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NMDA receptors are selectively partitioned into complexes and supercomplexes during synapse maturation

René A.W. Frank, Noboru H. Komiyama, Tomás J. Ryan, Fei Zhu, Thomas J. O’Dell & Seth G.N. Grant

How neuronal proteomes self-organize is poorly understood because of their inherent molecular and cellular complexity. Here, focusing on mammalian synapses we use blue-native PAGE and ‘gene-tagging’ of GluN1 to report the first biochemical purification of endogenous NMDA receptors (NMDARs) directly from adult mouse brain. We show that NMDARs partition between two discrete populations of receptor complexes and ~1.5 MDa supercomplexes. We tested the assembly mechanism with six mouse mutants, which indicates a tripartite requirement of GluN2B, PSD93 and PSD95 gate the incorporation of receptors into ~1.5 MDa supercomplexes, independent of either canonical PDZ-ligands or GluN2A. Supporting the essential role of GluN2B, quantitative gene-tagging revealed a fourfold molar excess of GluN2B over GluN2A in adult forebrain. NMDAR supercomplexes are assembled late in postnatal development and triggered by synapse maturation involving epigenetic and activity-dependent mechanisms. Finally, screening the quaternary organization of 60 native proteins identified numerous discrete supercomplexes that populate the mammalian synapse.
S
ynapses contain highly complex proteomes that when disrupted by mutations cause over 100 brain diseases\textsuperscript{1-3}. Since the function of almost all cellular proteins is dependent on their higher-order assembly into molecular machines\textsuperscript{3,4}, it is imperative to characterize the self-assembly of synapse proteins and the impact of mutations. However, defining the organization of the myriad synapse proteins is poorly understood largely because of the technical challenges imposed by the cellular and molecular complexity of the brain.

Canonical models of the higher-order assembly of proteins are hierarchical (Fig. 1a) with individual constituent subunits forming oligomeric complexes that may further associate into mega-Dalton sized supercomplexes (complexes of complexes)\textsuperscript{5}. Amongst the most highly studied examples of synaptic multiprotein machines are those comprising the N-methyl-D-aspartic acid receptors (NMDAR) and Dlg/MAGUK proteins, which are key mediators of synaptic plasticity, cognitive functions and implicated in multiple brain disorders\textsuperscript{6-11}. The finding that PDZ-ligands in GluN2 subunits of NMDARs bind the PDZ domains of Dlg/MAGUK proteins has underpinned the general paradigm that neurotransmitter receptors and other classes of synaptic proteins require this interaction for the purpose of anchoring and assembly with additional proteins\textsuperscript{12-15}. However, it is not known if the promiscuity of PDZ interactions is sufficient for assembly \textit{in vivo} or if other mechanisms select for the assembly of specific combinations of synaptic proteins\textsuperscript{16}.

The study of higher-order protein assemblies in mammals is further complicated by the evolution of more complex synapse proteomes in the vertebrate lineage\textsuperscript{17}. A major feature distinguishing vertebrate from invertebrate synapses is the expansion in proteome complexity that arose following two genome duplications in early chordates\textsuperscript{18-20}. The increase in number of subunits may have had a multiplicative impact on the diversity of potential vertebrate complexes and supercomplexes. This is exemplified by the NMDAR-Dlg/MAGUK assembly: many invertebrate genomes encode a single-GluN2 and -Dlg protein\textsuperscript{21}, whereas mammals express four, each retaining the conserved PDZ binding capacity and thereby permitting \textit{N}\textsubscript{10-30%} MDa complexes indicated by filled and open arrowheads, respectively (hereafter used to label all figures). The expected size of each protein in monomeric form indicated with pink rectangle. On left side, non-denaturing molecular mass indicated in MDa. On right side, non-denaturing molecular mass indicated in mega-Daltons (MDa). (b) Native protein complexes of GluN1, GluN2A and GluN2B detected in BNP immunoblot screen of fresh human cortical biopsy samples from the inferior frontal (inf. front.), inferior temporal (inf. temp.) and superior frontal (sup. front.) lobes. Mouse forebrain extract supernatant shown for comparison. These data show the ~0.8 and ~1.5 MDa NMDA receptor complexes (1.5-NR and 0.8-NR) were conserved between mouse and humans.

**Figure 1 | Native supermolecular assembly of NMDAR subunits in the mammalian brain.** (a) Schematic showing the higher-order assembly from individual proteins to complexes and supercomplexes (complexes of complexes). (b) Native protein complexes of GluN1, GluN2A and GluN2B detected in BNP immunoblot screen of mouse forebrain extracts. Approximately 0.8 and ~1.5 MDa complexes indicated by filled and open arrowheads, respectively (hereafter used to label all figures). The expected size of each protein in monomeric form indicated with pink rectangle. On left side, non-denaturing molecular mass indicated in mega-Daltons (MDa). (c) BNP GluN1 immunoblot of fractions from glycerol gradient (10-30%) ultracentrifugation. ‘IN’, inferior frontal extract supernatant. On right side, non-denaturing molecular mass indicated in MDa. (d) BNP GluN1 immunoblot of fresh human cortical biopsy samples from the inferior frontal (inf. front.), inferior temporal (inf. temp.) and superior frontal (sup. front.) lobes. Mouse forebrain extract supernatant shown for comparison. These data show the ~0.8 and ~1.5 MDa NMDA receptor complexes (1.5-NR and 0.8-NR) were conserved between mouse and humans.

RESULTS

Two supermolecular NMDAR complexes in mice and humans.

Numerous reports using co-immunoprecipitation and \textit{in vitro} methods have shown that NMDARs are associated with upwards of 50 different synaptic proteins, which are expected to affect the Ca\textsuperscript{2+}-dependent synaptic signalling, anchoring and trafficking of NMDARs. How these apparently numerous binary interactions relate to the assembly of native NMDARs is unknown. For example, does the native forebrain NMDAR complex contain all complexes/supercomplexes remains unknown. Furthermore, whether the redundant PDZ-ligand interactions confer resilience to mutations in individual GluN2 or Dlg paralogs is unknown because of the lack of studies testing paralog mutations on complex formation. These issues are fundamental to our understanding of the molecular organization of the synapse as well as the interpretation of the growing body of literature that has identified disease-causing mutations in NMDAR-Dlg/MAGUK complexes and other postsynaptic proteins\textsuperscript{22}.

