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Activation of dopamine neurons is critical for aversive conditioning and prevention of generalized anxiety

Larry S Zweifel[1–3,8], Jonathan P Fadok[3,4,8], Emmanuela Argilli[5], Michael G Garelick[4], Graham L Jones[1,2], Tavis M K Dickerson[3], James M Allen[6], Sheri J Y Mizumori[7], Antonello Bonci[5] & Richard D Palmiter[3]

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 orientation 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 NMDA receptors on dopamine neurons. We discovered that altering the magnitude of excitatory responses by dopamine neurons in response to an aversive stimulus was associated with impaired conditioning to a cue that predicts an aversive outcome. Impaired conditioning by these mice was associated with the development of a persistent, generalized anxiety-like phenotype. These data are consistent with a 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 that are important for emotional processing. Dopamine signaling in these target regions modulates both acute physiology and synaptic plasticity, implicating this neurotransmitter as a key facilitator of stimulus processing, learning and memory[1]. In response to aversive stimuli, dopamine neurons have been shown 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, whereas 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 nonthreatening 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 NMDA receptors (NMDARs)[13–15], and mice lacking functional NMDARs selectively on dopamine neurons have impaired phasic activation (or burst firing)[16]. We found that activation of dopamine neurons in response to an aversive stimulus (tail pinch) was substantially attenuated in the absence of functional NMDAR signaling. Pavlovian fear conditioning was impaired in knockout mice, and these animals developed an anxiety-like phenotype following fear conditioning. Viral-mediated restoration of functional NMDARs 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 are dependent on NMDAR signaling, we recorded the activity of these neurons in mice lacking an essential subunit of the NMDAR (NR1, *Grin1*) exclusively in dopamine-producing neurons. *Grin1*$^{\Delta/loxP}$; *Slc6a3*$^{cre/+}$ (knockout) mice were generated as described previously[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 on the basis of their sensitivity to quinpirole, a dopamine D2 receptor agonist (0.2 mg per kg of body weight, intraperitoneal), at the end of each recording session. During baseline recordings, firing rate and burst patterns of activation in quinpirole-sensitive neurons (control, $n = 21$ of 32 cells; knockout, $n = 19$ of 28) were impaired in knockout compared with control mice (**Supplementary Fig. 1b–e**), similar to previous observations[16].

[1]Department of Pharmacology, University of Washington, Seattle, Washington, USA. [2]Department of Psychiatry and Behavioral Sciences, University of Washington, Seattle, Washington, USA. [3]Department of Biochemistry and Howard Hughes Medical Institute, University of Washington, Seattle, Washington, USA. [4]Graduate Program in Neurobiology and Behavior, University of Washington, Seattle, Washington, USA. [5]Ernest Gallo Clinic and Research Center and Department of Neurology, University of California, San Francisco, San Francisco, California, USA. [6]Department of Neurology, University of Washington, Seattle, Washington, USA. [7]Department of Psychology, University of Washington, Seattle, Washington, USA. [8]These authors contributed equally to this work. Correspondence should be addressed to L.S.Z. (larryz@uw.edu) or R.D.P. (palmiter@uw.edu).

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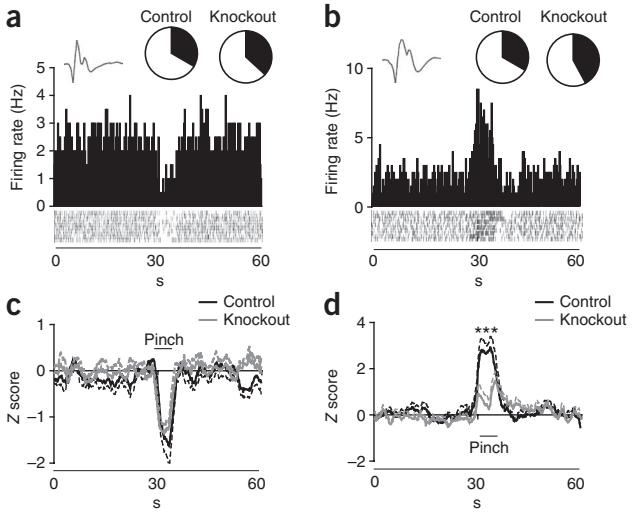

