The mGLU2/3 receptor antagonist LY341495 stimulates waking and fast EEG power and blocks the effects of the mGLU2/3 receptor agonist LY379268 in rats.

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Abbreviations:
REM – rapid eye movement
NREM – non-rapid eye movement
mGlu – metabotropic glutamate

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Abstract

The highly selective mGlu2/3 receptor agonist LY379268 completely suppresses REM sleep and strongly depresses theta (6-10 Hz) and high frequency (10-60 Hz) power in the waking and NREM EEG, effects consistent with depressed brain excitation (arousal). We hypothesized the selective mGlu2/3 receptor antagonist LY341495 given alone would (1) increase arousal, producing sleep-wake EEG effects opposite those of LY379268 and (2) block/reverse the effects of LY379268 when the drugs are coadministered. Rats with implanted electrodes were injected with 1, 5, or 10 mg/kg of LY341495 at hour 5.5 of the dark period. In the coadministration study the rats received the same dose of LY341495 followed 30 min later by 1 mg/kg LY379268. LY341495 alone increased waking by reducing NREM and REM sleep. LY341495 also depressed low frequency and stimulated high frequency EEG power. It produced a sharp spike in theta power in waking but not NREM sleep, a striking state-dependent difference in pharmacological response. These changes indicate that blocking mGlu2/3 receptors increases brain arousal. Moreover, they show that mGlu2/3 receptors actively support arousal even in the absence of heightened glutamate excitation. The coadministration experiment demonstrates that LY341495 is selective *in vivo* since it dose-dependently attenuates or reverses the sleep-wake EEG effects of the highly selective mGlu2/3 receptor agonist LY379268. The capacity of mGlu2/3 receptor agonists and antagonists to alter the sleep wake balance suggests they could be developed to enhance sleep or sustain arousal. Their opposing actions on theta EEG could test the putative role of these oscillations in memory consolidation.

The level of brain excitation is largely determined by the balance of inhibitory and excitatory neurotransmission by GABA and glutamate. Most clinically used hypnotic and anxiolytic drugs increase inhibition by stimulating the $GABA_A$-benzodiazepine receptor complex. Although these drugs remain our most effective hypnotics, they have serious limitations. They induce tachyphyllaxis and can produce addiction and life-threatening withdrawal syndromes. These drugs also distort the sleep EEG, reducing delta power in non-rapid eye movement (NREM) sleep and increasing power in sigma and beta frequencies (cf Feinberg et al., 2000).

Drugs that reduce brain excitation by decreasing glutamate excitation rather than increasing GABA inhibition might also have useful hypnotic and anxiolytic properties. Moreover, drugs that reduce glutamate excitation might produce novel clinical profiles since they act through qualitatively different mechanisms that presumably impact different structures and synaptic distributions. Ideally, drugs that depress glutamate activity would have improved therapeutic ratios with less tachyphylaxis and lower liability for addiction-withdrawal syndromes. They might also induce sleep with less distortion in the EEG.

Glutamate exerts its actions through both ligand-gated ion channels and G-protein-coupled metabotropic receptors (mGlu receptors). The mGlu receptors are classified into Groups I, II and III based on their molecular structures, second messenger systems and pharmacological profiles. Group II subtypes (mGlu2/3 Receptors) are located presynaptically outside the active axon terminals (Schoepp, 2001). Presumably, when glutamate escapes the synaptic space these receptors are activated to exert negative feedback that prevents or diminishes the effects of excessive glutamate release. Stimulation of mGlu2/3 receptors on post-synaptic neurons also dampens glutamate excitation by altering ion channels and inducing long-term depression (for review see Schoepp, 2001). Systemically administered mGlu2/3 receptor agonists reduce

glutamate-mediated neural excitation, raising the possibility that they could have several therapeutic applications (Schoepp and Marek, 2002).

For the past decade, our laboratory has been investigating the effects of altered glutamate neurotransmission on sleep. We first exploited the fact that NMDA ion channel blockade increases waking limbic system metabolism. According to our homeostatic model (Feinberg, 1974) an increase in the metabolism of these plastic brain structures should increase deep sleep. This prediction was strongly confirmed: Ketamine and MK-801 produced massive increases in NREM delta (Feinberg and Campbell, 1993; Campbell and Feinberg, 1996). We recently extended our studies to examine the direct sleep and EEG effects of a potent and selective mGlu2/3 receptor agonist, LY379268 (Monn et al., 1999). This compound is structurally related to LY354740, an earlier mGlu2/3 receptor agonist with demonstrated anxiolytic properties (Helton et al., 1998). LY379268 is even more effective than LY354470 in some animal models of psychosis and neuroprotection (Schoepp and Marek, 2002).

Injected subcutaneously in rats, 1 mg/kg LY379268 profoundly suppresses REM sleep (Feinberg et al., 2002). In the 6 h after administration, REM sleep is totally abolished and it is reduced by 80% in the next 6 h. NREM delta power is increased and theta and fast (10-50 Hz) EEG power are strongly decreased in both NREM sleep and waking. Although overall motor activity appears somewhat reduced at higher doses, LY379268 does not cause abnormal movements.

We interpret the sleep-wake and EEG changes induced by LY379268 as the result of depressed brain arousal. The remarkably strong REM suppression of LY379268 suggests that mGlu2/3 receptors directly participate in sleep regulation. It is interesting that the EEG effects of this mGlu2/3 agonist are so different from those produced by GABAergic hypnotics. Although

GABAergics also depress arousal, they reduce rather than increase NREM delta power and they increase rather than decrease fast EEG power.

Here, we investigated the sleep-wake and EEG effects produced by blocking mGlu2/3 receptors with LY341495, a selective mGlu2/3 antagonist (Kingston et al., 1998). We tested two hypotheses: (1) mGlu2/3 receptors tonically maintain the brain's level of endogenous glutamate excitation; if so, blocking these receptors in normal animals will increase waking and EEG arousal i.e. produce effects opposite those produced by the agonist. Specifically, REM time will increase, low frequency (delta) EEG power will decrease and theta and fast frequency power will increase.

Hypothesis (2) was that LY341495 would selectively block mGlu2/3 receptors *in vivo*. Selectivity would be demonstrated if coadministration of LY341495 blocks or reverses the effects of the highly selective mGlu2/3 receptor agonist LY379268.

