The Short- and Long-Term Impact of Gamma–Hydroxybutyrate (GHB)-Induced Comas on Cognition in a Rat Model

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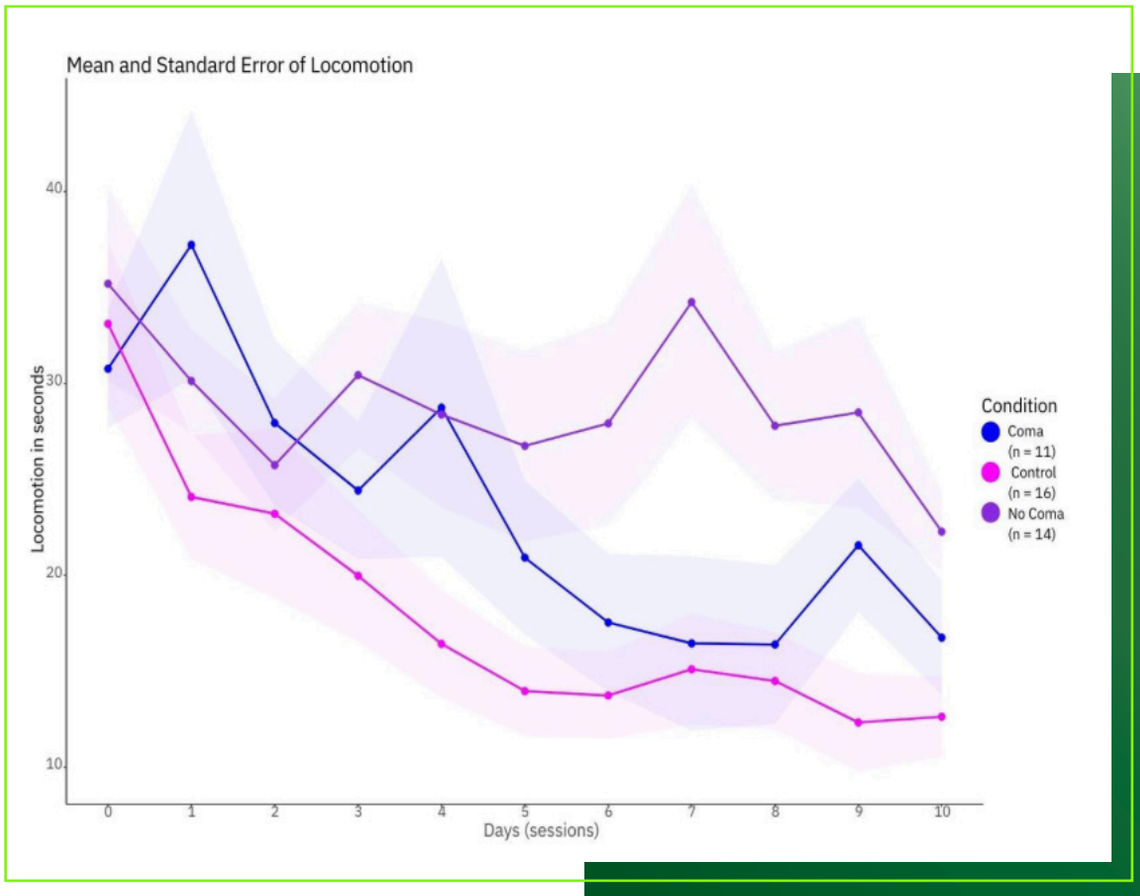
Mean and Standard Error of Locomotion
Locomotion in seconds
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
Days (sessions)
Condition
Coma
(n = 11)
Control
(n = 16)
No Coma
(n = 14)

Abstract

Gamma-Hydroxybutyrate (GHB), a substance with both therapeutic applications and potential for abuse, has raised concerns due to increased recreational use over the past two decades and the occurrence of GHB-induced comas. The absence of causal studies on GHB impact on cognition, particularly affecting memory and learning, underscores the need for further research. This study investigates the short and long-term effects of GHB on cognition using a rat model, with a focus on visuospatial working memory and impulse control. Utilizing a longitudinal design, rats were subjected to varying doses of GHB to induce comas and assess their cognitive functions through the Trial-Unique Non-Matching to Location task. Behavioral changes during GHB intoxication such as locomotion, rearing, and grooming were automatically measured using a sensor-based setup. The results indicate dose-dependent behavioral alterations during GHB intoxication and long-term cognitive effects. Specifically, animals who received coma-inducing doses of GHB exhibited reduced impulse control following GHB exposure, while animals that received low doses of GHB showed improved visuospatial working memory performance. These findings contribute to the understanding of GHB's cognitive impact, yet they contrast with previous research, highlighting the need for further investigation into the cognitive effects of GHB-induced comas.

Keywords: Addiction; Gamma-Hydroxybutyrate; GHB use disorder; Working Memory; Cognition;

1 Introduction

Gamma-hydroxybutyrate (GHB), a psychoactive substance employed therapeutically for narcolepsy and alcohol addiction, has become a widely abused recreational drug over the past decade (Giorgetti et al., 2022). In the last century, GHB was widely used as an anesthetic, but its current utilization for anaesthesia is now restricted to specific countries (Felmlee et al., 2021). GHB has also been marketed as a growth hormone enhancer, sleep aid, and dietary supplement (Giorgetti et al., 2022). Nowadays, GHB is used as a medication to treat narcolepsy and cataplexy in several countries. Furthermore, GHB is used as a treatment for alcohol addiction in Italy and Austria. However, this application remains a subject of controversy, given the addictive properties associated with GHB (Brunt et al., 2021).

Despite its therapeutic potential, GHB has gained popularity as a recreational drug due to its euphoric and sedative effects. Studies have shown that approximately 0.1% to 1.3% of adults in Western countries, engage in recreational GHB use (Brunt et al., 2021). Certain population subgroups, such as men who have sex with men (MSM), report even higher estimates of up to 5% (Tay et al., 2022). Repeated use can lead to GHB substance use disorder (GUD), as classified by the Diagnostic and Statistical Manual of Mental Disorders (DSM-5). GUD is marked by continued consumption and craving for the drug despite negative outcomes, including high relapse rates and severe withdrawal symptoms, which challenge the effective management of the addiction (American Psychiatric Association, 2013).

GHB withdrawal, defined as prolonged abstinence from GHB after its repeated use, presents challenges in managing GHB addiction effectively due to high relapse rates. GHB withdrawal syndrome includes severe symptoms, such as epileptic seizures and delirium upon sudden abstinence for patients with a dependence on GHB (Wolf et al., 2021). Inpatient treatment is often utilized to manage withdrawal symptoms associated with GHB addiction, yet approximately 50% of patients relapse within three months after detoxification and successful completion of treatment (Dijkstra et al., 2021). Contributing factors to these relapse rates include the positive attitude towards GHB from GHB users and the long-lasting withdrawal symptoms post-detox. The positive attitude towards GHB compared to other illicit drugs can lead to an underestimation of its addictive potential and associated risks. This perception and the long-lasting withdrawal symptoms can result in complacency and increased relapse risk, perpetuating the cycle of addiction (Beurmanjer et al., 2019; Wolf et al., 2021).

Consumption of high doses of GHB can lead to sedation, respiratory depression, or even coma, affecting both recreational users and patients with GUD. It has been observed that some patients with GUD do not consider GHB-induced comas as unpleasant experiences. This lack of aversion to comas, despite their severe implications, may further fuel the inclination to engage in reckless GHB consumption (Beurmanjer et al., 2019). The absence of residual negative effects after regaining consciousness may contribute to the false belief that these comas are harmless, encouraging continued

recreational use despite the inherent dangers (Dijkstra et al., 2021). GHB was reported as one of the primary drugs seen in hospital emergency departments in Europe in 2017 due to severe withdrawal symptoms and GHB-induced comas (Dijkstra et al., 2021; Kamal et al., 2016).

