A hypothalamic circuit for the circadian control of aggression

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'Sundowning' in dementia and Alzheimer's disease is characterized by early-evening agitation and aggression. While such periodicity suggests a circadian origin, whether the circadian clock directly regulates aggressive behavior is unknown. We hypothesize that a daily rhythm in aggression propensity in male mice is gated by GABAergic subparaventricular zone (SPZGABA) neurons, the major postsynaptic targets of the central circadian clock, the suprachiasmatic nucleus. Optogenetic mapping revealed that SPZGABA neurons receive input from vasoactive intestinal polypeptide suprachiasmatic nucleus neurons and innervate neurons in the ventrolateral part of the ventromedial hypothalamus (VMH), which is known to regulate aggression. Additionally, VMH-projecting dorsal SPZ neurons are more active during early day than early night, and acute chemogenetic inhibition of SPZGABA transmission phase-dependently increases aggression. Finally, SPZGABA-recipient central VMH neurons directly innervate ventrolateral VMH neurons, and activation of this intra-VMH circuit drove attack behavior. Altogether, we reveal a functional polysynaptic circuit by which the suprachiasmatic nucleus clock regulates aggression.
Fig. 1 | Aggression follows a daily rhythm in mice that is directly regulated by SPZGABA neurons. 

**a**, Fluorescent immunohistochemistry for VIP (turquoise) and AVP (red) delineating the mouse SPZ (white ovals). Representative of 3 mice. 3V, third ventricle. **b**, GFP-injected controls show a rhythm in aggression propensity with highest total time attacking at ZT13 and lowest at ZT1 (blue; n = 16, two-way repeated-measures ANOVA, interaction: $F_{3,90} = 7.63$, $P = 0.0001$; post hoc: ZT13 vs. ZT1 *$P = 0.0067$). SPZ Vgat-deleted mice (red, $n = 16$) show a disrupted rhythm with increased total time attacking at ZT1 (post hoc: deleted vs. intact *$P = 0.0055$). **c**, Similar rhythms in numbers of attacks were found in controls (blue; $n = 12$, two-way repeated-measures ANOVA, main effect of time: $F_{3,90} = 6.25$, $P = 0.0159$; post hoc: ZT1 vs. ZT13 †$P = 0.0244$), but lost in Vgat-deleted mice (red; $n = 16$, post hoc: ZT1 vs. ZT13 $P = 0.99$). **d**, Attack latency showed a significant interaction effect (two-way repeated-measures ANOVA, $F_{3,90} = 4.98$, $P = 0.0362$) with a trend towards differences at ZT1 and ZT13 in controls (blue; $n = 16$, post hoc, ZT1 vs. ZT13 $P = 0.0605$) and towards decreased latency at ZT1 in Vgat-deleted mice (red; $n = 16$, post hoc: deleted vs. intact $P = 0.0683$). **e**, Heat map showing injection sites across Vgat-deleted mice ($n = 16$), each weighted based on magnitude of difference from controls in total time attacking at ZT1. a.u., arbitrary units; OT, optic tract; F, fornix. **f**, Double-plotted actograms of entrained (12-h:12-h light:dark cycle, LD; yellow and black bars) and free-running (constant darkness, DD) LMA rhythms. **g**, SPZ Vgat-deleted mice (red, $n = 8$) exhibited decreased LMA during the dark period compared to controls (blue; $n = 8$, two-way repeated-measures ANOVA, main effects of SPZ condition: $F_{1,14} = 11.34$, $P = 0.0046$; time: $F_{23,322} = 12.58$, $P = 0.0001$; interaction: $F_{23,322} = 1.61$, $P = 0.0393$, post hoc: ZT12 *$P = 0.0204$, ZT13 *$P = 0.0014$, ZT23 *$P = 0.0066$). Notably, SPZ Vgat-deleted mice did not show increased LMA at ZT1 (turquoise arrow, post hoc: $P = 0.99$). **h**, SPZ Vgat-deleted mice ($n = 6$) also showed lower day–night differences of plasma corticosterone compared to controls ($n = 5$; two-way repeated-measures ANOVA, main effect of time: $F_{13} = 18.02$, $P = 0.0022$, post hoc: deleted ZT1 vs. ZT13 *$P = 0.21$; intact ZT1 vs. ZT13 *$P = 0.005$). Data are shown as mean ± s.e.m.; ns, not significant.
Vgat-deleted mice and their intact littermates (injected with AAV-VGAT-GFP into the SPZ, which does not delete Vgat), using the resident–intruder model (a well-established assay for territorial aggression in male mice)\(^\text{28,34,35}\), at zeitgeber times (ZT1), ZT7, ZT13, and ZT19 (where ZT0 = lights-on, in a 12-h:12-h light:dark cycle).

We found that aggression propensity in intact male mice toward a male mouse intruder exhibited a daily rhythm, with the highest total time (s) spent engaged in attack behavior at ZT13 and the lowest at ZT1 (ZT13: 62.16 ± 12.52, ZT1: 24.95 ± 7.29, \(P = 0.0067\); Fig. 1b). Intact GFP-fused mice also exhibited the highest number of attacks at ZT13 and the lowest number at ZT1 (ZT13: 19.38 ± 3.72, ZT1: 8.75 ± 2.30, \(P = 0.0244\); Fig. 1c). This aggression propensity rhythm was completely lost in SPZ Vgat-deleted mice (number of attacks, ZT13: 18.63 ± 4.37, ZT1: 16.38 ± 2.21, \(P = 0.99\); Fig. 1c) and was accompanied by a significant increase in total time attacking (s) at ZT1 compared to intact GFP-fused controls (deleted: 65.98 ± 8.13, intact: 24.94 ± 7.29, \(P = 0.0055\); Fig. 1b). Additionally, we found that attack latency (s) was significantly affected (time \(\times\) condition interaction, \(P = 0.0362\), with intact GFP-fused mice exhibiting a trend towards lower latencies at ZT13 compared to ZT1 (ZT13: 158.26 ± 41.32; ZT1: 284.26 ± 57.81, \(P = 0.0065\); Fig. 1d) and SPZ Vgat-deleted mice exhibiting a trend towards decreased latency to attack at ZT13 compared to intact GFP-fused controls (deleted: 142.34 ± 22.95; intact: 284.26 ± 57.81, \(P = 0.0063\); Fig. 1d). Notably, we also found that the lower propensity for aggression during the early resting phase in intact mice was not simply due to the direct effects of light. Specifically, control mice tested under free-running conditions in constant darkness showed similar temporal differences in total time (s) attacking between early subjective day and early subjective night (circadian time (CT): 12.47 ± 7.38; CT13: 27.30 ± 8.23, \(P = 0.0305\); Supplementary Fig. 3), suggesting these rhythms are circadian in nature.

