HHS Public Access

Author manuscript

Neuroscience. Author manuscript; available in PMC 2016 February 10.

Published in final edited form as:

Neuroscience. 2006 ; 137(2): 593–605. doi:10.1016/j.neuroscience.2005.08.059.

Gene Expression in the Rat Brain during Sleep Deprivation and Recovery Sleep: An Affymetrix GeneChip® Study

A. Terao[1,*], **J.P. Wisor**[1], **C. Peyron**[2,#], **A. Apte-Deshpande**[1], **S.W. Wurts**[3], **D.M. Edgar**[3,@], and **T.S. Kilduff**[1]

[1]Molecular Neurobiology Laboratory, Biosciences Division, SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025

[2]Department of Biological Sciences, Stanford University, Stanford CA 94305

[3]Department of Psychiatry and Behavioral Sciences, Stanford University, Stanford CA 94305

Abstract

Previous studies have demonstrated that macromolecular synthesis in the brain is modulated in association with the occurrence of sleep and wakefulness. Similarly, the spectral composition of electroencephalographic activity that occurs during sleep is dependent on the duration of prior wakefulness. Since this homeostatic relationship between wake and sleep is highly conserved across mammalian species, genes that are truly involved in the electroencephalographic response to sleep deprivation (SD) might be expected to be conserved across mammalian species. Therefore, in the rat cerebral cortex, we have studied the effects of SD on the expression of immediate early gene (IEG) and heat shock protein (HSP) mRNAs previously shown to be upregulated in the mouse brain in SD and in recovery sleep (RS) after SD. We find that the molecular response to SD and RS in the brain is highly conserved between these two mammalian species, at least in terms of expression of IEG and HSP family members. Using Affymetrix Neurobiology U34 GeneChips®, we also screened the rat cerebral cortex, basal forebrain, and hypothalamus for other genes whose expression may be modulated by SD or RS. We find that the response of the basal forebrain to SD is more similar to that of the cerebral cortex than to the hypothalamus. Together, these results suggest that sleep-dependent changes in gene expression in the cerebral cortex are similar across rodent species and therefore may underlie sleep history-dependent changes in sleep electroencephalographic activity.

Keywords

Taqman analysis; sleep deprivation; immediate early genes; basal forebrain; cerebral cortex; hypothalamus

Address correspondence to: Dr. Thomas S. Kilduff, Molecular Neurobiology Laboratory, SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025, Voice: 650-859-5509, FAX: 650-859-5535, thomas.kilduff@sri.com.

*Current address: Department of CNS Pharmacology, Pharmaceutical Research Division, Takeda Pharmaceutical Company, 17-85, Jusohonmachi 2-Chome, Yodogawa-ku, Osaka 532-8686 Japan.

#Current address: Laboratoire de pathophysiologie des réseaux neuronaux controllant le cycle veille-sommeil, CNRS UMR 5167, Université Claude Bernard, Lyon1, France.

@Current address: Hypnion, Inc., 500 Patriot Way, Lexington, MA 02421, USA.

Sleep is a homeostatic process in that the time spent asleep and the continuity of sleep states are directly related to the duration of prior wakefulness. Although sleep duration and the time spent in each of its stages are parameters commonly measured in sleep studies, sleep also has an intensity dimension, measurable by slow wave activity (SWA) in the electroencephalogram (EEG) during non-rapid eye movement (NREM) sleep. The amplitude of EEG SWA is directly proportional to the duration of prior wake and consequently has been proposed as a marker for the homeostatic regulation of sleep in mammals (Borbely and Achermann, 2000). Accordingly, sleep need, measurable as EEG SWA once sleep is initiated, is thought to accrue during wakefulness. Conversely, the decline of EEG SWA amplitude across a sleep bout is thought to reflect the diminution of the sleep-dependent "Process S" that reflects recovery from prior waking activities.

The temporal dynamics of the sleep-dependent discharge of sleep need (reflected in the decay of the sleep-dependent Process S) is conserved among genetically distinct rodent strains (Franken et al., 2001). The conserved nature of Process S supports the concept that EEG SWA may be an electrophysiological marker of restorative neurochemical processes that occur during sleep. Although the nature of these neurochemical processes - what exactly is restored during sleep, if anything – remains unknown, the time courses for the buildup and discharge of sleep need occur on the order of hours and are thus consistent with the involvement of macromolecular synthesis. Consequently, we and others (Cirelli and Tononi, 2000b, Cirelli et al., 2004) have tested the hypothesis that gene-specific changes in macromolecular synthesis occur in the brain during sleep as a response to prior waking activities. In a survey of gene expression in the C57BL/6 mouse brain during recovery sleep (RS) after a period of sleep deprivation (SD), we found that fewer than 1% of monitored genes increased their mRNA levels during RS and that such increases were anatomically restricted within the brain (Terao et al., 2003a, Terao et al., 2003b). Changes in the rate of protein synthesis, if not the synthesis of specific proteins, have also been documented in association with sleep-wake states (Ramm and Smith, 1990, Nakanishi et al., 1997).

Since a homeostatic relationship between activity and rest is highly conserved throughout evolution (Tobler, 1983, Shaw et al., 2000), changes in gene expression that are truly involved in the response to SD might also be expected to be conserved across species. Therefore, we have undertaken a SD study in the rat similar in design to our previous mouse studies. First, we undertook a candidate gene approach in the rat to evaluate the expression of genes previously found to be upregulated in mouse cerebral cortex (Cx) during RS (Terao et al., 2003a, Terao et al., 2003b). In the second part of this study, we used Affymetrix GeneChips® to identify other genes whose expression increases in SD and RS in the Cx and in two brain regions thought to be involved in the regulation of sleep, the basal forebrain (BF) and hypothalamus (Hy).

METHODS

Sleep Physiology

Male Wistar rats (Charles River Laboratories, Wilmington, MA) aged 2–3 months were surgically prepared, recorded, and subjected to SD as described (Terao et al., 2000). Rats were randomly assigned to one of four groups: (1) SD from light onset (ZT0) to ZT6 (n=11);

(2) control rats for the SD group (*n*=12); (3) SD from ZT0 to ZT6 followed by RS from ZT6 to ZT8 (*n*=13); (4) control rats for the RS group (*n*=13). SD and control animals were sacrificed at ZT6, just after a 6 h SD period; groups 3 and 4 were sacrificed at ZT8. Brains from six rats in each group were rapidly removed and dissected into multiple regions which were flash-frozen on dry ice. Cx was peeled away from underlying tissue and processed as a whole. The BF dissection included the septum (medial and lateral), the vertical and horizontal limb of the diagonal band of Broca, the rostral part of the preoptic area and the ventral pallidum. The Hy dissection was limited from the chiasma (−0.3mm from bregma) to the mammillary bodies (−4.5 mm from bregma). The dorsal limit of the hypothalamus was the roof of the third ventricle and its lateral limit was the amygdala (Paxinos and Watson, 2004). The brains from the remaining rats in each group were kept intact for subsequent histological studies. All animal use procedures were approved by the Animal Care and Use Committees of SRI International and Stanford University.

Assessment of the Expression of Candidate Genes

On the basis of our previous studies (Terao et al., 2003a, Terao et al., 2003b), thirteen genes were selected for real-time fluorescence detection analysis: six members of the Fos/Jun family (c-*fos*, *fra-1*, *fra-2*, *junB*, *c-jun* and *junD*), four other IEGs *(arc*, *egr-1*, *egr-3*, and *nur/77)*, and three chaperone proteins *(erp72*, *grp78* and *grp94)*. (One additional IEG that is upregulated in the mouse Cx as a result of SD, *fosB*, was not determined in the rat Cx). The primer and probe sequences used to measure *fra-1*, *fra-2*, *junB*, and *egr-3* were designed to be useful for both mouse and rat and have been reported previously (Terao et al., 2003a). The primer and probe sequences used for the other 9 genes plus *glyceraldehyde-3-phosphate dehydrogenase* (*g3pdh*) were chosen from the coding regions of the genes and are shown in Table 1. Primers and probes for the candidate genes were chosen using Primer Express v.1.0 Software (Perkin-Elmer Applied Biosystems, Foster City, CA). The primer and probe sequences for *arc* in Table 1 amplify DNA from both mouse and rat; the remaining sequences are rat-specific. To confirm the specificity of the nucleotide sequences chosen for the primers and probes and the absence of DNA polymorphisms, BLASTN searches were conducted against the dbEST and nonredundant set of Genbank, EMBL, and DDBJ databases.

Quantitation of mRNA levels was carried out using a fluorescence detection method using real-time amplification kinetics (Taqman; ABI Prism 7700 Sequence Detection System, Perkin-Elmer Corp., Foster City, CA) as described previously (Terao et al., 2000, Terao et al., 2003a, Terao et al., 2003b). Total RNA was prepared using the Atlas™ Pure Total RNA Labeling System (CLONTECH, Palo Alto, CA). To remove any genomic DNA contamination, total RNA was treated with RNase-free DNase I (Epicentre, Madison, WI) in the presence of anti-RNase (Ambion, Austin, TX). First-strand cDNA was prepared from the Cx, Hy and BF of six rats from each of the four experimental conditions (72 individual cDNA syntheses) using the Advantage™ RT-for-PCR kit (CLONTECH, Palo Alto, CA). For each reaction, a “target” cDNA of interest and the reference cDNA (*g3pdh*) were simultaneously PCR-amplified as described previously (Terao et al., 2003a, Terao et al., 2003b). To measure *arc* expression, cDNAs were subjected to Taqman analysis and purified

RNAs were subjected to Northern blot analysis in parallel with mouse cDNAs and RNAs from our previous studies (Terao et al., 2003a, Terao et al., 2003b).

Affymetrix GeneChip® Hybridization

Total RNAs from the rat BF, Cx and Hy samples described above were combined into 12 RNA pools (3 brain regions × 4 groups); each pool was comprised of 5 μg of total RNA from each of 6 rats per condition. Approximately 30 μg total RNA was purified from each of the 12 pools using an RNeasy mini spin column (Qiagen, Valencia, CA). Double-stranded cDNA was synthesized using the Superscript Choice System (Gibco/Invitrogen, Grand Island, NY) and an HPLC-purified primer encoding poly(dT) and the T7 RNA polymerase promoter sequence (Genset, La Jolla, CA). The cDNA was visualized using a 1% agarose gel to confirm size distribution.

