Neuropsychopharmacology (2014) 39, 1508–1518

www.neuropsychopharmacology.org

Deleterious Effects of a Low Amount of Ethanol on LTP-Like Plasticity in Human Cortex

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Ingesting ethanol (EtOH) at low doses during social drinking is a common human behavior for its facilitating effects on social interactions. However, low-dose EtOH may have also detrimental effects that so far are underexplored. Here we sought to test the effects of low-dose EtOH on long-term potentiation (LTP)-like plasticity in human motor cortex. Previous cellular experiments showed that low-dose EtOH potentiates extrasynaptic GABAAR and reduces NMDAR-mediated currents, processes that would limit the expression of LTP. Paired associative transcranial magnetic stimulation (PAS_{LTP}) was employed in nine healthy subjects for induction of LTP-like plasticity, indexed by a long-term increase in motor-evoked potential input–output curves. Synaptic α1-GABAAR function was measured by saccadic peak velocity (SPV). Very low doses of EtOH (resulting in blood concentrations of <5 mM) suppressed LTP-like plasticity but did not affect SPV when compared with a placebo condition. In contrast, 1 mg of alprazolam, a classical benzodiazepine, or 10 mg of zolpidem, a non-benzodiazepine hypnotic, decreased SPV but did not significantly affect LTP-like plasticity when compared with placebo. This double dissociation of low-dose EtOH *vs* alprazolam/zolpidem effects is best explained by the putatively high affinity of EtOH but not alprazolam/zolpidem to extrasynaptic GABAARs and to NMDARs. Findings suggest that enhancement of extrasynaptic GABAAR-mediated tonic inhibition and/or reduction of NMDAR-mediated neurotransmission by EtOH blocks LTP-like plasticity in human cortex at very low doses that are easily reached during social drinking. Therefore, low-dose EtOH may jeopardize LTP-dependent processes, such as learning and memory formation.

Neuropsychopharmacology (2014) **39,** 1508–1518; doi:10.1038/npp.2013.350; published online 22 January 2014

Keywords: GABAA receptor; tonic inhibition; human motor cortex; LTP-like plasticity; paired associative stimulation; transcranial magnetic stimulation

INTRODUCTION

Although ingestion of ethanol (EtOH) at low doses during social drinking is a common human behavior for its facilitating effects on social interactions, possible detrimental effects of low-dose EtOH remain underexplored. Cellular studies showed that low-dose EtOH ($\leqslant 30$ mM) produces mainly two effects: potentiation of extrasynaptic gamma-aminobutyric acid type A receptor (GABAAR)-mediated tonic inhibition (Sundstrom-Poromaa *et al*, 2002; Wallner *et al*, 2003; Wei *et al*, 2004) (for review, (Olsen *et al*, 2007)), although this has not been unanimously supported (Borghese *et al*, 2006; Yamashita *et al*, 2006), and *N*-methyl-D-aspartate receptor (NMDAR) inhibition (Lovinger *et al*, 1989, 1990; Weitlauf and Woodward, 2008).

These effects may disrupt long-term potentiation (LTP) of synapses and LTP-dependent processes such as learning and memory formation. In slice preparations of rat motor cortex (M1), LTP induction depends on disinhibition by application of a synaptic GABAAR antagonist and can be disrupted by NMDAR blockade (Aroniadou and Keller, 1995; Castro-Alamancos *et al*, 1995; Fritsch *et al*, 2010; Hess *et al*, 1996). At the systems level of human M1, LTP-like plasticity, indexed by a long-term increase in motor-evoked potential (MEP) amplitude, can be induced by paired associative stimulation (PAS_{LTP}) (Cooke and Bliss, 2006; Müller-Dahlhaus *et al*, 2010; Stefan *et al*, 2002; Stefan *et al*, 2000; Ziemann *et al*, 2004). This LTP-like increase in MEP amplitude shows tight similarities to cellular LTP because it is associative, input specific, and blocked by dextromethorphan, a non-competitive NMDAR antagonist (Stefan *et al*, 2002; Stefan *et al*, 2000; Wolters *et al*, 2003). Furthermore, PAS_{LTP}-induced LTP-like plasticity interacts homeostatically with prior or subsequent motor learning (Elahi *et al*,

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Received 13 October 2013; revised 28 December 2013; accepted 28 December 2013; accepted article preview online 2 January 2014

2013; Jung and Ziemann, 2009; Kang *et al*, 2011; Rosenkranz *et al*, 2007; Stefan *et al*, 2006; Ziemann *et al*, 2004), indicating its mechanistic importance in learning processes.

Here we sought to investigate the effects of two very low doses of EtOH (resulting in blood concentrations of <5 and <20 mM, respectively) on PAS_{LTP}-induced LTP-like plasticity in healthy volunteers, and to contrast them with those of two specific positive modulators of synaptic GABAAR-mediated inhibition (alprazolam, a classical benzodiazepine, and zolpidem, a non-benzodiazepine hypnotic with preferential α1-GABAAR affinity) (Möhler *et al*, 2002). We measured increases in GABAAR-mediated inhibition by slowing of saccadic peak velocity (SPV) (de Visser *et al*, 2003), rather than by other available techniques such as pharmacoelectroencephalography (Valle *et al*, 2002) or pharmacotranscranial magnetic stimulation (Paulus *et al*, 2008) because changes in SPV reflect changes specifically in synaptic α1-GABAAR- rather than α2- and α3-GABAAR-mediated inhibition (de Haas *et al*, 2009; de Haas *et al*, 2010) and, therefore, are expected to capture the sedative effects of alprazolam and zolpidem.

We show that low-dose EtOH abolished the PAS_{LTP}-induced LTP-like plasticity obtained in a placebo session, but had no effect on SPV. In contrast, alprazolam and zolpidem decreased SPV but exerted no significant effect on PAS_{LTP}-induced LTP-like plasticity. This provides strong evidence that LTP-like plasticity in human cortex is highly efficiently blocked by very low doses of EtOH that are easily reached during social drinking. The cellular mechanisms of this detrimental low-dose EtOH effect cannot be disentangled at the systems level but likely relate to the potentiation of tonic inhibition mediated by extrasynaptic GABAARs and/or blockade of glutamatergic neurotransmission through NMDARs. Findings have potentially significant impact at the behavioral level, as acute EtOH ingestion impairs LTP-dependent processes such as learning and memory formation (Lister *et al*, 1991; Lowy, 1970; Mattila *et al*, 1998).

