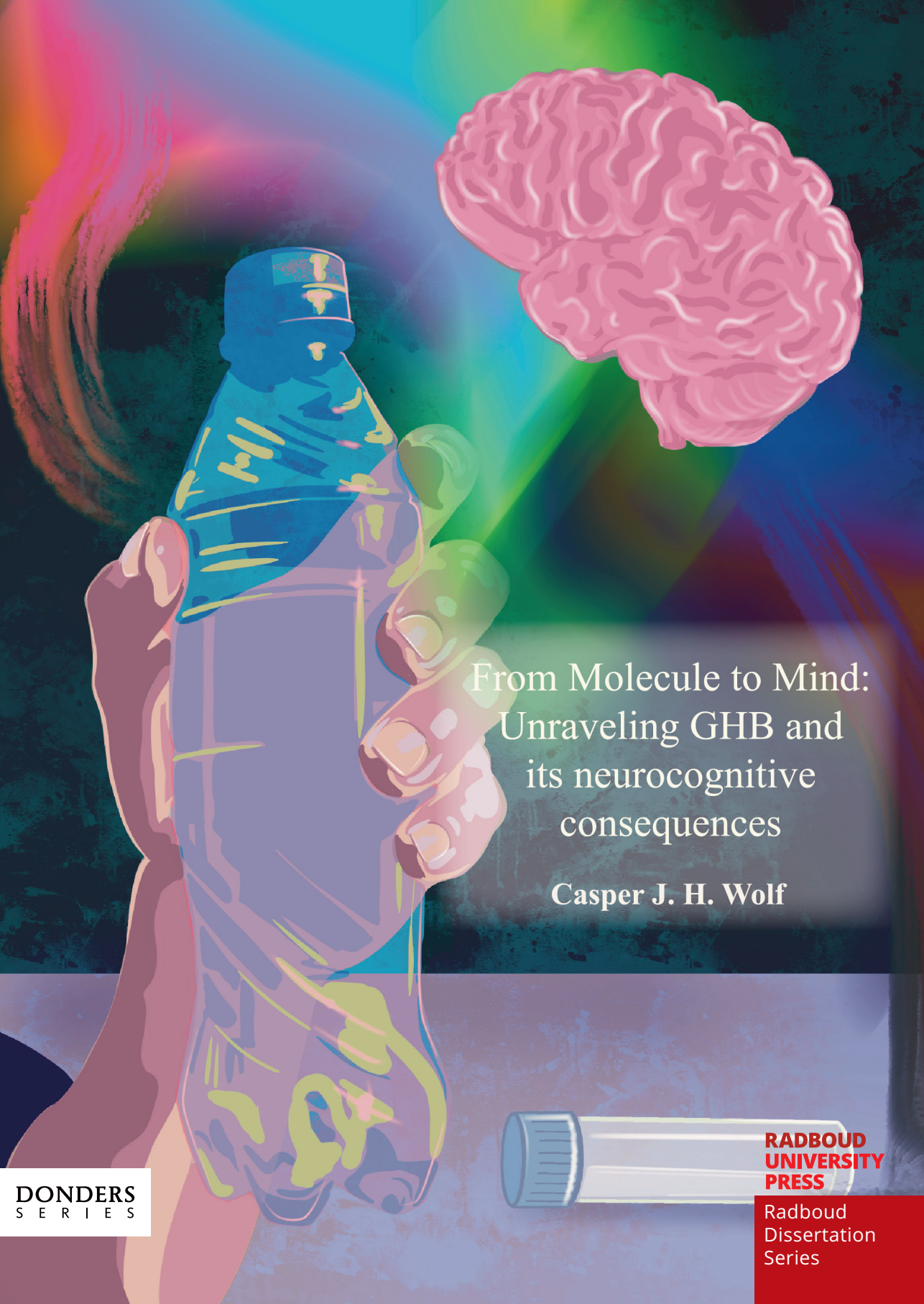

From Molecule to Mind: Unraveling GHB and its neurocognitive consequences

Casper J. H. Wolf

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Radboud Dissertation Series
ISSN: 2950-2772 (Online); 2950-2780 (Print)

Published by RADBOUD UNIVERSITY PRESS
Postbus 9100, 6500 HA Nijmegen, The Netherlands
www.radbouduniversitypress.nl

Design: Proefschrift AIO | Annelies Lips
Cover: A. elegans design
Printing: DPN Rikken/Pumbo

ISBN: 9789465152202
DOI: 10.54195/9789465152202
Free download at: https://doi.org/10.54195/9789465152202

RADBOUD UNIVERSITY PRESS

From Molecule to Mind

Unraveling GHB and its neurocognitive consequences

Proefschrift ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. J. M. Sanders,
volgens besluit van het college voor promoties
in het openbaar te verdedigen op

woensdag 25 maart 2026
om 10:30 uur precies

door

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geboren op 23 januari 1995
te Amersfoort

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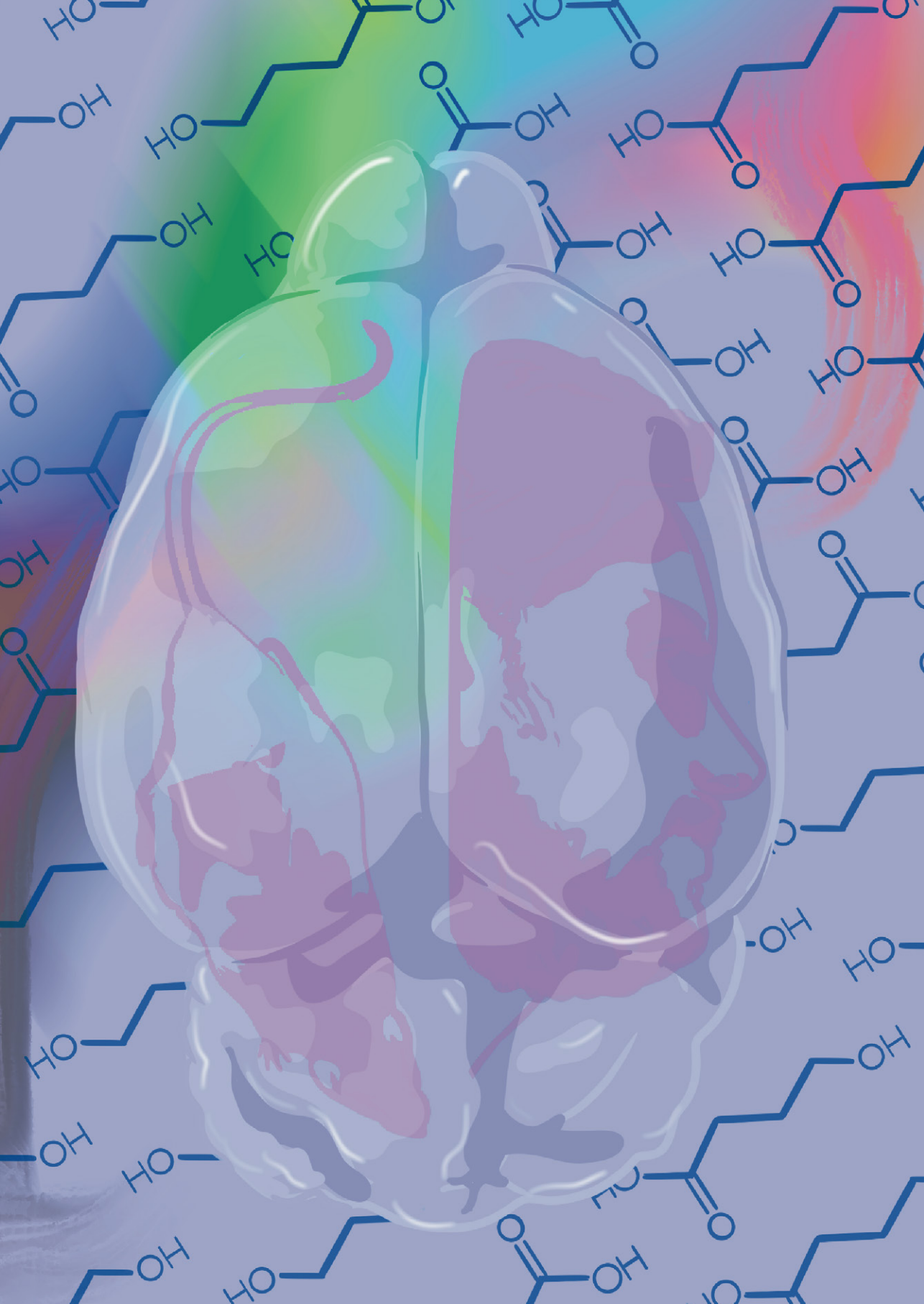


Chapter 1

General introduction

Gamma-hydroxybutyric acid (GHB) is an endogenous precursor and metabolite of the brain's main inhibitory neurotransmitter gamma-aminobutyric acid (GABA) (Laborit, 1964). GHB is used as a prescription drug for the treatment of the sleep disorder narcolepsy (Busardò et al. 2015), but in The Netherlands it is better known as a recreational drug. In The Netherlands, the use of GHB as a recreational drug has slowly gained popularity over the last 30 years, with an estimated 20.000-60.000 (~0.2%) adult GHB users in 2023 (Trimbos-instituut, 2025). Recreational GHB use is also reported in countries as the United States, Australia and several European countries (Kam & Yoong, 1998; EMCDDA, 2018; Karlsson et al., 2021). GHB has dose-dependent euphoric, disinhibiting and sedative effects, and is also used to increase sexual experience (Stein et al., 2011; Bosch et al., 2015).

In the 1960's, butyric acid was initially studied for its role in cell metabolism (Laborit et al., 1960). Due to metabolic processing, butyric acid was rapidly converted into an inactive compound, leaving the study of butyric acid *in vivo* unsatisfactory. To circumvent metabolic processing, an OH group was added to butyric acid in order to enable *in vivo* studies, leading to the synthesis of sodium 4-hydroxybutyrate, or Na-GHB (Laborit, 1964). Laborit published an English review in 1964 (Laborit, 1964), summarizing several French articles reporting low toxicity of GHB, hypnotic properties, and anti-convulsion- and hypothermic effects, all without the occurrence of oxidative processes or ventilatory depression. This review paved the way for research into the therapeutic effects of GHB, eventually leading to the use of GHB as an anaesthetic agent, performance-enhancing drug and later as a treatment for alcohol withdrawal, narcolepsy and idiopathic hypersomnia (Giorgetti et al., 2022).

GHB's euphoric and disinhibiting effects also made it a popular recreational drug. Concurrent with its rising popularity, the addictive potential of GHB became more apparent. Repeated use of GHB can lead to GHB use disorder (GUD) (Beurmanjer et al., 2019), and is a public health concern in several Western countries (Corkery et al., 2015; van Noorden et al., 2017; Arunogiri et al., 2020). Despite the low prevalence of GHB use, GHB is the second drug-related cause underlying emergency service invocation in Europe, only surpassed by opioids (EMCDDA, 2018). The contribution of GHB-related incidents to drug-related emergency care is primarily caused by the relative frequent occurrence of GHB-induced comas and severe GHB withdrawal (Kim et al., 2007; Munir et al., 2008; Krul & Girbes, 2011; Wood et al., 2013; Ogeil et al., 2023).

(Accidental) overdosing on GHB frequently occurs due to the steep dose-response curve of the drug between its euphoric, disinhibiting effects and its sedative effects. GHB overdosing usually results in a temporary coma, and may lead to respiratory

depression or death (Abanades et al., 2007; van Amsterdam et al., 2012). Patients with GUD may also deliberately overdose in order to get some sleep (Beurmanjer et al., 2019). Surprisingly, people undergoing GHB-induced coma usually experience no residual complaints upon awakening, contributing to the popular belief of its users that GHB-induced comas are not harmful (Van Sassenbroeck et al., 2007; Beurmanjer et al., 2019).

Patients with GUD can experience severe withdrawal symptoms upon sudden cessation of GHB use. As with other addictive substances, GUD develops from an initial positive reinforcement phase, when people use GHB for its positive effects, to a dose escalation phase, when GHB use becomes necessary to function in normal life, to the negative reinforcement stage, when GHB is mainly used to prevent negative effects. During the negative reinforcement phase, patients with GUD need to consume GHB every 2-3 hours to prevent withdrawal symptoms (Beurmanjer et al., 2019). GHB withdrawal syndrome without adequate treatment can lead to symptoms such as tremor, tachycardia, anxiety and hallucinations, with over >50% of patients experiencing delirium (McDonough et al., 2004). The severity of the GHB withdrawal syndrome can be strongly reduced with adequate treatment (Beurmanjer et al., 2020), resulting in a safe detoxification and preventing the development of severe withdrawal symptoms.

Besides the addictive properties of GHB, there is growing concern on the possible neurotoxic effects of GHB use (van Amsterdam et al., 2012; Brunt et al., 2016; van Amsterdam et al., 2022). A handful of papers indicate that repeated excessive GHB use may exert negative effects on cognition and may lead to neurotoxicity. While the majority of (animal) studies focus on the acute cognitive effects of GHB under intoxication, it is suggested that long-term excessive GHB use may also lead to prolonged effects on memory performance (van Nieuwenhuijzen, Long, et al., 2010; Beurmanjer et al., 2022). A recent study found an association between the occurrence of GHB-induced comas and altered brain structure and decreased memory performance, indicating possible negative effects specific to these comas (Raposo Pereira et al., 2018). Coincidentally, this research is complemented by a recent surge in literature examining the possible neuroprotective effects of low doses of GHB or GHB analogues, specifically following ischemia or brain trauma (Leurs et al., 2021; Griem-Krey et al., 2023; Gauger et al., 2025).

Outline of this chapter

In this chapter, I will first explain the neuropharmacological effects of GHB in the brain in order to better understand the behavioral effects of the drug. Also, I will zoom in on the indirect neuropharmacological effects of GHB that underly its reinforcing effects, which are responsible for the addictive properties of GHB and the emergence of GUD. Hereafter, I will provide an overview of current literature that examined the neurotoxic and cognitive effects of GHB. Finally, I will address current knowledge gaps and introduce my research that composes the main body of this thesis.

Pharmacokinetics of GHB

GHB is a short-chain fatty acid that can cross the blood–brain barrier (BBB) in a pH-dependent manner. GHB is transported across the BBB via the saturable monocarboxylate transporter MCTs 1-4 (Bhattacharya & Boje, 2004; Felmlee et al., 2021). The metabolism and renal reabsorption of GHB also employ a saturable mechanism, contributing to its nonlinear kinetics in both humans and rats (Lettieri & Fung, 1979; Palatini et al., 1993; Morris et al., 2005). Although the presence of nonlinear kinetics is similar across species, the specific characteristics, such as absorption, distribution, metabolism, and excretion, differ between humans and rats and across administration routes (Felmlee et al., 2021). Absolute plasma concentrations in the rat are ~two-fold higher for intracardial vs intravenous administration, and intravenous administration results in a three-fold higher plasma concentration compared to oral administration (Lettieri & Fung, 1979). GHB typically reaches its maximum plasma concentration within 25-40 minutes (Tmax) following oral ingestion, although Tmax increases with higher doses of GHB due to the saturable absorption mechanism (Lettieri & Fung, 1976; Morse & Morris, 2013). GHB and GHB-binding/-transport structures are also present in numerous peripheral tissues (Nelson et al., 1981; Maitre, Klein, & Mensah-Nyagan, 2016), although this will not be addressed in this thesis.

In addition to being consumed as a drug, GHB is also endogenously synthesized within the body. Endogenous GHB is synthesized from succinic semialdehyde (SSA), a metabolite of GABA. Around 1-2% of GABA is eventually converted to GHB (Gold & Roth, 1977). The exogenous drugs of abuse gamma-butyrolactone (GBL) and 1,4-Butadeniol (1,4-BD) are also converted into GHB following ingestion (Wong et al., 2004). GHB is cleared from the body primarily through metabolism (either directly or following renal reabsorption), with a half-life (T½) of 30-60 minutes (Brenneisen et al., 2004). GHB is metabolized into SSA via GHB dehydrogenase or GHB transhydrogenase, after which

SSA is further metabolized into GABA, or metabolized through mitochondrial pathways (Vayer et al., 1985; Snead et al., 1989). Only a small amount of GHB (2%) is secreted unchanged in the urine (Brenneisen et al., 2004; Jazz, 2020). However, the proportion of unchanged GHB that is excreted in the urine increases after administration of higher doses of GHB, due to the saturated renal reabsorption mechanism (Morse et al., 2012; Morse & Morris, 2013; Vijay et al., 2015). These distinct, non-linear pharmacokinetic properties contribute to the disparity in GHB-related effects found in (pre-)clinical GHB literature, which will be discussed later in this chapter.

Receptor systems of GHB

GHB has a bidirectional effect in the brain, underlying the disperse behavioral effects of the drug. At low doses, it can bind to several high-affinity targets, commonly referred to as the "GHB receptor". At high doses, GHB can bind to the metabotropic $GABA_B$ receptor, leading to an increase of the inhibitory neurotransmitter GABA and leading to reduced glutamatergic signaling (Lingenhoehl et al., 1999; Andriamampandry et al., 2007; Bay et al., 2014).

High-affinity GHB targets (GHB receptor)

Several research groups have aimed to identify the high-affinity target of GHB. Initially, the dominant theory existed that the GHB receptor was a metabotropic receptor, which was hypothesized to exist in both humans (GHBh1) and rats (C12K32) (Benavides et al., 1982; Andriamampandry et al., 2003). These receptors are primarily located post-synaptically within the prefrontal cortex and the hippocampus, and to a lesser extent within the striatum, thalamus and cerebellum (Snead, 2000; Kemmel, Miehe, Roussel, Taleb, Nail-Boucherie, et al., 2006). Activation of high-affinity GHB-receptors by low concentrations of GHB was shown to increase glutamatergic signaling and to indirectly activate glutamatergic (NMDA and to a lesser extent AMPA) receptors (Banerjee & Snead, 1995; Ferraro et al., 2001; Castelli et al., 2003; Li et al., 2007). Although GHB can bind to these receptors, the existence of the GHBh1 and C12K32 receptors have not been verified as a native GHB receptor.

Another high-affinity GHB target is the neuronal kinase CamKIIa (Leurs et al., 2021). CamKIIa is known to be involved in hippocampal long-term potentiation and spatial learning (Zalcman et al., 2018). Additionally, activation of the CamKIIa subunit by a GHB analog has been shown to cause neuroprotective effects following ischemia or other types of neural damage, providing a mechanistic explanation of the neuroprotective properties of GHB, which will be discussed later in this chapter (Leurs et al., 2021).

$GABA_A$

Although it has been established that GHB can indirectly bind to $GABA_A$ receptors through conversion of GHB into GABA, it has been suggested that GHB can bind to specific $GABA_A$ receptors with high affinity in *Xenopus laevis* (Absalom et al., 2012; Bay et al., 2014). The $GABA_A$ receptor is an ionotropic receptor, causing chloride ions (Cl^-) transportation into the cell upon receptor activation, leading to hyperpolarization of the cell membrane. This reduces the action potential frequency, leading to neuronal inhibition, similar to $GABA_B$ receptor activation. Photolinking studies and proteomic analyses identified possible $GABA_A$ targets for GHB. A subsequent screening of various recombinant $GABA_A$ receptors and subsequent voltage clamping and radioligand binding showed that GHB activated α4β1δ receptors with high affinity. In addition, $GABA_A$ with different β subunits (α4β2/3δ) were bound by GHB with slightly lower affinity (Absalom et al., 2012).

A paper by Connelly, Errington, and Crunelli (2013) did not find evidence for such affinity by GHB for δ-subtype containing $GABA_A$ receptors (δ-subunit is only present in combination with α4- and α6-subunits) (Connelly, Errington, et al., 2013). In three types of neuronal cell types, they examined the effects of GHB on synaptic or extrasynaptic $GABA_A$ receptors and found that GHB did not induce $GABA_A$ receptor mediated currents or modulated inhibitory synaptic currents. However, in another paper they did show that GHB affects extrasynaptic δ subunit-containing $GABA_A$ receptors through $GABA_B$ receptors (Connelly, Fyson, et al., 2013). Although an interesting finding, this can also not explain the results found by Absalom et al. (2012). As Connelly, Errington, et al. (2013) suggests, the remaining options are that GHB is not an agonist of native α4β1δ receptors, the receptors are expressed in rare cell populations, or that the receptor is not expressed in the examined brain regions.

$GABA_B$ receptor

There is wide consensus on the inhibitory effect of high doses of GHB through direct activation of the $GABA_B$ receptor in the CNS, or to a lesser extent indirectly through conversion of GHB to GABA (Xie & Smart, 1992; Banerjee & Snead, 1995; Mathivet et al., 1997; Lingenhoehl et al., 1999). The $GABA_B$ receptor is a metabotropic G-protein coupled receptor (GPCR), causing an intracellular signaling cascade upon activation (Luscher & Slesinger, 2010) (Fig. 1). Presynaptic $GABA_B$ receptor activation leads to neuronal inhibition through decreased excitatory neurotransmitter release, or to neuronal disinhibition through auto receptor activation and a subsequent decrease in GABAergic signaling. Postsynaptic $GABA_B$ receptor activation leads to neuronal inhibition through suppression of (spontaneous) excitatory activity by hyperpolarizing the postsynaptic cell (Cruz et al., 2004). Hyperpolarization of the cell

reduces the frequency of action potentials and thereby leading to neuronal inhibition (Madden & Johnson, 1998; Bettler et al., 2004). GHB, in contrast to $GABA_B$ agonist baclofen, primarily affects NMDA receptor-mediated signaling over AMPA receptor-mediated signaling through (dis)inhibitory mechanisms (Li et al., 2007). GHB overdosing, leading to comas, is a consequence of $GABA_B$ receptor activation (Carai, Colombo, Brunetti, Melis, Serra, Vacca, Mastinu, Pistuddi, Solinas, Cignarella, et al., 2001; Follman & Morris, 2022). Considering the role of the $GABA_B$ receptor in the adverse effects of GHB-use and GHB-addiction, the $GABA_B$ receptor is a target of interest for the reduction of sudden withdrawal symptoms and relapse risk (Kamal et al., 2015; Lingford-Hughes et al., 2016; Beurmanjer et al., 2018).

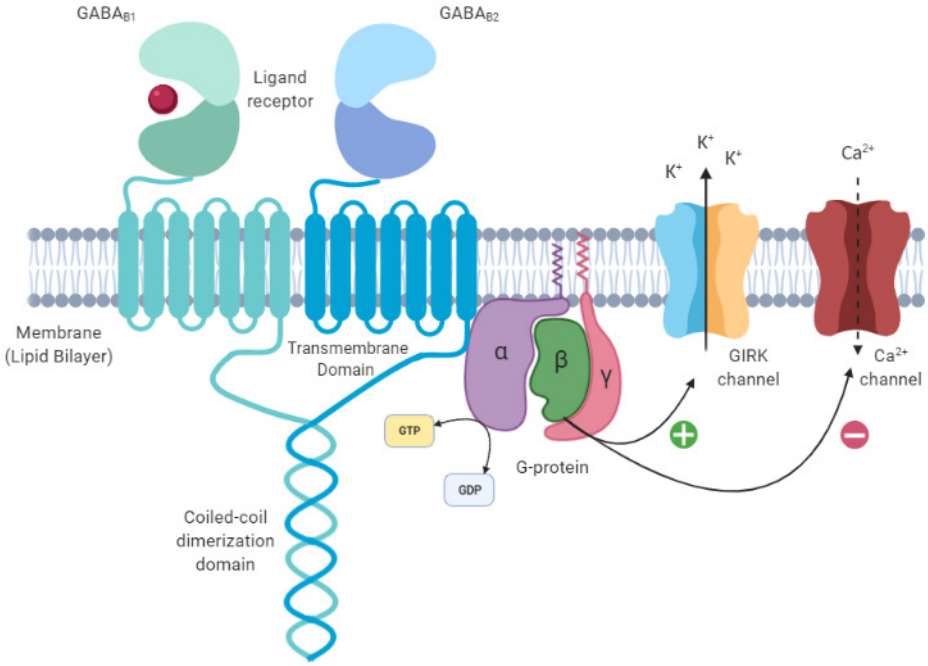


Figure 1. Schematic representation of the $GABA_B$ receptor and associated ion channels. The $GABA_B$ receptor is composed of two extracellular subunits: $GABA_{B1}$ and $GABA_{B2}$. The $GABA_{B1}$ subunit contains the ligand binding site, whereas the transmembrane structure of the $GABA_{B2}$ subunit is coupled to the G-protein. The G-protein, able to bind GDP and GTP, generally consists of an α, β and γ subunit. Upon receptor ligand binding (e.g. GHB), GDP bound to the protein is exchanged for a GTP, allowing for the dissociation of the G-protein into the individual α subunit and the βγ subunit complex/structure. This βγ subunit complex/structure in turn binds to G protein-coupled inwardly-rectifying potassium (K^+) channels (GIRK channels) and calcium (Ca^{2+}) channels. This causes to decreased Ca^{2+} influx and increased K^+ efflux, leading to hyperpolarization of the cell.

Reinforcing effects of GHB in the brain

Next to GHB's direct effect on GABA receptors and the GHB receptors, GHB exerts a neuromodulatory effect on dopaminergic signaling and, to a lesser extent, serotonergic, noradrenergic, cholinergic and opioid signaling (Kamal, van Noorden, et al., 2016). Over the last decade, it has become apparent that GHB is an addictive substance (Brunt et al., 2014; Kamal, Dijkstra, et al., 2017). As seen with all major addictive substances, the addictive properties of GHB are primarily mediated by mesolimbic dopamine signaling. Low and medium doses of GHB in the rat (<400mg/kg) can increase the firing rate of dopaminergic neurons in the substantia nigra pars compacta (SNc), a key structure in dopamine production and signaling (Diana et al., 1991). In contrast, higher doses of GHB (1000-1500mg/kg) inhibit dopaminergic cell firing in the SNc (Diana et al., 1991). Although the effects of specific doses are dependent on administration route and are subject to large individual variability (Diana et al., 1993), it indicates the dual dopaminergic effects of GHB in the brain.

GHB-induced changes in the firing pattern of dopaminergic neurons are mainly regulated by $GABA_B$ receptors (Erhardt et al., 1998; Pistis et al., 2005). However, compounds that also activate the $GABA_B$ receptor, such as the $GABA_B$ agonist baclofen, appear to be less addictive than GHB (Chick & Nutt, 2012). The highly addictive profile of GHB can be explained by two separate mechanisms.

First, the effect of GHB on GABAergic signaling plays a key role in the distinctive rewarding and addictive properties of GHB. Upon activation of $GABA_B$ receptors, K^+ channels are opened, which leads to hyperpolarization of the neuron. These K^+ channels, or G protein-coupled inwardly-rectifying potassium (GIRK) channels, differ in their subunit composition and localization. In the mammalian brain, the subunits GIRK1, GIRK2 and GIRK3 are expressed throughout the brain, forming tetrameric channels. There are three splice variants of GIRK2 (GIRK2a-GIRK2c), able to form both homomeric (GIRK2/2) and heteromeric (GIRK1/2; GIRK2/3) channels. GIRK1 and 3 are unable to form functional homomeric channels, leading to the composition of heteromeric channels containing GIRK1 or GIRK3 (GIRK1/2; GIRK1/3; GIRK2/3) (Luscher & Slesinger, 2010).

GIRK channels are expressed in different compositions on neuronal membranes in the mesolimbic system (Luján & Aguado, 2015). This difference in subunit composition, next to the differences in membrane localization of the $GABA_B$ receptors, causes a bidirectional (increasing/decreasing) effect of GHB on the mesolimbic dopamine system (Cruz et al., 2004). In the SNc, GIRK channels consisting exclusively of GIRK2

subunits are predominantly found. In the ventral tegmental area (VTA), another structure pivotal in dopaminergic signaling and involved in mediating the rewarding effects of drugs of abuse, GIRK1/2 channels are primarily expressed on GABAergic neurons. In contrast, dopamine-containing neurons in the VTA do not express GIRK1 containing channels and mainly express GIRK2c/3 channels (Fig. 2).

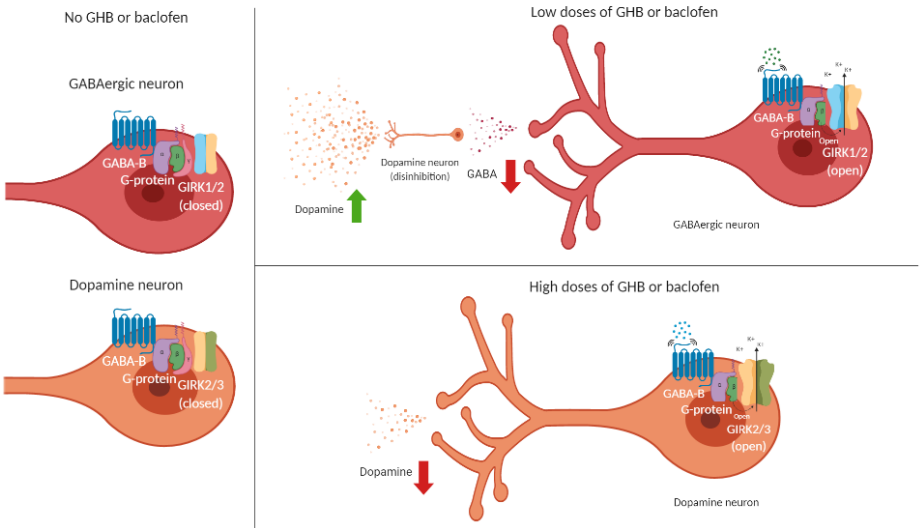


Figure 2. GABAergic VTA neuron in an inactivated (upper left) and activated (upper right) state. When low doses of GHB or the $GABA_B$ agonist binds to the $GABA_B$ receptor on a GABAergic neuron in the VTA, a GIRK1/2 channel will open and will hyperpolarize the cell. Hyperpolarization of the GABAergic neuron will decrease GABA release and will therefore disinhibit dopaminergic neurons, leading to increased dopamine release. In the bottom panel, a dopaminergic neuron in the VTA (inactivated, lower left; activated, lower right) are shown. When high doses of GHB or baclofen bind to a $GABA_B$ receptor on this type of neuron, the cell will be hyperpolarized through opening of a GIRK2/3 channel. This will lead to decreased dopamine release.

The reason why this subunit expression is relevant for the effects of GHB, is related to under which specific conditions these different $GABA_B$ receptors are activated. $GABA_B$ receptors hyperpolarize GIRK1/2 channels with a smaller effective dose (ED) of GHB compared to GIRK2/3 channels (Cruz et al., 2004; Crunelli et al., 2006; Lüscher & Ungless, 2006; Kamal, van Noorden, et al., 2016). This means that GHB preferentially inhibits GABAergic neurons compared to dopaminergic neurons in the VTA, causing a disinhibition of VTA dopaminergic output (Snead & Gibson, 2005; Lüscher & Ungless, 2006; Kamal, van Noorden, et al., 2016) (Fig. 2). Baclofen, a $GABA_B$ agonist, activates GIRK channels in both VTA GABAergic and dopaminergic neurons, leading to less dopamine release in the mesolimbic system (Fig. 3). At higher concentrations of GHB, $GABA_B$ receptors on dopaminergic neurons will also be activated, leading to decreased

dopamine output from the VTA to the nucleus accumbens (NAc) and a less rewarding/ reinforcing effect of GHB compared to lower concentrations of GHB (Fig. 3).

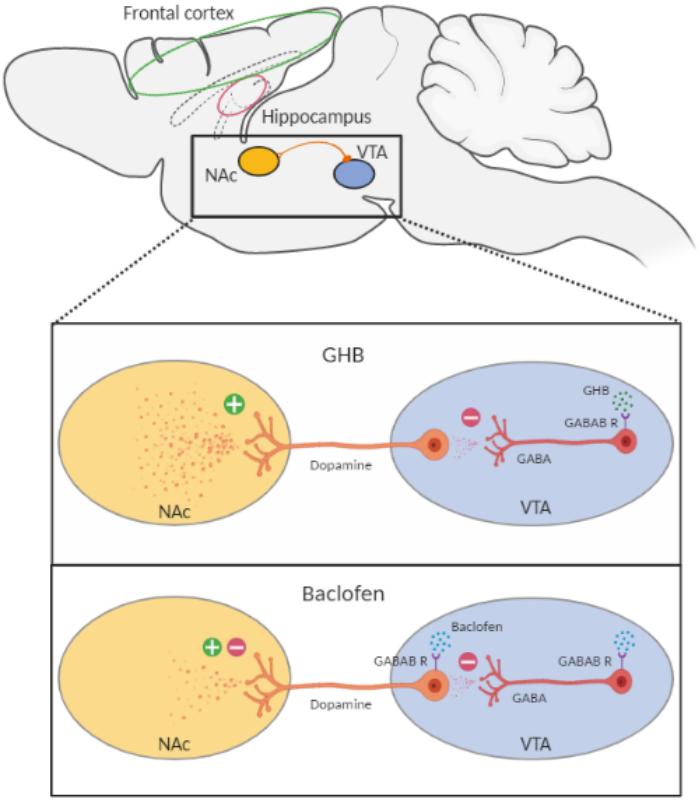


Figure 3. Mesolimbic dopaminergic pathway underlying the addictive properties of GHB. GHB predominantly activates GABAergic neurons in the VTA through binding of $GABA_B$ receptors, leading to increased dopamine release in the NAc, a region involved in the reinforcing effects of drugs. When baclofen binds to a $GABA_B$ receptor on a GABAergic neuron, the same process will occur. However, since it also activates $GABA_B$ receptors on dopaminergic neurons in the VTA, dopamine release in the NAc is reduced as compared to GHB-induced dopamine release.

These processes change following prolonged exposure to GHB, for example with GUD. After chronic exposure to GHB, $GABA_B$-GIRK channel coupling efficiency in dopamine neurons is increased, leading to a lower required ED of $GABA_B$-coupled GIRK2c/3 channels. This means that also low doses of GHB in its turn would lead to decreased dopamine release from the VTA into the NAc, reducing the reinforcing effects of GHB (Labouebe et al., 2007). This may contribute the shift in the use of GHB for its rewarding effects to the use of GHB to prevent negative effects such as

withdrawal symptoms (Beurmanjer et al., 2019). With other addictive substances, like cocaine, an increase in inhibitory $GABA_B$-dependent GIRK signaling is absent. With cocaine, there is even a decrease in inhibitory $GABA_B$-GIRK signaling after repeated cocaine injections, limiting tonic inhibition of dopaminergic neurons and further enhancing the output of VTA dopaminergic neurons to the NAc (Arora et al., 2011).

Next to its reinforcing effect through altering GABAergic signaling, GHB has also been shown to increase glutamatergic transmission in the brain. Glutamatergic signaling plays a key role in the initiation and expression of addiction-like behavior (Kalivas et al., 2009; Derman & Ferrario, 2018). The hippocampus, a pivotal structure for processes as spatial memory and working memory with longer temporal scales and long-term memory, is interconnected with mesolimbic structures involved in the rewarding processes of drugs of abuse, such as the VTA and NAc (Kahn & Shohamy, 2013). Through GHB-induced increases of glutamatergic signaling in the hippocampus (enhancing memory of reward, (Ferraro et al., 2001), together with an increase in dopamine release in the mesolimbic circuit through GABAergic processes (rewarding effect of drug, (Cruz et al., 2004), addiction-like behavior can be developed and strengthened (Belujon & Grace, 2011; Kutlu & Gould, 2016). Next to this synergistic effect of GABAergic and glutamatergic signaling on addictive behavior, it is suggested that GHB also directly excites dopaminergic neurons (Kamal, van Noorden, et al., 2016). Low doses of GHB that exclusively activate GHB receptors in rats (10mg/kg) have been shown to activate the majority of examined dopaminergic cells in the VTA, which was largely prevented by the application of GHB-receptor antagonist NCS-382 (Tremblay et al., 1998). Furthermore, GHB-producing neurons have been found to be surrounded by dopaminergic terminals in the basal ganglia (Hédou et al., 2000). This fosters the possibility that GHB directly excites dopamine neurons to promote dopamine release in the basal ganglia.

These direct and indirect dopaminergic effects of GHB manifest itself in the addictive properties of GHB. In humans, repeated GHB use leads to physical dependence, but also causes a strong psychologically reinforcing effect due to increased dopaminergic signaling, depending on the dose. In animal models, it has also been shown that GHB has rewarding effects. Animals preferred the area where they previously received a specific dose of GHB compared to a neutral area where they did not receive GHB (called conditioned place preference), indicating a rewarding effect of that dose of GHB (Martellotta et al., 1997; Watson et al., 2010). Altogether, the involvement of multiple GHB receptor targets is likely to contribute to rewarding and addictive characteristic of GHB, contributing to the positive reinforcement phase of the development of GUD.

Prolonged neurobiological effects of GHB

Despite the neuroprotective properties of GHB, it has also been suggested that GHB use is able to lead to neurotoxicity. Especially the use of higher doses of GHB, as often seen in patients with GUD, is suggested to be able to lead to cognitive impairment (van Amsterdam et al., 2022). This highlights the importance of investigating the long-term effects of GHB use on cognition and neurotoxicity and understanding the potential dual nature of GHB's effects on the brain.

Due to the pharmacological profile of GHB described above, the dose in which GHB is administered is of high relevance to the observed effects. Endogenous GHB levels in rats vary from 0.4 µM to 4.6 µM, depending on brain regions investigated, with maximum brain concentrations of 11-25 µM in the human brain (Maitre, 1997). It is generally accepted that GHB in nanomolar concentrations exclusively activates the high-affinity GHB binding sites in the brain, while micromolar concentrations are increasingly able to bind $GABA_B$ receptors, and millimolar GHB concentrations exclusively activate $GABA_B$ receptors, depending on the brain region (Lingenhoehl et al., 1999).

That said, there are several reasons why generalized statements on the receptor activation of systemic GHB injections are troubled, including 1) non-homogenous distribution of GHB in the brain (Klein et al., 2009), 2) the saturated transport across the blood-brain barrier (Felmlee et al., 2021), 3) the strong inter-individual differences in GHB pharmacokinetics (Lettieri & Fung, 1978), 4) inter-species differences in dose-effects (Wong et al., 2004), 5) differences between routes of administration (parenteral vs enteral) (Lettieri & Fung, 1978), 6) the non-homogenous distribution of high-affinity GHB binding sites (Maitre, 1997), and 7) the subtle-bidirectional effect of GHB on different types of $GABA_B$ receptors (Cruz et al., 2004).

Even when assessing the literature examining the conversion of systemic GHB injections to GHB brain concentrations, there is large variation between studies. Studies have shown a conversion of systemic injections of 400 mg/kg GHB to 960 µM GHB in the brain, 800 mg/kg to 1300 µM (Lettieri & Fung, 1978), 750 mg/kg to 463 µM (with variations from 333 – 533 between brain regions), 1000 mg/kg to 875 µM(with variations from 605 – 1495 µM) (Klein et al., 2009) and 548 mg/kg to 800 µM(Raybon & Boje, 2007). While literature on the conversion of lower doses of GHB is lacking, we assume that doses of <100 mg/kg roughly lead to nanomolar concentrations in the brain (low doses corresponding with GHB receptor activation), doses between >100 mg/kg and <400 mg/kg lead to micromolar doses in the brain (medium doses, corresponding with partial GHB- and $GABA_B$ receptor activation), and doses of

>400 mg/kg lead to millimolar concentrations in the brain (high doses, corresponding with $GABA_B$ receptor activation).

We identified 9 studies that assessed the prolonged neurological effects of GHB, see Table 1. Studies examining acute neuronal effects (sacrifice within 12 hours following GHB exposure) or GHB-induced effects following trauma were excluded.

Table 1. Studies examining the histological and functional effects of GHB.

Paper	Dose (mg/kg) x # injections	Brain region	Time sacrifice after injection	Effect
Sircar and Basak (2004)	10, 50, **100** x 5	FC, HC	4 weeks	Decreased NMDA receptor binding after 100mg/kg GHB in the FC, but not in HC.
Pedraza et al. (2009)	**10**, **100** x 15	PFC, HC	?	Decreased number of neurons in CA1 with 10 and 100mg/kg GHB and in PFC with 10mg/kg GHB. Increased number of non-neuronal cells with 10mg/kg GHB.
Sircar et al. (2011)	100 x 6	FC	?	Decreased NMDA subunit (NR1 and NR2B) protein expression
Johansson et al. (2014)	50, 300 x 16	Whole brain	24 hours	Increased IGF-1 receptor density in amygdala, superior colliculus and geniculate nucleus with 50mg/kg, decreased IGF-1 receptor density in HC (DG, CA1) with 300mg/kg, Decreased GABAB receptor density in HC (DG, CA1, CA3), thalamus, hypothalamus and amygdala with 300mg/kg.
Ren and Mody (2006)	500 x 1 / 500 x 29	HC	24, 72, 120 hours	No change in phosphorylation of cAMP-responsive element-binding (pCREB) protein or cytosolic cAMP-dependent protein kinase activity (PKA)
Youn et al. (2015)	100, 500 x 10	HC	?	Dose-dependent decrease in Ca^{2+} expression, reduced NMDAR expression and pCREB immunoreactivity, increased lipid peroxidation, accumulated intracellular Na^{+} levels, impaired COX expression (increased oxidative stress).
Chen et al. (2017)	**500** x 10	HC	10 days	Increased MDA (marker for oxidative stress) cholesterol in HC, increased Nfr2 in nucleus/cytoplasm, decreased anti-oxidative enzymes superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase in HC.

Table 1. Continued

Paper	Dose (mg/kg) x # injections	Brain region	Time sacrifice after injection	Effect
van Nieuwenhuijzen, Long, et al. (2010)	**500** x 10	PFC, striatum, hypothalamus	8 weeks	No change in NA, 5-HT or 5-HIAA in PFC after GHB, and no change in NA, DA, DOPAC, 5-HT or 5-HIAA in striatum. GHB leads to an increase in oxytocin receptor in the hypothalamus
van Nieuwenhuijzen, Kashem, et al. (2010)	**500** x 10	HC	8 weeks	GHB caused an upregulation of protein Neuronal Pentraxin-1 (7029), and a downregulation of proteins ZH-14 (cytoskeletal), Tropomyosin-α 3 chain (cytoskeletal), Astrocytic phosphoprotein PEA-15 (apoptosis) and glutathione-S-transferaseYb4.

Doses causing a significant effect are put in bold. FC = Frontal Cortex; PFC = Prefrontal Cortex; HC = Hippocampus; VTA = Ventral Tegmental Area; SNc = Substantia Nigra pars compacta.

In table 1, we not only see differential effects between doses, but also between brain (sub)regions. GHB differentially affected NMDA receptor binding and IGF-1 and GABAB receptor densities between different brain regions such as the prefrontal cortex and hippocampus, whereas it exerted specific subregional neurotoxic effects in the hippocampus (Pedraza et al., 2009; Johansson et al., 2014). This corresponds to the non-homogenous distribution of GHB in the brain follow systemic administration and is in line with the differential expression of GHB and $GABA_B$ receptors throughout the brain. Overall, it appears that subregions within the hippocampus are susceptible to changes induced by GHB administration, in line with the high density of $GABA_B$ and putative GHB receptors in that region. Low doses seem to lead to a decrease in neuronal cell count and affect glutamatergic signaling through alterations in NMDA receptor expression. Medium and high doses of GHB appear to negatively affect the brain through oxidative stress, although evidence remains scarce.

Dose-dependent decreases in bio-energetic processes have also been observed following GHB administration. This aligns with research on GHB's potential neuroprotective properties, suggesting that the induction of an energy-conserving cellular state by GHB may contribute to neuroprotection during ischemia-reperfusion. However, as discussed above, in studies conducted under non-ischemic conditions, an increase in oxidative stress and/or neuronal cell death may be observed. These opposing outcomes highlight the importance of the brain's baseline state (hypoxic vs. normal) in modulating the neuronal effects of GHB. This factor should be considered when interpreting findings related to the neuroprotective effects of GHB.

Altogether, the neurobiological effects of GHB are disperse and largely dependent on dose and brain region. Several studies indicate that GHB can lead to neurotoxicity in the (pre)frontal cortex and hippocampus. Other neurobiological effects presented in Table 1 are not neurotoxic per se, but may provide a mechanistic explanation for the other effects of GHB, such as neuroprotection. Future studies should further explore the neurotoxic effects of systemic GHB administration, as it may hold strong implications for the use of GHB as a recreational- and prescription drug.

Prolonged cognitive effects of GHB administration

The neurobiological changes and neurotoxic effects following repeated GHB administration have the potential to manifest themselves as cognitive aberrations. A study in patients with GUD employed a cognitive screener, the Montreal Cognitive Assessment (MoCa), testing patients both before and after inpatient detoxification (Beurmanjer et al., 2022). They found that impairment on the (long-term) memory domain was most frequent (~60%), but was not associated with GUD severity or number of comas. Performance on long-term memory performance slightly improved following detoxification (58.8 -> 70.4% correct). Interestingly, working memory performance was not affected in these patients (83.3% performance pre-detox, 85.5% post-detox). 56.3% of patients screened positive for cognitive impairment before detoxification, which dropped to 30.6% after detoxification, indicating strong intoxication effects on cognition and the potential reversibility of cognitive deficits in these patients.

Another clinical study investigated the working memory performance of GHB users that experienced <4 comas, GHB users that experienced >4 comas and polydrug users that did not use GHB (control) (Raposo Pereira et al., 2018). GHB users that experienced <4 comas did not differ in their working memory performance compared to the control group. The coma group also did not show decreased performance on a visuospatial working memory task, but the coma group performed worse on a "verbal" working memory task compared to the control group. This might be an indication that GHB-induced comas may play a role in the cognitive effects of GHB.

Although these studies provide insight in the occurrence of cognitive deficits in GUD patients, they do not provide information on the causal cognitive effects of GHB consumption, GUD or GHB induced comas. We identified 4 studies that assessed the prolonged causal cognitive effects of (repeated) GHB administration, see Table 2. A large number of animal studies that assessed cognitive effects of GHB, performed

cognitive tests within two hours of GHB administration (Sircar & Basak, 2004; Kueh et al., 2008; Laraway et al., 2008; Sircar et al., 2008; Sircar et al., 2010; Johansson et al., 2014; Sircar & Ishiwari, 2014), examining acute intoxication effects of GHB. I only discuss studies that examined prolonged cognitive effects of GHB (>2 hours post-administration), since Beurmanjer et al. has demonstrated that acute cognitive deficits found in GUD patients may naturally attenuate upon cessation of GHB use (Beurmanjer et al., 2022).

Table 2. Behavioral studies examining the effect of GHB on different cognitive tasks.

Paper	Age	Dose (mg/kg)	Number of injections	Task	Effect
Pedraza et al. (2009)	Adult (age unknown)	15 x **10**, 100	10X without task, 120 min prior to task (15X)	MWM, hole board test	No effect on spatial learning and memory (MWM) or long-term memory (hole board test), negative effect on working memory (hole board test)
van Nieuwenhuijzen, Long, et al. (2010)	Unknown	10 x 500	10X without task, tested 6 weeks later	Novel object recognition (1h interval)	Reduced time spent exploring novel object
Klein et al. (2015)	PND90	1,2% w/v (~3000mg/kg per day)	Continuous for 2 months, tested 6 weeks / continuous for 4 months, tested 6 weeks later	Spatial recognition task (3 min interval)	No effect
Chen et al. (2017)	Adolescent (age unknown)	10 x 500	10X without task, tested 5 days later	MWM	Negative effect on spatial learning and spatial memory

Two studies assessed the long-term cognitive effects of GHB (van Nieuwenhuijzen, Long, et al., 2010; Klein et al., 2015). After 10 daily IP injections of 500mg/kg GHB, animals were tested 6 weeks later for their performance on the novel object recognition (NOR) task (van Nieuwenhuijzen, Long, et al., 2010), a hippocampal-dependent task (Cohen & Stackman Jr, 2015). Performance on the NOR task was significantly decreased following repeated high dose administration, indicating impairments in (long-term) recognition memory. Klein et al. employed a partially similar cognitive test, based on the natural tendency of rodents to explore displaced objects vs objects that did not move (Klein et al., 2015). Klein et al. did not find any effects of GHB self-administration on working memory in the spatial recognition task. Despite the similarities between tasks of Klein et al. and Nieuwenhuijzen et al.,

one key difference is the duration between the acquisition trial and the testing trial (3 minutes vs 1 hour). These studies are assessing different components of memory (working memory vs long-term memory), although the difference in species, dose or route of administration could also have played a role in the findings.

Pedraza et al. did find a negative effect of repeated GHB administration on working memory performance, albeit with very low concentrations (10 mg/kg) (Pedraza et al., 2009). Important to note is that, surprisingly, a low dose of GHB caused a decreased grasping reflex, indicating signs of sedation, which was not observed with 100 mg/kg GHB. Pedraza et al. did not find an effect of GHB on spatial learning and spatial memory but found altered navigation strategies in animals that were treated with GHB (either low or medium doses) (Pedraza et al., 2009). Similarly, Chen et al. (2017) found altered navigation strategies in animals that were treated with (high-dose) GHB (Chen et al., 2017). This was accompanied with decreased spatial learning and -memory performance in GHB-treated animals, in contrast to findings by Pedraza et al.

Despite the limited availability of studies assessing the prolonged cognitive effects of GHB, it appears that GHB has the potential to negatively affect specific cognitive domains. As it is known that systemic GHB administration can differentially affect specific brain regions, GHB can possibly affect specific cognitive domains. Additionally, the non-linear pharmacokinetics of GHB, the differences between administration routes, and the differences between species hamper conclusive statements on the effects of specific doses of GHB. A more suited approach may be to mimic clinical situations as GUD or the occurrence of GHB-induced comas in animals, to establish translationally valid study designs and enhance translation of findings in animals to clinical reality.

Gaps in our understanding and overview of my thesis

Recreational GHB use and GUD lead to various adverse health effects, including frequent overdosing and severe withdrawal symptoms. However, the drug itself is commonly perceived as “safe” by users. There are several reasons for this perception, including that 1) GHB is used as a therapeutic compound for specific disorders such as narcolepsy, 2) GHB use and GHB-induced comas do not leave residual negative effects, and 3) the increasing scientific evidence for neuroprotective effects of GHB. The adverse effects of GHB are difficult to study in a clinical setting due to intoxication of patients, frequent relapse following treatment, volatility of patients, etc. Additionally, GHB users rarely use GHB in isolation, limiting the ability to draw

definitive conclusions regarding the effects of GHB use in clinical populations. Although clinical literature demonstrated associations between GHB use and cognitive deterioration, it is unclear whether there are indications for causality in the observed association. Animal studies are able to demonstrate causal effects of GHB and enable the study of possible pharmacological interventions. Although (self-administration) animal models for drugs such as alcohol and cocaine have been around for decades, a GHB self-administration model is still lacking, which is also indicative of the limited research into GHB and GUD.

In order to grasp the complex phenomenon of GUD, and to examine this in a controlled setting, we employed a translational approach. We do this by exploring the GHB receptor through a bioinformatics approach, assessing GHB self-administration in animals, exploring the neurobiological effects of GHB self-administration, examining the effects of GHB use and GHB-induced comas on cognition in animals, and characterizing GHB withdrawal in patients.

Chapter 2
Although the pharmacodynamics and receptor systems of GHB have been studied thoroughly, the high-affinity target(s) of GHB remain elusive and a lively topic of debate. Increasing our understanding of these high-affinity receptors would enhance our understanding of the wide variety of effects of GHB. In chapter 2, I present a narrative review on the identity of a subtype of the GHB receptor in order to complement our understanding of the receptor mechanisms of GHB. Additionally, I will discuss existing literature on the structures that are suggested to have high-affinity for GHB, and discuss possible implications of these findings.

Chapter 3
As discussed previously in this chapter, the existing clinical literature is unable to demonstrate causal relations between GHB use (disorder)/GHB-induced comas and cognitive/neurotoxic effects. An animal model for chronic GHB self-administration that would enable the study of such factors is currently lacking. In chapter 3 of this thesis, I will describe the development of the first voluntary GHB self-administration model in rats and examine the effects of chronic GHB self-administration on long-term memory performance.

Chapter 4
Comas are highly common in GHB use disorder. GHB-induced comas have been implicated in decreased verbal short-term memory performance in patients. However, it is unknown whether these effects are causal, or may have been confounded by factors

such as heavier GHB use or co-morbid drug use. To determine whether GHB-induced comas cause (additional) negative effects on memory performance compared to GHB use without comas, used a separate animal model. We compared a coma group that experienced 2 comas with a no-coma group that received a similar total amount of GHB, and a control group, on working memory performances and impulsivity.

Chapter 5

Our findings on the effects of GHB on different memory domains suggest brain-region specific effects that may underlie these cognitive findings. As we find that long-term memory performance is affected by chronic GHB use, whereas working memory performance and impulsivity was not affected by GHB-administration, we hypothesized that GHB primarily affected hippocampal structures. To explore potential mechanisms behind our results on the effects of GHB on memory performance, I zoom in on the neurobiological effects of chronic GHB administration in the hippocampus of rats that self-administered GHB.

Chapter 6

Clinical studies are highly valuable in understanding the many complex facets of GUD. Currently, the characteristics of GHB withdrawal and treatment strategies for relapse in patients with GUD are two key issues that remain understudied. Despite a review on several case studies and case series on the withdrawal symptoms of patients with GUD, the characteristics of the GHB withdrawal syndrome in an inpatient setting are currently unknown. In chapter 6 of this thesis, I will describe the GHB withdrawal syndrome under inpatient GHB tapering. Additionally, I will explore the relation between withdrawal symptoms and vital parameters, and investigate gender differences in experienced withdrawal symptoms.

Chapter 7

This thesis discusses many different facets of GHB, including behavioral, cognitive, neurobiological and structural studies. Despite the many approaches used to examine the possible negative effects of GHB, many outcomes of our studies are linked to each other. In chapter 6, I will summarize our findings presented in the previous chapters, highlight the associations between our findings and discuss the overarching implications of our research in light of the current literature.

Bibliography

Abanades, S., Farré, M., Barral, D., Torrens, M., Closas, N., Langohr, K., Pastor, A., & De La Torre, R. (2007). Relative abuse liability of γ-hydroxybutyric acid, flunitrazepam, and ethanol in club drug users. *Journal of clinical psychopharmacology*, *27*(6), 625-638.

Absalom, N., Eghorn, L. F., Villumsen, I. S., Karim, N., Bay, T., Olsen, J. V., Knudsen, G. M., Brauner-Osborne, H., Frolund, B., Clausen, R. P., Chebib, M., & Wellendorph, P. (2012). alpha4betadelta GABA(A) receptors are high-affinity targets for gamma-hydroxybutyric acid (GHB). *Proc Natl Acad Sci U S A*, *109*(33), 13404-13409. https://doi.org/10.1073/pnas.1204376109

Andriamampandry, C., Taleb, O., Kemmel, V., Humbert, J. P., Aunis, D., & Maitre, M. (2007). Cloning and functional characterization of a gamma-hydroxybutyrate receptor identified in the human brain. *FASEB J*, *21*(3), 885-895. https://doi.org/10.1096/fj.06-6509com

Andriamampandry, C., Taleb, O., Viry, S., Muller, C., Humbert, J. P., Gobaille, S., Aunis, D., & Maitre, M. (2003). Cloning and characterization of a rat brain receptor that binds the endogenous neuromodulator γ-hydroxybutyrate (GHB). *FASEB J*, *17*(12), 1691-1693. https://doi.org/10.1096/fj.02-0846fje

Arora, D., Hearing, M., Haluk, D. M., Mirkovic, K., Fajardo-Serrano, A., Wessendorf, M. W., Watanabe, M., Luján, R., & Wickman, K. (2011). Acute cocaine exposure weakens GABAB receptor-dependent G-protein-gated inwardly rectifying K+ signaling in dopamine neurons of the ventral tegmental area. *Journal of Neuroscience*, *31*(34), 12251-12257.

Arunogiri, S., Moayeri, F., Crossin, R., Killian, J. J., Smith, K., Scott, D., & Lubman, D. I. (2020). Trends in gamma-hydroxybutyrate-related harms based on ambulance attendances from 2012 to 2018 in Victoria, Australia. *Addiction*, *115*(3), 473-479.

Banerjee, P. K., & Snead, O. C., 3rd. (1995). Presynaptic gamma-hydroxybutyric acid (GHB) and gamma-aminobutyric acidB (GABAB) receptor-mediated release of GABA and glutamate (GLU) in rat thalamic ventrobasal nucleus (VB): a possible mechanism for the generation of absence-like seizures induced by GHB. *J Pharmacol Exp Ther*, *273*(3), 1534-1543.