In vitro transcription of double-stranded cDNA was performed in the presence of biotinylated UTP and CTP, using T7 RNA polymerase and the Enzo BioArray High Yield RNA Transcription Labeling Kit (Affymetrix, Santa Clara, CA and Enzo Diagnostics, Farmingdale, NY). Biotin-labeled cRNA was purified and fragmented using 200 mM Tris-acetate, pH 8.1, 500 mM potassium acetate and 150 mM magnesium acetate in DEPC-treated water. Purified cRNA was visualized on 1% agarose gels to confirm adequate fragmentation. RNA fragment sizes ranged from approximately 35 to 200 bases. Target cRNA was hybridized to replicate Affymetrix Rat Neurobiology U34 GeneChips® (a total of 24 GeneChips®), two per brain region (BF, Hy or Cx) per condition (SD, ZT6 control, Rec, ZT8 control). Hybridization, washing, staining with streptavidin-phycoerythrin, and antibody amplification were performed according to the Affymetrix GeneChip® Expression Analysis Manual. The arrays were scanned with an Affymetrix GeneArray Scanner at the Stanford University Protein and Nucleic Acid (PAN) Facility (Stanford, CA).

GeneChip® data analysis

For each brain region, the ZT6 and ZT8 control groups were merged into a single control group (n = 4 per region) to which the SD (n=2) and RS (n=2) chips were compared. Data were analyzed with Microarray Suite 4.0 (Affymetrix) and GeneSpring (version 6, Silicon Genetics, Redwood City, CA). Expression of each mRNA species was assessed by a series of DNA oligonucleotide probes (‘gene elements’) arrayed on the Affymetrix chip at known locations. Each gene element includes one probe that is complementary to the cDNA corresponding to the mRNA of interest (‘perfect match’=PM) and one that is complementary to the cDNA at all bases but one (‘mismatch’=MM). The former of these two probes measures the gene-specific signal and the latter measures background signal. General background signal intensity caused by autofluorescence of the array surface and nonspecific binding of target or dye molecules to that surface is subtracted from the intensities of all of the probes. The average difference (Avg Diff) value for every gene element represented on the chip was calculated by taking the difference in signal intensity between the PM and the MM, as detailed in the Affymetrix GeneChip® Expression Analysis Manual. The Affymetrix software automatically labels each gene element as being “present”, “marginal” or “absent” based on the Avg Diff value.

Two methods were used to identify gene elements exhibiting sleep state-dependent changes in expression. To evaluate qualitative ("all or none") changes in expression between conditions, gene elements were identified that met one of the four following criteria: 1) scored "present" in three of four control GeneChips® and absent in both SD GeneChips®; 2) scored "present" in three of four control chips and absent in both RS chips; 3) scored "absent" in three of four control chips and present in both SD chips; 4) scored "absent" in three of four control chips and present in both RS chips. Rather than imposing a more stringent requirement of "present201D in all four control GeneChips®, we decided to use the criterion of "present" in three of four control GeneChips® for this analysis to allow for the possibility of local hybridization problems within any of the four control GeneChips®.

To evaluate quantitative changes in expression between conditions, the GeneSpring Cross Gene Error Model (CGEM; http://www.silicongenetics.com/Support/GeneSpring/GSnotes/analysis_guides/error_model.pdf) was used to identify those gene elements that were expressed in all eight chips for a given brain region at a level of intensity sufficient to be considered reliable. Subsequent quantitative analyses were limited to the subset of gene elements identified by this procedure. Data values less than 0.01 were assigned a value of 0.01. The expression level for each gene element was normalized to the 50th percentile of the expression level of all gene elements on the same GeneChip® and subsequently to the median percentile of expression for that gene element across all chips from the same brain region (per GeneSpring's recommended normalization procedures). The normalized values of the four control GeneChips® in each brain region were averaged and those of the two experimental GeneChip® groups (SD and RS) in each brain region were averaged to provide a single expression value for each gene element in each condition for each brain region. These averaged values were used to calculate the percent change value between the experimental and the pooled control groups (SD vs. control and RS vs. control) where % change = (Experimental value − Control Value) / Control Value. Those gene elements for which the ratio of the average experimental value to the average control value (SD/control or RS/control) was greater than 1.5 were defined as being upregulated in the experimental group. Those gene elements for which the ratio of the average experimental value to the average control value (SD/control or RS/control) was less than 0.67 were defined as being downregulated in the experimental group. We report here all genes that show qualitative ("all or none") changes across experimental conditions and those that are up- or downregulated by 50% during either SD or RS relative to the pooled control group in any of the three brain regions studied.

Those genes that were upregulated or downregulated in response to SD or RS were subsequently categorized according to function in a modified version of the GeneSpring ontological structure for gene categorization (Ashburner et al., 2000). The analyses presented herein are based on hybridizations to the probesets on the Affymetrix Rat Neurobiology U34 GeneChip®, each of which was designed to correspond to a particular Genbank accession number. In some cases, the Genbank accession numbers represent expressed sequence tags (ESTs). To identify the genes to which each of the ESTs actually correspond, we utilized the GeneSpider function of GeneSpring, which integrates the UniGene, Genbank and Locus Link databases. Since these databases have not been fully curated to eliminate redundancies, our analyses based on probesets may identify two or

more genetic elements as unique entities that, in fact, correspond to the same gene. Based on our knowledge of the literature, we have made every effort to eliminate such redundancies. For example, *egr-1* (Genbank #M18416) is recognized as being synonymous with *krox-24* (Genbank #M18416), but it is possible that there may still be other synonyms in our gene lists.

Confirmation of Candidate Genes

On the basis of the GeneChip® results, two additional genes were selected for further analysis using the real-time fluorescence detection method as described above: and nuclear receptor subfamily 4, group A, member 3 (*nr4a3*; also known as *nor-1*) and the immediate early gene *ngfi-b*. The primer and probe sequences used are also shown in Table 1.

Statistical analysis

Real-time fluorescence detection PCR measurements were analyzed using Statview 5.0 (Abacus Concepts, Berkeley, CA). Data were initially analyzed by ANOVA with alpha set at 0.05 to determine whether significant effects occurred in any of the parameters. Significant main effects determined by ANOVA were followed by the Tukey-Kramer *post hoc* test to identify which experimental groups differed from their respective controls.

RESULTS

Sleep Physiology

Figure 1 presents total sleep time in Wistar rats across a 32 h recording period. A 24 h baseline recording period was followed by 6 h SD and then 2 h recovery. The 6 h SD period from ZT0–6 resulted in a 90% reduction of total sleep time (TST) relative to the corresponding period on the baseline day in both the SD and RS groups (SD: −229 ± 7 min; RS: −230 ± 10 min). During the 2-h recovery period, the RS group gained 15 ± 4 min (16.28%) over baseline ($p = 0.0014$; $t=3.621$; $df=24$). The ZT8 control animals for the RS group lost −4 ± 3 min of sleep relative to the baseline day, which was within normal baseline variance ($p=0.161$; $t=1.445$; $df=24$). After 6 h of SD, significant increases in sleep bout length and EEG delta power were also observed during the subsequent RS period, indicating that a sleep debt had been incurred as documented previously (Terao et al., 2000).

Candidate Gene Expression

To determine whether the genomic response to SD and RS was conserved between mouse and rat, we examined the expression of twelve genes that increased in expression during SD and/or RS in our previous studies in the mouse (Terao et al., 2003a, Terao et al., 2003b), as well as *arc* mRNA which has been reported to change in response to SD in the rat (Cirelli and Tononi, 2000a). Of particular interest from these studies were four genes that exhibited elevated expression during RS in the mouse: *fra-2*, *egr-3*, *grp78* and *grp94*. Figure 2 presents mRNA levels of these four genes in the rat Cx. ANOVA revealed a highly significant variation across the four experimental conditions for these genes (*fra-2*: $p < 0.0001$; *egr-3*: $p < 0.0001$; *grp78*: $p = 0.0009$; and *grp94*: $p < 0.0086$). Post hoc tests established that, as in the mouse Cx, all four of these genes showed significantly increased mRNA levels in the rat Cx during both SD and RS.

The immediate early gene *arc*, associated with synaptic plasticity, has been shown by others to exhibit increased expression during SD (Cirelli and Tononi, 2000a). Consequently, we also examined the expression of this gene in both rat and mouse Cx. Figure 3A shows expression of this gene in the Cx of the two species as detected by Northern analysis. As is evident from the autoradiographs, increased *arc* expression occurred in both species when subjected to 6 h SD. ANOVA on the intensity of autoradiographic signal revealed that the expression changes were significant in both species (mouse: $p < 0.0001$, $F= 26.510$; $df= 3, 12$; rat: $p = 0.0003$, $F= 14.193$; $df= 3, 12$). The significance levels determined by Northern analysis were also confirmed by Taqman analysis (Figure 3B; mouse: $p < 0.0001$, $F= 13.696$; $df= 3,24$; rat: $p < 0.0001$, $F= 38.931$; $df= 3, 18$).

Table 2 compares the direction and magnitude of expression changes of 13 genes in the rat Cx to previously documented changes in the mouse Cx (Terao et al., 2003a). All IEGs previously shown to be upregulated in the mouse Cx during SD or RS were also upregulated in the rat Cx. Two IEGs that were unaffected by SD in the mouse Cx, *grp94* and *fra2*, were increased in the rat Cx in response to SD. However, these two transcripts exhibited increased expression in the RS group relative to time of day controls in both species. Thus, of 26 possible comparisons between the two species, similar responses were observed in 24 cases.

Rat Neurobiology U34 GeneChip® Hybridizations

Of the 1322 genetic elements represented on the Affymetrix Rat U34 Neurobiology GeneChip, 568 were found to be expressed in at least three of the four control chips from the BF, 518 in the Cx, and 590 in the Hy. Figure 4 presents comparisons of the region specific patterns of gene expression in the SD vs. control chips (Figure 4, left panels) and the RS vs. control chips (Figure 4, right panels) for the 500+ genes that were present in a majority of control chips in each brain region.