Figure 1 NMDARs control amplitude of activation of dopamine neurons in response to tail pinch. (**a**,**b**) Assessment of NMDAR-dependent activation of dopamine neurons during tail pinch. Peri-event time histograms of representative dopamine neurons illustrating inhibitory (**a**) and excitatory (**b**) responses to tail pinch ($n = 5$ control mice and $n = 6$ knockout mice). Inserts, average waveform for representative neurons is shown on the left and pie charts showing the proportion of quinpirole-sensitive neurons inhibited or activated by tail pinch (black shade) for control and knockout mice are shown on the right. (**c**,**d**) Average *Z* score corrected inhibitory (**c**) and excitatory (**d**) responses to tail pinch (dashed lines represent s.e.m., s.e.m.). Excitatory responses are significantly reduced in knockout mice compared to controls (Bonferroni post-tests, $^{***}P < 0.001$).

After establishing baseline recordings, we determined responses of quinpirole-sensitive and quinpirole-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 quinpirole-insensitive neurons displayed differential responses to tail pinch (**Fig. 1** and **Supplementary Fig. 2a**,**b**). We observed nearly equivalent proportions of quinpirole-sensitive neurons activated (control, $n = 7$ of 20; knockout, $n = 8$ of 19), inhibited (control, $n = 7$ of 20; knockout, $n = 7$ of 19) or unaltered (control, $n = 6$ of 20; knockout, $n = 4$ of 19) by tail pinch. The magnitude of tail pinch–induced inhibition in either quinpirole-sensitive or quinpirole-insensitive neurons did not differ between control and knockout mice (**Fig. 1c** and **Supplementary Fig. 2a**). In contrast, excitation of quinpirole-sensitive neurons from knockout mice was markedly attenuated compared with quinpirole-sensitive neurons from control mice (two-way repeated-measures analysis of variance (ANOVA), genotype × time interaction, $F_{297,\,4,455} = 4.16$, $P < 0.0001$; **Fig. 1d**). No difference in activation was observed between quinpirole-insensitive neurons from knockout mice compared to control mice (**Supplementary Fig. 2b**). Because a subset of dopamine neurons have been described as being unresponsive to quinpirole[20] and a small proportion of non-dopamine neurons are inhibited by quinpirole[21], it is possible that our findings are biased as a result of selection criteria. We therefore 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 with 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 on the basis of 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 foot shock and FPS was assessed 10 min following training, as described previously[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]. Notably, knockout mice displayed significantly greater potentiation in both the cue and non-cue conditions compared to controls (repeated-measures ANOVA, genotype × conditioning interaction, $F_{3,45} = 4.22$, $P = 0.01$; **Fig. 2a**). Enhanced ASR in the non-cue condition precluded FPS detection in knockout mice (percentage potentiation: control, 47.2 ± 15.9 s.e.m.; knockout, 7.4 ± 8.0 s.e.m.; $P = 0.02$, two-tailed Student's *t* test).

To assess whether cued FPS is impaired in knockout mice as a result of 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 ASRs of conditioned mice. ASRs at 120 dB were significantly higher in non-conditioned knockout mice than the ASR at 105 dB following conditioning (two-tailed Student's *t* test, $P = 0.04$; **Fig. 2b**). In addition, no detectable differences were observed in behavioral response to foot shock during conditioning between control and knockout mice (**Fig. 2b**).

