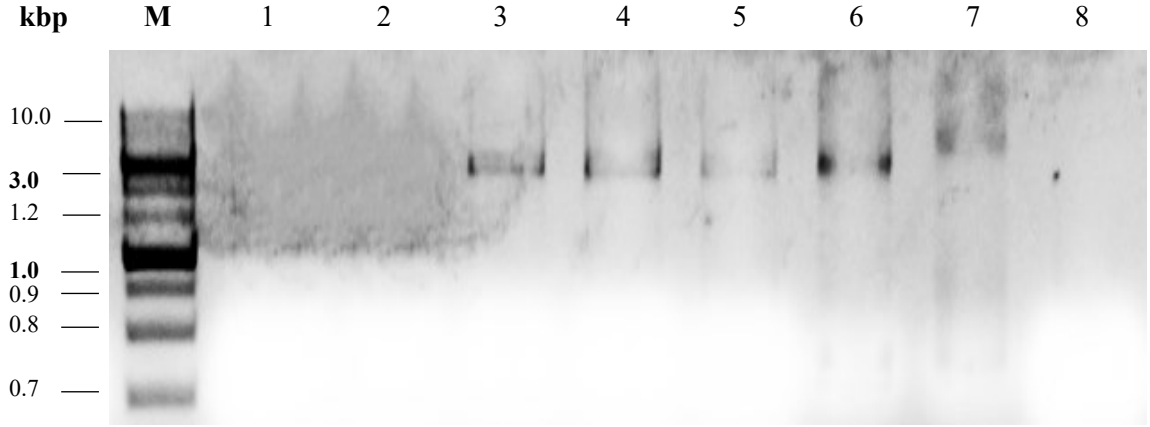


Fig. 36. **Colony PCR**; *GRIN2B*-2xStrep tag-pMT BiP V5-His*; transformed colonies sent for sequencing at size around 3.0 kbp (3, 4, 5 and 6)

Selected colonies were used for colony PCR to detect positive clones containing *GRIN2B*-pMT BiP V5-His*. The clone N° 4 (Fig. 36) was confirmed by sequencing as positive containning *GRIN2B*-pMT BiP V5-His* plasmid, where 2xStrep tag was added, was created (Fig. 37).

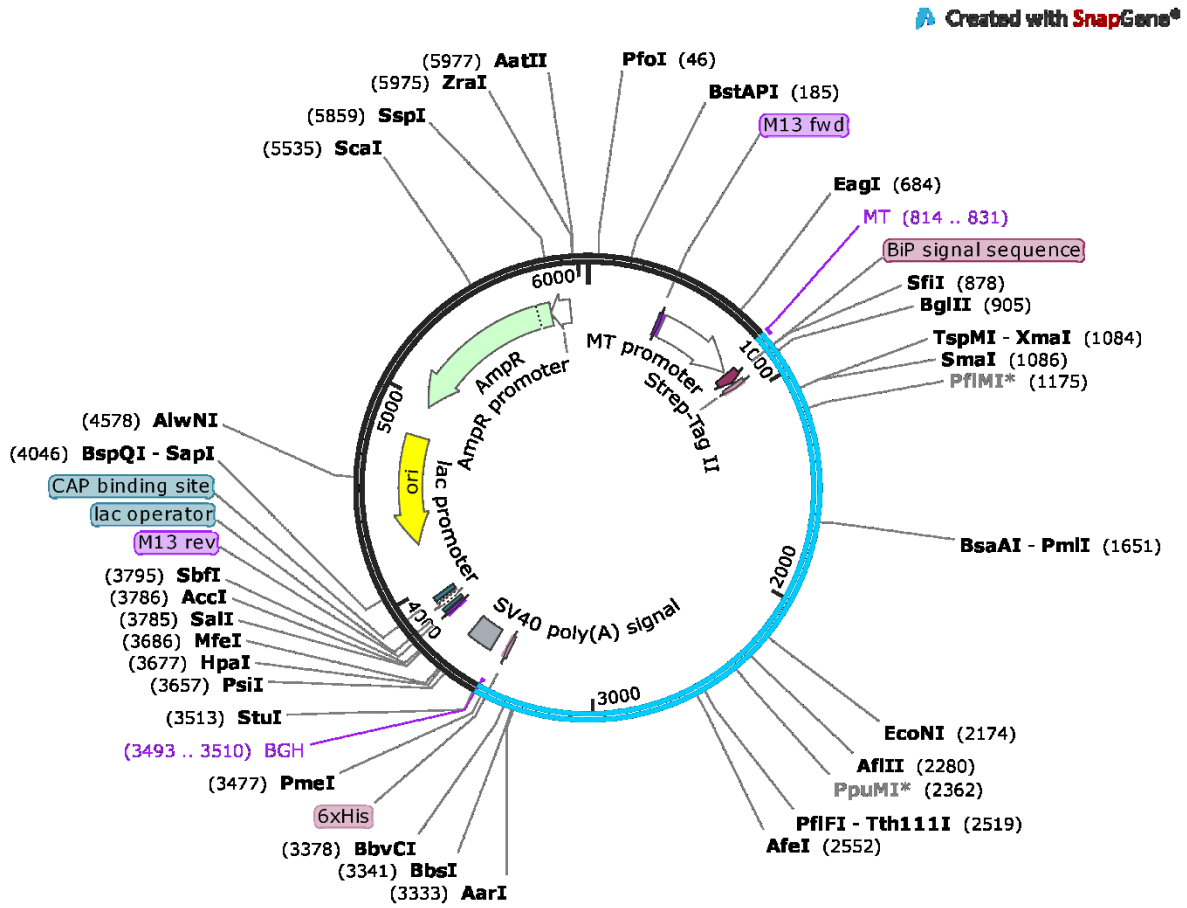


Fig. 37. **Map of *GRIN2B*-2xStrep tag-pMT BiP V5-His* plasmid**; MT Forward and BGH Reverse primers (violet) highlighted.

7.4.2 Verification of the expression of functional NMDARs in Schneider's Drosophila Line 2 cells

To verify expression of NMDA receptors in suspension Schneider's Drosophila Line 2 cells, several experiments were planned: alike primarily designed experiments for HEK293T cells, electrophysiology recording, immunochemistry and mass spectrometry analysis. Additionally, cytotoxicity test, indicating glutamate (NMDAR) mediated cell death, was performed. Due to specific characteristics of Schneider's Drosophila Line 2 cells, such as low surface adhesion and certain equipment for large scale cultivation (rotator), some of these methods are yet to be done and optimized (namely electrophysiology recording).

7.4.2.1 Cytotoxicity test

The expression of NMDA receptors in transfected Schneider's Drosophila Line 2 cells (see Fig. 38) was tested using the propidium iodide assay (cytotoxicity test). Agonists (glycine and NMDA) were added to the suspension culture, leading to the NMDAR ion channel opening. That induced cell death in cells expressing functional NMDAR enabling the propidium iodide dye to enter into dead cells.

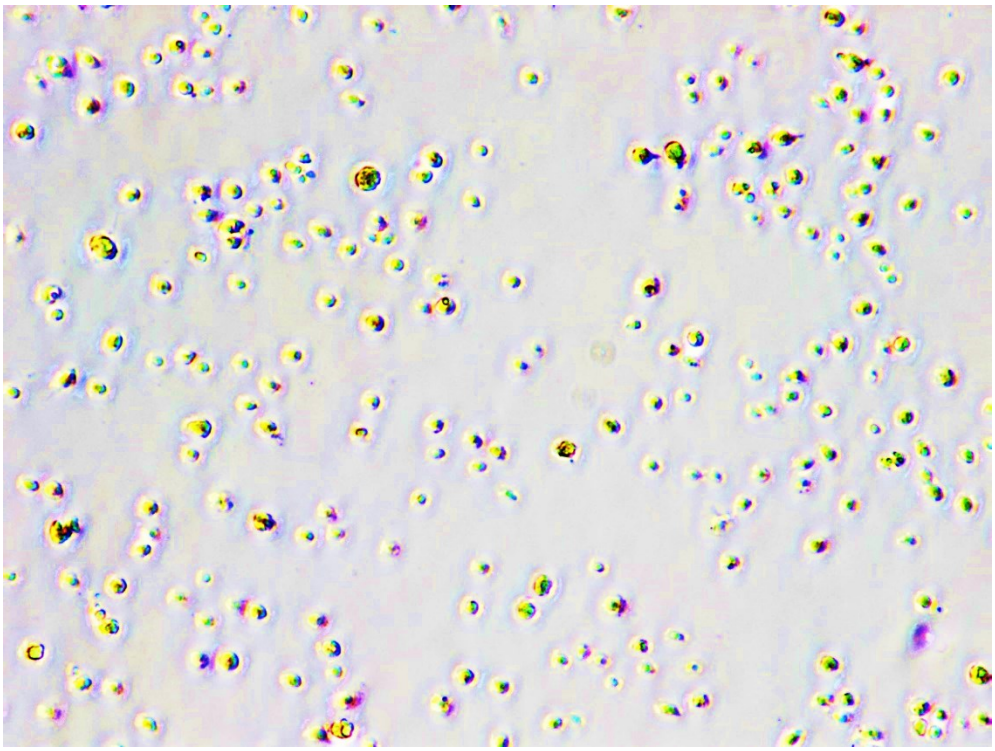


Fig. 38. **Schneider's Drosophila Line 2 cells** in bright-light field (100x magnified; DMi8 microscope (Leica)).

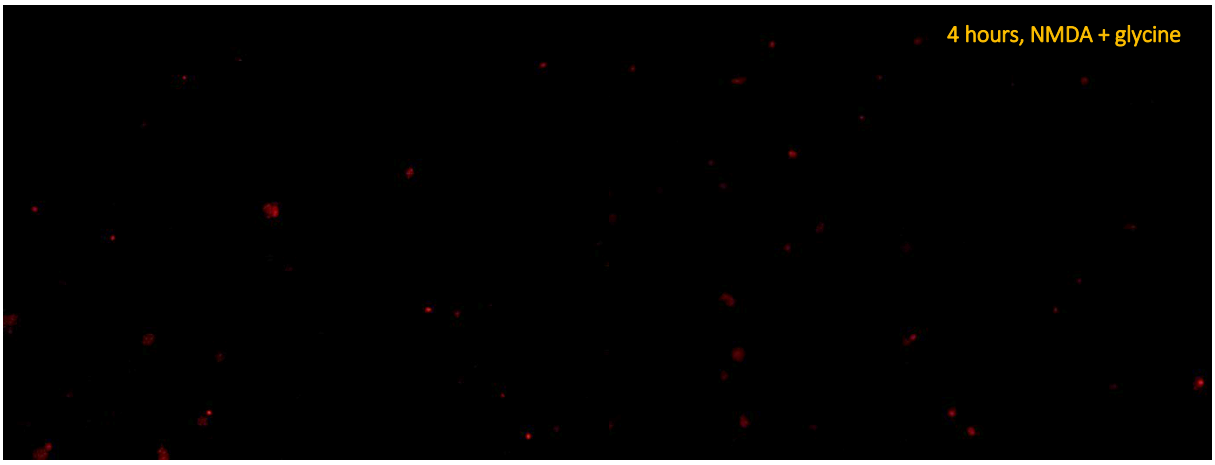


Fig. 39. **Schneider's Drosophila Line 2 cells – non-tranfected**; in red-light field (100x magnified; DMi8 microscope (Leica)).

