Supplemental Information

Activity-Induced Notch Signaling in Neurons Requires *Arc/Arg3.1* and Is Essential for Synaptic Plasticity in Hippocampal Networks

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

The methods described below are an expanded version of what is included with the manuscript and as such there is redundant text.

Animals

All mice were maintained in accordance with the Institutional Animal Care and Use Committee (IACUC) at Johns Hopkins University School of Medicine. The TNR mouse line (Mizutani et al., 2007) was generated by the Johns Hopkins Transgenic Core facility and is maintained and distributed by The Jackson Laboratory (Bar Harbor, Maine). Generation of *Arc/Arg3.1* mutant mice has been previously described (Plath et al., 2006). Unless otherwise stated (i.e. TNR, floxed *Notch1*, *Arc/Arg3.1* mutants), the mice used for tissue staining or cell cultures were CD1 (Charles River Labs) or CD1;C57BL/6 interbreds. Notch1cKO and wild-type littermate control (Notch1$^{flox/+}$, Notch1$^{flox/flox}$ and control CamKII-Cre), mice were obtained by crossing Notch1$^{flox/flox}$ mice on CD1 background to the CamKII-Cre (T29-1) mouse line on C57BL6/129 background (Tsien et al., 1996). Genotyping was done using the following primer sets: for the floxed allele, antisense 5'-CTTATTTCCCTTCCGGCTTCA-3', sense: 5'-ACTCCGACACCCAATACCTG-3'; for the Cre, antisense 5'-TGC CAC GAC CAA GTG ACA GCA ATG-3' and sense 5'-ACC AGA GAC GGA AAT CCA TCG CTC-3'. All mice used for

morphological, electrophysiological, and behavioral analyses were obtained from brother sister

matings in a closed colony, and control mice were always non-mutant siblings.

Behavioral experiments

Novel spatial exploration: All animals were caged individually, were habituated to daily

handling, and calorie-restricted (to increase exploratory behavior)(Kentros et al., 2004) for

seven consecutive days before exploration. Upon placement into the novel environment the

animals readily engaged in exploratory behavior. Cage control mice (t = 0 hr) were killed

directly from their home cages, whereas the experimental mice performed a five-minute

exploration session, were returned to their home cage, and were subsequently killed at the

indicated time point and processed to evaluate gene expression. The novel environment was a

61 x 61 cm box with 24 cm high walls containing visual cues and an open top.   Two open 50 ml

conical tubes were included to provide objects for the animals to explore. Two different

experiments were performed (see Figure S3 and Figure 3C,D). For the first experiment (Figure

S3), three-month old TNR mice were used as follows: cage control (n=4), 1.5 hr (n=3) or 8 hr

(n=4). For the second experiment (Figure 3C,D), five-week old *Arc/Arg3.1* mutants and control

mice were used: cage control (n=3 for each genotype); 45 minutes (n=3 for each genotype).

For characterization of the behavioral phenotype of the Notch1 cKO we did a battery of

tests that included the open field test, the elevated plus maze, novel object recognition, the Y-

maze, social interaction, the Morris water maze, and contextual fear conditioning. Each test

was followed by 5-7 days of rest.

The Open Field test was carried out for 60 minutes utilizing the PAS Open Field activity

chambers (SD, Instruments). Central and peripheral locomotor activity was automatically

tracked over time.

The Elevated Plus Maze test was carried out as previously described (Walf and Frye, 2007) for 10 min. The mice were videotaped and scored for time spent in each arm using the stopwatch+ software (http://www.cbn-atl.org/research/stopwatch.shtml).

The Novel Object Recognition test was done accordingly to a published protocol (Bevins and Besheer, 2006). The mice were videotaped and scored for interaction time with each object using the stopwatch+ software.

The Y-maze was made of Plexiglas, with elevated walls, and the internal surface was covered with washable non-transparent tape. The Y maze was placed on a support about 20 cm from the ground. On day one the animals were placed at the center of the maze, facing the wall and were left to explore the Y maze for 5 minutes. Alternation rate was scored. The next day one arm was blocked (hidden arm, c) and the animals were allowed to explore the remaining arms for 5 minutes. Twenty minutes after the 2-arm exploration, all arms were left open and the mice were reintroduced in the Y maze for another 5 minutes. The mice were videotaped and scored for number of entries and time spent in each arm using stopwatch+ software.

