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Independent hypothalamic circuits for social and predator fear

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The neural circuits mediating fear to naturalistic threats are poorly understood. We found that functionally independent populations of neurons in the ventromedial hypothalamus (VMH), a region that has been implicated in feeding, sex and aggression, are essential for predator and social fear in mice. Our results establish a critical role for VMH in fear and have implications for selective intervention in pathological fear in humans.

Studies in laboratory animals have routinely used freezing behavior elicited by exposure to cues associated with electric foot shock to study the neural circuits underlying fear. However, evidence suggests that fear behaviors elicited by other types of threat may not depend on these circuits[1]. In particular, c-Fos mapping studies have shown that exposure to a predator or an aggressive conspecific recruits the medial hypothalamus, a region that has been implicated in motivated behaviors such as feeding, sex and aggression[2]. Notably, exposures to predator and aggressive conspecific activate non-overlapping nuclei in the medial hypothalamus, suggesting that predator and social fear may depend on separate circuits[3]. However, it remains unclear whether the different brain regions recruited by foot shock, predator and aggressive conspecific reflect truly independent fear circuits or arise as a result of differences in the behaviors elicited, differences between innate and learned fear, or differences in testing methodology.

We developed a behavioral test in which similar patterns of fear behavior are elicited in mice by exposure to either a predatory rat, an aggressive mouse or an electric foot shock (**Fig. 1a**). The apparatus consisted of two chambers separated by a narrow corridor. Mice were housed in one chamber and, each day, a door was opened to allow brief access to the corridor and second chamber. On the fourth day, the mouse was confined to the second chamber and briefly exposed to a predatory rat, an aggressive conspecific, a foot shock or a fake toy rat. The door was reopened and defensive behaviors (immobility, flight, stretch postures and locomotion) were recorded. On the following day, mice were again allowed free access to the corridor and second chamber in the absence of threat and defensive behaviors were scored as a measure of contextual fear. Mice showed an increase in stretch postures, immobility and flight and a decrease in locomotion following exposure to all threats when compared with their behavior during habituation (**Fig. 1b–d** and **Supplementary Fig. 1**). Exposure to the conditioned context also elicited an increase in stretch postures, immobility and flight and a decrease in locomotion (**Fig. 1b–d** and **Supplementary Fig. 1**), whereas exposure to a toy rat did not elicit increases in stretch postures, immobility or flight, but did result in a decrease in locomotion following acute exposure, suggesting that some of the decreased locomotion to threat is a result of the novelty of the stimulus. These data validate our test as a robust method to examine similar acute and learned fear responses to foot shock, predator and social threat.

To investigate whether distinct neural activation patterns are induced by foot shock, predator and aggressive conspecific under conditions of similar testing methodology and behavior, we performed c-Fos mapping. c-Fos was induced in different brain regions in accordance with previous reports[2,3]. In particular, predator exposure significantly activated (P = 0.045) the dorsomedial division of the VMH (VMHdm), whereas exposure to an aggressive conspecific significantly activated (P = 0.012) the ventrolateral VMH (VMHvl; **Fig. 1e–g**). Neither control mice nor mice exposed to foot shock showed activation in VMH, indicating that the medial hypothalamus is selectively recruited during predator and social fear and that similar fear behaviors recruit different brain circuits. Notably, these data suggest that VMHdm, a region that has been extensively implicated in the control of energy homeostasis and metabolism[4], is involved in predator fear, whereas VMHvl, a region that has been implicated in sexual and aggressive behavior[4–6], is involved in social fear.

