Effects of Pregabalin and Environmental Familiarity upon Hippocampal Theta

MIRANDA MICHELLE HINES

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Durham University, Department of Psychology

Miranda Michelle Hines

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5-HT	5-hydroxytryptamine (serotonin)
ANOVA	analysis of variance
BIS	behavioural inhibition system
BST	bed nuclei of the stria terminals
CDP	chlordiazepoxide
CA	cornu ammonis
cm	centimetre
DG	dentate gyrus
DPN	Diabetic peripheral neuropathy
EEG	Electroencephalogram
EPM	Elevated plus maze
GABA	γ-aminobutyric acid
GAD	Generalised anxiety disorder
HPA	Hypothalamic-pituitary-adrenal axis
HCN	Hyperpolarisation-activated cyclic nucleotide gated
HAM-A	Hamilton anxiety scale
Hz	Hertz
ISAP	192 IgG-saporin
KA	Kainic acid
f_0	Intercept (of the theta-frequency-to-speed relationship)
ITI	Inter-trial-interval
i.p.	Intraperitoneal (injection)
i.m.	Intramuscular (injection)

kg	Kilograms
kHz	kilohertz
LEC	Lateral entorhinal cortex
LIA	Large irregular activity
mGluR	Metabotropic glutamate receptor
MS/DBB	Medial septum and diagonal band of Broca
MEC	Medial entorhinal cortex
MEG	Magnetoencephalography
μm	Microns
mg	Milligram
ml	Millilitre
ms	Millisecond
mm	Millimetre
μV	Microvolts
μs	Microseconds
NMDA	*N*-methyl-D-aspartate
nPO	Nucleus pontis oralis
OF	Open field
PIL	Personal licence
PTSD	Post-traumatic stress disorder
PHN	Post-herpetic neuralgia
PGB	Pregabalin
REM	Rapid eye movement
RPO	Rostral pontine region
SAL	saline
SSRI	Selective serotonin reuptake inhibitor

SNRI	Serotonin/norepinephrine reuptake inhibitor
<β>	Slope (of the theta-frequency-to-speed relationship
SIA	Small irregular activity
SEM	Standard error mean
s.c.	Subcutaneous (injection)
SUM	Supramammilary nucleus

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Abstract

The hippocampal formation is heavily involved in two major functions; spatial cognition and anxiety. Septo-hippocampal theta (5-10 Hz) is a very prominent oscillation in rodents and is believed to be a physiological substrate that plays a major role in processing spatial cognition and anxiety. Hippocampal theta can be recorded from awake rodents during voluntary movement (often called Type 1 theta) and during alert immobility (called Type 2 theta). Hippocampal theta frequency changes have been implicated in anxiety, spatial cognition, and environmental novelty (Jeewajee et al, 2008b). Theta frequency increases broadly linearly with running speed, and a computational model (Burgess, 2008) has theorised that the slope of this relationship relates to type 1 theta and spatial cognition, while its y intercept (i.e. at 0cm/s) relates to type 2 theta and thus potentially variables such as arousal and anxiety. The model predicts that instantaneous theta frequency is the sum of these two dissociable components.

In the present thesis, I examined the effects the anxiolytic pregabalin has on intercept and slope of the running-speed-to-theta-frequency relationship in freely moving rats. I find that pregabalin reduces intercept, further generalising the observation in Wells et al. (2013), whereby two known anxiolytic drugs, and one putative anxiolytic drug, reduced theta intercept. Additionally, this study examined the effects of environmental familiarisation upon the slope component of the theta-frequency-to-speed relationship. The Burgess 2008 model predicts that theta slope is reduced in environmental novelty and increases as the environment becomes familiar. I observed that slope was indeed flatter in novelty and steeper in later exposures. In summary, consistent with the predictions of Burgess 2008 and Wells et al 2013, the anxiolytic pregabalin reduced theta intercept, while environmental familiarisation increased theta slope.

1 Overview of Research Question

The experiments presented within this thesis are related to two aspects of hippocampal function. The hippocampal formation is believed to play key roles in two distinct brain functions. First, it is believed to play a role in spatial cognition and spatial memory (Tulving, 1983). Second, it is believed to play a role in the modulation of anxiety (Bannerman et al., 2004; Gray & McNaughton, 2003; Kjelstrup et al., 2002). This introduction will briefly explain how the hippocampal formation plays a role in spatial cognition as well as spatial learning and memory; along with the hippocampal formation's role in anxiety modulation. This introduction will also expand upon the notion that these two functions of the hippocampus (spatial cognition and anxiety) separately effect the shared physiological substrate of the septo-hippocampal theta when introduced to an anxiolytic drug and when familiarising a novel environment.

1.1 Hippocampus involvement in spatial learning and cognition

The hippocampal formation's involvement in spatial cognition and spatial memory can be traced to early laboratory rodent studies which demonstrated spatial learning during exploration without an explicit reward (Blodgett, 1929; Tolman, 1948). In 1929, Blodgett investigated the efficiency of maze learning in rats when unaccompanied by reward compared to maze learning when accompanied by reward. The author reported three main findings: (1) the rats that ran the maze under the 'non-reward' condition learned slower than the rats that ran the maze under the 'reward' conditioned; (2) the rats in the non-reward group showed significant improvement when a reward was introduced; and (3) during the 'non-reward' period of maze exploration, rats developed latent learning of the maze which manifested once a reward was introduced. In another study, rats demonstrated goal-directed actions based upon prior spatial knowledge (Spence, Bergmann, & Lippitt, 1950). Spence and colleagues had rats in two separate groups experience a T-maze where one group was water-

deprived with water as the goal whilst the other group was food-deprived with food as the goal. This studied demonstrated the rats' ability to navigate an environment without the need to 'practice.' These studies, which demonstrated spatial learning without explicit reward and goal-directed spatial learning, led Tolman (1948) to the assumption that a sort of 'cognitive map' is established in the rat's brain during the learning of an environment. Tolman assumed this 'cognitive map' provided flexible cognitive function when navigating an environment. One example of this is a rat's ability to form novel routes and shortcuts when the familiar routes are unavailable (Roberts, Cruz, & Tremblay, 2007). Roberts and colleagues performed a study which required rats to travel selected routes in an enclosed cross-maze. The authors were able to control for intrinsic and extrinsic cues to the rat's location within the maze; leaving only the internal geometry of the maze to be learned. Following an initial exploration period, the rats were placed in the maze leaving only peripheral alleys to travel from one end of the box to the next. The rats correctly chose the novel path or shortcut; demonstrating an ability to compute these novel paths/shortcuts based on their limited experience in the maze. Further support to Tolman's 'cognitive map' theory came after the discovery of place cells (O'Keefe & Dostrovsky, 1971). Using freely-moving rats, O'Keefe and Dostrovsky recorded individual hippocampal pyramidal cells which displayed a high rate of firing when the rat was in a specific area of the test environment; these cells are referred to as place cells. Additionally, O'Keefe and colleagues (O'Keefe, Nadel, Keightley, & Kill 1975) found that lesions to the fimbria/fornix, which is a major afferent/efferent pathway of the hippocampus, resulted in impaired spatial learning whilst leaving cue learning intact. This result, combined with the finding of hippocampal place cells, lead to the development of a cognitive map theory, which states that the hippocampus is involved in spatial memory by constructing cognitive maps that aid in spatial navigation in familiar environments (O'Keefe & Nadel, 1978). One behavioural procedure specifically designed to test the 'cognitive map' theory is

the Morris Water Maze (O'Keefe & Nadel, 1978). In a Morris Water Maze task, rodents are placed in a tank filled with opaque water, with a small hidden platform beneath the surface of the water. The hidden platform cannot be found using olfactory, visual or auditory cues, so the animals must learn to escape the tank by happening upon the hidden platform using only tactile cues; i.e., by bumping into the platform. When first placed in the Morris Water Maze, the rodents typically swim randomly until happening upon the hidden platform, and with repeated exposure to the apparatus, the rats gradually learn using available visual cues and with intrinsic navigation/self-motion. The accuracy in which the animals find the hidden platform typically improves quickly. Hippocampal lesions impair navigation accuracy, consistent with a view that the hippocampus provides a cognitive map of the local environment for navigation (Poulter, Hartley, & Lever, 2018).

Extension of the cognitive map theory to humans places more emphasis upon temporal signalling which allows the hippocampus to support spatiotemporal context-dependent/episodic-like memory (Andersen, Morris, Amaral, Bliss, & O'Keefe, 2007). Since the discovery of hippocampal place cells, other types of spatial cells have been identified within the rodent hippocampal formation. Some of these types of spatial cells are briefly summarised below.

1.1.1 Hippocampal theta and spatial cognition

One method in studying hippocampal function is the recording of local field potentials or LFP. This method allows for the study of the hippocampus in terms of its global rhythmical pattern, also known as hippocampal electroencephalograph (EEG). Hippocampal theta is a 5-10 Hz sinusoidal oscillation and is a widely studied component of the hippocampal EEG (Korotkova et al., 2017; O'Keefe, 2007). Theta rhythms in the hippocampus appear to be important in spatial cognition in rodents. Research has shown that temporary inactivation and lesions of the medial septum and fornix abolishes hippocampal theta, which results in

impaired performance on spatial navigation tasks (Brioni, Decker, Gamboa, Izquierdo, & McGaugh, 1990; Chrobak, Stackman, & Walsh, 1989; Mizumori, Barnes, & McNaughton, 1989; Winson, 1978). Hippocampal theta in the rat has also been found to play a major role in novelty detection, which aids in the encoding of new spatial information. For instance, theta frequency reduces in environmental novelty and then theta frequency gradually increases with familiarity (Jeewajee, Lever, Burton, O'Keefe, & Burgess, 2008).

1.1.2 Hippocampal spatial cells

This section will discuss four major types of spatial cells that can be found in the hippocampal formation; place cells, head directions cells, grid cells, and boundary vector cells. There is also a brief section other types of spatial cells, such as speed cells and theta cells.

1.1.2.1 Hippocampal place cells

As mentioned previously, hippocampal place cells were initially discovered in freely-moving rats by O'Keefe and Dostrovsky in 1971. Since then, place cells have been mainly recorded from the hippocampus proper, including the CA1, CA3 and the dentate gyrus, and within multiple regions of the hippocampal formation, including the subiculum, presubiculum, parasubiculum and the entorhinal cortex (O'Keefe, 2007). Characteristically, place cells fire at a low rate throughout most of an environment. However, each place cell shows increased firing when the animal is within a specific region of an environment which is known as its place field (Hartley, Lever, Burgess, & O'Keefe, 2014).

Place fields develop within minutes of novel environmental exposure and remain broadly stable over time in an unchanged environment (Frank, Stanley, & Brown, 2004; O'Keefe & Nadel, 1978; Wilson & McNaughton, 1993). However, when located in an altered or different environment, place cell firing patterns may change, which is known as "remapping" (Knierim, 2002; Leutgeb, Leutgeb, Treves, Moser, & Moser, 2004; Lever, Wills, Cacucci,

Burgess, & O'Keefe, 2002). The size and shape of place fields vary depending upon situational factors such as the shape and size of an environment, and anatomical factors, notably from which portion of the hippocampus along the long axis the recordings are taken (O'Keefe, 2007). For example, research has shown that place cells recorded at dorsal sites of the hippocampal formation tend to have small place fields, whilst place cells recorded ventrally tend to have broader place fields (Jung, Wiener, & McNaughton, 1994; Kjelstrup et al., 2008). Another characteristic of hippocampal place cells that has been reported is the tendencies of place fields recorded in a rectangular box to cluster closer to the walls rather than fire centrally (Hetherington & Shapiro, 1997).

O'Keefe and colleagues (O 'Keefe, Burgess, Donnett, Jeffery, & Maguire, 1998) reported that hippocampal place cells, irrespective of environmental type (rectangular, cylindrical, etc), located near each other are no more likely to produce place fields next to each other than those places located further away. Conversely, Eichenbaum and colleagues (Eichenbaum, Wiener, Shapiro, & Cohen, 1989) found that place cells in close proximity produced place fields closer to each other.

1.1.2.2 Head direction cells

Head direction cells were initially discovered by Ranck in 1984. Where place cells represent where an animal is located within an environment, head direction cells are named so because each cell fires rapidly when the head of a freely moving rat is pointing in a restricted range of angles (Ranck, 1984; Taube, Muller, & Ranck, 1990). Head direction cells are found in the entorhinal cortex, dorsal presubiculum and can also be found outside the hippocampal formation; in the anterior dorsal thalamic nucleus and retrosplenial cortex, for example (Hartley et al., 2014). Each head direction cell has its own preferred direction which corresponds to a compass-like direction. In the rodent, head direction cells code for azimuth, i.e. the horizontal component of head direction. This allows for the representation of the full

range of directions, such that when a subset of head direction cells is firing simultaneously one can reconstruct the animal's heading fairly accurately (Hartley et al., 2014; Johnson, Seeland, & Redish, 2005). When environmental cues are constant, the preferential direction of head direction cells are stable. However, the preferential direction of head direction cells can be rotated by moving prominent visual cues. Those changes in the directional preference of head direction cells can affect the location of place fields. For example, in a cylindrical environment the rotation of a singular visual cue that results in the rotation of head direction cell tunings can induce similar rotations of place field locations (Hartley et al., 2014). Further research has shown that lesions to the head direction network can impede the ability of visual cues to control the orientation of place cells in a cylindrical environment (Calton et al., 2003). This suggests that place cells rely on the head direction system for directional information (Hartley et al., 2014).

1.1.2.3 Grid cells

Grid cells, which were initially identified by the Moser group in 2005, can be found in the medial entorhinal cortex (Hafting, Fyhn, Molden, Moser, & Moser, 2005), and in the pre and parasubiculum . Like place cells, grid cells fire in specific locations in an environment. Unlike place cells, an individual grid cell has multiple firing fields which tessellate an environment in a hexagonal pattern forming the grid field. These grid fields can be described as having three properties; scale, orientation and spatial phase. The grid field scale refers to the distance between adjacent firing rate peaks; orientation refers to the grid axes as it relates to a reference direction; and spatial phase refers to the grid field's relation to an external reference point (Hartley et al., 2014). Like place cells, the scale tuning of grid cells varies along the dorsal-ventral axis of the hippocampal formation. Grid fields recorded from the dorsal medial entorhinal cortex (MEC) are smaller and closer together, whilst grid fields

recorded from the ventral MEC are larger and more spread out (Fyhn, Hafting, Witter, Moser, & Moser, 2008).

Grid cells that were recorded from the same animal were initially thought to show the same orientation, and gird cells recorded from the same portion of the entorhinal cortex were believed to have the same scale (Hafting et al., 2005). However, there is evidence that suggests that rather than grid cells forming a continuous scale, that they form discrete subset of scales (Barry, Hayman, Burgess, & Jeffery, 2007). Studies involving large number of grid cells that were recorded from the same animal have shown that grid cells form modules with diverse combinations of scale and orientation tunings. Although these grid cells anatomically overlap, they still demonstrate the tendency to increase in scale in the dorsal-ventral direction. Across the entire population of grid cells, it appears that the orientation varies more between modules, which suggests that each module operates independently (Hartley et al., 2014).

1.1.2.4 Boundary cells

Early research into the behaviour of place cells often focused on how locational firing patterns seemed specific to the environment; that a place cell would fire in the south-east of a rectangular environment but would fire in the centre of spherical environment (Muller & Kubie, 1987). However, it was observed that when the environmental geometry was manipulated, place cells would typically fire at the corresponding locations in the geometrically different environment (e.g., square vs circle). Those 'corresponding' locations also tended to maintain the distance of that nearest to the wall of each environment (Lever et al., 2002). This would suggest spatial tuning of place cells may be determined by distance to the boundaries of the environment (Hartley, Burgess, Lever, Cacucci, & O 'Keefe, 2000; Lever, Burgess, Cacucci, Hartley, & O'Keefe, 2002). In turn, this led to models predicting the existence of 'boundary vector cells' coding for preferred distances to environmental

boundaries in specific allocentric directions (Hartley et al, 2000). The directional component of the vector would be determined by the head direction system (Hartley et al., 2014). Boundary vector cells or BVCs are predicted to have extended firing fields that run parallel to the edges of an environment, along with additional fields where a new barrier is inserted. This prediction is based off the computational model in which place cells were modelled under a variety of geometric manipulations as the threshold sum of a small number of the putative BVC inputs (Hartley et al., 2000, 2014). An example of this could be a BVC that fires whenever there is a wall or barrier present approximately 5 cm to the south of a rat. This cell would be expected to fire along the southern perimeter of an enclosed environment and fire along the northern side of a barrier introduced into the same environment (Hartley et al., 2014). Cells with these characteristics have been discovered in the subiculum, MEC, and the pre-and parasubiculum (Barry et al., 2006; Boccara et al., 2010; Lever, Burton, Jeewajee, O ’Keefe, & Burgess, 2009; Savelli, Yoganarasimha, & Knierim, 2008; Solstad, Boccara, Kropff, Moser, & Moser, 2008).

The very existence of boundary cells would suggest that cues resulting from environmental geometry may be an important source of external information supporting cognitive mapping in the hippocampal formation (Hartley et al., 2014). As such, it has been established that environmental boundaries influence place cell firing, with subsequent evidence supporting the importance of environmental boundaries upon grid cell firing (Barry et al., 2007; Derdikman et al., 2009; Hardcastle, Ganguli, & Giocomo, 2015; Krupic, Bauza, Burton, Lever, & O ’Keefe, 2014).

1.1.2.5 Speed cells and other cells

It is worth noting that there are a number of classes of cells whose firing properties do not fully comply with the category of cells discussed above; nevertheless, these cells may be found to play an important role in the hippocampal formation (Hartley et al., 2014). Many

spatial cells found in the entorhinal cortex show both locational (grid, boundary) and direction (head-direction) tendencies (Cacucci, Lever, Burgess, & O'Keefe, 2000; Sargolini et al., 2006). An example of this would be if a task constrained the animal's movement, like walking along a track, would see hippocampal place cells, which would normally be insensitive to direction, become directional and fire majorly in one direction (Hartley et al., 2014; McNaughton, Barnes, & O'Keefe, 1983; Muller et al., 1994). Another type of spatial cell found in the entorhinal cortex are 'speed cells.' Speed cells may support the 'path integration' function of grid cells in the medial entorhinal cortex, helping to convert signals of self-motion into signals of spatial translation. In order for this spatial translation to occur, grid cells must have continuous access to instantaneous running speed. Research suggests that the animal's running speed could be represented by a dedicated population of theta-modulated entorhinal neurons, which increase linearly with running speed, and are distinct from other cell populations within the local circuit; e.g. grid, head-direction, and boundary vector cells (Kropff, Carmichael, Moser, & Moser, 2015).

1.1.2.6 Hippocampus involvement in spatial memory

The hippocampus involvement in spatial learning and navigation strongly implicates its involvement in spatial memory (Olton & Samuelson, 1976; Steele & Morris, 1999). The mechanisms involved in a rat translating spatial learning into spatial memory are thought to involve hippocampal oscillations and spatial cells such as hippocampal place cells. There are various oscillations in the hippocampus that are associated with spatial learning. Ripples, which occur in a range between 140-220 Hz (Suillivan et al., 2011), are thought to represent an offline consolidation of spatial and episodic memory, with theta representing online learning, of location sequencing. Replay, the process in which place cells fire during ripple events, is thought to occur in order to consolidate cognitive maps of an environment. This can occur during sleep (Lee & Wilson, 2002; Nádasdy, Hirase, Czurkó, Csicsvari, & Buzsáki,

1999) and stationary waking states (Jackson, Johnson, & Redish, 2006; O'Neill, Senior, & Csicsvari, 2006). A clear example of replay is known as reverse replay. Reverse replay is the sequential replay of place cell firing during an awake state immediately after a spatial experience. This type of replay is unique in that it replays the spatial experience in temporal reverse order (Diba & Buzsáki, 2007; Foster & Wilson, 2006). Diba & Buzsáki (2007) were able to demonstrate reverse replay immediately following a rat's run on a track, and sequential replay in anticipation of running on the track again. The authors propose that this 'bi-directional' replay contributes to associations in episodic memory.

1.1.2.7 Spatial representation; allocentric information

An important aspect of translating hippocampal spatial cells into a functioning cognitive map involves the representation of space as it relates to either the body (egocentric) or the external world (allocentric). Hippocampal spatial cells are the 'building blocks' of allocentric representation (Poulter et al., 2018). An allocentric reference frame is formed by the representation of a place in an environment based on external cues or landmarks (Poulter et al., 2018) which is in contrast to framing a location in an environment by its position relative to the observer; egocentric representation (Holdstock et al., 2000). Hippocampal representations of allocentric self-location are derived from basically two sources of information: 1) cues in the external world such as boundaries and landmarks; 2) cues relating to the subject's self-motion, such as its heading and speed (Poulter et al., 2018). O'Keefe (1976) explicitly considered both of these input types as supporting hippocampal cognitive mapping and following the work of Vanderwolf (1969) in rats linking theta to movement, O'Keefe & Nadal (1978) proposed that hippocampal theta was indexing spatial translation.

1.1.3 Theta rhythm and the coding of running speed

An important aspect examined within this thesis is the relationship between theta frequency and running speed. In addition to coding for location, experimental data has also indicated

that there is a relationship between theta frequency and running speed (Korotkova et al., 2017). Studies have shown the amplitude and frequency of hippocampal theta rhythm increases with running speed (Jeewajee, Barry, O'Keefe, & Burgess, 2008; Maurer, Vanrhoads, Sutherland, Lipa, & Mcnaughton, 2005), which suggests a role for theta in the coding of speed. Actually, this thesis shows the relationship is variable, but in a familiar environment, LFP theta frequency can increase by up to 0.5Hz from 5 cm/s to 30 cm/s (Korotkova et al., 2017). The coherence of theta oscillations (Royer, Sirota, Patel, & Buzsáki, 2010; Sabolek et al., 2009) and the relationship between theta power and locomotion speed (Hinman et al., 2011) has been shown to decrease along the dorsoventral (septo-temporal) axis (Patel et al., 2012), with theta power being significantly lower in the ventral (temporal) portion of the hippocampus (Royer et al., 2010). Indeed, it has been suggested for over a decade that the ventral portion of the hippocampus may be more linked to anxiety, including unconditioned anxiety, rather than spatial cognition, e.g. (Bannerman et al., 2004; Bannerman, Grubb, et al., 2002). The idea that the dorsal (posterior in primates) portion of the hippocampus might support spatial memory, and the ventral portion (anterior in primates) might support anxiety, has been important in trying to reconcile two ostensibly divergent views of hippocampal function; one linked to spatial memory, and the other to anxiety. Below considers literature linking the hippocampus to anxiety.

1.2 Hippocampus: emotional regulation and anxiety

The hippocampus has been associated with emotional processes since Papez (1937) theorised that the hippocampus was included as part of the limbic system, which was the first major theory of the neurology of emotion. Papez theorised that the hippocampus' involvement in the regulation of emotion was due to its connections with other structures in the limbic system. These structures, which include the prefrontal cortex, septum, amygdala and several hypothalamic nuclei (Herman et al., 2005) form the limbic circuit which is activated by

stressors such as fear conditioning, restraint and novelty (Herman & Cullinan, 1997). Within the limbic system, the hippocampus is involved in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis. Stress can result in the increased release of cortisol from the adrenal glands. Glucocorticoid secretion in response to stress is accomplished by the HPA stress axis, with the hippocampus possessing a high level of glucocorticoid binding and a high level of glucocorticoid receptors (Herman & Cullinan, 1997). Hippocampal lesions have demonstrated an impairment to the control of hormonal stress response (L. Jacobson & Sapolsky, 1991), implying that the hippocampus is vulnerable to stress/stressors (Sapolsky, 1996).

1.2.1 Gray and McNaughton: septo-hippocampal theory

Gray (1982) suggested that the hippocampus plays a crucial role in anxiety regulation (Gray, 1982; Gray & McNaughton, 2000). Gray (1982) and Gray & McNaughton (2000) posited that the hippocampus, together with the septum, form the septo-hippocampal 'Behavioural Inhibition System' (BIS). The BIS is a system theorised to control anxiety-related responses, such as inhibition of ongoing behaviours in the presence of predator-predicting cues, increasing vigilance and arousal, and promoting risk assessment. These behaviours can be produced by stimuli associated with pain, punishment, novelty and uncertainty. Septo-hippocampal theta is theorised to be a crucial substrate of the BIS, discussed further below. Gray (1982) proposed that the BIS would be overactive in those with high trait anxiety and generalized anxiety disorder, positing that those with low anxiety and psychopathic traits would have an underactive BIS (Fowles, 1980).

1.2.2 Hippocampal theta and anxiety modulation

As mentioned previously (section 1.1.2), one method in studying hippocampal function is through the recording of LFP; permitting the study of hippocampal EEG. Hippocampal theta's link to anxiety-like behaviours is discussed in Gray & McNaughton's (2003) theory of

the septo-hippocampal system. They emphasised three main points as to the involvement of the septo-hippocampal system in the action of anxiolytic drugs:

- Gray & McNaughton's (2003) first point regards the parallel between lesions to the septo-hippocampal system and the behavioural effects of anxiolytic drugs. This point was initially described by Gray (1970) on the basis that the combination of hippocampal and septal lesions and the administration of barbiturate sodium amytal resulted in similar behaviour in rodents; reduced anxiety. Further experiments involving classical anxiolytics, including barbiturates, benzodiazepines and ethanol (Gray, 1977), and several hundred lesion studies (Gray & Mcnaughton, 1983) proved a more robust data set to their first point.
- Their second point regarded experiments performed in their laboratories with anxiolytic drugs. They found that despite their different chemical structures, all tested anxiolytic drugs reduce hippocampal theta frequency. They also noted that these specific signatures, notably theta frequency reduction, have been found together for all classical anxiolytics but were not exhibited together with any other class of drugs.
- Their third point stated that "selectively reproducing parts of the septo-hippocampal electrographic signatures of the anxiolytic drugs reproduces parts of the behavioural profile which they share with septal and hippocampal lesions," (Gray & McNaughton, 2003, pg.13). Gray and colleagues (Gray, McNaughton, James, & Kelly, 1975) demonstrated this point by reproducing septally elicited anxiolytic drug action by selectively lesioning of the dorsal ascending noradrenergic bundle, which pathway arises from the locus coeruleus in the brain stem to innervate in the forebrain which includes the septo-hippocampal system (Gray & McNaughton, 2003). This point is important as it suggests that the electrophysiological signatures described above do not incidentally correlate with the clinical action of anxiolytic drugs, rather they

represent the functional effects of anxiolytic drugs upon the septo-hippocampal system (Gray & McNaughton, 2003).

The McNaughton and colleagues (McNaughton, Kocsis, & Hajós, 2007) review of the effects of systemically administered drugs on hippocampal theta elicited by high-frequency stimulation provided further support of hippocampal theta's link to anxiety-like behaviour proposed in Gray and McNaughton's theory of the septo-hippocampal system. In McNaughton and colleagues' review, the authors point out that high frequency trains of stimulation of the midbrain reticular formation can elicit hippocampal theta (Green & Arduini, 1954), and that the increase of stimulation produces a linear increase in theta frequency in both anaesthetised rats and freely moving rats (McNaughton et al., 2007; Siok, Taylor, & Hajós, 2009). They also argued that all known clinically dissimilar anxiolytic drugs reduce the frequency of reticular-elicited theta and this reduction is a good in-vivo method of detecting anti-anxiety drugs independent of their behavioural characteristics (McNaughton et al., 2007). This is important to note as the effects of anxiolytic drugs on hippocampal theta frequency is the primary focus of Study 1A, which examines if the gabapentinoid pregabalin, systemically administered, can reduce hippocampal theta frequency.

1.2.3 Anxiolytic drugs impair spatial learning

Discerning the efficacy of anxiolytic drugs has historically been explored through animal models of behaviour. The most common animal models used to test anxiolytic drugs are the elevated plus maze and the open field (Griebel & Holmes, 2013), which are unconditioned models of behaviour. However, many anxiolytic drugs have properties outside of anxiolysis. For example, aspects of classic anxiolytic drugs have been examined in areas such as their effects on spatial learning. Chlordiazepoxide (CDP) has been shown to impair spatial learning in a Morris Water Maze task (Pan & McNaughton, 1997). Deng and colleagues (Deng et al., 2009) have noted that GABA receptors (benzodiazepines are GABAergic) have

been shown to play a crucial role in spatial navigation. Buspirone, which is a drug that enacts on serotonin receptors, has also been shown to impair spatial navigation in the Morris Water Maze (McNaughton & Morris, 1992; Rowan, Cullen, & Moulton, 1990). Buspirone and CDP are two drugs that are neurochemically dissimilar and yet, both drugs have been shown to impair spatial leaning. One explanation in the impairment of spatial learning from these two separate anxiolytics is that they are mere side effects of the drugs themselves. However, another explanation is that the spatial learning impairment demonstrates changes in hippocampal function which may underline specific components of clinical anxiolytics. Knowing that both Buspirone and CDP have been shown to reduce hippocampal theta frequency in anaesthetised animals, and that hippocampal theta play a key role in spatial cognition, the 'underlining' component of clinical anxiolytics might reflect the functional change in the hippocampus are specific characteristics of hippocampal theta.

1.3 Dual functionality of the hippocampal formation: spatial cognition and anxiety

In general, there is no consensus in terms of the functionality associated with the hippocampus. However, as briefly outlined above, two sets of functional associations of the hippocampus tend to dominate theoretical overviews of the hippocampal role in the brain. One set of theories emphasises the role of the hippocampus in cognition, the other set emphasises its role in emotion (Korotkova et al., 2017). With the cognitive set, the hippocampus is mainly associated with the support of spatial cognition, context-dependent and episodic memory. These functions are usually linked to coding for novel contexts/environments (Burgess, Maguire, & O'Keefe, 2002; O'Keefe & Nadel, 1978; Schiller et al., 2015), and includes the discovery of hippocampal spatial cells, which were briefly discussed above (section 1.1.3). With the emotional set, the hippocampus plays a crucial role in anxiety modulation, likely linked to roles in stress and depression (Korotkova et al., 2017). These functions are linked to anxiety-modulating effects following hippocampal

disruptions (Gray & McNaughton, 2003), depression (Santarelli et al., 2003) and the hippocampal control over the HPA axis (Herman & Cullinan, 1997).

Perhaps surprisingly, there are few theoretical studies that try and bridge the gap between the cognitive and emotional functionality of the hippocampus. One influential approach to the proposed duality of the hippocampal function has been to regard the dorsal and ventral hippocampus as distinct structures. Space and memory are associated with the dorsal hippocampus, whilst the ventral hippocampus is associated with anxiety (Bannerman et al., 2004; Fanselow & Dong, 2010). Evidence to support the dissociation of the dorsal and ventral hippocampus supported by lesion studies in rodents (Bannerman et al., 2004; Kjelstrup et al., 2002), and neuroimaging studies in primates and humans (Bach et al., 2014; Loh et al., 2016). For instance, Bannerman and colleagues (2004) reviewed behavioural effects of hippocampal lesions spatial cognition and anxiety modulation. In their review, the authors initially defined the dorsal hippocampus as 50% of the hippocampal volume beginning from the septal pole and defined the ventral hippocampus as 50% of the hippocampal volume beginning from the temporal pole. This anatomical dissociation was strongly supported with studies demonstrating the dorsal-dependent and ventral-dependent functions. For instances, Moser and colleagues' showed that aspiration dorsal hippocampal lesions disrupt spatial learning in the Morris Water Maze, whilst ventral hippocampal lesions left spatial learning intact (Moser, Moser, & Andersen, 1993). Moser and colleagues were able to replicate this finding by using fibre-sparing ibotenic acid lesions. In this study, the lesion varied in size from 20 to 100% of the hippocampal volume which extended from either the septal or temporal pole. They found that if ~26% of the dorsal hippocampus remained intact, so too did spatial learning in Morris Water Maze. Conversely, they found that sparing as much as 60% of the ventral hippocampus impaired spatial learning in the Morris Water Maze (Moser, Moser, Forrest, Andersen, & Morris, 1995). On the other hand, lesions to the

ventral hippocampus have been shown to effect anxiety modulation (Richmond et al., 1999). For example, McHugh and colleagues (McHugh, Deacon, Rawlins, & Bannerman, 2004) found that cytotoxic ventral hippocampal lesions produced anxiolytic effects in four unconditioned tests of anxiety in rats; social interaction test, black/white box, hyponeophagia, and the elevated plus maze. Dorsal hippocampal lesions did not produce anxiolytic effects in either of the four tests.

Although there is substantial literature which supports the notion that the hippocampal functions can simply be separated into the dorsal and ventral hippocampus, the anatomy of the hippocampus suggests that there is not only an intermediate hippocampus, but the intermediate hippocampus has its own role in hippocampal functionality. Fanselow and Dong (2010) define the intermediate hippocampus based on the entirety of Ammon's horn appearance as an elongated C-shaped cylinder. While the two free ends comprise the dorsal and ventral 'domains,' the intermediate hippocampus, lying between the dorsal and ventral hippocampus, occupies the vertical domain of the 'C' (Fanselow & Dong, 2010). The intermediate hippocampus is thought to have overlapping behavioural characteristics of the dorsal and ventral hippocampus (Fanselow & Dong, 2010), with studies showing its importance in rapid-place learning (Bast, Wilson, Witter, & Morris, 2009) and anticipatory behaviour (Burton, Hok, Save, & Poucet, 2009). The present thesis uses recordings in both the dorsal and intermediate hippocampus.

Additional to the importance of examining the intermediate hippocampus, the approach of a stark duality of hippocampal function runs into further definitive problems in terms of its physiology. Hippocampal theta is found throughout the dorso-ventral axis and it appears to travel the dorso-ventral axis in a single wave (Lubenov & Siapas, 2009; Patel et al., 2012). In other words, while the amplitude may vary along the dorso-ventral axis (Hinman et al., 2011; Patel et al., 2012), frequency is essentially the same in the dorsal and ventral hippocampus in

an intact rat (Lubenov & Siapas, 2009; Patel et al., 2012). Indeed the vast majority of the research used to support Gray and McNaughton's (2003) theory of anxiety in which anxiolytic drugs are shown to reduce theta frequency have placed the recording electrodes in the dorsal hippocampus (Engin, Stellbrink, Treit, & Dickson, 2008; Gray & McNaughton, 2003; McNaughton et al., 2007; Siok et al., 2009; Yeung, Treit, & Dickson, 2012). In summary, how the hippocampus supports processing of two different functions remains relatively unclear.

Although the hippocampal processing of space/cognition and anxiety is seemingly unclear, the shared physiological substrate that these two functions have in common, i.e. septo-hippocampal theta, may provide that bridge into understanding how these functions operate within the hippocampus. One theoretical approach points to the assumption that the processing of one set of functions will not interfere with the processing of the other (Korotkova et al., 2017). An approach to this theory was highlighted by Wells and colleagues' (Wells et al., 2013) using freely moving rats. This built upon the Burgess, (2008) model, which suggested that theta frequency has two components; one corresponding to the slope of theta-frequency-to-running-speed-relationship and the other corresponding to the intercept of theta-frequency-to-speed relationship, Wells and colleagues made specific predictions involving spatial cognition and anxiety by combining the aforementioned Burgess (2008) model with the insights highlighted by Gray and McNaughton (2003) in regards to anxiolytic drugs. They predicted that a novel spatial environment would reduce the slope component of the theta-frequency-to-speed relationship without significantly affecting the intercept component, whilst the introduction of anxiolytics would reduce the intercept component of the theta-frequency-to-speed relationship without significantly affecting the slope component. Wells and colleagues tested two clinically established anxiolytic drugs (chlordiazepoxide and buspirone) and tested one novel anxiolytic drug (O-2545, a CB1

agonist). They found that at the doses tested, all anxiolytic drugs reduced the intercept component of the theta frequency-to-speed-relationship without affecting the slope component. Furthermore, Wells and colleagues found that the introduction of a novel environment decreased the slope component of the theta-frequency-to-speed relationship, without affecting the intercept component.

To try to shed light on common vs preferential functions associated with the long axis, the present thesis uses recordings in both the dorsal and intermediate hippocampus. The work by Wells et al (2013) predicting and observing that anxiolytic drugs would reduce the y-intercept (i.e. 0 cm/s intercept) of the theta frequency to running speed relationship was based on combining two theoretical frameworks: the Gray and McNaughton (2000) model, and the Burgess (2008) model, which is described further below.

1.3.1 The Burgess model

The Burgess (2008) model of grid cell formation posits the dissociation of two components of theta. The model extends a previous oscillatory interference model of grid cell formation (Burgess, Barry, & O'Keefe, 2007). Importantly, it makes certain testable predictions about Type 1 and Type 2 theta (defined in Chapter 3) in relation to the theta-frequency-to-speed relationship.

Speed modulated head-direction cells send inputs to velocity-controlled oscillators which have a membrane-controlled oscillation that increases with depolarisation. The grid scale is inversely proportional to the velocity-controlled oscillator's frequency in response to depolarisation (i.e., as velocity-controlled oscillator decreases, grid scale increases). The overall average of velocity-controlled oscillators, identified as theta, reflects two components: a baseline theta frequency at 0 cm/s (intercept) and the rate at which velocity-controlled oscillators' frequency increases with depolarisation (slope; Hz/cm/s). The model assumes that the relationship between theta frequency and running speed is essentially linear, and that its

'slope' would correspond to Type 1 theta and its y 'intercept' at 0 cm/s to Type 2 theta. The model is largely concerned to explain spatial cells, notably grid cells, and systematically relates changes in slope to spatial scale. Thus, in more dorsal portions of the hippocampus and associated entorhinal cortex, there is a steeper slope between running speed and cellular theta frequency, resulting in smaller scale space: the distance between grid nodes is smaller, grid node width is smaller, and hippocampal place fields are smaller. In contrast, in more ventral portions of the hippocampus and associated entorhinal cortex, there is a flatter slope between running speed and cellular theta frequency, resulting in larger scale space: the distance between grid nodes is larger, grid node width is larger, and hippocampal place fields are larger. These are the key anatomy-related predictions of the model. The Burgess model also makes context-related predictions, noting that cellular theta frequency could vary according to states such as novelty as well as anatomical variation. The model specifically predicts that larger grid scale in novel (compared to familiar) contexts, as shown in Barry and colleagues (Barry, Ginzberg, O'Keefe, & Burgess, 2012) is due to lower theta frequency in novelty, and specifically lower *theta slope* in novelty, as shown in Wells et al (2013). Consistent with the Burgess model, Wells et al (2013) found a correlation between hippocampal LFP theta slope, and hippocampal place field size. Thus, predictions can be made from the model systematically relating theta variables to spatial cognition and ultimately spatial behaviour (Burgess, 2008).

The above discussion emphasises spatial cognition, in line with the focus of the Burgess (2008) model. However, this model also suggested a y-intercept component of theta frequency, and speculatively linked this to type 2 theta mechanisms, which is typically associated with arousal. Wells and colleagues (Wells et al., 2013) combined the theta-frequency-reduction model of anxiolysis with the (Burgess, 2008)Burgess (2008) model, and assuming a reasonable link between anxiety and arousal, derived the specific prediction that

anxiolytic drugs would reduce the y-intercept of the theta frequency to running speed relationship, which they confirmed.

