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Journal Article

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REGULAR RESEARCH ARTICLE

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Abstract

Background: Gamma-hydroxybutyrate (GHB; or sodium oxybate) is an endogenous GHB-/gamma-aminobutyric acid $_B$ receptor agonist. It is approved for application in narcolepsy and has been proposed for the potential treatment of Alzheimer's disease, Parkinson's disease, fibromyalgia, and depression, all of which involve neuro-immunological processes. Tryptophan catabolites (TRYCATs), the cortisol-awakening response (CAR), and brain-derived neurotrophic factor (BDNF) have been suggested as peripheral biomarkers of neuropsychiatric disorders. GHB has been shown to induce a delayed reduction of T helper and natural killer cell counts and alter basal cortisol levels, but GHB's effects on TRYCATs, CAR, and BDNF are unknown.
Methods: Therefore, TRYCAT and BDNF serum levels, as well as CAR and the affective state (Positive and Negative Affect Schedule [PANAS]) were measured in the morning after a single nocturnal dose of GHB (50 mg/kg body weight) in 20 healthy male volunteers in a placebo-controlled, balanced, randomized, double-blind, cross-over design.
Results: In the morning after nocturnal GHB administration, the TRYCATs indolelactic acid, kynurenine, kynurenic acid, 3-hydroxykynurenine, and quinolinic acid; the 3-hydroxykynurenine to kynurenic acid ratio; and the CAR were significantly reduced ($P < 0.05$–0.001, Benjamini-Hochberg corrected). The quinolinic acid to kynurenic acid ratio was reduced by trend. Serotonin, tryptophan, and BDNF levels, as well as PANAS scores in the morning, remained unchanged after a nocturnal GHB challenge.

Significance statement

Gamma-hydroxybutyrate (GHB) is used medically and experimentally in neuropsychiatric disorders, such as narcolepsy, Parkinson's disease, Alzheimer's disease, and depression. These disorders involve neuro-immune and neuro-inflammatory processes, especially of the tryptophan catabolites (TRYCATs) pathway. We found that nocturnal GHB reduces the next morning's metabolite levels of the kynurenine branch of the TRYCAT pathway and the cortisol-awakening response in healthy subjects. This may be a mechanism by which the drug exerts its clinical effects in the above-mentioned disorders.

Conclusions: GHB has post-acute effects on peripheral biomarkers of neuropsychiatric disorders, which might be a model to explain some of its therapeutic effects in disorders involving neuro-immunological pathologies. This study was registered at ClinicalTrials.gov as NCT02342366.

Introduction

Gamma-hydroxybutyrate (GHB, or sodium oxybate) is a short-chain fatty acid that occurs naturally in the mammalian brain (Bessman and Fishbein, 1963). It is a gamma-aminobutyric acid (GABA) metabolite that binds as an agonist, with high affinity to so-called GHB receptors and with a much lower affinity to GABA$_B$ receptors (Benavides et al., 1982). GHB unfolds unique sleep-augmenting properties and, unlike other sleep medications, it has proven efficacy in the treatment of narcolepsy, a debilitating sleep-regulation disorder (Boscolo-Berto et al., 2012). Its potential to treat other neuropsychiatric disorders associated with insomnia is currently being investigated in patients suffering from Parkinson's disease (Büchele et al., 2018), Alzheimer's disease (Klein et al., 2015), fibromyalgia (Spaeth et al., 2012), and depression (Mamelak, 2009; Bosch et al., 2012). Interestingly, insomnia not only represents a cardinal symptom in those disorders, but is also being discussed as a main driver of pathological alterations in systems engaged with neuro-inflammation, stress processing, and neuroplasticity (Maes, 2011; Maurovich-Horvat et al., 2014; Qin et al., 2016; Tsilioni et al., 2016; Decourt et al., 2017). Thus, it has been argued that GHB may beneficially modulate the disease course by restoring the homeostatic functions physiological sleep has on those systems (Mamelak, 2009; Bosch et al., 2012). Interestingly, neuro-inflammation, stress processing, and neuroplasticity have been found to be substantially related to each other, with inflammation being a key trigger of pathological alterations in the stress processing and neuroplasticity. Several neuropsychiatric pathologies are characterized by increased levels of pro-inflammatory cytokines, excitotoxicity, oxidative stress, and impaired mood (Maes et al., 2011). Thereby, an important downstream effect of increased pro-inflammatory cytokine release is the activation of the kynurenine pathway (KYNP). The KYNP has been proposed as a biochemical hub, linking inflammatory processes, excitotoxicity, and impaired mood in neuropsychiatric disorders (Maes et al., 2011). The activation of the KYNP triggers the degradation of tryptophan to partly neurotoxic metabolites, thereby reducing the available amount of serotonin in the brain; serotonin is a major mood-stabilizing neurotransmitter (Maes et al., 2011). The entry point of the KYNP are 2 rate-limiting enzymes: indoleamine 2,3-dioxygenase (IDO) and tryptophan dioxygenase (TDO; Myint and Kim, 2014). IDO is activated by neuro-inflammatory processes: primarily by interferon gamma (IFN- γ), but also by tumor necrosis factor-alpha (TNF-α; Takikawa et al., 1988; Pemberton et al., 1997). However, TDO is activated by psychophysiological stress via cortisol release (Myint and Kim, 2014). Both enzymes metabolize tryptophan to kynurenine, which is metabolized to kynurenic acid. This metabolic pathway constitutes the neuroprotective branch, because kynurenic acid acts as an antagonist at glutamatergic N-methyl-D-aspartate receptors and reduces glutamate-related neuronal excitotoxicity (Klein et al., 2013). There is also a neurotoxic branch, in which kynurenine is metabolized to 3-hydroxykynurenic acid and quinolinic acid. Both of these metabolites are supposed to be neurotoxic, to induce oxidative stress with depressogenic and anxiogenic effects, and to serve as biomarkers for neurodegeneration (Maes et al., 2011).

