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LOCUS COERULEUS CONTROL OF STATE-DEPENDENT GENE EXPRESSION

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Abstract

Wakefulness and sleep are accompanied by changes in behavior and neural activity, as well as by the upregulation of different functional categories of genes. However, the mechanisms responsible for such state-dependent changes in gene expression are not known. Here we investigate to what extent state-dependent changes in gene expression depend on the central noradrenergic system, which is active in wakefulness and reduces its firing during sleep. We measured the levels of ~5000 transcripts expressed in the cerebral cortex of control rats and of rats pretreated with DSP-4, a neurotoxin that removes the noradrenergic (NA) innervation of the cortex. We found that NA depletion reduces the expression of ~20% of known wakefulness-related transcripts. Most of these transcripts are involved in synaptic plasticity and in the cellular response to stress. By contrast, NA depletion increased the expression of the sleep-related gene encoding the translation elongation factor 2. These results indicate that the activity of the central NA system during wakefulness modulates neuronal transcription to favor synaptic potentiation and counteract cellular stress, while its inactivity during sleep may play a permissive role to enhance brain protein synthesis.

Introduction

Wakefulness and sleep differ strikingly not only in behavior and neural activity, but also in gene expression. We recently found that ~5% of the transcripts in the cerebral cortex change their expression according to behavioral state independent of time of day (Cirelli et al., 2004). Moreover, we found that transcripts upregulated during wakefulness and sleep are associated with different cellular processes. Wakefulness-related transcripts code for transcription factors, mitochondrial proteins, heat shock proteins, and proteins involved in synaptic potentiation. Sleep-related transcripts code for proteins involved in protein synthesis, cholesterol synthesis, membrane trafficking, and synaptic depression. These findings have provided new insights about the functional consequences of wakefulness and sleep at the cellular level (Cirelli et al., 2004). However, the mechanisms underlying such pervasive changes in cortical gene expression are unknown. One possibility is that they are controlled directly by the membrane potential of cortical cells, which is steadily depolarized during waking and undergoes slow oscillations during non rapid eye movement (NREM) sleep (Steriade et al., 2001). Another possibility is that they result from the action of neuromodulatory systems with diffuse projections whose firing rate is high in wakefulness and low in sleep.

The noradrenergic neurons of the locus coeruleus (LC) and the serotonergic neurons of the nucleus raphe dorsalis are tonically active during wakefulness, reduce their firing rate during NREM sleep, and cease firing during REM sleep (Aston-Jones and Bloom, 1981a; McGinty and Harper, 1976). Moreover, the firing rate of LC neurons, but not that of raphe dorsalis neurons, increases phasically in response to salient events (Aston-Jones and Bloom, 1981b; Rasmussen et al., 1986) and in relation to the decision to act (Clayton et al., 2003). In a first series of studies we showed that an intact LC is required for the high expression during wakefulness of five genes involved in synaptic plasticity (Cirelli et al., 1996; Cirelli and Tononi, 2000b). Specifically, after lesioning LC with the neurotoxin DSP-4 (Fritschy and Grzanna, 1989) we found that the cortical expression of *Arc*, *BDNF, c-fos*, *NGFI-A*, and P-CREB during wakefulness was decreased and often as low as during sleep. By contrast, a neurotoxic lesion of the serotonergic system had no effect on the expression of these genes (Tononi et al., 2000).

But does LC control the expression of other wakefulness-related or sleep-related transcripts? If so, which are these genes, and what are their functions? Here we have measured the expression of ~5000 transcripts in the cerebral cortex of normal rats and of rats pretreated with DSP-4. Taking advantage of our database of gene expression changes associated with wakefulness and sleep (Cirelli et al., 2004), we then performed a conjunction search to determine which state-dependent genes are sensitive to NA depletion. We found that NA positively modulates the expression of ~20% of all known wakefulness-related transcripts. Most of these transcripts are involved in synaptic plasticity and in the cellular response to stress. By contrast, the sleep-related transcript for the translation elongation factor 2 was the only known transcript whose expression increased after cortical NA depletion. Thus, LC activity during wakefulness modulates neuronal transcription to favor synaptic potentiation and memory acquisition and to counteract cellular stress, while LC inactivity during sleep may play a permissive role to enhance brain protein synthesis.