To determine whether VMH harbors functionally independent circuits for predator and social fear, we used the hM4D–clozapine-*N*-oxide (CNO) pharmacogenetic neural inhibition tool[7] to rapidly and selectively inhibit neurons in VMHdm and VMHvl in behaving mice. Stable expression of hM4D in VMHdm neurons was achieved by constructing transgenic mice in which *hM4D* was driven by the *Nr5a1* gene promoter[8] (*Nr5a1*::*hM4D-2A-TomatoF*; **Fig. 2a**). Reporter gene expression in the transgenic mice was found in the dorsomedial and central divisions of VMH, in VMH efferents of the supraoptic commisure and in all known VMH target areas, including dorsal periaqueductal gray (dPAG)[9] (**Fig. 2b–d** and **Supplementary Figs. 2** and **3**). hM4D was selectively expressed in Nr5a1-expressing neurons in the transgenic mice (**Fig. 2e–g** and **Supplementary Fig. 4**). *In vitro* patch-clamp electrophysiology confirmed a significant reduction in spontaneous firing and membrane potential in VMHdm neurons in brain slices from transgenic mice (firing rate = −32% ± 6, P = 0.0013, N = 8 recorded neurons from 5 mice; membrane potential = −3.35 mV ± 1.07, P = 0.0074, N = 15 recorded neurons from 6 mice), but not non-transgenic littermates (firing rate = 7.6% ± 25.2, P = 0.78, N = 8 recorded neurons from 5 mice; membrane potential = 0.71 mV ± 0.94, P = 0.47, N = 10 recorded neurons from 5 mice) treated with CNO, a selective agonist of hM4D that is otherwise biologically inert[10] (**Fig. 2h**). Systemic treatment of *Nr5a1*::*hM4D-2A-TomatoF* transgenic mice, but not non-transgenic littermate control mice, with CNO before threat exposure resulted in a significant decrease in defensive behaviors (P < 0.0001) and an increase

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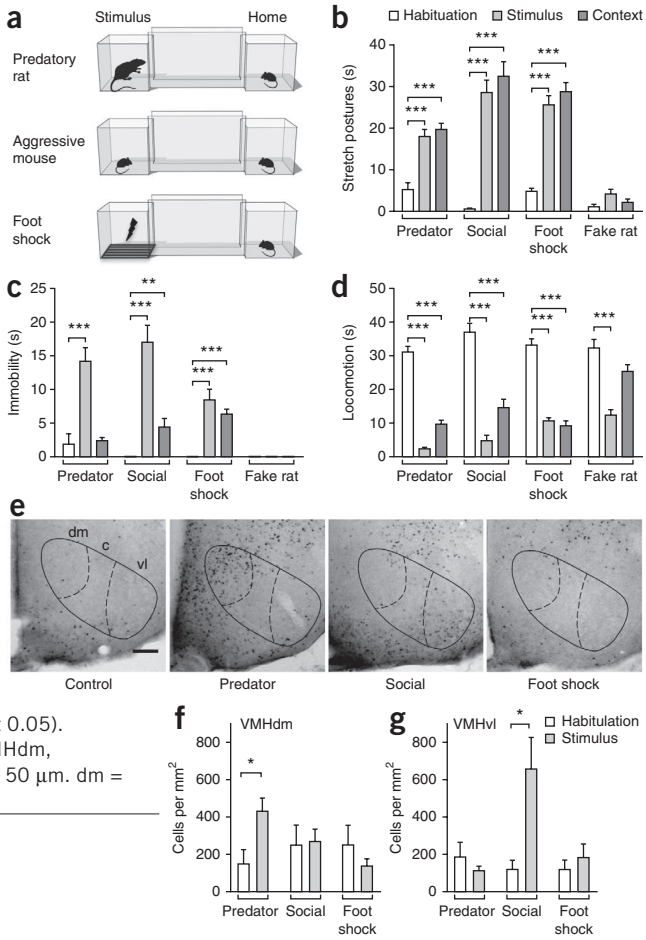

