Supporting Information

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SI Text

Generation of Transgenic Mice. A 1.4-kb fragment of mouse NgR1 cDNA was cloned into the *Hin*dIII-*Xba*I site of pTRE2 (BD Biosciences Clontech) and microinjected into pronuclei of fertilized mouse eggs from C57BL6 (MouseCamp, Karolinska Institutet). Transgenic mice carrying CamKII-tTA (Jackson Laboratories) and pTRE-NgR1 were crossbred to obtain NgR1 overexpressing mice. Doxycycline (Sigma-Aldrich) was administered dissolved in drinking water (100 μg/mL) to turn off transgene expression to mice carrying both the CamKII-tTA and the pTRE-NgR transgenes as well as to monotransgenic controls. Experiments in this study were approved by the Stockholm Animal Ethics committee.

In Situ Hybridization. In situ hybridization (modified from ref. 1) was performed using ^{33}P-labeled oligonucleotide DNA probes: Nogo-A: (5′-GCT CTG GAG CTG TCC TTC ACA GGT TCT GGG GTA CTG GGG AAA GAA GCA-3′), NgR1: (5′-AGT GCA GCC ACA GGA TGG TGA GTA TCC GGC ATG ACT GGA AGC TGG C-3′), transgene-specific NgR1: (5′-GGA GGC TGG ATC GGT CCC GGT GTC TTC TAT GGA GGT CAA AAC AGC GTC-3′), endogenous-specific NgR1: (5′-TTC GGG GTC GAG CGG GGC GCG TCG GGC ACT GGA AGC GGC TTC GGG GCG-3′), Lingo-1: (5′-TCC AAG ACC TTG AGT CGG TAC AGC CTC TTG AAG GAG TAG TCC CGG ATG GC-3′), Troy: (5′-TTT ATT CCT GCT ACT CGC CAG TGC TGT GCT CCA GAC TCA CGC TTT CCG-3′), p75 (5′-GGC CAC AAG GCC CAC GAC CAC AGC AGC CAG GAT GGA GCA ATA GAC AGG-3′) and BDNF (5′-CTC CAG AGT CCC ATG GGT CCG CAC ACC TGG GTA GGC CAA GCT GCC TTG-3′). When possible, specificity was ascertained by the use of two different probes for each mRNA species, targeting different areas of the mRNA and generating identical hybridization patterns, as well as by comparison with known patterns of mRNA expression. After air-drying, sections were exposed for 4–21 days to X-ray film (Biomax, Eastman Kodak) for quantification. Quantification of probe distribution as seen on the X-ray films was accomplished by digital scanning and measurements of optical density of regions of interest, using an image analysis program (ImageJ v. 1.32j, http://rsb.info.nih.gov/ij/). A ^{14}C step standard (Amersham) was included to calibrate optical density readings and to convert measured values into nCi/g. Measurements were performed on representative sections from each region studied, and a mean value calculated for each animal. Data were expressed as mean ± SEM.

Immunoblotting. Adult mouse tissues were dissected and immediately frozen until used. Tissues were sonicated in 1% sodium dodecyl sulfate and boiled for 10 min. Aliquots (100 μL) of the homogenate were used for protein content determination using the bicinchoninic acid protein assay method (Pierce). Equal amounts of protein (60 μg) from each sample were loaded onto 10% polyacrylamide gels, separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidine difluoride membranes (Amersham Pharmacia Biotech). The membranes were immunoblotted using an affinity-purified goat polyclonal antibody that selectively detects NgR1 (R&D Systems). Anti-body binding was revealed by incubation with affinity-purified goat IgG antibody diluted 1:10,000 (Rockland) and Odyssey System immunoblotting detection system. In a second experiment, to measure NgR1 protein in a more linear manner, and monitor time course of doxycycline effects, fresh frozen mouse tissues were lysed and sonicated in 0.5% Triton X-100, 3% SDS, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM EDTA containing protease inhibitor mixture (Sigma), NaF, and phosphatase inhibitor cocktails 1 and 2 (Sigma). Samples were analyzed by SDS/PAGE (NuPage 4–12% Bis-Tris Gels, Invitrogen) followed by immunoblotting. Membranes were incubated with primary antibodies against NgR1 (R&D Systems), GAPDH (Abcam), and N-Cadherin (Novus Biological), followed by labeled secondary antibodies (Alexa-680, Invitrogen or IRDye-800, Licor). The membranes were scanned using an infrared scanner (Odyssey, Licor).

