

Genetically Induced Retrograde Amnesia of Associative Memories After Neuroplastin Ablation

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ABSTRACT

BACKGROUND: Neuroplastin cell recognition molecules have been implicated in synaptic plasticity. Polymorphisms in the regulatory region of the human neuroplastin gene (*NPTN*) are correlated with cortical thickness and intellectual abilities in adolescents and in individuals with schizophrenia.

METHODS: We characterized behavioral and functional changes in inducible conditional neuroplastin-deficient mice.

RESULTS: We demonstrate that neuroplastins are required for associative learning in conditioning paradigms, e.g., two-way active avoidance and fear conditioning. Retrograde amnesia of learned associative memories is elicited by inducible neuron-specific ablation of *Nptn* gene expression in adult mice, which shows that neuroplastins are indispensable for the availability of previously acquired associative memories. Using single-photon emission computed tomography imaging in awake mice, we identified brain structures activated during memory recall. Constitutive neuroplastin deficiency or *Nptn* gene ablation in adult mice causes substantial electrophysiologic deficits such as reduced long-term potentiation. In addition, neuroplastin-deficient mice reveal profound physiologic and behavioral deficits, some of which are related to depression and schizophrenia, which illustrate neuroplastin's essential functions.

CONCLUSIONS: Neuroplastins are essential for learning and memory. Retrograde amnesia after an associative learning task can be induced by ablation of the neuroplastin gene. The inducible neuroplastin-deficient mouse model provides a new and unique means to analyze the molecular and cellular mechanisms underlying retrograde amnesia and memory.

Keywords: Associative memory, Knockout mouse model, Learning impairment, Neuroplastin, Retrograde amnesia, Synaptic plasticity

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Learning and memory, in particular associative memories, determine successful interaction with the environment. Memory loss (amnesia) characterizes dementias and other disorders of brain function, e.g., posttraumatic stress disorder. Learning and memory processes depend on synaptic architecture and plasticity. Cell adhesion molecules (CAMs) communicate extracellular and intracellular events, and neuronal CAMs such as neuroplastins, neuroligins, neural CAM, and L1 are involved in synapse formation, modulation, and plasticity (1–4).

Neuroplastin isoforms (Np55 and Np65) are encoded by a single gene. Polymorphisms in the regulatory region of the human *NPTN* gene are correlated with cortical thickness and intellectual abilities in adolescents (5) and individuals with schizophrenia (6). Np55 shows widespread expression in various organs and cell types, whereas Np65 is brain-specific and restricted to neurons and undergoes *trans*-homophilic

binding (1,7). Recombinant Np65- or Np65-specific antibodies can block long-term potentiation (LTP) in the hippocampal CA1 and CA3 (1). Furthermore, kainate induced seizures or induction of LTP increase Np65 in postsynaptic densities (1). Neuroplastins are binding partners of fibroblast growth factor receptors (8) and gamma-aminobutyric acid type A (GABA_A) receptors (9). Np65 interacts with the GABA_A receptor α_2 subunit, and neuroplastin-deficient neurons exhibit impaired inhibitory transmission (9,10).

We generated inducible neuroplastin-deficient mice and analyzed the dependence of learning and memory and synaptic plasticity on neuroplastins. We show that neuroplastins are required for associative learning and memory, LTP expression, and hormonal homeostasis. Furthermore, inducible neuroplastin-deficient mice enabled us to elicit and investigate molecular mechanisms of retrograde amnesia.

METHODS AND MATERIALS

Statistical Analysis

Statview (SAS Institute, Inc., Cary, NC) and SPSS 19 (IBM Corp., Armonk, NY) were used for analysis of variance, post hoc analysis (Scheffé or Fisher's protected least significant difference), repeated-measures analysis of variance, and *t* tests. $p < .05$ was considered significant.

Animals

Mice were kept with a 12-hour light/dark cycle and food and water ad libitum. All procedures were in accordance with institutional, state, and government regulations. For the generation of mutant mice see [Supplemental Figures S1–S10](#), [Supplemental Tables S1 and S2](#), and [Supplemental Methods](#). *Nptn*^{+/-} mice were backcrossed for more than 10 generations, and *Nptn*^{lox/+} mice for more than five generations to C57BL/6-Crl. *Nptn*^{lox/+} crossed with prion promoter CreERT mice (11) were maintained by inbreeding *Nptn*^{lox/lox} and *Nptn*^{lox/loxPrCreERT} mice. CreERT was activated by daily intraperitoneal injection of 200 μ L tamoxifen (10 mg/mL medical oil, T 5648; Sigma-Aldrich, St. Louis, MO) for 10 days.

Antibodies

Primary Antibodies. Polyclonal antisera against immunoglobulin 1–3 and immunoglobulin 2–3 detecting Np65 and Np55 are described (1). Purchased antibodies were rat polyclonal homer (Acris Antibodies GmbH, Herford, Germany); rabbit polyclonal glucocorticoid receptor (Abcam, Cambridge, United Kingdom); mouse monoclonal glucocorticoid receptor; β -actin; glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz Biotechnology, Inc., Heidelberg, Germany); goat polyclonal Np65 isoform-specific; sheep polyclonal neuroplastin-detecting Np65 and Np55 (R&D Systems, Minneapolis, MN); mouse monoclonal microtubule-associated protein 2; gephyrin; guinea pig polyclonal synapsin 1,2; vesicular GABA transporter; rabbit polyclonal synaptophysin 1 (Synaptic Systems GmbH, Göttingen, Germany); and mouse monoclonal pan-plasma membrane Ca^{2+} adenosine triphosphatase (PMCA) clone 5F10 (Abcam).

Secondary Antibodies. Secondary antibodies were anti-mouse horseradish peroxidase (Dako Cytomation, Hamburg, Germany) and Cy5, anti-rabbit and anti-goat horseradish peroxidase and Cy5, anti-guinea pig Cy3 and Cy5, anti-sheep Cy3 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), anti-goat Alexa Fluor 568, anti-rat and anti-mouse Alexa Fluor 488 (Molecular Probes Life Technologies Corporation, Grand Island, NY), and anti-rabbit Cy3 (Abcam).

Protein Analysis

Dissected organs were homogenized and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting as described elsewhere (12). Protein concentrations were determined by amido black assay. Representative Western blots were reproduced more than five times with different animals and in different laboratories.

Immunofluorescent Staining

Anaesthetized animals were perfused with phosphate-buffered saline (PBS) (pH 7.4) followed by 4% paraformaldehyde (10 mL/min, 10 minutes). Brains were postfixed in 4% paraformaldehyde (4°C overnight), serially incubated with 0.5 and 1 mol/L saccharose, frozen in methyl butane, and stored at -80°C . Free-floating cryostat sections (20 and 40 μ m) from four or more animals per genotype were blocked with 5% bovine serum albumin or 20% horse serum in PBS, incubated with primary antibodies (0.3% Triton [Serva, Heidelberg, Germany], 10% horse serum in PBS, 36–48 hours, 4°C), washed, probed with secondary antibodies, washed, and mounted using Mowiol (Sigma-Aldrich, Taufkirchen, Germany) or Vectashield (Vector Laboratories, Burlingame, CA) with 4',6-diamidino-2-phenylindole. Stainings were reproduced in three or more sections per animal.

Corticosterone Enzyme-Linked Immunosorbent Assay

Corticosterone concentrations were determined using an enzyme-linked immunosorbent assay kit (DEV9922; Demediatec Diagnostics, Kiel, Germany) according to the manufacturer's instructions.

