Global slowing of network oscillations in mouse neocortex by diazepam

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A B S T R A C T
Benzodiazepines have a broad spectrum of clinical applications including sedation, anti-anxiety, and anticonvulsive therapy. At the cellular level, benzodiazepines are allosteric modulators of GABA_A receptors; they increase the efficacy of inhibition in neuronal networks by prolonging the duration of inhibitory postsynaptic potentials. This mechanism of action predicts that benzodiazepines reduce the frequency of inhibition-driven network oscillations, consistent with observations from human and animal EEG. However, most of existing data are restricted to frequency bands below ~ 30 Hz. Recent data suggest that faster cortical network rhythms are critically involved in several behavioral and cognitive tasks. We therefore analyzed diazepam effects on a large range of cortical network oscillations in freely moving mice, including theta (4–12 Hz), gamma (40–100 Hz) and fast gamma (120–160 Hz) oscillations. We also investigated diazepam effects over the coupling between theta phase and the amplitude fast oscillations. We report that diazepam causes a global slowing of oscillatory activity in all frequency domains. Oscillation power was changed differently for each frequency domain, with characteristic differences between active wakefulness, slow-wave sleep and REM sleep. Cross-frequency coupling strength, in contrast, was mostly unaffected by diazepam. Such state- and frequency-dependent actions of benzodiazepines on cortical network oscillations may be relevant for their specific cognitive effects. They also underline the strong interaction between local network oscillations and global brain states.

1. Introduction
Benzodiazepines, like diazepam (DZ), are positive allosteric modulators of the GABA_A receptor that act by potentiating the agonistic potency of the natural ligand GABA (for a recent review, see Tan et al., 2011). At the cellular and synaptic level, benzodiazepines can enhance the amplitude of inhibitory postsynaptic events, prolong their duration and increase tonic inhibition. At the network level this results in reduced excitability and in characteristic alterations of rhythmic activity patterns. Increased cycle length of inhibition-driven activity reduces the frequency of network oscillations, while increased inhibition efficacy enhances coherence of the multi-neuronal rhythmic activity (Whittington et al., 1996). In line with this, DZ decreases the frequency of hippocampal theta oscillations during REM sleep (Monmaur, 1981), walking (Caudarella et al., 1987) and during active exploratory behavior (Van Lier et al., 2004).

However, mammals express a large variety of different region- and state-dependent network patterns, most of which involve an important role of inhibition (Whittington and Traub, 2003; Mann and Paulsen, 2007). These patterns cover a wide spectrum of frequencies and support different behavioral and cognitive functions (Buzsáki, 2006). Therefore, multiple frequency domains have to be analyzed to assess the effects of benzodiazepines at the network level and relate them to their behavioral or cognitive actions. Moreover, different oscillations can occur simultaneously and can be systematically coupled. For example, multiple fast oscillations co-exist in hippocampal CA1 of freely moving rats, with a layer-specific coupling to theta phase (Scheffer-Teixeira et al., 2012; Belluscio et al., 2012). Interactions between different rhythms are related to task-specific cognitive performance both in humans (Axmacher et al.,...
2.1. Ethics statement

Methods and materials (Canolty and Knight, 2010; Fell and Axmacher, 2011) and four to nine animals (Tort et al., 2008, 2009). All efforts were made to minimize animal suffering and to reduce the number of animals used. Due to the behavioral aspect of the study, alternatives to in vivo techniques were not available.

2.2. Animal care and housing conditions

Male C57BL/6J mice were purchased at 28 or 45 days of age from Charles River (Sulzfeld, Germany). For a minimum of two weeks they were housed in groups of four to five inside a ventilated Scantainer (Scanbur BK A/S Denmark) on an inverted 12/12-h light/dark cycle with light on between 8:00 p.m. and 8:00 a.m. Animals had free access to water and food. After electrode implantation, mice were housed individually throughout the experiment.

