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Role of wake-promoting basal forebrain and adenosinergic mechanisms in sleep promoting effects of ethanol

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Abstract

BACKGROUND—Ethanol intake has significant impact on sleep. However, the cellular substrates responsible for sleep promotion following ethanol intake are unknown. The purine nucleoside, adenosine, is responsible for mediating many neuronal and behavioral responses to ethanol. Studies performed in cell cultures suggest that ethanol inhibits equilibrative nucleoside transporter 1 to block the reuptake of adenosine resulting in increased extracellular adenosine. Adenosine also has a pivotal role in sleep regulation. Adenosine acts via A1 receptor to inhibit the wake-promoting neurons of the basal forebrain (BF) resulting in the promotion of sleep. Is ethanol induced sleep associated with the inhibition of the BF wake-promoting neurons? Do adenosinergic mechanisms in the BF have a role in sleep-promoting effects of ethanol?

METHODS—To address these questions, we performed three experiments in Sprague Dawley rats. First, we verified the effect of ethanol on sleep promotion. Second, we evaluated the effect of ethanol on c-Fos expression (a marker of neuronal activation) in the BF wake-promoting neurons and third we monitored the effects of A1 receptor blockade in the BF on ethanol induced sleep.

RESULTS—Significant increase in non-rapid eye movement (NREM) sleep with a concomitant decrease in wakefulness was observed during first 12 hours post- ethanol. REM sleep remained unaffected. Ethanol administration caused a significant decrease in the number of BF wake-promoting neurons with c-Fos immunoreactivity. Bilateral microinjections of a selective A1R receptor antagonist 8-cyclopentyl-1, 3-dipropylxanthine into the BF significantly attenuated sleep-promoting effects of ethanol.

CONCLUSION—These results suggest that the inhibition of BF wake-promoting neurons by adenosinergic mechanism may be responsible for the sleep promoting effects of ethanol. We believe our study is the first to investigate the cellular mechanisms responsible for the somnogenic effects of ethanol.

Keywords

Adenosine; Basal Forebrain; Ethanol; Microdialysis; Rat; Sleep

INTRODUCTION

Ethanol intake has profound impact on sleep (Brower, 2001; Roehrs and Roth, 2001). However, very little is known about the cellular substrates responsible for mediating the effects of ethanol on sleep. Considerable evidence suggest that adenosine is a key mediator of many behavioral and neuronal responses to ethanol including ataxia, anxiety, tremors and seizures (Dar, 1993;

Concas et al., 1996; Phan et al., 1997; Dar, 1997; Barwick and Dar, 1998; Jarvis and Becker, 1998; Kaplan et al., 1999; Dunwiddie and Masino, 2001; Hack and Christie, 2003; Batista et al., 2005; Prediger et al., 2006; Newton and Messing, 2006). Studies performed in cell cultures suggest that ethanol inhibits equilibrative nucleoside transporter 1 (ENT1) to block the reuptake of adenosine resulting in increased extracellular adenosine (Nagy et al., 1990; Krauss et al., 1993). The ENT1-null mice showed decreased adenosinergic tone resulting in increased ethanol consumption coupled with reduced hypnotic (loss of righting reflex) and ataxic responses to ethanol. While treatment with an A1R agonist decreased ethanol consumption in ENT1-null mice, A2AR agonist was ineffective (Choi et al., 2004).

Strong and compelling evidence suggest that adenosine is a homeostatic regulator of sleep (Radulovacki et al., 1984; Thakkar and Mallick, 1996; Portas et al., 1997; Thakkar et al., 2003b). During wakefulness, adenosine, a byproduct of metabolism, builds up in the wake-promoting basal forebrain (BF; Porkka-Heiskanen et al., 1997; Murillo-Rodriguez et al., 2004). Increased extracellular adenosine in the BF acts via A1 receptors to inhibit the cholinergic and non-cholinergic wake-promoting neurons in the BF (Alam et al., 1999; Thakkar et al., 2003a; Arrigoni et al., 2006) resulting in the promotion of sleep (Basheer et al., 2004; McCarley, 2007).

Is ethanol induced sleep associated with the inhibition of BF wake-promoting neurons? Do the BF adenosinergic mechanisms have a role in sleep-promoting effects of ethanol?