Moreover, inflammation has been related to enhanced cortisol secretion, via the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Bellavance and Rivest, 2014). The cortisol-awakening response (CAR) represents a reliable method to assess HPA axis activity (Stalder et al., 2016), and has been shown to be increased in depression (Boggero et al., 2017). Intriguingly, increased HPA axis activity not only triggers the KYNP, but also reduces levels of the brain-derived neurotrophic factor (BDNF; Masi and Brovedani, 2011), a neurotrophin that promotes neuroplasticity and has been theorized to substantially contribute to the pathogenesis of the above-mentioned disorders (Lu et al., 2014; Xu et al., 2015; Barbaccia et al., 2004). Indeed, a possible mechanism of action of classical antidepressants may rely partly on the induction of BDNF expression and subsequent increased neuroplasticity (Autry and Monteggia, 2012).

Interestingly, GHB unfolds immunomodulating and neuroprotective properties. In humans, it induces a delayed reduction of T-helper and natural-killer cell levels: both are activators of pro-inflammatory processes, by releasing cytokines such as IFN- γ and TNF-α (Pichini et al., 2010). In animals, GHB was shown to protect neuronal tissue against oxidative stress (Gao et al., 2008). Moreover, GHB appears to have homeostatic regulatory effects on the HPA axis; that is, it increases blunted cortisol levels and reduces enhanced levels (Van Cauter et al., 1997; Nava et al., 2007). Despite its promising effects on neuro-immune processes, to date it is unknown how a nocturnal dose of GHB affects downstream neuroinflammatory processes, such as the KYNP, BDNF expression, and HPA-axis activity, on the next day.

To this end, we investigated the effects of a sleep-inducing dose of GHB (50 mg/kg orally) versus a placebo on the morning serum levels of tryptophan catabolites (TRYCATs), including kynurenic acid, kynurenine, xanthurenic acid, 3-hydroxy-kynurenine,

quinolinic acid, serotonin, and tryptophan, as well as BDNF levels and the CAR, in 20 healthy, male volunteers. Additionally, the Positive and Negative Affect Schedule (PANAS) was applied to assess potential effects on mood. We used a post-acute assessment, because the immunomodulating effects of GHB have been reported to start with a delay of 2–4 hours (Pichini et al., 2010) and because nocturnal dosing, followed by therapeutic effects during daytime, is the standard pattern in the treatment of narcolepsy (Boscolo-Berto et al., 2012). The study followed a placebo-controlled, balanced, randomized, double-blind, cross-over design. Due to the above-mentioned effects of GHB on blood biomarkers of neuropsychiatric disorders, we expected that the drug would reduce KYNP metabolite levels and the CAR, while increasing serotonin, tryptophan, and BDNF levels and enhancing mood.

## Materials and Methods

Permission

The study was approved by the Swissmedic and Cantonal Ethics Committee of Zurich and registered at ClinicalTrials.gov (NCT02342366). All participants provided written informed consent, according to the declaration of Helsinki.

Participants

There were 20 healthy, non-smoking, Caucasian, male students (mean age 25.8 ± 2.45 years) who participated in the study. The following criteria were required for inclusion: male sex, to avoid the potential impact of a menstrual cycle on blood chemical variables; age within the range of 18 to 30 years; the absence of any somatic or psychiatric disorders; no first-degree relatives with a history of psychiatric disorders; non-smoking; and without a history of drug abuse (lifetime use >5 occasions, with the exception of occasional cannabis use). None of the participants reported previous experiences with GHB in their life. Before inclusion into the study, each subject was screened with all-night polysomnography in the sleep laboratory, to exclude sleep-related disorders such as sleep apnea, restless leg syndrome, sleep-onset rapid-eye movement (REM) sleep, and insufficient sleep efficiency (<80%). All participants were recruited via advertisements on the online student job platform of the University of Zurich. All subjects received monetary compensation for the completion of the study.

