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Activation of Dopamine Neurons is Critical for Aversive Conditioning and Prevention of Generalized Anxiety

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Abstract

Generalized anxiety is thought to result, in part, from impairments in contingency awareness during conditioning to cues that predict aversive or fearful outcomes. Dopamine neurons of the ventral midbrain exhibit heterogeneous responses to aversive stimuli that are thought to provide a critical modulatory signal to facilitate orienting to environmental changes and assignment of motivational value to unexpected events. Here, we describe a mouse model in which activation of dopamine neurons in response to an aversive stimulus is attenuated by conditional genetic inactivation of functional N–methyl–D–aspartate–type glutamate receptors (NMDARs) on dopamine neurons. We discovered that altering the magnitude of excitatory responses by dopamine neurons in response to an aversive stimulus is associated with impaired conditioning to a cue that predicts an aversive outcome. Impaired conditioning by these mice is associated with development of a persistent, generalized anxiety–like phenotype. These data are consistent with a

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Competing Interests Statement

The authors declare that they have no competing financial interests.

Author Contributions

LSZ and JPF designed experiments. LSZ performed in vivo recordings with assistance from GLJ and SJYM. LSZ and JPF performed behavioral experiments with assistance from MGG and TMKD. EA performed slice physiology with support from AB. RDP constructed the AAV1–fs–HA–NR1 viral vector. JMA purified AAV1–fs–HA–NR1. The manuscript was written by LSZ with assistance from JPF and RDP.

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role for dopamine in facilitating contingency awareness that is critical for the prevention of generalized anxiety.

Dopamine neurons of the ventral midbrain innervate numerous limbic structures important for emotional processing. Dopamine signaling in these target regions modulates both acute physiology and synaptic plasticity; thus, implicating this neurotransmitter as a key facilitator of stimulus processing, learning, and memory [1]. In response to aversive stimuli, dopamine neurons have been demonstrated to be either inhibited or excited [2–8]. These two modalities have been proposed to impart distinct signals to limbic structures. Inhibitory responses are proposed to signal motivational value of the unexpected aversive event, while excitations provide a motivational salience signal for orienting to environmental changes [9]. Thus, valence and salience coding by dopamine neurons are thought to work coordinately to assign value and signal awareness of unexpected outcomes – two features critical for learning contingencies.

Knowledge that a specific cue predicts an aversive outcome requires orienting attention to the predictive cue during conditioning, a process referred to as contingency awareness [10]. Impairments in learning conditioned stimulus–unconditioned stimulus associations during fear conditioning in humans can lead to generalized fear responses and elevated reports of anxiety under non–threatening conditions [11]. Because the dopamine neurotransmitter system is important for gating attention and facilitating conditioned stimulus associations during fear conditioning [9,12], we predicted that alterations in the activation of dopamine neurons would impair fear conditioning and result in generalized fear or anxiety.

Excitation of dopamine neurons is facilitated by glutamate signaling through NMDARs[13–15] and mice lacking functional NMDARs selectively on dopamine neurons (knockout mice) have impaired phasic activation (or burst firing) [16]. Here, we demonstrate that activation of dopamine neurons in response to an aversive stimulus (tail pinch) is significantly attenuated in knockout mice. Pavlovian fear conditioning was impaired in knockout mice and these animals developed an anxiety–like phenotype following fear conditioning. Viral–mediated restoration of functional NMDAR to dopamine neurons of the ventral tegmental area (VTA) prevented the development of the anxiety–like phenotype.

RESULTS

NMDARs on dopamine neurons facilitate excitatory responses

To determine whether excitatory responses of dopamine neurons to an aversive stimulus is dependent upon NMDAR signaling, we recorded the activity of these neurons in knockout mice lacking the essential subunit of the NMDAR (NR1) exclusively in dopamine–producing neurons. Knockout mice were generated as described [16,17]. Neural activity was recorded from the ventral midbrain, predominantly the VTA, of freely–moving mice using chronically–implanted microwire tetrodes [16,18] (Supplementary Fig. 1a). Putative dopamine neurons were identified based on sensitivity to quinpirole, a dopamine D2 receptor agonist (0.2 mg/kg i.p.), at the end of each recording session. During baseline recordings, firing rate and burst patterns of activation in quinpirole–sensitive neurons (n = 21/32 cells, control and

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n = 19/28, knockout) were impaired in knockout compared to control mice (Supplementary Fig. 1b–e), similar to that described [16].

After establishing baseline recordings, we determined responses of quinpirole–sensitive and –insensitive neurons to an aversive stimulus by continuously recording neural activity while administering ten 5–s tail pinches separated by 1 min. This stimulus has been previously reported to evoke either excitation or inhibition of distinct dopamine neurons in rats [19]. Both quinpirole–sensitive and —insensitive neurons displayed differential responses to tail pinch (Fig. 1b–e, and Supplementary Fig. 2a and b). We observed nearly equivalent proportions of quinpirole–sensitive neurons activated (n = 7/20, control and n = 8/19, knockout), inhibited (n = 7/20, control and n = 7/19, knockout), or unaltered (n = 6/20, control and n = 4/19, knockout) by tail pinch. The magnitude of tail pinch–induced inhibition in either quinpirole–sensitive or —insensitive neurons did not differ between control and knockout mice (Fig. 1d, and Supplementary Fig. 2a). In contrast, excitation of quinpirole–sensitive neurons from knockout mice was dramatically attenuated compared to quinpirole–sensitive neurons from control mice (Fig. 1e; two–way repeated measures analysis of variance (RM ANOVA): genotype X time interaction $F_{(297,4455)} = 4.16$, $P < 0.0001$). No difference in the level of activation was observed between quinpirole–insensitive neurons from knockout mice compared to control mice (Supplementary Fig. 2c). Because a subset of dopamine neurons have been described as unresponsive to quinpirole [20] and a small proportion of non–dopamine neurons are inhibited by quinpirole [21], it is possible that our results are biased due to selection criteria. Therefore, we repeated our analysis based on the method of waveform duration and firing rate [3], as well as a combination of waveform duration, firing rate, and quinpirole sensitivity [18]. Putative dopamine neurons identified by either of these methods demonstrated a similar attenuation in tail–pinch response in knockout mice compared to control mice (Supplementary Fig. 2c–e). We did not observe any discernable differences in waveform duration, firing rate, burst rate, or burst duration in quinpirole–sensitive cells segregated based on response to tail pinch (Supplementary Fig. 2f–h).

