Tactile Discrimination Learning in Mice

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A Thesis Submitted to Cardiff University

For the Degree of Doctor of Philosophy

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A special mention goes to my husband Matthew and my family whose constant support and encouragement enabled me to believe in myself, learn and grow as a person and a scientist.

Publications

Some of the research in Chapters 2 and 3 have been presented at conferences and described in two recent papers. Chapter 2 includes material briefly described in the selective review by Pacchiarini, Fox and Honey (2017), while Chapter 3 closely follows the paper by Pacchiarini, Berkeley, Fox and Honey (2019).

Peer-reviewed papers:

- Pacchiarini, N., Berkeley, R., Fox, K., & Honey, RC. (2019). Whisker-mediated texture discrimination learning in freely moving mice. *Journal of Experimental Psychology: Animal Learning and Cognition.*
- Pacchiarini, N., Fox, K., & Honey, RC. (2017). Perceptual learning with tactile stimuli in rodents: Shaping the somatosensory system. *Learning & Behavior*, *45*, 107-114.

Conference presentations:

- Pacchiarini, N., Fox, K., & Honey, RC. (2018) Discrimination learning with tactile stimuli in rodents. Poster presentation at the *48th Annual Meeting of the Society for Neuroscience,* San Diego, USA, 3-7 November.
- Honey, RC., & Pacchiarini, N. (2018) Developing a model system to understand perceptual learning: Texture discrimination in rodents.

Work presented by Honey, RC at the *Associative Learning Symposium,* Gregynog, 19-21 March.

- Pacchiarini, N., Honey, RC., Fox, K. (2018) Perceptual learning with tactile stimuli in rodents. Poster presentation at the *UK-Japan Neuroscience Symposium*, Royal Society, London, 5-6 March.
- Pacchiarini, N., Honey, RC., Fox, K. (2016) Investigating perceptual learning in the mouse. Poster presentation at the *Autumn School in Cognitive Neuroscience*, University of Oxford, 29-30 September.
- Pacchiarini, N., Honey, RC., Fox, K. (2016) Investigating perceptual learning in the mouse. Poster presentation at the *I.M.A Neural Imaging in Neuroscience Conference*, Peterhouse College, Cambridge, 21-23 March.

Summary

In order to investigate neuronal activity associated with learning, it is important to use robust behavioural tests that are rapidly acquired, long-lasting and reliant on the sensory modality under investigation. Chapter 1 provides an overview of the whisker-barrel system and currently available behavioural tasks used to assess texture-based learning in mice. A selective review is then presented which highlights the current understanding of synaptic plasticity in the barrel cortex. Chapter 2 reports the findings of a modified novel object recognition task that uses 3-D printed stimuli to assess texture recognition memory. The experiments revealed that mice can retain a memory of tactile stimuli across a 24-hour period, however, the procedure required a large number of animals. Chapter 3 describes the development of a texture-based two-choice discrimination task. The experiments demonstrated that mice can learn rapidly a texture-based discrimination and remember the discrimination over a 24-hour interval. The experiments also revealed that discrimination learning is whisker-dependent: whisker trimming impaired the animals' ability to learn the texture-discrimination but had no effect on an odour-discrimination. Chapter 4 explored the role of the barrel cortex in the new texture discrimination procedure by reducing the activity of neurons in layer 4 of the barrel cortex using the chemogenetic DREADD technology. The results revealed that reducing activity of these neurons resulted in an inability to acquire the texture discrimination, but did not impair performance on an equivalent odour discrimination task. Analysis of cFos levels, as a proxy for neuronal activity, revealed that in cases infected with DREADDs and activated by CNO, cFos levels were increased in PV cells

and decreased in non-PV cells. Together, these experiments suggest a critical role for the barrel cortex in whisker-dependent texture discrimination learning. Chapter 5 explores the broader implications of these results and makes recommendations for future experiments investigating the neural basis of texture discrimination.

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Abbreviations

Substances, Methods:

ChR2	Channelrhodopsin-2
CNO	Clozapine-*N*-Oxide
DMSO	Dimethyl Sulfoxide
DREADDs	Designer Receptors Exclusively Activated by Designer Drugs
PBS	Phosphate Buffered Saline
PBST	Triton X-100 in Phosphate Buffered Saline
PFA	Paraformaldehyde
PLA	Polylactic Acid
PV	Parvalbumin
TTX	Tetrodotoxin

Brain structures:

A1	Primary Auditory Cortex
ALM	Anterior Lateral Motor Area
AP	Anterior - Posterior
DV	Dorsal - Ventral
LD	Latero - Dorsal
M1	Primary Motor Cortex
M2	Secondary Motor Cortex
ML	Medial - Lateral

Pom	Medial Posterior Nucleus of the Thalamus
PPC	Posterior Parietal Cortex
PrV	Primary Trigeminal Nucleus
PSD	Postsynaptic Density
SpV	Spinal Trigeminal Nucleus
SpVi	Spinal Trigeminal Nucleus pars interpolaris
SpVic	Spinal Trigeminal Nucleus pars interpolaris (Caudal)
SpVir	Spinal Trigeminal Nucleus pars interpolaris (Rostral)
SpVo	Spinal Trigeminal Nucleus pars oralis
TEA	Temporal Association Area
V1	Primary Visual Cortex
VPMdm	Dorsomedial part of the Ventral Posterior Medial Nucleus
VPMh	Head area of Ventral Posterior Medial Nucleus
VPMvl	Ventrolateral part of Ventral Posterior Medial Nucleus
wM1	Whisker Motor Cortex
wS1	Whisker part of Primary Somatosensory Cortex
wS2	Whisker part of Secondary Somatosensory Cortex

Behavioural elements:

DNMS	Delayed Non-Matching to Sample
LTP	Long Term Potentiation
NOR	Novel Object Recognition
VPC	Visual Paired Comparisons

Chapter 1. General Introduction

1.1. Background

During learning, precise synaptic circuits form connections between hundreds of thousands of neurons. These neural circuits are influenced by sensory experiences and moulded by spontaneous and sensory-evoked activity (Katz & Shatz, 1996). Plasticity in the cortex allows us to remember past events, change our behaviour following experience and learn new skills (Fox, 2002). The first description of plasticity was reported by Bliss and Lomo (1970) who described long-term potentiation (LTP). LTP is caused by the coordinated spiking of pre- and postsynaptic neurons, which cause synaptic strengthening and thus increase the likelihood of an action potential. Synaptic strengthening is thought to underlie the cellular mechanism for learning and memory and the rodent barrel cortex is a system frequently used to investigate this type of plasticity. The barrel cortex stores representations of sensory stimuli, with each whisker represented by its own barrel column. This intrinsic connectivity makes the barrel cortex particularly useful as the peripheral sensory input can be easily manipulated (Fox, 2002). In order to investigate the role of the cortex in learning and memory, measurement of neuronal activity must be combined with rigorous behavioural tests based on precisely controlled stimuli. This thesis investigates the neural basis of texture discrimination learning in mice by using a novel behavioural procedure. There is a large range of genetic tools available for use in mice that enable the manipulation of neuronal activity at the cellular and molecular level (for review, see Feldmeyer et al., 2013). In

order to maximise these tools, precise behavioural methods must be developed.

This introduction will begin by presenting a summary of the whisker-barrel system, highlighting the major anatomical pathways involved. Next, the behavioural procedures that have been used to study texture learning in rodents will be reviewed. These procedures involve the use of textured materials as test stimuli in order to study texture learning in rodents. However, in these studies, the sensory basis of the texture discrimination is not addressed: The experiments do not control for other sensory elements that could be contributing to learning. For example, although learning may be based on the whisker system, it could also be based on other senses (e.g., olfactory or visual) which makes interpretation of behavioural effects and their neural bases less secure. The final section of the introduction consists of a selective review focusing on plasticity in the barrel cortex. This section describes the current understanding of dendritic spine plasticity, a process thought to underlie learning and memory. The overarching aim of this thesis was to develop a behavioural procedure for use in combination with imaging techniques to assess the role of dendritic spine activity in learning and memory. The procedure needed to result in rapid learning, which was long-lasting and based on the texture of the stimuli as opposed to other sensory modalities.

1.2. Whisker to Cortex

The somatosensory system is an important system for perception of the world in rodents. Rodents rely on somaesthetic senses in order to process

information relating to both the physical properties and spatiotemporal arrangement of objects (Hartmann, 2011; Tiest, 2010). Tactile stimulation is also closely linked with social and emotional behaviour (Gonzalez, Lovic, Ward, Wainwright, & Fleming, 2001; Hertenstein, Keltner, App, Bulleit, & Jaskolka, 2006; Imanaka et al., 2008); and there has been extensive research on the mechanisms that are essential for somatic sensation in a number of mammals (Bowden & McNulty, 2013; Guo et al., 2014; Reed-Geaghan & Maricich, 2011).

Vincent (1912) first demonstrated that a rat's whiskers are crucial for navigation and that touch represents a primary channel for navigating the environment. Whiskers are thin tapered rods (approximately 30 mm/3000 μm in length) located on each side of the rodents' snout that serve as sensors for acquiring tactile information (Brecht, Preilowski, & Merzenich, 1997; Sofroniew, Cohen, Lee, & Svoboda, 2014). Rodents have two sets of whiskers, the macrovibrissae (a large matrix of about 25 moveable sensors on either side of the snout) and microvibrissae (the shorter whiskers around the mouth, chin and nose of the animal) (Brecht et al., 1997; Deschenes, Moore, & Kleinfeld, 2012). The whiskers are organised in a grid made up of 5 rows labelled A to E, and numbered arcs, this allows each whisker to be recognised by its row and arc coordinates - for example C2 (Figure 1) (Diamond & Arabzadeh, 2013).

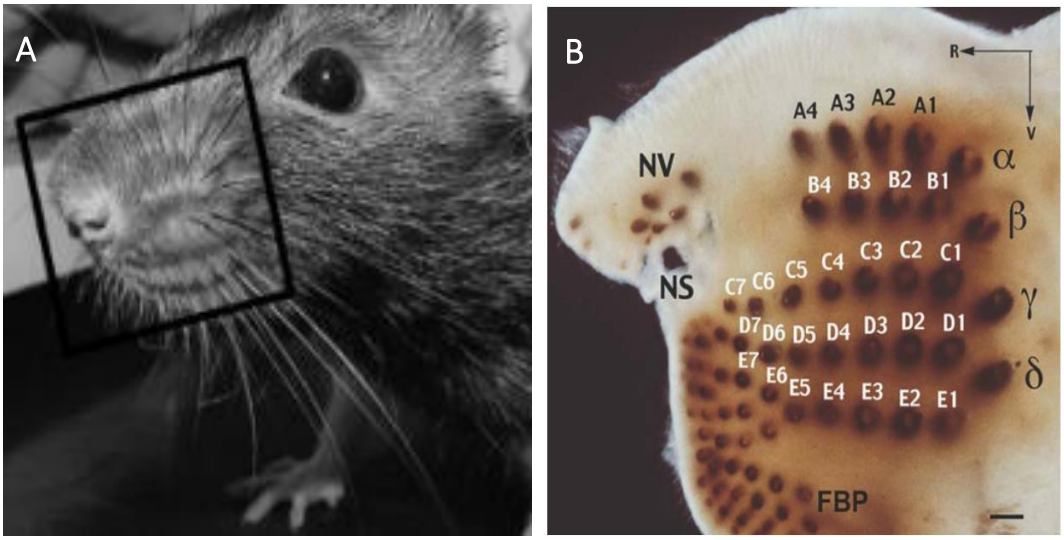

Figure 1. Rodents have two sets of whiskers: macrovibrissae located on the snout and smaller microvibrissae on the chin and nose. (A) Photograph of the rat head; mystacial whiskers and pad are indicated by the black box (Grant, Haidarliu, Kennerley, & Prescott, 2013) (B) Xylene-cleared section of the macrovibrissal follicles in the rat (Haidarliu, Simony, Golomb, & Ahissar, 2010). Images have been used with permission from authors.

As rodents are nocturnal animals that live in burrows it is believed that the whisker system developed to compensate for the low light levels and lack of visual information (for review, see Petersen, 2007). Consequently, when exploring an environment, rodents palpate objects through an active process known as 'whisking': this is a motor process whereby rodents make fast large-amplitude rhythmic sweeping motions (Carvell & Simons, 1990; Knutsen, Derdikman, & Ahissar, 2005; Mitchinson et al., 2011). The sweeping motions are typically a backward and forward movement whereby the whisker bends and exerts forces within the mechanosensory receptors at the base of the whisker when in contact with an object (Sofroniew et al., 2014). These forces open stretch-activated ion channels which drive action potential firing in whisker sensory neurons, and ultimately allow rodents to

recognise the size, shape and surface texture of an object (Petersen, 2014). This information is then used by the animal to build up a spatial representation of the environment (Diamond, von Heimendahl, Knutsen, Kleinfeld, & Ahissar, 2008; Vincent, 1912). Whisker processing in the brain is highly organised and each whisker is represented by a discrete structure in layer 4 of the primary somatosensory “barrel” cortex (Woolsey & Van der Loos, 1970). The cortex is a six-layered structure with the barrels located in layer 4. Each whisker is directly represented in the barrels, which can be recognized by the distinctive pattern in the cortex (Figure 2)(Feldmeyer et al., 2013; Fox, 2008; Vincent, 1912). Approximately 13% of the mouse cortical surface area and 69% of the somatosensory cortex is represented by the barrel cortex, demonstrating the large innervation levels of the whisker follicles (Fox, 2008).

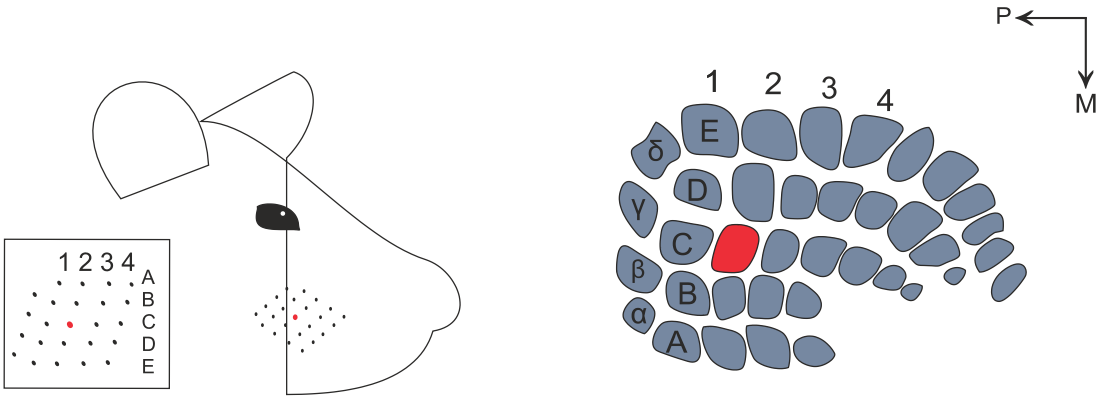

Figure 2. The barrels in the neocortex are located in layer 4 of the primary somatosensory cortex and consist of rows A-E and arcs 1,2,3 etc. The C2 whisker follicle and C2 barrel are highlighted in red.

The barrels are made up of dense clumps of thalamic axons separated by thalamocortical afferent branches that make up the gaps between the barrels, known as septae. The outer walls of the barrels have a higher cell density than the centre and contain approximately 2000 neurons with 75% excitatory and 25% inhibitory cell types (Ren, Aika, Heizmann, & Kosaka, 1992). The larger barrels are located in the posterior medial barrel subfield and the smaller ones in the anterior lateral section (Woolsey & Van der Loos, 1970). The barrels are predominately isolated from lateral connections, connecting mainly with other cells within the barrel, however, the septa represent the main channels of intracortical projections arising from neighbouring barrel and septal columns (Kim & Ebner, 1999). The septal regions receive innervation from the posterior medial thalamic nucleus as well as callosal input from the barrel area in the other hemisphere. These pathways convey information relating to vibrassal movement and thus form a complementary stream of information for tactile processing (Petersen, 2007). It is important to note, however, that in the mouse, unlike the rat, the septa are much smaller with fewer cells indicating that there may be differences in the cortical circuitry. The research in this thesis is directed to understanding the mouse system. Studies have demonstrated that a single whisker has the strongest influence on a given neuron's firing and there is a clear 'whisker-to-barrel' connection (Diamond, von Heimendahl, Knutsen, et al., 2008; Hutson & Masterton, 1986). The barrel cortex therefore serves as an ideal network for investigating dynamic function whilst undertaking specific whisker-related behaviours.

1.3. Anatomical Pathways of Whisker Movement

The cortical areas involved in sensing whisker movements have been widely investigated and a number of key areas have been identified: primary somatosensory cortex (S1), secondary somatosensory cortex (S2), primary motor cortex (M1), secondary motor cortex (M2), anterior lateral motor area (ALM) and posterior parietal cortex (PPC) (Figure 3). These areas are connected via neuronal circuits and mis-wiring of these circuits is associated with neurological disorders such as autism and schizophrenia (Luscher & Huber, 2010; Rubenstein, 2011; Wu, Ballester Rosado, & Lu, 2011). The whisker-to-barrel system described earlier has been used as a system for understanding cortical neural circuits. The pathway from whisker to barrel will be briefly described before evaluating the effect sensory experience has on the neural network in Section 1.5.

An individual whisker is innervated by the trigeminal ganglion where nerve endings convert mechanical energy from the whisker deflection into action potentials (Dörfl, 1985). These signals continue to the sensory trigeminal nuclei of the brainstem where they ascend to the thalamus. The primary trigeminal nucleus (PrV) lies anterior to the spinal trigeminal nucleus (SpV), which comprises of an oral (SpVo), an interpolar (SpVi), and a caudal section (SpVic). The SpVc and PrV contain a discrete group of neurons which receive direct input from one particular whisker, this clear anatomical map (termed barelettes) can be visualised in the same manner as in the cortex (Erzurumlu, Murakami, & Rijli, 2010). The thalamus is essential for transmission of whisker stimuli to the whisker part of S1 (wS1).

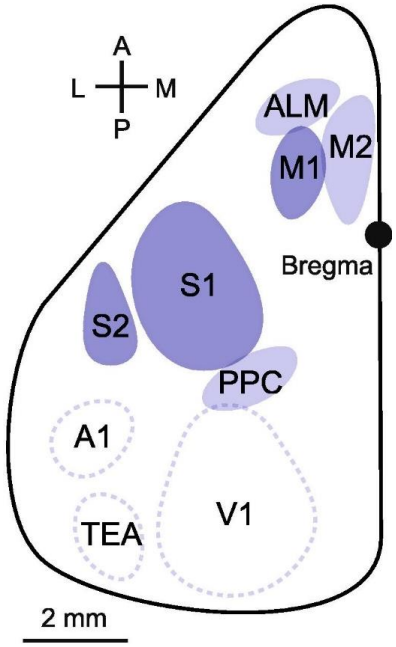

Figure 3. A top view of the left hemisphere of mouse cortex, schematic taken with permission from Helmchen, Gilad, and Chen (2018). There are a number of important areas for whisker-based discrimination. S1: primary somatosensory cortex, S2: secondary somatosensory cortex, M1: primary motor cortex, M2: secondary motor cortex, ALM: anterior lateral motor area, PPC: posterior parietal cortex; also shown are A1: primary auditory cortex, V1: primary visual cortex, and TEA: temporal association area.

There are three parallel pathways connecting the whisker to wS1: the lemniscal, the paralemniscal and the extralemnsical. The lemniscal pathway links the barelettes of the PrV to wS1 via the dorsomedial part of the ventral posterior medial nucleus (VPMdm) of the thalamus to layers 4, 5b and 6 of the cortex (Erzurumlu, Bates, & Killackey, 1980; Veinante & Deschenes, 1999) (Figure 4; orange line). The lemniscal pathway is the main pathway which conveys single-whisker input and the cells in VPMdm respond to deflections of a single principle whisker (Armstrong-James & Callahan, 1991; Simons & Carvell, 1989). The paralemniscal pathway ascends through the

medial division of the posterior nucleus (Pom) of the thalamus to layers 1 and 5a of wS1, whisker part of S2 (wS2) and layer 5A of the whisker M1 (wM1). The Pom receives input from multi-whisker cells in the rostral part of SpVi (SpVie) (Figure 4; blue line) (Chmielowska, Carvell, & Simons, 1989; Lu & Lin, 1993). Finally, the extralemniscal pathway synapses in the ventrolateral part of VPM (VPMvl) where the barreloids are not as clearly seen as in VPMdm (Figure 4; green line). The inputs for the extralemniscal pathway are found within the interbarelette cells of the SpVic and convey information form multi-whiskers. Layers 4 and 6 of wS2 receive the output from VPMvl, as well as the septal columns of wS1 (Bosman et al., 2011).

In addition to these main pathways, multi-whisker information is also conveyed to the barrel cotex from the interbarelette cells of PrV which project to Pom and the head area of VPM (VPMh) (Figure 4; pink line) (Veinante & Deschenes, 1999). From here, the barreloid cells project to the septal areas of wS1. Finally, Figure 4 depicts two other less characterised pathways, the first, shown in red, projects from SpVi to the thalamic laterodorsal nucleus (LD) which projects sparsely to wS1 (Bezdudnaya & Keller, 2008). The final line shown in Figure 4, in brown, depicts a pathway which originates from SpVo and projects to Pom (Jacquin & Rhoades, 1990).

As well as trigemino-thalamo-cortical connections, sensory processing is also conveyed to other cortical areas via cortico-cortical connections (Chakrabarti & Alloway, 2006; Welker, Hoogland, & Van der Loos, 1988). The projections from S1 barrel cortex have been extensively studied, and research indicates that it projects to M1, S1, S2, insular, perirhinal,

ectorhinal, auditory and visual cortex (Fabri & Burton, 1991; Hoffer, Hoover, & Alloway, 2003; Petrof, Viaene, & Sherman, 2015; Reep, Goodwin, & Corwin, 1990; Smith & Alloway, 2013; Zakiewicz, Bjaalie, & Leergaard, 2014). Furthermore, the barrel cortex also contains subcortical projections that terminate in the basal ganglia, thalamus, red nucleus, superior colliculus and pontine nuclei and contralaterally in the trigeminal nuclei and spinal cord (Alloway, Crist, Mutic, & Roy, 1999; Fabri & Burton, 1991; Hoogland, Welker, & Van der Loos, 1987; Zakiewicz et al., 2014).

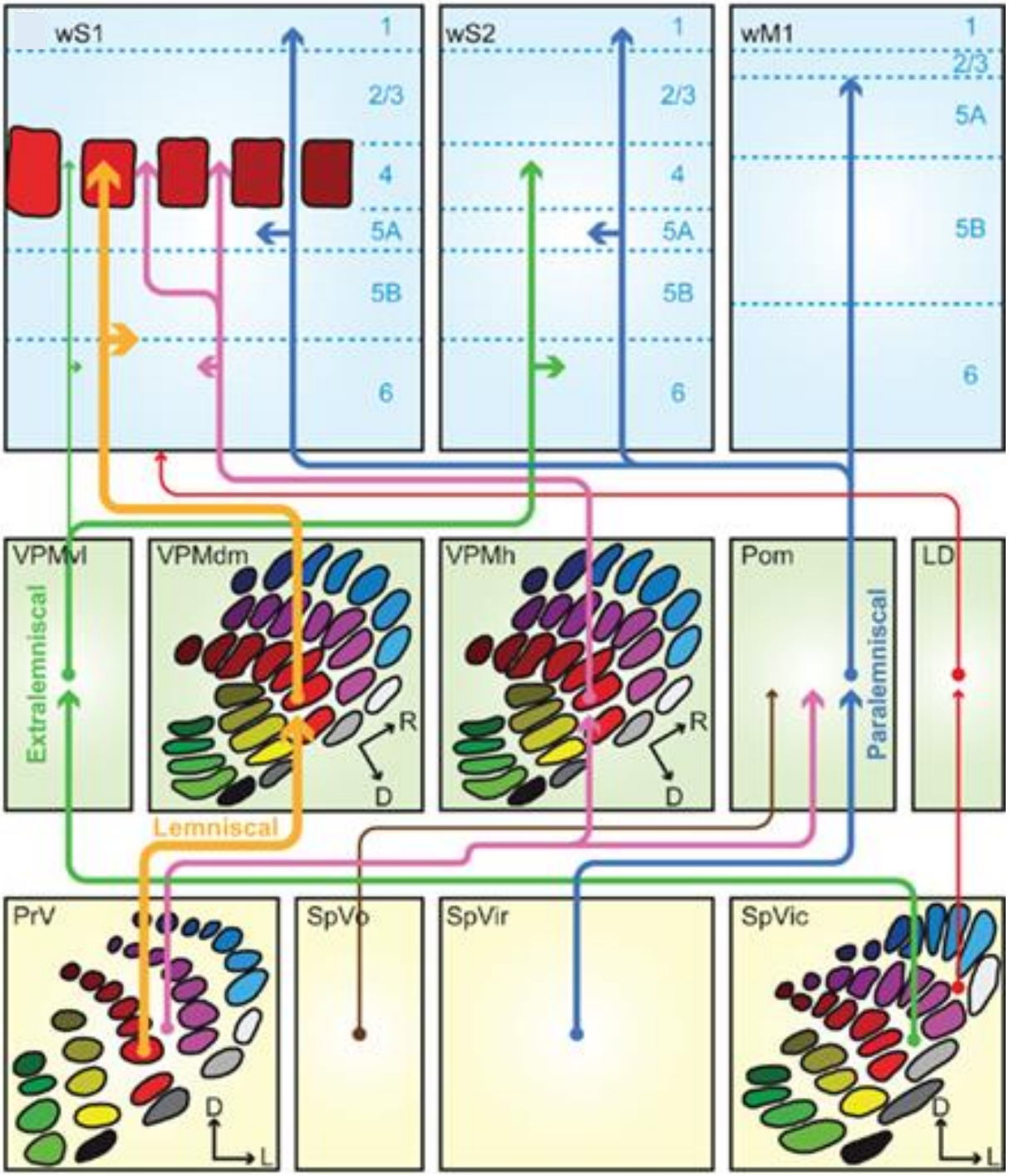
wS1
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Figure 4. Schematic drawing of the trigemino-thalamo-cortical pathways, image taken with permission from Bosman et al., (2011). The termination areas of the axons are indicated by the arrowheads and the relative importance of the pathway is indicated by the thickness of the line. The barreloids in VPM are indicated in an oblique coronal slice, the barrelettes of the trigeminal nuclei in coronal slices. D = dorsal; L, lateral; LD, laterodorsal nucleus of the thalamus; Pom, medial posterior nucleus of the thalamus; PrV, primary trigeminal nucleus; R, rostral; SpVic, caudal part of spinal trigeminal nucleus pars interpolaris; SpVir, rostral part of spinal trigeminal nucleus pars interpolaris; SpVo, spinal trigeminal nucleus pars oralis; VPMdm, dorsomedial part of the ventroposterior medial nucleus of the thalamus; VPMh, “head” area of VPM; VPMvl, ventrolateral part of VPM; wM1, whisker motor cortex; wS1, whisker part of primary somatosensory cortex; wS2, whisker part of secondary somatosensory cortex.

1.4. Tactile Behavioural Tasks

In order to investigate the neural basis of whisker-dependent behaviours, it is essential to develop well-defined tasks that provide an insight into the perceptual basis of decisions. This section will first describe the most commonly used whisker-based behavioural tasks and then go on to provide an analysis of the effectiveness and specificity of these tasks for future studies on neural activity.

One of the earliest examples of a barrel cortex dependent behavioural task was described by Hutson and Masterton (1986). This classic study required a rodent to perch on an elevated platform and use its whiskers to locate and move to another platform where it would receive a reward. The authors demonstrated that this ‘gap crossing task’ could be performed without visual input and required the whisker-to-barrel pathway for successful completion. They also showed that the task could be completed using a single whisker.

This single whisker preparation has been frequently utilised in the field and many behavioural tasks use this method for quantifying sensory learning with sensory processing. The technique often requires the animal to be trained in a head-fixed position, immobilized using implanted rods. The most frequently used whisker-based discrimination tasks are object localisation, texture discrimination, frequency discrimination and aperture discrimination tasks which will be described individually (Figure 5)(for review on whisker based tasks, see Helmchen et al., 2018). These experiments usually require the animal to be water restricted and extensively habituated to the head restraint. Following this, they are trained on different types of stimulus sampling: 'detection' or 'discrimination'. In detection tasks, the animal is required to simply detect the presence of a specific object or event whereas in a discrimination task, the animal is required to alter its behaviour when presented with numerous stimuli which each indicate different outcomes. In order to succeed in these tasks, the animal must encode and consolidate in memory a representation of the target stimulus and use this in subsequent trials to guide behaviour by comparing the stored representation to the current stimulus (Helmchen et al., 2018).

A frequently used behavioural paradigm is the 'pole localization' task, which uses a go/no-go procedure. This task involves presentation of a pole to the whiskers at varying rostro-caudal positions. The animal is required to lick for a water reward when the pole is in the target position as opposed to the distractor positions (Figure 5A)(Campagner et al., 2019; Hong, Lacefield, Rodgers, & Bruno, 2018; Huber et al., 2012; O'Connor et al., 2010; Pammer et al., 2013). This procedure has also been used to assess texture

discrimination. In this setup, the animal is trained to judge the roughness of a texture: usually different grades of sandpaper. As in the object localization task, the texture discrimination task requires the animal to behave in response to one texture and behave differently when presented with a different texture. This behaviour is often enforced by punishing the non-target behavior using loud noise, delay periods or light air puffs to the body (Figure 5B)(Chen, Margolis, et al., 2015; Isett, Feasel, Lane, & Feldman, 2018). The discrimination paradigm has also been used to train animals to detect different frequencies applied to a single whisker on each side of the snout (Mayrhofer et al., 2013). The animal is required to detect which side the higher frequency occurs and lick the corresponding spout for the reward. This method has advantages over the go/no-go paradigm as one could investigate the attentional and motivational levels of the animal. In a discrimination task, the animal is required to respond on every trial, therefore, when the animal does not respond it is distinguished as a ‘miss’. In the go/no-go paradigm however, a ‘miss’ is not distinguishable from a correct rejection (Figure 5C). The discrimination paradigm has also been used to detect aperture and width, in these experiments, the animal runs on a wheel between a virtual corridor and is required to estimate the position of the wall relative to the whisker length (Figure 5D)(Krupa, Matell, Brisben, Oliveira, & Nicolelis, 2001; Miyashita & Feldman, 2012; Sofroniew et al., 2014; Zainos, Merchant, Hernández, Salinas, & Romo, 1997). These methods are frequently utilised to investigate the involvement of the barrel cortex in learning. A recent study by Hong et al. (2018) employed the pole localization task to test whether optogenetic and lesioning of the barrel cortex impacted

task performance. The authors found that these manipulations have a transient disruptive effect which is restored following subsequent training and they conclude that the barrel cortex may be redundant for active detection. The study contrasts with previous research which suggests that the barrel cortex is a critical structure for whisker-dependent behaviours (Hutson & Masterton, 1986; O'Connor et al., 2010). One possibility for the differing results may be in the behavioural tasks employed. Current whisker-dependent tasks do not identify the behavioural conditions for which the barrel cortex is indispensable. It is important to rigorously assess the involvement of both the whiskers and the barrel cortex when using behavioural tasks for assessing the role of the brain structures in learning processes.