METHODS

Subjects

Written informed consent was obtained prior to participation. The experiments conformed to the *Declaration of Helsinki* and were approved by the ethics committee of the hospital of the Goethe-University of Frankfurt am Main, Germany. All subjects completed the adult safety screen questionnaire (Keel *et al*, 2001). Nine healthy right-handed (Oldfield, 1971) subjects (mean (±SD) age, 26.2 ± 4.3 years; mean (±SD) body length, 180.3 ± 7.6 cm; mean (±SD) body weight, 80.9 ± 15.1 kg; 6 males) were enrolled. None of the subjects had a history of neurological or psychiatric disease or was on CNS-active drugs at the time of the experiments as confirmed by comprehensive urine analysis. None of the subjects ever took alprazolam or zolpidem before, or consumed EtOH regularly. All subjects were non-smokers, as nicotine may alter PAS_{LTP}-induced plasticity (Thirugnanasambandam *et al*, 2011). The participating women used a hormonal contraception to avoid possible menstrual cycle-related alteration of M1 excitability and plasticity (Smith *et al*, 2002). Thirty subjects were screened. In a first screening step, resting motor threshold (RMT) was determined. Only those subjects with RMT $\leqslant$ 50% of maximum stimulator output ($n = 22$) were retained for a second screening step (PAS_{LTP} screening) because RMT $>$ 50% of maximum stimulator output is associated with a low probability for a LTP-like response after PAS_{LTP} (Müller-Dahlhaus *et al*, 2008). After a second screening step, nine subjects were retained and enrolled into this study that exhibited a significant PAS_{LTP}-induced increase in MEP amplitude $\geqslant$ 1.2 (ratio of MEP amplitude post-PAS/pre-PAS) (Heidegger *et al*, 2010; Korchounov and Ziemann, 2011). Therefore, this selection excluded subjects with a long-term depression-like MEP decrease or no MEP change following PAS_{LTP} (Müller-Dahlhaus *et al*, 2008), as the explicit aim of this study was to study drug effects on LTP-like plasticity, a process with significant relation to motor learning (Jung and Ziemann, 2009; Kang *et al*, 2011; Rosenkranz *et al*, 2007; Ziemann *et al*, 2004), rather than exploring drug effects on a great variety of magnitudes and directions of PAS_{LTP}-induced plasticity in the general population.

EMG Recordings

Subjects were seated in a comfortable reclining chair with their arms and hands lying relaxed on the armrests. All transcranial magnetic stimulation (TMS) measurements were obtained by surface electromyography (EMG) from the resting abductor pollicis brevis (APB) muscle of the dominant right hand by using wafer electrodes attached to the muscle belly (active electrode) and the proximal phalanx of the index finger (reference electrode). Reproducibility of exact electrode placement within each visit was assured by marking the electrode spots after baseline measurement with a waterproof marker. The EMG raw signal was amplified and band-pass filtered (20 Hz to 2 kHz; Digitimer D360 8-channel amplifier, Digitimer, Welwyn Garden City, UK), digitized at an A/D rate of 5 kHz per channel (CED Micro 1401; Cambridge Electronic Design, Cambridge, UK) and stored in a laboratory computer for online visual display and later offline analysis using customized data collection and conditional averaging software (Spike 2 for Windows, Version 3.05, CED). All measurements were conducted during complete voluntary muscle relaxation, which was monitored audio-visually by high-gain (50 μV/Div) EMG.

Stimulation Procedures

Focal TMS of the hand area of the left primary motor cortex (M1) was performed with a figure-of-eight coil (diameter of each wing, 70 mm) and a Magstim 200 magnetic stimulator (Magstim Company, Carmarthenshire, Wales, UK) with monophasic current waveform. The optimal coil position over the hand area of the left M1 for eliciting MEPs in the right APB was determined as the site where TMS at a slightly suprathreshold intensity consistently produced the largest MEPs. This site was marked with a soft-tipped pen on the scalp in order to assure a constant placement of the coil throughout the experiment. The coil was held tangential to the scalp with the handle pointing backwards and 45 degrees away from the midline. This orientation induced a lateral-posterior to medial-anterior current in the brain,

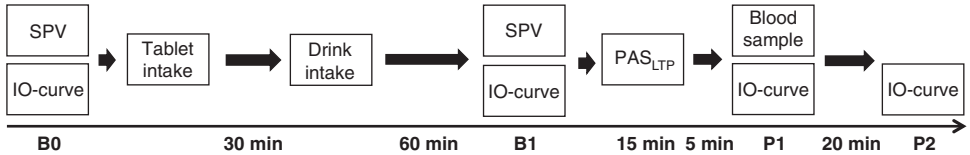

Figure 1 Time line of experimental sessions. Saccadic peak velocity ((SPV), marker of sedation and α1-(gamma-aminobutyric acid type A receptor) GABAAR-mediated inhibition) and motor-evoked potential input–output curves (IO-curves), marker of corticospinal excitability) were measured at baseline (B0). Then the study drug (alprazolam tablet, zolpidem tablet, ethanol low- or high-dose drink, placebo) was administered in a double blind, double dummy design (first tablet, 30 min later drink, see also Table 1). After 60 min waiting (to reach peak plasma concentrations of study drug), SPV and IO-curve were re-tested (B1) to measure the effects of the study drug on these markers. Then, PAS_{LTP} was applied and IO-curve was re-tested 5 min later (P1) and 30 min later (P2) to investigate the long-term potentiation (LTP)-like increase in corticospinal excitability. In addition, a blood sample was taken at P1 to measure the blood concentration of the study drug.

activating the corticospinal system preferentially transsynaptically via horizontal corticocortical connections (Di Lazzaro *et al*, 2008).

RMT was defined to the nearest 1% of maximum stimulator output (MSO) as the lowest stimulus intensity that elicited small MEPs ($\geqslant 50\,\mu$V) in at least five out of ten consecutive trials in the relaxed APB, starting determination from a slightly suprathreshold intensity (Groppa *et al*, 2012).

MEP input–output (IO)-curves were centered on a stimulus intensity that evoked an MEP of 1 mV peak-to-peak amplitude (SI_{1mV}). Eight MEPs each were recorded in randomized order with stimulus intensities of 0.5, 0.7, 0.8, 0.9, 1.0 ($=SI_{1mV}$), 1.1, 1.2, 1.3, and 1.5 times SI_{1mV} (Rosenkranz *et al*, 2007). The peak-to-peak MEP amplitude was analyzed in the single trials and then conditional averages were calculated. MEP IO-curves were determined at baseline (time point B0, before drug intake), at B1 (90 min after tablet and 60 min after drink intake, immediately prior to PAS_{LTP}, time point B1) and 5 and 30 min after PAS_{LTP} (time points P1 and P2) (cf. time line of the experiment in Figure 1). Stimulus intensities were kept constant throughout a given experimental session.

