Diazepam and ethanol differently modulate neuronal activity in organotypic cortical cultures

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Abstract

Background: The pharmacodynamic results of diazepam and ethanol administration are similar, in that each can mediate amnestic, sedative-hypnotic effects. Although each of these molecules effectively reduces the activity of central neurons, diazepam does so through modulation of a more specific set of receptor targets ($GABA_A$ receptors containing a γ-subunit), while alcohol is less selective in its receptor bioactivity. Our investigation focuses on divergent actions of diazepam and ethanol on the firing patterns of cultured cortical neurons. Method: We used electrophysiological recordings from organotypic slice cultures derived from Sprague-Dawley rat neocortex. We exposed these cultures to either diazepam (15 and 30 μM) or ethanol (30 and 60 mM) and recorded the electrical activity at baseline and experimental conditions. For analysis, we extracted the episodes of spontaneous activity, i.e., cortical up-states. After separation of action potential and local field potential (LFP) activity, we looked at differences in the number of action potentials, in the spectral power of the LFP, as well as in the coupling between action potential and LFP phase. Results: While both substances seem to decrease neocortical action potential firing in a similar fashion, diazepam seems to increase the spectral power of the up-state without impacting the spectral composition, whereas ethanol does not change the spectral power but the oscillatory architecture of the up-state. Further, the action potential to LFP-phase coupling reveals a synchronizing effect of diazepam and a (rather weak) de-synchronizing effect for ethanol. Conclusion: Diazepam and ethanol, induce specific patterns of network depressant actions. Diazepam, via gamma subunit containing $GABA_A$ receptors, induces cortical network inhibition and increased synchronicity. Ethanol, via a wider span of molecular targets, also induces cortical network inhibition, but without an increase in synchronicity.

Background

Diazepam and ethanol are widely used central depressants with similar pharmacological properties. Behaviorally, they produce sedation, amnesia and, at higher concentrations, unconsciousness. These effects are at least partially mediated by neurons in the cerebral cortex. Both agents significantly reduce the excitability of cortical neurons when administered within a behaviorally relevant range of concentrations [1, 2]. Besides their common properties, distinct differences exist. Diazepam almost exclusively binds to $GABA_A$ receptors containing α1-, α2-, α3-, or α5-subunits typically with a γ-subunit [3]. Especially α1-containing $GABA_A$ receptors seem associated with the sedative effects of benzodiazepines such as diazepam [4, 5]. Ethanol on the other hand is less selective in its molecular targets, in addition to $GABA_A$ receptors containing δ-subunits other pre- and postsynaptic targets are glutamate receptors, $GABA_B$ receptors, and potassium channels. Ethanol effects receptor trafficking through changes in receptor phosphorylation and also neurosteroid synthesis are influenced by alcohol [6-13].

Although both diazepam and ethanol influence inhibitory transmission among interneurons it is inaccurate to assume their effects on network activity is identical, especially given the multitude of pharmacologic targets available to ethanol. Electroencephalographic study of event related potentials

differ between ethanol and diazepam [14] but a detailed examination of the differences in activity of neocortical neuronal populations has not been undertaken. Neurons of the neocortex take a central role in the generation of cortical oscillations in the theta and gamma frequency range. It is possible that enhancing the strength of inhibitory synapses by diazepam not only results into a decrease in the average discharge rates but also modifies correlated firing of cortical neurons.

Synchronous oscillatory activity in the neocortex is a form of correlated neuronal firing that is involved in working memory tasks and sensorimotor integration. [15] Electroencephalogram recordings *in vivo* can help to investigate synchrony in neocortical oscillatory activity. But the impact of subcortical structures like the thalamus on these oscillations is difficult to interpret. *Ex vivo* models lacking subcortical structures, e.g., cultured brain slices from the neocortex [16], present an approach to evaluate the spontaneous neuronal activity recorded as local field potential (LFP) in the isolated neocortex. The neocortex is among the most important structures in the brain to induce sedation, hypnosis or general anesthesia by benzodiazepines and ethanol [17]. For that reason, we decided to probe for differential actions of diazepam and ethanol in organotypic neocortical slice cultures from rats. The firing patterns of cultured cortical neurons is characterized by phases of high-frequency action potential firing, called up-states, halted by neuronal silence, termed down states [18-20]. In order to identify substance-specific effects on spontaneous up-states activity, we evaluated the change of power spectral density (PSD) of the up-states as well as the changes in synchronization between action potentials (AP) and the phase of the LFP-up-state using the analytical signal.

Methods

Preparation of organotypic cortical slice cultures

All procedures were approved by the Animal Care Committee (Eberhard-Karls-University, Tuebingen, Germany) and were in accordance with the institutional and federal guidelines of the German Animal Welfare Act (TierSchG). We put in a great deal of effort to reduce the number and suffering of animals. We prepared organotypic slice cultures from the neocortex of P 3 – 5 rats as described earlier [16, 21]

In brief, six P3 – P5 Sprague-Dawley rat pups of both sexes (Charles River, Sulzfeld, Germany) were put into a see-through plastic container and anesthetized with 4 Vol% halothane using high air flow (Draeger Vapor 19.3, Draegerwerk, Luebeck, Germnany). Animals were decapitated well after loss of righting reflex, but before cardio-respiratory depression appeared. We withdrew the cortical hemisphere, removed the meninges, and cut 300 µm thick coronal slices, which we transferred onto glass coverslips and embedded them in a plasma clot. We transferred the coverslips into plastic tubes containing 750 µl of nutrition medium to be incubated in a roller drum at 37°C. After one day in culture, we added antimitotics and we renewed the suspension and the antimitotics twice a week. For our experiments, we used the cultures after two weeks *in vitro*.

Electrophysiologic recordings

We performed the extracellular recordings in a recording chamber mounted on an inverted microscope. Therefore, we perfused the slices with an artificial cerebrospinal fluid (ACSF) consisting of (in mM) NaCl 120, KCl 3.3, NaH_2PO_4 1.13, $NaHCO_3$ 26, $CaCl_2$ 1.8 and glucose 11, bubbled with 95% oxygen and 5% carbon dioxide. We positioned ACSF-filled glass electrodes with a resistance of about 3 to 5 MΩ on the surface of the slices and advanced into the tissue until extracellular spikes exceeding 100 µV in amplitude were visible. All experiments were conducted at 34°C. For preparation of the test solutions we dissolved diazepam (B.Braun, Melsungen, Germany) and ethanol (99%, university pharmacy) in the ACSF to yield the desired concentration. We applied the drugs via bath perfusion using syringe pumps (ZAK, Marktheidenfeld, Germany) at a flow rate of approximately 1 ml min-1. After switching to experimental drug-containing solutions, at least 95% of the medium in the experimental chamber was replaced within 2 min. Effects on the spike patterns were stable about 5 min later. To ensure steady state conditions, we carried out the recordings during anesthetic treatment 10 min after commencing the change of the perfusate using a personal computer with the Digidata 1200 AD/DA interface and Axoscope 9 software (Axon Instruments, Union City, CA).