1.4 Summary

In summary, the findings of Wells et al. (2013), which dissociated intercept reduction and slope reduction, provided useful empirical support for the two component model of hippocampal theta (Burgess, 2008). The main purpose of this thesis is to extend this line of work by testing the relatively novel anxiolytic drug pregabalin's effect on the intercept component of the theta-frequency-to-speed relationship and examine the effects of environmental familiarisation upon the slope component of the theta-frequency-to-speed relationship by recording in both the dorsal and intermediate portions of the hippocampus. The purpose of recording in both the dorsal and intermediate portion of the hippocampus is to discern if the anatomical duality (dorsal hippocampus modulating space/memory and ventral hippocampus modulating anxiety) would factor into the physiological nature of hippocampal theta when examining the effects of anxiolytics and environmental familiarisation. On the basis of the travelling wave results (Lubenov & Siapas, 2009; Patel et al., 2012), the prediction is that both dorsal and intermediate hippocampal theta would respond in the same way as demonstrated in the Wells and colleagues' (2013) study; pregabalin will reduce the intercept component of the theta-frequency-to-speed relationship and environmental familiarisation will increase the slope component of the theta-frequency-to-speed relationship, with each component having no significant interaction with other. Further discussion (methodologies) are detailed in Chapter 6 (Study 1A, intercept component) and Chapter 7 (Study 1B, slope component).

Additionally, pregabalin's anxiolytic efficacy will be examined in two animal models of behaviour; the elevated plus maze (EPM) and the open-field (OF). Chlordiazepoxide will be used a positive control of anxiolysis, with spatiotemporal and ethological measures analysed

to test anxiolysis. Interestingly, this is the first time, that electrophysiology in freely behaving rats, on the one hand, and behavioural tests, on the other hand, can be directly compared to test the idea in McNaughton et al. (2007) study that theta frequency reduction is a better predictor of anxiolysis than behavioural assays. Further discussion (methodologies and experimental aims) are detailed in Chapter 8.

2 Anatomy of the Hippocampus

This chapter will describe the important anatomical details of the hippocampal formation. This will aid in the understanding of the connectivity crucial to the mechanisms of the generation of hippocampal theta which will be addressed in Chapter 3.

2.1 Nomenclature

There has been some debate regarding the nuances between the definition of the *hippocampus proper* and the *hippocampal formation* (Amaral & Lavenex, 2007). This thesis adopts the nomenclature where the hippocampus proper is comprised of the cornu ammonis (CA) fields (CA1, CA2, and CA3) and the dentate gyrus (DG). The hippocampal formation includes the hippocampus proper and the subiculum. The adjacent parahippocampal formation is consists of the entorhinal cortex, pre- and para-subiculum (Furtak, Wei, Agster, & Burwell, 2007).

The distinction between the hippocampus proper and the hippocampal formation is based on their differing laminar organisation and connectivity. The hippocampal formation is part of the allocortex, which is characterised by less than six layers of organisation. The hippocampal formation is commonly considered as exhibiting a mostly tri-laminar organisation. Its connectivity is largely unidirectional (from one hippocampal field to another). In terms of connectivity, parahippocampal formation is more neocortical, in that it possesses six layers with many reciprocal connections. One connects the entorhinal cortex and the peri- and post-rhinal cortices, and the other connects the entorhinal cortex and the pre- and para-subiculum. For this reason, the parahippocampal formation is often regarded as a transition between the allo- and neo-cortices.

2.2 Gross Morphology

The position of the hippocampal formation differs between the rodent and primate/human brain, in part, due to the expansion of the primate/human cerebral cortex, which "forced" the hippocampus into a more ventral portion of the medial temporal lobe (Amaral & Lavenex, 2007). In rodents, the hippocampal formation occupies a large portion of the brain, where the hippocampus proper represents roughly half the cortical volume.

Figure 2.1 illustrates the position of the hippocampus within the rodent brain after removal of the occipital and temporal neocortex. The hippocampus is elongated and banana-shaped in appearance and extends along two orthogonal axes. The septo-temporal (or dorso-ventral) axis goes from the midline of the brain near the septal nuclei (rostrodorsally) over and behind the thalamus into the incipient temporal lobe (caudoventrally). The medio-lateral axis is often referred to as the transverse axis. The structural connectivity shows some differences along these two axes. The hippocampus is arbitrarily divided into a dorsal portion (located just behind the septum), a posterior portion (located at the start of the ventrolateral curvature) and a ventral portion (located within the temporal portion).

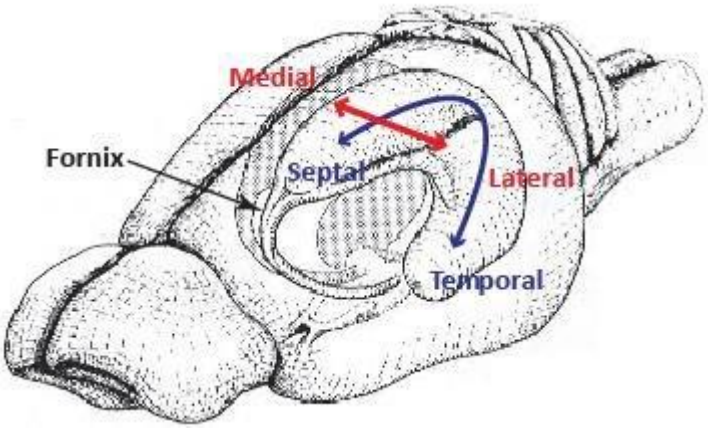


Figure 2.1 Three-dimensional drawing illustrating the position of the hippocampal formation in the rodent brain from a lateral point of view. The septo-temporal axis (blue arrows) runs between the septal and temporal poles (labelled). The transverse axis (red arrows) runs from medial to lateral. Drawing adapted from Amaral & Witter (1995).

The fimbria-fornix is the fibre pathway that links the hippocampus and several subcortical regions. The fimbria-fornix originates in the alveus, a thin sheet of afferent and efferent fibres covering septal/ventral surface of the hippocampus. These fibres collect at temporal/dorsal levels of the hippocampus and become progressively thicker from the temporal to septal levels. Some of these fibres will form the alvear pathway, innervating the entorhinal cortex as an example. The remaining fibres are referred to as the fornix as they leave the hippocampus and descend into the forebrain. The fornix innervates regions including the septum, nucleus accumbens, anterior and posterior hypothalamus, and the anterior thalamic nuclei. Ascending inputs, from the septum and raphe nuclei, also travel along the fornix bundle to innervate the hippocampus (Amaral & Lavenex, 2007).

2.3 Organisation of the Hippocampal Formation and the Parahippocampal Formations

Figure 2.2 shows a horizontal section of the hippocampal and parahippocampal formations. This figure illustrates the trilaminar organisation of the hippocampal structures along with the emergence of new layers at the border between the subiculum and presubiculum. The darkly stained areas highlight two interlocked C-shaped structures: the granule layer of the DG and the pyramidal layers of the CA3-1. Below these layers are the moderately cell-free layer known as the *stratum oriens*. The deep layers of the pre- and parasubiculum appear to be a continuation of the pyramidal layer of the subiculum. The boundary between the subiculum and presubiculum is marked by the emergence of a more superficially positioned cortical sheet. The increase in layers represents the border between the hippocampal formation and the parahippocampal formation. The sheet consists of the superficial layers of the pre- and para-subiculum in which the lamina dissecans, a cell-free zone, separates the deep layers. The lamina dissecans disappears at the border between the entorhinal and perirhinal or postrhinal/parahippocampal cortex and gives way to a more homogenously layered cortex

that resembles the six-layered neocortex, the only difference being that the peri- and post-rhinal cortices lack a marked granule cell layer IV.

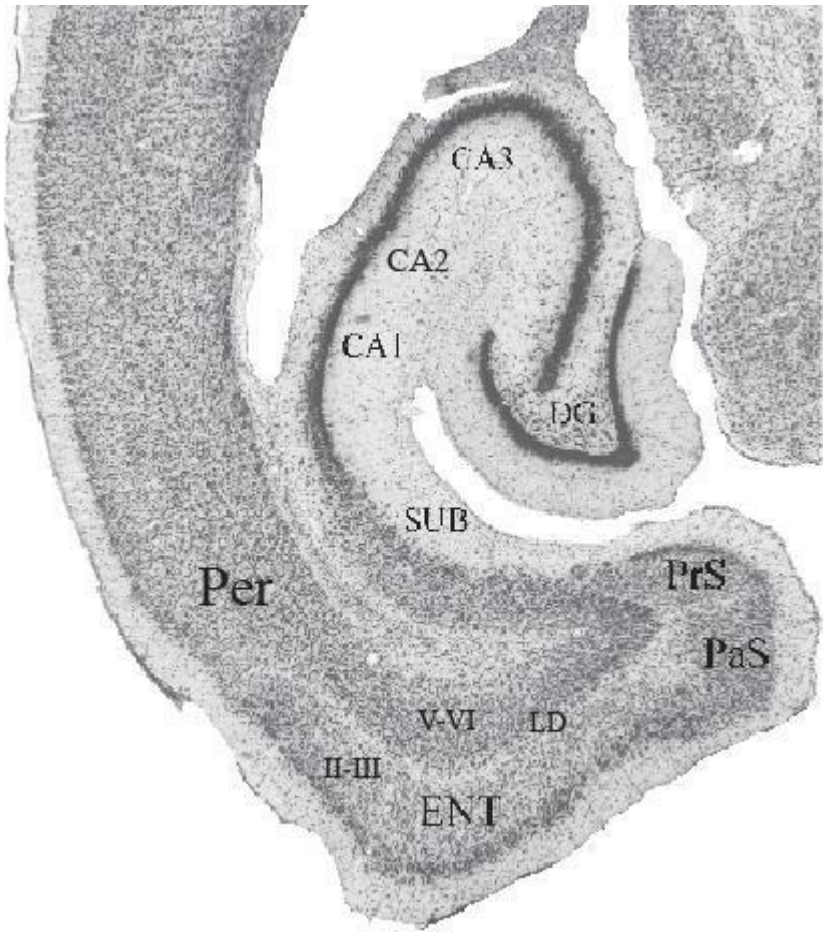


Figure 2.2 Horizontal section through the hippocampal formation and parahippocampal region of the rodent brain. The trilaminar (three layered) structures that comprise the hippocampal formation are CA1, CA2, C3, DG and subiculum (SUB). The parahippocampal region is composed of the presubiculum (PrS), parasubiculum (PaS), entorhinal cortex (ENT) and perirhinal cortex (Per). The parahippocampal region begins where there is an abrupt increase in the number of layers. Layers II and III form the superficial parts of these structures while the deep layers (V-VII) are a continuation of the subiculum. From Witter, Naber, & Haeften (2000).

2.4 Cytoarchitectonic organisation

2.4.1 Laminar organisation of the CA fields

The principal cellular layer is the *pyramidal cell layer*. The pyramidal cell layer is densely packed in CA1; less so in CA2 and CA3. The cell-free layer located beneath the pyramidal cell layer is called the *stratum oriens*, which contains the basal dendrites of the pyramidal

cells as well as some interneurons. This region also contains some of the CA3 autoassociational connections and the CA3 to CA1 Schaffer Collateral connections. In the CA3 field there is a narrow acellular zone, the *stratum lucidum*, where mossy fibres that originate from DG terminate. The *stratum radiatum* is located superficial to the stratum lucidum in CA3 and immediately above the pyramidal cell layer in CA1 and CA2. This region also contains some of the CA3 autoassociational connections and CA1 to CA3 Schaffer collateral connections, as well as interneurons.

The most superficial layer of the hippocampus is the *stratum lacunosum-moleculare*, which is where the performant pathway fibres from neurons of the superficial layers of the entorhinal cortex terminate. These terminate on the distal apical dendrites of CA1 pyramidal cells. Afferent projections from other regions (the distal dendrites project into the stratum lacunosum-moleculare), such as thalamic regions reuniens nucleus also terminate in the stratum lacunosum-moleculare. This layer also contains interneurons.

In summary, the pyramidal cells of the CA fields have two dendritic trees extending above and below the cell bodies layer: the basal and apical dendrites in the oriens and radiatum/l-m layers respectively. Their axons leave the CA fields via the alveus (Amaral & Lavenex, 2007).

2.4.2 Laminar organisation of the dentate gyrus

The DG comprises three layers. The *stratum moleculare* is a relatively cell-free layer and is the superficial layer in which the granule cell dendrites are located and receive their input from the entorhinal cortex layer II. The stratum granulosum, the intermediate layer, is densely packed with somas of the granule cells. These are the principal type of dentate cells and the only ones protruding outside the DG; they synapse with the CA3 dendrites in the *stratum lucidum*. Polymorphic cell layer (sometimes referred as the hilus) is the deepest layer of the

DG which contains various types of neurons. These neurons include mossy cells that project back to the granule cells.

2.4.3 Laminar organisation of the subiculum

The layers of the subiculum are very similar to that of DG. *Molecular*, the first layer, receives entorhinal projections in its superficial portion whilst the deep part of the layer is innervated by CA1. The middle layer, *pyramidale*, encloses the pyramidal cells somas. The third layer, *oriens*, contains the basal dendrites.

2.4.4 Laminar organisation of the entorhinal cortex

The entorhinal cortex contains six layers, which are often grouped into the superficial (layers I-III) and deep (IV-VI) layers. The superficial layers contain stellate (layer II) and pyramidal (layer II-III) cells. The neurons from the entorhinal cortex layer II project to the DG and CA3. Neurons from entorhinal cortex layer III project to CA1 and the subiculum.

2.4.5 Cell types within the hippocampal formation

The hippocampal formation contains various types of neurons, but the pyramidal cells comprise the majority of the cells in the pyramidal layer. Pyramidal cells derive their name from the pyramid-like shape of their soma. In the CA fields, these cell bodies are aligned densely in the pyramidal layer while their dendrites extend in two opposite directions. Pyramidal cells have a basal dendritic tree that extend into the stratum oriens and apical dendritic tree that extends to the hippocampal fissure.

The principal cell type of the DG are granule cells. The dentate intermediate layer contains mostly a tight alignment of granule somas. The granule cell projects its axons collaterals mostly to CA3 via the mossy fibres where it forms asymmetric excitatory synapses.

Another important cell type within the hippocampal formation is interneurons. Unlike the largely uniform population pyramidal, interneurons are heterogeneous in their morphological features, physiological characteristics, expression of neurochemical makers and transcription

factors (Vida, 2010). In the past, interneurons were ascribed as generally inhibiting neuronal activity; however, they are increasingly recognised as a crucial component of information processing within the hippocampal formation, with increased focus being placed on their heterogeneous nature (Amaral & Lavenex, 2007). For example, residing in or close to the pyramidal cell layer, interneurons known as basket cells have dendrites which extend into the stratum oriens, stratum radiatum, and stratum lacunosum-moleculare. Most of the excitatory inputs to the basket cells are from pyramidal cells with approximately 2000 pyramidal cell inputs contributing to the excitation of a single basket cell, which innervates over 1000 pyramidal cells (Amaral & Lavenex, 2007).

2.5 General Connectivity

The hippocampal formation differs from the parahippocampal regions in traits such as laminar organisation and connectivity. The hippocampal formation lacks much of the strong, reciprocal projections that exist between neocortical areas (Felleman & Van Essen, 1991). Its internal circuitry is predominantly unidirectional, connecting the components in a serial manner via the trisynpatic perforant path. This set of projection fibres make up the major of excitatory input to the hippocampus proper. The main source of input into the hippocampus originates in the superficial layers (II and III) of the entorhinal cortex and reaches the DG by "perforating" the subiculum. The second synapse is formed between the axon terminals of the dentate granule cells or mossy fibres, and the dendrites of the CA3 pyramidal cells. The final synapse is between the projections from the CA3 pyramidal cells and the CA1 dendrites, with CA3 projections which terminate in CA1, called the Schaffer collaterals.

CA1 projects mostly to the subiculum, the deep entorhinal layers and some subcortical nuclei. The subicular projections reach mainly the pre- and para-subiculum, various subcortical structures and the deep layers of the entorhinal cortex. Therefore, the hippocampal processing loop is mediated by parallel loops that begin in the superficial

entorhinal layers (II and III) and end in the deep layers (V and VI) (Amaral & Lavenex, 2007).

2.5.1 Afferents to the hippocampal formation

The most prominent cortical extrinsic input of the hippocampus originates in the superficial layers of the entorhinal cortex through the perforant path (Amaral & Lavenex, 2007). The perforant pathway into the hippocampus can be subdivided into two streams. Initially, projections that originate in layer II of the entorhinal cortex project to DG, CA3 and CA2. In the DG, they form a synapse mainly with the granule dendrites in the stratum moleculare, while in CA3, they terminate mostly on the distal apical dendrites of the pyramidal cells (stratum l-m). In terms of the second stream, projections from layer II project to the CA1 and subiculum, with projections to the CA1 terminating in the stratum lacunosum-moleculare. Projections from lateral entorhinal cortex terminate in distal CA1 and those from medial entorhinal cortex terminate in proximal CA1. Thus, entorhinal cortex input to CA1 varies depending upon which part of the entorhinal cortex is the source (Knierim, Neunuebel, & Deshmukh, 2014), with lateral entorhinal information being more item and object based, and medial entorhinal information being more related to spatial cognition and self-motion. Projections to the subiculum terminate in the molecular layer (Witter, 1993).

Rhythmic excitation of the apical dendrites of CA1 pyramidal cells by the entorhinal afferents is thought to be an important site involved in the generation of intrahippocampal theta (Buzsáki, 2002). Research has suggested that entorhinal input to the distal dendrites may be carrying speed-related information (Burgess, 2008a; Sargolini et al., 2006). It has also been suggested that the apical dendrites of CA1 pyramidal cells are a putative site for the generation of a speed-related component of the hippocampal theta rhythm (Burgess, 2008; Giocomo, Zilli, Fransén, & Hasselmo, 2007; Hu, Vervaeke, Graham, & Storm, 2009; Narayanan & Johnston, 2007). The apical dendrites of CA1 pyramidal cells also possess a

high-density pace-making HCN channels. The I_h current that flows through these channels has been suggested to support theta-band membrane potential oscillations in CA1 pyramidal cells (Hu et al., 2009; Hu, Vervaeke, & Storm, 2002; Narayanan & Johnston, 2007).

2.5.2 Subcortical afferents to the hippocampal formation

Five major subcortical domains input into the hippocampal formation; the thalamus, the amygdalar complex, the supramammillary hypothalamus, the brain stem and the medial septum. The last three are of particular interest within the context of this thesis as they are all involved in the generation of the hippocampal theta rhythm (Buzsáki, 2002; Vertes, Hoover, & Prisco, 2004).

2.5.2.1 Medial septum

Part of the basal forebrain, the septum projects to the hippocampus mainly from the medial septal nucleus and an associated region called the nucleus of the diagonal band of Broca (MS/DBB) (Amaral & Lavenex, 2007). Septal fibres innervate all areas of the hippocampal formation, but are particularly prominent in the DG, where cholinergic fibres from the medial septum innervate mossy cells. The medial septum is also a major source of subcortical input to CA3, and there is also some input to CA1.

Hippocampal input from the medial septum is considered to be crucial for the generation of hippocampal theta (Petsche, Stumpf, & Gogolak, 1962). For instance, hippocampal theta is permanently abolished by lesioning the MS/DBB and the fimbria-fornix, which connects the medial septum to the hippocampal formation (Buzsáki, Lai-Wo S., & Vanderwolf, 1983; Gray, 1971; Green & Arduini, 1954; Petsche et al., 1962). It has also been shown that hippocampal theta can be temporarily abolished through short-term inactivation of MS/DBB using microinfusion of the local anaesthetic procaine (Lawson & Bland, 1993; Mizumori et al., 1989). Further discussion of the importance of the medial septum to hippocampal theta physiology is in Chapter 3 (section 3.3.1.4).

2.5.2.1.1 Supramammillary nucleus

The supramammillary nucleus (SUM), part of the hypothalamus, consists of a population of large cells partially surrounding the medial mammillary nuclei of the hypothalamus (Amaral & Lavenex, 2007). The termination patterns of its projection in the hippocampus are relatively specific and target mostly the DG, subiculum and CA2, whilst projecting weakly to CA3 (Vertes et al., 2004). The SUM also projects to the medial septum nucleus. This connection is important for the generation of theta oscillations (Kirk & McNaughton, 1991). Neurons within the SUM fire in synchronous bursts with theta and appear to occur independently to its connections with the medial septum. Research has shown that the firing of SUM neurons are not disrupted by microinfusions of the anaesthetic procaine into the septum (Kirk & McNaughton, 1991). The SUM also appears to be involved with the conversion of non-bursting/non-theta synchronous firing of the reticular regions into phasic theta-bursting patterns (Kirk & McNaughton, 1991; Kirk et al.,1996). Further discussion of the importance of the supramammillary nucleus to hippocampal theta physiology is in Chapter 3 (section 3.3.1.3).

2.5.2.1.2 Brain stem

The brain stem reticular formation consists of several structures, which include the rostral pontine region (RPO), pedunculopontine tegmental nucleus and the raphe nuclei. The RPO and pedunculopontine tegmental nucleus are both considered to be part of the theta circuit. Projections from these structures innervate MS/DBB via the SUM (Oddie & Bland, 1998). Hippocampal theta can be elicited by high frequency electrical stimulation of the rostral pontine region and by microinjections of the cholinergic agonist carbachol in both the RPO and pedunculopontine tegmental nucleus in anaesthetised rats (Vertes, Colom, Fortin, & Bland, 1993). Further discussion of the importance of brain stem reticular formation to hippocampal theta physiology is in Chapter 3 (section 3.3.1.1).

2.5.3 Intrinsic connectivity of the hippocampal formation

The connectivity within the hippocampal formation is mainly constituted of five sets of projections. First, the mossy fibres from DG to CA3 which arise from the dentate granule cells and terminate just above the CA3 and CA2. Second, CA2 and CA3 are connected by recurrent collaterals. Third, numerous CA3 recurrent collaterals, terminating in the stratum radiatum both ispi- and contra-laterally, form a strong autoassociational network in CA3, which a large proportion of CA3 pyramidal cells can excite each other (Amaral & Witter, 1995; Ishizuka, Weber, & Amaral, 1990). Therefore, CA3 can be viewed functionally as a single network. This recurrent network might also act as an intrahippocampal theta generator (Bragin et al., 1995; Kocsis, Bragin, & Buzsáki, 1999). Fourth, the Schaffer collaterals project to CA1 (strata radiatum and oriens) from the same CA3 pyramidal cells that form the CA3 recurrent network. The fifth and final projection, the CA1 pyramidal cells send their axons to the molecular layer of the subiculum.

2.5.4 Efferents from the hippocampal formation

The subiculum and the CA1 both project to the deep layers of the entorhinal cortex. This gives rise to a number of other efferents to cortical and subcortical regions. For instance, the CA1 and subiculum project to medial prefrontal cortex, retrosplenial cortex and perirhinal cortex. Ventral CA1 projects to areas that include the basal amygdala, olfactory bulb, anterior and dorsomedial hypothalamus. The subiculum is a major output region of the hippocampal formation.

To summarise, information enters the hippocampus via the superficial layers of the entorhinal cortex and is processed within the hippocampal formation. This is then relayed to the deep layers of the entorhinal cortex. Through the connections between the deep and superficial layers, there can be feedback into the hippocampus or relays to the cortical mantle either directly or indirectly through peri- and postrhinal cortices (Amaral & Witter, 1995).

2.6 Parahippocampal Regions

2.6.1 The entorhinal cortex

The entorhinal cortex is crucial to the functioning of the hippocampal formation as both the main entry and exit points of the hippocampal and parahippocampal processing loops. This acts as a hub between the neocortex and the hippocampal regions. The entorhinal cortex can be subdivided into lateral (LEC) and medial (MEC) portions, which are cytoarchitetectonically distinct (Amaral & Witter, 1995).

2.6.1.1 Entorhinal afferents

The entorhinal cortex provides the majority of the cortical input to the hippocampal formation via the perforant pathway. The peri- and post-rhinal cortices are the two major neocortical inputs to the entorhinal cortex, projecting to the superficial layers of the LEC and MEC respectively (Burwell & Amaral, 1998b). It is believed that the MEC and LEC provide specific input into the hippocampus; with the MEC providing spatial input and the LEC providing non-spatial input (Knierim et al., 2014). Despite the fact that both perirhinal and postrhinal cortices are strongly innervated by uni- and poly-modal neocortical association areas, the postrhinal region receives stronger visual and visuo-spatial input, whilst the perirhinal region is more olfactory-orientated (Burwell & Amaral, 1998a). Additionally, the pre- and para-subiculum also project to the entorhinal cortex. Presubiculum receives projections from visuospatial neocortical areas and parasubiculum receives similar cortical inputs. Both pre- and para-subiculum receive input from the subiculum and project heavily into the entorhinal cortex, whilst the parasubiculum projects directly to DG. This functional loop may be important in re-directing hippocampally-processed information back into the hippocampus (Amaral & Lavenex, 2007).

2.6.1.2 Entorhinal efferents

The entorhinal cortex projects mainly to the neocortex relaying the hippocampal information received in its deep layers. Insausti, Herrero, & Witter (1997) report that there is a very

limited portion of layer V neurons in LEC in the lateral frontal (motor), parietal (somatosensory), temporal (auditory), occipital (visual), anterior insular and cingulate cortices. The subcortical targets of the entorhinal projections partly overlap with the CA1 and the subiculum, projecting to the septum. The entorhinal cortex also projects to the amygdala (especially the basal nucleus), nucleus accumbens and olfactory tubercle, but appears to not project to the thalamus or brain stem (Amaral & Lavenex, 2007).

2.6.2 Parasubiculum and presubiculum

The parasubiculum projects to the hippocampal formation and its efferent to the thalamus project back to the hippocampal formation. The presubiculum projects to the hippocampal formation along with the restrosplenial and perirhinal cortices. Therefore, neither the presubiculum nor the parasubiculum appear to have an important output region.

2.7 Dorsal-Intermediate-Ventral Hippocampus and their Proposed Behavioural Correlates

The dorsal/septal and ventral/temporal axis in rodents have different connectivities with cortical and subcortical areas (Strange, Witter, Lein, & Moser, 2014). As mentioned in Chapter 1, there is evidence to suggest that the dorsal, intermediate and ventral portions of the hippocampus modulate specific behavioural correlates, respectively. This section will describe the main functional differences of the dorsal, intermediate and ventral hippocampus further supporting the notion that each portion of the hippocampus plays an important role in the modulation of emotional regulation and spatial cognition.

2.7.1 Three hippocampal compartments

2.7.1.1 Dorsal Hippocampus

The dorsal hippocampus, which contains the greatest density of place cells (Jung et al., 1994), projects to the dorsal subiculum, presubiculum and postsubiculum (Amaral & Witter, 1995; Amaral, Dolorfo, & Alvarez-Royo, 1991; Swanson & Cowan, 1977); all of which contain the most head direction cells (Taube, Muller, & Ranck, 1990; Taube, 2007). The major cortical

projections coming from the dorsal CA1, along with the dorsal subicular, are the retrosplenial and anterior cingulate. These two regions are heavily involved in the cognitive processing of visuospatial information, memory processing and spatial navigation in rats, monkeys and humans (Frankland, Bontempi, & Talton, 2004; Han, O'Tuathaigh, & Trigt, 2003; Lavenex, Amaral, & Lavenex, 2006). Additionally, the dorsal subicular projects to the medial and lateral mammillary nuclei and the anterior thalamic complex, which contains the most navigation-related neurons (Taube, 2007).

Furthermore, the dorsal CA1, along with the dorsal CA3, selectively project the caudal part of the lateral septal nucleus and to the dorsal portion of the medial and rostral part of the lateral septal nucleus, which projects to the medial septal complex and supramammillary nucleus (Risold & Swanson, 1996). These two structures control and generate hippocampal theta rhythm during locomotion (Kocsis & Vertes, 1997; Stewart & Fox, 1990). Additionally, the dorsal subiculum and lateral band of the lateral and medial entorhinal cortex project to the rostrolateral part of the nucleus accumbens and rostral caudoputamen; both of which innervate the ventral tegmental area and/or reticular part of the substantial nigra (Groenewegen, Wright, & Beijer, 1996). The ventral tegmental plays a crucial role in locomotion and the substantial nigra mediates the orientation of movements of eyes, head, neck (Swanson & Kalivas, 2000).

Overall, the dorsal hippocampus-subiculum complex forms a cortical network that is believed to be crucial in mediating cognitive processes like memory, learning, navigation and exploration (Fanselow & Dong, 2010).

2.7.1.2 Ventral Hippocampus

The ventral CA1 projects to the olfactory bulb and other primary olfactory cortices. The ventral CA1 and ventral subiculum also bidirectionally connect with the amygdalar regions (Cenquizca & Swanson, 2007). These projections are thought to play a role in depression-like

symptoms (D. Wang, Noda, Tsunekawa, & Zhou, 2007). The ventral CA1 and ventral subiculum share bidirectional connectivity with the amygdalar nuclei, which receives olfactory sensory inputs from structures like the posterior amygdalar, posteromedial cortical amygdalar, posterior basomedial amygdalar nuclei, post-piriform transition area and medial amygdalar nuclei (Cenquizca & Swanson, 2007). The ventral CA1/subiculum, along with these amygdalar nuclei share bidirectional connectivity with the infralimbic, prelimbic and agranular insular cortices (A. Roberts, Tomic, & Parkinson, 2007). These ventral hippocampal/subicular-amygdalar-medial prefrontal cortical structures project directly or indirectly through the lateral septum, the medial and central amygdala nuclei and bed nuclei of the stria terminals (BST). These structures innervate the periventricular and medial portions of the hypothalamus, which are the primary structures involved in the control of neuroendocrine activities; which are associated with three behaviours strongly motivated by emotion: ingestion, reproduction and defence (Dong & Swanson, 2006). Projections from the ventral hippocampus to the anteromedial group of the BST may be crucial in understanding neuroendocrine dysfunction associated with disorders like anxiety, depression and PTSD (Dong & Swanson, 2006).

Additionally, the ventral CA1 and ventral subiculum project directly to the central amygdalar nucleus (Cenquizca & Swanson, 2007), which is thought to mediate the ventral hippocampus contribution to fear learning (Maren & Holt, 2004). The ventral CA1/subiculum also receive inputs from the lateral amygdalar and basolateral amygdalar nuclei (Petrovich, Canteras, & Swanson, 2001), which, combined with the central nucleus, are essential to Pavlovian fear conditioning (Fanselow & Poulos, 2005).

In summary, the circuits of the ventral hippocampus described above are believed to play a crucial role in regulating the impact of emotional experiences (Fanselow & Dong, 2010).

2.7.1.3 Intermediate Hippocampus

The intermediate hippocampus proper and dentate gyrus receive inputs from the intermediate band of the lateral and medial entorhinal cortex (Burwell, 2000). The intermediate CA1 projects to the anterior olfactory nucleus and dorsal tenia tecta along with inframlimbic and prelimbic areas of the medial prefrontal cortex (Cenquizca & Swanson, 2007). These sites also receive projections from the ventral hippocampus, as mentioned above, however, the intermediate CA1 does not project to the amygdala, BST or hypothalamus (Cenquizca & Swanson, 2007).

The intermediate portion of the subiculum inputs to several amygdalar nuclei, including the lateral, basolateral and basomedial amygdalar nuclei (Pitkänen & Pikkarainen, 2000). These amygdalar nuclei projects to the intermediate subiculum and to the intermediate CA1 and CA3 (Petrovich et al., 2001). It also appears that the neuronal input from several amygdalar nuclei terminates in the intermediate portion of the lateral entorhinal cortex (Petrovich et al., 2001; Pitkänen & Pikkarainen, 2000) and reaches the intermediate parts of the hippocampus proper and subiculum. Additionally, just as with the dorsal subiculum, projections from the hypothalamus from the intermediate subiculum mainly go through the postcommissural fornix pathway (Kishi et al., 2000). Furthermore, separate parts of the intermediate subiculum input into the anterior hypothalamic, supramammillary and medial mammillary nuclei (Kishi et al., 2000). The specific connectivity patterns of the intermediate hippocampus and subiculum remain to be further defined with little information known about the intermediate's specific functions (Fanselow & Dong, 2010). Nevertheless, the connectivity of the intermediate hippocampus as described above is believed to play a specific role in the translation of new and rapid learning into efficient behavioural performance (Bast et al., 2009) and to play a role in anticipatory behaviour (Burton et al., 2009).

2.8 Conclusion

The hippocampal formation receives highly processed multimodal information. Hippocampal efferents project to a wide range of cortical and subcortical areas, further supporting the role of the hippocampal formation in spatial cognition and emotional regulation. Within the context of this thesis, the role of the intermediate hippocampus in emotional regulation is explored. As mentioned above, the literature suggests that the dorsal hippocampus modulates spatial cognition, whilst the ventral hippocampus modulates emotional regulation; namely anxiety (Bannerman et al., 2004, 2002; Fanselow & Dong, 2010; Korotkova et al., 2017; McHugh, Deacon, & Rawlins, 2004; Moser, Moser, & Andersen, 1993). The intermediate hippocampus, lying between the dorsal and ventral hippocampus, is where accurate place encoding (strongest in the dorsal hippocampus) meets connections (strongest in the ventral hippocampus) to behavioural control areas, such as the prefrontal cortex (Strange et al., 2014). Lesions solely to the intermediate hippocampus have shown an impairment in rapid place learning (Bast et al., 2009), implying that its functionality is more in line with the dorsal hippocampus. However, other research has shown that the bilateral lesioning of both intermediate and ventral hippocampus largely abolishes anticipatory behaviour (Burton et al., 2009). Anticipatory behaviour can be seen as important in anxiety (Boissy et al., 2007; Spruijt, van den Bos, & Pijlman, 2001; Treit, 1985) which would imply that the intermediate hippocampus could, in fact, play a role in both the modulation of anxiety and the modulation of spatial cognition.

3 Hippocampal Theta and its Behavioural and Pharmacological Correlates

3.1 Overview

This chapter reviews some of the physiological properties of the hippocampus. It will mainly address the global hippocampal electroencephalograph (EEG) and hippocampal theta rhythm as it is the focus of Study 1A.

3.1.1 Rhythmical and non-rhythmical activity

In the hippocampus of the freely moving rat, there are six prominent EEG patterns; four rhythmical and two non-rhythmical. The rhythmic patterns include theta (6-12 Hz), beta (12-30 Hz), gamma (30-100 Hz) and ripples (100-200 Hz). The non-rhythmic patterns include large irregular amplitude activity (LIA) and small irregular amplitude activity (SIA). Some of these patterns can occur at the same time (i.e., theta and gamma patterns), whilst other cannot (i.e., LIA, SIA and theta patterns). The various EEG patterns are thought to provide information on different aspects of the hippocampal function, with each correlating with a specific set of behaviours (O'Keefe, 2007).

3.1.2 Hippocampal EEG patterns and their behavioural correlates

In a freely moving rat, the most prominent and exclusive hippocampal EEG states are that of LIA and theta. LIA patterns are more random and characterised by sharp waves and are typically observed when the rat is performing stationary behaviours such as grooming and eating (Figure 3.1). Theta, a sinusoidal oscillation that varies in frequency between 6-12 Hz, is prominent during behaviours such as transitional movement (Vanderwolf, 1969) or during arousal and anxiety (Green & Arduini, 1954). SIA has been described as an 'intermediate

state' between LIA and theta (Lever, Kaplan, & Burgess, 2014). SIA occurs during sleep and during transitions of alertness (Jarosiewicz & Skaggs, 2004; Vanderwolf, 1969). Beta oscillations, which range between 12-20 Hz, have been reported in CA3 and the CA1 pyramidal layer, and is highly modulated by environmental novelty (Berke, Hetrick, Breck, & Greene, 2008). Beta can occur alone or in combination with theta, LIA and SIA (O'Keefe, 2007). Gamma oscillations, which range between 20-100 Hz, co-occur with theta and LIA and can be modulated by theta (Lever et al., 2014).

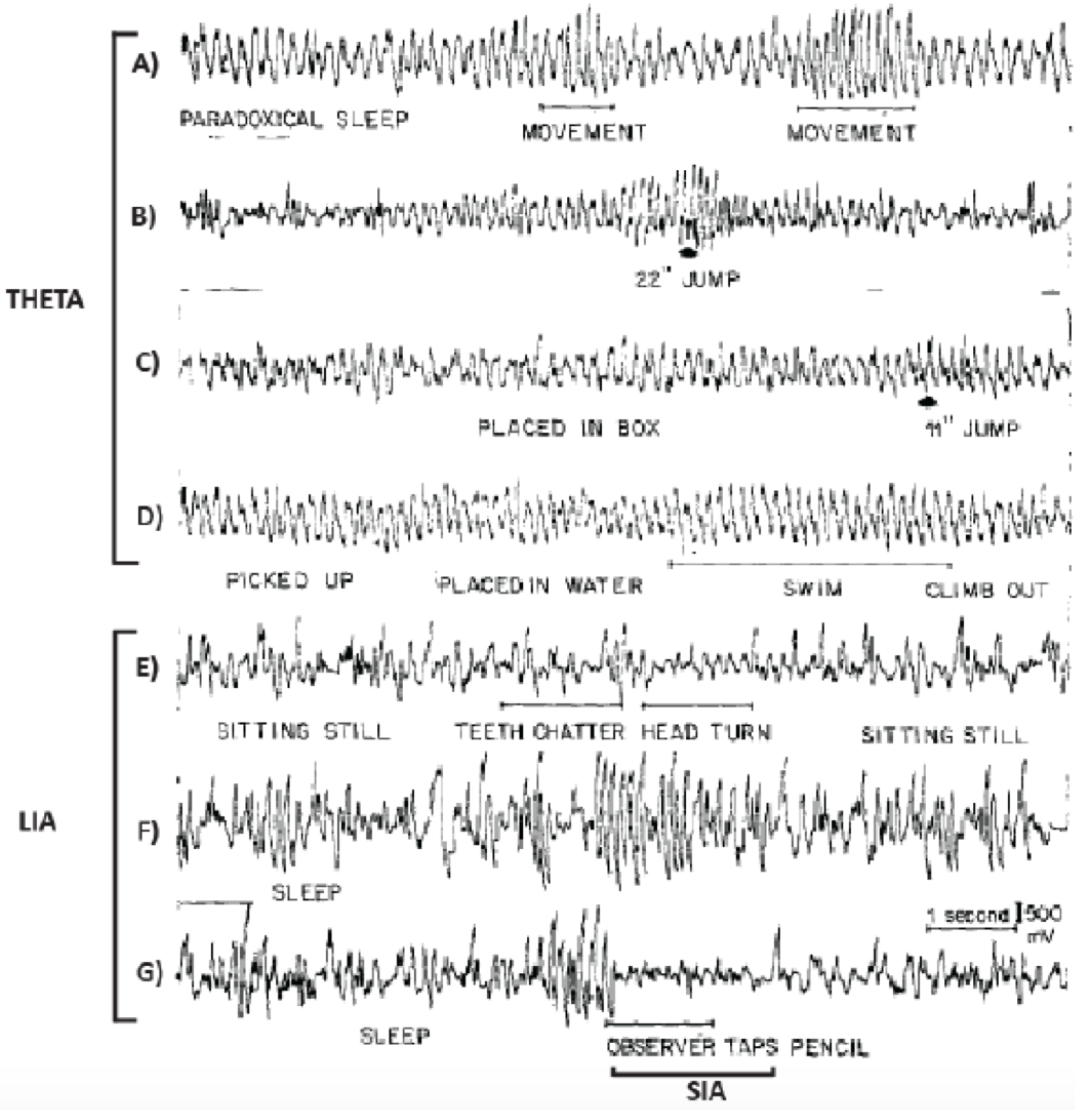


Figure 3.1 Hippocampal EEG traces. A) Theta during rapid-eye-movement (REM) sleep; **B) & C)** Jumping; **D)** Swimming; **E)** Large irregular activity (LIA) during quiet sitting; F) & G) LIA during slow-wave sleep. Some small irregular amplitude activity (SIA) during a brief period of arousal from slow-wave-sleep after a pencil tap. Adapted from Whishaw & Vanderwolf (1973).

