RNA-seq analysis of galaninergic neurons from ventrolateral preoptic nucleus identifies expression changes between sleep and wake

CURRENT STATUS: UNDER REVISION

Xiaofeng Guo
University of Pennsylvania

ORCiD: https://orcid.org/0000-0002-3098-5184

Xiaoling Gao
Peking University People's Hospital

Brendan T. Keenan
University of Pennsylvania

Jingxu Zhu
University of Pennsylvania

Dimitra Sarantopoulou
University of Pennsylvania

Jie Lian
University of Pennsylvania

Raymond J. Galante
University of Pennsylvania

Gregory R. Grant
University of Pennsylvania

Allan I. Pack

pack@pennmedicine.upenn.edu Corresponding Author

ORCiD: https://orcid.org/0000-0002-2879-0484

DOI:
10.21203/rs.2.21230/v1

SUBJECT AREAS
Epigenetics & Genomics
KEYWORDS
ventrolateral preoptic nucleus, galaninergic neurons, functions of sleep, sleep deprivation, next-generation RNA-sequencing
Abstract
Background: Previous studies show that galanin neurons in ventrolateral preoptic nucleus (VLPO-Gal) are essential for sleep regulation. Here, we explored the function of the VLPO-Gal neurons in sleep by comparing their transcriptional responses between sleeping mice and those kept awake, sacrificed at the same diurnal time.

Results: RNA-sequencing (RNA-seq) analysis was performed on eGFP(+) galanin neurons isolated using laser captured microdissection (LCM) from VLPO. Expression of Gal was assessed in our LCM eGFP(+) neurons via real time qPCR and showed marked enrichment when compared to LCM eGFP(-) neurons and to bulk VLPO samples. Gene set analysis utilizing data from a recent single-cell RNA-seq study of the preoptic area demonstrated that our VLPO-Gal samples were highly enriched with galanin-expressing inhibitory neurons, but not galanin-expressing excitatory neurons. A total of 263 genes were differentially expressed between sleep and wake in VLPO-Gal neurons. When comparing differentially expressed genes in VLPO-Gal neurons to differentially expressed genes in a wake-active neuronal region (the medial prefrontal cortex), evidence indicates that both systemic and cell-specific mechanisms contribute to the transcriptional regulation in VLPO-Gal neurons. In both wake-active and sleep-active neurons, ER stress pathways are activated by wake and cold-inducible RNA-binding proteins are activated by sleep. In contrast, expression of DNA repair genes is increased in VLPO-Gal during wakefulness, but increased in wake-active cells during sleep.

Conclusion: Our study identified transcriptomic responses of the galanin neurons in the ventrolateral preoptic nucleus (VLPO) during sleep and sleep deprivation. Data indicate that VLPO contains mainly sleep-active inhibitory galaninergic neurons. The VLPO galanin neurons show responses to sleep and wake similar to wake-active regions, indicating these responses, such as ER stress and cold-inducible RNA-binding proteins, are systemic affecting all neuronal populations. Region-specific differences in sleep/wake responses were also identified, in particular DNA repair, suggesting these could be driven by neuronal activity. Our study expands knowledge about the transcriptional response of a distinct group of neurons essential for sleep.

Background
The preoptic area (POA) of the hypothalamus, particularly the ventrolateral preoptic area (VLPO), is essential for sleep regulation (Saper, Chou et al. 2001). Damage to POA causes insomnia-like sleep disturbances in rats and cats, and the severity of sleep disturbances are correlated to the degree of damage (McGinty and Sterman 1968, Szymusiak and Satinoff 1984, John and Kumar 1998). Single neuron recordings in POA, including VLPO, demonstrated elevated discharge during NREM and REM sleep compared to waking, and the degree of increase reflected sleep depth, indicating their possible involvement in sleep homeostasis (Koyama and Hayaishi 1994, Szymusiak, Alam et al. 1998, Suntsova, Szymusiak et al. 2002). Studies using c-Fos immunoreactivity to indicate recent neuronal activities demonstrated increased numbers of c-Fos positive neurons in VLPO following consolidated sleep compared to wake, and the number of c-Fos positive neurons increased with recovery sleep after sleep deprivation, and showed a positive correlation with the amount of sleep before sacrifice (Sherin, Shiromani et al. 1996, Gong, Szymusiak et al. 2000, Gong, McGinty et al. 2004). These sleep-active neurons, particularly from VLPO, project to histaminergic tuberomammillary nucleus (TMN), serotonergic dorsal raphe (DR), and noradrenergic locus coreleus (LC), and the majority are GABAergic inhibitory neurons, suggesting that during sleep they inhibit multiple monoamine arousal systems (Sherin, Elmquist et al. 1998, Steininger, Gong et al. 2001).

Studies show that ~80% of the neurons that project to TMN from VLPO express galanin (Sherin, Elmquist et al. 1998), and this is conserved across multiple mammalian species (Gaus, Strecker et al. 2002). In a recent study, optogenetic and chemogenetic tools were applied to specifically activate or inhibit VLPO galanin neurons (VLPO-Gal), demonstrating that VLPO-Gal neurons were sleep-active and sleep-promoting (Kroeger, Absi et al. 2018). Chung et al. had conflicting results regarding the function of VLPO-Gal neurons (Chung, Weber et al. 2017); however, this is likely related to the optogenetic stimulation being at too high a frequency, resulting in depolarization block (Kroeger, Absi et al. 2018).

In the present study, to gain further insights into their function we studied these galaninergic neurons in VLPO by examining their behavioral state dependent transcriptional regulation between sleep and wake.

Galanin neuron localization using immunohistochemistry is challenging because galanin peptide is not
mainly located in the cell body, and requires the use of colchicine to block axonal transport. However, this induces cellular stress (Xu, Cortes et al. 1992, Landry, Roche et al. 1997). Hence, we utilized Tg(Gal-EGFP)HX109Gsat mice that express eGFP (enhanced green fluorescent protein) under control of the galanin promoter to aid identification of galanin-expressing neurons in VLPO. Laser capturing microdissection (LCM) was used to isolate individual eGFP-expressing cells. We used our previous design (Mackiewicz, Shockley et al. 2007), comparing gene expression at 3, 6, 9, and 12 hours after lights-on (7AM) between sleeping mice and those kept awake by gentle handling (Franken, Dijk et al. 1991). The same study design has been used in multiple published transcriptomics studies, including surveying bulk tissues of multiple brain regions [cortex and hypothalamus (Mackiewicz, Shockley et al. 2007)] and peripheral tissues [lung and heart (Anafi, Pellegrino et al. 2013)], as well as LCM isolated cholinergic neurons in basal forebrain using microarrays (Nikonova, Gilliland et al. 2017). The same design was also used in a recent bulk tissue study of medial prefrontal cortex using RNA-seq (Guo, Keenan et al. 2019). The similarity of the approach used here and in these prior studies allowed us to compare genes differentially expressed between sleep and wake in VLPO-Gal neurons to genes found differentially regulated in different regions and cell-types.