The number of genes that were found to be upregulated or downregulated by at least 50% in response to 6 h SD in one or more brain regions is presented in Figure 5. The nuclear factors *ngfi-b* and *nr4a3* and an apoptosis-related transcript, *BH3 interacting domain 3*, were the only transcripts to be upregulated in common across the three brain regions examined (Figure 5A and Table 3A). The BF (n=54) and Cx (n=72) showed a much larger number of genes upregulated in response to SD than did the Hy (n=30). In addition, the BF and Cx exhibited more genes commonly upregulated (n=16) in response to SD than did Hy with either Cx (n=1) or BF (n=3; Table 3). The number of transcripts downregulated by at least 50% (Figure 5B) was roughly equivalent in BF (n=30), Cx (n=34) and Hy (n=35; see Supplementary Table 2). *Hydroxysteroid (17-beta) dehydrogenase 10*, encoding an amyloid peptide binding protein, was the only transcript to be downregulated in common across the three brain regions examined (Figure 5B and Table 3B). The Cx showed four gene elements to be downregulated in common with the BF (*metabotropic glutamate receptor 5*, *immune complement component 4a*, *nestin*, and *insulin-like growth factor binding protein*) and three (*glutamate receptor subunit 4c*, *amiloride-sensitive cation channel 2*, *and somatostatin receptor 4*) in common with the Hy (Figure 5B). The full lists of genes upregulated in

response to SD in each of the three regions are presented in Supplementary Table 1. The list of genes downregulated in each of the three regions is presented in Supplementary Table 2.

The number of genes that were found to be upregulated in BF (n=44), Cx (n=34), Hy (n=55) by at least 50% in recovery sleep are presented in Figure 6A. During recovery sleep, only *glyceraldehyde-3-phosphate dehydrogenase* increased in common across the three brain regions examined (Table 4A). Approximately equivalent numbers of genes were downregulated by at least 50% in BF (n=37), Cx (n=23) and Hy (n=28), during RS (Figure 6B), and downregulation of only one transcript, encoding the transcriptional regulator and immediate early gene *junB*, occurred in common across the three brain regions (Table 4B). Table 4 provides the list of genes that were up- or downregulated in common between any two brain regions. The full list of genes upregulated during RS in each of the three regions is presented in Supplementary Table 3. The lists of genes downregulated in each of the three regions during RS are presented in Supplementary Table 4.

Confirmation of Candidate Genes Arising from GeneChip® Hybridizations

On the basis of the GeneChip® results, two genes found to be upregulated in the Cx in response to SD were selected for further analysis. Figure 7 presents results obtained using real-time fluorescence detection for *nr4a3* (Figure 7A) and *ngfi-b* (Figure 7B) in all three brain areas. For *nr4a3*, ANOVA revealed significant variation ($p < 0.0001$) in all three areas; *post hoc* tests determined that *nr4a3* expression was increased during SD in each of the three areas. Quantitative PCR also established that *nr4a3* levels were elevated during RS in the Cx and BF (Figure 7A). For *ngfi-b*, ANOVA revealed significant variation across treatment groups ($p < 0.0001$) in the Cx and BF only; *post hoc* tests determined that *ngfi-b* levels were increased during SD but not during RS. While this observation contradicted the results of microarray analysis for one *ngfi-b* probe set (U17254_g_at) which was classified as upregulated in all three brain regions, another *ngfi-b* probe set, U17254_at, was classified as upregulated only in BF and Cx (Table 3) in agreement with Taqman results.

DISCUSSION

The purposes of this study were to determine whether the genomic response to SD previously described in the mouse is conserved in the rat and to identify additional genes whose expression is increased during RS after SD. The hypothesis that there should be conservation of sleep loss-dependent changes in gene expression was influenced by the observation that EEG SWA is sleep history-dependent in a number of mammalian species (Tobler and Jaggi, 1987, Trachsel et al., 1989, Dijk et al., 1990, Lancel et al., 1991, Franken et al., 2001). If sleep history-dependent changes in SWA reflect a restorative process that is manifest at the level of gene expression in the Cx, RS should enhance this molecular response.

Candidate Gene Study

To characterize the macromolecular effects of sleep loss and recovery, we measured the expression of 13 genes in the rat Cx during SD and RS whose expression had previously been shown to change in response to SD and RS in the mouse Cx (Terao et al., 2003a, Terao

et al., 2003b). Among the 26 possible inter-specific comparisons listed in Table 2, there is agreement between the rat and mouse in 24 cases. The two exceptions are *grp94* and *fra-2,* which did not significantly change in response to SD in the mouse Cx (Terao et al., 2003b), but were significantly increased in the rat Cx in response to SD. Thus, from this admittedly small sample, the genomic response of the mouse and rat Cx to SD and RS appears to be similar. Given the differences in environmental conditions under which the SD occurred and differing durations of the RS period in the two experiments -- 4 h in the mouse studies (Terao et al., 2003a, Terao et al., 2003b) and 2 h in the current rat study -- it is remarkable that such similar results were obtained in these two species.

Affymetrix GeneChip® study

Our GeneChip® study was based on replicate hybridizations of pooled RNA samples (n=6/group) for each brain region within each experimental condition. This experimental design reduces the inter-individual variation within a group that results from experimental or biological variation but precludes statistical analyses that are possible when individual samples are hybridized to single GeneChips®. Consequently, our analyses focused on intergroup comparisons in which the –fold change was the primary criterion. Since these numbers were obtained from pooled samples, there can be no certainty that any particular gene whose expression change exceeded the 50% threshold represented a statistically significant change. Thus, the genes listed in Tables 3 and 4 (and Supplementary Tables 1–4) can be thought of as candidates to be confirmed by measurement of individual samples with other less costly techniques such as Taqman, Northern analysis or *in situ* hybridization.

Using the 50% change as criterion, our analyses focused on identification of genes that changed in common across brain regions in the two experimental conditions. Of the three genes identified in microarray analysis as upregulated 50% or more in all three brain regions during SD (Table 3), we chose two for confirmation by Taqman (Figure 7). *Nr4a3* mRNA was found to change in expression level during SD consistently across the Cx, Hy and BF by both Taqman and the GeneChip® analyses (Table 3A and Figure 7A). Taqman analysis of *ngfi-b* expression, however, demonstrated an increase during SD only in the BF and Cx. This observation resolved a discrepancy in the GeneChip® results: one *ngfi-b* probe set (U17254_g_at) was classified as upregulated in all three areas by SD, while another (U17254_at) was upregulated only in BF and Cx (Table 3A; Supplementary Tables 1A–C). Accurate assessment of gene expression by Taqman is complicated by the fact that the internal standard used in these experiments, *g3pdh*, was found in GeneChip® experiments to be upregulated during RS in all three brain regions studied (Table 4A). In subsequent Taqman analyses that used β-*actin* as an internal standard, *g3pdh* expression did not statistically differ between experimental groups, although there was a trend toward upregulation in each brain region (93% in BF, 12% in Cx and 16% in Hy). Therefore, the relative expression of some transcripts, such as U17254_at in Hy, may be underestimated in Taqman analyses when *g3pdh* is used as an internal standard.

Differential gene expression among brain regions is consistent with our previous studies in the mouse (Terao et al., 2003a, Terao et al., 2003b) and with a report indicating that sleep and wakefulness have divergent effects on gene expression between the rat cerebral Cx and

cerebellum (Cirelli et al., 2004). The BF and Cx were more similar to each other in response to SD than either region was to the Hy by several criteria. First, there were approximately twice as many genes upregulated as downregulated in response to SD in the Cx and BF but the numbers of SD-upregulated and -downregulated transcripts were about equal in the Hy (Figure 5). Second, while over 20% of genes upregulated in Cx during SD (19 of 72) were also upregulated in BF, only four were upregulated in common in Cx and Hy during SD (including *ngfi-b, BH3 interacting domain 3* and *nr4a3*, which were common to all three brain regions; Table 3A). Third, the number of SD-upregulated genes was approximately two-fold greater in both Cx and BF than in Hy. Finally, similarities in the functional categorization of genes affected by SD, as delineated by GeneSpring's ontological structure for gene categorization (Ashburner et al., 2000, Cirelli et al., 2004), are more common in comparisons between the BF and Cx than in comparisons between Hy and Cx (Table 5). For example, genes associated with neurotransmission, such as the *5HT-7 serotonin receptor*, the *NMDA (glutamate) receptor 3A* and the *alpha 1d adrenergic receptor*, make up fully one quarter of SD-upregulated transcripts in both BF and Cx but less than 10 percent of such transcripts in Hy. Similarly, more than one third of SD-downregulated transcripts in BF and Cx, but less than one quarter of SD-downregulated transcripts in Hy, are associated with neurotransmission. These changes are mirrored by changes in gene expression during RS: genes associated with neurotransmission are the most common category of downregulated genes in Hy during RS (36%), while neurotransmission-related genes constitute lower percentages of RS-downregulated genes in BF (16%) and Cx (26%) during RS.

Differences between BF and Hy in their gene expression profiles may reflect their distinct roles in relation to the cerebral Cx. BF neurons project broadly to the Cx and have a direct and profound influence on the cortical oscillations that define sleep and wakefulness. The activities of the BF and Cx are thus intimately coupled (McCormick, 1992, Manns et al., 2003, Jones, 2004, Steriade, 2004). In contrast, the Hy contains a diversity of neurochemically distinct nuclei, the activity of which is not uniformly sleep- or wake-related (Saper et al., 2001). Some hypothalamic nuclei are more active during sleep than wake (Sherin et al., 1996, Suntsova et al., 2002) while others are more active during wake than sleep (Steininger et al., 1999, Torterolo et al., 2003) and still others are active mostly as a function of the time of day (Yamazaki et al., 1998, Chou et al., 2003). Given the diversity in their neurochemical identities and their activity patterns, changes in gene expression are not likely to be uniform across hypothalamic nuclei. Whether individual hypothalamic nuclei exhibit sleep-wake dependent changes in gene expression similar to those of the BF and/or Cx cannot be determined from the current study.

Two wake-related genes that we identified in microarray analysis and confirmed by Taqman analysis as being upregulated in SD, *ngfi-b* and *nr4a3*, had been previously identified as such by Cirelli et al. (Cirelli et al., 2004). In addition, the activity and neurotransmitter-induced early genes *ania-1* and *ania-3* (aka *homer-1*) and *early growth response 1 (egr-1;* also known as *ngfi-a* and *krox-24*) were upregulated in the present study; these genes were also identified as "wake-related" in rat Cx (Cirelli et al., 2004). Other studies have previously found *egr-1/ngfi-a/krox-24* to be upregulated in the rodent Cx either during spontaneous wakefulness (Pompeiano et al., 1994) or in response to SD (O'Hara et al., 1993,

Terao et al., 2003a) and *homer-1* to be upregulated in response to SD (Nelson et al., 2004). In addition, levels of *arc* mRNA, which encodes a cytoskeletal protein associated with activity-dependent synaptic plasticity (Lyford et al., 1995, Guzowski et al., 2000), are high during wakefulness and decrease during sleep (Cirelli and Tononi, 2000b).

