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Stress and CRF gate neural activation of BDNF in the mesolimbic reward pathway

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Mechanisms controlling release of brain-derived neurotrophic factor (BDNF) in the mesolimbic dopamine reward pathway remain unknown. We report that phasic optogenetic activation of this pathway increases BDNF amounts in the nucleus accumbens (NAc) of socially stressed mice but not of stress-naive mice. This stress gating of BDNF signaling is mediated by corticotrophin-releasing factor (CRF) acting in the NAc. These results unravel a stress context–detecting function of the brain's mesolimbic circuit.

BDNF has numerous important functions in the developing and mature brain[1,2]. Among these is an essential role in the mesolimbic dopamine reward pathway in mediating responses to severe social stress[3]. BDNF activity is upregulated by chronic social-defeat stress in the NAc[4,5], a key component of the mesolimbic pathway that receives projections from ventral tegmental area (VTA) dopaminergic (DA) neurons[6–8]. Deletion of *Bdnf* in this VTA-NAc pathway (NAc-projecting VTA neurons) of adult mice reduces susceptibility to the deleterious effects of stress and induces antidepressant-like responses[4,5]. Conversely, microinjection of BDNF into the NAc increases stress susceptibility[5].

Release of BDNF from neuronal processes depends on neural activity[1,9]. VTA DA neurons display two types of firing patterns *in vivo*: single-spike, tonic firing and high-frequency, phasic firing[6,10,11]. Phasic firing of VTA DA neurons is essential for reward behaviors[10,12]. Previous work revealed that chronic social-defeat stress increases firing rate of VTA DA neurons in susceptible animals but not in resilient animals[5,11]. Furthermore, optogenetically mimicking this increased phasic firing in the VTA-NAc pathway of mice exposed to subthreshold stress induces reduced social interaction[13]. Here, using the same *in vivo* model, we combined retrograde viral vectors, pseudorabies virus vector expressing Cre recombinase (PRV-Cre) and adeno-associated virus vector expressing channelrhodopsin-2 fused with eYFP from a double *loxP*-flanked (DIO) Cre recombinase–dependent locus (AAV-DIO-ChR2-eYFP) to specifically express ChR2 in VTA-NAc neurons to explore the relationship of firing patterns and BDNF regulation in this pathway.