Study Design

The study followed a placebo-controlled, randomized, balanced, cross-over design. There were 2 experimental nights, separated by a washout phase of 7 days. Each experimental night was preceded by an adaptation night to habituate subjects to the laboratory environment (Figure 1).
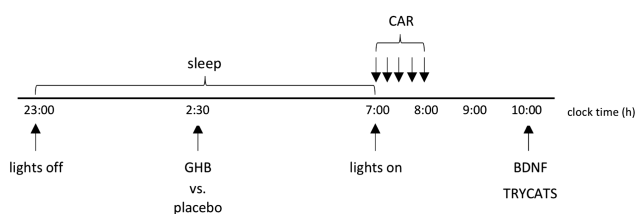
Figure 1. Session design. Abbreviations: BDNF, brain-derived neurotrophic factor; CAR, cortisol-awakening response; GHB, gamma-hydroxybutyrate; TRYCATs, tryptophan catabolites.

Participants were instructed to refrain from illegal drugs for at least 2 weeks and from caffeine for 1 week prior to the first night and until the second experimental night. No alcohol was allowed 24 hours before each experimental night. Participants had to keep a regular sleep-wake rhythm, with a bedtime of 8 hours, from 11:00 p.m. to 07:00 a.m., for 1 week prior to the first experimental night and during the week between the 2 experimental nights. To ensure adherence to these instructions, participants wore an actimeter on their non-dominant arm and kept a sleep-wake diary. All participants received monetary compensation for the completion of the study.

Drug Administration

GHB is currently the first-line, Food and Drug Administration–approved treatment for cataplexy and excessive daytime sleepiness in patients with narcolepsy (Boscolo-Berto et al., 2012). GHB-induced neuro-immunological alterations were previously shown to start at 2–4 hours after ingestion (Pichini et al., 2010). The drug is typically administered twice, in an initial dose at bedtime and a second dose 2.5–4 hours later, to stabilize the severely disturbed sleep continuity in narcolepsy patients (Boscolo-Berto et al., 2012). Therfore, a single, oral dose of GHB was administered 3.5 hours after bedtime. All study participants were awoken at 02:30 am and received 50 mg/kg of GHB, dissolved in 2 dl of orange juice, or a placebo, matched in appearance and taste. Immediately after GHB ingestion, subjects were allowed to return to sleep. The used dose represented the starting dose used for the treatment of narcolepsy (Boscolo-Berto et al., 2012).

Urine Drug Screening

Urine samples were taken upon arrival in order to ensure that all participants abstained from illegal drug use (Drug-Screen Multi 12-AE, Nal von Minden GmbH, Regensburg, DE). Additional urine samples were collected in the morning following an experimental night, to confirm GHB ingestion. Morning urine samples were analyzed with a cloned enzyme donor immunoassay (CEDIA, Indiko Plus, Thermo Fisher Scientific, San Jose, CA) for GHB, with a cut-off concentration of 40 mg/L for GHB.

Blood Collection

Venous blood was sampled from the ante-cubital vein at 10:00 a.m. After a coagulation time of 30 minutes, the blood was centrifuged for 10 minutes at 2000 relative centrifugal force (RCF) to obtain clear serum. The supernatant serum was then distributed to Eppendorf tubes and immediately stored at -80°C.

Tryptophan Catabolites

TRYCATs were analyzed using a ultra–high performance liquid chromatography system (Thermo Fisher, San Jose, CA), coupled to a 5500 linear ion trap quadrupole mass spectrometer (Sciex, Darmstadt/Germany). The method was validated according to the guidelines of the German Society of Toxicology and Forensic Chemistry (Peters et al., 2007). For details, see the Supplementary Material and Figure 2.