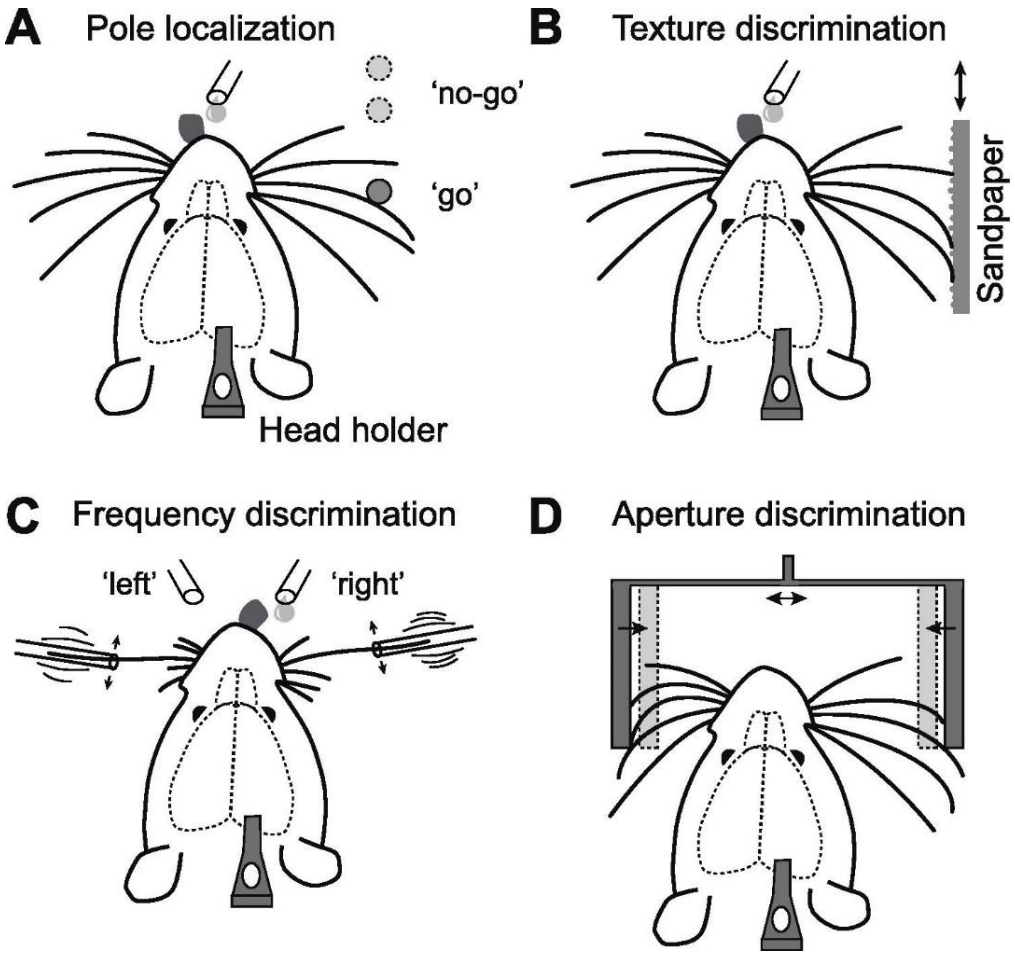


Figure 5. Schematic of the whisker-based tasks developed in head-fixed rodents, image taken with permission from Helmchen et al. (2018). (A) Object localization, the animal is presented with a vertical pole and the animal is required to judge its position. (B) Texture discrimination, the animal is required to make a decision based on the texture presented, varying degrees of sandpaper roughness are frequently used. (C) Bilateral frequency discrimination task, the animal is presented with two different stimulation frequencies on each side of the snout. (D) Aperture discrimination, the centrality and width of an aperture is evaluated.

The head-fixed preparation allows for a great level of experimental control over sensory inputs and motor output, however, this technique has some restrictions, most prominently in terms of ethological relevance. The head-fixed preparation requires the animal to maintain an unnatural physical position and sometimes only use a single whisker. This serves as a

significant limitation, as planning and decision making is often expressed via both head and body movements (for review on head fixed procedures, see Schwarz et al., 2010). Although work has been undertaken to incorporate some aspects of whole-body movements involved in rodent behaviour (e.g. whisking; Bermejo, Houben, & Zeigler, 1998; Jadhav, Wolfe, & Feldman, 2009), licking; (Han, Zhang, Zhu, Chen, & Li, 2018; Hentschke, Haiss, & Schwarz, 2006), running and fore-paw movements (Giovannucci et al., 2018; Sanders & Kepecs, 2012), rodents are still not able to employ their full range of natural movements. In order to investigate the brain changes that underpin learning and memory as it routinely occurs, it seems reasonable to use tasks that tap into the animal's natural behavioural repertoire as opposed to using preparations that are more analytically tractable but less ethologically relevant. The head-fixed tasks are not naturalistic and may be impacting the normal behaviour of the animal, which might make it difficult to draw conclusions about whether any neural changes that are observed are representative.

The head-fixed experimental set up also has practical limitations. It is very time consuming to prepare and the surgeries are high risk, particularly when undertaken on juvenile rodents who are still developing. Following the surgery, there is a minimum 2-week recovery period where the animal is at risk of infection and drop-out. Once recovered, the animal then undergoes an extensive habituation period (1-2 weeks) where it is accustomed to the head-fixed position and test environment. This stage of training can be prolonged if the rodent is stressed, as additional habituation sessions would be required. Finally, when training commences, head-fixed studies often require a large

number of trials to establish learning. This can be detrimental for studying short-term neural changes, which may no longer be present over the course of numerous weeks.

The alternative to head-fixed tasks are tasks in which the animal is free to move around in a relatively unrestricted manner. The task requirements are relatively similar to those used in head-fixed paradigms, but they are advantageous as they do not involve surgical procedures and allow the animal to move in a natural way. One can investigate trained or spontaneous behaviours without manipulation and physical restriction. As mentioned earlier, the gap-crossing task, originally developed to assess cortical whisker barrel function, consists of a number of trials requiring the rodent to navigate across a gap of variable distances (Hutson & Masterton, 1986) This behavioural task was utilised in a pioneering study by von Heimendahl, Itskov, Arabzadeh, and Diamond (2007) to characterize the response differences in barrel cortex during tactile discrimination. In this study, the animal encountered a texture and was required to undertake an appropriate response for a water reward. Further studies also utilised this task to test tactile learning by requiring the animal to assess which platform to jump to according to the tactile cues presented (Barneoud, Gyger, Andres, & van der Loos, 1991; Carvell & Simons, 1990; Guic-Robles, Jenkins, & Bravo, 1992; Morita, Kang, Wolfe, Jadhav, & Feldman, 2011; Papaioannou, Brigham, & Krieger, 2013; Tsytsarev, Arakawa, Zhao, Chédotal, & Erzurumlu, 2017). Although the gap-crossing task is a useful discrimination task, it is very time consuming as the animal must undergo numerous training stages to accustom it to jumping over the gap (taking approximately 2 weeks). The

training is extensive as the gap initially starts very small and is gradually extended by 0.5cm increments. Furthermore, the task requires animals to have a good level of muscular strength, motor coordination and balance as the animal needs to learn how to initially transfer from one platform to the second. The reliance on sensorimotor integration for success at this task also limits the use of the procedure for investigating one sensory system in isolation. With regard to the specificity of the gap-crossing task, some studies sought to eliminate sensory cues that may have been guiding behaviour. For example, early studies sought to remove visual and auditory cues by testing the animals following blinding by bilateral enucleation or deafening by bilateral destruction of the cochleae (Barneoud et al., 1991; Guic-Robles et al., 1992; Hutson & Masterton, 1986). Although it was assumed that the animals could not see or hear, such a severe manipulation may have affected the animals' natural behaviour both in general and in the task. Further, in the study undertaken by Barneoud et al. (1991), they found that unilateral ablation of the barrel cortex resulted in deficits during the gap-crossing task which were recovered when retested 10 weeks later. Similarly, Papaioannou et al. (2013) report that early sensory deprivation by whisker plucking did not affect performance on the gap crossing task as mice used their nose and paws to discriminate between the textures. Taken together, these findings suggest that the task may not be whisker or barrel cortex specific: the animals can utilise alternative behavioural strategies to acquire the 'texture' discriminations.

Frequently used alternatives to the gap crossing task are the radial arm, Y and T-maze tasks (Lipp & Van der Loos, 1991). The maze setup was

originally designed to assess spatial learning and working memory by placing cues around the room to distinguish the location of the arms (for reviews, see Olton, 1987 and Crawley, 2008). The animal is taught that a food reward is associated with a specific arm and the time taken to complete the task and number of errors is recorded. These tasks can be modified to assess texture learning. For example, the T-maze apparatus was modified to create a two alternative forced choice task where rats were trained to run between two tactile stimuli attached to the walls. This setup forced the animal to sample the texture with their whiskers and required them to learn an association and make a choice for a food reward (Kerekes, Daret, Shulz, & Ego-Stengel, 2017). Griffin, Owens, Peters, Adelman, and Cline (2012) also developed a tactile-visual version of the T-maze task whereby different textures were presented to the animal via removable floor inserts. More recently, the Y-maze was modified by Hu, Urhie, Chang, Hostetler, and Agmon (2018) to create a 3-D object discrimination task which relied on both visual and tactile cues. Although useful for assessing working memory (Y/T-maze) and long-term memory (radial arm maze), there are some drawbacks to these behavioural assays. The maze setup requires extensive training as the animal must first be trained to travel to the bottom of the arm prior to learning the discrimination rule. For example, Kerekes et al. (2017) report that training takes an average of 8.4 weeks in order for rats to learn the texture discrimination. The long training period would therefore not be suitable for use with mice which have high baseline anxiety and low activity levels (e.g. Tg2576/129S6) as they may not perform enough trials for successful completion of the task (Wolf, Bauer, Abner, Ashkenazy-Frolinger, & Hartz,

2016). Additionally, for those interested in monitoring rapid neural changes, an 8-week training period would be unsuitable as synaptic changes can appear and disappear within this time frame.

More generally, in order to draw inferences about the relationship between rodent behaviour and neuronal activity, it is important to ensure that the behaviour is explicitly reliant on the neural system from which activity is being measured. To study the barrel cortex, for example, studies must control for other sensory cues so to rule out the availability of olfactory, visual and auditory prompts. In the experiments mentioned earlier, attempts were made to remove visual cues by fitting opaque contact lenses to the rodents (Barneoud et al., 1991) or mounting metal caps over their eyes (Carvell & Simons, 1990). Although innovative, these techniques do not make certain that all visual cues are removed and require unnecessary surgical procedures. Other studies address the possibility of visual cues by removing all light sources and recording activity using non-visible infrared light (Lipp & Van der Loos, 1991; Zuo, Perkon, & Diamond, 2011). In addition to visual cues, olfactory cues are often present on the materials used as stimuli. In the study undertaken by Tsytsarev et al. (2017), materials such as sponge, terry cloth and cardboard are used as textures, and the floor inserts used by Griffin et al. (2012) are made from metal and mesh. Each of these materials will have associated odours, which rodents could use to acquire the discriminations. Some studies have attempted to avoid odour cues by using sandpapers with varying grit size (Guic-Robles et al., 1992; Morita et al., 2011). Unfortunately, these attempts do not make certain that all olfactory cues are removed as the grit size is determined by the amount of adhesive

glue present, which could serve as an olfactory cue individually. Moreover, even when using stimuli made from the same materials, individual objects may have tactile and non-tactile cues associated with them as developed throughout testing. It is therefore important to develop a large set of stimuli so to use different copies to avoid learning specific details of one particular surface.

It is clear that there are limited texture-based behavioural tasks available for use with mice, which do not require head-fixation. One aim of this thesis was to develop a robust behavioural task, which can be used to assess rapidly acquired texture learning in mice. It was important to determine that the task was both whisker and barrel cortex dependent and that it was not being undertaken using other sensory modalities. Development of a robust behavioural task will enable investigation into how multi-whisker information is used during learning and how that is represented and processed in the brain.

1.5. Synaptic plasticity of Barrel Cortex

Neural plasticity is the nervous systems' ability to reorganise its structure, functions or connections in response to intrinsic or extrinsic stimuli (Mateos-Aparicio & Rodríguez-Moreno, 2019). This is achieved through modification of the strength and efficacy of synaptic transmission. The barrel cortex is an ideal system for investigating plasticity as the peripheral sensory input can be easily manipulated (Fox, 2002). Numerous studies have demonstrated that removal of a subset of whiskers causes a decrease of the cortical area associated with the removed whiskers and a subsequent increase in

neuronal number for the spared whiskers (Diamond, Armstrong-James, & Ebner, 1993; Glazewski, McKenna, Jacquin, & Fox, 1998; Lebedev, Mirabella, Erchova, & Diamond, 2000; Wallace & Fox, 1999). Although this activity-dependent plasticity is more robust during development, plasticity is also observed in adulthood (Darian-Smith & Gilbert, 1994; Greenhill, Juczewski, et al., 2015).

Adult plasticity is theorised to be reliant on alterations in the strength of established synaptic connections. However, this rewiring also involves structural modifications in synapse formation and synapse elimination (Holtmaat et al., 2005). Research has focused on dendritic spines as potential substrates of plasticity (Grutzendler, Kasthuri, & Gan, 2002; Gu et al., 2014; Holtmaat et al., 2005; Segal, 2016). First described by Ramon Y Cajal, dendritic spines are tiny protrusions that receive excitatory synapses and undergo vast structural remodelling in response to synaptic stimulation (Ma et al., 2016). Spines consist of a number of specialized subdomains which each have a role in synaptic transmission and plasticity (Yuste & Denk, 1995). The spine head is the site for a single glutamatergic synapse and beneath this lies the postsynaptic density (PSD). The PSD is a cytoskeletal component located beneath the postsynaptic membrane where a number of subcomponents required for plasticity are found (for review on the structure of the dendritic spine, see Rochefort & Konnerth, 2012). The dendritic shaft is connected to the spine head by a thin neck, which can be used to classify spines into specific morphologies. The maturity of the spine progresses from left to right and is classified as follows: filopodium, thin,

stubby, mushroom and cup/branched shaped (Figure 6)(Chang & Greenough, 1984; Harris, Jensen, & Tsao, 1992; Hering & Sheng, 2002).

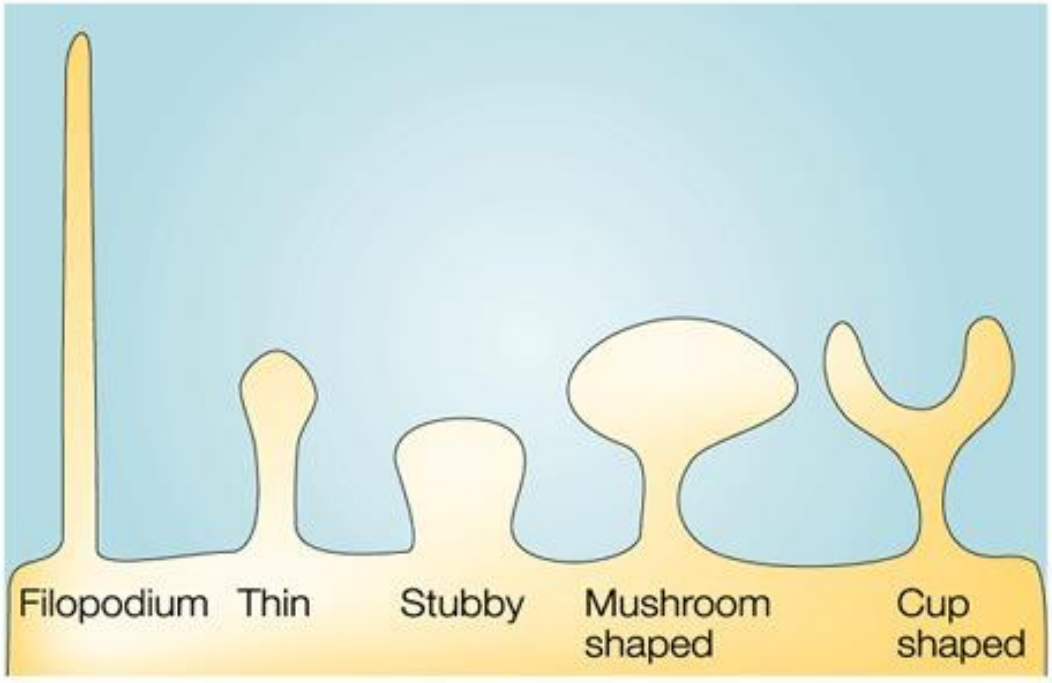


Figure 6. Schematic taken from Hering and Sheng (2002) of the morphological classification of dendritic spines.

These classifications have however been shown to underestimate the scope of spine morphology and studies have shown that the classification of spines has been limited by the resolution of the microscopy methods available. For example, Tønnesen, Katona, Rózsa, and Nägerl (2014) showed that the 'stubby' spine is in fact a 'mushroom' spine and misclassification was due to imaging techniques lacking the resolution to visualise a clear neck structure in shorter spines. Moreover, it is suggested that some spines are sampled during an intermediate phase where the spine does not naturally fall into any of the available classifications (Arellano, Benavides-Piccione, DeFelipe, & Yuste, 2007; Ruszczycki et al., 2012). Nevertheless, spine structure is of great interest for the study of plasticity as a positive correlation has been

shown between the volume of spine head, PSD area and synaptic strength (Dunaevsky, Blazeski, Yuste, & Mason, 2001; Harris & Stevens, 1989; Holtmaat et al., 2005; Matsuzaki et al., 2001; Trachtenberg et al., 2002).

Early studies of dendritic spine plasticity used electrical stimulation to induce changes in spine morphology. Fifková and Van Harreveld (1977) found that spines in the dendate gyrus became 15% larger immediately following tetanic stimulation and increased by 38% up to an hour after stimulation. These changes were also shown by Desmond and Levy (1983) who used a similar approach and found an increase in spine density of concave-shaped spines and a decrease in those with simple and ellipsoid shapes. Although key to our current understanding, the aforementioned studies were limited as they relied on fixed tissue and used two different populations of cells. It was therefore not possible to conclude whether changes were occurring in existing spines or whether larger spines were being formed following LTP. These challenges were overcome with the use of time-lapse imaging of living tissue using optical imaging and two-photon laser scanning microscopy (Hosokawa, Rusakov, Bliss, & Fine, 1995; Maletic-Savatic, Malinow, & Svoboda, 1999). Two-photon imaging allows researchers to repeatedly monitor spine dynamics *in vivo* over time (Sala & Segal, 2014). It is advantageous compared to one-photon confocal microscopy as it allows researchers to observe deep structures without causing as much photodynamic damage and bleaching of the tissue (Yuste & Bonhoeffer, 2001). One of the early studies utilising these methods was conducted by Trachtenberg et al. (2002) who demonstrated a marked increase of spine turnover following sensory deprivation. In addition to this, the authors also

provided results regarding the natural turnover of spines stating that although the spine density was stable, the turnover was extremely variable with around 50% of spines surviving for more than 30 days. These findings were reinforced by numerous studies, which supported the view that spine density and size are critically involved in long term memory (Fu, Yu, Lu, & Zuo, 2012; Hofer, Mrsic-Flogel, Bonhoeffer, & Hübener, 2009; Roberts, Tschida, Klein, & Mooney, 2010). The high turnover of dendritic spines adds to the challenges of examining the effect of learning on spine plasticity. The original reason that this thesis first sought to develop a selective behavioural task, where learning is rapid and persists overnight, was to enable the same spines to be monitored before and after learning (and prior to their natural degradation). However, the development of a suitable texture discrimination task was ultimately used to address the related issue of the role of the barrel cortex in texture discrimination learning, and the neural changes associated with such learning.

The link between learning and spine plasticity has been examined using classical conditioning experiments where whisker stimulation is paired with an aversive and/or rewarding outcome. These studies demonstrate a large expansion of the functional representation of the trained whiskers in S1 (Galvez, Weible, & Disterhoft, 2007; Siucinska & Kossut, 1996). Interestingly, unlike the results found in the whisker trimming studies mentioned earlier, the expansion was only present in the trained whisker row and not surrounding rows, indicating that the change was due to the learning as opposed to sensory stimulation alone. The link between spine plasticity and learning has mostly been demonstrated using motor learning tasks. For

example, Xu et al. (2009) showed that training on a seed-reaching task resulted in a rapid increase in spine turnover in layer 5 of M1. The spines generated following learning were preferentially stabilised during later training sessions and remained stable when training was concluded. This finding was also demonstrated in M1 by Yang, Pan, and Gan (2009) who trained mice on a rota-rod task and found a significant increase in spine formation after two days of training. These findings were later extended to reveal that different behavioural motor tasks (running backwards vs. running forwards) resulted in spine formation on different dendritic branches, further showing the task-specific nature of the spine changes (Yang et al., 2014). These studies show a clear link between motor learning and dendritic spine activity. However, the majority of this research focuses on spines located in M1. There are few studies examining spine plasticity in the barrel cortex following whisker-learning. One reason for this may be the lack of robust behavioural tasks which specifically rely on the whisker system. It is important to develop well-defined tasks so to further investigate sensory processing. As mentioned in section 1.4, early studies have utilised the single whisker preparation to investigate the cortical representation of learning (Harris, Petersen, & Diamond, 1999). For example, Kuhlman, O'Connor, Fox, and Svoboda (2014) used a head-fixed object localisation paradigm to explore spine plasticity in layer 2/3 barrel cortex. The authors reported a 67% increase in spine formation during the first 8 days of the training period. They also found that animals that learned more quickly had a larger number of newly formed spines on day four of training compared to animals demonstrating a slower learning rate. However, as noted earlier, this

setup has a number of drawbacks, predominantly that it is not representative of a rodent's natural behaviour.

Whilst there is a clear link between spine plasticity and learning, it must be noted that some studies have found opposing results when using fear conditioning paradigms. Sanders, Cowansage, Baumgärtel, and Mayford (2012) studied hippocampal neurons and found that fear-conditioned animals had a reduced spine density compared to controls. This finding was also reported by Lai, Franke, and Gan (2012) in the frontal association cortex where fear conditioning resulted in an elimination of dendritic spines. These findings were recently extended to reveal that the newly formed spines induced by fear conditioning, were eliminated following fear extinction. Moreover, fear reconditioning undertaken following the extinction paradigm resulted in reformation of new spines in close proximity to the original spines sites (Lai, Adler, & Gan, 2018). Although there is a significant correlation between spine plasticity and learning, there is little evidence for a causal relationship. This was addressed by Hayashi-Takagi et al. (2015) who developed a synaptic optoprobe: AS-PaRac1 (Activated Synapse-targeting PhotoActivatable Rac1). This probe is advantageous as it can label recently enlarged/newly formed dendritic spines as well as induce shrinkage of these spines following the presentation of blue light. The authors injected AS-PaRac1 in M1 and trained mice in a hind limb motor task. In line with previous studies, they found substantial changes in spine size and formation following motor learning. The authors report that once the learning had taken place, activation of Rac1 resulted in elimination of these recently potentiated spines and an associated deterioration of the learned behaviour. Moreover,

learned behaviour of a different motor task, which utilised the same cortical area, was not disrupted. These findings indicate that different learning tasks utilise different neuronal ensembles, which can be modified to affect behaviour thus providing a direct link between dendritic spines and learning. The study by Hayashi-Takagi et al. (2015) is valuable as it demonstrates the link between dendritic spines and learning. The behavioural tasks utilised in their study are well established tasks that are known to rely on M1 for completion (Costa, Cohen, & Nicolelis, 2004; Dunham & Miya, 1957; Shelton et al., 2008). The causal link between learning and spine plasticity in the barrel cortex has not been established. As a first step to doing this, this thesis sought to develop a robust, behavioural task which can be used to assess rapidly acquired texture learning in mice.

There is some evidence that dendritic spine plasticity is involved in learning (Holtmaat, De Paola, Wilbrecht, & Knott, 2008). However, as described earlier, some studies indicate a functional role in learning, but the evidence is mixed and often not conclusive. One reason for this might be the non-selective nature of the behavioural tasks that have been used – the tasks may not be directly reliant on the brain area under investigation or the animals might be using other dimensions of the stimuli. Given the fact that alterations in spine density and morphology have been linked to diseases such as Alzheimer's disease, Schizophrenia and Parkinson's disease (Boros et al., 2017; Moyer, Shelton, & Sweet, 2015; Zaja-Milatovic et al., 2005); it is important that robust and accurate behavioural tasks are developed to test the relationship between learning and spine plasticity in model systems.

1.6. Thesis Aims and Objectives

The overreaching aim of this thesis was to develop a behavioural task procedure in which learning is rapid and demonstrably whisker based; in order to investigate whether there are rapid changes in the barrel cortex that support learning. As mentioned earlier, current behavioural tasks are restricted in either their ethological relevance, when the rodent is head fixed, or in that sensory modalities other than texture may be directly involved. It was therefore essential that the procedure excluded visual and olfactory cues, and was acquired in a short period of time.

Chapter 2 describes the development of a long-term, texture-based novel object recognition task (NOR). The NOR task is a widely used test of recognition memory used in a range of sensory systems: rodents show a preference to explore a novel object when given a choice between that object and a familiar object. The task does not require head-fixation and is rapid. In Chapter 2, a whisker-based version of the NOR task is presented, where the possibility that mice are discriminating on the basis of odour or visual cues is excluded. The experiments revealed that mice show a preference for novel textures, which is rapidly acquired and retained overnight. However, the preferences were relatively weak, variable and as a consequence the number of mice required to establish a statistically significant preference was relatively large (i.e., 20). This number is too large to be useful in the context for which it was designed: to investigate the neural bases of learning. Chapter 3 describes the development of an alternative behavioural task: the two-choice texture and odour discrimination task, which

requires only 4 mice to achieve statistically significant and replicable effects. The task requires the mouse to learn that a pot with one texture on its outer surface (e.g., grooved) contains a reward whereas another pot with a different surface (e.g., smooth) does not. Learning is revealed through the animal displacing the sawdust in the rewarded bowl to uncover the buried reward. The series of experiments presented in Chapter 3 demonstrate that learning is rapid, retained overnight and whisker dependent. The role of the barrel cortex in the two-choice discrimination task is then examined in Chapter 4 using chemogenetic technology to decrease neuronal activity prior to undertaking the texture discrimination task. The study demonstrates that the two-choice discrimination task is barrel cortex dependent, and an analysis of the expression of immediate early gene, cFos, was undertaken to evaluate which neurons were selectively active during the task. Finally, Chapter 5 discusses the theoretical implications of the new results, their limitations and the future directions that they afford.

Chapter 2. The Novel Object Recognition Task

2.1. Summary

The novel object recognition (NOR) task has been widely used to test memory in a range of animals. However, the task is mainly used to investigate learning that relies on the visual system, and few studies have developed the task to investigate areas within the somatosensory system. Chapter 2 consists of three experiments with the main aim of adapting the original NOR task developed by Wu et al., (2013) so that it assessed long-term rather than short-term NOR. The experiments reported here were modified from the original study by testing mice in the dark, using stimuli constructed from the same materials and requiring the mice to retain the memory overnight. Experiment 1 comprised two pilot studies aimed at optimising the apparatus and procedure. The results of Experiment 1 revealed that mice spend more time with the stimulus when they were placed in the corners of the arena and also, reducing the size of the arena resulted in fewer anxious tendencies as demonstrated by an increase contact time. Experiments 2 and 3 involved further modifications to the procedure. The results of these experiments demonstrated that mice show a preference to explore a novel texture, which is retained over a 24-hour retention interval.

2.2. Introduction

As described in Chapter 1, current tasks established to test learning and memory can be grouped into two categories, head fixed and freely moving tasks. Head fixation is a common option for researchers who utilise the

technique for neural recording or stimulus control (Guo et al., 2014). Head fixed, choice-based tasks often use go/no go procedures and object localisation protocols, which require mice to make decisions to respond based on sensory stimuli (Mayrhofer et al., 2013; Mehta, Whitmer, Figueroa, Williams, & Kleinfeld, 2007; Sofroniew et al., 2014). These tasks allow researchers to precisely monitor movement and have a high degree of stimulus control. However, the tasks require a high number of trials to reach criterion. Tasks where rodents are free to move, on the other hand, usually employ gap crossing tasks, Y- or T- maze tasks to train mice to reach a food reward (Carvell & Simons, 1996; Lipp & Van der Loos, 1991). Mice need to palpate two textures presented at a junction and consequently choose an arm or platform to navigate towards. Although Y- and T- maze tasks are relatively simple and have the advantage that they are based on the animals' natural tendency to explore and forage, they still require a relatively large number of trials and are prone to motivational problems (e.g., the mice becoming sated during the course of a training session).