Separation of local field potential and action potential activity and signal preprocessing

We included n=7 and n=12 cultures in the diazepam and ethanol group, respectively. For each culture, we recorded spontaneous LFP activity during control conditions as well as in the presence of either ethanol or diazepam. The recorded electrophysiological data was band-pass filtered to separate AP activity from LFP activity. Filter settings for AP traces were 200 – 2000 Hz. For LFP the filter settings were 1 – 10 Hz [22] .For the identification of AP spikes and their time of occurrence we used a self-programmed MATLAB routine. The routine annotates the time point of a spike based on a defined amplitude threshold that was defined as three times the standard deviation of baseline noise. We also used MATLAB to extract episodes of cortical up-state activity from the LFP recordings. We only used recordings with valid data for all concentration levels to have a paired design for statistical analysis. Figure 1 presents an exemplary LFP with corresponding spiking activity.

<< *Figure 1 around here*>>

Analysis of local field potential activity

The recorded LFP present the cumulative activity of neuronal activity in proximity of the recording electrode. In order to extract relevant information from the cortical up-states we restricted our analyses to up-states that were longer than 2.5 s in order to adequately calculate the spectral power distribution of the up-state. The analysis of LFP episodes during neuronal up-states started after the initial LFP peak had returned to zero amplitude after the negative peak, otherwise the predominance of this part of the signal would have made it impossible to perform any kind of analysis. Hence, we excluded the first 1 s of each up-state that contained the initial LFP peak of the up-state for analysis of general up-state properties and looked at the initial LFP peak features separately. This step was necessary because the strength and amplitude of the initial LFP peak would have biased the up-state analysis (Figure 1). We measured the peak-to-peak amplitude of the initial up-state to quantify possible drug-induced effects. For

the analysis of the initial up-state amplitude, we had to exclude one diazepam experiment because we only observed short up-states in one concentration stage of this recording. For the same reasons, we excluded four ethanol experiments.

Further, we excluded the last 0.2 s of each up-state to prevent a bias due to the transition back to a cortical down-state at the end of the up-state.

We used the MATLAB *pmtm* function that applies the Thomson's multitaper method with 256 data points and time-halfbandwidth product to default for PSD calculation. We also calculated the normalized PSD (nPSD), by dividing the total power by the sum of power between 2 Hz and 30 Hz. While this approach provides information regarding changes in the spectral distribution with increasing drug concentrations, we used the information of AP times and LFP phase to evaluate possible changes in AP to LFP-phase locking.

Action potential probability at distinct field potential phase

We assessed the LFP phase with the Hilbert transform [23]. Using this method, an analytical signal X(t) is generated from the original trace, here the LFP up-state episode. X(t) is complex and the real part complies with the original trace and the imaginary part is the original trace after a ninety-degree phase shift. The analytic signal corresponds to the envelope of the original trace. The analytic phase Φ(t) can be obtained from . In order to correctly determine Φ(t), the trace has to be filtered to a narrow frequency range. Here, we analysed frequencies up to 16 Hz in non-overlapping 2 Hz steps. We followed a 5-degree raster of binning the AP to the phase. By matching the AP to the analytic phase we are able to evaluate possible (de-) synchronizing effects between AP and LFP-phase.

Statistical analysis

To describe diazepam- or ethanol induced effects on cortical up-state activity we applied different statistical approaches. To statistically describe possible changes in peak-to-peak amplitude of the initial LFP-spike, the number of AP, as well as in PSD and nPSD, we applied the Friedman test with pairwise Wilcoxon signed rank tests and a Bonferroni correction. For unpaired comparisons, we used the Mann-Whitney U test. For outlier analysis, we applied the MATLAB *isoutlier* function, defining elements that are greater than three scaled median absolute deviations away from the median as outlier. For changes in PSD and nPSD we only considered changes to be significant if they occured in at least two neighboring frequencies [24]. Being aware of the limited sample size in our experiments, wesupplemented the signed rank test with Hedges' g tests as effect size using the MATLAB-based MES toolbox [25]. We further used the Kolmogorov-Smirnov test to detect changes in the distribution of action potentials in relation to the LFP phase. We performed all descriptive and inference statistical tests with MATLAB.

Results

Effects of diazepam and ethanol on action potential firing of cultured cortical neurons

The depression of neocortical spike activity by diazepam and ethanol had been reported earlier [1, 2]. In the current study, the number of spikes decreased for both diazepam (X^2=8; p=0.0183; n=7) and ethanol (X^2=11.17; p=0.0013 n=11), excluding one outlier in the ethanol group (Figure S1A). For the diazepam experiments, the spike rate per 180 s recording time was 2070 [1470 4654] (median and 1st and 3rd quartile) for control conditions, 1259 [950 1541] for 15 µM, and 740 [579 904] for 30 µM diazepam. For the ethanol experiments, the spike rate was 2009 [824 2798] at control conditions, 1076 [435 1703] in the presence of 30 mM ethanol, and 673 [253 2210] with 60 mM ethanol. Table 1 contains the detailed statistical information regarding the substance-induced effects. In short, diazepam significantly and/or strongly reduced the firing rate in a concentration-dependent fashion. Ethanol reduced the AP rate, but did not have this concentration-dependent effect.

The boxplots in Figure 2 depict the relative change in the number of spikes which was 53% [37% 100%] for 15 µM and 36% [22% 62%] for 30 µM diazepam when compared to control conditions. The relative reduction of spike rate when compared to control conditions was 71% [41% 91%] for 30 mM and 41% [26% 77%] for 60 mM ethanol. Even though we could observe a substance-induced reduction of AP, there was no significant difference (p=0.659, Mann-Whitney U) in the reduction of spiking activity for the low concentrations of diazepam (15 µM) and ethanol (30 mM) versus the respective control conditions (p=0.659, Mann-Whitney U). Hence, we considered these concentrations as nearly equipotent.

<< *Figure 2 around here*>>

Actions of diazepam and ethanol on the number of neuronal up-states

Diazepam significantly reduced the number of up-states from 28 [12 44] during control to 5 [4.3 19] at 15 µM and 4 [3.3 12.8] at 30 µM (p=0.011; Chi-Sq=8.96). At the same time, the up-state duration did not reveal a significant difference among groups (p=0.1561, Chi-Sq=3.71), but the effect size analysis revealed a strong effect of 15 µM and 30 µM diazepam on up-state duration as presented in Table 1. The median up-state duration was 2.3 s [1.5 3.4] s at control conditions, 4.3 s [2.6 8.0] s at 15 µM and 6.0 [3.0 10.2] at 30 µM diazepam. Figure 3 displays the relative change in up-state duration by diazepam and ethanol, respectively. For the investigation of the effect of ethanol we did not observe a significant change in the number up-states (p=0.174; Chi-Sq=3.5) from 23 [14 32] at control conditions to 13.5 [8 22] at 30mM and 14.5 [9 27] at 60 mM ethanol. The effect of ethanol on the number of up-states was medium for 30 mM ethanol and XXX for 60 mM. For the evaluation of the duration of up-states in the ethanol experiments, we had to exclude two experiments detected as outliers as shown in the boxplots in Figure S1B in the supplement. Ethanol significantly affected the up-state duration (p=0.0247, Chi-sq=7.4). Median up-state duration was 2.6 s [2.1 3.9] s at control conditions, 3.3 s [1.7 5.0] s with 30 mM ethanol, and 2.1 s [1.4 3.4] s with 60 mM ethanol.