Cortisol-Awakening Response

Saliva of each subject was sampled at time points 7:00 (immediately after awakening), 7:15, 7:30, 7:45, and 8:00 a.m. Thereby, subjects were instructed to chew a swab for 60 seconds and then
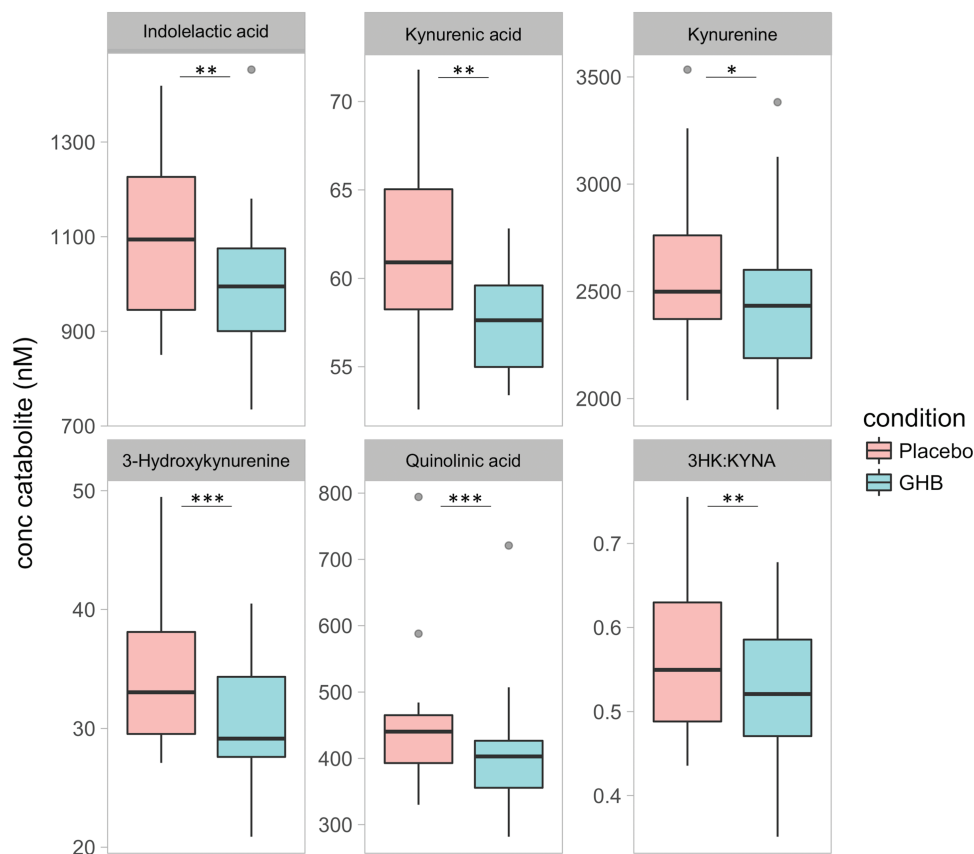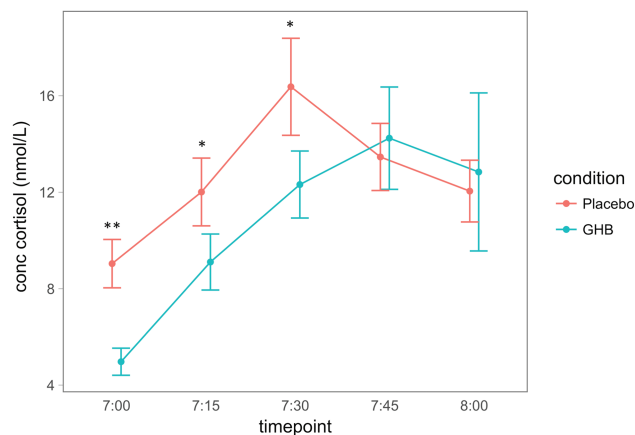
Figure 2. TRYCATs serum concentrations at 3 h post-awakening/7.5 h post–GHB challenge with placebo vs GHB. *P < .05, **P < .01, ***P < .001 (Benjamini-Hochberg correction). Abbreviations: 3HK, hydroxykynurenine; GHB, gamma-hydroxybutyrate; KYNA, kynurenic acid; TRYCATs, tryptophan catabolites.

return it in to the Salivette tube (Sarstedt, Germany). After sampling, tubes were immediately stored on ice until final storage at -80°C. For cortisol detection, tubes were defrosted and centrifuged for 5 minutes at 5000 rotations per minute (rpm) to yield clear saliva in the conical tube. We had to exclude 3 subjects, as the amount of saliva yielded from the swabs was insufficient for further analysis. The swab was removed from the conical tube and the yielded saliva was used for further analysis. A liquid-liquid extraction was carried out by adding 1.5 mL ethyl acetate to 265 μL of the saliva sample and 50 μl internal standard (IS) (Cortison-D_7 0.1 ng/μL). The resulting mixture was subsequently shaken for 10 min at 5 Hz. The samples were centrifuged for 5 min at 9000 rpm and then placed in a freezer (-20° C) for approximately 60 min. The ethyl acetate layer was poured off and dried under nitrogen at 35° C. The dry residue was re-suspended using a solution of 150 μL methanol (MeOH) and 350 μL ammonium formate (5 mM), which was used for liquid chromatography-tandem mass spectrometry analysis, following a recently published method using $^{13}C_3$-labeled cortisol as a surrogate analyte for calibration (Binz et al., 2016). The method was validated according to the guidelines of the German Society of Toxicology and Forensic Chemistry (Peters et al., 2007). The limit of detection for cortisol was 0.55 nmol/L and the limit of quantification was 1.1 nmol/L (Figure 3).