To address these issues we developed a biochemical and mouse genetic approach to characterize the supermolecular organization of proteomes \textit{in vivo}, focusing on native NMDAR complexes. First, using a simple biochemical screen we identify that all native NMDAR from the mouse forebrain are partitioned between ~0.8 and ~1.5 MDa complexes. Next, we report the knock-in of a high-affinity peptide tag into the gene encoding GluN1 subunit of the NMDAR in mouse. This enables the purification of all endogenous NMDARs, as well as the identification and quantification of constituents. Subunit tagging combined with multiple other mutant mice revealed a novel tripartite mechanism for assembly between NMDAR, PSD95 and PSD93 supercomplexes. This mechanism regulates assembly late in postnatal development. Supercomplex assembly is also driven by synapse maturation involving epigenetic and activity-dependent palmitoylation. Finally, we used a biochemical screen to survey 60 diverse proteins from within the synapse proteome identifying their higher-order organization into 190 novel complexes and supercomplexes consistent with those containing NMDARs. These findings reveal specific mechanisms that restrict the association of synapse proteins into specific supercomplexes and identify a role in synapse maturation.
of these components all of the time? To address this question we employed blue-native polyacrylamide gel electrophoresis (BNP) to explore the size distribution of the total population of NMDARs in the mouse forebrain. In contrast to co-immunoprecipitation, this permitted us to identify the distinct complexes within which each brain protein reside, without requiring prior knowledge of their constituents. BNP showed native NMDARs (containing GluN1, GluN2A and GluN2B) were distributed between only two distinct complexes with apparent median masses of ~840 kDa and ~1.5 MDa (Fig. 1b), for simplicity hereafter referred to as 0.8-NR and 1.5-NR, respectively. Although the detergent-lipid micelle surrounding 0.8-NR and 1.5-NR likely contributes to the observed masses, both were also separable on the basis of size by ultracentrifugation through a glycerol gradient (Fig. 1c), confirming these molecular species were distinct. The existence of NMDARs in these two forms has not been reported previously and we therefore examined whether both were detectable in human brain. BNP immunoblots probed with GluN1 (Fig. 1d) confirmed both 0.8-NR and 1.5-NR in extracts from neurosurgical biopsies resembling the four abundant bands, identified by peptide mass fingerprinting of the human neocortex. These data suggest that two distinct supermolecular forms differing in size constitute native NMDARs of the adult mammalian forebrain.

Isolation of native NMDAR complexes from GluN1-TAP mice. To understand the molecular basis for the partitioning of NMDARs into these supermolecular forms requires identification of constituents that distinguish 0.8-NR from 1.5-NR. Purification of scarce native assemblies from complex tissues typically suffers from low yields and the absence of reliable controls. To avert this problem, we used mouse genetics to insert a tandem affinity purification (TAP) tag into the first protein-coding exon of the Glun1 subunit gene (Glun1TAP) by homologous recombination in embryonic stem cells (Fig. 2a). Because most proteomic methods rely on denaturation to elute purified complexes, which prevents individual complexes from being separated from one another, we included a 3xFlag tag. This design permitted gentle elution (by competition with 3xFlag peptide) of intact individual complexes for further separation and characterization (Supplementary Fig. 1a).

Following germline transmission of this TAP-GluN1 knock-in mutation (GluN1TAP/+) mice were intercrossed to produce viable GluN1TAP/TAP homozygotes. Next, these mice were examined in biochemical, anatomical and electrophysiological assays, which indicated that the TAP-tagged NMDAR functioned normally (Supplementary Fig. 1b–g). Optimization of the purification conditions resulted in almost complete solubilization of TAP-GluN1, GluN2A and GluN2B, thus providing material that was representative of essentially all endogenous NMDARs (Fig. 2b, lanes 5 and 6 from left and Supplementary Fig. 2a). A single step of Flag-affinity purification (Fig. 2b and Supplementary Fig. 2b) yielded 10–25 µg protein from five adult mice, which corresponds to ~10^{12} receptors per mouse forebrain.

Purified TAP-NMDARs complexes were directly visible as coomassie-stained BNP bands equivalent to those detected in forebrain extract by BNP immunoblots (Fig. 2c, left lane), indicating the native NMDARs were purified intact. No bands were visible in control (wild type (WT)) samples (Fig. 2c, left lane), indicating the specificity of the purification. To determine the molecular constituents that might differentiate 0.8-NR from 1.5-NR, we excised each BNP band and profiled their composition using mass spectrometry (BNP-MS, Supplementary Table 1). A sample of these MS-identified proteins was validated by BNP immunoblot (Supplementary Fig. 2c,d), suggesting the 0.8-NR band was composed solely of NMDAR subunits and represents the expected ‘classical’ tetrameric channel complex. In contrast, the 1.5-NR band contained the NMDAR complex in association with 50 other proteins (see Supplementary Table 1 for full list) including other ion channel complexes (Kir2.3), trans-synaptic adhesion (Adam22), scaffold (PSD95, PSD93) and signalling proteins (CaMKII). Thus, the size and composition suggest 1.5-NR represent a sub-population of NMDAR supercomplexes.

To explore the higher-order assembly of synaptic receptor complexes further we used the same TAP-tag and BNP-MS strategy to analyse an available Psd95ΔTAP ΔTAP knock-in mice line24 and found TAP-purified PSD95 was readily visible on coomassie-stained BNP within a single ~1.5 MDa band (Fig. 2c, right lane). Thus, essentially all PSD95 assembles into ~1.5 MDa complexes (hereon called 1.5-PSD95). Moreover, MS of the gel-excised 1.5-PSD95 band identified 89 proteins, of which 87% were the same as those in 1.5-NR (Supplementary Table 2). The constituents of 1.5-NR and 1.5-PSD95 were consistent with the numerous proteins reported to interact with either NMDAR or PSD95 (refs 24,25). Importantly, these associations shown here were all contained within a discrete subset of NMDARs in the mega-Dalton size range, which is inconsistent with models of these synaptic proteins co-assembled into either a single very large complex or distributed within a network.

We therefore reasoned that the majority of 1.5-NR constituents would be in low abundance. Accordingly, Coomassie-staining of purified NMDAR separated by SDS-polyacrylamide gel electrophoresis (PAGE) revealed only four major bands (with apparent molecular weights of 180, 130, 105 and 85 kDa), and many more bands of lower abundance (Fig. 2d). The principal constituents of the four abundant bands, identified by peptide mass fingerprinting, were GluN2A/GluN2B, TAP-GluN1, PSD93 and PSD95, respectively. These data support a model in which there are many different 1.5-NR complexes each with different constituents and also suggest a central role for PSD95 and PSD93. We confirmed the minimal requirements for the assembly of 0.8-NR and 1.5-NR by heterologous expression of subunits in HEK cells. Transient transfection of HEK293 cells with complementary DNAs encoding GluN1 and GluN2B in HEK293 produced 0.8-NR (Supplementary Fig. 2e), whereas transient transfection with complementary DNAs encoding GluN1, GluN2B, PSD95 and PSD93, produced 0.8-NR and 1.5-NR (Supplementary Fig. 2f). Other constituents of forebrain 1.5-NR are likely to increase the range of masses as shown by BNP immunoblots of proteins co-purified with TAP-GluN1 (Supplementary Fig. 2d). To test the specificity and mechanism of assembly we next used mouse genetics to mutate constituents of 1.5-NR in vivo.

A tripartite mechanism for the assembly of ~1.5 MDa NMDARs. On the assumption that in vitro GluN2 binds interchangeably with either PSD95 or PSD93 via the same PDZ-ligand12,13 we reasoned that a knockout of either MAGUK would not prevent NMDAR recruitment into ~1.5 MDa supercomplexes. However, Fig. 3a shows that in Psd95Δ−/− knockout mice 1.5-NR was essentially absent from the whole forebrain. Perhaps more intriguing was the discovery that in Psd93Δ−/− knockout mice 1.5-NR also failed to assemble (Fig. 3a). Thus, the interaction of NMDAR with either PSD95 or PSD93 was not sufficient to recruit NMDARs into ~1.5 MDa complexes; instead both PSD95 and PSD93 were essential.

