University of Reading

The Effect of Dietary Supplementation of Vitamin B12 on Behavioural and Neural Activity in Healthy Rats

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Abstract

Background: Vitamin B12 is essential for brain function, including neurotransmitter synthesis and myelination. Although vitamin B12 deficiency is associated with cognitive and sensory impairments, its effects on sensory perception and sensory signal processing in healthy individuals remain unclear. Investigating such effects requires behavioural paradigms that allow reliable repeated assessment of sensory function; however, suitable protocols for repeated texture-based whisker discrimination in rodents have not been well established for repeated assessment.

Objectives: This study aimed (i) to establish an appropriate methodological framework for repeated assessment of whisker mediated texture discrimination using the textured novel object recognition test (tNORT), and (ii) to apply this framework to examine the effects of vitamin B12 supplementation on whisker sensitivity and sensory-evoked neural responses in healthy rats.

Methods: A methodological study was first conducted to determine suitable object properties and inter test intervals for repeated tNORT using eight male Lister Hooded rats. For the main study, twenty-eight male Lister Hooded rats were randomly assigned to a control group (n = 14) or a vitamin B12 group (n = 14). The B12 group received vitamin B12 enriched drinking water, with the concentration gradually increased to a final level of 4000 µg/kg diet. Behavioural assessments using the validated tNORT protocol were performed before and after a three-week supplementation period.

Following supplementation, *in vivo* electrophysiological recordings of sensory evoked field potentials (LFPs) were recorded in the ventral posteromedial nucleus (VPM) of the thalamus and the barrel cortex under varying stimulus intensities, and stimulation frequencies. In addition, *ex vivo* recordings were performed to assess input-output relationships, short-term plasticity, and sensory adaptation within cortical networks.

Results: The methodological study identified task parameters that enabled reliable repeated assessment of whisker-mediated texture discrimination without positional or exploration bias. For the main study, vitamin B12 supplementation significantly increased serum cobalamin levels; however, behavioural performance in the tNORT did not show significant group differences in healthy rats.

In vivo electrophysiological recordings revealed no marked effects of vitamin B12 supplementation on input-output relationships or short-term sensory adaptation in either the thalamus or barrel cortex.

Ex vivo recordings similarly showed no significant supplementation related changes in baseline synaptic transmission, presynaptic release probability, or overall adaptation strength. A subtle, frequency-specific difference was detected at 10 Hz stimulation, where evoked responses in the supplemented group exhibited greater attenuation across successive pulses; however, this effect did not generalise across frequencies or stimulation paradigms.

Conclusions: Together, these findings indicate that vitamin B12 supplementation does not robustly alter whisker-dependent behaviour or sensory processing in the barrel cortex of healthy, non-deficient rats. Any potential effects appear subtle, frequency-dependent, and restricted to specific circuit states. This thesis highlights the importance of methodological validation in behavioural neuroscience and suggests that the neurophysiological consequences of vitamin B12 supplementation in healthy systems are context-dependent rather than uniform.

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Publication

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Declaration of Original Authorship

Declaration: I confirm that this is my own work and the use of all material from other sources have been properly and fully acknowledged.

Yurie Hayashi

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List of Abbreviations

ACSF. *artificial cerebrospinal fluid*

AMPA. *alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid*

ART ANOVA. *aligned rank transformed analysis of variancel*

CSD. *current source density*

DI. *discrimination index*

EEG. *electroencephalogram*

FWHM. *full width at half maximum*

EPSP. *excitatory postsynaptic potential*

GABA. *gamma-aminobutyric acid*

IPSP. *inhibitory postsynaptic potential*

IQR. *interquartile range*

ISI. *inter stimulus interval*

LFP. *Local field potential*

MUA. *multi-unit activity*

NMDA. *N-methyl-D-aspartate*

NOR. *novel object recognition*

OSBT. *object shape bias test*

POm. *posterior thalamic nucleus*

PSD. *power spectral density*

RDA. *recommended dietary allowance*

S1. *primary somatosensory cortex*

tNORT. *textured novel object recognition test*

VPM. *ventral posterior medial*

Chapter 1 General Introduction

1.1 Functions and Health Implications of Vitamin B12

Vitamin B12 is a water-soluble vitamin that plays essential roles in cellular metabolism, red blood cell formation, and the maintenance of neurological function. This section outlines its physiological importance, examines the consequences of deficiency, and considers emerging evidence regarding the effects of supplementation—even in individuals without diagnosed deficiency.

Section 1.1.1 introduces the physiological roles of vitamin B12, in neural development and function, with particular attention to the neurological consequences of deficiency during early life. Section 1.1.2 reviews the broader neurological and psychiatric consequences of vitamin B12 deficiency, focusing on its associations with cognitive impairment, dementia, and depression. It also discusses the mixed evidence regarding the effect of supplementation. Finally, Section 1.1.3 highlights recent findings from both human and animal studies suggesting that vitamin B12 supplementation may influence neural activity and sensory processing, even in individuals without clinical deficiency.

1.1.1 The Role of Vitamin B12 in Neural Development and Function

This section focuses on the crucial role of vitamin B12 in neural development and function, particularly during early life. It is particularly essential for brain development, and deficiencies in vitamin B12 can lead to several neurological symptoms (Reynolds, 2006, Chalouhi et al., 2008, Grober et al., 2013, Stabler, 2013, Ata et al., 2020). Firstly, in the peripheral nervous system, vitamin B12 deficiency is associated with symptoms such as cutaneous sensory loss, reduced reflexes, symmetric weakness, and paraesthesia (Savage and Lindenbaum, 1995, Reynolds, 2006, Stabler, 2013, Hunt et al., 2014, Sarwar et al., 2020, Hassouneh et al., 2021).

In contrast, in the central nervous system, several studies reported the association between vitamin B12 deficiency and neurodegeneration and brain atrophy (Kamei et al., 2011, Ekici et al., 2016, Deng et al., 2017, Rabensteiner et al., 2020). These effects highlight the importance of maintaining adequate vitamin B12 levels for both central and peripheral nervous system function.

Adequate maternal intake of vitamin B12 during pregnancy and lactation is critical for infant immune and neurodevelopmental health (Dror and Allen, 2008, Duggan et al., 2014, Siddiqua et al., 2016, Dubaj and Czyż, 2020, Hasbaoui et al., 2021). Case studies have shown that vitamin B12 deficiency in exclusively breastfed infants can lead to severe symptoms such as somnolence and developmental regression, with the potential for long-term psychomotor impairment if diagnosis is delayed (Dubaj and Czyż, 2020). These findings highlight the importance of early screening and intervention, even in the absence of overt maternal deficiency symptoms. In addition to structural and behavioural impairments, vitamin B12 deficiency may also affect neural activity at the electrophysiological level.

Vitamin B12 deficiency has also been associated with abnormal brain activity patterns, such as hypsarrhythmia observed in infantile spasm cases. Case reports indicate that such EEG abnormalities are reversible following vitamin B12 supplementation (Walton et al., 1954, West and Ellis, 1966, Arican et al., 2016).

These findings collectively underscore the essential role of vitamin B12 in maintaining normal neural development and function. This is especially relevant for cognitive function and mental health in older adults, who are at increased risk of vitamin B12 deficiency and age-related neurological disorders.

1.1.2 Health Consequences of Vitamin B12 Deficiency

Vitamin B12 plays an essential role in the normal functioning of the nervous system, particularly in maintaining cognitive function. Numerous studies have reported associations between vitamin B12 deficiency and various neurological and psychiatric disorders, including cognitive decline, Alzheimer’s disease, and depression. This section reviews the health consequences of vitamin B12 deficiency, focusing on its impact on cognitive performance and the potential effects of supplementation.

(1) Associations between Vitamin B12 Deficiency and Dementia or Psychiatric Disorders

Several studies have found that individuals with Alzheimer’s-type dementia tend to have lower serum vitamin B12 levels compared to those with other types of dementia (Cole and Prchal, 1984; Wang et al., 2001; Shen, 2015). Furthermore, low serum levels of vitamin B12 and folate have been linked to an increased risk of Alzheimer’s disease and cognitive impairment (Goebels and Soyka, 2000; Hunt et al., 2014; Vinueza Veloz et al., 2022), as well as depression (Syed et al., 2013; Peppard et al., 2019; Khosravi et al., 2020).

(2) Evidence Supporting the Effects of Supplementation

Several studies have shown that vitamin B12 supplementation, often in combination with folic acid, can improve specific cognitive domains in older individuals. These include improvements in episodic memory (immediate and delayed recall), attention, and visuospatial processing, and executive functions such as working memory and calculation abilities (Walker et al., 2012; Ma et al., 2019; Wang et al., 2022, Zhou et al., (2023)).

These findings suggest a potential role for vitamin B12 supplementation in supporting frontal and parietal lobe-related cognitive processes.

(3) Studies Reporting Limited Effects of Supplementation

In contrast, some studies have reported no significant cognitive or neurological benefits from vitamin B12 supplementation. For example, Kwok et al. (2008) and Dangour et al. (2015) found that oral supplementation in older adults with moderate deficiency failed to improve cognitive performance. Specifically, Dangour et al. (2015) reported that 12 months of daily oral vitamin B12 supplementation (1 mg/day) in older adults with serum levels between 107 and 210 pmol/L and no anemia did not improve nerve conduction or cognitive outcomes.

Martin et al. (1992) found that only 11 out of 18 patients with low serum cobalamin concentrations (<150 pmol/L) showed cognitive improvement following high-dose B12 therapy for at least six months. This observation suggests that there may be a critical window during which intervention is most effective.

(4) Considerations for Interpretation and the Role of Biomarkers

These mixed findings highlight the importance of considering several factors when interpreting the effects of vitamin B12 supplementation, including baseline vitamin status, dosage and duration of treatment, and the presence or absence of neurological symptoms (Tardy and Pouteau, 2020). Moreover, metabolic biomarkers such as methylmalonic acid (MMA) and homocysteine may serve as more sensitive indicators of functional B12 status than serum concentrations alone. Elevated levels of these metabolites have been associated with reduced brain volume, impaired memory, and compromised white matter integrity (Tangney et al., 2011).

Conclusion

Taken together, the evidence indicates that vitamin B12 supplementation may support cognitive function in individuals with confirmed deficiency. However, its effectiveness in asymptomatic individuals or those with marginal B12 levels remains inconclusive. Further research is needed to determine the optimal timing and target populations for intervention, as well as the most reliable biomarkers for identifying functionally significant deficiency.

1.1.3 Impact of Vitamin B12 Supplementation on Brain Activity in Subjects without Diagnosed Vitamin B12 Deficiency

Vitamin B12 supplementation in young individuals without diagnosed vitamin B12 deficiency has been shown to modulate neural activity. Smith et al. (2017) investigated whether dietary intervention aimed at increasing gamma-aminobutyric acid (GABA) levels could influence neural responses to sensory stimuli. In a double-blind study involving 28 healthy adults, participants consumed either a yeast extract product rich in glutamate and B vitamins or a placebo for one month. Using steady-state EEG, the study found that the neural response to flickering visual patterns was significantly reduced in the yeast extract group but not in the placebo group. These results suggest that dietary components associated with GABA synthesis—including vitamin B12—can modulate cortical excitability. Prior research indicates that vitamin B12 may enhance GABA production and suppress glutamatergic activity (Ikeda et al., 1997), potentially accounting for the observed changes in visual cortical responses. These results suggest that dietary components associated with GABA synthesis—including vitamin B12—can modulate cortical excitability. However, as the yeast extract contained various B vitamins—including B6 and B12—as well as glutamate, the specific contribution of each component remains unclear.

To isolate the role of vitamin B12, Kang et al. (2019) conducted a study using a rodent to examine the effects of high-dose vitamin B12 supplementation alone on neural responses. In this experiment, 29 healthy rats received either standard water or water enriched with vitamin B12, and sensory-evoked potentials were recorded from the barrel cortex following whisker stimulation (Kang et al., 2019). Results showed that the peak sensory-evoked potential in the B12 group was 25% higher than in the control group. These findings suggest that B12 may support neural function even in healthy individuals, highlighting its potential neuroprotective role.

Notably, while Smith et al. (2017) observed a reduction in visual cortical responses following intake of a B vitamin-rich yeast extract, Kang et al. (2019) reported an increase in the amplitude of sensory-evoked responses in the barrel cortex following high-dose vitamin B12 supplementation alone. These divergent results may reflect differences in stimulus modality (visual vs. tactile), the type of neural activity measured (steady-state vs. event-related responses), the cortical area studied (visual vs. somatosensory), or species-specific and nutritional differences. Such observations underscore the complexity of vitamin B12's effects on the nervous system and highlight the need for further research to clarify the conditions under which B12 modulates neural excitability.

Given these findings and the remaining questions regarding the effects of vitamin B12 supplementation on sensory processing, the present study investigated whether vitamin B12 supplementation enhances whisker sensitivity and modulates neural activity in different brain regions such as the thalamus and barrel cortex and intra-cortical circuits. To this end, a behavioural test was conducted to assess whisker sensitivity, and *in vivo* and *ex vivo* electrophysiological recordings were conducted to examine the effects of B12 supplementation on neural responses across regions (*in vivo*) and on intra cortical signal processing (*ex vivo*).

The following section provides an overview of the whisker sensory pathways, which is essential for interpreting both the behavioural and neural data obtained in this study.

1.2 Neural Basis of Whisker Sensory Processing

This section outlines the anatomical and physiological basis of whisker sensory processing in rodents and introduces the experimental techniques used to investigate neural responses. The rodent whisker system is a highly organised tactile system that enables precise detection and discrimination of environmental stimuli, making it an ideal model for studying sensory coding and plasticity in the mammalian brain. Furthermore, because the thalamocortical circuit underlying whisker processing is well characterised both anatomically and functionally, it provides a powerful framework for linking cellular mechanisms to behaviour.

Section 1.2.1 describes the whisker sensory pathway from peripheral receptors to the barrel cortex, highlighting the topographic and hierarchical structure of the thalamocortical circuit. Section 1.2.2 introduces *in vivo* extracellular recording techniques, with a focus on local field potentials (LFPs) as a tool to study population-level neural activity. Section 1.2.3 explains *ex vivo* electrophysiological methods for assessing synaptic function and plasticity within intra-cortical circuits using acute brain slices. Finally, Section 1.2.4 discusses sensory adaptation in the whisker system, a fundamental phenomenon in which neural responses decrease with repetitive stimulation. Together, these subsections provide essential background for understanding how vitamin B12 supplementation might modulate sensory function at both behavioural and neural levels.

1.2.1 Whisker Sensory Signal Pathway

In rodents, the whisker system serves as a highly specialised and spatially organised tactile apparatus that enables efficient environmental exploration and object recognition (Adibi, 2019). Each whisker on the mystacial pad is innervated by a dense array of mechanoreceptors and associated afferent fibres, which transmit sensory information to the brainstem via the trigeminal nerve.

The primary relay station in the brainstem is the principal sensory trigeminal nucleus (Pr5), where whisker signals are topographically mapped as “barrelettes” (Erzurumlu et al., 2010). These signals are subsequently transmitted to the contralateral thalamus, specifically the ventral posteromedial nucleus (VPM), which maintains this somatotopic arrangement as “barreloids” (Haidarliu and Ahissar, 2001; Kitazawa and Rijli, 2018). VPM neurons then project primarily to layer 4 of the primary somatosensory cortex (S1), within a distinct region known as the barrel cortex (Woolsey and Van der Loos, 1970; El-Boustani et al., 2020).

Each barrel in layer 4 of the barrel cortex corresponds to a single whisker, forming a one-to-one representation that facilitates high-resolution spatial processing (Jones, 1998; Feldmeyer et al., 2013). Layer 4 neurons receive direct thalamic inputs and relay information to layers 2/3 for further integration and intracortical processing (Brecht, 2007; Wimmer et al., 2010).

In addition to the VPM, the posteromedial complex (PoM) of the thalamus provides modulatory input to S1, projecting mainly to layers 1 and 5A. Compared to VPM, PoM neurons have broader receptive fields and integrate information from multiple whiskers, contributing to temporal integration and bilateral sensory representation (Castejon et al., 2021; Sermet et al., 2019). This hierarchical and highly organised pathway—from the whisker pad to the barrel cortex—supports precise temporal and spatial coding of tactile stimuli. Therefore, it provides an ideal model for investigating how sensory information is processed in the mammalian brain.

To better understand how sensory signals are represented and processed within the thalamocortical circuit, it is essential to measure the electrical activity generated by neuronal populations. The next section introduces extracellular recording techniques, with a particular focus on local field potentials (LFPs), which provide valuable insights into population-level synaptic activity.

1.2.2 *In vivo* Extracellular Recordings

To investigate how sensory information is processed in the brain, electrophysiological recordings such as extracellular field recordings are widely employed. Extracellular recordings capture voltage fluctuations outside neurons and can be separated into low-frequency local field potentials (LFPs) and high-frequency multi-unit activity by applying appropriate filters (Buzsáki et al., 2012). Among these, LFPs are typically obtained by low-pass filtering the signal below ~300 Hz (Burns et al., 2010; Gawne, 2010; Lindén et al., 2011; Buzsáki et al., 2012; Einevoll et al., 2013; Dähne et al., 2014; Ahmadi, 2021), and are thought to primarily reflect synchronised synaptic activity from local neuronal populations, especially pyramidal cells arranged in an open-field geometry (Okun et al., 2010).

LFPs arise from transmembrane currents that form electrical dipoles, such as during excitatory postsynaptic events, in which sodium or calcium ions flow into the neurons to create a current sink, and exit through other membrane regions, forming a source (Dähne et al., 2014). Although LFPs are commonly recorded in layered structures like the neocortex, they can also be observed in the thalamus, which lacks such laminar organisation. Studies suggest that early components of thalamic LFPs reflect local synaptic activity, whereas later components may be influenced by cortical feedback (Temereanca and Simons, 2003, Yang et al., 2013, Anderson et al., 2017, Średniawa et al., 2023). These features make LFP recordings a powerful approach for examining neural processing across both cortical and subcortical circuits.

While *in vivo* LFP recordings offer valuable insights into population-level synaptic activity under physiological conditions, they do not allow for precise manipulation or isolation of specific cortical circuits. To address this, the following section introduces *ex vivo* electrophysiological recordings, which enable targeted investigation of intracortical synaptic mechanisms under well-controlled experimental conditions.

In the present study, *in vivo* recording s were conducted under isoflurane anaesthesia. Anaesthetic depth was assessed using a modified Guedel classification, in which Stage III is subdivided into four planes (III-1 to III-4) of increasing anaesthetic depth (Friedberg, 1999). As anaesthetic depth can influence sensory responsiveness and network activity, recordings were done across Stage III-3 and deeper anaesthetic planes and stage to examine state-dependent neural responses.

1.2.3 *Ex vivo* Electrophysiological Recording

To further investigate the intrinsic properties of local neural circuits and synaptic connectivity in a more controlled environment, *ex vivo* electrophysiological recordings in brain slices are commonly employed. This approach enables the stimulation and recording of specific cortical layers under well-controlled physiological conditions, allowing for precise dissection of intra-cortical processing and synaptic mechanisms.

To evaluate sensory processing and plasticity mechanisms in the neocortex, stimulation of thalamorecipient layer 4 and simultaneous recording from supragranular layers, particularly layer 2/3, has been widely employed in *ex vivo* slice preparations. This setup enables precise assessment of synaptic efficacy and circuit-level responses within the canonical feedforward pathway of the barrel cortex (Feldmeyer et al., 2002; Bender et al, 2006). In particular, stimulating layer 4 while recording from layer 2/3 enables the characterisation of input–output (I/O) relationships, which reflect synaptic strength and connectivity under varying stimulus intensities (Feldmeyer et al., 2002).

Furthermore, the use of paired-pulse stimulation at different inter-stimulus intervals is a well-established method to assess short-term synaptic plasticity, including facilitation and depression, within this pathway (Bender et al, 2006; Feldman, 2009). These short-term dynamics are not only essential for sensory signal transmission but also contribute to the temporal filtering properties of the cortex.

Sensory adaptation, typically assessed by delivering trains of stimuli at varying frequencies (e.g., 2–20 Hz), reveals frequency-dependent suppression or modulation of synaptic responses, providing insight into the system's ability to dynamically regulate its responsiveness to repeated sensory inputs (Khatri et al., 2004; Gabernet et al., 2005). The layer 4 – layer 2/3 projection is particularly relevant in this context, as it forms a major conduit for the transmission and modulation of tactile information in the rodent whisker system.

Therefore, the present study employed electrical stimulation of layer 4 with extracellular recordings from layer 2/3 to systematically evaluate I/O response characteristics, short-term synaptic plasticity, and frequency-dependent adaptation. This paradigm builds on and extends previous studies by combining these measurements in both control and intervention groups, thereby allowing for a comprehensive assessment of cortical function under experimental manipulation.

Building upon the investigation of synaptic responses in both *in vivo* and *ex vivo* settings, the next section focuses on sensory adaptation —a dynamic process in which the neural system adjusts its responsiveness to repeated stimulation.

1.2.4 Sensory Adaptation in the Whisker System

Sensory adaptation, also known as neuronal adaptation, refers to the process by which neural circuits reduce their response to a sustained or repetitive stimulus over time. The phenomenon is considered essential for preventing sensory overload and conserving neural resources, allowing organisms to maintain sensitivity to their environment (Barros-Zulaica and Villa, 2019). This reduction in responsiveness allows the nervous system to focus on new or changing stimuli, ensuring that only important or novel information is prioritised for further processing. Sensory adaptation was first demonstrated in cats by Adrian and Zotteman in 1926 (Adrian and Zotterman, 1926, Castro-Alamancos, 2004, Rodriguez et al., 2022). It occurs in all sensory modalities, and in various stages of sensory processing, from trigeminal ganglion through the brainstem and sensory thalamic nuclei to somatosensory cortex (Adibi and Lampl, 2021). The phenomenon has been well studied in rodents' barrel cortex (Castro-Alamancos, 2004, Katz et al., 2006, Martin-Cortecero and Nuñez, 2014, Kheradpezhouh et al., 2017, Barros-Zulaica and Villa, 2019, Rodriguez et al., 2022).