Potentiated startle is independent of context and persistent

Shock sensitization has been shown to be context dependent and to persist for only brief periods following foot shock conditioning[24]. We asked whether the enhanced ASR in knockout mice following fear conditioning was dependent on the context in which conditioning

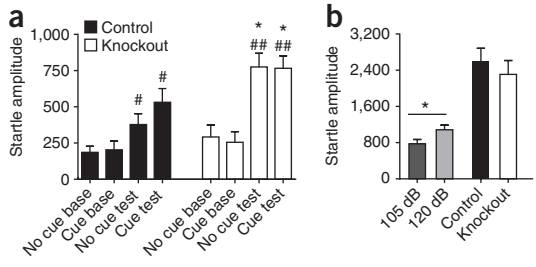

Figure 2 Cue-dependent fear conditioning is impaired in knockout mice. (**a**) Startle amplitude was enhanced 10 min after cue–foot shock pairings to a greater extent in knockout ($n = 8$) than control ($n = 9$) mice in both the presence and absence of the cue (Bonferroni post-tests, $P < 0.05$ and $P < 0.01$ post-conditioning compared with pre-conditioning in the presence or absence of the cue, $^{*}P < 0.05$ knockout compared with control no cue and cue tests, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ test compared to baseline). (**b**) Amplitude of startle response in conditioned knockout mice at 105 dB was lower than the startle response of unconditioned knockout mice at 120 dB. Startle amplitude to foot shock during conditioning trials was not different between groups. Error bars represent s.e.m.

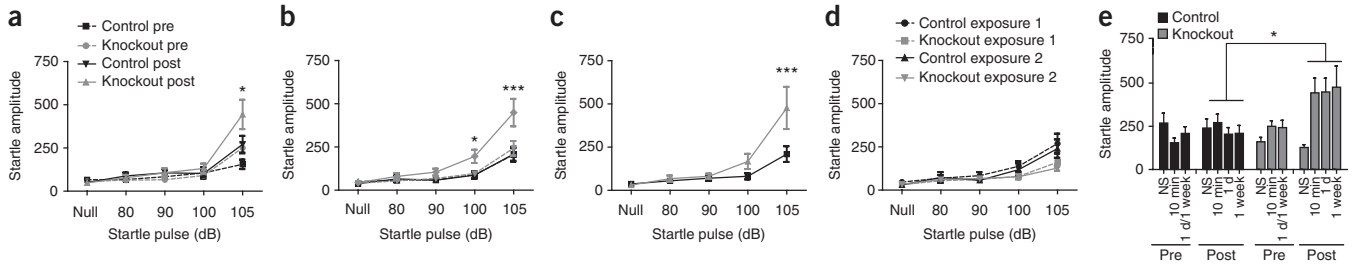

Figure 3 Sensitization of ASR following fear conditioning in knockout mice is context independent. (**a**) ASR before (dashed line) and 10 min after foot shock conditioning (solid line) in a distinct environmental context (control, $n = 13$; knockout, $n = 11$; Bonferroni post-tests, $^{*}P < 0.05$, knockout post-shock versus knockout pre-shock and control pre- and post-shock). (**b**) ASR (control, $n = 14$; knockout, $n = 14$) before (dashed line) and 1 d post-shock (solid line) was elevated in knockout mice following shock (Bonferroni post-tests, $^{***}P < 0.001$ and $^{*}P < 0.05$, knockout post-shock versus knockout pre-shock and control pre- and post-shock). (**c**) Same groups of mice as in **b** 1 week following conditioning in novel context. We observed persistent elevation of the ASR in knockout mice following foot shock (Bonferroni post-tests, knockout post-shock versus knockout pre-shock and control pre- and post-shock). (**d**) ASR following repeated exposure to ASR chamber without conditioning (control, $n = 8$; knockout, $n = 7$) 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; Bonferroni post-tests, knockout post-shock versus knockout pre-shock and knockout post-shock versus control pre- and post-shock). Error bars represent s.e.m.

