A highly collateralized thalamic cell type with arousal-predicting activity serves as a key hub for graded state transitions in the forebrain

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Sleep cycles consist of rapid alterations between arousal states, including transient perturbation of sleep rhythms, microarousals, and full-blown awake states. Here we demonstrate that the calretinin (CR)-containing neurons in the dorsal medial thalamus (DMT) constitute a key diencephalic node that mediates distinct levels of forebrain arousal. Cell-type-specific activation of DMT/CR+ cells elicits active locomotion lasting for minutes, stereotyped microarousals, or transient disruption of sleep rhythms, depending on the parameters of the stimulation. State transitions could be induced in both slow-wave and rapid eye-movement sleep. The DMT/CR+ cells displayed elevated activity before arousal, received selective subcortical inputs, and innervated several forebrain sites through highly branched axons. Together, these features enable DMT/CR+ cells to mediate subcortical arousal information and effectively transfer it as a rapid, synchronous signal to several forebrain regions to modulate the level of arousal.

The mechanisms of state transitions during sleep or between sleep and wakefulness are complex and poorly understood. Sleep itself is a highly dynamic state that consists of rapid transitions between slow-wave sleep (SWS) and rapid eye-movement (REM) sleep, with fluctuating levels of arousal that manifest, for example, as cyclic alternating patterns or microarousals. Control of these brain state changes appears to involve an ever-increasing number of interacting brain centers located mainly in the brainstem and the hypothalamus. It is still unclear, however, how the final output of these centers is summated and transferred rapidly to the forebrain as a coordinated, graded signal, that is, how arousal is controlled in a fast and synchronous manner in the forebrain.

Earlier studies using traditional tracing techniques suggested that cells in the DMT receive inputs from the main hypothalamic and brainstem arousal centers and innervate several cortical and subcortical regions in the forebrain. DMT utilizes fast glutamatergic transmission and thus is in a position to mediate rapid responses in forebrain structures. Indeed, lesions involving DMT in humans have been linked to hypersomnia and altered vigilance states. Thalamic neurons that are functionally related are often not confined to a single nucleus, and thalamocortical cells with distinct properties may intermingle. Moreover, the DMT region includes various nuclei with irregular shapes and sizes, which complicates traditional approaches for anatomical or functional interrogation. As a result, it is still unclear which thalamic neuron population, if any, mediates forebrain arousal and what neuronal activity governs concerted state changes among forebrain areas.

In both rodents and humans, DMT contains large populations of CR+ cells scattered across the various nuclei of this region. In this study, we tested whether this DMT/CR+ neuronal population plays a specific role in forebrain arousal. Using cell-type-specific approaches, we investigated DMT/CR+ neurons’ arousal-related activity, connectivity, and impact on arousal. We also investigated their inputs in the equivalent human DMT region and compared the properties of arousals elicited by DMT/CR+ cells and sensory thalamic nuclei. Predictive coding before sleep–wake transitions, graded arousal responses, and widespread, synchronous impact on forebrain targets identified DMT/CR+ cells as a key mediator of forebrain arousal.

Results
Arousal-related activity of DMT/CR+ neurons. Neurons in the DMT are known to display diurnal and stress-related c-Fos protein expression. In addition, this thalamic region is known to contain high numbers of CR+ neurons. Thus, to identify whether CR is a reliable marker for the activity-dependent DMT cell population, we perfused mice during the light (Zeitgeber time 2.5, sleep) or dark (Zeitgeber time 14.5, wake) phase of their diurnal cycles and tested the CR content and c-Fos expression of DMT cells (Fig. 1a–c). The DMT of mice contained significantly higher numbers of c-Fos+ neurons during the dark phase than the light phase (Fig. 1b–d and Supplementary Table 1), similar to findings in rats. The vast majority (~91%) of these neurons co-expressed CR in both states (Fig. 1e and Supplementary Table 1). The c-Fos+CR+ neurons...
were present in the major nucleus of the DMT (the paraventricular nucleus) but were also dispersed in adjacent portions of the anterior intralaminar and mediodorsal nuclei. Since this neuronal population was not confined to a single nucleus, we will refer to it as ‘DMT/CR+’ cells throughout this study.

Next, the DMT/CR+ cells were optogenetically tagged using short pulses of blue light (473 nm) in Calb2-Cre (CR-Cre) mice injected with AAV-DIO-ChR2 (Fig. 1f,g and Supplementary Fig. 1a–d), and their firing rates were extracellularly monitored during sleep–wake state changes for several hours. Thirty-one of 65 well-isolated units displayed elevated firing rates to the tagging protocol and were thus considered CR+ (Fig. 1h and see Methods). The activity of 29 of these 31 DMT/CR+ cells (93.5%) was correlated with changes in electromyogram (EMG) activity accompanying arousal from sleep. Twenty of the 31 DMT/CR+ cells (64.5%) started to significantly increase their firing rate up to 5–10 s before the onset of EMG activity and maintained elevated activity for tens of seconds after EMG activation (Fig. 1h and Supplementary Fig. 1g). In studies of brainstem neurons, a similar anticipatory elevation of firing rate several seconds before EMG activation has been considered the best indicator for their involvement in arousal. The other 8 DMT/CR+ neurons (25.8%) increased their firing at the onset of EMG activity, but not before, and remained active during it (Supplementary Fig. 1e,f). Of the remaining 3 DMT/CR- cells, one decreased its firing during EMG activation and two showed no changes (Supplementary Fig. 1h).
Thirty-four of the original 65 DMT neurons did not react to the tagging protocol and thus were regarded as putative CR+ cells (Fig. 1j). Among these, only 8 of 34 (23.5%) cells increased their firing before EMG activity, while the rest did not (Supplementary Fig. 1i). As a consequence, at the population level, DMT/CR− neurons did not show anticipatory activity, in sharp contrast to the DMT/CR+ population (Fig. 1j). Increased firing of DMT/CR− cells at the onset of the EMG signal was also shorter and lasted only for 1–2 s, not for >10 s as in the case of DMT/CR+ cells (Fig. 1j,k). These data show that DMT/CR+ cells selectively displayed arousal-related, predictive firing activity.

To analyze whether, under arousing conditions during the awake state, such as stress, DMT neurons are also CR+, we subjected three groups of animals to increasingly stressful situations (handling condition, habituation to a novel environment, and footshock) before perfusion. The number of c-Fos+ neurons significantly increased in the DMT in situations eliciting increasingly elevated arousal (Fig. 2a–c,e and Supplementary Table 1). When tested for CR expression, the vast majority of footshock-activated c-Fos+ cells expressed CR (Fig. 2d,e, Supplementary Fig. 2, and Supplementary Table 1).

To assess the response to a painful arousing signal, we measured the firing response of individual, juxtaocularly recorded and labeled DMT/CR+ cells to tail pinch under anesthesia (Supplementary Fig. 3). Tail pinch caused a reduction in the delta power of frontocortical local field potentials in all cases. Six of 13 DMT/CR+ cells significantly increased their activity during tail pinch, and activity remained elevated even after its termination (Supplementary Fig. 3; one-way ANOVA, $F_{1,22} = 5.3735, P = 0.0174$; Fisher’s least significant difference test, $F_{1,22} = 3.4945, P = 0.0481$). Bar graphs are means ± s.e.m.; open circles in e and i represent data for individual animals; horizontal lines in box plots indicate medians, box limits indicate first and third quantiles, and vertical whisker lines indicate minimum and maximum values. *$P < 0.05$; ** $P < 0.01$, ***$P < 0.001$.

