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Supramammillary glutamate neurons are a key node of the arousal system


Basic and clinical observations suggest that the caudal hypothalamus comprises a key node of the ascending arousal system, but the cell types underlying this are not fully understood. Here we report that glutamate-releasing neurons of the supramammillary region (SuM\textsuperscript{vglut2}) produce sustained behavioral and EEG arousal when chemogenetically activated. This effect is nearly abolished following selective genetic disruption of glutamate release from SuM\textsuperscript{vglut2} neurons. Inhibition of SuM\textsuperscript{vglut2} neurons decreases and fragments wake, also suppressing theta and gamma frequency EEG activity. SuM\textsuperscript{vglut2} neurons include a subpopulation containing both glutamate and GABA (SuM\textsuperscript{vglut2/vgat}) and another also expressing nitric oxide synthase (SuM\textsuperscript{Nos1/vglut2}). Activation of SuM\textsuperscript{vglut2/vgat} neurons produces minimal wake and optogenetic stimulation of SuM\textsuperscript{vglut2/vgat} terminals elicits monosynaptic release of both glutamate and GABA onto dentate granule cells. Activation of SuM\textsuperscript{Nos1/vglut2} neurons potently drives wakefulness, whereas inhibition reduces REM sleep theta activity. These results identify SuM\textsuperscript{vglut2} neurons as a key node of the wake−sleep regulatory system.
In the 1930s, von Economo proposed that damage to the diencephalic-mesencephalic junction was responsible for marked somnolence in patients with epidemic encephalitis lethargica, and Ranson was able to reproduce this somnolence with lesions of the caudal hypothalamus in monkeys. Supporting this observation, Moruzzi and Magoun found that stimulation of the caudal hypothalamic region could elicit wakefulness and electrocortical low-voltage fast activity at a threshold comparable to brainstem arousal sites, and at a lower threshold than for the thalamus. This induced arousal was seen even after destruction of the thalamus, while caudal hypothalamic lesions or inactivation resulted in marked and often prolonged somnolence. These experiments and similar observations in humans supported the existence of a key node of the arousal system within the caudal hypothalamus.
While orexin neurons in the lateral hypothalamus and tuberomamillary histaminergic neurons both promote arousal, lesions of these caudal hypothalamic cell groups have not been found to cause the level of hypersomnolence produced by larger posterior hypothalamic lesions. Based upon its anatomical connectivity and cellular composition, we hypothesized that the supramamillary nucleus (SuM), a region lying just dorsal to the mammillary body in the caudal hypothalamus, might comprise a key site for wake-promotion. The SuM provides extensive innervation of the cerebral cortex, wake-promoting basal forebrain, and the hippocampus. Neurons in the SuM are known to play a role in driving hippocampal theta rhythm, displaying theta band field potentials in freely moving rats, and unit discharges that correlate with this theta modulation. Previous work has examined the relationship of SuM activity to hippocampal field potentials and learning, and suggested a role for SuM neurons in REM sleep, but the SuM has not been studied at all in relation to wakefulness, nor with respect to the possibly distinct roles of SuM subpopulations in REM sleep and wakefulness. Whereas most neurons in the SuM region are glutamatergic, there is anatomical evidence that some of these neurons may contain other neuromodulators including a subpopulation of SuM neurons targeting the hippocampus that contain anatomical markers of both GABA and glutamate release. Here we used a combination of targeted genetic and viral vector technologies in behaving mice to determine whether SuM neurons contribute to electrocortical and behavioral arousal. We then employed in vitro optogenetics and application of a novel non-parametric mapping technique to delimit and characterize three distinct cell populations in the SuM region, each of which differentially contribute to cortical and hippocampal activation and behavioral wake. Our results show that SuM neurons are necessary for normal wakefulness, with activation potently promoting theta and gamma EEG activity and wakefulness. SuM Vglut2 neurons can promote wake when activated and are also necessary for normal REM theta activity. SuM Vglut2 neurons provide dense innervation of the dentate gyrus and CA2, monosynaptically releasing both GABA and glutamate on to the proximal dendrites of dentate granule cells, but only minimally affecting sleep–wake and,
interestingly, do not as potently promote theta EEG activity despite targeting the hippocampal network. In concert with these observations and the described anatomical connectivity of the SuM, these results suggest that SuM\textsuperscript{v glut2} neurons comprise a key nod of the sleep–wake regulatory system that is positioned to control both the hippocampal system and arousal.
Results
SuM\textsuperscript{gglut2} neurons drive wakefulness. In our first experiment, we tested whether glutamate neurons of the caudal hypothalamus drive wakefulness. In order to selectively activate caudal hypothalamic glutamate neurons, including SuM glutamate (SuM\textsuperscript{gglut2}) neurons, we first made large stereotaxic injections of an adenovirus-associated viral vector (AAV) that conditionally expressed an excitatory modified human G-protein coupled muscarinic receptor (hM3Dq)\textsuperscript{44, 45} fused to the mCherry fluorescent protein (AAV-hM3Dq), into the caudal hypothalamus of vglut2\textsuperscript{IREScre} mice (Fig. 1a). Functional transduction of the SuM neurons was shown by administration of the hM3Dq ligand clozapine-n-oxide (CNO, 0.3 mg/kg in 10 μl/g normal saline vehicle i.p.), which drove cFos expression in the transduced neurons (Fig. 1b, upper right). Administration of CNO after injection of AAV-hM3Dq in non-Cre-expressing control littermates, or injections of vehicle (Fig. 1c) after injection of AAV-hM3Dq in Cre-expressing mice was without effect on sleep–wake or cFos expression. In contrast, CNO-driven activation of hM3Dq-expressing SuM\textsuperscript{gglut2} neurons potently promoted continuous wakefulness, in some cases for periods as long as 12 h, during the animals’ normal sleep period (Fig. 1d; administration 3 h after lights-on (Zeitgeber (ZT) 3, 10 AM; Latency to sleep: 0.00014, n = 9, see group data Fig. 1g). In these mice, CNO also caused a significant increase in higher range theta activity (HTtheta 7–13 Hz, paired t-test with Bonferroni correction, p = 0.0148, n = 5, spectral peak at about 9 Hz), and decrease in lower frequency theta EEG activity (low range theta (LTheta) 4–7 Hz, p = 0.0027) (Fig. 1e, f, h, i) with a significant increase in gamma frequency EEG activity (gamma 30–120 Hz, p = 0.0005). Bipolar recording from the hippocampus (n = 6) revealed similar EEG spectral changes, confirming concomitant hippocampal activity with a spectral peak at 8.5–9 Hz (Fig. 1i upper right). Importantly, the repertoire of behavior after CNO in these mice was normal (Supplementary Movie 1), and the amount or speed of locomotor activity did not account for theta activity (mice were often stationary, e.g., see raw data in Fig. 1j, k with periods of low EMG in both examples, but increased theta power (red) in Fig. 1k; Supplementary Movie 1). Given the known role of the SuM in the genesis of the theta activity, we hypothesized that the wake-promoting cell group was either identical with or adjacent to the SuM neurons that promote theta.