Cue–dependent fear conditioning is impaired in knockout mice

To determine whether NMDAR–dependent activation of dopamine neurons is important for the acute processing of aversive information, we measured Pavlovian fear conditioning using fear–potentiated startle (FPS) [22]. Mice were conditioned with 30 pairings of a light cue with a footshock and FPS was assessed 10 min following training as described [23]. Following conditioning, control and knockout mice demonstrated a potentiation of the acoustic startle response (ASR) in the presence of the cue. In addition, both groups demonstrated a potentiation of the ASR in the non–cue condition, a context–dependent phenomenon referred to as shock sensitization [24]. Remarkably, knockout mice displayed significantly greater potentiation in both the cue and non–cue conditions compared to controls (Fig. 2a; RM ANOVA: genotype X conditioning interaction $F_{(3,45)} = 4.22$, $P = 0.01$). Enhanced ASR in the non–cue condition precluded FPS detection in knockout mice (% potentiation = 47.2 ± 15.9 s.e.m, control vs. 7.4 ± 8.0 s.e.m., knockout; $P = 0.02$, two–tailed Student's t test).

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To assess whether cued FPS is impaired in knockout mice due to a ceiling effect on ASR, we measured the startle responses to a 120–dB startle pulse which results in the maximal acoustic startle amplitude in mice [23] and compared those responses to the ASR of conditioned mice. ASR at 120 dB was significantly higher in non–conditioned knockout mice than the ASR at 105 dB following conditioning (Fig. 2b; two–tailed Student's *t* test: $P = 0.04$). Additionally, no detectable differences were observed in behavioral response to footshock during conditioning between control and knockout mice (Fig. 2b).

Potentiated startle is independent of context and persistent

Shock sensitization has been demonstrated to be context dependent and to persist for only brief periods following footshock conditioning [24]. Therefore, we asked whether the enhanced ASR in knockout mice following fear conditioning was dependent on the context in which conditioning occurred and whether this sensitization was short–lived or persistent. We monitored ASR before and after footshock using a repeated measures experimental design. Importantly, footshock conditioning was performed in a context distinct from the acoustic startle apparatus. Baseline startle was measured at multiple startle pulse intensities (null, 80, 90, 100, and 105 dB). The following day, mice received ten, 0.2–mA footshocks in a context distinct from the acoustic startle chamber and were returned to their home cages. ASRs were subsequently monitored either 10 min or 1 day and 1 week following conditioning. Results from two, independent groups of mice revealed that baseline ASR did not differ between control and knockout mice; however, ASRs were significantly potentiated in knockout mice compared to control mice 10 min following footshock conditioning in a novel context (Fig. 3a,e; RM ANOVA: genotype X conditioning interaction $F_{(12,176)} = 5.58$, $P < 0.0001$). To explore the persistence of sensitized startle in knockout mice, we shocked two additional cohorts of mice and measured ASRs both 1 day and 1 week following conditioning in a novel context. Startle was elevated in knockout mice at both 1 day and 1 week following conditioning (Fig. 3b,c and e; RM ANOVA: genotype X conditioning interaction $F_{(12,280)} = 2.82$, $P = 0.001$ and $F_{(4,112)} = 3.06$, $P = 0.02$, respectively). In both knockout and control mice, handling and repeated exposure to the startle chamber did not alter ASR (Fig. 3e).

To determine whether potentiation of the ASR in knockout mice can be induced by other aversive stimuli, we monitored the ASR of control and knockout mice before and after presentation of 10 tail pinches, identical to that described for electrophysiological recordings. Similar to footshock, ASRs were sensitized in knockout mice 10 min and 1 day following tail pinch (Supplementary Fig. 3b, c).

Enhanced anxiety–like behavior in knockout mice

Sensitization of the ASR, such as that observed in knockout mice following aversive conditioning has been reported in human subjects with associative–learning deficits and is also associated with generalized anxiety [11,25]. To determine whether knockout mice display other evidence of generalized anxiety–like behavior following aversive conditioning, we tested two cohorts of mice in both an elevated–plus maze and open–field task. We designed an experiment in which measurements were taken before and after footshock conditioning. Testing in each apparatus was performed in pseudo–random order. Additional cohorts of

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non–shocked mice were repeatedly measured for elevated–plus maze and open–field activity to control for potential novelty habituation effects. Before footshock, all groups showed similar behavior in both tasks (Fig. 4a,c). In the elevated–plus maze task, knockout mice that received shock had significantly fewer open arm entries (Fig. 4a,b; RM ANOVA: genotype X test interaction, pre– vs. post–shock $F_{(3,48)} = 4.07$, $P = 0.01$) than all other groups. Consistent with the reduction in open arm entries, total time spent in the open arm was significantly reduced in knockout mice following footshock (Supplementary Fig. 3d). Knockout mice demonstrated a reduction in center crossings in the open field following footshock compared to all other groups (Fig. 4c and d; RM ANOVA: genotype X test interaction $F_{(3,46)} = 2.84$, $P = 0.05$). Repeated testing in the elevated–plus maze and open–field task was associated with a decrease in total distance traveled in both behavioral assays across all groups (Supplementary Fig. 3e,f). Further analysis of distance traveled within the open arms of the elevated–plus maze revealed a significant difference between knockout mice that had received shock compared to all other groups (Fig. 4e; RM ANOVA: genotype X test interaction $F_{(3,48)} = 2.80$, $P = 0.05$). No such effect was observed for distance traveled in the closed arms (Fig. 4f). Distance traveled within the center of the open field arena was significantly affected by genotype and repeated testing; however, no interaction between the knockout and shock was observed (Supplementary Fig. 3g).

To determine whether the trend toward a reduction in overall activity in knockout mice that had received shock is due to a general reduction in locomotor activity, we monitored day–night activity of control and knockout mice before and after shock in locomotion chambers that resembled the home cage. There was no significant effect of shock on ambulatory activity in either group (Supplementary Fig. 3h).

PPI, cortisol and monoamine levels are unimpaired in knockout mice

Sensory–motor gating, as monitored by pre–pulse inhibition (PPI) of the ASR, is thought to reflect cortico–striatal modulation of the tri–synaptic startle reflex circuit [26]. PPI is disrupted in numerous psychiatric disorders and is modulated by dopamine [27,28]. To determine whether non–selective potentiation of ASR in KO mice is associated with an alteration in sensorimotor gating, we measured PPI in control and knockout mice at three different intensities, before and after footshock, as well as in seperate groups repeatedly tested without receiving shock (knockout n = 7, control n = 7, knockout non–shocked n = 7, control non–shocked n = 7). Startle was significantly affected by pre–pulse intensity (RM ANOVA: $F_{(2,48)} = 46.09$, $P < 0.0001$), but was not affected by genotype or footshock (Fig. 5a).

