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Chronic Desipramine Treatment Rescues Depression-Related, Social and Cognitive Deficits in *Engrailed-2* Knockout Mice

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Abstract

Engrailed-2 (En2) is a homeobox transcription factor that regulates neurodevelopmental processes including neuronal connectivity and elaboration of monoaminergic neurons in the ventral hindbrain. We previously reported abnormalities in brain noradrenergic concentrations in *En2* null mutant mice that were accompanied by increased immobility in the forced swim test, relevant to depression. An *EN2* genetic polymorphism has been associated with autism spectrum disorders (ASD), and mice with a deletion in *En2* display social abnormalities and cognitive deficits that may be relevant to multiple neuropsychiatric conditions. The present study evaluated the ability of chronic treatment with desipramine (DMI), a selective norepinephrine reuptake inhibitor and classical antidepressant, to reverse behavioral abnormalities in *En2* −/− mice. DMI treatment significantly reduced immobility in the tail suspension and forced swim tests, restored sociability in the three-chambered social approach task, and reversed impairments in contextual fear conditioning in *En2* −/− mice. Our findings indicate that modulation of brain noradrenergic systems rescues the depression-related phenotype in *En2* −/− mice and suggest new roles for norepinephrine in the pathophysiology of the social and cognitive deficits seen in neuropsychiatric disorders such as autism or schizophrenia.

Keywords

knockout mouse; depression; social behavior; autism; desipramine; norepinephrine

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INTRODUCTION

Engrailed-2 (En2) is a homeobox transcription factor that coordinates multiple aspects of CNS development, including formation of midbrain and hindbrain structures, cerebellar patterning and connectivity, and development and maintenance of monoaminergic neurons (Joyner, 1996; Sillitoe *et al.*, 2010; Simon *et al.*, 2005). We previously reported abnormalities in the noradrenergic system of mice lacking *En2* (Genestine *et al.*, 2011; Lin *et al.*, 2010). As compared to wildtype adult mice, *En2* null mutants displayed ~25% reductions in the levels of norepinephrine (NE) in the forebrain including hippocampus and cerebral cortex, which may contribute to a depression-relevant phenotype. Indeed, *En2* null mutants displayed high immobility in the forced swim test (Lin *et al.*, 2010), a finding replicated in two additional *En2* cohorts (Brielmaier *et al.*, 2012). Considerable experimental and clinical evidence supports a fundamental role for NE in the etiology of depression (Bunney & Davis, 1965; Nemeroff, 2002). Compounds that inhibit NE reuptake, that presumably replete functional deficiencies, have been used to treat major depression (Dell'Osso *et al.*, 2011), and decrease immobility in rodent forced swim and tail suspension assays (Lucki *et al.*, 2001; Steru *et al.*, 1985; Wong *et al.*, 2000). The depression-related phenotype in *En2* null mutants may therefore result from *En2* modulation of brain noradrenergic systems.

Eight association studies have demonstrated significant association for *EN2* with ASD (reviewed in Choi *et al.*, 2011), supporting the interpretation that *EN2* is an autism susceptibility gene. Autism spectrum disorders (ASD) affect approximately 1% of the population (Centers for Disease Control, 2012). Diagnosis is behaviorally defined by impairments in reciprocal social interactions and social communication, and repetitive behaviors with restricted interests (American Psychiatric Association, 2000; Lord *et al.*, 2000). Consistent with an earlier publication (Cheh *et al.*, 2006), we recently reported social and cognitive deficits in mice lacking *En2* (Brielmaier *et al.*, 2012). Though the autism-associated *EN2 rs1861972-rs1861973* A-C haplotype produces a gain in function (Choi *et al.*, 2012), investigations using mouse models of neurodevelopmental disorders indicate that mutations that produce either a loss or a gain of function can result in similar behavioral impairments (Auerbach *et al.*, 2012; Ramocki & Zoghbi, 2008). For example, both *En2* knockout and transgenic over-expressing mice display cerebellar phenotypes similar to those in ASD (Baader *et al.*, 1998; Courchesne, 1997; Kuemerle *et al.*, 1997; Ritvo *et al.*, 1986). *EN2* is also situated in a CNV duplication that has been associated with schizophrenia (Malhotra *et al.*, 2011). The robust social and cognitive deficits in *En2* null mutants thus offer a translational model for investigating pharmacological treatments for selected symptoms of autism and other disorders marked by social, cognitive and depressive endophenotypes.

Given the documented role of noradrenergic function in the pathophysiology of depression and antidepressant drug action (Dell'Osso *et al.*, 2011) we first tested the hypothesis that treatment with desipramine (DMI), a selective NE reuptake inhibitor and an early classical antidepressant (Dell'Osso *et al.*, 2011; Lucki *et al.*, 2001; Steru *et al.*, 1985), would rescue the depression-related phenotype in *En2* null mutants. Due to the delayed onset of antidepressant action associated with DMI treatment in clinical studies (Katz *et al.*, 1987;

Nierenberg, 2001), a chronic three week treatment regimen was employed. We additionally evaluated the ability of chronic DMI treatment to rescue social and cognitive deficits in mice lacking *En2* given the relevance of these behavioral phenotypes to autism and other neuropsychiatric conditions.

MATERIALS AND METHODS

Mice

En2$^{tm1Alj/\ tm1Alj}$ (*En2* −/− mice), generated on a 129S2/SvPas background as previously described (Joyner *et al.*, 1989), backcrossed into C57BL6/J (B6) into an unknown number of generations, were purchased from The Jackson Laboratories (Bar Harbor, ME). The line was backcrossed into B6 for at least 3 more generations at the University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School (UMDNJ-RWJMS) in Piscataway, NJ. *En2* heterozygous breeding pairs were subsequently imported from UMDNJ to the National Institute of Mental Health (NIMH) in Bethesda, using a het-het breeding scheme. Heterozygotes were bred in a conventional mouse vivarium using harem breeding trios. Mice were genotyped by PCR analysis of tail DNA using standard PCR methods. Briefly, 0.5–1 cm tail snips were digested using the Promega Wizard SV Genomic DNA Purification System (Promega, Madison, WI). 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. Identification of offspring was done by paw tattooing at age 2–4 days. Pups were kept with the dam until weaning at postnatal day 21. After weaning, juveniles were housed by sex in groups of two to four. Mice were housed in standard plastic cages in a colony room maintained at approximately 20°C, on a 12:12 light:dark cycle with lights on at 06:00 hours.

Drug Administration

Desipramine hydrochloride (DMI; 10, 20, and 30 mg/kg/day, salt; Sigma-Aldrich Corporation, St. Louis, MO) was made available *ad libitum* in the drinking water using procedures previously described (Dulawa *et al.*, 2004; Holick *et al.*, 2008) Vehicle-treated mice received plain (tap) drinking water. Doses of desipramine (DMI) were chosen based on previous behavioral pharmacology studies in mice (Lucki et al. 2001; Holick et al. 2008) and pilot data from our laboratory. The concentration of each drug in drinking water was determined by the average daily water consumption (ml per mouse per day) and the average body weight per mouse in grams to achieve the desired doses. Drinking water was administered in standard polycarbonate water bottles (Techniplast USA, Exton, PA) wrapped in aluminum foil to protect drug solutions from light. Animals were weighed and fresh drug solutions were prepared every 3–4 days. Both body weight and average water consumption were measured every 3 days in a subset of mice to ensure equal water consumption across drug treatment groups.

