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Ionotropic glutamate receptor dysfunction in pediatric neurodevelopment

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Summary

The N-methyl-*D*-aspartate (NMDA) receptor is a ligand gated ion channel and belongs to the ionotropic glutamate receptor (iGluR) family. The NMDA receptors play a unique role among the iGluRs because of their high Ca^{2+} permeability and their heterotetrameric assembly. They are central to development and function of the nervous system and are involved in processes like learning and memory. Impairment of NMDA receptors is associated with a wide spectrum of neurological diseases and because of their crucial role in brain development they are of great interest in pathophysiological conditions. NMDA receptors are composed of two obligatory GluN1 subunits and two GluN2(A-D) or GluN3(A,B) subunits. Each subunit has modular domains which are responsible for controlling distinct functions. The extracelluar N-terminal domain (NTD) has a modulatory function and contains binding sites for allosteric ligands like zinc. The ligand binding domain (LBD) contains the agonist binding sites and is connected with the transmembrane domains (TMD). The three TMDs (M1, M2, M3) and the re-entrant pore loop (P-loop) contribute to the formation of the core of the ion channel. The pore loop includes the QRN site, which builds the narrow constriction of the pore and is essential for permeation properties of the receptor.

One objective of this work was to analyze the NMDA receptor in pathological conditions, especially the influence of the allosteric modulation mediated through the NTD and the pore region referring to Mg^{2+} block and Ca^{2+} permeability. In cooperation with clinical groups we got information about mutations in NMDA receptor subunits from patients with different forms of epilepsy. Selected mutations in the NTD and the ion channel pore were used for site-directed mutagenesis and subsequent functional characterization by two-electrode-voltage-clamp in *Xenopus laevis* oocytes.

The investigation of different mutations revealed strong effects on ion channel function. Modifications of the NTD and the channel pore region led to a gain of function through impairment of different inhibition mechanisms. In the case of the NTD mutation GluN2A p.Ala243Val a loss of Zn^{2+} inhibition occurred. The pore mutations GluN2A p.Asn615Lys, GluN2B p.Asn615Ile and p.Val618Gly revealed also a reduced Mg^{2+} block and higher relative Ca^{2+} permeability. The gain of ion channel function could be a reasonable molecular correlate for the epilepsy syndromes because it results in an overactivation of the receptors, which in neuronal networks could lead to hyperexcitation in excitatory neurotransmission. This is in concordance with the

hypothesis that epilepsy proceeds from hyperexcitation.

Thus GluN1/GluN2A,B NMDA receptors antagonists are promising tools for treatment of neurological disorders, which results from hyperexcitation, like epilepsy. To validate the potential use of GluN1/GluN2 antagonists in disorder treatment, the effect and mechanism of different GluN1 antagonist at the excitatory GluN1/GluN3A NMDA receptor subtype was analyzed. In contrast to conventional NMDA receptors, antagonizing the GluN1 binding site results in GluN1/GluN3A receptors in an enhanced receptor activation. The analysis of several GluN1 antagonists showed a correlation of the extend of GluN1/GluN3A receptor current increase and the affinity of the respective antagonist. Thus, the results revealed a direct relationship between antagonist affinity and potentiation efficacy.

In summary this work presents mutations in the GluN2A and GluN2B subunit of the NMDA receptors as important genetic determinants of some severe forms of age-related epilepsies. The findings reveal strong evidence of the strong contribution of altered NMDA receptor signaling to epileptogenesis. Subsequent the data creates the possibility for NMDA receptors as new targets in epilepsy treatment with NMDA blockers and antagonists, but also reveal the complexity of antagonist impact on the different NMDA receptor subtypes.

Zusammenfassung

Der N-methyl-*D*-aspartate (NMDA) Rezeptor ist ein ligandengesteuerter Ionenkanal und gehört zur Familie der ionotropen Glutamatrezeptoren (iGluR). Aufgrund seiner hohen Ca^{2+}-Leitfähigkeit und seiner heterotetrameren Assemblierung spielt der NMDA Rezeptor innerhalb der iGluRs eine besondere Rolle. Für die Funktion sowie in der Entwicklung des zentralen Nervensystem, spielen diese Rezeptoren eine zentrale Rolle. Weiterhin sind sie an Prozessen wie Lernen und Gedächtnisbildung beteiligt. Eine Schädigung von NMDA Rezeptoren wird mit einer Reihe von neurologischen Erkrankungen in Verbindung gebracht und aufgrund ihrer wichtigen Rolle für die Gehirnentwicklung sind sie von großem Interesse in pathologischen Zusammenhängen.

NMDA Rezeptoren sind aus zwei obligaten GluN1 und zwei GluN2(A-D) oder GluN3(A,B) Untereinheiten aufgebaut. Jede dieser Untereinheiten weist einen modularen Aufbau mit verschiedenen Domänen auf, welche für unterschiedliche Funktionen zuständig sind. Die extrazellulär gelegene N-terminale Domäne (NTD) hat eine modulierende Funktion und besitzt Bindestellen für allosterische Modulatoren, wie beispielsweise Zink. Die Liganden-Bindungs-Domäne (LBD) beinhaltet die Agonisten-Bindestellen und ist mit den Transmembrandomänen (TMD) verbunden. Die drei Transmembrandomänen (M1, M2, M3) und die Wiedereintrittsschleife (P-Loop) bilden die Ionenkanalpore. Die Pore wird durch die QRN Stelle verengt, welche entscheidend für die Permeationseigenschaften des Rezeptors ist.

Ein Ziel der Arbeit war die Analyse des NMDA Rezeptors in pathologischen Zusammenhängen. Dabei wurde der Einfluss der allosterischen Modulation, welcher über die NTD vermittelt wird, untersucht. Weiterhin wurde der Einfluss der Porenregion bezüglich des Mg^{2+}-Blocks und der Ca^{2+}-Permeabilität analysiert. In Zusammenarbeit mit klinischen Arbeitsgruppen erhielten wir Informationen über Mutationen in verschiedenen NMDA Rezeptor Untereinheiten, aus Patienten mit verschiedenen Epilepsie-Syndromen. Für die Analyse wurden einzelne Mutationen in der NTD und der Ionenkanalregion ausgewählt und mittels zielgerichteter Mutagenese eingeführt. Anschließend erfolgte die funktionale Charakterisierung der ausgewählten Mutationen mittels der Zwei-Elektroden-Spannungsklemme (Two-electrode-voltage-clamp, TEVC) an *Xenopus laevis* Oozyten.

Die elektrophysiologischen Analysen der verschiedenen Mutationen in der NTD und

der Kanalpore zeigten eine Verstärkung (gain of function) der Ionenkanalfunktion durch eine Verminderung verschiedener Inhibitionsmechansimen. Die Mutation GluN2A p.Ala243Val in der NTD führte zu einem Verlust der Zn^{2+}-Inhibition. Die Mutationen in der Kanalregion führten zu einer starken Verringerung des spannungsabhängigen Mg^{2+}-Blocks sowie höheren, relativen Ca^{2+}-Permeabilitäten. Diese Verstärkung (gain of function) der Ionenkanalfunktion könnte ein molekulares Korrelat für die Epilepsie-Syndrome darstellen, wodurch es zu einer Überaktivierung der Rezeptoren kommt. In neuronalen Netzen könnte dies zu einer Übererregung (Hyperexzitabilität) der exzitatorischen Neurotransmission führen. Dies würde mit der Hypothese übereinstimmen, dass Epilepsie durch Übererregung ausgelöst werden kann.
Aus diesem Grund sind GluN1/GluN2A,B NMDA Rezeptor Antagonisten für die Behandlung neurologischer Erkrankungen, welche durch eine Übererregung ausgelöst werden, wie beispielsweise. Epilepsie, ein vielversprechender Ansatz. Um den möglichen Einsatz von GluN1/GluN2 Antagonisten in der Behandlung von neurologischen Erkrankungen zu validieren, wurden der Effekt und der Mechanismus verschiedener GluN1 Antagonisten an exzitatorischen GluN1/GluN3A NMDA Rezeptoren untersucht. Im Gegensatz zu den konventionellen NMDA Rezeptoren führt eine Antagonisierung der GluN1-Bindestelle zu einer verstärkten Rezeptoraktivierung. Die elektrophysiologische Analyse verschiedener GluN1 Antagonisten zeigte eine Korrelation zwischen der Potenzierung der GluN1/GluN3A Rezeptor-Ströme und der Affinität des jeweiligen Antagonisten. Diese Ergebnisse zeigten somit einen direkten Zusammenhang zwischen der Antagonistenaffinität und der Potenzierungseffizienz.

Zusammenfassend präsentiert diese Arbeit Mutationen in der GluN2A und GluN2B Untereinheit des NMDA Rezeptors als wichtige, genetische Determinanten, in einigen schweren, altersabhängigen Epilepsien. Diese Ergebnisse sind deutliche Hinweise für eine starke Beteiligung von veränderter NMDA-Rezeptor-Signalübertragung an der Epileptogenese. Weiterhin eröffnen diese Daten die Möglichkeit NMDA Rezeptoren als neue Ziele in der Epilepsie-Behandlung zu nutzen und mit Blockern und Antagonisten zu behandeln, aber zeigt zugleich die Komplexibilität des Einflusses von Antagonisten auf NMDA Rezeptoren.

1 Introduction

Glutamate receptors are responsible for the fast excitatory synaptic transmission in the central nervous system (CNS) and are involved in many brain functions. They are activated through glutamate and are subdivided in two main classes: the metabotropic and the ionotropic glutamate receptors. The ionotropic glutamate receptors again are grouped into four classes based on the pharmacology and structural homology: the (RS)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl) propionic acid receptors (AMPARs), the kainate receptors (KARs) and the *N*-methyl-D-aspartate receptors (NMDARS). The NMDA receptors are essential in development and play a major role in neuronal excitation in the CNS. Impairment of NMDA receptors is associated with a spectrum of neurological diseases like schizophrenia, Alzheimer disease, cognitive impairment, depression and pain. Several characteristic properties of NMDA receptors distinguish them from other ionotropic receptors.

1.1 Characterization of NMDA receptors

NMDA receptors are encoded by seven different genes: GRIN1, GRIN2A-D, GRIN3A und GRIN3B (Cull-Candy et al., 2001; Dingledine et al., 1999). Whereas the GRIN1 gene undergoes alternative splicing to generate eight different isoforms (Monyer et al., 1994), the other subunits are encoded by six separate genes. NMDARs are integral membrane proteins, which assemble as heterotetrameric complexes and form a cation selective ion channel. The so called “conventional“ NMDA receptors are composed of two obligate glycine-binding GluN1 subunits and two glutamate-binding GluN2 subunits (Monyer et al., 1992; Furukawa et al.,2005). The agonist glutamate and glycine are both required to activate GluN1/GluN2 receptors. The channel pore of these receptors conduct Na^+ and K^+ and is highly permeable to Ca^{2+} ions. The influx of Ca^{2+} leads to an activation of Ca^{2+}-dependent second messenger cascades and is important for processes like learning and memory (Burnashev N, 1995 ; Mayer and Westbrook, 1987). Furthermore NMDAR currents display a strong voltage dependency due to the Mg^{2+} block in the channel pore and in comparison with AMPA and kainate receptors, NMDARs show slow activation and deactivation kinetics. A further hallmark of NMDA receptors is the wide spectrum of allosteric modulation. There are various extracellular compounds to modulate NMDA receptor activity such as zinc (Zn^{2+}), ifenprodil,

polyamines and protons (H^+), acting as allosteric modulators and voltage dependent channel blockers MK-801, ketamine and memantine.

The last detected GluN3A and GluN3B subunits are the third member of the NMDA family, which shares many characteristics of the NMDA family, but also provides different properties to NMDA receptors in comparison to the conventional ones. These "unconventional" NMDA receptors display a different pharmacological profile. The GluN3 subunits can coassemble with GluN1 subunits and because of the absence of a glutamate-binding site, these receptors can be activated solely by glycine (Awobuluyi et al., 2007; Chatterton et al., 2002; Madry et al., 2007). Therefore, they are also called excitatory glycine receptors. Furthermore, when coassembled with GluN1 and GluN2 subunits, GluN3A exerts a dominant-negative effect on NMDA properties. Incorporating of the GluN3A subunit decreases single channel conductance, Ca^{2+} permeability and Mg^{2+} block *in vitro* and *in vivo* (Cavara et al., 2010; Chatterton et al., 2002; Matsuda et al., 2003; Perez-Otano et al., 2001; Sasaki et al., 2002; Tong et al., 2008). Also the modulation of GluN1/GluN3A receptors shows differences. Coapplication of Zn^{2+}, which acts on GluN2 subunits and inhibit the conventional NMDA receptors, can act as a positive modulator and also as an agonist at GluN1/GluN3A receptors (Madry et al., 2008). Furthermore, GluN1 antagonists, which inhibit GluN1/GluN2 receptors, lead to an enhancement of GluN1/GluN3A receptor activation (Madry et al., 2007). In comparison to GluN1/GluN2 NMDA receptors the role of the GluN3 subunits is less well defined and further studies are needed to elucidate the role of these GluN1/GluN3 receptors.

1.2 NMDA receptor subunit structure

Like AMPA and Kainate receptor, all NMDA subunits have a typical modular organization consisting of four functional domains: the N-terminal domain (NTD), the ligand binding domain (LBD), a transmembrane domain (TMD) comprised of three transmembrane helices (M1, M2, M3) and a re-entrant pore loop (P-loop), and an intracellular C-terminal domain (CTD) (Fig 1.1). The amino acid sequences of the NTD and LBD are homologous to two families of bacterial binding proteins (Armstrong et al., 1998; Nakanishi et al., 1990; O'Hara et al., 1993), what suggests a common phylogenetic origin. The NTD is related to the leucine-isoleucine-valine binding protein (LIVBP) and has a modular function. This domain shows a bilobed, clamshell like

structure which, is formed by an upper R1 and a lower R2 lobe and is involved in subunit assembly (Karakas 2009, Farina 2011). Furthermore, the GluN2 NTDs contain binding sites for allosteric modulators such as Zn^{2+} and Ifenprodil. The LBD shows sequence homology with the bacterial glutamine-binding protein and contains the agonist binding sites. Crystallographic studies revealed the typical bilobed, clamshell-like structure with the agonist glycine in GluN1 and GluN3 and glutamate in GluN2 subunits. The clamshell-like structure is formed by two extracellular segments, S1 and S2. The LBD is connected through three short linkers to the TMD. The TMD contributes to the formation of the core of the ion channel and has high sequence homology with the inverted ion channel domain of K^+ channels (Kuner et al., 2003; Wo and Oswald, 1995). The region lining the pore is highly conserved, important for permeation properties and the affinity for the pore blocker Mg^{2+}. The intracellular CTD is the most diverse domain showing no sequence homology to any known protein and varying in length. This domain is important for the interaction with intracellular proteins and mediates the anchoring of the receptor with membrane scaffold proteins and proteins that control the signal transmission in the intracellular field.

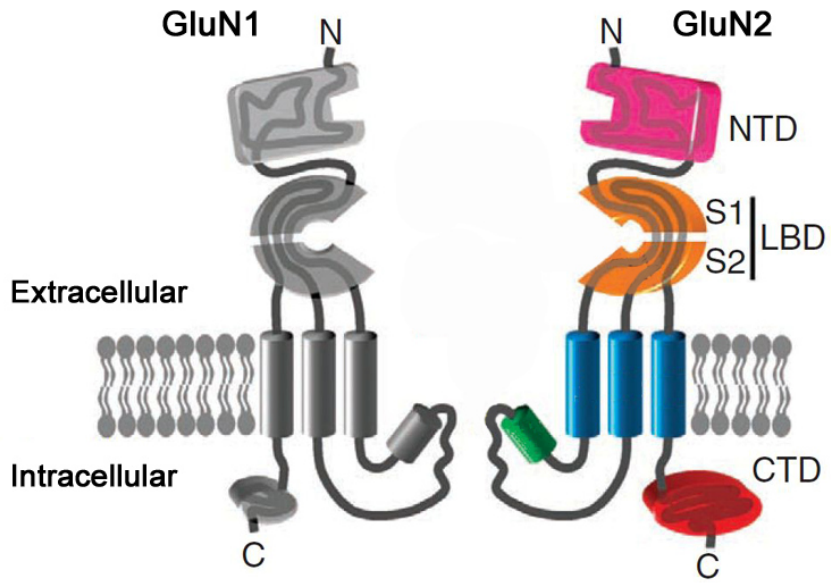

Figure 1. 1: Topology model of the NMDA receptor subunits GluN1 and GluN2. The N-terminal domain (NTD, pink) and the ligand binding domain (LBD, orange) including the S1 and S2 peptide segments are extracellularly located. The three transmembrane domains (blue) and the re-entrant P-loop (green) build the ion channel pore followed by the intracellular C-terminal domain (CTD, red) (modified from Endele et al, 2010).

1.3 Principles of NMDA receptor activation

Activation of the conventional NMDA receptors requires the presence of two agonists; presynaptically released glutamate and extracellular glycine as co-agonist (Johnson and Ascher, 1987). Furthermore NMDA receptors are blocked through extracellular Mg^{2+} ions at physiological membrane potential (Mayer et al., 1984; Nowak et al., 1984). For the efficient activation of GluN1/GluN2 NMDARs two conditions are required. First, membrane depolarization through non-NMDA receptors to release the Mg^{2+} ion out of the pore and second, the binding of glutamate and glycine. Thus, the conventional NMDA receptors are called coincidence detectors, linking neurotransmitter activation with the electrical state of the neuron.

Functional and crystallographic studies revealed the molecular structure of ionotropic glutamate receptors and lead to a better understanding of the activation mechanism. The ligand binding domain is composed of two lobes, S1 and S2, which are arranged in a back-to-back-conformation and form a bilobed or clamshell like structure (Furukawa and Gouaux, 2003). The agonist binding pocket is located in the interface between the two S1 and S2 segments. Agonist binding leads to a clamshell closure and stabilizes a closed cleft conformation. This conformational change in the LBD leads to a transition to the ion channel followed by separation of the transmembrane domain linkers, which finally induce channel opening of the receptor.

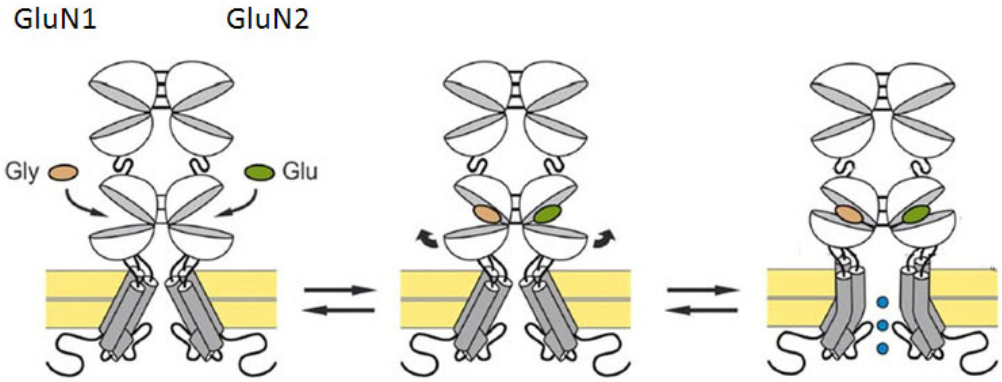

Figure 1. 2: Structural mechanism of NMDA receptor activation. Both agonists, glycine and glutamate, are required for NMDAR channel opening. The agonist binding leads to a closure of the LBD, which results in a separation of the transmembrane domain linkers. This induces channel opening of the receptor (modified from Paoletti, 2011).

1.4 Spatial and temporal expression of NMDA subunits

The subunit composition and localization of NMDA receptors seem to play an essential role for normal brain development. The expression of the NMDA subunits underlies different spatial and temporal expression patterns and is developmentally regulated (Paoletti, 2011). The obligate GluN1 subunit is ubiquitously expressed in the embryonic and adult CNS. Interestingly the expression pattern of the GluN2 subunits depends on brain region and developmental stage. The GluN2B subunit is already expressed in the embryonic rodent CNS, whereas the GluN2A subunit shows a gradually increase after birth. In the adult CNS the expression of the GluN2B subunit becomes restricted to forebrain areas, whereas the GluN2A subunit is abundantly expressed in the entire brain. The GluN2C subunit appears late in development and the GluN2D subunit is expressed already in the early development. The GluN3 subunits show also an unique expression profile. The GluN3A subunit reaches maximum expression in the early postnatal life followed by a decrease to low levels in adulthood. In contrast the GluN3B subunit expression is low in early stages of development with an increase and maximum in the adult CNS.

Furthermore, the subunit composition and cellular localization seem to play a crucial role for cell survival and cell death. NMDA receptors are localized at synapses, but also extrasynaptically and based on their localization they exhibit different physiological roles (Hardingham and Bading, 2003). GluN2A containing NMDA receptors are preferentially localized at synapses and promote cell survival. In contrast GluN2B containing receptors are extrasynaptically localized and activate pro-death signaling pathways.

Detailed knowledge of the mechanism behind the GluN2A containing receptor induced neuroprotection is currently lacking. But it has been demonstrated that calcium influx through synaptic GluN2 containing receptors activates two signaling pathways. Both activate the cAMP-response-element-binding protein (CREB) through phosphorylation, which results in an activation of pro-survival gene and expression of the highly protective brain-derived neurotrophic factor (BDNF). Through activation of extrasynaptic GluN2B containing receptors the activation is inhibited and CREB gets dephosphorylated, which results in a deactivation of CREB. Thus extrasynaptic localized GluN2B containing receptors activate a CREB shut-off pathway that block the induction of BDNF expression.

1.5 Architecture of NMDA receptors

Structural knowledge of NMDA receptors has been advanced in recent years with isolated N-terminal domains and ligand binding domains (Farina et al., 2011; Furukawa and Gouaux, 2003; Furukawa et al., 2005; Inanobe et al., 2005; Karakas et al., 2009; Karakas et al., 2011). The X-ray structure of the homomeric Glu2A AMPA receptor provided a structural template for development of a NMDA receptor model (Sobolevsky et al., 2009). But the modes of subunit and domain arrangement of intact heterotetrameric NMDA receptors were not elucidated till recently two groups reported the crystal structures of the GluN1/GluN2B NMDA receptor. The published crystal structures of GluN1/GluN2B NMDA receptors from Karakas and Furukawa and Lee and colleagues are the first crystal structures of an NMDA receptor including the NTD, LBD and TMD and though give more insight about the architecture, symmetry and domain organization of NMDA receptors.

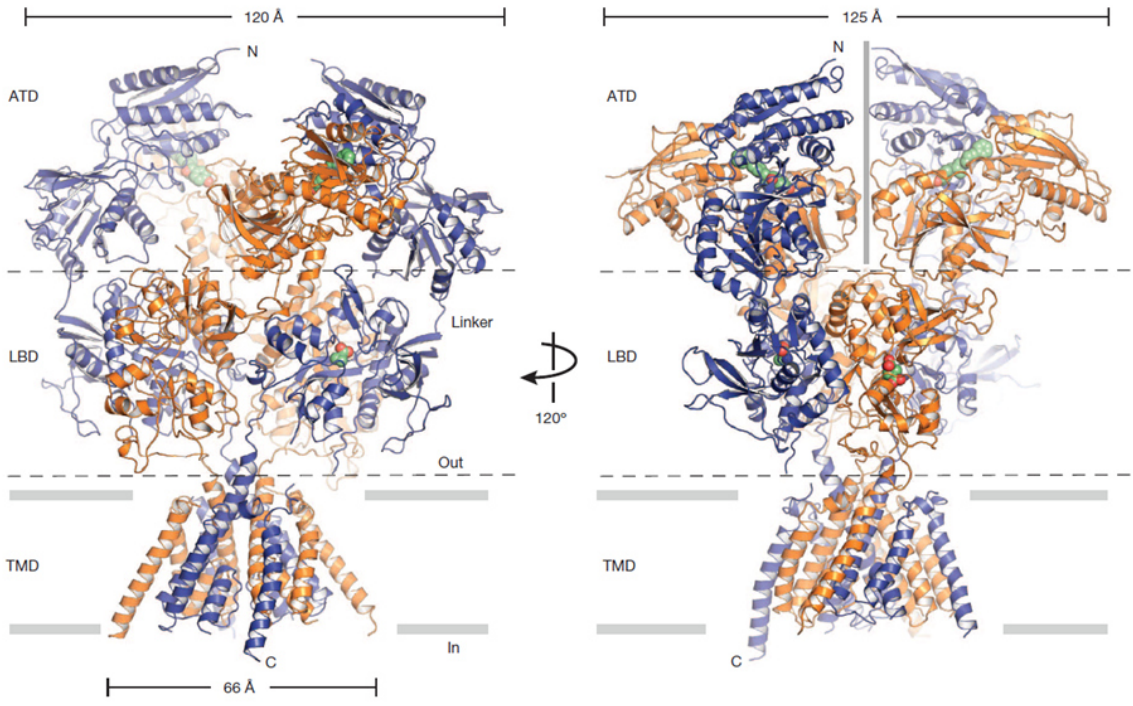

Figure 1. 3: Architecture of the GluN1/GluN2B NMDA receptor. Overall structure of NMDA receptor complex is shown with GluN1 subunits in blue and the GluN2B subunits in orange. The receptor domains are organized in three layers with the NTD, LBD and the TMD. (left) View of receptor complex parallel to the membrane and (right) rotated ~ 120° around the overall twofold axis (Lee et al., 2014).

The overall architecture of the GluN1/GluN2B crystal structures were described as a `hot-air ballon` or a `mushroom` with the receptor domains organized into three layers with the NTD layer at the top, the LBD layer in the middle and the TMD layer at the bottom. Both structures confirmed the GluN1-GluN2-GluN1-GluN2 (1-2-1-2) alternating arrangement in the heterotetramer, which was supposed in previous studies (Riou et al., 2012; Sobolevsky et al., 2009). The NTDs and LBDs are organized as local heterodimers and the extracellular domains are more compact in contrast to the homomeric Glu2A AMPA receptor.

1.5.1 The role of the N-terminal domain

An important key feature of NMDA receptors is their capacity for allosteric regulation by small molecules such as Zn^{2+}. This allosteric regulation is mediated through the large NTDs which form mobile regulatory domains. The overall architecture of the NTDs is shaped like a bilobed clamshell composed of the upper lobe (R1) and the lower lobe (R2) similar to the bilobed LBD with S1 and S2 lobes. In comparison to Non-NMDA receptors the NTDs of the NMDA receptors are substantially twisted. In the full-length crystal structure the AMPA receptor subunits are organized as a dimer of dimers where the dimer pairs are relatively far apart from each other. In NMDA receptors the NTDs also build dimer pairs, like in AMPA receptors, but in contrast the NMDA receptor dimer pairs are much more compact (Karakas et al., 2011). This is in concordance with the crystal structures from Lee and colleagues (Lee et al., 2014).

An important allosteric modulator of NMDA receptors is the divalent cation Zn^{2+}. It is the most prevalent trace element in the body, which occurres endogenously in the brain and has many functional roles (Paoletti et al., 2009). It has been demonstrated that Zn^{2+} is localized in synaptic vesicles at glutamatergic presynaptic terminals (Frederickson et al., 2005; Salazar et al., 2005) and the release takes place in an activity dependent manner (Smart et al., 2004). Zn^{2+} can reach concentrations of nearly 1 µM in the synaptic cleft (Smart et al., 2004).

Selective inhibition by low micromolar concentration of Zn^{2+} at NMDA receptors was first demonstrated in cultured hippocampal neurons (Perez-Clausell and Danscher, 1985; Westbrook and Mayer, 1987). Zn^{2+} acts as an allosteric inhibitor in a voltage-independent fashion (Choi and Lipton, 1999; Christine and Choi, 1990). Furthermore the voltage-independent Zn^{2+} sensitivity is influenced by the subunit composition.

GluN1/GluN2A receptors show high sensitivity in the nanomolar range (Choi and Lipton, 1999) whereas GluN1/GluN2B receptors show a 100 fold lower affinity (Rachline et al., 2005). The presence of Zn^{2+} in synaptic vesicles and the abundance of potential synaptic targets, especially NMDA receptors, indicate Zn^{2+} a crucial role as modulator of synaptic transmission.

1.5.2 The NMDA receptor transmembrane domains and ion channel pore

The assembly of the different NMDA receptor subunits results in NMDA receptors with multiple functions, because the subunit composition determines the functional properties of the receptors. Permeation and gating properties of the NMDA receptors differ strikingly between the different subunit compositions. GluN2A- and GluN2B-containing receptors show a higher Ca^{2+} permeability than GluN2C and GluN2D containing receptors (Burnashev N, 1995). Furthermore, receptors containing the GluN2A or GluN2B subunits show a higher sensitivity to Mg^{2+} as GluN2C and GluN2D containing receptors (Kuner and Schoepfer, 1996; Qian et al., 2005). Structural determinants that control the permeation are located in the P-loop within the narrowest constriction of the channel pore. In NMDA receptors the narrow constriction is formed by the QRN site asparagine and asparagines adjacent to the QRN site (QRN + 1 site) in GluN2 (Wollmuth et al., 1998; Wollmuth et al., 1996).

The recently published crystal structures of a GluN1/GluN2B NMDA receptor reveal nearly a complete representation of the ion channel pore and the putative selectivity filter (Lee et al., 2014). The pathway through the ion channel pore can be structured into three main sections: gate, vestibule and filter. The gate is the first constriction located at the extracellular ends of M3, which adopt a pyramid-like shape. This represents a physical constriction to the ion channel permeation pathway at the extracellular side of the membrane. Residues in the highly conserved SYTANLAAF motif define this narrowest constriction, which is implicated in modifying ion channel gating properties. Through bundle crossing of the M3 helices at the SYTANLAAF motif the ion penetration pathway is occluded.

The following central vestibule is a cavity flanked by the M3 helices on the side and helices with the tips of the pore loop on the bottom. Immediately below the central vestibule there is a second constriction of the pore, which builds the selectivity filter. This section is defined by residues at the beginning of the pore loop and includes the QRN site. Subsequent the pore expands to the cytoplasmic space.

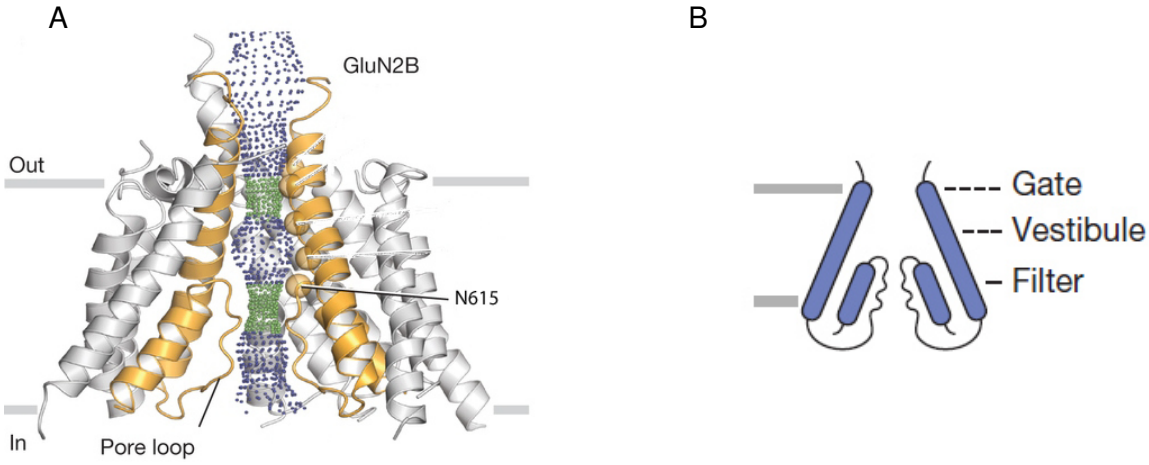

Figure 1. 4: Transmembrane domain architecture. (A) View of the ion channel pore with the solvent accessible surface along the pore axis. The pore region can be divided into three main sections; gate, vestibule and filter. For GluN2B, residues located at the gate, vestibule and the filter are shown as yellow spheres. The asparagine 615 is the crucial residue at the second constriction of the pore and defines the QRN site. (B) Schematic view of the channel pore and the three main sections. (modified from Lee et al., 2014).