Figure 1 Similar fear behavior elicited in mice by different classes of threat. (**a**) The behavioral testing apparatus consisted of two chambers connected by a narrow corridor. An experimental mouse was continuously housed in one chamber (home) and allowed to freely explore the corridor and second chamber (stimulus) once daily for 20 min. At the end of the free exploration period on the fourth day, the door to the stimulus chamber was briefly closed to confine the mouse, which was then exposed to either a predatory rat (predator, <5 s), aggressive mouse (Social, 10 min), electric foot shock (foot shock, 1 min, 4 × 0.5 s, 0.5 mA) or toy rat (fake rat, <5 s), after which free exploration continued for an additional 10 min. (**b**–**d**) Time spent performing stretch postures (**b**), immobility (**c**) and locomotion (**d**) was measured during the pre-stimulus (habituation) and post-stimulus (stimulus) free exploration periods, as well as on the day following stimulus exposure (context). Stretch postures and immobility were significantly increased after exposure to predator ($N = 15$, $P < 0.0001$), aggressive conspecific ($N = 9$, $P < 0.0001$) and foot shock ($N = 6$, $P < 0.0001$), but not to toy rat ($N = 6$; stretch postures, $P = 0.005$) when compared with the habituation session. Locomotion was significantly decreased after exposure to all stimuli. Re-exposure to the context associated with social, predator and foot shock, but not fake rat (stretch postures, $P = 0.45$; locomotion = 0.042, ns) threats, elicited a significant increase in stretch postures ($P < 0.0001$) and immobility (predator: $P = 0.195$, social: $P = 0.048$, foot shock: $P = 0.0007$) and a decrease in locomotion ($P < 0.0001$). $^{**}P < 0.01$, $^{***}P < 0.001$). (**e**–**g**) Quantification of c-Fos immunohistochemistry in brain sections from mice exposed to predator and aggressive conspecific in the two-chambered apparatus revealed a significant increase in the number of c-Fos cells labeled in VMHdm (predator, $P = 0.045$; social, $P = 0.87$; **f**) and VMHvl (predator, $P = 0.95$; social, $P = 0.012$; **g**), respectively, when compared with the habituation condition (predator, $N = 5$; social, $N = 4$; foot shock, $N = 3$–4; $^{*}P < 0.05$). Negligible neural activation was seen in VMH following foot shock exposure (VMHdm, $P = 0.49$; VMHvl, $P = 0.48$) or in home cage control mice. Scale bar represents 50 μm. dm = dorsomedial, c = central, vl = ventrolateral. Error bars represent s.e.m.

in locomotion ($P = 0.001$) to predator. Similar treatment had no effect on fear behaviors elicited by exposure to aggressive conspecific or foot shock (**Fig. 3a**,**b**). These data indicate that VMHdm has an essential and selective role in the expression of predator fear behavior.

Expression of hM4D in VMHvl neurons was achieved by local infection with adeno-associated virus (AAV) expressing hM4D (AAV-*Syn*::*Venus-2A-hM4D*; **Fig. 3c**,**d** and **Supplementary Figs. 5a–f** and **6a–f**). CNO treatment of AAV-*Syn*::*Venus-2A-hM4D*–infected mice before threat exposure resulted in a significant decrease in defensive behaviors ($P = 0.006$) and an increase in locomotion ($P = 0.02$) to an aggressive conspecific when compared with vehicle-treated controls, but no change in fear behavior was elicited by predator (**Fig. 3e**,**f**). In some cases, expression of hM4D in virally infected mice extended to the VMHdm (**Supplementary Fig. 6b**) and tuberal nucleus (**Supplementary Fig. 6d**) and we cannot completely rule out that inhibition of cells in these nuclei contributed to the behavioral effects seen. The observation that expression in these structures was significantly lower ($P = 0.035$) than in VMHvl (**Supplementary Fig. 6e**) and that this infection was not associated with a reduction in predator fear, suggests that this ectopic expression was not sufficient to modulate fear behavior. Expression outside the VMH was sparse (**Supplementary Fig. 6c**). Notably, CNO treatment did not affect the number of attacks received nor the submissive behavior during the direct encounter with the aggressor