Behavioral tests

Behavioral testing began at 10–12 weeks of age, following 21 days of administration of drug in the drinking water or plain water vehicle, which began at 7–9 weeks of age. *En2* wildtype (+/+), heterozygote (+/−) and null mutant (−/−) male and female littermates were randomly assigned to groups such that variable members of each litter were assigned to either plain water vehicle control or a particular compound dose, thus eliminating the confound of litter effects. A previous study in our laboratory documented no sex differences in depression-related behaviors, sociability, or cognitive abilities (Brielmaier et al., 2012). Therefore, male and female mice were used in approximately equal proportions. We used a between-subjects design such that each mouse received a single chronic dose of DMI or plain water vehicle. Treatment groups consisted of 8–16 mice per genotype for each dose of drug or plain water vehicle. The number of subjects tested in a given assay was chosen based on the throughput capacity of the assay as well as the number of animals needed to achieve sufficient statistical power. Drug doses, paw tattoo patterns, and digital videotapes were coded to ensure that investigators were blind to genotype and drug treatment conditions. Order of testing was as follows: 1) tail suspension test, 2) forced swim test, 3) three-chambered social approach test, 4) novel object recognition, 5) fear conditioning, 6) pain sensitivity. Mice were left undisturbed in their home cages with the exception of water bottle changes for at least 3 days between behavioral tests. A detailed experimental timeline is depicted in Figure 1. Experiments were conducted in dedicated testing rooms during the standard light phase, usually between 10:00 and 16:00. Prior to all experiments, mice were acclimated to the behavioral testing area for at least 60 minutes. All procedures were conducted in strict compliance with the NIH Guidelines for the Care and Use of Laboratory Animals and approved by the National Institute of Mental Health Animal Care and Use Committee.

Tail suspension test—The tail suspension test was conducted as previously described (Brielmaier *et al.*, 2012; Steru *et al.*, 1985). Mice were securely fastened by taping the distal end of the tail to the edge of a metallic shelf, and suspended in a visually isolated area. A CCTV camera (Security Cameras Direct, Luling, TX) placed approximately 1 m in front of the shelf recorded each session for subsequent scoring of time spent immobile. The presence or absence of immobility, defined as the absence of limb movement, was sampled every 5 seconds over a 6 minute test session by a highly trained observer who remained blind to genotype and drug treatment. The shelf was cleaned with 70% ethanol between subjects.

Forced swim test—The Porsolt forced swim test was conducted as previously described (Brielmaier *et al.*, 2012; Lucki *et al.*, 2001). Mice were gently placed in a transparent Plexiglas cylinder (20 cm in diameter) filled to a depth of 15 cm with tap water (24 ± 1°C). A CCTV camera (Security Cameras Direct, Luling, TX) placed 30–40 cm in front of the cylinder recorded each session for subsequent scoring of time spent immobile. The presence or absence of immobility, defined as the cessation of limb movements except minor movement necessary to keep the mouse afloat, was sampled every 5 seconds during the last 4 minutes of a 6 minute test session by a highly trained observer blind to genotype and drug treatment.

Sociability—Adult sociability was tested in our automated three-chambered social approach apparatus using methods previously described (Brielmaier *et al.*, 2012; Yang *et al.*, 2011). The apparatus was a rectangular box made of clear polycarbonate, divided into a center chamber and two side chambers (each 20 cm length × 40.5 cm width × 22 cm height). Retractable doors (10 cm width × 5 cm height) built into the two dividing walls allowed access to the side chambers. Number of entries and time spent in each of the three chambers were detected by photocells embedded in the doorways and automatically recorded by the software. Equipment and the Labview software program were designed and built by George Dold and coworkers, Section on Instrumentation, NIH, Bethesda, MD. A top mounted CCTV camera (Security Cameras Direct, Luling, TX) was positioned over the box to record the session, for subsequent scoring of the videos for time spent sniffing the novel mouse and time spent sniffing the novel object.

The subject mouse was acclimated to the apparatus before sociability testing, beginning with a 10 minute habituation session in the empty center chamber, followed by a 10 minute habituation to all three empty chambers. The second habituation session served to confirm a lack of innate side chamber preference. Following the two habituation sessions, the subject mouse was briefly confined to the center chamber of the apparatus while a novel object (inverted wire pencil cup, Galaxy, Kitchen Plus, http://www.kitchen-plus.com) was placed in one side chamber and a novel mouse contained inside an identical inverted wire cup was placed in the other side chamber. Stimulus mice were enclosed in a wire cup to ensure that all social approach was initiated by the subject, and to avoid complications of fighting and sexual activity, while allowing visual, olfactory, auditory, and partial tactile contact through the wire bars. Mice used as the novel mouse stimuli were age- and sex-matched 129S1/SvImJ mice obtained from The Jackson Laboratory (Bar Harbor, ME), a strain that is relatively inactive. Stimulus mice were habituated to the inverted wire cup enclosure within a side chamber for 15 minutes during 2 habituation sessions a day for 3 days prior to the start of experiments. The location (left or right) of the novel object and novel mouse alternated across subjects. Weighted paper cups were placed on the top of the inverted wire cups, to prevent climbing and sitting on top of the inverted wire cup during the sociability phase of the test.

After the novel mouse and empty cup were positioned, the two side doors were lifted and the subject mouse was allowed access to all three chambers for 10 minutes. Time spent in each chamber and number of entries was automatically recorded. Number of entries served as a within-task control for levels of general exploratory locomotion. Cumulative time spent sniffing the novel mouse and novel object were later scored by a trained observer uninformed of genotype and drug treatment. The apparatus was cleaned with 70% ethanol and water between subjects. At the end of each testing day, the boxes were thoroughly washed with soap and warm water and air dried.

Novel object recognition—The novel object recognition test was conducted in the open field arena using methods previously described (Bevins & Besheer, 2006; Brielmaier *et al.*, 2012). The objects used consisted of a plastic coral, treasure chest, fish, and polar bear (Safari Ltd., Miami Gardens, FL) ranging from 4–7 cm in width and height. Previous studies in our laboratory demonstrated that mice do not display a spontaneous preference for any of

these objects. The experiment took place over two days. On day 1, each subject was habituated to a clean empty open field arena for 30 minutes. Twenty-four hours later, each mouse was returned to the open field for a second habituation phase lasting 10 minutes. The mouse was then removed from the open field and placed in a clean temporary holding cage for approximately 2 minutes, during which time two identical objects were placed in the arena. Each subject was returned to the open field and allowed to freely explore the objects for 10 minutes. After the object familiarization session, subjects were returned to their holding cages. The open field was cleaned with 70% ethanol and let dry. One clean familiar object and one clean novel object were placed in the arena, where the two identical objects had been located during the familiarization phase. Thirty minutes after the familiarization session, each subject was returned to its open field for a 5 minute object recognition test, during which time it was allowed to freely explore the familiar object and the novel object. Object investigation was defined as time spent sniffing the object when the nose was in contact with the object or within ≤ 2 cm from the object. The familiarization session and recognition test were videotaped and subsequently scored by a highly trained investigator uninformed of genotype and drug treatment. Recognition memory was defined as spending significantly more time sniffing the novel object than the familiar object. Time spent sniffing two identical objects during the familiarization phase confirmed lack of an innate side bias.