1.1 Decoding the Mechanisms of Action of GHB

GHB is a short-chain fatty acid, functioning both as a naturally occurring neurotransmitter and neuromodulator, implicated in various central nervous system activities, from energy homeostasis to neuroprotection and neurotoxicity (Felmlee et al., 2021; Wolf et al., 2023). Understanding GHB's pharmacological actions is essential for comprehensively addressing GUD and GHB withdrawal syndrome. This knowledge is crucial for developing effective treatment strategies and informing policy decisions, aiming to maximize therapeutic benefits and minimize potential harm. Its pharmacological significance stems from its dual role in therapeutic applications for conditions like narcolepsy and its abuse potential, facilitated by its dose-dependent euphoric, disinhibiting, and sedative properties. GHB's interactions within the brain's neurochemical framework are intricate, as it directly binds to GABA-B receptors and several GHB-specific receptors, thereby eliciting its sedative and euphoric effects. GHB also serves as both a precursor and metabolite of GABA, the brain's primary inhibitory neurotransmitter (Felmlee et al., 2021). Additionally, a specific subtype of the rat GHB receptor is primarily located in the hippocampus, frontal cortex, piriform cortex, and cerebellum, with lower expression in the striatum, olfactory bulb, and thalamus. This distribution highlights the receptor's role in modulating glutamatergic signaling, further complicating GHB's impact on the central nervous system and its pharmacological profile (Kamal et al., 2016; Wolf et al., 2023). Beyond direct interactions, GHB also indirectly influences neurotransmitters and hormones like adrenaline, growth hormones, neurosteroids, oxytocin, serotonin, and opioids, contributing to the rewarding properties and attractiveness of GHB for abuse (Kamal et al., 2016).

Recent findings have emphasized the existence of multiple GHB receptor subtypes, including the GHBh1 receptor, which shares a 100% amino acid sequence overlap with the riboflavin transporter, suggesting a dual-function (transceptor) structure (Wolf et al., 2021). This unique overlap points to a broader functional scope of GHB beyond its known pharmacological effects, potentially involving mechanisms of neuroprotection under energy-deprived conditions like ischemia, paralleled by riboflavin's similar protective roles (H. Wu et al., 2018). Moreover, the dose-dependent activation of the GABA-B receptor by exogenous GHB contrasts with the activation of GHB receptors by endogenous low doses, which increase glutamatergic signaling, highlighting the intricate balance GHB maintains between its therapeutic and addictive potentials. The GHBh1 receptor's structural analysis, indicating 11 transmembrane units and at least one intrinsically disordered region (IDR), further complicates the understanding of GHB's interaction with the central nervous system (Wolf et al., 2023).

The high affinity of GHB for the GHBh1 receptor, combined with the receptor's rapid and prolonged desensitization, suggests that GHBh1 is unlikely to be involved in the GABAergic response to high exogenous doses of GHB typically seen in GUD, including comas, severe withdrawal symptoms, or high relapse rates. However, GHBh1 may play a role in the cellular effects of exogenous GHB, with conflicting evidence regarding its impact on neuronal health and cognitive functions. While some studies indicate GHB use leads to neuronal damage and cognitive deterioration (F. Raposo Pereira et al., 2018a F. Raposo Pereira et al., 2018b), GHB is also safely used for treating narcolepsy and has been tested for alcohol use disorder treatment, indicating a complex interaction between GHB, GHBh1 receptor activation, and cellular effects that requires further investigation (Wolf et al., 2023).

1.2 Protective or Destructive: The Duality of the Effects of GHB on the Brain

Research has shown that GHB has neuroprotective and neurotoxic properties, which are suggested to be dose-dependent. GHB may exert beneficial neuroprotective effects at low doses on the brain, whereas higher doses or chronic abuse of GHB can lead to neurotoxicity and cognitive impairments (Beurmanjer et al., 2022; Brunt et al., 2021). This underscores the importance of investigating the long-term effects of GHB use on cognition and neurotoxicity. Understanding the potential dual nature of the effects of GHB on the brain is crucial for evaluating its overall impact and determining safe usage guidelines. The following paragraphs will explain neuroprotection and neurotoxic evidence on GHB use, respectively.

1.3 Safeguarding the Brain: The Neuroprotective Potential of GHB

GHB has neuroprotective effects at low doses (van Amsterdam et al., 2012; **vanLaar2012**). Neuroprotection, defined as the ability to prevent or reduce damage to the nervous system, is a critical aspect of the pharmacological profile of GHB. This neuroprotective capacity becomes particularly evident in scenarios involving neurotoxic events, such as cerebral ischemia, neurodegeneration, and cell death (Vergoni et al., 2000; Wendt et al., 2014). For example, in experimental conditions mimicking cerebral ischemia, GHB is beneficial. Administering GHB two hours after an ischemic insult in rats has been shown to reduce sensory-motor, learning, and memory impairments. Moreover, it reduces hippocampal damage caused by transient global brain ischemia (Vergoni et al., 2000). Besides cerebral ischemia, GHB exhibits promise in protecting against neurodegeneration and cell death. A study by Wendt et al. (2014) demonstrated the cytoprotective effects of GHB against hydrogen peroxide-induced cell death in human neuroblastoma cells. The protective action extended to both normal and genetically modified cells, indicating a broad applicability of GHB in mitigating apoptotic markers and apoptosis induced by oxidative stress.

While the precise mechanisms underlying the neuroprotective effects of GHB are still under investigation (Leurs et al., 2021; **wolf2023**), some potential pathways have been identified. The impact

of GHB on energy metabolism, oxygen radical scavenging, and modulation of neurotransmitters through GABAb receptors are notable contributors to its neuroprotective properties. GHB appears to influence energy metabolism and oxygen radical scavenging, reducing the utilization of cerebral glucose and high-energy phosphates. This decrease in energy demand helps protect neurons from damage and maintain their overall function (MacMillan, 1978; Wolfson et al., 1977). Additionally, the ability of GHB to reduce cellular metabolism decreases oxygen demand in cerebral tissue, crucial in mitigating damage from oxygen-derived free radicals generated during reperfusion in ischemic tissue (MacMillan, 1978; Ottani et al., 2003; Wolfson et al., 1977).

The recent works of Wolf et al. (2023) and Leurs et al. (2021) provide additional insights into the potential mechanisms through which GHB exerts its neuroprotective effects. Wolf et al. (2023) offer a bioinformatics perspective, shedding light on the putative structural and functional properties of the GHBh1 receptor subtype. The GHBh1 receptor, characterized by 11 transmembrane helices and an intracellular intrinsically disordered region (IDR), exhibits a 100% amino acid sequence overlap with the Riboflavin transporter. This suggests a possible dual-function (transceptor) structure, opening avenues for understanding the neuroprotective properties of GHB through receptor interactions. Leurs et al. (2021), on the other hand, unravel the neuroprotective potential of GHB analogs through specific interaction with the CaMKIIα hub domain. The study identifies CaMKIIα, a key neuronal signaling protein, as the high-affinity target for GHB analogs. Through selective binding within the central hub domain, GHB analogs stabilize the hub oligomer complex, providing sustained neuroprotection. This finding underscores the potential of GHB as a selective and high-affinity therapeutic candidate for conditions involving CaMKIIα activation, such as cerebral ischemia.