A gene whose expression is affected in opposite directions by SD and RS might constitute a molecular basis for the buildup of sleep need during wakefulness and the discharge of this need during sleep. Several such genes were identified in the current study (Table 6). For instance, the gene encoding the *shal1* potassium channel polypeptide was upregulated during SD and downregulated during RS in Cx, and seven others exhibited the same pattern in either BF or Cx. Conversely, ten genes were downregulated in SD and upregulated in RS in a region-specific manner. Although all of these genes are arguably candidates for the molecular basis of state-dependent sleep need build-up and decline in local brain regions, none of these candidates consistently changed its expression in all three brain regions in conjunction with either SD or RS.

In summary, we found that SD- and RS-dependent changes in IEG and heat shock protein expression in the Cx are conserved between two rodent species. In addition, a subset of genes reported elsewhere to be modulated in response to sleep-wake state were similarly modulated in the current study. If there is a molecular restorative function of sleep, the fact that changes in the expression of very few genes are detected in common among the three brain regions (a total of 6 genes listed in Tables 3 and 4 and illustrated in Figures 5 and 6) is surprising. Either there is only a loose functional relationship between gene expression and molecular restoration during RS, or this relationship is not common across all brain regions. Alternatively, the functional consequences of SD and RS may be mediated by changes in gene expression within specialized cell populations. Indeed, recent studies have suggested that the effects of prior waking on sleep EEG are manifested locally within the cerebral cortex, rather than as a global response (Huber et al., 2004, Krueger and Obal, 2002). Specialized cell populations within the hypothalamus, basal forebrain or brainstem regions where sleep is controlled would be other candidate areas where a closer relationship between sleep restoration and gene expression may exist.

Since the information obtained in the present study represents a fragmentary sampling of the transcriptome (no more than 1,322 of the ~30,000 mRNAs), the absence of any clearly activated pathway in association with sleep restoration in the present study should not be surprising. It is possible that a restorative function does not involve mRNA and protein synthesis or suppression, but could instead involve protein modifications, for example, cessation of protein cleavage by calpains, caspases or the proteasome. The degree to which changes at the mRNA level are accompanied by changes in protein synthesis, and the roles of specific proteins in mediating these functional consequences, remain to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Research supported by RO1 HL/MH59658.

REFERENCES

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet. 2000; 25:25–29. [PubMed: 10802651]

Borbely, AA.; Achermann, P. Sleep homeostasis and models of sleep regulation. In: Kryger, MH., et al., editors. Principles and Practice of Sleep Medicine. Philadelphia: W.B. Saunders; 2000. p. 377-390.

Chou TC, Scammell TE, Gooley JJ, Gaus SE, Saper CB, Lu J. Critical role of dorsomedial hypothalamic nucleus in a wide range of behavioral circadian rhythms. J Neurosci. 2003; 23:10691–10702. [PubMed: 14627654]

Cirelli C, Gutierrez CM, Tononi G. Extensive and divergent effects of sleep and wakefulness on brain gene expression. Neuron. 2004; 41:35–43. [PubMed: 14715133]

Cirelli C, Tononi G. Differential expression of plasticity-related genes in waking and sleep and their regulation by the noradrenergic system. J Neurosci. 2000a; 20:9187–9194. [PubMed: 11124996]

Cirelli C, Tononi G. Gene expression in the brain across the sleep-waking cycle. Brain Res. 2000a; 885:303–321. [PubMed: 11102586]

Dijk D-J, Brunner DP, Beersma DG, Borbely AA. Electroencephalogram power density and slow wave sleep as a function of prior waking and circadian phase. Sleep. 1990; 13:430–440. [PubMed: 2287855]

Franken P, Chollet D, Tafti M. The homeostatic regulation of sleep need is under genetic control. J Neurosci. 2001; 21:2610–2621. [PubMed: 11306614]

Guzowski JF, Lyford GL, Stevenson GD, Houston FP, McGaugh JL, Worley PF, Barnes CA. Inhibition of activity-dependent arc protein expression in the rat hippocampus impairs the maintenance of long-term potentiation and the consolidation of long-term memory. J Neurosci. 2000; 20:3993–4001. [PubMed: 10818134]

Huber RM, Felice Ghilardi M, Massimini M, Tononi G. Local sleep and learning. Nature. 2004; 430:78–81. [PubMed: 15184907]

Jones BE. Activity, modulation and role of basal forebrain cholinergic neurons innervating the cerebral cortex. Prog Brain Res. 2004; 145:157–169. [PubMed: 14650914]

Krueger, JM.; Obal, F. Function of Sleep. In: Lee-Chiong, TL.; Sateia, MJ.; Carskadon, MA., editors. Sleep Medicine. Philadelphia: Hanley & Belfus, Inc; 2002.

Lancel M, van Riezen H, Glatt A. Effects of circadian phase and duration of sleep deprivation on sleep and EEG power spectra in the cat. Brain Res. 1991; 548:206–214. [PubMed: 1868336]

Lyford GL, Yamagata K, Kaufmann WE, Barnes CA, Sanders LK, Copeland NG, Gilbert DJ, Jenkins NA, Lanahan AA, Worley PF. Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. Neuron. 1995; 14:433–445. [PubMed: 7857651]

Manns ID, Alonso A, Jones BE. Rhythmically discharging Basal forebrain units comprise cholinergic, GABAergic, and putative glutamatergic cells. J Neurophysiol. 2003; 89:1057–1066. [PubMed: 12574480]

McCormick DA. Neurotransmitter actions in the thalamus and cerebral cortex and their role in neuromodulation of thalamocortical activity. Prog Neurobiol. 1992; 39:337–388. [PubMed: 1354387]

Nakanishi H, Sun Y, Nakamura RK, Mori K, Ito M, Suda S, Namba H, Storch FI, Dang TP, Mendelson W, Mishkin M, Kennedy C, Gillin JC, Smith CB, Sokoloff L. Positive correlations between cerebral protein synthesis rates and deep sleep in Macaca mulatta. Eur J Neurosci. 1997; 9:271–279. [PubMed: 9058047]

Nelson SE, Duricka DL, Campbell K, Churchill L, Krueger JM. Homer1a and 1bc levels in the rat somatosensory cortex vary with the time of day and sleep loss. Neurosci Lett. 2004; 367:105–108. [PubMed: 15308308]

O'Hara BF, Young KA, Watson FL, Heller HC, Kilduff TS. Immediate early gene expression in brain during sleep deprivation: preliminary observations. Sleep. 1993; 16:1–7. [PubMed: 8456228]

Paxinos, G.; Watson, C. The rat brain in stereotaxic coordinates. San Diego: Academic; 2004.

Pompeiano M, Cirelli C, Tononi G. Immediate-early genes in spontaneous wakefulness and sleep: expression of c-fos and NGFI-A mRNA and protein. J Sleep Res. 1994; 3:80–96. [PubMed: 10607112]

Ramm P, Smith CT. Rates of cerebral protein synthesis are linked to slow wave sleep in the rat. Physiol Behav. 1990; 48:749–753. [PubMed: 2082375]

Saper CB, Chou TC, Scammell TE. The sleep switch: hypothalamic control of sleep and wakefulness. Trends Neurosci. 2001; 24:726–731. [PubMed: 11718878]

Shaw PJ, Cirelli C, Greenspan RJ, Tononi G. Correlates of sleep and waking in *Drosophila melanogaster*. Science. 2000; 287:1834–1837. [PubMed: 10710313]

Sherin JE, Shiromani PJ, McCarley RW, Saper CB. Activation of ventrolateral preoptic neurons during sleep. Science. 1996; 271:216–219. [PubMed: 8539624]

Steininger TL, Alam MN, Gong H, Szymusiak R, McGinty D. Sleep-waking discharge of neurons in the posterior lateral hypothalamus of the albino rat. Brain Res. 1999; 840:138–147. [PubMed: 10517961]

Steriade M. Acetylcholine systems and rhythmic activities during the waking--sleep cycle. Prog Brain Res. 2004; 145:179–196. [PubMed: 14650916]

Suntsova N, Szymusiak R, Alam MN, Guzman-Marin R, McGinty D. Sleep-waking discharge patterns of median preoptic nucleus neurons in rats. J Physiol. 2002; 543:665–677. [PubMed: 12205198]

Terao A, Greco MA, Davis RW, Heller HC, Kilduff TS. Region-specific changes in immediate early gene expression in response to sleep deprivation and recovery sleep in the mouse brain. Neuroscience. 2003a; 120:1115–1124. [PubMed: 12927216]

Terao A, Peyron C, Ding J, Wurts SW, Edgar DM, Heller HC, Kilduff TS. Prepro-hypocretin (prepro-orexin) expression is unaffected by short-term sleep deprivation in rats and mice. Sleep. 2000; 23:867–874. [PubMed: 11083595]

Terao A, Steininger TL, Hyder K, Apte-Deshpande A, Ding J, Rishipathak D, Davis RW, Heller HC, Kilduff TS. Differential increase in the expression of heat shock protein family members during sleep deprivation and during sleep. Neuroscience. 2003b; 116:187–200. [PubMed: 12535952]

Tobler I. Effect of forced locomotion on the rest-activity cycle of the cockroach. Behav Brain Res. 1983; 8:351–360. [PubMed: 6871020]

Tobler I, Jaggi K. Sleep and EEG spectra in the Syrian hamster (Mesocricetus auratus) under baseline conditions and following sleep deprivation. J Comp Physiol [A]. 1987; 161:449–459.