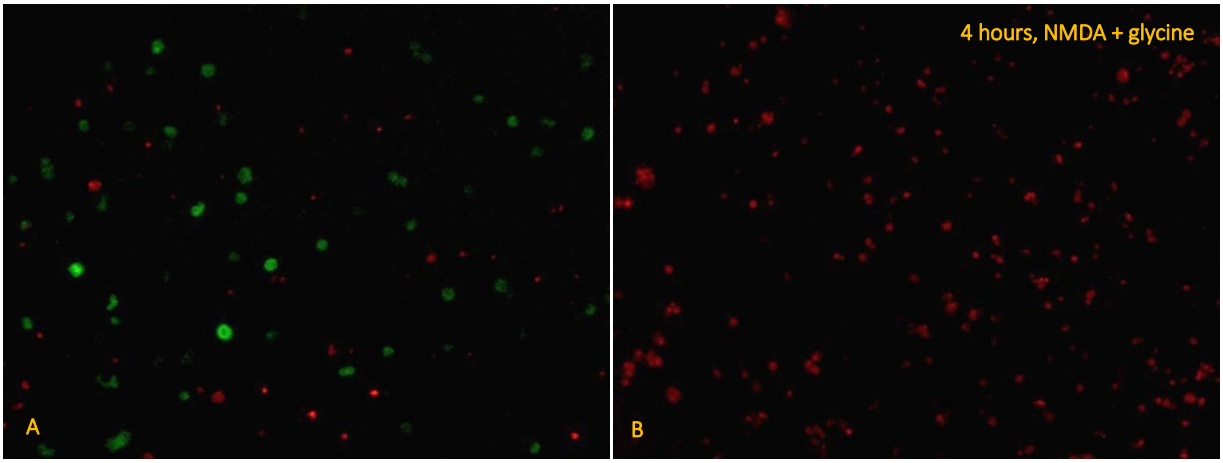


Fig. 40. **Schneider's Drosophila Line 2 cells**: **A** – transfected with **eGFP**; **B** – transfected with **GluN1/GluN2B** (100x magnified; DMi8 microscope (Leica)).

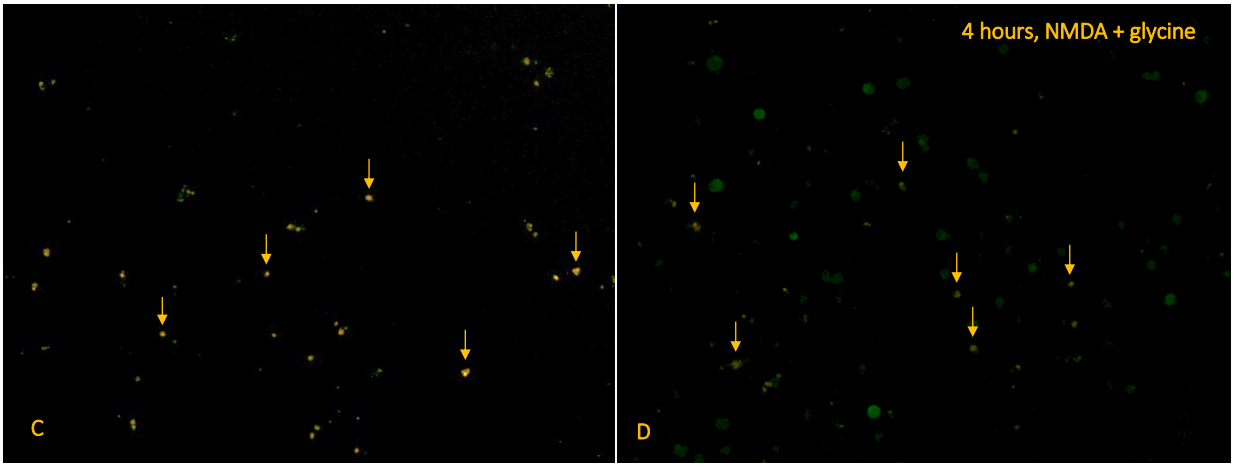


Fig. 41. **Schneider's Drosophila Line 2 cells**: **C** – transfected with **GluN1/GluN2B/eGFP**; **D** – transfected with **GluN1/GluN2B-2xStrep tag/eGFP** – indicated by yellow arrows (50x/100x magnified; DMi8 microscope (Leica)).

Figures 39-41 document the level of NMDAR/eGFP expression in Schneider's Drosophila Line 2 cells, assayed four hours after agonist were added to the media (Mg^{2+} free). Propidium iodide is able to permeate the plasmatic membrane of the dead cells, and allows to visualize them in red. As shown in Fig. 42, the number of dead cells, that were transfected with GluN1/GluN2B NMDAR, rose in time. Fig. 43 shows the percentage of dead cells, of all-positively trasfected (expressing eGFP) cells in time. Assumably these were expressing GluN1/GluN2B, or GluN1/GluN2B-Strep tag NMDAR subunits. After 4 hours only 5 percent of the cells expressing the GluN1/GluN2B-Strep tag/eGFP survived, which supports functional expression of NMDARs.

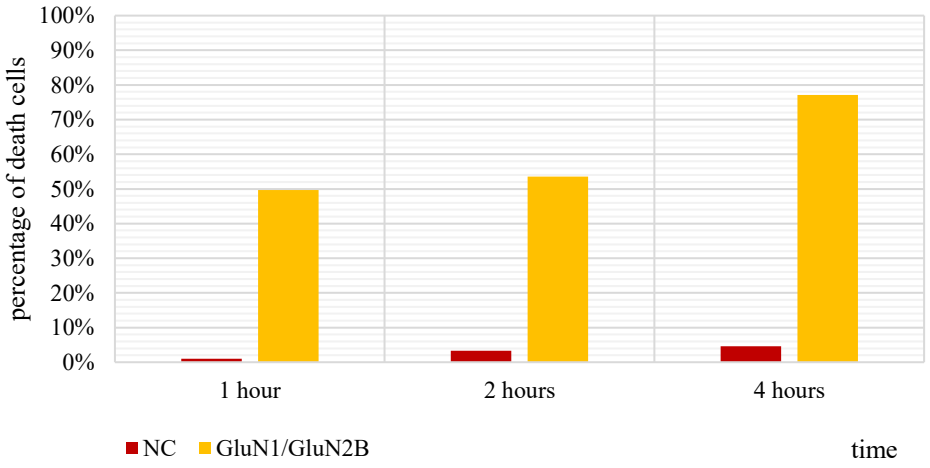


Fig. 42. **Cytotoxicyty test – GluN1/GluN2B**; the percengae of cells stained with propidium iodide (dead cells), counted in time intervals; **NC** – non-transfected Schneider's Drosophila Line 2 cells (negative control), with GluN1/GluN2B NMDAR subunits transfected Schneider's Drosophila Line 2 cells

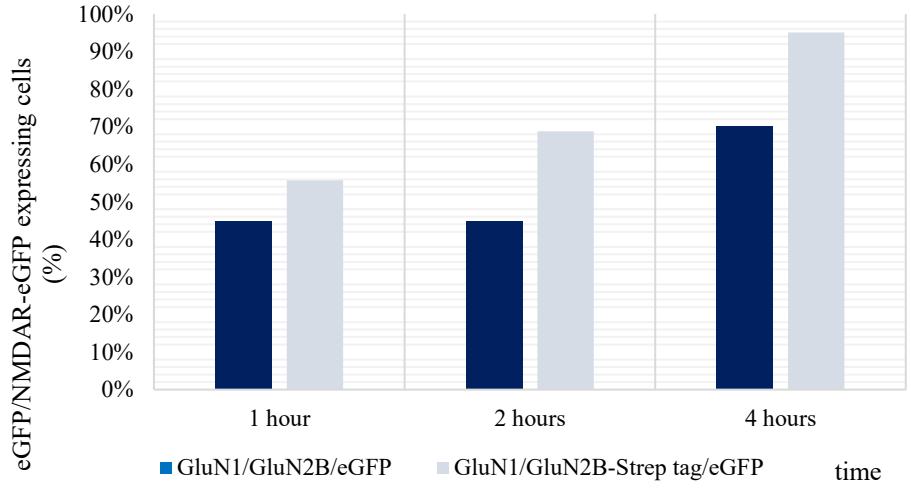


Fig. 43. **Cytotoxicyty test – GluN1/GluN2B**; percentage of propidium iodide stained (dead) cells, expressing NMDA receptor subunits (GluN1/GluN2B; GluN1/GluN2B-Strep tag), from the cells of all-successfuly transfected cells (expressing eGFP).

7.4.3 Mass spectrometry

The pilot measurement and identification of proteins collected from Schneider's Drosophila Line 2 cells on mass spectrometer was performed. Cells transfected with NMDAR subunits, grown in 25 ml suspension, were harvested and lysed (as described in 6.2.2.14.2). Lysates were separated on SDS-PAGE and protein bands were visualised with Coomassie blue stain. Bands of corresponding size to NMDAR, were excised and subjected to mass spectrometry analysis, using Bruker Daltonics 15T-Solarix XR FT-ICR mass spectrometer. Data analysis was performed by Dr. Petr. Pompach using Mascot software (Matrix Science). Unforutnately, yet no NMDA receptor subunit was identified (go to supplementary for full MS I record). The protein found in appropriate size, was identified as glycoprotein 93 (Drosophila melanogaster).

These negative results could mean, that NMDA receptors are expressed by Schneider's Drosophila Line 2 cells, but still due to low amount of cell suspension, concentration of NMDAR in lysate was under detectable limit of Bruker Daltonics 15T-Solarix XR FT-ICR mass spectrometer. Therefore, we continue to solve this problem by preparation and analysis of larger amount of suspension cells, expressing NMDA receptors.

7.5 NMDAR expression in the HEK293S GnTI- cells

Alongside with the Schneider's Drosophila Line 2 cells expression system, we tested the possibility of higher level of protein expression in genetically modified line of HEK293T cells – HEK293S GnTI- cells. Beside the protein overexpression, they do allow us to electrophysiologically assess any NMDAR subunit clones (e.g. mutations of some AA to L-Methionine or lysine before specific crosslinking experiment). HEK293S GnTI- cells feature some characteristics, which include the lack of N-acetyl-glucosaminyltransferase I (thus lack of complex N-glycans) (Reeves *et al.*, 2002).

