Supporting Online Material for

Npas4 Regulates a Transcriptional Program in CA3 Required for Contextual Memory Formation

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Supporting online material (SOM)

Materials and Methods

Mice

Npas4$^{-/-}$ and Npas4$^{flx/flx}$ mutants were previously generated (*47*). All mouse lines were backcrossed at least 9 times into the C57Bl6 background (Charles River Laboratory). Heterozygous mice were bred to produce Npas4$^{-/-}$ and Npas4$^{+/+}$ littermates and Npas4$^{flx/flx}$ mice were bred as homozygotes. Mice were weaned at postnatal day 20, housed by sex in groups of 3-5, and used for experiments at 8-12 weeks of age. For gene expression and IHC experiments mice were housed individually for one week prior to conditioning. All mice were housed with a 12 hour light-dark schedule and received food and water *ad libitum*. Animal protocols were performed in accordance with NIH guidelines and approved by the Massachusetts Institute of Technology Committee on Animal Care.

Fear conditioning. On day 1, mice (8-12 weeks old) were trained in one of the following conditions:

Contextual Fear Conditioning. Mice were placed in the chamber, allowed to explore for 58s and then given three 2s 0.55mA footshocks at 58s intervals. Following the last shock mice were left in the chamber for 1 minute and then returned to their home cage.

Auditory Delay Conditioning. Mice were placed in the chamber, allowed to explore for 1 minute and then given three tone stimuli (85dB, 20s, 2.8kHz) at 58s intervals that co-terminated with a 2s 0.55mA footshock. Following the last shock mice were left in the chamber for 1 minute and then returned to their home cage.

Context Only. Mice were placed in the training chamber for 4min and then returned to their home cage.

Immediate Shock. Mice were placed in the training chamber, immediately given a 6s shock (0.55mA) and then returned to their home cage.

5min, 1hr or 24hr after training, mice were returned to the conditioning chamber for 4min to test memory recall. Separate sets of subjects were used at each time point to prevent extinction effects. Memory for the context was measured by recording freezing behavior, defined as the total absence of movement aside from that required for respiration. Memory for the tone was measured by recording freezing in a novel context during presentation of the tone. Training and testing sessions were video recorded and behavioral scoring was conducted by scorers blind to the experimental genotypes. In every case, the chamber was cleaned with 70% ethanol between subjects. Subjects used for gene expression or immunohistochemical analysis were sacrificed at various time points after the conditioning. Genotypes were compared using a one-way ANOVA followed by Holm-Sidak posthoc test or Student's t-test.

Elevated Plus Maze

The elevated plus maze consisted of two open arms, two closed arms, and a center. Animals were always placed in the same orientation and observed for 5min. Genotypes were compared using Student's t-test.

Open Field Activity

Open field activity in a novel context was measured using a Versamax Activity Monitor. One hour prior to testing, mice were placed in the testing room to habituate to the room. Mice were placed in the open field and were monitored for 10 minutes. Genotypes were compared using Student's t-test.

Foot Shock Sensitivity Assay

Assay was performed similar to (*48*). Responses to a range to footshocks (0.1mA to 0.7mA, 0.1mA steps) were measured by assigning a numerical value by a blinded scorer. 0 – no response, 1 - move, 2 – flinch, 3 – run, 4 – jump, 5- maximum response. Genotypes were compared using a two-way repeated measures ANOVA.

Immunohistochemistry

Mice were overdosed with avertin (1000mg/kg, 1.25% 2,2,2-tribromoethanol and 2.5% 2-methyl-2-butanol) and perfused with 4% paraformaldeyde in PBS. Brains were removed and post-fixed for 12hr then cryo-protected in 30% sucrose overnight. Subsequently, brains were immersed in OCT-Tissue Tek, flash frozen on dry ice, and sectioned on a cryostat at 50μm thickness. All sections were blocked for 1hr at room temperature in a solution containing 0.3% Triton X-100, 0.2% Tween-20, 3.0% BSA, and 3.0% goat serum then incubated with primary antibody overnight at 4°C. The next day, sections were rinsed in PBS and incubated in secondary antibody for 1hr at room temperature. Sections were mounted on Superfrost slides.

The following antibodies were used: c-Fos (rabbit, 1:500, Santa Cruz sc-52), Cre (mouse, 1:100, Millipore MAB3120), GFP (chicken, 1:1000, Aves GFP-1020), NeuN (mouse, 1:1000, Millipore MAB377), Parvalbumin (mouse, 1:1000, Sigma P3088), and VGAT (rabbit, 1:1000, Synaptic Systems 131002). The Npas4 antibody (rabbit, 1:10,000) was produced and validated as previously described (*47*).

Immunohistochemistry Image Analysis:

DAPI and IEG (c-Fos or Npas4) images were obtained from coronal hippocampal sections using an Olympus BX51 fluorescent microscope with a 10x objective. Image analysis was performed using MetaMorph software (Molecular Devices, Sunnyvale, CA)

employing a custom journal. Briefly, the area of interest (CA1 or CA3) was defined, creating a mask of the cells within that area based on a nuclear DAPI stain. This mask was then applied to the IEG image confining analysis to specific cell populations. A cell was considered "IEG positive" if the cell's fluorescent intensity was above background and if the area above background was greater than 25 pixels. Background was independently determined for each image in a blinded fashion. IEG density was defined as the number of counted cells divided by the DAPI mask area.

Viral Vectors

HSV vectors were prepared as previously described (*49-51*). Npas4, ΔNpas4, or Cre cDNA were cloned into the bicistronic p1005+ vector and driven by the constitutive promoter immediate-early gene IE 4/5. EGFP was expressed from a CMV promoter.

Viral Injection Surgery

Mice were anesthetized with avertin (250mg/kg, 1.25% 2,2,2-tribromoethanol and 2.5% 2-methyl-2-butanol) and monitored for depth of anesthesia throughout the procedure. Animals were secured in a stereotax (Kopf Instruments) and the skull was exposed. After resection, holes were drilled bilaterally overlying dorsal CA3 (AP -2.0, ML +/- 2.3; DV -2.3) or CA1 (AP -2.0; ML +/- 1.6; DV -1.4) using bregma as a reference point. Injections consisted of 1μL of virus ($1.5x10^8$ infectious units/mL) delivered at a rate of 50nL/minute and the needle was left in place for 20min post-injection. Incision sites were closed using Vet-Bond glue (3M) and treated with topical antibiotic and anesthetic. Mice were given i.p. injections of Buprenex (1mg/kg) and allowed to recover for 3d before behavior or expression experiments were conducted, at the point of maximum HSV viral vector expression (*49-51*). Following testing, GFP-immunoflourescence was used to

verify proper targeting of the virus. Only mice with bilateral expression of virus into the target structure (CA3 or CA1) were included for analysis. Multiple sections were taken from each animal and only animals with at least 50% of the target structure hit in the section with the highest GFP expression were included. Additionally, any mice exhibiting GFP expression within hippocampus, but outside of the target region were excluded. In total, five out of 62 mice from the Cre condition and six out of 81 mice from the rescue condition were removed from analysis following histological verification.