Torterolo P, Yamuy J, Sampogna S, Morales FR, Chase MH. Hypocretinergic neurons are primalrily involved in activation of the somatomotor system. Sleep. 2003; 26:25–28. [PubMed: 12627728]

Trachsel L, Tobler I, Borbely AA. Effect of sleep deprivation on EEG slow wave activity within non-REM sleep episodes in the rat. Electroencephalogr Clin Neurophysiol. 1989; 73:167–171. [PubMed: 2473885]

Yamazaki S, Kerbeshian MC, Hocker CG, Block GD, Menaker M. Rhythmic properties of the hamster suprachiasmatic nucleus *in vivo*. J Neurosci. 1998; 18:10709–10723. [PubMed: 9852606]

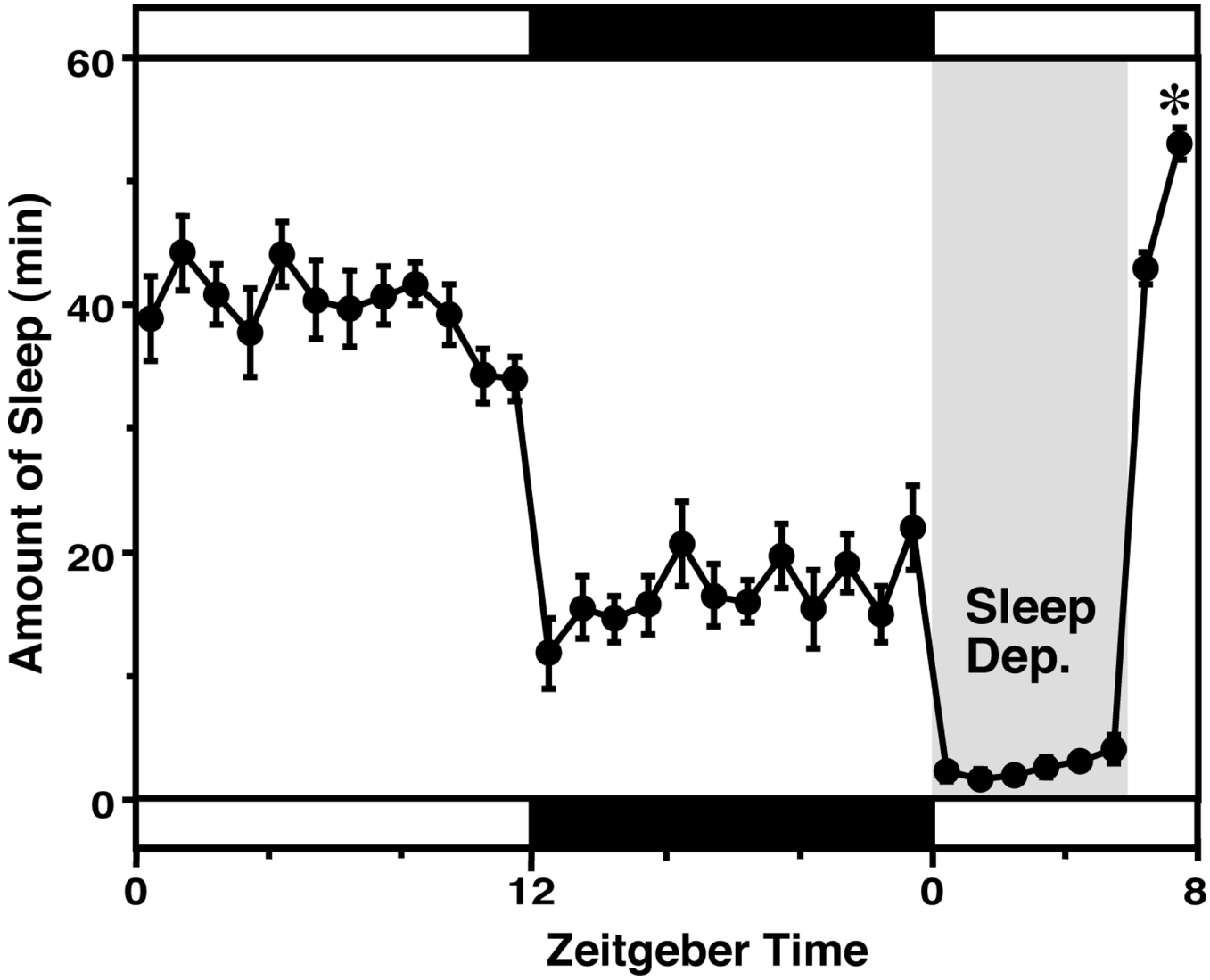

Figure 1.

Hourly total sleep time values (mean ± S.E.M.) in male Wistar rats (n=13) over a 32 h period: 24 h baseline followed by 6 h of SD and a subsequent 2 h recovery period. Black horizontal bars indicate dark periods, and white bars indicate light period. Asterisk denotes significant difference (*$p < 0.05$) between experimental and corresponding control group by Student-Newman-Keuls post-hoc test followed by one-way ANOVA.

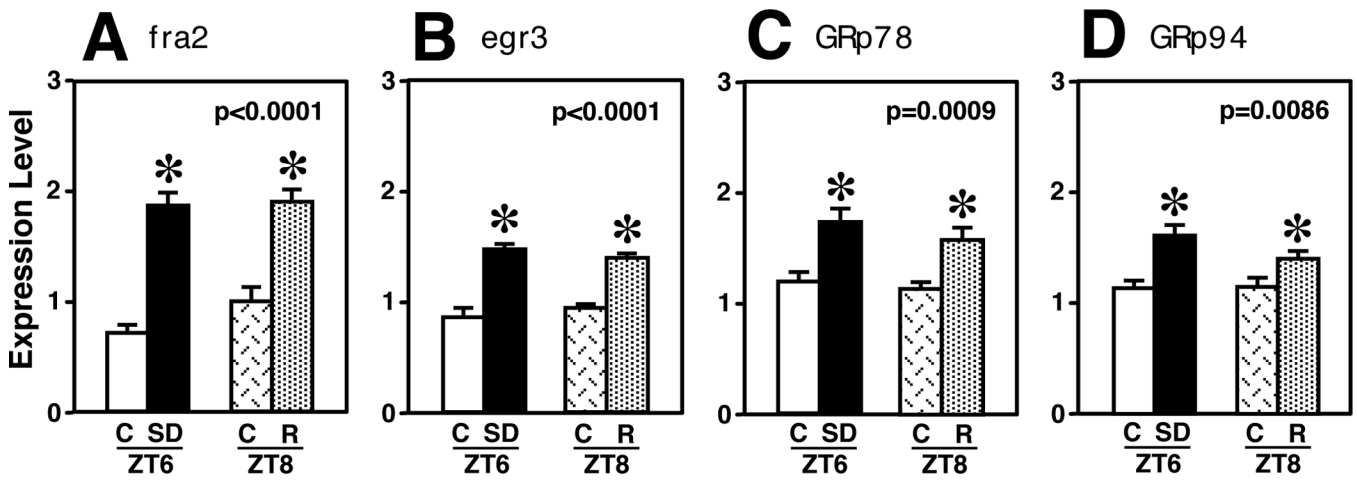

Figure 2.

Expression of *egr3*, *fra2*, *grp78* and *grp94* mRNA in the rat cerebral cortex across the four experimental conditions determined by real-time RT-PCR. *g3pdh* expression was used as an internal standard. Values are mean ± S.E.M. *P* values are based on ANOVA; asterisks denote significant differences (*$p < 0.05$) between experimental and corresponding control group by Tukey-Kramer post-hoc test. Abbreviations: C, control; R, recovery sleep; SD, sleep deprived; ZT, zeitgeber time; NS, no significant difference.

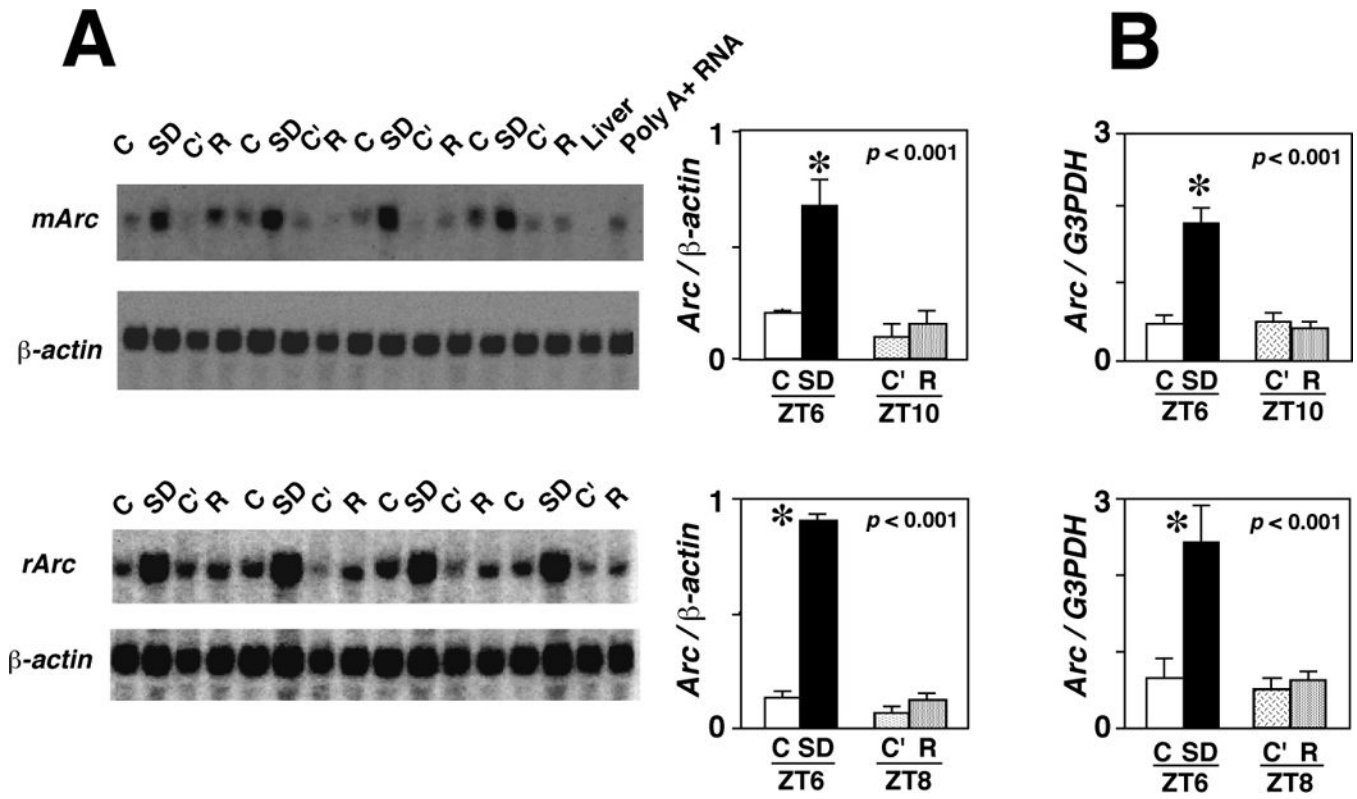

Figure 3.