The above issues may be overcome by using a novel object recognition (NOR) task. This task has been increasingly used to understand the neural basis of memory (e.g., Robertson, Eacott, and Easton (2015)). This task is based on research described by Berlyne (1950) who demonstrated that rats prefer to explore 'novel' stimuli as opposed to previously seen 'familiar' stimuli. Ennaceur and Delacour (1988) developed the NOR test to investigate this preference for novelty. The NOR test harnesses the rodents' natural propensity to explore novel stimuli and differs from other tasks in that it does not require extensive training (e.g., to approach one stimulus rather

than another to gain access to reward; Ennaceur, 2010). In the task, an object is presented and after a delay period, it is presented again with a second, different object (Hu et al., 2018). The preference to explore the novel object demonstrates that exposure to the familiar object has resulted in a reduction in exploration of that object. In this sense, the animal can be said to have recognized the familiar object.

The NOR task has been used to demonstrate rapid learning in a variety of neural systems, (for review, see Warburton & Brown, 2015). However, few studies have studied texture NOR; and typically, these studies utilise objects which have multimodal features, for example, objects may vary in odour, height, width, colour and texture (Albasser et al., 2013; Mitchnick et al., 2018). In order to investigate a sensory system engaged in a behaviour, it is critical that the sensory cues used by the mice to perceive and discriminate between objects are controlled (Blaser & Heyser, 2015). The experiments presented in this thesis address this issue by eliminating cues other than texture through presenting the stimuli in dim red light and 3-D printing the test stimuli using the same material. Another issue with current NOR test procedures using stimuli including textures is the relatively short interval periods used between the familiarization phase and the test phase. Studies with mice typically utilise retention intervals ranging from minutes to hours (Ennaceur & Delacour, 1988). These tests can be regarded as involving relatively short-term memory as opposed to long-term memory (e.g., lasting overnight). Studies using rats have assessed long-term memory by increasing the retention period to 24 hours (Albasser, Davies, Futter, & Aggleton, 2009; Clark, Zola, & Squire, 2000; Gaskin et al., 2010; Goulart et

al., 2010), 48 hours (Ennaceur & Delacour, 1988) and even 1-10 weeks (Iwamura, Yamada, & Ichitani, 2016; Mumby, Glenn, Nesbitt, & Kyriazis, 2002). However, few studies have tested longer intervals using mice (Hale & Good, 2005; Wang et al., 2007) or combined a longer interval with test stimuli that rely on a single sensory system.

The overreaching aim of the first series of experiments was to adapt the NOR procedure developed by Wu et al. (2013) to demonstrate long-term texture memory in mice. Wu et al. (2013) developed a NOR task which involved mice discriminating between different textures (sandpaper gradients) with the finding that mice spend more time with the novel texture. The task used here was adapted to overcome a number of the potential limitations of the Wu et al. (2013) study: sandpaper was replaced with 3-D printed bowls which differed only in the outer texture, visual cues were removed by conducting the experiments in the dark, and the retention period was increased to 24 hours. Experiment 1 comprised two pilot studies in which the apparatus and basic procedure were amended in order to maximise contact times with the tactile plates. Experiment 2 and 3 involved further methodological changes, to examine whether or not mice show a preference for a novel stimulus, when its familiar counterpart had been presented 24 hours before.

2.3. Experiment 1: Texture based NOR task

2.3.1. Method

Ethical Consideration

A full ethical evaluation was undertaken and all procedures were approved by the ethical review committee at Cardiff University, UK, and all procedures were performed in accordance with the guidelines set out by the UK Animals (Scientific Procedures) Act (1986). Throughout this thesis, care was taken to comply with the 3Rs (Replacement, Reduction and Refinement) with the aim to reduce the number of animals used whilst maintaining a suitably powered experiment. It is noted that although important to reduce the number of animals used, the study must not fail to provide a robust answer to the research question due to a limited sample size/inadequate statistical power. Wu et al. (2013) undertook a power calculation, which included provision for a 20% drop-out rate. The authors recommend testing a minimum of 6 animals per group in order to reach 90% power. As the behavioural task was intended to be used to investigate the neural underpinnings of texture learning, the calculation was retaken with the power set at 95%. This increased the minimum number of mice to 8 per group in order to account for potential dropouts due to surgical complications.

Animals

Experiment 1a and 1b each used 16 C57BL/6J male mice obtained from Envigo, (UK) and transferred to Cardiff University where they were given one week of habituation to the new housing environment. The age, sex and

breed of the mice were matched to the original study so to enable comparison of results (Wu et al., 2013). All mice were housed in groups of four per cage with two cardboard nesting tubes for enrichment. They were maintained on standard laboratory diet and water *ad libitum* and were kept on a 12-h/12-h light/dark cycle (lights on at 7 am) and in a humidity and temperature-controlled environment (55-65% and 19-21°C). Experimental testing began at 8 weeks old.

Apparatus

The testing arena in Experiment 1a was custom-made from clear Perspex sheets and measured 40cm x 60cm x 60cm (H x W x D). The floor of the arena was made from a removable wooden base which in its centre held four stands which held the tactile plates upright. In Experiment 1b a number of modifications were made to the apparatus. Firstly, the wooden base of the arena was replaced with a Perspex base. This reduced the possibility of olfactory scents from the mice being absorbed into the wooden material. Secondly, the overall size of the arena was reduced to 50cm x 50cm x 50cm (H x W x D). It was also noted that the arena used in Experiment 1a resembled the arena used in the open field test, a test widely used to measure anxiety in mice (Gould, Dao, & Kovacsics, 2009; Walsh & Cummins, 1976). The open field test is designed to assess anxiety by reproducing the natural aversion of rodents to exposed fields (Carola, D'Olimpio, Brunamonti, Mangia, & Renzi, 2002). The arena used in Experiment 1a had the same dimensions as those used in some open field studies. The reduction in the arena was intended to reduce the signs of

anxiety (e.g., freezing, repetitive behaviours (stereotypies), and increased grooming) (Bailey & Crawley, 2009) that were evident in Experiment 1a. The final modification made in Experiment 1b was the placement of the tactile plates. The plates were moved from the middle of the arena to the four corners. This modification was made following observations that the mice spent the majority of their time adjacent to the walls of the arena (i.e., displayed thigmotaxis; see Simon, Dupuis & Costentin, 1994) as opposed to the middle area.

The target objects (tactile plates) used for tactile discriminations were created using 3-D printer technology (Ultimaker B.V., The Netherlands). The material used to create these plates was printer filament Polylactic Acid (PLA; 2.85mm 1kg), which is a biodegradable thermoplastic derived from renewable resources which makes it environmentally friendly and safe for the animals. PLA can withstand alcohol-based cleaning solutions and can be easily cleaned between trials as it dries rapidly and unlike sandpaper, is non-absorbent. The tactile plates were all 80mm x 80mm square and 3mm thick. The spatial frequency of the grooves printed into one side of the plates differed. The plates with high spatial frequency had grooves that were 0.4mm deep, 0.6mm wide and 0.6mm apart (Figure *7*A). The plates with low spatial frequency had grooves which were 1.0mm deep, 0.6mm wide, 1.9mm apart (Figure *7*B). The novel and familiar stimuli were distinguished by changing the spatial frequency (high vs. low) or the orientation of the grooves (i.e., horizontal vs. vertical).

In both pilot studies, the arena was situated on an elevated table in a quiet behavioural testing room. The entire procedure was carried out in the dark with the addition of infrared illumination in order to record behaviour. Exploration was recorded using an 850nm infrared Sony Bullet Camera, 600TVL Resolution, 3.6mm fixed focal lens. The animal activity was visualised on a television monitor and recorded using a Panasonic DMR EX83 DVDR recorder.

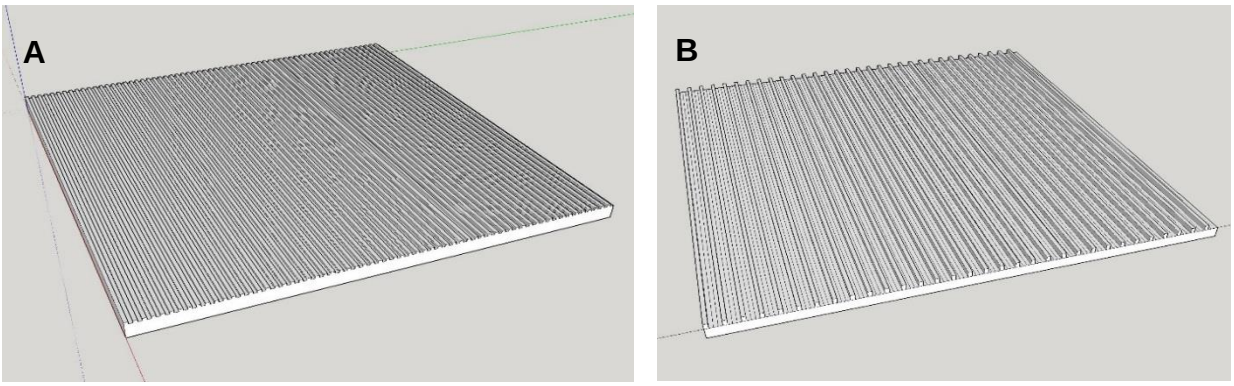


Figure 7. Schematic of tactile plates created using 3-D printer technology. (A) Tactile plate with high spatial frequency. (B) Tactile plate with low spatial frequency.

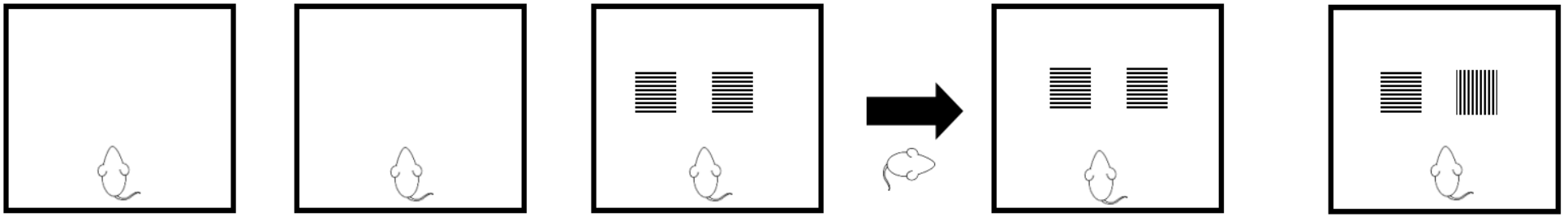
Behavioural procedure

The procedures utilised in both pilot studies were derived and modified from Hall et al., (2016) and Wu et al., (2013). All behavioural testing was undertaken between 09:00 and 17:00. One week before testing, mice were handled daily for 5 minutes each for 7 days. During this period, the animals were also habituated to the behavioural testing room for 30 min on three consecutive days. During the testing week, the mice were given an habituation period of 10 min in the test arena for two consecutive days, the aim of this was to encourage exploratory behaviour on the testing days by acclimatising the mice to the arena. The mice were transported from their

home cage to the testing arena using a holding cage, which contained bedding and an enrichment tube for comfort. The holding cage was cleaned after each mouse with 70% ethanol wipes to remove any animal olfactory cues and familiarise the animals with the scent of the cleaning agent.

Training days consisted of a ‘learning phase’ and a ‘test phase’. In the learning phase of Experiment 1a, the mouse was positioned opposite but facing away from the two identically textured plates. In Experiment 1b, the animal was positioned in the centre of the arena at an equal distance away from each plate in order to avoid any bias. The learning phase consisted of two 10-min learning phases with a 10-min interval (spent alone in the holding cage away from the other mice). Following a 24-hr retention period, mice undertook the test phase where the mouse was placed back in the arena and allowed to explore the textured plates for 10 min. In Experiment 1a, the two identical plates were replaced with one identical texture plate and a second novel texture plate with either a different orientation or different spatial frequency. In Experiment 1b, the plates consisted of two stimuli identical to those presented in the learning phase and two novel stimuli (two vertical and two horizontal plates). In both pilot studies, eight mice were tested in the spatial frequency condition and eight mice in the orientation condition. A visual schematic of the procedure is shown in Figure 8. The order in which the mice were tested was counterbalanced so to avoid the time of day impacting a specific condition. Specifically, the testing order alternated between a subject in the spatial frequency condition and a subject from the orientation condition so that half of each condition were tested in the morning and the other half in the afternoon. Additionally, the texture plates that served

as novel or familiar were also counterbalanced in order to eliminate spatial biases and order effects. For example, for the group of mice trained and tested with different spatial frequencies, half received high spatial frequency plates in the learning phase and a novel, low spatial frequency plate in the test phase. The remainder of the mice in this group received low spatial frequency plates in the learning phase and a novel high spatial frequency plate in the test phase. This counterbalancing was also undertaken for mice trained with plates differing in orientations whereby half of the mice received vertical orientation plates in the learning phase and a novel horizontal orientation plate in the test phase whilst the remainder received horizontal orientation plates in the learning phase and a novel vertical orientation plate in the test phase. The arena and plates were thoroughly cleaned with 70% ethanol wipes and dried with paper tissue between each trial. The habituation days, learning and test phases were all conducted in darkness so to ensure the mice were not using the visual system to discriminate between the plates.



10 min	10 min	10 min	10 min	10 min	10 min
Day 1	Day 2	Day 3			Day 4
			Learning Phase		Test Phase
Habituation			Testing		

Figure 8. Schematic of the novel object recognition task used in Experiment 1a. The procedure takes a total of four days and consists of two habituation days, one learning phase and one test phase. During the learning phase the animal is exposed to two identical textures (in this case horizontal) for two 10-min periods split with a 10-min interval. The test day was conducted 24 hours later when a familiar horizontal plate and a novel vertical plate were presented. This figure depicts the orientation condition whereby the novel texture is a vertical texture, which replaced the two identical horizontal textures. This figure is adapted from Wu et al., (2013).

Analysis and statistical methods

The time spent actively exploring the objects was the dependent variable. Exploration was defined as directing the nose towards the object with a distance of 2-4 cm to the plate. This was intended to capture occasions when the whiskers were being used. Touching the nose to the object, grooming, climbing or digging next to the object was not classified as exploration. Furthermore, mice that showed no exploratory activity during the learning phase or only explored one of the tactile plates during the test phase were omitted from the analysis. In Experiment 1a, one mouse was excluded from analysis due to a lack of exploratory activity in the learning phase. The full group of mice were included for analyses in Experiment 1b. The recordings of each phase were inputted into EthoVision (Noldus Information Technology) for automated tracking. Ethovision 3.0 was used in Experiment 1a and EthoVision XT 10.0 was used from Experiment 1b onwards.

Figure 9 illustrates the tracking areas that were defined for analysis. The ‘area of interest’ with respect to whisker exploration is depicted in orange and ‘area to ignore’ in red. The ‘area to ignore’ is the zone nearest the object; as noted above, it was assumed that if the mouse was in this area they would be using their nose or paws to investigate the object. This experiment aimed to only account for exploration using the whiskers alone therefore the orange area represents the approximate distance for whisker use (between 2-4 mm distance from the plate). Consequently, for the analysis, the total duration of exploration for each plate was calculated as: area of interest minus area to ignore. In Experiment 1a, the automated analysis was combined with manual

observation, to examine how frequently Ethovision tracked the animal as exploring the plate when its hindquarters were in the area of interest. These instances occurred if the animal was stationary next to the plate, these were very rare. A manual observation was not required in Experiment 1b as the EthoVision XT 10.0 software includes a feature whereby the head and tail of the animal can be marked prior to tracking. Using this feature, the animal is not automatically tracked as exploring when in proximity but facing away from the plate.

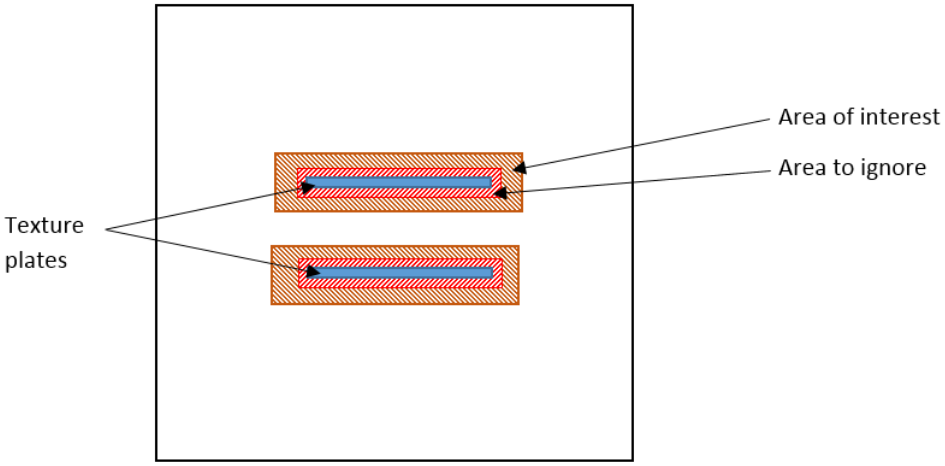


Figure 9. Schematic diagram showing an aerial view of the arena used in Experiment 1a tracked using Ethovision software. Tracking activity of interest (orange) and areas to ignore (red). Tactile plates are represented by the blue and are not to scale.

Statistical analysis was conducted on the entire 10-minute test period to examine whether there was an overall preference to explore the novel plate during the test. Subsequently, the data was binned into minutes relating to the early, middle and end of the test period to assess whether the preference was evident at different points during the test (minutes: 1-3, 4-6, and 7-10). The analysis was undertaken in this way to allow a more in depth view of the data that did not include the high variability present when analysing each

minute individually. A conventional recognition index was used to examine the preference for the novel object. It took the following form: Time spent investigating the novel object (T_N) divided by the total time spent investigating the novel and familiar objects (T_N+T_F). A ratio greater than 0.5 indicates a preference for the novel stimulus whereas a value less than 0.5 indicates a preference for the familiar stimulus. It is noted that the conclusions gained from the ratio analysis should be undertaken with caution as they may be obscured by cases where exploration was very low. Nevertheless, the recognition index adopted in this chapter allows for differences in total exploration to be accounted for which reduces the impact of these cases. Statistical analyses were undertaken using SPSS (Version 23.0). The α level was set as $p < 0.05$ for all analyses. One sample *t*-tests were used to assess whether the discrimination rations were different from chance (i.e., 0.5).

2.3.2. Results and Discussion

In Experiment 1a, a total of 15 mice were used for analysis, seven completed the spatial frequency condition and eight completed the orientation condition. One mouse was omitted due to a lack of exploratory behaviour during the learning phase. In Experiment 1b, the data from all 16 mice were included.

The contact times for each group of mice during the 10-min test phase are shown in Table 1. Inspection of Table 1 indicates that in Experiment 1a, mice did not demonstrate a great deal of exploratory behaviour. In fact, as already mentioned, re-analysis of the tapes revealed that the mice spent the majority of the time around the walls and in the corners of the arena. In contrast,

Experiment 1b reveals a marked increase in contact time with the tactile plates when positioned in the corners of the arena. As a result, the plates were positioned in the corners for all subsequent NOR experiments. However, what is also clear from inspection of this table is that in neither experiment did mice show a preference for the novel object; in fact, preferences tended to be at or below 0.50 irrespective of whether the test stimuli differed in spatial frequency of orientation.

Table 1.

Mean total contact time (±standard error of mean) with the tactile plates and mean recognition index (±standard error of mean) during the test phase for Experiment 1a and 1b.

Condition	Test Phase (s)	Recognition Index
Experiment 1a		
Spatial Frequency	39.4 ± 5.06	0.50 ± 0.14
Orientation	58.1 ± 6.16	0.44 ± 0.08
Experiment 1b		
Spatial Frequency	117.6 ± 11.9	0.43 ± 0.06
Orientation	134.4 ± 15.9	0.40 ± 0.05

The novel recognition index for the full 10 minutes of Experiment 1a did not differ from chance (0.50) in either the spatial frequency condition (M = 0.50, SEM = 0.14; $t(6) = 0.04$, p = .97, d = 0.15) or the orientation condition (M = 0.44, SEM = 0.08; $t(7) = -0.63$, p = .54, d = 0.23). Subsequent analysis of Experiment 1a in bins of 0-3, 4-6 and 7-10 minutes did not reveal a significant preference for the novel or familiar texture at any part of the test phase (Table 2; largest $t(7) = -2.10$, p = .07, d = 0.62).

Analysis of the full 10 minute test phase of Experiment 1b also demonstrated that there was no preference in either the spatial frequency condition (M = 0.43, SEM = 0.06; $t(7) = -1.02$, p = .34, d = 0.35) or the orientation condition (M = 0.40, SEM = 0.05; $t(7) = -1.84$, p = .10, d = 0.57). Experiment 1b was also further analysed in bins and results of this analysis did not reveal a significant preference for the novel or familiar texture during any phase of the test phase (Table 2; largest $t(7) = -1.87$, p = .10, d = 0.57).

Table 2.

Mean recognition ratio (± standard error of mean) for the test phase of Experiment 1a and Experiment 1b for minutes 0-3 (Bin 1), 4-5 (Bin 2) and 7-10 (Bin 3).

Condition	Recognition Index		
	Bin 1 (Minutes 0-3)	Bin 2 (Minutes 4-6)	Bin 3 (Minutes 7-10)
Experiment 1a			
Spatial Frequency	0.42 ± 0.14	0.49 ± 0.13	0.57 ± 0.10
Orientation	0.60 ± 0.12	0.30 ± 0.09	0.53 ± 0.11
Experiment 1b			
Spatial Frequency	0.53 ± 0.02	0.36 ± 0.01	0.41 ± 0.02
Orientation	0.38 ± 0.01	0.52 ± 0.06	0.43 ± 0.02

The results of Experiment 1b show that far from a preference for the novel plate, mice showed a tendency to prefer to explore the familiar plate. Further examination of the full test period of Experiment 1b revealed a non-significant and weak preference for the familiar texture in both the spatial frequency and orientation condition. The basis for this tendency is not clear, and given the fact that it was not significant requires only brief mention. Certainly, exposure to a stimulus can produce sensitization as well as habituation, but in this case the sensitizing effect of exposure to the stimulus would need to be evident 24-hr later (Horn & Hinde, 1978). Detailed analyses of Experiment 1b also indicated that during the spatial frequency condition, the preference for the familiar texture tended to be stronger when the familiar plate was vertical (*M* = 0.37, *SEM* = 0.08) than when it was horizontal (*M* = 0.50, *SEM* = 0.04). Additionally, when discriminating between orientations, the mice showed a stronger preference when the familiar spatial frequency was low (*M* = 0.37, *SEM* = 0.06) rather than when it was high (*M* = 0.52, *SEM* = 0.04). These observations might reflect the kinematics of the whisker palpation. Studies have shown that whisking kinematics are tuned to task requirements and varying whisking techniques are utilised during texture palpation (Carvell & Simons, 1990). More specifically, whisking techniques have been shown to change in response to different spatial frequencies (Zuo et al., 2011). Consequently, during the present task, a difference in spatial frequency may be more readily noticed when the plates are in the vertical position due to the whisker sweeping across the plate rather than in a less natural upwards direction when the grooves run horizontally. Similarly, a low

spatial frequency would make differences in orientation more salient to the mice when whisking (Carvell & Simons, 1995).

Although a significant effect was not found in Experiment 1, it is acknowledged that a non-significant result derived from null hypothesis significance testing (NHST), is not substantial evidence for a lack of effect to be concluded. This thesis uses this methodology throughout and it is noted that it carries some limitations; namely that by failing to reject the null it assumes that the H0 is true. This is problematic as the lack of evidence for rejecting the null cannot be used as evidence of a lack of effect. Consequently, the conclusions gained from the NHST presented throughout this thesis (whereby the null hypothesis is not rejected) should be taken with caution. The strength of the null effect can be established using tests of equivalence or Bayesian approaches which are not presented in this thesis.

2.4. Experiment 2: Procedural modification of the NOR task

Leaving aside the speculation above, the possibility that extended exposure to the stimuli might result in a general tendency not to explore the plates, suggested one modification that might yield a preference (e.g., for the novel stimulus). Namely, to reduce the periods of exposure during the learning phase from 10 minutes to 5 minutes, and to reduce the test periods in the same way.

2.4.3. Method

Animals

The subjects were 20 C57BL/6J male mice purchased from Envigo (UK). 10 of the mice were six weeks old on arrival and 10 were eight weeks old, they were given one week of habituation to the new housing environment and tested in two consecutive batches.

Apparatus

The apparatus was the same as used in Experiment 1b, however, a modification was made to the lighting conditions. In addition to the infrared light, the room was also illuminated with a dim red light as mice have poor spectral sensitivity for acute vision in red light (Chalupa & Williams, 2008; Hitchcock, 2009). Mice lack red cone opsins and can perceive a maximum wavelength of 620nm; red light has a wavelength range of 620 – 750nm (Jacobs, Williams, & Fenwick, 2004); it was therefore assumed that the mice were unable to obtain visual information from the tactile plates.

Behavioural procedure

The protocol used in Experiment 2 replicated that used in Experiment 1b, except for a change to the experimental timings. The habituation days remained 10 minutes each day however the time periods in the learning and test phase were changed from 10 minutes to five minutes. More specifically, the learning phase was changed to two, 5-min sample phases with a 5-min interval and the test phase was modified to one 5-min period. A 24-hour retention interval was maintained as previously. 20 mice undertook the task

with low spatial frequency plates, which changed in orientation from four vertical in the learning phase to two vertical and two horizontal in the test phase. Of note, the textures used in Experiment 2 were not counterbalanced in order to fully explore the findings described in Experiment 1. This leaves open the possibility that the mice had a preference for the horizontal orientation rather than a novel preference. This limitation was subsequently rectified in the replication study described in Experiment 3 whereby the textures were counterbalanced. The results of Experiment 3 demonstrated that the animals did not have a preference for one orientation over another which could be generalised to the findings described in Experiment 2.

2.4.4. Results and Discussion

Two mice were excluded from analysis due to a lack of exploratory activity and the remaining results were analysed for the full five-minute test period. Exploration times were consistent with those observed in Experiment 1b (mean exploratory time = 131.26s, SEM ± 9.77s).

The mean recognition ratio for the 5-min test period was 0.56 (*SEM* = 0.03) and the individual scores upon which this mean was based differed from chance (i.e., .50 $t(17) = 2.45$, $p < .05$, $d = 0.51$). These results are consistent with those reported by Wu et al., (2013), and show that the effect of exposure to a texture is retained overnight. However, it should be acknowledged that the effect was numerically small and required a relatively large number of mice, which means that it is perhaps not ideally suited to use in the context of advanced neuroscience techniques that are labour intensive and expensive.

2.5. Experiment 3: The effect of experimental timing

The aim of Experiment 3 study 1, was to replicate the previous result from Experiment 2 with further modifications to the methodology following a paper published by Miyamoto et al., (2016). The authors trained mice in a floor-texture recognition task and found that mice had a preference for the novel texture, which lasted two days. This preference declined when the mice were sleep deprived immediately after the learning phase, but not when sleep deprivation took place 6 to 7 hours later. This finding suggests that the time period immediately after the learning phase is important for consolidation of memories. Experiment 3 therefore sought to avoid causing such disruption by amending the testing times to ensure a cage was not disturbed in the period following the learning phase.

2.5.5. Method

Animals and apparatus

The subjects used in Experiment 3 were 20, 8 week old C57BL/6J male mice purchased from Envigo (UK).

The apparatus and experimental conditions were the same as in Experiment 2, where the low spatial frequency plates were used and the orientation of the plates serves as the stimuli. In this experiment however, the orientations were counterbalanced; half of the subjects received low spatial frequency plates which changed from the vertical orientation in the learning phase to two vertical and two horizontal in the test phase and the other half received the opposite contingencies. There was no difference in the discrimination

ratios between the two counterbalanced groups (horizontal to vertical ratio: 0.64; and vertical to horizontal ratio: 0.64). This provides a remedy for the caveat mentioned in Experiment 2.

Behavioural procedure

The procedure was the same as in Experiment 2. In order to avoid disrupting the animals' sleeping patterns, the experiment was carried out in batches and testing was undertaken between 09:00 and 11:00 as opposed to throughout the whole day as it had been in Experiments 1 and 2. The schedule was designed to ensure that once the learning phase had been undertaken for one mouse, it would be returned to its cage and left undisturbed for the rest of the day. On each day, except Day 1 (as there is no test phase on Day 1), the test phase for one mouse was undertaken first and a different animal from the same cage would then undertake their learning phase. For example, the first subject from cage A was mouse 1 undertaking its test phase followed by mouse 2 of cage A undertaking its learning phase. Once mouse 2 from cage A had been tested, the whole cage of animals would be returned to the holding room and testing would continue with mouse 1 of cage B etc. This ensured that an animal was not disturbed for the rest of the day once they had undertaken their learning phase.

2.5.6. Results and Discussion

One mouse was omitted from the full analysis due to a lack of exploratory activity across the test phase. This mouse was, however, included in the 30-second analysis as the animal had visited every corner in the first instance and the reduction in exploration occurred during the final minutes of the test

phase. Analysis of the full five minute test period revealed that exploration times were in line with previous experiments with a mean exploratory time of 157.74s (SEM =18.47s). The ratios, with a mean of 0.50 (*SEM* = 0.03) did not differ from chance (i.e., .50; $t(18) = .25$, $p > .05$, $d = 0.05$). The results were further examined by evaluating the behaviour during the first 30-seconds of the test period during which the mice visited more than one corner (often the first epoch). During this epoch, the mice demonstrated a preference for the novel plate (*M* = 0.64, *SEM* = 0.03; $t(19) = 2.54$, $p < .05$, $d = 0.50$). These results replicate those of Experiment 2 and extend those of Wu et al., (2013). However, the effect does not appear to be sufficiently robust to begin to directly analyse its reliance on the whisker system or barrel cortex.

2.6. Concluding Remarks

The aim of these experiments was to develop a whisker-based discrimination procedure for use with mice. The study utilised the modified NOR task developed by Wu et al., (2013). The present task differs from the traditional NOR task as it tests the ability of mice to discriminate between textures using their whiskers rather than relying on the visual system. The current study also modified the NOR procedure undertaken by Wu et al. (2013) by ensuring that the stimuli could not be discriminated on the basis of visual cues (by conducting the experiment in the dark) or olfactory cues (by using the same materials to create the stimuli).