Paired Associative Stimulation (PAS_{LTP})

PAS_{LTP} was performed according to a protocol originally described by (Stefan *et al*, (2000)) and later on slightly modified by our group (Müller *et al*, 2007; Müller-Dahlhaus *et al*, 2008). It consisted of 225 pairs of electrical stimulation of the right median nerve at the wrist followed by TMS delivered at a rate of 0.25 Hz (duration: 15 min). Electrical stimulation was applied through a bipolar electrode (cathode proximal) at an intensity of three times the perceptual threshold. The intensity of TMS was adjusted to SI_{1mV} in the resting APB when given without the preceding median nerve stimulus. To produce a long lasting LTP-like increase in MEP amplitude the interstimulus interval was equal to the individual N20-latency of the median nerve somatosensory-evoked cortical potential plus 2 ms (N20 + 2 ms) (Müller *et al*, 2007). The mean (± SD) interstimulus interval was 21.9 ± 0.6 ms. Attention may have considerable effects on the magnitude of the PAS_{LTP} effect (Kamke *et al*, 2012; Stefan *et al*, 2004). Therefore, to control the level of attention, a randomly flashing light emitting electrode was attached to the back of the stimulated hand. Subjects were instructed to count the flashes and to report the number at the end of the PAS intervention.

Saccadic Peak Velocity Measurements

Visually guided SPV is a biomarker of sedation mediated through the α1-GABAAR (de Haas *et al*, 2009; de Haas *et al*, 2010; de Visser *et al*, 2003). We were interested in obtaining this marker at baseline (time point B0) and after drug intake (time point B1, cf. time line in Figure 1) to estimate the contribution of GABAAergic sedation to drug effects on LTP-like plasticity. Subjects sat in front of a screen (eyes-to-screen distance, 90 cm) and were instructed to make visually guided saccades in response to a white dot subtending an angle of view of 1° on a black screen while the head was maintained in straight position. The dot jumped at randomized intertrial intervals of 2–3 s (to prevent anticipation of the next event) horizontally from one lateral edge to the opposite edge of the screen, subtending an angle of view of 40°. Per time point 90 s were recorded, resulting on average in 36 trials. Saccade recordings were obtained by electronystagmography using surface wafer electrodes placed at the outer canthus of each eye. The electronystagmography raw signals were amplified and band-pass filtered (20 Hz to 2 kHz; Digitimer D360), digitized at an A/D rate of 5 kHz per channel (CED Micro 1401) and stored in a laboratory computer for online visual display and later offline analysis using customized data collection and conditional averaging software (Spike 2 for Windows, Version 3.05). The raw data were exported into MATLAB (version 6.1; Natick, MA). Software written in-house was used for manually setting markers of saccade onsets and offsets. SPV (in °/s) was determined by automatic identification of the maximum value between a pair of markers of saccade onset and offset using a third-order polynomial fit of the raw signal (Velazquez-Perez *et al*, 2004). Conditional SPV averages were calculated for left- and rightward saccades and a grand mean was finally calculated for each individual, session, and time point.

Pharmacokinetic Measurements

Pharmacokinetic measurements were performed in the Institute for Forensic Toxicology, Goethe-University Frankfurt/Main using well-established chromatographic-mass spectrometric screening and target compound analyses. At each visit, a urine sample before drug intake was screened

for medical drugs and drugs of abuse to ensure that subjects were free of the study drugs or any other psychoactive substance at baseline. Blood samples were taken at time point P1 to measure the plasma levels of the study medications.

Experimental Design

We performed a randomized double blind, double dummy, placebo (PBO)-controlled five-period crossover study (Table 1). Study visits always started at 8:00 a.m. to exclude diurnal variability of PAS_{LTP} effects (Sale *et al*, 2007), and were separated by at least 1 week to prevent carry-over effects between sessions. Subjects were in a fastening state and received during each visit one tablet immediately after the MEP IO-curve and SPV measurements at time point B0 and one drink 30 min later (cf. Table 1 and time line in Figure 1). Study medications were: alprazolam (APZ, 1 mg), zolpidem (ZLP, 10 mg), 96% ethanol in two different dosages (resulting in blood concentrations of <5 mM and <20 mM, respectively, and hence termed $EtOH_{<5mM}$, and $EtOH_{<20mM}$), and PBO tablet and PBO drink (mixture of orange juice and bitter syrup to imitate ethanol content). $EtOH_{<5mM}$ and $EtOH_{<20mM}$ doses were individually calculated according to the Widmark formula to reach a peak blood ethanol concentration of 0.35‰ and 0.65‰, respectively. Male subjects received ethanol doses of 0.29 and 0.55 g/kg, and female subjects 0.25 and 0.47 g/kg in the $EtOH_{<5mM}$ and $EtOH_{<20mM}$ conditions, respectively. Due to the fast metabolism of alcohol, we expected plasma levels of $\sim$0.2‰ ($\approx$4.6 mM) and 0.5‰ ($\approx$11.6 mM) at the time of determination (time point P1, Figure 1). One blood sample in the $EtOH_{<5mM}$ condition could not be retrieved for analysis. Dosages of APZ and ZLP were selected according to previously shown sedative effects indexed by significant slowing of SPV (Blom *et al*, 1990; de Haas *et al*, 2010), while selection of dosages of $EtOH_{<5mM}$ and $EtOH_{<20mM}$ was based on their specific action on recombinant $\alpha4\beta3\delta$- and $\alpha6\beta3\delta$-GABAARs (Wallner *et al*, 2003). Timing of B1 and PAS_{LTP} after tablet and drink intake (cf. Figure 1) was planned according to the expected times of peak plasma concentration of APZ, ZLP, and EtOH in healthy young adults (de Haas *et al*, 2010; Greenblatt and Wright, 1993; Welling *et al*, 1977).

Table 1 Summary of Conditions in the Randomized, Double Blind, Double Dummy (PBO Controlled Tablet and Drink) Experimental Crossover Design

Condition	Tablet	Drink
1	APZ (1 mg)	PBO
2	ZLP (10 mg)	PBO
3	PBO	$EtOH_{<5mM}$
4	PBO	$EtOH_{<20mM}$
5	PBO	PBO

Abbreviations: APZ, alprazolam; $EtOH_{<5mM}$ and $EtOH_{<20mM}$, ethanol resulting in blood concentrations of <5 mM and <20 mM, respectively; PBO, placebo; ZLP, zolpidem.

Statistics

Statistical testing was performed with IBM SPSS Statistics (Version 20.0.0). Drug effects on SPV were analyzed with a mixed repeated measures analysis of variance (rmANOVA) with DRUG as between-subject effect (5 levels: PBO, APZ, ZLP, $EtOH_{<5mM}$, $EtOH_{<20mM}$) and TIME (2 levels: B0, B1) as within-subject effect. *Post hoc* rmANOVAs compared the effects of each drug (APZ, ZLP, $EtOH_{<5mM}$, $EtOH_{<20mM}$) pairwise with the effects of PBO on SPV with DRUG as between-subject effect (2 levels: drug *vs* PBO) and TIME (2 levels: B0, B1) as within-subject effect. *Post hoc* two-tailed *t*-tests were performed in case of significant DRUG * TIME interactions.