The Social Interaction testing was carried out in three sessions using a three-chambered box with openings between the chambers. After a habituation session in the middle box for 5 minutes, the doors to the right and left box were opened and for 10 minutes the subject was allowed to explore a novel subject under a metallic grid cup, placed in one box, and an empty cup in the other box. The test subject was then returned to its home cage for 30 minutes. Then the test subject was reintroduced into the middle box of the three-chamber arena, and for 10 minutes the mouse was allowed to explore the first subject under the same metallic grid cup (familiar), as well as a new subject under the previously empty cup (novel). The mice were videotaped, and scored time spent interacting/sniffing each cup in the two sessions.

The fear-conditioning test was carried out in a chamber with a metal grid bottom (Colburn, Med Associate, USA) for 5 min. During that time two shocks of 0.3 mA intensity and 2 second length were delivered with a 15 seconds interval. Twenty-four hours later the mice were

reintroduced in the chamber for 5 min without any shock. The percentage of freezing during the 2 sessions was recorded using Freezscan (CleverSys, USA).

The Morris Water Maze test was done using a published protocol (Vorhees and Williams, 2006). Latency, path length, average speed, target crossing, and quadrant occupancy were videotaped using the automated system Smart 2.5 program (SD Instruments, USA).

The Rotarod test was performed according to (Gantois et al., 2007; Mazarakis et al., 2005) using the Rotamex-2 tracking system (Columbus Instruments), and performance was expressed as latency to fall.

Significant effects of time or treatment and genotype were explored with two way-ANOVA for arm occupancy in the Y-maze, latency curves and quadrant occupancy in the Morris Water Maze, and latency to fall in the Rotarod test, otherwise for all other behavioral tests student T-test, or one-way ANOVA was used for comparison of effects between the two genotypes. A p value less than 0.05 was considered significant. Error bars show standard error if not otherwise specified.

Cell culture and *in vitro* manipulation

Neuronal cultures were prepared from the hippocampus of E17.5 embryos and plated on poly-L-lysine coated 60 mm dishes or 18 mm glass cover slips. Cultures were maintained in Neurobasal media supplemented with B27 (Invitrogen) and 10% horse serum as previously described (Banker and Cowan, 1977). Neurons were exposed to pharmacological manipulations after 14 days *in vitro* (DIV). These included a 3 min pulse of 20 μM NMDA (Sigma) in a controlled salt solution (120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 20 mM glucose, 10 μM glycine), or a 3 min pulse of 150 μM EDTA in Hepes HBSS. After either NDMA or EDTA treatment, neurons were returned to Neurobasal media for 1.5 hr before analysis. Other cultures were exposed to 40 μM bicuculline (Tocris), or 50 μM APV (Tocris) for 2-6 hr.

Actinomycin-D (Sigma) was used alone, or in combination with bicuculline (which was added 30 minutes later), at 1µM. Cultures were fixed using 4% paraformaldehyde (PFA), lysed in ice cold RIPA buffer, or collected in RNAlater solution (Qiagen).

For Sindbis virus infection, the pSinRep5 vector (Invitrogen) was used to generate viruses expressing either full-length Arc/Arg3.1, or a non-functional form with residues 91-100 deleted (Chowdhury et al., 2006). Cultured neurons, derived from the E17.5 hippocampus, were infected 2 weeks after plating, and Western Blots were performed 12–16 hr after infection.

Subcellular Fractionation

Synaptosomal fractions were prepared as previously described (Blackstone et al., 1992). All procedures were performed at 4°C, and all reagents included proteinase inhibitor cocktails (Roche). Cerebral cortical tissue from adult wild type mice was homogenized in 10 volumes of HEPES-buffered sucrose (0.32 M sucrose, 4 mM HEPES/NaOH, pH 7.4) with a glass-Teflon homogenizer. The homogenates were centrifuged at 1000 g for 10 min to remove the nuclear fraction and unbroken cells. The supernatant (S1) was spun at 10,000 g for 15 min to yield the crude synaptosomal fraction in the pellet (P2), and the supernatant (S2). The pellet was resuspended in 10 volumes of HEPES-buffered sucrose, and re-spun at 10,000 g for 15 min. The pellet (P2') was lysed by hypo-osmotic shock in water, rapidly adjusted to 4 mM HEPES, and mixed constantly for 30 min. The lysate was then centrifuged at 25,000 g for 20 min to yield the supernatant and a pellet (P3) containing the lysed synaptosomal membrane fraction.