7.5.1 Verification of the expression of NMDAR subunits in HEK293S GnTI- cells

To confirm successful expression and localization of NMDA receptors to the plasmatic membrane (trafficking), in specific line of HEK293S GnTI-, two methods were performed up-to-date – Calcium imaging assay and pilot immunochemistry with specific primary antibodies.

7.5.1.1 Ca^{2+} imaging

Calcium imaging assay was kindly performed by Dr. Jan Krůšek (Institute of Physiology), as presented in methods (see 6.2.2.12). Cells, specifically HEK293S GnTI- cells, expressing GluN1/GluN2B subunits; were positively responding to added agonists (Glycine (100 mM) + Glutamate (1 mM)) by increased level of intracellular concentration of Ca^{2+} (see Fig. 44). During measurements, cells were bathed in Mg^{2+}-containing (before and after agonist application) or Mg^{2+}-free media (for agonists application). These results indicate: 1. the successful transfection, expression and plasmatic membrane localization of NMDA receptors; 2. the functional NMDA receptor subunits, without significant alternation, when N-glycosylation is avoided.

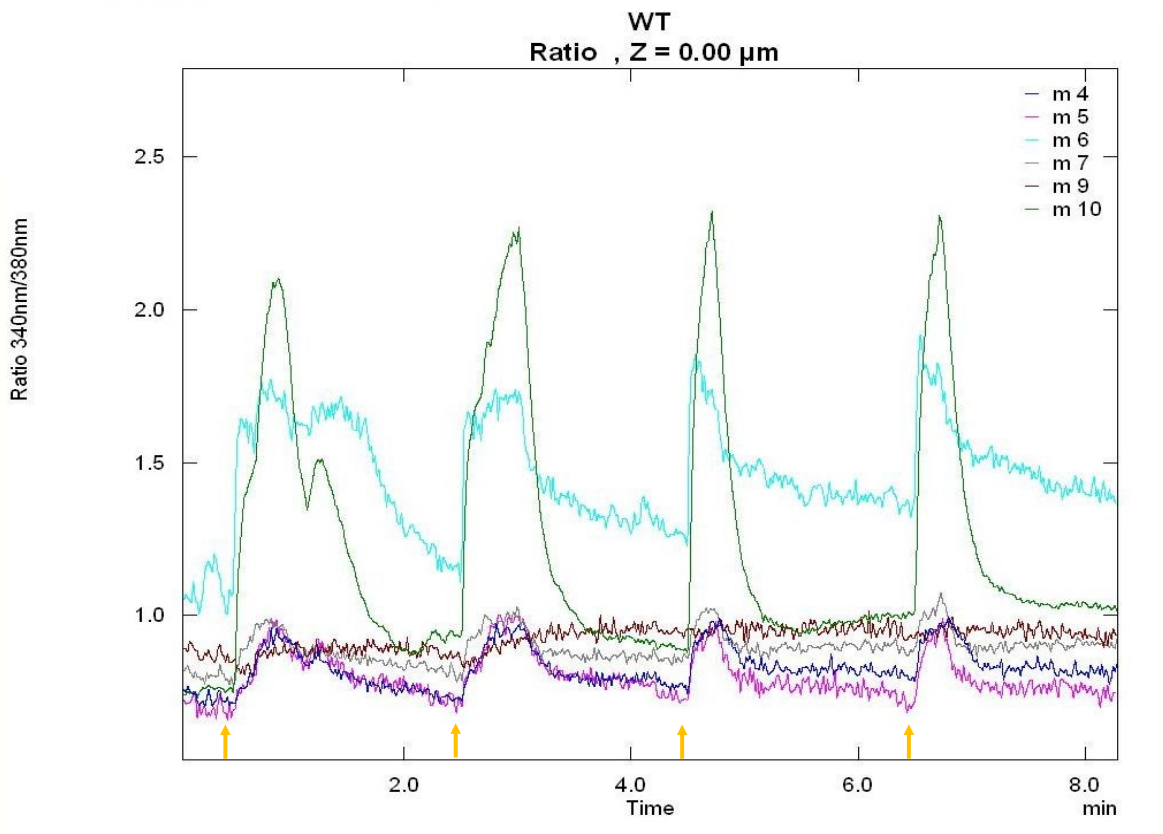


Fig. 44. **Ca^{2+} imaging respose of GluN1/GluN2B**; arrows – agonist added to the media; colored curves – single cell responses in time

7.5.2 Immunodetection of the NMDARs expression in HEK293S GnTI- cells

HEK293S GnTI- cells were transfected with cDNA encoding different subunits of NMDA receptor – GluN1-6x His tag or GluN2B. Cells were harvested, lysed and analysed on Western blot (see Fig. 45) using 6x-His Tag primary antibody (ThermoFisher Scientific™) and Anti-NR2B primary antibody (NeuroMab). More bands present in western blot, are caused by unspecifity of the primary antibody.

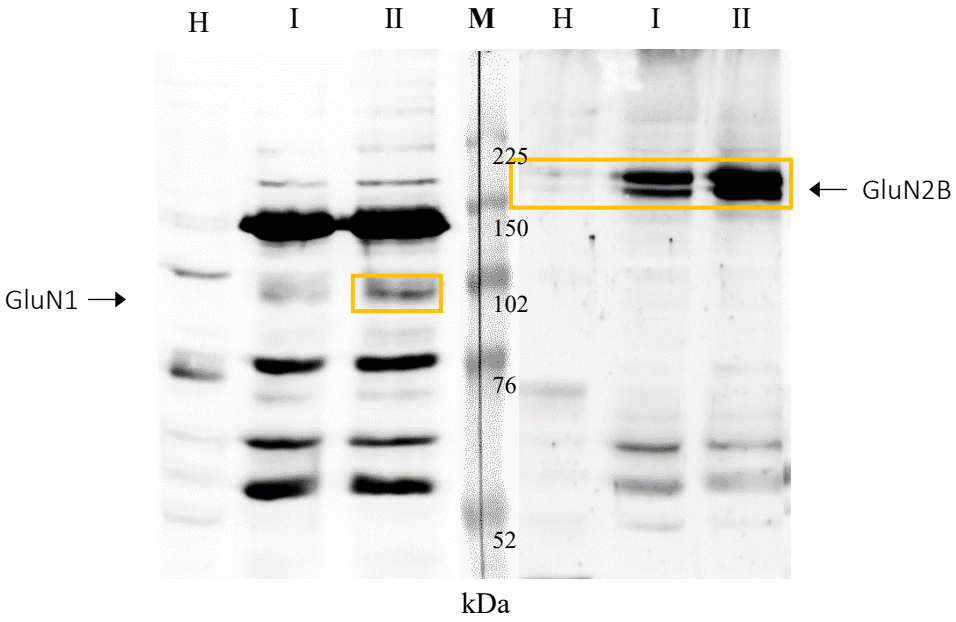


Fig. 45. **Western blot of transfected HEK293S GnTI- cell lysates**; cells expressing:
I – GluN1/GluN2B, **II** – GluN1-6xHis tag/GluN2B subunits of NMDA receptor; **H** – rat hippocampal lysate control

As shown in Fig. 45, the HEK293S GnTI- cells expressed both subunits (GluN1 and GluN2B) of NMDA receptor.

8 DISCUSSION

Ionotropic glutamate receptors present proteins, that mediate exctitatory neurotransmission within the mammalian central nervous system. Two subtypes of iGluRs – the AMPA receptor and NMDA receptor share similar features and both of them do participate in the process of memory and learning. With these the processes of long-term potentiation and long-term depression, triggered by AMPAR and NMDAR, are closely related. The altered function of the NMDAR, overactivation, leads to the excitotoxicity, therefore to the progress of neurodegenerative disorders. The hypo-activity of NMDAR is then associated with neuropsychiatric disorders. The pharmacological treatment affecting NMDAR is still accompanied by adverse effects, due to the principal role of NMDAR in the nervous sytem. Therefore, the fullfilled understanding of the receptor structure would lead to the development of new suitable drugs with less side effects. There were three main aims in the beinning of this work. To collect data about NMDA receptor structure, using computational modelling; the establishment of perspective expression system for efficient NMDAR/AMPAR expression and protein sample preparation followed by comparison of NMDAR/AMPAR at different functional states, using, for the first time, the mass spectrometry approach.

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8.1 NMDA receptor structural data and molecular modelling.

In the first part of this project I collected the available crystal structure data referring to iGluRs. Several recently-published crystal structures of the NMDA receptor, are still missing some high-resolution data. Karakas and Furukawa, 2014, obtained the first crystal structure of the whole NMDA receptor, but in order to get these data, they performed several changes in the DNA sequence (deletions, mutations, cys-bridges) for protein stabilization, which enabled successful protein crystallization. Due to this, final observation of the receptor may be a bit altered from the native state. Another successful crystallization of NMDA receptor performed by Lee *et al.*, 2014, also brought in more details of the NMDAR structure. Since new technologies became available, cryo-EM NMDAR structure shows the most-likely structure of NMDAR. In this case, the triheteromeric NMDA receptor and also structures of GluN1/GluN2B in several states resembling specific NMDAR functional status, were obtained (Tajima *et al.*, 2016). We have decided to combine three available crystal structures of NMDA receptors (*Rattus norvegicus*, *Xenopus laevis*), and using Modeller 9v14, we were able to assort structures, creating the native-like structure of the homologous NMDAR. Resulting from this

model, we have designed *in vitro* experiments, that would fully confirm our predicted modelling data. Among these drawn up experiments, the UV or chemical crosslinking of proteins belongs. For the analysis, using UV crosslinking, all L-Methionine residues were selected and further looked out for their precise localization within the receptor structure. Results showed possible L-Methionine residues in the NMDA receptor model (Fig. 19). For chemical crosslinking experiments, lysine, arginine and glutamate residues were further selected and remitted to analysis. The conditions for selection were more rigorous than with UV crosslinking. Their localization within the receptor needed to be in position accessible for chemical crosslinker – EDC for "zero length" crosslinking, that links together carboxyl groups (aspartate, glutamate) and primary amines (lysines) within distance 12 Å. Two others chemical crosslinkers – DSS and DSG connect two groups of primary amines (lysines) within distance 20 Å or 24 Å respectively. Lysines thought for this method are shown in the receptor model structure (Fig. 20). The distance between selected residues was measured with reference to the residues position. Fig. 16 and 18, show the distribution of individual amino acid residues in red, where additonal residues could also participate in chemical crosslinking, due to the high flexibility of receptor substructures (linkers between LBD and TMD). With this methodical approach, we would be able to stabilize NMDA receptor in a certain state – opened or closed, depending on the presence of agonists. These *in silico* findings lay the solid ground for planned – *in vitro* experiments.