### Results

**LCM enriched galanin expressing neurons from VLPO**

eGFP(+) cells in VLPO were dissected using LCM as shown in Fig. 1. To validate enrichment of galanin expressing neurons in our LCM samples, we compared expression of Gal among the eGFP(+) neurons, eGFP(-) neurons, and the bulk tissue surrounding VLPO. Significant differences among the three neuron groups were observed for expression of both Gal (Kruskal-Wallis exact p-value = 0.0005) and Aldh1l1 (p = 0.0038). As shown in Fig. 2a, expression of Gal in eGFP(+) samples showed a median fold-increase of 116.9 (95% confidence interval [CI]: 59.5, 497.0; p = 0.0286) when compared to VLPO bulk tissues, whereas the expression of Gal in eGFP(-) samples showed a median fold-reduction of 13.4 (95% CI: 2.0, 34509.4; p = 0.0286) when compared to the VLPO bulk tissue. On the other hand, expression of Aldh1l1, a specific marker of astrocytes, was significantly decreased in both the eGFP(+) (median [95% CI] fold-decrease = 72.5 [4.4, 386.2]; p = 0.0286) and the eGFP(-) (median
[95% CI] fold-decrease = 301.0 [179.4, 1252.9]; p = 0.0286) samples when compared to the VLPO bulk tissue (Fig. 2b). Thus, results indicate effective enrichment of galanin expressing neurons and removal of contaminating astrocytes in our samples using LCM.

Differentially regulated genes in VLPO-Gal neurons between sleep and sleep deprivation

We assessed differentially expressed genes (DEGs) in VLPO-Gal neurons between mice sleep deprived (SDep) for 3, 6, 9 or 12 hours and their time-matched sleep controls (SS) using a moderated F-test in LimmaVoom. A total of 184 and 79 genes were up-regulated by SDep and SS, respectively, based on an FDR of 5% (Fig. 3a). The SDep up-regulated genes were strongly enriched for GO terms related to protein folding, including response to unfolded protein and response to ER stress, whereas the genes up-regulated during sleep were enriched for nucleosome assembly, circadian rhythm, and positive regulation of translation (Fig. 3b). The complete list of differentially expressed genes and DAVID gene ontology analysis results are provided in Additional file 1.

Comparison to the genes differentially regulated between sleep and sleep deprivation in bulk tissue from mPFC

To gain further understanding of the sleep and SDep regulation in VLPO-Gal neurons and explore differences from other brain regions, we utilized data collected from mPFC in a previous RNA-seq study using the same experimental design (i.e. SS vs. SDep over four time-points) (Guo, Keenan et al. 2019). Despite a number of dissimilarities between the two datasets, including brain regions (VLPO vs. mPFC), cell types (enriched galanin neuron vs. bulk tissue), and tissue preparation (micro-punch vs. LCM), 13,068 genes were detected in both studies, corresponding to 85% of the total detected genes across the two studies. As expected, Gal and galanin receptor 1 (Galr1) are among the 788 genes uniquely expressed in VLPO-Gal neurons, and cholinergic receptors (Chrna1-5) and microglia-specific genes involved in immune systems, such as C-type lectin family members (Clec5a and Clec4a2), are among the 1,588 genes uniquely expressed in mPFC samples.

Among the 13,068 genes commonly detected between the two studies, 254 (1.9%) and 6,179 (47.3%) genes were significantly differentially regulated between sleep and SDep in VLPO-Gal neurons and
mPFC, respectively. Among the 254 genes differentially regulated in VLPO-Gal neurons, 181 genes were up-regulated by SDep and 73 genes were up-regulated by sleep. Surprisingly, large proportions of these genes were also regulated by sleep or SDep to the same direction in mPFC, a region that has increased neuronal activity during wake. Specifically, 127 (70.0%) genes were commonly up-regulated by SDep in both VLPO-Gal and mPFC, and 40 (54.8%) genes were found commonly up-regulated by sleep in both VLPO-Gal and mPFC (Table 1 and Additional file 2). As depicted in the heatmap of the fold-changes between SDep and sleep across the four time-points from both studies (Fig. 4a), genes strongly up-regulated by SDep in both VLPO-Gal neurons and mPFC are involved in protein folding/ER stress and transcription regulation, involving multiple heat shock genes (Hspa8 and Dnajb1) and transcription factors (Egr1 and Fosl2). Genes up-regulated by sleep in both VLPO-Gal neurons and mPFC are involved in regulation of translation (cold-inducible RNA-binding proteins Rbm3 and Cirbp), cell differentiation (Gli1, Sox9, and Spata24), beta-alanine transport (Slc6a6), and circadian rhythm (Dbp).

| DEGs | VLPO-Gal | mPFC | Direction of change |
|------|----------|------|---------------------|
| 127  | ↑        | ↑    | up-regulated with SDep |
| 42   | ↑        | ↓    |                      |
| 12   | ↑        | ↑    |                      |
| 40   | ↓        | ↓    | down-regulated with sleep |
| 29   | ↓        | ↑    |                      |
| 4    | ↓        | ↑    |                      |
| 3259 | –        | ↑    | – not significant    |
| 2937 | –        | ↓    |                      |

Comparison was made among the 13,068 genes commonly detected between the two studies (for detailed identities of the genes, see Extended Data Table <link rid="tb1">1</link>-1).

On the other hand, a subset of genes were regulated in opposite directions by sleep and SDep between VLPO-Gal neurons and mPFC (Fig. 4b). Among the 54 genes up-regulated by SDep in VLPO-Gal neurons only, twelve were activated during sleep in mPFC (Table 1). These genes are involved in DNA repair (Herc2 and Mlh3), nervous system development (Epha4, Oprk1, and Srrm4), cellular response to cAMP (Akap9 and Jun), and neuroprotective signaling (Lifr). Similarly, among the 33 genes up-regulated by sleep only in VLPO-Gal neurons, four genes (Mnt, Cry2, Lrrc23, and Igsf11) were activated by SDep in mPFC (Table 1). Max-binding protein (Mnt) is involved in regulation of transcription and cell cycle, and cryptochrome 2 (Cry2) is critical to glucose homeostasis and other
functions related to circadian clock. Therefore, multiple mechanisms exist controlling transcription between sleep and SDep in VLPO-Gal neurons. Some of these mechanisms may be systemic (e.g., due to neurohormonal effects or secondary to temperature change), while others could result from neuronal activation.

VLPO-Gal samples show significant enrichment of gene sets expressed in galanin-enriched inhibitory neurons clusters identified in a single-cell RNA-seq study.