Bay, T., Eghorn, L. F., Klein, A. B., & Wellendorph, P. (2014). GHB receptor targets in the CNS: focus on high-affinity binding sites. *Biochem Pharmacol*, *87*(2), 220-228. https://doi.org/10.1016/j.bcp.2013.10.028

Belujon, P., & Grace, A. A. (2011). Hippocampus, amygdala, and stress: interacting systems that affect susceptibility to addiction. *Ann N Y Acad Sci*, *1216*, 114-121. https://doi.org/10.1111/j.1749-6632.2010.05896.x

Benavides, J., Rumigny, J. F., Bourguignon, J. J., Cash, C., Wermuth, C. G., Mandel, P., Vincendon, G., & Maitre, M. (1982). High affinity binding sites for gamma-hydroxybutyric acid in rat brain. *Life Sci*, *30*(11), 953-961. https://doi.org/10.1016/0024-3205(82)90624-5

Bettler, B., Kaupmann, K., Mosbacher, J., & Gassmann, M. (2004). Molecular structure and physiological functions of GABA(B) receptors. *Physiol Rev*, *84*(3), 835-867. https://doi.org/10.1152/physrev.00036.2003

Beurmanjer, H., Asperslag, E. M., Oliemeulen, L., Goudriaan, A. E., De Jong, C. A. J., Schellekens, A. F. A., & Dijkstra, B. A. G. (2019). A Qualitative Approach in Understanding Illness Perception and Treatment Needs in Patients with Gamma Hydroxybutyrate Use Disorder. *Eur Addict Res*, *25*(5), 248-255. https://doi.org/10.1159/000500807

Beurmanjer, H., Bruijnen, C. J. W. H., Greeven, P. G. J., De Jong, C. A. J., Schellekens, A. F. A., & Dijkstra, B. A. G. (2022). Cognitive Impairments in Patients with GHB Use Disorder Predict Relapse in GHB Use. *Eur Addict Res*, *28*(5), 350-357. https://doi.org/10.1159/000525507

Beurmanjer, H., Kamal, R. M., de Jong, C. A. J., Dijkstra, B. A. G., & Schellekens, A. F. A. (2018). Baclofen to Prevent Relapse in Gamma-Hydroxybutyrate (GHB)-Dependent Patients: A Multicentre, Open-Label, Non-Randomized, Controlled Trial. *CNS Drugs*, *32*(5), 437-442. https://doi.org/10.1007/s40263-018-0516-6

Beurmanjer, H., Luykx, J. J., De Wilde, B., van Rompaey, K., Buwalda, V. J. A., De Jong, C. A. J., Dijkstra, B. A. G., & Schellekens, A. F. A. (2020). Tapering with Pharmaceutical GHB or Benzodiazepines for Detoxification in GHB-Dependent Patients: A Matched-Subject Observational Study of Treatment-as-Usual in Belgium and The Netherlands. *CNS Drugs*, *34*(6), 651-659. https://doi.org/10.1007/s40263-020-00730-8

Bhattacharya, I., & Boje, K. M. (2004). GHB (γ-hydroxybutyrate) carrier-mediated transport across the blood-brain barrier. *The Journal of pharmacology and experimental therapeutics*, *311*(1), 92-98.

Bosch, O. G., Eisenegger, C., Gertsch, J., von Rotz, R., Dornbierer, D., Gachet, M. S., Heinrichs, M., Wetter, T. C., Seifritz, E., & Quednow, B. B. (2015). Gamma-hydroxybutyrate enhances mood and prosocial behavior without affecting plasma oxytocin and testosterone. *Psychoneuroendocrinology*, *62*, 1-10.

Brenneisen, R., Elsohly, M. A., Murphy, T. P., Passarelli, J., Russmann, S., Salamone, S. J., & Watson, D. E. (2004). Pharmacokinetics and excretion of gamma-hydroxybutyrate (GHB) in healthy subjects. *J Anal Toxicol*, *28*(8), 625-630. https://doi.org/10.1093/jat/28.8.625

Brunt, T., van Noorden, M., Kamal, R., Dijkstra, B., & de Jong, C. (2016). Epidemiology of gamma-hydroxybutyrate (GHB) use and misuse and characteristics of GHB-dependent inpatients. In *Neuropathology of Drug Addictions and Substance Misuse* (pp. 491-499). Elsevier.

Brunt, T. M., van Amsterdam, J. G. C., & van den Brink, W. (2014). GHB, GBL and 1, 4-BD Addiction. *Current Pharmaceutical Design*, *20*(25), 4076-4085.

Carai, M. A., Colombo, G., Brunetti, G., Melis, S., Serra, S., Vacca, G., Mastinu, S., Pistuddi, A. M., Solinas, C., Cignarella, G., Minardi, G., & Gessa, G. L. (2001). Role of GABA(B) receptors in the sedative/hypnotic effect of gamma-hydroxybutyric acid. *Eur J Pharmacol*, *428*(3), 315-321. https://doi.org/10.1016/s0014-2999(01)01334-6

Castelli, M. P., Ferraro, L., Mocci, I., Carta, F., Carai, M. A., Antonelli, T., Tanganelli, S., Cignarella, G., & Gessa, G. L. (2003). Selective γ-hydroxybutyric acid receptor ligands increase extracellular glutamate in the hippocampus, but fail to activate G protein and to produce the sedative/hypnotic effect of γ-hydroxybutyric acid. *Journal of neurochemistry*, *87*(3), 722-732.

Chen, L. Y., Renn, T. Y., Liao, W. C., Mai, F. D., Ho, Y. J., Hsiao, G., Lee, A. W., & Chang, H. M. (2017). Melatonin successfully rescues hippocampal bioenergetics and improves cognitive function following drug intoxication by promoting Nrf2-ARE signaling activity. *J Pineal Res*, *63*(2). https://doi.org/10.1111/jpi.12417

Chick, J., & Nutt, D. J. (2012). Substitution therapy for alcoholism: time for a reappraisal? *Journal of Psychopharmacology*, *26*(2), 205-212.

Cohen, S. J., & Stackman Jr, R. W. (2015). Assessing rodent hippocampal involvement in the novel object recognition task. A review. *Behavioural brain research*, *285*, 105-117.

Connelly, W. M., Errington, A. C., & Crunelli, V. (2013). gamma-Hydroxybutyric acid (GHB) is not an agonist of extrasynaptic GABAA receptors. *PLoS One*, *8*(11), e79062. https://doi.org/10.1371/journal.pone.0079062

Connelly, W. M., Fyson, S. J., Errington, A. C., McCafferty, C. P., Cope, D. W., Di Giovanni, G., & Crunelli, V. (2013). GABAB receptors regulate extrasynaptic GABAA receptors. *Journal of Neuroscience*, *33*(9), 3780-3785.

Corkery, J. M., Loi, B., Claridge, H., Goodair, C., Corazza, O., Elliott, S., & Schifano, F. (2015). Gamma hydroxybutyrate (GHB), gamma butyrolactone (GBL) and 1, 4-butanediol (1, 4-BD; BDO): A literature review with a focus on UK fatalities related to non-medical use. *Neuroscience & Biobehavioral Reviews*, *53*, 52-78.

Crunelli, V., Emri, Z., & Leresche, N. (2006). Unravelling the brain targets of γ-hydroxybutyric acid. *Curr Opin Pharmacol*, *6*(1), 44-52. https://doi.org/10.1016/j.coph.2005.10.001

Cruz, H. G., Ivanova, T., Lunn, M.-L., Stoffel, M., Slesinger, P. A., & Lüscher, C. (2004). Bi-directional effects of GABAB receptor agonists on the mesolimbic dopamine system. *Nature neuroscience*, *7*(2), 153-159.

Derman, R. C., & Ferrario, C. R. (2018). Enhanced incentive motivation in obesity-prone rats is mediated by NAc core CP-AMPARs. *Neuropharmacology*, *131*, 326-336.

Diana, M., Mereu, G., Mura, A., Fadda, F., Passino, N., & Gessa, G. (1991). Low doses of gamma-hydroxybutyric acid stimulate the firing rate of dopaminergic neurons in unanesthetized rats. *Brain Res*, *566*(1-2), 208-211. https://doi.org/10.1016/0006-8993(91)91700-b

Diana, M., Pistis, M., Muntoni, A., & Gessa, G. (1993). Heterogeneous responses of substantia nigra pars reticulata neurons to gamma-hydroxybutyric acid administration. *Eur J Pharmacol*, *230*(3), 363-365. https://doi.org/10.1016/0014-2999(93)90574-2

EMCDDA. (2018). *European Drug Report 2018: Trends and Developments* (TD-AT-18-001-EN-N). P. O. o. t. E. Union.

Erhardt, S., Andersson, B., Nissbrandt, H., & Engberg, G. (1998). Inhibition of firing rate and changes in the firing pattern of nigral dopamine neurons by γ-hydroxybutyric acid (GHBA) are specifically induced by activation of GABAB receptors. *Naunyn-Schmiedeberg's archives of pharmacology*, *357*, 611-619.

Felmlee, M. A., Morse, B. L., & Morris, M. E. (2021). gamma-Hydroxybutyric Acid: Pharmacokinetics, Pharmacodynamics, and Toxicology. *AAPS J*, *23*(1), 22. https://doi.org/10.1208/s12248-020-00543-z

Ferraro, L., Tanganelli, S., O'Connor, W. T., Francesconi, W., Loche, A., Gessa, G. L., & Antonelli, T. (2001). γ-Hydroxybutyrate modulation of glutamate levels in the hippocampus: An in vivo and in vitro study. *Journal of neurochemistry*, *78*(5), 929-939.

Follman, K. E., & Morris, M. E. (2022). Treatment of gamma-Hydroxybutyrate (GHB) Overdose with the GABA(B) Antagonist SGS742. *J Pharmacol Exp Ther*, *382*(1), 21-30. https://doi.org/10.1124/jpet.122.001108

Gauger, S. J., Palmelund, L. B., Tian, Y., Marek, A., Namini, M. R., Griem-Krey, N., Kickinger, S., Mortensen, J. S., Frolund, B., & Wellendorph, P. (2025). Allosteric factors in the calcium/calmodulin-responsive kinase II hub domain determine selectivity of GHB ligands for CaMKIIa. *bioRxiv*, 2025.2001. 2019.633782.

Giorgetti, A., Busardò, F. P., & Giorgetti, R. (2022). Toxicological characterization of GHB as a performance-enhancing drug. *Frontiers in Psychiatry*, *13*, 846983.

Gold, B. I., & Roth, R. H. (1977). Kinetics of in vivo conversion of gamma-[3H]aminobutyric acid to gamma-[3H]hydroxybutyric acid by rat brain. *J Neurochem*, *28*(5), 1069-1073. https://doi.org/10.1111/j.1471-4159.1977.tb10670.x

Griem-Krey, N., Klein, A. B., Clausen, B. H., Namini, M. R. J., Nielsen, P. V., Bhuiyan, M., Nagaraja, R. Y., De Silva, T., Sobey, C. G., & Cheng, H. C. (2023). The GHB analogue HOCPCA improves deficits in cognition and sensorimotor function after MCAO via CaMKIIα. *Journal of Cerebral Blood Flow & Metabolism*, *43*(8), 1419-1434.

Hédou, G., Chasserot-Golaz, S., Kemmel, V., Gobaille, S., Roussel, G., Artault, J. C., Andriamampandry, C., Aunis, D., & Maitre, M. (2000). Immunohistochemical studies of the localization of neurons containing the enzyme that synthesizes dopamine, GABA, or γ-hydroxybutyrate in the rat substantia nigra and striatum. *Journal of Comparative Neurology*, *426*(4), 549-560.

Jazz. (2020). *XYREM® (sodium oxybate) oral solution, CIII* [Prescription Package Leaflet].

Johansson, J., Grönbladh, A., & Hallberg, M. (2014). Gamma-hydroxybutyrate (GHB) induces cognitive deficits and affects GABAB receptors and IGF-1 receptors in male rats. *Behavioural brain research*, *269*, 164-174.

Kahn, I., & Shohamy, D. (2013). Intrinsic connectivity between the hippocampus, nucleus accumbens, and ventral tegmental area in humans. *Hippocampus*, *23*(3), 187-192. https://doi.org/10.1002/hipo.22077

Kalivas, P. W., Lalumiere, R. T., Knackstedt, L., & Shen, H. (2009). Glutamate transmission in addiction. *Neuropharmacology*, *56 Suppl 1*(Suppl 1), 169-173. https://doi.org/10.1016/j.neuropharm.2008.07.011

Kam, P., & Yoong, F. (1998). Gamma-hydroxybutyric acid: an emerging recreational drug. *Anaesthesia*, *53*(12), 1195-1198.

Kamal, R. M., Dijkstra, B. A., de Weert-van Oene, G. H., van Duren, J. A., & De Jong, C. A. (2017). Psychiatric comorbidity, psychological distress, and quality of life in gamma-hydroxybutyrate-dependent patients. *Journal of Addictive Diseases*, *36*(1), 72-79.

Kamal, R. M., Schellekens, A. F. A., De Jong, C. A. J., & Dijkstra, B. A. G. (2015). Baclofen as relapse prevention in the treatment of gamma-hydroxybutyrate (GHB) dependence: an open label study. *BMC psychiatry*, *15*, 1-8.

Kamal, R. M., van Noorden, M. S., Franzek, E., Dijkstra, B. A. G., Loonen, A. J. M., & De Jong, C. A. J. (2016). The neurobiological mechanisms of gamma-hydroxybutyrate dependence and withdrawal and their clinical relevance: a review. *Neuropsychobiology*, *73*(2), 65-80.

Karlsson, A., Sutherland, R., & Peacock, A. (2021). Use of GHB amongst sentinel samples of people who regularly use illicit drugs in Adelaide, South Australia, 2020. *Drug Trends Bulletin Series. Sydney: National Drug and Alcohol Research Centre, UNSW Sydney*.

Kemmel, V., Miehe, M., Roussel, G., Taleb, O., Nail-Boucherie, K., Marchand, C., Stutz, C., Andriamampandry, C., Aunis, D., & Maitre, M. (2006). Immunohistochemical localization of a GHB receptor-like protein isolated from rat brain. *J Comp Neurol*, *498*(4), 508-524. https://doi.org/10.1002/cne.21072

Kim, S. Y., Anderson, I. B., Dyer, J. E., Barker, J. C., & Blanc, P. D. (2007). High-risk behaviors and hospitalizations among gamma hydroxybutyrate (GHB) users. *The American journal of drug and alcohol abuse*, *33*(3), 429-438.

Klein, C., Kemmel, V., Taleb, O., Aunis, D., & Maitre, M. (2009). Pharmacological doses of gamma-hydroxybutyrate (GHB) potentiate histone acetylation in the rat brain by histone deacetylase inhibition. *Neuropharmacology*, *57*(2), 137-147.

Klein, C., Mathis, C., Leva, G., Patte-Mensah, C., Cassel, J. C., Maitre, M., & Mensah-Nyagan, A. G. (2015). gamma-Hydroxybutyrate (Xyrem) ameliorates clinical symptoms and neuropathology in a mouse model of Alzheimer's disease. *Neurobiol Aging*, *36*(2), 832-844. https://doi.org/10.1016/j.neurobiolaging.2014.10.003

Krul, J., & Girbes, A. R. (2011). Gamma-hydroxybutyrate: experience of 9 years of gamma-hydroxybutyrate (GHB)-related incidents during rave parties in The Netherlands. *Clinical Toxicology*, *49*(4), 311-315.

Kueh, D., Iwamoto, K., Poling, A., & Baker, L. E. (2008). Effects of γ-hydroxybutyrate (GHB) and its metabolic precursors on delayed-matching-to-position performance in rats. *Pharmacology Biochemistry and Behavior*, *89*(2), 179-187.

Kutlu, M. G., & Gould, T. J. (2016). Effects of drugs of abuse on hippocampal plasticity and hippocampus-dependent learning and memory: contributions to development and maintenance of addiction. *Learn Mem*, *23*(10), 515-533. https://doi.org/10.1101/lm.042192.116

Laborit, H. (1964). Sodium 4-hydroxybutyrate. *International journal of neuropharmacology*, *3*(4), 433-IN438.

Laborit, H., Jouany, J., Gerard, J., & Fabiani, F. (1960). Summary of an experimental and clinical study on a metabolic substrate with inhibitory central action: sodium 4-hydroxybutyrate. *La Presse medicale*, *68*, 1867-1869.

Labouebe, G., Lomazzi, M., Cruz, H. G., Creton, C., Lujan, R., Li, M., Yanagawa, Y., Obata, K., Watanabe, M., Wickman, K., Boyer, S. B., Slesinger, P. A., & Luscher, C. (2007). RGS2 modulates coupling between GABAB receptors and GIRK channels in dopamine neurons of the ventral tegmental area. *Nat Neurosci*, *10*(12), 1559-1568. https://doi.org/10.1038/nn2006

Laraway, S., Snycerski, S., Baker, L. E., & Poling, A. (2008). Gamma-hydroxybutyrate (GHB) reduces operant behavior without impairing working memory in rats responding under fixed-consecutive-number schedules. *Pharmacology Biochemistry and Behavior*, *88*(3), 205-212.

Lettieri, J., & Fung, H. (1978). Improved pharmacological activity via pro-drug modification: comparative pharmacokinetics of sodium gamma-hydroxybutyrate and gamma-butyrolactone. *Research communications in chemical pathology and pharmacology*, *22*(1), 107-118.

Lettieri, J., & Fung, H. L. (1976). Absorption and first-pass metabolism of 14C-gamma-hydroxybutyric acid. *Res Commun Chem Pathol Pharmacol*, *13*(3), 425-437.

Lettieri, J. T., & Fung, H.-L. (1979). Dose-dependent pharmacokinetics and hypnotic effects of sodium gamma-hydroxybutyrate in the rat. *The Journal of pharmacology and experimental therapeutics*, *208*(1), 7-11.

Leurs, U., Klein, A. B., McSpadden, E. D., Griem-Krey, N., Solbak, S. M. Ø., Houlton, J., Villumsen, I. S., Vogensen, S. B., Hamborg, L., & Gauger, S. J. (2021). GHB analogs confer neuroprotection through specific interaction with the CaMKIIα hub domain. *Proceedings of the National Academy of Sciences*, *118*(31), e2108079118.

Li, Q., Kuhn, C. M., Wilson, W. A., & Lewis, D. V. (2007). Effects of gamma hydroxybutyric acid on inhibition and excitation in rat neocortex. *Neuroscience*, *150*(1), 82-92.

Lingenhoehl, K., Brom, R., Heid, J., Beck, P., Froestl, W., Kaupmann, K., Bettler, B., & Mosbacher, J. (1999). Gamma-hydroxybutyrate is a weak agonist at recombinant GABA(B) receptors. *Neuropharmacology*, *38*(11), 1667-1673. https://doi.org/10.1016/s0028-3908(99)00131-8

Lingford-Hughes, A., Patel, Y., Bowden-Jones, O., Crawford, M. J., Dargan, P. I., Gordon, F., Parrott, S., Weaver, T., & Wood, D. M. (2016). Improving GHB withdrawal with baclofen: study protocol for a feasibility study for a randomised controlled trial. *Trials*, *17*(1), 472. https://doi.org/10.1186/s13063-016-1593-9

Luján, R., & Aguado, C. (2015). Localization and targeting of GIRK channels in mammalian central neurons. *International review of neurobiology*, *123*, 161-200.

Luscher, C., & Slesinger, P. A. (2010). Emerging roles for G protein-gated inwardly rectifying potassium (GIRK) channels in health and disease. *Nat Rev Neurosci*, *11*(5), 301-315. https://doi.org/10.1038/nrn2834

Lüscher, C., & Ungless, M. A. (2006). The mechanistic classification of addictive drugs. *PLoS medicine*, *3*(11), e437.

Madden, T. E., & Johnson, S. W. (1998). Gamma-hydroxybutyrate is a GABAB receptor agonist that increases a potassium conductance in rat ventral tegmental dopamine neurons. *J Pharmacol Exp Ther*, *287*(1), 261-265.

Maitre, M. (1997). The γ-hydroxybutyrate signalling system in brain: organization and functional implications. *Progress in neurobiology*, *51*(3), 337-361.

Maitre, M., Klein, C., & Mensah-Nyagan, A. G. (2016). Mechanisms for the Specific Properties of gamma-Hydroxybutyrate in Brain. *Med Res Rev*, *36*(3), 363-388. https://doi.org/10.1002/med.21382

Martellotta, M. C., Fattore, L., Cossu, G., & Fratta, W. (1997). Rewarding properties of gamma-hydroxybutyric acid: an evaluation through place preference paradigm. *Psychopharmacology*, *132*, 1-5.

Mathivet, P., Bernasconi, R., De Barry, J., Marescaux, C., & Bittiger, H. (1997). Binding characteristics of gamma-hydroxybutyric acid as a weak but selective GABAB receptor agonist. *Eur J Pharmacol*, *321*(1), 67-75. https://doi.org/10.1016/s0014-2999(96)00916-8

McDonough, M., Kennedy, N., Glasper, A., & Bearn, J. (2004). Clinical features and management of gamma-hydroxybutyrate (GHB) withdrawal: a review. *Drug Alcohol Depend*, *75*(1), 3-9. https://doi.org/10.1016/j.drugalcdep.2004.01.012

Morris, M. E., Hu, K., & Wang, Q. (2005). Renal clearance of γ-hydroxybutyric acid in rats: increasing renal elimination as a detoxification strategy. *The Journal of pharmacology and experimental therapeutics*, *313*(3), 1194-1202.

Morse, B. L., & Morris, M. E. (2013). Effects of monocarboxylate transporter inhibition on the oral toxicokinetics/toxicodynamics of gamma-hydroxybutyrate and gamma-butyrolactone. *J Pharmacol Exp Ther*, *345*(1), 102-110. https://doi.org/10.1124/jpet.112.202796

Morse, B. L., Vijay, N., & Morris, M. E. (2012). gamma-Hydroxybutyrate (GHB)-induced respiratory depression: combined receptor-transporter inhibition therapy for treatment in GHB overdose. *Mol Pharmacol*, *82*(2), 226-235. https://doi.org/10.1124/mol.112.078154

Munir, V. L., Hutton, J. E., Harney, J. P., Buykx, P., Weiland, T. J., & Dent, A. W. (2008). Gamma-hydroxybutyrate: a 30 month emergency department review. *Emergency Medicine Australasia*, *20*(6), 521-530.

Nelson, T., Kaufman, E., Kline, J., & Sokoloff, L. (1981). The extraneural distribution of γ-hydroxybutyrate. *Journal of neurochemistry*, *37*(5), 1345-1348.

Ogeil, R. P., Faulkner, A., Beard, N., Killian, J. J., Arunogiri, S., Phan, V., Wilson, J., Smith, K., Lubman, D. I., & Scott, D. (2023). Adverse outcomes of GHB use: analysis of ambulance attendances in metropolitan and regional Victoria. *International Journal of Drug Policy*, *111*, 103932.

Palatini, P., Tedeschi, L., Frison, G., Padrini, R., Zordan, R., Orlando, R., Gallimberti, L., Gessa, G., & Ferrara, S. (1993). Dose-dependent absorption and elimination of gamma-hydroxybutyric acid in healthy volunteers. *European Journal of Clinical Pharmacology*, *45*, 353-356.

Pedraza, C., Garcia, F. B., & Navarro, J. F. (2009). Neurotoxic effects induced by gammahydroxybutyric acid (GHB) in male rats. *Int J Neuropsychopharmacol*, *12*(9), 1165-1177. https://doi.org/10.1017/S1461145709000157

Pistis, M., Muntoni, A., Pillolla, G., Perra, S., Cignarella, G., Melis, M., & Gessa, G. (2005). γ-Hydroxybutyric acid (GHB) and the mesoaccumbens reward circuit: Evidence for GABAB receptor-mediated effects. *Neuroscience*, *131*(2), 465-474.

Raposo Pereira, F., McMaster, M. T. B., Polderman, N., de Vries, Y., van den Brink, W., & van Wingen, G. A. (2018). Adverse effects of GHB-induced coma on long-term memory and related brain function. *Drug and alcohol dependence*, *190*, 29-36.

Raybon, J. J., & Boje, K. M. (2007). Pharmacokinetics and pharmacodynamics of γ-hydroxybutyric acid during tolerance in rats: effects on extracellular dopamine. *The Journal of pharmacology and experimental therapeutics*, *320*(3), 1252-1260.

Ren, X., & Mody, I. (2006). γ-Hydroxybutyrate induces cyclic AMP-responsive element-binding protein phosphorylation in mouse hippocampus: An involvement of GABAB receptors and cAMP-dependent protein kinase activation. *Neuroscience*, *141*(1), 269-275.

Sircar, R., & Basak, A. (2004). Adolescent γ-hydroxybutyric acid exposure decreases cortical N-methyl-D-aspartate receptor and impairs spatial learning. *Pharmacology Biochemistry and Behavior*, *79*(4), 701-708.

Sircar, R., Basak, A., & Sircar, D. (2008). Gamma-hydroxybutyric acid-induced cognitive deficits in the female adolescent rat. *Ann N Y Acad Sci*, *1139*, 386-389. https://doi.org/10.1196/annals.1432.044

Sircar, R., Basak, A., Sircar, D., & Wu, L. C. (2010). Effects of gamma-hydroxybutyric acid on spatial learning and memory in adolescent and adult female rats. *Pharmacol Biochem Behav*, *96*(2), 187-193. https://doi.org/10.1016/j.pbb.2010.04.028

Sircar, R., & Ishiwari, K. (2014). Systemic administration of gamma-hydroxybutyric acid in adolescent rat impairs contextual fear conditioning, but not cued conditioning. *J Drug Alcohol Res*, *3*, 235801.

Sircar, R., Wu, L. C., Reddy, K., Sircar, D., & Basak, A. K. (2011). GHB-Induced Cognitive Deficits During Adolescence and the Role of NMDA Receptor. *Curr Neuropharmacol*, *9*(1), 240-243. https://doi.org/10.2174/157015911795017038

Snead, O. C., 3rd. (2000). Evidence for a G protein-coupled gamma-hydroxybutyric acid receptor. *J Neurochem*, *75*(5), 1986-1996. https://doi.org/10.1046/j.1471-4159.2000.0751986.x

Snead, O. C., 3rd, Furner, R., & Liu, C. C. (1989). In vivo conversion of gamma-aminobutyric acid and 1,4-butanediol to gamma-hydroxybutyric acid in rat brain. Studies using stable isotopes. *Biochem Pharmacol*, *38*(24), 4375-4380. https://doi.org/10.1016/0006-2952(89)90645-x

Snead, O. C., 3rd, & Gibson, K. M. (2005). Gamma-hydroxybutyric acid. *N Engl J Med*, *352*(26), 2721-2732. https://doi.org/10.1056/NEJMra044047

Stein, L., Lebeau, R., Clair, M., Martin, R., Bryant, M., Storti, S., & Monti, P. (2011). A web-based study of gamma hydroxybutyrate (GHB): Patterns, experiences, and functions of use. *The American Journal on Addictions*, *20*(1), 30-39.

Tremblay, H., Godbout, R., Girodias, V., Schmitt, M., & Bourguignon, J.-J. (1998). Effects of gamma-hydroxybutyrate on ventral tegmental unit activity in the rat: considerations on REM sleep control. *Sleep Research Online*, *1*(4), 152-158.

Trimbos-instituut. (2025). *Nationale Drug Monitor*. Trimbos-instituut.

van Amsterdam, J. G. C., Brunt, T. M., McMaster, M. T. B., & Niesink, R. J. M. (2012). Possible long-term effects of γ-hydroxybutyric acid (GHB) due to neurotoxicity and overdose. *Neuroscience & Biobehavioral Reviews*, *36*(4), 1217-1227.

van Amsterdam, J. G. C., Brunt, T. M., Pereira, F. R., Crunelle, C. L., & van den Brink, W. (2022). Cognitive impairment following clinical or recreational use of Gammahydroxybutyric acid (GHB): a systematic review. *Current Neuropharmacology*, *20*(4), 809-819.

van Nieuwenhuijzen, P. S., Kashem, M. A., Matsumoto, I., Hunt, G. E., & McGregor, I. S. (2010). A long hangover from party drugs: residual proteomic changes in the hippocampus of rats 8 weeks after gamma-hydroxybutyrate (GHB), 3,4-methylenedioxymethamphetamine (MDMA) or their combination. *Neurochem Int*, *56*(8), 871-877. https://doi.org/10.1016/j.neuint.2010.03.002

van Nieuwenhuijzen, P. S., Long, L. E., Hunt, G. E., Arnold, J. C., & McGregor, I. S. (2010). Residual social, memory and oxytocin-related changes in rats following repeated exposure to gamma-hydroxybutyrate (GHB), 3,4-methylenedioxymethamphetamine (MDMA) or their combination. *Psychopharmacology (Berl)*, *212*(4), 663-674. https://doi.org/10.1007/s00213-010-1986-5

van Noorden, M. S., Mol, T., Wisselink, J., Kuijpers, W., & Dijkstra, B. A. (2017). Treatment consumption and treatment re-enrollment in GHB-dependent patients in The Netherlands. *Drug and alcohol dependence*, *176*, 96-101.

Van Sassenbroeck, D. K., De Neve, N., De Paepe, P., Belpaire, F. M., Verstraete, A. G., Calle, P. A., & Buylaert, W. A. (2007). Abrupt awakening phenomenon associated with gamma-hydroxybutyrate use: a case series. *Clinical Toxicology*, *45*(5), 533-538.

Vayer, P., Mandel, P., & Maitre, M. (1985). Conversion of gamma-hydroxybutyrate to gamma-aminobutyrate in vitro. *J Neurochem*, *45*(3), 810-814. https://doi.org/10.1111/j.1471-4159.1985.tb04065.x

Vijay, N., Morse, B. L., & Morris, M. E. (2015). A Novel Monocarboxylate Transporter Inhibitor as a Potential Treatment Strategy for gamma-Hydroxybutyric Acid Overdose. *Pharm Res*, *32*(6), 1894-1906. https://doi.org/10.1007/s11095-014-1583-0

Watson, J., Guzzetti, S., Franchi, C., Di Clemente, A., Burbassi, S., Emri, Z., Leresche, N., Parri, H. R., Crunelli, V., & Cervo, L. (2010). Gamma-hydroxybutyrate does not maintain self-administration but induces conditioned place preference when injected in the ventral tegmental area. *International Journal of Neuropsychopharmacology*, *13*(2), 143-153.

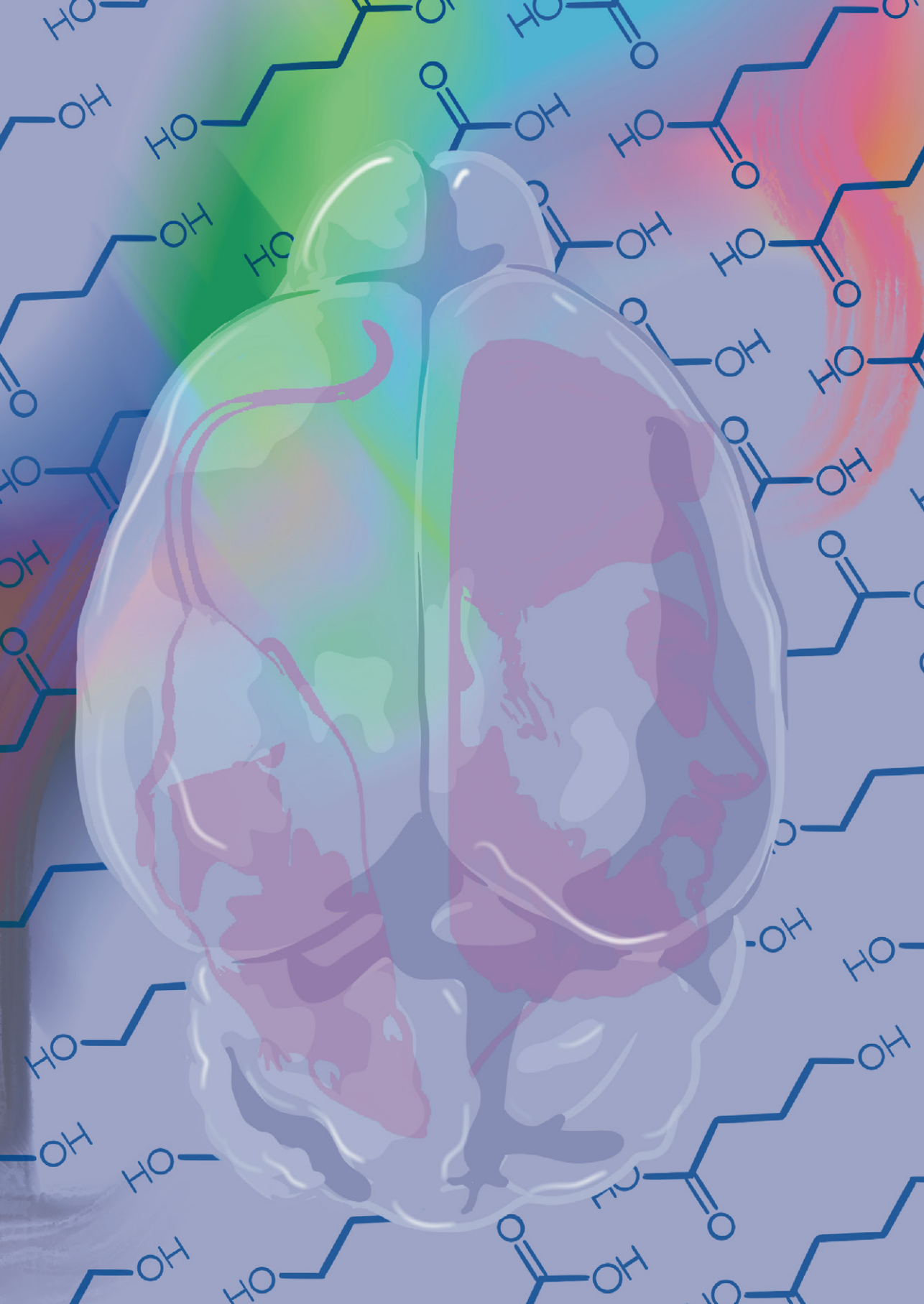
Wong, C. G., Gibson, K. M., & Snead, O. C., 3rd. (2004). From the street to the brain: neurobiology of the recreational drug gamma-hydroxybutyric acid. *Trends Pharmacol Sci*, *25*(1), 29-34. https://doi.org/10.1016/j.tips.2003.11.001

Wood, D. M., Greene, S. L., & Dargan, P. I. (2013). Five-year trends in self-reported recreational drugs associated with presentation to a UK emergency department with suspected drug-related toxicity. *European Journal of Emergency Medicine*, *20*(4), 263-267.

Xie, X., & Smart, T. G. (1992). Gamma-hydroxybutyrate hyperpolarizes hippocampal neurones by activating GABAB receptors. *Eur J Pharmacol*, *212*(2-3), 291-294. https://doi.org/10.1016/0014-2999(92)90347-7

Youn, S.-C., Chen, L.-Y., Chiou, R.-J., Lai, T.-J., Liao, W.-C., Mai, F.-D., & Chang, H.-M. (2015). Comprehensive application of time-of-flight secondary ion mass spectrometry (TOF-SIMS) for ionic imaging and bio-energetic analysis of club drug-induced cognitive deficiency. *Scientific Reports*, *5*(1), 18420.

Zalcman, G., Federman, N., & Romano, A. (2018). CaMKII Isoforms in Learning and Memory: Localization and Function. *Front Mol Neurosci*, *11*, 445. https://doi.org/10.3389/fnmol.2018.00445



Chapter 2

An overview of the putative structural and functional properties of the GHBh1 receptor through a bioinformatics approach

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Published in: Life

Abstract

The neurotransmitter gamma-hydroxybutyric acid (GHB) is suggested to be involved in neuronal energy homeostasis processes, but the substance is also used as a recreational drug and as a prescription medication for narcolepsy. GHB has several high-affinity targets in the brain, commonly generalized as the GHB receptor. However, little is known about the structural and functional properties of GHB receptor subtypes. This opinion article discusses the literature on the putative structural and functional properties of the GHBh1 receptor subtype. GHBh1 contains 11 transmembrane helices and at least one intracellular intrinsically disordered region (IDR). Additionally, GHBh1 shows a 100% overlap in amino acid sequence with the Riboflavin (vitamin B2) transporter, which opens the possibility of a possible dual-function (transceptor) structure. Riboflavin and GHB also share specific neuroprotective properties. Further research into the GHBh1 receptor subtype may pave the way for future therapeutic possibilities for GHB.

Introduction

Gamma-hydroxybutyric acid (GHB) is a neurotransmitter that is both a precursor and metabolite for the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). GHB has a high direct affinity for various binding sites in the brain, commonly referred to as the GHB receptor, next to a low direct affinity for the inhibitory $GABA_B$ receptor (Maitre, Klein, & Mensah-Nyagan, 2016). Endogenous GHB occurs in the mammalian brain in concentrations that exclusively activate the GHB receptor and is implicated in energy homeostasis processes on both a micro- (neuronal energy homeostasis) and macro level (sleep-wake homeostasis) (Mamelak, 1989). A specific subtype of the rat GHB receptor is primarily located in the hippocampus, frontal cortex, piriform cortex and cerebellum, and in a lesser extent in the striatum, olfactory bulb and thalamus (Kemmel, Miehe, Roussel, Taleb, Nail-Boucherie, et al., 2006). Low (endogenous) doses of GHB have been shown to increase glutamatergic signaling through GHB receptor activation (Banerjee & Snead, 1995; Ferraro et al., 2001; Castelli et al., 2003). In contrast, exogenous GHB dose-dependently activates the $GABA_B$ receptor, and is used medically for the treatment of narcolepsy (Carter, Koek, et al., 2009). GHB is also used for recreational purposes due to its dose-dependent euphoric, disinhibiting and sedative properties, and may lead to a severe substance use disorder (Dijkstra et al., 2017; Beurmanjer et al., 2019; Dijkstra et al., 2021). Although the structural and functional properties of the $GABA_B$ receptor have been extensively studied, there is no clear overview of the structural and functional properties of the GHB receptor.

There are several structures that have a high affinity for GHB (or its analogs NCS-382/Ph-HBTA) (Maitre et al., 1990; Tian et al., 2022), indicating the existence of several GHB receptor subtypes (Crunelli et al., 2006; Andriamampandry et al., 2007). Recently, an elegant paper was published on a high-affinity target of GHB, identified as the CamKIIα hub domain (Leurs et al., 2021). However, already in 2002, an incorrectly spliced form of a GHB-binding structure containing eight transmembrane domains was identified through a human genome database search (Takeda et al., 2002). In 2003, the full-length variant of this GHB-binding structure was first identified as a porcine endogenous retrovirus receptor (Ericsson et al., 2003), that later was classified as a (closely related) subtype of the human GHB (GHBh1) receptor. The authors claimed that this full-length protein contained 10 or 11 transmembrane helices (Ericsson et al., 2003). In the same year, Andriamampandry and colleagues cloned a putative rat GHB receptor that had high affinity for GHB, but did not correspond with the pharmacological properties or localization of native GHB sites (Andriamampandry et al., 2003; Crunelli et al., 2006; Bay et al., 2014). Finally, Andriamampandry et al. cloned a GHB receptor (GHBh1) in 2007 using polymerase chain reaction (PCR), based on human frontal cortex cDNA and

primers from the putative rat GHB receptor (Andriamampandry et al., 2007). Through subsequent DNA sequencing and database searches (basic local alignment search tool (BLAST) at NCBI), the GHBh1 receptor was identified as a G-protein coupled receptor 172A, although several studies could not find an activation of G protein by GHB (Castelli et al., 2003; Odagaki & Yamauchi, 2004). Despite the identification of the GHBh1 receptor, the identity of high-affinity GHB binding sites is still under debate (Bay et al., 2014; Maitre, Klein, & Mensah-Nyagan, 2016; Leurs et al., 2021), where it is possible that high-affinity GHB binding sites in the brain consist of multiple subpopulations of various protein structures.

In vitro it has been established that the GHBh1 receptor is activated by low doses of GHB and NCS-382, a ligand that specifically binds to GHB binding sites (Castelli et al., 2004; Andriamampandry et al., 2007). Nonetheless, the functional significance of GHBh1 in terms of the pharmacological effects of GHB in humans is unclear and remains speculative. In 2010, Yao et al. classified this structure as a riboflavin transporter (hRFT3), which was suggested to have 10 TM domains (Yao et al., 2010; Subramanian et al., 2015; "UniProt: The universal protein knowledgebase in 2021," 2021). However, there is currently still limited knowledge on GHB high-affinity binding sites and the putative GHBh1 in specific. Both the localization and function of the GHBh1 receptor is unknown, and its structural properties are unclear. The aim of the current work is to provide an overview of the structural and functional properties of the GHBh1 receptor, by using a bioinformatics approach and employing a literature search. Specifically, we will describe the transmembrane structure and intrinsically disordered regions (IDRs) of the GHBh1 receptor, and examine the functional overlap between the GHBh1 receptor and the riboflavin transporter. To further explore possible functional properties of the GHBh1 receptor, we also provide an overview of proteins that may be able to interact with the GHBh1 receptor. Finally, we speculate on the possible clinical role of the GHBh1 receptor.

Structural properties of the GHBh1 receptor

We used the amino acid sequence of the GHBh1 receptor, published by Andriamampandry et al. (2007), to assess the putative structure of this receptor (accession code Q9HAB3) (Andriamampandry et al., 2007). Several predictors, next to the Uniprot/Swissprot database, were used to identify various characteristics of the receptor, including the number of transmembrane (TM) helices, terminus orientation and IDRs (see Table 1). Using Protter, an interactive protein visualization tool based on the Uniprot database and Phobius predictor, the two-dimensional receptor structure of the GHB receptor was visualized (Omasits et al., 2014) (Fig. 1).

The majority of predictors showed that the GHBh1 receptor was composed of 11 TM helices. Uniprot and two predictors indicated the presence of either one or two IDRs (see Table 1). IDRs are characterized by a large degree of structural adaptability, allowing for the interaction with multiple and structurally diverse ligands. This is in contrast to structured proteins or structured regions that are only able to interact with one specific structure, according to the lock & key principle (Wright & Dyson, 2015). All predictors showed an intracellular N-terminus orientation. With an intracellular N-terminus orientation, it is likely that the loop between TM5 and TM6 serves as a ligand binding site, whereas the intracellular loop between TM6 and TM7 might be involved in intracellular signaling cascades. The long extracellular tail containing the C-terminus might also serve as a ligand binding domain. The predictions of the secondary structure of the GHB receptor shown in Table 1 all showed a large overlap with the structure presented in Figure 1.

Table 1. Overview of the number of transmembrane helices, terminus orientation and IDRs assessed by various predictors and the Uniprot / Swissprot database

Predictor / database	**Transmembrane helices**	**N-terminus intra-cellular/ extracellular**	**Intrinsically disordered regions**
Alphafold 2 (Jumper et al., 2021)	11	NA	NA
Phobius (Käll et al., 2007)	10	Extracellular	NA
Predict Protein (Bernhofer et al., 2021)	11	NA	1 (intracellular, 237-261, between TM6 and TM7)
Psipred (Jones & Cozzetto, 2015; Buchan & Jones, 2019)	11	Intracellular	2 (intracellular, 1-11, before TM 1, and 223-270, between TM6 and TM7)
MEMSAT 3 (Jones, 2007)	11	Intracellular	NA
TMHMM (Krogh et al., 2001)	11	Intracellular	NA
TM-pred (Hofmann, 1993)	10	Intracellular	NA
Uniprot ("UniProt: The universal protein knowledgebase in 2021," 2021)	11	NA	1 (intracellular, 228-264, between TM6 and TM7)

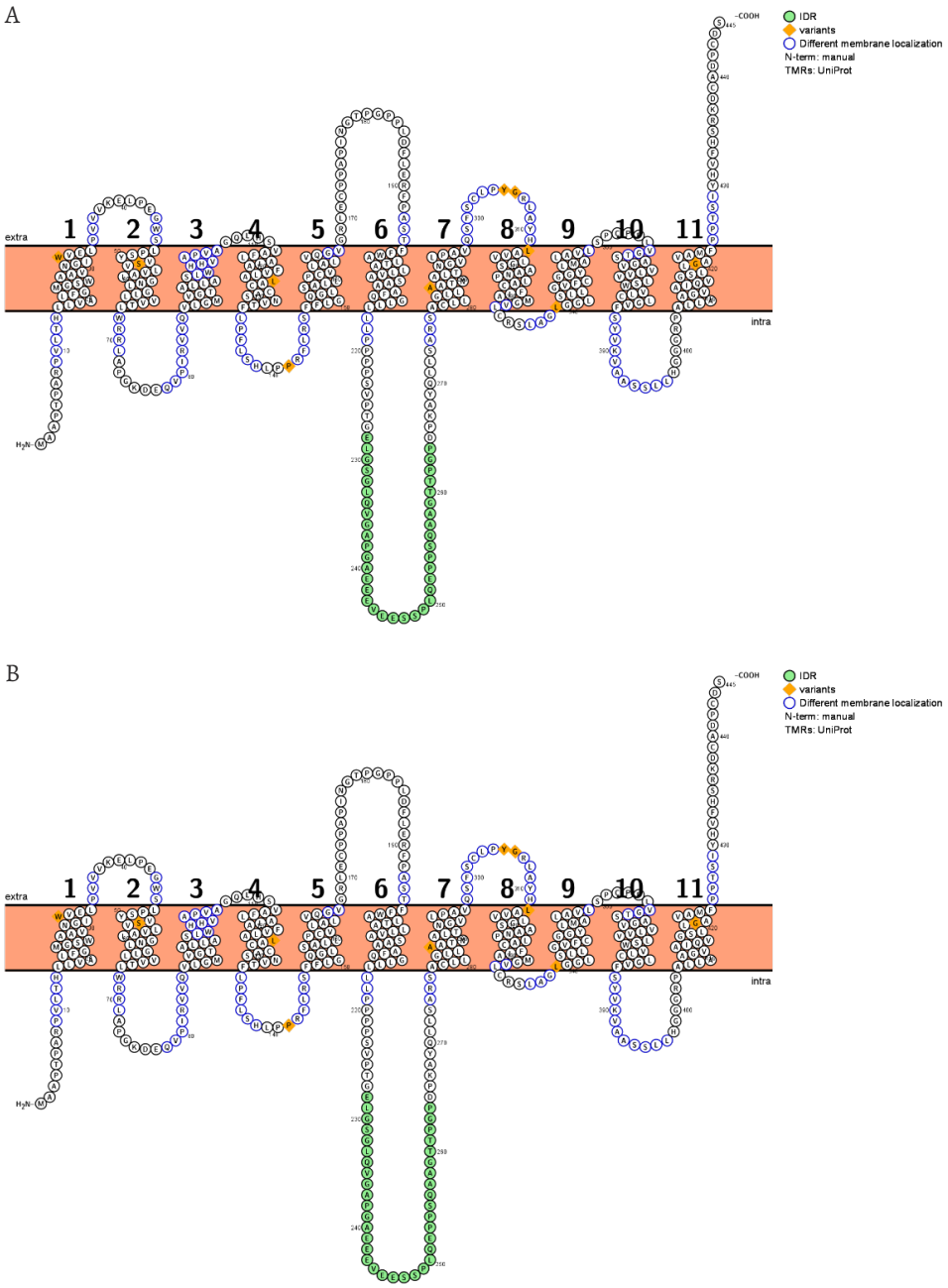


Figure 1. Two-dimensional structure of the GHBh1 receptor based on the A) Uniprot database, and the B) Alphafold 2 model, both with an intracellular N-terminus (Omasits et al., 2014). IDR is highlighted in green. Each letter represents a single amino acid. Differences in TM localization of amino acids are circled in blue. The orange bar represents the cell membrane, and numbers above the cellular membrane represent the number of transmembrane units. Extra = extracellular, intra = intracellular.

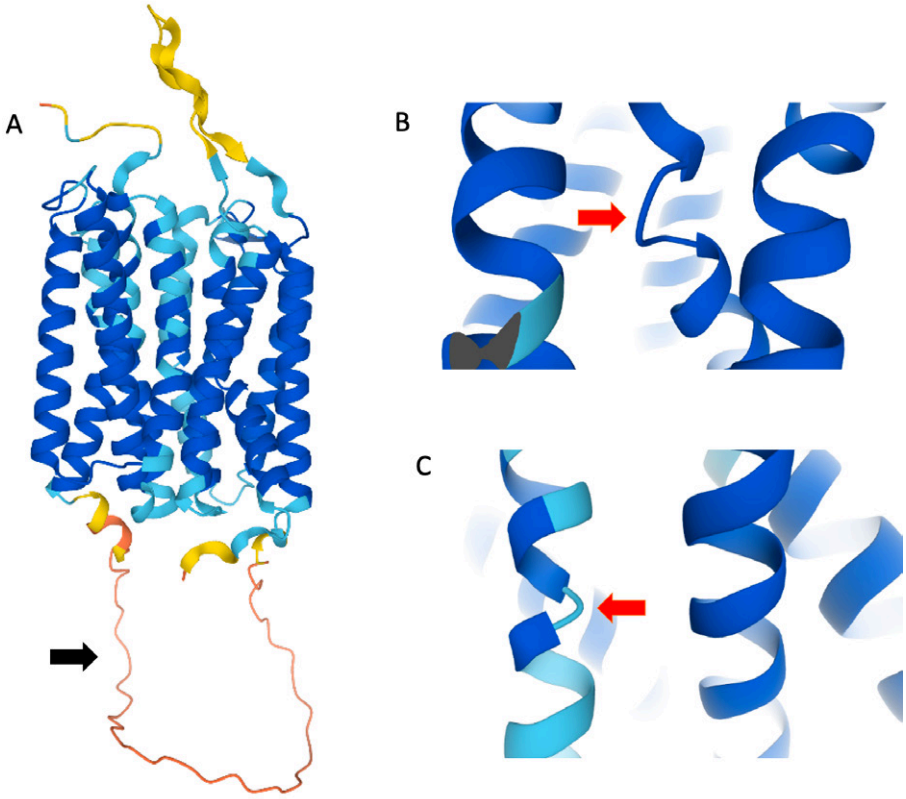


Figure 2. A) Three-dimensional structure of the GHBh1 receptor (Jumper et al., 2021; Varadi et al., 2022). The loop between TM6 and TM7 is indicated with a black arrow. B) Close-up of the bend present in TM1, indicated with a red arrow. C) Close-up of the bend present in TM2, indicated with a red arrow. Colors indicate per-residue confidence scores ranging from 0-100 (pLDDT). Dark blue: very high (pLDDT > 90), light blue: high (90 > pLDDT > 70), yellow: low (70 > pLDDT > 50), orange: very low (pLDDT < 50). The shown structures were arbitrarily selected from the 3D model to visualize the TM6-TM7 loop, TM1 bend and TM2 bend in the clearest way possible.

In addition to the two-dimensional predictions of the GHBh1, the recently published Alphafold 2 model allows us to also assess the three-dimensional structure of the GHBh1 receptor (Fig. 2A) (Jumper et al., 2021). In accordance with the majority of other predictors, the Alphafold 2 model confirms that the GHB receptor contains 11 transmembrane helices (Varadi et al., 2022). It appears that the start- and endpoints of the TM helices slightly differ between the Alphafold 2 model and the Uniprot database (Fig. 1). This in its turn leads to a difference in the length of some loops between TMs. Alphafold 2 shows very low confidence in the structural prediction of the large loop between TM6-TM7, suggesting that the conformation of these residues will likely not resemble the model and indicating the presence of an IDR (Fig. 2A). An IDR between TM6-TM7 is also predicted by Psipred and Predict Protein (Table 1).

When we further zoom in on the three-dimensional properties of the receptor, we observe a bend within both TM1 (Fig. 2B) and TM2 (Fig. 2C). These bends are common in transmembrane helices, and are believed to be involved in helix flexibility and conformational changes (Cao & Bowie, 2012; Högel et al., 2018). Overall, the Alphafold 2 model shows primarily overlapping characteristics with other (two-dimensional) predictions of the GHBh1 receptor, and provides more detailed information on the three-dimensional conformation of the GHBh1 receptor.

GHB receptor and riboflavin transporter

The GHBh1 receptor amino acid sequence showed a 100% overlap in identity with the Riboflavin transporter sequence, or the solute carrier family 52, member 2 (SLC52A2), also known as human riboflavin transporter 3 (hRFT3) (Andriamampandry et al., 2007; Yao et al., 2010). A recent study employed homology modeling with three template structures to construct a three-dimensional model of hRFT3, revealing strong similarities with the Alphafold2 model of the GHBh1 receptor (Console et al., 2022).

Riboflavin, also known as vitamin B2, is a water-soluble micronutrient that is not naturally present in the human body, but can be obtained through the intestinal absorption of several food products, such as dairy products, meat, fish, fruit and vegetables (Powers, 2003). Following intestinal absorption, riboflavin is eventually transported from the extracellular environment into the cell through a membrane transport protein (or transporter). Riboflavin is thought to be involved in a variety of cellular energy homeostasis processes in peripheral- and brain tissue. More specifically, it plays a vital role in several pathways in mitochondria, such as oxidative phosphorylation of the electron transport chain, the Kreb's cycle and the tricarboxylic acid cycle (TCA) cycle. These key pathways are involved in the production of ATP, responsible for membrane stability and energy-related cellular functions (Powers, 2003; Udhayabanu et al., 2017).

The hRTF3 structure is primarily present in the brain and salivary gland, and has been implicated in neurological disorders such as spinocerebellar ataxia and Brown -Vialleto-Van Laere (BVVL) syndrome (Babanejad et al., 2018). hRFT3 and has been previously characterized as both a 10 and 11 TM structure (Yonezawa & Inui, 2013; Jin & Yonezawa, 2022). hRFT3 can also bind, but not transport, the riboflavin co-enzymes Flavin Adenine Dinucleotide (FAD) and Flavin Mononucleotide (FMN) with low affinity (Yonezawa & Inui, 2013). FMN, but also lumiflavin and Mg2+ are able to inhibit riboflavin binding and transport (Console et al., 2019). Riboflavin transport

through hRTF3 is suggested to be Ca2+ dependent (Console et al., 2019). hFRT3 has a strong homology with other riboflavin transporters including hRFT1 and hRFT2, which are primarily present in placenta/small intestine and testis/small intestine/prostate respectively (Yonezawa & Inui, 2013).

At first sight, it is surprising that a GHB receptor and a riboflavin transporter consist of an identical amino acid sequence, since transporters and receptors strongly differ in functionality. A transporter mediates the uptake of a specific ligand by the cell, after which the ligand itself can act as an intracellular signaling molecule. In contrast, a receptor interacts with an external ligand, leading to a conformational change of the receptor and inducing downstream intracellular processes. However, there is evidence for the existence of a structure functionally linking transporters and receptors, called a transceptor (Diallinas, 2017).

The first evidence of the concept transceptors was presented by Johan Thevelein at a conference in Spain in 1999 (and later published by his group in 2003), where he showed evidence of a cellular sensing mechanism (Gap1) that operates through transporters in *Saccharomyces cerevisiae* (Donaton et al., 2003). Transceptors combine both transporter and receptor functions, while these two functions are generally independent from each other. Known transceptors include structures specific for phosphate (Pho84), ammonium (Mep2), sulfate (Sul1 and Sul2), iron (Ftr1) and zinc (Zrt1). Transceptors are usually highly induced upon micronutrient starvation, and downregulated by substrate-induced endocytosis (Diallinas, 2017).

Transceptors generally have partially overlapping binding sites in the same substrate-binding pocket. The subsequent effect depends on the specific ligand that binds. The IDR between TM6 and TM7 might play a crucial role in the ability of the GHB/riboflavin transceptor to mediate the dual-function of the structure. Figure 1 shows that the IDR between TM6 and TM7 is likely situated within the cytoplasm, as with the majority of transmembrane IDRs (Minezaki et al., 2007; Xue et al., 2009). The intracellular orientation of the IDR makes the role of a ligand-binding site unlikely, but indicates a role of the IDR in the flexible initiation of intracellular signaling cascades. Upon interaction of the IDR with an intracellular compound, the IDR might form a stable structure around the compound. When multiple compounds are bound to the IDR, it may even mediate and accelerate interaction between these ligands (Oldfield & Dunker, 2014).

Intracellular IDRs have been implicated in the regulation of transmembrane protein function and -activity. These IDRs are likely involved in the recruitment of

proteins that are activated by signaling molecules, such as kinases and phosphatases (Kjaergaard & Kragelund, 2017). One hypothesis could be that the extracellular binding ligand determines which intracellular proteins are bound to the IDR, and consequently determines which intracellular pathways are triggered upon transceptor activation.

