Refined anatomical isolation of functional sleep circuits exhibits distinctive regional and circadian gene transcriptional profiles


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ABSTRACT

Powerful new approaches to study molecular variation in distinct neuronal populations have recently been developed enabling a more precise investigation of the control of neural circuits involved in complex behaviors such as wake and sleep. We applied laser capture microdissection (LCM) to isolate precise brain nuclei from rat CNS at opposing circadian time points associated with wake and sleep. Discrete anatomical and temporal analysis was performed to examine the extent of variation in the transcriptional control associated with both identifiable anatomical nuclei and with light/dark cycle. Precise isolation of specific brain nuclei regulating sleep and arousal, including the LC, SCN, TMN, VTA, and VLPO, demonstrated robust changes in gene expression. Many of these differences were not observed in previous studies where whole brain lysates or gross dissections were used to probe for changes in gene expression. The robust and differential profiles of genomic data obtained from the approaches used herein underscore the requirement for careful anatomical refinement in CNS gene expression studies designed to understand genomic control within behaviorally-linked, but functionally isolated brain nuclei.

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1. Introduction

Sleep and wake behavioral arousal states are established through very disparate levels of neuronal activity among functionally interconnected and well-characterized brain nuclei (Saper et al., 2005). A major challenge for understanding genetic control points that could shape complex sleep and wake behavior arises from the hierarchical interaction of behaviorally-related brain nuclei within complex neural networks modulated by unique molecular divergence within each nucleus to modulate the overall behavior. This complexity presents challenges for many of the technologies typically utilized to unravel these neural networks, including microarray-based mRNA profiling, which typically requires several...
Fig. 1 - LCM capture of brain nuclei and RNA isolation. (A) Shown are representative brain sections from which LC, SCN, TMN, VLPO and VTA were captured. Left panel shows sections before LCM dissection, middle panel shows section after removal of area of interest by LCM, and right panel shows region captured by LCM for profiling. (B) Agilent Bioanalyzer traces of RNA recovered from LCM isolated brain regions. (C) 28S/18S ratios and average total RNA yields recovered by LCM capture of each region.
Fig. 2 – mRNA profiles cluster by brain region regardless of animal cohort or microarray platform. (A) Principal component analysis (PCA) of each cohort separated samples based on brain region. For cohort 3, ZT-7 and ZT-19 samples were also resolved by PCA. (B) All 69 SCN, TMN, VTA, VLPO and LC samples were combined and clustered by 2-dimensional agglomerative clustering of all gene models. Note that three independent experiments and animal cohorts are represented, and that some samples from each region were hybridized to Affymetrix and some to Agilent arrays. All samples from each experiment/cohort were ratioed against a control pool of all samples and regions for that experiment/cohort. All samples, with the exception of two SCN samples, clustered correctly by region, and the region signature dominated over the differences between microarray platforms.
micrograms of RNA, and thus relatively large brain tissue samples for analysis. To overcome this requirement experimentally, more tissue, and thus more anatomically and functionally diverse tissues are often utilized (Stansberg et al., 2007). Under this condition, though the anatomically localized fold-changes in mRNA expression may be large (several fold), no change or minor fold-changes (<2-fold) are traditionally detected by microarray profiling of whole brain regions, such as hypothalamus (Letwin et al., 2006; Pavlidis and Noble, 2001). This difficulty is a widespread concern when profiling brain tissue, given the extensive functional and cellular heterogeneity throughout the brain.

Here we describe results of transcriptional profiling obtained through the application of laser capture microscopy (LCM) combined with ultra low input (1 ng) RNA amplification (ULI), which overcomes the difficulties of profiling brain tissue by allowing mRNA profiling of individual brain nuclei and cell populations. We report the light/dark cycle transcriptional variation of 5 different components of a neuronal circuit that controls aspects of sleep and wakefulness, namely the locus coeruleus (LC), suprachiasmatic nucleus (SCN), tuberomammillary nucleus (TMN), ventrolateral preoptic nucleus (VLPO) and the ventral tegmental area (VTA). These studies demonstrate both the specificity and sensitivity of this approach for differentiating brain nuclei according to their mRNA profiles and show that transcriptional changes are associated with physiological activities relevant to the light/dark cycle. Importantly, the characterization of regional and light/dark-responsive signature genes in this study reveals previously unrecognized genomic regulation within these discrete brain nuclei. The substantial gene expression changes detected in each discrete nucleus highlight the complexity of transcriptional regulation in the network of components governing the sleep–wake system and underscore the need for more refined efforts when addressing functional-anatomical circuit oriented gene profiling studies.