2.3. Animal preparation

Ten male C57BL/6J mice were anesthetized with isoflurane in medical oxygen (4% isoflurane for induction, 1.5–2.5% for maintenance, flow rate: 1 l per min). Anesthetized animals were placed in a stereotactic apparatus with a custom-made inhalation tube. For analgesia, 0.1 mg/kg of buprenorphin was injected subcutaneously. Anesthetized animals were placed in a stereotactic apparatus with a custom-made inhalation tube. For analgesia, 0.1 mg/kg of buprenorphin was injected subcutaneously. Anesthetized animals were placed in a stereotactic apparatus with a custom-made inhalation tube. For analgesia, 0.1 mg/kg of buprenorphin was injected subcutaneously. Anesthetized animals were placed in a stereotactic apparatus with a custom-made inhalation tube. For analgesia, 0.1 mg/kg of buprenorphin was injected subcutaneously.

2.4. Electrophysiology and recording of behavior

One week after surgery, experiments began with a 2-h session in a Phenotyper home cage (Noldus Information Technology, Wageningen, Netherlands) measuring 30 cm × 30 cm with free access to food and water. EEG recordings were performed with a miniaturized data logger (Neurologger 2A), an advanced version of the device previously described (Vyssotski et al., 2006, 2009) with the reference configuration on the ground. The dimension and shape of all recording channels of reference was larger than 30 MΩ. Four channels of EEG signals were bandpass-filtered (1–700 Hz, –3 dB, attenuation –6 dB/octave) and digitized by an on-board A/D-converter (6400 Hz per channel) after amplification at ×1000 (input range of ±1 mV). Samples were stored in the on-board 512 MB memory at a rate of 1600 Hz. The dimensions of the neurologger were 23 × 15 × 13 mm and the total weight was 3.6 g with two batteries (Renata ZA 10; Itingen, Switzerland). This weight includes the neurologger itself (1.4 g), the add-on accelerometer/infrared synchronization board (0.4 g), batteries (0.6 g), battery holders as well as protective casing. The head implant (electrodes, wires, contacts and dental acrylic) had an additional weight of 0.7 g. Special care was taken to habituate the mice to the recording chamber and weight of data logger. The movement of the mice was not visibly altered (for details, see Branka et al., 2010). Prior to the first full-length recording of 10 h, all animals went through 3 habituation sessions. These were performed during the light period and contained epochs of data logger recordings with increasing duration from 2–3 h to 4 h in the third night. After each trial, the phenotyper cage was carefully wiped with 70% EtOH to remove all odor traces of previous animals, the cage bedding was removed and stored in a clean box so that it could be used again for the same subject. At the end of the experiment, data was downloaded onto a personal computer for further analysis. Animal behavior was continuously recorded by the video tracking system Ethovision XT 7.1 (Noldus Information Technology, Wageningen, Netherlands).

2.5. Drug preparation

Diazepam (Sigma–Aldrich) was prepared in a 10% (2-Hydroxypropyl)–β-cyclohextrin solution (Sigma–Aldrich). The same solvent was used as vehicle in control experiments. Diazepam was injected intraperitoneally in doses of 1, 2, or 4 mg/kg (Straub et al., 2010) in a random order with inter-injection intervals of at least 48 h. The injected volume was 10 μl/kg. Each injection was followed by data acquisition for a period of 10 h, starting 30 min after injection of drug or vehicle.