Brain-Derived Neurotrophic Factor

The quantification of serum BDNF levels was conducted at the Department of Clinical Psychology and Psychotherapy at the University of Zurich, using a 96-Well MULTI-ARRAY BDNF Assay

Figure 3. Cortisol-awakening response at 3 h post-awakening/7.5 h post–GHB challenge with placebo vs GHB. *P < .05, **P < .01 (Benjamini-Hochberg correction). Abbreviations: GHB, gamma-hydroxybutyrate.

(Meso-Scale Discovery, Rockville, MD). The analysis was performed according to the manufacturer's instructions (Figure 4).

Positive and Negative Affect Schedule

Each participant's post-awakening mood was assessed at 10 a.m. using the PANAS, a questionnaire in which the participant is asked to rate the occurrence and intensity of 20 mood states (comprised of 10 positive and 10 negative adjectives) in the moment of the rating on a 5-point Likert scale (Watson et al., 1988).

Statistical Analysis

All analyses were conducted using RStudio Version 1.0.136 (RStudio, Inc.). We used 3 independent linear mixed effects (LME) models for TRYCATS, CAR, and BDNF levels. Post hoc testing was carried out using the R package *emmeans* (Version 1.2.1). Individual averages for each log-transformed TRYCAT level, the 3-hydroxykynurenine to kynurenic acid ratio, and the quinolinic acid to kynurenic acid ratio were entered in a LME model, where the possible factors were condition (GHB vs placebo) and metabolite (type of TRYCAT). Furthermore, statistical differences ($P < 0.05$) of each TRYCAT were tested using post hoc *t*-tests and applying the Benjamini-Hochberg correction to adjust for multiple comparisons (Hochberg and Benjamini, 1990). Individual log-transformed cortisol levels were entered in an LME, where the possible factors were condition (GHB vs placebo) and time point (7:00, 7:15, 7:30, 7:45, and 8:00 a.m.). Furthermore, statistical differences ($P < 0.05$) in cortisol levels at each time point were tested using post hoc *t*-tests, applying the Benjamini-Hochberg correction to adjust for multiple comparisons. Individual log-transformed BDNF levels were entered in an LME, with condition (GHB vs placebo) as a possible factor. Furthermore, statistical differences ($P < 0.05$) in BDNF levels were
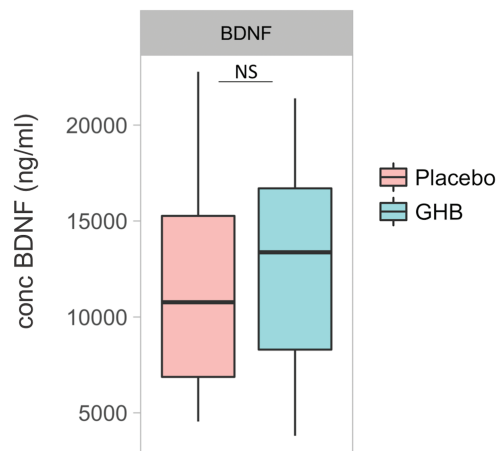
tested using post hoc *t*-tests, applying the Benjamini-Hochberg correction to adjust for multiple comparisons.

For all applied models, normal Q-Q plots were applied, demonstrating normality of the residuals. Moreover, the assumption of homoscedasticity and linearity was verified using a Tukey-Anscombe (residuals vs fitted) plot.

Results

Urine Drug Screening

Drug screening revealed no positive test results for any subjects on either experimental nights Morning urine analyses revealed a positive result exclusively for GHB in the drug condition of all investigated subjects.

Tryptophan Catabolites

A statistical analysis revealed a significant main effect for condition (F[1,475] = 28.81; $P < 0.001$; $\eta^2 = 0.057$). Post hoc tests further revealed reduced levels of indolelactic acid ($P < 0.01$), kynurenine ($P < 0.05$), kynurenic acid ($P < 0.01$), 3-hydroxykynurenine ($P < 0.001$), quinolinic acid ($P < 0.001$), and the 3-hydroxykynurenine to kynurenic acid ratio ($P < 0.01$) in the GHB condition, compared to placebo (corrected for multiple comparisons). Tryptophan and the quinolinic acid to kynurenic acid ratio were reduced on a trend level (both *P* values = 0.085). All other metabolites remained unaffected by the drug. Results of the statistical analysis are summarized in Table 1.

Cortisol-Awakening Response

A statistical analysis revealed a significant main effect for condition (F[1, 125.41] = 4.08; $P < 0.05$; $\eta^2 = 0.029$). Post hoc testing further revealed reduced cortisol levels at time points 7:00, 7:15, and 7:30 a.m. in the GHB condition, compared to placebo (corrected for multiple comparisons). By contrast, cortisol levels at time points 7:45 and 8:00 a.m. remained unaffected by the drug.

