Neuroligin-1 regulates excitatory synaptic transmission, LTP and EPSP-spike coupling in the dentate gyrus in vivo

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Abstract Neuroligins are transmembrane cell adhesion proteins with a key role in the regulation of excitatory and inhibitory synapses. Based on previous in vitro and ex vivo studies, neuroligin-1 (NL1) has been suggested to play a selective role in the function of glutamatergic synapses. However, the role of NL1 has not yet been investigated in the brain of live animals. We studied the effects of NL1-deficiency on synaptic transmission in the hippocampal dentate gyrus using field potential recordings evoked by perforant path stimulation in urethane-anesthetized NL1 knockout (KO) mice. We report that in NL1 KOs the activation of glutamatergic perforant path granule cell inputs resulted in reduced synaptic responses. In addition, NL1 KOs displayed impairment in long-term potentiation. Furthermore, field EPSP-population spike (E-S) coupling was greater in NL1 KO than WT mice and paired-pulse inhibition was reduced, indicating a compensatory rise of excitability in NL1 KO granule cells. Consistent with changes in excitatory transmission, NL1 KOs showed a significant reduction in hippocampal synaptosomal expression levels of the AMPA receptor subunit GluA2 and NMDA receptor subunits GluN1, GluN2A and GluN2B. Taken together, we provide first evidence that NL1 is essential for normal excitatory transmission and long-term synaptic plasticity in the hippocampus of intact animals. Our data provide insights into synaptic and circuit mechanisms of neuropsychiatric abnormalities such as learning deficits and autism.

Keywords Dentate granule cells · Perforant path · Excitability · Synaptic plasticity · Paired-pulse inhibition · Cell adhesion molecule

Introduction

Neuroligin-1 (NL1) is a cell adhesion protein of the neuroligin family. There are four neuroligin proteins (NL1, 2, 3, 4) that transsynaptically bind to presynaptic neurexins (Ichtchenko et al. 1996). NL1 is a postsynaptic transmembrane molecule that is targeted specifically to excitatory synapses (Song et al. 1999). There is now a considerable body of evidence indicating that NLs play a critical role in the formation, maintenance and function of synapses (Knight et al. 2011; Krueger et al. 2012; Südhof 2008). Intriguingly, mutations in NL genes have been associated with autism and other neuropsychiatric disorders (Bourgeron 2009; Südhof 2008). Therefore, understanding the role of NLs in the brain is important not only for a fundamental understanding of synaptic physiology but also for elucidating the pathophysiology of cognitive deficits (Bourgeron 2009).

Consistent with its synaptic localization, overexpression of NL1 has been shown to increase the number of excitatory synapses and their responses in cultured neurons.
(Chih et al. 2005; Chubykin et al. 2007; Prange et al. 2004). Somewhat unexpectedly, subsequent studies on NL1 deletion in mice revealed that NL1 is not essential for synaptogenesis as the loss of NL1 does not lead to reduced synapse numbers (Blundell et al. 2010; Varoqueaux et al. 2006). However, suppression of NL1 expression has been demonstrated to decrease AMPA receptor excitatory post-synaptic currents (EPSCs), NMDA receptor EPSCs and the NMDA/AMPA current ratio (Budreck et al. 2013; Chubykin et al. 2007; Mondin et al. 2011; Shipman et al. 2011). Conversely, enhanced expression of NL1 in transgenic mice has been reported to increase excitatory transmission (Dahlhaus et al. 2010; Ko et al. 2009; Schnell et al. 2012; see also Chubykin et al. 2007). In line with the decrease in the NMDA/AMPA ratio, deletion of NL1 impaired long-term potentiation (LTP) at thalamic (but not cortical) inputs to the amygdala (Jung et al. 2010; Kim et al. 2008) and at Schaffer collateral inputs to the CA1 area of the hippocampus in vitro (Blundell et al. 2010; see also Shipman and Nicoll 2012). The alterations in LTP were also accompanied by deficits in learning and memory (Blundell et al. 2010; Kim et al. 2008). Similarly, learning deficits and impaired synaptic plasticity have been observed following NL1 overexpression in mice (Dahlhaus et al. 2010).