Experiment 1 involved two pilot studies where custom-made tactile plates were created. These plates varied either in spatial frequency or orientation

and were used as the test stimuli. The use of 3-D printed tactile plates is unique compared with previous studies, which commonly utilise sandpaper as the test stimuli (Diamond, von Heimendahl, & Arabzadeh, 2008; Morita et al., 2011; Wolfe, 2008; Wu et al., 2013). The use of sandpaper is problematic due to the difficulty in accurately replicating the varying sandpaper grades. Moreover, sandpaper roughness cannot be accurately quantified to ensure consistent conditions between studies or even between one presentation and another. A further consideration when using different sandpapers as stimuli is which manufacturer is used. Sandpaper manufacturers use different chemicals in generating the abrasive, which can make it difficult to attribute results to texture learning rather than odour learning. The use of 3-D printed plates allows for precise reproduction of tactile stimuli. The results of Experiment 1a revealed that the mice did not explore the tactile plates a great deal, perhaps because they spent little time in the centre of the arena. The observation that they tended to spend the majority of the time in close proximity to the walls resulted in moving the plates to the four corners in Experiment 1b, which increased the contact times but did not result in a preference for the novel plate during the test.

During Experiment 2, the duration of the learning and test sessions were reduced from 10 minutes to 5 minutes. In this case, a preference for the novel texture plate was found. This result is consistent with the results reported by Wu et al. (2011), and suggested that mice are able to recognize a previously experienced tactile plate using their whiskers alone after a 24-hour retention period. Experiment 3, sought to replicate the findings of Experiment 2 with changes to the details of the timings of the training and

test periods to avoid disruption of sleep between the learning and test sessions. In particular, the method was the same as Experiment 2 with the exception that testing was only undertaken between the hours of 09:00 and 11:00, and a mouse was left undisturbed following the learning phase so to allow it to sleep immediately after the sampling phase. In keeping with the results of Experiment 2, there was a preference for the novel texture plate. However, it was only evident during the first 30-second epoch in which more than one corner was visited. The effect seen in Experiment 3 certainly appeared to be no more robust than that observed in Experiment 2.

Although the results of Experiments 2 and 3 are promising there are a number of caveats to consider. Firstly, there is a possibility that the mice could discriminate between plates using their nose or paws rather than their whiskers alone. The protocol eliminates any visual or olfactory cues, but does not exclude the possibility that the mice were using other somatosensory information to investigate the plates. Of interest, in the original study by Wu et al. (2013), the authors plucked out the full set of whiskers to assess whether mice still showed a preference for a novel texture. They observed that mice with intact whiskers could successfully distinguish between the novel and familiar textures, but those without were unable to do so, even with the use of their nose and paws. Although an important control, there remains the possibility that whisker use was not directly involved in the preference. For example, plucking the whiskers and administering anesthetic may have had a general effect on performance rather than a selective effect on whisker discrimination. The experiments presented in Chapter 3 contrasted the effects of whisker trimming on a

texture learning procedure and an otherwise equivalent odour learning procedure to evaluate whether the whisker system was specifically involved in texture based learning. These experiments were based on a different procedure to those adapted in Experiment 1-3, and required fewer mice to generate robust effects. The procedures developed for mice in Chapter 3 were based on those first described in a paper by Birrell and Brown (2000) that used rats. As will become clear, the results presented in Chapter 3 represent a firm basis upon which to analyze the role of plasticity in the barrel cortex in texture learning.

Chapter 3. Discrimination Learning with Textures

3.1. Summary

There is a paucity of selective behavioural assays for whisker-dependent texture discrimination learning in freely moving rodents: where the textures are not discriminable on the basis of other sensory features, and learning is long-lasting and demonstrably whisker dependent. The results presented in Chapter 2 showed that a novel texture discrimination procedure required a large number of mice to reveal a relatively small effect. Chapter 3 describes the results of a series of experiments which exploits rodents' natural foraging preferences in a behavioural task originally developed by Birrell and Brown (2000). In Experiments 4-9, mice were placed in a maze illuminated with dim red light and were presented with two 3-D printed plastic bowls. The outer surface of the bowls had 3-D printed textures (grooved or smooth). One bowl contained a reward buried in sawdust while the other did not. Within a 60-min session, mice learned to dig in the bowl containing the reward, this learning was retained overnight (Experiment 4 and 5), and whisker trimming disrupted learning based on the texture of the bowls (Experiment 6), but not an equivalent discrimination based on the odour of the sawdust in bowls with the same outer surface (Experiment 7). Learning is robust across different manipulations including headplate attachment (Experiment 8) and an intraperitoneal injection administered 60 minutes before testing (Experiment 9). The new assay requires fewer mice than previous methods in order to detect robust and statistically significant results, and learning is rapid.

3.2. Introduction

Rodents are eclectic learners: They are capable of learning about a broad range of stimulus dimensions. This characteristic enables them to adapt effectively to their environments, but it often complicates interpretation of even the most simple of experiments. For example, if one is interested in the capacity of rodents to use their whisker system to learn about different textures then one might use different grades of sandpaper (Montuori & Honey, 2016) or different media in which rodents dig to find a reward (Birrell & Brown, 2000). However, as mentioned in Chapter 1, these stimuli are also discriminable on the basis of other visual and/or olfactory cues. Isolating the sensory system that is used in the context of a given task is often a secondary concern to those who study learning and memory at a behavioural level. However, this is not the case for those interested in the neural mechanisms that underpin these processes, where the possibility that learning involving different sensory domains might involve different principles has greater importance. Given the considerable interest in investigating the behavioural and neural mechanisms that underpin whisker-based learning in rodents as a model system, it is important to establish that behavioural indices of learning are specific to the whisker system rather than involve, for example, the visual or olfactory systems. Research aimed at understanding the role of the barrel cortex in texture learning (Diamond, von Heimendahl, Knutsen, et al., 2008; Stüttgen & Schwarz, 2017) has been limited by the restricted number of behavioural assays involving freely moving mice (Pacchiarini, Fox, & Honey, 2017). The head-fixed behavioural procedures described in Chapter 1 are not necessarily representative of how rodents

naturally use their whiskers, and the generality of the results might therefore be limited (Hong et al., 2018; Mayrhofer et al., 2013; O'Connor et al., 2010; Sofroniew et al., 2014).

This chapter describes the development of a behavioural assay that allows the selective assessment of long-term whisker-based learning in freely moving mice. It is adapted from the attentional set shifting task originally reported by Birrell and Brown (2000) in which rodents learned to dig in one bowl rather than another in order to find a reward. This task resulted in rapid learning and was used to great effect to investigate, among other things, the neural mechanisms involved in attentional set shifting. They used textures as a convenient dimension that could be combined with others: The outer surfaces of the bowls could be covered with fine or coarse sandpaper, waxed or grain paper, and the front and reverse surfaces of velvet, which could be combined with odours and different digging media. The rats might well have used their whiskers to discriminate between each pair of textures (or their paws), but it is also possible that they were discriminable on the basis of their olfactory or visual properties.

The series of experiments in Chapter 3 first assess the ability of mice to learn and retain overnight a tactile and odour discrimination (Experiments 4 and 5). Tactile cues were provided by the texture printed on the outer surface of the bowls (grooved or smooth) that were made of the same material. In both experiments, mice in the control groups received the same discrimination on days 1 and 2 (e.g., grooved->reward and smooth->no reward), whereas for those in the reversal groups, the stimuli that signalled reward and no reward

were reversed between days 1 and 2 (e.g., day 1: grooved->reward and smooth->no reward; and day 2: smooth->reward and grooved->no reward). If the mice retained overnight what they had learnt on the first day (i.e., for approximately 24 hours), then the control groups should perform more accurately than those in the reversal group on the second day. Reversing the rewarded texture provides an additional means of ruling out the possibility that the mice were simply basing their choice to dig on the presence of the odour of the reward. If the mice were using the odour as a cue there would be no reason to predict a difference in their behaviour on the second day between control and reversal groups. The next series of experiments assessed whether the whiskers were used selectively for the tactile discrimination (Experiment 6) and not the odour discrimination (Experiment 7) by trimming the whiskers in one group of mice but not another. The reliability of the results were established in mice given a headplate attachment (Experiment 8), and an intraperitoneal injection administered 60 minutes before testing (Experiment 9). These experiments demonstrate that the procedure has several appealing features: learning is rapid, long-term, and requires relatively few mice to produce reliable results.

3.3. Experiment 4 and 5: Texture and odour discrimination learning

3.3.1. Method

Animals

Experiment 4 and 5 each used 8 C57BL/6J mice (Charles River, Margate, Kent, UK; mean *ad libitum* weight: 27.1g; range: 23.0g - 31.0g). The mice

were housed individually in 25 × 45 × 15 cm Plexiglass cages. In Experiments 4 and 5, the anticipated discrimination learning effects with textures and odours were based on 8 mice. A power analysis showed that with an expected effect size of .90, 8 mice allows a one-sample t test to detect a difference with power of .74 using a one-tailed test: There is no good reason to expect hungry mice to dig consistently in an unbaited bowl (e.g., with a smooth outer texture) when they have only received reward in a baited bowl (e.g., with a grooved outer texture). However, as will be demonstrated, statistically significant results can be demonstrated consistently with $N = 4$ in both texture discrimination learning and odour discrimination learning. The mice were maintained on standard laboratory diet and water *ad libitum* and were kept in a humidity- and temperature-controlled environment. Experimental testing began at 8 weeks of age and was conducted in the light phase of a 12 hr light/dark cycle (lights on at 7 A.M.). Mice were weighed before each training session and were maintained at 85% of their *ad libitum* weights by being given 3-4 g of food per day at the end of the day. Water was freely available in the home cage. All procedures were approved by the ethical review committee at Cardiff University, UK, and were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act (1986).

Apparatus

The apparatus was based on that described in Huang et al. (2014) and is depicted in Figure 10. It was placed on a table in a small experimental room that was illuminated with dim red light. The arena was constructed from

opaque acrylic, with an acrylic base and walls, and two transparent Plexiglas guillotine doors (25 x 20 x 15 cm). The apparatus included a waiting compartment (20 x 10 cm), which allowed access to two equally sized choice compartments via guillotine doors (15 x 10 cm). Cylindrical digging bowls (45 mm in diameter, 25 mm in height) were created using 3-D printer technology (Ultimaker B.V., The Netherlands) and RS 3-D Printer Filament Polylactic Acid (PLA; 2.85mm 1kg). A drinking bowl was also creating using Wood PLA, which was placed in the waiting compartment filled with water. One digging bowl was placed in each choice compartment. The bowls could be baited with a small piece of cereal (30 mg; Coco Pops, Kellogg; nutrient composition: whole white rice (59%), sugar, cocoa (3%), minerals (calcium carbonate, iron, zinc oxide), salt, flavours, dextrose, barley malt extract, vitamins (vitamin C, niacin, thiamin, riboflavin, folate). The cereal was then fully covered with sawdust (mixed with 2% Coco Pop cereal blended to form a dust) and mice were required to dig to retrieve the hidden food reward. In Experiment 4, the bowls were discriminated by their outer surface (smooth or grooved) and both contained cereal dust (to mask any odour generated by the reward), whereas in Experiments 5, the bowls had the same outer surface (smooth for half of the mice and grooved for the remainder) and the two were discriminated by their odour (either the presence or absence of 2% Coco Pop cereal dust in one set of 4 mice (Davies, Greba, & Howland, 2013); or the presence of 0.5% ginger or 0.5% cinnamon for another set of 4 mice)(Grieves, Jenkins, Harland, Wood, & Dudchenko, 2016).

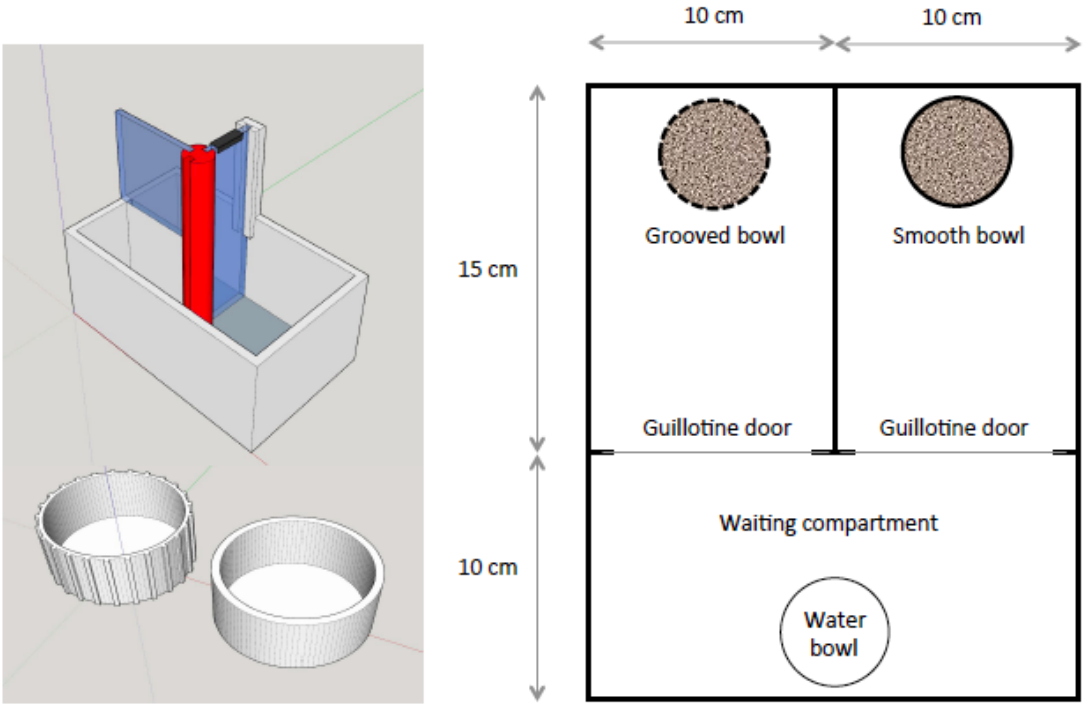


Figure 10. Schematic (left) and plan (right) of the two-choice discrimination apparatus including two digging bowls one of which had a grooved outer surface (grooves: 0.9 mm deep × 1.5 mm across, with 1.6 mm spaces) and the other a smooth outer surface. These two bowls were filled with sawdust, and one (e.g., the grooved bowl) was baited with a small piece of cereal irrespective of whether it was placed in the left or right choice compartments. There was a third bowl in the waiting compartment that was made of a different material and filled with water.

Habituation and pretraining

The general details of the procedure were modified from previous studies (Birrell & Brown, 2000; Colacicco, Welzl, Lipp, & Wurbel, 2002; Garner, Thogerson, Würbel, Murray, & Mench, 2006; Huang et al., 2014). One week prior to testing, mice were gradually accustomed to the experimenters through regular handling. Before each training session, mice were weighed and transferred to the testing room for a 30-min period to acclimatise them to

the room. In order to habituate the mice to the digging bowls and to train them to retrieve the reward by digging in the bowls, a baited digging bowl, filled with sawdust, was left in their home cage overnight for two nights. On the two days before the start of training, mice were given 10 minutes to explore the empty arena. Immediately after the 10-minute periods, mice received two consecutive trials with free access to two (baited) bowls until both rewards were consumed. For half of the mice the baited bowl was grooved and for the remainder it was smooth; the identity of the bowl that was baited during pretraining was the same as during day 1 of discrimination training. This protocol ensured that the mice would dig in the bowls on the subsequent training days.

Training

A trial was initiated by lifting the guillotine doors, thereby allowing the mice access to the two choice compartments containing the bowls (Figure 10). The identities of the bowls that were baited or unbaited were counterbalanced, for example, half of the mice received a baited grooved bowl and the remainder a baited smooth bowl. The first day of training began with 4 exploratory trials (trials 1-4) in which mice were allowed to dig in both bowls and to self-correct if an incorrect choice was made. On trials 5-24 they were only allowed to dig in one bowl per trial. If a mouse began to dig in the baited bowl (e.g., grooved) then they were allowed to retrieve and consume the reward before returning to the waiting compartment, whereas if they began to dig in the unbaited bowl (e.g., smooth), an error was recorded and the next trial was initiated by returning them to the waiting compartment.

Digging was defined as moving the sawdust with the paws or nose; climbing over the bowl was not included as digging. Each of these training sessions lasted approximately 1 hour for each mouse. Fixed pseudo-random sequences were generated to counter-balance the number of times the baited bowl was in the left or right compartment. These sequences were mirror images of one another and had the constraint that there were no more than 3 trials in succession where the baited bowl was in a given compartment (Fellows, 1967). Each of the textured bowls was regularly substituted throughout a training session to ensure that mice did not use cues attached to one specific bowl. Furthermore, any possible olfactory cues left by the animal was removed by cleaning the bowls with 70% alcohol. Once testing was complete the mice were returned to their home cages and given their daily food quotient. On the second day of training, the 4 exploratory trials were omitted and the mice received 20 standard training trials. For mice in the control group, the contingencies on the second day were the same training as on the first day of training (e.g., grooved->reward and smooth->no reward), whereas for those in the reversal group the previously baited bowl (e.g., grooved) became unbaited and the previous unbaited bowl (e.g., smooth) became baited (Table 3).

Table 3.

Design of Experiments 4 and 5

Group	Day 1: Blocks 1 and 2	Day 2: Blocks 3 and 4
Control	Grooved ➔ reward	Grooved ➔ reward
	Smooth ➔ no reward	Smooth ➔ no reward
Reversal	Grooved ➔ reward	Grooved ➔ no reward
	Smooth ➔ no reward	Smooth ➔ reward

Note: The experimental design involved two days of training. Mice in the Control and Reversal groups received the same training across blocks 1 and 2 on Day 1: digging in the bowl with a grooved outer surface was rewarded with a single Coco Pop whereas digging in the bowl with the smooth outer surface was not. For the Control group, the same discrimination continued during blocks 3 and 4 on Day 2, whereas for the Reversal group the discrimination was reversed. This design was also used in Experiment 5 where the bowls were discriminated on the basis of the odour of the digging medium. In both experiments, the identities of the rewarded and nonrewarded bowl were counterbalanced across mice.

Analysis and statistical methods

The percentage of correct choices are binned into two consecutive blocks on day 1 (blocks 1 and 2), and day 2 (blocks 3 and 4). All mice completed at least 16 trials on both days, and on both days the blocks contained the first and second sets of 8 completed trials. Trials on which mice abstained tended

to be towards the end of the session on both days, and presumably reflected satiation and/or fatigue. Of the subjects which completed the full 20 trials, performance reflected that of the previous 4 trials, e.g. if they had completed 75% correct on the previous 4 trials, they performed equivalently on the final (omitted) 4 trials. Similarly, if the animal had performed poorly on the previous 4 trials (e.g. at 50%) the final (omitted) block of training also reflected this poor performance. If performance on day 2 was determined solely by the effects of training on day 1 (at least initially), then all 8 mice in Experiments 4 and 5 should continue to dig in the bowl that was baited on day 1. This could be assessed by coding the accuracy of the choices of both groups of mice on day 2 with respect to the bowl that was correct on day 1, and using one-sample t test to assess the difference from chance levels (i.e., 50%). Similarly, the accuracy of all mice could be coded with respect to the contingencies that were in force on day 2 and compared between the control groups and the reversal groups. In this case, the clear prediction is that those in the control groups should perform more accurately than those in the reversal groups. This was the approach taken in both Experiments 4 and 5, which allows the effects of texture and odour learning on day 1 to be assessed with *Ns* = 8, and for the critical differences between the control and reversal groups on day 2 to be replicated with different stimulus modalities (textures and odours).

3.3.2. Results and Discussion

The texture learning results from mice in the control and reversal groups in Experiment 4 are depicted in Figure 11. Inspection of Figure 11 shows that

the percentages of correct choices increased between the first and second block of training on the first day, and that this increase was similar in both groups, which was to be expected as they had received the same training up to this point. On the second day, the performance of mice in the control group was more accurate than those in the reversal group. ANOVA with day, block and group as factors confirmed that there was no effect of group, $F(1, 6) = 1.96$, $p > .05$, $\eta_p^2 = .24$, no effect of day, $F < 1$, a significant effect of block, $F(1, 6) = 6.18$, $p < .01$, $\eta_p^2 = .50$ and a significant interaction between group and day, $F(1, 6) = 9.07$, $p < .05$, $\eta_p^2 = .60$. Analysis of the overall percentages of correct choices on the first day confirmed that the groups did not differ ($t(6) = -.928$, $p > .05$); and a one-sample t test confirmed that the percentages of correct choices on day 1 were significantly higher than chance (i.e., 50.00%; $M = 57.03\%$, $SEM = 4.16$; $t(7) = 13.57$, $p < .001$, $d = 0.59$). Analysis of performance on the second day confirmed that the percentage of correct choices was significantly higher in the control group ($M = 62.50\%$, $SEM = 4.41$) than in the group for which the rewarded and nonrewarded textures were reversed ($M = 40.62\%$, $SEM = 4.03$); $t(6) = 3.65$, $p < .05$, $d = 0.83$). This difference is important for two reasons. First, it shows that the mice were not simply using the odour of the Coco Pop to guide digging; had they been doing so then there should have been no difference between groups control and reversal. Second, it demonstrates that what was learned on the first day was retained overnight. The observation that differences in performance are evident with group sizes as small as 4 suggests that the training procedure used on day 1 is very effective.

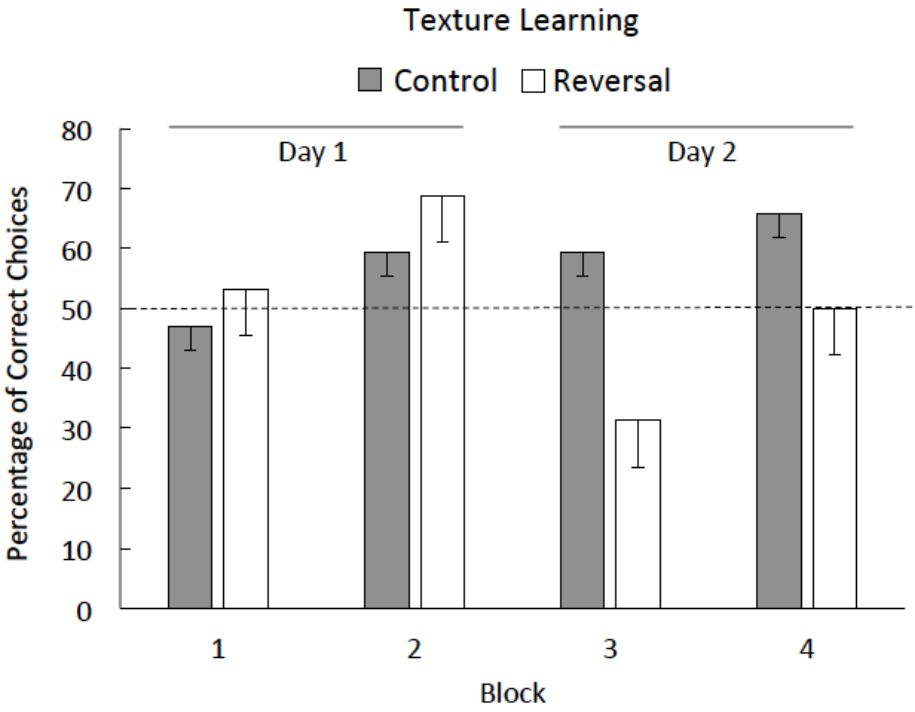


Figure 11. Texture learning in Experiment 4. Mean percentage of correct choices (-SEM) for the control group (grey bars), for which the rewarded and nonrewarded textures were the same on days 1 and 2, and for group reversal, where the rewarded and nonrewarded textures were reversed between days 1 and 2 (white bars). The dotted line indicates chance level.

The odour learning results from mice in the control and reversal groups of Experiment 5 are depicted in Figure 12, and were similar to those from the texture learning experiment. The results are pooled across the odour pairs that we used (i.e., presence and absence of Coco Pops; ginger and cinnamon) because they did not affect the pattern of results (percent correct over day 1; $t(6) = 0.66$, $p > .05$). Comparison of the results from Experiment 5 with those from Experiment 4 suggests that the discrimination involving

odours was acquired somewhat more readily than the discrimination involving textures. On the first day, the mice in Experiment 5 rapidly learned to dig in the bowl with the reward, there being some tendency for those in group reversal to perform somewhat better than those in the control group; in spite of the fact that on this day both received the same training. On the second day, the mice in group control performed at above chance level (i.e., 50.00%), while those in group reversal performed at below chance level. ANOVA showed that there was no effect of group, $F(1, 6) = 3.59$, $p > .05$, $\eta_p^2 = .37$, a significant effect of day, $F(1, 6) = 8.61$, $p < .05$, and a significant effect of block, $F(1, 6) = 9.32$, $p < .05$, $\eta_p^2 = .60$. There was no significant interaction between day and block, $F < 1$, but there was a significant interaction between group and day, $F(1, 6) = 11.59$, $p < .05$, $\eta_p^2 = .59$, and no interaction between group, day and block, $F < 1$. Analysis of the percentages of correct scores on the first day revealed no difference between the groups ($t(6) = -2.11$, $p > .05$, $d = 0.65$); and a one-sample t test confirmed that the percentages of correct choices were significantly higher than chance (i.e., 50.00%; $M = 81.25\%$, $SEM = 3.73$, $t(7) = 21.61$, $p < .001$, $d = 0.99$). Analysis of performance on the second day confirmed that the scores in the control group ($M = 77.08\%$, $SEM = 9.08$) were significantly higher than those in the group that received the reversal of the reward contingencies ($M = 30.00\%$, $SEM = 11.42$), $t(6) = 2.83$, $p < .05$, $d = 0.75$). The difference in performance between the control and reversal groups on the second day confirms that the mice were not simply basing their choice to dig on the presence of the odour of the Coco Pop reward, and that what had been learned on the first day was retained overnight. The pattern of results

from Experiment 4 involving textures was replicated in Experiment 5 using odours.

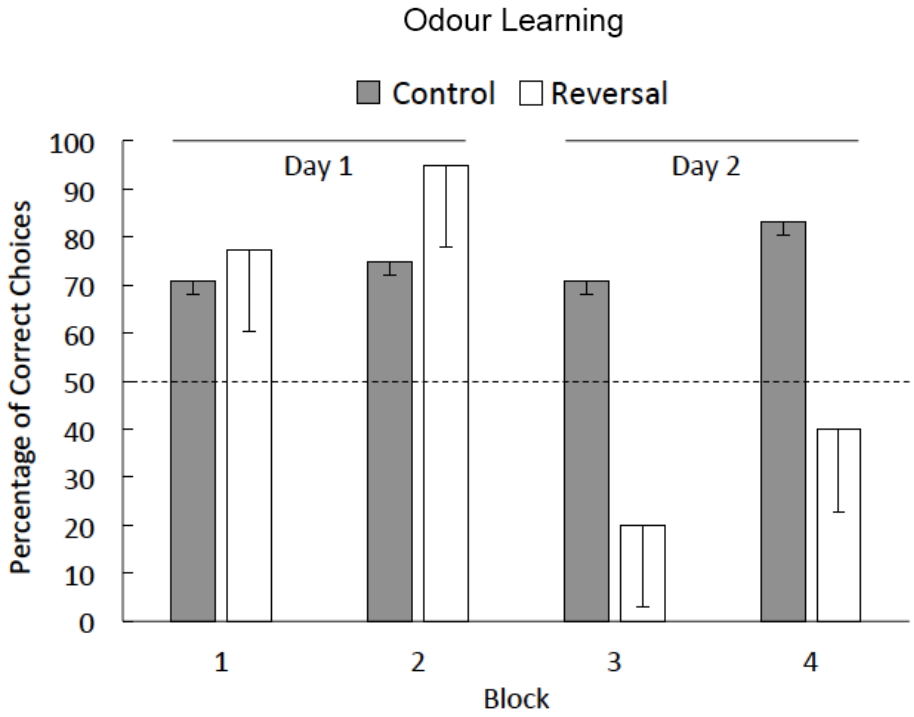


Figure 12. Odour learning in Experiment 5. Mean percentage of correct choices (-SEM) for the control group (grey bars), where the rewarded and nonrewarded odours were the same on days 1 and 2, and for group reversal, where the rewarded and nonrewarded odours were reversed between days 1 and 2 (white bars). The dotted line indicates chance level.

3.4. Experiment 6 and 7: The effect of whisker trimming

3.4.3. Method

Animals

Experiment 6 and 7 each used 8, 8 week old C57BL/6J male mice (Charles River, Margate, Kent, UK; mean *ad libitum* weight: 30.6g; range: 28.0g -

33.0g). In Experiments 6 and 7, the anticipated effects were based on groups of 4 mice (4 in the deprived condition and 4 in the control condition). In these cases, the manipulation (whisker trimming) should make a texture discrimination based on whiskers impossible, but should have no effect on an odour discrimination.

Training

The behavioural procedures used in Experiments 6 and 7 were the same as for day 1 of Experiments 4 and 5, respectively. In Experiment 6 the bowls were discriminated by their outer surface (smooth or grooved) and both contained cereal dust (to mask any odour generated by the reward), whereas in Experiment 7, the bowls had the same outer surface (smooth for half of the mice and grooved for the remainder) and the two bowls could be discriminated by their odour.

Whisker Deprivation

For the deprived group, all macrovibrissae were trimmed bilaterally to the base of the snout (< 1mm), whereas for the control group the whiskers were lightly stimulated by brushing against them for two minutes each side to simulate the stimulation produced by the trimming procedure but without the trimming. Both groups of mice were lightly anesthetized with isoflurane (1.5 % isoflurane in O2, 0.75 l/min) after the two habituation days and 24 hours before behavioural training, but only mice in the deprived groups had their whiskers trimmed.