The question remains how both GHB and riboflavin, which are two structurally distinct compounds, can bind to one structure. It is possible that the C-terminus tail may also act as a ligand binding site, since it has been shown that the deletion of the C-terminal region of hRFT3 leads to impairment of transport function (Subramanian et al., 2015). However, in this study, the C-terminal was predicted to be situated in the cytoplasm, hampering direct translation of the results. Future studies should examine the binding characteristics of GHB and riboflavin at the transceptor and experimentally confirm the binding site localization and dual function of this structure.

Functional properties of GHBh1 binding proteins

Although the existence of a dual-function binding site for GHB and riboflavin has not been experimentally verified, GHB and riboflavin independently show overlapping functional properties. Riboflavin and GHB are both suggested to have neuroprotective properties under energy-deprived circumstances, such as oxygen deprivation (ischemia). It has been shown that riboflavin protects tissue against ischemia-reperfusion injury in the brain (Hultquist et al., 1993; Betz et al., 1994; Mack et al., 1995). Riboflavin, in combination with other vitamin supplements, has also been shown to lead to improvement in Erythrocyte Glutathione Reductase (EGR) during alcohol withdrawal, increasing levels of the anti-oxidant glutathione and decreasing oxidative stress (Brown et al. 1982). Similar to riboflavin, GHB protects against ischemia-reperfusion damage, through improved cerebral blood flow, cerebral vascular density, and improved pCO_2 and pO_2 in the ischemic region (Wu et al., 2018). It has been shown that GHB can exert its neuroprotective effects following ischemia through CamKIIα activation (Leurs et al., 2021). Although GHB is used as a pharmaceutical drug, it is unknown whether GHB is able to (indirectly) influence antioxidant activity. The role of the GHBh1 receptor in the neuroprotective properties of GHB and riboflavin remains elusive.

In order to better understand the functions of the GHBh1 receptor, we can take a look at other proteins that may bind the GHBh1 receptor. Using the publicly

available database Biological General Repository for Interaction Datasets (BioGRID) (Oughtred et al., 2021), we summarized a list of proteins with known functions that are suggested to interact with GHBh1 receptor (Table 2). We did not show interactors that were identified based on genetic interactions, nor structures that interacted with possible downstream proteins of the GHBh1 receptor. Consequentially, Table 2 shows putative interactors identified through either a protein-fragment complementation assay (PCA), affinity capturing, or a combination of Transcriptional activation domain (TAD) + DNA binding domain (DBD) interaction.

The putative GHBh1 receptor interactors can be roughly divided into 3 classes: interactors involved in the immune response (SPPL2B, TRIM25), proteins involved in the cell cycle (CDC23, UPK1A), and mitochondrial proteins (ATP13A1 and GHITM), which is in line with the key role of riboflavin in several mitochondrial pathways. The functional implications of other interactors are not intuitive based on our current knowledge of GHB, riboflavin or the GHBh1 receptor, but may encourage further exploration of these protein interactions.

Table 2. Overview of possible interactors of the GHBh1 receptor structure (Oughtred et al., 2021)

Interactor	Description	Function
SPPL2B (Huttlin et al., 2017; Huttlin et al., 2021)	Signal peptide peptidase like 2B	Involved in immune response by cleaving TNFα in dendritic cells
ATP13A1 (Huttlin et al., 2021)	ATPase type 13A1	Mediates removal/extraction of mislocalized mitochondrial transmembrane proteins from the endoplasmic reticulum membrane
CDC23 (Rolland et al., 2014)	Cell division cycle 23	Part of a ubiquitin ligase that controls progression through mitosis
FAM209A (Luck et al., 2020)	Family with sequence similarity 209, member A	May play a role in sperm acrosome biogenesis
GHITM (Huttlin et al., 2021)	Growth hormone inducible transmembrane protein	Plays a role in apoptosis through mediating mitochondrial morphology and cytochrome c release
TRIM25 (Choudhury et al., 2017)	Tripartite motif containing 25	Ubiquitin ligase regulating the innate immune response
UPK1A (Huttlin et al., 2017)	Uroplakin 1A	Mediating signal transduction events involved in regulating cell development, activation, growth and motility

Clinical implications and limitations

To speculate on the role of GHBh1 in the pharmacological effects of GHB in humans, we can take a look at the cellular response characteristics of GHBh1. GHB patch clamping experiments have shown that activation of the GHBh1 by a low dose of GHB leads to a clear cellular response, which is rapidly followed by a prolonged period of desensitization (Andriamampandry et al., 2007). This indicates slow recovery kinetics following desensitization or internalization, as also often observed with transceptors upon repeated substrate binding (Diallinas, 2017). The high affinity of GHB for GHBh1, in combination with the rapid and long desensitization of the receptor, make it improbable that the GHBh1 receptor is involved in the (GABAergic) response following high, exogenous doses of GHB. Considering the use of high doses of GHB in patients with GHB use disorder (GUD), it is unlikely that GHBh1 receptor activation plays a significant role in the characteristics of GUD (such as comas, severe withdrawal symptoms or high relapse rates). However, the GHBh1 receptor may play a role in the cellular effects of exogenous GHB. Opposing results have been reported on the cellular effects of exogenous GHB. On the one hand, there are indications that GHB use leads to neuronal damage and cognitive deterioration (van Nieuwenhuijzen, Long, et al., 2010; Johansson et al., 2014; Raposo Pereira et al., 2018). On the other hand, GHB is safely used as a treatment for the neurological disorder narcolepsy, and tested for the treatment of alcohol use disorder (Boscolo-Berto et al., 2012; Guiraud et al., 2021). Future studies should examine the functional significance of GHBh1 in terms of the pharmacological effects of GHB in humans.

The findings and interpretations presented in this opinion article should be considered in the light of some limitations. In this paper, we discussed the GHBh1 amino acid sequence as retrieved by Andriamampandry et al. (2007) as a high-affinity GHB receptor (Andriamampandry et al., 2007). It should be noted that other subtypes of the GHB receptor exist, such as C12K32 and CamKIIα, which will exhibit distinct structural and functional characteristics (Andriamampandry et al., 2007). This opinion article solely addresses the GHBh1 subtype, and our findings are likely not to be translatable to other GHB receptor subtypes. Additionally, the GHBh1 subtype has not been verified as a native GHB receptor. Although the GHBh1 receptor has been shown to bind low doses of GHB and the GHB receptor-specific antagonist NCS-382, it should be further examined whether the GHBh1 receptor shows overlap with native GHB sites. Despite the large overlap in results between predictors, some discrepancy can be found regarding the number of predicted TM helices. Phobius and TM-pred predicted 10 transmembrane helices with high certainty. It appears that TM-pred does not predict a TM helix within the region of 81-103, while Phobius

does not predict a TM helix within the region of 14-34. Instead, Phobius predicts a long extracellular amino acid chain that includes a signaling peptide chain at the N-terminus. Nevertheless, the majority of predictors, including the Alphafold 2 model, show a presence of 11 TM domains and show a large overlap in their outcomes, providing a solid basis for the findings that are presented in this opinion article. Future studies should experimentally verify the exact structure of the GHBh1 receptor, in order to further understand the functional mechanism of this structure.

In conclusion, the GHBh1 receptor contains 11 transmembrane units and contains at least one IDR. The amino acid sequence of the GHBh1 receptor is identical to the sequence of the riboflavin transporter, indicating the existence of a dual-function GHB/riboflavin transceptor. Proteins involved in the cell cycle, immune response, and mitochondrial processes may also interact with the GHBh1 receptor. This research provides a theoretical framework regarding the functional properties of the GHBh1 receptor, allowing experimental verification of our findings, which may lead to a further understanding of the possible (neuro)biological role of the GHBh1 receptor.

Bibliography

Andriamampandry, C., Taleb, O., Kemmel, V., Humbert, J. P., Aunis, D., & Maitre, M. (2007). Cloning and functional characterization of a gamma-hydroxybutyrate receptor identified in the human brain. *FASEB J*, *21*(3), 885-895. https://doi.org/10.1096/fj.06-6509com

Andriamampandry, C., Taleb, O., Viry, S., Muller, C., Humbert, J. P., Gobaille, S., Aunis, D., & Maitre, M. (2003). Cloning and characterization of a rat brain receptor that binds the endogenous neuromodulator γ-hydroxybutyrate (GHB). *FASEB J*, *17*(12), 1691-1693. https://doi.org/10.1096/fj.02-0846fje

Babanejad, M., Adeli, O. A., Nikzat, N., Beheshtian, M., Azarafra, H., Sadeghnia, F., Mohseni, M., Najmabadi, H., & Kahrizi, K. (2018). SLC52A2 mutations cause SCABD2 phenotype: A second report. *International journal of pediatric otorhinolaryngology*, *104*, 195-199.

Banerjee, P. K., & Snead, O. C., 3rd. (1995). Presynaptic gamma-hydroxybutyric acid (GHB) and gamma-aminobutyric acidB (GABAB) receptor-mediated release of GABA and glutamate (GLU) in rat thalamic ventrobasal nucleus (VB): a possible mechanism for the generation of absence-like seizures induced by GHB. *J Pharmacol Exp Ther*, *273*(3), 1534-1543.

Bay, T., Eghorn, L. F., Klein, A. B., & Wellendorph, P. (2014). GHB receptor targets in the CNS: focus on high-affinity binding sites. *Biochem Pharmacol*, *87*(2), 220-228. https://doi.org/10.1016/j.bcp.2013.10.028

Bernhofer, M., Dallago, C., Karl, T., Satagopam, V., Heinzinger, M., Littmann, M., Olenyi, T., Qiu, J., Schuetze, K., & Yachdav, G. (2021). PredictProtein-Predicting Protein Structure and Function for 29 Years. *bioRxiv*.

Betz, A. L., Ren, X., Ennis, S., & Hultquist, D. (1994). Riboflavin reduces edema in focal cerebral ischemia. In *Brain Edema IX* (pp. 314-317). Springer.

Beurmanjer, H., Asperslag, E. M., Oliemeulen, L., Goudriaan, A. E., De Jong, C. A. J., Schellekens, A. F. A., & Dijkstra, B. A. G. (2019). A Qualitative Approach in Understanding Illness Perception and Treatment Needs in Patients with Gamma Hydroxybutyrate Use Disorder. *Eur Addict Res*, *25*(5), 248-255. https://doi.org/10.1159/000500807

Boscolo-Berto, R., Viel, G., Montagnese, S., Raduazzo, D. I., Ferrara, S. D., & Dauvilliers, Y. (2012). Narcolepsy and effectiveness of gamma-hydroxybutyrate (GHB): a systematic review and meta-analysis of randomized controlled trials. *Sleep medicine reviews*, *16*(5), 431-443.

Buchan, D. W., & Jones, D. T. (2019). The PSIPRED protein analysis workbench: 20 years on. *Nucleic Acids Research*, *47*(W1), W402-W407.

Cao, Z., & Bowie, J. U. (2012). Shifting hydrogen bonds may produce flexible transmembrane helices. *Proceedings of the National Academy of Sciences*, *109*(21), 8121-8126.

Carter, L. P., Koek, W., & France, C. P. (2009). Behavioral analyses of GHB: receptor mechanisms. *Pharmacology & therapeutics*, *121*(1), 100-114.

Castelli, M. P., Ferraro, L., Mocci, I., Carta, F., Carai, M. A., Antonelli, T., Tanganelli, S., Cignarella, G., & Gessa, G. L. (2003). Selective γ-hydroxybutyric acid receptor ligands increase extracellular glutamate in the hippocampus, but fail to activate G protein and to produce the sedative/hypnotic effect of γ-hydroxybutyric acid. *Journal of neurochemistry*, *87*(3), 722-732.

Castelli, M. P., Pibiri, F., Carboni, G., & Piras, A. P. (2004). A Review of Pharmacology of NCS-382, a Putative Antagonist of γ-Hydroxybutyric Acid (GHB) Receptor. *CNS drug reviews*, *10*(3), 243-260.

Choudhury, N. R., Heikel, G., Trubitsyna, M., Kubik, P., Nowak, J. S., Webb, S., Granneman, S., Spanos, C., Rappsilber, J., & Castello, A. (2017). RNA-binding activity of TRIM25 is mediated by its PRY/SPRY domain and is required for ubiquitination. *BMC biology*, *15*(1), 1-20.

Console, L., Tolomeo, M., Colella, M., Barile, M., & Indiveri, C. (2019). Reconstitution in proteoliposomes of the recombinant human riboflavin transporter 2 (SLC52A2) overexpressed in E. coli. *International Journal of Molecular sciences*, *20*(18), 4416.

Console, L., Tolomeo, M., Cosco, J., Massey, K., Barile, M., & Indiveri, C. (2022). Impact of natural mutations on the riboflavin transporter 2 and their relevance to human riboflavin transporter deficiency 2. *IUBMB life*, *74*(7), 618-628.

Crunelli, V., Emri, Z., & Leresche, N. (2006). Unravelling the brain targets of γ-hydroxybutyric acid. *Curr Opin Pharmacol*, *6*(1), 44-52. https://doi.org/10.1016/j.coph.2005.10.001

Diallinas, G. (2017). Transceptors as a functional link of transporters and receptors. *Microbial cell*, *4*(3), 69.

Dijkstra, B. A. G., Beurmanjer, H., Goudriaan, A. E., Schellekens, A. F. A., & Joosten, E. A. G. (2021). Unity in diversity: A systematic review on the GHB using population. *Int J Drug Policy*, *94*, 103230. https://doi.org/10.1016/j.drugpo.2021.103230

Dijkstra, B. A. G., Kamal, R. M., van Noorden, M. S., de Haan, H., Loonen, A. J. M., & De Jong, C. A. J. (2017). Detoxification with titration and tapering in gamma-hydroxybutyrate (GHB) dependent patients: the Dutch GHB monitor project. *Drug and alcohol dependence*, *170*, 164-173.

Donaton, M. C., Holsbeeks, I., Lagatie, O., Van Zeebroeck, G., Crauwels, M., Winderickx, J., & Thevelein, J. M. (2003). The Gap1 general amino acid permease acts as an amino acid sensor for activation of protein kinase A targets in the yeast Saccharomyces cerevisiae. *Molecular microbiology*, *50*(3), 911-929.

Ericsson, T. A., Takeuchi, Y., Templin, C., Quinn, G., Farhadian, S. F., Wood, J. C., Oldmixon, B. A., Suling, K. M., Ishii, J. K., & Kitagawa, Y. (2003). Identification of receptors for pig endogenous retrovirus. *Proceedings of the National Academy of Sciences*, *100*(11), 6759-6764.

Ferraro, L., Tanganelli, S., O'Connor, W. T., Francesconi, W., Loche, A., Gessa, G. L., & Antonelli, T. (2001). γ-Hydroxybutyrate modulation of glutamate levels in the hippocampus: An in vivo and in vitro study. *Journal of neurochemistry*, *78*(5), 929-939.

Guiraud, J., Addolorato, G., Aubin, H.-J., Batel, P., de Bejczy, A., Caputo, F., Goudriaan, A. E., Gual, A., Lesch, O., & Maremmani, I. (2021). Treating alcohol dependence with an abuse and misuse deterrent formulation of sodium oxybate: Results of a randomised, double-blind, placebo-controlled study. *European Neuropsychopharmacology*, *52*, 18-30.

Hofmann, K. (1993). TMbase-A database of membrane spanning proteins segments. *Biol. Chem. Hoppe-Seyler*, *374*, 166.

Högel, P., Götz, A., Kuhne, F., Ebert, M., Stelzer, W., Rand, K. D., Scharnagl, C., & Langosch, D. (2018). Glycine perturbs local and global conformational flexibility of a transmembrane helix. *Biochemistry*, *57*(8), 1326-1337.

Hultquist, D. E., Xu, F., Quandt, K. S., Shlafer, M., Mack, C. P., Till, G. O., Seekamp, A., Betz, A. L., & Ennis, S. R. (1993). Evidence that NADPH-dependent methemoglobin reductase and administered riboflavin protect tissues from oxidative injury. *American journal of hematology*, *42*(1), 13-18.

Huttlin, E. L., Bruckner, R. J., Navarrete-Perea, J., Cannon, J. R., Baltier, K., Gebreab, F., Gygi, M. P., Thornock, A., Zarraga, G., & Tam, S. (2021). Dual proteome-scale networks reveal cell-specific remodeling of the human interactome. *Cell*, *184*(11), 3022-3040. e3028.

Huttlin, E. L., Bruckner, R. J., Paulo, J. A., Cannon, J. R., Ting, L., Baltier, K., Colby, G., Gebreab, F., Gygi, M. P., & Parzen, H. (2017). Architecture of the human interactome defines protein communities and disease networks. *Nature*, *545*(7655), 505-509.

Jin, C., & Yonezawa, A. (2022). Recent advances in riboflavin transporter RFVT and its genetic disease. *Pharmacology Therapeutics*, *233*, 108023.

Johansson, J., Grönbladh, A., & Hallberg, M. (2014). Gamma-hydroxybutyrate (GHB) induces cognitive deficits and affects GABAB receptors and IGF-1 receptors in male rats. *Behavioural brain research*, *269*, 164-174.

Jones, D. T. (2007). Improving the accuracy of transmembrane protein topology prediction using evolutionary information. *Bioinformatics*, *23*(5), 538-544.

Jones, D. T., & Cozzetto, D. (2015). DISOPRED3: precise disordered region predictions with annotated protein-binding activity. *Bioinformatics*, *31*(6), 857-863.

Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Žídek, A., & Potapenko, A. (2021). Highly accurate protein structure prediction with AlphaFold. *Nature*, *596*(7873), 583-589.

Käll, L., Krogh, A., & Sonnhammer, E. L. (2007). Advantages of combined transmembrane topology and signal peptide prediction—the Phobius web server. *Nucleic Acids Research*, *35*(suppl_2), W429-W432.

Kemmel, V., Miehe, M., Roussel, G., Taleb, O., Nail-Boucherie, K., Marchand, C., Stutz, C., Andriamampandry, C., Aunis, D., & Maitre, M. (2006). Immunohistochemical localization of a GHB receptor-like protein isolated from rat brain. *Journal of Comparative Neurology*, *498*(4), 508-524.

Kjaergaard, M., & Kragelund, B. B. (2017). Functions of intrinsic disorder in transmembrane proteins. *Cellular and Molecular Life Sciences*, *74*(17), 3205-3224.

Krogh, A., Larsson, B., Von Heijne, G., & Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *Journal of molecular biology*, *305*(3), 567-580.

Leurs, U., Klein, A. B., McSpadden, E. D., Griem-Krey, N., Solbak, S. M. Ø., Houlton, J., Villumsen, I. S., Vogensen, S. B., Hamborg, L., & Gauger, S. J. (2021). GHB analogs confer neuroprotection through specific interaction with the CaMKIIα hub domain. *Proceedings of the National Academy of Sciences*, *118*(31), e2108079118.

Luck, K., Kim, D.-K., Lambourne, L., Spirohn, K., Begg, B. E., Bian, W., Brignall, R., Cafarelli, T., Campos-Laborie, F. J., & Charloteaux, B. (2020). A reference map of the human binary protein interactome. *Nature*, *580*(7803), 402-408.

Mack, C. P., Hultquist, D. E., & Shlafer, M. (1995). Myocardial flavin reductase and riboflavin: a potential role in decreasing reoxygenation injury. *Biochemical and biophysical research communications*, *212*(1), 35-40.

Maitre, M., Hechler, V., Vayer, P., Gobaille, S., Cash, C. D., Schmitt, M., & Bourguignon, J. J. (1990). A specific gamma-hydroxybutyrate receptor ligand possesses both antagonistic and anticonvulsant properties. *Journal of Pharmacology Experimental Therapeutics*, *255*(2), 657-663.

Maitre, M., Klein, C., & Mensah-Nyagan, A. G. (2016). Mechanisms for the Specific Properties of γ-Hydroxybutyrate in Brain. *J Medicinal Research Reviews*, *36*(3), 363-388.

Mamelak, M. (1989). Gammahydroxybutyrate: an endogenous regulator of energy metabolism. *Neuroscience & Biobehavioral Reviews*, *13*(4), 187-198.

Minezaki, Y., Homma, K., & Nishikawa, K. (2007). Intrinsically disordered regions of human plasma membrane proteins preferentially occur in the cytoplasmic segment. *Journal of molecular biology*, *368*(3), 902-913.

Odagaki, Y., & Yamauchi, T. (2004). γ-Hydroxybutyric acid, unlike γ-aminobutyric acid, does not stimulate Gi/Go proteins in rat brain membranes. *Basic & clinical pharmacology & toxicology*, *94*(2), 89-98.

Oldfield, C. J., & Dunker, A. K. (2014). Intrinsically disordered proteins and intrinsically disordered protein regions. *Annual review of biochemistry*, *83*, 553-584.

Omasits, U., Ahrens, C. H., Müller, S., & Wollscheid, B. (2014). Protter: interactive protein feature visualization and integration with experimental proteomic data. *Bioinformatics*, *30*(6), 884-886.

Oughtred, R., Rust, J., Chang, C., Breitkreutz, B. J., Stark, C., Willems, A., Boucher, L., Leung, G., Kolas, N., & Zhang, F. (2021). The BioGRID database: A comprehensive biomedical resource of curated protein, genetic, and chemical interactions. *J Protein Science*, *30*(1), 187-200.

Powers, H. J. (2003). Riboflavin (vitamin B-2) and health. *The American journal of clinical nutrition*, *77*(6), 1352-1360.

Raposo Pereira, F., McMaster, M. T. B., Polderman, N., de Vries, Y., van den Brink, W., & van Wingen, G. A. (2018). Adverse effects of GHB-induced coma on long-term memory and related brain function. *Drug and alcohol dependence*, *190*, 29-36.

Rolland, T., Taşan, M., Charloteaux, B., Pevzner, S. J., Zhong, Q., Sahni, N., Yi, S., Lemmens, I., Fontanillo, C., & Mosca, R. (2014). A proteome-scale map of the human interactome network. *Cell*, *159*(5), 1212-1226.

Subramanian, V. S., Kapadia, R., Ghosal, A., & Said, H. M. (2015). Identification of residues/sequences in the human riboflavin transporter-2 that is important for function and cell biology. *Nutrition & metabolism*, *12*(1), 1-10.

Takeda, S., Kadowaki, S., Haga, T., Takaesu, H., & Mitaku, S. (2002). Identification of G protein-coupled receptor genes from the human genome sequence. *FEBS letters*, *520*(1-3), 97-101.

Tian, Y., Shehata, M., Gauger, S. J., Veronesi, C., Hamborg, L., Thiesen, L., Bruus-Jensen, J., Royssen, J. S., Leurs, U., & Larsen, A. S. (2022). Exploring the NCS-382 Scaffold for CaMKIIα Modulation: Synthesis, Pharmacology, and Biophysical Characterization of Ph-HTBA as a Novel High-Affinity Brain-Penetrant Stabilizer of the CaMKIIα Hub Domain.

Udhayabanu, T., Manole, A., Rajeshwari, M., Varalakshmi, P., Houlden, H., & Ashokkumar, B. (2017). Riboflavin responsive mitochondrial dysfunction in neurodegenerative diseases. *Journal of Clinical Medicine*, *6*(5), 52.

UniProt: The universal protein knowledgebase in 2021. (2021). *Nucleic Acids Research*, *49*(D1), D480-D489.

van Nieuwenhuijzen, P. S., Long, L. E., Hunt, G. E., Arnold, J. C., & McGregor, I. S. (2010). Residual social, memory and oxytocin-related changes in rats following repeated exposure to gamma-hydroxybutyrate (GHB), 3,4-methylenedioxymethamphetamine (MDMA) or their combination. *Psychopharmacology (Berl)*, *212*(4), 663-674. https://doi.org/10.1007/s00213-010-1986-5

Varadi, M., Anyango, S., Deshpande, M., Nair, S., Natassia, C., Yordanova, G., Yuan, D., Stroe, O., Wood, G., & Laydon, A. (2022). AlphaFold Protein Structure Database: massively expanding the structural coverage of protein-sequence space with high-accuracy models. *Nucleic Acids Research*, *50*(D1), D439-D444.

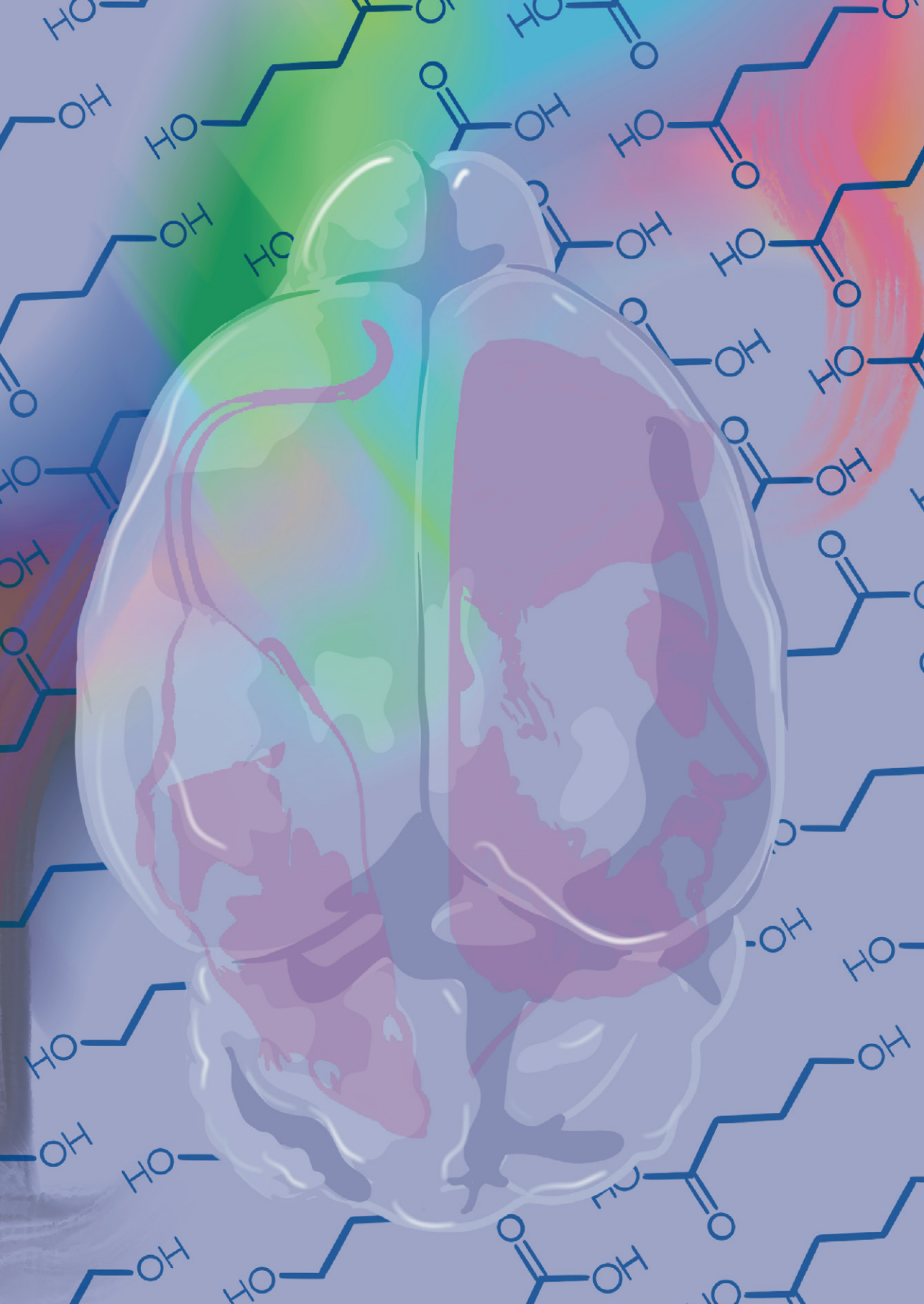
Wright, P. E., & Dyson, H. J. (2015). Intrinsically disordered proteins in cellular signalling and regulation. *Nature reviews Molecular cell biology*, *16*(1), 18-29.

Wu, H., Guo, P., Li, X., Jin, Z., Yang, X., & Wang, Y. (2018). Hydroxybutyrate promotes the recovery from cerebral infarction by activating Amp-activated protein kinase signaling. *Experimental and therapeutic medicine*, *16*(2), 1195-1202.

Xue, B., Li, L., Meroueh, S. O., Uversky, V. N., & Dunker, A. K. (2009). Analysis of structured and intrinsically disordered regions of transmembrane proteins. *Molecular bioSystems*, *5*(12), 1688-1702.

Yao, Y., Yonezawa, A., Yoshimatsu, H., Masuda, S., Katsura, T., & Inui, K.-i. (2010). Identification and comparative functional characterization of a new human riboflavin transporter hRFT3 expressed in the brain. *The Journal of nutrition*, *140*(7), 1220-1226.

Yonezawa, A., & Inui, K.-i. (2013). Novel riboflavin transporter family RFVT/SLC52: identification, nomenclature, functional characterization and genetic diseases of RFVT/SLC52. *Molecular aspects of medicine*, *34*(2-3), 693-701.



Chapter 3

Individual differences in GHB consumption in a new voluntary GHB self-administration model in outbred rats

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Published in: Psychopharmacology

Abstract

The use of the recreational drug gamma-hydroxybutyric acid (GHB) has increased over the past decade, concomitantly leading to a higher incidence of GHB use disorder. Evidence-based treatment interventions are hardly available and cognitive effects of long-term GHB use remain elusive. In order to study the development of GUD and the causal effects of chronic GHB consumption, a GHB self-administration model is required. Long Evans rats had access to GHB in their home cage according to a two-bottle choice procedure for three months. Intoxication and withdrawal symptoms were assessed using an automated sensor-based setup for longitudinal behavioral monitoring. Rats were trained in an operant environment according to a fixed ratio (FR) 1, 2 and 4 schedule of reinforcement. Addiction-like behaviors were assessed through progressive ratio-, non-reinforced- and quinine-adulterated operant tests. In addition, the novel object recognition test and elevated plus maze test were performed before and after GHB self-administration to assess memory performance and anxiety-like behavior, respectively. All rats consumed pharmacologically relevant levels of GHB in their home cage, and their intake remained stable over a period of three months. No clear withdrawal symptoms were observed following abstinence. Responding under operant conditions was characterized by strong inter-individual differences, where only a subset of rats showed high motivation for GHB, habitual GHB-seeking, and/or continued responding for GHB despite an aversive taste. Male rats showed a reduction in long-term memory performance three months after home-cage GHB self-administration. Anxiety-like behavior was not affected by GHB self-administration. The GHB self-administration model was able to reflect individual susceptibility for addiction-like behavior. The reduction in long-term memory performance upon GHB self-administration calls for further research into the cognitive effects of chronic GHB use in humans.

Introduction

Gamma-hydroxybutyric acid (GHB) is a neurotransmitter, and a precursor and metabolite of the main inhibitory neurotransmitter gamma-aminobutyric acid (GABA). Next to its use as a pharmaceutical drug for narcolepsy and patients with an alcohol use disorder (AUD) (Busardò et al., 2015), GHB is increasingly being used as a recreational drug for its disinhibiting and anxiolytic effects. The majority of GHB users consume GHB recreationally, without adverse effects (Dijkstra et al., 2021). However, due to its addictive properties, 4-21% of frequent users develop GHB use disorder (GUD) (Dijkstra et al., 2021). GUD is characterized by frequent overdosing and comas, a severe and erratic withdrawal syndrome, and high relapse rates (Kamal, van Noorden, et al., 2017).

GUD has been associated with (verbal) short-term memory deficits (Raposo Pereira et al., 2018; Beurmanjer et al., 2022). In addition, patients with GUD often experience co-morbid anxiety- and mood-related symptoms (Beurmanjer et al., 2019). It is difficult to determine whether these cognitive and emotional problems are pre-existing (and increase GUD susceptibility), or whether these symptoms are caused by chronic GHB use. Animal studies provide the opportunity to study these relationships and its causality in an experimental model. The majority of previous pre-clinical GHB studies primarily studied the effects of acute and forced, non-voluntary GHB administration on neurotoxicity and cognition (Sircar & Basak, 2004; Kueh et al., 2008; Laraway et al., 2008; Johansson et al., 2014; Chen et al., 2017). In contrast to the established preclinical alcohol- and cocaine self-administration models, a model for prolonged voluntary GHB self-administration is currently lacking.

The establishment of a GHB self-administration model is crucial to assess the causes and consequences of GUD, to understand the neurotoxic effects of GHB, and to eventually examine novel pharmacological interventions to e.g. reduce relapse rates. Previous studies have shown that GHB administration induces rewarding effects in rodents (Martellotta et al., 1997; Itzhak & Ali, 2002; Watson et al., 2010), allowing for the development of a GHB self-administration model. Colombo et al. (1995) initially demonstrated home-cage binge-like GHB consumption in outbred rats (Colombo et al., 1995). GHB self-administration has further been examined in ethanol-preferring rats, leading to increased GHB intake compared to outbred rats (Colombo et al., 1998). In mice, voluntary intravenous GHB self-administration for 14 days leads to an initial increase in GHB intake, followed by a subsequent decrease (Watson et al., 2010). However, it is unknown to what extent these paradigms serve as a model for GUD, since extensive characterization is lacking.

The aim of the current study was to develop an animal model for prolonged voluntary home-cage- and operant GHB self-administration to examine potential GUD-like characteristics. We first performed a pilot study in rats, in which we determined the optimal GHB self-administration parameters and assessed whether (accidental) GHB overdosing occurred in the rats. Specifically, we 1) examined GHB intake patterns in a home-cage environment, 2) characterized the behavioral phenotype during GHB intoxication and withdrawal, and 3) tested multiple addiction-like behaviors in an operant setting. In addition, we explored the predictive effect of baseline anxiety-like behavior on GHB intake, and assessed the effects of chronic GHB self-administration on cognition and anxiety-like behavior.

Methods

The methods of this study are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020; Curtis et al., 2022).

Animals

We employed rats in this study because the rat is the preferred species for preclinical addiction research (Homberg et al., 2017). All rats were pair-housed in enriched type IV cages (59 × 38 × 20 cm; Tecniplast 1500U) with corncob bedding (GM 12 irradiated, Bio Services) under conventional conditions (no filtertops). When GHB was available in the home cage, rats were separated with a perforated plexiglass divider in order to measure individual GHB- and water intake. The divider enabled rats to be in closer proximity and in a less stressful environment compared to individual housing. On each side of the divider, one bottle of water and one bottle of GHB was freely available. The rats had *ad libitum* access to food (dried pellets of standard chow food [ssniff RM V1534-703, Bio Services]) and water. The rats were maintained on a reversed light-dark schedule (lights off at 08:00h) in temperature- (21 ± 1°C) and humidity-controlled (55% ± 5%) rooms. The experimental procedures were performed under a project license from the Central Committee on Animal Experiments (Centrale Commissie Dierproeven, The Hague, The Netherlands), in full compliance with the legal requirements of Dutch legislation on the use and protection of laboratory animals (Animal Testing Act). All efforts were made to reduce the number of animals used and their suffering.

Pilot study

We first conducted a pilot study to 1) determine under which conditions the rats showed maximum voluntary GHB intake, and 2) assess the possible risk for GHB overdosing during home-cage GHB consumption.

Animals

In our pilot study, we used 10 Long Evans rats (50% female) (Janvier, France, PND28 on arrival). No power analysis was performed for this pilot study. We based our sample size on GHB consumption seen in Colombo et al. 1995. The weight of the rats was monitored twice-daily during the GHB consumption period.

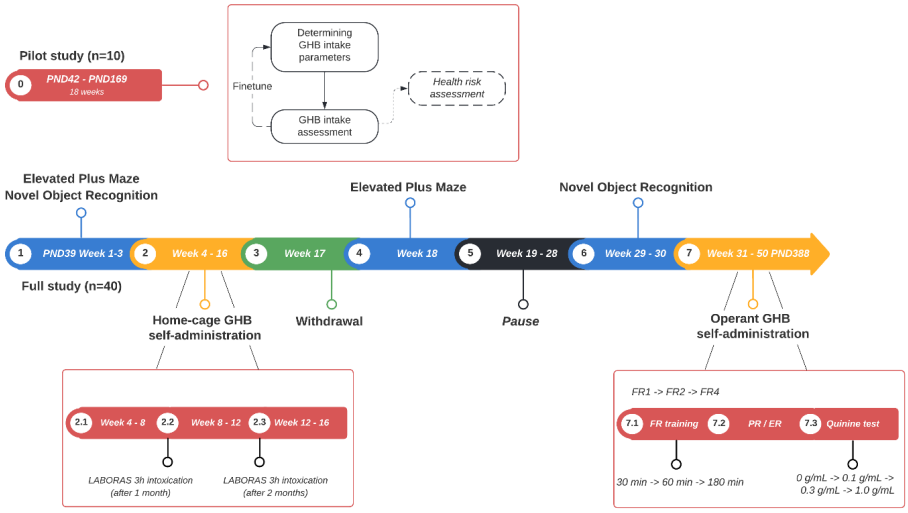


Figure 1. Overview and timeline of the experimental procedures that were performed in this study. Behavioral tests are represented in blue, GHB self-administration is represented in yellow, the pilot study and self-administration details are represented in red, the withdrawal period is represented in green, and a temporary cessation of the study due to construction work in the animal facility is represented in black

GHB self-administration

All rats had access to GHB in water in their home cage according to a two-bottle choice procedure (GHB and water). On Mondays, the GHB concentration was increased from 1 to 2, 3, 4 and 5% GHB (weight (w) / volume (v)) (Serra et al., 2002). On Mondays and Tuesdays, GHB was available for six hours per day and rats were monitored for possible GHB overdosing. On Wednesdays, Thursdays and Fridays, rats had access to GHB for 24 hours. GHB and water consumption were measured hourly over a six-hour period by measuring bottle weights. In the weekends, rats only had access to water. The location of the water- and GHB bottle were switched every day to prevent side bias.

The concentration that resulted in the highest volume consumed (5% GHB (w/v)) was used in subsequent voluntary GHB intake tests. First, a, 5% GHB (w/v) was dissolved

in a 5% sucrose solution (w/v) for one week to increase palatability of GHB and to examine whether this would increase GHB intake. This sucrose/GHB solution was presented in addition to a regular water bottle.

Hereafter, continuous versus intermittent access of a 5% GHB (w/v) solution (without sucrose) was measured, based on earlier work of Spoelder, et al. (2015). First, continuous 5% GHB consumption was assessed for 14 days. Then, intermittent GHB consumption was assessed by providing a GHB bottle for 24 hours on Mondays, Wednesdays and Fridays for two weeks. The remaining days, rats only had access to water.

Following the access schedule test (continuous versus intermittent), increasing concentrations of GHB were assessed to examine whether increasing GHB concentrations would automatically yield higher GHB intake. We increased GHB doses from 4% onwards to 5, 6, 7 and 8%, until we observed a lack of a further increase in GHB intake at 8% GHB (w/v). Finally, we examined if the temporary removal of the water bottle (3h per day) would lead to increased GHB intake (forced GHB), as observed in Colombo et al. (1995).

Full study

Animals

We used 40 Long Evans rats (50% female) (Janvier, France, PND28 on arrival) to establish a GHB self-administration paradigm. As a part of our ethical approval application, we performed an *a-priori* sample size calculation with novel object recognition memory as the main outcome parameter. Based on findings by (van Nieuwenhuijzen, Long, et al., 2010) (standardized effect size = 1.64), with a power of >0.80 and an α of 0.05, a sample size of 10 rats per group was required. In order to also be able to assess time effects and interactions, and assuming a large effect size of 0.5, a sample size of 18 rats per group was required. Accounting for possible drop-out, we used a total group size of 40 rats. Rats were assigned to either the GHB group or a control group according to a counterbalanced design, based on baseline elevated-plus maze (EPM) and novel object recognition (NOR) memory. This lead to similar baseline EPM and NOR scores between treatment groups (male GHB n = 10, female GHB n = 10, male control n = 10, female control n = 10). Cage location in the housing room was randomized to prevent unwanted effects caused by the environment. Throughout the study, four rats (1 male control, 1 female control, 2 female GHB) were taken out of the study due to reaching the humane endpoints (HE) unrelated to GHB consumption (e.g. a broken leg, bacterial infection) (HE1: The animal experiences

more discomfort than justified for the purpose of the experiment approved by the local Animal Ethical Committee (unrelievable adverse behavioral changes, unrelievable adverse body condition, weight loss >20%); HE2: The animal experiences more than little, additional, discomfort as a result of conditions not related to the experiment). The exact number of rats that was used in each experiment is provided in the respective figure legends.

Home-cage GHB consumption

Rats in the GHB group had continuous access to 7% (w/v) GHB (Xyrem 500mg/mL, Jazz Pharmaceuticals, Athlone, Ireland) in water according to a two-bottle choice procedure. GHB and water were presented in drinking bottles with stainless steel dual ball-bearing drinking spouts for three months. These GHB access parameters were determined in the pilot study. The location of the GHB bottle and water bottle were switched from left to right (or vice versa) once per week to prevent side bias. The drinking bottles were weighed daily on Mondays until Fridays and the body weight of the rats was measured once a week.

Behavioral assessment of GHB intoxication and withdrawal

To assess potential GHB intoxication, individual behavioral parameters (locomotion, immobility, grooming, rearing, drinking and eating) and the aberrant behaviors wet dog shakes, head shakes and head twitching were automatically classified and assessed during GHB access in regular Type-IIIH cages using LABORAS (Metris B.V., Hoofddorp, The Netherlands); Laboratory Animal Behavior Observation Registration and Analysis System (Castagné et al., 2012). The intoxication measurements were conducted during two three-hour sessions, performed after one- and two months of home-cage GHB access. Throughout these sessions, the rats had free access to food. The GHB group had access to GHB, while the control group had access to water. The measurements were performed during the rats' active phase.

To assess withdrawal, GHB was removed from the home cage directly following the three-month GHB consumption period, and behavior was assessed using LABORAS for 22 hours.

Operant GHB self-administration

Rats that previously had access to GHB in the home cage underwent behavioral training and testing in operant boxes (29.5 cm L, 24 cm W 25 cm H; Med Associates, Georgia, VT), situated in light- and sound-attenuating cubicles equipped with a ventilation fan. Each box was equipped with an active and an inactive 4.8-cm-wide retractable lever, placed 11.7 cm apart and 6 cm from the grid floor. The location of the

active and inactive lever was counterbalanced between rats. A cue light was present above each lever and a liquid dipper was located between the levers. Upon an active lever press, the dipper cup containing GHB (0.1 mL, 7% w/v) was raised for 10 seconds. Simultaneously, a cue light lit up for 15 seconds, during which additional presses on the active lever had no consequences. Pressing the inactive lever was recorded, but had no programmed consequences. Head entries in the reward receptacle were measured through infrared beam breaks.

Rats were habituated to the operant box for three daily 30-minute habituation sessions, during which no levers or cues were present. Rats were subsequently trained to press the lever according to fixed ratio 1 (FR1), FR2 and FR4 schedules of reinforcement (7-14 sessions per schedule) (Fig. 2). The response requirement was increased after a minimum of seven sessions, and when rats earned at least one reward in three subsequent sessions. To increase behavioral output, the duration of the FR4 operant sessions was increased from 30 minutes (7-14 sessions), to 1 hour (3 sessions) and 3 hours (3 sessions with 7% GHB (w/v)). Seven additional 3-hour FR4 sessions were conducted using a 2% GHB (w/v) concentration to examine whether rats would increase their lever active presses, seeking comparable GHB effects as observed with the 7% GHB concentration (Fig. 2). The rats were tested five days per week, once per day. Rats that did not earn a reward in three subsequent sessions (n=6) were excluded from further training and testing.

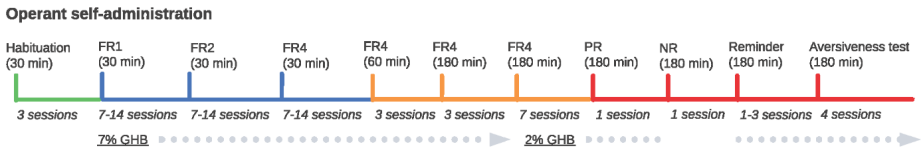


Figure 2. Schematic representation of the operant self-administration experiments.

Assessment of motivation to use, habitual drug-seeking and use despite negative consequences

The motivation to use GHB was tested on a three-hour progressive ratio (PR) schedule of reinforcement, during which the number of required active lever presses was calculated according to the formula . Following PR, a three-hour non-reinforced session (NR) was performed, in which an active lever press had no programmed consequences. After the NR session, one to three 30-min FR4 reminder sessions were performed to reinstate GHB-seeking behavior. When a rat earned at least one

GHB presentation in a reminder session, we examined GHB use despite negative consequences. We adulterated GHB with increasing doses of quinine, from 0.0 to 0.1, 0.3 and 1.0 g/L quinine in 2% GHB (w/v). Each three-hour quinine session was performed under a FR4 schedule of reinforcement.

Elevated plus-maze

Anxiety-like behavior was measured before and directly after home-cage GHB consumption using the elevated plus maze (EPM) (Fig. 1) (Walf & Frye, 2007). The maze, elevated 50 cm from the floor, consisted of two open arms (50 × 10 cm, 10 lux) and two closed arms (50 × 10 cm) that were enclosed by a side wall. Rats were placed in the center of the maze, facing the open arm and were allowed to explore the apparatus for five minutes, while being recorded by a camera suspended above the center of the maze.

Novel object recognition

Object recognition was tested before, and 13 weeks after home-cage GHB consumption using the novel object recognition (NOR) test. The second assessment of the NOR (after GHB exposure) was preceded by a 10-week reconstruction period at the animal facility (Fig. 1). Habituation, training and testing were performed according to (Genzel et al., 2019). In short, rats were habituated to a white MDF testing box (80 x 80 x 80 cm) during five daily 10-minute sessions. During the first three sessions, no cues or objects were present in the testing box. In session four and five, two objects (made from Duplo blocks, not used in main experiment) were placed in the testing box and rats were allowed to explore. On the bottom side of the floor of the testing box, four magnets were placed in fixed locations for consistent placement of the objects. The objects were attached to square metal plates. During training, two identical objects were placed in the testing box and rats were allowed to explore the testing box for 10 minutes. The object type during training and the location of the objects were counterbalanced between rats. Following an inter-trial interval of 24 hours, one of the objects was replaced with another object, after which the rats were again allowed to explore the testing box for 10 minutes. Rats were recorded using an overhead video camera system.

Data and statistical analysis

The data and statistical analyses complied with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2022). All rats tested were treated as independent values, there were no technical replicates.

Performance on the NOR task was determined by calculating the Discrimination Index (DI). The DI was calculated as the difference between the time exploring the

novel object and the old object, divided by the total exploration time. Videos were analyzed using the automated tracking software DeepLabCut combined with a custom-written Python script, providing unbiased data analysis (Mathis et al., 2018). Exploration time was determined as the total duration that the nose of the rat was within eight pixels of the object. When the center of the body was in the object area (i.e. rat was sitting on the object) it was not counted as exploration time.

For the EPM, total time spent in the center, open- and closed arms and latency to enter the open arms were automatically quantified using Observer Ethovision (Noldus, Wageningen, The Netherlands).

The effect of baseline anxiety-like behavior (EPM) on home-cage GHB consumption was analyzed with a linear regression model, using the AUC for the three-month home-cage GHB consumption as the dependent variable and the relative time spent in the closed arms as the predictor variable.

Typical withdrawal symptoms (wet dog shakes, head shakes, heat twitches and scratching) during the first three hours of abstinence in GHB rats were compared with a three-hour LABORAS session of control rats (performed 2 months after home-cage self-administration).

Performance on operant sessions was represented as active lever presses, GHB presentations (number of successful completions of the schedule of reinforcement), and GHB consumptions (head entries directly following a GHB presentation). An addiction severity score was computed based on home-cage GHB intake (area under the curve (AUC) for GHB intake (mg/kg)), habitual drug-seeking (active lever presses under non-reinforced conditions divided by active lever presses under reinforced conditions), motivation to use (active lever presses during PR) and use despite negative consequences (AUC for GHB intake (mg/kg) under increasing concentrations of quinine) (Belin et al., 2009). The four measures were normalized by subtracting the mean of all rats from the score of every individual rat. Thereafter we divided this ratio by the standard deviation of the whole group. The sum of three out of four measures was calculated for every combination. For every combination, the highest quartile (n = 3) was identified as exhibiting addiction-like behavior. For a more detailed explanation, see (Smeets et al., 2022).

Where appropriate, each parameter was tested for normality with a Shapiro–Wilk test. Mauchly's test of sphericity was used to test whether variances of the differences between treatment levels were equal. If the assumption of sphericity was

violated, or when dealing with repeated measures, a Geisser-Greenhouse correction was applied. Normally distributed behavioral data were analyzed via two-sample t-tests, one-, two- or three-way ANOVAs with time or session as within-subject factor, and drug (and sex where appropriate) as between-subject factors, unless indicated otherwise. Correlation analyses were performed between home-cage GHB consumption, motivation to use GHB, habitual drug-seeking and use despite negative consequences, resulting in six individual correlation analyses. Bonferroni correction was performed to correct for multiple comparisons ($p = 0.05 / 6 = 0.0083$). For correlation analyses involving motivation to use GHB, one rat was identified as an outlier and was excluded from the analyses (deviating >2x SD from the group mean). Bonferroni post-hoc analyses were also performed following planned comparisons or following significant ANOVA interactions, and were only performed if data were normally distributed and if there was no inhomogeneity of variance. Experimenters were blinded during manual data analysis. The threshold for statistical significance was set at $p < 0.05$. All data are presented as mean ± SEM. Statistical analyses were conducted using GraphPad Prism (version 10.0).

Results

Pilot study

We did not witness GHB overdosing following home-cage GHB consumption. Maximum average GHB intake was established at 7% GHB in a water (w/v) solution under continuous free-choice access, without addition of sucrose (Suppl. Fig. 1). These access parameters were employed in the full study.

Full study

Home-cage GHB consumption

Rats showed pharmacologically relevant (>~87.5 mg/kg per acute dose, (Martellotta et al., 1997) levels of daily GHB intake during home-cage consumption (males: $\mu = 809.7$ mg/kg, SD = 226.7; females: $\mu = 660.6$ mg/kg, SD = 92.0), although the dose for each consumption is difficult to accurately estimate. GHB intake fluctuated over a period of 12 weeks, and the GHB intake of males was higher compared to females during week 4 (Fig. 3a: two-way ANOVA, time x sex interaction, $F_{(11, 198)} = 2.769$, $p < 0.01$, Bonferroni post-hoc test week 4, $p < 0.05$). No difference in overall GHB intake was observed between males and females over a period of 12 weeks. Male rats exhibited a higher preference for GHB versus water compared to females (Fig. 3b: two-sample t-test, $p < 0.01$).

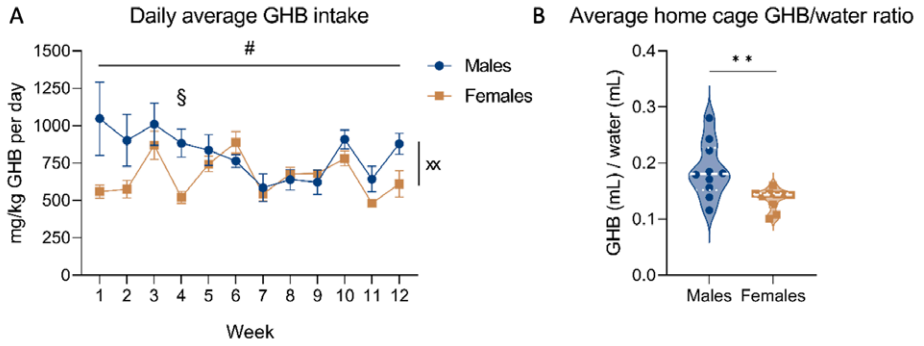


Figure 3. Home-cage GHB consumption over a period of 12 weeks. A) mg/kg GHB intake during the self-administration period of 12 weeks, divided by sex. B) GHB preference normalized by water intake for males and females, averaged over the entire self-administration period. Males n = 10, females n = 10. # = $p < 0.05$ two-way RM ANOVA main effect of time; xx = $p < 0.01$ time x sex interaction; § = $p < 0.05$ Bonferroni multiple comparison test; ** = $p < 0.01$ two-sample t-test

Behavioral assessment of GHB intoxication and withdrawal: Intoxication

No clear intoxication symptoms were visible during the three-hour sessions of GHB consumption in LABORAS (Suppl. Fig. 2, three-way ANOVA, no effect of drug, $p > 0.05$). No sex-specific drug effects were observed (Suppl. Fig. 2, three-way ANOVA, no sex x drug interaction, $p > 0.05$). However, we did see a differential effect of GHB on home-cage behaviors (Suppl. Fig. 2, three-way ANOVA, behavior x drug interaction, $F_{(5, 216)} = 6.567$, $p < 0.0001$). Therefore, we compared GHB vs control for every behavior with males and females grouped. GHB rats spent less time eating compared to controls (Suppl. Fig. 2, Bonferroni post-hoc comparison, GHB vs control eating, $p < 0.05$).

Rats showed stable consumption of GHB during three-hour GHB sessions, both after one month of home-cage GHB consumption (Session 1, Fig. 4A) and two months of home-cage GHB consumption (Session 2, Fig. 4B). During session 1, water consumption in the control group exceeded GHB consumption in the GHB group, which did not have access to water during the LABORAS sessions (Fig. 4A, two-way ANOVA, main effect of group, $F_{(1, 38)} = 9.799$, $p < 0.01$). Time spent drinking GHB varied from ~50 seconds up to ~500 seconds between rats during a three-hour period (Fig. 4C session 1, $\mu = 219.0$ seconds, SD = 119.4; Fig. 4D session 2, $\mu = 199.7$ seconds, SD = 118.5). Additionally, some rats showed binge-like periods of GHB consumption, i.e. distinct peaks in GHB consumption (Suppl. Fig. 3).

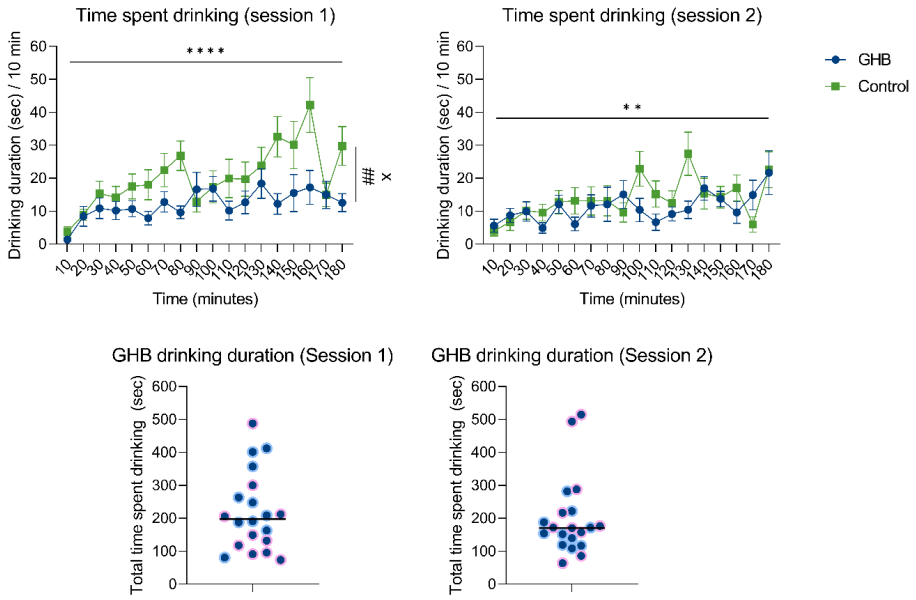


Figure 4. A) Time spent drinking during three-hour LABORAS sessions after one month (session 1) in 10-minute bins. B) Time spent drinking during three-hour LABORAS sessions after two months (session 2) in 10-minute bins. GHB rats only had access to GHB, while control rats only had access to water. C) total time spent drinking GHB for each individual GHB rat during session 1. D) total time spent drinking GHB for each individual GHB rat during session 2. Blue dots represent male rats, pink dots represent female rats. GHB n = 20, control n = 20. ** = $p < 0.01$, **** = $p < 0.0001$ two-way RM ANOVA main effect of time; ## = $p < 0.01$ main effect of drug, x = $p < 0.05$ time x drug interaction

Behavioral assessment of GHB intoxication and withdrawal: Withdrawal

We did not observe a difference in the number of wet dog shakes, head shakes, heat twitches or scratches during the first 3 hours of GHB abstinence compared to a control group (Fig. 5, Bonferroni post-hoc comparisons, male GHB vs male control, $p > 0.05$; female GHB vs female control, $p > 0.05$). Upon visual inspection of the 22h development of locomotion, rearing, grooming, wet dog shakes, head shakes and head twitches following abstinence, no clear indications of withdrawal were observed (Suppl. Fig. 4). Both males and females showed a decrease in all behaviors during the dark phase, indicating a regular day-night rhythm unaffected by abstinence. Females showed higher behavioral output compared to males during withdrawal, as similar during intoxication (Suppl. Fig. 2).