There is an oscillatory band, which is higher than gamma frequency, known as 'ripples.' Ripples oscillations occur in a range between 140-220 Hz (Suillivan et al., 2011). Pyramidal cells are highly active during ripples. If an electrode is placed within the CA1 pyramidal layer, it can be seen that many pyramidal cells fire simultaneously with ripples. Buzsáki (1989) suggested that theta may represent the online learning state and that ripples represent

the consolidation state offline. Marr (1991) proposed a model of hippocampal function in which information is transferred from the hippocampus to the neocortex during sleep, and Buzsaki suggested that this process occurred through ripples.

In summary, researchers have shown that there are two main EEG states: LIA and theta state. Ripples can co-occur with theta, whilst beta oscillations can co-occur with theta. Gamma activity has been shown to co-occur with both LIA and theta activity. Theta may also represent encoding and retrieval consolidations, whilst ripples represent offline consolidation, as stated previously (Lever et al., 2014).

3.2. Characterising hippocampal theta: Type 1 and Type 2 Theta

Studies that have examined theta amplitude and theta power have noted that hippocampal theta has an atropine-sensitive and atropine resistant component (Kramis, Vanderwolf, & Bland, 1975). It has been shown that lesions of the MS/DBB abolish theta activity. Additionally, the injection of scopolamine, which is an anticholinergic agent, into the medium septum abolishes the theta activity that is observed during arousal and anxious states, whilst leaving theta that is observed during movement virtually unaffected (Buzsáki, 2002; Kramis et al., 1975). From this, the concept has emerged that are two 'types' or two components of hippocampal theta; 'Type 1' (atropine-resistant) and 'Type 2' (atropine-sensitive) theta. The two subtypes of theta activity have been characterised best in rats. 'Type 1' theta which is also known as atropine-resistant or translational-movement theta, and 'Type 2' theta which is affected by drugs that act at cholinergic synapses or atropine-sensitive theta (O'Keefe, 2007).

The behavioural correlates associated with 'Type 1' theta occur during movements which change the spatial relation between the animal's head and its environment; such as jumping, swimming, walking and exploratory movements (Bland, Seto, & Rowntree, 1983; Harper, 1971). The behavioural correlates associated with Type 2 are not as well defined, but Type

2 theta can be observed during REM sleep and also during periods of immobile attention or arousal and is associated with anxiety-like behaviours (Sainsbury, Heynen, & Montoya, 1987). In rats, Type 2 theta is rarely seen on its own and is usually only observed during moments of freezing in reaction to adverse stimuli, or when the animal is preparing to move. The theta observed under anaesthesia is considered to be Type 2 theta (O'Keefe, 2007). Research has also shown when an animal is under anaesthesia, type 2 theta frequency can be reduced when an anxiolytic is administered (Gray & McNaughton, 2003; McNaughton et al., 2007). The two types of theta do not occur absent of one another. Type 2 theta can co-occur with Type 1 when an animal is engaged in movement behaviours. Because of this, when recording from freely-moving rats, the theta recorded is considered to be comprised of both types.

Surgical isolation or complete surgical removal of the entorhinal cortex eliminates atropine-resistant theta ('Type 1' theta) in the hippocampal formation, whilst leaving atropine-sensitive theta ('Type 2' theta) intact. Cholinergic projections from MS/DBB appear to be required for Type 2 theta (Yoder & Pang, 2005). Kramis and colleagues (Kramis et al., 1975) were first to demonstrate the cholinergic nature of Type 2 theta. The authors demonstrated that atropine sulphate injections abolished theta activity in anesthetised and immobile rats, whilst injections of atropine methyl nitrate, which does not cross the blood-brain barrier, did not abolish theta activity. A recent study has demonstrated that the stimulation of glutamatergic neurons in the MS/DBB induces Type 1 theta in the hippocampus which demonstrated its independence of the cholinergic system (Fuhrmann et al., 2015).

The following sections outline the mechanisms of theta generation as it is currently understood.

3.3 Hippocampal theta physiology

This section examines some of the different subcortical structures that contribute to the generation of hippocampal theta.

3.3.1 External theta generators

The circuitry that is believed to be integral to the generation of hippocampal theta includes the medial septum and the diagonal band of Broca (MS/DBB), the supramammillary nucleus (SUM), the posterior hypothalamus, and the reticular formation which includes the rostral pons in the nucleus reticularis pontis oralis (RPO) and the pedunculopontine tegmental nucleus (PPT). As illustrated in figure 3.2, the reticular formation projects to both the SUM and posterior hypothalamus, with the posterior hypothalamus projecting directly to the SUM. The SUM and posterior hypothalamus are reciprocally connected to the medial septum, which is reciprocally connected to the hippocampus. The following sections will briefly describe the structures involved in the external theta circuitry, where it will conclude that the medial septum is crucial to the generation of hippocampal theta. These sections will begin with the reticular formation and work its way up the circuitry to the hippocampus.

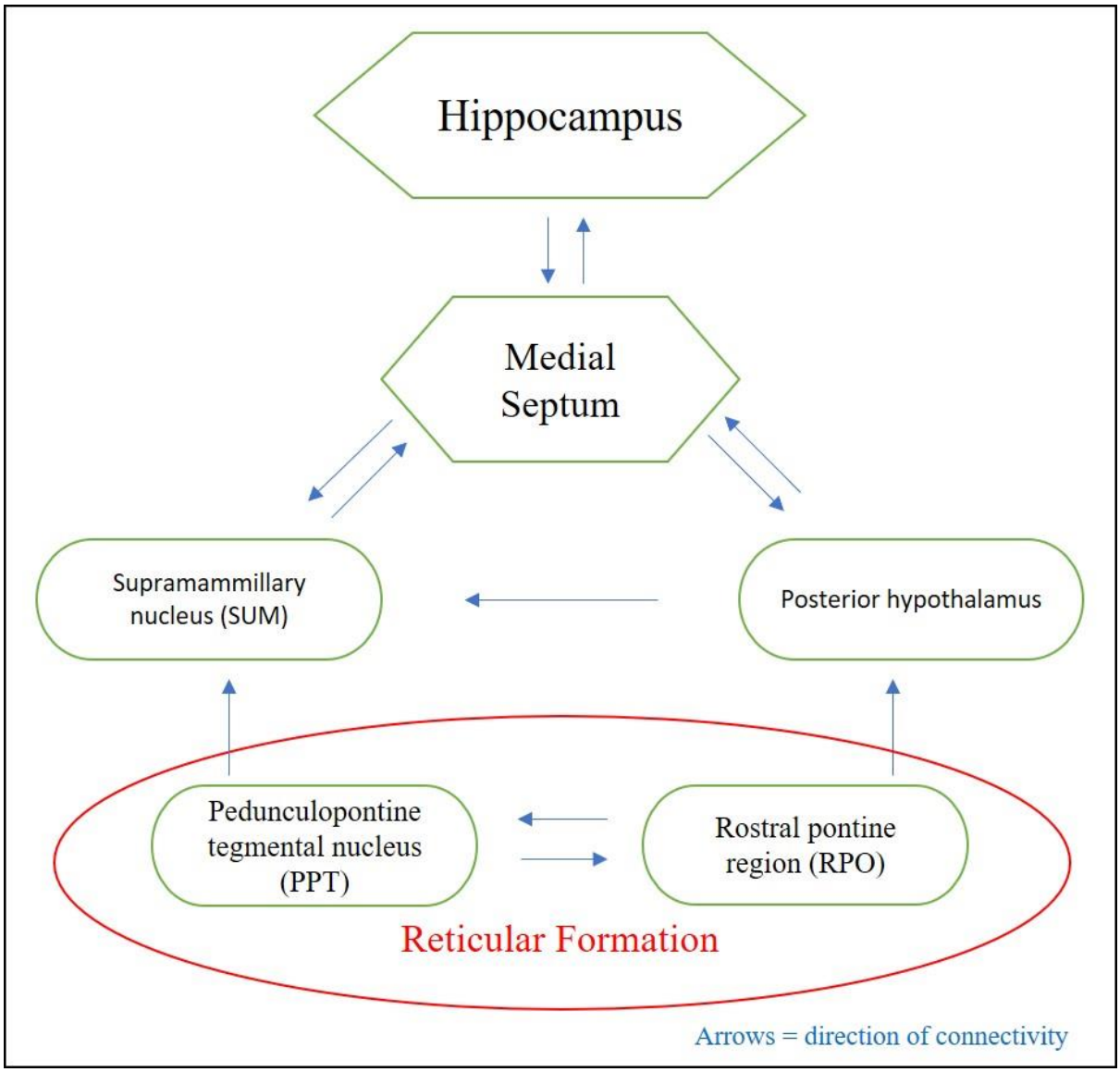


Figure 3.2 The main regions believed to be involved in the external generation of hippocampal theta.

3.3.1.1 The reticular formation

As mentioned above, the reticular formation is directly involved in the generation of theta rhythm. Studies have shown that the originating pathways involved in the external theta circuit can be traced back to two reciprocal nuclei in the reticular formation; the rostral pontine oralis (RPO) and the pedunculopontine tegmental nucleus (Vertes et al., 1993). Previous studies illustrated their role in the generation of theta rhythms by demonstrating that electrical stimulation of the reticular formation in an anesthetised rat results in synchronised

septal firing and hippocampal theta (Petsche, Gogolak, & van Zwieten, 1965; Petsche et al., 1962; Vertes et al., 1993). Research has also shown that increasing the level of reticular stimulation linearly increases theta frequency in both anaesthetised and freely-moving rats (McNaughton & Sedgwick, 1978). Hippocampal theta rhythm can also be produced by injecting carbachol in both the RPO and PPT, suggesting that a cholinergic input modulates the reticular formation's generation of theta rhythm (Bland, Oddie, Colom, & Vertes, 1994; Petsche et al., 1965; Vertes et al., 1993). Although the RPO and PPT are reciprocally connected, they each project to differing subcortical regions involved in the generation of theta rhythm. The RPO projects to the posterior hypothalamus, whilst the PPT projects to the SUM and the septum.

3.3.1.2 Posterior hypothalamus

The posterior hypothalamus, which is part of the caudal diencephalic region, receives projections from the reticular formation and projects through the supramammillary nucleus (SUM) on its way to the medial septum. Microinfusions of procaine or the lesioning of the posterior hypothalamus abolishes hippocampal or septal theta rhythms (Bland et al., 1994; Bland, Konopacki, Kirk, & Dickson, 1995; Oddie, Bland, Colom, & Vertes, 1994; Robinson & Wishaw, 1974). Same as with the reticular formation, electrical stimulation of the posterior hypothalamus effects theta rhythms in the hippocampus and the medial septum (Bland, Colom, & Ford, 1990; Bland & Vanderwolf, 1972).

There is evidence to suggest that the posterior hypothalamus plays a role in the locomotion component of 'Type 1' theta (Kramis et al., 1975). Research has demonstrated a linear relationship between the intensity of electrical stimulation in the posterior hypothalamus, wheel-running speed and hippocampal theta frequency. Lesions to the posterior hypothalamus reduces locomotion speed and hippocampal theta, whilst microinfusions of procaine impedes upon stimulation-induced wheel-running and theta frequency in the

posterior hypothalamus (Oddie, Stefanek, & Kirk, 1996). In freely moving rats, studies have shown that the lesioning of the posterior hypothalamus not only reduces theta frequency, but it also reduced voluntary movement whilst keeping 'involuntary' movement intact (Robinson & Wishaw, 1974). Taken together, these studies appear to support the notion that the posterior hypothalamus plays a major role in 'Type 1' theta generation.

3.3.1.3 Supramammillary nucleus

Previous research indicated that the medial septum was the only region in which tonic (non-bursting, non-theta synchronous firing) input from the reticular formation was converted to a rhythmical pattern that was relayed to the hippocampal formation to generate theta rhythms (Petsche et al., 1962). Subsequent research, however, found that neurons in the supramammillary nucleus (SUM) fire in burst that synchronise with theta and are undisturbed by the blocking of the medial septum (Bland et al., 1995; Kirk & McNaughton, 1991). The cells within SUM demonstrate this rhythmicity, whilst cells in the posterior hypothalamus do not. This would suggest that the SUM is an important bridge between the reticular formation and the medial septum in the generation of hippocampal theta.

Research using anaesthetised rats found that microinfusions of procaine into the SUM resulted in the reduction of hippocampal theta frequency and amplitude of an RPO-stimulated rat, whilst microinfusions of procaine to the medial septum reduced theta amplitude but had little effect on theta frequency. This result would suggest that the SUM play a major role in the encoding of theta frequency in an anaesthetised rat (Kirk & McNaughton, 1993). In freely-moving rats, injections of chlordiazepoxide into the SUM reduces theta frequency (McNaughton et al., 1995). Lesion studies, however, suggests that the SUM cannot be the only nuclei involved in the generation of theta rhythm as these studies have resulted in a minor reduction in frequency of reticular-stimulated theta (McNaughton et al., 1995).

The SUM is also thought to be involved in the 'Type 2' or immobility/arousal theta rather than in 'Type 1" movement theta. This can be seen with the microinfusions of procaine into the SUM of anaesthetised rats resulting in the reduction of theta frequency of reticular-elicited theta (Kirk & McNaughton, 1993), whilst the injection of procaine in a freely-moving rat appears to have no effect on theta frequency (Kirk, 1998). Additionally, lesions of the SUM have produced increases in approach and aggression in social interactions (Olivier et al., 1983) and produces changes in defensive behaviours in fear conditioning tasks (Pan & McNaughton, 2002). Taken together, the data supports the notion that the SUM is more involved in 'Type 2' theta mechanisms than 'Type 1.'

3.3.1.4 Medial septum and diagonal band of Broca

The medial septum and diagonal band of Broca's (MS/DBB) connection to the hippocampus is considered to be the minimal requirement to drive hippocampal theta activity (Bland & Oddie, 2001). Lesioning of the MS/DBB, along with the lesioning of the fimbra-fornix, which connects the medial septum to the hippocampus, completely abolishes theta rhythms (Buzsáki et al., 1983; Green & Arduini, 1954; Petsche et al., 1962; Stewart & Fox, 1990). Theta rhythms can also be temporarily abolished by microinfusions in the MS/DBB by the anaesthetic procaine (Bland & Oddie, 1998; Kirk & McNaughton, 1993).

Cholinergic and GABAergic inputs in the MS/DBB both play a major role in the generation of hippocampal theta. GABAergic neurons of the medial septum generate and maintain hippocampal theta rhythms by pacing GABAergic interneuron and pyramidal cells in the hippocampus (Freund & Antal, 1988; Goutagny, Jackson, & Williams, 2009), whilst cholinergic cells are thought to modulate hippocampal theta amplitude (Lee, Chrobak, Sik, Wiley, & Buzsáki, 1994). GABAergic neurons in the septum send inhibitory projections and cholinergic neurons send excitatory projections to the hippocampal formation (Lee et al., 1994). GABAergic neurons in the MS/DBB are thought to be responsible for maintaining the

septum's synchronicity by inhibiting cholinergic neurons (Brazhnik & Fox, 1997). Although it appears that GABAergic neurons are important in generating hippocampal theta rhythms (Brazhnik & Fox, 1997; Lee et al., 1994), the exact mechanisms involved in the pacemaking activity is relatively unknown (Sotty et al., 2003; Zhang, Lin, & Nicolelis, 2010, 2011). One possible explanation pertains to GABAergic septo-hippocampal neurons having the pacemaking current I_h. I_h current is known to contribute to the expression of rhythmic bursting and network oscillations throughout the brain (Dickson et al., 2000; Lüthi & McCormick, 1998; Thoby-Brisson, Telgkamp, & Ramirez, 2000), and with GABAergic neurons in the medial septum it has been shown that the application of an I_h blocker (ZD7288) reduces spontaneous firing of GABAergic neurons in rat brain slices (Xu, Datta, Wu, & Alreja, 2004).

Cholinergic and GABAergic neurons in the medial septum also appear to play a major role in the modulation of 'Type 1' and 'Type 2' hippocampal theta. As mentioned previously, Kramis and colleagues (Kramis et al. 1975), on the basis of pharmacological studies, defined 'Type 1' theta as atropine-resistant, which occurs during voluntary movement, whilst 'Type 2' theta is defined as atropine-sensitive, associated with immobility and occurs during urethane anaesthesia. Initially, it was thought that cholinergic neurons in the septum mediated 'Type 2' theta, which was supported by several observations (Smythe, Colom, & Bland, 1992). However, subsequent studies found that both 'Type 1' and 'Type 2' theta remains present after the lesioning of cholinergic neurons of the septo-hippocampus. The prevailing idea was then that hippocampal theta generation is modulated by the co-activation of cholinergic and GABAergic neurons in the medial septum (Smythe et al., 1992; Stewart & Fox, 1990). This idea finds support in a study performed by Yoder & Pang (2005), in which the authors examined the role of cholinergic and GABAergic septo-hippocampal neurons on hippocampal theta activity during urethane anaesthesia and locomotion. The authors

intraseptally administered kainic acid (KA) to selectively eliminate GABAergic MS-DBB neurons and 192 IgG-saporin (ISAP) to selectively eliminate cholinergic MS-DBB neurons. They found that either intraseptal KA or ISAP significantly reduced hippocampal theta activity in urethane anaesthetised rats, suggesting that both cholinergic and GABAergic neurons in the MS-DBB are integral for 'Type 2' hippocampal theta activity. By contrast, the authors found that either KA or ISAP treated rats did not see a significant reduction in hippocampal theta activity during locomotion, suggesting that 'Type 1' theta remains intact despite the loss of either cholinergic or GABAergic MS-DBB neurons. Taken together, the data supports the notion that the MS-DBB plays an important role in 'Type 2' hippocampal theta activity and is involved in the modulation of 'Type 1' hippocampal theta activity.

3.3.1.5 Theta-ON and Theta-OFF cells

Within the rodent hippocampal formation there is a major class cells known as theta cells. Theta cells were initially classified as such based on their phase correlation to EEG theta pattern and by the increase of the firing rate during theta. The class of theta cells expanded to include cells which firing rates decreased during theta (O'Keefe, 2007). Colom & Bland (1987) identified four classes of theta cell, which include theta-ON and theta-OFF, which were subdivided into two subtypes; phasic and tonic. Theta-ON cells firing rates increase during theta activity and normally occur during locomotive behaviours such as walking, running or swimming, whilst Theta-OFF cells firing rates decrease during theta activity and have the opposite behavioural correlates to that of Theta-ON cells.

The phasic and tonic subtypes are characterised by the firing pattern each cell produces during theta activity. Phasic firing is defined as having a consistent phase relation to theta activity, whilst tonic firing is defined by a cell's non-bursting discharge pattern with no steady phase relation to theta activity (Colom & Bland, 1987).

The pharmacological component of theta-ON and theta-OFF cells appear to be crucially modulated by cholinergic and GABAergic projections from the MS-DBB (Colom & Bland, 1987). GABAergic projections to the hippocampus reduce inhibition levels by inhibiting theta-OFF cells, whilst cholinergic projections provide the afferent circuitry drive for theta-ON cells (Colom & Bland, 1987).

3.3.2 Intrinsic oscillatory properties in hippocampal neurons

Cells within the hippocampus possess an intrinsic ability to oscillate. The membrane potential oscillation (MPO) of a cell is the natural frequency at which the membrane potential of the cell fluctuates. Evidence that hippocampal neurons act as oscillators can be found in hippocampal slice studies which showed that depolarization via injected currents causes membrane potentials oscillations in the theta-frequency without the need for action potentials and that the oscillation frequency varies with injected currents (Leung & Yim, 1991). A cell's resonance is described as the ability of that cell to selectively respond to inputs at preferred frequencies (Hutcheon & Yarom, 2000). Hippocampal CA1 cells have been shown to demonstrate hippocampal resonance at 3-10 Hz with injected sinusoidal current (Leung & Yu, 1998). Whole-cell patch-clamp recordings with applied sinusoidal inputs (1-100 Hz) to CA1 pyramidal cells also preferentially fired at a 2-7 Hz range (Pike et al., 2000). It should be noted that oscillations of CA1 hippocampal cells have been shown to not be affected by blockade of glutamatergic and GABAergic transmission suggesting that the oscillations were not synpatically driven (Chapman & Lacaille, 1999).

3.3.3 Theta rhythms in non-human primates and humans

Hippocampal theta is relatively easy to record from animals such as rabbits, rats, mice and cats. However, stable recordings of hippocampal theta from non-human primates and humans has been more challenging. For example, in urethane anaesthetised monkeys, 7-9 Hz theta-like patterns, which were shorter in durations compared to urethane anaesthetised rats, has

been recorded from the hippocampal formation (Stewart & Fox, 1991). The theta recorded from these urethane-anaesthetised monkeys was abolished by the administration of atropine which suggests the theta recorded is, in some respects, similar to the atropine-sensitive theta found in rats under urethane anaesthesia.

In terms of recording human hippocampal theta, it has been difficult establishing its presence in the human brain. For one, recording directly from the human hippocampus is quite challenging. The studies in which this has been done used patients with epilepsy who require therapeutic implantation of depth electrodes (Caplan et al., 2003). Another issue that arises from recording theta from non-human primates and humans is when the subject is immobile which is not an ideal condition in which to record Type 1 theta. It should also be noted that EGG is generally recorded from electrodes placed on the scalp of the subject which may act as a sort of barrier to detecting theta activity (O'Keefe, 2007).

Using a variety of different techniques, researchers have been able to record hippocampal theta activity in humans. For example, using magnetoencephalography (MEG), Tesche & Karhu (2000) reported theta activity in the hippocampus accompanying subjects performing a working memory tasks. As mentioned previously, researchers have performed studies using subdural or depth electrodes on patients with epilepsy. In a number of these studies, patients performed virtual movement (i.e., driving a taxi, virtual Morris Water Maze) using a virtual reality video game, with researchers finding theta oscillations increasing as patients performed these virtual movements (Cornwell, Johnson, Holroyd, Carver, & Grillon, 2008; Ekstrom et al., 2005; Kahana, Sekuler, Caplan, Kirschen, & Madsen, 1999).

As briefly discussed in chapter 1 (section 1.3), there are two sets of functional associations of the hippocampus that tend to dominate theoretical overviews of the hippocampal role in the brain, with one set emphasising the role of the hippocampus in cognition, and the other set

emphasising its role in emotion. The use of virtual reality navigation tasks to examine human hippocampal theta activity in epileptic patients using depth electrodes has provided researchers with the means to use analogous rodent spatial memory models (Burgess et al., 2002). Kahana and colleagues (Kahana et al., 1999) published the first study which examined theta activity related to movement in the human hippocampus. Subsequent studies found neural firing in the human hippocampus correlated with direction, goal and place tasks (Ekstrom et al., 2003; Jacobs, Kahana, Ekstrom, Mollison, & Fried, 2010). Researchers have also attempted to categorise the human equivalent of 'Type 1' hippocampal theta activity using intracranial EEG or iEEG studies (Ekstrom et al., 2005) and MEG studies (Cornwell et al., 2008). Research has found that movement-related theta is highest in amplitude at the initiation of movement, similar to rodent hippocampal theta activity. They also found that theta power rather than frequency (as seen in rodents) reduces in a novel environment (Kaplan et al., 2012).

Researchers have also attempted to interpret the spatial navigation-related theta results for clinical application. For example, Cornwell and colleagues (Cornwell et al. 2010) used a virtual navigation task and compared healthy participants to patients with depression, and found that hippocampal theta was reduced in depressed patients compared to the healthy participant control group during virtual navigation. Cornwell et al. (2012) also found whilst investigating threatening (unpredictable shocks) versus non-threatening (no shocks) conditions during virtual navigation of a Morris water maze that self-reported anxiety during the threatening navigation condition correlated with increased left anterior (temporal) hippocampal theta (2-6 Hz activity), which was consistent with the region's involvement in modulating conditioned and innate fear.

With the increasing use of MEG and iEEG techniques in cognitive neuroscience, researchers may be able to bridge the gap in electrophysical data between non-human primates and human hippocampal theta activity with rodent hippocampal theta activity.

3.4 Theta and anxiety

A considerable amount of research has been dedicated to the examination of the role the hippocampus plays in anxiety. The hippocampus has been associated with emotional processes since Papez (1937) theorised that the limbic system plays a major role in the regulation of emotions. More specifically, Gray (1982) and Gray & McNaughton (2000) have suggested that, together, the septum and hippocampus form the septohippocampal system. They theorised that the septohippocampal system functions as a 'behavioural inhibition system' (BIS), with the role of comparing anticipated anxiety with the perceived threat level; the BIS either inhibits or permits a fear/anxiety response. Anxiolytic drugs are thought to impair the function of BIS and the septohippocampal system.

Gray (1982) originally proposed that anxiolytic drugs reduce anxiety by the impairment of theta activity. Lesions to the hippocampus have been shown to reduce anxiety-like behaviour in animal models, such as the elevated plus maze, open field, light-dark box and shock-probe test (Bannerman et al., 2003; Briley, Chopin, & Moret, 1990; Deacon, Bannerman, & Rawlins, 2001; Kjelstrup et al., 2002; Pesold & Treit, 1992). Similar to the lesion studies, Gray and McNaughton (2000) proposed that the effects of anxiolytics drugs also impaired anxiety-like behaviours.

As mentioned previously, Type 2 hippocampal theta is sometimes associated with anxiety-like behaviours in animals. To reiterate, Type 2 theta rhythms occur during anaesthesia and periods of immobility (Kramis et al., 1975). Sainsbury and colleagues were able to induce type 2 theta in rodents in a high state of arousal and vigilance during anxiety tests which

involved the presence of a predator's smell (Sainsbury et al., 1987). Another example of anxiogenic stimuli inducing Type 2 theta involved exposing guinea pigs to a random auditory stimulus coupled with arousing owl sounds (Sainsbury & Montoya, 1984).

Subsequent research into the relationship between hippocampal theta and anxiety has mostly focused on the effects of arousing stimuli and/or anxiolytic drugs upon reticular stimulated hippocampal theta *frequency* (Green & Arduini, 1954; McNaughton & Sedgwick, 1978; Siok et al., 2009; Stumpf, 1965). One of the most comprehensive reviews on the effects of anxiety on elicited hippocampal theta focused on the effects of systemically administrated anxiolytic drugs (McNaughton et al., 2007). McNaughton and colleagues pointed out that high frequency trains of stimulation of the reticular formation not only elicit hippocampal theta, but also that the increase of stimulation produces a linear increase of theta frequency. The authors found that all known classes of drugs that are clinically effective in treating anxiety (barbiturates, benzodiazepines, 5-HT_{1A} receptor agonists, SSRIs) decrease reticular-elicited theta frequency in anaesthetised rats (McNaughton et al., 2007). More recently, Wells and colleagues (Wells et al., 2013) demonstrated a reduction in theta frequency in freely-moving rats when administered an anxiolytic. In this study, the researchers adapted the Burgess (2008) model, which suggests that theta frequency has two components; one corresponding to the slope of the theta-frequency-to-speed relationship and the other corresponding to the intercept component of the theta-frequency-to-speed relationship. Using this model, Wells and colleagues predicted that the administration of anxiolytic drugs would reduce the intercept component of the theta-frequency-to-speed relationship whilst having no affecting on the slope component. During the experiment the researchers had rats forage for blacken sweet rice in a black square-walled arena for 10 minutes per trial (four trials total), with a 30-minute inter-trial-interval. After the third trial, each rat was given an injection (i.p) of saline on non-test days, or an injection of an anxiolytic on test days. The researchers tested two

clinically established anxiolytic drugs (Chlordiazepoxide and buspirone) along with one putative anxiolytic drug (O-2545, a CB_1 agonist). They found that at the doses test, all anxiolytic drugs reduced the intercept component of the theta-frequency-to-speed relationship without affecting the slope component. Taken together, the McNaughton and colleagues' review, and Wells and colleagues' study empirically demonstrate that hippocampal theta plays a role in the modulation of anxiety.

One important aspect in the understanding of the relationship between hippocampal theta and anxiety comes from the finding that all known classes of anxiolytic drugs reduce reticular-stimulated theta (McNaughton et al., 2007). To further understand the significance of this finding, the following sections briefly outline the properties of different classes of anxiolytic drugs.

3.4.1 Classical anxiolytics

Anxiolytic drugs, in its most basic definition, is a drug that inhibits anxiety. Currently, there are eight recognised syndromes of anxiety: separation anxiety disorder, selective mutism, specific phobia, social anxiety disorder (social phobia), panic disorder, agoraphobia, generalised anxiety disorder (GAD), and anxiety disorder due to another medical condition ("American Psychiatric Association," 2013). Initially, when treating anxiety, the use of barbiturates was considered the sole option, even though barbiturates had limited efficacy and presented with adverse side effects, including sedation and addiction (Gray and McNaughton, 2000). Since its discovery in the mid-1950s, benzodiazepines have become the benchmark for anxiolytic drugs (Griebel & Holmes, 2013). Benzodiazepines, which are γ-aminobutyric acid (GABA) agonist, exert its effect by allosterically activating specific $GABA_A$ receptor subtypes to promote inhibitory neurotransmission in the brain. Although benzodiazepines like chlordiazepoxide and diazepam are efficacious in the acute treatment of generalised anxiety disorder (GAD), social anxiety disorder, and panic disorders (Baldwin, Ajel, & Garner, 2009;

Baldwin & Garner, 2008), the long-term use of benzodiazepines are also associated with adverse side effects such as anterograde amnesia, physical dependency, and decreased motor performance (Barker, Greenwood, Jackson, & Crowe, 2004; Grimsley & Jann, 1992; Gudex, 1991; Vgontzas, Kales, & Bixler, 1995). The therapeutic limitations of benzodiazepines have led to the search for compounds that were chemically dissimilar to benzodiazepines, with the addition of a more specific therapeutic action without the unwanted side effects.

The serotonin (5-HT) system has long been implicated in the modulation of anxiety (Griebel, 1995). Research has shown that genetic variations in humans and knockout mice in the 5-HT_{1A} receptor influence anxiety traits, and show increased anxiety-related behaviours, respectively (Belzung & Griebel, 2001; Hariri & Holmes, 2006; Jacobson & Cryan, 2009; Lesch et al., 1996). Goldberg & Finnerty (1979) described the 5-HT_{1A} receptor partial agonist buspirone as the first pharmacotherapeutic alternative to benzodiazepines for the treatment of GAD. Rickles and colleagues (Rickels et al., 1982) were the first to show its anxiolytic efficacy in a clinical trial. It is proposed that 5-HT_{1A} partial agonist exerts its anxiolytic action through the activation of 5-HT_{1A} heteroreceptors in forebrain areas (Goodfellow, Benekareddy, Vaidya, & Lambe, 2009; Zhang et al., 2010). Although effective in treating GAD, 5-HT_{1A} receptor targeted drugs are limited due to their first-pass hepatic metabolism (Gammans, Mayol, & Labudde, 1986), meaning their concentration is greatly reduced before reaching the systemic circulation.

Serotonin reuptake inhibitors (SSRIs) are antidepressants that have been shown to possess anxiolytic properties (Kent, Coplan, & Gorman, 1998). It is believed that SSRIs exert their therapeutic effects through the increase of extracellular 5-HT levels (Gartside, Umbers, Hajós, & Sharp, 1995). SSRIs, such has fluoxetine, have been shown to be effective in the treatment of GAD (Baldwin et al., 2009; Baldwin & Garner, 2008), and are among the most

commonly prescribed anxiolytics psychiatrist utilise (Griebel & Holmes, 2013). However, SSRIs are not effective in all patients and are associated with adverse effects such as sexual dysfunction, dermatological symptoms and psychiatric symptoms (Kennedy, Eisfeld, Dickens, & Bagby, 2000; Vaswani, Linda, & Ramesh, 2003).

3.4.2 Novel anxiolytics

As with the case of discovering benzodiazepines, 5-HT_{1A} partial agonist and SSRIs, research into clinically effective anxiolytic has brought forth a new wave of drugs. These 'novel' drugs enact on different neurotransmission systems, which has the potential to provide further knowledge into understanding anxiety modulation. Recently, there has been an increase in preclinical research on the anxiolytic properties of the glutamate and endocannabinoid systems (Griebel & Holmes, 2013). As it pertain to the glutamate system, in rodents, glutamate levels are altered in the presences of stressors; whilst in humans, abnormal glutamate levels have been observed in patients with anxiety disorders (Krystal et al., 2010). For example, metabotropic glutamate receptors (mGluRs), specifically mGluR1, mGluR2, mGluR3, and mGluR5 have been shown to have a role in the modulation of anxiety behaviours, with agonists at the specified mGluRs demonstrating anxiolytic effects across various rodent assays, such as the Vogel conflict test and elevated plus maze (Krystal et al., 2010). Another example can be found with NMDA (*N*-methyl-D-aspartate) receptors, which are glutamate-gated cation channels found in nerve cells (Blanke & VanDongen, 2009). Ketamine, an NMDA receptor antagonist, has been shown to be effective in the treatment of major depression (Sanacora, Zarate, Krystal, Manji, & Manji, 2008), which has led to preclinical studies into the potential anxiolytic effects of NMDA receptors. For instance, the NMDA receptor channel blocker MK-801 has shown anxiolytic effects across multiple assays (Criswell, Knapp, Overstreet, & Breese, 1994; Jessa, Nazar, Bidzinski, & Plaznik, 1996; Koek & Colpaert, 1991; Xie & Commissaris, 1992). Another potential glutamate-actin

target for anxiety is D-cycloserine, which is a partial NMDA agonist of the glycine site that indirectly increases glutamatergic activity in previously 'quiet' synapse, has been shown effective in treating various anxiety disorders (Gomperts, Rao, Craig, Malenka, & Nicoll, 1998; Norberg, Krystal, & Tolin, 2008; Ressler et al., 2004).

Endocannabinoids are endogenous lipid-based retrograde neurotransmitters that bind to cannabinoid receptors, which are expressed throughout the brain; particularly in regions that mediate anxiety, such as the hippocampus (Herkenham et al., 1990; Wang, Xie, & Dey, 2006). There is evidence that shows abnormalities in the endocannabinoid principal central nervous system (CNS) receptor, cannabinoid 1 (CB_1) is implicated in anxiety disorders (Gunduz-Cinar et al., 2013; Neumeister et al., 2013). Preclinical research into the effects of CB_1 agonists, antagonist and inverse agonists in behavioural models of anxiety have yielded some positive results (Griebel, Stemmelin, & Scatton, 2005; Haller, Varga, Ledent, & Freund, 2004; Moreira & Wotjak, 2009). However, there are complexities in the anxiety-related effects associated in the manipulations of CB_1 receptors (Griebel & Holmes, 2013). One potential explanation for these complexities comes from the diverse neurotransmitter systems. For instance, cannabinoids could be inhibiting GABA and glutamate in the brain, resulting in the modulation of neurotransmitters, altering their function on anxiety (Moreira & Wotjak, 2009).

Gabapentinoid is another 'novel' system being explored for its potential anxiolytic properties. Gabapentin and pregabalin are currently the only clinically-used gabapentinoid (Patel & Dickenson, 2016). Their mechanism of action is different from the pre-defined classic and novel anxiolytics in that they bind to $\alpha_2\delta$ subunit of the presynaptic neuron's voltage-gated calcium channel (Kavoussi, 2006). For the interest of this thesis, the anxiolytic efficacy of

pregabalin will be the focus, with a more detailed review of pregabalin's anxiolytic efficacy detailed in Chapter 4.

3.4.3 Classic and novel anxiolytic drugs reduce theta frequency

As mentioned previously (section 3.4), research into the effects of anxiolytic drugs have demonstrated that neurochemically dissimilar anxiolytics, like barbiturates, benzodiazepines, 5-HT_{1A} receptor agonists and SSRIs reduce the frequency of *reticular-elicited* theta. It should be noted that drugs that are not anxiolytic (e.g. antipsychotic and sedative) do not reduce the frequency of reticular-elicited. This action of theta frequency reduction is thought to be the best animal-based predictor of a clinically effective anxiolytic drug (John, Kiss, Lever, & Erdi, 2014). In fact, McNaughton and colleagues (McNaughton et al., 2007) argue that the anxiolytic reduction of theta frequency has produced no false positives or negatives, and has predicted anxiolytic effect of several drugs, thus far. For instance, Siok and colleagues (Siok et al., 2009) were able to demonstrate the pre-defined novel anxiolytic pregabalin reduced the frequency of reticular-elicited theta. More recently, research into the effects of anxiolytics on hippocampal theta frequency has examined the effects of anxiolytics on naturally occurring theta. Woodnorth & McNaughton (2005) were able to demonstrate a reduction of hippocampal theta frequency on naturally occurring theta using a classic anxiolytic, chlordiazepoxide (CDP, benzodiazepine). Wells and colleagues (Wells et al., 2013) were also able to demonstrate a reduction of hippocampal theta frequency in naturally occurring theta using the classic anxiolytic buspirone (a partial 5-HT_{1A} receptor agonist), as well as CDP. Importantly, the Wells and colleagues' study was also able to demonstrate that the novel endocannabinoid O-2545 (CB_1 agonist) reduced hippocampal theta frequency in naturally occurring theta as well. These two studies, along with the McNaughton and colleagues comprehensive review, demonstrates six neurochemically dissimilar drugs reduce hippocampal theta.

One important aspect to consider is how anxiolytic drugs have been screened. Classic behavioural animal models have had vary degrees of success when predicting the clinically efficacy a non-GABAergic modulating anxiolytic (Rodgers, 1997). An example of this would be buspirone. Buspirone, as described above (section 3.4.1), is a partial 5-HT_{1A} receptor agonists that has successfully been shown to reduce the frequency of reticular-elicited theta (McNaughton & Coop, 1991) despite its anxiolytic effects not being detected using classic animal models (Taylor & Moon, 1991). One explanation for classic animal models producing false positive and false negatives is that those models (e.g. EPM and open field) have been validated mostly by benzodiazepines and therefore, may be a sensitive test for specific type of anxiety that is mediated by GABAergic mechanisms (Green & Hodges, 1991). Thus, reiterating the viewpoint that the screening for changes in hippocampal theta frequency may be a more effective technique in preclinical testing of anxiolytic drugs (McNaughton et al., 2007).