The interaction of GHB with GABAb receptors and its modulation of excitatory amino acids, such as glutamate, play a crucial role in the context of ischemia-induced neuronal damage. Through GABAb receptor activation and the augmentation of potassium conductance, GHB contributes to the hyperpolarization of hippocampal neurons. This aligns with current research exploring drugs enhancing GABAergic transmission and potassium channel openers for stroke treatment, positioning GHB as a potential regulator of neurotransmitter activity to mitigate excitotoxicity during brain ischemia (Ottani et al., 2003; Ottani et al., 2004).

In conclusion, GHB has shown neuroprotective effects against cerebral ischemia and oxidative stress-induced cell death. Its capabilities in reducing tissue damage, modulating metabolism, scavenging free radicals, and neurotransmitter receptor interaction highlight its potential in neuronal health preservation. Further research is required to clarify its mechanisms and set guidelines for clinical use (Ottani et al., 2004).

1.4 The Dark Side of GHB: Neurotoxic Effects

GHB can pose a threat to the brain when consumed in high doses (van Amsterdam et al., 2012). Neurotoxicity, defined as the capacity of a substance to inflict harm on neurons or nerve cells in the brain, may be a consequence of GHB abuse. This neurotoxicity is primarily linked to the agonistic effect of GHB on GABAb receptors when administered at high doses (e.g. GHB-induced comas, Brunt et al., 2021).

High doses of GHB are correlated with altered gene expression patterns and structural changes in the brain. Kemmel et al. (2010) studied the impact of a single acute pharmacological dose of GHB on gene expression patterns in the rat hippocampus and frontal cortex, revealing substantial alterations in gene expression in the hippocampus and prefrontal cortex. However, the study did not explicitly address neuronal death or provide insights into impairments in working memory and cognition. Furthermore, Raposo Pereira et al. (2020) demonstrated that the occurrence of GHB-induced comas is associated with structural changes in the brain. This study exposed microstructural alterations in the white matter of individuals who experienced GHB-induced comas. Notably, these changes were not observed in individuals who engaged in repeated GHB use without experiencing comas. The alterations in white matter were associated with heightened impulsivity, aligning with abnormalities in the white matter tracts responsible for response inhibition, located in prefrontal and limbic brain areas. Importantly, the experiments by Kemmel et al. (Kemmel et al., 2010) and Raposo Pereira et al. (P. Raposo Pereira et al., 2020) establish a correlation between GHB use and neurotoxicity, prompting further exploration of the underlying causal mechanisms. In line with the findings by Raposo Pereira et al. (2020), Brunt et al. (2021) suggests that GHB itself may not be neurotoxic. Instead, the observed neurotoxicity may result from comas and subsequent hypoxia during recreational use.

The neurotoxic effects of GHB on cognitive functions have been explored through various animal models, revealing a complex impact on memory and behavior. Studies have consistently shown that both acute and chronic exposure to GHB can impair spatial learning and memory. For instance, Kueh et al. (Kueh et al., 2008) found that acute administration of GHB and its precursors disrupts working memory in rats, with some tolerance developing over time to these effects. Similarly, Sircar et al. (Sircar et al., 2010) reported that repeated GHB administration in adolescent female rats impaired their spatial learning and memory, an effect that appears to be age-specific. Long-term consequences of GHB exposure have also been documented, showing that chronic GHB administration led to residual changes in protein expression in the hippocampus, which could underlie lasting cognitive impairments (van Nieuwenhuijzen et al., 2010). Moreover, additional studies have suggested that repeated GHB exposure may result in neurological damage and memory impairments in male rats, indicative of its neurotoxic potential (Pedraza et al., 2009). Similarly, cognitive deficits and alterations in GABAb and IGF-1 receptor densities were observed following GHB treatment, providing insight into the potential mechanisms underlying the cognitive effects of GHB (Johansson et al., 2014).

Despite the known acute impairments of GHB on cognitive functions, one critical research gap involves the lack of studies examining the causal relationship between GHB-induced comas and their long-term effects on cognition. Understanding the potential causal long-term consequences of GHB-induced comas on cognition is essential for identifying the risks associated with GHB use, particularly considering its narrow therapeutic window, which can lead to unintentional overdoses and comas. The current state of knowledge, especially regarding GHB-induced comas and their subsequent effects on cognition is notably limited, leaving significant areas understudied. This insufficiency underscores the urgency for comprehensive investigations to establish a causal relationship between GHB-induced comas and their impact on cognitive function and brain health. The absence of validated pharmaceutical treatments for GHB dependence, high relapse rates post-treatment, and the increasing prevalence of GHB use impact cognition by potentially exacerbating cognitive deficits and impeding recovery processes, thereby underscoring the need for further research (Brunt et al., 2021).

1.5 This Project

A previous pilot study on GHB-induced coma in rats revealed its health consequences, the health effects of GHB administration through oral gavage, and the number of comas to have basal cognitive effects. In this study, we investigated the short and long-term effects of GHB at repeated low doses and GHB-comas on cognition in adult rats. We investigate the cognitive effects of GHB administration after one day and two months of withdrawal, concerning visuospatial working memory. As a secondary analysis, we examined the behavioral effects of GHB intoxication, assessed through automated animal behavior recognition cages.

2 Methods

2.1 Research Design

This longitudinal study investigated the effects of GHB on cognitive functions by evaluating outcomes at two-time points: one day and two months post-administration of either GHB or saline. The research spanned two years and involved two separate cohorts of animals, each comprising 24 subjects. Animals were allocated to one of the three conditions (control, coma, or no-coma) based on their individual learning rates in the trial-unique nonmatching-to-location (TUNL) task.

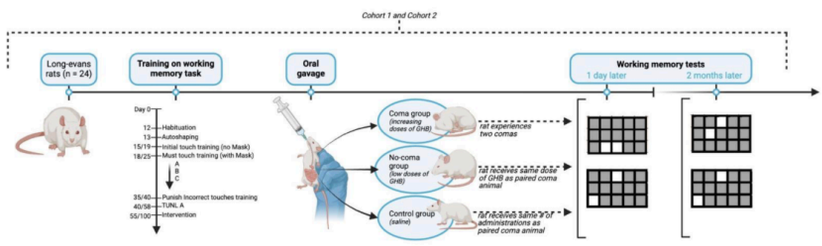

Figure 1: Graphical overview of the methods for the first (n=24) and second (n=24) cohorts.

2.2 Animals

In this study, 48 Long Evans rats were used (50% males in the first cohort, and 33% in the second cohort). After arrival, animals were housed in Rat Eurostandard type IV cages, with a grouping of four per cage. Their housing environment maintained consistent temperature and humidity levels, alongside a reversed 12-hour light/dark cycle (lights off at 08:00 h, on at 20:00) in temperature- (21±1 °C) and humidity-controlled (55%±5%) rooms. Upon arrival at the animal facility, animals were allowed to acclimate to the housing cage for seven days without interaction from the researcher, after which they were handled for another two weeks. The animals had free access to water throughout the experiment. When animals were not cognitively trained or tested, they received ad libido food (dried pellets of standard chow food [ssnif RM V1534- 703, Bio Services]). During the training and testing periods, animals were food-restricted during weekdays to enhance motivation during the TUNL task. Over the weekend, a predetermined quantity of food, surpassing their consumption capacity, was provided. Subsequently, the actual weekend consumption was measured to calculate 90% of their daily intake. This calculated portion was then dispensed throughout the subsequent week for behavioral testing sessions. This regimen was consistently applied each week to ensure the animals sustained a healthy body weight without hindering their development. 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). The study was conducted following ethical guidelines and regulations, in alignment with the principles of the three Rs that were integrated into the study design to minimize harm and ensure the ethical treatment of animals. All procedures and methods involving animals adhered to European and Dutch laws and received approval from the Animal Experimentation Committee of the Royal Netherlands Academy of Arts and Sciences.