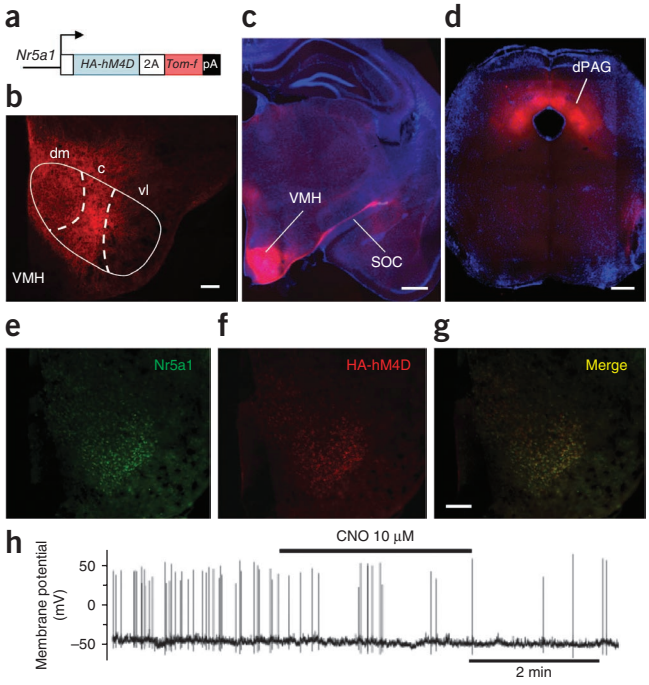

Figure 2 Generation and validation of *Nr5a1*::*hM4D-2A-tomatoF* transgenic mice. (**a**–**d**) Mice carrying a transgene in which the HA-tagged hM4D pharmacogenetic neural inhibition tool and a farnesylated Tomato fluorescent protein (Tom-f) were expressed under the control of the *Nr5a1* gene promoter (**a**) showed expression of Tom-f in the dorsomedial (dm) and central (c), but not ventrolateral (vl) divisions of the VMH (**b**; scale bar represents 100 μm), in the supraoptic commissure (soc; **c**), and in the dPAG (**d**). Scale bars in **c** and **d** represent 500 μm. (**e**–**g**) Double immunofluorescence staining with (**e**) antibody to Nr5a1 (**e**) and HA (**f**) confirmed selective and robust expression of the transgene in Nr5a1-positive cells (**g**). Scale bar represents 100 μm. (**h**) Sample trace from *in vitro* patch-clamp electrophysiological recordings in VMHdm neurons confirmed a reduction of firing rate and membrane potential following CNO treatment in brain slices from *Nr5a1*::*hM4D*-2A-*tomatoF* mice, but not control mice.