It is indicated that the subcortical areas, such as Pr5 and VPM, exhibit less pronounced adaptation and reach a steady state more quickly than the barrel cortex (Martin-Cortecero and Nuñez, 2014). For example, at 5 Hz whisker stimulation, neuronal responses in the Pr5 decreased by approximately 20%, while the VPM thalamus and somatosensory cortex exhibited reductions of around 50% and 80%, respectively (Castro-Alamancos, 2002, Martin-Cortecero and Nuñez, 2014). It is suggested that the adaptation in the thalamus may arise from synaptic depression, potentially caused by input from trigemino-thalamic synapses.

Therefore, understanding the dynamics of sensory adaptation across different brain regions provides a crucial foundation for investigating whether vitamin B12 supplementation modulates neural responsiveness and enhances whisker-based sensory perception.

Building on the electrophysiological evidence of sensory adaptation in the thalamocortical circuit, it is also important to assess whether these neural changes translate into perceptible differences in behaviour. The following section introduces behavioural paradigms used to evaluate whisker-based sensory perception in rodents, providing a complementary perspective to the neural findings.

1.3 Behavioural Assessment of Whisker Sensitivity in Rodents.

Behavioural tests are essential for assessing how rodents gather and respond to sensory information through their whiskers. Some behavioural tests evaluate sensory discrimination and neural plasticity, providing insights into the mechanisms underlying perception and learning. A common approach involves tasks that measure the ability of rodents to differentiate between various textures, relying on their whisker-mediated sensory system.

There are two main approaches used for behavioural testing of whisker sensitivity. One approach involves prolonged training of animals (Carvell and Simons, 1990, Prigg et al., 2002, von Heimendahl et al., 2007, Chen and Margolis, 2015, Vecchia et al., 2020). In such studies, animals are deprived of food or water and trained to discriminate between textures. When they correctly identify a texture, they receive a food or water reward. These tests require significant preparation, including animal training and sometimes surgery, which can be time-consuming and often involves specialised techniques and equipment.

Another approach uses spontaneous behavioural paradigms, such as the textured novel object recognition test (tNORT), which require less training and utilises the innate behaviours of rodents, making them less time-intensive (Wu et al., 2013, Kwak et al., 2020, Sabzalizadeh et al., 2021, Balasco et al., 2022). This test leverages natural tendency of rodents to spend more time exploring a novel object than a familiar one (Ibañez et al., 2023). Specifically, animals are placed in a test arena containing one familiar textured object and one novel textured object for a few minutes. The time spent exploring each object is recorded, and a discrimination index (DI) is calculated to measure sensitivity (Antunes and Biala, 2012). The tNORT requires only basic equipment, such as a test arena, objects, and sandpaper to cover the objects. It does not require training or food (or water) deprivation.

However, existing tNORT protocols lack established guidelines for repeated testing. Since the tNORT relies on rodents' natural tendency to explore novel objects longer than familiar ones, it requires sufficient object exploration to yield valid results. Repeated sessions may, however, reduce the animals' interest in the textured objects, potentially resulting in shorter exploration times. To date, there has been insufficient investigation into optimal inter-test intervals and object complexity to maintain consistent exploration behaviour.

For our project, repeated tNORT sessions are crucial to assess whether vitamin B12 supplementation enhances whisker sensitivity. By evaluating varying levels of texture differences across multiple sessions, we aim to determine whether vitamin B12 improves sensory perception. In a methodological study, we modified the existing tNORT protocol to enable repeated measures, facilitating this investigation prior to testing the effect of vitamin B12 supplementation on whisker sensitivity in rats. This modified repeated tNORT protocol has been published (Hayashi et al., 2024).

Building upon these behavioural assessments, the present study aimed to investigate the effect of vitamin B12 supplementation on whisker sensitivity and its underlying neural mechanisms.

Together, the findings from electrophysiological and behavioural assessments provide a strong foundation for investigating the potential effects of vitamin B12 on whisker-based sensory function. The following section outlines the central hypothesis, research objectives, and experimental approaches employed to address this question.

1.4 Research Hypothesis, Objectives and Approaches

The overall aim of this research is to investigate the effects of vitamin B12 supplementation on whisker-based sensory processing and neural activity in healthy rats, integrating behavioural, *in vivo*, and *ex vivo* electrophysiological approaches. Based on the literature reviewed in Sections 1.1-1.3, vitamin B12 has the potential to modulate neural excitability, enhance sensory processing, and improve perceptual discrimination. These considerations led to the following hypotheses:

Hypotheses:

1. **Enhanced Whisker Sensitivity:**

   Vitamin B12 supplementation improves whisker-based texture discrimination in rats, as indicated by a higher discrimination index in the tNORT compared to control group.

2. **Enhanced Sensory Signal Processing in Different Brain Regions:**

   Vitamin B12 supplementation increases the amplitude and/or reduces the latency of sensory-evoked LFPs in both the thalamus and barrel cortex under Stage III-3 anaesthesia, as assessed through input-output relationships across stimulation intensities and sensory adaptation across stimulation frequencies. Group comparisons were conducted separately within each brain region to directly assess the supplementation effects.

3. **Strengthened Intracortical Signal Processing:**

   Vitamin B12 supplementation facilitates synaptic transmission across cortical layers involved in whisker sensory processing, reflected in changes to input-output relationships, short-term plasticity and sensory adaptation in *ex vivo* recordings. This section will enabled us to tease apart central from peripheral effects of the supplementation.

To test these hypotheses, a combination of behavioural and electrophysiological experiments was conducted.

Overview of Study Design:

Prior to the main experiments, a methodological study was conducted to develop a reliable version of the tNORT suitable for repeated behavioural assessments. The methodological study evaluated object shapes, grit discrimination, and the effects of repeated trials, and its findings are presented in Chapter 2. These results informed the selection of sandpaper grit textures used as tactile stimuli in the main behavioural experiment.

The main study consisted of three experimental approaches:

1. **Behavioural assessment of whisker sensitivity:**

   Rats received vitamin B12 supplementation or vehicle treatment. tNORT was performed before and after the supplementation using selected sandpaper grits. Improved performance after B12 supplementation was expected to indicate enhanced sensory discrimination.

2. ***In vivo* electrophysiology of the thalamus and barrel cortex:**

   Sensory-evoked LFPs were recorded simultaneously from the thalamus and barrel cortex in response to whisker pad stimulation. Recordings were conducted under multiple anaesthetic depths, stimulation intensities, and frequencies. However, statistical analyses were restricted to Stage III-3 anaesthesia to simplify comparisons. Within this condition, analyses focused on input-output relationships and sensory adaptation with group comparisons performed separately for each brain region.

3. ***Ex vivo* assessment of intracortical processing:**

   Field potentials were recorded from cortical slices to evaluate layer-specific signal processing. Stimuli varied in intensity, inter-stimulus interval and frequency. Differences between groups were interpreted with reference to the proposed effects of B12 on cortical network function, enabling assessment of synaptic strength, short-term plasticity and sensory adaptation.

Novelty and Significance of the Study:

This study is the first to systematically examine the effects of vitamin B12 supplementation on whisker-based sensory discrimination and on sensory processing in both the thalamus and barrel cortex, as well as intracortical circuits, in healthy rats. By combining behavioural assessments with *in vivo* and *ex vivo* electrophysiology, the research provides an integrated understanding of how B12 influences sensory perception and neural circuit function. The findings are expected to contribute to a broader understanding of the non-deficiency-related roles of vitamin B12 in the nervous system.

1.5 Chapter Outline

Chapter 2 presents the methodological study conducted to adapt the tNORT for repeated assessments of whisker-based texture discrimination in rats. The chapter describes the development of complex-shaped objects and the use of both the sandy and laminar sides of the same sandpaper to minimise olfactory cues. Using an inter-test interval of seven days, the study determined the sandpaper grit threshold for reliable discrimination, providing a protocol suitable for evaluating sensory changes before and after supplementation.

Chapter 3 reports the main behavioural experiments investigating the effects of vitamin B12 supplementation on whisker-based texture discrimination using the optimised tNORT protocol. Discrimination indices were compared between vitamin B12-supplemented and control groups before and after the intervention.

Chapter 4 details the *in vivo* electrophysiological experiments examining sensory-evoked LFPs in the thalamus and barrel cortex. Recordings were performed under Stage III-3 anaesthesia, focusing on input–output relationships across stimulation intensities and sensory adaptation across stimulation frequencies, with group comparisons conducted separately for each brain region.

Chapter 5 describes the *ex vivo* electrophysiological recordings in barrel cortex slices, assessing layer-specific signal transmission from layer 4 to layers 2/3. Analyses included input–output relationships, short-term synaptic plasticity, and sensory adaptation, enabling evaluation of intracortical processing differences between groups.

Finally, Chapter 6 integrates the behavioural and electrophysiological findings, discusses their implications for understanding the role of vitamin B12 in sensory processing under non-deficiency conditions, and outlines potential directions for future research.

Chapter 2 Behavioural Tests in the Methodological Study

This chapter is based on a published article:

Hayashi, et al. (2024). An effective textured Novel Object Recognition Test (tNORT) for repeated measure of whisker sensitivity of rodents. Behavioural Brain Research, 472, 115153. Minor formatting adjustments have been made for consistency with the thesis.

This study aimed to establish a reliable behavioural method for identifying the sensitivity threshold of the whisker system in rats. An adapted version of the textured Novel Object Recognition Test (tNORT) was used, which allowed repeated testing without loss of exploratory motivation, thereby enabling precise assessment of the minimum detectable texture difference. The findings informed the design and rationale of the subsequent experiments described in Chapter 3.

2.1 Introduction

The whisker-mediated texture discrimination task has been used to investigate both cognitive function and neural mechanisms underlying the sensory networks of rodents (Carvel and Simons., 1990, Diamond et al., 2008, Wolfe et al., 2008, Morita et al., 2011, Garion et al., 2014, Chen et al., 2015., Grant and Goss., 2022). The firing rate of neurons in the barrel cortex was shown to impact directly on the rodent's judgement of texture (von Heimendahl et al., 2007), with average firing rate increasing as the roughness of the texture increased. Furthermore, activity of layer 5 pyramidal neurons was shown to impact behavioural reaction time in a whisker-based texture discrimination task (Vecchia et al 2020). It was suggested that the activation of a specific inhibitory circuit underlie such control. A recent study used an array of neurophysiological methods and behavioural assessments including tNORT and demonstrated that increasing tonic inhibition in the thalamus enhanced tactile acuity through texture discrimination in mice (Kwak et al., 2020). Specifically, they conducted the tNORT using two textures with subtle differences and showed that the control group failed the test while the experimental group with enhanced tonic inhibition was able to complete the test successfully. These findings suggest that a measure of the threshold of whisker sensitivity to texture discrimination between groups of rodents, or a change of such measure within a group before and after an intervention, may reflect differences in the effectiveness of neural inhibition underlying sensory perception, with potential implications on altered balance between neural excitation and inhibition.

Historically, the whisker-mediated texture discrimination task required a prolonged training period involving food/water rewards. More recently, a simpler texture discrimination paradigm was developed, based on the novel object recognition (NOR) test (Wu et al., 2013). The paradigm exploited an innate preference of rodents for novelty, i.e., a rodent will spend more time exploring novel rather than familiar objects (Berlyne et al., 1950, Ennaceur et al., 1998, Bevins et al., 2006, Ennaceur et al., 2010., Chao et al., 2022). Instead of using object shapes, the proposed paradigm used the surface texture of objects to distinguish a novel object from a familiar one. Due to this innate preference, the paradigm required no food/water deprivation and only limited training consisting of two 10-min sessions (one session per day) to habituate rodents to the test arena prior to the day of the test. Since its publication, the textured novel object recognition test (tNORT) has been used to investigate texture discrimination learning (Pacchiarini et al., 2020), cortical-hippocampal connectivity in somatosensory processing in mice displaying autistic-like behaviours vs controls (Balasco et al., 2022a, Balasco et al., 2022b) and other neural and genetic mechanisms underlying sensory information processing (Sabzalizadeh et al., 2021, Kuang et al., 2022, Kwak et al., 2020).

An important consideration when conducting a texture discrimination experiment in rodents is the selection of appropriate textures for the objects. If two textures are too similar, the discrimination task will be less likely to succeed. On the other hand, if they are too different and easy to discriminate, comparison or improvement of whisker sensitivities between or within groups will not be possible. When investigating the sensitivity threshold of the whisker system, iterative tNORT plays a pivotal role in identifying the minimum particle size difference that a specific group of rodents may be able to distinguish.

In many studies, sandpapers of varying grit sizes are used to cover the surface of objects in tNORT to provide a range of textures. The difference between the average particle diameter of a pair of sandpapers is used as an indicator of difficulty associated with the discrimination task (Kwak et al., 2020, Wu et al., 2013, Sabzalizadeh et al., 2021, Balasco et al., 2022a, Balasco et al., 2022b). However, it is possible to select different pairs of sandpapers with the same difference in particle diameters, while the roughness of the sandpapers themselves is different. For example, the difference in average particle diameters (ϕ) between sandpapers P40 (ϕ = 35 μm) and P60 (ϕ = 25.8 μm) is the same as that between sandpapers P800 (ϕ = 21.8 μm) and P1500 (ϕ = 12.6 μm). It has been shown that the roughness of the sandpapers themselves plays a role in the discrimination task (Morita et al., 2011).

Another consideration associated with tNORT is the length of exploration time with respect to the objects used. If the animal is not interested in an object during the task, possibly due to its size, the complexity of its shape, or its familiarity, the object will be ignored, or the exploration time will be very brief. Such events will need to be excluded from the study, resulting in reduced animal numbers and less robust calculations of the discrimination index. This issue was closely examined in several NOR studies. It has been shown that if a NOR test was conducted repeatedly with the same group of rodents within a short time frame (e.g., daily), the total exploration time decreased markedly (Broadent et al., 2010). However, if the NOR task was repeated about 1 week apart, total exploration time could be maintained (d'Isa et al., 2014). Furthermore, total exploration time was shown to increase if more complex objects were used in NOR tasks (Aubele et al., .2008, Chrzanowska et al., 2022). Very limited studies using tNORT have examined the effect of repeated tests on the exploration time. We conducted a methodological study of tNORT (not published) which indicated that a tNORT repeated in three consecutive days markedly decreased the exploration time of rats. By the third test, over 50 % of the rats explored one of the objects less than 2 s within a 5 mins duration. Our methodological study suggested that to conduct repeated tNORTs to investigate whisker sensitivity to texture discrimination, careful consideration is needed in designing its protocol.

Here, we propose a modified tNORT paradigm which is robust to repeated tests. We increased the complexity of the object shapes by combining several simple objects and increased the inter-test-interval to one week. Instead of selecting two different sandpapers for each test, we used both sides (i.e., sandy and laminar) of the same sandpaper, with one side acting as the familiar texture, and the other as the novel texture, thus removing the need to select two different sandpapers for a tNORT while simultaneously eliminating possible olfactory cues. We suggest that the modified tNORT is sufficiently robust to repeated testing, allowing whisker sensitivity to texture to be investigated and compared across multiple groups of rodents.

2.2 Materials and Methods

Behavioural tests in rodents are important to evaluate a huge variety of neurological assessments such as locomotor activity, depression-like behaviour and memory. In this section, several behavioural tests are introduced, and general considerations for behavioural assessment in rodents are described.

2.2.1 Animals

Eight male Lister hooded rats (weighing 309 ~ 329 g at purchase) were used. Rats were caged in pairs for three days to acclimatise to the environment after arrival, with ad libitum access to food and water and housed under controlled temperature (21◦C), humidity (50 ± 10 %) and 12-hr light-dark cycle (lights off 6:00–18:00 hr). All procedures were conducted in the morning under dim red-light illumination to minimize stress and visual cues contributing to task performance (Wu et al., 2013). All habituation and behaviour tests were conducted in the same room as the rats were housed.

2.2.2 Construction of Complex Objects

Eight complex objects were constructed from four simple shapes 3D printed in-house (Figure 2.1A). Each complex object combined three simple objects (Figure 2.1B) to create different shapes, with multiple copies made for each complex shape so that no single object was used more than once in a tNORT to eliminate any lingering olfactory cues.

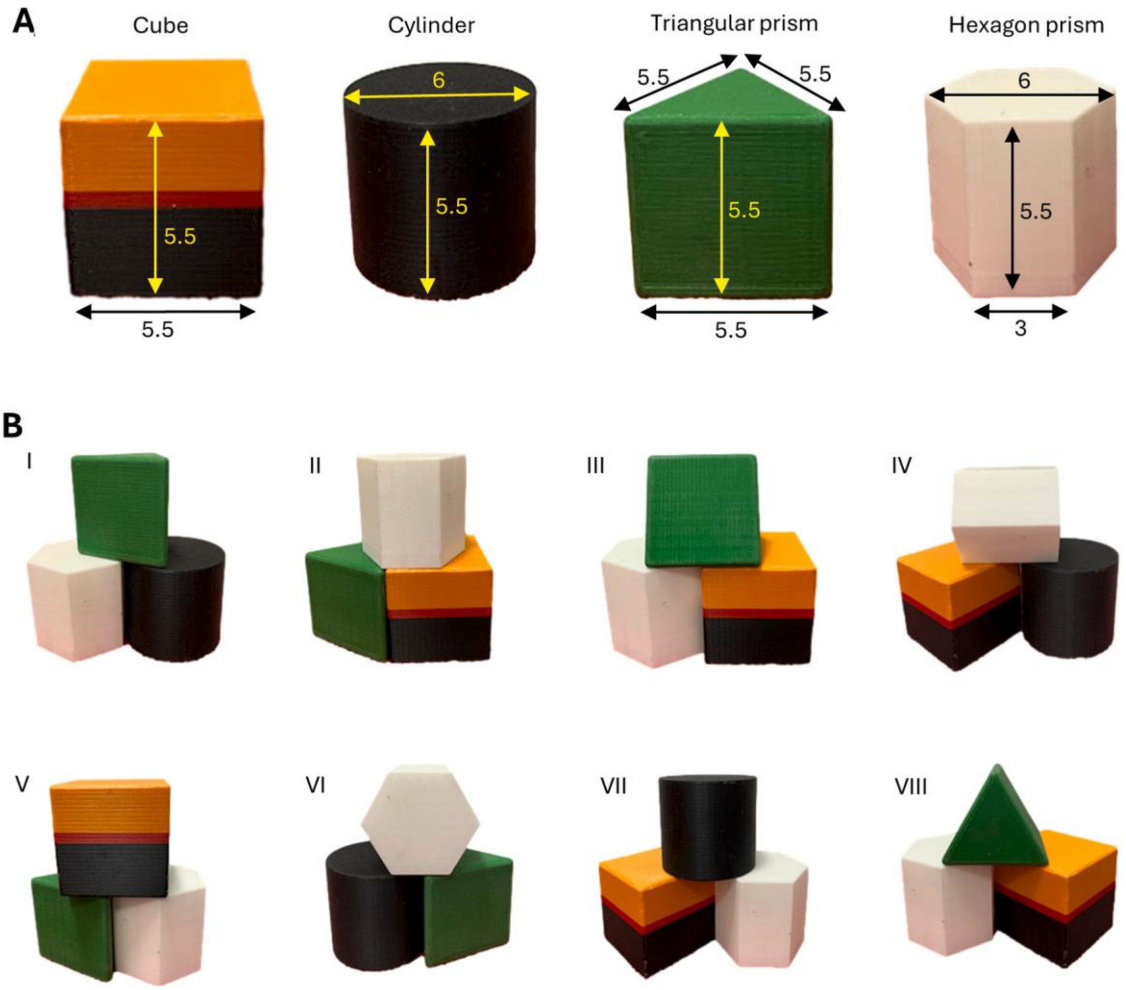

Figure 2.1 Design of objects. (A) Photos and dimensions of four simple objects. (All dimensions are in cm.) (B) Photos of eight complex objects made from three simple objects. Note that the colour of each object is irrelevant to the task as all objects were covered in sandpaper during experiments

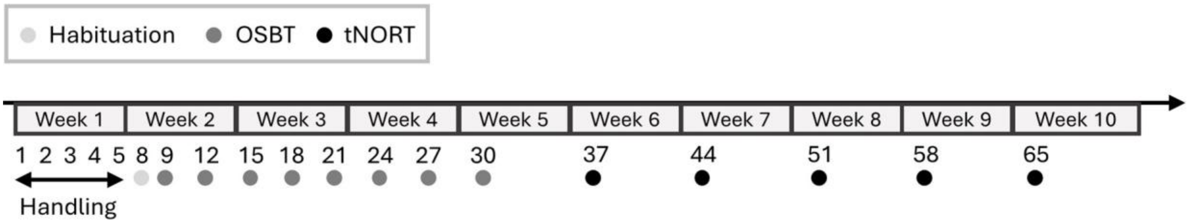

Figure 2.2 Overall schedule from the first day of rat handling to the completion of the tNORT test. OSBT: Object Shape Bias Test. tNORT: textured Novel Object Recognition Test.

2.2.3 Experimental Protocols

Two sets of experiments were conducted. In the first study, we conducted object shape bias tests (OSBTs) to investigate if rats explored certain objects more than others among the eight complex objects we constructed. In the second study, we performed repeated tNORTs, with each test using a different complex object covered in sandpapers of different grit sizes chosen prior to the experiment. The repeated tNORT stopped when rats failed the texture discrimination task in two tests with sandpapers of finer particle sizes.

The overall schedule is shown in Figure 2.2. Week 1 is defined as the week immediately after the acclimatisation period; and Day 1 refers to the first day of Week 1. Animals were handled by an experimenter for the first five consecutive days in Week 1 to familiarise them to the experimenter and the environment, thus minimising anxiety during behavioural tests (Bevins et al., 2006). The handling protocol was as follows. For Days 1 and 2, rats were handled in their cage for 10 mins. The experimenter first put their hands in the cage to allow rats to sniff them. After a few minutes, the experimenter scooped one rat with both hands at a time, allowing it to jump between hands. For the following two days, rats were handled in their cage in a similar manner for 5 mins before two rats housed from the same cage were transferred to the empty test arena and handled in that environment for 5 mins by allowing them to jump on and off the experimenter's hands placed inside the arena and to explore the arena itself. On the last day of handling, rats were individually transferred and handled in the test arena for 5 mins. On Day 8, 24 hrs prior to the first OSBT, rats were individually put in the empty test arena for 10 mins without any handling (Bevins et al., 2006).