Membrane Fractionation. Fresh frozen mouse tissues were homogenized using a Dounce homogenizer in hypotonic buffer (10 mM NaHCO3 with protease inhibitor and phosphatase inhibitors cocktails). After 10-min incubation on ice, nuclei and cell debris were removed by centrifugation at 1,200 × g for 10 min. The supernatant was then centrifuged at 21,600 × g for 30 min in a Beckman L7 ultracentrifuge to remove internal membranes, followed by centrifugation at 150,000 × g for 2 h to isolate plasma membranes. The pellet containing the plasma membrane was washed by resuspension in hypotonic buffer and re-centrifuged at 150,000 × g for 2 h. The plasma membrane was then solubilized in extraction buffer containing 0.50% Triton-X-100, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% SDS, and protease inhibitor and phosphatase inhibitors cocktails. The expression level of NgR in the plasma membrane fraction was determined by SDS/PAGE (NuPage 3–8% Tris-acetate Gels, Invitrogen) followed by Western blotting.

RhoA Activity Assays. A commercially available ELISA-based RhoA activity assay (G-LISA; Cytoskeleton) was used to measure the relative RhoA activity of hippocampus from L1 and control mice.

Immunohistochemistry. Mice were perfused with 4% paraformaldehyde and processed for indirect immunohistochemistry using a goat anti-Nogo receptor antibody (R&D Systems) visualized using a Cy3-conjugated donkey anti-goat antibody (Jackson Laboratory).

Spine Counts. A rapid Golgi stain (FD Rapid Golgi kit, MTR Scientific) was used according to the manufacturer's protocol. Spines were counted on the apical dendrite of pyramidal cells in parietal cerebral cortex, at least 50 μm from the soma, for a distance of 10 μm. Spines were visually categorized as mushroom shaped or non-mushroom shaped. At least 15 neurons per animal were measured, and animal averages generated from eight to nine animals per group for statistical analysis.

HPLC. Concentrations of monoamines and their metabolites in brain tissue samples were determined by high-performance liquid chromatography (2). Separations were performed using a reverse-phase column (Reprosil-Pur, C18-AQ). Monoamines and metabolites were detected using an electrochemical detector system with a high sensitivity analytical cell and appropriate software (ESA Coulochem III and EZ Chrom Elite; ESA, Dalco Chromtech AB). Tissue level values were expressed as ng/g wet weight.

Electrophysiology. Brains were rapidly removed and immersed in cold (4 °C), oxygenated media of the following composition (mM): NaCl, 87; KCl, 2.5; $MgCl_2$, 7; $CaCl_2$, 0.5; NaH_2PO_4, 1.25; glucose, 25; sucrose 45; and $NaHCO_3$, 25. Transverse slices (280 μm) were made using a vibrating tissue slicer (VT1000S, Leica Instruments), and incubated in normal media consisting of (mM): NaCl, 126; KCl, 3.0; $MgCl_2$, 1.0; $CaCl_2$, 2.4; NaH_2PO_4, 1.2; glucose, 11.0; and $NaHCO_3$, 25, saturated with 95% O2 and 5% CO_2. Individual brain