2.3 Intervention

Animals were administered GHB or saline through oral gavage. The coma group received escalating doses of GHB (300 mg/kg, 700 mg/kg, 1100 mg/kg, 1500 mg/kg, etc.) through oral gavage until two GHB-induced comas were observed. Comas were determined as a loss of righting reflex and unresponsiveness to a painful stimulus. In short, when the coma animals were not moving in response to the researcher entering the room, touching the cage, and physical touch of the animal we assessed the occurrence of comas by the absence of movement after placing animals on their backs for two minutes and response to a toe pinch. The no-coma and control condition animals were each paired with a coma animal. Therefore, the no-coma group received one dose of 300 mg/kg and subsequently, a fixed dose of 700 mg/kg of GHB through oral gavage, until they had received the same total amount of GHB as the paired coma animal. The control group received the same number and volume of saline (Natriumchloride 0.9% w/v) administrations as the paired coma animal's GHB administration. Due to procedural complications with the oral gavage method, three no-coma animals from the second

cohort were administered their final doses of GHB by mixing them with crushed pellets. These GHB pellets were provided in individual cages and made available for consumption over up to two hours.

Behavioral Data Collection During GHB-Intoxication With LABORAS. The effects of GHB administration on behavior were evaluated using LABORAS cages. LABORAS is an automated, non-invasive system that accurately records and analyzes rat behaviors in a home cage setting, without the need for human observers. Utilizing pattern recognition technologies, it provided detailed behavioral data and precise position tracking of the following behaviors: locomotion, drinking, eating, rearing, grooming, and speed average behaviors. Animals were temporarily relocated to the testing room for a 30-minute habituation period before oral gavage. After, they were relocated to individual testing cages, not exceeding 2 hours and 30 minutes. The animals had one individual habituation session in the LABORAS cages. In the sessions following habituation, we administered either (a) increasing doses of GHB until two comas were experienced, (b) low and fixed doses of GHB or (c) saline, through oral gavage. The no-coma animals were tested in the LABORAS cage for the same number of sessions as their paired coma animals before continuing to receive GHB oral gavage in their housing rooms.

Cognitive Data Collection Post-Intoxication With the TUNL Test. To assess visuospatial working memory and impulsivity, the TUNL task was used, a validated hippocampus-dependent tool (Talpos et al., 2010). On training and testing days, the animals were transported to the designated TUNL room and allowed a 30-minute acclimatization period in preparation for their training or testing sessions. After, they were relocated to individual training and testing cages, not exceeding 45 minutes for each session. The TUNL task training lasted approximately two months (range = 60-105 days, mean = 67 days), depending on the learning rate of the animals. During training, animals underwent autoshaping, learning to eat 25 pellets in two sessions. In subsequent stages, the animals were taught to touch targets first without a visual mask and then with one. Each stage required 25 correct responses in consecutive sessions. A subsequent stage introduced penalties for mistakes, necessitating an 80% success rate over two consecutive sessions. Proficiency was determined in the TUNL A stage, where animals must hit a 70% correct response threshold over three sequential sessions, after a minimum of 15 sessions. When these conditions were met, animals were assigned to one of three conditions: GHB-coma, GHB no coma, or a control group. Animals failing to meet this benchmark within 50 sessions were removed from the study (n=2). Animals who met this benchmark started GHB/saline administration and LABORAS behavioral tracking (see table A1). After completion of GHB/saline administration, cognition was assessed at two time points: one day after GHB/saline administration to examine short-term effects and again at the two-month mark to investigate potential long-term effects. This involved a series of three schedules with different levels of complexity (TUNL A, B and C) over thirteen consecutive working days for each time mark (one day and two months). The sequence began with a one-off, 30-minute TUNL A reminder session, serving as a refresher. This was succeeded by six 45-minute sessions of TUNL A with two short and four long delays comprising several large distances between squares. Subsequently, animals were tested on 45-minute sessions of TUNL

B and C (Difficulty I and II, respectively), where animals underwent three consecutive sessions for each, with medium and narrow square separations. Detailed information on the TUNL task stages and distances used can be found in the tables A2, and A3.

2.4 Statistical Analysis

Cohort Comparisons. To test the hypothesis that there is no difference between cohorts 1 and 2, a linear mixed model (LMM) was utilized, with the individual animal as a random effect to account for intra-animal correlation. The fixed effects in the model included the cohort variable (Cohort 1 and Cohort 2). Three datasets for TUNL A, differentiated by schedule ('reminder', 'short', and 'long'), were analyzed using Restricted Maximum Likelihood (REML) with Satterthwaite's method for degrees of freedom estimation. We applied the following formula:

$$\text{correct} \sim \text{Cohort} + (1 \mid \text{Animal}) \tag{1}$$

Behavioral Data Analysis. This study employed a linear mixed model to evaluate the effect of conditions and sessions on six behavioral metrics—(1) locomotion, (2) drinking, (3) eating, (4) grooming, (5) rearing, and (6) average speed. The model incorporated random effects to accommodate individual variability among the animals and conditions. The data for each behavior were analyzed using a linear mixed-effects model to determine the effects of condition and session, as well as their interaction on the measured behaviors. The model included random effects to account for variability between animals nested within each condition. The model used is represented as follows:

$$\text{lmer}(\text{parameters} \sim \text{condition} * \text{session} + (1 \mid \text{Animal}/\text{condition})) \tag{2}$$

This model structure allows for the assessment of fixed effects (condition, session, and their interaction) on each behavior, while accounting for random effects at the level of individual animals within each condition.

Cognitive Data Analysis. The linear mixed effect model focused on working memory and impulsivity measures for reminder, short, long, difficulty I, and II schedules. Working memory performance was assessed through percentage correct and correct latency, while impulsivity was assessed through Inter-Trial Interval (ITI) touches, and Blank touches. These parameters were evaluated at two distinct time points: one day after the last intervention (i.e. oral gavage) and at a two-month follow-up. The statistical models were tailored to each TUNL stage. The statistical significance level was set at $\alpha = 0.05$ for TUNL measurements, without adjusting for multiple comparisons. The analysis of TUNL A Trials is structured around three main sessions: Reminder, Short delay, and Long delay trials. For the reminder sessions, the following linear mixed-effects model was applied:

$$\text{lm}(\text{parameter} \sim \text{Condition}) \tag{3}$$

For the other TUNL stages (short, long, difficulty I, difficulty II), we accounted for the nested structure of the data:

$$\text{lmer}(\text{parameter} \sim \text{Session} * \text{Condition} + (1 \mid \text{Condition}/\text{Animal})) \tag{4}$$

The assessment of Difficulty I and II trials focused on categorizing difficulty levels according to the distances between large and small squares. Difficulty I trials were classified into easy, intermediate, and difficult levels based on square distances. Conversely, Difficulty II trials, while also divided into easy, intermediate, and difficult categories, consistently featured the shortest distances among the squares. In both difficulty levels, some animals completed fewer choice trials within a session in comparison to other animals, resulting in less reliable success rates compared to animals with many trials. Despite this variability, all animals were included in the analysis. These LMM models compared the primary conditions of interest (i.e., control vs GHB-groups), and post-hoc Tukey's HSD tests assessed the other contrasts. Statistical analysis of the collected data from the LABORAS and TUNL measurements was performed using the R programming language and associated packages.

3 Results

3.1 Cohort Comparisons

There was no difference in TUNL performance between Cohort 1 and Cohort 2 (TUNL A % correct response, $p > 0.05$). Therefore, in further analyses, cohorts 1 and 2 were grouped.