Although there is considerable evidence indicating that galanin cells in VLPO are sleep-active and sleep-promoting (Sherin, Shiromani et al. 1996, Sherin, Elmquist et al. 1998, Gong, McGinty et al. 2004, Kroeger, Absi et al. 2018), a recent study suggests that there might be a mixture of sleep-active and wake-active galanin neurons in VLPO (Chung, Weber et al. 2017). To further assess cellular heterogeneity, we utilized data from a recent single cell RNA-seq study performed in the preoptic area of hypothalamus (Moffitt, Bambah-Mukku et al. 2018). A number of galanin-enriched inhibitory (n = 5) and excitatory (n = 3) neuron clusters were identified. Among them, three inhibitory clusters (i8, i16, i18) and one excitatory cluster (e3) were localized at or near VLPO using multiplexed error-robust FISH (MERFISH). Genes highly expressed in these clusters were selected as gene sets (see Additional file 3 and Methods) and tested for enrichment in our LCM isolated VLPO-Gal samples. Two other inhibitory cell clusters were also considered: i2 [possibly containing the Tac1/Pdyn expressing sleep-active neurons identified by Chung et al. (Chung, Weber et al. 2017)] and i5 (enriched with Pou3f3 instead of galanin near VLPO). The gene sets informed by the galanin-expressing inhibitory neuron clusters i8 and i18 were the most significantly enriched in our VLPO-Gal samples (q = 1.9 × 10^-5 and 7.9 × 10^-5, respectively; Table 2), whereas gene sets based on the galanin-expressing excitatory neuron cluster e3 and the Tac1/Pdyn-expressing inhibitory neuron cluster i2 were not enriched in our VLPO-Gal samples (q = 0.15 and 0.052) (Table 2). These results supported that our LCM isolated samples were enriched with galanin-expressing inhibitory neurons at or near VLPO, but not with galan-expressing excitatory neurons or Tac1/Pdyn expressing inhibitory neurons primarily found outside of VLPO.
Table 2
Gene set enrichment analysis of gal-expressing neuron clusters identified in a single-cell RNA-seq study of preoptic area of hypothalamus (Moffitt, Bambah-Mukku et al. 2018).

| Neuron Cluster | Description    | Number of Genes | p-value       | FDR           |
|----------------|----------------|-----------------|---------------|---------------|
| i8             | Gal-inhibitory | 25              | $3.17 \times 10^{-6}$ | $1.90 \times 10^{-5}$ |
| i18            | Gal-inhibitory | 27              | $2.64 \times 10^{-4}$ | $7.92 \times 10^{-4}$ |
| i5             | Pou3f3-inhibitory | 39          | $1.75 \times 10^{-3}$ | $3.50 \times 10^{-3}$ |
| i16            | Gal-inhibitory | 31              | $7.78 \times 10^{-3}$ | $1.17 \times 10^{-2}$ |
| i2             | Tac1/Pdyn-inhibitory | 54         | $4.38 \times 10^{-2}$ | $5.25 \times 10^{-2}$ |
| e3             | Gal-excitatory | 30              | $1.52 \times 10^{-1}$ | $1.52 \times 10^{-1}$ |

Neuron cluster gene sets were adapted from (Moffitt, Bambah-Mukku et al. 2018) using criteria as described in Method. Description indicates the marker gene enriched in the cluster and if the neuron cluster is inhibitory (starts with “i”) or excitatory (starts with “e”) (for detailed identities of the genes, see Extended Data Table 2–1). P-value and FDR values are two-tailed p-value and Benjamini-Hochberg controlled FDR values obtained from the camera results, respectively.

Additionally, in the mice allowed spontaneous sleep, we observed a moderate, albeit statistically non-significant, positive correlation between the Fos expression and amount of sleep in the last hour before sacrifice (Pearson’s correlation coefficient = 0.33, p = 0.120; Fig. 5). Thus, results suggest a moderately large association between increased levels of Fos and greater sleep amounts in VLPO-Gal neurons, based on definitions provided by Cohen (Cohen 1988). This was found despite the limited range in sleep amounts among mice in our spontaneous sleep group (72–97%; Fig. 5), which were required to have ≥70% in the last hour prior to sacrifice (see Methods). In contrast, in mPFC, Fos showed a moderate negative correlation with the percentages of sleep in the last hour before sacrifice (Pearson’s correlation coefficient = -0.38, p = 0.063; Fig. 5). Together, these results suggest that our VLPO-Gal samples were primarily made up of sleep-active inhibitory neurons.

Discussion
A total of 263 genes were differentially expressed between sleep and sleep deprivation in VLPO-Gal neurons. Pathways related to protein folding/ER stress and regulation of transcription were enriched among the 184 genes up-regulated during sleep deprivation, whereas nucleosome assembly and regulation of translation were functions enriched among the 79 genes up-regulated during sleep. A recent single-cell RNA-seq study of the preoptic area identified a number of galanin-enriched neuron clusters situated at or near VLPO (Moffitt, Bambah-Mukku et al. 2018). Our gene set enrichment analysis demonstrated that our VLPO-Gal samples were highly enriched with genes expressed in inhibitory neuron cluster i8 (close to the core of VLPO), but not enriched with excitatory neuron...
cluster e3 (overlap with VLPO) nor the Tac1/Pdyn expressing inhibitory neuron cluster i2 [outside of VLPO and possibly the sleep-active neurons identified by Chung et al. (Chung, Weber et al. 2017)]. These results indicate that our LCM samples were enriched with galanin-expressing inhibitory neurons in VLPO, which are most likely to be the same group of neurons demonstrated by Kroeger et al. to be sleep-active and sleep-promoting when activated optogenetically at the appropriate stimulation frequency (Kroeger, Absi et al. 2018). Additionally, Fos expression showed a moderate positive correlation with the amount of sleep the animals experienced during the one hour before sacrifice, suggesting that our VLPO-Gal samples contained primarily sleep-active inhibitory neurons.

Genes involved in protein folding and ER stress pathways (Hspa8, Hsph1, and Hspa5) were significantly upregulated by SDep in VLPO-Gal neurons. Previous studies from us and others have demonstrated that these same genes/pathways are consistently up-regulated by wakefulness across multiple brain regions [cortex & hypothalamus (Mackiewicz, Shockley et al. 2007), hippocampus (Vecsey, Peixoto et al. 2012), mPFC (Guo, Keenan et al. 2019), and in wake-active Chat neurons of basal forebrain (Nikonova, Gilliland et al. 2017)], as well as in multiple peripheral tissues [liver (Maret, Dorsaz et al. 2007), lung & heart (Anafi, Pellegrino et al. 2013), and pancreas (Naidoo, Davis et al. 2014)]. This confirms our previous hypothesis that a systemic mechanism might be involved in the activation of protein-folding genes in response to extended wakefulness (Nikonova, Gilliland et al. 2017). The activation of protein-folding genes is not specific to sleep deprivation or related to glucocorticoid signaling. Results from Cirelli et al. demonstrated the same heat-shock genes were also elevated during spontaneous wakefulness (Cirelli, Gutierrez et al. 2004). Similarly, heat shock genes were upregulated in sleep deprived adrenalectomized mice in which glucocorticoid signaling was maintained constant (Mongrain, Hernandez et al. 2010). One possible mechanism for this molecular response is suggested by studies in C. elegans (Taylor and Dillin 2013) that examined the effect of increasing expression of the spliced form of XBP-1 (XBP-1 s), one component of the unfolded protein response (UPR) in neurons. XBP-1 s is a transcription factor that regulates expression of a number of genes involved in ER proteostasis (Shen, Ellis et al. 2005, Acosta-Alvear, Zhou et al. 2007). Transgenic expression of XBP-1 s in neurons in worms results in a cell-non-autonomous response in which the
UPR is activated in multiple tissues (Taylor and Dillin 2013). While the basis of this effect was not fully identified, the mechanism involved neurotransmitter release from small vesicles, since the cell-non-autonomous UPR induction was attenuated in worms lacking UNC-13, which have a deficiency of release from small neuronal vesicles. The investigators did not, however, identify the actual signaling molecule (Taylor and Dillin 2013).