Brain-Derived Neurotrophic Factor

Statistical analysis revealed no significantly different BDNF levels between the GHB and the placebo condition (F[1,18.07] = 0.29; $P = 0.59$; $\eta^2 = 0.01$).

Figure 4. BDNF serum levels at 3 h post-awakening/7.5 h post–GHB challenge with placebo vs GHB. Abbreviations: BDNF, brain-derived neurotrophic factor; GHB, gamma-hydroxybutyrate; NS, not signficant.

Table 1. Means and Standard Deviations of Assessed Metabolites

Metabolites	Placebo		GHB		t Value	Cohen's d	P Value
	Mean	SD	Mean	SD			
Indolelactic acid	1100	180	990	160	-3.37	1.18	<0.01**
Serotonin	1000	370	940	370	-1.78	1.54	0.11
Kynurenic acid	61	4.9	58	2.9	-4.28	0.75	<0.01**
Tryptophan	90 000	13 000	85 000	8700	-2.05	0.75	0.085
Kynurenine	2600	390	2400	380	-2.90	0.78	<0.05*
Xanthurenic acid	25	7.9	36	49	0.97	-1.44	0.37
3-Hydroxykynurenine	35	6.7	30	5.4	-6.00	1.53	<0.001***
Hydroxyindoleacetic acid	39	8.2	38	5.1	-0.86	0.65	0.40
Quinolinic acid	450	100	410	97	-5.22	1.35	<0.001***
3HK:KYNA ratio	0.57	0.09	0.53	0.09	-3.33	0.77	<0.01**
QUIN:KYNA ratio	7.4	1.6	7.0	1.7	-2.00	0.60	0.085

Data are for the placebo and GHB conditions (*n* = 20), and are shown as tryptophan catabolite levels (nM) and ratios. *P < .05, **P < .01, ***P < .001 (Benjamini-Hochberg correction).Abbreviations: 3HK, 3-hydroxykynurenine; GHB, gamma-hydroxybutyrate; KYNA, kynurenic acid; QUIN, quinolinic acid; SD, standard deviation.

Positive and Negative Affect Schedule

A statistical analysis revealed no significant difference in the PANAS ratings between the GHB and the placebo condition ($F[1,56.99] = 0.02$; $P = 0.87$; $\eta^2 = 0.0004$).

Discussion

Here, we investigated the effects of the mixed GHB-/GABA$_B$ receptor agonist GHB on peripheral biomarkers of neuropsychiatric disorders, assessing serum levels of TRYCATs and BDNF, the CAR from saliva, and subjective mood effects with the PANAS. We found that in healthy, male subjects, a nocturnal dose of 50 mg/kg p.o. GHB reduces peripheral levels of the KYNP metabolites indolelactic acid, kynurenic acid, kynurenine, 3-hydroxykynurenine, and quinolinic acid 7.5 h after GHB intake, while BDNF, serotonin, and tryptophan levels, as well as PANAS scores, remained unchanged. Moreover, GHB reduced the CAR 4.5 h after drug intake.

Sleep disturbances represent a cardinal symptom in several neuropsychiatric disorders (Khurshid, 2018) and may represent a main driver of pathological alterations in systems engaged with neuro-inflammation, stress processing, and neuroplasticity (Krysta et al., 2017; Irwin and Piber, 2018; Irwin and Vitiello, 2019). GHB is an internationally established treatment of narcolepsy, but has also been experimentally used and investigated to treat Parkinson's disease (Büchele et al., 2018) and Alzheimer's disease (Klein et al., 2015), as well as depression (Mamelak, 2009; Bosch et al., 2012). The therapeutic efficiency of GHB in these disorders has been classically attributed to its unique sleep consolidating effects, as evening/nocturnal doses of the drug strongly increase deep sleep and reduce daytime sleepiness and fatigue during the next day (Van Cauter et al., 1997; Büchele et al., 2018; Dornbierer et al., 2019). Given the important relationship between sleep and neuro-immunological functioning, it has been argued that GHB may beneficially modulate neuro-inflammation and its downstream effects by restoring sleep's regenerating functions on the immune system (Gao et al., 2008; Mamelak, 2009; Klein et al., 2015).