Methods

Our methods for studying sleep in the rat have been detailed elsewhere (Feinberg et al., 2002) and will be briefly summarized here.

Animals: Male Sprague-Dawley rats (Simonsen Labs.), 300-350 g at the start of the experiment. N=18.

Surgery: Under deep pentobarbital anesthesia (65 mg/Kg), rats were implanted with flexible wire EMG electrodes in the nuchal muscles, a stainless steel screw ground electrode over the olfactory bulb, and six cortical screw electrodes over the frontal, frontoparietal and parietal cortices. Electrode leads were inserted into a small connector cemented to the rat's skull with dental acrylic. Rats were given 2 weeks to recover from the surgery. The animals were then

trained to the recording system, a counterbalanced cable and commutator that permitted free movement about the cage. During training, EEG was recorded from all possible ipsilateral pairs of electrodes, and the three cleanest signals were used for recording in the experiment. EEG analysis was performed on the cleanest of these three signals. The rats were recorded in their home cage with temperature (20-22^{o}C) and light (12:12) controlled. The UC Davis Animal Use and Care Advisory Committee approved all procedures.

EEG recording and analysis: EEG and EMG signals were amplified and filtered with Grass amplifiers. Amplifier filters were set at 0.3 Hz high pass and 100 Hz low pass for EEG and 3 Hz high pass and 500 Hz low pass for EMG. Notch (60 Hz) filters on the amplifiers were disabled at all times as they attenuate amplitudes over a wide frequency range. PASS PLUS (Delta software, St. Louis) digitized the amplified signals at 256 Hz and performed power spectral analyses with the Fast Fourier Transform (FFT) and simultaneous zero-cross and zero first derivative period amplitude analyses. PASS PLUS was calibrated before each recording session with a 200-µV 10 Hz sine wave. PASS PLUS scaled the period-amplitude and FFT measurements on each channel to this signal. FFT windows were 4-second Welch tapered windows with 2-second overlap, yielding five windows per 10-second epoch. FFT analysis yields frequency bands that differ from integer values. With these FFT parameters, delta was 1.25-4.25 Hz rather than 1-4 Hz. For simplicity of presentation, integer values will be used in this report. Frequency bands were 1 Hz wide for 1-4 Hz, 2 Hz wide for 4-12 Hz, 12-15 Hz, 5 Hz wide for 15-35 Hz, and the highest band analyzed was 35-50 Hz. For statistical analyses, these 13 bands were collapsed into the following *a priori* determined bands: 1-4, 4-6, 6-10, 10-20, 20-30, and 30-50 Hz.

Scoring of vigilance states: The digitized EEG and EMG signals were displayed on a computer monitor and each 10-second epoch was scored visually as NREM, REM or wake. Using PASS PLUS the scorer could also view hourly plots of period-amplitude analyzed wide band EMG and FFT power in delta (1-4 Hz) and *rho* frequencies. Delta peaks contributed to the detection of NREM and *rho* peaks to the detection of REM. Rho power was 25-30 or 30-35 Hz, depending on the animal. Rho helps detect REM sleep because rho power is elevated in REM above its levels in NREM and waking (Campbell and Feinberg, 1993). Displaying *rho* power obviates the need for a hippocampal (theta) electrode for REM detection.

We used the following criteria to classify the vigilance state of each 10 sec epoch: Wake: low amplitude-high frequency EEG with high EMG activity; NREM: high amplitude-low frequency EEG with low EMG activity; REM: low amplitude high frequency cortical EEG (often dominated by theta) with high rho spectral power and very low EMG activity. All vigilance state scoring was checked by a second scorer. Epochs containing artifacts (low frequency movement artifacts and high frequency eating artifacts) were excluded from FFT analyses but included in state durations. The vigilance state scores and computer-analyzed EEG databases were linked so that FFT power could be summed in each frequency band for each hour of each vigilance state.

Behavioral observations: Each animal's behavior was observed for 2 h post-injection and notations were made in the laboratory notebook.

Drugs; LY379268, (−)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate was synthesized as described by (Monn et al., 1999) (E. Lilly & Co. Inc). LY341495, (2S-2-amino-2-(1S,2S-2-carboxycyclopropyl-1-yl)-3-(xanth-9-y l)propanoic acid), was synthesized as described

by (Ornstein et al., 1998). Solutions of both compounds were prepared prior to each usage by dissolving the drug in equimolar NaOH and adjusting the pH to 7 with HCl.

Drug administration and recording: The 18 animals were randomly divided into 3 groups of six. One animal in the 10 mg/Kg group became ill during recording and was removed from the study, leaving 5 animals in that group. Each animal was recorded continuously during two 3-day experiments that were separated by at least one week (see Fig 1). In both experiments, the first day was a saline injection(s), the second day was a drug injection(s), and the third day was a recovery day with no injections. One experiment was a single drug study to assess the effects of the antagonist alone. The other experiment was a blocking experiment to determine the ability of the antagonist to block the pronounced sleep-wake effects of 1 mg/Kg LY379268, an mGlu2/3 receptor agonist. The single drug and the blocking experiment were performed in counterbalanced order. In the analyses below, we also used data from six rats studied in a previous experiment (Feinberg et al., 2002) with LY379268 alone. This was also a 3-day experiment with methods of recording and analysis identical to those employed here.

In the single drug experiment, one group received 1 mg/kg of LY341495, one received 5 mg/kg of LY341495 and the third group received 10 mg/kg on the drug day. As with the preceding saline injection on day 1, the drugs were given subcutaneously at hour 5.5 of the dark period on day 2.

In the blocking experiment, the mGlu2/3 antagonist LY341495 was injected subcutaneously at hour 5.5 of the dark period of day 2 using the same dose of LY341495 the rat had received (or would receive) in the single drug experiment. This was followed 30 min later by injection of 1 mg/kg of LY379268. Figure 1 outlines the experiments.

Statistical analyses: For the single drug experiment, the sleep and EEG effects of LY341495 were evaluated by ANOVAs followed by t-tests. For the blocking experiment in which LY341495 was followed by 1 mg/kg of LY379268, we performed a separate set of ANOVAs. For these analyses, we included data from our previous study in which six rats received 1 mg/kg of LY379268 subcutaneously alone at hour 6 of the dark period. These data were included so that the vigilance state and EEG effects of the three blocking doses of LY341495 could be compared to the animals' responses to 1 mg/kg of LY379268 alone i.e. in the absence of a previous injection of an mGlu2/3 receptor antagonist. All analyses were performed on data from dark period hours 7-12, i.e. we examined the acute sleep-wake and EEG responses in the 6 hours following drug injection(s).

