

Narp Mediates Antidepressant-Like Effects of Electroconvulsive Seizures

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Growing recognition of persistent cognitive defects associated with electroconvulsive therapy (ECT), a highly effective and commonly used antidepressant treatment, has spurred interest in identifying its mechanism of action to guide development of safer treatment options. However, as repeated seizure activity elicits a bewildering array of electrophysiological and biochemical effects, this goal has remained elusive. We have examined whether deletion of Narp, an immediate early gene induced by electroconvulsive seizures (ECS), blocks its antidepressant efficacy. Based on multiple measures, we infer that Narp knockout mice undergo normal seizure activity in this paradigm, yet fail to display antidepressant-like behavioral effects of ECS. Although Narp deletion does not suppress ECS-induced proliferation in the dentate gyrus, it blocks dendritic outgrowth of immature granule cell neurons in the dentate molecular layer induced by ECS. Taken together, these findings indicate that Narp contributes to the antidepressant action of ECT and implicate the ability of ECS to induce dendritic arborization of differentiating granule cells as a relevant step in eliciting this response.

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INTRODUCTION

Depression is a growing problem worldwide and 30% of patients do not adequately respond to existing pharmacologic treatment (Rush *et al*, 2006a). As a result, electroconvulsive therapy (ECT), which is the most effective and has the most rapid onset of all FDA-approved therapies available for treatment-resistant depression (Segman *et al*, 1995; Husain *et al*, 2004; Rush *et al*, 2006b), is widely used despite its limitations, including the requirement for repeated anesthesia and development of significant cognitive side effects. These drawbacks have stimulated interest in identifying the molecular mechanisms mediating ECT's antidepressant action to guide development of improved treatment regimens, a daunting challenge given the wide ranging effects of repeated seizure activity on biochemical signaling pathways in neurons and glia throughout the brain.

As multiple immediate early genes are rapidly and robustly induced by electroconvulsive seizures (ECS) (Cole *et al*, 1990) and have been shown to play key roles in enduring forms of synaptic plasticity (Xiao *et al*, 2000; Shepherd *et al*,

2006), we considered the possibility that this transcriptional response could contribute to the long-lasting antidepressant effect of ECT. In exploring this hypothesis, we chose to focus on Narp (Tsui *et al*, 1996a), an immediate early gene induced by ECS, for several reasons: (1) Glutamatergic dysregulation has been implicated in the pathophysiology of depression (Krishnan and Nestler, 2008), and Narp, which is secreted from pre-synaptic terminals (Reti *et al*, 2002a, 2008b), regulates AMPA receptor clustering (O'Brien *et al*, 1999). (2) Narp plays a key role in BDNF-dependent synaptic modulation (Mariga *et al*, 2015), and BDNF regulates antidepressant responses (Castren and Rantamaki, 2010a). (3) Narp is expressed in multiple afferents to the nucleus accumbens (NAc; Johnson *et al*, 2010a), a key regulator of mood. (4) Unlike most other immediate early gene products induced by ECS which show transient increases in protein levels following ECS, Narp protein stays elevated for nearly 24 h and accumulates in the hippocampus with repeated ECS (Reti and Baraban, 2000). To test the role of Narp in mediating the antidepressant effects of ECT, we evaluated the effect of repeated ECS or sham treatment on the performance of Narp KO and WT mice on the forced swim test (FST) and tail suspension test (TST), widely used measures of antidepressant efficacy (Cryan *et al*, 2005; Petit-Demouliere *et al*, 2005). In addition, we assessed whether Narp deletion impairs biochemical or histological responses that have been linked to ECS-mediated antidepressant action.

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MATERIALS AND METHODS

Animals

Since repeated ECS treatments have a high rate of mortality in many mouse strains commonly used for behavioral studies (Torchiana and Stone, 1959), we generated Narp KO mice (Johnson *et al*, 2007) on a CF-1 background, as this strain withstands repeated ECS well. There is no evidence that this strain has altered pain sensitivity that could affect strain response on FST and TST (Gebhart and Mitchell, 1973; Seale *et al*, 1996). Narp KO mice were backcrossed to the outbred CF-1 strain (Charles River, Wilmington, MA) for five generations. WT and KO mice on a CF-1 background were generated by breeding Narp heterozygotes. Male CF-1 mice aged 8–12 weeks (25–45 g) were used for all the experiments. With the exception of mice undergoing chronic stress, mice were housed 2–5 per cage, in a mouse-only animal room maintained at 71 ± 4 °F, 23–48% humidity, and under a 12 : 12 h light:dark cycle, with lights on at 0500 hours. Unless otherwise stated, mice had *ad libitum* access to food and water, and behavioral experiments took place between 0900 hours and 1500 hours. Prior to experimentation, mice were acclimatized to the animal room for a minimum of 2 weeks and handled for at least 3 days. All experiments were approved by The Johns Hopkins University Animal Care and Use Committee.

Electroconvulsive Seizure

Electrical pulses used to induce ECS were administered using padded earclip electrodes soaked in saline. Pulse trains were generated by a rodent electroconvulsive device (ECT Unit model 57800; Ugo Basile, Collegeville, PA) and set to the following parameters for all treatments: 0.5 ms pulse width, 1 s stimulus duration, frequency of 100 Hz, and current of 40 mA (Chang *et al*, 2016). The stopwatch was started as soon as the stimulation train stopped. All mice treated with ECS exhibited a tonic-clonic seizure lasting approximately 20 s. Sham ECS was performed by attaching saline-soaked earclips to the mice but without passing any current. All courses of ECS consisted of five daily treatments as preliminary studies showed that five sessions, but not one, was sufficient to trigger a significant antidepressant-like response on the FST (Supplementary Figure S1).

Pharmacological Antidepressants

The following drugs were administered by intraperitoneal (i.p.) injection. Imipramine 20 mg/kg (Sigma-Aldrich, St Louis, MO) was dissolved in saline and administered daily for 22 days. The TST was run 1 h after treatment on day 21, followed approximately 2 h later by a pre-test FST. The FST was carried out 1 h after injection on day 22. Ketamine 10 mg/kg (Sigma-Aldrich) was dissolved in saline and administered once. The mice were subjected to an FST pre-test 2 h later. The TST and FST were performed the next day, approximately 23 and 29 h after treatment, respectively. Fluoxetine 20 mg/kg (Santa Cruz Biotechnology, Santa Cruz, CA) was dissolved in 1% dimethyl sulfoxide in saline and administered daily for 29 days. The TST and FST were run as for imipramine.