Graded arousal elicited by DMT/CR+ cells. To directly test whether selective activation of DMT/CR+ neurons can initiate state transitions in freely sleeping animals, we first checked the reliability of their optogenetic responses (Fig. 3a). CR-Cre mice were injected with AAV-DIO-ChR2-eYFP (Supplementary Fig. 1a–d) and

**Figs. 2 |** c-Fos content and optogenetic inhibition of DMT/CR+ cells in situations with distinct arousal levels. a–c, Schematic drawing of the experimental design (top) and representative images of c-Fos expressions (bottom) in DMT following (a) handling, (b) habituation (no shock), and (c) footshock. d, Representative confocal image of the colocalization of c-Fos and CR in DMT cells in footshock. e, Left: normalized data for c-Fos+ DMT cells in control (H), habituation (H), and shock (Sh) situations ($n = 4$ mice per group; control: 100 ± 40%; habituation: 179 ± 24%; shock: 249 ± 12%; two-tailed unpaired $t$ test, C vs. H, $t_{6} = -3.339, P = 0.0156$; H vs. Sh, $t_{6} = -5.152, P = 0.0021$; C vs. Sh, $t_{6} = -7.043, P = 0.0004$). Right: CR content (right) of c-Fos+ cells in Sh. Yellow bar, CR+c-Fos+ cells (1,393 of 1,433 neurons, 97.2%; $n = 4$ mice); green bar, CR−c-Fos+ cells (40 of 1,433, neurons, 2.8%). f, Schematic drawing for optogenetic inhibition of DMT/CR+ in a novel environment. g, h, Representative data for short immobile states (red dots) evoked by optogenetic silencing during the exploration of a novel box (gray) in (g) a YFP (control) mouse and (h) a SwichR-injected mouse. i, Population data for the number of immobile states during the pre-OFF (3 min), ON (3 min) and post-OFF (3 min; see Methods) periods in the YFP ($n = 6$ mice; pre-OFF $n = 12.2 ± 3.0$ states; ON $n = 11.2 ± 1.7$ states, post-OFF $n = 9.3 ± 1.6$ states) and SwichR-injected animals ($n = 7$ mice; pre-OFF $n = 18.9 ± 1.9$ states; ON $n = 29.9 ± 4.4$ states, post-OFF $n = 23.3 ± 2.7$ states; repeated-measures ANOVA with Fisher’s least significant difference test, $F_{2,21} = 3.4945, P = 0.0481$). Bar graphs are means ± s.e.m.; open circles in e and i represent data for individual animals; horizontal lines in box plots indicate medians, box limits indicate first and third quantiles, and vertical whisker lines indicate minimum and maximum values. *$P < 0.05$; ** $P < 0.01$, ***$P < 0.001$. 

**Fig. 3.** Demonstration of optogenetic silencing of DMT/CR− cells. a, b, Representative data for short immobile states (red dots) evoked by optogenetic silencing during the exploration of a novel box (gray) in (a) a YFP (control) mouse and (b) a SwichR-injected mouse. e, Representative data for short immobile states (red dots) evoked by optogenetic silencing during the exploration of a novel box (gray) in (a) a YFP (control) mouse and (b) a SwichR-injected mouse. i, Population data for the number of immobile states during the pre-OFF (3 min), ON (3 min) and post-OFF (3 min; see Methods) periods in the YFP ($n = 6$ mice; pre-OFF $n = 12.2 ± 3.0$ states; ON $n = 11.2 ± 1.7$ states, post-OFF $n = 9.3 ± 1.6$ states) and SwichR-injected animals ($n = 7$ mice; pre-OFF $n = 18.9 ± 1.9$ states; ON $n = 29.9 ± 4.4$ states, post-OFF $n = 23.3 ± 2.7$ states; repeated-measures ANOVA with Fisher’s least significant difference test, $F_{2,21} = 3.4945, P = 0.0481$). Bar graphs are means ± s.e.m.; open circles in e and i represent data for individual animals; horizontal lines in box plots indicate medians, box limits indicate first and third quantiles, and vertical whisker lines indicate minimum and maximum values. *$P < 0.05$; ** $P < 0.01$, ***$P < 0.001$. 

**Fig. 4.** Decrease in delta power during increased arousal in DMT/CR− cells. A, B, Representative EMG recordings from the main body in a control mouse (A) and a SwichR-injected mouse (B) during the exploration of a novel box (gray). C, EMG activity in Sh (shock) and C (control) conditions ($n = 2$ per group; control: 129 ± 53%; Sh: 25 ± 20%; two-tailed unpaired $t$ test, C vs. Sh, $t_{4} = -5.152, P = 0.0021$). D, EMG activity in Sh (shock) and C (control) conditions ($n = 2$ per group; control: 129 ± 53%; Sh: 25 ± 20%; two-tailed unpaired $t$ test, C vs. Sh, $t_{4} = -5.152, P = 0.0021$).
subjected to juxtacellular recording and labeling under urethane anesthesia. When tested with 1 ms laser light, all DMT neurons post hoc identified as channelrhodopsin-2 (ChR2)-eYFP+ (n=4 cells) were able to follow 20 Hz stimulation for up to 10 s with short response latency (1.8±1.1 ms), low jitter, and very high probability (0.997±0.005; Fig. 3b–e).

Next, we injected either AAV-DIO-ChR2-eYFP or AAV-DIO-eYFP into the DMT of CR-Cre mice and subsequently photostimulated DMT with 10 s, 10 Hz light pulse trains (see Methods) via chronically implanted optic fibers, in drug-free conditions (Fig. 3f–g). We used 10 Hz stimulation, as in our freely moving experiments all recorded DMT/CR+ cells (n=31) were able to fire at this frequency and 66% of them were able to sustain this activity for at least 1 s during awakening.

Ten-second activation of DMT/CR+ neurons faithfully induced prolonged behavioral arousal accompanied by active locomotion in all ChR2-injected mice during non-REM (NREM) sleep (Fig. 3h and Supplementary Video 1). Parameters of arousal were measured based on the EMG signal (Supplementary Fig. 5a). Evoked arousal lasted the stimulation by several minutes (range: 2.17 to 17.89 min; average: 8.9±5.6 min). The photostimulation of DMT/CR+ cells first induced an immediate drop in delta power (Fig. 3h,i), followed by an abrupt increase in EMG activity with a latency of 1.34±0.64 s (Fig. 3h and Supplementary Fig. 5b). During the first 180 s following the stimulation, the animals spent 78.66% (141.59±21.43 s) of their time in the active, wake state (EMG ON). The same value for the prestimulation period was 3.65% (6.58±3.54 s). In the control eYFP-injected animals, no arousal was evoked (Fig. 3; prestimulation EMG ON state, 2.07%; 3.72±0.74 s; poststimulation EMG ON state 2.87%; 5.17±3.54 s). These data show that activation of DMT/CR+ cells represents a rapid, strong arousal signal that results in a prolonged arousal state.

Optogenetic stimulation of DMT/CR+ cells for only 1 s (10 Hz) induced transient arousals (Fig. 3i). These transient interruptions of sleep, known as microarousals, are considered part of normal sleep behavior both in humans and rodents12,13,27. During these events, the animals stayed in their nests and displayed only brief head and neck movements lasting for only a few seconds (3.69±1.31 s, probability: 0.66±0.19; Fig. 3i, Supplementary Fig. 5c, and Supplementary Video 2). The onset of the EMG activity was 2.75±1.48 s. As in the 10 s stimulations, electroencephalogram (EEG) delta power dropped sharply; however, in these events it returned to baseline within 30 s (Fig. 3i). To identify whether the primary response to the activation of DMT/CR+ cells was a change in the EEG or a change in the EMG activity, we grouped the responses according to the onset of EMG ON states and examined the corresponding change in the drop of delta activity. Regardless of the onset of the EMG activity, the onset of the change in delta power was instantaneous and preceded the corresponding EMG change (Supplementary Fig. 6a,b).

In addition, measurements of time differences between the onset of reduction in delta power and the onset of EMG activity in individual arousal events demonstrated that the primary response following DMT/CR+ activation is a cortical arousal followed by a change in muscle activity (Supplementary Fig. 6c). These observations argue for a top-down cortical effect on behavior, not for a direct action of DMT/CR+ cells on motor centers.