MATERIALS AND METHODS

Experimental Design

We performed three experiments to address these questions and understand the cellular mechanisms responsible for sleep induction following acute ethanol intake. Our first experiment verified the effects of acute ethanol treatment on sleep-wakefulness. Our second experiment examined the effects of acute ethanol treatment on the activation of BF wake-promoting neurons by monitoring c-Fos expression in BF cholinergic neurons. Our third experiment evaluated the effects of A1 receptor antagonist infusion in the BF on sleep inducing effects of ethanol.

All the experiments described in this report were performed during the dark period because rats are awake and maximally active during dark period, which allows us to easily isolate anatomical substrates/cellular mechanisms involved in ethanol induced sleep. In addition, ethanol induced increase in non-rapid eye movement (NREM) sleep can be observed without any “ceiling” effects during the dark period (Thakkar et. al., 2003b)

Animals

Adult male Sprague–Dawley rats (200 – 400 gm) were housed under constant temperature, with *ad libitum* access to food and water, and with 12-hr light/dark cycle at least 10 days before surgery. Every effort was made to minimize animal suffering and to reduce the number of animals used. All animals were treated in accordance with the American Association for Accreditation of Laboratory Animal Care’s policy on care and use of laboratory animals. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and all experimental protocols were approved by the Animals Committee of the Harry S. Truman Memorial Veterans Hospital.

Chemicals

Ethanol—The 200 proof ethyl alcohol (Acros Organics) was purchased from Fischer Scientific. A 35% (v/v) solution (in sterile water) was prepared fresh on the day of the

experiment. Since rats do not readily consume alcohol, acute ethanol administration was performed by intragastric intubation with a gavage tube. We used a 3 g/Kg dose of ethanol.

Adenosine A1 receptor antagonist—We used 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), a selective A1 receptor antagonist (Lohse et al., 1987; Klotz, 2000) to examine the role of adenosine A1 receptor in ethanol induced sleep. DPCPX was purchased from Tocris Biosciences Ellisville, Missouri and dissolved in dimethyl sulphoxide (DMSO) to make stock solution of 2 mM. Subsequent dilutions were made in saline and prepared fresh on the day of the experiment. A detailed description of each experiment is as follows:

Experiment 1: Effects of acute ethanol treatment on behavioral states in freely behaving rats

Surgery—Under sterile conditions and using standard surgical protocol, the animals were implanted with sleep recording electrodes as described previously [for details see (Thakkar et al., 1999)]. Briefly, under sterile conditions rats were anesthetized with inhalation (isoflurane) anesthesia. Two screw electrodes were stereotactically implanted on the skull to record the electroencephalogram (EEG). One indifferent electrode (ground) was implanted on one side of the nasal suture. Two electrodes were implanted in the nuchal muscle to record the electromyogram (EMG). The EEG and EMG electrodes were connected to an electrical connector and secured to the skull with dental cement.

Post- surgical recovery and habituation—Experiments were conducted with the same light conditions with food and water available, *ad libitum*. After 3 days of post-operative recovery, the rats were tethered to a lightweight recording cable and habituated to the recording setup for at least 7 days.

Habituation to the gavage feeding tube insertion—Each animal was habituated with the insertion of the gavage needle for 3 days before the experiment. The protocol is as follows: The rat was disconnected from the recording cable and swaddled in a towel. The length of the gavage tube to be inserted (from the oral cavity to the end of the caudal rib) was measured. The gavage tube was attached to a syringe and placed into the right lateral side of oral cavity and gently inserted into the esophagus. As soon as the desired length was inserted, sterile water (10 ml/Kg) was slowly injected. On completion, the animal was reconnected to the recording cable.

Experimental Protocol—Following habituation to the recording cable and insertion of gavage tube, the experiment was begun. Sterile water (10 mL/Kg) was administered at dark onset on day 1, while ethanol was administered at dark onset on day 2. Sleep-wakefulness was continuously recorded for 48 hr beginning after sterile water treatment on day 1.