Thus, several lines of evidence support the relevance of NL1 in excitatory synapse stabilization and maturation. Based on these in vitro and ex vivo studies, NL1 has been suggested to play a selective role in the function of excitatory synapses. However, since all previous electrophysiological analyses have been performed in cultured neurons or acute slices, it has remained unclear whether NL1 is essential for synaptic function in neuronal circuits of live animals. In addition, little is known about potential region or pathway-specific effects of NL1. We studied the effects of NL1 deficiency on synaptic transmission in the dentate gyrus using recordings of field potentials evoked by perforant path stimulation in anesthetized NL1 knockout (KO) mice. Our data indicate that NL1 is required for normal excitatory transmission and LTP in the dentate circuit in vivo.

Methods

Surgery and electrophysiology

Experiments were performed on 2- to 3-month-old adult wild type (WT) and NL1 knockout (KO) littermate mice from heterozygote interbreedings (Chubykin et al. 2007; Varoqueaux et al. 2006; Wittenmayer et al. 2009). Experiments were carried out in accordance with German laws governing the use of laboratory animals. Analyses were performed on male age-matched littermates. Electrophysiological procedures to characterize NL1 KO and WT mice were carried out as described before (Jedlicka et al. 2009, 2011a, b). Briefly, mice were anesthetized with an intraperitoneal injection of urethane (Sigma-Aldrich, St. Louis, MO, USA; 1.2 g/kg body weight, supplemental doses of 0.3–1 g/kg s.c. as needed). The body temperature of mice was constantly monitored through a rectal probe and maintained at 37 °C with a heating pad. The animal was placed in a stereotactic frame for the insertion of electrodes. For local anesthesia, procainhydrochloride with adrenalin 1:200,000 (Xylonest 1 %, AstraZeneca, Germany) was injected subcutaneously into the tissue surrounding the incision prior to surgery. Holes were drilled in the skull and after removal of the dura mater a bipolar stimulation electrode (NE-200, 0.5 mm tip separation, Rhodes Medical Instruments, USA) was positioned in the angular bundle of the perforant path (3.7 mm posterior to bregma, 2.5 mm lateral to midline, 1.8 mm from the brain surface). A tungsten recording electrode (TM33A10KT, World Precision Instruments, Sarasota, FL, USA) was placed in the granule cell layer of the dentate gyrus (1.7 mm posterior to bregma and 1 mm lateral to midline; 1.7 mm approximate depth from the brain surface). The recording electrode was lowered into the brain in 0.05–0.1 mm increments while monitoring the laminar profile of the response waveform evoked by a 500 μA test stimulus applied at 0.1 Hz until the waveform indicated that the recording electrode had reached the granule cell layer, usually 1.6–2.0 mm from the brain surface. Recordings and stimulations were made in the granule cell layer of the dentate gyrus and in the perforant path, respectively. Current pulses (30–800 μA, 0.1 or 0.2 ms duration) were generated using a stimulus generator STG1004 (Multichannel Systems, Reutlingen, Germany). Recorded granule cell field potentials were amplified with a Grass AC P55 preamplifier (Grass Technologies, West Warwick, RI, USA), and digitized at 10 kHz with a Digidata 1440A interface (Molecular Devices, Union City, CA, USA) for offline-analysis. Electrophysiological data were analyzed using Clampfit 10.2 (Molecular Devices, Union City, MA, USA). Stimulus–response relationships were determined with stimulation intensities ranging from 30 to 800 μA (0.1 ms pulse width). Three responses at each stimulus intensity were collected and averaged. The amplitude of the population spike was defined as the average of the amplitude from the first positive peak (a) to the first negative peak (b) and the amplitude from the negative peak (b) to the second positive peak (c); \[ (a - b) + (c - b) \right/2. For the analysis of the slope of fEPSPs, only the early component of the waveform was measured to avoid contamination by the population spike. To quantify EPSP–population spike (E–S) coupling, the E–S curve...
from each measurement was fitted with the Boltzmann function. Only curves with the goodness of fit \( R^2 \) larger than 0.8 were further analyzed and the v50 value (i.e., the slope eliciting half-maximal spike amplitude) was calculated. To measure paired-pulse facilitation (PPF) of the field excitatory postsynaptic potential (fEPSP) amplitude, two pulses were applied at intensities below population spike threshold, with interpulse intervals ranging from 15 to 100 ms. To study paired-pulse inhibition and disinhibition (PPI/PPDI) of the population spike, strong (800 μA/0.2 ms) and weak stimulation intensities (evoking 1.5–2 mV population spikes; 120–290 μA/0.2 ms) were used (interpulse intervals 15–1,000 ms). PPI/PPDI curves were fitted using the Boltzmann function to obtain the mean interpulse interval at which equal amplitudes of the first and second population spike could be observed. LTP was induced by strong theta-burst stimulation (TBS), i.e., six series of six trains of six stimuli at 400 Hz, with 200 ms between trains and 20 s between series (Cooke et al. 2006; Jedlicka et al. 2009; Jones et al. 2001), or by weak TBS, i.e., three series of six trains of six stimuli at 400 Hz, with 200 ms between trains and 20 s between series followed by the strong (“six series”) TBS 30 min after the weak TBS. Pulse width and stimulus intensity was doubled in comparison to baseline recordings. Baseline fEPSP slopes were calculated from the average of responses over the 10 min prior to TBS. Baseline stimulus intensity was set to evoke a population spike of approximately 1.5 mV before LTP induction. The potentiation of the fEPSP slope was expressed as a percentage change relative to the baseline. Only mice that showed successful LTP induction and provided a stable recording throughout the experiment were included in the analysis. All statistical analyses were performed using the software GraphPad Prism 5.03 for Windows. Electrophysiological data were tested for statistical significance using unpaired Student’s \( t \) test or two-way analysis of variance (ANOVA) with Bonferroni’s multiple-comparison post-tests as specified in the text. A two-tailed \( p \) value lower than 0.05 was considered to be significant. Group values are reported as mean ± SEM.

