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Erasure of Fear Memories is Prevented by Nogo Receptor 1 in Adulthood

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Abstract

Critical periods are temporary windows of heightened neural plasticity early in development. For example, fear memories in juvenile rodents are subject to erasure following extinction training, while after closure of this critical period, extinction training only temporarily and weakly suppresses fear memories. Persistence of fear memories is important for survival, but the inability to effectively adapt to the trauma is a characteristic of post-traumatic stress disorder. We examined whether Nogo Receptor 1 (NgR1) regulates the plasticity associated with fear extinction. Loss of NgR1 function in adulthood eliminates spontaneous fear recovery and fear renewal, with a restoration of fear reacquisition rate to equal that of naïve mice; thus mimicking the phenotype observed in juvenile rodents. Regional gene disruption demonstrates that NgR1 expression is required in both the basolateral amygdala (BLA) and infralimbic (IL) cortex to prevent fear erasure. NgR1 expression by parvalbumin expressing interneurons is essential for limiting extinction-dependent plasticity. NgR1 gene deletion enhances anatomical changes of inhibitory synapse markers after extinction training. Thus, NgR1 robustly inhibits elimination of fear expression in the adult brain and could serve as a therapeutic target for anxiety disorders, such as post-traumatic stress disorder (PTSD).

INTRODUCTION

Early in life, neural circuits are remarkably plastic, such that synapses are frequently formed, lost, or modified by experience. During developmental critical periods, the neural circuits underlying behaviors, such as language and vision, undergo significant remodeling. When the critical period closes, these neural circuits and the behaviors they mediate are stabilized and are resistant against experience-dependent plasticity.

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Conflict of Interest: S.M.S. is a cofounder of Axerion Therapeutics, seeking to develop PrP- and NgR-based therapeutics.

Fear memories can be formed in juvenile rodents and undergo complete erasure following extinction training[1–3]. In this context, fear erasure refers to the complete absence of cue-elicited fear behavior following extinction training; it does not imply an underlying neural mechanism for removal of the fear memory trace itself. In contrast, pairing a neutral tone (conditioned stimulus, CS) with an aversive foot shock (unconditioned stimulus, US) results in a permanent fear memory in adult rodents. Although extinction training in adults reduces cue-elicited fear expression, the original fear memory is still intact. Rather, extinction provides a new, parallel memory that temporarily inhibits the original fear memory[4, 5]. The permanence of fear memories after extinction or fear recovery, which is the return of the fear response, can be observed by spontaneous recovery of the fear response, fear renewal when exposed to the CS in a novel context, or changes in reacquisition rates compared to naïve mice[4–8]. Critically, juvenile rodents do not show spontaneous recovery, fear renewal, or changes in reacquisition rate compared to naïve mice after extinction training[1–3].

Interestingly, the closure of the critical period for fear erasure coincides with the formation of myelin and perineuronal nets (PNNs), extracellular matrix structures composed of chondroitin sulfate proteoglycans (CSPGs), in the basolateral amygdala (BLA)[3]. Nogo Receptor 1 (NgR1), a neuronal receptor for myelin-associated inhibitors (MAIs)[9, 10] and CSPGs[11], was first identified as a myelin-dependent inhibitor of axonal regeneration[9]. NgR1 is localized on the axonal membrane, and also at pre- and post-synaptic sites[12]. Two MAIs, Nogo A and oligodendrocyte myelin glycoprotein (OMgp), are located at the synapse, as well as being expressed by oligodendrocytes[12, 13]. Thus, NgR1-regulated plasticity may occur through protein interactions with oligodendrocytes, neurons, and perineuronal nets. More recently, NgR1 has been recognized to restrict experience-dependent plasticity in adulthood. NgR1 is essential in closing the critical period for visual cortex plasticity after monocular deprivation. Specifically, the visual cortex of NgR1 null adult mice exhibits increased electrophysiological responsiveness to the non-deprived eye relative to the deprived eye[14]. Furthermore, NgR1 signaling has also been shown to increase the threshold for experience-dependent anatomical plasticity of synapses in the adult brain[15, 16]. Relatively little is known about the behavioral impact of NgR1 in the adult brain. Previous work showed that overexpression of NgR1 at supraphysiological levels impairs long-term spatial memory[17], but loss of NgR1 function does not alter a range of behavioral assays, including passive avoidance learning[18].

In order to uncover the functional and behavioral relevance of NgR1 loss in adults, we sought to determine whether blocking NgR1 signaling reinstates juvenile-like fear erasure in adulthood. We hypothesize that if NgR1 expression restricts neural plasticity in adulthood, then blocking NgR1 would restore juvenile-like patterns of extinction learning. Here, we report that extinction-trained, adult NgR1 null mice do not exhibit spontaneous recovery, fear renewal, or changes in fear reacquisition rates compared to naïve mice. Thus, adult NgR1 null mice recapitulate the fear erasure phenotype that has been observed in juvenile rodents. Deleting NgR1 specifically in the IL and BLA robustly eliminates fear expression after extinction training. NgR1 deletion from parvalbumin positive inhibitory interneurons also restores juvenile-levels of extinction plasticity. Lastly, blocking NgR1 during extinction training is correlated with more robust anatomical changes of inhibitory synapses in the amygdala. Therefore, NgR1 closes the critical period for fear erasure. In this way,

endogenous NgR1 expression maintains fear memories in adults, which is important for survival. However, in anxiety disorders, such as post-traumatic stress disorders (PTSD), the NgR1 signaling pathway provides a novel target to enhance behavioral therapy.

METHODS

Animals

Adult male C57BL6/J mice (4–6 months old) were group housed (2–5 mice per cage) throughout all experiments. All animals were kept under a 12 h light/dark cycle and provided with food and water *ad libitum*. All behavioral experiments were performed during the light cycle. All animal procedures were performed in accordance to the Yale Animal Care and Use Committee. NgR1 null, Nogo A/B null, MAG/OMgp double null, conditional mutants have been described previously[19, 20]. *PV-Cre* were obtained from Jackson Laboratories (Jax #017320)[21]. All targeted deletion and transgenic mice were on the C57BL6/J strain background, except the mixed background of the Nogo-A/B strain and litter-matched controls, as described. For conditional deletion experiment, intraperitoneal tamoxifen was given for three consecutive days 2 weeks prior to extinction to ensure complete knockdown of NgR1, as previously demonstrated[22].

