Effects of chronic ethanol and nicotine consumption on sleep-wakefulness in rats

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Abstract
Ethanol and nicotine are the two most widely used and abused substances in the world and are often consumed together. However, the effects of chronic co-consumption of ethanol and nicotine as well as their withdrawal on sleep-wakefulness remain poorly understood. We examined effects of chronic treatments of rats with ethanol, nicotine, and ethanol + nicotine and their withdrawals on sleep-wake architecture. Saline, ethanol (3g/kg), nicotine (3mg/kg), or their mixture was injected intraperitoneally at early light-on phase for 28 days in four different group of rats. Sleep-wake parameters were recorded for 6 hours on treatment day-1, day-14, and day-28, followed by one day without any treatment (day-29 or withdrawal day). The findings suggest that while ethanol acutely increased sleep, its chronic use produced only marginal effects as rats developed a tolerance for ethanol. However, sleep decreased severely on withdrawal day. Nicotine acutely suppressed sleep, but like ethanol, rats developed tolerance and nicotine’s effectiveness was attenuated with its chronic use. Sleep, especially, REM sleep, however, increased significantly on withdrawal day. When given together, ethanol and nicotine nullified each other’s effects on sleep-wake parameters. These findings are discussed in the light of known interactions between ethanol and nicotine in influencing sleep.

Keywords: Ethanol, nicotine, non-rapid eye movement sleep, rapid eye movement sleep, wakefulness

1. Introduction
Sleep is an essential behavior across the animal kingdom ranging from fruit flies to humans [1-4]. Its importance is evident from the fact that it is a homeostatic behavior and we spent nearly 30% of our lives sleeping. A sound sleep is critical to health and wellness, whereas, chronic sleep disturbance increases the risk for a number of diseases including obesity, type-2 diabetes, cognitive decline, and many psychiatric disorders [1, 2, 5, 6]. Sleep consists of two alternating stages called non-rapid eye movement (NREM) and REM sleep [1, 2, 7]. Sleep onset normally occurs through NREM sleep, which accounts for most of the sleep amount. NREM sleep is characterized by EEG synchronization or high voltage and low-frequency electroencephalogram (EEG) activity, little or no eye movements, and low muscle tone. NREM sleep progress into another state of sleep called REM sleep, which is characterized by EEG desynchronization or high frequency and low amplitude EEG wave, frequent eye movements, and loss of muscle tone. Typically, the duration of waking, NREM, and REM sleep varies with the species and health condition [1, 2, 4, 5]. For example, humans are monophasic sleepers, i.e., sleep is organized into a single nocturnal bout with about 90 min NREM-REM sleep cycles that occur repetitively throughout the night. Rodents (e.g., rat), on the other hand, are polyphasic sleepers and sleep is organized into many short bouts of about 15 min NREM-REM cycles [1, 2, 4, 5] that occur throughout the 24-hour light-dark phase. However, since rats are nocturnal, sleep predominantly occurs during lights-on phase or day time, although rats still spent 30-40% of the time in wakefulness during the lights-on phase. Ethanol is one of the most commonly used legal and recreational substance, which is often taken to self-medicate for insomnia. This is because ethanol consumption by healthy non-alcoholics reduces sleep onset latency and increases time spent in deep NREM sleep or sleep with slow wave activity (SWA), and decreases time spent in REM sleep during the first half of the night [6-10]. However, during the second half of the night sleep becomes disturbed due to increased wakefulness and REM sleep. The findings from animal studies also confirm that an acute ethanol treatment reduces sleep latency, increases NREM sleep and SWA [11-13]. The findings on REM sleep, however, are inconsistent ranging from a decrease to no change in REM sleep [14-16]. On the other hand, both human and animals studies suggest that chronic exposure to ethanol causes sleep disturbances, i.e., increases sleep latency, reduces sleep...
during sleep period [8, 14, 16] but decreases wakefulness and increases sleep during the active period [14, 17, 18]. Nicotine, which is the principal pharmaco-active substance in tobacco smoke, is another most consumed legal drugs, which is often taken for maintaining alertness. Globally, about 35% of adults use tobacco. Both human and animal studies suggest that nicotine consumption adversely affects sleep architecture. In general, tobacco smoking, which is driven by nicotine, by nonsmokers, causes an increased sleep latency, and a decrease in NREM sleep amount as well as SWA during sleep period [19-21]. While the effects of nicotine consumption on REM sleep has been inconsistent, in general, nicotine consumption has been shown to suppress REM sleep [21]. Animal studies also support that nicotine treatment causes increased sleep onset latency, increased sleep fragmentation, decreased NREM sleep, and a dose-dependent effect on REM sleep with its higher doses decreasing REM sleep time [22, 23]. Epidemiological studies suggest that a close relationship exists between smoking and alcohol consumption and that the two drugs are often consumed together [24]. Today, there are more than a billion tobacco users around the world and people who smoke often drink alcohol and vice versa [24, 25]. Evidence suggests that the amount of alcohol consumed and the extent of alcohol-dependence are positively correlated with the amount of tobacco used [29]. Studies also suggest that sleep disturbance itself is a risk factor for relapse in addiction [26]. However, the effects of chronic co-consumption of both ethanol and nicotine on sleep-wake architecture including REM sleep, and during acute withdrawal period remains poorly understood. Therefore, in this study, we determined changes in sleep-wakefulness, after acute (treatment day-1) and chronic treatments (two weeks and four weeks) and during acute withdrawal (day-1 without drugs) of rats with effective doses of ethanol, nicotine, and both nicotine and ethanol given together during the lights-on phase. The drugs were given intraperitoneally so that animals received the same amount of both drugs in any treatment condition.