The test arena, made of clear acrylic, has a dimension 52x52x80 cm (WxDxH), with its base divided into a 16-square grids, each square being 13×13 cm (Figure 2.3A). The grid was drawn on the outside base of the arena to ensure they were in clear view of the camera throughout the study. For all experiments describe here, two complex objects were placed at the base of the arena, secured with double-sided tapes, 26 cm apart and 13 cm away from each adjacent wall (Figure 2.3A and B). The walls of the arena were covered with cardboards to reduce the influence of any cues from the external environment during experiments. All experiments were conducted between 6 am and 8 am on each experimental day to minimise potential acoustic contamination from the background.

The test arena was lit by four red light bulbs attached to two floor lamps, with two bulbs in each lamp. The lamps were placed opposite of each other on two sides of the test arena. The positions and angles of the light bulbs were adjusted to provide as a uniform illumination as possible on the base of the arena, with luminance values ≤ 14 lux measured at the base. The position of the arena in the test room and the positions of the two lamps were kept consistent throughout all experiments presented here.

Prior to each experiment, the experimenter made sandpaper covers for all objects to be used. These covers were precisely measured, and the edges were fixed together by double-sided tapes. They were made to fit each object securely without using further tapes or glue so that they could be removed from the object quickly after each test. During an experiment, double-sided tapes were used to combine three simple objects, covered with sandpapers, together to create the desired complex object (Figure 2.3C). Care was taken to ensure no tape was exposed on the outer surface of the objects. For all the tests presented here, no cover was removed or destroyed by the animals.

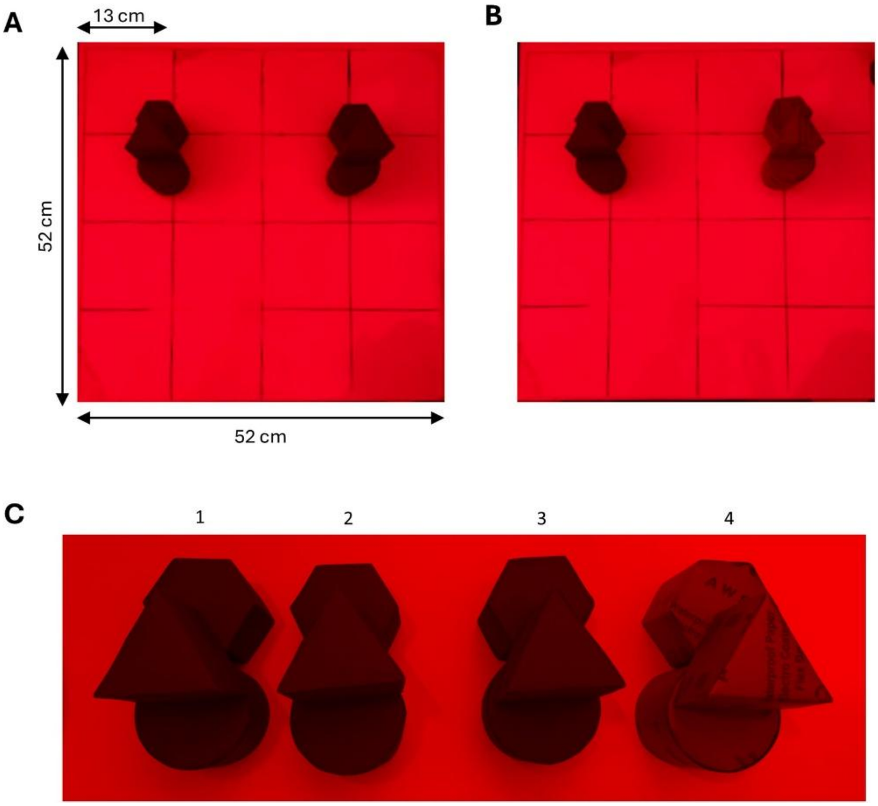

Figure 2.3 Experimental set up for OSBT and tNORT under red light illumination (A) Two objects having the same shape and cover were placed securely on the base of the test arena. This set up was used for OSBT as well as the sample phase of the tNORT. (B) Two objects having the same shape but different covers were placed securely on the base of the test arena. This set up was used for the test phase of the tNORT. (C) A close up of objects covered with sandpapers of the same grit using the sandy side (Objects 1, 2 and 3) and the laminar side (Object 4).

Selection of object shapes based on animal preference

OSBTs started on Day 9. All rats went through eight tests each separated by 72 hrs. For each test, two of the same shaped complex objects covered by the same grit-sized sandpaper P1200 (ϕ = 15.3 μm) were secured in the test arena. This extra fine sandpaper was selected primarily based on two considerations. First, finer sandpapers were easier to fold to make object covers. Second, the sandpaper used for OSBT was not used for the subsequent tNORTs to avoid bias due to familiarisation. Once the two objects were positioned inside the arena, the rat was placed close to the midpoint of the wall opposite, facing away from the objects, and left to explore the objects and the arena for 5 mins. The rat was then returned to its home cage. Sandpaper covers were discarded after the test, and the objects and the test arena cleaned with 70 % ethanol to remove olfactory cues before objects with new sandpaper covers were placed in the arena for the next test. For each test day, all eight objects (Figure 1B) were used, one for each rat (see Table 1 for the allocation of object shapes for all OSBTs). After eight test days, all rats had been exposed to all eight objects once. By Week 5, OSBTs were complete.

All tests were recorded with a video camera placed above the test arena. Exploration times were measured using the Observer software (Noldus Information Technology). The exploration time was defined as the time during which a rat's nose was less than 2 cm away from an object, excluding when the rat was resting, grooming, or playing with its tail near or on top of the objects (Ennaceur and Delacour., 1988, Bevins and Besheer., 2006, Wu et al., 2013,). Before using the Observer software, the experimenter was trained to code the rat's exploratory behaviour as follows. First, a video of a rat exploring two objects in the arena was randomly selected. Three types of exploratory behaviour were coded: no exploration, exploration of the left object, and exploration of the right object. The video was coded in real-time first by multiple users with an accuracy level set at 98 % for inter-rater reliability. Once this was reached, the video was coded again until an intra-rater reliability of 98 % was achieved. The experimental data was only coded when these criteria were met.

Table 2.1 Allocation of objects used for each rat of each experimental day

Rat #	Day 9 (Tue)	Day 12 (Fri)	Day 15 (Mon)	Day 18 (Thu)	Day 21 (Sun)	Day 24 (Wed)	Day 27 (Sat)	Day 30 (Tue)
1	I	II	III	IV	V	VI	VII	VIII
2	II	V	I	VII	III	VIII	IV	VI
3	III	I	IV	II	VI	V	VIII	VII
4	IV	III	VII	I	VIII	II	VI	V
5	V	VI	II	VIII	I	VII	III	IV
6	VI	IV	VIII	III	VII	I	V	II
7	VII	VIII	V	VI	II	IV	I	III
8	VIII	VII	VI	V	IV	III	II	I

Repeated tNORT to investigate whisker sensitivity

Repeated tNORT started in Week 6, with one tNORT conducted per week for each rat. The tNORT consisted of two phases, the sample phase and the test phase. For each tNORT, four of the same shaped complex objects were prepared and covered with the appropriate sandpaper covers (Figure 2.3C). In the sample phase, the two objects had the same sandpaper cover (Figure 2.3A). In the test phase, one object, known as the familiar object, had an identical cover as that used in the sample phase (the cover was changed between sample and test phase to prevent use of olfactory cues), while the other, known as the novel object, was covered by sandpaper of a different texture (Figure 2.3B). At the start of both phases, the rat was placed in the test arena equidistant to, and facing away from, the two objects and was allowed to explore for 4 mins. In between the two phases, the rat was moved to the holding cage for 2 mins (Taglialatela et al., 2009), known as the delay period. We set the delay period as short as possible to minimise hippocampal-mediated learning (Wu et al., 2013). During the delay period, both objects in the sample phase were removed, the sandpaper covers discarded, and the test arena cleaned with 70 % ethanol to remove olfactory cues. The two new objects prepared for the test phase were then placed in the arena. After each tNORT, all objects were cleaned with 70 % ethanol, and the apparatus was thoroughly cleaned.

2.2.4 Selection of Familiar and Novel Textures

When selecting sandpapers for familiar and novel textures in the tNORT, instead of selecting two different sandpapers with different grit sizes, we used the same sandpaper but assigned at random the sandy and the laminar sides of the paper as familiar (used for both objects in the sample phase and one object in the test phase) and novel (one object in the test phase) textures, thus eliminating olfactory cues from using different types of sandpapers. The texture selection protocol was balanced across all eight rats used for tNORTs.

2.2.5 Whisker Sensitivity Test

To test whisker sensitivity to textures, we conducted a set of tNORTs using different grit size sandpapers with a criterion for stopping the experiment if rats failed the tNORT for two discrimination tests using sandpapers of finer particle diameters, an indication that a breakpoint was reached where the animals could no longer discriminate between two textures. Figure 2.4 shows the five sandpapers used and the corresponding particle diameters. The sandpaper selection procedure was based on the statistical analysis of the tNORT data with respect to each selection and will be described in the Results section.

The position (left or right) and texture (sandy or laminar) of the novel object were pseudo-randomised for each rat and balanced throughout. Table 2 shows the sandpaper grit and object shape used for each tNORT in the whisker sensitivity study

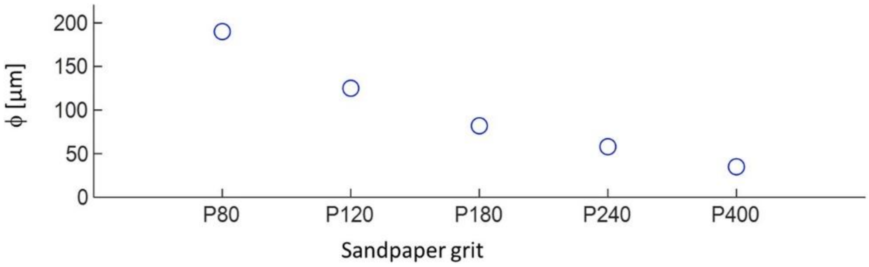

Figure 2.4 Sandpaper average particle diameters ϕ used for the five tNORTs

Table 2.2 Allocation of sandpaper grits and objects for the five tNORTs over five weeks
Note that both the sandy and laminar sides of sandpapers acted as the familiar and novel textures in a balanced manner. Thus, only one sandpaper type was selected for each tNORT.

	Day 37	**Day 44**	**Day 51**	**Day 58**	**Day 65**
Grit size	P80	P240	P120	P180	P400
Object	VII	I	III	II	VIII

2.2.6 Data Analysis and Statistics

All data analysis was performed in MATLAB (The MathsWork, Natick, MA, USA). To analyse the tNORT, the discrimination index (DI) d was calculated separately for the sample phase (d_s) and test phase (d_t), as follows (Antunes and Bera., 2012). In the sample phase,

$$d_s = \frac{T_L - T_R}{T_L + T_R} \qquad (2.1)$$

where TL and TR were the exploration times for the left and right objects respectively; whilst for the test phase,

$$d_t = \frac{T_N - T_F}{T_N + T_F} \qquad (2.2)$$

where T_N and T_F were the exploration times for the novel and familiar objects respectively. Rats with total exploration time (Ttot) less than 2 s were excluded from the analysis (Wu et al., 2013). From its definition, the value of DI would be 0 if the exploration times for the left and right objects, or the familiar and novel objects, were identical.

To determine if T_{tot} was significantly different across test days and between eight objects, all data were first processed to identify outliers (MATLAB function ‘filloutliers’). They were then tested for normality using the Jarque-Bera goodness-of-fit test (Jaque and Bera., 1987) (MATLAB function: ‘jbtest’). As no outliers were identified and all data were normally distributed, a one-way repeated measure ANOVA was performed in each case (MATLAB function ‘RMAOV1’; (Trujillo-Ortiz et al., 2004). For pairwise multiple comparisons with Bonferroni corrections, the MATLAB function ‘multcompare’ was used.

2.3 Results

2.3.1 Total Exploration Time was Maintained by Increasing Object Complexity

Figure 2.5A shows T_{tot} for the eight test days of OSBTs, each with an interest-interval of 72 hrs. We observed that T_{tot} for the first test day appeared to be longer than the other test days and repeated measures ANOVA using data from all eight test days showed a significant difference in T_{tot} ($p < .001$)

When multiple comparison tests were conducted for pairwise comparison between the eight days with Bonferroni correction, it revealed that the significant difference was indeed the result of significantly longer exploration time on Day 1 compared with all other days, apart from Day 5.

We also noted that all eight tests had a mean $T_{tot} > 30$ s, indicating that increasing object complexity could maintain rats' interests in object exploration over 8 repeated tests with an inter-test-interval of three days, provided that different shaped objects with sufficient complexity were used. The range of exploration times found in this study was like other NOR studies in the literature [13,14,26,30–32] where only a single NOR test was conducted.

We subsequently investigated if rats had preference for certain object shapes over others by comparing T_{tot} across the eight complex objects (Figure 2.5B). Again, all data were tested for normality before a repeated measure ANOVA was conducted. No significant difference was found ($p=0.814$), suggesting that rats had no preference to any of the objects. Nevertheless, for the subsequent repeated tNORTs, we chose five objects (I, II, III, VII, and VIII) which produced slightly higher T_{tot} across the eight objects.

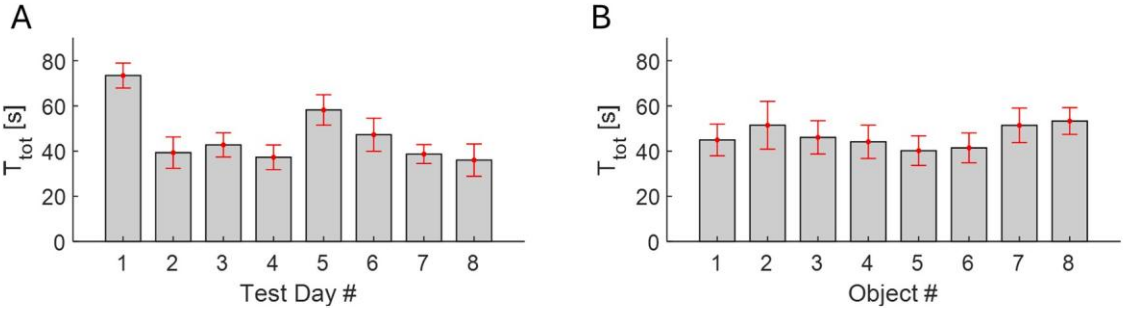

Figure 2.5 Total exploration time in OSBT. (A) Total exploration time in each test day. (B) Total exploration time for each object. Error bars indicate the standard error of the mean

2.3.2 Modified tNORT was Robust for Repeated Measures

We performed five tNORTs using five sandpaper grit sizes, with an inter-test interval of seven days. The sandpaper selection process was as follows. The first tNORT used the rather coarse sandpaper P80 (ϕ=201 μm) to ensure successful discrimination of the novel texture from the familiar one. A one-sample t-test on the DIs obtained from the test phase confirmed that the animals were indeed able to discriminate be- tween the familiar and novel textures (p=0.012). For the second tNORT, a finer sandpaper with grit size P240 (ϕ = 58.5 μm) was used. Statistical analysis showed that rats failed to detect the novel from the familiar texture (p=0.149). For the third and fourth tNORT, we used sandpapers P120 (ϕ = 125 μm) and P180 (ϕ = 82 μm) respectively and found that rats successfully detected the novel object during both tNORTs, with p=0.008 and p=0.024 respectively. For the fifth test, we further decreased the sandpaper particle size by using P400 (ϕ = 35 μm) and found that rats were unable to detect the novel texture at this finer scale (p=0.065). Thus, the experiment was terminated.

Figure 2.6A shows the exploration times for individual rats for the left and right objects in the sample phase (top) and familiar and novel objects in the test phase (bottom) respectively. The panels from left to right were arranged in the order of decreasing sandpaper particle diameters. We noted that all exploration times with respect to a single object were greater than 2 s, thus no data was excluded from the DI calculations. To examine if rats' interest in exploring the objects decreased significantly over the five tNORTs, we conducted repeated ANOVA for T_{tot} for the sample and test phases respectively over the five weeks (Figure 2.6B). No significant difference was found in either the sample phase (p=0.059) or the test phase (p=0.447). In addition, the mean T_{tot} for the sample and test phases were > 30 s for all sessions, suggesting that rats' interests in exploring these complex objects were maintained across the five weeks of testing. Figure 2.6C shows the DIs calculated for each tNORT for both the sample (top) and test (bottom) phases. It showed that during the sample phase, all DIs were not significantly different from zero, suggesting no significant bias in exploration time between the L and R objects. On the other hand, during the test phase, DIs were significantly different from zero for the three tests when the sandpapers were coarser (P80, P120, and P180). As the grit size increased (P240 and P400), or the particle size of the sandpaper decreased, the DIs became smaller and the group mean values showed no significant difference from zero, indicating that rats were unable to discriminate between the sandy and the laminar sides of sandpapers when the particle size of the sandpaper was less than 58.5 μm (P240). (For particle diameter information, see (Arabzadeh et al., 2005, Morita and Kang., 2011, Montuori et al., 2016).)

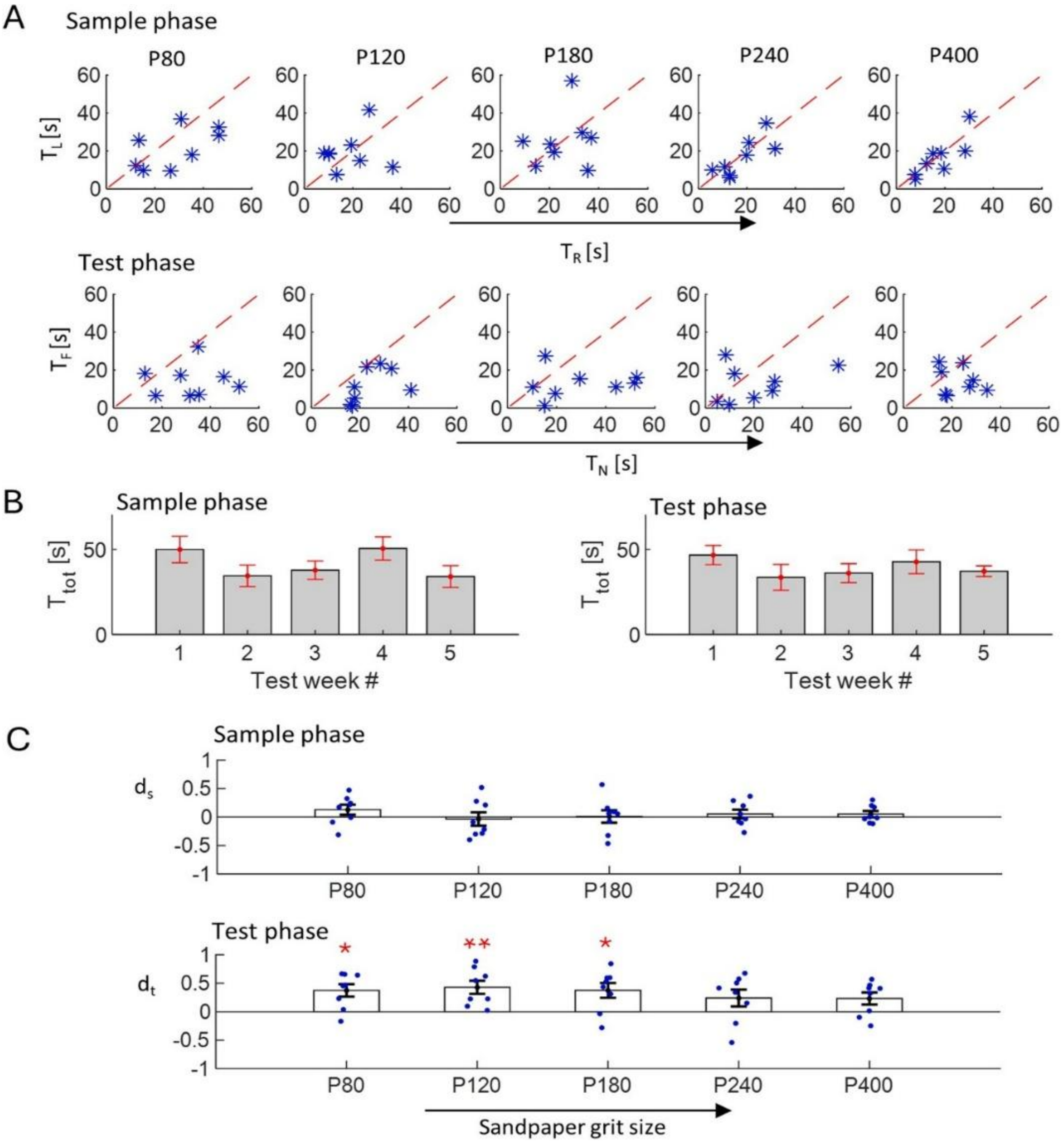

Figure 2.6 Exploration time and DI in tNORT associated with complex objects (A) Exploration times T_L vs T_R in the sample phase (top) and T_F vs T_N in the test phase (bottom) for individual rats using sandpaper of five grit sizes. The broken diagonal line represents equal exploration time for both objects. (B) Total exploration time over the five tNORTs for the sample phase (left) and the test phase (right). Error bars indicate the standard error of the mean. (C) Top: DI in each test during the sample phase. Bottom: DI in each test during the test phase. The DI for each rat was displayed as a blue dot. The height of each bar indicates the mean of DIs over all rats for each test, and error bars indicate the standard error of the mean. * and ** indicate $p < 0.05$ and $p < 0.01$ respectively.