Effects on vigilance state durations were initially tested with an ANOVA with dose as a grouping factor and condition (saline or drug) as a repeated measure. If the ANOVA produced a significant condition or dose by condition interaction, we performed paired t-tests between saline and drug conditions for each dose. We tested for significant dose response with an ANOVA using dose as a grouping factor and saline value as a covariate.

Effects on the EEG spectrum could only be determined for the waking and NREM EEG. The drugs suppressed REM sleep so strongly that the REM EEG data were insufficient for analysis. Effects on the waking and NREM EEG spectra were initially tested with an ANOVA using dose as a grouping factor and frequency and condition as repeated measures. ANOVAs with significant condition or interactions between any factors were followed by ANOVAs for each frequency band with dose as a grouping factor and condition as a repeated measure. Significant condition or dose by condition interactions were followed by paired t-tests and dose response ANOVAs as for the vigilance state analyses.

Results

Table 1 gives the means (SE) for waking, NREM and REM durations in dark period hours 7-12 for the 3 groups of rats receiving LY341495 in doses of 1, 5, and 10 mg/kg alone. Table 2 shows the same data for coadministered LY341495 and LY379268. The responses of these vigilance states to LY341495 are presented as percent baseline (saline) values for vigilance states in Figure 2A and the responses to coadministered drugs are presented in Figure 2B. LY341495 effects on the waking and NREM EEG power spectra are shown as percent change from baseline (Figure 3). Significance tests for NREM and Wake EEG responses to LY341495 alone are summarized in Table 3 and are illustrated in Figure 3. The significance results for the EEG effects in the coadministration study are listed in Table 4 and illustrated in Figure 4.

Vigilance state and EEG responses to LY341495 alone:

Vigilance states: Figure 2A shows that LY341495 significantly increased waking. The magnitude of the increase was similar for the 1 and 5 mg/kg doses; both increased waking above baseline by about 40%. The 10 mg/kg dose had a stronger effect than the two smaller doses, increasing waking by about 70%. Waking increased because both REM and NREM sleep decreased. The 1 and 5 mg/kg doses produced similar reductions in REM, decreasing it by about 70%. The 10 mg/kg dose almost completely eliminated REM sleep. Following both the 1 and 5 mg/kg doses NREM decreased by about 40%. As was the case for REM, 10 mg/kg of LY341495 produced a greater NREM suppression than 1 and 5 mg/kg doses (65 vs. 40%). It was the greater response to 10 mg/kg that accounted for the statistically significant dose-response F test indicated in Table 1.

Response of EEG spectral power in NREM and Waking to LY341495: The vigilance state data show that REM sleep durations were so strongly depressed that they were insufficient for EEG analysis. EEG analyses were therefore performed only for NREM and waking.

NREM EEG Spectra: Figure 3A shows that LY341495 produced strong effects on NREM EEG. The three doses decreased low frequency power (<10 Hz) by similar amounts. The slower the frequency below 10 Hz, the greater was the depression in power by all three doses. LY341495 also stimulated high frequency (>20 Hz) NREM power. The higher the frequency above 20 Hz, the greater was the increase in power. The overall shapes of the NREM power spectra for 1, 5 and 10 mg/kg LY341495 were similar. Power was depressed below baseline in the lowest frequencies and increased to near baseline at 6-10 Hz. Power then remained near baseline to about 20 Hz and then increased steadily. While 1 mg/kg suppressed low frequency EEG about as much as the two higher doses, it did not produce the clear-cut high frequency stimulation found with the two higher doses. In 10-50 Hz, stimulation was greater for 10 than 5 mg/kg, accounting for the significant dose-dependence.

Wake EEG Spectra: The three doses of LY341495 increased power in the fast (>20 Hz) frequencies of waking EEG. The pattern of change in fast EEG power closely resembled that in NREM sleep. Power following the 5 and 10 mg/kg doses steadily increased in 20-50 Hz with the increase stronger for 10 than for 5 mg/kg.

Below 20 Hz, LY341495 produced strikingly different effects on the wake spectra from those it produced in NREM. In contrast to NREM, wake power in the lowest frequencies (1-2, 2-3 Hz) was not depressed, remaining near baseline. Power was slightly below baseline in 3-6 Hz. For both the 5 and 10 mg/kg doses, this slight decrease was followed by a sharp spike in power at about 8 Hz (i.e. in the theta band) reaching about 38% above baseline. This was followed by

an equally sharp decline to slightly below baseline at about 12 Hz. The theta spike was virtually identical for the 5 and 10 mg/kg doses. There was no theta spike for 1 mg/kg LY341495 although power did increase from the depressed 4 Hz level to baseline at 8-10 Hz.

Blocking experiment: Effects on sleep-wake EEG of the mGlu2/3 receptor agonist LY379268 injected 30 min after 1, 5, or 10 mg/kg of the mGlu2/3 receptor antagonist LY341495

Vigilance states: Fig. 2 B includes, as 0 mg/Kg LY341495, the data for six rats who received 1 mg/kg LY379268 in our previous study. Fig 2B shows 1 mg/kg of LY379268 did not significantly affect time awake, although NREM duration tended to increase. The effect of LY379268 on REM was dramatic; 1 mg/kg LY379268 totally eliminated REM sleep in the 6 h after injection.

Following 1 mg/kg of LY341495, 1 mg/kg of LY379268 increased NREM duration above baseline. When LY379268 was preceded by 5 mg/kg LY341495, NREM duration decreased below baseline. NREM duration was further reduced when LY379268 was preceded by 10 mg/kg LY341495.

REM duration under the drug combination was suppressed by about the same amount as by 1, 5 and 10 mg/kg of LY341495 alone. This result appears paradoxical because 1 mg/kg LY379268 totally suppresses REM. Nevertheless, when preceded by LY341495, which itself markedly suppresses REM (Fig. 2A), REM duration was *higher* than with LY379268 alone. The three doses of LY341495 blocked the LY379268 REM suppression to the same extent, permitting levels of REM that were at about 5% of baseline. For this reason, the dose-response data for REM were non-significant, Table 2.