1.6 Physiological and pathophysiological role of NMDA receptors

NMDA receptors are critical for normal brain function (Dingeldine 1999) and play a crucial role in physiological and pathophysiological processes in the CNS. Because of the high calcium permeability of NMDA receptors, they play an important role in increasing the intracellular Ca^{2+} concentration. Calcium is a key intracellular signaling molecule involved in many types of neuronal plasticity (Malenke and nicoll 1999). But it is also a key mediator of excitotoxic cell death if present in excess (Mattson 2003). Under physiological conditions NMDA receptors play a crucial role in synaptic plasticity. The Ca^{2+} influx, which is a consequence of receptor activation, contributes to the stabilization of the synaptic contacts during the development of the nervous system (Nakanishi and Okazawa, 2006). Their dysfunction is implicated in several neurodegenerative disorders such as schizophrenia, Alzheimer disease and epilepsy. These diseases have been mainly attributed to an overstimulation of the glutamatergic transmission. The resulting, excessive Ca^{2+} influx into the post-synapse, which is mostly carried by NMDA receptors, plays a crucial role in neuronal cell death.

In recent years there has been a large amount of research, which advances the understanding of the contribution of excitatory glutamatergic transmission to seizures. It has been accepted that the overstimulation of glutamatergic transmission and thereby the activation of glutamate receptors may be of significant relevance for its clinical manifestation (Ghasemi and Schachter, 2011). The involvement of excitatory NMDA receptors in the etiology of epilepsy is of main interest in the research of neurological disorders because a regulated balance of excitation and inhibition is fundamental for proper neurological functions in the CNS. Imbalance in this complex circuit of neuronal transmission could lead to neurological disorders and is a hallmark of epilepsy. Epilepsy is a disorder of neuronal network excitability and one of the most common neurological disorders affecting approximately one percent of the population (Hauser, 1994). The most prominent characteristics are spontaneously appeared seizures caused by synchronized electrical discharges of neurons, which lead to alteration in the electroencephalogram (EEG).

This work presents for the first time that mutations in the NMDA receptor can cause variable epilepsy syndromes, which indicates a strong involvement of NMDARs in the etiology of epilepsy.

1.7 Aim of the study

Ca^{2+} permeability and allosteric modulation are fundamental characteristics of NMDA receptors. Because of these special features, among ionotropic glutamate receptors, NMDA receptors on the one hand trigger neurotoxicity and are involved in different neurological disease. On the other hand for this reason they represent promising targets for regulation and treatment of various neurological diseases. The functional properties of NMDA receptors are strongly influenced by the GluN2 subunit. The aim of the project was to investigate the Mg^{2+} block, the Ca^{2+} permeability and the allosteric modulation of GluN1/GluN2 receptors with epilepsy associated mutations. In cooperation with clinical groups we got information about various mutations in GRIN2A and GRIN2B, which encode the GluN2A and GluN2B subunits of the NMDA receptor. All mutations were detected in patients with different forms of epilepsy. The work focused the analysis on mutations in the NTD and the ion channel pore. For this purpose we used molecular biology and electrophysiological methods, i.e. site-directed mutagenesis and two-electrode-voltage-clamp (TEVC) on *Xenopus laevis* oocytes. This analysis gives us an idea of the involvement of the NTD and the ion channel pore on NMDA receptor function in pathological states, especially age-related epilepsy syndromes.

For the treatment of neurological disorders like epilepsy, NMDA antagonists are a promising tool. Beside allosteric antagonists, glycine site antagonists are under investigations for several neurological disorders, also in epilepsy (Jansen and Dannhardt, 2003). In clinical trials a main problem with antagonists are the occurrence of servere side effects. Due to the use of antagonists in disorder treatment it is necessary to investigate the effect and mechanism of antagonists at different receptor subtypes. A further objective of this work was the functional characterization of different GluN1 antagonist at the excitatory GluN1/GluN3A NMDA receptors. The GluN1/GluN3A receptors reveal different features in comparison to conventional NMDA receptors and antagonizing of the GluN1 glycine binding site results in enhanced receptor activation. This effect would be counteractive in epilepsy treatment. The aim of this study was to investigate the potentiation effect, especially the influence of high and low affinity GluN1 antagonists on GluN1/GluN3 receptor currents and the effect on potentiation strength.

2 Manuscript:

Mutations in GRIN2A and GRIN2B encoding regulatory subunits of NMDA receptors cause variable neurodevelopmental phenotypes (published online 3 of October 2010 in Nature Genetics)

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2.1 Abstract

N-methyl-D-aspartate (NMDA) receptors mediate excitatory neurotransmission in the mammalian brain. Two glycine-binding NR1 subunits and two glutamate-binding NR2 subunits each form a highly Ca^{2+}-permeable cation channels which are blocked by extracellular Mg^{2+} in a voltage-dependent manner (Cull-Candy et al., 2001).
Either *GRIN2B* or *GRIN2A*, encoding the NMDA receptor subunits NR2B and NR2A, was found to be disrupted by chromosome translocation breakpoints in individuals with mental retardation and/or epilepsy. Sequencing of *GRIN2B* in 468 individuals with mental retardation revealed four *de novo* mutations: a frameshift, a missense and two splice site mutations. In another cohort of 127 individuals with idiopathic epilepsy and/or mental retardation, we discovered a *GRIN2A* nonsense mutation in a three-generation family. In a girl with early-onset epileptic encephalopathy, we identified the *de novo GRIN2A* mutation c.1845C>A predicting the amino acid substitution p.N615K. Analysis of NR1/NR2A^{N615K} (NR2A subunit with the p.N615K alteration) receptor currents revealed loss of the Mg^{2+} block and a decrease in Ca^{2+} permeability. Our findings suggest that disturbances in the neuronal electrophysiological balance during development result in variable neurological phenotypes depending on which NR2 subunit of NMDA receptors is affected.

2.2 Paper

N-methyl-D-aspartate (NMDA) receptors are neurotransmitter-gated ion channels involved in regulation of synaptic function in the central nervous system (Cull-Candy et al., 2001). The identity of the NR2 subunit (A, B, C, or D) determines many of the physiological and pharmacological properties of NMDARs (Cull-Candy and Leszkiewicz, 2004). Inappropriate activation of NMDA receptors has been implicated in several neurological conditions, such as Alzheimer's disease and schizophrenia (Lau and Zukin, 2007), however, single gene mutations in subunits of the NMDA receptors have not yet been reported in Mendelian disorders. Many human disease genes have been identified by breakpoint mapping of chromosome rearrangements (Kurotaki et al., 2002; Najm et al., 2008; Ray et al., 1985; Tonkin et al., 2004). We ascertained two male patients with *de novo* chromosome translocations, 46,XY,t(9;12)(p23;p13.1) and 46,XY,t(10;12)(q21.1;p13.1), and a common breakpoint in 12p13.1. Subject 1 was referred at the age of 2 years because of mild to moderate mental retardation (MR), behavioral anomalies, and abnormal electroencephalogram (EEG). Subject 2 was a

12-year-old boy with a more complex phenotype including severe mental retardation, behavioral and EEG anomalies, and ophthalmological and other manifestations (Table 2.1). Using serial fluorescence *in situ* hybridization (FISH) experiments to delineate translocation breakpoints, we found both breakpoints in 12p13.1 to disrupt *GRIN2B*, which encode the NR2B subunit of NMDA receptors (Fig. 2.1a,b and Supplementary Figs. 2.1 and 2.2).

In light of the neurodevelopmental phenotype of both translocation subjects with translocation, we hypothesized *GRIN2B* to be the phenocritical gene. In mice, Nr2b is required for neuronal pattern formation and synaptic plasticity in general and for channel function and formation of dendritic spines in hippocampal pyramidal cells in particular (Akashi et al., 2009; Cull-Candy et al., 2001). Transgenic overexpression of *Grin2b* in the forebrain of mice and in the cortex and hippocampus of rats results in superior performance in various tests of learning and memory (Tang et al., 1999; Wang et al., 2009). Thus, *Grin2b* seems to represent a rate-limiting genetic factor in gating NMDA receptor's function in the developing and adult mammalian brain strongly suggesting involvement of NR2 subunits in human brain function and cognition.

This assumption was further corroborated by our finding of a third translocation, t(16;17)(p13.2;q11.2), disrupting GRIN2A, the gene encoding the NR2A subunit. The 16;17 translocation was found to co-segregate with epilepsy and variable degree of cognitive impairment in a family (Fig. 2.1c-e and Supplementary Fig. 2.3). The index subject, referred to as 3-1, was a 26-year-old male with a history of febrile seizures followed by tonic-clonic seizures and severe mental retardation. His father (subject 3-2), paternal aunt (subject 3-3), and cousin (subject 3-4) had a history of grand-mal seizures with onset at the end of their first decade that spontaneously decreased during adolescence. All four family members had behavioral problems, and subject 3-4 also had moderate mental retardation (Table 2.2). *GRIN2A* represented an excellent candidate gene based on the crucial role of NMDA receptors in neuronal maturation and excitatory synaptic transmission. In addition, submicroscopic deletions in 16p13 encompassing *GRIN2A* among other genes have recently been associated with intellectual disability and epilepsy (Reutlinger et al., 2010a).

Grin2a knockout mice show increased spontaneous locomotor activity and deficits in contextual fear conditioning and spatial learning, along with reduced hippocampal long-term potentiation that is the cellular basis for learning and memory (Kiyama et al., 1998; Sakimura et al., 1995). The phenotype of *Grin2b*-deficient mice is more severe as they die perinatally due to severe developmental brain

defects (Kutsuwada et al., 1996; Sakimura et al., 1995). These data together with our finding of disrupted *GRIN2A* or *GRIN2B* in individuls with variable neurodevelopmental phenotypes strongly suggest that any disturbance in the number and/or composition of NMDA receptors has profound effects on neuronal development and activity in humans.

We further substantiated this hypothesis through detection of point mutations in individuals with mental retardation and/or epilepsy. Two cohorts of affected individuals were screened for mutations in *GRIN2B*: the first cohort consisted of 315 individuals with mental retardation (185 patients with mild to moderate mental retardation and 130 with moderate to severe mental retardation) and the second cohort consisted of 153 individuals (73 with mild to moderate mental retardation and 57 moderate to severe mental retardation; and in 23 individuals the severity of mental retardation was unspecified). We sequenced the *GRIN2A* in a third cohort comprising 127 individuals with a history of idiopathic epilepsy and/or abnormal EEG and variable degree of mental retardation. We identified four individuals with moderate mental retardation and behavioral anomalies who had a heterozygous *de novo GRIN2B* mutation: c.411+1G>A in subject 4, c.2044C>T in subject 5, c.2360-2A>G in subject 8, and c.803_804delCA in subject 9 (all parental identities were confirmed; Table 2.1 and Supplementary Tables 2.1-2.3). The c.2044C>T mutation was absent in 1,080 control chromosomes and the other three mutations observed were absent in 360 control chromosomes. *In silico* analysis using splice site prediction programs confirmed that c.411+1G>A and c.2360-2A>G disrupt the splice donor and acceptor site, respectively, and likely result in altered splicing(Wang and Cooper, 2007) (Supplementary Table 2.3). Using RNA analysis, we could not detect aberrant *GRIN2B* transcripts in subject 4 (c.411+1G>A) or subject 8 (c.2360-2A>G) (data not shown). However, heterozygosity of *rs7301328* (c.366C>G) in exon 2 of *GRIN2B* was only found in genomic DNA (gDNA) and not in cDNA of both of these individuals (Fig. 2.2a). This suggests monoallelic expression of the wild-type allele due to nonsense-mediated mRNA decay of aberrantly spliced *GRIN2B* transcripts. In contrast, we identified *GRIN2B* mRNAs with a premature stop codon (PTC) in subject 9 carrying the 2-bp deletion c.803_804delCA (resulting in p.T268SfsX15), indicating that *GRIN2B* transcripts with a PTC are not efficiently degraded (Fig. 2.2b). The c.2044C>T mutation predicts the amino acid substitution p.R682C affecting a highly conserved arginine within the glutamate-binding NR2B ligand binding domain (LBD)(Cull-Candy et al., 2001) (Fig. 2.3a and Supplementary Fig. 2.4a). Our model of the NR2B LBD predicts strong intra-

LBD interactions between the guanidinium group of p.R682 with the carboxyl group of p.D727 and a backbone carbonyl oxygen of an adjacent helical structure (Fig. 2.3b). The p.R682C alteration results in a loss of three hydrogen bonds, thereby destabilizing the tertiary structure of the NR2B LBD (Fig. 2.3b, inset), which may affect glutamate affinity similar to the substitution of neighboring residues (Laube et al., 2004; Laube et al., 1997). However, analysis of agonist dose-response curves revealed no differences in affinities of both glutamate and glycine of wild-type NR1/NR2B and mutant NR1/NR2B^{R682C} NMDA receptors (Supplementary Fig. 2.5). This data suggests that the p.R682C substitution may alter NMDA receptor function through other mechanisms.

We likewise identified two mutations in *GRIN2A* (Table 2.1 and Supplementary Tables 2.1-2.3): First, we found the heterozygous c.652C>T transition, resulting in a premature stop codon (p.Q218X) in a 4-year-old individual (subject 6-1) with mild to moderate mental retardation and abnormal EEG. His mother (subject 6-2) and maternal grandmother (subject 6-3) also carry the c.652C>T mutation. Both the subject`s mother and grandmother had a history of seizures during early childhood (Table 2.2). RNA analysis in subject 6-1 revealed monoallelic expression of the wild-type allele, suggesting that *GRIN2A* transcripts with the PTC were efficiently degraded by nonsense-mediated mRNA decay (Fig. 2.2c). The second mutation we identified was the heterozygous *de novo* c.1845C>A transversion found in a 3-year-old girl (subject 7; whose parental identity was confirmed) with early-onset epileptic encephalopathy, abnormal EEG, and severe developmental delay (Table 2.2). The c.652C>T mutation was absent in 360 control chromosomes and the c.1845C>A mutation in 1,080 control chromosomes. The latter change predicts the substitution of an evolutionary conserved asparagine for lysine (p.N615K) in the membrane re-entrant loop (the P-loop) of the NR2A subunit (Fig. 2.3a and Supplementary Fig. 2.4b), which determines the narrow constriction and ion selectivity of the channel (Mayer, 2006). Because conversion of the polar residue N615 at the tip of the pore loop to an uncharged side chain has been shown to alter inward-current rectification by Mg^{2+} (Wollmuth et al., 1998), p.N615K likely has a strong impact on NMDA channel properties (Fig. 2.3c). Indeed, analyzing voltage dependence of NR1/NR2A^{N615K} receptor currents revealed loss of the Mg^{2+} block and decrease in Ca^{2+} permeability (Fig. 2.3d,e). Co-expression of wild-type NR2A and mutant NR2A^{N615K} resulted in intermediate effects (Fig. 2.3d), indicating a negative impact of p.N615K on the channel properties of heterooligomeric NR1/NR2A/NR2A^{N615K} NMDA receptors.

Taken together, disruption of *GRIN2A* in the translocation subjects and the

nonsense alteration p.Q218X most likely lead to functional null alleles associated with a relatively mild phenotype comprising epilepsy and variable cognitive impairment. However, the more severe phenotype in subject 7 with p.N615K can be explained by a dominant negative effect on NMDA receptor function.

The functional properties of NMDA receptors depend on subunit composition, with the NR2 subunits controlling channel kinetics and synaptic signalling(Lau and Zukin, 2007). The composition of native NMDA receptors undergoes a developmental change from heterotetramers containing predominantly NR2B at the early stages of development to those containing NR2B, NR2A, or both subunits at mature stage(Cull-Candy et al., 2001). Regulation of the NR2A:NR2B ratio of synaptic NMDA receptors is thought to be a major determinant of the developmental and experience-dependent properties of synaptic plasticity (Yashiro and Philpot, 2008). The concept that the remodeling of the number and composition of synaptic NMDA receptors is important for neuronal activity and development is reflected by the different neurodevelopmental phenotypes in individuals with heterozygous *GRIN2A* and *GRIN2B* mutations: whereas mutations in the prenatally expressed *GRIN2B* lead to cognitive defects, mutations in the postnatally expressed *GRIN2A* cause epilepsy as the most consistent clinical feature. Recently, risk haplotypes of the *GRIN1*, encoding the NR1 subunit of NMDA receptors, have been associated with infantile spasms further indicating an involvement of NMDA receptors in epileptogenesis (Ding et al., 2010). The precise pathophysiological consequences of the *GRIN2A* and *GRIN2B* mutations remain to be elucidated. We hypothesize that both loss-of-function mutations causing a change in NMDA receptor composition and/or number and missense mutations leading to abnormal NR2 subunit function may affect neuronal ion flux and electrical transmission between neurons in the human brain.

Accession codes. The cDNA sequence and the genomic reference sequence for the *GRIN2B* gene can be found under accession number NM_ 000834.3 and NC_000012.11, respectively. The cDNA sequence and the genomic reference sequence for the *GRIN2A* gene can be found under accession number NM_001134407.1 and NC_000016.9, respectively.

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AUTHOR CONTRIBUTIONS

Mutation analysis: S.E., B.P, and G.Ro. Transcript analysis: K.K. and G.Ro. Functional analysis of NMDA receptors: K.G. and B.L. NMDA receptor modeling: C.T. and B.L. Patient ascertainment: Y.H., L.V.M., M.M., U.M., G.Ra., A.Ra., S.v.S., I.S., N.V., L.V., D.W., B.Z. and M.Z. FISH analysis and breakpoint mapping: A.F., V.M.K., F.K., F.K.P. and H.H.R. Array CGH analysis: J.K. and H.T. Manuscript writing: K.K., V.M.K, B.L., L.V.M., A.Re., G.Ro. and D.W. Study design: K.K., L.V.M., A.Re., G.Ro. and D.W. All authors contributed to the final version of the paper.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

2.3 Figures

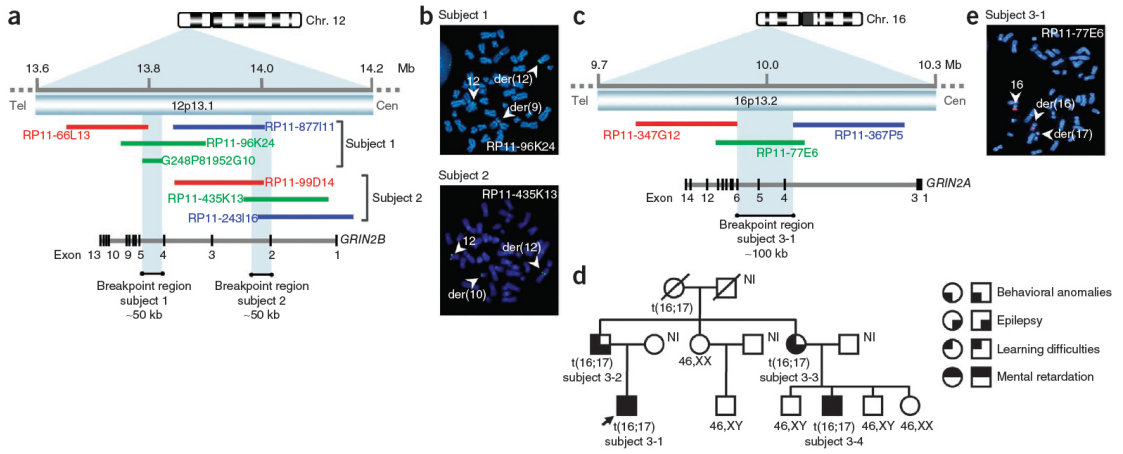

Figure 2. 1: Disruption of *GRIN2A* and *GRIN2B* in subjects with chromosome translocations and different neurodevelopmental phenotypes. (a) Physical map of 12p13.1. BAC (RP11-) and fosmid (G248P8) clones used for mapping the breakpoints of subjects with the 9;12 translocation (subject 1) and the 10;12 translocation (subject 2) are indicated by colored bars and the names are given. Red, mapped distal to the translocation breakpoint; green, spanned the breakpoint; blue, mapped proximal to the breakpoint. Exons of GRIN2B are indicated by vertical bars, exon numbers are given and breakpoint regions are shown. (b) FISH with BAC clones RP11-96K24 and RP11-435K13 on metaphase spreads from lymphocytes of subjects 1 and 2, respectively, yielded split signals. Wild-type and derivative (der) chromosomes are indicated by arrowheads. (c) Physical map of 16p13.2. BAC (RP11-) clones used for mapping the breakpoint of subject 3-1 with the 16;17 translocation are indicated by colored bars and the names are given. Color codes are the same as in (a). Exons of GRIN2A are indicated by vertical bars and the exon numbers are given. The breakpoint region is indicated. (d) Segregation of the 16;17 translocation in members of the family with epilepsy. t(16;17) denotes individuals carrying the chromosomal rearrangement, whereas 46,XX and 46,XY denote individuals with normal karyotype. The index subject is marked by an arrow. NI, chromosomes not investigated. (e) FISH with BAC clone RP11-77E6 on metaphase spread from lymphocytes from subject 3-1 yielded split signals. Wild-type and derivative (der) chromosomes are indicated by arrowheads.

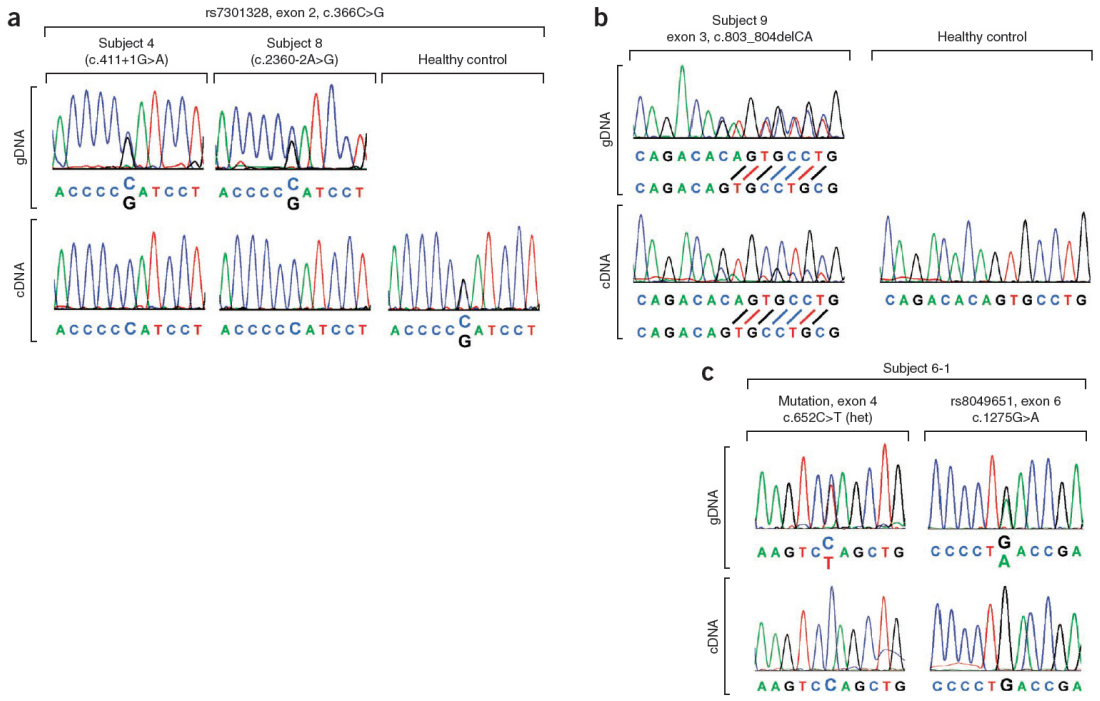

Figure 2. 2: Transcript analysis for the mutations c.411+1G>A, c.2360-2A>G, and c.803_804delCA in *GRIN2B* and c.652C>T in *GRIN2A*. (**a**) Partial sequence electropherograms of *GRIN2B* exon 2 obtained from gDNA and cDNA from subjects 4 (c.411+1G>A) and 8 (c.2360-2A>G) and a healthy individual. Both subjects are heterozygous for *rs7301328* (c.366C>G) at the gDNA level (upper panel, left and middle). In contrast, we observed monoallelic expression of one SNP allele in cDNA of subjects 4 and 8 (lower panel, left and middle). A control individual showed biallelic expression of the SNP alleles in *GRIN2B* cDNA (lower panel, right). (**b**) Partial sequence electropherograms of *GRIN2B* exon 3 obtained from gDNA and cDNA of subject 9 (c.803_804delCA). This subject is heterozygous for the 2-bp deletion in gDNA and cDNA. The respective wild-type cDNA sequence from a healthy individual is shown on the right. (**c**) Partial sequence electropherograms of exons 4 and 6 of the *GRIN2A* obtained from gDNA and cDNA of subject 6-1. The subject is heterozygous for both the c.652C>T (p.Q218X) nonsense mutation and the *rs8049651* (c.1275G>A) at the genomic level (upper panel). Sequence analysis of cDNA-derived amplicons revealed monoallelic expression (lower panel).

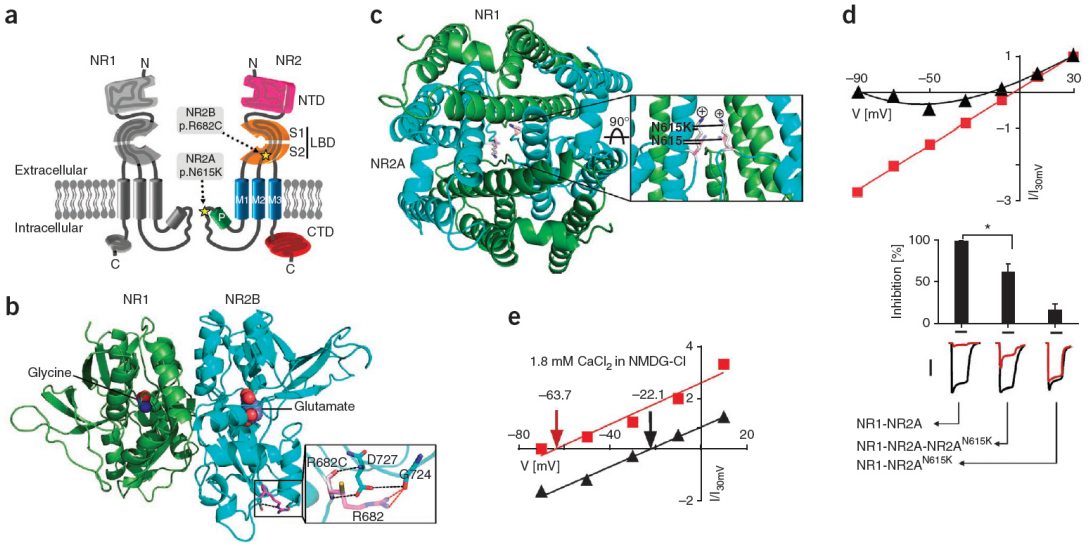

Figure 2. 3: Structural and functional consequences of missense mutations in *GRIN2B* and *GRIN2A* found in subjects with mental retardation and/or epilepsy. (**a**) Topology model of an NR1 and an NR2 subunit.Positions of the two alterations p.R682C and p.N615K are indicated by yellow asterisks in the NR2 subunit consisting of an amino-terminal domain (NTD), the ligand-binding domain (LBD) including the S1 and S2 peptide segments, three transmembrane segments (M1, M2, and M3), a re-entrant pore loop (P), and an intracellular carboxy-terminal domain (CTD). Residue R682 in NR2B lies within the glutamate binding domain and N615 in NR2A in the ion channel pore. N, NH_2-terminus; C, COOH-terminus(**b**) Model of the LBDs of the NR1/NR2B NMDA receptor shows residue p.R682 (magenta) within the glutamate-binding NR2B LBD (cyan) together with an adjacent glycine-binding NR1 LBD (green). Enlargement shows the loss of the stabilizing side chain interactions of p.R682 with p.D727 and the carbonyl oxygen of p.G724 upon mutation to cysteine. (**c**) Transmembrane arrangement of the NMDA receptor composed of NR1 (green) and NR2A (cyan) subunits (top view). Enlargement highlights the predicted repulsive effect of the positive side chains of p.N615K on cation permeability in the pore forming region (side view). (**d**) Current-voltage (I–V) relationships of NR1/NR2A receptors (upper panel). Linear I–V curves of NR1/NR2A^{N615K} (red squares) receptor currents reveal a loss in Mg^{2+}-mediated outward rectification of NR1/NR2A (black triangles) receptors. Currents of NR1/NR2A, NR1/NR2A/NR2A^{N615K} and NR1/NR2A^{N615K} receptors show a gradual loss of voltage-dependent Mg^{2+} inhibition (lower panel). (**e**) Differences in the reversal potential (indicated by arrows) of NR1/NR2A (black triangles) and NR1/NR2A^{N615K} (red squares) receptor currents reveal a decrease in Ca^{2+} permeability of the mutant receptor.

Table 2. 1: Clinical data from subjects with mutations in GRIN2B

	Subject 1 ES06E1083	Subject 2 ES10E0186	Subject 4 ES07E0211	Subject 5 ER14077	Subject 8 HDMR187	Subject 9 HDMR179
GRIN2B mutation	Translocation breakpoint disrupting *GRIN2B*	Translocation breakpoint disrupting *GRIN2B*	c.411+1G>A	c.2044C>T (p.R682C)	c.2360-2A>G	c.803_804delCA (p.T268SfsX15)
Inheritance	*De novo*	*De novo*	*De novo*	*De novo*	*De novo*	*De novo*
Ethnic origin	Germany	Germany	Germany	Germany	European descent	Germany
Sex	Male	Male	Male	Male	Female	Female
Age at last follow up[a]	5	12	10	13	41	13
Microcephaly	–	+	–	–	–	–
Cognitive development	Moderate MR	Severe MR	Moderate MR	Moderate MR	Mild MR	Moderate MR
Seizures	–	–	–	–	–	–
EEG anomalies	Left-sided sharp-wave complexes	Irregular slow dysrhythmia, occipital abortive sharp waves	Irregular slow dysrhythmia	–	–	–
Behavioral anomalies	+	+	+	+	+	+
MRI scan anomaly	–	+	–	–	NA	NA
Facial dysmorphism	–	–	–	–	–	–
Eye anomalies	NA	+	–	–	–	–
Other abnormalities	–	Choanal atresia, cryptorchidism, inguinal hernia, flat feet	–	–	Hypothyroidism	–

+, present; –, absent; MR, mental retardation, NA, not analyzed.
[a]Age at last follow up in years.

Table 2. 2: Clinical data from subjects with mutations in GRIN2A

	Subject 3-1	Subject 3-2	Subject 3-3	Subject 3-4	Subject 6-1 D57-09	Subject 6-2 D86-10	Subject 6-3 D125-10	Subject 7 1110-SaJa
GRIN2A mutation	Translocation breakpoint disrupting *GRIN2A*	Translocation breakpoint disrupting *GRIN2A*	Translocation breakpoint disrupting *GRIN2A*	Translocation breakpoint disrupting *GRIN2A*	c.652C>T (p.Q218X)	c.652C>T (p.Q218X)	c.652C>T (p.Q218X)	c.1845C>A (p.N615K)
Inheritance	Inherited	Inherited	Inherited	Inherited	Inherited	Inherited	NA	*De novo*
Ethnic origin	Belgium	Belgium	Belgium	Belgium	Germany	Germany	Germany	France
Sex	Male	Male	Female	Male	Male	Female	Female	Female
Age at last follow up[a]	25	46	62	39	4	21	49	3
Microcephaly at birth	–	ND	ND	ND	+	ND	ND	–
Small for gestational age	+	ND	ND	–	–	ND	ND	–
Postnatal microcephaly	–	ND	ND	–	+	–	–	–
Postnatal short stature	+	–	+	+	–	–	–	ND
Cognitive development	Severe MR	Learning difficulties	Learning difficulties	IQ 40	Mild to moderate MR	Learning difficulties	Learning difficulties	Severe MR
Type of seizures and age of onset	Febrile seizures at 10 months followed by tonic-clonic crises until 17 years	Tonic-clonic crises from 9 to 18 years	Tonic-clonic crises from 16 to 25 years	Tonic crises from 8 to 20 years	–	First complicated febrile seizures at the age of 11 months, focal seizures until age of 3 years	Seizures within the first year of life, seizures disappeared in adolescence	First seizure at the age of 3 months; epileptic spasms, myoclonies and frequent massive myoclonies until the present
EEG anomalies	Paroxysmal diffuse bilateral spike-wave bursts (3–4 c/sec) with temporal predominance	Diffuse slow and rapid dysrhythmia (4–9 c/sec, amplitude 40–120 microvolts), slowing with hyperpnea	NA	Paroxysmal diffuse triphasic spike-waves (3–6 c/sec), accentuated by hyperpnea, anterior and temporal predominance	Bilateral independent centro-temporal spikes, activation in sleep, but no electrical status in sleep (ESES)	Bilateral independent centro-temporal spikes	ND	Generalized slowing, bilateral independent posterior spikes
Behavioral anomalies	+	+	+	+	+	–	–	+
MRI scan anomaly	–	NA	NA	NA	–	NA	NA	–
Muscular hypotonia	Moderate	–	–	Moderate	+	ND	ND	+
Facial dysmorphism	Short nose	–	–	–	+	–	–	–
Metabolic screen	Normal	NA	NA	NA	Normal	NA	NA	Normal
Other abnormalities	Pes cavus	–	–	–	–	–	–	–

+, present; –, absent; MR, mental retardation; IQ, intelligence quotient; NA, not analyzed; ND, not documented.
[a]Age at last follow up in years.