Hypothalamic–pituitary–adrenal (HPA) axis function is correlated with ASR magnitude [29] and startle potentiation in individuals with post–traumatic stress disorder (PTSD) [30], as well as in non–human primates following rearing stress [31]. To determine whether footshock altered HPA axis function in knockout mice, we measured serum corticosterone (Cort) levels. The experimental groups included control and knockout mice that were left in home cages (basal, knockout n = 4; control n = 3), and those that were placed in startle chamber with no shock, or with shock. These groups were further subdivided and serum was obtained immediately (t=0, knockout non–shocked n = 4; knockout shocked n = 4; control non–

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shocked n = 3; control shocked n = 3), 1 hr (knockout shocked n = 4; control shocked n =4), 1 d (knockout shocked n = 4; control shocked n =4), or 1 wk later (knockout shocked n = 4; control shocked n =4). Mice exposed to the startle chamber with or without footshock displayed elevated Cort levels at t = 0 and 1 h following exposure (Fig. 5b; ANOVA P = 0.004). Cort levels were similar between control and knockout mice under all conditions.

Alterations in brain monoamine levels can also disrupt ASR [32]. To explore whether footshock conditioning alters brain monoamine levels or metabolites, we performed HPLC analysis of whole brains from shocked (24 h post–shock; knockout n = 3, control n = 3) and non–shocked knockout (n = 3) and control (n = 3) mice. No statistically significant differences in the neurotransmitters dopamine (DA), norepinephrine (NE), serotonin (5–HT), or the metabolites dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), or 5–hydroxyindoleacetic acid (5–HIAA) were observed across groups (Fig. 5c).

Prevention of anxiety phenotype by viral restoration

To determine which population of dopamine neurons is sufficient for preventing generalized anxiety, we developed a viral rescue strategy that allows for conditional re–expression of the NR1 subunit of the NMDA receptor in a cell–specific manner. An adeno–associated viral vector (AAV1) containing a hemaglutinin (HA)–tagged, floxed–stop *Grin1* cDNA cassette (AAV1–fs–HA–NR1) was generated (Supplementary Fig. 4a). Because of the specific localization of Cre expression to dopamine neurons in knockout mice [17], only dopamine cells at the site of injection should allow re–expression of the NR1 subunit.

AAV1–fs–HA–NR1 was injected into the VTA of knockout mice to restore NMDAR function to dopamine neurons (Supplementary Fig. 4b). Histological analysis revealed a high degree of restoration to tyrosine hydroxylase–positive neurons of the VTA that was significantly higher than the marginal restoration to the adjacent tyrosine hydroxylase positive neurons of the substantia nigra pars compacta (SNc) (VTA 69.51 ± 8.19% vs. SNc 13.89 ± 4.32%, Student's t–test: $P < 0.0001$; Fig. 6a,b). HA immunoreactivity was highly co–localizatized with tyrosine hydroxylase (95.57 ± 1.31% s.e.m., Fig. 6b) indicating that HA–NR1 was largely expressed in dopamine neurons. To confirm restoration of NMDAR–mediated function, excitatory postsynaptic currents (EPSCs) were recorded as described [33]. NMDAR–mediated EPSCs were present in virally–rescued dopamine neurons from knockout and control mice, but not non–injected knockout mice (Fig. 6c). Quantitatively, AMPAR to NMDAR ratios in dopamine neurons from virally–rescued knockout were similar to that from controls (0.40 ± .05 vs. 0.55 ± 0.16; control, n = 11 and virally rescued knockout, n =4). Firing–rate and burst–firing parameters were also largely restored in virally–rescued mice (Supplementary Fig. 4c,d). Similarly, tail–pinch evoked excitations and inhibitions were indistinguishable between virally–rescued knockout mice and control mice (Supplementary Fig. 4e,f).

To determine the extent to which viral restoration of NR1 in knockout mice prevents anxiety–like behavior following fear conditioning, we monitored pre– and post–shock ASR, open–field, and elevated–plus maze exploration in two cohorts of virally–injected control (n = 11) and knockout (n = 11) mice. Restoration of functional NMDAR signaling to dopamine

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neurons of the VTA resulted in indistinguishable behavior between virally–rescued knockout and control mice in all tests (Fig. 6d–f).

DISCUSSION

We have demonstrated that the magnitude of activation of a population of dopamine neurons in the ventral midbrain is dependent upon functional NMDAR signaling. Attenuating this activation is associated with impaired cue–conditioning to an aversive stimulus. Disrupting the dopamine–dependent component of aversive conditioning resulted in the sensitization of the ASR and development of a generalized anxiety–like phenotype. These phenotypes were prevented by selective restoration of functional NMDAR signaling to dopamine neurons of the VTA indicating that activation of the mesolimbic dopamine system is important for aversive conditioning and the prevention of generalized anxiety.

Our observation that NMDAR signaling in dopamine neurons plays an important role in behaviorally–evoked, transient activation of these neurons is consistent with previous studies implicating NMDARs in phasic dopamine neuron activity [15,16]. Based on the observations that not all dopamine neurons conform to the commonly used selection criteria [20,34], we cannot rule out the possibility that some dopamine neurons were excluded from the analysis and some non–dopamine neurons were included. Nonetheless, using three different selection criteria, we observed a significant overall effect of NMDAR inactivation on tail pinch–induced firing by presumptive dopamine neurons. It has been reported that dopamine neurons activated by aversive stimuli are anatomically segregated based on dorsal–ventral positioning [4,7]. Although we were unable to precisely determine the dorsal/ventral position of the drivable tetrodes used here due to the nature of the electrode configuration, activated and inhibited neurons were rarely observed together on a single tetrode during a given recording session (2/11 tetrodes), indicating some anatomical segregation of the two populations.

Inactivation of functional NMDAR signaling in dopamine neurons is associated with impairment in the early stages of conditioning to a fear–predictive cue. This finding is consistent with previous reports implicating a role for dopamine in fear conditioning [12,23]. We discovered that impaired fear conditioning in knockout mice is associated with the development of context–independent, persistent sensitization of the ASR. This was surprising in light of previous findings that shock sensitization is context–dependent and short–lived [24]. Intriguingly, in humans, sensitization of the ASR is associated with some anxiety disorders, such as PTSD; however, whether sensitized ASRs develop coincidentally with the onset of anxiety in patients or are a pre–existing condition has been difficult to ascertain [25]. Our results suggest that, at least in mice, these two phenomena can be invoked coincidentally.

It has been proposed that generalized anxiety is the manifestation of inappropriate fear processing in which aversive expectancies do not accurately predict the outcome resulting in the inability to distinguish between periods of safety and threat [25]. Development of generalized anxiety–like behavior by the knockout mice reported here is consistent with this hypothesis and demonstrates a critical role for the dopamine system. The simultaneous

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activation and inhibition of subpopulations of dopamine neurons during aversive outcomes has been proposed to reflect the involvement of dopamine neurons in encoding two distinct aspects of the stimulus. Activation is thought to reflect a salience, or attention–orienting signal, whereas inhibition is thought to reflect motivational value, or valence [9]. In order for a conditioned stimulus to acquire significance it must elicit an orienting response [10]; thus, we propose that the deficits in conditioned fear in knockout mice reflect an impairment in dopamine–dependent salience detection that prevents development of the appropriate conditioned stimulus-unconditioned stimulus association. Such a failure to associate a predictive stimulus with the aversive outcome, in turn, results in generalization and the perception of threat during non–threatening conditions.