2.6. Data analysis

Continuous EEG recordings of 10 h duration from the circadian quiet phase (from 9:00 p.m. to 7:00 a.m.) of ten male animals were used for analysis. Data was imported into a MATLAB-based program (The Mathworks Inc., Natick, MA) using both built-in- and custom-written routines. Visual classification of REM-sleep, non-REM-sleep (NREM), quiet waking (qWk) and active wakefulness (aWk) was based on: 1) three-dimensional accelerometer activity (aWk distinguished by prominent signals); 2) the amount of high amplitude-low frequency delta activity in the neocortex (characteristic for NREM); 3) regular theta (4–12 Hz) oscillations (indicative of REM or aWk). For a detailed description of behavioral staging, see Branka et al., 2010). Waking states with movement were reliably and automatically detected by crossing a threshold in the summed integral of all three accelerometer dimensions. The remaining time corresponded to NREM sleep, drowsiness or immobile waking. Manual scoring in two animals revealed that less than 1% of the time spent in immobility consisted of qWk while most of the immobility corresponded to drowsiness and NREM sleep. For analyzing the sleep-wake cycle we therefore did not distinguish waking immobility as a separate state in the present study. REM sleep is heterogeneous and can be divided into tonic and phasic (Sakai et al., 1973), the latter characterized by brief periods of increased theta amplitude and frequency, ponto-geniculo-occipital spikes, muscle twitches, increased eye movements and vegetative arousal (Montgomery et al., 2008). Phasic REM was detected with a MATLAB program based on methods adapted from Mizusaki et al. (2011) and covered less than 3% of total REM duration (for detailed analysis of phasic versus tonic REM, see Branka et al., 2012). In addition, latency from vehicle or drug application to the first occurrence of NREM and REM sleep was identified. Baseline data on aWk, tonic and phasic REM in the absence of diazepam have been described elsewhere (Branka et al., 2012). Power spectral density (PSD) estimation was done by means of the Welch periodogram method using the built-in MATLAB function “pwelch” from the Signal Processing Toolbox. We employed 50% overlapping Hamming windows with a length of 4 s. To estimate “peak frequency” in the gamma and fast gamma range we first removed a 1/f fit from the PSD, and then smoothed the remaining PSD using a 20-Hz moving average. For an illustration of this procedure see Supplementary Fig. 1. Band power was defined as the area under the curve of the corresponding frequency domain.

The stability of theta oscillations was estimated by the width of the peak observed in the PSD (see Fig. 3B). Power values between 0 and 30 Hz were fitted by a Gaussian curve. For this procedure, we only took into account values above a minimal level chosen to provide the best fit (usually 20 or 30% of peak theta power). Theta peak width was then defined as the standard deviation of the Gaussian fit. For general estimation of DZ effects on oscillation frequency, we calculated the mean frequency in the 0–20 Hz and 20–100 Hz ranges, respectively (see Fig. 5). Power values were transformed into probabilities Pj by dividing the power of each...
frequency $F_j$ by the area under the curve for the analyzed band. The mean frequency is then obtained by $S_j = \sum F_j P_j$.

To measure the intensity of phase-amplitude coupling, we used the modulation index (MI) as described in Tort et al. (2010). The MI is capable of assessing coupling between two frequency ranges of interest: a slower phase-modulating ($f_p$) and a faster amplitude-modulated ($f_a$) frequency. The comodulation map is obtained by expressing the MI of multiple frequency pairs in a pseudo color scale (for further details, see Tort et al., 2008 and Tort et al., 2010).

### 2.7. Statistics

Data are expressed as means and standard error of the mean (S.E.M.). For group comparisons of normally distributed data (Kolmogorov–Smirnov test), we used repeated measures ANOVA and as a post test Tukey’s Multiple Comparison Test. For data with non-Gaussian distribution, we used the nonparametric Friedman test and as a post test Dunn’s Multiple Comparison Test. Different significance levels are shown in figures with one to three asterisks (*: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$).

### 3. Results

Effects of diazepam (DZ) on vigilance and EEG activity were analyzed by injecting single doses of the drug or vehicle, respectively, in 10 freely behaving male mice. DZ was tested at 1, 2 and 4 mg/kg of body weight.

#### 3.1. Diazepam effects on sleep-wake cycle

At all concentrations, DZ significantly shortened the latency to the first NREM episode (Fig. 1A). In contrast, REM latency was largely unaffected (Fig. 1B). Consistent with a sedating action of DZ, all three doses strongly suppressed waking time (Fig. 1C) and significantly prolonged NREM sleep (Fig. 1D). REM sleep was also significantly prolonged by all three doses of DZ (Fig. 1E). In contrast to total REM duration, the fraction of phasic REM (see Methods) was strongly suppressed by DZ from $2.4 \pm 0.1\%$ to $1.3 \pm 0.1\%$ (Fig. 1F). Interestingly, most effects of DZ were not dose-dependent (see Fig. 1A–E), indicating that 1 mg/kg of body weight is already a saturating dose for its effects on vigilance. However, this ceiling effect was not apparent for the fraction of phasic REM within REM sleep, which was linearly related to DZ dose (Fig. 1F).