Another set of genes that showed highly consistent changes between VLPO-Gal neurons and other brain regions were cold-inducible RNA-binding proteins (Cirbp and Rbm3). These genes were also elevated during sleep in peripheral tissues (Anafi, Pellegrino et al. 2013). It has been speculated that expressions of these genes are controlled by the small drop in body temperature during sleep (Nikonova, Gilliland et al. 2017). The change in body temperature across the day in mouse is largely caused by differences in behavioral state (sleep/wake) (Hoekstra, Emmenegger et al. 2019). Another possible explanation of the systemic regulation of Cirbp across multiple brain regions and cell-types is its important function in circadian gene expressions (Morf, Rey et al. 2012). A recent study demonstrated that Cirbp altered the changes in clock-gene expression produced by sleep deprivation (Hoekstra, Emmenegger et al. 2019). Moreover, the amount of REM sleep during recovery following sleep deprivation is attenuated in Cirbp knockout mice. Cirbp is at the core of the interaction between circadian gene expression and sleep homeostasis.

Interestingly, a list of genes were regulated between sleep and wake in the opposite direction in VLPO-Gal when compared to our previously published RNA-seq analysis of mPFC (Guo, Keenan et al. 2019). These genes included two genes involved in DNA repair, Herc2 and Mlh3, which were elevated during sleep deprivation in VLPO-Gal samples, but activated during sleep in mPFC. This suggests that the expression of these genes is increased during relative quiescence of the relevant neurons (e.g., during sleep in mPFC and wake in galanin cells in VLPO). Herc2 is an important mediator of DNA damage response and is critical for the ubiquitin-dependent retention of the DNA repair factors on damaged chromosomes (Bekker-Jensen, Rendtlew Danielsen et al. 2010). Mlh3 is important for DNA mismatch repairs (Lipkin, Wang et al. 2000). Both genes have been implicated to be important in cancer and neurodegenerative diseases (Korhonen, Vuorenmaa et al. 2008, Pinto, Dragileva et al.
2013, Bonanno, Costa et al. 2016, Zheng, Huang et al. 2016), indicating their essential roles in maintaining DNA integrity. Recent studies have established direct relationships between neuronal activity and DNA breaks, and DNA breaks in-turn facilitate the induction of immediate early genes (Madabhushi, Gao et al. 2015). Another study used time-lapse imaging of chromosomal markers of live zebrafish and revealed accumulation of DNA breaks during wakefulness and increased chromosome dynamics (essential for DNA damage repair) during sleep (Zada, Bronshtein et al. 2019). This is consistent with our finding of elevated DNA repair pathways during sleep in mPFC. In contrast, VLPO-Gal neurons show increased activity during sleep, and reduced activity during wakefulness (Sherin, Shiromani et al. 1996, Gong, McGinty et al. 2004). This is likely the reason why we observed increased DNA repair during wakefulness in VLPO-Gal neurons.

By comparing the genes differentially regulated between sleep and wake in mPFC to those in VLPO-Gal neurons, there was a large reduction in the extent of transcriptional regulation in VLPO-Gal cells. Among the 13,068 genes commonly detected between the two studies, 6,179 (47.3%) had significantly altered expressions between sleep and sleep deprivation in mPFC, whereas 254 genes (1.9%) were differentially regulated in VLPO-Gal cells at the same FDR cutoff of 5%. Similarly, in our previous microarray studies, 3,988 and 823 genes were identified to be differentially regulated between sleep and wake in cortex and hypothalamus bulk tissues, respectively (Mackiewicz, Shockley et al. 2007). A previous study of Chat neurons in basal forebrain also revealed a small number of genes (n = 10) meeting an FDR cutoff of 5% (Nikonova, Gilliland et al. 2017). These results are consistent with the findings from an in-situ hybridization whole-brain mapping of the effect of sleep deprivation (Thompson, Wisor et al. 2010), which demonstrated that hypothalamus and brainstem are relatively less responsive at the transcriptional level compared to cortex and hippocampus.

Another challenge of studying gene expression regulation of sleep and wake in hypothalamus is the heterogeneous nature of this region. A single-cell RNA-seq study of hypothalamus identified 34 neuronal clusters (Chen, Wu et al. 2017). Another single-cell RNA-seq study of the preoptic area of hypothalamus identified 43 inhibitory neuron clusters and 23 excitatory neuron clusters (Moffitt, Bambah-Mukku et al. 2018). This highlights the need to study cell-type specific gene expression...
changes in highly heterogeneous regions; our LCM study serves as an initial approach in this direction. However, while more uniform than bulk tissue, a limitation in our data is that the results are still obtained from pooling of hundreds of cells. A future single-cell RNA-seq study is desired to further decipher on the single-cell level the transcriptomic changes responding to sleep and wake.

In conclusion, our results indicate that at the transcriptional level both wake-active and sleep-active neurons show increased expression of genes in the endoplasmic reticulum unfolded protein response during wakefulness, while during sleep they show increased expression of genes for cold-induced RNA binding proteins. Thus, these effects are systemic and indeed found in organs other than brain (Maret, Dorsaz et al. 2007, Anafi, Pellegrino et al. 2013, Naidoo, Davis et al. 2014). They are likely not being driven by neuronal activity. In contrast, we found that expression of DNA repair genes is increased in VLPO-Gal cells during wakefulness, but increased in wake-active cells during sleep (Bellesi, Bushey et al. 2016, Guo, Keenan et al. 2019). The increase in expression of these genes during the cell-type-specific quiescent periods suggests expression may be driven by neuronal activity – DNA breaks occur when neurons are active and are repaired when neurons are relatively inactive. The role of these changes in regulation of sleep and wake remains to be determined.

Methods

Mouse experiments

Male Tg(Gal-EGFP)HX109Gsat mice on a Swiss-Webster background that express eGFP under the control of galanin promoter were obtained from the Mutant Mouse Resource & Research Centers. Mice at 2–3 months of age were housed individually in a pathogen-free, temperature (22 °C), and humidity (45–55%) controlled room with a 12-hour/12-hour light/dark cycle with lights-on at 7AM (Zeitgeber time zero or ZT0). Water and food were available ad libitum. Animals were acclimated for 14 days to individual housing and the presence of experimenter prior to the experiment. There were two groups of mice—one that was sleep deprived by gentle handling and one that was allowed to sleep spontaneously. On the days of the experiments, sleep deprivation (SDep) was initiated using gentle handling (Franken, Dijk et al. 1991) at lights-on, and mice were collected following 3, 6, 9, and 12 hours of SDep (n = 5–6 at each time point). For mice that were allowed spontaneous sleep (SS) at
matching time-points (n = 5–6 at each time point), sleep was monitored starting from lights-on; only mice that slept for ≥70% of the last one hour before sacrifice were included in the SS group. Sleep was monitored using the AccuScan infrared monitoring system that detects movement when the mouse crosses electronic beams, and based on previous validation studies performed with Swiss-Webster male mice of 3 months of age using EEG/EMG, sleep was defined as ≥50 seconds of continuous inactivity (Nikonova, Gilliland et al. 2017). Additional mice (n = 6) were also chosen randomly for “baseline” assessment of gene expression and sacrificed at ZT0.