Methods

Animal groups and sleep recording

Male inbred WKY rats were pretreated with the selective serotonin uptake inhibitor fluoxetine (10mg/kg, i.p.) to minimize the effects of DSP-4 on serotoninergic terminals and injected 30-min later with either saline or DSP-4 (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine, RBI, 50 mg/kg, i.p.; see below). One week later, under pentobarbital anesthesia (60-75 mg/kg, i.p.), rats were implanted with screw electrodes on the dura over the frontal, parietal and occipital cortex to record the electroencephalogram (EEG) and silver electrodes in the neck muscles of both sides to record the electromyogram. After surgery rats were housed individually in sound-proof recording cages where lighting and temperature were kept constant (LD 12:12, lights on at 10am, 24±1°C, food and drink *ad libitum*). Immediately after recovery from anesthesia rats were connected by means of a flexible cable and a commutator (Airflyte, Bayonne, NJ) to a Grass electroencephalograph (mod. 15LT, Astro-Med. Inc., West Warwick, RI) and recorded continuously for 1-2 weeks. Each day from 10 to 10:30am all rats were gentle handled and exposed to a new object to become familiar with the sleep deprivation procedure. At least 7 days after surgery, saline-treated and DSP-4 treated rats were killed at 6pm after being kept awake for 8 hours by introducing novel objects in their cages. Every new object was delivered just following the first signs of synchronization in the EEG signal, and triggered exploratory behavior in all animals. Animal protocols followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were in accordance with institutional guidelines.

Lesions of the noradrenergic system

To lesion the NA system we used injections of the neurotoxin DSP-4. Lesions of the central noradrenergic system need to be selective, leaving other aminergic systems intact. Moreover, it is important that only the central noradrenergic system be affected, to avoid confounding factors due to the involvement of the peripheral nervous system. Injections of two neurotoxins, DSP-4 and 6-OHDA, represent the methods of choice for selective central noradrenergic lesions. 6-OHDA does not cross the blood-brain barrier, and therefore must be infused directly into the brain. The i.c.v. injection of 6-OHDA causes a rapid and long-lasting depletion of brain noradrenaline (Bloom, 1971), but the dopaminergic system is also affected (Hedreen and Chalmers, 1972), so that a selective depletion of cortical NA fibers can only be achieved by 6-OHDA injections in the dorsal noradrenergic bundle (Robbins and Everitt, 1995). DSP-4, on the other hand, can be given i.p. DSP-4 is a site-directed alkylating agent with affinity for the neuronal uptake transporter (for a review, see Dudley et al., 1990). It accumulates within the nerve endings and appears to deplete catecholamines by affecting their transport, possibly by reducing mitochondrial function. As confirmed in this study, when given with fluoxetine, which protects serotonergic fibers, DSP-4 can act as a selective neurotoxin for NA neurons, with little effect on the serotonergic system and no effect on the dopaminergic system. DSP-4, however, has some potential limitations. A few weeks after DSP-4 treatment damaged NA fibers are capable of restoring some of their lost functions by turnover (Fritschy and Grzanna, 1992). Moreover, DSP-4 affects the vast majority of noradrenergic fibers, which originate in the LC, but not the small percentage of fibers originating in cell groups A1 and A2 (Fritschy and Grzanna, 1989). Finally, the efficacy of the DSP-4 lesion can vary from animal to animal and an incomplete lesion (NA depletion of less than 80%) can cause "paradoxical" effects due to

compensatory mechanisms such as the upregulation of adrenergic receptors (Robbins and Everitt, 1995). To overcome these limitations, all animals in this study were killed within the first 3 weeks after DSP-4 treatment and HPLC measurements were used to confirm that cortical NA levels were reduced more than 80%.

Assay of noradrenaline (NA), dopamine (DA), serotonin (5-HT) and metabolites

Rats were deeply anesthetized with isoflurane (within 2 min) and decapitated. The head was cooled in liquid nitrogen and the whole brain was rapidly removed and placed on ice. Cerebral cortex, hippocampus, and cerebellum were dissected out and frozen immediately by immersion of the tubes in liquid nitrogen. All samples were stored at -80ºC until the time of the assay. Levels of NA, DA and its metabolites DOPAC (3,4-dihydroxyphenylacetic acid) and HVA (homovanillic acid), and 5-HT and its metabolite 5-HIAA (5-hydroxyindoleacetic acid) were measured in the left cerebral cortex using high performance liquid chromatography with electrochemical detection using the method of Fuertes et al. (2000). Briefly, the left cerebral cortex from each rat was homogenized in 0.02 M perchloric acid using a motor driven Potter-Elvehjem homogenizer. After centrifugation at 13,000 rpm for 5 minutes at 4°C, the clear supernatant was filtered through a 0.22µm GV (Millipore, Bedford, MA) and 15µl were injected into a 3µm C18 reverse-phase column (ESA Inc, Chelmsford, MA) in HPLC system (ESA Inc, Chelmsford, MA) where NA, DA and 5-HT and metabolites were analyzed. The mobile phase consisted of 0.1M sodium phosphate, 50mM citric acid, 1.8mM 1-Octanesulfonic acid, 10% acetonitrile, adjusted to pH 3.01 and the flow rate was 0.5ml/min. Values were expressed as ng/ml of tissue homogenate, based on internal standard measurements using dihydroxybenzylamine. The nonparametric Mann-Whitney U-test was used for the statistical analysis of the results.