Preparation of hippocampal synaptosomal fractions and Western blot analysis

Synaptosomal fractions were prepared according to a protocol modified from Carlin et al. (1980). Mice were sacrificed by rapid decapitation, brains were removed, and hippocampi were dissected out on ice. All further steps were performed at 4 °C. Hippocampi were homogenized in solution A (0.32 M sucrose, 1 mM MgCl₂, 0.5 mM CaCl₂, 1 mM HEPES, pH 7.4, containing freshly added protease inhibitor cocktail) using a motor-operated Teflon/glass homogenizer (Potter S, Sartorius, Göttingen, Germany) with 12 strokes at 900 rpm. Homogenates were centrifuged for 10 min at 3,000 rpm (approx. 1,000 × g), and supernatants were saved. Pellets were resuspended in solution A, re-homogenized with 3 strokes at 900 rpm, and once again centrifuged for 10 min at 3,000 rpm. The supernatants from the two centrifugation steps were pooled and centrifuged for 10 min at 10,000 rpm (approx. 10,000 × g). Pellets were resuspended in solution B (0.32 M sucrose, 1 mM HEPES, pH 7.4 containing freshly added protease inhibitor cocktail, EMD Biosciences, Darmstadt, Germany) with 4–5 strokes at 900 rpm and layered over a sucrose gradient consisting of 0.85 M/1.0 M/1.2 M sucrose layers (each containing 1 mM HEPES and protease inhibitor cocktail). Gradients were centrifuged for 2 h at 21,600 rpm (approx. 82,500 × g) using an SW 40 Ti rotor (Beckman Coulter, Krefeld, Germany). The band at the interface between 1.0 and 1.2 M sucrose containing the synaptosomes was collected. SDS was added to a final concentration of 1 %, and lysed synaptosomes were stored at −80 °C until further processing.

For immunoblot analysis, protein concentration was assessed using a BCA assay (Thermo Scientific, Waltham, MA, USA), and 1–5 μg total protein per sample was used for immunoblotting. Samples were boiled in 1 × loading buffer (2 % SDS, 62.5 mM Tris, 10 % glycerol, 1 % β-mercaptoethanol, 0.01 % bromophenol blue, pH 6.8) for 5 min, resolved on 10 % SDS-PAGE gels, transferred onto nitrocellulose membranes, and stained for total protein using a Memcode assay (Thermo Scientific, Waltham, MA, USA). Membranes were blocked in 50 % LiCor blocking buffer (LiCor Biosciences, Lincoln, NE, USA) in PBS for 1 h, and were subsequently incubated in primary antibody in 1 % antibody dilution buffer (50 % LiCor blocking buffer and 0.1 % Tween-20 in PBS) at 4 °C overnight. The following primary antibodies were used: PSD-95 (NeuromAB, Davis, CA, USA), GluA1, GluN2B (both from Chemicon, Temecula, CA, USA), GluN2A (Millipore, Billerica, MA, USA), GluA2, GluN1, VGlut1, Gephyrin (all from Synaptic Systems, Göttingen, Germany) and actin (Sigma-Aldrich, St. Louis, MO, USA). Membranes were washed 4 × in PBS with 0.1 % Tween-20, then incubated with secondary antibody (Goat anti-M-IRDye800 and Goat anti-Rb-IRDye680, Licor Biosciences, Lincoln, NE, USA., and Goat-anti-GP-IRDye700, Rockland Immunochemicals, Gilbertsville, PA, USA) in 2 % antibody dilution buffer (50 % LiCor blocking buffer, 0.1 % Tween-20 and 0.01 % SDS in PBS) for 1 h at 4 °C. Blots were washed as above, scanned on an Odyssey Infrared Imager (LiCor Biosciences, Lincoln, NE, USA), and the signal intensity for each sample was quantified using the Odyssey 2.1 software. Each sample value was divided by the total protein loading value for the corresponding lane, and then normalized to the average sample value of all lanes on the same blot to