2. Materials and methods

Experiments were conducted on male Wistar rats weighing between 225-275 grams. These rats were maintained at 12:12 hour light:dark cycles with lights on at 08:00AM. The details of the experimental procedure are described in earlier publications [27].

2.1 Surgical procedure: Briefly, rats were prepared for chronic recording of their electroencephalogram (EEG), electromyogram (EMG), and electrooculogram (EOG) to objectively quantify their sleep-wakefulness as used in earlier studies [23, 28]. The surgical procedure was done in aseptic condition, using sterilized instruments, and under isoflurane anesthesia. After anesthetization, rat’s head was secured on the stereotaxic apparatus, and its scalp was exposed by making a longitudinal incision on the skin over the scalp. Two stainless steel screw electrodes were threaded through the skull (2 mm anterior to and 4 mm lateral to the bregma and midsagittal suture, respectively) for recording EEG. One screw electrode was also threaded on the midline over the frontal bone to serve as ground. Flexible insulated wires (except at the tip, which was not insulated for picking up signal) were inserted into the dorsal cervical neck muscles to record EMG or muscle tone and to the muscles near the external canthus of eye for recording EOG or eye movements. Leads from EEG, EMG, and EOG electrodes were soldered to an electrical connector, which was fixed on the skull with dental cement. The wound was sutured. Rats were allowed ten days of recovery from the surgical procedure and then were acclimatized with the recording setup for 2-3 days.

2.2 Data Acquisition: Experiments were done during lights-on phase or day time between 08.00AM and 05.00PM. On the day of the experiment, rats were hooked up with the recording systems and their EEG, EMG and EOG were amplified by Grass Polygraph and displayed and recorded on the computer using the software “spike-2”. Before the experiment, baseline EEG, EMG, and EOG were recorded for at least 60 minutes to check if rats showed a normal sleep-wake pattern. After confirming that rats exhibited a normal sleep-wake pattern, the following four sets of experiments were conducted.

Experiment-1: Chronic effects of saline treatment on sleep-wakefulness: As a control, in one group of seven rats, 1ml of saline was injected intraperitoneally, every day for 28 days and their EEG, EMG, and EOG signals were recorded for 6 hours as a control on day-1, day-14, and day-28. On the day-29, rats were not injected with saline but were handled the same way they were handled for saline injection and their EEG, EMG, and EOG were recorded for 6 hours. This served as a control for comparing the effects of ethanol, nicotine, or ethanol + nicotine withdrawals on sleep-wake parameters.