mRNA differential display

The study was performed on 6 saline-treated rats and 6 DSP-4 treated rats, none of which was used for microarray analysis. mRNA differential display was performed as previously described (Cirelli and Tononi, 1998, 2000a). Briefly, total RNA (2µg) from the right cerebral cortex of each animal was individually reverse transcribed using Superscript II reverse transcriptase (GIBCO-BRL, Gaithersburg MD) and one of three 3' composite anchor primers $E1T_{12}M$ (E1=5'CGGAATTCGG, M=A, C, or G). Each reverse transcription reaction was then amplified by PCR in the presence of [α-^{33}P]dATP (New England Nuclear-Du Pont, Natick, MA). The primers used were one of the three 3' anchor primers used for the reverse transcription and one of thirty-five E2-AP 5' arbitrary primers, where E2 = CGTGAATTCG and AP is a sequence of 10 bp with a presence of 50% G+C and A+T and an absence of uninterrupted self-complementarity of more than two nucleotides. PCR conditions were as previously described. Radiolabeled PCR products were separated on 6% denaturating polyacrylamide gels and visualized by autoradiography. PCR reactions were performed in duplicate for each individual animal and run in parallel in the same gel. Differentially expressed bands were quantified with a phosphorimager (Amersham Biosciences, Piscataway, NJ) by measuring the average volume of each band for each lane and subtracting the background. The mean density within and among conditions was calculated and significant differences among conditions were evaluated by ANOVA followed by Bonferroni correction. Differentially expressed bands were recovered from dried gels, reamplified by PCR using the same primers and subcloned into pCR^{TM}II vectors using the TA cloning kit (Invitrogen, Carlsbad, CA). Plasmid DNA sequencing of cloned cDNAs

was carried out using the Cy5™ AutoRead sequencing kit (Pharmacia Biotech, Sweden). Sequences were screened using the BLAST program and the EMBL and GenBank databases.

Microarrays: labeling, hybridization and data analysis

Microarrays analysis was performed on pooled RNA from 7 saline-treated rats and 7 DSP-4 treated rats, while subsequent real time qPCR was performed both on pooled RNA from the same animals (technical verification) as well as on pooled RNA from an independent groups of 4 saline-treated and 4 DSP-4 treated rats (biological verification). Total RNA was isolated from right cerebral cortex of each animal by using Trizol (GIBCO-BRL) according to the manufacturer's instructions. Final RNA concentrations were determined spectrophotometrically. An equal mass amount of total RNA from the cerebral cortex of each animal was pooled from 7 rats within each experimental group (20 μg/pool), converted into first-strand cDNA using Superscript II Rnase H- reverse transcriptase (Invitrogen), and the second strand was synthesized, all according to the Affymetrix Gene Expression manual (Affymetrix, Inc., Santa Clara, CA). cDNA was then converted to biotinylated cRNA using the ENZO BioArray High Yield In Vitro Transcription kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's instructions. The cRNA was fragmented at 0.5 μg/μl final concentration in 1X fragmentation buffer (40 mM Tris-acetate, pH 8.1, 100 mM potassium acetate, 30 mM magnesium acetate). The size range of cRNA before (0.5 kb and longer) and after (35-200 base fragments) fragmentation was checked by denaturing agarose electrophoresis. The hybridization reaction and the automated hybridization procedure were performed according to Affymetrix instructions. Briefly, the procedure involved a 16 h hybridization followed by two washes and staining with Streptavidin-Phycoerythrin (SAPE, Affymetrix) stain solution. The staining was followed by another wash, antibody amplification with biotinylated antibody, a second SAPE staining, and a final wash (Affymetrix GeneChip Expression Analysis Technical Manual). Each sample was hybridized to an Affymetrix GeneChip U34A, B or C, scanned and normalized according to the Affymetrix GeneChip Expression Analysis Technical Manual [see https://www.affymetrix.com/Download/manuals/expression_ever_manual.pdf]. Quality of the cDNA and cRNA syntheses was determined by the 3'/5' ratio of housekeeping genes within the array (ubiquitin, glyceraldehyde 3-phosphate dehydrogenase, β-actin, and hexokinase). For each cDNA represented in the array, a ratio of intensities and its statistical significance were calculated. Statistical analysis was performed using paired non-parametric tests (MicroArray Suite 5.0, Affymetrix; Bolstad et al., 2003). Statistical analysis was also performed using a Discrete Bayesian Approach (DBA, SBI-Moldyn, Inc, Karlov, V.I., Padilla C.E., Maggio, E., Billingsley, F., Kasten, B., Discrete Bayesian Approach for Diagnosing Unapparent Diseases from Common Clinical Tests, US Patent application, submitted May 1, 2002). DBA, which is based on Bayesian nonlinear statistical analysis and extensive Monte Carlo cross-validation, confirmed >90% of the NA-modulated genes identified by MicroArray Suite 5.0.