8.2 Expression system of NMDA receptors establishment and receptor presence confirmation

Pilot experiments were proceeded using HEK293T cell expression system, which, at first, seemed as sufficient for protein expression and more then suitable for electrophysiological confirmation of the NMDAR expression, as well as for planned UV or chemical crosslinking. HEK293T cell expression system is also long-term used in our laboratory. We were able to express NMDAR subunits and confirm the protein expression with immunochemical and electrophysiological methods. Moreover, the protein purification via 6xHis tag, added to the N-terminal domain of the receptor was succesfuly performed. In parallel, the expression of the receptor with L-Methionine, substituted by photo-L-Methionine, usable for UV crosslinking, was performed and confirmed. These results led to the designed mass spectrometry analysis. Despite of the effort, there was not enough of NMDAR subunits to be detected using the Bruker Daltonics 15T-Solarix XR mass spectrometer. However, the wide range mass spectrometer, analysing the whole cell lysate, identified the GluN2B subunit and eGFP protein in the sample.

Unfortunately, no GluN1 subunit, but considering other experimental results, the GluN1 subunit was expressed in lesser amount, because of unknown reason.

Therefore, we have decided, for the expression system change, in order to obain sufficient amount of expressed NMDAR subunits. According to several advantages, including the production of proteins in native conformation and high levels of cell densities without toxic metabolite issues, we chose the Schneider's Drosophila Line 2 cell expression system. Two native NMDAR subunits – the GluN1 and GluN2B were, despite of some difficulties, succesfuly cloned into suitable vector – pMT BiP V5-His*, and also one of two tagged subunits – the GluN2B-Strep tag (with 2xStrep tag on the N-terminal domain of the subunit). The 6xHis tagged GluN1 subunit is still to be cloned into the pMT BiP V5-His* vector. To achieve this, the process of cloning must be further optimized, using more different approaches, such as NMDAR subunits pre-cloning step into the pGem®-T Easy Vector. With these GluN1/GluN2B pGem®-T Easy Vector prepared plasmids, we performed the Schneider's Drosophila Line 2 cell transfection. The expression of functional NMDAR was confirmed by Cytotoxicity assay. Even though, the immunochemistry and electrophysiology have to be optimized. Some features of Schneider's Drosophila Line 2 cells – small size, the non-adhesivity to the surface, do impede these methods. The mass spectrometry, which should follow, still requires higher level of the NMDAR expression/expressing cells. Simultaneously, we also tried to exploit the HEK293S GnTi- cell expression system, which features of the lack of N-acetyl-glucosaminyltransferase I, that leads to higher protein expression. Some NMDAR protein samples for crystallography experiments were successfully produced in the HEK293S GnTi- cell lines (Goehring *et al.*, 2014; Lee *et al.*, 2014). Currently performed experiments on HEK293S GnTi- cells seem promising and could be, in parallel, applied for the NMDAR subunits expression. Beside the more accessible electrophysiology (HEK293S GnTi- cells are adhesive cell culture), the pilot positive immunochemistry results show up successful expression of NMDAR. As well as with the Schneider's Drosophila Line 2 cell expression system, we plan to further continue with HEK293S GnTi- cell expression system and hopefuly, obtain first results from mass spectrometry analysis. But for that, the optimalization of the protein expression in larger scale needs to be performed. Subsequently, the AMPA receptor expression and verification is to be done. AMPA receptors do feature several structural similarities to the NMDA receptors, and their expression could be easier, thanks to the possibility of the expression of the homomeric (one-subunit composition) receptor. This could be also one of the explanations of low NMDAR expression in HEK293T cell expression system. The composition of NMDAR needs to be of

two different subunits, forming tetramer. Therefore, the expression and following cell membrane localisation and formation of the transmembrane receptor, could be more demanding. Moreover, the cell culture media contains NMDAR agonists, leading to the ion-channel opening and increase of the intracellular level of Ca^{2+} even to cell death. Hence specific NMDAR inhibitors (D-AP5, $MgCl_2$ and ketamine) must be added to the cell culture media.

When the ample amount of subunits expression will be achieved, the third of our aims could be fulfilled – the stabilization of NMDAR in certain functional state using UV and/or chemical crosslinking in native composition (without DNA modifications in construct for expression, that needed to be performed for protein crystallography) and sequent comparison will give us more details on receptor gating.

9 SUMMARY

The NMDA receptors are crucial in glutamatergic excitatory neurotransmission. Revealing their structure would bring in new aspects on the receptor function. Up-to-date available structures presented a great shift, but some details are still missing. In order to fill these gaps, we designed pathways, leading towards the expression of NMDA receptors and their further structural analysis with mass spectrometry. One fundamental part of this project was in-silico analysis, using the molecular model of the NMDAR.

Firstly, we prepared NMDA receptor model from available crystal structure data and employed it in consequent experiments, where we analysed the presence of a functional properties of L-Methionines in context of the planned UV-crosslinking experiment. Similar analysis was performed for prospective chemical crosslinking experiments, with the „zero-lengh“ cross-linker 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC), which couples carboxyles to amines (within 12Å distance); and disuccinimidyl suberate and disuccinimidyl glutarate (DSS and DSG), which both couple two amino groups (20Å/24Å). Lysines, glutamates or aspartates accessible for chemical crosslinking were selected and the distance was measured in our homology models. According to these obtained results, further experiments will be designed.

In parallel, we use several approaches to establish appropriate expression system for iGluRs, especially GluN1/GluN2B heteromeric NMDAR. Pilot experiments with HEK293T cells were performed, and all results were in line with our expectations. However, mass spectrometry analyzis was yet not successful.

We assumed, that we are facing to the problem of low amount of NMDA receptor expressed. Therefore, Schneider’s Drosophila Line 2 cells were chosen as a new expression system used in other laboratories. Cloning of two NMDA receptor subunits – GluN1 and GluN2B (*Rattus norvegicus*) was draught and optimized within the process for successful cloning into pMT BiP V5-His* vector and one of them – GluN2B was additionally 2xStrep tagged. Identically to the cDNA encoding gene for NMDAR subunits used for HEK293T cells transfection, NMDAR subunit cDNAs were designed with truncated CTD, and that did not affect the protein expression neither trafficking to the membrane. We were able to verify the functional protein expression with cytotoxicity assay in Schneider’s Drosophila Line 2 cells, but yet not with immunochemistry nor mass spectrometry analysis.

Simultaneously, another cell type – HEK293S GnTI- cells, lacking of N-acetyl-glucosaminyltransferase I, suitable for mammalian protein expression in larger scale was used to overcome a potential problem with the level of protein expression and electrophysiological recording. The primary results obtained from immunochemistry or functional analysis using calcium imaging, are promising, thus we are going to express NMDAR in large scale cell cultures, for protein purification and consequent mass spectrometry analysis.

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11 SUPPLEMENTARY

pMT BiP V5-His* – MSC editing underlined

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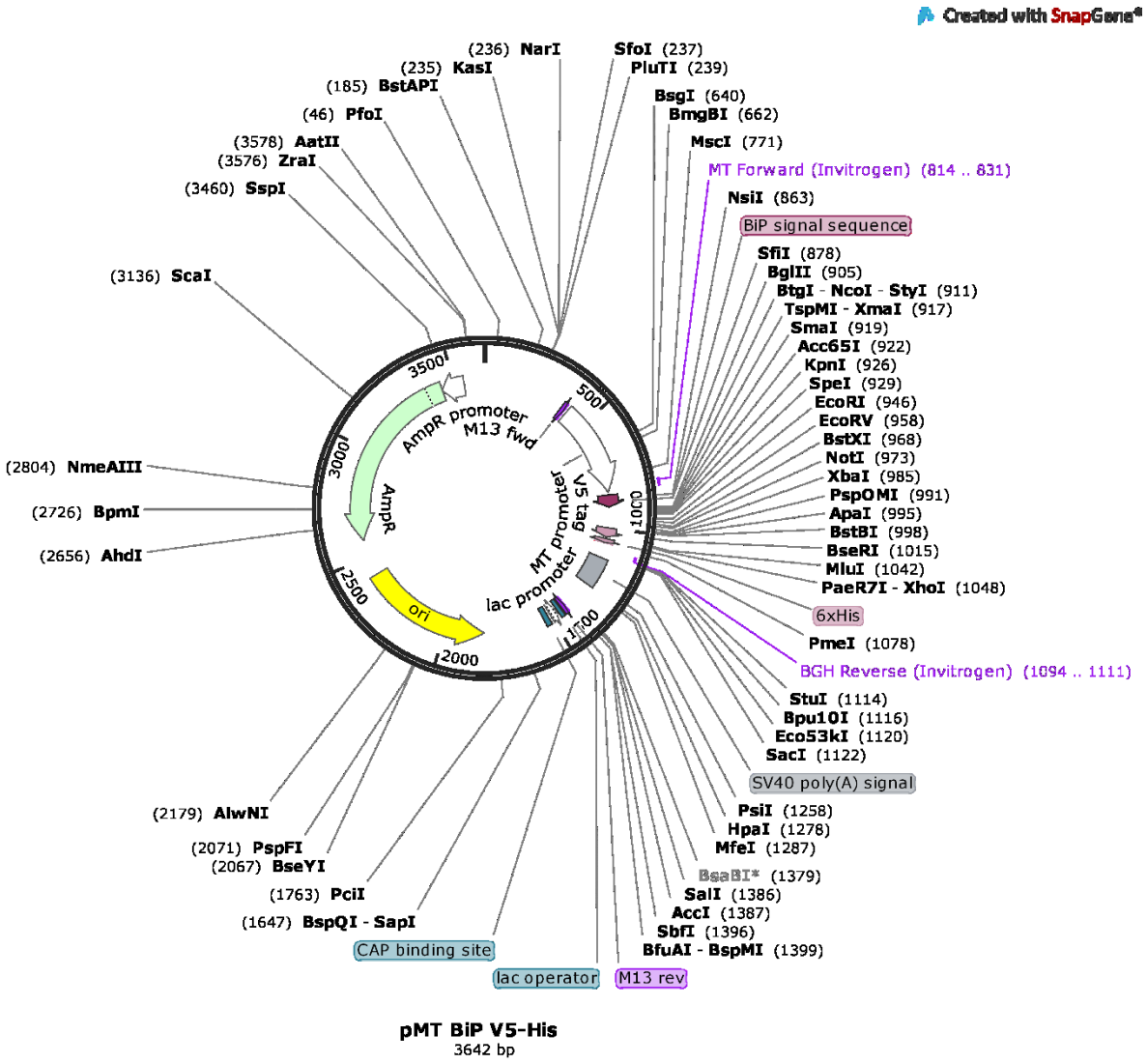
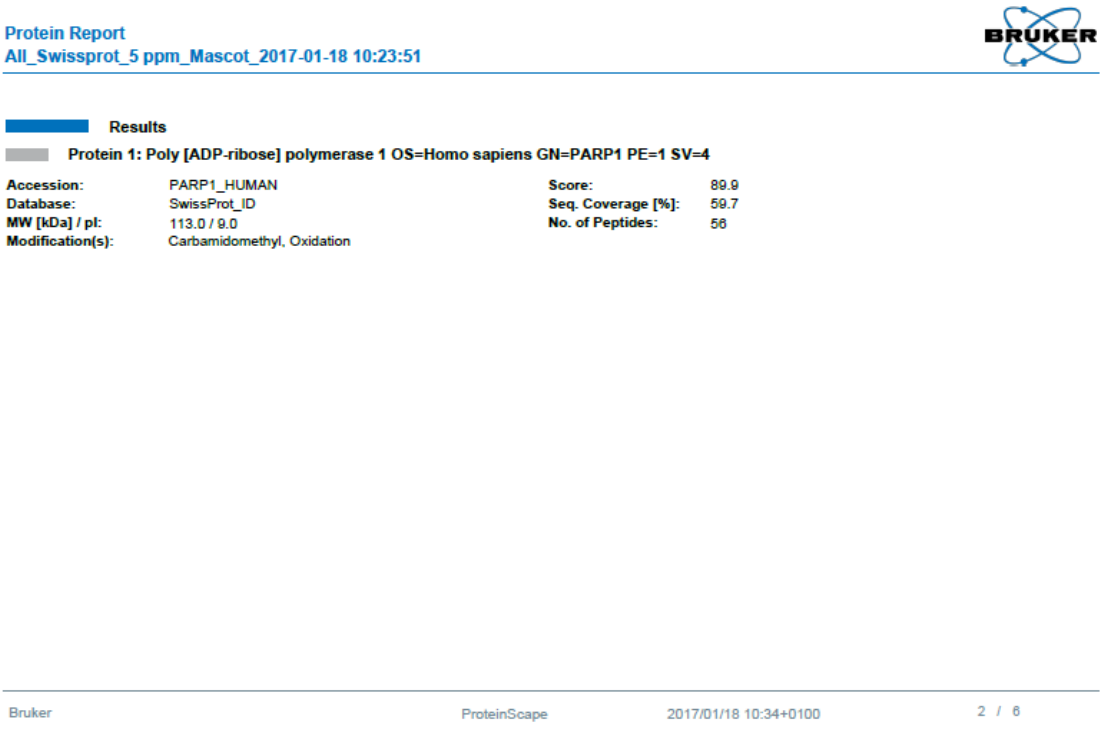