slices were transferred to a recording chamber, continuously perfused with normal media (3 mL/min), and maintained at 30–32 °C. Recordings were performed in the CA1 region of hippocampus, and the experimenter was blind to the genotype of the animal during the recordings. Extracellular fEPSPs were recorded in stratum radiatum using glass micropipette electrodes filled with 3M NaCl, and an AC amplifier (A-M Systems Model 1800). The signals were high- (10 Hz) and low-pass (10 kHz) filtered, and acquired to a personal computer at 4 kHz via an A/D board (National Instruments PCI 6251), using appropriate software (WinLTP v0.95b, courtesy of Dr. William A. Anderson, University of Bristol, Bristol, U.K.). fEPSPs were elicited by electrical stimulation of stratum radiatum at a frequency of 0.033 Hz using single, 0.1-ms pulses, delivered through a bipolar electrode constructed using formvar-insulated nichrome wire. After obtaining an input-output relationship (stimulus intensity versus peak fEPSP amplitude) for each response, the stimulus intensity was adjusted to produce a baseline fEPSP with a peak amplitude of 0.5–1 mV (30–40% of the maximum response). After at least 10 min of stable baseline, LTP was induced by either (1) high frequency stimulation (HFS) consisting of three trains of 1-s duration at 100 Hz, delivered at 20-s intervals; or (2) theta-burst stimulation (TBS) consisting of 10 bursts of five pulses at 100 Hz, with a 200-ms inter-burst interval. Both HFS and TBS were delivered at the stimulus intensity used to elicit the baseline responses. Peak amplitude and slope of the initial (1–2 ms) rising phase of the fEPSP were calculated using the acquisition software, and changes in the synaptic response were normalized to the baseline period. De-potentiation was induced by delivery of 1-Hz stimulation for either 5 or 15 min, beginning 5 min following TBS.

Rotarod Performance. Mice were assessed for balance and motor coordination on an accelerating rotarod (Ugo-Basile, Stoelting Co.). Training session 1 consisted of three rotarod encounters, with the first serving to habituate mice with a stationary rod and the next two carried out with the rod rotating at a fixed speed (4 rpm). Each trial was 180 s with 60 s inter-trial rest. Next day, during training session 2, the rod was set to rotate at an accelerating speed of 4–40 rpm. Each mouse was trained twice for 180 sec/training episode with 60 sec between the two tests. The following day, the test session consisted of 2 trials with the speed accelerating from 4–40 rpm during 300 s and with 60 s rest between trials. Latency to fall, or to rotate off the top of the turning barrel, was measured by the rotarod timer and averaged for the two test runs.

Locomotion and Open Field Tests. Spontaneous locomotion was evaluated using an automated system (AccuScan VersaMax, Accuscan Instruments). Mice were placed individually in 42 × 42 × 30 cm perspex chambers (center zone 33 × 33 cm) and locomotor activity (total distance, cm/5 min) was recorded for 60 min.

Elevated Plus-Maze. Mice were given one 5-min trial in the plus maze, which had two closed arms with gray tinted semitransparent Perspex walls 20 cm in height, and two open arms. The maze was elevated 60 cm from the floor, and the arms were 30 cm long and 5 cm wide. Mice were placed in the centre section (5 × 5 cm) and time spent in, and number of entries into the open and closed arms was recorded, using a video camera-based system (Ethovision, Noldus).

Step-Through Passive Avoidance. Passive avoidance behavior was studied as previously described (3, 4). Briefly, animals were placed in the light compartment of a two-compartment box (Ugo Basile) with a door to the dark compartment closed. Following 60 s of exploration, the door was opened. When a mouse enters the dark compartment the door closes and it receives one single shock (0.3 mA, 2 s). Retention tests were conducted after 24 h, 7 days, and 4 weeks and the latency for the animal to enter the dark compartment with all four paws was recorded. No shock was delivered. Maximum retention latency of 300 s was assigned if the animal did not enter the dark compartment. In other experiments, another passive avoidance system was instead used (TSE Systems). The first passive avoidance learning session was identical to the one described above, but during the memory test trials, the door to the dark compartment opened after 10 s and then remained open, allowing mice to enter and exit the dark compartment several times during the 300-s test period. Total time spent in the light compartment was recorded.

Running Wheel Behavior. Mice were single-housed in cages (22 × 16 × 14 cm) with free access to running wheels (circumference 12.4 cm; one revolution corresponding to 39 cm) for 5 weeks. Running activity was recorded continuously. Animals had free access to food and water.