Acquisition and analysis of sleep–wakefulness data—The EEG and EMG signals were recorded differentially from two bilateral electrodes and amplified by a Quad Neuroamplifier (Model 15A54) housed in a 16 channel, bipolar Physiodata Amplifier System (Model 15LT; Grass Technologies, West Warwick, RI). The EEG signal was filtered between 0.3 Hz and 100 Hz, whereas the EMG signal was filtered between 30 to 300 Hz. In addition, both EEG and EMG signals were filtered at 60 Hz with a notch filter, digitized and acquired (128 Hz/channel) by Icelus data acquisition software (build 071503, authored by Mark Opp, University of Michigan, Ann Arbor, MI, USA) and stored in Dell Desktop computer. Sleep-wake analysis was performed by using the Icelus data analysis software. The behavior of animals was visually scored (10 sec epochs) and divided into three states: (1) wakefulness, (active and quiet wakefulness) determined by the presence of low amplitude, high frequency desynchronized EEG with the concomitant presence of active muscle tone; (2) NREM sleep; determined by the presence of low frequency, high amplitude, synchronized EEG with low

EMG tone; and (3) rapid eye movement (REM) sleep, determined by complete absence of muscle tone along with desynchronized EEG. Sleep-wake data was grouped separately for dark and light period. We calculated latency to NREM and REM sleep. Latency to NREM sleep was defined as the amount of time between ethanol administration and first non-interrupted 60 sec bout of NREM sleep, whereas, the amount of time between ethanol administration and first non-interrupted 30 sec bout of REM sleep was considered as latency to REM sleep (Thakkar et al., 2008).

Statistical analysis—The sleep-wake data grouped in 12 hr bins (dark and light period) was used for statistical analysis. Statistical analysis was performed with paired t-test. The significance level of $p < 0.05$ was accepted.

Experiment 2: Effects of acute ethanol administration on c-Fos expression in the cholinergic neurons of the BF

Experimental Protocol—Two groups of rats were used in this experiment. At dark onset, sterile water (10 mL/Kg) was administered to Group 1 rats and ethanol (3 g/Kg) was administered to Group 2. The animals were left undisturbed for 2 hr. Subsequently, the rats were euthanized under deep phenobarbital anesthesia and perfused transcardially with 200 mL of 0.9% saline followed by perfusion of 200 ml 10% formalin (Sigma Chemicals, St. Louis MO). The brains were removed, blocked and post-fixed in the same fixative overnight, and stored in 20% sucrose at 4°C until equilibrated.

Histology and immunohistochemistry—Brains were cut into 40 μ m sagittal sections on a freezing microtome. The c-Fos and ChAT immunohistochemistry was performed in every third section. The protocol in detail is as follows: **Day 1:** Sections were washed (3×10 min) in 100 mM phosphate buffered saline (PBS, pH 7.4) and incubated in blocking solution [a mixture of PBS, 3% Normal donkey serum (Jackson ImmunoResearch, West Grove, PA) and 0.3% Triton X-100 (Sigma)] for 2 hr. Next, the sections were incubated in a mixture containing rabbit anti-c-Fos antibody (1: 50; SantaCruz) and blocking solution overnight at room temperature. **Day 2:** After washing in PBS (3×10 min), the sections were incubated in donkey anti-rabbit secondary antibody conjugated with FITC (1:400, Jackson ImmunoResearch) for 1 hr. The sections were washed in PBS (3×10 min) and incubated in a mixture containing goat anti-ChAT antibody (1: 200; Chemicon) and blocking solution overnight at room temperature. **Day 3:** The sections were washed in PBS (3×10 min) and then incubated in donkey anti-goat secondary antibody conjugated with Cy3 (1:400, Jackson ImmunoResearch) for 1 hr followed by washing in PBS (3×10 min). After washing, the sections were mounted on glass slides and cover-slipped using Vectashield (Vector Labs) mounting medium.

Cell counting and analyses—A single person blind to the treatment conditions performed manual counting of immunoreactive neurons in the horizontal diagonal band/substantia innominata (HDB/SI) region of the BF, identified by the presence of a dense population of ChAT+ve neurons in the ventral forebrain below the anterior commissure. We chose the HDB/SI region because this BF region is known to contain the wake-promoting neurons (Basheer and Shiromani, 2001; Greco et al., 2000) and where adenosine acts to promote sleep (Thakkar et al., 2003b; Thakkar et al., 2003a; Szymusiak and McGinty, 2008).