2.4 Online methods

Subjects. Detailed information on the patients is found in the **Supplementary Note**. This study was approved by all institutional review boards of the participating institutions, and written informed consent was obtained from all participants or their legal guardians.

Genome-wide array comparative genomic hybridization (aCGH). *Subject 1.* We obtained genomic DNA from a healthy male 46,XY (G147A, Promega). aCGH was performed using commercial Agilent oligo CGH arrays (244K arrays, Agilent Technologies) consisting of ~244,000 *in situ* synthesized 60-mer oligo- nucleotides spanning the entire genome, resulting in an average genomic dis- tance of ~12 kb. These probes included both coding and non-coding sequences on each human chromosome. For hybridization, we digested 3 µg of genomic DNA from the reference sample (46,XY) and 3 µg of genomic DNA from subject 1 with *Alu*I (20 units) and *Rsa*I (20 units) (Promega). All restrictions were done for a minimum of 2 h at 37 °C then heat inactivated at 65 °C for 20 min and verified by agarose gel electrophoresis. Labeling reactions were performed with 3 µg of restricted DNA and the Agilent Genomic DNA Labeling Kit PLUS (Agilent Techologies) according to the manufacturer's instructions in a total volume of 50 µl with a modified dNTP pool containing dATP, dGTP, dCTP and dTTP, and Cy5-dUTP (for subject 1) or Cy3-dUTP (for the 46,XY reference). Labeled targets were subsequently filtered twice with ×1 TE-buffer at pH 8.0 (Promega) through a Centricon YM-30 column (Millipore) and concentrated to 80.5 µl. The amount and specific activity of the Cy3- and Cy5-labeled samples were determined by using the Nanodrop ND-1000 UV-VIS Spectrophotometer (Peqlab) and the microarray measurement protocol. The manufacturer's recom- mendations (Agilent Technologies) were followed for selecting or rejecting the probes for hybridization. Hybridization and features extraction were performed as described (Barrett et al., 2004; Spitz et al., 2006), and data were visualized by means of the CGH Analytics 3.4 software (Agilent Technologies).

Subject 2. Whole-genome aCGH analysis was performed using a 400K oligo- nucleotide array (Agilent) according to protocols provided by the manufacturer. Image analysis, normalization and annotation were based on Feature Extraction 9.1 (Agilent) using the default settings, and visualization of data was performed with the CGHPRO software (Chen et al., 2005).

Subject 3-1. aCGH was performed using the Human Genome CGH Microarray 244A platform (Agilent Technologies). Experimental procedures were performed according to the manufacturer's instructions with slight modifications. One microgram of DNA from subject 3-1 and one microgram of reference DNA from a pool of 10 healthy donors with a normal male karyotype were hybridized. The array was scanned with the Axon GenePix 4000B microarray scanner (Axon Instruments) at a resolution of 5 µm/pixel. Signal intensities from the generated images were measured and evaluated with the Feature Extraction v9.5.3 and CGH Analytics v4.0.81 software packages (Agilent Technologies)

Lymphoblastoid cells. Lymphoblastoid cell lines were established according to standard protocols and cultured in RPMI medium (Invitrogen) supplemented with
15% FCS medium, 580 mg/l L-glutamine, 110 mg/l sodium pyruvate, penicillin and streptomycin (100 U/ml and 100 µg/ml, respectively) and incubated at 37 °C in a humidified atmosphere with 10% CO_2. Two hours before preparation of metaphase spreads, cells were incubated with 0.2 µg/ml colcemide (Sigma Aldrich) **Lymphoblastoid cells.** Lymphoblastoid cell lines were established according to standard protocols and cultured in RPMI medium (Invitrogen) supplemented with 15% FCS medium, 580 mg/l L-glutamine, 110 mg/l sodium pyruvate, penicillin and streptomycin (100 U/ml and 100 µg/ml, respectively) and incubated at 37 °C in a humidified atmosphere with 10% CO_2. Two hours before preparation of metaphase spreads, cells were incubated with 0.2 µg/ml colcemide (Sigma Aldrich).

Fluorescence *in situ* hybridization (FISH). Metaphase spreads from peripheral blood lymphocytes or lymphoblastoid cells were prepared by standard procedures. FISH experiments for the delineation of the 9p23, 10q21.1, 12p13.1, 16p13.2 and 17q11.2 breakpoints were performed with BAC and fosmid clones (**Supplementary Tables 2.4–2.6**). BACs (RPCI-11 libraries 753 and 737) were obtained from ImaGenes or from the BACPAC Resources Center (BPRC). Fosmid clones (library WIBR-2) were obtained from the BPRC. BAC and fosmid DNA was prepared using the NucleoBond Xtra Midi kit (Machery-Nagel). DNA was labeled by nick translation using the CGH Nick Translation Kit and Spectrum Green-dUTP or Spectrum Orange-dUTP (Vysis) according to the protocols provided. Chromosomes were counterstained using 4′,6-diamidino-2-phenylindole (DAPI) (Serva Feinbiochemica) and mounted in antifading solution (Vector Labs). Slides were evaluated using an epifluorescence

microscope (Leica DMRA) that was fitted with different single band-pass filter sets for DAPI, Spectrum Green and Spectrum Orange fluorescence. Images were captured using a cooled charged-couple device (CCD) camera (Pieper) and Cyto Vision software (Applied Imaging).

Mutation analysis. *GRIN2B.* Genomic DNA was isolated from blood lym- phocytes according to standard procedures. DNA samples from the first cohort of 315 individuals with mental retardation and the second cohort of 153 individuals with mental retardation were amplified by whole genome amplification using the GenomiPhi V2 DNA amplification kit (GE Healthcare). The DNA samples were screened for *GRIN2B* mutations by unidirectional sequencing of all *GRIN2B* exons (1–13), including flanking intronic regions, using the ABI BigDye Terminator Sequencing Kit (Applied Biosystems) and an automated capillary sequencer (ABI 3730, Applied Biosystems). PCR and sequencing reaction cleanup were performed with the Biomek NXP workstation using the Agencourt AMPure PCR purification system and the Agencourt CleanSEQ Dye Terminator Removal kit (Beckman-Coulter). Mutations were confirmed by independent PCR and bidirectional sequencing using the subjects' original DNA samples. Sequence analysis was done with the Sequencing Analysis v.3.6.1 (Applied Biosystems) and the Sequencher 4.9 (Gene Codes Corporation) software packages. Primer sequences (**Supplementary Table 2.7**) were determined using the Exon Primer program from the UCSC genome browser (see URLs) and the primers were supplied by Thermo Fisher Scientific.

GRIN2A. We analyzed a cohort of 127 individuals with a history of epilepsy, an abnormal EEG pattern and a variable degree of mental retardation for *GRIN2A* mutations. We amplified the coding region, including the flanking intronic sequences, of *GRIN2A* (14 exons in total) from genomic DNA. Primer sequences are summarized in **Supplementary Table 2.8**. Amplicons were directly sequenced either as described above or using the ABI BigDye Terminator Sequencing Kit (Applied Biosystems) and an automated capillary sequencer (ABI 3130, Applied Biosystems). Sequence electropherograms were analyzed using Sequence Pilot software (JSI medical systems).

Where mutations were shown to have arisen *de novo*, we verified self-reported relationships by genotyping both parents and the subject at a minimum of fifteen microsatellite loci.

Computational analyses. Splice site prediction of intronic variants identified in *GRIN2A* and *GRIN2B* were calculated by using the online tools HSF V2.4 (Desmet et al., 2009), SpliceView, the Berkeley *Drosophila* Genome Project (BDGP) (Reese et al., 1997) and the NetGene2 server (Hebsgaard et al., 1996). See URLs.
Prediction of potential deleterious effects of missense mutations detected in *GRIN2A* and *GRIN2B* was performed using the software tools SIFT (Kumar et al., 2009), PolyPhen (Ramensky et al., 2002), SNAP (Bromberg and Rost, 2007) and PANTHER 7.0 Beta (Thomas et al., 2003). See URLs

***GRIN2A* and *GRIN2B* transcript analysis.** RNA was isolated from fresh blood leukocytes from subjects 4, 6-1, 8 and 9 using the PAXgene Blood RNA System (PreAnalytics) according to the manufacturer's recommendations. First-strand cDNA was synthesized from 1–2 µg of RNA using the Omniscript Reverse Transcriptase Kit (Qiagen) and gene-specific oligonucleotides. Of a total of 20 µl of volume from the reaction, 1 µl was used as template to amplify transcripts by using primer pairs spanning different exon-exon junctions in nested PCR reactions. Amplicons were directly sequenced or cloned into pCR2.1 TOPO TA Cloning Vector (Invitrogen). *Escherichia coli* clones were subjected to colony PCR, and PCR products from individual clones were sequenced.

Molecular modeling of NMDA receptor domains. Sequence alignment of the NMDA receptor subunits was taken from a previous study (Sobolevsky et al., 2009) or performed using ClustalX 2.0.12 (see URLs). Molecular modeling of the transmembrane domains of NR1-NR2A and the LBDs of NR1-NR2B receptors was based on the crystal structure of GluR2 (Brookhaven Protein Data Bank entry 3KG2) (Sobolevsky et al., 2009) and of the NR1-NR2A LBD (PDB ID 2A5T) (Furukawa et al., 2005), respectively, by using Modeller 9v6 (Sali lab at the University of California, San Francisco) (Fiser and Sali, 2003) and lsqman 9.7.9 (Uppsala Software Factory) (Kleywegt, 1996). Models were subjected to short term molecular dynamics simulations using the Charmm27 force field, which is implemented in the Tinker 4.2 molecular modeling software (see URLs). Figures were made using PyMOL 1.2 (see URLs).

cDNA constructs, oocyte expression and electrophysiology. cDNA constructs encoding the mouse NR1-1a, NR2A and NR2B subunits have been described previously (Schuler et al., 2008). We introduced mutations with the QuikChange site-

directed mutagenesis kit (Stratagene, Agilent Technologies) using mutagenesis primers and identified positive clones by DNA sequencing. We obtained and injected collagenase-defolliculated *Xenopus laevis* oocytes (stages 5 or 6) with capped cRNAs as described previously (Haeger et al., 2010)). We kept oocytes at 19 °C in sterile frog Ringer's solution (ORi: 90 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM HEPES at pH 7.4) supplemented with 50 mg/l gentamy- cin. At 1–3 days after cRNA injection, we measured glycine and glutamate dose-response curves in the presence of saturating concentrations of the corresponding agonist (10 µM and 100 µM, respectively) by two-electrode voltage clamping (TEVC) at a holding potential of −70 mV as described previously (Haeger et al., 2010). Concentration-response curves and current traces shown in the figures were drawn using KaleidaGraph (Synergy Software). To monitor the voltage dependence of NR1-NR2A receptor combinations, whole-cell current- voltage relationships of saturating glutamate- and glycine-induced currents were recorded in 20 mV-intervals ranging from −90 mV to +30 mV and nor- malized to the current value obtained at +30 mV above the respective reversial potential (E_{rev}). Data points were aligned by using a third order polynomial fit (Madry et al., 2010). The relative divalent to monovalent permeability (P_{div}/P_{mono}) was calculated by the Goldman-Hodgkin-Katz constant field (GHK) voltage equation assuming no anion permeability as described (Madry et al., 2010). The internal concentrations of Na^+ and K^+ used in the calculations were 20 mM and 150 mM, respectively (Madry et al., 2010). Permeability ratios were calculated for each oocyte and then averaged. In order to avoid the activation of the oocytes' native Ca^{2+}-sensitive chloride currents, all experiments were carried out in oocytes incubated for 30 min at 20–25 °C with the membrane-permeant Ca^{2+} chelator 1,2-bis(2-amino-phenoxy)ethane-N,N,N′,N′-tetraacetic acid-acetoxymethyl ester (BAPTA- AM, 100 µM) before electrophysiological recordings. Mg^{2+} inhibition (1 mM) was evaluated in the presence of 1.8 mM Ca^{2+} at a holding potential of −70 mV upon application (5 s) of saturating glycine (10 µM) and glutamate (100 µM) concentrations.

Statistical analyses. Values given represent means ± standard deviation. Statistical significance was determined at the $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) levels using a Student's two-tailed unpaired *t*-test.

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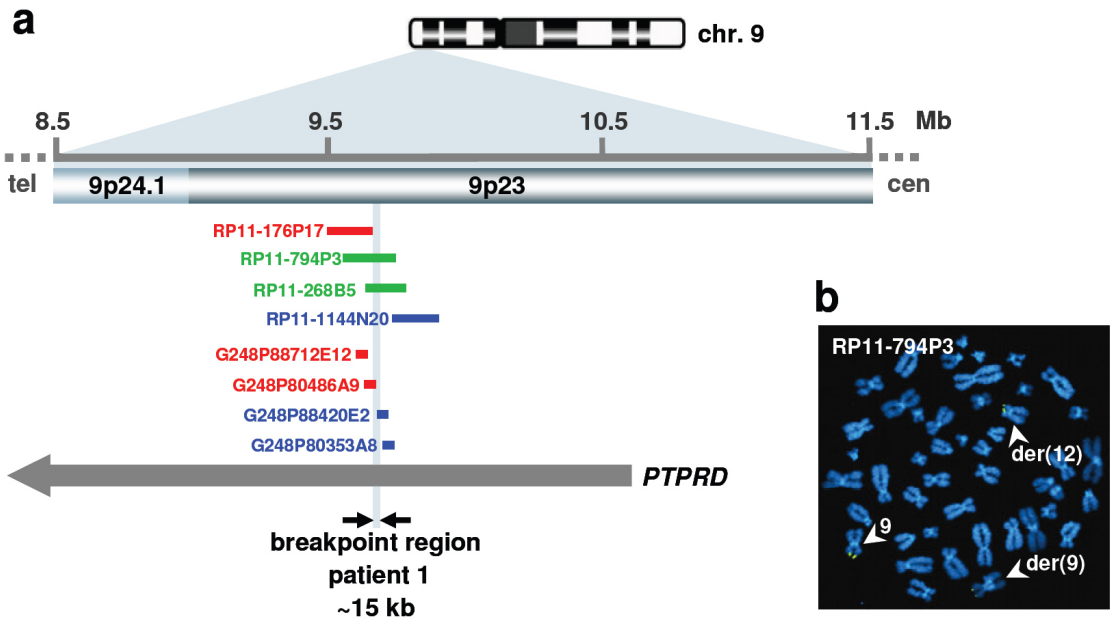
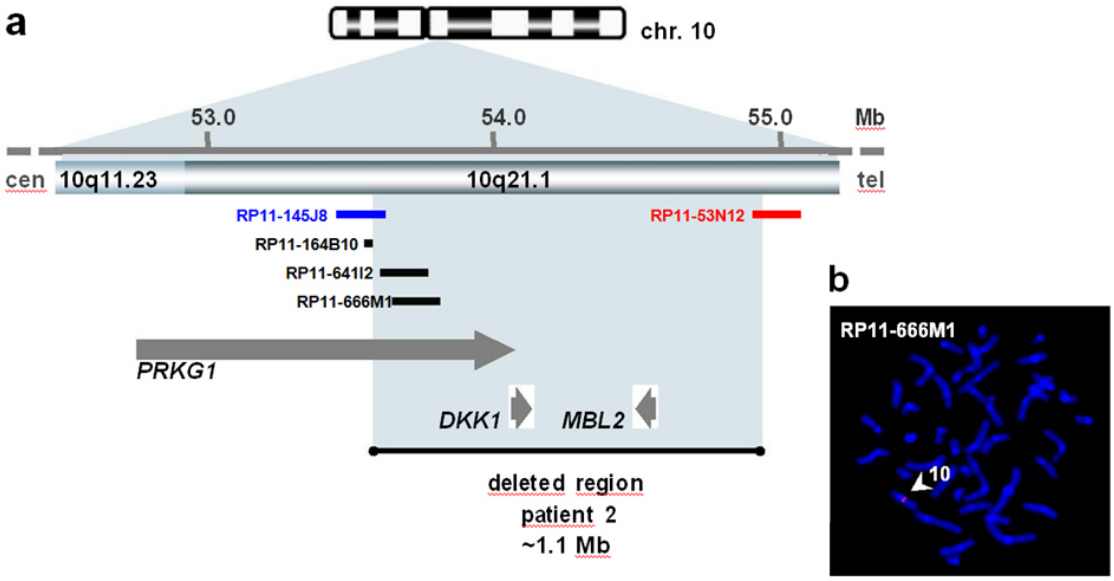
2.6 Supplementary Information



Supplementary Figure 2. 1: Delineation of the translocation breakpoint in 9p23 of patient 1 (see also Supplementary Table 4). Chromosome analysis showed a de novo 9;12 translocation (karyotype 46,XY,t(9;12)(p23;p13.1)dn). A genome-wide array comparative genomic hybridization (aCGH) did not reveal any clinical relevant genomic imbalance. (a) Physical map of 9p23. BAC (RP11-) and fosmid (G248P8) clones hybridizing distally and proximally to the breakpoint are represented by red and blue bars, respectively. Breakpoint spanning BACs are indicated by green bars. The 15-kb breakpoint region is represented by a vertical grey bar and flanked by black arrows. Names of clones are given. The PTPRD gene is represented by a grey arrow indicating 5'→3' orientation. (b) FISH with BAC clone RP11-794P3 on metaphase spread from lymphocytes of patient 1 yielded split signals. Wild-type and derivative (der) chromosomes are indicated by arrow heads.

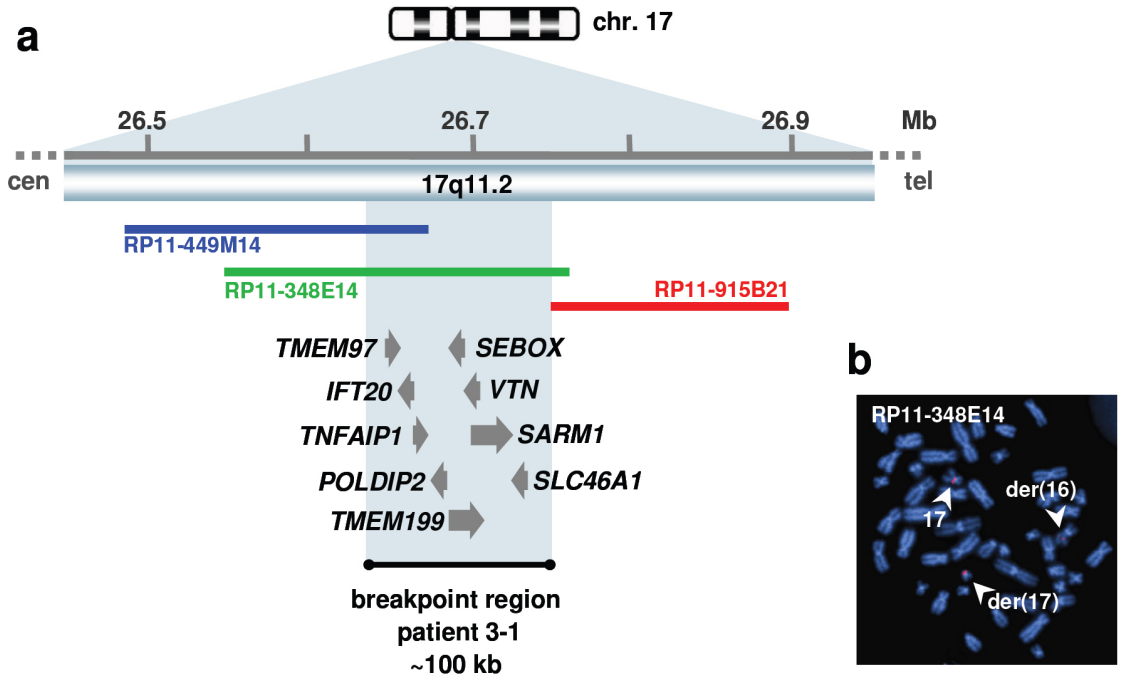
PTPRD encodes the receptor-like protein tyrosine phosphatase (PTP) delta. Human receptor-type PTPs play an important role in cancer development and progression as somatic mutations of *PTPRD* have been identified in various cancers (Cox et al., 2005; Ostman et al., 2006; Sjoblom et al., 2006; Solomon et al., 2008; Weir et al., 2007). Recently, strong evidence has been provided that *PTPRD* is a human tumor suppressor gene (Solomon et al., 2008). However, Ptprd was also found to be involved in motoneuron axon targeting (Uetani et al., 2006) and synaptic plasticity (Uetani et al., 2000)[7]. Although we can not exclude that disruption of *PTPRD* in patient 1 contributed to his phenotype, interruption of *GRIN2B* most likely accounts for mental retardation and behavioural anomalies in this individual.



Supplementary Figure 2. 2: Delineation of the translocation breakpoint in 10q21.1 of patient 2 (see also **Supplementary Table 2.5**). He carried a *de novo* 10;12 translocation (46,XY,t(10;12)(q21.1;p13.1)dn). (**a**) Physical map of 10q21.1. BAC (RP11-) clones hybridizing distally and proximally to the breakpoint are represented by red and blue bars, respectively, and BAC clones yielding only one signal on chromosome 10 are shown as black bars. Genes are represented by grey arrows indicating their 5'→3' orientation. Names of genes and BAC clones are given. The maximum deleted region of ~1.1 Mb is indicated in light grey. (**b**) FISH with BAC clone RP11-666M1 on metaphase spread from lymphocytes of patient 2 yielded only one signal on chromosome 10. A genome-wide aCGH revealed four additional *de novo* chromosome 12 deletions larger than 150 kb (see below).

PRKG1 encodes the cyclic guanosine monophosphate-dependent protein kinase I (cGKI). To date, haploinsufficiency of this gene has not been associated with a clinical phenotype in humans and its function in human brain is presently unknown. In the mouse, Prkg1 plays an important role in synaptic plasticity and learning (Hofmann et al., 2006). DKK1 is an inhibitor of Wnt signalling and counteracts Wnt-mediated effects on bone differentiation and adipogenesis. *Dkk1* knockout mice are not viable and show morphological defects including lack of anterior head structures and abnormalities of limb digits (Mukhopadhyay et al., 2001). More recently, it has been shown that Dkk1 contributes to the pathophysiology of ischemic neuronal damage (Mastroiacovo et al., 2009). *MBL2* is presently known to play a role in innate immune defense. Reports from several groups indicate that there is a high frequency of dominantly expressed MBL2 alleles resulting in

low levels of the corresponding protein in several ethnic groups (Garred, 2008). For chromosome 12, four deletions >150 kb were found, one did not harbour a gene (UCSC hg 18, 16,875,976-17,078,092), two others which both overlap with published CNVs contained genes (*ABCC9* and *CMAS*, 21,980,820-22,161,796, and the 5' region of some *SOX5* transcript variants, 24,133,205-24,335,999). The fourth deletion contained the 3' region of all *SOX5* transcript variants (23,140,567-23,664,990). Sox5 is expressed in neurons, oligodendrocytes, melanocytes, and chondrocytes and plays a role in chondrogenesis as well as development of neuronal cell types by controlling the timing of critical cell fate and differentiation decisions (Lefebvre, 2010). In summary, we cannot rule out that reduced *SOX5*, *PRKG1* and/or *DKK1* expression contributes to the clinical phenotype of patient 2. Nonetheless, disruption of *GRIN2B* likely explains his neurological symptoms.



Supplementary Figure 2. 3: Delineation of the translocation breakpoint in 17q11.2 of patient 3-1 (see also **Supplementary Table 2.6**). Chromosome analysis revealed a 16;17 translocation (t(16;17)(p13.2;q11.2)). Genome-wide aCGH did not show any clinical relevant genomic imbalance. (**a**) Physical map of 17q11.2. BAC (RP11-) clones hybridizing distally and proximally to the breakpoint are represented by red and blue bars, respectively. The breakpoint spanning BAC RP11-348E14 is indicated by a green bar. Names of clones are given. The 100-kb breakpoint region is shown by a black bar. Genes are represented by grey arrows indicating their 5'→3' orientation, and names of genes are given. (**b**) FISH with BAC clone RP11-348E14 on metaphase spread from lymphoblastoid cells of patient 3-1 yielded split signals. Wild-type and derivative (der) chromosomes are indicated by arrow heads. *(i)TMEM97* (transmembrane protein 97) codes for an integral membrane protein of unknown function. TMEM97 plays a role in controlling cellular cholesterol levels, and its up-regulation has been implicated in the pathogenesis of ovarian cancer (Bartz et al., 2009; Wilcox et al., 2007). (ii)The intraflagellar transport 20 homolog, encoded by *IFT20*, has been suggested to function in the delivery of ciliary membrane proteins from the Golgi apparatus to the cilium (Follit et al., 2006). *(iii)TNFAIP1* [tumor necrosis factor, alpha-induced protein 1 (endothelial)] was identified as a gene whose expression can be induced by tumor necrosis factor (TNF) alpha in umbilical vein endothelial cells (Wolf et al., 1992). Increased *TNFAIP1* transcript levels have been detected in brains from patients with Alzheimer's disease (Link et al., 2003). *(iv)POLDIP2* codes for polymerase (DNA-directed), delta interacting protein 2 which interacts with the DNA polymerase delta p50 subunit and inhibits DNA polymerase delta activity by about

50% (Klaile et al., 2007; Xie et al., 2005). It also binds to the proliferating cell nuclear antigen (Liu et al., 2003). (v)The function of the transmembrane protein 199, encoded by *TMEM199*, is unknown. (vi) *SEBOX* (SEBOX homeobox) is the human orthologue of mouse *sebox* that is expressed in maturing mouse oocytes, eggs, zygotes, and two-cell embryos, in adult mouse brain, skin, ovary, and liver (Cinquanta et al., 2000; Kim et al., 2008). Homeodomain proteins such as Sebox play a key role in coordinating gene expression during development. The human SEBOX protein has substitutions at the invariant residues asparagine 51 and arginine 53 suggesting that it may be nonfunctional (Cinquanta et al., 2000). (vii)The glycoprotein VTN (vitronectin) is a member of the pexin family. VTN is found in serum and tissues and promotes cell adhesion and spreading, inhibits the membrane-damaging effect of the terminal cytolytic complement pathway, binds to several serpin serine protease inhibitors, and modulates antithrombin III-thrombin action in blood coagulation (Felding-Habermann and Cheresh, 1993; Preissner and Seiffert, 1998). Altered expression of VTN has been associated with various diseases such as cancer, hemorrhagic fever with renal syndrome, and coronary artery disease (Ekmekci et al., 2002; Liu et al., 2009; Ryschich et al., 2009). (viii)SARM1 (sterile alpha and TIR motif containing 1) is an almost ubiquitously expressed adaptor protein which negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling (Carty et al., 2006; Nagase et al., 1998). It has been suggested that *SARM1* is a candidate gene for hereditary infectious/inflammatory diseases (Mink and Csiszar, 2005).(ix) *SLC46A1* (solute carrier family 46 (folate transporter), member 1) codes for a transmembrane proton-coupled folate transporter protein that facilitates the movement of folate and antifolate substrates across cell membranes in acidic pH environments. This protein is expressed in the brain and choroid plexus where it transports folates into the central nervous system. It further functions as a transmembrane heme transporter in duodenal enterocytes and, potentially, in other tissues like liver and kidney. Its localization to the apical membrane or cytoplasm of intestinal cells is modulated by dietary iron levels. Mutations in this gene cause the autosomal recessive hereditary folate malabsorption (HFM) disease. HFM is characterized by folate deficiency due to reduced intestinal folate absorption and subsequent anemia, hypoimmunoglobulinemia, and recurrent infections (Qiu et al., 2006). None of the nine genes seems to be a strong phenocritical candidate for epilepsy/MR in patient 3-1.

Supplementary Table 2. 1: *GRIN2A* and *GRIN2B* sequence alterations not annotated in dbSNP

gene	nucleotide change	gene region	protein consequences	frequency of rare allele in patients	inheritance	frequency of rare allele in control chromosomes
GRIN2B	c.117C>T (het)	exon 2	p.=	1/306	maternal	–
	c.189C>T (het)	exon 2	p.=	1/630	maternal	–
	c.228C>T (het)	exon 2	p.=	2/936	maternal/paternal	–
	c.411+1G>A (het)	intron 2	–	1/630	*de novo*	0/360
	c.803_804delCA (het)	exon 3	p.T268SfsX15	1/306	*de novo*	0/360
	c.1126-12A>G (het)	intron 4	–	2/630	maternal/nd	–
	c.1560G>A (het)	exon 7	p.=	1/630	maternal	–
	c.1768G>A (het)	exon 8	p.A590T	2/936	maternal/paternal	–
	c.1780+9G>A (het)	intron 8	–	1/630	nd	–
	c.2044C>T (het)	exon 10	p.R682C	1/630	*de novo*	0/1080
	c.2360-2A>G (het)	intron 11	–	1/306	*de novo*	0/360
	c.2599-29T>C (het)	intron 12	–	1/630	maternal	–
	c.2628G>A (het)	exon 13	p.=	1/630	nd	–
	c.3552C>T(het/hom)	exon 13	p.=	3/306	paternal/nd	–
	c.3799G>T (het)	exon 13	p.A1267S	1/630	paternal	–
	c.3993G>A (het)	exon 13	p.M1331I	1/630	nd	0/360
	c.*70G>A (het)	exon 13	–	1/630	paternal	–
	c.*388C>T (het)	exon 13	–	1/630	maternal	–
	c.*611T>C (het)	exon 13	–	1/630	maternal	–
	c.*1116A>G (het)	exon 13	–	1/630	nd	–

het, heterozygous; hom, homozygous; p.= indicates that there is no effect on protein level expected; nd, not determined.

Supplementary Table 2.1 continued.

gene	nucleotide change	gene region	protein consequences	frequency of rare allele in patients	inheritance	frequency of rare allele in control chromosomes
GRIN2A	c.652C>T (het)	exon 4	p.Q218X	1/254	familial	0/360
	c.1007+36A>C (het)	intron 4	–	1/254	nd	–
	c.1122+51A>G (het)	intron 5	–	1/254	nd	–
	c.1757G>A (het)	exon 9	p.R586K	1/254	maternal	0/360
	c.1845C>A (het)	exon 10	p.N615K	1/254	*de novo*	0/1080
	c.2008-32_c.2008-31dupCT (het)	intron 10	–	2/254	nd	–
	c.3190A>G (het)	exon 14	p.T1064A	2/254	maternal/nd	0/360
	c.3228C>G (het)	exon 14	p.N1076K	2/254	nd	7/360

het, heterozygous; p.= indicates that there is no effect on protein level expected; nd, not determined.