The MEP data were not normally distributed according to Wilk–Shapiro testing. Therefore, a logarithmic transformation was applied to obtain a normal distribution of the MEP data (Bland and Altman, 1996). All statistical tests were performed on these transformed MEP data. The effects of DRUG on MEP IO-curve were tested in a mixed rmANOVA with DRUG as between-subject effect (5 levels: PBO, APZ, ZLP, $EtOH_{<5mM}$, $EtOH_{<20mM}$) and TIME (2 levels: B0, B1) and Stimulus Intensity (SI, 9 levels: $0.5\times SI_{1mV}$, $0.7\times SI_{1mV}$, $0.8\times SI_{1mV}$, $0.9\times SI_{1mV}$, $1.0\times SI_{1mV}$, $1.1\times SI_{1mV}$, $1.2\times SI_{1mV}$, $1.3\times SI_{1mV}$, $1.5\times SI_{1mV}$) as within-subject effects. Similarly, the effects of DRUG on PAS_{LTP}-induced changes in MEP IO-curve were tested by a mixed rmANOVA with DRUG (5 levels) as between-subject effect, and TIME (3 levels: B1, P1, P2) and SI (9 levels) as within-subject effects. *Post hoc* rmANOVAs compared the effects of each drug (APZ, ZLP, $EtOH_{<5mM}$, $EtOH_{<20mM}$) pairwise with the effects of PBO on PAS_{LTP}-induced changes in MEP IO-curve.

In order to obtain a single measure of the drug and PAS_{LTP}-induced changes in the MEP IO-curves, the area under logarithmically transformed MEP IO-curves (AUIOC) was calculated for each subject, time point, and experimental session. The AUIOC is a highly reliable and valid measure to characterize the excitability state of the corticospinal projection to hand muscles (Carson *et al*, 2013). To correlate drug blood concentrations (determined at time point P1, cf. Figure 1) and changes in SPV with drug effects on the PAS_{LTP}-induced changes in AUIOC, the following index was calculated: $(AUIOC(P1)\text{-}AUIOC(B1))_{DRUG}$—$(AUIOC(P1)\text{-}AUIOC(B1))_{PBO}$. This way, the PAS_{LTP}-induced change in AUIOC in any of the DRUG conditions is related to the PAS_{LTP}-induced AUIOC increase in the PBO condition. This index is referred to as normalized ΔAUIOC in this paper. Correlation analyses of the normalized ΔAUIOC with drug blood concentrations and changes in SPV (difference between time points B1–B0) were performed by linear regression.

For all rmANOVAs, Mauchly's test was applied to test for sphericity and in case of violation of sphericity, the degrees of freedom were corrected by the Greenhouse–Geisser test. Significance was assumed when $p<0.05$. All data are reported as means ± SEM, unless stated otherwise.

RESULTS

Experimental procedures and study drugs were generally well tolerated except for ZLP, which caused nausea and

vomiting in one subject. Other common adverse events were mild to moderate sedation or dizziness, and did not limit full compliance of the subjects with the requirements of this study.

Baseline Excitability Data (RMT, SI_{1mV} at Time Point B0)

ANOVAs did not show differences between DRUG conditions for RMT or SI_{1mV} at time point B0 (all $p>0.75$, Table 2).

Plasma Concentrations of Drugs at Time Point P1

The following plasma concentrations were measured in the respective drug conditions: APZ: 9.61 ± 0.48 ng/ml; ZLP: 98.2 ± 37.1 ng/ml; $EtOH_{<5mM}$: 0.13 ± 0.02‰ (≈3.01 ± 0.46 mM; range: 0.46–4.40 mM); $EtOH_{<20mM}$: 0.55 ± 0.05‰ (≈12.74 ± 1.16 mM; range, 5.79–18.30 mM). The $EtOH_{<5mM}$ *vs* $EtOH_{<20mM}$ concentrations were significantly different (unpaired two-tailed *t*-test, $t_{15}=6.15$, $p<0.001$).

Drug Effects on Saccadic Peak Velocity (Comparison of SPV at Time Points B1 *vs* B0)

In the PBO condition, there was no effect of TIME on SPV (two-tailed paired *t*-test, $t_8=0.26$, $p=0.80$, Figure 2). The mixed rmANOVA for all drug conditions revealed significant effects of TIME ($F_{1,40}=25.62$, $p<0.001$) and the DRUG * TIME interaction ($F_{4,40}=3.08$, $p=0.027$) but not DRUG ($F_{4,40}=1.70$, $p=0.17$) (Figure 2a–d). *Post hoc* pairwise rmANOVAs demonstrated significant DRUG * TIME interactions for APZ *vs* PBO ($F_{1,16}=7.40$, $p=0.015$, Figure 2a) and ZLP *vs* PBO ($F_{1,16}=12.08$, $p=0.003$, Figure 2b), but not for $EtOH_{<5mM}$ *vs* PBO ($F_{1,16}=0.21$, $p=0.65$, Figure 2c) or $EtOH_{<20mM}$ *vs* PBO ($F_{1,16}=3.11$, $p=0.10$, Figure 2d). The significant DRUG * TIME interactions were explained by a reduction in SPV post-drug compared with PBO (all $p<0.05$ in two-tailed unpaired *t*-tests, indicated by asterisks in Figure 2a and b). These data demonstrate that only APZ and ZLP, but not $EtOH_{<5mM}$ or $EtOH_{<20mM}$ resulted in significant SPV reduction, indicative of a sedative effect mediated by positive modulation at the α1-GABAAR by APZ and ZLP, but not by $EtOH_{<5mM}$ or $EtOH_{<20mM}$.

Table 2 Baseline Motor Cortical Excitability in the Five Experimental Drug Conditions

Drug	RMT (%MSO)	$F_{4,40}$	*p*	SI_{1mV} (%MSO)	$F_{4,40}$	*p*
PBO	38.0 ± 1.6			46.7 ± 1.9		
APZ	36.0 ± 1.8			46.7 ± 2.1		
ZLP	35.6 ± 2.3	0.33	0.86	44.5 ± 3.0	0.45	0.77
$EtOH_{<5mM}$	36.7 ± 1.4			45.0 ± 1.8		
$EtOH_{<20mM}$	35.4 ± 2.0			43.1 ± 2.5		

Abbreviations: APZ, alprazolam; $EtOH_{<5mM}$ and $EtOH_{<20mM}$, ethanol resulting in blood concentrations of <5 mM and <20 mM, respectively; MSO, maximum stimulator output; PBO, placebo; RMT, resting motor threshold; SI_{1mV}, stimulus intensity needed to induce a motor-evoked potential of 1 mV in peak-to-peak amplitude, ZLP, zolpidem.