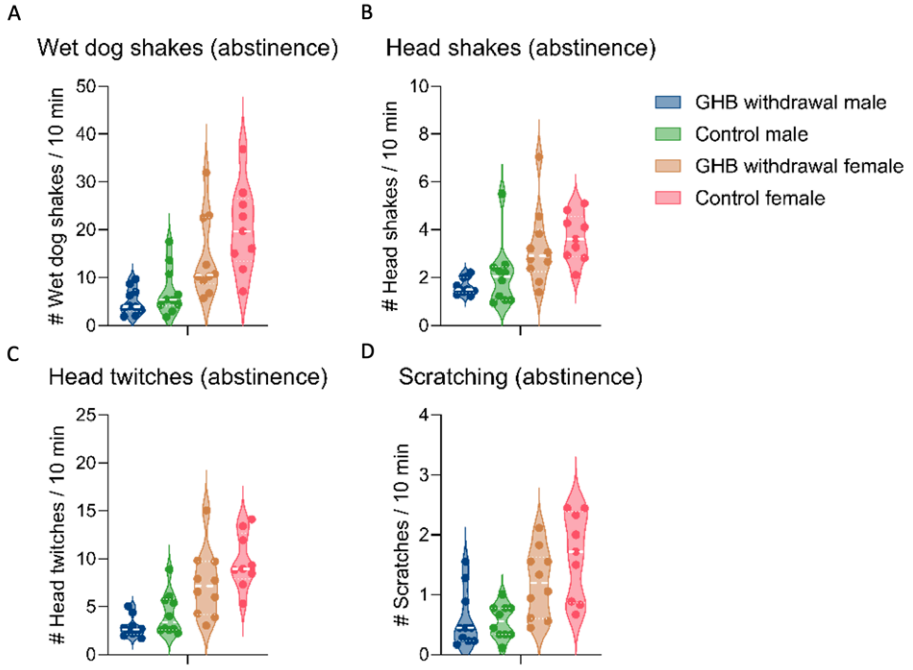


Figure 5. Aberrant behaviors during three hours of sudden abstinence of the GHB group, compared to a three-hour session of control rats. A) Number of wet dog shakes averaged per 10 minutes, B) Number of head twitches averaged per 10 minutes, C) Number of head shakes averaged per 10 minutes, D) Number of scratches averaged per 10 minutes

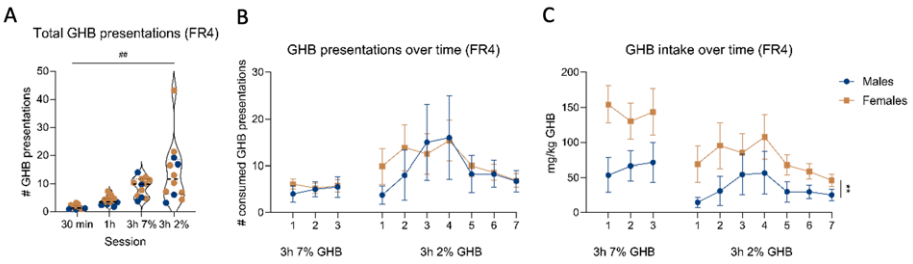


Figure 6. GHB presentations and intake during three-hour FR4 sessions. A) Total GHB presentations during different FR4 sessions. Blue data points correspond to males, pink data points correspond to females. B) Consumed GHB presentations per 3h session, divided by sex. C) mg/kg GHB intake per 3h session, divided by sex. Males n = 4, females n = 8. ## = $p < 0.01$ one-way ANOVA main effect. ** = $p < 0.01$ Welch's two-sample t-test males vs females

Operant GHB self-administration

The number of GHB presentations increased when the operant session duration increased (Fig. 6A, one-way ANOVA, main effect of session, $F_{(1.158, 12.73)} = 13.36$, $p < 0.01$). Males and females showed a similar number of (consumed) GHB presentations (Fig. 6B). Due to a lower bodyweight of the female rats, this resulted in a higher mg/kg GHB consumption for females compared to males (Fig. 6c, Welch's two-sample t-test, males vs females, $p < 0.01$). Throughout the operant FR4 sessions, active lever presses were higher than inactive lever presses (Suppl. Fig. 5: two-way ANOVA, main effect of lever type, $F_{(2, 35)} = 11.04$, $p < 0.001$). After switching from 7% GHB to 2% GHB, an increase and subsequent decrease in GHB presentations and GHB intake over sessions was observed (Fig. 6B, Fig. 6C, Suppl. Fig. 5). The number of GHB presentations and GHB intake during 3h 7% GHB sessions remained stable (Fig. 6B, Fig. 6C).

Rats that finished operant training (n=12) were tested for several addiction-like behaviors. During PR testing, one rat showed extremely high responding (489 active lever presses during a three-hour session) (Fig. 7A). Active lever responses of the majority of rats during the PR schedule of reinforcement was comparable to active lever responses during FR4 (varying between 13 and 67) (Fig. 7B). Responding under non-reinforced conditions was slightly decreased compared to reinforced responding, although responding was still maintained (average ratio of 0.81) (Fig. 7C). Quinine-adulterated GHB decreased operant responding on the active lever in a dose-dependent manner. Two rats still showed responding with 0.1g/L and 0.3g/L quinine in GHB, whereas responding for GHB completely diminished in all rats with 1.0g/L quinine (Fig. 7D). Motivation to use GHB strongly correlated with use despite negative consequences ($r = 0.76$, $p < 0.0083$) (Fig. 7E). Other addiction-like behaviors were not correlated with each other (Suppl. Fig. 6).

To assess and quantify the presence and consistency of addiction-like behavior, we calculated the addiction severity score for every individual rat. One rat was represented in the highest quartile of all addiction severity score computations (one female), two rats were represented in the highest quartile of two addiction severity score computations (one male, one female), while four rats were represented in the highest quartile in one of the four of the addiction severity score computations (two males, two females). 42% of the tested rats (5/12) were not represented in the highest quartile of any of the addiction severity score computations (one male, four females) (Fig. 7F).

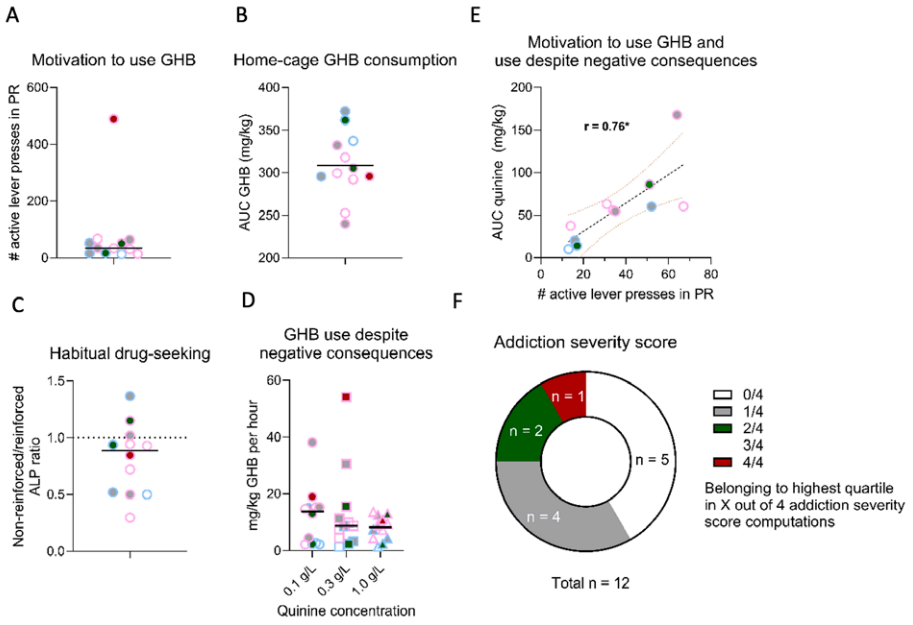


Figure 7. Addiction-like behaviors for individual rats. A) Area-under-curve (AUC) for three-month home-cage GHB intake. B) Motivation to use GHB, represented by the active lever presses during the three-hour progressive ratio (PR) schedule of reinforcement test. C) Habitual drug-seeking, calculated by the active lever presses during the non-reinforced session compared to the amount of active lever presses (ALP) during previous reinforced FR4 schedule of reinforcement. D) GHB use despite negative consequences, represented by GHB consumption during three-hour operant sessions with different doses of quinine-adulterated GHB. E) Correlation between motivation to use GHB and use despite negative consequences ($n = 11$). * = $p < 0.0083$, Bonferroni correction. Black line represents the regression line, curved dotted lines represent the 95% confidence interval. F) Distribution of addiction severity scores, represented by the frequency of belonging to the highest quartile in the 4 addiction severity score computations. Data points represent individual animals. Symbol border color corresponds to the sex of the individual rat. Addiction severity score colors are used as symbol colors in a-e.

Object recognition memory and anxiety-like behavior

We explored the effect of home-cage GHB intake on object recognition memory. Baseline object recognition memory was similar between the GHB group and the control group (Fig. 8, three-way ANOVA, no effect of group $p > 0.05$). Three-month home-cage GHB intake led to decreased object recognition memory after 13 weeks of abstinence in males, whereas object recognition performance was unaffected in females (Fig. 8: three-way ANOVA, group x sex x intervention interaction, $F_{(1, 34)} = 7.621$, $p < 0.01$, Bonferroni post-hoc comparison, GHB male pre-GHB vs post-GHB, $p < 0.001$). Home-cage GHB intake was not predictive of novel object recognition performance (Suppl. Fig. 7, linear regression, $p > 0.05$). We also explored the effect of baseline anxiety-like behavior on home-cage GHB intake and vice versa.

We did not observe a predictive effect of baseline anxiety-like behavior on home-cage GHB intake (Suppl. Fig. 8a). Home-cage GHB consumption and subsequent abstinence did not affect anxiety-like behavior (Suppl. Fig. 8b, c, d, e).

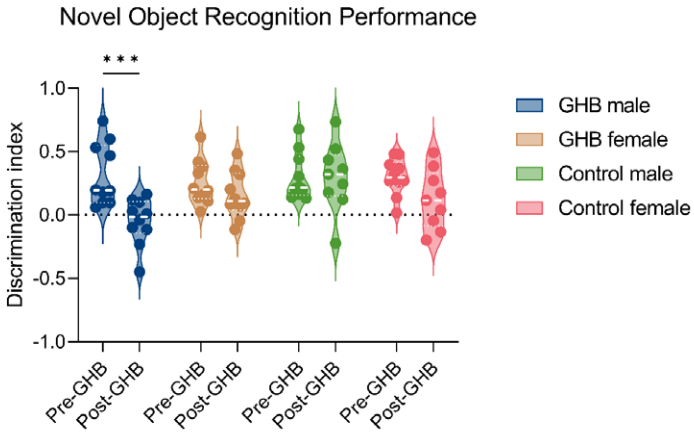


Figure 8. Novel object recognition (NOR) performance before and after a three-month home-cage GHB self-administration period. Baseline (pre-GHB) NOR was tested in the two weeks prior to home-cage GHB access. Post-GHB NOR performance was tested under abstinence, 13 weeks after home-cage GHB self-administration. GHB male n = 10, GHB female n = 10, control male n = 10, control female n = 10 (n = 9 for female control post-GHB). *** = $p < 0.001$ Bonferroni multiple comparison

Discussion

This study set out to assess the characteristics of oral GHB self-administration in outbred rats. GHB self-administration was primarily characterized by strong inter-individual differences between rats. All rats showed intake of pharmacologically relevant levels (>87.5 mg/kg) of GHB in a home-cage environment. We did not observe escalation in GHB intake, physical intoxication symptoms, or withdrawal symptoms after sudden GHB abstinence. A subset of rats showed motivation for GHB or GHB use despite negative consequences, while over half of the rats showed habitual GHB-seeking in an operant environment. Chronic home-cage GHB intake led to a decrease in long-term memory performance in male rats. Baseline anxiety-like behavior was not predictive of home-cage GHB intake, while GHB did not affect anxiety-like behavior following abstinence.

Over a period of 12 weeks of home-cage GHB access, rats showed stable consumption of pharmacologically relevant amounts of 7% GHB (w/v) (~700 mg/kg/day) with an

average GHB preference of ~15-20%. This stable GHB intake over time is in line with the majority of human GHB users that do not show escalation (Dijkstra et al., 2021). In addition, patients who daily consume GHB for medical purposes (e.g. narcolepsy patients) also reportedly do not show signs of tolerance or dose escalation (Wang et al., 2009). Our findings are also largely in line with preclinical GHB literature. A study by Colombo et al. (1995) found no increase in 1% GHB (w/v) consumption during 20 days of free-choice GHB access in outbred Wistar rats (Colombo et al., 1995). In selectively-bred ethanol-preferring male rats, 1% GHB (w/v) intake did increase over a period of 28 days (from ~200 mg/kg/day up to ~600mg/kg/day), but GHB intake in non-ethanol-preferring male rats remained stable over time (~200 mg/kg/day) (Colombo et al., 1998). GHB preference varied from ~20% in non-ethanol-preferring rats (as observed in our study) to ~50% in ethanol-preferring rats at the end of the 28-day period. Altogether, this indicates that escalation in GHB use may only occur in a subset of rats that are susceptible for the development of addiction-like behavior, similar to the human situation. Future studies should examine the use of selectively-bred animals to assess GHB escalation and tolerance in a self-administration model.

Besides decreased food consumption, no other GHB intoxication symptoms were detected after one or two months of GHB self-administration. Rats showed clear individual differences in total GHB consumption and GHB consumption pattern (binge-like drinking vs stable consumption) during three-hour LABORAS sessions after one and two months of home cage GHB consumption. One other preclinical GHB study characterized the (hourly) drinking pattern of GHB, reporting binge-like behavior in some rats on some days (Colombo et al., 1995). Unfortunately, the study does not report whether these binges are representative for the entire sample. This binge-like drinking behavior is also observed in patients with GUD, in order to prevent withdrawal symptoms (Beurmanjer et al., 2019). Although we see binge-like consumption in some rats, the majority of rats in our sample exhibit controlled, stable GHB consumption. This may resemble the recreational GHB consumption pattern that is seen in the broader population of GHB users, unrelated to the occurrence and prevention of withdrawal (Dijkstra et al., 2021).

GHB rats did not show clear withdrawal symptoms when comparing the first three hours of abstinence with a control group. No aberrant behaviors across a 22-hour period of abstinence were observed. In rats, it has been shown that abstinence following 10 repeated injections of 500mg/kg GHB induces anxiety-like behavior in male rats (van Nieuwenhuijzen, Long, et al., 2010), which is also seen in patients with GUD undergoing controlled detoxification (Wolf et al., 2021). Additionally, 24-hour abstinence following 18 twice-daily doses of 1.5-3.5 g/kg GHB in selectively-

bred alcohol-preferring male rats was shown to induce audiogenic seizures in 60% of the rats (Carai et al., 2005). Seizures are a hallmark of sudden GHB withdrawal in patients with GUD consuming high doses of GHB (McDonough et al., 2004) and are also observed following withdrawal from other sedatives like alcohol and benzodiazepines (Rogawski, 2005). However, when rats were tested for different types of withdrawal symptoms (including hyperlocomotion, anxiety-like behavior, tremors and head shakes), we observed no clear signs of GHB withdrawal. Despite differences in administration procedures and the specific parameters that were assessed, our results correspond to the lack of withdrawal symptoms in recreational GHB users in contrast to patients with GUD. This is in line with the suggestion that our model does not resemble the phenotype of GUD, but may resemble recreational, habitual GHB use instead.

We were able to establish consistent and stable operant responding for GHB in two-thirds of the rats (n=12) that were exposed to GHB in an operant environment. Motivation to use GHB and use despite negative consequences were strongly correlated in our model, indicating internal consistency in (operant) GHB addiction-like behavior. Only one rat (~10%) showed consistent addiction-like behavior across all four tests. Smeets et al. (2022) employed a similar approach to assess several addiction-like behaviors in an alcohol self-administration model. They did not find a correlation between motivation to use and use despite negative consequences. However, despite differences in experimental design, they found that ~10% of the rats showed consistent addiction-like behavior. Similar to our study, the majority of their rats did not exhibit any addiction-like behaviors despite the availability of a reinforcing drug. Other pre-clinical studies also found clear differences in addiction-like behavior between outbred rats with access to reinforcing drugs (e.g. high vs low responders, high-drinking animals vs low-drinking animals) (Bell et al., 2006; Kabbaj, 2006; Spoelder et al., 2015). This variance in addiction-like behavior is also observed in the general population, where it is known that a small subset of people that recreationally use e.g. alcohol develop a substance use disorder (~15%) (Grant et al., 2017). Specifically, for GHB users, three subpopulations are described in the literature: people using GHB recreationally without adverse effects (most prevalent), people using GHB recreationally with adverse effects (less prevalent), and people with dependence on GHB (least prevalent) (Dijkstra et al., 2021). These subgroups may be represented in our model, with the majority of rats consuming GHB without measurable adverse effects. Future studies should further develop our model to allow the study of more prominent addiction-like behavior. Additionally, future studies may also explore the subset of rats that do not show any signs of addiction-like behavior, despite the availability of a drug with abuse potential. This may provide

us novel insights regarding the resilience in development of GUD and substance use disorder in general.

Despite the limited presence of GUD-like characteristics, three-month home-cage GHB access led to a sex-specific decrease in novel object memory compared to a control group. Memory performance following a 24-hour interval was tested 13 weeks after the final exposure to GHB, indicating a long-lasting residual effect on long-term memory performance (Akkerman et al., 2014). In line with our findings, another preclinical study found that ten daily injections of 500 mg/kg GHB in rats (similar to the daily intake observed in our study) caused decreased spatial long-term memory performance five days after the final injection (Chen et al., 2017). In patients with GUD, long-term memory performance has not yet been studied, in contrast to other memory domains. GUD patients showed impairment in verbal short-term memory performance, unrelated to severity of GUD or number of comas (Beurmanjer et al., 2022). Another study could not find this association between GHB use and verbal short-term memory impairment, or an association between GHB use and spatial short-term memory impairment (Raposo Pereira et al., 2018). However, in this study GHB-induced comas were associated with decreased verbal short-term memory (Raposo Pereira et al., 2018). Altogether, it appears that long-lasting and possibly irreversible cognitive effects can arise following chronic GHB administration. It remains unknown through what mechanisms these cognitive effects occur. The current model provides the means to study these mechanisms in a controlled setting, and allow for the exploration of potential preventive measures. Future studies should examine the underlying mechanisms of the residual negative effects on long-term memory performance, and further study potential negative cognitive effects of chronic GHB use in a patient population.

The current findings should be viewed in light of several study limitations. Responding during the operant sessions (under FR, PR and NR) was relatively low compared to other addictive substances, such as alcohol (Spoelder et al., 2015; Smeets et al., 2022). This may give rise to the impression that GHB may be less reinforcing than alcohol. However, an important factor to take into account is that here, we established one of the first oral GHB self-administration paradigms in outbred rats, while operant alcohol self-administration has been studied and optimized for decades. It has been suggested that especially the use of selectively bred alcohol-preferring rats is of high value in studying AUD (Borruto et al., 2021), indicating that the use of GHB-preferring rats can be of value in future GHB-related experiments (Lobina et al., 2004).

Since our control rats were not placed in LABORAS following the three-month self-administration period, we compared symptoms during the first three hours of abstinence/withdrawal to a three-hour LABORAS session in control rats performed one month earlier. Due to the lack of 22h data from the control group during abstinence, it is possible that we overlooked the presence of withdrawal symptoms. Our interpretation of the results may therefore be an underestimation of GHB withdrawal in our paradigm.

Finally, we quantified operant GHB consumption by registering the number of obtained GHB rewards, and verified whether they were followed-up with a head entry to collect the GHB reward. We opted for this output parameter instead of measuring GHB in the GHB holding container, since evaporation of GHB strongly contributed to the amount of GHB removed from the holding container. Using the number of rewards and the timing of head entries to calculate the amount of consumed GHB might lead to a slight overestimation of the actual amount of consumed GHB. However, in earlier work of operant (alcohol) self-administration, Spoelder et al. (2015) has shown that the number of rewards strongly correlates with the actual volume of consumed 20% alcohol solution (w/v). Based on these results, we expect that the number of rewards and the actual GHB that rats consumed would also correlate. Therefore, we believe that the impact of the possible overestimation of consumed GHB on our results is minimal.

Conclusion

Oral voluntary GHB self-administration in outbred rats leads to habitual, controlled consumption characterized by inter-individual differences, which is also observed with other addictive substances such as alcohol. In contrast to human GHB users, rats consuming GHB did not show overdosing and subsequent GHB-induced comas. Despite the chronic intake of pharmacologically relevant levels of GHB, no clear intoxication or withdrawal symptoms were observed. Only few rats showed consistent addiction-like characteristics. Our model may thus represent a genetically diverse population sample where the majority does not develop addiction-like behavior. This is further supported by the absence of a relation between anxiety and GHB consumption. Chronic GHB exposure led to residual long-term memory effects in rats, calling for further research into the potential negative cognitive effects of chronic GHB use in humans. This voluntary GHB self-administration model in rats allows for the study of the mechanisms involved in the development of addiction-like GHB use, and enables the exploration of potential preventive strategies.

Bibliography

Akkerman, S., Blokland, A., & Prickaerts, J. (2014). Mind the gap: delayed manifestation of long-term object memory improvement by phosphodiesterase inhibitors. *J Neurobiology of learning memory*, *109*, 139-143.

Belin, D., Balado, E., Piazza, P. V., & Deroche-Gamonet, V. (2009). Pattern of intake and drug craving predict the development of cocaine addiction-like behavior in rats. *J Biological psychiatry*, *65*(10), 863-868.

Bell, R. L., Rodd, Z. A., Lumeng, L., Murphy, J. M., & McBride, W. J. (2006). The alcohol-preferring P rat and animal models of excessive alcohol drinking. *J Addiction biology*, *11*(3-4), 270-288.

Beurmanjer, H., Asperslag, E. M., Oliemeulen, L., Goudriaan, A. E., De Jong, C. A. J., Schellekens, A. F. A., & Dijkstra, B. A. G. (2019). A Qualitative Approach in Understanding Illness Perception and Treatment Needs in Patients with Gamma Hydroxybutyrate Use Disorder. *Eur Addict Res*, *25*(5), 248-255. https://doi.org/10.1159/000500807

Beurmanjer, H., Bruijnen, C. J. W. H., Greeven, P. G. J., De Jong, C. A. J., Schellekens, A. F. A., & Dijkstra, B. A. G. (2022). Cognitive Impairments in Patients with GHB Use Disorder Predict Relapse in GHB Use. *Eur Addict Res*, *28*(5), 350-357. https://doi.org/10.1159/000525507

Borruto, A. M., Stopponi, S., Li, H., Weiss, F., Roberto, M., & Ciccocioppo, R. (2021). Genetically selected alcohol-preferring msP rats to study alcohol use disorder: Anything lost in translation? *Neuropharmacology*, *186*, 108446.

Busardò, F., Kyriakou, C., Napoletano, S., Marinelli, E., & Zaami, S. (2015). Clinical applications of sodium oxybate (GHB): from narcolepsy to alcohol withdrawal syndrome. *Eur Rev Med Pharmacol Sci*, *19*(23), 4654-4663.

Carai, M. A., Quang, L. S., Atzeri, S., Lobina, C., Maccioni, P., Orrù, A., Gessa, G. L., Maher, T. J., & Colombo, G. (2005). Withdrawal syndrome from γ-hydroxybutyric acid (GHB) and 1, 4-butanediol (1, 4-BD) in Sardinian alcohol-preferring rats. *Brain research protocols*, *15*(2), 75-78.

Castagné, V., Wolinsky, T., Quinn, L., & Virley, D. (2012). Differential behavioral profiling of stimulant substances in the rat using the LABORAS™ system. *Pharmacology Biochemistry Behavior*, *101*(4), 553-563.

Chen, L. Y., Renn, T. Y., Liao, W. C., Mai, F. D., Ho, Y. J., Hsiao, G., Lee, A. W., & Chang, H. M. (2017). Melatonin successfully rescues hippocampal bioenergetics and improves cognitive function following drug intoxication by promoting Nrf2-ARE signaling activity. *J Pineal Res*, *63*(2). https://doi.org/10.1111/jpi.12417

Colombo, G., Agabio, R., Balaklievskaia, N., Diaz, G., Lobina, C., Reali, R., & Gessa, G. L. (1995). Oral self-administration of γ-hydroxybutyric acid in the rat. *European journal of pharmacology*, *285*(1), 103-107.

Colombo, G., Agabio, R., Diaz, G., Fà, M., Lobina, C., Reali, R., & Gessa, G. L. (1998). γ-Hydroxybutyric acid intake in ethanol-preferring sP and-nonpreferring sNP rats. *Physiology & behavior*, *64*(2), 197-202.

Curtis, M. J., Alexander, S. P. H., Cirino, G., George, C. H., Kendall, D. A., Insel, P. A., Izzo, A. A., Ji, Y., Panettieri, R. A., & Patel, H. H. (2022). Planning experiments: Updated guidance on experimental design and analysis and their reporting III. *British Journal of Pharmacology*, *179*(15), 3907-3913.

Dijkstra, B. A. G., Beurmanjer, H., Goudriaan, A. E., Schellekens, A. F. A., & Joosten, E. A. G. (2021). Unity in diversity: A systematic review on the GHB using population. *Int J Drug Policy*, *94*, 103230. https://doi.org/10.1016/j.drugpo.2021.103230

Genzel, L., Schut, E., Schröder, T., Eichler, R., Khamassi, M., Gomez, A., Navarro Lobato, I., & Battaglia, F. (2019). The object space task shows cumulative memory expression in both mice and rats. *PLoS Biology*, *17*(6), e3000322.

Grant, B. F., Chou, S. P., Saha, T. D., Pickering, R. P., Kerridge, B. T., Ruan, W. J., Huang, B., Jung, J., Zhang, H., & Fan, A. (2017). Prevalence of 12-month alcohol use, high-risk drinking, and DSM-IV alcohol use disorder in the United States, 2001-2002 to 2012-2013: results from the National Epidemiologic Survey on Alcohol and Related Conditions. *JAMA psychiatry*, *74*(9), 911-923.

Homberg, J. R., Wöhr, M., & Alenina, N. (2017). Comeback of the rat in biomedical research. *ACS Chemical Neuroscience*, *8*(5), 900-903.

Itzhak, Y., & Ali, S. F. (2002). Repeated administration of gamma-hydroxybutyric acid (GHB) to mice: assessment of the sedative and rewarding effects of GHB. *Annals of the New York Academy of Sciences*, *965*(1), 451-460.

Johansson, J., Grönbladh, A., & Hallberg, M. (2014). Gamma-hydroxybutyrate (GHB) induces cognitive deficits and affects GABAB receptors and IGF-1 receptors in male rats. *Behavioural brain research*, *269*, 164-174.

Kabbaj, M. (2006). Individual differences in vulnerability to drug abuse: the high responders/low responders model. *CNS Neurological Disorders-Drug Targets*, *5*(5), 513-520.

Kamal, R. M., van Noorden, M. S., Wannet, W., Beurmanjer, H., Dijkstra, B. A. G., & Schellekens, A. F. A. (2017). Pharmacological treatment in γ-hydroxybutyrate (GHB) and γ-Butyrolactone (GBL) dependence: detoxification and relapse prevention. *CNS Drugs*, *31*(1), 51-64.

Kueh, D., Iwamoto, K., Poling, A., & Baker, L. E. (2008). Effects of γ-hydroxybutyrate (GHB) and its metabolic precursors on delayed-matching-to-position performance in rats. *Pharmacology Biochemistry and Behavior*, *89*(2), 179-187.

Laraway, S., Snycerski, S., Baker, L. E., & Poling, A. (2008). Gamma-hydroxybutyrate (GHB) reduces operant behavior without impairing working memory in rats responding under fixed-consecutive-number schedules. *Pharmacology Biochemistry and Behavior*, *88*(3), 205-212.

Lobina, C., Colombo, G., Gessa, G. L., & Carai, M. A. (2004). Completion by the 10th generation of the bidirectional selective breeding of GHB-sensitive and GHB-resistant rats. *Brain research protocols*, *13*(1), 53-56.

Martellotta, M. C., Fattore, L., Cossu, G., & Fratta, W. (1997). Rewarding properties of gamma-hydroxybutyric acid: an evaluation through place preference paradigm. *Psychopharmacology*, *132*, 1-5.

Mathis, A., Mamidanna, P., Cury, K. M., Abe, T., Murthy, V. N., Mathis, M. W., & Bethge, M. (2018). DeepLabCut: markerless pose estimation of user-defined body parts with deep learning. *Nature neuroscience*, *21*(9), 1281-1289.

McDonough, M., Kennedy, N., Glasper, A., & Bearn, J. (2004). Clinical features and management of gamma-hydroxybutyrate (GHB) withdrawal: a review. *Drug Alcohol Depend*, *75*(1), 3-9. https://doi.org/10.1016/j.drugalcdep.2004.01.012

Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., Browne, W. J., Clark, A., Cuthill, I. C., & Dirnagl, U. (2020). The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *Journal of Cerebral Blood Flow and Metabolism*, *40*(9), 1769-1777.

Raposo Pereira, F., McMaster, M. T. B., Polderman, N., de Vries, Y., van den Brink, W., & van Wingen, G. A. (2018). Adverse effects of GHB-induced coma on long-term memory and related brain function. *Drug and alcohol dependence*, *190*, 29-36.

Rogawski, M. A. (2005). Update on the neurobiology of alcohol withdrawal seizures. *Epilepsy Currents*, *5*(6), 225-230.

Serra, S., Colombo, G., Melis, S., Vacca, G., Carai, M. A., & Gessa, G. L. (2002). Gamma-hydroxybutyric acid versus alcohol preference in Sardinian alcohol-preferring rats. *J Alcohol Alcoholism*, *37*(2), 128-131.

Sircar, R., & Basak, A. (2004). Adolescent γ-hydroxybutyric acid exposure decreases cortical N-methyl-D-aspartate receptor and impairs spatial learning. *Pharmacology Biochemistry and Behavior*, *79*(4), 701-708.

Smeets, J. A., Minnaard, A. M., Ramakers, G. M., Adan, R. A., Vanderschuren, L. J., & Lesscher, H. M. B. (2022). On the interrelation between alcohol addiction–like behaviors in rats. *Psychopharmacology*, *239*(4), 1115-1128.

Spoelder, M., Hesseling, P., Baars, A. M., Lozeman-van 't Klooster, J. G., Rotte, M. D., Vanderschuren, L. J., & Lesscher, H. M. B. (2015). Individual variation in alcohol intake predicts reinforcement, motivation, and compulsive alcohol use in rats. *Alcoholism: Clinical Experimental Research*, *39*(12), 2427-2437.

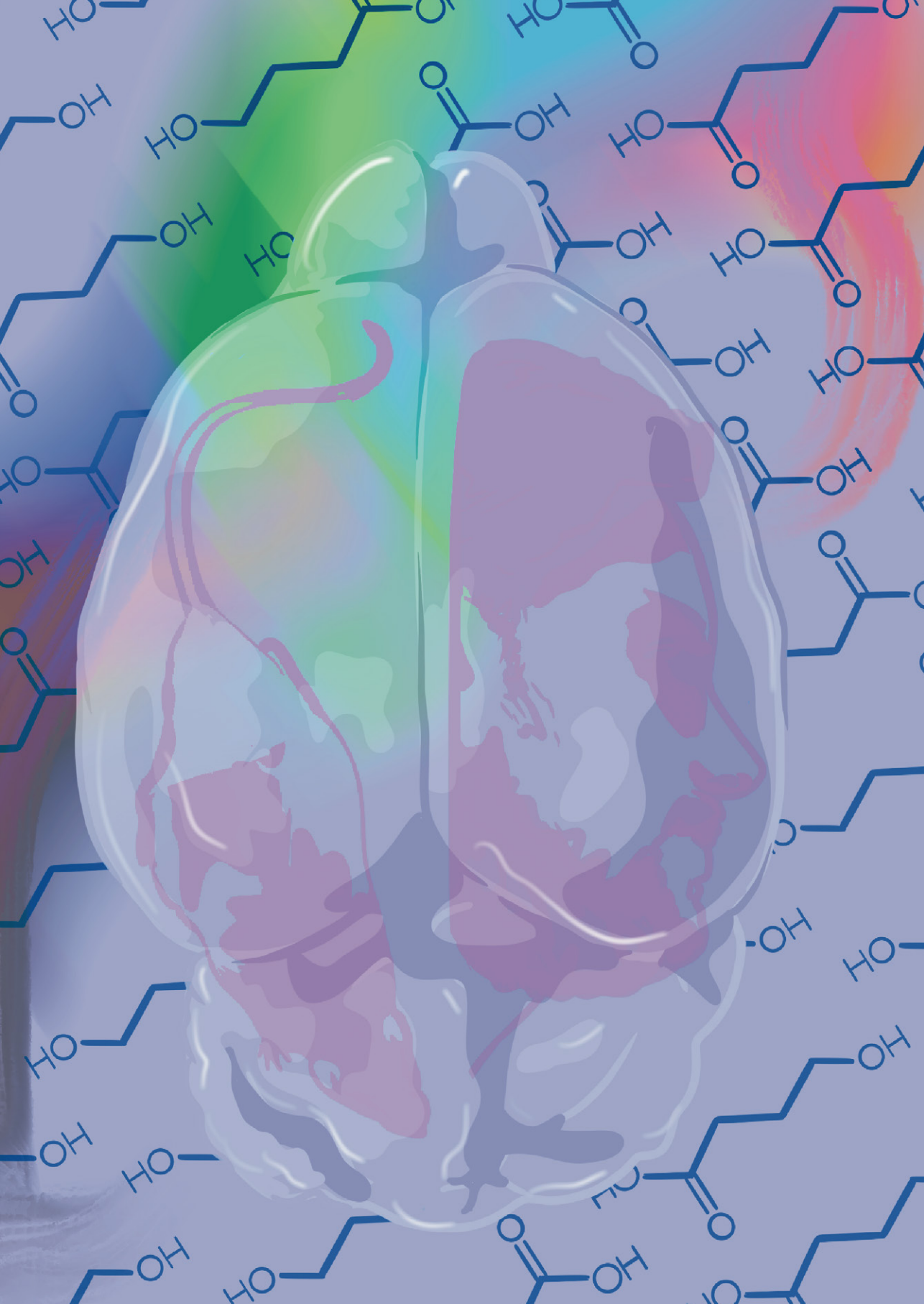
van Nieuwenhuijzen, P. S., Long, L. E., Hunt, G. E., Arnold, J. C., & McGregor, I. S. (2010). Residual social, memory and oxytocin-related changes in rats following repeated exposure to gamma-hydroxybutyrate (GHB), 3,4-methylenedioxymethamphetamine (MDMA) or their combination. *Psychopharmacology (Berl)*, *212*(4), 663-674. https://doi.org/10.1007/s00213-010-1986-5

Walf, A. A., & Frye, C. A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nature protocols*, *2*(2), 322-328.

Wang, Y. G., Swick, T. J., Carter, L. P., Thorpy, M. J., & Benowitz, N. L. (2009). Safety overview of postmarketing and clinical experience of sodium oxybate (Xyrem): abuse, misuse, dependence, and diversion. *Journal of Clinical Sleep Medicine*, *5*(4), 365-371.

Watson, J., Guzzetti, S., Franchi, C., Di Clemente, A., Burbassi, S., Emri, Z., Leresche, N., Parri, H. R., Crunelli, V., & Cervo, L. (2010). Gamma-hydroxybutyrate does not maintain self-administration but induces conditioned place preference when injected in the ventral tegmental area. *International Journal of Neuropsychopharmacology*, *13*(2), 143-153.

Wolf, C. J. H., Beurmanjer, H., Dijkstra, B. A. G., Geerlings, A. C., Spoelder, M., Homberg, J. R., & Schellekens, A. F. A. (2021). Characterization of the GHB Withdrawal Syndrome. *J Clin Med*, *10*(11). https://doi.org/10.3390/jcm10112333



Chapter 4

The short- and long-term effects of GHB use and GHB-induced comas on working memory and impulsivity in outbred rats

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Abstract

The use of the recreational drug Gamma-Hydroxybutyric acid (GHB) can lead to accidental overdosing and concurrent GHB-induced comas due to its steep dose-response curve. GHB use has been implicated in decreased long-term memory performance. GHB-induced comas in humans have also been associated with reduced memory performance and increased impulsivity. However, these findings may be confounded by co-morbid drug use, co-morbid psychiatric disorders, or heavier GHB use in the coma group. Here, we aimed to elucidate the short- and long-term effects of GHB use and GHB-induced coma on working memory performance and impulsivity in rats. Using touchscreen operant cages, we first trained outbred rats (n=48) on the trial-unique, delayed nonmatching to location (TUNL) working memory task. Rats then received increasing doses of GHB leading to a total of two comas (coma group), an equivalent total dose divided over multiple low doses of GHB (no coma group), or isovolumetric saline (control group) through oral gavage. Working memory performance and impulsivity were assessed before, one day after, and two months after the final administration of GHB. We found no residual effects of GHB intake, or GHB-induced comas on varying difficulties of the TUNL task or on proxies of impulsivity. Our results suggest that GHB or GHB-induced comas do not have a causal negative effect on working memory and impulsivity in rats. Negative cognitive effects of GHB may be restricted to hippocampal-dependent transition from working- to long-term memory, while leaving non-hippocampal-dependent memory performance intact. Future studies should examine possible dose-dependent effects of GHB use on other memory domains, and the effects of more than two GHB-comas, in order to further complete our understanding of GHB use on cognition.

Introduction

Gamma-hydroxybutyric acid (GHB) is a recreational and therapeutic drug with addictive potential. GHB can lead to dose-dependent stimulating, disinhibiting and sedative behavioral effects (Kamal, van Noorden, et al., 2016). Low doses of GHB can activate the GHB receptor, while high doses of GHB increasingly activate the inhibitory $GABA_B$ receptor. Due to the steep dose-response curve of GHB, over 50% of recreational users and over 80% of patients with GHB use disorder (GUD) overdose on GHB (Oene et al., 2013; Ogeil et al., 2023). Symptoms linked to GHB overdose vary from nausea, vomiting and headaches, to respiratory depression, bradycardia and coma. These symptoms are primarily driven by $GABA_B$ receptor activation.

Despite a last-year use prevalence of ~1.6% in several Western countries, GHB was involved in over 25% of all drug-related emergency care visits, primarily due to GHB-induced coma (EMCDDA, 2023). Due to the short half-life of GHB (30-60 minutes), GHB-induced comas typically last 1-3 hours and leave no residual physical or behavioral effects (Borgen et al., 2004; Brenneisen et al., 2004; Liechti et al., 2006; Korf et al., 2014; Miró et al., 2017). Consequentially, patients with GUD generally do not consider these comas as harmful (Beurmanjer et al., 2019).

GHB-induced comas have been implicated in impaired cognitive performance. Raposo-Pereira et al. (2018) showed that a group of GHB users reporting more than 4 comas exhibited decreased verbal memory performance and increased impulsivity compared to a group of GHB users experiencing less than 4 comas and compared to a group of poly-drug users (no GHB) (Raposo Pereira et al., 2018). However, no effects were found for spatial memory performance (Raposo Pereira et al., 2018; Raposo Pereira et al., 2020). Possibly, decreased verbal memory performance in the coma group has been confounded by lower premorbid verbal intelligence and heavier GHB use in the coma group. Due to the cross-sectional associative nature of the studies, it is difficult to pinpoint whether the negative effects on working memory and impulsivity were a consequence of GHB-induced comas or contributed to the frequent occurrence of GHB-induced comas. A causal link between GHB-induced comas and decreased memory performance has not been established or examined.

Besides GHB-induced comas, GHB intoxication and GUD in humans have also been linked to different types of deficits in memory function (Carter et al., 2006; Beurmanjer et al., 2022). Deficits in (spatial) working memory performance have been found in rats, although only low doses of GHB were tested (Pedraza et al. 2009). More recently, animal studies demonstrated impaired long-term memory performance

following higher doses of GHB administration, but did not include GHB-induced coma's (Chen et al., 2017; Wolf et al., 2024). Taken together, whether GHB-induced comas contribute to memory deficits remains a topic of debate (van Amsterdam et al., 2022) and has not been studied in controlled animal experiments, where it is possible to control GHB exposure and to conduct before- and after measurements.

In this study, we aim to differentiate the effects of GHB use from GHB-induced comas on working memory and impulsivity in an animal model. We hypothesize that GHB-induced comas negatively impact these cognitive functions, whereas repeated low-dose GHB administration has no significant effect. To test this, we employed a touchscreen-based, trial-unique, delayed nonmatching-to-location (TUNL) task in rats. This allowed us to evaluate the effects of GHB exposure, with and without comas, on working memory performance and impulsivity at varying levels of task difficulty.

Material and methods

The methods of this study are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020; Curtis et al., 2022).

Animals

We used 48 Long Evans rats (58% female) (Janvier, France, PND28 upon arrival). We employed rats in this study because the rat is the preferred species for preclinical addiction research (Homberg et al., 2017). Rats were pseudo-randomly assigned to groups based on the order they met the training criterion: the first to a coma group (receiving increasing doses of GHB), the second to a no-coma group (receiving low, constant doses of GHB), the third to a control group (receiving saline), the fourth to the coma group, etc. Males and females were balanced between groups.

As a part of our ethical approval application, we performed an *a priori* sample size calculation with visuospatial working memory in a TUNL task as a main outcome parameter. Based on previous findings (Talpos et al., 2010; Davies et al., 2017) (standardized effect size = 0.74), and with a power of >0.80 and an α of 0.05, a sample size of 5 rats per group was required to compare memory performance between the three groups. To also be able to perform *post hoc* comparisons between groups, using identical parameters, a sample size of 14 rats per group was required when correcting for multiple comparisons. Accounting for rats that will likely not acquire the task (~10%, based on unpublished data from our lab), we used 16 rats per group, leading to a total of 48 rats.

This study involved two separate batches of 24 rats each, undergoing identical procedures. During the first batch, 12 males and 12 females were tested. Due to high mortality in the male coma group (3 out of 4) and no mortality in the female coma group, we decided to only include females in the coma group of the second batch. This resulted in the use of 16 females and 8 males in the second batch, totaling to 28 females and 20 males. In total, six rats in the coma group died, either due to GHB toxicity (one female, three males) or improper oral gavage administration (two females). Four rats in the no-coma group died due to improper oral gavage administration (one female, two males) or unknown cause (one female). Considering the drop-out of rats that did not acquire the TUNL task, the coma group consisted of seven female rats, the no-coma group included 12 rats (seven female, five male), and the control group comprised 15 rats (nine female, six male).

Upon arrival, rats were habituated to their housing room for 14 days. All rats were pair-housed in enriched type IV cages (59 × 38 × 20 cm; Tecniplast 1500U) with corncob bedding (GM 12 irradiated, Bio Services) under conventional conditions (no filtertops). Cage location in the housing room was randomized to prevent unwanted effects caused by the environment. The rats had *ad libitum* access to food (dried pellets of standard chow food [ssniff RM V1534-703, Bio Services]) and water during the GHB/saline administration period. During cognitive task training and testing, rats were food-restricted by providing *ad libitum* food on Fridays, weighing the remaining food on Monday, and providing 90% of their 24-hour intake every weekday. This food restriction regimen allowed both increased motivation for the TUNL task and unrestricted development of the rats. Rats were maintained on a reversed light-dark schedule (lights off at 08:00h) in temperature- (21 ± 1°C) and humidity-controlled (55% ± 5%) rooms.

The experimental procedures were performed under a project license from the Central Committee on Animal Experiments (Centrale Commissie Dierproeven, The Hague, The Netherlands; AVD1030020198688), in full compliance with the legal requirements of Dutch legislation on the use and protection of laboratory animals (Animal Testing Act). All efforts were made to reduce the number of animals used and their suffering.

GHB administration

Rats first underwent training on a visuospatial working memory task (Cognitive testing, described in detail below). Then, rats were subdivided into three groups: 1) coma, 2) no-coma, 3) control. Depending on the group, GHB (Xyrem, Jazz Pharmaceuticals, 500mg/mL) or saline (0.9% NaCl) was administered through oral

gavage. GHB coma rats received daily increasing doses of GHB (starting at 0.3g/kg), with increments of 0.4 g/kg GHB per administration (Suppl. Table 1). No-coma rats initially received 0.3g/kg oral GHB administrations once or twice per day, with a minimum of 5 hours in between administrations. The no-coma dose was later increased to 0.7 g/kg per administration to reach the matching total GHB dose of coma rats faster. No comas were observed with the 0.7 g/kg dose. Due to complications following GHB oral gavages, three rats were fed GHB through food in the final GHB administration sessions to decrease the risk of adverse events.

Behavioral measurement and comas

Directly following GHB/saline administration, rats were placed in regular Type-III cages, where their behavior was automatically recorded for 2.5 hours using the sensor-based system LABORAS (Metris B.V., Hoofddorp, The Netherlands); Laboratory Animal Behavior Observation Registration and Analysis System (Castagné et al., 2012). Rats were habituated to the LABORAS cage for one session, without receiving oral gavage. After the injection, the rats were continuously monitored by an expert observer through a live video recording for 2.5 hours. The coma group underwent two GHB-induced comas, manually assessed by examining the loss-of-righting reflex (LORR). If a marked decrease in locomotion was observed, rats were placed on their side. In case a rat was unable to right itself within two minutes, we assessed the response to a painful stimulus (toe pinch). Coma was confirmed if no auditory- or motor response to the painful stimulus was observed. Due to limited availability of the LABORAS cages, no-coma rats were placed in their home cage following GHB administration after 10 LABORAS sessions.

Cognitive testing

Visuospatial working memory performance and impulsivity were tested using the TUNL task (Talpos et al., 2010). The task was performed in sound-attenuated Bussey-Saksida Touch Screen Chambers for rats (33.2 x 24 x 30 cm (l x w x h)) (Campden Instruments, Loughborough, England), running on Whisker Standard Software and ABET II software for Operant Control (Cardinal & Aitken, 2010). The chambers contained a touchscreen on one side of the cage, and a pellet dispenser on the opposite side. A polycarbonate mask, dividing the touchscreen in 15 equal squares, was placed in front of the touch screen from the 'Must touch'-phase onwards. Rats were trained according to the learning schedule described in Table 1 until they reached a 70% correct score in three consecutive TUNL sessions (large separation / short delay). During the TUNL task, one square was illuminated (sample phase), which the rat needed to press to get a food reward in 33% of trials. After a head entry into the food magazine, both the previous square and a new square were illuminated

(choice phase). The rat needed to press the new square to receive a food reward. All training sessions lasted 30 minutes.

Table 1. Different stages of cognitive testing and training.

TUNL training			
Learning stage	**Criteria**	**Description**	
Habituation	Complete one session	The rat was provided with 25 pellets at variable time intervals.	
Autoshaping	All 25 pellets eaten	In order to learn the association between the stimulus and the reward, the rat received a reward regardless of whether it touched the stimulus and would receive the reward immediately when it touched the active stimulus.	
Must touch	Two sessions 25 correct responses and all 25 pellets eaten	The rat had to touch the active stimulus to receive a reward, otherwise no reward was given.	
Punish incorrect	At least 70% correct responses for two sessions	When the active stimulus was touched, the rat received a food reward. When an inactive stimulus was touched, the rat received negative feedback (house light on) and no food reward was provided.	
TUNL large separation / short delay	At least 15 sessions & at least 70% correct responses for three consecutive sessions	The rat had to touch a single active stimulus. After a delay of one second, two squares are active.	
GHB / saline administration			
TUNL test			
Testing stage	**Duration**	**Days post-GHB (Initial test)**	**Days post-GHB (two-month follow-up)**
Reminder (30 minutes)	1 day	D0	D60
Large separation / Long delay (45 minutes)	2 days	D1-2	D61-62
Large separation (45 minutes)	2 days	D3-4	D63-64
Large separation / Long delay (45 minutes)	2 days	D5-6	D65-66
Large + medium separation (45 minutes)	3 days	D7-9	D67-69
Large + medium + small separation (45 minutes)	3 days	D10-12	D70-72

Following the GHB/saline administration, rats were tested on different variants of the TUNL test (Table 1). The delay between the sample phase and choice phases was either short (1 second) or long (6 seconds). A longer delay increases the difficulty of the trial since rats have to retain the newly provided information for six seconds instead of one second (Talpos et al. 2010). Additionally, the distance between squares varied both across and within sessions to adjust the task's difficulty, with closer squares being more difficult. The active (white) stimuli during large separation trials were separated by two inactive (black) squares, which were regarded as easy trials (Suppl. Fig. 3 G-H). The active stimuli during medium separation trials were separated by one inactive stimulus, and were regarded as medium difficult trials (Suppl. Fig. 3C-F). Finally, active stimuli during small separation trials were adjacent to each other (either horizontally or diagonally) and were considered difficult trials (Suppl. Fig. 3A-B). After completion of the first testing period (day 0 – day 12), rats were placed in their home cage for two additional months, after which they were re-tested according to the same testing schedule (Day 60 – day 72) to assess the long-term effects of GHB. During testing, total responses, correct responses, inter-trial interval (ITI) touches and blank touches were recorded. ITI- and blank touches had no programmed negative consequences.

Statistical analysis

The data and statistical analyses complied with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2022). Rats were treated as independent values, there were no technical replicates.

The percentage of correct responses, i.e. TUNL performance, was calculated as correct choice trials divided by total choice trials. Differences in training performance (% correct and number of sessions to reach criterion) between groups were analyzed using one- and two-way RM ANOVAs. Differences in TUNL performance between study cohorts were analyzed using a linear mixed model with cohort as a fixed effect and individual animal as a random effect. Two linear mixed models with session and group as fixed effects, and animal nested in group as a random effect were performed for the entire first and second testing period. Performance on individual TUNL stages were assessed with linear mixed models using group as fixed effect and animal nested in group as random effect. The effect of the two-month intermission between the two testing periods was assessed by comparing performance during the first- and second testing period with a linear mixed model (group and session as a fixed effect, and animal nested within treatment group as a random effect). Sessions with <5 choice trials were excluded from analysis.

As a proxy for impulsivity, the parameters blank touches and ITI touches were used. These touches reflect incorrect and premature responses. These parameters were

analyzed using linear mixed models for both the entire first- (D0-D12) and the entire second (D60-D72) testing period. Group (between-subject) and session (within-subject) were used as fixed effects, and animal nested within treatment group as a random effect. Where appropriate, each parameter was tested for normality using a Shapiro–Wilk test. Mauchly's test of sphericity was used to test whether variances of the differences between treatment levels were equal. If the assumption of sphericity was violated, or when dealing with repeated measures, a Geisser-Greenhouse correction was applied. Tukey post-hoc analyses were performed upon significant interactions between fixed effects. Post-hoc tests were only performed if data were normally distributed and if there was no inhomogeneity of variance. The threshold for statistical significance was set at $p < 0.05$. All data are presented as mean ± SEM. Statistical analyses were conducted using R (version 4.2.1), and graphs were created using GraphPad Prism (version 10.0).

Results

Training

All groups showed a similar increase in performance during the initial 15 training sessions of the TUNL, prior to GHB administration (Fig. 1A, two-way RM ANOVA, main effect of time, $F_{(7.994, 247.8)} = 4.541$, $p < 0.0001$; no effect of group, $p > 0.05$; no group x session interaction, $p > 0.05$). Groups did not differ in their performance during the last training session before the start of GHB/saline administration (Fig. 1B). There were no differences between groups in the number of sessions required to reach the criterion (three subsequent sessions of >70% correct) (Fig. 1C). Altogether, this demonstrates comparable baseline performance between groups. Four male rats did not reach the criterion and were therefore excluded from subsequent analyses.

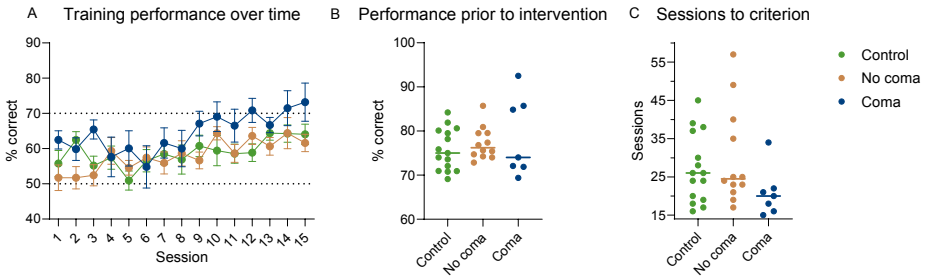


Figure 1. TUNL training performance of the three groups. A) performance during the first 15 TUNL training sessions. B) Average performance on the final three training sessions before drug exposure, expressed as % correct responses. C) number of sessions required to meet the criterium of three times >70% correct before drug exposure.

GHB-induced comas

The rats required high doses of GHB to experience comas, varying from 1,9 g/kg to 4,7 g/kg (μ = 3,60 g/kg, SD = 1,15 g/kg). The number of administrations varied from 9 to 28 injections for the control and coma rats, and from 24 to 83 injections for the no-coma rats to match the total dose of the coma rats. The total GHB dose administered to the coma and no-coma rats ranged from 20,6 g/kg to 78,6 g/kg (μ = 32,1 g/kg, SD = 16,1 g/kg).

Effect of GHB and GHB-induced comas on working memory

Effects of GHB on working memory performance during long delay trials

Because no differences between the two cohorts were detected, the cohorts were grouped in subsequent analyses ($p > 0.05$). When we examined the performance on large separation trials over the entire first (D0-D12) testing period, the interaction between group (coma versus no-coma) and session nearly reached significance (Suppl. Fig. 2A: Linear mixed model, group x time interaction, $t = -1.798$, $df = 356.48$, $p = 0.07$). Groups showed similar performance over time for the second testing period (D60-D73) (Suppl. Fig. 2B).

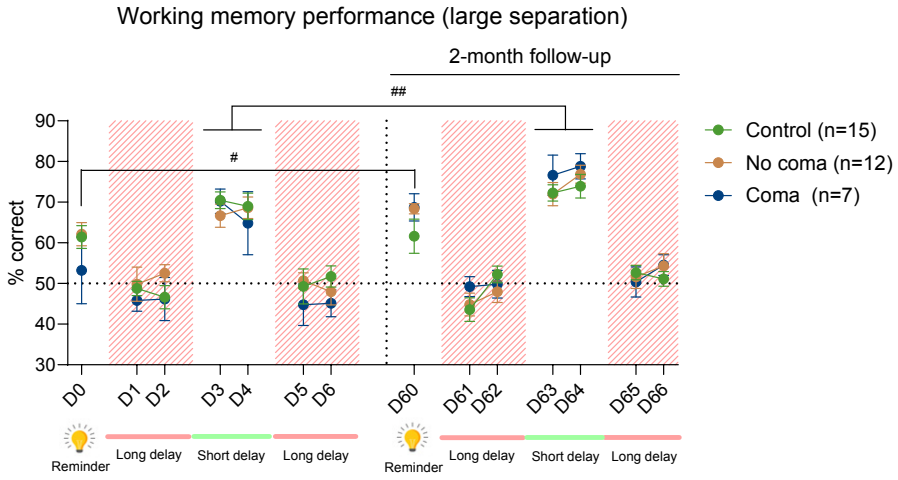


Figure 2. Working memory performance under either the short- or long delay between the sample and choice phase of the TUNL task. # = main effect of time $p < 0.05$, ## = main effect of time $p < 0.01$. Data points represent average ± SEM of % correct responding.

Zooming in on the different stages of the TUNL test, no differences between the groups were found for working memory performance after drug exposure on the reminder session, the long delay sessions or the short delay sessions with large separation (Fig. 2: linear mixed model, no effect of group, $p > 0.05$). All rats showed increased performance

after two months on the reminder session compared to the first reminder session (Fig. 2: linear mixed model, main effect of time, $t = 2.292$, $p < 0.05$), and performed better on the short sessions on D63/64 compared to the sessions on D3/4 (Fig. 2: linear mixed model, main effect of time, $t = 3.028$, $df = 102.19$, $p < 0.01$).