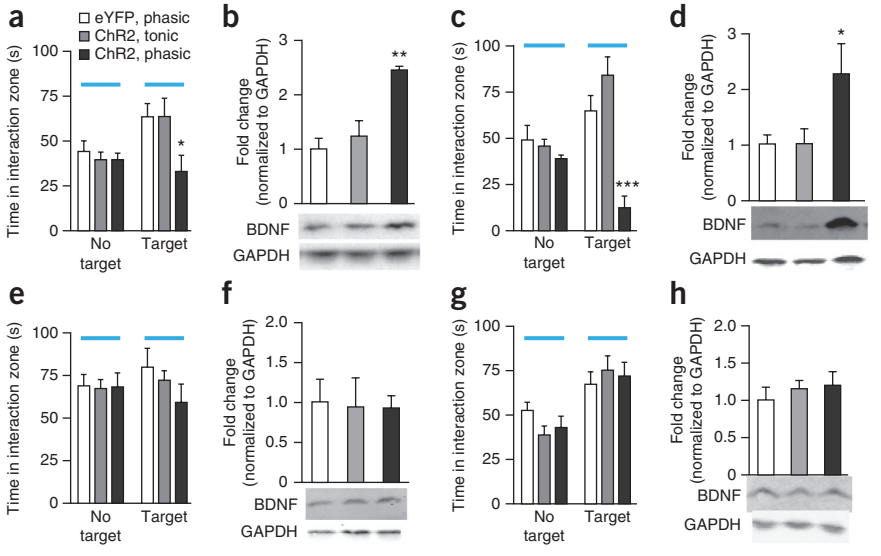

Figure 1 Optogenetic phasic activation of VTA-NAc pathway increases BDNF amounts in the NAc of socially stressed mice but not of stress-naive mice. (**a**) Duration of social interaction during tonic and phasic stimulation of VTA-NAc cells ($F_{2,20} = 4.56$, $^{*}P = 0.0231$, $n = 7,7,9$ mice). eYFP and ChR2 represent control and AAV-DIO-ChR2-eYFP vectors, respectively. 'No target' and 'target' denote absence and presence, respectively, of a social target (CD1 mouse) during behavior. Blue bars represent activation with light during social interaction. (**b**) NAc BDNF amounts (top) and western blot gels (bottom) 24 h after stimulation ($F_{2,19} = 14.55$, $^{**}P = 0.0058$, $n = 7,7,8$ mice). (**c**) Durations of social interaction of Th^{cre} mice during tonic and phasic stimulation of VTA-NAc DA neurons ($F_{2,15} = 19.35$, $^{***}P < 0.0001$, $n = 6,6,6$ mice). (**d**) NAc BDNF amounts in Th^{cre} mice 24 h after stimulation ($F_{2,15} = 3.69$, $^{*}P = 0.0497$, $n = 6,6,6$ mice). (**e**) Duration of social interaction of stress-naive mice during tonic or phasic stimulation ($F_{2,18} = 1.12$, $P = 0.3473$, $n = 6,7,8$ animals). (**f**) NAc BDNF amounts in stress-naive mice 24 h after stimulation ($F_{2,18} = 0.03$, $P = 0.9730$, $n = 6,7,8$ mice). (**g**) Duration of social interaction of stress-naive mice after 5 d of 20 min/day repeated stimulation ($F_{2,24} = 0.30$, $P = 0.7437$, $n = 9,10,8$ mice). (**h**) NAc BDNF amounts 24 h after social interaction ($F_{2,21} = 0.38$, $P = 0.6883$, $n = 8,8,8$ mice). Full-length blots are available in **Supplementary Figure 10**. All bars represent mean ± s.e.m. *P* values were calculated using one-way analysis of variance (ANOVA).

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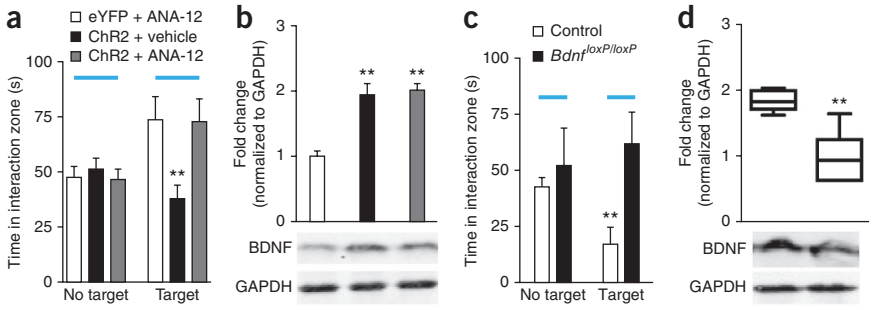

Figure 2 BDNF is necessary for the optogenetically induced susceptible phenotype. (**a**) Duration of social interaction of mice that received intra-NAc infusions of TrkB inhibitor ANA-12 with phasic stimulation, as in **Figure 1a** ($F_{2,23} = 5.59$, $^{**}P = 0.0105$, $n = 6,10,10$ mice). (**b**) NAc BDNF amounts (top) and western blot gels (bottom) after infusions of ANA-12 and phasic stimulation ($F_{2,22} = 16.18$, $^{**}P = 0.0204$, $n = 8,9,8$ mice). *P* values were calculated using one-way analysis of variance (ANOVA) (**a**,**b**). (**c**) Duration of social interaction during phasic stimulation of *Bdnf*$^{loxP/loxP}$ compared to controls (unpaired *t*-test, $t_{11} = 2.9$, $^{**}P = 0.0154$, $n = 6,7$ mice). (**d**) NAc BDNF amounts (top) and western blot gels (bottom) 24 h after stimulation (unpaired *t*-test, $t_9 = 4.78$, $^{**}P = 0.0010$, $n = 5,6$ mice). Bars in **a**–**c** represent mean ± s.e.m. In **d**, center line shows the median; box limits indicate s.e.m.; whiskers indicate minimum and maximum values.