SPZ Vgat deletions also reduced the amplitude of entrained (under a 12-h:12-h light:dark cycle) and free running (in constant darkness) rhythms of locomotor activity (LMA; Table 1 and Fig. 1f,g), as well as the difference in day–night levels of plasma corticosterone (Fig. 1h). In the 12-h light:dark cycle, SPZ Vgat deletions significantly blunted the elevated LMA counts per 5 min observed in control mice during the dark period (Fig. 1g), strongly so at ZT12 (deleted: 4.64 ± 0.66, intact: 7.84 ± 0.86), ZT13 (deleted: 4.61 ± 0.66, intact: 8.48 ± 2.13), and ZT23 (deleted: 3.48 ± 0.47, intact: 6.98 ± 1.53). Blunted elevation of plasma corticosterone levels (ng/mL) at ZT13 as compared to controls was also observed (deleted: 13.82 ± 4.54, intact: 29.24 ± 8.31). Crucially, SPZ Vgat deletions did not significantly increase LMA counts per 5 min (deleted: 1.24 ± 0.24, intact: 1.43 ± 0.26; \(P = 0.99\)) or plasma corticosterone (deleted: 2.61 ± 0.72, intact: 1.98 ± 0.62; \(P = 0.09\)) during the early light period (ZT1) when peak changes in attack behavior were observed, suggesting that the SPZ mediation of aggression propensity is independently regulated from LMA and plasma corticosterone rhythms. We should note that, although in some mice AAV-iCre-2A-Venus transduced cells in the PV (Fig. 1e), a structure extensively implicated in corticosterone regulation\(^\text{41,42}\), the mouse PV contains relatively few Vgat\(^\text{+}\) neurons and hence its function was unlikely to be affected by Cre injections. Interestingly, we did not find any differences in entrained or free-running rhythms of body temperature (Tb; Table 1 and Supplementary Fig. 4), which is consistent with previous work demonstrating the dissociability of individual circadian rhythms\(^\text{43}\).

### Table 1 | SPZ Vgat deletions disrupt entrained and free-running rhythms of LMA, but not body temperature

| SPZ groups | LMA amplitude (counts per 5 min) | LMA Tau (h) | Tb amplitude (°C) | Tb Tau (h) |
|------------|---------------------------------|-------------|-------------------|-----------|
| AAV-iCre-2A-Venus (n = 7) | (LD) 1.99 ± 0.13\(^\text{1}\) | (LD) 24.00 ± 0.12 | (DD) 0.52 ± 0.02 | (LD) 23.83 ± 0.00 |
| (DD) 1.84 ± 0.16\(^\text{1}\) | (DD) 23.95 ± 0.06 | (DD) 0.51 ± 0.02 | (DD) 23.87 ± 0.04 |
| AAV-GFP (n = 7) | (LD) 2.76 ± 0.20\(^\text{1}\) | (LD) 23.93 ± 0.12 | (DD) 0.58 ± 0.03 | (LD) 23.83 ± 0.00 |
| (DD) 2.86 ± 0.25\(^\text{1}\) | (DD) 23.95 ± 0.06 | (DD) 0.56 ± 0.01 | (DD) 23.83 ± 0.00 |

SPZ\(^\text{GABA}\) neurons inhibit VMH neurons and receive input from SCN\(^\text{VIP}\) neurons. Given that the SPZ functions largely as a post-synaptic relay for the circadian clock\(^\text{28,43}\), we sought to define the functional synchronizing circuitry by which the SPZ may control daily rhythms of aggression. We first asked whether SPZ\(^\text{GABA}\) neurons have functional synaptic connections with neurons of the VMH (Fig. 2a), a hypothalamic region known to regulate attack behavior\(^\text{10,12–15}\). To do this, we placed stereotaxically guided injections of an AAV containing the gene for Cre-dependent expression of channelrhodopsin2 (ChR2; AAV-DIO-ChR2-mCherry) into the SPZ of mice engineered to express Cre in VGAT\(^+\) cells (VGat-IRES-Cre mice)\(^\text{26}\). We then optogenetically mapped projections from SPZ\(^\text{GABA}\) neurons (Fig. 2b) by recording light-evoked inhibitory postsynaptic currents (IPSCs) from randomly selected VMH neurons (ChR2-assisted circuit mapping, or CRACM)\(^\text{29,30}\). We detected light-evoked IPSCs, which were completely abolished by the GAB\(_A\)-receptor antagonist bicuculline, in almost all (25 of 28) VMH neurons, confirming functional synaptic connectivity between SPZ\(^\text{GABA}\) and VMH neurons. We next sought to establish functional synaptic connectivity between SCN\(^\text{VIP}\) and VMH-projecting SPZ\(^\text{GABA}\) neurons (SPZ\(^\to\)VMH; Fig. 2c). To do this, we placed stereotoxic injections of a Cre-dependent ChR2 fused with EYFP (AAV-DIO-Chr2-EYFP) into the SCN of VIP-IRES-Cre\(^\text{+}\) mice and a fluorescent retrograde tracer (CtB-555) into the VMH (to label SPZ\(^\to\)VMH neurons) of the same mice. We detected light-evoked GABAergic IPSCs in the majority of SPZ\(^\to\)VMH neurons (5 of 9), confirming functional synaptic connectivity between SCN\(^\text{VIP}\) and SPZ\(^\text{GABA}\) neurons that project to the VMH. Our CRACM results therefore show that SCN\(^\text{VIP}\) neurons provide GABAergic input to SPZ neurons that innervate the VMH and that SPZ\(^\text{GABA}\) neurons subsequently inhibit the VMH. This pathway provides a possible circuit substrate by which the SCN may modulate the activity of VMH neurons, including VMH neurons that regulate aggression.

To obtain a more detailed anatomic understanding of the SPZ\(^\to\)VMH pathway, we injected an AAV coding for Cre-dependent expression of the anterograde tracer humanized Renilla GFP (AAV-FLEX-hrGFP) into the SPZ of Vgat-IRES-Cre mice (Fig. 2d–f). Consistent with the optogenetic mapping experiments described above, we found that SPZ\(^\text{GABA}\) neurons densely innervated the central VMH (VMHc), and, to a lesser extent, the dorsomedial VMH (VMHdm) and VMHvl. Using subtraction methods across mice with different hrGFP injection sites, we found that VGAT\(^+\) neurons within the dSPZ predominately target the VMH, whereas VGAT\(^+\) neurons within the more ventral portions of the SPZ predominately target the dorsomedial hypothalamic nucleus (Supplementary Fig. 5). A similar topographic organization of SPZ...
projections was previously reported in rats\(^\text{23}\). When we constructed a heat map showing the regions of the SPZ in which Vgat-deletion produced maximal disruption of aggression rhythms, the most critical region included the dSPZ, which maximally innervates the VMH (Fig. 1e). We also noted that SPZ GABA projections show ramification and dense synaptic boutons among estrogen receptor 1 (Esr1)-expressing neurons within the VMHvl (Fig. 2f), which have previously been shown to regulate attack behavior in male mice\(^\text{12,14}\).

To examine whether SPZ projections to these cells form GABAergic synaptic connections we performed CRACM experiments using Esr1-2a-Cre mice\(^\text{14}\). Toward this end, Cre-independent ChR2 (AAV-CAG-ChR2-mCherry) was targeted to SPZ neurons and Esr1\(^+\) neurons were transduced with AAV-FLEX-hrGFP for their identification in brain slices (Fig. 2g). We detected light-evoked IPSCs, which were completely abolished by bicuculline, in 10 of 10 Esr1\(^+\) neurons, confirming direct functional synaptic connectivity between the SPZ neurons (which are nearly all GABAergic) and the VMHvl aggression locus.