Behavior

Sex- and age-matched littermate *Nptn*^{+/-} mice served as controls for *Nptn*^{-/-} and *Nptn*^{+/-} mice, and *Nptn*^{lox/lox} mice as controls for *Nptn* ^{Δ lox/loxPrCreERT} mice. The experimenter was not aware of the genotype. For initial characterization of *Nptn*^{-/-} and *Nptn* ^{Δ lox/loxPrCreERT} mice, the following tests were conducted sequentially during the light phase: neurologic examination, grip strength, rotarod, open field, O-maze, light/dark avoidance, water maze, shuttle box, and startle-prepulse inhibition, as described elsewhere (13–17). Grip strength was measured with a force sensor (TSE Systems GmbH, Bad Homburg, Germany). The latency to fall off the rotarod (TSE Systems GmbH) was determined in two training sessions (3-hour interval) with increasing speed (4–40 rpm, 5 minutes) and 4 days later at 16, 24, 32, and 40 rpm constant speed. Open-field (50 $\times$ 50-cm) exploration for 15 minutes was analyzed for path length, speed, and time spent in the center and corners and at the walls (VideoMot2 software; TSE Systems GmbH). Mice were placed in an O-maze (San Diego Instruments, San Diego, CA) for 5 minutes. Entries, time, speed, and distance in closed or open areas were analyzed (VideoMot2). For light/dark avoidance behavior, mice were placed in an illuminated compartment (250 lux, 25 $\times$ 25 cm) adjacent to a dark compartment (12.5 $\times$ 25 cm). Time spent within compartments and transitions between them were analyzed for 10 minutes. Reduced latencies entering the dark at a later exposure (on the last day of behavioral experiments) indicate long-term memory (15). Spatial learning was assessed in the hidden platform Morris water maze as described elsewhere (13) using VideoMot2 and Wintrack software (<http://www.dpwolfer.ch/wintrack/index.html>) (18). Associative learning was assessed by two-way active avoidance in a two-chambered shuttle-box (TSE Systems GmbH) with 10 seconds

of light as conditioning (CS) and electrical foot shock as unconditioned stimulus (5 seconds, 0.5 mA pulsed) delivered after the CS (80 trials/day, 5–15 seconds of stochastically varied intertrial intervals for 5 consecutive days). Compartment changes during CS were counted as conditioned avoidance reactions. The acoustic startle response to a stimulus (50 ms, 120 dB) and its inhibition by prepulses (PPI) (30 ms; 100 ms before startle stimulus with eight different intensities, 73–94 dB, 3-dB increments, 70 dB white noise background) was analyzed in a startle-box system (TSE Systems GmbH). Habituation (3 minutes) was followed by two startle trials and in pseudorandom order by 10 startle trials and five trials at each prepulse intensity with stochastically varied intertrial intervals (5–30 seconds). The maximal startle amplitude was measured by a sensor platform. Fear conditioning was conducted as described elsewhere (19) using distinct cohorts of mice. Mice were conditioned in an operant chamber (San Diego Instruments) by exploration (2 minutes) and auditory cue presentation (15 seconds), followed by a foot shock (2 seconds, 1.5 mA unpulsed) with one repetition. Twenty-four hours later, mice were placed in the training chamber (context, 5 minutes) and then returned to their home cage. One hour later, mice were placed in a novel environment (3 minutes) and then the auditory cue (CS) was presented (3 minutes). Freezing behavior (immobility) was recorded during all sessions. For memory tests after *Nptn* ablation, the procedure described for the second day was conducted again at 4 and 10 weeks after induction. Social interactions were analyzed by the three-chamber test as described elsewhere (20). Briefly, during three test phases (10 minutes each), the mouse could explore all compartments. In phase 1, the mouse was alone. In phase 2, an unfamiliar C57BL/6Crl mouse (same sex, stranger 1) was placed in one of the wire cups. In phase 3, another unfamiliar C57BL/6Crl mouse (same sex, stranger 2) was placed in the other cup. Time spent in each compartment, time in contact with strangers, and transitions between compartments were recorded. The tail suspension test was performed as described (21) with mice secured at the distal part of the tail. Duration of active struggling behavior (mobility time) was scored for 6 minutes, not considering passive limb or head movements or swinging motion as active struggling.

Memory Assessment After Induced *Nptn* Ablation

Mice were trained in the water maze (5 consecutive days, 6 trials per day, hidden platform, fixed position), the light/dark avoidance paradigm, and the two-way active avoidance paradigm (80 trials per day until they reached $\geq 75\%$ performance). Then mice were injected with tamoxifen for 10 days and were tested 8 weeks later in the water maze (two trials), light/dark avoidance, and shuttle box (80 trials). Mice analyzed by single-photon emission computed tomography (SPECT) or for relearning were subjected only to the two-way active avoidance paradigm.

Electrophysiology in the CA1 Region of Hippocampal Slices

Preparation and methods applied were as detailed elsewhere (22). Briefly, the right hippocampus of 3- to 5-month-old mice (both sexes) killed by cervical dislocation was dissected out. Transverse slices (400 μm) prepared from the dorsal area were

maintained at 32°C continuously perfused with artificial cerebrospinal fluid (ACSF) (2.2 mL/min, in mmol/L: NaCl, 124; KCl, 4.9; NaH_2PO_4 , 1.2; NaHCO_3 , 25.6; CaCl_2 , 2; MgSO_4 , 2; glucose, 10; saturated with 95% O_2 and 5% CO_2 , pH 7.3–7.4). A tungsten electrode was placed in the CA1 stratum radiatum for stimulation. Evoked field excitatory postsynaptic potentials (fEPSPs) were recorded with a glass electrode (filled with ACSF, 3–7 M Ω). The descending slope of the fEPSP was used as a measure of this potential. Stimulation strength, adjusted eliciting a fEPSP slope of 35% of the maximum (determined by input–output curves), was kept constant. Paired-pulse facilitation was investigated applying two pulses in rapid succession (interpulse intervals of 10, 20, 50, 100, 200, and 500 ms, respectively) at 120-second intervals. During baseline recording, three single stimuli (0.1-ms pulse width; 10-second intervals) were measured every 5 minutes and averaged. To induce strong LTP, theta-burst stimulation (10 bursts of four stimuli at 100 Hz, applied every 200 ms; pulse width of 0.2 ms) was repeated three times every 10 minutes, with evoked responses at 1, 4, and 7 minutes during the three conditioning protocols. Thereafter, responses were recorded every 5 minutes for 2 hours.

Postsynaptic currents from single CA1 pyramidal cells were recorded in transverse vibratome slices (400 μm , Microm HM650V; Thermo Scientific, Waltham, MA) from the medial hippocampus placed for 90 minutes in an incubation chamber containing ACSF and continuously perfused (95% O_2 /5% CO_2 , room temperature). Whole-cell voltage clamp recordings were performed at 32°C (MultiClamp 700B patch-clamp amplifier; Molecular Devices, Sunnyvale, CA). Data were collected with pClamp software (Axon Instruments, Union City, CA). Borosilicate glass recording electrodes were filled with the following solution (in mmol/L): 135 CsMeSO₄, 4 NaCl, 4 Mg-adenosine triphosphate, 0.5 ethylene glycol bis-2-aminoethyl ether-N,N',N'',n'-tetraacetic acid-Na, 0.3 Na-guanosine triphosphate, 10 K-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 5 QX-314; pH 7.3 (pipette resistance, 3–5 M Ω). Access resistance was 10–20 M Ω and then compensated to 75%. If input resistance changed more than 25%, the neuron was excluded.