Effects of GHB on working memory during medium- and large separation trials

On days 7-9 and days 67-69 after GHB/saline administration, large- and medium separation trials were randomly alternated within each TUNL session with short delay. Rats performed better on the large separation trials during the 2-month follow-up (D67-D69) compared to D7-D9 (Fig. 3, linear mixed model, main effect of time, $t = 2.937$, $df = 143.59$, $p < 0.01$). Performance improved for the medium separation trials over D7-D9, but did not exceed chance level (Fig. 3, linear mixed model, main effect of time, $t = 2.232$, $df = 54.60$, $p < 0.05$). No differences between groups were found for either the large separation or the medium separation trials (Fig. 3).

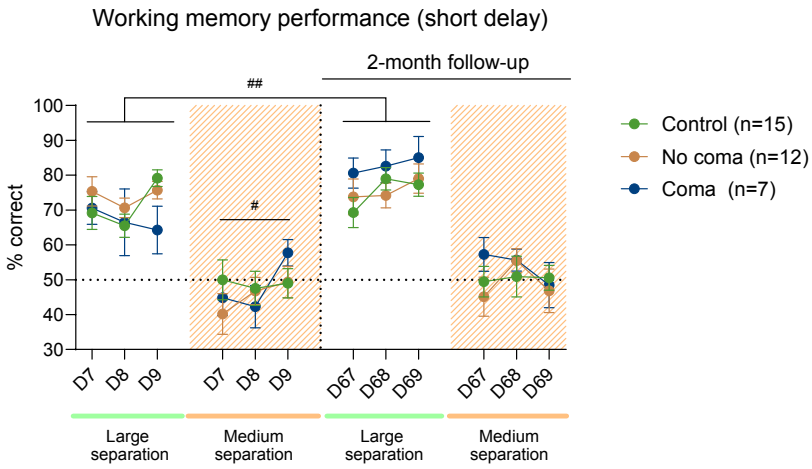


Figure 3: Working memory performance under different difficulties of the TUNL task, containing a short (1s) delay between the sample- and choice phase. Rats were tested shortly after GHB/saline administration (D7-D9) and two months following GHB/saline administration (D67-D69). Easy and medium trials were randomly alternated during each session. ## = main effect of time $p < 0.01$. Data points represent average ± SEM of % correct responding.

Effect of GHB on working memory during medium- and small separation trials

On days 10-12 after GHB/saline administration, large-, medium- and small separation trials were randomly alternated within one TUNL session. For the small separation trials, groups showed differences in their performance and performed differently over time, which was largely driven by better performance of the coma group during

days 10 and 11 and a decrease in performance on day 12 (Fig. 4, linear mixed model, main effect of group, t = -2.579, df = 77.00, p < 0.05; main effect of session, t = -2.151, df = 76.04, p < 0.05; group x session interaction, t = 2.528, df = 76, p < 0.05). Post-hoc comparisons revealed that indeed the coma group performed better than the no-coma and control groups (Tukey's post hoc test, coma vs no coma p < 0.05, coma vs control p < 0.05). No differences between groups were found >2 months after GHB/saline administration (Fig. 4).

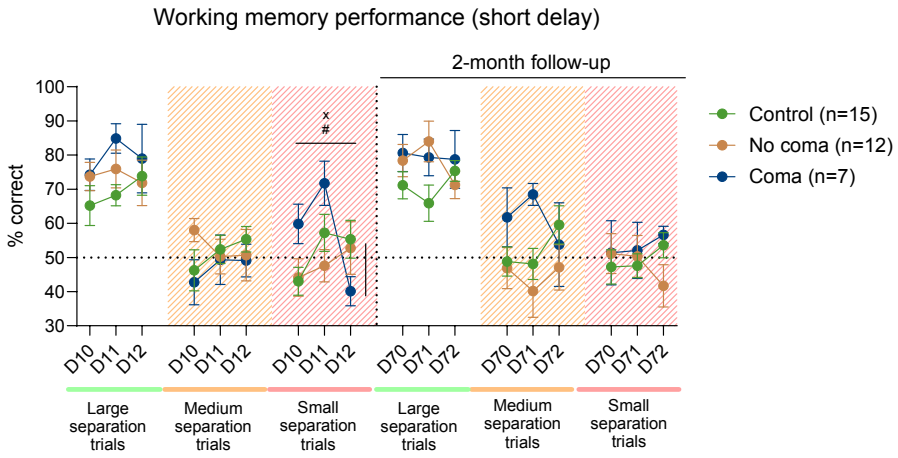


Figure 4. Working memory performance under different difficulties of the TUNL task, containing a short (1s) delay between the sample- and choice phase. Rats were tested on D10-D12 and on D70-D72 after GHB/saline administration. Easy, medium and difficult trials were randomly alternated during one session. Data points represent average ± SEM.

Effect of GHB on impulsivity

We did not find significant differences between groups in either blank touches or ITI touches across both the first testing period (D0-D12) and the second testing period (D60-D72) (Fig. 5A-D: p > 0.05). Although it appears that the coma group showed higher blank- and ITI-touches compared to the no-coma- and control groups, this is largely driven by the variance of individual animals within groups. In other words, for all four analyses, the random effect of individual rats nested within their experimental group explained a large fraction of the variance found in the linear mixed models compared to the fixed effects group and session (Fig. 5A, blank touches D0-D12: R^2 group * session: 0.10, R^2 including random effect: 0.57; Fig. 5B, blank touches D60-D76: R^2 group * session = 0.04, R^2 including random effect: 0.30; Fig. 5C, ITI touches D0 – D12: R^2 group * session: 0.08, R^2 including random effect: 0.41; Fig. 5D, ITI touches D60 – D72: R^2 group * session: 0.05, R^2 including random effect: 0.37).

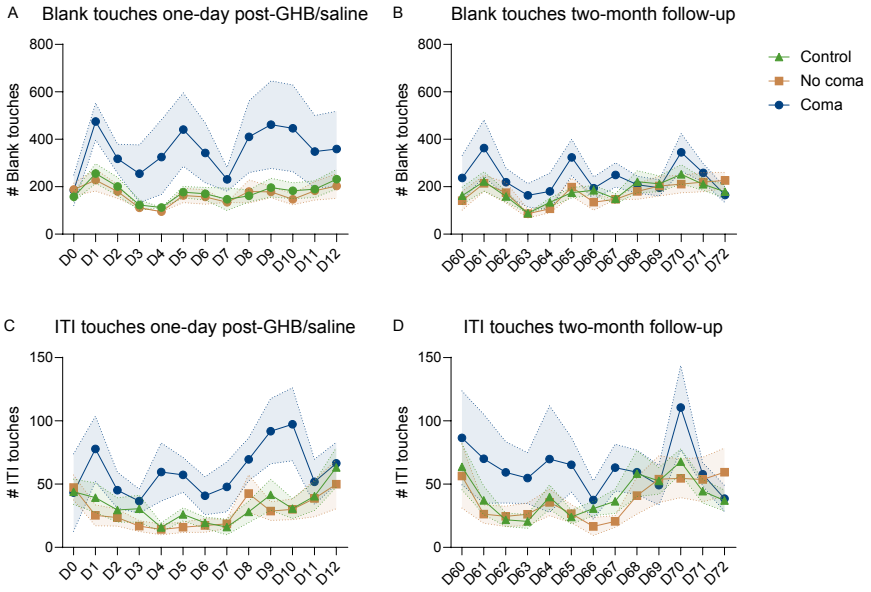


Figure 5. A-B) Total number of touchscreen responses on non-active stimuli per individual TUNL session. Blank touches were registered during both the sample phase (one active stimulus) and the choice phase (two active stimuli). C-D) Total number of touchscreen responses during the inter-trial interval (ITI), i.e. between the choice phase and a new sample phase. D0 and D60 = the reminder sessions (large separation short delay), D1-2, D5-6, D61-62 and D65-66 = large separation long-delay sessions, D3-4 and D63-64 = large separation short delay sessions, D7-9 and D67-69 = large + medium separation, short delay sessions, D10-12 and D70-72 = large + medium + small separation short delay sessions. Data points correspond to averages per group, shaded area represents standard error of the mean.

Discussion

This study aimed to assess the causal effects of GHB administration and GHB-induced comas on working memory performance and impulsivity in rats. We were not able to find effects of repeated GHB administration or GHB-induced comas on working memory performance during the first two weeks after GHB-administration, or after two months follow-up. Increasing the task's difficulty, either by increasing the time between trials or decreasing the distance of the pattern separation, reduced performance of all groups. However, the coma group performed better after drug exposure compared to the other groups during trials with small pattern separation. No effects of GHB or GHB-induced comas on proxies of impulsivity were found.

We observed improved performance in the coma group compared to the no-coma and control groups during difficult trials, contrary to our initial hypothesis. To our

knowledge, no previous studies have reported a cognitively enhancing effect of drug-induced comas, and there is currently no clear (neuro)biological explanation for this unexpected result. During the training phase, we found no significant differences between groups in the number of sessions required to reach the criterion or in the average percentage of correct responses upon reaching the criterion. However, the way the experimental groups were composed may have introduced a (non-significant) performance bias in the coma group. Specifically, the fastest-learning animal in each group of three was assigned to the coma group. Additionally, in the second cohort, only females were included in the coma group due to animal welfare considerations. Females appeared to acquire the task more quickly than males. These two factors could have collectively contributed to the coma group's superior performance during testing when difficult trials were introduced.

Performance during test sessions conducted 1-14 days after GHB appeared to be worse compared to the two-month follow-up, potentially due to stress-related effects. All animals underwent at least 9 oral gavages, a procedure known to induce mild stress in rats. This initial stress-effect may have attenuated over time, contributing to the improved performance at the two-month follow-up, regardless of group. Further support for this attenuation of the putative stress effects is the improved performance after a two-month-interval for the first nine test sessions following oral gavage, but not for the final three test sessions. Together, these findings suggest that GHB performance may have been impacted by stress, which attenuated over time.

We were not able to detect negative effects of repeated GHB administration on working memory performance. Existing preclinical literature on the effects of GHB on memory, particularly working memory (active manipulation of information) and short-term memory (passive retention of information), is limited. One study examining the residual effects of GHB on spatial working memory (30-second interval) using a spatial hole board test, found a negative effect of repeated (15x) low-dose GHB administration (10 mg/kg / 100 mg/kg) (Pedraza et al., 2009). However, other animal studies do not find effects of GHB on short-term (passive information retention) memory performance with a spatial recognition task (3-5 minute interval) (Pedraza et al., 2009; Klein et al., 2015). Studies using a larger (1h) interval demonstrated decreased object recognition memory following repeated (500mg/kg) GHB administration (van Nieuwenhuijzen, Long, et al., 2010). More recently, preclinical studies found a causal, long-lasting negative effect of GHB on long-term (12-24h) memory performance in tasks where the hippocampus is involved (Chen et al., 2017; Wolf et al., 2024). Spatial memory tasks, and the transition from working- to long-term memory, rely on brain circuits involving the dorsal hippocampus

(Goodrich-Hunsaker et al., 2008; Cohen & Stackman Jr, 2015). The TUNL task has been shown to depend on hippocampal integrity for small- and medium separation trials, where hippocampal-dependent pattern separation plays a critical role (Bakker et al., 2008; Talpos et al., 2010). Our findings showed chance-level performance for the control group with medium and small separation trials, limiting the detection of potential negative effects of GHB on hippocampal-dependent TUNL performance. Taken together, we speculate that repeated GHB administration negatively impacts the hippocampal-dependent transition from working- to long-term memory, while leaving non-hippocampal-dependent memory performance intact. Future animal and human studies should further explore this hypothesis.

In humans, one study examining cognition in patients with GUD after GHB detoxification did not find working memory/attention impairments using a cognitive screener (Beurmanjer et al., 2022). However, memory performance with a ~5-min interval was shown to be affected in patients with GUD before detoxification, which partially recovered during detoxification (Beurmanjer et al. 2022). This cognitive screener is designed to detect robust cognitive impairments and might therefore may not be sensitive enough to detect more subtle working memory impairments. Nevertheless, these results are in line with the absence of clear residual working memory effects of GHB found in our study.

In our study, we were not able to detect negative effects of GHB-induced comas on working memory using the TUNL task. A human study that examined the association between GHB-induced comas or GHB use and performance on the two-dimensional spatial recognition memory (SRM) test, a test that is similar to the TUNL test, also did not find any effects of GHB (Raposo Pereira et al., 2018). Pereira et al. (2018) did find an association between the occurrence of GHB-induced comas in patients with GUD and verbal memory performance (Raposo Pereira et al., 2018). Due to the associative nature of their study, these findings may have been confounded by co-morbid drug use, co-morbid psychiatric disorders or heavier GHB use in the coma group. In addition, decreased baseline memory performance, as seen with e.g. people with (mild) intellectual disability, may have contributed to a higher frequency of comas, for example, due to a limited capacity to recall “safe” doses. Our study, eliminating the possibility of reverse causality and other confounders, was not able to replicate a GHB-coma effect on memory performance.

We did not find effects of GHB use or GHB-induced comas on proxies for impulsivity. Pereira et al. (2020) found increased impulsivity in GHB users who experienced repeated comas compared to GHB users that did not experience repeated comas,

or to a control group. Due to the associative nature of their study, it is difficult to pinpoint whether the increased impulsivity was pre-existent in the coma group, or if it was a consequence of the repeated comas. For other substances, e.g. alcohol (Dick et al., 2010), it has been shown that impulsivity can both be a determinant and a consequence of substance use (De Wit, 2009). In light of our results, the association between GHB-induced comas and increased impulsivity may have been a result of reverse causality, where people with increased impulsivity are more likely to overdose and experience GHB-induced comas.

In the studies by Pereira et al., the coma group experienced a minimum of four comas (with the majority experiencing >20 comas), which is similar to the clinical situation. In contrast, the rats experienced only two comas in our study. To pinpoint whether the occurrence of only two (GHB-induced) comas would have been sufficient to demonstrate cognitive effects, we take a look at other studies examining the cognitive effects of comas. Coma caused by the toxic compound hydrogen sulfide (H2S) resulted in a decreased use of spatial search strategies in a spatial memory task in rats (Sonobe et al., 2015), although H2S has also been shown to lead to dose-dependent cognitive effects (Struve et al., 2001). Comas caused by hypoglycemia in type 1 diabetic patients can lead to cognitive deterioration associated with neuronal death in the hippocampus (Languren et al., 2013). Hypoglycemia without comas also leads to neurotoxicity, and is associated with impaired performance in memory and attention tasks (Languren et al., 2013). This suggests that coma itself is not likely an isolated component that leads to brain damage or cognitive decline. In addition, general anesthesia during surgery, defined by complete unconsciousness and no responses to external stimuli, does not lead to further residual cognitive effects compared to local anesthesia, showing that drug-induced unconsciousness does not automatically lead to residual cognitive effects (Davis et al., 2014). Altogether, it appears that the occurrence of one coma is sufficient to induce cognitive effects, although these effects are likely to be dependent on the toxic properties of the chemical. Therefore, we expect that possible toxic effects of GHB-induced comas would have been greater with a greater number of comas, while we also believe that two GHB-induced comas would have been able to induce cognitive effects in case of a clear toxic effect of GHB-induced comas.

The current findings should be viewed in light of several study limitations. We observed a large drop-out in our study following the administration of high doses of GHB. This may have introduced a bias in our results, since rats that were most sensitive to the sedative effects of GHB were not represented anymore in this study. It is imaginable that these rats were also more sensitive to possible cognitive

effects of GHB, although this remains speculation. The large drop-out also further emphasizes the need for caution in the use of GHB in both clinical and preclinical studies. Additionally, in this study we did not use a cognitive task specifically designed for impulsivity. Instead, we used ITI touches and blank touches as proxies for impulsivity. However, the outcome of these proxies may have been confounded by factors as hyperactivity and increased arousal. Future studies should verify our results on the effects of GHB on impulsivity with a task specifically designed to test impulsivity. Due to limitations in sample size, we were not able to make sex comparisons in this study. The residual decrease in long-term memory performance specific for male rats previously found by our group (Wolf et al., 2024) calls for a further examination of sex-specific effects of GHB on cognition, and should be taken into account in future research.

Altogether, we were not able to find residual effects of GHB use on working memory performance and proxies for impulsivity in rats. We did not find evidence for an additive negative effect of GHB-induced comas on working memory performance, opening up the possibility for a dose-dependent effect on cognition rather than an additive effect of GHB-induced comas. We speculate that negative effects of GHB use and GHB-induced comas may be specific for hippocampal-dependent processes and long-term memory performance, which should be further examined. Besides the possible cognitive effects of GHB, high doses of GHB and associated GHB-induced comas are inherently associated with serious health risks such as respiratory depression and death, and the occurrence of comas should therefore be minimized. Future studies should further explore the effects of GHB use on hippocampal-dependent memory processes in humans.

Bibliography

Bakker, A., Kirwan, C. B., Miller, M., & Stark, C. E. (2008). Pattern separation in the human hippocampal CA3 and dentate gyrus. *science*, *319*(5870), 1640-1642.

Beurmanjer, H., Asperslag, E. M., Oliemeulen, L., Goudriaan, A. E., De Jong, C. A. J., Schellekens, A. F. A., & Dijkstra, B. A. G. (2019). A Qualitative Approach in Understanding Illness Perception and Treatment Needs in Patients with Gamma Hydroxybutyrate Use Disorder. *Eur Addict Res*, *25*(5), 248-255. https://doi.org/10.1159/000500807

Beurmanjer, H., Bruijnen, C. J. W. H., Greeven, P. G. J., De Jong, C. A. J., Schellekens, A. F. A., & Dijkstra, B. A. G. (2022). Cognitive Impairments in Patients with GHB Use Disorder Predict Relapse in GHB Use. *Eur Addict Res*, *28*(5), 350-357. https://doi.org/10.1159/000525507

Borgen, L. A., Okerholm, R. A., Lai, A., & Scharf, M. B. (2004). The pharmacokinetics of sodium oxybate oral solution following acute and chronic administration to narcoleptic patients. *The Journal of Clinical Pharmacology*, *44*(3), 253-257.

Brenneisen, R., Elsohly, M. A., Murphy, T. P., Passarelli, J., Russmann, S., Salamone, S. J., & Watson, D. E. (2004). Pharmacokinetics and excretion of gamma-hydroxybutyrate (GHB) in healthy subjects. *J Anal Toxicol*, *28*(8), 625-630. https://doi.org/10.1093/jat/28.8.625

Cardinal, R. N., & Aitken, M. R. (2010). Whisker: a client—server high-performance multimedia research control system. *Behavior research methods*, *42*(4), 1059-1071.

Carter, L. P., Richards, B. D., Mintzer, M. Z., & Griffiths, R. R. (2006). Relative abuse liability of GHB in humans: A comparison of psychomotor, subjective, and cognitive effects of supratherapeutic doses of triazolam, pentobarbital, and GHB. *Neuropsychopharmacology*, *31*(11), 2537-2551. https://doi.org/10.1038/sj.npp.1301146

Castagné, V., Wolinsky, T., Quinn, L., & Virley, D. (2012). Differential behavioral profiling of stimulant substances in the rat using the LABORAS™ system. *Pharmacology Biochemistry Behavior*, *101*(4), 553-563.

Chen, L. Y., Renn, T. Y., Liao, W. C., Mai, F. D., Ho, Y. J., Hsiao, G., Lee, A. W., & Chang, H. M. (2017). Melatonin successfully rescues hippocampal bioenergetics and improves cognitive function following drug intoxication by promoting Nrf2-ARE signaling activity. *J Pineal Res*, *63*(2). https://doi.org/10.1111/jpi.12417

Cohen, S. J., & Stackman Jr, R. W. (2015). Assessing rodent hippocampal involvement in the novel object recognition task. A review. *Behavioural brain research*, *285*, 105-117.

Curtis, M. J., Alexander, S. P. H., Cirino, G., George, C. H., Kendall, D. A., Insel, P. A., Izzo, A. A., Ji, Y., Panettieri, R. A., & Patel, H. H. (2022). Planning experiments: Updated guidance on experimental design and analysis and their reporting III. *British Journal of Pharmacology*, *179*(15), 3907-3913.

Davies, D. A., Hurtubise, J. L., Greba, Q., & Howland, J. G. (2017). Medial prefrontal cortex and dorsomedial striatum are necessary for the trial-unique, delayed nonmatching-to-location (TUNL) task in rats: role of NMDA receptors. *Learning Memory*, *24*(6), 262-266.

Davis, N., Lee, M., Lin, A. Y., Lynch, L., Monteleone, M., Falzon, L., Ispahany, N., & Lei, S. (2014). Postoperative cognitive function following general versus regional anesthesia: a systematic review. *Journal of neurosurgical anesthesiology*, *26*(4), 369-376.

De Wit, H. (2009). Impulsivity as a determinant and consequence of drug use: a review of underlying processes. *Addiction biology*, *14*(1), 22-31.

Dick, D. M., Smith, G., Olausson, P., Mitchell, S. H., Leeman, R. F., O'Malley, S. S., & Sher, K. (2010). Understanding the construct of impulsivity and its relationship to alcohol use disorders. *Addiction biology*, *15*(2), 217-226.

EMCDDA. (2023). *European Drug Report 2023: Trends and Developments*.

Goodrich-Hunsaker, N. J., Hunsaker, M. R., & Kesner, R. P. (2008). The interactions and dissociations of the dorsal hippocampus subregions: how the dentate gyrus, CA3, and CA1 process spatial information. *Behavioral neuroscience*, *122*(1), 16.

Homberg, J. R., Wöhr, M., & Alenina, N. (2017). Comeback of the rat in biomedical research. *ACS Chemical Neuroscience*, *8*(5), 900-903.

Kamal, R. M., van Noorden, M. S., Franzek, E., Dijkstra, B. A. G., Loonen, A. J. M., & De Jong, C. A. J. (2016). The neurobiological mechanisms of gamma-hydroxybutyrate dependence and withdrawal and their clinical relevance: a review. *Neuropsychobiology*, *73*(2), 65-80.

Klein, C., Mathis, C., Leva, G., Patte-Mensah, C., Cassel, J. C., Maitre, M., & Mensah-Nyagan, A. G. (2015). gamma-Hydroxybutyrate (Xyrem) ameliorates clinical symptoms and neuropathology in a mouse model of Alzheimer's disease. *Neurobiol Aging*, *36*(2), 832-844. https://doi.org/10.1016/j.neurobiolaging.2014.10.003

Korf, D. J., Nabben, T., Benschop, A., Ribbink, K., & van Amsterdam, J. G. C. (2014). Risk factors of γ-hydroxybutyrate overdosing. *J European Addiction Research*, *20*(2), 66-74.

Languren, G., Montiel, T., Julio-Amilpas, A., & Massieu, L. (2013). Neuronal damage and cognitive impairment associated with hypoglycemia: an integrated view. *Neurochemistry international*, *63*(4), 331-343.

Liechti, M. E., Kunz, I., Greminger, P., Speich, R., & Kupferschmidt, H. (2006). Clinical features of gamma-hydroxybutyrate and gamma-butyrolactone toxicity and concomitant drug and alcohol use. *Drug alcohol dependence*, *81*(3), 323-326.

Miró, Ò., Galicia, M., Dargan, P., Dines, A. M., Giraudon, I., Heyerdahl, F., Hovda, K. E., Yates, C., Wood, D. M., & Liakoni, E. (2017). Intoxication by gamma hydroxybutyrate and related analogues: clinical characteristics and comparison between pure intoxication and that combined with other substances of abuse. *Toxicology letters*, *277*, 84-91.

Oene, G., Schellekens, A., Dijkstra, B., Kamal, R., & de Jong, C. (2013). Detoxification of patients with GHB dependence.

Ogeil, R. P., Faulkner, A., Beard, N., Killian, J. J., Arunogiri, S., Phan, V., Wilson, J., Smith, K., Lubman, D. I., & Scott, D. (2023). Adverse outcomes of GHB use: analysis of ambulance attendances in metropolitan and regional Victoria. *International Journal of Drug Policy*, *111*, 103932.

Pedraza, C., Garcia, F. B., & Navarro, J. F. (2009). Neurotoxic effects induced by gammahydroxybutyric acid (GHB) in male rats. *Int J Neuropsychopharmacol*, *12*(9), 1165-1177. https://doi.org/10.1017/S1461145709000157

Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., Browne, W. J., Clark, A., Cuthill, I. C., & Dirnagl, U. (2020). The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *Journal of Cerebral Blood Flow and Metabolism*, *40*(9), 1769-1777.

Raposo Pereira, F., McMaster, M. T. B., Polderman, N., de Vries, Y., van den Brink, W., & van Wingen, G. A. (2018). Adverse effects of GHB-induced coma on long-term memory and related brain function. *Drug and alcohol dependence*, *190*, 29-36.

Raposo Pereira, F., McMaster, M. T. B., Schellekens, A. F. A., Polderman, N., de Vries, Y., van den Brink, W., & van Wingen, G. A. (2020). Effects of recreational GHB use and multiple GHB-induced comas on brain structure and impulsivity. *Frontiers in Psychiatry*, *11*, 166.

Sonobe, T., Chenuel, B., Cooper, T. K., & Haouzi, P. (2015). Immediate and long-term outcome of acute H2S intoxication induced coma in unanesthetized rats: effects of methylene blue. *PLoS One*, *10*(6), e0131340.

Struve, M. F., Brisbois, J. N., James, R. A., Marshall, M. W., & Dorman, D. C. (2001). Neurotoxicological effects associated with short-term exposure of Sprague–Dawley rats to hydrogen sulfide. *Neurotoxicology*, *22*(3), 375-385.

Talpos, J., McTighe, S., Dias, R., Saksida, L., & Bussey, T. (2010). Trial-unique, delayed nonmatching-to-location (TUNL): a novel, highly hippocampus-dependent automated touchscreen test of location memory and pattern separation. *Neurobiology of learning memory*, *94*(3), 341-352.

van Amsterdam, J. G. C., Brunt, T. M., Pereira, F. R., Crunelle, C. L., & van den Brink, W. (2022). Cognitive impairment following clinical or recreational use of Gammahydroxybutyric acid (GHB): a systematic review. *Current Neuropharmacology*, *20*(4), 809-819.

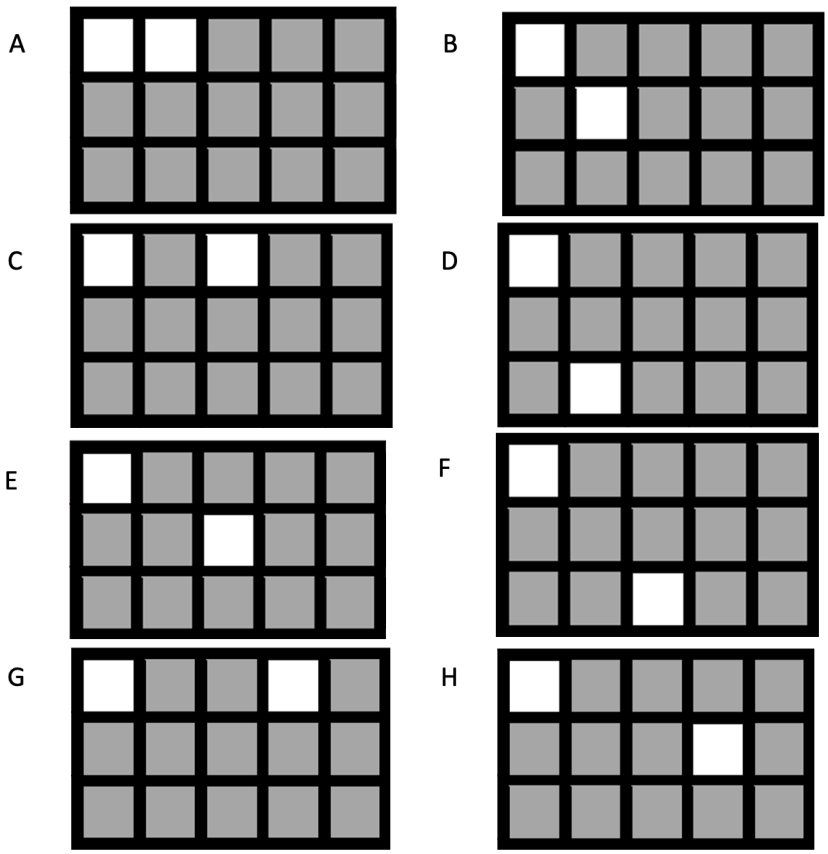
van Nieuwenhuijzen, P. S., Long, L. E., Hunt, G. E., Arnold, J. C., & McGregor, I. S. (2010). Residual social, memory and oxytocin-related changes in rats following repeated exposure to gamma-hydroxybutyrate (GHB), 3,4-methylenedioxymethamphetamine (MDMA) or their combination. *Psychopharmacology (Berl)*, *212*(4), 663-674. https://doi.org/10.1007/s00213-010-1986-5

Wolf, C. J. H., Spoelder, M., Beurmanjer, H., Bulthuis, R., Schellekens, A. F. A., & Homberg, J. R. (2024). Individual differences in GHB consumption in a new voluntary GHB self-administration model in outbred rats. *Psychopharmacology (Berl)*, *241*(3), 613-625. https://doi.org/10.1007/s00213-024-06537-5

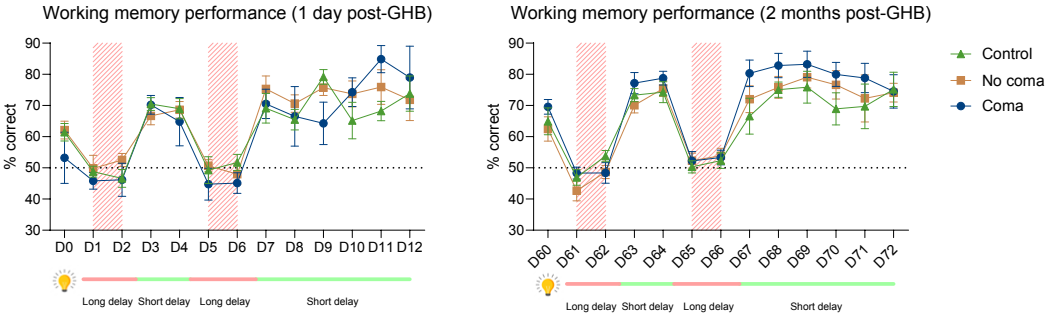
Supplementary files

Supplementary Table 1. Experimental group details

Group	GHB dose	Criterium to advance to TUNL test
GHB coma	Starting dose: 0.3 g/kg GHB (daily) Increment (no coma observed: 0.4 g/kg Increment (coma observed): 0.2 g/kg	2x GHB-induced coma (assess manually through loss of righting reflex and non-responsivity to painful stimulus)
GHB no coma	Dose: 0.3 g/kg / 0.7 g/kg (daily or bi-daily)	Equal total dose of GHB compared to paired GHB coma rat
Control	Saline (equivolumar to GHB coma, daily)	Equal amount of oral gavage administrations compared to paired GHB coma rat

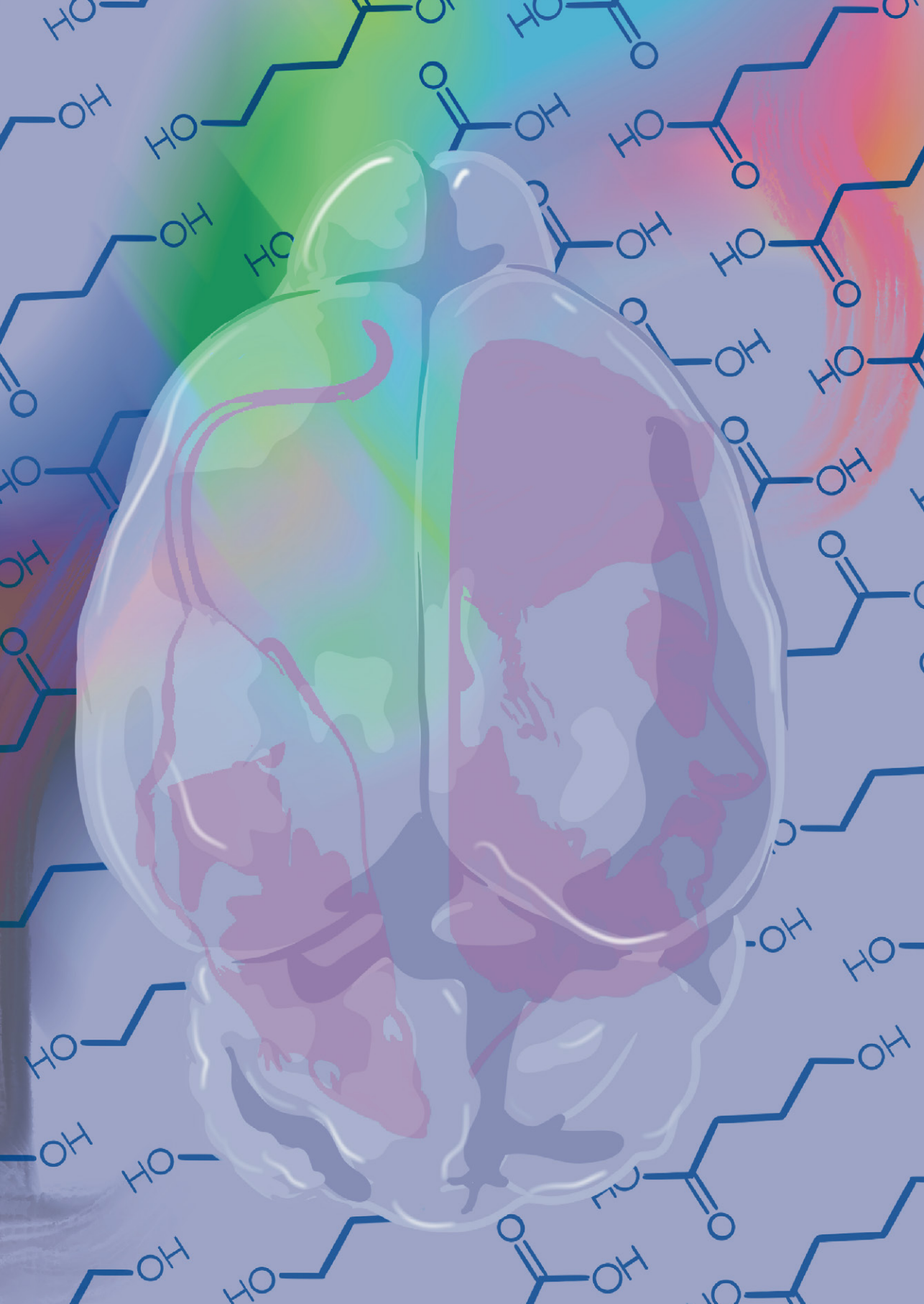


Supplementary Figure 1. A-B) Examples of difficult trials (small separation). C-F) Examples of medium trials (medium separation). G-H) Examples of large distances. All distances can appear on the entire screen and are not limited to the example locations shown here. All stacked distances were omitted due to a bias towards the bottom image found in previous studies.



Supplementary Figure 2. Working memory performance for large separation trials. TUNL test on day 1, 2, 5 and 6, and day 61, 62, 64 and 65 consist of a long delay (6s) between the sample- and choice phase. All other days consist of a short delay (1s) between the sample- and choice phase. The 50% line corresponds to chance-level performance. Data points represent average ± SEM.

4



Chapter 5

The neurobiological effects of voluntary GHB administration in the rat dorsal hippocampus

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Abstract

Gamma-hydroxybutyrate (GHB) is therapeutically used for sleep disorders, but also frequently misused as a recreational drug, potentially leading to cognitive impairments. Chronic GHB exposure has been associated with memory deficits, specifically within hippocampal-dependent memory domains. However, the causal effects of chronic voluntary GHB self-administration on the hippocampus remain unclear. This study investigated the neurobiological consequences of prolonged voluntary GHB self-administration on neuronal markers (NeuN), GABAergic markers (GAD67), glutamatergic markers (vGLUT1), and microglial activation (IBA-1) in subregions of the dorsal hippocampus of Long-Evans rats. A negative correlation was found between cumulative home-cage GHB intake and neuronal cell count in the CA1 and dentate gyrus subregion. However, no group-level differences between GHB-exposed and control animals were detected in the expression of neuronal, GABAergic, glutamatergic, or microglial markers. These findings highlight possible region-specific neurotoxicity linked to high-dose GHB consumption and emphasize the need for further studies examining dose-dependent effects and subregional differences of GHB-induced neurobiological changes.

Introduction

Gamma-hydroxybutyrate (GHB) is a psychoactive substance used to treat the sleep disorders narcolepsy and idiopathic hypersomnia (Xu et al., 2019). Over the past two decades, GHB has gained popularity as a recreational drug due to its euphoric and anxiolytic effects (Sumnall et al., 2008). This is especially the case in Western countries such as The Netherlands, Australia, Sweden and the United Kingdom, where the prevalence of recreational GHB use has increased up to 1.7% among young adults (EMCDDA, 2022, 2023, 2024). Repeated use of GHB can lead to GHB use disorder (GUD), which is characterized by the physical and psychological dependence on GHB, loss of control over GHB use, and a fast developing withdrawal syndrome (Brunt et al., 2013; Wolf et al., 2021).

Overdosing on GHB, resulting in temporary coma, is common amongst people who use GHB in a recreative manner. Users themselves often see this as innocent as they feel no residual effects of the overdose (Beurmanjer et al., 2019). However, GHB -induced comas and GHB use have been linked to cognitive effects and neurobiological changes (Raposo Pereira et al., 2018; Raposo Pereira et al., 2020; Beurmanjer et al., 2022; van Amsterdam et al., 2022). In recent years, several studies have focused on mapping the effects of GHB use and GHB induced comas on cognitive functions, especially its effects on memory.

Beurmanjer et al. (2022) detected impaired memory performance in patients with GUD. Additionally, Raposo-Pereira et al. (2018) demonstrated a memory-domain dependent association between the occurrence of GHB-induced comas and impaired "verbal" memory performance in GHB users, whereas no effects were found for short-term spatial- or recognition memory tests (Raposo Pereira et al. 2018). This memory-domain specificity is also reflected in animal models, with a recent study by our group demonstrating a causal negative effect of chronic GHB use on hippocampal-dependent long-term memory performance in male rats (Wolf et al., 2024). These cognitive effects may be a consequence of structural or functional alterations in specific brain regions such as the hippocampus. In another study we found no causal effects of repeated high doses of GHB on non-hippocampal dependent working memory or impulsivity-like behavior (Wolf et al., under rev.), potentially implying that specifically hippocampus-dependent cognitive functions are affected by GHB exposure.

GHB can exert dose-dependent bi-directional effects (Klein et al., 2009). Low doses of GHB can activate the so-called GHB receptors (an umbrella term for several high-

affinity targets), while high doses of GHB can also activate the $GABA_B$ receptor (Wolf et al., 2023). High-affinity binding sites of GHB are primarily located in the cortex and in subregions of the (dorsal) hippocampus, in accordance with GHB's distribution pattern (Hechler et al., 1992; Castelli et al., 2000; Klein et al., 2016). Due to the strong dose-dependent effects of GHB, GHB administration can lead to various neurobiological effects, both stimulating and sedating.

Repeated administration of low doses of GHB have been shown to decrease the number of neurons in the hippocampus and prefrontal cortex measured ~3 hours after the final GHB administration (Pedraza et al., 2009), whereas neuronal loss following high-dose GHB administration has not been studied so far. GHB has also been shown to increase binding of pCREB and to increase/decrease IGF-1, structures involved in energy homeostasis (Ren & Mody, 2006; Johansson et al., 2014). Other studies found that repeated administration of GHB resulted in increased oxidative stress in the hippocampus (van Nieuwenhuijzen, Long, et al., 2010; Youn et al., 2015; Chen et al., 2017). In contrast, an increasing body of evidence shows neuroprotective effects of GHB against ischemia through the activation of high-affinity GHB receptors (Leurs et al., 2021; Griem-Krey et al., 2023). The effects of GHB administration on neurobiological parameters remain unclear, leaving a debate on the exact effects of GHB in the brain.

Recently, our group established a GHB self-administration paradigm in rats (Wolf et al., 2024), which allowed us to study the causal effects of GHB consumption on behavior and cognition. There was substantial heterogeneity in GHB intake and addiction-like behavior, and we observed a negative effect of GHB consumption on long-term memory performance in male rats. In this study, we aimed to assess the neurobiological and possible neurotoxic effects of GHB self-administration in rats. Due to the complex pharmacological profile of GHB and its implication in both glutamatergic and GABAergic neuronal signaling, we determined the effects of GHB on neuronal levels, GABAergic neurons and glutamatergic neurons, in addition to the effect of GHB on microglia. More specific, we studied the effects of GHB self-administration on the intensity of the neuronal markers NeuN, GAD67, vGLUT1 and IBA-1 in the CA1, CA2, CA3 and dentate gyrus regions of the dorsal hippocampus.

Methods

The methods of this study are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020; Curtis et al., 2022).

Animals

36 wildtype Long-Evans rats (50% female) (Janvier, France, PND28 on arrival) were used for this study (see Wolf et al. 2024). We employed rats because the rat is the preferred species for preclinical addiction research (Homberg et al., 2017). 18 rats had access to GHB (GHB rats), and 18 rats did not have access to GHB (control rats). All rats were pair-housed within their experimental group in enriched type IV cages (59 × 38 × 20 cm; Tecniplast 1500U) with corncob bedding (GM 12 irradiated, Bio Services) under conventional conditions (no filtertops). The rats had ad libitum access to food (dried pellets of standard chow food [ssniff RM V1534-703, Bio Services]) and water. The rats were maintained on a reversed light-dark schedule (lights off at 08:00h) in temperature- (21 ± 1°C) and humidity-controlled (55% ± 5%) rooms. Due to technical and experimental limitations, we were not able to include all rats that were behaviorally tested in the study, leading to the use of 16 GHB rats and 14 control rats in this study. The experimental procedures were performed under a project license from the Central Committee on Animal Experiments (Centrale Commissie Dierproeven, The Hague, The Netherlands), in full compliance with the legal requirements of Dutch legislation on the use and protection of laboratory animals (Animal Testing Act). All efforts were made to reduce the number of animals used and their suffering.

GHB administration paradigm

For an extensive description of the behavioral paradigms of the animals used in this study, see Wolf et al. (2024). In short, GHB rats had access to ad libitum GHB in their home cage for three months. The average daily intake of GHB was 809.7 (± 226.7) mg/kg (males) and 660.6 (± 92.0) mg/kg (females), resulting in a total dose of ~72.9 g/kg and ~59,5 g/kg respectively. Following several behavioral tests and a period of forced abstinence, all animals had access to GHB under operant conditions. Control animals had only access to water in their home cage and did not undergo operant training and testing. GHB animals were sacrificed 2 weeks after the final operant test, and 34 weeks after the final home cage GHB exposure. Control animals were sacrificed at the same timepoints as the GHB animals.

Histology

Following perfusion, brains were removed and post-fixated in 4% paraformaldehyde (PFA) for 24 hours. Brains were then transferred to a 30% sucrose solution for three days and frozen at -80° until brain slicing. 40 µm coronal sections were obtained using a cryostat (Leica Biosystems, Germany) from Bregma -1.92 mm to -4.36 mm according to the rat brain in stereotaxic coordinates by Paxinos and Watson (Paxinos & Watson, 2006). Slices were stored at 4°C in 1x phosphate-buffered saline (PBS) + 0.01% sodium azide (Na-Az) until further use.

Immunohistochemical staining

Brain slices were brought up to room temperature and were washed three times for 10 minutes in 1x phosphate buffered saline (PBS) on a horizontal shaker. Then, the slices were pre-incubated in PBS-BT on the horizontal shaker for 30 minutes. Afterwards, the slices were incubated overnight on the horizontal shaker in PBS-BT together with the primary antibodies NeuN (1:1000, Merck, ABN91), GAD67 (1:1000, Sigma, MAB5406), vGlut1 (1:1000, Synaptic Systems, 135 302) and IBA-1 (1:1000, Thermo Fisher, MA5-27726).

18 hours following primary antibody incubation, the slices were washed three times in 1x PBS for 10 minutes on the horizontal shaker. Then, the slices were incubated in PBS-BT with the secondary antibodies AlexaFluor 488 Donkey anti-Mouse, AlexaFluor 555 Goat anti-Chicken, and AlexaFluor 647 Donkey anti-Guinea Pig for three hours. DAPI, a marker for cell nuclei, was added during the last 15 minutes of the incubation. After the incubation with the secondary antibodies, the slices were washed in 1x PBS three times for 10 minutes on the horizontal shaker. The slices were then mounted on microscope slides, and a coverslip was applied using FluorSave™.

Full staining protocol is available upon request.

Image analysis

Microscope images of the brain slices were processed to calculate the cell count and signal intensity in the different regions of interest (ROIs) of the dorsal hippocampus using FIJI (Fiji is just ImageJ). ROIs were manually drawn according to the images corresponding to the bregma coordinates (Paxinos & Watson, 2006). The granular layer from the dentate gyrus was excluded when drawing the ROIs. The number of cells stained with DAPI and NeuN, and the number of GAD67-positive spots were detected and quantified using ComDet v.0.5.5 plugin for ImageJ (Katrukha, 2020). The surface area of each ROIs was automatically measured in each image. The number of cells stained with NeuN and the number of individual GAD67 spots were corrected for total number of cells measured with DAPI, to obtain the ratio of neurons / total cells and the ratio of GAD67 / total cells. The total intensity for vGLUT1 and IBA-1 was corrected for the surface area to obtain average signal intensity within one brain subregion. Cell counts and signal intensity were measured in the CA1, CA2, CA3 and the molecular and polymorph layer of the dentate gyrus of one hemisphere. All settings, including exposure time, were identical between all images when capturing the images. Comdet settings and FIJI macros for image preprocessing, signal intensity measurement and cell count are available upon request.

Statistical analysis

Image analysis was performed in a blinded manner, i.e. the researcher was not aware of the experimental group of the image that was analyzed. Cell counts and signal intensity for each individual antibody were averaged over slices per region per animal for subsequent analyses. The association between NeuN/GAD67 cell count, and vGLUT1 / IBA1 signal intensity versus home cage GHB administration was analyzed using Pearson's correlation for each subregion. Cell counts and signal intensity were compared between groups using a linear mixed model, with group and region (repeated element) as fixed effects, and animal nested within treatment group as a random effect. Due to the low n for vGLUT1 and IBA-1 stainings, exploratory, hypothesis-forming linear model analyses were performed with group and region as fixed effects. Significant interactions between fixed effects were followed by Tukey post-hoc analyses. Post-hoc tests were only performed if data were normally distributed and if there was no inhomogeneity of variance. The threshold for statistical significance was set at $p < 0.05$. All data are presented as mean ± SEM. Statistical analyses were conducted using R (version 4.2.1), and graphs were created using GraphPad Prism (version 10.0).

Results

NeuN

Relative number of NeuN-positive cells was negatively associated with the amount of GHB that was consumed during the home-cage self-administration period. This is the case for CA1 (Fig. 1A, r = -0.54, p < 0.05), and DG (Fig. 1D, r = -0.52, p <0.05). No correlation between operant GHB intake and the number of NeuN-positive cells was observed.

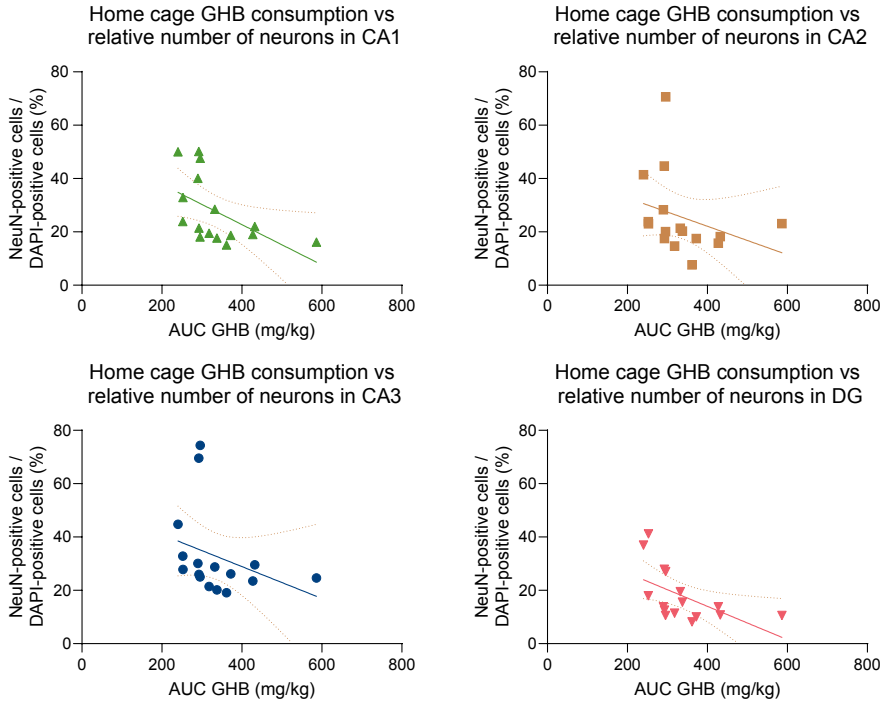


Figure 1. Correlation between relative NeuN cell count and GHB consumed in the home cage for the CA1 (A), CA2 (B), CA3 (C) and DG (D) subregion of the hippocampus. Solid line corresponds to correlation coefficient, dotted lines correspond to 95% confidence interval.

We did not find differences in the relative number of NeuN positive cells between control and GHB animals across all four hippocampal regions (CA1, CA2, CA3, dentate gyrus) (Fig. 2, p > 0.05). No sex-specific GHB effects were found.

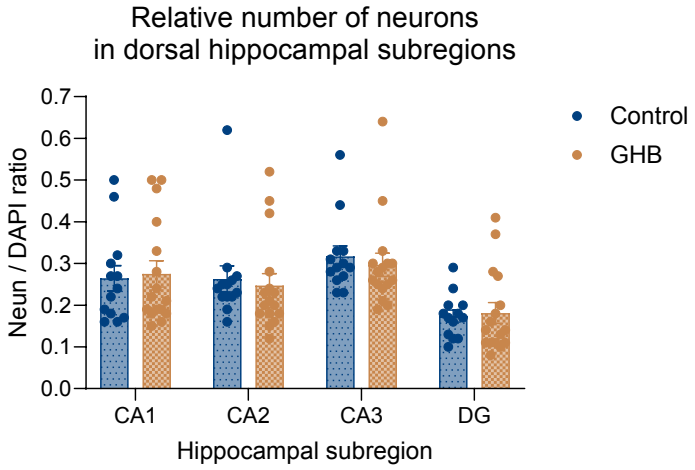


Figure 2. Relative NeuN cell count in CA1, CA2, CA3 and dentate gyrus (DG) subregion of the dorsal hippocampus. Data points represent individual animals. The bars represent means +/- standard error of the mean.

GAD67, vGLUT1 and IBA-1

We did not find differences in number of GAD67-positive spots between control and GHB animals for any of the hippocampal subregions (Fig. 3). No correlation between home cage GHB consumption and number of GAD67-positive cells was observed (not shown).

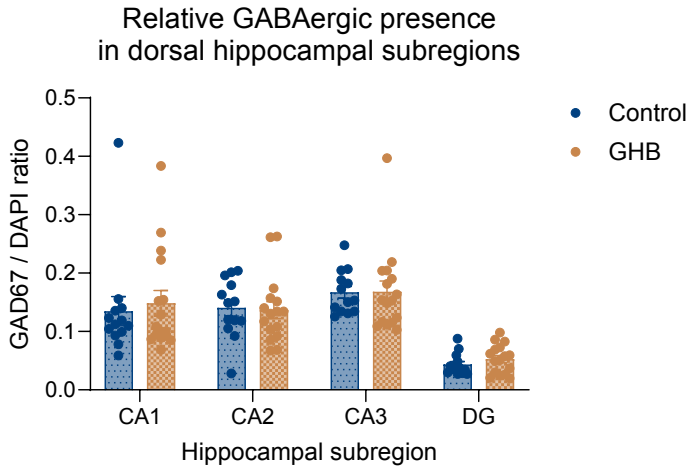


Figure 3. Relative GAD67 presence in CA1, CA2, CA3 and dentate gyrus (DG). Data points represent individual animals. The bars represent means +/- standard error of the mean.

Exploratory analyses for vGLUT1 and IBA-1 were also not able to detect any differences between GHB animals and control animals for any of the subregions (Fig. 4, Fig. 5).

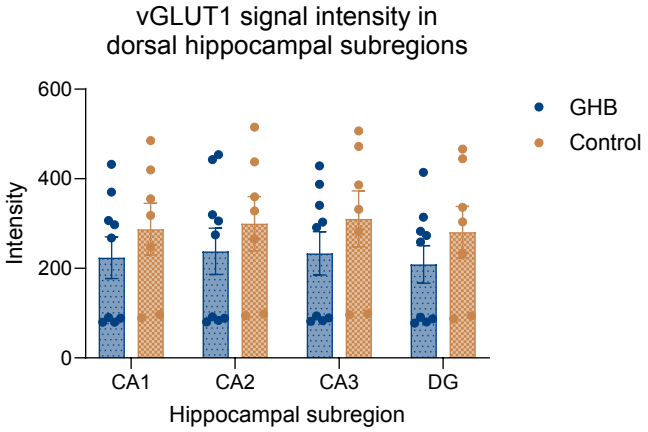


Figure 4. Intensity of the glutamatergic marker vGLUT1 in CA1, CA2, CA3 and and dentate gyrus (DG) subregions of the dorsal hippocampus. Data points represent individual animals. The bars represent means +/- standard error of the mean.

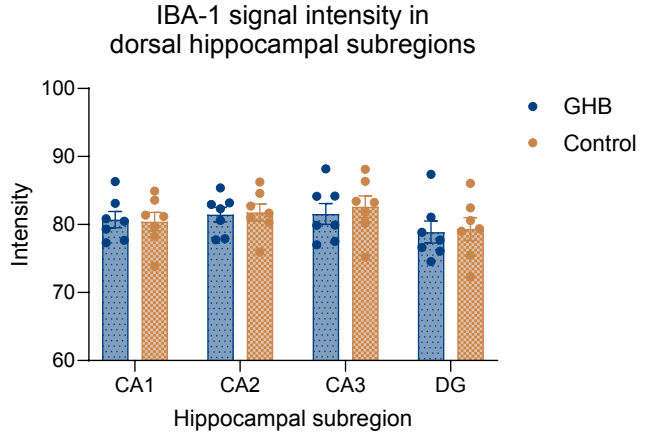


Figure 5. Intensity of the microglial marker vGLUT1 in CA1, CA2, CA3 and dentate gyrus (DG) subregions of the dorsal hippocampus. Data points represent individual animals. The bars represent means +/- standard error of the mean.

Discussion

This study set out to examine the neurobiological changes in the dorsal hippocampus following GHB self-administration. We observed a negative correlation between the amount of consumed GHB and the number of neuronal nuclei in the DG and CA1 region of the hippocampus measured 36 weeks after home cage GHB consumption. This association was not present for GAD67. We did not observe differences between GHB rats and control rats in the number of neurons or specifically GABAergic neurons, regardless of hippocampal subregion. Exploratory assessment of glutamatergic transmission and the presence of microglia did not reveal differences between groups.

We observed a moderate negative correlation between neuronal cell count and amount of GHB consumed in the home cage for the CA1 and DG. This finding is in line with a previous finding showing that GHB leads to reduced NeuN signaling compared to a control group (Pedraza et al., 2009).The neurotoxic effect by Pedraza et al. (2009) was specific for the CA1 region of the hippocampus and occurred after repeated (10x) administration of both 10mg/kg and 100mg/kg GHB. This is a lower concentration of GHB than our animals consumed, which was approx. 735mg/kg GHB daily intake over 12 weeks (84 days). The negative correlation found in this study would suggest that low concentrations of GHB, as used in the study by Pedraza et al. (2009), would be associated with less neuronal signal loss. There are several key differences that could play a role in explaining the different results, such as the difference in interval between GHB administration and sacrifice (1 day versus 34 weeks) and administration method (intraperitoneal vs oral). Although we were not able to show a difference between the GHB group and the control group in this study, this may have been due to the small proportion of rats that consumed higher doses of GHB. Overall, our results suggest that higher doses of GHB may be involved in hippocampal neuronal loss. Future research should examine in on high-dose effects of GHB on the hippocampal subregions.