In each animal, we selected three representative sections ($\sim 120 \mu$ m apart), encompassing the regions with maximum numbers of ChAT +ve neurons, (red fluorescent cytoplasm) in the HDB/SI region. These sections were used for the quantification of c-Fos immunoreactivity in ChAT+ve neurons. Under 100 $\times$ magnification (10 $\times$ objective and 10 $\times$ eye piece, Leica microscope, DM 4000B) and with same focus, two photomicrographs were taken with Spot camera (version 4.0.9; Diagnostic Instruments, Inc., Sterling Heights, MI, USA), one

visualizing ChAT +ve neurons (red fluorescent cytoplasm) and the second visualizing c-Fos labeling (green fluorescent nuclear staining). Subsequently, the two photomicrographs were merged and counted with the Adobe Photoshop CS3 software counting tool. All single labeled ChAT +ve neurons along with double labeled ChAT and c-Fos +ve neurons were counted from the HDB/SI regions in three sections from each animal. The counts were summed and the percentage of ChAT +ve neurons with c-Fos immunoreactivity was calculated for each rat and tabulated separately for water and ethanol treated animals and used for statistical analysis.

Statistical analysis—Statistical analysis was performed with independent t-test. Significance level of $p < 0.05$ was accepted.

Experiment 3: Effects of A1 receptor blockade in the BF on ethanol induced sleep in freely behaving rats

Surgery—The third experiment evaluated the effects of A1 receptor blockade in the BF on ethanol induced sleep. The protocol used for the surgery was identical to the one described above (experiment 1) except that in addition to sleep recording electrodes, intracerebral guide cannulas, for later insertion of injector cannulas, were also implanted bilaterally at a 90 degree angle above the target site in the HDB/SI region (Thakkar et al., 2003b; Thakkar et al., 2003a). The target coordinates (Paxinos and Watson, 2007) for the tips of the microinjections were: AP= P 0.3, ML= L2.5, DV= V9.0: relative to bregma and skull surface at bregma (Thakkar et al., 2001).

Experimental Protocol—Following post-operative recovery and habituation the experiment was begun. On day 1, 30 min before dark onset, vehicle (10% DMSO/250 nL) was bilaterally microinjected into the BF (Thakkar et al., 2008). Subsequently, ethanol was administered at dark onset. Sleep-wakefulness was recorded for the next 12 hr. There was no treatment on days 2 to 5. On day 6, 30 min before the dark onset, DPCPX (50 pmol/250 nL) was bilaterally microinjected into the BF followed by ethanol administration at dark onset. Sleep-wakefulness was recorded for subsequent 12 hr.

Localization of the injection sites—On completion of the experiment, the animals were euthanized under deep phenobarbital anesthesia and perfused with 0.9% saline followed by perfusion of 10% formalin. The brains were isolated, blocked and processed for ChAT immunohistochemistry to illustrate the locations of cholinergic neurons relative to the drug injector cannula track (Thakkar et al., 2001).

Statistical Analysis—The sleep-wake data was grouped into 12 hr bin (dark period) and used for statistical analysis (paired t-tests). The significance level of $p < 0.05$ was accepted.

Blood ethanol levels—Since drawing of blood required restraining the animals and can affect sleep-wakefulness, we measured blood ethanol levels in a separate group of rats ($N=5$). These rats were housed individually in the recording cages and under same light- dark condition, however, there were no surgeries or sleep recordings. A 3 g/Kg dose of ethanol was administered at dark onset. Blood collection was performed after 1 and 6 hr. The blood (approximately 400 μ L) was collected from the tail vein in a small vial (containing heparin) and centrifuged at 800 g for 5 min to prepare plasma. Subsequently the plasma was used to measure blood ethanol levels with an Ethanol Assay Kit (Biomedical Research Service, Buffalo, NY) following manufacturer's instructions.

RESULTS

Acute ethanol treatment promotes NREM sleep in freely behaving rats

As described in Figure 1 (**Panel A**), acute ethanol treatment at dark onset significantly increased NREM sleep ($t=2.5$; $df=6$; $p<0.05$; paired t test; $N=7$) with a concomitant decrease in wakefulness ($t=10.3$; $df=6$; $p<0.01$; paired t test; $N=7$) during the dark period (first 12 hr) as compared to sterile water treatment. REM sleep remained unaffected ($t=0.22$; $df=6$; $p>0.05$; paired t test; $N=7$). Ethanol treatment significantly reduced the latency to NREM sleep (Figure 1, Panel B; $t=2.77$; $df=6$; $p<0.05$; paired t test; $N=7$). REM sleep latency remained unaffected (data not shown). Interestingly, ethanol treated rats had their first uninterrupted 60 sec bout of NREM sleep in < 9 min after ethanol treatment. There was no change in the amount of time spent in any behavioral state during the next 12 hour (light period; Figure 1, Panel C).