Real time qPCR

Real time qPCR was performed as already described (Cirelli and Tononi, 2000a; Sequence Detection System 5700, Perkin Elmer, Wellesley, MA). Briefly, reverse transcription reactions were carried out in parallel on DNase I digested pooled total RNA from saline-treated and DSP-4 treated rats. Prior to reverse transcription, total RNA was confirmed to be free of contaminating DNA sequences by PCR using rat β-actin specific primer pairs designed to differentiate between cDNA, genomic DNA, and pseudogene genomic DNA. Eight reverse transcription reactions

were performed for each experimental group. Reverse transcription reactions were as follows: 100 ng total RNA, 2.5 µl oligo dT_{16} (500 µg/ml), 5 µl dNTPmix (10mM each dNTP), 1pg artificial transcript (IDT, Inc., Coralville, IA), H_2O to 29.75µl. Samples were incubated at 70 °C for 10 min, put briefly on ice, and then incubated at 42 °C for 2-5 min. Mix #2 (10 µl 5X Superscript II First Strand Buffer, 5 µl 0.1 M DTT, 4 µl 25 mM $MgCl_2$ and 1.25 µl Superscript II RNAse H^- Reverse Transcriptase 200 U/µl) was added, mixed, and samples were immediately returned to incubate at 42 °C for 1 hour. Reactions were stopped by incubation at 70 °C for 15 min. PCR reactions to measure levels of artificial transcript were done to confirm uniformity of reverse transcription within sample groups and between samples. Comparable reverse transcription reactions within a sample group were pooled. Each PCR reaction contained specific forward and reverse primers (200-750nM final concentration), 2X SYBR Green Master Mix (used at 3.2X), 5µl of a 1:10 dilution of pooled reverse transcription product and H_2O to a total volume of 25µl. A two step PCR profile was used: 10 min at 95°C denaturation and Amplitaq gold activation, followed by 40 cycles alternating between 95°C for 15 sec and 60°C for 60 sec. Dilution series (1:2, 1:10, 1:50, 1:250, 1:1250) standard curves were performed in quadruplicate for each primer pair using reverse transcription products from generic rat brain total RNA. PCR was done in quintuplicate for each sample condition assayed and relative quantities determined based on the equation of the line of best fit derived from the standard curve (R^2>=0.985).

Results

All animals recovered overnight from the DSP-4 injection, and increased their body weight during the 3 weeks following the treatment. All rats were recorded continuously for several days after adaptation to the recording environment to establish baseline percentages and distributions of wakefulness, NREM sleep, and REM sleep. The last day of the experiment all animals had been kept awake from 10am to 6pm, except for short (<30 sec) episodes of NREM sleep that could not be avoided at the end of the 8-h period of exposure to novel objects.

Effects of DSP-4 treatment on brain monoamine levels

Figure 1 shows that, in line with previous work (Dudley et al., 1990), DSP-4 lesions were effective and quite selective for NA. Cortical NA levels were reduced by more than 85% in all DSP-4 treated animals relative to saline-treated controls. By contrast, 5-HT content was only slightly reduced, and DA levels were preserved.

Effects of DSP-4 treatment on behavioral states

All rats were asleep for most of the light period and awake for most of the dark period, as expected under a 12h:12h light-dark schedule. As in previous studies (Cirelli et al., 1996; Cirelli and Tononi, 2000b), overall daily percentages of behavioral states 2-3 weeks after the DSP-4 injection did not differ between saline-treated and DSP-4 treated animals (Fig. 2). Also in agreement with previous reports (Cirelli et al., 1996; Cirelli and Tononi, 2000b), overall EEG power did not differ between the 2 experimental groups in any frequency bin (data not shown).

mRNA differential display

In an initial set of experiments, we used mRNA differential display to evaluate differences in gene expression among individual animals. One hundred and five primer combinations were

used, for a total of 105 gels. As in previous studies (Cirelli and Tononi, 1998, 2000a) the mean number of PCR products visualized in each gel was at least 50, thus theoretically allowing the evaluation of more than 5,000 RNA species. The band patterns obtained in separate experiments utilizing the same primers were highly reproducible, whereas patterns were markedly different when different primer pairs were used. As in previous studies (Cirelli and Tononi, 1998, 2000a), the vast majority of bands were similarly expressed in all individual animals and in both conditions, indicating that inter-individual variability in these inbred strains is low. Visual inspection identified 12 bands that were differentially expressed between conditions, all with higher expression in saline-treated rats (Fig. 3). All bands were excised from the gel, reamplified, cloned, and sequenced. Partial DNA sequence analysis revealed flanking sites complementary to the PCR primers in all cases, and all transcripts were 200-700 bp in size. Three transcripts corresponded to expressed sequence tags (ESTs). The remaining bands with higher expression levels in saline-treated animals corresponded to 8 known genes coding for alpha crystallin B, Arc, aryl sulfotransferase, BDNF, BiP, c-fos, HSP70, and NGFI-A. NGFI-A was isolated twice using 2 different sets of primers. Thus, this screening identified at least 8 known genes whose expression is positively modulated by NA. For four of them, *Arc, BDNF, c-fos*, and *NGFI-A*, the current results confirm previous findings obtained using a combination of ribonuclease protection assays (RPAs), in situ hybridization, and cDNA microarrays (Cirelli et al., 1996, 2000b).