Waking was slightly below baseline with 1 mg/kg LY341495 and then showed a stepwise increase to 5 and 10 mg/kg. For these latter doses, the waking increases were produced mainly by the NREM declines. The decrease in REM accounted for a small part of the increased waking.

NREM EEG spectra: Figure 4 includes data from our previous study of LY379268. It shows that this compound had strong effects on the NREM spectrum. Power in 1-2 Hz increased by 50%. Power then declined precipitously so that by 10-11 Hz it was about 40% below baseline. Power in 10-50 Hz was depressed to 50-60% of baseline.

In the coadministration experiment, 1 mg/kg of LY341495 blocked the LY379268 increase in low frequency power, so that it was slightly decreased. Although 1 mg/Kg LY341495 completely blocked the low frequency increase in power produced by LY379268 alone, it only slightly attenuated the LY379268 depression of 10-50 Hz power. The 5 mg/kg dose of LY341495 reversed the LY379268 elevation of low frequency power, producing about a 25% reduction at 1-2 Hz. From 4 to about 20 Hz the reduction was roughly 20%. Power then increased steadily from 20 to 50 Hz, so that it was reduced by only about 10% at 50Hz. The shape of the frequency spectrum for the 10 mg/kg blocking dose of LY341495 paralleled the curve for 5 mg/kg dose. Low frequency EEG was depressed by the same amount as with 5 mg/kg. Above 10 Hz, 10 mg/kg LY341495 stimulated EEG power more strongly than 5 mg/kg. At 25 Hz, power was at baseline levels and at 50 Hz it was about 20% above baseline. The shapes of the spectral curves for the 5 and 10 mg/kg doses were similar and paralleled those for the same doses given alone.

Wake EEG spectra: Fig. 4 shows that waking spectral power with LY379268 alone was slightly above baseline in 1-4 Hz. It then declined steeply to about 75% of baseline at 8 Hz. The

decrease was about 20% at 12 Hz and stayed close to this level until 35 Hz. Between 35-50 Hz power gradually declined to a 25% reduction at 50 Hz.

With prior administration of 1 mg/kg LY341495, power in 1-6 Hz was below that with 1 mg/kg LY379268 alone. Above 8 Hz, the spectral curve with the 1 mg/kg dose of LY341495 paralleled but was slightly above the curve for LY379268 alone.

The 5 and 10 mg/kg doses of LY341495 completely reversed the LY379268 effects on the waking EEG spectrum. The theta spike in wake EEG seen with LY341495 alone was present when LY341495 (5 or 10 mg/Kg) was given in combination with 1 mg/Kg LY379268. The spike was again almost identical in size and shape for the two larger doses of LY341495. Above 15 Hz, 10 mg/kg increased power more than 5 mg/kg, as was the case for LY341495 alone. The waking EEG spectra for 5 and 10 mg/kg LY341495 combined with LY379268 had the same shape as the spectrum for LY341495 alone except that the size of the theta spike and the increases in fast frequency power were both somewhat smaller.

Behavioral observations: Observations of behavior were documented for the two hours following each injection. LY341495 did not produce any discernable changes, either when given alone or with LY379268. No motor abnormalities were seen.

Discussion

This experiment, along with our previous findings with LY379268, demonstrates that stimulation and blockade of mGlu2/3 receptors profoundly alters sleep-wake states and their EEG spectra. The changes they induce are among the strongest pharmacologic effects on sleep-wake EEG yet observed. Except with respect to the complex responses of REM sleep, the results with LY3421495 directly support our two *a priori* hypotheses: (1) the mGlu2/3 receptor

antagonist LY341495 will produce effects on sleep-wake EEG opposite those produced by the agonist LY379268; and (2) LY341495 will demonstrate high *in vivo* selectivity by attenuating and/or reversing the sleep-wake and EEG effects of the highly selective agonist LY379268.

Effects of LY341495 on waking and REM duration: LY341495 increases time awake by decreasing both NREM and REM sleep. Most of the waking increase results from the NREM reduction. Although LY341495 depresses REM sleep proportionately more than NREM, REM makes up only about 10% of the rat's dark period sleep.

Despite a five-fold difference in dose, 1 and 5 mg/kg of LY341495 increase waking equally. However, when the dose increases from 5 to 10 mg/kg, wake time almost doubles, indicating that LY341495 crosses some biological threshold between 5 and 10 mg/kg. LY341495 is known to block mGlu8 receptors at higher doses (Schoepp, 2001). The 10 mg/kg dose might therefore add arousing effects of mGlu8 receptor blockade to those of mGlu2/3 receptor antagonism, indicating that the *in vivo* selectivity for mGlu2/3 receptors might be overridden at high doses. There are other possibilities. The higher dose might extend the blockade of presynaptic mGlu2/3 receptors to those on post-synaptic neurons or bring into play arousal systems with higher thresholds for responding to mGlu2/3 blockade.

Our previous experiment showed that 1 mg/kg of the mGlu2/3 agonist LY379268 totally eliminates REM sleep in the six hours following injection. Here we show that 1 and 5 mg/kg of LY341495 also substantially reduce REM sleep and that 10 mg/kg suppresses REM almost completely. That REM sleep is suppressed rather than increased by the mGlu2/3 receptor antagonist apparently contradicts the opposing actions we predicted. This apparent contradiction can be reconciled by the "one-stimulus" model of NREM-REM alternation (Feinberg and March, 1988, 1995). According to this model, REM sleep is an intermediate state of arousal that emerges

when the strength of a hypothetical inhibitory stimulus that initiates NREM sleep falls below a critical level. At this point, neurons escape from inhibition, discharging intensely but irregularly in many brain structures (Evarts, 1967). This inhibitory escape *is* REM sleep. It is an arousal level intermediate between the high arousal of waking and the low arousal of NREM. REM therefore could be depressed by drugs that increase neural excitation and arousal (e.g. LY341495); such drugs could convert REM to waking. Conversely, REM could also be depressed by drugs that decrease excitation level and arousal (e.g. LY379268); such drugs could convert REM to NREM. In fact, the coadministration experiment demonstrates that although LY341495 by itself suppresses REM, it partially blocks the REM suppression of LY379268, an effect consistent with the opposing actions we expected.