Dorsal SPZ\(^{\rightarrow}\)VMH neurons are active at ZT1 and acutely inhibiting them increases aggression. To determine whether low levels of aggressive behavior at ZT1 are associated with increased activity of SPZ\(^{\rightarrow}\)VMH neurons, we examined expression of c-Fos, a marker of neuronal excitation. First, we perfused control mice 90 min after ZT1 and found a high number of c-Fos-labeled cells in the dSPZ (Supplementary Fig. 6). Using retrograde tracing with CTb-555, we next showed that these dSPZ neurons specifically project to the VMH (Fig. 3a) and were significantly more active at ZT1 compared to ZT13 (Fig. 3b,c; \(t_{16} = 3.903, P = 0.0013\)), together suggesting that low levels of aggression at ZT1 are gated by this dSPZ\(^{\rightarrow}\)VMH pathway.
Fig. 3 | SPZ<sup>−/−</sup>VMH neurons are more active at ZT1 than ZT13, and chemogenetic inhibition of SPZ<sup>GABA</sup> transmission increases aggression at ZT1 but not ZT13. 

a. Retrograde tracer CTb-555 injected into the VMH (right) labels neurons (red, left and center) in the dSPZ that also show c-Fos expression (turquoise) at ZT1. Representative of 4 mice. 

b. dSPZ neurons are significantly more active at ZT1 (n=9) compared to ZT13 (n=9; unpaired two-tailed t test: t<sub>7</sub> = 3.903, *P= 0.0013). 

c. Sections depicting differences in dSPZ c-Fos expression at ZT1 (left, representative of 9 mice) and ZT13 (right, representative of 9 mice). d. Construct of AAV-FLEX-hGlyR<sup>-</sup>mCherry inhibitory vector, containing a FLEX cassette in reverse orientation and flanked by loxP sites. Cre-recombinase excises these sites, and the cassette flips and locks into the correct orientation, permitting expression of hGlyR<sup>-</sup>mCherry in Cre<sup>+</sup> cells. e. At ZT1, Vgat-IRES-Cre mice (n=8) injected with AAV-FLEX-hGlyR<sup>-</sup>mCherry in the SPZ show increased total time attacking (left; paired two-tailed t tests, t<sub>7</sub> = 4.404, *P= 0.0031), increased number of attacks (center; paired two-tailed t test, t<sub>7</sub> = 4.615, *P= 0.0024), and decreased attack latency (right; paired two-tailed t tests, t<sub>7</sub> = 5.686, **P= 0.0007), following administration of IVM compared to VEH. f. In additional Vgat-IRES-Cre mice (n=8) injected with AAV-FLEX-hGlyR<sup>-</sup>mCherry into the SPZ, IVM administration did not significantly increase aggression propensity compared to VEH at ZT13 (paired two-tailed t tests; time attacking, t<sub>7</sub> = 0.5467, nsP= 0.6016; number of attacks, t<sub>7</sub> = 1.93, nsP= 0.0949; attack latency, t<sub>7</sub> = 1.118, nsP= 0.3005). g. Heat map depicting overlapping injection sites within the SPZ, each weighted according to the magnitude of difference in total time attacking for the IVM condition compared to the mean VEH response (a.u., arbitrary units). h. Vgat-IRES-Cre mice injected with AAV-FLEX-hGlyR<sup>-</sup>mCherry into the SPZ were injected with IVM (n=3) or VEH (n=3) at ZT1 and then perfused 90 min after ZT1 on the following day. Mice receiving IVM showed significantly fewer neurons containing dsRed (a marker for neurons injected with AAV-FLEX-hGlyR<sup>-</sup>mCherry, brown) that expressed c-Fos (black) compared to mice receiving VEH (unpaired two-tailed t test, t<sub>6</sub> = 3.497, *P= 0.025). i. Representative sections depicting decreased dsRed<sup>c-Fos</sup> double-labeling at ZT1 in the dSPZ (center boxes) following IVM (right, representative of 3 mice) compared to VEH (left, representative of 3 mice) administration. Data are shown as mean ± s.e.m.; ns, not significant.
Fig. 4 | VMHc neurons strongly excite VMHvl neurons and drive behavioral aggression. a, Schematic of VMH connectivity. b, Pacap-ires-Cre mice were injected with AAV-DIO-Synaptophysin-mCherry, which expresses the synaptic vesicle protein synaptophysin fused with mCherry in a Cre-dependent fashion. Top: expression of AAV-DIO-Synaptophysin-mCherry in VMHCpacAP neurons and VMHCpacAP terminals. Bottom: expression of AAV-DIO-Synaptophysin-mCherry of VMHCpacAP terminals in VMHvl (left). Very few VMHCpacAP terminals were found in VMHdm (right). Representative of 3 mice. c, Top: schematic shows connections being tested using CRACM. VMHCpacAP neurons in Pacap-ires-Cre mice were transduced with AAV-DIO-ChR2-mCherry. Light-evoked EPSCs were detected in mCherry-negative neurons in the VMHc (representative of 2 mice from different litters) and VMHvl (representative of 2 mice from different litters), but not in the VMHdm (representative of 2 mice from different litters). d, VMHCpacAP neurons in Pacap-ires-Cre mice were bilaterally transduced with AAV-DIO-hM3Dq-mCherry. Representative of 6 mice. e, CNO+hM3Dq-mediated stimulation of VMHCpacAP neurons in Pacap-ires-Cre mice increased total time attacking (n = 6, paired two-tailed t test, t = 3.337, P = 0.0206). f, CNO administration 2 h before perfusion produced a robust c-Fos response in VMHCpacAP neurons expressing AAV-DIO-hM3Dq-mCherry (immunostained for dsRed) as well as in hM3Dq-VMHvl neurons. Representative of 3 mice.

Next, we examined the effects of acutely disrupting SPZ activity on aggression at ZT1 compared to ZT13. To inhibit SPZ neurons, we used a newly developed inhibitory viral vector that employs a mutated human glycine receptor in a Cre-dependent configuration (AAV-FLEX-hGlyR-mCherry; hGlyR-AAV; Fig. 3d). The hGlyR receptor is mutated to respond to the antiparasitic drug ivermectin (IVM) instead of its normal ligand, glycine. We placed small bilateral injections of the hGlyR-AAV into the SPZ of Vgat-IRES-Cre mice and then tested the effects of acute inhibition of SPZGABA neurons on aggression at either ZT1 or ZT13. At ZT1, mice showed significantly higher total time attacking (Fig. 3e; t = 4.404, P = 0.0031), higher numbers of attacks (Fig. 3f; t = 4.615, P = 0.0024), and lower attack latencies (Fig. 3e; t = 5.686, P = 0.0007) after the administration of IVM (5 mg/kg in propylene glycol) compared to vehicle (VEH). In contrast, at ZT13, IVM did not significantly change the total time attacking (Fig. 3f; t = 0.5467, P = 0.6016), number of attacks (Fig. 3f; t = 1.93, P = 0.0949), or attack latency (Fig. 3f; t = 1.118, P = 0.3005) compared to VEH. We also found that this effect was not due to a phase-dependent effect of IVM at ZT1, as control animals did not show increased aggression compared to VEH conditions at this time (Supplementary Fig. 7).

To verify that the hGlyR-AAV and IVM inhibited SPZGABA neurons, we examined c-Fos expression in the dSPZ (which was among the most heavily weighted areas in the heat maps of our injection sites; Fig. 3i). Crucially, we found that Vgat-IRES-Cre mice injected with hGlyR-AAV in the SPZ and receiving IVM showed a significant decrease in the number of c-Fos+ cells at ZT1 in the dSPZ compared to mice receiving VEH (Fig. 3i; t = 3.497, P = 0.0025), indicating that hGlyR-AAV and IVM successfully inhibited dSPZGABA neurons. Although Vgat-IRES-Cre mice as a strain were less aggressive overall than VgatloxP/loxP mice, the acute, reversible neuronal inhibition permitted a within-animal design, which improved the sensitivity of the aggression assay and demonstrated that inhibition of dSPZGABA neurons increased aggression at ZT1, recapitulating the results following chronic disruption of SPZGABA neurotransmission.