#### 3.2. EEG power spectra and cross-frequency coupling under baseline conditions

Neocortical field potentials (EEG) are strongly state-dependent. In our recordings, five different states were detected (see Methods):
Fig. 2. Power spectral content (A) and theta phase coupling of fast oscillations (B) vary strongly across the sleep-wake cycle. Five different states are shown: active waking (aWk, black solid lines and black filled bars), quiet waking (qWk, black dotted lines, black open bars), Non REM sleep (NREM, blue solid lines, blue filled bars), tonic REM sleep (toREM, red solid lines, red filled bars) and phase REM sleep (phREM, red dotted line, red open bars). (A) The upper row shows mean power spectra for theta, gamma and fast gamma frequency ranges ($N = 10$). Notice prominent theta, gamma and partly fast gamma power peaks present only in the three “theta states” (aWk, toREM and phREM) but not in the two “non-theta states” (qWk, NREM). The middle row depicts peak frequencies (means ± S.E.M.). The lower row depicts band power values. The “theta states” differ significantly both in frequency and power of theta, gamma and fast gamma from the “non-theta states” (*: significances in reference to aWk; #: in reference to toREM; +: in reference to phREM; §: in reference to qWk). (B) Upper left: Theta phase coupling strength for frequencies between 20 and 200 Hz. Upper middle and right panels: Theta-gamma coupling strength is significantly larger during “theta states” compared to “non-theta states”. Theta-fast gamma coupling is significantly larger during REM sleep states compared to aWk, qWk and NREM. Lower panels: peak frequency values for theta phase (left), gamma (middle) and fast gamma amplitudes (means ± S.E.M.; symbols of significances as in A). Baseline data during aWk, toREM and phREM have been previously published in Branka/et al. (2012).
active wakefulness (aWk), quiet wakefulness (qWk), Non REM (NREM), tonic and phasic REM sleep. Power spectra were computed from 30 s of activity per animal and behavioral state. Prominent peaks in the theta range (4–12 Hz) were present during aWk and both types of REM sleep, while qWk and NREM showed greater power in the delta (1–4 Hz) range (Fig. 2A). Peak frequency of theta power was 9.6 Hz in phasic REM, 8.4 Hz in aWk, and 6.4 Hz in tonic REM. Similar to theta, oscillations in the gamma (40–100 Hz) and fast gamma (120–160 Hz) ranges were largely restricted to aWk and the two REM states, while spectra from NREM sleep and qWk followed a 1/f distribution, with no prominent activity of fast oscillations. Theta band power was larger during phasic REM as compared to tonic REM ($p < 0.0005$) and aWk ($p < 0.05$). Gamma band power was larger during aWk ($p < 0.0005$) and phasic REM ($p < 0.005$) compared to qWk, NREM and tonic REM (Fig. 2A). Clear power peaks were apparent in the fast gamma range (120–160 Hz) during both REM states. Power of fast gamma was larger in phasic and tonic REM than in qWk ($p < 0.0005$) and NREM ($p < 0.005$).

Recent findings indicate the functional significance of temporal relationships between superimposed network oscillations (Tort et al., 2008; Axmacher et al., 2010; Canolty and Knight, 2010). We next estimated cross-frequency coupling strength between slow and fast oscillations across the sleep-wake cycle (Fig. 2B). Again, there were clear differences between vigilance states, with highest theta-to-high frequency coupling in tonic REM ($p < 0.0005$), followed by phasic REM ($p < 0.05$) and aWk (see also Scheffzük et al., 2011 and Branka et al., 2012). As expected, states with low or negligible theta power (NREM, qWk) showed low coupling strength. We focused our further analysis on the three states with prominent peaks in power spectra and cross-frequency coupling (aWk, phasic REM, tonic REM).

### 3.3. Effects of diazepam on theta, gamma and fast gamma oscillations

We next investigated how DZ affects spectral content during different vigilance states. Fig. 3A shows the effects of DZ on power spectra for aWk, tonic REM and phasic REM. Mean and S.E.M. are shown. Significances: *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$ compared to vehicle.