Contextual and cued fear conditioning—Standard delay contextual and cued fear conditioning was conducted using an automated fear conditioning chamber (Med Associates, St. Albans, VT) as previously described (Brielmaier *et al.*, 2012). The conditioning chamber (32 × 25 × 23 cm, Med Associates, St. Albans, VT) was interfaced to a PC installed with VideoFreeze software (version 1.12.0.0, Med Associates) and enclosed in a sound-attenuating cubicle (64 × 76 × 42 cm, Med Associates). Training consisted of a 2 minute acclimation period followed by three tone-shock (CS-US) pairings (80 dB tone, duration 30 seconds; 0.5 mA footshock, duration 1 second; intershock interval 90 seconds) and a 2.5 minute period during which no stimuli were presented. Cumulative time spent freezing before and after the CS-US pairings was quantified by VideoFreeze (Med Associates) software. A 5 minute test of contextual fear conditioning was performed 24 hours after training, in the absence of the tone and footshock. The conditioning chamber and test room environments were identical to those used on the training day. Cumulative time spent freezing during the 5 minute test was quantified by the software. Cued fear conditioning, conducted 48 hours after training, was assessed in a novel environment with distinct visual, tactile and olfactory cues. The cued test consisted of a 3 minute acclimation period followed by a 3 minute presentation of the tone CS and a 90 second exploration period. Cumulative time spent freezing before and after the auditory CS presentation was quantified by the software. The chamber was cleaned with 70% ethanol between subjects.

Pain sensitivity—Antinociceptive effects of DMI and other tricyclic antidepressants are documented in the literature (Lund *et al.*, 1989; Rojas-Corrales *et al.*, 2003). To control for the possibility that drug-induced changes in nociception may have influenced perception of the footshock US during fear conditioning, we tested the effects of chronic DMI treatment on pain sensitivity. Responsiveness to painful stimuli was assessed using the hot plate and

tail flick tests as previously described (Brielmaier *et al.*, 2012). For the hot plate test, the mouse was placed on the surface of a hot plate apparatus (Columbus Instruments, Columbus, OH) maintained at 55°C. Latency to the first paw lick, jump or vocalization was measured by an observer uninformed of genotype and drug treatment. A maximum cut-off latency of 30 seconds was used to prevent the risk of tissue damage to the paws. For the tail flick test, mice were gently restrained with the tail lying in the groove of a tail flick apparatus (Columbus Instruments). Thermal stimulation was provided by application of an intense photobeam. The latency for the mouse to move its tail out of the path of the beam was recorded by an observer uninformed of genotype and drug treatment. A maximum cut-off latency of 10 seconds was used to prevent the risk of tissue damage.

Statistical Analysis

Average daily water consumption and body weights were analyzed using between-groups repeated measures ANOVAs, to compare water consumption and body weights across DMI treatment groups. Tail suspension, forced swim and contextual fear conditioning were analyzed using two-way genotype × drug dose analyses of variance (ANOVAs). Social approach results were analyzed using within groups repeated measures ANOVAs, to compare time spent in the two side chambers, and time spent sniffing the novel mouse versus the novel object for each genotype within each drug dose. Time spent in the center chamber is shown in the graphs for illustrative purposes only. Novel object recognition results were also analyzed using within groups repeated measures ANOVAs, to compare time spent sniffing the novel object versus the familiar object, within each genotype and drug dose. Cued fear conditioning was analyzed with a between groups repeated measures ANOVA. Significant ANOVA results were followed by Student-Newman-Keuls *post hoc* tests. Data were analyzed using StatView software (SAS Institute, Cary, NC) and are presented as means ± SEMs.

RESULTS

No Differences in Water Consumption and Body Weights in *En2* Mice During Chronic DMI Treatment

Body weight and average daily water consumption were measured every 3 days in a subset of mice (3 cages per DMI treatment group; N = 11 for plain water vehicle; N = 9 for 10 mg/kg DMI; N = 8 for 20 mg/kg DMI; N = 10 for 30 mg/kg DMI). A repeated measures ANOVA revealed that all four DMI treatment groups displayed an increase in body weight over the course of the 21-day dosing period ($F_{(6,204)} = 206.0$, $p > .001$). No body weight differences were detected between DMI treatment groups. The repeated measures ANOVA for average daily water consumption did not did not reveal any significant differences between DMI dose groups. Average daily consumption was as follows: plain water vehicle: 3.67 ± 0.18; 10 mg/kg DMI; 3.32 ± 0.61; 20 mg/kg DMI; 3.95 ± 0.29; 30 mg/kg DMI: 3.67 ± 0.24.

Chronic DMI Treatment Reduced Depression-Related Behaviors in *En2* −/− Mice

Figure 1 illustrates immobility in the tail suspension test after chronic treatment with 10, 20 or 30 mg/kg DMI per day in the drinking water as compared to no-drug drinking water (a–

c). Overall two-way ANOVA revealed a statistically significant main effect of DMI dose ($F_{(3,172)} = 4.11$, $p = 0.008$) and a genotype × DMI dose interaction ($F_{(6,172)} = 2.64$, $p = 0.018$). DMI treatment had no significant effects on percent immobile observations in wildtypes (a). A trend towards a significant effect of DMI dose was seen for heterozygotes (b; $F_{(3,59)} = 2.47$, $p = 0.071$). Chronic DMI treatment significantly reduced percent immobile observations in null mutants (c; $F_{(3,56)} = 6.13$, $p = 0.001$), at treatment doses of 10 mg/kg ($p = 0.001$), 20 mg/kg ($p = 0.16$), and 30 mg/kg ($p = 0.002$) as compared with plain water vehicle treatment. Figure 1 further illustrates depression-related immobility in the forced swim test after chronic treatment with DMI or plain water vehicle (d–f). A statistically significant main effect of genotype ($F_{(3,169)} = 12.40$, $p < 0.0001$) and a genotype × DMI dose interaction ($F_{(6,169)} = 2.30$, $p = 0.037$) were detected. DMI treatment had no significant effects on percent immobile observations in wildtypes (d) and heterozygotes (e). Chronic DMI treatment significantly reduced percent immobile observations in null mutants (f; $F_{(3,54)} = 4.66$, $p = 0.006$) at doses of 10 mg/kg ($p = 0.001$), 20 mg/kg ($p = 0.006$), and 30 mg/kg ($p = 0.009$) as compared with plain water vehicle.

Chronic DMI Treatment Rescued Social Deficits in *En2* −/− Mice

Figure 2 illustrates the sociability scores from the automated three-chambered social approach task after chronic treatment with DMI or plain water vehicle. In wildtypes and heterozygotes, sociability and social sniffing were not different among groups treated with DMI and plain water vehicle. Sociability, defined as spending more time in the chamber with the novel mouse than in the chamber with the novel object, was intact after chronic treatment with plain water vehicle and each dose of DMI for these two genotypes (a and e; $p < 0.05$ for all comparisons). Social sniffing, defined as spending more time sniffing the cup containing the novel mouse than the empty cup, was similarly intact after all doses of DMI and plain water vehicle for wildtypes and heterozygotes (b and f; $p < 0.05$ for all comparisons). Specific *F* and *p* values for sociability chamber time and social sniffing in wildtype and heterozygote mice are listed in Table 1.