The temporal distribution of the percent time spent in NREM sleep following water and ethanol treatment is described in Figure 2, Panel A. The maximal effect was observed on NREM sleep during the first 6 hours after ethanol treatment. There was no change in NREM sleep during the next 12 hr (light period) suggesting that ethanol treatment did not have any persistent effects.

The percent time spent in wakefulness across 24 hr is described in **Panel B** (Figure 2). Maximal reduction in wakefulness was observed during the first six hours after ethanol treatment (Figure 2, Panel B). Ethanol treatment did not have any effect on wakefulness during the subsequent light period. REM sleep did not show any change at any time point (Figure 2, Panel C).

Ethanol suppresses c-Fos expression in cholinergic neurons of the BF

Representative photomicrographs describing the effects of water (**Panel A**) and ethanol (**Panel B**) treatment on c-Fos immunoreactivity in BF cholinergic (ChAT +ve) neurons are displayed in Figure 3. There was a significant reduction in ($t = 3.0$; $df=7$; $p<0.05$; independent t -test) the percentage of BF ChAT +ve neurons with c-Fos immunoreactivity in ethanol treated rats as compared to water treated animals (Figure 3, Panel C). Out of a total of 683 ChAT +ve neurons in water treated animals ($N=4$), 57% were ChAT and c-Fos double labeled neurons. In contrast, out of 1104 ChAT +ve neurons in ethanol treated rats ($N=5$), 44% were ChAT and c-Fos +ve.

A1 receptor blockade in the BF attenuates ethanol induced sleep

Localization of the injection sites—Figure 4, Panel A describes the localization of microinjection sites for each of the animals in the coronal schematic ($N=6$, adapted from Paxinos and Watson 2007). All injection sites were in the cholinergic BF between AP levels -0.3 and -0.6 . A representative photomicrograph illustrating bilateral injection sites, surrounded by cholinergic neurons in the BF is shown in Figure 4, Panel B.

Microinjection of DPCPX attenuates ethanol induced sleep—Bilateral microinjection of 50 pmol of DPCPX into the BF produced a significant reduction in ethanol induced NREM sleep ($t=3.4$; $df=5$; $p<0.05$; $N=6$; paired t -test; see Figure 4 Panel C) along with a concomitant significant increase in wakefulness ($t=2.8$; $df=5$; $p<0.05$; $N=6$; paired t -test) as compared to vehicle injections. REM sleep remained unaffected ($t=1.0$; $df=5$; $p=0.39$; $N=6$; paired t -test). The hourly distribution of NREM sleep, wakefulness and REM sleep is depicted in Figure 5.

Blood ethanol levels—Administration of 3 g/kg of ethanol produced a mean ($\pm$ SEM; $N=5$) blood alcohol levels of $134.2 (\pm 50.3)$ mg/dL at 1 hr. There was no alcohol in the blood at 6 hr.

DISCUSSION

We performed 3 experiments designed to understand the cellular mechanisms mediating sleep promoting effects of ethanol. The major findings of our study are that the NREM sleep promoting effects of acute ethanol are: 1) associated with the inhibition of BF wake-promoting neurons, and 3) attenuated by local application of A1 receptor antagonist in the wake-promoting BF.

Our study is the first attempt to understand the neurochemical substrates associated with sleep promoting effects of ethanol. In order to understand the cellular mechanisms associated with sleep promoting effects of ethanol and to be consistent across experiments, we performed all our experiments during the dark period when the rats are maximally awake.

Our first experiment verified the effect of ethanol treatment on sleep-wakefulness. Our results were similar to the results observed by other investigators (Kubota et al., 2002; Mendelson and Hill, 1978; Hill and Reyes, 1978). The primary effect of acute ethanol treatment was observed on NREM sleep. Ethanol treatment reduced NREM sleep latency and increased the amount of time spent in NREM sleep. The induction of sleep after ethanol treatment was rapid. The first 60 sec non-interrupted bout of NREM sleep occurred in less than 9 min suggesting that ethanol is a potent somnogen. The maximal effect was observed during the first six hours after ethanol treatment. Interestingly, ethanol treatment had no effect on sleep onset at the beginning of light period (12 hr after ethanol treatment) or during the subsequent light period suggesting that the somnogenic effects of ethanol do not persist.