In our study, the negative association between NeuN signal intensity and home cage GHB consumption was present for CA1 and DG, but not for CA2 and CA3. This suggests that the effects of GHB consumption on the hippocampus are specific for hippocampal subregions in the dorsal hippocampus. The CA1 and DG are rich in high-affinity GHB receptors (Klein et al., 2016). Considering the high doses of ~500 mg/kg GHB that were consumed in our study, it is uncertain if the observed effects could have been a mere consequence of GHB receptor activation. $GABA_B$ receptor activation by GHB in hippocampal subregions was demonstrated by

Johansson et al. (2014). They showed that 16 daily doses of 300 mg/kg GHB lead to decreased $GABA_B$ receptor density in the DG, CA1 and CA3 regions of the male rat hippocampus, whereas CA2 was not examined. This reduction in receptor density was concurrent with reduced $GABA_B$ receptor binding in these regions, suggesting a desensitization and internalization of $GABA_B$ receptors upon GHB exposure. These effects were not observed following daily 50 mg/kg injections (Johansson et al., 2014). Although the duration of these effects is unknown, we speculate that the association between high doses of GHB and decreased neuronal intensity observed in our study was influenced by $GABA_B$ receptor activation.

In our study, we did not see differences in the number of GAD67 spots between the GHB- and control group, nor a correlation between number of GAD67 spots and home cage GHB intake. To the best of our knowledge, no other papers have studied the change of GAD65/67 in the rat hippocampus upon GHB (self-)administration, although it has been established that GHB activates the $GABA_B$ receptor. Electrophysiological studies demonstrated $GABA_B$ receptor activation by micromolar doses of GHB (Ferraro et al., 2001; Cruz et al., 2004; Li et al., 2007), whereas binding studies showed effects of systemic GHB administration on $GABA_B$ receptor density and function (Johansson et al., 2014). Other drugs of abuse affecting GABAergic signaling, such as alcohol, have also been shown to affect electrophysiological properties of GABAergic neurons in the hippocampus (Fleming et al., 2012; Fleming et al., 2013). Ethanol-induced cell death has been specifically shown in hippocampal GAD67 neurons. These ethanol-effects were highly dependent on the age of the animals during alcohol administration (Ogievetsky et al., 2017). This may suggest that robust parameters of neurotoxicity, such as apoptosis and cell death, only emerge upon neonatal drug exposure. More subtle parameters, such as desensitization and internalization of $GABA_B$ receptors, were not possible to detect with our GAD67 measurements, and are also more likely to emerge temporally closer to GHB administration. Future studies should further examine the association between oral GHB intake and altered $GABA_B$ function and related structural changes.

We were not able to detect differences in intensity of glutamatergic signaling. Previous studies have shown that higher doses of GHB decrease glutamatergic signaling via increased GABAergic transmission (Castelli et al., 2003). As we did not observe altered GABAergic signal intensity, decreased glutamatergic signaling intensity is also less likely. It has been previously suggested that GHB can also lead to increased glutamatergic signaling in the hippocampus, either through NMDA receptor activation (Ferraro et al., 2001; Sircar et al., 2011) and through the so-called GHB receptor. Considering the high doses of GHB consumed, it is unlikely that we

would have observed increased glutamatergic signaling through NMDA receptor activation and GHB receptor activation.

We also did not find differences in the presence of microglia between the GHB- and control group. This is in contrast with a study by Pedraza et al. (2009), showing increased microglia levels in the dorsal CA1 following 15 days of GHB administration. Pedraza and colleagues observed this increase in microglia after repeated 10 mg/kg GHB administration, not after 100 mg/kg administration, suggesting this effect may have been driven by high-affinity GHB receptor activation. Interestingly, an increasing body of evidence shows that GHB can have neuroprotective properties, especially following toxic circumstances such as ischemia or excitotoxicity (Leurs et al., 2021; Griem-Krey et al., 2023). This effect is suggested to be driven by activation of CaMKIIα receptors, identified as a high-affinity GHB target (Leurs et al., 2021). It is uncertain whether this neuroprotective effect is exclusively present following a toxic intervention, or that a neuroprotective effect of GHB can be exerted in an undamaged brain. Future studies should examine the conditions in which GHB can either lead to neurotoxic, neutral or neuroprotective effects in the hippocampus.

The current findings should be viewed in the light of several study limitations. The interval between the cognitive assessment and the histological assessment of 32 weeks may have been sufficient for the brain to (partially) recover from neurobiological effects exerted by GHB. Nevertheless, it is remarkable that we find an association between home cage GHB and neuronal cell intensity. This may have even been stronger with a shorter interval between GHB consumption and histological assessment. The vGLUT1 and IBA-1 antibodies used in this study were quantified through signal intensity measurements instead of cell count measurements. The markers of vGLUT1 and IBA-1 are not per se representative of individual cells, hence the choice to assess signal intensity. The animals used in this study underwent many procedures as a part of the GHB self-administration paradigm (Wolf et al., 2024), making it difficult to see home cage self-administration as a stand-alone factor that is associated with decreased neuronal signal intensity in the hippocampus. Interestingly, operant GHB self-administration, where consumed GHB concentrations more closely corresponded to the doses observed in Pedraza et al. (2009) (~10 mg/kg) and was closer in time to the moment of sacrifice (~3 weeks), did not correlate with NeuN signal intensity. This further strengthens the argument that high home-cage GHB self-administration is a component that is associated with decreased neuronal signal intensity. However, contribution of other factors within the self-administration paradigm to the association between home-cage self-administration and neuronal cell intensity cannot be ruled out.

In conclusion, our findings revealed a negative correlation between GHB intake and number of neurons in the CA1 and DG subregion of the dorsal hippocampus. While these effects suggest potential GHB-induced neuronal loss, no significant changes were observed in GABAergic markers, degree of glutamatergic transmission, or microglia cell intensity, possibly due to methodological limitations, including the long interval between GHB exposure and histological analysis. The exact mechanisms of the possible negative effects of high-dose GHB on the brain remain elusive, especially considering the potential neuroprotective properties of GHB in different contexts. Future research should further explore these mechanisms, particularly the high-dose effects of GHB on different neuronal populations in the hippocampus, as well as the temporal nature of these changes, to clarify the complex interaction between GHB and hippocampal function.

Bibliography

Beurmanjer, H., Asperslag, E. M., Oliemeulen, L., Goudriaan, A. E., De Jong, C. A. J., Schellekens, A. F. A., & Dijkstra, B. A. G. (2019). A Qualitative Approach in Understanding Illness Perception and Treatment Needs in Patients with Gamma Hydroxybutyrate Use Disorder. *Eur Addict Res*, *25*(5), 248-255. https://doi.org/10.1159/000500807

Beurmanjer, H., Bruijnen, C. J. W. H., Greeven, P. G. J., De Jong, C. A. J., Schellekens, A. F. A., & Dijkstra, B. A. G. (2022). Cognitive Impairments in Patients with GHB Use Disorder Predict Relapse in GHB Use. *Eur Addict Res*, *28*(5), 350-357. https://doi.org/10.1159/000525507

Brunt, T. M., Koeter, M. W., Hertoghs, N., van Noorden, M. S., & van den Brink, W. (2013). Sociodemographic and substance use characteristics of gamma hydroxybutyrate (GHB) dependent inpatients and associations with dependence severity. *Drug and alcohol dependence*, *131*(3), 316-319.

Castelli, M. P., Ferraro, L., Mocci, I., Carta, F., Carai, M. A., Antonelli, T., Tanganelli, S., Cignarella, G., & Gessa, G. L. (2003). Selective γ-hydroxybutyric acid receptor ligands increase extracellular glutamate in the hippocampus, but fail to activate G protein and to produce the sedative/hypnotic effect of γ-hydroxybutyric acid. *Journal of neurochemistry*, *87*(3), 722-732.

Castelli, M. P., Mocci, I., Langlois, X., Gommeren, W., Luyten, W. H., Leysen, J. E., & Gessa, G. L. (2000). Quantitative autoradiographic distribution of γ-hydroxybutyric acid binding sites in human and monkey brain. *Molecular brain research*, *78*(1-2), 91-99.

Chen, L. Y., Renn, T. Y., Liao, W. C., Mai, F. D., Ho, Y. J., Hsiao, G., Lee, A. W., & Chang, H. M. (2017). Melatonin successfully rescues hippocampal bioenergetics and improves cognitive function following drug intoxication by promoting Nrf2-ARE signaling activity. *J Pineal Res*, *63*(2). https://doi.org/10.1111/jpi.12417

Cruz, H. G., Ivanova, T., Lunn, M.-L., Stoffel, M., Slesinger, P. A., & Lüscher, C. (2004). Bi-directional effects of GABAB receptor agonists on the mesolimbic dopamine system. *Nature neuroscience*, *7*(2), 153-159.

Curtis, M. J., Alexander, S. P. H., Cirino, G., George, C. H., Kendall, D. A., Insel, P. A., Izzo, A. A., Ji, Y., Panettieri, R. A., & Patel, H. H. (2022). Planning experiments: Updated guidance on experimental design and analysis and their reporting III. *British Journal of Pharmacology*, *179*(15), 3907-3913.

EMCDDA. (2022). *European Drug Report 2022: Trends and Developments*.

EMCDDA. (2023). *European Drug Report 2023: Trends and Developments*.

EMCDDA. (2024). *European Drug Report 2024: Trends and Developments*.

Ferraro, L., Tanganelli, S., O'Connor, W. T., Francesconi, W., Loche, A., Gessa, G. L., & Antonelli, T. (2001). γ-Hydroxybutyrate modulation of glutamate levels in the hippocampus: An in vivo and in vitro study. *Journal of neurochemistry*, *78*(5), 929-939.

Fleming, R. L., Acheson, S. K., Moore, S. D., Wilson, W. A., & Swartzwelder, H. S. (2012). In the rat, chronic intermittent ethanol exposure during adolescence alters the ethanol sensitivity of tonic inhibition in adulthood. *Alcoholism: Clinical and Experimental Research*, *36*(2), 279-285.

Fleming, R. L., Li, Q., Risher, M. L., Sexton, H. G., Moore, S. D., Wilson, W. A., Acheson, S. K., & Swartzwelder, H. S. (2013). Binge-pattern ethanol exposure during adolescence, but not adulthood, causes persistent changes in GABAA receptor-mediated tonic inhibition in dentate granule cells. *Alcoholism: Clinical and Experimental Research*, *37*(7), 1154-1160.

Griem-Krey, N., Klein, A. B., Clausen, B. H., Namini, M. R. J., Nielsen, P. V., Bhuiyan, M., Nagaraja, R. Y., De Silva, T., Sobey, C. G., & Cheng, H. C. (2023). The GHB analogue HOCPCA improves deficits in cognition and sensorimotor function after MCAO via CaMKIIα. *Journal of Cerebral Blood Flow & Metabolism*, *43*(8), 1419-1434.

Hechler, V., Gobaille, S., & Maitre, M. (1992). Selective distribution pattern of γ-hydroxybutyrate receptors in the rat forebrain and midbrain as revealed by quantitative autoradiography. *Brain research*, *572*(1-2), 345-348.

Homberg, J. R., Wöhr, M., & Alenina, N. (2017). Comeback of the rat in biomedical research. *ACS Chemical Neuroscience*, *8*(5), 900-903.

Johansson, J., Grönbladh, A., & Hallberg, M. (2014). Gamma-hydroxybutyrate (GHB) induces cognitive deficits and affects GABAB receptors and IGF-1 receptors in male rats. *Behavioural brain research*, *269*, 164-174.

Katrukha, E. (2020). *ComDet plugin for ImageJ*. In (Version v0.5.5) Zenodo.

Klein, A., Bay, T., Villumsen, I., Falk-Petersen, C., Marek, A., Frølund, B., Clausen, R., Hansen, H., Knudsen, G., & Wellendorph, P. (2016). Autoradiographic imaging and quantification of the high-affinity GHB binding sites in rodent brain using 3H-HOCPCA. *Neurochemistry international*, *100*, 138-145.

Klein, C., Kemmel, V., Taleb, O., Aunis, D., & Maitre, M. (2009). Pharmacological doses of gamma-hydroxybutyrate (GHB) potentiate histone acetylation in the rat brain by histone deacetylase inhibition. *Neuropharmacology*, *57*(2), 137-147.

Leurs, U., Klein, A. B., McSpadden, E. D., Griem-Krey, N., Solbak, S. M. Ø., Houlton, J., Villumsen, I. S., Vogensen, S. B., Hamborg, L., & Gauger, S. J. (2021). GHB analogs confer neuroprotection through specific interaction with the CaMKIIα hub domain. *Proceedings of the National Academy of Sciences*, *118*(31), e2108079118.

Li, Q., Kuhn, C. M., Wilson, W. A., & Lewis, D. V. (2007). Effects of gamma hydroxybutyric acid on inhibition and excitation in rat neocortex. *Neuroscience*, *150*(1), 82-92.

Ogievetsky, E., Lotfullina, N., Minlebaeva, A., & Khazipov, R. (2017). Ethanol-induced apoptosis of interneurons in the neonatal GAD67-GFP mouse hippocampus. *BioNanoScience*, *7*(1), 151-154.

Paxinos, G., & Watson, C. (2006). *The rat brain in stereotaxic coordinates: hard cover edition*. Elsevier.

Pedraza, C., Garcia, F. B., & Navarro, J. F. (2009). Neurotoxic effects induced by gammahydroxybutyric acid (GHB) in male rats. *Int J Neuropsychopharmacol*, *12*(9), 1165-1177. https://doi.org/10.1017/S1461145709000157

Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., Browne, W. J., Clark, A., Cuthill, I. C., & Dirnagl, U. (2020). The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *Journal of Cerebral Blood Flow and Metabolism*, *40*(9), 1769-1777.

Raposo Pereira, F., McMaster, M. T. B., Polderman, N., de Vries, Y., van den Brink, W., & van Wingen, G. A. (2018). Adverse effects of GHB-induced coma on long-term memory and related brain function. *Drug and alcohol dependence*, *190*, 29-36.

Raposo Pereira, F., McMaster, M. T. B., Schellekens, A. F. A., Polderman, N., de Vries, Y., van den Brink, W., & van Wingen, G. A. (2020). Effects of recreational GHB use and multiple GHB-induced comas on brain structure and impulsivity. *Frontiers in Psychiatry*, *11*, 166.

Ren, X., & Mody, I. (2006). γ-Hydroxybutyrate induces cyclic AMP-responsive element-binding protein phosphorylation in mouse hippocampus: An involvement of GABAB receptors and cAMP-dependent protein kinase activation. *Neuroscience*, *141*(1), 269-275.

Sircar, R., Wu, L. C., Reddy, K., Sircar, D., & Basak, A. K. (2011). GHB-Induced Cognitive Deficits During Adolescence and the Role of NMDA Receptor. *Curr Neuropharmacol*, *9*(1), 240-243. https://doi.org/10.2174/157015911795017038

Sumnall, H. R., Woolfall, K., Edwards, S., Cole, J. C., & Beynon, C. M. (2008). Use, function, and subjective experiences of gamma-hydroxybutyrate (GHB). *Drug Alcohol Depend*, *92*(1-3), 286-290. https://doi.org/10.1016/j.drugalcdep.2007.07.009

van Amsterdam, J. G. C., Brunt, T. M., Pereira, F. R., Crunelle, C. L., & van den Brink, W. (2022). Cognitive impairment following clinical or recreational use of Gammahydroxybutyric acid (GHB): a systematic review. *Current Neuropharmacology*, *20*(4), 809-819.

van Nieuwenhuijzen, P. S., Long, L. E., Hunt, G. E., Arnold, J. C., & McGregor, I. S. (2010). Residual social, memory and oxytocin-related changes in rats following repeated exposure to gamma-hydroxybutyrate (GHB), 3,4-methylenedioxymethamphetamine (MDMA) or their combination. *Psychopharmacology (Berl)*, *212*(4), 663-674. https://doi.org/10.1007/s00213-010-1986-5

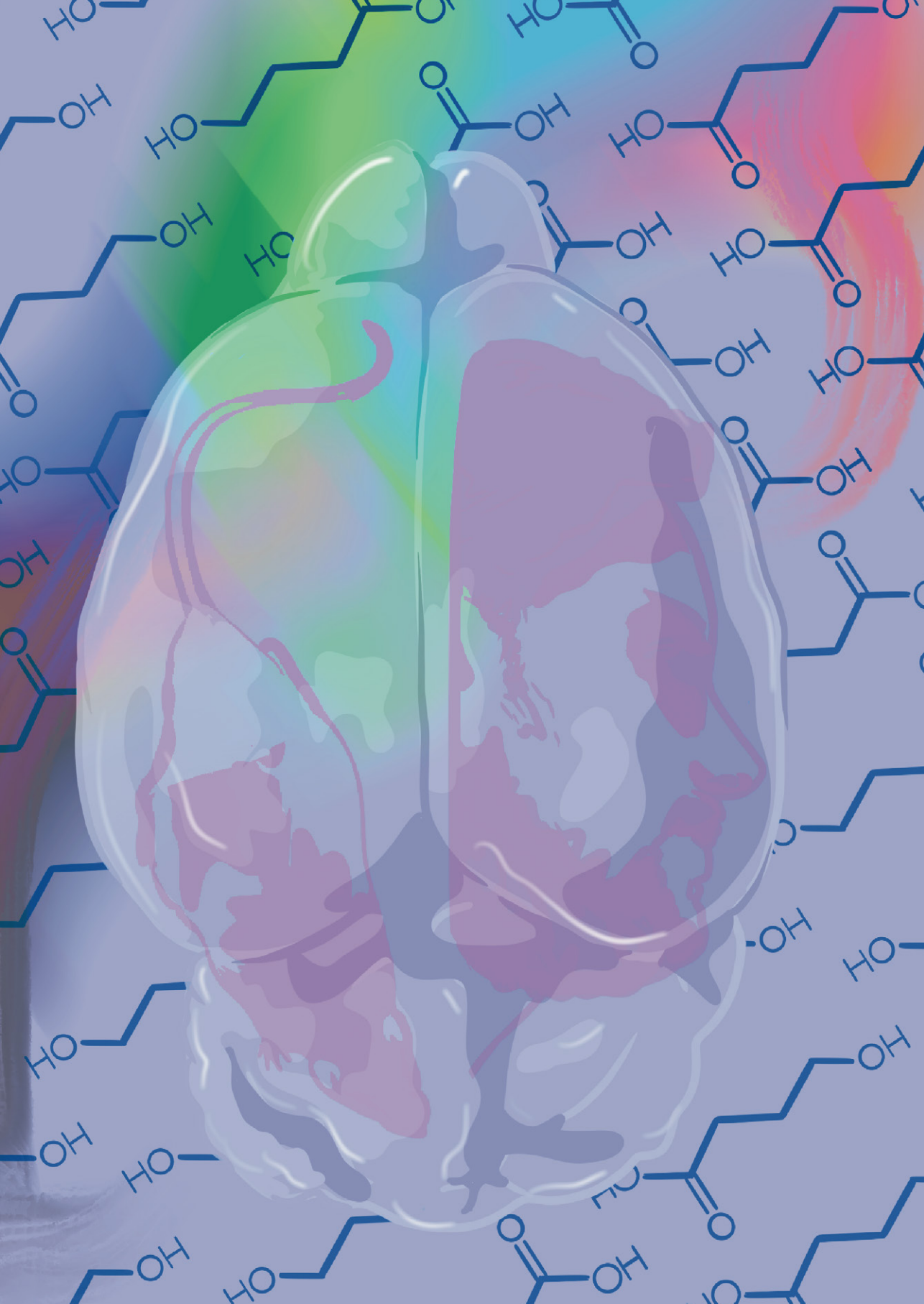
Wolf, C. J. H., Beurmanjer, H., Dijkstra, B. A. G., Geerlings, A. C., Spoelder, M., Homberg, J. R., & Schellekens, A. F. A. (2021). Characterization of the GHB Withdrawal Syndrome. *J Clin Med*, *10*(11). https://doi.org/10.3390/jcm10112333

Wolf, C. J. H., Spoelder, M., Beurmanjer, H., Bulthuis, R., Schellekens, A. F. A., & Homberg, J. R. (2024). Individual differences in GHB consumption in a new voluntary GHB self-administration model in outbred rats. *Psychopharmacology (Berl)*, *241*(3), 613-625. https://doi.org/10.1007/s00213-024-06537-5

Wolf, C. J. H., Venselaar, H., Spoelder, M., Beurmanjer, H., Schellekens, A. F. A., & Homberg, J. R. (2023). An overview of the putative structural and functional properties of the GHBh1 receptor through a bioinformatics approach. *Life*, *13*(4), 926.

Xu, X. M., Wei, Y. D., Liu, Y., & Li, Z. X. (2019). Gamma-hydroxybutyrate (GHB) for narcolepsy in adults: an updated systematic review and meta-analysis. *Sleep Med*, *64*, 62-70. https://doi.org/10.1016/j.sleep.2019.06.017

Youn, S.-C., Chen, L.-Y., Chiou, R.-J., Lai, T.-J., Liao, W.-C., Mai, F.-D., & Chang, H.-M. (2015). Comprehensive application of time-of-flight secondary ion mass spectrometry (TOF-SIMS) for ionic imaging and bio-energetic analysis of club drug-induced cognitive deficiency. *Scientific Reports*, *5*(1), 18420.



Chapter 6

Characterization of the GHB Withdrawal Syndrome

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Published in: Journal of Clinical Medicine

Abstract

The gamma-hydroxybutyric acid (GHB) withdrawal syndrome can have a fulminant course, complicated by severe complications like delirium or seizures. Detoxification by tapering with pharmaceutical GHB is a safe way to manage GHB withdrawal. However, a detailed description of the course of the GHB withdrawal syndrome is currently lacking. This study aimed to 1) describe the course of GHB withdrawal symptoms over time, 2) assess the association between vital signs and withdrawal symptoms, and 3) explore sex differences in GHB withdrawal. In this observational multicenter study, patients with GHB use disorder (n = 285) were tapered off with pharmaceutical GHB. The most reported subjective withdrawal symptoms (SWS) were related to craving, fatigue, insomnia, sweating and feeling gloomy. The most prevalent objective withdrawal symptoms (OWS) were related to craving, fatigue, tremor, sweating, and sudden cold/warm feelings. No association between vital signs and SWS/OWS was found. Sex differences were observed in the severity and prevalence of specific withdrawal symptoms. Our results suggest that the GHB withdrawal syndrome under pharmaceutical GHB tapering does not strongly differ from withdrawal syndromes of other sedative drugs. The lack of association between vital signs and other withdrawal symptoms, and the relative stability of vitals over time suggest that vitals are not suitable for withdrawal monitoring. The reported sex differences highlight the importance of a personalized approach in GHB detoxification.

Introduction

The repeated use of the recreational drug γ-hydroxybutyric acid (GHB) can lead to GHB use disorder (GUD) (EMCDDA, 2019; Arunogiri et al., 2020). Similar to other substance use disorders (SUDs), GUD is characterized by a loss of control over GHB intake and physical dependence on GHB (APA, 2013). In 2019, the prevalence of GHB use in European countries varied from 0.1% in adults (16-64 year old) to 1.7% among young adults (16-34 year old) (EMCDDA, 2019). Although GUD has a relatively low prevalence compared to other SUDs, its societal and financial impact are disproportionally high. GHB use is involved in ~12% of drug-related emergency care cases in Europe, caused by accidental overdosing or severe withdrawal symptoms upon sudden abstinence (Dines et al., 2015; EMCDDA, 2019).

Due to the rapid onset of action (Tmax = 25-40 minutes) and the short half-life (T½ = 30-60 minutes) of GHB, patients with GUD typically consume GHB every 2-3 hours to prevent withdrawal symptoms (Busardo & Jones, 2015; Dijkstra et al., 2017). Cessation of GHB use results in a severe withdrawal syndrome, characterized by an erratic and fulminant course. Reported symptoms of GHB withdrawal are tremor, agitation, anxiety, hallucinations, psychoses and delirium (McDonough et al., 2004; Dijkstra et al., 2017). Abrupt GHB withdrawal without adequate treatment leads to delirium in over 50% of cases (McDonough et al., 2004).

Treatment of GHB withdrawal during detoxification aims to reduce the severity of withdrawal symptoms. Two commonly used methods for GHB detoxification are benzodiazepine tapering and the more extensively studied pharmaceutical GHB tapering. Benzodiazepine administration increases GABAergic signaling through the GABAA receptor, and requires dose regimens of up to six times per day (Kamal, van Noorden, et al., 2017; Beurmanjer et al., 2020). In contrast, pharmaceutical GHB activates both the GABAB- and GHB receptor, and is administered to the patient every two to three hours (Kamal, van Noorden, et al., 2016).

Some studies suggest that pharmaceutical GHB tapering is a safer method for detoxification compared to benzodiazepines (Dijkstra et al., 2017; Beurmanjer et al., 2020). This might be related to the complex pharmacological profile of GHB. Studies have shown that low doses of GHB primarily affect the metabotropic GHB receptor, causing an increase in glutamatergic signaling and a decrease in GABAergic signaling (Gobaille et al., 1999; Hu et al., 2000). In contrast, high doses of GHB also activate the GABAB receptor, decreasing glutamatergic signaling and increasing GABAergic signaling (Carai, Colombo, Brunetti, Melis, Serra, Vacca, Mastinu, Pistuddi, Solinas,

& Cignarella, 2001; Liechti et al., 2016). Benzodiazepines, acting at GABAA receptors, might therefore not sufficiently suppress GABAB-mediated GHB withdrawal, leading to an increased risk for adverse events during benzodiazepine detoxification, such as delirium (Beurmanjer et al., 2020).

Despite the existing evidence for the safety of pharmaceutical GHB tapering, several important issues regarding this approach remain to be elucidated (de Jong et al., 2012; Dijkstra et al., 2017). For instance, little is known about the development of individual withdrawal symptoms over time during GHB detoxification. Additionally, some suggest to base the speed of tapering on the monitoring of vital signs, such as blood pressure and heart rate (Sivilotti et al., 2001; Hack et al., 2006; Ling et al., 2009), whereas others propagate the monitoring of (subjective) withdrawal symptoms (de Jong et al., 2012; Sachdeva et al., 2014). Understanding the development of individual withdrawal symptoms over time, and their relationship with vital signs, could facilitate effective dosing and monitoring of the detoxification process.

The GUD population is characterized by a substantial proportion of women of about one third (Dijkstra et al., 2017; Vasilenko et al., 2017). Women with SUDs are known to show higher rates of internalizing psychiatric symptoms, such as depression and anxiety, whereas men with SUD show higher rates of externalizing symptoms, such as antisocial personality (Zilberman et al., 2003; Seedat et al., 2009; McHugh et al., 2018). It has been shown that women with GUD experience stronger withdrawal compared to men with GUD (Dijkstra et al., 2017). However, detailed information on the exact nature of these sex differences is lacking.

The aim of the current study is to further our understanding of the GHB withdrawal syndrome, in order to improve medical treatment of this condition. We characterize the course of the GHB withdrawal syndrome during inpatient pharmaceutical GHB detoxification in a large database of clinical observations in patients with GUD. Specifically, we analyze 1. the course of individual withdrawal symptoms over time, 2. the association between vital signs and subjective withdrawal symptoms, and 3. sex differences in the course of the GHB withdrawal syndrome.

Materials and Methods

Study design

We used data from two large observational multicenter studies in patients with GUD. The main focus of these studies was to assess the safety of detoxification with pharmaceutical GHB, as published elsewhere (Dijkstra et al., 2017; Beurmanjer et al., 2018). Both studies had similar inclusion criteria, treatment paradigms, and outcome measures. The Medical Ethical Research Committee Twente and Central Committee on Research Involving Human Subjects approved the study protocols and considered that the study did not fall under the scope of the Medical Research Involving Human Subjects Act (WMO). Off-label use of pharmaceutical GHB for GHB detoxification was approved by the Dutch Health Care Inspection.

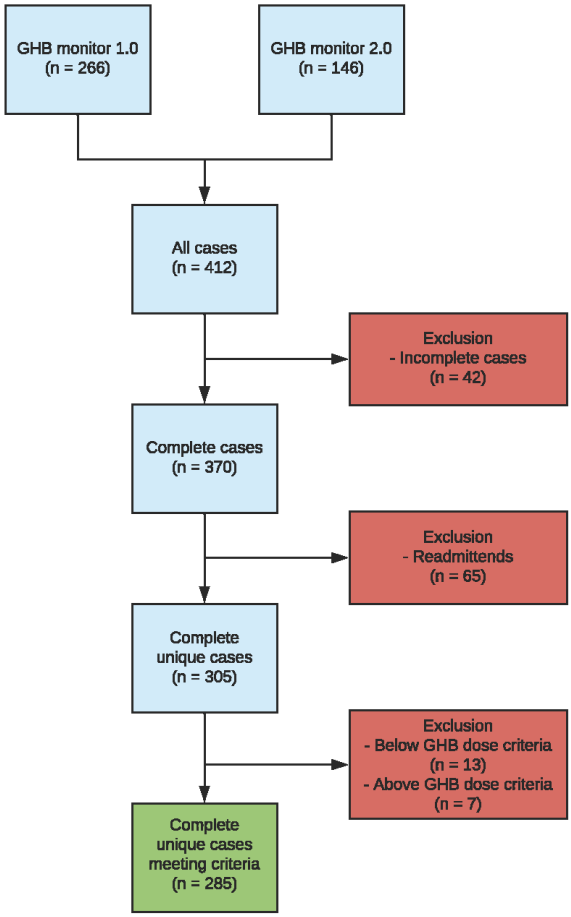

Figure 1. Flowchart of participants included in the study.

Participants

Inpatients being treated for GUD (n=412) at one of the seven participating addiction treatment centers in The Netherlands (Novadic-Kentron, Tactus, IrisZorg, Victas, Verslavingszorg Noord-Nederland, Brijder and Mondriaan GGZ) were included between 2011 and 2015. Patients were between 18 and 60 years old. All patients were classified with GHB dependence according to the Diagnostic and Statistical Manual of Mental Disorders IV-TR (APA, 2000) general criteria for psychoactive substance dependence. Patients were excluded from the study if they could not speak or read the Dutch language, if they suffered from a severe co-morbid psychiatric condition that required immediate attention (e.g. psychosis, manic episode, or suicidal ideation), or in case of pregnancy (Dijkstra et al., 2017). Patients were excluded from data analyses if they had less than three tapering days, or if their GHB dose before admission was below 30 milliliters (since these patients should have been treated ambulatory) or above 240 milliliters (since these patients showed an aberrant, non-representative GHB withdrawal syndrome under pharmaceutical GHB tapering). The threshold of >240mL was determined by adding 2.5 SDs to the daily average consumption volume. If patients were included in both monitors, data of the first treatment episode were used. This resulted in a database of 285 complete, unique patients with GUD undergoing inpatient GHB detoxification (Figure 1).

Instruments

Demographics and other clinical data were obtained from chart reviews (admission data, discharge data and the discharge summary). Measurements in the Addictions for Triage and Evaluation (MATE) section 1 was used to assess current substance use (past 30 days), lifetime substance use, and classification of substance dependence according to DSM-IV (Schippers et al., 2010). In Dutch addiction treatment centers, the MATE is the standard clinical assessment tool, and has proven to have good psychometric quality (Schippers et al., 2010). The GHB questionnaire, specifically assessing the pattern of previous GHB use, was used in addition to the MATE (Dijkstra et al., 2017). The GHB questionnaire consists of 23 parameters, including total years of use, daily dose, volume per dose and time interval between doses. The questionnaire is commonly used in Dutch addiction treatment centers that treat patients with GUD.

Subjective withdrawal scale

The subjective withdrawal scale consists of 33 items representing individual withdrawal symptoms. Patients indicate to what degree they experience each symptom on a 5-point Likert scale (0 = not at all, 1 = a little, 2 = moderately, 3 = quite a bit, 4 = extremely). The subjective withdrawal scale is based on the format of

the Subjective Opiate Withdrawal Scale (Handelsman et al., 1987), extended with subjective withdrawal symptoms of other psychoactive substances as described in the DSM-IV-TR (APA, 2000). Its Dutch translation has good psychometric properties in opioid dependent inpatients (Dijkstra et al., 2007).

Objective withdrawal scale

The objective withdrawal scale consists of 34 observable signs of withdrawal. It is composed of symptoms included in the Objective Opiate Withdrawal Scale (Handelsman et al., 1987) and objective withdrawal symptoms of other psychoactive substances as described in the DSM-IV-TR (APA, 2000). The objective withdrawal scale is filled in by health professionals (mostly nursing staff), where symptoms are classified as present (1) or absent (0). The objective- and subjective withdrawal scale have been reported in several previous studies assessing GHB withdrawal (Dijkstra et al., 2017; Beurmanjer et al., 2020), and are the standard GHB withdrawal assessment scales in addiction treatment centers in The Netherlands. As a result, clinical staff is experienced with applying these instruments in their daily routine. Furthermore, prior to the data collection of both samples, all nursing staff received instructions and training in how to handle the withdrawal scales.

Vitals

Vital signs (heart rate, systolic- and diastolic blood pressure) were measured by the nursing staff. Vitals were annotated under the objective withdrawal scale.

Procedure

Upon admission to the addiction treatment center, information on GHB use and GUD was acquired by trained study nurses through the above-mentioned questionnaires. The detoxification procedure consisted of three phases: titration, tapering, and recovery. During the titration phase, patients were treated with pharmaceutical GHB that was 70% of the reported self-administered illicit GHB dose (based on an average 'street' concentration of 650 mg/ml). The GHB dose was increased in case of withdrawal and decreased in case of sedation, until the pharmaceutical GHB dose was found on which patients were stable and experienced neither withdrawal or sedation. This usually took between one and two days, after which the tapering phase started. During the tapering phase, the GHB dose was lowered by 300 mg of GHB per dose per day. The interval between doses was usually two to three hours. Symptoms were assessed 30 minutes prior to each GHB dose. The tapering phase lasted 11 days on average. The recovery phase started when the pharmaceutical GHB was tapered to 0, which lasted six days on average. For a more detailed description of the protocol, see Dijkstra et al. (2017) (Dijkstra et al., 2017).

Data analysis

Demographics were summarized using descriptive statistics and compared between men and women using one-way MANOVAs (GHB-use characteristics (including age, age at first GHB use, mean years of GHB use, mean days of GHB use, mean daily GHB dose, mean interval between two GHB doses) and co-morbid substance use) and Pearson chi-square test (medication).

To describe the general course of GHB withdrawal, we examined the first 11 days of tapering, since the average tapering period lasted 10.3 days. Linear Mixed Model analysis was performed to assess the development of total SWS/OWS scores over time. Mean daily SWS/OWS scores were used as dependent variables.

We visualized symptom severity and prevalence using heat maps. Average relative symptom severity and -prevalence were calculated by dividing the average symptom score on the respective scale by the maximum possible score on that scale. To examine the development of individual withdrawal symptoms over time, we performed descriptive statistics. Pearson correlation analysis was performed to assess the association of individual withdrawal symptoms between both scales. Bonferroni corrections were applied to correct for multiple comparisons.

Linear Mixed Model analysis was performed to assess the development of vital signs over time. Mean daily scores of the vital parameters were used as dependent variables. Pearson correlation analysis was performed to assess the association of vital signs with daily average SWS/OWS scores. Bonferroni corrections were applied to correct for multiple comparisons.

Finally, to explore sex differences in the course of withdrawal symptoms over time, we performed Linear Mixed Model analysis. Daily average SWS and OWS scores were used as dependent variables, and sex was used as an independent variable. Sex differences in severity and prevalence of individual withdrawal symptoms were analyzed using one-way MANOVA, using average scores per patient per symptom across the entire tapering period.

ANOVAs, Chi-square tests, correlations, heat map analyses and Linear Mixed Model analyses were carried out with Statistical Package for the Social Sciences (SPSS) (25.0) and with GraphPad Prism (9.0). Significance was set at $p < 0.05$.

Results

Demographics

Demographic characteristics of participants (n=285) are presented in Table 1. Men and women differed in GHB-related characteristics (Table 1: One-way MANOVA, $F_{(6, 174)} = 2.227$, $p < 0.05$; Wilk's $\lambda = 0.929$). Men included in the analysis were older and started using GHB at a later age compared to women (Table 1: Age ($F_{(1,179)} = 8.978$, $p < 0.01$; Age at first GHB use ($F_{(1,179)} = 6.797$, $p < 0.01$). Men and women did not differ in rates of co-morbid substance use or in prevalence of medication use.

Table 1. Patients with GUD characteristics of unique patients (n=285).

Characteristics	Male (n=206)	Female (n=79)
Sex	72.3%	27.7%
Mean age in years (SD) **	29.34 (6.44)	26.63 (6.67)
Mean age at first GHB use (SD) **	25.05 (6.67)	21.96 (6.62)
Mean years of GHB use (SD)	4.18 (2.62)	3.83 (2.96)
Mean days of GHB use in last 30 days (SD)	29.69 (1.74)	29.91 (0.71)
Mean daily GHB dose before admission in mL (SD)	92.01 (48.75)	76.44 (43.05)
Mean interval between two GHB doses in hours (SD)	2.30 (5.88)	1.85 (0.64)
Mean number of days of co-morbid substance use in last 30 days (SD)		
Alcohol	4.95 (8.87)	2.96 (5.77)
Nicotine	20.97 (13.55)	23.04 (12.64)
Cannabis	6.68 (11.56)	5.73 (10.60)
Stimulants	5.46 (10.23)	5.35 (10.07)
Cocaine	2.11 (5.97)	1.64 (0.95)
Sedatives	8.16 (12.88)	8.87 (12.92)
Medication		
Anti-psychotics	15.53%	12.66%
Beta blockers	3.88%	2.53%
Benzodiazepines	40.78%	41.77%
Sleep medication	17.48%	15.19%
Other	24.27%	29.11%

** = $p < 0.01$.

Development of withdrawal symptoms over time during GHB detoxification

Total SWS and OWS scores gradually decreased across the tapering phase (Fig. 4: Linear Mixed Models, SWS: main effect of time, $F_{(10, 198)} = 12.185$, $p < 0.0001$; OWS, main effect of time, $F_{(10, 209)} = 9.639$, $p < 0.0001$). The most severely experienced SWS were "craving", "fatigue", "insomnia" and a "gloomy" and "slow, sluggish feeling" (Fig. 2). The most often reported OWS by nurses were "craving" and "fatigue", next to the symptoms "shaking hands", "sweating" and a "sudden cold/warm feeling" (Fig. 2).

The SWS "muscle aches", "muscle twitches", "tensed, stressed feeling", "experiencing a fast heart rate" and "abdominal cramps" were most severe in the first part of detoxification (first three days). Over the first four days, a >70% decrease in severity was reported for these symptoms. In contrast, several SWS ("sweating", "tremor", "sleeps a lot", and "restless feeling") remained stable over time (<25% decrease in severity over 11 days). On average, there were no SWS that became more severe during the tapering phase.

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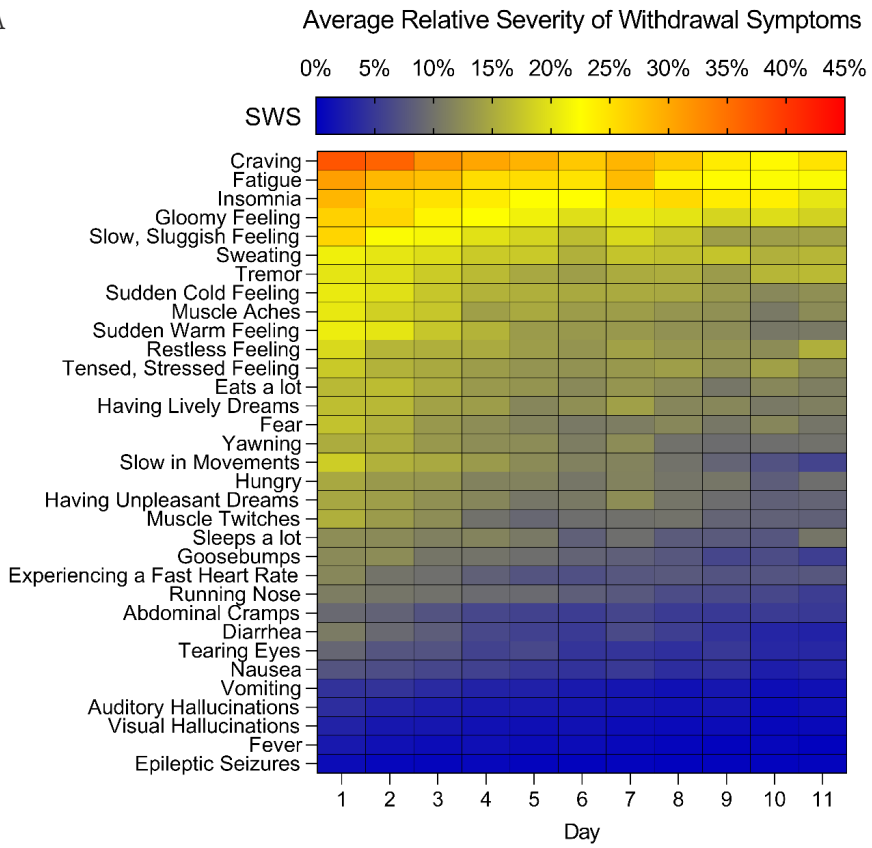

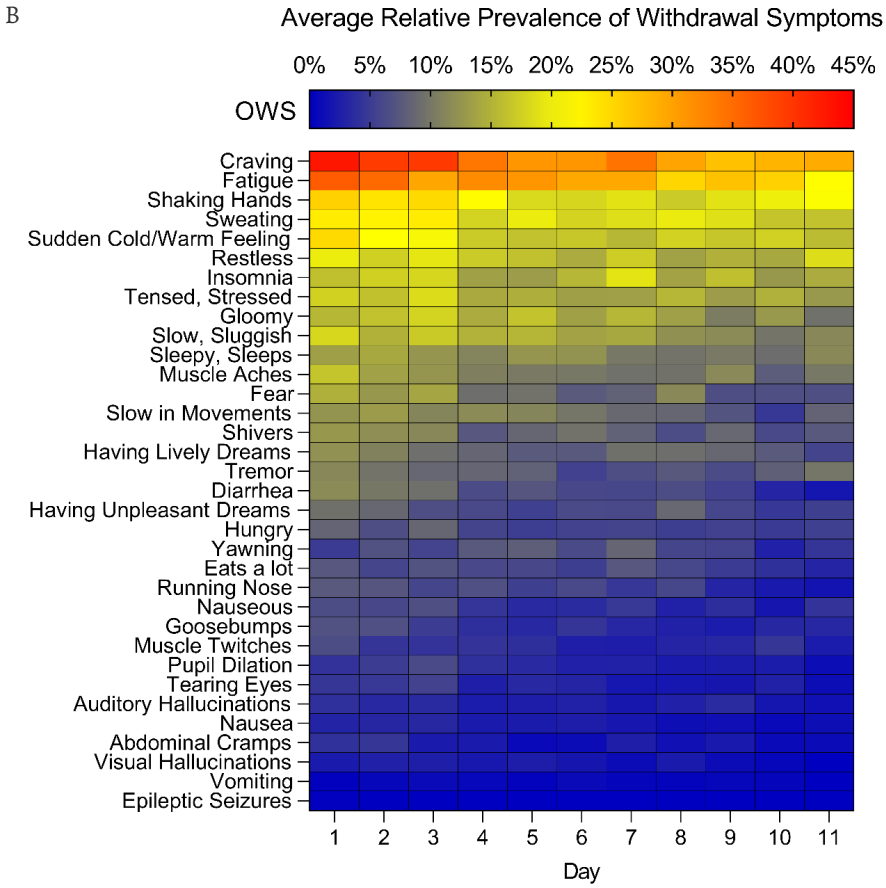

Figure 2. A) Heat map of all 33 SWS over time for the first 11 detoxification days. Symptoms are ranked based on the average severity of the symptom over all days for males and females combined. B) Heat map of all 34 OWS over time for the first 11 detoxification days. Symptoms are ranked based on the average presence of the symptom over all days for males and females combined.

The OWS "sweating", "sudden cold/warm feeling", "muscle aches", "tensed, stressed feeling", "shivers", "having unpleasant dreams", "hungry", and "goosebumps" were primarily present in the first part of detoxification (first 3 days). Over the first four days, a >70% decrease in prevalence was observed for these symptoms. The OWS "tremor", "shaking hands", "sleepy, sleeps", "insomnia", "restless" and "yawning" were consistently present over time (<25% decrease in presence over 11 days). The OWS "yawning", "insomnia", "gloomy" and "visual hallucinations" were on some days more prevalent compared to day 1. The majority of SWS and OWS that appeared on both scales showed a moderate to strong correlation with the corresponding symptom on the other scale ($r = 0.226$ to 0.826, $p < 0.0015$ after Bonferroni correction) (Table A1).

The average heart rate gradually increased over time during detoxification from 87.9 to 91.1 bpm (Linear Mixed Models, heart rate: main effect of time: $F_{(10, 192)} = 3.509$, $p < 0.001$). The average systolic and diastolic blood pressure gradually decreased over time from 132.5 to 127.3 mmHg, and from 84.1 to 80.4 mmHg, respectively (Linear Mixed Models, systolic blood pressure main effect of time: $F_{(10, 196)} = 5.848$, $p < 0.0001$; diastolic blood pressure main effect of time: $F_{(10, 183)} = 10.095$, $p < 0.0001$), see Figure 3.

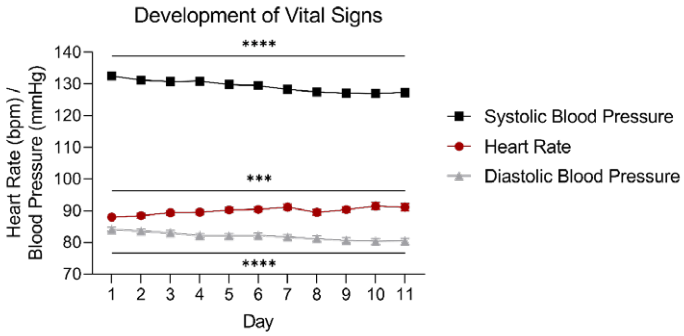

Figure 3. Average vital signs over time for the first 11 detoxification days. Data are presented as the average of all patients measured on the respective days ± SEM (n = 108 – 263). Error bars may lie under the symbol of the graph. *** = main effect of time $p < 0.001$; **** = main effect of time $p < 0.0001$.

Association between vital signs and subjective and objective symptoms of GHB withdrawal

Overall, no correlations were observed between vital signs and daily average SWS scores (Table A2) or between vital signs and daily average OWS scores after correction for multiple testing ($p > 0.0015$), see Table A3.

Differences in GHB withdrawal syndrome between men and women

In contrast to what we expected, there was no difference in total SWS and OWS scores between men and women across the tapering phase (Fig. 4: Linear Mixed Models, no effect of sex), and both sexes showed a similar decrease in total OWS score over time (Fig. 4: Linear Mixed Models, OWS no significant interaction). Men and women showed a slightly different course of total SWS score over time (Fig. 4: Linear Mixed Models, SWS time x sex interaction, $F_{(10, 198)} = 2.038$, $p < 0.05$).

One-way MANOVA showed that men and women differed in severity of individual SWS ($F_{(33,233)} = 2{,}550$, $p < 0.001$; Wilk's $\lambda = 0.735$). Specifically, women scored higher on fear ($F_{(1, 265)} = 4.531$, $p < 0.05$), gloomy feeling ($F_{(1, 265)} = 7.507$, $p < 0.01$), yawning ($F_{(1, 265)} = 6.132$, $p < 0.05$), goosebumps ($F_{(1, 265)} = 6.272$, $p < 0.05$), sweating ($F_{(1, 265)} = 7.583$, $p < 0.01$), tearing

eyes ($F_{(1, 265)} = 6.863$, $p < 0.01$), muscle aches ($F_{(1, 265)} = 9.357$, $p < 0.01$), nausea ($F_{(1, 265)} = 4.700$, $p < 0.05$), craving ($F_{(1, 265)} = 7.519$, $p < 0.01$), sudden cold feelings ($F_{(1, 265)} = 13.248$, $p < 0.001$), and sudden warm feelings ($F_{(1, 265)} = 7.979$, $p < 0.01$), whereas men more often reported to eat a lot during detoxification compared to women ($F_{(1, 265)} = 14.059$, $p < 0.001$), see Fig. 2).

One-way MANOVA showed that men and women differed in prevalence of individual OWS ($F_{(34,234)} = 2.004$, $p < 0.01$; Wilk's $\lambda = 0.774$). Women showed more shivering ($F_{(1, 267)} = 16.046$, $p < 0.0001$), sudden cold/warm feelings ($F_{(1, 267)} = 5.664$, $p < 0.05$, abdominal cramps ($F_{(1, 267)} = 6.665$, $p < 0.05$), nausea ($F_{(1, 267)} = 10.103$, $p < 0.01$) and vomiting ($F_{(1, 267)} = 4.492$, $p < 0.05$), while men showed more insomnia ($F_{(1, 267)} = 5.024$, $p < 0.05$) and eat a lot ($F_{(1, 267)} = 7.382$, $p < 0.01$). Additionally, men showed a higher blood pressure than women (Fig. A1).

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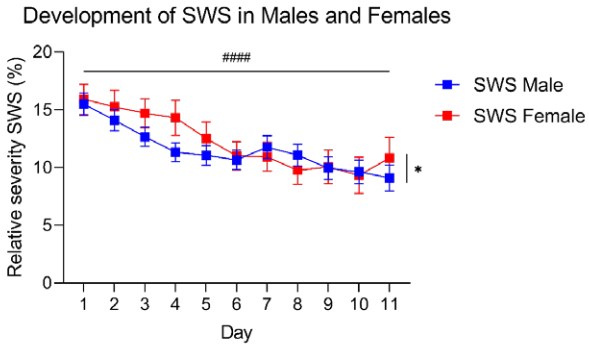

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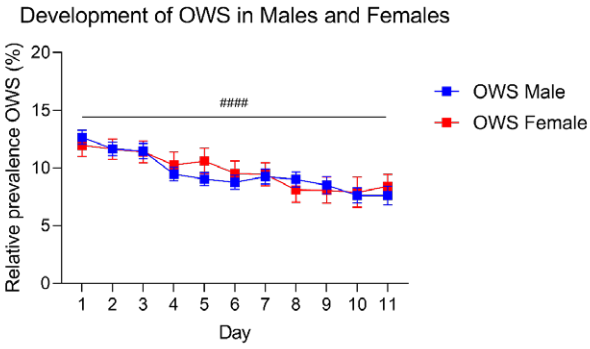

Figure 4. A) Development of subjective withdrawal symptoms (SWS) during GHB detoxification, divided by sex. SWS are presented as the average severity of all 33 measured SWS during the day (males n = 74 – 184, females n = 31 – 76). B) Development of objective withdrawal symptoms (OWS) during GHB detoxification, divided by sex. OWS are presented as the average presence of all 34 measured objective withdrawal symptoms during the day (males n = 77 – 191, females n = 31 – 72). Data are presented as mean ± SEM. #### = main effect of time $p < 0.0001$; * = time x sex interaction $p < 0.05$.

Discussion

This study set out to analyze the course of the GHB withdrawal syndrome in patients with GUD during inpatient detoxification with pharmaceutical GHB. The GHB withdrawal syndrome was primarily characterized by sleep-related symptoms, mood-related symptoms and several physiological symptoms, including sweating and tremor. The majority of symptoms steadily declined in severity over time, while some symptoms (e.g. tremor, sleeps a lot) were not strongly affected by GHB tapering. Vital signs did not correlate with other withdrawal symptoms. Women showed a different pattern of withdrawal symptoms compared to men.

The most prominent withdrawal symptoms that decreased over time include "craving", "fatigue", "insomnia", "gloomy", "slow, sluggish", "sudden cold/warm feeling", "muscle aches" and "tensed, stressed", and might represent the core symptoms of GHB withdrawal. Other withdrawal symptoms that were frequently present during detoxification include "sweating", "tremor" and "restlessness". Withdrawal syndromes of other sedatives, such as alcohol- and benzodiazepine withdrawal, show overlap with symptoms seen in this study, such as anxiety/fear, tremor, sweating, insomnia (alcohol/benzodiazepines), restlessness and muscle twitches (benzodiazepines) (Ashton, 2005; Perry, 2014). Other characteristic alcohol withdrawal symptoms such as hypertension, tachycardia, and fever were hardly seen in our sample (Quaglio et al., 2012; Mirijello et al., 2015). Similarly, severe GHB withdrawal symptoms as epileptic seizures, hallucinations and delirium, were rare in our sample, probably because GHB tapering dampened the overall severity of withdrawal symptoms. Future studies should address whether other withdrawal scales, for instance the Clinical Institute Withdrawal Assessment for Alcohol (CIWA-Ar) tool, can also reliably be used to guide tapering (Sullivan et al., 1989). Indeed, the CIWA-Ar has also been used to assess GHB withdrawal in two case reports (Liao et al., 2018; Habibian et al., 2019).

Several symptoms present at the start of detoxification were not strongly affected by GHB tapering ("sweating", "tremor", "sleeps a lot", "sleepy, sleeps", "restless feeling", "restless", "yawning" and "shaking hands"). This may reflect a more long-term dysregulation of autonomic processes, for instance due to a chronically disrupted sleep pattern. Several of these symptoms were also still present upon discharge of GHB detoxification treatment, including "craving" and "insomnia". The presence of several symptoms following detoxification, including sleep-related disturbances, might also contribute to the high relapse rates seen with GUD, which is also observed with other SUDs like alcohol-, cocaine- and opioid use disorder (Garcia & Salloum,

2015). Aftercare following detoxification should therefore aim at reducing these symptoms that persist after detoxification, such as sleep-related issues and craving.

Contrary to other substance withdrawal syndromes, we did not find an association between vital signs and objective- or subjective GHB withdrawal symptoms (Kattimani & Bharadwaj, 2013; Arroyo-Novoa et al., 2020). In several other substance withdrawal syndromes, vitals are associated with withdrawal symptom severity and therefore used as an indicator for overall withdrawal severity. For instance, in alcohol, vital signs are used to determine titration- and tapering regimes during detoxification (APA, 2013; Kattimani & Bharadwaj, 2013; Maldonado et al., 2014; Schuckit, 2014). Our findings suggest that changes in vital signs during GHB detoxification may not be suitable for monitoring of GHB withdrawal severity.

Additionally, both the increase in heart rate (from 87.9 to 91.1 bpm on average) and the decreases in systolic and diastolic blood pressure (from 132.5/84.1 to 127.3/80.4 mmHg on average) we observed here were rather small and of little clinical relevance, despite being statistically significant. Yet, with sudden GHB withdrawal, tachycardia and hypertension are often observed (McDonough et al., 2004). Our results indicate that GHB tapering might have prevented a derailment of vital signs, implicating that a change in withdrawal symptoms may precede a derailment of vital signs during GHB detoxification, as also suggested by Beurmanjer et al. (2020) (Beurmanjer et al., 2020).

Men and women showed different types of withdrawal symptoms. Specifically, women scored on average higher on a large variety of (mainly subjective) individual withdrawal symptoms. This is also observed with other SUDs like opioids, cannabis and nicotine (Leventhal et al., 2007; Back et al., 2011; Herrmann et al., 2015). The differences in GHB withdrawal symptoms between males and females may be partially related to differences in co-occurring psychiatric conditions. Dijkstra et al. (2017) showed that patients with GUD with higher baseline levels of depression, anxiety and stress experienced higher levels of subjective withdrawal (Dijkstra et al., 2017). In addition, co-morbid mood- and anxiety disorders are more common in women with SUD compared to men with SUD (Zilberman et al., 2003; Seedat et al., 2009; McHugh et al., 2018), possibly explaining the increased severity of several individual withdrawal symptoms in women during GHB detoxification compared to men. In contrast to the findings of Dijkstra et al. (2017), we did not find a difference in total SWS severity between men and women across the tapering phase (Dijkstra et al., 2017). This may be explained by the fact that Dijkstra et al. (2017) included the titration-, tapering- and recovery days, whereas we only focused on the tapering phase. Limited suppression of (comorbid) symptoms during titration and recovery

days might account for the observed differences. The reported differences between men and women suggest that women might benefit from more gradual tapering strategies compared to men.

In the assessment of GHB withdrawal severity, both objective and subjective symptoms were measured. There is a large overlap between the objective- and subjective withdrawal scales regarding the type of symptoms assessed. It can be questioned whether both scales are required to obtain a complete picture of withdrawal severity. The current data show that subjective withdrawal severity generally parallels clinical observations by nursing staff, as also seen with e.g. opioid withdrawal (Dijkstra et al., 2007). However, the subjective withdrawal scale seems more sensitive to a change in withdrawal severity, probably as a result of the 5-point Likert scale design compared to the dichotomous objective withdrawal scale. It might thus be sufficient to focus on self-reported withdrawal severity to monitor GHB detoxification.