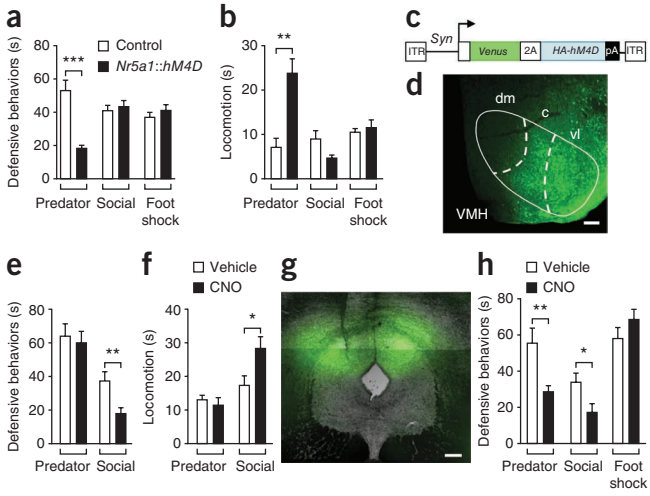

Figure 3 Functional dissociation of fear in VMH and PAG. (**a**,**b**) *Nr5a1*::*hM4D*-2A-*tomatoF* transgenic mice, but not non-transgenic littermates, showed a significant inhibition of cumulative defensive responses (**a**) and an increase of locomotion elicited by exposure to a predatory rat (predator), but not an aggressive conspecific (social) or electric foot shock (foot shock, 4 × 0.5 s, 0.5 mA) (**b**), following systemic administration of CNO (3 mg per kg, intraperitoneal; predator: $N = 7–8$, total defense, $P = 0.0001$; locomotion, $P = 0.001$; social: $N = 7–8$, total defense, $P = 0.72$; locomotion, $P = 0.04$; foot shock: $N = 6–8$, total defense, $P = 0.42$; locomotion, $P = 0.68$). (**c**,**d**) Mice locally infected with an AAV expressing the Venus fluorescent protein and HA-tagged hM4D pharmacogenetic neural inhibition tool (HA-hM4D) under the control of the *synapsin-1* (*Syn1*) gene promoter (**c**) showed expression in the VMHv1 (**d**). Scale bar represents 100 µm. (**e**,**f**) AAV-*Syn*::*Venus*-2A-*hM4D* infected mice showed a significant inhibition of cumulative defensive responses (**e**) and an increase of locomotion elicited by exposure to an aggressive conspecific (social), but not predatory rat (predator) (**f**), following systemic administration of CNO when compared with vehicle-treated mice (predator: $N = 17–18$, defensive responses, $P = 0.58$; locomotion, $P = 0.54$; social: $N = 17–19$, defensive responses, $P = 0.006$; locomotion, $P = 0.02$). (**g**,**h**) Mice locally infected with AAV-*Syn*::*Venus*-2A-*hM4D* in the dPAG (**g**) displayed a significant decrease of cumulative defensive responses elicited by exposure to an aggressive conspecific (social) or a predatory rat (predator), but not to an electrical foot shock (foot shock, 4 × 0.5 s, 0.5 mA) (**h**) following systemic administration of CNO when compared with similarly infected vehicle-treated mice (predator: $N = 5–13$, $P = 0.003$; social, $N = 9–10$, $P = 0.031$; foot shock, $N = 13–14$, $P = 0.67$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Scale bar represents 100 µm. Error bars represent s.e.m.

(**Supplementary Fig. 7a**,**b**). These data suggest a double dissociation of VMH circuits supporting fear behavior to predator and social threats.

Finally, we examined whether fear of predator, aggressive conspecific and foot shock were also functionally dissociable at the level of the PAG, a downstream structure that is involved in motor pattern initiation and has been shown to be critical for the expression of fear responses[11]. Both VMHdm and VMHvl projected prominently to the dPAG[9], and CNO-treated mice with local infection of AAV-*Syn*::*Venus-2A-hM4D* (**Fig. 3g**) in dPAG showed significantly reduced predator ($P = 0.003$) and social ($P = 0.031$), but not foot shock, fear when compared with vehicle-treated, similarly infected control mice (**Fig. 3h**). Although infection often included the overlying superior colliculus (**Supplementary Fig. 8**), treatment of mice explicitly infected in superior colliculus with CNO did not result in a change in fear behavior (**Supplementary Fig. 9**). Although CNO treatment did not affect the number of attacks received, a decrease in submissive behavior was observed during the direct encounter with the aggressor (**Supplementary Fig. 10a**,**b**), suggesting that dPAG is involved in supporting passive defensive behaviors during conspecific encounters. These data indicate that the neural circuits supporting defensive behaviors to distinct threats are also dissociable at the level of downstream motor initiation centers.

Our findings demonstrate that VMH is a multi-modal hub for the control of motivated behaviors and physiological homeostasis. Nr5a1-expressing cells in VMHdm are leptin responsive and essential for supporting metabolic responses to dietary challenge[4], and our data suggest that a link between metabolic regulation and predator fear may occur at the level of the VMHdm. Consistent with an evolutionarily conserved role for VMHdm in fear, electrical stimulation of VMHdm in humans elicits panic attacks[12]. On the other hand, the dual role of VMHvl in aggression[5,6] and social fear suggests that it functions as a key threat processing circuit during social encounters. Our observation that dPAG is critical for predator and social, but not foot shock, fear further supports the existence of independent fear circuits at both the level of fear processing and expression. These data suggest that fear of different classes of threat are processed in distinct circuits and open the possibility for the selective pharmacological blockade of fear. Finally, our data provide, to the best of our knowledge, the first functional dissection of the neural circuits supporting social fear, an important risk factor for mental illness.