To specifically express ChR2 in VTA-NAc neurons, we injected PRV-Cre into the NAc and AAV-DIO-ChR2-eYFP into the VTA (**Supplementary Fig. 1**). Optical activation of VTA cell bodies in this pathway with a phasic but not tonic stimulation, after a subthreshold social stress[5,13], rapidly induced reduced social interaction (**Fig. 1a** and **Supplementary Fig. 2**). Consistent with the behavior, phasic stimulation increased NAc BDNF amounts 24 h after stimulation and a social-interaction test (**Fig. 1b**), the time point when BDNF was previously upregulated by chronic social defeat[4,5]. In contrast, subthreshold social stress without stimulation had no effect on social behavior[13] or the amount of BDNF (**Supplementary Fig. 3a**,**b**) compared to a stress-naive control. To selectively target VTA DA neurons that project to the NAc, we injected a Cre recombinase–inducible AAV vector that expresses ChR2 fused with eYFP (AAV2/5-DIO-ChR2), which undergoes retrograde transport, into the NAc of tyrosine hydroylase (*Th*^{cre}) transgenic mice (**Supplementary Fig. 4a**). Validation confirmed the viability of using AAV2/5-DIO-ChR2 to express functional ChR2 in VTA-NAc DA neurons (**Supplementary Fig. 4b**,**c**). Consistent with our initial findings, phasic activation of VTA-NAc DA neurons induced social avoidance (**Fig. 1c**) and an increase in NAc BDNF amounts 24 h after stimulation (**Fig. 1d**). These findings demonstrate firing pattern–dependent regulation of BDNF in the VTA-NAc circuit, mediated specifically by DA neurons. Furthermore, this DA neuron–dominated effect on BDNF regulation in this pathway is consistent with our previous observation that the vast majority (95%) of ChR2-expressing VTA-NAc neurons express tyrosine hydroylase[13].

Mesolimbic DA neurons respond to rewarding and aversive stimuli, and can mediate divergent behavioral outputs[5,6,8,11,14–16]. This suggests a possible context-specific role of this circuit in encoding behaviors. Thus, we next tested whether the phasic stimulation–induced increase in NAc BDNF amounts was contextually dependent on stress. We repeated our first experiments in stress-naive mice (**Supplementary Fig. 5a**). Consistent with previous work[13], optical stimulation of VTA-NAc neurons of stress-naive mice had no effect on social interaction behavior (**Fig. 1e**) or NAc BDNF amounts (**Fig. 1f**), suggesting that phasic stimulation alone is not sufficient to induce this adaptation and its behavioral sequelae. These data highlight that the context of a stress combined with phasic firing of mesolimbic DA neurons is critical for these pathological adaptations. We next determined whether repeated stimulation of these neurons (20 min each day for 5 d) is sufficient to produce an effect (**Supplementary Fig. 5b**) and found no changes in social interaction (**Fig. 1g**) or NAc BDNF amounts (**Fig. 1h**). These findings demonstrate that both contextual stress and phasic activation of the VTA-NAc pathway are required to induce BDNF signaling in the NAc.

To determine whether increased NAc BDNF amounts have a causal role in the optogenetically induced avoidance behavior, we blocked the BDNF receptor TrkB using the TrkB antagonist ANA-12 (1 μg)[17], administered into the NAc 1 h before the optical stimulation and social interaction (**Supplementary Fig. 6a**). ANA-12 completely blocked the ability of phasic stimulation to induce social avoidance (**Fig. 2a**), without affecting BDNF amounts (**Fig. 2b**). Furthermore, we performed similar experiments in *Bdnf*$^{loxP/loxP}$ mice[4] (**Supplementary Fig. 6b**), where *Bdnf* was deleted selectively in the VTA-NAc pathway