These genetic interdependencies for 1.5-NR assembly were highly selective because in Psd95Δ−/− mice 1.5-PSD93 remain and in Psd93Δ−/− most 1.5-PSD95 were still present (Supplementary Fig. 3). This result also indicates that a large population of PSD95 and PSD93 must exist in complexes that are independent of NMDARs.
Figure 2 | Generation of TAP-tagged GluN1 (Glun1TAP/TAP) knock-in mice for purification of native NMDARs. (a) Genetic engineering of TAP-tags into GluN1. Left, schematic shows tetrameric NMDAR with two GluN1 subunits (grey) engineered with tandem affinity peptide (TAP-tags, magenta) on their extracellular N-termini. GluN2 subunits (GluN2A, cyan; GluN2B, orange) shown with C-terminal cytoplasmic ESDV motifs/PDZ-ligands. TAP-tag encodes 3xFlag and His16. Right, shows gene-targeting vector carrying TAP-tag sequence in Glun1 exon1, 5′ and 3′ regions of homology with genome and resultant targeted allele below. The neomycin selection cassette was subsequently deleted using Cre/loxP. Grey-filled boxes, exons; magenta, TAP cassette; brown box, neo neomycin resistance cassette; brown triangle, loxP site; dotted lines, homology arms. (b) Purification of native NMDARs from Glun1TAP/TAP mouse forebrains. Left, schematic of purification steps and samples corresponding to right, immunoblots of NMDAR subunits, PSD95, actin. Dissected mouse forebrains were homogenized and fractionated. Crude membrane fraction was solubilized (membrane extract) and separated by centrifugation to produce supernatant and pellet. Supernatant was incubated with Flag-affinity resin capturing TAP-tagged receptors with some residual Flow-through. High yields of native receptor were released (eluate) by peptide-antigen exchange. Following elution no material remained (exchanged resin). Right, purification from Glun1TAP/TAP (TAP) and WT control mouse shows receptor subunits (GluN1, GluN2A, GluN2B), PSD95, and actin detected by SDS–PAGE immunoblots from indicated fractions. Note the higher molecular weight of TAP-engineered GluN1 compared with WT. IB, immunoblotting antibody; MW, molecular weight markers. SDS, SDS–PAGE. (c) Coomassie-stained BNP of TAP-purified NMDAR and PSD95 complexes from Glun1TAP/TAP and Psd95TAP/TAP mice, respectively. TAP-purified complexes isolated from forebrain extracts from control (WT), Glun1TAP/TAP and Psd95TAP/TAP mice were separated on BNP gel and Coomassie stained. Supplementary Fig. 2a shows excised bands used in native proteomic analysis (TAP-BNP-MS). Filled arrow indicates ~1.5 MDa complexes and open arrow indicates 0.8-NR. Molecular weight in MDa shown on left and in stained ladder in first and last gel lanes. (d) Coomassie-stained SDS–PAGE of TAP-purified NMDARs from Glun1TAP/TAP and control (WT) mice. Abundant constituents of bands 1–4 were identified by MALDI-MS: GluN2A/B, TAP-GluN1, PSD95 and PSD95, respectively. MW shown in kDa on left.

One potential explanation for the dual genetic dependency of PSD95 and PSD93 for 1.5-NR assembly is that the PDZ-ligand interactions with GluN2A/B are not sufficient in vivo. To test directly the necessity of PDZ-ligands in vivo we engineered double mutant mice that lacked both GluN2A and GluN2B PDZ-ligands in their C-termini. First, we targeted a point mutation that deletes the C-terminal valine from the gene encoding GluN2B, producing Glun2bdV mice (Supplementary Fig. 4a). Glun2bdV mice were viable and fertile. Next, to render forebrain NMDARs completely devoid of C-terminal PDZ-ligands on both GluN2A and GluN2B, we crossed Glun2bdV mice with another available line in which the entire carboxy-terminal domain (CTD) of GluN2A was deleted (Glun2adC mice)26. The resulting NR-APDZlig double knock-in mice were crossed with Glun1TAP to produce Glun2bdV/GluN2adC/Glun1TAP mice with triple homozygous knock-in mutants that enable purification of receptors from NR-APDZlig mice (Fig. 3b, left). Remarkably, in NR-APDZlig mice the assembly of NMDARs into 1.5-NR was preserved (Fig. 3b, right). We confirmed the association between NMDARs, PSD95 and PSD93 in the absence of PDZ-ligands by detecting PSD95 and PSD93 in 1.5-NR purified from NR-APDZlig mice (Fig. 3c). Although the PDZ-ligand was not essential for the assembly of 1.5-NR, we observed a notable reduction in the total...
expression levels of all NMDAR subunits (GluN1, GluN2A and GluN2B) in NR-ΔPDZlig mice (Supplementary Fig. 4b), suggesting the PDZ-ligand influences receptor biogenesis or trafficking.27

Having established that the PDZ-ligand interaction was dispensable, we reasoned that the mechanism of NMDAR assembly with MAGUKs could be mediated by sequences other than the conserved ESDV motif within the GluN2 CTDs (ref. 28). The CTD is the most divergent domain between GluN2A and GluN2B paralogs21, raising the possibility that only one of these two paralogs was capable of directing assembly of NMDARs into 1.5-NR. To test the role of GluN2A and GluN2B CTDs we examined two knock-in mutant mice lines. In the first mutant, Glun2b2A/(CTR)/2A(CTR), mice express only GluN2A CTDs (the exon encoding the GluN2B CTD has been replaced with a copy of the exon that encodes the GluN2A CTD)20.

In the forebrains of homozygous Glun2b2A(CTR)/2A(CTR) mice the NMDAR was almost completely absent from the ~1.5 MDa supercomplexes, leaving only 0.8-NR (Fig. 3d). Thus, GluN2A CTDs were insufficient to replace GluN2B CTDs for assembling NMDARs with PSD95 in 1.5-NRs. In accordance, in Glun2a2B(CTR)/2B(CTR) knock-in mice that only express the GluN2B CTD (the exon encoding the GluN2B CTD is inserted into the GluN2A gene) we found NMDARs assembled into both ~1.5 and ~0.8 MDa complexes (Fig. 3d and Supplementary Fig. 3). Thus, the CTD of GluN2B specifically assembles NMDAR-Dlg/MAGUK supercomplexes independent of its PDZ-ligand.

These genetic findings make the prediction that the higher-order assembly of receptor tetramers into 1.5-NRs is not promiscuous because it is essential for any given tetramer to contain a minimum of one GluN2B subunit; those receptors...
containing only GluN2A subunits will be excluded from the population of 1.5-NRs (Fig. 3d). We tested this biochemically by depleting all GluN2B-containing receptors from extracts and, as predicted, BNP immunoblots showed no detectable 1.5-NR (Fig. 3e, immunodepletion controls in Supplementary Fig. 4c). Indeed, most receptors including 0.8-NR were also removed, indicating the majority of native NMDARs contained at least one GluN2B in the adult forebrain. In contrast, subunit-depleting GluN2A-containing complexes, removed only a small fraction of 1.5-NR (Fig. 3e), which corresponded to heterotetramers containing a single GluN2A and GluN2B subunit. Similarly, when both PSD95 and PSD93 were immuno-depleted, all detectable 1.5-NR was removed from the sample (Fig. 3e), while the amount of 0.8-NR remained unaffected. To deplete just PSD95 and rule out the possibility that PSD95 antibodies were non-specific, we used a knock-in mouse carrying an in-frame, C-terminal GFP-tag targeted to the gene encoding PSD95 (Psd95EGFP/EGFP, reported elsewhere). Immuno-depleting GFP from the brain extracts of Psd95EGFP/EGFP-GluN1TAP/TAP double knock-in mice also removed all 1.5-NR (Fig. 3e). Therefore, every 1.5-NR must contain at least one molecule of PSD95. Together these genetic and biochemical results indicate a mechanism of 1.5-NR assembly that was applicable across the mouse forebrain and required three components: PSD95, PSD93 and the CTD of GluN2B (Fig. 3f).

