Structure of Excitatory Synapses and GABA<sub>A</sub> Receptor Localization at Inhibitory Synapses Are Regulated by Neuroplastin-65*  

Received for publication, August 30, 2013, and in revised form, January 22, 2014. Published, JBC Papers in Press, February 19, 2014, DOI 10.1074/jbc.M113.514992  

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**Background:** Synaptic excitatory to inhibitory ratio is crucial for brain function but its regulation is poorly understood.  
**Results:** The cell adhesion molecule neuroplastin-65 regulates contact and stability of excitatory synapses and localization of GABA<sub>A</sub>R α2 subunits at inhibitory synapses.  
**Conclusion:** Neuroplastin-65 mediates specific neuronal connections and regulates the number and function of synapses in hippocampal neurons.  
**Significance:** Correct neuronal network activity in specific brain regions may depend on neuroplastin-65.  

Formation, maintenance, and activity of excitatory and inhibitory synapses are essential for neuronal network function. Cell adhesion molecules (CAMs) are crucially involved in these processes. The CAM neuroplastin-65 (Np65) highly expressed during periods of synapse formation and stabilization is present at the pre- and postsynaptic membranes. Np65 can translate into synapses in response to electrical stimulation and it interacts with subtypes of GABA<sub>A</sub> receptors in inhibitory synapses. Here, we report that in the murine hippocampus and in hippocampal primary culture, neurons of the CA1 region and the dentate gyrus (DG) express high Np65 levels, whereas expression in CA3 neurons is lower. In neuroplastin-deficient (Np<sup>−/−</sup>) mice the number of excitatory synapses in CA1 and DG, but not CA3 regions is reduced. Notably this picture is mirrored in mature Np<sup>−/−</sup> hippocampal cultures or in mature CA1 and DG wild-type (Np<sup>+/+</sup>) neurons treated with a function-blocking recombinant Np65-Fc extracellular fragment. Although the number of GABAergic synapses was unchanged in Np<sup>−/−</sup> neurons or in mature Np65-Fc-treated Np<sup>+/+</sup> neurons, the ratio of excitatory to inhibitory synapses was significantly lower in Np<sup>−/−</sup> cultures. Furthermore, GABA<sub>A</sub> receptor composition was altered at inhibitory synapses in Np<sup>−/−</sup> neurons as the α1 to α2 GABA<sub>A</sub> receptor subunit ratio was increased. Changes of excitatory and inhibitory synaptic function in Np<sup>−/−</sup> neurons were confirmed evaluating the presynaptic release function and using patch clamp recording. These data demonstrate that Np65 is an important regulator of the number and function of synapses in the hippocampus.  

The correct connectivity and function of neuronal networks in the brain is a delicate process that requires the carefully controlled formation, maturation, maintenance, and plastic modulation of synaptic contacts between neurons. Neuronal cell adhesion molecules (CAMs)<sup>5</sup> are crucially involved in these processes (1) as evidenced, for example, in studies of synaptic function and long-term plasticity in hippocampal slices (2–7). More recent findings document CAMs as mediators of specificity in synapse formation and stabilization between neuronal subtypes <i>in vivo</i> and <i>in vitro</i> (8–10), which is of great importance for the proper function of networks. Here, we report on neuroplastin-65 as a new candidate to participate in these processes.  

Neuroplastin-55 (Np55) and -65 are members of the immuno-globulin (Ig) superfamilly of single-pass transmembrane CAMs that arise from a single gene by alternative splicing. Np65 possesses three Ig domains, whereas Np55 lacks the N-terminal Ig1 domain.
Np65-mediated Synaptic Ratio

(15, 16). Np65, but not Np55, is involved in the adhesion of pre- and postsynaptic elements as: 1) it is localized both presynaptically and postsynaptically (17, 18), 2) it is prominently expressed during the major period of synapse formation in cortex and hippocampus (19), and 3) the Ig1 domain mediates homophilic trans-interactions, which modulate synaptic activity (20, 21). Furthermore, Np65 interacts with GABA<sub>A</sub> receptor (GABA<sub>A</sub>R) subunits in hippocampal inhibitory synapses (22). Np65 is also involved in the maintenance of hippocampal CA1 synaptic plasticity and its association with the postsynaptic density protein fraction is regulated by synaptic activity (20, 23). Therefore, it is likely that Np65 regulates formation and/or functional reorganization of excitatory and inhibitory synapses under conditions of basal and increased neuronal activity.

The main focus of the present study was to establish clear roles for the neuroplastins in synapse formation and stabilization and in functional synaptic connectivity. Therefore, we analyzed the numbers of synapses in the CA1, CA2/3, and DG regions of the hippocampus in Np<sup>−/−</sup> mice. To obtain insight into the underlying subcellular processes and mechanisms we utilized knock-out, fusion protein- and antibody-treated primary hippocampal cultures at different developmental stages to study in detail the role of Np65 in: 1) the formation, stability, morphology, and density of synaptic contacts and spines; 2) the distribution of pre- and postsynaptic protein markers for glutamatergic and GABAergic synapses; 3) the distribution and composition of GABA<sub>A</sub> receptors; and 4) the function of both excitatory and inhibitory synapses as determined by patch clamp recording of excitatory and inhibitory postsynaptic currents and by a synaptotagmin uptake assay.

Our data show that Np65 plays a key role in maintaining the structural integrity of excitatory, but not inhibitory synapses. We also provide evidence that Np65 mediates alignment of GABA<sub>A</sub>R α2 subunits at postsynapses with vesicular amino acid transporter (VIAAT)-positive presynapses to ensure the correct composition of GABA<sub>A</sub>Rs. Our data suggest that Np65 is an important regulator of the number and activity of synapses in the hippocampus.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Primary antibodies used: rabbit anti-GABA<sub>A</sub>R α2 subunit (generously supplied by Dr. W. Sieghart); rat anti-homer (Acris, Herford, Germany); guinea pig anti-synapsin 1,2, guinea pig anti-GAP65, mouse anti-gephyrin, rabbit and guinea pig anti-VIAAT, mouse Cy3-conjugated anti-synaptotagmin-1, guinea pig anti-GABA α2 subunit, and rabbit anti-GABA α1 subunit; guinea pig, rabbit, and mouse anti-microtubule-associated protein 2 (MAP2) (Synaptic Systems, Goettingen, Germany); goat anti-Np65 antibody (RandD Systems, Abingdon, UK); mouse anti-Prox1 (clone 4G10) (Millipore, Schwabach, Germany); rat anti-Ctip2 (clone 25B6) and mouse anti-GFP (Abcam, Cambridge, UK); mouse anti-β-actin (C4, Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-Np Ig 1–3 (20); and rabbit anti-bassoon (24). Secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA).

**Generation of Neuroplastin-deficient Mice**—The first exon of the neuroplastin gene encoding the translational start codon and the signal sequence was flanked with 2 lox sites by homologous recombination in embryonic stem cells. Chimeric mice were generated by blastocyst injection of the targeted embryonic stem cells (Karolinska Institute, Stockholm, Sweden). Offspring carrying the floxed neuroplastin allele were crossed with transgenic mice expressing Cre recombinase under control of the CMV promoter (25). Cre recombinase-mediated excision resulted in mice carrying a neuroplastin allele missing the first exon and, thus, lacking the expression of both Np isoforms. These mice, transmitting the deletion in the germline, were backcrossed to establish heterozygote (Np<sup>+/−</sup>) mice without Cre recombinase transgene. Heterozygote (Np<sup>+/−</sup>) mice were crossed to obtain Np−deficient (Np<sup>−/−</sup>) and wild-type (Np<sup>++/+</sup>) littermates. To confirm the lack of Np expression, protein extracts from brains (Fig. 1A) and cultured hippocampal cells were prepared and analyzed by Western blot using standard procedures as described (26).

