Behavioural Brain Research 413 (2021) 113466

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Engrailed 2 deficiency and chronic stress alter avoidance and motivation behaviors

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ARTICLE INFO

Keywords:
Chronic stress
En2
Social defeat
Norepinephrine
Motivation
Reward
Avoidance
Autism spectrum disorder
Engrailed 2
Gene x stress

ABSTRACT

Autism spectrum disorder (ASD) is a pervasive neurodevelopmental disorder characterized by impairments in social interaction, cognition, and communication, as well as the presence of repetitive or stereotyped behaviors and interests. ASD is most often studied as a neurodevelopmental disease, but it is a lifelong disorder. Adults with ASD experience more stressful life events and greater perceived stress, and frequently have comorbid mood disorders such as anxiety and depression. It remains unclear whether adult exposure to chronic stress can exacerbate the behavioral and neurodevelopmental phenotypes associated with ASD. To address this issue, we first investigated whether adult male and female *Engrailed-2* deficient (*En2*-KO, *En2*−/−) mice, which display behavioral disturbances in avoidance tasks and dysregulated monoaminergic neurotransmitter levels, also display impairments in instrumental behaviors associated with motivation, such as the progressive ratio task. We then exposed adult *En2*-KO mice to chronic environmental stress (CSDS, chronic social defeat stress), to determine if stress exacerbated the behavioral and neuroanatomical effects of *En2* deletion. *En2*−/− mice showed impaired instrumental acquisition and significantly lower breakpoints in a progressive ratio test, demonstrating *En2* deficiency decreases motivation to exert effort for reward. Furthermore, adult CSDS exposure increased avoidance behaviors in *En2*-KO mice. Interestingly, adult CSDS exposure also exacerbated the deleterious effects of *En2* deficiency on forebrain-projecting monoaminergic fibers. Our findings thus suggest that adult exposure to stress may exacerbate behavioral and neuroanatomical phenotypes associated with developmental effects of genetic En2 deficiency.

1. Introduction

Neurodevelopmental disorders such as autism spectrum disorders (ASD) are characterized by a high degree of genetic heterogeneity [1–3]. Core features of ASD include impairments in social communication and restricted, repetitive sensory-motor behaviors. Though deficits such as altered brain development, dysregulation of monoamine neurotransmission, and impaired neural organization are observed, reliable biomarkers are not currently available [4–8]. As a result, ASD diagnosis is often based on behavioral phenotypes in different social communication subdomains. While typically studied as a neurodevelopmental disorder, ASD is a lifelong illness, and adults with ASD experience more stressful life events, and greater perceived stress, cognitive deficits, and abnormal behaviors. Adults with ASD often suffer from comorbid anxiety and/or depression as well [9–17]. Rates of social anxiety among adults with ASD are reported to be as high as 28 % [18]. However, it remains unclear whether chronically stressful life events can exacerbate the long-lasting neurodevelopmental and behavioral phenotypes associated with ASD.

Directly studying the rich heterogeneity of complex human disorders such as ASD in mice is impossible. However, some strains of mice with mutations in genes implicated in ASD do show neurodevelopmental deficits, including monoamine deficiencies, and altered behaviors [5–7, 19,20]. In this study, we focus on *Engrailed*-2 deficient ($En2^{-/-}$) mice. *Engrailed-2* is a homeobox transcription factor critical for midbrain to hindbrain regionalization, cerebellar development, and neural growth and maturation [4,22,23]. *En2* genetic polymorphisms (both rare and

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https://doi.org/10.1016/j.bbr.2021.113466
Received 8 January 2021; Received in revised form 18 June 2021; Accepted 9 July 2021
Available online 13 July 2021

common variants) may be associated with ASD [24–33]. Loss of cerebellar Purkinje neurons is a neuropathological feature in the majority of ASD patients [34–38]. Recent fMRI studies suggest that disrupted cerebro-cerebellar circuit activity may be a target for therapeutic intervention [39,40]. Human brain postmortem studies and neuroimaging analysis have also reported variable degrees of cerebellar atrophy and cortical anomalies of dopaminergic/serotoninergic innervation in ASD brains [41–46]. Importantly, two independent studies of human cerebellum, including 13 and 29 ASD samples [21–23] found abnormal levels of *En2* mRNA and protein in postmortem tissues, suggesting a correlation between *En2* gene dysregulation and ASD.

Human neuropathology, brain imaging, genetic studies, and mouse experimental systems, support the idea that the *En2*$^{-/-}$ mouse is a relevant experimental system for studying neurodevelopmental deficits [21–30,35–37,39,47–49]. *En2*$^{-/-}$ mice exhibit behavioral alterations and abnormalities in hindbrain development and monoaminergic neural circuitry. Specifically, *En2*$^{-/-}$ mice show impairments in social interaction, memory tasks, and sensory-motor gating, as well as reductions in juvenile play, social sniffing, and aggressiveness [50–52]. During the early postnatal period, mice lacking *En2* (*En2*$^{-/-}$) show dysregulated levels of monoaminergic neurotransmitter levels and dramatic loss of norepinephrine innervation of the forebrain [4].

However, it remains unknown whether *En2* deficiency leads to deficits in acquisition and performance on reward based instrumental tasks. Decreased valuation of social reward, both in the anticipated and actual pleasure received, can lead to blunted processing of rewarding social stimuli and contexts [53–55]. Furthermore, mood disorders such as major depressive disorder, which are highly comorbid with ASD, also decrease motivated behavior [56]. Therefore, we first investigated whether *En2*$^{-/-}$ mice also display disrupted motivated behaviors.

We also asked whether chronic adult exposure to stressful experiences can exacerbate *En2* deficiency-induced alterations in behavior and monoaminergic fibers. Chronic stressful experiences are a major risk factor for mood disorders in humans, and rodents exposed to chronic stress paradigms display behavioral disturbances indicative of an altered affective state [57–60]. One widely used chronic stress paradigm is chronic social defeat stress [61,62](CSDS). In CSDS, an experimental mouse (e.g. C57BL/6 J) is exposed to a larger and more aggressive conspecific (e.g. CD-1) for 5 min per day for 10 days. These exposures result in multiple incidents of attack and defeat by the more aggressive conspecific, and subsequent changes in avoidance, reward, and motivated behaviors as well as neuroanatomical and neuroendocrine measures [63,64]. We hypothesized that adult exposure to CSDS would exacerbate phenotypes associated with developmental *En2* deficiency. This gene x stress hypothesis should also have relevance for understanding how adults with ASD respond to chronically stressful life experiences.

2. Methods and materials

All experimental protocols were conducted in compliance with NIH laboratory animal care guidelines and approved by the Rutgers University Institutional Animal Care and Use Committee (IACUC number: ID999900478). All mice were maintained on a 12 L:12D schedule where the lights were on from 6:00 a.m. to 6:00 p.m. Mice were housed in standard plastic cages in a colony room maintained at approximately 20 °C, with ad libitum access to food and water.

2.1. Experimental subjects

En2 tm1Alj/tm1Alj (En2−/− mice), generated on a 129S2/SvPas background as previously described [23,26,65], were purchased from Jackson Laboratories (Bar Harbor, ME, USA). *En2* heterozygous offspring on the B6/Pas hybrid genetic background were intercrossed to non-littermates to maintain the line. Pups were kept with the dam until weaning at postnatal day (P) 21. After weaning, juveniles were housed by sex in groups of two to five. All experiments were conducted using adult male and female littermates of the *En2* wildtype (+/+; *En2*-WT) and null mutant (−/−; *En2*-KO) genotypes generated from crosses of En2 heterozygous (+/−) parents.