The current findings should be viewed in light of several study limitations. A relatively high proportion of patients in our study showed comorbid substance use (Table 1), possibly contributing to the observed withdrawal symptoms. However, the observed prevalence of comorbid substance use is representative for the population of patients with GUD (Kamal, Dijkstra, et al., 2016). On the one hand, the high rates of co-morbid substance use hamper firm conclusions about the specific effects of GHB withdrawal. On the other hand, clinical reality is that people with GUD often have comorbid SUDs, thus making our observations clinically relevant (Kamal, Dijkstra, et al., 2016).

The withdrawal scales used in this study were originally based on opioid withdrawal scales (Handelsman et al., 1987), and complemented with other symptoms based on the DSM-IV (APA, 2000). Although a total of 38 individual withdrawal signs and symptoms were assessed, it is still possible that withdrawal symptoms that are unique for GHB withdrawal were not assessed with the current withdrawal scales. Throughout detoxification, patients repeatedly mentioned to have an itchy feeling. This symptom may be considered to be included in the GHB withdrawal scale.

During GHB detoxification, pharmacological treatment for co-morbid psychiatric disorders, such as benzodiazepines, selective serotonin reuptake inhibitors (SSRIs) or anti-psychotics, continued. We speculate this may have dampened the severity of GHB withdrawal, possibly causing our results to be an underestimation of the severity of GHB withdrawal compared to when only pharmaceutical GHB is provided,

as also suggested in previous reports (Kamal, van Noorden, et al., 2017; Cappetta & Murnion, 2019). The effects of other medications on the course of the GHB withdrawal syndrome requires further study.

It is also important to note that the current findings do not generalize to other methods for GHB detoxification, such as benzodiazepine tapering (different receptor systems), or acute unassisted GHB detoxification (cold turkey). Severe withdrawal symptoms, such as epileptic seizures and psychotic symptoms that were hardly observed here, might be more common in such cases (McDonough et al., 2004; Beurmanjer et al., 2020).

Conclusions

The GHB withdrawal syndrome during pharmaceutical GHB tapering is characterized by a variety of symptoms that fade over time, and which are also commonly observed during alcohol- and benzodiazepine withdrawal. The observed lack of association between vitals and subjective- or objective withdrawal symptoms, and limited variation of vitals over time, question their relevance as an indicator for GHB withdrawal severity during detoxification. Finally, women experience qualitatively different GHB withdrawal symptoms during GHB detoxification compared to men. Our research suggests that the subjective withdrawal scale may serve as a basis to personalize tapering speed in order to minimize withdrawal severity, and account for sex differences in the GHB withdrawal syndrome.

Bibliography

APA. (2000). *Diagnostic and statistical manual of mental disorders (4th ed.)*. American Psychiatric Association.

APA. (2013). *Diagnostic and statistical manual of mental disorders (5th ed.)*. American Psychiatric Association.

Arroyo-Novoa, C. M., Figueroa-Ramos, M. I., Balas, M., Rodríguez, P., & Puntillo, K. A. (2020). Opioid and benzodiazepine withdrawal syndromes in trauma ICU patients: a prospective exploratory study. *Critical care explorations*, *2*(4).

Arunogiri, S., Moayeri, F., Crossin, R., Killian, J. J., Smith, K., Scott, D., & Lubman, D. I. (2020). Trends in gamma-hydroxybutyrate-related harms based on ambulance attendances from 2012 to 2018 in Victoria, Australia. *Addiction*, *115*(3), 473-479.

Ashton, H. (2005). The diagnosis and management of benzodiazepine dependence. *Current opinion in Psychiatry*, *18*(3), 249-255.

Back, S. E., Payne, R. L., Wahlquist, A. H., Carter, R. E., Stroud, Z., Haynes, L., Hillhouse, M., Brady, K. T., & Ling, W. (2011). Comparative profiles of men and women with opioid dependence: results from a national multisite effectiveness trial. *The American journal of drug and alcohol abuse*, *37*(5), 313-323.

Beurmanjer, H., Kamal, R. M., de Jong, C. A. J., Dijkstra, B. A. G., & Schellekens, A. F. A. (2018). Baclofen to Prevent Relapse in Gamma-Hydroxybutyrate (GHB)-Dependent Patients: A Multicentre, Open-Label, Non-Randomized, Controlled Trial. *CNS Drugs*, *32*(5), 437-442. https://doi.org/10.1007/s40263-018-0516-6

Beurmanjer, H., Luykx, J. J., De Wilde, B., van Rompaey, K., Buwalda, V. J. A., De Jong, C. A. J., Dijkstra, B. A. G., & Schellekens, A. F. A. (2020). Tapering with Pharmaceutical GHB or Benzodiazepines for Detoxification in GHB-Dependent Patients: A Matched-Subject Observational Study of Treatment-as-Usual in Belgium and The Netherlands. *CNS Drugs*, *34*(6), 651-659. https://doi.org/10.1007/s40263-020-00730-8

Bhattacharya, I., & Boje, K. M. (2004). GHB (γ-hydroxybutyrate) carrier-mediated transport across the blood-brain barrier. *The Journal of pharmacology and experimental therapeutics*, *311*(1), 92-98.

Busardo, F., & Jones, A. (2015). GHB pharmacology and toxicology: acute intoxication, concentrations in blood and urine in forensic cases and treatment of the withdrawal syndrome. *Current Neuropharmacology*, *13*(1), 47-70.

Cappetta, M., & Murnion, B. P. (2019). Inpatient management of gamma-hydroxybutyrate withdrawal. *Australasian Psychiatry*, *27*(3), 284-287.

Carai, M. A., Colombo, G., Brunetti, G., Melis, S., Serra, S., Vacca, G., Mastinu, S., Pistuddi, A. M., Solinas, C., & Cignarella, G. (2001). Role of GABAB receptors in the sedative/hypnotic effect of γ-hydroxybutyric acid. *European journal of pharmacology*, *428*(3), 315-321.

de Jong, C. A., Kamal, R., Dijkstra, B. A., & de Haan, H. A. (2012). Gamma-hydroxybutyrate detoxification by titration and tapering. *European addiction research*, *18*(1), 40-45.

Dijkstra, B. A. G., Kamal, R. M., van Noorden, M. S., de Haan, H., Loonen, A. J. M., & De Jong, C. A. J. (2017). Detoxification with titration and tapering in gamma-hydroxybutyrate (GHB) dependent patients: the Dutch GHB monitor project. *Drug and alcohol dependence*, *170*, 164-173.

Dijkstra, B. A. G., Krabbe, P. F. M., Riezebos, T. G. M., van der Staak, C. P. F., & De Jong, C. A. J. (2007). Psychometric evaluation of the Dutch version of the Subjective Opiate Withdrawal Scale (SOWS). *European addiction research*, *13*(2), 81-88.

Dines, A. M., Wood, D. M., Yates, C., Heyerdahl, F., Hovda, K. E., Giraudon, I., Sedefov, R., Dargan, P. I., & Group, E.-D. R. (2015). Acute recreational drug and new psychoactive substance toxicity in Europe: 12 months data collection from the European Drug Emergencies Network (Euro-DEN). *Clinical Toxicology*, *53*(9), 893-900.

EMCDDA. (2019). *European Drug Report 2019: Trends and Developments*. P. O. o. t. E. Union.

Felmlee, M. A., Morse, B. L., & Morris, M. E. (2021). gamma-Hydroxybutyric Acid: Pharmacokinetics, Pharmacodynamics, and Toxicology. *AAPS J*, *23*(1), 22. https://doi.org/10.1208/s12248-020-00543-z

Garcia, A. N., & Salloum, I. M. (2015). Polysomnographic sleep disturbances in nicotine, caffeine, alcohol, cocaine, opioid, and cannabis use: a focused review. *The American Journal on Addictions*, *24*(7), 590-598.

Gobaille, S., Hechler, V., Andriamampandry, C., Kemmel, V., & Maitre, M. (1999). γ-Hydroxybutyrate modulates synthesis and extracellular concentration of γ-aminobutyric acid in discrete rat brain regions in vivo. *Journal of Pharmacology and Experimental Therapeutics*, *290*(1), 303-309.

Habibian, S., Ahamad, K., McLean, M., & Socias, M. E. (2019). Successful management of gamma-hydroxybutyrate (GHB) withdrawal using baclofen as a standalone therapy: a case report. *Journal of addiction medicine*, *13*(5), 415.

Hack, J. B., Hoffman, R., & Nelson, L. S. (2006). Resistant alcohol withdrawal: does an unexpectedly large sedative requirement identify these patients early? *Journal of medical toxicology*, *2*(2), 55-60.

Handelsman, L., Cochrane, K. J., Aronson, M. J., Ness, R., Rubinstein, K. J., & Kanof, P. D. (1987). Two new rating scales for opiate withdrawal. *The American journal of drug and alcohol abuse*, *13*(3), 293-308.

Herrmann, E. S., Weerts, E. M., & Vandrey, R. (2015). Sex differences in cannabis withdrawal symptoms among treatment-seeking cannabis users. *Experimental and clinical psychopharmacology*, *23*(6), 415.

Hu, R., Banerjee, P., & Snead Iii, O. (2000). Regulation of γ-aminobutyric acid (GABA) release in cerebral cortex in the γ-hydroxybutyric acid (GHB) model of absence seizures in rat. *Neuropharmacology*, *39*(3), 427-439.

Kamal, R. M., Dijkstra, B. A., Loonen, A. J., & De Jong, C. A. (2016). The effect of co-occurring substance use on gamma-hydroxybutyric acid withdrawal syndrome. *Journal of addiction medicine*, *10*(4), 229-235.

Kamal, R. M., van Noorden, M. S., Franzek, E., Dijkstra, B. A. G., Loonen, A. J. M., & De Jong, C. A. J. (2016). The neurobiological mechanisms of gamma-hydroxybutyrate dependence and withdrawal and their clinical relevance: a review. *Neuropsychobiology*, *73*(2), 65-80.

Kamal, R. M., van Noorden, M. S., Wannet, W., Beurmanjer, H., Dijkstra, B. A. G., & Schellekens, A. F. A. (2017). Pharmacological treatment in γ-hydroxybutyrate (GHB) and γ-Butyrolactone (GBL) dependence: detoxification and relapse prevention. *CNS Drugs*, *31*(1), 51-64.

Kattimani, S., & Bharadwaj, B. (2013). Clinical management of alcohol withdrawal: A systematic review. *Industrial psychiatry journal*, *22*(2), 100.

Lettieri, J., & Fung, H. L. (1976). Absorption and first-pass metabolism of 14C-gamma-hydroxybutyric acid. *Res Commun Chem Pathol Pharmacol*, *13*(3), 425-437.

Lettieri, J. T., & Fung, H.-L. (1979). Dose-dependent pharmacokinetics and hypnotic effects of sodium gamma-hydroxybutyrate in the rat. *The Journal of pharmacology and experimental therapeutics*, *208*(1), 7-11.

Leventhal, A. M., Waters, A. J., Boyd, S., Moolchan, E. T., Lerman, C., & Pickworth, W. B. (2007). Gender differences in acute tobacco withdrawal: Effects on subjective, cognitive, and physiological measures. *Experimental and clinical psychopharmacology*, *15*(1), 21.

Liao, P.-C., Chang, H.-M., & Chen, L.-Y. (2018). Clinical management of gamma-hydroxybutyrate (GHB) withdrawal delirium with CIWA-Ar protocol. *Journal of the Formosan Medical Association*, *117*(12), 1124-1127.

Liechti, M. E., Quednow, B. B., Liakoni, E., Dornbierer, D., von Rotz, R., Gachet, M. S., Gertsch, J., Seifritz, E., & Bosch, O. G. (2016). Pharmacokinetics and pharmacodynamics of γ-hydroxybutyrate in healthy subjects. *British journal of clinical pharmacology*, *81*(5), 980-988.

Ling, W., Hillhouse, M., Domier, C., Doraimani, G., Hunter, J., Thomas, C., Jenkins, J., Hasson, A., Annon, J., & Saxon, A. (2009). Buprenorphine tapering schedule and illicit opioid use. *Addiction*, *104*(2), 256-265.

Maitre, M., Klein, C., & Mensah-Nyagan, A. G. (2016). Mechanisms for the Specific Properties of gamma-Hydroxybutyrate in Brain. *Med Res Rev*, *36*(3), 363-388. https://doi.org/10.1002/med.21382

Maldonado, J. R., Sher, Y., Ashouri, J. F., Hills-Evans, K., Swendsen, H., Lolak, S., & Miller, A. C. (2014). The "Prediction of Alcohol Withdrawal Severity Scale"(PAWSS): systematic literature review and pilot study of a new scale for the prediction of complicated alcohol withdrawal syndrome. *Alcohol*, *48*(4), 375-390.

McDonough, M., Kennedy, N., Glasper, A., & Bearn, J. (2004). Clinical features and management of gamma-hydroxybutyrate (GHB) withdrawal: a review. *Drug Alcohol Depend*, *75*(1), 3-9. https://doi.org/10.1016/j.drugalcdep.2004.01.012

McHugh, R. K., Votaw, V. R., Sugarman, D. E., & Greenfield, S. F. (2018). Sex and gender differences in substance use disorders. *Clinical psychology review*, *66*, 12-23.

Mirijello, A., D'Angelo, C., Ferrulli, A., Vassallo, G., Antonelli, M., Caputo, F., Leggio, L., Gasbarrini, A., & Addolorato, G. (2015). Identification and management of alcohol withdrawal syndrome. *Drugs*, *75*(4), 353-365.

Morris, M. E., Hu, K., & Wang, Q. (2005). Renal clearance of γ-hydroxybutyric acid in rats: increasing renal elimination as a detoxification strategy. *The Journal of pharmacology and experimental therapeutics*, *313*(3), 1194-1202.

Morse, B. L., & Morris, M. E. (2013). Effects of monocarboxylate transporter inhibition on the oral toxicokinetics/toxicodynamics of gamma-hydroxybutyrate and gamma-butyrolactone. *J Pharmacol Exp Ther*, *345*(1), 102-110. https://doi.org/10.1124/jpet.112.202796

Nelson, T., Kaufman, E., Kline, J., & Sokoloff, L. (1981). The extraneural distribution of γ-hydroxybutyrate. *Journal of neurochemistry*, *37*(5), 1345-1348.

Palatini, P., Tedeschi, L., Frison, G., Padrini, R., Zordan, R., Orlando, R., Gallimberti, L., Gessa, G., & Ferrara, S. (1993). Dose-dependent absorption and elimination of gamma-hydroxybutyric acid in healthy volunteers. *European Journal of Clinical Pharmacology*, *45*, 353-356.

Perry, E. C. (2014). Inpatient management of acute alcohol withdrawal syndrome. *CNS Drugs*, *28*(5), 401-410.

Quaglio, G., Pattaro, C., Gerra, G., Mathewson, S., Verbanck, P., Des Jarlais, D. C., & Lugoboni, F. (2012). High dose benzodiazepine dependence: description of 29 patients treated with flumazenil infusion and stabilised with clonazepam. *Psychiatry research*, *198*(3), 457-462.

Sachdeva, A., Chandra, M., & Deshpande, S. N. (2014). A comparative study of fixed tapering dose regimen versus symptom-triggered regimen of lorazepam for alcohol detoxification. *Alcohol and Alcoholism*, *49*(3), 287-291.

Schippers, G. M., Broekman, T. G., Buchholz, A., Koeter, M. W. J., & van den Brink, W. (2010). Measurements in the Addictions for Triage and Evaluation (MATE): an instrument based on the World Health Organization family of international classifications. *Addiction*, *105*(5), 862-871.

Schuckit, M. A. (2014). Recognition and management of withdrawal delirium (delirium tremens). *New England Journal of Medicine*, *371*(22), 2109-2113.

Seedat, S., Scott, K. M., Angermeyer, M. C., Berglund, P., Bromet, E. J., Brugha, T. S., Demyttenaere, K., De Girolamo, G., Haro, J. M., & Jin, R. (2009). Cross-national associations between gender and mental disorders in the World Health Organization World Mental Health Surveys. *Archives of general psychiatry*, *66*(7), 785-795.

Sivilotti, M. L., Burns, M. J., Aaron, C. K., & Greenberg, M. J. (2001). Pentobarbital for severe gamma-butyrolactone withdrawal. *Annals of emergency medicine*, *38*(6), 660-665.

Sullivan, J. T., Sykora, K., Schneiderman, J., Naranjo, C. A., & Sellers, E. M. (1989). Assessment of alcohol withdrawal: the revised clinical institute withdrawal assessment for alcohol scale (CIWA-Ar). *British journal of addiction*, *84*(11), 1353-1357.

Vasilenko, S. A., Evans-Polce, R. J., & Lanza, S. T. (2017). Age trends in rates of substance use disorders across ages 18–90: Differences by gender and race/ethnicity. *Drug and alcohol dependence*, *180*, 260-264.

Zilberman, M. L., Tavares, H., Blume, S. B., & El-Guebaly, N. (2003). Substance use disorders: sex differences and psychiatric comorbidities. *The Canadian Journal of Psychiatry*, *48*(1), 5-13.

Supplementary files

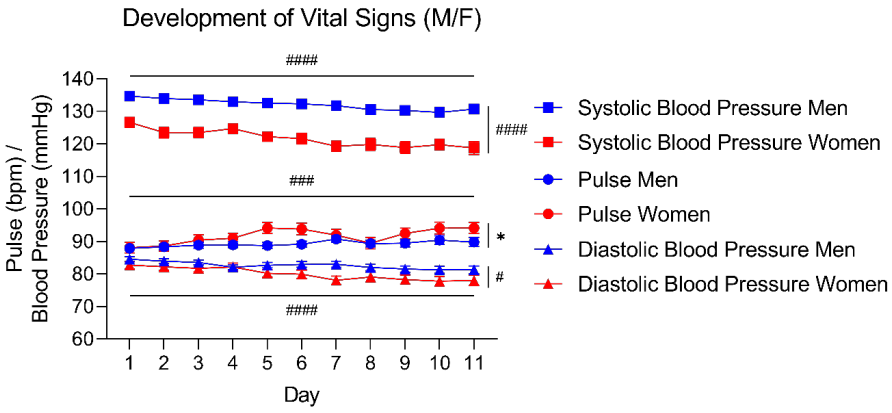

Figure A1. Development of vital signs (systolic blood pressure, diastolic blood pressure, heart rate) in both males and females during the first 11 days of GHB tapering. Data presented as mean ± SEM. Error bars are not shown if they are shorter than the size of the symbol. # = main effect of sex, $p < 0.05$; ### = main effect of time, $p < 0.001$; #### = main effect of time/sex, $p < 0.0001$; * = time x sex interaction, $p < 0.05$.

Table A1. Correlation between similar withdrawal symptoms measured with both the subjective- and objective withdrawal scale

SWS	OWS	Pearson's r
Craving	Craving	0.602***
Fatigue	Fatigue	0.488***
Insomnia	Insomnia	0.479***
Gloomy feeling	Gloomy	0.532***
Slow, sluggish feeling	Slow, sluggish	0.403***
Sweating	Sweating	0.534***
Tremor	Tremor / shaking hands	0.313*** / 0.409***
Sudden cold feeling	Sudden cold/warm feeling	0.533***
Muscle aches	Muscle aches	0.629***
Sudden warm feeling	Sudden cold/warm feeling	0.528***
Restless feeling	Restless	0.443***
Tensed, stressed feeling	Tensed, stressed	0.511***
Having lively dreams	Having lively dreams	0.679***
Eats a lot	Eats a lot	0.512***
Fear	Fear	0.660***
Yawning	Yawning	0.318***
Slow in movements	Slow in movements	0.403***
Having unpleasant dreams	Having unpleasant dreams	0.687***
Hungry	Hungry	0.470***
Muscle twitches	Muscle twitches	0.516***
Sleeps a lot	Sleepy/sleeps	0.399***
Goosebumps	Goosebumps	0.370***
Experience a fast heart rate	Heart rate (vital sign)	0.370***
Running nose	Running nose	0.461***
Abdominal cramps	Abdominal cramps	0.564***
Diarrhea	Diarrhea	0.800***
Tearing eyes	Tearing eyes	0.162 NS
Nausea	Nausea / Nauseous	0.479*** / 0.657***
Vomiting	Vomiting	0.226**
Auditory hallucinations	Auditory hallucinations	0.826***
Visual hallucinations	Visual hallucinations	0.725***
Epileptic seizures	Epileptic seizures	-0.017 NS
Fever		
	Shivers	
	Pupil dilation	

Correlation is significant if $p < 0.0015$ (Bonferroni correction, 2-tailed). ** = $p < 0.001$; *** = $p < 0.0001$; NS = non-significant

Table A2. Daily average subjective withdrawal symptoms correlated with vitals

	Heart Rate	Systolic Blood Pressure	Diastolic Blood Pressure	N
Tapering day 1	.164	-.035	.003	242
Tapering day 2	.137	-.078	.038	234
Tapering day 3	.159	-.170	-.023	239
Tapering day 4	.103	-.137	.011	219
Tapering day 5	.173	-.089	-.013	213
Tapering day 6	.159	-.094	.016	203
Tapering day 7	.127	-.093	.002	169
Tapering day 8	.190	-.006	.051	152
Tapering day 9	.193	-.107	.100	138
Tapering day 10	.071	-.053	.007	116
Tapering day 11	.167	-.036	.171	98

Shown values are Pearson r.

Table A3. Daily average objective withdrawal symptoms correlated with vitals

	Heart Rate	Systolic Blood Pressure	Diastolic Blood Pressure	N
Tapering day 1	.141	-.021	.046	263
Tapering day 2	.133	-.079	.030	254
Tapering day 3	.123	-.005	.086	255
Tapering day 4	.050	-.101	.039	241
Tapering day 5	.155	-.081	.033	233
Tapering day 6	.055	-.078	.018	219
Tapering day 7	.065	-.091	.037	185
Tapering day 8	.114	.047	.134	168
Tapering day 9	.172	-.063	.036	150
Tapering day 10	-.040	-.021	.078	128
Tapering day 11	.063	-.043	.157	108

Shown values are Pearson r.

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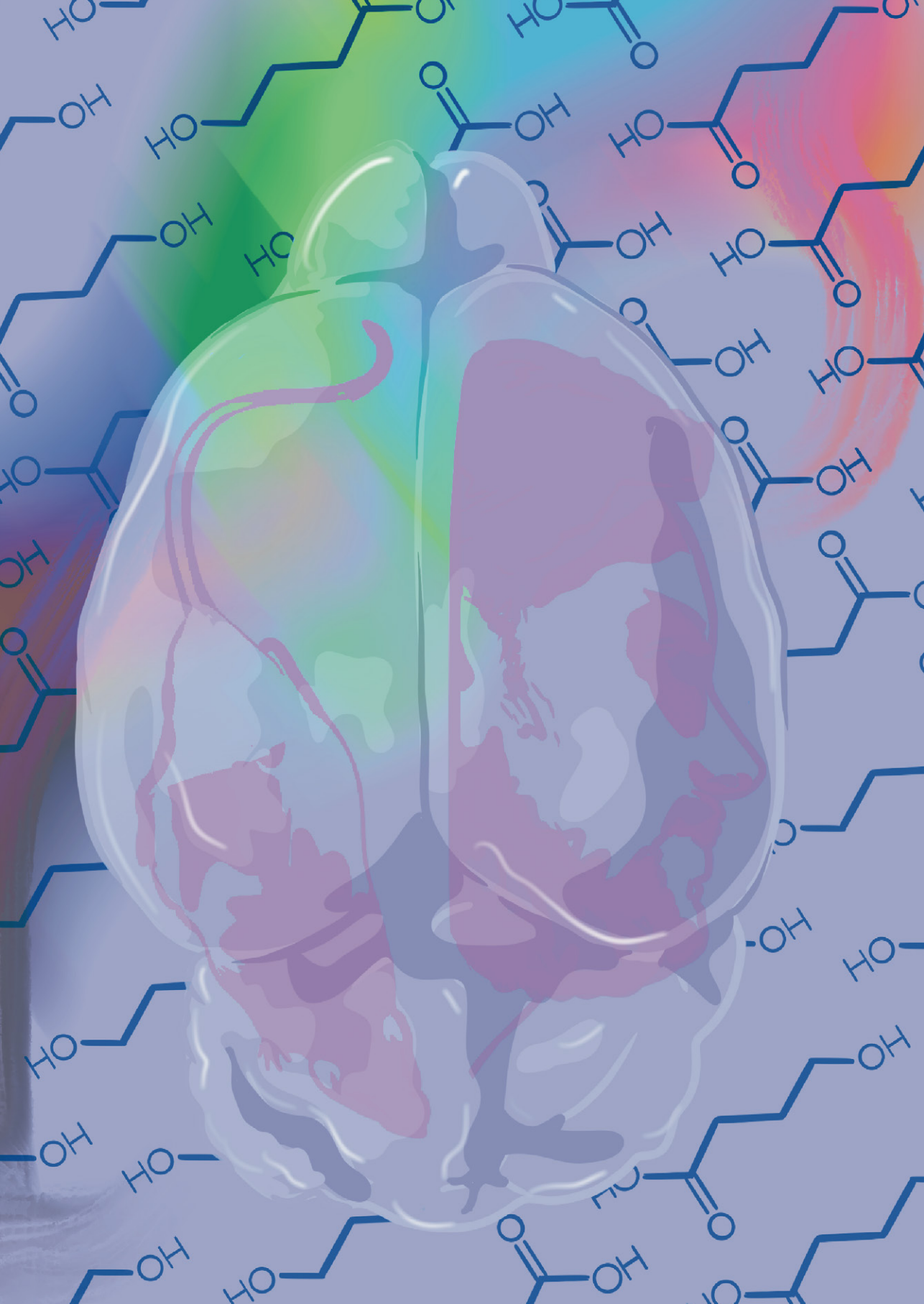


Chapter 7

Summary and discussion

The aim of this thesis was to 1) to provide a hypothesis for the identities of the high-affinity GHB receptor, 2) establish a GHB self-administration paradigm in rats, 3) examine the cognitive and neurobiological effects of GHB and 4) characterize the GHB withdrawal syndrome in humans. In this chapter, I will outline and discuss the key findings and their implications. I will begin with a summary of the included chapters, then move on to the overall conclusions, clinical implications, recommendations for future research and broader societal issues.

Chapter 2: GHB receptor structure

For decades, GHB has been assumed to target two distinct receptors, the $GABA_B$ receptor and the so-called GHB receptor. The $GABA_B$ receptor has been extensively studied, but little is known on the GHB receptor, and the exact identity of the GHB receptor remains debated. In our review, we used a bioinformatics approach to visualize a subtype of the GHB receptor, showing its putative two-dimensional and three-dimensional structure. This GHB receptor subtype is also known to function as a riboflavin (vitamin B2) transporter, opening the possibility of a dual-function structure, or a transceptor. In addition, we discuss the different high-affinity targets of GHB, including the CamKIIα kinase and the $GABA_A$ α4βδ receptor subtype, possibly providing an explanation for the neuroprotective properties of GHB following ischemia.

Chapter 3: GHB self-administration in rats

Human studies on GHB use have provided many insights into GHB use disorder, including the risks associated with GHB use, effective detoxification strategies and associations between GHB and cognition. In order to assess the causal effects of GHB consumption on a variety of potential outcome measures, an animal model is required. To develop such GHB self-administration model, we first determined GHB consumption parameters such as GHB concentration, administration interval and addition of sucrose that led to maximal GHB intake in rats. According to the optimal parameters, we achieved consistent consumption of pharmacologically relevant levels of GHB in the home-cage. These animals were subsequently trained in an operant cage to self-administer GHB through a lever press, enabling us to study addiction-like behavior. Two-thirds of the GHB rats established operant responding for GHB, of which a subset of animals, primarily females, exhibited addiction-like behavior. The majority of animals exhibited habitual GHB consumption. Home cage GHB self-administration led to a reduction of long-term memory performance in male rats, observed 3 months after the last consumption of GHB. Although GHB is suggested to reduce anxiety, we were not able to demonstrate an anxiolytic effect of home cage GHB consumption in our model.

Chapter 4: The cognitive effects of GHB-induced comas in rats

The occurrence of comas is common in GHB use disorder, yet the cognitive effects of these comas have hardly been studied. One study demonstrated an association between the occurrence of frequent GHB-induced comas and verbal short-term memory performance and impulsivity, contributing to the belief that especially high doses of GHB are harmful. We examined the effects of GHB-induced comas and GHB use without comas on working memory performance in rats. We did not find an effect of either GHB use or GHB-induced comas on non-hippocampal dependent working memory performance. Also, no effect was found on proxies for impulsivity, suggesting that an association between GHB-induced comas and impulsivity may be due to reversed causality.

Chapter 5: Neurobiological effects of GHB self-administration

Due to the strong dose-dependent effects of GHB and the multifaceted properties of the GHB receptor, many contrasting findings exist on the neurobiological effects of GHB. In this study, we used brain tissue of the rats used in the GHB self-administration study to examine the neurobiological effects of voluntary GHB self-administration. We showed a moderate negative correlation between home cage GHB intake and neuronal intensity in several subregions of the dorsal hippocampus. This correlation was not present for GABAergic, glutamatergic or glial markers. When comparing the GHB group with the control group, we did not find a difference, likely due to the large variance in home cage self-administration between animals.

Chapter 6: Characterization of the GHB withdrawal syndrome in humans

GHB withdrawal can be severe and highly erratic if left untreated and should therefore always be guided by healthcare professionals. There are multiple detoxification methods available for prevention and treatment of GHB withdrawal. In this chapter we characterized the GHB withdrawal syndrome during treatment with pharmaceutical GHB. The most commonly self-reported withdrawal symptoms consisted of craving, sleep-related symptoms such as fatigue and insomnia, mood-related symptoms such as gloominess, and physical symptoms such as sweating and tremor. Although pharmaceutical GHB tapering safely reduced most withdrawal symptoms, many symptoms were still present to some degree following the average duration of 11 days of pharmaceutical GHB tapering. Vital signs such as heart rate and blood pressure did not correlate with the severity of GHB withdrawal symptoms, in contrast to e.g. alcohol withdrawal. Men and women showed a similar degree of withdrawal severity, but showed a different pattern of withdrawal symptoms, with women scoring higher on mood-related symptoms and physical symptoms, whereas men more often reported to eat a lot.

GHB withdrawal syndrome and detoxification

A fulminant withdrawal syndrome is one of the hallmarks of GHB use disorder and can lead to life-threatening complications (McDonough et al., 2004). GHB withdrawal syndrome without guided detoxification is characterized by tremor, tachycardia, anxiety and hallucinations, with over >50% of patients experiencing delirium (McDonough et al., 2004). Two common detoxification methods to reduce the severity of the GHB withdrawal syndrome is benzodiazepine tapering or pharmaceutical GHB tapering. Although widely used, the use of benzodiazepine treatment of GHB detoxification still occasionally leads to severe withdrawal symptoms, likely due to a mismatch in receptor systems between GHB ($GABA_B$) and benzodiazepines ($GABA_A$). Compared with detoxification with benzodiazepines, detoxification with pharmaceutical GHB has been shown to lead to a less severe withdrawal syndrome and to lead to a less frequent occurrence of adverse events (Beurmanjer et al., 2020).

In chapter 2 we describe the symptoms experienced by patients with GUD undergoing detoxification through pharmaceutical GHB tapering. The most experienced- and observed withdrawal symptoms consisted of sleep-related symptoms, mood-related symptoms and physical symptoms. These symptoms are generally observed with other sedative withdrawal syndromes like alcohol and benzodiazepines. This confirms the hypothesis that GHB, and GUD specifically, shares several characteristics with other (sedative) SUDs (Beurmanjer, 2021).

GHB withdrawal is generally perceived as a complex phenomenon to adequately treat. Several case reports and case series have been published on the treatment of GHB withdrawal syndrome. A recent clinical observational study observed over 30% of cases with delirium during GHB detoxification with benzodiazepines, and over 20% of intensive care admissions (Neu et al., 2023). Similar numbers were observed by (Harjanto et al., 2025), where 30% of the patients required intensive care treatment, 13% required intubation, and in 17% of the cases delirium or seizures occurred following benzodiazepine treatment. Another example of complex GHB detoxification is shown by Gupta and colleagues (2025), where a patient was admitted to the hospital thrice with intravenous sedation and tracheal intubation, and eventually treated with benzodiazepine and baclofen tapering (Gupta et al., 2025).

Our study confirms that GHB detoxification through titration and tapering with pharmaceutical GHB is a safe and reliable method, yet the use of $GABA_A$ agonist appears to remain the most frequently used treatment strategy internationally. However, $GABA_B$ receptor-based drugs or pharmaceutical GHB might be more favorable for prevention

and treatment of GHB withdrawal, with lower risk of complications. Pharmaceutical GHB tapering is already the preferred method in The Netherlands for GHB detoxification.

Based on the effective and safe GHB detoxification protocol, Wood and colleagues have recently published a novel, GHB-specific withdrawal questionnaire (Wood et al., 2025). This GHB withdrawal symptom questionnaire (GWSQ) focuses on patient-reported withdrawal severity (subjective withdrawal), while maintaining good psychometric properties such as reliability and concurrent-/convergent validity (Wood et al., 2025). The assessment of GHB-specific withdrawal symptoms that are representative of GHB withdrawal syndrome, as proposed in the GWSQ, may decrease the burden of withdrawal-monitoring for both patients and nursing staff, without compromising on assessment quality. This promising questionnaire should be further evaluated to determine its validity and usefulness in clinical practice to assess GHB withdrawal.

Another way to further improve GHB detoxification is to explore other compounds targeting $GABA_B$ receptors. In 2016, a study protocol has been published on a randomized controlled trial to treat GHB withdrawal with baclofen, although this has not been followed up to our knowledge (Lingford-Hughes et al., 2016). Future studies should further explore the use of baclofen or other $GABA_B$ agonists in the treatment of GHB withdrawal.

GHB consumption pattern and the role of sleep

Next to the GHB withdrawal syndrome, physical and psychological dependence on GHB are other hallmarks of GHB use disorder, similar to other substance use disorders. However, in contrast to other substance use disorders, there is no existing animal model for GHB use disorder. Chapter 3 describes the first study to date that extensively characterizes GHB self-administration in an animal model.

The typical consumption pattern observed in patients with GHB use disorder (GUD) is characterized by GHB consumption every two to three hours, also during the night (Beurmanjer et al., 2019). This binge-like pattern was not replicated in our animal model. This discrepancy may be attributed to practical limitations, such as the challenge for humans to consistently consume GHB throughout the day. However, in another animal study, a binge-like consumption pattern was observed, more closely mimicking the consumption pattern observed in humans (Colombo et al., 1995). In the pilot study described in chapter 4, we observed GHB binging after adding sucrose to the GHB solution. We observed a three- to four-fold increase in GHB intake, which

was followed by a cessation of consumption. This suggests that higher doses of GHB may be aversive to rats, potentially explaining why high-dose self-administration or binge-like consumption was not consistently observed when GHB without sucrose was available. Although the home cage consumption pattern in our model does not directly reflect the dosing behavior seen in patients with GUD, the model remains a valuable tool for studying the effects of GHB self-administration and for investigating the mechanisms underlying GHB consumption behavior.

The distinct consumption pattern in patients with GUD, primarily driven by the short half-life of GHB, leads to a highly disrupted sleeping pattern. This disrupted sleeping pattern may be a strong confounder in the negative effects observed in patients with GHB use disorder (Beurmanjer et al., 2022). During detoxification, sleep-related problems are strongly present (Wolf et al., 2021), which can linger even several months after successful detoxification (Beurmanjer et al., 2019). This confounding effect of sleep deprivation has been hardly studied, due to the difficulty to disentangle GHB dependence and disrupted sleep.

In our GHB self-administration model, we were not able to assess nightly GHB intake. We estimated average consumption of GHB outside working hours based on before- and after measurements, which appeared to be slightly lower than during working hours. As rats are polyphasic sleepers, it is difficult to compare disruption of sleeping patterns without controlled nightly measurements (Simasko & Mukherjee, 2009). Future studies should examine whether chronic home-cage GHB consumption in rats disturbs sleeping patterns to separate the effects of GHB consumption and sleep disruption.

Treatment strategies aiming to restore disrupted sleeping should be explored. For instance, a longer-acting GHB substitute may contribute to the quality of life of patients with GUD by reducing sleep disruption, social isolation and supporting a healthy daily routine. Additionally, studies into the beneficial effects of Xyrem on sleep in patients with narcolepsy may provide novel insights in promoting a healthy sleeping architecture that can be applied in patients with GUD. Eventually, such studies may contribute and enable sustainable abstinence from GHB.

Individual variability and sex differences in addiction models

Individual variability is a key component in substance use and substance use research. Although it is difficult to estimate the susceptibility to substance use disorder in

the general population, an estimated ~10% of the population of 12 years and older is suspected to have an alcohol use disorder, shining a light on the addiction prevalence on widely available substances (SAMHSA, 2024). Despite similar opportunities to access illicit substances between humans, not every individual is equally susceptible to substance use and substance use disorders. Many factors contribute to this divergence, including socio-economic conditions, cultural circumstances, psychiatric divergence, genetic factors.

There is a strong difference in illicit drug use between men and women, with in the US ~13% of men reporting illicit drug use, in contrast to ~8% of women (CBHSQ, 2017). Interestingly, sex does not seem to be the determining factor in explaining this difference, since the likelihood of substance use between men and women does not differ when controlling for access (Caris et al., 2009). However, as an interaction of bio-environmental factors, men have higher rates of substance dependence than women based on substance use treatment admissions (65% male vs 35% female) (SAMHSA, 2023, 2024). Reported gender differences, such as women showing a later onset of substance use compared to men, distinct underlying motivation to use, and earlier treatment admission in women compared to men are suggested to be driven by the influence of hormones, brain dimorphisms, and drug pharmacokinetics and pharmacodynamics (Fattore et al., 2008).

In chapter 6, we observe clear sex-differences in both prevalence and type of withdrawal symptoms during pharmaceutical GHB tapering. In chapter 3, we observe sex-differences between animals, both in GHB/water preference, development of addiction-like behavior and memory performance before and after GHB administration. One of the few studies examining differences in the pharmacokinetics of GHB between sexes did not find any differences in peak concentration, AUC or half-life in humans (Borgen et al., 2003), making it unlikely that the sex differences found in our studies are driven by differences in GHB pharmacokinetics between males and females. Although not directly examined in our study, we hypothesize that the majority of these differences are at least partially driven by hormonal regulation (Soldin & Mattison, 2009; Fonseca et al., 2021).

In animal models, clear sex differences in (the development of) substance dependence are present. For example, it has been shown that female rats acquire consistent drug self-administration more rapidly, escalate their drug intake more rapidly, showed more drug-seeking behavior during non-reinforced withdrawal, and show greater rates of relapse compared to their male counterparts (Becker & Koob, 2016). These phenomena are also observed in our study described in chapter 3, where 8 out of

12 animals that acquired operant responding for GHB were female, which also exhibited most prominent addiction-like behavior. It has been shown that the estrous cycle plays a critical role in determining these sex differences in operant behavior (Becker & Koob, 2016).

Sex differences in memory performance were also observed in our study described in chapter 3. We expect this finding can be a consequence of the initial sex-bias of the cognitive test that was used. Female rats have been shown to perform better than male rats on the novel object recognition test, depending on the estrous cycle phase (Sutcliffe et al., 2007). The possibility for estrous-cycle-dependent, and therefore time-dependent, performance on the novel object recognition test may have led to an underestimation of the GHB effects in female GHB group. Future studies should examine the role of the estrous cycle in the assessment of the cognitive effects of GHB, and further explore possible sex-specific cognitive effects by GHB.

In preclinical research, selectively bred animal models have been developed to exclude the genetic variety underlying differences in drug intake, development of drug addiction, and relapse vulnerability. Our findings on sex differences throughout this thesis emphasize the need for a focus on sex-differences in substance use research and in the development of treatment strategies. While the use of selectively bred models and the exclusive use of male animals can increase behavioral output and decrease variance, it minimizes translatability to the clinical reality. And still, even within genetically homogenous populations that may increase behavioral output, individual differences in drug intake behavior can be observed (Colombo et al., 1998). This suggests that environmental, epigenetic, and neurobiological factors also play a role in mediating susceptibility to substance use and the development of addiction. Despite the use of selectively-bred animals, (behavioral) preclinical addiction research has yielded limited clinically relevant output (Corley et al., 2024). Animals models mimicking specific pathologies or a subset of patients are highly valuable in acquiring phenotype-specific knowledge, yet studies employing subjects with a mixed genetic background may improve translatability and clinical relevance of preclinical studies.

Our studies highlight the critical role of sex differences and individual variability in substance use and dependence. Therefore, future research should continue to incorporate genetically diverse models to enhance the clinical relevance of preclinical findings. Future studies considering sex-specific responses will not only advance our understanding of addiction mechanisms, but also support the development of tailored treatment approaches accounting for individual susceptibility and substance use phenotypes.

The effects of GHB on memory and cognition

GHB has been approved by the FDA and EMA as a medicinal drug for the treatment of narcolepsy. As a requirement for this approval, several safety-studies have been published (Xyrem, 2002, 2003, 2005; Strunc et al., 2021; Junnarkar et al., 2022). Although no direct long-term cognitive tests were performed, most studies reported outcomes such as "brain fog", "half-asleep state", "lack of alertness", "decrease in mental agility" following GHB administration (Abad, 2019). Since GHB was identified as a treatment for the sleeping-disorder narcolepsy, there was a huge confounder of sleep in the assessment of cognition. One study that examined cognition in healthy controls (and therewith excluding the effect of sleep on the results) only focused on the acute cognitive effects of GHB during intoxication, showing acute memory impairment following higher doses (Carter et al., 2006). Controlled studies examining the long-term cognitive effects of GHB are currently lacking.

Animal studies focusing on the residual, i.e. non-acute cognitive and memory effects of GHB, including the studies described in chapter 2 and 3, report counterintuitive findings. Studies on the effects of GHB on memory performance report both null findings (Pedraza et al., 2009; Klein et al., 2015) and negative effects on varying memory domains (Pedraza et al., 2009; van Nieuwenhuijzen, Long, et al., 2010; Chen et al., 2017). To properly link the studies and findings on the memory effects of GHB, a deeper understanding of the currently dominant theory on memory is required.

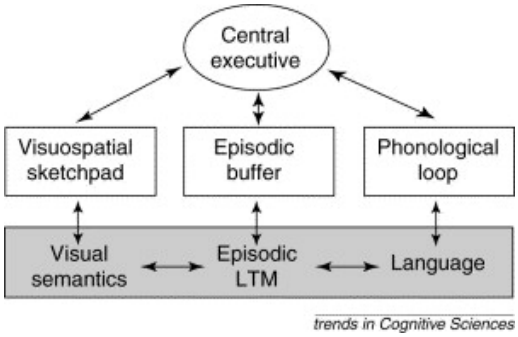


Figure 1. Baddeley & Hitch's model for working memory and transition to long-term memory. Adapted from Baddeley, Trends in Cognitive Sciences, 2000.

The most common and widely accepted theory on working memory and long-term memory is the one of Baddeley and Hitch, already proposed in 1974 (Baddeley, 1974). In short, Baddeley and Hitch state that working memory is composed of multiple interacting components, namely 1) the central executive system, responsible for

directing attention, prioritizing and coordinating activities of other components, 2) phonological loop, holding and refreshing auditory information, 3) visuospatial sketchpad, processing and temporarily storing visual and spatial information. The fourth component, the episodic buffer, integrates information of the phonological loop and visuospatial sketchpad, together with long-term memory, into a (sequence of) episodes, bridging working memory and long-term memory (Fig. 1). Studies suggest that the Baddeley & Hitch working memory model largely translates to animals (Keeler & Robbins, 2011), although results on the language-aspect of the phonological loop are impossible to translate due to obvious reasons.

Several factors that trouble the interpretation and comparison of studies on the memory effects of GHB are the temporal borders of the transition between working memory and long-term memory, and the role of verbal components. It is unclear when mechanisms of working memory (consolidation) are in place, and when a transition to long-term memory occurs. Tests that use different time intervals find discrepant results (such as a novel object recognition test with an interval of 1 vs 5 vs 15 minutes), making it difficult to determine what type of memory is affected (Antunes & Biala, 2012). Together with the strong dose-dependent effects of GHB, differences in pharmacokinetics between species and administration methods, bold, generalized statements on the effects of GHB on cognition are often too simplistic. In the section below, we aim to interpret our cognitive findings in light of existing literature on the cognitive effects of GHB, considering the many facets that may influence the properties of GHB and the translation of cognitive animal studies to humans.

There is strong evidence for negative acute effects of GHB on both working memory and episodic / long-term memory (Carter et al., 2006; Carter, Griffiths, et al., 2009). These effects were primarily observed with relatively high doses, resp. 4 g / 70 kg and 8 g / 70 kg (Carter et al., 2006) and 4.5 g / 70 kg (Carter, Griffiths, et al., 2009), doses that are also used for the treatment of narcolepsy (Xyrem, 2002). Upon GHB administration, disorientation, decreased focus and drowsiness occurred, showing an effect on the central executive system and likely affecting cognitive outcomes (Carter et al., 2006; Carter, Griffiths, et al., 2009). These studies also showed that there is strong individual susceptibility to the subjective effects of GHB, reflected by individual dosing paradigms and differential behavioral responses to similar doses of GHB in this study (Carter et al., 2006). It is unclear whether GHB also affects cognition through other routes than impairing the central executive system based on these human studies.

Animal studies also find acute impairments in spatial learning and working memory performance in rats during GHB intoxication (Sircar & Basak, 2004; Sircar et al.,

2011). In these studies, similar doses of GHB were administered between animals, ignoring possible individual susceptibility to GHB but confirming findings from human studies. The doses used in these studies exceed doses commonly used in human studies, in line with the differential conversion of systemic GHB administration into plasma levels between humans and rats (Felmlee et al., 2021). Despite the fact that the comparison of dose-effects between species is troubled, it appears that GHB intoxication may negatively affect learning and memory processes.

Residual effects of GHB are not easy to measure in a patient population, due to the fluidity of the GUD patient population and the confounding effects of sleep-deprivation, psychiatric comorbidity, polydrug use patterns, dose discrepancies, etc. There are less than a handful of human studies that try to detect residual memory deficits in GHB users. Following detoxification, GHB users show impaired short-term (5-min) phonological memory deficits (Beurmanjer et al., 2022). Interestingly, Beurmanjer and colleagues find an improvement of memory performance following a period of abstinence, allowing for the possibility for GHB-related memory impairment to be temporary to some extent (Beurmanjer et al., 2022). Another study did not find effects of GHB use on verbal or spatial short-term (5 min) memory performance (Raposo Pereira et al., 2018). Interestingly, specifically the experience of >4 GHB-induced comas were associated with decreased verbal, but not spatial, memory performance (Raposo Pereira et al., 2018).

The transition from working memory storage to (episodic) long-term memory storage is fluid and relies on many factors such as context, repetition, stimulus strength etc. The results following the ~5-minute interval used in the human studies of Beurmanjer et al. (2021) and Pereira et al. (2019) discussed above are therefore difficult to compare to studies examining working memory performance (~5s interval) or studies where the transition to long-term memory has likely occurred (>1h interval). Nevertheless, these studies seem to indicate that high-intensity GHB use (as seen in GUD patients by Beurmanjer et al., and in the >4 coma group by Pereira et al.) could be involved in the decreased transition from (verbal) working to long-term memory, in a reversible manner.

The residual cognitive effects of GHB, e.g. cognitive effects that are still present when GHB is not present in the body, are highly relevant from a clinical perspective. Our studies described in chapter 3 and 4 show that chronic oral GHB administration negatively affects long-term recognition memory performance, whereas it does not affect non-hippocampal-dependent spatial working memory performance. Existing literature in rodents shows mixed results on the effect of GHB on spatial learning and

memory (Pedraza et al., 2009; Chen et al., 2017), but did not find effects of GHB on spatial recognition performance (Klein et al., 2015). GHB did decrease novel object recognition performance or long-term memory performance on the hole-board test (Pedraza et al., 2009), although all these studies use different animal models, administration methods, doses, frequency of administration and memory modality, etc. Placing our results within this context, it appears that GHB especially affects the transition from (recognition) working memory to long-term memory, and GHB has the potential to affect spatial memory performance depending on dose and administration frequency.

In conclusion, while GHB has established medicinal uses, its cognitive effects—both (sub)acute and residual —are complex and nuanced, with varying findings across human and animal studies. Applying the Baddeley & Hitch working memory model suggests that GHB may not only affect the central executive network, but could also impair the transition from working memory to long-term memory in susceptible individuals, especially with frequent or high-dose use. While animal models have demonstrated memory deficits that seem dose-dependent, translating these findings to humans is complicated by pharmacokinetic differences and confounding factors like sleep and comorbidities among GHB users. The observed impairments in phonological memory in heavy GHB users imply that certain memory functions could be more vulnerable, with possible (but uncertain) reversibility after abstinence. Altogether, future studies should further examine hippocampal-dependent memory performance in human users to better understand GHB's long-term cognitive risks, guiding both clinical use and harm reduction strategies for recreational GHB users and patients with GUD.

Neurobiology and neuroprotection

As mentioned in the introduction, GHB was initially identified as a non-toxic compound (Laborit, 1964), in line with the endogenous presence of GHB in the brain. With the emergence of GHB use disorder, the focus concurrently shifted from the therapeutic effects of GHB to the negative effects of GHB. Thus far, we have discussed the negative effects of GHB use and GHB use disorder. In line with chapter 2, where we touched upon the possible neuroprotective effects through GHB receptor subtypes and its overlap with the riboflavin transporter, we will now focus on the line of evidence on the neuroprotective properties of GHB. We will also zoom in on the proposed underlying neurobiological mechanisms, and the overlap and separation with the putative mechanisms behind the neurotoxic effects of GHB.

GHB has been suggested to exert neuroprotective effects following cerebral ischemia (Vergoni et al., 2000). Neuroprotection refers to the ability of a substance to prevent or reduce damage to the nervous system and promote its overall health and functioning. Administration either before or 10 minutes after arterial occlusion, followed by twice-daily GHB injections for 10 days, strongly reduced neuronal death in the CA1 region of the hippocampus (~20% survival -> ~85% survival). This neuronal effect was accompanied by a rescue of sensory-motor performance, spatial learning and memory performance 27 days after ischemia (Vergoni et al., 2000). Similar findings were observed in a model for middle cerebral artery occlusion (MCAO) (Sadasivan et al., 2006) or when GHB was administered even 2 hours after the ischemic episode (Ottani et al., 2004). Inducing focal cerebral damage through ischemia or excitotoxicity (instead of four-vessel occlusion and reperfusion / middle cerebral artery occlusion (MCAO)), and using a similar GHB treatment paradigm (2h post-ischemia, twice-daily injections for 10 days), cell death and memory performance were also largely rescued (Ottani et al., 2003). These neuroprotective effects do not seem to be specific for ischemia or excitotoxicity, since GHB can also rescue cells following hydrogen-peroxide exposure through inhibition of hydrogen peroxide-induced apoptosis (Wendt et al., 2014). Collectively, these results suggest that GHB may hold therapeutic potential for brain damage.

$GABA_B$ receptor activation has been associated with neuroprotection against apoptosis and biochemical damage, preserving cognitive processes (Tu et al., 2010; ali Shah et al., 2013; Kumar et al., 2017). The anti-apoptotic effect by activation of $GABA_B$ receptors is suggested to involve transactivation of the IGF-1 receptor, a receptor involved in learning and memory processes (Nyberg, 2000; Tu et al., 2010). IGF-1 also plays a significant role in the rescue of neuronal loss after hypoxic-ischemic injury (Gluckman et al., 1992; Guan et al., 1996; Peruzzi et al., 1999; Sizonenko et al., 2001). In 2014, Johansson and colleagues reported a dose-dependent decrease in IGF-1 receptor density in the hippocampus in rats receiving GHB, but an increase of IGF-1 receptor density in other brain regions (Johansson et al., 2014). In addition, one-week GHB administration in rats lead to downregulation of IGF-1 mRNA expression (Johansson et al., 2014). Future research should disentangle the effects of GHB on IGF-1 in neuroprotection following ischemic/excitotoxic conditions on the one hand, and the effects of GHB on IGF-1 in possible neurotoxicity following non-pathogenic conditions on the other hand.

More recently, another mechanism through which GHB could exert neuroprotection was demonstrated. Binding of micromolar concentrations of GHB and analogs to the binding hub of CamKIIa stabilizes CamKIIa, and provides protection in neuronal cell

lines against excitotoxicity, and in a mouse model of ischemia (Leurs et al., 2021). This was found for both cortical and hippocampal cell cultures, and in cortical areas *in vivo* (Leurs et al., 2021). There were no signs that these neuroprotective properties were driven by $GABA_B$ receptor activation (Leurs et al., 2021). Additionally, the mechanisms underlying the observed neuroprotective effect were not activated by GHB analogs under nonpathological conditions.

It appears that these neuroprotective properties were highly dependent on timing of administration. Application of a GHB-analog directly or 30-min after the occurrence of excitotoxicity did not lead to improved cell survival. However, administration of GHB analogs >1h post-trauma dose-dependently improved cell survival (Leurs et al., 2021). The activation of CamKIIa through GHB analogs following ischemia, using effective timing and dose parameters, resulted in an improvement of working memory and sensorimotor performance

Our findings on the structural properties of a GHB receptor subtype and the overlap with the riboflavin transporter shed new light on the possible neuroprotective effects of GHB. Although these findings should be experimentally confirmed, the overlap between the GHB receptor subtype and the riboflavin transporter allows for further neuroprotective potential of GHB. Riboflavin has previously been shown to exert neuroprotective properties in specific neurotoxic conditions such as ischemia (Silva-Araújo et al., 2024). Riboflavin transporter deficiency type 2, which has a similar structure as the putative GHB receptor subtype, leads to severe motor- and sensory neuronal damage (Jaeger & Bosch, 2016), although cognitive functions remain intact. It should be further investigated whether riboflavin exerts an additional neuroprotective effect in addition to GHB, and to what extent this affects cognitive functions. In addition to the increased focus of the neuroprotective properties of GHB through interaction with CamKIIα, future research should explore the role of riboflavin and the riboflavin transporter in the neuroprotective properties of GHB.

Overall, GHB has shown to be able to exert neuroprotective effects following ischemia, excitotoxicity or hydrogen peroxide-induced cell death. Its ability to reduce ischemic tissue damage, modulate cellular metabolism, scavenge oxygen radicals, and to interact with $GABA_B$ receptors underscores its potential for preserving neuronal health and mitigating the consequences of brain ischemia and cell death. As negative effects of GHB are more likely to emerge following higher doses, or in individuals with higher sensitivity to GHB, the dose-dependent effects may play a key role in the different functions of GHB, next to the healthy/pathological state of the brain. The absence of involvement of the neuroprotective mechanisms upon GHB/GHB-analog

administration under nonpathological conditions may allow for the existence of parallel negative and positive effects of GHB on cognition and brain function.

The future of GHB: pros versus cons and alternative routes

Throughout this thesis, I have discussed the many facets of GHB in an experimental and clinical context, including its development from a therapeutic agent to a drug of abuse. However, this dual identity of GHB as both a drug of abuse with negative effects on cognition, and as a medicinal drug with potential neuroprotective effects, asks for consideration of both the positive and negative effects of GHB in the public debate. The current debate surrounding GHB's future boils down to whether its medical benefits will outweigh the risks and negative effects that come with its (illicit) use, or whether these two sides can co-exist.

GHB's classification as a Schedule I substance, together with its notoriety as a "date rape" drug has impacted its public perception, although evidence for common use of GHB as a date rape drug remains scarce (Németh et al., 2010). GHB remains a drug that is experienced as dangerous, and patients with GUD are generally regarded as difficult to treat. The research presented in this thesis points out that one of the hallmarks of GUD, GHB withdrawal syndrome, can be effectively treated with pharmaceutical GHB tapering, reducing all measured withdrawal symptoms while minimizing adverse events. Additionally, our self-administration model has demonstrated that GHB in preclinical models share many similarities with other substances, such as alcohol. In addition, we also show memory-domain specific impairment, and a dose-dependent association between GHB intake and hippocampal neuronal intensity, in line with existing literature demonstrating memory impairments in animal models and humans. Increasing knowledge on GHB and GHB use disorder provides handles in the (education of) treatment and management of GUD. The challenge for healthcare providers is to ensure that the risk of abuse and negative effects on cognition and the brain are minimized in patients that use GHB as a medicinal drug, people that use GHB recreationally or patients that suffer from GUD.