We next scattered small injections of AAV-hM3Dq throughout the SuM and adjacent regions to better understand the precise location of the wake-promoting neurons (n = 15). We studied each injection site in detail, noting which nuclei were wholly or partially involved in the injection site and their degree of transduction. We noted that small injection sites flanking the SuM were associated with minimal wakefulness after CNO injection, while those involving the whole of the SuM were associated with the most prolonged wakefulness (see examples, Fig. 2a). To analyze these data in an objective fashion, we made a three-dimensional (3D) map of the injection site, plotted as 30 μm cubic voxels on to a template derived from a high resolution mouse brain MRI scan (Fig. 2b). We then used a non-parametric permutation test of voxels containing transduced neurons and correlated this with the duration of wakefulness resulting from SuM activation (Fig. 2c, n = 21; Methods section). This produced an anatomical z-score, revealing that the combination of voxels that was statistically associated with increased wakefulness corresponds best to the SuM region (false discovery rate < 0.01 and corresponding to a z ≥ 3.216, n = 21). Conversely, caudal hypothalamic regions immediately surrounding the SuM region did not significantly contribute to prolonged wakefulness. Involvement of somewhat further cell groups such as the lateral and perifornical regions may have accounted for the most prolonged duration of wakefulness with the largest injection sites, given the known wake-promoting effect of orexin neurons that also express vglut2 in that region. Smaller injections with transduction constrained within the SuM resulted in just over 5 h of wakefulness (saline 29 ± 5 min versus CNO 310 ± 40 min, n = 6, p = 0.0012), indicating that this caudal hypothalamic cell group independently promotes wakefulness.

SuM actions depend on glutamate. Given the presence of other mediators such as nitric oxide, GABA, and several peptides\textsuperscript{23, 38–41} in the SuM, we next sought to determine whether glutamate is necessary for the wake-promoting effect of SuM neuronal activation. To test this hypothesis, and whether the action of upregulated GABA release might decrease wakefulness, we placed stereotaxic injections of both AAV-Cre and a conditional AAV-hM3Dq into the SuM region of vglut2\textsuperscript{nuc/ox} mice, to concurrently disrupt synaptic glutamate release and express hM3Dq in transduced neurons (Fig. 3a). Because only neurons that had been transduced with the AAV-Cre (and therefore deleted the vglut2 gene) would also express the hM3Dq, CNO would only drive neurons that lacked functional VGLUT2 protein. Histologically, there was effective co-expression of hM3Dq and cFos after CNO over a large part of the SuM (Fig. 3b), with correlated loss of vglut2 in situ hybridization signal (compare Fig. 3c, d). Activation of these SuM neurons resulted in a brief period of wakefulness that was not statistically significant from vehicle injection (vehicle 21 ± 4 (SE) min versus CNO 49 ± 12 min, p = 0.06, n = 6, Fig. 3e–h, summary data Fig. 3i), and was far shorter than comparable activations of neurons without vglut2 deletion (Fig. 1c, d), suggesting that glutamate release from SuM neurons is required for the bulk of the wake-promoting effects of SuM activation. Furthermore, EEG spectral changes associated with activation of SuM vglut2-only neurons were abolished, with no changes in individual spectral bands in any state (p > 0.99 in wake, n = 6, see wake spectra Fig. 3g, h, j).