2.2. Genotyping

Mice were genotyped by PCR analysis of tail DNA using standard PCR methods provided by Jackson Laboratories (Bar Harbor, ME, USA). Briefly, 0.5–1 cm tail snips were digested using a PureLink™ Genomic DNA Mini Kit (Product#K182001; Invitrogen/ lifetechnologies.com). The following primers were utilized in the PCR reaction: GTTCACAGTCCTGTGAAATGCAGC, a sequence common to both *En2*+/+ and *En2*−/− mice; (2) ACCAACAGGTACCTGACAGAGC, a sequence specific for the *En2*+/+ homeobox; and (3) CTTGGGTGGAAGGGCTATTC, a sequence in the neomycin gene in the *En2*−/− mutation. These primers amplify a 600-bp band in *En2*+/+ mice, a 950-bp band in *En2*−/− mice, and one band of each size in *En2*+/− mice.

2.3. Behavioral tests

Behavioral experiments were conducted between 9:00 and 16:00 in dedicated testing rooms, using methods previously described [66–68]. Cohorts of adult males and females were used for all behavioral experiments, and data from both sexes were combined [50].

2.3.1. Avoidance behaviors

We first used elevated plus maze and open field, which historically were called anxiety-related behaviors, but actually assess innate avoidance of aversive novel contexts. These behaviors are thus referred to as avoidance behaviors [85] throughout the manuscript.

2.3.1.1. Elevated plus-maze. The elevated plus-maze (EPM) test was performed as previously described [66,67].The apparatus was comprised of two open arms (28 cm) and two closed arms (28 cm) that extended from a common central platform (5 × 5 cm). Each mouse was individually placed in the center facing an open arm and allowed to freely explore the apparatus for 5 min. The illumination on the open arms was approximately 300 lx. The 5-minute session was recorded using a video camera mounted overhead above each EPM arena. The apparatus was cleaned with 70 % ethanol and water between subjects. Time spent in the open arms and numbers of open and closed arm entries were scored using a commercial software package, EthoVision (Noldus Information Technology, Leesburg, VA, USA).

2.3.1.2. Open field. General exploratory activity in a novel open field (OF, measuring 40.64 × 40.64 cm, session total time: 30 min) was quantified via infrared photobeams using Motor Monitor software (Kinder Scientific, Poway, CA, USA). The computer software predefined grid lines that divide each OF chamber into center and periphery regions, with the center being a 20.32 × 20.32 cm square 10.16 cm equidistant from all four walls. Automated scoring was used to measure distance traveled (cm), both entries into the center and time in the center of the OF. Test chambers were cleaned with 70 % ethanol and water between subjects.

2.3.2. Instrumental conditioning

As previously described [69], mice were trained and tested in standard mouse operant chambers (Med Associates, Fairfax, VT, USA) housed in sound-attenuating cubicles, in a designated behavioral testing room. The operant chambers were coupled to a power control and interface connected to a computer running Med-PC IV software (Med Associates, Fairfax, VT, USA). Reinforcement was grain-based food pellet reinforcers (20 mg food pellet, Bio-Serv, Flemington, NJ, USA), delivered from each hopper into the delivery port. Throughout

behavioral procedures, mice were food-restricted, with daily supplements provided to maintain at ~85–90% ad lib weight.

One cohort of mice was trained to lever press on a continuous reinforcement (CRF) schedule followed by fixed ratio (FR) schedules that require an invariant number of responses per reinforcer (FR1, FR5). A second cohort of mice was trained on a progressive ratio (PR) schedule of reinforcement where the response requirement increments with each reinforcer series achieved, modified from [70]. The number of responses required to earn a food reward follow the order: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, and so on. The final ratio completed is the breakpoint. Total active lever presses were measured in each session (20 min in duration).

2.3.3. Chronic social defeat stress

In chronic social defeat stress (CSDS), the experimental mouse is exposed to a larger and more aggressive conspecific (e.g. CD-1) for 5 min per day for 10 days. These exposures result in multiple incidents of attack and defeat by the more aggressive conspecific as described previously [61]. Briefly, after the screening procedure, the aggressors are housed in a cage with a transparent divider for 24 h before the social defeat begins. On the first day, an experimental mouse was placed on the same side of the cage as an aggressor for 5 min. After the defeat, the experimental (defeated) mice are placed on the empty side of a divider in an aggressor's housing cage. On the second day the experimental mice faced a different aggressor. The defeated mice were moved from the compartment in the aggressor's cage it faced the day prior and it is placed on the same side of the cage as the new aggressor is housed on. This cycle is repeated for a total of 10 days, with the defeated mice facing a different aggressor each day and being housed on one side of the divider next to an aggressor overnight. For the experimental control mice, they did not meet nor were housed alongside an aggressor. Control mice are housed two per cage, with one mouse on each side of a perforated plexiglas divider for the duration of the 10-day CSDS without any physical contact. The mice were rotated daily between control cages. After the last defeat session, the experimental mice were housed two per cage, with a divider separating them. For CSDS of females, we used CSDS variants described by [66,71].

2.3.4. Social interaction test

The social interaction test (SIT) evaluates social interaction in the presence of a novel, aggressive mouse. In the social interaction test, one novel aggressor (CD-1) was used per 20 mice being tested. The arena is the same size as the open field arena. The aggressive CD1 mouse was placed into a perforated plastic enclosure centered against one wall of the arena. The social interaction zone was a defined area flanking the aggressor. The corners were on the opposite side of the arena from the plastic enclosure. There were two 150 s trials performed. First, the experimental mouse was placed into the arena with no target mouse (CD-1 mouse not present, Trial 1). During the second 150 s trial, the CD-1 mouse was present inside the plastic enclosure (CD-1 mouse present, Trial 2). The time spent in the social interaction zone (with and without CD-1 present) and within the corners opposite the CD-1 mouse were recorded by an overhead camera placed above each arena and analyzed via EthoVisionXT software (Noldus, Leesburg, VA, USA).

2.4. Tissue fixation, histology, and immunohistochemistry

2.4.1. Brain collection

Following behavioral testing, brains were collected from all experimental mice. Mice were anesthetized with EUTHASOL® Euthanasia Solution (pentobarbital sodium and phenytoin sodium, 80 mg/kg) and perfused transcardially with PBS followed by 4% paraformaldehyde. Brains were collected, stored in 4% paraformaldehyde overnight at 4 °C, and then switched to 30 % sucrose 0.1 % sodium azide (NaN_3) in PBS solution until sectioned.

2.4.2. Sectioning

Neuroanatomical methods and analyses were previously described [4]. Briefly, 20 μm frozen cryosections for immunostaining were collected in a 1:3 series, discarding 2 and retaining the 3rd, and distributed onto 5 parallel series of 15 slides per mouse brain. The first five slides encompassed the area of the brain containing the mPFC (dorsally) and the nucleus accumbens (ventrally), and the remaining ten slides encompassed the area containing the amygdala. The mPFC/nucleus accumbens slides began at Interaural 5.76 mm and Bregma 1.97 mm, and the BLA slides began at Interaural 3.20 mm and Bregman -0.059 mm.

2.4.3. Fluorescence immunochemistry

Sections from male mice from all four groups were immunostained simultaneously. Following overnight incubation with the primary NET mouse antibody (MAb Technologies, Inc., Stone Mountain, GA, USA (#NET05-2); dilution factor of 1:1000 in 0.3 % Triton and 2% NGS) and three washes, sections were exposed to Goat Anti-Mouse IgG H&L (Alexa Fluor® 594) antibody with a dilution of 1:1000 (Invitrogen, Carlsbad, CA, USA) and then counterstained with DAPI. This mouse monoclonal antibody has been fully characterized by Matthies et al., 2009 [86] https://doi.org/10.1186/1471-2202-10-65 in mouse brain where it colocalizes with tyrosine hydroxylase (TH) in the locus coeruleus and with vesicular monoamine transporter-2 (VMAT-2) in hippocampal dentate gyrus. Further, in cultured sympathetic neurons, the monoclonal labels cell soma, axonal fibers, and synaptic VMAT + vesicles, but signal is absent in the NET knock out ganglion cells. In our studies, processing of brain region sections with vehicle alone lacking primary antibody, resulted in no fiber staining after incubation with secondary antibody.