occurred and whether this sensitization was short-lived or persistent. We monitored ASRs before and after foot shock using a repeated-measures experimental design. Notably, foot shock conditioning was performed in a context that was 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 foot shocks in a context that was distinct from the acoustic startle chamber and were returned to their home cages. ASRs were subsequently monitored either 10 min or 1 d and 1 week following conditioning. Results from two independent groups of mice revealed that baseline ASRs did not differ between control and knockout mice; however, ASRs were significantly potentiated in knockout mice compared with control mice 10 min after foot shock conditioning in a novel context (repeated-measures ANOVA, genotype × conditioning interaction, $F_{12,176} = 5.58$, $P < 0.0001$; **Fig. 3a**). To explore the persistence of sensitized startle in knockout mice, we shocked two additional cohorts of mice and measured ASRs both 1 d and 1 week following conditioning in a novel context. Startle was elevated in knockout mice at both 1 d and 1 week following conditioning (repeated-measures ANOVA, genotype × conditioning interaction: 1 d, $F_{12,280} = 2.82$, $P = 0.001$; 1 week, $F_{4,112} = 3.06$, $P = 0.02$; **Fig. 3b**). In both knockout and control mice, handling and repeated exposure to the startle chamber did not alter the ASR (**Fig. 3d**,**e**).

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 ten tail pinches, identical to that described for electrophysiological recordings. Similarly to foot shock, ASRs were sensitized in knockout mice 10 min and 1 d 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 foot shock conditioning. Testing in each apparatus was performed in pseudorandom order. Additional cohorts of nonshocked mice were repeatedly measured for elevated-plus maze and open-field activity to control for potential novelty habituation effects. Before foot shock, all groups showed similar behavior in both tasks (**Fig. 4**). In the elevated-plus maze task, knockout mice that received shock had significantly fewer open arm entries (repeated-measures ANOVA: genotype × test interaction, pre-shock versus post-shock, $F_{3,48} = 4.07$, $P = 0.01$; **Fig. 4a**,**b**) 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 foot shock (repeated-measures ANOVA: genotype × test interaction, pre-shock versus post-shock, $F_{3,48} = 2.83$, $P = 0.04$; **Supplementary Fig. 3c**). Knockout mice demonstrated a reduction in center crossings in the open field following foot shock compared with all of the other groups (repeated-measures ANOVA: genotype × test interaction, $F_{3,46} = 2.84$, $P = 0.05$; **Fig. 4c**,**d**). 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. 3d**,**e**). Further analysis of distance traveled in the open arms of the elevated-plus maze revealed a significant difference between knockout mice that had received shock compared with all of the other groups (repeated-measures ANOVA: genotype × test interaction, $F_{3,48} = 2.80$, $P = 0.05$; **Fig. 4e**). No such effect was observed for distance traveled in the closed arms (**Fig. 4f**). Distance traveled in the center of the open field arena was significantly affected by genotype (repeated-measures ANOVA: $F_{3,46} = 4.3$, $P = 0.03$) and repeated testing (repeated-measures ANOVA, $F_{1,48} = 120.43$, $P < 0.0001$); 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 a result of 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 effect of shock on ambulatory activity in either group (**Supplementary Fig. 3g**).

PPI, cortisol and monamines are unaltered in knockouts

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 nonselective potentiation of ASR in knockout mice is associated with an alteration in sensorimotor gating, we measured PPI in control and knockout mice at three different intensities, before and after foot shock, as well as in separate groups repeatedly tested