2.4 Discussion

The tNORT is a valuable behaviour test to examine rodents' whisker system sensitivity with little training. In the present study, we extended the existing tNORT to facilitate its repeated use, and successfully identified, after five iterative tNORTs, a breakpoint where the animals could no longer discriminate between two textures. We showed that T_{tot} of the animals was consistently above 30 s in both the sample and the test phases across the five tNORTs with an inter-test-interval of seven days. In addition, exploration time with respect to individual objects for all animals was above the threshold of 2 s, thus no animal was excluded from the subsequent texture discrimination analysis. We suggest that the modified tNORT provides a viable protocol for the investigation of texture discrimination abilities of rodents at progressively finer spatial scale.

2.4.1 Effect of Other Sensory Cues on tNORT Performance

Although it has been shown that rodents could use their whisker system alone to perform texture discrimination (Guic-Robles et al., 1989, Guic-Robles et al., 1992), other sensory systems may also contribute to the task (Ennaceur., 2010). This was examined carefully by Wu et al. in their original tNORT study (Wu et al., 2013). To determine the extent to which visual cues may contribute to the tNORT due to different sandpapers having different visual appearances even under dim red-light conditions, objects were covered with plastic transparent film to create 'texture-less' objects while maintaining visual differences between them. It was found that mice with intact whisker system were unable to discriminate between these objects. Furthermore, Wu et al. investigated if mice used their paws to aid their ability to discriminate textures. They grouped mice into those that used their paws and those that did not during the tNORT. Both groups were found to be able to distinguish between the familiar and novel textures that differed by 25 μm, suggesting that tactile sensation from paws was not a major contributor to texture discrimination. Finally, it was demonstrated that mice with their mystacial vibrissae removed bilaterally were unable to discriminate between novel and familiar textures, and that the removal did not predict the number of mice that used their paws.

Evidence that the sensory system responsible for texture discrimination was primarily the whisker system was also presented in a texture discrimination study of rats with prolonged training period (52–90 days) involving rewards (Morita and Kang., 2011). It was shown that a whisker trimmed rat reduced its texture discrimination performance to chance level. The study also found that rats had better ability for texture discrimination with respect to a fine sandpaper (P1500) than to a coarse sandpaper (P150), adding another dimension to the complex neurophysiological mechanisms underlying texture discrimination.

The assumption that rodents were functionally blind under red-light illumination was challenged by a recent study (Nikbakht and Diamond., 2021). Using a visual stimulus in the form of a black and white square-wave grating, the study examined the performance of rats in categorising the stimulus orientation under conditions of white light illumination and red-light illuminations (using two red light wavelengths 626 nm and 652 nm respectively) among other wavelengths. No significant difference in the average performance was found between the white light and two red-light illuminations, with success rates reaching 87 %, 84 % and 86 % respectively. However, the performance was achieved after a lengthy training period of 4–6 weeks, with one session per day, and with rewards.

Although the above study demonstrated the range of illumination wavelengths, including red light illumination, within which the rat's visual perception capacity was intact, there were distinct differences between the above study and the tNORT paradigm presented here. First, the object contrast used in the above study was much stronger than those used for common tNORTs. The visual appearance between different grit sandpapers and between the sandy and laminar sides of the same sandpaper have much less contrast compared to the white-black grating (Figure 2.3). This difference in object contrast may affect the strength of the visual cues in the tNORT, thus significantly reduce the ability of the animal to utilise vision for the task (Schnell et al., 2023). Secondly, it is unclear, in the above study, if the level of performance of the rats under red light illumination was partly due to prolonged training. The tNORT protocol is a one-trial object recognition test. With little training, rodents are unlikely to discriminate colours of objects under dim red-light illumination (Ennaceur., 2010).

For our modified tNORT, we also noted that the smoothness of the laminar side of the sandpaper made it more reflective compared with the sandy side. To minimise this, we took extra care in positioning the red-light bulbs so that they pointed away from the arena, and the red-light illumination was dim (<14 lux). Although we cannot be certain if visual cues contributed to the successful texture discrimination for the three tNORTs using rougher sandpapers, the consistent failure of tNORTs corresponding to the two finer sandpaper grits suggested that the rodent whisker system was the dominant sensory system for texture discrimination.

2.4.2 Key Parameters Influencing the Performance of Repeated tNORTs

As far as we are aware, most objects used in published tNORT studies were simple objects such as 2-dimentional rectangular boards (Wu et al., 2013, Sabzalizadeh et al., 2021, Kuang et al., 2022), simple cubes (Kwak et al., 2020), or simple cylinder-shaped objects (Balasco et al., 2022a, Balasco et al., 2022b). This may be due to the fact that covering complex shaped objects using sandpaper was difficult. However, our own methodological study suggested that repeated tNORTs using simple shaped objects were likely to lead to significantly reduced object exploration time. To minimise exclusion of data due to short exploration time, we designed eight complex objects by combing three simple shaped objects to maintain the interest of rats in object exploration in repeated tNORTs. The OSBT indicated that the mean T_{tot} for all objects exceeded 30 s. Notably, after the first test when T_{tot} was the highest, the exploration time remained consistent statistically across the subsequent seven repetitions at 72-hr intervals. Furthermore, when these objects were used in iterative tNORTs five times with an inter-test-interval of seven days, the mean T_{tot} was maintained above 30 s. The sandpaper covers for the objects were not difficult to make but it was time consuming, as each tNORT involved 12 simple objects to be covered. One possible modification to our protocol would be to combine two instead of three simple shaped objects into a complex object, thus reducing the preparation time for conducting a tNORT. However, this simplification would need to be assessed to ensure sufficiently long and consistent T_{tot} after repeated tNORTs.

In addition to object shapes that can enhance the robustness of repeated tNORTs, the inter-test-interval also plays a key role. Repeated tNORTs separated by 24 hrs are likely to lead to significant reduction in T_{tot}, similar to that found in NOR studies (d'Isa et al., 2014). Our OSBT had an inter-test-interval of three days, with each test consisting of a single exploration session with a 5 mins duration, whereas our repeated tNORTs had an inter-test-interval of seven days, with each test consisting of two exploration sessions. The seven-day interval was chosen to ensure that the rat's interest in the objects was maintained while experimental day did not fall to a weekend for practical reasons.

One feature of the repeated tNORT protocol presented here was that instead of using two different sandpapers in a tNORT, we used both sides of a sandpaper, one acting as a familiar texture, the other as a novel texture. This approach was adopted to minimise potential olfactory cues from two different grit sandpapers while simplifying the process of making object covers.

A potential source of bias in the tNORT protocol was associated with the manual classification of the rat's exploratory behaviour by watching videos recorded during the sample and test phases. There were situations when the rat's behaviour was ambiguous and difficult to judge. For example, if a rat was sitting on an object with its head and nose facing downwards towards the object, it would be difficult to judge the distance between its nose and the object. During the software training period, experimenters would agree a set of criteria when dealing with difficult situations. We found that inter-rater agreement (98 %) on exploration time was typically reached within one week, and the sub- sequent intra-rater agreement (98 %) reached within one day. The training does not imply the elimination of bias during behaviour coding. However, such bias should be statistically the same regardless of the object being familiar or novel. As the DI reflects the difference in exploration time between the two objects, such bias should not impact significantly on the value of DI. Instead of manual classification, an alternative for behavioural coding of rodent is to adopt automated software (e.g., EthoVision), with the advantage of consistency and faster analysis.

In conclusion, the tNORT protocol presented here is a cost-effective way of investigating whisker sensitivity of rodents based on their behaviour. It has been demonstrated to be robust to repeated tests, thus allowing a range of textures to be used to identify the sensitivity threshold of rodent whisker system for texture discrimination. As the whisker sensitivity of rodents has been shown to be altered by the manipulation of tonic inhibition, the repeated tNORT protocol may be used as a tool to examine whisker sensitivity difference between different groups of rodents, or within a single group before and after an intervention. Thus, the threshold for whisker-mediated texture discrimination may be used as a marker for shifted balance between neural excitation and inhibition.

Ethics approval

All experiments were carried out in accordance with the British Home Office regulations (Animals (Scientific Procedures) Act, 1986) and approved by the Research Ethics Committee at the University of Reading, UK.

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Credit authorship contribution statement

Yurie Hayashi: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. Najeeba Alamir: review & editing. Tamagnini: Supervision. Yoshikatsu Hayashi: Supervision, Resources. Claire Williams: Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. Ying Zheng: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Data availability

Data, videos and Matlab codes supporting the results reported in this paper are openly available from the University of Reading Research Data Archive at https://doi.org/10.17864/1947.000538.

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Chapter 3

Behavioural Tests in the Main Study

3.1 Introduction

Vitamin B12 supplementation may influence sensory processing even in individuals without diagnosed deficiency. Previous studies have shown that vitamin B12 intake can modulate sensory-evoked responses in both humans and animals, although the direction of this modulation appears to depend on factors such as sensory modality, cortical region, and experimental design. For example, in humans, Smith et al. (2017) reported a reduction in steady-state visual cortical responses following dietary intake of a yeast extract rich in B vitamins, including B12. In contrast, using a rodent model, Kang et al. (2019) found that high-dose vitamin B12 supplementation increased the amplitude of whisker-evoked potentials in the barrel cortex. These contrasting findings suggest that, under certain conditions, vitamin B12 could enhance whisker-mediated tactile discrimination in rodents.

To assess whisker sensitivity, the present study employed the textured Novel Object Recognition Test (tNORT), a spontaneous behavioural paradigm that capitalises on rodents' natural preference for novel objects over familiar ones (Wu et al., 2013; Kwak et al., 2020; Sabzalizadeh et al., 2021; Balasco et al., 2022). The discrimination index (DI) is calculated from exploration times and serves as a measure of texture discrimination ability (Antunes and Biala, 2012). However, conventional tNORT protocols lack guidelines for repeated testing, which is necessary for longitudinal assessment of sensory function. Therefore, in a preceding methodological study (Chapter 2; Hayashi et al., 2024), the tNORT protocol was optimised by modifying object design and texture presentation to maintain exploration interest across sessions.

Building on the optimised protocol, the main behavioural experiment compared DI values between vitamin B12-supplemented and control rats before and after supplementation. This allowed evaluation of whether B12 intake improved whisker-based texture discrimination under repeated testing conditions.

This chapter presents the behavioural component of the main study, which forms part of a broader experimental framework combining behavioural testing, *in vivo* electrophysiology (Chapter 4), and *ex vivo* electrophysiology (Chapter 5) to investigate the effects of vitamin B12 supplementation on sensory processing at multiple levels of the nervous system.

3.2 Overview of the Main Study

This section provides an overview of the experimental design of the main study, including details of the animals used, housing conditions, and the overall experimental schedule. These details are presented here to provide context for the behavioural, *in vivo*, and *ex vivo* experiments reported in Chapters 3–5.

All experimental protocols complied with the regulations set forth by the United Kingdom Home Office (Animals (Scientific Procedures) Act, 1986) and were approved by the Research Ethics Committee at the University of Reading, UK.

3.2.1 Animals and Housing

Twenty-eight male Lister Hooded rats (Envigo, UK), weighing between 286 and 337 g (aged 9-12 weeks) at the time of purchase, were randomly allocated to either the control or the B12 groups. All procedures complied with the UK Animals (Scientific Procedures) Act (1986). The study was conducted at the University of Reading Biological Resource Unit.

Rats were pair-housed in standard cages with environmental enrichment consisting of shredded paper for nesting, two wooden gnawing blocks, and a red transparent plastic tunnel. Food (standard commercial diet (RM3(E), 801066, Special Diets Services, UK, containing 26.78µg/kg of vitamin B12) and water were available ad libitum. The housing environment was maintained at 21°C with a relative humidity of 50 ± 10% and a 12-hour reversed light-dark cycle (lights off from 05:00 to 17:00).

Following arrival on a Friday, rats were left undisturbed for 2–3 days to acclimatise to the housing environment. Subsequently, they were handled daily for one week by the experimenter to habituate them to human contact and reduce stress before experimental procedures commenced. Handling procedures were identical to those described for the methodological study (Section 2.2.3). Individual identification was performed without ear tags or microchips, using distinctive coat patterns to reliably recognise each animal throughout the study. All other housing and husbandry conditions matched those used in the methodological study.

3.2.2 Schedule of the Main Study

The experimental schedule for each animal extended over nine weeks (Figure 3.1). Week 0 (Days 1-7) consisted of daily handling sessions to familiarise the rats with the experimenter and laboratory environment. On Day 8, rats underwent habituation to the tNORT. The first behavioural assessment was conducted on Day 9 (Week 1), followed by additional tests on Day 16 (Week 2) and Day 23 (Week 3), each using different grit sandpapers.

Following the third test on Day 23, the water provided to the vitamin B12 group was replaced with vitamin B12-enriched water at 25% of the target concentration. Over the next three days, the concentration was gradually increased to 50%, 75%, and finally 100% of the target level, equivalent to 80 times the recommended daily allowance (RDA) for rats (RDA = 50 µg/kg diet), resulting in a final concentration of 4000µg/kg diet. This gradual increase was implemented to minimise potential changes in water palatability, which could otherwise reduce water consumption, and to avoid possible effects on hydration status or general health. Supplementation continued until the end of the experiment.

Week 4 and 5 involved no behavioural testing. From Week 6 onwards, the effect of vitamin B12 supplementation on whisker sensitivity was evaluated by repeating the tNORT on Day 44 (Week 6), Day 51 (Week 7), and Day 58 (Week 8). *In vivo* electrophysiological recordings were performed either on the same day as the final re-test (Day 58) or within the following three days. *Ex vivo* electrophysiological experiments were conducted immediately after the *in vivo* recordings in the same animal.

Exclusion criteria differed for each experimental component and are described in the respective Materials and Methods sections. For example, in the tNORT, data from animals with insufficient total exploration times were excluded from analysis. In the electrophysiological experiments, exclusions were based on technical issues (e.g., equipment malfunction) or procedural complications (e.g., anaesthesia-related problems).

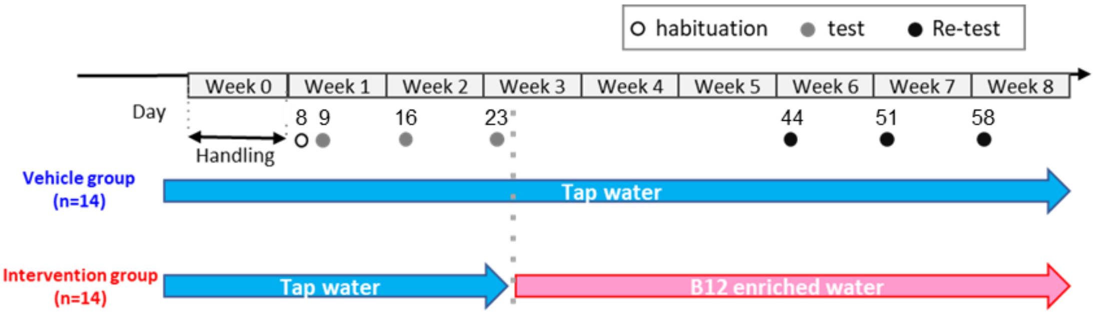


Figure 3.1 Experimental schedule for the main study

3.2.3 Power calculation

A priori power calculation was not conducted prior to data collection. Instead, a post hoc calculation was performed using the peak amplitude of sensory-evoked LFPs recorded under stage III-3 anaesthesia at 1.2mA stimulation as the primary outcome. A large effect size (Cohen's d = 0.8) was adopted because a previous study using comparable stimulation parameters reported ~25% larger peak amplitudes in the vitamin B12 supplemented group relative to controls. Note that percent change does not by itself determine the standardised effect size; however, typical variability in sensory evoked potential amplitudes reported in similar preparations, d = 0.8 represents a reasonable, literature-informed target.

Based on d = 0.8, α = 0.05 (two-tailed) and power (1−β) = 0.80, the required sample size was n ≈ 26 per group (two-sample t-test; G*Power-equivalent calculation). In practice, 14 rats were allocated to each group, informed by sample sizes used in previous studies employing comparable experimental paradigms, which had successfully detected group differences in peak amplitudes. These considerations should be kept in mind when interpreting null findings.

3.3 Materials and Methods

3.3.1 Experimental Design

The tNORT in the main study was conducted under the same environmental conditions and apparatus as described for the methodological study in Chapter 2 (Section 2.2.3). The experimental structure was identical to that of the methodological study, consisting of a Sample phase and a Test phase separated by a Delay period. However, the objects and grit sizes used in the main study were selected based on the methodological study results, representing a refinement of the protocol to optimise sensitivity for detecting whisker-mediated texture discrimination.

Specifically, six objects (I, II, III, IV, VII, and VIII in Figure 2.1B) were chosen because they elicited longer exploration times in the object shape bias test (OSBT) during the methodological study. Objects with higher baseline exploration times were considered more suitable for eliciting measurable novelty responses and thus for detecting potential supplementation effects. Each rat was presented with a different shaped object in every test to avoid decreased exploration due to familiarity with the same object shape

All objects, object covers, and the test arena were handled exclusively while wearing laboratory gloves to prevent olfactory contamination. The texture and position of the novel object, the grit size of the sandpaper, and the object shape were pseudo-randomised across animals and tests. Randomisation was constrained so that no rat was exposed to the same grit size or novel object position in consecutive tests. Three grit sizes were selected: P120, P400, and P1200. P120 served as a positive control to confirm task engagement, while P400 and P1200 were used to evaluate potential improvements in whisker-mediated texture discrimination following vitamin B12 supplementation. The selected objects were then prepared with textured covers as described below for use in the main study.

3.3.2 Apparatus

The test arena, lighting conditions, and methods for attaching sandpaper to the objects were identical to those described in the methodological study (Section 2.2.3). Each object was covered with sandpaper of the same grit size, using either its abrasive surface or its non-abrasive backing to provide different tactile properties. The placement of the abrasive or non-abrasive side was determined in a pseudorandom manner for each trial. These prepared objects were then used in the tNORT according to the procedure described in the following section.

3.3.3 Procedure

In each trial, four identical-shaped objects were used: three were covered with the same texture (familiar) and one with a different texture (novel). During the Sample phase (4 min), two familiar-textured objects were presented. After a Delay period of 2 min, the Test phase (4 min) was conducted, in which one of the Sample phase objects was replaced with a new familiar-textured object, and the other was replaced with the novel-textured object. This ensured that both objects in the Test phase were physically new to the rat, eliminating potential olfactory cues from prior exploration. The position of the novel object (left or right) was pseudorandomised to avoid positional bias. The sequence of grit size presentation across the three weekly tests was randomised for each animal, and the same grit sequence was used for both pre-supplementation and post-supplementation testing. The behavioural responses during each phase were recorded for subsequent analysis.

3.3.4 Outcome Measures and Data Analysis

All behavioural tests were recorded using a video camera positioned above the test arena and analysed using The Observer software (Noldus Information Technology, Wageningen, Netherlands). Exploration time was defined as the duration during which the rat’s nose was within 2 cm of an object, excluding periods of resting, grooming, or playing with its tail.

The experimenter, who had been trained and validated before the methodological study, coded all videos in the main experiment following the same procedure as in the methodological study. Each video was coded twice by the same experimenter, and the agreement between the two sets of codes was confirmed to exceed 98%. If agreement fell below this threshold, the coding was repeated. The mean exploration time from the two matched codes was used for analysis. Measurements were conducted by the experimenter, who was aware of the treatment group (control or B12) but blinded to which object was the novel one in each trial.

To analyse the tNORT, the discrimination index (DI), d was calculated separately for the sample phase (d_s) and test phase (d_t), as follows (Antunes and Bera., 2012). In the sample phase,

$$\mathrm{d_s} = \frac{\mathrm{T}_{L}-\mathrm{T_R}}{\mathrm{T_L}+\mathrm{T_R}} \qquad (3.1)$$

where T_L and T_R were the exploration times for the left and right objects respectively; whilst for the test phase,

$$\mathrm{d_t} = \frac{\mathrm{T_N}-\mathrm{T_F}}{\mathrm{T_N}+\mathrm{T_F}} \qquad (3.2)$$

where T_N and T_F were the exploration times for the novel and familiar objects respectively. Rats with total exploration time (T_{tot}) less than 2 s were excluded from the analysis (Wu et al., 2013). From its definition, the value of DI would be 0 if the exploration times for the left and right objects, or the familiar and novel objects, were identical.

3.3.5 Statistical Analysis

Statistical analyses of the tNORT data were conducted using SPSS Statistics version 27 (IBM Corp., Armonk, NY, USA). The significance level was set at $p < .05$. Data distribution was assessed using the Shapiro–Wilk test, and normality was assumed if this test was non-significant. For all ANOVAs, the *F* statistic and *p* value were reported. To evaluate the efficacy of the tNORT and to examine the potential effects of vitamin B12 supplementation, two separate analyses were conducted.

Validation of tNORT Performance (Sample vs Test Phase Comparison)

To confirm that exploration time was unbiased between the left and right objects in the Sample phase but shifted towards the novel object in the Test phase, a two-way repeated measures ANOVA was conducted with phase (Sample vs Test) and grit size (P120, P400, P1200) as within subject factors.

Supplementation groups and time points were collapsed to focus on the main effect of phase and its interaction with grit size. We expected a significant main effect of phase, indicating an overall increase in the discrimination index from the Sample to the Test phase regardless of grit size. If a significant phase × grit size interaction was observed, Bonferroni-adjusted post hoc tests were performed to identify specific grit sizes contributing to the effect.

Effect of Supplementation on Texture Discrimination (Control vs B12 Group Comparison)

To examine whether supplementation (Control vs B12) had differential effects on exploratory behaviour depending on grit size and measurement time (before or after supplementation), separate three-way repeated measures ANOVAs were conducted for the Sample and Test phases. In each analysis, grit size (P120, P400, P1200) and time (before vs after supplementation) were treated as within-subject factors, and supplementation (Control vs B12) as the between-subject factor. A significant main effect of supplementation would indicate an overall difference between groups, whereas significant supplementation × grit size or supplementation × time interaction would indicate that the supplementation effect varied depending on grit size or measurement time. Bonferroni-adjusted post hoc tests were applied when significant main effects or interactions were identified.