**Cultures of Hippocampal Neurons**—Hippocampal neurons were prepared using published protocols (27, 28) with some modifications. To obtain neuronal cultures from genetically modified mice, hippocampi of P0 pups were trypsinized at 37 °C for 8 min and then gently dissociated in minimal essential medium supplemented with 10% horse serum (Invitrogen). Cells were seeded onto poly-d-lysine-coated coverslips using a 1:3 mixture of astroglial conditioned medium and Neurobasal medium supplemented with B27 (Invitrogen). After 1 h, seeding medium was carefully replaced by fresh mixed glial medium. Cultures of rat hippocampal neurons and glia were prepared following published protocols (28). After 14 days, confluent glial cultures in 75-cm<sup>2</sup> flasks were depleted of microglial cells by shaking for 15 min and washing with excess Ca<sup>2+</sup>/Mg<sup>2+</sup>-free Hanks’ balanced salt solution (Invitrogen). Conditioned medium was obtained by maintaining the monolayer of astrocytes in 10 ml of Neurobasal medium supplemented with B27 for 54–56 h.

**Treatment with Neuroplastin Fusion Proteins**—Mature neuronal cultures were treated with 0.1 μg/ml of neuroplastin-55-Fc (Np55-Fc, fusion protein comprising Np Ig domains 2–3 fused to human Ig Fc fragment) or neuroplastin-65-Fc (Np65-Fc, Ig domains 1–3 fused to the Fc fragment) or Fc only for 30 min at 37 °C or 15 min at room temperature following published protocols (20, 23). Then cells were carefully washed once with PBS and fixed for fluorescent immunostaining.

**Immunostaining of Synaptic Markers and Image Acquisition**—Living neurons were incubated with antibodies for 20 min and fixed with 4% p-formaldehyde in Hanks’ balanced salt solution buffer for 8 min at room temperature. For staining of intracellular proteins, cultures were fixed and then washed with a solution containing 10% bovine fetal serum, 0.1 mxs glycine, and 0.1% Triton X-100 in Hanks’ balanced salt solution four times for 5 min. To visualize excitatory synaptic contacts, neurons were stained with rat anti-homer (1:500), guinea pig anti-synapsin 1,2 (1:500), or rabbit anti-bassoon (1:500), and mouse anti-MAP2 (1:1000) antibodies for 1 h at room temperature. For inhibitory synapses, staining was done with guinea pig anti-VIAAT (1:1000), mouse anti-gephyrin (1:500), and rabbit anti-MAP2 (1:1000) for 2 h at room temperature. Alternatively, rabbit anti-GABA<sub>A</sub>R α2 subunit (5 μg/ml), and guinea pig anti-VIAAT (1:1000) were used for staining. Subsequently, samples...
were incubated with anti-mouse Cy5-, anti-rabbit Alexa 488-, anti-rat Alexa 488-, and/or anti-guinea pig Alexa 568-conjugated secondary antibodies (1:1000), all of them generated in donkey. Staining of recombinant proteins adhering to the neuronal cell surface was performed following published protocols (28). For Np65 staining in brain, hippocampal sections were prepared from 2-month-old C57BL6/J mice. Slices were fixed using 4% p-formaldehyde followed by 15% ethanol, blocked with 10% horse serum and 0.1% Triton X-100 for 30 min, and incubated with goat anti-Np65-specific antibody overnight. Subsequently, sections were incubated with secondary antibodies, washed, and mounted with Mowiol. Fluorescence was visualized using a Zeiss AXIO Imager A2 microscope equipped with a CCD camera (VisiTron Systems) using ×10 (0.45 NA) and ×63 (1.4 NA) objectives. Image acquisition was performed using VisiView software (VisiTron Systems, camera binning = 1, pixel size = 0.10238 × 0.10238 μm, pixel depth = 16 bytes). Hippocampal slices of Np\(^{+/+}\) and Np\(^{-/-}\) mice were prepared, incubated with rat anti-homer (1:500), guinea pig anti-synapsin 1.2 (1:500), or goat anti-bassoon (1:500) antibodies, and mounted as published (28). Slices were photographed using an oil-immersion (HCX APO ×63/1.40 NA) objective coupled to a TCS SP5 confocal microscope under sequential scanning mode and a 6.0-fold digital magnification to reach the Nyquist criteria (80.2 × 80.2 × 209.8 nm voxel dimensions). Z-stacks (41.01 × 41.01 × 5 μm physical lengths) were digitalized in a 512 × 512 pixels format file. Pictures were taken for the CA1 field 50 μm from the midline of the somata and for CA3 75 μm of the stratum radiatum (30). For the DG field, we imaged and analyzed areas of the molecular layer surrounding the granule cell layer. For Fig. 3C, nonlinear correction was applied to original pictures for improved visualization by minimal adjustment in γ settings to 0.9 using Photoshop CS3 version 10.

Identification of Hippocampal Neuronal Type—Neuronal cultures were fixed, permeabilized, and incubated with goat anti-Np65 (1:100), rat anti-homer (1:500), guinea pig anti-MAP2 (1:1000), rat anti-Ctip2 (1:500), and mouse anti-Prox1 (1:500) antibodies for 2 h at room temperature. Subsequently, samples were incubated with anti-rat Alexa 488-, anti-goat Alexa 568-, anti-guinea pig Cy5-, and anti-mouse Alexa 350-conjugated donkey secondary antibodies (1:1000). In some cases, anti-mouse Alexa 488 and anti-rat Cy3-conjugated secondary antibodies (1:1000) were used. In addition, some samples were incubated with 1% DAPI in PBS for 10 min at room temperature.

Image Processing and Puncta Match Quantification—Z-stack images of hippocampal sections were deconvoluted using the Huygens Professional software (Scientific Volume Imaging B.V., The Netherlands) and then segmented using the “3D object counter” plugin in the Fiji software (31). Then, from maximal intensity projections, we generated 1-bit mask for each fluorescent channel as described above. For images taken from cultured neurons, each fluorescent image was deconvoluted using the adaptive PSF method (10–15 iteration cycles, low-medium noise level) with AutoQuant X2 software (Media Cybernetics, Inc.), and then segmented using Otzu’s algorithm. During image processing the original brightness/contrast settings were not modified. For a segmented image of each synaptic marker, 1-bit masks were created using the “analyze particle” in the Fiji software (range of particle size = 0.04 – 0.5 μm\(^2\) approximately). To quantify the synaptic match, we used binary masks and the “Box_puncta_Ex” and “Match_Set” functions in OpenView software (32). To do this, all puncta in a mask (either postsynaptic to quantify post- to presynaptic match or presynaptic for pre- to post-synaptic match) were detected, and individually boxed in a 1.75 × 1.75 μm matching area centered on their respective center of mass (maximal distance for match detection = 1.25 μm approximately). Each boxed punctum was classified as matched when at least one complementary marker site was detected in the matching area described above. Raw data of distances between matched puncta were automatically generated as an Excel-compatible file for further statistical analysis (for calculations total boxed puncta were considered as 100%). When non-related masks derived from different fluorescent pictures were used, very few false-positive cases of postsynaptic “random match” (6.1 ± 0.9%) were detected. Thus, we opted to use this procedure as it was robust in detecting tightly matched complementary synaptic markers. Use of a bigger matching area (2.75 × 2.75 μm, maximal distance for detection = 2 μm approximately) increased the number of apparent matches due to detection both of synaptic markers belonging to the same synapse but also those belonging to neighboring synapses. The random match detection increased up to 40% in many cases and, therefore, the larger matching was not used for analysis. To normalize the number of synaptic puncta, the length of dendrite segments was quantified using the semiautomatic tracing tool in the NeurOJ application of ImageJ.