2. Results

2.1. Isolation, amplification and profiling of RNA from specific nuclei involved in sleep

Laser capture microdissection was used to isolate LC, SCN, TMN, VLPO and VTA (Fig. 1A) from rats sacrificed during the light period (7 h after lights-on, referred to as ZT-7) and during the dark period (7 h after lights-off, referred to as ZT-19). These times correspond with the middle of normal inactive phase (ZT-7) and mid active phase (ZT-19) of the rat. Regions were identified based on standard anatomical landmarks and stereotaxic coordinates (see Supplemental Table 3) (Paxinos and Watson, 1998). Extraction of high quality RNA was achieved as shown in Fig. 1, with average yields ranging from 10–50 ng of total RNA per region, showing distinct 185s.

Fig. 3 – Identification of transcripts differentially expressed in sleep nuclei. (A) Differentially expressed transcripts were identified by 3-way ANOVA controlling for brain region, animal cohort/array platform and time of day. 10,647 gene models were differentially expressed between brain regions at a p-value of <0.001, and these were clustered by cosine divisive clustering along the horizontal axis. All 69 hybs are shown and are ordered along the vertical axis by brain region, cohort and time of day. Log ratio values are normalized to the average of each gene’s expression in LC, SCN, TMN, VLPO and VTA for each cohort. (B) Top plot indicates the number of the 10,647 gene models in A, expressed differentially between each region at Scheffe post hoc p-value <0.05. Bottom plot gives estimated FDRs for each comparison.
and 28S bands (Figs. 1B, C). 1 ng of RNA isolated from each tissue sample was amplified by two rounds of reverse transcriptase in vitro transcription, consistently yielding more than 10 μg of amplified RNA prior to hybridization to either Affymetrix or Agilent custom genome-wide microarrays.

This experimental design was repeated independently for three separate rat cohorts (4–8 rats per arm). The first cohort of four animals was sacrificed at ZT-7 and then profiled on custom rat 44K Agilent arrays. LC, SCN, TMN, VLPO and VTA were profiled from each animal (4 animals × 5 regions = 20 samples). The second cohort of four animals was also sacrificed at ZT-7 but samples were hybridized to custom rat Affymetrix arrays. LC, SCN, TMN, VLPO and VTA were profiled from each animal (4 animals × 5 regions = 20 samples). The third cohort consisted of 8 animals, four of which were sacrificed at ZT-7 and four of which were sacrificed at ZT-19, and was hybridized to custom rat 44K Agilent arrays. LC, SCN, VLPO and VTA were profiled from each animal (4 animals × 4 regions × 2 time points = 32 samples). A total of 72 samples were profiled. Three of the 72, namely one VLPO sample from cohort one, one TMN sample from cohort 2 and one VTA sample from cohort 2, were outliers in either RNA or array quality control metrics and were excluded from all analysis. For each or the 69 remaining individual samples, ratio experiments were generated by comparing against a pool of all samples for that cohort.

### 2.2. Determination of the transcriptional profiles of brain nuclei involved in response to light/dark phase and arousal

Principal component analysis tightly clustered each of the remaining samples from each cohort according to brain region (Fig. 2A) demonstrating the ability of this approach to differentiate the profiled regions. To further examine the quality of the profiles and regional differences, ratio experiments from all cohorts, array platforms, time points and regions were combined at the gene level, and clustered by unsupervised 2-dimensional cosine divisive clustering (Fig. 2B). All 69 samples, with the exception of 2 SCN samples, clustered correctly by brain region demonstrating that regional expression differences were the predominant source of variation between the profiles, and that similar results were obtained between animal cohorts and across both Agilent and Affymetrix platforms. Within regions, profiles clustered by platform as expected due to differences in probe design and hybridization chemistry. Together, these findings indicated that large transcriptional profiles differentiated the brain nuclei profiled, and confirmed consistent performance of the LCM-ULI samples on either the Agilent or Affymetrix platforms.