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

B.A.S. designed, carried out and analyzed all of the experiments, except for some of the behavioral experiments, which were carried out and analyzed by C.M. and P.K., and the electrophysiology experiments, which were designed, carried out and analyzed by E.M. and D.R. Viruses were produced and tested by A.I. and V.G. The project was conceived by B.A.S. and C.T.G. with critical input from N.S.C. The manuscript was written by B.A.S. and C.T.G. with input from D.R. and N.S.C.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. All mice were derived from local European Molecular Biology Laboratory breeding colonies. Non-transgenic experimental subjects were adult C57BL/6N mice. Predators were adult male SHR/NHsd rats (Harlan). Aggressive conspecifics were adult male CD1 mice selected for elevated aggression as previously described[13]. All animals were housed at 22–25 °C on a 12-h light-dark cycle with water and food *ad libitum*. Males were used for all experiments except for data in **Figure 3a**,**b**, where both males and females were tested. No sex difference in behavioral responses was observed. All animals were handled according to protocols approved by the Italian Ministry of Health (#231/2011-B, #121/2011-A).

Behavioral testing. The experimental apparatus (adapted from ref. 14) was made of clear Plexiglas and composed of similar detachable home and stimulus chambers (25 × 25 × 25 cm) that were connected by an opening (2.0 cm wide, 2.0 cm high) to a narrow corridor (12.5 cm wide, 60 cm long, 30 cm high). Both openings could be closed by a manual sliding door. The experimental subject was continuously housed in the home chamber with access to food and water for the entire test. Each day the home cage was carried from the housing room to the testing room and attached to the apparatus, and the sliding door opened to give the mouse access to the entire apparatus for 20 min (habituation period). In case of foot shock, a metal grid connected to a scrambled electric shock generator (Med Associates) was placed into the stimulus compartment. On day 4, following 10 min of exploration, the experimental mouse was confined to the stimulus compartment by closing the door and either a rat or an aggressive mouse was placed into the stimulus compartment and allowed to interact (rat, <5 s; mouse, 10 min) before the door was re-opened to allow the experimental mouse to escape. The door was immediately re-closed in the case of the stimulus mouse to prevent escape. In case of foot shock, a scrambled electric current was delivered to the grid over a period of 1 min (0.5 mA every 15 s) before the door was re-opened. To prevent injury to the experimental mouse, the experimenter held the rat during the direct encounter. Defensive behaviors were scored during the first 3 min of free exploration each day and during the first 3 min of the post-stimulus period. CNO (3 mg per kg of body weight, intraperitoneal, in 0.9% saline (wt/vol); Enzo Life Sciences) or vehicle was injected 30 min before the beginning of the test. On day 5, the experimental mouse was given access to the entire apparatus as on the habituation days. Between each subject the apparatus was cleaned first with 50% ethanol (vol/vol) and then detergent and the bedding was changed. The apparatus was washed in an automatic cage washer between testing days to eliminate odors. All the testing was performed during the dark phase under red light illumination (40 W).

Mice were naive to the testing apparatus except in the case of the data shown in **Figure 3e**,**f**,**h**, where predator- and foot shock–exposed mice had been previously tested. In these cases, pseudo-randomization of drug treatment groups was performed and no influence of multiple drug treatment was observed. Behavior was scored from videotape using Observer software (Noldus) by an experimenter blind to genotype and treatment. Behaviors were scored as follows: immobility, subject motionless; stretch postures, body stretched forward without movement or mouse moving slowly toward stimulus compartment in an elongated posture; flight, subject quickly running toward home cage; locomotion, ambulatory movement not characterized by stretch posture. Defensive behavior was the sum of stretch postures and immobility. In the case of mice tested for social fear, the number of biting attacks received and the time spent in an upright/submissive posture during the direct encounter was also scored. In two cases, the experimental mouse performed a pronounced number of attacks toward the intruder (>70% of all attacks) and was excluded from the analysis.

Statistical analysis. Data analysis was performed with PRISM software (GraphPad). All data are reported as mean ± s.e. measurement. Statistical significance was determined by repeated-measures ANOVA with behavior during habituation, stimulus and context considered as repeated measures coupled to Bonferroni *post hoc* analysis in case of significance (**Fig. 1b–d** and **Supplementary Fig. 1**), two-way ANOVA (**Fig. 1f**,**g**), *t*-test (**Figs. 2** and **3**, and **Supplementary Figs. 5e**, **6**, **8** and **9**) or MANCOVA[15] followed by pairwise correlation analysis (**Supplementary Fig. 5**). In the *in vitro* electrophysiology experiments, the changes from baseline in firing rate and membrane potential were calculated by *t* test. No statistical methods were used to predetermine group sizes. The sample sizes that we chose are similar to those used in previous publications.