Based on reversal potential, miniature excitatory postsynaptic currents (mEPSCs) and miniature inhibitory postsynaptic currents (mIPSCs) were mostly measured consecutively from the same neurons (23–25). First, mEPSCs were recorded at the reversal potential for GABA_A receptor-mediated events (–60 mV); then mIPSCs were recorded at the reversal potential for glutamatergic currents (+10 mV) with tetrodotoxin (1 $\mu\text{mol/L}$) present. Blocking mEPSCs by 20 $\mu\text{mol/L}$ 6-cyano-7-nitroquinoxaline-2,3-dione and 10 $\mu\text{mol/L}$ d-aminophosphonovalerate verified their glutamatergic nature. The mIPSCs were blocked by 100 $\mu\text{mol/L}$ picrotoxin. Data were low-pass filtered at 2 kHz and acquired at 10 kHz using Digidata 1440 and pClamp 10 software (Molecular Devices). The mEPSCs and mIPSCs offline analysis used MiniAnalysis software (version 6.0.7; Synaptosoft, Decatur, GA).

SPECT Imaging

We used SPECT imaging of regional cerebral blood flow for in vivo mapping of spatial patterns of neuronal activity in the brains of awake behaving mice (26). Catheters were placed into

the right external jugular veins 2 days before experiments (26). In the shuttle box, mice were injected during habituation (3 minutes) with saline, followed by ^{99m}Tc -hexamethylpropyleneamineoxime (^{99m}Tc -HMPAO) (250 μL , 25 $\mu\text{L}/\text{min}$) during the initial 10 minutes of the paradigm consisting of 80 trials. The average injected dose was 67.5 MBq of ^{99m}Tc at 250 μL per animal. After the shuttle box experiment, animals were anesthetized and scanned with a four-head NanoSPECT/CT scanner (Mediso, Hungary) as described elsewhere (26). The SPECT images were reconstructed at an isotropic voxel size of 338 μm using the

manufacturer's software (HiSPECT; SCIVIS, Göttingen, Germany) and aligned with a high-resolution magnetic resonance mouse brain data set (27,28) (MPI-Tool 6.36; Advanced Tomo Vision, Kerpen, Germany). The SPECT brain data were cut out of the SPECT data in Osirix (64-bit, version 5.7.1; Pixmeo SARL, Bernex, Switzerland) using a whole-brain volume of interest made from the template by Ma and colleagues (27,28). Brain SPECT data were global mean normalized using MPI-Tool software. In the voxelwise analysis, unpaired t tests were made to compare brain tracer distribution in $Nptn^{\Delta lox/loxPrCreERT}$ ($n = 7$)