\[ \text{Baseline fEPSP} \]

\[ \text{Baseline stimulus} \]

\[ \text{Pulse width} \]

\[ \text{Interpulse intervals} \]

\[ \text{Strong stimulation intensities} \]

\[ \text{Weak stimulation intensities} \]

\[ \text{Gradient} \]

\[ \text{SDS-PAGE} \]

\[ \text{Nitrocellulose membranes} \]

\[ \text{Total protein} \]

\[ \text{Primary antibodies} \]

\[ \text{Secondary antibodies} \]

\[ \text{Odyssey 2.1 software} \]

\[ \text{Normalization} \]
correct for blot-to-blot variance. Data were tested for statistical significance using the Wilcoxon signed-rank test. A two-tailed p value lower than 0.05 was considered to be significant. Data are expressed as percent WT (mean ± SEM).

Immunohistochemistry

Mice were transcardially perfused with a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4. Brains were removed and postfixed up to 18 h in 4% paraformaldehyde in 0.1 M PBS. Serial frontal sections of the hippocampus (50 μm) were cut with a vibratome, washed in PBS, and stored at -20°C in cryoprotection solution (30% ethylene glycol, 25% glycerin in PBS). After a blocking step to reduce non-specific staining (for 1 h at room temperature in 5% bovine serum albumin), free-floating sections were incubated for 48 h at room temperature in primary antibody solution, containing 2% bovine serum albumin, 0.25% Triton X-100, 0.1% NaN₃ in 0.1 M PBS. The following primary antibodies were used: Anti-GluA1 (rabbit, polyclonal, 1:500, Chemicon, Ctl. AB1504) and Anti-GluA2 (mouse, monoclonal, 1:500, Chemicon, Ctl. MAB397). For fluorescence-immunohistochemical detection, sections were incubated with secondary fluorescence-labeled antibodies (1:1,000; Alexa 568, Vector Labs., Burlingame, CA, USA) for 24 h at room temperature.

Histological quantification

For each animal, 24 z-stacks (5 images, interval 2 μm, starting at 10 μm below the surface) were acquired from 1 frontal section of the dentate gyrus (granular cell layer: 6 z-stacks; inner molecular layer: 6 z-stacks; outer molecular layer: 12 z-stacks) using a confocal microscope (Nikon Eclipse 80i) and a 60× oil immersion objective (N.A. 1.4), tenfold field zoom (image area 21.22 μm²). Mean fluorescence intensity of images was measured with ImageJ. For counting GluA1 and GluA2 clusters images were processed by application of the following commands in ImageJ (1.47b). Images were enhanced and filtered with “Subtract background” (rolling ball radius 20), “Remove outliers” (radius 2.0, threshold 50) and “Mean filter” (radius 2.0). Contrast of images was set to 150–700 for GluA1 and 100–700 for GluA2. Binary images were created with “Auto local threshold” (type Bernsen, radius 50) and further processed with the binary operations “Open” and “Watershed”. Clusters were then identified and counted by utilization of “Analyze particles” (size 0.05–2.00 μm², circularity 0.20–1.00). Data management and graphs were performed with Matlab R2010a and GraphPad Prism 6.02. Statistical comparisons were calculated with the nonparametric Kruskal–Wallis test and Dunn’s multiple-comparison test using GraphPad Prism 6.02. Significance level was set at p < 0.05. Group values are reported as mean ± SEM.