Tissue collection
Mice were euthanized by cervical dislocation and brains were quickly removed and rinsed in nuclease-free 1x phosphate buffered saline (PBS). A 3 mm coronal section containing the ventrolateral preoptic (VLPO) region (Bregma – 1.5 to 1.5) was incubated in ice-cold 4% paraformaldehyde (pH 7.4) for 5 minutes, rinsed twice with PBS, frozen in cryo-embedding medium OCT (Tissue-Tek), and stored at -80 °C until sectioning. 10 µm sections were made in a cryostat kept at -20 °C. Slices were then captured on regular glass microscope slides, 2–3 sections per slide, and stored at -80ºC until use of LCM.

Laser capture microdissection (LCM)
LCM was performed as described previously (Nikonova, Gilliland et al. 2017). Briefly, brain sections were dehydrated immediately before using the LCM protocol: air dry (30 seconds), 75% ethanol (30 seconds), RNase-free water (15 seconds), 75% EtOH (30 seconds), 95% EtOH (30 seconds), 1st 100% ethanol (30 seconds), 2nd 100% ethanol (1 minute), 1st xylene (1 minute), and 2nd xylene (3 minutes), and finally air dried in hood for 5 minutes to remove residual xylene. Between each transition from buffer to buffer, the slides were quickly tapped against the tube rim to remove excess liquid. Dehydrated slides were kept in clean and dried microscope slide boxes containing molecular sieves until ready for LCM. Using the Arcturus XT LCM system (Leica Microsystems, Germany), Gal-eGFP(+) neurons from VLPO were dissected using CapSure HS caps under green fluorescence. The thermoplastic film on the cap was carefully peeled off and immersed in 100 µl lysis buffer (10 mM Tris-HCl [pH 7.5], 50 mM EDTA [pH 8.0], 0.2 M NaCl, 2.2% SDS, RNase inhibitor and 1000 mg/ml
proteinase K) and incubated at 55 °C for 30 minutes with gentle shaking (Khodosevich, Inta et al. 2007). Each sample contained at least 200 cells, and the tube was stored at -80 °C until RNA extraction.

**RNA purification, sequencing and bioinformatics**

RNA extraction was performed using an RNAqueous-Micro Total RNA Isolation Kit (Invitrogen) and on-column DNase digestion was performed using an RNase-Free DNase Set (Qiagen) following the manufacturers' protocols. RNA concentration and qualities were accessed by Agilent 2100 Bioanalyzer RNA 6000 Pico chip. All samples had RIN values above 7.0, with a mean (standard deviation) RIN of 8.21 (0.52). Library preparation was performed using a SMARTer® Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian by Takara Bio, and sequencing done with 150 base-pair paired-end reads on Illumina HiSeq 4000. Raw reads were aligned to the mouse genome build mm9 by STAR version 2.5.3a, and quantified at the gene level using scripts from the PORT pipeline (github.com/itmat/Normalization -v0.8.4-beta). Low-expressing genes were removed by keeping the genes with mean of 10 counts or higher across all samples. A total of 13,856 genes with unique ensemble IDs that passed this filtering criteria were normalized using the “Trimmed Mean of M-values” (TMM) method in the edgeR package (Robinson, McCarthy et al. 2010), and differential expression analyses were performed using the LimmaVoom package (Ritchie, Phipson et al. 2015) (see also Differential gene expression and functional analysis, below).

**Test enrichment of galanin in eGFP(+) samples**

eGFP(-) neurons were collected from the adjacent region using LCM, and, in addition, VLPO region bulk tissue (including the eGFP(+) cells) was punched, as previously described (Guo, Keenan et al. 2019). Enrichment of galanin expressing neurons as well as depletion of astrocytes in eGFP(+) samples were tested by comparing expression of Gal and Aldh1l1 in eGFP(+) samples (n = 4), eGFP(-) samples (n = 4), and the VLPO bulk tissue (n = 3). Real time PCR was performed using the following TaqMan assays: Gal (Mm00439056_m1), Aldh1l1 (mm03048957_m1), Tbp (mm00446971_m1), and Hprt (mm01545399_m1). Relative expressions of Gal and Aldh1l1 were calculated using the deltaCt method (Schmittgen and Livak 2008) with the reference values being the geometric means of the two
housekeeping genes, Tbp and Hprt. Comparisons of Gal and Aldh1l1 expression were made among the LCM samples [eGFP(+) or eGFP(-)] and the VLPO bulk samples on the –deltaCt level using an exact p-value from the Kruskal-Wallis test. To test for enrichment of Gal in eGFP(+) samples, two separate pairwise comparisons were made: eGFP(+) vs. eGFP(-) and eGFP(+) vs. VLPO bulk samples, using a one-tailed Wilcoxon exact test, with median and 95% confidence intervals of differences derived using the Hodges-Lehmann estimate. Similarly, pairwise comparisons of eGFP(+) vs. VLPO bulk and eGFP(-) vs. VLPO bulk were performed to test for depletion of Adlh1l1 in LCM samples compared to the bulk tissue. Analyses were performed using R (www.r-project.org) and SAS Version 9.4 (SAS Institute, Cary, NC).

Differential gene expression and functional analysis
Using the LimmaVoom package in R software (Law, Alhamdoosh et al. 2016), we evaluated the differentially expressed genes (DEGs) between sleep deprived and spontaneous sleep behavioral states (SDep vs. SS) across all time points. Specifically, SDep samples were compared with their time-matched sleep controls at each of the four time durations (3, 6, 9, and 12 hours), and a moderated F-test was used to assess any significant differences at any of the time points. Multiple comparisons correction for statistical significance was applied using the method of Benjamini and Hochberg (Benjamini and Hochberg 1995) to control the overall false discovery rate (FDR) at 5%.

Functional gene ontology (GO) analysis was performed using DAVID Bioinformatics Resources 6.8 (Huang, Sherman et al. 2009). Unless otherwise noted, enrichment of GOs was performed against GOTERM_BP_Direct (biological process). The 13,856 genes used for identifying DEGs were used as the background in all cases. Enrichment of GO terms was defined as a p < 0.05 and containing at least 3 genes.