Drug Effects on MEP IO-Curve (Comparison of Time Points B1 *vs* B0)

The rmANOVA revealed no effects of DRUG ($F_{4,40}=0.98$, $p=0.43$), or the DRUG * TIME ($F_{4,40}=0.61$, $p=0.66$) or DRUG * TIME * SI interactions ($F_{14.77,147.68}=1.19$, $p=0.28$) (Figure 3a–d). This is an important nil finding because it indicates that there were no significant drug effects on MEP IO-curve at time point B1 (immediately prior to PAS_{LTP}) that could have potentially confounded interpretation of the PAS_{LTP} data.

Drug Effects on PAS_{LTP}-Induced Changes of MEP IO-Curve (Comparison of Time Points P1 and P2 *vs* B1)

At time point B1 (immediately before PAS_{LTP}, cf. Figure 1), there was no difference in MEP IO-curves between drugs, ie there were no significant effects of DRUG ($F_{4,40}=0.57$, $p=0.69$) or DRUG * SI ($F_{9.48,94.78}=0.66$, $p=0.75$) (Figure 4). This is an important nil finding as there were no differences in corticospinal excitability prior to intervention (PAS_{LTP}) that could have accounted for the differential drug effects (see below) on the PAS_{LTP}-induced changes in MEP IO-curves.

In the PBO condition, the effects of TIME ($F_{2,16}=4.92$, $p=0.022$) and the TIME * SI interaction ($F_{5.42,43.35}=2.91$, $p=0.021$) were significant (Figure 4). *Post hoc* testing

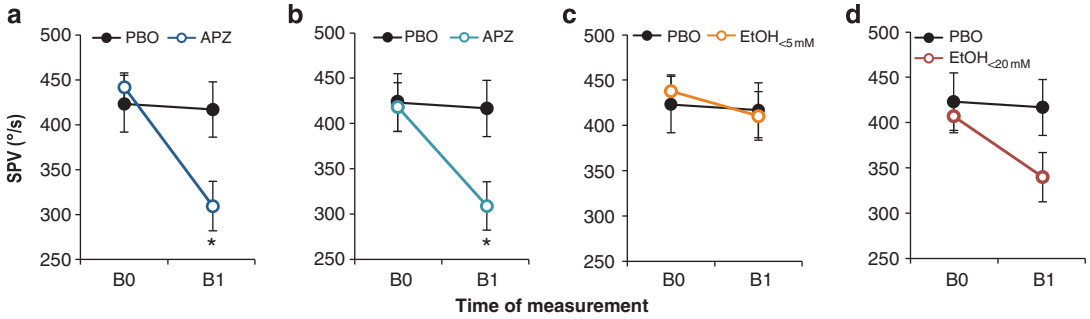

Figure 2 Saccadic peak velocity (SPV, in °/s) at time points B0 (before drug intake) and B1 (after drug intake) in the alprazolam (APZ) (a), zolpidem (ZLP) (b), ethanol-low ($EtOH_{<5mM}$) (c), and ethanol-high ($EtOH_{<20mM}$) (d) condition compared with the placebo (PBO) condition (black circles in a–d). All data are means ($n=9$) ± SEM. Asterisks indicate significant difference to PBO ($p<0.05$).

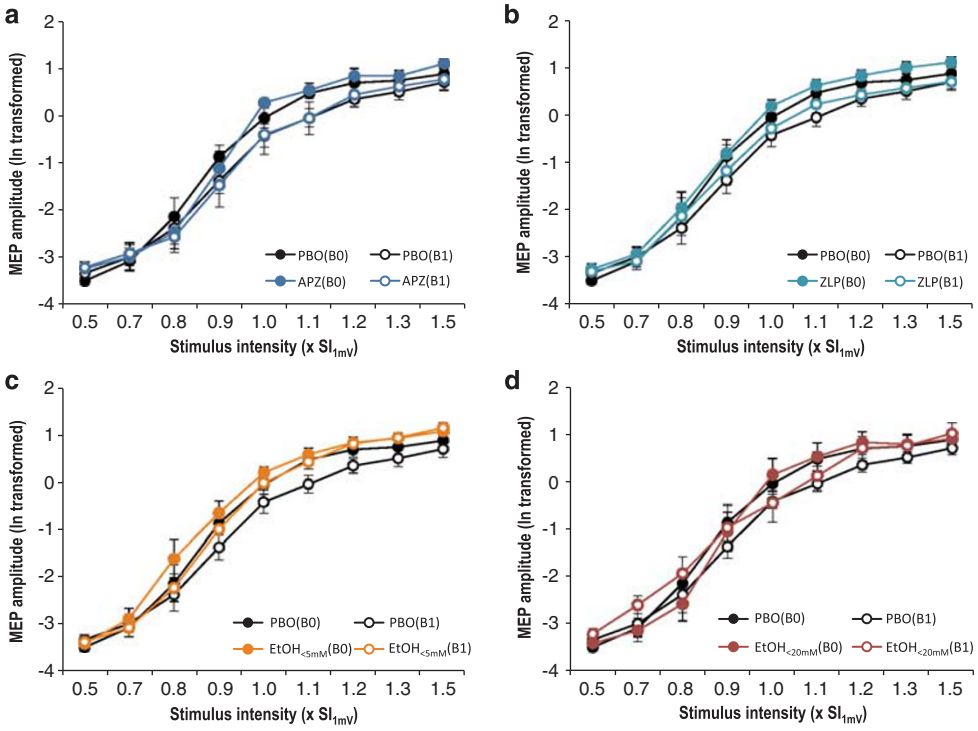

Figure 3 Logarithmically transformed motor-evoked potential (MEP) input–output (IO)-curves at time points B0 (before drug intake, filled symbols) and B1 (after drug intake, open symbols) as a function of stimulus intensity (in multiples of SI_{1mV}) in the alprazolam (APZ) (a), zolpidem (ZLP) (b), ethanol-low ($EtOH_{<5mM}$) (c) and ethanol-high ($EtOH_{<20mM}$) (d) condition compared to the placebo (PBO) condition (black circles and lines in a–d). All data are means ($n = 9$) ± SEM. Note that drugs had no significant effect on MEP IO-curve in comparison to PBO.

revealed that PAS_{LTP} resulted in increased MEP IO-curves at P1 (TIME: $F_{1,8} = 6.70$, $p = 0.032$, TIME * SI interaction: $F_{2.75,22.00} = 4.91$, $p = 0.011$) and P2 (TIME * SI interaction: $F_{2.89,23.11} = 3.83$, $p = 0.024$).