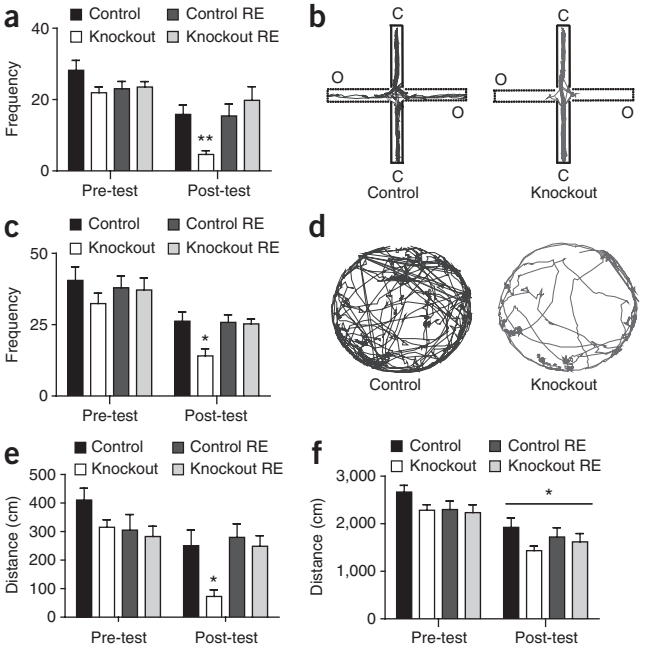

Figure 4 Anxiety-related behavior is enhanced in knockout mice following foot shock conditioning. (**a**) Frequency of open-arm entries in an elevated-plus maze following foot shock was significantly reduced in knockout ($n = 14$) compared with control mice ($n = 13$) or mice repeatedly exposed (RE) to the elevated-plus maze (Bonferroni post-tests, $^{**}P < 0.01$, knockout post-shock versus all other groups). (**b**) Representative activity traces from control (left) and knockout mice (right) in elevated-plus maze test following foot shock (C, closed arm; O, open arm). (**c**) Frequency of center crossings in an open-field apparatus by knockout mice ($n = 14$) following foot shock conditioning was reduced compared to control mice ($n = 13$) or mice repeatedly exposed to the open field (knockout, $n = 13$; control, $n = 10$; Bonferroni post-tests, $^{*}P < 0.05$, knockout post-shock versus all other groups). (**d**) Representative activity traces from control (left) and knockout mice (right) in open field following foot shock conditioning. (**e**) Distance traveled in the open arm of the elevated-plus maze was significantly reduced in knockout mice following foot shock compared with other groups (Bonferroni post-tests, $^{*}P < 0.05$, knockout post-shock versus all other groups). (**f**) Distance traveled in closed arm of elevated-plus maze was not significantly reduced in knockout mice that received foot shock. $^{*}P < 0.05$. Error bars represent s.e.m.

without receiving shock (knockout, $n = 7$; control, $n = 7$; knockout nonshocked, $n = 7$; control nonshocked, $n = 7$). Startle was significantly affected by pre-pulse intensity (repeated-measures ANOVA, $F_{2,48} = 46.09$, $P < 0.0001$) but was not affected by genotype or foot shock (**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 nonhuman primates following rearing stress[31]. To determine whether foot shock altered HPA axis function in knockout mice, we measured serum corticosterone (Cort). 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 nonshocked, $n = 4$; knockout shocked, $n = 4$; control nonshocked, $n = 3$; control shocked, $n = 3$), 1 h (knockout shocked, $n = 4$; control shocked, $n = 4$), 1 d (knockout shocked, $n = 4$; control shocked, $n = 4$) or 1 week later (knockout shocked, $n = 4$; control shocked, $n = 4$). Mice exposed to the startle chamber with or without foot shock displayed elevated Cort at $t = 0$ and 1 h following exposure (ANOVA, $P = 0.004$; **Fig. 5b**). Cort levels were similar between control and knockout mice under all conditions.

Alterations in brain monoamine levels can also disrupt the ASR[32]. To explore whether foot shock conditioning alters brain monoamine levels or metabolites, we performed high-performance liquid chromatography analysis of whole brains from shocked (24 h post-shock: knockout, $n = 3$; control, $n = 3$) and nonshocked knockout ($n = 3$) and control ($n = 3$) mice. No statistically significant differences in the neurotransmitters dopamine (ANOVA, $P = 0.12$), norepinephrine (ANOVA, $P = 0.33$), serotonin (ANOVA, $P = 0.88$), or the metabolites dihydroxyphenylacetic acid (ANOVA, $P = 0.08$), homovanillic acid (ANOVA, $P = 0.06$) or 5-hydroxyindoleacetic acid (ANOVA, $P = 0.46$) were observed across groups (**Fig. 5c**,**d**).