Fig. 3 Wake-promotion after SuM activation depends on glutamate. a Cartoon showing that after co-injection of AAV-FLEX-hM3Dq-mCherry and AAV-Cre in a vglut2\textsuperscript{floox} mouse, that vglut2 is disrupted in neurons expressing Cre, with Cre positive neurons also able to drive hM3Dq expression. Thus, the only neurons activated by CNO are neurons that lack glutamate release. b mCherry-like immunoreactivity and cFos expression after CNO administration (left) showing where hM3Dq was transduced, and cFos expression consistent with cellular activation after CNO. c Normal expression of vglut2, revealed by in situ hybridization, and d in situ hybridization showing the loss of vglut2-expression in the injection site in the same mouse shown in b (area with near-complete signal loss shown in yellow, area with deletion in some neurons is outlined in orange). e, f Hypograms of two mice showing marked attenuation of wake-promoting effects of SuM activation, consistent with substantial dependence on glutamate release. g, h CSA showing the effect of vehicle and the near-absent effect of activation with CNO in the period shown by the blue and red boxes in c and d, respectively (0–100 Hz, black bar shows the dark period, short white bars represent 20 min and are spaced 1 h apart). f Pooled data (mean ± SEM in 1 h bins, n = 6) after saline and CNO administration, with post hoc comparisons showing that more of the first hour is spent awake after CNO (Bonferroni corrected comparison for 1 h p = 0.0153, all other hours p > 0.99). J Spectral changes associated with SuM activation are also abolished (wake from 6 h after injection shown, mean ± SEM, n = 6)
Chronic vglut2 deletion in the SuM had little effect on amounts of wake, REM, and NREM sleep, all of which remained within normal limits for mice (wake 45.2 ± 2.5%, NREM 48.4 ± 2.4%, REM 6.4 ± 0.2%, n = 6). However, like the ibotenic acid lesions of the rat SuM by Renouard et al. 35, the deletions in these experiments never covered the entirety of the SuM, and because of the time it took for the full deletion to occur and for the animals to recover, there was ample time for compensation. We therefore tested the effect of acute inhibition of the SuM vglut2 cells on wakefulness.
**SuM**<sup>vglut2</sup> neuronal inhibition causes somnolence. To examine the effect of acute inhibition the SuM<sup>vglut2</sup> neurons, we used a conditionally expressed modified inhibitory human muscarinic 4 receptor (hM4Di)<sup>44, 45</sup>. We made large injections of an AAV-FLEX-hM4Di-mCherry (AAV-hM4Di) vector in the posterior hypothalamus of vglut2-IREScre mice (Fig. 4a), deliberately including as much as possible of the SuM<sup>vglut2</sup> cell population (Fig. 4b). We validated that CNO was able to inhibit transduced neurons by recording SuM neurons in vitro in the cell attached mode. CNO (5 µM) reduced the firing frequency of SuM<sup>vglut2</sup> neurons that express hM4Di (firing frequency in control 0.89 ± 0.27 Hz, CNO 0.09 ± 0.05 Hz; p = 0.02 paired t-test, n = 6) (Fig. 4c–g). CNO had no effect in SuM<sup>vglut2</sup> neurons that only express mCherry (firing frequency in control 1.2 ± 0.6 Hz, CNO 1.35 ± 0.65 Hz; p = 0.42 paired t-test, n = 5) (Fig. 4h–j).

In freely behaving mice, increased sleep and fragmentation of wakefulness and sleep were present after CNO injection (Fig. 4k–n). Increased NREM sleep was significant, elevated by about 20% over the 6 h following CNO administration (injection at ZT12, typical time of maximal wakefulness, saline 33.8 ± 2.80% (SEM) versus CNO 41.95 ± 1.49%, 2-way ANOVA, p = 0.0199, n = 6, Fig. 4o). This was at the expense of both wakefulness and REM sleep, although neither reduction reached statistical significance (2-way ANOVA by state; wake—62.4 ± 2.85 versus 55.8 ± 1.94%, p = 0.0659, n = 6; REM —33.1 ± 0.34 versus 23.1 ± 0.72%, p = 0.2614). There was an increased number of sleep bouts in the six hours following injection, as well as a reduction in the maximal time for which wakefulness was maintained (saline 24.5 ± 2.56 (SEM) versus 37.3 ± 2.90 bouts, paired t-test, p = 0.0194, n = 6, Fig. 4p), with the longest wake bouts after saline being significantly longer than after CNO (44.5 ± 7.83 versus 28.2 ± 4.98 min, p = 0.0152, n = 6, Fig. 4q). The spectral power changes were inverse to those of activation, i.e., we saw a significant decrease in high frequency theta and gamma power during wakefulness (HTheta p < 0.0001, and Gamma p = 0.0472, n = 5, Fig. 4r). There was also an increase in the power of low-frequency (4–7 Hz), but not high-frequency (7–13 Hz) theta during REM sleep (p < 0.0001, n = 5, Fig. 4s), with no effect on other REM spectral bands (p > 0.16 for all bands), nor in the power spectrum of NREM sleep (p = 0.38 to > 0.99 for all bands, Fig. 4t). These changes in power spectrum and sleep architecture are consistent with sleepiness during the wake state and the inability to sustain longer bouts of wakefulness, but normal NREM sleep.

**SuM**<sup>vglut2/glat2</sup> neurons. GABAergic neurons surround the mammillothalamic tract as it penetrates the SuM<sup>vglut2</sup>, and spread medially and laterally in the more posterior SuM. The terminals of these neurons in the hippocampus have been reported to contain markers of both GABA and glutamate release<sup>42, 43</sup>. Given that these neurons are within our region of interest, we sought to understand if they were also capable of driving cortical and hippocampal activation and/or behavioral arousal. We first sought to verify the exact location of the GABA/glutamate neurons in the SuM. We performed in situ hybridization for vglut2 mRNA in a vglut2-IREScre reporter mouse (L100 reporter line, see Methods), revealing vglut2 expression in all of the GABAergic neurons surrounding the mammillothalamic tract in the SuM—thus neurons in the SuM are of either SuM<sup>vglut2</sup> or SuM<sup>vglut2/glat2</sup> phenotype (Fig. 5a). The latter neurons are larger than the adjacent ones expressing only vglut2 (20.54 ± 0.64 (SEM) µm longest axis of soma, versus 13.93 ± 0.50 µm, unpaired t-test, p < 0.0001, n = 12,12), and correspond with what has been termed the granicellular field (SuMg)<sup>G</sup>, forming a clear subset of larger neurons in the SuM (Fig. 5b). There were no examples noted of neurons that were only vglut positive within the SuM.