3.4 Results

3.4.1 Data Exclusion

Fourteen rats were initially assigned to each group. In the control group, two rats were excluded: one due to damage to the object during testing and one for interacting only with the novel object. In the B12 group, one rat was excluded due to damage to the object. The final sample sizes were therefore 12 rats for the control group and 13 rats for the B12 group. These numbers were used in all subsequent analyses.

3.4.2 Validation of tNORT Performance (Sample vs Test Phase Comparison)

To validate the performance of the tNORT, a two-way repeated measures ANOVA with phase (Sample vs Test) and grit size (P120, P400, P1200) as within-subject factors was conducted.

The analysis revealed a significant main effect of phase, $F(1,24) = 40.09$, $p < .001$, $np^2 = .626$, indicating that the discrimination index was significantly higher in the Test phase than in the Sample phase. There was also a significant main effect of grit size, $F(2,48) = 3.49$, $p = .038$, $np^2 = .127$. However, Bonferroni-adjusted pairwise comparisons showed no significant differences between individual grit sizes (all ps > .05). The phase × grit size interaction was not significant, $F(2,48) = 0.05$, $p = .948$, $np^2 =.002$.

These results confirm that the tNORT successfully detected novelty preference in the Test phase, thereby validating its use as a measure of whisker-mediated texture discrimination. Although grit size exerted a small overall effect, the absence of significant pairwise differences suggests that this factor did not substantially influence performance in the present study.

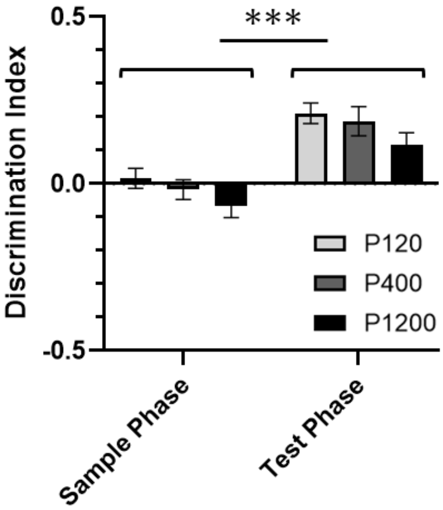


Figure 3.2. Validation of the tNORT

Mean discrimination index during the Sample and Test phases across three sandpaper grit sizes (P120, P400, P1200). A two-way repeated measures ANOVA revealed a significant main effect of phase, with higher discrimination in the Test phase compared to the Sample phase. No significant differences were observed between grit sizes. Error bars represent SEM (n = 25, pooled across Control and B12 groups).

3.4.3 Baseline Exploration in the Sample Phase (Control vs B12 Group Comparison)

To ensure that exploration during the Sample phase was unbiased across groups, time points, and grit sizes, a three-way repeated measures ANOVA was conducted with grit size (P120, P400, P1200) and time (before vs after supplementation) as within-subject factors, and supplementation (Control vs B12) as the between subject factor.

The analysis revealed no significant main effects of grit size, F(2, 46) = 1.65, p = .203, ηp^2 = .067, time, F(1, 23) = 0.21, p = .651, np2 = .009, or supplementation group, F(1, 23) = 0.20, p = .663, np2 = .009. No significant interactions were observed: grit × group, F(2, 46) = 1.10, p = .342, np2 = .046; time × group, F(1, 23) = 2.51, p = .127, np2 = .098; grit × time, F(2, 46) = 0.29, p = .749, np2 = .012; grit × time × group, F(2, 46) = 0.38, p = .683, np2 = .016.

These results indicate that exploration behaviour during the Sample phase was unbiased across groups, time points, and grit sizes, confirming that no systematic differences influenced baseline exploration (Figure 3.2).

Although mean d_s values at P1200 appeared slightly negative in some conditions, this reflects a mild side preference (right > left exploration) rather than object preference, and no statistically significant effects involving grit size were detected.

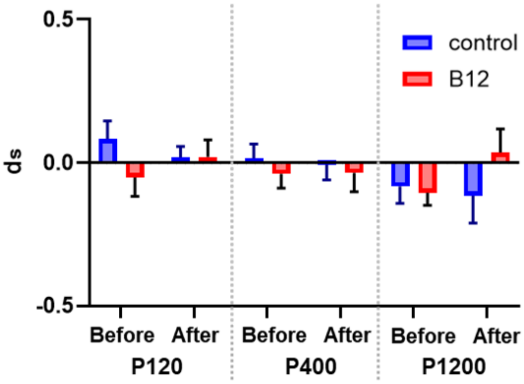


Figure 3.3. Discrimination index during the Sample phase (ds) across grit sizes, supplementation period and groups

Mean d_s values (± SEM) are shown for the control (blue) and the B12 group (red) before and after supplementation at grit sizes P120, P400, and P1200. No significant main effects of grit size, time, or supplementation, and no significant interactions, were observed in the three-way repeated measures ANOVA. Final sample sizes were n = 12 (control) and n = 13 (B12).

3.4.4 Baseline Exploration in the Sample Phase (Control vs B12 Group Comparison)

To examine whether vitamin B12 supplementation influenced texture discrimination during the Test phase, a three-way repeated measures ANOVA with grit size (P120, P400, P1200) and time (before vs after supplementation) as within-subject factors, and supplementation (Control vs B12) as the between-subject factor, was conducted on the discrimination index in the Test phase (d_t).

The analysis revealed no significant main effects of grit size, F(2, 46) = 1.85, p = .173, np^2 = .074, time, F(1, 23) = 1.12, p = .302, np^2 = .046, or supplementation group, F(1, 23) = 0.24, p = .628, np2 = .010. Similarly, no significant interactions were observed between grit size and group, F(2, 46) = 1.03, p = .364, np2 = .043, time and group, F(1, 23) = 0.95, p = .340, np2 = .040, or grit size and time, F(2, 46) = 0.49, p = .616, np2 = .021. The three-way interaction (grit × time × group) was also non-significant, F(2, 46) = 0.29, p = .752, np2 = .012.

These results indicate that novelty preference during the Test phase was consistently observed across grit sizes and time points, but vitamin B12 supplementation did not significantly influence discrimination performance (Figure 3.3).

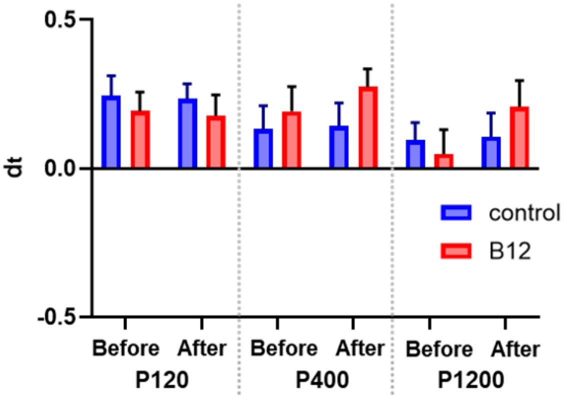


Figure 3.4. Discrimination index during the Test phase (d_t) across grit sizes, supplementation period and groups

Mean d_t values (± SEM) are shown for the control (blue) and the B12 group (red) before and after supplementation at grit sizes P120, P400, and P1200. No significant main effects of grit size, time, or supplementation, and no significant interactions, were observed in the three-way repeated measures ANOVA. Final sample sizes were n = 12 (control) and n = 13 (B12).

3.5 Discussion

3.5.1 Validation of tNORT Performance

The validation analysis showed that the tNORT reliably detected novelty preference: discrimination indices were significantly higher in the Test than in the Sample phase across grit sizes. This confirms the suitability of tNORT as a behavioural assay of texture discrimination under the present conditions. A small main effect of grit size was observed, but pairwise contrasts were non-significant and the phase × grit interaction was absent, indicating that novelty preference was robust at all tested grits and not driven by one specific texture condition.

These findings align well with prior work on whisker-based texture discrimination. Classic behavioural studies demonstrated that rats can discriminate surface features and task demands (Carvell and Simons, 1990; von Heimendahl et al., 2007). In spontaneous object recognition oparadigms, rondents spend longer exploring a novel than a familiar object, a principle leveraged by NORT/tNORT variants to index perceptual sensitivity without training (reviews: Antunes & Biala, 2012). More recent tNORT implementations similarly report reliable novelty preference when object features and test scheduling are appropriately controlled (Hayashi et al., 2024). Our results are consistent with this literature: we observed a robust phase effect (novelty preference) and only a modest, non-specific contribution of grit.

The present data also converge with our methodological study, which indicated a perceptual threshold for whisker-mediated texture discrimination: rats distinguished coarser textures (e.g., P80, P120, P180), but not finer ones (e.g., P240, P400). Within the main istudy's grit range (P120, P400, P1200), the small main effect of grit likely reflects a general tendency for coarser textures to yield slightly higher discrimination, whereas the lack of a phase × grit interaction shows that the novelty effect was expressed consistently across conditions.

In sum, the validation confirms that the optimised tNORT protocol provides a stable and sensitive read-out of whisker-based texture discrimination suitable for repeated testing. This offers a solid behavioural foundation for the subsequent analyses examining whether vitamin B12 supplementation improves texture discrimination relative to the control group.

3.5.2 Absence of Exploration Bias in the Sample Phase

In the Sample phase, no systematic bias was observed in exploration between the left and right objects across groups, grit sizes, or time points. This finding confirms that the rats did not exhibit positional or object-related preferences when only familiar textures were presented. Such an absence of bias is consistent with the design principles of the Novel Object Recognition Test (NORT), in which exploration during the Sample phase is typically balanced (Antunes & Biala, 2012). By ensuring that baseline exploration was unbiased, the present results validate the use of the tNORT in this study and support the interpretation that differences observed in the Test phase reflect genuine novelty preference, rather than pre-existing biases in exploratory behaviour.

3.5.3 Lack of Vitamin B12 Supplementation Effect on Texture Discrimination

The present analysis revealed no significant effects of vitamin B12 supplementation on texture discrimination during the Test phase. Neither main effects of grit size and measurement time nor their interactions with supplementation were significant, indicating that novelty preference was consistently expressed across conditions, but was not altered by B12 supplementation.

This null result suggests that B12 supplementation did not enhance whisker-mediated texture discrimination in healthy rats. One possible explanation is that the animals were not deficient in B12 at baseline, leaving little room for measurable improvement. Indeed, previous human studies have shown that B12 supplementation most effectively improves cognitive and sensory functions in populations with low baseline B12 status, whereas evidence for benefits in healthy individuals is limited (Martin et al., 1992).

Another consideration is task sensitivity. While the methodological study indicated that rats were able to discriminate coarse textures (P80, P120, P180) but not finer ones (P240, P400), preliminary data also suggested that P1200 could not be discriminated. However, because only six animals were tested at P1200 due to attrition, these results were not included in the published methodological study. In the present main study, we therefore included P1200 as a challenging condition to further probe the limits of whisker-based discrimination. Interestingly, no significant main effect of grit size or phase × grit interaction was observed in the repeated-measures ANOVA. This apparent discrepancy between the methodological study and main findings may be explained by methodological differences: the methodological study relied on chance-level comparisons within single conditions, whereas the main study used multifactorial ANOVA across repeated tests. Furthermore, repeated exposure and larger inter-individual variability in the main experiment may have masked subtle differences across grit sizes.

Moreover, although prior work and our methodological study indicated that discrimination declines as grit size differences become smaller (Carvell and Simons, 1990), the range of grit sizes used in the present study (P120, P400, P1200) may not have been optimal to detect subtle supplementation-related changes. In addition, statistical power was limited by the available sample size, so that small-to-moderate effects could not be ruled out.

Future studies should therefore examine B12 effects under conditions of dietary deficiency, test a broader range of texture differences, and consider larger sample sizes to improve sensitivity. These refinements may help determine whether B12 has a measurable impact on sensory processing in rodents.

3.5.4 Summary

Together, these analyses confirm the validity and reliability of the adapted tNORT protocol for assessing whisker-mediated texture discrimination. The task successfully elicited novelty preference without positional or exploration bias, thereby providing a robust behavioural paradigm. Importantly, vitamin B12 supplementation did not enhance discrimination performance in healthy rats, suggesting that potential effects of supplementation may emerge only under conditions of deficiency or with more sensitive testing protocols. These behavioural findings provide a solid foundation for the subsequent electrophysiological analyses presented in Chapters 4 and 5.

Chapter 4 *In vivo* Electrophysiology

4.1 Introduction

The primary aim of this chapter is to investigate whether vitamin B12 supplementation alters sensory-evoked neural responses *in vivo*. Local field potentials (LFPs) were selected as the main measure, as they reflect the synchronised synaptic activity of local neuronal populations and provide valuable insights into neural processing at the circuit level (Buzsáki et al., 2012, Einevoll et al., 2013). LFPs are typically obtained by low-pass filtering extracellular recordings below ~300 Hz (Burns et al., 2010, Gawne, 2010, Lindén et al., 2011).

To evaluate whisker-evoked responses, two fundamental properties of sensory systems were examined: intensity dependence and sensory adaptation. Intensity dependence reflects input–output gain control, a core feature of sensory encoding that adjusts neural responsiveness according to stimulus strength (Brooks et al., 201,; Jääskeläinen et al., 2012). Sensory adaptation, in turn, is expressed as a progressive reduction in response amplitude to repeated stimulation and is considered essential for filtering redundant inputs and enhancing the detection of novel stimuli (Chung et al., 2002, Khatri et al., 2004). Together, these measures provide complementary information about how sensory signals are processed in cortical and thalamic circuits under varying stimulation conditions.

Previous studies have suggested that vitamin B12 supplementation may modulate cortical excitability, even in the absence of overt deficiency. In humans, Smith et al. (2017) reported a reduction in steady-state visual cortical responses following intake of a yeast extract enriched with B vitamins, including B12. In contrast, Kang et al. (2019) demonstrated that high-dose B12 supplementation increased whisker-evoked potentials in the barrel cortex of healthy rats. Importantly, however, Kang et al. assessed sensory adaptation only through a paired-pulse paradigm, which captures short-term synaptic dynamics but does not fully characterise adaptation across stimulus trains. Thus, the broader question of whether B12 influences canonical features of sensory processing such as intensity dependence and sustained adaptation remains unresolved.

Building on the behavioural findings presented in Chapter 3, the present *in vivo* electrophysiological experiments were designed to determine whether vitamin B12 supplementation modifies whisker-evoked LFP responses in the thalamus and barrel cortex. While LFPs can also be recorded from subcortical regions such as the thalamus, where laminar organisation is less pronounced, they nevertheless provide valuable information about local synaptic activity. Early components of thalamic LFPs are thought to reflect thalamic input, whereas later components may be influenced by corticothalamic feedback (Temereanca & Simons, 2003, Yang et al., 2013, Średniawa et al., 2023). Although the present study did not directly dissect feedforward or feedback interactions, recording LFPs from both the thalamus and barrel cortex enabled us to characterise local sensory-evoked response properties across different nodes of the thalamocortical system. By focusing on intensity dependence and sensory adaptation, this chapter aims to provide a systems-level perspective on how B12 may influence thalamocortical processing of tactile information.

4.2 Materials and Methods (*In vivo* Electrophysiology)

4.2.1 Preparation for *In vivo* Neuronal Recordings

All surgical and recording procedures were performed under sterile conditions in accordance with the United Kingdom Animals (Scientific Procedures) Act (1986) and were approved by the University of Reading Research Ethics Committee. Rats were anaesthetised with isoflurane (induction: 5%; maintenance: ~3%) delivered in oxygen (flow rates summarised in Table 4.1). Body weight was recorded prior to surgery. Animals were placed on a thermostatically controlled heating pad (Harvard Apparatus) and secured in a stereotaxic frame (World Precision Instruments) using ear and tooth bars. Body temperature was continuously monitored via a rectal probe coated with petroleum jelly and maintained at ~37 °C. To prevent corneal drying, eye lubricant (Optixcare, CLC Medica) was applied. Adequate anaesthetic depth was confirmed by the absence of the hind-paw withdrawal reflex.

Table 4.1 Isoflurane (ISO) level and oxygen flow during experiment

	ISO level (%)	Oxygen flow (L/min)
Induction	5	2
Surgery	3	0.5~0.6
Recording (stage III3)	0.4~1.7	0.5~0.6
Recording (stage III4)	0.9~2	0.5~0.6
Recording (stage IV)	3	0.5~0.6

4.2.2 Surgical Procedures and Electrode Placement

The cranial region was shaved and cleaned with antiseptic solution (Hibiscrub, Mölnlycke). Local anaesthesia (lidocaine, topical application) was administered before a 3 cm midline incision was made. The skull surface was cleaned with saline, and the temporalis muscle was retracted. A craniotomy was performed using a micro-drill under a surgical microscope to expose the barrel cortex (2.5 mm posterior to bregma, 6 mm lateral to the midline) and the ventral posteromedial (VPM) thalamic nucleus (3 mm posterior to bregma, 3 mm lateral; Paxinos & Watson, 2006) (Figure 4.1A).

For recordings, 16-channel linear silicon probes (site spacing: 100 µm; site area: 177 µm²; NeuroNexus Technologies) were used. One electrode was inserted into the barrel cortex at ~20–30° to the vertical axis to a depth of 2 mm, and a second was advanced vertically to 6 mm into the VPM (Figure 4.1B). Reference electrodes were placed in the neck incision. The exposed cortical surface was covered with saline-moistened cotton to prevent drying. Electrodes were connected to a preamplifier (Tucker Davis Technologies) and linked to a data acquisition system via fibre-optic cables.

If evoked responses to 1.2 mA single-pulse stimulation at stage III-4 anaesthesia were <1 mV, electrode positions were adjusted (maximum of three reinsertions). If no adequate responses were obtained, the recording was excluded.

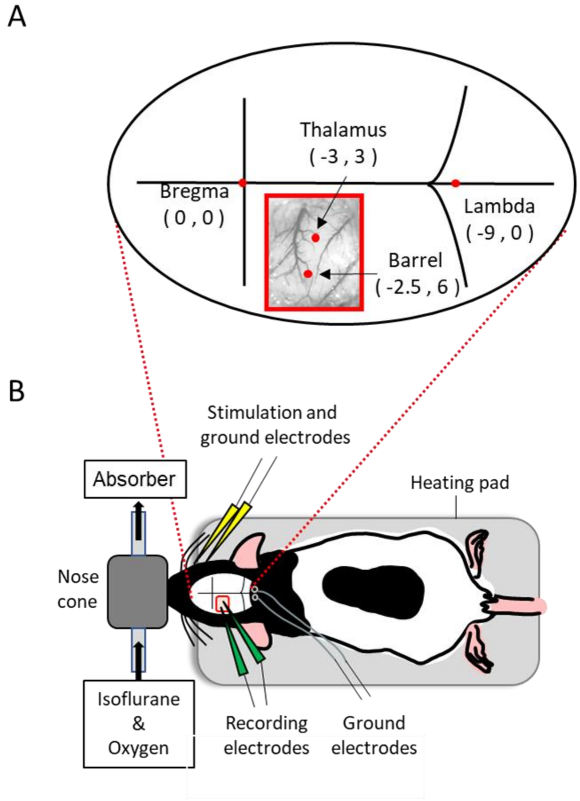


Figure 4.1 Schematic of *in vivo* neuronal recording

(A) Schematic of rat's skull and the points where recording electrodes were inserted. (B) Schematic of neuronal recording setup. Rat was placed on the heating pad. Isoflurane and oxygen were administered through a nose cone.

4.2.3 Stimulation Paradigm and Anaesthesia level

Whisker stimulation was delivered via bipolar electrodes inserted into the right whisker pad at ~1 cm separation. Rectangular current pulses (0.3 ms duration) were applied under different stimulation paradigms to assess specific response properties of the thalamocortical system. Stimulation parameters are summarised in Table 4.2.

Three paradigms were employed:

1. Anaesthetic depth:

   Single-pulse stimulation (1.2 mA) was applied under three anaesthetic stages (III-3, III-4, IV) to examine potential depth-dependent effects on evoked and spontaneous activity. Although these recordings were collected, they were not included in the main statistical analyses in order to maintain clarity and avoid unnecessary complexity.

2. Intensity dependence:

   Under stage III-3 anaesthesia, trains of whisker pad stimulation were delivered at 5 Hz for 2 s with amplitudes of 0.4, 0.8, and 1.2 mA. To focus on input–output relationships, only the first evoked response of each train was analysed.

3. Sensory adaptation:

   Under stage III-3 anaesthesia, whisker pad stimulation was delivered at 1.2 mA for 2 s at frequencies of 2, 5, 10, and 20 Hz. For analysis, the peak amplitude of each response was expressed as a ratio to the first response within the train, enabling assessment of adaptation dynamics across repeated stimuli. Sensory adaptation was evaluated only in the barrel cortex, as the relatively low signal-to-noise ratio (SNR) of thalamic LFPs under these conditions precluded reliable quantification of adaptation.

For sensory-evoked analyses, neural activity was extracted from 0–2 s following stimulus onset. Resting-state activity was assessed during 5–9 s of single-pulse runs and 9–19 s of multi-pulse runs to confirm anaesthetic depth. Anaesthetic depth was titrated to stages III-3, III-4, and IV based on established criteria derived from characteristic patterns of spontaneous neuronal activity (Friedberg et al., 1999). Recordings commenced 10–20 min after each adjustment to ensure stable physiological conditions.

Table 4.2 Recording parameters for *in vivo* electrophysiology

Run	Anaesthesia stage	(Hz)	(mA)	(second)	(second)
1	III4	single pulse	1.2	-	10
2~7 (Randomised)	III3	2	1.2	2	20
		5	0.4		
		5	0.8		
		5	1.2		
		10	1.2		
		20	1.2		
8	III3	single pulse	1.2	-	10
9	IV	single pulse	1.2	-	10

4.2.4 Sample Collection and Termination

To verify the efficacy of vitamin B12 supplementation, blood samples were collected immediately after the completion of *in vivo* recordings. Cardiac puncture was performed under deep isoflurane anaesthesia (5% in oxygen, confirmed by slow respiration and loss of hind-paw pinch reflex) using a 10 ml syringe with a 23-gauge 5/8-inch needle (Terumo Corporation). Approximately 5 ml of blood was withdrawn from the left ventricle and transferred to a 15 ml centrifuge tube. After 30 min of clotting at room temperature, samples were centrifuged at 3300 rpm for 15 min. Serum was separated, stored at −80 °C, thawed prior to vitamin B12 quantification, and subsequently refrozen before shipment to the Royal Veterinary College (Hertfordshire, UK) for analysis. Samples were not protected from light, as previous work demonstrated no significant effect of light exposure on vitamin B12 stability (Clement and Kendall, 2009).