Expression of *arc* mRNA in mouse and rat cerebral cortex after 6 h SD and after recovery from SD. **A.** Autoradiographs of Northern blots of total RNA from mouse cerebral cortex (upper panel) and rat cerebral cortex (lower panel) hybridized to [^{32}P]-labeled *arc* and β-*actin* probes. Based on OD_{260} readings, approximately 5 μg total RNA was loaded in each lane. Abbreviations: SD, animals deprived of sleep for 6 h; C, control animals sacrificed 6 h after lights on (ZT6); R, animals allowed 2 h (rat) or 4 h (mouse) recovery sleep after 6 h SD; C', control mice sacrificed 10 h after lights on (ZT10; upper panel) or control rats sacrificed eight hours after lights-on (ZT8; lower panel). Graph based on optical density ratios from resultant autoradiographs; values are mean ± S.E.M. **B.** Taqman analysis of *arc* expression in mouse (upper panel) or rat (lower panel) cortex. *P* values are based on ANOVA; asterisks denote significant differences (*$p < 0.05$) between experimental and corresponding control group by Tukey-Kramer post-hoc test.

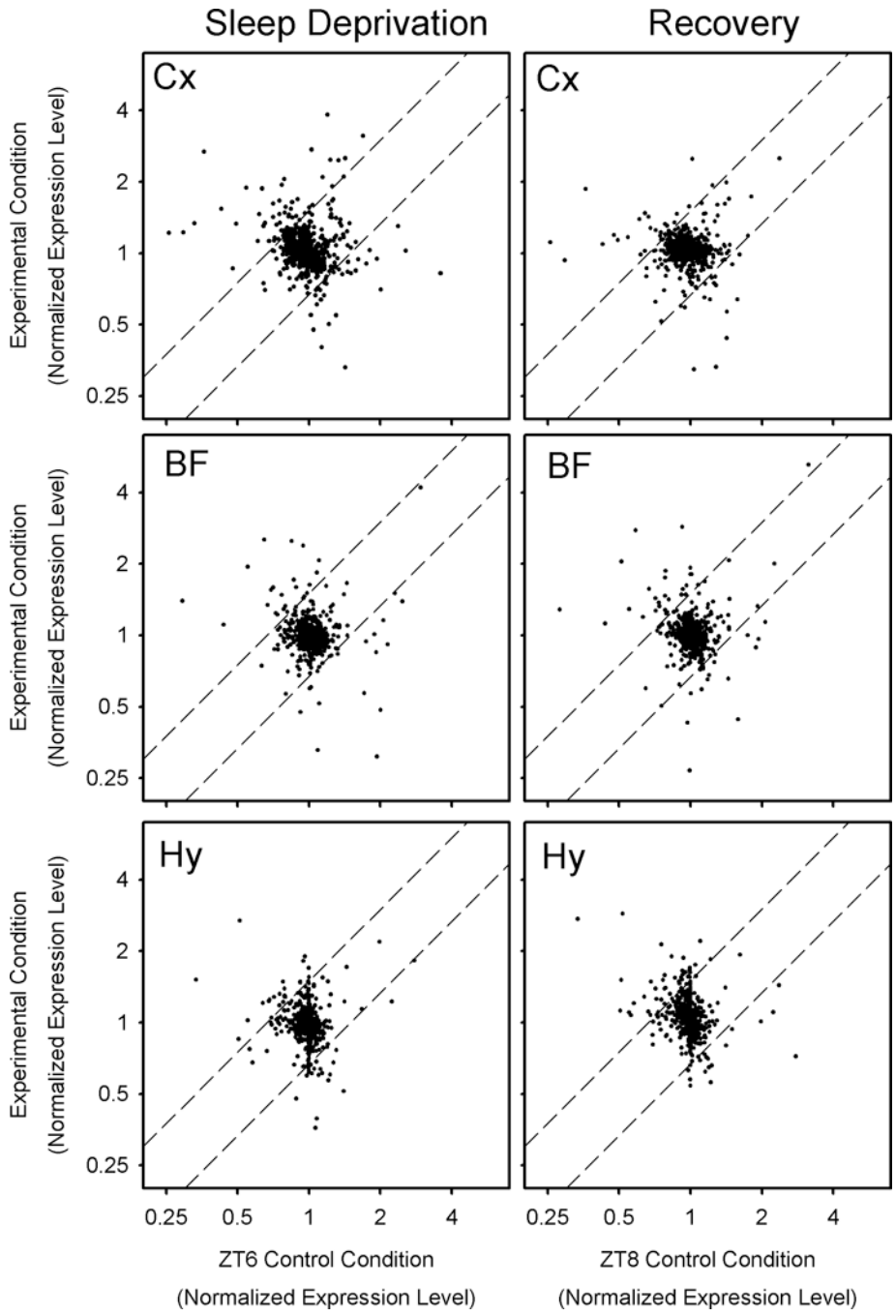

Figure 4.

Comparison of normalized intensity values obtained in SD vs. ZT6 control (left column) and RS vs. ZT8 control (right column) comparisons for the cerebral cortex (**A**), basal forebrain (**B**), and hypothalamus (**C**). For each gene, the mean signal intensity value for the experimental group (SD or RS; y-axis) is plotted against the mean of the corresponding control group (control; x-axis). The dotted lines in each graph demarcate the threshold for 1.5-fold upregulation or downregulation in the experimental group relative to the control group. Accordingly, points that lie above the upper dotted line in each graph represent genes

that underwent an increase in expression more than 1.5-fold relative to control values; points that lie below the lower dotted line underwent a decrease in expression more than 1.5-fold relative to control values. Values for which there was a qualitative difference between experimental and control groups (i.e., present in control chips and absent in experimental chips, or vice versa) are not plotted. Abbreviations: BF=basal forebrain; Cx=cerebral cortex; Hy=hypothalamus.

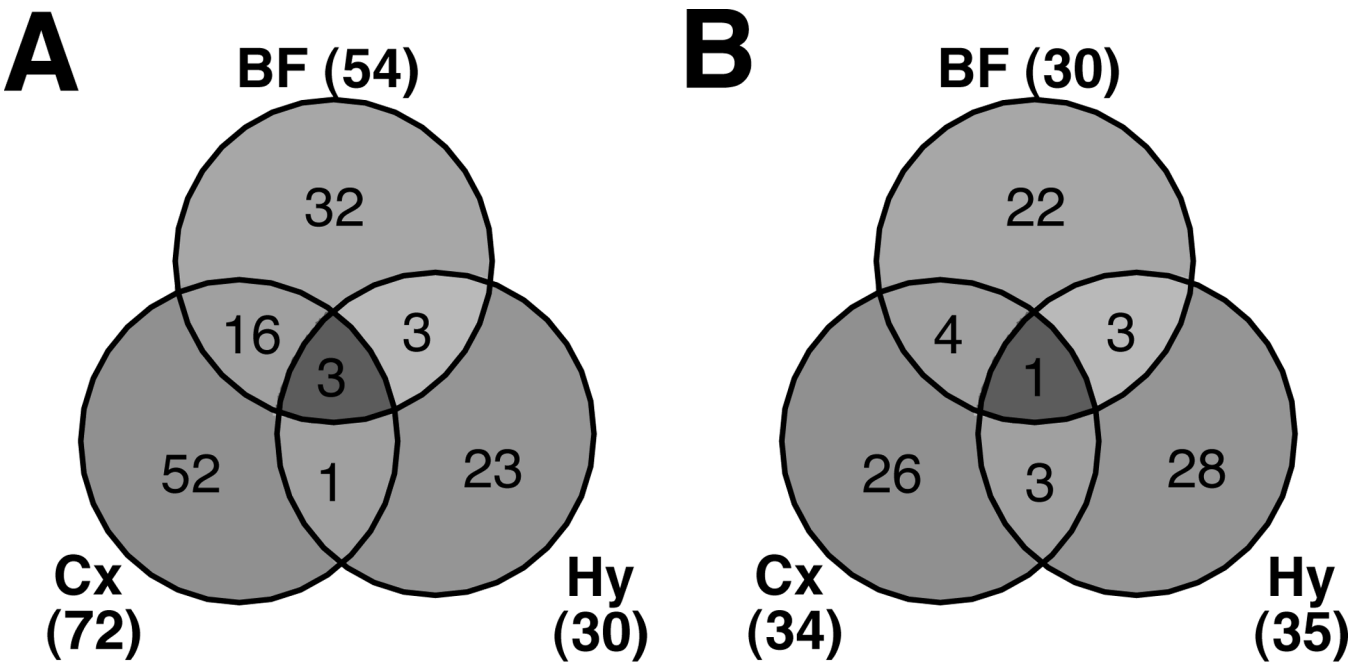

Figure 5.

Venn diagrams illustrating the number of genes up-regulated (**A**) or down-regulated (**B**) at least 1.5-fold in each of the three brain regions at ZT6 during SD. Abbreviations: BF=basal forebrain; Cx=cerebral cortex; Hy=hypothalamus.