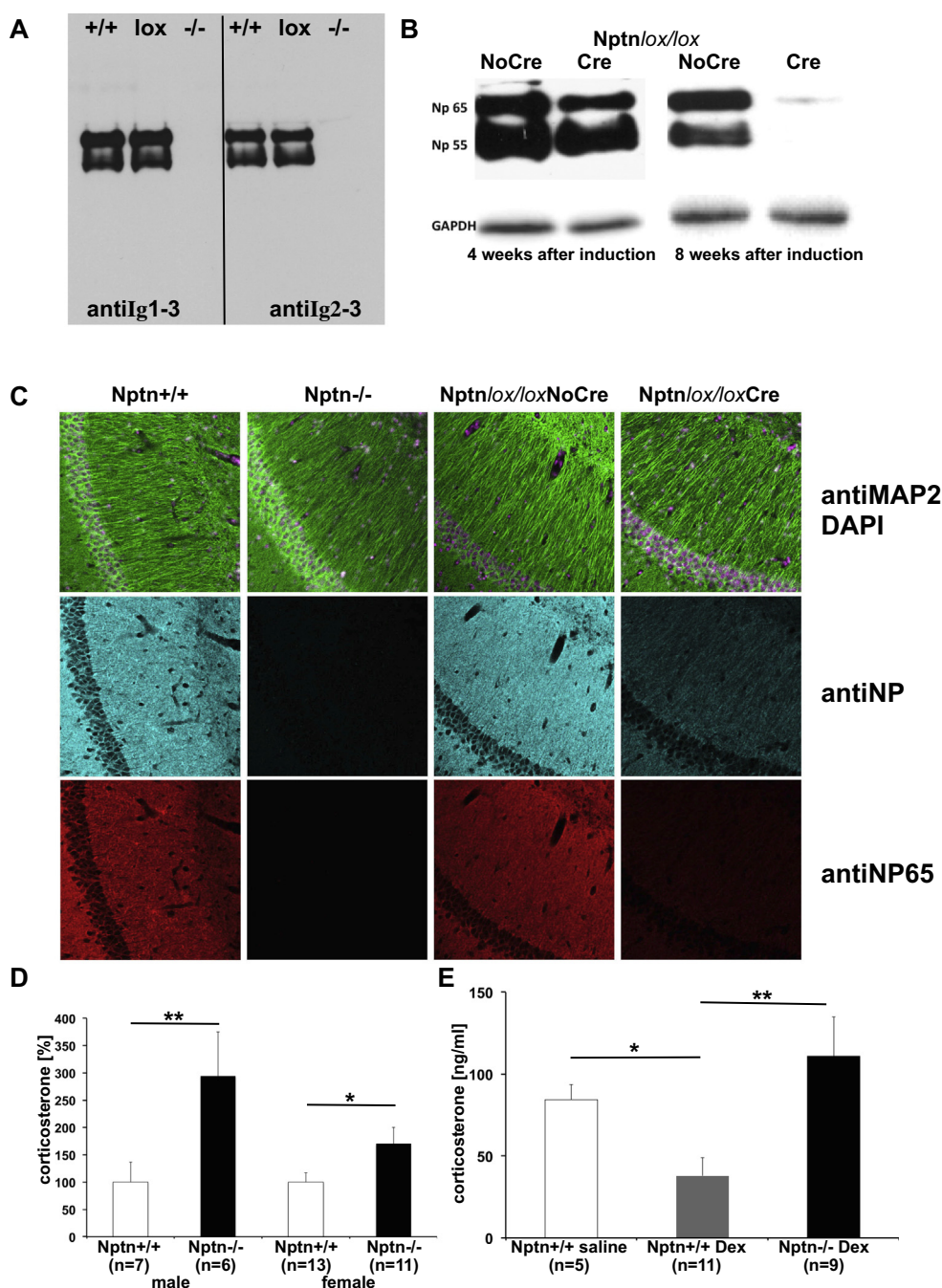


Figure 1. Characterization of $Nptn^{-/-}$ mice and impaired corticosterone regulation. **(A)** Western blot of membrane fractions (20 μg protein per lane) from brains of wild-type ($Nptn^{+/+}$), $Nptn^{lox/lox}$ (lox), and $Nptn^{-/-}$ mice. Polyclonal antibodies against immunoglobulin domains 1–3 (left) or 2–3 (right) of neuroplastin detecting the Np65 and Np55 isoforms were used. **(B)** Western blot of membrane fractions (20 μg protein per lane) from brains of $Nptn^{\Delta lox/loxPrCreERT}$ (Cre) or control $Nptn^{lox/lox}$ mice (NoCre) 4 and 8 weeks after induction by tamoxifen probed with antibodies against immunoglobulin domains 2–3 of neuroplastin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as loading control. **(C)** Immunohistochemistry for anti-microtubule-associated protein 2 (antiMAP2) (green) and 4',6-diamidino-2-phenylindole (DAPI) (magenta) (top row), anti-neuroplastins (antiNP) (cyan) (middle row), or anti-neuroplastin 65 isoform (antiNP65) (red) (bottom row) in the hippocampus of wild-type ($Nptn^{+/+}$), $Nptn^{-/-}$, and $Nptn^{\Delta lox/loxPrCreERT}$ ($Nptn^{lox/lox}$ or $Nptn^{lox/loxCre}$) or control $Nptn^{lox/lox}$ mice ($Nptn^{lox/loxNoCre}$) 8 weeks after induction by tamoxifen. **(D)** Elevated morning serum corticosterone levels in neuroplastin-deficient mice ($Nptn^{-/-}$) compared with wild-type ($Nptn^{+/+}$) analyzed by enzyme-linked immunosorbent assay. **(E)** After a dexamethasone challenge (Dex), serum corticosterone levels are higher in male neuroplastin-deficient ($Nptn^{-/-}$) mice. All data are presented as means $\pm$ SEM; all p values are derived from Scheffé post hoc test after one-way analysis of variance (* $p < .05$; ** $p < .01$; *** $p < .001$).

versus *Nptn^{lox/lox}* control mice ($n = 9$) using MagnAn software (version 2.4; BioCom, Uttenreuth, Germany). Following common procedures in small-animal radionuclide imaging (29–31), uncorrected p values were used. Results were illustrated using Osirix and Photoshop (version CS4; Adobe Systems Software, San Jose, CA).

RESULTS

Lox sites were introduced into the *Nptn* gene (*Nptn^{tmloxexon1lox}* [*Nptn^{lox}*]), allowing Cre-recombinase-mediated permanent or inducible deletion of exon 1 encoding the start codon and the signal sequence (Supplemental Figure S1). Constitutively

neuroplastin-deficient mice (*Nptn^{-/-}*) were generated by intercrossing heterozygous mice (*Nptn^{+/-}*) obtained after germ line excision of exon 1 (*Nptn^{tmΔexon1}*) by a constitutively expressed Cre-recombinase (32). In *Nptn^{-/-}* mice, neuroplastins were undetectable in the brain and other organs (Figure 1A, C and Supplemental Table S1). *Nptn^{-/-}* mice had a reduced life span (Supplemental Figure S2), body size, and weight, and male-specific incompetency to sire offspring (not shown). The gross brain architecture was not affected and magnetic resonance imaging morphometry showed normal anatomy of *Nptn^{-/-}* brains with no significant size abnormalities (Supplemental Table S2, Supplemental Figure S3). Several endocrinologic factors, e.g., blood glucose levels, insulin regulation, and

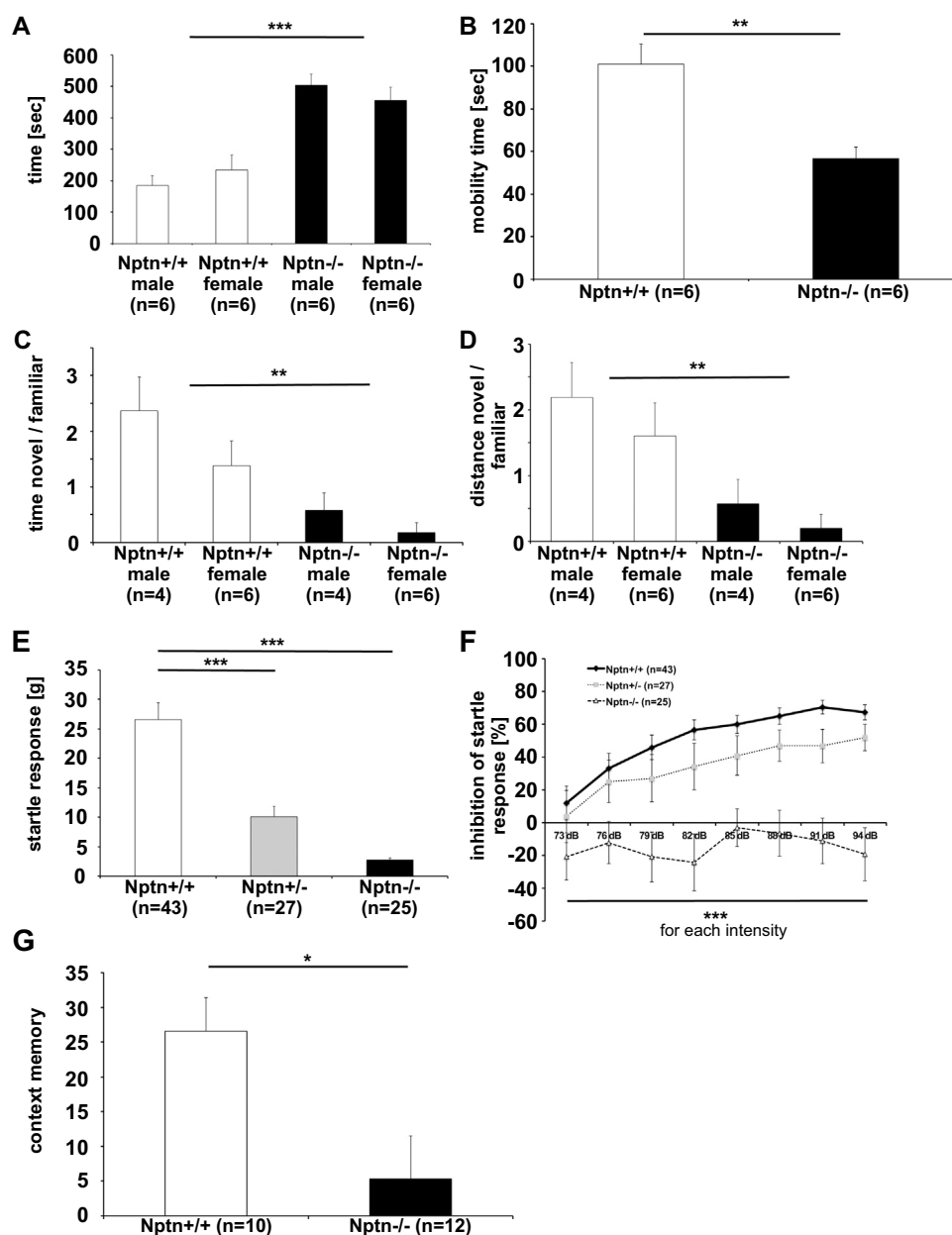


Figure 2. Behavioral deficits in *Nptn^{-/-}* mice. (A) Increased preference for the center of an open field by *Nptn^{-/-}* mice compared with wild-type (*Nptn^{+/+}*) mice. (B) Reduced active mobility time during the tail suspension test of male *Nptn^{-/-}* mice. (C, D) Impaired social interaction of *Nptn^{-/-}* mice in three-chamber assays. (C) Social novelty recognition (interaction with novel wild-type mouse of the same sex vs. familiarized wild-type mouse of the same sex). (D) Activity in the compartment of the strange mouse. (E) Reduced magnitude of the startle response of heterozygous (*Nptn^{+/-}*) and *Nptn^{-/-}* mice. (F) Inhibition of the startle response by prepulses (PPI) with the given intensity as percentage of the startle response for *Nptn^{+/+}*, *Nptn^{+/-}*, and *Nptn^{-/-}* mice (one-way ANOVA for all prepulse intensities: $p < .001$). (G) Reduced context memory (% freezing time shock minus neutral context) after fear conditioning in *Nptn^{-/-}* mice. All data are presented as means $\pm$ SEM; all p values are derived from Scheffé post hoc test after one- or two-way analysis of variance (* $p < .05$; ** $p < .01$; *** $p < .001$).

thyroid (T3 and T4) and growth hormone levels were normal (Supplemental Figure S4). However, *Nptn*^{-/-} mice had altered hypothalamic-pituitary-adrenal (HPA) axis correlates, namely elevated corticosterone levels, both basal and after a dexamethasone suppression test (Figure 1D, E) and decreased corticotropin-releasing hormone messenger RNA and glucocorticoid receptor levels in brain (Supplemental Figure S4).