Dissociated Neuron Culture

Dissociated cortical and hippocampal neurons were prepared from P1 mouse pups and maintained in a humidified incubator with 5% CO_2 at 37˚C, as previously described (*47*). Cultures were maintained in Neurobasal A medium supplemented with B27 (Invitrogen) and glutamine. Neurons were plated at 100,000 per well in a 24 well plate, 1,000,000 per well in a 6 well plate, or 8,000,000 per 10cm plate. Plates were coated with poly-D-lysine. Neurons were transfected using calcium phosphate precipitation on DIV5/6. For viral transfection, neurons were infected overnight on DIV 6 with 1μL of $1.5x10^8$ infectious units/mL per 1,000,000 cells and collected in the morning on DIV 7. For stimulation experiments neurons were depolarized for 1h for RNA, 2h for western blots and ChIP, and 6h for luciferase with KCl (55mM), forskolin (10μM), BDNF (50ng/ml), NT3 (50ng/ml), NT4 (50ng/ml), NGF (100ng/ml), or EGF (100ng/ml).

RNA Extraction and Quantitative PCR (qPCR)

RNA was purified from dissociated neurons (1,000,000 neurons/prep) or dorsal hippocampal tissue (bilateral dorsal hippocampus/prep). 1000ng of RNA was reverse transcribed to cDNA using iScript Reverse Transcriptase and qPCR was performed using

SybrGreen Supermix on an iQ5 thermal cycler. Primers were verified with standard curves to ensure reliability. Optimal primer pairs were then used to evaluate levels of cDNA samples. Genes of interest were normalized to Gapdh and presented as fold changes over baseline using the delta-delta CT method (*52*). "n" represents the number of mice used. Data were complied from independent experiments each conducted in triplicate. For fear conditioning experiments a one-way or repeated measures ANOVA followed by a Holm- Sidak or Dunnett's test was used to compare expression across conditions. For culture experiments a one-way ANOVA followed by a Dunnett's test was used to compare experimental conditions to control and a two-way ANOVA followed by a Holm Sidak posthoc test was used to compare the condition (No Stim or KCl) and virus (Uninfected, GFP, or Cre).

Primers:

Npas4: forward 5'- CTGCATCTACACTCGCAAGG-3', reverse 5'- GCCACAATGTCTTCAAGCTCT-3'

c-Fos: forward 5'- ATGGGCTCTCCTGTCAACACAC-3', reverse 5'- ATGGCTGTCACCGTGGGGATAAAG-3'

Arc: forward 5'- TACCGTTAGCCCCTATGCCATC-3', reverse 5'- TGATATTGCTGAGCCTCAACTG-3'

Zif268: forward 5'- TATGAGCACCTGACCACAGAGTCC-3', reverse 5'- CGAGTCGTTTGGCTGGGATAAC-3'

GAPDH: forward 5'- CATGGCCTTCCGTGTTCCT-3', reverse 5'- TGATGTCATCATACTTGGCAGGTT-3'

Chromatin Immunoprecipitation (ChIP)

For *in vitro* experiments eight million cortical neurons were used per ChIP. For *in vivo* experiments bilateral hippocampi were used per ChIP. Samples were fixed in 1% formaldehyde, quenched with 2M glycine, and washed with cold PBS + cocktail protease inhibitor tablets. Samples were lysed in 10% SDS lysis buffer then sonicated (10% output, 5s on/30s off repeated 24 times). Samples were precleared with agarose beads for 1h at 4˚C, and then supernatant was incubated with primary antibody overnight at 4˚C. The next day, samples were incubated with agarose beads for 1hr at 4˚C. Beads were washed 2 times each with low salt, high salt, LiCl, and TE solutions. Samples were eluted in Elution Buffer then reverse crosslinked at 65˚C for at least 6hr. Samples were purified using a PCR Purification Kit (Qiagen) and processed by qPCR. "n" represents the number of mice used. Each qPCR was conducted in triplicate and normalized to a negative control region. Genotypes were compared using Student's t-test.

The following antibodies were used: RNA Polymerase II (mouse, 1:500, Convance 8WG16), and the Npas4 antibody (rabbit, 1:500).

Primers:

BDNF promoter I: forward: 5′-GTGCCTCTCGCCTAGTCATC-3′, reverse: 5′-AGGGAACAACTGCGTGAATC-3′

c-Fos promoter: forward: 5'-GCCCAGTGACGTAGGAAGTC-3', reverse: 5'-GTCGCGGTTGGAGTAGTAGG-3'

c-Fos E2: forward: 5'- CACAGATGACATCGCTCCAT-3', reverse: 5'-GCCGACGTCCTGACACTAA-3'

ß-actin promoter: forward: 5'- CCCATCGCCAAAACTCTTCA-3', reverse: 5'-GGCCACTCGAGCCATAAAAG-3'

Negative control region: forward 5'- GGACAATTCAACCGAGGAAA-3', reverse 5'-TGAACTGGTTTGGTGTGCTC-3'

Luciferase Assay

Plasmids were transfected on DIV 5/6. TK-Renilla, which expresses renilla luciferase, was co-transfected in every experiment to control for transfection efficiency. Prior to stimulation, neuronal activity was block for 1h or overnight with TTX (1μM) and APV (100μM). On DIV 7 cells were depolarized (55mM KCl, 6hr) and lysed in passive lysis buffer. Firefly luciferase levels were measured and normalized to renilla luciferase levels. Data were compiled from separate experiments each conducted in triplicate. Two-way ANOVA followed by a Holm-Sidak posthoc test was used to compare conditions (no stimulation vs KCl depolarization) and plasmids (pcDNA3 vs Cre).

Gel Electrophoresis and Immunoblotting

Samples were lysed in Laemmli buffer, separated by SDS-PAGE, and transferred to 0.45μm nitrocellulose membrane. Following transfer, the membrane was blocked with 10% nonfat milk in TBST for 1hr at 25°C and probed with specific antibodies overnight. The following day membranes were washed 3 times with TBST and incubated with horseradish peroxidase-labeled secondary antibody for 1h at room temperature. After 3 more washes, the membranes were incubated in ECL Plus reagent for 5min and then developed. The following antibodies were used: c-Fos (rabbit, 1:1000, Santa Cruz sc-52 for cultured neurons), c-Fos (rabbit, 1:1000, Synaptic Systems 226033 for tissue samples), phosphor-serine133 CREB (mouse, 1:2000, Upstate) and the Npas4 antibody (rabbit, 1:10,000).