Experiment-2: Chronic effects of ethanol treatment on sleep-wakefulness: In the second group of rats (n=7), after baseline confirmation that rats exhibited normal sleep-wake pattern, 1ml of saline containing an effective dose of ethanol, i.e., 3g/kg was injected intraperitoneally every day for 28 days and their EEG, EMG, and EOG signals were recorded for 6 hours on day-1, day-14, and day-28. On day-29, rats were not injected with ethanol but were handled the same way they were handled for ethanol injection and their EEG, EMG, and EOG were recorded for 6 hours. These days were chosen so that the acute (day-1), mild-chronic (14 days of treatment, day-14), and chronic (28 days of treatment, day-28) effects of ethanol exposure as well as its withdrawal (day-29) on sleep-wake architecture could be examined. We chose a daily dose of 3g/kg of ethanol since in our earlier study we found that this dose was acutely effective in increasing NREM sleep and suppressing REM sleep in initial hours [27]. This dose was found to be effective in other studies as well [14, 29, 30].

Experiment-3: Chronic effects of nicotine treatment on sleep-wakefulness: In the third group of rats (n=7), after confirming that they had a normal sleep-wake pattern, rats were injected intraperitoneally with 3mg/kg of nicotine in 1ml of saline for 28 days, and their EEG, EMG, and EOG were recorded for 6 hours on day-1, day-14, and day-28. On day-29, rats were not injected with nicotine but were handled the same way they were handled for nicotine injection and their EEG, EMG, and EOG were recorded for 6 hours. Like ethanol, the same recording days were chosen so that the acute (day-1), mild-chronic (day-14), and chronic (day-28) effects of nicotine treatments as well as its withdrawal (day-29) on sleep-wake architecture could be examined. We chose a daily dose of 3mg/kg of nicotine since in our earlier study we found that this dose was acutely effective in inducing wakefulness and suppressing both NREM and REM sleep [27].
Experiment-4: Chronic effects of ethanol + nicotine treatment on sleep-wakefulness: After confirming that rats exhibited a normal sleep-wake pattern, a group of seven rats was injected intraperitoneally with a mixture of ethanol (3gm/kg) + nicotine (3mg/kg) for 28 days, and their EEG, EMG, and EOG were recorded for 6 hours on day-1, day-14, and day-28. On day-29, rats were not injected with drugs but were handled the same way they were handled for drug injection and their EEG, EMG, and EOG were recorded for 6 hours. These days were chosen so that the acute (day-1), mild-chronic (day-14), and chronic (day-28) effects of ethanol + nicotine treatment as well as their withdrawal (day-29) on sleep-wake architecture could be examined. At the end of the experiment, rats in all experimental groups were euthanized by injecting pentobarbital @ 100mg/kg.

2.3 Analysis: All analyses were performed off-line from the stored data files using “Spike 2” software for sleep-wake scoring and Sigmaplot software for statistical analysis.

Sleep-wake scoring: Sleep-wake states were scored manually in 10-second epochs on the basis of EEG, EMG, and EOG patterns using standard criteria. Sleep-wake stages were scored in terms of waking (active- and quiet-waking), NREM sleep, and REM sleep. The criteria used were similar to those used by many other researchers as well as in our earlier study [1, 2, 28] and included:
1. Active-waking: EEG desynchronization, i.e., high frequency and low amplitude EEG waves, higher muscle tone with movements, and occasional eye movements.
2. Quiet-waking: EEG desynchronization with occasional synchronization (less than 25% of time), higher muscle tone but without movements, and occasional eye movements, if any.
3. NREM sleep: EEG synchronization, i.e., high amplitude and low-frequency EEG waves (>25% of the time bin), reduced muscle tone, and no eye movements.
4. REM sleep: EEG desynchronization, no muscle tone, and frequent eye movements.

Sleep-onset latency: was defined as the time from injection to the first appearance of sleep for at least 30 sec in duration. REM sleep latency: was defined as the time from injection to the appearance of the first REM sleep episode.

Statistical analysis: The statistical significance of sleep-wake changes due to chronic injections of saline, ethanol, nicotine, or ethanol + nicotine on day-1, day-14, day-28, and day-29 were determined by One Way Repeated Measures ANOVA followed by a post-hoc Holm-Sidak test for each treatment group. Changes in sleep-wake parameters after ethanol, nicotine, or ethanol + nicotine treatments each day were compared with sleep-wake parameters on respective days after saline treatment using t-test.