Antibodies

Antibodies used to detect Notch1: rabbit anti-NICD1 (Cat. No. 2421, Cell Signaling), goat anti-Notch1 (Cat. No. sc-6014, Santa Cruz Biotechnology), rabbit anti-Notch1 (Cat. No. 07-220, Millipore), mouse anti-Notch1 (Cat. No. N6786, Sigma). Unless use of the anti-NICD1 antibody is indicated, the goat anti-Notch1 was used for immunostaining of cells and tissue sections, and

the mouse anti-Notch1 was used for Western blots. Other antibodies used were rabbit anti-

Jagged1 (Cat. No. sc-8303, Santa Cruz Biotechnology), goat anti-EGFP (Cat. No. 600-102-215,

Rockland), rabbit anti-Arc/Arg3.1 (Worley lab), mouse anti-Arc/Arg3.1 (Worley lab), mouse anti-

β-tubulin (Cat. No. sc-5274, Santa Cruz Biotechnology), mouse anti-β-actin (Cat. No. A4700,

Sigma), mouse anti-MAP2 (Cat. No. AB5622, Millipore), mouse anti-PSD95 (Cat. No. 75-028,

UC, Davis, NeuroMab facility), rabbit anti synaptophysin (Cat. No. A01307, Genescript) and

mouse anti-Synapsin-1 (gift of V. Dawson, JHU). Secondary antibodies were obtained from

Jackson ImmunoResearch, and in some cases the TSA kit (Perkin Elmer) was used for signal

amplification. All secondary antibodies used were raised in donkey, and when no amplification

was used, these were conjugated to Alexa 488, Alexa 555, and Alexa 652.

Immunoprecipitation

Fractionated tissue homogenates from adult cerebral cortex were and supplemented

with 0.2% deoxycholate and sonicated. The homogenates were incubated for 1.5 hours with 10

μg of rabbit anti-Notch1 or with 10 μg of purified rabbit IgG (Santa Cruz Biotechnology). Protein

G beads were added to the samples, which were incubated for another 1.5 hours. The beads

were then washed 3 times (0.1% Triton, 50 mM Tris-HCL pH.7.5, 300 mM NaCl). The fourth

wash was performed using the washing buffer containing 0.2% SDS, and the fifth wash with

PBS containing 0.1% Triton. The beads were eluted using 50 μl of 2x Laemmli Buffer (BioRad).

Western blot

Standard Western blot protocols were used. Lysates from neuronal cultures or brain

tissue were obtained using ice-cold RIPA buffer, and protein concentrations were determined

using the BCA method (BioRad). Protein samples were subjected to denaturing SDS-PAGE

and then transferred from the gel to an Immun-Blot PVDF membrane (BioRad). Membranes

were probed with primary antibodies, and HRP-conjugated secondary antibodies. A
chemiluminescent substrate (ECL+, GE Amersham) and film were used to visualize the HRP
signal. Densitometric analysis to quantitate the intensity of individual protein bands was done
using ImageJ software. Values were averaged between experiments, and Student's T-test was
used to compare treated and untreated samples, and wild type and *Arc/Arg3.1* mutant samples.

Immunostaining of tissue sections and neuronal cultures

Tissue sections were selected from the medial portion of the dorsal hippocampus (-
2.055;-2.48 mm posterior to bregma; Allen Brain Atlas), and from the primary somatosensory
cortex (-1.855;-2.055 mm). Brain tissue and neuronal cultures were fixed in 4% PFA, and post-
fixed in ice-cold acetone-methanol (1:1) at -20°C for 10 minutes. The immunostainings with
rabbit anti-Arc/Arg3.1 and anti-Notch1 antibodies were performed using the TSA fluorescence
amplification kit (Perkin Elmer). Nuclei were counterstained with DAPI (Roche). Cover slips
were mounted on slides with DABCO. Stainings were visualized using a Zeiss Axioskop with an
Axiocam, or a Zeiss LSM 510. Images were processed using Adobe Photoshop.

In situ hybridization

In situ RNA hybridization was performed on 20 μm thick coronal brain sections with
digoxigenin-labeled RNA probe for Notch1 (provided by J. Rossant) overnight at 72ºC in buffer
containing 50% formamide, and detected using an anti-DIG-AP antibody according to
manufacturer's instructions (Roche) (Wilkenson, 1992). Expression was detected by
colorimetric reaction using the alkaline phosphatase substrates: NBT (nitroblue tetrazolium
chloride) and BCIP (5-bromo-4-chloro-3-indolylphosphate p-toluidine) (Roche). Sections were
dehydrated and mounted using DPX (Fluka). Images were taken using an Axioskop 2
microscope connected to an Axiocam (Zeiss).