Results

Excitatory transmission at perforant path synapses

We first tested whether a lack of NL1 expression affects excitatory transmission in the dentate gyrus. To investigate synaptic transmission at excitatory perforant path-granule cell synapses, we recorded local field excitatory postsynaptic potentials (fEPSPs) evoked by stimulating the perforant path in anesthetized wild type (WT) and NL1 knockout (KO) mice. Since the slope of the fEPSP is a measure of excitatory synaptic strength, we analyzed fEPSP slopes across a range of stimulation intensities (30–800 μA; Fig. 1a). The analysis of stimulus–response curves revealed that the fEPSP slope was significantly reduced in NL1 KO mice as compared to their WT littermates. Two-way ANOVA with subsequent Bonferroni’s multiple-comparison tests revealed that fEPSPs of significantly reduced slope amplitudes were elicited by 225–800 μA stimulation intensities in NL1 KO mice as compared with NL1 WT mice (225, 275 μA: p < 0.05; 300, 350, 600 μA: p < 0.01; 400–550 and 650–800 μA: p < 0.001). Thus, excitatory synaptic transmission is reduced at perforant path synapses of NL1 mutant mice in vivo.

To examine the presynaptic properties of excitatory perforant path-granule cell synapses in the NL1 KO dentate gyrus, we measured presynaptic short-term plasticity in the form of paired-pulse facilitation of fEPSPs in NL1 WT and NL1 KO mice. Paired-pulse facilitation was determined as the ratio of two fEPSP amplitudes induced by two successive stimuli at 15–100 ms interpulse intervals (Fig. 1b). The stimulation intensity was subthreshold for the population spike to avoid the firing of granule cells and activation of their postsynaptic partners. We found no significant differences in paired-pulse facilitation between mutant and WT mice (two-way ANOVA with Bonferroni’s multiple-comparison tests; p > 0.05). These results indicate that NL1 deletion impairs excitatory transmission at perforant path inputs from the entorhinal cortex to the dentate gyrus without significantly changing their presynaptic function.

Dentate granule cell firing and fEPSP-spike coupling

Next, we studied whether the loss of NL1 affects the ability of granule cells to generate action potentials. For this purpose, we compared population spike amplitudes in NL1 KO mice and WT littermates, Fig. 2a). NL1 deletion did not lead
to pronounced changes in granule cell firing since no significant differences in population spikes were detected by Bonferroni’s multiple-comparison post-tests across the whole range of stimulation intensities ($p > 0.05$, two-way ANOVA). These data indicate that despite of their impaired input excitation, granule cells preserve their firing capability in the absence of NL1. This was also confirmed by fEPSP–population spike (E–S) coupling analysis. We plotted fEPSP slopes against population spike amplitudes (E–S plot, Fig. 2b; see “Methods”). Comparison of E–S plots revealed that in mutants, the course of EPSP-spike curves was significantly altered as shown by a leftward shift of the NL1 KO curve with respect to the WT curve. Quantification showed that the slope generating 50% of maximal spike amplitude ($v_{50}$) was significantly lower in NL1 KO mice as compared to WT animals ($n = 14$). $t$ test: **$p < 0.01$

Long-term synaptic plasticity in the dentate gyrus

Because changes in NL1 expression have been linked to synaptic plasticity deficits as revealed by recordings in acute slices (Blundell et al. 2010; Dahlhaus et al. 2010;
Shipman and Nicoll 2012), we set out to test LTP in NL1 KO and WT mice in our in vivo experiments. In the first set of experiments, we induced LTP using a strong theta-burst stimulation (sTBS) protocol consisting of six series of six trains of six pulses at 400 Hz, 200 ms between trains, 20 s between series (Fig. 3a). When comparing KO to WT animals, no significant difference was observed in the amount of LTP produced by this stimulation. Both mutant and KO mice showed strong potentiation of the fEPSP slope (1–10 min: $151.2 \pm 6.4\%$ in WT and $153.4 \pm 8.3\%$ in KO mice; $p > 0.05$, $t$ test; 51–60 min: $139.5 \pm 9.5\%$ in WT and 135.2 $\pm$ 4.6 $\%$ in KO mice; $p > 0.05$, $t$ test). Although sTBS-evoked LTP was normal in NL1 KO mice, we hypothesized that a combination of different stimulation protocols (c.f. Seabrook et al. 1999) might uncover more subtle deficits in synaptic plasticity in mutant animals. Therefore, we tested the effects of NL1 deletion on synaptic potentiation induced by a weak theta-burst stimulus (wTBS; 3 series of 6 trains of 6 pulses at 400 Hz, 200 ms between trains, 20 s between series) followed 30 min later by a strong theta-burst stimulus (sTBS). The comparison of KO and WT mice revealed an unchanged initial induction of LTP but a significant impairment in maintenance of synaptic potentiation (Fig. 3b, c) that was detectable already after the first weak stimulation in NL1 KO mice (21–30 min: $135.3 \pm 3.3\%$ in WT and $125.1 \pm 2.6\%$ in KO mice; $p < 0.05$, $t$ test). Reduced potentiation was further accentuated following subsequent sTBS (31–40 min: $165.5 \pm 3.4\%$ in WT and $145.8 \pm 3.6\%$ in KO mice; $p < 0.01$, $t$ test; 81–90 min: $149.7 \pm 5.1\%$ in WT and 132.2 $\pm$ 4.1 $\%$ in KO mice; $p < 0.05$, $t$ test). These results indicate that NL1 is involved in LTP regulation mechanisms in vivo.