The rmANOVA for all drug conditions revealed a significant DRUG * TIME interaction ($F_{8,80} = 2.20$, $p = 0.036$), while the effects of TIME ($F_{2,80} = 2.29$, $p = 0.06$), DRUG ($F_{4,40} = 1.81$, $p = 0.15$), and the DRUG * TIME * SI interaction ($F_{25.04,250.37} = 0.86$, $p = 0.66$) were not significant. *Post hoc* pairwise rmANOVAs revealed significant DRUG * TIME interactions for $EtOH_{<5mM}$ *vs* PBO ($F_{2,32} = 4.25$, $p = 0.023$, Figure 4c) and $EtOH_{<20mM}$ *vs* PBO ($F_{2,32} = 4.95$, $p = 0.013$, Figure 4d) but not for APZ *vs* PBO ($F_{2,32} = 2.11$, $p = 0.14$, Figure 4a) and ZLP *vs* PBO ($F_{2,32} = 0.59$, $p = 0.56$, Figure 4b).

These effects are explained by abolition of LTP-like effects in the $EtOH_{<5mM}$ and $EtOH_{<20mM}$ conditions but significant, although weak LTP-like effects in the APZ (at time point P2) and ZLP (at time point P1) conditions (see also Figure 5a).

The selection of PAS_{LTP} LTP-responders (see methods) may have biased the drug effects toward suppression of the LTP-like increase in MEP IO-curve obtained in the PBO condition. However, this was unlikely in the present study, as linear regression analyses of $(AUIOC(P1)\text{-}AUIOC(B1))_{PBO}$ *vs* $(AUIOC(P1)\text{-}AUIOC(B1))_{Drug}$ did not reveal negative correlations (all $p > 0.10$). Only the linear regression of $(AUIOC(P1)\text{-}AUIOC(B1))_{PBO}$ *vs* $(AUIOC(P1)\text{-}AUIOC(B1))EtOH_{<5mM}$ revealed a non-significant trend, but toward a positive correlation ($r = 0.54$, $p = 0.13$), ie the strongest PAS_{LTP} LTP-responders in the PBO condition had the weakest suppressive effect in the $EtOH_{<5mM}$ condition. Therefore, preselection of PAS_{LTP} LTP-responders did not set a bias toward drug suppression of LTP-like plasticity in this study.

Relation of Sedation and Drug Levels to PAS_{LTP}-Induced Changes of AUIOC (Comparison of Time Points P1 *vs* B1)

The AUIOC group data are displayed in Figure 5a. Linear regression analyses showed that the blood concentrations of $EtOH_{<5mM}$ correlated negatively with the normalized ΔAUIOC ($EtOH_{<5mM}$: $r = -0.71$, $p = 0.048$, Figure 5b). This means that higher ethanol concentrations were associated with a stronger suppression of the LTP-like plasticity obtained in the PBO condition. In contrast, the blood concentrations of APZ, ZLP and $EtOH_{<20mM}$ did not correlate with the normalized ΔAUIOC (all $p > 0.25$). Finally, changes in SPV as a biomarker of sedation mediated through the α1-GABAAR did not correlate with the normalized ΔAUIOC in any of the drug conditions (all $p > 0.1$).

DISCUSSION

The pharmacological effects of this study showed double dissociation: enhancement of synaptic GABAAR-mediated inhibition by APZ and ZLP resulted in sedation indexed by a decrease of SPV but no significant effect on

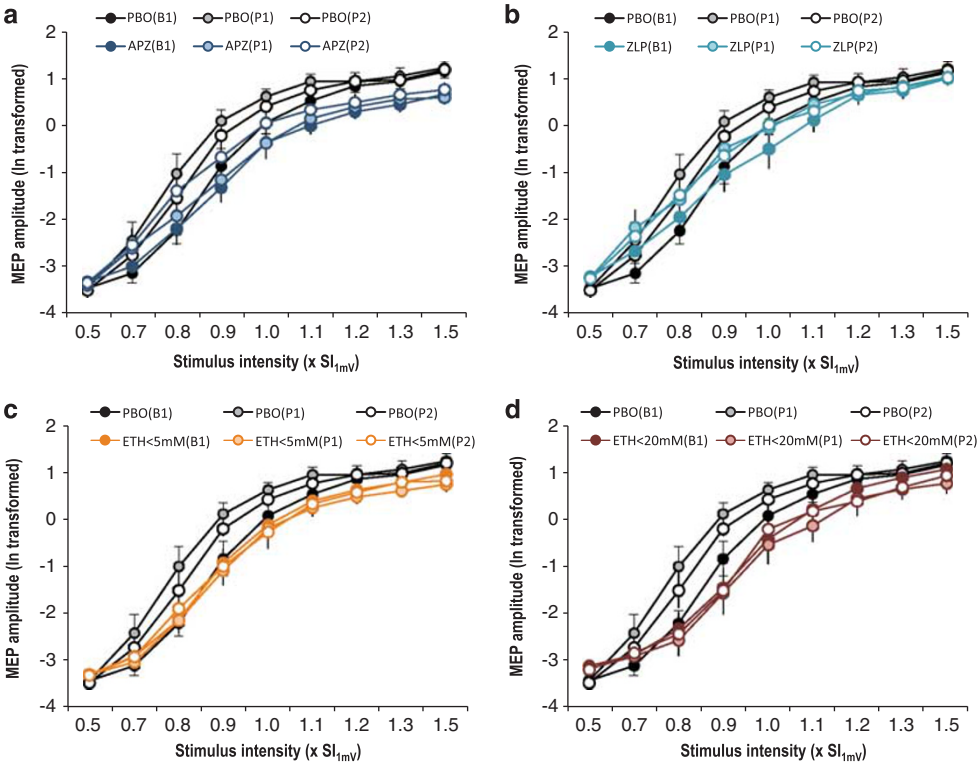

Figure 4 Logarithmically transformed motor-evoked potential (MEP) input–output (IO)-curves at time points B1 (after drug intake and before PAS_{LTP}, dark-filled symbols), P1 (5 min after PAS_{LTP}, light-filled symbols) and P2 (30 min after PAS_{LTP}, open symbols) as a function of stimulus intensity (in multiples of SI_{1mV}) in the alprazolam (APZ) (a), zolpidem (ZLP) (b), ethanol-low ($EtOH_{<5mM}$) (c) and ethanol-high ($EtOH_{<20mM}$) (d) condition compared with the placebo (PBO) condition (black circles and lines in a–d). All data are means ($n = 9$) ± SEM. Note that the significant increase in MEP IO-curves after PAS_{LTP} in the PBO session was significantly suppressed in the $EtOH_{<5mM}$ and $EtOH_{<20mM}$ sessions.