Morris Water Maze. Spatial learning and memory were assessed in the Morris water maze. A circular water filled tank (180 cm in diameter) with a hidden 15 cm platform placed in the target quadrant of the tank, surrounded by several external cues, was used. Mice were trained by being subjected to four trials per day during 7 days. In each trial, the mouse was put into the water from one of four starting points (north, east, south and west) in a semirandom order. The trials lasted 90 s or until the mice reached the platform (escape latency). The platform location remained constant over the training period. During each trial, several parameters were recorded, and latency, swim speed and quadrant data were used for analysis (Water Maze Software). A probe trial (60 s), where the platform was removed from the tank, was performed 24 h after the last training session. To measure long-term memory, mice were tested in the water maze again (with the platform in its original position) at day 60 four times following the retention test. In an additional test, a separate group of L1 and control mice were trained as above, and subjected to a probe trial (90 s) at day 39.

Statistical Analysis. One-way ANOVA (GraphPad Prism 4.0, GraphPad Software Inc.) and generalized estimating equations (SPSS) with an independent correlation matrix and pair wise Bonferroni corrected comparisons between groups were used. Student's two-tailed *t*-test was used to analyze data from the retention tests and on individual days in the long-term memory tests.

1. Dagerlind A, Friberg K, Bean AJ, Hokfelt T (1992) Sensitive mRNA detection using unfixed tissue: Combined radioactive and non-radioactive in situ hybridization histochemistry. *Histochemistry* 98:39–49.
2. Andersson H, Luthman J, Lindqvist E, Olson L (1995) Time-course of trimethyltin effects on the monoaminergic systems of the rat brain. *Neurotoxicology* 16:201–210.
3. Madjid N, et al. (2006) 5-Hydroxytryptamine 1A receptor blockade facilitates aversive learning in mice: Interactions with cholinergic and glutamatergic mechanisms. *J Pharmacol Exp Ther* 316:581–591.
4. Stiedl O, Misane I, Spiess J, Ogren SO (2000) Involvement of the 5-HT1A receptors in classical fear conditioning in C57BL/6J mice. *J Neurosci* 20:8515–8527.

Fig. S1. Kainic acid down-regulates NgR and up-regulates BDNF mRNA in adult mouse brain. Mice were given 30 mg/kg i.p. of kainic acid, and rated for seizure-like behavior. Brains were collected at the indicated time points and sections hybridized for detection of NgR and BDNF mRNA. (*A*) Examples of film autoradiograms of NgR and BDNF mRNA hybridization patterns in wild-type mice before and 4 h after kainic acid injection. (*B*) Quantitation of mRNA signals in cortex cerebri, CA3 of hippocampus and the dentate gyrus. For definition of areas of interest, see Fig. S6). There is a rapid and marked decrease of NgR mRNA in the dentate gyrus and similar, although less pronounced, decreases in CA3 and cortex cerebri. BDNF mRNA changes markedly in the opposite direction in the same areas at the same time points. Means ± SEM. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ANOVA with Games-Howell post hoc test compared to saline treated mice). n: 0 h = 5; 0.5 h = 6; 2 h, 4 h, 24 h = 4.