cDNA microarrays

In a second set of experiments, performed on an independent group of animals, we used cDNA microarrays to comprehensively evaluate gene expression changes between conditions. In order to properly compare the effects of NA depletion with state-dependent gene expression, microarray hybridization and analysis were performed exactly as in our recent study of gene expression in sleep and wakefulness (Cirelli et al., 2004). Briefly, total RNA from the cerebral cortex of 7 saline-treated and 7 DSP-4 treated rats was analyzed using Affymetrix high-density oligonucleotide arrays (Rat GeneChip RGU34 A) to screen for the expression of ~ 7,000 annotated sequences and 1,000 ESTs. The analysis was performed using a set of statistical and empirical parameters aimed at minimizing the number of differentially expressed transcripts while maximizing the number of positive controls (see Methods). Transcripts whose expression level was too low in both saline-treated and DSP-4 treated rats were called "absent" and removed from the analysis. Approximately 62% of the transcripts remained after this step (4978/8000). To limit technical variability, we pooled RNA from 7 animals for each experimental group and performed 3 independent replicas of all the steps of the experiment, from first strand cDNA synthesis to chip hybridization. As in our previous study (Cirelli et al., 2004), outlier effects are unlikely due to the use of pooled RNA from a homogeneous tissue (whole cerebral cortex) and because of the choice of inbred WKY rats fully adapted to the recording environment and carefully selected for behavioral state (see Methods). Most importantly, the results obtained with mRNA differential display indicate that inter-individual differences in cortical gene expression patterns among animals belonging to the same group are minimal.

Significant expression changes were determined with paired non-parametric tests (MicroArray Suite 5.0, Affymetrix; Bolstad et al., 2003). Transcripts whose expression was considered up- or down-regulated after DSP-4 treatment had to be called "increased" or "decreased", respectively, in at least 2 out of 3 non-parametric statistical comparisons between controls and DSP-4 groups. A technique called Discrete Bayesian Approach (DBA, SBI-Moldyn,

Inc) was also used as an independent method for the identification of differentially expressed transcripts. DBA, which is based on Bayesian nonlinear statistical analysis and extensive Monte Carlo cross-validation, confirmed >90% of the transcripts identified by MicroArray Suite 5.0.

To validate microarray results we first examined positive controls, defined as transcripts that had been identified as NA-dependent in different sets of animals either in previous reports (Cirelli et al., 1998, 2000b) or in this study using mRNA differential display. Out of 8 previously identified positive controls (the genes coding for alpha crystallin B, Arc, arylsulfotransferase, BDNF, BiP, c-fos, HSP70, NGFI-A), 7 (all except BDNF) were represented on the chip, one (*c-fos*) was called absent, while the expression of five out of the remaining six (all except HSP70, whose differential expression also failed to be confirmed with qPCR), was identified as "decreased" in DSP-4 treated rats by our analysis.

To further validate the microarray results and control for biological variability we used 8 new animals (4 saline-treated and 4 DSP-4 treated rats). qPCR was used to confirm the differential expression of 32 transcripts identified by the microarray analysis as "increased" in DSP-4 (n=3), "decreased" in DSP-4 (n=25) or "no change" (n=4). Altogether, ~81% (26/32) of the results of microarray analysis examined were validated by qPCR experiments performed on independent sets of rats. Confirmation rate was ~90% when the same transcripts were measured by qPCR using pooled RNA from the same sets of animals used in the microarray experiments (technical verification). Thus, based on qPCR results, < 20% of our selected genes can be ascribed to noise related to biological variability, similarly to what has been described in other recent studies (Storch et al., 2002; Cirelli et al., 2004). Because in our hands real time qPCR had a global absolute error of <20%, we only report transcripts for which the average percentage change was > 20% (see Table 1). As measured by qPCR, expression changes for most NA-dependent transcripts were between 30% and 60%, similar to the range of modulation reported for mammalian transcripts affected by circadian time (Akhtar et al., 2002; Panda et al., 2002; Storch et al., 2002; Ueda et al., 2002) and by behavioral state (Cirelli et al., 2004).

Out of the 4978 transcripts called present, 97 (including 70 known transcripts and 27 ESTs) were differentially expressed between DSP-4 treated rats and saline-treated controls. Table 1 shows that in almost all cases mRNA levels were lower in DSP-4 treated rats relative to saline-treated controls, with only 2 transcribed sequences (the gene coding for EF2, the eukaryotic elongation factor 2, and one EST) showing higher mRNA levels in LC-lesioned animals. Most genes whose expression was decreased by the DSP-4 treatment could be grouped in a few functional categories, including neural plasticity, signal transduction, energy and lipid metabolism, and cellular response to stress.