Statistical Analysis

All data are shown as mean ± s.e.m. Data were analyzed with one-way, two-way, or repeated measure ANOVA followed by Holm-Sidak posthoc or Dunnett's post hoc tests. Student's *t*-test was used when two groups were compared.

Detailed Captions

Fig. 1. Npas4 expression is selectively induced by neuronal activity in vitro and by learning in vivo.

(A) Quantitative RT-PCR (qPCR) showing that Npas4 mRNA expression in cultured mouse hippocampal neurons (DIV 7) is selectively induced by depolarization (55mM KCl, 1hr), but not by BDNF (50ng/ml, 1hr), forskolin (10μM, 1hr), NT3 (50ng/ml, 1hr), NT4 (50ng/ml, 1hr), NGF (100ng/ml, 1hr), or EGF (100ng/ml, 1hr). Induction of Npas4 is prevented by pretreatment with the Ca^{2+} chelator EGTA (5mM, 10min), but not by treatment with cycloheximide (CHX, 10ug/ml, 1 hr). Neurons were stimulated in the presence of TTX (1μM) and APV (100μM). n = 4 independent cultures. *$p < 0.001$ compared to control, one-way ANOVA followed by Dunnett's test.

(B) Western blot showing that Npas4 protein expression in cultured mouse hippocampal neurons (DIV 7) is selectively induced by depolarization, but not by forskolin, BDNF, NT3, NT4, NGF, and EGF. All treatments were the same as in (A) except applied for 2h. Npas4 protein expression is prevented by pretreatment with the Ca^{2+} chelator EGTA (5mM, 10min), and by treatment with cycloheximide (CHX, 10ug/ml, 2 hr).

(C) qPCR analysis of IEG expression in DH after CFC. Separate groups of mice were sacrificed 5min (n = 8), 30min (n = 9-11), 1hr (n = 6), or 4.5hr (n = 5) after CFC and compared to naive home cage mice (HC, n = 10). Values are plotted relative to peak time point, please note differences in scale. Npas4 mRNA reaches peak expression 5 minutes after CFC, while c-Fos reaches peak expression 30 min after CFC. *$p < 0.001$ one-way ANOVA followed by Holm-Sidak posthoc test.

(D) Schematic of experimental design and behavioral outcomes. C+S: context + shock; C: context exposure; S: immediate shock; HC: home cage. Mice trained in CFC (C+S) showed significantly higher freezing behavior in comparison to all groups 24 hours later. Immediate shock (S), and context exposure (C) failed to produce significant freezing behavior. *$p < 0.001$, one-way ANOVA followed by Holm-Sidak posthoc test.

(E) Npas4 mRNA expression is induced under C+S (n = 8-10) and C (n = 8) conditions in comparison to S (n = 8) and HC (n = 10) conditions. c-Fos and Arc mRNA are induced by all conditions in comparison to HC. All groups were sacrificed 30min after training and compared to naive home caged mice. Please note differences in scale. * $p < 0.001$ one-way ANOVA followed by Holm-Sidak posthoc test.

Fig. 2. Npas4 global knockout mice exhibit impaired hippocampal-dependent STM and LTM.

(A,B) $Npas4^{-/-}$ and $Npas4^{+/+}$ littermates exhibit similar freezing during the training session (A) and 5min after training (B). $p = 0.879$, Student's t-test.

(C,D) 1h (C) and 24h (D) after CFC $Npas4^{-/-}$ mice freeze at a significantly lower level than $Npas4^{+/+}$ littermates. *$p \leq 0.001$, Student's t-test.

(E) 24h after auditory delay conditioning, $Npas4^{-/-}$ mice exhibit similar freezing to $Npas4^{+/+}$ mice during a tone memory test. $p = 0.859$, Student's t-test.

Fig. 3. Npas4 expression in CA3 is required for contextual fear conditioning.

(A) Upper panel: Npas4 protein expression is increased in CA3 and to a lesser extent in the dentate gyrus after CFC. Lower panel: c-Fos expression is induced in all subregions following CFC. Seizure induces Npas4 and c-Fos in all subregions of hippocampus

(kainic acid, 12mg/kg, 2h). Npas4 and c-Fos are shown in red, overlaid with blue DAPI stain. DAPI stain is not shown for seizure condition for easier viewing.

(B) Upper panel: Western blot quantification of Npas4 and c-Fos expression in DH at various time points after CFC. Npas4 is significantly expressed 30 minutes and 1h after CFC, while c-Fos is significantly induced 1.5h after CFC. n = 5 mice/condition. Values are plotted relative to peak time point. *$p < 0.04$, compared to home cage. Lower panel: Representative western blot with a cohort of two animals per condition.

(C) Immunostaining showing viral injection specifically targeted to CA3 or CA1. Images were taken 3 days after injection. Injection of Cre into wild type animals does not impair the expression of Npas4, while injection into $Npas4^{flx/flx}$ animals abolishes Npas4 expression.

(D) Mice were injected with HSV-Cre on day 0 and trained in CFC 3 days post-injection. Memory tests were given 1h and 24h (day 4) after training. 1h after CFC all groups freeze at similar levels. $p = 0.212$, one-way ANOVA followed by Holm-Sidak posthoc test. 24h after CFC $Npas4^{flx/flx}$ animals injected with Cre in CA3 exhibit impaired freezing in comparison to CA1-injected or WT controls. *$p < 0.001$, one-way ANOVA followed by Holm-Sidak posthoc test.

Fig. 4. Npas4 regulates the expression of several IEGs.

(A) Conditional deletion of Npas4 in CA3 results in a loss of c-Fos expression in CA3 *in vivo* (kainic acid, 12mg/kg, 2h).

(B) All IEGs are significantly induced by KCl treatment (55mM, 1h) in both uninfected and HSV-GFP-infected $Npas4^{flx/flx}$ hippocampal neurons (7DIV), however no induction is observed when Npas4 is deleted by HSV-Cre. Data were compiled from 3 independent

cultures, each conducted in triplicate. Please note that the basal level of Arc was elevated by viral treatment, but the fold induction of Arc following KCl depolarization is very similar in the uninfected and GFP infected conditions, suggesting that the virus is affecting the baseline expression of Arc but not preventing its activity-regulated expression. *$p < 0.001$, two-way ANOVA followed by Holm-Sidak posthoc test.