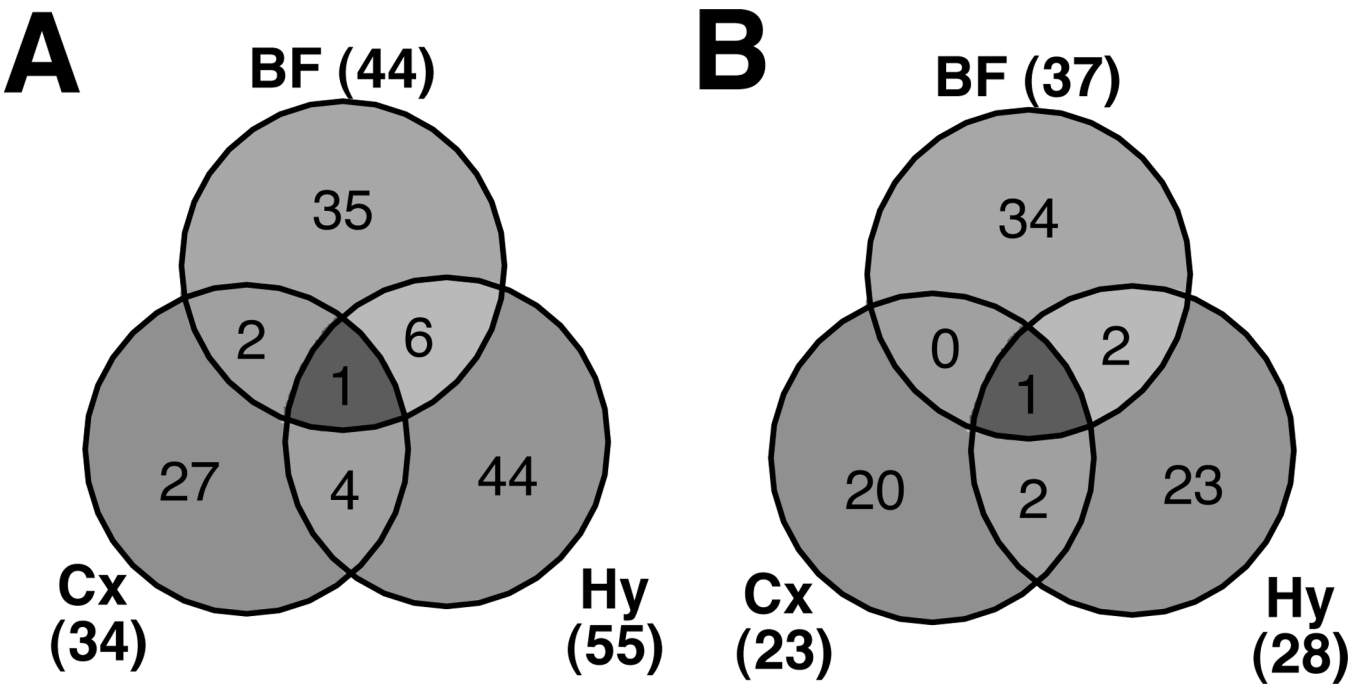

Figure 6.

Venn diagrams illustrating the number of genes up-regulated (**A**) or down-regulated (**B**) at least 1.5-fold in each of the three brain regions at ZT8 during RS. Abbreviations: BF=basal forebrain; Cx=cerebral cortex; Hy=hypothalamus.

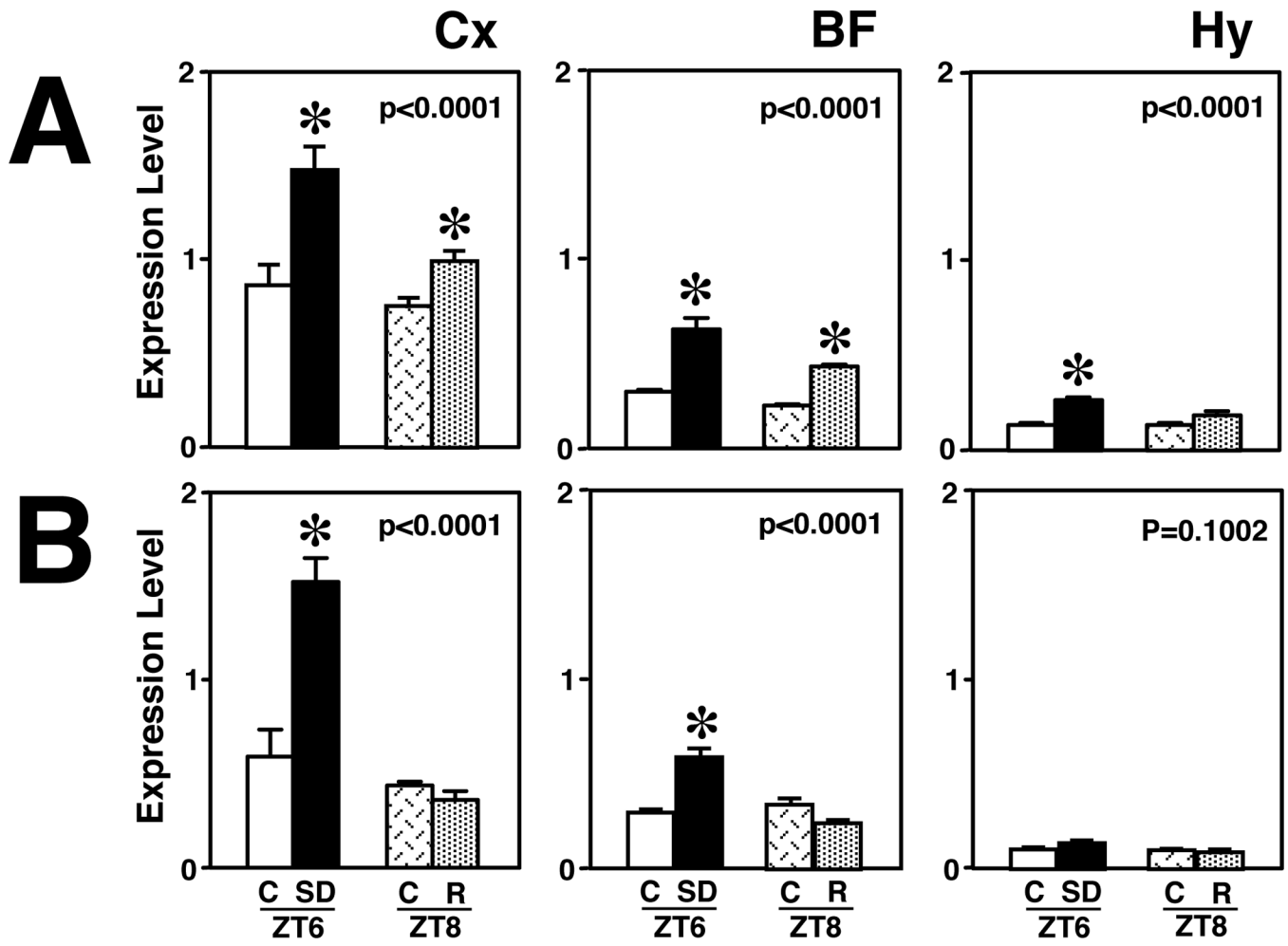

Figure 7.

Expression of *nr4a3* (A) and *ngfi-b* (B) mRNAs in the cerebral cortex (Cx), basal forebrain (BF), and hypothalamus (Hy) across the four experimental conditions. *g3pdh* expression was used as an internal standard. Values are mean ± S.E.M. *P* values are based on the ANOVA; asterisks denote significant differences (**p* < 0.05) between experimental and corresponding control group by Tukey-Kramer post-hoc test. Abbreviations: C, control; R, recovery sleep; SD, sleep deprived; ZT, Zeitgeber time.

Table 1

Primer and probe sequences used for quantitative real-time PCR analyses.

Gene name	Amplicon (bp)	Forward (F) and reverse (R) primers and probe (P) sequence
arc	68	F: GCAGGTGGGTGGCTCTGA
		R: TCTTGGCTGGCCCATTCA
		P: 6FAM5'-AATATTGGCTGTCCCAGATCCAGAACCAC-3'TAMRA
c-fos	64	F: ATTCCCCAGCCGACTCCTT
		R: TCTGCGCAAAAGTCCTGTGT
		P: 6FAM5'-CCAGCATGGGCTCCCCTGTCAA-3'TAMRA
c-jun	68	F: AATGGGCACATCACCACTACAC
		R: TGCTCGTCGGTCACGTTCT
		P: 6FAM5'-ACCCCCACTCAGTTCTTGTGCCCC-3'TAMRA
egr-1	70	F: GGCGCTGGTGGAGACAAGT
		R: GAAGCGGCCAGTATAGGTGATG
		P: 6FAM5'-TCCCAGCCAAACTACCCGGTTGC-3'TAMRA
ERp72	67	F: ATGTTGGCCAGCAAATTCG
		R: CAGCCTGTCCCTTCTTCAGG
		P: 6FAM5'-TGTGAGTGGCTACCCCACCATCAAGA-3'TAMRA
G3PDH	60	F: GGGCAGCCCAGAACATCA
		R: TGACCTTGCCCACAGCCT
		P: 6VIC5'-CCCTGCATCCACTGGTGCTGCC-3'TAMRA
GRp78	59	F: CGTCCAACCCGGAGAACA
		R: ATTCCAAGTGCGTCCGATG
		P: 6FAM5'-CGTCTTCGACGCCAAGCGCC-3'TAMRA
GRp94	64	F: GTGGGTGCTGGGCCTCT
		R: GACTTCATCGTCAGCTCTCACAA
		P: 6FAM5'-CTGCGTCCTGCTGACCTTCGGG-3'TAMRA
junD	69	F: CGCAAGCTGGAGCGTATCTC
		R: CAGCTCGGTGTTCTGGCTTT
		P: 6FAM5'-CGCCTGGAGGAGAAAGTCAAGACCCTC-3'TAMRA
ngfi-b	65	F: CACCCACCTCTCCGAACTGT
		R: GGCCTTGGTGGAGGTTACG
		P: 6FAM5'-CACTTCCGGCATTCTGGACGC-3'TAMRA
nr4a3	68	F: CGCCCTTGTCCGAGCTTTA
		R: CGGTGGGACAGTATCTGGAGTAA
		P: 6FAM5'-CAGACGCAACGCCCAGAGACCTTG-3'TAMRA
nur77	65	F: CACCCACCTCTCCGAACTGT
		R: GGCCTTGGTGGAGGTTACG
		P: 6FAM5'-CACTTCCGGCATTCTGGACGCA-3'TAMRA

F: forward primer, R: reverse primer, P: TaqMan® probe

Table 2

Magnitude of gene expression change between experimental and control conditions for the 13 genes examined in mouse and rat.

	SD		RS	
Gene Name	**Mouse**	**Rat**	**Mouse**	**Rat**
c-fos	↑↑↑	↑↑↑	-	-
fosB	↑↑	**?**	-	**?**
fra-1	-	-	-	-
fra-2	-	↑↑↑	↑↑	↑↑
junB	↑↑	↑↑	-	-
c-jun	-	-	-	-
junD	-	-	-	-
egr-1/NGFI-A	↑↑↑	↑↑	-	-
egr-3	↑↑	↑↑	↑	↑↑
nur77/NGFI-B	↑↑	↑↑	-	-
Arc	↑↑↑↑	↑↑↑↑	-	-
ERp72	↑↑	↑	-	-
GRp78	↑↑	↑	↑	↑
GRp94	-	↑	↑↑	↑

Number of arrows indicates x-fold change as determined by real-time RT-PCR analysis:

↑	indicates gene is significantly upregulated but the magnitude is 1.5-fold or less.
↑↑	indicates gene is significantly upregulated and the magnitude is 1.5- to 2.5-fold.
↑↑↑	indicates gene is significantly upregulated and the magnitude is 2.5- to 3.5-fold.
↑↑↑↑	indicates gene is significantly upregulated and the magnitude is 3.5- to 4.5-fold.
-	No change relative to time-matched control.
?	Not determined in rat.