Forced Swim Test

The FST (Porsolt *et al*, 1977a,b) was performed by placing each animal in a glass cylindrical container filled with water (maintained at 24 ± 1 °C) at a depth of 20 cm to ensure the animals could not rest their tails or hindpaws on the floor of the container. A large, 21 cm-diameter container was used, as larger swimming areas may provide greater sensitivity to antidepressant-like effects on the FST (Sunal *et al*, 1994). Water was changed between mice. Twenty-four hours after the last ECS, animals were each exposed to a 10-min 'pre-test' swim. After the pre-test swim, mice were dried with paper towels, placed in a warming chamber for 15 min, and returned to their home cages. After an additional 24 h, mice were exposed to a 6 min swim which was videotaped from a top-view camera angle. Videos were manually scored for immobility by two independent raters blind to genotype and treatment using the computer program Hindsight v.1.5. Immobility times were determined for the last 4 min of the 6-min swim session (Porsolt *et al*, 1978, 2001). Immobility times were averaged between both raters. In the event the raters' scores differed by more than 15% for a particular mouse, a third rater scored the video and an average across the three reviewers was used for analysis. Different cohorts were used for the forced swim, tail suspension and open field (see below) tests.

Tail Suspension Test

In unstressed mice, the TST was performed approximately 24 h after the last ECS. In stressed mice, the TST was run 48 h after the last ECS. A full description of experiments utilizing stressed mice can be found in Supplementary Materials. The TST (Steru *et al*, 1985) was performed by suspending mice for 6 min about 12 inches in the air using adhesive tape to fix the end of their tails to a metal frame. Tests were videotaped and scored as described for the FST, except that immobility scores were determined for the entire 6 min of the test (Porsolt *et al*, 2001; Steru *et al*, 1985).

Open-Field Test

Twenty-four hours after the fifth daily ECS or sham treatment, mice were subjected to a 10 min pre-test FST. After another 24 h (48 h after the last ECS or sham), locomotor activity was monitored in an open field measuring 40 × 40 cm. Distance traveled was measured by an infrared tracking system (Digiscan Animal Activity Monitor, Accuscan Instruments Inc, Columbus, Ohio) over a 30 min time period.

Immunohistochemistry

Cell markers of neuronal activity, proliferation, and maturation were monitored following the 5-day ECS regimen. For analyses of c-Fos neuronal activation following ECS, mice were killed 1 h after the last ECS. To monitor the rate of cell division triggered by ECS, mice were injected with BrdU (B5002; Sigma, St Louis, MO; 50 mg/kg i.p.) every 12 h for 5 days beginning on day 3 of the ECS course, and killed 12 h after the last BrdU injection. Finally, for morphological analyses of newly born doublecortin-positive cells, animals were killed 24 h after the last ECS treatment. For all these experiments, animals were anesthetized with chloral hydrate

(C8383; Sigma; 400 mg/kg i.p.) prior to perfusion with 4% paraformaldehyde (P6148; Sigma). Brains were maintained in 4% paraformaldehyde overnight, and subsequently transferred to 25% sucrose.

Sections were processed for immunohistochemistry as previously described (Reti and Baraban, 2003). Primary antibodies were: rabbit anti-c-Fos (1 : 1000; PC05; Millipore, Billerica, MA), mouse anti-BrdU (1 : 100; B8434; Sigma), and goat anti-doublecortin (DCX; 1 : 250; SC8066; Santa Cruz Biotechnology, Santa Cruz, CA).

Cell Counting

Stained cells were counted by two raters who were blind to genotype and treatment, and the areas used for counting were the same size across all animals for each region. Digital images were captured at six representative coronal sections containing each brain region of interest. For c-Fos and BrdU quantification, images were visualized and captured with a Zeiss microscope fitted with a Retiga 2000R camera (QImaging, Surrey, BC Canada). For c-Fos quantification, we selected sections through the basolateral amygdala (BLA), dentate gyrus, NAc shell, NAc core, ventromedial hypothalamus (VMH), and medial prefrontal cortex (mPFC), located approximately -1.34 , -2.30 , 1.42 , 1.42 , -1.70 , and 2.10 mm from bregma, corresponding to plates 42, 50, 19, 19, 46, and 14, respectively, of the Paxinos and Franklin mouse brain atlas (Paxinos and Franklin, 2001). For BrdU and DCX analyses, cell counts were limited to the dentate gyrus. Reported BrdU-positive cell counts were normalized by the cumulative average for sham-treated WT mice. For quantification of DCX-positive cells, we used stereological optical fractionation at $\times 100$ magnification from multiple sample frames of $40\ \mu\text{m} \times 40\ \mu\text{m}$ that were distributed over the delineated region of interest. Images were captured with a Zeiss AxioImager.

RNA Isolation and Quantitative PCR (qPCR)

WT and NARP KO adult male mice ($n = 4$ per genotype/condition) were killed by cervical dislocation 90 min or 24 h after completion of the last ECS session and hippocampal tissues were collected and placed in RNAlater (ThermoFisher). Total RNA was isolated and extracted using TRIzol (Life Technologies, Carlsbad, CA). RNA was subsequently purified using an RNeasy minicolumn (Qiagen, Valencia, CA) and quantified using a NanoDrop spectrophotometer (Agilent Technologies, Savage, MD). RNA concentration was normalized and reverse transcribed into single stranded cDNA using Superscript III (Life Technologies). Quantitative PCR was performed using a Realplex thermocycler (Eppendorf, Hamburg, Germany) using GEMM mastermix (Life Technologies) with 40 ng of synthesized cDNA. PCR efficiencies of *Bdnf* primers were examined by standard curve of serial-diluted cDNA and melting-curve functionality (Maynard *et al*, 2016; Sakata *et al*, 2009). Individual mRNA levels were normalized for each well to *Gapdh* mRNA levels.

Hippocampal BDNF Levels

BDNF protein assays were conducted as described previously (Matsumoto *et al*, 2008). Briefly, mice were killed by

decapitation 24 h after the fifth daily ECS or sham treatment, when BDNF levels have been shown to reach maximal levels following repeated ECS (Altar *et al*, 2003). Hippocampi were then dissected and flash-frozen in a 50 : 50 mixture of isopentane and dry ice. Hippocampal samples were subsequently homogenized in 1.5 ml of RIPA buffer (50 mM Tris-HCl (pH7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton-X 100, 1% Na deoxycholate, and 0.1% SDS) containing 4% protease inhibitor cocktail (complete, Mini; Roche Diagnostics Corp., Indianapolis, IN). Tissue lysates underwent immunoprecipitation with monoclonal mouse anti-BDNF antibody (1 μg /sample; BDNF-#9; Developmental Studies Hybridoma Bank, Iowa City, IA) bound to protein G beads (10 μl /sample; 10003D; Thermo Fisher Scientific, Waltham, MA), and final purified samples were solubilized and boiled for 2 min in 30 μl Laemmli solution containing 5% β -Mercaptoethanol. Non-precipitated sample lysates were saved in the same manner to normalize total protein levels across samples.