<< *Figure 3 around here*>>

<< Table 1 around here>>

Effects of diazepam and ethanol on absolute amplitude of the initial up-state

For both substances, we did not observe a significant effect on the initial amplitude. The Friedman test revealed a p=0.513 (Chi-Sq=1.33) for diazepam and p=0.687 (Chi-Sq=0.75) for ethanol.

Spectral properties of LFP up-states after the initial action potential

For diazepam we observed an increase in the PSD of the up-state episodes over the entire frequency range observed. We did not find a difference between the concentration levels. The nPSD did no change significantly, indicating a preserved oscillatory architecture in the up-state. Figure 4A-B highlight these findings. We found contrasting results for ethanol, which did not significantly change the PSD of the up-states. Ethanol had an effect on nPSD in certain frequency ranges indicative of an altered oscillatory architecture of the up-states, but only for the low, 30mM concentration. Figure 4C-D present the findings for ethanol.

There were only changes in nPSD in the very low frequencies. For ethanol we observed PSD changes only in a limited frequency range, whereas the frequency composition as evaluated by nPSD changed as well.

<< *Figure 4 around here*>>

Actions of diazepam and ethanol on the AP firing to LFP phase relationship

Diazepam induced a stronger effect on AP to LFP phase coupling than ethanol in the 2-16 Hz range. This effect was concentration-dependent. Higher concentrations of diazepam caused a stronger concentration of AP in a limited range of LFP phase. Ethanol in contrast did not affect the AP to LFP-relationship in this way. The effects were weaker and the high dose of ethanol caused a more uniform distribution of AP among the LFP phase. Table 2 presents the results of the statistical analysis. Figure 5 shows the polar plots of AP to LFP-phase distribution for diazepam and Figure 6 the AP to LFP-phase distribution for ethanol.

<< *Figure 5 around here*>> ; << *Figure 6 around here*>>

<< Table 2 around here>>

Discussion

In the current study we could show, that diazepam and ethanol both depress spontaneous cortical activity in cultures neocortical slices from rat. However, a detailed analysis reveals that diazepam and ethanol affect spontaneous firing patterns in a different fashion. While diazepam has a synchronizing effect on local field potential activity, ethanol only caused a small change towards desynchronization of spiking activity.

Relevance of used concentrations

For the current *ex vivo* study concentrations of diazepam in the micromolar range and concentrations of ethanol in the millimolar range were chosen to induce clear cut effects. Both, diazepam in the µM range and ethanol in the mM range roughly lead to a 50%-reduction of the spontaneous action potential firing rate and can therefore be considered as nearly equipotent. We previously described that a 50%-reduction of the spontaneous firing rate in rodent cultured cortical slices induced by benzodiazepines and other common drugs of anesthesia corresponds quite well with the EC_{50} of loss of righting reflex [2], which in turn presents a surrogate measure for loss of consciousness in humans. For ethanol a spike rate 50% effective concentration of 38.6 mM in cultured murine neuronal networks has been described [26]. Furthermore, Draski et al. reported blood ethanol concentrations of 64 mM at loss and 81 mM at return of righting reflex in rats [27, 28]. A blood ethanol concentration of 0.08%, the limit of legal driving in some countries, would correspond to 17 mM [29]. Therefore, the concentrations of diazepam and ethanol used for the current study are neither “low”, nor “intoxicating” [8, 30], but correspond approximately to induction of hypnosis *in vivo*.

Diazepam and ethanol differently influence spiking and up-state behavior

With our analytical approach we could identify differential effects of diazepam and ethanol at concentrations inducing comparable depression of overall network activity, perhaps indicating non-overlapping molecular targets. These findings may present a consequence of diazepam and ethanol targeting different subtypes of $GABA_A$ receptors [9, 10, 31].Our analyses investigating the relationship between the instantaneous phase of the LFP and the occurrence of an action potential revealed a phase to AP synchronization with diazepam and a weaker effect towards desynchronization with ethanol. The relative change in the spectral composition of the recorded up-state activity as evaluated by our multitaper-PSD analysis supports the finding. Diazepam does not affect the architecture of up-states activity, but the amplitudes of the up-states, also an indicator of a synchronization process. Ethanol in contrast has no effect on up-state amplitude but leads to faster oscillatory activity in the up-states, as sign for desynchronization. Our results further indicated a decrease in the number of up-states with diazepam. But the fewer up-states became longer. This finding supports the results regarding network synchronization; because once an up-state was initiated the synchronized activity could maintain neuronal activity for a longer time. For ethanol, we observed a different effect. We did not observe a significant reduction in the number of up-states with ethanol, and a decrease in up-state time with 60 mM of ethanol. Ethanol-induced desynchronizing mechanisms could cause the up-state to fade earlier.

Putative mechanisms of differential actions

Experimental studies suggest, that synchronous firing of inhibitory interneuronal networks present the source of neuronal networks oscillations [32-34]. Diazepam may alter the firing frequency of neurons by specifically modulating the decay of synaptic responses via specific $GABA_A$ receptors (containing □-subunits) and cause neuronal network activity to synchronize [32, 35]. This selective action of diazepam on $GABA_A$ receptors may lead to an increased AP to LFP phase locking caused by (subtle) inhibitory action on neuronal network activity resulting in more synchronized firing patterns that lead to increased

AP to LFP-phase locking. The more heterogeneous effect of ethanol may lead to effects on neuronal network activity, very different to diazepam. Further, ethanol increases in mediated via $GABA_A$ receptor-mediated inhibition mainly caused by δ-subunit-containing receptors, but other types may be upregulated as well [36, 37]. Increasing tonic inhibition dampened the (low-frequency) oscillatory activity of excitatory cells in an *in silico* model [35].

Furthermore, diazepam and ethanol may act via receptors that differ in their desensitization features. Desensitization of $GABA_A$ receptors (the main molecular target of diazepam) could play a key role in altering the ability of inhibitory networks to synchronize [32]. One exception from this is the δ-subunit containing $GABA_A$ receptor, which does not demonstrate desensitization. This $GABA_A$ receptor subtype is diazepam-insensitive but has been proposed as a target for ethanol [10].

Conclusion

In conclusion, we could present new evidence that the depression of spontaneous neuronal activity in the neocortex by substances inducing anxiolysis, sedation, hypnosis and addiction is not uniform. Depending on the specific molecular targets, diazepam and ethanol, induce specific patterns of network depressant actions. Diazepam, acting mostly through $GABA_A$ receptors containing the gamma-subunit, induces cortical network inhibition and increased synchronicity, whereas ethanol, acting via a much wider range of molecular targets, also induces cortical network inhibition, but without an increase in synchronicity.