Determining the anatomical target(s) of dopamine neurons activated by aversive stimuli and the type of dopamine receptors required for fear processing will provide a greater understanding of fear generalization. We have previously demonstrated impairments in fear conditioning in mice that lack the ability to synthesize dopamine and in mice that lack the dopamine D1 receptor [23]. However, whether these mice develop a sensitization of the ASR or anxiety–like behavior following conditioning was not thoroughly investigated and should be the subject of future investigations. Anatomically, the amygdala provides a compelling target for the dopamine signal generated by aversive stimulus–induced activation. Conditioned stimuli evoke differential activity patterns in discrete subdivisions of the amygdala following cued–fear conditioning that are important for learning [35]. Additionally, dopamine release increases in the amygdala during stress and modulates plasticity within multiple subdivisions of this brain region [12]. Thus, impaired dopamine release in the amygdala of knockout mice during fear conditioning could cause inappropriate stimulus processing or plasticity within amygdala circuitry resulting in the inability to appropriately ascribe potential threat levels.

We did not observe alterations in PPI, Cort, or monoamine levels following fear conditioning in knockout mice. PPI is frequently used to model psychiatric disorders such as schizophrenia [36] and is modulated by dopamine [28]. The lack of impairment in PPI in knockout mice suggests that modulation of sensorimotor reflexes is not dependent upon the same mechanisms responsible for non–selective potentiation of the ASR. Cortisol levels in human subjects are proportional to reported anxiety state and startle potentiation [29]. Whether sensitized ASRs are the result of altered stress responses associated with experimentation is not clear [25]. Our findings indicate that alterations in Cort levels in mice are not required for sensitization of the ASR or elevated anxiety. Moreover, we found that exposure to the acoustic startle chamber led to similar, elevated Cort levels as footshock, yet animals exposed to the testing apparatus without shock did not develop sensitized ASR responses or altered elevated–plus maze or open field responses. Thus, stress alone does not appear to be sufficient to elicit generalized fear– or anxiety–related behavior. Nonetheless, our results do not rule out the possibility that enhanced Cort levels or activation of the HPA axis can be a contributing factor to anxiety state or startle potentiation. We also did not observe significant alterations in total brain monoamine levels associated with enhanced anxiety measures. Thus, fear–induced enhancement of anxiety and startle do not appear to be dependent on gross changes in reflexive circuits, stress hormones, or neurotransmitters associated with anxiety. Taken together, our findings provide evidence for a dopamine–

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dependent component of aversive outcome processing that, when compromised, may be an underlying cause of some generalized anxiety disorders such as PTSD.

Methods

Mice

Male *Grin1* $^{/+}$*Slc6a3*$^{Cre/+}$ mice were bred with female *Grin1*$^{lox/lox}$ mice. All mice were on the C57BL/6 background (> 10 generations backcrossed). Knockout (*Grin1* $^{/lox}$*Slc6a3*$^{Cre/+}$) and control (*Grin1*$^{lox/+}$*Slc6a3*$^{Cre/+}$) mice were identified by PCR of tail DNA as described[17]. Male and female mice between the ages of 8–12 weeks were used for all behavioral experiments. Male mice between the ages of 10–14 weeks were utilized for in vivo electrophysiology recordings. All procedures were approved by the University of Washington, Institutional Animal Care and Use Committee.

Tetrode recordings

4–tetrode microdrives (Neuralynx) were implanted in anesthetized mice by using stereotaxic coordinates for the VTA (3.5 mm A–P, 0.5 mm M–L, and 4.5 mm D–V). Stereotaxic coordinates for the A–P plane were normalized using a correction factor (F = Bregma–Lambda distance/4.21). Two weeks after surgery, mice were connected through an HS–16 headstage preamplifier to an ERP27 patch panel, signals were amplified (200– and 8,000–fold) and filtered (300–6,000 Hz) using a Lynx–8 programmable amplifier. Data were acquired by using Cheetah acquisition software (Neuralynx). Tetrodes were lowered by 50–μm increments each day until putative DA neurons were identified by sensitivity to quinpirole (Sigma; 0.2 mg/ml i.p.; > 70% inhibition of baseline frequency). Baseline DA neuron firing properties were recorded for 5 min, followed by treatment with quinpirole. Tetrode placement was assessed postmortem by cresyl violet staining of midbrain sections. Neural units were isolated by cluster analysis using Offline Sorter software (Plexon). Clustered waveforms were subsequently analyzed by using MATLAB software (Mathworks). Baseline activity was used to calculate burst sets (burst onset, ISI of 80 ms; burst offset, ISI of 160 ms), burst set rate (burst sets/s), burst duration, and firing frequency (total spikes/s).

Tail pinch analysis

PETHs (200–ms bins) were generated for ten 5–s tail pinches presented with an average inter–trial interval of 1 min utilizing MATLAB software (Mathworks, Inc., Natick, MA). We chose tail pinch as the aversive stimulus, as opposed to footshock, because the electrical interference generated by shock is difficult to eliminate in freely moving mice. The proportion of putative DA neurons we found to be activated by tail pinch is similar to that previously described for footshock in anesthetized rats in the VTA[4], where electrical noise is more easily controlled. For all mice, tail pinches were administered by the same individual, blinded to genotype, using padded hemostats. The time at which the pinch was delivered was flagged by an independent investigator during the recording session. Though the magnitude of the tail pinch could not be precisely controlled, the pressure applied to each animal was the minimal force required to suspend the animal by the tail such that the hind limbs did not touch the floor of the recording chamber. Neurons were characterized as

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activated or inhibited by comparing the spike count per 200–ms bin for each trial (1 to 10) to the average number of spikes per bin during the first and last 10 s of all 10 trials, followed by Wilcoxon signed–rank test for significance (GraphPad Prism, La Jolla, CA). Data were normalized by calculating the Z–score for 200 ms bins ((Bin_x − <baseline>)/s.d.) and smoothed using a 3–bin sliding average.

Startle Response Apparatus

Sound–attenuating startle chambers (SR–Lab, San Diego Instruments, San Diego, CA) were used to measure pre–pulse inhibition, startle responses, and fear–potentiated startle. For startle responses, 65 1–ms readings were taken, starting at pulse onset. To measure the response to footshock, 500 1–ms readings were taken, starting at shock onset. The peak amplitude of the response was used to calculate prepulse inhibition, startle responses, fear–potentiated startle, and shock reactivity. White noise was produced by a high–frequency speaker located in the ceiling of the chamber. Background sound was maintained at a constant 65–dB level. Sound levels were measured in decibels (A scale) using a sound–level reader (RadioShack, Fort Worth, TX). A calibration unit was used to ensure the integrity of the startle response readings (San Diego Instruments, San Diego, CA). An 8–watt light mounted on the rear wall of the startle box was used as a cue.