The processed tissue was analyzed under a Zeiss Apotome microscope at 20x magnification. Images were taken of the area of interest. Following the imaging, 5 × 4 grids were superimposed on the image. Fiber densities were quantified using stereological measures of labeled fibers crossing overlaid grids using Stereo Investigator software. Grid densities were determined blind based on pilot studies of fiber crossings, so that the coefficient of variance is <0.05, and were applied to the prefrontal cortex and amygdala. To quantify density, the number of times the NET fibers crossed each horizontal and vertical grid line was counted. This was repeated for each 20 μm section per region of interest, per animal subject. To determine the overall density of fibers for that brain region per animal per treatment, the values were then averaged across all sections counted.

2.5. Statistical analyses

Significant differences and P values were calculated using a 2 × 2 ANOVA test (unless noted otherwise) with Origin Pro 2020b. Kolmogorov-Smirnoff test was used to compare the distribution of responses and performance in the instrumental tasks. Mean ± standard error of the mean was reported unless stated otherwise. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$. Males and females were combined for analyses as we had no *a priori* expectations of sex differences and there was no statistical indication of sex differences from the data. The D'Agostino-Pearson normality test and the Grubbs' test for outliers were also used. However, in all cases reanalyses with outliers removed did not change the significant differences observed or interpretations. Thus, no outliers were removed from the data presented throughout.

2.6. Data sharing

The raw data that support the findings of this study are available from the corresponding author upon reasonable request.

3. Results

3.1. Effects of En2 deficiency on instrumental responding and motivated behaviors

We began by assessing *Engrailed-2* knockout ($En2^{-/-}$) mice in an operant task involving instrumental responding. Adult male and female mice were combined into mixed sex cohorts for analyses since we had no *a priori* expectations of sex differences [50] and no statistical indication of sex differences from the data.

En2 mice ($N = 13_{En2-/-}; 7_{En2+/+}$) were trained on a continuous schedule of reinforcement (CRF) to press a lever to receive a food reward

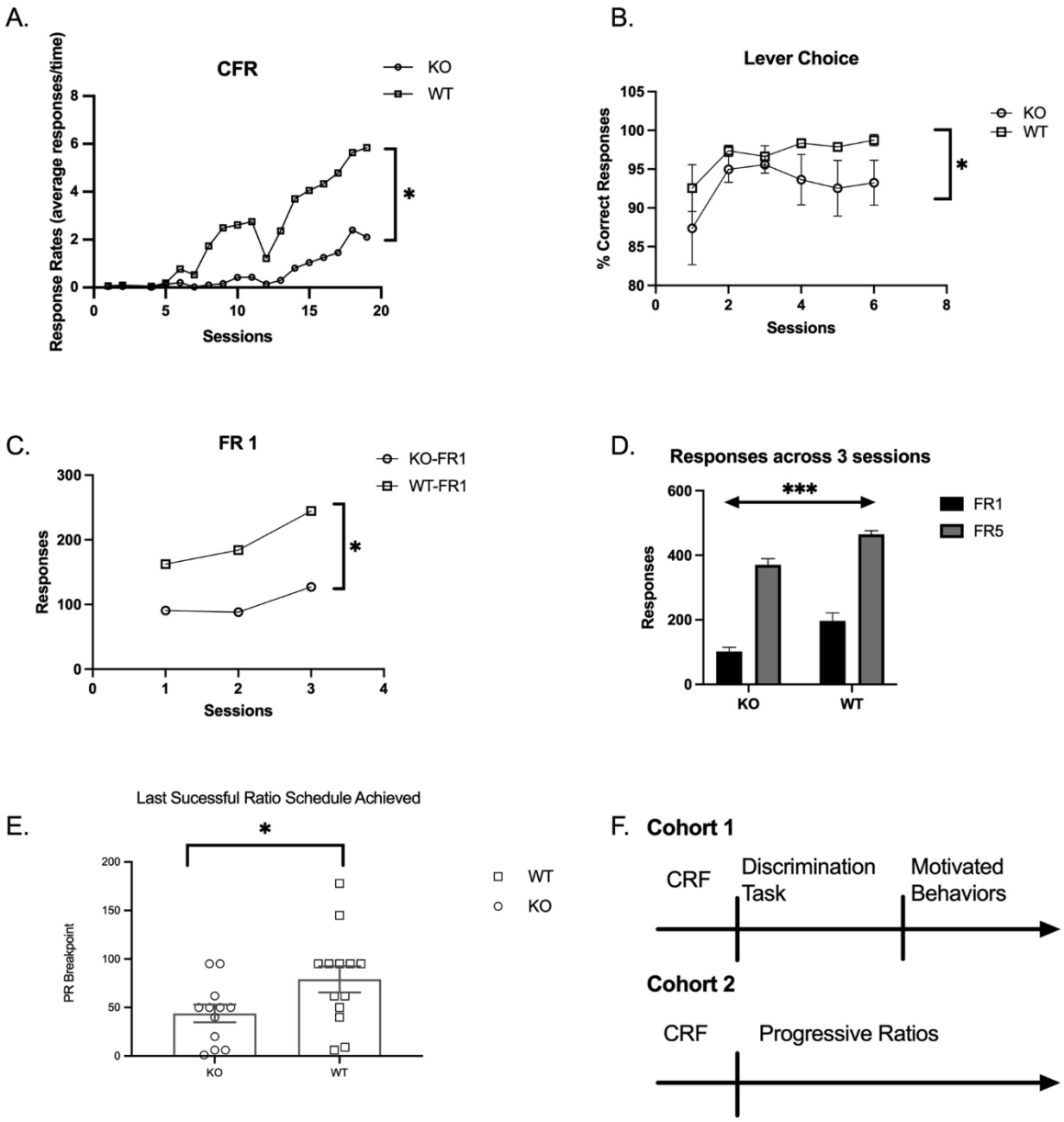

Fig. 1. Instrumental task acquisition rates and motivated behaviors negatively impaired in the $En2^{-/-}$. **A,B.** $En2^{-/-}$ displayed lower rates of acquisition on an instrumental lever pressing task (Fig. 1A: continuous reinforcement schedule, CRF) and impaired performance on a lever discrimination task (Fig. 1B: mouse had to choose between an active lever which dispensed a reward or an inactive lever which did not) as compared to $En2^{+/+}$. Across 18 session days, $En2^{+/+}$ mice exhibited a faster rate of acquiring the operant task on a continuous schedule of reinforcement as compared to $En2^{-/-}$ mice ($N = 13_{En2-/-}; 7_{En2+/+}$; mean rate $_{En2-/-} = 0.616$, mean rate $_{En2+/+} = 2.4$; $D = 0.5$; $Z = 1.5$; Exact Prob>|D| = 0.02075, Kolmogorov-Smirnov Test). When tasked with discriminating between two levers to receive a food reward, $En2^{+/+}$ mice had fewer errors in choosing the correct lever as compared to $En2^{-/-}$ mice (mean correct response$_{En2-/-} = 93$ %; mean correct response$_{En2+/+} = 97$ %; $D = 0.83$; $Z = 1.4$; Exact Prob>|D| = 0.0259, Kolmogorov-Smirnov Test). **C,D.** $En2^{-/-}$ mice emitted significantly fewer responses across all ratios of reinforcement as compared to the $En2^{+/+}$ mice. This concomitant decrease in responses indicated that the $En2^{-/-}$ mice were not able to adjust to increasing task demands. Mice ($N = 13_{En2-/-}; 7_{En2+/+}$) were sequentially trained to press a lever to receive a food reward on increasing FR schedules of reinforcement (FR1,5). Responses were analyzed with a 2 (Genotype: $En2^{-/-}$, $En2^{+/+}$) x 2 (FR1, FR5) ANOVA. The main effect of genotype was significant, $F(1,11) = 28.62$, $p = 6.86087E\text{-}4$. The main effect of schedules of reinforcement was significant, $F(1,11) = 229.8$, $p = 3.5524E\text{-}7$. There was not a significant interaction between genotype and the reinforcement schedules. **E.** When challenged with increasing task demands on a progressive ratio task, $En2^{-/-}$ mice exhibited decreased motivated behaviors to press a lever for a food reward. This lower breakpoint in the $En2^{-/-}$ (unpaired t-test, $En2^{-/-}{}_{n=13} = 41$; $En2^{+/+}{}_{n=13} = 79$; $t = 2.356$; $df = 24$; $p = 0.027$) is not reflective of a lack of motivation to feed (see Supplemental Fig. S1C). **F.** Timeline of the sequence of instrumental tasks. CRF = continuous reinforcement schedule; Discrimination task: active vs inactive lever; Motivated Behaviors: fixed ratio task.