Supplementary Table 2. 2: *In silico* protein analysis of amino acid substitutions in *GRIN2A* and *GRIN2B.*

protein	amino acid change	SIFT		PolyPhen		PANTHER		SNAP	
		score	prediction	score	prediction	score	$P_{deleterious}$	accuracy	prediction
GRIN2B	p.A590T	0.17	tolerated	0.519	benign	na		92%	neutral
	p.R682C	0.00	affects protein function	2.695	probably damaging	-7.10378	0.98376	78%	non-neutral
	p.A1267S	0.85	tolerated	0.077	benign	na		78%	neutral
	p.M1331I	0.34	tolerated	0.843	benign	na		85%	neutral
GRIN2A	p.R586K	0.04	affects protein function	1.670	possibly damaging	-3.03053	0.50763	58%	non-neutral
	p.N615K	0.00	affects protein function	2.296	probably damaging	-3.8302	0.6964	78%	non-neutral
	p.T1064A	0.59	tolerated	0.180	benign	na		78%	neutral
	p.N1076K	0.48	tolerated	1.737	possibly damaging	na		78%	neutral

Amino acid substitutions in GRIN2B and GRIN2A were analyzed by the SIFT, PolyPhen, PANTHER, and SNAP programs. When the program offered a choice of parameter settings, defaults were used. Tolerated, benign, and neutral indicate that the amino acid substitution is unlikely to affect protein function. SIFT prediction scores that fall below 0.05 are predicted to affect protein function. High values computed by the PolyPhen software indicate that the substituted amino acid residue is rarely or never observed in the protein family and therefore more likely to affect protein function. PANTHER scores are continuous values from 0 (neutral) to about −10 (most likely to be deleterious), and $P_{deleterious}$ indicates the probability of functional impairment. SNAP accuracy illustrates the likelihood that a prediction for a functional effect of an amino acid change is correct. na, not applicable (position does not align to Hidden Markov Model).

Supplementary Table 2. 3: *In silico* splice site prediction analysis of sequence alterations in GRIN2A and GRIN2B.

gene	nucleotide change	HSF 2.4		SpliceView		NetGene2		BDGP	
		wild-type	mutated	wild-type	mutated	wild-type	mutated	wild-type	mutated
GRIN2B	c.117C>T[a]	ASS 92.1 DSS 84.6	ASS 92.1 DSS 84.6	ASS 0.92 DSS 0.85	ASS 0.92 DSS 0.85	ASS 0.97 DSS 0.93	ASS 0.97 DSS 0.93	ASS 0.99 DSS 0.98	ASS 0.99 DSS 0.98
	c.189C>T[a]	ASS 92.1 DSS 84.6	ASS 92.1 DSS 84.6	ASS 0.92 DSS 0.85	ASS 0.92 DSS 0.85	ASS 0.97 DSS 0.93	ASS 0.97 DSS 0.93	ASS 0.99 DSS 0.98	ASS 0.99 DSS 0.98
	c.228C>T[a]	ASS 92.1 DSS 84.6	ASS 92.1 DSS 84.6	ASS 0.92 DSS 0.85	ASS 0.92 DSS 0.85	ASS 0.97 DSS 0.93	ASS 0.97 DSS 0.93	ASS 0.99 DSS 0.98	ASS 0.99 DSS 0.98
	c.411+1G>A	**DSS 84.55**	**DSS 57.71**	**DSS 0.85**	**no DSS**	**DSS 0.93**	**no DSS**	**DSS 0.98**	**no DSS**
	c.1126-12A>G	ASS 89.62	ASS 89.81	ASS 0.86	ASS 0.87	ASS 1.00	ASS 1.00	ASS 0.95	ASS 0.96
	c.1560G>A[a]	ASS 87.3 DSS 85.9	ASS 87.3 DSS 85.9	ASS 0.86 DSS 0.84	ASS 0.86 DSS 0.84	ASS 0.96 DSS 0.55	ASS 0.95 DSS 0.55	ASS 0.98 DSS 0.89	ASS 0.98 DSS 0.89
	c.1768G>A	ASS 90.0 DSS 96.3	ASS 90.0 DSS 96.3	ASS 0.93 DSS 0.91	ASS 0.93 DSS 0.91	ASS 1.00 DSS 1.00	ASS 1.00 DSS 0.99	ASS 1.00 DSS 0.99	ASS 1.00 DSS 0.99
	c.1780+9G>A	DSS 96.31	DSS 96.31	DSS 0.91	DSS 0.91	DSS 1.00	DSS 0.99	DSS 1.00	DSS 1.00
	c.2044C>T	ASS 94.6 DSS 91.9	ASS 94.6 DSS 91.9	ASS 0.92 DSS 0.89	ASS 0.92 DSS 0.89	ASS 0.96 DSS 0.89	ASS 0.95 DSS 0.89	ASS 1.00 DSS 0.98	ASS 1.00 DSS 0.98
	c.2360-2A>G	**ASS 94.2**	**no ASS**	**ASS 0.95**	**no ASS**	**ASS 1.00**	**no ASS**	**ASS 0.99**	**no ASS**
	c.2599-29T>C	ASS 94.8	ASS 94.8	ASS 0.90	ASS 0.90	ASS 0.95	ASS 0.95	ASS 0.87	ASS 0.87
	c.2628G>A[a,b]	ASS 94.8	ASS 94.8	ASS 0.90	ASS 0.90	ASS 0.95	ASS 0.95	ASS 0.87	ASS 0.87
	c.3552C>T[a,b]	ASS 94.8	ASS 94.8	ASS 0.90	ASS 0.90	ASS 0.95	ASS 0.95	ASS 0.87	ASS 0.87
	c.3799G>T[b]	ASS 94.8	ASS 94.8	ASS 0.90	ASS 0.90	ASS 0.95	ASS 0.95	ASS 0.87	ASS 0.87
	c.3993G>A[b]	ASS 94.8	ASS 94.8	ASS 0.90	ASS 0.90	ASS 0.95	ASS 0.95	ASS 0.87	ASS 0.87

Supplementary Table 2.3 continued

gene	nucleotide change	HSF 2.4		SpliceView		NetGene2		BDGP	
		wild-type	mutated	wild-type	mutated	wild-type	mutated	wild-type	mutated
GRIN2A	c.1007+36A>C	DSS 81.9	DSS 81.9	DSS 0.80	DSS 0.80	DSS 0.90	DSS 0.90	DSS 0.88	DSS 0.88
	c.1122+51A>G	DSS 95.71	DSS 95.71	DSS 0.91	DSS 0.91	DSS 1.00	DSS 1.00	DSS 0.99	DSS 0.99
	c.2008-32_2008-31dupCT	ASS 94.58	ASS 94.58	ASS 0.92	ASS 0.92	ASS 0.71	ASS 0.75	ASS 0.98	ASS 0.98

Splice site prediction for the intronic nucleotide changes in *GRIN2B* and *GRIN2A*. Donor and acceptor splice site prediction scores were calculated for wild-type and mutated sequences by using the computer programs HSF 2.4, SpliceView, NetGene2, and Berkeley Drosophila Genome Project (BDGP). High and low scores indicate strong and weak splice sites, respectively. Nucleotide changes resulting in loss of splice site are marked by red letters. [a], silent change; [b], this nucleotide change is located in the last exon of the gene; DSS, donor splice site; ASS, acceptor splice site.

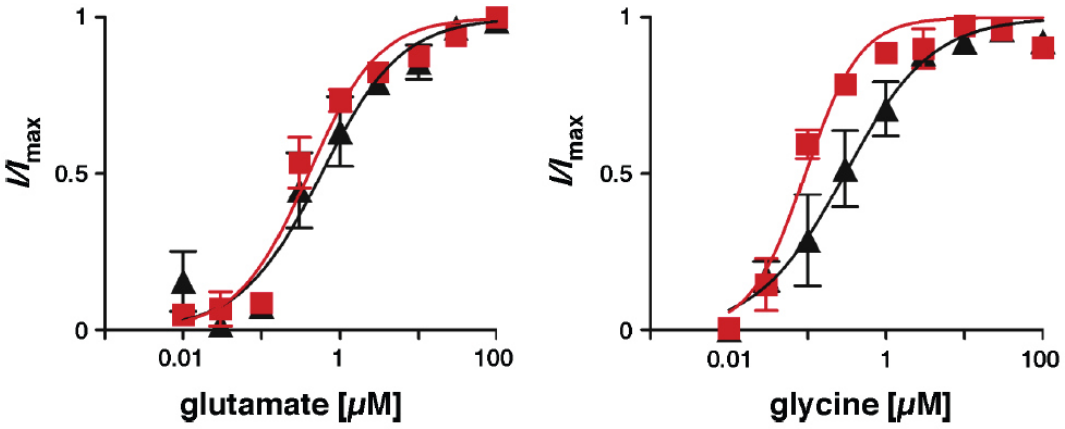
a evolutionary conservation of arginine 682 in NR2B (GRIN2B)

species	gene	prot. acc. no.	amino acid sequence
Human (*Homo sapiens*)	*GRIN2B*	NP_000825.2	QRPNDFSPPFRFGTVPNGSTE
Chimpanzee (*Pan troglodytes*)	*GRIN2B*	XP_528744.2	QRPNDFSPPFRFGTVPNGSTE
Rhesus monkey (*Macaca mulatta*)	*GRIN2B*	XP_001088140.1	QRPNDFSPPFRFGTVPNGSTE
Common marmoset (*Callithrix jacchus*)	*GRIN2B*	XP_002752166.1	QRPNDFSPPFRFGTVPNGSTE
Pig (*Sus scrofa*)	*NMDA2B*	XP_001924243.1	QRPNDFSPPFRFGTVPNGSTE
Dog (*Canis lupus familiaris*)	*GRIN2B*	NP_001008719.1	QRPNDFSPPFRFGTVPNGSTE
Horse (*Equus caballus*)	*NMDAR2B*	XP_001501691.2	QRPNDFSPPFRFGTVPNGSTE
Cow (*Bos taurus*)	*GRIN2B*	XP_617989.2	QRPNDFSPPFRFGTVPNGSTE
Rat (*Rattus norvegicus*)	*Grin2b*	NP_036706.1	QRPNDFSPPFRFGTVPNGSTE
Mouse (*Mus musculus*)	*Grin2b*	NP_032197.3	QRPNDFSPPFRFGTVPNGSTE
Chicken (*Gallus gallus*)	*GRIN2B*	XP_416204.2	QKPNDFSPPFRFGTVPNGSTE
Zebra finch (*Taeniopygia guttata*)	*GRIN2B*	XP_002195885.1	QKPNDFSPPFRFGTVPNGSTE
Clawed frog (*Xenopus laevis*)	*grin2b*	NP_001106368.1	QRPNDFSPAFRFGTVPNGSTE
Zebra fish (*Danio rerio*)	*NMDA2B*	NP_001121809.1	QNPNDFSPPFRFGTVPNGSTE
Knifefish (*Apteronotus leptorhynchus*)	*NR2B*	AAN65280.1	QRPNDFSPPFRFGTVPNGSTE
Roundworm (*Caenorhabditis elegans*)	*nmr-2*	NP_506694.3	NFPHDQKPPFRFGTVDGGNTH
Fruitfly (*Drosophila melanogaster*)	*Nmdar2*	NP_001014715.1	VHPFSHKPSFKFGTIPYSHTD

b evolutionary conservation of aspargine 615 in NR2A (GRIN2A)

species	gene	prot. acc. no.	amino acid sequence
Human (*Homo sapiens*)	*GRIN2A*	NP_001127879.1	IWLLWGLVFNNSVPVQNPKGT
Chimpanzee (*Pan troglodytes*)	*GRIN2A*	NP_001029361.1	IWLLWGLVFNNSVPVQNPKGT
Orangutan (*Pongo abelii*)	*GRIN2A*	XP_002826155.1	IWLLWGLVFNNSVPVQNPKGT
Common marmoset (*Callithrix jacchus*)	*GRIN2A*	XP_002755928.1	IWLLWGLVFNNSVPVQNPKGT
Dog (*Canis lupus familiaris*)	*GRIN2A*	XP_547132.2	IWLLWGLVFNNSVPVQNPKGT
Cow (*Bos taurus*)	*GRIN2A*	XP_874010.3	IWLLWGLVFNNSVPVQNPKGT
Opossum (*Monodelphis domestica*)	*GRIN2A*	XP_001366920.1	VWLLWGLVFNNSVPVQNPKGT
Rabbit (*Oryctolagus cuniculus*)	*NMDA2A*	XP_002711833.1	IWLLWGLVFNNSVPVQNPKGT
Rat (*Rattus norvegicus*)	*Grin2a*	NP_036705.3	IWLLWGLVFNNSVPVQNPKGT
Mouse (*Mus musculus*)	*Grin2a*	NP_032196.2	IWLLWGLVFNNSVPVQNPKGT
Platypus (*Ornithorhynchus anatinus*)	*NMDA2A*	XP_001506052.1	VWLLWGLVFNNSVPVQNPKGT
Chicken (*Gallus gallus*)	*GRIN2A*	XP_425252.2	VWLLWGLVFNNSVPVQNPKGT
Zebra finch (*Taeniopygia guttata*)	*GRIN2A*	XP_002194954.1	VWLLWGLVFNNSVPVQNPKGT
Clawed frog (*Xenopus laevis*)	*grin2a*	NP_001106367.1	VWLLWGLVFNNSVPVQNPKGT
Zebra fish (*Danio rerio*)	*NMDA2A*	XP_699070.4	VWLLWGLVFNNSVPVQNPRGT
Roundworm (*Caenorhabditis elegans*)	*nmr-2*	NP_506694.3	YWLVWATLFSASVSTDVPKST
Fruitfly (*Drosophila melanogaster*)	*Nmdar2*	NP_001014715.1	YWLVWAVLFQAAVHVDSPRGF

Supplementary Figure 2. 4: Conservation of amino acids R682 in human NR2B and N615 in human NR2A. Species, species-specific gene names, protein database accession numbers (www.ncbi.nlm.nih.gov/protein) and partial amino acids sequences are given. Orthologues residues identical to human NR2B R682 or human NR2A N615 are indicated in green letters and nonidentical in red letters. Both amino acid sequence alignments demonstrate high evolutionary conservation of R682 in NR2B (**a**) and N615 in NR2A (**b**).



Supplementary Figure 2. 5: Pharmacological characterization of the apparent glutamate and glycine affinities of wild-type NR1/NR2B and mutant NR1/NR2B^{R682C} NMDA receptors. Glutamate (left) and glycine (right) dose-response curves of wild-type NR1/NR2B (black triangles) and mutant NR1/NR2B^{R682C} (red squares) NMDA receptors were measured upon heterologous expression in *Xenopus laevis* oocytes by two-electrode voltage clamping (TEVC). The results revealed similar glutamate concentrations required for a half-maximal response (EC_{50} value) for wild-type NR1/NR2B and mutant NR1/NR2B^{R682C} receptors (left panel). Only a slight (2-fold) reduction in the EC_{50} value for glycine was seen for mutant NR1/NR2B^{R682C} NMDA receptors in the presence of saturating concentrations of glutamate (right). This indicates no major effect of the substitution R682C in the NR2B subunit on apparent agonist affinities of NR1/NR2B NMDA receptors. Values present data from 3–6 oocytes for each subunit combination.

Supplementary Table 2. 4: FISH data for patient 1 with t(9;12)(p23;p13.1).

Clone	FISH results		
	distal	spanning	proximal
9p23 (tel→cen)			
RP11-244O6	x		
RP11-176P17	x		
G248P88712E12	x		
G248P80486A9	x		
RP11-794P3		x	
RP11-268B5		x	
G248P88420E2			x
G248P80353A8			x
RP11-1144N20			x
RP11-784G11			x
RP11-702P22			x
RP11-348I12			x
RP11-382G4			x
12p13.1 (tel→cen)			
RP11-357D3	x		
RP11-66L13	x		
RP11-96K24		x	
G248P81952G10		x	
G248P86254B10			x
G248P89997E6			x
RP11-877I11			x
RP11-435K13			x
RP11-933E15			x
RP11-1083O6			x

Supplementary Table 2. 5: FISH data for patient 2 with t(10;12)(q21.1;p13.1).

Clone	FISH results		
	distal	spanning/deleted	proximal
10q21.1 (cen→tel)			
RP11-512N4			x
RP11-47O13			x
RP11-431O2			x
RP11-145J8			x
RP11-164B10		deleted	
RP11-641I2		deleted	
RP11-666M1		deleted	
RP11-53N12	x		
RP11-29J13	x		
12p13.1 (tel→cen)			
RP11-253I19	x		
RP11-392P7	x		
RP11-118K19	x		
RP11-99D14	x		
RP11-435K13		spanning	
RP11-243I16			x
RP11-103D14			x
RP11-515B12			x
RP11-495A12			x

Supplementary Table 2. 6: FISH data for patient 3-1 with t(16;17)(p13.2;q11.2).

Clone	FISH results		
	distal	spanning	proximal
16p13.2 (tel→cen)			
RP11-616M22	x		
RP11-95P2	x		
RP11-243A14	x		
RP11-788K23	x		
RP11-347G12	x		
RP11-77E6		x	
RP11-367P5			x
RP11-153N18			x
RP11-788A3			x
RP11-14K2			x
17q11.2 (cen→tel)			
RP11-663N22			x
RP11-1145F2			x
RP11-449M14			x
RP11-348E14		x	
RP11-915B21	x		
RP11-832J20	x		
RP11-1007K22	x		
RP11-1095J4	x		

Supplementary Table 2. 7: Primers for mutation analysis of *GRIN2B.*

exon	amplicon	amplicon size (bp)	forward primer name	forward primer 5'→3'	reverse primer name	reverse primer 5'→3'
1	AP_1	331	GRIN2B_e01_F	TTGTGAGCTGCTCTCCATACC	GRIN2B_e01_R	AAGATCCACTTGAGGGACTCG
2_1	AP_2	362	GRIN2B_e02_1_F	GGAGATAAGGTCCTTGAATTGC	GRIN2B_e02_1_R	GTGATGATGCTCTTTGGGTC
2_2	AP_3	371	GRIN2B_e02_2_F	GCATTGGCATTGCTGTCATCCTC	GRIN2B_e02_2_R	ATGCATGGTTTAGTCCTCAGC
3_1	AP_4	427	GRIN2B_e03_1_F	TCTCCATTACCATCTTTGGTTTG	GRIN2B_e03_1_R	ACGATCCACGTGTAGCCATAG
3_2	AP_5	424	GRIN2B_e03_2_F	CTCAAGAAACTTCAAAGCCCC	GRIN2B_e03_2_R	AACTTCCAACCCCAGTCTTTG
4	AP_6	305	GRIN2B_e04_F	TCTCCAGCCAGCTTTCTCTC	GRIN2B_e04_R	TAGGGACAAAAGCCAAAGGAC
5	AP_7	440	GRIN2B_e05_F_old	GGCTGTTTGTTATGTTGTGGC	GRIN2B_e05_R	AGCTGGACAGAACGAGACATC
6	AP_8	459	GRIN2B_e06_F	AAATTGTGCTGAGCTGTGAAG	GRIN2B_e06_R	CAGCCTATCAGTGGTTTTCTTTG
7	AP_9	284	GRIN2B_e07_F	CTGTATTTTCCTTGTGGTGGTCC	GRIN2B_e07_R	CCTTATTTCACTTCCCCATCC
8	AP_10	376	GRIN2B_e08_F	CTGAAGGCAGACAACACATTG	GRIN2B_e08_R	CACCTGAGGGTTCCTTTTCAG
9	AP_11	505	GRIN2B_e09_F	TAAAAGGGCTTGGTTTTAGCG	GRIN2B_e09_R	GACTTCTACTCCCATGTTCCAATAC
10	AP_12	430	GRIN2B_e10_F	GGGTCCCTTCCATTATAAATCC	GRIN2B_e10_R	GGAAATCCATAAAGAGCAAATG
11	AP_13	366	GRIN2B_e11_F	GCTTCATATTAATGGCCCCACAG	GRIN2B_e11_R	GGAAATGCACAGGTTAAAGAAATGG
12	AP_14	619	GRIN2B_e12_F	CATGTGTTCATATCGCCTGC	GRIN2B_e12_R	CATGATGTGGTTTCTTGCTTG
13_1	AP_15	547	GRIN2B_e13_01_F	GCAATTATTGGTGGGAGAGTG	GRIN2B_e13_02_R	CTTCTTGCTGATGGACCTGG
13_2	AP_16	558	GRIN2B_e13_03_F	AGGACAGCAACGTGTACCAAG	GRIN2B_e13_04_R	AGTCATCACTCCGCTCCTTG
13_3	AP_17	546	GRIN2B_e13_05_F	TACGGGACTTCTACCTGGACC	GRIN2B_e13_06_R	GCAGGTCCACGAAGGTGTC
13_4	AP_18	602	GRIN2B_e13_07_F	ACTCCCTGCAGGAACTGGAC	GRIN2B_e13_08_R	GTTAGGCACACAGGGGTTGG
13_5	AP_19	622	GRIN2B_e13_09_F	ACTTTTGGGGACGACCAGTG	GRIN2B_e13_10_R	GGAGCTCTTCCACACCAGGA
13_6	AP_20	552	GRIN2B_e13_11_F	GCACCATCTCTCCTCCTTTTC	GRIN2B_e13_12_R	CACAATCCACCTGCTCTTGTC
13_7	AP_21	519	GRIN2B_e13_13_F	TGGTGGTGAACAGAACAAAAG	GRIN2B_e13_14_R	GCTTTGTAGAATCTTTTGCTCCC
13_8	AP_22	602	GRIN2B_e13_14_F	CTTTCAATATCCCCAAGCAGTGTG	GRIN2B_e13_15_R	TCCCCTCTTTTCGTACCTCC

Supplementary Table 2. 8: Primers for mutation analysis of *GRIN2A.*

exon	amplicon	amplicon size (bp)	forward primer name	forward primer 5'→3'	reverse primer name	reverse primer 5'→3'
3	AP_3	664	GRIN2AEx_3F	CCGTGATCCCAGCAGCCTATCCTG	GRIN2AEx_3R	TCACATCAAGACAGATTCTAGGGG
4	AP_4	847	GRIN2AEx_4F	AGTAGATGCTCAGGAATGTCTGC	GRIN2AEx_4R	GTAAGTAAACACATAACATTCTGC
5	AP_5	365	GRIN2AEx_5F	GGCTTCTTGTGATGAATATAGCATTG	GRIN2AEx_5R	GTGAGATGGGATCTAGAGCAGCTC
6	AP_6	468	GRIN2AEx_6F	TGCATATGTTGAGATATGTGGCAG	GRIN2AEx_6R	GTATTATACCTACTATAACGACGTG
7/8	AP_7/8	720	GRIN2AEx_7F	GTGTGTCCTTGGGAAAGCCACTTC	GRIN2AEx_8R	CCTCTGAAATATGCTGCCATGGCC
9	AP_9	320	GRIN2AEx_9F	GAGAAAATATTTTTGGCGAGCCTGC	GRIN2AEx_9R2	TGGGATTACAGGCGTGAGCCACC
10	AP_10	486	GRIN2AEx_10F	GGGCAATCACAGGACACAACTATG	GRIN2AEx_10R	GGCTTTTGTGCATTCGAGTTGATG
11	AP_11	421	GRIN2AEx_11F	GCCCTAATGCATGCATTTACCTCC	GRIN2AEx_11R	GCCCATCTGGACCACAGTCACTC
12	AP_12	459	GRIN2AEx_12F	AGGCCATGAGTCATCATCACCAGC	GRIN2AEx_12R	CCATGGTTTCATGTGACAGGAACTC
13	AP_13	634	GRIN2AEx_13F	GAATAAGTTTGCTATACTCTCTCTC	GRIN2AEx_13R	CTTGTGACATGCCCAAGAAAGGC
14_1	AP_14	695	GRIN2AEx_14F	CTGTTTCTCCAGGGCTCCTGCAAG	GRIN2AEx_14R2	CATCTCTTCTGGAAGATACCTAGG
14_2	AP_15	915	GRIN2AEx_14F2	GCAGCTGTGGAAGAAATCCGTGG	GRIN2AEx_14R3	CTGTCCTTGAGGCTTATGCTCC
14_3	AP_16	702	GRIN2AEx_14F3	AGGACTGGGCACAGAACAATGCCC	GRIN2AEx_14R	TACCTCCCTACATCTTCTTCCTC

Supplementary note regarding human research subjects

Patients and their parents were clinically assessed by experienced clinical geneticists and/or neurologists. Blood samples were collected from patients and parents after obtaining informed consent. The study was approved by all Institutional Review Boards of the participating institutions. Clinical information pertinent to the diagnosis and, if applicable, results from laboratory testing were provided by the parents or legal guardians for minors, or by the evaluating authors (Y.H., L.V.M., M.M., U.M., A.Ra., S.v.S., I.S., L.V., N.V., D.W., B.Z., M.Z.). The 540 control probands were German (white) healthy blood donors as describe elsewhere (Huffmeier et al., 2009)[32].

Subjects

The first cohort for mutational screening of *GRIN2B* consisted of 315 consecutive patients seen either in the clinic of the University Departments of Human Genetics in Erlangen or Essen (Germany). 185 had mild to moderate (IQ>50) and 130 moderate to severe mental retardation (MR) (IQ<50). 187 were male and 128 female patients. 19 patients had a positive family history for MR, 296 patients were isolated cases. 60 patients had seizures, including one patient with absences, two patients with tonic clonic seizures, three with febrile convulsions and one with focal seizures. In the remaining patients the seizures were not classified in more detail. Standard evaluation consisted of detailed clinical investigation, normal chromosome analysis and subtelomeric screening, exclusion of fragile-X syndrome and any clinically recognizable syndrome. 70% of patients had molecular karyotyping using either Affymetrix 100K, 250K or 6.0 arrays without any obvious pathogenic aberration.

The second cohort for mutational screening of *GRIN2B* consisted of 153 patients evaluated at the University Department of Human Genetics in Heidelberg (Germany). 73 had mild to moderate (IQ>50), 57 moderate to severe MR (IQ<50), and in 23 patients the severity of MR was not specified. The male to female ratio was 90 to 63, including one patient with a positive family history for MR. 35 patients suffered from seizures: eight patients had complex focal seizures, another eight patients presented with BNS seizures, five patients with focal seizures, six patients with generalized seizures, one patient had multifocal seizures, one patient had partial epilepsy, one had myoclonic seizures and in five patients the seizures were not further classified. Standard evaluation consisted of detailed clinical investigation, normal chromosome analysis, exclusion of fragile-X syndrome and any clinically recognizable syndrome. All patients had molecular karyotyping using the Affymetrix 6.0 array without any obvious

pathogenic aberration.

The third cohort of patients comprised 127 individuals with a history of epilepsy and/or an abnormal EEG pattern, and in most of them a variable degree of MR. These patients were evaluated in the clinics of the University Departments of Neuropediatrics and Human Genetics in Kiel and Lübeck (Germany), Marseille (France), Liege and Antwerp (Belgium). For 99 patients detailed clinical data were available: 51 patients had mild to moderate (IQ>50), 44 moderate to severe (IQ<50) MR, and four patients had normal intelligence. 50 were males and 49 were females. 25 patients had a positive family history for seizures and/or epileptic activity in EEG, 74 patients were isolated cases. 40 patients were diagnosed with an epileptic encephalopathy including patients with “suppression-burst” pattern, West and/or Lennox-Gastaut syndrome and Dravet syndrome or severe idiopathic generalized epilepsy of infancy. In addition, 23 patients had a focal epilepsy, 27 patients suffered from a generalized epilepsy, four from absences and five patients had no obvious seizures but epileptic activity in EEG. Previous genetic testing in this cohort included array CGH analysis in 63 patients (all negative), *SCN1A* mutation screening in 13 cases and screening for copy number changes in *GRIN2A* in four patients (Reutlinger et al., 2010a). This cohort was analyzed for mutations in the *GRIN2A* gene (see Mutation analysis, *GRIN2A*).

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2.8 Declaration of own achievement within the publication

I designed, performed and analyzed all functional experiments on the mutations GluN2A p.N615K and GluN2B p.R682C with previous mutagenesis. The results are represented in the listed figures:

- Figure 2.3 d + e
- Supplementary Figure 2.5

I did not write the original manuscript, but I was involved in the rewriting-process within the revision. Additionally, I was involved in proofreading the manuscript during the submission- and revision-process.

3 Manuscript:

Mutations in GRIN2A cause idiopathic focal epilepsy with rolandic spikes (published online 11 of August 2013 in Nature Genetics)

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3.1 Abstract

Idiopathic focal epilepsy (IFE) with rolandic spikes is the most common childhood epilepsy comprising a phenotypic spectrum from rolandic epilepsy (also benign epilepsy with centrotemporal spikes, BECTS) to atypical benign partial epilepsy (ABPE), Landau-Kleffner syndrome (LKS) and epileptic encephalopathy with continuous spike-and-waves during sleep (CSWS) (Berg et al., 2010; Gobbi et al., 2006). The genetic basis is largely unknown. We detected novel heterozygous mutations in *GRIN2A* in 27/359 patients from two independent cohorts with IFE (7.5%; p=4.83x10^{-18}, Fisher's exact test). Mutations occurred significantly more frequent in the more severe phenotypes with mutation detection rates ranging from 12/245 (4.9%) in patients with BECTS to 9/51 (17.6%) in patients with CSWS (p=0.009, Cochran Armitage test for trend). In addition, exon-disrupting microdeletions were found in 3/286 patients (1.0%; p=0.004, Fisher's exact test). These results establish alterations of the gene encoding the NMDA-receptor NR2A subunit as a major genetic risk factor for IFE.

3.2 Paper

Within the spectrum of idiopathic (genetic) focal epilepsy (IFE) with rolandic spikes BECTS is characterized by focal and secondarily generalized seizures, which usually remit by puberty. The EEG shows rolandic spike and wave discharges (mainly centrotemporal spikes, CTS) as the characteristic hallmark(Stephani, 2000). ABPE, CSWS and LKS refer to more severe disorders with various seizure types and/or a highly pathological (sleep) EEG (Gobbi et al., 2006; Hughes, 2011) as well as cognitive, language and behavioral deficits.

The genetic causes of IFE are largely unknown, although family studies have provided evidence for a presumed autosomal dominant inheritance of CTS (Bali et al., 2007; Vadlamudi et al., 2006). Linkage studies identified loci for CTS on chromosomes 15q14 (Neubauer et al., 1998) and 11p13 (Pal et al., 2010). Association of CTS with markers in *ELP4* has been replicated, but no causative mutation has yet been identified (Strug et al., 2009). Following hints from a microdeletion study published by our group that *GRIN2A,* encoding the alpha-2-subunit (NR2A) of the N-methyl-D-aspartate (NMDA)-selective glutamate receptor, could be an interesting candidate gene in IFE (Reutlinger et al., 2010b), we here identify various mutations in *GRIN2A* in two large independent cohorts comprising the whole spectrum of IFEs.

We first performed a mutation analysis in cohort I (*screening cohort*) comprising 39

patients with IFE and CTS (for all cohorts: Supplementary Table 1). One patient (index patient 1, 1/39, 2.6%) with BECTS and learning difficulties was identified carrying a novel missense mutation (c.728C>T, p.Ala243Val, family history and segregation unknown, parents unavailable) in *GRIN2A* predicted to be located in the Zn^{2+}-binding domain of the glutamate-gated NR2A (Fig. 3.1a,b). Maximal inducible currents, agonist affinities and relative open probabilities of mutant NR1-NR2A^{Ala243Val} receptors were not significantly different upon heterologous expression from those of the respective receptors containing the wild-type NR2A subunit (Fig. 3.1c,d). In contrast, currents for NR1-NR2A, NR1-NR2A-NR2A^{Ala243Val} and NR1-NR2A^{Ala243Val} receptors show a gradual loss of high-affinity inhibition by 0.1 µM Zn^{2+} ($p<0.01$, Fig. 3.1e), suggesting an increased activation *in vivo* due to impaired tonic inhibition of NR1/NR2A^{Ala243Val} receptors at physiological concentrations of Zn^{2+}. Furthermore, two different mutations in *GRIN2A* were identified by *next generation sequencing* (NGS) of >300 known and suggested epilepsy genes for diagnostic purposes (Lemke et al., 2012) in two patients with Landau-Kleffner syndrome not included in cohort I. Index patient 2 carries a novel truncating mutation c.2041C>T, p.Arg681* (pedigree shown in Fig. 2), whilst index patient 3 has a novel splice site mutation c.1007+1G>A (family history positive, segregation unknown, parents unavailable), both predicting non-functional proteins.