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**Fig. 4** - Taqman confirmation of regional differences identified by microarray. Relative mRNA expression in VTA and VLPO measured by Taqman (bottom) for 13 genes identified as differentially expressed between VTA and VLPO by microarray (top). Microarray results for all 13 genes examined were confirmed by Taqman. Genes in heatmap are aligned with bar graph data. 15 VTA samples and 15 VLPO samples are ordered along the vertical axis by region and array platform. Log ratio values are normalized to the average of each gene’s expression in all regions for each cohort.
To identify transcripts that were differentially expressed between the five brain regions, we combined all three cohorts of animals and analyzed the profiles at the gene level by 3-way ANOVA controlling for platform, time and brain region. This analysis identified a total of 10,647 transcripts that were differentially expressed at an ANOVA *p*-value of <0.001 between the five regions examined (Table S1, Fig. 3A). The false discovery rate was estimated by Monte Carlo analysis to be less than 0.3%. Fig. 3B shows the number of these transcripts that were differentially expressed between each region as well as the estimated false discovery rate of each comparison. For example, 2795 transcripts were differentially expressed between VTA and VLPO with Scheffe post-hoc analysis *p* <0.05. The estimated false discovery rate of this comparison is <0.16% or <6 transcripts.

We used Taqman on a separate cohort of animals to validate a number of the transcripts identified by microarray as differentially expressed. 13 genes with consistent expression at both time points were selected from the gene list with 3-way ANOVA *p*<0.001 and post-hoc *p*-value<0.05 between VTA and VLPO with no additional filters applied. Additional genes that are in opposing light/dark phases between the regions can be seen in Figs. 8B,9B and 10.

2.3. Identification of biomarkers of discrete brain nuclei

We further filtered the set of 10,647 gene clusters to identify transcripts expressed uniquely in one region compared to each of the other 5 regions. Differences in expression between these nuclei could serve to define unique functions for these discrete regions and are potential markers for labeling specific brain structures. We identified a number of transcripts that were expressed higher in LC (843), SCN (221), TMN (251), VLPO (147) and VTA (502) with a post-hoc ANOVA *p*<0.05 relative to each of the other 4 brain regions (Fig. 5, Table 1, Table S1). Of these 1964 transcripts, 1266 (64%) and 713 (36%) exhibited a greater than 1.5-fold or 2.0-fold expression difference, respectively, between at least two regions.

Based on the large number of genes expressed within each LCM-isolated nucleus, we hypothesized that transcripts uniquely expressed within discrete brain nuclei may not be detected when profiling gross brain regions as is traditionally done for gene expression profiling. To test this hypothesis we compared the transcripts identified above to be unique in one of 5 nuclei to their expression observed in gross dissections of brain and peripheral tissues in a previously reported atlas of rat gene expression (Fig. 6) (Bleasby et al., 2006). We limited the analysis to the 713 transcripts identified above as expressed >2-fold more in one LCM isolated region versus one or more other regions. This ensured probe functionality and that these transcripts were robustly expressed above background in at least one of the five brain nuclei profiled. 411 (58%) of these 713 transcripts were profiled in the rat body atlas. Of these, 251 (61%), were expressed greater than 2-fold above background in