Quantification of Somatic and Dendritic α2, α1, and Gephyrin Clusters—Living Np\(^{+/+}\) and Np\(^{-/-}\) cultured hippocampal mouse neurons were stained with guinea pig anti-GABA\(_A\)R α2 subunit (1:250), rabbit anti-GABA\(_A\)R α1 subunit (1:500) for 20 min, washed, fixed, permeabilized, and incubated with mouse anti-gephyrin (1:500) for 2 h. Subsequently, samples were incubated with anti-rabbit Alexa 488-, anti-mouse Alexa 568-, and anti-guinea pig Cy5-conjugated donkey secondary antibodies (1:1000) for 1 h. Z-stack images of soma and secondary/tertiary dendrites were independently acquired using an oil-immersion (HCX APO ×63/1.40 NA) objective coupled to a TCS SP5 confocal microscope under sequential scanning mode with a 4.0-fold digital magnification, and digitalized in a 512 × 512 pixels format file (61.51 × 61.51 μm physical lengths). We carefully set the lasers power and detection to avoid any fluorescence intensity saturation. All parameter were strictly maintained during the imaging procedure. For these experiments, the size and number of gephrin puncta were quantified using analyze particle in the Fiji software by setting the next parameters: brightness and contrast range = 30 to 255; color threshold filter pass range = 70 to 255; range of particle size = 0.04 – 0.6 μm\(^2\).

To quantify localization of the gephrin marker, 1-bit masks were created using the “analyze particle” in the Fiji software (range of particle size = 0.04 – 0.5 μm\(^2\) approximately). To quantify the synaptic match, we used binary masks and the “Box_puncta_Ex” and “Match_Set” functions in OpenView software (32). To do this, all puncta in a mask (either postsynaptic to quantify post- to presynaptic match or presynaptic for pre- to post-synaptic match) were detected, and individually boxed in a 1.75 × 1.75 μm matching area centered on their respective center of mass (maximal distance for match detection = 1.25 μm approximately). Each boxed punctum was classified as matched when at least one complementary marker site was detected in the matching area described above. Raw data of distances between matched puncta were automatically generated as an Excel-compatible file for further statistical analysis (for calculations total boxed puncta were considered as 100%). When non-related masks derived from different fluorescent pictures were used, very few false-positive cases of postsynaptic “random match” (6.1 ± 0.9%) were detected. Thus, we opted to use this procedure as it was robust in detecting tightly matched complementary synaptic markers. Use of a bigger matching area (2.75 × 2.75 μm, maximal distance for detection = 2 μm approximately) increased the number of apparent matches due to detection both of synaptic markers belonging to the same synapse but also those belonging to neighboring synapses. The random match detection increased up to 40% in many cases and, therefore, the larger matching was not used for analysis. To normalize the number of synaptic puncta, the length of dendrite segments was quantified using the semiautomatic tracing tool in the NeuroJ application of ImageJ.
**Np65-mediated Synaptic Ratio**

**Synaptotagmin Uptake Assay**—We monitored spontaneous presynaptic activity in hippocampal neurons (21 DIV) pre-treated with pre-immune goat IgG or goat anti-Np65 antibody (5 ng/ml) for 15 min at 37 °C. Then cells were briefly washed once with warm Tyrodes buffer (119 mM NaCl, 2.5 mM KCl, 25 mM HEPES, pH 7.4, 30 mM glucose, 2 mM MgCl₂, 2 mM CaCl₂) and immediately incubated with a mouse Cy3-labeled anti-synaptotagmin-1 antibody (24) in the presence of control goat IgG or goat anti-Np65 antibody for 20 min at 37 °C. Upon uptake, cells were washed twice with Tyrodes buffer, fixed, and stained with rabbit anti-VIAAT (1:1000) and guinea pig anti-synapsin 1,2 (1:500) primary antibodies for 1 h at room temperature. Subsequently, samples were incubated with anti-rabbit Alexa 488-, anti-mouse Cy3-, and anti-guinea pig Cy5-conjugated donkey secondary antibodies (1:1000) for 1 h. Z-stack images of soma and secondary/tertiary dendrites were acquired as described under “Quantification of Somatic and Dendritic α2, α1, and Gephyrin Clusters.” We quantified the synaptotagmin-associated fluorescence co-localizing with 1-bit masks derived from VIAAT-positive (inhibitory presynapses) or VIAAT-negative synapsin-positive (excitatory presynapses) puncta using the “image calculator” in the Fiji software. During image processing the original settings of the synaptotagmin channel were not modified. For a segmented image of each presynaptic marker, 1-bit masks were created using the analyze particle in the Fiji software (range of particle size = 0.08 – 0.5 μm² approximatively). At least 300 presynapses from 10 neurons per experiment were analyzed.

**Electrophysiology**—Miniature excitatory postsynaptic current (mEPSC) and miniature inhibitory postsynaptic current (mIPSC) from Np⁺/⁻ and Np⁻/⁻ cultured hippocampal mouse neurons (17–24 DIV) were recorded with the patch clamp method in the whole cell configuration. In all cases the experimenter did not know the genotype. Patch electrodes produced from borosilicate glass with a horizontal Brown-Flämming pipette puller (Sutter, Novato, CA) had a pipette resistance of 3–4 megaohm. Intra- and extracellular solutions had the following composition: intracellular 140 mM K-glutamate, 2 mM MgCl₂, 4 mM NaATP, 0.1 mM EGTA, 10 mM HEPES, pH was adjusted to 7.25 by 1 M KOH; extracellular 145 mM NaCl, 2.5 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES, and 10 mM d-glucose, pH 7.4. To block spontaneous network activity 1 μM tetrodotoxin was added to the extracellular solution. AMPA receptor-mediated mEPSCs were recorded at a holding potential of −70 mV in the presence of 10 μM bicuculline and 10 μM (2R)-amino-5-phosphovaleric acid to block GABA_A and NMDA receptors, respectively. GABA_B-mediated mIPSCs were recorded at a holding potential of −40 mV in the presence of 10 μM (2R)-amino-5-phosphovaleric acid and 10 μM 6-cyano-7-nitroquinazoline-2,3-dione. Recordings were performed at room temperature using an EPC 10 patch clamp amplifier (HEKA Electronics, Lambrecht, Germany). Data were acquired and stored using Patchmaster software (HEKA Electronics) and analyzed with Mini-Analysis (Synaptosoft Inc., Decatur, GA).

**Statistical Analysis**—Raw data distributions were compared using one-way (experimental condition as factor with Kruskal-Wallis H-test) analysis of variance (ANOVA) or two-tailed t-test using Origin software. Normalized data classes were evaluated with non-parametric Mann-Whitney U analysis followed by a Kolmogorov-Smirnov test. Statistical significance and corresponding statistical tests are indicated in each figure and in the text.

**RESULTS**

**Reduction of CA1 and DG Excitatory Synapses in Hippocampal Slices of Np⁻/⁻ Mice**—First, we confirmed that Np⁻/⁻ mice do not express detectable levels of Np65 or Np55. Whereas Western blots of brain homogenates prepared from wild-type (wt) mice and mice carrying the floxed neuroplastin allele (lox) show readily detectable levels of both neuroplastins, homoge-
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NP65-MEDIATED SYNAPtic RATIO

FIGURE 2. Impaired numbers and matches of excitatory post- and presynaptic sites in NP<sup>−/−</sup> neurons. Neurons were stained for homer (red), synapsin (green), and MAP2 (blue) at 12 (A, I–IV) or 21 (B, I–IV) DIV and photographed using confocal microscopy. A, I, maximum intensity projections of fluorescent staining are shown. Note that in NP<sup>−/−</sup> neurons several enclosed homer-positive puncta lack a synapsin-positive presynaptic counterpart. Arrowheads, contacts of homer-positive puncta with a presynaptic partner in mutant and wild-type neurons. B, I, three-dimensional perspectives of Z-stack pictures. Arrows indicate well structured synaptic contacts around dendrites. Asterisks in NP<sup>−/−</sup> neurons indicate malformed contacts with diffusely spread presynaptic markers. Quantification of data from neurons lacking NP (black bars and squares) or wild-type (gray bars and squares), cultivated for 12 DIV (A, I–IV) and 21 DIV (B, I–IV). The percent mismatch of homer to synapsin and synapsin to homer was quantified (A, II and B, II). Size distribution of numbers of homer (A, II and B, III) or synapsin puncta (A, IV and B, IV), and insets with the corresponding group summary for each puncta size distribution are shown. Data are mean ± S.D. from 5 independent cultures. **, p < 0.01 or *, p < 0.05 between genotypes. C, NP<sup>−/−</sup> and NP<sup>+/+</sup> neurons (21 DIV) were stained with 568-conjugated phalloidin (red) and anti-MAP2 antibodies (green), and photographed using confocal microscopy. C, I, maximum intensity projections of fluorescent staining are shown. Indicated digital magnifications of F-actin staining are shown in C, II. Scale bar = 6 μm. Data are mean ± S.D. from 5 independent cultures. **, p < 0.01 comparing NP<sup>−/−</sup> versus NP<sup>+/+</sup> neurons.