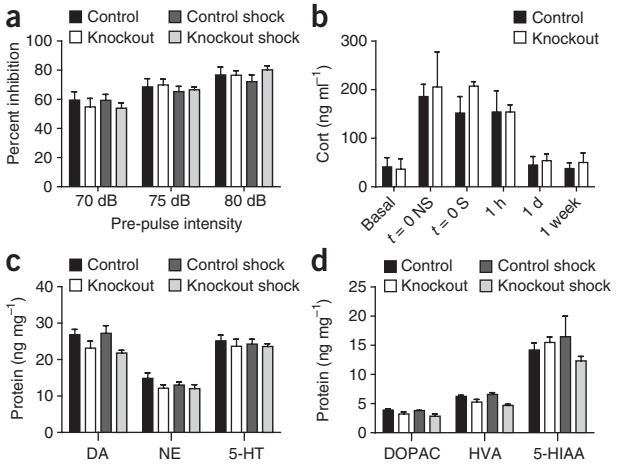

Figure 5 Sensory motor gating, peripheral stress response and monoamine levels are not altered following foot shock conditioning. (**a**) Increasing pre-pulse intensities led to greater PPI of ASRs, but was not altered in knockout mice following foot shock. (**b**) Cort was increased immediately ($t = 0$) after exposure to the startle chamber with shock (S) or without shock (NS) and was elevated 1 h following shock, but not 1 d or 1 week later. (**c**) Whole-brain monoamine as measured by high-performance liquid chromatography were not different in knockout or control mice that had received shock. DA, dopamine; NE, norepinephrine; 5-HT, serotonin. (**d**) Monoamine metabolites were unaltered by foot shock conditioning. 5-HIAA, 5-hydroxyindoleacetic acid; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid. Error bars represent s.e.m.

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 hemagglutinin (HA)-tagged *Grin1* cDNA cassette with a *loxP*-flanked stop codon (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

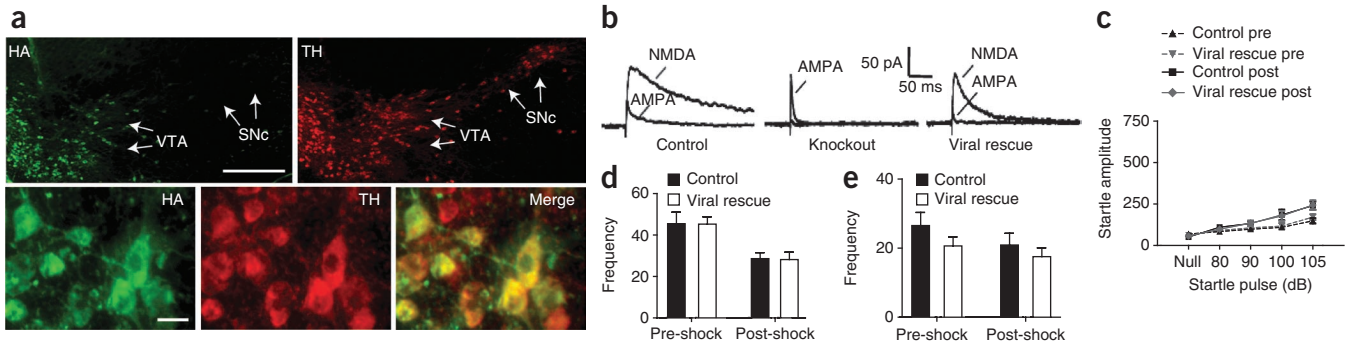

Figure 6 Conditional restoration of NMDAR signaling to ventral midbrain dopamine neurons prevents generalized anxiety-like behavior. (**a**) Low magnification of ventral midbrain (top). HA-NR1 (green) was predominantly localized to the tyrosine hydroxylase (TH)-positive region of the VTA and not the SNc. Scale bar represents 500 μm. High-magnification (bottom) images reveled that HA-NR1 colocalized with tyrosine hydroxylase–positive neurons. Scale bar represents 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 prevented generalized anxiety-like behavior: ASR (**c**), frequency of open arm entries in the elevated-plus maze (**d**), and frequency of center crossing in the open field (**e**) before and after foot shock conditioning in novel context (control, $n = 11$; virally rescued knockout, $n = 11$). Error bars represent s.e.m.

tyrosine hydroxylase–positive neurons of the substantia nigra pars compacta (SNc; VTA, 69.51 ± 8.19%; SNc, 13.89 ± 4.32%; Student's *t* test, $P < 0.0001$; **Fig. 6a**,**b**). HA immunoreactivity was highly colocalized 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 previously[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 ± 0.05 versus 0.55 ± 0.16; control, $n = 11$; 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 neurons of the VTA resulted in indistinguishable behavior between virally rescued knockout and control mice in all tests (**Fig. 6c**–**e**).