To determine whether these neurons co-release both neurotransmitters, we conditionally transduced channelrhodopsin-2 (ChR2) in the SuM of vglut-IREScre mice (SuM<sup>vglut2</sup>; Fig. 5c), then made whole-cell recordings from dentate granule cells in hippocampal brain slices (Fig. 5d). Photostimulation of Vglut positive axons and terminals originating from the SuM (as shown in the cartoon in Fig. 3d) evoked fast postsynaptic currents in granule cells (15/18 neurons). Application of bicuculline or DNXQ reduced the amplitude of, but did not eliminate, photo-evoked postsynaptic currents. The combination of bicuculline and DNQX completely blocked postsynaptic responses, suggesting mediation by release of both GABA and glutamate, respectively activating GABA-A and AMPA receptors (Fig. 5e). Selective blockade with these two antagonists revealed that GABA-A-mediated postsynaptic currents had typical slower kinetics than AMPA-mediated currents<sup>16</sup> (Fig. 5f; decay time constants: 34.57 ± 5.99 ms for the GABA-A-mediated current and 5.15 ± 1.06 ms for the AMPA-mediated currents, fitting with a single exponential; n = 3). Reversal potentials were −49.74 ± 3.65 mV (SEM) and +11.94 ± 2.02 mV (n = 4 neurons from four brain slices from four mice; p < 0.001, paired t-test), compatible with GABA-A-mediated chloride and AMPA-mediated sodium currents, respectively (Fig. 5g, h). Importantly, both the AMPA-mediated and the GABA-A-mediated photo-evoked postsynaptic currents had a short onset delay characteristic of photostimulation-evoked release (GABA-A-mediated: 5.04 ± 0.37 ms and AMPA-mediated: 4.33 ± 0.23 ms, n = 8, 9 neurons from 3 vglut2-Cre and 5 vglut-Cre mice, respectively), p = 0.002, paired t-test) supporting monosynaptic release of both GABA and glutamate from SuM<sup>vglut2/glat2</sup> terminals onto dentate granule cells (Fig. 5i, j). Overall, photostimulation of the SuM<sup>vglut2/glat2</sup> → DG pathway evoked GABA-A-mediated and AMPA-mediated synaptic responses in a large proportion of dentate granule cells (82%) whereas it evoked solely GABA-A-mediated or AMPA-mediated responses in only 9% and 9% of granule cells (n = 18), respectively. We also conditionally expressed ChR2 in SuM<sup>vglut2</sup> neurons, with

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**Fig. 4** Inhibition of the SuM results in somnolence and wake fragmentation. a Cartoon showing AAV-FLEX-hM4Di-mCherry recombining in vglut2 neurons that express Cre. b Conditional transduction of inhibitory hM4Di in vglut2-IREScre mice (red—brown staining outlined in red; scale bar 500 µm). c–g CNO (5 µM) inhibits the firing of SuM<sup>vglut2</sup> neurons that express hM4Di-mCherry (c current-clamp recordings; d cell attached recordings; e–g expanded traces of the outlined region from d, show firing in control, during CNO application and in washout). h–j CNO (5 µM) has no effect on the firing of SuM<sup>vglut2</sup> neurons that only express mCherry (h; firing frequency, 1-min bin, red arrows indicate the 1-min bins represented in i, j, j, cell attached recordings of the neuron in h). k, j Hypnograms from two mice over 12 h after vehicle and CNO, showing increased sleep and fragmentation of sleep and wakefulness after inhibition of the SuM. m, n CSA after vehicle and CNO from the periods shown by the blue and red boxes, respectively, in c and d (0–100 Hz, black bar shows the dark period, short white bars represent 20 min and are spaced 1h apart). o There is increased overall NREM sleep in the 6 h after CNO administration (~20%); 2-way ANOVA for 6-hourly bins between vehicle and CNO, graph with mean ± SEM, p = 0.0199, p wake-sleep fragmentation with an increase in sleep bouts over the 6 h after CNO when compared with vehicle (paired t-test, mean ± SEM, p = 0.0194). q The length of the longest episode of wakefulness in the same time period was also significantly reduced (paired t-test, mean ± SEM, p = 0.0152). r Power spectrum (band mean ± SEM) with a significant suppression of waking high theta activity (2-way repeated measures ANOVA, Bonferroni ***p < 0.0001) and gamma activity (**p = 0.0472), and s an unexpected increase in low theta power during REM sleep (**p < 0.0001), with t no change in the NREM spectrum.
photostimulation of terminals again evoking both GABA-A-mediated and AMPA-mediated synaptic responses in 90% of dentate granule cells, with AMPA responses alone in 10% of granule cells (n = 9). This is consistent with previous studies indicating that the SuM hippocampal projection arises from the neurons adjacent to the mammillothalamic tract. The mean amplitude and mean onset delay of the photo-evoked glutamate-mediated currents were $-5.36 \pm 1.06$ pA and $4.55 \pm 0.25$ ms (Vh = $-70$ mV) and for the GABA-A-mediated currents were $+7.11 \pm 0.89$ pA and $5.84 \pm 0.25$ ms (Vh = 0 mV; n = 8), again consistent
with a photic stimulation-evoked monosynaptic response (Fig. 5j). Together, these results suggest that the SuM input to the dentate gyrus predominantly originates from neurons that express both vesicular transporters for glutamate and GABA (SuMvgat/vgat2) and that these SuMvgat/vgat2 neurons co-release both transmitters at their synaptic terminals.

We next examined whether SuMvgat/vgat2 neurons, representing about 15% of SuM neurons (see below), were wake-promoting, by activating these neurons in vglut2-IRES-Cre mice after transduction with AAV-hM3Dq (Fig. 6a). Injection sites covering the region of vglut expression in the SuM had a modest but significant effect on the duration of wakefulness following CNO injection (saline 14 ± 3.6 (SE) min, versus CNO 92 ± 10 min, paired t-test, n = 4, p = 0.0027; Fig. 6c–g). Thus, activation of these neurons was significantly less wake-promoting than activation of the entire population of SuMvgat2 neurons (duration of wakefulness, CNO in vglut2-Cre mice 92 ± 10 min, vglut2-Cre mice 310 ± 40 min, n = 4, 6, p = 0.0028).