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21 DIV, Np\textsuperscript{+/+} neurons displayed numerous well formed synaptic contacts around their dendrites, whereas many flattened synaptic contacts were observed in Np\textsuperscript{−/−} neurons (Fig. 2B, I). To visualize the F-actin-based cytoskeleton we used 568-conjugated phalloidin and detected numerous malformed dendritic spines in Np\textsuperscript{−/−} neurons (Fig. 2C). Together, these results indicate that inactivation of the Np gene may produce a disturbed synaptogenesis due to either reduced formation or reduced stabilization of excitatory synaptic contacts. To evaluate these possibilities we analyzed and compared the presence and the organization of protein markers of excitatory synapses at an early stage of synapse development (12 DIV), and in mature (21 DIV) Np\textsuperscript{+/+} and Np\textsuperscript{−/−} hippocampal cultures by analysis of immunostained cultures. Briefly, images of homo- and synapsin-positive immunofluorescence were deconvolved, segmented to generate independent binary masks, and used to classify each punctum. By this procedure, a synapse is defined as a match of a pre- and postsynaptic punctum due to close proximity, whereas all non-matching puncta are counted as a match of a pre- and postsynaptic punctum due to close proximity, whereas all non-matching puncta are counted as a match of a pre- and postsynaptic punctum due to close proximity, whereas all non-matching puncta are counted as a match of a pre- and postsynaptic punctum due to close proximity, whereas all non-matching puncta are counted as

![Image 1](https://via.placeholder.com/150)

**FIGURE 3.** Np65 is highly expressed in CA1 and DG neurons in hippocampal cultures. Rat hippocampal neurons were stained at 21 DIV for MAP2, homer, and Np65 as well as for Prox1 and Ctip2 to identify neuronal subtypes as indicated below. A (left), arrows indicate a dendritic segment with numerous Np65-positive Homer puncta, whereas in A (right) a dendrite from a different neuron displays lower Np65 expression. Scale bar = 5 μm. B, identification of CA1 (Ctip2-positive, Prox1-negative) and DG (Ctip2-positive and Prox1-positive) neurons. Nuclei were stained with DAPI. No Neu indicates a MAP2-negative non-neuronal cell. In C, note that anti-Ctip2 and anti-homer primary antibodies were detected using the same anti-rat Alexa 488-conjugated secondary antibody. Distinguishing the staining for the two antigens was straightforward due to the specificity of the primary antibodies and the characteristic subcellular location of each of these proteins (nuclear versus synaptic). C, I, shows CA1 neurons expressing very high levels of Np65, whereas in C, II, two Np65-positive DG neurons are next to a Prox1- and Ctip2-negative neuron expressing low levels of Np65. In B and C, scale bar = 20 μm. C, III, digital magnifications taken from C, I and II show Np65 (red) co-localizing with homer (green) in CA1 (a) and DG (b) neurons (arrows). Note that most postsynapses of a Prox1- and Ctip2-negative neuron (c) do not display Np65 (asterisk) in contrast to a small number of others (# symbol). Scale bar = 1 μm. Representative pictures from 4 independent experiments are shown.

In mature (21 DIV) Np\textsuperscript{−/−} neurons we found a postsynaptic mismatch of 67.2 ± 4.2% compared with a mismatch of 53.5 ± 4.2% in Np\textsuperscript{+/+} neurons (p < 0.01 for KO versus WT, Mann-Whitney U test; Fig. 2A, II). Thus, at 12 DIV Np\textsuperscript{−/−} neurons formed a higher number of non-matching homer-containing clusters than Np\textsuperscript{+/+} neurons, whereas the number of excitatory contacts remained unchanged. Surprisingly at 21 DIV, the number of synapses was reduced from 5.21 ± 0.13 for Np\textsuperscript{−/−} to 4.09 ± 0.49 for Np\textsuperscript{−/−} neurons (120 neurons from 5 independent cultures, p < 0.05 for KO versus WT, ANOVA). Moreover, the number and percentage of mismatched postsynaptic sites in Np\textsuperscript{−/−} neurons were lower than in the wild-type neurons (Fig. 2B, II). The post-synaptic mismatch was 36.4 ± 4.7% in mutant compared with 48.6 ± 1.8% in wild-type neurons (p < 0.01 for KO versus WT, Mann-Whitney U test; Fig. 2B, II). Thus the mean number of postsynapses per 10 μm² of dendrite area was 10.1 ± 0.5 for Np\textsuperscript{+/+} neurons compared with 7.4 ± 0.4 for Np\textsuperscript{−/−} neurons (p < 0.05 for KO versus WT, ANOVA; inset in Fig. 2B, III). In contrast, no statistically significant difference for the number of presynapses was found between Np\textsuperscript{+/+} and Np\textsuperscript{−/−} neurons (Fig. 2B, IV). These data indicate that the lower number of synapses is accompanied by a decrease of postsynapses and a change in contact with a matching presynaptic site in mature Np\textsuperscript{−/−} neurons.

**Differential Expression of Np65 in Hippocampal Neuronal Types**—The abundance of Np65 varies between rat hippocampal areas, being highest in the CA1, less in the DG, and lowest in the CA2/3 region (Fig. 1B). Thus, we sought to establish whether this differential expression of Np65 is an intrinsic signature that also occurs in dissociated hippocampal neurons in

![Image 2](https://via.placeholder.com/150)
culture. We found that Np65 expression varies from highly abundant to sparse between individual neurons in culture (Fig. 3A). To evaluate whether the differential Np65 expression is associated with a subset of excitatory synapses present on different neuronal subtypes, we used antibodies to stain Np65, homer, and MAP2 in combination with an anti-Ctip2 antibody, which labels CA1 pyramidal neurons and many DG neurons (8, 36), and anti-Prox1 that selectively labels DG neurons (37). Using this combination of antibodies, we were able to distinguish CA1 neurons (Ctip2-positive, Prox1-negative nuclei) and DG neurons (Ctip2-positive and Prox1-positive nuclei) from other neuronal types (Ctip2-negative and Prox1-negative nuclei) (Fig. 3, B and C). Np65 was abundant in CA1 (Fig. 3C, I) and DG neurons (Fig. 3C, II) in contrast to the low Np65 levels detected in other neurons (Fig. 3C, II). Digital magnifications confirmed that Np65 is tightly associated with CA1 and DG postsynaptic sites (arrows in Fig. 3C, III, a and b) even though postsynaptic sites in other neuronal types contained no (asterisk in Fig. 3C, IIIc) or only low levels (hash sign in Fig. 3C, IIIc) of Np65. Thus, as for the in vivo situation, CA1/DG hippocampal neurons express higher levels of Np65 and can localize it to their synaptic contacts in vitro.