Image analysis

ImageJ software (NIH) was used to quantify fluorescence intensity of immunostainings with NICD1 (Figure 2A), EGFP (Figure S3B) and Notch1 (see Figure 3C,D legend). Student's T-test was used to determine p values. For analysis of the novel environmental exposure experiment presented in Figure S3, 20 z-planes were taken from 20 μm sections. On the basis of the nuclear counterstain (DAPI), neurons and glia were distinguished. Only neurons found in the middle 20% of each z-stack were included in the analyses. For Arc/Arg3.1 and Notch1 protein analysis, the cells were classified as either positive or negative. Notch1 was scored as localized to the nucleus if immunoreactivity was detected in most of the nucleus in at least three z-planes. Images of Arc/Arg3.1 immunostaining were assessed as previously described (Vazdarjanova et al., 2002). For each animal, at least four regions within CA1 from four different sections were scored. Image analysis was done by two people blind to sample identity.

Golgi Cox staining and spine imaging and analysis.

Brains from Notch1 cKO and control mice (3-5 months old) were immersed in Golgi-cox solution (FD NeuroTechnologies) and processed according to the manufacturer instructions. Dendritic length and spines density on apical and basal dendrites of CA1 pyramidal neurons were examined and imaged using a Zeiss confocal microscope and camera in Bright-field at 40x and 63x (1.5x zoom) magnification, respectively. Images were visualized using LSM Meta5 software. Six cells were imaged from each of 4 animals for each genotype. Dendrite and spine lengths and widths were measured using Reconstruct software by the Neural Systems Laboratory (http://www.bu.edu/neural/Reconstruct.html). Spine morphology analysis was done on randomly selected segments ranging from 10-20 µm in length (n = 56 segments for WT, n = 62 segments for Notch1 cKO) according to (Auffret et al., 2009). Dendritic length, spine density and morphology data were analyzed with Microsoft Excel 2007 software. Comparisons between genotypes were carried out using the Student t-test. Spine length and width data was analyzed

using the Kolmogorov-Smirnov statistical test. Data are presented as the mean ± standard

error. Values were considered significant at $p < 0.05$.

Hippocampal slice preparation

Transverse hippocampal slices (350 μm) were prepared from Notch1 cKO and control

mice, and maintained in artificial cerebrospinal fluid (120 mM NaCl; 2.5 mM KCl; 1.25 mM

NaH_2PO_4; 26 mM $NaHCO_3$; 1.3 mM $MgSO_4$; 2.5 mM $CaCl_2$ and 10 mM glucose; pH 7.4) at room

temperature. Slices were allowed to equilibrate for 1 hour prior to electrophysiological

recordings, and kept in a holding chamber up to 6 hours. The osmolality was adjusted to 290

mmol/kg, using a Vapro Pressure Osmometer (Wescor).

Electrophysiology

All recording solutions for LTP and LTD studies contained 50 μM picrotoxin to block

$GABA_A$ activity. Slices were maintained at 30-32°C during recording. Only slices having a steep

input-output curve were used for the study. LTP in acute hippocampal slices using a MED64

probe: Each hippocampal slice was placed in a MED64 probe, and stable connections were

established between the surface of the hippocampal slice and the probe's 64 planar

microelectrodes (probe dimensions: array size: 1 x 1 mm; electrode size: 50 μm; interpolar

distance: 150 μm). A stable baseline was acquired after the slice was in contact with the

electrode array for ~30 minutes and continuous perfusion with oxygenated ACSF. Theta burst

stimulation (10-100 Hz bursts of 4-400 μs pulses, each burst separated by 200 ms)(Figure 2G-

I), or high frequency stimulation (HFS, 100 Hz, 1 sec)(Figure 4I and Figure S2) were used to

induce LTP in Schaffer collateral axons of hippocampal slices. Slices were then placed in a

holding chamber with oxygenated ACSF for 3.5 hours, prior to fixing with 4% paraformaldehyde.

Field excitatory postsynaptic potentials (fEPSPs) were recorded in CA1 stratum radiatum as described previously (Wang et al. 2004). Stimuli (30 μs duration every 20 s) were delivered through a fine bipolar tungsten electrode to activate Schaffer collateral/commissural afferents. LTD was induced with 900 pulses at 1 Hz, with stimulation intensity that evoked about 30-40% of the maximum of fEPSP. The plots were normalized to the initial slope of the fEPSPs; each data point represents the averaged values for 1 minute (three consecutive sweepings with an interval of 20 seconds). Values shown are mean ± SEM. Data were collected using an Axopatch 1D amplifier (Molecular Device); signals were filtered at 2 kHz, digitized at 10 kHz and analyzed using pCLAMP 8 software (Molecular Device).