pMT BiP V5-His
3642 bp

MASS SPECTROMETRY RECORDS:

- **MS I** (HEK293T)



Protein Report
All_Swissprot_5 ppm_Mascot_2017-01-18 10:23:51

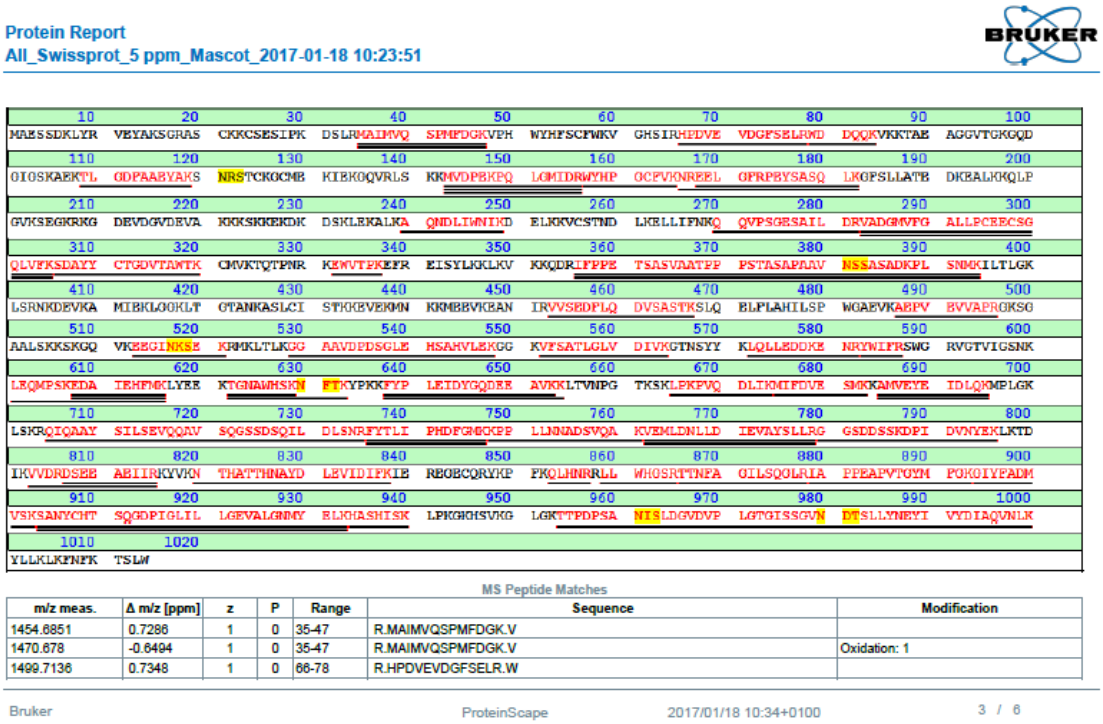
BRUKER

Results

Protein 1: Poly [ADP-ribose] polymerase 1 OS=Homo sapiens GN=PARP1 PE=1 SV=4

Accession:	PARP1_HUMAN	Score:	89.9
Database:	SwissProt_ID	Seq. Coverage [%]:	59.7
MW [kDa] / pI:	113.0 / 9.0	No. of Peptides:	56
Modification(s):	Carbamidomethyl, Oxidation		

Bruker ProteinScape 2017/01/18 10:34+0100 2 / 6



Protein Report
All_Swissprot_5 ppm_Mascot_2017-01-18 10:23:51

BRUKER

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MAESSDKLYR	VEYAKSGRAS	CKKCSESIPK	DSLRMAIMVQ	SPMFDGKVPH	WYHFSCFWKV	GHSIRHPDVE	VDGFSELPWD	DQQKVKKTAE	AGGVTGKGQD
110	120	130	140	150	160	170	180	190	200
GIQSKAEKTL	GDPAAEVAKS	NRSTCKGCME	KIEKQQVRLS	KKMVDPEKPQ	LGMIDRWYHP	GCFVKNREEL	GFRPEYSASQ	LKGFSLLATE	DKEALKKQLP
210	220	230	240	250	260	270	280	290	300
GVKSEGKRKG	DEVDGVDEVA	KKKSKKEKDK	DSKLEKALKA	QNDLIWNIKD	ELKKVCSTND	LKELLIFNKQ	QVPSGESAIL	DRVADGMVFG	ALLPCEECSG
310	320	330	340	350	360	370	380	390	400
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410	420	430	440	450	460	470	480	490	500
LSRNKDEVKA	MIEKLGGKLT	GTANKASLCI	STKKEVEKMN	KKMEEVKEAN	IRVVSEDFLQ	DVSASTKSLQ	ELFLAHILSP	WGAEVKAEPV	EVVAPRGKSG
510	520	530	540	550	560	570	580	590	600
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610	620	630	640	650	660	670	680	690	700
LEQMPSKEDA	IEHFMKLYEE	KTGNAWHSKN	FTKYPKKFYP	LEIDYGQDEE	AVKKLTVNPG	TKSKLPKPVQ	DLIKMIFDVE	SMKKGAMVEYE	IDLQKMPLGK
710	720	730	740	750	760	770	780	790	800
LSKRQIQAAY	SILSEVQQAV	SQGSSDSQIL	DLSNRFYTLI	PHDFGMKKPP	LLNNADSVQA	KVEMLDNLLD	IEVAYSLLRG	GSDDSSKDPI	DVNYEKLKTD
810	820	830	840	850	860	870	880	890	900
IKVVDRDSEE	AEIIRKYVKN	THATTHNAYD	LEVIDIFKIE	REGECQRYKP	FKQLHNRRLL	WHGSRTTNFA	GILSQGLRIA	PPEAPVTGYM	FGKQIYFADM
910	920	930	940	950	960	970	980	990	1000
VSKSANYCHT	SQGDPIGLIL	LGEVALGNMY	ELKHASHISK	LPKGKHSVKG	LGKTTPDPSA	NISLDGVDVP	LGTGISSGVN	DTSLLYNEYI	VYDIAQVNLK
1010	1020								
YLLKLKFNFK	TSLW								

MS Peptide Matches

m/z meas.	Δ m/z [ppm]	z	P	Range	Sequence	Modification
1454.6851	0.7286	1	0	35-47	R.MAIMVQSPMFDGK.V	
1470.678	-0.6494	1	0	35-47	R.MAIMVQSPMFDGK.V	Oxidation: 1
1499.7136	0.7348	1	0	66-78	R.HPDVEVDGFSELR.W	

Bruker ProteinScape 2017/01/18 10:34+0100 3 / 6

Results

Protein 1: Lon protease homolog, mitochondrial OS=Homo sapiens GN=LONP1 PE=1 SV=2