List Of Abbreviations

ACSF artificial cerebrospinal fluid

AP action potential

CNT control

DZP diazepam

EtOH ethanol

GABA γ-aminobutyric acid

LFP local field potential

PSD power spectral density

Declarations

Ethics approval and consent to participate: All procedures were approved by the Animal Care Committee (Eberhard-Karls-University, Tuebingen, Germany) and were in accordance with the institutional and federal

guidelines of the German Animal Welfare Act (TierSchG). We put in a great deal of effort to reduce the number and suffering of animals.

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Authors' contributions:

MK: Performed data and statistical analyses, wrote paper

PSG: Discussed the data, helped to write the paper

VBW: Performed the experiments, helped to analyze data

RC: Performed the experiments, helped to analyze data

GS: Discussed the data, helped to write the paper

BA: Designed the study, helped to analyze data, helped to write the paper

BD: Designed the study, helped to analyze data, wrote the paper

All authors gave their consent for publication

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References

1. Antkowiak B: Different actions of general anesthetics on the firing patterns of neocortical neurons mediated by the GABA(A) receptor. *Anesthesiology* 1999, 91(2):500-511.

2. Drexler B, Zinser S, Hentschke H, Antkowiak B: Diazepam decreases action potential firing of neocortical neurons via two distinct mechanisms. *Anesth Analg* 2010, 111(6):1394-1399.

3. Mohler H: GABA(A) receptor diversity and pharmacology. *Cell Tissue Res* 2006, 326(2):505-516.

4. García PS, Kolesky SE, Jenkins A: General anesthetic actions on GABAA receptors. *Curr Neuropharmacol* 2010, 8(1):2.

5. McKernan RM, Rosahl TW, Reynolds DS, Sur C, Wafford KA, Atack JR, Farrar S, Myers J, Cook G, Ferris P *et al*: Sedative but not anxiolytic properties of benzodiazepines are mediated by the GABA(A) receptor alpha1 subtype. *Nat Neurosci* 2000, 3(6):587-592.

6. Criswell HE, Breese GR: A conceptualization of integrated actions of ethanol contributing to its GABAmimetic profile: a commentary. *Neuropsychopharmacology* 2005, 30(8):1407-1425.

7. Korpi ER, Debus F, Linden AM, Malecot C, Leppa E, Vekovischeva O, Rabe H, Bohme I, Aller MI, Wisden W *et al*: Does ethanol act preferentially via selected brain GABAA receptor subtypes? the current evidence is ambiguous. *Alcohol* 2007, 41(3):163-176.

8. Weiner JL, Valenzuela CF: Ethanol modulation of GABAergic transmission: the view from the slice. *Pharmacol Ther* 2006, 111(3):533-554.

9. Sundstrom-Poromaa I, Smith DH, Gong QH, Sabado TN, Li X, Light A, Wiedmann M, Williams K, Smith SS: Hormonally regulated alpha(4)beta(2)delta GABA(A) receptors are a target for alcohol. *Nat Neurosci* 2002, 5(8):721-722.

10. Wallner M, Hanchar HJ, Olsen RW: Ethanol enhances alpha 4 beta 3 delta and alpha 6 beta 3 delta gamma-aminobutyric acid type A receptors at low concentrations known to affect humans. *Proceedings of the National Academy of Sciences of the United States of America* 2003, 100(25):15218-15223.

11. Glykys J, Peng Z, Chandra D, Homanics GE, Houser CR, Mody I: A new naturally occurring GABA(A) receptor subunit partnership with high sensitivity to ethanol. *Nat Neurosci* 2007, 10(1):40-48.

12. Paul SM: Alcohol-sensitive GABA receptors and alcohol antagonists. *Proceedings of the National Academy of Sciences of the United States of America* 2006, 103(22):8307-8308.

13. Kumar S, Porcu P, Werner DF, Matthews DB, Diaz-Granados JL, Helfand RS, Morrow AL: The role of GABA(A) receptors in the acute and chronic effects of ethanol: a decade of progress. *Psychopharmacology (Berl)* 2009, 205(4):529-564.

14. Krull KR, Smith LT, Parsons OA: Simple reaction time event-related potentials: effects of alcohol and diazepam. *Prog Neuropsychopharmacol Biol Psychiatry* 1994, 18(8):1247-1260.

15. Miller EK, Lundqvist M, Bastos AM: Working Memory 2.0. *Neuron* 2018, 100(2):463-475.

16. Gahwiler BH, Capogna M, Debanne D, McKinney RA, Thompson SM: Organotypic slice cultures: a technique has come of age. *Trends Neurosci* 1997, 20(10):471-477.

17. Rudolph U, Antkowiak B: Molecular and neuronal substrates for general anesthetics. *Nature Reviews Neuroscience* 2004, 5(9):709-720.

18. Sanchez-Vives MV, McCormick DA: Cellular and network mechanisms of rhythmic recurrent activity in neocortex. *Nat Neurosci* 2000, 3(10):1027-1034.

19. Seamans JK, Nogueira L, Lavin A: Synaptic Basis of Persistent Activity in Prefrontal Cortex In Vivo and in Organotypic Cultures. *Cereb Cortex* 2003, 13(11):1242-1250.

20. Hoffman KL, Battaglia FP, Harris K, MacLean JN, Marshall L, Mehta MR: The Upshot of Up States in the Neocortex: From Slow Oscillations to Memory Formation. *J Neurosci* 2007, 27(44):11838-11841.

21. Gahwiler BH: Organotypic Monolayer-Cultures of Nervous-Tissue. *J Neurosci Methods* 1981, 4(4):329-342.

22. Drexler B, Roether CL, Jurd R, Rudolph U, Antkowiak B: Opposing actions of etomidate on cortical theta oscillations are mediated by different gamma-aminobutyric acid type A receptor subtypes. *Anesthesiology* 2005, 102(2):346-352.

23. Freeman W: Hilbert transform for brain waves. Scholarpedia 2 (1): 1338. In.; 2007.

24. Akeju O, Westover MB, Pavone KJ, Sampson AL, Hartnack KE, Brown EN, Purdon PL: Effects of sevoflurane and propofol on frontal electroencephalogram power and coherence. *Anesthesiology* 2014, 121(5):990-998.

25. Hentschke H, Stüttgen MC: Computation of measures of effect size for neuroscience data sets. *Eur J Neurosci* 2011, 34(12):1887-1894.

26. Xia Y, Gross GW: Histiotypic electrophysiological responses of cultured neuronal networks to ethanol. *Alcohol* 2003, 30(3):167-174.

27. Draski LJ, Spuhler KP, Erwin VG, Baker RC, Deitrich RA: Selective breeding of rats differing in sensitivity to the effects of acute ethanol administration. *Alcohol Clin Exp Res* 1992, 16(1):48-54.

28. Little HJ: The contribution of electrophysiology to knowledge of the acute and chronic effects of ethanol. *Pharmacol Ther* 1999, 84(3):333-353.

29. Harris RA, Mihic SJ: Alcohol and inhibitory receptors: unexpected specificity from a nonspecific drug. *Proceedings of the National Academy of Sciences of the United States of America* 2004, 101(1):2-3.

30. Grobin AC, Matthews DB, Devaud LL, Morrow AL: The role of GABA(A) receptors in the acute and chronic effects of ethanol. *Psychopharmacology (Berl)* 1998, 139(1-2):2-19.