SuMvgat1/vglut2 neurons drive wake. As previously described in cats and rats50, 51, the mouse SuM contains a subpopulation of Nos1-containing neurons (Fig. 7a, b; see also Allen Mouse Brain Atlas52). Given that cortical Nos1-expressing neurons have been previously implicated in behavioral state control, albeit in the regulation of slow-wave-sleep53, we sought to further understand the anatomic distribution, cellular co-localization and physiology of SuMNos1 neurons. We first examined Nos1 immunoreactivity in Vglut2-Cre-L10 reporter mice, and found Nos1 immunoreactive neurons to be scattered throughout the SuM, representing about 15–20% of vglut2-positive neurons at all rostrocaudal levels (Fig. 7a, b). We then used Nos1-IRES-Cre mice and stereotaxic microinjection of AAV-hM3Dq into the SuM (Fig. 7c) to selectively activate these neurons with CNO (see cFos in transduced neurons, Fig. 7d). Activation of these neurons potently drove sustained wakefulness (latency to sleep after saline 33 ± 4 (SEM) min versus CNO 215 ± 27 min, n = 7, p = 0.0007, Fig. 7e, f), with post hoc comparisons (Bonferroni corrected) showing a significantly elevated wakefulness for four hours after CNO injection (Fig. 7j). While activation of SuMNos1/Vglut2 neurons resulted in lesser wakefulness than activation of all SuMvgat2 neurons in the cases with large injection sites (Fig. 1), the duration of wakefulness was more than twice as long as after activating a similarly numerous population of SuMvgat/Vglut2 neurons, and similar to the amount of wakefulness driven by activation of SuMvgat2 neurons with smaller injection sites constrained within the SuM (see above).

SuMNos1/Vglut2 neurons contribute to REM theta. To examine the necessity of SuMNos1/Vglut2 neurons for normal wakefulness, we made stereotaxic injections of AAV-hM4Di into the SuM of Nos1-IRES-Cre mice (Fig. 8a), with transduction in the SuMNos1/Vglut2 cellular field (Fig. 8b). There was no significant difference in wake, NREM and REM sleep duration when mice were injected with saline (vehicle) or CNO (0.3 or 0.9 mg/kg) (Fig. 8c–h), no overall increase in sleep over the first 6 h (p = 0.31, two-way ANOVA for dose, n = 4, F (2, 6) = 1.423), nor fragmentation of sleep-wake as measured by the number of wake bouts over the 6 h after injection (p = 0.7588, n = 4, t = 0.3363, df = 3) nor changes in the duration of longest wake in this period (saline 26 bouts ± 5.1 versus CNO 0.9 mg/kg 23.75 bouts ± 2.7, p = 0.6028, n = 4, t = 0.5797, df = 3), which had all been noted after chemogenetic inhibition of all SuM glutamate neurons (Fig. 4). However, SuMNos1/Vglut2 neurons by our estimate account for no more than 20% of the SuMvgat2 population, so inhibition of such a small percentage of SuMvgat2 neurons may not have been sufficient to affect overall wake-sleep. On the other hand, powerspectral analysis revealed a significant decrease in high theta power (7–13 Hz) during REM sleep (Fig. 8i) in these mice when given CNO, but without changes in spectra during wakefulness (p = 0.1744, two-way ANOVA, saline versus CNO 0.9 mg/kg, n = 4, F (1, 18) = 1.999; Bonferroni post hoc comparisons by hand p > 0.9999 except for gamma p = 0.2335), suggesting a particularly important role for SuMNos1/Vglut2 neurons in normal REM theta activity (Fig. 8i).

Discussion
Prior studies in humans and animals have indicated that a key, but hitherto uncharacterized, node of the arousal system resides in the caudal hypothalamus1–16. Prior neuroanatomical tracing and lesion studies were consistent with the SuM playing a role in arousal, but the results of the present study for the first time demonstrate that the SuM is a major source of ascending arousal tone. Furthermore, we demonstrate that release of glutamate from SuM neurons is necessary for normal amounts of wake and EEG spectral activity in the high-theta and gamma bands and for consolidation of sleep–wake; and that acute inhibition of SuMvgat2 neurons results in an increase in NREM sleep and fragmentation of wakefulness.

With acute inhibition in mice, we also found no changes in REM sleep amounts, although we did note an increase in low frequency REM theta activity when all SuM neurons were inhibited. SuMNos1/Vglut2 neurons were capable of promoting wakefulness when activated, but inhibition of these neurons did not alter sleep-wake or the waking power spectra, although it did result in decreased theta activity in REM sleep. While chronic cell-specific lesions of the SuM in rats have been reported to have little effect on sleep-wake17, 18 including REM amounts, a recent paper by Renouard et al.35 reported that SuM lesions reduced 7–10 Hz power in the EEG (high theta in the rat) during REM sleep, and that dentate gyrus cFos expression during REM sleep

![Figure 5](https://example.com/figure5.png)
was reduced. Based on our data, the loss of SuM^{Nos1/Vglut2} neurons may have been responsible for decreased REM theta power.

While the effect of inhibition may appear modest, it was actually striking in relation to the effect of acute inhibition of other components of the arousal system with increased NREM sleep and fragmentation of wakefulness. Inhibition or destruction of other accepted components of the ascending arousal system such as the locus coeruleus, tuberomammillary histamine neurons, midline thalamus, or lateral hypothalamic orexin neurons has little or no effect of sleep wake while stimulation in some of these studies can show potent arousal responses. While it is tempting to conclude that the ascending arousal network is highly redundant, it may instead indicate a specificity of function for individual components of the arousal network and it does not mean that these nodes are not playing an important role in intact animals. For example, locus ceruleus acute activation promotes wakefulness, but destruction of these neurons only prevents wake maintenance in a novel environment, without changing baseline sleep–wake.