3.4.4. Results and Discussion

The effects of whisker trimming on texture learning (Experiment 6) and odour learning (Experiment 7) are depicted in the left-hand and right-hand panels of Figure 13, respectively. It is clear that while whisker trimming had a dramatic effect on the acquisition of the texture discrimination (left-hand panel) it had no effect on odour discrimination learning (right-hand panel). In Experiment 6, there was a marked increase in the percentage of correct choices between blocks 1 and 2 in the control group but not in the group deprived of their whiskers; but the corresponding groups in Experiment 7 showed an equivalent increase in the percentages of correct choices between blocks 1 and 2. ANOVA conducted on the results from Experiment 6 confirmed that there was a significant effect of group, $F(1, 6) = 8.44$, $p < .05$, $\eta_p^2 = .58$, no effect of block, $F < 1$, and a significant interaction between these factors, $F(1, 6) = 5.17$, $p < .05$, $\eta_p^2 = .46$. Additional analyses confirmed that the groups did not differ on block 1, $t(6) = .45$, $p > .05$, $d = 0.15$, but differed significantly on block 2, $t(6) = 3.16$, $p < .05$, $d = 0.79$. In fact, training in Experiment 6 continued for a second day, with the same reward contingencies in place, and in the same way as for group control in Experiment 7. On this second day, the mice in the deprived group continued to perform at chance levels on both block 3 ($M = 46.87\%$, $SEM = 3.12$) and block 4 ($M = 53.12\%$, $SEM = 3.12$), whereas the scores in the control group were above chance on both block 3 ($M = 62.50\%$, $SEM = 5.10$) and block 4 ($M = 71.87\%$, $SEM = 3.12$). ANOVA confirmed that there was an effect of group, $F(1, 6) = 33.00$, $p < .01$, $\eta_p^2 = .85$, no significant effect of block, $F(1, 6) = 3.26$, $p > .10$, $\eta_p^2 = .35$, and no interaction between these factors, Fs < 1.

A parallel ANOVA conducted on the results from Experiment 7 revealed that there was no significant effect of group, $F < 1$, a significant effect of block, $F(1, 6) = 22.23$, $p < .01$, $\eta_p^2 = .78$, and no significant interaction between these factors, $F < 1$. Further analysis revealed that the percentages of correct choices on block 1 ($M = 64.06\%$, $SEM = 3.68$) and block 2 ($M = 90.62\%$, $SEM = 3.91$) were significantly higher than chance, $t(7) = 17.23$, $p < .001$, $d = 0.98$, and $t(7) = 23.00$, $p < .001$, $d = 0.99$, respectively.

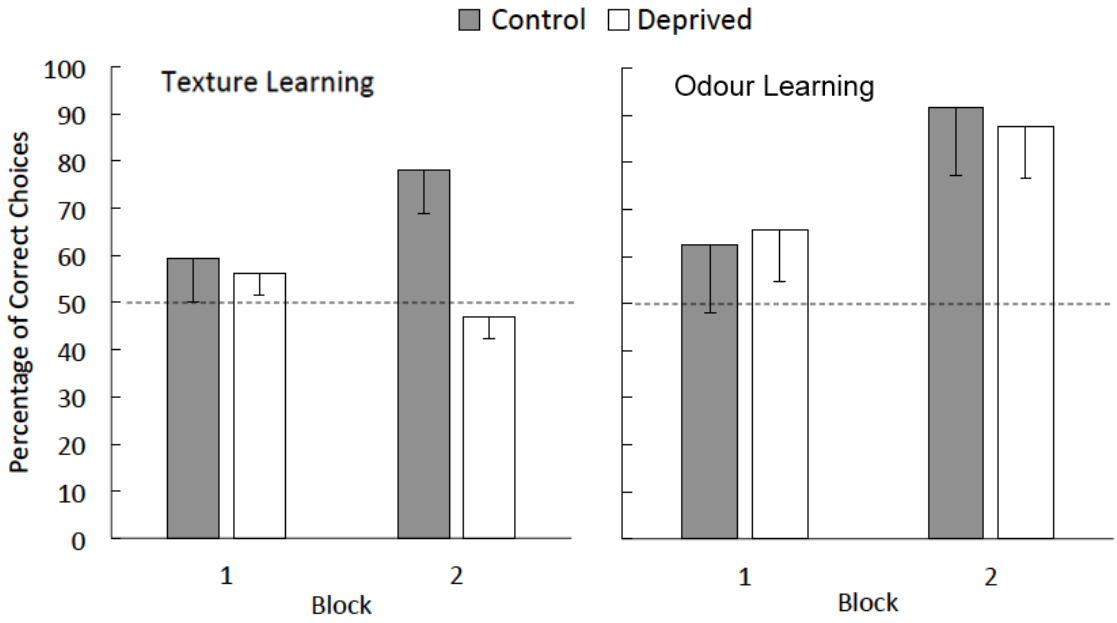


Figure 13. The effect of whisker trimming on texture learning (left-hand panel, Experiment 6) and odour learning (right-hand panel, Experiment 7). Mean percentage correct choices (-SEM) for groups Control and Deprived. The dotted lines indicate chance level.

3.5. Experiment 8 and 9: Texture discrimination learning is reliable and robust

Experiments 8 and 9 were conducted to assess how robust the texture discrimination procedure was in mice that had received two procedures: a

surgical intervention (Experiment 8) and an intraperitoneal injection (Experiment 9). If the texture learning procedure is to be used in the intended context (investigating the neural mechanisms that underpin whisker-based learning) then it is important to establish that learning remains possible when mice have received manipulations regularly used in that context. For example, in order to investigate the cellular underpinnings of experience-dependent plasticity, it is necessary to analyse neurons in the intact brain over a prolonged period of time. The methodology, originally pioneered by Svoboda, allows for high resolution, *in vivo* imaging of dendritic spines in the mouse. The procedure involves creating a chronic cranial window that provides optical access to the mouse cortex for a prolonged period (Mostany & Portera-Cailliau, 2008). Due to the opacity of the mouse skull, imaging the brain requires the bone to be partly removed and replaced with a transparent coverglass. The coverglass is then secured and a clear view of the brain is provided (Holtmaat et al., 2009). This method has been used by a number of groups to image for many weeks to months (Chow et al., 2009; Keck et al., 2008; Lee et al., 2006; Majewska, Newton, & Sur, 2006; Mizrahi & Katz, 2003; Svoboda, Denk, Kleinfeld, & Tank, 1997).

3.5.5. Method

Animals

The subjects used in Experiment 8 and 9 were 10, 8 week old C57BL/6J male mice (Charles River, Margate, Kent, UK; mean *ad libitum* weight: 26.4g; range: 24.0g - 29.0g). Experiment 8 used 6 mice in total and Experiment 9 used 4 mice in total.

Surgical Procedure

In Experiment 8, mice underwent a headplate attachment surgery. The procedure was replicated from Holtmaat et al. (2009) with some minor amendments. Mice underwent this procedure 10 days before the start of food deprivation and weighed at least 18.6 g prior to the surgery so to ensure they could support the weight of the headplate (0.92 g). Surgical tools were sterilized using an autoclave and a hot bead sterilizer prior to the surgery (Germinator 500, Braintree Scientific, USA). On the day of surgery, all tools, equipment and gloves were soaked and cleaned with 70% ethanol solution to ensure good aseptic technique was maintained. The mice were initially anaesthetised in an anaesthetic chamber (Teva UK Limited, UK) with 5% isoflurane in medical oxygen (2L/min). Glucose-saline was injected intraperitoneally to maintain blood sugar levels (10 mL/kg). Following this, subcutaneous injections of metacam (5mg/kg; Boehringer Ingelheim, DE) and dexamethasone (at 2 mg/kg; Colvasone; Norbrook, UK) were injected into the scruff of the neck to reduce pain and reduce inflammation. The scalp was shaved using clippers (miniARCO; Kent Scientific, USA) and two 50µl injections of lidocaine were subcutaneously injected under the scalp to provide a topical anaesthetic. Throughout the surgery, mice were maintained under anaesthetic with 1.2 - 1.7% isoflurane carried by oxygen at 0.75 L/min. Breathing and temperature was regularly checked and anaesthetic depth was monitored throughout the procedure by ensuring abolition of the hind-limb and tail withdrawal reflex. Once the animal had been prepared for surgery, it was transferred to a viral hood and positioned in a Kopf

stereotaxic frame using cheek bars. Opthalmic ointment (Lacri-Lub; Allergan Ltd.) was applied to protect the eyes and the scalp surface was disinfected with 6 alternating swabs of povidone iodine solution (Betadine, Betadine Inc., UK) and 70% ethanol. An incision was made using iris scissors and approximately 1.0cm incision was made along the midline on the skull from the ears to bregma and the right hand skin cut away to reveal the temporalis muscle. The periosteum was removed and the skull cleaned with cortex buffer (distilled water containing: 125 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES buffer, 2 mM $CaCl_2$, and 2 mM $MgSO_4$, pH adjusted to 7.4). Stereotaxic coordinates (1.5 mm posterior and 3.0 mm lateral to bregma) were used to expose the skull approximately over the D1 barrel in S1 cortex. A headplate (UCL, UK; Figure 14) was attached using tissue adhesive (Vetbond; 3M, US) and dental cement (Prestige Dental, UK) with care taken to avoid brain damage by applying too much pressure. Following the surgery, isoflurane was removed and the animal left on the heating pad with pure oxygen until it regained its righting reflex. Mice were then left to recover in a heating chamber (33 degrees Celsius) with wet mash for a minimum of one hour. Prior to transfer back to its home cage, the mouse was inspected for signs of recovery for example, locomotion, eating and grooming. The mouse was then left undisturbed in the home cage for 10 days before behavioural testing began. The animal was monitored daily for seven days post-surgery to observe signs of pain or skin irritation.

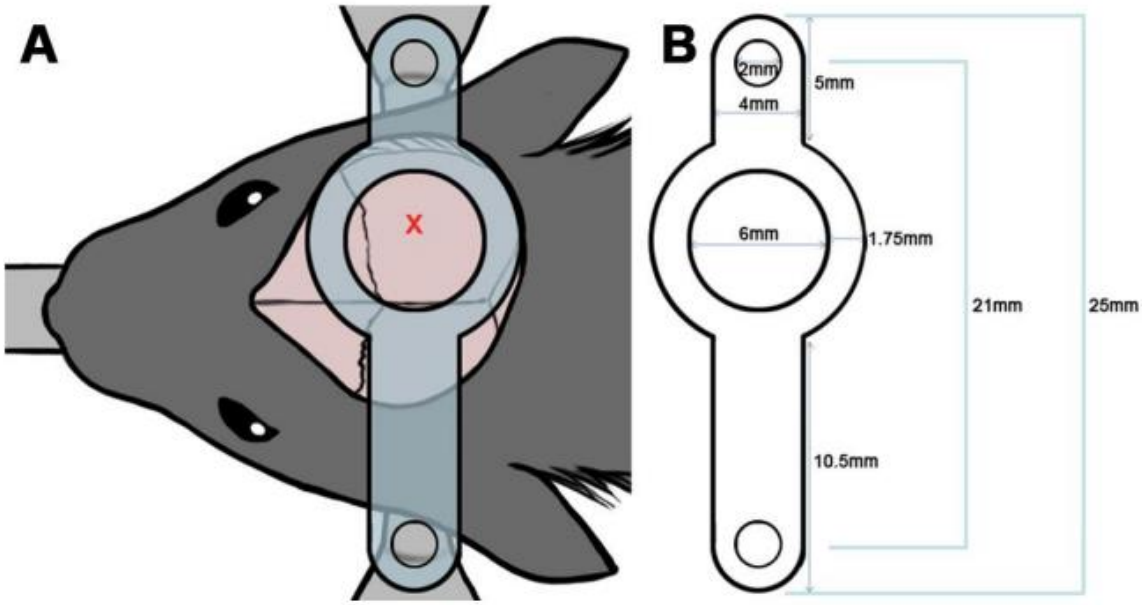


Figure 14. Schematic of the headplate attachment on a mouse. Used with permission from Annelies de Haan (unpublished). (A) The metal headplate attachment is positioned approximately over the D1 barrel indicated here by a red ‘x’. (B) Schematic depicting the measurements of the headplate attachment.

For Experiment 9, a second group of mice were given an IP injection (10 uL/g of CNO in saline) administered an hour before testing on day 1 and 2.

Behavioural procedure

In both Experiment 8 and 9, the procedure was the same as in Experiment 4 except that the mice received two days of testing in which the same reward contingencies were in force.

3.5.6. Results and Discussion

The results of Experiment 8 revealed that in the headplate group, the percentage of correct choices was significantly higher than chance on both

day 1 (M = 63.54%, SEM = 1.92, $t(5) = 32.82$, $p < .001$, $d = 0.99$), and day 2 (M = 70.83%, SEM = 3.84, $t(5) = 18.30$, $p < .001$, $d = 0.99$). Experiment 9 also revealed similar results for the IP injection group. The percentage of correct choices was significantly higher than chance for both day 1 (M = 68.75%, SEM = 5.70, $t(3) = 11.96$, $p < .01$, $d = 0.98$), and day 2 (M = 71.87%, SEM = 4.03, $t(3) = 17.69$, $p < .001$, $d = 0.99$). The novel texture learning procedure is clearly robust.

3.6. Concluding Remarks

The study of learning and memory in rodents often requires considerable ingenuity in generating stimuli that they can both learn about readily and can be combined in such a way that the perception of stimuli from one dimension is not affected by the presence of stimuli from another dimension. The procedures described by Birrell and Brown (2000) represent a good example of such ingenuity. However, it is often the case that the way in which animals are processing the stimulus dimensions within studies of learning and memory is a secondary consideration. This fact can limit further analysis of the requisite behavioural and brain mechanisms. The task procedure was adapted from Birrell and Brown (2000) to investigate whether freely moving mice use their whiskers to learn a texture discrimination.

The discriminations took place in dim red light and the textures were made of the same material (3-D printed PLA). The mice learned within a single session which of the two textured bowls contained a reward and this learning was evident the next day (Experiment 4). The development of an equivalent procedure in which the bowl that contained the reward was signalled by the

odour in the digging medium (Experiment 5), allowed the demonstration that whisker trimming had an impact on discrimination learning involving the texture of the bowls but not the odour of the digging medium (Experiments 6 and 7). The task was further validated in mice which had received an experimental procedure: a surgical intervention (Experiment 8) and an intraperitoneal injection (Experiment 9) establishing that both these groups were able to successfully complete the task.

These experiments directly implicate the whisker system in the discrimination of texture in the digging paradigm developed by Birrell and Brown (2000), which might operate in a synergistic fashion with stimuli from other senses (e.g., information from the paws) when both are available. The fact that the bowls were 3-D printed provides a ready means of changing the discriminability of the textures (e.g., the spatial frequency of the grooves). It may be noted that in the texture discrimination task there was a slight drop in performance from the end of the first day to the beginning of the second day. This decrease in performance is consistent throughout the experiments undertaken in Chapter 3. For example, in Experiment 6, the texture control group decreased in performance from 78.12 ± 9.37 on block 2 to 62.5 ± 5.10 on block 3. Similarly, in Experiment 8, the group decreased from 70.83 ± 2.63 to 68.70 ± 5.35 and in Experiment 9 from 78.12 ± 5.98 to 68.75 ± 3.60. This decrease is believed to be due to satiation and/or motivational reasons. If the mice perform well on Day 1 they receive a large number of Coco-Pop rewards and are therefore not as hungry at the start of the following Day 2 as they would have been at the beginning of Day 1 following the food deprivation schedule. In order to avoid this, future studies could allocate

smaller quantities of the Coco-Pop reward in order to reduce this effect. Informal observations of the behaviour of mice towards the end of training in Experiments 4-9 suggested that on trials where they happened to sample the rewarded stimulus first they rapidly started digging, whereas on trials when they encountered the nonrewarded stimulus then any digging was initiated less rapidly. If the measurement of latencies could be automated then they would provide an additional index of learning, which could also be useful in gauging whether a given manipulation (e.g., a lesion) had a general impact (e.g., on impulsivity).

A final important feature of Experiments 4-9 is that robust and reliable learning effects were observed with a small number of mice; in fact, the minimum number of mice (i.e., 4) that is necessary to conduct an experiment that counterbalances the nature of the rewarded stimulus (e.g., grooved or smooth) and the side of the arena on which it was first positioned during training (Pacchiarini et al., 2017). The development of a procedure that produces rapid learning, which is retained overnight, will allow future research to investigate the neural systems that underpin texture learning and memory in freely moving mice (Diamond, von Heimendahl, Knutsen, et al., 2008; Stüttgen & Schwarz, 2017); complementing research using head-fixed mice where the sensory features of the stimuli can be manipulated (Hong et al., 2018). The fact that our procedures result in reproducible effects with small-N-designs (Smith & Little, 2018) is consistent with the 3Rs agenda (e.g., nc3rs.org.uk) and also enables the efficient assessment of mice with rare or costly mutations.

Chapter 4. The Barrel Cortex and Discrimination Learning

4.1. Summary

A large number of studies have investigated the neural systems involved in learning and memory (for review, see Aggleton & Pearce, 2001). However, many studies have employed behavioural learning tasks that may not be dependent on the systems under investigation; specifically, studies investigating the role of the barrel cortex in learning may not be using behavioural tasks which are specifically reliant on tactile properties of the stimuli employed (Griffin et al., 2012; Guic-Robles et al., 1992; Guic-Robles, Valdivieso, & Guajardo, 1989; Hutson & Masterton, 1986; Tsytsarev et al., 2017). The following series of experiments aimed to establish whether the barrel cortex is critical for the texture and odour discrimination learning tasks developed in Chapter 3. In order to investigate this, the chemogenetic approach was employed. Designer Receptors Exclusively Activated by Designer Drugs (DREADD) were used to reduce the activity of excitatory neurons in the barrel cortex during learning. Expression was limited to PV cells by using a PV-cre line and floxed DREADDs. Experiment 10a consists of a pilot experiment to assess whether the inhibitory hM4DGi or the excitatory hM3DGq DREADD would be more effective at inhibiting the activity of surrounding excitatory cells with which they synapse. The findings revealed that the average evoked responses of nearby excitatory neurons were significantly lower following activation of PV cells using the hM3DGq DREADD. Experiment 10b investigates whether silencing the barrel cortex prior to the behavioural test days would have an effect on the animals' ability

to learn the discrimination tasks involving textures and odours used in Chapter 3. The results indicate that mice that had increased PV interneuron activity in the barrel cortex were unable to learn the texture discrimination task whereas a different group, with the same treatment, were unaffected in the odour discrimination task.

To assess the effect of the DREADD treatment on neuronal activity in the barrel cortex, Experiment 11 used immunohistochemistry on barrel cortex tissue of the subjects involved in Experiment 10b. The tissue was stained against the immediate early gene cFos as a proxy for neuronal activity (Chaudhuri, 1997). The experiment showed that cases where neurons were infected with DREADDs and activated by CNO had a significantly higher density of PV cFos positive cells and a significantly lower density of cFos positive non-PV cells compared with the control groups. Further investigation revealed that within the DREADD and CNO groups, there was a significant difference between cFos positive cell density when comparing an infected and uninfected area of the barrel field. This finding suggests that increasing activity of barrel cortex PV cells causes activity of cells in the infection area to decrease but has no effect on a non-infected surrounding area still within the barrel cortex. Experiments 10 and 11 reveal that the texture discrimination learning is barrel cortex dependent and that decreasing the activity of excitatory neurons in barrel cortex results in a specific inability to acquire the texture discrimination task, but has no significant effect on an odour discrimination task.

4.2. Introduction

In order to ascertain whether a brain area is necessary for a specific behaviour, studies have altered neural circuits within isolated brain regions and observed the consequences on behaviour (for review, see Garner & Mayford (2012)). If an effect is observed, then it is typically concluded that the brain area is directly involved in the behaviour. A commonly used method of altering neural regions is via lesion or ablation techniques. Ablation/lesion experiments involve removal of the tissue from a specific brain region, usually chemically or via surgery (Goldstein, 2014). A great deal of information has been obtained using these techniques. However, cases where there is no effect of a lesion may be explained by a recovery of function due to compensatory processes during the extensive recovery period (more than one week) (Cimadevilla, Wesierska, Fenton, & Bures, 2001) or redundancy in the neural processes involved in a given behavioural function. Specifically, neural reorganization may result in the animal employing different brain regions and/or strategies to compensate for the lesioned area. In addition to this, lesions can disrupt other, perhaps more general processes involved in learning and memory tasks such as arousal, attention, motivation and emotional processes. In order to link a brain area to a specific learning or memory process, it must be shown that it is not involved in these additional processes (Gallo, 2007). More recently, studies have used optogenetic and pharmacological methods to inactivate brain regions in a temporally and spatially selective manner (Boyden, Zhang, Bamberg, Nagel, & Deisseroth, 2005). The use of reversible methods to disrupt a brain structure are advantageous as they are more selective and

any compensatory processes would need to act very rapidly (Cimadevilla et al., 2001).

Optogenetic inactivation involves the use of light to selectively alter the electric state of neurons based on the light-sensitive proteins expressed in the cell membrane (Boyden et al., 2005; Kasparov, 2012). A number of recombinant proteins have been developed to control different brain systems making the technique commonly utilised. The most frequently used protein is Channelrhodopsin-2 (ChR2), a non-selective cation that reacts to the presentation of blue light. The technique has high spatio-temporal precision and a number of studies have used it to elucidate the neural mechanisms of whisker movement (Auffret et al., 2018; Khateb, Schiller, & Schiller, 2017; Liu et al., 2019; Sofroniew, Vlasov, Hires, Freeman, & Svoboda, 2015). Although very useful, the technique also has some disadvantages. When injecting ChR2 for optogenetic manipulation, care must be taken not to overexpress ChR2 as it can perturb the organization of cortical circuits (Miyashita, Shao, Chung, Pourzia, & Feldman, 2013). It is also important to monitor the effects of ChR2 as it can cause large calcium transients which may lead to plastic changes due to an increased probability of neurotransmitter release (Schoenenberger, Schärer, & Oertner, 2011). Furthermore, a recent study by Tyssowski and Gray (2019) showed that blue light exposure, in the absence of ChR2, affected the expression of neuronal-activity-regulated genes in cultured cortical neurons. From a practical viewpoint, there are also further disadvantages. Optogenetic technology requires the delivery of light to simultaneously activate whole populations of cells. This light delivery requires a tethered setup to enable the fiber optics, which is challenging with

behavioural tasks in which the animals are free to move and involve apparatus with sliding door mechanisms (see Chapter 3). The alternative to tethering is to place a light on top of the head, which is powered by a battery pack. This technique is also problematic as it may cause the mouse to behave in a less naturalistic way due to the extra equipment.

Another commonly used method of transient inactivation is via pharmacological procedures, which typically involve the delivery of a pharmacological agent via injection cannula connected to a microsyringe (for review, see Martin & Ghez, 1999). This review provides an analysis of the advantages and disadvantages of the technique. To summarize their main conclusions: These methods typically inhibit action potential initiation and transmission via sodium channel blockers such a tetrodotoxin (TTX) and local anaesthetics (such as lidocaine and procaine). Alternative pharmacological techniques also include neurotransmitter agonists and antagonists such as the GABA-A agonist muscimol. Pharmacological manipulations also have a number of limitations. The technique requires stereotaxic surgery and infusion procedures. The injection cannula and microinjection procedures can cause tissue damage, particularly following repeated administration of the agent. During the infusion of the agent, it is also very difficult to ensure the injection cannula is not occluded, which can subsequently alter the desired injected volume. Moreover, it is not possible to monitor the extent of the inactivation as histological analysis only reveals the location of the injection track. Together, these problems can result in high variability of injection spread within and between subjects. Additionally, the effects of agonists/antagonists can last from 12 to 24 hours which is

relatively long-lasting for behavioural experiments (as noted by Martin and Ghez,1999).

An alternative method, adopted here, is the chemogenetic DREADD approach. A stimulatory (hM3Dq) and inhibitory (hM4Di) DREADD have been developed to activate or inhibit neuronal activity respectively (Armbruster, Li, Pausch, Herlitze, & Roth, 2007). DREADDs are modified human muscarinic receptors, which do not bind to endogenous ligands such as acetylcholine but respond to synthetic ligand, clozapine-*N*-oxide (CNO) (Armbruster et al., 2007; Vlasov, Van Dort, & Solt, 2018). When CNO is bound to the hM4Di DREADD, it produces a hyperpolarization of the cell through activation of G-protein-coupled inwardly-rectifying potassium channels (Urban & Roth, 2015).The hM3Dq DREADD on the other hand, when bound to CNO, causes membrane depolarization through phospholipase C/PIP2-mediated inhibition of voltage-activated KCNQ potassium channels (Alexander et al., 2009; Armbruster et al., 2007). Chapter 4 uses the hM3Dq DREADD to examine the role of the barrel cortex in whisker-dependent discrimination learning.

In the following experiments, anatomical specificity of DREADD expression was achieved by stereotaxic injection of viral vectors encoding the proteins. Cell specific expression was achieved using a Cre-driven viral vector with a double-floxed gene encoding the DREADD. The Cre enzyme catalyzes recombination between two LoxP sites, therefore gene expression only occurs in cells containing Cre (Figure 15)(Vlasov et al., 2018). The DREADD system is advantageous for altering neural activity compared with the

aforementioned techniques, because it causes a transient effect that can be tightly controlled via the administration of CNO via several routes (injection, food, water) (Whissell, Tohyama, & Martin, 2016). Following peripheral administration of CNO, studies have demonstrated that plasma levels of the drug peak within 30 minutes and decrease over the following 2 hours (Anaclet, Griffith, & Fuller, 2018; Guettier et al., 2009). However, although the plasma levels decline during this period, the behavioural effects may be present up to 6 hours later (Alexander et al., 2009). For the purpose of the present study, this period is ideal for behavioural testing over consecutive days. Moreover, unlike optogenetics and pharmacological methods, the DREADD technique does not require additional equipment such as the setup of fiber optics or injection cannula.

It is important to note that recent work has indicated some limitations with the DREADD methodology. It has been reported that DREADDs are not activated by CNO, but rather, its metabolite, clozapine (Chang et al., 1998; Gomez et al., 2017; Jann, Lam, & Chang, 1994). This has led to the suggestion that CNO may not penetrate the blood brain barrier and that clozapine may be acting as the DREADD activator (Gomez et al., 2017; Hellman, Aadal Nielsen, Ek, & Olsson, 2016; Manvich et al., 2018; Raper et al., 2017). This finding is significant as clozapine is a commonly used antipsychotic drug which has sedative effects at high doses (MacLaren et al., 2016). However, a number of studies have shown that both the hM4Di and hM3Dq receptors are biologically inert in the absence of the ligand (Alexander et al., 2009; Armbruster et al., 2007; Bender, Holschbach, & Stöcklin, 1994), and other studies have demonstrated that there is not a

significant effect of CNO on a variety of behaviours in non-DREADD expressing animals (Anaclet et al., 2018; Mahler et al., 2014; Robinson et al., 2014; Sano et al., 2014; Stachniak, Ghosh, & Sternson, 2014; Zhu et al., 2014). Moreover, a recent mouse study undertaken by Jendryka et al. (2019) demonstrated that CNO can penetrate the blood brain barrier and this unbound CNO is sufficient to activate DREADDs. Furthermore, the authors did not find any significant off-target behavioural effects (Jendryka et al., 2019). With this in mind, the experiments presented in Chapter 4 used a low dose of CNO and included a number of control experiments in the experimental design (Mahler & Aston-Jones, 2018; Roth, 2016; van der Peet et al., 2018). This approach allowed for observation of DREADD-specific effects as opposed to a nonspecific effect of CNO or its metabolite clozapine (Gomez et al., 2017).

The purpose of the experiments described in Chapter 4 was to establish the role of the barrel cortex in texture learning using the behavioural procedures developed in Chapter 3. In order to investigate this, the hM3Dq DREADD was expressed in the inhibitory PV cells of layer 4 barrel cortex. PV is a calcium-binding protein found in inhibitory interneurons responsible for modulating intracellular calcium dynamics (Celio, 1986; Ren et al., 1992). Circa 12% of neurons in the cortex are interneurons, and within Layer 4, 8% are interneurons (Meyer et al., 2011). Among the Layer 4 interneurons, circa 65% are PV positive (Tremblay, Lee, & Rudy, 2016). It was theorised that activation of PV neurons using the hM3Dq DREADD would result in an overall inhibitory effect on local circuitry by supressing neuronal activity. Following the results in Chapter 3, which indicated that the texture

discrimination learning task is whisker dependent, it was hypothesised that animals injected with DREADD and CNO would not be able to successfully complete the texture discrimination, but would be unaffected in the odour discrimination.

Chapter 4 also describes an investigation of the expression of the cFos protein in the barrel cortex. cFos is an immediate-early gene induced by the increased calcium levels caused by cell activity. It is a widely used marker of functional activity and considered a marker of task-related activity (Chaudhuri, 1997; Dragunow & Faull, 1989; Filipkowski, Rydz, Berdel, Morys, & Kaczmarek, 2000; Sagar, Sharp, & Curran, 1988; Sakata, Kitsukawa, Kaneko, Yamamori, & Sakurai, 2002). The immediate early gene cFos is transcribed extremely quickly, often minutes after stimulation (Ryser, Fujita, Tortola, Piuz, & Schlegel, 2007). Critically, the presence of Fos, the protein product of the *cFos* gene, does not merely reflect neuronal activity. Studies measuring activity using 2-deoxyglucose have found that it is not necessarily accompanied by the Fos protein suggesting that the expression of cFos is task related (Cullinan, Herman, Battaglia, Akil, & Watson, 1995; Dragunow & Faull, 1989; Jørgensen, Wright, & Gehlert, 1989). The barrel cortex has a relatively low baseline level of cFos expression in the adult (Mack & Mack, 1992; Melzer & Steiner, 1997) and it has been demonstrated that the gene expression of cFos can be reduced by whisker deprivation (Steiner & Gerfen, 1994) or increased following stimulation (Mack & Mack, 1992). Expression of cFos is also proportional to the intensity of the stimulus, with a higher stimulus intensity resulting in higher levels of cFos (Melzer & Steiner, 1997). Moreover, cFos expression has been used as an indirect

marker for processes related to learning and memory (Gore et al., 2015; Guzowski, 2002; Tischmeyer & Grimm, 1999). This is due to the repeated finding that cFos activity increases during discrimination training (Aggleton, Brown, & Albasser, 2012; Chowdhury & Caroni, 2018; Hess, Gall, Granger, & Lynch, 1997) and blocking the production of the Fos protein results in memory impairment (de Hoz et al., 2018; Fleischmann et al., 2003; Guzowski, 2002; Seoane, Tinsley, & Brown, 2012). It was hypothesised that the mice expressing DREADDs in their PV interneurons and receiving an injection of CNO would a) have a higher cFos positive cell density within the PV cells of the barrel cortex and b) have a lower cFos positive cell density within the non-PV cells, when compared to the control groups.