Fig. 3. Diazepam (DZ) alters the spectral content of neocortical EEG. (A) The effect of DZ on power spectral densities in the theta (left column), gamma (middle column) and fast gamma (right column) ranges are shown for the three states with prominent theta oscillations: active waking (aWk), tonic REM (toREM) and phasic REM (phREM). All graphs show means ($\pm$ S.E.M.) of ten mice. Color line code: vehicle – black; 1 mg/kg DZ – red; 2 mg/kg DZ – blue; 4 mg/kg DZ – green. (B) DZ did not change theta power peak width (see Methods) during aWk (left); DZ decreased theta peak width in toREM (middle) and increased it in phREM (right). Means and S.E.M. are shown. Significances: *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$ compared to vehicle.
frequencies, we calculated the mean frequency in two spectral bands (0–20 Hz and 20–160 Hz, respectively). These values were significantly decreased by DZ at all three doses in all three vigilance states (Fig. 5). To assess the effect of DZ on the regularity of theta oscillations, we estimated the width of the theta peak by fitting a Gaussian curve to the PSD (see Methods). Narrower peak widths mean that theta oscillations vary less in instantaneous frequency compared to wider peaks. We found that DZ caused a significant narrowing of the theta peak during tonic REM (Fig. 3B). In contrast, DZ induced a widening of the theta peak in phasic REM, while having no effect on theta peak width during aWk. Therefore, the frequency of theta oscillations becomes more regular with DZ during tonic REM, and more variable during phasic REM. This result suggests that the network mechanisms giving rise to theta oscillations in phasic and tonic REM have different dependence on GABA<sub>A</sub> receptors.

DZ differentially affected oscillatory power depending on vigilance state and frequency band (Fig. 5). To assess the effect of DZ on theta oscillations, we estimated the width of the theta peak by fitting a Gaussian curve to the PSD (see Methods). Narrower peak widths mean that theta oscillations vary less in instantaneous frequency compared to wider peaks. We found that DZ caused a significant narrowing of the theta peak during tonic REM (Fig. 3B). In contrast, DZ induced a widening of the theta peak in phasic REM, while having no effect on theta peak width during aWk. Therefore, the frequency of theta oscillations becomes more regular with DZ during tonic REM, and more variable during phasic REM. This result suggests that the network mechanisms giving rise to theta oscillations in phasic and tonic REM have different dependence on GABA<sub>A</sub> receptors.

3.4. State-specific effects of diazepam on theta phase coupling

As outlined above, fast and slow oscillations were superimposed in a phase-dependent temporal order, with prominent cross-frequency coupling between theta and both gamma bands. Consistent with previous reports (Scheffzik et al., 2011; Brankack et al., 2012)
Benzodiazepine effects on sleep-wake cycle and EEG spectra have been investigated over the last thirty years (Krijzer and van der Molen, 1987; Van Lier et al., 2004). However, recent evidence shows the importance of high-frequency network oscillations (Canolty et al., 2006; Gaona et al., 2011), which remained largely unexplored in previous studies. Here we show that diazepam (DZ) causes an overall slowing of slow and fast field oscillations. Using a series of new analysis tools in combination with a carefully conducted vigilance/behavioral staging, we found that DZ-induced changes in spectral power depend on vigilance state, whereas peak frequencies of all major oscillations are decreased by DZ independently of vigilance state. We also found that the strength of the coupling between the amplitude of fast oscillations and the phase of the theta rhythm was largely unaffected by DZ, in spite of its effects on frequency and power of multiple neuronal oscillations.

4. Discussion

Benzodiazepine effects on sleep-wake cycle and EEG spectra have been investigated over the last thirty years (Krijzer and van der Molen, 1987; Van Lier et al., 2004). However, recent evidence shows the importance of high-frequency network oscillations (Canolty et al., 2006; Gaona et al., 2011), which remained largely unexplored in previous studies. Here we show that diazepam (DZ) causes an overall slowing of slow and fast field oscillations. Using a series of new analysis tools in combination with a carefully conducted vigilance/behavioral staging, we found that DZ-induced changes in spectral power depend on vigilance state, whereas peak frequencies of all major oscillations are decreased by DZ independently of vigilance state. We also found that the strength of the coupling between the amplitude of fast oscillations and the phase of the theta rhythm was largely unaffected by DZ, in spite of its effects on frequency and power of multiple neuronal oscillations.