Motivated by these findings and to evaluate the significance of these preliminary results, we recruited cohort II (*validation cohort*) including 119 additional independent IFE patients. Sanger sequencing of *GRIN2A* revealed mutations in 8/119 patients (6.7%) that were not listed in dbSNP, 1000Genomes or the Exome Variant Server (EVS) databases. Regarding the different sub-entities, mutations were found in 0/3 patients with isolated CTS, 1/48 patients with BECTS (2.1%), 1/17 patients with ABPE (5.9%), 0/17 patients with LKS and 6/34 patients with CSWS (17.6%) (Table 3.1, Supplementary Tables 1-3, Supplementary Fig. 1). We finally replicated these findings in cohort III (*replication cohort*) comprising 240 additional patients who underwent whole-exome sequencing and subsequent validation by Sanger sequencing. The proportion of *GRIN2A* mutation carriers in the replication cohort was similar to that of the validation cohort, with an overall mutation rate of 19/240 (7.9%). Mutations were identified in 0/2 patients with isolated CTS, 11/197 patients with BECTS (5.6%), 4/20 patients with ABPE (10.0%), 1/4 patients with LKS (25.0%) and 3/17 patients with CSWS (17.6%) (Table 3.1, Supplementary Tables 1-3, Supplementary Fig. 1).

We then combined cohorts II and III for subsequent statistical analysis, providing a total of 359 patients. Mutations occurred at significantly higher frequency in our patient

cohort (27/359; 7.5%) than in the EVS (37/6503; 0.6%) that was used as a reference of unaffected controls (p=4.83×10^{-18}, Fisher's exact test). Restricting the comparison to patients and controls of European ancestry yielded similar results (26/315 *vs* 27/4300, p=1.18×10^{-16}, Fisher's exact test). The frequency of mutation carriers significantly increased towards more severe phenotypes (p=0.009; Cochran Armitage test for trend, Supplementary Fig. 2). Furthermore, the severity of phenotypes showed substantial association with the type of mutation (Pearson's corrected contingency coefficient C_{corr}= 0.52; Supplementary Fig. 3). For index patient 2 and 19 cases from cohorts II and III, additional family information was available. Of these, two cases were found to have different *de novo* mutations. The remaining 17 cases each exhibited a novel mutation that co-segregated with a phenotype of different epileptic disorders (often but not exclusively associated with CTS) and various degrees of intellectual disability within the family (Fig. 3.2). The *GRIN2A* locus showed significant linkage to this phenotype in these 17 families (two-point parametric LOD score of 3.55 under a dominant risk model with reduced penetrance of 80% and complete linkage).

In addition, 286 IFE patients were screened for copy number variations (CNVs) in *GRIN2A* using the Illumina HumanOmniExpress BeadChip® (Illumina Inc., San Diego, CA) (cohort IV, *CNV cohort*). This cohort included all patients from cohort III (i.e. an overlap of 83.9%) and 46 additional IFE patients. Out of 286 patients, three (1.0%) were identified with exon-disrupting microdeletions within *GRIN2A* (Supplementary Fig. 4). An additional intronic duplication was found to segregate with the phenotype but remains of unknown significance (Supplementary Fig. 4). CNVs of *GRIN2A* occurred significantly more often in patients than in controls (3/286 *vs* 0/1520, p=0.004).

Our investigations have revealed mutations as well as exon-disrupting CNVs within *GRIN2A* in a significant subset of IFE patients. We therefore postulate that genetic alterations in *GRIN2A* are not only a major genetic risk factor, but are compatible with a monogenetic trait for IFE in up to 7.5% of patients. However, additional modifying factors might explain phenotypic variability. Similar to other idiopathic epilepsies (Helbig et al., 2009), mutations were occasionally identified in apparently non-affected relatives suggesting a mosaic status or incomplete penetrance (although EEG abnormalities and rare seizures might have been missed in these individuals). A significant trend towards higher mutation rates in more severe phenotypes was demonstrated, which is similar to other epilepsy genes such as *SCN1A* (Claes et al., 2009; Marini et al., 2007).

NMDA receptors are tetrameric ligand-gated ion channels, composed of two NR1

subunits and two of four NR2 subunits (NR2A-D), which bind glutamate and determine the NMDA receptor subtype location as well as functional properties of synaptic transmission and plasticity (Paoletti, 2011). Changes in NMDA receptor function have been demonstrated in animal models of temporal lobe epilepsy (Di Maio et al., 2012; Frasca et al., 2011; Niimura et al., 2005) and the Stargazer mouse model of idiopathic absence epilepsy (Lacey et al., 2012). Earlier studies also suggested evidence for a role of *GRIN2A* alterations in individual patients with epilepsy (Endele et al., 2010; Lesca et al., 2012; Reutlinger et al., 2010b). NMDA receptors are tonically inhibited by Zn^{2+}, a mechanism which has been shown to protect neurons against NMDA receptor mediated over-excitation and glutamate toxicity *in vitro*.

Functional analysis of the missense mutation p.Ala243Val (index patient 1) demonstrated impaired reduction of receptor currents by low concentrations of Zn^{2+} suggesting increased activation of the NR1/NR2A heteromer due to reduced high-affinity Zn^{2+}-mediated inhibition *in vivo*. Accordingly, relief of NMDA receptors from tonic Zn^{2+} inhibition results in a higher susceptibility and enhanced Ca^{2+} influx. This mechanism should be particularly effective at synaptically localized NR1/NR2A receptors, due to their high-affinity Zn^{2+}-binding site. This finding is in stark contrast to the loss of function predicted by truncating mutations, frameshift mutations or deletions of *GRIN2A (Lesca et al., 2012; Reutlinger et al., 2010)*. However, different molecular alterations of NMDA receptor subunit genes might lead to similar changes in subunit composition resulting in comparable changes of electrophysiological properties of the receptor (Balu and Coyle, 2011). Moreover, similar phenomena are known from other epilepsy-associated genes such as mutations and deletions of *SCN1A* in Dravet syndrome (Marini et al., 2011).

IFE is characterized by an age-dependent clinical phenotype. NMDA receptor subunit composition is also age-dependent with a switch from predominantly NR2B expression in early development to more prominent NR2A expression at later stages (Paoletti, 2011). Therefore, alterations in NR2A may become relevant only in specific age groups (Henson et al., 2012).

In summary, we report genetic alterations in *GRIN2A* in 7.5% of patients with IFE rendering alterations of *GRIN2A* a major genetic risk factor. This is of particular importance as NMDA receptors are promising targets for epilepsy treatment (Ghasemi and Schachter, 2011).

URLs

dbSNP build 135 (http://www.ncbi.nlm.nih.gov/projects/SNP/)

1000Genomes database (http://www.1000genomes.org/)

Exome Variant Server (http://evs.gs.washington.edu/EVS/)

PolyPhen2 (http://www.genetics.bwh.harvard.edu/pph2)

MutationTaster (http://www.mutationtaster.org)

SpliceView (http://zeus2.itb.cnr.it/~webgene/wwwspliceview_ex.html)

HSF2.4 (http://www.umd.be/HSF)

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Author Contributions

Study design: J.R.L., S.v.S., I.H., S.B., E.M.R., F.Z., D.L., B.A.N., H.L. Subject ascertainment and phenotyping: I.H., J.A.J., H.M., U.S., R.B., W.v.P, R.C., N.F., M.A., S.W., P.DeJ., J.L., R.S.M., H.H., L.A., S.T., E.H., D.K.P., K.V., U.V., T.T., P.D., R.G.L., J.M.S., T.L., A.-E.L., S.B., G.W., J.K., A.N.D., S.R., M.W., B.F., G. Ku., G. Kl., A.H., D.E.H., C.K., J.S., F.B., Y.G.W., H.L., M.F., H.S., B.N., G.M.R., U.G.-S., J.G., F.Z., B.A.N., J.R.L., S.v.S. Mutation analysis of cohort 1: S.v.S., I.H., K.F., Ma.S., A.F. NGS-Panel analysis of index patients: I.S., S.B. Mutation analysis of cohort 2: C.W., J.R.L., S.B. Segregation Analysis of cohort 2: C.W., S.B. Mutation analysis of cohort 3: E.M.R., D.L., J.A., M.R.T., H.T., P.N. Segregation Analysis of cohort 3: E.M.R., D.L. CNV control cohort: P.H., S.H. Statistical Analysis: M.N. Functional analysis of *GRIN2A* missense mutation: Mi.S., K.G., B.L. Data interpretation: H.L., R.J.H., Mi.S., B.L., J.R.L., I.H., S.v.S., S.B., D.L., E.M.R., M.N., B.A.N., F.Z. Manuscript writing: J.R.L., S.v.S., S.B., B.L., M.N., E.M.R., F.Z., D.L., B.A.N. All authors contributed to the final version of the manuscript.

Competing Financial Interest

The authors declare no competing financial interests.

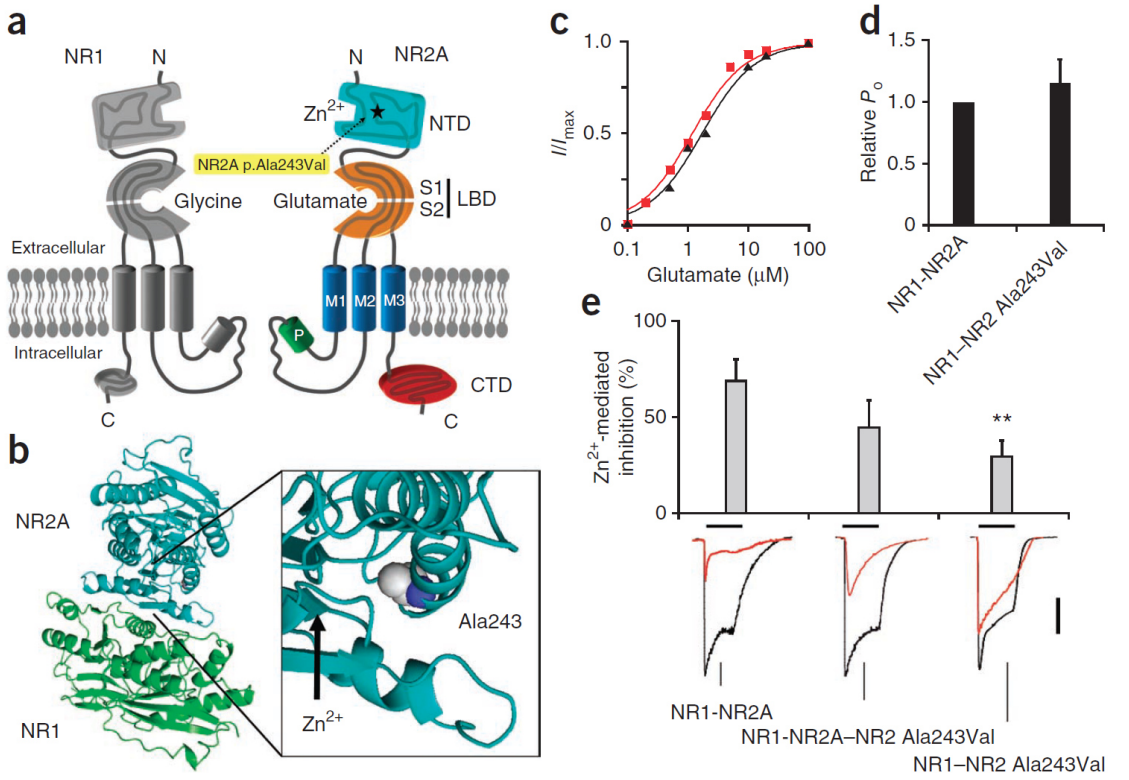

Figure 3. 1: Structural and functional consequences of missense mutation p.Ala243Val in *GRIN2A*

Functional analysis of the missense mutation p.Ala243Val (index patient 1) revealed a significant reduction of high-affinity Zn^{2+} inhibition whereas current amplitude, glutamate/glycine affinities or relative open probability remained unchanged. (**a**) Topology model of an NR1 and an NR2A subunit. Position of p.Ala243Val is indicated by asterisk in the NR2A subunit consisting of an amino-terminal domain (NTD), the ligand-binding domain (LBD) including the S1 and S2 peptide segments, three transmembrane segments (M1, M2 and M3), a re-entrant pore loop (P) and an intracellular carboxy-terminal domain (CTD). Residue Ala243 lies within the Zn^{2+}-binding NTD in NR2A. N, NH_2-terminus; C, COOH-terminus. (**b**) Model of the NR2A NTD (cyan) together with an adjacent NR1 NTD (green). Enlargement shows residue Ala243 within the Zn^{2+}-binding NR2A NTD. (**c**) Pharmacological characterization of the apparent agonist affinities of wild-type NR1/NR2A and mutant NR1/NR2A^{Ala243Val} NMDA receptors. Glutamate and glycine dose-response curves of wild-type NR1/NR2A (black triangles) and mutant NR1/NR2A^{Ala243Val} (red squares) NMDA receptors were measured upon heterologous expression in *Xenopus laevis* oocytes by two-electrode voltage clamping (TEVC; n=5). The results revealed similar glutamate and glycine (not shown) concentrations required for a half-maximal response (*EC*50 value; means ± standard deviation) for wild-type and mutant receptors. (**d**) Maximal current responses and kinetics of the open channel blocker MK-801 to wild-type NR1/NR2A and mutant NR1/NR2A^{Ala243Val} NMDA receptors reveal

similar channel activity. Maximal agonist-inducible currents and rate kinetics of MK-801-mediated inhibition were used for determining the relative open probability (P_o) of wild-type NR1/NR2A vs. mutant NR1/NR2A^{Ala243Val} NMDA receptors. (**e**) Inhibition of agonist-evoked currents by low concentrations of Zn^{2+} at wt and mutant NR1/NR2A receptors. Currents for NR1-NR2A, NR1-NR2A-NR2A^{Ala243Val} and NR1-NR2A^{Ala243Val} receptors show a gradual loss of high-affinity inhibition by 0.1 µM Zn^{2+} (n=5; $p<0.01$; Student's *t*-test). Traces show currents for NR1-NR2A, NR1-NR2A-NR2A^{Ala243Val} and NR1-NR2A^{Ala243Val} receptors in the absence (black) and presence of 0.1 µM Zn^{2+} (red traces).

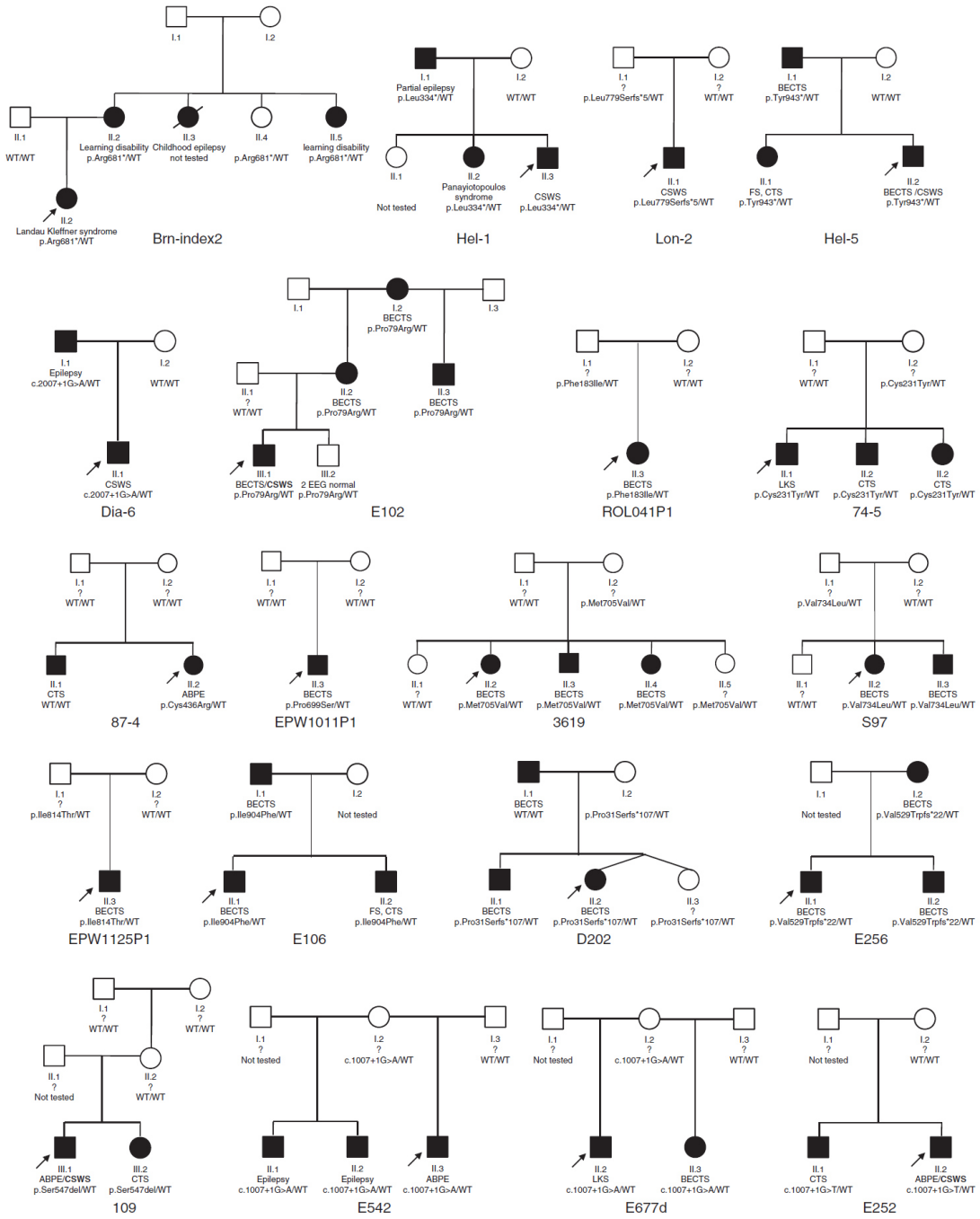

Figure 3. 2: Pedigrees of patients with available family information

Analysis of possible segregation of the respective mutation in family members could be performed for index patient 2 and 19/27 mutation carriers of cohorts II and III where DNA samples of family members were available. The respective *GRIN2A* mutation segregated with a variable phenotype of seizures, pathologic EEG patterns and/or intellectual disability in family members. A few individuals carried the respective familial mutation but did not present any clinical features, indicating incomplete penetrance of the mutations or mosaicism. However, subclinical phenotypes (e.g. EEG patterns) have not been investigated in these individuals. Pedigree 87-4 is suggestive for a phenocopy in the proband's brother.

Table 3. 1: Novel mutations detected in *GRIN2A*

ID	Case	Epilepsy syndrome	DNA mutation	Protein alteration
	K_i-index1	BECTS	c.728C>T	p.Ala243Val
	Brn-index2	LKS	c.2041C>T	p.Arg681*
	Brn-index3	LKS	c.1007+1G>A	IVS4, p.?
1	Ant-18	BECTS	c.1108C>T	p.Arg370Trp
2	Ant-11	CSWS	c.2140G>A	p.Glu714Lys
3	K_i-11	CSWS	c.2927A>G	p.Asn976Ser
4	K_i-40[a]	ABPE	c.594G>A	p.Trp198*
5	Hel-1	Panayiotopoulos/**CSWS**	c.1001T>A	p.Leu334*
6	Lon-2[b]	ABPE/**CSWS**	c.2334_2338delCTTGC	p.Leu779Serfs*5
7	Hel-5	CSWS	c.2829C>G	p.Tyr943*
8	Dia-6	CSWS	c.2007+1G>A	IVS7, p.?
9	E102	BECTS/**CSWS**	c.236C>G	p.Pro79Arg
10	ROL 041P1	BECTS	c.547T>A	p.Phe183Ile
11	74-5	LKS	c.692G>A	p.Cys231Tyr
12	EPW 1109P1	BECTS	c.869C>T	p.Ala290Val
13	87-4	ABPE	c.1306T>C	p.Cys436Arg
14	EPW 1011P1	BECTS	c.2095C>T	p.Pro699Ser
15	3619	BECTS	c.2113A>G	p.Met705Val
16	EPW 1083P1	BECTS	c.2179G>A	p.Ala727Thr
17	S97	BECTS	c.2200G>C	p.Val734Leu
18	EPW 1128P1	ABPE	c.2314A>G	p.Lys772Glu
19	EPW 1125P1	BECTS	c.2441T>C	p.Ile814Thr
20	E106	BECTS	c.2710A>T	p.Ile904Phe
21	ROL 057P1	ABPE	c.2927A>G	p.Asn976Ser
22	D202	BECTS	c.90delTins(T)2	p.Pro31Serfs*107
23	E256	BECTS	c.1585delG	p.Val529Trpfs*22
24	109-4	ABPE/**CSWS**	c.1637_1639delCTT	p.Ser547del
25	E542	ABPE	c.1007+1G>A	IVS4, p.?
26	E677d	BECTS	c.1007+1G>A	IVS4, p.?
27	E252	ABPE/**CSWS**	c.1007+1G>T	IVS4, p.?
28	NB3	ABPE	CNV deletion	CNV deletion
29	72-3	BECTS	CNV deletion	CNV deletion
30	EPW 1111P1	BECTS	CNV deletion	CNV deletion
31	145	CSWS	CNV duplication	CNV duplication

BECTS, benign epilepsy with centrotemporal spikes (rolandic epilepsy); Panayiotopoulos, Panayiotopoulos syndrome; ABPE, atypical benign partial epilepsy; LKS, Landau-Kleffner syndrome; CSWS, epileptic encephalopathy with continuous spike and wave during sleep. Phenotype appears in bold for individuals who had either BECTS or ABPE and electrical status in sleep, which were subsumed under the phenotype of CSWS.
[a]Array-based CGH (aCGH) analysis identified a duplication at 8q11.23 and a duplication at 15q26.1q26.2. [b]aCGH analysis identified a *de novo* microduplication at 22q11.21 and a small maternally inherited intronic deletion of *NRXN1*.

3.3 Methods

Study Design.

The overall study design is demonstrated in Supplementary Figure 5. Following reports on microdeletions including *GRIN2A* in patients with complex neurodevelopmental phenotypes, epilepsy and centrotemporal spikes as a common feature of the EEG (Reutlinger et al., 2010b), we started sequencing a small cohort of 39 patients with idiopathic focal epilepsies (*screening cohort*) and identified a first index patient with BECTS, learning difficulties and a missense mutation (p.Ala243Val) in *GRIN2A*. In addition, consecutive *Next Generation Sequencing (NGS) Epilepsy Panel Analysis* for diagnostic purposes revealed two additional index patients with LKS and mutations in *GRIN2A* (p.Arg681* and c.1007+1G>A, IVS4, p.?). In these patients, molecular genetic analysis of 323 genes that are known to be involved in epilepsy was performed using a targeted NGS approach (*Epilepsy Panel Version 2*) as recently described (Lemke et al., 2012). No other patients with mutations in *GRIN2A* were identified using this analysis method to date.

Motivated by these findings, we analyzed a second cohort (*validation cohort*) of 119 patients with idiopathic focal epilepsies and confirmed our findings by analysis of a third cohort (*replication cohort*) of additional 240 patients. For statistical analysis, data from cohorts II and III were combined giving a total cohort of 359 patients with idiopathic focal epilepsies of childhood and compared to publically available sequence control data of the *Exome Variant Server* (see below).

In addition to sequence analysis, we performed CNV analysis in a fourth cohort (*CNV cohort*) and compared these data to CNV data from 1520 platform- and ethnicity-matched in-house controls.

Patients.

Cohort I (*screening cohort*) comprised patients with idiopathic focal epilepsies with rolandic spikes recruited at the Department of Neuropediatrics at the University Hospital Schleswig-Holstein (Kiel, Germany) and the Northern German Epilepsy Center for Children and Adolescents (Schwentinental/Raisdorf, Germany).

For follow-up studies, study cohort II (*validation cohort*) was recruited by partners at European and Argentinian epilepsy centers, children's hospitals and departments of neuropediatrics and neurology.

For replication, patients for study cohort III (*replication cohort*) were recruited by collaborating centers of the EuroEPINOMICS-CoGIE (Complex genetics of idiopathic epilepsies) initiative (www.euroepinomics.org).

Cohort IV for CNV analysis (*CNV cohort*) included all patients of cohort III as well as additional patients recruited at the participating centers.

A summary of cohorts I - IV is given in Supplementary Table 4.

Phenotyping was performed according to the 2001 and 2010 ILAE classification schemes (Berg et al., 2010; Engel, 2001). For analysis, the following epilepsy syndromes were used: benign childhood epilepsy with centrotemporal spikes (BECTS, also rolandic epilepsy), atypical benign partial epilepsy (ABPE), Landau-Kleffner syndrome (LKS) and epileptic encephalopathy with continuous spike-waves during sleep (CSWS). Rare forms of benign occipital epilepsy (Panayiotopoulos syndrome, Gastaut syndrome) were subsumed under BECTS as these syndromes often show overlapping features. Patients who had either BECTS or ABPE and electrical status in sleep were subsumed under the phenotype of CSWS as this is a rather atypical feature in these epilepsy syndromes and will influence and probably change the clinical outcome. In LKS, electrical status in sleep is a frequent symptom of the syndrome. Here, CSWS was used synonymously with “electrical status epilepticus in slow wave sleep”, ESES (Fernandez et al., 2012).

All patients and/or their legal guardians gave written informed consent. The study protocol was approved at all sequencing centers (Kiel, Tübingen, Cologne, all: Germany). Approval for patient recruitment and inclusion in epilepsy genetics studies is available at all participating centers.

Control cohorts.

For statistical analysis of mutation frequencies, control data were derived from the *Exome Variant Server* (NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (http://evs.gs.washington.edu/EVS/), release (ESP6500SI), accessed 11/2012).

Data from CNV analysis were compared to 1520 in-house controls matched for analysis platform and geographic origin. This cohort was drawn from the HNR (Heinz Nixdorf RECALL) population-based epidemiological study consisting of males and females aged 45 to 75 years from an unselected urban population from the Ruhr area in Germany (Schmermund et al., 2002).

DNA-extraction from blood samples.

DNA from individual blood samples was extracted locally at the recruitment centers using commercially available kits.

Mutation screening and CNV analysis.

Sequence analysis of cohorts I and II:

For cohort I and II, mutation analysis of *GRIN2A* including all coding exons and exon-intron boundaries was performed using primers given in Supplementary Tables 5a and 5b. PCR amplification and bidirectional sequencing were done following standard protocols(Franke et al., 2008). New and known polymorphisms as well as InDels were identified by NovoSNP(Weckx et al., 2005) and Sequence Pilot^{CE} (JSI Medical Systems GmbH, Kippenheim, Germany).

Sequence analysis of cohort III:

Sequence analysis of cohort III was performed using next generation sequencing techniques. In brief, DNA was fragmented using sonification technology (Covaris, Woburn, MA, USA) and fragments were end repaired and adaptor ligated. SeqCap EZ Human Exome Library® v2.0 (Roche NimbleGen, Madison, WI, USA) was used for enrichment and samples were analyzed on the Illumina HiSeq 2000® sequencer. Only exome data with an average coverage >30x for 85% of the target sequences were included in the analysis. Data were filtered using Illumina Realtime Analysis® (RTA) software v1.8 and mapped to the human genome reference build hg19 via the ELANDv2 alignment algorithm on a multinode compute cluster. PCR duplicates were excluded using CASAVA v1.8. Variant calling was performed by SAMtools (version 0.1.7) for InDel detection. Scripts developed in-house at the Cologne Center for Genomics (Cologne, Germany) were applied to detect protein changes, affected splice sites, and overlaps with known variants. In particular, variants were filtered for high-quality unknown variants in *GRIN2A* compared to an in-house variation database, *dbSNP* build 135 (www.ncbi.nlm.nih.gov/projects/SNP/), *1000Genomes database* (www.1000genomes.org/) and the *Exome Variant Server*.

Pathogenic implications of identified coding variants were assessed by different *in silico* analysis programs (PolyPhen2: www.genetics.bwh.harvard.edu/pph2 and MutationTaster www.mutationtaster.org). For intronic SNPs, splice site analysis was performed using SpliceView (http://zeus2.itb.cnr.it/~webgene/wwwspliceview_ex.html) and HSF2.4 (www.umd.be/HSF).

CNV analysis.

Whole blood DNA was genotyped for 730,525 markers using the Illumina HumanOmniExpress BeadChip® (Illumina Inc., San Diego, CA) according to the manufacturer's protocol. Genotypes were analyzed with the Illumina Genome Studio® genotyping module (v.2011). CNV calls were generated by using the PennCNV software (Wang et al., 2007) by the use of the log R ratio (LRR) and B allele frequency (BAF) for all probes included on the genotyping chips. Analysis was restricted to CNVs larger than 30kb and coverage of at least five consecutive probes. All potential microdeletions were manually inspected for the regional SNP heterozygosity state and log2 ratios of the signal intensities to exclude technical artifacts. Subsequently, *GRIN2A* CNV validation of the index patient and CNV segregation in the family was conducted by MLPA® (MRC-Holland) and CNV analysis of WES data, respectively.

Statistical analysis. *Sequencing studies.*

For statistical analysis of sequencing data, patients from cohorts II and III (i.e. *validation* and *replication cohorts*) were combined. Due to the small sample size of cohort I and isolated index patients 2 and 3, patients of this *screening cohort* were excluded from statistical analysis.

Fisher's exact test was used to compare mutation frequencies in cases and EVS controls. To exclude potential population stratification effects, an additional analysis was performed comparing only patients with European ancestry (geographic origin determined by surname and/or self-reported ethnicity; excluding patients from Turkey and Russia as these countries cross borders between Europe and Asia and non-European countries) against the EVS-European-American controls.

A Cochran Armitage test for trend was used to test the hypothesis of higher mutation frequencies in more severe phenotypes and a Pearson's corrected contingency coefficient was calculated to demonstrate an association between the severity of phenotypes and the type of mutation. All tests were carried out using the R statistical environment v2.15.1 (www.R-project.org).

Two-point linkage analysis was performed using the LINKAGE package(Lathrop et al., 1984). We assumed a dominant mode of inheritance with a reduced penetrance of 80% and complete linkage between *GRIN2A* and the disease locus.

CNV studies:

A Fisher's exact test was used to compare the frequency of exon-disrupting CNVs in cases and in-house controls.

Functional studies.

For *Xenopus laevis* oocyte experiments, GRIN2A and GRIN1 constructs and capped cRNAs were generated as described previously (Endele et al., 2010). Individual stage V to VI oocytes were obtained from anaesthetized frogs and isolated by collagenase treatment. 10 ng of total GRIN1/2A cRNA were injected into oocytes. Following injection, oocytes were kept at 17 °C in ND96 solution (in mM) (96 NaCl, 2 KCl, 1.8 CaCl2, 1 MgCl2, 5 HEPES, pH 7.4). Glutamate, glycine and Zn^{2+}-dose-response curves of wild-type NR1/NR2A and mutant NR1/NR2A^{Ala243Val} NMDA receptors were analyzed by two-electrode voltage clamp recording. Molecular modeling of the NMDA receptor subunits was taken from a previous study (Endele et al., 2010).

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3.5 Declaration of own achievment within the publication

I performed and analyzed part of the functional experiments on the mutation GluN2A p.Ala243Val with previous mutagenesis.

The results are represented in Figure 3.1 c + e.

4 Manuscript:

GRIN2B mutations in West syndrome and intellectual disability with focal epilepsy (published 18 of November 2013 in Annals of Neurology)

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4.1 Abstract

Objective: To identify novel epilepsy genes using a panel approach and describe the functional consequences of mutations.

Methods: Using a panel approach, we screened 357 patients comprising a vast spectrum of epileptic disorders for defects in genes known to contribute to epilepsy and/or intellectual disability (ID). After detection of mutations in a novel epilepsy gene, we investigated functional effects in *Xenopus laevis* oocytes and screened a follow-up cohort.

Results: We revealed *de novo* mutations in *GRIN2B* encoding the NR2B subunit of the NMDA receptor in 2 individuals with West syndrome and severe developmental delay as well as 1 individual with ID and focal epilepsy.

The patient with ID and focal epilepsy had a missense mutation in the extracellular glutamate-binding domain (p.Arg540His) whereas both West syndrome patients carried missense mutations within the NR2B ion channel-forming re-entrant loop (p.Asn615Ile, p.Val618Gly). Subsequent screening of 47 patients with unexplained infantile spasms did not reveal additional *de novo* mutations, but detected a carrier of a novel inherited *GRIN2B* splice site variant in close proximity (c.2011-5_2011-4delTC).