Effects of LY341495 on the EEG power spectrum: As predicted by hypothesis (1) the mGlu2/3 receptor antagonist increases EEG arousal. In NREM sleep, LY341495 depresses power in the low EEG frequencies that characterize low arousal and deeper sleep. It increases power in the higher EEG frequencies that normally indicate heightened arousal. These effects are opposite those of the agonist LY379268.

The low and high EEG frequencies of NREM sleep respond differently to the different doses of LY341495. Power in the low frequencies (<6 Hz) is depressed to the same extent by 1, 5 and 10 mg/kg of LY341495 despite the ten-fold difference between the lowest and highest dose. In contrast, high frequency EEG power (20-50 Hz) dose-dependently increased following LY341495 with the increase greater in higher frequencies. This differential effect supports the idea that different brain systems control low and high frequency EEG oscillations.

Evidence that different brain systems control high and low EEG frequencies is also provided by the theta (6-10 Hz) responses. Theta power is strikingly stimulated by 5 and 10

mg/kg LY341495 in waking but not in NREM. In contrast, high frequency EEG (>15 Hz) shows highly similar responses to LY341495 in both NREM and waking with dose-dependent increases in power that produce virtually parallel spectral curves. The stimulation of theta spikes in waking but not in NREM is a noteworthy illustration of a state-dependent pharmacologic response.

We interpret the depression of theta power by LY379268 and its increase by LY341495 as manifestations of decreased and increased brain arousal level. Many theorists have proposed that the theta oscillations perform a critical role in memory consolidation (cf Graves et al., 2001; Kahana et al., 2001). LY341495 and LY379268 could test this hypothesis since they strongly stimulate and depress waking theta power without motor impairment. However, even if theta power were shown to correlate positively with memory acquisition/consolidation, it would be necessary to distinguish non-specific effects of arousal level from a direct role of the theta oscillations themselves. Arousal level is a well-known determinant of memory acquisition and consolidation.

Effects of coadministration of LY341495 and LY379268: These results support hypothesis (2): that LY341495 would show *in vivo* selectivity by blocking/reversing the sleep-wake EEG effects of the highly selective mGlu2/3 receptor agonist LY379268. Although 1 mg/Kg LY341495 combined with LY379268 significantly increases NREM sleep, coadministering 10 mg/kg LY341495 strongly reduces NREM duration and both 5 and 10 mg/Kg increase waking. At 10 mg/kg, waking increases by about 50%.

The effects of coadministered LY341495 and LY379268 on REM are complex. REM sleep is totally eliminated by 1 mg/kg LY379268. LY341495 alone also strongly depresses REM sleep: 1 and 5 mg/kg LY341495 decreased REM by about 75% and the 10 mg/kg dose decreased it by over 95%. However, the REM suppression is not additive when the drugs are

coadministered. There is less REM suppression when 1 mg/kg LY379268 is injected after 1, 5 and 10 mg LY341495 than when 1 mg/kg LY379268 is given alone. Thus, although LY341495 itself suppresses REM, it partially blocks the REM suppression of LY379268. We outlined above how these responses could be explained by the one-stimulus model of NREM-REM sleep.

In NREM, 1 mg/kg LY379268 increases low frequency (<6 Hz) EEG power and profoundly suppresses power in 10 - 50 Hz. LY341495 at 1 mg/kg is sufficient to block the low frequency stimulation of EEG power by LY379268. 5 and 10 mg/kg LY341495 reverse the LY379268 stimulation of low frequency power, producing about a 25% reduction. Above 10 Hz, LY341495 dose-dependently counteracts the suppression of EEG power by LY379268; for the 5 and 10 mg/kg dose, the reversal of LY379268 EEG suppression becomes greater in the higher frequencies. It is interesting that LY341495 does not itself increase power above baseline in the middle EEG frequencies (10-20 Hz). Nevertheless, it dose-dependently blocks the pronounced LY379268 depression of power in these frequencies.

In waking, LY379268 alone depresses power in the theta band. It also depresses high frequency power but somewhat less so than in NREM. Coadministered LY341495 in 1 mg/kg does not block the LY379268 theta suppression and has little effect on its depression of high frequency EEG. Coadministration of 5 or 10 mg/kg LY341495 reverses the theta suppression and produces spikes in theta power similar to but slightly smaller than those produced by LY341495 alone. In high EEG frequencies, 5 mg/kg LY341495 returns the markedly depressed LY379268 spectrum to baseline or slightly above and 10 mg/kg increases power above baseline, with the response greater in the higher frequencies. Thus, LY341495 attenuates or reverses the sleep and EEG effects of the highly selective agonist LY379268, suggesting but not definitively demonstrating *in vivo* selectivity of LY341495.

Implications: Perturbations of mGlu2/3 receptors induce changes in many brain systems (Schoepp, 2001). It would be difficult or impossible to predict the consequences of these multiple discrete actions for global brain physiology. The vigilance state and EEG responses described here represent integrated resultants of these effects and could help elucidate the role of mGlu2/3 receptors in brain function.

The ability of mGlu2/3 receptor antagonists to increase arousal has potential clinical applications. Such agents might be useful for normal individuals who must maintain prolonged vigilance. Both the arousing and REM suppressing effects of mGlu2/3 receptor antagonists could be therapeutic in disorders of excessive daytime sleepiness, including narcolepsy-cataplexy. With careful adjustment of dose, the extraordinary capacity of mGlu2/3 receptor agonists like LY379268 to suppress REM might be used to treat cataplexy, a condition in which the atonia of REM intrudes into waking. We have previously noted (Feinberg et al., 2002) that the sleep EEG effects of mGlu2/3 agonists raise the possibility of potential hypnotic or antidepressant actions. In addition to their clinical implications, the findings here suggest that these G protein coupled metabotropic receptors play a role in the regulation of sleep.

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Footnotes

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Legends for Figures

Figure 1. Time line showing the recording schedule and injection times for LY341495 alone (top line) and in combination with LY379268 (bottom line). EEG was recorded in 3 consecutive 24 hour sessions from rats on a 12:12 light:dark schedule. LY341495 was injected subcutaneously at hour 5.5 of the dark period. LY379268 was injected subcutaneously at hour 6 of the dark period. The EEG and vigilance state responses presented here are for hours 7-12 of the dark period on the control and drug days.