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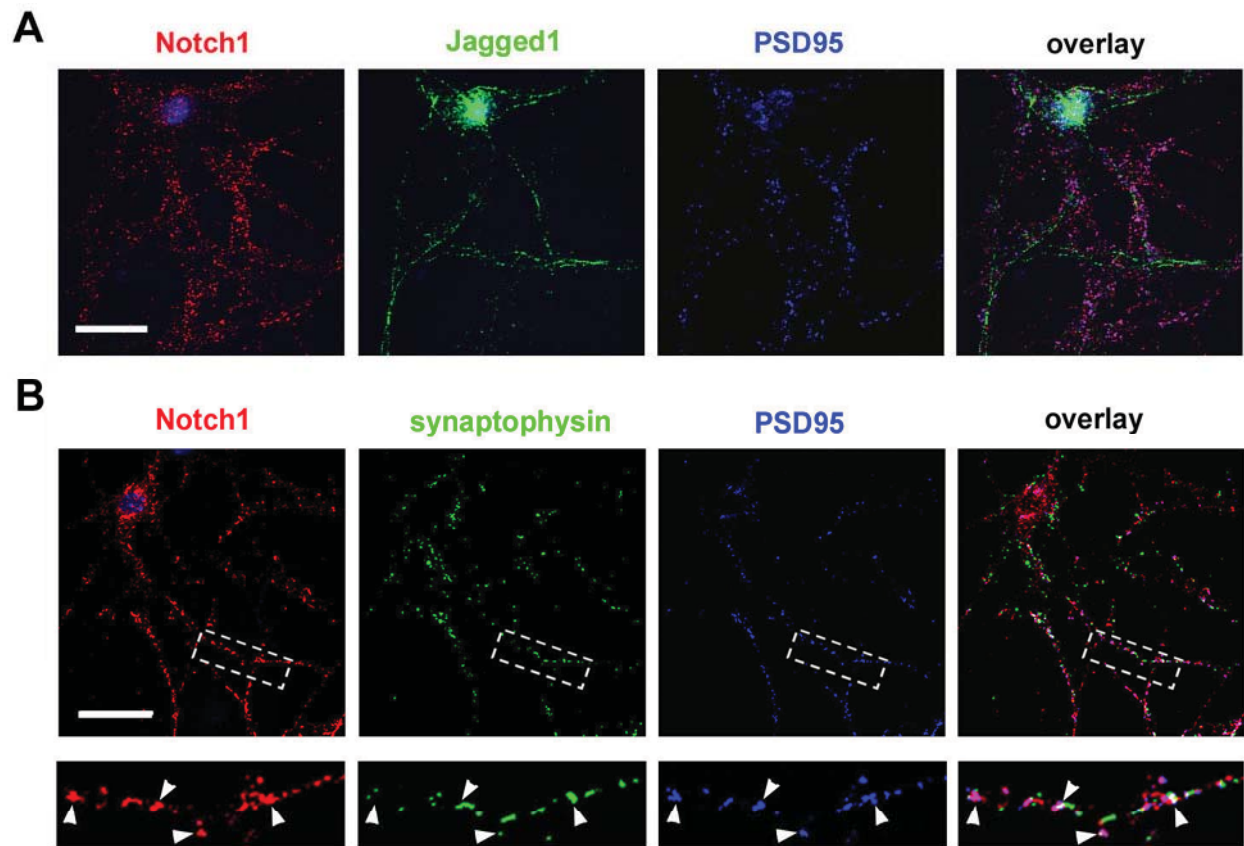
Figure S1