3.5 Theta and spatial cognition

3.5.1 Theta and movement

Hippocampal theta occurring during voluntary movement was initially reported by Vanderwolf (1969). Subsequently, the two types of theta were defined based on their behavioural and pharmacological correlates. ‘Type 1’ theta is observed during transitional movement, whilst ‘Type 2’ theta is observed during REM sleep or alert immobility (Kramis et al., 1975). O’Keefe & Nadal (1978) suggested that Vanderwolf’s characterisation of movement related theta (‘Type 1’) needed to be reconceptualised as a class of movement rather than just movement. As it has been mentioned before, Type 1 theta is observed during movement related behaviours, such as in walking, jumping and swimming. These types of movements can be described as translation through space. Thus, O’Keefe & Nadel (1978)

suggestion that the behavioural correlate observed during Type 1 theta is present during behaviours in which the animals head is translated through space.

3.5.2 Theta frequency and speed relationship

The relationship between theta frequency and speed of movement was initially reported by McFarland, Teitelbaum, & Hedges (1975) when they found that hippocampal theta increased linearly with running speed when a rat was running on a treadmill. Rivas et al, (1996) also reported a positive linear relationship between hippocampal theta frequency and running speed with guinea pigs, also running on a treadmill. Rivas and colleagues recorded theta frequency from dorsal CA1 in the guinea pigs and found that the frequency increase co-occurred with an increase in mean firing rate of neurons. They suggested that the increase in theta frequency was influenced by the excitation of the neurons. Theories surrounding the proposed input of speed into the hippocampus has implicated the posterior hypothalamus (Oddie et al., 1996). Oddie and colleagues implanted rats in the posterior hypothalamus with the purpose of electrical stimulation. They also implanted a hippocampal recording electrode and cannula into the medial septum. They placed rats in a wheel and simultaneously stimulated the posterior hypothalamus and recorded from the medial septum. They reported a positive linear relationship between the stimulation of the posterior hypothalamus and running speed. They also found that when they infused the local anaesthetic procaine into the medial septum, it not only disrupted wheel running, but also hippocampal theta. The stimulation of the posterior hypothalamus 30-60 minutes post-infusion of procaine gradually restored wheel running and hippocampal theta. The infusion of saline to the medial septum and procaine to the lateral septum and paraventricular thalamic nucleus had no effect on wheel running and hippocampal theta frequency. Similarly, Sławińska & Kasicki (1998) stimulated the posterior hypothalamus and found that the increase of stimulation increased

both locomotion and hippocampal theta. These two studies demonstrated that a linear relationship between that of running speed and hippocampal theta frequency.

An important concept within this thesis is that the two forms of hippocampal theta, 'Type 1' and 'Type 2', are not exclusive from one another. As noted previously, 'Type 2' theta is thought to be observed during periods of immobility and associated with anxiety modulation, whilst 'Type 1' theta is observed during translational movement and associated with spatial cognition. However, a more nuanced view is possible, that both forms of theta are simultaneously active, implying that both spatial cognition and anxiety functions are 'in action' during locomotion. As detailed above, there is a relationship between theta frequency and speed; that hippocampal theta frequency increases as speed increases. This is the basis of deriving the two components of theta frequency, the slope component associated with spatial cognition and the intercept component associated with anxiolysis. Some care needs to be taken in deriving the two measures of slope and intercept. Wells and colleagues analysed locomotion by breaking up speeds into 10 relatively small bins (2.5cm/s wide) and by applying both upper (30cm/s) and lower limits (5cm/s) of speed. Using several small bins ensures consistency across conditions. The use of too few bins which are large in size (e.g. (10 cm/s wide) could result in mean speed within a bin being different across conditions (e.g. drug vs saline). The use of too many could result in too few samples. Importantly, intercept is derived as an extrapolation of the theta-frequency to running speed regression line to 0cm/s, rather than as the theta frequency when the rat is stationary, the point being that the hippocampus typically falls into a different oscillatory state during stationary behaviour; LIA, with the frequent presence of high frequency ripple states. Applying an upper limit helps to exclude artefacts due to headshake and avoids the problem of too few samples at rather high speeds. In this thesis, the same analytical methodology as Wells et al (2013) was applied and is briefly explained in Chapter 5 (section 5.7.5).

3.5.3 Theta and phase precession

Phase precession was initially discovered when O'Keefe & Recce (1993) observed a relationship between CA1 place cells and hippocampal theta. Upon placing a rat on a linear track, they observe that hippocampal place cells spikes fired late relative to a cycle of theta EEG as the rat entered the place field (Figure 3.2). As the animal progressed through the field, the neurons discharged progressively earlier in the EEG theta cycle. Thus, the theta-phase of firing correlated with the location of the rat within the place field, independently of the running speed.

It is believed place cells fire rhythmically at a frequency higher than LFP theta (O'Keefe, 2007). This phase-location relationship led to the idea that the firing activity of a neuron would code information not only with its rate but also on a temporal dimension (Huxter, Burgess, & O'Keefe, 2003). The timing of the discharge with respect to the phase of the ongoing network oscillation would provide further, independent information.

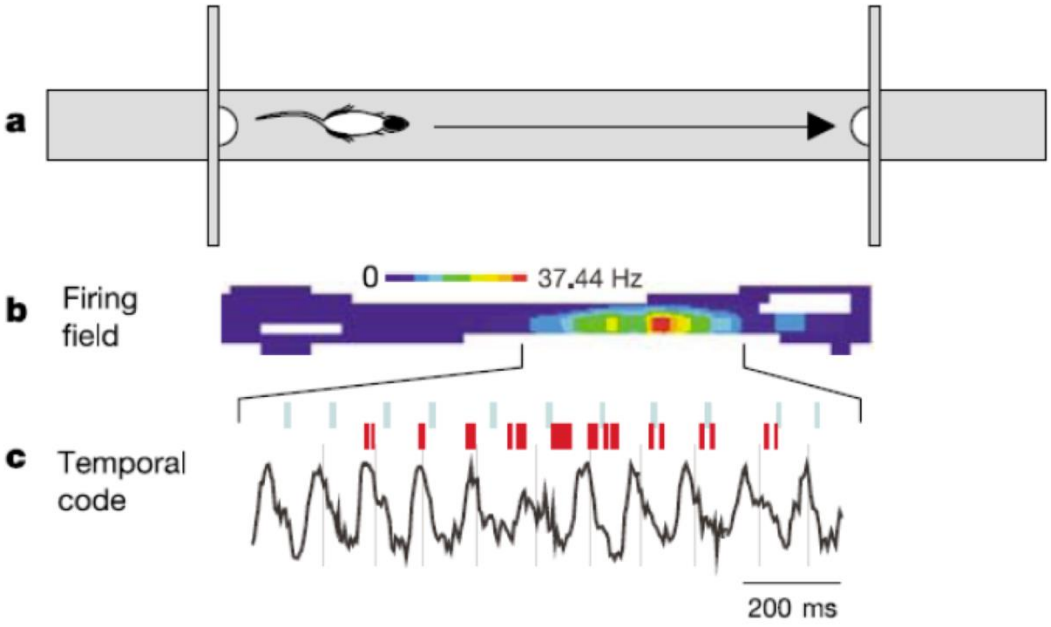


Figure 3.3 **Phase precession of place cell firing as a rat runs along a linear track. A)** the rat is motivated to run back and forth to collect food rewards placed at both ends; **B)** The firing field was created from multiple runs from west to east; **C)** Theta rhythm and place cell firing (the cell spikes are represented by the red lines) for the same cell on a single eastward run. The grey lines above the spikes indicate + to – zero crossings (0/360°) for each theta cycle. Line through each theta cycle indicate 270°. Bursts of spikes occur at higher than theta frequency, causing each successive burst to move to an earlier phase of the theta cycle. Reprinted from (Huxter et al., 2003)**.**

3.5.4 Theta frequency and temperature

Previous studies have shown a positive relationship between theta frequency and brain temperature (Whishaw & Vanderwolf, 1971). For instance, Deboer (2002) used male Djungarian hamsters to show that moderate changes in brain temperatures can significantly influence EEG frequencies during REM sleep under spontaneous euthermic changes. More recently, Wells et al. (2013) demonstrated a positive correlation between brain temperatures and theta frequency. Wells and colleagues examined the relationship between theta frequency and temperature by first examining if the application of anxiolytic drugs correlated between the intercept component of the theta-frequency to speed relationship and changes in brain temperatures. They concluded that the intercept-reducing properties of anxiolytic drugs could

not be attributed to the effect of changes in brain temperatures. Secondly, the researchers examined the relationship temperature and the intercept and slope components in drug-free conditions. They found that changes in brain temperature did not correlate with the intercept component, but the slope component and temperature were positively correlated. This would suggest that the increase in movement should be seen in the changes in brain temperatures of freely-moving rats.

3.6 Dissociating two component model: Anxiety and Environmental Familiarity

As mentioned earlier, studies that have focused on theta amplitude/power have noted that hippocampal theta has atropine-sensitive and atropine-resistant components that have led to the concept of two components of theta: 'Type 1' and 'Type 2'. Type 1 theta is attributed to movement whilst Type 2 theta is attributed to immobile attention/arousal. A recent model of oscillatory interference (Burgess, 2008) has presented certain testable predictions in relation to Type 1 and Type 2 theta component of the theta-frequency-to speed relationship. Burgess (2008) proposes that both components of theta simultaneously contribute to theta during locomotion rather than the two components being mutually exclusive. It has been noted that there is a broadly linear relationship between theta frequency and running speed; as in as running speeds increase, so does theta frequency. The Burgess (2008) model links the Type 1 and Type 2 theta to dissociable components of the relationship of theta frequency to ($f_\theta(t)$) to running speed (s(t)):

$$f_\theta(t) = f_0 + < \beta > s(t)$$

where the rate of increase with running speed (< β >, 'slope') reflects the presence of 'velocity-controlled oscillators' in the septo-hippocampal system (Burgess, 2008): neurons whose firing shows theta-band modulation who frequency increase with running speed, as also seen in place (Geisler, Robbe, Zugaro, Sirota, & Buzsáki, 2007) and grid cells (Jeewajee

et al., 2008a) This slope component is identified with Type 1 theta or movement related theta (entorhinal cortex dependent). The second component (f_0, 'intercept') is identified with Type 2 theta; independent of both movement and entorhinal cortex.

In summary, taking into account the broadly linear relationship between theta frequency and running speed and by deriving a regression line from said relationship, the slope and intercept at 0 cm/s would correspond with Type 1 and Type 2 theta components. Burgess' (2008) model predicts a dissociation between the intercept component, related to arousal/anxiety and the slope component, related to spatial representation in translational movement at 0 cm/s.

As previously stated, Gray & McNaughton (2000) have reported that a wide range of neurochemically dissimilar anxiolytic drugs commonly reduce the frequency of reticular-elicited theta. Woodnorth & McNaughton (2005) have also reported the benzodiazepine chlordiazepoxide reduces naturally occurring theta. This brings forth the notion that the frequency reduction previously reported could reflect a reduction of the frequency of Type 2 theta specifically. Thus, a prediction could be made that the theta mechanism represented by f_0 (intercept) is reduced by anxiolytic drugs. This prediction was originally tested by Wells et al, (2013) using the benzodiazepine chlordiazepoxide and the partial 5-HT_{1A} receptor agonists buspirone in freely moving rats. Study 1A of this thesis aims to replicate the results from Wells et al, (2013) using the novel anxiolytic pregabalin. An example of how this is predicted to look is shown in figure 3.4.

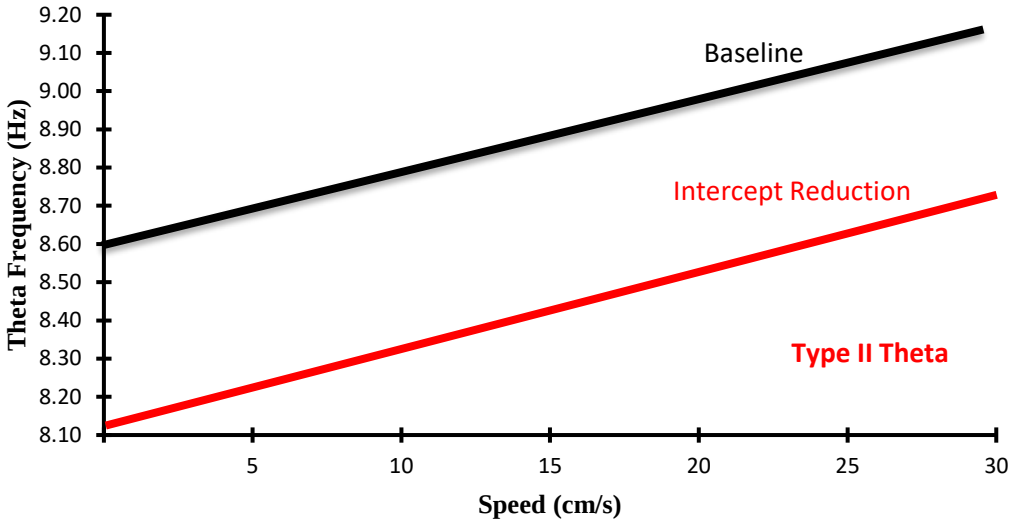


Figure 3.4 Hypothetical example of intercept reduction of the theta-frequency-to-speed relationships. The baseline (black) represents the pre-injection trial. The intercept reduction (red) represents the post-injection trial, after systemic administration of the anxiolytic drug.

As with anxiolytics, environmental novelty has been shown to reduce average hippocampal theta frequency (Jeewajee et al., 2008b). Burgess' (2008) model relates slope (< β >) inversely to grid spatial scale with grid spatial scale increasing in a novel environment (Barry, Ginzberg, O'Keefe, & Burgess, 2012). Thus, a prediction could be made that novelty would specifically decrease the slope (< β >) component of the frequency-to-speed relationship. As with the first predictor of anxiolytic drugs reduces that intercept (f_0) of the frequency-to-speed-relationship, Wells et al., (2013) also tested this prediction. They were able to show that novelty decreased slope of the frequency-to-speed relationship. Figure 3.5 illustrates how this is predicted to look.

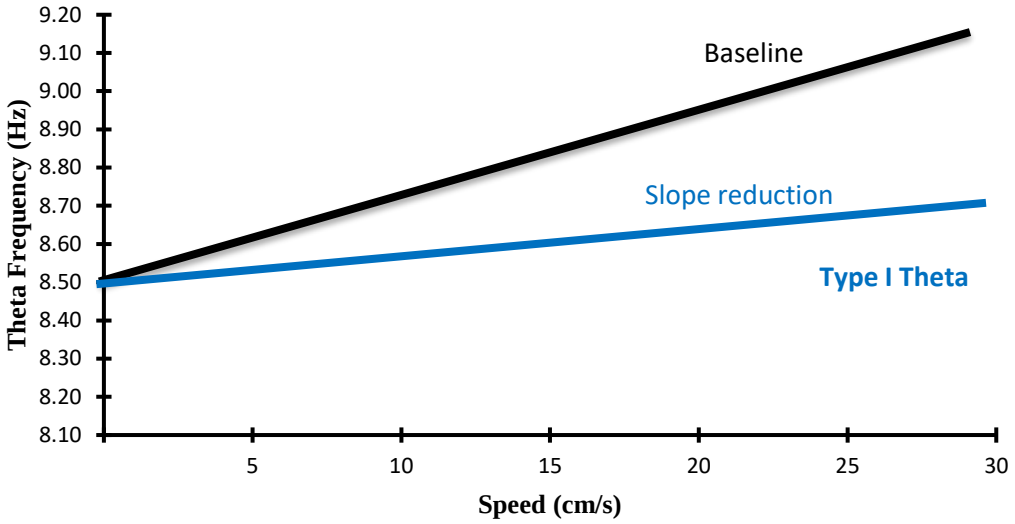
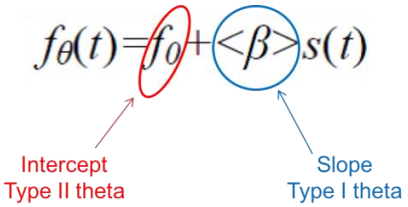


Figure 3.5 Hypothetical example of a reduction of slope of the theta-frequency-to-speed relationship. The baseline (black) represents the familiar environment. The 'slope reduction' represents the predicted effect of exposure to a novel environment.

In summary, Burgess' (2008) two-component model suggest that the slope and intercept components of the theta-frequency-to-speed relationship are correspond with Type 1 and Type 2 theta, respectively.



3.8 Aims of Study 1A and 1B

Research into the effect of anxiolytic drugs in the reduction of theta frequency has been conducted for more than two decades (McNaughton et al., 2007), however the exploration of the effects of various anxiolytic drugs on the slope and intercept theta-frequency-to-speed relationship has not been widely tested. Wells et al. (2013) firmly demonstrated that there is a

dissociable relationship between the slope (sensitive to novelty) and intercept (sensitive to anxiety).

The aim of Study 1A (Chapter 6) and Study 1B (Chapter 7) is to expand upon the research of Wells et al, (2013) by testing the Burgess (2008) model using the anxiolytic drug pregabalin. Briefly, pregabalin has a proposed mechanism of action that is different from any known anxiolytic (e.g., barbiturates, benzodiazepines, SSRIs, 5-HT_{1A} receptor agonists). Pregabalin selectively binds to the $\alpha_2\delta$ subunits of the *presynaptic* neuron's voltage gated calcium channels. Pregabalin's efficacy in the treatment of anxiety varies across models (human and animal models) and across doses. The purpose of Study 1A, following the model of Wells et al, (2013), is to test the effects of pregabalin on slope and intercept component of the theta-frequency-to-speed relationship. The experimental prediction being that pregabalin will reduce the intercept component, whilst having no effect on the slope component of the theta-frequency-to-speed relationship. Study 1A also examines the effect of pregabalin upon spatial properties of hippocampal place cells with the prediction that pregabalin will have no significant effect on hippocampal place cells. The basis of this prediction is that a purely intercept-affecting effect should not, in and of itself, affect spatial coding. Rather the Burgess 2008 model links the slope component, not the intercept component, to spatial coding.

Study 1B analyses environmental familiarity's (Chapter 7) effect on the slope component of the theta-frequency-to-speed relationship by examining the slope recorded from the initial day of exposure through to the final experimental day. The prediction is that the slope component should increase with environmental familiarisation. Study 1B also examines the prediction that slope should predict spatial stability.

In preparation for Study 1A (Chapters 6) and Study 1B (Chapter 7), Chapter 4 presents a review of pregabalin and Chapter 5 presents the general methods used in the experiment.

4 Pregabalin

For over 50 years, the most preferred method in the pharmacological treatment of anxiety disorders has been the use of gamma-Aminobutyric acid (GABA) agonists (benzodiazepines), antidepressants that act on serotonin neurotransmitters (serotonin-reuptake inhibitors or SSRIs) and partial 5-HT_{1A} receptor agonists, such as buspirone (Kavoussi, 2006). Long-term use of benzodiazepines has been associated with anterograde amnesia, physical dependence, and decreased motor performance and co-ordination (Barker et al., 2004; Grimsley & Jann, 1992; Gudex, 1991; Vgontzas et al., 1995). SSRIs have been associated with neurological symptoms, psychiatric symptoms, gastrointestinal symptoms, insomnia, dermatological symptoms, sexual dysfunction, paraesthesia, headache and diarrhoea (Kennedy et al., 2000; Spigset, 1999; Trindade, Menon, Topfer, & Coloma, 1998); whilst partial 5- HT_{1A} agonist are associated with gastrointestinal issues. In part, the adverse effects that are associated with these medications have motivated some researchers to explore alternative pharmacological mechanisms in treating anxiety disorders.

Pregabalin (Lyrica®), has been described as "an analogue of the neurotransmitter γ-aminobutyric acid (GABA) which has analgesic, anticonvulsant and anxiolytic effects," (Tassone, Boyce, Guyer, & Nuzum, 2007). Despite the original associations with GABA, pregabalin has a calcium-channel mechanism of action that is different from any other anxiolytic. This mechanism of action is believed to be what gives pregabalin its analgesic and antiepileptic effects (Kavoussi, 2006). This chapter will briefly investigate the original conception of pregabalin, its proposed novel mechanism of action, and the pre-clinical and clinical data, which supports this notion.

4.1 Pregabalin: a brief history

Pregabalin is closely related to an anticonvulsant drug known as Gabapentin (Neurontin®, (Dworkin & Kirkpatrick, 2005)). Gabapentin, which interacts with the L-amino acid transport system (Sills, 2006) was manufactured as a γ-aminobutyric acid (GABA) mimetic. Engineering gabapentin as a GABA mimetic was based on previous research which showed that the dysfunction of GABAergic neurons had implications in many types of seizures disorders (Dworkin & Kirkpatrick, 2005). Further research in clinical trials revealed that gabapentin could also be used to treat chronic pain (Kimos et al., 2007; R. A. Moore, Wiffen, Derry, & McQuay, 2009; R. A. Moore, Wiffen, Derry, Toelle, & Rice, 2014), and had the potential to be used in the treatment of behavioural disorders (Bryans & Wustrow, 1999; Herrmann, Lanctôt, & Myszak, 2000; Miller, 2001; Pollack, Matthews, & Scott, 1998). The manufacturing of pregabalin was developed with the purpose to improve upon the mechanisms demonstrated by gabapentin (Dworkin & Kirkpatrick, 2005). Where gabapentin excelled as an anticonvulsant, pregabalin has been used, primarily, as an effective pain reducer from certain chronic disorders, such as fibromyalgia (Arnold et al., 2008; Häuser, Bernardy, Üçeyler, & Sommer, 2009; Mease et al., 2008; Smith & Moore, 2012) . Prior to the use of pregabalin as a treatment for fibromyalgia, the pharmacological treatments generally consisted of medications that have a neuromodulatory factor (Crofford et al., 2005). For example, selective serotonin reuptake inhibitors (SSRIs) and serotonin/norepinephrine reuptake inhibitors (SNRIs) were often used treat pain associated with fibromyalgia.

Briefly, SSRIs are antidepressants that inhibit the reuptake of serotonin transmitters thus increasing the amount of serotonin (Hyttel, 1994), whilst SNRIs are non-selective serotonin and norepinephrine reuptake inhibitors (LoVecchio & Mattison, 2011). Although SSRIs and SNRIs have been shown to be effective in treating patients with fibromyalgia, their strength is in managing the depressive symptoms associated with having a chronic illness (Baker &

Barkhuizen, 2005). Considering SSRIs and SNRIs are, in fact, antidepressants, this effect is not surprising. Pregabalin, on the other hand, has been shown to relieve pain symptoms associated with fibromyalgia, along with the reduction of anxiety (Crofford et al., 2005).

Although gabapentin and pregabalin were designed to be GABAergic drugs, research has shown that neither drug has a significant effect on $GABA_A$ and $GABA_B$ receptors (Pande et al., 2003), nor do they act upon glutamate receptors like SSRIs (Kavoussi, 2006). It has been shown, however, that despite their similar chemical structure to GABA, both gabapentin and pregabalin inhibit the release of neurotransmitters by *presynaptically* binding at calcium channel alpha$_2$-delta ($Ca_v\alpha_2$-δ) proteins (C. P. Taylor, 2009, p. 13).

4.2 The mechanism of action of pregabalin

As previously stated, pregabalin has a different mechanism of action compared to that of commonly used pharmacological treatments of anxiety. As stated previously, pregabalin binds to the $\alpha_2\delta$ subunit of the presynaptic neuron's voltage-gated calcium channels (Kavoussi, 2006). Voltage-gated calcium channels are formed of different subunit proteins, which include the aforementioned $\alpha_2\delta$ subunits. There are four $\alpha_2\delta$ subunit proteins expressed in the brain. Type-1 $\alpha_2\delta$ subunit has been expressed in the forebrain, dorsal horn of the spinal cord, skeletal muscle and cardiac muscle; type-2 $\alpha_2\delta$ subunit is expressed in the forebrain (basal ganglia, habenula, inhibitory neurons) and cerebellum; type-3 $\alpha_2\delta$ subunit is expressed in the brain; and type-4 $\alpha_2\delta$ subunit is expressed in the brain, pituitary, adrenal glands and intestine (Dooley, Taylor, Donevan, & Feltner, 2007; Nasca et al., 2013).

Pre-clinical research involving receptor autoradiography (Li et al., 2011) and replacement mutations has shown that pregabalin selectively binds to $\alpha_2\delta$ subunits of the voltage-gated calcium channels (Bian et al., 2006). Voltage-gated calcium channels are cell membranes that permeate to calcium ions, which control the action potential of selective neurons. A major

characteristic of pregabalin is that its action is state dependent. This is to say, in excitatory neurons pregabalin decreases the release of excitatory neurotransmitters and returns the excitatory neurons to their standard state. Thus, when calcium channels enter neurons through voltage-gated calcium channels and activate the release of neurotransmitters, they allow the vesicles to join with the cell membrane of the synaptic cleft (Figure 4.1). When these neurons are overly excited, a surplus entry of calcium ions occurs. Due to pregabalin's strong binding to alpha$_2$-delta subunits, this surplus of calcium ions entry is reduced, which thus attenuates the release of neurotransmitters, such as aspartate, glutamate, norepinephrine, and substance P (Kavoussi, 2006). The alpha$_2$-delta subunits in which pregabalin selectively binds are found to be highly expressed in the corticolimbic structures of the brain (Millan, 2003), which include areas such as the amygdala and the hippocampus (Kavoussi, 2006). It is the decrease of activation of excitatory neurotransmitters that is believed to be the source of pregabalin's anxiolytic properties (Stahl, 2004).

The proposed anxiolytic effects of pregabalin provides researchers with a few unique questions. For instances, since pregabalin's binding to calcium channel $\alpha_2\delta$ subunits is unlike any well-known anxiolytic (i.e., benzodiazepines, SSRIs, and SNRIs) this could suggest that pregabalin's binding mechanism may provide a new path to anxiolysis. Described below is a brief review of the animal models and clinical experiments that have successively demonstrated pregabalin's anxiolytic effect.

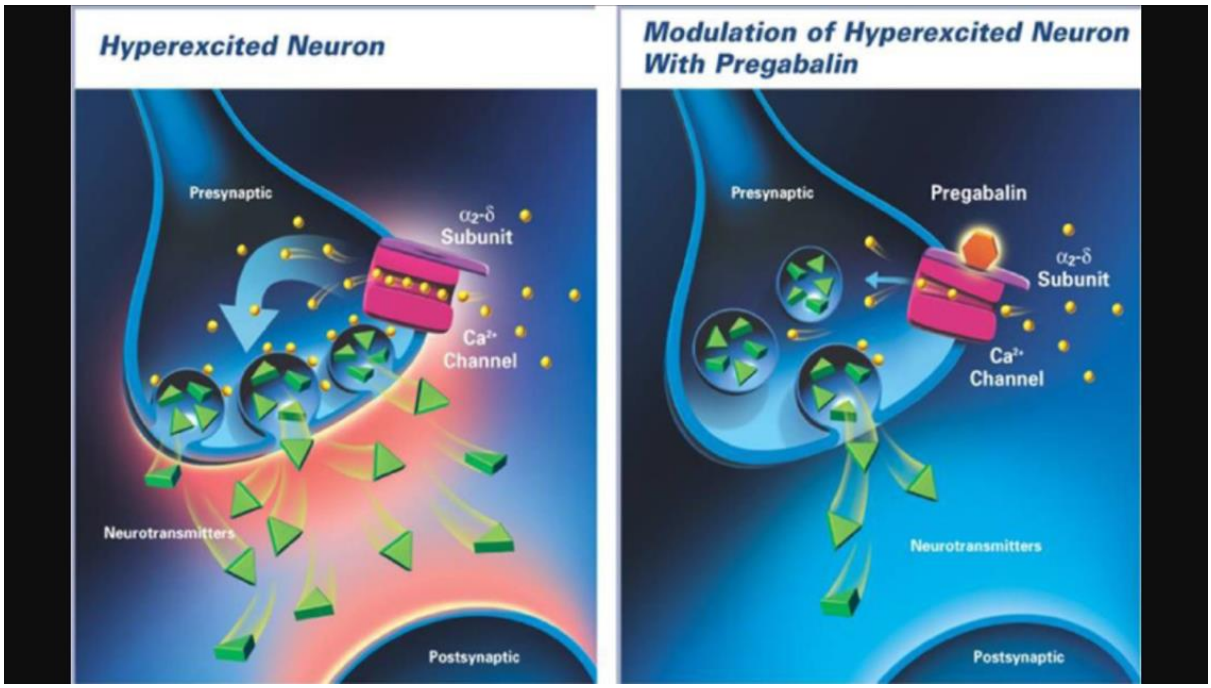


Figure 4.1 Pregabalin's mechanism of action. The left side of the image illustrates when neurons enter through voltage-gated calcium channels and activate the release of neurotransmitters allow the vesicles to join with the cell membrane of the synaptic cleft. a surplus entry of calcium ions occurs. The right side of the image illustrate pregabalin's strong binding to alpha$_2$-delta subunits (Shim, 2011).

4.3 Pregabalin's efficacy in clinical trials

4.3.1 Generalised Anxiety Disorder

Researchers have been examining pregabalin's effects on treating patients with generalized anxiety disorder (GAD). Briefly, GAD is can be characterized as "excessive and inappropriate worrying that persists (lasting 6 months or more) and is not restricted to particular circumstance," (Pande et al., 2003). The pharmacological interventions used in treating anxiety disorders for the past 56 years have mostly involved the use of benzodiazepines, SSRIs and 5-HT$_{1A}$ receptor agonists, as previously stated. Although the efficacy of these therapeutic interventions has been demonstrated time and again, much is still unclear concerning the neurobiology of GAD. For instance, changes in GABA, serotonin, and noradrenaline are paramount to treating GAD. Any disturbances in these specific neurotransmitters may, in fact, underline the pathophysiology associated with GAD

(Baldwin & Ajel, 2007). Hence, the use of benzodiazepines, SSRIs, and 5-HT_{1A} receptor agonists have persisted irrespective of the many adverse effects associated with their use. However, since the 1990s, studies involving genetically modified mice revealed that the subunits of α_1, α_2, α_3 and α_5 rendered pharmacological interventions that are $GABA_A$ receptors, such as benzodiazepines, ineffective (Skolnick, 2012). For example, Engin and colleagues (Engin et al 2016) used diazepam on region-specific knockout mice and demonstrated that the inhibition of principal neurons of the CA1, CA3 and dentate gyrus via α_2-containing $GABA_A$ receptors is required for the suppression of anxiety and fear in rodent models (e.g., elevated plus maze, light/dark box, Vogel conflict test and fear-potentiated startle).

Despite the discovery of the anxiolytic properties associated with α-subunits, there is still controversy surrounding the efficacy of its use. Independent studies involving genetically altered mice were conducted to determine if benzodiazepines mediated by $GABA_A$ receptors α_1 subtypes anxiolytic effects would be rendered ineffective (Skolnick, 2012). Despite the studies showing that drugs, such as diazepam, still elicited anxiolytic properties in mutant mice (McKernan, 2000), researchers are beginning to focus their efforts away from $GABA_A$ receptor-based anxiolytic drugs (Skolnick, 2012). Pregabalin's mechanism of action involves binding to alpha$_2$-delta subunits of voltage-gated calcium channels, making its perceived anxiolytic properties a focus of many researchers.

4.3.1.1 Efficacy of pregabalin compared to benzodiazepines in short-term clinical trails

Short-term clinical studies (here defined as ≤ 12 week-long administration) involving pregabalin's anxiolytic effects have shown pregabalin to be as effective as benzodiazepines. For example, Pande and colleagues performed a double-blind study involving both pregabalin and lorazepam (Pande et al., 2003). Patients were randomly assigned to receive pregabalin

(150 mg/day or 600 mg/day), lorazepam (6mg/day) or placebo, with a 1-week placebo lead-in followed by 4 weeks of treatment and ending with a 1-week dose taper. The researchers found that both 150mg/day of pregabalin and 6mg/day of lorazepam significantly reduced patient's anxiety as measured by the patients' self-report on the Hamilton anxiety scale (HAM-A). Additionally, this study compared the adverse effects of using both lorazepam and pregabalin in their treatment for anxiety. They found that no patients within the pregabalin treatment group experienced a serious adverse event compared to those patients within the lorazepam treatment group, concluding that 600mg/day of pregabalin was just as effective in treating anxiety as 6mg/day of lorazepam without the addition of serious side effect. Comparatively, Rickles and colleagues performed a double-blind study involving pregabalin and alprazolam (Rickels et al., 2005). In this study, patients were randomised to 4-weeks of treatments with either pregabalin (300mg/day, 450mg/day or 600mg/day), alprazolam (1.5 mg/day) or placebo. The researchers found that patients treated with pregabalin 300mg/day and 600mg/day along with alprazolam 1.5mg had a significant HAM-A improvement. However, patients treated with pregabalin 450mg/day did not significantly reduce HAM-A. The researchers also measured the tolerability of pregabalin compared to alprazolam and found that pregabalin was better tolerated. Overall, the researchers found that patients treated with pregabalin 300mg/day had the highest impact on HAM-A compared to pregabalin 600mg/day and alprazolam.

In comparing the studies described above, both researchers found pregabalin to be as effective in treating patients with anxiety as previously effectively proven benzodiazepines. The researchers also found that pregabalin is better tolerated by patients than benzodiazepines. This appears to be the case across the board (Table 4.1).

Table 4.1 Efficacy of pregabalin in generalised anxiety disorders

Duration	Dose	Number of participants	Effective Dose	Assessment(s)	Outcomes	Reference
6 weeks	150mg/day	69	600mg/day	HAM-A CGI-C	HAM-A scale scores were significantly higher in all dose groups	Pande et al., 2003
	600mg/day	70				
	Placebo	69				
4 weeks	300mg/day	91	300mg/day 600mg/day	HAM-A	300 mg/day and 600mg/day reduced anxiety scores (HAM-A) compared to placebo.	Rickels et al., 2005
	450mg/day	90				
	600mg/day	89				
	Placebo	91				
6 weeks	200mg/day	78	450mg/day	HAM-A	Improved HAM-A scales score compared to placebo.	Pohl, Feltner, Fieve, & Pande, 2005
	400mg/day	89				
	450mg/day	88				
	Placebo	86				
11 weeks	300mg/day	49	600mg/day	LSAS	600mg/day significantly reduced mean LSAS score compared to placebo.	Feltner, Liu-Dumaw, Schweizer, & Bielski, 2011
	450mg/day	57				
	600mg/day	50				
	Placebo	52				
8 weeks	Flexible doses	121	300-	HAM-A	Pregabalin significantly reduced HAM-A	Kasper et al., 2009

	(300-600mg/day)		600mg/day	HADS-A HADS-D	and HADS-A scores.	
	Placebo	128				
4-6 weeks	150mg	210	300-600mg/day	HAM-A	Pregabalin significantly reduced HAM-A scores.	Lydiard et al., 2010
	300-450mg	455				
	600mg	406				

Abbreviations: *HAM-A*, Hamilton Anxiety Rating Scale; *HAM-D*, Hamilton Depression Rating Scale; *CGI-I*, Clinical Global Impression Improvement Scale; *CGI-C*, Clinical Global Impression-Change; *LSAS*, Liebowitz Social Anxiety Scale; *(C)*, Control; *HADS-A*, Hospital Anxiety Scale; *HADS-depression*, Hospital Depression Scale

4.3.1.2 Efficacy of pregabalin compared to benzodiazepines in long-term clinical studies

Long-term clinical studies (here defined as > 12 week-long administration) examining the efficacy of pregabalin have shown that pregabalin is not only as effect as a benzodiazepine, but it is also an effective anxiolytic replacement for long-term benzodiazepine use (Kasper et al., 2014). For example, Kasper and colleagues (Kasper et al., 2014), performed a combined short-term (12-week) and long-term (24-week) double-blind study involving both pregabalin and lorazepam. Participants were given one of three doses; 150-300mg/day, 450-600mg/day of pregabalin and 3-4mg/day of lorazepam. The researchers found that there was a significant difference in HAM-A between the placebo group, both pregabalin groups and the lorazepam group during the 12-week, short-term trial. The participants that continued onto the 24-week saw a maintained improvement in anxiety levels on the HAM-A scale in the pregabalin and lorazepam groups without any significant difference between the two pregabalin groups nor the lorazepam group.

Reviews that have examined the literature of pregabalin's effectiveness in treating patients with anxiety have found different efficacy levels of pregabalin in treating patients with anxiety (Feltner et al., 2003; Pande et al., 2003; Pohl, Feltner, Fieve, & Pande, 2005; Rickels et al., 2005). The reviews also examined the tolerability of pregabalin across different studies (Baldwin & Ajel, 2007; D. J. Stein, Baldwin, Baldinetti, & Mandel, 2008). One review also reported that pregabalin was well tolerated across the studies, with dizziness and somnolence being the more frequently reported adverse symptoms (Baldwin & Ajel, 2007). Overall, the current clinical data on pregabalin supports the notion that pregabalin is a safe and effective option.

4.3.1.3 Pregabalin's efficacy in neuropathic pain trials

Briefly, this section will discuss pregabalin's use in neuropathic pain clinical trials not only to provide a clear review of the use of pregabalin but to also demonstrate pregabalin's use in acute pain relief which is linked to pregabalin's use in acute anxiety trials (section 4.4) Pregabalin is often used as a pain reliever for individuals suffering from different types of neuropathic pain. Defined by the International Association for the Study of Pain, neuropathic pain is described as "pain caused by a lesion or disease of the somatosensory nervous system." Most notably, pregabalin has been shown to be effective in the management of chronic pain associated with fibromyalgia and perioperative pain management (Eipe, Penning, Ansari, Yazdi, & Ahmadzai, 2012, Table 4.2).

Pregabalin's use in fibromyalgia is well documented, but pregabalin's use to treat perioperative pain is a relatively recent clinical endeavour, with some researchers attributing pregabalin's effectiveness in managing perioperative pain to its anxiolytic and sedative properties (Eipe et al., 2012). And it is those properties that has led other researchers to examine pregabalin's efficacy in treating acute anxiety in surgical patients (section 4.4).