Surgery

Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Body temperature was maintained with a heating pad and breathing was monitored throughout surgery. Mice were then head-fixed on a stereotaxic frame and bilaterally injected with adeno-associated virus (AAV) to the following regions: basal lateral amygdala (approximately 1.6 mm posterior to bregma; 3.1 mm later to midline and 3.1 ventral from cortical surface); infralimbic prefrontal cortex (approximately 1.7 mm anterior to bregma, 0.5 mm from midline, and 2.5 from cortical surface); prelimbic prefrontal cortex (approximately 2 mm anterior to bregma, 0.5 mm from midline, and 1.5 mm from cortical surface); and barrel cortex (approximately 3 mm posterior to bregma, 2 mm from midline and 0.5 mm from cortical surface). Mice receiving NgR(310)ecto-Fc protein or PBS were secured to a stereotaxic frame and a cannula was introduced to the right lateral ventricle at coordinates 0.6 mm from bregma, 1.2 mm from midline. Cannula was secured with cyanoacrylate and the skin was then sutured over the cannula (Alzet brain infusion kit 3).

Viral Infusions

For region-specific knockdown of NgR1, mice were bilaterally injected with 200 nl of either AAV-CMV-GFP or AAV-CMV-Cre-GFP, serotype 2/3. Delivery was completed using a glass pipette (tip diameter ~25 microns) connected to a Microsyringe and driven by a Microsyringe Pump Controller (Micro4; World Precision Instruments). Location of the viral injection was assessed after the behavioral experiment. Incorrect placement analysis resulted in the exclusion of one out of a total of 40 mice.

Pumps

Mice receiving NgR(310)ecto-Fc protein or PBS were implanted with a cannula that was connected to an micro-osmotic pump (Alzet Model 1004; 0.11 μl per hour) containing either

100 μl of protein (0.2 mg) or PBS. Recombinant protein administration and the resulting widespread CNS protein levels have been described[22–24].

Behavior

Mice were fear conditioned and extinguished in standard operant boxes in an isolated behavior room in two different contexts (context A and context B, respectively). The chambers were thoroughly cleaned with 70% ethanol or 1% acetic acid between each animal during fear conditioning and extinction, respectively. An experimenter blind to experimental conditions scored freezing behavior by measuring time spent freezing during 30 s CS+ (defined by complete immobility with the exception of respiratory movements). An additional experimenter blind to experimental condition scored a representative sample. The inter-rater reliability coefficient was ≤0.85.

Fear Conditioning—On Day 1 mice were placed into context A and presented with 5 CS + (2.5 kHz, 30 s, 80 dB), which each co-terminated with a foot shock (1 s, 0.6 mA). The CS − (30 s, white noise) was presented after CS-US pairing and was never reinforced. The time between fear conditioning and extinction varied between 24 hours and 26 days, depending on experiment.

Extinction Training—Extinction training consisted of 2 training sessions separated by 24 hours. Mice were placed into context B and were presented with 12 CS+ and 4 CS− over a 30-minute session.

Extinction Retrieval—Mice were returned to context B seven days after extinction training for extinction retrieval and presented with 2 CS+. Conditional knockout mice were returned 2 hours after extinction retrieval to context C (a novel context) to test fear renewal.

Fear Reacquisition—Most experimental groups were returned 7 days after extinction retrieval to context A for fear reacquisition, which was the identical protocol of fear conditioning.

Cue Selectivity—To test extinction selectivity to the cue, mice were fear conditioned to two distinct CS (tone (2.5 kHz, 30 s, 80 dB) or white noise (30 s)). On Day 1 mice were fear conditioned to CS1 (4 CS-US), and Day 2 mice were fear conditioned to CS2 (4 CS-US). There was no CS− used in these experiments. On Days 3–5 mice were injected with tamoxifen once per day. On Days 12–13 mice were extinguished to CS1 by presenting 12 CS1 during Day 12, and 10 CS1 during Day 13. Day 20 freezing was measured to CS1 and CS2 during a test session where mice were exposed to 2 CS1 and then 2 CS2.

Immunohistochemistry

Mice were transcardially perfused with saline and then 4% paraformaldehyde. Brains were extracted and post-fixed for 24 hours with 4% paraformaldehyde, and then equilibrated with 30% sucrose for 3 days. Brains were then sliced on a cryostat and collected as 40-micron free-floating sections. Sections were collected in phosphate buffered saline and stained with: Myelin Basic Protein (Calbiochem, NE1019, 1:1000), biotinylated *Wisteria Floribunda*

Lectin (Vector Laboratories, B1355, 1:500), NeuN (abcam, ABN91, 1:1000), Myelin Associated Glygoprotein (Millipore, MAB1567, 1:1000), Pavalbumin (abcam, ab11427, 1:1000), Gephyrin (Synaptic Systems, 147-021, 1:1000). Images for Fig 1, 3, and 5 were taken with Zeiss LSM 710 confocal microscope, and images for Fig. 4 were taken with Zeiss Axio Imager Z. All quantification was conducted using NIH ImageJ software. Images were first thresholded and then percentage of area was measured for several BLA regions from at least 2 brain slices per mouse. Imaging conditions were the same for each slice.

Immunoblot

Medial prefrontal cortex and amygdala were dissected and homogenized in RIPA buffer to measure protein expression. Protein was separated by SDS-PAGE and transferred to a nitrocellulose membrane that was then probed with anti-NgR1 (R&D Systems, AF1440, 1:1000), Gephyrin (Synaptic Systems, 147-021, 1:1000), and/or anti-Actin (Sigma-Aldrich, 3700, 1:5000). Subsequent treatments with IRDye 700CW and 800CW secondary antibodies were used (Li-Cor Biosciences, 1:10,000) to analyze the blots on an Odyssey infrared imaging system (Li-Cor Biosciences).

Statistics

All data are reported as the mean ± s.e.m. All data were analyzed by two-way ANOVA, two-way repeated measured ANOVA or *t*-test using GraphPad Prism software and IBM SPSS Statistics software. Significant main effects or interactions were followed by Bonferroni or LSD post-hoc tests as appropriate. All analyses used two-tailed tests. The criterion for statistical significance was set at $P<0.05$. Previous experiments informed the number of animals used for each subsequent experiment to ensure adequate statistical power. All data met assumptions for the specific statistical test that was performed.

RESULTS

Deleting NgR1 restores juvenile-like erasure of fear memories in adulthood

Perineuronal nets (PNNs) in the basolateral amygdala (BLA) begin to form as the critical period for fear erasure closes at postnatal day 21 (P21)[3]. However, the maturation of oligodendrocyte and MAIs has not been described in parallel. We observed minimal myelin basic protein staining in the BLA at P16, but well developed myelination by P21 and through adulthood (Fig. 1A–D and Supplementary Fig. 1). Based on this temporal pattern, we sought to determine whether NgR1 null mice exhibit fear behaviors subsequent to extinction training, or whether they show evidence for juvenile-like fear erasure. Therefore, we tested spontaneous recovery, fear renewal, and fear reacquisition in adult NgR1 null male mice after fear extinction training (Fig. 1E). Our previous studies have verified that fear conditioning and extinction of constitutive *ngr1*−/− mice is equal to wild type mice, although the rate of extinction training is more rapid in the NgR1 null mice[15]. Interestingly, NgR1 null mice do not express any evidence of spontaneous recovery of fear expression, in stark contrast to WT adult mice (Fig. 1F). One week after extinction, mice were placed back into the original fear conditioning context and tested for fear renewal, which was measured during the first tone prior to foot shock, and then subsequently measured for rate of fear reacquisition. NgR1 null mice showed no fear renewal in comparison to WT mice (Fig. 1G).