![Fig. 5](image-url) - Genes uniquely expressed in one of 5 nuclei. 1964 gene models were expressed higher in one brain region than in any of the other 4 regions examined (post hoc *p*<0.05 for each region). These genes are sorted by region of highest expression along the horizontal axis. All 69 hybs are shown and are ordered along the vertical axis by brain region, cohort and time of day. Log ratio values are normalized to the average of each gene’s expression in LC, SCN, TMN, VLPO and VTA for each cohort.
at least one of the 9 gross brain regions in the atlas (whole brain, brain stem/cerebellum, cerebellum, forebrain, hippocampus, hypothalamus, medulla oblongata/pons, mesencephalon, telencephalon), indicating that these transcripts were detected in the gross brain region profiles. However, 126 (31%) of the 411 transcripts had probe intensities less than 1.5 fold above background in all of the gross brain regions indicating these transcripts were not reliably detected as expressed in the gross region profiles. 96 (76%) of the 126 transcripts not detected in the gross brain region profiles were detected as expressed (greater than 2-fold above background) in at least one of 52 peripheral tissues or cell lines in the rat body atlas, indicating that the microarray probes were functional and that these transcripts are expressed in rat cells. Taken together,
this analysis confirms that profiling of gross brain regions fails to detect transcripts confined to discrete brain nuclei as well as localized differences in gene expression within gross regions. Thus, these experiments demonstrate the importance of finely mapping gene expression throughout the brain, and provide scientific rationale for further refined analysis of anatomically distinct regions.

2.4. Identification of light/dark-regulated transcripts in sleep circuits

To assess whether we could detect nuclei-enriched light/dark cycle differences in gene expression using the LCM-ULI approach, we compared the mRNA profiles of each region 12 h apart; during the middle of the normal active (ZT-19) and inactive (ZT-7) phase. A large number of unique and overlapping signatures associated with ZT-7 or ZT-19 were detected across the regions profiled. Interestingly, the VTA had the largest number of regulated signature genes (1369, error weighted ANOVA p-values <0.001) whereas the LC, SCN and VLPO had similar numbers of signature gene models (564, 633 and 579 respectively, error weighted ANOVA p-values <0.001) (Fig. 7). The estimated false discovery rates at this p-value threshold are 7%, 27%, 20% and 13%, for VTA, LC, SCN and VLPO, respectively, and also demonstrate the robustness of the VTA signature relative to the other areas. In total, 2766 time-regulated genes were identified with p<0.001 in one or more regions, and 29 of these were regulated (post-hoc p<0.05) in each of the 4 brain regions (see Tables 2 and S2). Light/dark regulated transcripts with estimated FDR<5% are highlighted for each region in Tables 2 and S2. These findings are remarkable in that they demonstrate a level of transcriptional variation within and between fine brain structures regulating sleep that has not been previously
Fig. 7 – Identification of sequences differentially expressed during the light and dark cycles. Bar graph indicates the number of sequences differentially expressed (1-way error weighted ANOVA p-value <0.001) between light cycle (ZT-7) and dark cycle (ZT-19) in LC, SCN, VLPO and VTA. Within each bar, heatmaps show the relative expression of each gene observed in the four ZT-19 and four ZT-7 samples (stacked vertically). Sequences are ordered by K-means (2 group) clustering and stacked along the horizontal axis.

3. Discussion

Analysis of transcriptional changes within gross brain regions has substantially increased the understanding of key factors governing sleep, circadian signaling and regional differences including recent investigations of CNS gene expression in normal and sleep deprived models (Cirelli and Tononi, 1999, 2000; Cirelli et al., 2004; Fukuhara and Tosini, 2008; Greco et al., 1999; Kilduff et al., 2008; Mackiewicz et al., 2008; Tafti and Franken, 2007; Terao et al., 2003a,b, 2006). New microarray studies examining normal sleep and wake, and the effects of sleep deprivation on cortical and hypothalamic function have yielded interesting connections with circadian, biosynthetic and metabolic pathways (Mackiewicz et al., 2007; Wisor et al., 2008). In agreement with these reports, we observed global gene expression changes between the light and dark phases across brain regions for genes responsible for synaptic plasticity, lipid metabolism, cholesterol biosynthesis, cell-cell signaling, intracellular transport, and circadian regulation. Although informative of robust changes within a tissue, many of these earlier studies have focused on hand dissection of relatively large brain structures or CNS regions which may not account for nuclei or sub-nuclei specific gene changes that would be minimized by more overwhelming changes in larger tissue volumes. In fact, we and others have observed that transcriptional profiling of large heterogeneous brain regions such as hippocampus, midbrain and hypothalamus may not detect transcriptional differences due to the diverse cell populations contained within those structures (Hovatta et al., 2007; Sandberg et al., 2000; Zapala et al., 2005). The potential utility of more regional dissections has recently been proposed for studying biological rhythms (Peirson et al., 2007; Porterfield et al., 2007) and some elegant work has been completed examining changes in circadian clock genes following sleep deprivation across mouse strains (Wisor et al., 2008). By limiting tissue collection to discrete brain nuclei with LCM we have detected a large number of significant regional and diurnal transcriptional signatures that were not apparent when collecting larger samples of tissue.