Table 4.2 Studies which demonstrate the effectiveness of pregabalin in treating neuropathic pain

Type of Pain	Dose	Duration	Adverse effects/Outcomes	References
Fibromyalgia	150-600 mg/day	4-12 weeks	Significant pain relief Dizziness, peripheral oedema, somnolence and dry mouth	Crofford et al., 2005 Straube, Derry, Moore, & McQuay, 2010 Pauer, Atkinson, Murphy, Petersel, & Zeiher, 2012 Arnold et al., 2012 Lloyd, Boomershine, Choy, Chandran, & Zlateva, 2012
Post-operative Pain	300-600 mg/day	Pre/post-operative	Reduction of acute pain and reduction of the consumption of opioid	Jokela, Ahonen, Tallgren, Haanpää, & Korttila, 2008 Kim et al., 2010 Burke & Shorten, 2010 Hegarty et al., 2011 Choi et al., 2013

4.3.2 Pregabalin's efficacy in acute anxiety

Another method in clinical testing of pregabalin's efficacy in anxiety is in acute anxiety, more specifically, preoperative anxiety (Table 4.3). Evidence has shown that the majority of adults admitted into hospital for elective surgery experience preoperative anxiety (Badner, Nielson, Munk, Kwiatkowska, & Gelb, 1990). Accounting for preoperative anxiety has shown to be critical in controlling for intra-operative anaesthetic requirements (Padmanabhan, Hildreth, & Laws, 2005) and the increase postoperative pain (Nasr & Abdellatif, 2014). Studies have examined whether or not the administration of pregabalin, as opposed to benzodiazepines, would be effective in reducing preoperative anxiety, and, consequently, post-operative pain (Ghai et al., 2012; Rath, Yadav, & Chaturvedi, 2012; Spreng et al., 2011). For instance, Spreng et al. (2011) gave a single dose (150mg) of pregabalin prior to the administration of anaesthesia before lumbar discectomy to twenty-two patients. Spreng and colleagues two endpoints were pain at rest 120 minutes post-surgery and pre-operative anxiety. They found that those who received a single dose (150mg) of pregabalin reduced pre-operative anxiety and had reduced pain scores [visual analogue scale (VAS)] compared to the placebo group 4 hr post-surgery. They also found that there was a positive correlation between pre-operative anxiety score and post-operative pain 120 minutes post-surgery.

Although, studies like Spreng et al (2011) demonstrate the reduction of preoperative anxiety and postoperative pain, there are studies those show pregabalin's efficacy in preoperative anxiety with no effect on postoperative pain. Gonano et al (2011), gave a single dose (300mg) of pregabalin 1hr prior to the administration of anaesthesia before minor orthopaedic surgery to twenty patients. They found that pregabalin reduced preoperative anxiety compared to the placebo group, whilst the researchers found that there was no significant

difference in postoperative pain scores (VAS) between the pregabalin group and placebo group.

Within this thesis, clinical studies such as Spreng and colleagues (Spreng et al, 2011) and Gonano and colleagues (Gonano et al, 2011) are more relevant due to their demonstration of pregabalin's efficacy in reducing anxiety with a single dose. Clinical testing of acute anxiety is more akin with animal models of anxiety (section 4.5) than traditional clinical experiments such that traditional clinical models have patients taking pregabalin over the course of several weeks versus the singles administration of pregabalin to test its effectiveness as an anxiolytic. Nevertheless, pregabalin's efficacy in acute anxiety in the form of pre-operative anxiety is not entirely universal (White, Tufanogullari, Taylor, & Klein, 2009). As briefly detailed above, studies have had vary degrees of success in demonstrating pregabalin's effect upon acute anxiety along with its efficacy in postoperative pain.

Table 4.3 Efficacy of pregabalin of acute anxiety

Dose	Number of patients	Effective Dose	Administration	Outcomes	References
150mg	20	150mg	Orally 1hr prior to anaesthesia	150 mg 1 hr before operation **reduced** preoperative anxiety	Nasr & Abdellatif, 2014
150mg	30	150mg 300mg	Orally 2 hr prior anaesthesia	Both pregabalin groups **reduced** preoperative anxiety	Rath, Yadav, & Chaturvedi, 2012
300mg	30				
300mg	45	300mg	Orally 1 hr prior to anaesthesia	300mg 1hr before surgery **reduced** preoperative anxiety	Ghai, Gupta, Rana, & Wadhera, 2012
150mg	22	150mg	Orally 1 hr prior to surgery	150mg 1hr before surgery **reduced** preoperative anxiety	Spreng, Dahl, & Raeder, 2011
75mg	27		Orally 60-90 prior to anaesthesia	All doses (75mg, 150mg, and 300mg**) failed to reduce** preoperative anxiety	White, Tufanogullari, Taylor, & Klein, 2009
150mg	27				
300mg	27				
300mg	20	300mg	Orally ~1hr prior to anaesthesia	300mg **reduced** preoperative and postoperative anxiety	Gonano et al., 2011

4.4 Pregabalin's efficacy in animal models

Researchers have found pregabalin to be effective in reducing anxiety-like behaviour using multiple rodent models (Table 4.4). As demonstrated within the clinical models, research using rodent models of anxiety have found varying doses of pregabalin have successfully reduced anxiety-like behaviour in rodents. For example, in the elevated plus maze models, researchers have demonstrated reduced anxiety-like behaviour in rats given pregabalin at doses as small as 10 mg/kg to as high as 300 mg/kg (Baastrup, Jensen, & Finnerup, 2011; Field, Oles, & Singh, 2001; Zohar, Matar, Ifergane, Kaplan, & Cohen, 2008). However, like researchers moving away from the commonplace use of $GABA_A$ receptor interventions, researchers are beginning to examine the effects of anxiolytics on theta oscillations in the hippocampus in the rodent. Briefly, theta activity can be described as "an approximately sinusoidal electroencephalogram (EEG) activity that can be recorded at amplitudes as great as 1000μV through gross electrodes placed almost anywhere in and around the hippocampal formation…," (McNaughton et al, 2007 pg. 330). As mentioned previously (Chapter 1 and Chapter 3), theta activity in the hippocampus has been associated with mobility related processes and with anxiolytic drugs. Irrespective of the neurochemical nature of the anxiolytic, whether it is a classical GABAergic anxiolytic, like a benzodiazepine, or a non-sedative anxiolytic, like buspirone, when an animal is moving, research has demonstrated that anxiolytic drugs reduce the frequency of theta activity in the hippocampus (Coop & McNaughton, 1991; Wells et al., 2013). Researchers have also found that reticular stimulated hippocampal theta activity in anaesthetised rat can be reduced by the administration of anxiolytics (Sainsbury et al., 1987).

At present, there is one study that has examined the effects of pregabalin on hippocampal theta frequency. Siok and colleagues performed a study in which they examined the effects of pregabalin on stimulus-induced hippocampal theta activity in anaesthetised rats (Siok et al.,

2009). They recorded hippocampal theta from the dorsal portion of the hippocampus and induced theta activity by stimulating the nucleus pontis oralis (nPO). The experimenters stimulated the nPO at 0.03ms pulses over 6s at a rate of 250 Hz, with the current beginning at 0.06 mA and increasing at 0.02 mA increments until a maximum of 0.16 mA was reached over a 10-minute period. This pattern of stimulation was repeated without interruption over the duration of the experiment. The rats were either administered pregabalin, diazepam or saline (vehicle) 30 minutes prior to the start of the experiment. They found that both diazepam and pregabalin reduce stimulation-induced theta frequency. Diazepam effectively reduced theta frequency at 0.32 mg/kg and 1.0 mg/kg compared to vehicle. Pregabalin effectively reduced theta frequency at 32 mg/kg, whilst 10 mg/kg and 17.8 mg/kg of pregabalin did not significantly reduce stimulation-induced theta frequency compared to vehicle. The researchers also noted that diazepam's effects were present 15-minutes post-administration, whilst pregabalin's effectiveness was more gradual, reaching significance 30 minutes post-administration.

Table 4.4 Anxiolytic effects of pregabalin, animal models

Experimental Methods/ Model		Animal	Administration/Time		Dose	Effective Dose	Effects	Reference
Punished/Unpunished Food Reinforcement	Anxiety	Rat	s.c.	40 min prior	3 mg/kg 10 mg/kg 30 mg/kg	30 mg/kg	Significant effect on unpunished/ punished responses at 30.0mg/kg	Evenden, Duncan, & Ko, 2006
Elevated Plus Maze (EPM)	Anxiety	Rat	i.p.	45 min prior	30 mg/kg	30 mg/kg	Significant decrease of PEAP behaviour in response to PGB in EPM	Baastrup, Jensen, & Finnerup, 2011
	Anxiety	Rat	s.c.	40 min prior	3-30 mg/kg	10 mg/kg	Significant increase time spent on the open arms at 10mg/kg	Field, Oles, & Singh, 2001
	Anxiety	Rat	i.p.	60 min prior	30 mg/kg 100 mg/kg 300 mg/kg	100 mg/kg 300 mg/kg	Significant increase in open arm exploration and entries.	Zohar, Matar, Ifergane, Kaplan, & Cohen, 2008
Rat Conflict Test (RCT)	Anxiety	Rat	s.c.	40 min prior	1-100 mg/kg	30 mg/kg	Significant increased lever press during the punished period at 30mg/kg	Field et al., 2001
Ultrasound Induced Defensive Behaviour	Anxiety Fear	Rat	p.o.	60 min prior	10 mg/kg 30 mg/kg 100 mg/kg	100 mg/kg	Significantly altered distanced travelled. Significantly reduced escape.	Nicolas, Klein, & Prinssen, 2007
Acoustic Startle Response	Anxiety	Rat	i.p.	60 min prior	30 mg/kg 100 mg/kg	30 mg/kg 100 mg/kg	Significantly decreased the mean startle amplitude.	Zohar et al., 2008

(ASR)					300 mg/kg	300 mg/kg		
Vogel Conflict Protocol	Anxiety	Mice	i.p.	120 min prior	3.2mg/kg 10 mg/kg 32 mg/kg 100 mg/kg 320 mg/kg	10 mg/kg 32 mg/kg 100 mg/kg 320 mg/kg	Baseline level shock-supressed drinking (0.4mA) was achieved for WT mice and $\alpha_2\delta$-1 mice. $\alpha_2\delta$-2 mice required 0.7mA to supress drinking on the same level.	Lotarski et al., 2011
Stimulation-induced Hippocampal Theta in Anesthetized Rats	Anxiety	Rats	s.c.	30 min prior	10 mg/kg 17.8 mg/kg 32 mg/kg	32 mg/kg	Significant increase in theta power in response to increase nPO stimulation (0.06-0.16 mA). Significant decrease in average theta, dose-dependent administration of diazepam and pregabalin.	Siok, Taylor, & Hajós, 2009

Abbreviations: *S.C.*, Subcutaneous injection; *P.O.*, Orally; *PGB*, Pregabalin; *PEAP*, Place escape/avoidance paradigm; *EPM*, Elevated plus maze; *ASR*, Acoustic startle response; *I.P.*, Intraperitoneal injection; *nPO*, nucleus pontis oralis; *mA*, milliamps.

4.5 Pregabalin's efficacy in hippocampal theta frequency reduction

As mentioned previously, there is only one study which examines the effects of pregabalin on hippocampal theta frequency. Siok and colleagues' (Siok et al. 2009) elicited hippocampal theta frequency through reticular-stimulation and administered either 10 mg/kg, 17.8 mg/kg or 32 mg/kg of pregabalin. Only the higher dose (32 mg/kg) reduced theta frequency. The research within this thesis aims to not only further the Siok and colleagues' study, but to expand upon the Wells and colleagues' study, in part, by demonstrating pregabalin's effect upon hippocampal theta frequency in freely-moving rats in both a low dose (17.5 mg/mg) and a high dose (35 mg/kg). The methodology used for the electrophysiological experiment is described in Chapter 5 and the results of pregabalin's effect upon hippocampal theta frequency is described in Chapter 6.

5 General Methods of Electrophysiological Experiment

This chapter describes the methods used in the electrophysiology experiment (Experiment 1) featured in Chapters 6 (Study 1A) and 7 (Study 1B). The methods and procedures for Experiment 2 and Experiment 3 (strictly behavioural) are outlined in Chapter 8.

5.1 Ethics

All procedures described in this experiment were carried out in accordance with the Animals (Scientific Procedures) Act 1986. This experiment was conducted under a project licence held by Dr Colin Lever (PPL 40/2935) and personal licence held by Miranda Hines (l1EE35DOA).

5.2 Subjects

Seven adult male Lister Hooded rats (Envigo, Huntington, UK) were used, weighing between 300g-420g at the time of surgery. After arrival, the animals were housed in groups of five per cage for at least 1 week whilst they acclimatised to the local conditions. At this stage, a rat later undergoing surgery would be housed with some rats who did not undergo surgery. Cage dimensions were (1500cm^2), with the cage floor covered in woodchip bedding. Water was available *ad libitum*. After surgery, each rat was housed individually in cages of [53 x 42.5 x 20cm], with the cage floor being covered by woodchip bedding. Each animal was introduced to food restriction at least 1-week post-surgery (85% free-feeding bodyweight). Each animal was maintained under a 12:12 reverse light: dark schedule. All subjects were handled and scruffed (in preparation for i.p. injections) daily, which began soon after each animal's arrival in the laboratory.

5.3 Drugs and administration

The drug used in the electrophysiological experiment was pregabalin (35 mg/kg and 17.5 mg/kg; Sigma-Aldrich, Poole, UK), which was dissolved in a vehicle of physiological saline solution (0.9% NaCl). Saline was used as a control condition. Pregabalin and saline was administered intraperitoneally (i.p.) at a volume of 1ml/kg bodyweight. The drug solution was prepared on each testing day that required the drug injection. The interval between the injection and the following test was 30 minutes.

The first dose tested in this study was 35 mg/kg, which was chosen based on previous research showing that it had an anxiolytic effect in both conditioned and unconditioned behavioural tests, and in an *in vivo* experiment (Evenden et al., 2006; Field et al., 2001; Siok et al., 2009). However, earlier research using pregabalin showed pregabalin could effectively reduce anxiety behaviour in rodent models at doses as low as 10 mg/kg (Field et al., 2001). Based on this previous research, it was decided that the use of 17.5 mg/kg (half of 35 mg/kg) of pregabalin to tests its efficacy as well.

5.4 Materials

5.4.1 Testing arena and laboratory layout

Figure 5.1 shows the laboratory layout. This layout was consistent across subjects for the electrophysiology experiment. The testing environment was a wooden square, black-painted, open-field environment (60 x 60 x 50 cm) with a black Plexiglas floor (Figure 5.2). The open-field environment was placed on a table elevated 48 cm off the ground. The holding platform was a small wood box (90 x 44 x 44 cm) which contained woodchip bedding.

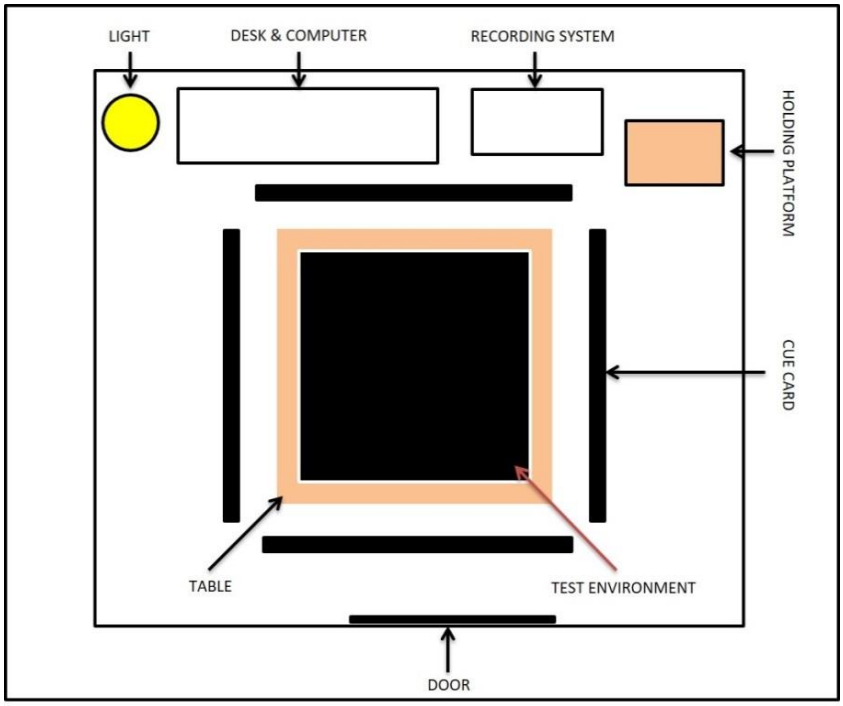


Figure 5.1 **Bird's eye view of the laboratory setup.** This illustrates the general overview of the testing facility. Each animal experienced all trials of the experiment in the testing environment (see Figure 5.2). Animals were placed on the holding platform behind one of the cue cards during screening and testing to shield the animal's view of the testing environment.

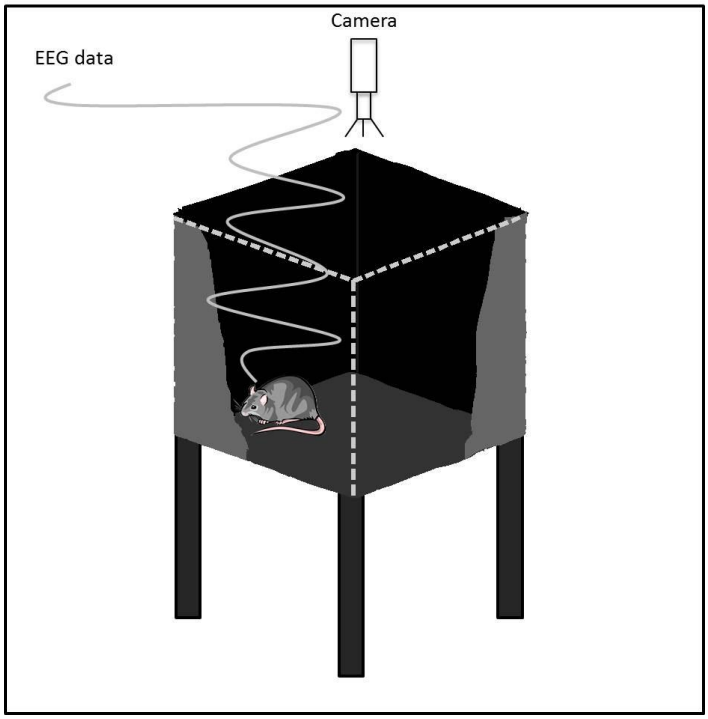


Figure 5.2 **an illustration (not to scale) of the test environment; a square, walled open field (used in Experiment 1).**

5.4.2 Recording electrodes

The electrodes were made of HM-L-coated platinum/iridium (90%/10%) wire (California Fine Wire, Grover Beach, CA) of 25-µm-diameter. To improve upon the electrodes' quality of signal discrimination, the electrodes were assembled into a tetrode configuration (Harris et al., 2000). Tetrodes were constructed by measuring out a piece of wire (usually 20 cm in length) and sticking the ends together on a small piece of medical tape, creating a loop. The loop was hung on a smooth round metal bar and twisted together from beneath the bar. Once twisted, the end was cut from the medical tape and the tetrode was carefully remove from the metal bar, loop intact. Using an alcohol burner, the insulation from the two untwisted ends was removed. Once removed, the loop was cut in the centre creating four tips which were used to wrap around the post of the microdrive. Silver conductive paint was put on the post, followed by nail varnish, which was used to cover the post and microdrive for protection.

5.4.3 Microdrive

Tetrodes were loaded into a moveable 16-channel microdrive. See Figure 5.3 for a diagram of a standard microdrive. The two metal posts were used for the attachment of the microdrive to the skull using dental cement, making them stationary relative to the brain. One of the metal posts carried a fine-threaded screw which was fitted over a nut made of heat shrink. Attached to this, carrying the tetrodes, was the cannula. This could be moved up and down using the screw turner. One 360° anticlockwise turn moved the tetrodes down 200µm. Protecting the cannula and the tetrodes was a sleeve, which was marginally longer (approximately 1.5mm) than the cannula and was fitted over the cannula. During surgery, the sleeve was pulled down over the cannula and tetrodes, with the bottom half fixed to the skull using dental cement, thus, keeping the tetrodes from being exposed.

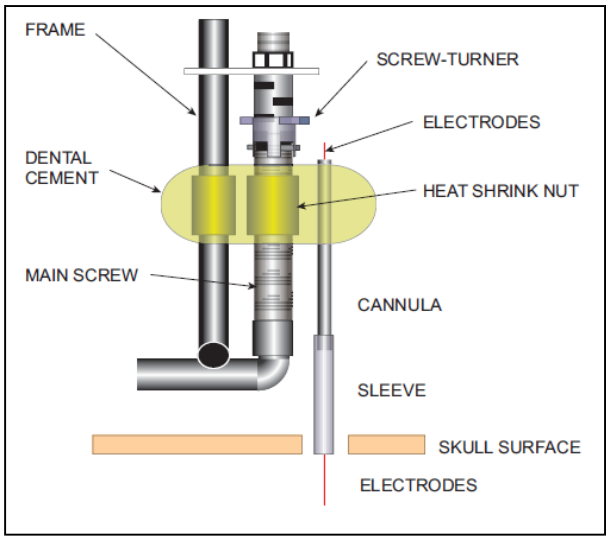


Figure 5.3 A microdrive and its main features. The microdrive was fixed to the skull using dental cement. Turning the screw-turner results in movement of the cannula containing the electrode bundle. Adapted from a diagram by John Huxter.

5.5 Surgery

The general anaesthesia was induced by a combination of oxygen (flow rate: 3 litre/min) and isoflurane (3% of gas volume). The animal's head was then shaved using electric clippers, after which the animal was placed into the stereotaxic frame and the ear bars fitted. An analgesic [buprenorphine (Vetergesic), Reckitt Beckinser, Hull, UK), 0.1ml, i.p.] and an antibiotic [enrofloxacin (Baytril), Bayer, Newbury, UK), 0.3ml, s.c.] were then administered. At this point, the isoflurane was reduced to 1-2% and the animal's breathing was monitored (breathes per minute) every 30 minutes to check the anaesthesia's stability; he normal respiratory rate for a rat is approximately 65-150 breaths per minute and under anaesthesia about 30 – 75 breaths per minute (Thomas & Lerche, 2017). Vaseline was applied to the animal's eyes to prevent them from drying out, followed by the application of the topical antiseptic Betadine (Seton Healthcare LTD, Oldham, UK) to the incision site and left in situ for a couple of minutes.

The skin was then incised, and the skull was exposed, and the stability of the skull was check along with making sure the skull was parallel with the base plate of the stereotaxic frame. Seven holes were drilled into the skull using a trephine drill bit. Seven stainless steel screws (3mm diameter, Precision Technology Supplies, East Grinstead, UK) were screwed into the

holes. This was done to aid the attachment of the microdrive to the skull. Two of these screws served as the ground screw attachment using a short piece of pre-soldered wire.

Following this, two-holes (1.5mm diameter) were drilled into each hemisphere. The coordinates for the left hemisphere were chosen to reach either the dorsal or intermediate portion of the CA1. The same range of coordinates were chosen for the right hemisphere (dorsal: -3.6 to -4.5 mm antero-posterior and 3.0 mm medio-lateral; intermediate: -6.2 mm antero-posterior and -4.5 mm medio-lateral). After the two holes were drilled, the right hole was plugged with a sterile cotton bud soaked in saline. This was done to protect the brain surface from drying and dental cement from touching the surface of the brain. A loaded microdrive was sterotaxically positioned over the left hemisphere leaving the tip of the tetrode configuration just above the surface of the brain at the target coordinates. The microdrive was slowly lowered into the brain (~1.5 mm deep above dorsal CA1; ~ 2.4 mm deep above intermediate-ventral CA1). The protective sleeve around the cannula was also lowered so that the bottom of the sleeve rested on the surface of the brain. The exposed brain and skull around the sleeve were covered with sterile Vaseline. Dental cement was then applied around the sleeve, drive feet, and screws whilst avoiding the hole in the left hemisphere; thus, fixing the microdrive to the skull. Once the dental cement had dried, the same process was undertaken on the right hemisphere. Lastly, each drive ground wire was carefully soldered to the ipsilateral ground screw. The ground screws were then cemented so prevent the animal from accidentally detaching the ground connections. A wing screw, which is used to hold the head-stage that links to the microdrives to the recording system, was fixed at the base of the skull and left to dry.

5.6 Screening

Screening for theta began approximately 1-week post-surgery. The animal was screened whilst on a holding platform (Figure 5.1). The tetrodes were slowly lowered, typically no

larger than 100µm (2 x 50µm increments) per day to discern the presence of high-frequency ripples. Ripples have maximal amplitude in or just below the pyramidal layer (O'Keefe & Nadel, 1978). Ripples are the main physiological marker used for the hippocampus. Once the presence of ripples has been established, the tetrodes were lowered at 25µm increments, until complex spikes and relatively good theta activity had been detected. Cell activity was recorded once detected; however, the main focus was on hippocampal EEG and theta.

5.7 Data recording

5.7.1 Experimental trials

The experiment began once clear high amplitude theta was being recorded. The experiment used the basic design of 5 x 10-minute trials per day over 5 days in a square, walled environment with black walls and floor (Figure 5.2), with an inter-trial interval of 30 minutes. Detailed discussion of these issues is provided in the relevant experimental chapter (Chapter 6).

5.7.2 EEG recording and data collection

During recording, each subject was connected to the recording system by a head-stage amplifier which could be plugged into the microdrive. The head-stage cables were both light and flexible which enabled to the animal to move around the arena freely. The head-stage amplifiers were unity-gain buffers, which isolated the electrodes from the wires transmitting their signals to the recording system. The implanted electrodes were AC-coupled to these amplifiers. Light-weight wires 2-3 metres long connected the head-stage to a preamplifier. The EEG signals were amplified 4000-8000 times, band pass filtered at 0.34-125Hz and sampled at 250Hz.

EEG sampling in the present thesis occurs at 250Hz. As mentioned previously, the raw signal from the hippocampus passes through a low-pass filter (0.34 to 125 Hz). After 6-12 Hz offline filtering, the signal is Hilbert transformed, as shown in Figure 5.4.

The subjects' head position and orientation were video tracked using two arrays of small LEDs that were attached to the head-stage, so they were positioned on the rat's head. The two sets of LEDs were separated by 5cm and were differentiated by the tracking system on the basis on their different brightness. All EEG recording equipment was custom built (Axona, St Albans).

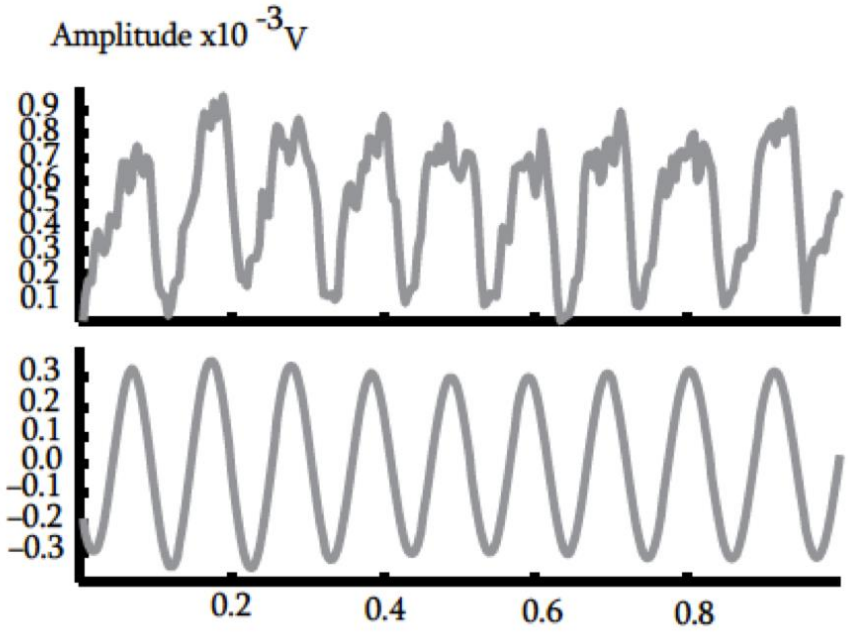


Figure 5.4 The processing of raw EEG trace (1 sec). The top panel shows 'raw' EEG (filtered at 0.34-125 Hz). The bottom panel is the same EEG trace after the application of theta-band filtering and a Hilbert transform. Smoothing was achieved by using Gaussian kernel (filtering to remove noise) with a standard deviation of 0.375 Hz.

5.7.3 Single-units

Signals on the channels dedicated to single-cell recording were amplified (10,000-20,000) and band-pass filtered (500 Hz-7kHz). All the channels of a given tetrode were recorded differently with respect to a single-cell channel on another tetrode within the same hemisphere. Using this method of referencing was helpful in removing most of the common background activity, which increased the signal readability. Each channel was monitored at a sampling rate of 50kHz. Action potentials were stored as 50 points per channel (1ms, with 200µs pre-threshold and 800µs post-threshold) whenever the signal from any of the pre-

specified recording channels exceed a given threshold. Gains and references for each channel were set each day before recording and are kept the same for all trials of that day. Threshold was rarely changed.

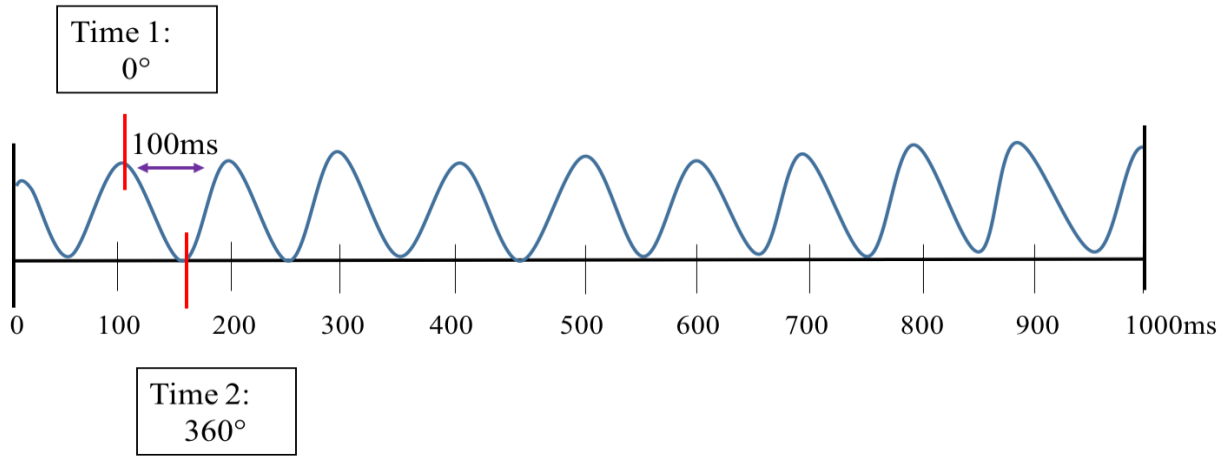
5.7.4 Instantaneous theta frequency from recorded EEG

The recorded EEG signal was filtered using a 6-12 Hz, 251-tap, Blackman window erred, band-pass sinc (sine cardinal). The 6-12 Hz filtering removed non-theta-band frequencies (reduces noise). Windowing the filter achieves good stop-band attenuation and small pass-band ripple (dampens the undesired frequencies which remain after initial filtering). A Hilbert transform was then applied (giving the analytic representation of the EEG signal). Figure 5.4 illustrates an example of the effects of using theta-band filtering and the Hilbert transform upon the 'raw' EEG.

Instantaneous theta frequency was derived by calculating theta phase differences between the adjacent time points (using 4ms intervals). The phase of the analytic signal at a given time step gave the phase of theta at that time step and the difference in phase between each time step defined the instantaneous frequency. As spectral analysis is much more common, this non-spectral procedure is explained in detail below using worked examples:

EXAMPLE 1

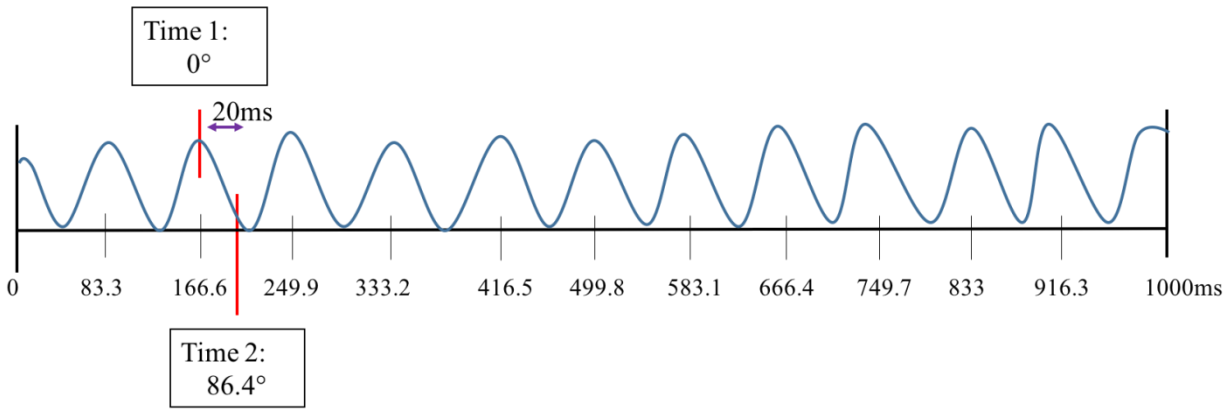
If we sample 100ms of EEG and the phase difference between Time 1 ('zero') and Time 2 (100ms later) is 360°. As 1s = 10 x 100ms, then there are 10 cycles per second; (10 x 360°)/360 = 10 Hz. Thus, the instantaneous frequency of the 100ms sample is 10 Hz.



In the same respect, if the sampling occurred every 20ms, using this example, the phase difference between TIME 1 (‘zero’) and TIME 2 (20m later) would be 72°. With 1 second = 50 x 20ms, the instantaneous frequency of the 20ms sample [(50 x 72°)/360°] = 10 Hz.

EXAMPLE 2

If we sample 20ms of EEG and the phase difference between Time 1 (‘zero’) and Time 2 (20ms later) of 86.4°. We know that 1 second = 50 x 20ms, there would be 12 cycles per second (50 x 86.4°/360°) = 12 Hz. Therefore, the instantaneous frequency of 20ms is 12 Hz.



The rat’s position was sampled every 20ms. Knowing the position of the rat at the time point t and t + 20ms later enabled the calculation of instantaneous running speed.

The EEG sampling rate in Experiment 1 was every 4 ms (250 Hz) and was therefore five times that position. As such, instantaneous frequency was averaged over every five consecutive values corresponding to each position sample. In conclusion, concurrent measurements of speed and EEG theta frequency were produced every 20 ms. In this way, frequency calculation took into account the dynamic relationship between theta frequency and running speed.

5.7.5 Theta-frequency-to-speed plots

To quantify the linear relationship between theta frequency and speed on a given trial, a regression line was filtered to the frequency-speed data points for speeds between 5 and 30 cm/s. The two variables were derived from this regression line: a) the slope and b) the intercept of the regression line extended to the frequency axis at 0 cm/s (Figure 5.5).

Speeds that were below 5 cm/s were excluded in order to avoid non-theta behaviours, such as grooming. Variability of behaviour is generally highest for those behaviours when displacement is minimal. During immobility/slow speed, the rat could be during a variety of different behaviours, whereas at higher speeds, the rat could only be walking/running. Speeds above 30 cm/s were excluded as at higher speeds theta sampling is more prone to error. Some high-speed samples are likely to represent erratic head movement and LED reflection near walls.

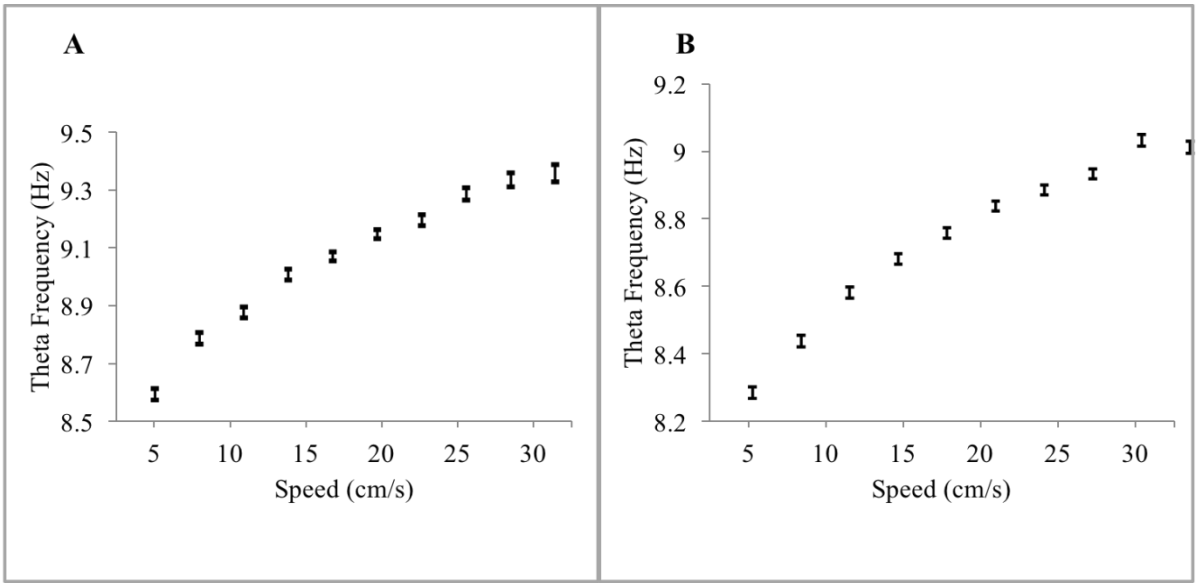


Figure 5.5 The approximate linear relationship between theta frequency and running speed from 5 - 30cm/s. Each point shows the mean ±SEM of theta frequency within each speed bin. A) Data is from a non-injection trial from rat 3 in Experiment 1. B) Data is from a non-injection trial from rat 5 in Experiment 1.

5.8 Histology

After completion of the experiment, each rat was euthanized with an overdose of sodium pentobarbital [(Euthalal), Merial, Harlow, UK; 1ml i.p.] and perfused transcardially with saline solution, followed by 4% paraformaldehyde. Brains were sliced coronally into 40-µm-thick sections, which were mounted and stained using freshly violet solution to aid visual toon of tetrode tracks and tips.

5.9 Data processing

5.9.1 Cluster-cutting

Cluster-cutting consists of isolating the action potentials that have similar features. The resulting clusters of spikes are assumed to represent single neurons. The analysis was performed manually using custom-made software (TINT; Axona, St. Albans, UK), which ran an algorithm to separate the clusters.

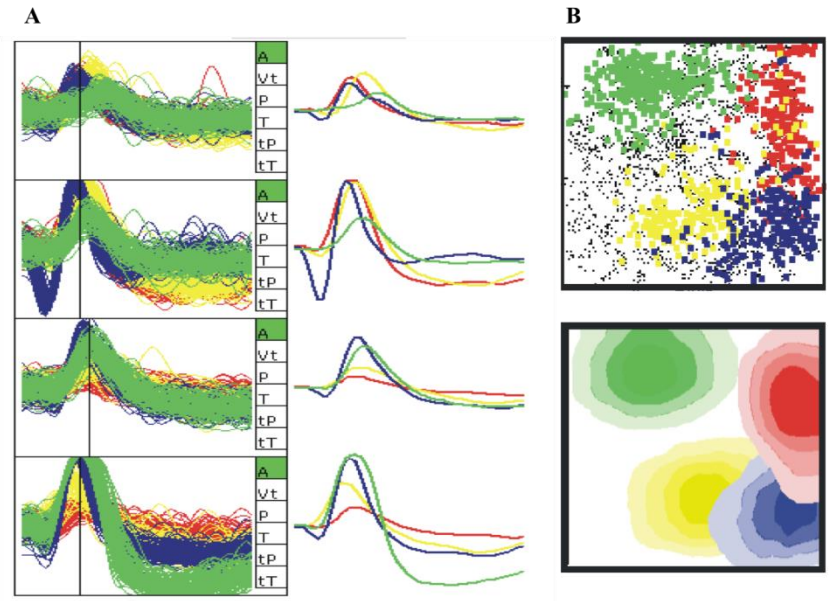


Figure 5.6 Example of cluster-cutting of a tetrode. Each cluster (colour) is assumed to represent different neurons. A) shows the waveforms of all the neuron's spikes and their averages (next to them). B) shows the clusterplots and heat map of each neuron.