3. Results
3.1 Effects of saline treatment on sleep-wakefulness: The effects of saline on sleep-wakefulness during the 6 hours of post-injection recording period on the first day of treatment (day-1), two weeks of treatment (day-14), and four weeks of treatment (day-28) as well as without any treatment on day-29 are shown in Figure-1A. In a saline-treated group, like earlier studies [1, 2, 28], rats spent more time in sleep including NREM and REM sleep during the post-injection period. The waking, NREM, and REM sleep amounts on different treatment days (day-1, day-14, day-28) and on no-treatment day (day-29) were comparable and not significantly different (Figure-1A).

![Fig 1](image-url): Bar diagrams showing mean percent (±SD) waking, NREM, and REM sleep on day-1, day-14, day-28, and day-29 during the 6 hours of post-injection recording period after saline (A), ethanol (B), nicotine (C), and ethanol + nicotine (D) treatments. A. saline had no effects on sleep-wake amounts on different treatment days as well as on withdrawal day. B. ethanol increased sleep acutely (day-1), but this effect weakened with chronic treatments (days 14 and 28), and its withdrawal caused severe sleep disruption. C. nicotine decreased sleep acutely (day-1), but this effect was attenuated during chronic treatments (days 14 and 28), whereas, its withdrawal caused a decrease in wakefulness and increase in REM sleep. D. when given together, ethanol and nicotine attenuated each other’s effect on sleep-wakefulness. *, p<0.05; **, p<0.01 (One Way Repeated Measures ANOVA followed by Holm-Sidak test); NS, not significant.
3.2 Effects of chronic ethanol treatment on sleep-wakefulness: The effects of ethanol on sleep-wakefulness during the 6 hours of post-injection recording period on the first day of treatment (day-1), two weeks of treatment (day-14) and four weeks of treatment (day-28) as well as on withdrawal day (day-29) are shown in Figure-1B. There was a significant effect of ethanol treatment and its withdrawal on sleep-wakefulness within the treatment group with ethanol producing sleep on day-1 and causing sleep suppression and increased wakefulness on withdrawal day.

The effects of ethanol after various days of treatment as well as its withdrawal, as compared to respective saline treatment days, are shown in Figure-2. As compared to saline, 3g/kg of ethanol injection significantly increased NREM sleep and decreased wakefulness on day-1. It also decreased REM sleep, which was significant for the first 2 hours, however, for the entire 6 hours it missed significance level. The sleep latency or the occurrence of the first episode of NREM sleep was reduced significantly (19.2 ± 3.6 min vs. 9.8 ± 2.9 min, p<0.01), i.e., rats fell asleep significantly faster after ethanol treatment as compared to saline control. REM sleep latency did not differ significantly (35.9 ± 3.8 vs. 42.3 ± 5.7).

However, as compared to respective saline treatment, ethanol treatment produced only marginal changes in wakefulness, NREM sleep, and REM sleep on day-14 and day-28. Overall, there was a small increase in wakefulness and decreases in NREM and REM sleep amounts that were not significant. The NREM sleep and REM sleep onset latencies also did not differ significantly on day-14 and day-28 after saline and ethanol treatments. Only marginal changes in sleep-wake architecture after chronic ethanol treatment suggest that rats developed a tolerance for ethanol with its repeated and chronic use.

On withdrawal day, as compared to saline-treated rats, chronic ethanol-treated rats exhibited a significant increase in wakefulness and decreases in NREM and REM sleep amounts. The NREM sleep-onset latency (16.8 ± 4.8 min vs. 36.2 ± 4.2 min, p<0.01) as well as REM sleep onset latency also increased significantly (34.2 ± 3.8min vs. 56.4 ± 6.1 min, p<0.01). These findings indicate that on the withdrawal day, as compared to saline treated rats, ethanol-treated rats were predominantly awake during the 6 hours of the recording period (Figure-2).

3.3 Effects of chronic nicotine treatment on sleep-wakefulness: The effects of nicotine on sleep-wakefulness during the 6 hours of post-injection recording period on the first day of treatment (day-1), two weeks of treatment (day-14) and four weeks of treatment (day-28) as well as on withdrawal day (day-29) are shown in Figure-1C. There was a significant effect of nicotine treatment and its withdrawal on sleep-wakefulness within the treatment group, with animals exhibiting decreased NREM and REM sleep and increased wakefulness with acute nicotine treatment and increased NREM and REM sleep and decreased wakefulness after its withdrawal.