An intra-VMH circuit receives circadian input and drives aggression. While SPZGABA neurons project directly to VMHvl neurons, our mapping experiments show that SPZGABA neurons most densely innervate the VMHc (Fig. 2 and Supplementary Fig. 5). Therefore, we asked whether VMHc neurons engage and consequently excite VMHvl neurons, which then drive aggressive behavior (Fig. 4a), since the vast majority of VMHc neurons are glutamatergic (Vglut2+) and hence excitatory. Notably, such a SCN→SPZ→VMHc→VMHvl circuit could thus form a parallel pathway that also regulates daily rhythms of aggression propensity. To investigate this possibility, we used Pacap-ires-Cre mice (PACAP, peptide pituitary adenylate cyclase activation polypeptide), which express Pacap (Adcyap1), and hence Cre-recombinase, in many VMHc neurons, but in many fewer VMHdm or VMHvl neurons. We first assessed axonal projections of PACAP neurons in the VMHc (VMHcPACAP neurons) by stereotaxically injecting the anterograde tracer AAV-DIO-Synaptophysin-mCherry into the VMHc of Pacap-ires-Cre mice. We found that both the VMHc and the VMHvl contained many VMHcPACAP terminals (Fig. 4b). In contrast, we found only a few VMHcPACAP terminals in the VMHdm (Fig. 4b).
We next ascertained functional synaptic connectivity between VMHcPACAP and VMHdm, VMHc, or VMHvl neurons using CRACM (Fig. 4c). For this, we placed injections of AAV-DIO-ChR2-mCherry into the VMHc of Pacap-IRESCre mice and recorded light-evoked excitatory postsynaptic currents (EPSCs) from ChR2-mCherry terminals on VMHvl neurons (Fig. 4c). Consistent with the tracing data, we detected light-evoked EPSCs in almost all VMHc and VMHvl neurons (13 of 15 neurons; Fig. 4c). In contrast, we detected light-evoked EPSCs in only 1 of 13 VMHdm neurons (Fig. 4c). Thus, VMHcPACAP neurons densely innervate and excite VMHc and VMHvl neurons, but not VMHdm neurons.

To determine whether acute stimulation of VMHcPACAP neurons drives behavioral aggression, we placed bilateral injections of a Cre-dependent excitatory chemoreceptor (AAV-DIO-hM3Dq-mCherry; hM3Dq) into the VMHc of Pacap-IRESCre mice (Fig. 4d). Administration of the hM3Dq ligand, clozapine-N-oxide (CNO, 1 mg/kg in saline), significantly increased total time attacking compared to VEH (Fig. 4e; t1 = 3.357, P = 0.0206). Strong induction of c-Fos served as further confirmation that CNO effectively activated VMHcPACAP neurons (Fig. 4f). In agreement with our tracing and CRACM experiments, acute activation of VMHcPACAP neurons also increased c-Fos expression in VMHvl neurons, strongly suggesting that the increase in aggression was driven by this intra-VMH connection.

**Discussion**

Here we demonstrate, for the first time, that aggression propensity in male mice exhibits a daily rhythm. We then show, using genetically targeted disruption and inhibition, that this rhythm in aggression propensity requires normal functioning of SPZGABA neurons and is independent of LMA and plasma corticosterone rhythms. Using the CRACM approach23–25, we uncover a previously undescribed, functional polysynaptic circuit connecting the SCN clock with VMHvl neurons known to regulate attack behavior. We also show a parallel pathway from the SPZ through PACAP+ neurons within the VMHc, highly interconnected with the VMHvl and forming an intra-VMH circuit that, upon activation, also drives attack behavior.

Agnostic encounters between conspecific males are characterized by the progression from an investigative motivational phase15, in which the sex, size, and strength of the opponent are determined, to a consummatory phase comprising attack behavior or retreat2. This progression is generally conceptualized as an escalation in the intensity of a continuous internal emotional state26,27. Our data strongly suggest that input from the SCN clock to VMHvl neurons, either directly from SPZ neurons or indirectly through VMHc neurons, may increase or decrease the rate of this progression and the intensity or duration of consummatory attack behavior, depending on the time of day of the encounter. Indeed, because the VMHc has also been shown to be relevant in other emotionally related behaviors associated with fear and anxiety28,29, it is possible that this circuit may modulate circadian rhythms in the propensity for such behaviors as well30,31.

Since SCN activity in mice is high during the day and lower at night32, it is notable that dSPZ→VMH neurons, which receive GABAergic input from the SCN (Fig. 2a–c), are more active at ZT1 compared to ZT13 (Fig. 3a–c). We therefore believe that it is the summation of inhibitory GABAergic SCN→dSPZ input across the day that leads to low levels of dSPZ→VMH inhibition, and therefore increased aggression propensity, during the end of the light period and the early dark period (CT11 and CT13, Supplementary Fig. 3; ZT13, Fig. 1b–d). Furthermore, we believe the reduction of SCN activity during the night allows for the summation of GABAergic dSPZ→VMH inhibition over the dark period, leading to the lowest aggression propensity during the early light period (ZT1). A previous study examining SCN and SPZ multiunit activity in mice reported that SPZ multiunit activity was highest during the dark period33, antiphase to the day-active SCN, but the recording sites depicted in a supplementary figure for that paper appear to be rostral and ventral to the dSPZ region, which we show is highly active at ZT1. The SPZ is known to be composed of multiple subregions with differing input and output pathways34, and these subregions appear to modulate dissociable circadian rhythms35. Thus, the temporal pattern of activity for each SPZ subregion may be influenced by several other factors, such as intra-SPZ connectivity from other subregions36, direct input from the retina37, and peptidergic input from different subpopulations within the SCN (VIP, AVP, etc.)38, as well as input from other neural systems. Regardless of the underlying cause of increased dSPZ activity at ZT1, our results demonstrate that GABAergic transmission from these neurons serves to inhibit the propensity for aggression during the early resting period, most likely via their projections to the VMH.

A better understanding of how the SCN clock and its primary synaptic relay, the SPZ, modulate aggressive behavior will have important implications for treating patients with neurologi- disorders associated with circadian dysfunction and physi- cal and verbal aggression. For instance, patients suffering from dementia and Alzheimer’s disease frequently exhibit sundowning syndrome39–41, a poorly understood clinical phenomenon characterized by agitation, aggression, and delirium during the early evening hours. These symptoms take a tremendous toll on the patient’s caregivers and represent a common reason for institutionalization42. Our results showing that disruption of SPZGABA output produces increased behavioral aggression during the early resting phase (early in the light period, for mice) are temporally consistent with the increased aggression seen during sundowning, suggesting that the SCN→SPZ→VMH pathway may be compromised in such neurodegenerative diseases. An examination of neuropathological changes to this pathway in Alzheimer’s disease and other neurological conditions may provide insight into future interventions that could greatly improve the quality of life for both patients and caregivers.

**Methods**

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41593-018-0126-0.