Each discrete region had a unique transcriptome that reflects the known biology of each anatomical structure. For example, transcripts unique to LC include genes involved in norepinephrine synthesis and transmission such as Ddbh, Adora2b, Slc6a2 as well as genes associated with serotonin signaling including Htr1d, Htr3b, Maa, Slc6a4 and Tph2. Additionally, SCN unique transcripts are enriched for genes involved in circadian rhythm signaling including Vip, Avp, Per2 and Cry1. TMN uniquely expresses HDC, an enzyme that catalyzes histamine from histadine correlating with the role of TMN in governing wakefulness. Transcripts unique to VTA include genes involved in dopamine synthesis and transmission such as Ddc, Drd2 and Slc6a3 (DAT), as well as genes associated with locomotor responsiveness such as Chrna4 (Labarca et al., 2001), Chrna5 (Salas et al., 2003), and Cadps2 (Sadakata et al., 2007) and learning including Ptprd (Jutani et al., 2000), Ctmd2 (Israely et al., 2004) and Drd2 (Glickstein et al., 2002). Many additional transcripts with highly enriched expression were identified for each of the regions for example Abca7, Diabio, Kcnk1 and Pvalb (LC); Gabrb1, Cldn3 and Kcng2 (SCN); Kpl2, Slit2 and Tekt1 (TMN); Crabp2, Foxg1 and Gbx2 (VLPO) and Kcn3, Pde8a and Ntn1 (VTA). The regional enrichment of these transcripts may point to previously unrecognized roles for these brain nuclei. For example, 27 genes involved in neurogenesis were identified in LC including Avil, Pou4F1, Fez2 and Shox2, even though LC is not an area described. Importantly, this refined anatomical approach to identifying alterations in gene expression between distinct brain nuclei enables a better understanding of the level of complexity controlling interacting circuits of the mammalian sleep and wake behaviors.

We further analyzed the light/dark cycle expression of 94 genes categorized as circadian phase-responsive in the Gene Ontology (GO) database (Gene Ontology Consortium, 2001; http://www.geneontology.org/). Of these phase-responsive genes, 37 (44%) were significantly regulated in one or more of the 4 brain regions (t-test p-value<0.05; 19 out of 94 expected by chance), with 13 genes regulated in the same direction across all four regions (Fig. 8A) and 24 regulated in different directions between the 4 brain regions (Fig. 8B). Results from the examination of all light/dark cycle regulated transcripts identified a cohort of genes regulated similarly between multiple regions (Fig. 9A) and a set of genes regulated in different directions between regions (Fig. 9B). Among all regions compared, the differences between VTA and SCN were particularly striking. Out of the 31 genes in Figs. 8 and 9 that were significantly regulated (p<0.05) in both VTA and SCN, 15 were regulated in opposite directions. We also observed genes that were regulated between diurnal phases in one region, but not in any of the other three regions, and VTA had the most uniquely regulated transcripts (Fig. 10). Taken together these findings demonstrate that this approach enabled the detection of robust light/dark cycle signature genes in discrete brain regions, and avoided the signal dampening due to cellular heterogeneity observed in studies examining whole brain lysates or large brain regions across time points.
that has typically been associated with neurogenesis. The identification of 31 genes enriched in the VTA linked with lipid metabolism and cholesterol biosynthesis including FASN, ACAT2, APOA1, EBP, FDF1, DHCR7 and HPGD is particularly intriguing given the recent connections between fatty acid signaling in the CNS and nicotine addiction (Mellis et al., 2008). In addition to defining new functions and subpopulations with these structures, the expression of regionally enriched transcripts may serve as regional markers for further experimental analyses. There were significant differences detected between regions directly networked together. For example, there were 2717 signature genes between VLPO and TMN (FDR <0.32%), 4155 signature genes between VLPO and LC (FDR <0.07%) and 4190 signature genes between LC and TMN (FDR <0.22%).