4.3. Experiment 10: The effect of decreasing barrel cortex activity on learning

Experiment 10 consists of two experiments. Experiment 10a is a pilot study that assessed the effect of CNO on neuronal spike rate for hM3D(Gq) and hM3D(Gi) DREADD injected mice. Experiment 10b is a behavioural study utilising the hM3D(Gq) DREADD to investigate the role of the barrel cortex in the discrimination task.

4.3.1. Method

Animals

Experiment 10a used three male C57BL/6 mice for the hM3D(Gi) experiment (Charles River, Margate, Kent, UK; mean *ad libitum* weight: 26.3g; range: 24.2g – 27.5g) and two female and one male PV-Cre mice for the hM3D(Gq)

experiment (mean *ad libitum* weight: 27.2g; range: 24.8g – 29.8g). Experiment 10a also used one male C57BL/6 mouse as a non-DREADD injected control (Charles River, Margate, Kent, UK; *ad libitum* weight 29.0g). Experiment 10b used a total of 24 PV-Cre mice (12 male and 12 female, mean *ad libitum* weight: 24.6g; range: 20.0g - 30.0g). All PV-Cre mice were bred in house. These mice have Cre recombinase expressed under the endogenous PV promoter. The excitatory hM3D(Gq) DREADD was floxed so it was only expressed in a Cre-dependent manner and identified by its red fluorescence (reporter mCherry; Figure 15). The mice were virally injected at 4 weeks old and either were employed for *in vivo* electrophysiology (Experiment 10a) or tested in the two-choice discrimination task (Experiment 10b) at 8 weeks old.

Virus injection

The viruses used in Experiment 10 are shown in Table 4. In Experiment 10a mice were sterotactically injected bilaterally in the barrel cortex with one of two DREADD viruses. The DREADD viruses were obtained from The Viral Vector Facility of the Neuroscience Center Zurich (Zurich, Switzerland). The first virus was an excitatory synapsin driven adeno-associated viral vector used to transduce barrel cortex neurons with hM3D: AAV8-hSyn1-dlox-hM3D(Gq)_mCherry(rev)-dlox-WPRE-hGHp(A) (hereafter referred to as hM3D(Gq) mice). The second virus was an inhibitory CaMKII driven adeno-associated viral vector used to transduce barrel cortex neurons with hM4D: ssAAV8-mCaMKIIa-hM4D(Gi)_mCherry-WPRE-hGHp(A). These DREADD viruses were both tagged with a mCherry reporter to allow visualisation of

expression (Figure 15). In Experiment 10b, mice were stereotactically injected bilaterally in the barrel cortex with one of two viruses: The first virus was the above excitatory hM3D(Gq) DREADD. The second virus was a floxed GFP control virus: AAV1-CAG-Flex-eGFP-WPRE-bGH (hereafter referred to as GFP mice) obtained from the Penn Vector Core of the University of Pennsylvania (Philadelphia, Pennsylvania, USA). All viruses were diluted to achieve a 1 x 10^{12} particles/ml titre.

Table 4.

Viral constructs used in Experiment 10.

Viral Construct	Titre (gc/mL)	Dilution	Injection Volume (nL per site)	Effect	Source
AAV8-hSyn1-dlox-hM3D(Gq)_mCherry(rev)-dlox-WPRE-hGH	1×10^{12}	N/A	200	Excitatory	The Viral Vector Facility of the Neuroscience Center Zurich
AAV8-mCaMKIIa-hM4D(Gi)_mCherry-WPRE-hGH	2.6×10^{12}	1:100	200	Inhibitory	The Viral Vector Facility of the Neuroscience Center Zurich
AAV1-CAG-Flex-eGFP-WPRE-bGH	1×10^{13}	1:1000	200	None	Penn Vector Core of the University of Pennsylvania

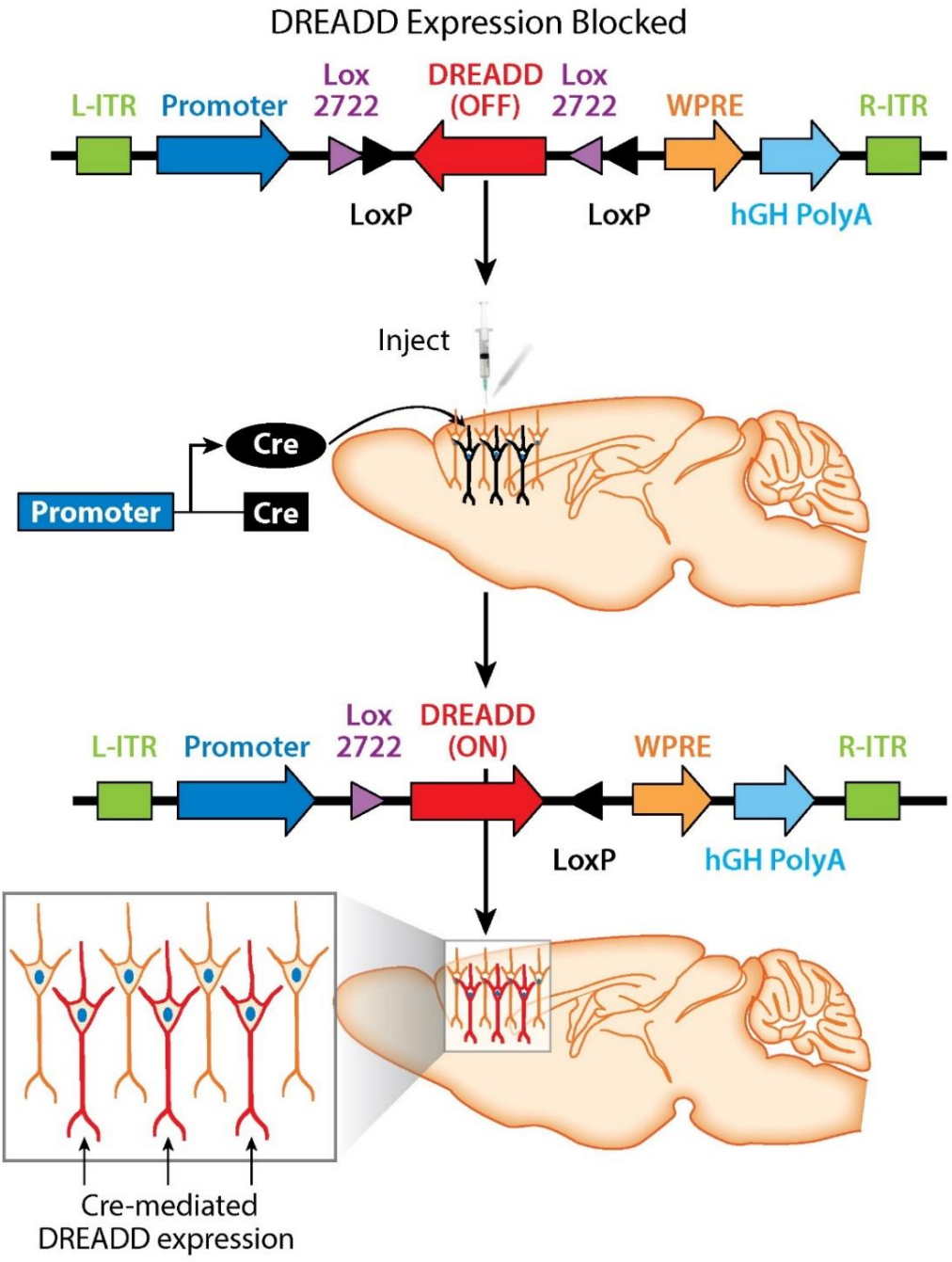


Figure 15. Experimental design of the DREADD virus experiments. Cell type specificity is achieved using the FLEX-switch whereby the DREADD is in an inverse orientation. When the virus infects cells that express Cre recombinase (black) the two lox sites are cut which corrects the orientation of the DREADD for selective expression in Cre-expressing cells (red). Figure used with permission from Urban and Roth (2015).

The initial surgical procedure was as described in Chapter 3, Experiment 8 (Section 3.5.5). However, once the midline scalp incision was made, three small holes (approximately 1mm diameter) were drilled in the skull of each hemisphere estimated to be above the D2, D6 and B2 barrels. Viruses (200 nL per site) were pressure injected bilaterally using a glass micropipette (25-30 um diameter opening) into the barrel cortex at the following coordinates: +1.3mm posterior to bregma and ±3.0mm medial and lateral to midline (approximately barrel D2); 1.05mm posterior to bregma and ±3.3mm medial and lateral to midline (approximately barrel D6); 1.6mm posterior to bregma and ±3.2mm medial and lateral to midline (approximately barrel B2; Table 5). All injections were made 400µm below brain surface and infused at a rate of 25nl/min and the micropipette left to settle for 10 minutes following infusion at each site. Following injections, mice were sutured with nylon suture 5-0 and monitored postoperatively for 7 days for any sign of pain or distress. Mice were returned to their cages and allowed to recover for four weeks prior to behavioural testing to allow for maximal viral expression.

Table 5.

Stereotactic coordinates for viral injections into barrel cortex. Measurements are in millimeteres for AP, ML and DV and were derived from bregma, midline and skull surface respectively and made from Paxinos and Watson (1998).

Anterior – Posterior (AP)	**Medial – Lateral (ML)**	**Dorsal – Ventral (DV)**
- 1.3	± 3.0	-0.4
- 1.0	± 3.3	-0.4
- 1.6	± 3.2	-0.4

Treatment groups

Table 6 shows the experimental design used in Experiment 10b. The primary group of interest was the experimental group referred to as 'hM3D(Gq)-CNO'. This group received an intracranial injection of the hM3D(Gq) DREADD and an intraperitoneal injection of CNO prior to behavioural testing. In addition to the experimental group, additional control groups were tested in the experiment, which are indicated under the 'Controls' section in Table 6. The control groups were important to include due to the aforementioned findings by Gomez et al., (2017), which demonstrated off target effects of CNO. The groups were as follows: group GFP-CNO received intracranial

injections of GFP-Flex and received CNO during behavioural testing. The purpose of this group was to assess whether any behavioural effect was due to the CNO injection, which may have been altering the behavioural state of the animal. Group GFP-Saline received intracranial GFP-Flex injections and saline prior to behavioural testing. This group allowed investigation of whether or not the injection of a virus into the barrel cortex affected discrimination learning. It also allowed investigation of whether the behaviour was affected by possible nonspecific damage caused by the intracranial injections. Group hM3D(Gq)-Saline received intracranial injections of the hM3D(Gq) DREADD and saline at test. This group served to establish whether the DREADD itself was producing an effect without direct activation via CNO. All mice were perfused 90 minutes following test day 2 and the brains collected for immunohistochemistry procedures. These methods are described in Experiment 11 (Section 4.4.3). Post-hoc histological analyses relative to the barrels revealed whether viral injection placement had been inaccurate. Two cases where fluorescence in the barrel cortex was detected in only one hemisphere and one case where the virus injection was unsuccessful in both hemispheres (i.e. there was no visible fluorescence) have been excluded from analysis.

Drugs and injection protocol

Four weeks after the intracranial injection surgery, mice received texture discrimination training as described in Section 3.3.1. As shown in Table 6, all mice undertook the texture discrimination task except four mice in the hM3D(Gq)-CNO group which undertook the odour discrimination. The

purpose of this group was to investigate whether the activated DREADDs have a specific effect on texture discrimination learning or would be non-selective and affect any discrimination. CNO (purchased from Sigma Aldrich, Gillingham, UK) was initially dissolved in dimethyl sulfoxide (DMSO) and stored at -18 degrees Celsius. On the day of testing, mice received intraperitoneal injection of 4 mg/kg CNO (0.3 mg/ml, 1.3% DMSO, diluted in 0.9% saline). This is within the safe range of DMSO for mice (0.5-5%) (Brayton, 1986; Castro, Hogan, Benson, Shehata, & Landauer, 1995; Gad, Cassidy, Aubert, Spainhour, & Robbe, 2006). The drug was administered 30 minutes into recording in Experiment 10a and 60 minutes prior to behavioural testing on test day 1 and test day 2 in Experiment 10b. This allowed peak activation of DREADD receptors by CNO. For the control groups, the same ratio of DMSO and saline was administered without the CNO.

Table 6.

Treatment groups and sample sizes.

Group	**Surgical Treatment**	**Injection prior to testing**	**Behavioural Discrimination**	**Final sample sizes (n)**
Experimental				
hM3D(Gq)-CNO	hM3D(Gq)	CNO	Texture	6
Controls				
GFP-CNO	GFP-FLEX	CNO	Texture	4
GFP-Saline	GFP-FLEX	Saline	Texture	4
hM3D(Gq)-Saline	hM3D(Gq)	Saline	Texture	4
hM3D(Gq)-CNO	hM3D(Gq)	CNO	Odour	3

In vivo electrophysiology

The aim of Experiment 10a was to establish the effect of CNO on whisker-stimulation-evoked-responses to mechanical movement of the principal whisker. The method was taken from Greenhill, Ranson, and Fox (2015). Mice were anaesthetised with urethane (1.5g/kg body weight) and unlike in the aforementioned study, the sedative acepromazine was not used as it is an antagonist of muscarinic receptors, which may have interfered with the binding of CNO to the DREADDs. Anaesthesia depth was monitored by observing breathing rate, cortical activity and hindlimb and corneal reflexes. Due to a light anaesthetic depth, one mouse in group hM3D(Gq) was also maintained under anaesthesia using isoflurane (1.5 % isoflurane in O2, 0.75 l/min). Topical analgesic (lidocaine) was applied to the ears and scalp and the mice were placed in a stereotaxic frame (Narashige, Japan) fitted with a thermostatically controlled heating blanket (Harvard Apparatus, Kent, UK) to maintain body temperature at 37ºC. An electric drill was used to thin a 2x2mm section of the cranium over the barrel cortex (0-2mm caudal from bregma, 2-4mm lateral from midline) and a small hole was made by removing a fleck of thinned skull using a 30G hypodermic needle. A carbon-fibre electrode (Fox, Armstrong-James, & Millar, 1980) was inserted into the hole to enable recording in the layer 4 barrels. A Neurolog system isolated the spikes using a window discriminator to provide single-unit recordings (Digitimer, Welwyn Garden City, UK) amplified the action potentials, which were digitised using a CED 1401 and Spike2 software (CED, Cambridge, UK). The location of the electrode within the barrel field was established by individually stimulating the whiskers using a glass rod attached to a piezo

wafer driven by a Digitimer DS-2 isolated stimulator. Stimuli were applied as single 10ms 200µm upward deflections at 1Hz, repeated 50 times. See Figure 16 for a schematic illustrating this apparatus. The recording location was confirmed by micro-lesions (1 µA DC, tip negative, 10 seconds, estimated depth of 350µm) made at the end of each recording session from post-mortem histology. The mice were perfused and tissue stained for cytochrome oxidase activity by reaction with diaminobenzidine and cytochrome (Wong-Riley, 1979). The lesions made during recording were then correlated with the histology to confirm in which barrel each cell was recorded (Figure 17B).

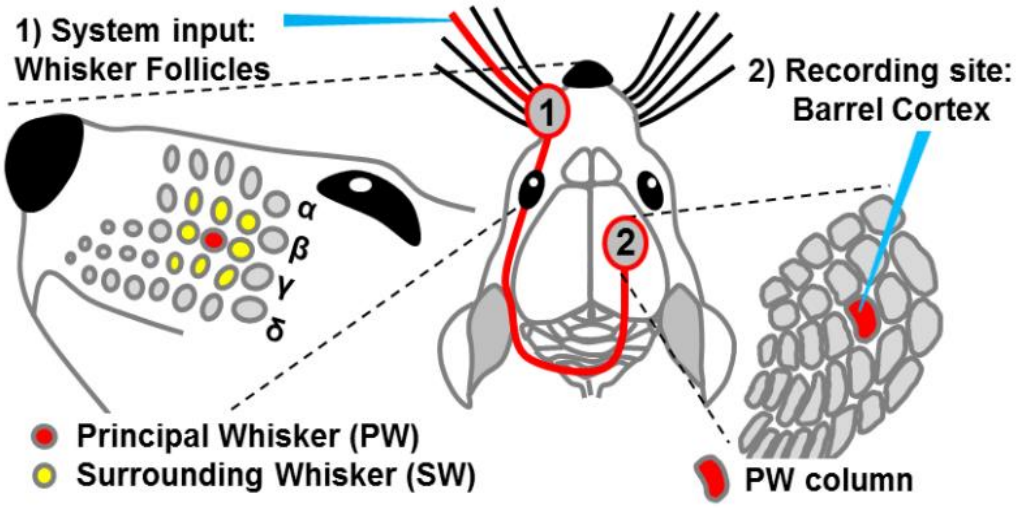


Figure 16. Schematic taken with permission from Juczewski (2017) illustrating the *in vivo* electrophysiology experimental setup. A piezo-electric stimulator was used to mechanically move individual whiskers whilst recording activity in the barrel cortex column corresponding to the principle whisker (PW).

Statistical methods

In Experiment 10a, due to natural differences in baseline in firing rate between cells, the average evoked responses were normalised using the GraphPad Prism 'Normalize' function (GraphPad Software, La Jolla, California, USA). This function defines the smallest value in the dataset as zero and the maximum value as 100 and presents the dataset as percentages. The groups were then analysed using paired samples *t*-tests to compare the firing rates pre and post-CNO administration.

The statistical analysis of Experiment 10b followed that used in Chapter 3: the dependent measure of interest was the percentage of correct responses on each block of training. The behavioural results were analysed using a repeated-measures ANOVA to investigate whether the hM3D(Gq)-CNO group differed from each control group (GFP-CNO, GFP-Saline and hM3D(Gq)-Saline). Following this, the control groups were combined and analysed as there was not a statistically significant difference between groups (percent correct over days 1 and 2) as determined by one-way ANOVA ($F(2,11) = 1.21$, $p > .05$). A repeated-measures analysis of variance with appropriate factors and Bonferroni corrected post-hoc tests were used to compare the behavioural score of the hM3D(Gq)-CNO group with the pooled control group.

4.3.2. Results and Discussion

The aim of Experiment 10a was to investigate the effect of CNO (0.3 mg/ml) on evoked responses following infection of either excitatory cells with hM4D(Gi) or inhibitory cells with hM3D(Gq) DREADD. The results from

Experiment 10a were used to inform which DREADD was used in the full study (Experiment 10b).

Figure 17 depicts the normalised average evoked responses from the recording of the principal whisker barrel for: the hM4D(Gi) and the hM3D(Gq) groups combined (Figure 17A), the hM4D(Gi) group (Figure 17C) and the hM3D(Gq) group (Figure 17D). Electrode penetrations were directed to be close to the virus injection sites where it was possible to see them. Neurons were recorded at a mean depth of 312.8 ± 19.72 µM (group hM4D(Gi) *M* = 316.6, *SEM* = 28.48 µM; group hM3D(Gq) *M* = 313.33, *SEM* = 43.33 µM). A paired-samples t-test was conducted to compare the mean evoked principal whisker response at set time points during the recording period. The mean evoked response for minutes 25 and 30 (pre-CNO administration) was compared to the mean evoked response for minutes 95 and 100 (60 minutes post-CNO administration). The 25-30 minute period was chosen as it allowed sufficient time for the response of the cell to stabilise after the recording electrode had moved into position to record it. The CNO injection was administered immediately after the 30-minute reading had been completed. The 95-100 minute period was chosen as this simulated the experimental design used in Experiment 10b where the mouse was tested in the discrimination task 60 minutes post CNO injection. In the hM3D(Gq) group, there was a significant difference in the mean evoked responses for minutes 25-30 (*M* = 66.78, *SEM* = 2.79) compared to minutes 95-100 (*M* = 11.14, *SEM* = 4.09); $t(2) = 9.37$, $p < .05$, $d = 0.99$. Analysis of group hM4D(Gi) revealed that there was no significant difference in the mean evoked responses between minutes 25-30 (*M* = 53.59, *SEM* = 5.01) and minutes 95-

100 (*M* = 29.23, *SEM* = 21.11); *t*(2) = 1.24, *p* > .05, *d* = 0.62. The single mouse, which did not receive a DREADD injection but received an intraperitoneal injection of CNO, did not demonstrate a decrease in its mean evoked response throughout the testing period (minutes 25-30: *M* = 27.27; minutes 95-100: *M* = 21.81). Visual inspection of Figure 17C-D also confirmed that the decrease in firing rate was more dramatic in the hM3D(Gq) group. Consequently, the hM3D(Gq) DREADD was used in Experiment 10b.

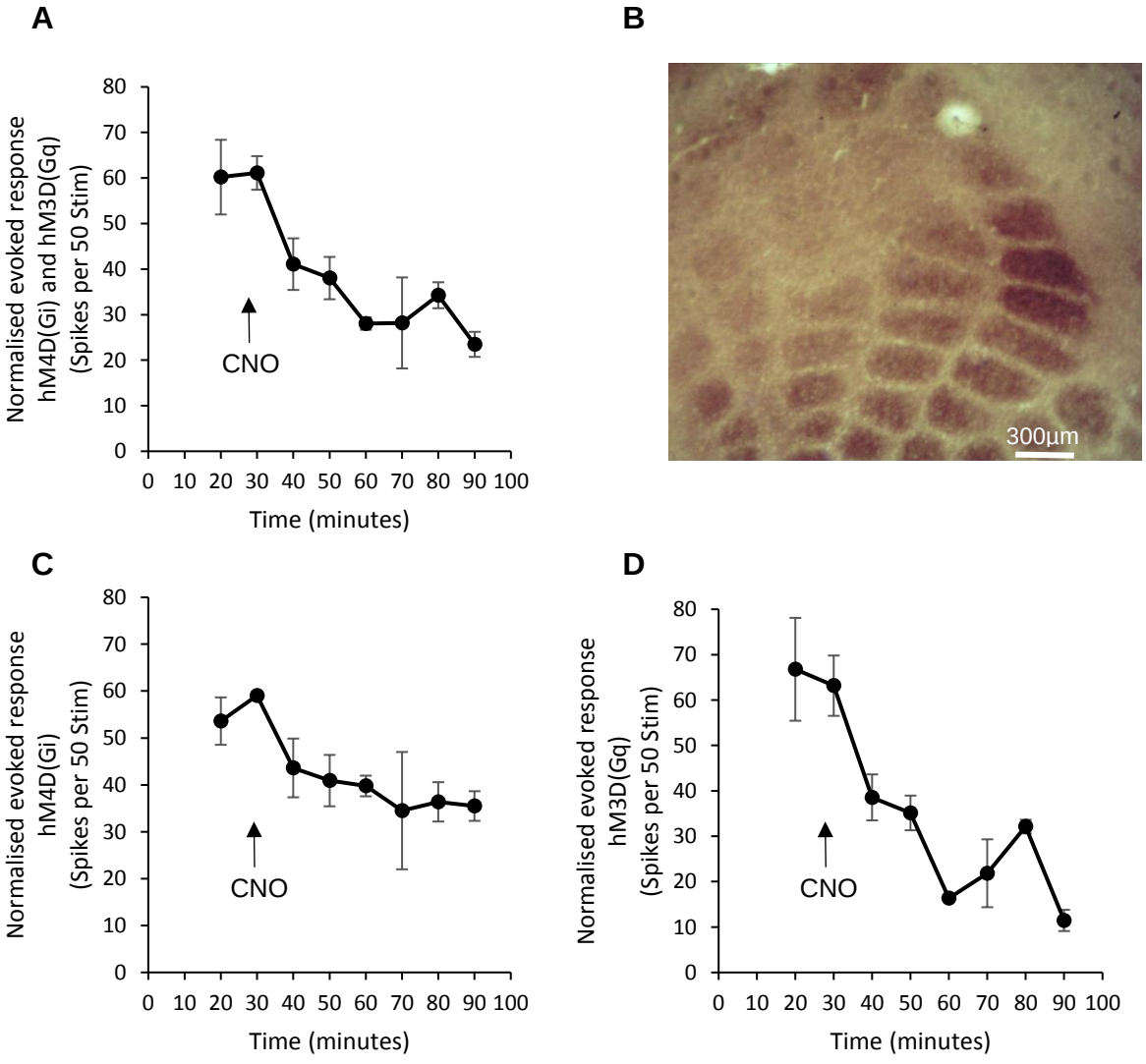


Figure 17. (A) Normalised mean spike response from principal whisker stimulation from six cases injected with DREADDs (B) Example picture of the microlesion made in the delta barrel to mark the location of the recording penetration (C) Normalised mean spike response from principal whisker stimulation from three cases injected with the inhibitory hM4D(Gi) DREADD (D) Normalised mean spike response from principal whisker stimulation from three cases injected with the excitatory hM3D(Gq) DREADD. Values show mean ± SEM.

The effects of decreasing neuronal activity on texture and odour learning (Experiment 10b) are depicted in the top and bottom panels of Figure 18, respectively. Inspection of Figure 18 shows that the hM3D(Gq)-CNO group were not able to acquire the texture discrimination task (top panel) while a different hM3D(Gq)-CNO group were able to acquire the odour discrimination task (bottom panel). The results of the ANOVA revealed that there was a significant effect of group, $F(1, 14) = 4.68$, $p < .05$, $\eta_p^2 = .50$, a significant effect of block, $F(3, 42) = 4.51$, $p < .01$, $\eta_p^2 = .24$, and a significant interaction between block and group, $F(9, 42) = 2.17$, $p < .05$, $\eta_p^2 = .31$. Post hoc analysis revealed that the hM3D(Gq)-CNO group performed significantly worse (M = 49.47%, SEM = 4.86) than the hM3D(Gq)-saline group (M = 75.00%, SEM = 5.95; $p < .01$) and the GFP-saline group (M = 71.87%, SEM = 5.95; $p < .05$). There was no significant difference between the hM3D(Gq)-CNO group and the GFP-CNO group (M = 61.71%, SEM = 5.95; $p > .05$). Although there was no significant difference in behavioural performance between these groups, in group GFP-CNO, the percentage of correct choices was significantly higher than chance on both day 1 (M = 62.50%, SEM = 7.65, $t(3) = 8.10$, $p < .01$, $d = 0.97$), and day 2 (M = 60.93%, SEM = 5.33, $t(3) = 11.32$, $p < .01$, $d = 0.98$).

The results of the pooled group analysis of Experiment 10b over day 1 and day 2 confirmed that there was a significant effect of group, $F(1, 16) = 10.84$, $p < .01$, $\eta_p^2 = .40$, no effect of block, $F < 1$, and no significant interaction between these factors, $F(3, 48) = 2.73$, $p < .054$, $\eta_p^2 = .14$. Further analysis revealed that in the pooled control group, the percentage of correct choices was significantly higher than chance on day 1 ($M = 75.52\%$, $SEM = 4.51$, $t(11) = 5.65$, $p < .001$, $d = 0.86$) and day 2 ($M = 63.54\%$, $SEM = 3.91$, $t(11) = 3.46$, $p < .05$, $d = 0.76$). In the hM3D(Gq)-CNO treated group, the statistical analysis revealed that the percentage of correct choices was not significantly higher than chance on either day 1 ($M = 48.95\%$, $SEM = 5.20$, $t(5) = -.20$, $p > .05$, $d = 0.08$) or day 2 ($M = 49.65\%$, $SEM = 4.94$, $t(5) = -.07$, $p > .05$, $d = 0.03$). Of note, the two hM3D(Gq)-CNO (texture) cases excluded from the overall analysis due to only infecting the barrel cortex in one hemisphere, also demonstrated an inability to acquire the texture discrimination task ($M = 45.31\%$, $SEM = 4.68$). Further studies could investigate the effect of single hemisphere inactivation of barrel cortex on performance on the discrimination task (this is discussed in Section 5.4).

A parallel analysis conducted on the results from the hM3D(Gq)-CNO mice given the odour discrimination task revealed that the percentage of correct choices was significantly higher than chance on both day 1 ($M = 79.16\%$, $SEM = 11.59$, $t(2) = 6.78$, $p < .05$, $d = 0.97$), and day 2 ($M = 79.16\%$, $SEM = 11.59$, $t(2) = 6.78$, $p < .05$, $d = 0.97$). This suggests that activating Gq DREADDs in the barrel cortex with CNO has a selective effect on the texture discrimination task and not the odour discrimination task. It is noted however, that the extensive control groups used for the texture discrimination

experiment were not utilised in the odour discrimination task. Consequently, it is not possible to directly demonstrate that the DREADD or CNO manipulations had no effect on performance of the odour discrimination. However, the performance demonstrated in Experiment 10 was equivalent to that shown in Chapter 3 (Experiment 5 and Experiment 7) which suggests that the manipulations of the barrel cortex were not materially impacting on the odour discrimination.