Replicating previous findings (Brielmaier *et al.*, 2012), null mutant mice did not display significant sociability on the parameters of chamber time and social sniffing. DMI treatment rescued this sociability deficit in *En2* −/− mice. *En2* −/− treated with plain water vehicle did not show sociability. Time spent in the novel mouse chamber was greater than time spent in the novel object chamber after chronic treatment with 10, 20 and 30 mg/kg DMI (i; $p < 0.05$ for all comparisons). *En2* −/− did not display social sniffing. Time spent sniffing the novel mouse was greater than time spent sniffing the novel object after chronic treatment of *En2* −/− with 10, 20, and 30 mg/kg DMI, indicating a rescue of the social sniffing deficit by chronic DMI treatment (j; $p < 0.05$ for all comparisons). Specific *F* and *p* values for sociability chamber time and social sniffing in −/− are listed in Table 1. To control for general exploratory activity, entries into the left and right side chambers were scored. Entries into the side chambers were not affected by DMI in wildtype (c), heterozygote (g) or null mutant (k) mice, indicating that the drug treatment had no direct effect on exploratory locomotion during the task. No innate side preference was present in wildtype (d), heterozygote (h), or null mutant (l) mice, as shown by similar amounts of time in the left and

right side chambers during the 10 minute habituation session that preceded the start of social testing.

Chronic DMI Treatment Rescued the Contextual Fear Conditioning Deficit in *En2* −/− Mice

Figure 3 illustrates contextual and cued fear conditioning after chronic treatment with DMI or plain water vehicle. Repeated measures ANOVA revealed high levels of freezing subsequent to the CS-US pairings on the training day among all treatment groups of wildtypes, heterozygotes and null mutants (a–c; main effect of training phase, $F_{(1,151)} = 863.43$, $p < 0.0001$). No significant effects of genotype or drug group were detected for the training day. Overall two-way ANOVA for contextual fear conditioning revealed a significant effect of DMI dose ($F_{(3,151)} = 9.56$, $p < 0.0001$) and a significant genotype × DMI dose interaction ($F_{(6,151)} = 2.30$, $p = 0.037$). Repeated measures ANOVA for cued fear conditioning revealed significant main effects of genotype ($F_{(2,151)} = 7.33$, $p < 0.001$) and DMI dose ($F_{(3,151)} = 3.12$, $p = 0.028$). One-way ANOVA did not reveal any significant differences between the +/+ plain water vehicle and DMI treatment groups for contextual fear conditioning (a). A significant difference between the plain water vehicle and DMI treatment groups was found for heterozygotes (b; $F_{(3,51)} = 9.90$, $p < 0.0001$). Chronic DMI treatment significantly increased contextual freezing at the 30 mg/kg treatment dose ($p < 0.0001$) as compared with plain water vehicle treatment.

A significant difference between the plain water vehicle and DMI treatment groups was also found for null mutants (c; $F_{(3,49)} = 4.54$, $p = 0.007$). Chronic DMI treatment significantly increased contextual freezing at treatment doses of 10 mg/kg DMI ($p = 0.004$), 20 mg/kg DMI ($p = 0.014$), and 30 mg/kg DMI ($p = 0.002$) as compared with plain water vehicle treatment. Repeated measures ANOVA revealed high levels of freezing following presentation of the CS on the cued day as compared to before the CS presentation in wildtype mice (a; main effect of cue, $F_{(1,51)} = 184.18$, $p < 0.0001$). No differences were detected between the +/+ plain water vehicle and DMI treatment groups on the cued day. Main effects of cue (b; $F_{(3,51)} = 202.94$, $p < 0.0001$) and DMI dose ($F_{(3,51)} = 2.84$, $p = 0.047$) were detected for heterozygote mice. *Post hoc* comparisons, however, did not reveal any significant differences in cued freezing between drug treatment groups. A main effect of cue was detected for null mutant mice (c; $F_{(1,49)} = 66.78$, $p < 0.0001$), but no differences were detected between the plain water vehicle and DMI treatment groups.

Chronic DMI Treatment Rescued Novel Object Recognition Memory Deficits in *En2* +/− but Not in *En2* −/− Mice

Figure 4 illustrates novel object recognition memory after chronic treatment with a single dose of DMI or plain water vehicle. Significant novel object recognition was detected in +/+, but not in *En2* +/− or *En2* −/−. No innate preference for object position was exhibited among the treatment groups of wildtype, heterozygote, or null mutant mice, as indicated by similar amounts of time spent sniffing the left and right objects during the 10 minute familiarization session (a–c). Specific *F* and *p* values for the object familiarization phase in +/+, +/− and −/− are listed in Table 2. In +/+ mice, normal novel object recognition, defined as spending more time sniffing a novel object than a familiar object, was not significantly different among doses of DMI and plain water vehicle (d). Time spent sniffing the novel

object was significantly greater than time spent sniffing the familiar object for all treatment groups ($p < 0.05$ for all comparisons). *En2* +/− mice treated with plain water vehicle failed to display a preference for the novel object over the familiar object during the test phase (e). *En2* +/− mice treated with each dose of DMI spent more time sniffing the novel object as compared to the familiar object, indicating novel object recognition ($p < 0.05$ for all comparisons). Replicating our previous finding (Brielmaier *et al.*, 2012), *En2* −/− mice failed to display novel object recognition (f). Treatment with DMI did not reverse the object recognition memory deficit. Similar amounts of time spent sniffing the familiar and novel objects was seen for −/− mice chronically treated with 10, 20 and 30 mg/kg DMI. Specific *F* and *p* values for the novel object recognition test in +/+, +/− and −/− are listed in Table 2.

Chronic DMI Treatment Did Not Alter Pain Sensitivity in *En2* Mice

Pain sensitivity was tested to control for the possibility that drug-induced changes in nociception influenced perception of the footshock US during fear conditioning. Figure 5 illustrates responsiveness to painful stimuli in the hot plate and tail flick tests after chronic treatment with DMI or plain water vehicle. Overall two-way ANOVAs did not reveal any significant differences between genotypes or DMI dose groups for hot plate pain sensitivity (a–c). Differences were also not detected for tail flick pain sensitivity (d–f).

DISCUSSION

Robust phenotypes in mouse models offer a powerful translational tool for discovering mechanisms underlying the pathophysiology of psychiatric disorders. Mutant mouse models have provided valuable leads in understanding the neurobiology of depression-related behavior and detection of pharmacological agents with antidepressant activity (Cryan & Mombereau, 2004). Mice with a deletion in the autism susceptibility gene *En2* display a depression-related phenotype (Brielmaier *et al.*, 2012; Lin *et al.*, 2010) as well as social and cognitive deficits (Brielmaier *et al.*, 2012; Cheh *et al.*, 2006) that may be relevant to multiple neuropsychiatric conditions. Given the role of *En2* in development and maintenance of monoaminergic systems (Simon *et al.*, 2005) and our previous finding of NE system abnormalities in *En2* null mutant mice (Genestine *et al.*, 2011; Lin *et al.*, 2010), we sought to determine whether an antidepressant would rescue behavioral phenotypes in mice lacking *En2*. DMI, a classical tricyclic antidepressant that selectively blocks reuptake of norepinephrine and reduces depression-relevant behaviors in mice (Lucki *et al.*, 2001; Steru *et al.*, 1985), was employed to test the hypothesis that elevated synaptic norepinephrine would reduce behavioral abnormalities in *En2* null mutant mice.