In addition, NgR1 null mice fear condition the second time at a similar rate to naïve mice, while WT mice show increased freezing during fear reacquisition (Fig. 1G). Since fear expression is higher in WT mice compared to *ngr1*−/− mice prior to receiving the first US, a direct comparison of the rate of acquisition between the two groups is not possible. The absence of fear expression is dependent on extinction training, not spontaneous forgetting, since *ngr1*−/− mice exhibit a stable fear memory over 14 days (Fig. 1H). Thus, global absence of NgR1 allows robust and efficient elimination of the conditioned fear response following extinction training.

We next sought to determine whether NgR1 deletion in adulthood after fear conditioning in the presence of NgR1 could restore juvenile-like fear erasure. To assess the temporal role of NgR1, we used tamoxifen-inducible gene recombination. The *ngr1*$^{flox/flox}$ *cre* mice ubiquitously express estrogen-regulated Cre fusion protein, and this strain yields complete global loss of NgR1 protein and mRNA within 6 days of tamoxifen treatment[22]. The *ngr1*$^{flox/flox}$ mice with or without Cre were fear conditioned with intact NgR1 expression, and then treated with tamoxifen two weeks prior to extinction training (Fig. 2A). The 17-day delay between fear conditioning and extinction creates a strong fear memory, such that control mice show slow and limited extinction of fear behavior[25]. The *ngr1*$^{flox/flox}$ *cre* mice show a 3-fold decrease in freezing to the CS during late extinction compared to *ngr1*$^{flox/flox}$ mice without Cre expression (Fig. 2B). In addition, the *ngr1*$^{flox/flox}$ *cre* mice do not express any evidence of spontaneous recovery or fear renewal (Fig. 2C). We conclude that NgR1 is required during or after adult extinction training in order to eliminate fear expression.

Next, we examined whether the sustained loss of fear expression after extinction training in NgR1 null mice is cue specific. In WT adult rodents, extinction learning is selective to the CS that is presented and does not transfer to a CS that was not explicitly presented during extinction training[26–28]. In order to test learning selectivity, *ngr1*$^{flox/flox}$ mice with or without Cre were fear conditioned to two different CS (tone and white noise) on two separate days (Day 1: CS1-US; Day 2: CS2-US) (Supplementary Fig. 2A–B). One week following a three-day tamoxifen treatment, extinction training for CS1 only was performed on two consecutive days. One week after the last day of extinction for CS1, mice were presented with CS1 and CS2 to test freezing levels (Supplementary Fig. 2C). Both groups show selective extinction learning for CS1 compared to CS2 during the test on Day 20. However, unlike *ngr1*$^{flox/flox}$ mice without Cre, the *ngr1*$^{flox/flox}$ mice expressing Cre exhibit no spontaneous fear recovery (Supplementary Fig. 2D). Therefore, deleting NgR1 during extinction training produces a CS selective erasure of fear expression that depends on extinction training.

Multiple NgR1 Ligands Contribute to the Adult Extinction Phenotype

Next, we considered whether NgR1 ligands are involved in limiting extinction. First, we blocked the three MAIs known to interact with NgR1 (Nogo A, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp)) by infusing NgR(310)ecto-Fc intracerebroventricularly[22–24, 29–31]. This reagent does not contain the CSPG binding site[11] and therefore is specifically blocking NogoA/OMgp/MAG interactions with endogenous NgR1. Following extinction training, mice treated with NgR(310)ecto-Fc

do not exhibit spontaneous recovery or fear renewal, and show comparable rates of reacquisition to naïve mice (Fig. 3A, B). These data indicate that one or more of the MAIs acting via NgR1 limits juvenile-like fear erasure in adults in a CSPG-independent manner.

We sought to determine which NgR1 ligands are involved in regulating fear recovery after extinction training in adults. The *nogoA/B*−/− mice fail to show spontaneous recovery after extinction training, which is significantly different from WT controls (Fig. 3D). We also investigated the role of MAG and OMgp in this paradigm, noting that MAG is expressed by oligodendrocytes in the BLA (Fig. 3C). The *mag/omgp*−/− mice exhibit no evidence of spontaneous recovery, in contrast to WT controls (Fig. 3E). Thus, elimination of either Nogo-A or MAG/OMgp results in long-term suppression of fear expression. These results elucidate the molecular specificity of ligands involved in restricting fear extinction through NgR1; however, the cellular specificity, i.e. neuron-to-neuron interaction versus neuron-to-oligodendrocyte interaction, is not resolved.

Infralimbic and Basolateral Amygdala Expression of NgR1 is Necessary to Block Fear Expression from Erasure

Since NgR1 and MAIs are expressed at high levels throughout the brain and regulate plasticity broadly, we aimed to identify the neuroanatomical locus for NgR1 control of extinction. At the regional level, the BLA is known to be the epicenter for fear acquisition and extinction processes[32–34], and the infralimbic (IL) cortex regulates extinction retrieval and recall through interactions with the amygdala[35–37]. We used *ngr1*$^{flox/flox}$ mice and injected AAV-Cre-GFP or AAV-GFP into either BLA, IL, prelimbic (PL) or barrel cortex (Fig. 4A, B, E, H and Supplementary Fig. 3D). Deleting NgR1 expression from cells in either BLA or IL after fear conditioning eliminates spontaneous fear recovery in contrast to AAV-GFP treated mice and yields rates of fear reacquisition equivalent to naïve animals (Fig. 4B–G and Supplementary Fig. 3A–B). The cortical domain is specific for IL, because injection of AAV-Cre-GFP into adjacent PL or more distant barrel cortex causes no change in fear expression after extinction training (Fig. 4H–J and Supplementary Fig. 3C–J). Therefore, deletion of NgR1 within IL and BLA circuits is sufficient to produce robust and sustained loss of fear expression following extinction training.