(C) Activity of BDNF promoter I (PI_{BDNF}), Npas4 promoter (P_{Npas4}), CREB (CRE) and MEF2 (MRE) reporter constructs. $Npas4^{flx/flx}$ hippocampal cells were transfected (DIV 5) with one of the reporter constructs, with or without Cre. Neurons were treated with TTX (1μM) and APV (100μM) 1hr prior to KCl treatment for 6hr. PI_{BDNF} reporter is significantly induced by depolarization, but the activity of the reporter is abolished in the absence of Npas4. P_{Npas4}, CRE, and MRE reporters show similar induction in the presence or absence of Npas4. Data were compiled from 4 independent cultures each conducted in triplicate. *$p \leq 0.001$, two-way ANOVA followed by Holm-Sidak posthoc test.

(D) ChIP experiments showing that under depolarized conditions (cultured cortical neurons, DIV 7, 55mM KCl, 2hr) Npas4 binds to PI_{BDNF} and enhancer II of c-Fos (c-Fos E2). No binding is observed at the c-Fos promoter, the β-actin promoter, or a negative control region.

Fig. 5. Npas4 is required for the recruitment of RNA Polymerase II to enhancer and promoter regions of activity-regulated genes.

(A) Localization of Pol II to PI_{BDNF} and c-Fos enhancer region E2 is dependent on Npas4. Acute deletion of Npas4 results in reduced binding of Pol II at these regions. No change is observed in Pol II binding at the c-Fos or β-actin promoter.

(B) Npas4$^{flx/flx}$ cortical cells were transfected (DIV 5) with luciferase reporters driven by either PI_{BDNF} or c-Fos promoter, with or without Cre. Neurons were treated with TTX (1μM) and APV (100μM) overnight prior to KCl treatment for 6hr on DIV 7. The activity of PI_{BDNF} reporter is significantly induced by depolarization (55mM, 6h), but its activity is abolished when Npas4 is removed by Cre. The activity of c-Fos promoter is not attenuated, but rather heightened, in the absence of Npas4. Data were compiled from 4 independent experiments each conducted in triplicate. *$p < 0.001$, two-way ANOVA followed by Holm-Sidak posthoc test.

(C) qPCR analysis of ChIP samples from seized Npas4$^{-/-}$ and Npas4$^{+/+}$ littermates (kainic acid, 12mg/kg, 2h, hippocampus). Npas4$^{-/-}$ samples showed diminished Pol II binding to PI_{BDNF} (*$p \leq 0.008$, Student's t-test, n = 7/genotype) and c-Fos E2 (*$p \leq 0.048$, Student's t-test, n = 6/genotype) relative to Npas4$^{+/+}$ littermates. No change is observed in Pol II binding at the c-Fos promoter ($p = 0.333$, Student's t-test, n = 6/genotype). Data are normalized to a negative control region and are presented as mean ± s.e.m. from separate experiments run in triplicate.

Fig. 6. Acute expression of Npas4 in CA3 reverses STM and LTM deficits observed in Npas4$^{-/-}$ mice.

(A) Immunostaining showing specific targeting and expression of Npas4 in CA3 of Npas4$^{-/-}$ mice using HSV-Npas4.

(B) Expression of HSV-Npas4 in CA3 of Npas4$^{-/-}$ mice resulted in activation of c-Fos, But expression of ΔNpas4 in CA3 did not.

(C) Expression of Npas4 resulted in activation of a PI_{BDNF} reporter. PI_{BDNF} reporter was co-transfected into $Npas4^{flx/flx}$ hippocampal cells (DIV 5) with Cre and either Npas4 or ΔNpas4. Neurons were treated with TTX (1μM) and APV (100μM) 1hr prior to KCl treatment for 6hr. Depolarization significantly induced PI_{BDNF} when Npas4 was present. Expression of Npas4, but not ΔNpas4, drives activity of PI_{BDNF} independent of KCl depolarization. *$p \leq 0.001$, two-way ANOVA followed by Holm-Sidak posthoc test. Data were compiled from 4 independent cultures each conducted in triplicate.

(D) Mice were injected on day 0 and trained in CFC 3 days post-injection. Memory tests were given 1h and 24h (day 4) after training. $Npas4^{-/-}$ mice with Npas4 injected into CA3 freeze at similar levels to $Npas4^{+/+}$ mice injected with GFP 1hr and 24hrs after training. CA1 injection of Npas4 or CA3 injection of ΔNpas4 did not rescue the memory deficit. *$p < 0.001$, one-way ANOVA followed by Holm-Sidak posthoc test.

Supplemental Data

Fig. S1. Control behaviors in Npas4$^{-/-}$ and Npas4$^{+/+}$ littermates. No differences were observed between Npas4$^{-/-}$ and Npas4$^{+/+}$ littermates in open field (A), elevated plus maze (B), and footshock sensitivity (C) assays.

(A) Open field: time moving ($p = 0.713$), time resting ($p = 0.718$), distance traveled ($p = 0.354$), and number of movements ($p = 0.841$), Student's t-test.

(B) Elevated plus maze: similar amounts of time were spent in the open ($p = 0.427$), closed ($p = 0.097$), and middle (0.100) arms, Student's t-test.

(C) Footshock reactivity: response to increasing shock intensity was not different across genotypes ($p = 0.395$), two-way repeated measures ANOVA.

Fig. S2. There are no gross alterations in hippocampal morphology in Npas4$^{-/-}$ mice as detected by NeuN, DAPI, VGAT, or parvalbumin staining.

Fig. S3. Immunostaining showing that Npas4 was expressed in several brain regions 1h after CFC. Expression was observed in hippocampus (CA3), amygdala, and various cortical regions including entorhinal cortex.

Fig S4. Immunostaining showing higher levels of Npas4 in dorsal CA3 in comparison to ventral CA3 following CFC. *$p = 0.003$, Student's t-test.

Fig. S5. Validation of the P_{Npas4} construct. P_{Npas4} was selectively activated by membrane depolarization. Cultured hippocampal neurons were transfected with P_{Npas4} at DIV 5 and

at DIV 7 treated with DMSO (1:1000), KCl (55mM), forskolin (10μM), BDNF (50ng/ml), NT3 (50ng/ml), or NT4 (50ng/ml) for 6h in the presence of TTX (1 μM) and APV (100 μM). Only KCl treatment significantly activated P_{Npas4}. Data were compiled from 2 independent cultures each conducted in triplicate.

Fig. S6. ChIP from seized $Npas4^{-/-}$ and $Npas4^{+/+}$ littermates (kainic acid, 12mg/kg, 2h, hippocampus) showing that localization of Pol II to the β-actin promoter is not altered in $Npas4^{-/-}$ mice. Results from two representative pairs of animals are shown here.