Np65 Stabilizes Excitatory Synaptic Contacts of CA1 and DG Neurons—Np65 maintains synaptic plasticity of CA1 synapses in rat hippocampal slices as incubation with Np65-Fc fusion protein impairs the maintenance of long term potentiation (20). To enable detailed analysis of synaptic matching, we perturbed Np function by addition of Np-Fc fusion proteins to hippocampal cultures. In particular, we evaluated whether incubation with Np65-Fc or Np55-Fc perturbs the contact of pre- and postsynaptic structures in mature Np+/+ neurons. Np55-Fc was not able to modify the contact between homer-positive and bassoon-positive post- and presynaptic compartments because they were similarly abundant and matched as well as under control conditions (Fc protein) (Fig. 4A). In contrast, Np65-Fc reduced the amount of synaptic contacts and produced a striking increase of decoupled pre- and postsynaptic sites (Fig. 4A). This observation was confirmed by quantifying the post- to presynaptic mismatch as described for Fig. 2 (Fig. 4B). The post- to presynaptic mismatch was 61.4 ± 8.5% in Np65-Fc-treated compared with 43.1 ± 7.4% in Fc-treated Np+/+ neurons (100 neurons from 4 independent cultures, p < 0.01, Mann-Whitney U test; inset in Fig. 4B). In control neurons, 64.7 ± 4.2% of matched puncta were formed by pre- and postsynapses as close as 0.25 μm in proximity, whereas in Np65-Fc-treated neurons only 48.1 ± 5.6% of contacts showed the same proximity (100 neurons from 4 independent cultures, p < 0.05, Mann-Whitney U test; Fig. 4B). Furthermore, Np65-Fc-treated neurons displayed more synaptic contacts in the higher range of pre- to postsynaptic distances than control neurons (Fig. 4B). To assess the robustness of our quantification and statistics, we calculated the cumulative frequency for matched synaptic contacts as cumulative puncta match (Fig. 4C). Np65-Fc-treated neurons presented a lower pre- to postsynaptic match than control neurons throughout the whole range of pre- to postsynaptic distances (4 independent experiments, F_{1,149} = 33.345, p = 0.023, one-way ANOVA; Fig. 4C). Moreover, particular classes of pre- to postsynaptic distances were significantly

FIGURE 4. Binding of Np65-Fc to CA1 and DG neurons causes dissociation of excitatory synaptic contacts. A–C, and G, Np+/+ or Np−/− mouse hippocampal neurons (21 DIV) were treated with Fc, Np65-Fc, or Np55-Fc for 30 min at 37 °C, fixed, stained for pre- (Bsn, bassoon, green) and postsynaptic (homer, red) markers, photographed, and analyzed as described in the legend to Fig. 2. Representative cells are shown, and digital magnifications of different regions from each experimental condition are included. B, the inset shows the percent of mismatched puncta for Np65-Fc- and Fc-treated cultures. C, cumulative frequencies of matched puncta versus distance between synaptic markers were plotted taking the value of the number of matched puncta as 100%. D, after treatment with recombinant proteins, rat hippocampal neurons were fixed, permeabilized, and classified by immunostaining as described in the legend to Fig. 3. White arrows indicate clusters of fusion proteins detected using an Fc fragment-specific FITC-conjugated anti-human IgG antibody (green). E, quantification of the number of neurons positive for aggregates of recombinant protein was performed following published protocols (28). F, magnification of wild-type or mutant neurons after treatment with Np65-Fc detected using an Fc fragment-specific FITC-conjugated anti-human IgG antibody. In A and D, scale bar = 20 μm. In F, scale bar = 6 μm. B and C, data are mean ± S.D. from 4 independent cultures, *p < 0.05 comparing Np65-Fc-treated versus Fc-treated control neurons. G, data are mean ± S.D. from 3 independent cultures, **p < 0.01 comparing Fc-treated Np−/− and Np+/+ neurons, §§, p < 0.01 comparing Np65-Fc-treated with Fc-treated Np−/− neurons.
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decreased in Np\textsuperscript{−/−} compared with Np\textsuperscript{+/+} neurons as indicated in Fig. 4C (4 independent experiments, Mann-Whitney \textit{U} test). Thus, when Np65-Fc, but not Np55-Fc, was added to Np\textsuperscript{+/+} hippocampal neurons, a subset of pre- andpostsynaptic sites dissociates yielding the observed pattern. These data strongly suggest a specific and acute involvement of Np65 in the structural stability and/or integrity of a population of excitatory synapses.

To evaluate which hippocampal neurons are affected by Np65-Fc, we identified the neuronal subtype to which Np65-Fc binds. After treatment with Fc, Np55-Fc or Np65-Fc neurons were washed, fixed, and the recombinant protein bound to the neuronal surface was detected with FITC-conjugated anti-human IgG antibody specific for the Fc fragments (green, Fig. 4, \textit{D} and \textit{E}). Additionally, the neuronal type was determined according to the presence of Ctip2 and/or Prox1 as described above. Aggregates of Np65-Fc were observed decorating the dendritic arborization of CA1 and DG neurons (Fig. 4D). In contrast, non-identified neuronal types bound lower amounts of adherent Np65-Fc. Quantification of classifiable neurons decorated with recombinant proteins revealed that indeed CA1 and DG neurons were preferential targets for Np65-Fc, which formed puncta-like aggregates along the dendritic shaft. In contrast, binding of Np55-Fc was comparable with nonspecific binding of control Fc fragments for all the analyzed neuronal types (Fig. 4E).

The existence of further cell membrane-bound element(s) binding Np65-Fc is suggested by the fact that residual attachment of the Np65-Fc construct could be detected at the surface of the dendritic shaft of Np\textsuperscript{−/−} neurons (Fig. 4F). Therefore, we tested whether Np65-Fc and/or Np55-Fc can affect synaptic stability in Np-deficient neurons. Np\textsuperscript{−/−} and Np\textsuperscript{+/+} neurons were treated with the recombinant proteins, stained with antibodies against Homer, Synapsin, and MAP2, and the homo to synapsin mismatch was quantified. As expected, the mismatch was increased by 23% in Np65-Fc-treated compared with Fc-treated Np\textsuperscript{+/+} neurons (75 neurons from 3 independent experiments, \textit{p} < 0.01, Mann-Whitney \textit{U} test; Fig. 4G). Importantly, Np65-Fc was not able to further enhance the already increased synaptic mismatch in Np\textsuperscript{−/−} neurons (\textit{p} = 0.366, Mann-Whitney \textit{U} test; Fig. 4G) indicating that endogenous Np65 is required by Np65-Fc to destabilize synaptic contacts. In addition, incubation with a function-blocking Np65-specific Ig domain antiserum (AS Ig1, 20) drastically decreased both the number of and match between Homer- and synapsin-positive synaptic sites in rat hippocampal neurons at 21 DIV (data not shown). In summary these results add further support to the presence of Np65 in GABAergic synapses was established recently (22). We therefore investigated whether matching of somatic and dendritic GABAergic synapses is also affected in Np\textsuperscript{−/−} neurons. We immunostained cultures of 12 or 21 DIV for inhibitory postsynaptic ( gephyrin (gep, red), VIAAT (green), and MAP2 (blue), photographed, and puncta mismatch was independently quantified for dendrites and soma as described in the legend to Fig. 2. E and F, Np\textsuperscript{+/+} neurons were exposed to recombinant Np65-Fc or Fc (control) for 30 min at 37 °C, fixed, and stained. E, quantification of the puncta match (fraction of all matched puncta = 100%) versus the distance between pre- and postsynaptic sites. Data are mean ± S.D. from at least 5 independent cultures. G, quantification of the number of excitatory (total homer to synapsin matched contacts per 10 \mu m, green, Np\textsuperscript{+/+} and red Np\textsuperscript{−/−}, triangles) and inhibitory (total gephyrin to VIAAT matched contacts per 10 \mu m, in green, Np\textsuperscript{+/+} and in red Np\textsuperscript{−/−}, squares) synapses in neurons derived from 6 Np-deficient and 5 wild-type mice. Lines connect excitatory and inhibitory contacts from the same culture from a single animal. H, the ratio of the number of excitatory to inhibitory synapses (ratio E/I) for each genotype is shown. In \textit{G} and \textit{H}, * \textit{p} < 0.05; n.s.d., non-significant statistical difference both comparing genotypes. Scale bars in \textit{A} = 20 \mu m, in \textit{B} = 5 \mu m, and in \textit{F} = 6 \mu m.
9 and data not shown). This suggests that Np is neither needed to form nor to maintain inhibitory pre- to postsynaptic contacts. To interfere with already established interactions of Np65 in inhibitory synaptic contacts, Np65+/- neurons (21 DIV) were treated with Fc protein (control) or Np65-Fc fusion protein for 30 min and stained for gephyrin and VIAAT. Both Fc- and Np65-treated neurons presented well matched inhibitory synapses in dendrites (Fig. 5F) and somata (not shown). No statistically significant changes of the pre-postsynaptic site proximity were found after treatment with Np65-Fc. Moreover, the 81.3 ± 5.9% in Np65-Fc-treated and the 88.1 ± 5.5% in Fc-treated neurons of inhibitory contacts were formed by very close apposition (0.25 μm) of pre- and postsynaptic sites (5 independent experiments, p = 0.148, Mann-Whitney U test Fig. 5E). Thus, neither Np deficiency nor acute treatment with Np65-Fc perturb the match of inhibitory synaptic contacts.