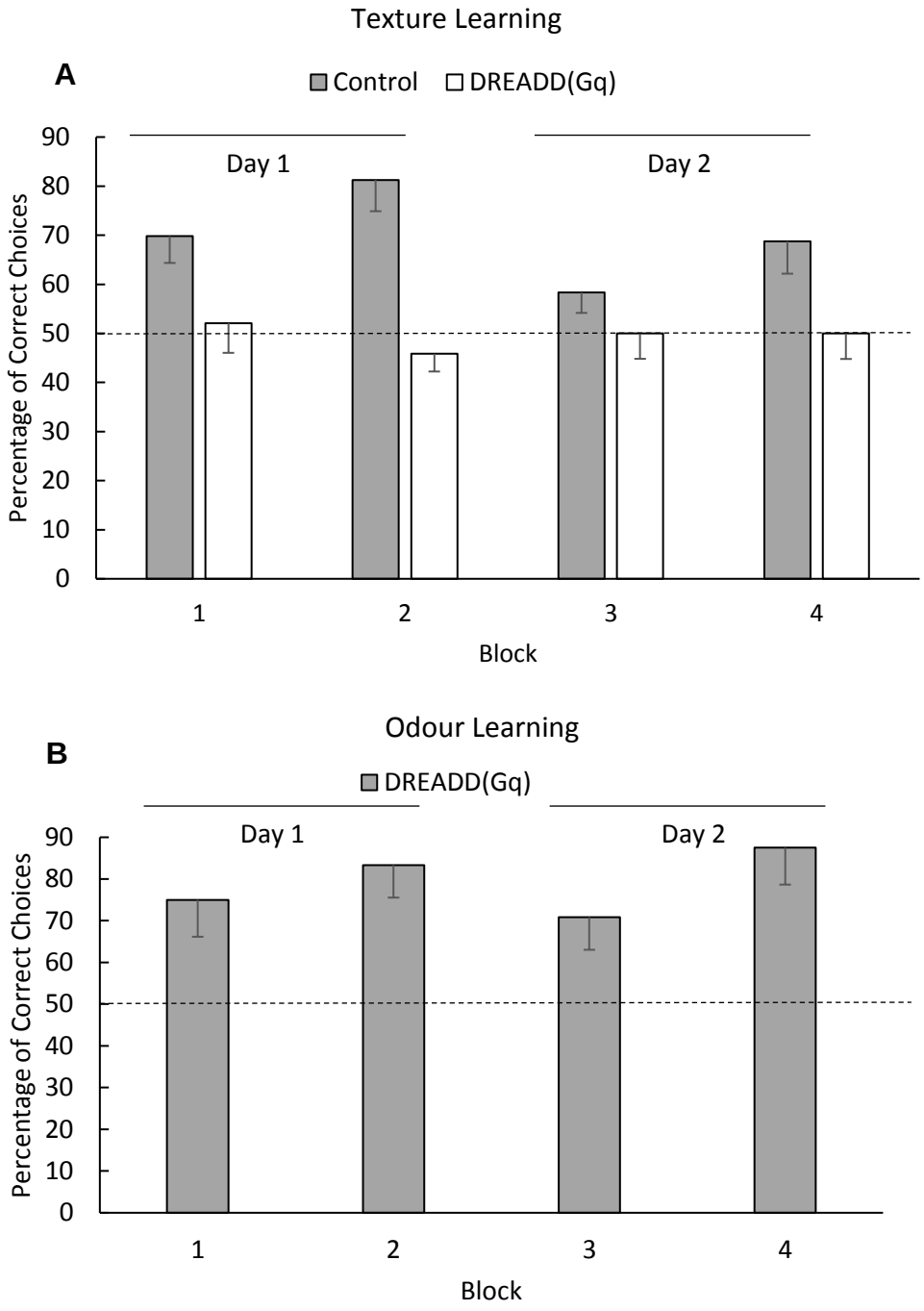


Figure 18. The effect of DREADDs on discrimination learning. (A) Mean percentage correct choices (-SEM) in the texture discrimination task for groups hM3D(Gq)-CNO and control groups: GFP-CNO, GFP-Saline and hM3D(Gq)-Saline combined. (B) Mean percentage correct choices (-SEM) in the odour discrimination task for group hM3D(Gq)-CNO. The dotted lines indicate chance level.

4.4. Experiment 11: Neural activity during the discrimination task

The following section describes a series of immunohistochemistry experiments aimed to further elucidate the underlying neural activity in the barrel cortex during the discrimination task. These experiments served to confirm the effectiveness of the DREADDs by indicating whether the activity of PV cells in the hM3D(Gq)-CNO groups had been enhanced. The experiments also allowed investigation of whether or not cFos activity in the barrel cortex was altered during discrimination training.

4.4.3. Method

Immunohistochemistry

Following completion of behavioural training, mice were left in a dark room in their home cages for 90 minutes to allow maximal expression of the cFos protein (Cullinan et al., 1995). Mice were then given a lethal dose of pentobarbital (Euthatal, Boehringer Ingelheim Animal Health UK Ltd.) and immediately perfused transcardially with 0.1M phosphate buffered saline (PBS; pH 7.4), followed by 4% paraformaldehyde (PFA) in PBS. The brains were removed and, for a horizontal view of cortex, the cortex was dissected free of subcortical structures and gently flattened between two glass slides (Armstrong-James & Fox, 1987; Lauer, Schneeweiß, Brecht, & Ray, 2018). The sample was then post-fixed for 24 hours at 4 ºC in 4% PFA and equilibrated in PBS containing 25% sucrose at 4 ºC. Fixed brains were then cut tangentially into 35 µm sections using a microtome (Leica Biosystems SM200 R) and sections stored at -20 degrees in a cryoprotectant solution (50% sucrose, 1% polyvinyl pyrrolidone and 30% ethylene glycol in 0.1M

PBS). For the immunohistochemistry, individual floating sections were thoroughly rinsed in PBS solution and blocked for 1 hour with 2% goat serum and permeabilized with 0.05% Triton X-100 in PBS (PBST). The slices were then incubated at 4 ºC for two days with a mixture of the following antibodies, which are also listed in Table 7: rabbit anti-cFos polyclonal antibody (1:5,000; Synaptic Systems), and guinea-pig polyclonal anti-VGluT2 primary antibody (1:2,000 Synaptic Systems). To confirm the cell type infected with DREADD, a second subset of DREADD injected slices were also incubated in rabbit anti-PV polyclonal antibody (1:2,000; Swant Inc.) and incubated at 4 ºC for one day. After incubation, slices were washed thoroughly in PBST and incubated in a solution of Alexa Fluor 647-conjugated anti-rabbit (1:1,000; Abcam) and either Alexa Fluor 488 conjugated anti-guinea pig antibody (1:500; Abcam) or Alexa Fluor 568-conjugated anti-guinea pig antibody (1:500; Abcam) in the 2% blocking solution for 2 hours at room temperature. The second batch of anti-PV slices were incubated in a solution of Alexa Fluor 488-conjugated anti-rabbit antibody (1:500, Abcam). All slices were then washed in PBST and incubated in DAPI (1:15,000; Sigma Aldrich, Gillingham, UK) in PBS for 10 minutes. Slides were washed in PBS, air dried and mounted in Fluoromount Aqueous Mounting Medium (Sigma Aldrich, Gillingham, UK) and coverslipped using Vectashield (Vector Laboratories). The following control solutions were used for both protocols: (1) a solution without the primary antibody, (2) a solution without the secondary antibody and (3) a solution without any antibody. Compared with the normal solution, no fluorescence was detected (data not shown).

Table 7.

Antibodies used in Experiment 11. Dilutions, sources and product codes.

Antibody	**Dilution**	**Source**	**Identifier**
Rabbit polyclonal anti-cFos	1:5,000	Synaptic Systems	226 003
Guinea-pig polyclonal anti-VGluT2	1:2,000	Synaptic Systems	135 404
Rabbit anti-PV polyclonal	1:2,000	Swant Inc.	PV 27
Alexa Fluor 647-conjugated anti-rabbit	1:1,000	Abcam	ab150079
Alexa Fluor 488 conjugated anti-guinea pig antibody	1:500	Abcam	ab150185
Alexa Fluor 568-conjugated anti-guinea pig antibody	1:500	Abcam	ab175714
Alexa Fluor 488-conjugated anti-rabbit	1:500	Abcam	ab150077

Imaging and analysis of colocalization

Tissue was visualized using a confocal laser scanning microscopy (Zeiss LSM880) and the location of fluorescent neurons outlined using Adobe Photoshop (CS4) and analysed using Imaris (Bitplane) Image Software (Oxford Instruments, Abingdon, Oxon, UK). Brain regions were outlined by comparing the slice images with the corresponding atlas maps (Paxinos & Watson, 1998; Wang, Sporns, & Burkhalter, 2012). Once the area was measured, Fos-immunopositive neurons were counted using the automated Dot Quantification Analysis feature of Imaris. As with previous studies, cFos neurons were counted only when clear immunostained nuclei were co-localized with DAPI staining (Oshitari, Yamamoto, & Roy, 2014; Yokoyama et al., 2013). DREADD and GFP infected neurons were readily visualized with native fluorescence from the mCherry/GFP expression in the PV-Cre mice. One representative section per brain region from each mouse was used for quantification, and both hemispheres were quantified for each mouse

In order to compare the cFos expression in specific regions within the barrel cortex (infected vs. uninfected barrel cortex ROIs), a new channel was created in the Bitplane program, which only included colocalized cells (virus expression (either DREADD or GFP) plus cFos signal present). Cells were detected using pixel intensity threshold whereby all pixels with a value higher than the threshold value are classified as target pixels, and pixels with a lower value classified as background pixels. These thresholds were used to generate the new Bitplane channel, which only contained the colocalized

voxels and excluded channels outside the region that exhibited no correlation (Costes et al., 2004). The colocalized channel was manually inspected to ensure background had not been included, if this occurred, the thresholds were altered to correct for it. Following the creation of the colocalization channel, the Imaris spot detection feature was used to detect spots within a region of interest (ROI) of either 400 x 400µm or 200 x 200µm. Within the ROI, the mean density (cells/mm^2) was calculated as the number of colocalized cells in one region divided by the mean area size of that region (Lin et al., 2018). In addition to this, a 'minimum area of super-threshold' filter (4.15µm) and a 'background subtraction' filter was applied; this feature adds a Gaussian filtered channel that filters by ¾ of the spot radius. The intensity centre of the spot is then used to detect the spot for the channel of interest (Costes et al., 2004). All spot detection images were manually inspected to ensure the background was not being registered, as a result, some spots were manually removed or the spot detection thresholds altered in sections that contained a large amount of background staining.

To provide an independent measure that the chemogenetic expression of DREADDs did indeed only take place in PV cells, two cases of DREADD infection in PV-cre mice were stained against PV. Two ROIs of 1000 x 1000µm area were defined (one encompassing the DREADD injection sites and one with no DREADD injection viral spread). Imaris (Bitplane) was used to automatically detect the colocalization in the two channels (DREADD and PV). As before, colocalization was determined using a pixel intensity threshold. The same 'quality' and 'background subtraction' filter as described

above was applied to remove noise signal and the spot detection feature was used to count cells.

Statistical methods

The statistical analysis of the cFos imaging experiments matched that used in Experiment 10. The cFos density results were analysed using a one-way ANOVA to investigate whether the hM3D(Gq)-CNO group differed from each control group (GFP-CNO, GFP-Saline and hM3D(Gq)-Saline). Following this, the control groups were pooled for further analysis as there was no statistically significant difference between the control groups as determined by one-way ANOVA for the PV cFos positive cell density: $F(2,11) = 3.02$, $p > .05$). In order to further normalise the data, a difference measure was calculated as the cFos positive cell density in an uninfected barrel cortex ROI minus the cFos positive cell density if an infected barrel cortex ROI. This difference was calculated for each subject in order to reduce individual variability (e.g., due to differences in tissue staining). For the analysis of the non-PV cFos positive difference measure, the control groups were initially analysed separately and then pooled as there was no significant differences between the difference measure between groups GFP-CNO, GFP-Saline and hM3D(Gq)-Saline: $F(2,11) = .12$, $p > .05$. A repeated-measures analysis of variance with appropriate factors and Bonferroni corrected post-hoc tests were used.

4.4.4. Results and Discussion

PV-cre mice received bilateral DREADD viral injections and immunohistological analyses confirmed the selective expression of Gq-

DREADD in PV neurons. First, the relative proportion of PV interneurons in layer 4 of the barrel area was assessed by a nuclear stain and PV immunofluorescence. A mean of 3521.87 ± 178.12 cells/mm^2 were stained positive for DAPI and from these, a mean of 131.25 ± 6.25 cells/mm^2 were stained positive for PV. This indicates that 3.73 ± 0.01% of the total DAPI stained cells were stained positive for PV, which is consistent with previous research (Lefort, Tomm, Sarria, & Petersen, 2009; Yang et al., 2017). Second, the cell type specificity of the DREADD expression was assessed. Within the injection sites, 91.67 ± 1.17% of PV positive cells expressed mCherry, indicating a high rate of viral infection and DREADD expression (Figure 19A).

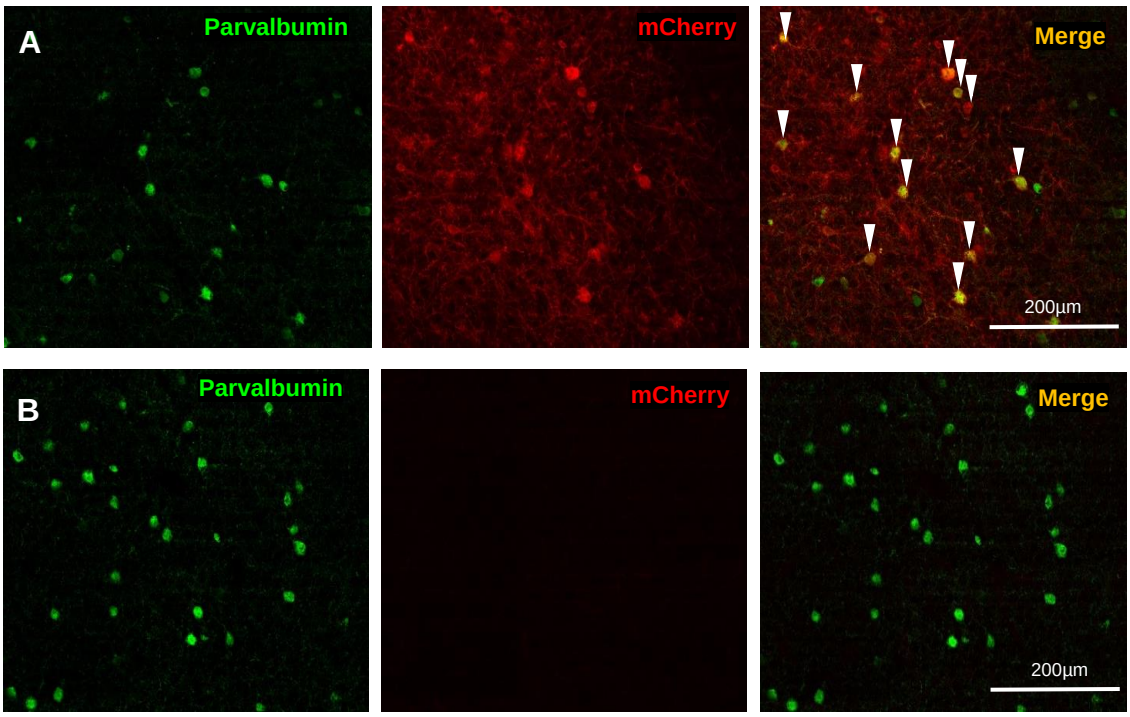


Figure 19. Cell type specificity of DREADD expression in mouse barrel cortex. Representative confocal images of tangential sections of barrel cortex from PV-cre mice injected with AAV-floxed-hM3D(Gq)-mCherry. PV positive neurons shown in green, DREADD expression shown in red and a merge shown in yellow. (A) Expression of DREADD and PV within the intracranial injection site. (B) Expression of DREADD and PV outside the intracranial injection site but still within the barrel cortex. White arrowheads indicate colocalization between channels.

In order to estimate whether the DREADD infected cells had been activated by CNO, the immediate early gene cFos was used to indicate recent neuronal activity. The spread of the virus was confirmed (Figure 20A-B) and the images analysed for colocalization of cFos and the viral vector (Figure 20C-D). Visual inspection of Figure 20C-D indicated that there was high cFos expression in cells infected with DREADD that were activated by CNO and low expression in the PV cells infected with GFP.

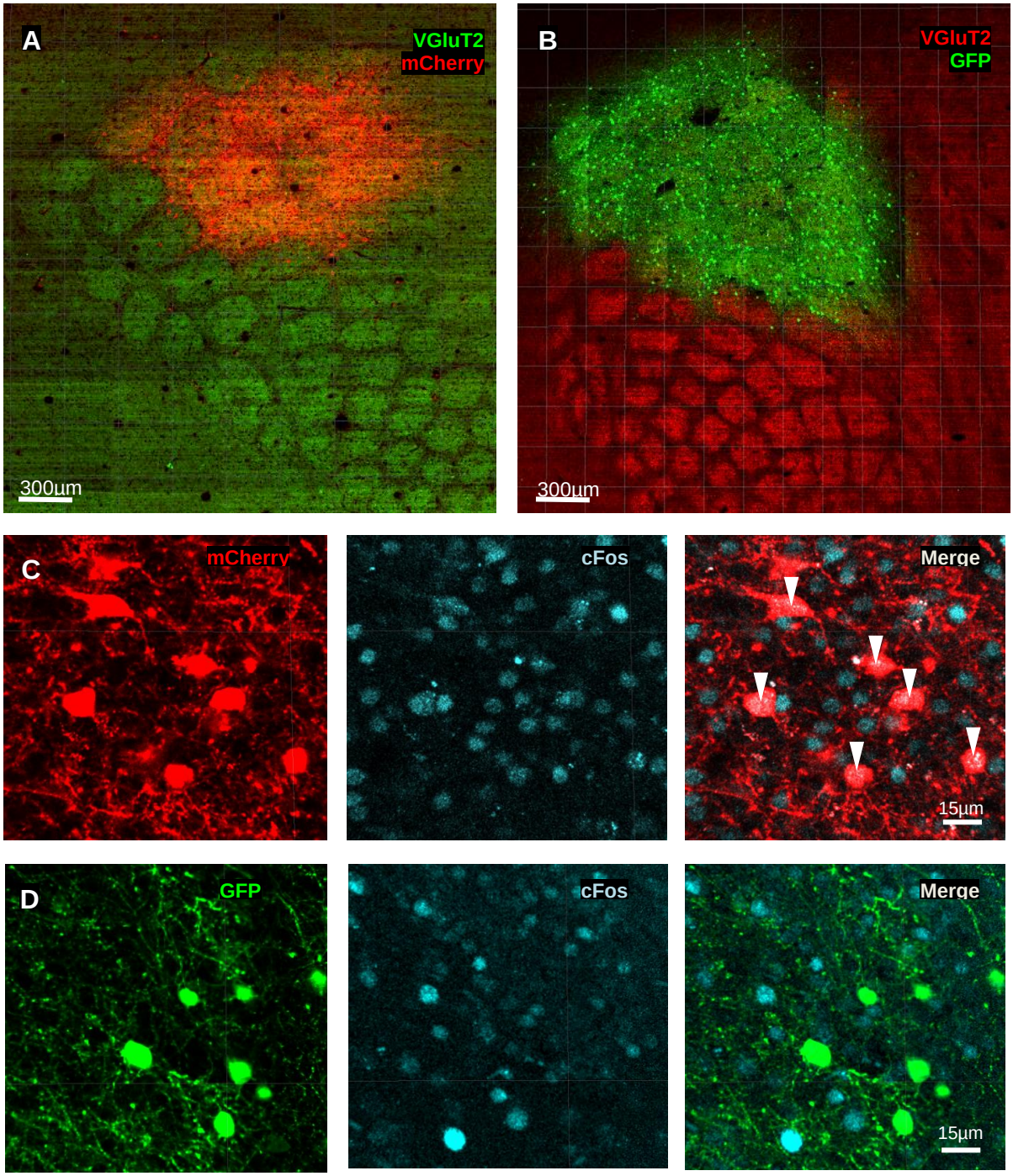


Figure 20. Validation of chemogenetic activation of barrel cortex PV neurons. PV-Cre mice were injected with (A) AAV-hSyn-DIO-hM3D(Gq)-mCherry and (B) GFP-Flex. (C) Representative image of excitatory DREADD (Gq) mCherry viral reporter expression (red) and cFos immunoreactivity (blue).(D) Representative image of GFP viral reporter expression (green) and cFos immunoreactivity (blue). White arrowheads indicate double-positive neurons.

Statistical analysis of the cFos positive cell count in PV cells confirmed that there was a significant effect of group, $F(3, 18) = 13.98$, $p < .001$, $\eta_p^2 = .75$. Additional analysis of the PV cells cFos count indicated that the cell density was significantly higher in the hM3D(Gq)-CNO group ($M = 246.35$, $SEM = 37.58$) compared to the GFP-CNO ($M = 18.75$, $SEM = 32.59$, $p < .001$), GFP-Saline ($M = 21.87$, $SEM = 32.59$, $p < .001$) and hM3D(Gq)-Saline ($M = 85.93$, $SEM = 32.59$, $p < .01$) group. There was no statistically significant difference between the texture and odour hM3D(Gq)-CNO groups ($M = 246.35$, $SEM = 37.58$ and $M = 275.00$, $SEM = 35.50$; $p > .05$).

Statistical analysis of the pooled cFos positive cell count in PV cells confirmed that there was a significant effect of treatment group, $F(2, 18) = 29.56$, $p < .001$, $\eta_p^2 = .76$ (Figure 21). Additional analysis of the PV cells cFos count indicated that the cell density was significantly higher in the texture discrimination hM3D(Gq)-CNO group ($M = 246.35$, $SEM = 37.58$) compared to the combined control group ($M = 42.18$, $SEM = 21.13$); $t(16) = 6.13$, $p < .001$, $d = 0.83$. Similarly, the odour discrimination hM3D(Gq)-CNO group ($M = 275.00$, $SEM = 13.01$) had significantly higher levels of cFos in PV cells compared to the combined control group; $t(13) = 7.55$, $p < .001$, $d = 0.90$. There was no statistically significant difference between the texture and odour hM3D(Gq)-CNO groups ($p > .05$).

These findings are consistent with the hypothesis that injection of CNO activates DREADDs and does not cause an increase in activity in the absence of DREADD. Moreover, cFos levels are not affected by DREADDs which are not activated by CNO.

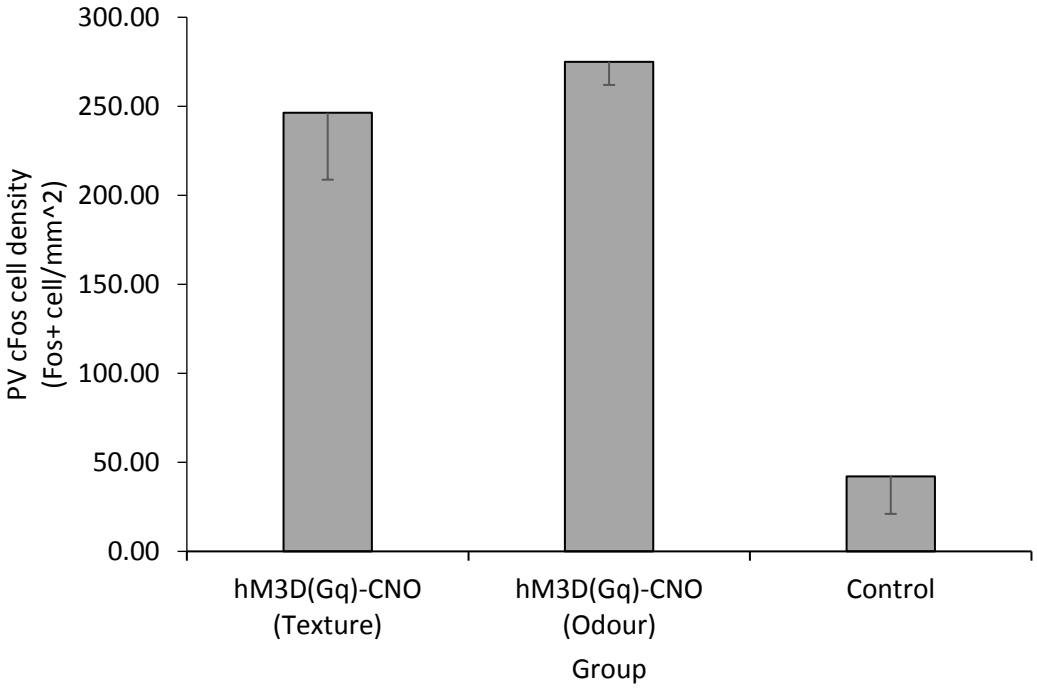


Figure 21. cFos positive PV cell density (Fos+ cells/mm2) -SEM for the hM3D(Gq)-CNO texture discrimination task group, the hM3D(Gq)-CNO odour discrimination task group and the combined control groups.

The next stage of analysis involved the evaluation of cFos in non-PV cells. This was calculated as the total cell density minus the PV cell density. Inspection of Figure 22 suggests that in the hM3D(Gq)-CNO groups, the non-PV cFos cell density is lower in the barrel cortex infected area compared to the barrel cortex uninfected area. This was not the case in the control groups where there is no difference in non-PV cFos cell density between the infected and uninfected barrel cortex ROIs. Analysis of the non-PV cFos positive cell density difference measure (cell density of the barrel cortex uninfected area minus the cell density of the barrel cortex infected area) revealed a significant effect of group, $F(3, 18) = 6.45$, $p < .01$, $\eta_p^2 = .58$. Additional analysis of the non-PV cFos difference measure indicated that the cell density was significantly higher in the hM3D(Gq)-CNO group (*M* =

613.02, *SEM* = 163.34) compared to the GFP-CNO (*M* = -73.43, *SEM* = 152.0, $p < .01$), GFP-Saline (*M* = -6.25, *SEM* = 152.04, $p < .01$) and hM3D(Gq)-Saline (*M* = -80.46, *SEM* = 152.04, $p < .01$) group. There was no statistically significant difference between the texture and odour hM3D(Gq)-CNO groups (*M* = 613.02, *SEM* = 163.34 and *M* = 756.25, *SEM* = 430.33; $p > .05$).

Analysis of the pooled non-PV cFos positive cell density difference measure revealed a significant effect of group, $F(2, 18) = 14.28$, $p < .001$, $\eta_p^2 = .79$ (Figure 22). Additional analysis of the non-PV cells' cFos difference measure indicated that this was significantly greater in the texture discrimination hM3D(Gq)-CNO group (*M* = 613.02, *SEM* = 163.34) compared to the control groups (*M* = -53.38, *SEM* = 214.79); $t(16) = 4.66$, $p < .001$, $d = 0.75$. Similarly, the odour discrimination hM3D(Gq)-CNO group (*M* = 756.25, *SEM* = 248.45) had a significantly higher difference measure compared to the control groups; $t(13) = 4.82$, $p < .001$, $d = 0.80$. There was no statistically significant difference between the texture and odour hM3D(Gq)-CNO groups ($p > .05$). This suggest that activation of barrel cortex PV cells in groups hM3D(Gq)-CNO caused the activity of cells in the infection area to decrease but had no effect on a non-infected surrounding area still within barrel cortex.

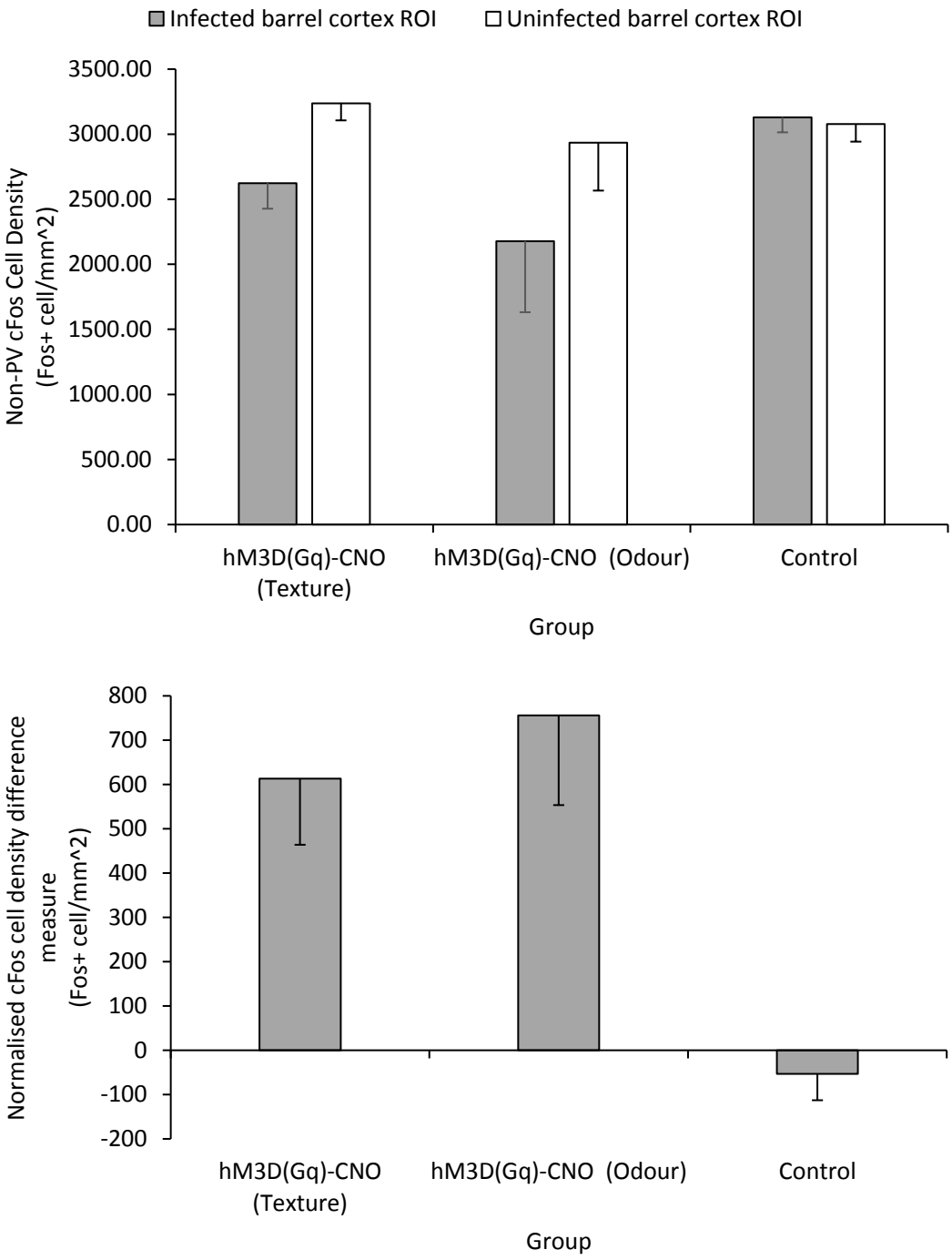


Figure 22. Cell density (Fos+ cells/mm^2) -SEM for non-PV cells in an infected and uninfected barrel cortex ROI for the hM3D(Gq)-CNO and combined control groups. The top panel shows the cell density (Fos+ cell/mm^2). The bottom panel shows the normalised cell density calculated as the uninfected barrel cortex ROI cell density minus the infected barrel cortex ROI cell density; this gives a difference value.