Expression of NgR1 by PV^{+} Interneurons is Required to Suppress Extinction Efficacy

Separate from neuroanatomical specificity, we considered cell-type specificity for NgR1 regulation of fear circuit plasticity in adult mice. Recently, several reports have demonstrated that parvalbumin (PV) positive cells, which are fast spiking inhibitory interneurons, regulate fear expression and fear learning[38–41]. However, their role in extinction training and fear erasure is not as well defined. To examine the role of NgR1 in this cell type, *ngr1*$^{flox/flox}$ mice expressing Cre from the PV promoter (NgR1 flox; PV Cre) were fear conditioned and extinguished as previously described (Fig. 1E)[21]. We found that mice lacking NgR1 on PV^{+} cells do not show spontaneous recovery one-week post extinction, unlike *ngr1*^{flox} controls (Fig. 5A). The control *ngr1*^{flox} mice exhibit fear renewal during fear reacquisition one-week post extinction retrieval and enhanced fear reacquisition compared to naïve mice (Fig. 5B). However, experimental *ngr1*^{flox}: *PV-cre* mice show a statistically significant reduction of fear renewal, and reacquisition rates are similar to naïve mice (Fig. 5B). Therefore, NgR1

expression by PV^+ interneurons is required for full adult blockade of fear erasure following extinction training.

We considered the cellular basis of these NgR1 effects at the biochemical and anatomical level. As a first step, we assessed whether fear extinction altered protein expression of NgR1 itself. While there is evidence that increased neural activity via seizures or exercise down-regulates NgR1 mRNA expression[42], extinction training has no effect of NgR1 protein levels in either the amygdala or medial prefrontal cortex 30 minutes after the Late Extinction (Supplementary Fig. 4). Because NgR1 is known to gate downstream actin-based cytoskeletal changes triggered by activity and necessary for anatomical rearrangement of circuits[13, 15, 16], we assessed structural plasticity of inhibitory processes. Since knocking out NgR1 from PV^+ cells produces robust and sustained extinction, we measured PV and gephyrin puncta in the BLA (Fig. 5C–N). Co-staining with gephyrin reveals that many PV puncta are adjacent to gephyrin puncta, consistent with synaptic structures (Fig. 5K–N). The *ngr1*−/− mice show increases of PV and gephyrin puncta 24 hours following extinction training, while WT mice do not show changes in such PV or gephyrin puncta after extinction (Fig. 5O–P). The extinction-induced changes in gephyrin were confirmed with immunoblot analysis from extracted amygdala tissue (Fig. 5Q–R). The *ngr1*-null-selective changes in inhibitory processes following extinction support the hypothesis that increasing inhibitory inputs in the BLA is critical for efficient extinction. In addition to greater extinction-responsiveness of inhibitory synapse markers in *ngr1*−/− mice, there is a statistically non-significant trend to a decrease of baseline gephyrin expression in naïve *ngr1*−/− mice by immunohistology and by immunoblot, consistent with previous literature[43]. Together, the data support the hypothesis that NgR1 limits experience-dependent structural plasticity of inhibitory processes in the amygdala.

DISCUSSION

The major finding of the current study is that blockade of a single receptor, NgR1, allows robust extinction-dependent elimination of the conditioned fear response, thereby restoring juvenile-like erasure of fear expression in adults. Specifically, deleting NgR1 expression, prior to or following fear conditioning, strongly enhances extinction compared to control mice. During fear reacquisition in a second conditioning session after extinction, NgR1 null mice exhibit fear acquisition comparable to naïve mice, whereas WT mice exhibit higher levels of freezing during fear reacquisition. These phenotypes are achieved by a selective loss of NgR1 in BLA or IL cortex. In addition, increasing plasticity in PV^+ cells by removal of NgR1 restores juvenile-like levels of extinction efficiency.

While our data demonstrate a powerful role for NgR1 in long-term loss of fear expression, we cannot conclude whether these changes are a result of erasure of the original fear memory, enhanced extinction learning, impaired reconsolidation during the first day of extinction training, or some combination of these mechanisms. Although the extinction mechanism for juvenile rodents is described as erasure of the original fear memory, we do not conclude that loss of NgR1 reverts the adult brain to juvenile-like mechanisms of fear extinction. Adult *ngr1*−/− mice may utilize distinct neural mechanisms from juvenile-aged rodents, even though the behavioral output is similar following extinction training.

Our results define a molecular specificity for MAIs as NgR1 ligands in fear extinction. However, the cell-type specificity of these effects remains to be fully elucidated. Since Nogo A and OMgp are expressed by neurons and oligodendrocytes, their actions may occur at the synapse via interneuronal interactions, or through oligodendrocyte contacts with neurons, or some combination. Creating cell-specific Nogo and OMgp conditional knockouts will address the cellular mechanism of their action through NgR1 to regulate extinction of fear memories.

While global NgR1 signaling is an essential molecular regulator of extinction-dependent elimination of fear memories in the adult brain, we demonstrate that the protein inhibits extinction-induced plasticity in a region and sub-region-specific manner. Our data indicate that deleting NgR1 from the amygdala permits robust elimination of fear expression following extinction training. It is striking that NgR1 deletion from IL alone is also sufficient to produce permanent fear extinction. Rodent IL cortex correlates most closely with human ventromedial prefrontal cortex (vmPFC), a region strongly implicated in human fear extinction, post-traumatic stress disorder (PTSD) and other psychiatric conditions[44]. Although the amygdala in both rodent and human studies is the source of fear, the vmPFC executes extinction of the fear memory[45]. Functional imaging studies found PTSD patients exhibit hypoactivity in vmPFC and hyperactivity in the amygdala[46]. In healthy adults, functional imaging studies revealed increased activation during extinction learning in vmPFC was correlative with increased extinction success[47]. The robust effect of NgR1 deletion during extinction training suggests a therapeutic potential for targeting NgR1 in anxiety disorders, including PTSD, to enhance the efficacy of cognitive behavioral therapies. This possibility is supported by intervention with NgR(310)ecto-Fc, a pharmacological tool that blocks the action of the MAIs, Nogo A/B, MAG, and OMgp, but not CSPGs. In contrast, previous studies showed that degrading CSPGs only before fear conditioning, but not after, allowed for erasure of the original fear memory after extinction[3], limiting translational relevance of CSPG modulation.

The role of specific cell types in fear expression, extinction, and behavioral flexibility has only recently been investigated and is still not fully understood. Several reports have shown that PV^+ cells are key players in fear expression, conditioning, and extinction learning[38–41]. In fact, increasing PV^+ cell activity during the CS enhanced fear learning and resulted in stronger response in principal neurons in the BLA[41]. We found that eliminating NgR1 expression from PV^+ cells enhances extinction learning in the adult mouse. Therefore, removing NgR1 from PV^+ cells alone is sufficient to permanently diminish the fear response following extinction training.