Protein samples were resolved by electrophoresis using 4–12% gradient SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. For western blotting, membranes were blocked in 0.05% Tween 20 in Tris-buffered saline containing 5% non-fat milk and $1 \times$ Protein G blocker (M01014; Genscript, Piscataway, NJ). Membranes were then incubated at 4 °C overnight in either polyclonal rabbit anti-BDNF (1 : 200; SC546; Santa Cruz Biotechnology, Santa Cruz, CA), or monoclonal rabbit anti-ERK (1 : 1000; #4695; Cell Signaling Technology, Danvers, MA), followed by overnight incubation with horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (NA934; GE Healthcare Life Sciences, Marlborough, MA) at room temperature. The signals were visualized using an electrochemiluminescence system (ECL; RPN2232; GE Healthcare Life Sciences).

Quantification of raw signal intensities was conducted using ImageJ software (Schindelin *et al*, 2015). Raw intensity values for pro- and mature-BDNF signals were sequentially normalized for: (1) protein levels, using the ERK signal intensity of each individual sample, and (2) comparisons to WT sham levels using the average intensity of the WT sham samples in the same film.

DCX-positive Dendritic Outgrowth

DCX-positive dendritic outgrowth in the suprapyramidal blade of the dentate gyrus was quantified in mice killed 24 h following five consecutive days of ECS. For each animal, 5 μm -thick z-stack projections were obtained using the Zeiss Axiovision extended focus module from three representative coronal sections corresponding to -1.82 , -2.06 , -2.30 mm from bregma (Paxinos and Franklin, 2001). Images were captured with a Zeiss AxioImager M2.

A simplified Sholl analysis was used to analyze the extent of dendritic growth towards the hippocampal fissure. We conducted our analyses at the midpoint of the molecular layer, where projection fibers from the entorhinal cortex terminate upon the DCX-positive dendrites that extend into the outer molecular layer. Thus, for each coronal section analyzed, we quantified the number of dendritic intersections crossing the midline of the molecular layer for the entire suprapyramidal blade of the dentate gyrus in the manner outlined below.

Using MCID Core Software (Focus Imaging Ltd, Linton, UK), relative optical density (ROD) profiles were obtained along the midline of the molecular layer and the granule cell layer. Scanning across the midline of these layers, the line of analysis was largely composed of low amplitude ROD values corresponding to background staining, with only intermittent spikes corresponding to true DCX-positive dendritic intersections. Therefore, the weighted mean ROD for each section roughly corresponded to the threshold intensity between signal and noise. However, we chose a more conservative signal threshold obtained by adding three times the standard deviation of the ROD values for sham-treated WT mice to the weighted mean ROD value. Since sham-treated WT mice represent the 'baseline' minimum for dendritic intersections at the midpoint of the molecular layer, the range of their ROD values also provided the most consistent standard deviation value to adjust the threshold. Although it would have been more straightforward to use each individual section's ROD standard deviation, this would have preferentially raised the threshold for ECS-treated WT mice (which have more dendritic crossings) to more conservative values. Following signal binarization, the number of discrete dendritic intersections were counted and divided by the total width of molecular layer segment analyzed. The described thresholding algorithm yielded 92% accuracy compared with manual counts (data not shown).

Statistical Methods

Data were analyzed using two-way analysis of variances (ANOVAs). When appropriate, *post hoc* analyses were performed using Bonferroni or Fisher's least significant difference test. All values are expressed as means \pm SEM. *p*-values < 0.05 were considered statistically significant.

RESULTS

Narp Deletion Blocks Antidepressant-Like Effects of ECS in FST and TST

Consistent with our hypothesis, Narp KO mice fail to show reduced immobility following ECS compared to sham controls in the FST. In contrast, WT mice display the expected reduction in immobility in this assay in response to ECS (Figure 1a; two-way ANOVA shows a significant interaction between genotype and treatment: $F(1, 35) = 5.08$, $p = 0.031$; *post hoc* analysis of WT sham vs ECS groups, $p = 0.002$; Narp KO sham vs ECS groups, $p = 0.63$).

Analysis of the TST data yielded a similar pattern of results as WT, but not Narp KO, mice show reduced immobility following ECS treatment (Figure 1b; two-way ANOVA shows a significant interaction between genotype and treatment: $F(1, 37) = 7.83$, $p = 0.00813$; *post hoc* analysis of WT sham vs ECS groups, $p < 0.0001$; Narp KO sham vs ECS groups, $p = 0.67$). In pilot studies, we found that CF-1 mice do not show reduced sucrose preference following chronic stress precluding us from using this assay to compare the antidepressant-like effects of ECS in WT and KO mice. However, as reported above for naïve mice, in stressed mice, Narp deletion blocked the ability of ECS to decrease immobility in the TST observed in WT mice (Supplementary Figures S2).