31. Mody I, Glykys J, Wei W: A new meaning for "Gin & Tonic": tonic inhibition as the target for ethanol action in the brain. *Alcohol* 2007, 41(3):145-153.

32. Baker PM, Pennefather PS, Orser BA, Skinner FK: Disruption of coherent oscillations in inhibitory networks with anesthetics: role of GABA(A) receptor desensitization. *J Neurophysiol* 2002, 88(5):2821-2833.

33. Traub RD, Whittington MA, Colling SB, Buzsaki G, Jefferys JG: Analysis of gamma rhythms in the rat hippocampus in vitro and in vivo. *J Physiol* 1996, 493 (Pt 2):471-484.

34. Whittington MA, Traub RD, Jefferys JG: Synchronized oscillations in interneuron networks driven by metabotropic glutamate receptor activation. *Nature* 1995, 373(6515):612-615.

35. Maex R, De Schutter E: Synchronization of golgi and granule cell firing in a detailed network model of the cerebellar granule cell layer. *J Neurophysiol* 1998, 80(5):2521-2537.

36. Speigel I, Bichler EK, Garcia PS: The Influence of Regional Distribution and Pharmacologic Specificity of GABAAR Subtype Expression on Anesthesia and Emergence. *Front Syst Neurosci* 2017, 11:58.

37. Liang J, Suryanarayanan A, Abriam A, Snyder B, Olsen RW, Spigelman I: Mechanisms of reversible GABAA receptor plasticity after ethanol intoxication. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2007, 27(45):12367-12377.

Tables

diazepam	CNT vs 15 uM		CNT vs 30 uM		15 vs 30 uM	
	p	g	p	g	p	g
AP Depression	0.078	0.89 [0.34 1.94]; strong	0.031	1.20 [0.70 2.59]; strong	0.0156	1.20 [0.72 2.57]; strong
# of up-states	0.063	1.18 [0.53 2.70]; strong	0.031	1.33 [0.78 2.84]; strong	0.141	0.20 [0.02 0.84]; weak
up-state duration	0.031	-0.88 [-0.43 -1.77]; strong	0.11	-1.25 [-0.54 -2.75]: strong	0.578	-0.04 [-0.80 0.47];
ethanol	CNT vs 30 mM		CNT vs 60 mM		30 vs 60 mM	
	p	g	p	g	p	g
AP Depression	0.054	0.43 [-0.08 1.16];	0.003	0.69 [0.31 1.26]; medium	0.042	0.22 [-0.11 0.51];
# of up-states	0.079	0.75 [0.13 1.60]; medium	0.311	0.43 [-027 1.28];	1	-0.28 [-0.70 0.12];
up-state duration	0.432	-0.14 [-0.82 0.32]	0.0488	0.40 [-0.11 1.05]; weak	0.0195	0.56 [0.28 1.05]; medium

Table 1: p-values and effect sizes for the comparisons between the concentration levels of diazepam and ethanol for the depression of action potentials (AP), the number of LFP up-states, and the up-state duration.

REQ [Hz]	CNT vs DZP 15µM	CNT vs DZP 30µM	DZP 15µM vs DZP 30µM	CNT vs EtOH 30mM	CNT vs EtOH 60mM	EtOH 30mM vs EtOH 60mM
-2	0.7409	0.2461	0.4615	**0.0105**	0.2461	0.1713
-4	0.0484	0.0105	0.0018	0.7409	0.2461	0.4615
-6	**0.0009**	**0.0005**	**0**	0.3427	**0.006**	**0.006**
-8	**0.0484**	**0.0033**	**0.0001**	**0.0484**	0.3427	**0.006**
-10	**0.018**	**0.0002**	**0**	0.1158	0.2461	**0.0033**
0-12	0.1713	**0**	**0**	0.4615	0.1158	**0.0033**
2-14	0.5982	**0**	**0.0009**	0.9561	0.1713	0.1158
4-16	0.3427	**0**	**0.0033**	0.8685	0.1158	0.1158

Table 2: p-values of the Kolmogorov-Smirnov test evaluating possible differences in the distribution of AP to LFP-phase. Diazepam (left columns) causes a concentration-dependent change towards a less uniform distribution, i.e., the AP are occurring at a higher rate at the same LFP phase. Ethanol in contrast (right columns) had a weaker, opposite effect towards a more uniform distribution of AP to LFP-phase. Significant differences are indicated as bold p-values

Supplemental Figure Legend

Figure S1: We excluded one experiment (depicted by '+') as outlier, as defined by the MATLAB boxplot and function, for the analyses regarding the change in the number of spikes (A) as well as two experiments regarding the duration of the up-states (B).

Figures

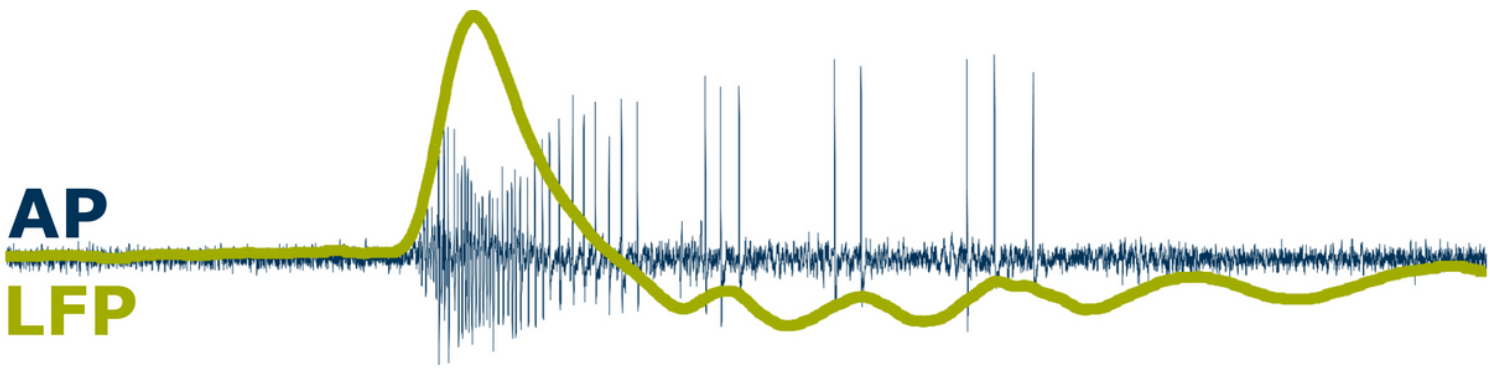

Figure 1

This plot shows an example of the association between the action potential (AP) firing (blue) and the filtered local field potential (LFP, green) during a cortical up-state. The AP and LFP trace were derived from the same recorded signal and separated by adequate filtering.