Following blood collection, animals were euthanised. Cervical dislocation was used in all cases, except for one rat in which isoflurane depth could not be adequately maintained; this animal was euthanised with pentobarbital.

4.2.5 Statistical Analysis

All statistical analyses were performed using SPSS Statistics version 27 (IBM Corp., Armonk, NY, USA). The significance threshold was set at $p < .05$. Data normality was assessed using the Shapiro–Wilk test. When the assumption of sphericity was violated, the Greenhouse–Geisser correction was applied. For data sets that did not meet normality assumptions, non-parametric aligned rank transform (ART) ANOVA was employed, allowing for factorial analyses equivalent to mixed-design ANOVA while accommodating non-normal distributions.

Effect of Stimulus Intensity on Whisker-Evoked LFP Responses

To evaluate input–output relationships, the peak amplitude of the first evoked response in each 5 Hz train was analysed using a 2 (group: Control vs B12; between-subjects) × 3 (stimulus intensity: 0.4, 0.8, 1.2 mA; within-subject) mixed-design repeated measures ANOVA or ART ANOVA. Separate analyses were conducted for neural data recorded in the VPM and in the barrel cortex.

Primary analysis (adaptation pattern):

To evaluate whether vitamin B12 supplementation alters adaptation dynamics, response amplitudes within each 2-s train (1.2 mA at 2, 5, 10, or 20 Hz) were expressed as ratios relative to the first pulse. For each frequency separately, we ran a mixed repeated-measures ANOVA or ART ANOVA with group (Control vs B12; between-subjects) and stimulus number (2nd to Nth pulse; within-subjects) as factors. While the main effect of stimulus number indexes the presence of adaptation, our a priori focus was on the group main effect and group × stimulus number interaction, which test whether supplementation changes the overall level or the pattern of adaptation, respectively.”

Supplementary analysis (concise summary index):

To provide a succinct frequency-resolved index of overall adaptation depth, we averaged the ratios across all pulses within each frequency, yielding one value per frequency per rat. These mean ratios were compared using a mixed repeated-measures ANOVA with group (between-subjects) and frequency (2, 5, 10, 20 Hz; within-subjects).

Serum cobalamin concentration:

To evaluate whether vitamin B12 supplementation increased serum cobalamin levels, group differences in serum cobalamin levels were assessed using independent-samples t-tests when normality assumptions were met, or Mann–Whitney U tests otherwise.

4.3 Results (*In vivo* Electrophysiology)

4.3.1 Data Exclusion

A total of 28 rats were included in the study, with 14 assigned to the control group and 14 to the B12 group. However, several datasets were unavailable or excluded due to technical or biological issues such as equipment malfunction, unstable anaesthetic depth or electrode placement error.

VPM recordings

In the control group, one rat was excluded due to equipment malfunction and four due to insufficient evoked neural activity. In the B12 group, one rat was excluded because of unstable anaesthetic depth and five due to insufficient evoked activity. Consequently, 17 rats (control: 9; B12: 8) were included in the VPM analyses.

Barrel cortex recordings

In the control group, one rat was excluded because of equipment malfunction, one due to insufficient evoked responses, and one due to electrode placement error. In the B12 group, two rats were excluded due to insufficient evoked responses and one because of unstable anaesthesia. Consequently, 22 rats (control: 11; B12: 11) were included in the barrel cortex analyses.

Serum cobalamin concentration.

Of the serum samples collected, 5 out of 14 per group coagulated and became unusable after centrifugation, leaving 9 samples per group available for analysis. These exclusions were due to technical issues during sample processing and were not related to group allocation or experimental outcome.

4.3.2 VPM – Stimulus intensity

To determine whether vitamin B12 supplementation influenced the input-output relationship of sensory-evoked responses, we analysed the effect of stimulus intensity on VPM peak LFP amplitude. Peak amplitude did not differ significantly between groups (F(1,15) = 1.11, p = .308, np2 = .069), and no Group × Intensity interaction was observed (F(2, 30) = 0.47, p = .630, np2 = .030). As expected, amplitude increased with stimulus intensity (F(2,30) = 15.86, p < .001, np2 = .514；Figure 4.2).

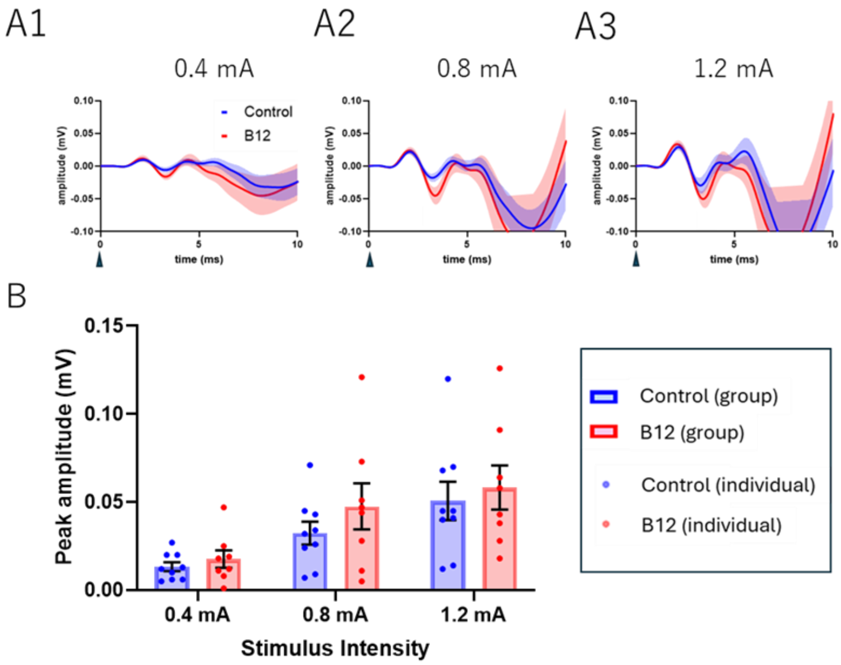


Figure 4.2 Stimulus intensity and whisker-evoked LFP responses in the VPM

(A1-A3) Group-averaged traces (mean ± SEM) of whisker-evoked LFPs in the VPM at 0.4, 0.8 and 1.2 mA stimulation intensities for the Control (blue) and B12 (red) groups. Black triangles indicate the stimulus onset. (B) Peak amplitude of the first evoked response in each intensity. Sample size: N =9 for the control group and N = 8 for the B12 group.

4.3.3 Barrel Cortex – Stimulus intensity

To determine whether vitamin B12 supplementation influenced the input–output relationship in the barrel cortex, we analysed the effect of stimulus intensity on peak LFP amplitude using an aligned rank transform (ART) ANOVA. There was no significant main effect of Group (F(1,20) = 0.67, p = .423, np2 = .032) and no Group × Intensity interaction (F(2,40) = 0.38, p = .684, np2 = .019). As expected, peak amplitude increased significantly with stimulus intensity (F(2,40) = 116.76, p < .001, np2 = .848; Figure 4.3).

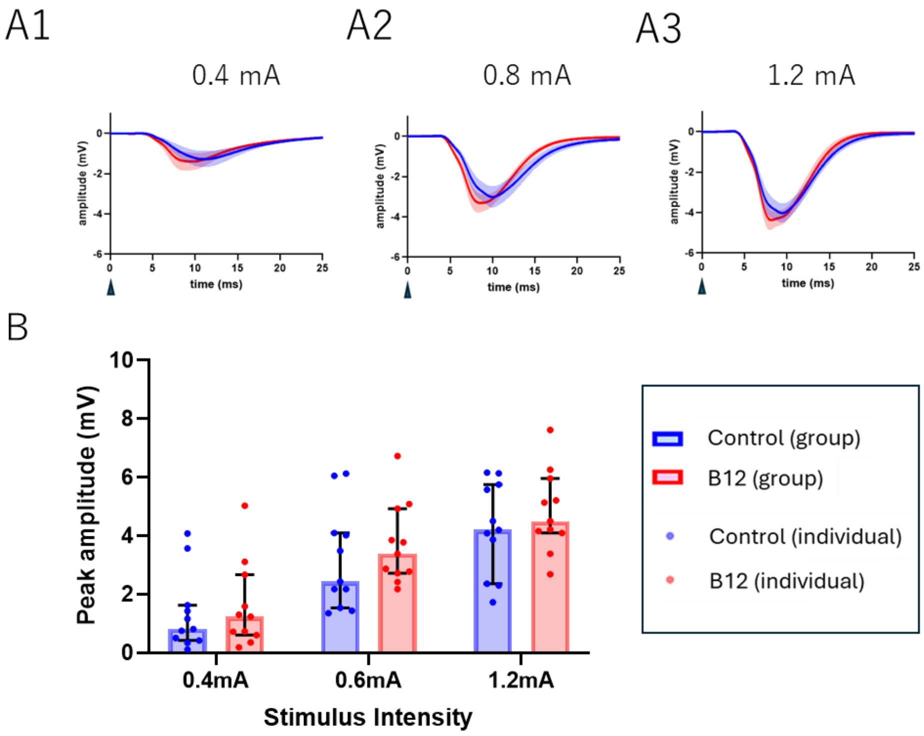


Figure 4.3 Stimulus intensity and whisker-evoked LFP responses in the barrel cortex

(A1-A3) Group-averaged traces (mean ± SEM) of whisker-evoked LFPs at 0.4, 0.8 and 1.2 mA stimulation intensities for the Control (blue) and B12 (red) groups. Black triangles indicate the stimulus onset. (B) Peak amplitude of the first evoked response (median ±IQR) in each intensity. Sample size: N =11 for each group.

4.3.4 Barrel Cortex – Sensory adaptation

To examine whether vitamin B12 supplementation altered adaptation dynamics in the barrel cortex, we conducted mixed repeated-measures ANOVAs (or ART ANOVAs) with Group (Control vs B12) and Stimulus number as factors, separately for each stimulation frequency. No significant main effects of Group or Group × Stimulus number interactions were observed at any frequency (Table 4.3). A significant main effect of Stimulus number was found at 5 Hz, $F(2.49,49.83) = 17.84$, $p < .001$, $np^2 = .471$, at 10 Hz, $F(18,360) = 22.96$, $p < .001$, $np^2 = .534$, and at 20 Hz, $F(38,760) = 29.77$, $p < .001$, $np^2 = .598$, reflecting the presence of adaptation across repeated pulses (Figure 4.4).

Table 4.3 Results of repeated-measures ANOVAs testing sensory adaptation in the barrel cortex. Mixed model repeated-measures ANOVAs were used for 2 and 5 Hz. For 10 and 20 Hz, ART ANOVAs were applied.

Frequency	Effect	F(df1, df2)	P value	ηp^2
2 Hz	Group	F(1,20) = 0.010	.920	.000
	Group × Stimulus num	F(1.18,23.50) = 0.469	.530	.023
	Stimulus num	F(1.18,23.50) = 0.653	.451	.032
5 Hz	Group	F(1,20) = 0.023	.881	.001
	Group × Stimulus num	F(2.49,49.83) = 0.758	.487	.036
	Stimulus num	F(2.49,49.83) = 17.839	< .001	.471
10 Hz	Group	F(1,20) = 0.211	.651	.010
	Group × Stimulus num	F(18,36) = 0.549	.933	.215
	Stimulus num	F(18,36) = 22.958	< .001	.920
20 Hz	Group	F(1,20) = 0.138	.741	.007
	Group × Stimulus num	F(38,76) = 0.348	1.000	.148
	Stimulus num	F(38,76) = 29.765	< .001	.937

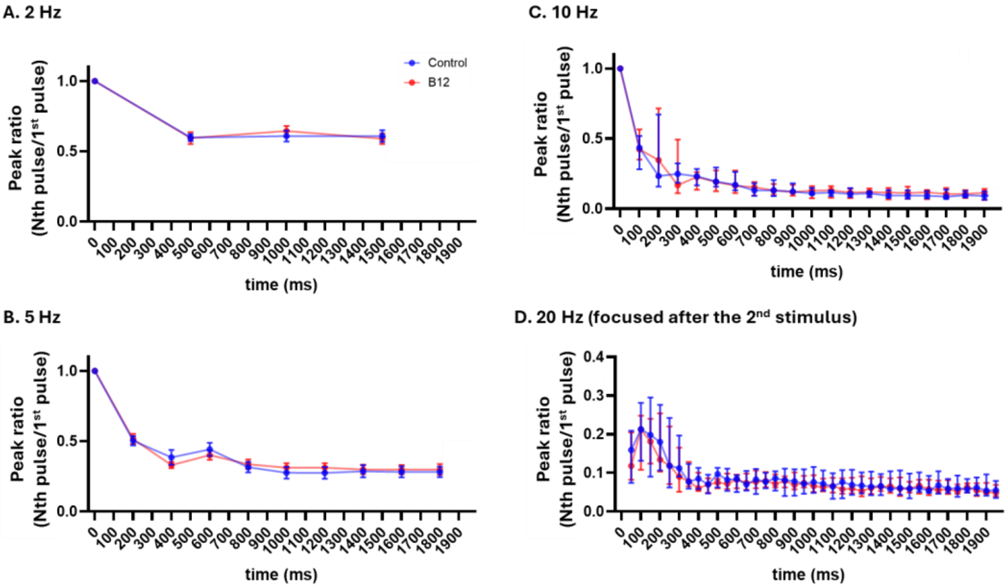


Figure 4.4 Sensory adaptation in the barrel cortex (primary analysis)

(A–D) Response amplitudes expressed as ratios relative to the first pulse, plotted across stimulus trains at 2, 5, 10, and 20 Hz (Control: blue, B12: red; mean ± SEM for A and B, median ± IQR for C and D). Sample size: N =11 for each group.

Barrel cortex – Sensory adaptation (supplementary analysis)

To provide a summary index of adaptation depth, mean peak ratios were calculated for each frequency and analysed using a mixed repeated-measures ANOVA with Group (Control vs B12) and Frequency as factors. There was no significant main effect of Group, F(1,20) = 0.005, p = .943 np^2 = .000, and no Group × Frequency interaction, F(2.04,40.81) = 0.087, p = .920 np^2 = .004. A significant main effect of Frequency was observed, F(2.04,47.81) = 258.76, p < .001 np^2 = .917, indicating stronger adaptation at higher stimulation frequencies (Figure 4.5)

Together, these analyses indicate that while robust adaptation occurred in the barrel cortex across stimulation frequencies, vitamin B12 supplementation did not alter either the dynamics or the overall depth of adaptation.

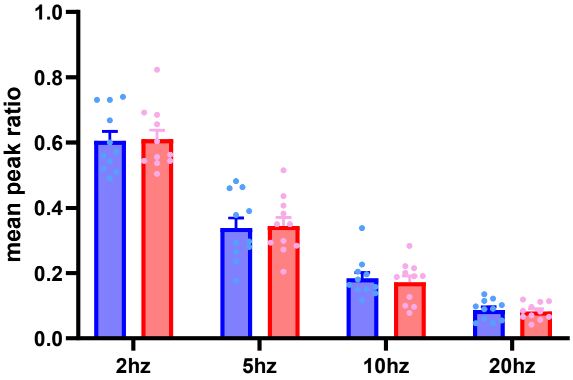


Figure 4.5 Sensory adaptation in the barrel cortex (supplementary analysis)

Mean peak ratios (response amplitude relative to the first pulse, averaged across all pulses within each frequency) for Control (blue) and B12 (red) groups at 2, 5, 10, and 20 Hz. Bars indicate group means ± SEM (Control: light blue; B12: pink), and dots represent individual animals (Control: dark blue; B12: red). Sample size: N =11 for each group.

4.3.5 Serum cobalamin concentration

To confirm the efficacy of supplementation, serum cobalamin levels were compared between groups. As expected, concentrations were significantly higher in the B12 group (mean ± SEM: 1.99 ± 0.05 ng/mL) than in the Control group (1.27 ± 0.10 ng/mL), t(16) = –6.21, p < .001 (Figure 4.6).

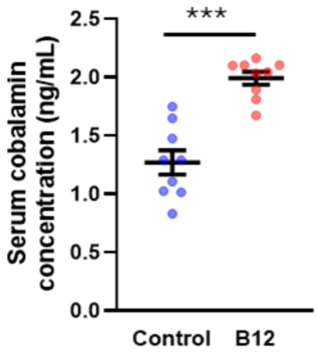


Figure 4.6 Serum cobalamin concentration

Serum cobalamin levels in the Control (blue) and B12-supplemented (red) groups. Each dot represents an individual animal, and bars indicate group means ± SEM..Sample size: N = 9 for each group.

4.4 Discussion (*In Vivo* Electrophysiology)

4.4.1 VPM and Barrel Cortex – Stimulus Intensity

In both the VPM and the barrel cortex, vitamin B12 supplementation did not alter the input–output relationship of sensory-evoked LFPs. Peak amplitudes rose monotonically with stimulus intensity, consistent with recruitment of additional neuronal populations, yet no group differences or group × intensity interactions emerged. Under our conditions, supplementation therefore did not modify the stimulus-dependent responsiveness of thalamic or cortical circuits.

This contrasts with Kang et al. (2019), who reported larger whisker-evoked LFPs in the barrel cortex of healthy female rats after supplementation, while spontaneous activity was unchanged. Differences in supplementation efficacy are a plausible explanation. Although control-group serum B12 appeared broadly comparable across studies, the supplemented group in Kang et al. shows a visibly greater rise (based on inspection of their figures; absolute values are not reported), suggesting that bioavailability, metabolism, or protocol details may have yielded stronger effective dosing in their cohort.

Recent findings indicate that cobalamin deficiency alters the balance between pro-inflammatory tumour necrosis factor-α and neurotrophic epidermal growth factor, and that supplementation restores their levels in parallel with clinical improvement (Scalabrino and Peracchi, 2006). These results suggest that vitamin B12 exerts regulatory functions beyond its classical coenzyme role. Moreover, oral absorption of vitamin B12 is known to be dose-dependent and subject to saturation, and intervention trials have reported considerable variability in serum responses even at comparable doses (Seal et al., 2002, Kashyap et al., 2024). Taken together, these observations suggest that interindividual differences in absorption and metabolism may contribute to variability in the outcomes of nutritional intervention studies.

A second consideration is sex. Kang et al. studied female rats, whereas our study used male rats. There is now wide recognition that sex can shape neural phenotypes and experimental outcomes, motivating its explicit inclusion as a biological variable (Shansky and Woolley., 2016; Margalit et al, 2018). Moreover, large cross-sectional data indicate sex-linked differences in B12 status (e.g., a higher risk of deficiency in men), implying that baseline physiology and endocrine milieu could interact with supplementation (Margalit et al., 2018). Future work that directly compares males and females under identical regimens will be valuable.

Beyond methodological factors, our null effect should be interpreted in the context of broader mechanisms linking B12 to neural excitability. Deficiency impairs myelin integrity and neural conduction and is associated with characteristic central and peripheral neuropathology; conversely, repletion can restore signalling. Previous literature converges on demyelination and slowed conduction as key features of B12 lack (Scalabrino., 2001, Briani et al., 2013).

At the cellular level, vitamin B12 exerts neurotrophic actions: methylcobalamin promotes neurite outgrowth and functional recovery in nerve injury models, partly through the activation of intracellular signalling pathways (Okada et al., 2010). Vitamin B12 can also influence neurotransmitter pathways; for example, it has been shown to reduce glutamate release from cortical neurons by modulating calcium-dependent mechanisms (Hung et al., 2009). These findings provide plausible routes by which B12 might alter cortical responsiveness even in the absence of overt deficiency (reviewed by Baltrusch., 2021).

Taken together, the literature identifies credible mechanisms through which B12 could influence thalamo-cortical responsiveness (myelin/axonal conduction, trophic signalling, and glutamatergic drive). However, our results indicate that such effects are not readily detectable in healthy, non-deficient animals when assessed via input–output functions of sensory-evoked LFPs. Subtle synaptic or cellular changes may require more sensitive assays (e.g., single-unit electrophysiology), or effects may be more pronounced under deficiency, injury, or increased trophic demand. Systematic studies varying dose, sex, and nutritional status (deficient vs. replete) will be essential to define the conditions under which vitamin B12 measurably modulates sensory-evoked responses in thalamocortical circuits.

4.4.2 Barrel Cortex – Sensory adaptation

In the barrel cortex, we observed robust frequency-dependent attenuation of evoked LFP responses across pulse trains, confirming that our paradigm reliably captured sensory adaptation as described in previous work (Adibi and Lampl, 2021). However, neither the primary analysis (pulse-by-pulse amplitude ratios) nor the supplementary analysis summarising overall adaptation depth revealed significant Group effects or Group × Stimulus interactions at any frequency. Thus, under the present experimental conditions, vitamin B12 supplementation did not measurably alter short-term adaptation dynamics at the population-synaptic level reflected in LFPs.

Kang et al. (2019) reported that vitamin B_{12} supplementation in healthy rats weakened sensory adaptation in the barrel cortex, reducing the attenuation of neural responses to repeated stimuli. However, it is important to note a methodological difference: the present study assessed adaptation using response amplitude ratios across multiple pulses within a stimulus train, whereas Kang et al. employed the paired-pulse ratio (PPR). PPR primarily reflects presynaptic mechanisms and very short-latency inhibitory effects at a single inter-pulse interval, while train-based adaptation integrates cumulative processes such as progressive synaptic depression and recruitment of inhibitory circuits over longer sequences (Hanse and Gustaffsson., 2001, Kirischuk et al., 2002, Waldbaum and Dudek., 2010). Therefore, changes detected with PPR do not necessarily predict alterations in adaptation measured across longer pulse trains.