3.2 Automated Animal Behavior During GHB Intoxication

Motor behavior decreased over time for all conditions (estimate = -1.71, SE = 0.26, $t(399.35) = -6.68$, $p < .001$, Figure 2), while locomotion was higher in the no-coma condition over time than the other conditions (estimate = 1.03, SE = 0.37, $t(399.35) = 2.75$, $p = .006$; Figure 2).

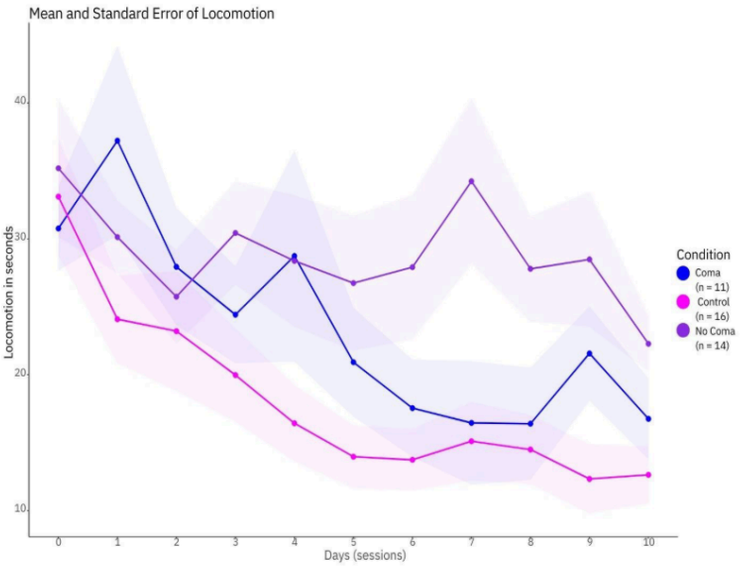

Figure 2: Average motor activity comparison over initial 10 sessions across groups. Data points represent average +/- SEM for each animal. The no-coma condition moderated motor behavior over sessions (p = .006).

Animals in the coma group exhibited reduced motor behavior during the sessions in which coma was induced, compared to the sessions before and after the coma induction (Figure B2). The velocity of movement also declined over time for all conditions (estimate = -178,368, SE = 40,137, t(1.05e+13) = -4.44, p < .001). Further, rearing behavior declined over time for coma animals (estimate = -4.87, SE = 1.75, t(401.66) = -2.56, p = 0.01). Drinking, eating, and grooming behaviors did not change across conditions after adjustment for multiple comparisons (p > .0083).

3.3 Working Memory Performance

The working memory performance (i.e., percentage correct) was higher in the no-coma condition in comparison to control in the reminder trial at the two-month mark (intercept = 13.86, SE = 5.53, t = 2.51, p = .018, Rm2 = 0.16; Figure 3).

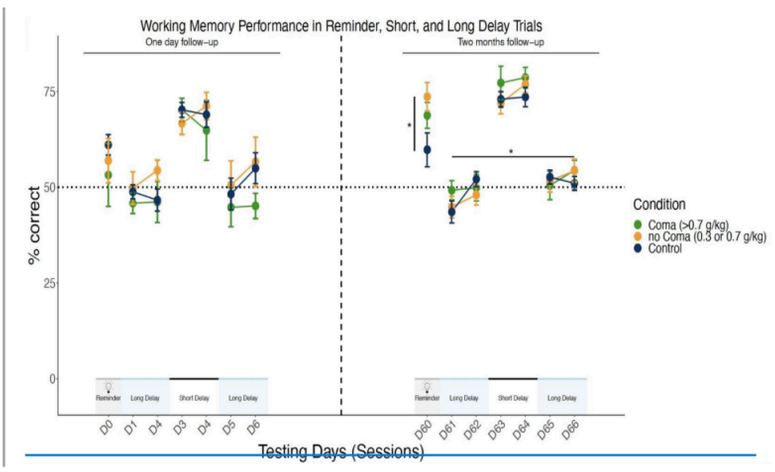

Figure 3: Working memory performance (%correct trials) across reminder (gray), short delay (white), and long delay (blue) sessions in TUNL A trials at one day (D0-D6) and two months (D60-D66) after the last intervention. The reminder and short delay trials involved a 1s delay between sample-and-choice phases, while the long delay included a 5s delay. All trials included large separation square distances only. Data points represent average +/SEM of % correct responding. * indicate p-values < 0.05. Vertical lines show differences between no coma and control conditions, while horizontal lines show differences between long delay sessions.

Furthermore, the working memory performance increased over time for all conditions in the long delay at the two-month mark (intercept = 1.129, SE = 0.515, t(98.318) = 2.190, p = .031; Figure 3). All other conditions, sessions, and their interactions in reminder, short, long, difficulty I, and II tasks at the two-month mark did not differ (p > .05; Figures 3, 4a, and 4b). Low effect sizes (Rm2 = 0.088, Rc2 = 0.234) across trial types support the conclusion that the experimental conditions, session, and their interaction had a limited overall impact on trial performance.

In assessing correct latency across reminder, short, long, difficulty I and II trials at both one day and two months, there were no differences between conditions, sessions, or their interactions (p > 0.05). The effect size values were generally low. Therefore, the results do not support any correct latency differences between conditions, sessions, or their interactions.

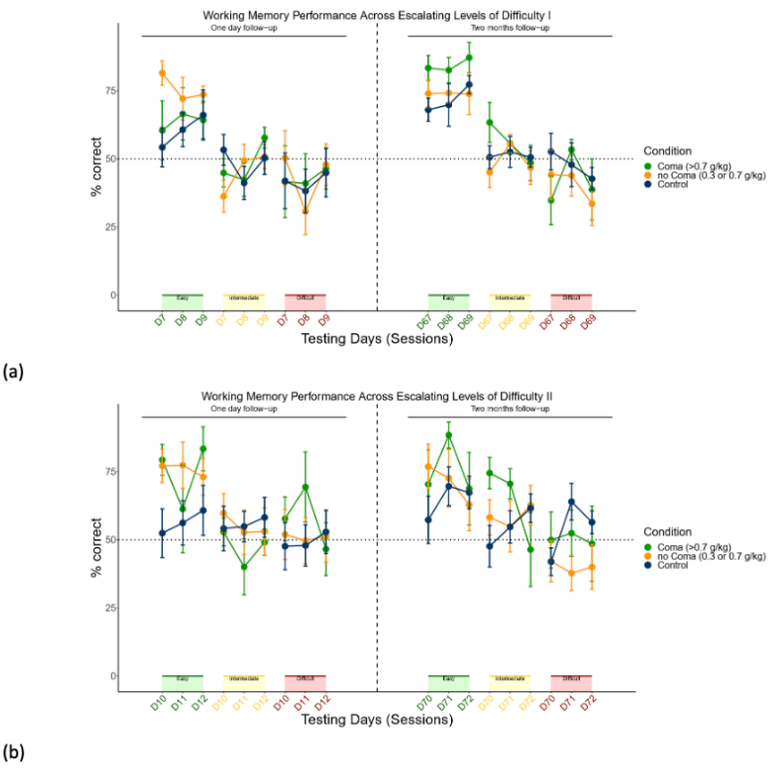

Figure 4: Working memory performance in increasing levels of (a) difficulty I trials at one day (D7-D9) and two months (D67-D69), and in (b) Difficulty II trials at one day (D10-D12) and two months (D70-D72) after the last intervention, showing the percentage of correct trials across easy (large square separation), intermediate (moderate square separation), and difficult (narrow square separation) trials. Trials were randomly alternated in each session, and the task involved a short (1s) delay between sample-and-choice phases. Data points represent average +/- SEM of % correct responding."