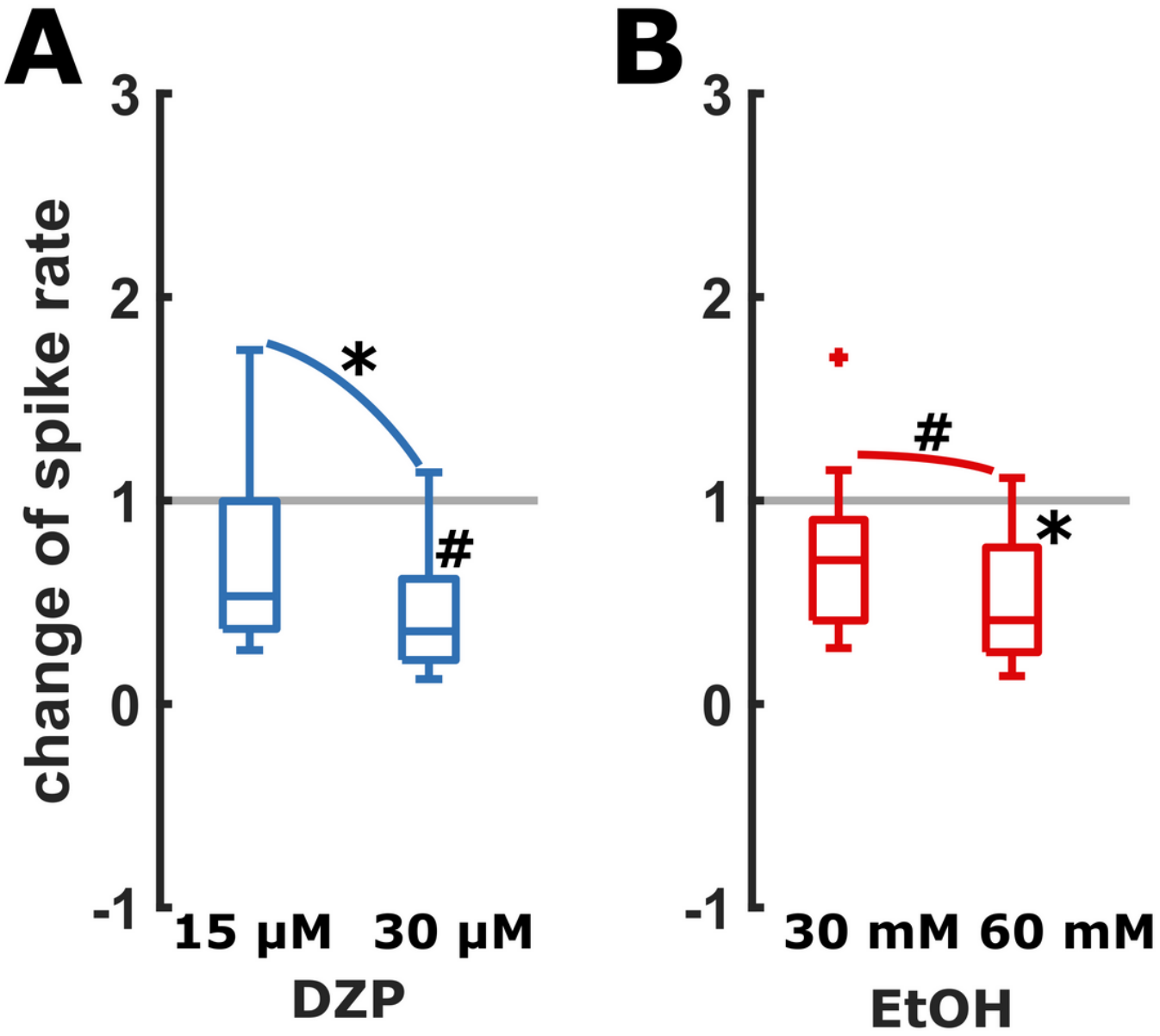

Figure 2

Relative change in the number of action potentials for (A) diazepam (DZP) and (B) ethanol (EtOH). Both diazepam (blue, left) and ethanol (red, right) decreased the number of action potentials in a concentration-dependent manner. A) 30 µM diazepam had a strong effect on the spiking rate. Diazepam caused a decrease in the number of action potentials as indicated by Hedge's g (g=1.20 [0.70 2.59]) that was not significant after Bonferroni correction (p=0.031, uncorrected) The decrease in spike rate from 15 µM to 30 µM diazepam was significant and strong (p=0.0156; g= 1.20 [0.72 2.57]). B) While 30mM

ethanol did not show a significant reduction of spike rate, 60 mM significantly reduced the spiking rate (p=0.003; g=0.69 [0.31 1.26]). The change from 30 mM to 60 mM ethanol was weak and not significant after Bonferroni correction (p=0.042, uncorrected; g=0.22 [-0.11 0.51]). * p<0.05 Bonferroni corrected; #: p<0.05 uncorrected