Figure 2. Vigilance state effects of 3 doses of LY341495 alone (A) and when followed by 1 mg/Kg LY379268 (B). Data are means (+/- s.e.) of dark period hours 7-12 totals expressed as a percent of the corresponding hours on the saline day. LY341495 dose dependently depressed NREM and REM and increased wake durations. LY379268 alone (0 mg/Kg LY341495) completely suppressed REM sleep, slightly increased NREM sleep and did not significantly affect waking. LY379268's effects outweighed the LY341495 effects at 1 mg/Kg on NREM and waking, but at higher doses the LY341495 effect predominated. The drug combination greatly reduced but did not completely suppress REM sleep. Significant differences from saline values are indicated by asterisks (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

Figure 3. EEG effects of LY341495 alone on EEG power density in NREM (top) and Wake (bottom). Data for each frequency band are means of power/min in dark period hours 7-12 expressed as a percent of power/min in corresponding hours on the saline day. All doses depressed low frequency EEG power in NREM but not waking. The 5 and 10 mg/Kg LY341495 doses produced pronounced spikes in theta power (6-10 Hz) EEG in waking but not NREM. The

5 and 10 mg/Kg doses also increased high frequency EEG power. Significant differences from saline values for each frequency band and each dose are indicated by dark bars below the x-axis (line – not significant, thin bar – $p<0.05$, thick bar – $p<0.01$).

Figure 4. NREM and Wake EEG effects of LY341495 doses in combination with 1 mg/Kg LY379268. Format as in Fig. 3. LY379268 alone (0 mg/Kg LY341495) elevated NREM slow wave EEG power, suppressed theta power in the waking EEG, and suppressed mid and high frequency EEG in both NREM and waking. All doses of LY341495 blocked the LY379268-induced slow wave elevation. The 5 and 10 mg/Kg LY341495 doses reversed the theta depression. Although LY341495 did not affect 10-20 Hz EEG in waking, the higher doses blocked the LY379268 induced depression of these frequencies. 5 mg/Kg LY341495 blocked the high frequency suppression caused by LY379268 and 10 mg/Kg reversed it.

Table 1. Vigilance state effects of LY341495 injected at hour 5.5 of the dark period. Mean durations in each state are shown for hours 7-12 of the dark period for the saline control day and the drug day. The results of the dose response ANOVA are also listed.

Vigilance state		Saline and LY341495 listed by dose						ANOVA	
		Sal	1 mg/Kg	Sal	5 mg/Kg	Sal	10 mg/Kg	$F_{2,13}$	p
NREM (min)	Mean	153	96[a]	142	85[a]	167	61	4.53	0.032
	s.e.	10	17	17	14	15	12		
REM (min)	Mean	20.1	5.4[a]	16.4	3.8[a]	20.3	0.4	4.27	0.038
	s.e.	2.2	1.6	3.9	1.0	2.0	0.3		
Wake (min)	Mean	186	256[a]	201	272[a]	173	298	4.52	0.032
	s.e.	11.7	19.4	18.8	14.3	15.7	12.2		

[A] A common superscript on the drug averages indicates that covariate adjusted means do not differ at α=0.05. Significant dose responses were due to larger effects of the 10 mg/Kg dose.

Table 2. Vigilance state effects of LY341495 injected at hour 5.5 and LY379268 (1 mg/Kg) injected at hour 6 of the dark period. Mean durations in each state are shown for hours 7-12 of the dark period for the saline control day and the drug day. The results of the dose response ANOVA are also listed.

		Saline and combination of LY379269 (1mg/Kg) + LY341495 listed by dose								ANOVA	
		Sal	0 mg/Kg	Sal	1 mg/Kg	Sal	5 mg/Kg	Sal	10mg/Kg	$F_{3,18}$	p
NREM (min)	Mean	138	164[a]	147	189[a]	136	107[b]	145	70[b]	18.28	<0.0001
	s.e.	4.7	15.3	13	11	12	9	9	12		
REM (min)	Mean	16.0	0.0	20.1	1.7	20.0	1.9	22.1	0.9	1.20	0.34
	s.e.	1.5	0.0	3.3	0.7	2.8	0.9	3.0	0.9		
Wake (min)	Mean	204	195[a]	192	167[a]	203	248[b]	192	288[b]	16.70	<0.0001
	s.e.	5.7	15	16	12	14	9	11	13		

[A] A common superscript on the drug averages indicates that covariate adjusted means do not differ at α=0.05.

Table 3. Statistical tests of dose effects of LY341495 alone on the NREM and Wake EEG in dark period hours 7-12. Results of ANOVAs with dose as a grouping factor and saline values as covariates. Dashes indicate where dose response ANOVAs were not conducted because ANOVA with dose as a grouping factor and condition (saline and drug) as a repeated measure did not yield a significant condition or dose x condition interaction.

	NREM		Wake	
Frequency	$F_{2,13}$	P	$F_{2,13}$	p
1-4 Hz	0.43	0.66	-	-
4-6 Hz	0.69	0.52	0.50	0.62
6-10 Hz	0.36	0.70	3.69	0.054
10-20 Hz	0.02	0.98	-	-
20-30 Hz	2.28	0.14	4.87	0.026
30-50 Hz	8.44	0.0045	13.18	0.0007

Table 4. Statistical tests of dose effects of LY341495 followed by an injection of 1 mg/Kg LY379268 on the NREM EEG in dark period hours 7-12. Results of ANOVAs with dose as a grouping factor and saline values as covariates.