Accession:	LONM_HUMAN	**Score:**	41.9
Database:	SwissProt_ID	**Seq. Coverage [%]:**	53.2
MW [kDa] / pI:	106.4 / 6.0	**No. of Peptides:**	43
Modification(s):	Carbamidomethyl, Oxidation		


```
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       110        120        130        140        150        160        170        180        190        200
AGAGEGEVIT ALTPMTIPDV FPHLPLIAIT RNPVFPRFIK IIEVKNKKLV ELLRRKVRLA QPYVGVFLKR DDSNESDVVE SLDEIYHTGT FAQIHEMQDL
       210        220        230        240        250        260        270        280        290        300
GDKLRMIVMG HRRVHISRQL EVEPEEPEAE NKHKPRRKSK RGKKEAEDEL SARHPAELAM EPTPELPAEV LMVEVENVVH EDFQVTEEVK ALTAEIVKTI
       310        320        330        340        350        360        370        380        390        400
RDIIALNPLY RESVLQMMQA GQRVVDNPIY LSDMGAALTG AESHELQDVL EETNIPKRLY KALSLLKKEF ELSKLQQRLG REVEEKIKQT HRKYLLQEQL
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KIIKKELGLE KDDKDAIEEK FRERLKELVV PKHVMDVVDE ELSKLGLLDN HSSEFNVTRN YLDWLTSIPW GKYSNENLDL ARAQAVLEED HYGMEDVKKR
       510        520        530        540        550        560        570        580        590        600
ILEFIAVSQL RGSTQGKILC FYGPPGVGKT SIARSIARAL NREYFRFSVG GMTDVAEIKG HRRTYVGAMP GKIIQCLKKT KTENPLILID EVDKIGRGYQ
       610        620        630        640        650        660        670        680        690        700
GDPSSALLEL LDPEQNANFL DHYLDVPVDL SKVLFICTAN VTDTIPEPLR DRMEMINVSG YVAQEKLAIA ERYLVPQARA LCGLDESKAK LSSDVLTLII
       710        720        730        740        750        760        770        780        790        800
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       810        820        830        840        850        860        870        880        890        900
EVTGQLGEVM KESARIAYTF ARAFLMQHAP ANDYLVTSHI HLHVPEGATP KDGPSAGCTI VTALLSLAMG RPVRQNLAMT GEVSLTGKIL PVGGIKEKTI
       910        920        930        940        950        960
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MS Peptide Matches

m/z meas.	Δ m/z [ppm]	z	P	Range	Sequence	Modification
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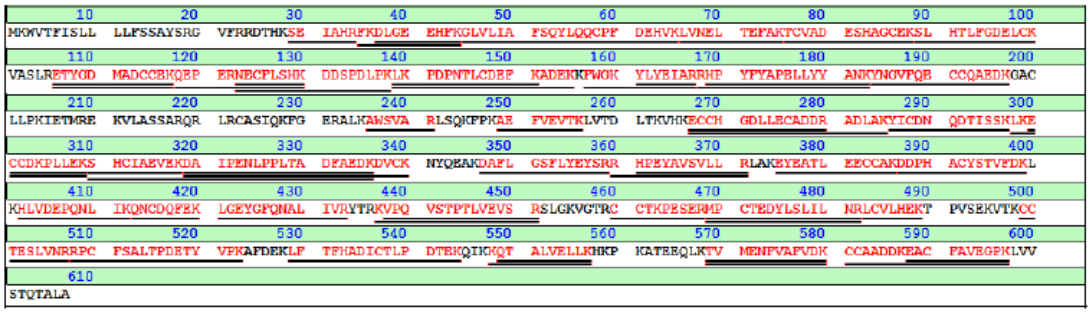
3893.7427	0.5275	1	1	170-203	K.RDDSNESDVVESLDEIYHTGTFAQIHEMQDLGDK.L	
3737.636	-0.9458	1	0	171-203	R.DDSNESDVVESLDEIYHTGTFAQIHEMQDLGDK.L	
875.4223	-0.3482	1	0	206-212	R.MIVMGHR.R	Oxidation: 1, 4
767.4634	-0.1060	1	1	213-218	R.RVHISR.Q	

Results

Protein 1: Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4

Accession:	ALBU_BOVIN	**Score:**	303.0
Database:	SwissProt_ID	**Seq. Coverage [%]:**	75.5
MW [kDa] / pI:	69.2 / 5.8	**No. of Peptides:**	53
Modification(s):	Carbamidomethyl, Oxidation		



MS Peptide Matches

m/z meas.	Δ m/z [ppm]	z	P	Range	Sequence	Modification
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974.4577	-0.08914	1	0	37-44	K.DLGEEHFK.G	

- **MS II** (HEK293T)

MS II record (totally 5861 proteins/protein fragments detected)

- First row – positively identified **GFP protein**
- Second row – positively identified **GluN2B subunit**

12	6	3	1	C: Only identified by site	C: Reverse	C: Potential contaminant
NaN	NaN	25,3297	NaN			+
NaN	NaN	NaN	23,6838			

N: Peptides	N: Razor + unique peptides	N: Unique peptides	N: Sequence coverage [%]	N: Unique + razor sequence coverage [%]	N: Unique sequence coverage [%]	N: Mol. weight [kDa]
4	4	4	32,5	32,5	32,5	28,106
2	2	2	2,6	2,6	2,6	166,03

N: Q-value	N: Score	N: Intensity	N: MS/MS count	N: Intensity 12	N: Intensity 6	N: Intensity 3
0	33,128	45429000	4	0	0	45429000
0,000405	3,8407	34287000	4	6700200	13793000	318220

N: Intensity 1	N: MS/MS count 12	N: MS/MS count 6	N: MS/MS count 3	N: MS/MS count 1	N: Identification type 12	N: Identification type 6
0	0	0	4	0	NaN	NaN
13475000	0	1	0	3	NaN	NaN

N: Identification type 3	N: Identification type 1	N: Razor + unique peptides 12	N: Razor + unique peptides 6	N: Razor + unique peptides 3
NaN	NaN	0	0	4
NaN	NaN	1	2	1

N: Razor + unique peptides 1	N: Peptides 12	N: Peptides 6	N: Peptides 3	N: Peptides 1
0	0	0	4	0
2	1	2	1	2

T: Protein IDs	T: Majority protein IDs	T: Protein names	T: Gene names	T: Fasta headers
CON__Q9U6Y5;balik_gfp	CON__Q9U6Y5;balik_gfp			;
balik_NR2BNP_036706;Q13224	balik_NR2BNP_036706;Q13224	Glutamate receptor ionotropic, NMDA 2B	GRIN2B	;Glutamate receptor ionotropic, NMDA 2B OS

- **MS I** (Schneider's Drosophila Line 2)

MASCOT Search Results

Protein View: NP_651601.1

glycoprotein 93 [Drosophila melanogaster]

Database:	NCBIprot
Score:	83
Expect:	0.71
Monoisotopic mass (M_r):	90296
Calculated pI:	4.93
Taxonomy:	Drosophila melanogaster

This protein sequence matches the following other entries:

- AAF56765.1 from Drosophila melanogaster
- AAL68222.1 from Drosophila melanogaster
- ACL90853.1 from synthetic construct
- AGQ06282.1 from synthetic construct

Sequence similarity is available as an NCBI BLAST search of NP_651601.1 against nr.

Search parameters

Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Variable modifications:	Acetyl (Protein N-term), Oxidation (M)
Mass values searched:	263
Mass values matched:	30

Protein sequence coverage: 44%

Matched peptides shown in ***bold red***.

```
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101 KKLIGNASDA IDKIRLLALS NSKELETNVE LKIKIKADKK NKALSIMDSS
151 IGMTHQDLIN NLNTIAKSGT ADFLAKMQDP SKEESLDMKD MISQFGVGFT
201 SAFLVADKVV VTTKHNDDKQ TIMEKDANSF SITEDFKSGT LKRGSVISLY
251 LKEEAQDFLE EDTVKELIKK TSQFINFVIK MNSSKTVEEE VPVEEEAEYE
301 KSEDVEEED AEVEEAEDEK PKTEKVSKTT WDWTLINDSK PIWTRKPAEV
351 TEDEYTKFYK SLTKDSSEPL TQTHFIAEGE VTFKSLLYVP KVQPSESYNK
401 YGTKSDNIKL YVRRVFITDE FNDMMPNYLS FIKGVVDSED LPLNVSRETL
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501 RSRLAKLLRF QTSNGKGVTS LAEYKERMKA KQEHIYYIAG ASRAEVEKSP
551 FVERLLKKGY EVLYLVEAVD EYCIQALPEF DGKKFQNVAK EGFQLNESEK
601 SKKNFESLKS TYKPLVKWLN DVALKDQISK AQVSERLSNS PCALVASVFG
651 WTGNMERLAM SNAHQKSDDP QRTYYLNQKK TLEINPRHPL MKELLRRVEA
701 DEADDTAKDM AVMMFRTATL RSGYMLQETS QFADSIEQMM RQTLGVSQDE
751 QVKPDKKEED DAKETATGHQ EDGNADDEKE EQQHDEL
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Unformatted sequence string: 787 residues (for pasting into other applications).

Sort by ◉ residue number ○ increasing mass ○ decreasing mass
Show ◉ matched peptides only ○ predicted peptides also

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
50 - 66	1926.0302	1925.0229	1925.0218	0.59	1	K.KEEEAIQLDGLNVAQLK.E
51 - 66	1769.9280	1768.9208	1768.9207	0.048	0	K.EEEAIQLDGLNVAQLK.E
75 - 83	1141.5649	1140.5576	1140.5564	1.09	0	K.FTFQTKVNK.G
75 - 86	1547.7397	1546.7325	1546.7272	3.39	1	K.FTFQTKVNKGMK.L + Oxidation (M)
124 - 134	1350.7026	1349.6954	1349.6939	1.09	0	K.ELETNVELKIK.I
143 - 167	2663.3719	2662.3646	2662.3571	2.83	0	K.ALSIMDSSIGMTHQDLINNLNTIAK.S
143 - 167	2679.3651	2678.3578	2678.3520	2.17	0	K.ALSIMDSSIGMTHQDLINNLNTIAK.S + Oxidation (M)
143 - 167	2695.3581	2694.3508	2694.3469	1.46	0	K.ALSIMDSSIGMTHQDLINNLNTIAK.S + 2 Oxidation (M)
183 - 208	2839.3061	2838.2988	2838.2993	-0.17	0	K.EESLDMKDMISQFGVGFTSAFLVADK.V
220 - 237	2157.9747	2156.9674	2156.9651	1.08	0	K.QTIMEKDANSFSITEDFK.S
253 - 265	1580.7085	1579.7012	1579.7002	0.65	0	K.EEAQDFLEEDTVK.E
270 - 280	1412.7698	1411.7625	1411.7612	0.93	1	K.KTSQFINFVIK.M
271 - 280	1284.6747	1283.6674	1283.6662	0.91	0	K.TSQFINFVIK.M