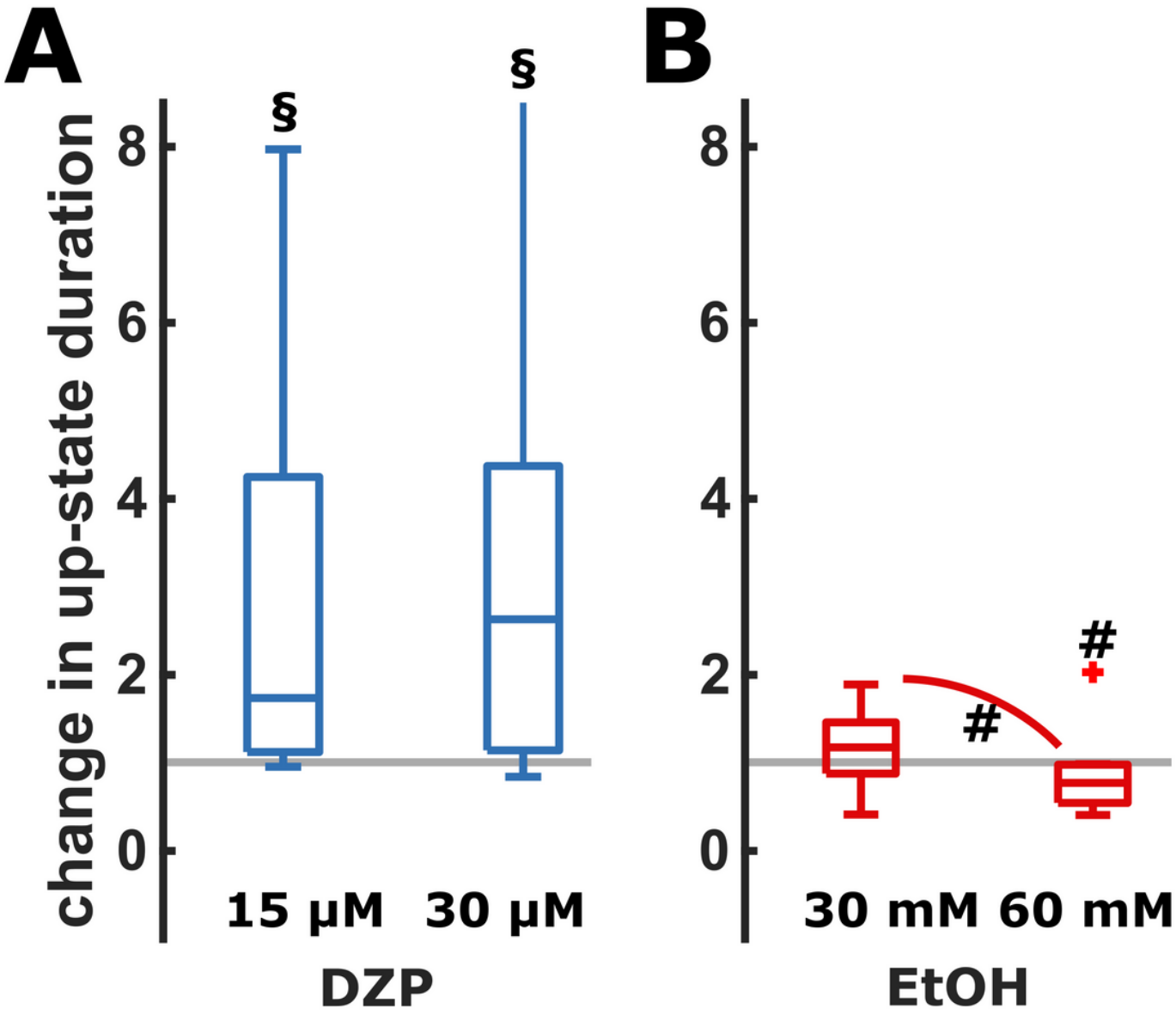

Figure 3

Relative change in the duration of up-states for (A) diazepam (DZP) and (B) ethanol (EtOH). A) Diazepam had a strong effect on the duration of up-states (15 µM: (g=-0.88 [-0.43 -1.77]); 30 µM diazepam (g=-1.25 [-0.54 -2.75])) B) While 30mM ethanol had no effect on up-state duration, 60 mM ethanol had a weak, but significant (p=0.0488, uncorrected) effect (g=0.40 [-0.11 1.05]) on up-state duration. Further, 60 mM ethanol had a medium effect causing shorter up-states (p=0.0195, uncorrected; g=0.55 [0.28 1.05), when compared against 30 mM ethanol. #: p<0.05 uncorrected; §: strong effect

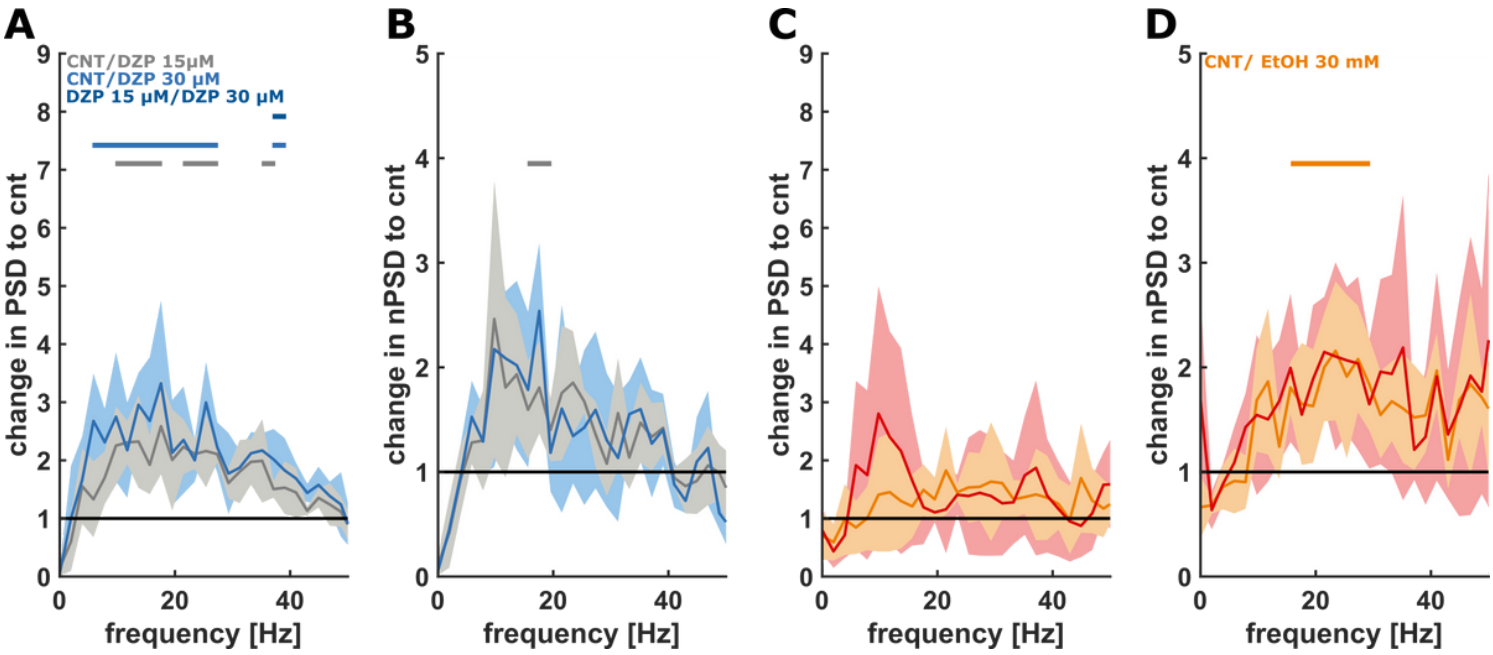

Figure 4

Relative changes in absolute power spectral density (PSD) or normalized PSD (nPSD) as induced by diazepam (DZP) or ethanol (EtOH) A) DZP-induced changes in PSD: DZP concentration-dependently increases the power (i.e., the amplitude) in a wide range of frequencies. The grey and blue horizontal line indicate a significant effect of low (15 µM, grey) or high (30 µM, blue) DZP concentration vs. control (CNT). A horizontal bar in dark blue indicates a significant difference between 15 µM and 30 µM DZP. B) DZP-induced changes in nPSD: The oscillatory composition did not change in a significant fashion, except for a narrow frequency range around 20 Hz for 15 µM DZP. C) EtOH-induced changes in PSD: EtOH does not affect the power (i.e., the amplitude) in LFP oscillations. D) EtOH-induced changes in nPSD: The oscillatory composition changed in a significant fashion towards a stronger contribution of higher frequencies above 10 Hz for the low EtOH concentration (30 mM) as indicated by the horizontal bars The solid trend lines indicate the median and the shaded areas the median absolute deviation.