Gene set enrichment analysis
Gene sets of different neuronal clusters were chosen based on the preoptic area single-cell RNA-seq data by Moffitt et al. (Moffitt, Bambah-Mukku et al. 2018). The following neuron clusters were chosen: galanin-enriched inhibitory neurons at or near VLPO (i8, i16, i18), gal-enriched excitatory neurons near VLPO (e3), Tac1/Pdyn-enriched inhibitory neurons in horizontal limb of the diagonal band of
Broca (HDB) (i2), and Pou3f3-enriched inhibitory neurons at VLPO (i5). Genes detected from the neuron clusters were included in the gene sets if they expressed in > 25% of cells in the cluster and were expressed at least 10% higher than the mean expression averaged across all six chosen clusters. The resulted gene sets were shown in Additional file 3. Gene set enrichment was run using Camera from the Limma package using default parameters against a gene list ranked based on mean expression averaged across all VLPO-Gal samples (Wu and Smyth 2012). Previous studies relying on c-Fos immunoreactivity demonstrated increased c-Fos expression in VLPO with greater amounts of sleep (Sherin, Shiromani et al. 1996, Gong, McGinty et al. 2004). Here, we calculated Pearson’s correlations to test for an association between Fos expression and the percentage of estimated sleep in the last hour prior to sacrifice.

**Comparison to RNA-seq data of medial prefrontal cortex (mPFC)**

Data was obtained from a previously published RNA-seq study performed on mPFC using the same experimental design (Guo, Keenan et al. 2019). Differentially regulated genes between sleep and SDep in mPFC identified from the young animals (2–4 months) were used to compare with data obtained from this study. The mPFC data is publicly available at Gene Expression Omnibus (GSE128770).

**Declarations**

**Ethics approval and consent to participate**

The mice used in this study were purchased from Mutant Mouse Resource & Research Centers. All animal husbandry and procedures were approved by the Institution of Animal Care and Use Committee of the University of Pennsylvania and were carried out in accordance with all National Institutes of Health guidelines.

**Consent for publication**

Not applicable.

**Availability of data and materials**

All raw and processed RNA-Seq data generated in this study have been submitted to the NCBI Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE132308.
**Competing interests**

The authors declare no competing financial interests.

**Funding**

Research was supported by NIH grant P01 AG017628.

**Author's contributions**

XF.G. performed the LCM and RNA-seq experiments and data analysis, drafted and revised the manuscript. XL.G. performed the LCM experiments. B.T.K assisted data analysis and revised the manuscript. J.Z. assisted the mouse and LCM experiments. D.S. and G.R.G. contributed to data analysis. J.L. and R.J.G. contributed to the mouse experiments. A.I.P. designed the study and revised the manuscript. All the authors have read and approved the final version.

**Acknowledgements**

We thank Dr. Jonathan Schug for his assistance on the RNA-Seq experiment, May Chen for animal breeding, and Lin Zhang and May Chen for helping with the sleep deprivation experiment.

**Abbreviations**

DEGs
differentially expressed genes
DR
dorsal raphe
EEG/EMG
electroencephalogram/electromyography
eGFP
enhanced green fluorescent protein
ER
Endoplasmic reticulum
FDR
false discovery rate
Gal
galanin
GO
gene ontology
HDB
horizontal limb of the diagonal band of Broca
LC
locus coreleus
LCM
laser captured microdissection
MERFISH
multiplexed error-robust fluorescence in situ hybridization
mPFC
medial prefrontal cortex
NREM
non-rapid eye movement
POA
preoptic area
REM
rapid eye movement
RNA-seq
RNA-sequencing
SDep
sleep deprivation
SS
spontaneous sleep
TMN
tuberomammillary nucleus
VLPO
ventrolateral preoptic nucleus

References

Acosta-Alvear, D., Y. Zhou, A. Blais, M. Tsikitis, N. H. Lents, C. Arias, C. J. Lennon, Y. Kluger and B. D. Dynlacht (2007). "XBP1 controls diverse cell type- and condition-specific transcriptional regulatory networks." Mol Cell 27(1): 53-66.

Anafi, R. C., R. Pellegrino, K. R. Shockley, M. Romer, S. Tufik and A. I. Pack (2013). "Sleep is not just
for the brain: transcriptional responses to sleep in peripheral tissues." **BMC Genomics** **14**: 362.

Bekker-Jensen, S., J. Rendtlew Danielsen, K. Fugger, I. Gromova, A. Nerstedt, C. Lukas, J. Bartek, J. Lukas and N. Mailand (2010). "HERC2 coordinates ubiquitin-dependent assembly of DNA repair factors on damaged chromosomes." **Nat Cell Biol** **12**(1): 80-86; sup pp 81-12.

Bellesi, M., D. Bushey, M. Chini, G. Tononi and C. Cirelli (2016). "Contribution of sleep to the repair of neuronal DNA double-strand breaks: evidence from flies and mice." **Sci Rep** **6**: 36804.

Benjamini, Y. and Y. Hochberg (1995). "Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing." **Journal of the Royal Statistical Society Series B-Methodological** **57**(1): 289-300.

Bonanno, L., C. Costa, M. Majem, J. J. Sanchez, I. Rodriguez, A. Gimenez-Capitan, M. A. Molina-Vila, A. Vergnenegre, B. Massuti, A. Favaretto, M. Rugge, C. Pallares, M. Taron and R. Rosell (2016). "Combinatory effect of BRCA1 and HERC2 expression on outcome in advanced non-small-cell lung cancer." **BMC Cancer** **16**: 312.

Chen, R., X. Wu, L. Jiang and Y. Zhang (2017). "Single-Cell RNA-Seq Reveals Hypothalamic Cell Diversity." **Cell Rep** **18**(13): 3227-3241.

Chung, S., F. Weber, P. Zhong, C. L. Tan, T. N. Nguyen, K. T. Beier, N. Hormann, W. C. Chang, Z. Zhang, J. P. Do, S. Yao, M. J. Krashes, B. Tasic, A. Cetin, H. Zeng, Z. A. Knight, L. Luo and Y. Dan (2017). "Identification of preoptic sleep neurons using retrograde labelling and gene profiling." **Nature** **545**(7655): 477-481.

Cirelli, C., C. M. Gutierrez and G. Tononi (2004). "Extensive and divergent effects of sleep and wakefulness on brain gene expression." **Neuron** **41**(1): 35-43.

Cohen, J. (1988). *Statistical Power Analysis for the Behavioral Sciences*. New York, NY: Routledge Academic.

Franken, P., D. J. Dijk, I. Tobler and A. A. Borbely (1991). "Sleep-Deprivation in Rats - Effects on Eeg Power Spectra, Vigilance States, and Cortical Temperature." **American Journal of Physiology** **261**(1): R198-R208.

Gaus, S. E., R. E. Strecker, B. A. Tate, R. A. Parker and C. B. Saper (2002). "Ventrolateral preoptic
nucleus contains sleep-active, galaninergic neurons in multiple mammalian species." *Neuroscience* **115**(1): 285-294.

Gong, H., D. McGinty, R. Guzman-Marin, K. T. Chew, D. Stewart and R. Szymusiak (2004). "Activation of c-fos in GABAergic neurones in the preoptic area during sleep and in response to sleep deprivation." *J Physiol* **556**(Pt 3): 935-946.