c-Fos immunohistochemistry. 90 min after exposure to the stimulus (predator, conspecific or foot shock), the experimental mouse was deeply anesthetized with Avertin (Sigma-Aldrich), perfused trans-cardially (4.0% paraformaldehyde (wt/vol), 0.1 M phosphate buffer, pH 7.4), and the brain was removed, postfixed (4% paraformaldehyde overnight) and cryoprotected (20% sucrose (wt/vol), PBS, 4 C, overnight). The brains were frozen and 40-µm coronal sections were cut with a sliding cryostat (Leica Microsystems) and processed for immunohistochemistry with rabbit antibody to Fos (1:20,000, Ab-5, Calbiochem). The primary antiserum was localized using a variation of the avidin-biotin complex system (Vector Laboratories)[16]. In brief, sections were incubated for 90 min at 22–25 °C in a solution of biotinylated goat antibody to rabbit IgG (PK-6101, Vector Laboratories) and then placed in the mixed avidin-biotin horseradish peroxidase complex solution (ABC Elite Kit, Vector Laboratories) for the same period of time. The peroxidase complex was visualized by a 5-min exposure to chromogen solution (0.05% 3,30-diaminobenzidine tetrahydrochloride (wt/vol, Sigma-Aldrich), 0.4 mg ml^{-1} nickel ammonium sulfate, 6 µg ml^{-1} glucose oxidase (Sigma-Aldrich), 0.4 mg ml^{-1} ammonium chloride in PBS) followed by incubation in the same solution with 2 mg ml^{-1} glucose to produce a blue-black product. The reaction was stopped by extensive washing in PBS. Sections were dehydrated and coverslipped with quick mounting medium (Eukitt, Fluka Analytical).

Fluorescent protein detection. Mice were trans-cardially perfused (4.0% paraformaldehyde, 0.1 M phosphate buffer, pH 7.4) and brains were removed and left overnight in fixative. Coronal sections (70 µm) were cut on a vibratome (Leica Microsystems). All sections were imaged for Venus, TomatoF and DAPI fluorescence with a motorized wide-field microscope (Leica Microsystems).

Double immunostaining. Mice were perfused trans-cardially (4.0% paraformaldehyde, 0.1 M phosphate buffer, pH 7.4) and brains were removed, postfixed (4% paraformaldehyde overnight) and cryoprotected (20% sucrose, PBS, 4 C, overnight). The brains were frozen and 40-µm coronal sections were cut with a sliding cryostat (Leica Microsystems) and processed for immunohistochemistry with antibody to HA raised in rat (1:200, 11867423001, Roche) and antibody to Nr5a1 in rabbit (1:200, K0611, Trans Genic). Before incubation with primary antibodies, the sections were boiled for 10 min in citrate buffer (10 mM) and incubated with blocking solution (1% BSA, 5% NGS in PBS and 0.4% Triton X-100; vol/vol) for 1 h. Primary antibodies were detected with fluorescent-labeled secondary antibodies (1:800, Alexa Fluor 488 goat antibody to rabbit, A-11034; 1:800, Alexa Fluor 647 goat antibody to rat, A-21248; Invitrogen).

Generation of transgenic mice. Recombineering was used to insert a HA-hM4D-2A-TomF-FRT-kan/neo-FRT cassette replacing the translational start of the *Nr5a1* gene in a bacterial artificial chromosome (BAC) clone (RP23-225F7, CHORI-BACPAC). The hemagluttinin-tagged hM4D sequence (HA-hM4D) was excised from pcDNA-5FRT-HA-hM4D (a gift from B. Roth, University of North Carolina). A farnesylation domain (KLNPPDESGPGCMSCKCVLS[17]) was added to the C terminus of the Tomato open reading frame and the viral P2A[18] sequence was inserted between hM4D and TomatoF to produce separate peptides from a single open-reading frame. Modified BAC DNA was prepared (Large-Construct kit, Qiagen), diluted in injection buffer (30 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 100 mM NaCl), and microinjected into the pronucleus of fertilized one-cell stage B6x(B6xD2) embryos. One of two founders showed stronger reporter gene expression was used in all studies and backcrossed to C57BL/6N. Transgenic mice were genotyped by PCR (forward: 5′-CAATCCAGCTGTGTGCCCTACTTCGCC-3′, reverse: 5′-GGCCATAGCGTAATCTGGAACATCGTATGGGTA-3′).