(Fig. 1A; see Fig. 1F for experimental groups and timeline). Across 18 session days, *En2*$^{+/+}$ mice exhibit a faster rate of acquiring the operant task as compared to *En2*$^{-/-}$mice (mean rate$_{En2-/-}$ = 0.62, mean rate-$_{En2+/+}$ = 2.4; D = 0.5; Z = 1.5; Exact Prob>|D| = 0.02075, Kolmogorov-Smirnov Test). Following the CRF sessions, mice were required to discriminate between two levers to receive a food reward (Fig. 1B). Though both genotypes performed above 90 % on the discrimination task, *En2*+/+ mice made fewer errors in choosing the correct lever as compared to *En2*$^{-/-}$mice (mean correct response $_{En2-/-}$ = 93 %; mean correct response$_{En2+/+}$ = 97 %; D = 0.83; Z = 1.4; Exact Prob>|D| = 0.0259, Kolmogorov-Smirnov Test). We did not observe any differences in total lever presses (active, inactive, active + inactive) (Supplemental Fig. 1A). Thus, *En2*$^{-/-}$ mice were able to learn and perform instrumental responses, but at a reduced learning rate and at decreased performance level as compared to WT (*En2*$^{+/+}$) littermates.

We next tested motivated behavior. En2$^{-/-}$ mice (N = 13 $_{En2-/-}$; 7$_{En2+/+}$) were sequentially trained to press a lever to receive food reward on increasing Fixed Ratio (FR) schedules of reinforcement (FR 1 or FR 5). Both En2$^{-/-}$and En2$^{+/+}$ mice were able to adjust to increasing task demands (Fig. 1C, D). However, En2$^{+/+}$ mice showed significantly higher responses across all ratios of reinforcement as compared to En2$^{-/-}$ mice (unpaired t-test, t = 3.43, df = 4, p = 0.026). A 2 (Genotype: En2$^{-/-}$, En2$^{+/+}$) x 2 (FR1, FR5) ANOVA found main effects of genotype ($F(1,11) = 28.62$, $p < 0.0001$) and reinforcement schedule ($F(1,11) = 229.8$, $p < 0.0001$), but not an interaction (Fig. 1D). These effects could be attributable to differences in hunger levels or body weights. However, we also measured the baseline weights and weights following the fixed ratio tasks. The weights were not significantly different between the groups [(En2$^{-/-}$$_{pre}$ = 25.96 +/-0.64 g; En2$^{-/-}$$_{post}$ = 22.46 +/-0.61 g); (En2$^{+/+}$$_{pre}$ = 25.46+/-0.98 g; En2$^{+/+}$$_{post}$ = 21.54+/-0.97 g); Supplemental Fig. S1B]. Additionally, the % drop in weight due to food restriction (p = 0.083, Mann-Whitney test) was comparable between the En2$^{+/+}$ (96 %) and En2$^{-/-}$ (92 %). In the open field test, we measured exploratory locomotor behaviors and calculated the total distance travelled in the periphery (En2$^{+/+}$$_{control}$ = 6327.7 +/- 636.49 SEM; En2$^{-/-}$$_{control}$ = M = 6694+/- 365.4SEM; Fig. 3C). In the control condition, similar to the condition tested in the operant tasks, both the En2$^{+/+}$ and En2$^{-/-}$ travelled similar distances in the periphery.

In a separate cohort of adult mice, we next used a progressive ratio reward schedule to further investigate motivation to exert increasing effort for reward. *En2*$^{-/-}$ mice exhibited a lower breakpoint compared to *En2*$^{+/+}$ mice (unpaired t-test, *En2*$^{-/-}$$_{n=13}$ = 41; *En2*$^{+/+}$$_{n=13}$ = 79; t = 2.36; df = 24; p = 0.027; Fig. 1E). This lower breakpoint was not reflective of a lack of motivation to feed, as *En2*$^{-/-}$mice do not display differences from *En2*$^{+/+}$ littermates in latency to eat a pellet in the home cage following a 18 -h food deprivation that mimics the ~85–90% of baseline body weight used throughout instrumental conditioning or progressive ratio (unpaired t-test, *En2*$^{-/-}$$_{n=10}$ = 16.7 s; *En2*+/+ $_{n=8}$ = 41.5 s; t = 1.99; df = 16; p = 0.06; Supplemental Fig. 1C). Taken together, these data indicate that *En2* deficiency results in deficits in instrumental responding and motivated behaviors.

3.2. The behavioral effects of chronic social defeat stress on *En2*$^{+/+}$ and *En2*$^{-/-}$ mice

We next exposed a different cohort of En2−/− and En2+/+ littermates to either control or chronic social defeat stress (CSDS) for 10 days. 24 h after the last CSDS session, we exposed the experimental mice to a social interaction test (SIT, Fig. 2A). A two-way repeated measures ANOVA assessing the *En2*$^{-/-}$ mice revealed a significant treatment (control vs CSDS) × target (CD-1 aggressor vs empty enclosure) interaction, (Fig. 2B; $F(1, 20) = 7.69$, p= 0.01172). Post hoc comparisons using the Bonferroni test ($t = 3.65$, $p = 0.0096$) indicated that, following CSDS, *En2*$^{-/-}$ mice spent more time in the SIT zone in the target present condition (M = 68.3 S, Std. Error = 14.7) as compared to the no target present condition (M = 34.23 S, Std. Error = 5.92). *En2*$^{-/-}$ mice without CSDS did not spend more time in the SIT zone in the target present condition (M = 46.4 S, Std. Error = 7.18) as compared to the no target present condition (M = 34.40 S, Std. Error = 4.76). By contrast, a repeated-measures two-way ANOVA in the *En2*$^{+/+}$mice revealed no interaction or main effects of either treatment or target. Therefore, CSDS had larger effects on social interaction in *En2*$^{-/-}$ than in *En2*$^{+/+}$ littermates.

We also examined the social interaction ratio (SI ratio (%) = interaction zone time of "target" trial/interaction zone time of "no target" trial) of *En2*$^{+/+}$ and *En2*$^{-/-}$ mice in SIT. SI ratio equal to 1 indicates that equal time is spent in the presence versus absence of a social target. These data indicated that all mice spent more time in the interaction zone in the "target" present trial (n $_{En2-/-\ Control}$ = 20; SI ratio = 1.4 +/- 1.1 SD; n $_{En2-/-\ CSDS}$ = 19; SI ratio = 2.1 +/- 1.8 SD; n $_{En2+/+Control}$ = 17; SI ratio = 1.7 +/- 1.1 SD; n $_{En2+/+CSDS}$ = 21; SI ratio = 1.6 +/- 0.94 SD; Fig. 2C). We also quantified the cumulative duration (%) of the time spent in the corners during SIT. The data indicate no significant differences among the groups (Target $_{En2-/-\ Control}$ = 33.34+/- 7.38 ; No Target $_{En2-/-\ Control}$ = 36.5+/- 6.03 ; Target $_{En2-/-\ CSDS}$ = 40.65+/- 7.97; No Target $_{En2-/-\ CSDS}$ = 34.13+/- 7.33; Target $_{En2+/+\ Control}$ = 33.74+/- 7.3; No Target $_{En2+/+\ Control}$ = 29.43+/-5.31; Target $_{En2+/+\ CSDS}$ = 23.3+/-8.36; No Target $_{En2+/+\ CSDS}$ = 23.52+/-6,44; nested 1way ANOVA with multiple comparisons, n.s.; Fig. 2D).