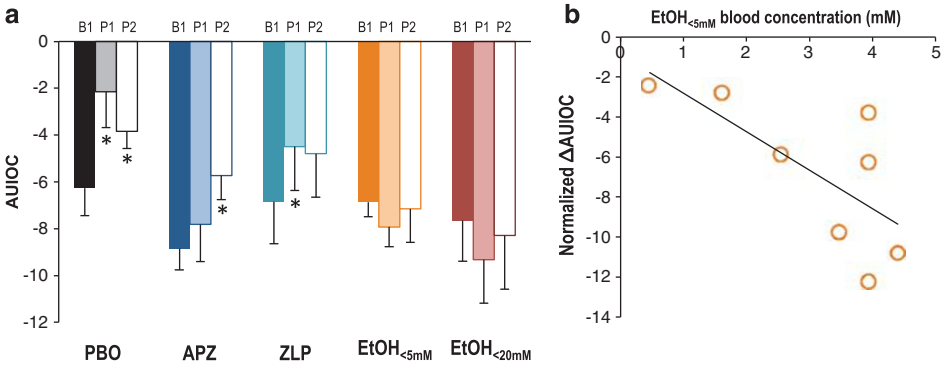

Figure 5 (a). Areas under the logarithmically transformed motor-evoked potential (MEP) input–output curves (AUIOCs) are shown for time points B1 (before PAS_{LTP}), P1 (5 min after PAS_{LTP}) and P2 (30 min after PAS_{LTP}) for the five different drug conditions. The significant PAS_{LTP}-induced increase in AUIOC in the placebo (PBO), alprazolam (APZ) and zolpidem (ZLP) conditions (one-tailed paired *t*-tests, $^*p < 0.05$) was suppressed in the ethanol-low ($EtOH_{<5mM}$) and ethanol-high ($EtOH_{<20mM}$) conditions. (b). The normalized ΔAUIOC in the $EtOH_{<5mM}$ condition correlated negatively with $EtOH_{<5mM}$ blood concentration ($r = -0.71$, $p < 0.05$), ie, higher EtOH concentrations were associated with a stronger suppression of the LTP-like plasticity obtained in the PBO condition.

PAS_{LTP}-induced LTP-like plasticity when compared with the PBO condition. Conversely, rather low doses of EtOH led to abolition of LTP-like plasticity but not to sedation, ie SPV remained unchanged. We argue that these findings suggest detrimental effects of low doses of EtOH on mechanisms of learning and memory and support the importance of extrasynaptic GABAAR-mediated tonic inhibition and/or glutamatergic neurotransmission through NMDARs in regulating plasticity in neuronal networks of human cortex.

Drug Effects on Saccadic Peak Velocity (SPV)

SPV is an established biomarker of sedation mediated through the α1-GABAAR (de Visser *et al*, 2003). Accordingly, classical

benzodiazepines and also ZLP decrease SPV in a sigmoid dose-dependent manner (de Haas *et al*, 2009; de Haas *et al*, 2010) while a specific agonist at the α2- and α3-GABAAR did not induce sedation or a decrease in SPV (de Haas *et al*, 2009). This is consistent with a wealth of data in support of the notion that the different synaptic GABAAR subtypes mediate different functions: α1-GABAAR activation results in sedative and anticonvulsant effects, α2-GABAAR activation leads to anxiolytic and muscle relaxant effects, while the biological functions of the α3-GABAAR are as of yet unclear (Möhler, 2007; Möhler *et al*, 2002). EtOH at the low doses tested here had no effect on α1-GABAAR-mediated currents in acutely dissociated cells (Criswell *et al*, 2003) and did not affect SPV in monkeys (Fuster *et al*, 1985) while higher doses $\geqslant 0.6‰$ (≈ 13.9 mM) resulted in SPV slowing (Fransson *et al*, 2010). Therefore, the lack of any SPV slowing in the $EtOH_{<5mM}$ (Figure 2c) condition provides further evidence that EtOH at very low doses has no significant action at the synaptic α1-GABAAR.

Drug Effects on MEP IO-Curve

The nil findings of this study on corticospinal excitability as tested by MEP IO-curve are largely consistent with previous studies. While the effects of APZ and ZLP on MEP IO-curve have not been tested previously, other benzodiazepines such as lorazepam or diazepam had either no effect (Ilic *et al*, 2002; Ziemann *et al*, 1996) or they produced a moderate MEP IO-curve depression (Boroojerdi *et al*, 2001). EtOH at a higher blood concentration (16.5 ± 2.3 mM) than in the present study had no effect (Ziemann *et al*, 1995). Therefore, MEP IO-curve used for testing the PAS_{LTP} effects on corticospinal excitability was not affected *per se* by any of the drugs at the dosages tested in the present experiments.

Drug Effects on PAS_{LTP}-Induced LTP-Like Increase of MEP IO-Curve

EtOH but not APZ or ZLP significantly suppressed the PAS_{LTP}-induced LTP-like increase in MEP IO-curve seen in the PBO condition. This effect was obtained already at the lowest EtOH dose (the $EtOH_{<5mM}$ condition; blood concentration, 3.01 ± 0.46 mM), and in this condition the suppressive effect on LTP-like plasticity correlated with the individual EtOH blood concentration. The non-significant trends toward suppression of LTP-like plasticity by APZ and ZLP are consistent with a similarly non-significant trend toward a suppressive effect of diazepam in one previous study (Heidegger *et al*, 2010). Of note, others have demonstrated in animal studies that diazepam can suppress neocortical LTP, but this may require high dosages $\geqslant 5$ mg/kg (Komaki *et al*, 2007; Trepel and Racine, 2000). Therefore, the present findings should not be interpreted as an all-or-none dissociation of suppression of LTP-like plasticity in human cortex by benzodiazepines *vs* EtOH, but they point to a mechanism particularly sensitive to EtOH. The present findings are not in disagreement with the lack of an effect of acute EtOH exposure on MEP increase during a 5 Hz-train of 10 TMS pulses in one previous study (Conte *et al*, 2008), as this MEP increase is very short-lasting (<1 s), thus reflecting short-term synaptic enhancement rather than LTP (Ziemann *et al*, 2008).