Next, we examined the transitions between microarousals and prolonged arousals using various stimulus durations (0.5, 1, 2, and 10 s), while keeping laser power constant, during NREM sleep. The probability of arousal increased with increasing stimulus duration (Fig. 3). The mean durations of evoked EMG ON states in the first 60 s following stimulus onset also increased with longer stimuli (Fig. 3k,l and Supplementary Fig. 5c). The average duration of microarousals evoked by 1 s stimulations did not differ from the duration of spontaneous microarousals recorded in control periods (Fig. 3k). This indicates that the 1 s optical stimulations evoked a behaviorally relevant arousal pattern. Together, these data show that graded recruitment of DMT/CR+ cells elicits distinct, graded, natural arousal patterns.

We also examined whether any alterations in cortical EEG can be observed when the 1 s photostimulation of DMT/CR+ cells did not induce arousal as detected by EMG activity (that is, ‘sleep-throughs’);28 Fig. 3m–q). We compared changes in delta and sigma powers following the stimulations that resulted in microarousals or sleep-throughs. A sharp drop in delta power with comparable size could be observed both in microarousals and sleep-throughs. However, this perturbation recovered much faster in sleep-throughs than in microarousals (see Methods and Fig. 3m–o). A large drop in sigma power with comparable size was also evident in both microarousals and sleep-throughs, but, in contrast to delta, sigma power returned to baseline slowly in both cases (Fig. 3p–q). These data indicate that, even in the absence of overt behavioral (EMG) activity,
activation of DMT/CR⁺ cells can disrupt ongoing sleep oscillations and, thus, can induce cortical arousal. The extent of this perturbation was different in the two main frequency bands of NREM sleep.

Finally, to determine whether cortical states differ in stimulations resulting in microarousals versus stimulations resulting in sleep-throughs, we compared the cortical evoked responses after DMT/CR⁺ stimulations in these two cases, but found no difference (Supplementary Fig. 7a). We also examined the EEG powers preceding the laser activation (Supplementary Fig. 7b,c). Prestimulation power values up to 40 s before the laser activation did not differ between sleep-throughs and microarousals in the delta and sigma bands. These data show that failure of EMG activation following
EEG changes in cases of sleep-throughs is not the consequence of overt differences in cortical states or receptivity to DMT/CR⁺ activation, but rather a result of the variable efficacy of cortical arousal over the motor responses.

State transitions during REM sleep. Microarousals are also prevalent at the REM–NREM state transitions. In our recording conditions, mice expressed higher spontaneous rates of microarousals during (or after) REM than during NREM sleep (0.012 ± 0.003 Hz versus 0.007 ± 0.001 Hz, respectively, n = 8 mice, two-tailed paired t test, t₁ = −5.451, P = 0.0009). The duration of REM-linked microarousals were significantly longer (7.19 ± 4.4 s for REM versus 3.23 ± 1.27 s for NREM, n = 8, two-tailed paired t test, t₁ = 2.576, P = 0.037). In most cases, the animals returned to NREM following REM-linked microarousals.

One-second photostimulation of DMT/CR⁺ cells during REM sleep evoked microarousals in 4 of 6 animals, with an average probability of 0.57 ± 0.21 (n = 4; Fig. 3m–o). Duration of evoked microarousals during REM was longer than during NREM (5.41 ± 2.3 s versus 3.03 ± 0.75 s, n = 4, two-tailed paired t test, t₁ = 2.82, P = 0.067), mimicking the spontaneous condition. Following evoked microarousals during REM, animals switched to NREM sleep as shown by a gradual increase of delta power (Fig. 3o). This activity pattern recapitulated the spontaneous REM–microarousal–NREM transitions.

These data together demonstrate that graded activation of DMT/CR⁺ neurons is able to evoke distinct, behaviorally relevant arousal patterns such as full-blown persistent arousal, microarousals, and subthreshold disruption of sleep rhythms, as well as state transitions from SWS to wake and REM to SWS.

Distinct arousal via DMT/CR⁺ and sensory nuclei. Arousal from sleep may occur spontaneously, in the absence of any particular sensory stimuli, or as a result of certain sensory stimulation (for example, tactile or acoustic). To compare these two types of arousals under similar experimental conditions, we optogenetically activated the ventrobasal complex (VB), which contains the main somatosensory relay nuclei of the thalamus. We injected Syn-AAV-ChR2 into VB of CR-Cre mice and applied unilateral photostimulation with the 1 s, 10 Hz stimulation protocol (Fig. 4a–c). VB stimulation evoked microarousals in NREM sleep with high probability (Fig. 4c–f and Supplementary Fig. 7d). Microarousals evoked by VB had longer durations (VB, n = 7 mice, 4.55 ± 0.3 s versus DMT, n = 6 mice, 3.69 ± 1.31 s; 2 × one-tailed Mann–Whitney test, P = 0.029) and shorter latencies (VB, 0.36 ± 0.28 s versus DMT: 2.72 ± 1.43 s; 2 × one-tailed Mann–Whitney test, P = 0.0003). However, in contrast to DMT/CR⁺ stimulation, VB stimulations were ineffective during REM sleep (Fig. 4d–f), indicating a qualitative difference between the two conditions. During NREM sleep, the VB stimulations that did not result in EMG activation (that is, sleep-throughs) also evoked transient changes in case of sigma powers (Supplementary Fig. 7e,f).

To study how microarousal properties depend on graded parameters of photostimulation, we established intensity–response curves for both DMT/CR⁺ and VB stimulations by using different laser intensities and plotting arousal probabilities, latencies, and...
Next, we tested whether widespread, effective forebrain outputs. By mapping the axons of DMT/CR+ axons in the mouse forebrain. Injection site of AAV-DIO-ChR2-eYFP in DMT of a CR-Cre mouse (in b). Similar data were obtained in 29 mice. k–m. Normalized peri-event time histogram of evoked MUA (eMUA) responses in PrL (k), NAc (l) and basolateral amygdala (BLA; m) at 1 Hz light stimulation of DMT/CR+ (blue line). Bins in red are substantially larger (+2× s.d.) than the 50-ms prestimulation baseline (green). n. Population data for latencies of eMUA in PrL (± 1.26 ms, n = 6), NAc (± 1.83 ms, n = 4), and BLA (9.75 ± 2.22 ms; n = 4; two-tailed unpaired t test, PrL vs. BLA, t = -2.526, P = 0.0354). o–q. Normalized peri-event time histogram of eMUA responses in PrL (o), NAc (p), and BLA (q) at 10 Hz light stimulation (blue dotted lines) of DMT/CR+. r–t. Normalized heatmap showing peak latencies of eMUA at 10 Hz in PrL (r), NAc (s) and BLA (t). Horizontal lines in box plots indicate medians, box limits indicate first and third quartiles, and vertical whisker lines indicate minimum and maximum values. *P < 0.05. Ac, anterior commissure; BNST, bed nucleus of the stria terminalis; CeA, central amygdala; Cg, cingulate cortex; Ent, entorhinal cortex; Hyp, hypothalamus; IC, insular cortex; M1, primary motor cortex; NB, nucleus basalis; PTA, parietal association cortex; RSA, retrosplenial agranular cortex; S1, primary somatosensory cortex; Sub, subiculum; TeA, temporal association cortex; Tu, olfactory tubercle; vHipp, ventral hippocampus.

Widespread, effective forebrain outputs. Next, we tested whether DMT/CR+ cells have the necessary connectivity and sufficiently strong and synchronous impact on their targets that could support a generalized function like arousal. By mapping the axons of AAV-DIO-ChR2-eYFP infected cells, we found that these cells provided widespread projection to extensive cortical as well as subcortical forebrain targets (Fig. 5a–q and Supplementary Fig. 9a–k). We observed profuse axon arborizations in several layers of the prefrontal (PrL), insular, perirhinal, and entorhinal cortices, as well as in the subiculum. In addition, layer 6 of almost every cortical region was innervated at a lower density. Rich innervation reached the core and shell of nucleus accumbens (NAC), the olfactory tubercle, the basolateral and central amygdala (AMY), and the lateral septum. In addition, the hypothalamus, dorsal striatum, and bed nucleus of stria terminalis also received substantial amounts of fibers.