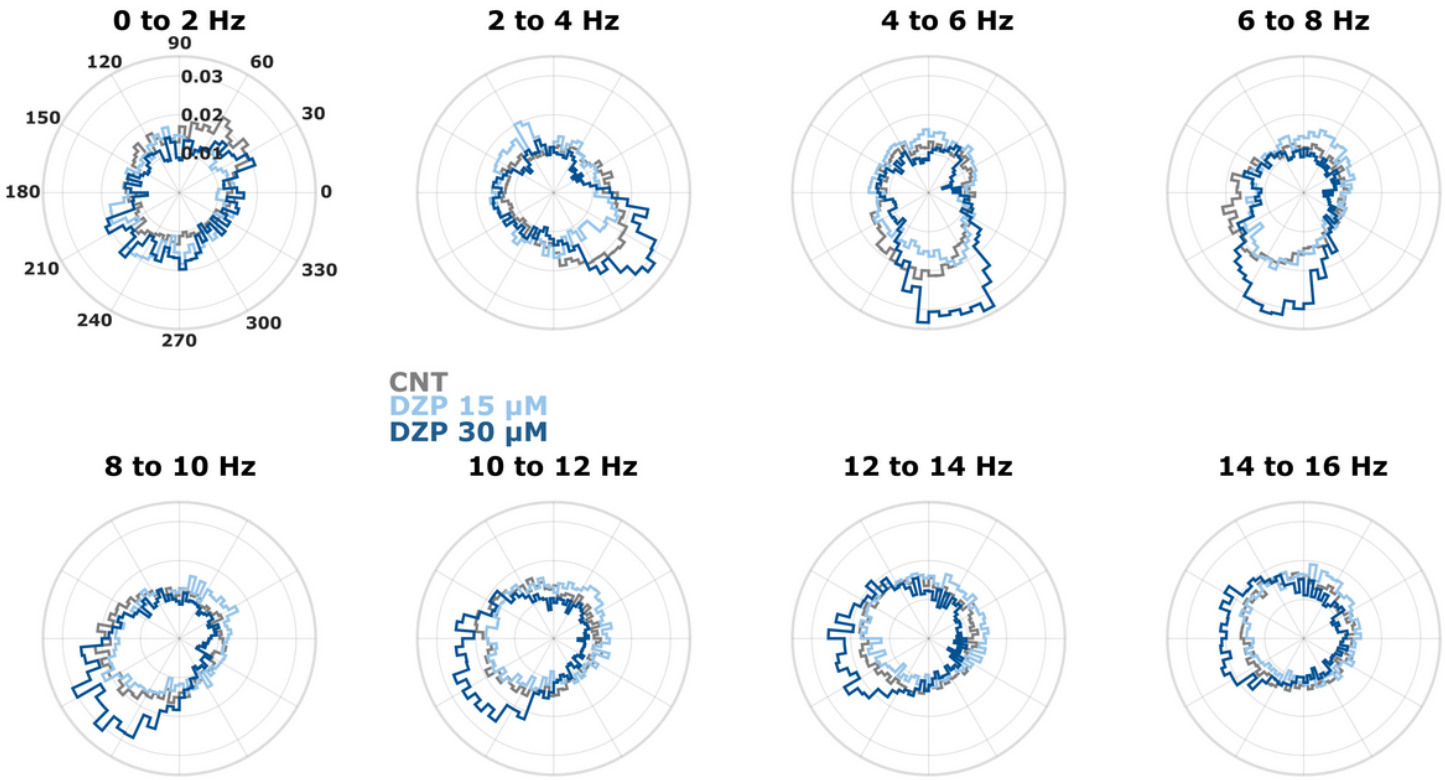

Figure 5

Diazepam–induced changes of the action potential to local field potential phase relationships. Especially at the high diazepam concentration (dark blue) peaks in the distribution develop that are indicative of a strong spike to phase locking. DZP: diazepam; CNT: control conditions

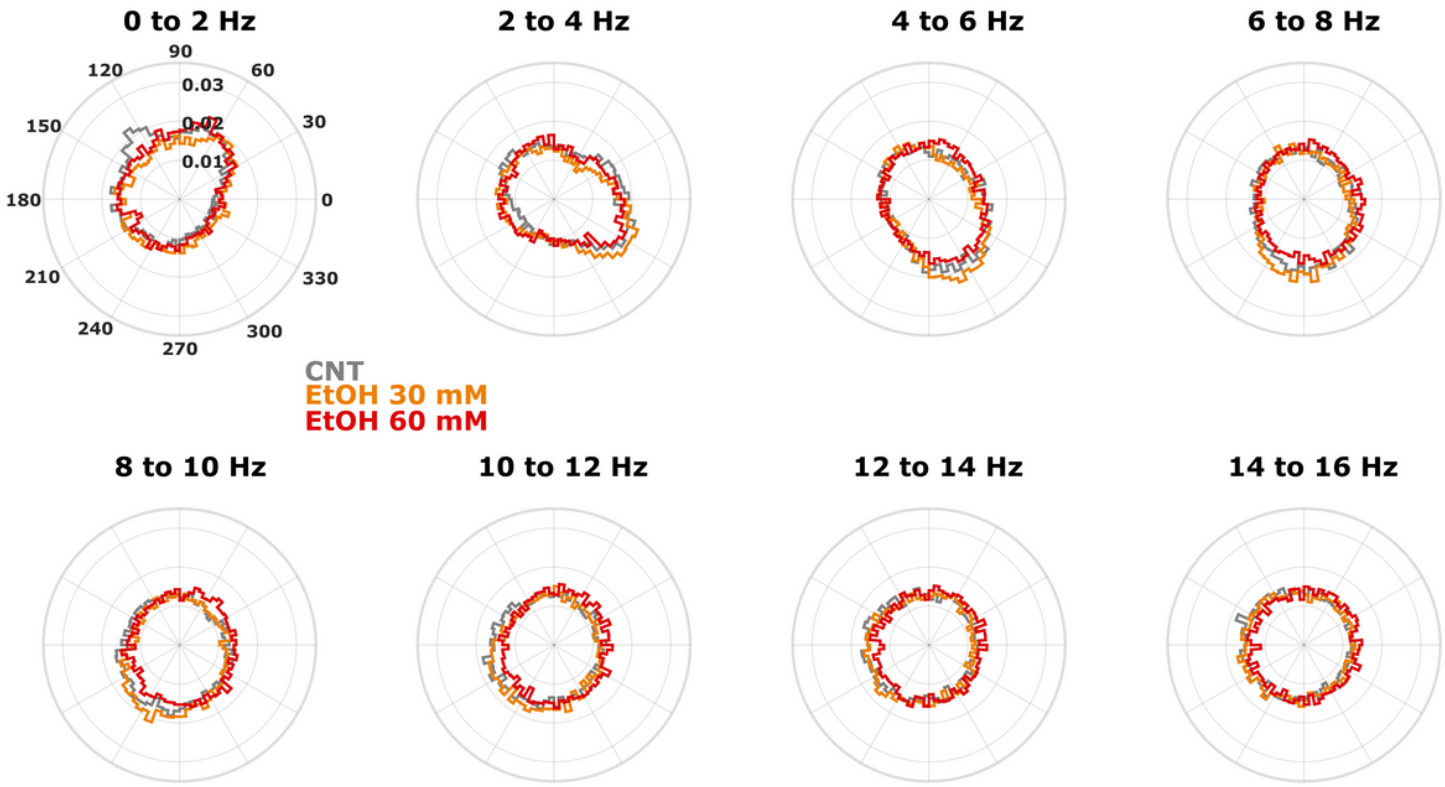

Figure 6

Ethanol-induced changes of the action potential to local field potential phase relationships. Application of ethanol (EtOH) leads to more uniform distribution of AP and LFP phase relationships. CNT: control conditions

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement1.docx
- supplement1.xlsx
- supplement3.png