DISCUSSION

We found that the magnitude of activation of a population of dopamine neurons in the ventral midbrain is dependent on 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 is important role for behaviorally evoked, transient activation of these neurons is consistent with previous studies implicating NMDARs in phasic dopamine neuron activity[15,16]. On the basis of 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 on the basis of dorsal-ventral positioning[4,7]. Although we were unable to precisely determine the dorsal/ventral position of the drivable tetrodes that we used because of the nature of the electrode configuration, activated and inhibited neurons were rarely observed together on a single tetrode during a given recording session (2 of 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 found 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]. Notably, 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 these knockout mice is consistent with this hypothesis and demonstrates a critical role for the dopamine system. The simultaneous 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]. 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 nonthreatening 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]. In addition, dopamine release increases in the amygdala during stress and modulates plasticity in 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 in 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 on the same mechanisms responsible for nonselective potentiation of the ASR. Cortisol levels in humans 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 similarly elevated Cort as foot shock, but 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 elevated Cort 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-dependent component of aversive outcome processing that, when compromised, may be an underlying cause of some generalized anxiety disorders such as PTSD.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

L.S.Z. and J.P.F. designed the experiments. L.S.Z. performed *in vivo* recordings with assistance from G.L.J. and S.J.Y.M. L.S.Z. and J.P.F. performed behavioral experiments with assistance from M.G.G. and T.M.K.D. E.A. performed slice physiology with support from A.B. R.D.P. constructed the AAV1-fs-HA-NR1 viral vector. J.M.A. purified AAV1-fs-HA-NR1. The manuscript was written by L.S.Z. with assistance from J.P.F. and R.D.P.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. Male *Grin1*$^{\Delta/+}$; *Slc6a3-cre* mice were bred with female *Grin1*$^{loxP/loxP}$ mice. All mice were on the C57BL/6 background (>10 generations backcrossed). Knockout (*Grin1*$^{\Delta/loxP}$; *Slc6a3-cre*) and control (*Grin1*$^{loxP/+}$; *Slc6a3-cre*) mice were identified by PCR of tail DNA as described previously[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 used for *in vivo* electrophysiology recordings. All procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Tetrode recordings. Four-tetrode microdrives (Neuralynx) were implanted in anesthetized mice using stereotaxic coordinates for the VTA (3.5 mm anterior-posterior, 0.5 mm medial-lateral and 4.5 mm dorsal-ventral). Stereotaxic coordinates for the anterior-posterior plane were normalized using a correction factor (F = Bregma-Lambda distance/4.21). Mice were connected through an HS-16 headstage preamplifier to an ERP27 patch panel 2 weeks after surgery, 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 dopamine neurons were identified by sensitivity to quinpirole (Sigma, 0.2 mg ml^{-1}, intraperitoneal, >70% inhibition of baseline frequency). Baseline dopamine 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 (MathWorks). Baseline activity was used to calculate burst sets (burst onset, interspike interval of ≤ 80 ms; burst offset, interspike interval of ≥160 ms), burst set rate (burst sets per s), burst duration and firing frequency (total spikes per s).

Tail pinch analysis. Peri-event time histograms (200-ms bins) were generated for ten 5-s tail pinches presented with an average intertrial interval of 1 min using MATLAB. We chose tail pinch as the aversive stimulus, as opposed to foot shock, because the electrical interference generated by shock is difficult to eliminate in freely moving mice. The proportion of putative dopamine neurons that we found to be activated by tail pinch was similar to that previously described for foot shock 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, blind to genotype, using padded hemostats. The time at which the pinch was delivered was flagged by an independent investigator during the recording session. Although 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 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 ten trials, followed by Wilcoxon signed-rank test for significance (GraphPad Prism). Data were normalized by calculating the Z score for 200-ms bins ((Bin_x – <baseline>)/s.d.) and smoothed using a three-bin sliding average.