GluN2B is fourfold more abundant than GluN2A in forebrain. The immuno-depletion experiments indicated that almost all NMDARs contain a GluN2B subunit and that very few contain GluN2A (Fig. 3e), yet prevailing models suggest GluN2A is the most abundant GluN2 subunit in the adult forebrain,29,30 or that the major population of NMDARs are triheteromeric 11,31,32. To investigate this discrepancy further, a genetic tagging assay was devised to measure the molar ratio of GluN2A/GluN2B expression in vivo using the Glun2b2A(CTR)/2A(CTR) knock-in mice. First, we showed by immunoblotting the N-terminal domain that replacing the CTD of GluN2B with that of GluN2A in Glun2b2A(CTR)/2A(CTR) mice caused no change in the total expression levels of GluN2A, GluN2B or GluN1 compared with WT (Fig. 4a, bottom three panels). Next, we measured in total forebrain extracts the relative amount of the GluN2A CTD and found it was fivefold greater in Glun2b2A(CTR)/2A(CTR) compared with WT (Fig. 4a, top panel). This indicates that the molar ratio of GluN2A to GluN2B was 1:4 ± 1 (mean ± s.d.) in the adult forebrain (Fig. 4b,c and Methods section ‘Quantification of subunit molar ratios in vivo’).

As a further control, we performed the reciprocal measurement of the GluN2B CTD in Glun2b2B(CTR)/2B(CTR) mice.20 In accordance with the observation that GluN2A subunits were expressed at much lower abundance than GluN2B in the adult forebrain, the amount of GluN2B detected with antibodies that recognize the GluN2B CTD was only slightly greater in Glun2b2B(CTR)/2B(CTR) compared with WT (Fig. 4a, second from top panel, Fig. 4b–c).

Different brain regions might have different molar ratios of GluN2A to GluN2B that could skew the overall average; therefore, we measured the molar ratio of GluN2A to GluN2B within eight distinct brain regions of the adult mouse. As expected, GluN2A was more abundant than GluN2B within the cerebellum (Fig. 4d,e). In the cortex and hippocampus, GluN2B was six- and threefold more abundant than GluN2A, respectively. In the hindbrain, GluN2A and GluN2B were present at near equimolar amounts. In all other brain regions a three- to sixfold excess of GluN2B over GluN2A was detected (Fig. 4e). Quantification of the excess of GluN2B over GluN2A is therefore widespread and consistent with the importance of GluN2B for the assembly of 1.5 MDa supercomplexes in the forebrain.

Supercomplex assembly in synapse maturation. Having established the composition and genetic dependencies, we next sought to understand the functional context of NMDAR and PSD95 supercomplexes. An extensive literature has highlighted the importance of NMDAR and PSD95 in the postnatal development and synapse maturation of the mammalian brain.33 We hypothesized that the two NMDAR complexes found in the adult brain may be subject to differential regulation during development.

We compared the native assembly of NMDAR complexes at multiple postnatal ages, from birth (P1) to adulthood (P69) in the mouse forebrain, a period marked by profound synaptogenesis and synapse maturation. At all ages we observed 0.8-NR complexes (Fig. 5a), whereas significant amounts of 1.5-NR were only seen from P16 onwards (Fig. 5b). In keeping with the triheteromeric mechanism of assembly, 1.5-PSD95 and 1.5-PSD93 were absent in the first postnatal week and increase significantly from the second postnatal week onwards (Fig. 5a). Thus, 0.8-NR is constitutively expressed at all ages, whereas 1.5-NR assembly is developmentally regulated and characteristic of the mature forebrain.

We therefore wondered if the late postnatal re-organization of synaptic proteins into supercomplexes was a property of synapse maturation34–38. Epigenetic mechanisms involving histone deacetylases have been reported to regulate synapses maturation39, stabilize memory and enhance memory retrieval in mouse models of Alzheimer’s disease40,41. To test the role of supermolecular assembly in synapse maturation we used an inhibitor of HDAC1/2, trichostatin A (TSA), in primary neuronal cultures39,42. We found DIV7 primary neuronal cells, similar to the early postnatal forebrain, contain only trace amounts of 1.5-NR. Following 18–24 h TSA treatment, we observed a significant recruitment of PSD95 from its monomeric form into 1.5-PSD95 supercomplexes over control cells (Fig. 6a,b). This TSA-stimulated synapse maturation also caused a significant decrease of 0.8-NR and increase of 1.5-NR (Fig. 6c,d). These findings suggest epigenetic mechanisms can regulate the assembly of 1.5-NR and 1.5-PSD95 during synapse maturation.

The punctate distribution of PSD95 that is characteristic of mature synapses can be reversed using inhibitors of palmitoylation43. To test a structural role of palmitoylation in 1.5-PSD95 assembly we chemically de-palmitoylated proteins in forebrain extracts by nucleophilic thiolysis (with 40 mM MESNA -mercaptoethanol)44, which had no effect on 1.5-PSD95 assembly 1.5-PSD95 (Supplementary Fig. 5a). Thus, palmitoylation per se is not necessary for maintaining PSD95 within 1.5 MDa supercomplexes. Since palmitoylation also regulates trafficking45, we tested if instead palmitoylation had an indirect influence on the assembly of 1.5-PSD95. Treating DIV14 primary neurons for 8 h with an inhibitor of palmitoylation, 2-bromopalmitate (2BP) caused a dramatic disassembly of 1.5-PSD95 (Fig. 6g,f), without affecting total expression of PSD95 (Fig. 6g). The effect of 2BP on 1.5-NR could not be determined because primary neurons cultured from 14 to 28 days contain only trace amounts of 1.5-NR compared with neurons within the intact forebrain (Fig. 6c). The 2BP-dependent decrease in 1.5-PSD95 was rescued by blocking activity with 1 µM TTX or inhibiting NMDARs with 50 µM AP5 (Fig. 6i,h), suggesting activity-dependent palmitoylation cycling46 regulates the dynamics of 1.5-PSD95 assembly. The 2BP-dependent decrease in 1.5-PSD95 coincided with a reduction in the punctate distribution of PSD95 (Supplementary Fig. 5b) as
**Figure 4** | The GluN2A/GluN2B relative molar abundance using mouse genetics and quantitative tagging. (a) SDS–PAGE immunoblot of WT, Glun2b2A(CTR)/2A(CTR) and Glun2a2B(CTR)/2B(CTR) forebrain extract. Top panel, detected the GluN2A C-terminal domain (CTD). Second panel down, GluN2B CTD. Glun2a2B(CTR)/2B(CTR) and Glun2b2A(CTR)/2A(CTR) provided negative controls for the specificities of the GluN2A CTD and GluN2B CTD antibodies, respectively. Third, fourth and fifth panels down, detected GluN2A NTD, GluN2B NTD subunits and GluN1, respectively. Blue and orange labels indicate chimeric Glun2a and Glun2b knock-in mutations, respectively. (b) Dilution series of Glun2a2B(CTR)/2B(CTR) forebrain extract into that of Glun2b2A(CTR)/2A(CTR) indicated sensitivity of quantification. Immunoblots detected GluN2A CTD (upper panel) and GluN2B CTD (lower panel). (c) Quantification of GluN2A CTD and GluN2B CTD immunoblots. Measurements from Glun2b2A(CTR)/2A(CTR) (P<0.001) and Glun2a2B(CTR)/2B(CTR) (t-test, P<0.02) were normalized to WT. (d) SDS–PAGE immunoblot of WT, Glun2b2A(CTR)/2A(CTR) extracts from left to right: cortex, hippocampus and cerebellum. Top panels, detected the GluN2A C-terminal domain (CTD). Lower panels, detected GluN2A NTD. (e) Quantification of GluN2A relative to GluN2B in eight different brain regions from top to bottom: olfactory bulb, caudate putamen, hippocampus, cortex, colliculus, cerebellum, thalamus and hindbrain. GluN2A was measured using GluN2A CTD antibodies against WT extracts (n = 3). GluN2B was measured by measuring GluN2A CTD antibodies against Glun2b2A(CTR)/2A(CTR) extracts (n = 3) and subtracting the intensity from that of WT. Error bars indicate 1 s.d.