The role of grandcellular SuM^{V-glut/} neurons remains to be determined. They are apparently less involved than the SuM^{Nos1} cells in the control of wakefulness, but given the extraordinary density of SuM^{V-glut} terminals on the proximal dendrites of dentate granule cells in the supragranular layer, an effect on temporal precision of “dentate gating” and perhaps mnemonic processes appears likely. Furthermore, they may play a role in the plastic changes in the dentate gyrus that occur after status epilepticus and during epileptogenesis, as the SuM terminals sprout and take on an aberrant distribution in the dentate gyrus in a rat pilocarpine model of post-status epilepticus medial temporal lobe epilepsy.

Based on the anatomical connectivity of the SuM, as well as these physiological effects, our findings strongly suggest that the

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**Fig. 6** Activation of SuM^{vgat} neurons results in brief wakefulness. **a** Cartoon showing AAVx-FLEX-hM3Dq-mCherry recombining in vgat neurons that express Cre. **b** An example injection site showing hM3Dq transduction (red outline) in the region containing GABA-glutamate neurons in the SuMg (lower left—duration of wakefulness after CNO in this mouse, scale bar 500 µm). **c, d** Hypnograms showing sleep–wake in two mice with saline, then CNO, with a transient increase in wakefulness. **e, f** Example CSA’s corresponding to the periods shown by the blue and red boxes in **c** and **d**, respectively (0–100 Hz, black bar shows the dark period, short white bars represent 20 min and are spaced 1 h apart).
SuM is a key node in the ascending arousal network, but the precise neuroanatomical pathway mediating these wake-promoting effects is not yet proven. Overall, there are three major groups of projections from the SuM: First, the most appreciated output innervates components of the hippocampal network including the recently described entorhinal-dentate-CA2 circuit, the septal area and diagonal band, subiculum and entorhinal cortex, as well as the medial prefrontal cortices. Second, while not as evident in the Vertes study, there is significant topographic innervation of the cerebral cortex. Finally,
particularly in the lateral-most SuM, there are projections to the upper brainstem and the more lateral basal forebrain components of the arousal system25–27, 52. Thus, particularly to the lateral SuM, there are strong reciprocal connections with upper brainstem and midbrain components of the arousal system36, including the parabrachial complex, locus coeruleus, dorsal raphe and laterodorsal tegmental nuclei30, 32, 51, 57, 60. Ascending connections include basal forebrain GABAergic and cholinergic neurons43; cell groups recently shown to mediate arousal and suppress cortical slowing, respectively. Wakefulness seems most likely due to activation of the second or third group of projections, reliant on the wide distribution of SuM efferents in the cortex and the basal forebrain, similar to those of the locus ceruleus34. Notably, prolonged wake was always associated with increased theta activity, so the activation of these pathways by SuMglut2 or SuMNos1/Vglut2 neurons may not be independent. Consistent with this observation there are both spatial learning impairments32, 33 and amnesia16 after SuM inactivation or lesions in animal models or humans, and, as with the locus ceruleus, reciprocal connection of the SuM with medial prefrontal regions that subserve volitionally directed attention and the volitional maintenance of wakefulness39, 20, 23, 57. Our data taken together with previous studies, is most consistent with SuMglut2 neurons being the long-sought caudal hypothalamic node of the arousal system, playing a role in wakefulness, as well as attention and mnemonic processes.

Methods

Mice. We used adult male heterozygous Vglut2-ires-Cre, Vglut-IRES-Cre, Nosi-ires-Cre, non-expressing littersmates and adult male homozygous Vglut2lox/lox mice (8–12 weeks, 20–25 g; total n = 58 in vivo and n = 8 in vitro), generated as described elsewhere32–34 and obtained from the Lowell and Jackson Laboratories. For Cre mouse lines, Cre-recombine was targeted just distal to the stop codon of the vgat or vglut2 genes, respectively, using an optimized internal ribosomal entry sequence (IRES), with endogenous gene promoters therefore driving Cre-recombine expression via a bicistronic message. Nosi-ires-Cre mice were generated by insertion of an internal ribosome entry site (IRES) along with Cre recombinase into the 5' untranslated region of Nos1. Vglut2 conditional knockout mice have loxp sites flanking the second exon of the Vglut2 gene. Each strain was originally derived from 129S mice or embryonic stem cells, but were back-crossed with C57Bl/6j mice for two or more generations. All mice were bred and genotyped, before and after use, in-house and maintained on a 12 h light–dark cycle with food and water ad libitum. Procedures met National Institute of Health and Guide for the Care and Use of Laboratory Animals standards; all protocols were approved by the Beth Israel Deaconess Medical Center IACUC.

Vectors. We used the pAAV-Irsyn-DIO-kmd3-mCherry and pAAV-Irsyn-DIO- hM4Di-mCherry plasmids (from Dr. Brian Roth, University of North Carolina) to produce the Cre-dependent hM3Dq and hM4Di transgene, packaged by standard triple transfection protocol to generate helper virus-free pseudotyped AAV2/10 virus. An AAV2/10 rep/cap plasmid provided AAV2 replicate and AAV10 capsid functions, while adenoval helper functions were supplied by the pHPel plasmid (Stratagene, La Jolla, CA). Briefly, AAV-293 cells were transfected via calcium phosphate precipitation with 1.33 pmol of pHPel and 1.15 pmol each of AAV2/10 and an AAV vector plasmid (double-fluxed or FLEXED configuration)25. The cells were collected 72 h later and the pellets resuspended in four volumes of PBS, incubated at 4 °C, and centrifuged for 15 min at 200 × g. The supernatant was discarded, and the pellets redissolved three times and centrifuged to produce a clarified viral lysate. Vector stocks were titered by rTqCR (Eppendorf Realplex), ranging from 1 × 1012 to 1 × 1015 copies/ml. The necessity for Cre-enabling for hM3Dq and hM4Di expression was confirmed in vitro using HEK293-Cre cells. These vectors are referred to as AVV-hM3Dq and AVV-hM4D. For experiments in vglut2lox/lox mice, we used an AAV serotype 10 vector expressing Cre-recombine fused to GFP (AAV-Cre; Dr Caroline Bass, University at Buffalo).