Cluster plots are two-dimensional section of a multidimensional space. Each spike is represented as a dot that is derived by plotting the peak-trough amplitude on one electrode against the peak-trough amplitude of another tetrode. Therefore, one tetrode is plotted simultaneously on six cluster plots comparing each of the four channels (Figures 5.6 and 5.7). This helps in separating the spikes from two different neurons due to the number of spikes being fired are likely to be very similar across all channels. There are several criteria which are used in determining and isolating a place cell cluster; which includes peak-to-trough amplitude and interval, and a 40-ms autocorrelogram.

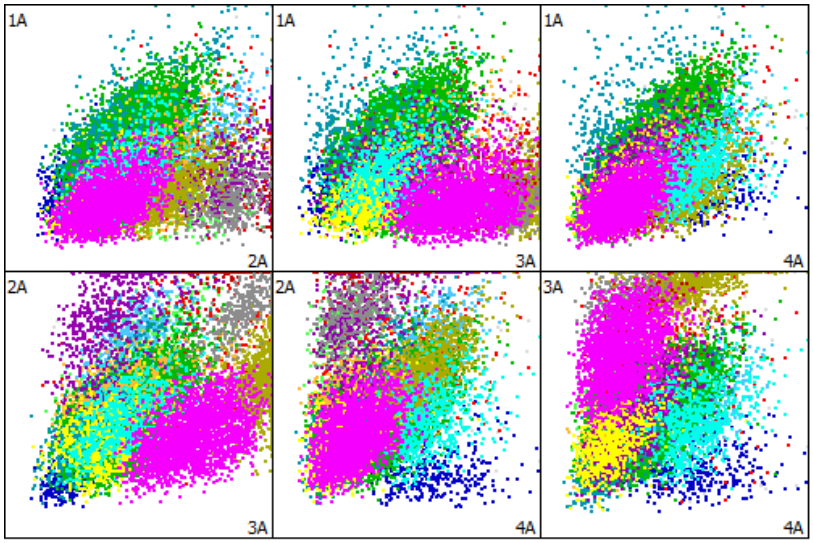


Figure 5.7 Example of six cluster plots comparing each of the four channels on tetrode 2 from rat 5.

5.10 Analysis of data

Experiment 1 (Study 1A, Chapter 6) was analysed using paired sampled t-test to compare pre-injection and post-injection trials on the intercept and slope theta-frequency-to-speed relationship. For example, to analyse theta intercept, let's assume for the saline day that the intercept value for trial four was 8.4 Hz and the intercept value for trial five was 8.5 Hz. On the pregabalin test day, let's assume that the trial four intercept value is 8.3 Hz and the intercept value for trial five is 7.8. The comparison would then be of the saline intercept value of +0.1 to the pregabalin intercept value of -0.5 (trial 5-trial 4). These numerical differences would represent a single rat's saline and pregabalin intercept values and would subsequently be used in the paired samples t-test analysis. This method was also applied for part of the analysis of cell data, which examined Skaggs spatial information (bits/spike), global mean rate (number of spikes fired divided by trial time in seconds), and locational peak rate (Hz). The cell data was recorded from five of the seven animals and was analysed cell-by-cell (independent samples t-test) and rat-level (paired samples t-test). Change in field size was analysed using an independent samples t-test. The change in field size was calculated by using a MATLAB function to obtain the field size of each cell recorded in bins. The field size

was converted from bins to cm^2 (for method of conversion, see section 6.5.1.4). Once converted, a change index score was calculated in the same way a D2 score for an object recognition experiment is calculated. For this experiment, the change index was calculated as follows: $(Trial\ 5 - Trial\ 4)/(Trial\ 5 + Trial\ 4)$. Further explanation of statistical analysis is described in Chapter 6.

Experiment 1 (Study 1B, Chapter 7) was analysed using repeated measure ANOVAs to examine the effects of environmental familiarisation on slope and intercept. Due to a technical error, the recording for first trial of the first day from the first animal used in Experiment 1 was not taken. To account for this missing data, the slope and intercept values for trial 1-rat 1 was interpolated. This was accomplished by taking the average of trials 2-4 on the first day for each animal, subtracting that value from their trial 1 value and averaging those values into one a single number. That number was then subtracted by the average of trials 2-4 for rat 1, leaving the interpolated value for trial 1-rat 1. The average slope and the average intercept values for each pre-injection trial (trials 1-4) of all subjects (n = 7) was divided into dorsal slope, dorsal intercept (n = 6), and intermediate slope, intermediate intercept (n = 6). Initially, a mixed model ANOVA was used to analyse the pre-injection trials of dorsal slope, dorsal intercept, and intermediate slope, intermediate intercept across days (days 1 – 3). Dorsal slope, dorsal intercept and intermediate slope, intermediate intercept of the pre-injection trials was then analysed across all test days (n = 5) to examine the robustness of any effects found. The cell data for Study 1B were cells recorded from five of the seven animals and were recorded from days 1 – 3. Mixed model ANOVAs were performed on global mean rate, locational peak rat, Skaggs spatial information, the change in distance of place cells, and place cell field size change. The change in distance of place cells was calculated by converting the pixel coordinates (x, y) of each cell to centimetres and calculating the difference from each cell between each of the adjacent pre-injection trials by

using the distance formula ($d = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}$, as applied to (trial 2 – trial 1), (trial 3 – trial 2), and (trial 4 – trial 3)). Place cell field size change was calculated in the same way as in Chapter 6 (see above for detail) and was analysed using a repeated measures ANOVA. Additionally, the average place field size for cells recorded on days 1 – 3 were correlated with the average slope from animals with cell recordings for each day. Further correlational analysis was performed on the normalised area and average slope. Normalised area was calculated by using change index formula for each individual cell recorded during the pre-injection trials (e.g., Cell 1; trial 2-trial 1, trial 3-trial 2, and trial 4-trial 3). For example, the change index of trial 2 and trial 1 was calculated as: $(Trial\ 2 - Trial\ 1)/(Trial\ 2 + Trial\ 1)$. To normalise the values from the change index, the average was taken for each trial for days 1 – 3. Those values were then correlated with the average slope recorded for each trial across day (e.g., Rat 1, Day 1, normalised area for trial 2 compared to Rat 1, Day 1, average slope for Trial 2, etc).

Experiment 2 (Elevated Plus Maze, Chapter 8) was analysed using a one-way between-subjects ANOVA. The elevated plus maze (EPM) was run at the same time as the open field (OF, Experiment 3) experiment, with subjects participating in the EPM experiment first before participating in the OF experiment. Three groups were tested; saline group, chlordiazepoxide (CDP) group, and pregabalin group. The percent amount of time spent in the open and closed arms were analysed, along with the frequency of open and closed arm entries. Additionally, ethological measures were analysed to further assess anxiety-like behaviour. The frequency of the stretch attenuated posture (SAP), head-dips, rearing, and faecal boli were recorded and were also analysed using a one-way between-subjects ANOVA. Further explanation for the statistical analysis of Experiment 2 is detailed in Chapter 8.

Experiment 3 (OF, Chapter 8) was analysed using a one-way between-subjects ANOVA. The OF was run directly after Experiment 2 and utilised the same three groups: saline, CDP and pregabalin. The percent time spent in the inner zone and the frequency of inner zone entries were analysed. Additionally, the frequency of the ethological measures faecal boli and rears were analysed; along with the time spent grooming by using one-way between-subjects ANOVA. Further explanation for the statistical analysis of Experiment 3 is detailed in Chapter 8

6 Effects of Pregabalin on Hippocampal Theta

6.1 Aim of experiment and background information

This chapter presents and examines Study 1A of Experiment 1. The aim of Study 1A was to examine the effects of pregabalin on hippocampal theta and hippocampal place cells in freely moving rats.

As discussed in Chapter 3, there is an abundance of research that implies that there is a relationship between hippocampal theta frequency and anxiety. A wide range of anxiolytics, which include benzodiazepines (alprazolam, diazepam), SSRIs (fluoxetine, sertraline) and 5-HT_{1A} agonists (buspirone) have been shown to reduce Type 2 theta frequency in rats during electrical stimulation of the medial septum and reticular formation (Coop & McNaughtocn, 1991; Munn & McNaughton, 2008). As mentioned in Chapter 4, Siok and colleagues (2009) performed a study that examined the effects of pregabalin in stimulation-induced hippocampal theta activity in anesthetised rats and found that pregabalin, along with diazepam, reduced stimulation-induced theta frequency. More recently, Wells and colleagues (Wells et al., 2013) have been able to demonstrate neurochemically dissimilar anxiolytic drugs reduce theta frequency in freely moving rats. As stated in Chapter 1 (section 1.3), Wells and colleagues (2013) built upon the Burgess (2008) model, which states that theta frequency has two components; the intercept and slope component of the theta-frequency-to-speed relationship. Wells and colleagues made specific predictions regarding the two components. For this chapter the prediction of interest is related to the intercept component, in which Wells and colleagues predicted that anxiolytic drugs would reduce the intercept component of the theta-frequency-to-speed relationship whilst having no affect upon the slope component. Wells and colleagues tested two well established anxiolytics

(chlordiazepoxide and buspirone) and one putative anxiolytic (0-2545, a CB1 agonist) and found that, at the doses used, the anxiolytic drugs reduced the intercept component of theta-frequency-to-speed relationship without affecting the slope component. Thus, the first aim for this study (Study 1A) is to expand upon the Wells and colleagues and Siok and colleagues' studies by examining the effects of pregabalin on the intercept component of the theta-frequency-to-speed relationship.

One key aspect of both the Wells and colleagues, and Siok and colleagues' studies is that both recorded hippocampal theta frequency from the dorsal hippocampus. In the present study, hippocampal theta frequency is recorded from both the dorsal and intermediate hippocampus. This was done to explore the duality of the hippocampus. As stated in Chapter 1 (section 1.3), there is anatomical evidence which supports the notion that the dorsal hippocampus plays a role in spatial cognition and the ventral hippocampus plays a role in anxiety modulation (Bannerman et al., 2004; Fanselow & Dong, 2010; Korotkova et al., 2017). However, hippocampal physiology characteristics suggest a more complex mode of operation, which includes the intermediate hippocampus. Previous research has shown that the intermediate hippocampus plays a role in both spatial cognition and anticipatory behaviours in rats (Bast et al., 2009; Burton et al., 2009). This would suggest that the intermediate hippocampus should be further explored in terms of its role in the modulation of anxiety and spatial cognition. However, on the basis that hippocampal theta is found throughout the dorso-ventral axis and appears to travel along this axis in a single wave (Patel et al., 2012), there should be no discernible differences in the characteristics of dorsal and intermediate hippocampal theta. Therefore, it is predicted that pregabalin will reduce the intercept component of the theta-frequency-to-speed relationship in both the dorsal and intermediate hippocampus without affecting the slope component.

In terms of hippocampal place cells, the second aim of Study 1A was to further define the proposed duality of the hippocampus. On the basis that the intercept component of the theta-frequency-to-speed relationship corresponds with anxiety and the slope component corresponds with spatial cognition, one could assume, due to hippocampal place cell's importance to spatial cognition, that the administration of an anxiolytic would have no significant effect on hippocampal place cells. Thus, the prediction is that pregabalin will not affect hippocampal place cells.

6.1.1 Anxiolytic drugs and the intercept of the theta-frequency-to-speed relationship

One behavioural correlate that is related to theta frequency is running speed. Hippocampal theta frequency increases with running speed and a regression line can be fitted to this linear relationship (see Chapters 3). The Burgess (2008) model proposes that the slope and intercept of the broadly linear relationship between theta frequency and running speed can be thought of as reflecting the components of Type 1 and Type 2 theta components, respectively. The model predicts that the independent manipulations of Type 1 and Type 2 theta results in separate effects on the slope and intercept components of the theta-frequency-to-speed relationship. Using the Burgess model, with the knowledge that Type 2 theta has been associated with anxiety modulation, a prediction can be made which stipulates that anxiolytic drugs will reduce the intercept component of the theta-frequency-to-speed relationship exclusively, whilst having no effect on the slope component. Wells and colleagues (Wells et al., 2013) were able to demonstrate this prediction of the model (anxiolytic reduction of the intercept component) by using the classic anxiolytics CDP and buspirone and the novel anxiolytic O-2545 (a CB1 agonist). The current study aims to demonstrate that pregabalin will also reduce the intercept component of the theta-frequency-to-speed relationship whilst having no effect on the slope component.

6.2 Summary of Study 1A

The aim of this experiment was to test the predication that the novel anxiolytic pregabalin will reduce the intercept component of the theta-frequency-to-speed relationship, whilst having no effect on the slope component. The effects of the 'higher' doses (35 mg/kg) and the 'lower' dose (17.5 mg/kg) of pregabalin were examined. Additionally, analyses were performed to examine pregabalin's effects on dorsal and intermediate intercept, and dorsal and intermediate slope. The effect of pregabalin upon hippocampal place cell properties were also analysed.

6.3 Method

General methods detailing the subjects, surgery, wiring, microdrives and drug administration are detailed in chapter 5.

6.3.1 Subjects

Subjects were 7 male Lister-hooded rats. See chapter 5 for details on housing and laboratory conditions. All 7 rats were implanted with 2 x 16 channel microdrives and were either implanted in the dorsal and/or intermediate portion of the hippocampus, with the hippocampal EEG (electroencephalography) recorded from both the dorsal and intermediate hippocampus, which was analysed separately. Not all animals received the same dose of pregabalin; a higher dose of pregabalin (35 mg/kg) was given to rats 1-3, whilst a lower dose (17.5 mg/kg) was given to rats 4-7 (Table 6.1).

Table 6.1 Each subject's pregabalin dose and implanted hippocampal region

Animal/Subject ID	**Dosage**	**Hemisphere**	**Hippocampal region (Dorsal/Intermediate)**
Rat1/R401	High (35 mg/kg)	Left Left	Dorsal Intermediate
Rat2/R402	High (35 mg/kg)	Left Left	Dorsal Intermediate
Rat3/R403	High (35 mg/kg)	Left Right	Dorsal Dorsal
Rat4/R411	Low (17.5 mg/kg)	Left Right	Dorsal Intermediate
Rat5/R422	Low (17.5 mg/kg)	Left Right	Dorsal Intermediate
Rat6/R434	Low (17.5 mg/kg)	Left Right	Intermediate Intermediate
Rat7/R468	Low (17.5 mg/kg)	Left Right	Dorsal Intermediate

6.3.2 Task design

The experimental procedure consisted of 5 x 10-minute trials per test day in a square-walled open field (Chapter 5, Figure 5.1). The experiment lasted for 5 days with the rats receiving saline injections for at least the first 3 days and receiving the drug injection on either the fourth or fifth day of the experiment. The purpose of counterbalancing the day of drug administration was ascertain if giving the drug on the fourth day of the experiment would result in an effect on the fifth day of the experiment. Previous research has suggested that between trial differences in both behaviour and hippocampal theta frequency would more than likely be minimal across the last two trials of the day which contain multiple exposures to the same environment (Jeewajee, Barry, et al., 2008; Lever, Burton, & O'Keefe, 2006),

therefore it was decided that the drug and saline injections would be performed immediately following the fourth trial (see Table 6.2 for experimental procedure timeline).

Table 6.2 Experimental procedure timeline.

Day	1	2	3	4	5
Trial	1	1	1	1	1
Trial	2	2	2	2	2
Trial	3	3	3	3	3
Trial	4	4	4	4	4
	 V	 V	 V	 V or D	 V or D
Trial	5	5	5	5	5

V = Vehicle Injection

D = Drug Injection

6.3.3 Procedure

By the start of each experiment, all rats were habituated to the experimenter, the experimental room and holding platform. Each animal had been screened daily on the holding platform for at least 2 weeks prior to the start of the experiment (Chapter 5, Figure 5.1). Each animal was scruffed with a towel daily following their surgery to get them accustomed to the potentially stressful style of handling for intraperitoneal (i.p.) injections.

On each test day, the rats were brought into the experimental room 60-75 minutes before the first trial of the day (2pm) to enable pre-test screening and recording setup. Approximately 1 minute before the start each trial the aural temperature was taken three times, then the rat was placed back on the holding platform, and then immediately placed in the centre of the test arena for 10 minutes. The purpose of taking the rat's temperature was twofold. First, to examine any side-effects upon temperature of pregabalin. Second, to examine the possible relationship between brain temperature (taken aurally) and hippocampal theta frequency (see Chapter 3, section 3.5.4). To encourage continuous movement, the experimenter threw a

piece of black-dyed sweeten rice approximately every 20-30 seconds. At the end of each trial, the rat was removed from the test arena. The rat's aural temperature was taken another three times and then placed back on the holding platform for the 30-minute inter-trial interval. The rationale for implementing a 30-minute inter-trial-interval was influenced by studies such as Siok et al. (2009) which found a theta-frequency reducing effect of pregabalin after 30 minutes of systemic administration in rats. As with Wells et al (2013), the desired latency for a drug effect (30 minutes) in the post-injection trial was used as the inter-trial interval for all trials (30 minutes). The inter-trial protocol was identical for the first four trials of the day. The inter-trial interval between trial 4 (pre-injection trial) and trial 5 (post-injection trial) differed in that the rat was immediately injected with either the drug or saline following the recording of the aural temperature at the end of the fourth trial. Each rat was then placed on the holding platform for 30 minutes before the commencing the fifth trial (post-injection trial).

6.3.4 Temperature measurement

To examine the possible relationship between brain temperature and hippocampal theta frequency, all 7 rats in this experiment had their aural temperatures taken using a thermometer. The temperature readings were taken by restraining each rat in a towel and placing the thermometer probe in the rat's ear until a reading was given, which took approximately 5 seconds. Three readings were taken approximately 1 minute before the start of each trial (pre-trial) and another 3 readings were taken at the end of each trial (post-trial) for all experimental days. The highest temperature of the pre-trial and the highest temperature of the post-trial were averaged together and was used in subsequent analyses.

6.3.5 Statistical analysis

6.3.5.1 Unit of measurement: different values

To examine the effects of pregabalin, the value of the fifth trial ('post-injection' trial) was subtracted from the value of the fourth trial ('pre-injection' trial) to give a single value to show the change in intercept, slope, and running speed between pregabalin and saline days, which were analysed using paired samples t-tests. The effects of pregabalin on hippocampal place cells was analysed using an independent samples t-test for a 'cell-by-cell' analysis and paired sample t-tests for a 'rat-level' analysis of Skaggs spatial information (bits/spike), global mean rate (number of spikes fired divided by trial time in seconds), and locational peak rate (Hz) between pregabalin and saline days. These values were obtained on a specific saline day, which was directly prior to the drug day (day 3 or day 4) and the drug administration day (day 4 or day 5). Temperature effect of pregabalin was analysed by paired sample t-tests, whilst the relationship between slope and intercept with temperature was examined using correlational analysis. Effect size was calculated with Cohen's D and reported with each analysis.

6.4 Results

6.4.1 Electrode localisation

Theta was recorded from the dorsal (c. 3.00 – 4.5 mm behind bregma) and intermediate hippocampus (c. 6.24 mm behind bregma). Figures 6.1 and 6.2 illustrate the histology of three rats. At the time of writing, other brains could not be located. Due to a misunderstanding between the lab and staff of Durham's LSSU, a few brains in a refrigerator were accidentally thrown away, and it is possible that these brains were part of that missing set.

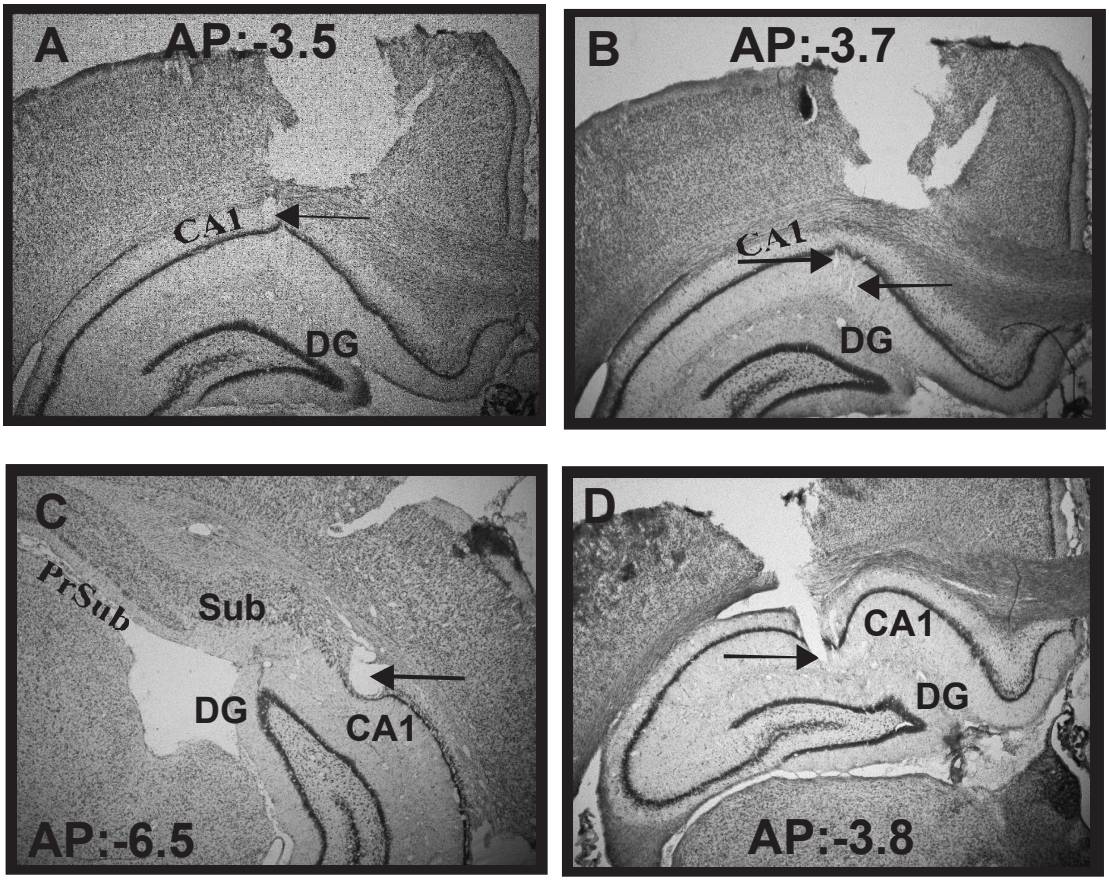


Figure 6.1 Photomicrographs of examples of electrode recording locations in the hippocampus. A-C) show estimated recording sites in the dorsal hippocampus (A, B) and intermediate hippocampus (C) from Rat 411. D) shows recording site in the dorsal hippocampus from Rat 468. All sections are coronal. AP numbers are estimates of distance in millimetres of given coronal section behind bregma. Arrows point to electrode tracks. Estimated recording sites are: A) pyramidal layer/stratum radiatum in dorsal CA1; B) pyramidal layer/stratum radiatum in dorsal CA1 (left track) and stratum radiatum/stratum lacunosum moleculare in dorsal CA1 (right track); C) stratum oriens in intermediate CA1; D) stratum radiatum/stratum lacunosum moleculare in dorsal CA1.

Abbreviations. CA1: Cornu Ammonis Field 1; DG: Dentate Gyrus; Sub: Subiculum; PrSub: Pre- subiculum.

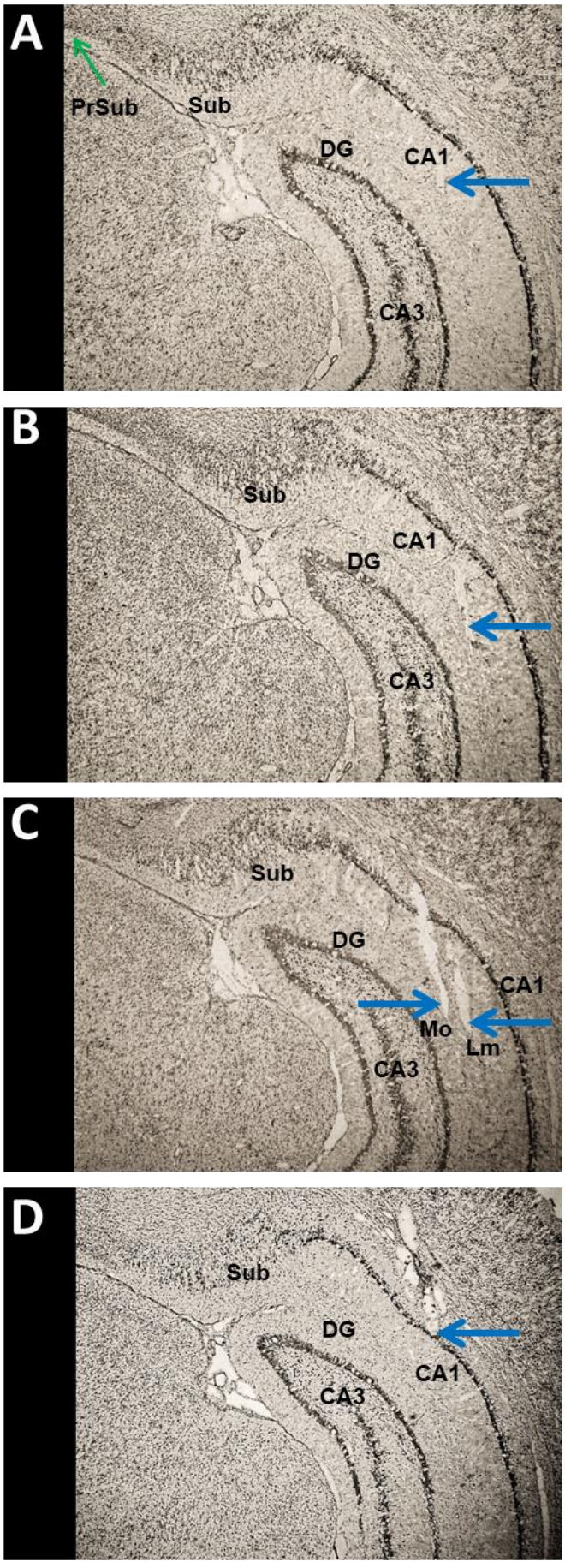

A
PrSub
Sub
DG
CA1
CA3
B
Sub
CA1
DG
CA3
C
Sub
DG
CA1
Mo
Lm
CA3
D
Sub
DG
CA1
CA3

Figure 6.2 Photomicrographs showing estimated locations for recording electrodes implanted in the intermediate hippocampus for rat 422. Arrows show tetrode tracks. An estimated location in brain: -6.2mm posterior to bregma. **A**-**B**) show most posterior signs of electrodes tracks in intermediate hippocampus. Note signs of dorsal presubiculum in the top left of image suggesting posterior extent of section; **B**) is 50µm anterior to *A*; **C**) shows likely ventral-most tip of two theta-recording tetrodes. Electrodes are likely in lacunosum moleculare of CA1 and molecular layer of dentate gyrus and is 150µm anterior to *A*; **D**) shows estimated location of tetrode recording cells from CA1 layer and is 300µm anterior to C.

6.4.2 Pregabalin's effects on mean speed

To examine the effects of high and low dose on the change in speed, an examination in the change of speed (trial 5 – trial 4) between the saline days and pregabalin days for all animals, low dose animals and high dose animals was performed. There was no significant difference between saline post-injection and pregabalin post-injection running speed for low dose animals ($t_3 = 0.349$, $p = 0.750$), nor was there a significant difference between saline post-injection and pregabalin post-injection running speed for high dose animals ($t_2 = 1.447$, $p = 0.285$), nor across all animals ($t_6 = 1.377$, $p = 0.282$). Figure 6.2 shows the average change in speed for both low dose and high dose animals. The effect size of change in running speed for low dose animals was -0.226, with observed power of 0.06. The effect size of change in running speed for high dose animals was -1.67, with observed power of 0.33. The effect size of change in running speed for all animals was 0.867, with observed power of 0.462.

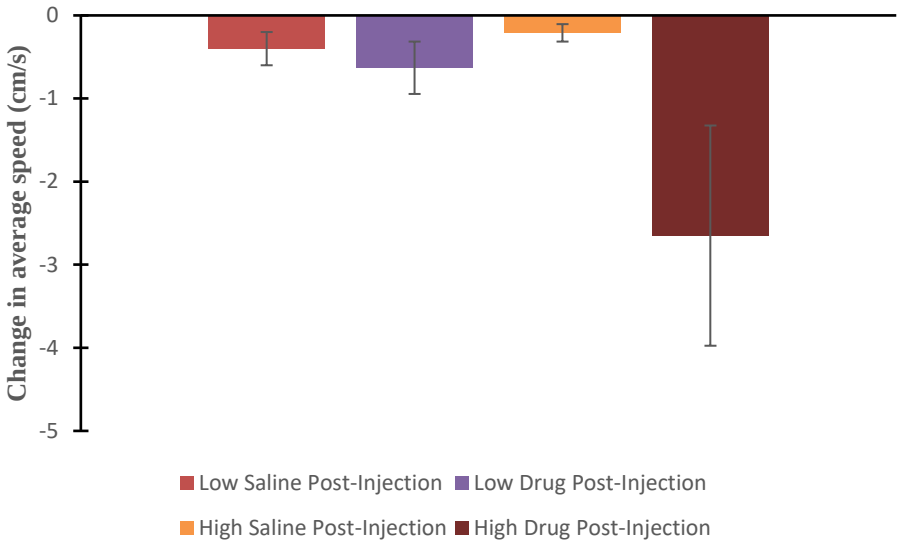


Figure 6.3 The average change in speed of saline, low and high dose of pregabalin. The bars show the average change in speed on the post-injection drug trial compared to the post-injection saline trials (low dose and high dose). Error bars show standard error.

6.4.3 Pregabalin's effect of average intercept and slope

Initially, an analysis was performed to examine the effect pregabalin across all subjects. An average of hippocampal theta frequency recorded from each tetrode for each animal was taken from the day the drug was administered and the day before the drug was administrated (baseline, saline day); dorsal and intermediate combined. This was used to analyse the effect of pregabalin on the intercept and slope component of the theta-frequency-to-speed relationship. A paired samples t-test showed that there was a significant reduction of the intercept component (t_6 = 4.010, p = 0.007), with pregabalin (-0.4059±0.07 Hz reduction) significantly reducing the intercept component compared to saline (0.0388±0.0532 Hz reduction). There was no significant effect of slope (t_6 = 1.169, p = 0.287). The effect size of average intercept reduction for pregabalin was 2.238, with observed power of 0.998. The effect size of average slope reduction for pregabalin was 0.715, with observed power of 0.357. The sample size used in this analysis was n=7, with the appropriate sample size for average intercept reduction with minimum power of 0.8 being n = 4 and the appropriate sample size for average slope reduction being n = 18. Figure 6.3 illustrates the reduction of

the intercept component of the theta-frequency-to-speed relationship for an individual animal (rat 3) and 6.4 shows the reduction of theta intercept across all subjects.

Subsequent analysis of the average dorsal intercept showed a significant effect ($t_5 = 4.205$, $p = 0.008$) with pregabalin (-0.459±0.0731 Hz reduction) significantly reducing the intercept component compared to saline (0.0525±0.0617 Hz reduction). Pregabalin had no significant effect on dorsal slope ($t_5 = 0.981$, $p = 0.372$). The effect size of average dorsal intercept reduction for pregabalin was 2.455, with observed power of 0.997. The effect size of average dorsal slope reduction for pregabalin was 0.654, with observed power of 0.257. The sample size used in this analysis was n = 6, with the appropriate sample size for average dorsal intercept reduction with minimum power of 0.8 being n = 4 and the appropriate sample size for average dorsal slope reduction being n = 21.

Analysis of the average intermediate intercept showed a significant effect ($t_5 = 5.000$, $p = 0.004$) with pregabalin (-0.3477±0.0628 Hz reduction) significantly reducing the intercept component compared to saline (-0.0075±0.0253 Hz reduction). Pregabalin had no significant effect on intermediate slope ($t_5 = 1.115$, $p = 0.316$). The effect size of average intermediate intercept reduction for pregabalin was 2.902, with observed power of 0.998. The effect size of average intermediate slope reduction for pregabalin was 0.521, with observed power of 0.181. The sample size used in this analysis was n = 6, with the appropriate sample size for average intermediate intercept reduction with minimum power of 0.8 being n = 4, and the appropriate sample size for average intermediate slope reduction being n = 31. The power-related calculations were performed using G*Power, version 3.1.9.2, with α set to 0.05, two-tailed.

As argued in the Wells and colleagues' (Wells et al., 2013) study, the dissociation observed with an anxiolytic drug reducing intercept component whilst not affecting the slope

component is somewhat conditional. In other words, anxiolytics may, in fact, affect the slope component of the theta-frequency-to-speed relationship in certain conditions. In that respect, the above reports of the effect size, power and sample size demonstrate to what degree in which the conditions tested within this thesis, anxiolytic drugs and environmental familiarisation, are dissociable. Although the above analysis is across dosage groups (high, 35 mg/kg; low, 17.5 mg/kg), it can be argued that not only does pregabalin show a single dissociation (intercept component significantly reducing theta frequency whilst not affect the slope component), but that dissociation appears to be more sensitive in the intermediate portion of the hippocampus. To further investigate this dissociation, the analysis detailed below strictly examines the effects of low dose pregabalin (17.5 mg/kg).

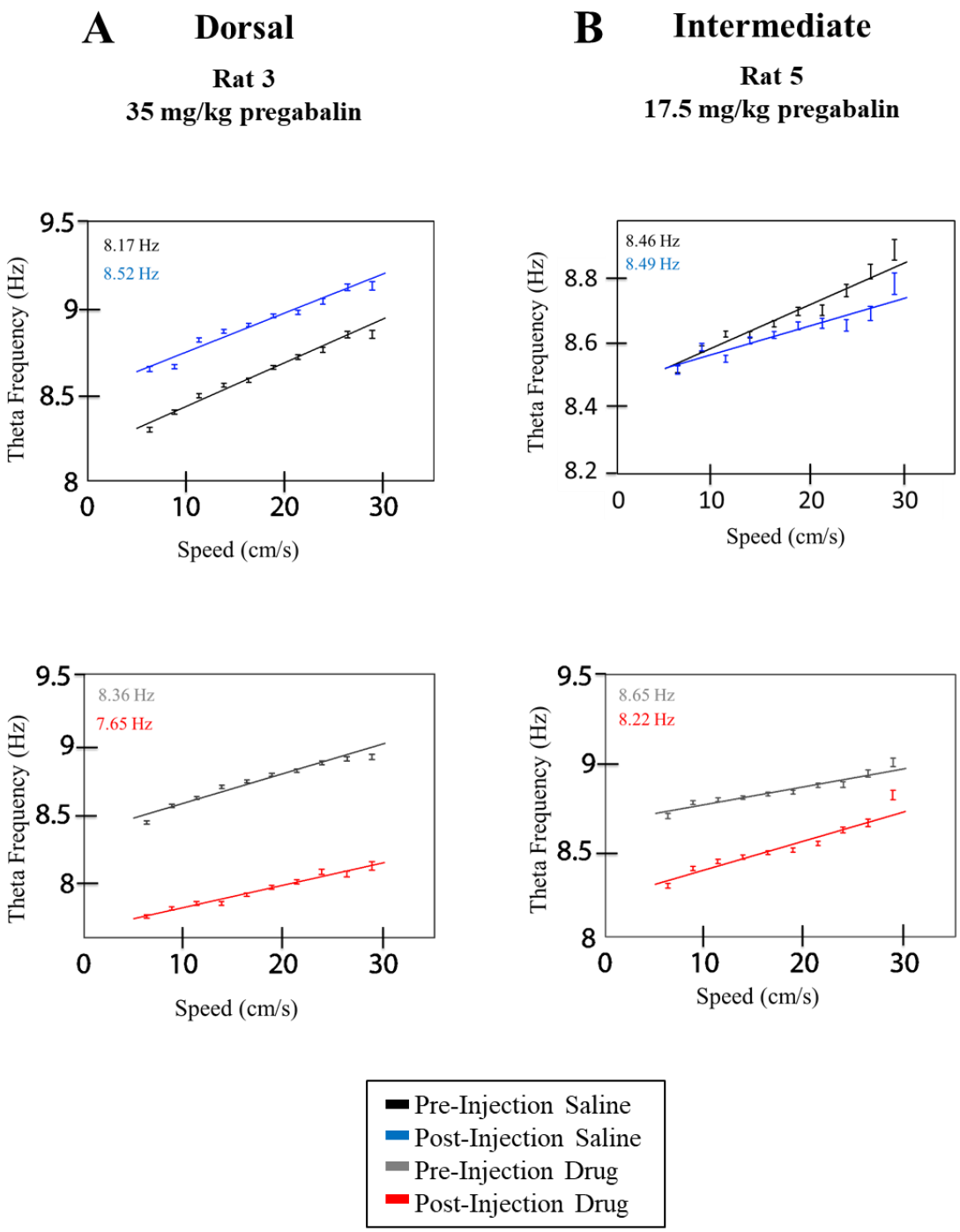


Figure 6.4 Pregabalin (high and low dose) reduces intercept of the theta frequency-to-speed-relationship. A) The graphs shown here are from the ***dorsal*** hippocampus of rat 3 (***high dose*** animal, 35 mg/kg), with the ***top*** graph showing theta frequency regression line from Saline day, whilst the ***bottom*** graph shows theta frequency regression line from Drug Day. The numbers in the upper left corner of each graph are the frequency values. The differentiating colours correspond to the key located at the bottom of the figure; B) The graphs shown here are from the ***intermediate*** hippocampus of rat 5 (***low dose*** animal, 17.5 mg/kg), with the ***top*** graph showing theta frequency regression line from Saline day, whilst the ***bottom*** graph shows theta frequency regression line from Drug day. The numbers in the upper left corner of each graph are the frequency values. The differentiating colours corresponds to the key located at the bottom of the figure.