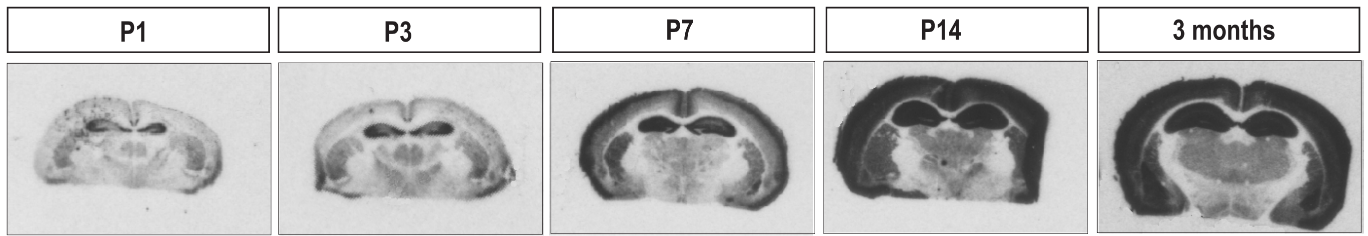

Fig. S2. Transcriptional activity of the CamKII gene in developing mice. CamKII mRNA in the brain was detected by in situ hybridization. A degree of CamKII mRNA hybridization is already seen in the newborn (P1) hippocampus. A major increase of CamKII mRNA occurs 7–14 days after birth, when the cortical mantle also becomes strongly positive. Note that nerve cell body layers are not distinguishable in the hippocampal formation, due to the well-known abundant presence of CamKII mRNA also in the dendritic fields.

Fig. S3. Schematic illustration of strategy used to obtain mice with inducible (tet-off) expression of a NgR1 transgene in forebrain neurons. Mice expressing the tetracycline transactivator under the CamKII promoter (upper left box) were crossed with mice carrying a construct consisting of a miniCMV promoter followed by the tetracycline transactivator responsive element and a NgR1 gene (upper right box). Resultant bitransgenic mice (lower left box) will express the transactivator in forebrain neurons and, because they also carry the NgR transgene construct, will express the transgene. When given tetracycline (doxycycline in the drinking water, red symbol in lower right box) transgene transcription will stop.

Fig. S4. Monoamines and monoamine metabolites in NgR overexpressing mice and RhoA activation. (*A–D*) Noradrenaline (NA), DOPAC, dopamine (DA), 5-hydroxyindoleacetic acid (5HIAA), homovanillic acid (HVA), and serotonin (5HT) levels in the substantia nigra area, striatum, hippocampus and cortex cerebri of L1 NgR overexpressing mouse brains are shown as percent of littermate control brain levels. There are no marked changes of any monoamine or metabolite levels in any of the investigated areas, although the 15.4 and 12.2% decreases of serotonin and 5HIAA in hippocampus are statistically significant. In the substantia nigra area there was a significant 24.5% increase in noradrenaline. NgR $n = 6$; controls $n = 6$. Means ± SEM. (*E*) Activation of RhoA. There is no significant difference in amount of phosphorylated RhoA between L1 NgR1 overexpressing and control hippocampus as determined by ELISA of brain tissue obtained from hippocampus. Means ± SEM.

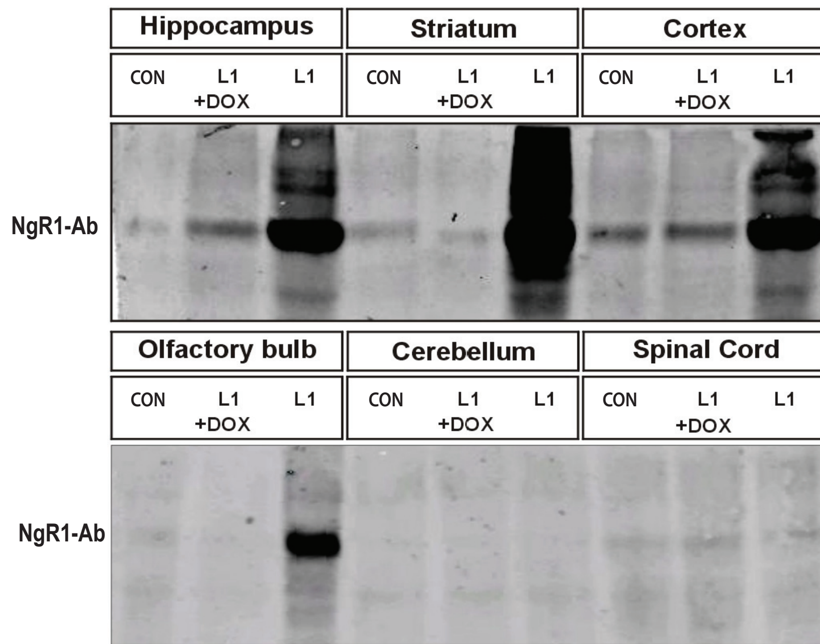

Fig. S5. NgR1 protein levels and effects of doxycycline. L1 NgR1 overexpressing mice have high amounts of protein in hippocampus, striatum, cortex cerebri, and the olfactory bulb, while levels in cerebellum and the spinal cord are below the detection level of the method. Doxycycline (DOX), here given for a month, effectively shuts down transgene activity in all four areas where it is found.