Startle response apparatus. Sound-attenuating startle chambers (SR-Lab, San Diego Instruments) were used to measure PPI, startle responses and fear-potentiated startle. For startle responses, sixty-five 1-ms readings were taken, starting at pulse onset. To measure the response to foot shock, five hundred 1-ms readings were taken, starting at shock onset. The peak amplitude of the response was used to calculate PPI, 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. Sound levels were measured in decibels (A scale) using a sound-level reader (RadioShack). A calibration unit was used to ensure the integrity of the startle response readings (San Diego Instruments). An 8-W 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 seven trials with escalating sound levels: from 80 to 120 dB, with an intertrial interval of 30 s. This series was presented ten times for a total of 70 trials. In all trials, except for null trials in which there was no sound, the sound pulse was 40 ms.

PPI. Animals were given a 10-min habituation period after which subjects were presented with five 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-ms duration prepulse of 70-, 75- or 80-dB intensity, which preceded the 40-ms, 120-dB pulse by 100 ms. PPI was calculated for each prepulse level using the following formula: percentage of 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-plus maze (Med Associates), time spent in these arms, as well as distance traveled, was monitored for 10 min 1–3 d before foot shock conditioning and 1–3 d following conditioning using a video acquisition system (Canopus MediaCruise). Data were analyzed using Ethovision software (Noldus Information Technology).

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 1–3 d before footshock conditioning and 1–3 d following conditioning using a video acquisition system as above. Mice were tested in 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 pseudorandomly 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: percentage 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 three HA tag sequences preceded by a *loxP*-flanked SV40 late polyadenylation site and seven 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 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 (anterior-posterior, 3.25 mm; medial-lateral, 0.5 mm; dorsal-ventral, 4.5 mm)[37]. A lambda-bregma correction factor described above was used to facilitate appropriate targeting. The injection needle (32 GA) was inserted 5 mm dorsal-ventral and drawn back to 4.5 mm to create an injection pocket. 0.5 µl of virus (~4×10^9 particles per µl^{-1}) was injected at a rate of 0.25 µl min^{-1}. 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 d.

Slice electrophysiology. VTA slices were obtained from halothane-anesthetized male mice that ranged in age from postnatal day 25 to 35. Horizontal slices were prepared (190 mm) in ice-cold low Ca^{2+} artificial cerebrospinal fluid (ACSF, 126 mM NaCl, 1.6 mM KCl, 1.2 NaH_2PO_4 mM, 1.2 $MgCl_2$ mM, 2.5 $CaCl_2$ mM, 18 $NaHCO_3$ mM and 11 mM glucose) and subsequently equilibriated at 31–34 °C for at least 1 h. All solutions were bubbled with 95% O_2/5% CO_2 and perfused over the slice at a rate of 2.5 ml min^{-1}. Whole-cell recordings were performed using an Axopatch 1D amplifier (Axon Instruments). EPSCs recordings were acquired using electrodes (2–6 MU) contained in 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 $GABA_A$ receptor–mediated inhibitory postsynaptic currents.

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(−)-2-amino-5-phosphonovaleric acid(AMPAR only) from that recorded in its absence.

Immunohistochemistry. Proteins were detected with primary antibodies to tyrosine hydroxylase (rabbit polyclonal antibody 1:1,000, Millipore) and hemagglutinin (polyclonal antibody 1:1,000; Applied Biological Materials). Primary antibodies were detected using CY2- or CY3-conjugated, goat antibody to mouse and goat antibody to rabbit antibodies (1:200, Jackson Immunolabs). 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 tyrosine hydroxylase–positive neurons were scored for overlapping protein expression using merged images.

Statistical analyses. Statistical analysis was performed using GraphPad Prism (GraphPad Software). Repeated-measures ANOVA was used for multivariate analysis and Bonferroni post-tests were performed where appropriate. Student's *t* tests were performed using two-tailed distribution.

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