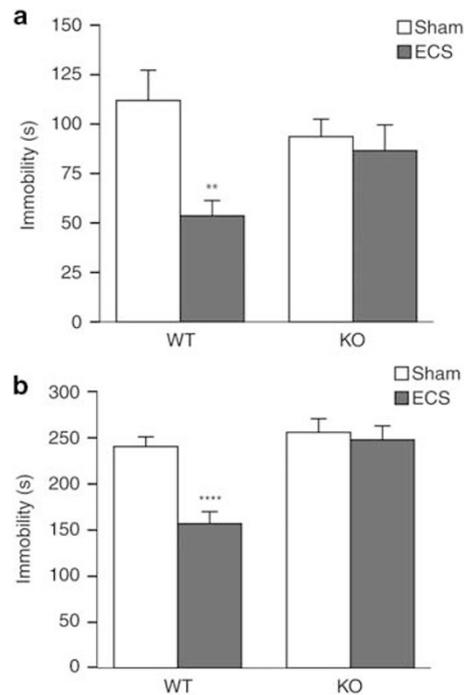


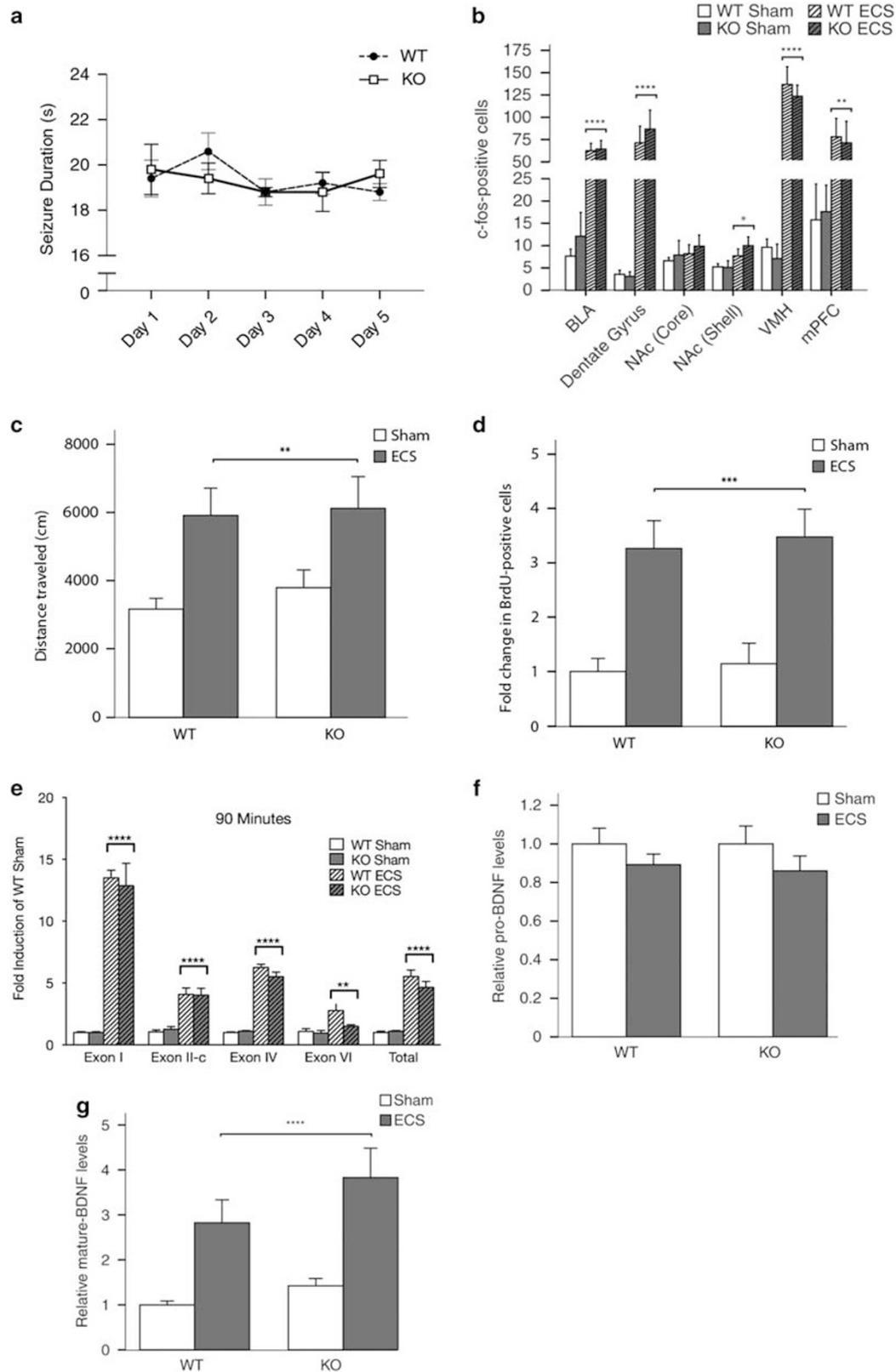
Figure 1 Effect of ECS on the FST (a) and TST (b) in Narp KO and WT mice. The ability of ECS to decrease immobility scores in both the FST and TST is blocked by Narp deletion. Two-way ANOVAs showed a significant interaction between genotype and treatment on immobility in the FST ($p < 0.05$, $n = 8-12$ in each group) and the TST ($p < 0.01$, $n = 10-11$ in each group). Comparison of sham vs ECS-treated WT mice in FST and TST, ** $p < 0.01$ and **** $p < 0.0001$, respectively (Fisher's *post hoc* test). Bars represent means (\pm SEM) in this and subsequent figures. ECS, electroconvulsive seizures; FST, forced swim test; TST, tail suspension test; KO, knockout; WT, wild-type; ANOVA, analysis of variance.

Narp KO Mice Display Normal Electrically Induced Seizure Activity

Seizure duration. As these behavioral results indicate that Narp deletion blocks the antidepressant-like effects of ECS, we proceeded to check whether it may do so by impairing the ability of electric stimuli to elicit seizure activity. Accordingly, we compared ECS-induced seizure duration in WT and Narp KO mice.

Evidence from human studies suggests that seizure duration can play a role in the efficacy of ECT (Kimball *et al*, 2009). However, seizure duration was unchanged in Narp KO mice (Figure 2a; two-way repeated measures ANOVA showed no effect of genotype: $F(1, 8) = 0.49$, $p = 0.831$, nor an interaction between genotype and treatment: $F(4, 32) = 0.56$, $p = 0.69$).

c-Fos induction. To gauge whether Narp deletion may impair seizure activity *per se*, we also assessed whether it affects several responses to ECS: induction of c-Fos, increased locomotor activity, enhanced hippocampal neurogenesis and BDNF induction. Analysis of the number of c-Fos-positive cells in multiple brain regions did not reveal any effect of Narp deletion (Figure 2b). Two-way ANOVA revealed a main effect of treatment on c-Fos induction in the BLA ($F(1, 15) = 35.98$, $p < 0.0001$), dentate gyrus ($F(1, 16) = 28.54$, $p < 0.0001$), NAc (shell only) ($F(1, 14) = 5.23$, $p = 0.038$), VMH ($F(1, 15) = 106.32$,



$p < 0.0001$) and mPFC ($F(1, 16) = 12.04, p = 0.003$). In all areas except the core of the NAc, ECS resulted in an increase in c-Fos-positive cells. There was no interaction between genotype

and treatment in this experiment for any of the brain regions examined, suggesting that ECS produces equivalent activation of neurons in Narp KO and WT mice.