Surgery. Mice were anesthetized with ketamine/xylazine (100/10 mg/kg i.p.) and placed in a stereotaxic frame. To selectively transduce hM3Dq receptors in glutamatergic or GABAergic neurons of the SuM (SuMglut2 and SuMnos1, respectively), we placed bilateral injections of AAV-hM3Dq or AAV-hM4D (13–15 nl) side into the caudal hypothalamus (coordinates: AP = −2.45 to −2.7 mm, L ± 0.4 mm, V = −4.9 mm)46 (of Vglut-IRES-Cre and Vglut2-IRES-Cre mice. For controls we injected AAV-hM3Dq and AAV-hM4D in non-Cre-expressing littermates, with no changes in sleep–wake physiology, nor transduction of hM3Dq or hM4D, as we have previously reported31, 66. To transduce hM3Dq only in neurons with disrupted vglut2 expression, we mixed equal portions of AAV-Cre and AAV-hM3Dq, injecting this as above into the SuM of vglut2lox/lox mice. Microinjections were made using a compressed air delivery system67. After injections, headsets were implanted, consisting of a six pin plug (Hedlund Electronics, Inc., Willington, MA) soldered to four EEG screws (Pinnacle Technologies, Lawrence, KS) and two silver pad electrodes, then secured with dental cement. Electrodes were 1 mm rostral and lateral to Bregma, 2 mm caudal and lateral to Bregma, with EMG electrodes over nuchal muscles. In some, a twisted-pair electrode was placed in the dorsal hippocampus unilaterally, with contacts either side of the dentate gyrus pyramidal cell layer (Plastics One, Wallfording, CT). Mice were kept warm until normal activity resumed then housed singly thereafter.

Sleep–wake recordings. Four to 6 weeks later, mice were placed in transparent acrylic barrels in well-ventilated, light-tight and sound attenuating isolation chambers on a 12 h light–dark cycle and at 22 ± 1 °C, with typical bedding material and ad libitum food and water. Mice were connected to a commutator with a triaxial biotelemetry unit (8202ZL, Pinnacle Technologies, Lawrence, KS), and habituated for 72 h. Amplifer units (8200-K1-SL) were connected to a recording computer running Sirenia Acquisition (Pinnacle Technologies) acquiring EEG (one oblique (frontal contralateral parietal) and one longitudinal (frontal parietal) derivation, and bipolar nuchal EMG), sampled at 400 or 600 Hz, with a hardware high-pass filter of 0.5 Hz, most with synchronized infrared video. CNO and vehicle were given in random order in early experiments with large sites of vector transduction (Fig. 1c–f), and no order effect was noted.

Histology. Prior to perfusion, mice received CNO (0.3 mg/kg, IP 10 AM) before deep anesthesia with chloral hydrate (200 mg/kg) and transcardial perfusion (20 ml saline then 100 ml of neutral phosphate-buffered formalin (4%, Fisher Scientific, Pittsburgh, PA). Brains were removed, incubated in 20% sucrose at 4 °C for at least 48 h, then sectioned on a vibratome at 50 μm. Immunostaining was carried out in sections mounted on gelatin coated slides and permeabilized with 0.2% Triton X100 and 5% normal donkey serum. Primary antibodies used were rabbit anti-cFos (1:200; Santa Cruz, CA), and donkey anti-rabbit biotinylated (Invitrogen; 1:500). Sections were incubated overnight with primary antibody followed by 1 h incubation with secondary (goat anti-rabbit Vectastain ABC kit, Vector Laboratories), rinsed in standard saline citrate (SSC) wash, then resonated in standard saline citrate (SSC) wash, then incubated with 0.06% solution of 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich), sometimes with 0.05% CoCl2 and 0.01% NiSO4 (NH4), in PBS plus 0.02% H2O2 for about 5 min (based on observed reaction product). The rabbit polyclonal Fos antibody was raised against synthetic human cFos (4–17) (OncoGence Sciences, rabbit polyclonal cat#AB5). Rabbit polyclonal antibody anti-mCherry was raised against DsRed (Clontech cat#632496) with specificity indicated by the lack of immunostaining in control mice. For secondary antibody immunohistochemical controls, primary antibodies were omitted and the tissue showed no background immunoreactivity. Secondary antibodies included donkey anti-rabbit Alexa Fluor 546 (Invitrogen; 1:500) and donkey anti-rabbit biotinylated (Invitrogen; 1:500).

In situ hybridization revealed the distribution of SuM neurons co-expressing markers of GABA and glutamate release42, 43, using a vgat-IRES-Cre mouse, RNA. Sections were incubated overnight with hybridization buffer containing a 52.5 °C (gift from Dr. Shigafumi Yokota, Shimane University, Izumo, Japan; see Niu et al.,60 for sense controls), rinsed in standard saline citrate (SSC) wash, then 50% formamide after rinsing in Tris-buffered saline (TBS) pH 7.5.

Fig. 7 Activation of SuMnos1 neurons promotes wakefulness. a Nos1 immunohistochemistry (left, white scale bar 50 μm applies to all panels of a and b) reveals a subpopulation of SuM neurons that only rarely contain vglut, shown as gray-white neurons on the merged image (right). b Nos1 immunohistochemistry (left) in a vglut-Cre L10 reported mouse (center) showing that Nos1 neurons are a subset of vglut2 neurons, with no examples of neurons being the long-sought caudal hypothalamic node of the arousal system, playing a role in wakefulness, as well as attention and mnemonic processes.