Furthermore, our null result suggests that any B12-related changes in initial synaptic release probability or fast inhibitory gating—as might be captured by PPR—do not necessarily translate into altered cumulative depression or inhibitory recruitment over sustained stimulation. Given that whisking in behaving rodents involves prolonged trains at 5–20 Hz (Berg and Kleinfeld, 2003), train-based measures may provide a more behaviourally relevant index of adaptation. Future studies combining PPR and train adaptation within the same preparation could clarify how B12 influences the transition from short- to longer-term synaptic dynamics. Additionally, pairing LFPs with single-unit recordings or optogenetic dissection of excitatory and inhibitory circuits could reveal subtle, cell-type–specific effects not visible at the population level.

4.4.3 Summary – *In vivo* electrophysiology

The present *in vivo* electrophysiological experiments showed that vitamin B_{12} supplementation did not alter the input–output relationship of whisker-evoked LFPs in either the VPM or barrel cortex and did not measurably affect short-term sensory adaptation within the barrel cortex. These results contrast with earlier reports of enhanced paired-pulse responses after supplementation (Kang et al., 2019), but methodological differences—particularly our use of multi-pulse train adaptation rather than single-interval PPR—and variability in achieved serum B_{12} levels may partly explain the discrepancy.

Future studies should first determine whether B_{12} supplementation influences thalamocortical responses under conditions of dietary deficiency or low baseline status, as deficiency is known to impair myelination and synaptic transmission. In addition, employing complementary measures of sensory adaptation (e.g., combining paired-pulse ratio and train-based indices in the same animals) and higher-resolution electrophysiology could help reveal subtle or circuit-specific effects that were not detectable with LFPs alone. Careful control of sex, dosing regimen, and achieved serum levels will further clarify the conditions under which B_{12} may modulate whisker system processing.

Chapter 5 *Ex Vivo* Electrophysiology

5.1 Introduction

The primary aim of this chapter is to examine whether vitamin B_{12} supplementation influences intracortical synaptic transmission and short-term plasticity within the barrel cortex using *ex vivo* electrophysiological recordings. *In vivo* electrophysiology, as described in the previous chapter, captures the integrated activity of large neuronal populations under physiologically intact conditions. In contrast, *ex vivo* recordings permit mechanistic investigation of defined synaptic connections within a precisely controlled environment.

Within the barrel cortex, excitatory projections from layer 4 to layer 2/3 constitute a key feedforward pathway for processing whisker-derived sensory information (Feldmeyer et al., 2002; Bender et al, 2006). Investigating this pathway in cortical slices provides a mean to evaluate how sensory input is integrated and transmitted across cortical layers while maintaining tight control over physiological parameters such as ionic composition and temperature. Together, these complementary approaches help bridge the gap between large-scale network and local circuit perspectives on how vitamin B_{12} may influence neural processing.

To characterise the functional properties of this pathway, three complementary electrophysiological measures were employed: 1. input–output (I/O) relationships, which index synaptic efficacy and connectivity as stimulus intensity increases. 2. paired-pulse ratio (PPR), which assesses short-term synaptic plasticity reflecting presynaptic release probability and immediate inhibitory modulation (Hanse & Gustafsson, 2001; Kirischuk et al., 2002; Feldman, 2009); and 3. frequency-dependent adaptation, which reflects the cumulative depression or facilitation of synaptic responses across stimulus trains, a process thought to underlie sensory filtering and response tuning in cortical circuits (Gabernet et al., 2005; Khatri et al., 2004).

The present study sought to determine whether vitamin B_{12} supplementation alters the strength, dynamics, or adaptability of intracortical transmission within the canonical barrel cortex microcircuit. Together with the behavioural and *in vivo* findings presented in the previous chapters, these experiments provide a circuit-level perspective on how vitamin B_{12} may influence sensory processing in the rodent whisker system.

5.2 Materials and Methods (*Ex vivo* Electrophysiology)

5.2.1 *Ex vivo* Electrophysiological Recordings

Brain slices were prepared for extracellular recordings in the barrel cortex to evaluate intracortical synaptic transmission and short-term plasticity.

Brain extraction and transport:

Immediately after completion of the *in vivo* recording, animals were euthanised by cervical dislocation, and the brain was rapidly extracted and rinsed with ice-cold oxygenated artificial cerebrospinal fluid (ACSF). The ACSF composition was (in mM): NaCl, 124; KCl, 3; NaHCO3, 26; CaCl2, 2; NaH2PO4, 1.25; MgSO4, 1; and D-glucose, 10. The brain was gently blotted on filter paper (Fisher Scientific), weighed, and immersed in ~4°C oxygenated ACSF contained in a 100 mL borosilicate glass vessel surrounded by ice. The tissue was kept in this condition for approximately 45 min while serum samples were collected. It was then transported to another building for *ex vivo* recording, a transfer that took approximately 10-15 min, with continuous maintenance in ice-cold oxygenated ACSF throughout the procedure.

Slice preparation:

Upon arrival, the brain was rapidly submerged in an ice-cold sucrose-based cutting solution composed of (in mM): sucrose, 189; D-glucose, 10; NaHCO3, 26; KCl, 3; MgSO4, 5; CaCl2, 0.1; NaH2PO4, 1.25. This solution was continuously aerated with carbogen (95% O2, 5% CO2) to maintain oxygenation and pH stability. The use of sucrose solution minimises osmotic stress and protects neural tissue during slicing by preventing cell swelling and mechanical damage.

The cerebellum and anterior forebrain regions anterior to bregma (> 0 mm) were removed using a single scalpel cut. The trimmed brain was affixed caudal side down to a metal stage with cyanoacrylate glue (RS Components) and placed in the cutting chamber of a Leica VT1200 vibratome filled with ice-cold cutting solution. Coronal slices (300 μm thickness) were cut at an angle of approximately 45° and a speed of 0.10 mm/s.

Freshly cut slices were collected using a wide-bore pipette and transferred to a holding chamber containing oxygenated ACSF. The slices were gradually warmed to 34 °C for 30 min, then maintained at room temperature (~22–24 °C) for at least 1 h before recording.

5.2.2 Stimulation Paradigm

The *ex vivo* electrophysiology setup consisted of a submerged-style recording chamber, stimulating and recording electrodes, a perfusion system, a microscope, an amplifier Multiclamp 700A (Molecular Devices, UK), a stimulator (current) isolation unit (Digitimer, UK), an analogue-to-digital converter (Digidata 1550B, Molecular Devices, UK), and a computer for data acquisition. The chamber was mounted on a vibration isolated table and enclosed within a grounded Faraday cage to minimise electrical noise.

Brain slices were transferred to the recording chamber using a wide-mouth pipette and stabilised with a U-shaped platinum wire strung with nylon fibres (“harp”). The stimulating electrode (twisted bipolar tungsten, quadruple PTFE coated wire, 50 μm wide) was positioned in layer 4 of the barrel cortex, and borosilicate glass pipettes (5-10 MΩ, filled with ACSF) were used to record field potentials from layer 2/3 (Figure 5.1).

Three stimulation paradigms were used to assess the effects of vitamin B12 supplementation on intracortical network properties (Table 5.1).

Paradigm 1: **Input-Output Relationship**

To assess synaptic efficacy across stimulus intensities, single pulses stimulations (0 to 300 μA in 50 μA increments) were applied to layer 4. Each intensity was repeated five times with an inter-trial interval of 20 s. The mean amplitude across five trials was used for analysis.

Paradigm 2: **Paired-Pulse Ratio (PPR)**

To evaluate short-term synaptic plasticity, paired-pulse stimulation was applied with inter-stimulus intervals of 10, 20, 50, 100, 200, 500, and 1000 ms. The PPR was calculated as the ratio of the second to the first response amplitude.

Paradigm 3: **Frequency-Dependent Adaptation**

To investigate frequency-dependent sensory adaptation, trials of electrical pulses were delivered at 2, 5, 10, and 20 Hz for 2 s. Each frequency was repeated five times with a 20 s inter-trial interval. Averaged responses were analysed to determine the frequency dependence of adaptation.

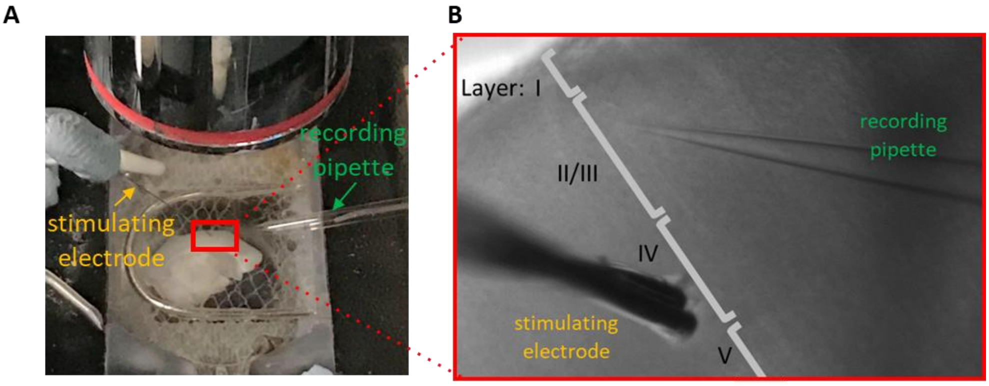
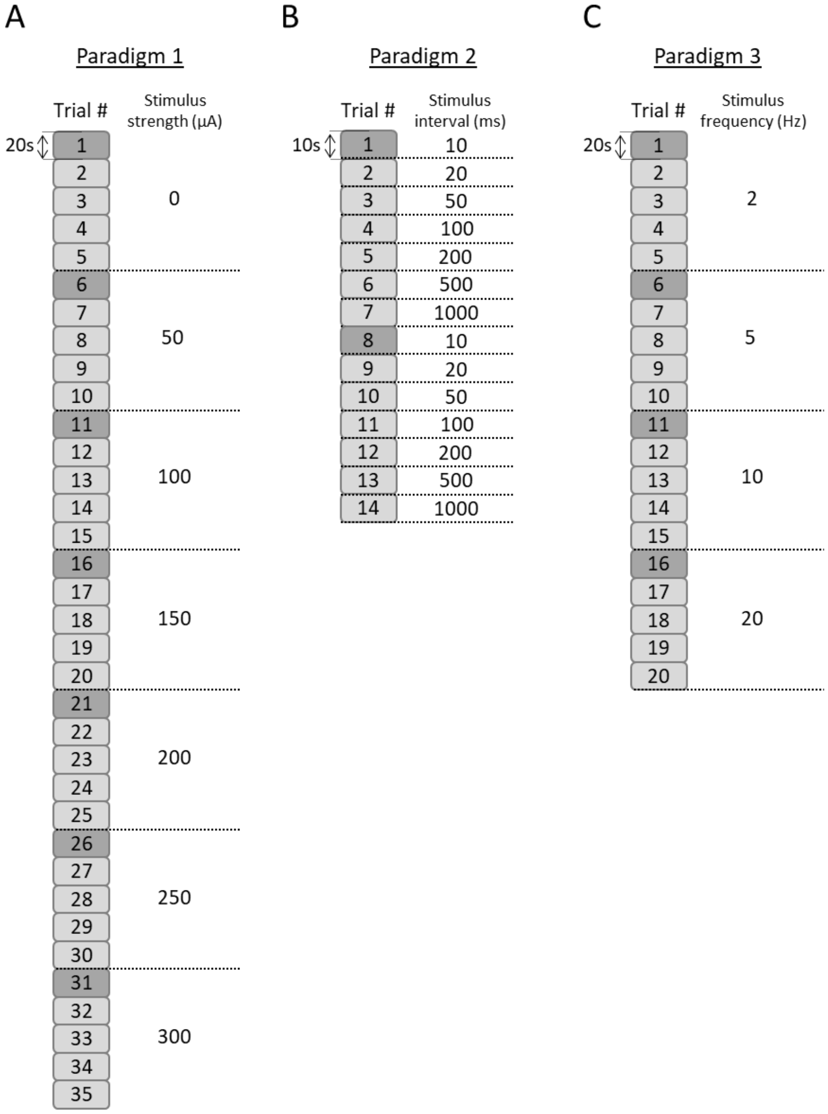


Figure 5.1 Pictures of neural recording in *ex vivo*

(A)Picture of recording set up. (B) Close-up picture of the recording area.

Table 5.1 Stimulation paradigm for *ex vivo* experiment



A

Paradigm 1

	Trial #	Stimulus strength (μA)
20s	1	0
	2	
	3	
	4	
	5	
	6	50
	7	
	8	
	9	
	10	
	11	100
	12	
	13	
	14	
	15	
	16	150
	17	
	18	
	19	
	20	
	21	200
	22	
	23	
	24	
	25	
	26	250
	27	
	28	
	29	
	30	
	31	300
	32	
	33	
	34	
	35	

B

Paradigm 2

	Trial #	Stimulus interval (ms)
10s	1	10
	2	20
	3	50
	4	100
	5	200
	6	500
	7	1000
	8	10
	9	20
	10	50
	11	100
	12	200
	13	500
	14	1000

C

Paradigm 3

	Trial #	Stimulus frequency (Hz)
20s	1	2
	2	
	3	
	4	
	5	
	6	5
	7	
	8	
	9	
	10	
	11	10
	12	
	13	
	14	
	15	
	16	20
	17	
	18	
	19	
	20	

5.2.3 Statistical Analysis

All statistical analyses were performed using SPSS Statistics version 27 (IBM Corp., Armonk, NY, USA). The significance threshold was set at $p < .05$. Data normality was assessed using the Shapiro–Wilk test, and when the assumption of sphericity was violated, the Greenhouse–Geisser correction was applied. For data sets that did not meet normality assumptions, non-parametric aligned rank transform (ART) ANOVA was employed, allowing for factorial analyses equivalent to mixed-design ANOVA while accommodating non-normal distributions.

Paradigm 1: Input-Output Relationship

To examine whether vitamin B12 supplementation affected intracortical synaptic transmission, the peak amplitude of layer 2/3 responses evoked by layer 4 stimulation was analysed using a two-way mixed-design ANOVA with group (Control vs B12; between-subjects) and stimulus intensity (50, 100, 150, 200, 250, 300 µA; within-subject) as factors. The main effect of stimulus intensity reflects the recruitment of synaptic responses as stimulation strength increases, while group-related effects test whether supplementation modifies synaptic efficacy.

Paradigm 2: Paired-Pulse Ratio (PPR)

Short-term synaptic plasticity was evaluated using the paired-pulse ratio (PPR), calculated as the amplitude of the second response divided by the first.

Data normality was first assessed using the Shapiro-Wilk test. As some data sets did not meet normality assumptions, aligned rank transform (ART) ANOVA was used to perform a two-way mixed-design analysis with group (Control vs B12; between-subjects) and inter-stimulus interval (10, 20, 50, 100, 200, 500, 1000 ms; within-subject) as factors. While the main effect of interval indexes the general facilitation or depression pattern, group-related effects test whether B12 alters short-term synaptic plasticity.

Paradigm 3: Frequency-Dependent Adaptation

To assess sensory adaptation within intracortical networks, layer 4 was stimulated at four frequencies (2, 5, 10 and 20 Hz) for 2 s.

Primary analysis (adaptation pattern):

Response amplitudes within each stimulus train were normalised to the first pulse (ration form). For each frequency, a mixed repeated-measures ANOVA or ART ANOVA was conducted with group (control vs B12; between subjects) and stimulus number (2nd to Nth pulse: within subjects) as factors. The main effect of stimulus number reflects adaptation, and the group × stimulus number interaction tests whether supplementation alters its pattern.

Supplementary analysis (summary index):

To provide a concise, frequency resolved index of overall adaptation depth, ratios were averaged across pulses within each frequency, yielding one value per frequency per slice. These mean ratios were analysed using a two-way mixed-design ANOVA with group (between subjects) and frequency (within subjects).

Finally, given the number of statistical comparisons conducted across paradigms, results are interpreted with caution. Where multiple tests were performed, the risk of inflated Type I error is acknowledged, and significant findings are considered in the context of this limitation.

5.3 Results (*Ex vivo* Electrophysiology)

5.3.1 Data Exclusion

Ex vivo recordings were obtained from a subset of animals used for the *in vivo* experiments (10 rats per group). Due to time constraints following the completion of *in vivo* recordings, *ex vivo* measurements were not performed for all animals.

Recordings were not conducted when slices failed to exhibit evoked responses following test stimulation. As a result, evoked responses were recorded from 15 slices taken from 6 rats in the control group, and 15 slices from 5 rats in the B12 group. All data were included for the analysis of Paradigm 1. However, some signal loss occurred during the recordings in certain slices, so 12 slices from the control group and 13 slices from the B12 group were used for the analyses of Paradigms 2 and 3.

5.3.2 *Ex vivo* Paradigm1 – Input-Output Relationship

Evoked field potentials recorded from layer 2/3 following layer 4 stimulation are shown in Figure 5.2A. Both groups exhibited a clear negative deflection whose amplitude generally increased with stimulus intensity, although the change tended to plateau at higher intensities. The mean peak amplitudes across different stimulus intensities are summarised in Figure 5.2B. Although the B12 group showed slightly higher mean amplitudes than the control group at all intensities, this difference was not statistically significant.

A two-way mixed ANOVA with Greenhouse Geisser correction revealed a significant main effect of stimulus intensity (F(1.77, 49.70) = 33.49, p < .001 np^2 = .544), indicating increasing responses with stronger stimulation, but neither the main effect of group (F(1, 28) = 1.85, p = .190 np^2 = .062) nor the group × intensity interaction (F(1.77, 49.67) = 0.16, p = .830 np^2 = .006) reached significance.

These results indicate that vitamin B12 supplementation did not alter the input-output relationship of layer 4-2/3 synaptic transmission under *ex vivo* conditions.

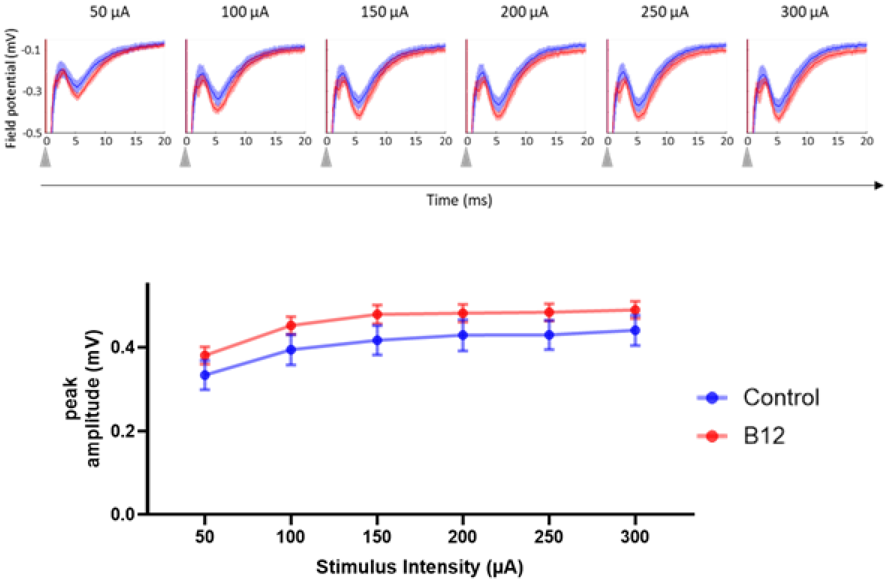


Figure 5.2 Input-output relationship of synaptic responses in layer II/III in the barrel cortex

(A) Representative field potentials recorded in layer 2/3 evoked by layer 4 stimulation at different current intensities (50-300 µA, 50 µA steps) for the Control (blue) and B12 (red) groups. Gray triangles indicate stimulus onset. (B) Mean (± SEM) peak amplitudes across stimulus intensities. Sample size: 15 slices from control group and 15 slices from B12 group

5.3.3 *Ex vivo* Paradigm2 – Effect of Vitamin B12 supplementation on Short-Term Synaptic Plasticity

To examine whether vitamin B12 supplementation alters short-term synaptic plasticity within the barrel cortex, paired-pulse stimulation was applied at ISI of 10-1000 ms. The PPR, defined as the amplitude of the second response divided by the first, was used as an index of short-term synaptic efficacy. Data were analysed using ART ANOVA and are reported as median ± IQR.

Figure 5.3 shows the median PPRs and individual data for each ISI in both groups. In both the control and B12 groups, the PPR increased with longer ISIs, reflecting a gradual reduction in short-term synaptic depression. However, no differences between the two groups were observed. The ART ANOVA revealed no significant main effect of group (F(1, 23) = 0.04, p = .84 np^2 = .002) and no significant group × ISI interaction (F(6, 138) = 0.32, p = .92 np^2 = .014). A significant main effect of ISI was found (F(6, 138) = 16.79, p < .001 np^2 = .422), indicating expected recovery of synaptic responses with increasing interval duration.

Overall, vitamin B12 supplementation did not significantly influence short-term synaptic transmission efficiency as indexed by the PPR, suggesting no measurable effect on intracortical presynaptic dynamics under *ex vivo* conditions.

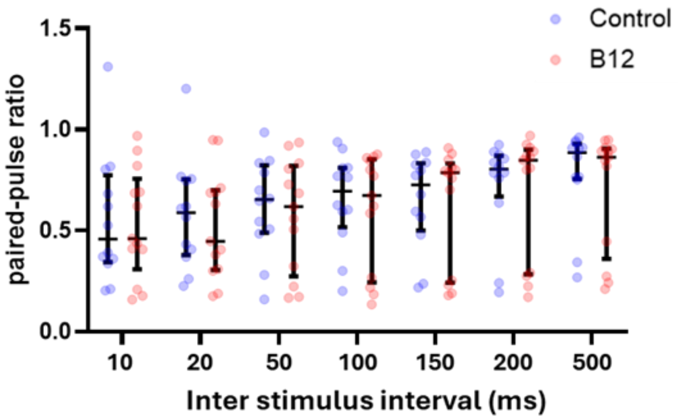


Figure 5.3 Short-term synaptic plasticity assessed by PPR in layer II/III in the barrel cortex

Median (± IQR) PPR values for the Control (blue) and B12 (red) groups across ISIs. Sample size: 12 slices from the control group and 13 slices from the B12 group.