Mutations p.Asn615Ile and p.Val618Gly cause a significantly reduced Mg^{2+} block and higher Ca^{2+} permeability leading to a dramatically increased Ca^{2+} influx, whereas p.Arg540His caused less severe disturbance of channel function, corresponding to the patient's milder phenotype.

Interpretation: We identified *GRIN2B* gain-of-function mutations as a cause of West syndrome with severe developmental delay as well as of ID with childhood-onset focal epilepsy. Severely disturbed channel function corresponded to severe phenotypes underlining the important role of facilitated NMDA-receptor signaling in epileptogenesis.

4.2 Introduction

Epileptic encephalopathies (EE) constitute a group of disorders in which the epileptic activity itself is considered to contribute to severe cognitive impairment or decline above and beyond what might be expected from the underlying pathology alone. (Berg et al., 2010) West syndrome belongs to this heterogeneous group of disorders presenting with distinctive clinical and electrophysiological features usually manifesting between 3 and 12 months as clusters of infantile spasms (IS) and a characteristic EEG pattern called hypsarrhythmia.(Roger, 2005) The etiology of West syndrome is very diverse, and a substantial subgroup is considered to have a genetic origin. West syndrome has been associated with mutations in *ARX*, *CDKL5*, *STXBP1*, *ST3GAL3* as well as various copy number variations (CNVs).(Edvardson et al., 2013; Kalscheuer et al., 2003; Paciorkowski et al., 2011; Saitsu et al., 2010; Stromme et al., 2002) However, in many cases the genetic defect remains unresolved.

Mutations in *GRIN2A* and *GRIN2B* encoding the alpha and beta-2-subunits (NR2A and NR2B) of the glutamate-activated N-methyl-D-aspartate (NMDA) receptor are associated with several neurodevelopmental disorders. Mutations in *GRIN2A* have recently been detected in idiopathic focal epilepsy with rolandic spikes and related epileptic encephalopathies, that is, in Landau-Kleffner syndrome, epilepsy with continuous spike-and-waves during slow sleep syndrome and in non-syndromic epilepsy associated with ID.(Carvill et al., 2013; Endele et al., 2010; Lemke et al., 2013b; Lesca et al., 2013) By contrast, *GRIN2B* has not been described as epilepsy gene to date but has repeatedly been considered as a putative candidate gene for seizures (Endele et al., 2010; Epi et al., 2013) and mutations were detected in patients with ID, autism spectrum disorders (ASD) and schizophrenia.(de Ligt et al., 2012; Endele et al., 2010; Freunscht et al., 2013; Kenny et al., 2013; O'Roak et al., 2011; O'Roak et al., 2012; Tarabeux et al., 2011)

4.3 Materials and Methods

We used a targeted massive parallel resequencing approach to diagnostically screen 357 individuals (Cohort A) with a broad range of epilepsy phenotypes. The panel contained 50 known genes comprising EE genes, plus genes for severe ID not associated with seizures, but nevertheless suspected to be involved in epileptogenesis (e.g. voltage-sensitive and ligand-gated ion-channel genes). Analysis was performed

as described previously.(Lemke et al., 2012) Ninety-one of the 357 individuals were diagnosed with EE. Detailed clinical information necessary for a more specific epilepsy syndrome classification was not available for many of these 91 patients. After detecting *de novo* mutations in *GRIN2B* within Cohort A, we subsequently screened 47 patients with unexplained IS (Cohort B) by conventional methods. Three patients were diagnosed with Ohtahara syndrome, 38 with West syndrome, and 6 patients had a nonsyndromic early onset EE with IS during the course of the disease.

Sequence analysis

We performed direct Sanger sequencing to detect point mutations/small indels as well as multiplex amplicon quantification (MAQ) to detect CNVs in DNA extracted from peripheral blood. All 13 exons and intron-exon boundaries of *GRIN2B* were analyzed by bidirectional sequencing with the BigDye Terminator v3.1 Cycle Sequencing kit on an ABI3730 DNA Analyzer (Applied Biosystems, Foster City, CA; primers available upon request).

Additionally, the genomic region containing *GRIN2B* was screened for CNVs by use of an in-house developed technique for MAQ (http://www.multiplicom.com/multiplex-amplicon-quantification-maq) in Cohort B. This assay comprises a multiplex polymerase chain reaction (PCR) amplification of fluorescently labeled target and reference amplicons, followed by fragment analysis on the ABI3730 DNA Analyzer.(Suls et al., 2006) The comparison of normalized peak areas between the test individual and the average of 5 control individuals results in the target amplicon doses indicating the copy number of the target amplicon (using the in-house developed Multiplex Amplicon Quantification Software ; http://www.multiplicom.com/maq-s). The multiplex PCR reaction consists of 10 test amplicons located in the genomic region of *GRIN2B* and 6 reference amplicons randomly located on different chromosomes (primer mix is available upon request).

In silico prediction

Pathogenic implications of identified coding variants were assessed by different *in silico* analysis programs (PolyPhen2, http://www.genetics.bwh.harvard.edu/pph2 and MutationTaster, http://www.mutationtaster.org). (Table 4.1) For intronic single nucleotide polymorphism, splice site analysis was performed using HSF2.4 (http://umd.be/HSF/). The mutation of the ligand-binding domain in *GRIN2B* encoding human NR2B was analyzed using the x-ray crystal structure (Furukawa et al., 2005) of

rat NR2A (Protein Data Bank # 2A5S). Rat NR2A and human NR2B share 82% sequence identity and 88% sequence similarity in their glutamate-binding regions, making the rat NR2A structure a useful template for the analysis of mutations in *GRIN2B*. The interactive visualization program University of California, San Francisco (UCSF) Chimera(Pettersen et al., 2004) was used for structural analysis. The '*swapaa*' command was used to substitute amino acids, selecting the side-chain from the Dunbrack backbone-dependent rotamer library based on lowest number of clashes, highest number of hydrogen bonds and highest probability.

Molecular modelling of the transmembrane domains of NR1/NR2B receptors was based on the crystal structure of GluR2 (Brookhaven Protein Data Bank entry 3KG2) using Modeller 9v6 (UCSF Sali lab) and lsqman 9.7.9 (Uppsala Software Factory) as described.(Furukawa et al., 2005) Models were subjected to short-term molecular dynamics simulations using the Charmm27 force field, which is implemented in the Tinker 4.2 molecular modelling software (http://dasher.wustl.edu/tinker/). Figures were made using PyMOL 1.2 (http://www.pymol.org).

Functional investigations

For Xenopus laevis oocyte experiments, NR1-1a and NR2B constructs and capped cRNAs were generated as described previously.(Endele et al., 2010) Mutations were introduced into these constructs using the QuikChange site-directed mutagenesis kit (Stratagene, Agilent Technologies, santa Clara, CA) and confirmed by Sanger DNA sequencing. Individual stage V to VI oocytes were obtained from anaesthetized frogs and isolated by collagenase treatment. 10 ng of total NR1/NR2B cRNA were injected into oocytes. Following injection, oocytes were kept at 17 °C in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, pH 7.4). Glutamate and glycine dose-response curves of wild-type NR1/NR2B and mutant NMDA receptors were analyzed by 2-electrode voltage-clamp recording as described.(Laube et al., 1997) Concentration-response curves and current traces shown in the figures were drawn using KaleidaGraph (Synergy Software, Reading, PA). To monitor the voltage dependence of NR1-NR2B receptor combinations, whole-cell current-voltage relationships of saturating glutamate- and glycine-induced currents were recorded in 20 mV intervals ranging from –90 mV to +30 mV and normalized to the current value obtained at +30 mV above the respective reversial potential (E_{rev}) as described previously.(Madry et al., 2010) The relative divalent to monovalent permeability was calculated by the Goldman-Hodgkin-Katz constant field voltage equation assuming no

anion permeability. The internal concentrations of Na^+ and K^+ used in the calculations were 20 mM and 150 mM, respectively.(Madry et al., 2010) Permeability ratios were calculated for each oocyte and then averaged. Mg^{2+} inhibition (1 mM) was evaluated in the presence of 1.8 mM Ca^{2+} at a holding potential of −70 mV upon application (5 s) of saturating glycine (10 µM) and glutamate (100 µM) concentrations.

Statistical analyses

Values given represent means ± standard error of the mean. Statistical significance was determined at the $p < 0.05$, $p < 0.01$ and $p < 0.001$ levels using a Student's 2-tailed, unpaired *t* test.

4.4 Results

Sequence analysis and functional investigations

Among the 357 patients of Cohort A, we identified 2 individuals with West syndrome (Patients 1 and 2) carrying novel heterozygous *de novo* mutations in *GRIN2B* (2 of 91 EE cases, 2.2%) affecting key amino acids (p.Val618Gly and p.Asn615Ile) within the NR2B ion channel-forming re-entrant loop, as well as a patient with ID and childhood-onset focal epilepsy (Patient 3) carrying a novel heterozygous *de novo* mutation (p.Arg540His) within the NR2B glutamate-binding domain. (see Table 4.1, Fig 4.1)

Mutation p.Asn615Ile (Patient 2) affects 1 of 2 paired asparagines (Asn615, Asn616 in NR2B) in the re-entrant pore-forming loop implicated in Mg^{2+} block, whilst p.Val618Gly (Patient 1) is in close vicinity. Expression of NR1/NR2B^{Asn615Ile} and NR1/NR2B^{Val618Gly} heteromers revealed a significant loss of ion-channel block by extracellular Mg^{2+} and a dramatically increased Ca^{2+} permeability (Fig 4.2), consistent with a gain of function and consequent hyperexcitability. By contrast, p.Arg540His (Patient 3) affects a highly conserved residue located in the extracellular glutamate-binding region. Mutation of p.Arg540His is predicted to abolish the hydrogen bonding with the backbone of Cys746 and His802, and a cation-pi interaction with His802, possibly leading to a relaxed fold in this region. (Fig 3.3) Curiously, rather than affecting glutamate binding, p.Arg540His also resulted in a decrease of Mg^{2+} block and increase of Ca^{2+} permeability, implying an allosteric effect for this mutation. However, the functional impacts were less severe, in line with the milder phenotype of the patient. (see Fig 4.2)

Among the 47 patients of Cohort B, we detected 1 additional West syndrome patient (Patient 4) with a novel paternally inherited heterozygous splice-site variant deleting 2 base pairs of the splice acceptor site of exon 10, which encodes parts of the ion channel pore domain of *GRIN2B*. *In silico* prediction suggests a consecutive alternative splice acceptor site 6 base pairs downstream of the mutated splice site that is only marginally weaker compared to the wild-type splice acceptor site. Use of this alternative splice acceptor site is predicted to result in an in-frame deletion of amino acids Phe671 and Gln672 (p.Phe671_Gln672del) of the *GRIN2B* pore complex which would be in line with the location of the mutations of the two West syndrome patients described above. However, the latter putative splice variation was inherited from the patient's healthy father and no fresh sample or tissue was available to functionally confirm the potentially aberrant splicing. Screening for copy number variations by MAQ did not reveal additional deletions or duplications affecting *GRIN2B* in Cohort B.

Patient phenotypes

Patient 1 was a male born at term after an uneventful pregnancy. Myoclonic jerks and infantile spasms occasionally occurring in clusters at the age of 4 months led to the diagnosis of West syndrome. At 6 months, old he had no eye contact and presented with episodic hyperextension of axial muscels. The result of first EEG was not available for review. EEG at age 8 months showed multifocal bursts of irregular spike waves as well as rhythmic bilateral generalized spike waves with a frequency of 4 to 5 per second reminiscent of modified hypsarrhythmia. During sleep, there was rare irregular high-amplitude epileptiform activity with a hypsarrhythmialike pattern. Treatment with vigabatrine and pyridoxine led to a slight clinical improvement. Replacement of vigabatrine by levetiracetam improved neither seizures (predominantly of myoclonic type) nor EEG pattern, whereas implementation of valproate finally led to a significant reduction of seizure frequency. At last follow up at age of 2 years and 1 month, the boy's length was at P90, weight at P25 and head circumference at P50. He had severe axial hypotonia with episodic hyperextension and could not sit independently. He could hold eye contact only briefly before drifting away. At this time, the boy presented additionally with dystoniclike movements of his fingers and still no verbal communication.

Patient 2 was a female born at term after an uneventful pregnancy. At the age of 7 weeks she presented with infantile spasms. She held no eye contact and showed

muscular hypotonia and showed muscular hypotonia. She had episodic hyperextension of axial muscles. These episodes were not recorded on video. Brain magnetic resonance imaging (MRI), magnetic resonance spectroscopy, cerebrospinal investigation, and metabolic workup were normal. EEG showed typical hypsarrhythmia. Treatment with vigabatrine, sulthiame and topiramate failed to decrease seizures, whereas implementation of steroid pulse therapy led to an improvement. At last follow-up at age of 5 years and 3 months, the girl is not able to sit independently. She presented with autisticlike behavior and no verbal expression, severe feeding difficulties and mild microcephaly. The EEG showed increased theta activity without epileptic discharges. The girl still exhibits series of epileptic spasms as well as occasional generalized tonic-clonic seizures.

Patient 3 was a female born at 41 weeks after an uneventful pregnancy and conception by *in vitro* fertilization. Early development was delayed, as she sat at 11 months, walked at 19 months and used her first words at 18 months. At the age of 3 years, she was diagnosed with global developmental delay. There was no evidence for stagnation or even regression of development. Since the age of 9 years and 9 months she had focal dyscognitive seizures with postictal paresis of the right arm as well as occasional bilateral convulsive seizures and status epilepticus. Postictal EEG showed slowing over left frontoparietal region, and MRI of the brain showed postictal diffusion restriction in the same region that resolved over time. Lumbar puncture and metabolic screening were normal. At the last follow up at the age of 10 years and 6 months she had a mild intellectual disability and occasional seizures with postictal paresis.

Patient 4 was a male born at term after an uneventful pregnancy. At the age of 2 months epileptic spasms occurred, which soon evolved into asymmetric tonic seizures. EEG at the age of 7 months showed hypsarrhythmia, and multifocal epileptic activity was seen at the age of 14 months. MRI of the brain, metabolic workup and lumbar puncture were all normal. At last follow up at the age of 4 years, he still had daily therapy-resistant tonic seizures and focal motor seizures. He had severe intellectual disability with hypotonic muscle tone and was unable to talk or walk.

4.5 Discussion

Our investigations revealed *de novo GRIN2B* missense mutations in 2 of 91 patients with unexplained EE (2.2%) and in 1 patient with ID and childhood-onset focal epilepsy. We also detected 1 novel inherited putative splice variant in 1 of 47 patients with IS. The observation of a possibly reduced penetrance of an inherited splice variant in Patient 4 is in agreement with previous observations (Lemke et al., 2013b) in families carrying mutations and putative splice variants in *GRIN2A*. However, much larger data sets are needed to prove a role of inherited *GRIN2B* variants as risk factors for these disorders.

Interestingly, the very recent study of the Epi4K consortium revealed 1 additional case of a *de novo* mutation in *GRIN2B* in 1 of 115 individuals with epileptic encephalopathy of Lennox-Gastaut type.(Epi et al., 2013) (see Fig 4.1) The patient shows parallels to Patient 3 of our present study. Both mutations p.Arg540His (Patient 3) and p.Cys461Phe (Epi4K patient) are located in the extracellular glutamate-binding region and both patients present with primary developmental delay and childhood-onset epilepsy.

NMDA receptors are tetrameric ligand-gated ion channels permeable to Na^+, K^+ and Ca^{2+} composed of 2 glycine binding NR1 subunits and 2 glutamate binding NR2 subunits (NR2A, NR2B, NR2C, NR2D).(Laube et al., 1998; Paoletti et al., 2013) Subunit composition of NMDA receptors is spatially and temporally regulated with a switch from predominant NR2B expression in early development to more prominent synaptically localized NR2A expression at later stages,(Hardingham and Bading, 2010; Paoletti, 2011) which might explain the tendency towards earlier-onset epilepsy phenotypes in *GRIN2B* versus *GRIN2A* mutation carriers.

NMDA receptor subunits are organized into multiple structural domains (see Fig 4.1) including a signal peptide, and amino-terminal domain involved in receptor assembly, S1 and S2 segments that form the ligand binding domain, 3 membrane-spanning domains M1-M3, and a re-entrant pore-forming loop. Lastly, a large intracellular C-terminus and PDZ domain-binding motif mediate interactions with intracellular proteins such as PSD95. In the Exome Variant Server (EVS, National Heart, Lung, and Blood

Institute Exome Sequencing Project, http://evs.gs.washington.edu/EVS/), 38 missense and no putative essential splice variants are listed in *GRIN2B* in 6503 healthy controls. The described variants are unevenly distributed with the vast majority (27 of 38) of variants being positioned within the NR2B C-terminus. Only 9 of 38 variants were detected repeatedly, and again 7 of these are within the C-terminus. Curiously, the only described *de novo* aberration within the NR2B C-terminus was detected in an apparently healthy control subject(Rauch et al., 2012) whereas all known pathogenic *GRIN2B* mutations causing neurodevelopmental disorders are found within the N-terminal region, ligand-binding S1 and S2 segments and the re-entrant pore-forming loop.(de Ligt et al., 2012; Endele et al., 2010; Freunscht et al., 2013; Kenny et al., 2013; O'Roak et al., 2011; O'Roak et al., 2012; Tarabeux et al., 2011) (see Fig 4.1) This suggests that pathogenic mutations affecting key functional motifs have a greater impact on protein function and can negatively influence neurodevelopment and brain excitability. This is also reflected by the finding that variants outside the C-terminus of *GRIN2B* occurred significantly more frequently in alleles of EE individuals of Cohorts A and B compared to the EVS controls (p=0.0027, Fisher's exact test).

Interestingly, 2 of the West syndrome patients presented gain-of-function mutations (Patient 1, p.Val618Gly and Patient 2, p.Asn615Ile) in the re-entrant pore-forming loop. By contrast, Patient 3 with ID, childhood-onset focal epilepsy, and less severe developmental delay carried a milder gain-of-function mutation (p.Arg540His), positioned in the extracellular glutamate-binding S1 domain. Additionally, in support of the hypothesis that epilepsy is caused by gain-of-function mutations in *GRIN2B*, truncating and thus predicted loss-of-function mutations have only been described in patients with ID and/or ASD so far.(Kenny et al., 2013) These data suggest a distinct genotype-phenotype correlation, although more mutations and functional studies are needed to verify this assumption.

In summary, we postulate that genetic alterations in *GRIN2B* are responsible for ~2% (2 of 91) of EE cases, preferentially causing IS and West syndrome. This further strengthens the concept that West syndrome comprises a heterogeneous group of several disease entities, causing increased brain excitability with a similar and age-related EEG and seizure pattern. Additionally, our findings reveal further evidence of the contribution of altered NMDA receptor signaling to epileptogenesis and establish *GRIN2B* as another key player in epileptic encephalopathies. In the patients described

above, the severity of phenotypes corresponds to the electrophysiological severity of gain of ion channel function. This is of particular interest as existing NMDA receptor blockers such as memantine represent promising drugs to selectively restore the altered NMDA receptor function in patients with gain of function mutations in NR2 subunits, putting NMDA receptors more in focus in the search for new targets in epilepsy treatment.(Ghasemi and Schachter, 2011)

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Potential Conflicts of Interest

Nothing to report.

Table 4. 1: Mutations detected in *GRIN2B.*

TABLE 1. Mutations Detected in *GRIN2B*

Patient	Phenotype	Mutation	Prediction (MutationTaster/ Polyphen-2)	Origin	Domain	Functional Effect
1	West syndrome	c.1853T>G, p.Val618Gly	Disease causing/damaging	De novo	Channel pore	Gain of function
2	West syndrome	c.1844A>T, p.Asn615Ile	Disease causing/damaging	De novo	Channel pore	Gain of function
3	Focal epilepsy & ID	c.1619G>A, p.Arg540His	Disease causing/damaging	De novo	Glutamate-binding domain	Gain of function (mild)
4	West syndrome	c.2011-5_2011-4delTC	Not applicable	Paternal	Not applicable	Potential splice defect

ID = intellectual disability.

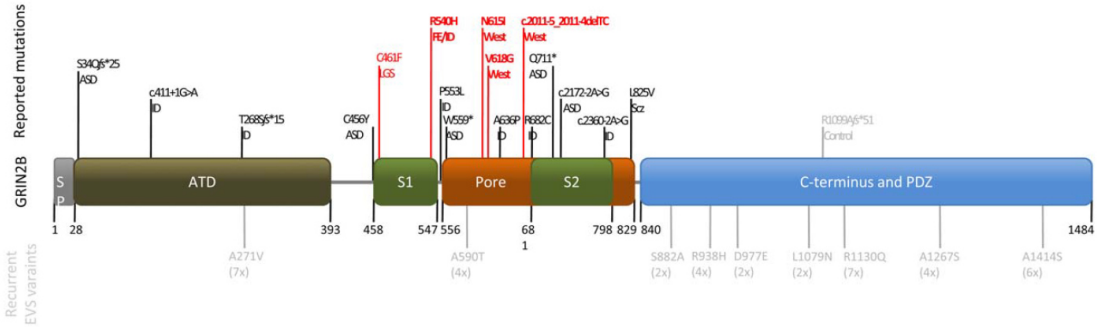

Figure 4. 1: Location of *GRIN2B* mutations in a schematic illustration of the conserved domains of the NR2B subunit (SP = signal peptide; ATD = amino terminal domain, involved in receptor assembly; S1 and S2 form the ligand-binding domain; Pore = re-entrant pore-forming and transmembrane spanning domains; PDZ = PDZ domain binding motif). All reported *de novo* mutations and their according phenotypes (ASD = autism spectrum disorders, FE = focal epilepsy, ID = intellectual disability, LGS = Lennox-Gastaut syndrome, Scz = schizophrenia) are listed in the top row. Mutations causing phenotypes without seizures are labeled in black, mutations in epilepsy patients are in red. So far, no pathogenic variants have been observed in the C-terminal region of NR2B. Mutations causing West syndrome cluster within re-entrant pore-forming domain, whereas the mutation causing ID and focal epilepsy was observed in the glutamate-binding domain S1, similar to a recently described LGS case. Non-synonymous variants that are believed not to be associated with abnormal phenotypes (grey) and are reported more than once (in brackets) in the Exome Variant Server (EVS) are listed in the bottom line.

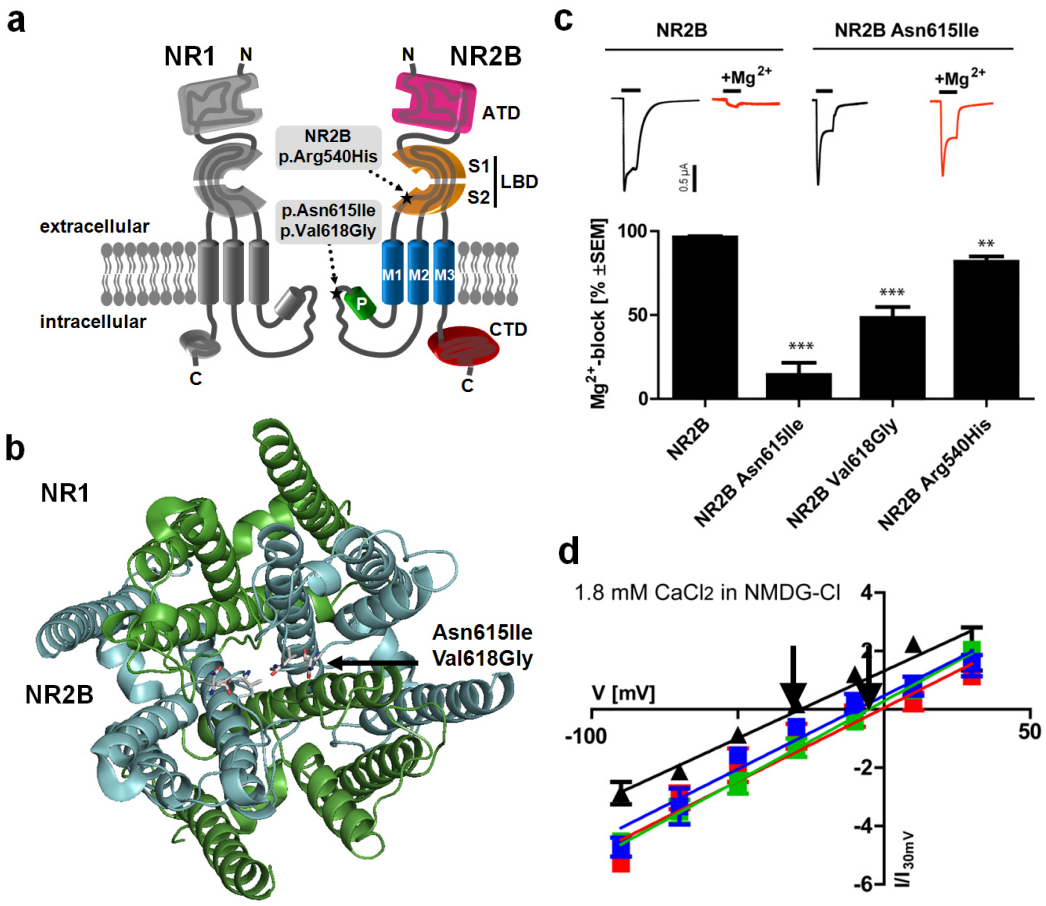

Figure 4. 2: Structural and functional consequences of missense mutations in GRIN2B. (a) Topology model of an NR1 and an NR2B subunit. Positions of the alterations p.Arg540His, p.Asn615Ile and p.Val618Gly are indicated by asterisks in the NR2 subunit consisting of an amino-terminal domain (ATD), the ligand-binding domain (LBD) including the S1 and S2 peptide segments, 3 transmembrane segments (M1, M2 and M3), a re-entrant pore loop (P) and an intracellular carboxy-terminal domain (CTD). Residue Arg540 lies within the glutamate-binding domain, and Asn615 and Val618 in the ion channel pore. N, NH_2-terminus; C, COOH-terminus. (b) Model of the transmembrane arrangement of the NMDA receptor composed of NR1 (green) and NR2B (cyan) subunits (top view). The arrow highlights the side chains of p.Asn615Ile and p.Val618Gly in the pore forming region. (c) Gradual loss of Mg^{2+} inhibition of NR1-NR2B wild-type and NR1-NR2B mutant receptor currents at -70 mV. Respective sample traces of NR1-NR2B and NR1-NR2B^{Asn615Ile} are shown above with inhibition of receptor currents by 1 mM Mg^{2+} of NR1-NR2B (96 ± 0.9 %, n=4) and mutant NR1-NR2B^{Asn615Ile} (14 ± 7.2 %, p<0.0001, n=3), NR1-NR2B^{Val618Gly} (48 ± 6.5 %, p=0.0003, n=3) and NR1-NR2B^{Arg540His} (81 ± 3.2 %, p=0.005, n=5) receptors. (d) Effect on Ca^{2+} permeability of NR1-NR2B wil-type and NR1-NR2B mutant receptor currents. Current-voltage (I–V) relationships of NR1-NR2B receptors in the absence of Mg^{2+} in Na^{+}-free extracellular solution reveal significant

differences in the reversal potential (indicated by arrows) of NR1-NR2B (-31 ± 1.7 mV, n=4, black triangles) and mutant NR1-NR2B^{Asn615Ile} (-1.0 ± 6.8 mV,p=0.004, n=3, red squares), NR1-NR2B^{Val618Gly} (-5.4 ± 3.7 mV, p<0.001, n=3, green squares) and NR1-NR2B^{Arg540His} (-9.4 ± 6.5 mV, p=0.013, n=3, blue squares) receptor currents. (NMDG-Cl, N-methyl-D-glucamine chloride) Calculation of the relative divalent to monovalent cation permeability PCa/PNa by the Goldman-Hodgkin-Katz voltage equation revealed a >3-fold increase in Ca^{2+} permeability of the mutant NMDA receptors (PCa/PNa for NR1-NR2B: 0.86, NR1-NR2B^{Asn615Ile}: 5.22, NR2B^{Val618Gly}: 3.12 and NR2B^{Arg540His}: 3.23).

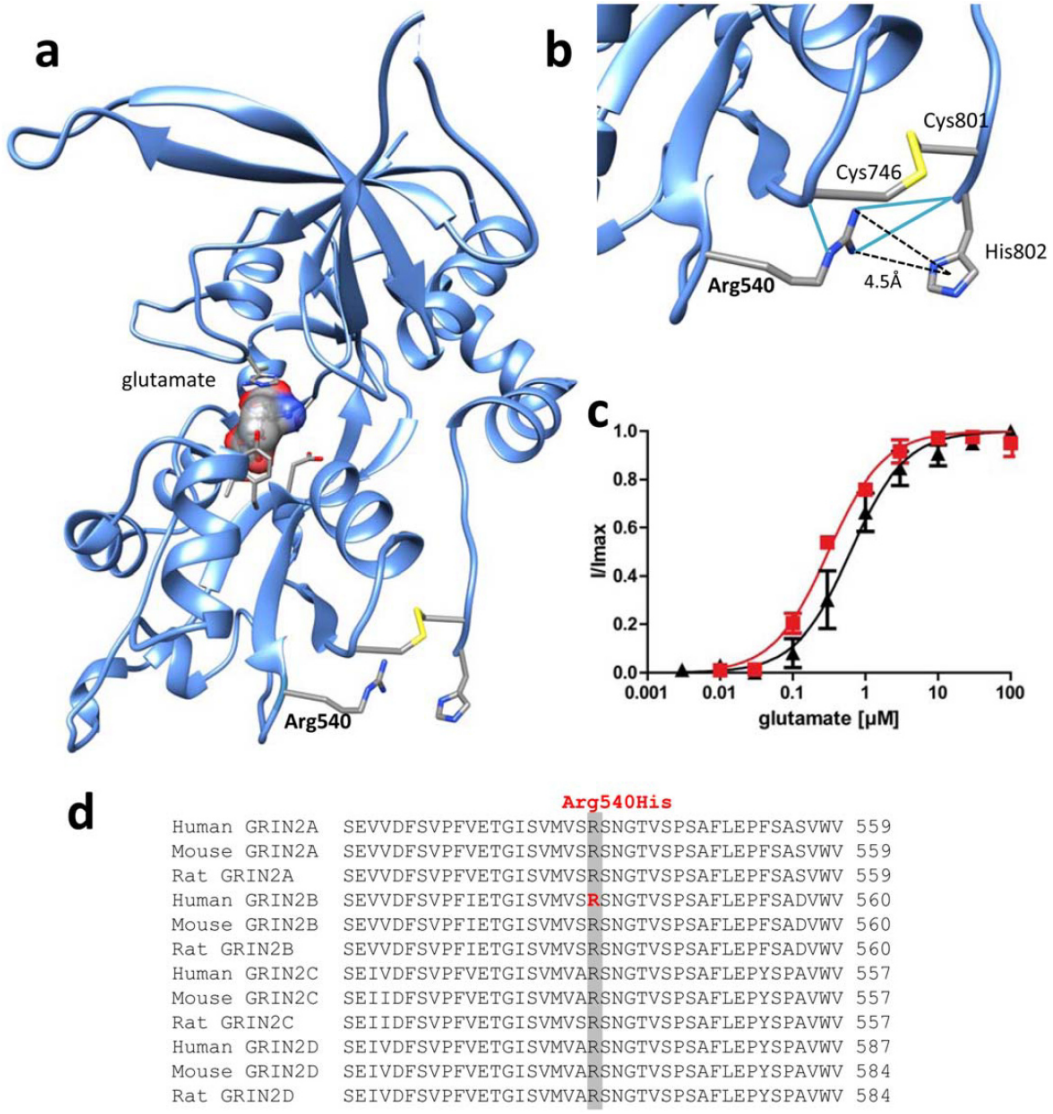

Figure 4. 3: Structural and functional analyses of the glutamate binding-domain mutation Arg540His. (a) Residue 540 is predicted to be located within the glutamate-binding S1 domain, and is significant in the stabilization of the tertiary structure of the glutamate binding-domain. (b) Substitution p.Arg540His is likely to abolish hydrogen bonding (blue lines) with the backbone of Cys746 and His802 and a cation-pi interaction with His802, possibly leading to a relaxed fold in this region. (c) Pharmacological characterization of the apparent agonist affinities of wild-type NR1-NR2B (black triangles) and mutant NR1-NR2B^{Arg540His} (red squares) NMDA receptors measured after heterologous expression in *Xenopus laevis* oocytes by two-electrode voltage-clamping revealed similar glutamate concentrations were required to elicit half-maximal response (EC50-value 0.72 ± 0.22 µM and 0.31 ± 0.02 µM, p=0.14, n=3, respectively). (d) Arg540 is a highly conserved residue within NR2A-D subunits.