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
286 - 301	1841.9015	1840.8943	1840.8942	0.050	0	K.TVEEEVPVEEEAAQVEK.S
302 - 322	2406.0318	2405.0245	2405.0241	0.16	1	K.SEDDVEDEDAEVEEAEDEKPE.T
346 - 360	1806.8429	1805.8356	1805.8359	-0.16	0	K.EEAEVTEDEYTTSFTK.S
365 - 384	2236.0797	2235.0724	2235.0696	1.27	0	K.DSSEELTQTNFIAESEVTYK.S
392 - 400	1063.5177	1062.5104	1062.5094	0.97	0	K.VQFSEEFNK.T
415 - 433	2384.0980	2383.0908	2383.0865	1.81	0	K.VFITTDEFMDMMGPNTLAFIR.S + 2 Oxidation (M)
434 - 447	1485.7558	1484.7486	1484.7471	0.98	0	R.GVVDSEGLPLNVEK.E
492 - 503	1360.6671	1359.6598	1359.6565	2.44	1	K.LGVMEDPEEKGR.L
530 - 543	1633.8450	1632.8377	1632.8372	0.30	1	K.AEQEKITTIAGANR.A
532 - 543	1434.7142	1433.7070	1433.7051	1.28	0	K.QEKITTIAGANR.A
559 - 583	2879.3730	2878.3657	2878.3623	1.20	0	K.GTEVLTLVEAVDEYCIAALPEPDGK.E
637 - 657	2282.0679	2281.0606	2281.0620	-0.61	0	K.LSSSPCALVAAPVPGNTGSMEK.L + Oxidation (M)
697 - 708	1319.6088	1318.6015	1318.6001	1.12	1	K.NVEAIEKADTAE.D
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722 - 741	2368.0285	2367.0212	2367.0181	1.32	0	K.SGTMLQETSQFADSIEQMMK.Q + Oxidation (M)
722 - 741	2384.0194	2383.0121	2383.0130	-0.39	0	K.SGTMLQETSQFADSIEQMMK.Q + 2 Oxidation (M)
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No match to: 590.3197, 737.0486, 740.7239, 746.3926, 842.5095, 870.5413, 1348.6645, 1357.7193, 1381.7450, 1394.7072, 1404.7272, 1417.6873, 1423.6802, 1452.8176, 1460.8227, 1474.7346, 1479.7123, 1480.7557, 1487.7029, 1493.7363, 1494.7964, 1499.6772, 1513.7871, 1515.7501, 1516.7031, 1523.7837, 1526.7492, 1527.7454, 1529.7328, 1537.6407, 1549.8404, 1550.7019, 1552.7757, 1553.7395, 1569.6316, 1572.8499, 1593.8133, 1596.8171, 1599.8194, 1600.9179, 1601.8227, 1606.7947, 1608.8510, 1610.7705, 1616.8765, 1619.8531, 1630.8324, 1631.8351, 1636.8295, 1638.8617, 1639.9401, 1646.8252, 1655.7875, 1659.8959, 1670.8983, 1686.7874, 1687.8902, 1691.7274, 1691.8272, 1706.8816, 1707.7716, 1710.8592, 1718.8629, 1743.9030, 1749.8671, 1750.7442, 1756.9219, 1759.8844, 1763.8009, 1766.9283, 1766.7822, 1773.9517, 1778.7828, 1779.9067, 1779.9898, 1780.8005, 1784.9981, 1790.8934, 1791.7283, 1792.7978, 1794.8162, 1799.8968, 1808.7930, 1810.8077, 1816.7973, 1816.9704, 1818.9187, 1820.8788, 1821.8873, 1824.9522, 1825.9939, 1826.8066, 1832.7675, 1833.7827, 1842.7950, 1844.7945, 1848.7866, 1850.1347, 1851.9282, 1856.0527, 1860.9369, 1861.8609, 1868.8881, 1870.0652, 1878.8419, 1880.9214, 1887.9738, 1902.0476, 1907.9007, 1920.0813, 1922.9265, 1940.9365, 1954.0658, 1958.9820, 1960.9133, 1962.9184, 1965.9735, 1966.1417, 1969.0722, 1969.9903, 1976.9051, 1977.9456, 1978.9833, 1981.9437, 1983.8576, 1984.9415, 1987.0793, 1996.0151, 2003.0764, 2011.9750, 2016.9436, 2043.0391, 2044.9135, 2048.0004, 2060.9087, 2083.0106, 2104.9899, 2113.0380, 2120.0058, 2134.1358, 2134.9150, 2134.9829, 2135.0865, 2140.9440, 2153.0337, 2169.0464, 2179.9521, 2180.1249, 2189.9629, 2196.1988, 2197.0502, 2208.1002, 2211.1057, 2215.0672, 2221.1278, 2225.1216, 2230.2148, 2231.0641, 2233.0857, 2239.1368, 2246.2105, 2247.1028, 2249.0554, 2253.1565, 2260.9735, 2261.1186, 2263.0737, 2267.1755, 2277.0877, 2283.1839, 2284.9894, 2285.0547, 2285.1717, 2297.1987, 2298.1823, 2306.0789, 2310.1776, 2311.0866, 2313.1932, 2314.0569, 2315.1805, 2316.9206, 2347.0588, 2383.9570, 2400.1503, 2409.2148, 2410.2122, 2419.2628, 2421.2473, 2457.0982, 2469.4006, 2475.2240, 2501.2579, 2511.2529, 2586.3675, 2589.3417, 2658.8682, 2659.3711, 2666.8697, 2696.2511, 2698.3458, 2706.2466, 2733.2833, 2752.2749, 2774.3298, 2780.3044, 2780.4498, 2790.9425, 2787.9386, 2788.4422, 2808.3200, 2827.3980, 2872.3987, 2916.5107, 2969.4314, 3011.5747, 3077.6112, 3094.6109, 3095.6107, 3111.6147, 3338.7521, 3345.3946, 3346.6808, 3347.6754, 3354.7575, 3393.8204, 3515.7085, 3543.7435, 3961.9509, 4229.9279, 5315.7074, 5331.7299, 5574.8808

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VERSION     NP_651601.1
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KEYWORDS    RefSeq.
SOURCE      Drosophila melanogaster (fruit fly)
  ORGANISM  Drosophila melanogaster
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            Muscomorpha; Ephydroidea; Drosophilidae; Drosophila; Sophophora.
REFERENCE   1  (residues 1 to 787)
  AUTHORS   Hoskins,R.A., Carlson,J.W., Kennedy,C., Acevedo,D., Evans-Holm,M.,
            Frise,E., Wan,K.H., Park,S., Mendez-Lago,M., Rossi,F.,
            Villasante,A., Dimitri,P., Karpen,G.H. and Celniker,S.E.
  TITLE     Sequence finishing and mapping of Drosophila melanogaster
            heterochromatin
  JOURNAL   Science 316 (5831), 1625-1628 (2007)
   PUBMED   17569867
REFERENCE   2  (residues 1 to 787)
  AUTHORS   Smith,C.D., Shu,S., Mungall,C.J. and Karpen,G.H.
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TITLE The Release 5.1 annotation of Drosophila melanogaster heterochromatin
JOURNAL Science 316 (5831), 1586-1591 (2007)
PUBMED 17569856
REMARK Erratum:[Science. 2007 Sep 7;317(5843):1325]
REFERENCE 3 (residues 1 to 787)
AUTHORS Quesneville,H., Bergman,C.M., Andrieu,O., Autard,D., Nouaud,D., Ashburner,M. and Anxolabehere,D.
TITLE Combined evidence annotation of transposable elements in genome sequences
JOURNAL PLoS Comput. Biol. 1 (2), 166-175 (2005)
PUBMED 16110336
REFERENCE 4 (residues 1 to 787)
AUTHORS Hoskins,R.A., Smith,C.D., Carlson,J.W., Carvalho,A.B., Halpern,A., Kaminker,J.S., Kennedy,C., Mungall,C.J., Sullivan,B.A., Sutton,G.G., Yasuhara,J.C., Wakimoto,B.T., Myers,E.W., Celniker,S.E., Rubin,G.M. and Karpen,G.H.
TITLE Heterochromatic sequences in a Drosophila whole-genome shotgun assembly
JOURNAL Genome Biol. 3 (12), RESEARCH0085 (2002)
PUBMED 12537574
REFERENCE 5 (residues 1 to 787)
AUTHORS Kaminker,J.S., Bergman,C.M., Kronmiller,B., Carlson,J., Svirskas,R., Patel,S., Frise,E., Wheeler,D.A., Lewis,S.E., Rubin,G.M., Ashburner,M. and Celniker,S.E.
TITLE The transposable elements of the Drosophila melanogaster euchromatin: a genomics perspective
JOURNAL Genome Biol. 3 (12), RESEARCH0084 (2002)
PUBMED 12537573
REFERENCE 6 (residues 1 to 787)
AUTHORS Misra,S., Crosby,M.A., Mungall,C.J., Matthews,B.B., Campbell,K.S., Hradecky,P., Huang,Y., Kaminker,J.S., Millburn,G.H., Prochnik,S.E., Smith,C.D., Tupy,J.L., Whitfield,E.J., Bayraktaroglu,L., Berman,B.P., Bettencourt,B.R., Celniker,S.E., de Grey,A.D., Drysdale,R.A., Harris,N.L., Richter,J., Russo,S., Schroeder,A.J., Shu,S.Q., Stapleton,M., Yamada,C., Ashburner,M., Gelbart,W.M., Rubin,G.M. and Lewis,S.E.
TITLE Annotation of the Drosophila melanogaster euchromatic genome: a systematic review
JOURNAL Genome Biol. 3 (12), RESEARCH0083 (2002)
PUBMED 12537572
REFERENCE 7 (residues 1 to 787)
AUTHORS Celniker,S.E., Wheeler,D.A., Kronmiller,B., Carlson,J.W., Halpern,A., Patel,S., Adams,M., Champe,M., Dugan,S.P., Frise,E., Hodgson,A., George,R.A., Hoskins,R.A., Laverty,T., Muzny,D.M., Nelson,C.R., Pacleb,J.M., Park,S., Pfeiffer,B.D., Richards,S., Sodergren,E.J., Svirskas,R., Tabor,P.E., Wan,K., Stapleton,M., Sutton,G.G., Venter,C., Weinstock,G., Scherer,S.E., Myers,E.W., Gibbs,R.A. and Rubin,G.M.
TITLE Finishing a whole-genome shotgun: release 3 of the Drosophila melanogaster euchromatic genome sequence
JOURNAL Genome Biol. 3 (12), RESEARCH0079 (2002)
PUBMED 12537568
REFERENCE 8 (residues 1 to 787)
AUTHORS Adams,M.D., Celniker,S.E., Holt,R.A., Evans,C.A., Gocayne,J.D., Amanatides,P.G., Scherer,S.E., Li,P.W., Hoskins,R.A., Galle,R.F., George,R.A., Lewis,S.E., Richards,S., Ashburner,M., Henderson,S.N., Sutton,G.G., Wortman,J.R., Yandell,M.D., Zhang,Q., Chen,L.X., Brandon,R.C., Rogers,Y.H., Blazej,R.G., Champe,M., Pfeiffer,B.D., Wan,K.H., Doyle,C., Baxter,E.G., Helt,G., Nelson,C.R., Gabor,G.L., Abril,J.F., Agbayani,A., An,H.J., Andrews-Pfannkoch,C., Baldwin,D., Ballew,R.M., Basu,A., Baxendale,J., Bayraktaroglu,L., Beasley,E.M., Beeson,K.Y., Benos,P.V., Berman,B.P., Bhandari,D., Bolshakov,S., Borkova,D., Botchan,M.R., Bouck,J., Brokstein,P., Brottier,P., Burtis,K.C., Busam,D.A., Butler,H., Cadieu,E., Center,A., Chandra,I., Cherry,J.M., Cawley,S., Dahlke,C., Davenport,L.B., Davies,P., de Pablos,B., Delcher,A., Deng,Z., Mays,A.D., Dew,I., Dietz,S.M., Dodson,K., Doup,L.E., Downes,M., Dugan-Rocha,S., Dunkov,B.C., Dunn,P., Durbin,K.J., Evangelista,C.C., Ferraz,C., Ferriera,S., Fleischmann,W., Fosler,C., Gabrielian,A.E., Garg,N.S., Gelbart,W.M., Glasser,K., Glodek,A., Gong,F., Gorrell,J.H., Gu,Z., Guan,P., Harris,M., Harris,N.L., Harvey,D., Heiman,T.J., Hernandez,J.R., Houck,J., Hostin,D., Houston,K.A., Howland,T.J., Wei,M.H., Ibegwam,C., Jalali,M., Kalush,F., Karpen,G.H., Ke,Z., Kennison,J.A., Ketchum,K.A., Kimmel,B.E., Kodira,C.D., Kraft,C., Kravitz,S., Kulp,D., Lai,Z., Lasko,P., Lei,Y., Levitsky,A.A., Li,J., Li,Z., Liang,Y., Lin,X., Liu,X., Mattei,B., McIntosh,T.C., McLeod,M.P., McPherson,D., Merkulov,G., Milshina,N.V., Mobarry,C., Morris,J., Moshrefi,A., Mount,S.M., Moy,M., Murphy,B., Murphy,L., Muzny,D.M., Nelson,D.L., Nelson,D.R., Nelson,K.A., Nixon,K., Nusskern,D.R., Pacleb,J.M., Palazzolo,M., Pittman,G.S., Pan,S., Pollard,J., Puri,V., Reese,M.G., Reinert,K., Remington,K., Saunders,R.D., Scheeler,F., Shen,H., Shue,B.C., Sidén-Kiamos,I., Simpson,M., Skupski,M.P., Smith,T., Spier,E., Spradling,A.C., Stapleton,M., Strong,R., Sun,E., Svirskas,R., Tector,C., Turner,R., Venter,E., Wang,A.H., Wang,X., Wang,Z.Y., Wassarman,D.A., Weinstock,G.M., Weissenbach,J., Williams,S.M., Woodage,T.