Network inhibition

To assess network excitability in the hippocampus of NL1 KO mice, we measured paired-pulse inhibition (PPI) of the population spike. The PPI test is commonly used to estimate granule cell inhibition, mediated mainly by GABAergic interneurons in the dentate circuit (Jedlicka et al. 2010; Sloviter 1991). Double-pulse stimulation of perforant path inputs results in population spike inhibition at short interstimulus intervals (PPI) followed by disinhibition at longer intervals (paired-pulse disinhibition, PPDI). First, we used a strong double-pulse stimulation intensity of 800 $\mu$A/0.2 ms to recruit as much GABAergic inhibition as possible. This stimulation protocol revealed weaker PPI in NL1 knockout mice since their PPI/PPDI curve was shifted leftward in relation to the WT curve (Fig. 4a). The interpulse interval at which PPI turned to PPDI was significantly shorter in NL1 KO mice ($38.85 \pm 1\ ms$) as compared to their WT littermates ($43.75 \pm 1\ ms$; $p < 0.01$). Next, to determine whether
this difference was dependent on stimulus intensity, we measured the PPI of the population spike also at lower double-pulse stimulation intensities evoking 1.5–2 mV population spikes (see “Methods”). We did not find significantly reduced PPI in NL1 KOs (n = 5) relative to WTs (n = 6), although a trend toward diminished interpulse intervals at which PPI turned into PPDI was observed (KOs, 34.51 ± 0.98 ms; WTs, 37.33 ± 1.19 ms; p > 0.05, t test). These data indicate that the deletion of NL1 results in reduced inhibition and increased dentate network excitability in vivo.

Finally, we attempted to uncover the synaptic mechanisms underlying the alterations in synaptic transmission and changes in LTP in NL1 KOs. For this purpose, we determined expression levels of pre- and postsynaptic markers (VGlut1, PSD95, GluA1, GluA2, GluN1, GluN2A, GluN2B, gephyrin) in adult NL1 KO mice by quantitative Western blot analyses of synaptosome preparations (Fig. 5 and Suppl. information). Significantly reduced synaptic GluA2, GluN1, GluN2A and GluN2B protein levels were detected in synaptosomes from NL1 KO mice (GluA2: n = 15 pairs, p = 0.001; GluN1: n = 17 pairs, p < 0.05; GluN2A: n = 14 pairs, p < 0.05; GluN2B: n = 14 pairs, p < 0.01). These results indicate that decreased glutamatergic responses upon NL1 deletion are most likely attributable to reduced levels of synaptic AMPA and NMDA type glutamate receptors.

Immunohistochemical analysis revealed that wTBS followed by sTBS (c.f. Fig. 3b, c) induced a significant increase in the GluA2-immunofluorescence intensity and the number of GluA2 clusters in WT mice as compared to WT controls and sham operated mice (Fig. 6 and Fig. S1 in the Suppl. information), while there was no difference between theta-burst stimulated NL1 KO and naive NL1 KO-control mice. The increase was most pronounced in the outer molecular layer where perforant path synaptic contacts are located (Fig. 6), but was also present in the inner molecular layer and granule cell layer (Fig. S1 in the Suppl. information). In contrast, GluA1-immunofluorescence intensity and the number of GluA1 clusters within the dentate gyri were unaltered.
in WT and NL1 KO mice and showed no significant changes following application of wTBS and sTBS (Fig S2 in the Suppl. information). Taken together, our functional and immunohistochemical data indicate impaired recruitment of AMPA receptors following LTP induction in the NL1 KO dentate gyrus.