**Figure 5** | 1.5-NR and 1.5-PSD95 assemble late in postnatal development. (a) BNP immunoblots show WT mouse forebrain extracts at developmental time points probed with antibodies against GluN1, GluN2A, GluN2B, PSD95 and PSD93. Filled arrow, ~1.5 MDa complexes that only assemble from P16 onwards. Sample load was normalized by forebrain mass. Molecular weight in MDa shown on right. (b) Quantification of changing abundance of 0.8-NR and 1.5-NR from P12 (n = 3) to P16 (n = 3) using BNP immunoblots of GluN1. 0.8-NR increased by 25%, whereas 1.5-NR increased by 445%; t-test, P > 0.01; Error bars indicate s.e.m.
A higher-order molecular architecture of the synapse. Finally, we asked if the organizational principles identified for NMDARs were consistent with the higher-order assembly of 60 diverse proteins within the synaptic proteome. Total mouse forebrain synaptoneurosomes were solubilized in a screen of five buffers (with different detergents of varying stringency), then rapidly resolved by blue non-denaturing-PAGE (BNP), and proteins detected by immunoblotting (see Methods section for details). As shown in Fig. 7, we examined proteins from six major functional modalities of the synapse: neurotransmitter receptors, trans-synaptic/adhesion, ion channels, signalling enzymes, scaffolds/adaptors and immediate-early/local translation.

Strikingly, every protein was tethered at least one complex, typically 5–20x its monomeric size, consistent with expectations that almost all individual proteins function within higher-order assemblies. After correcting for the potential over-estimation of mass (≤100 kDa) as a consequence of the contribution of the detergent/lipid micelle, we found only 13 bands corresponded to the size of native proteins unassembled in monomeric form. This left 220 reproducible and separable protein bands that likely represent the major populations of the candidate proteins. Each band was assigned as a complex and compared with previously published results (Supplementary Data 1). Seventeen bands corresponded to the size of known oligomeric complexes including multiple native AMPA receptor and auxiliary subunits previously reported. Together these results show the late postnatal assembly of supercomplexes is determined by the tripartite mechanism and can be modulated by epigenetic, palmitoylation and activity-dependent processes.

Figure 6 | 1.5-NR and 1.5-PSD95 assembly is correlated with synapse maturation. (a) HDAC1/2 inhibition with TSA causes increased assembly if 1.5-PSD95. Cultured primary cortical neurons (DIV14) were treated with DMSO (control; lanes 1 and 2) or 0.25 μM TSA (lanes 3 and 4) for 18–24 h before cells were harvested and analysed by BNP PSD95 immunoblot. (b) Quantification shows TSA treatment increased 1.5-PSD95 to 166% (n = 6) of control. t-test, P < 0.001; Error bars indicate s.e.m. (c) HDAC1/2 inhibition causes decreased and increased assembly of 0.8-NR and 1.5-NR, respectively. Cultured primary cortical neurons (DIV14) were treated with DMSO (control; lanes 1 and 2) or 0.25 μM TSA (lanes 3 and 4) for 18–24 h before cells were extracted and analysed by BNP GluN2B immunoblot. (d) Left, quantification shows TSA treatment decreased 0.8-NR to 28% (n = 6) of control. P < 0.001; Error bars indicate s.e.m. Right, TSA treatment increased 1.5-NR to 251% (n = 6) of controls. (e) Inhibition of palmitoylation with 2-bromopalmidate (2-BP) decreased 1.5-PSD95. Cultured primary cortical neurons (DIV14) were treated with DMSO (control; lanes 1 and 2) 10 μM 2-BP (lanes 3 and 4) for 8 h before cells were harvested and analysed by BNP PSD95 immunoblot. (f) Quantification shows 2-BP treatment decreased 1.5-PSD95 to 14% (n = 6) of control. (g) Inhibition of palmitoylation with 2-BP does not change the expression levels of PSD95 or GluN2B. (h) 2-BP-mediated disassembly of 1.5-PSD95 is activity dependent. Cultured primary cortical neurons (DIV14) were treated with DMSO (control; lanes 1, 2 and 3) 10 μM 2-BP (lanes 4, 5 and 6), 10 μM 2-BP with 1 μM TTX (lanes 7, 8 and 9), 10 μM 2-BP with 50 μM APV (lanes 10, 11 and 12) for 8 h before cells were harvested and analysed by BNP PSD95 immunoblot. Filled arrow indicates 1.5-PSD95. Molecular weight in MDa shown on right. (i) Quantification shows 2-BP treatment decreased 1.5-PSD95 to 14% of control (n = 6, P < 0.001). TTX rescued 2-BP-dependent disassembly (n = 6, P < 0.001) to 74% of control. AP5 rescued 2-BP-dependent disassembly (n = 6, P < 0.001) to 69% of control; error bars indicated s.e.m.
indicated in mega-Daltons (MDa).

open arrowheads, respectively. The expected size of each protein in monomeric form indicated with pink rectangle. Non-denaturing molecular mass (independent of its PDZ-ligand). Although our findings do not

PSD95 and/or PSD93, whereas GluN2B alone was sufficient

cytoplasmic domain of GluN2A could not assemble with

assembly of NMDAR with PSD95). Third, we found that the

in vivo

the GluN2A and GluN2B ESDV ligands were not required

prevailing model that 1.5-NR assembly is mediated by C-terminal

molecular forms using a battery of mutant mice revealed 1.5-NR

mechanisms controlling the higher-order assembly of proteins

within the mammalian synapse proteome and novel super-
molecular forms of the NMDAR. Endogenous NMDARs were

partitioned within two discrete populations of 0.8 and 1.5 MDa,

which were biochemically, genetically and developmentally

separable. The former was expressed at all postnatal ages and

the latter first appeared late in postnatal development and driven

by synapse maturation. Genetic dissection of the two super-
molecular forms using a battery of mutant mice revealed 1.5-NR

assembly was critically dependent on a tripartite requirement of

the receptor before assembly with Dlg/MAGUKs into synaptic

supercomplexes. Moreover, both the developmental and genetic

investigations of 0.8-NR and 1.5-NR show that the PDZ-ligand

interaction with Dlg/MAGUKs is not required for synaptic

localization of NMDARs: 1.5-NRs were absent during the first 12

postnatal days (when NMDA function is essential) and the three

mutants lacking 1.5-NRs in adulthood have all been reported to

have normal synaptic NMDAR currents in hippocampus CA1

region40,47–49.