3.4 Impulsivity

At the two-month time mark for short delay trials, the number of ITI touches over time increased for all groups (intercept = 19.64, SE = 7.36, t(22.999) = 2.669, p = .014, Figure 5). Moreover, coma animals had higher ITI touches than in the other conditions in the two-month short delay trials (intercept = 593.61, SE = 235.78, t(23.281) = 2.518, p = .019, Figure 5).

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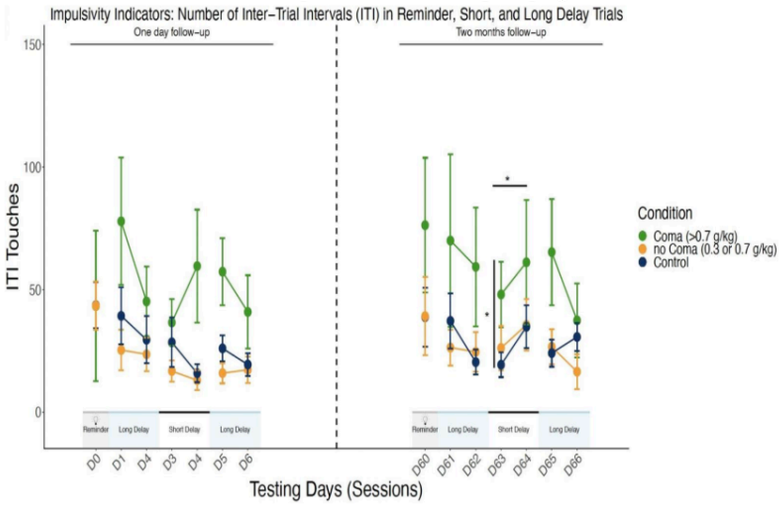

Figure 5: Impulsivity (Inter-trial Interval touches) across reminder, short delay, and long delay sessions in TUNL A trials at one day (D0-D6) and two months (D60-66) time points. Data points represent the average +/- SEM of ITI touches. * indicate p-values < 0.05. Vertical lines show differences between coma and control conditions, while horizontal lines show interactions of the coma condition over sessions.

Furthermore, the number of ITI touches changed over time in Difficulty II trials at the one-day sessions for all conditions (intercept = 30.25, SE = 12.27, t(67.58) = 2.4, p = .016;). Post-hoc comparisons indicated that the ITI touches were higher in the coma condition compared to control (intercept = −24.20, SE = 10.07, t(65.000) = −2.403, p = .019). Conversely, reminder, long, and difficulty I and II at the two-month mark and short trials at the one-day mark did not differ between conditions and over time (p > 0.05; Figures 5 and 6).

Furthermore, the analysis revealed low to moderate explanatory power. For short trials at the two-month mark, the models had a low effect size (Rm2 = 0.052, Rc2 = 0.752). Similarly, the effect size was moderate for Difficulty II at one-day trials (Rm2 = 0.080, Rc2 = 0.732), and for Difficulty II at two-month trials (Rm2 = 0.109, Rc2 = 0.480). These findings indicate that while ITI touches at times differ in the coma condition, the overall impact of these factors is limited, as reflected in the low to moderate effect sizes.

In the short two-month trials, blank touches increased over time for the coma condition (intercept = −73.72, SE = 33.28, t(23.000) = −2.21, p = .03; Figure 7).

At the one-day mark, blank touches increased over time for the coma condition in Difficulty I trials

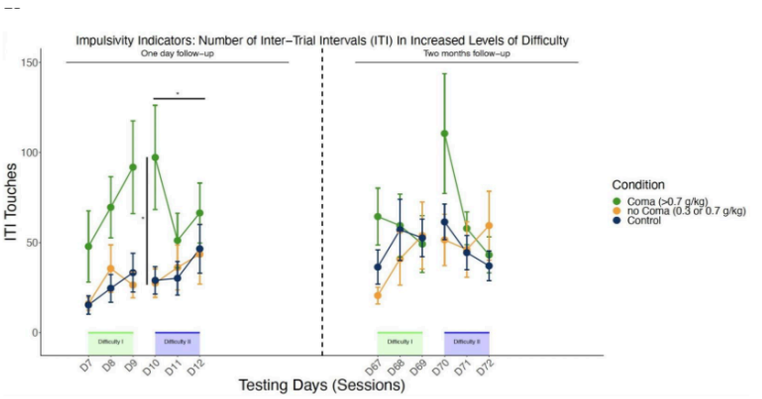

Figure 6: Graphical overview of the methods for the first (n=24) and second (n=24) cohorts.

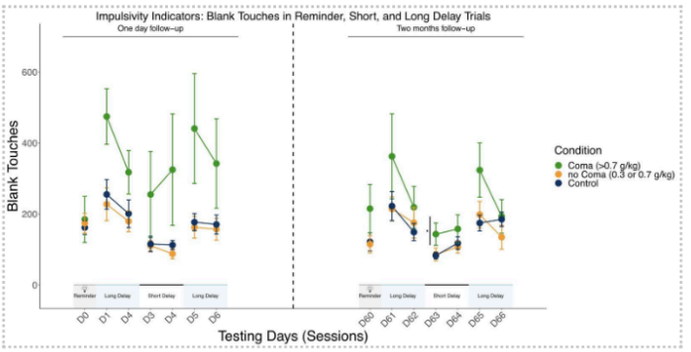

Figure 7: Impulsivity (blank touches) across reminder, short delay, and long delay sessions in TUNL A trials at one day (D0–D6) and two months (D60–D66) time points. Data points represent the average ± SEM of blank touches. * indicate p-values < 0.05.

(intercept = 97.79, SE = 43.51, t(64.999) = 2.247, p = .028). For Difficulty II trials at one day, the coma condition had higher blank touches than the control (intercept = 825.81, SE = 374.71, t(73.3) = 2.20, p = .031).

At the two-month time mark in the Difficulty I trials, blank touches decreased over time for the coma condition (intercept = 31.1, SE = 15.34, t(64.999) = 2.027, p = .047). Moreover, the coma condition had higher blank touches at day 67 than the control (intercept = 1338.30, SE = 572.95, t(65.792) = 2.336, p = .023; Figure 8). In the analyses of the reminder, long, difficulty I, and II schedules, there were no differences between conditions at either time point (p > .05); Figure 8). The effect sizes ranged from low to moderate and the corresponding post-hoc comparisons across all assessments failed to identify any differences (p > .05).

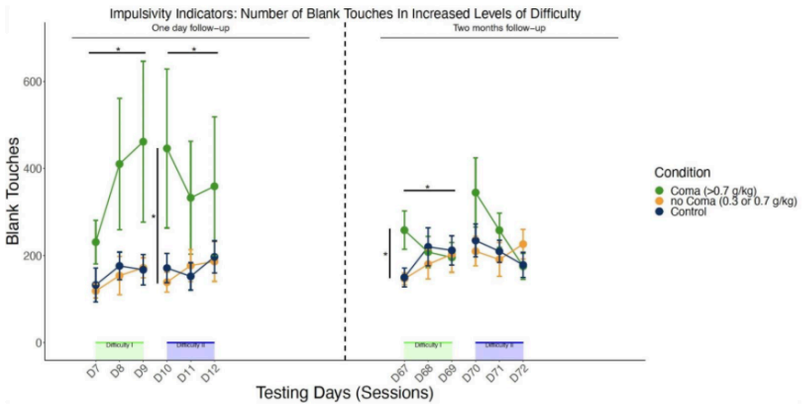

Figure 8: Impulsivity (blank touches) across increased levels of difficulty sessions one day (D7–D12) and two months (D67–D72) post GHB administration. Difficulty I included fewer trials with narrow distances between squares in comparison to difficulty II. All sessions alternated randomly at various large and narrow distances. Data points represent the average ± SEM of blank touches. * indicate p-values < 0.05. Vertical lines show differences between coma and control conditions, while horizontal lines show interactions of the coma condition over sessions.