Discussion

The major findings of this study can be summarized as follows: 1. Deletion of NL1 results in a significant impairment of excitatory transmission in the dentate gyrus of live animals. 2. Decreased excitatory synaptic responses are compensated by reduced network inhibition and enhanced excitability of NL1 KO dentate granule cells. 3. The lack of NL1 interferes with the magnitude of long-term synaptic potentiation of excitatory perforant path inputs. 4. Deletion of NL1 leads to a significant reduction in hippocampal synaptosomal expression levels of the AMPA receptor subunit GluA2 and the NMDA receptor subunits GluN1, GluN2A and GluN2B, and to impaired GluA2 upregulation upon LTP induction.

NL1 deletion reduces excitatory transmission at perforant path synapses

Significant changes of the input–output curve for fEPSP slopes were seen in NL1 KO mice as compared to WT littermates, indicating that the lack of NL1 affects basal...
glutamate-mediated synaptic transmission. These results are consistent with recent patch-clamp experiments in acute slices, which reported reduced excitatory AMPA receptor- and NMDA receptor-mediated currents upon lentiviral-mediated knockdown of NL1 in the adult rat dentate gyrus (Shipman and Nicoll 2012). Interestingly, knockdown of NL1 reduced AMPA receptor- and NMDA receptor-mediated currents only in young but not in adult CA1 hippocampal area (Shipman and Nicoll 2012). These findings indicate age- and region-specific differences in the involvement of NL1 in the maintenance of functional excitatory transmission (see also Blundell et al. 2010; Chen et al. 2010; suppl. Fig. 4 in Chubykin et al. 2007). In line with this, reduced AMPA receptor currents associated with recent experiments showing that neurexin/NL1 contacts are involved in the recruitment and trafficking of functional AMPA receptors (Heine et al. 2008; Mondin et al. 2011; Nam and Chen 2005; Zeidan and Ziv 2012).

Reductions in fEPSP slopes can be attributed to a decrease in the number of glutamatergic receptors at synapses or in the number of functional synapses or in both. Previous immunolabeling experiments revealed a normal density of glutamatergic presynaptic terminals in NL1 KO mice (Blundell et al. 2010). In agreement with this, unaltered numbers of synapses were previously detected in the brain of NL1, NL2, NL3 triple KOs (Varoqueaux et al. 2006; see also Soler-Llavina et al. 2011). In contrast to the cell wide deletion of NL1 in NL1 KO mice (Blundell et al. 2010), knockdown of NL1 in subsets of neurons leads to a loss of synapses (Shipman and Nicoll 2012; Shipman et al. 2011). Similarly, a recent comparison of global versus sparse loss of NL1 in cortical pyramidal cells has revealed that the excitatory synapse number is not regulated by absolute NL1 levels but rather depends on relative NL1 expression across cells (Kwon et al. 2012). Since our experiments were performed in animals with a homogeneous NL1 deletion in all granule cells, a decrease in the number of excitatory synapses is not a likely mechanism behind the observed changes in perforant path responses. Instead, given that our Western blot data from hippocampal synaptosome preparations of NL1 KO mice showed significantly reduced expression levels of glutamate receptor subunits, it is likely that the impaired glutamatergic transmission in the NL1 KO dentate gyrus results from changes in the number of receptors per synapse. However, we cannot rule out that a minor loss of excitatory synapses might have contributed to the fEPSP slope reduction as suggested by the trend toward reduced synaptic protein levels of PSD95 and VGlut1. Taken together, the present in vivo data add to the accumulating evidence that NL1 is a key regulator of basal excitatory transmission.

NL1 deletion enhances E–S coupling and alters network inhibition

Although NL1 KO mice exhibited smaller fEPSP slopes than WT mice, no difference in population spike amplitudes was seen between the two genotypes. E–S analysis confirmed that population spikes of similar amplitudes were generated by significantly lower fEPSP slopes in NL1 KO mice. The E–S curve was shifted to the left indicating that NL1 KO granule cells fire more readily in response to an EPSP of a given size. What could be the potential mechanism of such an increase in the responsiveness of granule cells to the EPSP (i.e. in the E–S coupling) upon NL1 deletion? E–S coupling depends on two key factors, the intrinsic neuronal excitability (Wathey et al. 1992) and the ratio of excitation to inhibition (Abraham et al. 1987; Marder and Buonomano 2004). Field potential recordings cannot conclusively show which of these mechanisms are responsible for the enhanced input–output function of NL1 KO granule cells (Bowden et al. 2012). However, the most parsimonious explanation is that NL1 deletion affected not only excitatory perforant path inputs to granule cells but also to inhibitory interneurons, thereby diminishing feed-forward inhibition and increasing the excitatory/inhibitory ratio. Importantly, previous studies in NL1 KOs have not found any changes in the number of inhibitory synapses or in inhibitory currents recorded from cortical or hippocampal pyramidal cells (Blundell et al. 2010; Chubykin et al. 2007; see also Shipman et al. 2011) indicating that GABAergic synapses are not perturbed by NL1 deletion (but see Giannone et al. 2013).