Next, we tested to what extent the DMT/CR+ cells are responsible for thethalamic inputs to three main forebrain targets. We found that 95–98% of the retrogradely labeled neurons from PrL, AMY, and NAC displayed CR immunoreactivity (Supplementary Fig. 9l–n and Supplementary Table 2), indicating that the CR+ cells provide the vast majority of the total DMT inputs to these forebrain sites.

To assess the impact of DMT/CR+ cells on their targets, we simultaneously recorded in vivo multiunit activity in the PrL, AMY,
and NAc, while optical stimulation was delivered to DMT under urethane anesthesia, following AAV-DIO-ChR2-eYFP injection into the DMT of CR-Cre mice. We found that 1 Hz stimulation reliably activated neurons in all three postsynaptic targets with fast onset (<10 ms), consistent with a monosynaptic glutamatergic pathway (Fig. 5k−n). Additionally, 10 Hz stimulation was effective in driving the targets and did not cause a delay in the timing of response (Fig. 5o−t). The magnitude of the response depended on stimulus intensity. The multiunit and cortical local field potential signal displayed depression at 10 Hz (Supplementary Fig. 10a−d). These data show that DMT/CR+ have widespread projections and can effectively drive their main cortical and subcortical targets.

To determine whether these widespread signals are broadcasted by highly collateralized DMT/CR+ cells or rather by separate populations that project to distinct regions, we used three methods to assess the extent of DMT/CR+ collateralization among multiple target regions. Dual injections of retrograde tracers to PrL−AMY, PrL−NAc, and AMY−NAc resulted in 7−30% of dual-labeled cells (Fig. 6a−c, Supplementary Fig. 11a−l, and Supplementary Table 3), confirming earlier results in rat[29,30]. Dual retrograde tracing is, however, known to grossly underestimate neurons with branching axons. Thus, we labeled isolated DMT/CR+ neurons in 7 mice (9 neurons) with an RNA construct (Pal-eGFP-Sindbis). This method resulted in individual axon arbors branching to reach multiple targets in every case (Supplementary Table 4). Neurons projecting to more than one target (among PrL, AMY, and NAc) were exceedingly rare in other brain regions (Supplementary Fig. 11q,r).

Finally, to quantify DMT/CR+ fibers in one target area that derived from neurons projecting to another, we used a quantitative retro-anterograde tracing method (also called collateral labeling[31]) using the AAV-DIO-ChR2-eYFP virus in CR-Cre animals (see Methods). We systematically examined the collateralization DMT/CR+ cells projecting to PrL (Fig. 6d−f). First, we measured the length of axon arbors in PrL resulting from direct anterograde labeling of the DMT/CR+, thalamus−PrL pathway in a 100-µm-wide cortical slab (11.389 ± 1.00 mm per 10,000 µm3; Fig. 6g). Next, we measured what proportion of these PrL axons originated from neurons that simultaneously projected to other targets. Injecting the same AAV vector into NAc, following retro-anterograde transport...
of the virus, the fibers in PrL were 70 ± 4% (7.956 ± 0.475 mm per 10,000 μm²; Fig. 6g) of the direct DMT→PrL anterograde labeling. These data clearly show that the vast majority of DMT/CR+ axons in PrL arise from cells that also project to NAc. The same retro-antegrade approach applied to AMY labeled 32 ± 11% (3.637 ± 1.637 mm per 10,000 μm²) of the total anterograde fiber length in PrL (Fig. 6f,g), indicating less-wide spread but still substantial collateralization among these two targets.

To test the efficacy of these branching axons to drive postsynaptic targets, we used ‘antidromic–orthodromic’ experiments (see Methods), assuming that antidromic spikes evoked in one part of the axon arbor will invade axon branches targeting another region in an orthodromic manner. Thus, we optogenetically activated DMT/CR+ fibers in NAc and recorded the evoked multiunit activity (MUA) in PrL (Fig. 6h). These experiments measured whether DMT/CR+ cells that have collaterals in NAc are able to drive the activity of their PrL target cells. Indeed, antidromic–orthodromic activation successfully evoked elevated MUA in PrL with short latency (< 10 ms; Fig. 6i). Reliable antidromic–orthodromic responses could also be evoked in basolateral amygdala after NAc stimulation (Fig. 6i,j). Only minor antidromic–orthodromic responses could be detected on the contralateral PrL (Fig. 6i) after NAc stimulations, confirming the low abundance of interhemispheric collateralization (Supplementary Table 4). Antidromic–orthodromic MUA had similar latency to the orthodromically evoked MUA both in PrL and basolateral amygdala. These data show that single DMT/CR+ neurons axons target and are able to simultaneously drive multiple forebrain regions. Such cellular features are optimal to elicit a generalized, brain-wide effect like arousal.

**Selective inputs of DMT/CR+ in mice and humans.** To provide arousal-specific inputs to the forebrain, DMT/CR+ cells might be expected to receive selective inputs from subcortical cell networks. DMT is known to be contacted by many hypothalamic and brainstem afferents3–5, some of which contain glutamate7 or orexin6. Both of these substances play a role in arousal6,33. Thus, as a representative example, here we examined the association of these two major subcortical input systems (orexinergic and glutamatergic) and DMT/CR+ cells in mice and, for comparison, in humans.

In mice thalami, orexin-immunopositive fibers provided a highly selective innervation of DMT/CR+ cells irrespective of the exact nuclear position (Fig. 7a–c). CR+ cells located both in the paraventricular nucleus, as well as those scattered in the rostral intralaminar nuclei, received dense orexinergic inputs, whereas nearby DMT...
regions were devoid of orexin+ fibers. Similar observations were made for subcortical glutamatergic terminals labeled by vesicular glutamate transporter 2 (vGLUT2; Fig. 7d–f).

To study the DMT/CR+ system in humans and its selective subcortical innervation, we performed parallel experiments in postmortem human tissue. In humans (n = 4 brains), CR+ cells were distributed along the ventricular wall of the thalamus17,35 (Fig. 7g–l). As in mice, a substantial number of CR+ cells were also distributed in the intralaminar nuclei. Irrespective of the shape or size of the DMT/CR+ region in humans, orexinergic axon terminals selectively innervated the CR+ cell groups (Fig. 7g–l) in a pattern similar to that observed in mice. Also as in mice, heat maps of vGLUT2 fiber density displayed high values in midline and intralaminar regions in correspondence with the distribution of DMT/CR+ cells, whereas the adjacent regions of the mediodorsal nucleus were practically free of any vGLUT2+ axons, demonstrating highly selective innervation of the DMT/CR+ cells (Fig. 7j–l).

Discussion
In this study, we demonstrated several features of DMT/CR+ neurons that identify them as a key thalamic cell population controlling spontaneous forebrain arousal. DMT/CR+ cells received selective subcortical inputs and provided widely branching, effective glutamatergic outputs to several major forebrain centers. In freely sleeping conditions, DMT/CR+ cells displayed anticipatory, arousal-related activity several seconds before spontaneous behavioral arousal, a major feature of neurons involved in state changes24. Their optogenetic manipulations were able to bidirectionally modulate arousal levels. Graded activation of DMT/CR+ neurons evoked biologically relevant graded arousal patterns and state transitions (sleep–waking, microarousals, persistent arousals) that were qualitatively different from arousal elicited by activation of a sensory system. Based on these data, we propose that DMT/CR+ cells represent a highly specialized neuronal hub that is able to summate and simultaneously transfer brainstem arousal signals to a wide array of subcortical and cortical forebrain structures.

Behavioral patterns elicited by DMT/CR+ cells were biologically relevant. Evoked NREM microarousals were indistinguishable from spontaneous microarousals. Evoked REM microarousals were longer than evoked NREM microarousals, as in the spontaneous condition, and the sequence of state changes induced during REM sleep (REM–microarousal–SWS sequence) also mimicked the natural pattern. Long (10 s) stimulation evoked prolonged, active locomotion for up to tens of minutes, similar to spontaneous arousals which can be observed at the end of the sleep phase.