Depression is associated with high cortisol levels, the analog of mouse corticosterone, and HPA axis dysregulation (33). Therefore, we tested *Nptn*^{-/-} mice for core features of depression-related behavior including stress resilience, anxiety, social interaction, motivation, and despair. *Nptn*^{-/-} mice displayed less anxious behavior in open-field (Figure 2A, Table 1) and light/dark avoidance tests (Supplemental Figure S5D) and higher preference for a familiarized over an unfamiliar mouse, which indicated altered social interactions (Figure 2C, D). Although male *Nptn*^{-/-} mice did not exhibit consummatory anhedonia in the sucrose preference test (not shown), lack of motivation in the alternating T maze (not shown) and higher immobility in the tail suspension test indicated depressive-like behavior (Figure 2B).

While fundamental functions of the nervous system, e.g., reflexes and sensory abilities, appeared normal, neurologic deficits were apparent in specific tests including reduced motoric capabilities (grip strength and rotarod) (Supplemental Figure S5C, D) and aberrant swimming behavior (diving) in the Morris water maze. Furthermore, sensorimotor gating as examined by the startle response and its PPI (Figure 2E, F), associative learning as analyzed by fear conditioning (Figure 2G), and active avoidance learning (Figure 3A) revealed evident cognitive deficits in *Nptn*^{-/-} mice.

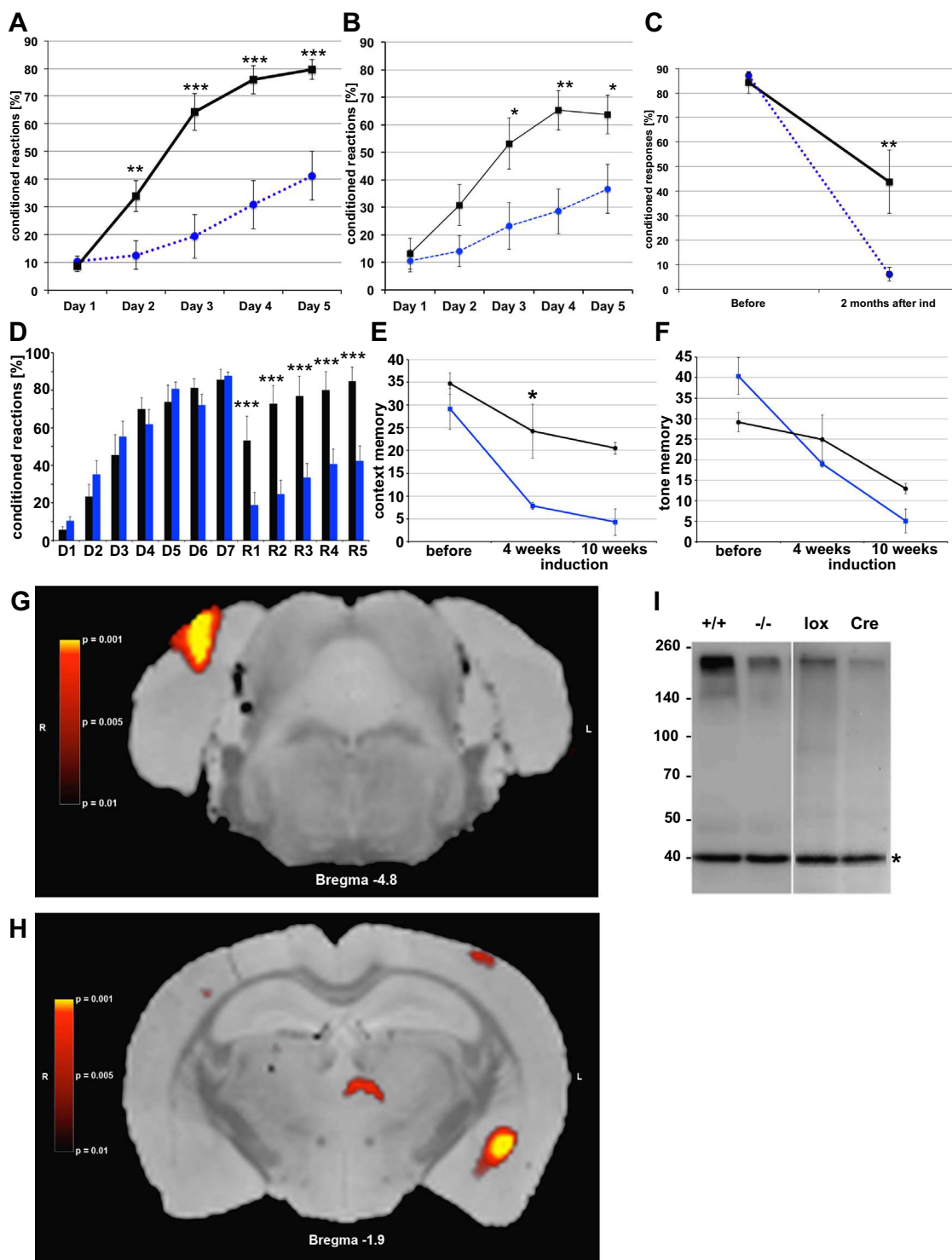
These cognitive deficits suggested important roles of neuroplastins in learning and memory. To differentiate developmental from mature neuronal functions, we ablated neuronal neuroplastin expression in adult *Nptn*^{lox/lox} mice by activating prion promoter-driven CreERT recombinase (11) with tamoxifen

(*Nptn*^{Δlox/loxPrCreERT}). Immunoblot analysis showed about 65% reduced neuroplastin levels in the brain 4 weeks after induction, which further decreased to just detectable levels after 2 months (Figure 1B). When the behavior of *Nptn*^{Δlox/loxPrCreERT} mice was analyzed more than 4 weeks after induction, their performance was similar to that of control mice without Cre with respect to grip strength, social interaction, motor abilities, and startle response (Supplemental Figure S6, Table 1). However, PPI of the startle response was slightly affected (Supplemental Figure S6E). Significantly reduced startle response and PPI in heterozygous *Nptn*^{+/-} mice (Figure 2E, F) indicated that 50% of less of neuroplastin expression, either throughout life or after ablation in adulthood, affects PPI, a candidate endophenotype of schizophrenia.