Previous reports have shown that PV^+ cells undergo structural remodeling following extinction learning and that extinction training increases levels of gephyrin mRNA and protein[38, 48, 49]. In addition, changes in the GABAergic system have been implicated with the regulation of critical periods[50]. Critically, extinction training increases both presynaptic PV and post-synaptic gephyrin selectively in the BLA of mice lacking NgR1, and not in WT mice. Thus, removing NgR1 during extinction training enhances anatomical changes of inhibitory processes in the amygdala, which may be involved in the sustained loss or suppression of fear expression. In addition, we noted a statistically non-significant trend to a

decrease of gephyrin expression in *ngr1*−/− naïve mice by immunohistology and by immunoblot. It has been reported that *ngr1*−/− mice have decreased spontaneous inhibitory post-synaptic potential (sIPSC) frequency in visual cortex[43]. Therefore, it is possible that there are decreases or changes of inhibitory synapses in naïve *ngr1*−/− mice compared to naïve WT mice, in addition to pronounced alteration in responsiveness to extinction training.

While NgR1 is required in PV^+ cells to prevent complete loss of fear expression after extinction, NgR1 may be required in other cells as well. Future studies deleting the protein from other cell types implicated in the fear memory circuits will reveal whether NgR1 is also required in components of the fear circuit other than the PV^+ cells. The possibility to employ enhanced structural plasticity for therapeutic purposes in neuropsychiatric disorders, such as PTSD disorder provides fertile ground for new investigations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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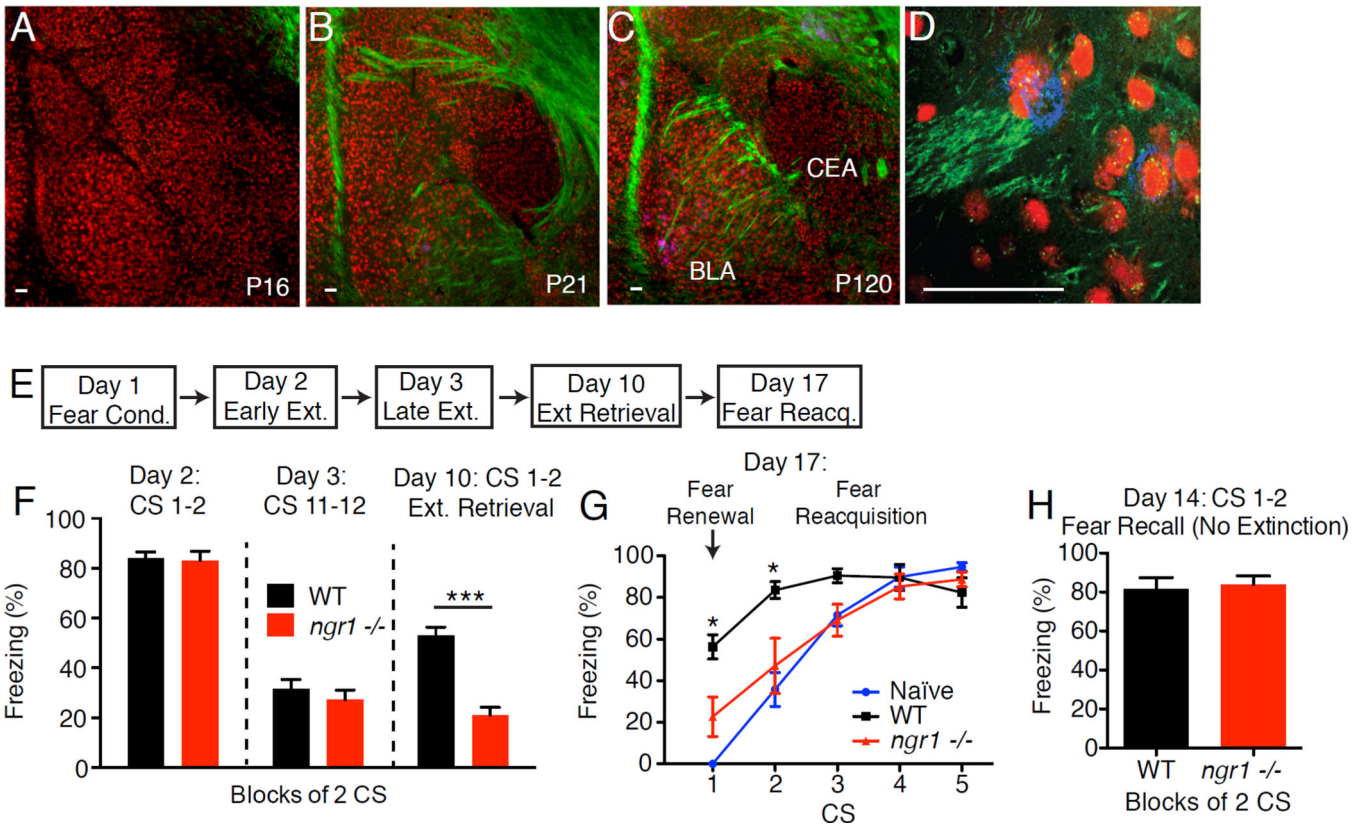

Figure 1. Elimination of NgR1 permits robust loss of fear expression in adult mice

(A–D) Coronal sections of adult BLA contain high level of both myelin (green; MBP) and PNNs (blue; WFA) surrounding neurons (red; NeuN) in low (left) or high (right) magnification views of immunohistology. Scale bars, 50 μm. **(E)** Schematic of behavioral paradigm for experiments in F-G. **(F)** One week after extinction, *ngr1*−/− mice exhibit no spontaneous recovery (n=18, WT; n=15, *ngr1*−/− mice) (WT: 52.9 ± 3.5, *ngr1*−/−: 21.1 ± 3.2; $t(31)=6.672$; $P<0.0001$, two-tailed unpaired t-test), **(G)** fear renewal (tested in the original fear conditioning context), or changes in reacquisition rates compared to naïve mice (n=5, naïve; n=6, WT; n=5, *ngr1*−/−) (two-way ANOVA with repeated measures, (group × time); group: $F(2, 13)=11.9$, $P=0.001$; time: $F(4, 52) = 57.0$, $P<0.0001$; interaction between group and time: $F(8, 52) = 6.5$, $P<0.0001$). Bonferroni corrected *post hoc* analysis reveals significant differences at indicated time points. (H) Both groups show equal rates of freezing 14 days after fear conditioning (n=6, WT; n=8, *ngr1*−/− mice). *P<.05, **P<0.01, ***P<.001; error bars represent SEM.