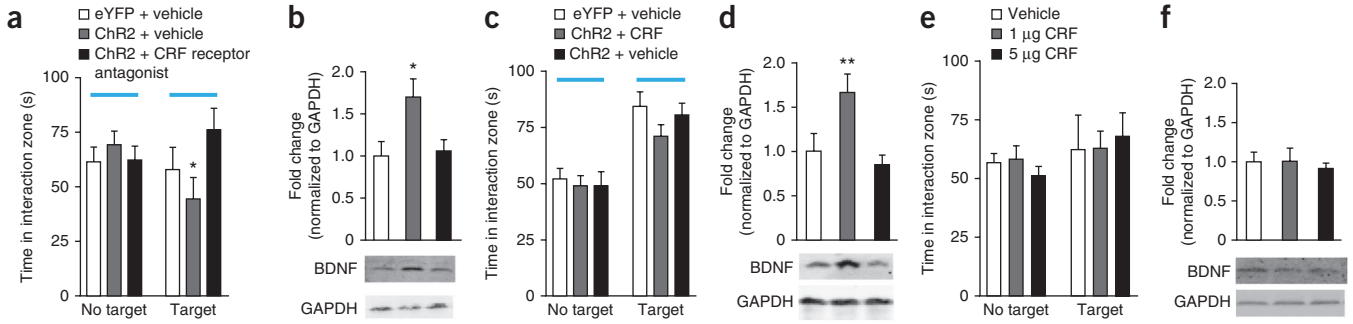

Figure 3 CRF is required for phasic firing induced increase in the NAc BDNF in the context of stress. (**a**) Duration of social interaction of stressed mice with infusion of intra-NAc CRF receptor antagonist (alpha-helical CRF) or vehicle 1 h before behavior with phasic stimulation of VTA-NAc neurons, as in **Figure 1a** ($F_{2,21} = 3.99$, $^{*}P = 0.0348$, $n = 6,8,9$ mice). (**b**) NAc BDNF amounts (top) and western blot gels (bottom) 24 h after stimulation ($F_{2,18} = 4.66$, $^{*}P = 0.0234$, $n = 6,7,8$ mice). (**c**) Duration of social interaction of stress-naive mice that received intra-NAc CRF infusions or vehicle 1 h before behavior and phasic stimulation ($F_{2,23} = 0.58$, $P = 0.5674$, $n = 8,9,9$ mice). (**d**) NAc BDNF amounts (top) and western blot gels (bottom) 24 h after stimulation ($F_{2,19} = 5.88$, $^{**}P = 0.0103$, $n = 6,8,8$ mice). (**e**) Duration of social interaction of stress- and stimulation-naive mice that received intra-NAc CRF or vehicle infusions 1 h before behavior and phasic stimulation ($F_{5,12} = 0.082$, $P = 0.9221$, $n = 5,5,5$ mice). (**f**) NAc BDNF amounts 24 h after stimulation ($F_{2,12} = 0.186$, $P = 0.8329$, $n = 5,5,5$ mice). All bars represent mean ± s.e.m. *P* values were calculated using one-way analysis of variance (ANOVA).

of adult mice, and saw blockade of the optically induced avoidance behavior (**Fig. 2c**) and no increase in NAc BDNF amounts (**Fig. 2d**). Collectively, these results suggest that firing pattern–dependent regulation of BDNF in the VTA-NAc circuit is functionally important in mediating social-avoidance behavior.