Our second experiment examined the effects of acute ethanol treatment on neuronal activation in the BF. Since its discovery almost 20 year ago, the immediate early gene product, c-Fos has been extensively used as a tool for determining neuronal activation. Since neuronal activation results in the translocation of c-Fos protein into the nucleus, identification of the neuronal phenotype is possible with double labeling immunohistochemistry. This characteristic of c-Fos protein has been extensively used to identify wake and sleep promoting neurons. For example, activation of the BF cholinergic neurons during wakefulness was identified by measuring c-Fos activation (Basheer and Shiromani, 2001; Greco et al., 2000).

The BF is a critical wake-promoting site and contains cholinergic and non-cholinergic wake-promoting neurons that are maximally active during wakefulness and minimally during NREM sleep (Datta and Maclean, 2007; Szymusiak and McGinty, 2008). The BF non-cholinergic neurons can be either wake or sleep-promoting and therefore, difficult to differentiate. For example, GABA-containing neurons in the BF can be either wake or sleep-promoting (Hassani et al., 2009). In contrast, the cholinergic neurons are exclusively wake-promoting and can serve as a representative group for the entire BF wake-promoting neurons. Therefore, we examined the effects of acute ethanol treatment on c-Fos expression in the BF cholinergic neurons using double labeled immunohistochemistry. Although, Western-blot technique can provide a good quantitative measure of c-Fos expression, it cannot provide the information about the neuronal activation or neuronal phenotype in which c-Fos is expressed. In contrast, the double labeled immunohistochemistry is an appropriate technique that will allow selective discrimination and quantification of activated neurons in the BF.

Since rats are maximally awake at dark onset, we performed the experiment at dark onset. To enhance the sensitivity and avoid any ambiguity in c-Fos labeling, we used two color fluorescence immunohistochemistry. Our data revealed that acute ethanol treatment reduced the number of ChAT +ve neurons with c-Fos immunoreactivity in the BF suggesting that ethanol treatment inhibited the BF wake-promoting neurons. This data suggest that ethanol treatment reduces neuronal activation of the BF wake-promoting neurons. Reduced activity of BF wake-promoting neurons is likely to result in the promotion of sleep.

Our third experiment evaluated the effects of adenosine A1 receptor blockade on ethanol induced sleep. While both A1 and A2a receptors subtypes are implicated in mediating sleep, the A2a receptor subtype has been suggested to mediate sleep via the activation of sleep promoting neurons in the ventrolateral preoptic region (Gallopín et al., 2005). In contrast, the wake-promoting BF only contains A1 receptor subtype and is responsible for mediating sleep by inhibiting the wake-promoting neurons of the BF (Basheer et al., 2004). Therefore, we chose to examine only the A1 receptor subtype in this study. We performed bilateral microinjections of a selective A1 receptor antagonist DPCPX into the BF followed by intragastric administration of ethanol. The dose used in this report was selected based on previous microinjection studies (Saeed, 2006; Dar, 2001). Non-specific effects of microinjections were controlled by performing vehicle injections in the same animal.

Microinjection of 50 pmol of DPCPX into the BF significantly attenuated the sleep inducing effects of ethanol. These results implicate A1 receptors in the BF to play a role in mediating the sleep promoting effects of ethanol. These results clearly demonstrate the importance of adenosinergic mechanisms in ethanol induced sleep promotion. This is not surprising because there is convincing evidence to suggest that ethanol increases extracellular adenosine in the brain (Sharma et al., 2010; Newton and Messing, 2006), and increased extracellular adenosine in the BF promotes sleep. Thus, based on our results, we believe that acute ethanol intake induced increase in extracellular adenosine is responsible for the inhibition of BF wake-promoting neurons resulting in sleep promotion.

Ethanol has profound effects on sleep. While acute ethanol intake promotes sleep in human subject, recovering alcoholics suffer from severe insomnia during acute withdrawal (Brower, 2001; Brower et al., 2001; Brower, 2003). Based on our results, we believe that chronic ethanol intake may impair adenosine release and/or adenosine transmission resulting in increased activation of wake-promoting BF neurons. Increased activation of BF neurons is likely to reduce sleep and produce insomnia-like symptoms. Indeed, in vitro studies, performed in cell cultures have demonstrated that chronic ethanol affects adenosine transport (Nagy et al., 1990).