An important pro-inflammatory downstream effect of increased cytokine release is the activation of the KYNP, which has been proposed as biochemical hub, linking inflammatory processes and impaired mood in neuropsychiatric disorders (Anderson and Maes, 2014; Maes and Anderson, 2016; Ogyu et al., 2018). The metabolization of tryptophan down the KYNP is initiated by the enzymes IDO and TDO, thereby reducing neuronal levels of tryptophan, serotonin, and melatonin. IDO is strongly activated by inflammatory cytokines, such as IFN-γ and TNF-α, and the inhibition of this rate-limiting enzyme prevents the development of inflammation-induced depression- and anxiety-like behaviors (O'Connor et al., 2009; Salazar et al., 2012). Moreover, the products of this process are partly neurotoxic and pro-inflammatory, such as 3-hydroxykynurenine and quinolinic acid (Chiarugi et al., 2001). Intriguingly, in our study, GHB induced an inhibition of the putatively neurotoxic KYNP metabolites 3-hydroxykynurenine and quinolinic acid, but also of kynurenine and the putative neuroprotective kynurenic acid. Intriguingly, this effect outlived the acute drug phase, given the short half-life of GHB (30–50 min) and the late time point of blood sampling (7.5 h). Inhibition of KYNP by GHB may occur on several levels, but most evidence points towards indirect interactions of the drug with the function of the 2 rate-limiting enzymes of tryptophan degradation: IDO and TDO.

First, GHB was found to induce a delayed reduction of T helper and natural killer cell levels in humans (Pichini et al., 2010). These lymphocytes are major cytokine producers, and it is likely that GHB reduces the plasma cytokine load via this mechanism. Second, direct GABA$_B$ receptor stimulation of astrocytes (Gould et al., 2014) and microglia (Kuhn et al., 2004) were likewise found to reduce the release of the pro-inflammatory cytokines TNF-α and interleukin 6, both of which were identified as the most reliable inflammatory biomarkers of major depression (Miller and Raison, 2016). The IDO gene is predominantly activated by IFN-γ, whereas TNF-α synergistically increases the transcriptional stimulation of IDO synthesis in response to IFN-γ by up to 300% (Robinson et al., 2005). Increased progesterone release is a third mechanism via which GHB might indirectly inhibit IDO activity (Bosch et al., 2015). Progesterone attenuates IFN-γ–mediated KYNP metabolite synthesis in human macrophages *in vitro*, leading to decreased levels of quinolinic acid and increased kynurenic acid levels (de Bie et al., 2016). Thus, GHB-induced lymphocyte reduction and astrocyte activation might reduce cytokine levels and, thus, IDO activity, while progesterone release seems to directly inhibit neurotoxic KYNP metabolite formation in macrophages.

The other rate-limiting enzyme of the KYNP is TDO. The reduction of TDO activity was hypothesized to be an antidepressant treatment mechanism (Lapin and Oxenkrug, 1969), as TDO degrades approximately 99% of tryptophan in the periphery (Watanabe et al., 1980). Although TDO activity is generally stable and mainly controlled by the tryptophan level itself, stress-related cortisol release can enhance the TDO degradation of tryptophan (Nakamura et al., 1987). The GHB-induced CAR reduction found in our study indicates a down-regulation of HPA axis activity and may be another mechanism by which the drug inhibits the KYNP. In fact, the enhancement of IDO activity by cytokines and the enhancement of TDO activity by cortisol are hypothesized to be the core mechanisms of KYNP metabolite–mediated degenerative neuropsychiatric disorders (Myint and Kim, 2014), and both seem to be inhibited by GHB in humans.

The 2 putatively neurotoxic products of the KYNP are 3-hydroxykynurenine and quinolinic acid. The serum levels of both molecules were reduced the next morning after GHB ingestion in our subjects. It has been shown that 3-hydroxykynurenine is a free radicals producer, potentially involved in the mediation of oxidative stress and neuronal cell death (Chiarugi et al., 2001). Furthermore, 3-hydroxykynurenine can cause oxidative protein damage by generating superoxide and hydrogen peroxide (Goldstein et al., 2000). Several studies found associations of 3-hydroxykynurenine with neuropsychiatric and neurodegenerative pathologies: memory deficits were associated with the molecule in patients with bipolar disorder (Platzer et al., 2017) and major depressive disorder (Young et al., 2016). A study with 20 patients suffering from Alzheimer's disease revealed elevated 3-hydroxykynurenine plasma levels, compared to controls (Schwarz et al., 2013). Another study with 48 patients with Parkinson's disease found an increase of 3-hydroxykynurenine by 30% in a metabolomics analysis of cerebrospinal fluid (Lewitt et al., 2013).

The other potentially neurotoxic KYNP metabolite, quinolinic acid, acts as an agonist at NR2A and NR2B subtypes of glutamatergic N-methyl-D-aspartate receptors and, thereby, mediates excitotoxicity (de Carvalho et al., 1996). It is also a free radicals producer, as it induces nitric oxide synthase and excessive nitric oxide–mediated free radical damage (Braidy et al., 2009). As with 3-hydroxykynurenine, elevated quinolinic acid (Guillemin et al., 2005) and kynurenic acid (Jacobs et al., 2019)

levels were associated with Alzheimer's disease, Parkinson's disease (Nemeth et al., 2006), and depression (Ogyu et al., 2018).