In addition to greater E–S coupling, reduced feed-forward inhibition due to impaired excitation of interneurons in NL1 KO mice is also the most likely explanation for the impairment in network inhibition, which was observed in our PPI measurements. Computational modeling indicates that perisomatic feed-forward and feedback inhibition mediated by basket cells contributes strongly to PPI (Jedlicka et al. 2010; see also Lømo 2009). Therefore, decreased strength of excitatory inputs to basket cells might lead to the shift of the NL1 KO PPI curve to the left. Nevertheless, PPI is a network phenomenon which can also be affected by changes in additional parameters, such as intrinsic excitability of granule cells (Jedlicka et al. 2010).

NL1 deletion impairs LTP

Our LTP experiments showed that excitatory synapses in NL1 KO animals are potentiated to a lesser extent than in
WT controls. Synaptic potentiation induced by wTBS and by subsequent sTBS was diminished in NL1 KO dentate gyrus. On the other hand, sTBS on its own did not lead to changes in LTP in NL1 KO mice. Since the degree of NMDA receptor activation and subsequent LTP induction strongly depends on the effectiveness of the tetanus in depolarizing the postsynaptic membrane (Nicol and Roche 2013), the observed increased E–S coupling in NL1 KOs might have masked an LTP impairment in the sTBS experimental group. Collectively, the LTP data indicate that NL1 KO animals have a higher threshold for LTP induction and lower capacity for potentiation induced by repeated tetanization. In agreement with our data, Shipman and Nicoll (2012) have recently reported that perforant path LTP was abolished in hippocampal slices prepared from rats with lentiviral-mediated knockdown of NL1 in the dentate gyrus. Similarly, LTP at Schaffer collateral inputs to the CA1 region as well as at thalamic but not cortical inputs to the amygdala has been found to be NL1-dependent (Blundell et al. 2010; Budreck et al. 2013; Dahlin and Deller et al. (Frankfurt, Germany) for continuous support and critical reading of the manuscript. The present work was supported by the LOEWE-Program “Neuronal Coordination Research Focus Frankfurt” (NeFF to S.W.S.), the Max Planck Society, and the European Commission. We thank Thomas Deller (Frankfurt, Germany) for continuous support and critical reading of the manuscript. The present work was supported by the LOEWE-Program “Neuronal Coordination Research Focus Frankfurt” (NeFF to S.W.S.), the Young Investigators Grant (from the Faculty of Medicine, Goethe-University, Frankfurt to P.J.), by the BMBF Grant (Germany–USA Collaboration in Computational Neuroscience to P.J., No. 01GQ1203A), the Max Planck Society, and the European Commission (EUROPSPIN and SynSys consortia, N.B.). N.B. was further supported by EU-AIMS (European Autism Interventions), which receives support from the Innovative Medicines Initiative Joint Undertaking under Grant Agreement No. 115300, the resources of which are composed of financial contributions from the European Union’s Seventh Framework Programme (Grant P7/2007–2013), from the European Federation of Pharmaceutical Industries and Associations companies’ in-kind contributions, and from Autism Speaks. DDK is a recipient of a fellowship of the Alexander von Humboldt Foundation and a Marie Curie International Reintegration Grant of the European Commission.

Clinical relevance

NL1 KO mice show increased repetitive (stereotypic) behaviors which are key symptoms of autism spectrum disorders, as well as learning and memory deficits (Blundell et al. 2010; Kim et al. 2008). Similar to NL1 KOs, mice with deletions of postsynaptic proteins of the ProSAP/Shank family display alterations in glutamatergic transmission combined with altered repetitive behavior (Schmeisser et al. 2012; Yang et al. 2012). Intriguingly, the synaptic function of ProSAP2/Shank3 has recently been linked to neurexin–neuroligin transsynaptic signaling (Arons et al. 2012; see also Gkogkas et al. 2013). These findings indicate that deficits in excitatory synaptic function caused by the loss of proteins at glutamatergic inputs may play a major role in the neural basis of complex psychiatric diseases including autism spectrum disorders.

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