	NREM		Wake	
Frequency	$F_{3,18}$	p	$F_{3,18}$	p
1-4 Hz	9.08	0.0007	4.47	0.016
4-6 Hz	4.93	0.011	4.81	0.013
6-10 Hz	2.14	0.13	7.86	0.0015
10-20 Hz	4.07	0.023	4.13	0.022
20-30 Hz	8.29	0.0011	10.05	0.0004
30-50 Hz	13.97	<0.0001	13.55	0.0001

Figure 1

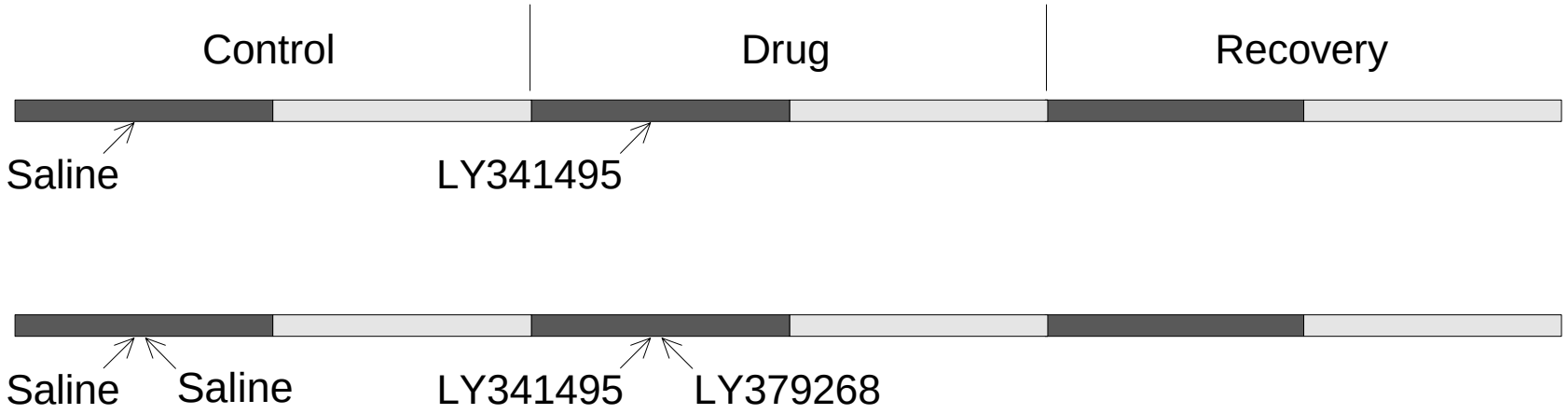

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Figure 2

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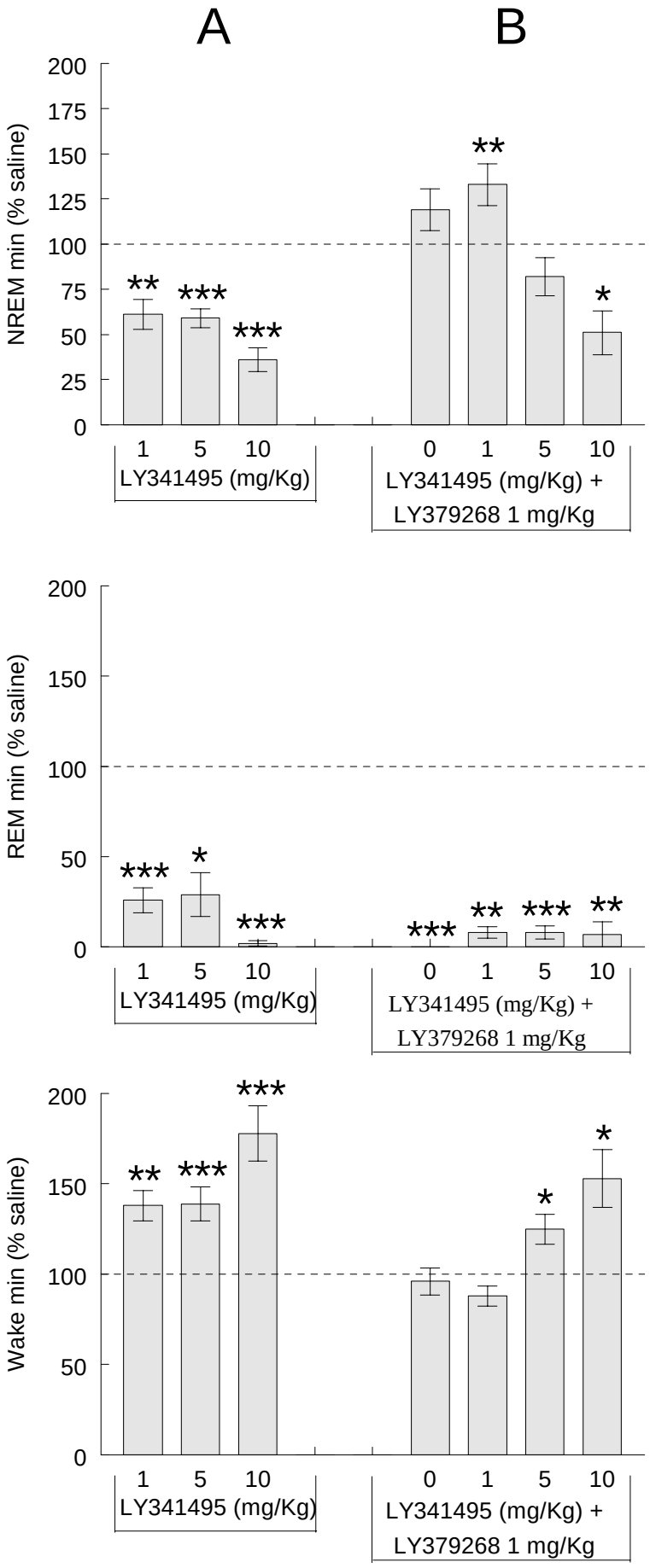