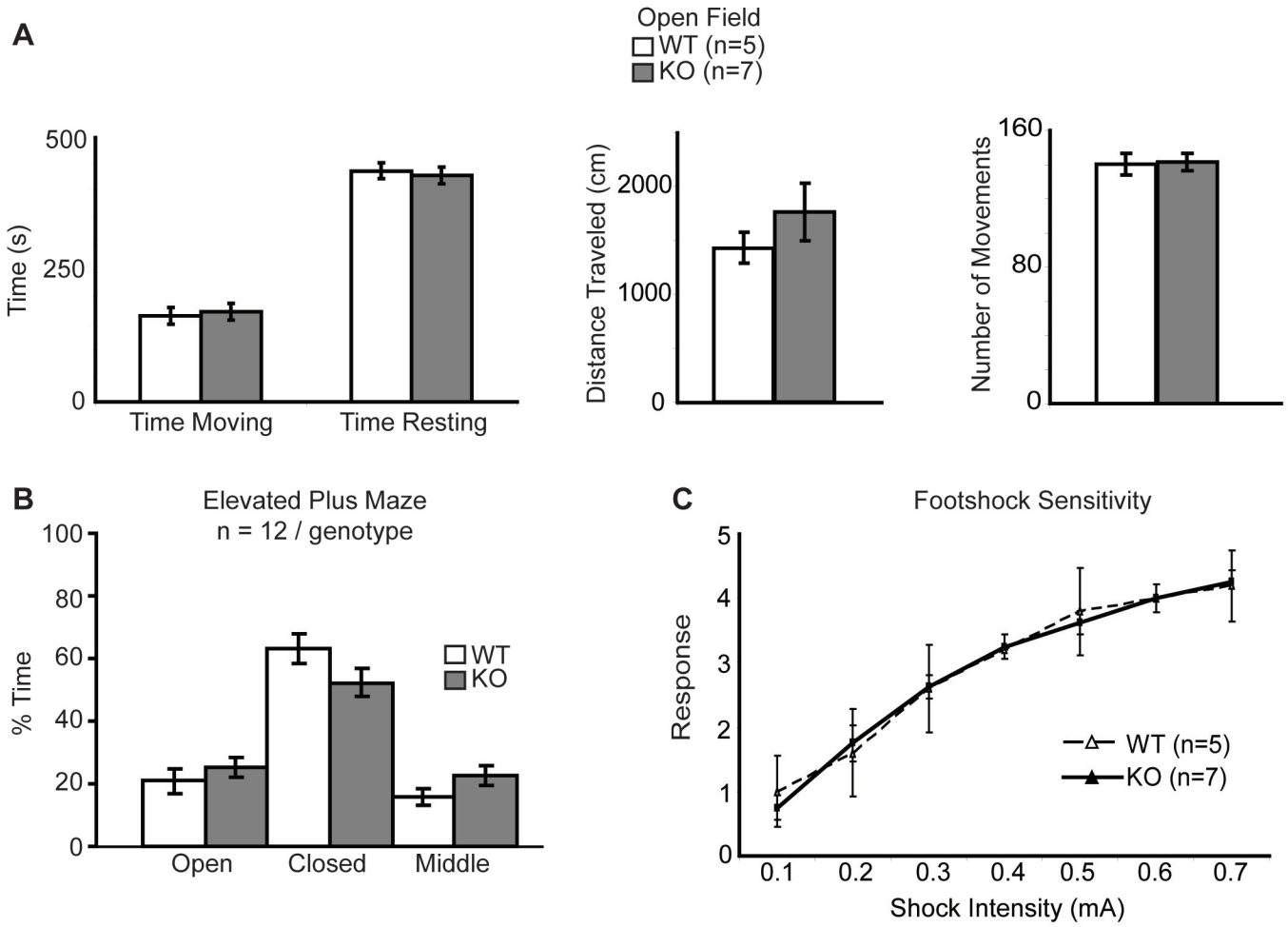
Fig. S7. Expression of the activity-regulated genes Arc, BDNF, and Narp is attenuated in Npas4 global knockouts.

(A) Mice were dark-reared for one week and then light stimulated for 1h. Induction of Arc mRNA in response to light stimulation was impaired in $Npas4^{-/-}$ mice. *p = 0.002, two-way ANOVA followed by Holm-Sidak posthoc test. WT: dark n = 2, light n = 2, KO: dark n = 2, light n = 3.

(B) The expression of BDNF and Narp mRNA in response to KCL (55mM) is impaired in cortical neurons cultured from $Npas4^{-/-}$ mice. Data are from two independent cultures each conducted in triplicate. Plotted data are normalized to the WT KCl condition. *p < 0.009, two-way ANOVA followed by Holm-Sidak posthoc test.