The connectivity of DMT/CR+ cells was highly specialized and distinct from that of DMT/CR- cells. DMT/CR- cells received selective subcortical inputs in both mice and humans. The similarities in two mammalian species that diverged over 80 million years ago are consistent with an evolutionary ancient role for DMT/CR+ cells in relaying arousal-related information from subcortical centers to the forebrain. Furthermore it supports the notion that CR content, rather than the location of these cells in a specific thalamic nucleus, is the key trait for anatomically defining this system. The highly collateralized output of DMT/CR+ cells could simultaneously activate several forebrain regions. Our antidromic–orthodromic experiments unambiguously demonstrated that axon potentials elicited by optogenetic activation of the axon arbor in one brain regions invaded collaterals that innervated other regions, and hence this method is a useful tool for assessing collateralization.

Arousals elicited by DMT/CR+ always followed a fixed sequence of events. Disruption of EEG rhythms (that is, cortical arousal) was the first and immediate response. This can be attributed to the strong, widespread activation of the postsynaptic forebrain targets with short response latencies (< 10 ms) via the highly collateralized efferent connectivities of these cells. Both delta- and sigma-band activities displayed sharp drops after stimulation. When delta activity returned to baseline with fast kinetics, no behavioral response could be observed (sleep-through). However, if delta activity remained low, EMG activity—that is, behavioral arousal—ensued with a delay of 2–3 s. These data clearly dissociated the electrophysiological and motor components of the arousal (EEG and EMG).

The observed EMG changes are likely the consequence of a multisynaptic top–down influence of the aroused forebrain on brainstem motor centers rather than resulting from direct DMT action on muscle activity, for the following reasons: (i) the altered EEG activity following DMT/CR+ activation always preceded the change in EMG activity, (ii) DMT/CR+ activation was able to alter EEG activity even in the absence of EMG arousal (sleep-throughs), and (iii) DMT/CR+ cells did not have direct descending collaterals to brainstem motor centers. Shorter EMG arousal onset was observed after VB stimulations, which may indicate a different route to motor responses in another arousal system.

Brief DMT stimulations qualitatively changed arousal responses from persistent to microarousals. In these short stimulations, stronger laser intensities (that is, recruiting more DMT/CR+ neurons) had a higher probability of evoking microarousals, but these activations never resulted in prolonged arousal. This indicates that DMT/CR+ neurons may constitute a crucial filter to protect sleep integrity against brief, random increases in brainstem activity during sleep.

In the absence of microarousals, activation of DMT/CR+ cells could still perturb ongoing sleep oscillations. During these subthreshold responses, the two major sleep rhythms (delta and sigma) displayed distinct sensitivity to the thalamic activation. Sleep spindles were more sensitive to perturbations, probably due to the highly intricate network mechanism responsible for their generation, whereas the more robust, globally generated delta activity was more resistant. However, when delta activity was perturbed for longer duration, it was tightly linked to altered EMG activity.

Our data together demonstrate that graded recruitment of DMT/CR+ cells determines a precise behavioral outcome and suggest that the variable optogenetic stimulation we used here imitated the graded activation of DMT/CR+ cells during arousal. Indeed, the increased spontaneous activity of optically tagged DMT/CR+ (but not DMT/CR-) cells anticipated the onset of EMG activity in animals arousing from sleep by several seconds, which to our knowledge has not been described in the forebrain.

DMT has previously been proposed to play important role in arousal4,13. This idea, however, was criticized later, due to the artifacts of electrical stimulation used in the original experiments, and almost entirely abandoned4,14,41. Recent investigations have linked the DMT nuclei to wide range of brain functions including fear learning22,23,42, reward43–45, feeding behavior46,47, and social interactions48. Our present data demonstrate that besides the above specific functions, the highly collateralized DMT/CR+ neurons are involved in arousal, which is a necessary component for the active execution of any given behavior49. It should also be noted that although the above-mentioned studies ascribed various roles to specific DMT pathways (for example, DMT–AMY or DMT–NAc), our present data demonstrate that DMT neurons projecting to a single target are exceedingly rare, if they exist at all. The differences between DMT/CR+ and DMT/CR- in terms of connectivity, activity, and c-Fos expression clearly indicate that it is the cell’s phenotype rather than its location in a particular thalamic nucleus15 that is the critical variable in DMT neuronal functions, underlining the importance of cell-type-specific approach in DMT. Whether specialized and generalized roles are linked to the same or different neuronal subpopulations of DMT/CR+ neurons remains to be established.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and
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Acknowledgements

We thank Z.J. Huang (CSHL, NY, USA) for providing us with the Calb2-Cre mice and C. Cepeda and C. Ballesteros (ITMA, University of Navarre, Spain) for synthesizing the Sindbis-Pal-eGFP RNA construct. The technical help of K. Faddi, K. Varga, A. Jász and E. Szabo-Egyud is acknowledged. The authors thank the Nikon Microscopy Center at IEM, Nikon Austria GmbH, and Auro-Science Consulting Ltd for kindly providing the National Office for Research and Technology (NKT/ANR-09-BLAN-0401, Neurogen to L.A.F : K119650 to P.B.; FK124434 to E.M.; PD124034 to B.B.), "Lendület".

NATURE NEUROSCIENCE | VOL 21 | NOVEMBER 2018 | 1551-1562 | www.nature.com/natureneuroscience
Program of the Hungarian Academy of Sciences (LP2012-23; B.B.), Hungarian Korean Joint Laboratory Program, Hungarian Brain Research Program (grants no. KTIA_NAP_13-2-2015-0010 to F.M., KTIA_NAP_13-2-2014-0016 to P.B. and KTIA_13_NAP-A-1/1 to L.A.), ERC (FRONTHAL, 742595 to L.A.), and HBP-FLAG-ERA (118886 to L.A.).

**Author contributions**

F.M. and L.A. designed the experiments; F.M. and Á.B. performed the anatomical experiments; F.M. and G.K. performed the freely moving EEG recordings; G.K. performed the freely moving data analysis with support from P.B.; K.K. and A.M. performed the freely moving unit recording and data analysis; F.M., V.K., and B.B. performed the behavioral experiments and data analysis; C.D. performed the axon analysis in PrL and the human histology; C.P. performed the electroporation with support from F.C.; I.S. provided the human thalamic samples; F.M., G.K., and L.A. wrote the paper, which was edited by all authors.

**Competing interests**

The authors declare no competing interests.

**Additional information**

Supplementary information is available for this paper at https://doi.org/10.1038/s41593-018-0251-9.

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Methods

Experimental models. Adult (>2 months of age) CR-(Calb2)-Cre (a gift from Z.J. Huang) and CBA/B6f mice from both sexes were used for the experiments. Female mice were used only in the anatomical experiments. Mice were housed in groups of 3–5 mice in transparent Plexiglass cages (367 × 140 × 207 mm) in a humidity- and temperature-controlled environment. During testing, mice were caged individually. Mice were entrained to a 12-h light/dark cycle (light phase from 7:00) with food and water available ad libitum. Testing occurred in the light phase.

Control human thalamic tissues (n = 4) were obtained from male subjects (55–77 years old) who died from causes not linked to brain diseases. None of them had a history of neurological disorders. The four subjects were processed for autopsy in the Department of Pathology, Szent Borbála Hospital, Tatabánya, Hungary. Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. Tissue was obtained and used in a manner compliant with the Declaration of Helsinki.

All procedures were approved by the Research and Institutional Committee of Science of Experimental Medicine of the Hungarian Academy of Sciences, Research Centre for Natural Sciences and the Autonoma University in Madrid and Research Ethics of Scientific Council of Health (ETT TUKB 31443/2011/EKU (518/PI/11)). The experiments were approved by the National Animal Research Authorities of Hungary and Spain.