Although the current study focused specifically on two time points to coincide with the middle of the normal rat inactive phase (ZT-7) and active phase (ZT-19), a substantial number of differentially expressed transcripts were identified within differently expressed between light and dark cycles from the 2766 time regulated genes in Table S2. Findings with FDR estimates <5% are in bold. Max [LogR] column lists the absolute value of the maximum log10(ZT-7/ZT-19) observed across the four regions. Min ANOVA p-val column gives the minimum 1-way ANOVA p-value observed across the four regions. For each region, ANOVA p-values, average log10(ZT-7/ZT-19), post-hoc p-values and average normalized probe intensities are given.
specific brain regions. Studies of the molecular components governing circadian oscillations have identified many points of transcriptional control with regional contributions, (Zheng and Sehgal, 2008). Importantly, in comparing expression changes between the light and dark phases, many genes previously linked with circadian regulation were found to vary and were detected across the regions examined. Specific circadian genes that were up-regulated uniformly during the light phase include DBP, Timeless, and NR1D1 in agreement with previous findings (Lopez-Molina et al., 1997; Onishi et al., 2002; Tischkau et al., 1999). There was also a strong alignment with characteristic circadian genes upregulated during the dark phase including Hcrtr1, Homer1, HTR2a and Kcnip1.

The connection between circadian dysregulation and psychiatric diseases has been observed for more than 40 years (Elithorn et al., 1966; Knapp et al., 1967), however in most cases the underlying molecular components and CNS circuits involved have not been established. Recently, genetic
Fig. 8 – GO circadian rhythm genes regulated between light and dark cycles. 36 of the 94 genes in the GO circadian rhythm gene set were significantly (t-test $p < 0.05$) regulated in one or more regions between light cycle (ZT-7) and dark cycle (ZT-19). Heatmap and bar graph data represent the average difference between the four ZT-7 and four ZT-19 log ratios. Error bars represent standard error. (A) GO circadian genes regulated in the same direction in all four brain regions. (B) GO circadian genes regulated in opposite directions across the four brain regions.

Fig. 9 – Time-regulated genes between light and dark cycles. Named genes that were robustly regulated (t-test $p < 0.05$ in two or more regions, ANOVA $p < 0.001$, fold change $> 1.5$ and FDR $< 5\%$ in at least one region) between ZT-7 and ZT-19. Heatmap and bar graph data represent the average difference between the ZT-7 and ZT-19 log ratios. Error bars represent standard error. (A) Genes regulated in the same direction in all four brain regions. (B) Genes regulated in opposite directions across the four brain regions.
and transcriptional profiling approaches have been combined to link the transcription factor DBP to multiple psychiatric diseases in addition to known roles in circadian regulation and sleep behaviors (Franken et al., 2000; Le-Niculescu et al., 2008). We observed expression changes in response to the light/dark phase for several other genes across all brain regions associated with psychiatric diseases including schizophrenia (Timeless, Htr2a and Homer1), bipolar disorder (Htr2a and Timeless), Parkinson’s Disease (Htr2a), migraine (Htr2a) and multiple sclerosis (HRH3) (Carter, 2007; Teuscher et al., 2007). It is interesting to note that all of these diseases share disturbances in sleep patterns and circadian rhythms.