A

striatum

B

cortex
CA1
DG
CA3
thalamus
amygdala

C Lingo-1 mRNA

D Nogo mRNA

E BDNF mRNA

F Troy mRNA

Fig. S6. Areas used for quantitative in situ hybridization, and Lingo-1, Nogo, BDNF, and Troy mRNA in different brain regions. (*A*) Brain level and area (brown box) from which densitometry readings for striatal mRNA signals were taken. (*B*) Brain level and colored areas from which densitometry readings were taken for: red: cortex cerebri, green: CA1, blue: CA3, light brown: dentate gyrus, yellow: thalamus, and purple: amygdala, as indicated. Not shown is the olfactory bulb, from which densitometry readings were taken from full coronal sections of the bulb. (*C*–*F*) Quantitative in situ hybridization of Nogo, NgR coreceptors and BDNF mRNA. No marked changes of (*C*) Lingo-1, (*D*) Nogo, (*E*) BDNF, or (*F*) Troy mRNA levels in olfactory bulb (OB), cortex, thalamus, striatum, CA1, CA3, dentate gyrus (DG), or amygdala were noted in L1 NgR overexpressing mice compared to littermate controls. L1 was chosen for these comparisons because the transgene overexpression was higher than in the L2 line. $n = 3$–4 animals in each group. Means ± SEM.

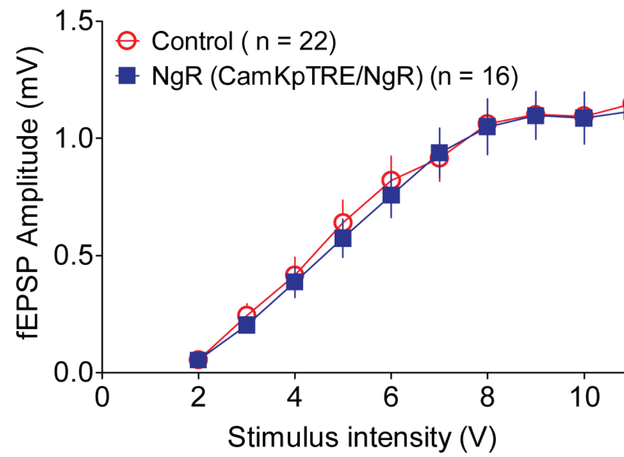

Fig. S7. fEPSP amplitudes at different stimulus intensities. Input-output relationship between stimulus intensity and fEPSP peak amplitude (means ± SEM.) in hippocampal brain slices from control and NgR mice. There is no difference between transgenic and control mice.

Fig. S8. Running wheel behavior of control and NgR transgenic mice. Total number of revolutions/week for mice with continuous access to running wheels differed between L1 and controls. Means ± SEM. (##, $P < 0.01$; ###, $P < 0.001$ compared to week 1; *, $P < 0.05$ Con vs. L1; ***, P, < 0.001 Con vs. L1.)

A

B

C

Fig. S9. Swim maze behavior of 18–19 months old control and NgR transgenic mice. (*A*) An L1 group of mice reintroduced to the swim maze at 18 months of age performed worse than controls (genotype*time $P = 0.02$). When these aged L1 mice (*B* and *C*) were again tested 30 days later, they had a tendency to spend less time in the platform quadrant (*B*) and significantly longer escape latencies compared to littermate aged controls (*C*). Means ± SEM. *, $P < 0.05$.