Received: 17 October 2017; Accepted: 9 February 2018;
Published online: 9 April 2018

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Methods

Animals. Adult male VgatloxP/loxP, Vgat-IRES-Cre, VIP-IRES-Cre, Earl1-2a-Cre, Pacap-IRES-Cre, and C57BL6/J mice were used. VgatloxP/loxP mice, Vgat-IRES-Cre mice, VIP-IRES-Cre, and Pacap-IRES-Cre mice were described previously27,28,29. Earl1-2a-Cre mice were obtained from Jackson Laboratories (stock No. 017911). Vgat-IRES-Cre and VgatloxP/loxP lines had been backcrossed to the C57BL6/ strain, whereas Pacap-IRES-Cre mice were mixed background. C57BL6/J mice were obtained from Jackson Laboratories. All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal care and use protocols were obtained from the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center. Efforts were made to minimize pain and the number of mice used. Additional information about the following procedures can be found in the Life Sciences Reporting Summary for this paper.

AAV-FLEX-hGlyR-mCherry generation. We used a human alpha-1 glycine receptor gene, modified by Lynagh and Lynch and previously tested in vitro30, in which A288G and F207A mutations were induced to render the channel almost insensitive to glycine. This construct was placed within a DIO/FLEX cassette and packaged within an AAV, serotype 10 by P-MF.

Other vectors used. AAV-cerebPV-A-Venus contains genes for Cre and Venus, a FP, connected via a self-processing 2 A viral peptide bridge25. Plasmid construct was placed within a DIO/FLEX cassette and packaged within an AAV, serotype 10 by P-MF. Other vectors used.

Surgeries. Mice were anesthetized with ketamine/xylazine (100 and 10 mg/kg respectively, intraperitoneally (IP)) and placed in a stereotaxic apparatus. Injections were placed into SPZ (AP = −0.4 mm, ML = ±0.2 mm, DV = −5.1 mm), SCN (AP = −0.4 mm, ML = ±0.1 mm, DV = −5.5 mm), or VMH (AP = −1.6 mm, ML = −0.95 mm, DV = −5.6 mm) using a compressed air delivery system.

AAV-FLEX-hGFP or AAV-DIO-Chr2-mCherry (10–15 nL) was injected unilaterally into the SPZ of Vgat-IRES-Cre mice. AAV-FLEX-hGFP or AAV-FLEX-hGFP (60–60 nL) and CTb-555 (5 nL) were injected unilaterally into the SPZ and VMH of Earl1-2a-Cre mice. AAV-cerebPV-A-Venus or AAV-GFP (15–10 nL) was injected bilaterally into the SPZ of Vgat-IRES-Cre mice. AAV-cerebPV-A-Venus or AAV-FLEX-hGFP (30 nL) was injected into the SPZ and VMH of Earl1-2a-Cre mice. AAV-cerebPV-A-Venus or AAV-GFP (10–15 nL) was injected bilaterally into the SPZ of Vgat-IRES-Cre mice. AAV-cerebPV-A-Venus or AAV-GFP (10–15 nL) was injected bilaterally into the VMH of Pacap-IRES-Cre mice.

AAV-DIO-hM3Dq-mCherry (15 nL) was injected unilaterally into the VMH of Pacap-IRES-Cre mice. AAV-DIO-hM3Dq-mCherry (15 nL) was injected bilaterally into the VMH of Pacap-IRES-Cre mice. AAV-cerebPV-A-Venus or AAV-GFP (10–15 nL) was injected bilaterally into the VMH of Pacap-IRES-Cre mice. AAV-DIO-synaptophysin-mCherry (10 nL) was injected unilaterally into the VMH of Pacap-IRES-Cre mice. To allow time for AAV expression, all mice were given at least 4 weeks before experiments.

Immediately following brain injections, VgatoxPlox mice used in LMA and Tb recordings were implanted IP with biotelemetry transmitters (Data Sciences International, St. Paul, MN). Incisions were sutured and treated with topical antibiotic, and all mice received meloxicam, an analgesic, for 48 h during recovery.

Resident–intruder model. All ‘resident’ mice (VgatoxPlox, C57BL6/J, Vgat-IRES-Cre, or Pacap-IRES-Cre) were sexually experienced adult males (4–6 months old at testing), singly housed and isolated from other males for at least 1 month, which produces a territorially aggressive phenotype. For behavioral tests, we habituated resident mice to an open field with a home corner (diameter, 20 cm; height, 25 cm) for at least 5 d. All residents were maintained in these home containers inside light−light isolation chambers under a 12:12-lightdark (LD) cycle with lights on at 08:00 (zeitgeber time 0, ZT0). Chambers provided ventilation, ambient temperature of 22±2 °C, and visual isolation from other mice. Mice received food (Lab Diet) and water ad libitum. Ambient lighting was provided by a white-light LED matrix, containing 18 single LEDs (light peak 490/540 nm) using a phosphor layer (yttrium aluminium garnet) on the surface of a blue (gallium nitride) chip (the LED Light, Inc.). For each test, resident were moved, in their home containers, to an adjacent testing room illuminated with red light (GE lighting, Red LED, A15, 620–640 nm), which does not alter circadian phase nor disrupt affective behaviors in nocturnal rodents18, and given 5 min to acclimate. A male group−housed and sexually naïve ‘intruder’ mouse (C57BL6/J), maintained on the same LD cycle in a separate chamber, was introduced into the resident’s home container and behavioral interactions were video−recorded for 10 min using two cameras from different lateral angles. No other mice were in the testing room during each test. Residents and intruders were always novel to one another and were of similar ages and weights.

We tested these mice under red light conditions, instead of under normal room lighting, so that only the dark, in order to maintain the same level of visual cues across all timepoints. For instance, half of our behavioral tests performed in the light (at ZT1 and ZT7), resident mice would have had additional visual cues to aid them in attacking the intruder. On the other hand, we had instead put mice into a lighted environment during their normal dark phase (at ZT13 and ZT19), we might have actually induced disinhibition (a phenomenon called negative masking) since mice are nocturnal, which would not have been conducive for measuring aggression. We do not believe that taking these mice out of a lighted environment and testing them under only red light biased our observed day−night (ZT1 vs. ZT13; Fig. 1 b−d) differences in aggression, because we saw similar temporal differences in attack behavior between CT1 and CT13 (Supplementary Fig. 1B). All control mice that had not been housed in constant darkness and then moved to red light conditions for testing in free−running conditions (see below).

Littermate VgatloxPlox mice (n=40) injected with AAV-cerebPV-A-Venus or AAV−GFP into the SPZ were tested at one of four different timepoints: ZT1 (between 08:45 and 09:30), ZT7 (between 14:45 and 15:30), ZT13 (between 20:45 and 21:45), and ZT19 (between 02:45 and 03:30). The order of timepoints was counterbalanced across all residents using a Latin square design. Following each test, home containers were changed and mice were habituated to the new containers for 3−5 d before being tested again at the next timepoint, until all four trials were completed. Mice were randomly assigned to experimental/control groups and to the order in which they were tested.

Littermate Vgat−IRES-Cre mice (n=23) injected with AAV−FLEX–hGlyR–mCherry into the SPZ received either IVM (5 mg/kg in propylene glycol) or VEH, administered via IP injection at ZT1 or ZT13. Because IVM has a long half−life and minor off-target effects (drowsiness) that are no longer present after 24 h30, we tested these mice using the resident−intruder assay described above, at ZT1 or ZT13, respectively, on the following day. We allowed 2 weeks following each test to ensure drug clearance, and then mice were injected with the other compound (IVM or VEH) and tested again 24 h later, serving as within−animal controls. Half of each group of mice received IVM first and half received VEH first, with mice being randomly assigned to each group. As an additional control for the phase−dependent effects of IVM, we tested an additional group of Vgat−IRES-Cre mice (n=10) injected with Chr2 into the SPZ (which does not respond to IVM) at ZT1 using the same methods described above.