Our results show that DMI effectively reduced depression-related behaviors in *En2* null mutant mice. Doses of 10, 20 and 30 mg/kg administered chronically in the drinking water reduced immobility in both the tail suspension and forced swim tests, two assays that detect antidepressant drug activity in mice (Cryan *et al.*, 2005; Lucki, 1997). In both tests, the elevated number of percent immobile observations in *En2* −/− was reduced by DMI to levels that were not significantly different from those of +/+ controls. This finding is in line with the large literature on antidepressant effects of DMI in these assays in various inbred strains of mice and rodent models of depression (e.g. Lucki *et al.*, 2001; Steru *et al.*, 1985). The

efficacy of DMI in reducing tail suspension and forced swim immobility in *En2* null mutants is a novel finding which suggests that the depression-related phenotype may be a functional readout of disrupted noradrenergic pathways resulting from *En2* deletion.

Chronic treatment with DMI unexpectedly reversed the social approach deficits in *En2* null mutants. Sociability, the more general parameter measured by time spent in the novel mouse chamber versus the novel object chamber, and social sniffing, a specific parameter measured as time spent sniffing the novel mouse versus time spent sniffing the novel object, were both restored by DMI treatment at doses of 10, 20 and 30 mg/kg. Normal social sniffing and sociability in +/+ and +/− were not affected by DMI treatment. Number of entries into the side chambers was not significantly different between DMI and plain water vehicle treatment groups, indicating that the rescue of social deficits in null mutants was not due to changes in exploratory locomotor activity. This novel finding suggests that the robust social deficits in *En2* null mutants (Brielmaier *et al.*, 2012; Cheh *et al.*, 2006) may be a consequence of NE abnormalities. Noradrenergic neurons mainly originate in the locus coeruleus and the ventral noradrenergic bundle and project to multiple brain regions mediating social behaviors, including the hippocampus, amygdala, septum, thalamus, hypothalamus and cerebral cortex (Moore & Bloom, 1979). Studies in patients with ASD have indicated that DMI reduces hyperactivity, an associated symptom of autism, without affecting the core symptom domain of social abnormalities (Gordon *et al.*, 1993; Gordon *et al.*, 1992). This dissociation between the effects of DMI in patients with autism and *En2* −/− mice may be due to the fact that *En2* −/− mice display social deficits that are potentially relevant to autism as opposed to being a model of autism *per se*, and are best conceptualized as a mouse model that can be used to investigate the basic neurobiology of social behavior. Results from our social approach task thus suggest new directions for understanding the role of NE in neuroanatomical circuits involved in social behaviors.

Chronic DMI treatment rescued the deficit in contextual fear conditioning in *En2* null mutants. Doses of 10, 20 and 30 mg/kg per day increased contextual freezing in −/− mice to levels comparable to that seen in +/+ mice. Increased contextual freezing was also seen in +/− mice treated with 30 mg/kg DMI per day as compared to plain water vehicle treatment. Pain sensitivity in the hot plate and tail flick tests was also unaffected by DMI treatment, indicating that increases in contextual fear memory were not a consequence of reduced nociception during presentation of the footshock unconditioned stimulus during training. In contrast, cued fear conditioning was not affected by DMI treatment in any of the three genotypes. Intact function of the hippocampus is essential for contextual but not cued fear conditioning, whereas the amygdala is involved in both contextual and cued fear conditioning (Phillips & LeDoux, 1992). Chronic treatment with antidepressants, including DMI, is known to increase cell proliferation and neurogenesis in the hippocampus (Balu *et al.*, 2009), which contributes to contextual fear conditioning (Saxe *et al.*, 2006). It is interesting to speculate that chronic DMI treatment improved contextual fear memory in *En2* −/− mice through mechanisms involving hippocampal neurogenesis. In immature *En2* −/− mice, aspects of developmental neurogenesis including neural stem cell proliferation and survival are abnormally regulated (Genestine *et al.*, 2011; Lin *et al.*, 2010), though effects of noradrenergic drugs remain to be fully defined.

Both *En2* −/− and +/− displayed deficits on the novel object recognition task, as previously reported (Brielmaier *et al.*, 2012). Absence of novel object recognition memory in *En2* null mutants was not reversed by DMI treatment. *En2* heterozygotes treated with 10, 20 and 30 mg/kg DMI per day displayed restored novel object recognition. It is possible that DMI treatment is only effective in improving novel object recognition impairments when the impairment is less severe, as was observed in *En2* +/−, as compared to the more severe deficits seen in −/− mice. Our previous investigations of brain noradrenergic concentrations in *En2* mice (Genestine *et al.*, 2011; Lin *et al.*, 2010) only included widtypes and null mutants. It would be interesting to include heterozygotes in future investigations, to determine whether there are potential gene-dose effects on brain noradrenergic abnormalities that correspond to our novel object recognition findings. Inactivation studies in rodents indicate that novel object recognition memory is mediated by the perirhinal cortex (Winters & Bussey, 2005) and that the hippocampus is only involved when a longer retention interval of at least 24 hours is used (Hammond *et al.*, 2004). It is interesting to speculate that DMI improved contextual fear conditioning but not novel object recognition due to differing effects on noradrenergic function in brain regions mediating these two distinct types of memory. Investigations of changes in noradrenergic function in brain regions mediating cognitive abilities following chronic DMI treatment could shed light on the specific mechanisms underlying DMI enhancement of contextual fear conditioning but not cued fear conditioning, and a lesser effect on novel object recognition.

It is interesting to note that DMI-induced improvements in depression-related behaviors, social deficits and contextual fear memory impairments in *En2* −/− mice in our experiments did not display a clear dose-response curve. Doses of 10, 20 and 30 mg/kg DMI per day all rescued phenotypes in tail suspension, forced swim, social approach and contextual fear conditioning. Previous studies that demonstrated dose-dependent reductions of tail suspension and forced swim immobility following DMI treatment employed acute intraperitoneal injections of DMI (e.g. Lucki *et al.*, 2001; Steru *et al.*, 1985). The chronic treatment regimen and drinking water route of administration in the present experiments may have reached higher effective steady-state levels of DMI in the brain than in acute intraperitoneal treatment experiments. Lower doses of DMI in the drinking water may be required to elaborate dose-response curves for the behaviors tested in the present study.

Our results demonstrate that chronic treatment with the tricyclic antidepressant DMI ameliorates the depression-related phenotype in *En2* null mutant mice, consistent with their reported abnormalities in noradrenergic levels in hindbrain and forebrain (Genestine *et al.*, 2011; Lin *et al.*, 2010). Improvements in sociability and contextual fear memory in null mutants, and an improvement in novel object recognition in heterozygotes, following DMI treatment suggests that compounds which block NE reuptake may have additional potential for treating social and cognitive deficits that are core features of psychiatric disorders including autism, schizophrenia or depression. The present findings provide new insight into the role of *En2* modulation of brain noradrenergic systems in mediating complex behaviors relevant to psychiatric and neurodevelopmental disorders, and suggest new directions for mechanistically-based therapeutics for specific symptom domains of psychiatric disorders that present with social and cognitive endophenotypes.