What are the mechanisms through which low-dose EtOH exerted its suppressive effects on LTP-like plasticity in the present study? The δ-subunit containing extrasynaptic α4ß3δ and α6ß3δ GABAARs are uniquely sensitive to EtOH and show significant increases in GABA-related currents at EtOH concentrations of 3 mM or less (Sundstrom-Poromaa *et al*, 2002; Wallner *et al*, 2003). As this was the mean blood concentration in the $EtOH_{<5mM}$ condition, the suppressive effects on LTP-like plasticity could have been mediated through these GABAARs. The α6ß3δ GABAAR is exclusively located on granule cells in the cerebellum (McKernan and Whiting, 1996; Nusser *et al*, 1998), while the α4ß3δ GABAAR is expressed with decreasing abundance in thalamus, the dentate gyrus, the striatum, and the outer layers of neocortex (Pirker *et al*, 2000). We cannot tell with certainty if any of these two receptor subtypes was more likely to mediate the observed EtOH effects on LTP-like plasticity. However, it was recently shown that increasing cerebellar excitability by anodal transcranial direct current stimulation or intermittent theta-burst stimulation abolished PAS-induced LTP-like plasticity (Hamada *et al*, 2012; Popa *et al*, 2013), but this suppressive effect was seen only with PAS_{25ms} (ie with the interval between the electrical stimulus to the median nerve and TMS of the contralateral M1 equaling 25 ms) and not with $PAS_{21.5ms}$ (Hamada *et al*, 2012). The mean PAS interstimulus interval in our study was 21.9 ms. Therefore, it is very likely that we have investigated PAS_{LTP}-induced LTP-like plasticity that is not influenced by processing of sensory afferent information in the cerebellum. This indirectly supports the notion that the effect of low-dose EtOH was mediated by enhancement of tonic inhibition through the α4ß3δ GABAAR. In summary, this would be first indirect evidence that extrasynaptic GABAAR-mediated tonic inhibition has an exquisite role in regulating LTP-like plasticity in human cortex. However, these statements deserve caution, given that the systems level approach of our experiments does not permit direct insights into the cellular mechanisms. Furthermore, the high sensitivity of extrasynaptic GABAARs to low doses of EtOH has not been unanimously replicated (Borghese *et al*, 2006; Yamashita *et al*, 2006).

Of particular note are possible alterations of neurotransmission through the NMDAR by EtOH because the NMDAR is a major target of EtOH (Kumari and Ticku, 2000). EtOH at low concentrations of $\leqslant 25$ mM resulted in NMDAR inhibition in various experimental preparations (Lovinger *et al*, 1989, 1990; Weitlauf and Woodward, 2008), and in significant suppression of LTP (Blitzer *et al*, 1990; Morrisett and Swartzwelder, 1993), although other studies failed to reported significant LTP suppression by low concentrations of EtOH (Pyapali *et al*, 1999; Schummers *et al*, 1997). Taken together, NMDAR inhibition may have contributed to the suppressive effect of low-dose EtOH on LTP-like plasticity in our study, in agreement with previous studies that have demonstrated suppression of LTP-like plasticity by specific pharmacological NMDAR blockade (Stefan *et al*, 2002; Wankerl *et al*, 2010).

Finally, EtOH may exert excitability-depressant actions through a variety of other receptors such as non-NMDA glutamate receptors (Badanich *et al*, 2013; Frye and Fincher,

2000) or G protein-coupled inwardly rectifying potassium channels (Lewohl *et al*, 1999), but these effects were consistently observed only at high, intoxicating EtOH concentrations >50 mM and, therefore, are unlikely to have contributed to our findings.

Together, our findings are consistent with the notion that enhancement of tonic inhibition through the extrasynaptic $\alpha4\beta3\delta$ GABAAR has contributed to the acute suppressive effect of low-dose EtOH on LTP-like plasticity. Of note, others have shown that NMDAR-dependent hippocampal LTP can be attenuated by tonic inhibition through the $\alpha4\beta3\delta$ GABAAR (Shen *et al*, 2010). Tonic inhibition is mediated through extrasynaptic GABAARs that are exquisitely sensitive to low concentrations of ambient GABA (Farrant and Nusser, 2005). Increase of tonic inhibition shifts the input–output relationship of cells to the right, ie the probability of action potential generation to a given excitatory input is decreased (Mitchell and Silver, 2003). The input–output relationship of corticospinal cells in human motor cortex can be shifted to the right or left by cortex polarization through cathodal or anodal transcranial direct current stimulation, respectively (Nitsche and Paulus, 2000). A shift to the right by cathodal stimulation abolished PAS_{LTP}-induced LTP-like plasticity, while a shift to the left by anodal stimulation enhanced it (Nitsche *et al*, 2007). Similarly, cathodal *vs* anodal stimulation of rat hippocampal slices respectively suppressed or enhanced subsequent LTP induction (Ranieri *et al*, 2012).

PAS_{LTP}-induced LTP-like plasticity shares common mechanisms with motor skill learning (Elahi *et al*, 2013; Jung and Ziemann, 2009; Kang *et al*, 2011; Rosenkranz *et al*, 2007; Stefan *et al*, 2006; Ziemann *et al*, 2004) and acute EtOH ingestion has deleterious effects on memory formation and learning (Lister *et al*, 1991; Lowy, 1970; Mattila *et al*, 1998). Therefore, the present findings suggest a negative impact of EtOH on memory formation and learning at doses as low as reached by a single drink, but this will need to be tested in further experiments.

On the other hand, NMDAR-dependent LTP has also been implicated in the induction and maintenance of alcohol addiction (for reviews, (Krystal *et al*, 2003; Ron and Wang, 2009)). Basic experiments suggested that this is caused by facilitation rather than inhibition of LTP in the presence of high concentrations of EtOH (100 mM) and that blockade of this aberrant LTP facilitation can attenuate operant self-administration of EtOH in rats (Wang *et al*, 2007). TMS can be used to investigate and therapeutically interfere with this aberrant plasticity in alcohol addicts (Barr *et al*, 2011; Naim-Feil and Zangen, 2013).

In conclusion, very low, non-sedating doses of EtOH show deleterious effects on LTP-like plasticity at the systems level of human motor cortex, contrasting with non-significant effects on LTP-like plasticity by sedating doses of alprazolam and zolpidem. We argued that one possibility to explain this double dissociation is enhanced tonic inhibition through the extrasynaptic $\alpha4\beta3\delta$ GABAAR by low-dose EtOH. Findings may stimulate more extensive research of the physiological importance of tonic inhibition in regulating excitability and plasticity of cortical neuronal networks, and its potential role in abnormalities of these processes in neurological disorders such as epilepsy (Semyanov *et al*, 2004; Walker and Semyanov, 2008).

FUNDING AND DISCLOSURE

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was funded by the Interdisciplinary Center for Neuroscience Frankfurt (ICNF), an intramural funding program of the Goethe-University Frankfurt. This work was funded by an intramural grant from Goethe-University, Frankfurt. The authors declare that over the past three years UZ has received compensation for professional advice from CorTec GmbH Freiburg, Germany and Biogen Idec GmbH Ismaning, Germany; LK was supported by the grant ZI 542/5-1 from the German Research Foundation (DFG), and after completion of the work reported here LK was employed at EyeSense GmbH Großostheim, Germany. All of these compensations are unrelated to the research presented in this paper. Apart from this, all authors declare that, except for income received from the primary employer, no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional service and that there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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