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Figure 3

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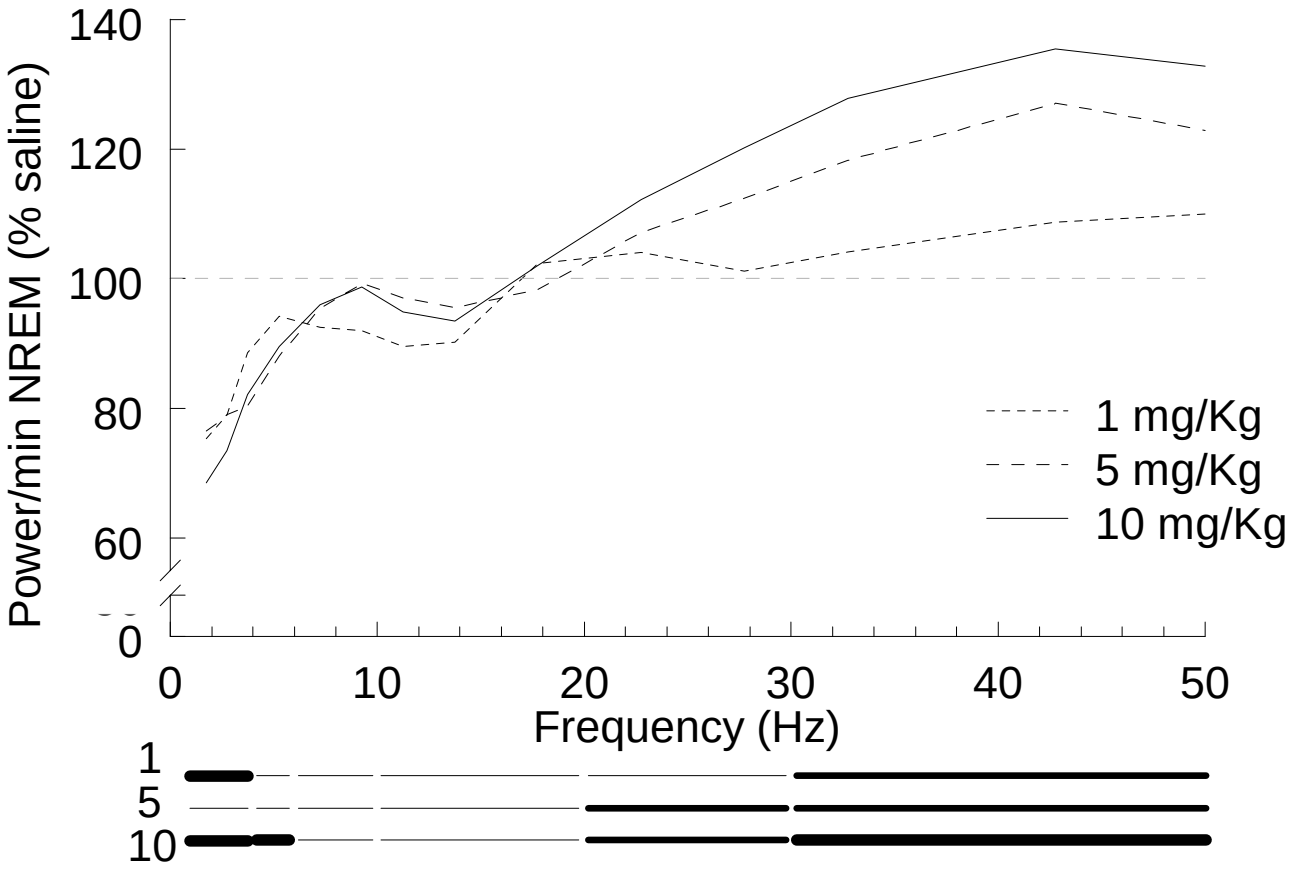

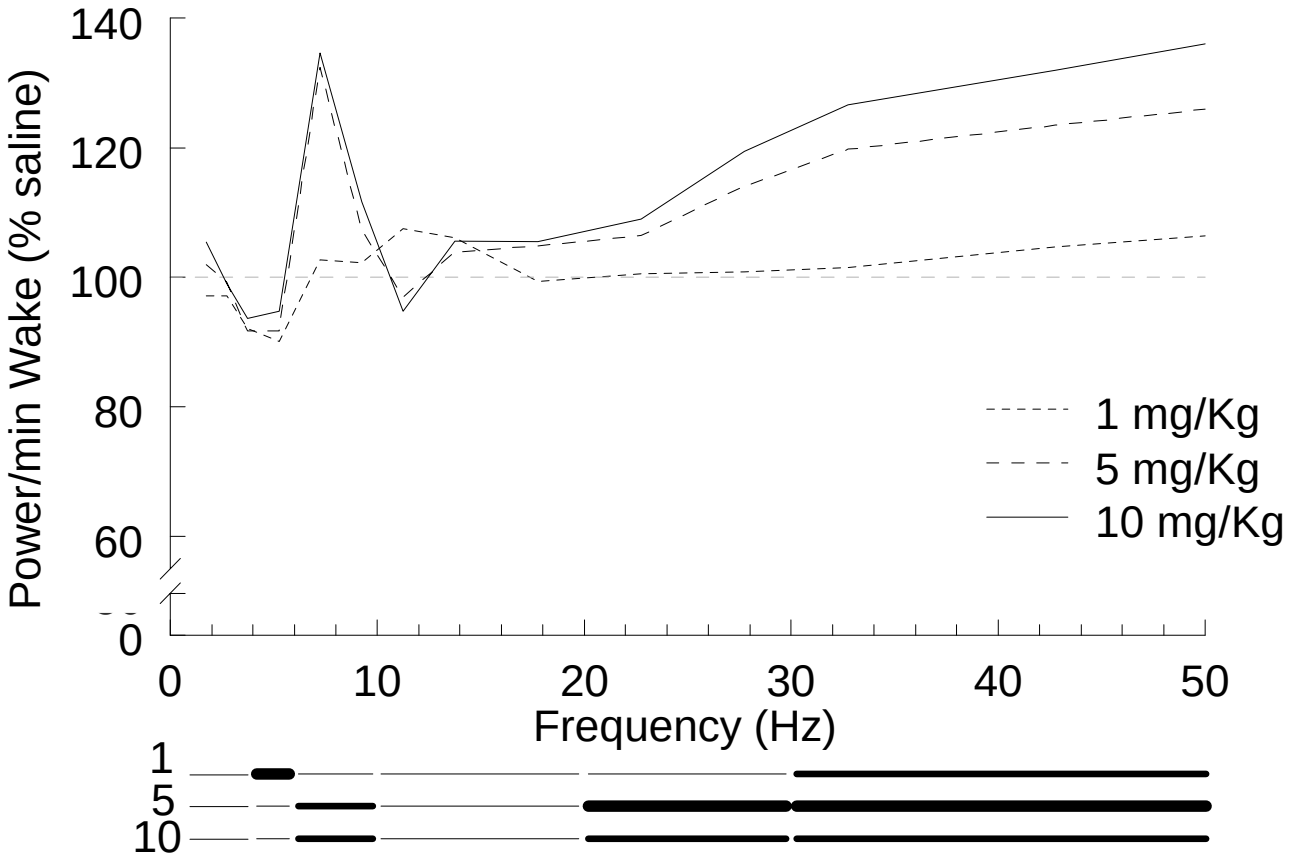

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Figure 4

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