Littermate Pacap−IRES−Cre mice (n=n+6) injected with AAV−DIO−hM3Dq−mCherry into the VMH received CNO (1 mg/kg in saline) or VEH, administered IP; 1 h before being tested with resident−intruder assays at ZT1 as described above. Mice received 3−5 d of rest for drug clearance and were then injected with the other compound and tested again, serving as within−animal controls. Half of these mice received CNO first and half received VEH first, with mice being randomly assigned to each group. Previous studies have shown that CNO has no effect on aggressive behavior in either WT C57BL6 mice or control mice injected with an AAV not containing hM3Dq.

Littermate intact C57BL6/J mice (n=15) were used to assess rhythms of aggression propensity under free−running conditions (constant darkness, DD). Mice were put into DD, extending from the end of their normal dark period, for 2 full days and then tested at one of four timepoints: CT1 (between 08:45 and 09:30), CT7 (between 14:45 and 15:30), CT13 between 20:45 and 21:45, and CT19 between 02:45 and 03:30. The order of tests for each mouse was counterbalanced using a Latin square design, with mice randomly assigned to each order. Although we would expect the internal phase of each mouse to drift in DD due to its period not being exactly 24h, our data for average period length in DD (Table 1) from intact mice on a C57BL6 background would suggest an average drift of only about 15 min across 3 d (using the following equation: (24 h − period × 2 °C, and visual isolation from other mice. Mice received food and water ad libitum. Recorded videos of resident–intruder tests were manually scored by experimenters blinded to the condition of each resident mouse and the time of day of the test. Videos were sequenced and the total time each resident spent engaging in attack behavior (including biting, chasing, lunging, wrestling, and boxing) was quantified on a frame−by−frame basis using the ‘Behavior annotator’ Matlab script developed by researchers at the California Institute of Technology [http://wwwision.caltech.edu/Video_Datasets/CRIM13/CRIM13/Main.html]). The resident’s total number of attack bouts and the latency to its first attack were also calculated. Behaviors of intruders, which did not display attack behavior, were not scored.

LMA and Tb rhythm recordings. A subset of littermate VgatloxPlox mice (n=16), injected with AAV−cerebPV-A-Venus or AAV−GFP into SPZ, also underwent Tb and LMA telemetry recordings at least 2 weeks after completion of all resident−intruder tests. Mice were individually housed in standard plastic cages inside isolation chambers as described above with ad libitum food and water. Cages were placed...
atop telemetry receivers interfaced to a microcomputer data acquisition system (Data Sciences International, New Brighton, MN, USA). Tba values were recorded for 5-min intervals, and LMA data were collected in 25-min bins. Cages were changed every 2 weeks, and health checks were performed daily. For time-series analysis. We collected at least 10 d of LMA and Tb data under 12:12 light-dark (LD) conditions, and at least 3 weeks of data in constant darkness (DD).

**Day-night plasma corticosterone.** A subset of littermate VgatloxP/loxP mice (n = 11), injected with an AAV-Cre, 2A Venus or AAV-GFP into the Vgat-IRES-Cre mice were over-night fasting for aggression as described above, also underwent assessment of day–night plasma corticosterone levels. Mice were housed in standard laboratory cages with ad libitum food and water, and maintained undisturbed in isolation chambers as described above for at least 2 weeks under LD conditions. Blood was collected via tail snap at ZT1 or ZT13 and again 12 h later (half-underwent collection at ZT1 first and half at ZT13) with mice randomly assigned to each group. Blood collection and plasma corticosterone levels assays were as described above. About 10 μL of blood was collected in EDTA-coated microvette tubes within 60 s after touching the cage. Samples were centrifuged, and 4 μL plasma was collected and diluted in 200 μL of steroid diluent. Plasma corticosterone was determined using a 125I radioimmunoassay kit from MP Biomedicals. All samples were analyzed in duplicate in the same assay to avoid inter-assay variability.

**Histology and immunohistochemistry.** Deeply anesthetized mice were transcardially perfused with saline and 1:10 formalin. Brains were removed postfixed in cryoprotectant solution overnight, and then cut in 30-μm coronal sections using a freezing microtome. Sections were washed in phosphate buffered saline (PBS) and incubated in primary AAV, VgatloxP/loxP, Cre, dsRed, Ctb, or c-Fos antisera diluted in PBS containing 0.3% Triton X-100 and 0.2% sodium azide, overnight at room temperature (22 ± 2 °C). The AVP antibody (1:20,000, Peninsula, cat#F-5094) was a guinea pig polyclonal raised against synthetic AVP (1-38-Tyr-Cys-Phe-Gln-Ala-Arg-Pro-Glu-NH₂). The VIP antibody (1:10,000, ImmunoStar, cat#20077) was a rabbit polyclonal raised against porcine VIP conjugated to bovine thyroglobulin. The hrGFP antibody (1:20,000, Stratagene, cat#240142) was a rabbit polyclonal raised against full-length recombinant hrGFP from Escherichia coli. The Er1 antibody (1:3,000, Millipore, cat#06-935) used was a rabbit polyclonal raised against rat Er1β (TYYIPPEAGFPNTI). The GFP antibody (1:20,000, Invitrogen, cat#A-6455) was a rabbit polyclonal raised against 27-kD GFP isoform derived from jellyfish Aquorea victoria. The Cre antibody (1:5,000, Novagen, cat#69050) was used was a rabbit polyclonal raised against bacteriophage P1 Cre recombinase (35 KD). The dsRed antibody (1:50,000, Invitrogen, cat#A-19628) was a rabbit polyclonal raised against DsRed-Express, a variant of Dicosoma sp. red fluorescent protein. The Ctb antibody (1:10,000, List Biological, cat#703) was a goat polyclonal raised against cholecoglin. The c-Fos antibody (1:20,000, −55 kD, Oncogene, cat#Ab5) was a rabbit polyclonal raised against residues 4–17 human v-Fos. Additional validation details are available from the manufacturer for each antibody used. Sections were washed in PBS and incubated in biotinylated secondary antisera (against appropriate species, 1:500) in PBS containing 0.3% Triton X-100 for 1 h, washed in PBS, and incubated in ABC reagents for 1 h. Sections were washed and incubated in a solution of 0.06% 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma) and 0.02% H₂O₂. Nickel–cobalt solution was added to the substrate to produce the color. The Ctb antibody was a rabbit polyclonal raised against dsRed, CTb, or c-Fos antibodies diluted in PBS containing 0.3% Triton X-100 for 1 h, washed in PBS, and incubated in ABC reagents for 1 h. The c-Fos antibody labeled typical patterns in uninjected animals, that c-Fos antibody labeled typical patterns in uninjected animals, and that AvP, VIP, and Er1 antibodies labeled typical patterns in PV, SCN, and VMH, respectively

**Cell counting.** All cell counts were analyzed blind to the time of day of perfusion and the condition of each animal. For quantification of day–night differences in c-Fos expression, adult male Vgat-IRES-Cre mice, housed on a 12:12 LD cycle in chambers as described above, were perfused 90 min after ZT1 or ZT13 (n = 9 per group). Bilateral images of the SPZ from sections immunostained for c-Fos were imported into ImageJ (NIH) and labeled neurons were counted in the last two sections containing the dSPZ sections using the ‘cell counter’ plugin. To quantify differences in c-Fos expression following chemogenetic inhibition, a subset of Vgat-IRES-Cre mice injected with AAV-FLEX-hGlyR-mCherry into the SPZ and tested for aggression, as described above, were given IVm (n = 3) or VEH (n = 3) at ZT1, and then perfused 90 min after ZT1 on the following day. These mice were housed on a 12:12 LD cycle as described above, and were given at least 2 weeks after aggression testing before these perfusions. Unilateral images of the SPZ from sections immunostained for dsRed and Ctb were imported into ImageJ. Double-labeled neurons were counted in the last two sections containing the dSPZ sections using the ‘cell counter’ plugin. An Abercrombie correction was applied to all cell counts.