Functional insight was apparent from the regulated assembly of

1.5-NR, and 1.5-PSD95 late in postnatal development from

~P12 onwards. This time point coincides with eye opening and

a period of marked synaptogenesis within the forebrain33,34,35.

Consistent with this developmental regulation we also identified a

role for 1.5-NR and 1.5-PSD95 in synapse maturation. Blockade

of histone deacetylases in immature primary neurons that has

been reported to accelerate synapse maturation39, increased the

assembly of 1.5-NR and 1.5-PSD95. These data indicate that

epigeneric mechanisms influence the assembly of synaptic

supercomplexes.

A defining property of maturation is the punctate enrichment of

PSD95 within the postsynaptic membrane, which is regulated

by palmitoylation cycles43. Blockade of palmitoylation signalled

an activity-dependent disassembly of 1.5-PSD95, which as

previously reported43, caused a dramatic decrease in PSD95

puncta. Thus the present data suggest 1.5-PSD95 supercomplexes

are the molecular species responsible for the punctate appearance

of PSD95 that indicates a functionally mature synapse52.

Fig. 5c) revealing several different temporal profiles of super-
molecular assembly, and thus suggesting the regulated assembly

of synaptic supercomplexes is a widespread property of postnatal
development.

Discussion

This integrative biochemical and genetic analysis identifies

mechanisms controlling the higher-order assembly of proteins

within the mammalian synapse proteome and novel super-
molecular forms of the NMDAR. Endogenous NMDARs were

partitioned within two discrete populations of 0.8 and 1.5 MDa,

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Figure 7 | Screen of native protein assemblies of 60 brain proteins detected by BNP (blue non-denaturing PAGE) immunoblot of mouse forebrain extracts. Complexes were extracted with a panel of five different buffers. For most candidate proteins, different buffers extracted complexes of a similar size (see Methods section for details and Supplementary Data 1 for list of all complexes). Novel and previously described complexes indicated by filled and open arrowheads, respectively. The expected size of each protein in monomeric form indicated with pink rectangle. Non-denaturing molecular mass indicated in mega-Daltons (MDa).
The quantification of GluN2A and GluN2B within populations of postsynaptic complexes and supercomplexes impact on models that suggest differential functions for these subunits. We have shown that in vivo the adult forebrain contains fourfold molar excess of GluN2B over GluN2A. Most GluN2A was associated with GluN2B (in so called triheteromeric receptors) and GluN2A is dependent on GluN2B for assembly into ~1.5 MDa NMDAR-MAGUK supercomplexes. Moreover, we expect further subpopulations of these complexes since additional receptors, ion channels, signalling and trans-synaptic adhesion proteins were detected as constituents of 1.5-NRs and in discrete 1.5 MDa complexes by BNP immunoblot (Fig. 7). The brain region-specific differences in the molar ratio of GluN2A to GluN2B raise the possibility that supercomplexes with different compositions are allocated into anatomically distinct synapses.

This study provides new insight into the higher-order molecular organization of the synapse proteome. Almost all surveyed proteins were found in novel complexes and supercomplexes. It was also striking that many related proteins (within the same paralog family) did not all co-migrate suggesting they were part of larger, more complex assemblies. The four members of the MAGUK/Dlg family have each been reported to interact promiscuously with numerous synaptic proteins (including NMDAR subunits). However, in our screen we found MAGUKs were differentially organized with PSD95 and PSD93 in genetically separable 1.5 MDa supercomplexes, whereas the largest population of Sapo7/Dlg1 and Sapo102/Dlg3 were retained within smaller (0.2–0.4 MDa) complexes. Similar patterns emerge in comparing proteins within 10 out of 12 paralog families surveyed in our screen (Fig. 7 and Supplementary Data 1). Interestingly, paralog-mediated restriction together with the modular arrangement of proteins apparent within our BNP screen (Fig. 7) are two mechanisms that diminish the combinatorial molecular complexity of the synapse and thereby overcome a theoretical ‘complexity brake’ on systems with high numbers of components such as the brain.

Although many disease-associated mutations have been reported in proteins co-purifying in the 1.5 MDa TAP-NR and TAP-PSD95 supercomplexes (including GluN2A, GluN2B and PSD93/Dlg2), this study provides the first direct evidence that mutations disrupt their supermolecular organization at the synapse. We found the 1.5-NRs were exquisitely sensitive to mutations in PSD95, PSD93 and GluN2B. There is abundant evidence of physiological and behavioural changes in mice carrying mutations in these genes. Recurrent de novo mutations in PSD93 and GluN2 genes are now widely reported in schizophrenia as are mutations in the sets of proteins isolated in 1.5-NR and 1.5-PSD95 complexes. Thus, future studies that dissect the postsynaptic complexes could shed light on the synaptic mechanisms of brain disorders.

Methods

BNP screen of synaptic complexes. Forebrains (hippocampus and cortex) were dissected from adult (P56–70) mice and homogenized (12 strokes with a Teflon-glass pestle and mortar) in 5 ml ice-cold buffer H (1 mM Na HEPES pH7.4, 320 mM sucrose with protease inhibitors). The homogenate pellet was collected by centrifugation at 1,000g (MLA-80, 5,000 r.p.m.) at 2°C for 10 min and re-homogenized (6 strokes) in 2 ml buffer H and centrifuged as before. The first and second 1,000g supernatants were pooled and centrifuged at 18,500g (MLA-80, 19,000 r.p.m.) to pellet the crude membranes. The 18,500g supernatant was discarded. Extraction conditions were screened using the crude membrane from 50 to 60 mg mouse forebrain re-suspended in 0.5 ml buffer H, to which different detergents in 0.5 ml buffer X (100 mM NaCl, 50 mM tris.Cl pH8) were mixed for 1 h at 4°C. Final detergent concentrations were 1% w/v, 1% v/v for 1 h at 4°C. Conditions selected for use (8% w/v, 0.8% v/v) were 1% w/v sodium deoxycholate (Figs 1–3 and 5–7), 3% (3-cholamidopropyl)-dimethylammonio-1- propanesulfonate (Fig. 6), n-Dodecyl-N,N-Dimethyl-3- Ammonio-1-Propanesulfonate (Fig. 7), 7% 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (Fig. 8). The first detergent was used for immuno-blot to detect the native complexes of NMDAR subunits (Fig. 1b) and 60 additional brain proteins (Fig. 7). In general different detergent buffers gave similarly sized complexes and the less stringent detergent buffers were less efficient at solubilizing native complexes. Antibodies and all the native complexes detected with each detergent buffer are detailed in Supplementary Data 1.

BNP immunoblot and immuno-depletions. BNP was run according to Schagger. For immuno-depletions, extract supernatant were incubated with antibodies to deplete subunits at 6–10°C for 2–16 h. To capture antibody-antigen complexes, 3 mg protein G magnetic beads were added for 30 min at 6–10°C. Additional immunoblots ensured the completeness of immuno-depletions (Supplementary Fig. 4c).