3.3. Open field

Following CSDS and SIT, we next assessed avoidance and exploratory locomotor behaviors in a novel open field arena. First, percent time spent in the center and total center entries were used to measure avoidance of the aversive center of the arena. For percent time in center, we found a significant interaction between genotype (*En2*$^{+/+}$ vs *En2*$^{-/-}$) and treatment (control vs CSDS), $F(1,72) = 6.37$, $p = 0.0139$ (Fig. 3A). Post hoc comparisons using the Bonferroni test revealed that *En2*−/− mice$_{n=23}$ following CSDS significantly spent less time in the center (M = 0.772+/- 0.18SEM) than Control *En2*−/− mice$_{n=21}$(M = 2.03+/- 0.37SEM); $p = 0.008$. By contrast, *En2*$^{+/+}$ mice did not differ in % time spent in the center following CSDS ($M_{control}$ = 0.958+/- 0.18SEM; M_{CSDS} = 1.19 +/- 0.24SEM; $p = 1$). These differences were not due to reduced center entries, as the number of center entries were not significantly different among the groups (Fig. 3B; *En2*$^{-/-}$ $_{control}$ = 13.57 +/- 3.46SEM; *En2*$^{-/-}$ $_{CSDS}$ = 8.3+/- 2.23SEM; *En2*$^{+/+}$$_{control}$ = 12.4 +/- 3.20SEM; *En2*$^{+/+}$$_{CSDS}$ = 12.23+/- 2.25SEM). For periphery distance travelled (cm), we found a main effect of treatment ($F(1,72) = 5.89$, $p = 0.0178$). Planned Bonferroni post hoc analyses suggested that *En2*$^{-/-}$ mice$_{n=23}$ exposed to CSDS displayed significantly decreased (p = 0.026) periphery distance traveled relative to Control *En2*$^{-/-}$ mice$_{n=21}$ (Fig. 3C). By contrast, *En2*+/+ mice did not differ in periphery distance travelled following CSDS (*En2*$^{+/+}$$_{control}$ = 6327.7 +/- 636.49 SEM; *En2*$^{+/+}$$_{CSDS}$ = 5768.14 +/- 479.60SEM; p = 0.90). Taken together, these data demonstrate that chronic stress exposure increases avoidance and decreases exploration (even of the non-aversive periphery) more prominently in *En2*$^{-/-}$ than in *En2*$^{+/+}$ mice, suggesting a gene x stress interaction.

3.4. Elevated plus maze

We next investigated the effects of CSDS on *En2*$^{-/-}$ and *En2*$^{+/+}$ mice in another avoidance behavior test, the elevated plus maze (EPM). We found nuanced effects of CSDS in EPM, where there were no clear differences in the mean amount of time spent in the aversive open (data not shown) or the non-aversive closed arms. However, when we compared the distributions of % time spent in the closed arms using the Median Test (Fig. 4A), our results indicated that the *En2*$^{-/-}$ mice following CSDS$_{n=23}$ spent more time in the closed arms (median = 92.5 s) as compared to *En2*$^{-/-}$ Control mice$_{n=21}$ (median = 83.6 s, Chi-square = 4.46; p = 0.03). Fig. 4A plots the median with interquartile range with