Startle response curves

Following a 5–min habituation period, mice were presented with a series of 7 trials with escalating sound levels: from 80 to 120 dB, with an ITI of 30 s. This series was presented 10 times for a total of 70 trials. In all trials, except null trials in which there was no sound, the sound pulse was 40 ms.

Pre–pulse inhibition

Animals were given a 10–min habituation period after which subjects were presented with 5 40–ms, 120–dB, pulse–alone trials. Mice were then presented with 50 trials of either a startle pulse–alone trial, one of three prepulse trials (5, 10, and 15 dB above background), or a null trial in which there was no acoustic stimulus. The ITI averaged 15 s (range 5–25 s). A startle trial consisted of a 40–ms, 120–dB pulse of white noise. Prepulse trials consisted of a 20–msec duration prepulse of 70, 75, or 80–dB intensity, which preceded the 40–ms 120–dB pulse by 100 ms. Prepulse inhibition was calculated for each prepulse level using the following formula: % inhibition=[(average startle response on prepulse trial/average startle response on pulse–alone trial)×100].

Elevated–plus maze

Entries into the open and closed arms of an elevated plusmaze (Med Associates Inc., St. Albans, VT), time spent in these arms, as well as distance traveled was monitored for 10 min one to three days before footshock conditioning and one to three days following conditioning using a video acquisition system (Canopus MediaCruise, Tokyo, Japan). Data were analyzed using Ethovision software (Noldus Information Technology, Leesburg, VA).

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Open field

Number of center crossings and distance traveled in an open field (75 cm diameter tub with laminated floor designating the center of the arena) was monitored for 10 min one to three days before footshock conditioning and one to three days following conditioning using a video acquisition system as above. Mice were tested in the both the elevated–plus maze and open field in pseudorandom order on different days. Two control mice were eliminated from open field analysis due to technical failures during video acquisition.

Fear–potentiated startle

On day 1 (baseline), following a 5–min habituation period, mice were given a pseudo–randomly ordered series of 20 trials, split evenly between cue and no–cue conditions. For no–cue trials, animals were presented with a 40–ms, 105–dB acoustic pulse. For cue trials, the animals were presented with a 10–s light cue, which co–terminated with a 40–ms, 105–dB pulse. The ITI averaged 120 s (range 60 to 180 s). On day 2, following a 5–min habituation period, mice were given 30 pairings of a 10–s cue light, which co–terminated with a 0.5–s, 0.2–mA footshock. The ITI averaged 120 s (range 60 to 180 s). Following training, the mice were placed into their home cages for 10 min before testing. The following formula was used to calculate fear–potentiated startle: %potentiation=[(average of responses on cue trials/average of responses on–no cue trials−1)×100].

Generation of AAV1–fs–HA–NR1 and viral injections

The virus was generated by inserting 3 HA tag sequences preceded by a loxP–flanked SV40 late polyadenylation site and 7 in–frame termination codons after amino acid 31 of the rat NMDAR1–3a splice variant. Following Cre–mediated recombination, NR1 expression is driven by the cytomegalovirus–chicken β–actin (CBA) promoter. The open reading frame is followed by the bovine growth hormone polyadenylation sequence. Viral injections were performed by targeting the ventral midbrain using coordinates (AP: 3.25 mm, ML: 0.5 mm, and DV: 4.5 mm; Paxinos and Franklin, 2001). A lambda–bregma correction factor described above was utilized to facilitate appropriate targeting. The injection needle (32 GA) was inserted 5 mm DV and drawn back to 4.5 mm to create an injection pocket. 0.5 μl of virus (~4×10^9 particles/μl) was injected at a rate of 0.25 μl/min. For behavioral experiments mice were allowed to recover at least 1 week following surgery. For slice electrophysiology experiments mice were tested 3–5 d following surgery. For tetrode recordings, microdrives were implanted immediately following viral injections and mice were allowed to recover from surgery for 10–14 days.

Slice electrophysiology

VTA slices were obtained from halothane–anesthetized male mice that ranged in age from p25 to p35. Horizontal slices were prepared (190 mm) in ice–cold low Ca2+ artificial cerebrospinal fluid (ACSF, 126 mM NaCl, 1.6 mM KCl, 1.2 NaH2PO4 mM, 1.2 MgCl2 mM, 2.5 CaCl2 mM, 18 NaHCO3 mM, and 11 mM glucose) and subsequently equilibriated at 31–34 C for at least 1 hr. All solutions were bubbled with 95% O2/5% CO2 and perfused over the slice at a rate of 2.5 ml/min. Whole–cell recordings were performed using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA). EPSCs recordings were

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acquired using electrodes (2–6 MU) contained in mM: 120 mM cesium methansulfonic acid, 20 mM HEPES, 0.4 mM EGTA, 2.8 mM NaCl, 5 mM TEA–Cl, 2.5 mM MgATP, and 0.25 mM MgGTP (pH 7.2–7.4). Picrotoxin (100 mM) was added to the ACSF to block GABAA receptor–mediated IPSCs. NMDAR or AMPAR traces were constructed by averaging 15 EPSCs elicited at +40 mV or at –70 mV. NMDAR responses were calculated by subtracting the average response in the presence of 50 mM D–APV (AMPAR only) from that recorded in its absence.

Immunohistochemistry

Proteins were detected with primary antibodies to tyrosine hydroxylase (rabbit polyclonal antibody 1:1000, Millipore, Temecula, CA) and hemagglutinin (polyclonal antibody 1:1000; Applied Biological Materials). Primary antibodies were detected using CY2– or CY3–conjugated, goat anti–mouse and goat anti–rabbit antibodies (1:200, Jackson Immunolabs, West Grove, PA). For quantification, three serial sections were stained from each mouse. Images were acquired using a Nikon Eclipse E600 microscope and processed using Adobe Photoshop. HA–NR1 and TH–positive neurons were scored for overlapping protein expression using merged images.