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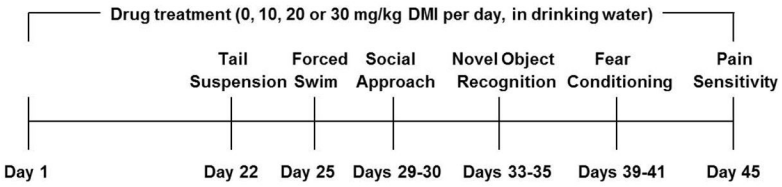

Figure 1.

Experimental timeline. *En2* +/+, +/− and −/− mice were treated with DMI or plain water vehicle for 21 days before the beginning of behavioral testing. Dosing continued until the final day of behavioral testing. More details are provided in the Methods section.

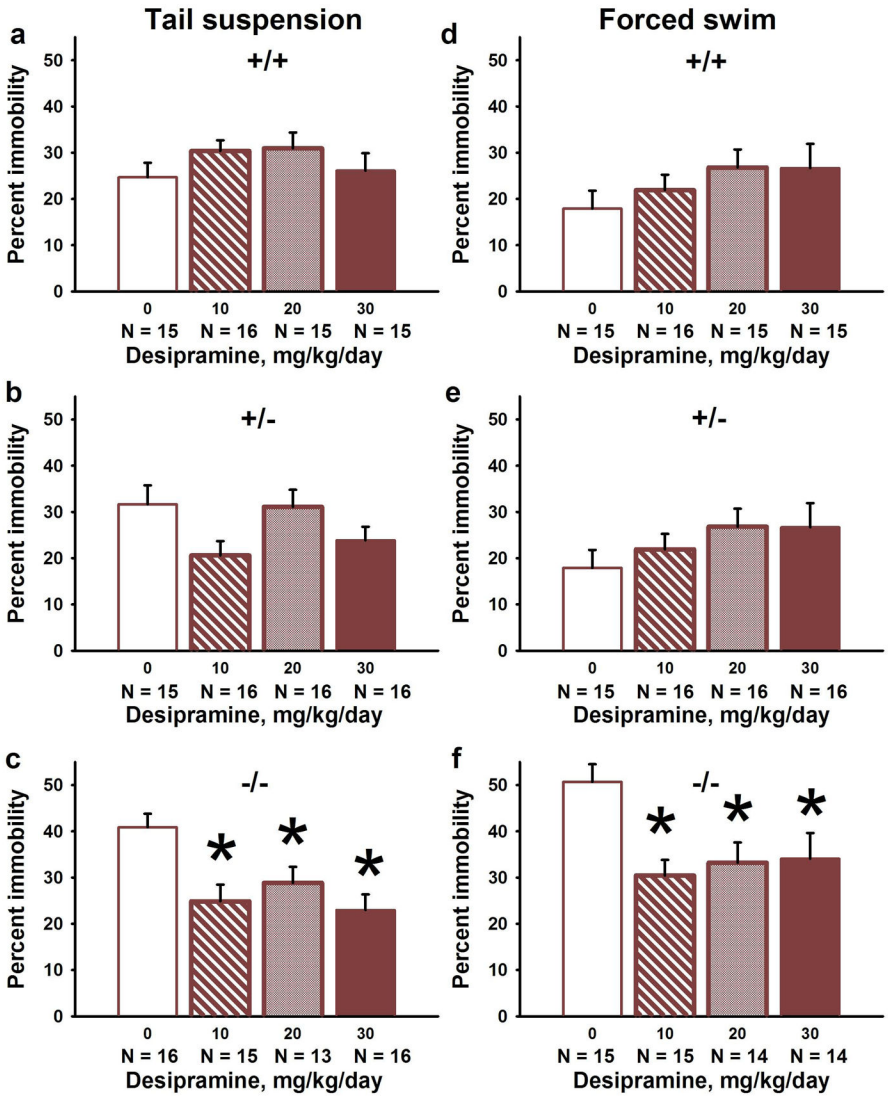

Figure 2.

Chronic desipramine (DMI) treatment rescued depression-related behaviors in *En2* −/− mice. *En2* null mutants (−/−) treated with plain water vehicle (white bars, Panels a–f) displayed high levels of depression-related immobility in the tail suspension and forced swim tests, as compared to heterozygote (+/−) and wildtype (+/+) littermates. (a–b) *En2* +/+ and +/− did not show any significant differences in the percentage of observations in which immobility was displayed in the tail suspension test after chronic treatment with plain water vehicle or DMI at doses of 10 mg/kg, 20 mg/kg or 30 mg/kg per day. (c) *En2* −/− showed significant reductions in their high levels of tail suspension immobility after chronic treatment with DMI at doses of 10 mg/kg, 20 mg/kg, and 30 mg/kg per day. (d–e) *En2* +/+ and +/− did not show any significant differences in percent immobile observations in the forced swim test after chronic treatment with plain water vehicle and each dose of DMI. (f) *En2* −/− showed significant reductions in their high levels of forced swim immobility after chronic treatment with DMI at doses of 10 mg/kg, 20 mg/kg, and 30 mg/kg per day. N = 13–16 per dose for each genotype. *$p < 0.05$ vs. plain water vehicle.

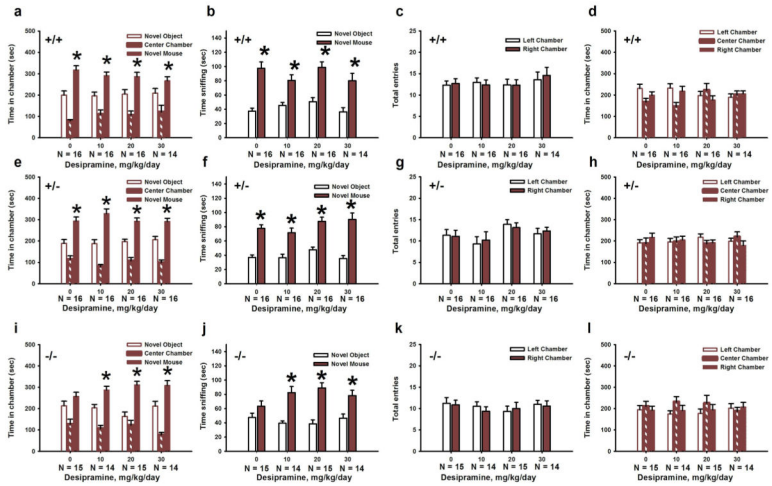

Figure 3.

Chronic DMI treatment rescued social approach deficits in *En2* −/− mice. (a) *En2* +/+ mice displayed social sniffing, defined as more time spent sniffing the novel mouse as compared to the novel object, at each dose of DMI and plain water vehicle. (b) *En2* +/+ displayed normal sociability, defined as spending more time with the novel mouse as compared to the novel object, at each dose of DMI and plain water vehicle. (c) Number of entries into the side chambers during the sociability phase and (d) time spent in each chamber during the habituation phase were not different between +/+ mice treated with each dose of DMI and plain water vehicle. (e) *En2* +/− spent more time sniffing the novel mouse as compared to the novel object at each dose of DMI and plain water vehicle. (f) *En2* +/− also displayed normal sociability at each dose of DMI and plain water vehicle. (g) Number of entries into the side chambers and (h) time spent in each chamber were not different between +/− mice treated with each dose of DMI and plain water vehicle. (i) *En2* −/− exhibited the previously demonstrated lack of social sniffing, that is, did not spend more time sniffing the novel mouse as compared to the novel object, after treatment with plain water vehicle. Following chronic treatment with 10 mg/kg, 20 mg/kg and 30 mg/kg of DMI, −/− displayed significant social sniffing. (j) *En2* −/− exhibited the previously demonstrated lack of sociability, that is, did not spend more time sniffing the novel mouse as compared to the novel object, after treatment with plain water vehicle. Following treatment with each dose of DMI, −/− displayed significant sociability. (k) Number of entries into the side chambers and (1) time spent in each chamber were not different between −/− mice treated with each dose of DMI and plain water vehicle, indicating normal locomotion and exploration in all treatment groups. N = 14–16 per dose for each genotype. *p < 0.05 vs. novel object.