**Heat map generation.** Each experimental mouse was assigned an aggression score based on the magnitude of its total time attacking at ZT1 compared to the control mean at this time. Each injection site showing the extent of transduced neurons were counted using imaging software (NIH Image) in Vgat-IRES-Cre mice. Vgat-IRES-Cre mice was digitally projected onto representative templates at three rostral–caudal levels throughout the anterior hypothalamus, constructed using immunolabeling against VIP and AVP (to better delineate the SCN and PV). Each mouse’s injection site was weighted by its aggression score, and weighted sites for the SPZ and AVP were selected against using a custom script (https://www.pyborean.org). The aggression score was summed over the group (pixel-by-pixel over each overlaid image), and heat maps were created demarcating brain regions most associated with increased aggression at ZT1, as previously described.

**Digoxigenin (DIG)-labeled RNA probe in situ hybridization.** Sections were cut in RNase-free conditions, preserved in RNAlater RNA stabilization solution, and frozen until use. Sections were washed in RNase-free PBS containing diethyl pyrocarbonate (DEPC) then incubated in hybridization buffer for 1 h at 53 °C. The Vgat probe was denatured at 80 °C for 10 min then incubated in the hybridization buffer overnight at 53 °C. Sections were successively washed in 2× standard saline citrate (SSC) with 50% formamide solution and in 1×SSC 50% with formamide solution, both at 53 °C. After Tris-buffered saline (TBS, pH 7.5) washes, sections were incubated in 1% blocking reagent (Roche Applied Science, Penzberg, Germany) for 30 min, then incubated overnight in peroxidase-conjugated DIG antibody (1:500, Roche Applied Science, Penzberg, Germany). Following TBS washes, sections were reacted with tyramide signal amplification (TSA) Cy3 (1.50, Perkin Elmer, Waltham, MA) for 30 min. Vgat probes were prepared by S. Yokota, University School of Medicine, Japan.

**Statistical analyses.** All data were imported into Prism 7 (Graphpad) and normality was assessed using D’Agostino–Pearson and Kolmogorov–Smirnov tests, to verify the appropriateness of the following statistical analyses. For Vgat-IRES-Cre mice, aggression data scored during resident–intruder testing were compared across time and between SPZ conditions using two-way repeated-measures ANOVA, with Siddak’s tests for multiple comparisons serving as post hoc analyses. Sample sizes were not predetermined using formal statistical tests for Vgat-IRES-Cre mice, but post hoc power calculations (G’Power software) using means and standard deviations from these analyses revealed that this study had sufficient power to detect respective effect sizes with ≥90% reliability. Because of natural variability within strains, not all mice show aggression, and we set a criteria before these experiments of at least one attack bout in at least half (50%) of all tests for inclusion in statistical analyses. Notably, only 2 Vgat-deleted mice failed to meet criteria for inclusion, whereas 6 intact controls failed. Overall, 80.0% of Vgat-IRES-Cre mice met criteria for inclusion.

**Aggression data from Vgat-IRES-Cre mice was compared between IVM and VEH conditions using separate paired t tests for ZT1, ZT13, and ChR2-injected control groups. For Pacap-IRES-Cre mice, aggression data were compared between CNO and VEH conditions using paired t tests. These mice were not predetermined, but post hoc power calculations using means and standard deviations from Vgat-IRES-Cre and Pacap-IRES-Cre mice revealed that we had enough power to detect effect sizes with ≥90% and 80%, reliability, respectively. For C57BL/6j mice, differences in aggression data between CT1 and CT13 were compared using planned comparisons (paired t tests). Sample sizes for C57BL/6j mice were predetermined (G*Power software) using the effect size, means, and standard deviations of previously tested intact control mice on a C57BL6/J background and a power level of 80%. We set the same criteria (at least one attack bout in 50% of test trials) for inclusion in these analyses. Overall, 72.7% of C57BL/6j mice, 78% met criteria for inclusion (24 of 33), while 9 failed and were excluded. All 6 Pacap-IRES-Cre mice were included. For C57BL/6j mice, 80% met criteria (12 of 15), while 3 failed and were excluded.

For cell counting in Vgat-IRES-Cre mice, differences in c-Fos expression were compared between ZT1 and ZT13 conditions and differences in dsRed/c-Fos-labeled cells were compared between IVM and VEH, conditions using unpaired t tests. All data shown are mean ± s.e.m. unless otherwise noted. For all tests, alpha was P < 0.05.
Electrophysiology. Mice (9–12 weeks old at testing) were deeply anesthetized and decapitated, and brains were quickly removed into ice-cold cutting solution consisting of (in mM): 72 sucrose, 83 NaCl, 2.5 KCl, 1 NaH2PO4, 26 NaHCO3, 22 glucose, 5 MgCl2, 1 CaCl2, oxygenated with 95% O2/5% CO2, measured osmolarity 310–320 mOsm/L. We cut 30-μm-thick coronal sections with a Leica VT1000S vibratome and incubated them in oxygenated cutting solution at 34°C for 45 min. Slices were transferred to oxygenated aCSF (126 mM NaCl, 21.4 mM NaHCO3, 2.5 mM KCl, 1.2 mM NaH2PO4, 1.2 mM MgCl2, 2.4 mM CaCl2, 10 mM glucose) and stored in the same solution at room temperature (20–24°C) for 60 min prior to recording. A single slice was placed in the recording chamber where it was continuously superfused at a rate of 3–4 mL per min with oxygenated aCSF. Neurons were visualized with an upright microscope (SliceScope, Scientifica) equipped with infrared-differential interference contrast and fluorescence optics. Borosilicate glass microelectrodes (5–7 MΩ) were filled with internal solution. Light-evoked EPSCs and IPSCs were recorded in whole-cell voltage-clamp mode, with membrane potential clamped at $V_h = -70$ mV. Light-evoked IPSCs were recorded using a CsCl-based internal solution consisting of (in mM): 140 CsCl, 1 BAPTA, 10 HEPES, 5 MgCl2, 2 Mg-ATP, and 0.3 Na2-GTP (pH 7.3 adjusted with CsOH; 295 mOsm·kg−1). Light-evoked EPSCs were recorded using Cs+-based internal solution consisting of (in mM): 135 CsMeSO4, 10 HEPES, 1 EGTA, 4 MgCl2, 4 Na2-ATP, 0.4 Na+2-GTP, 10 Na+2-phosphocreatine (pH 7.3 adjusted with CsOH; 295 mOsm·kg−1) in the presence of bicuculline (10 μM). The light-evoked EPSC/IPSC detection protocol consisted of four blue light pulses (473 nm wavelength, 0.5–5 ms) administered 1 s apart during the first 4 s of an 8-s sweep, repeated for a total of 30 sweeps. Evoked EPSCs/IPSCs with short latency (≤5 ms) upon light stimulation were considered light-driven. As discussed by others, such currents are most likely monosynaptic58. Light-evoked IPSCs were recorded in the presence of CNQX (10 μM) and d-AP5 (50 μM) to block polysynaptic glutamatergic excitation (Fig. 2). Due to the high local connectivity, light-driven monosynaptic EPSCs recorded from VMH neurons (Fig. 4) could be influenced by intranucleus polysynaptic glutamatergic excitation. It is noteworthy that this, however, does not change our overall finding that VMHc neurons are much more likely to connect to VMHvl neurons (13 of 15 neurons; Fig. 4c) rather than VMHdm neurons (1 of 13 neurons; Fig. 4c). Numbers of animals used for CRACM experiments: SPZ → VMH1, n = 3; SCN → SPZ, n = 2; VMHc → VMHc, VMHdm or VMHvl, n = 2 each; and SPZ → VMHesr1, n = 4. All recordings were made using a Multiclamp 700B amplifier, and data were filtered at 2 kHz and digitized at 10 kHz. To photostimulate ChR2 fibers, an LED light source (473 nm) was used. Blue light was focused onto the back aperture of the microscope objective, producing widefield exposure around recorded cell of 10–15 mW per mm2. Light power at the specimen was measured using a PM100D optical power meter (Thorlabs). Light output was controlled by a programmable pulse stimulator, Master-8 (A.M.P.I.) and pClamp 10.2 software (Axon Instruments). All recordings were analyzed offline using Clampfit 10.