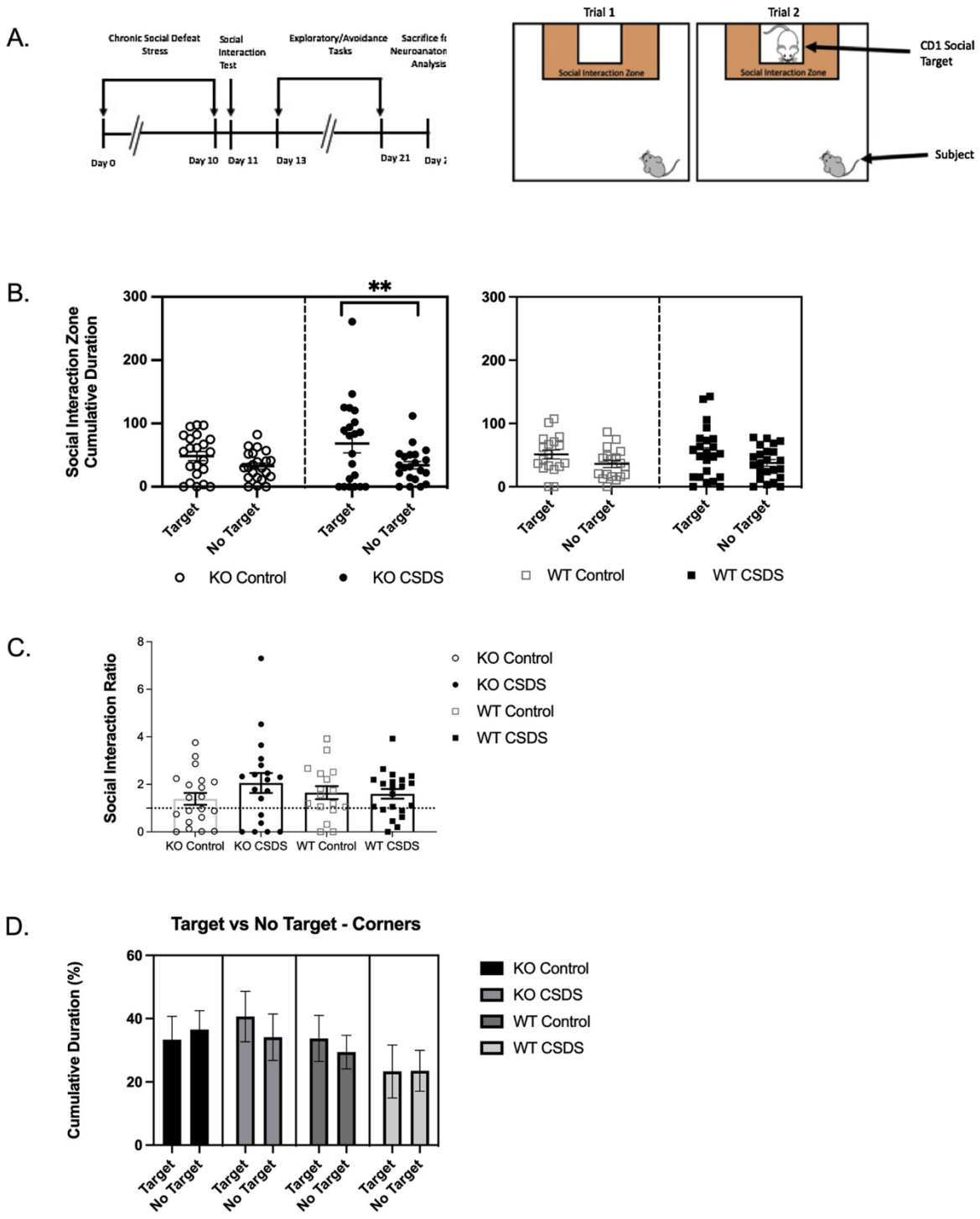

Fig. 2. Following CSDS, *En2*$^{-/-}$ mice displayed altered behaviors. **A.** Outline of the timeline of behavioral assessments (left panel). The schematic of the Social Interaction Test illustrates the trial types (right panel). The mouse explores the same testing arena across two trials (2.5 min per trial). In trial 1, the mouse is alone with an empty plexiglass enclosure (no target). In trial 2, the mouse paired with a social target, housed in the plexiglass enclosure (novel CD1). **B.** *En2*$^{-/-}$ mice exhibit altered social behaviors following Chronic Social Defeat Stress (CSDS). Post hoc comparisons using the Bonferroni test ($t = 3.65$, $p = 0.0096$) indicated that only the *En2*$^{-/-}$ mice, following CSDS, significantly spent more time in the social interaction zone (SIT) in the target present condition (M = 68.3 S, Std. Error = 14.7) as compared to the no target present condition (M = 34.23 S, Std. Error = 5.92). *En2*$^{-/-}$ mice in the control condition did not significantly spent more time in the SIT zone in the target present condition (M = 46.4 S, Std. Error = 7.18) as compared to the no target present condition (M = 34.40 S, Std. Error = 4.76). Following CSDS, *En2*$^{+/+}$ mice did not significantly spend more time in the SIT zone as compared to the control mice, irrespective of the presence and absence of a social target. **C.** Social interaction ratio data (SI ratio (%) = interaction zone time of "target" trial/interaction zone time of "no target" trial) demonstrate that all mice usually spend more time in the interaction zone in the "target" present trial. A SI ratio equal to 1 indicates equal time was spent in the presence versus absence of a social target (dashed line). **D.** Analysis of the cumulative duration (%) of the time spent in both corner 1 and corner 2 indicate that all mice spend comparable amount of times in the corners in both trial conditions (nested 1way ANOVA with multiple comparisons, n.s).

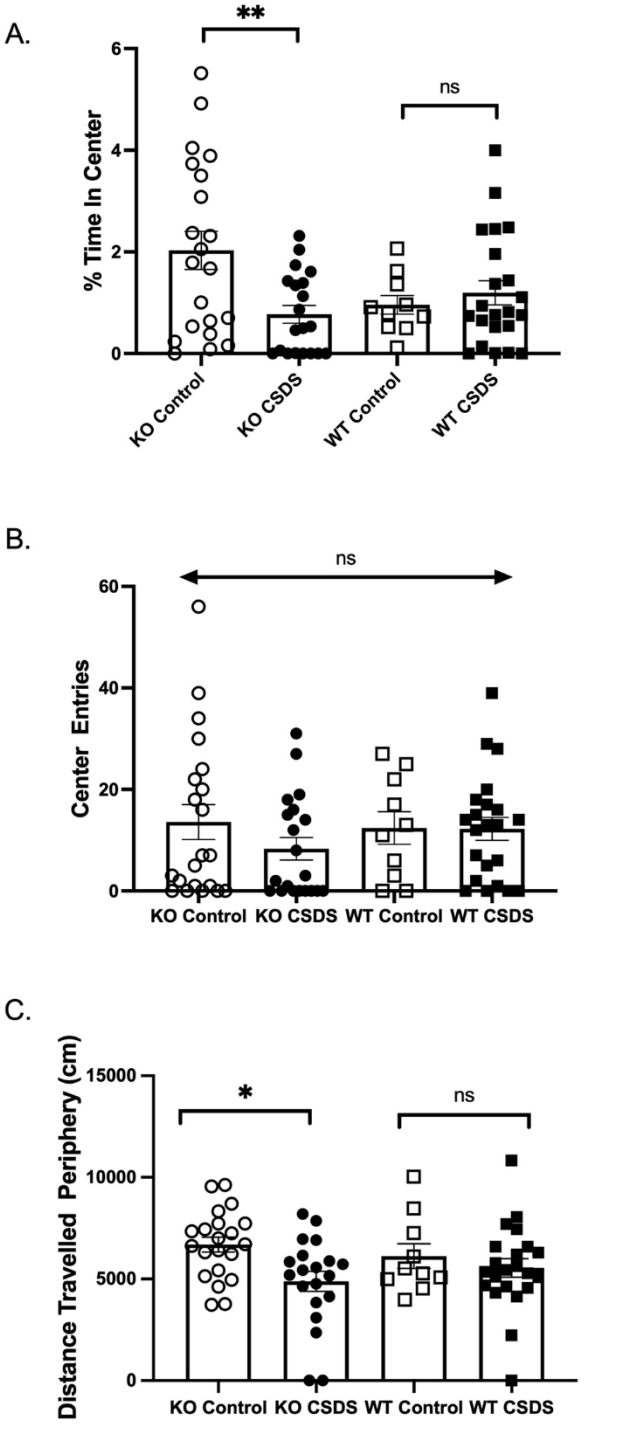

Fig. 3. Following CSDS, *En2*$^{-/-}$ mice displayed increased avoidance and decreased exploration. **A.** Mice were tested in an open field arena. *En2*$^{-/-}{}_{n=23}$ following CSDS significantly spent less time in the center (M = 0.772; SEM = 0.18) as Control *En2*$^{-/-}{}_{n=21}$(M = 2.03 ; SEM = 0.375); p = 0.008, Bonferroni test. There was a significant interaction between genotype (En2+/+ vs En2−/−) and treatment (control vs CSDS), F(1,72) = 6.37, p = 0.0139. The *En2*$^{+/+}$ mice did not differ in percent time spent in the center following CSDS. The increased avoidance measured was not reflective of differential center entries. The number of center entries was not significantly different among the groups **(B). C.** CSDS resulted in decreased exploratory behaviors in *En2*$^{-/-}$. *En2*$^{-/-}{}_{n=23}$ following CSDS showed significantly less periphery distance travelled (M = 4885 cm; SEM = 493.1) as Control *En2*$^{-/-}$ mice$_{n=21}$(M = 6694 cm; SEM = 365.4); p = 0.03. In contrast, the *En2*$^{+/+}$ mice did not differ in periphery distance travelled following CSDS. For periphery distance travelled (cm), there was a main effect of treatment ($F(1,72) = 5.89$, $p = 0.0178$).

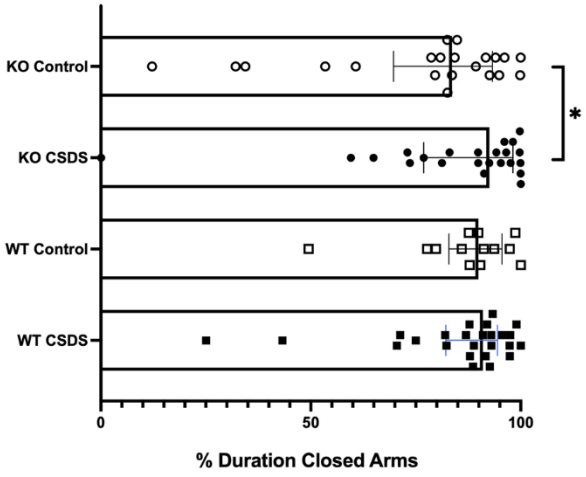

Fig. 4. Following CSDS, *En2*$^{-/-}$ displayed increased avoidance behavior in the elevated plus maze. Analysis of the distribution of duration in the closed arms using the Median Test indicated that the *En2*$^{-/-}$ following CSDS$_{n=23}$ spent more time in the closed arms (median = 92.5 s) as compared to the Control mice$_{n=21}$ (median = 83.6 s), Chi-square = 4.46; p = 0.03. There were not significant differences in time spent in the closed arms following CSDS for the *En2*$^{+/+}$ mice (CSDS$_{median}$ = 91 s; Control$_{median}$ = 90 s). Data indicate median with interquartile range.

individual data overlaid. The distribution of the individual data set for the *En2*$^{-/-}$ mice following CSDS is biased towards more time spent in the closed arms than *En2*$^{-/-}$ control mice. By contrast, there was not a significant difference in time spent in the closed arms following CSDS for the *En2*$^{+/+}$ mice (CSDS$_{median,\ n=25}$ = 91 s; Control$_{median,\ n=13}$ = 90 s, Chi-square = 0.12; $p = 0.73$).