Overlap between state-dependent transcripts and transcripts modulated by noradrenaline

This study of NA-dependent gene expression in the cerebral cortex was specifically designed with the aim of enabling a conjunction analysis with our previous study of state-dependent gene expression (Cirelli et al., 2004). We used rats of the same inbred strain, age, and sex as in Cirelli et al. 2004. Moreover, as in that study, rats were kept awake during the first 8 hours of the light period using exposure to novel objects. Finally, both studies used the same Affymetrix rat chips to profile the whole cerebral cortex and applied the same combination of paired non-parametric and Bayesian –based approaches to perform the statistical analysis. Thus, we were able to cross-reference cortical transcripts whose expression was previously identified as wakefulness-related or sleep-related with transcripts identified in this study and whose

expression changes after cortical depletion of NA. For the current study, we used chip RGU34A, which includes the great majority (>90%) of all the known transcripts represented in the Affymetrix set (chips B and C mostly include ESTs).

In our study of state-dependent gene expression (Cirelli et al., 2004), out of the ~5000 known transcripts included in the RGU34A chip and called present in the cerebral cortex, 95 were identified as wakefulness-related and 107 as sleep-related. By comparing the two analyses we found that 25% (18/72, including *c-fos* and *BDNF*) of all the known transcripts whose expression was modulated by NA had been previously identified as modulated by behavioral state, including 16 wakefulness-related and 2 sleep-related transcripts coding for the proteasome subunit RCX and EF2 (Figure 4A; see also Table 1). Interestingly, all 16 wakefulness-related known transcripts decreased their expression after cortical NA depletion while the sleep-related transcript EF2 was the only known transcript identified by the microarray analysis as "increased" after DSP-4 treatment. qPCR confirmed a significant increase (84%) in EF2 mRNA levels after DSP-4 treatment. Figure 4B shows that the majority (10/16) of the transcripts positively modulated by both wakefulness and cortical NA are involved in neural plasticity and in the cellular response to stress. On the other hand, transcripts modulated by NA represented ~9% (18/202) of all known state-dependent genes, and 17% (16/95) of all known wakefulness-related genes.

Discussion

In a previous study, we showed that gene expression in the cerebral cortex is markedly modulated by behavioral state (Cirelli and Tononi, 2004). Specifically, we found that 95 transcripts are expressed at higher levels in wakefulness than in sleep, whereas 107 behave in the opposite manner. In this study, we asked to what extent state-dependent changes in gene expression can be accounted for by the action of neuromodulators that are released in a state-dependent manner. In this study, using the same approach as in our previous report, we compared gene expression profiles in the cerebral cortex of control rats and rats in which cortical NA fibers had been destroyed by DSP-4. We found that NA depletion caused the downregulation of ~20% (16/95) of the known wakefulness-related genes in the cerebral cortex, most of which involved in synaptic plasticity and in the cellular response to stress. Other transcripts positively modulated by both NA and wakefulness are involved in the regulation of extracellular glutamate levels (GLAST-1), in the dopamine receptor signaling pathway (ania-4), in the activation of the mitochondrial pyruvate dehydrogenase complex (pyruvate dehydrogenase phosphatase 1) and in the catabolism of catecholamines (minoxidil(aryl)sulfotransferase). By contrast, only one gene, coding for EF2, the translation elongation factor 2, had previously been identified as sleep-related and was one of the two that increased their transcript levels after the cortical noradrenergic innervation had been destroyed.

Noradrenaline and plasticity

The largest functional category of genes whose expression is positively modulated by NA comprises genes involved in neural plasticity. Compelling evidence for a role for NA in plasticity has been obtained both in vitro and in vivo. Studies in vitro have shown that NA enhances the magnitude, duration, and probability of induction of long-term potentiation (LTP) in the cerebral cortex and in the hippocampus, an action mediated largely via beta adrenoceptors (Hopkins and Johnson, 1984; Stanton and Sarvey, 1985; Huang and Kandel, 1996; Thomas et al., 1996; Katsuki et al., 1997). Studies in vivo have shown that NA innervation of the cortex is

needed for ocular dominance plasticity (reviewed in Gu, 2002) and other forms of plasticity (Levin et al., 1988). Again, the effect of NA is mainly mediated by beta1-adrenoceptors, suggesting an LTP-related effect.