The effects of nicotine and saline treatments on sleep-wakefulness on various days of treatments as well as on withdrawal day are shown in Figure-3. As compared to saline injection, 3mg/kg of nicotine injection significantly increased wakefulness and suppressed NREM and REM sleep. The NREM sleep latency (19.2 ± 3.6 min vs. 35.5 ± 5.2 min, p<0.01) as well as latency to REM sleep (35.9 ± 3.8 vs. 54.5 ± 6.1 min, p<0.01) also increased significantly, i.e., it took longer for rats to fall asleep as well as to enter REM sleep. However, as compared to respective saline treatment, nicotine treatment produced only marginal changes in wakefulness, NREM sleep, and REM sleep on day-14 and day-28. In both saline and nicotine-treated groups, the NREM and REM sleep onset latencies showed only smaller differences on day-14 and day-28 that were statistically not significant. These sleep-wake changes suggest that rats developed a tolerance for nicotine with its repeated and chronic use.

On withdrawal day, as compared to saline treated rats, chronic nicotine-treated rats exhibited a significant decrease in wakefulness and increase in sleep, especially REM sleep. The increase in NREM sleep was significant for first 2 hours, however, for the entire 6 hours, it was not significant. The NREM sleep latency decreased slightly (16.8 ± 4.8 min vs. 12.10 ± 4.2 min, NS) but REM sleep onset latency decreased significantly (34.2 ± 3.8 min vs. 24.4 ± 4.10 min, p<0.05) in nicotine-treated rats, as compared to saline control, on withdrawal day (Figure-3).
3.4 Effects of chronic ethanol + nicotine treatment on sleep-wakefulness:

The effects of ethanol + nicotine on sleep-wakefulness during the 6 hours of post-injection recording period on the first day of treatment (day-1), two weeks of treatment (day-14) and four weeks of treatment (day-28) as well as on withdrawal day (day-29) are shown in Figure-1D. There was no significant effect of ethanol + nicotine treatment and its withdrawal on waking, NREM sleep, or REM sleep in this treatment group. The effects of ethanol + nicotine vs. saline treatment on sleep-wakefulness on various days of treatments as well as on withdrawal day are shown in Figure-4. As compared to saline injection, ethanol + nicotine injection did not produce significant changes in waking, NREM, and REM sleep on any of the treatment days as well withdrawal day. Abbreviations are same as in figure-2.

5. Discussion

First of all, the effects of acute as well as chronic treatments of ethanol, nicotine, or their combination on sleep-wake states, as observed in this study, were specific pharmacological effects and not due to non-specific reasons like animal handling, or injection procedure. This conclusion is based on the following observations: a) that after saline injection, which was used as a control, rats spent almost similar amount of time in waking, NREM sleep, and REM sleep on treatment day-1, day-14, and day-28, as well as on day-29 without any treatment; b) that the effects of ethanol and nicotine were opposite on waking and sleep; c) that in earlier study, we showed that the effects were time and dose-dependent [27]; d) that acute and chronic treatment, as well as withdrawal of ethanol and/or nicotine, produced differential effects on sleep-wakefulness; e) that when given together, ethanol and nicotine attenuated each other’s effects on sleep-wakefulness; and f) that similar effects of ethanol and nicotine have also been observed in other studies using different approaches.

The findings of this study suggest that acute, repeated and chronic use of ethanol and its withdrawal produced significantly different effects on sleep-wake architecture. While ethanol acutely reduced waking, increased NREM sleep and slightly suppressed REM sleep, its chronic treatment caused attenuation of the sleep-promoting effects of ethanol in a time-dependent manner. However, the first day without ethanol treatment, the animals showed significant withdrawal effect, which was marked by increased wakefulness, increased sleep latency, a decrease in NREM sleep, increased REM sleep latency and decreased REM sleep. These findings are consistent with human and animals studies showing that acute ethanol withdrawal causes insomnia and sleep disruptions [8, 11, 14-16, 18, 22].