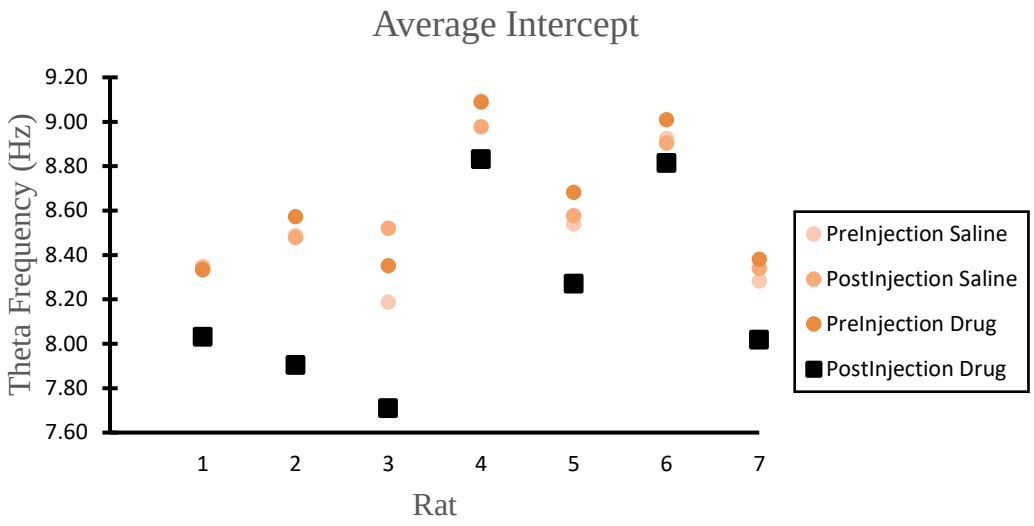


Figure 6.5 Average intercept pre-injection saline, post-injection saline, pre-injection drug and post-injection drug values for all seven rats. The black (square) points in the graph illustrate the reduction in the intercept component for post-injection drug trials of the theta-frequency-to-speed relationship compared to the pre- and post-injection saline trials, and the pre-injection drug trials.

6.4.3.1 Low dose effect of intercept and slope

Although there are 3 animals that received a high dose (35 mg/kg) of pregabalin, it was determined that the sample size of 3 animals meant the analysis was underpowered. For comparison, effect sizes of drug-elicited intercept reduction in the Wells et al., (2013) study for CDP and Buspirone were 2.09 and 2.06 respectively. Using Cohen's D to determine effect size, G*Power calculations showed that for matched pairs t-test, with two-tailed alpha p value set at 0.05, and power at 0.80, a sample size of three animals can only detect very high effect sizes (> 2.297). (For the sake of comprehensivity, the results are noted here: the effect size of average intercept reduction for high dose of pregabalin was 2.15, with observed power of 0.517. In contrast, the effect size of average intercept reduction for low dose of pregabalin was 3.34, with observed power of 0.987). It was decided that analysis of the lower dose of pregabalin was sufficient with four animals, the sample size in Wells et al., (2013).

6.4.3.1.1 Average low dose intercept and slope

Paired samples t-test analysis of average low dose intercept showed a significant intercept-reducing effect of pregabalin (-0.3072±0.0491 Hz reduction; t_3 = 3.687, p = 0.035). There was no significant effect of slope (-0.0156±0.2479 Hz/m/s; t_3 = -0.117, p = 0.915). The effect size of average low dose intercept reduction for pregabalin was 3.34, with observed power of 0.987. The effect size for average low dose slope reduction for pregabalin was 0.097, with observed power of 0.05. The sample size used in this analysis was n = 4, with the appropriate sample size for average low dose intercept reduction with minimum power of 0.8 being n = 3, and the appropriate sample size for average low dose slope reduction being n = 828. Therefore, to detect an effect of low dose pregabalin on slope reduction would require 275X more subjects than it would to detect the same dose of pregabalin on intercept.

Figure 6.5 shows the reduction of theta intercept for low dose animals.

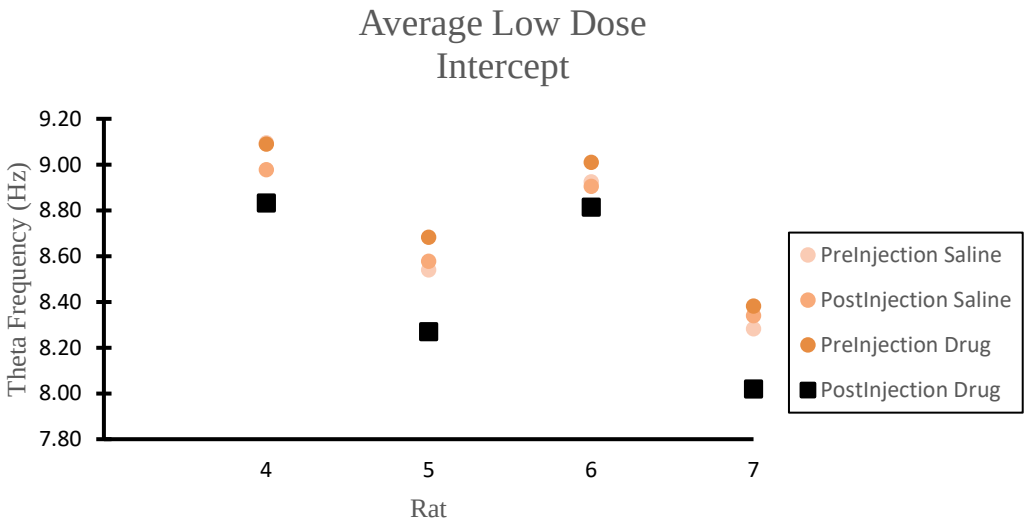


Figure 6.6 Average low dose average intercept pre-injection saline, post-injection saline, pre-injection drug and post-injection drug values for rats 4-7. The black (square) points in the graph illustrate the reduction in the intercept component for post-injection drug trials of the theta-frequency-to-speed relationship compared to the pre- and post-injection saline trials, and the pre-injection drug trials.

6.4.3.2.2 Average low dose dorsal intercept and slope

Paired samples t-test analysis of average low dose dorsal intercept showed no significant intercept-reducing effect of pregabalin (-0.3650±0.0483 Hz reduction; $t_2 = 3.642$, $p = 0.068$), nor was there a significant difference in low dose dorsal slope between pregabalin (0.0017±0.3577 Hz/m/s) and saline (-0.1225±0.3191 Hz/m/s; $t_2 = -0.219$, $p = 0.847$). The effect size of average low dose dorsal intercept reduction for pregabalin was 3.982, with observed power of 0.998. The effect size of average low dose dorsal slope reduction for pregabalin was 0.206, with observed power of 0.056. The sample size used in this analysis was n = 3, with the appropriate sample size for average low dose dorsal intercept reduction with minimum power of 0.8 being n = 3, and the appropriate sample size for average low dose dorsal slope reduction being n = 188.

6.4.3.2.3 Low dose average intermediate intercept and slope

Paired samples t-test analysis of low dose intermediate intercept showed a significant intercept-reducing effect of pregabalin (-0.2909±0.045 Hz reduction; $t_3 = 3.894$, $p = 0.030$), see (Figure 6.6 and Figure 6.7). There was no significant effect on intermediate slope ($t_3 = 0.055$, $p = 0.960$; -0.0613±0.239 Hz/m/s). The effect size of average low dose intermediate reduction for pregabalin was 3.43, with observed power of 0.989. The effect size of average low dose intermediate slope reduction for pregabalin was 0.046, with observed power of 0.050. The sample size used in this analysis was n = 4, with the appropriate sample size for average low dose intermediate intercept reduction with minimum power of 0.8 being n = 3, and the appropriate sample size for average intermediate slope reduction being n = 3653.

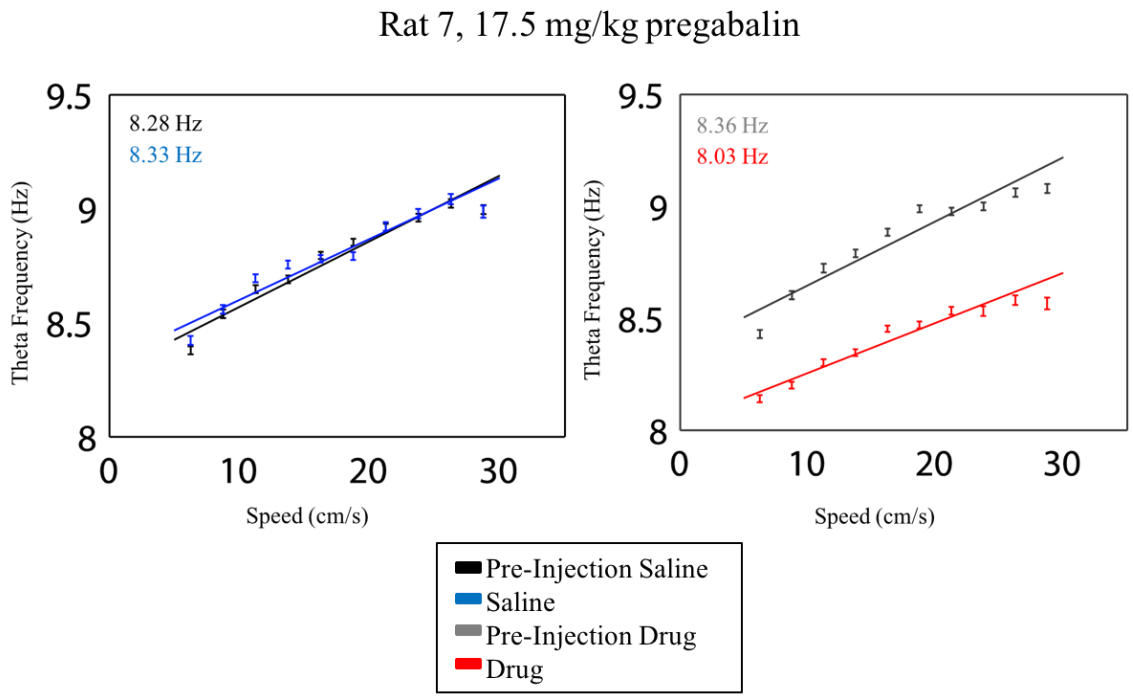


Figure 6.7 Low dose pregabalin significantly reduces intermediate intercept of the theta-frequency-to-speed relationship. Rat 7, 17.5 mg/kg (low dose) 0.33 Hz reduction in comparison to the pre-injection drug trial. Each point shows the mean, the error bars show SEM. The regression lines are fitted to each trial.

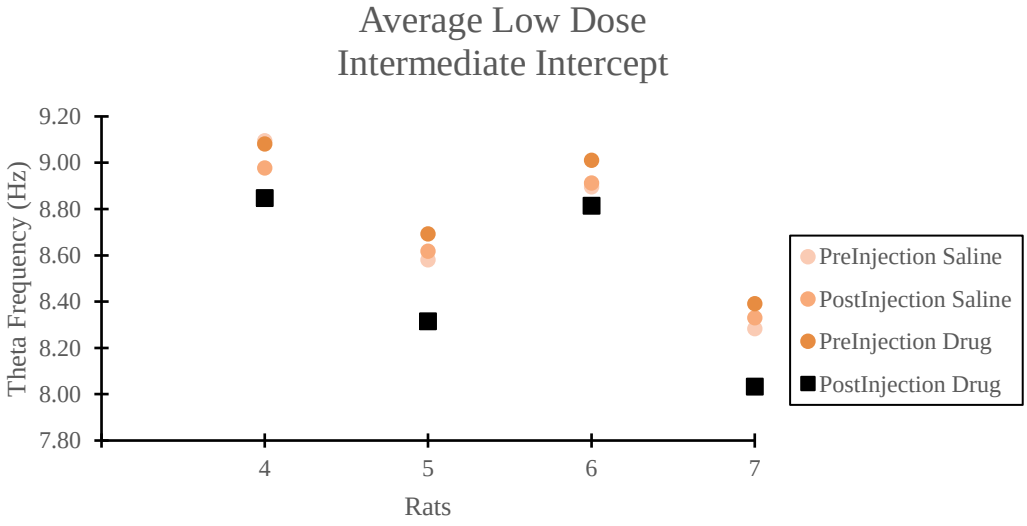


Figure 6.8 Average low dose intermediate intercept pre-injection saline, post-injection saline, pre-injection drug and post-injection drug values for rats 4-7. The black (square) points in the graph illustrate the reduction in the intercept component for post-injection drug trials of the theta-frequency-to-speed relationship compared to the pre- and post-injection saline trials, and the pre-injection drug trials.

6.4.4 The effect of pregabalin on temperature, and effects of temperature on intercept and slope

There are limited studies that examine the relationship between theta frequency and temperature in rodents. Of the studies that do examine the relationship between theta frequency and temperature there appears to be a positive relationship (Deboer, 2002; Whishaw & Vanderwolf, 1973). There are also few studies that examine how anxiolytic drugs might affect aural temperature. Wells and colleagues (2013) found that though both buspirone and CDP reduced the intercept of the theta-frequency-to-speed relationship, only buspirone was found have an effect upon temperature by reducing the aural temperatures in rats. Additionally, Wells and colleagues found a positive correlation between aural temperature and slope recorded in pre-injection trials.

As with the results found in the Wells et al., (2013) study, the present study found that pregabalin reduced the intercept component of the theta-frequency-to-speed relationship. Based on their study, the examination of aural temperature was done to ascertain if: 1) pregabalin affected temperature and 2) if temperature affected the slope and intercept components of the theta-frequency-to-speed relationship.

6.4.4.1 The effect of pregabalin on temperature

The effect of drug on aural temperature was analysed by comparing the net temperature change from post-injection drug and pre-injection drug to post-injection saline and pre-injection saline (irrespective of drug dose), in the same way that the effect of drug is examined in theta frequency (post-injection trial – pre-injection trial; trial 5 – trial 4; pregabalin compared to saline).

A paired samples t-test was performed for all seven rats and found that there was no significant effect of pregabalin upon aural temperature (saline temperature change: (0.21±0.272 °C); pregabalin temperature change (-0.31±0.172 °C); $t_6 = 1.511$, $p = 0.182$).

6.4.4.2 Temperature effect on intercept and slope

Contrary to the Wells et al. (2013) study, there was no evidence a correlation between temperature and the slope component. Correlational analysis was performed for all subjects' average intercept and slope values, dorsal intercept and slope values, and intermediate intercept and slope values (Table 6.3 and Table 6.4) The correlational analysis was conducted using the pre-injection trials (1-4) from test days 1-5.

Interestingly, one animal (Rat 5) did show a positive correlation between temperature and average intercept (r = 0.777, *p* = 0.0004), dorsal intercept (r = 0.76, p = 0.001*) and intermediate intercept (r = .778, p = 0.0004*), whilst the remaining 6 animals did not show an effect on slope and intercept.

Table 6.3 Correlation results; slope and temperature

Rat ID	Average	Dorsal	Intermediate
R401 (Rat 1)	r = .34, p = .201	r = .25, p = 0.352	r = .31, p = .244
R402 (Rat 2)	r = .25, p = 0.342	r = .28, p = 0.291	r = .22, p = 0.418
R403 (Rat 3)	r = -.022, p = 0.936	r = -.022, p = 0.936	----
R411 (Rat 4)	r = .09, p = 0.735	r = .19, p = 0.485	r = .06, p = 0.829
R422 (Rat 5)	r = .23, p = 0.396	r = .22, p = 0.411	r = .22, p = 0.408
R434 (Rat 6)	r = -.15, p = 0.573	----	r = -.15, p = 0.573
R468 (Rat 7)	r = .31, p = 0.243	r = .31, p = 0.249	r = .31, p = 0.248

Table 6.4 Correlation results; intercept and temperature

Rat ID	Average	Dorsal	Intermediate
R401 (Rat 1)	r = .38, p = 0.144	r = .25, p = 0.352	r = .40, p = 0.121
R402 (Rat 2)	r = .36, p = 0.171	r = .29, p = 0.280	r = .42, p = 0.103
R403 (Rat 3)	r = -.15, p = 0.572	r = -.15, p = 0.572	——
R411 (Rat 4)	r = -.002, p = 0.976	r = -.062, p = 0.819	r = .05, p = .854
R422 (Rat 5)	r = .777, p = 0.0004*	r = 0.76, p = 0.001*	r = .778, p = 0.0004*
R434 (Rat 6)	r = .35, p = 0.189	——	r = .35, p = 0.189
R468 (Rat 7)	r = .31, p = 0.240	r = .39, p = 0.201	r = .26, p = 0.323

6.5 Pregabalin's effect on hippocampal place cells

Of the seven rats tested, five rats yielded 127 pyramidal cells (Table 6.5) recorded in the dorsal and intermediate hippocampus CA1 field. To examine the effect of pregabalin on hippocampal place cells, cell-by-cell analysis (independent samples t-tests) and rat-level analysis (paired sampled t-tests) were performed. Depicted after the analysis are hippocampal place fields recorded during Experiment 1. Figure 6.15 shows the rate maps of place cells recorded from rat 3 and rat 5, with figure 6.15A shows a large-scale version of a rate map. Figure 6.15B shows cells recorded on the baseline saline day, Figure 6.15C showing cells recorded on the drug day, and Figure 6.15D showing cells recorded on the second saline day of rat 3. Figure 6.15E showing cells recorded baseline saline day, Figure 6.15F showing cells recorded on the pregabalin day and Figure 6.15G showing cells recorded on the second saline day.

Table 6.5 The number of cells, recorded region for each animal and total number of cells recorded by region

Rat ID	Region	Number of Cells	Total # by region	
R401 (Rat 1)	Dorsal	1	Dorsal	Intermediate
	Intermediate	14	**80**	**52**
R402 (Rat 2)	Dorsal	2		
	Intermediate	0		
R403 (Rat 3)	Dorsal	40		
R411 (Rat 4)	Dorsal	3		
	Intermediate	10		
R422 (Rat 5)	Dorsal	34		
	Intermediate	28		

6.5.1 Cell-by-cell analysis: pregabalin's effect on global mean rate, locational peak rate and spatial information

The initial analysis examining the effect of pregabalin on pyramidal cells was performed on the saline day before the drug day (3rd or 4th day) and the drug (4th or 5th day). It should be noted that a portion of the animals were given a second saline or baseline day (see Table 6.2 for experimental procedure). Across five animals, there were 38 cells recorded on the baseline saline day and 51 cells recorded on the pregabalin day. To determine if pregabalin effected hippocampal place fields, the global mean rate, locational peak rate, Skaggs spatial information and the place cell's field size were examined (post-injection saline compared to post-injection pregabalin).

6.5.1.1 Global mean rate

The global mean rate was calculated by taking the number of spikes a cell fired and dividing that by time of each trial (600 seconds). An independent samples t-test found that there was no significant effect of pregabalin on global mean rate (saline: -0.02±0.11 Hz; pregabalin: -0.07±0.41 Hz); $t_{87} = 0.445$, $p = 0.657$. The effect size for an effect of pregabalin on global mean rate was 0.092, with observed power of 0.138. The sample size used in this analysis was n = 89, with the appropriate sample size for an effect of pregabalin on global mean rate with minimum power of 0.8 being n = 929. Figure 6.9 illustrates there's no significant different in global mean rate between saline and pregabalin days.

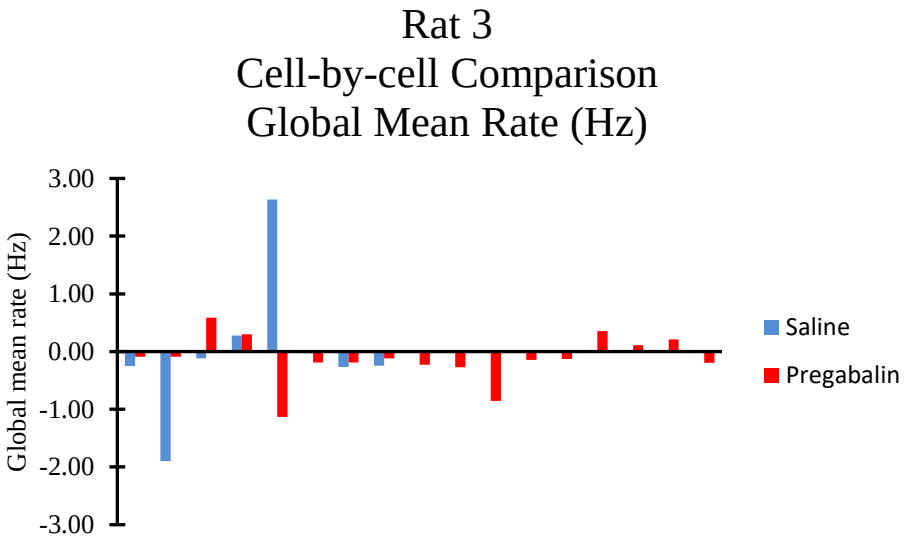


Figure 6.9 Cell-by-cell comparison global mean rate; Rat 3. Each bar represents a cell's change in global mean rate (Hz; trial 5 – trial 4). The blue bars are cells recorded on the baseline saline day (n = 8) and the red bars are cells recorded on the pregabalin day (n = 17).

6.5.1.2 Locational peak rate

An independent samples t-test found that there was no significant effect of pregabalin on locational peak rate (saline: -0.81±0.42 Hz; pregabalin: -0.52±0.38 Hz; $t_{87} = -0.510$, $p = 0.611$). The effect size of locational peak rate was 0.11, with observed power of 0.176. The

sample size used in this analysis was n = 89, with the appropriate sample size for an effect of pregabalin on locational peak rate being n = 653. Figure 6.10 illustrates there's no significant difference in locational peak rate of between saline and pregabalin days.

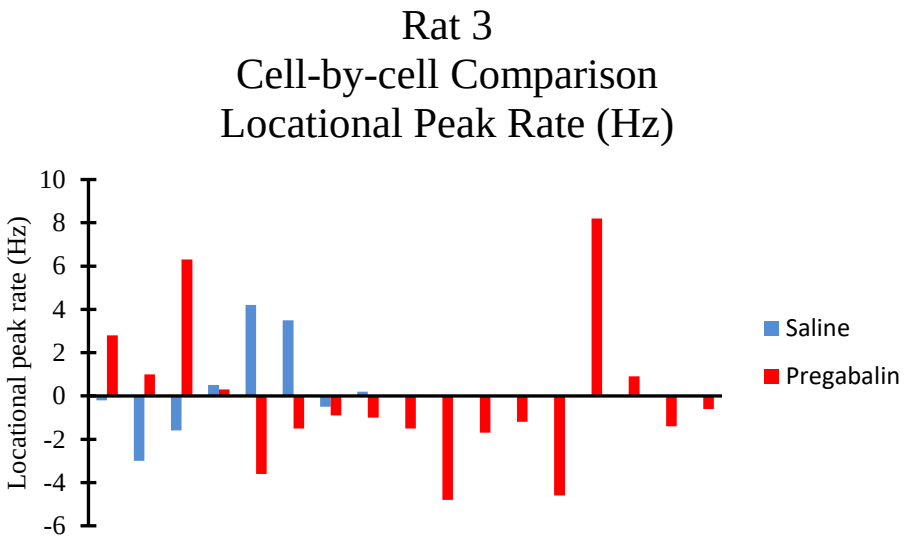


Figure 6.10 Cell-by-cell comparison locational peak rate; Rat 3. Each bar represents a cell's change in locational peak rate (Hz; trial 5 – trial 4). The blue bars are cells recorded on the baseline saline day (n = 8) and the red bars are cells recorded on the pregabalin day (n = 17).

6.5.1.3 Spatial information

Briefly, Skaggs spatial information is based on a formula used to determine the average rate of chance in neuron firing in response to potential manipulations and other changes (Skaggs et al., 1993). An independent samples t-test found that there was no significant effect of pregabalin on spatial information (saline: -0.10±0.067 bits/spike; pregabalin: -0.06±0.048 bits/spike; t_{87} = -0.516, p = 0.607). The effect size of Skaggs spatial information was 0.109, with observed power of 0.174. The sample size used in this analysis was n = 89, with the appropriate sample size being n = 661. Figure 6.11 illustrates there's no significant difference in Skaggs spatial information between saline and pregabalin days.

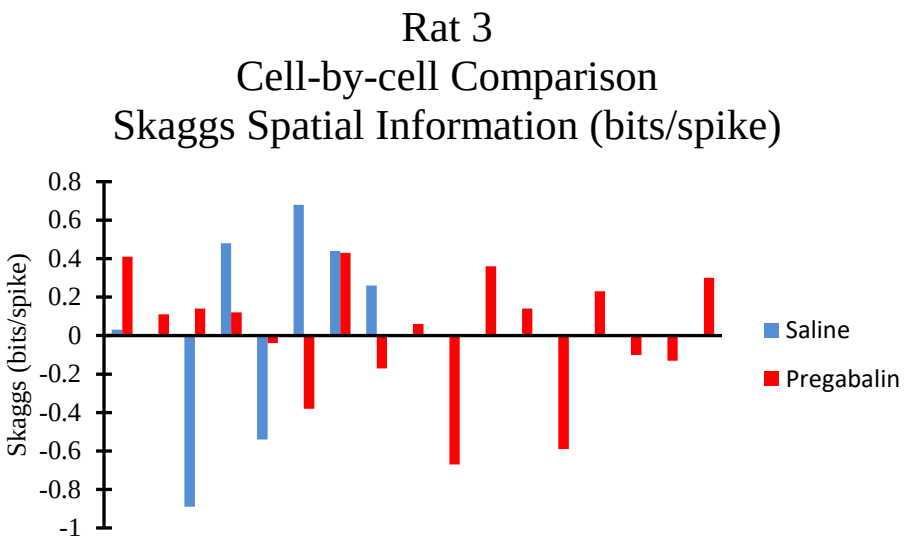


Figure 6.11 Cell-by-cell Comparison Skaggs Spatial Information; Rat 3. Each bar represents a cell's change in Skaggs spatial information (bits/spike; trial 5 – trial 4). The blue bars are cells recorded on the baseline saline day (n = 8) and the red bars are cells recorded on the pregabalin day (n = 17).

6.5.1.4 Field Size

To determine the field size of each cell, a MATLAB function was used to obtain the field size in bins. The field size was converted from bins to cm^2 by taking the length of the testing apparatus (60 cm) and dividing it by the length of the x-axis of the figure produced by the MATLAB function (25 bins). That value (2.4) was squared which provides the number of square centimetres per bin (5.76). Once the field sizes have been converted, a change index was calculated. Just like with calculating the D2 scores in objection recognition experiments, the change index was calculated by subtracting the trial 4 field size from the trial 5 field size and dividing that number by the value of trial 4 field size plus trial 5 field size. An independent samples t-test was performed using the change index values to ascertain if pregabalin affected hippocampal place cell field size. The t-test showed that there was no significant difference between saline (0.027±0.042 cm^2) and pregabalin (-0.02±0.024 cm^2); $t_{87} = 1.032$, $p = 0.305$. The effect size of hippocampal place cell field size was 0.215, with observed power of 0.517. The sample size used in this analysis was n = 89, with the

appropriate sample size being n = 173. Figure 6.12 illustrates there's no significant difference in place cell field size between saline and pregabalin days.

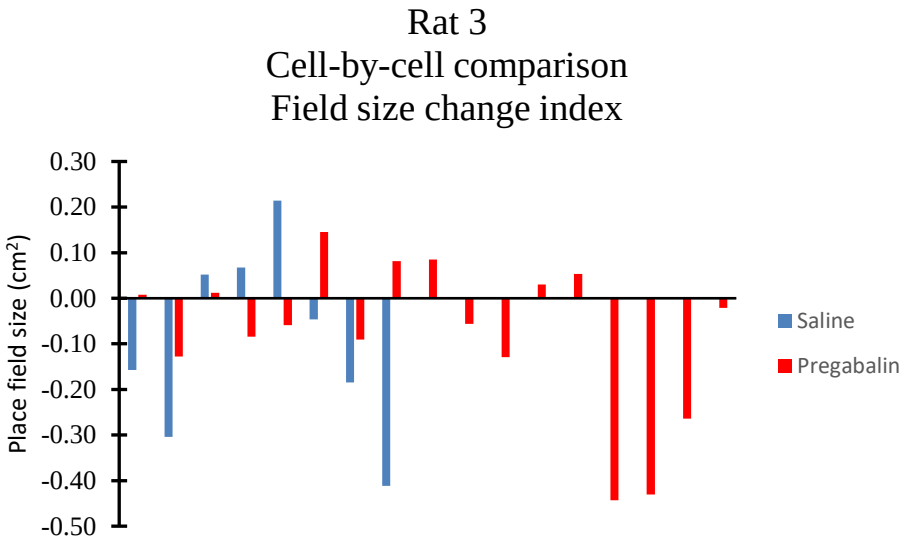


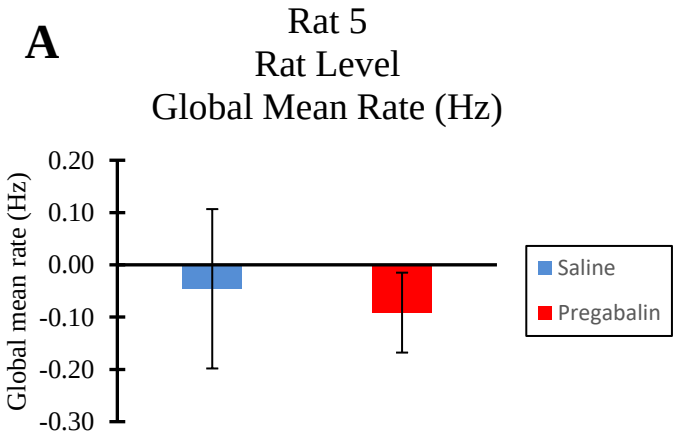
Figure 6.12 Cell-by-cell Comparison Field Size Average Change Index; Rat 3. Each bar represents a cell's post-injection change index (cm^2; trial 5 – trial 4). The blue bars are cells recorded on the baseline saline day (n = 8) and the red bars are cells recorded on the pregabalin day (n = 17).

6.5.2 Rat-level analysis: pregabalin's effect on average global mean rate, locational peak rate and spatial information

The effect of pregabalin on hippocampal place cells was also analysed by looking at the difference between saline and pregabalin days for each rat. Individual cell values within a rat were averaged to provide a single post-vs-pre-injection value for the saline and pregabalin day for each rat. Paired sample t-tests were performed to examine the effects of pregabalin on global mean rate, locational peak rate, spatial information and field size change index.

Paired samples t-test analysis of the change in average global mean rate found that there was no significant effect of pregabalin (saline: 0.01±0.04 Hz; pregabalin: 0.13±0.21 Hz; t_4 = 0.662, p = 0.544) The effect size of global mean rate was 0.394, with observed power of

0.091. There was no significant effect of pregabalin on the change in average Skaggs spatial information (saline: -0.19±0.12 bits/spike; pregabalin: -0.09±0.05 bits/spike; t_4 = -0.745, p = 0.498). The effect size of Skaggs spatial information was 0.444, with observed power of 0.116. There was no significant effect of pregabalin on the average place field size change index (saline:0.045±0.04 cm^2; pregabalin:0.005±0.026 cm^2; t_4 = 1.515, p = 0.204). The effect size of average place field was 0.530, with observed power of 0.152. The sample size used for theses paired sample t-test analysis was n = 5, with the appropriate sample size for global mean rate with minimum power of 0.8 being n = 70, the appropriate sample size for locational peak rate being n = 16, the appropriate sample size for Skaggs spatial information being n = 44, and the appropriate sample size for place field size being n = 30. Figure 6.13 further illustrates there was no difference in effect between saline and pregabalin on hippocampal place cells.



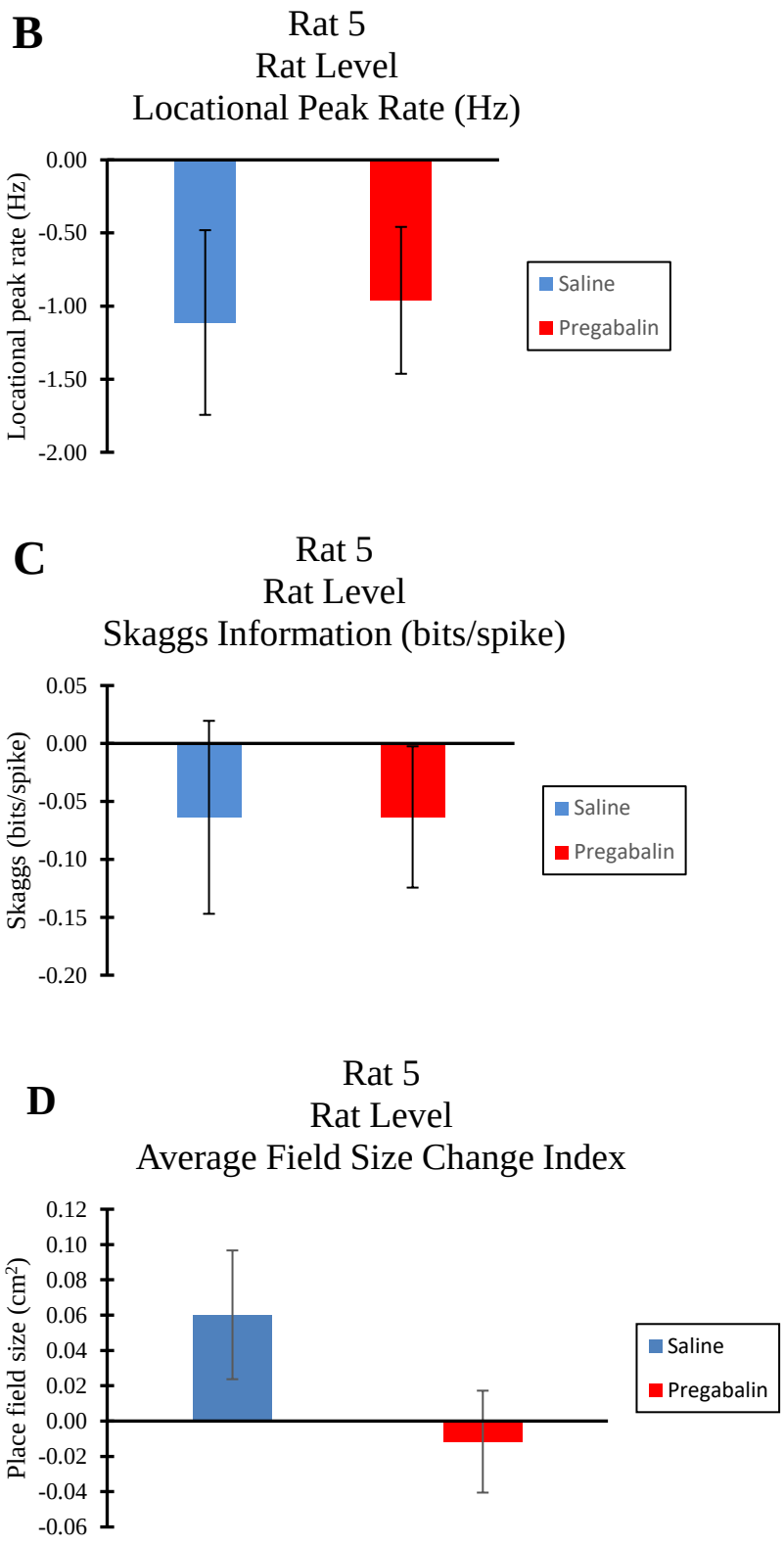


Figure 6.13 There was no significant difference in hippocampal place cell characteristics between saline and pregabalin days; Rat 5. Each graph illustrates that average change (trial 5 – trial 4) of hippocampal cell properties on Saline (blue bar) and Pregabalin (red bar) days for Rat 5; (**A**) average global mean rate (Hz), (**B**) average locational peak rate (Hz), (**C**) average Skaggs spatial information (bits/spike), and (**D**) average field size change index.

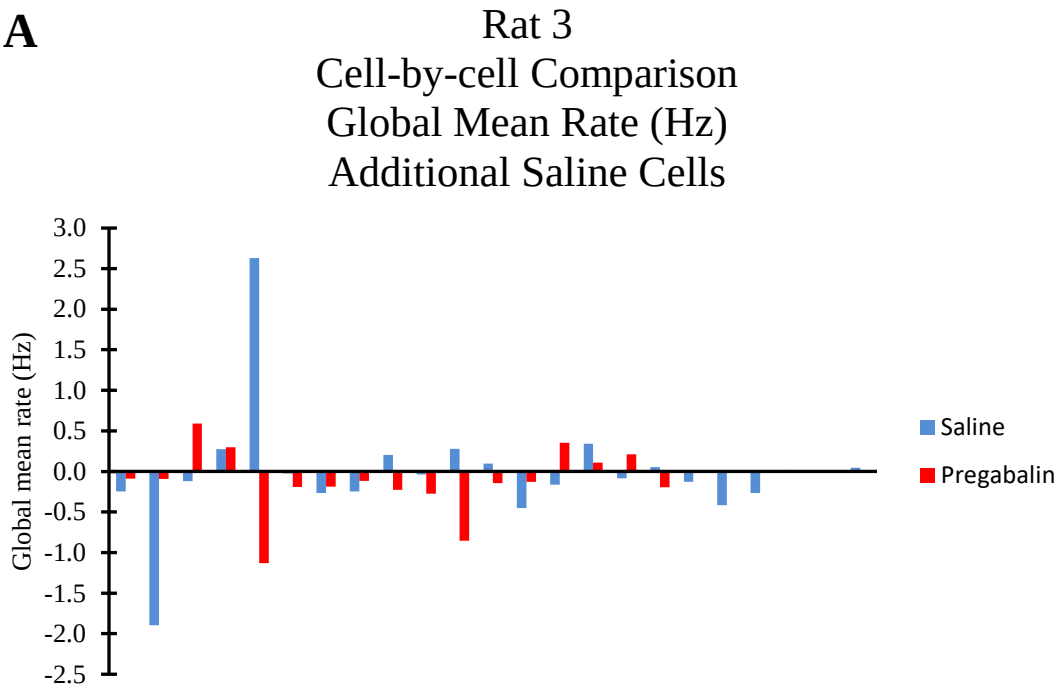
6.5.3 Pregabalin's effect of place cells, additional saline day

As stated previously, the initial analysis consisted of values taken from the saline day directly before the drug day (baseline saline day) and the drug day itself. A portion of the animals were tested for an additional saline day (Day 5). Additional analysis was performed with the values from the second saline day included. Thirty-eight additional cell values were added to the number of saline cells recorded (saline, n = 76; pregabalin, n = 51).

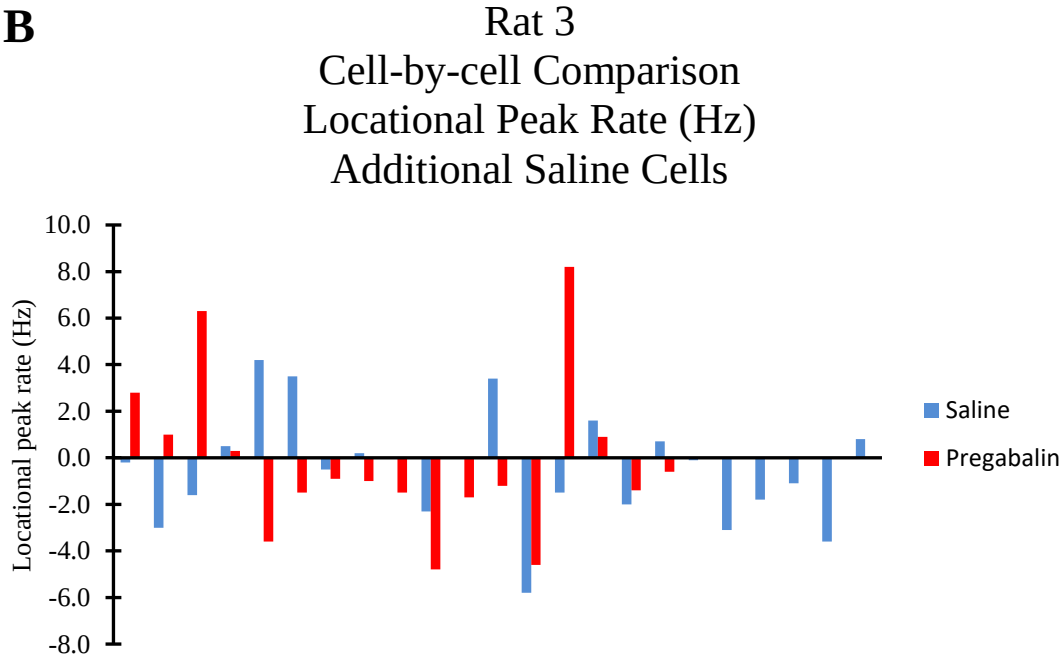
The results of the additional cell-by-cell independent samples t-test analysis with the additional saline values were roughly the same as the initial analysis (figure 6.13). There was no significant effect of pregabalin on global mean rate (saline change: -0.02±0.06 Hz; pregabalin change: -0.07±0.06 Hz; $t_{125} = 0.529$, $p = 0.598$ Hz), locational peak rate (saline change: -0.74±0.29 Hz; pregabalin change: -0.52±0.38Hz); $t_{125} = -0.460$, $p = 0.646$), on Skaggs spatial information (saline change: -0.10±0.04 bits/spike; pregabalin change: -0.06±0.05 bits/spike; $t_{125} = -0.535$, $p = 0.594$), nor on the field size change index (saline change: 0.052±0.029 cm^2; pregabalin change: -0.02±0.024 cm^2; $t_{125} = 1.795$, $p = 0.075$).