3.5. Exposure to CSDS results in neuroanatomical and structural changes to noradrenergic fibers

In addition to the behavioral effects of *En2* deficiency described above and elsewhere, *En2*$^{-/-}$ mice also exhibit developmental deficits in dorsal forebrain monoamine systems, especially norepinephrine (NE) levels, which are reduced by 35 % at three postnatal weeks [4]. Changes in locus coeruleus (LC) fibers are especially prominent, with 73 % less tyrosine hydroxylase (TH) positive fibers and 65 % less NE reuptake transporter (NET) positive fibers in the hippocampus. By P60 (adulthood), NE levels and fibers in *En2*$^{-/-}$ mice are partially recovered. Interestingly, chronic stress exposure activates both the LC-NE system and the HPA axis [72,73]. Therefore, we next asked whether CSDS exposure exacerbated the developmental deficits seen in noradrenergic fibers in limbic regions. Following CSDS, male (cohort mean age: P118, SEM 5.11; for CSDS, n = 6 $_{En2\text{-}/\text{-}\rightarrow En2+/+}$; for control, n = 5 $_{En2\text{-}/\text{-}\rightarrow En2+/+}$) mouse brains were processed for NET fiber immunostaining. Strikingly, we found that 10 days of CSDS led to a ~20 % +/-5 SEM reduction in *En2*$^{-/-}$ NET fibers in the amygdala (BLA) compared to *En2*$^{-/-}$ littermates not exposed to stress (Fig. 5A; quantification of NET positive fibers that crossed standardized grids as described in Material and Methods; average number of fibers: control = 49.72 +/- 5.67 SEM; CSDS = 39.16 +/- 4.31 SEM). *En2*$^{+/+}$ mice following CSDS show a similar number of NET positive fibers in the amygdala compared to *En2*$^{+/+}$ littermates not exposed to stress. Surprisingly, we did not find a genotype (*En2*$^{-/-}$ vs *En2*$^{+/+}$) x treatment (control vs CSDS) interaction, but instead only saw a main effect of stress treatment ($F(1,21) = 8.1$, $p = 0.01$) (Fig. 5D, first panel). In contrast, there were no significant differences in the NET + fibers in the mPFC among all comparisons (Fig. 5C,D, second panel). However, when we quantified the number of NET + fibers in the nucleus accumbens, we observed an intriguing but not significant trend. *En2*$^{-/-}$ mice following only 10 days of CSDS

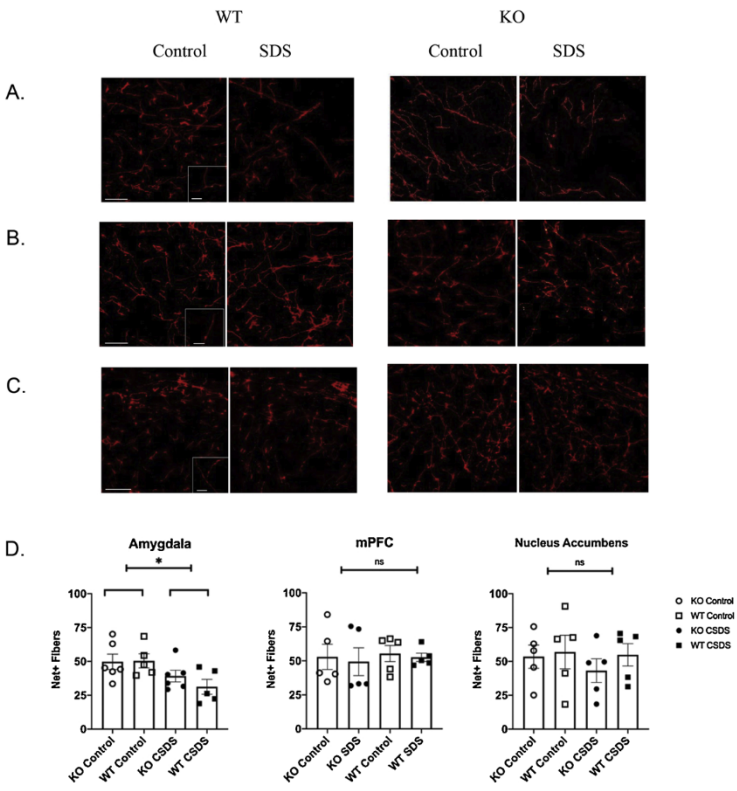

Fig. 5. Quantification of NET positive fibers in the amygdala, nucleus accumbens and medial PreFrontal Cortex of *En2*$^{-/-}$ and *En2*$^{+/+}$ mice. **A.** Following CSDS and associated negative valence behaviors and behaviors alone (control conditions) indicate that CSDS (left) reduced the number of fibers as compared to control conditions (right) in BLA for both *En2* $^{-/-}$ and *En2*$^{+/+}$. This same reduction was not observed in Nucleus Accumbens **(B.)** and the medial PreFrontal Cortex **(C.)** For each area the *En2*$^{+/+}$ Control is first column on the left, and this has a scale bar (100 μm) that applies to all the other 3 groups (horizontally) for that region. In addition, each *En2*$^{+/+}$ Control has an inset (bottom right) at higher magnification. The low power images are at 20x, at which they were quantified, and the higher power images are at 40x to show focused fibers. **D.** NET + fibers in the amygdala, nucleus accumbens, and medial PreFrontal Cortex were analyzed with a 2 (Genotype: *En2*$^{-/-}$, *En2*$^{+/+}$) x 2 (treatment: CSDS, Control) ANOVA. Though a main effect of genotype was not significantly different, the main effect of treatment was significantly different, $F(1,21) = 8.1$, $p = 0.01$, indicating that 10 days of CSDS significantly reduced the density of NET fibers measured in the amygdala. However, quantification of NET positive fibers in the nucleus accumbens and the medial PreFrontal Cortex of *En2*$^{-/-}$ indicated no significant differences in the NET + fibers among all the groups.

showed a 21 % (control = 53.47 +/- 8.58 SEM; CSDS = 43.15 +/- 8.78 SEM) reduction in the number of NET + in the nucleus accumbens; *En2*$^{+/+}$ only exhibited a 6% decreased (control = 56.92 +/- 12.42 SEM; CSDS = 54.84 +/- 8.28 SEM; Fig. 5B,D, third panel). These data thus describe what may be an additive effect of CSDS and *En2* deficiency on NET fibers and, subsequently, on noradrenergic neural circuitry.

4. Discussion

Our results indicate that *En2* deficiency decreases performance in motivated behaviors, and that a gene x stress interaction exacerbates avoidance behaviors observed in *En2* deficient mice. Specifically, *En2*-/- mice showed deficits in response acquisition, decreased accuracy in lever choices, lower responses across all FRs, and lower breakpoints overall compared to *En2*+/+ mice. *En2*-/- mice exposed to CSDS spent more time in the elevated plus maze closed arms and displayed reduced exploratory behaviors and time spent in the center of the open field paradigm. Strikingly, CSDS also resulted in a significant decrease in NET fibers in the amygdala (BLA) but not in medial prefrontal cortex in both *En2*+/+ and *En2*-/- mice.

4.1. En2-deficiency impairs effortful responding

En2$^{-/-}$ mice were able to learn and perform instrumental tasks using a continuous reinforcement schedule and a fixed ratio schedule. However, performance metrics, such as rate of task acquisition and correct responses, were impaired relative to *En2*+/+ littermates. The CRF tasks provide a reward after a fixed number of responses and are typically associated with motivated effort and instrumental learning. However, CRF tasks followed by progressively altering reinforcement may also offer some insight about resistance to change, behavioral rigidity to routines, and frustrative non-reward. In a progressive ratio task [56,69,74], we found a decreased breakpoint (last successful ratio completed) in *En2*$^{-/-}$ mice relative to *En2*+/+ littermates. Thus, En2-deficient mice display decreased effortful responding. These deficits in motivation could reflect an imbalance in monoaminergic neurotransmitter levels in *En2*-/- mice [4,52].