Figure 2 Effect of ECS on seizure duration (a), c-Fos expression (b), locomotor activity (c), hippocampal neurogenesis (d) and hippocampal BDNF expression (e–g) in Narp KO and WT mice. (a) Duration of motor seizure activity was measured daily over a course of five ECS treatments. There was neither a significant main effect of genotype nor an interaction between genotype and treatment on seizure duration on a two-way repeated measures ANOVA ($n=5$ in each group). (b) c-Fos-positive neurons were counted in mice killed 1 h after the fifth daily ECS or sham treatment. Two-way ANOVAs showed a significant main effect of treatment in the BLA, dentate gyrus, NAc (shell), VMH, and mPFC. There was no significant interaction between genotype and treatment on c-Fos expression in any of the regions examined ($n=3–6$ in each group). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ in ECS vs sham-treated mice. (c) Distance traveled in an open field over 30 min measured 48 h after the fifth daily ECS or sham treatment. A two-way ANOVA showed a significant main effect of ECS on locomotor activity ($n=5$ in each group). No interaction was found between treatment and genotype. (d) Hippocampal neurogenesis (represented as fold-change in BrdU-positive cells in the dentate gyrus vs sham-treated WT mice) following the fifth daily ECT or sham treatment. There was a significant main effect of treatment on BrdU-positive cells using a two-way ANOVA and no interaction between genotype and treatment ($n=3–4$ for each group). (e) Hippocampal *Bdnf* total RNA levels are represented as fold change vs sham-treated WT mice. A two-way ANOVA showed a significant main effect of ECS treatment compared to Sham on *Bdnf* mRNA transcripts. Notably, there was no significant effect of Narp genotype, nor an interaction effect between genotype and treatment ($n=4$ per group). (f) Hippocampal pro-BDNF levels were monitored by western blot and are represented as fold change vs sham-treated WT mice. A two-way ANOVA showed no significant effect of Narp deletion or ECS treatment on pro-BDNF protein levels ($n=4–5$ in each group). (g) Hippocampal mature BDNF levels were monitored by western blot and are represented as fold change vs sham-treated WT mice. A two-way ANOVA showed a significant main effect of ECS treatment on mature BDNF protein levels ($n=12–13$ per group). **** $p<0.0001$ in ECS vs sham-treated mice. Of note, there was no significant interaction between genotype and treatment in mature BDNF protein levels. ECS, electroconvulsive seizures; KO, knockout; WT, wild-type; ANOVA, analysis of variance; BLA, basolateral amygdala; NAc, nucleus accumbens; VMH, ventromedial hypothalamus; mPFC, medial prefrontal cortex; ECT, electroconvulsive therapy.

Locomotor activity. ECS increases locomotor activity in rodents (Li et al, 2006). Therefore, it is conceivable that if Narp deletion alters the impact of ECS on locomotor activity, this differential effect could potentially confound the interpretation of the FST and TST results reported above. However, by two-way ANOVA we found no interaction between genotype and treatment on locomotor activity ($F(1, 16)=0.96$, $p=0.76$, Figure 2c), although there was an expected significant main effect of treatment ($F(1, 16)=13.84$, $p=0.002$), such that both WT and KO mice display similar increases in locomotor activity following ECS compared to sham-treated controls.

Hippocampal neurogenesis. Hippocampal neurogenesis has been shown to be necessary for the antidepressant response to chemical antidepressants in rodents (Santarelli et al, 2003). Accordingly, we checked whether Narp deletion might impair the ability of ECS to increase hippocampal neurogenesis. Analysis of BrdU-positive cells by two-way ANOVA revealed a main effect of treatment ($F(1, 10)=24.33$, $p<0.001$) showing that mice given ECS had a significant increase in neurogenesis compared to sham-treated mice (Figure 2d). However, there was no significant interaction of genotype and treatment ($F(1, 10)=0.005$, $p=0.94$), suggesting that Narp deletion does not impair stimulation of hippocampal neurogenesis by ECS.

Hippocampal BDNF. As these studies thus far indicate that the ability of Narp deletion to block antidepressant-like effects of ECS occurs in the face of normal levels of seizure activity, we proceeded to check whether Narp deletion might impair the ability of ECS to induce hippocampal BDNF, a neurotrophic factor that has been strongly implicated in mediating therapeutic effects of antidepressant drugs (Hashimoto et al, 2004; Castren and Rantamaki, 2010a). Transcription of *Bdnf* is controlled by nine promoters, which drive expression of multiple transcripts containing alternative 5' untranslated exons (Timmusk et al, 1993; Aid et al, 2007). Promoters I, II, IV, and VI produce the majority of BDNF in the brain, and promoters I and IV contribute significantly to activity-dependent BDNF transcription (Aid et al, 2007; Pruunsild et al, 2007). Analysis of *Bdnf*

transcripts derived from promoters I, II, IV and VI as well as total *Bdnf* mRNA in WT and Narp KO hippocampus collected 90 min after the last treatment session revealed a main effect of treatment for all *Bdnf* transcripts analyzed (Figure 2e; two-way ANOVAs; Exon I: $F(1, 12)=162.3$, $p<0.0001$; Exon II: $F(1, 12)=54.93$, $p<0.0001$; Exon IV: $F(1, 12)=440.9$, $p<0.0001$; Exon VI: $F(1, 12)=9.501$, $p=0.0095$; Total *Bdnf*: $F(1, 12)=126.5$, $p<0.0001$). However, there were no interactions of genotype and treatment indicating that ECS induces similar elevations in *Bdnf* transcription in WT and Narp KO. We also examined *Bdnf* mRNA levels at 24 h after the last treatment session and found a similar persistence in the elevation of activity-induced *Bdnf* promoters I and II between WT and NARP KO mice (Supplementary Figure S4).

As BDNF transcription and translation are distinctly regulated (Lau et al, 2010), we also checked the effect of Narp deletion on BDNF protein levels in response to ECS. Moreover, given data suggesting that pro-BDNF is capable of facilitating hippocampal long-term depression (Woo et al, 2005), we assayed protein levels of pro- and mature-BDNF independently. Following 24 h after the last sham or ECS treatment, pro-BDNF protein levels remained identical across all WT and Narp KO mice treated in either condition (Figure 2f). Nonetheless, both the WT and Narp KO mice treated with repeated ECS exhibited a comparable two-fold increase in mature-BDNF protein levels compared to the levels of sham-treated mice (Figure 2g; two-way ANOVA shows a significant treatment effect: $F(1, 44)=24.88$, $p=0.00001$), but no effect of genotype nor a genotype \times treatment interaction. These results were confirmed by immunohistochemistry on WT and Narp KO transgenic mice featuring hemagglutinin (HA) epitope tagged BDNF (Yang et al, 2009). These mice exhibited comparable elevations in HA-BDNF immunoreactivity in the dentate gyrus and CA3 after repeated ECS (Supplementary Figure S4).