We next explored the molecular basis by which stress gates the VTA-NAc circuit. Recent work showed that the stress-induced neuropeptide CRF is functional both presynaptically and postsynaptically in the NAc[18], and increased CRF signaling in the NAc increases motivation for cued rewards[19]. Based on these findings, we investigated whether CRF is the contextual signal required for phasic stimulation of VTA-NAc neurons to induce expression of BDNF and social avoidance. After administering the subthreshold stress protocol (**Supplementary Fig. 2a**), we infused a CRF receptor antagonist (alpha-helical CRF, 1 μg), which blocks both CRF receptors 1 and 2, into the NAc 1 h before phasic stimulation and behavior testing (**Supplementary Fig. 7a**). The intra-NAc infusion of the CRF receptor antagonist effectively blocked the phasic firing–induced social avoidance, compared to vehicle controls (**Fig. 3a**). Mice that received intra-NAc infusions of the CRF receptor antagonist and phasic stimulation did not show changes in NAc BDNF amounts (**Fig. 3b**). Additionally, animals infused with control viral vector AAV-DIO-eYFP into VTA that received intra-NAc infusions of CRF receptor antagonist exhibited no changes in behavior or BDNF amounts (**Supplementary Fig. 7b**,**c**). We next investigated whether intra-NAc infusion of CRF (1 μg) before stimulation modulates BDNF amounts in the absence of stress (**Supplementary Fig. 8a**). In stress-naive mice, phasic stimulation increased NAc BDNF amounts in CRF-infused mice compared to vehicle-infused controls, but infusions of CRF had no effect on social behavior in these social stress-naive mice (**Fig. 3c**,**d** and **Supplementary Fig. 8b**,**c**). Additionally, in the absence of stress or optical stimulation, animals that received CRF infusion alone (1 μg or 5 μg) exhibited no changes in behavior (**Fig. 3e** and **Supplementary Fig. 8d**) or BDNF amounts (**Fig. 3f**). Together, these findings implicate CRF, acting in the NAc, in gating the ability of phasic firing, in the context of stress, to increase BDNF signaling in the NAc (**Supplementary Fig. 9a**).

Similar to the case with conventional neurotransmitters, release of BNDF from axons and dendrites depends on neuronal activity[1,9]. In this *in vivo* study, we demonstrated that neither acute nor 5 d of repeated phasic activation of VTA-NAc neurons induced an increase in BDNF amounts in the NAc of stress-naive mice. Further, BDNF upregulation was blocked in socially stressed and phasically activated mice given an intra-NAc infusion of CRF receptor antagonist. In contrast, the same phasic activation of this pathway induced upregulation of BDNF in the NAc of subthreshold-stressed mice, which was absent in unstimulated mice. This induction of BDNF signaling in the NAc was absent in mice that lacked BDNF selectively in VTA-NAc neurons. These results show that phasic firing alone is not sufficient to induce upregulation of BDNF in this circuit and suggest that mesolimbic DA neurons have a stress context–detecting function, which is mediated by CRF acting in the NAc (**Supplementary Fig. 9b**). These data unravel a gating function of the brain's mesolimbic reward circuitry in producing selective responses to environmental stimuli[20].

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.J.W., A.K.F., H.S., E.A.H., S.M.K, B.J., V.L.B., M.S.M.-R., D.F., S.A.G., J.W.K., D.C. and D.J.C. collected and analyzed data. L.P., J.M.F., S.J.R. and E.J.N. generated and provided viral vectors and Th^{cre} and $Bdnf^{loxP/loxP}$ mice. J.J.W., E.J.N. and M.-H.H. designed the study and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Experimental subjects. Male 7–8-week-old C57BL/6J mice, Th^{cre} mice[7,10], $Bdnf^{loxP/loxP}$ mice (B6129PF2/J background)[4], B6129PF2/J (control for $Bdnf^{loxP/loxP}$ mice) and 6-month-old CD1 retired breeders[4,5,11,13] were kept at 22–25 °C with a 12-h light-dark cycle and fed *ad libitum*. Some of the differences in stress responses are likely due to background differences. All experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committees at Icahn School of Medicine at Mount Sinai. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications[4,5]. A sample size of 5–10 animals is appropriate to see a significant difference both in behavior tests and western blots. A second party blinded the experimenter to the individual groups. Animals were randomized by cage before surgeries. For example, if there were 30 mice in an experiment, with five mice per cage, mice were randomly assigned to be in eYFP or ChR2 groups. Additionally, the order of the mice was randomized before behavioral tests. Experiments with three groups had mice from at least 3–4 litters/group. Experiments with two groups had mice from at least 2 litters/group.