The pathophysiological role of indolelactic acid, which was also reduced by GHB in our participants, is still unclear. Some evidence points towards increased indolelactic acid in liver toxicity (Manna et al., 2010).

Not only neurotoxic KYNP metabolites, such as quinolinic acid and 3-hydroxykynurenine, but also the putatively neuroprotective KYNP metabolite kynurenic acid was reduced after GHB administration in our participants. This was most likely due to an inhibition on the IDO and TDO activity, as all 3 molecules are downstream metabolites of these enzymes. However, it seems that this inhibition is stronger on the neurotoxic KYNP branch, as GHB also reduced the 3-hydroxykynurenine to kynurenic acid ratio significantly and the quinolinic acid to kynurenic acid ratio by trend. A comparable effect was already shown after electroconvulsive therapy (Guloksuz et al., 2015) and after antidepressant medication (Kocki et al., 2012) in depression.

The anti-oxidative effects of GHB are well established, as the drug limits the damage caused by reactive oxygen species, lipid peroxides, and other reactive molecules (for a review, see Mamelak, 2007)). However, the reduction of oxidative stress via the inhibition of 3-hydroxykynurenine and/or quinolinic acid has not been documented to date. It was shown that $GABA_B$ agonism reduces excitatory glutamatergic neurotransmission in the brain (Chalifoux and Carter, 2011). So, GHB seems to inhibit a major source of neuroinflammation-induced excitotoxicity and, via $GABA_B$ agonism, is also the target receptor of excitotoxic molecules.

Further evidence for a modulation of human neuroendocrine and neuro-immune pathways by GHB is given by our finding that the drug reduces saliva CAR. The stress hormone cortisol is the end product of the HPA axis, and the CAR reflects the steep increase of cortisol occurring immediately after awakening. Diverse factors, such as psychological functioning, psychiatric disorders, and alterations of the sleep-wake cycle, have an impact on the CAR, but there is considerable heterogeneity in the literature (Elder et al., 2014). However, evidence from meta-analyses point towards negative associations with fatigue, burnout and post-traumatic stress disorder and a positive association with depression and general life stress (Chida and Steptoe, 2009; Boggero et al., 2017). Consequently, GHB-induced CAR reduction may be interpreted in line with potential antidepressant and stress-reducing effects of the drug.

Another disease-relevant biomarker that has been found to be affected by increased inflammation and psychophysiological stress is BDNF (Masi and Brovedani, 2011; Calabrese et al., 2014; Barbaccia et al., 2004). It is a neurotrophin that promotes neuroplasticity and has been discussed to substantially contribute to the pathogenesis of neuropsychiatric disorders (Lu et al., 2014; Xu et al., 2015). Despite the modulatory effects of GHB on the KYNP and CAR observed in this study, we did not find any drug effects on BDNF levels. Several reasons may account for this, such as that changes in BDNF levels rely on the induction/inhibition of the BDNF gene expression, which may take several days or weeks to occur, as is the case for antidepressants (Autry and Monteggia, 2012). Moreover, it is not fully understood how peripheral blood levels of BDNF are related to its concentration in the central nervous system.

Subjective mood was assessed using the PANAS, but no significant post-acute drug effect was found. As we tested the drug in healthy participants, a potential antidepressant effect was not addressed. However, the overall tolerability of GHB was good, and no severe adverse effects occurred.

Our study has limitations. First, it was performed with healthy volunteers, and not in a clinical sample. Therefore, further studies with patients suffering from neuropsychiatric disorders involving neuro-immune pathologies should be performed to test whether GHB induces the same effects on TRYCATs and CAR; therapeutic effects should also be assessed in these subjects. Second, we only applied a single dose of GHB, instead of a continuous treatment, as is usually used in narcolepsy patients. Studies with repeated dosing should evaluate the course of blood levels of TRYCATs and BDNF and the CAR during GHB treatment in healthy participants and patient populations.

In summary, in healthy volunteers, a single nocturnal dose of GHB significantly reduces post-awakening serum levels of several KYNP metabolites, including indolelactic acid, kynurenine, kynurenic acid, 3-hydroxykynurenine, and quinolinic acid, as well as the 3-hydroxykynurenine to kynurenic acid ratio. Moreover, CAR was significantly reduced. These effects are most likely mediated by indirect inhibition of the rate-limiting enzymes of the KYNP, IDO, and TDO. IDO may be inhibited via multiple mechanisms that reduce the serum cytokine load, while TDO seems to be inhibited via the reduction of HPA axis activity. These effects show that GHB modulates neuro-inflammatory and neuro-immune pathways in humans, and suggest that this mechanism may be responsible for its existing and potential therapeutic effects in neuropsychiatric and neurodegenerative disorders, including narcolepsy, depression, Alzheimer's disease, and Parkinson's disease.

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