The future of GHB may partially depend on advancements in pharmacology, in combination with a stronger focus on individual susceptibility and its underlying social, psychological and genetic components. One potential avenue is the development of safer, modified versions of GHB that retain its therapeutic benefits

while reducing the risk of abuse. The development of GHB analogs such as the molecule HOCPA developed by the group of Leurs et al. (2021), is a promising first step into the development of compounds that yield the therapeutic properties of GHB, with reducing abuse potential (Li et al., 2025). Additionally, more precise prescribing guidelines and monitoring systems could help prevent misuse, ensuring that GHB is used effectively and safely in clinical settings. Besides the established dose-dependent effects of GHB, our study shows an association between GHB administration and neuronal signal intensity in the hippocampus, further emphasizing the role of dose in the possible negative effects of GHB. As the current treatment strategies with GHB in the context of narcolepsy show the use of a fixed dose of GHB per night (e.g. 6g or 9g per night), further refinement of the dosing strategy can be achieved, in order to maximally reduce the risk of adverse events or side effects.

In parallel to exploring and refining the therapeutic effects of GHB and its analogs, this thesis continuously emphasized the risks that are associated with GHB use. The future of GHB remains a balance between these risks of (illicit) GHB use, and the benefits it provides for patients for which GHB is the only treatment option left. Our findings presented in this thesis contribute a nuanced view on the negative effects of GHB, that should be considered in both the treatment of GHB use disorder, and the continued use of GHB as a medicinal drug. Future studies should contribute to a further understanding of the dose-dependent cognitive and neurobiological effects of GHB in humans, ultimately leading to a better understanding of the risks associated with GHB use.

Bibliography

Abad, V. C. (2019). An evaluation of sodium oxybate as a treatment option for narcolepsy. *Expert Opinion on Pharmacotherapy*, *20*(10), 1189-1199.

ali Shah, S., Ullah, I., Lee, H. Y., & Kim, M. O. (2013). Anthocyanins protect against ethanol-induced neuronal apoptosis via GABA B1 receptors intracellular signaling in prenatal rat hippocampal neurons. *Molecular neurobiology*, *48*, 257-269.

Antunes, M., & Biala, G. (2012). The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cognitive processing*, *13*, 93-110.

Baddeley, A. D. H., G. J. (1974). Working memory. In G. A. Bower (Ed.), *Recent Advances in Learning and Motivation* (pp. 47-89). Academic Press. https://doi.org/10.1016/s0079-7421(08)60452-1

Becker, J. B., & Koob, G. F. (2016). Sex Differences in Animal Models: Focus on Addiction. *Pharmacol Rev*, *68*(2), 242-263. https://doi.org/10.1124/pr.115.011163

Beurmanjer, H. (2021). *When the party is over: Addressing clinical challenges in patients with GHB use disorders*. Sl: sn.

Beurmanjer, H., Asperslag, E. M., Oliemeulen, L., Goudriaan, A. E., De Jong, C. A. J., Schellekens, A. F. A., & Dijkstra, B. A. G. (2019). A Qualitative Approach in Understanding Illness Perception and Treatment Needs in Patients with Gamma Hydroxybutyrate Use Disorder. *Eur Addict Res*, *25*(5), 248-255. https://doi.org/10.1159/000500807

Beurmanjer, H., Bruijnen, C. J. W. H., Greeven, P. G. J., De Jong, C. A. J., Schellekens, A. F. A., & Dijkstra, B. A. G. (2022). Cognitive Impairments in Patients with GHB Use Disorder Predict Relapse in GHB Use. *Eur Addict Res*, *28*(5), 350-357. https://doi.org/10.1159/000525507

Beurmanjer, H., Luykx, J. J., De Wilde, B., van Rompaey, K., Buwalda, V. J. A., De Jong, C. A. J., Dijkstra, B. A. G., & Schellekens, A. F. A. (2020). Tapering with Pharmaceutical GHB or Benzodiazepines for Detoxification in GHB-Dependent Patients: A Matched-Subject Observational Study of Treatment-as-Usual in Belgium and The Netherlands. *CNS Drugs*, *34*(6), 651-659. https://doi.org/10.1007/s40263-020-00730-8

Borgen, L. A., Okerholm, R., Morrison, D., & Lai, A. (2003). The influence of gender and food on the pharmacokinetics of sodium oxybate oral solution in healthy subjects. *The Journal of Clinical Pharmacology*, *43*(1), 59-65.

Caris, L., Wagner, F. A., Rios-Bedoya, C. F., & Anthony, J. C. (2009). Opportunities to use drugs and stages of drug involvement outside the United States: Evidence from the Republic of Chile. *Drug Alcohol Depend*, *102*(1-3), 30-34. https://doi.org/10.1016/j.drugalcdep.2008.12.004

Carter, L. P., Griffiths, R. R., & Mintzer, M. Z. (2009). Cognitive, psychomotor, and subjective effects of sodium oxybate and triazolam in healthy volunteers. *Psychopharmacology*, *206*, 141-154.

Carter, L. P., Richards, B. D., Mintzer, M. Z., & Griffiths, R. R. (2006). Relative abuse liability of GHB in humans: A comparison of psychomotor, subjective, and cognitive effects of supratherapeutic doses of triazolam, pentobarbital, and GHB. *Neuropsychopharmacology*, *31*(11), 2537-2551. https://doi.org/10.1038/sj.npp.1301146

CBHSQ, C. f. B. H. S. a. Q. (2017). *Results from the 2016 National Survey on Drug Use and Health: Detailed Tables*. S. A. a. M. H. S. Administration.

Chen, L. Y., Renn, T. Y., Liao, W. C., Mai, F. D., Ho, Y. J., Hsiao, G., Lee, A. W., & Chang, H. M. (2017). Melatonin successfully rescues hippocampal bioenergetics and improves cognitive function following drug intoxication by promoting Nrf2-ARE signaling activity. *J Pineal Res*, *63*(2). https://doi.org/10.1111/jpi.12417

Colombo, G., Agabio, R., Balaklievskaia, N., Diaz, G., Lobina, C., Reali, R., & Gessa, G. L. (1995). Oral self-administration of γ-hydroxybutyric acid in the rat. *European journal of pharmacology*, *285*(1), 103-107.

Colombo, G., Agabio, R., Diaz, G., Fà, M., Lobina, C., Reali, R., & Gessa, G. L. (1998). γ-Hydroxybutyric acid intake in ethanol-preferring sP and-nonpreferring sNP rats. *Physiology & behavior*, *64*(2), 197-202.

Corley, C., Craig, A., Sadek, S., Marusich, J. A., Chehimi, S. N., White, A. M., Holdiness, L. J., Reiner, B. C., & Gipson, C. D. (2024). Enhancing translation: A need to leverage complex preclinical models of addictive drugs to accelerate substance use treatment options. *Pharmacology Biochemistry and Behavior*, 173836.

Fattore, L., Altea, S., & Fratta, W. (2008). Sex differences in drug addiction: a review of animal and human studies. *Womens Health (Lond)*, *4*, 51-65. https://doi.org/10.2217/17455057.4.1.51

Felmlee, M. A., Morse, B. L., & Morris, M. E. (2021). gamma-Hydroxybutyric Acid: Pharmacokinetics, Pharmacodynamics, and Toxicology. *AAPS J*, *23*(1), 22. https://doi.org/10.1208/s12248-020-00543-z

Fonseca, F., Robles-Martínez, M., Tirado-Muñoz, J., Alías-Ferri, M., Mestre-Pintó, J.-I., Coratu, A. M., & Torrens, M. (2021). A gender perspective of addictive disorders. *Current addiction reports*, *8*, 89-99.

Gluckman, P., Klempt, N., Guan, J., Mallard, C., Sirimanne, E., Dragunow, M., Klempt, M., Singh, K., Williams, C., & Nikolics, K. (1992). A role for IGF-1 in the rescue of CNS neurons following hypoxic-ischemic injury. *Biochemical and biophysical research communications*, *182*(2), 593-599.

Griem-Krey, N., Klein, A. B., Clausen, B. H., Namini, M. R. J., Nielsen, P. V., Bhuiyan, M., Nagaraja, R. Y., De Silva, T., Sobey, C. G., & Cheng, H. C. (2023). The GHB analogue HOCPCA improves deficits in cognition and sensorimotor function after MCAO via CaMKIIα. *Journal of Cerebral Blood Flow & Metabolism*, *43*(8), 1419-1434.

Guan, J., Williams, C. E., Skinner, S., Mallard, E. C., & Gluckman, P. D. (1996). The effects of insulin-like growth factor (IGF)-1, IGF-2, and des-IGF-1 on neuronal loss after hypoxic-ischemic brain injury in adult rats: evidence for a role for IGF binding proteins. *Endocrinology*, *137*(3), 893-898.

Gupta, R., Moon, G., Bonomo, Y., & Pastor, A. (2025). A case of severe and prolonged γ-hydroxybutyrate (GHB) withdrawal syndrome successfully managed with a slow benzodiazepine and baclofen taper. *Drug and Alcohol Review*, *44*(1), 376-379.

Harjanto, R., Du Toit, A., Sperandei, S., Hallinan, R., McCaul, P., & Whitton, G. (2025). Severity and outcomes of admissions for gamma hydroxy-butyrate disorders before and after COVID-19 pandemic restrictions in South Western Sydney. *Journal of Substance Use*, *30*(1), 149-156.

Jaeger, B., & Bosch, A. M. (2016). Clinical presentation and outcome of riboflavin transporter deficiency: mini review after five years of experience. *Journal of inherited metabolic disease*, *39*, 559-564.

Johansson, J., Grönbladh, A., & Hallberg, M. (2014). Gamma-hydroxybutyrate (GHB) induces cognitive deficits and affects GABAB receptors and IGF-1 receptors in male rats. *Behavioural brain research*, *269*, 164-174.

Junnarkar, G., Allphin, C., Profant, J., Steininger, T. L., Chen, C., Zomorodi, K., Skowronski, R., & Black, J. (2022). Development of a lower-sodium oxybate formulation for the treatment of patients with narcolepsy and idiopathic hypersomnia. *Expert Opin Drug Discov*, *17*(2), 109-119. https://doi.org/10.1080/17460441.2022.1999226

Keeler, J., & Robbins, T. (2011). Translating cognition from animals to humans. *Biochemical pharmacology*, *81*(12), 1356-1366.

Klein, C., Mathis, C., Leva, G., Patte-Mensah, C., Cassel, J. C., Maitre, M., & Mensah-Nyagan, A. G. (2015). gamma-Hydroxybutyrate (Xyrem) ameliorates clinical symptoms and neuropathology in a mouse model of Alzheimer's disease. *Neurobiol Aging*, *36*(2), 832-844. https://doi.org/10.1016/j.neurobiolaging.2014.10.003

Kumar, K., Kaur, H., & Deshmukh, R. (2017). Neuroprotective role of GABAB receptor modulation against streptozotocin-induced behavioral and biochemical abnormalities in rats. *Neuroscience*, *357*, 67-74.

Laborit, H. (1964). Sodium 4-hydroxybutyrate. *International journal of neuropharmacology*, *3*(4), 433-IN438.

Leurs, U., Klein, A. B., McSpadden, E. D., Griem-Krey, N., Solbak, S. M. Ø., Houlton, J., Villumsen, I. S., Vogensen, S. B., Hamborg, L., & Gauger, S. J. (2021). GHB analogs confer neuroprotection through specific interaction with the CaMKIIα hub domain. *Proceedings of the National Academy of Sciences*, *118*(31), e2108079118.

Li, P., Shi, X., Liu, H., Feng, Y., Wang, X., Herb, M., Ji, H., Wagner, S., Vogt, J., & Prokosch, V. (2025). HOCPCA Exerts Neuroprotection on Retinal Ganglion Cells by Binding to CaMKIIα and Modulating Oxidative Stress and Neuroinflammation in Experimental Glaucoma. *Neuroscience Bulletin*, 1-18.

Lingford-Hughes, A., Patel, Y., Bowden-Jones, O., Crawford, M. J., Dargan, P. I., Gordon, F., Parrott, S., Weaver, T., & Wood, D. M. (2016). Improving GHB withdrawal with baclofen: study protocol for a feasibility study for a randomised controlled trial. *Trials*, *17*(1), 472. https://doi.org/10.1186/s13063-016-1593-9

McDonough, M., Kennedy, N., Glasper, A., & Bearn, J. (2004). Clinical features and management of gamma-hydroxybutyrate (GHB) withdrawal: a review. *Drug Alcohol Depend*, *75*(1), 3-9. https://doi.org/10.1016/j.drugalcdep.2004.01.012

Németh, Z., Kun, B., & Demetrovics, Z. (2010). The involvement of gamma-hydroxybutyrate in reported sexual assaults: a systematic review. *Journal of Psychopharmacology*, *24*(9), 1281-1287.

Neu, P., Danker-Hopfe, H., Fisher, R., & Ehlen, F. (2023). GHB: a life-threatening drug complications and outcome of GHB detoxification treatment-an observational clinical study. *Addict Sci Clin Pract*, *18*(1), 62. https://doi.org/10.1186/s13722-023-00414-w

Nyberg, F. (2000). Growth hormone in the brain: characteristics of specific brain targets for the hormone and their functional significance. *Frontiers in neuroendocrinology*, *21*(4), 330-348.

Ottani, A., Saltini, S., Bartiromo, M., Zaffe, D., Botticelli, A. R., Ferrari, A., Bertolini, A., & Genedani, S. (2003). Effect of γ-hydroxybutyrate in two rat models of focal cerebral damage. *Brain research*, *986*(1-2), 181-190.

Ottani, A., Vergoni, A. V., Saltini, S., Mioni, C., Giuliani, D., Bartiromo, M., Zaffe, D., Botticelli, A. R., Ferrari, A., & Bertolini, A. (2004). Effect of late treatment with γ-hydroxybutyrate on the histological and behavioral consequences of transient brain ischemia in the rat. *European journal of pharmacology*, *485*(1-3), 183-191.

Pedraza, C., Garcia, F. B., & Navarro, J. F. (2009). Neurotoxic effects induced by gammahydroxybutyric acid (GHB) in male rats. *Int J Neuropsychopharmacol*, *12*(9), 1165-1177. https://doi.org/10.1017/S1461145709000157

Peruzzi, F., Prisco, M., Dews, M., Salomoni, P., Grassilli, E., Romano, G., Calabretta, B., & Baserga, R. (1999). Multiple signaling pathways of the insulin-like growth factor 1 receptor in protection from apoptosis. *Molecular and cellular biology*, *19*(10), 7203-7215.

Raposo Pereira, F., McMaster, M. T. B., Polderman, N., de Vries, Y., van den Brink, W., & van Wingen, G. A. (2018). Adverse effects of GHB-induced coma on long-term memory and related brain function. *Drug and alcohol dependence*, *190*, 29-36.

Sadasivan, S., Maher, T. J., & Quang, L. S. (2006). γ-Hydroxybutyrate (GHB), γ-Butyrolactone (GBL), and 1, 4-Butanediol (1, 4-BD) Reduce the Volume of Cerebral Infarction in Rodent Transient Middle Cerebral Artery Occlusion. *Annals of the New York Academy of Sciences*, *1074*(1), 537-544.

SAMHSA, S. A. a. M. H. S. A. (2023). *Treatment Episode Data Set (TEDS) 2021: Admissions to and Discharges from Publicly Funded Substance Use Treatment*. S. A. a. M. H. S. Administration. https://www.samhsa.gov/data/sites/default/files/reports/rpt42794/2021-teds-annual-report.pdf

SAMHSA, S. A. a. M. H. S. A. (2024). *Key substance use and mental health indicators in the United States: Results from the 2023 National Survey on Drug Use and Health* (HHS Publication No. PEP24-07-021, NSDUH Series H-59). S. A. a. M. H. S. A. Center for Behavioral Health Statistics and Quality.

Silva-Araújo, E. R. d., Manhães-de-Castro, R., Pontes, P. B., Visco, D. B., Lacerda, D. C., José Cavalcanti Bezerra Gouveia, H., & Toscano, A. E. (2024). Effects of riboflavin in the treatment of brain damage caused by oxygen deprivation: an integrative systematic review. *Nutritional Neuroscience*, *27*(9), 989-1007.

Simasko, S. M., & Mukherjee, S. (2009). Novel analysis of sleep patterns in rats separates periods of vigilance cycling from long-duration wake events. *Behav Brain Res*, *196*(2), 228-236. https://doi.org/10.1016/j.bbr.2008.09.003

Sircar, R., & Basak, A. (2004). Adolescent γ-hydroxybutyric acid exposure decreases cortical N-methyl-D-aspartate receptor and impairs spatial learning. *Pharmacology Biochemistry and Behavior*, *79*(4), 701-708.

Sircar, R., Wu, L. C., Reddy, K., Sircar, D., & Basak, A. K. (2011). GHB-Induced Cognitive Deficits During Adolescence and the Role of NMDA Receptor. *Curr Neuropharmacol*, *9*(1), 240-243. https://doi.org/10.2174/157015911795017038

Sizonenko, S. V., Sirimanne, E. S., Williams, C. E., & Gluckman, P. D. (2001). Neuroprotective effects of the N-terminal tripeptide of IGF-1, glycine-proline-glutamate, in the immature rat brain after hypoxic–ischemic injury. *Brain research*, *922*(1), 42-50.

Soldin, O. P., & Mattison, D. R. (2009). Sex differences in pharmacokinetics and pharmacodynamics. *Clinical pharmacokinetics*, *48*(3), 143-157.

Strunc, M. J., Black, J., Lillaney, P., Profant, J., Mills, S., Bujanover, S., & Thorpy, M. J. (2021). The Xyrem((R)) (Sodium Oxybate) Risk Evaluation and Mitigation Strategy (REMS) Program in the USA: Results From 2016 to 2017. *Drugs Real World Outcomes*, *8*(1), 15-28. https://doi.org/10.1007/s40801-020-00223-6

Sutcliffe, J. S., Marshall, K. M., & Neill, J. C. (2007). Influence of gender on working and spatial memory in the novel object recognition task in the rat. *Behav Brain Res*, *177*(1), 117-125. https://doi.org/10.1016/j.bbr.2006.10.029

Tu, H., Xu, C., Zhang, W., Liu, Q., Rondard, P., Pin, J.-P., & Liu, J. (2010). GABAB receptor activation protects neurons from apoptosis via IGF-1 receptor transactivation. *Journal of Neuroscience*, *30*(2), 749-759.

van Nieuwenhuijzen, P. S., Long, L. E., Hunt, G. E., Arnold, J. C., & McGregor, I. S. (2010). Residual social, memory and oxytocin-related changes in rats following repeated exposure to gamma-hydroxybutyrate (GHB), 3,4-methylenedioxymethamphetamine (MDMA) or their combination. *Psychopharmacology (Berl)*, *212*(4), 663-674. https://doi.org/10.1007/s00213-010-1986-5

Vergoni, A. V., Ottani, A., Botticelli, A. R., Zaffe, D., Guano, L., Loche, A., Genedani, S., Gessa, G. L., & Bertolini, A. (2000). Neuroprotective effect of γ-hydroxybutyrate in transient global cerebral ischemia in the rat. *European journal of pharmacology*, *397*(1), 75-84.

Wendt, G., Kemmel, V., Patte-Mensah, C., Uring-Lambert, B., Eckert, A., Schmitt, M., & Mensah-Nyagan, A. (2014). Gamma-hydroxybutyrate, acting through an anti-apoptotic mechanism, protects native and amyloid-precursor-protein-transfected neuroblastoma cells against oxidative stress-induced death. *Neuroscience*, *263*, 203-215.

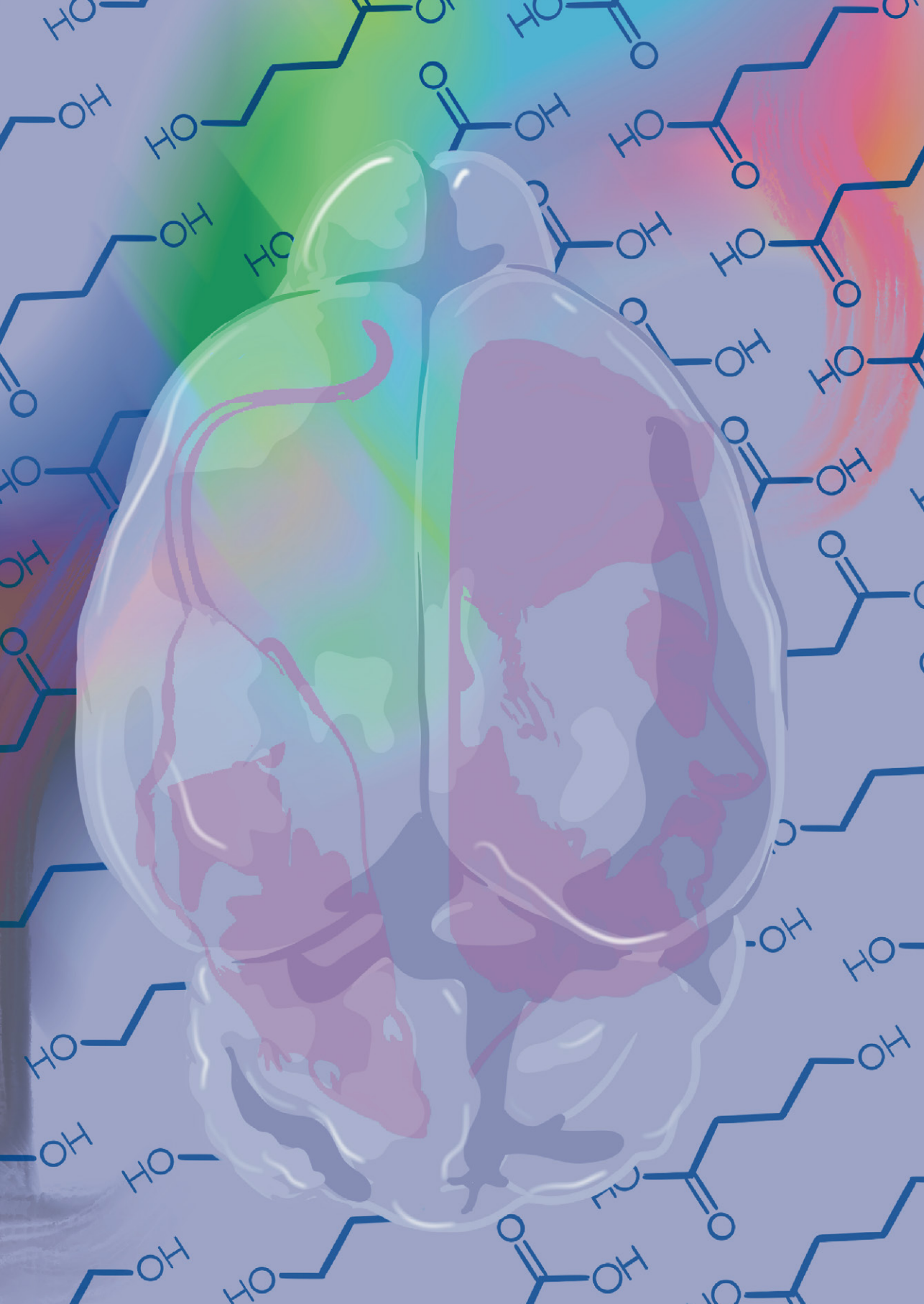
Wolf, C. J. H., Beurmanjer, H., Dijkstra, B. A. G., Geerlings, A. C., Spoelder, M., Homberg, J. R., & Schellekens, A. F. A. (2021). Characterization of the GHB Withdrawal Syndrome. *J Clin Med*, *10*(11). https://doi.org/10.3390/jcm10112333

Wood, A. M., Schellekens, A. F., Dijkstra, B. A., Wolf, C. J., & Beurmanjer, H. (2025). Development of the Gamma-hydroxybutyrate withdrawal symptom questionnaire (GWSQ). *European addiction research*.

Xyrem, U. X. M. S. G. (2002). A randomized, double blind, placebo-controlled multicenter trial comparing the effects of three doses of orally administered sodium oxybate with placebo for the treatment of narcolepsy. *Sleep*, *25*(1), 42-49.

Xyrem, U. X. M. S. G. (2003). A 12-month, open-label, multicenter extension trial of orally administered sodium oxybate for the treatment of narcolepsy. *Sleep*, *26*(1), 31-35.

Xyrem, U. X. M. S. G. (2005). A double-blind, placebo-controlled study demonstrates sodium oxybate is effective for the treatment of excessive daytime sleepiness in narcolepsy. *Journal of Clinical Sleep Medicine*, *1*(04), 391-397.



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Summary

This thesis contributes to a better understanding of the neurocognitive, pharmacological and clinical characteristics of GHB, a substance with a dual role as both a drug of abuse and a therapeutic compound. GHB has been studied across various domains, including observational cohorts, clinical trials focused on sleep regulation, and preclinical studies of its acute effects. Combining clinical observational studies, behavioral neuroscience and molecular biology, this thesis investigates the impact of GHB on cognition, neural function, and withdrawal symptoms, with a particular emphasis on patterns of use in recreational users and individuals with GHB use disorder (GUD).

In the first chapter, the introduction starts with an outline of the history of its clinical use, followed by a description of the use of GHB as a recreational drug and its associated health risks. I then zoom in on the reinforcing effects of GHB and its addictive potential, next to its pharmacological and pharmacokinetic properties. The chapter continues with an overview of the known cognitive and neurotoxic effects of GHB, while noting that its impact on cognition remains unclear.

Chapter 2 presents a detailed analysis of GHB's high-affinity targets through a bio-informatics and literature-based approach, challenging the traditional view of a single GHB receptor type. In this chapter, I focused on the structural properties of one putative subtype with complete sequence overlap with the riboflavin transporter, suggesting a dual-function structure. The implications of this overlap for the receptor's function and its potential relevance in respect to GHB's neuroprotective properties are discussed.

For many drugs of abuse, a self-administration paradigm has been established to study the drug's causal effects, which was not yet the case for GHB. In chapter 3, I describe the development and validation of a novel voluntary GHB self-administration model in rats. After establishing the optimal parameters for GHB intake, I assessed the effects of GHB self-administration on long-term memory performance. GHB use resulted in reduction of long-term memory performance in male rats, without affecting anxiety levels. In an operant setting, I observed widespread individual differences in addiction-like behaviors such as motivation, use despite negative consequences and habitual responding. Only a subset of animals displayed multiple addiction-like traits, reflecting the human situation where only a fraction of users develop GUD.

Following GHB self-administration, I did not observe characteristics of GUD for which high doses are required, such as sedation or GHB-induced comas. Therefore in chapter 4, I investigated the cognitive consequences of high-dose, GHB-induced comas.

Using a touchscreen-based task, rats were tested for working memory performance and impulsivity before and after GHB administration. Testing the animals on varying difficulties of the task did not reveal effects of GHB or GHB-induced comas on working memory performance or on proxies for impulsivity. Together with the findings from chapter 3, this suggests that GHB's effects on memory are domain-specific: working memory appears unaffected, while long-term memory is impaired.

These memory domain-dependent effects bring up the question what (regional) effects GHB exerts in the brain. Therefore in chapter 5, I quantified neuronal, GABAergic, glutamatergic and glial markers in subregions of the dorsal hippocampus of the rats that were part of the GHB self-administration study. While no differences were found between GHB and control animals, a negative correlation was found between GHB intake and neuronal cell counts in the CA1 and dentate gyrus subregions of the hippocampus, suggesting a dose-dependent effect of GHB that may have been obscured in group-level comparisons.

Chapter 6 shifts to a clinical context, characterizing the GHB withdrawal syndrome in patients undergoing detoxification with pharmaceutical GHB. Using an existing dataset, I analyzed the course of the subjective and objective withdrawal symptoms over time. Both subjective and objective withdrawal symptoms decreased over time, but some symptoms persisted after the average duration of detoxification, highlighting the importance of follow-up care. Vital signs such as heart rate and blood pressure remained stable, indicating that they are not a reliable parameter for tapering speed. Sex differences in the GHB withdrawal syndrome were clearly present: women reported more mood- and sleep-related symptoms, whereas men reported increased food craving.

In chapter 7, an overview of the thesis is presented, where I reflect on the findings and place them in the context of theories of (working) memory and addiction. I argue for a nuanced interpretation of GHB's effects, acknowledging the complex interplay between species, dose, administration route, individual susceptibility and environmental context. This thesis shows that GHB is a multifaceted compound with possible harmful properties, depending on dose and context. While GHB may hold therapeutic potential under controlled conditions, the insights presented in this thesis emphasize the potential negative cognitive- and health effects following GHB consumption.

Nederlandse samenvatting

Dit proefschrift draagt bij aan een beter begrip van de neurocognitieve, farmacologische en klinische karakteristieken van GHB, een middel dat dient als zowel een recreatieve- als medicinale drug. GHB is onderzocht in observationele studies, klinische trials gefocust op slaapregulatie, en in preklinische diermodellen die de acute effecten van GHB bestuderen. Middels een combinatie van klinische observationele studies, neurowetenschappen en moleculaire biologie onderzoekt deze thesis de impact van GHB op cognitie, de hersenen en onthouding. Hierin is een bijzondere aandacht voor het gebruikspatroon en de karakteristieken van gebruik bij zowel recreatieve gebruikers als patiënten met een stoornis in het gebruik van GHB.

Het eerste hoofdstuk, de introductie van de thesis, begint met een beschrijving van de geschiedenis van het medicinale gebruik van GHB, gevolgd door een beschrijving van GHB als recreatief middel en de geassocieerde gezondheidsrisico's. Vervolgens zoom ik in op de verslavende effecten van GHB en de farmacologische en farmacokinetische eigenschappen. Het hoofdstuk gaat verder met een overzicht van de tot zover bekende cognitieve en neurotoxische effecten, gepaard met de bevinding dat de impact van GHB op cognitie nog onduidelijk is.

Hoofdstuk 2 beschrijft een gedetailleerde analyse van de receptoren met hoge affiniteit voor GHB middels een bio-informatica en beschrijving van de huidige literatuur. Hieruit komt naar voren dat de zogenoemde "GHB-receptor" geen enkelvoudige structuur is, maar eigenlijk een groep van receptoren met hoge affiniteit voor GHB vertegenwoordigt. In hoofdstuk 2 beschrijf ik de structurele eigenschappen van een specifiek subtype van de GHB receptor die een complete aminozuur-overlap met de riboflavinetransporter heeft, wat duidt op een mogelijk dubbel-functioneel eiwit. De functionele implicaties van deze overlap worden besproken, waaronder een mogelijke rol van dit multifunctionele eiwit in neuroprotectie.

Voor veel drugs is er een diermodel ontwikkeld om de causale effecten de drug te kunnen bestuderen. Een dergelijk model is nog niet voor GHB ontwikkeld. In hoofdstuk 3 beschrijf ik de ontwikkeling en validatie van een nieuw diermodel voor vrijwillige orale zelftoediening van GHB. Na het vaststellen van de optimale parameters voor inname werden dieren getest op een taak voor lange-termijn geheugen. Mannelijke ratten vertoonden maanden na een periode van GHB inname een afname in langetermijngeheugen, terwijl er geen effect te zien was op angstniveaus. Vervolgens werden dieren getraind in een operante setting met GHB. In deze setting liet slechts een deel van de dieren verslavingsachtig gedrag liet zien,

zoals verhoogde motivatie, inname van GHB ondanks negatieve consequenties, en gewoontegedrag bij het consumeren van GHB. Deze interindividuele verschillen weerspiegelen het klinische beeld bij mensen, waarbij een minderheid van de gebruikers GUD ontwikkelt.

Wanneer ratten GHB vrijwillig consumeerden, waren er geen karakteristieken van GUD zoals sedatie en GHB-geïnduceerde coma's te zien. Daarom heb ik in hoofdstuk 4 de cognitieve gevolgen van GHB- geïnduceerde coma's onderzocht. Door middel van een touchscreentaak werden ratten getest op werkgeheugen en impulsiviteit voor en na GHB toediening. Het testen van de ratten op verschillende moeilijkheden van de touchscreentaak liet geen effect van GHB of GHB-geïnduceerde coma's zien op werkgeheugen of op indicatoren van impulsiviteit. Samen met de bevindingen uit hoofdstuk 3 suggereert dit dat GHB domeinspecifieke cognitieve effecten heeft: werkgeheugen blijkt onaangetast door GHB, terwijl lange-termijn geheugen verslechtert.

Deze domeinspecifieke cognitieve effecten roepen de vraag op welke (regionale) effecten GHB heeft in de hersenen. Daarom heb ik me in hoofdstuk 5 gericht op de neurobiologische gevolgen van GHB-gebruik. In hersenweefsel van de dieren uit hoofdstuk 3 werden markers van neuronen, GABAerge cellen, glutamarge cellen en gliacellen onderzocht. Er werden geen groepsverschillen gevonden, maar wel een negatieve correlatie tussen GHB-inname en het aantal neuronen in de CA1 en dentate gyrus subregio's van de hippocampus. Dit duidt op een dosisafhankelijk effect, welke niet zichtbaar was in de vergelijking tussen groepen.

In hoofdstuk 6 maak ik de verschuiving naar een klinische context, waar ik het onthoudingssyndroom bij mensen met GUD tijdens detoxificatie met farmaceutische GHB beschrijft. Middels een bestaande dataset volgde ik het verloop van zowel subjectieve als objectieve onthoudingssymptomen. Deze symptomen namen af in ernst over tijd, maar een deel van de symptomen bleef aanwezig na detoxificatie. De aanwezigheid van onthoudingssymptomen na detoxificatie kan het risico op terugval verhogen. Vitale parameters bleven stabiel gedurende de detoxificatie, en bleken daarmee ongeschikt als indicator voor afbouwsnelheid. Er werden ook sekseverschillen gevonden in GHB onthouding: vrouwen rapporteerden meer stemming- en slaapgerelateerde symptomen, terwijl mannen vaker honger ervaarden.

In hoofdstuk 7 worden de bevindingen van dit proefschrift besproken in het licht van huidige geheugentheorieën en verslavingsmechanismen. Ik pleit voor een genuanceerde benadering van GHB, waarbij rekening wordt gehouden met de invloed

van (dier)soort, dosering, manier van toediening, gebruikscontext en individuele kwetsbaarheid. Dit proefschrift laat zien dat GHB een veelzijdig molecuul is met mogelijk schadelijke eigenschappen, afhankelijk van de dosis en context. Hoewel GHB voor sommige aandoeningen gebruikt kan worden als medicijn, benadrukken de bevindingen in deze thesis de mogelijke negatieve effecten op cognitie en gezondheid door GHB consumptie.

Acknowledgements

Het zit er nu echt op. 6,5 jaar na mijn eerste werkdag van mijn eerste baan. Als een enthousiaste, positieve en ietwat naieve ex-student startte ik met mijn PhD. Een periode waarin ik vooral de eerste drie jaar met veel plezier heb ervaren, waarna toch ook tegenvallende resultaten, onrealistische ambities, en dringende tijdslijnen zijn weerslag op mijn enthousiasme begonnen te vinden. Na afloop van mijn contract heb ik, parallel aan mijn baan bij Zilveren Kruis, mijn proefschrift in mijn eigen tempo afgerond. En ik kan er nu eindelijk een streep onder zetten. Want dat heeft een periode buiten de academie me ook geleerd: af en toe wat pragmatischer zijn, waar goed ook goed genoeg is. Het was een mooi avontuur, wat ik niet had willen missen.

Het doen van een PhD heeft me naast veel vaardigheden, ook veel mooie interacties en vriendschappen opgeleverd. Een PhD-traject is in vele opzichten een solistisch, eenzaam pad dat bewandeld wordt. Maar zo'n traject wordt verrijkt en gekleurd door de mensen die je ontmoet, waarmee je samenwerkt, en waarmee je samen vreugde en smart deelt. Daarom wil ik iedereen die mijn periode als promovendus kleur heeft gegeven, en mij heeft laten groeien op zowel professioneel als persoonlijk vlak, hieronder graag bedanken.

Allereerst wil ik mijn promotoren Judith en Arnt bedanken. Een samenwerking tussen afdelingen en disciplines leidde tot een beurs voor mijn PhD-traject. Al tijdens de sollicitatie zag ik jullie kwaliteiten en de eerste tekenen van jullie manier van leidinggeven terug. Arnt, jij had al heel snel door waar mijn sterke en zwakke punten lagen, en liet mij deze op een natuurlijke manier ook zelf ontdekken. Je kon met één zin een gedachtegang van mij doorbreken, om me op het juiste pad te zetten. Ondanks jouw vele bezigheden en taken had ik altijd het gevoel dat ik bij je terecht kon wanneer dat nodig was. Judith, gedurende het grootste deel van mijn PhD maakte ik fysiek deel uit van jouw onderzoeksgroep. Een groep met vele verschillende onderzoeksrichtingen, achtergronden en persoonlijkheden. Je stond altijd voor me klaar met concrete feedback, maar gaf me ook op basis van vertrouwen de ruimte die ik nodig had. Ik had door jullie altijd een enorme schat aan kennis en ervaring naast me waar ik op terug kon vallen. Jullie stonden altijd voor me klaar met feedback, wisten me te motiveren op de juiste momenten en hielpen mij met jullie positieve stijl van leidinggeven en samenwerking mij optimaal floreren in de wetenschap. Dank voor alle kansen en mogelijkheden die jullie me hebben geboden.

Uiteraard wil ik ook de andere helft van mijn team bedanken: mijn co-promoteren Harmen en Marcia. Harmen, ik waardeer het enorm dat je continue de klinische

focus en de patiënt centraal stelde in de afgelopen 6,5 jaar. Jouw klinische kennis en kunde heeft mij ook anders leren denken en kijken naar het onderzoek. Daarnaast ben je ook op het vlak van persoonlijke ontwikkeling een mentor voor me geweest, waar je altijd een luisterend oor bood, ruimte bood voor een gesprek naast de wetenschappelijke inhoud, en waar je ook vooral oog voor mij, mijn belangen en ontwikkeling had. Marcia, in de eerste jaren heb ik enorm veel praktische en pre-klinische kennis van jou opgedaan. Maar ook jij liet me inzien dat er meer was dan wetenschap alleen, en leerde me om te relativeren. Ook al scheidde onze wegen op het Donders na enkele jaren, hebben we een basis gelegd waar ik mijn hele PhD profijt van heb gehad. Beiden hebben jullie, net als ik, een eigen pad bewandeld. Een pad welke het beste voelt, en daarmee waarschijnlijk ook het beste is.

Ik wil daarnaast graag de leden van de manuscriptcommissie bedanken: prof. Dr. Robert-Jan Verkes, prof. Dr. Taco de Vries en dr. Heidi Lesscher. Als examinatoren van de scripties van mijn studenten zijn jullie een deel van mijn werk al eerder tegengekomen, en ditmaal mochten jullie alles in samenhang lezen. Veel dank dat jullie de tijd en moeite hebben genomen om mijn volledige manuscript te lezen, en ook goed te keuren.

Al na drie maanden na de start van mijn PhD kreeg ik de rol van “dagelijks begeleider” toebedeeld, om de Master-stage van Izel te begeleiden. En deze rol heb ik gedurende mijn hele PhD mogen vervullen voor een groep enorm talentvolle, gedreven en intellectuele (ex-)studenten. Adina, Annelot, Anouk, Arawa, Claire, Eva, Izel, Jesse, Nele, Rhanna: jullie zijn van onschatbare waarde geweest in de projecten die mijn PhD vormden. Ik heb met enorm veel plezier met jullie samengewerkt, en jullie hebben mij ook laten groeien als begeleider en wetenschapper. Ik heb jullie hopelijk mogen inspireren om jullie eigen pad te vervolgen, voor sommigen van jullie binnen de academische wereld, en voor sommigen van jullie erbuiten. Ik weet zeker dat jullie er iets moois van gaan maken.

I would also like to thank all my fellow PhD-candidates from the Homberg lab and fellow CNS-labs for the scientific discussions and support. Roel, Alex, Helen, Nina, Henrik, Angelos, you all made the PhD “flex-room” feel like a “non-flex room”. An actual office with dear colleagues with whom I could chat, laugh, and have traditional office banter.

And when I crossed the campus, entered the hospital and took some flights of stairs down to the basement, I would enter my “other” office, the kelderkamer. Filled with both NISPA-colleagues, and other PhD candidates from the Department of

Psychiatry, I came here to write a METC application, work on one of the clinical projects or simply hang out with a great group of colleagues. Kelderkamer-colleagues, thank you for the fun times, the drinks we had after work and collectively eating the enormous amount of snacks in the local snack box.

Maar ook op andere locaties op de campus kwam ik NISPA collega's tegen. Boukje, Esther, Ilse, Louise, Anneleen, Sanne, Wiebren, Stijn, Arno, dank voor de leuke discussies tijdens NISPA forum, de gezellige etentjes bij Arnt thuis, en natuurlijk de memorabele retraites naar het Dominicanenklooster in Huissen. Het was altijd een klein feestje om daar met elkaar te ontbijten, lunchen, avondeten, schrijven en natuurlijk borrelen. En natuurlijk Elise en ons avontuur in Mannheim, waar we een paar hele gezellige dagen op de ECNP hebben gehad! En Amber, mijn mede GHB-lotgenoot, die het GHB-stokje zonder moeite overnam en een prachtig project aan het neerzetten is. Dank voor de samenwerking met het Novadic-Radboud project, en bedankt voor de gezellige momenten in Vught, Nijmegen en op de tennisbaan!

Halfway through my PhD I got the opportunity to spend one month at an Italian research facility to exchange knowledge on the neurobiology of addiction. I went to visit the research group of Guido Mannaioni and Alessio Masi at the University of Florence. Similar to the Radboud, the University of Florence has a strong link between the hospital and the (fundamental) research departments. Despite not speaking the language and only being in Florence for one month, I was kindheartedly welcomed by my new colleagues, and felt welcome from the moment I stepped into the Cubo research building. This set the foundation for an exciting exploration into the neuroprotective effects and interactions with GABAergic and GHB-receptor-related compounds, hopefully leading to a nice publication. Elisabetta, Guido, Alessio, but also Beppe, Lorenzo, Martina, Federica, Simone, Christiana, Antonino, thank you for your warmth, kindness, scientific insights and incredible Italian food.

Uiteraard ook veel dank aan de rotsen in de branding, degenen die altijd klaar stonden wanneer er iets opgelost moet worden. Michel, Sjef, Anthonieke en Ilse, wat enorm fijn dat ik de afgelopen jaren op jullie kon bouwen. Jullie stonden altijd voor me klaar als er iets mis was met de apparatuur, er iets geregeld moest worden op de afdeling of als ik een vraag had in het lab. Jullie zijn de ruggengraat van TNU en CNS, met een nuchtere kijk op zaken en altijd in voor een leuk gesprek of een grappig verhaal.

Daarnaast zijn er nog veel meer Radboud-collega's die mij hebben geholpen zowel binnen als buiten het lab. De medewerkers van het CDL, en met name Stef, Tim, Karin, Stephanie, Helma en Mayke, dank voor jullie extreem goede zorg voor de

dieren. Dierenwelzijn is in dieronderzoek het allerbelangrijkst, en jullie zorgen ervoor dat alle dieren een zo goed mogelijk leven hebben. En daarnaast wil ik jullie ook bedanken voor al het werk dat jullie hebben gedaan en de vaardigheden die jullie mij hebben aangeleerd. Er werd/wordt soms veel van jullie gevraagd, maar jullie werk wordt enorm gewaardeerd.

Naast alle fijne collega's die heb ik leren kennen vanaf 2019, heb ik ook veel enorm veel steun, enthousiasme en blijdschap gevoeld van mijn vrienden. Dewi, Martin, Kari, Maxime, Anneloes, beter bekend als lunch club aka coffee club (and tea), thanks for the countless hours of lunch, coffee breaks, pool nights at Piet Houseman and several hours of online D&D. Sharing laughter and pain, we were there for each other when it counted. Thank you for adopting me in your post-CNS group, a group where all our different characters formed a great synergy. I will treasure all the beers and breaks we shared, and it's a nice feeling to know that I always have friends in/nearby Nijmegen.

Hannah en Lisanne, de grootste reden waarom ik gedurende mijn PhD ook steeds meer in kelderkamer te vinden was. Lief en leed delen, en over alles behalve wetenschap praten. Jullie hebben de cacao bars in mijn leven geherintroduceerd, en hielden me draaiende met de snackbox en morele steun. Dankjewel voor het zo veel leuker maken van mijn PhD, het was altijd fijn om jullie te zien zitten als ik de kelderkamer binnenliep. Hopelijk blijven we elkaar zien om nog veel wijntjes met elkaar te drinken.

Alejandro, Carolina, Mariana, Nishant, Raimon, Helena, Tea, Greg, Fernando, what a great group of people you guys are. You bring such positivity and energy that makes every person smile. And you definitely made me smile countless of times. An absolute highlight of my life was to travel to Mexico together, and I immensely enjoyed all the late dinners, movie nights, drinks and church parties. I'm proud that I was/am your "Dutch" friend, and gracias for teaching me the great latino culture.

Ten slotte heb ik ook enorm veel steun en liefde gevoeld van mijn familie. Pap, mam, jullie hebben me vanaf de basisschool gestimuleerd, gemotiveerd en me trots laten voelen op mijn prestaties. Jullie hebben me, toen ik van de middelbare school in Amersfoort uitvloog naar de universiteit in Amsterdam, geholpen in mijn keuze voor biomedische wetenschappen. Achteraf was dit een keuze die, gezien alles wat er gebeurd is, niet beter kon. Sindsdien hebben jullie me volledig vrij gelaten en gestimuleerd om mijn eigen pad te zoeken en mijn potentie te benutten, zonder mij de waarde van goed-bestede vrije tijd uit het oog te laten verliezen. Ik kon dit niet

zonder de liefde die ik altijd van jullie heb gevoeld. En ook opa Henk, oma Els en opa Koos, mijn grootste supporters, waaraan ik zoveel mooie herinneringen heb en mooie herinneringen mee aan het maken ben, bedankt voor jullie steun en liefde. Imke, mijn lieve zus en die me qua denken en doen zo dicht bij me staat. Een voorbeeld waar ik me altijd aan kon optrekken, in alle manieren die je kan bedenken. Jelle, dank voor je relativerende blik en de rust die je deelt, want een stukje relativering en rust is zeker nodig om een promotietraject tot een gezond einde te brengen. En natuurlijk mijn lieve schoonfamilie: Emmy, Fred, Siem, Esmee, Ruud, Greet, wat fijn dat jullie via Elise ook in mijn leven zijn gekomen. Met jullie betrokkenheid en nieuwsgierigheid hebben jullie ook bijgedragen aan de totstandkoming van dit proefschrift.

En dan de liefde van mijn leven, lieve Elise. Door dik en dun stond/zat/lag je naast me, heb je alle mooie en minder mooie momenten van deze hindernisbaan wat de PhD was met me meegemaakt. Een luisterend oor, een motiverend woord, een confettikanon, je was er altijd voor me. En tijdens mijn PhD een heel leven samen opgebouwd, samenwonen, een huis kopen, trouwen en dan in mei of juni ons eerste kindje. Als ik zie en stilsta bij wat wij hebben, heb ik geen spijt, van niks. Want alle beslissingen die ik heb gemaakt, groot of klein, hebben me gebracht waar ik nu ben en waar ik het liefste wil zijn: naast jou!

Curriculum Vitae

Casper Jacob Hendrik Wolf received his Bachelor's degree in Biomedical Sciences in 2016 from the Vrije Universiteit, Amsterdam. During the bachelor, he completed a clinical internship at the Department of Radiology at the Leiden University Medical Center, focusing on a novel magnetic resonance technique to register brain activity through the detection of changes in creatine kinase cycle rate. Casper received his Master's degree in Neurobiology in 2019 from the Universiteit van Amsterdam. As a part of this Master's program, he followed two internships. One was performed at the Netherlands Insititute for Neuroscience at the group of Ingo Willuhn and Damiaan Denys, where he examined the contribution of different rat brain regions to compulsive behavior. In addition, he contributed to the validation of an automated classification tool to quantify grooming behavior in mice. For his second internship, he joined the lab of Carrie Ferrario at the Department of Pharmacology at the University of Michigan, MI, USA. Here, he performed a project on the effect of junk food in obesity-prone and obesity resistant rats on cognitive flexibility. In addition, he examined the effects of micro-injections of insulin in the nucleus accumbens on a Pavlovian learning paradigm.

In 2019, Casper started his PhD at the Donders Institute under the supervision of Judith Homberg and Arnt Schellekens. His doctoral research focused on the cognitive effects of GHB use and GHB use disorder. He aimed to employ a translational approach across two departments to answer clinical questions through an (animal-) experimental approach. During his PhD, Casper supervised 11 Master's and Bachelor students, guiding them through experimental- or data-analysis projects varying from 3 to 12 months. He also attended several national and international conferences, presenting his work through a poster- or oral presentation. Triggered by the joy of performing multi-faceted data-analysis, combined with the ambition to retain the link to psychiatry and mental health care, Casper started as a Data Analist for the Mental Health Care (GGZ) division of health care insurance company Zilveren Kruis in 2024, where he works to date.

List of publications

First author

Wolf, C.J.H., Beurmanjer, H., Dijkstra, B. A., Geerlings, A. C., Spoelder, M., Homberg, J. R., & Schellekens, A. F. (2021). Characterization of the GHB withdrawal syndrome. *Journal of Clinical Medicine*, *10*(11), 2333.

Wolf, C.J.H., Spoelder, M., Beurmanjer, H., Bulthuis, R., Schellekens, A. F., & Homberg, J. R. (2024). Individual differences in GHB consumption in a new voluntary GHB self-administration model in outbred rats. *Psychopharmacology*, *241*(3), 613-625.

Wolf, C.J.H., Venselaar, H., Spoelder, M., Beurmanjer, H., Schellekens, A. F., & Homberg, J. R. (2023). An overview of the putative structural and functional properties of the GHBh1 receptor through a bioinformatics approach. *Life*, *13*(4), 926.

Co-author

van den Boom, B. J., Pavlidi, P., Wolf, C.J.H., Mooij, A. H., & Willuhn, I. (2017). Automated classification of self-grooming in mice using open-source software. *Journal of neuroscience methods*, *289*, 48-56.

López-Toro, E., Wolf, C.J.H., González, R. A., van den Brink, W., Schellekens, A.F.A., Vélez-Pastrana, M. C., & AFP Working Collaborative Group. (2022). Network analysis of DSM symptoms of substance use disorders and frequently co-occurring mental disorders in patients with substance use disorder who seek treatment. *Journal of clinical medicine*, *11*(10), 2883.

Goudriaan, A.E., Crunelle, C.L., Wolf, C.J.H., & Schellekens, A. F. A. (2023). Neurale netwerken bij verslaving. *Tijdschrift voor Psychiatrie*, (2023/10), 609-612.

Wood, A. M., Schellekens, A.F.A., Dijkstra, B.A., Wolf, C.J.H., & Beurmanjer, H. (2025). Development of the Gamma-hydroxybutyrate withdrawal symptom questionnaire (GWSQ). *European Addiction Research*.

Submitted manuscripts

Wolf, C.J.H., Spoelder, M., Beurmanjer, H., Schellekens, A.F.A., & Homberg, J.R. - The short- and long-term effects of GHB use and GHB-induced comas on working memory and impulsivity in outbred rats

Research Data Management

This thesis research has been carried out under the research data management policy of the Donders Institute for Brain, Cognition and Behavior. This research followed the applicable laws and ethical guidelines. Research Data Management was performed according to the FAIR principles. The information below details how this was achieved.

Ethical Approval

This thesis is based on the results of animal studies, which were conducted in accordance with the European, Dutch and local regulations on the basis of the DEC Projects 2019-0024. The local Animal Welfare Body has approved the protocols for the present project 2019-0024-001, 2019-0024-002, 2019-0024-003, 2019-0024-004, 2019-0024-005 and 2019-0024-006. Chapters that did not involve animal experiments, where based on existing data. This research was funded by the Radboudumc Junior Researcher Grant 2019, which did not entail any financial interests of conflict.

Data collection and storage

Data for chapters 3, 4 and 5 were obtained from experiments involving animals. Raw and processed data and documentation are archived in a Data Sharing Collection (DSC) and a Research Documentation Collection (RDC) in the Radboud Data Repository. These secure storage options safeguard the availability, integrity and confidentiality of the data.

Data sharing according to the FAIR principles

All studies that are published have been published with open access. Chapter 5 is not published.

Chapter 4 has been submitted for publication and is currently under review.

Findability and Accessibility

The dataset from chapters 3, 4 and 5 are published in the Radboud Data Repository, with collection identifiers di.dcmn.DSC_fmmugnc_t0000517a_527 and di.dcmn.RDC_fmmugnc_t0000517a_411. Requests for access will be checked by the PI and the data steward of the department. Chapter 6 is based on existing data, which was obtained from NISPA.

Interoperability and Reusability

The data used for chapter 6 are not owned by Radboudumc. The data are archived by NISPA. Questions about the data can be addressed to sanne.heijnen-kamps@ru.nl.

The data used in the unpublished chapters 4 and 5 are archived with closed access via the Radboud Data Repository. Upon publication of the chapters, the data will be archived with open access.

The table below details where the data and research documentation for each chapter can be found on the Radboud Data Repository (RDR). All data archived as a Data Sharing Collection remain available for at least 10 years after termination of the studies.

Chapter	RDR - RDC	RDR - DSC	NAME DATA REPOSITORY	License or Data Use Agreement (DUA)
3		DOI: di.dcmn. DSC_fmmugnc_ t0000517a_527	From Molecule to Mind: Unraveling GHB and its neurocognitive consequences	RUMC-RA-DUA-1.0
4	DOI: di.dcmn. RDC_fmmugnc_ t0000517a_411		From Molecule to Mind: Unraveling GHB and its neurocognitive consequences	RUMC-RA-DUA-1.0
5	DOI: di.dcmn. RDC_fmmugnc_ t0000517a_411		From Molecule to Mind: Unraveling GHB and its neurocognitive consequences	RUMC-RA-DUA-1.0

RDR = Radboud Data Repository, RDC = Research Documentation Collection, DSC = Data Sharing Collection

For all data in these repositories long-lived file formats have been used, ensuring that data remains usable in the future. All data collections have been structured in a standardized way that is described in accompanying text files.

Donders Graduate School

For a successful research Institute, it is vital to train the next generation of scientists. To achieve this goal, the Donders Institute for Brain, Cognition and Behaviour established the Donders Graduate School in 2009. The mission of the Donders Graduate School is to guide our graduates to become skilled academics who are equipped for a wide range of professions. To achieve this, we do our utmost to ensure that our PhD candidates receive support and supervision of the highest quality.

Since 2009, the Donders Graduate School has grown into a vibrant community of highly talented national and international PhD candidates, with over 500 PhD candidates enrolled. Their backgrounds cover a wide range of disciplines, from physics to psychology, medicine to psycholinguistics, and biology to artificial intelligence. Similarly, their interdisciplinary research covers genetic, molecular, and cellular processes at one end and computational, system-level neuroscience with cognitive and behavioural analysis at the other end. We ask all PhD candidates within the Donders Graduate School to publish their PhD thesis in de Donders Thesis Series. This series currently includes over 750 PhD theses from our PhD graduates and thereby provides a comprehensive overview of the diverse types of research performed at the Donders Institute. A complete overview of the Donders Thesis Series can be found on our website: https://www.ru.nl/donders/donders-series

The Donders Graduate School tracks the careers of our PhD graduates carefully. In general, the PhD graduates end up at high-quality positions in different sectors, for a complete overview see https://www.ru.nl/donders/destination-our-former-phd. A large proportion of our PhD alumni continue in academia (>50%). Most of them first work as a postdoc before growing into more senior research positions. They work at top institutes worldwide, such as University of Oxford, University of Cambridge, Stanford University, Princeton University, UCL London, MPI Leipzig, Karolinska Institute, UC Berkeley, EPFL Lausanne, and many others. In addition, a large group of PhD graduates continue in clinical positions, sometimes combining it with academic research. Clinical positions can be divided into medical doctors, for instance, in genetics, geriatrics, psychiatry, or neurology, and in psychologists, for instance as healthcare psychologist, clinical neuropsychologist, or clinical psychologist. Furthermore, there are PhD graduates who continue to work as researchers outside academia, for instance at non-profit or government organizations, or in pharmaceutical companies. There are also PhD graduates who work in education, such as teachers in high school, or as lecturers in higher education. Others continue in a wide range of positions, such as policy advisors, project managers, consultants,

data scientists, web- or software developers, business owners, regulatory affairs specialists, engineers, managers, or IT architects. As such, the career paths of Donders PhD graduates span a broad range of sectors and professions, but the common factor is that they almost all have become successful professionals.

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