5.3.4 *Ex vivo* Paradigm3 – Sensory Adaptation

Ex vivo – Sensory Adaptation (primary analysis)

To examine whether vitamin B12 supplementation altered sensory adaptation dynamics within the intracortical pathway, mixed repeated-measures ANOVAs (or ART ANOVA) were conducted with Group (Control vs B12; between-subjects) and Stimulus number (within subjects) as factors, separately for each stimulation frequency (2, 5, 10, and 20 Hz).

No significant main effects of Group × Stimulus number interactions were observed at 2, 5, or 20 Hz, whereas a significant Group × Stimulus number interaction was found at 10 Hz with a relatively large effect size (np^2 = 228) (Table 5.3), indicating that adaptation dynamics differed between groups.

A significant main effect of Stimulus number was observed across all frequencies (Table 5.3), reflecting the expected adaptation to repeated stimulation (Figure 5.4).

Table 5.2 Results of repeated-measures ANOVAs testing sensory adaptation in the barrel cortex. Mixed model repeated-measures ANOVAs were used for 10 Hz. For other frequencies, ART ANOVAs were applied.

Frequency	Effect	F(df1, df2)	P value	np^2
2 Hz	Group	F(1,23) = 0.004	.952	.000
	Group × Stimulus num	F(2,46) = 0.794	.432	.033
	Stimulus num	F(2,46) = 9.308	< .001	.288
5 Hz	Group	F(1,23) = 0.141	.771	.006
	Group × Stimulus num	F(8,184) = 0.573	.799	.024
	Stimulus num	F(8,184) = 4.168	< .001	.153
10 Hz	Group	F(1,23) = 0.138	.741	.006
	Group × Stimulus num	F(3.844,88.415) = 6.776	< .001	.228
	Stimulus num	F(3.844,88.415) = 5.796	< .001	.201
20 Hz	Group	F(1,23) = 1.670	.209	.068
	Group × Stimulus num	F(38,874) = 1.273	.128	.052
	Stimulus num	F(38,874) = 4.840	< .001	.174

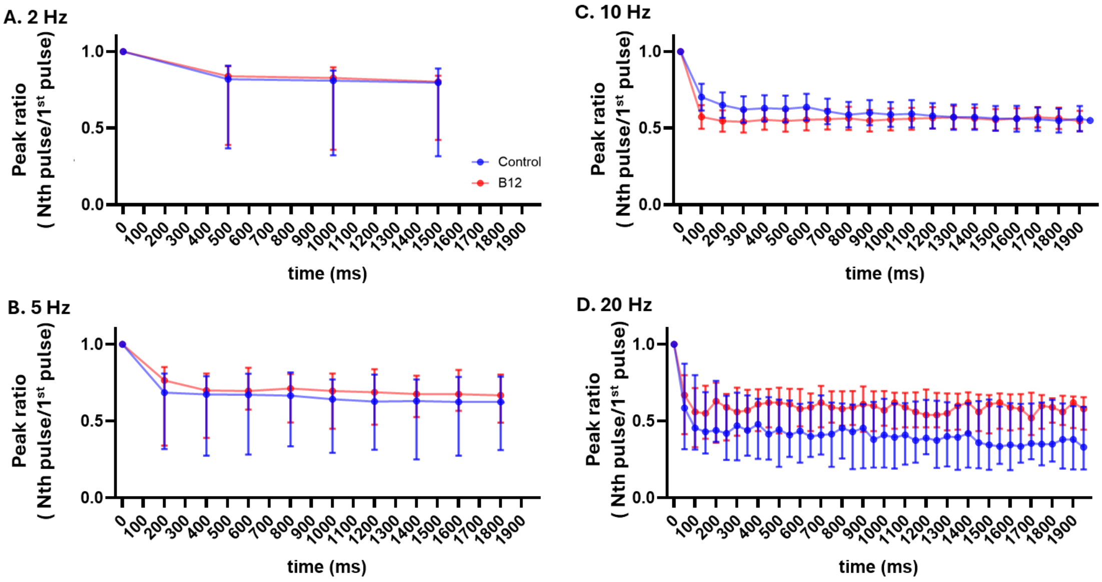


Figure 5.4 Sensory adaptation in layer II/III in the barrel cortex (primary analysis)

(A–D) Response amplitudes expressed as ratios relative to the first pulse, plotted across stimulus trains at 2, 5, 10, and 20 Hz (Control: blue, B12: red; mean ± SEM for C, median ± IQR for A, B and D). Sample size: 12 slices from the control group and 13 slices from the B12 group.

Ex vivo – Sensory Adaptation (supplementary analysis)

To provide a frequency-resolved summary measure of adaptation, mean peak ratios were calculated for each stimulation frequency (2, 5, 10, and 20 Hz) and analysed using a mixed repeated-measures ANOVA with Group (Control vs B12; between-subjects) and Frequency (within-subjects) as factors.

There was no significant main effect of Group (F(1,22) = 0.012, p = .913 np^2 = .001), and no Group × Frequency interaction (F(3,20) = 1.260, p = .315 np^2 = .159). A significant main effect of Frequency was observed, indicating storonger adaptation at higher sitmulation frequencies as expected (F(3,20) = 7.497, p = .001 np^2 = .529).

Totether, these analyses indicate that although adaptation depth varied systematically across stimulation frequencies, vitamin B12 supplementation did not alter the overall magnitude of adaptation in the intracortical pathway in the barrel cortex (Figure 5.5).

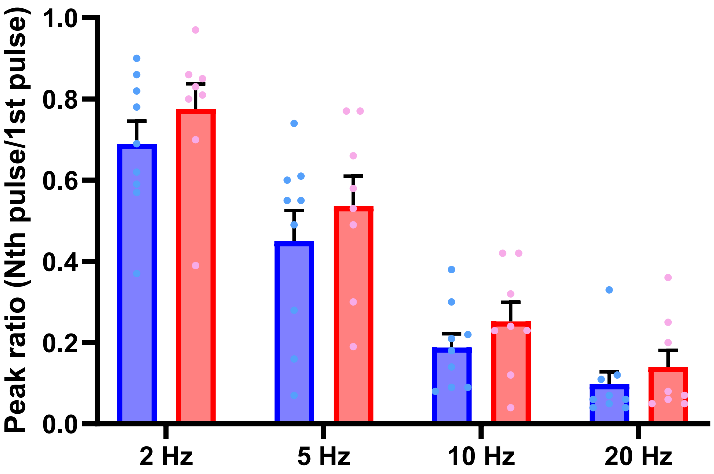


Figure 5.5 Sensory adaptation in layer II/III in the barrel acortex (supplementary analysis)

Mean peak ratios (response amplitude relative to the first pulse, averaged across all pulses within each frequency) for Control (blue) and B12 (red) groups at 2, 5, 10, and 20 Hz. Bars indicate group means ± SEM. Sample size: 12 slices from the control group and 13 slices from the B12 group.

5.4 Discussion (*Ex vivo* Electrophysiology)

5.4.1 Input/Output relationship

To In the layer 4 - layer 2/3 pathway of thess barrel cortex, vitamin B12 supplementation did not alter the input-output relationship of *ex vivo* evoked field potentials. Peak amplitudes increased with stimulation intensity, indicating normal recruitment of synaptic responses, but no group differences or Group × Intensity interactions were observed. These findings suggest that vitamin B12 does not modify basal synaptic gain within this feedforward circuit under the present experimental conditions.

The result is consistent with the idea that layer 4 - layer2/3 pathway exhibits relatively stable synaptic transmission properties in healthy animals (Feldmeyer et al., 2002; Lefort et al., 2009). Prior *ex vivo* studies have demonstrated that excitatory synapses from layer 4 to layer 2/3 show robust transmission, with limited variability in baseline synaptic strength across animals when no pathological or deprivation-induced changes are present. Thus, the absence of a supplementation effect may simply reflect that this pathway already operates close to its normal physiological range in healthy, non-deficient rats.

Importantly, these *ex vivo* findings differ from some *in vivo* reports howing enhanced cortical responsiveness following vitamin B12 supplementation —for example, the increased barrel cortex LFP amplitudes described by Kang et al. (2019). However, direct comparison between these studies must consider methodological differences. *In vivo* LFPs reflect the integrated activity of large neuronal populations, incorporating contributions from thalamocortical input, local recurrent excitation, long-range corticocortical signalling, and inhibitory networks. In contrast, *ex vivo* slice preparations isolate local synaptic circuits and remove neuromodulatory, vascular, and behavioural state influences. Therefore, the absence of vitamin B12 related chcanges *ex vivo* suggests that supplementation does not strongly alter the intrinsic synaptic strength of the layer 4-layer2/3 projection, even though circuit-level responsiveness *in vivo* may vary due to additional network interactions.

Overall, these findings indicate that vitamin B12 supplementation does not induce measurable changes in intracortical synaptic efficacy in healthy animals. Any potential supplementation effects may instead manifest at the systems or network level, or under conditions where synaptic transmission is compromised, such as during deficiency, injury, or heightened metabolic demand.

5.4.2 Short-Term Plasticity (Paired Pulse Ratio)

Short-term synaptic plasticity, as assessed by the PPR, provides insight into presynaptic release probability and short-latency inhibitory mechanisms within the layer 4 – layer 2/3 pathway. In the present study, PPR measured across inter-stimulus intervals from 10-500 ms showed typical interval-dependent modulation—strong depression at short intervals and recovery toward baseline at linger intervals—consistent with previous characterisations of this pathway (Bender et al., 2006; Feldman, 2009; Lefort & Petersen, 2017).

Across all intervals, however, no significant differences were observed between the control and B12 groups, indicating that vitamin B12 did not measurably alter presynaptic release probability in this intracortical circuit. These results are broadly consstent *in vivo* findings showing no ngroup differences in paired-stimulus responses when measured at the level of population LFPs.

To summarise, this finding indicate that vitamin B12 supplementation does not exert detectable effects on short-term synaptic plasticity in this intracortical pathway, implying that previously reported vitamin B12 related gains in sensory processing are unlikely to arise from changes in presynaptic release probability.

5.4.3 Frequency-Dependent Adaptation

Sensory adaptation in the layer 4-2/3 pathway was examined by assessing the attenuation of evoked responses during 2 s stimulus trains at four frequencis (2, 5, 10 and 20 Hz). Two complementary analyses were performed: a pulse-by-pulse analysis to assess the temporal profile of adaptation, and a summary analysis to evaluate overall adaptation depth at each frequency.

Pulse by Pulse Analysis (Main Analysis)

Both groups exhibited clear frequency-dependent adaptation across stimulation trains, consistent with the well-established properties of the layer 4-2/3 pathway, in which repeated activation induces short-term synaptic depression due to presynaptic resource depletion and reduced release probability (Feldmeyer et al., 2002; Gabernet et al., 2005). This pathway is particularly sensitive to repetitive input because excitatory synaptic depression and feed forward inhibition are rapidly engaged at physiologically relevant timescales (Higley and Contreas, 2006).

In the present study, no significant group effects or Group × Stimulus number interactions were observed at 2, 5, or 20 Hz, indicating that vitamin B12 supplementation did not alter the temporal profile of adaptation at these frequencies. At 10 Hz, however, a significant Group × Stimulus number interaction was detected, with the B12 group exhibiting stronger attenuation across successive pulses than the control group. This suggests that supplementation may selectively modulate the temporal dynamics of adaptation under specific stimulation regimes.

A frequency-specific effect at 10 Hz is notable given prior evidence that the 8-12 Hz range represents a transition zone in which excitatory synaptic depression and feedforward inhibition interact nonlinearly to shape cortical responsiveness (Gabernet et al., 2005; Higley and Contreras, 2006). Under such conditions, subtle circuit-level modulations introduced by vitamin B12 may become more detectable, as competing synaptic processes are more dynamically balanecd. In broader context, 10 Hz activity has also been implicated in somatosensory processing in humans, where transcranial alternating current stimulation at this frequency altered perceptual response bias

(Craddock et al., 2019). Although mechanistically distinct from the present ex vivo slice preparation, these findings are consistent with the view that 10 Hz may represent a functionally sensitive temporal regime within somatosensory networks. Importantly, however, the present effect was confined to a single frequency and did not generalise across stimulation conditions, warranting cautious interpretation.

Summary Index of Adaptation Depth (Supplementary Analysis)

To provode a frequency-resolved index of overall adaptation strength, peak ratios were averaged across all pulse at each stimulation frequency. Using this summary measure, no significant differences were observed between the control and B12 groups, and no Group × Frequency interaction were found. Both groups exhibited a pronounced frequency dependence, with stronger adaptation at higher stimulation frequencies, consistent with established models of short-term synaptic depression in cortical circuits (Abbott et al., 1997; Gabernet et al., 2005).

The absence of a group effect in the summary index indicates that vitamin B12 supplementation did not produce a systematic shift in overall adaptation magnitude. Instead, the supplementation effect observed in the pulse-by-pulse analysis at 10 Hz appears to represent a localised modulation of temporal dynamics, rather than a general enhancement or suppression of adaptation. Such a dissociation suggests that vitamin B12 may influence specific phases of response evolution without altering the accumulated depth of adaptation over the course of the stimulation train.

Taken together, these findings indicate that vitamin B12 supplementation does not uniformly alter intracortical sensory adaptation but may exert frequency-specific effects that emerge under particular temporal regimes. Whether these subtle effects reflect enhancement of inhibitory recruitment, alteration of presynaptic resource use, or other circuit level mechanisms remains to be determined.

5.4.4 Summary – *Ex vivo* Discussion

Across three complementary assays of synaptic function in the layer 4-2/3 pathway, vitamin B12 supplementation did not produce consistent or large-scale modifications of intracortical signal processing in healthy adult rats. Input-output relationships were comparable between groups, indicating that supplementation did not alter baseline synaptic gain within this feedforward projection. Short-term synaptic plasticity, assessed via PPR across a range of inter stimulus intervals, also did not differ between groups, suggesting that presynaptic release probability and early inhibitory mechanisms were unaffected by supplementation.

Analysis of frequency dependent adaptation revealed a generally similar adaptation profile between groups, with a robust increase in attenuation at higher stimulation frequencies, consistent with established models of short term synaptic depression in this pathway. The only difference was a frequency specific effect at 10 Hz, where the B12 group exhibited greater attenuation across successive pulses, although this effect did not translate into a group difference in overall adaptation depth.

Together, these findings suggest that vitamin B12 supplementation does not broakly modify intracortical synaptic transmission or short term plasticity under *ex vivo* conditions, but may exert subtle, frequency dependent effects that emerge only within specific temporal regimes. Importantly, the absence of widespread effects implies that previously reported supplementation related enhancements in sensory processing may arise from network level mechanisms present *in vivo* —such as neuromodulatory influences, multi layer interactions, or behavioural state —rather than intrinsic modifications of local synaptic connectivity in healthy tissue. Future work should therefore establish whether supplementation interacts with cortical processing under conditions of deficiency, altered metabolic demand, or behaviourally relevant sensory engagement.

Chapter 6 General Discussion

The present thesis examined the effects of vitamin B12 supplementation on whisker dependent behaviour, cortical sensory processing *in vivo*, and intracortical synaptic transmission *ex vivo*. Across these complementary levels of analysis, supplementation did not produce consistent or robust changes in behavioural performance, sensory evoked cortical responses, or short-term synaptic plasticity in healthy adult rats. Although frequency-specific differences were observed in the *ex vivo* analysis of sensory adaptation, with a relatively large effect size at 10 Hz ($F(3.844, 88.415) = 6.776$, $p < .001$, $np^2 = .228$), suggesting a condition-specific modulation of circuit dynamics. These effects were confined to a single stimulation condition and did not generalise across frequencies or analytical approaches. Taken together, these findings indicate that vitamin B12 supplementation does not markedly alter sensory processing in the rodent barrel cortex under non-deficient physiological conditions, and that any potential effects may depend on specific circuit states, stimulation regimes, or physiological constraints.

Behavioural outcomes and implications for neural processing

The adapted tNORT protocol demonstrated reliable sensitivity to texture novelty without positional or exploration bias, validating it as a behavioural tool for assessing whisker-based sensory discrimination. However, vitamin B12 supplementation did not improve discrimination performance in healthy rats. This suggests that, under conditions where baseline perceptual ability is already high, supplementation may not confer measurable behavioural benefits.

Importantly, the absence of behavioural effects does not necessarily imply an absence of neural consequences. The tNORT task requires only modest sensory precision and does not probe the limits of texture discrimination, raising the possibility that subtle neural modifications could remain behaviourally silent under the present testing conditions. Behavioural sensitivity may depend critically on the degree of sensory challenge imposed, or on conditions in which neural processing is compromised, such as dietary deficiency.

Systems level neural responses: *in vivo* electrophysiology

Consistent with the behavioural findings, *in vivo* recordings showed no measurable supplementation effect on input-output relationships or short-term sensory adaptation in the thalamus or barrel cortex. These results differ from reports of enhanced paired-pulse responses after supplementation (Kang et al., 2019), but several methodological distinctions may explain the discrepancy. They examined PPR following dietary supplementation, whereas the present study characterised multi pulse adaptation across stimulation trains, which may capture different aspects of cortical processing.

Moreover, *in vivo* LFPs reflect the integrated activity of distributed neuronal populations, shaped not only by synaptic connectivity but by neuromodulatory state, recurrent dynamics, and corticothalamic interactions. It remains possible that supplementation influences broader network properties in a manner that is not detectable with the measures used here.

Circuit level mechanisms: *ex vivo* electrophysiology

Slice recordings provided a mechanistic examination of intracortical transmission in the layer 4-2/3 pathway. Across three stimulation paradigms, supplementation did not significantly alter baseline synaptic gain, presynaptic release probability, or overall adaptation strength. These results indicate that, in healthy animals, vitamin B12 does not induce large scale modifications of local synaptic efficacy.

A single exception emerged at 10 Hz, where the B12 group exhibited greater attenuation of evoked responses across successive pulses. Notably, whisker-dependent behaviours in rodents are associated with specific frequency ranges: exploratory whisking typically occurs at 5-15 Hz, whereas object recognition and texture discrimination involve higher frequencies (15-25 Hz) (Berg and Kleinfeld, 2003). Moreover, cortical sensory representations can be reorganised in a frequency-specific manner (Alonso et al., 2008). Thus, subtle supplementation effects may have become detectable specifically at 10 Hz. As this difference did not generalise across frequencies or produce consistent changes in overall adaptation measures, it may reflect a localisedT rather than global, modulation of circuit dynamics.

Integrating behavioural and neural findings

Together, the behavioural, *in vivo*, and *ex vivo* results indicate that vitamin B12 supplementation does not produce robust enhancements of sensory processing in healthy rats. Any effects, if present, appear subtle, frequency-dependent, and confined to specific circuit states.

The pattern of findings is consistent with the view that texture discrimination, as assessed here, is supported by neural mechanisms already operating near optimal levels in healthy, non-deficient animals. Under such conditions, supplementation may neither enhance perceptual performance nor significantly modify neural function.

Moreover, the discrepancy between subtle neural effects (observed *ex vivo* at 10 Hz stimulation) and the absence of behavioural enhancement suggests that circuit-level modulation was insufficient to influence performance under the behavioural demands tested.

Critically, different aspects of whisker behaviour recruit different frequency regimes. During natural object recognition, activity in the 15-25 Hz range may be particularly relevant. Since the tNORT does not control whisker movement or impose temporal constraints on sensory sampling, it remains unclear whether supplementation might influence behaviour under conditions that more strongly engage frequency-dependent neural computation.

Relation to previous literature

Although previous studies have suggested that vitamin B12 can modulate cortical excitability, the direction and magnitude of these effects appear to be highly state-dependent.

For example, Smith et al. (2017) reported reduced visual cortical responses following intake of B vitamin rich supplement in healthy adults, consistent with an enhancement of inhibitory tone. In contrast, Kang et al. (2019) observed increased whisker-evoked responses in the barrel cortex of healthy rats following high dose vitamin B12 supplementation.

These findings, together with the present results showing little or no change in evoked activity in non-deficient male rats, indicate that vitamin B12 supplementation does not exert a uniform excitatory effect across species, brain regions, or experimental conditions.

Instead, its impact in non-deficient systems may be subtle and shaped by multiple interacting factors, including:

- Baseline vitamin B12 status and metabolic demand
- Sex dependent physiological differences
- The measure used (EEG, LFP, or behaviour)
- The balance of excitatory and inhibitory mechanisms engaged by each paradigm.

Thus, the present findings do not necessarily contradict earlier reports; rather, they highlight that the neurophysiological consequences of vitamin B12 supplementation ion healthy subjects are complex, context-dependent, and unlikely to follow a single mechanistic pathway.

Future directions

While the present findings indicate that vitamin B12 supplementation does not robustly alter sensory processing in healthy, non-deficient rats, they also point to several directions for future research.

First, given that vitamin B12 deficiency is known to impair myelination, synaptic transmission, and sensory function, it will be important to determine whether supplementation exerts clearer effects under conditions of dietary deficiency or metabolic challenge. Direct comparisons between deficient and replete animals may help clarify whether the absence of effects observed here reflects a ceiling phenomenon in healthy systems.

Second, the frequency-specific effect observed *ex vivo* at 10 Hz suggests that vitamin B_{12} supplementation may interact with specific temporal regimes of cortical processing. This is notable given broader evidence that activity around 10 Hz can influence somatosensory perception in humans, including altered response bias during tactile detection following transcranial alternating current stimulation (Craddock et al., 2019). Although distinct from the present rat behavioural paradigm, these findings support the possibility that the alpha band (8–12 Hz) range may represent a sensitive processing window within somatosensory networks. Future studies could therefore examine this frequency range more systematically using combined behavioural, *in vivo*, and circuit level approaches.

Third, higher-resolution approaches, such as single-unit recordings or cell-type specific manipulations, may be required to detect subtle circuit-level changes that are not captured by field potential measures alone.

Finally, behavioural paradigms that impose greater sensory demands or tighter temporal constraints on whisker use may be more sensitive to detecting functional consequences of such neural modulation.

Together, these approaches will help to define more precisely the physiological and experimental conditions under which vitamin B12 supplementation may influence sensory processing, and to place such effects in a clearer context for both basic neuroscience and nutritional intervention research.

Chapter 7 References

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