4 Discussion

In this study, we investigated the short- and long-term effects of GHB at low and coma-inducing doses on adult rats. We first assessed the behavioral effects of GHB intoxication and then examined the cognitive effects after one day and two months of GHB withdrawal, focusing on visuospatial working memory and impulsivity. Behavioral observations during intoxication showed that GHB increased locomotion at low doses but decreased it at high doses. Additionally, GHB reduced rearing behavior over time more than control, while essential behaviors like eating and drinking remained unaffected. Working memory performance was higher in the no-coma condition compared to controls at the two-month mark. Impulsivity is higher in coma animals compared to controls at both one day and two months of withdrawal. Although there were differences in impulsivity and working memory for the coma and no-coma conditions compared to control, respectively, these effects lacked consistency across different trial types.

The observed decline in motor behavior with GHB intoxication underlines its dose-dependent effects on motor functions. Decreases in locomotion over time, particularly in the coma condition, affirm the sedative impact of high GHB doses on the central nervous system. However, the reduced locomotion in the control condition over sessions may have created a ”floor effect,” making it difficult to observe further decreases in the coma condition (Brunt et al., 2021; Kemmel et al., 2010; Pedraza et al., 2009). Conversely, the no-coma condition, where low doses increased locomotion, highlights the stimulant properties of GHB at lower doses (Abanades et al., 2006; Brunt et al., 2021; Pedraza et al., 2009).

Rearing behavior was reduced for all conditions, especially for GHB-coma animals, this typically indicates increased anxiety and reduced exploratory inclination in rats (Figure B1a). Interestingly, the more pronounced decrease in rearing for the coma condition is not caused by a general decrease in mobility; locomotion was not decreased in the coma group compared to the control group (Grüner et al., 1999). This is particularly interesting because it suggests that the observed reduction in rearing behavior may be specifically related to anxiety and exploratory behavior rather than overall physical impairment. This aligns with a study by Navarro et al. (2008), which links GHB to central nervous system changes that influence anxiety and exploration. GHB-treated mice showed increased anxiety-like behaviors, spending less time in the lit area, more time in the dark, and displaying reduced rearing, transitions, and latency, suggesting the anxiogenic effects of GHB, possibly via GABA and/or dopaminergic receptor modulation (Abanades et al., 2006; Felmlee et al., 2021; Navarro et al., 2008). Speculatively, GHB may selectively affect behaviors requiring more cognitive and motor involvement while maintaining stability in basic functions like drinking, eating, and grooming. This hypothesized selectivity might be due to the differential sensitivity of neural circuits mediating complex behaviors versus those regulating basic survival behaviors (Beurmanjer et al., 2022; Navarro et al., 2008). It is plausible that complex behaviors such as exploration and rearing involve higher brain regions, which might be more susceptible to the neuromodulatory effects of GHB, particularly through its action on GABAergic systems (Felmlee et al., 2021). In contrast, eating, drinking, and grooming are often regulated by lower brain regions that may be less affected by GHB (Graybiel, 2008). Thus, it can be speculated that the effects of GHB may be more pronounced on behaviors that integrate higher cognitive processes and motor planning, while affecting less the autonomic and essential survival behaviors.

There were no differences in working memory performance between GHB-coma and control animals; however, low GHB doses increased working memory performance in the reminder session at the two-month GHB-withdrawal mark. To our knowledge, the only preclinical study addressing the effects of GHB on working memory reported that repeated administration of 10 mg/kg of GHB (i.e. low dose) causes neurological damage and decreases working memory in an animal model. However, their findings are somewhat ambiguous due to variations in the dosage and the specific neurological tests employed, which could lead to inconsistent interpretations of the neurotoxic impact of GHB (Pedraza et al., 2009) on memory. For instance, Pedraza et al. (2009) relied on the Morris water maze, which measures spatial learning rather than working memory (Othman et al., 2022), and the hole-board test, a non-sophisticated paradigm that has been used to assess long-term memory (Casarrubea et al., 2023). In short, these tasks do not fully capture the dynamic updating and manipulation of information that define working memory. Additionally, Pedraza et al. (2009) tested animals 2 hours after GHB administration, so their findings on decreased locomotion and increased anxiety-like behaviors align more with our behavioral findings during GHB intoxication and less with actual working memory performance and short-term effects. This suggests that the observed behavioral effects are likely due to the immediate pharmacological action of GHB, rather than any short-term or long-

term cognitive impairment. Additionally, their use of traditional observational methods introduced potential human error and bias. In contrast, our study employed the TUNL task to assess visuospatial working memory and impulse control, using automated systems for precise behavioral measurements. While Pedraza et al. (2009) used two GHB doses (10 mg/kg and 100 mg/kg), our study investigated a broader range of doses and included a longitudinal follow-up to understand the dose-response relationship and potential threshold effects. While Pedraza et al. (2009) provided valuable insights, the methodological rigor in our study allows for a more comprehensive and precise assessment of GHB's effects on working memory. The immediate working memory deficits observed by Pedraza et al. (2009) are likely related to acute pharmacological actions on locomotion and anxiety rather than short- and long-term cognitive impacts, highlighting the importance of precise cognitive tasks and timing in evaluating GHB's effects.

Furthermore, no difference in spatial working memory in GHB-coma groups compared to controls contrasts with clinical observations, which predominantly report cognitive impairments in GHB-coma users. For example, Raposo Pereira et al. (2018a) observed a decrease in verbal working memory performance, but not in spatial working memory, among individuals who had experienced multiple GHB comas. This discrepancy points to the nuanced nature of GHB's effects, which may vary based on the individual's exposure history. Perhaps, the variance in outcomes between this study and our study could be due to different study populations. Raposo's research concentrated on human polydrug users, contrasting with our controlled environment that featured non-human subjects exposed solely to GHB, thereby removing the confounding variables of polydrug use. Furthermore, human subjects with GUD may have personality traits and genetic factors that increase their susceptibility to addiction. For instance, Beurmanjer et al. (2022) demonstrated that cognitive impairments, particularly in memory, were highly prevalent among patients with GUD and could predict relapse. The study also found that memory impairments present before detoxification were associated with a higher risk of relapse after detoxification. This suggests that individuals predisposed to addiction might experience decreased memory performance due to more frequent and heavier drug use, as well as a greater number of GHB-induced comas, alongside pre-existing cognitive deficits. Additionally, Raposo Pereira et al. (2018b) reported that individuals in the GHB-Coma group had lower premorbid IQs compared to those in the GHB-NoComa group, despite similar age and education levels. This finding indicates that pre-existing cognitive differences might exacerbate the impact of GHB use on memory performance. However, it is also possible that the coma-inducing effects of GHB contributed to the lower premorbid IQ observed in this group. Therefore, while pre-existing cognitive differences could play a role, we cannot definitively determine whether decreased cognition is a cause or a consequence of GHB-induced comas. These factors underline the complex interplay between genetic predisposition, personality traits, and the detrimental cognitive effects of GHB, particularly concerning memory. To further highlight the nuanced effects of GHB, there may be a selective impact of GHB on verbal but not on visuospatial domains of working memory, which suggests that the cognitive consequences of GHB might be domain-specific. However, another study by Raposo

Pereira et al. (2018b) has found impairments in spatial working memory, using an n-back test, in GHB users who have experienced multiple comas. Possibly, the frequency of GHB-induced comas, as well as the spacing between these comas, is a differentiator; while studies have shown impairments in spatial and verbal working memory in humans after multiple GHB comas (F. Raposo Pereira et al., 2018a; F. Raposo Pereira et al., 2018b), our research induced two comas with varied intervals between them, which might not be sufficient to reveal cognitive deficits in visuospatial working memory. An increased number of comas within a short spacing period could potentially expose more profound cognitive dysfunctions. Thus, the discrepancies between our study and previous research highlight that the cognitive impacts of GHB are influenced by individual differences and exposure histories, suggesting that GHB may have a nuanced effect on working memory.