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4.7 Declaration of own achievment within the publication

I designed, performed and analyzed the functional experiments on the mutations GluN2B p.Asn615Ile, GluN2B p.Val618Gly and GluN2B p.Arg540His with the previous mutagenesis. The results are represented in Figure 4.2 and Figure 4.3 c.
I did not write the original manuscript, but I was involved in the rewriting-process within the revision. Additionally, I was involved in proofreading the manuscript during the submission- and revision-process.

5 Manuscript:

Electrophysiological characterization of high and low affinity GluN1 antagonists at GluN1/GluN3 receptors

Kirsten Geider and Bodo Laube

5.1 Abstract

N-methyl-*D*-aspartate (NMDA) receptors belong to the family of ionotropic glutamate receptors, which mediate most excitatory synaptic transmission in the mammalian brain. They are tetrameric cation channels composed of two glycine-binding GluN1or GluN3A subunits and two glutamate-binding GluN2 subunits. For efficient activation conventional GluN1/GluN2 NMDA receptors require both agonists, glutamate and glycine. In contrast recombinant receptors composed of two GluN1 and two glycine binding GluN3 subunits lack glutamate binding sites and can be activated by glycine alone. These so called “excitatory glycine receptors” showed a potentiation of receptor currents upon coapplication of GluN1 antagonists. The potentiation effect of GluN1/GluN3A receptors is still an open question. For further investigation, which determinants are important for this effect we analyzed several GluN1 antagonists in terms of potentiation strength. Two electrode voltage clamp recordings from GluN1/GluN3A expressing oocytes were measured upon co-application with low and high affinity GluN1 antagonists. The results showed a relationship between the affinity of GluN1 glycine site antagonists and the potentiation efficacy. Low affinity GluN1 antagonists result in a slide potentiation, whereas high affinity GluN1 antagonists reveal a strong increase of receptor currents. In summary all tested GluN1 antagonists turned out to be positive modulators of GluN1/GluN3A receptors and our results present strong evidence for a correlation between the affinity of the GluN1 antagonists and the potentiation strength.

5.2 Introduction

NMDA receptors are ligand gated ion channels that mediate excitatory neurotransmission in the brain. They are heterotetrameric membrane proteins comprised of the glycine-binding GluN1 and GluN3 subunits or the glutamate-binding GluN2 subunit. Each subunit shares a common modular structure characterized by the extracellular N-terminal domain (NTD), the ligand binding domain (LBD), three transmembrane domains (TMDs) plus an intracellular P-loop forming the ion channel pore, and an intracellular C-term domain (CTD) (Traynelis et al., 2010). Agonist recognition occurs in the ligand binding domain, which is composed of the two domains S1 and S2 (Furukawa and Gouaux, 2003; Laube et al., 1997). Agonist binding results in a clamshell closure. This ligand-induced movement of the LBD impacts the linker region, which is connected to the transmembrane domains and leads to channel opening.

The so called “conventional” NMDA receptors are composed of two GluN1 and two GluN2 subunits and need two agonists for efficient channel opening: glycine and glutamate, and the relief of Mg^{2+} ion out of the channel pore (Traynelis et al., 2010). The GluN3 subunits are the last detected members of the NMDA receptor gene family (Ciabarra et al., 1995; Nishi et al., 2001; Sucher et al., 1995). Assembly of GluN1 and GluN3 subunits results in tetrameric receptors, which were exclusively activated by glycine (Awobuluyi et al., 2007; Chatterton et al., 2002; Madry et al., 2007). In contrast to the conventional NMDA receptors GluN1/GluN3A receptors display a different pharmacological profile. Activation of GluN1/GluN3A receptors with glycine leads only to small and rapid desensitized currents and they are insensitive for Mg^{2+} (Chatterton et al., 2002) Functional studies revealed structural and pharmacological differences between GluN1 and GluN3, because of strikingly different binding affinities of agonists and antagonist. Yao and Mayer showed that soluble ligand binding domains from GluN3A subunits bind glycine with a 650 fold higher affinity than GluN1 subunits (Yao and Mayer, 2006). Furthermore, they revealed that glycine site antagonists e.g. 5,7-dichlorokynurenic acid exhibits strong selectivity for binding the GluN1 versus the GluN3A subunit (Yao and Mayer, 2006).

Concerning GluN1/GluN3 receptors, glycine seems to have a dual action at the GluN1 and GluN3 subunits with regards to the activation mechanism because it acts agonistically at the GluN3 subunit and inhibitory at the GluN1 subunit (Awobuluyi et al., 2007; Madry et al., 2007). Interestingly antagonizing the GluN1 subunit of GluN1/GluN3

receptors results in a potentiation of receptor currents (Madry et al., 2007). Coapplication of the high affinity GluN1 antagonist MDL ((E)-4,6-Dichloro-3-(2-phenyl-2-carboxyethenyl)indole-2-carboxylic acid (Baron et al., 1992) together with the agonist glycine, results in a 25 fold potentiation of receptor currents (Madry et al., 2007). Furthermore, introducing a mutation in the GluN1 LBD, which was shown to nearly abolish the glycine affinity of the GluN1 subunit, results in an enhancement of receptor currents, without coapplication of the antagonist MDL (Madry et al., 2007). Madry and colleagues proposed a model for the activation mechanism of GluN1/GluN3 receptors: glycine binding to the GluN3 subunit leads to a conformational change in the LBD and induces a closure of the clamshell. Binding of glycine to the low affinity GluN1 LBD weakens the interdomain interactions between the GluN3 and GluN1 LBDs, which results in receptor desensitization. Crystallographic studies of the soluble LBD revealed that antagonist binding stabilizes the binding domain clamshell in an open conformation (Furukawa and Gouaux, 2003). Antagonizing the GluN1 ligand binding pocket seems to affect the structure of the clamshell and lead to an open clamshell structure. This results in an potentiation of the small and rapid desensitizing glycine currents.

Because the determinants of the potentiation effect are not yet known, in the present study we investigated several GluN1-glycine site antagonists at GluN1/GluN3 receptors. We examined high and low affinity GluN1 antagonists and their effect on potentiation strength at GluN1/GluN3A receptors. The results showed an increase of efficacy of the GluN1/GluN3A receptor currents upon antagonizing the GluN1 LBD in different magnitude in correlation with the affinity of selected antagonist.

5.3 Methods

DNA Constructs and cRNA Synthesis

cDNAs encoding the GluN1-1a (Genbank accession no.U11418) were provided by Dr. S. Heinemann (Salk Institute , La Jolla, CA, USA) and GluN3A-1 (Genbank accession no. U29873) provided by Dr. D. Zhang (Stanford-Burnham Medical Research Institute, La jolla, CA, USA). For expression the cDNAs were linearized with restriction enzyme NotI and were used as a template for cRNA synthesis using sp6 mMessage mMachine kit (Ambion Life Technologies,Paisley,UK).

Xenopus Oocyte Preparation and Expression

For isolation of oocytes female Xenopus laevis frogs were anesthetized with 0,1 % tricain and the oocytes were transferred to ND96 solution [96 mM NaCl, 2 mM KCl, 1 mM CaCl, 1 mM $MgCl_2$ and 5 mM HEPES (pH 7.4)]. Individual oocytes were defolliculated by 2 h incubation in type IIA collagenase. After collagenase treatment the oocytes were washed with Ca^{2+}-free ND96 and stored in ND96 solution.

For heterologous expression of GluN1/GluN3A receptors, 25 ng of GluN1 and GluN3A cRNA was coinjected in selected stage V-VI oocytes and maintained at 18 °C in ND96 solution supplemented with 100 µg/ml gentamycin.

Two-Electrode Voltage-Clamp Recordings

Two-electrode voltage-clamp (TEVC) recordings were performed on Xenopus oocytes at room temperature 3-4 days after cRNA injection. Current responses were measured under continuous perfusion with Ca^{2+}-free frog ringer solution (pH 7,4). The microelectrodes used for voltage clamping were filled with 3 M KCl solution. For recordings the oocytes were clamped at -70 mV and currents were amplified with a Geneclamp 500B amplifier, digitized with Digidata 1322A interface and recorded with Clampex version 10.2 (Molecular Devices, Sunnyvale, CA).

GluN1 Antagonists

The GluN1 antagonists used in the experiments were purchased from Tocris Bioscience (Bristol, United Kingdom). Stock solutions were solved in DMSO or sigma water. Agonist and antagonist dilutions were made in frog ringer solutions. The used antagonists are: HA-966 (1-Hydroxy-3-aminopyrrolid-2-one) (Fletcher and Lodge, 1988; Foster and Kemp, 1989); Cycloleucine (1-Amino-1-cyclopentanecarboxylic acid) (Snell and Johnson, 1988) ;Kynurenic acid (4-Hydroxyquinoline-2-carboxylic acid) (Stone and Burton, 1988); 7-CKA (7-Chloro-4-hydroxyquinoline-2-carboxylic acid) (Kemp et al., 1988) ; 5,7-DCKA **(**5,7-Dichloro-4-hydroxyquinoline-2-carboxylic acid) (Baron et al., 1990) ; MDL (2-Carboxy-4,6-dichloro-1H-indole-3-propionic acid) (Baron et al., 1992); L-689560 (*trans*-2-Carboxy-5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline) (Leeson et al., 1992)

Statistical analysis

Values given represent means ± standard error of the mean. Statistical significance was determined at the $P < 0,05$ (*), $P < 0,01$ (**) and $P < 0,001$ (***)

5.4 Results

GluN1/GluN3 receptors are activated solely through glycine but generate only small currents upon agonist application. Antagonizing the GluN1 subunit leads to a potentiation of receptor currents. The high affinity GluN1 antagonist MDL is known to potentiate GluN1/GluN3A receptor currents upon coapplication with glycine (Madry et al., 2010; Madry et al., 2007). Because of the fact that the mechanism of this potentiation is not clear, we characterize further GluN1 antagonists at GluN1/GluN3A receptors. Figure 5.1 shows the structure of the GluN1 antagonists, analyzed in the present study. The selected antagonists have different affinities for the GluN1 subunit covering a broad spectrum from the milimolar to the nanomolar range. MDL, DCKA, 7-CKA and L-689560 are high affinity glycine site antagonists, whereas HA-966 and Cycloleucine, Kynurenic acid are weak antagonists. All selected GluN1 antagonists bind with higher affinity to the GluN1 than to the GluN3A subunit (Yao and Mayer, 2006).

To investigate the effects of the GluN1 antagonists on GluN1/GluN3A receptor currents we coapplicated the antagonists with saturating glycine concentration. Receptor currents were increased upon adding the antagonists (Fig. 5.2A) and showed a potentiation in different extent in dependence of the antagonist (Fig. 5.2B). The antagonist HA-966 revealed a slight increase of receptor currents after coapllication with glycine, which led to a 1.6 fold potentiation. Coapplication of the antagonists Cycloleucine and Kynurenic acid showed also a slight, but in comparsion with HA-966 a stronger potentiation. Cycloleucine led to a 2.84 fold potentiation, whereas the kynurenic acid coapplication resulted in a significant 4.49 fold potentiation. The high affinity glycine site antagonists 5,7-DCKA ,7-CKA, MDL and L-689560 showed a strong potentiation. Coaplication of 5,7-DCKA with glycine resulted in 23.45 fold potentiation, whereas the 7-CKA led to a 30.6 fold increase of receptor currents. Maximal potentiation was seen with the antagonists MDL (63.6 ±14.0) and L-689560 (73.4 ± 19.4). In summary all measured GluN1 antagonists resulted in a significant increase of receptor currents.

Because we used GluN1 antagonists with different affinities the next step was to verify the affinity of the GluN1 antagonists at the heterologoues expressed GluN1/GluN3 receptors. Therefore we measured the apparent affinity of GluN1 antagonist. The EC_{50}

values were determined in the presence of 10 mM glycine (Table 5.1). The results confirmed the different affinities of the GluN1 antagonists with the low affinity antagonists Cycloleucine, HA-966 and Kynurenic acid with EC_{50} values in the mM to µM range and the high affinity antagonists with EC_{50} values in the lower µM to nM range. The EC_{50} values are nearly in the range of the K_d values determined at the soluble GluN1 S1S2 (Yao and Mayer, 2006).

Previously, the GluN1 antagonist MDL has been shown to accompany by an increase of the apparent glycine affinity during coapplication (Madry et al., 2007). Therefore the pharmacological characterization of the apparent glycine affinity were analyzed in the presence of constant GluN1 antagonist concentrations and increasing glycine concentrations. The dose response curves showed an increase in the EC_{50} values of glycine in the presence of the antagonists, except HA-966 (Fig. 5.3). The coapplication of HA-966 showed no significant increase of the glycine EC_{50} (12.29 ± 4.1 µM) in comparison with the glycine EC_{50} without antagonist (8.49 ± 0,89 µM). The coapplication of the GluN1 antagonist Cycloleucine showed a slight but significant increase of 30 ± 3.7 µM. With Kynurenic acid coapplication the EC_{50} value of glycine incrases significantly to 742 ± 222 µM. Also a strong and significant shift of the apparent affinity revealed due to the coapplication of the high affinity GluN1 antagonists MDL (1.05 ± 0.38 mM), 7-CKA (1.34 ± 0.16 mM) and 5,7-DCKA (2.19 ± 0.11 mM). The maximal shift in the apparent affinity was shown with the high affinity GluN1 antagonist L-689560 (2.42 ± 0.58 mM). The apparent affinities with the high affinity antagonists are in a similar range and differ not significantly among each other.

The potentiation effect seems to be dependend on the GluN1 antagonist affinity. Because of this observation, we try to correlate the x-fold potentiation with the obtained GluN1 antagonist apparent affinity and also with the Kd values from the soluble GluN1 S1S2 (Fig. 5.4 A+B). Interestingly there is a correlation between the affinity of the GluN1 antagonist and potentiation strength. Increasing affinity of GluN1 antagonists results in an increase of receptor currents. In the case of the EC_{50} values for glycine in presence of constant GluN1 antagonist concentrations there is no obvious correlation (Fig. 5.4 C). Between the obtained apparent affinities in the heterologoues expression system and the affinities from the soluble GluN1 S1S2 domain, we could obtain a clear linear correlation (Fig 5.4 D).

In summary all selected GluN1 antagonists were positive modulators of GluN1/GluN3A receptor currents and coapplication with the agonist glycine resulted in a potentiation. This potentiation effect can be correlated with the affinity of the GluN1 antagonists. Weak GluN1 antagonist affinity resulted in a slight potentiation, whereas high affinity GluN1 antagonists resulted in a strong potentiation.

5.5 Figures

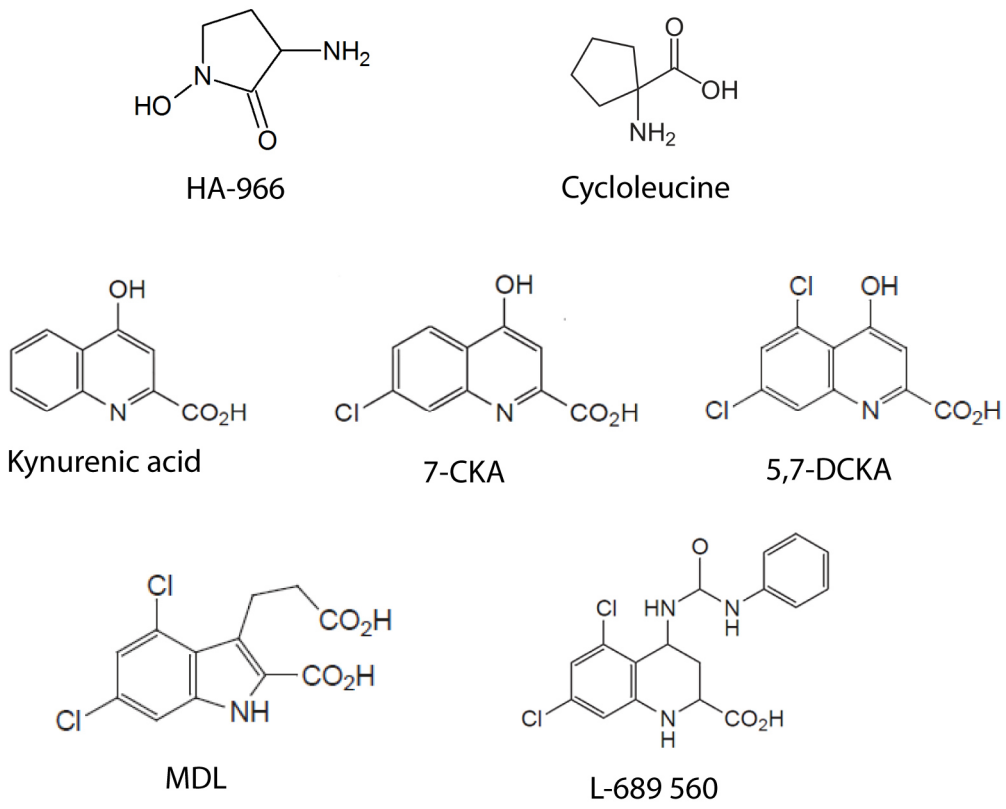

Figure 5. 1: Chemical structure of GluN1 antagonists. The selected GluN1 antagonists are competitive to the GluN1 glycine binding site and exhibit different affinities for the glycine binding site of the NMDA receptor. HA-966 is a low-efficacy partial agonist/antagonist of the glycine site with a Kd value of 162.58 µM (Miyazaki et al., 1999). The weak GluN1 antagonist Cycloleucine revealed an affinity for the glycine binding site with a Kd of 15.3 mM (Inanobe et al., 2005). The antagonists Kynurenic acid, 7-CKA , 5,7-DCKA, L-689560 and MDL revealed affinities with Kd values of 53 µM, 2,6 µM, 0,54 µM, 29,3 nM and 3,89 nM (Miyazaki et al., 1999; Yao and Mayer, 2006).

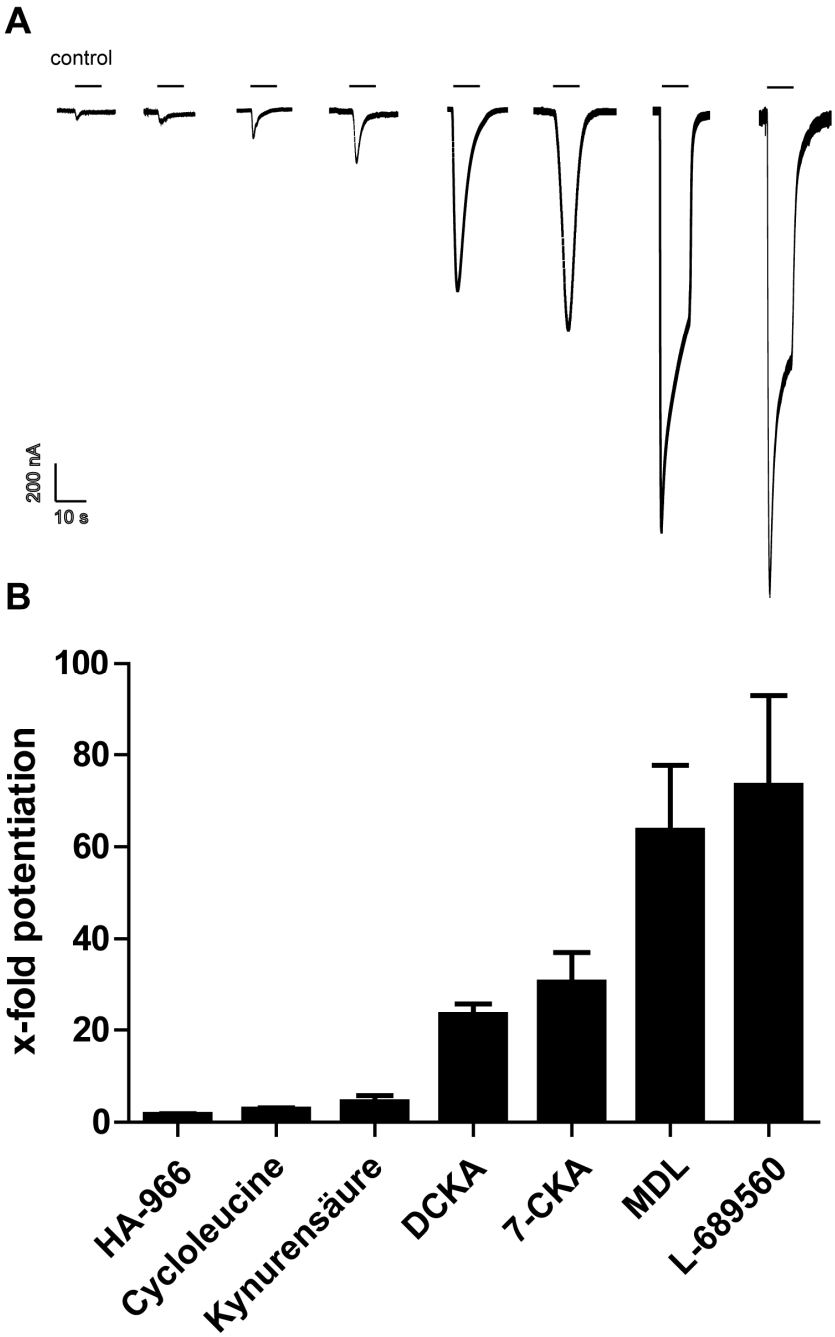

Figure 5. 2: Effects of GluN1 antagonists on GluN1GluN3A receptor currents. (A) Glycine induced currents were increased upon adding GluN1 antagonists. Sample traces at -70 mV were activated with saturating glycine concentration (10 mM) in absence and presence of GluN1 antagonists. Control represent the application with glycine alone. Glycine and GluN1 antagonist application is indicated as black bars. (B) Relative potentiation by GluN1 antagonists of GluN1/GluN3A receptor currents at -70 mV: HA-966: 1.6 ± 0.1 (n=10); Cycloleucine: 2.84 ± 0.2 (n=9); kynurenic acid: 4.49 ± 1,2 (n=8); 5,7-DCKA: 23.45 ± 2.4 (n=13); 7-CKA: 30.6 ± 6.4 (n=8); MDL: 63.6 ± 14.07 (n=7); L-689560: 73.4 ± 19,4 (n=7). Error bars indicate SEM.

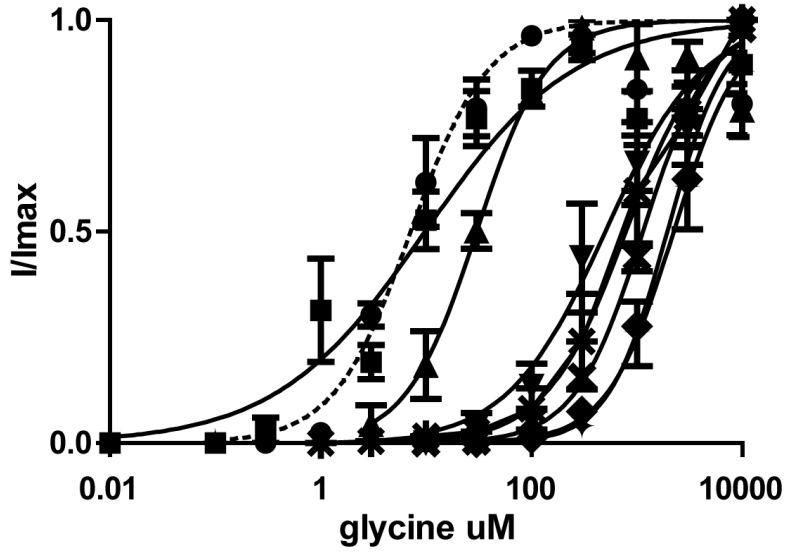

Figure 5. 3: Pharmacological characterization of the apparent glycine affinity upon GluN1 antagonist application. Coapplication of GluN1 antagonists result in a shift of the apparent glycine affinity. Wt (dashed line ●): 8.49 ± 0.89 µM (n=7); HA-966 (■): 12.29 ± 4.1 µM (n=6); Cycloleucine (▲): 30 ± 3.7 µM (n=4); Kynurenic acid (▼): 742 ± 222 µM (n=7); MDL (❋): 1.05 ± 0.38 mM (3); 5,7-DCKA (◆): 2.19 ± 0.11 mM (n=13); 7-CKA (✕): 1.34 ± 0.16 mM (n=8); L-689560 (✦): 2.42 ± 0.58 mM (n=3)

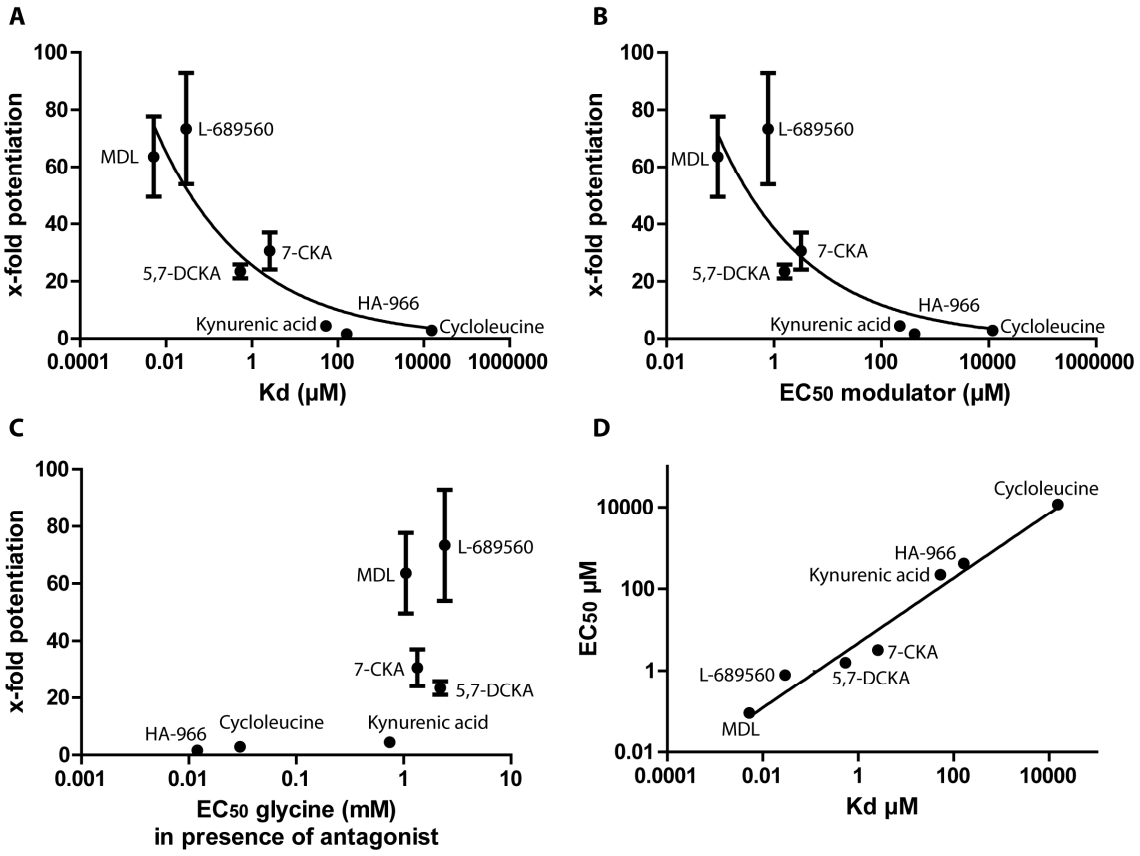

Figure 5. 4: Correlation of different parameters with the x-fold potentiation. The x-fold potentiation increased with higher affinity of the GluN1 antagonists. (A) K_d values obtained from the soluble GluN1 S1S2 in correlation with the x-fold potentiation (B) EC_{50} values of GluN1 antagonists measured during coapplication with saturating glycine in correlation with the x-fold potentiation. (C) EC_{50} values of glycine in presence of GluN1 antagonists. Low affinity GluN1 antagonists HA-966 and Cycloleucine showed no or only a slight reduction in the apparent affinity. The high affinity antagonists result in an increased EC_{50} value, thus in a strong reduction of apparent glycine affinity, but without obvious correlation between the x-fold potentiation and the altered apparent affinity of glycine. (D) Linear correlation between the obtained EC_{50} values of the GluN1 antagonists and the KD values from the soluble GluN1 S1S2.

Table 5. 1: Apparent affinity of GluN1 antagonists at GluN1/GluN3A receptors. GluN1 antagonists EC_{50} values were determined in the presence of 10 mM glycine (except HA-966, in presence of 100 µM glycine) 3-4 days after injection of GluN1 and GluN3A subunit cRNA. Values represent means ± SEM, n number of experiments.

GluN1 antagonist	EC_{50}	n
HA-966	414 ± 144 µM	3
Cycloleucine	11814 ± 871 µM	7
Kynurenic acid	220 ± 69 µM	4
5,7-DCKA	1.58 ± 0.87 µM	4
7-CKA	3,2 ± 1.6 µM	7
MDL	0.098 ± 0.01 µM	3
L-689560	0.78 ± 0.05 µM	5

5.6 Discussion

In this study we investigated several GluN1 antagonists at GluN1/GluN3A receptors and their potentiation effect on receptor currents. Madry and colleagues already showed a potentiation effect with the high affinity GluN1 antagonist MDL. The mechanism of potentiation of the small, rapid desensitized glycine induced currents through GluN1 antagonists is still unclear. For further investigation which determinants are important for this effect we analyzed different GluN1 antagonists, which exhibit different ligand affinities for the soluble GluN1 S1S2 LBD.

Crystallographic and functional studies showed that agonist activity is associated with binding within the LBD clamshell cleft that stabilizes the closed conformation. Antagonists prevent the interlobe closure (Armstrong and Gouaux, 2000; Furukawa and Gouaux, 2003; Inanobe et al., 2005). The crystal structure of the NMDA receptor ligand binding domain with the high affinity GluN1 antagonist 5,7-DCKA adopts a conformation in which domain S1 and S2 are open by 24° (Furukawa and Gouaux, 2003). 5,7-DCKA seems to stabilize an open cleft conformation, which is in concordance with previous observations of antagonists (Armstrong and Gouaux, 2000). In AMPA receptors there is a relationship between ligand binding domain closure and ion channel activation (Armstrong et al., 2003). The observed differences in potentiation strength with high and low affinity GluN1 antagonists at GluN1/GluN3A receptors possibly result from differences in domain configuration pertaining to closed or open state. But the ligand binding domain in complex with the weak GluN1 antagonist Cycloleucine adopts an open cleft conformation most similar to the GluN1 S1S2 complex with 5,7-DCKA (Inanobe et al., 2005). Consequently the potentiation effect of GluN1GluN3A receptors with GluN1 antagonists has to be independent of the degree of domain closure.

Our results present strong evidence for a correlation between the affinity of the GluN1 antagonists and the potentiation strength. With higher affinity of the GluN1 antagonist there is an increase of efficacy of GluN1GluN3A receptor currents. The high affinity GluN1 antagonists showed a strong potentiation in the range of 60-80 fold enhancement of receptor currents, whereas the low affinity GluN1 antagonist like kynurenic acid shows also potentiation effects but in the lower range of 4 fold potentiation. The correlation between the degree of potentiation and the affinity has to be elucidated with further GluN1 antagonists. Trough the correlation of the x-fold

potentiation with the GluN1 antagonist affinity, it could be possible to predict the extend of potentiation strength with other or new antagonists.