Viruses. AAV2/5-Ea1a-DIO-ChR2-eYFP, AAV2/5-Ea1a-DIO-SwichRCA-eYFP, and AAV2/5-Ea1a-DIO-eYFP viruses (50–100 nL; Penn Vector Core or UNC; titer: 5 × 10^{12} to 1 × 10^{13} GC/mL) were injected at a rate of 1 nL/s into the dorsal thalamic nuclei of anesthetized C57Bl/6 mice (Ap –0.9 to 1.0 L, 26.8 × 23.2 × 21.0 mm, brain surface) or into a target region: prelimbic cortex (PrL, AP 2, ML 0.3, DV 3.3 mm), nucleus accumbens (NAc; AP 1.4, ML 0.8, DV 4 mm), and amygdala (AMY, AP –1.5, ML 3.3, DV 4 mm). For anatomical analysis, after 3–8 weeks of survival time, mice were perfused first with saline, then with ~150 mL of fixative solution containing 4% PFA in 0.1 M phosphate buffer (pH 7). Tissue blocks were cut on a Vibratome (Leica) to 50-μm coronal sections and fluorescently counterstained for parvalbumin (PV; rabbit, Swant: PV27; 1:3,000), calretinin (CR; mouse, Swant: 683; 1:1,000–3,000), choline acetyltransferase (Chat; mouse, 1:500) and Orexin (Orx; goat, Santa Cruz: sc-8071; 1:2,000–5,000), with secondary antibodies conjugated with a fluorescent IgGs (Alexa Fluor 488 donkey anti-mouse IgG (H+L), Jackson ImmunoResearch, 715-455-150; Alexa Fluor 648 donkey anti-rabbit IgG (H+L), Jackson ImmunoResearch, 715-45-150; Molecular Probes, A21206; Alexa Fluor 488 donkey anti-goat IgG (H+L), Molecular Probes, A11055; CY3 donkey anti-rabbit IgG (H+L), Jackson ImmunoResearch, 715-161-512; CY3 donkey anti-mouse IgG (H+L), Jackson ImmunoResearch, 715-165-151; CY3 donkey anti-goat IgG (H+L), Jackson ImmunoResearch, 715-165-157; and Alexa Fluor 647 donkey anti-mouse IgG (H+L), Jackson ImmunoResearch, 715-605-151) to identify the DMT-targeted cortical and subcortical regions.

In vivo electrophysiology in anesthetized preparations. In vivo recordings were performed 4–8 weeks after the viral injections. For LFP recordings, 16-channel silicon probes were lowered in the PdL (AP +2, ML 2.5, DV 35.5 mm, and 55°) and primary somatosensory cortex (S1; AP 1.2; ML 3.2; DV 1.2 mm, inclined at 20°), Ventral striatal (NAc, AP 1.4, ML 0.8, DV 4 mm) and amygdalar (AMY, AP –1.5, ML 3.3, DV 4 mm) multunit activities (MUA) were monitored via 32-channel linear silicon probes (Neuronexus) labeled by DiI. Two different recording conditions were used. First, the optic fibers were lowered to DMT and classical orthodromic responses were recorded. Next, the optic fibers were repositioned to the NAc, DMT/CR fibers were activated, and the evoked MUA (eMUA) responses were detected in PrL and AMY. Under these latter conditions, action potentials first traveled antidromically, and at a putative branching they could turn to orthodromic direction as well; therefore we call this ‘antidromic–orthodromic’ activation. As NAc contains no CR fibers, and as NAc-projecting neurons are GABAergic and do not project to PdL, fast activation of PdL neurons is only possible via the branching collaterals of DMT/CR fibers.

Silicon probe signals were high-pass filtered (0.3 Hz), amplified (2,000 x) by a 256-channel amplifier, and digitized at 20kHz (Intron Technologies). Single-unit activity was traversed by cross-correlation analysis using microelectrode impedance (>40 MΩ) filled with 0.5 M NaCl and 2% neurobiotin (Vector Laboratories). Neuronal signals were amplified by a DC amplifier (Axoclamp 2B, Molecular Devices) and further amplified and filtered between 0.16 and 2 kHz to characterize EMG activity. Signals were then threshold-determined for each animal (+2.5–3.0× baseline)–each time bin was thus classified as either EMG ON or OFF. Two simple algorithms were applied to reduce fragmentation of EMG ON/OFF states. To reduce the detection of simple muscle twitches and favor to those with real head movements, EMG ON states longer than 0.5 s were kept, and those with shorter duration was regarded as EMG OFF. To reduce fragmentation of active states, EMG OFF states shorter than 2 s were converted to EMG ON states if they were embedded in an EMG ON state (Supplementary Fig. 5).

To reduce the probability of detecting a muscle twitch, we used the following criteria: once an EMG spike reached an amplitude >15% of the mean, the s.d. was calculated for each bin. Plotting a probability distribution for s.d. values of muscle activity, for each animal, we were able to determine a value (peak of the distribution) characteristic for muscle activity in sleep. Then, using a threshold—determined for each animal (+2.5–3.0× baseline)–each time bin was assigned either EMG ON or EMG OFF. Two simple algorithms were applied to reduce fragmentation of EMG ON/OFF states. To reduce the detection of simple muscle twitches and favor to those with real head movements, EMG ON states longer than 0.5 s were kept, and those with shorter duration was regarded as EMG OFF. To reduce fragmentation of active states, EMG OFF states shorter than 2 s were converted to EMG ON states if they were embedded in an EMG ON state (Supplementary Fig. 5).

Stimulus-induced arousals (probability, onset, duration) were evaluated within a 60 s time-window (if not stated otherwise) following stimulus onset. First, all trials were excluded if they (i) occurred with an EMG ON state within 10 s preceding the stimulation, (~15% of trials) or (ii) were transient, for example, no stimulus EMG or REM (protein) that were not preceded by a REM state (~15% of trials). Spontaneous arousals were evaluated by exactly the same criteria, but for nonstimulated periods (beginning of a 60 s time-window, at 61–101 s before stimulus onset). The vast majority of microarousals occurred within 10 s after stimulus onset (Supplementary Fig. 5b); thus, any arousal bout with longer latency...
was not considered here as evoked activity. Those with no evoked EMG activity during the 60 s periods were classified as sleep-throughs. Total stimulated trials containing all kind of stimulus durations and intensities used in the analysis after exclusions (see above) 262 trials for eYFP mice; 3,173 trials for 8 ChR2-DMT mice, and 3,168 trials for 4 VB mice. For microaerobic experiments, 0.5-, 1-, and 2 s stimulations were applied regularly every 3–5 min for 4–6 h per day. Stimulations at different laser intensities and durations were applied randomly. Long, 10 s stimulations were usually delivered only once at the beginning or end of a day. When animals awakened for longer periods, stimulation protocol was paused.

For Figs. 3f–l and 4a–f, we used high laser intensities (13–46 mW for 7 DMT animals) to achieve the possible highest arousal probability, and we used lower intensities (0.001–4.3 mW for 1 DMT stimulation and for 7 VB stimulations) when evoked microaerobic probability was higher than 90%. When subthreshold effect was tested (Fig. 3m–q), 1 s stimulation, we used lower laser intensities (d: 12.5 μm core diameter, NA = 0.22; Thorlabs), all tunnelled in a polyimide tube (0.008 ID, Neuralynx). The tetrode wires were attached to an electrode interface board (EIB-16, Neuralynx) using gold electrode contact pins (Neuralynx). The EMG electrode wire, as well as the ground and reference wires, was soldered to the EIB. Before implantation, tetrodes were cut to their final lengths (200–400 μm) and connected to the optic fiber and tetrode tips; impedances measured at 1 kHz were kept between 300–700 kΩ. Ground and reference screws were implanted in the occipital and parietal bones, respectively; an EMG wire was inserted into the neck muscle. Finally, all pieces were secured to the skull by multiple layers of dental acrylic (Paladur, Heraeus Kulzer). Mice were left at least 7 d to recover, and then handled for several days.