Viral vectors and stereotaxic surgeries for optogenetic approaches. A double *loxP*-flanked (DIO) Cre-dependent adeno-associated virus (AAV) vector expressing channelrhodopsin-2 (ChR2) fused with enhanced yellow fluorescent protein (eYFP) (AAV-DIO-ChR2-eYFP), a replication-defective version of the retrograde traveling pseudorabies virus expressing Cre (PRV-Cre) and a double *loxP*-flanked (DIO) Cre recombinase–dependent AAV2/5 vector that undergoes retrograde transport, expressing ChR2 fused with eYFP (AAV2/5-DIO-ChR2-eYFP), were used in this study for optical activation of the VTA-NAc pathway[13]. AAV-DIO-ChR2 was purchased from University of North Carolina Vector Core, PRV-Cre was engineered by researchers in the laboratory of J.M.F. and AAV2/5-DIO-ChR2-eYFP was purchased from University of Pennsylvania Vector Core. For surgeries, mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and positioned in a small-animal stereotaxic instrument (Kopf Instruments); the skull surface was exposed. 33-gauge syringe needles (Hamilton) were used to bilaterally infuse 0.5 µl of AAV-DIO-ChR2-eYFP or control viral vector AAV-DIO-eYFP at a rate of 0.1 µl/min into VTA (bregma coordinates: anteroposterior, −3.2; mediolateral, −0.5; dorsoventral, −4.6; 7° angle). 0.5 µl of retrograde PRV-Cre or AAV2/5-DIO-ChR2-eYFP was bilaterally infused into NAc (bregma coordinates: anteroposterior, +1.5; mediolateral, +1.6; dorsoventral, −4.4; 10° angle) at a rate of 0.1 µl/min. Optic fibers (ferrules) were implanted into VTA (bregma coordinates: anteroposterior, −3.2; mediolateral, −0.5; dorsoventral, −4.4; 7° angle). For drug infusions, a 26-gauge guide cannula, 3.6 mm in length from the cannula base, was implanted bilaterally into NAc (bregma coordinates: anteroposterior, +1.5; mediolateral, 0; anteroposterior, −3.9; 0° angle. Mice were allowed 4 d of recovery before drug infusions and stimulation.

Microinjections. One hour before the social-interaction test, mice received an intra-NAc infusion of a TrkB antagonist (ANA-12, 1 µg)[17], CRF receptor antagonist (alpha-helical CRF, 1 µg), CRF (1 µg)[18] or vehicle. Drugs were infused through an injector cannula for a total volume of 0.3 µl per hemisphere at a continuous rate of 0.1 µl per min using a microinfusion pump (Harvard Apparatus). Injector cannulae were removed 2 min after infusions were complete, and mice were allowed to sit undisturbed for 1 h before the social-interaction test. All drugs were purchased from Sigma.

Blue-light stimulation. Animals received either tonic or phasic stimulation of the VTA-NAc pathway during the social-interaction test (2.5 min) or chronically (20 min) for 5 d before the behavior test. Mice received either ChR2 (473 nm) or eYFP infusions into VTA and were given either a low-frequency tonic (0.5 Hz) or high-frequency phasic (20 Hz) stimulation, exposing cells to 5 spikes over each 10-s epoch as we previously used[12,13]. These protocols were established based on *ex vivo* studies that showed VTA DA neurons exhibit increased firing in susceptible animals[5] and *in vivo* studies that showed an increase in overall firing as well as burst firing in VTA DA neurons in susceptible animals[11].

Subthreshold social-defeat stress protocol. This is a well-established protocol, which has been used to detect the mechanisms that promote a susceptible, depression-like phenotype[5,13]. Otherwise, these mechanisms are difficult to explore under maximal conditions such as the chronic (10-d) social-defeat model. To measure the molecular and cellular factors that increase social avoidance behavior, we adapted this subthreshold defeat protocol. Under these conditions, male C57BL/6J mice were exposed to a novel CD1 male aggressor for 5 min, followed by 15 min rest in the home cage. Exposure to the CD1 aggressor occurred two times with 15-min intervals between each exposure. Twenty-four hours later, mice were assessed using the social-interaction test described below. This subthreshold defeat protocol does not induce social avoidance in stress-naive mice, but makes the mice more sensitive to additional stress.