Gong, H., R. Szymusiak, J. King, T. Steininger and D. McGinty (2000). "Sleep-related c-Fos protein expression in the preoptic hypothalamus: effects of ambient warming." *Am J Physiol Regul Integr Comp Physiol* **279**(6): R2079-2088.

Guo, X., B. T. Keenan, D. Sarantopoulou, D. C. Lim, J. Lian, G. R. Grant and A. I. Pack (2019). "Age attenuates the transcriptional changes that occur with sleep in the medial prefrontal cortex." *Aging Cell*: e13021.

Hoekstra, M. M., Y. Emmenegger, J. Hubbard and P. Franken (2019). "Cold-inducible RNA-binding protein (CIRBP) adjusts clock-gene expression and REM-sleep recovery following sleep deprivation." *Elife* **8**.

Huang, D. W., B. T. Sherman and R. A. Lempicki (2009). "Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources." *Nature Protocols* **4**(1): 44-57.

John, J. and V. M. Kumar (1998). "Effect of NMDA lesion of the medial preoptic neurons on sleep and other functions." *Sleep* **21**(6): 587-598.

Khodosevich, K., D. Inta, P. H. Seeburg and H. Monyer (2007). "Gene expression analysis of in vivo fluorescent cells." *PLoS One* **2**(11): e1151.

Korhonen, M. K., E. Vuorenmaa and M. Nystrom (2008). "The first functional study of MLH3 mutations found in cancer patients." *Genes Chromosomes & Cancer* **47**(9): 803-809.

Koyama, Y. and O. Hayaishi (1994). "Firing of neurons in the preoptic/anterior hypothalamic areas in rat: its possible involvement in slow wave sleep and paradoxical sleep." *Neurosci Res* **19**(1): 31-38.

Kroeger, D., G. Absi, C. Gagliardi, S. S. Bandaru, J. C. Madara, L. L. Ferrari, E. Arrigoni, H. Munzberg, T. E. Scammell, C. B. Saper and R. Vetrivelan (2018). "Galanin neurons in the ventrolateral preoptic area promote sleep and heat loss in mice." *Nat Commun* **9**(1): 4129.
Landry, M., D. Roche, E. Angelova and A. Calas (1997). "Expression of galanin in hypothalamic magnocellular neurones of lactating rats: co-existence with vasopressin and oxytocin." J Endocrinol 155(3): 467-481.

Law, C. W., M. Alhamdoosh, S. Su, X. Dong, L. Tian, G. K. Smyth and M. E. Ritchie (2016). "RNA-seq analysis is easy as 1-2-3 with limma, Glimma and edgeR." F1000Res 5.

Lipkin, S. M., V. Wang, R. Jacoby, S. Banerjee-Basu, A. D. Baxevanis, H. T. Lynch, R. M. Elliott and F. S. Collins (2000). "MLH3: a DNA mismatch repair gene associated with mammalian microsatellite instability." Nat Genet 24(1): 27-35.

Mackiewicz, M., K. R. Shockley, M. A. Romer, R. J. Galante, J. E. Zimmerman, N. Naidoo, D. A. Baldwin, S. T. Jensen, G. A. Churchill and A. I. Pack (2007). "Macromolecule biosynthesis: a key function of sleep." Physiological Genomics 31(3): 441-457.

Madabhushi, R., F. Gao, A. R. Pfenning, L. Pan, S. Yamakawa, J. Seo, R. Rueda, T. X. Phan, H. Yamakawa, P. C. Pao, R. T. Stott, E. Gjoneska, A. Nott, S. Cho, M. Kellis and L. H. Tsai (2015). "Activity-Induced DNA Breaks Govern the Expression of Neuronal Early-Response Genes." Cell 161(7): 1592-1605.

Maret, S., S. Dorsaz, L. Gurcel, S. Pradervand, B. Petit, C. Pfister, O. Hagenbuchle, B. F. O'Hara, P. Franken and M. Tafti (2007). "Homer1a is a core brain molecular correlate of sleep loss." Proceedings of the National Academy of Sciences of the United States of America 104(50): 20090-20095.

McGinty, D. J. and M. B. Sterman (1968). "Sleep suppression after basal forebrain lesions in the cat." Science 160(3833): 1253-1255.

Moffitt, J. R., D. Bambah-Mukku, S. W. Eichhorn, E. Vaughn, K. Shekhar, J. D. Perez, N. D. Rubinstein, J. Hao, A. Regev, C. Dulac and X. Zhuang (2018). "Molecular, spatial, and functional single-cell profiling of the hypothalamic preoptic region." Science 362(6416).

Mongrain, V., S. A. Hernandez, S. Pradervand, S. Dorsaz, T. Curie, G. Hagiwara, P. Gip, H. C. Heller and P. Franken (2010). "Separating the contribution of glucocorticoids and wakefulness to the molecular and electrophysiological correlates of sleep homeostasis." Sleep 33(9): 1147-1157.

Morf, J., G. Rey, K. Schneider, M. Stratmann, J. Fujita, F. Naef and U. Schibler (2012). "Cold-inducible
RNA-binding protein modulates circadian gene expression posttranscriptionally. "Science **338**(6105): 379-383.

Naidoo, N., J. G. Davis, J. Zhu, M. Yabumoto, K. Singletary, M. Brown, R. Galante, B. Agarwal and J. A. Baur (2014). "Aging and sleep deprivation induce the unfolded protein response in the pancreas: implications for metabolism." Aging Cell **13**(1): 131-141.

Nikonova, E. V., J. D. Gilliland, K. Q. Tanis, A. A. Podtelezhnikov, A. M. Rigby, R. J. Galante, E. M. Finney, D. J. Stone, J. J. Renger, A. I. Pack and C. J. Winrow (2017). "Transcriptional Profiling of Cholinergic Neurons From Basal Forebrain Identifies Changes in Expression of Genes Between Sleep and Wake." Sleep **40**(6).

Pinto, R. M., E. Dragileva, A. Kirby, A. Lloret, E. Lopez, J. St Claire, G. B. Panigrahi, C. Hou, K. Holloway, T. Gillis, J. R. Guide, P. E. Cohen, G. M. Li, C. E. Pearson, M. J. Daly and V. C. Wheeler (2013). "Mismatch repair genes Mlh1 and Mlh3 modify CAG instability in Huntington's disease mice: genome-wide and candidate approaches." PLoS Genet **9**(10): e1003930.

Ritchie, M. E., B. Phipson, D. Wu, Y. F. Hu, C. W. Law, W. Shi and G. K. Smyth (2015). "limma powers differential expression analyses for RNA-sequencing and microarray studies." Nucleic Acids Research **43**(7).

Robinson, M. D., D. J. McCarthy and G. K. Smyth (2010). "edgeR: a Bioconductor package for differential expression analysis of digital gene expression data." Bioinformatics **26**(1): 139-140.

Saper, C. B., T. C. Chou and T. E. Scammell (2001). "The sleep switch: hypothalamic control of sleep and wakefulness." Trends Neurosci **24**(12): 726-731.

Schmittgen, T. D. and K. J. Livak (2008). "Analyzing real-time PCR data by the comparative C(T) method." Nat Protoc **3**(6): 1101-1108.