Narp Deletion Impairs ECS-Mediated Increase in DCX+ Dendritic Arborization in the Dentate Molecular Layer

While ECS-triggered seizure activity, including neurogenesis monitored by BrdU, appears to be intact in Narp KO mice,

we explored whether proliferation and differentiation of neural progenitors in the hippocampal dentate gyrus were affected by Narp deletion. We stained for DCX which is a microtubule-associated protein expressed by migrating neuroblasts for around 2 weeks as the cells mature into

neurons (Lledo *et al*, 2006). We counted DCX+ dentate granule cells and monitored DCX+ dendritic outgrowth in the molecular layer in mice killed 24 h following five consecutive, daily ECS administrations.

Unlike the analysis of cell proliferation using BrdU, the number of DCX-positive progenitor cells was not significantly altered by either deletion of Narp or repeated ECS treatment at this time point (Figure 3a). However, we found that dendritic outgrowth beyond the midpoint of the molecular layer was markedly greater in WT mice treated with repeated ECS compared with sham; importantly, this ECS-mediated dendritic outgrowth was absent in Narp KO mice (Figure 3b–d; two-way ANOVA shows a significant interaction between genotype and treatment: $F(1,20) = 4.65$, $p = 0.0435$; *post hoc* analysis of WT sham vs ECS groups, $p = 0.000468$; Narp KO sham vs ECS groups, $p = 0.273322$). Of note, dendritic outgrowth after ECS monitored at the mid-point of the granule cell layer was unaffected by Narp deletion (two-way ANOVA shows no interaction between genotype and treatment: $F(1,24) = 0.007$, $p = 0.932$), although there is a main effect of treatment ($F(1,24) = 12.107$, $p = 0.002$) but not of genotype ($F(1,24) = 0.003$, $p = 0.957$). These data indicate Narp deletion does not influence DCX+ proximal dendritic outgrowth triggered by ECS.

Narp KO Mice Respond to Chemical Antidepressants

To determine if Narp deletion selectively affects the antidepressant response to ECS, we also tested its effects on several pharmacologic antidepressants. We monitored the effect of Narp deletion on the antidepressant response to imipramine (Figure 4a and b), fluoxetine (Figure 4c and d), and ketamine (Figure 4e and f; Li *et al*, 2012). Consistent with previous reports that the ability of chemical antidepressants to elicit decreased immobility in the FST and TST paradigms varies among mouse strains (Lucki *et al*, 2001; Jacobson and Cryan, 2007), we found that WT CF-1 mice only displayed decreased immobility in either of these paradigms for two of these drugs: imipramine reduced

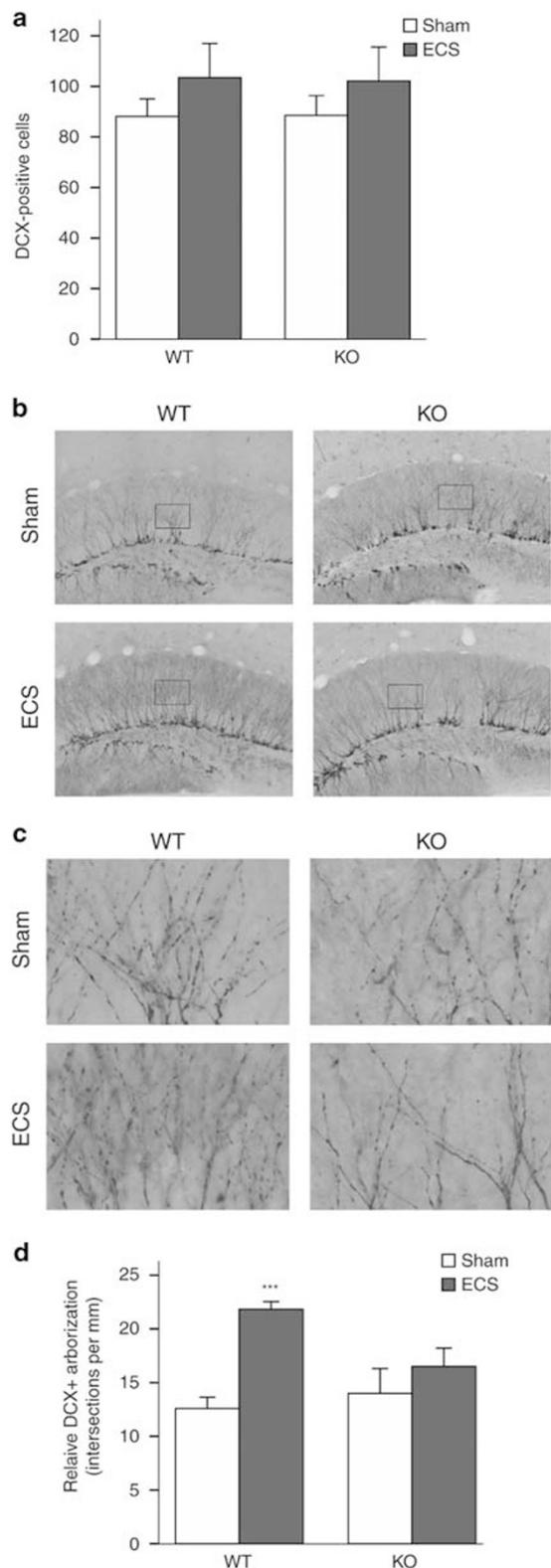


Figure 3 Narp modulates ECS-mediated DCX+ dendritic arborization in the molecular layer of the hippocampal dentate gyrus: (a) DCX+ cell count; (b) representative images of immunohistochemistry showing the distribution of DCX+ neurons and dendrites in the dentate gyrus; (c) representative images of inset DCX+ dendrites in the dentate gyrus; (d) DCX+ dendritic branching in the molecular layer of the dentate gyrus. (a) DCX+ cell counts were obtained within the granule layer of the dentate gyrus of WT and Narp KO mice killed 24 h after the fifth daily ECS or sham treatment. Two-way ANOVAs showed no significant main effects of either genotype or treatment ($n = 6$ in each group). (b) Representative histological images of DCX+ immunohistochemistry staining in the hippocampal dentate gyrus for each category of genotype and treatment. Square insets are shown enlarged in (c). (c) Higher resolution representation of DCX+ dendrite distribution in the molecular layer of the hippocampal dentate gyrus. (d) The extent of DCX+ dendrite branching was quantified at the middle of the molecular layer of the hippocampal dentate gyrus ($n = 12$ per group). Two-way ANOVA showed a significant main effect of treatment, as well as a significant interaction effect between genotype and treatment. Of note, *post-hoc* analysis with Fisher's LSD showed a significant difference in WT mice treated with either sham or ECS, but no corresponding difference in Narp KO mice. *** $p < 0.001$ in ECS vs sham-treated WT mice. ECS, electroconvulsive seizures; WT, wild-type; KO, knockout; LSD, least significant difference.