Strikingly, associative learning in the shuttle box was abolished in induced *Nptn*^{Δlox/loxPrCreERT} mice (Figure 3B). Investigating information acquisition, retention, access, and retrieval of memories, we first trained *Nptn*^{lox/loxPrCreERT} mice in the shuttle box to high performance (greater than 75% correct responses) before inducing gene ablation. Two months after induction (Figure 3C), tamoxifen-treated control mice (*Nptn*^{lox/lox} without Cre-recombinase) retained more than 50% of their previous performance, whereas *Nptn*^{Δlox/loxPrCreERT} mice induced after training performed as poorly as did naive mice, displaying complete retrograde amnesia. Like naive induced *Nptn*^{Δlox/loxPrCreERT} mice, the amnesic mice could not relearn the association even after extensive training (Figure 3D). These results demonstrate that neuroplastins are essential for learning and retention, and/or the retrieval of associative memories. Similar results were obtained using white noise instead of light as the conditioning stimulus (not shown), which showed stimulus modality independence of the retrograde amnesia. Furthermore, *Nptn*^{-/-} mice displayed deficits in the context memory after fear conditioning (Figure 2G), which suggests that associative learning and memory are impaired task-independently. In agreement with these findings, *Nptn*^{lox/loxPrCreERT}

Table 1. Behavior of *Nptn*^{-/-} and *Nptn*^{Δlox/loxPrCreERT} Mice

Test	<i>Nptn</i> ^{-/-}	<i>Nptn</i> ^{Δlox/loxPrCreERT} Ablation Before Tests	<i>Nptn</i> ^{Δlox/loxPrCreERT} Ablation After Training
Grip Strength	<i>Nptn</i> ^{-/-} reduced (Supplemental Figure S5A)	Not different (Supplemental Figure S6A)	
Rotarod	<i>Nptn</i> ^{-/-} reduced (Supplemental Figure S5B)	<i>Nptn</i> ^{Δlox/loxPrCreERT} slightly improved (Supplemental Figure S6B)	
Open Field	<i>Nptn</i> ^{-/-} more in center (Figure 2A)	Not different (not shown)	
O Maze	Not different (Supplemental Figure S5C)	Not different (not shown)	
Light/Dark Avoidance	<i>Nptn</i> ^{-/-} more transitions, more time in light (Supplemental Figure S5D)	Not different (not shown)	
Light/Dark Avoidance Memory	Not different (Supplemental Figure S5D)	Not different (not shown)	Not different (Supplemental Figure S7C)
Shuttle Box Two-Way Active Avoidance	<i>Nptn</i> ^{-/-} , do not acquire task (Figure 3A)	<i>Nptn</i> ^{Δlox/loxPrCreERT} , do not acquire task (Figure 3B)	<i>Nptn</i> ^{Δlox/loxPrCreERT} , retrograde amnesia (Figure 3C, D)
Startle Response and Prepulse Inhibition (PPI)	<i>Nptn</i> ^{-/-} reduced startle response, reduced PPI (Figure 2E, F)	Startle not different, <i>Nptn</i> ^{Δlox/loxPrCreERT} , slightly reduced PPI (Supplemental Figure S6D, E)	
Fear Conditioning	<i>Nptn</i> ^{-/-} reduced context memory (Figure 2G)		<i>Nptn</i> ^{Δlox/loxPrCreERT} reduced context memory (Figure 3E, F)
Social Interaction	<i>Nptn</i> ^{-/-} less time, novel/familiar, and distance (Figure 2C, D)	Not different (Supplemental Figure S6C)	
Tail Suspension	<i>Nptn</i> ^{-/-} less active mobility (Figure 2B)		



mice that are conditioned to fear before induction displayed significantly less context memory 4 weeks after induction (Figure 3E) but similar tone memory (Figure 3F) compared with control mice. The same *Nptn*^{lox/loxPrCreERT} animals trained in the shuttle box had been trained in water maze and light/dark-avoidance paradigms before induction. Interestingly, neuroplastin ablation did not affect water maze performance or memory for the dark compartment (Supplemental Figure S7), which demonstrated the specificity of retrograde amnesia for associative memories. Loss of memory retrieval capabilities might be associated with alterations in activity patterns of relevant brain regions. Therefore, we traced regional cerebral blood flow during memory recall in the shuttle box by ^{99m}Tc-HMPAO infusion and analyzed it by SPECT (26). Trained to 75% or greater correct responses before induction, at 8 weeks after induction *Nptn*^{Δlox/loxPrCreERT} and *Nptn*^{lox/lox} mice showed different activation patterns in the shuttle box (Figure 3G, H). As a key finding, ^{99m}Tc uptake in the right primary visual cortex of induced *Nptn*^{Δlox/loxPrCreERT} mice, compared with *Nptn*^{lox/lox} control mice, was significantly increased ($p < .001$). This difference argues for an increased workload in processing of the visual stimulus in the induced *Nptn*^{Δlox/loxPrCreERT} mice and might reflect an increase in task difficulty for these mice and/or differences in familiarity with the stimulus.

In *Nptn*^{-/-} mice, we previously observed about 30% less excitatory synapses in the hippocampal CA1 region and the dentate gyrus, areas with highest Np65 levels in wild-type mice, whereas in CA3, which normally expresses less Np65, the number of synapses was unaltered (10). Ultrastructurally, no obvious abnormalities of synapses are observed in the hippocampal CA1 region of *Nptn*^{-/-} mice (Supplemental Figures S8 and S9). Furthermore, the number of GABAergic synapses is not affected by constitutive loss of neuroplastins (Supplemental Figure S10). Therefore, part of the complex phenotype of *Nptn*^{-/-} mice may result from disbalance of excitatory glutamatergic and inhibitory GABAergic synapses. Two months after inducible ablation of neuroplastin expression in adult *Nptn*^{Δlox/loxPrCreERT} mice, we observed a slight increase in inhibitory CA1 synapses but no loss or disassembly of excitatory synapses after establishment of neuronal connectivity (Supplemental Figure S10).

Interestingly, we detected significantly reduced amounts of PMCA in both *Nptn*^{-/-} and *Nptn*^{Δlox/loxPrCreERT} mice

(Figure 3I). Because PMCA restores normal Ca^{2+} levels after neuronal activation (34), fewer PMCA may result in altered Ca^{2+} homeostasis and distribution in intracellular stores. Indeed, extrusion of calcium is an effective regulator of synaptic plasticity in neurons (35).