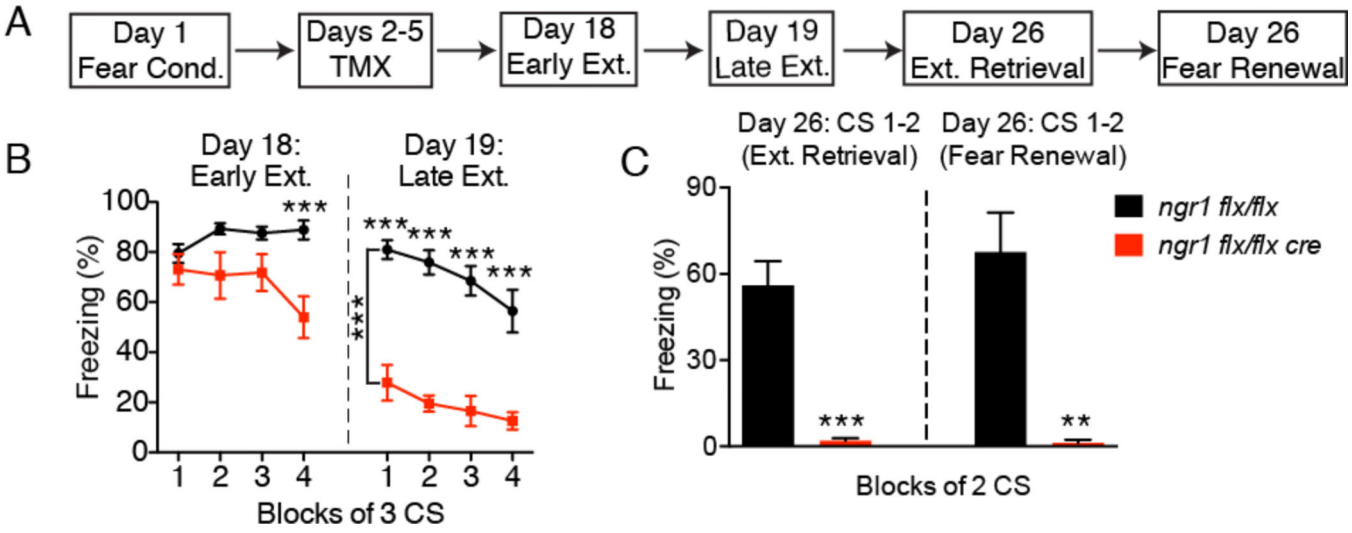

Figure 2. NgR1 deletion after fear conditioning produces juvenile-like levels of fear expression after extinction training

(A) Protocol for experiments in B–C. **(B)** ngr1 flox/flox *cre* mice show enhanced extinction acquisition, retrieval (two-way ANOVA with repeated measures, (group × time); group: $F(1, 19)=36.1$, $P<0.0001$; time: $F(7, 133)=38.8$, $P<0.0001$; interaction between group and time: $F(7, 133)=11.0$, $P<0.001$; n=12, ngr1 flox/flox; n=10, ngr1 flox/flox *cre*; Bonferroni corrected post hoc analysis reveals significant differences at indicated time points) and **(C)** no spontaneous recovery ($t(20)=5.79$; ngr1 flox/flox: 56.0 ± 8.4, ngr1 flox/flox *cre*: 2.0 ± 0.9; $P<0.0001$, two-tailed unpaired t-test; n=12, ngr1 flox/flox; n=10, ngr1 flox/flox *cre*) or fear renewal (tested in a novel context) ($t(8)=4.791$; ngr1 flox/flox: 67.5 ± 13.8, ngr1 flox/flox *cre*: 1.3 ± 1.1; $P=0.001$, two-tailed unpaired t-test; n=5 per group) compared to ngr1 flox/flox mice. **P<0.01; ***P<0.001, error bars represent SEM.