Several transcripts related to plasticity and modulated by NA, such those encoding Arc, N-cadherin, CDC42, and Homer 1c, affect the actin cytoskeleton. Arc interacts with cytoskeletal proteins and its mRNA localizes to those regions of the dendritic tree that have received synaptic stimulation (Link et al., 1995; Lyford et al., 1995; Steward et al., 1998). Moreover, Arc is specifically induced during the acquisition of a novel behavior (Kelly and Deadwyler, 2002), and the inhibition of Arc expression impairs the maintenance of LTP and the consolidation of long-term memory (Guzowski et al., 2000). The adhesion molecule N-cadherin regulates dendritic spine morphogenesis and is synthesized and recruited to activated synaptic sites during LTP (refs in Huntley et al., 2002). N-cadherin associates with molecules that control cytoskeletal rearrangements including the Rho family of small GTPases, of which CDC42 is one of the best studied (Huntley et al., 2002). CDC42 activation is required for the dendritic arbor growth driven by enhanced visual activity (Sin et al., 2002). Long isoforms of Homer 1 proteins, such as Homer 1c (also called PSDZip45) provides a physical link between NMDA and metabotropic glutamate receptors (mGluRs), inositol triphosphate receptors and the TRPC1 channel that mediates the replenishment of intracellular calcium stores (Yuan et al., 2003). Homer proteins also regulate the coupling of mGluRs to N-type calcium and M-type potassium channels (Kammermeier et al., 2000). Finally, Homer 1b and 1c promote growth and maturation of spines and synapses (Sala et al., 2003), and their overexpression enhances the accumulation of synaptic F-actin and induces synaptic enlargement (Sala et al., 2001; Usui et al., 2003).

Other plasticity-related genes positively modulated by NA, including Narp, the phosphatidylinositol 3-kinase p85α, and GIT1, regulate the activity and clustering of glutamate AMPA receptors. The activation of AMPA receptors is sufficient to maintain dendritic spines, and their insertion into synapses is essential for the induction and maintenance of LTP (Lamprecht and Ledoux, 2004). Moreover, LTP and LTD in vivo are associated with complementary changes in the number and phosphorylation levels of AMPA receptors (refs in Heynen et al., 2000, 2003). Narp is a member of the pentraxin family that localizes specifically to excitatory synapses. Narp transgenic expression increases the number of AMPA receptor clusters, while dominant-negative Narp reduces such number (refs in Xu et al., 2003). The phosphatidylinositol 3-kinase p85α/p110α is required for the insertion of AMPA receptors at activated synapses during LTP (Man et al., 2003). Finally GIT1, which associates with G-protein-coupled receptors and various focal adhesion molecules (refs in Ko et al., 2003), inhibits the ligand-induced endocytosis of several membrane proteins, including the β2-adrenergic receptors (Premont et al., 1998). Moreover, GIT1 interacts with liprin-alpha and GRIP to facilitate the clustering of AMPA receptors at the surface membrane (Ko et al., 2003).

Noradrenaline and cellular stress

The increased expression of chaperones and heat shock proteins during spontaneous or forced wakefulness is a consistent finding in flies, rats, and humans (Shaw et al., 2000; 2002; Cirelli et al., 2000a, 2004; Gutierrez et al., 2003; Terao et al., 2003). The present study shows that an intact noradrenergic system is essential for the induction of the brain cellular stress response, suggesting that NA may help to protect the brain against several forms of cellular insult. There is some evidence that NA can maintain the brain in a state refractory to inflammatory activation, and in particular may limit the expression of potentially damaging

cytokines and reactive oxygen species. One study, for instance, observed a decrease in the expression of 2 endogenous anti-inflammatory molecules, IkB and HSP70, after DSP-4 treatment, suggesting that NA maintains a protective heat shock response (Heneka et al., 2003). Moreover, NA can inhibit lipid peroxidation in vitro (Liu and Mori, 1993) and can promote the survival of dopaminergic neurons by directly reducing oxidant production (Troadec et al., 2001). We have recently shown that rats sleep deprived for several days show no signs of oxidative damage, neither in the brain nor in peripheral tissues (Gopalakrishnan et al., 2004). Ongoing experiments are examining whether this effect requires an intact noradrenergic system.

Noradrenaline and protein synthesis

Only 2 transcripts increased their expression when cortical NA levels were depleted. The level of one of them, coding for EF2, increased two-fold. We had previously shown that transcript levels of *EF2* and the initiation factor 4AII - both key components of the translational machinery – are specifically increased in the brain in relation to sleep, suggesting that sleep may favor brain protein synthesis (Cirelli et al., 2004). This conclusion is in agreement with previous studies showing that leucine incorporation is higher during NREM sleep relative to waking in both rats and monkeys (Ramm and Smith, 1990; Nakanishi et al., 1997). Thus, it appears that the increased of NA during wakefulness may inhibit brain protein synthesis, whereas the decreased release of NA during sleep may facilitate it.