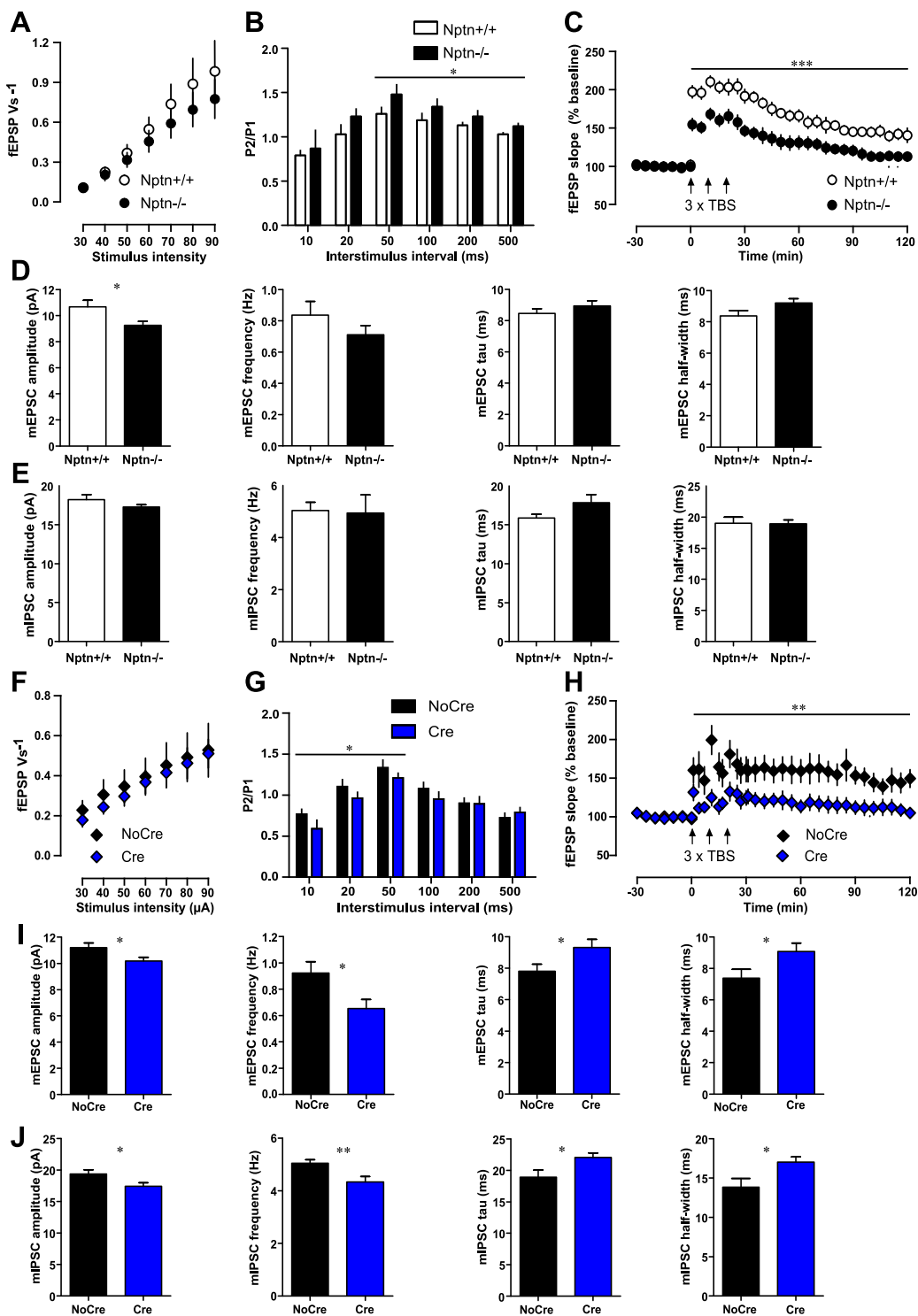
In the hippocampus of *Nptn*^{-/-} mice, basal synaptic transmission evaluated by fEPSPs was normal (Figure 4A). Analysis of short-term plasticity revealed significantly increased paired-pulse facilitation in *Nptn*^{-/-} synapses at longer interpulse intervals between 50 and 500 ms (Figure 4B). In agreement with a reduced excitatory-inhibitory ratio resulting from fewer glutamatergic synapses, LTP was impaired in *Nptn*^{-/-} mice (Figure 4C) and mEPSC amplitudes were reduced (Figure 4D). The mEPSC frequencies showed a similar reduction; however, they did not reach the level of significance owing to a higher variance. No differences were found in mIPSCs (Figure 4E).

To differentiate developmental from mature neuroplastin functions, we measured the same parameters after *Nptn* ablation in adult *Nptn*^{Δlox/loxPrCreERT} mice (Figure 4F–J). Basal synaptic transmission was normal (Figure 4F), but short-term plasticity was significantly reduced at short interpulse intervals between 10 and 50 ms (Figure 4G), which indicated affected GABAergic inhibition and presynaptic functions. As for *Nptn*^{-/-} mice, LTP was significantly impaired (Figure 4H) and mEPSC amplitudes and frequencies were reduced in *Nptn*^{Δlox/loxPrCreERT} mice, indicating presynaptic and postsynaptic changes (Figure 4I). The resulting reduced charge transfer per time and potential seems to be partially compensated by the slower decay and broader half-width of mEPSCs in *Nptn*^{Δlox/loxPrCreERT} mice. Strikingly, mIPSCs showed similar changes, i.e., reduced amplitudes and frequencies but delayed decay and increased half-width (Figure 4J). Neuroplastin ablation in mature animals resulted in pronounced differences. For most parameters a similar tendency, although weaker and mostly not statistically significant, was observed in *Nptn*^{-/-} mice. This is most likely explained by interference with developmental processes in *Nptn*^{-/-} mice.

DISCUSSION

Constitutive *Nptn*^{-/-} mice reveal essential functions of neuroplastins associated with pleiotropic effects on the animal.

Figure 3. Inducible reduction of neuroplastin disrupts associative learning and memory with retrograde amnesia. (A) Two-way active avoidance learning of wild-type (*Nptn*^{+/+}, $n = 13$, black) and *Nptn*^{-/-} ($n = 14$, blue) mice analyzed in a shuttle box with light as the conditioning stimulus. (B) Two-way active avoidance learning of *Nptn*^{lox/lox} ($n = 9$, black) or *Nptn*^{Δlox/loxPrCreERT} ($n = 11$, blue) mice 8 weeks after tamoxifen induction. (C) *Nptn*^{lox/lox} mice with prion CreERT ($n = 7$, blue) or without Cre ($n = 5$, black) were trained for two-way active avoidance to 75% or greater correct responses and then induced with tamoxifen for 10 days, and retested 2 months later. (D) *Nptn*^{lox/lox} mice with prion CreERT ($n = 8$, blue) or without Cre ($n = 6$, black) were trained for two-way active avoidance to more than 75% correct responses and then induced with tamoxifen for 10 days, and retrained 2 months later. (E, F) *Nptn*^{lox/lox} mice with prion CreERT ($n = 8$, blue) or without Cre ($n = 7$, black) before induction with tamoxifen were subjected to fear conditioning and analyzed for context (E) and tone (F) memories (before). Then, tamoxifen was administered for 10 days. At 4 and 10 weeks after onset of induction, context and tone memories were analyzed again. (A–F) All data are presented as means $\pm$ SEM; all p values are derived from Scheffé post hoc test after one- or two-way analysis of variance ($p < .05$; $**p < .01$; $***p < .001$). (G, H) *Nptn*^{lox/loxPrCreERT} ($n = 7$) and *Nptn*^{lox/lox} ($n = 9$) mice were trained before induction to more than 75% correct responses to the light stimulus in the two-way active avoidance paradigm. Then these mice were induced with tamoxifen for 10 days and retested 8 weeks later under intravenous injection with ^{99m}Tc-hexamethylpropyleneamineoxime during 10 minutes of ongoing behavior in the shuttle box. Single-photon emission computed tomography/computed tomography images were aligned with a high-resolution magnetic resonance mouse brain data set, and in voxelwise analysis unpaired t tests were made to compare brain tracer distribution. ^{99m}Tc brain uptake in induced *Nptn*^{Δlox/loxPrCreERT} mice compared with *Nptn*^{lox/lox} control mice increased significantly ($p < .0001$) in the right primary visual cortex, anterior olfactory nucleus, lateral septum, left primary somatosensory cortex, left basolateral amygdala, right lateral posterior nucleus of the thalamus/dorsal lateral geniculate nucleus, and right pedunculopontine tegmental areas, and decreased in the left motor cortex, ventromedial hypothalamus, and right ventral hippocampus/amygdalo-hippocampal regions. (I) Western blot of membrane fractions (20 μ g protein per lane) from brains of wild-type (*Nptn*^{+/+}), *Nptn*^{-/-}, *Nptn*^{lox/lox} (lox), and *Nptn*^{Δlox/loxPrCreERT} (Cre) mice using polyclonal antibodies detecting all forms of plasma membrane Ca^{2+} adenosine triphosphatases. As a control for equal loading, the blot was probed for actin (asterisk). Molecular mass (kDa) of protein standards is indicated on the left.