The proportion of the barrel cortex infected with the viral construct was also examined to evaluate whether the size of infection was correlated with performance on the discrimination task and to ensure that the groups were well matched. The analysis revealed that in the hM3D(Gq)-CNO (texture) group, a mean of 36.59 ± 4.81% of the barrel cortex was infected with DREADDs. Similarly, in the combined control group, 37.99 ± 3.59% of the barrel cortex was infected with the viral construct and in the hM3D(Gq)-CNO (odour) group, 51.53% ± 7.32% of the barrel cortex was infected with DREADDs. The proportion of the barrel cortex infected with the viral construct was not correlated with behavioural performance for any of the groups. It is important to note however, that in every case, the large barrels, corresponding to the long whiskers were infected. This suggests that the size of the infection area may not be as critical as the location of the DREADD injection for behavioural performance.

4.5. Concluding Remarks

The aim of the experiments presented in Chapter 4 was to establish whether decreasing neuronal activity in the barrel cortex had a selective effect on texture discrimination learning.

To test whether texture discrimination learning was disrupted when neurons in the barrel cortex were inhibited, Experiment 10 utilised the DREADD system to silence neurons. Experiment 10a established the methodology and Experiment 10b tested mice in the discrimination task. The results revealed that mice injected with DREADDs and CNO were unable to acquire the texture discrimination task, whereas an equivalently treated but different

cohort were able to acquire an odour discrimination task. This demonstrates that silencing neurons in barrel cortex results in a specific deficit in texture discrimination learning. Of note, the results of the individual group analysis revealed that the behavioural performance of group GFP-CNO was not significantly different from the hM3D(Gq)-CNO group. Although CNO alone was found not to have a behavioural effect in Chapter 3, Experiment 9 (*M* = 70.31%, *SEM* = 4.86), the findings of Experiment 10b indicate that CNO alone may have affected behavioural performance. Further analysis revealed that the behavioural score of group GFP-CNO differed significantly from chance, whereas the score of group hM3D(Gq)-CNO did not. As previously mentioned however, the absence of a significant effect is not sufficient evidence for conclusions to be made. Consequently, future studies utilising DREADDs should assess whether behavioural effects are present and utilise additional statistical analysis (e.g. Bayesian methods) to reveal whether CNO alone has any behavioural implications. Experiment 11 added to these findings by utilising the brain tissue from mice used in Experiment 10b and staining against immediate early gene cFos. The results revealed that the hM3D(Gq)-CNO group had a higher cFos cell density in PV neurons compared to each of the individual control groups as well as the pooled control group. This suggests that the inhibitory neurons had been recently active. Similarly, the cFos density in non-PV cells was also evaluated and the cFos density within and outside the infection spread was calculated. The results indicated that the hM3D(Gq)-CNO group had a larger difference in cell density between the two barrel cortex ROIs (infected and uninfected areas) compared to the individual and pooled control groups. Together these

results indicate that the texture discrimination task is sensitive to alterations in barrel cortex neuronal activity.

In sum, the findings from Chapter 4 reveal that texture discrimination, using the procedure developed in Chapter 3, is disrupted when neurons in the barrel cortex are silenced. Experiment 10a demonstrates that increasing activity in PV interneurons causes a decrease in evoked potentials in neighbouring pyramidal cells in the mouse barrel cortex *in vivo*. Experiment 10b revealed that animals in the DREADD and CNO group, undertaking the texture variant of the task, were unable to acquire the discrimination rule whereas those undertaking the odour variant were unaffected. These results suggest that reduction of neuronal activity in layer 4, barrel cortex, has a specific effect as opposed to a global effect on learning. It is noted that the results from the odour variant of the task did not include the CNO and DREADD control groups therefore conclusions regarding the effect of these manipulations on odour learning should be made with caution. Moreover, conclusions based on the failure to find an effect, especially when the sample sizes are small, should be drawn with caution. The findings from Experiment 11 further validate the chemogenetic technique and demonstrate that cFos levels were affected by DREADDs. These results provide the basis for future studies to examine the role of synaptic changes in the barrel cortex on a behavioural task known to be both whisker-dependent and barrel-cortex dependent. For example, the findings presented in these experiments demonstrate that the discrimination learning task could be employed to assess the functional role of dendritic spine plasticity in the barrel cortex.

This possibility will be developed in the future directions of the general discussion presented in Chapter 5.

Chapter 5. General Discussion

5.1. Overall Summary

The research presented in this thesis examined tactile discrimination learning in mice. The experiments addressed the need for a robust behavioural procedure where learning is rapid and whisker based, in order to examine the functional role of the barrel cortex in texture learning. In order to investigate learning related changes in the barrel cortex, an ethologically relevant tactile discrimination was developed. Chapter 2 focused on the development of a behavioural procedure modelled on novel object recognition (NOR), which has been extensively used to assess memory for objects primarily discriminated on the basis of their visual features. The procedure involved 3-D printed plates with different textures that were presented in an arena illuminated with red light. Mice showed a preference for a novel plate over a different plate that had been presented 24 hours before. However, the procedure produced a relatively modest preference and required a large sample size. Chapter 3 adopted a quite different procedure in which mice were required to forage in two pots with distinctive outer surfaces in order to retrieve a Coco pop reward in a chamber dimly illuminated with red light. Mice readily learned this discrimination and an equivalent odour discrimination. While the discrimination based on textures was disrupted by whisker trimming the odour discrimination was not. Chapter 4 showed that when neurons in the barrel cortex were silenced using the chemogenetic DREADD system mice were unable to learn the texture discrimination but not the odour discrimination. Further analysis of the tissue

from these mice revealed that cFos levels were also affected by the DREADD manipulations. In this concluding chapter, a summary of the main findings from Chapters 2-4 will be presented followed by a discussion on the implications of the work. The final section of Chapter 5 will focus on the future research directions from both a Psychology and Neuroscience perspective.

5.2. Summary of results

5.2.1. The Novel Object Recognition Task

Chapter 2 describes a series of experiments which adapted the NOR task procedure developed by Wu et al., (2013). These authors modified the original NOR task by using sandpaper as textured stimuli as opposed to different shaped/coloured objects. It was argued that mice were required to use the somatosensory system to discriminate between these stimuli on the basis of a series of subsidiary manipulations. For example, when the textures were covered with cling film disrupted mice no longer showed a preference. This result was taken to show that the preference was based on texture given the assumption that there visual characteristics were still available. Chapter 2 consisted of three experiments which detail the development of a texture based NOR procedures. The novel innovations in this chapter included the use of 3-D printed tactile plates as opposed to sandpaper. The tactile plates were deemed advantageous because they can be reproduced easily and are made from the same material. The results from Experiment 1a and 1b suggested that positioning the plates in the corner of the arena rather than in the centre resulted in an increase in contact times, but this increase

did not result in a preference for a novel plate. In Experiments 2 and 3, the duration of exposure to the plate was reduced from 10 minutes to 5 minutes. Under these conditions, mice had a significant preference for the novel plate indicating that they had a memory of the plate that had been presented 24 hours earlier. The successful experiments presented in Chapter 2 show that the 3-D generated stimuli can be discriminated, but the preference for a novel texture was not marked and required a large number of mice in order to obtain statistically significant results. Chapter 3 made use of aspects of the technical innovations from Chapter 2, but a quite different training procedure with the hope of generating a behavioural effect that could be used to study the functional significance of plasticity in the barrel cortex.

5.2.2. Discrimination Learning with Textures

The texture discrimination learning procedure was based on the original research reported by Birrell and Brown (2000). The task required mice to discriminate between bowls that differed in the texture of their outer surfaces. One of the bowls contained food and the other did not, and the food-deprived mice foraged in dim red light in the two textured bowls. The results of Experiment 4 demonstrate that mice are able to learn which of the two textured bowls to dig in to retrieve food within a 60-minute session delivered on Day 1 of training. The long-term nature of learning was evident from the fact that mice that received the same contingencies on Day 2 of training performed more accurately than when the contingencies were reversed. In the same way, Experiment 5 showed that mice also retained overnight a discrimination based on the odour of the bowls. In order to test whether the

learning was whisker dependent, a different group of mice either had their full set of whiskers trimmed or light whisker pad stimulation. Experiment 6 revealed that whisker-trimmed mice were unable to acquire the texture discrimination and Experiment 7 showed that whisker-trimmed mice were able to learn the odour discrimination. The fact that the whisker manipulation had a specific effect on texture learning provided converging evidence that the texture discrimination was based on the texture of the bowls as detected by the whisker system. As previously mentioned however, caution should be taken when drawing conclusions from a lack of effect. Further analysis using tests of equivalence or Bayesian statistics can be undertaken to assess the strength of this result. Finally, Chapter 3 established that the texture discrimination was robust and useful in the context of understanding the function of the barrel cortex. It did so by showing that mice with head plates (Experiment 8) and those who had just received an intraperitoneal injection (Experiment 9) could acquire the texture discrimination. Chapter 3 demonstrated that a modified version of the Birrell and Brown (2000) procedure was whisker-dependent and that reliable learning effects are shown with a small number of mice.

5.2.3. The Barrel Cortex and Discrimination Learning

Chapter 4 aimed to assess the role of the barrel cortex in the discrimination learning procedures developed in Chapter 3. Neurons in the barrel cortex were silenced using DREADDs. Excitatory DREADDs were injected into the barrel cortex, infecting only inhibitory PV neurons. Experiment 10a revealed that activation of PV cells using CNO caused a reduction in the average

evoked responses of nearby excitatory cells. Experiment 10b utilised the excitatory DREADD to silence neurons in the barrel cortex via activation of inhibitory PV neurons. As in Chapter 3, two experimental groups were tested in the discrimination learning procedures using textures and odours. Mice injected with DREADD and CNO were unable to learn the discrimination involving textures but could learn the discrimination involving odours. These results clearly implicate the barrel cortex in texture discrimination learning, however, it is noted that control groups were not utilised in the odour discrimination experiment and the conclusions drawn from this group should be made with caution as they are based on a lack of effect. Experiment 11 further explored these results by staining the barrel cortex tissue against the immediate early gene cFos. cFos is considered a marker of neuronal activity and the findings indicate that mice injected with DREADDs and CNO had a higher cFos cell density in PV cells compared to the control mice. Interestingly, the cFos in all other (non PV) cells was lower in these cases indicating that the PV cells may have had an inhibitory effect on cells within the infection area. Moreover, when comparing the cFos cell density of these non-PV cells within and outside the infection area, there was a greater difference in the experimental groups compared to the control groups. The experiments described in Chapter 4 confirm that the neuronal activity in the barrel cortex affects texture learning but not odour learning. This observation sets the stage for an analysis of the role of synaptic plasticity mechanisms within the barrel cortex in texture discrimination learning.

5.3. Implications of the results

The development of a procedure in which texture discrimination learning is rapid, occurs in freely moving mice, and is whisker-dependent is potentially useful for future studies investigating the neural basis of learning and memory. The task resolves some of the current limitations with the use of head-fixed behavioural setups and allows for the investigation of more ethologically relevant forms of learning. Moreover, the results presented in Chapter 4 indicate a critical role for the barrel cortex in the texture discrimination variant of the task. These findings are consistent with studies that have manipulated activity in the barrel cortex and found it is essential for learning a whisker based behavioural task (Hutson & Masterton, 1986; Miyashita & Feldman, 2012; O'Connor et al., 2010). More specifically, Experiment 10b showed that exciting PV neurons in the barrel cortex reduced the animals' ability to complete the texture discrimination task. This finding compliments the work by Sachidhanandam, Sermet, and Petersen (2016) who found that optogenetic inhibition of PV neurons resulted in an increase in behavioural performance in a tactile detection task. The authors propose that a reduction in PV neuron activity allows nearby excitatory cells to increase their firing rate on hit trials. The results are also consistent with recent work by Chaudhary and Rema (2018) who found that unilateral lesion of the barrel cortex resulted in drastic deficits in rats performing the gap crossing task. Their findings are in line with the anecdotal data described in Section 4.3.2 regarding the two excluded hM3D(Gq)-CNO (texture) cases where fluorescence from the intracranial injections was only detected in one hemisphere but performance on the texture task was still impaired. Future

studies could test one implication of these results. Namely, whether sensory input from one set of whiskers or the presence of one intact barrel cortex is insufficient for texture-based learning in the discrimination task.

However, the results from Chapter 4 contrast with the findings of a recent study by Hong et al. (2018) who showed that both optogenetic inactivation and lesioning of the barrel cortex caused only a transient deficit in performance on a whisker based pole detection task. Critically, this impaired performance was fully recovered in a subsequent session, which suggests that the barrel cortex is not required for success at the task. As the studies presented in Chapter 4 only test mice over two consecutive days, it is not possible to know whether the mice would have eventually acquired the discrimination rule if given further training. It is important to note, however, that the task used by Hong et al. (2018) is a passive task whereby the animal is head-fixed and only able to use a single whisker. As a result, the effects of manipulating the barrel cortex are not comparable to a task where the animal is free to move and use its full set of whiskers. Further, the task undertaken by Hong et al. (2018) requires many weeks of training (animals reached criterion in an average of 48 sessions) and includes a punishment for incorrect responses to no-go trials (consisting of a time out period with white noise). This differs critically with the present study whereby animals learn the discrimination rule in a single one hour session and do not receive an aversive stimulus when digging in the incorrect bowl. Hong et al. (2018) also found that the optogenetic manipulations caused a decrease in overall whisker movement and a reduction in overall responses. This suggests that the inactivation procedure may have also disrupted the physical process by

which mice detect objects as opposed to the result being due to the silencing of the brain area.

Although initially contrasting, the results presented in Chapter 4, in combination with those by Hong et al. (2018) may also be explained by the view that the cortex is involved differently depending on the demands of the task. For example, research indicates that discrimination of complex sensory features (e.g. object shape, texture) is dependent on the cortex whereas basic detection and discrimination (e.g. presence/absence of pole, passive touch) does not require the cortex (Chen, Margolis, et al., 2015; Krupa, Wiest, Shuler, Laubach, & Nicolelis, 2004; LaMotte & Mountcastle, 1979; Porter, Rosenthal, Ranasinghe, & Kilgard, 2011; Prusky & Douglas, 2004; Romo, Hernandez, Zainos, Lemus, & Brody, 2002; Zainos et al., 1997). This was also shown in a study by Chen, Carta, Soldado-Magraner, Schneider, and Helmchen (2013) who used two-photon calcium imaging to monitor neuronal activity in S1 neurons projecting to either S2 or M1. The study used a texture discrimination task (using sandpaper) and a pole-detection task to investigate whether the task requirements elicited differences in neuronal activity. The results revealed that the texture discrimination task utilised S1 neurons projecting to S2 whereas the pole detection task utilised S1 neurons projecting to M1. The activity from S1 is therefore selectively routed depending on the sensory processing requirements; with more complex tasks recruiting S1 to S2 neurons. An explanation for these distinctions may be taken from the primate research whereby ipsilateral cortico-cortical pathways emerging from S1 can belong to either the dorsal or ventral stream (Gardner, 2008). In primates, research indicates that processes relating to

motor planning, exploration and coordination relies on the dorsal stream (projections from S1 to M1, M2, PFC and PPC) whereas processes such as object recognition, multimodal integration and memory utilise the ventral stream (projections from S1 to S2, parietal ventral and parietal rhinal cortex)(Fox, 2018; Nishimura, Sawatari, Takemoto, & Song, 2015). Studies showing that barrel cortex is indispensable for more complex tasks may be explained by these pathways as S1 relays information from several whiskers to ventral areas which integrate the information and provide a route to areas such as the hippocampus (Chen, Margolis, et al., 2015; Krupa et al., 2004). These conclusions could be explored further using the texture discrimination task described in Chapter 3 alongside optical imaging approaches (e.g., Sych, Chernysheva, Sumanovski & Helmchen, 2019) (see Chapter 5.4 for future experiments).

It is important to note the limitations of the DREADD manipulations carried out in Chapter 4. First, although the rate of transfection was high (91.67 ± 1.17%), 8.3% of PV cells within the injection site were not infected with the viral construct. This leaves open the possibility that a subset of pyramidal neurons may not have been inhibited, which could have meant that the manipulation was not fully effective in silencing neurons in the barrel cortex. This might have resulted in a residual capacity for discrimination learning, but it did not. Furthermore, studies have shown that a single PV interneuron contacts nearly every local pyramidal neuron spanning multiple layers (Hu, Gan, & Jonas, 2014; Packer & Yuste, 2011). It is, therefore, likely that activation of a large subset of PV cells would have caused a widespread inhibition of pyramidal cells activity, which would compensate for the small

number of uninfected PV cells. Second, there is also a possibility that exciting PV cells in the barrel cortex could cause an increase in excitatory cell activity via disinhibition. Although vasoactive intestinal polypeptide (VIP) interneurons are considered the main disinhibitory cells, studies have shown that PV cells can converge on somatostatin and VIP interneurons (Figure 23) (Karnani, Agetsuma, & Yuste, 2014; Kepecs & Fishell, 2014). Consequently, increasing activity of PV cells would decrease activity of these inhibitory neurons, which in turn would favour excitation of their target cells (Kuhlman et al., 2013; Letzkus et al., 2011; Wolff et al., 2014). Studies investigating disinhibition have shown that it facilitates sensory processing and learning (Courtin et al., 2014; Lee, Kruglikov, Huang, Fishell, & Rudy, 2013; Poorthuis, Enke, & Letzkus, 2014; Wolff et al., 2014). This contrasts with the behavioural results presented in Experiment 10b where learning was impaired. Consequently, it is unlikely that disinhibition was exerting a strong effect on the overall circuit and behavioural output.

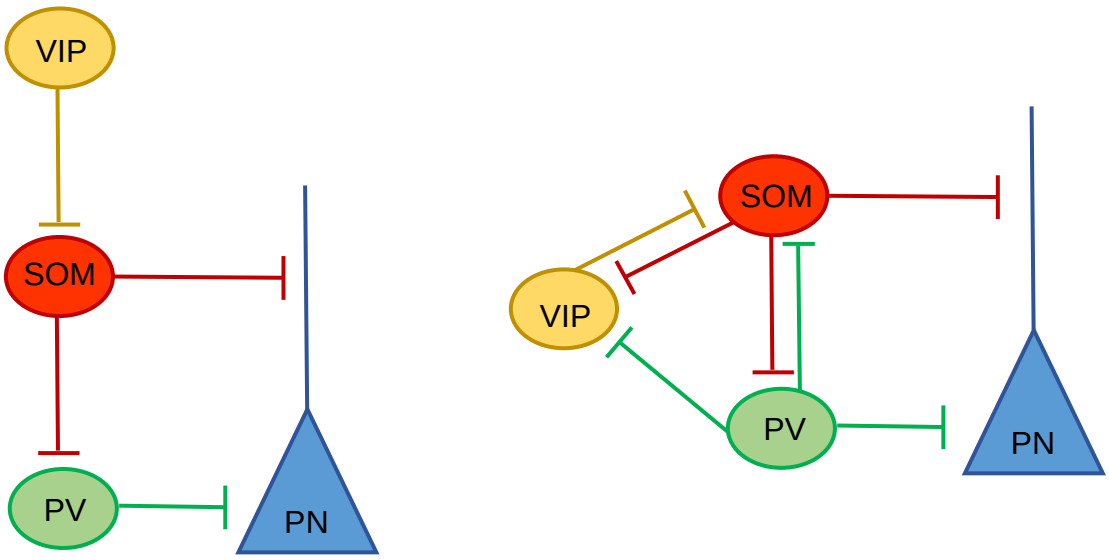


Figure 23. Schematic illustrating the interneuron patterns for disinhibition in the cortex. Left indicates the traditional hierarchical connectivity of interneurons whereas the right depicts a model based on interneuron cooperativity. Figure replicated from Artinian and Lacaille (2018).

Another technical caveat to consider is that in a small subset of cases, layer specificity of the viral expression was not achieved. Research shows that in S1, the largest population of PV interneurons are found in layer 4 and that they are virtually absent in layer 1 (Tremblay et al., 2016). Consistent with this, a small number of cases revealed some fluorescence in deeper layers which suggests that the behavioural effects may have been due to excitation of PV cells in multiple layers. Nonetheless, PV cells are the dominant inhibitory systems which mainly target thalamocortical axons in layer 4 (Rudy, Fishell, Lee, & Hjerling-Leffler, 2011). As all cases demonstrated predominant and widespread fluorescence in layer 4, it can be concluded that enhancing activity using the DREADD system would likely have created powerful inhibition of target neurons. This is consistent with studies that have shown that activation of PV interneurons significantly reduces firing of principal neurons (Atallah, Bruns, Carandini, & Scanziani, 2012) and limits excitatory drive (Khoshkhoo, Vogt, & Sohal, 2017) further strengthening the view that PV cells are key contributors to the control of excitation/inhibition balance in the cortex.

As mentioned earlier, recent studies have also highlighted some limitations with the use of CNO in DREADD studies. The main concern being that CNO is metabolized to clozapine, which is instead responsible for activating the DREADDs (Gomez et al., 2017). These findings are important as clozapine

is an antipsychotic drug with activity at multiple endogenous receptors (Ashby Jr & Wang, 1996; Selent, López, Sanz, & Pastor, 2008). If clozapine is present at concentrations high enough to affect endogenous receptors there may be additional effects as well as those mediated by DREADDs (MacLaren et al., 2016; Mahler & Aston-Jones, 2018). A number of alternative methods of DREADD activation have been proposed which include using subthreshold doses of clozapine or the newly developed DREADD agonists compound 21 and perlapine (Chen, Choo, et al., 2015; Gomez et al., 2017). Although promising, these compounds were not used in the present study as their effects *in vivo* had not been fully characterised. The current work therefore used a low concentration of CNO in combination with careful experimental design where a full range of control groups were in place to highlight any possible behavioural effects. This included a non-DREADD expressing group, which received intraperitoneal injection of CNO at test (group GFP-CNO). The results of Experiment 10a revealed no change in the mean evoked response from the mouse injected with CNO alone, however, Experiment 10b indicated that the behavioural score of group GFP-CNO was not significantly different to group hM3D(Gq)-CNO. This raises the possibility that CNO was impacting behavioural performance in the absence of DREADDs. However, unlike in group hM3D(Gq)-CNO, the behavioural performance of group GFP-CNO was significantly higher than chance and did not differ from the other control groups (GFP-Saline and hM3D(Gq)-Saline). Nevertheless, future work using DREADDs should employ additional statistical analysis to establish whether CNO alone has any behavioural implications. These findings add to the current literature, aimed to investigate

the presence of DREADD-independent behavioural effects of CNO (Anaclet et al., 2018; Mahler et al., 2014; Robinson et al., 2014; Sano et al., 2014; Stachniak et al., 2014; Zhu et al., 2014) and highlight the importance of using appropriate control groups. The cFos findings described in Experiment 11 also compliment the recent work by Jendryka et al. (2019) who reported that CNO is present at sufficient levels for DREADD activation.

It is also important to note the limitations with the immediate early gene cFos as a proxy for neuronal activity. The induction of cFos is mainly due to an influx of calcium, due to either voltage-sensitive calcium channels or glutamate binding to the NMDA receptor-calcium complex (Ghosh, Ginty, Bading, & Greenberg, 1994). These activate a number of calcium-dependent kinase cascades which activate transcription factors and induce the induction of target genes (Bito, Deisseroth, & Tsien, 1997). It is generally accepted that if cFos were a marker of all neuronal activity (due solely to depolarization), it would be heavily present under baseline conditions. Research has shown that this is not the case and that cFos is only found to be induced following significant alterations in afferent inputs and/or external stimuli (Luckman, Dyball, & Leng, 1994). Research also indicates that within the cortex, whisker stimulation causes an increase in cFos expression in excitatory pyramidal neurons but not in inhibitory PV cells (Chaudhuri, 1997; Filipkowski et al., 2000; Staiger et al., 2002). This suggests that the findings described in Experiment 11, whereby the hM3D(Gq)-CNO group displayed a lower cFos density in non-PV cells compared to the combined control group, were due to PV interneurons exerting their effect on excitatory cells. Given the multiple transduction pathways for cFos, it is not possible in the current

study, to establish a causal link between gene expression and learning as it is not possible to rule out that the induction of cFos was due to the sensory stimulus alone. Studies have addressed this issue by combining cFos with DREADDs/optogenetics to manipulate recently active populations of neurons (Liu et al., 2012). These studies show that memory acquisition can be manipulated by reactivation of the specific neuronal ensemble previously associated with the pre-stored memory (Garner et al., 2012; Liu et al., 2012; Ohkawa et al., 2015; Ramirez et al., 2013). This has also been shown in drosophila where blocking or activating the cFos-positive populations results in an inhibition or induction of memory-associated behaviours (Miyashita, Kikuchi, Horiuchi, & Saitoe, 2018). Future work could follow this experimental design using the texture discrimination procedure (this is described in more detail in Section 5.4).

5.4. Future Directions

The development of a behavioural assay for whisker-dependent texture discrimination learning in freely moving rodents allows for a variety of future experiments. As mentioned earlier, the idea that the complexity of learning may be related to the sensory processing could be further investigated. The texture discrimination learning procedure differs from popular head fixed tasks in that it requires the mice to utilise both full body, head and whisker movements. The texture task cannot be learned in the absence of whiskers and future work could investigate the multi-whisker patterns used to discriminate the textured bowls. Moreover, these patterns can be combined with analysis of synaptic plasticity in the barrel cortex. As described in

Section 1.5, the activity and dynamics of dendritic spines are thought to play an important role in learning and memory. The next steps are to longitudinally monitor these structures using *in vivo* two-photon microscopy. Repetitive imaging of the same spines at specific time points, prior to, during and post learning, would further elucidate their role in learning. Preliminary work has revealed that the task is not affected by a regular anaesthetic regime and that mice are able to perform normally with some minor alterations in the task procedure. This investigation would further reveal the neural underpinning of texture learning with the potential to manipulate specific spines at discrete time points.

The observation reported in Section 3.6 that mice do not successively sample both bowls prior to making a decision indicates that the mice are comparing the current bowl to a stored mental template of the two bowls. As mentioned briefly in Section 5.3, it would be interesting to selectively target the stored representation with the view to decreasing activity in this specific subset of neurons using a double transgenic approach. For example, as in Garner et al. (2012), hM3Dq^{fos} double transgenic mice could be used to selectively activate a specific neuronal ensemble. hM3Dq^{fos} mice carry two transgenes: the first transgene is under the control of a cFos promoter and expresses the tetracycline transcriptional activator (tTA). The activity of tTA can be inhibited by the antibiotic doxycycline (Dox). The second transgene allows expression of hM3Dq under the tet operator (tetO), which is activated upon binding of tTA. Removal of Dox enables tTA to bind to a Tet operator to allow transcription. hM3Dq^{fos} double transgenic mice could be trained in the discrimination task and using Dox, one could temporally control the

expression of DREADDs in a subset of neurons sufficiently active to drive the cFos promoter (Matsuo, Reijmers, & Mayford, 2008; Reijmers, Perkins, Matsuo, & Mayford, 2007). This approach could be used at various time points during the discrimination task to artificially reactivate the specific subset of neurons associated with the discrimination learning. Equally, one could also use the hM4D(Gi) DREADD to artificially silence these neurons to observe whether the learning is impacted. This technique has been mainly used in studies of fear conditioning to show that reactivation of a specific neuronal network is sufficient to retrieve a memory representation (for review, see Asok, Leroy, Rayman, & Kandel, 2019). Chemogenetic silencing of the neuronal network tagged during acquisition of the discrimination rule, may inhibit recall of the discrimination rule. Enhancing activity on the other hand, may improve performance when required to learn a new discrimination rule.

From a behavioural neuroscience perspective, the discrimination learning procedure might also prove useful for understanding a wide range of phenomena. For example, the 3-D printing technology could be developed to create an array of digging bowls which differ in their textured surface. The textured stimuli could be created so that they differ in complexity with some being very similar and others very different. Studies could use these stimuli to investigate whether repeated exposure and/or training results in improvements in performance when discriminating between similar textured bowls (as tested in rats; Montuori & Honey, 2016). These experiments could be coupled with neural imaging investigations to observe potential differences in spine activity and learning. Future studies could train the

animals in a series of increasingly difficult texture discriminations whilst longitudinally imaging the brain *in vivo* to monitor spine activity over the different stages of discrimination learning. These experiments could reveal whether different ensembles of spines are recruited depending on the level of difficulty required in the task. Moreover, the task could be further developed to investigate the experience-dependent neural changes across different cortical areas. The role of other brain regions in texture-learning is poorly understood and studies could utilise the task to explore connectivity and interactions within different brain networks during learning (Sych et al., 2019).

5.5. General Conclusions

There has been a longstanding interest in how tactile information is processed and coded in the brain. The barrel cortex serves as an ideal structure for investigating this due to the ability to manipulate the peripheral stimuli. Studies have assessed a large range of processes ranging from the behaviour of animals and the physical properties of whisker movement, to the cortical and subcortical processing of sensory information.

This thesis sought to resolve an important problem present with currently available behavioural tasks: the lack of sensory specificity in the stimuli used in procedures when mice are free to move around. The experiments described in this thesis demonstrate that mice can retain a memory of tactile information over a 24-hour period, both in a preference to explore a novel texture and in a rewarded texture discrimination. The experiments also demonstrate that the rewarded texture discrimination is both whisker and

barrel cortex dependent. These observations provide a foundation for future investigation into both the behavioural and neural basis of texture learning.

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