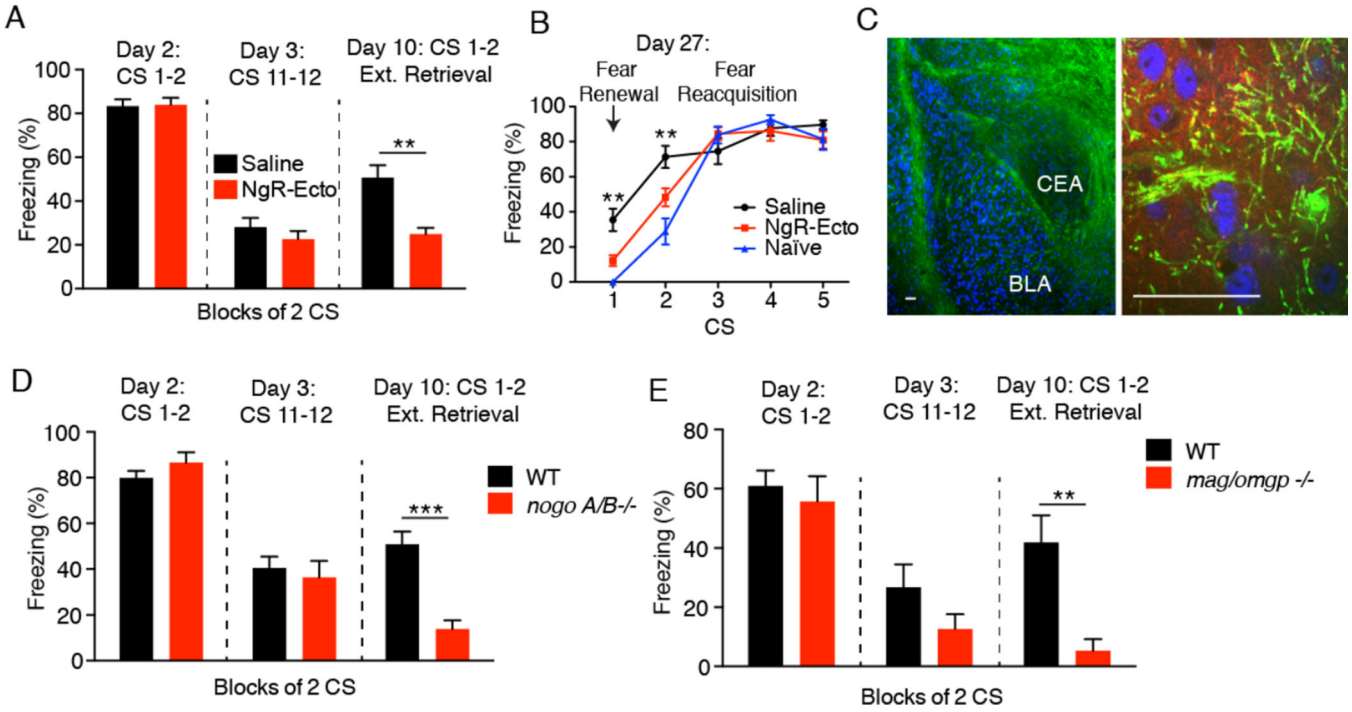

Figure 3. Myelin-associated ligands for NgR1 blocks fear erasure in adulthood

(A) Mice treated with NgR(310)ecto-Fc (n=21) or saline (n=20) intracerebroventricularly exhibit no spontaneous recovery during extinction retrieval (saline: 50.5 ± 5.2, NgR ecto: 28.2 ± 4.4; P=0.002, two-tailed unpaired t-test). **(B)** *nogo A/B* −/− mice exhibit no spontaneous recovery (t(11)=5.307; WT: 50.86 ± 5.6, *nogo A/B* −/−: 13.8 ± 3.8; P=0.0002, two-tailed unpaired t-test; n=7, WT; n=6 *nogo A/B* −/−), unlike WT mice. **(C)** High levels of MAG are detected immunohistologically in the vicinity of neurons (MAG, green; NeuN, red; synaptotagmin, red) observed in low (left) and high (right) magnification views of BLA. Scale bars, 50 μm. The *nogo A/B* −/− mice exhibit normal extinction learning (n=13, WT; n=12, *nogo A/B* −/−) but **(D)** no spontaneous recovery (t(11)=5.307; WT: 50.86 ± 5.6, *nogo A/B* −/−: 13.8 ± 3.8; P=0.0002, two-tailed unpaired t-test; n=7, WT; n=6 *nogo A/B* −/−), unlike WT mice. **(E)** *MAG/OMgp* double −/− mice do not exhibit spontaneous recovery, in contrast to WT mice (t(11)=3.898; WT: 41.9 ± 9.1, *MAG/OMgp* −/−: 5.2 ± 3.9; P=0.003, two-tailed unpaired t-test; n=6, WT; n=7, *MAG/OMgp* −/−). *P<0.05; **P<0.01; ***P<0.001, error bars represent SEM.

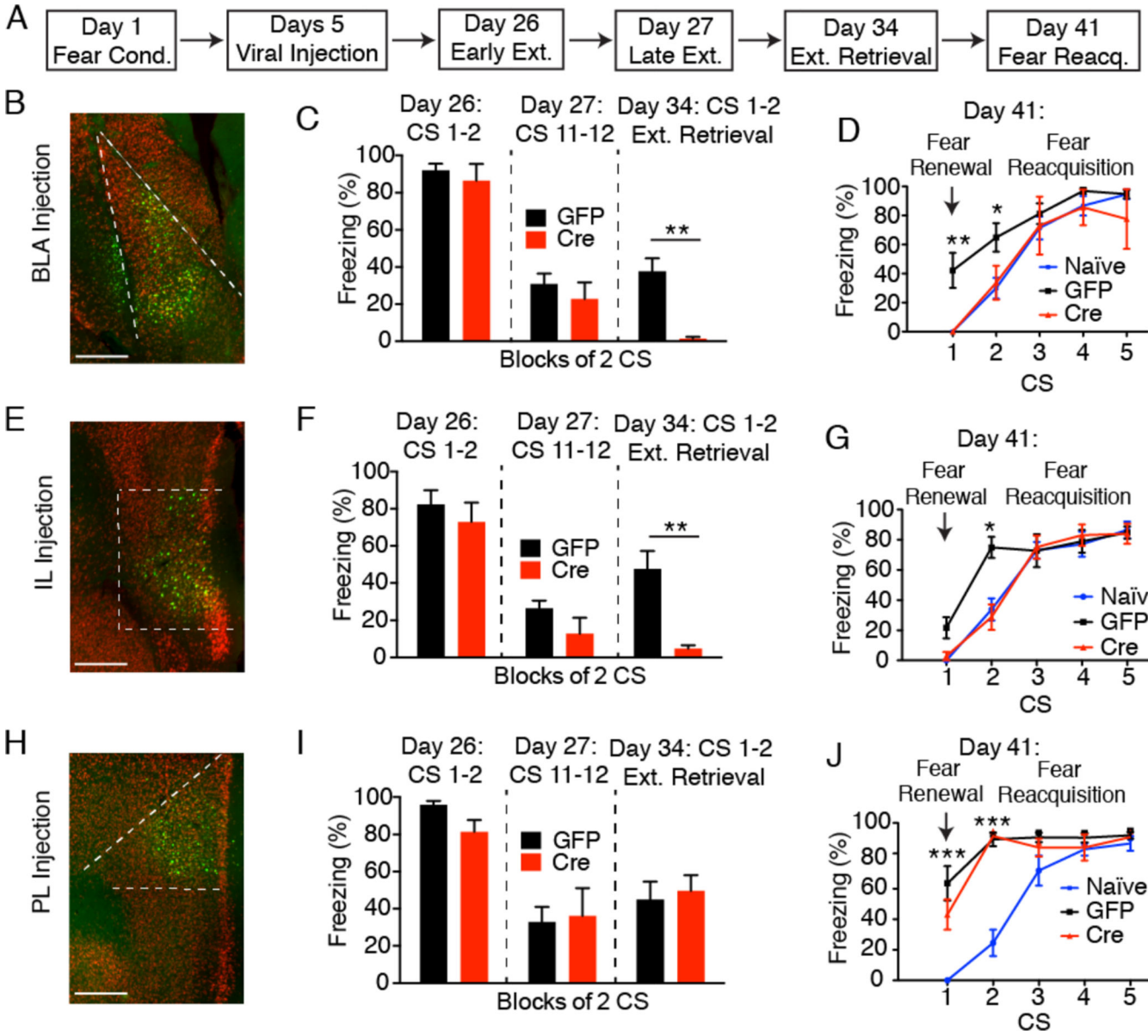

Figure. 4. NgR1 is required in amygdala and infralimbic cortex to restrict fear erasure

(A) Experimental protocol for region specific deletion of NgR1. **(B, F, J)** Mice were injected with AAV-Cre or GFP (green, AAV-Cre-GFP; red, neurons (NeuN)) in BLA **(B)**, IL **(E)**, or PL **(H)** after fear conditioning. Scale bars, 250 μm. **(B–D)** Mice injected with AAV-Cre (n=4) into the amygdala show no spontaneous recovery (AAV-GFP: 37.6 ± 7.0, AAV-Cre: 1.4 ± 1.0; P=0.0027, two-tailed unpaired t-test) and **(D)** the following week show no fear renewal or changes in reacquisition rate compared to naïve mice (two-way ANOVA with repeated measures, (group × time); group: $F(2, 11) = 2.9$, $P = 0.1$; time: $F(4, 44) = 68.7$, $P<0.0001$; interaction between group and time: $F(8, 44) = 2.2$, $P=0.04$, Bonferroni corrected *post hoc* analysis reveals significant differences at indicated time points), unlike AAV-GFP injected mice (n=5). **(E–G)** Injection of AAV-Cre into IL (n=5) produces complete elimination of fear expression during extinction retrieval (AAV-GFP: 47.6 ± 9.6, AAV-Cre:

4.7 ± 1.8; P=0.0024, two-tailed unpaired t-test) and **(G)** reacquisition (two-way ANOVA with repeated measures, (group × time); group: F(2, 12) = 1.9, P = 0.2; time: F(4, 48) = 115.9, P<0.0001; interaction between group and time: F(8, 48) = 5.2, P=0.0001, Bonferroni corrected *post hoc* analysis reveals significant differences at indicated time points), in contrast to mice injected with AAV-GFP (n=5). **(H–J)** Injection of AAV-Cre or AAV-GFP into PL (n=5) generates no differences in fear expression during **(I)** extinction retrieval or **(J)** reacquisition. *P<0.05; **P<0.01; ***P<0.001.