Generation of GluNTAP/TAP knock-in mice. Sequence encoding a TAP-tag was designed for in-frame insertion within exon 1 of the GluN1 gene, downstream of the brain-specific signal peptide (Fig. 2a). The GluN1 TAP knock-in mice (homozygous at both alleles) contained all of the amino-terminal domain (Pro23). The tag contained a linker (PSGSTG)-, a 3xFlag epitope tag (DYKDDDDK), linker containing a TEV protease consensus site (-GTENVLYFQGT-) (1) and a linker to the beginning of the amino-terminal domain (GAS). The 161-bp sequence encoding the TAP tag with flanking restriction sites (Apel and Nhel) was synthesized by ligase chain reaction. Upstream of the tag, 2.249 bp 5' homology arm was retrieved by recombinating with 500 bp mini-homology arms and mouse genomic BAC clone (bm2-32D16) in e. coli EL350. The mini-homology arms flanked by restriction sites (NotI and Scal) were synthesized by PCR from mouse genomic BAC clone. Downstream of the TAP tag, the 3' flank comprising the 3' end of exon 1 (280 bp) and the first 500 bp of intron 1 were retrieved with flanking restriction sites (NheI and Sfil) by PCR from mouse genomic BAC clone. Additional restriction sites (Ndel and BgRI) suitable for genomic southern genotyping were also included upstream of the Sfil restriction site in this product. The 3' mini homology arm, TAP-tagged with homology arms used to hybridize to the mouse genomic BAC clone by recombining in e. coli EL350. The targeting vector also contained a Diphtheria Toxin A cassette for negative selection of non-homologous integration.

The linearized targeting vector was electroporated into E14tg2a ES cells. ES clones mutated by homologous recombination were identified from genomic DNA samples by PCR. Six out of 216 ES clones were putative recombinants. Recombination was confirmed for 2 of these 6 clones by genomic southern hybridization to regions outside the 5' and 3' homology arms of Ndeltidigested genomic DNA. Positive ES clones were microinjected into C57Bl/6 Neomycin resistant (2) embryos, which generated chimeras containing 30–70% wild-type cells. Chimeras back-crossed onto 129S5 gave germ line transmission, which was genotyped by PCR and sequenced from ear biopsy samples.

The neomycin selection cassette was inserted into the first intron of GluN1 (Fig. 2a, right) was removed by backcrossing on a transgenic Cre recombinase-2,24 (Cre) mouse strain (homozygous containing GluNTAP/TAP with other mutations were also bred. Except for developmental experiments, all mice were adult, aged, gender- and background-matched.

Purification of native NMDARs from TAP-tagged knock-in mice. The crude membranes from 5 P56-P70 GluNTAP/TAP mouse forebrains were re-suspended in 12.5 ml buffer H and extracted with 12.5 ml 2% deoxycholate, 100 mM NaCl, 50 mM Tris.Cl pH8 for 1 h at 6°C. Total extract was centrifuged at 120,000g for 40 min at 8°C. Conditions for immuno-capture, wash and peptide-antigen exchange elution were screened using a high-throughput purification robot (MAGic sample processor, Invitrogen). For 25 ml GluNTAP/TAP extract supernatant, 80 µg mouse Flag antibody was coupled to 30 mg (300 µl) protein G magnetic beads (Invitrogen). Receptor was captured from extract supernatant for 40 min at 8°C. For 25 ml wash buffer (0.37% sodium deoxycholate, 0.05 mg.ml–1 lipids (1:1:3 POPC:POPE:POPG), 150 mM NaCl, 50 mM Tris.Cl pH8). Flag captured complexes were eluted with 600 µl wash buffer.
supplemented with 0.2 mg/ml -1 Flag peptide for 2 h at 6°C. Euate was buffer exchanged to remove the Flag elution peptide and concentrated with a 100-kDa MWCO centrifugal filter (Amicon) to 35 μl 0.1-0.25 mg/ml -1 native NMDAR.

**Native proteomics (TAP-BNP-LC-MS/MS).** Excised BN gel slices containing the purified proteins were prepared for mass spectrometric analysis by manual in situ enzyme digestion. Briefly, the excised gel protein pieces were placed in a well of a 96-well microtitre plate and de-stained with 5% v/v acetonitrile and 50 mM ammonium bicarbonate, reduced with 10 μM DTT and alkylated with 55 mM iodoacetamide. After alkylation, proteins were digested with 6 pg Trypsin (Promega, UK) overnight at 37°C. The resulting peptides were extracted in 2% v/v formic acid, 2% v/v acetonitrile. The digest was analysed by nano-scale capillary LC-MS/MS using an Ultimate U3000 HPLC (ThermoScientific Dionex, San Jose, USA) to deliver a flow of ~300 nl min -1. A C18 Acclaim PepMap100 5 μm, 100 μm × 20 mm nanoViper (ThermoScientific Dionex, San Jose, USA), trapped the peptides. Prior to separation on a C18 Acclaim PepMap100 75 μm × 150 mm nanoViper (ThermoScientific Dionex, San Jose, USA). Peptides were eluted with a gradient of acetonitrile. The analytical column outlet was directly interfaced via a modified nano-flow electrospray ion source, with a hybrid dual pressure linear ion trap mass spectrometer (Orbitrap Velos, ThermoScientific, San Jose, USA). Data-dependent analysis was carried out, using a resolution of 30,000 for the full MS spectrum, followed by 10 MS/MS spectra in the linear ion trap. MS spectra were collected over an m/z range of 300–2000. MS/MS scans were collected using a threshold energy of 35 for collision induced dissociation. LC-MS/MS data were then searched against a protein database (UniProt) containing all 65,000 sequences and a local protein search engine (MatrixScience, UK)41. Database search parameters were set with a precursor tolerance of 5 p.p.m. and a fragment ion mass tolerance of 0.8 Da. Two missed enzyme cleavages were allowed and variable modifications for oxidized methionine, carbamidomethyl cysteine, pyroglutamic acid, phosphorylated serine, threonine, tyrosine, tert-butylcarboxyl-lysine, N-formylmethionine, and prop-2-ynyl-lysine were included. MS/MS data were validated using the Scaffold programme (Proteome Software Inc., USA). All data were additionally interrogated manually. Peptide identifications were accepted if they could be established at >80.0% probability as specified by the Peptide Prophet algorithm2. Protein probabilities were assigned by the Protein Prophet algorithm42. Using these stringent identification parameters, the rate of false-positive identifications is <1%. All non-mouse proteins that were detected but could not be matched to a mouse protein (in Swissprot-TrEMBL) were not included in the analysis below (for example, human keratins).

The composition of ~1.5 MDA complexes from TAP-GluN1, PSD95-TAP and WT control were compared (Supplementary Table 1) using a stringent threshold of detection (≥ 2 unique peptides and ≥ 1 unique peptide in ≥ 2 replicate samples). This level of stringency was sufficient to detect 55 proteins in TAP-GluN1, 89 proteins in PSD95-TAP and only one protein in the WT control (IgG). Since it expected that the 'tails' of the bands may partially overlap, the differential composition of proteins in PSD95-TAP and only one protein in the WT control (IgG). Since it expected that the 'tails' of the bands may partially overlap, the differential composition of proteins in the ~0.8 and ~1.5 MDA TAP-GluN1 bands were validated by BN immunoblot of TAP-purified complexes (Supplementary Fig. 2d).

All MS data (raw data, mascot files and scaffold file) were processed with the PRIDE converter43 and deposited in the PRIDE database (http://www.ebi.ac.uk/pride/muscle/) with ProteomeXchange accession number: PXD000011.