In addition, there were many genes that showed significant variation between the active and inactive phases across all regions, but not previously described as having significant diurnal patterns or roles in arousal. Among these are genes involved in synaptic function (Grin2c, Ntrk1) (Herbst et al., 2002; Rossi et al., 2002), cell transformation (Frag1, Hspa1a, Ntrk1) (Gual et al., 2001; Hunt et al., 2004; Lorenzi et al., 1996), and inflammatory disorders (Creb3l1, Mmp14, Hspa1a) (Antony et al., 2007; Jarvis et al., 2006; Sternlicht and Werb, 2001). Although not on the GO list of circadian rhythm genes, Ntrk1 (TrkA) was significantly upregulated at ZT-7 in SCN, VLPO and VTA and mutations in the Ntrk1 gene have been associated with circadian abnormalities in humans (Ohto et al., 2004).

The observation of reduced immediate early gene expression (including Fos, Jun, Homer1) across regions, and particularly in SCN, during the inactive interval (ZT-7) may be an indication of reduced activity of these areas during normal sleep. Interestingly, several genes involved in memory and neuronal remodeling were upregulated across the regions during the inactive phase including Ntrk1, Grin2c, Nrl1d1 and Mmp14 (Chomez et al., 2000; Nakada et al., 2003; Rossi et al., 2002; Woolf et al., 2001). An interesting finding was that a number of genes linked to circadian functions were regulated in different directions between areas. For example, the transcription factor Egr1, associated with neuritogenesis and memory formation was upregulated in SCN during the middle of the active phase, but tended to be downregulated in the other areas at this time point (Li et al., 2007; Ravni et al., 2008).

BDNF expression was reduced at ZT-7 in VLPO but was increased significantly in the VTA. The downregulation of BDNF has been described for SCN, hippocampus, cortex and other regions primarily during wakefulness (Cirelli and Tononi, 2000; Liang et al., 1998). Within the VTA, BDNF is thought to play a role in sensitization to cocaine and amphetamine (Lu et al., 2004) and it may be linked to the pathogenesis of depression (Duman and Monteggia, 2006; Nestler and Carlezon, 2006). The bidirectional action of a common neurotransmitter in different brain nuclei is certainly intriguing and the common disruptions in circadian rhythms and sleep behaviors in both drug addiction and depression are well known (Krystal et al., 2008).

Numerous studies have demonstrated the role of the VLPO in regulating the transition to sleep. Dopamine signaling through the VTA is linked to sleep and wakefulness, and activation of neurons within the VTA during the active phase has recently been described (Luo et al., 2008; Monti and Monti,
In our studies the VLPO and VTA had many genes commonly upregulated during the inactive phase that were unchanged or down regulated in other regions, including Mgll, Ppp1r7, Galnt4 and Cntn3. Interestingly, Cplx2 which has been recently associated with schizophrenia was upregulated at the ZT-7 time point in VLPO, but downregulated or unchanged in all other regions (Carter, 2007). Several genes linked with synaptic function and neuronal plasticity were also differentially expressed across regions including Cplx2, Pacsin1 and Cntn3 (Anggono and Robinson, 2007; Eastwood and Harrison, 2005; Yoshihara et al., 1994). Some genes exhibited altered expression between the active and inactive phases in one region but not the other regions examined. For example, genes in LC such as Capb1, Cdkn2b and Abra were downregulated at ZT-7, but not significantly altered by light/dark phase in the other regions (Fig. 10).

Through the evaluation of gene expression changes in brain regions, these studies provide many new insights into the diverse roles of the sleep and wake circuitry. The combination of LCM, transcriptional profiling and pathway analysis sensitively detects both regional and diurnal variations in gene expression and provides a mechanistic basis for the relationships between the LC, SCN, VTA, VLPO and TMN. Importantly, this approach isolates all components of the brain nuclei and may contain neuronal, glia, astrocytic, and endothelial populations. Complementary methods, including flow cytometry, are being developed to further isolate neuronal populations within brain structures (Hempel et al., 2007). The gene expression differences between the middle of the light and dark periods are intriguing and support the expansion of this approach to look across the more time points throughout the circadian period and with coincident recording of sleep and wake activities. The knowledge of when and where gene expression changes occur within brain nuclei is the key to understanding the regulation of transcriptional networks in the CNS. A focus on whole brain lysates or gross regional dissections in studies of plasticity or sleep/wake regulation can result in the dampening of transcriptional signals and prevent the identification of core regulatory processes driven by critical brain nuclei. Continued mining of these data will provide additional insights into the functions of these circuits and may point to new connections between human diseases and sleep and wake dysregulation.