The potentiation effect through GluN1 antagonists is an interesting characteristic of GluN1/GluN3A receptors. Because the GluN1 antagonist kynurenic acid occurs endogenously in the brain, it is necessary to understand the impact of antagonist and their physiological relevance. Kynurenic acid is an end-product of the kynurenine pathway, in which more than 95 % of the amino acid tryptophan is metabolized (Vecsei et al., 2013). Kynurenic acid has proved to be a neuroprotective molecule in various experimental models of neurotoxicity (Winn et al., 1991). The halogenated derivatives 7-CKA and 5,7-DCKA are promising compounds in prodrug concepts. 4-chlorokynurenine is the blood brain barrier penetrante prodrug of 7-CKA and investigated in preclinical studies. It represents one of the most advanced L-kynurenine prodrug candidates for the use as a neuroprotective agent (Vecsei et al., 2013). The knowledge of antagonist agency at GluN1/GluN3A receptors is very important and could be essential in the future, because of the involvement of NMDA receptors in several neurological disorders and the use of NMDA antagonists in therapy treatment.

5.7 References

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5.8 Declaration of own achievment within the publication

I designed, performed and analyzed the functional experiments on GluN1/GluN3A receptors. I wrote the paper self dependent.

6 Discussion

6.1 Epilepsy and ion channels

It is well established that alterations in central inhibitory and excitatory neurotransmission play a crucial role in the etiology of epilepsy. In the last years a number of epileptogenic mutations were identified in genes, encoding for voltage – or ligand-gated ion channels (Noebels, 2003). There have been mutations in the nicotinic acetylcholine receptor subunits (De Fusco et al., 2000), in the voltage-gated potassium channel subunits (Leppert and Singh, 1999), in the $GABA_A$ receptor (Galanopoulou, 2010) and in the voltage-gated sodium channel genes (Oliva et al., 2012), which underlie different forms of genetic epilepsies. The focus on ion channels is reasonable because they play a crucial role in neurotransmission and are involved in excitatory and inhibitory signal transmission. A gain-of-function of ion channels in excitatory neurotransmission could lead to hyperexcitation while a loss-of-function in inhibitory transmission impairs the neuronal inhibitory system. Both can result in epileptic seizures.

Changes in NMDA receptor signaling have been demonstrated with animal models and the recent findings of mutations in the GRIN2 genes in patients with epilepsy syndromes provided evidence for a crucial role of NMDA receptors in epilepsy. The three publications (Endele et al., 2010; Lemke et al., 2013a; Lemke et al., 2013b) identified *de novo* mutations in the GRIN2A and GRIN2B gene, which encode the GluN2A and GluN2B subunits of the NMDA receptor. The mutations were found in patients with variable neurodevelopmental phenotypes e.g. epilepsy and/or mental retardation. They are located in different domains of the receptor and influence channel properties and allosteric modulation of the NMDA receptors. Because of the fundamental role of the GluN2 subunits for physiological and pathophysiological processes in the CNS and the unique role for receptor function, I focused my investigations on mutations in the GluN2A and GluN2B subunits, especially in the NTD and the pore region.

In Table 6.1 all analyzed mutations are listed with informations about the amino acid substitution, the affected domain, the functional results, the phenotype, the supposed functional impact and the origin of mutation.

Table 6. 1: Summary of the analyzed epilepsy associated mutations with information about the amino acid substitution, the affected domain, the functional results, the patient`s phenotype, the supposed functional impact and the origin of mutation. (n.d. = not determined)

	GluN2A		GluN2B		
mutation	Ala243Val	Asn615Lys	Arg540His	Asn615Ile	Val618Gly
affected domain	NTD	TMD P-loop QRN+1 site	LBD S1	TMD P-loop QRN site	TMD P-loop
apparent affinity of glutamate and glycine	no change	no change	no change	no change	no change
inhibition Mg^{2+} block	n.d.	16% ***	81% **	14% ***	48% ***
relative Ca^{2+}permeability	n.d.	10 fold decrease	3 fold increase	6 fold increase	3 fold increase
zinc inhibibition	significant reduction	n.d.	n.d.	n.d.	n.d.
gain or loss of function	gain	gain	gain (mild)	gain	gain
phenotype	BECTS & learning difficulties	early-onset encephalopathy	Focal epilepsy & ID	West Syndrome	West Syndrome
origin	newly identified, parents unavailable	de novo	de novo	de novo	de novo

6.2 The N-terminal domain and allosteric modulation

The NTD is the largest domain of the NMDA receptor and has modular influence on receptor function. Binding of allosteric modulators occurs at the NTD of GluN2 containing receptors, but the physiological role of the NTD of NMDA receptors is largely unkown. The results of the GluN2A NTD mutation may have implications for understanding how the NTD of NMDA receptors is involved in NMDA receptor function in pathological states. Sequence analysis revealed that the mutation c.728C>T in the GRIN2A, causes the substitution alanine to valine at the position 243 (p.Ala243Val) in the GluN2A subunit. This mutation was found in a patient with the epilepsy syndrome BECTS (benign epilepsy with centro-temporal spikes) and learning difficulties. The functional analysis revealed no changes in maximal inducible currents, agonist affinities and relative open probabilities of the mutated receptors but a loss of high-affinity inhibition by Zn^{2+} (Lemke et al, 2013b). The p.Ala243Val mutation is located in the Zn^{2+} binding NTD of the GluN2A subunit and leads to a reduced Zn^{2+} inhibition. Zn^{2+}

inhibition occurs at physiologically relevant zinc levels and/or during co-release of Zn^{2+} with glutamate, which implicates a crucial role in physiological and pathophysiological processes (Assaf and Chung, 1984; Howell et al., 1984). Because of the inhibitory effect of Zn^{2+} on recombinant GluN1/GluN2 receptors (Kutsuwada et al., 1992; Meguro et al., 1992) and the high Zn^{2+} sensitivity of GluN2A-containing receptors, it is conceivable that under physiological conditions the receptor is tonically inhibited and Zn^{2+} has protective function against NMDA receptor-mediated overexcitation and glutamate toxicity. The loss of the regulatory high affinity Zn^{2+} inhibition in the mutated receptor could lead to repeated activation and enhanced Ca^{2+} influx. It is possible that this gain of function of the receptor leads to an overactivation, which could finally result in epilepsy syndromes.

Mutagenesis studies showed that the bilobate NTD of the GluN2 subunit oscillates spontaneously between open-cleft and close-cleft conformations and influences the gating properties of the receptor (Gielen et al., 2009). Binding of allosteric modulators, e.g. Zn^{2+}, is thought to stabilize the closed conformation and thereby weakens the LBD-interaction, which results in a subsequent closure of the channel. The crystal structure of the isolated GluN2B NTD reveals the Zn^{2+} binding site to be within the cleft of the R1 and R2 lobes and stabilizes a closed cleft conformation (Karakas et al., 2009). Based on mutagenesis studies, it is known that the zinc binding site is located at the similar cleft of the GluN2A NTD (Fayyazuddin et al., 2000). Regarding the NTD mutation (GluN2A p.Ala243Val) the question arises, if there is a disturbance of zinc binding or a disturbance of the zinc inhibition mechanism in the mutated receptor complex, which leads to the reduction of zinc inhibition. Because the residue alanine 243 is not in direct contact with the Zn^{2+} ion, the substitution seems not to influence the zinc binding itself. Probably the substitution of alanine to valine changes the structure of the NTD or the binding pocket. The Zn^{2+} inhibition mediated by the NTDs has to be transduced into alterations in channel gate activity and the closure of the NTD is a crucial step in this inhibition mechansim. Perhaps the mutation destabilizes the architecture of the Zn^{2+} binding pocket or the entire NTD clamshell, so that it cannot persist in the closed conformation or cannot close completely, which results in reduced zinc inhibition. Another possibility could be that the substitution leads to a conformational change of the NTD so that the Zn^{2+} ions cannot bind efficently in the clamshell cleft. To clarify the exact mechanism of the reduced Zn^{2+} inhibition further experiments are required to establish the role of this NTD mutation site in allosteric zinc modulation. Nevertheless the GluN2A NTD mutation reveals the crucial role of Zn^{2+} inhibition in glutamatergic

neurotransmission and the involvment in pathological conditions.

The *in vivo* relevance of Zn^{2+} neuromodulation has already been shown in another ligand gated receptor, in the α1 glycine receptor, which belongs to the Cys loop receptor family. The Zn^{2+} binding site was shown to be localized in the N-terminal domain of glycine receptor α subunits (Laube, 2002; Lynch, 2004). A substitution of a single amino acid in the α1 subunit gene in the NTD eliminated the potentiating effect of Zn^{2+} on glycine receptor currents. Homozygous mice containing this mutation developed a severe neuromotor phenotype after birth, which leads to hyperekplexia also called startle disease (Hirzel et al., 2006).

6.3 The ion channel pore

The ion channel of glutamate receptors consists of a water-filled pore, which is divided in an external and internal cavity. These cavities are separated through the narrow constriction of the pore. The residues lining the pore build the permeation pathway and are the primary determinants of ion selectivity and conductance. The recently published crystal structure of the GluN1/GluN2B NMDA receptor refines the picture of the channel pore of NMDA receptors. The structure revealed a three-part arrangement in a gate, a vestibule and a filter section (Lee et al., 2014). A known important key determinant of conductance and permeation of all glutamate receptors is the identity of the residues at the apex of the P-loop, the so called QRN site. In AMPA and Kainate receptors there is a glutamine (Q) unedited or an arginine (R) edited, in GluN1 and GluN2 NMDA receptors an asparagine (N) and in GluN3 a glycine (G). The crystal structure of the full length NMDA receptor confirms these important localization and represents this residue at the second constriction of the channel pore at the turn between the end of the P helices and the beginning of the extended filter sequence (Lee et al., 2014).

Within the group of ionotropic glutamate receptors, NMDA receptors exhibit a unique permeation profile because of their high Ca^{2+} permeability and the voltage-dependent Mg^{2+} block. In GluN1/GluN2 NMDA receptors the QRN site asparagine of GluN1 and the asparagine adjacent to the QRN site (N+1) in GluN2 formed the narrow constriction of the ion channel (Wollmuth et al., 1996) and single amino acid substitutions have shown that interactions at or near the QRN site are critical for Ca^{2+} permeability and

Mg^{2+} block.

The detected *de novo* GRIN2A mutation Asn615Lys (c.1845C>A) (Endele et al., 2010) leads to the amino acid substitution asparagine to lysine at position 615 (p.Asn615Lys) in the mature protein. This mutation was found in a patient (3 year old girl) with early-onset encephalopathy. The mutation Asn615Lys is located in the transmembrane domain in the re-entrant loop (P-loop) and is the asparagine at the QRN+1 site (GluN2A Asn615). In the functional analyses the substitution results in a loss of the Mg^{2+} block and a decrease in Ca^{2+} permeability (Endele et al., 2010). Introducing a positive side chain through the substitution of the amino acid lysine in the channel pore at this critical position, could have a repulsive effect on cation permeability and could explain the reduced Ca^{2+} permeability. Furthermore, Wollmuth and colleagues showed volume specific effects on the pore size with mutations at the QRN+1 site in GluN1/GluN2A receptors (Wollmuth et al., 1996). Supposably the lysine leads to a decrease in pore size, which also could influence the channel properties, finally leading to the reduced Ca^{2+} permeability.

Interestingly sequence analysis of the GluN2B gene in two patients with West syndrome revealed *de novo* mutations in the channel pore also at the QRN site. One patient carries the *de novo* mutation c.1844 A>T in GRIN2B, which leads to the substitution asparagine to isoleucine at the position 615 in the GluN2B subunit (GluN2B p.Asn615Ile) (Lemke et al., 2013a). This substitution is the asparagine at the QRN site in GluN2B. The other patient carries the *de novo* mutation c.1853 T>G in GRIN2B, which results in a substitution of valine to glycine at the position 618 in the GluN2B subunit (GluN2B p.Val618Gly) (Lemke et al., 2013a). By functional analysis both mutations in the GluN2B revealed an increase in Ca^{2+} permeability and a reduced Mg^{2+} block.

The mutated asparagine residues at the QRN and QRN+1 site are situated in a position to project their side chains into aqueous vestibule in the GluN1/GluN2B crystal structure (Lee et al., 2014). The substitution of the uncharged and hydrophilic aspargine in the narrow constriction of the pore in the GluN2B subunit (p.Asn615Ile) to an also uncharged but more hydrophobic isoleucine could result in strong alteration of side chain orientation of the isoleucine away from the inner of the pore. Thus, could change the characteristics of the selectivity filter and the Mg^{2+} block.

The valine residue 618 is located in close vicinity to the QRN site but more in the middle of the pore loop. The influence in the electrophysiological analysis of this substitution is less than the QRN site substitution maybe because of the more distinct

location in the middle of the pore loop. Furthermore the substitution from the amino acid valine to glycine is not such a strong alteration of the side chain characteristics.
However, the results showed that not only substitutions at the QRN site had strong influence on receptor function but also further residues in the middle of the pore loop. The results demonstrate the fundamental role of the channel residues at the QRN site and residues in close vicinity lining the pore loop for appropriate receptor function in GluN2A and GluN2B containing receptors. These observations are in concordance with previous studies. In mouse models, a change of these residues in the GluN1 subunit is not tolerated and leads to early death (Seeburg et al., 2001). Introducing an arginine at this position in the GRIN1 leads to Mg^{2+} insensitive and Ca^{2+} impermeable receptors in the mouse model (Rudhard et al., 2003). Also in other ionotropic glutamate receptors the QRN site is fundamental for proper receptor function. Point mutations at the QRN site in AMPA receptors of the GluA2 subunit lead to neonatal epileptic seizures and increased Ca^{2+} permeability (Brusa et al., 1995; Feldmeyer et al., 1999) .

Through the mutations at the QRN site in both subunits (GluN2A and GluN2B) in the channel pore no coincidence detection is possible anymore and the coincidence-dependent calcium signaling through NMDA receptors, which is necessary for precise refinement of synaptic connectivity, is disturbed. The functional analysis of the epilepsy associated mutations at these critical residues in the pore region of GluN2A and GluN2B leads to a gain of ion channel function. In neuronal networks this could lead to hyperexcitation in excitatory neurotransmission, finally resulting in epilepsy.

In the GluN2B subunit, further mutations were found in the LBD of NMDA receptors. One *de novo* GRIN2B mutation was detected in the glutamate binding domain of the GluN2B subunit in a patient with moderate mental retardation. The c.2004C>T mutation leads to the amino acid substitution arginine to cysteine at the position 682 (p.Arg682Cys). This residue is highly conserved within the glutamate ligand binding domain. In the model of the GluN2B LBD the alteration of this residue results in a loss of three hydrogen bonds, which destabilizes the tertiary structure of the GluN2B LBD. This could affect the glutamate affinity, but the functional analysis revealed no changes in the apparent affinity of the agonist glutamate and glycine (Endele et al., 2010).
A further LBD mutation was detected in the GluN2B subunit in a patient with focal epilepsy and intellectual disability (ID). The mutation p.Arg540His leads to the substitution of a highly conserved arginine residue within GluN2A-D subunits. The

substitution to histidine influenced the Mg^{2+} block by a reduction of the Mg^{2+} sensitivityand led to an increased Ca^{2+} permeability. The structural analysis showed that the arginine 540 in GluN2B is essential in the stabilization of the tertiary structure of the glutamate binding domain. However there was no change in glutamate binding affinities. Unfortunately, the amino acid substitution Arg540His cannot be illustrated in the full length NMDA crystal structure because some residues in the loop connecting the LBD to the TMD are not resolved. Despite the localization in the LBD the GluN2B mutation p.Arg540His seems to have strong influence on the core region of the receptor. GluN2A and GluN2B-containing NMDA receptors are more sensitive to Mg^{2+} block than NMDA receptors that contain GluN2C and GluN2D subunits. But all four subunits possess an asparagine (N) residue at the QRN and QRN+1 site indicating that additional structural elements are required to determine the sensitivity of subtypes to block by Mg^{2+}. The mutation p.Arg540His seems to have an allosteric effect and chimeric approaches provide evidence that the LBD of GluN2 subunits not only influence the agonist binding and potency but also the voltage-dependent Mg^{2+} block (Wrighton et al., 2008). Recent findings revealed also a *de novo* mutation in the GluN2B subunit in the glutamate binding site at position 461, which substitutes a cysteine into a phenylalanine (p.Cys461Phe) (Allen et al., 2013). The patient with the mutation p.Arg540His patients and the patient with the mutation (p.Cys461Phe) displayed similar symptoms presented with primary developmental delay and childhood onset epilepsy. Unfortunately, no functional data from the p.Cys461Phe mutation were available. Therefore, it would be very interesting to investigate the functional consequences and the influence on Mg^{2+} sensitivity as well as Ca^{2+} permeation.

The recent findings focused on the GluN2 subunits of the NMDA receptor. Hamdan and colleagues identified also *de novo* mutations in the GRIN1, which encodes the essential GluN1 subunit of the NMDA receptor (Hamdan et al., 2011). The mutations were located in the transmembrane regions and lead to an increase of Ca^{2+} currents and an abolishment of receptor activity and were detected in patients with intellectual disability (ID) and epilepsy. Further GluN1 mutations were analyzed in our group in the context of Bachelor theses. The results showed a strong influence on receptor function of mutations in the transmembrane domains. These findings further support the involvement of further NMDA receptors subunits in epilepsy syndromes and implicated a strong influence of the core domains of the NMDA receptor subunits for receptor function and an involvement in pathological conditions.

6.4 NMDA mutation link to disease phenotype?

There is great interest in determining whether different NMDA receptor subtypes make specific contributions to pathological neuronal processes. All epilepsy associated mutations were detected in patients with age-related disease phenotypes and all pathological mutations were detected in the developmental regulated GluN2 subunits. The appearance of the age-related epilepsy syndromes in combination with the mutations in the developmental regulated GluN2 subunits lead to the conclusion that the stage of brain maturation plays a crucial role in the development of epilepsy syndromes. A disturbance of the precise regulation mechanisms during development results in serious impairment.

NMDA receptors are central in brain development and the calcium influx through NMDA receptors on the one hand plays a neuroprotective role but on the other hand could lead to excitotoxicity, which leads to cell death. Thus, the activation of these receptors has important implications for the survival or death of neurons, which is important especially in the developing brain. It was shown that blockade of NMDA receptors during late fetal or early neonatal life triggered apoptotic neurodegeneration in rats (Ikonomidou et al., 1999). Hence, it is conceivable that disturbance of the regulation mechanism, instead of blockade, could also lead to neurological impairment. Due to the gain of function mutations in the GluN2A and GluN2B subunits, it is likely that also increased activity during the development could result in strong impairment, like, epilepsy. The gain of ion channel function could be a reasonable molecular correlate for the epilepsy syndromes, because it results in an overactivation of the receptors, which is in concordance with the hypothesis that epilepsy proceeds from hyperexcitation.

Based on the different physiological roles of GluN2A and the GluN2B subunits it is not much surprising that there occurred differences in the patient phenotypes. One common characteristic of the different phenotypes of patients with GluN2 subunit mutations is the age-dependent occurrence during the childhood, which implicates the crucial role of correct regulation and function of the GluN2 subunits in development.

6.4.1 The GluN2A subunit

Since the first identification and functional investigation of epilepsy associated mutations in the GluN2A subunits of the NMDA receptor (Endele et al., 2010) several

further GluN2A mutations in different receptor domains were detected. Yuan and colleagues recently identified a *de novo* mutation in the linker region between the ligand binding domain and the transmembrane domain in GluN2A in a patient with early-onset epileptic encephalopathy and profound developmental delay (Yuan et al., 2014). Like the GluN2A mutation in the NTD and the ion channel pore of the NMDA receptor, this mutation also results in a gain of function through a strong impairment of receptor function with enhanced agonist potency, decreased sensitivity to magnesium, protons and zinc and an increased single-channel open probability. Further gain of function mutations in the GluN2A subunit were detected from Carvill et al. and Lesca and colleagues (Carvill et al., 2013; Lesca et al., 2013a). They identified mutations in patients with disorders of the epilepsy-aphasia spectrum.

Despite the information about the functional impact of several GluN2A mutations, to date it is difficult to build a clear correlation between the different GluN2A subunit mutations and the appeared phenotype. The GluN2A NTD mutation (p.Ala243Val) occurred in a patient with BECTS (Chapter 3). This epilepsy form is the most frequent epileptic syndrome in children (Holmes, 1993). According to the International League Against Epilepsy (ILAE) BECTS is classified as idiopathic focal epilepsy. Seizure onset is usually aged between 3-10 years (Beaumanoir et al., 1974). In most cases the children have a good prediction and after adolescence there are no further seizures or neurospychatric abnormalities. A more severe phenotype was detected in the patient with early-onset encephalopathy with first seizures at the age of 3 month with the pore mutation GluN2A p.Asn615Lys (Chapter 2). The differences pertaining to the phenotypes could be due to the fact that the gain of function arises from different mechanisms. In the case of the mutation in the NTD (GluN2A p.Ala243Val) appeared a loss of the Zn^{2+} inhibition. This inhibition mechanism is non-competitive and, unlike Mg^{2+} block, voltage independent. Although there is no doubt that during synaptic activity vesicular Zn^{2+} is released, there is no consensus about the time course and amplitude of Zn^{2+} concentrations in the synaptic cleft (Paoletti et al., 2009). But the important role of synaptic Zn^{2+} in the pathophysiology of epilepsy could point out in several observations (Frederickson, 1989). So a disturbance of the Zn^{2+} inhibition mechanism in the GluN2A subunit is likely to trigger epilepsy syndromes.

The pore mutation (GluN2A p.Asn615Lys) influences two main features of NMDA receptors in the functional analysis. On the one hand the mutation leads to a loss of the voltage dependent Mg^{2+} inhibition. On the other hand there is a reduced Ca^{2+} permeability. This mutation raises the question if this is a gain or loss of function. The

loss of the Mg^{2+} inhibition results in a higher susceptibility of the receptor and results in a loss of coincidence detection. The reduced Ca^{2+} permeability could result in a reduced pro-death signaling through a diminished phosphorylation of CREB. This could lead to neurodegeneration and cognitive impairment, especially in development.

6.4.2 The GluN2B subunit

Both mutations in the channel pore within the GluN2B subunit are found in patients with the West syndrome. The West syndrome is a severe form of epilepsy characterized by early onset (in the first year), a cluster of infantile spasms (IS) and a characteristic EEG pattern called hypsarrhythmia (Lagae et al., 2010; Pavone et al., 2013). In most cases the West syndrome has worse prediction and the treatment is relatively difficult. Another mutation in the GluN2B subunit, located in the LBD, was found in a patient with focal epilepsy and ID. The mutation in the LBD shows a milder gain of function in comparison to the pore mutations of the West Syndrome patients, which results in a stronger gain of function of the ion channel.
Especially, GluN2B containing receptors are critical for cortical development and function. This could be shown through an inversion of the developmental switch from GluN2B to GluN2A through a genetically replacement of the GluN2B subunit by the GluN2A. The NMDA current is then mediated by GluN2A containing receptors in the absence of GluN2B (Wang et al., 2011). The early expression of GluN2A instead of GluN2B is unable to rescue GluN2B loss of function. This indicates that GluN2B containing NMDA receptors activate unique cellular processes and that the period of predominance of the GluN2B subunit in development is critical for the developing brain. The mutations in the GluN2B subunit lead to a gain of function and results in a higher Ca^{2+} permeability. Referring to the extrasynaptic localization and subsequent deactivation of CREB, an overactivation would result in an enhanced pro-death signaling (Hardingham and Bading, 2010).

In summary differences in the mechanism of ion channel impairment, in regulation mechanisms during development and downstream pathways could be possible reasons for the differences in the occurred phenotypes. Generally it seems that mutations in the core domains of the receptor, independent from the subtype, results in stronger impairment of receptor function and to servere phenotypes, which are not

easily treatable and leads to long-lasting developmental impairment. In the case of the three GluN2B mutations there exists a correlation between the severity of phenotype and the electrophysiological impairment of receptor function. Although it is nearly impossible to make a prediction for the patients phenotype based on a detected mutation, the electrophysiological results could be useful for therapeutic treatment.

6.5 The NMDA receptor: a promising target for therapeutic intervention in epilepsy

The findings of epilepsy-associated mutations in the GRIN2A and GRIN2B lead to a new interest in more selective NMDA modulators in the future for possible therapeutic treatment. In the case of mutations, which result in a gain of function and finally result in an overactivity of the NMDA receptors, maybe there exists a chance to treat some forms of epilepsy with subunit selective antagonists. GluN2A and GluN2B subunits are potential targets and can be modulated by existing drugs or compounds, for example through the antagonist memantine. Memantine is a NMDA channel blocker and is already therapeutically used in Parkinson and Alzheimer therapy and shows only little side effects (Parsons et al., 1999; Witt et al., 2004)

A recent paper indicates therapy potential with memantine also in epilepsy syndromes. In a patient with a mutation in the GluN2A subunit and early-onset encephalopathy the medication with memantine reduces the patients seizure burden. The cognitive ability remained unchanged (Pierson et al., 2014). This case create the new possibility by combination of high-throughput screening, electrophysiological studies and subsequent therapeutic screening on the mutated protein find a specific antagonist, which use could lead to an improvement of the course of disease. In the future it is conceivable that an early treatment with an appropriate NMDA antagonist could improve the patients development or minimize the developmental impairment in some cases of NMDA associated forms of epilepsy. Based on the findings of epilepsy associated mutations in different NMDA receptor subunits, a genetic diagnosis could be possible in the future and the proof of genetic defects could lead to a definitive diagnosis. This can be important for the prognosis and probably influence therapeutic decisions. Furthermore, with the knowledge of genetic defects new treatment strategies of some forms of epilepsies become possible in the future and perhaps the defective protein can be used as new pharmacological target (Weber and Lerche, 2008).

In some cases, the use of NMDA antagonists seems to have good prospects in epilepsy therapy. A fundamental problem would be that many antagonists have strong adverse effects or could have opposite effects on other receptor subunit combinations (Chapter 5). The challenge in the future would be to find subunit-selective compounds, which correct the abnormal activity of the protein without disturbing/changing the NMDAR signaling in the whole brain.

6.6 The excitatory GluN1/GluN3A receptors

Although GluN1/GluN2 receptors and GluN1/GluN3 receptors belong to the same receptor family, they display essential different pharmacological properties. In contrast to the conventional GluN2 containing receptors they show reduced Ca^{2+} permeability and Mg^{2+} sensitivity. Because of the high Ca^{2+} permeability of conventional NMDA receptors an overstimulation can induce cell death due to excessive Ca^{2+} influx in the cell. The reduced calcium permeability of GluN3A subunits emerge the idea of a neuroprotective role for the GluN3A subunit (Pachernegg et al., 2012). The occurrence of native GluN1/GluN3A receptors is controversial, but there are hints for GluN1/GluN3A receptors in CNS myelin (Pina-Crespo et al., 2010). A problem in measuring GluN1/GluN3A receptor activity *in vivo* is the rapid desensitization in response to glycine (Awobuluyi et al., 2007; Chatterton et al., 2002) and because of the high glycine affinity the receptor might be saturated at physiological concentrations of glycine.

Despite the recent results of our findings, the exact mechanism of the potentiation effect of GluN1 antagonists at GluN1/GluN3A receptors, remains to be elucidated. We had shown the agonistic effect of GluN1 antagonists, which leads to enhanced receptor currents of GluN1/GluN3A receptors in an affinity dependent manner (Chapter5). These results of different influence on the magnitude of potentiation on receptor currents could be a useful tool for studying GluN1/GluN3A receptors *in vivo.*

Special attention should be paid to the GluN1 antagonist kynurenic acid, which occurred endogenously in the brain. Kynurenic acid may play a physiological role in blocking conventional NMDA receptor activation. In the case of GluN1/GluN3A receptors it can enhance receptor activation. This is of interest because the GluN1 antagonist kynurenic acid may play also a pathophysiological role in epilepsy. Studies

showed a decrease of kynurenic acid levels of the cerebrospinal fluid in patients with West syndrome (Yamamoto et al., 1995; Young et al., 1983). A chronic deficiency of the GluN1 antagonist could potentially lead to a developing NMDA excitotoxic process through conventional GluN1/GluN2 NMDA receptors, but also could have a negative effect through GluN1/GluN3A receptors.

6.7 Outlook

Understanding the NMDA receptor function in neurological disorders is fundamental and could be a promising approach for the improvement of therapeutic strategies. The detection of epilepsy associated mutations and the electrophysiological analysis revealed strong impairment of receptor function and identify the NMDA receptor as a key player in the incidence of epilepsy syndromes. The understanding of genotype-phenotype relationships prove to be challenging. Mutations in different domains of the receptor cause similar impairment of receptor function, but the mutations cause different phenotypes. Further investigations are needed to elucidate the role of mutations in different receptor subunits and a possible influence on receptor function. Knowledge of the genetic defect and their underlying mechanism can give rise to new therapeutic strategies. Further physiological characterization of epilepsy associated mutations in cell culture and animal models for studying hippocampal slices and brain physiology could be considered, because data of relative simple expression systems lack the complexity of neurons. Also the *in vivo* relevance for changes in ion channel behaviour observed *in vitro* has to be elucidated. Studies in knock in mice could be a possibility for deeper understanding of mechanisms not only *in vitro* but also *in vivo*. Especially, in regard to the developmental influence of mutations in the GluN2 subunits, *in vivo* studies in mice could recapitulate the human genotype and phenotype.

The results from this work put the NMDA receptors in perspective as novel therapeutic target in epilepsy syndromes in personalized therapy. Further experiments to detect and to understand the antagonist mechanism on different mutations in various receptor combinations could lead to promising therapeutic strategies. For example, the GluN2A p.Asn615Lys mutation responded in electrophysiological experiments not to the antagonist memantin, but to other antagonists (dextromethorphan and dextrorphan) (Pierson et al., 2014). This indicates a need for specific electrophysiological evaluation of the mutations and raises the possibility that treatment with the proper antagonist can help to select the best therapy.

7 References

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Ehrenwörtliche Erklärung

Ich erkläre hiermit ehrenwörtlich, dass ich die vorliegende Arbeit entsprechend den Regeln guter wissenschaftlicher Praxis selbstständig und ohne unzulässige Hilfe Dritter angefertigt habe.

Sämtliche aus fremden Quellen direkt oder indirekt übernommenen Gedanken sowie sämtliche von Anderen direkt oder indirekt übernommenen Daten, Techniken und Materialien sind als solche kenntlich gemacht. Die Arbeit wurde bisher bei keiner anderen Hochschule zu Prüfungszwecken eingereicht.

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List of Contributions

Parts of this work has already been published in journals.

Chapter 2: Mutations in GRIN2A and GRIN2B encoding regulatory subunits of NMDA receptors cause variable neurodevelopmental phenotypes.
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Chapter 3: Mutations in GRIN2A cause idiopathic focal epilepsy with rolandic spikes.
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Chapter 4: GRIN2B mutations in West syndrome and intellectual disability with focal epilepsy.

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List of Abbreviations

BAPTA-AM	1,2-bis(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid-acetoxymethyl ester
BECTS	Benign epilepsy with centro-temporal spikes
CNS	Central nervous system
CSWS	Epileptic encephalopathy with continuous spike-and-waves during sleep
7-CKA	7-Chloryknurenic acid
5,7-DCKA	5,7-Dichlorokynurenic acid
DMSO	Dimethyl sulfoxide
EC_{50}	Half maximal effective concentration
EE	Epileptic encephalopthies
EEG	Electroencephalogram
Glu	Glutamate
GluN1 = NR1	GluN1 subunit of the NMDA receptor
GluN2 = NR2	GluN2 subunit of the NMDA receptor
GluN3 = NR3	GluN3 subunit of the NMDA receptor
Gly	Glycine
HA-966	1-Hydroxy-3-aminopyrrolid-2-one
ID	Intellectual disabilty
IFE	Idiopathic focal epilepsy
ILAE	International League Against Epilepsy
IS	Infantile spams
LBD	Ligand binding domain

LKS	Landau Kleffner syndrome
L-689560	*Trans*-2-Carboxy-5,7-dichloro-4-phenylaminocarbonyl-amino-1,2,3,4-tetrahydroquinoline
MDL	(E)-4,6-Dichloro-3-(2-phenyl-2-carboxyethenyl)indole-2-carboxylic acid
NMDG-Cl	N-methyl-D-glucamine
NTD	N-terminal domain
SEM	Standard error of the mean
TEVC	Two electrode voltage clamp
TMD	Transmembrane domain

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