Reporting Summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The data that support the findings of this study are presented within this paper and its supplementary materials, or are available from the corresponding author upon reasonable request.

Code availability. The custom code used for creating the weighted heat maps of our injection sites is available in Supplementary Software.

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Experimental design

1. Sample size
   Describe how sample size was determined.

   For experiments in Vgatlox/lox, Vgat-IRES-Cre, Pacap-IRES-Cre mice, sample sizes were not determined using pre-established statistical tests. But, post hoc power analyses revealed these studies were powered to determine given effect sizes with >90% reliability in Vgatlox/lox mice and Vgat-IRES-Cre mice, and with 80% reliability in Pacap-IRES-Cre mice. For our supplementary experiment in C57BL6/J mice, sample size was determined with G*Power software using effect sizes, means, and standard deviations of data from intact mice on a C57 background and a power level of 80%.

2. Data exclusions
   Describe any data exclusions.

   We pre-established a criteria of 50% responding (at least one attack bout in half the RI tests administered to each mouse) for inclusion in our statistical analyses. Data from mice that failed to meet this criteria were excluded from these analyses. This information is detailed in the "Statistical Analyses" subsection within the "Methods" section.

3. Replication
   Describe whether the experimental findings were reliably reproduced.

   All attempts at replication were successful.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.

   For all experiments, mice were randomly assigned to experimental and control groups and to which order they were tested in.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

   All data was scored or counted by an investigator who was blind to the condition of the mouse and the time of day of each experiment.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

- **n/a**
- **Confirmed**

| Item                                                                 | Status   |
|----------------------------------------------------------------------|----------|
| The exact sample size \((n)\) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) | Confirmed |
| A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly | Confirmed |
| A statement indicating how many times each experiment was replicated | Confirmed |
| The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section) | Confirmed |
| A description of any assumptions or corrections, such as an adjustment for multiple comparisons | Confirmed |
| The test results (e.g. \(P\) values) given as exact values whenever possible and with confidence intervals noted | Confirmed |
| A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) | Confirmed |
| Clearly defined error bars | Confirmed |

See the web collection on statistics for biologists for further resources and guidance.

### Software

**Policy information about availability of computer code**

7. **Software**

Describe the software used to analyze the data in this study.

Data was analyzed with Graphpad 7, G*Power 3.1, Excel 14, Matlab (Behavior Annotator and Clocklab scripts), ImageJ (Cell Counter plugin), and Clampfit 10. A custom code was used in Python for generating heat maps.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

### Materials and reagents

**Policy information about availability of materials**

8. **Materials availability**

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

There are no restrictions on unique materials used in this study.

9. **Antibodies**

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

The AVP antibody (1:20K, Peninsula, cat#T-5048) used was a guinea pig polyclonal raised against synthetic AVP (H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH2). The VIP (1:10K, ImmunoStar, cat#20077) antibody used was a rabbit polyclonal raised against porcine VIP conjugated to bovine thyroglobulin. The hrGFP (1:20K, Stratagene, cat#240142) antibody used was a rabbit polyclonal raised against full-length recombinant hrGFP from E. coli. The Esr1 (1:3K, Millipore, cat#06-935) antibody was a rabbit polyclonal raised against rat ER\(\alpha\) (TYYIPPEAEGFPNTI). The GFP (1:20K, Invitrogen, cat#A-6455) antibody used was a rabbit polyclonal raised against 27kD GFP isolated from jellyfish Aequorea victoria. The Cre (1:5K, Novagen, cat#69050) antibody used was a rabbit polyclonal raised against bacteriophage P1 Cre Recombinase (35 kD). The dsRed (1:10K, Clontech, cat#632496) antibody used was a rabbit polyclonal raised against DsRed-Express, variant of Dicosoma sp. red fluorescent protein. The CTb antibody (1:10K, List Biological, cat#703) was a goat polyclonal raised against cholera enterotoxin. The c-Fos antibody (1:20K, ~55kD, Oncogene, cat#Ab5) used was a rabbit polyclonal raised against residues 4-17 human cFos. Additional validation details are available from the manufacturer for each antibody used. For controls, we verified that hrGFP, GFP, Cre, and CTb antibodies did not label cells in uninjected animals, that cFos antibody labeled typical patterns in unstimulated animals, and that AVP, VIP, and Esr1 antibodies labeled typical patterns in PV, SCN, and VMH, respectively (See Methods section for associated references).
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used. No eukaryotic cells lines were used
   b. Describe the method of cell line authentication used. No eukaryotic cells lines were used
   c. Report whether the cell lines were tested for mycoplasma contamination. No eukaryotic cells lines were used
   d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use. No eukaryotic cells lines were used

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals
   Provide details on animals and/or animal-derived materials used in the study.
   Adult male Vgatlox/lox, Vgat-IRES-Cre, VIP-IRES-Cre, Esr1-2a-Cre, Pacap-IRES-Cre, and C57BL6/J mice were used. Vgatlox/lox mice, Vgat-IRES-Cre mice, and Pacap-IRES-Cre mice were described previously21,24,26. VIP-IRES-Cre mice were provided by Dr. David Olson. Esr1-2a-Cre mice were obtained from Jackson laboratories (Stock No: 017911). Vgat-IRES-Cre and Vgatlox/lox lines had been backcrossed to C57BL6/J strain, whereas Pacap-IRES-Cre mice were mixed background. C57BL6/J mice were obtained from Jackson Laboratories. Mice were 4-6 months old for behavioral tests and 9-12 weeks old for electrophysiology experiments. All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and formal approval of our protocols was obtained from the Institutionary Animal Care and Use Committee at Beth Israel Deaconess Medical Center. All efforts were made to minimize pain and the number of mice used.

Policy information about studies involving human research participants

12. Description of human research participants
   Describe the covariate-relevant population characteristics of the human research participants.
   This study did not involve human research participants.