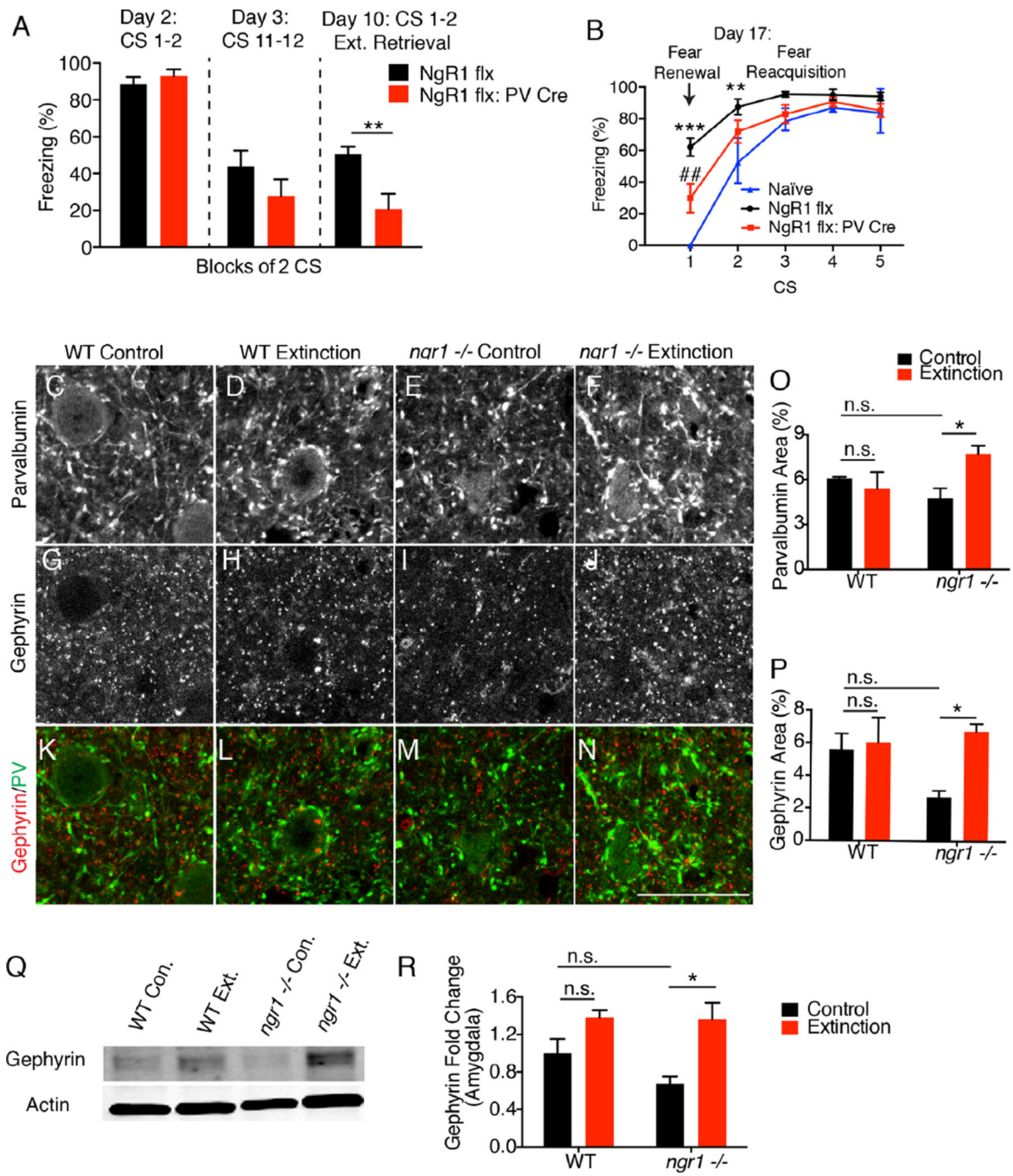

Figure 5. Plasticity of inhibitory interneurons is involved in fear recovery following extinction

(A) One-week after extinction, NgR1 flox: PV Cre mice (n=7) do not show spontaneous recovery, in contrast to NgR1 flox control mice (n=7) (two-tailed unpaired t-test reveals a significant difference between genotype, t(12)=3.220, P=0.007). **(B)** One-week after extinction retrieval, NgR1 flox, NgR1 flox: PV Cre and naïve mice were fear conditioned. NgR1 flox control mice show greater fear renewal and enhanced reacquisition as compared to NgR1 flox: PV Cre and naïve mice (two-way ANOVA with repeated measures, interaction between group and time: F(8, 60)=3.8, P=0.0011; n=7 per genotype, n=5 for

naïve mice, Bonferroni corrected *post hoc* analysis reveals significant differences at indicated time points **(C–N)** Parvalbumin (green) and gephyrin (red) were imaged immunohistologically in slices from the BLA of WT and *ngr1*−/− mice collected 24 hours after the second day of extinction, or without training. Scale bar, 50 μm. **(O, P)** The area of PV and gephyrin puncta is measured in the BLA from slices as in C-N as a function of training and genotype. For PV puncta, two-way ANOVA reveals statistically significant interaction between genotype and extinction: $F(1, 12)=6.59$, $P=0.02$, n=4/group. For gephyrin puncta, two-way ANOVA reveals statistically significant main effect of genotype: $F(1, 12)=5.67$, $P=0.03$ n=4/group. Post-hoc pairwise comparisons of PV and gephyrin puncta by ANOVA with Bonferroni correction are indicated. **(Q–R)** Immunoblot analysis confirms increases of gephyrin in amygdala of *ngr1*−/− mice after extinction. Post-hoc pairwise comparisons by ANOVA with Bonferroni correction are indicated. n=3–4/group. *P<0.05; **P<0.01; ***P<0.001, data are mean ± SEM.