Statistical Analyses

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA). Repeated measures analysis of variance (ANOVA) was used for multivariate analysis and Bonferoni posttests were performed where appropriate. Student's t–tests were performed using two-tailed distribution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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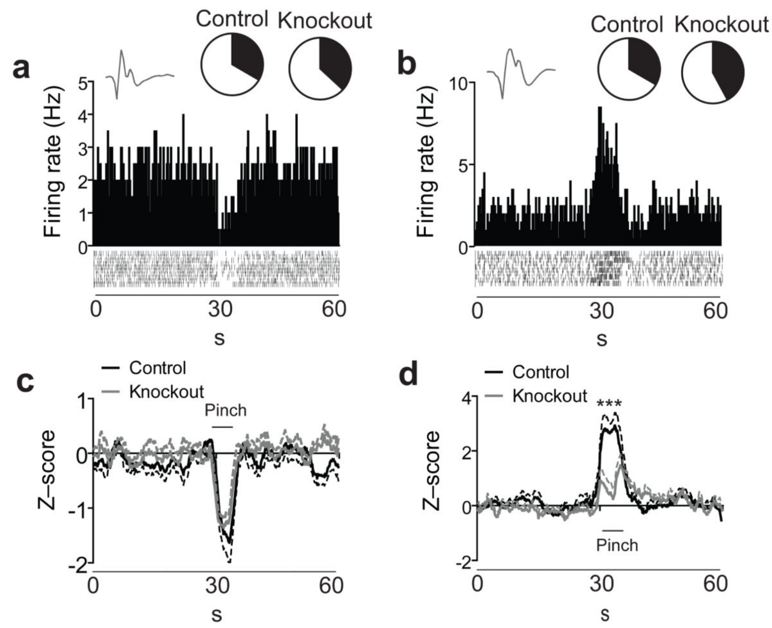

Figure 1.

NMDARs control amplitude of activation of dopamine neurons in response to tail pinch. (**a–d**) Assessment of NMDAR–dependent activation of dopamine neurons during tail pinch. (**a,b**) Peri–event time histograms (PETHs) of representative dopamine neurons illustrating inhibitory (**b**) and excitatory (**c**) responses to tail pinch (n = 5 control mice and n = 6 knockout mice). Inserts for **a** and **b,** left: average waveform for representative neurons, right: pie charts show proportion of QS neurons inhibited or activated by tail pinch (black shade) for control and knockout mice. **(c,d)** Average Z–score corrected (see Supporting Online Material) inhibitory (**c**) and excitatory (**d**) responses to tail pinch (dashed lines represent standard error of the mean, s.e.m.). Excitatory responses are significantly reduced in KO mice compared to controls (Bonferroni posttests ***$P < 0.001$).

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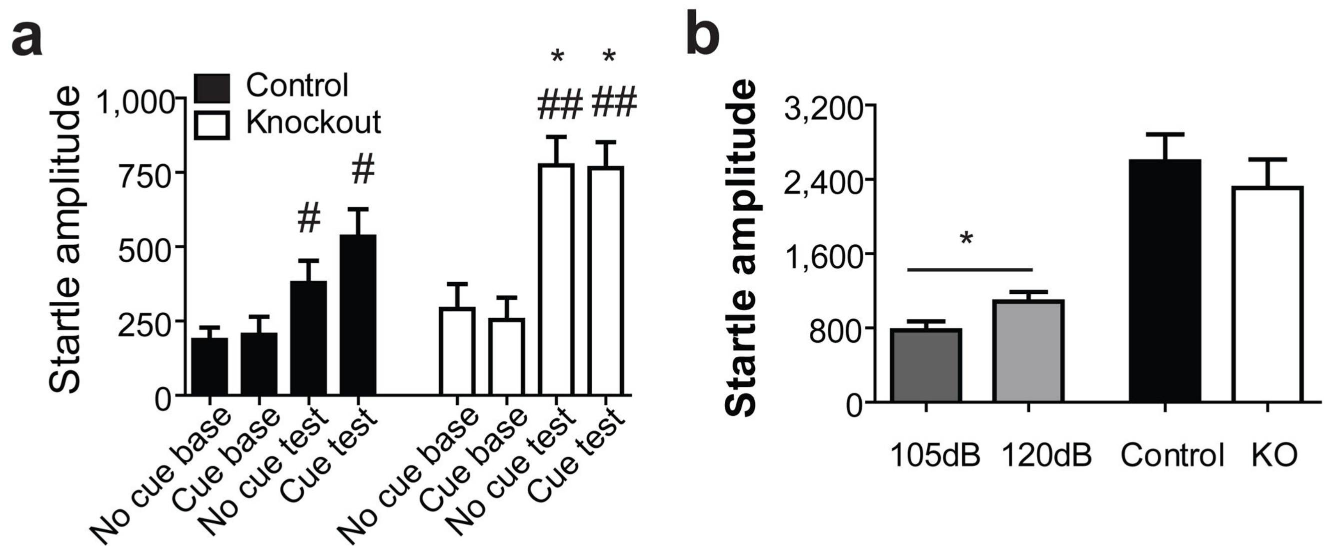

Figure 2.

Cue–dependent fear conditioning is impaired in knockout mice. **(a)** Startle amplitude is enhanced 10 min after cue–footshock pairings to a greater extent in knockout (n=8) compared than control (n=9) mice in both the presence and absence of the cue (Bonferroni posttests, #$P < 0.05$ and ##$P < 0.01$ post–conditioning compared to pre–conditioning in the presence or absence of the cue, *$P < 0.05$ knockout compared to control no cue and cue tests). **(b)** Amplitude of startle response in conditioned knockout mice at 105 dB is lower than the startle response of unconditioned knockout mice at 120 dB (*$P < 0.05$). Startle amplitude to footshock during conditioning trials is not different between groups. Error bars represent s.e.m.