If Np65 is necessary for maintenance of the contact of excitatory (Figs. 1–4), but not inhibitory (Fig. 5, A–F) synapses, it can be postulated that Np deficiency may affect the ratio of excitatory to inhibitory synapses. To test this hypothesis, cultured hippocampal neurons from individual Np-/- or Np65+/- neonate mice were immunostained for excitatory (homer and synapsin) or inhibitory synaptic markers (gephyrin and VIAAT) as well as for MAP2 at 21 DIV. First, we quantified the number of excitatory and inhibitory contacts for each wild-type and mutant culture as for Fig. 2 (Fig. 5G). All cultures from individual Np-deficient mice displayed a lower number of excitatory contacts, whereas the number of inhibitory synaptic contacts was essentially the same when compared with Np65+/- neurons from individual wild-type mice. We calculated the mean of the ratio of excitatory to inhibitory synapses for each genotype (ratio E/I of cultures derived from 6 deficient and 5 wild-type mice, Fig. 5H). Strikingly, this E/I ratio decreased by 30% in Np-/- compared with Np65+/- neurons at 21 DIV (p < 0.05, Mann-Whitney U test, Fig. 5H), confirming that Np65 is required for the maintenance of excitatory but not inhibitory contacts.

**Acute Treatment with Anti-Np65 Antibodies Differentially Affects Excitatory Versus Inhibitory Presynaptic Function**—As Np65 is acutely required for maintaining the structural ratio of excitatory to inhibitory synapses (Fig. 5), it is possible that Np65 is also involved in maintaining the correct excitatory to inhibitory synaptic function. To test this hypothesis we evaluated the spontaneous presynaptic activity, i.e. recycling of synaptic vesicles driven by endogenous network activity in the neural cultures. This was reported by the uptake of a Cy3-conjugated antibody against the luminal domain of synaptotagmin (24) in living mature hippocampal neurons where endogenous Np65 was acutely targeted. Therefore we preincubated neurons with an isoform-specific anti-Np65 antibody (see Fig. 8A), a manipulation known to block Np65 function (20). After the uptake assays, cells were fixed and stained for presynaptic markers to discriminate between VIAAT-positive inhibitory presynapses and VIAAT-negative synapsin-positive excitatory presynapses. Preparations were photographed using a confocal microscope to distinguish somatic and dendritic compartments (Fig. 6, A and C). In the somata, the fluorescence intensity of the endocy-
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FIGURE 7. Impaired excitatory and inhibitory synaptic transmission between Np-deficient hippocampal neurons. Np\(^+\) and Np\(^{-/-}\) neurons were impaled under whole cell configuration patch clamp and synaptic activity was recorded. A, representative traces of mEPSCs are shown. B, quantification of amplitudes of excitatory events versus mean cumulative fraction and the mean amplitude (inset) in Np\(^{-/-}\) (11 cells from 3 independent cultures, 11/3) and Np\(^{-/-}\) neurons (13/3). C, the mean mEPSC frequency (Hz) was calculated from the data set in B. D, traces of GABA\(_A\)-mediated mIPSCs corresponding to an average of 100 events from representative cells are shown. Mean cumulative fraction and mean amplitudes (E) as well as frequency of mIPSCs (F) were calculated from 10 wild-type and 9 mutant cells from 3 independent cultures. **, \(p < 0.01\), or *, \(p < 0.05\) between genotypes.

Np\(^{-/-}\) neurons (Fig. 7B). The mean amplitude for Np\(^{+/+}\) neurons was 15.33 ± 1.15 pA, but only 10.27 ± 0.46 pA for Np\(^{-/-}\) neurons (13 Np\(^{+/+}\) and 11 Np\(^^{-/-}\) neurons from 3 independent cultures, \(p < 0.01\), Student’s \(t\) test, inset in Fig. 7B). The frequency of synaptic events was 0.45 ± 0.37 Hz for Np\(^{+/+}\) neurons, but only 0.31 ± 0.05 Hz for Np\(^{-/-}\) neurons (\(p < 0.05\), \(t\) test, Fig. 7C). Kinetic parameters such as the rise time or decay time (\(\tau_{\text{decay}}\)) were undistinguishable between mutant (rise time = 0.51 ± 0.05 ms; \(\tau_{\text{decay}} = 3.92 ± 0.33\) ms) and wild-type cells (rise time = 0.55 ± 0.04 ms; \(\tau_{\text{decay}} = 4.45 ± 0.37\) ms). Thus, in good agreement with the anatomical data, spontaneous excitatory synaptic transmission events are reduced in both amplitude and number in Np\(^{-/-}\) compared with Np\(^{+/+}\) neurons.

We then studied GABA\(_A\)-mediated mIPSCs (representative traces in Fig. 7D) in Np\(^{-/-}\) and Np\(^{+/+}\) neurons at 17–24 DIV. Although there was no change in the number and alignment of inhibitory synapses (see above), the amplitude of the mIPSCs was increased by some 24% in Np\(^{-/-}\) compared with Np\(^{+/+}\) neurons (Fig. 7, D and E), whereas their frequency was unaffected (Fig. 7F). Surprisingly, \(\tau_{\text{decay}}\) was significantly reduced from 29.35 ± 2.28 in Np\(^{+/+}\) to 22.64 ± 1.12 in Np\(^{-/-}\) neurons (10 Np\(^{+/+}\) and 9 Np\(^{-/-}\) neurons from 3 independent cultures, \(p = 0.0198\), Student’s \(t\) test), whereas the rise time of 0.93 ± 0.29 in Np\(^{+/+}\) neurons remained unchanged at 0.85 ± 0.31 in Np\(^{-/-}\) neurons (\(p = 0.4902\), Student’s \(t\) test). Thus, inhibitory synaptic currents mediated by the GABA\(_A\)-R are perturbed in Np\(^{-/-}\) neurons.

Reduction of GABA\(_A\) Receptor \(\alpha_2\) Subunit in Inhibitory Synapses of Np-deficient Neurons—Because 1) loss of neuropoatin produces perturbed inhibitory synaptic currents (Fig. 7), 2) Np65 is normally expressed in glutamic acid decarboxylase (GAD65)-positive GABAergic axons (Fig. 8B), and 3) Np65 co-localizes with the GABA\(_A\)- receptor \(\alpha_2\) subunit at inhibitory synapses in hippocampal neurons (22), we tested whether the localization of \(\alpha_2\) subunits is altered in the mutant neurons. We assessed whether GABA\(_A\)-R \(\alpha_2\) subunits co-localize with Np65 on the surface of living neurons using an isoform-specific anti-Np65 antibody for native immunostaining (Fig. 8A), and we observed a robust co-localization of Np65 with GABA\(_A\)-R \(\alpha_2\) subunits on the surface of dendrites (Fig. 8C). Immunostaining and confocal microscopy revealed a clearly decreased matching between postsynaptic GABA\(_A\)-R \(\alpha_2\) subunits and presynaptic VIAAT on dendrites but not in the somata of Np\(^{-/-}\) compared with Np\(^{+/+}\) neurons at 7–12 DIV (Fig. 8D). On dendrites of Np\(^{+/+}\) neurons 23.3 ± 3.4% of the total \(\alpha_2\) subunit-associated immunoreactivity was detected co-localizing (below optic resolution distance) with VIAAT. This is in good agreement with previous reports (38). In contrast, GABA\(_A\)-R \(\alpha_2\) subunit staining was much more diffuse on dendrites of Np\(^{-/-}\) neurons where only 8.9 ± 2.9% was found co-localized with VIAAT (100 neurons from 4 independent experiments, \(p < 0.01\), Mann-Whitney \(U\) test, Fig. 8D). To assess the synaptic location of \(\alpha_2\) subunits on somata and dendrites, we calculated separately the \(\alpha_2\) to VIAAT match as described above for synaptic markers in these neuronal compartment (box size for matching = 0.875 \(\mu\)m). In dendrites, Np\(^{+/+}\) neurons displayed an \(\alpha_2\) to VIAAT match of 72.1 ± 4.9%, whereas this parameter was only 49.2 ±
5.2% for Np"/" neurons (p < 0.05, Mann-Whitney U test, Fig. 8D). In contrast, the somatic α2 to VIAAT match was not statistically different between Np"/" and Np"+/+ neurons (Fig. 8D). Thus, Np deficiency produces partial loss of synaptic location of the GABA<sub>A</sub>R α2 subunits confirming that Np65 is involved in their positioning at inhibitory synapses.