Figure S1. Notch1 and Jag1 expression in cultured neurons. (A) Immunocytochemistry on DIV21 hippocampal neuronal culture to detect Notch1, Jag1 and PSD95, shows that Notch1 strongly localizes with PSD95 puncta in dendrites, whereas Jag1 primarily localizes in long processes resembling axons and largely devoid of PSD95 staining. (B) Notch1 expression strongly co-localizes with PSD95 in puncta adjacent to synaptophysin positive pre-synaptic terminals (arrowheads). The lower panels show higher power views of the distal dendrite in the white box in the upper panels. Scale bars = 25 μm.
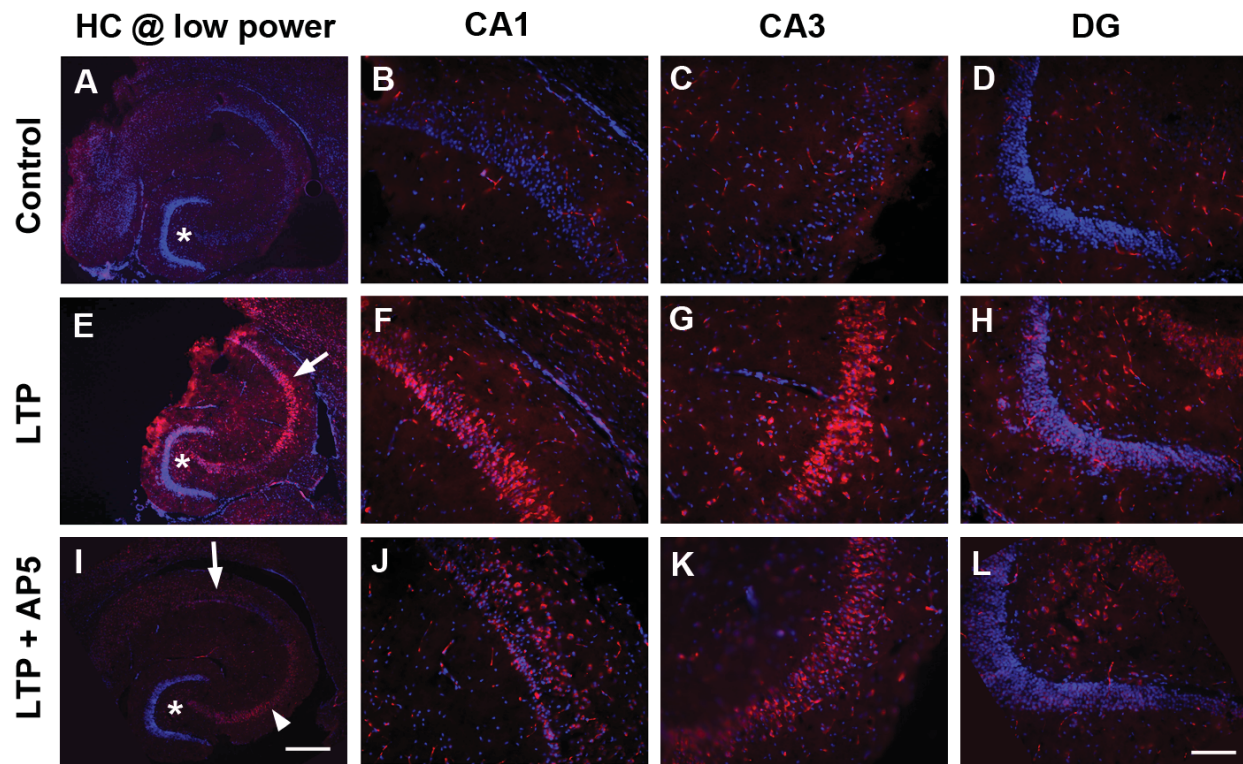
Figure S2

Figure S2. Induction of LTP increases Notch1 protein levels in hippocampal slice preparations. (A-D) Notch1 protein is present at very low levels in control mice taken directly from their home cage. (E-H) A dramatic increase in Notch1 expression occurs in both CA1 (arrow in E, F) and CA3 (G) in response to LTP induction along the Schaffer collateral pathway. (I-L) The increase in Notch1 expression in response to an LTP-inducing stimulation paradigm can be blocked by the NMDA receptor inhibitor AP5. This effect is more dramatic in CA1 (arrow in I, and compare F to J), than in CA3 (arrowhead in I, and compare G to K). The dentate gyrus is marked with an asterisk (A,E,I). Scale bars = 400 μm in I for A, E, I, and 100 μm in L for B-D, F-H, J-L. HC, hippocampus.
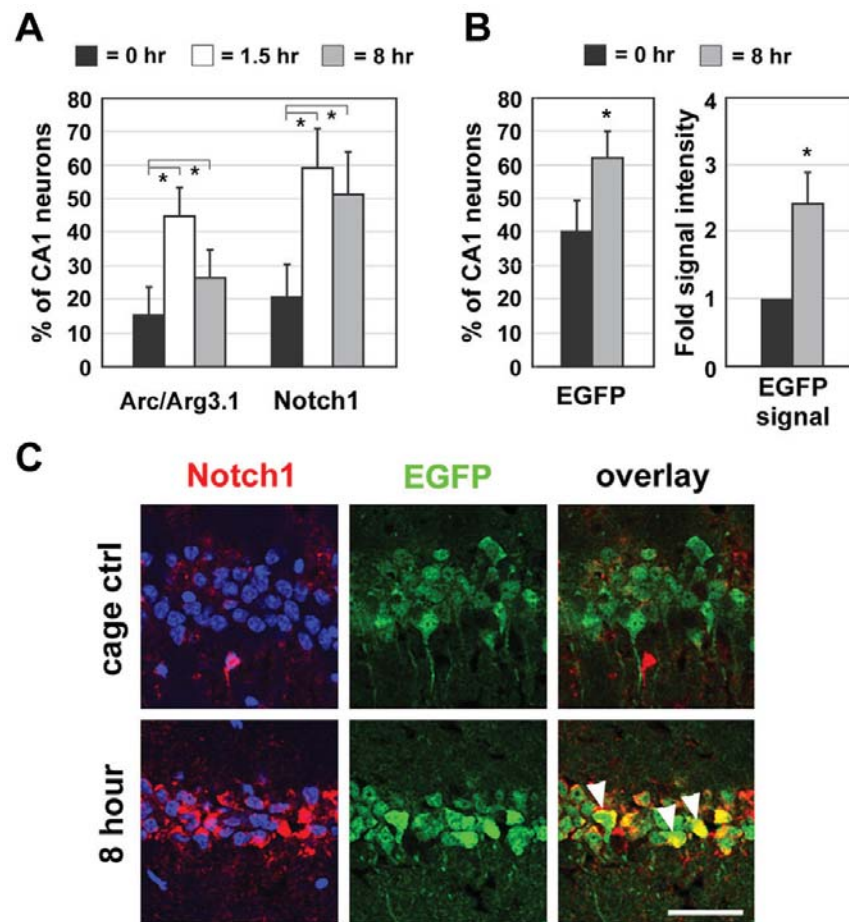
Figure S3