4.1. Diazepam effects on sleep

We found a significant effect of diazepam on the sleep-wake cycle in mice: NREM sleep latency decreased threefold, REM latency was not affected, the duration of active waking decreased, NREM and REM duration increased, and the percentage of phasic REM on total REM decreased. This picture corresponds well to findings by Radulovacki et al. (1984) who reported similar changes in the distribution of sleep-wake states in rats (although the increase in REM sleep duration was not significant in their study). Our behavioral results are also consistent with two other studies in rats, which however did not analyze REM sleep states separately (Coenen and Van Luijtenaar, 1989; Van Lier et al., 2004). Several studies reported that DZ decreases sleep latency (Stone, 1979; Gottesmann et al., 1998; Kopp et al., 2004b) and increases NREM duration (Stone, 1979; Carley et al., 1998; Maillet et al., 2001; Feng and Gu, 2005). One group also found an increase in REM sleep after DZ (Feng and Gu, 2005). The effects of DZ on phasic REM have been analyzed in three previous studies, all consistently reporting a suppression of phasic REM (Monmaur, 1981; Gandolfo et al., 1994; Gottesmann et al., 1998), which is in agreement with the results of the present study.

Other reports contrast with the results mentioned above and our own findings. Some studies found no significant effects of DZ on any stage of sleep-waking cycle (Monti et al., 1979; Tobler et al., 2001; Siok et al., 2012). Others reported no change of NREM sleep duration (Hashimoto et al., 1992; Renger et al., 2004; Kopp et al., 2003, 2004a), a decrease of REM sleep (Carley et al., 1998; Renger et al., 2004; Kopp et al., 2003, 2004a), or even an increase of the time spent in waking state (Hashimoto et al., 1992). The inconsistency among studies may be caused by the different doses used, ways of application, species and strains investigated, habituation procedures, recording conditions, or sleep stage classification. Of note, Gottesmann et al. (1998) separated REM sleep from a preceding intermediate transition state between NREM and REM sleep, which can only be distinguished from REM sleep by spindle activity in the frontal cortex. They found an increase of this intermediate state by DZ at the expense of REM sleep. Since we did not record from the frontal cortex, we were not able to distinguish this intermediate state from REM sleep. As a consequence, our REM sleep definition includes the intermediate state, which may explain why we observed a clear increase in REM sleep after DZ.

4.2. Diazepam effects on EEG power spectra

The most striking effect of DZ is an overall slowing of EEG frequencies. In accordance with our findings, DZ has been consistently reported to decrease theta frequency during active waking and REM sleep (Monmaur, 1981; Caudarella et al., 1987; Gottesmann et al., 1998; Siok et al., 2012). In addition, we showed that DZ differentially changes theta power and the regularity of
theta frequency (as inferred by peak width) depending on vigilance state. After DZ administration, we found that theta oscillations became more stable (as inferred by a narrower peak width) during tonic REM sleep and less stable (i.e., larger peak width) during phasic REM, whereas DZ did not change theta peak width in active waking. On the other hand, theta band power decreased with DZ treatment only during active waking.

Previous studies consistently found an increase in beta power by DZ that is restricted to active waking (Yamamoto, 1985; Krijzer and van der Molen, 1987; Valerio and Massotti, 1988; Coenen and Van Luijtelaar, 1989; Santucci et al., 1989; Van Lier et al., 2004; Siok et al., 2012). Our results suggest that this increase may result from a slowing of gamma frequencies, which shifts oscillation power into the beta band. We note that a similar interpretation had already been given by Van Lier et al. (2004). The slowing effect of DZ on gamma frequency is consistent with DZ effects in vitro (Whittington et al., 1996, 2000). The underlying cellular and network mechanisms of action of DZ are reviewed in Whittington et al. (2000). Shortly, benzodiazepines positively modulate most molecular subtypes of GABA\textsubscript{A} receptors, resulting in prolonged duration of inhibitory postsynaptic potentials. The duration of GABA\textsubscript{A}-mediated phasic inhibition can, in turn, control the frequency of gamma oscillations (Whittington et al., 2000).