Other effects of noradrenaline depletion on gene expression

Many genes whose levels were reduced after NA depletion did not change their expression between sleep and wakefulness. There are several possible explanations why NA depletion by DSP-4 produces changes in gene expression above and beyond those occurring between sleep and wakefulness in association with changes in LC activity. DSP-4 treatment per se may have caused some of these changes through some toxic effect unrelated to NA depletion. This is unlikely because i) some of the changes reported here were also observed after NA depletion caused by local injections of 6-OHDA, a neurotoxin with a different mechanism of action; ii) transcriptional changes after DSP-4 treatment affect only 2% of cortical genes, ruling out a global impairment of transcription; iii) DSP-4 does not cause nonspecific cytotoxic effects on brain cells (Dudley et al., 1990; Yamamoto et al., 2001). Finally, except for NA terminals, there is no evidence of synaptic loss in adult animals treated with DSP-4 (Matsukawa et al., 2003). A more likely explanation has to do with the cortical levels of NA during sleep vs. after DSP-4 lesions. Although LC activity during NREM sleep is much lower than during wakefulness, it is not zero (Aston-Jones and Bloom, 1981a). Thus, while there are no direct measurements of NA cortical levels during NREM sleep, it is plausible that they do not decrease as much as after DSP-4 treatment. Perhaps such low levels can only be reached during REM sleep, which accounts for only 10-20% of total sleep in rats. Another possible explanation is that the reduced firing of LC cells during sleep lasts for a few hours, while NA depletion after DSP-4 is nearly permanent. Indeed, knowing how brain gene expression changes after protracted NA depletion may be relevant when considering the effects of pathological conditions or pharmacological manipulations that interfere chronically with the functioning of the noradrenergic system.

Figure legends

Figure 1. Levels of norepinephrine (NA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) were measured in the left cerebral cortex using high performance liquid chromatography with electrochemical detection. Values (mean ± SEM) in DSP-4 treated rats are expressed as % relative to saline-treated controls = 100. NA = 14.2 ± 1.3; DA = 105.5 ± 10.3; DOPAC = 84.4 ± 9.0; HVA = 84.6 ± 9.2; 5-HT = 70.3 ± 5.7; HIAA = 87.6 ± 4.6. Average values refer to all experimental animals, including 17 controls (6 for mRNA differential display, 7 for microarrays analysis, and 4 for confirmation experiments) and 17 DSP-4 treated rats (6 for mRNA differential display, 7 for microarrays analysis, and 4 for confirmation experiments). Mann-Whitney U-test: NA, *p = 0.0017; DA, p = 0.277; DOPAC, p = 0.337; HVA, p = 0.11; 5-HT, *p = 0.0017; 5-HIAA, p = 0.14.

Figure 2. Wakefulness (W), NREM and REM sleep in saline-treated (controls, n = 17) and DSP-4 treated rats (n = 17). Values refer to the 24-h period preceding the final experimental day and are expressed as % of total recording time (mean ±SEM in parenthesis; Mann-Whitney U-test: W, p = 0.72; NREM, p = 0.67; REM, p = 0.08.

Figure 3. Examples of mRNA differential display analysis of cerebral cortex RNA in saline-treated (controls, lanes 1-6) and DSP-4 treated rats (lanes 7-12). Each lane corresponds to a single rat. The figure shows a small section of 3 different gels. The band indicated by an asterisk in the 2 upper gels corresponds to *NGFI-A*, the one on the lower gel to minoxidil (aryl) sulfotransferase.

Figure 4. (**A**) Cortical transcripts (GeneChip RGU34 A) regulated by sleep and wakefulness, by norepinephrine (NA), and their overlap. Numbers only include known genes and not ESTs. Up in wakefulness: genes whose mRNA levels increased in both spontaneously awake and sleep deprived animals relative to sleeping animals according to Cirelli et al., 2004. Up in sleep: genes whose mRNA levels increased in sleeping animals relative to both spontaneously awake and sleep deprived animals according to Cirelli et al., 2004. Up with NA: genes whose mRNA levels were decreased in DSP-4 animals, i.e. after cortical NA depletion. Down with NA: genes whose mRNA levels were increased in DSP-4 animals, i.e. after cortical NA depletion. The 16 overlapping genes up in wakefulness and with NA include *c-fos* and *BDNF*, which were identified using mRNA differential display. The only overlapping gene up in sleep and down with NA is *EF2*. (**B**) Biological functions associated with transcripts with higher expression in wakefulness (red box) and sleep (blue box), as in Cirelli et al., 2004. Functional categories listed in bold are those that include one or more transcripts whose expression is also modulated by NA. The tree on the left (dots and connecting paths) represents biological processes annotations according to the gene ontology hierarchy.

Table 1. Rat cortical genes exhibiting decreased or increased mRNA levels in DSP-4 treated rats relative to saline-treated animals. For each gene bank accession number (GBA#), molecular function and biological process annotations were based on an extensive analysis of the literature and, when available, on the gene ontology hierarchy. * = gene identified also, or only (in case of *c-fos* and *BDNF*) by mRNA differential display. W and S, wakefulness-related and sleep-related

genes according to Cirelli et al. 2004. SLR = signal to log ratio (average); %, average percentage change as measured by microarray analysis. % qPCR, average percentage change as measured by quantitative PCR. Note that only 2 transcripts show increased expression after DSP-4 treatment, including one known gene, EF2.