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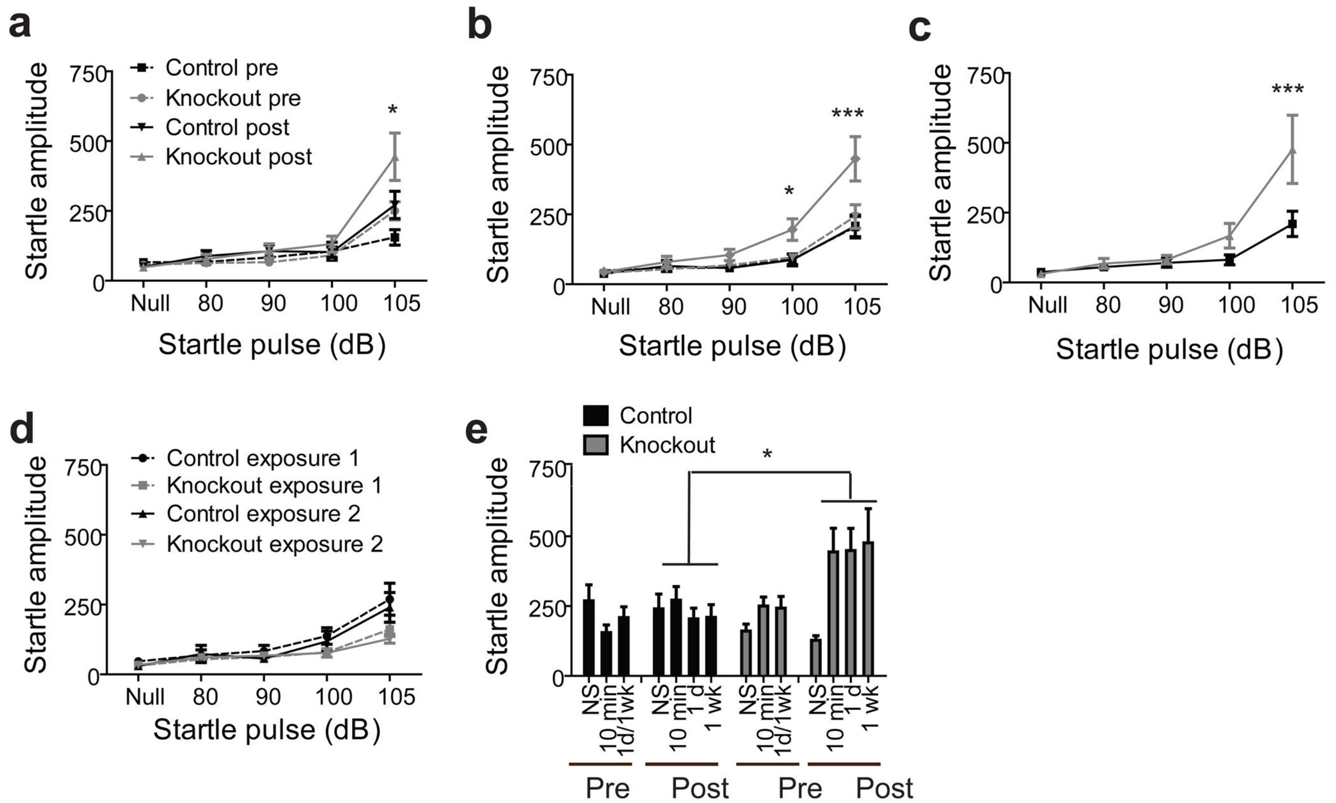

Figure 3.

Sensitization of ASR following fear conditioning in knockout mice is context independent. **(a)** ASR before (dashed line) and 10 min following footshock conditioning (solid line) in a distinct environmental context (n = 13, control and n = 11, knockout; Bonferoni posttests *P < 0.05, knockout post–shock vs. knockout pre–shock and control pre– and post–shock). **(b)** ASR (n = 14, control and n = 14, knockout) before (dashed line) and 1 day post–shock (solid line) is elevated in knockout mice following shock (Bonferoni posttests ***P < 0.001 and *P < 0.05, knockout post–shock vs. knockout pre–shock and control pre– and post–shock). (**c**) Same groups of mice as in (**b**) 1 week following conditioning in novel context demonstrating persistent elevation of the ASR in knockout mice following footshock (Bonferoni posttests ***P < 0.001, knockout post–shock vs. knockout pre–shock and control pre– and post–shock). **(d)** ASR following repeated exposure to ASR chamber without conditioning (n = 8, control and n = 7, knockout) is not different between groups. (**e**) Average ASR at 105 dB across all groups of mice pre– and post–conditioning (NS = no shock, repeated exposure group; Bonferoni posttests *P < 0.05, knockout post–shock vs. knockout pre–shock and knockout postshock vs. control pre– and post–shock). Error bars represent s.e.m.

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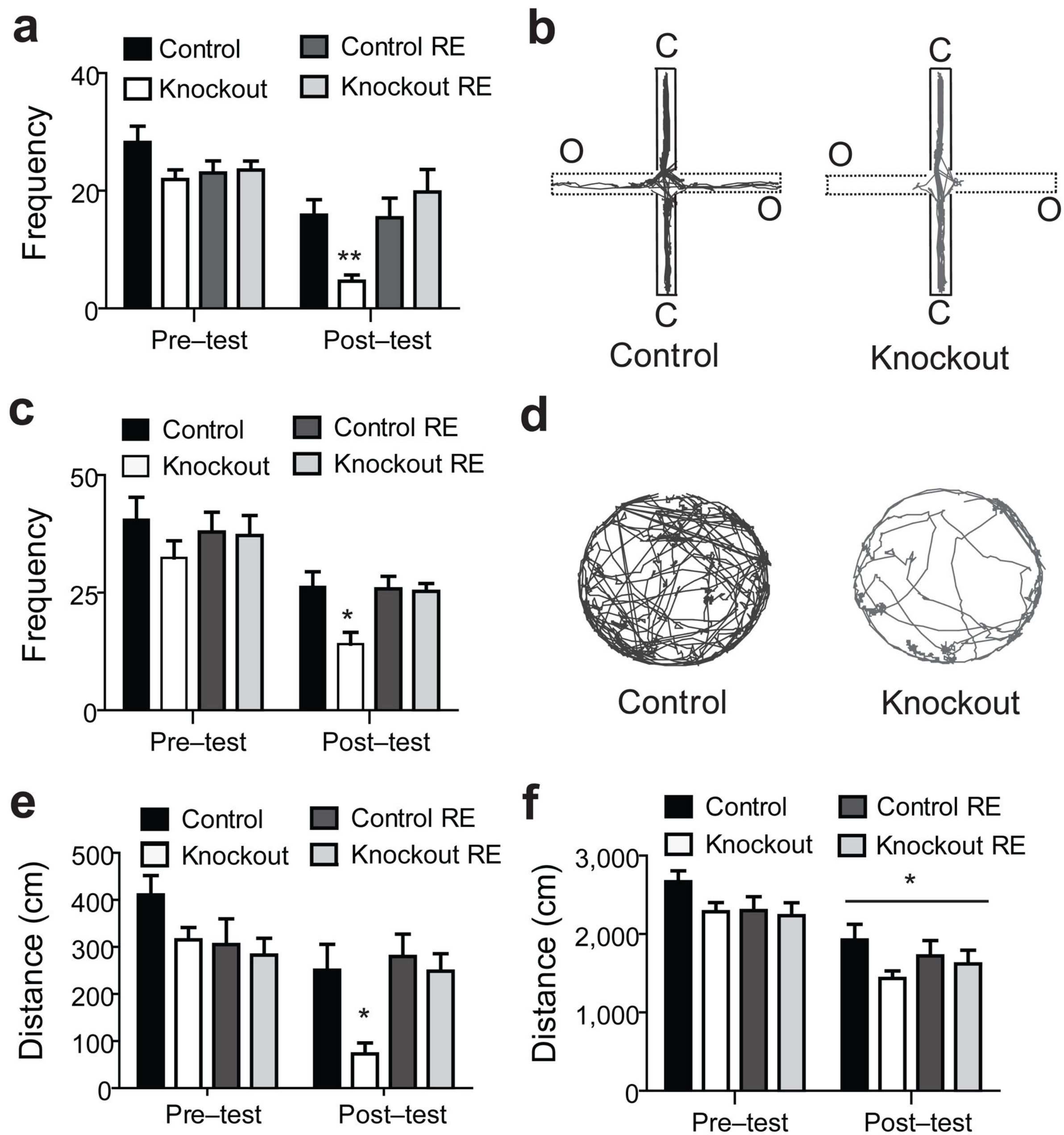

Figure 4.