During recordings, animals were left in their home cages to sleep during their light phase (9 a.m.–7 p.m.). Behavior of mice was also video recorded (30 fps). The apparatus consisted of a Plexiglas open field (40 cm × 40 cm × 40 cm). DMT of male CR-Cre mice (3–6 months old) were injected with either AAV2/5-E1A-DIO-SwischR2-CRE-eYFP or AAV2/5-E1A-DIO-eYFP (for controls), and an optic fiber was implanted above the DMT. After 4 weeks, the recording, mice were moved into the open field apparatus. The first minute served as habituation phase, followed by 3 × 3 min of testing periods (OFF–OFF–OFF). Based on the juxtacellular recordings (Supplementary Fig. 4) we applied 2 s of continuous laser-light illumination (10 mW) every 30 s during the ON period to inactivate the DMT/CR+ population. The number of brief behavioral immobility periods (pauses) was quantified as a sign of lowered arousal periods. Pauses longer than 2 s (< 1.7% of total time) were discarded. Within-group and between-groups comparisons were analyzed with repeated-measures ANOVA.

Open field behavior. The apparatus consisted of a Plexiglas open field (40 cm × 40 cm × 40 cm). DMT of male CR-Cre mice (3–6 months old) were injected with either AAV2/5-E1A-DIO-SwischR2-CRE-eYFP or AAV2/5-E1A-DIO-eYFP (for controls), and an optic fiber was implanted above the DMT. After 4 weeks, the mice were moved into the open field apparatus. The first minute served as habituation phase, followed by 3 × 3 min of testing periods (OFF–OFF–OFF). Based on the juxtacellular recordings (Supplementary Fig. 4) we applied 2 s of continuous laser-light illumination (10 mW) every 30 s during the ON period to inactivate the DMT/CR+ population. The number of brief behavioral immobility periods (pauses) was quantified as a sign of lowered arousal periods. Pauses longer than 2 s (< 1.7% of total time) were discarded. Within-group and between-groups comparisons were analyzed with repeated-measures ANOVA.

Retrograde tracing. Single retrograde tracings were carried out with cholesteric tetrades (CTB; List Biological Laboratories; 104) labeled with a chemically stable or radioactive probe (e.g., Fluorogold; FG; Fluorochrome). Both tracers were iontophoretically injected (7/7 s on/off duty cycle; 2–3 μA, for 10 min) into one of the following brain areas: Nac (n = 13), Prl (n = 15), or Bla (n = 15), under ketamine (75 mg/kg)/xylazine (5 mg/kg) anesthesia. After 1 week of survival time, the animals were perfused; brains were extracted and cut into 50 μm thick coronal sections. Free-floating sections of the sections were intensively washed with PBS and then treated with a blocking solution containing 10% NDS and 0.5% Triton-X for 30 min at room temperature. The primary antibodies against CTB (goat; List Biological Laboratories: 703; 1:20,000), FG (rabbit; Chemicon: AB153; 1:10,000), PV (mouse; Swant: PV 234; 1:3,000), and CR (mouse; Swant; 1:3,000) were diluted in PB containing 0.1% NDS and 0.1% Triton-X. After primary antibody incubation (1 d at room temperature or 2–3 days at 4°C), sections were treated with Alexa Fluor 488-conjugated donkey anti-rabbit IgG, Cy3-conjugated donkey anti-goat and Alexa Fluor 488-Cy5-conjugated secondary antibodies for 2 h at room temperature.

c-Fos experiments. Neuronal activation to distinct external stimuli was monitored via c-Fos expression. In wake-sleep cycle experiments, animals were perfused at T7.25 in the sleeping (light) phase and at T13.45 in the waking (dark) phase (n = 8, each). In experiments measuring the effect of increasing arousal, control animals (n = 4) were handled for 2 d; the habituation group (n = 4) was placed in the shock chamber for 5 min without receiving footshock after 2 d of handling, and shocked animals (n = 4) received 2 s, 1-mA footshocks every 30 s for 4 min in the same chamber. After 60 min, animals were perfused. c-Fos and CR double-staining were performed on 50 μm thick coronal sections containing DMT. The primary antibodies against c-Fos (rabbit; Calbiochem: Ab-5; 1:20,000) and calretinin (CR; mouse; Swant; 1:3,000) were diluted in PB containing 0.1% NDS and 0.1% Triton-X. Twenty-four to 48 h after sections were treated with Alexa Fluor 488 and Cy3-conjugated secondary antibodies for 2 h at room temperature. After further PBS washes, sections were mounted in Vectashield and imaged using a confocal microscope.

To quantify c-Fos density, anti-c-Fos was developed with DABNi as a chromogen. The section was dehydrated and then mounted with DePex (Serva, Heidelberg, Germany). All sections used for quantification were developed together for the same duration. Images were taken using a brightfield epifluorescent (Zeiss) or confocal microscope (Zeiss, Olympus, and Nikon). Three sections were analyzed per animal: one each from the rostral, middle, and caudal part of the DMT. Sections separated by 600 μm. The CR contents of single retrogradely labeled cells and CTB+ FG double-retrogradely labeled and c-Fos activated cells were analyzed manually in 60× confocal images. The number of c-Fos-labeled cells was analyzed using a custom-written ImageJ script.

Single-cell labeling and reconstruction. Single DMT neurons were transfected with an RNA construct driving the expression of eGFP associated with the palmitoylation signal GAP43, which specifically directs it to the axonal membrane. Transfections were carried out following a recently described method of in vivo RNA electroporation in a high-saline vehicle. Briefly, borosilicate micropipettes (20-μm tip) were backfilled with an RNA solution (1.8 μg/μl) in a high-saline vehicle (NaCl, DMEM, and 1% Pluronic-PSTH solution). The electrode was positioned into the DMT, and 50–100 nL of the RNA solution was slowly injected using a precision electroporation system (Picospritzer II, Parker Hannifin, Cleveland OH). Two to four 200 Hz trains of 1 ms negative–square pulses at 50 V were then applied using a CS20 stimulator (Ciberrec, Madrid, Spain). After 52–65 h survival, the
animals were perfused and serial 50 µm-thick coronal sections were obtained. First, the GFP signal was intensified with anti-GFP (rabbit, Millipore, 1:10,000) staining, and then sections were counterstained for CR. Finally, all the sections were immunostained, free-floating, in anti-GFP serum followed by incubation with a biotinylated goat anti-rabbit serum (1:300; Sigma-Aldrich, St. Louis, MO, USA) and an avidin-biotin–peroxidase kit (1:300; Vectastain Elite, Vector Laboratories, Burlingame, CA, USA). Sections were serially mounted, dehydrated, and coverslipped with DePeX. The axonal arbor of one cell was reconstructed using a Camera Lucida tube.

Parallel immunostainings of the human and mice thalamus. Postmortem human brains were removed 2–5 h after death. The internal carotid and the vertebral arteries were cannulated, and the brains were perfused first with physiological saline (1:5:1 in 30 min) containing heparin (5 mL), and then with a fixative solution containing 4% paraformaldehyde, 0.05% glutaraldehyde, and 0.2% picric acid (vol/vol) in 0.1 M PB, pH 7.4 (4–5 h; 1.5–2 h). The thalamus was removed after perfusion and was postfixed overnight in the same fixative solution minus the glutaraldehyde. Mouse brains were taken after mice were killed via perfusion. Subsequently, 50-µm-thick coronal sections were obtained for immunohistochemistry using a Leica VTS-1000 Vibratome (Leica Microsystems). The sections were incubated against CR, vGluT2 (mouse, Millipore; MAB5504, 1:3,000) and Orx. The signals were visualized with either DAB or DAB/Ni. Afterwards, in some cases, glucose (7%, wt/vol) was added to the OsO4 solution to preserve color differences. The sections were dehydrated and cover slipped with DePeX.