Increased α1/α2 Ratio in Larger Inhibitory Postsynapses in Np"/" Neurons—To further understand why GABA<sub>A</sub>R currents are perturbed in Np"/" neurons, we performed live-cell co-immunostaining of GABA<sub>A</sub>R α2 and α1 subunits, before fixation and permeabilization and subsequent staining for gephyrin. To extend the analysis of the GABA<sub>A</sub>R composition to somatic and dendritic inhibitory postsynapses in Np"+/+ and Np"/" neurons at 12 (Fig. 9A) and 21 DIV (Fig. 9B, I and II), we applied confocal microscopy. Although the fluorescence intensity of somatic gephyrin puncta was statistically higher in Np"/" compared with Np"+/+ neurons at 12 DIV (44.9 ± 1.4 arbitrary units for Np"/" and 38.1 ± 1.2 arbitrary units for Np"+/+, p < 0.05, ANOVA, Fig. 9C), no differences were found for this parameter at 21 DIV (Fig. 9D). At 12 DIV, in the somatic compartment, Np"+/+ neurons displayed 180.1 ± 21.1 clusters of gephyrin per somata including 10 µm of the initial segment of primary dendrites although this parameter was 204.5 ± 17.9 for Np"/" neurons (45 neurons, 3 independent cultures, p = 0.405, ANOVA, Fig. 9C). Np"+/+ neurons displayed 120.2 ± 35.1 and Np"/" neurons 136.4 ± 17.2 gephyrin-positive clus-
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C. 12 DIV

D. 21DIV

E.I. 12 DIV

E.II. 21 DIV
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At 12 DIV, we confirmed that α2, but not α1, is diminished in gephyrin-positive inhibitory postsynapses in dendrites, but not in soma, and at 21 DIV in both somata and dendrites of cultured Np+/− neurons (Fig. 9, A and B, and data not shown). This supports the results shown here in Fig. 8 as well as published data (22). Furthermore, we quantified the α1- and α2-associated fluorescent intensity in postsynaptic sites and calculated the α1/α2 intensity ratio (ratio α1/α2) for Np+/− and Np+/+ neurons at 12 (Fig. 9E, I) and 21 DIV (Fig. 9E, II). This parameter includes gephyrin-positive postsynapses where the GABAAR α2 subunit is still present, but not those where α2-associated fluorescence is completely absent (see arrows in Figs. 8C, and 9, B, I and II). At 12 DIV, Np+/− and Np−/− neurons displayed a similar somatic α1/α2 ratio of 0.96 ± 0.04 and 0.94 ± 0.03, respectively (45 neurons, 3 independent cultures, p = 0.798, Mann-Whitney U test, Fig. 9E, I). In dendrites, the α1/α2 ratio was 1.07 ± 0.06 and 0.71 ± 0.16 for Np+/+ and Np−/− neurons, respectively (p < 0.05, Mann-Whitney U test, Fig. 9E, II). Importantly, the α1/α2 ratio was increased in both somata and dendrites of Np−/− compared with Np+/+ at 21 DIV. For 21 DIV Np+/+ neurons the α1/α2 ratios were 1.15 ± 0.5 and 0.95 ± 0.09, whereas for Np−/− neurons the ratios were 1.59 ± 0.31 and 1.36 ± 0.06 in soma and dendrites, respectively (p < 0.05, Mann-Whitney U test, Fig. 9E, II). Thus, Np deficiency increases the relative abundance of α1 as compared with α2 subunits in inhibitory postsynapses.

DISCUSSION

Here we show that Np deficiency leads to a selective structural destabilization of glutamatergic but not GABAergic synapses. Although Np−/− neurons showed an altered localization of GABA receptorsα2 subunits, we observed no change either in number or matching of post-synaptic inhibitory compartments. The ratio of glutamatergic versus GABAergic synapses was significantly perturbed, and the electrophysiological studies confirmed that transmission at both inhibitory and excitatory synapses is altered as a consequence of Np deficiency. Acute incubation of mature wild-type neurons with recombinant Np65-Fc, but not Np55-Fc mimicked the mutant phenotype at excitatory synapses. Moreover, incubation with an isofrom-specific anti-Np65 antibody perturbed excitatory, but not inhibitory presynaptic function as evaluated by synaptotagmin uptake assay. Acute Np65-mediated synaptic stabilization could be assigned predominantly to CA1 and DG, but not CA3 neurons. Although Np65 is present in GABAergic terminals, our data argue for a more restricted and acute role for the trans homophilic binding of Np65 in the structural stability of excitatory synapses.

Np65 in Hippocampal Circuits—We show that Np65 is predominantly expressed in CA1 and DG fields of hippocampal slices, which is in good agreement with previous data (20, 23). The number of glutamatergic synaptic contacts was found to be reduced in the CA1 and DG, but not in the CA3 area, of Np-deficient mice. Thus, the reduction in synaptic connections resulting from the mutation occurs in the hippocampal fields where normally Np65 is abundant. Mimicking the in vivo situation, dissociated hippocampal neurons with a CA1 or DG signature expressed the highest levels of Np65 compared with other hippocampal neurons, and in cultures obtained from Np−/− mice these neurons displayed the most severe loss of glutamatergic synaptic contacts. Accordingly, the expression of Np65 by CA1 and DG neurons seems to be required for the establishment of both the correct number and function of glutamatergic synapses in specific hippocampal circuits. Similarly another single-pass transmembrane CAM, Cadherin-9, a prototypical class II cadherin, is required specifically for formation of DG to CA3 but not CA1 synapses in culture and in hippocampal slices loss of cadherin-9 disrupts mossy fiber bouton and thorny excrescence spine formation through trans-synaptic interactions (8). We therefore propose that, as for Cadherin-9 and others (9), Np65 is one such differentially expressed molecular cue, which contributes to the specific connection of excitatory hippocampal synapses (10).

Np65 Stabilizes Excitatory Synapses—At 12 DIV, we found no change in the number of newly formed excitatory contacts but more mismatched postsynaptic sites, and thus a decreased post- to presynaptic matching in Np−/− neurons. After 21 DIV, when synaptic contacts have matured the hallmark finding is that Np−/− neurons display less excitatory synapses, whereas the number of inhibitory contacts is unchanged, resulting in a decreased ratio of excitatory to inhibitory contacts.

Features observed in Np−/− neurons were mimicked by acute application of recombinant Np65-Fc, but not Np55-Fc, to mature wild-type neurons. Thus, we infer that the mutant phenotype reveals a direct acute function of Np65 in stabilization of excitatory synaptic contacts. Np65-Fc, but not Np55-Fc, predominantly attaches to the surface of CA1 and DG neurons.