![Cartoon showing AAVx-FLEX-hM3Dq-mCherry recombining in NOS1 Cre mice](https://example.com/cartoon.png)
After additional TBS rinses, sections were incubated for 30 min in 1% blocking reagent (Roche, cat#11 096 176 001) then overnight in peroxidase conjugated anti-digoxigenin antibody (1:500; Roche cat#11 207 733 910). Sections were rinsed in TBS then reacted with 1:50 Tyramide signal amplification fluorosent kit (TSA Plus-A-Cy3, Perkin Elmer cat#NEL744001KT) diluted in amplification diluent (30 min).

To determine neuronal size, one high power field of the SuM, immediately above the mammillothalamic tract before it emerges dorsally through the mammillary capsule was used to compare the size of SuM\(^{\text{vglut2}}\) and SuM\(^{vglut2}\) neurons. Neurons with clear nuclei and unambiguously distinguishable from adjacent neurons were included and measured through their long axis. Student’s t-test was used for parametric two-tailed comparison.

**Fig. 8** Inhibition of SuM\(^{\text{Nos1}}\) does not cause somnolence, but reduces REM theta activity. **a** Cartoon showing AAV-FLEX-hM4Di-mCherry recombining in Nos1 neurons that express Cre. **b** Conditional transduction of inhibitory hM4Di in Nos1-IRES-Cre mice (mCherry fluorescence with DAPI counterstain; scale bar 500 µm). **c, d** Hypnograms from two mice over 12 h after vehicle and CNO 0.9 mg/kg injected at ZT12 (7 PM, lights-off). Unlike inhibition of all glutamate neurons (Fig. 4), the hypnograms appeared similar regardless of treatment. **e, f** CSA after vehicle and CNO from the periods shown by the blue and red boxes, respectively, in **c** and **d** (0–100 Hz, black bar shows the dark period, short white bars represent 20 min and are spaced 1 h apart). **g, h** There is no change in the amount of wakefulness, NREM or REM sleep (separate two-way ANOVA, wake, REM, and NREM: F (2, 192) = 1.709, 0.8381, 2.04; p = 0.1837, 0.4341, 0.1329, respectively, n = 5). **i** Power spectra (band mean ± SEM) were only changed for REM sleep in post hoc comparisons, with a significant suppression of waking high theta activity (Bonferroni *p = 0.0332, all other bands p > 0.9999, n = 3, t = 3.048, df = 24).

**Data analysis.** Physiological Data Analysis was performed with Spike 2 (version 8.05, Cambridge Electronic Design, UK) using the Ratsleepauto script (version 2.02, Cambridge Electronic Design), with complete manual rescoring to correct any errors. Compressed spectral arrays were created by use of the sonogram feature of Spike 2 (settings 32768 points, Hanning window). Images of sonograms were imported into FIJI (ImageJ 2.0.0, NIH) despeckled before transfer to Inkscape (version 0.91) for figure creation. Spectral data for comparison of discrete spectral bands was performed by ‘Ratsleepauto’ script with 1024 points per Hanning window, saved as text and imported to Excel where 60 Hz artifact was removed by interpolation of points from 59.5 to 60.5 Hz, and spectral values were added into spectral bands (slow wave 0–2 Hz, delta 2–4 Hz, low theta (LTheta) 4–7 Hz, high theta (HTheta) 7–13 Hz, beta 13–30 Hz, gamma 30–120 Hz). Statistical analysis
In vitro experiments. vglut2-IRE-S-Cre (n = 5) and vglut2-IRE-S-Cre (n = 8) mice (8–12 weeks, 20–25 g) were used for in vitro electrophysiological recordings. Mice were injected unilaterally in the SuM (bregma: –2.55 mm; midline 0.4 mm; dorsal surface) with a concentration of 100 µg in a 1:1 mixture of adeno-associated virus (AAV) vectors Cre-dependent channelrhodopsin-2 (ChR2) (AAV-Flex-ChR2[R134H]-YP-FP vector; titer: 6 × 10^{12} genomic copies/ml; circuit mapping experiments) or with 75 nl of AAV-CMV-Cherry or AAV-Flex-mCherry (in vitro CNO experiments). Four to seven weeks after AAV injections, we performed in vitro recordings with further verification of the injection site after recording by fluorescence microscopy.

To prepare brain slices, mice were anesthetized with isoflurane (5% in O2) and a small incision was made on the skull to expose the left hemisphere. After removing the skull bone, the brain was rapidly removed and kept in the cold ACSF for 20 min. The brain was then cut into 250-µm-thick coronal sections using a vibratome (VT1200S, Leica, Bannockburn, IL, USA), then transferred into normal ACSF (in mM): 100 NaCl, 2.5 KCl, 10 HEPES, 1.24 NaH2PO4, 310 CaCl2, 1 EGTA, 5 Mg-ATP, and 0.3 Na-GTP (pH adjusted to 7.2 with KOH, 280 mOsm). CNO was dissolved in normal ACSF to final concentration of 5 µM on the day of the experiment. All drugs used in the in vitro experiments were purchased from Thermo Fisher Scientific (Waltham, MA) with the exception of Bicuculline methiodide (Tocris, Bristol, UK) and CNO (Sigma-Aldrich, St. Louis, MO).

Data availability. All data are available by contacting the corresponding author at npedes@emory.edu.

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Author contributions

NPP, N.V., E.A., P.M.F. and C.B.S. were responsible for study conception and design of experiments. N.P.P., L.F., J.L.W., A.S.A., E.A., and P.M.F. performed experiments.

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