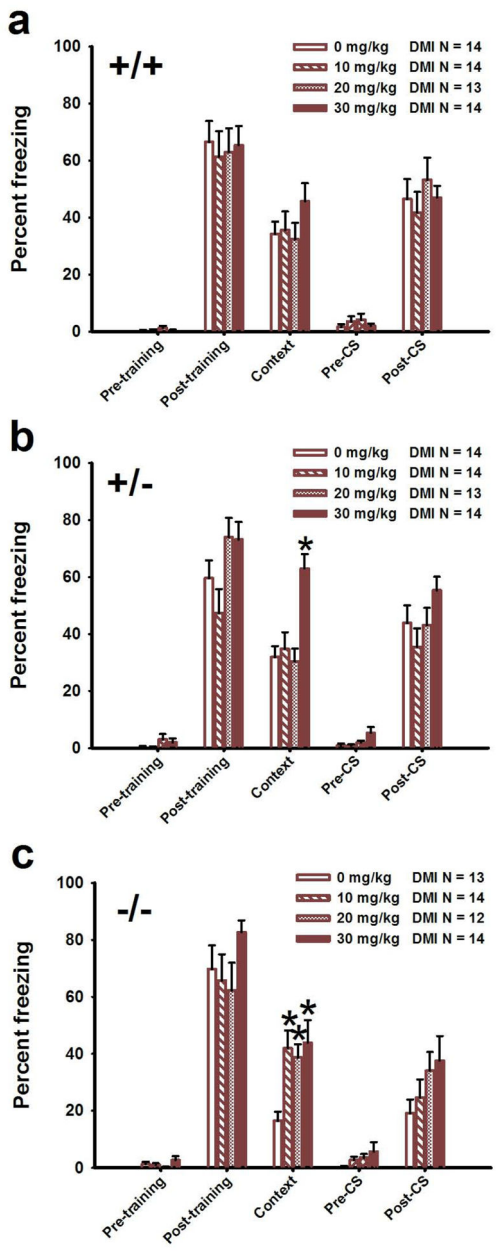

Figure 4.

Chronic DMI treatment rescued contextual fear conditioning deficits in *En2* −/− mice without affecting cued fear conditioning. As previously demonstrated, En2 −/− treated with plain water vehicle displayed significantly less freezing upon testing of contextual and cued fear conditioning as compared to +/+ and +/− mice treated with plain water vehicle, despite normal freezing during the training session. (a) Freezing during training, contextual, and cued sessions were not different among *En2* +/+ mice treated with plain water vehicle and each dose of DMI. (b) *En2* +/− mice chronically treated with the highest dose of DMI, 30 mg/kg per day, displayed increased contextual freezing as compared to +/− mice treated with plain water vehicle. During training and cued testing, freezing was not significantly different among groups of +/− mice treated with plain water vehicle and each dose of DMI. (c) *En2* −/− mice chronically treated with 10 mg/kg, 20 mg/kg, and 30 mg/kg DMI per day displayed increased contextual freezing as compared to −/− mice treated with plain water vehicle. Freezing during training and cued

sessions was not significantly different among groups of −/− mice treated with DMI or plain water vehicle. N = 12–14 per dose for each genotype. *p < 0.05 vs. plain water vehicle.

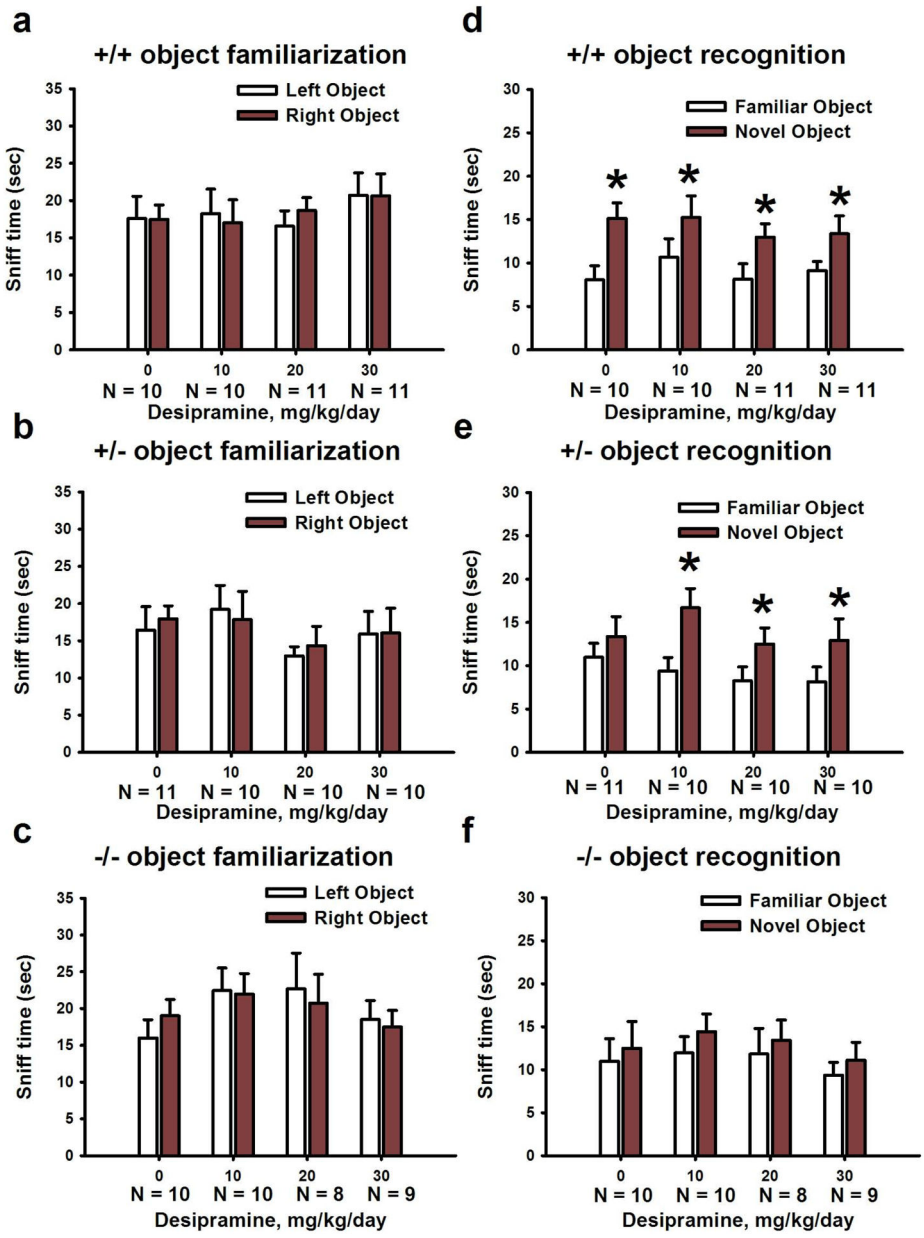

Figure 5.