**MALDI-MS.** To identify the principle NMDAR constituents (Fig. 2d), 35 μl concentrated TAP-purified samples were denatured in SDS loading buffer and separated on a 4–12% bis-tris polyacrylamide gel. The gel was fixed and stained with colloidal Brilliant Blue-G Coomassie (Sigma). The principle bands were identified by MALDI-MS mass fingerprinting. Gel bands were excised and incubated with colloidal Brilliant Blue-G Coomassie (Sigma). The principle bands were digested with 6 ng trypsin (Promega) in 50% acetonitrile/0.1% trifluoroacetic acid. Peptide mass were determined by the Protein Prophet algorithm62. Using these stringent identification parameters, the rate of false-positive identifications is 80.0% probability as specified by the Peptide Prophet algorithm2. Protein probabilities were assigned by the Protein Prophet algorithm42. Using these stringent identification parameters, the rate of false-positive identifications is <1%. All non-mouse proteins that were detected but could not be matched to a mouse protein (in Swissprot-TrEMBL) were not included in the analysis below (for example, human keratins).

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**Generation of Glun2b2BdV knock-in mice.** The Glun2b2BdV knock-in mice was engineered by standard mouse genetic protocols in which the Glun2b2BdV terminal valine (1,482) was deleted, leaving the last codon for Asp1471 before the stop codon (Supplementary Fig. 4a). In brief, 5.9 kb Bst EII and 1.8 kb Bst XBI homology arms were used to construct the gene targeting vector for Glun2b2BdV terminal valine (1,482) deletion (Fig. 4). Digestion restriction enzyme Bst EII was added. Digestion was saturated at 32°C. Peptides were recovered and desalted using uC18 ZipTip (Millipore) and eluted to a MALDI target plate using 2 μl 2-cyano-4-hydroxycinnamic acid matrix (Sigma) in 50% acetonitrile/0.1% trifluoroacetic acid. Peptide mass were determined using a MALDI micro MX mass spectrometer (Waters) in reflection mode and analysed with Masslynx software. Data searches of the mass fingerprint data were performed using Mascot (http://matrixscience.com).

**Quantification of subunit molar ratios in vivo.** Measurements of the molar ratios of GluN2A/GluN2B were performed using age-matched (P56-P69) total forebrain (cortex and hippocampus) samples. Quantifying the molar ratios of two different endogenous mouse proteins was achieved by using targeted mouse genetics to match an epitope for immuno-detection.

WT control with either Glun2b2BdV/C2(C176) or Glun2b2BdV/C2(C176) were used for two independent measurements of the GluN2A/GluN2B molar ratio (Fig. 4). In the first measurement of the GluN2A/GluN2B molar ratio was made using Glun2b2BdV/C2(C176) mice in which the CTD of GluN2A/GluN2B was Glun2b2BdV and Glun2b2BdV mutants were crossed with a Cre-deleter line and the neo cassette was removed. Inter-crossing heterozygous mice produced viable homozygous Glun2b2BdV knock-in mutants. The detailed phenotypic characterization of this mutant will be described elsewhere.

**Immunohistochemistry.** Homozygous Glun11P/TAP and wild-type mice were anesthetized with 60 mg g -1 avertin and perfused with 10 ml PBS, followed by 5 ml 4% paraformaldehyde at 2.5 ml min -1. Forebrains were dissected, post-fixed in 4% paraformaldehyde for 2–4 h and then stored at 4°C in 30% sucrose for up to one week. Sagittal sections (30 μm) were collected with a vibratome. Free-floating sections were incubated with 1:500 Rabbit Flag (Genscript, A000187) or 1:200 Rabbit GluN2A (Seropec, AHP1880) in buffer A (0.3% triton X-100, 100 mM NaCl, 50 mM trisció pH 7.4 overnight at 4°C. Following 3 × 10 min washes with buffer A at RT, sections were DAB stained with anti-rabbit-HRP using a kit (Vectorain). Digital photographs of 0.8–1 μm optical sections were collected at a Zeiss 710 confocal microscope with ×63/1.4 numerical aperture objective.

**Generation of Glun2b2BdV knockout mice.** The Glun2b2BdV knockout mice was engineered by standard mouse genetic protocols in which the Glun2b2BdV terminal valine (1,482) was deleted, leaving the last codon for Asp1471 before the stop codon (Supplementary Fig. 4a). In brief, 5.9 kb Bst EII and 1.8 kb Bst XBI homology arms were used to construct the gene targeting vector for Glun2b2BdV terminal valine (1,482) deletion (Fig. 4). Digestion restriction enzyme Bst EII was added. Digestion was saturated at 32°C. Peptides were recovered and desalted using uC18 ZipTip (Millipore) and eluted to a MALDI target plate using 2 μl 2-cyano-4-hydroxycinnamic acid matrix (Sigma) in 50% acetonitrile/0.1% trifluoroacetic acid. Peptide mass were determined using a MALDI micro MX mass spectrometer (Waters) in reflection mode and analysed with Masslynx software. Data searches of the mass fingerprint data were performed using Mascot (http://matrixscience.com).
exponential fits to the decay of synaptic currents measured at +40 mV were used to calculate the weighted mean decay time constant for the decay of NMDAR-mediated currents. All results are reported as mean ± s.e.m. The results for the same experiment on multiple slices or cells from the same animal were averaged and the N used for statistical comparisons (two-tailed Student’s t-tests) equals the number of animals in each group.

**Antibodies.** Primary antibodies (dilutions for immunoblot in parenthesis) mouse Flag (Sigma, F3165) mouse GluN1 (1:1,000, Invitrogen, 32–0500), rabbit GluN1 (1:1,500, Millipore, AB9864), mouse GluN2A (1:1,000, BD Bioscience, 612265), rabbit GluN2A (1:500, Serotec, AHP1800), mouse GluN2B (1:1,000, BD Bioscience, 610416/7), rabbit GluN2B (1:500, Invitrogen, 71–8,600), mouse PDS95/3 (1:1,000, Thermo scientific, MA1–045), mouse PSD93 (1:2,500, NeuroM, 75–057), rabbit PSD95 (1:1,000, Abcam, ab18238), mouse GFP (Invitrogen, A11120), rabbit GFP (Invitrogen, A11122). Conjugated antibodies: mouse Flag-HRP (1:40,000, Sigma, A8592), goat mouse-HRP (1:20,000, Millipore, 12–349) and goat rabbit-HRP (1:20,000, Millipore, 12–348). Antibodies used for Fig. 7 are listed in Supplementary Data 1.

**SDS-PAGE immunoblot.** Images in Figs 2b, 4a,b,d and 6h have been cropped for presentation. Full size images are presented in Supplementary Figs 6–8, respectively.

**Human brain samples.** Three cortical samples were obtained from neurosurgical operations. Tissue was immediately chilled in ice for 30–40 min after which they were frozen at –70 °C. Tissue was not defrosted until used extraction and BNP. In all cases prior written informed consent had been obtained, and the study approved by the local regional ethics committee (Lothian Region Ethics Committee /2004/4/16).

**Animals.** All animal experiments conformed to the British Home Office Regulations (Animal Scientific Procedures Act 1986, Project License PPL80/2,337 to Prof Seth Grant), local ethical approval, and NIH guidelines.

**References**


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Author contributions

R.A.W.F. and S.G.N.G. conceived the project, designed the experiments, analysed the data and wrote the manuscript. R.A.W.F. performed the experiments, except for electrophysiology. N.H.K. designed and generated Glun2a2B(CTR) knock-in mice. T.J.R. and N.H.K. provided Glun2b2A(CTR) and Glun2b2B(CTR) mice. F.Z. and N.H.K. designed and generated Psd95EGFP mice. T.J.O. performed the electrophysiology experiments.

Additional information

Accession codes: PRIDE database (http://www.ebi.ac.uk/pride/)64 with ProteomeXchange accession number: PXD000011.

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