Figure S3. Neuronal Notch signaling occurs *in vivo* in response to exploration. TNR mice (3 months old) explored a novel environment (see Methods) for five minutes, and were then returned to their home cage for 1.5 (n = 3) or 8 (n=4) hours prior to being sacrificed. These were compared to 'cage control' animals (0 hr) not exposed to the novel environment (n = 4). (A) 1.5 hours after exploration, the number of hippocampal CA1 neurons expressing Arc/Arg3.1 and Notch1 protein dramatically increased. Eight hours after exploration, while the number of Arc/Arg3.1+ neurons decreased as compared to the 1.5-hour time point, the number of Notch1+ neurons remained elevated. (B) Both the number of EGFP+ CA1 neurons and EGFP signal intensity (quantified using ImageJ software), increased after exploration, indicating elevated Notch signaling. (C) Representative images of Notch1 and EGFP expression in a cage control and 8 hours after exploration. Examples of cells expressing both Notch1 and EGFP are indicated (arrowheads). *$p < 0.03$. S.D. is shown. Scale bar = 50 μm.
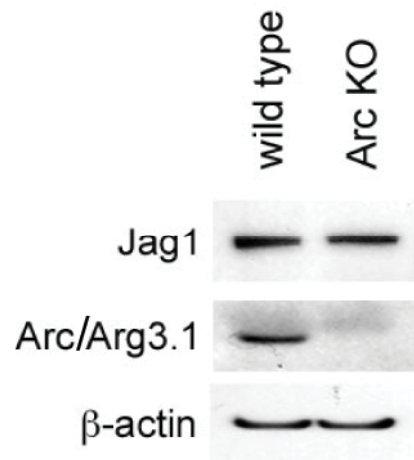
Figure S4

Figure S4. Jag1 expression is normal in neuronal cultures derived from *Arc/Arg3.1* mutants. Western blot analysis showing that neuronal cultures, derived from the E17.5 hippocampus of wild-type and *Arc.Arg3.1* knockout (KO) embryos, express the Notch ligand Jag1 at similar levels. This result suggests that the defect in Notch signaling seen in *Arc/Arg3.1* mutants is not a function of reduced ligand availability.

Figure S5

Figure S5. Notch1 S3 cleavage occurs normally during development in *Arc/Arg3.1* mutants. (A) Immunohistochemistry to detect NICD1 in E15.5 *Arc/Arg3.1* knockout (KO) or wild-type forebrains suggests that Notch signaling is normal in neural progenitors lacking Arc/Arg3.1. (B) Western blot showing that Notch1 processing is normal in the embryonic cerebral cortex of *Arc/Arg3.1* mutants. VZ, ventricular zone; CTX, cerebral cortex. Scale bar = 100 μm.