***In vitro* electrophysiology.** Coronal slices (250 µm) containing the VMH were cut at 4 °C using a vibratome (DSK, Dosaka EM) from brains incubated for 5–10 min in ice-cold oxygenated modified artificial cerebrospinal fluid (ACSF; 3 mM KCl, 2 mM $MgCl_2$, 1.6 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 2 mM $MgSO_4$, 26 mM $NaHCO_3$, 10 mM glucose, 200 mM sucrose) extracted from transgenic and control littermates that were anesthetized with halothane and decapitated. Slices were maintained for at least 1 h at 22–25 °C in oxygenated (95%/5% CO_2) ACSF (125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 10 mM glucose, pH 7.35). Recordings were performed at 22–25 °C in ACSF perfused at a rate of ~1.5 ml min^{-1}. CNO (10 µM) was applied to the slice by bath perfusion for 3 min. Whole-cell patch-clamp

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recordings in current clamp configuration were performed using borosilicate glass pipettes (3–5 MΩ) filled with 140 mM potassium gluconate, 2 mM $MgCl_2$, 5 mM BAPTA, 10 mM HEPES, 2 mM MgATP, 0.4 mM NaGTP, and pH corrected with KOH to pH 7.32. Recordings were performed using an Axopatch 200A amplifier (Molecular Devices); signal was low-pass filtered at 2 kHz, collected at 10 kHz using Clampex10 (Molecular Devices), and analyzed off-line with Clampfit10 software (Molecular Devices). In some experiments 10–50 pA of current were injected to induce firing. Recordings were discarded if membrane potential and/or firing rate were unstable. To determine changes in membrane potential, signals were digitized at 1 Hz and firing frequency was monitored using 30-s duration bins. In both cases, CNO response was assessed 4 min following the start of drug application.

Viral production. The Venus-P2A-HA-hM4D cassette was cloned so as to replace the open reading frame of pAAV-Syn-NpHR3.0-EYFP-WPRE (a gift from K. Deisseroth, Stanford University). Production and purification of recombinant AAV (chimeric capsid serotype 1/2) were as described[19]. Viral titers (>10^{10} genomic copies per µl) were determined with QuickTiter AAV Quantitation Kit (Cell Biolabs) and RT-PCR as previously described[20].

Stereotaxic viral injections. Bilateral injection of AAV aimed at the VMHvl (posterior, −0.95 mm; depth, −5.75 mm; lateral, ±0.65 mm to bregma; coordinates empirically adapted from ref. 21) or dPAG (posterior, −3.8 mm; depth, −2.3 mm lateral ±1.0; angle, 26 degrees) was performed using a glass pipette (intraMARK, 10-20 µm tip diameter, Blaubrand) connected to a syringe and a stereotaxic micromanipulator (Kopf Instruments) in deeply anesthetized mice (Ketavet, ketamine 100 mg per kg, xylazine 10 mg per kg, Intervet). We injected 0.3 µl of AAV-containing solution per side in VMHvl and 0.1 µl per side in dPAG. Behavioral experiments were performed 3–4 weeks after surgery.

Quantification of viral infection. The location of viral infection was determined in all mice injected with AAV. The mice were trans-cardially perfused with 4% paraformaldehyde within 3 d of behavioral testing. The exact position of the brain nucleus of interest was determined by overlaying a reference atlas grid[21] using white matter landmarks on the bright field fluorescent image. Venus signal was thresholded and quantified (ImageJ) and infection efficiency (either % area or total area) was calculated over the total area of the nucleus as determined from the atlas overlay. 15 dPAG-infected mice that showed less than 10% infection of the target area were excluded from the behavior analysis. No VMHvl-infected mice were excluded from the behavior analysis.

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