4. **Experimental procedures**

4.1. **Animals**

All experimental animals were housed and handled according to the Federal Animal Welfare guidelines and all studies were approved in advance by the Animal Care and Use Committee at Merck & Co., Inc. Male Sprague Dawley rats at 8 weeks of age were individually housed in 12:12 light:dark conditions with water and food available ad libitum prior to sacrifice by conscious decapitation at ZT-7 and ZT-19 time points to correspond with the middle of the inactive and active phases (n=4 per time point). Two additional independent cohorts (n=4 per cohort) were housed/treated equivalently and sacrificed at ZT-7. Non-perfused fresh brains were harvested using an RNase-free protocol and rapidly frozen.

4.2. **Tissue isolation and RNA extraction**

From the rat brains, 20 μM sections were prepared on PEN-membrane slides. The LC, SCN, TMN, VLPO and VTA regions were identified by cresyl violet staining using the LCM Staining Kit (Molecular Devices) and isolated by laser micro-dissection using the Veritas platform (Molecular Devices). Total RNA was isolated using the PicoPure RNA isolation kit (Molecular Devices). Total RNA was analyzed by PicoChip using the BioAnalyzer instrument (Agilent Technologies, Inc.) and the Nanodrop Fluorospectrometer (Thermo Scientific) using Ribogreen (Invitrogen) and stored at −70 °C until amplification. Samples from each cohort of animals were processed separately.

4.3. **Microarray profiling**

Samples were amplified and labeled using a custom 2 cycle version of the Amino Allyl or Biotin MessageAmp II™ aRNA Amplification kits from Ambion. Briefly, 1–5 ng of total RNA were added to the initial reaction mix together with 250 ng of pBR322 (Invitrogen). Following the first round IVT, the plasmid carrier was removed with a DNase1 treatment. The first round IVT products were purified using the Qiagen MinElute Kit (Qiagen). Input into the second round was normalized to the lowest yielding sample. Hybridizations to custom Agilent microarrays (containing 39,552 sequences) and normalization was completed as described (Hughes et al., 2001; Parrish et al., 2004; Weng et al., 2006). Hybridization to custom rat Affymetrix arrays (containing 43,686 probe-sets), labeling and scanning were completed following the manufacturer’s recommendations and profiles were normalized using RNA as described (Irizarry et al., 2003). Custom arrays were designed to monitor additional genes and poly A sites than commercially available microarrays. Sample amplification, labeling, and microarray processing were performed by the Rosetta Inpharmatics Gene Expression Laboratory in Seattle, Wash. Samples from each cohort of animals were processed and hybridized separately. Data analysis across microarray platforms was performed at the gene level. False discovery rates were estimated using the average results from 10 random permutations of the specified samples sets.

4.4. **Taqman analysis**

Amplified cDNA from 2 ng of total RNA was prepared by using the WT-Ovation™ Pico RNA Amplification System (NuGen Technologies, Inc.). The resulting cDNA samples were analyzed using the BioAnalyzer instrument (Agilent Technologies, Inc.) and stored at −20 °C until real-time quantitative PCR (qPCR) analysis. To determine transcript levels 20 ng of cDNA were analyzed using TaqMan™ Gene Expression Assays on the 7500 Fast System (Applied Biosystems). Reactions were performed in triplicate and GAPDH probe was used as a control and for data normalization using the 7500 Fast System software (Applied Biosystems).
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Appendix A. Supplementary data


References


Eastwood, S.L., Harrison, P.J., 2005. Decreased expression of vesicular glutamate transporter 1 and complexin II mRNAs in schizophrenia: further evidence for a synaptic pathology affecting glutamate neurons. Schizophr. Res. 73, 159–172.