Similar deficits in learning and motivation are observed in other mice used to study neurodevelopmental disorders. Male 16p11.2 hemideletion animals show a delay in the acquisition of a nose poke operant task and a reduced breakpoint relative to WT littermates [75]. Likewise, BTBR T^{+}Itpr3^{tf}/J mice (BTBR), which have impairments in social interactions, including high levels of repetitive self-grooming and minimal vocalization in social settings, display significantly lower breakpoints in a social motivation paradigm and make significantly fewer lever presses in an operant task when compared to C57BL/6 J (B6) WT mice [6]. Future work using a variable schedule of reinforcements may provide additional measures of flexibility and sensitivity to variable response contingencies and how deficits observed in neurodevelopmental disorders might be impacted [76]. Importantly, analogous reward and motivation behavioral tasks can be performed in both humans and rodents using similar data analyses [56]. These cross-species reward and motivation tasks (including progressive ratio) are therefore highly translationally relevant. Given that three strains of mice with neurodevelopmental deficits that are historically associated with ASD (16p11.2, BTBR, and as shown here, *Engrailed 2*-deficient mice) show impairments in motivated behaviors, it is critical for future work to assess the relevance of these behavioral impairments to neurodevelopmental disorders such as ASD. A starting point could be to assess these cross-species reward and motivation tasks in ASD patients. While humans suffering from mood disorders show impairments in several of these tasks [56], effects of ASD are currently unknown. Future work is also necessary to determine whether chronic stress exposure exacerbates the effects of Engrailed-2 deficiency on reward and motivation.

4.2. A nuanced gene x stress interaction affects behavior in Engrailed-2 deficient mice

Clinical observations suggest the negative impact of complex social challenges in ASD [17,77]. In humans, social conflict is a stressor and

associated with increased risk for developing mood disorders such as depression and anxiety [78]. In rodents, social stressors are modeled by repeated exposure to unavoidable aggression and defeat followed by sustained sensory threat. This well validated chronic social defeat stress (CSDS) paradigm leads to impaired social interactions, decreased preference for sucrose, and increased avoidance behaviors [58,61]. We found a gene x stress interaction in open field, as CSDS-exposed *En2*−/− mice spent significantly less time in the aversive center of the OF and even displayed reduced exploration of the non-aversive periphery of the OF in comparison to the control *En2* -/- mice. Effects in EPM were more nuanced, but *En2*-/- mice exposed to CSDS showed increased avoidance. DiCicco-Bloom and colleagues (2012,[50]) detailed comprehensive behavioral phenotyping of *En2*-/- in the absence of stress. They reported no significant genotype differences in time spent in the open arms in the EPM paradigm and in distance travelled and center entries in the OF assessment. These results indicate that stress may unmask avoidance behaviors in mice lacking *En2*. Surprisingly, during SIT we also observed that *Engrailed 2*-deficient mice exposed to CSDS spent more time in the social interaction zone when a CD-1 aggressor was present than when no other mouse was present. There are several possible explanations for this result. For example, there could be a social learning deficit in the Engrailed 2-deficient mice. Another possibility is that individual differences are leading to more varied responses within the Engrailed 2-deficient group. A larger experiment where mice can be divided into resilient and susceptible groups could be one way to investigate these possibilities.

Everyone encounters stressful experiences throughout their lives. However, it remains unknown whether ASD patients have a different susceptibility for developing mood disorders from these stressors. The increased prevalence of mood disorders in the ASD patient population indicates a maladaptive response to stress is possible. This study was a first step to assess whether stress exacerbates behavioral changes caused by neurodevelopmental deficits. Importantly, given the nuanced and mixed effects that we observed in avoidance behaviors (a gene x stress interaction in Open Field but not in Elevated Plus Maze), we cannot conclude whether the effects of chronic stress exposure on avoidance behaviors are synergistic with or additive to the neurodevelopmental effects of Engrailed-2 deficiency on avoidance behaviors. Continued studies are necessary to investigate this hypothesis.

4.3. CSDS results in decreased NET fibers in the amygdala

Interestingly, we found that chronic adult exposure to social defeat not only exacerbates *En2* deficiency-induced deficits in avoidance behaviors, but also reduces the density of monoaminergic fibers in the amygdala, but not in the mPFC nor the nucleus accumbens. This reduction was caused by CSDS exposure and was found in both *En2*+/+ and $En2^{-/-}$ mice. During the early postnatal period, mice lacking *En2* show dysregulated levels of monoaminergic neurotransmitter levels and dramatic loss of (or failure to elaborate) norepinephrine innervation of the forebrain. DiCicco-Bloom and colleagues (2015) found that *En2*-/- mice, under normative conditions, exhibit developmental deficits in dorsal forebrain monoamine systems, especially NE levels, which were reduced 35 % at three postnatal weeks. The NE deficits were paralleled by changes in locus coeruleus (LC) fiber innervation, with 73 % less tyrosine hydroxylase (TH) positive fibers and 65 % less NE reuptake transporter (NET) positive fibers in hippocampus. By P60 (adulthood), NE levels and fibers in the *En2* mice recovered, but only partially. We originally hypothesized that CSDS may exacerbate these neurodevelopmental effects in En2-/- mice specifically. Surprisingly, we observed not a gene x stress interaction, but only a main effect of stress. If anything, the effects of CSDS were more prominent in *En2*+/+ mice.

Though not significant, the NE deficits measured in the nucleus accumbens following 10 days of CSDS also provide additional insight on how exposure to stress can have deleterious effects on the monoaminergic system with respect to motivated behaviors. In *En2*-/- mice, we report delayed task acquisition and decreased effortful responding on reward-based tasks. Quantification of the NET + fiber showed reduction only in the *En2* -/- and not *En2*+/+ following 10 days of CSDS. The data are suggestive that the decreased dopaminergic input into the nucleus accumbens might blunt performance on reward-based tasks in the *En2* -/- and not *En2*+/+. Further studies will be needed to investigate these possible mechanisms.

These results indicate that chronic stress reduces LC fiber innervation into target regions such as the amygdala. Seminal findings from McEwen and others [79–82] have detailed prominent neuroanatomical effects of stress exposure, including pruning of dendrites and reductions in dendritic spine densities in hippocampus. However, to our knowledge, these chronic stress effects on LC-Amygdala circuitry are not previously described. Altered monoaminergic signaling in the forebrain, and the hippocampus in particular, is also associated with mood disorders and their treatments. Stress also activates the LC-NE system and the HPA axis [83,84]. It will be interesting for future studies to determine if altered LC-Amygdala signaling is necessary for mediating the effects of chronic stress on behavior. Furthermore, it will be interesting for future studies to assess whether there is a gene x stress interaction in En2-deficient mice that affects structural plasticity of dendrites and spines in amygdala.

Importantly, one weakness of this study is that we only assessed NET fibers in sections from male mice. While we did not see any statistical indication of sex differences in our behavior experiments, it is certainly possible that there are sex differences in the effects of chronic stress on monoaminergic projections.

Author statement

MLP: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – Original Draft, Writing - Review & Editing, Visualization.

TTL, MSV, MHM, IN, NJ, NP, JD, GS, RCR, WSK, and XZ: Investigation, Formal analysis, Visualization.

EDB and BAS: Conceptualization, Methodology, Formal analysis, Resources, Writing - Original Draft, Writing - Review & Editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

The authors thank members of the Samuels and DiCicco-Bloom labs for their assistance. This work was funded by a Rutgers - CEED Pilot Project Grant [NIEHS Center grant (ES005022)]

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bbr.2021.113466.

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