References

47. Y. Lin *et al.*, *Nature* **455**, 1198 (2008).
48. J. C. Alexander *et al.*, *Learn Mem* **16**, 167 (2009).
49. J. H. Han *et al.*, *Science* **316**, 457 (2007).
50. M. Barrot *et al.*, *Proc Natl Acad Sci U S A* **99**, 11435 (2002).
51. J. H. Han *et al.*, *Science* **323**, 1492 (2009).
52. K. J. Livak, T. D. Schmittgen, *Methods* **25**, 402 (2001).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80
0
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Elevated Plus Maze
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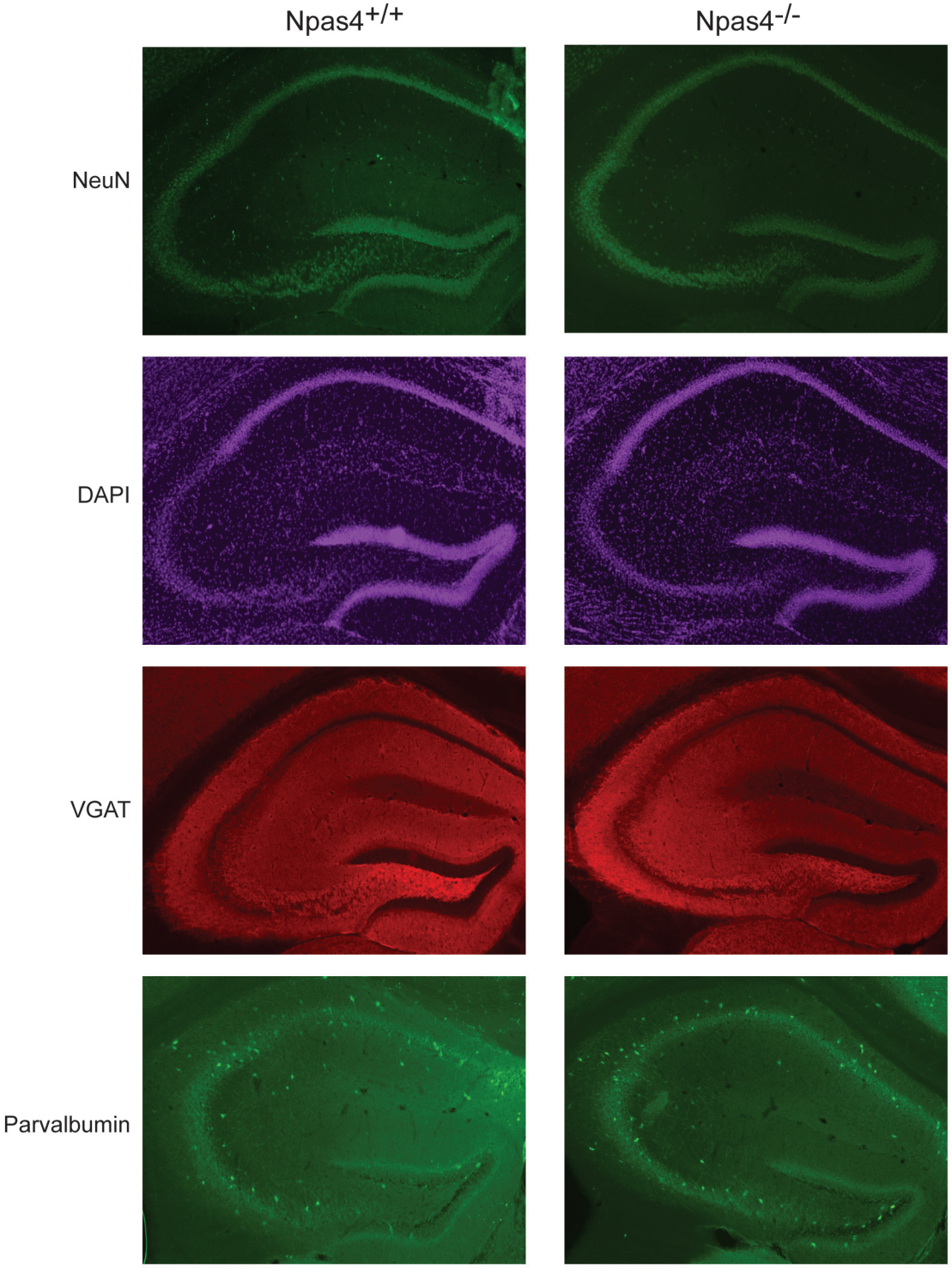
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Npas4-/-
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DAPI
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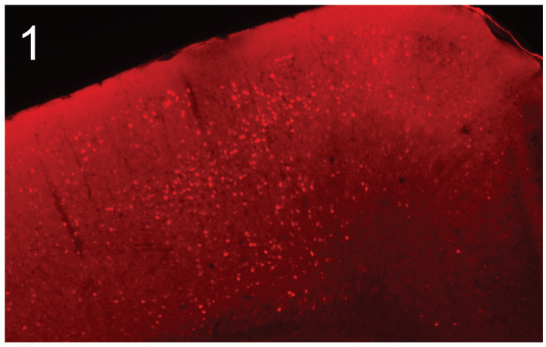
Parvalbumin

Parietal/Retrosplenial Cortex



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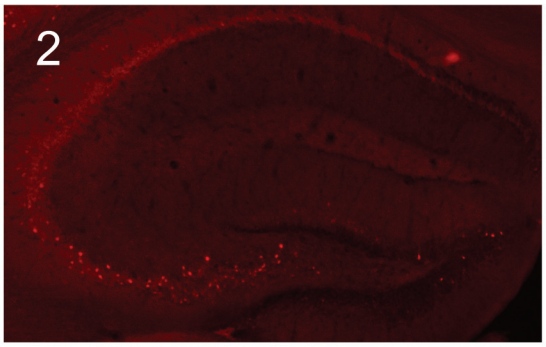
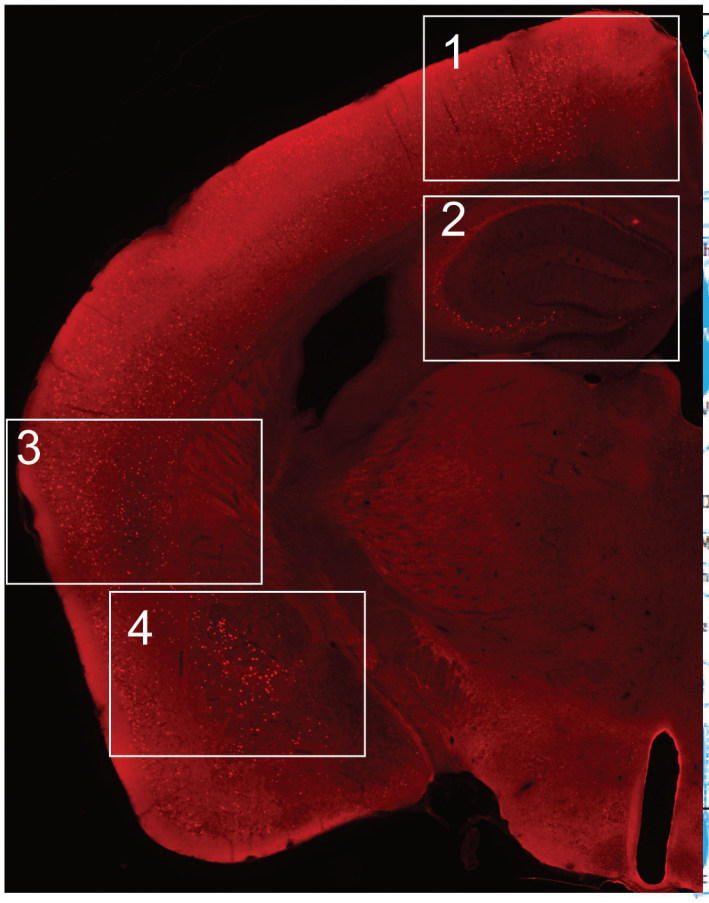
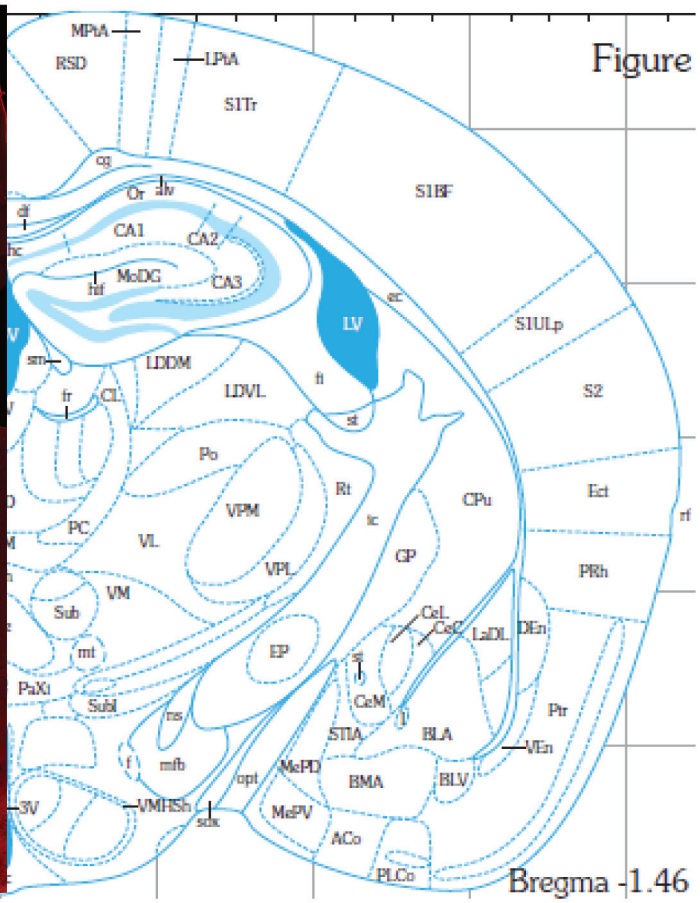
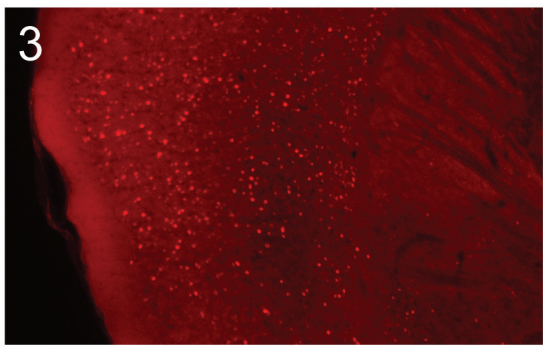