Social-interaction test. A social interaction test was performed on the day after administration of the subthreshold defeat protocol as described in our previous work[5,13]. For this test, an open field arena is divided into an interaction zone and two opposing corner zones. A mesh-plastic target box is placed into the interaction zone. A test mouse is allowed to roam around the open field arena for 2.5 min with no social target (CD1 mouse) in the mesh box (denoted as 'no target' in figures showing results of social-interaction experiments). After this, a novel CD1 mouse is placed in a metal mesh-plastic target box in the interaction zone (denoted as 'target' in figures showing results of social-interaction experiments) and the test mouse is placed back into the open arena for another 2.5 min. Using the Ethovision tracking software, the amounts of time spent in the interaction zone and corner zones are measured during phasic or tonic optical stimulation, 2.5 min each with or without social target. Mice that spend less time interacting with the social target and a significantly more time in the corner zones are determined as having social avoidance behavior[4,5]. This social interaction test also provides a measurement of distance traveled as a locomotor activity calculation.

Immunohistochemistry. For immunofluorescence experiments, tissue sections were fixed in 4% (vol/vol) paraformaldehyde in PBS. Perfused brains were kept in 10% formalin at 4 °C for 24 h, then transferred to 30% sucrose for 24 h and finally were transferred to PBS and sliced into coronal sections using a vibrating blade microtome (Leica Microsystems, model VT1000S). The tissue was blocked in PBS-T (0.3% Triton X-100) including 2% BSA (Sigma) and then exposed overnight to antibodies against GFP (Invitrogen, A-11122; 1:2,000) and tyrosine hydroxylase (Sigma, T1299; 1:5,000). The antibodies were labeled with donkey anti-rabbit Cy2 (Jackson Immunolab, 711-225-152; 1:500) and donkey anti-mouse Cy3 (Jackson Immunolab, 715-165-150; 1:500), respectively. Tissue was mounted with antifade solution, including DAPI (VectaShield; Vector Laboratories). Sections were imaged on an LSM 710 confocal microscope (Zeiss).

Western blotting. Bilateral 14-gauge NAc punches were obtained from 1 mm coronal NAc sections from mice 24 h after social interaction test with optical stimulation. Punches were then sonicated (Cole Parmer) in 30 µl of homogenization buffer containing 320 mM sucrose, 5 nM HEPES buffer, 1% (vol/vol) SDS, phosphatase inhibitor cocktails I and II (Sigma), and protease inhibitors (Roche). The concentration of protein was determined using a DC protein assay (Bio-Rad), and 30 µg of total protein was fractionated on a 18% gradient Tris-HCl polyacrylamide gel (Bio-Rad). Samples were then transferred onto a nitrocellulose membrane and then blocked in Odyssey blocking buffer (Li-Cor) for 1 h for Li-Cor analysis. After blocking, the same membrane was incubated at 4 °C overnight with either antibodies against BDNF (1:5,000, Santa Cruz sc546) to detect truncated BDNF or antibodies against GAPDH (1:10,000, Cell Signaling 2118) in Odyssey blocking buffer. After thorough washing with TBST, blots were incubated for 1 h at room temperature with IRDye 680 secondary antibodies (1:10,000; Li-Cor) in Odyssey blocking buffer. Blots were then scanned using Odyssey Infrared Imaging System (Li-Cor) and quantified using ImageJ (US National Institutes of Health).

Statistics. Normality was formally tested for all experiments. The data met the assumptions of the statistical tests used. Where appropriate, a Bartlett's test for equal variances was performed for the one-way analyses of variance (ANOVAs). The variance was similar between the groups. Few animals were excluded from analysis; however, if they were it was after behavioral tests when mice were killed to collect tissue for protein quantification. If no viral expression was seen, animals

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were excluded. In some cases, western blot data have information for one or two fewer animals compared to the behavioral data. This was due to the number of samples that could be run on a gel. One-way ANOVA was used for analysis in **Figures 1a–h**, **2a**,**b** and **3a–f**, and **Supplementary Figure 8b**,**c** with a *post hoc* Newman-Keuls Multiple Comparison test, when appropriate. Student's *t*-test was used for analysis of **Figures 2c**,**d**, **Supplementary Figures 3a**,**b** and **7b**,**c**. Main and interaction effects were considered significant at $P < 0.05$. All data are expressed as mean ± s.e.m.