Further, our study's findings indicate decreased impulse control in the short- and long-term in GHB-coma animals but not in animals receiving low GHB doses. This aligns with clinical research suggesting that high doses of GHB impair higher-order brain functions, such as the frontal cortex. For instance, Raposo Pereira et al. (2019) found that multiple GHB-induced comas affected brain microstructure, particularly white matter, and were associated with increased impulsivity. They observed decreased functional connectivity within the Central Executive Network and between this network and the Default Mode Network, crucial for cognitive control and decision-making. Furthermore, gene alterations in the prefrontal cortex and reduction of NMDA receptor subunit levels in the frontal cortex have been reported during and post-GHB intoxication (Kemmel et al., 2010; Sircar et al., 2011; van Nieuwenhuijzen et al., 2010). These findings can relate to Raposo Pereira et al. (2018b), who found that GHB-induced comas led to hyperactivation of the right dorsolateral prefrontal cortex (DLPFC) and increased functional connectivity with the left anterior cingulate and medial frontal gyrus, regions associated with impulsive control, decision-making, and reward processing (Ballard et al., 2018; Cho et al., 2010). Together, these alterations in prefrontal areas and subsequent disruptions in brain connectivity may underlie the observed impulse control reductions.

GHB-coma animals experienced prolonged unconsciousness, potentially leading to brain hypoxia, which may explain the increased impulsivity in this group compared to low-dose animals who did not experience such periods. For instance, overdosing on GHB, similar to binge drinking or high doses of ketamine, can result in profound coma, which is particularly neurotoxic (F. Raposo Pereira et al., 2018a; F. Raposo Pereira et al., 2018b; van Amsterdam et al., 2012). Prolonged unconsciousness and the resultant inadequate oxygen supply to the brain (i.e. hypoxia), can lead to severe oxidative stress and exacerbate neurotoxic effects, particularly in higher-order regions, such as the prefrontal cortex. This oxidative stress and neurotoxicity can impair cognitive functions, such as decision-making and impulsivity, and trigger neuronal apoptosis, especially during critical developmental periods. These neurobiological alterations may suggest that the observed behavioral changes in our study could result more from the effects of unconsciousness and hypoxia rather than from GHB itself, as GHB-coma but not GHB-Nocoma animals have reduced impulse control. This highlights the importance of considering the broader context of GHB exposure, including the duration of unconsciousness and

the resultant hypoxic conditions, when evaluating its impact on cognitive and behavioral functions (Brunt et al., 2021; van Amsterdam et al., 2012).

While this study provides valuable insights into the effects of GHB on cognition and neurotoxicity, it is essential to acknowledge its limitations, which point toward directions for future research. A primary limitation is the potential confounding impact of stress, motivation, and sensory-motor skills, which can alter cognitive task outcomes (Chattarji et al., 2015). This is a critical concern in both animal and human studies and controlling for these factors might be challenging (J. Wu and Yan, 2017), potentially leading to variability in one group compared to others (e.g., in this study the no coma group received more oral gavages than the other groups; Starcke et al., 2016). Behavioral outcomes under stress can manifest as detrimental effects on executive functions, motor coordination, and spatial memory, while chronic stress may influence neuroplasticity and the efficacy of cognitive training (Cerqueira et al., 2007; Liston et al., 2009; J. Wu et al., 2014). Furthermore, acute stress has been shown to affect attentional resource allocation and neural efficiency, indicating a direct relationship between stress-induced cognitive load and brain function (Schwabe and Wolf, 2010; Weymar et al., 2013). Secondly, the use of oral gavage introduces complexity due to the risk of gavage-related reflux, which can lead to serious respiratory effects and even mortality. This increased dropout rates and introduced bias (Damsch et al., 2011). Unscheduled mortality observed in our study may be linked to respiratory complications that stem from gavage errors or gastro-esophageal reflux causing aspiration of stomach contents, with a study noting respiratory tract necrosis in about 20% of rats subjected to high drug concentrations (Eichenbaum et al., 2011). Additionally, individual variability in baseline impulsivity, such as predispositions toward sign-tracking or goal-tracking behaviors, could be a confounder in our study since we did not select animals based on baseline impulsivity, biasing interpretations of GHB's impact on impulsivity. Lastly, the statistical significance level was set at $\alpha = 0.05$ for TUNL measurements, without adjusting for multiple comparisons. This decision, made due to the study's exploratory nature, low power, and the high number of comparisons increases the risk of Type I errors. Therefore, results should be interpreted carefully, recognizing the heightened likelihood of false positives. These limitations imply that while our results indicate dose-dependent effects of GHB on behavior during intoxication and cognitive impairments in GHB-coma animals, the findings should be interpreted with caution due to potential confounding factors such as stress, gavage-related complications, and individual variability in impulsivity, which might have influenced the observed outcomes.

Future research should expand cognitive assessments to include decision-making, reward processing, and emotional regulation while addressing the confounding effects of individual differences, sensory-motor skills, and stress on cognitive outcomes. Incorporating these broader assessments is especially important for investigating the long-term effects of GHB-induced coma, as it will help determine the generalizability of findings, and identify subgroups particularly susceptible to GHB's effects (Talpos et al., 2010). In terms of patient treatment, it is essential to focus on cognitive aspects due to the potential cognitive impairments following GHB use, particularly at coma-inducing doses. Treat-

ment should also address other facets of GUD, such as dependency and the potential for relapse. Furthermore, future studies should specifically examine individual variability in baseline impulsivity and reward processing. Understanding these individual differences is crucial to prevent bias, besides tailoring treatments and interventions for patients with GUD. Additionally, assessing sensory-motor abilities is necessary to ensure that observed cognitive deficits are not confounded by impairments in sensory-motor function, thereby isolating the specific cognitive effects of GHB. Lastly, addressing stress related to GHB administration is vital to avoid reducing sample size due to animal morbidity or mortality and to prevent stress-induced alterations in cognitive performance. Using flexible plastic gavage needles or red rubber feeding tubes for rats, which are less likely to damage the esophagus compared to stainless steel gavage needles, could minimize stress and complications (Herrod et al., 2023).

5 Conclusion

Coma and no-coma-inducing doses of GHB, administered through oral gavage in outbred rats, have a nuanced effect, influencing various cognitive and behavioral functions differently depending on the dose and individual variability of animals. The impact of GHB might be less pronounced than previously reported, as GHB-induced comas disrupted impulse control during one-day, and two-month withdrawal but not visuospatial working memory, while low doses of GHB increased working memory performance. This highlights the need for further research on the dose-dependent and long-term cognitive effects of GHB-coma on cognition. This rat model of short- and long-term effects of GHB allows for a thorough study of GHB-induced comas, differentiating their impact on cognition from low GHB doses and thus highlighting the importance of addressing both the therapeutic use and potential abuse of GHB leading to GHB-induced comas.

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