Figure S6

Figure S6. Conditional deletion of Notch1. RNA in situ hybridization was used to detect Notch1 mRNA in control animals (A), and in animals homozygous for a conditional (floxed) allele of Notch1 and heterozygous for CamKII-Cre (B). A dramatic reduction in signal is evident in the Notch1 cKO tissue in CA1 and CA3, but not in the dentate gyrus (DG) where recombination is not expected to occur with the Cre driver used. Scale bar = 150 μm

Figure S7

A

Time spent in each quadrant (%)

□ = control
■ = Notch1 cKO

p<0.001

LEFT   RIGHT   OPPOSITE   TARGET

B

Time spent in each quadrant (%)

□ = control
■ = Notch1 cKO

p<0.002

LEFT   RIGHT   OPPOSITE   TARGET

C

Test	Control	Notch1 cKO
Open Field – Total activity in chamber (beams/sec)	4218.9 ± 333.6	3748.1 ± 543.4
Open Field – (outer zone)/(inner+outer zones)(% of time)	38.2 ± 6.1	44.3 ± 9.7
Elevated Plus Maze – (open arm)/(open+closed arm)(% of time)	41.5 ± 4.4	48.8 ± 5.7
Contextual Fear Conditioning – Freezing (% of time)	48.9 ± 5.2	45.3 ± 4.3
Rotarod – Average latency to fall (sec)	80.8 ± 3.5	71.7 ± 6.1

Motor activity (Open Field, 9 Ctrl, 10 cKO), anxiety levels (Elevated Plus Maze, 10 Ctrl, 9 cKO), motor coordination (Rotarod, 12 Ctrl, 12 cKO), and spatial recognition (Fear Conditioning, 21 Ctrl, 17 cKO) in the Notch1 cKO mice are comparable to controls. For all pair wise comparisons p>0.05.
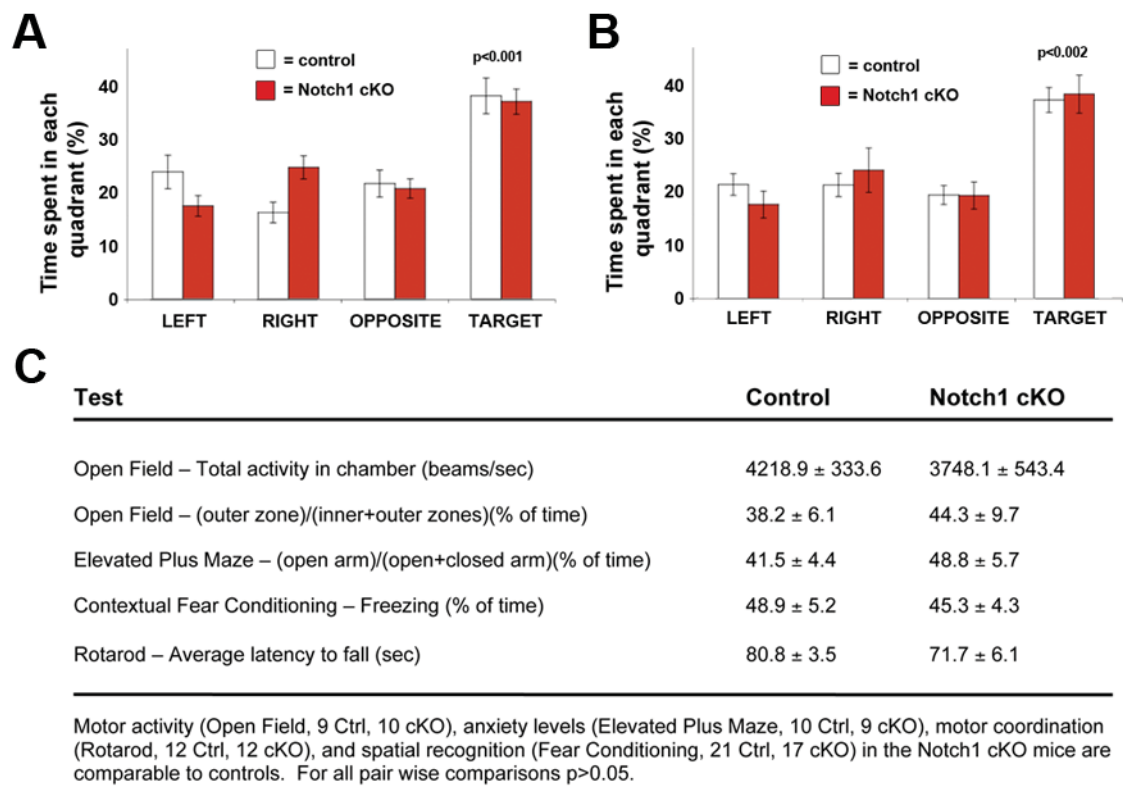
Figure S7. Notch1 cKO mice can learn after repetitive training. 24 hours after either initial (A) or reversal learning (B) both controls and Notch1 cKO animals exhibited the same increased occupancy preference for the target quadrant. (C) Behavioral tests for which there was no difference between wild type and Notch1 cKO animals.