Table 3

A. Genes found to be commonly upregulated by at least 50% in two or more brain regions in response to SD.

Brain Regions	Probeset Name	Gene Name (UniGene Abbreviation)	Genbank #
BF and Cx	AJ000557cds_s_at	Janus kinase 2 (Jak2)	AJ000557
	rc_AI230211_s_at	Potassium voltage gated channel, Shal-related family, member 3 (Kcnd3)	AI230211
	U17254_at	Immediate early gene transcription factor NGFI-B (Nr4a1)	U17254
	AF030088UTR#1_at	Homer, neuronal immediate early gene, 1 (ania-3)	AF030088
	X60769mRNA_at	CCAAT/enhancer binding protein (C/EBP), beta	X60769
	rc_AI229237_at	Opioid receptor-like (Oprl)	AI229237
	L13406_s_at	Calcium/calmodulin-dependent protein kinase II, delta (Camk2d)	L13406
	AF030086UTR#1_at	Rattus norvegicus activity and neurotransmitter-induced early gene 1 (ania-1) mRNA, 3'UTR.	AF030086
	S64320_at	K+ channel polypeptide; Shal1=K+ channel polypeptide [rats, hippocampus, mRNA, 3350 nt].	S64320
	Z38067exon_g_at	R.norvegicus DNA for c-myc, exon 2.	Z38067
	AF027954_at	Bcl-2-related ovarian killer protein (Bok)	AF027954
	U56261_s_at	Synaptosomal-associated protein 25 (Snap25)	U56261
	U67140_at	Disks large-associated protein 4 (Dlgap4)	U67140
	rc_AI045501_s_at	Neuronal pentraxin receptor (Nptxr)	AI045501
	S82649_s_at	Narp=neuronal activity-regulated pentraxin [rats, hippocampus, mRNA, 2562 nt].	S82649
	U75397UTR#1_s_at	Rattus norvegicus Krox-24 mRNA, 3' untranslated region, partial sequence.	U75397
BF and Hy	M54987_at	Rat corticotropin releasing hormone (CRH) gene, complete cds.	M54987
	U17995_at	Rattus norvegicus kappa opioid receptor gene, exon 4 and complete cds.	U17995
	U14647_g_at	Caspase 1 (Casp1)	U14647
Cx and Hy	U14005exon#1_s_at	Rattus norvegicus calcium channel alpha-1 subunit gene, partial cds.	U14005
BF, Cx and Hy	rc_AI102299_s_at	BH3 interacting domain 3 (Bid3)	AI102299
	rc_AI176710_at	Nuclear receptor subfamily 4, group A, member 3 (Nr4a3)	AI176710
	U17254_g_at*	Immediate early gene transcription factor NGFI-B (Nr4a1)	U17254

B. Genes found to be commonly downregulated by at least 50% in two or more brain regions in response to SD.

Brain Regions	Probeset Name	Gene Name (UniGene Abbreviation)	Genbank #
BF and Cx	U42719_at	Complement component 4a (C4a)	U42719
	D10891_at	Glutamate receptor, metabotropic 5 (Grm5)	D10891
	M34384_at	Nestin (Nes)	M34384
	M69055_at	Rat insulin-like growth factor binding protein (rIGFBP-6) mRNA, complete cds.	M69055
BF, Hy	AF053312_s_at	Small inducible cytokine subfamily A20 (Ccl20)	AF053312
	X13905cds_at	rab1B protein (AA 1 – 201); Rat cDNA for ras-related rab1B protein.	X13905
	M22357_at	Myelin-associated glycoprotein (Mag)	M22357
Cx and Hy	S94371_at	GluR-4c; glutamate receptor subunit 4c {alternatively spliced} [mRNA, 3452 nt].	S94371
	AJ006519_at	Amiloride-sensitive cation channel 2, neuronal (Accn2)	AJ006519
	U04738_at	Somatostatin receptor 4 (Sstr4)	U04738
BF, Cx and Hy	rc_AA945583_at	Hydroxysteroid (17-beta) dehydrogenase 10 (Hsd17b10)	AA945583

*Upregulation in hypothalamus during SD not confirmed by Taqman analysis.

Note also presence of this gene in BF and Cx category above under a different probeset name.

Table 4

A. Genes found to be commonly upregulated by at least 50% in two or more brain regions during recovery sleep.

Brain Regions	Probeset Name	Gene Name (UniGene Abbreviation)	Genbank #
BF and Cx	U50412_at	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (Pik3r1)	U50412
	S82649_s_at	Narp; neuronal activity-regulated pentraxin; precursor	S82649
BF and Hy	AF027954_at	Bcl-2-related ovarian killer protein (Bok)	AF027954
	rc_AI176710_at	Nuclear receptor subfamily 4, group A, member 3 (Nr4a3)	AI176710
	X51992_at	Gamma-aminobutyric acid A receptor, alpha 5 (Gabra5)	X51992
	rc_AI045501_s_at	Neuronal pentraxin receptor (Nptxr)	AI045501
	U39549_at	Synaptogyrin 1 (Syngr1)	U39549
	X01032_at	Cholecystokinin (Cck)	X01032
Cx and Hy	M91599mRNA_g_at	Rattus sp. fibroblast growth factor receptor subtype 4 (FGFR4) mRNA	M91599
	rc_AI102299_s_at	BH3 interacting domain 3 (Bid3)	AI102299
	rc_AI030286_s_at	Brain derived neurotrophic factor (Bdnf)	AI030286
	rc_AI227647_s_at	Chemokine (C-X3-C motif) ligand 1 (Cx3cl1)	AI227647
BF, Cx, Hy	AFFX_Rat_GAPDH_3_st	Glyceraldehyde-3-phosphate dehydrogenase (Gapd)	X02231

B. Genes found to be commonly downregulated by at least 50% in two or more brain regions during recovery sleep.

Brain Regions	Probeset Name	Gene Name (UniGene Abbreviation)	Genbank #
BF, Hy	AF031430_at	Syntaxin 7 (Stx7)	AF031430
	M22357_at	Myelin-associated glycoprotein (Mag)	M22357
Cx, Hy	X13905cds_at	Rat cDNA for ras-related rab1B protein.	X13905
	U08257_at	Glutamate receptor, ionotropic, kainate 4 (Grik4)	U08257
BF, Cx, Hy	X54686cds_at	R.norvegicus pJunB gene.	X54686

Table 5

Percent of genes in each functional category whose mRNA expression levels changed by at least 50% during SD or RS.

Biological Process Category	# Genes on Chip	% of mRNAs upregulated during SD			% of mRNAs upregulated during RS		
		BF (n=54)	Cx (n=72)	Hy (n=30)	BF (n=44)	Cx (n=34)	Hy (n=55)
Cell Adhesion	39	0	1	3	2	0	2
Cell Death	27	0	0	0	0	0	0
Maintenance/ Growth	420	17	31	27	27	29	27
Neurotransmission	290	26	25	7	25	15	22
Development	77	2	1	3	0	9	2
Intracellular Signal	132	15	8	7	14	6	5
Physiology	29	2	4	0	7	3	0
Other	308	39	29	53	25	38	42
Biological Process Category	# Genes on Chip	% of mRNAs downregulated during SD			% of mRNAs downregulated during RS		
		BF (n=30)	Cx (n=34)	Hy (n=35)	BF (n=37)	Cx (n=23)	Hy (n=28)
Cell Adhesion	39	3	0	3	5	4	7
Cell Death	27	0	3	0	0	4	0
Maintenance/ Growth	420	30	26	20	30	4	29
Neurotransmission	290	37	38	23	16	26	36
Development	77	7	9	3	11	9	7
Intracellular Signal	132	10	15	9	16	0	4
Physiology	29	3	3	0	3	0	4
Other	308	10	6	43	19	52	14

Table 6

A. Genes found to be upregulated by at least 50% during sleep deprivation and downregulated by at least 50% during recovery sleep.

Brain Regions	Probeset Name	Gene Name (UniGene Abbreviation)	Genbank #
BF	rc_AI229237_at	Opioid receptor-like (Oprl)	AI229237
	Z38067exon_g_at	R.norvegicus DNA for c-myc, exon 2.	Z38067
	U67140_at	Disks large-associated protein 4 (Dlgap4)	U67140
	AF030086UTR#1_at	activity and neurotransmitter-induced early gene 1 (ania-1), 3'UTR.	AF030086
Cx	X56306_s_at	Tachykinin 1 (Tac1)	X56306
	X13905cds_at	rab1B protein (AA 1 – 201); Rat cDNA for ras-related rab1B protein.	X13905
	S64320_at	Shal1=K+ channel polypeptide [rats, hippocampus, mRNA, 3350 nt].	S64320
	U52950_at	Microtubule-associated protein 1b (Map1b)	U52950

B. Genes found to be downregulated by at least 50% during sleep deprivation and upregulated during recovery sleep.

Brain Regions	Probeset Name	Gene Name (UniGene Abbreviation)	Genbank #
BF	AB016161cds_i_at	Gamma-aminobutyric acid (GABA) B receptor, 1 (Gabbr1)	AB016161
	U50412_at	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (Pik3r1)	U50412
	rc_AI137246_s_at	Ig VH193020=anti-insulin 193020 monoclonal antibody heavy chain variable region [mice, NOD].	S65980
Cx	S71597_s_at	Nitric oxide synthase 2, inducible (Nos2)	S71597
Hy	M91599mRNA_g_at	Rattus sp. fibroblast growth factor receptor subtype 4 (FGFR4) mRNA	M91599
	X01032_at	Cholecystokinin (Cck)	X01032
	Z11548_at	Glutamate receptor, ionotropic, kainate 2 (Grik2)	Z11548
	U67140_at	Disks large-associated protein 4 (Dlgap4)	U67140
	X07287cds_s_at	protein kinase C gamma (AA 1 – 697)	X07287
	M13707_at	Protein kinase C, gamma (Prkcc)	M13707