Reduced life span, corticosterone elevation, dysregulation of the HPA axis, male infertility, less anxious behavior, motivational deficits, altered social interaction, increased despair-like behavior, and learning deficits displayed by *Nptn*^{-/-} mice are potentially related to psychopathologic conditions such as depression, autism, and affective disorders. The role of CAMs in mediating chronic stress-induced signaling, for example, a contribution of neural CAM and L1 in cognitive impairment resulting from stress, has been discussed (36). However, CAM deficiency resulting in chronically elevated corticosterone levels and impaired feedback inhibition of the HPA axis has not been reported. *Nptn*^{-/-} mice display a disturbed balance between excitatory and inhibitory synaptic transmission. Similar imbalances have been implicated in stress-related pathologies including forms of major depression, chronic anxiety, and posttraumatic stress disorder (37–40). Np65 interacts with GABA_A receptor subunit α_2 and *Nptn*^{-/-} neurons exhibit impaired inhibitory transmission (9,10). Altered expression of GABA_A receptor subunits has been linked to depression, mood disorders, and schizophrenia (41). Here, we show that diminished neuroplastin expression (about 50% or less) throughout life or after loss in the adult affects PPI of the acoustic startle response, the candidate endophenotype of schizophrenia. Interestingly, a neuroplastin promoter mutation has been associated with schizophrenia (6). In addition, high glucocorticoid levels and/or other components of the stress response increase the risk of developing this disorder (42).

Our previous work confirmed that the differential expression of neuroplastins in the hippocampus could confer circuit specificity (10). Furthermore, neuroplastins exert multiple effects on glutamatergic and GABAergic synapses, resulting in altered electrophysiologic properties of inhibitory and excitatory synapses in *Nptn*^{-/-} and *NPTN* ^{Δ lox/loxPrCreERT} mice as demonstrated by whole-cell recordings of hippocampal CA1 neurons in this study. Inducible neuron-specific loss of neuroplastins, unlike the constitutive deficiency, did not affect the number of glutamatergic synapses, but both result in electrophysiologic deficits. This indicates that neuroplastins directly affect synaptic transmission that is distinguishable from developmental functions. Thus, neuroplastin deficiency may be regarded as a synaptopathy, a term recently coined for diseases and syndromes caused by synaptic malfunctions including some forms of autism and schizophrenia (43).

Neuroplastin deficiency, both constitutive and induced, affects specifically associative learning and memory, e.g., acquisition and retention or retrieval of learned associations, but does not impair all forms of memory. Retrograde amnesia after ablation of neuroplastins was consistently observed specifically for associative memory in the active avoidance and fear conditioning paradigms, but spared other memories. Our data suggest that circuits with highest neuroplastin expression, e.g., involved in nonspatial (associative) memories, are more strongly affected by ablation than are circuits with lower expression, e.g., involved in spatial memories (44), thus altering information processing affecting association-related but not all forms of memory.

Retrograde amnesia of an associative learning task after induced ablation of the neuroplastin gene is a remarkable phenotype not yet reported for any other gene. Although amnesia is a central pathologic trait common to various psychopathologic disorders, the underlying molecular mechanisms are still unknown. The inducible neuroplastin-deficient mouse model clearly singles out one neuronal protein as indispensable for recalling a previously learned associative task. To date, we cannot distinguish retrograde amnesia caused by loss of the memory trace (retention/storage deficit) or the inability to access the memory (retrieval deficit). Hence, brain region or neuron type-specific inactivation of neuroplastin may identify specific loci for associative memories and disentangle molecular mechanisms underlying amnesia. Our data identify neuroplastins as a novel therapeutic target for memory modulation after traumatic experiences and in post-traumatic stress disorder.

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Figure 4. Synaptic transmission and short-term and long-term synaptic plasticity in the hippocampus of *Nptn*^{-/-} and *Nptn* ^{Δ lox/loxPrCreERT} mice. **(A)** Basal synaptic transmission (evoked field excitatory postsynaptic potentials [fEPSP]) did not differ between wild-type (*Nptn*^{+/+}, open circles, *n* = 6) and *Nptn*^{-/-} (filled circles, *n* = 7). **(B)** Short-term plasticity as evaluated by paired-pulse facilitation significantly increased at intensities from 50 to 500 ms in *Nptn*^{-/-} mice (filled bars, *n* = 6) compared with wild-type mice (*Nptn*^{+/+}, open bars, *n* = 7) (effect of genotype: $F_{1,11}$ = 5.089; *p* = .045, repeated-measures analysis of variance [ANOVA]). **(C)** Long-term potentiation induced by triple theta burst stimulation (TBS) resulted in significantly reduced potentiation in *Nptn*^{-/-} mice (*p* = .017, repeated-measures ANOVA). **(D)** *Nptn*^{-/-} mice displayed a significantly reduced miniature excitatory postsynaptic current (mEPSC) amplitude (*n* = 10 per genotype; *p* = .029, *t* test) and a similar trend in mEPSC frequency. The kinetics of inactivation (decay-time constant τ , half-width) of both mEPSC and miniature inhibitory postsynaptic currents (mIPSCs) were not changed. **(E)** None of the mIPSC parameters was significantly affected. **(F)** Basal synaptic transmission (fEPSPs) was virtually the same in *Nptn* ^{Δ lox/loxPrCreERT} mice (Cre, blue diamonds) and *Nptn*^{lox/lox} control mice (NoCre, black diamonds). **(G)** Paired-pulse facilitation in *Nptn* ^{Δ lox/loxPrCreERT} (Cre, blue bars) was significantly reduced at interpulse intervals between 10 and 100 ms (effect of genotype: $F_{1,24}$ = 4.383; *p* = .047, repeated-measures ANOVA). **(H)** Long-term potentiation induced by triple TBS was significantly impaired in *Nptn* ^{Δ lox/loxPrCreERT} mice ($F_{1,13}$ = 7.084; *p* = .0196, repeated-measures ANOVA). **(I)** In *Nptn* ^{Δ lox/loxPrCreERT} mice (Cre, blue bars, *n* = 9), mEPSC amplitudes and frequencies were significantly reduced compared with *Nptn*^{lox/lox} (NoCre, black bars, *n* = 9) (amplitude: *p* = .0457; frequency: *p* = .0297, Welch test). The resulting reduced charge transfer per time and potential seemed to be partially compensated by a slower decay and broader half-width of mEPSCs in *Nptn* ^{Δ lox/loxPrCreERT} mice (τ : *p* = .0430; half-width: *p* = .0461, Welch test). **(J)** Strikingly, similar responses were observed for inhibitory responses, i.e., reduced mIPSC amplitudes and frequencies for *Nptn* ^{Δ lox/loxPrCreERT} (Cre, blue bars, *n* = 9) compared with *Nptn*^{lox/lox} (NoCre, black bars, *n* = 9) (amplitude: *p* = .0421; frequency *p* = .0128, Welch test), but delayed decay and increased mIPSC half-width (τ : *p* = .0370; half-width: *p* = .0307, Welch test) were seen.

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ARTICLE INFORMATION

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