Anxiety–related behavior is enhanced in knockout mice following footshock conditioning. (**a**) Frequency of open–arm entries in an elevated–plus maze following footshock is significantly reduced in knockout (n = 14) compared to control mice (n = 13) or mice repeatedly exposed (RE) to the elevated–plus maze (Bonferoni posttests, $^{**}P < 0.01$, knockout post–shock vs. all other groups). (**b**) Representative activity traces from control (left) and knockout mice (right) in elevated–plus maze test following footshock (C, closed arm and O, open arm). (**c**) Frequency of center crossings in an open–field apparatus by knockout mice (n=14) following footshock conditioning is reduced compared to control mice (n=13) or mice repeatedly exposed to the open field (knockout n=13, control n=10;

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Bonferoni posttests, *$P < 0.05$, knockout post–shock vs. all other groups). (**d**) Representative activity traces from control (left) and knockout mice (right) in open field following footshock conditioning. (**e**) Distance traveled in the open arm of the elevated–plus maze is significantly reduced in knockout mice following footshock compared to other groups (Bonferoni posttests, *$P < 0.05$, knockout post–shock vs. all other groups). (**f**) Distance traveled in closed arm of elevated–plus maze is not significantly reduced in knockout mice that received footshock. Error bars represent s.e.m.

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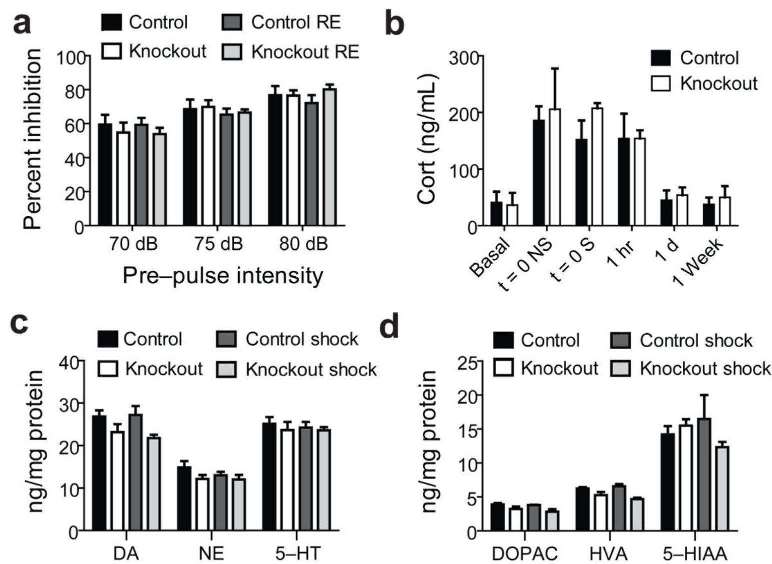

Figure 5.

Sensory motor gating, peripheral stress response, and monoamine levels are not altered following footshock conditioning. (**a**) Increasing pre–pulse intensities leads to greater PPI of ASRs but is not altered in knockout mice following footshock (pre–pulse effect, *** $P < 0.0001$). (**b**) Cort levels are increased immediately (t=0) after exposure to the startle chamber with shock (S) or without shock (NS) and are elevated 1 h following shock, but not 1 d or 1 wk later. There is no significant difference between genotypes. (**c**) Whole–brain monoamine levels as measured by HPLC are not different in knockout or control mice that had received shock. (**d**) Monoamine metabolites are unaltered by footshock conditioning. Error bars represent s.e.m.

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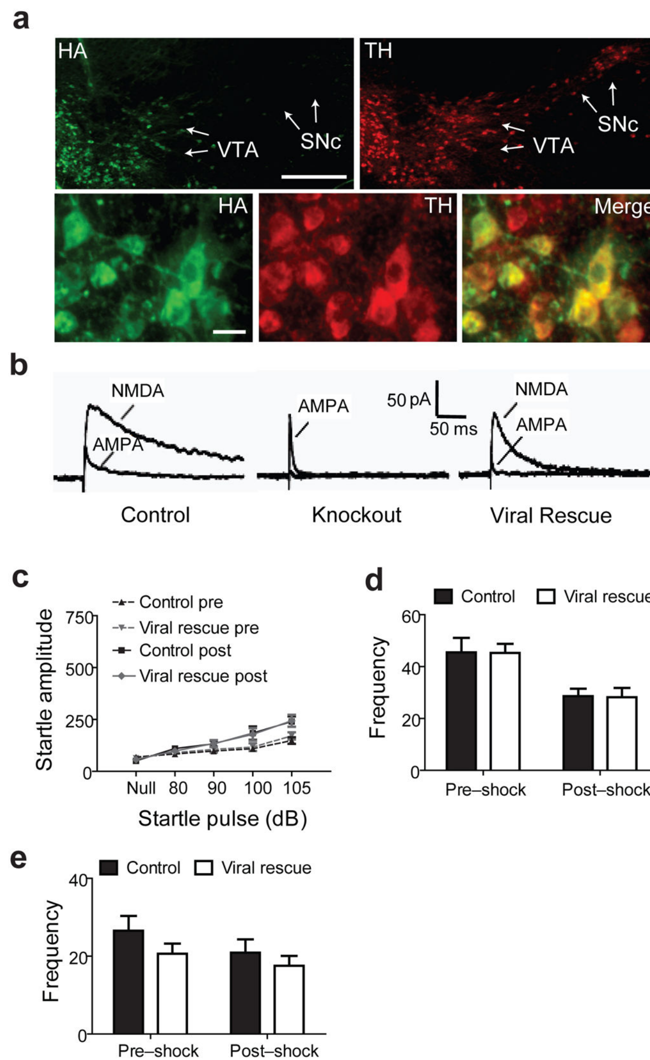

Figure 6.

Conditional restoration of NMDAR signaling to ventral midbrain dopamine neurons prevents generalized anxiety–like behavior. (**a**) Low magnification of ventral midbrain (top) demonstrating HA–NR1 (green) is predominantly localized to the tyrosine hydroxylase (TH)–positive region of the VTA and not the SNc (scale bar = 500 μm). High magnification (bottom) shows HA–NR1 co–localized with TH–positive neurons (scale bar = 25 μm). (**b**) Evoked AMPAR and NMDAR–mediated EPSCs from control, knockout, and virally rescued knockout mice. (**c–e**) Expression of HA–NR1 in knockout mice prevents generalized anxiety–like behavior: (**c**) ASR, (**d**) frequency of open arm entries in the elevated–plus maze, and (**e**) frequency of center crossing in the open–field before and after foot shock conditioning in novel context (control, n = 11 and virally rescued knockout, n = 11). Error bars represent s.e.m.