Here we demonstrate that DZ also decreases the frequency of fast gamma oscillations (Fig. 4C). Classical EEG studies hardly investigate frequencies larger than 50 Hz; most are limited to 25 or 30 Hz. In a few studies this upper limit was extended to 100 Hz (Krijzer and van der Molen, 1987; Van Lier et al., 2004). There are
only few reports about DZ effects on network oscillations above 100 Hz (Nishida et al., 2009). Diazepam was shown to affect hippocampal sharp-wave ripple oscillations (140–200 Hz) in vivo by decreasing their occurrence, amplitude, duration and peak frequency (Ponomarenko et al., 2004). Although ripple oscillations have an overlapping frequency range with the fast gamma oscillations (120–160 Hz) investigated here, both oscillation patterns can be distinguished. Most importantly, ripples and fast gamma oscillations occur during different behavioral states (ripples: aWk and NREM sleep; fast gamma: aWk and REM sleep) and in different brain regions (ripples: hippocampal pyramidal layer; fast gamma: stratum oriens-alveus and neocortex; see Scheffer-Teixeira et al., 2012 and Scheffzük et al., 2011). To our best knowledge, the present study is the first to report DZ effects on neocortical fast gamma oscillations.

Similar to the changes in theta power, we found that the effect of DZ on gamma power also depended on vigilance state. Namely, following DZ administration we observed that gamma power increased during active waking, did not change in tonic REM sleep, and decreased in phasic REM. Due to the simultaneous slowing of gamma frequencies, the increase in gamma power in active waking likely corresponds to the beta power increase described earlier (c.f. above). DZ decreased fast gamma power during the two REM sleep states whereas it did not change fast gamma power in active waking. While the cellular and network mechanisms underlying fast gamma oscillations remain to be established, our results suggest a critical role for the GABA<sub>A</sub> receptor in their generation.

**4.3. Diazepam effects on cross-frequency coupling**

To the best of our knowledge, no previous study has investigated the effects of benzodiazepines on cross-frequency interactions. Cross-frequency coupling has been previously suggested to play a role in the execution of several cognitive functions (for a review, see Canolty and Knight, 2010), including some that are impaired by DZ, such as attention and reaction times (Muñoz-Torres et al., 2011). However, here we found that the strength of phase-amplitude coupling was largely unaffected by DZ. The exception was a slight reduction in theta-gamma coupling strength induced by DZ during active waking. This could be explained by the fact that DZ decreases locomotor activity (Stanley et al., 2005) and that theta-gamma coupling correlates with running speed (Chen et al., 2011). In contrast, DZ did not affect the strength of coupling between theta and fast-gamma oscillations during active waking, suggesting that gamma and fast gamma oscillations originate from different mechanisms. DZ also did not affect theta-gamma (nor theta-fast gamma) coupling strength during phasic and tonic REM sleep, which shows that the DZ effect on theta-phase coupling depends on vigilance state. In contrast to preserved coupling strength during REM sleep, however, DZ changed the frequencies in which cross-frequency coupling occurs, mirroring the induced global slowing of oscillatory power. It is currently unclear whether such change in peak coupling frequencies would impact the cognitive functions associated with cross-frequency coupling.

In all, our data suggest that benzodiazepines produce a global slowing of network oscillations, while having different effects on the power and interaction of different frequency bands depending on vigilance state. It remains to be established whether the frequency- and state-specific effects observed at the network level (EEG) are related to the multiple different, though specific, cognitive and behavioral effects of GABA<sub>A</sub> receptor modulators (Korpi et al., 2002; Smith and Rudolph, 2012).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at [http://dx.doi.org/10.1016/j.neuropharm.2012.09.014](http://dx.doi.org/10.1016/j.neuropharm.2012.09.014).

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