An attenuation of the sleep-promoting ability of ethanol with its chronic treatment suggests that animal gradually developed...
tolerance to ethanol. In the brain, ethanol may act on sleep as well as wake regulatory systems via various mechanisms to produce its acute hypnotic effect [14, 17]. For example, ethanol amplifies GABAergic inhibitory tone on wake-promoting neurons, inactivates excitatory glutamate receptors, and increases adenosinergic inhibitory tone on wake-active neurons in the basal forebrain and posterior hypothalamus [14, 17, 31]. Therefore, a gradual weakening of ethanol’s effects on sleep-wakefulness and/or sleep disturbance during chronic ethanol consumption or its withdrawal could be due to the dysfunction of those systems that contributed to it sleep-producing effects. These may include a gradual desensitization of GABA receptor systems, glutamate receptors hypersensitivity, or attenuated adenosinergic actions on wake-promoting systems [14, 17]. The findings of insomnia on withdrawal day as observed in this study further supports findings of earlier studies. Abstinence from ethanol (withdrawal) in both human and animals are also marked by severe insomnia including increased sleep latency and decreased total sleep time (see Introduction). The findings on REM sleep, however, are inconsistent with two studies showing decreased REM sleep [14, 32] and two showing increased REM sleep during initial withdrawal period [11, 14, 16]. Our findings also suggest that REM sleep is reduced during withdrawal period. On the other hand, nicotine acutely increased waking and suppressed both NREM and REM sleep. Like ethanol, as compared to acute effects, chronic administration of nicotine progressively became less effective in inducing wakefulness and suppressing NREM sleep or REM sleep. During the withdrawal period, however, animals exhibited only a marginal increase in NREM sleep (which could be due to the ceiling effect because rats mostly sleep during the daytime), while REM sleep increased significantly. As regards the mechanisms, nicotine imitates the action of the neurotransmitter, acetylcholine, via its actions on nicotinic acetylcholine receptor that are expressed in wake-promoting regions like cholinergic neurons in the basal forebrain and hypocretin neurons in the hypothalamus. Consistent with this hypothesis, nicotine activates both basal forebrain and hypocretin neurons and promote wakefulness [23, 33]. Therefore, it is likely that the behavioral effects of nicotine treatment, as observed in this study, partly involved activation of both wake-promoting cholinergic and hypocretin systems. Evidence also suggest that with chronic consumption of nicotine, there is a gradual desensitization of nicotinic acetylcholine receptors, which potentially contributes to the weakening of its effects on sleep-wake responses during chronic treatment and differential response on withdrawal period. When ethanol and nicotine, with antagonistic effects on sleep-wakefulness, were given together, these two drugs attenuated each other’s effects on sleep-wakefulness. Such interactions persisted throughout the treatment as well as during withdrawal periods. These findings are consistent with an earlier study where the effects of ethanol was blocked by the injection of nicotine in the wake-promoting part of the brain, i.e., the basal forebrain [23]. The findings of this study that nicotine co-treatment attenuated the sleep-promoting effects of ethanol and thus may increase the pleasure experience, as hypothesized by other investigators regarding the reason for their co-consumption. Studies show that nicotine-treated animals or smokers consume more alcohol than control groups [34, 35] whereas, administration of nicotine receptor antagonist reduces alcohol consumption [36, 37]. Thus co-consumption of nicotine and ethanol, potentially creates an effect that is different from the effects of either drug taken alone, which explains as to why the co-consumption of these two drugs are so prevalent.

6. Conclusion
The findings of the present study suggest that while ethanol acutely decreased waking, increased NREM sleep and decreased REM sleep, its repeated and chronic use produced only marginal effects on sleep-wakefulness. However, both NREM and REM sleep were severely disrupted on withdrawal day. On the contrary, nicotine acutely increased waking and decreased both NREM and REM sleep, an effect which was attenuated with nicotine’s repeated and chronic use. However, waking was suppressed and sleep, especially REM sleep increased significantly on withdrawal day. These findings suggest that animals develop tolerance to both ethanol and nicotine with their repeated and chronic use. When given together, these drugs nullified each other’s adverse effects on sleep-wake parameters. These findings are consistent with known interactions between ethanol and nicotine in influencing sleep and explains as to why the co-consumption of these two drugs is so common.

7. References