Shen, X. H., R. E. Ellis, K. Sakaki and R. J. Kaufman (2005). "Genetic interactions due to constitutive and inducible gene regulation mediated by the unfolded protein response in C-elegans." Plos Genetics **1**(3): 355-368.

Sherin, J. E., J. K. Elmquist, F. Torrealba and C. B. Saper (1998). "Innervation of histaminergic tuberomammillary neurons by GABAergic and galaninergic neurons in the ventrolateral preoptic
nucleus of the rat." J Neurosci 18(12): 4705-4721.
Sherin, J. E., P. J. Shiromani, R. W. McCarley and C. B. Saper (1996). "Activation of ventrolateral preoptic neurons during sleep." Science 271(5246): 216-219.
Steininger, T. L., H. Gong, D. McGinty and R. Szymusiak (2001). "Subregional organization of preoptic area/anterior hypothalamic projections to arousal-related monoaminergic cell groups." J Comp Neurol 429(4): 638-653.
Suntsova, N., R. Szymusiak, M. N. Alam, R. Guzman-Marin and D. McGinty (2002). "Sleep-waking discharge patterns of median preoptic nucleus neurons in rats." J Physiol 543(Pt 2): 665-677.
Szymusiak, R., N. Alam, T. L. Steininger and D. McGinty (1998). "Sleep-waking discharge patterns of ventrolateral preoptic/anterior hypothalamic neurons in rats." Brain Res 803(1-2): 178-188.
Szymusiak, R. and E. Satinoff (1984). "Ambient temperature-dependence of sleep disturbances produced by basal forebrain damage in rats." Brain Res Bull 12(3): 295-305.
Taylor, R. C. and A. Dillin (2013). "XBP-1 Is a Cell-Nonautonomous Regulator of Stress Resistance and Longevity." Cell 153(7): 1435-1447.
Thompson, C. L., J. P. Wisor, C. K. Lee, S. D. Pathak, D. Gerashchenko, K. A. Smith, S. R. Fischer, C. L. Kuan, S. M. Sunkin, L. L. Ng, C. Lau, M. Hawrylycz, A. R. Jones, T. S. Kilduff and E. S. Lein (2010). "Molecular and anatomical signatures of sleep deprivation in the mouse brain." Front Neurosci 4: 165.
Vecsey, C. G., L. Peixoto, J. H. K. Choi, M. Wimmer, D. Jaganath, P. J. Hernandez, J. Blackwell, K. Meda, A. J. Park, S. Hannenhalli and T. Abel (2012). "Genomic analysis of sleep deprivation reveals translational regulation in the hippocampus." Physiological Genomics 44(20): 981-991.
Wu, D. and G. K. Smyth (2012). "Camera: a competitive gene set test accounting for inter-gene correlation." Nucleic Acids Research 40(17).
Xu, Z., R. Cortes, M. Villar, P. Morino, M. N. Castel and T. Hokfelt (1992). "Evidence for upregulation of galanin synthesis in rat glial cells in vivo after colchicine treatment." Neurosci Lett 145(2): 185-188.
Zada, D., I. Bronshtein, T. Lerer-Goldshtein, Y. Garini and L. Appelbaum (2019). "Sleep increases chromosome dynamics to enable reduction of accumulating DNA damage in single neurons." Nat Commun 10(1): 895.
Zheng, Q., T. Huang, L. Zhang, Y. Zhou, H. Luo, H. Xu and X. Wang (2016). “Dysregulation of Ubiquitin-Proteasome System in Neurodegenerative Diseases.” *Front Aging Neurosci* 8: 303.

**Figures**

![Figure 1](image)

Identification and dissociation of eGFP-galanin neurons from VLPO using LCM. The top left panel shows a representative 10µm brain section at approximately +0.15 Bregma under the bright field with VLPO region being highlighted and the dark black circle shows the placement of the CapSure HS cap; the top right panel shows an enlarged VLPO region under
fluorescence with red circles added facilitating locating of the eGFP-galanin cells to be removed (Before); the bottom left panel shows the captured cells on the CapSure HS cap (Cap) with red circles added to locate the eGFP (+) cells; the bottom right panel (After) shows the brain slide after the eGFP-galanin cells being removed (indicated by the purple circles, which were images direction taken from the instrument, showing the exact location of dissociation).

Figure 2

Confirmation for the enrichment of galanin-expressing neurons in eGFP(+) samples collected using LCM. a) eGFP(+) samples showed a median 116.9-fold increase in the expression of Gal when compared to the VLPO bulk tissues, indicating effective enrichment of galanin-expressing neurons in our eGFP(+) LCM samples. b) Expression of the astrocyte gene Aldh1l1 in both the eGFP(+) and eGFP(-) samples was significantly lower when compared to the VLPO bulk tissues, indicating effective removal of contaminating astrocytes from the neuron samples collected by LCM. Kruskal-Wallis tests were made for comparisons among all three groups. Separate pairwise comparisons were then made between the VLPO bulk tissues and the LCM samples (eGFP+ and eGFP-) using one-tailed Wilcoxon exact test (see Methods).
Differentially expressed genes between spontaneous sleep (SS) and sleep deprivation (SDep). a) 184 genes were identified to be significantly up-regulated by SDep (orange) and 79 genes were identified to be significantly up-regulated by sleep (green) with a cutoff of FDR<0.05. b) Selected Biological Processes GO terms enriched in the differentially expressed genes. Protein folding, response to unfolded protein, and regulation of gene expression are among the functions enriched from the genes up-regulated in SDep (red), whereas nucleosome assembly and regulation of translation are among the functions enriched from the genes up-regulated in sleep (green).
Figure 4

Heat map of fold-changes between sleep deprivation and sleep at each time point in VLPO-Gal neurons and mPFC. Cell colors with red indicates up-regulation with SDep and blue indicates up-regulation with sleep. Row annotations indicate biological functions. a) Genes involved in protein folding and transcription are commonly up-regulated with SDep in both VLPO-Gal and mPFC, whereas translation and cell differentiation are commonly up-regulated with sleep in both VLPO-Gal and mPFC. b) Sixteen genes show opposite direction of change between VLPO-Gal and mPFC cells. Twelve genes were up-regulated with SDep in VLPO-Gal neurons but up-regulated with sleep in mPFC. Functions played by these genes include DNA repair and neuronal development. Four genes (Mnt, Cry2, Lrrc23, and Igf11) were up-regulated with sleep in VLPO-Gal but up-regulated with SDep in mPFC.
Figure 5

Pearson’s correlation of Fos with the percentage of sleep in the last 1hr sleep before sacrifice in VLPO-Gal neurons and medial prefrontal cortex (mPFC). Fos expression in VLPO-Gal neurons had a moderate positive correlation with the percentage of sleep in the last hour before sacrifice, whereas Fos expression in mPFC had a moderate negative correlation with the percentage of sleep in the last hour before sacrifice.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

Additionalfile1.xlsx
NC3Rs ARRIVE Guidelines Checklist.XGuo.1.7.20.pdf
Additionalfile2.xlsx
Additionalfile3.xlsx