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                Worley,K.C., Wu,D., Yang,S., Yao,Q.A., Ye,J., Yeh,R.F.,
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                Zhong,F.N., Zhong,W., Zhou,X., Zhu,S., Zhu,X., Smith,H.O.,
                Gibbs,R.A., Myers,E.W., Rubin,G.M. and Venter,J.C.
  TITLE         The genome sequence of Drosophila melanogaster
  JOURNAL       Science 287 (5461), 2185-2195 (2000)
   PUBMED       10731132
REFERENCE       9  (residues 1 to 787)
  AUTHORS       Celniker,S., Carlson,J., Wan,K., Pfeiffer,B., Frise,E., George,R.,
                Hoskins,R., Stapleton,M., Pacleb,J., Park,S., Svirskas,R.,
                Smith,E., Yu,C. and Rubin,G.
  CONSRTM       Berkeley Drosophila Genome Project
  TITLE         Drosophila melanogaster release 4 sequence
  JOURNAL       Unpublished
REFERENCE       10 (residues 1 to 787)
  CONSRTM       NCBI Genome Project
  TITLE         Direct Submission
  JOURNAL       Submitted (14-MAR-2017) National Center for Biotechnology
                Information, NIH, Bethesda, MD 20894, USA
REFERENCE       11 (residues 1 to 787)
  CONSRTM       FlyBase
  TITLE         Direct Submission
  JOURNAL       Submitted (07-DEC-2016) FlyBase, Harvard University, Biological
                Laboratories, 16 Divinity Ave, Cambridge, MA 02138, USA
REFERENCE       12 (residues 1 to 787)
  CONSRTM       FlyBase
  TITLE         Direct Submission
  JOURNAL       Submitted (07-OCT-2015) FlyBase, Harvard University, Biological
                Laboratories, 16 Divinity Ave, Cambridge, MA 02138, USA
REFERENCE       13 (residues 1 to 787)
  AUTHORS       Celniker,S., Carlson,J., Kennedy,C., Wan,K., Frise,E., Hoskins,R.,
                Park,S., Svirskas,R. and Kaufman,G.
  TITLE         Direct Submission
  JOURNAL       Submitted (10-AUG-2006) Berkeley Drosophila Genome Project,
                Lawrence Berkeley National Laboratory, One #Cyclotron Roadone
                Cyclotron Road, MS 64-121, Berkeley, CA 94720, USA
  REMARK        Direct Submission
REFERENCE       14 (residues 1 to 787)
  AUTHORS       Celniker,S., Carlson,J., Wan,K., Frise,E., Hoskins,R., Park,S.,
                Svirskas,R. and Rubin,G.
  TITLE         Direct Submission
  JOURNAL       Submitted (10-AUG-2006) Berkeley Drosophila Genome Project,
                Lawrence Berkeley National Laboratory, One Cyclotron Road, MS
                64-121, Berkeley, CA 94720, USA
  REMARK        Direct Submission
REFERENCE       15 (residues 1 to 787)
  AUTHORS       Smith,C.D., Zhu,S., Mungall,C.J. and Karpen,G.H.
  CONSRTM       Drosophila Heterochromatin Genome Project
  TITLE         Direct Submission
  JOURNAL       Submitted (01-AUG-2006) Drosophila Heterochromatin Genome Project,
                Ernest Orlando Lawrence Berkeley National Laboratory, 1 Cyclotron
                Road, Mailstop 64-121, Berkeley, CA 94720, USA
REFERENCE       16 (residues 1 to 787)
  AUTHORS       Adams,M.D., Celniker,S.E., Gibbs,R.A., Rubin,G.M. and Venter,C.J.
  TITLE         Direct Submission
  JOURNAL       Submitted (21-MAR-2000) Celera Genomics, 45 West Gude Drive,
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COMMENT         REVIEWED REFSEQ: This record has been curated by FlyBase. The
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Mascot: http://www.matrixscience.com/

FASTA sequence of GluN1 (Organism *Rattus norvegicus*):

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norvegicus OX=10116 GN=Grin1 PE=1 SV=1

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EERITGINDPRLRNPSDKFIYATVKQSSVDIYFRRQVELSTMYRHMEKHNYESAAEAIQA
VRDNKLHAFIWDSAVLEFEASQKCDLVTTGELFFRSGFGIGMRKDSPWKQNVSLSILKSH
ENGFMEDLDKTWVRYQECDSRSNAPATLTFENMAGVFMLVAGGIVAGIFLIFIEIAYKRH
KDARRKQMQLAFAAVNVWRKNLQDRKSGRAEPDPKKKATFRAITSTLASSFKRRRSSKDT
STGGGRGALQNQKDTVLPRRAIEREEGQLQLCSRHRES

FASTA sequence of GluN2B (Organism *Rattus norvegicus*):

>sp|Q00960|NMDE2_RAT Glutamate receptor ionotropic, NMDA 2B OS=Rattus

norvegicus OX=10116 GN=Grin2b PE=1 SV=1

MKPSAECCSPKFWLVLAVLAVSGSKARSQKSPPSIGIAVILVGTSDEVAIKDAHEKDDFH
HLSVVPRVELVAMNETDPKSIITRICDLMSDRKIQGVVFADDTDQEAIAQILDFISAQTL
TPILGIHGGSSMIMADKDESSMFFQFGPSIEQQASVMLNIMEEYDWYIFSIVTTYFPGYQ
DFVNKIRSTIENSFVGWELEEVLLLDMSLDDGDSKIQNQLKKLQSPIILLYCTKEEATYI
FEVANSVGLTGYGYTWIVPSLVAGDTDTVPSEFPTGLISVSYDEWDYGLPARVRDGIAII
TTAASDMLSEHSFIPEPKSSCYNTHEKRIYQSNMLNRYLINVTFEGRNLSFSEDGYQMHP
KLVIILLNKERKWERVGKWKDKSLQMKYYVWPRMCPETEEQEDDHLSIVTLEEAPFVIVE
SVDPLSGTCMRNTVPCQKRIISENKTDEEPGYIKKCCKGFCIDILKKISKSVKFTYDLYL
VTNGKHGKKINGTWNGMIGEVVMKRAYMAVGSLTINEERSEVVDFSVPFIETGISVMVSR
SNGTVSPSAFLEPFSADVWVMMFVMLLIVSAVAVFVFEYFSPVGYNRCLADGREPGGPSF
TIGKAIWLLWGLVFNNSVPVQNPKGTTSKIMVSVWAFFAVIFLASYTANLAAFMIQEEYV
DQVSGLSDKKFQRPNDFSPPFRFGTVPNGSTERNIRNNYAEMHAYMGKFNQRGVDDALLS
LKTGKLDAFIYDAAVLNYMAGRDEGCKLVTIGSGKVFASTGYGIAIQKDSGWKRQVDLAI
LQLFGDGEMEELEALWLTGICHNEKNEVMSSQLDIDNMAGVFYMLGAAMALSLITFICEH
LFYWQFRHCFMGVCSGKPGMVFSISRGIYSCIHGVAIEERQSVMNSPTATMNNTHSNILR
LLRTAKNMANLSGVNGSPQSALDFIRRESSVYDISEHRRSFTHSDCKSYNNPPCEENLFS
DYISEVERTFGNLQLKDSNVYQDHYHHHHRPHSIGSTSSIDGLYDCDNPPFTTQPRSISK
KPLDIGLPSSKHSQLSDLYGKFSFKSDRYSGHDDLIRSDVSDISTHTVTYGNIEGNAAKR
RKQQYKDSLKKRPASAKSRREFDEIELAYRRRPPRSPDHKRYFRDKEGLRDFYLDQFRTK
ENSPHWEHVDLTDIYKERSDDFKRDSVSGGGPCTNRSHLKHGTGEKHGVVGGVPAPWEKN
LTNVDWEDRSGGNFCRSCPSKLHNYSSTVAGQNSGRQACIRCEACKKAGNLYDISEDNSL
QELDQPAAPVAVTSNASSTKYPQSPTNSKAQKKNRNKLRRQHSYDTFVDLQKEEAALAPR
SVSLKDKGRFMDGSPYAHMFEMPAGESSFANKSSVPTAGHHHNNPGSGYMLSKSLYPDRV
TQNPFIPTFGDDQCLLHGSKSYFFRQPTVAGASKTRPDFRALVTNKPVVVTLHGAVPGRF
QKDICIGNQSNPCVPNNKNPRAFNGSSNGHVYEKLSSIESDV