Estimation of the length of thalamic axons in prelimbic cortex using retro-antegrade viral labeling. We used the fact that in the CR-Cre mice, the Cre-dependent-AAV vectors used here propagated both anterogradely and retrogradely after a sufficiently long survival time (> 6 weeks). Thus, virus injection into target A of DMT/CR+ cells back-labeled CR+ neurons in a retrograde manner. If neurons projecting to target A had collaterals in target B, the virus propagated in an anterograde fashion and visualized axons in target B as well. Obviously, to demonstrate that these axons in target B belonged to the DMT cells and not to other calretinin neurons we should demonstrate that (i) target A contains no calretinin cells which project to target B and (ii) there are no other regions outside DMT that project to both target A and B. For this analysis we selected as the three main targets PrL, NAc, and AMY. Injection of the AAV virus into any of these targets (n = 8, 9, and 12 cases, for PrL, NAc, and AMY, respectively) labeled abundant cell populations in the DMT but no cell bodies could be found in the other two regions, demonstrating the lack of CR+ projecting cells among these three centers. The virus injection, however, did label scattered neurons in the dorsal/caudal hypothalamus and the VTA following PrL and NAc injections, indicating a minor CR+ projection arising outside the thalamus. Using sections from the double retrograde CTB + FG experiments described above, however, we found that only a small fraction of CR+ cells (< 2%) projected to any two of these three targets (Supplementary Fig. 11). Based on these data, we can firmly conclude that following virus injection to PrL, NAc, or AMY, the axons labeled in any other two regions are collaterals of branching DMT/CR+ axons. The experiments indeed demonstrated that injection to any of these three targets labeled abundant axon arbor in the other two.

Next, cortical projections of all DMT cells, as well as NAc- and AMY-projecting DMT cells, were analyzed in frontal cortical sections as follows. The native fluorescent signal was analyzed in 50 µm coronal sections. PrL cortex was divided into 50-µm-thick bins from the pia to the bottom of L6 that were positioned perpendicularly with the pia surface. In each bin, the image stacks were thresholded to optimally select the axonal branches containing tracer. The thresholded image was reduced to skeletons using the following Fiji plugin Plugins/Skeleton/ Skeletonize (2D/3D), then measured by plugin Analyze/Skeleton/Analyze Skeleton (2D/3D). This measured the lengths of the segments of the skeletonized structures. Lengths were summarized for a given area, and then the values were normalized to 10,000 µm³. Six sampling areas were investigated in each animal (n = 9); the results from the same animal were averaged and displayed as mean ± s.d.

The distribution of the vGluT2 terminals was mapped by the optical fractionators method: the numbers of vGluT2+ terminals were counted in 50 × 50 µm counting frames placed on grid points of a 500 × 500 µm sampling grid for human samples and 10 × 10 µm counting frames on grid points of a 50 × 50 µm sampling grid for mouse samples in the upper 5 µm of the section. The density of boutons were normalized to 1/1,000 µm³. Grid data were interpolated with Matlab (MathWorks) and displayed as a heat map. Distributions of CR+ cells were mapped with NeuroLucida (MBF Biosciences) and displayed dot plots (Fig. 7) on the top of the vGluT2 heat maps.

Statistical analysis. No statistical methods were used to predetermine sample size, but our sample sizes are similar to those reported in previous publications. Experiments and/or analysis described in Figs. 1a–e, 2a–e, 3i–k, o–q, 4d–i, 5k–q, and 6a–g and Supplementary Figs. 1e–i, 5b,c, 6–8, 9l–n, 10c,d, 11a–l, and 12p–r were randomized; all other experiments were not. In all experiments, investigators were blinded to allocation and outcome assessments except in the cases of tracer/viral tracing. Data in figures represent mean ± s.e.m. unless otherwise indicated. Data from independent experiments were pooled when possible. Sample sizes were chosen based on pilot experiments to accurately detect statistical significance as well as considering technical feasibility and ethical animal and sample use. Statistical significance was assessed using two-tailed t tests, Mann–Whitney U tests, or ANOVA after testing normality of the dataset, using Kolmogorov–Smirnov tests. Statistical analyses were performed using Statistica (Statsoft) or SPSS 15. Significance is labeled as *P < 0.05, **P < 0.01, ***P < 0.001; n.s., not significant.

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

Data and code availability. The data and code that support the findings of this study are available from the corresponding author upon reasonable request.

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Life Sciences Reporting Summary

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Experimental design

1. Sample size
   Describe how sample size was determined.
   No statistical methods were used to predetermine sample size but our sample sizes are similar to those reported in previous publications (Barthó et al., 2014; Giber et al., 2015)

2. Data exclusions
   Describe any data exclusions.
   Animals with incorrect viral/tracer injections or optic fibers implantation were excluded from the study. From the single-unit freely moving recordings units (n = 4) from different tetrodes which shared a symmetric crosscorrelogram as well as a similar action potential shape in order to avoid enumerating the same cell more than one time.

3. Replication
   Describe the measures taken to verify the reproducibility of the experimental findings.
   Each experiment was reproduced with similar results. To ensure experimental findings can be easily reproduced, we included detailed methods and sources of all reagents and protocols for experiments included in the manuscript.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   Experiments and/or analysis described in Figs 1a-e, 2 a-e, 3i-k, o-q, 4d-i, 5k-q, 6a-g and Supplementary Figs 1e-i, 5 b,c, 6-8, 9l-n, 10c,d, 11a-l and 12p-r were randomized.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   In all experiments investigators were blinded to allocation and outcome assessments except in the cases of tracer/viral tracing.

Note: all in vivo studies must report how sample size was determined and 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
   - Exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   - A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   - A statement indicating how many times each experiment was replicated
   - The statistical test(s) used and whether they are one- or two-sided
   - Only common tests should be described solely by name; describe more complex techniques in the Methods section.
   - A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   - Test values indicating whether an effect is present
   - Provide confidence intervals or give results of significance tests (e.g. P values) as exact values whenever appropriate and with effect sizes noted.
   - A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   - Clearly defined error bars in all relevant figure captions (with explicit mention of central tendency and variation)

See the web collection on statistics for biologists for further resources and guidance.
Describe the software used to analyze the data in this study. Custom MATLAB 2016 codes for single-unit and EEG data; custom ImageJ (FIJI) codes for anatomical data; BONSAI for animal tracking; SpikeDetekt, KlustaViewa and KlustaKwik for single-unit analysis; STATISTICA 13 and SPSS 15 for statistical analysis.

Materials and reagents

8. Materials availability

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

9. Antibodies

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

Secondary antibodies: Alexa488-donkey anti-mouse IgG (H+L), Jackson ImmunoResearch, 715-545-150; Alexa488-donkey anti-rabbit IgG (H+L), Molecular Probes, A21206; Alexa488-donkey anti-goat IgG (H+L), Molecular Probes, A11055; CY3-donkey anti-rabbit IgG (H+L), Jackson ImmunoResearch, 711-165-151; CY3-donkey anti-mouse IgG (H+L), Jackson ImmunoResearch, 715-165-151; Cy3-donkey anti-goat IgG (H+L), Jackson ImmunoResearch, 705-165-147; Alexa647-donkey anti-mouse IgG (H+L); Jackson ImmunoResearch, 715-605-151 (all in 1:500).

The specificity of the primary antibodies was validated by the manufacturer, unless otherwise stated. Specificity of the secondary antibodies was confirmed by verifying a lack of expression in tissue sections in which the primary antibody was omitted from the immunohistochemical protocol.

Animals and human research participants

11. Description of research animals

Provide all relevant details on animals and/or animal-derived materials used in the study.

Adult (> 2 months of age) CR-(Calb2)-Cre (a gift from Z. Josh Huang) and CBA/Bl6J mice from both genders were used for the experiments. Female mice were used only in case of the anatomical experiments.

The four subjects were processed for autopsy in the Department of Pathology, Szent Borbála Hospital, Tatabánya, Hungary. Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. Tissue was obtained and used in a manner compliant with the Declaration of Helsinki.