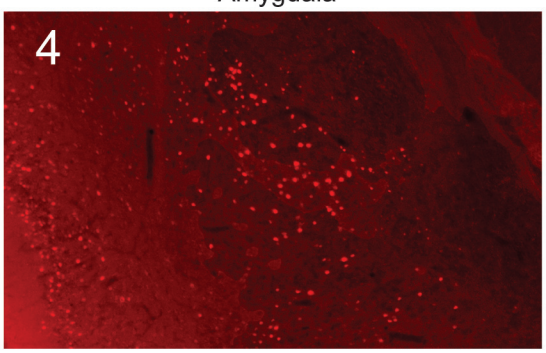


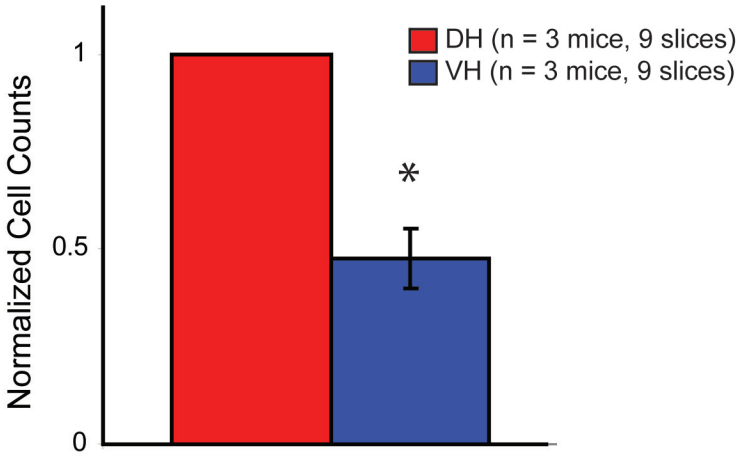


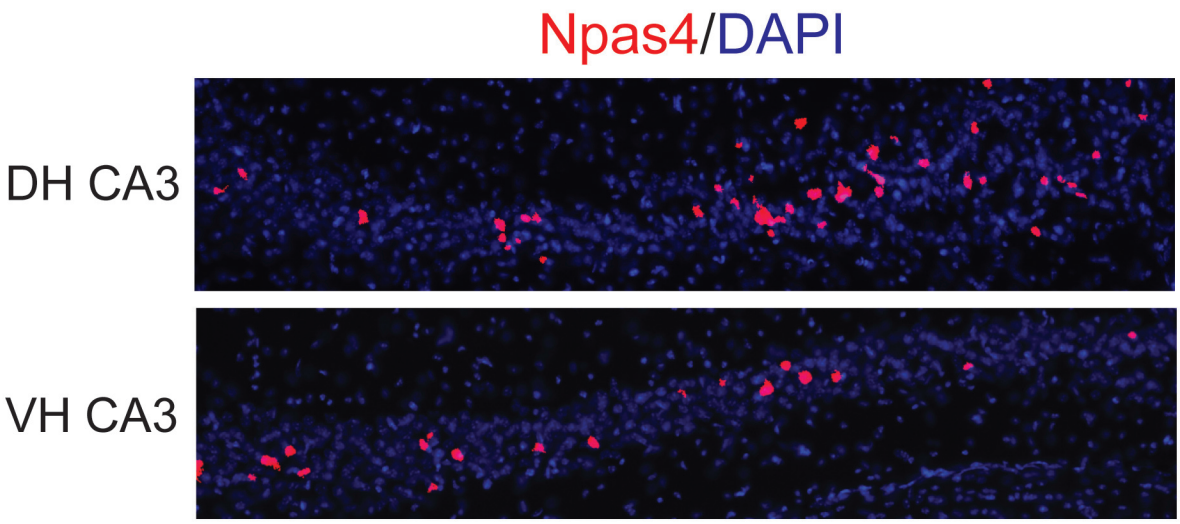
Entorhinal/Perirhinal Cortex

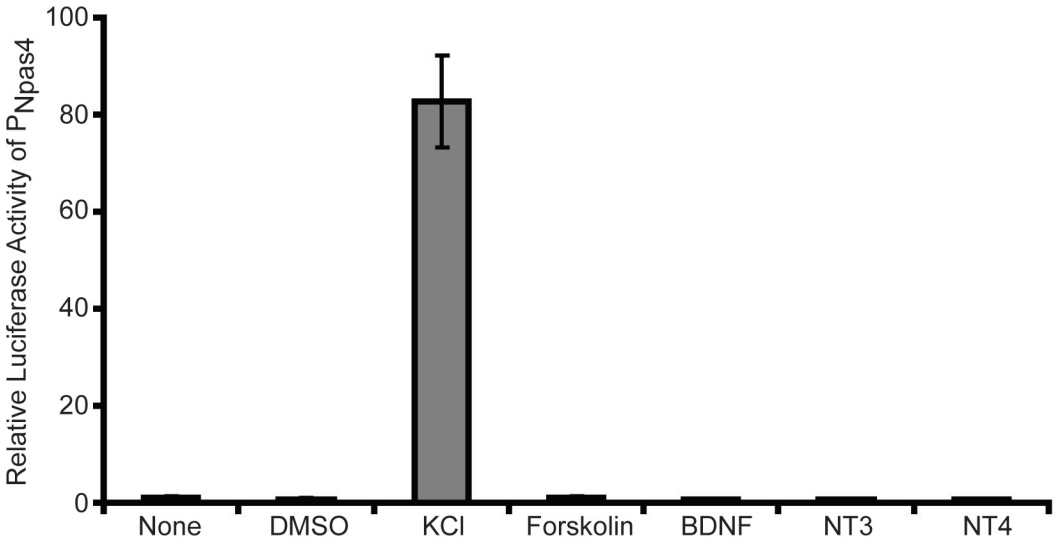


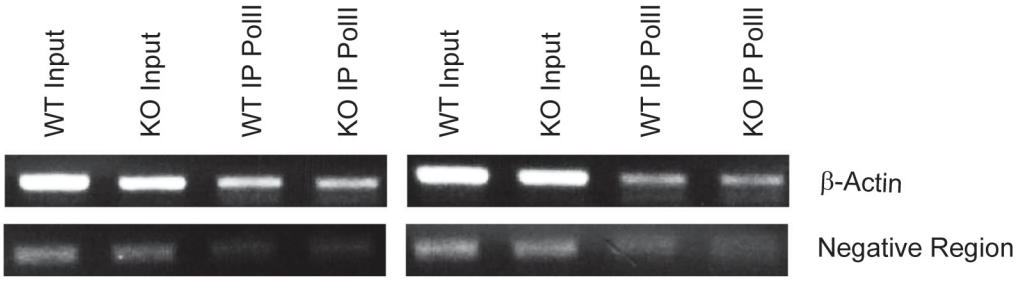
Amygdala

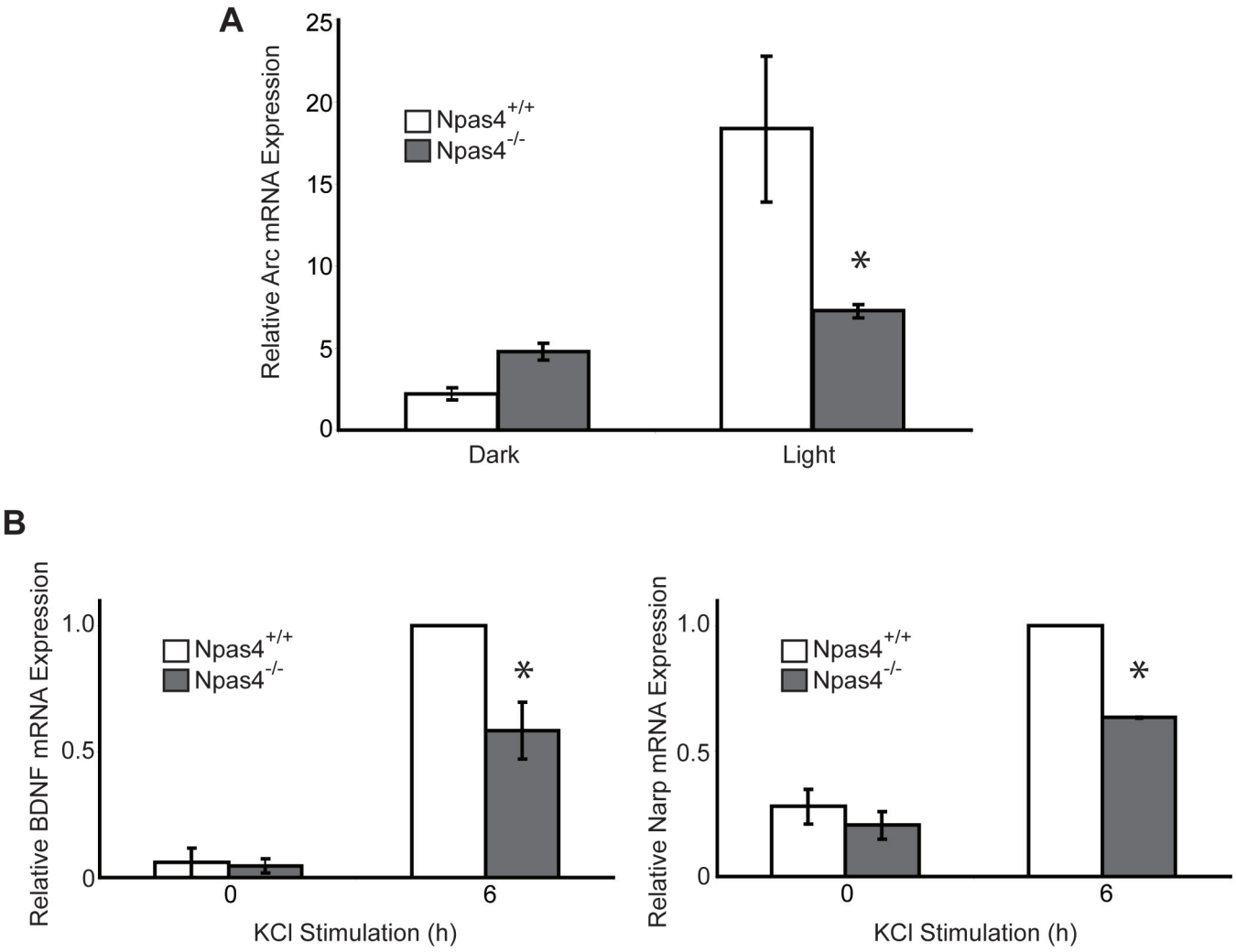



DH (n = 3 mice, 9 slices)
VH (n = 3 mice, 9 slices)
Normalized Cell Counts
1
0.5
0
*


Npas4/DAPI
DH CA3
VH CA3


Relative Luciferase Activity of P_{Npas4}
100
80
60
40
20
0
None
DMSO
KCl
Forskolin
BDNF
NT3
NT4


WT Input
KO Input
WT IP PolII
KO IP PolII
WT Input
KO Input
WT IP PolII
KO IP PolII
β-Actin
Negative Region


A
Relative Arc mRNA Expression
25
20
15
10
5
0
Npas4+/+
Npas4-/-
*
Dark
Light
B
Relative BDNF mRNA Expression
1.0
0.5
0
Npas4+/+
Npas4-/-
*
0
6
KCl Stimulation (h)
Relative Narp mRNA Expression
1.0
0.5
0
Npas4+/+
Npas4-/-
*
0
6
KCl Stimulation (h)