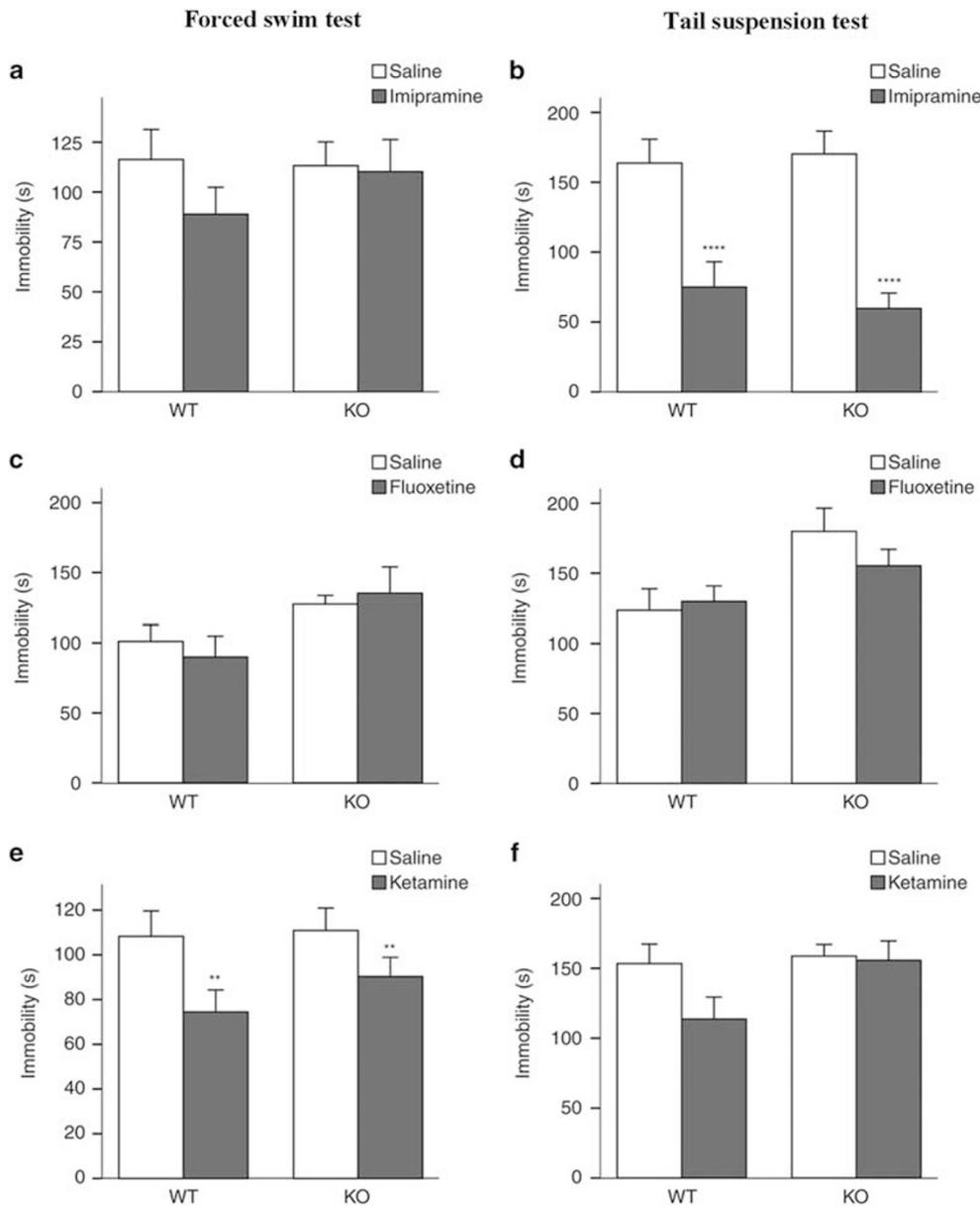


Figure 4 Effects of imipramine, fluoxetine, and ketamine on the FST and TST in Narp KO and WT mice. Immobility was scored during the last 4 min of a 6 min FST and over the entire 6 min TST. Imipramine (a,b) was administered at 20 mg/kg daily for 22 days and the TST and FST were performed 60 min following drug administration on days 21 and 22, respectively. Fluoxetine (c,d) was administered at 20 mg/kg daily for 29 days and the TST and FST were performed 60 min following drug administration on days 28 and 29, respectively. Ketamine (e,f) was administered only once at 10 mg/kg, with the TST and FST taking place approximately 23 and 29 h later, respectively. Two-way ANOVAs did not reveal an interaction between genotype and treatment in any of the cohorts ($n = 9-14$ in each group) but showed a main effect of treatment in the TST for mice treated with imipramine and in the FST for mice treated with ketamine. ** $p < 0.01$, **** $p < 0.0001$ vs saline-treated mice. FST, forced swim test; TST, tail suspension test; KO, knockout; WT, wild-type.

immobility on the TST; ketamine reduced immobility on the FST; fluoxetine did not affect either test. In both these informative cases, Narp KO mice displayed the same response as WT mice. In the imipramine cohort, two-way ANOVAs did not show any significant interaction of genotype and treatment in the TST ($F(1, 40) = 0.42$, $p = 0.52$). However, there was a significant main effect of treatment on immobility in the TST, whereby mice that received chronic imipramine had significantly less immobility compared to saline-treated controls ($F(1, 40) = 35.26$, $p < 0.0001$). In the ketamine cohort, a two-way ANOVA

showed that there was a significant main effect of ketamine on the FST ($F(1, 49) = 7.39$, $p = 0.009$), with no interaction of genotype and treatment ($F(1, 49) = 0.43$, $p = 0.51$).

DISCUSSION

In studies aimed at assessing whether Narp, which is induced by ECS, mediates its antidepressant actions, we have found that Narp deletion blocks the antidepressant-like behavioral effects of ECS. In particular, Narp KO mice fail to show the expected immobility reduction in the TST and FST after

ECS. In theory, these deficits could be due to the inability of Narp KO mice to mount a normal seizure in response to electroconvulsive stimulation. However, we have found that multiple responses elicited by seizure activity, including hippocampal neurogenesis and locomotor activity, as well as induction of *c-Fos* and BDNF are normal in Narp KO mice. Thus, these findings provide compelling evidence that Narp deletion blocks the ability of ECS to elicit an antidepressant effect without inhibiting seizure activity.