FIGURE 9. Enlarged postsynaptic sites and altered synaptic α2 to α1 GABAAR receptor subunit ratio of Np−/− neurons. Np+/− and Np−/− neurons were cultured for 12 (A, C, and E, panel I) and 21 DIV (B, D, and E, panel II), and stained for gephyrin (green, gray, α2 (cyan), and α1 (magenta) GABAAR subunits. Somata (A and B, panel I) and dendrites (A and B, panel II) were independently photographed using a confocal microscope and analyzed for the number, size, and fluorescence intensity of gephyrin puncta. E, the content of α1 and α2 in gephyrin-positive postsynapses (α1/α2 ratio per gephyrin punctum) was calculated using the fluorescence intensities of these GABAAR subunits co-localizing with gephyrin clusters. Representative pictures and data (mean ± S.E.) are from 3 independent experiments. Scale bar in A and B, panel I = 20 μm, and in B, panel II = 2 μm. **, p < 0.01, or *, p < 0.05 between genotypes.
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coinciding with the high level of endogenous Np65 in these neuronal types, indicating that endogenous Np65 can act as a receptor of Np65-Fc. This is consistent with Np65, but not Np65-containing glutamate receptors from the synaptic membrane (23). Although we observed some attachment of Np65-Fc to the neuronal surface even in Np65−/− neurons this is not directly responsible for synaptic destabilization as no further synaptic mismatch was induced by treatment of Np65−/− neurons with Np65-Fc. Thus, competition with Np65-Fc could rapidly destabilize pre-existing Np65-containing complexes producing impairment of synaptic function ultimately leading to the loss of functional and structural integrity of excitatory synapses. Consistent with this hypothesis, failed synapse stabilization can be responsible for a lower number of postsynaptic sites (39). Although our experiments argue for an acute function for Np65 at excitatory synapses, they do not fully exclude a potential involvement of extra-synaptic neuroplastin that would further contribute to the mutant phenotype.

Using F-actin we observed a lower number of postsynapses in Np65−/− compared with Np65+/+ neurons (Fig. 2C). Moreover F-actin seemed to be abnormally accumulated on the dendritic shaft of the mutant neurons. Actin is the major cytoskeletal component of excitatory spines, and it has been shown that the small RhoA GTPase is a key participant in the mechanism controlling actin polymerization (40), a plausible hypothesis is that Np65 regulates the RhoA activation/deactivation cycle to modulate actin polymerization and thus spine formation/stabilization.

Np65 at Inhibitory Synapses—GABAergic synapses containing α2-subunits co-localize with Np65 at the plasma membrane in rat hippocampal neurons. In Np65−/− neurons, more α2-immunoreactivity was detected outside VIAAT-positive synapses compared with wild-type indicating that Np65 may be required for the correct localization of α2-containing receptors at inhibitory synapses. Consistent with this result it has been suggested that Np65 may play a role in the localization of GABAergicRs to synapses (22). Here, we show that number and matching of VIAAT to gephyrin-positive synaptic contacts remained normal in Np65−/− neurons and Np65-Fc-treated mature Np65−/− neurons, indicating that the Np65−/−GABAergicRs α2-subunit association may not be crucial for maintenance of inhibitory synapses. In the same direction, a change in GABAergicRs composition or function is not necessarily accompanied by structural changes of inhibitory synapses (41).

Importantly, we found that inhibitory transmission is impaired in Np65−/− neurons in which IPSCs exhibit both increased amplitude and accelerated decay time. These results support a role for Np65 in modulation of GABAergicRs activity, most probably by coordinating proper α2 subunit-containing GABAergicRs localization at inhibitory synapses. Indeed, we observed delocalization of the α2 subunit and an increased synaptic α1/α2 ratio in Np65−/− neurons. The α2 subunit contributes to the slow decaying of GABAergicRs-mediated IPSCs as concluded from antisense-mediated α2 subunit down-regulation. This in turn results in up-regulation of α1-containing receptors in synapses and thereby accelerating kinetic decay (42). Indeed, α1 subunits can substitute the α2 subunits in hippocampal neurons from α2-deficient mice (43). Thus, we conclude that Np65 deficiency leads to α2 subunit delocalization thereby switching the composition of GABAergicRs to a more α1-enriched receptor population. It has been reported that altered GABAergicRs subunit protein levels, abnormal synaptic location, and modified GABAergicRs composition impair inhibitory transmission as observed in α1 (44, 45), α2 (43), and α3 null mice (46). These data suggest that interference with the expression of one specific GABAergicRs α-subunit may produce complex compensatory changes by the others.

Is Np65 a Regulator of the Ratio and Function of Excitatory and Inhibitory Synapses?—Our data suggest that Np deficiency alters the number ratio and the functionality of glutamatergic and GABAergic synapses by distinct and multiple mechanisms. It has been shown that, at 7 DIV when still excitatory synapses are not yet developed, α2-subunit delocalization from GABAergic contacts occurs in anti-Np65 shRNA-transfected rat hippocampal neurons (22). Thus, we conclude that Np deficiency is directly responsible for the phenotype at GABAergic synapses in Np65−/− neurons. However, an interaction of the observed GABAergic phenotypes yielding impaired excitatory synapses in Np65−/− neurons cannot be excluded. For instance, it is known that early during neuronal network formation GABAergic synapses can be excitatory (48, 49). Therefore, it is possible that the altered composition of GABAergicRs may secondarily affect the development of glutamatergic synapses in Np65−/− neurons. The observation that acute interference with Np65 function in excitatory synapses without altering GABAergic synapses adding Np65-Fc or anti-Np65 antibodies (Ref. 20 and 23 and this report) on Np65+/+ neurons mimics the glutamatergic synaptic phenotype of Np65−/− neurons (this report) argues for independent functions of Np65 in glutamatergic and GABAergic synapses.

Our data indicate that Np65 might be involved in the determination of the number of excitatory and inhibitory synapses in the adult brain. In this context, Np65 shows fundamental differences to neuroligins that have been reported as key regulators of the neuronal excitability by regulating E/I balance. First, heterophilic binding of postsynaptic neuroligins to presynaptic neuroligins recruits intracellular scaffolds (11, 13, 47). In contrast, postsynaptic Np65 may bind presynaptic Np65 in a homophilic fashion without direct recruitment of synaptic scaffolding proteins as its intracellular domain lacks any known motif able to bind these intracellular proteins. Alternatively, Np65 may structure synapses by organizing synaptic membrane protein complexes, i.e. GluA1-containing glutamate receptors (23), GABAergicRs receptor subunits (22), and others. Second, Np65 acts at a later stage of development and may therefore be implicated in stabilization and maintenance of synapses rather than in their formation as proposed for neuroligins (12, 13). Thus, Np65 function is not redundant but adds a new facet to the proper E/I ratio in neuronal circuits.

Potential Roles for Np65 in the E/I Balance and Brain Function—Disturbed E/I balance in specific brain areas, i.e. the hippocampal CA1 region, is a key hypothesis to explain the
development of schizophrenia in patients (50, 51). Interestingly, both Np as well as the GABA_A R subunit α2 have been proposed to play a role in schizophrenia (38, 52). Thus, Np65 is an attractive candidate to mediate the correct balance in specific brain areas whose dysfunction might be relevant for mental pathologies.

In summary, we show that Np65 regulates structural stability and contact of excitatory synapses and the localization of GABA_A R α2 subunits at inhibitory synapses. Our results strongly support a role for Np65 not only in establishing and maintaining of specific hippocampal connections but also in regulating the correct ratio of excitatory to inhibitory synaptic activity. As Np65 is implicated in synaptic plasticity and memory formation (16), it will be interesting to relate Np65-mediated structural stability of synapses and its role in maintaining the correct neuronal network activity in specific brain regions to the complex phenotype displayed by Np-deficient mice.

Acknowledgments—We gratefully acknowledge the assistance of Karla Sowa, Daniela Hill, and Soumeet Bhattacharya for providing mice. One of us (R. H-M.) thanks A.-C. Lehmann for critical comments and Dr. Angela Kolodziej for valuable help in staining of mouse sections.

Addendum—After submitting our article for review, we learned of an article to be published by Desrivières et al. (53). This new study provides evidence for a critical role of neuroplastin in individual intellectual differences and further supports the association of neuroplastin with schizophrenia. Furthermore, in human neuroplastin, as a target of FOXP2 (54), may be linked to speech development. Altogether the new data further support important functions of neuroplastin in the human brain. Our data, describing at the synaptic level the functional role of neuroplastin, are thus in our view of high interest to a broad readership.

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