In conclusion, our study is the first to examine the cellular mechanisms associated with ethanol induced sleep promotion. Our results suggest that the adenosinergic mechanisms and wake-promoting neurons of the BF may be key substrates responsible for sleep promoting effects of ethanol.

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Figure 1.

Describes the effects of acute ethanol treatment on sleep-wakefulness

Panel A: Acute ethanol treatment at dark onset produced a significant increase in NREM sleep (* = $p < 0.05$; paired t test) with a concomitant decrease in wakefulness (** = $p < 0.01$; paired t test) during the dark period (first 12 hr) as compared to sleep-wakefulness following water treatment. REM sleep remained unaffected.

Panel B: In addition to increase in NREM sleep, ethanol treatment significantly reduced the latency to NREM sleep (* = $p < 0.05$; paired t test).

Panel C: There was no change in the amount of time spent in any behavioral state during the next 12 hour (light period).

Figure 2.

The temporal distribution of sleep-wakefulness across 24 hr in 3 hr bins is described.

Panel A: The maximal effect was observed on NREM sleep during the first 6 hours after ethanol treatment. There was no change in NREM sleep during the next 12 hr (light period) suggesting that the somnogenic effects of ethanol do not persist.

Panel B: During first 6 hour after ethanol treatment the percent time spent in wakefulness was reduced as compared to wakefulness after sterile water treatment. No change in wakefulness was observed during the subsequent light period.

Panel C: Ethanol treatment had no effect on REM sleep at any time point.

Figure 3.

Expression of c-Fos in the wake-promoting cholinergic neurons in the BF.

Panel A: A representative photomicrograph depicting the ChAT and c-Fos double labeled neurons in the BF after sterile water treatment. Sterile water treatment was performed at dark onset. ChAT labeling was performed by using secondary antibody conjugated with Cy3, the red fluorescent dye. The c-Fos was labeled with secondary antibody conjugated with FITC, the green fluorescent dye. Many ChAT +ve neurons display c-Fos immunoreactivity after sterile water treatment suggesting that increased activation of ChAT +ve wake-promoting neurons during the dark controls. Calibration bar = 50 μ m.

Panel B: A representative photomicrograph depicting the ChAT and c-Fos double labeled neurons in the BF after ethanol treatment. ChAT and c-Fos labeling was performed as described above. Few ChAT +ve neurons show c-Fos immunoreactivity indicating the ethanol treatment at dark onset is responsible for inhibiting the ChAT +ve wake-promoting neurons. Calibration bar = 50 μ m.

Panel C: There was a significant reduction ($p < 0.05$; independent t-test) in the percentage ChAT and c-Fos double-labeled neurons in the BF region of ethanol treated rats as compared to water treated animals (see text for detail).

Figure 4.

Effect of A1 receptor blockade in the wake-promoting BF on ethanol induced sleep.

Panel A: Schematic representation of the anatomical location of histologically identified bilateral microinjection sites. All sites (black circles; N=15) were located between antero-posterior -0.3 and -0.6 and are mapped bilaterally onto one coronal brain section (-0.4 , adapted from Paxinos and Watson, 2007). Abbreviations: ac = anterior commissure; HDB = Horizontal diagonal band; MCPO = Magnocellularis preoptic; SI = Substantia inominata. For identification of unlabeled structures see Paxinos and Watson (2007).

Panel B: A representative photomicrograph depicting the bilateral lesions caused due to microinjections in the BF (large arrowhead) surrounded by ChAT +ve cholinergic neurons in the BF (small arrows). Abbreviations: ac = anterior commissure.

Panel C: Bilateral microinjections of 50 pmol dose of DPCPX into the BF produced a significant reduction in ethanol induced NREM sleep (* = $p < 0.5$; paired t-test) along with a significant increase in wakefulness (* = $p < 0.5$; paired t-test) as compared to vehicle injections. REM sleep remained unaffected.

Figure 5.
The hourly distribution of NREM sleep (**Panel A**), wakefulness (**Panel B**), and REM sleep (**Panel C**) following bilateral microinjections of 50 pmol dose of DPCPX in ethanol treated

rats is described. Local administration of DPCPX attenuated the NREM sleep promoting effects of ethanol (Panel A) and promoted wakefulness (Panel B). The maximal effect was observed during first six hours after treatment. REM sleep did not show any significant change.