To assess whether Narp deletion selectively blocks the antidepressant effect of ECS or also impairs the action of antidepressant drugs, we examined the effects of fluoxetine, imipramine, and ketamine on FST and TST in Narp KO mice. Unfortunately, these drugs only had limited effectiveness in reducing immobility in these assays in CF-1 WT mice. However, Narp deletion did not impair any of the antidepressant-like responses displayed by these mice, that is, imipramine on TST and ketamine on FST, indicating that Narp deletion selectively blocks the antidepressant response elicited by ECS. We did not evaluate whether Narp deletion impacts the antidepressant response to voluntary exercise. Sleiman *et al* (2016) demonstrate that voluntary exercise triggers activity at multiple BDNF promoters. Because Narp deletion regulates BDNF-mediated plasticity at the mossy fiber—CA3 synapse (Mariga *et al* (2015); see below), it is conceivable that Narp might play a role in the antidepressant effect of voluntary exercise.

Since BDNF is known to play a key role in mediating antidepressant responses (Castren and Rantamaki, 2010a), we have carefully evaluated whether Narp KO mice display deficits in BDNF induction elicited by ECS. To this end, we monitored expression of multiple BDNF transcripts, as well as mature and pro-BDNF proteins. However, none of these assays revealed a genotype \times treatment abnormality. On the other hand, Mariga *et al* (2015) reported bi-directional regulation of Narp by BDNF, and that BDNF directly regulates Narp to mediate glutamatergic transmission and mossy fiber plasticity. Taken together, these findings suggest that Narp deletion does not impair BDNF induction by ECS, but could interfere with its downstream effects.

Like BDNF, hippocampal neurogenesis has also been implicated in mediating antidepressant responses to both ECS and chemical agents (Santarelli *et al*, 2003; Schloesser *et al*, 2015). Utilizing a pharmacogenetic model (hGFAP^{tk}) to conditionally ablate adult-born neurons, Schloesser *et al* (2015) recently found that ECS was unable to promote antidepressant-like behavior in mice lacking adult neurogenesis. In our study, we found comparable increases in the number of BrdU labeled cells in the dentate gyrus of WT and Narp KO mice after ECS, suggesting that Narp does not affect ECS-induced increases in neurogenesis. The lack of increase in DCX+ cell number that we found 5 days after initiating ECS likely reflects insufficient time for the total number of DCX+ cells to be impacted by the increased number of new progenitor cells triggered by ECS. Although the number of DCX+ neurons was unaffected in Narp KO mice, the ability of ECS to increase their dendritic arborization was blocked. Therefore, our data suggest that the enhanced hippocampal neurogenesis induced by ECS is not sufficient to mediate the rapid antidepressant action of ECS but must be coupled with increased arborization of DCX+ granule cell neurons.

Since Narp is not present in DCX+ neurons (Supplementary Figure S5) and is secreted from nerve terminals (O'Brien *et al*, 1999), we hypothesize that its role in mediating ECS-induced dendritic outgrowth is due to the influence of Narp in afferents to DCX+ neurons. Detailed analysis of afferents to immature granule cells generated in adulthood has demonstrated that they receive axonal inputs from mature granule cells, which express Narp (Vivar and van Praag, 2013). In addition, Narp is robustly expressed in the entorhinal cortex (Oh *et al*, 2014), which sends projections to the dentate molecular layer via the perforant pathway. Narp secreted by these projections could promote dendritic outgrowth as previous studies have shown that recombinant Narp enhances growth of dendritic processes of neurons of cortical explants (Tsui *et al*, 1996a). However, it is also conceivable that the observed effects on DCX+ dendritic arborization could reflect indirect effects of Narp on dentate gyrus excitability. As reviewed in Ikrar *et al* (2013), dentate granule cells demonstrate feedback inhibition via the mossy fiber—CA3—hilar interneuron circuit which could be disrupted by Narp deletion as Narp is trafficked along mossy fibers after ECS (Reti and Baraban, 2000; O'Brien *et al*, 1999) and Narp regulates mossy fiber plasticity (Mariga *et al*, 2015).

Since the ability of ECS to elicit antidepressant-like effects is dependent on adult hippocampal neurogenesis (Schloesser *et al*, 2015), it is tempting to speculate that the increased dendritic arborization induced by ECS also plays a critical role. Then, the ability of Narp deletion to abolish the behavioral antidepressant effects of ECS could reflect its blockade of these morphological changes elicited by ECS. However, further studies are needed to test this hypothesis. The availability of conditional Narp KO mice would be particularly helpful in identifying which Narp-positive afferents mediate the ability of ECS to increase dendritic arborization of DCX+ neurons and if these play an essential role in eliciting the antidepressant response to ECS. In addition, further studies employing retroviral labeling of newborn granule cells and their afferents are warranted to confirm the effects observed on dendritic morphology using DCX immunostaining and to determine if these changes reflect a general increase in afferent innervation or selective increases in particular afferents. Furthermore, conditional deletion of Narp would also be helpful in assessing whether Narp expression in other limbic areas (Reti *et al*, 2002b, c, 2008a, 2012) may also contribute to mediating the antidepressant effects of ECS.

We are aware that concerns about whether the TST and FST provide a reliable measure of antidepressant efficacy have fostered the use of additional behavioral assays considered to have higher face validity, such as sucrose preference and novelty-induced suppression of feeding (Nestler and Hyman, 2010). However, in preliminary studies we found that WT CF-1 mice do not exhibit reduced sucrose preference following chronic stress rendering this assay uninformative in evaluating the impact of Narp deletion on the CF-1 background. However, while it is reasonable to be skeptical of the predictive validity of these immobility assays in evaluating novel antidepressant treatments, we are using them here in a different context, that is, to assess whether Narp deletion blocks the antidepressant activity of a well-established and highly efficacious antidepressant treatment.

Furthermore, we have found in pilot studies that a single ECS episode is not sufficient to reduce immobility on the FST in CF-1 mice indicating that this assay parallels the need to use multiple ECT treatments clinically.

In conclusion, as repeated seizure activity produces a bewildering array of electrophysiological and biochemical effects in brain, it has been unclear whether it would be feasible to identify which of these play a critical role in its highly effective antidepressant effects, information that would be valuable in developing improved treatment regimens. We submit that these findings provide an important starting point for deciphering the molecular basis of ECT by focusing attention on Narp, and its role in regulating dendritic outgrowth, as a potential mediator of its antidepressant action.

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