Chronic DMI treatment improved the novel object recognition memory impairment in *En2* +/− but not −/− mice. A lack of innate object preference was observed for *En2* +/+ (a), +/− (b) and −/− (c) treated with plain water vehicle and each dose of DMI. (d) *En2* +/+ mice displayed novel object recognition memory at each dose of DMI and plain water vehicle. (e) As previously demonstrated, +/− mice treated with plain water vehicle failed to display novel object recognition memory. Following daily chronic treatment with 10 mg/kg, 20 mg/kg and 30 mg/kg of DMI, +/− displayed novel object recognition. (f) *En2* −/− mice displayed the previously demonstrated deficit in novel object recognition memory after treatment with plain water vehicle and each dose of DMI. N = 8–11 per dose for each genotype. *$p < 0.05$ vs. familiar object.

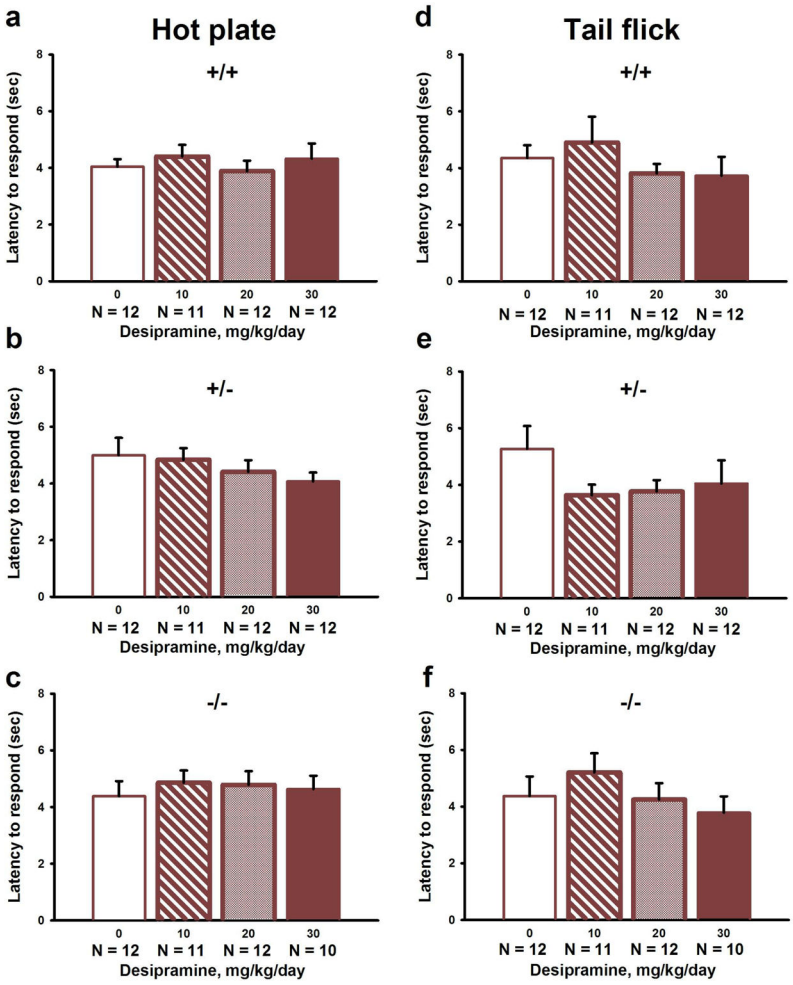

Figure 6.

Chronic DMI treatment did not alter pain sensitivity in *En2* mice. No differences in latency to hot plate were seen in (a) +/+, (b), +/− or (c) −/− mice treated with plain water vehicle and each dose of DMI. Similarly, no differences in latency to respond in the tail flick test were seen in (d) +/+, (e) +/−, or (f) −/− mice treated with plain water vehicle and each dose of DMI. N = 10–12 per dose for each genotype.

Table 1

Statistical results of social approach

Genotype	Drug dose	# of animals	Chamber time *F* and *p* values	Sniff time *F* and *p* values	Sociability	Figure
+/+	0	15	$F(1,14) = 9.60$ $p = 0.009$	$F(1,14) = 49.33$ $p < 0.0001$	Present	2a–b
	10	16	$F(1,15) = 11.35$, $p = 0.004$	$F(1,15) = 24.49$, $p < 0.001$	Present	
	20	16	$F(1,15) = 5.24$ $p = 0.037$	$F(1,15) = 37.15$, $p < 0.0001$	Present	
	30	14	$F(1,13) = 4.79$, $p = 0.008$	$F(1,13) = 24.27$, $p < 0.001$	Present	
+/−	0	15	$F(1,15) = 7.98$, $p < 0.001$	$F(1,14) = 49.33$, $p < 0.0001$	Present	2e–f
	10	16	$F(1,15) = 9.68$, $p = 0.007$	$F(1,15) = 24.49$, $p < 0.001$	Present	
	20	16	$F(1,15) = 17.98$, $p < 0.001$	$F(1,15) = 37.15$, $p < 0.0001$	Present	
	30	14	$F(1,15) = 10.92$, $p = 0.006$	$F(1,13) = 24.27$, $p < 0.001$	Present	
−/−	0	15	NS	NS	Absent	2i–j
	10	14	$F(1,13) = 7.31$, $p = 0.018$	$F(1,13) = 32.81$, $p < 0.0001$	Present	
	20	15	$F(1,14) = 24.42$, $p < 0.001$	$F(1,14) = 37.16$, $p < 0.0001$	Present	
	30	14	$F(1,13) = 4.90$, $p = 0.045$	$F(1,13) = 18.80$, $p = 0.001$	Present	

Summary of statistical results of social approach behaviors in *En2* +/+, +/− and −/− mice treated with DMI and plain water vehicle. Data are graphically presented in Figure 2. NS = non-significant.

Table 2

Statistical results of novel object recognition

Genotype	Drug dose	# of animals	Familiarization phase *F* and *p* values	Test phase *F* and *p* values	Novel object recognition	Figure
+/+	0	10	NS	$F(1,9) = 12.40$ $p = 0.007$	Present	4a, 4d
	10	10	NS	$F(1,10) = 5.68$, $p = 0.041$	Present	
	20	11	NS	$F(1,10) = 8.52$ $p = 0.015$	Present	
	30	11	NS	$F(1,10) = 6.35$, $p = 0.030$	Present	
+/−	0	11	NS	NS	Absent	4b, 4e
	10	10	NS	$F(1,9) = 14.89$, $p = 0.004$	Present	
	20	10	NS	$F(1,9) = 7.90$, $p = 0.020$	Present	
	30	10	NS	$F(1,9) = 8.04$, $p = 0.020$	Present	
−/−	0	10	NS	NS	Absent	4c, 4f
	10	10	NS	NS	Absent	
	20	8	NS	NS	Absent	
	30	9	NS	NS	Absent	

Summary of statistical results of novel object recognition testing in *En2* +/+, +/− and −/− mice treated with DMI and plain water vehicle. Data are presented graphically in Figure 4. NS = non-significant.