The effect size of global mean rate was 0.098, with observed power of 0.196; the effect size of locational peak rate was 0.083, with observed power of 0.152; the effect size of Skaggs spatial information was 0.098, with observed power of 0.195; and the effect size of place field size change index was 0.337, with observed power of 0.965. The sample size used in these independent sample t-tests was n = 127, with the appropriate sample size with minimum power of 0.8 for global mean rate being n = 815, for locational peak rate being n = 1150, for Skaggs spatial information being n = 822, and for place field size change index being n = 72. Figure 6.14 illustrates there was no difference in place cells between saline and pregabalin days even with the inclusion of cells from an additional saline day.

A


Rat 3
Cell-by-cell Comparison
Global Mean Rate (Hz)
Additional Saline Cells
Global mean rate (Hz)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
Saline
Pregabalin

B


Rat 3
Cell-by-cell Comparison
Locational Peak Rate (Hz)
Additional Saline Cells
Locational peak rate (Hz)
10.0
8.0
6.0
4.0
2.0
0.0
-2.0
-4.0
-6.0
-8.0
Saline
Pregabalin

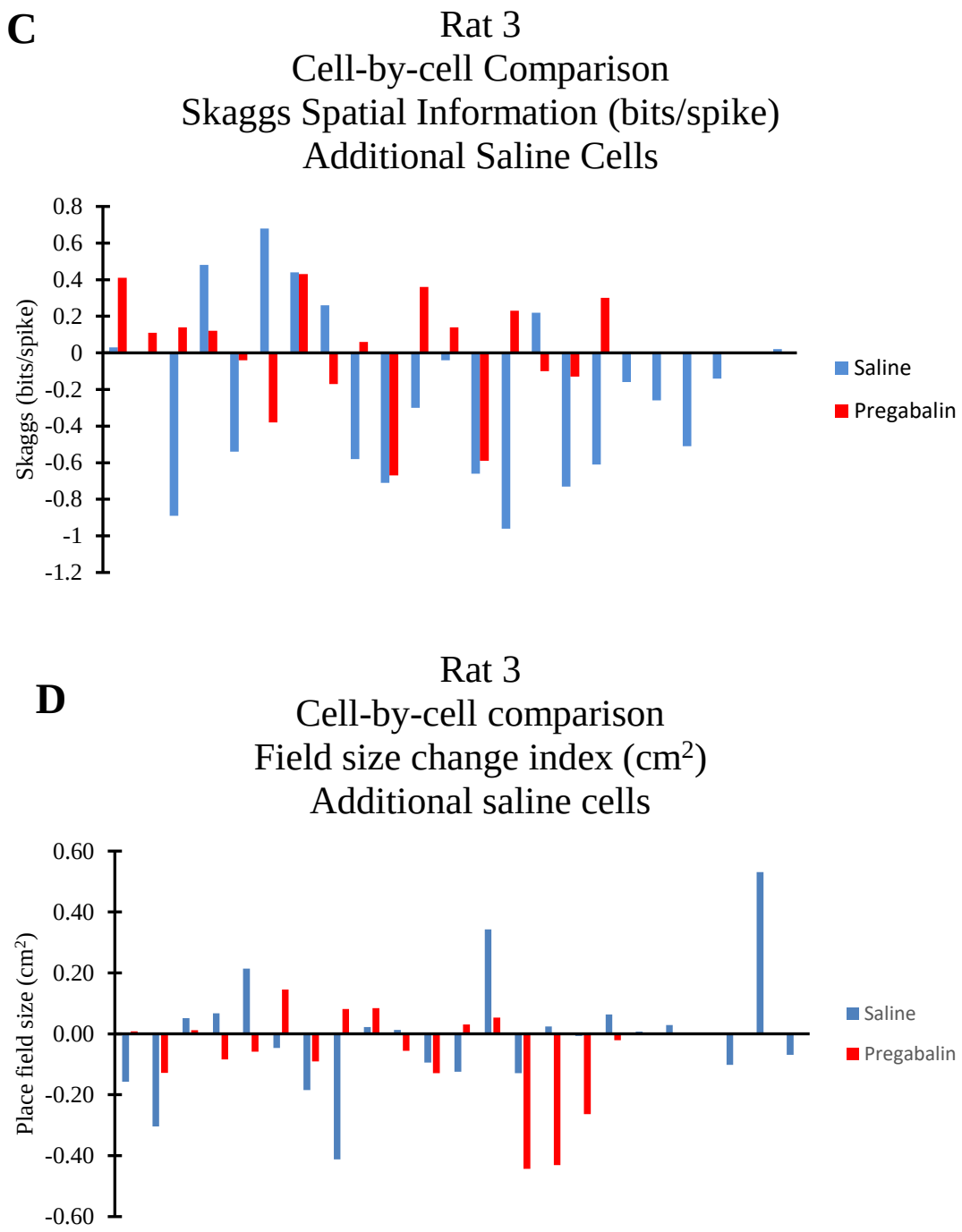
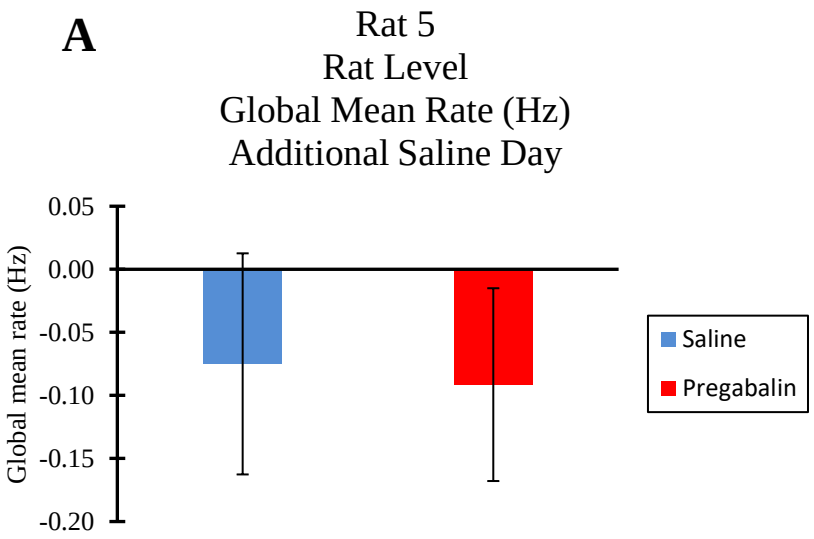
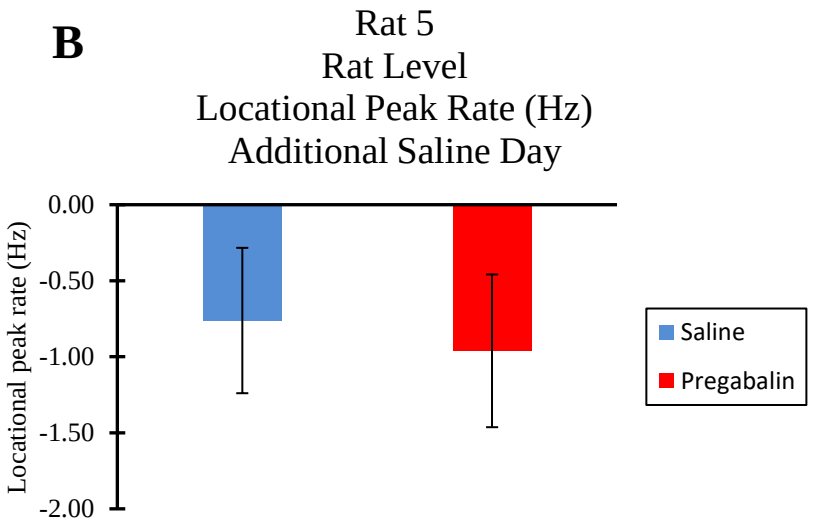


Figure 6.14 Cell-by-cell Comparison, additional Saline Cells; Rat 3. Each bar graph shows the cell's change in (**A**) global mean rate (Hz; trial 5 – trial 4), (**B**) locational peak rate (Hz; trial 5 – trial 4), (**C**) Skaggs spatial information (bits/spike; trial 5 – trial 4) with the additional saline cells, and (**D**) field size change index (cm^2; trial 5 – trial 4). The blue bars are cells recorded on both the baseline saline day and the additional saline day (n = 23), and the red bars are cells recorded on the pregabalin day (n = 17).

The analysis with the additional saline values at the rat level of the average change of global mean rate, locational peak rate, spatial information and field size change index also showed no effects of pregabalin: average global mean rate (saline change: 0.03±0.04 Hz; pregabalin change: 0.13±0.21 Hz; t_5 = -0.559, p = 0.600), average locational peak rate (saline change: -0.74±0.29 Hz; pregabalin change: -0.52±0.38 Hz; t_5 = -0.460, p = 0.646), average Skaggs spatial information (saline change: -0.10±0.04 bits/spike; pregabalin change: -0.06±0.05 bits/spike; t_5 = -0.535, p = 0.594), nor on average field size change index (saline: 0.09±0.05 cm^2; pregabalin: 0.005±0.03 cm^2); t_4 = 2.638, p = 0.058.

The effect size of global mean rate was 0.293, with observed power of 0.081; the effect size of locational peak rate was 0.879, with observed power of 0.327; the effect size of Skaggs spatial information was 0.477, with observed power of 0.132; and the effect size of place field size change index was 0.932, with observed power of 0.359. The sample size used in these paired t-tests was n = 5, with the appropriate sample size with minimum power of 0.8 for global mean rate was n = 94, for locational peak rate was n = 13, for Skaggs spatial information was n = 37, and for place field size index was n = 12. Figure 6.15 illustrates there was no difference in place cells between saline and pregabalin days even with the inclusion of cells from an additional saline day.


A
Rat 5
Rat Level
Global Mean Rate (Hz)
Additional Saline Day
Global mean rate (Hz)
0.05
0.00
-0.05
-0.10
-0.15
-0.20
Saline
Pregabalin


B
Rat 5
Rat Level
Locational Peak Rate (Hz)
Additional Saline Day
Locational peak rate (Hz)
0.00
-0.50
-1.00
-1.50
-2.00
Saline
Pregabalin

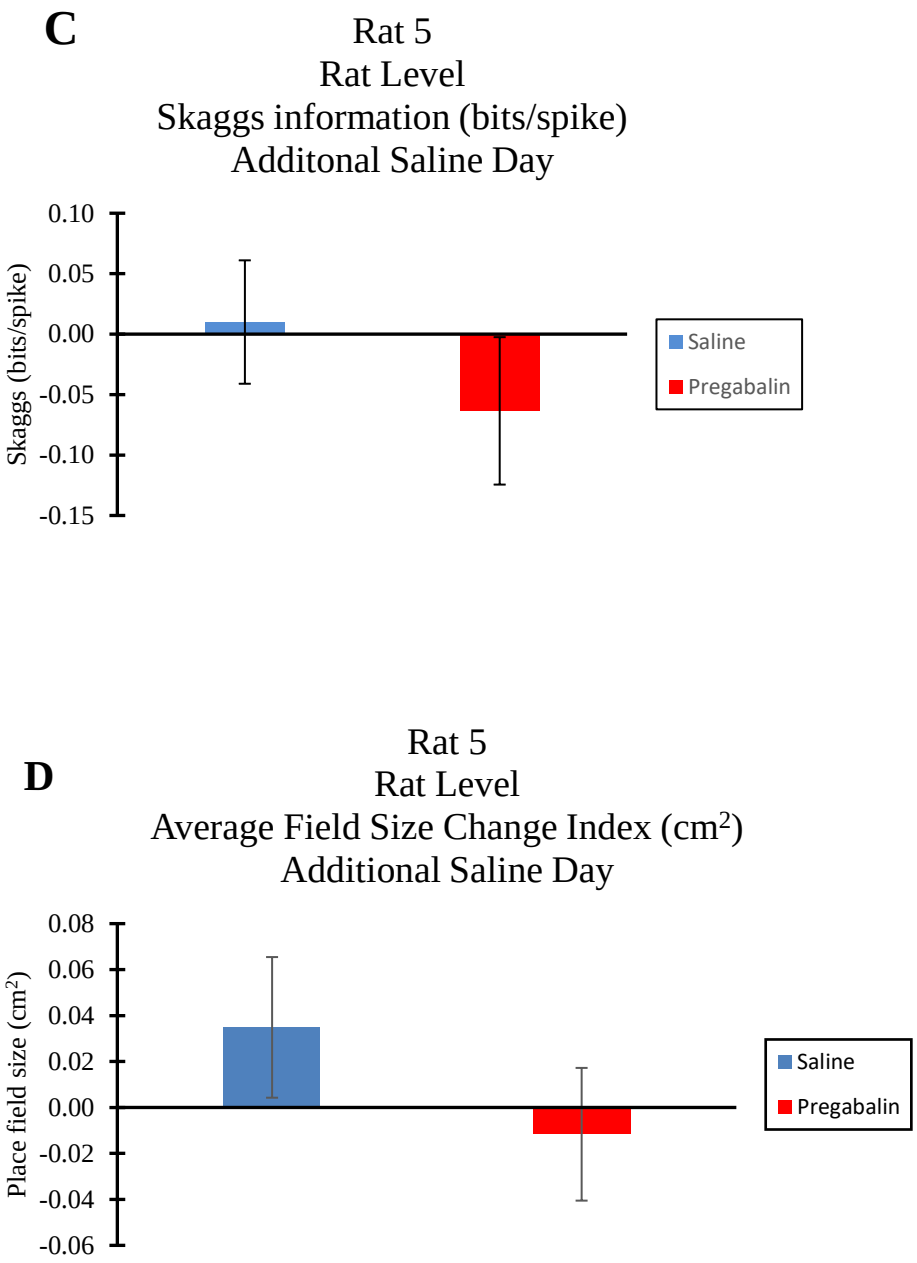
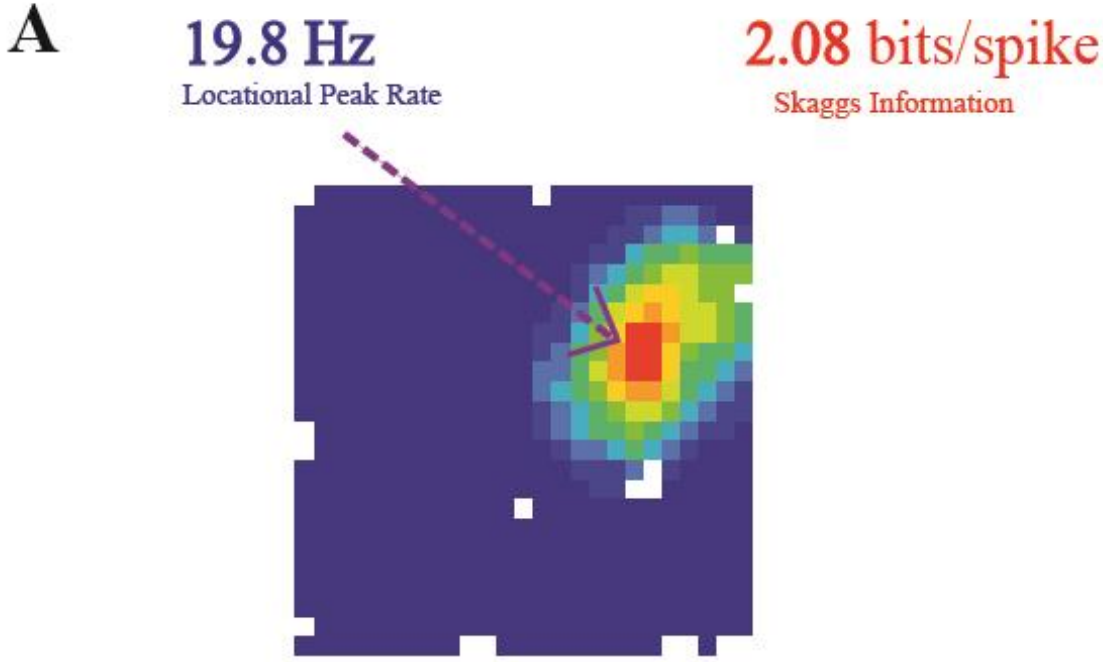
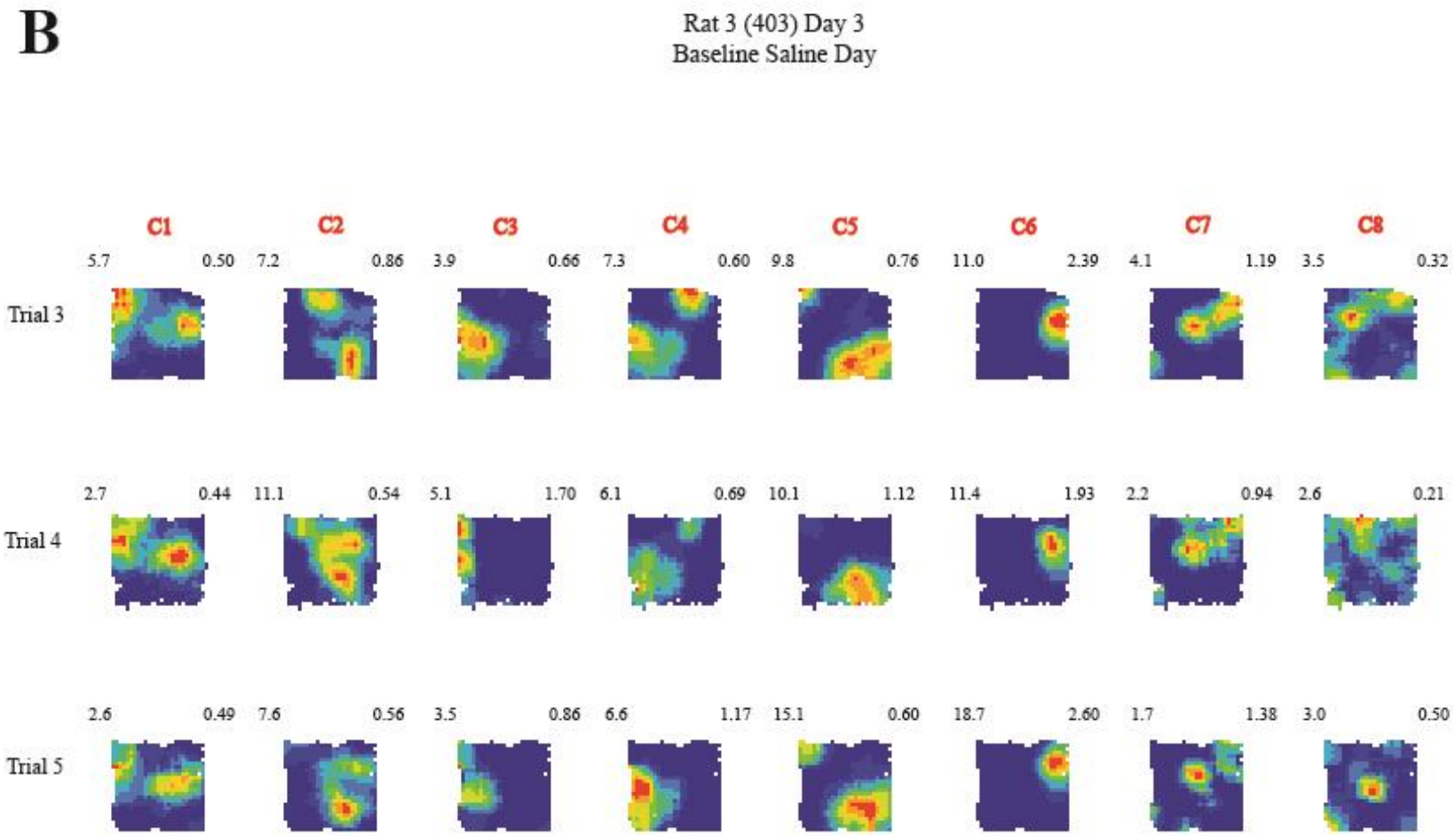


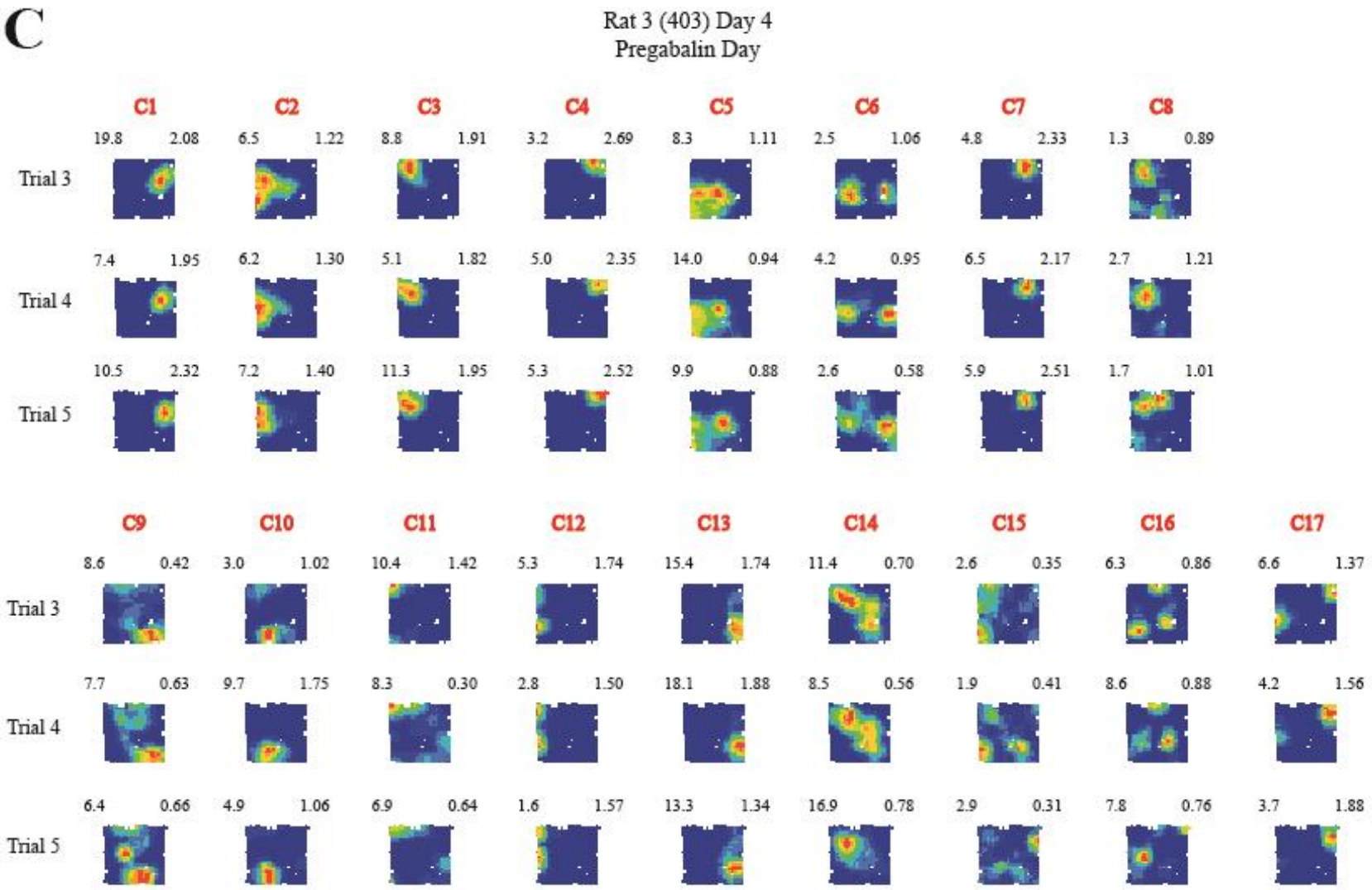
Figure 6.15 Rat Level, additional Saline Cells; Rat 5. With the second saline day cells added, each bar graph illustrates the change in (**A**) average global mean rate (Hz), (**B**) average locational peak rate (Hz), (**C**) average Skaggs spatial information (bits/spike), and (**D**) average field size change index for Rat 5. The blue bar is for both saline days and the red graph is for the pregabalin day.

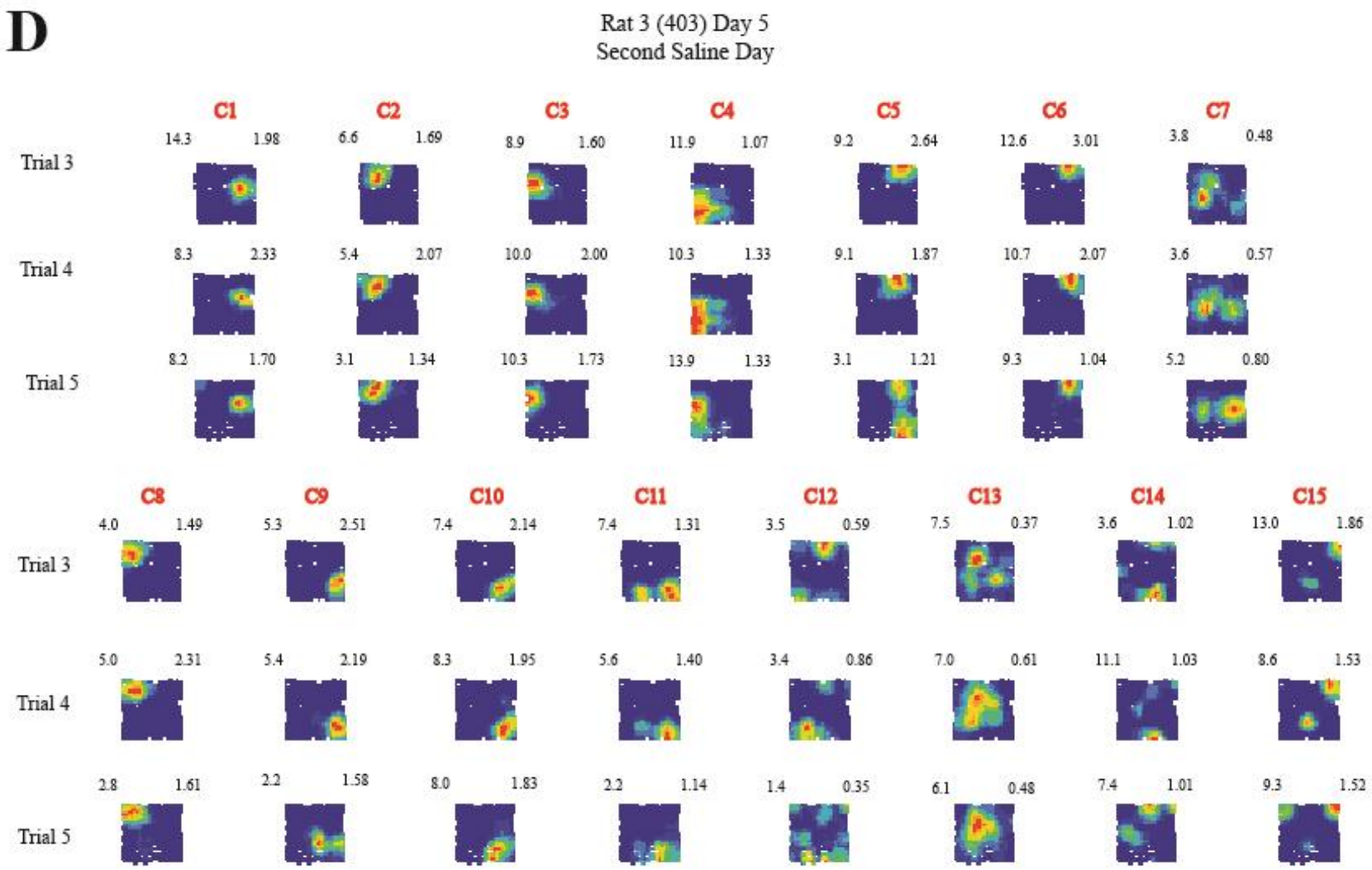
A

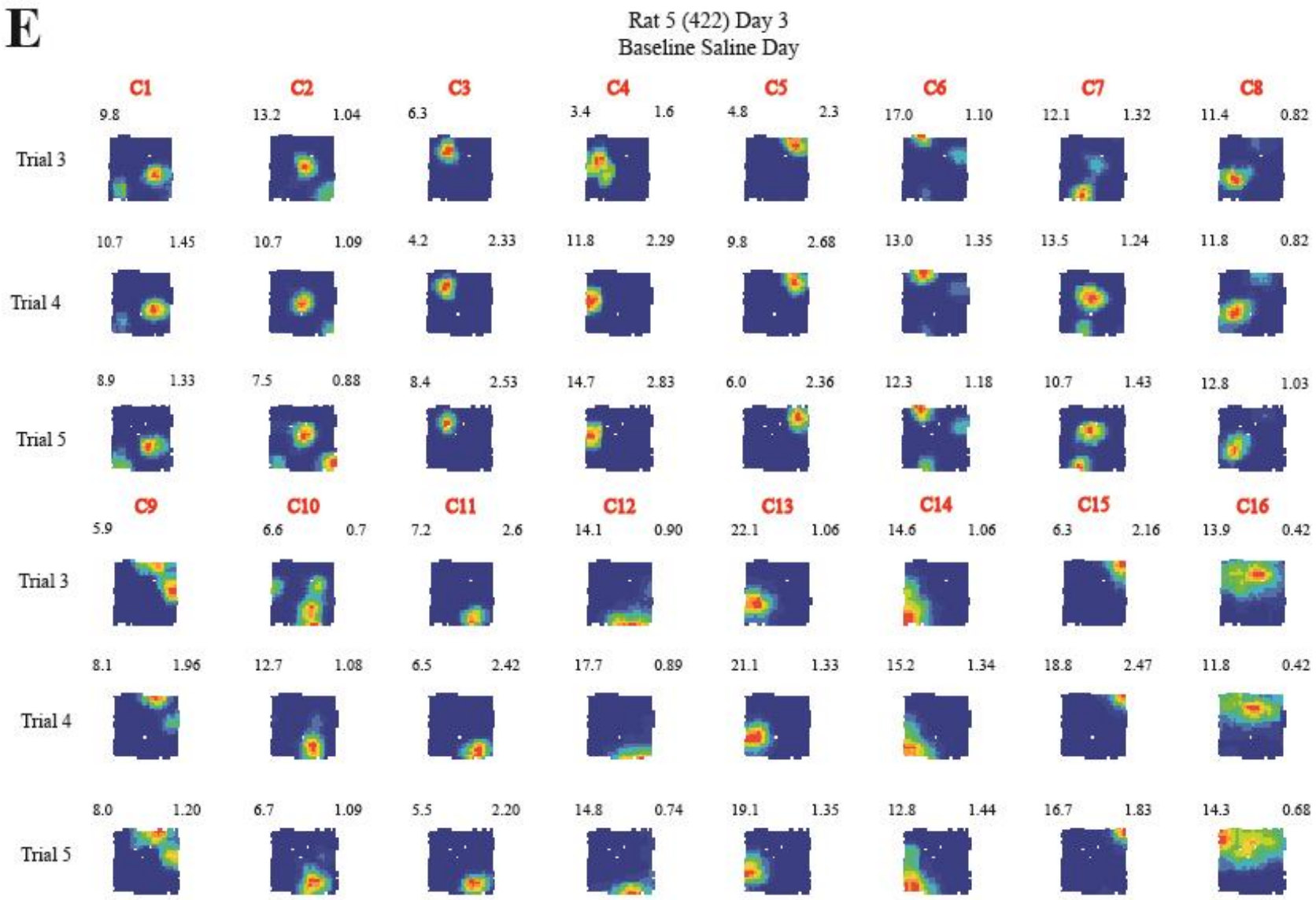


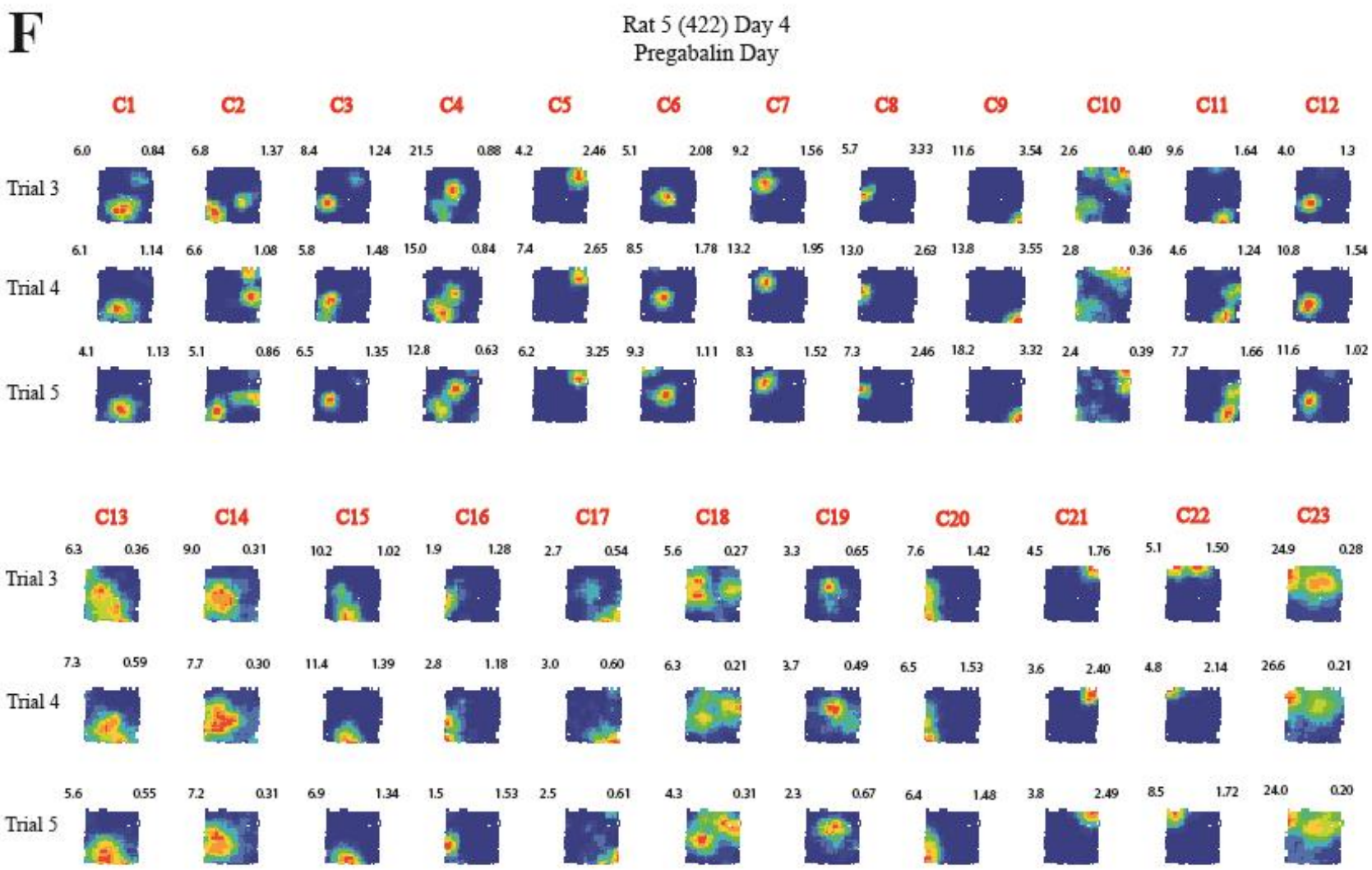
Depicted above is a large scale version of a rate map. Above the top left corner is the locational peak rate (Hz) and above the top right corner is Skaggs spatial information (bits/spike). For space reason, the rate maps depicted below only show the values of locational peak rate and skaggs spatial information, not the units.


B
Rat 3 (403) Day 3
Baseline Saline Day
C1
C2
C3
C4
C5
C6
C7
C8
5.7 0.50
7.2 0.86
3.9 0.66
7.3 0.60
9.8 0.76
11.0 2.39
4.1 1.19
3.5 0.32
Trial 3
2.7 0.44
11.1 0.54
5.1 1.70
6.1 0.69
10.1 1.12
11.4 1.93
2.2 0.94
2.6 0.21
Trial 4
2.6 0.49
7.6 0.56
3.5 0.86
6.6 1.17
15.1 0.60
18.7 2.60
1.7 1.38
3.0 0.50
Trial 5


C
Rat 3 (403) Day 4
Pregabalin Day
C1 C2 C3 C4 C5 C6 C7 C8
Trial 3
19.8 2.08 6.5 1.22 8.8 1.91 3.2 2.69 8.3 1.11 2.5 1.06 4.8 2.33 1.3 0.89
Trial 4
7.4 1.95 6.2 1.30 5.1 1.82 5.0 2.35 14.0 0.94 4.2 0.95 6.5 2.17 2.7 1.21
Trial 5
10.5 2.32 7.2 1.40 11.3 1.95 5.3 2.52 9.9 0.88 2.6 0.58 5.9 2.51 1.7 1.01
C9 C10 C11 C12 C13 C14 C15 C16 C17
Trial 3
8.6 0.42 3.0 1.02 10.4 1.42 5.3 1.74 15.4 1.74 11.4 0.70 2.6 0.35 6.3 0.86 6.6 1.37
Trial 4
7.7 0.63 9.7 1.75 8.3 0.30 2.8 1.50 18.1 1.88 8.5 0.56 1.9 0.41 8.6 0.88 4.2 1.56
Trial 5
6.4 0.66 4.9 1.06 6.9 0.64 1.6 1.57 13.3 1.34 16.9 0.78 2.9 0.31 7.8 0.76 3.7 1.88


D
Rat 3 (403) Day 5
Second Saline Day
C1
C2
C3
C4
C5
C6
C7
Trial 3
14.3 1.98
6.6 1.69
8.9 1.60
11.9 1.07
9.2 2.64
12.6 3.01
3.8 0.48
Trial 4
8.3 2.33
5.4 2.07
10.0 2.00
10.3 1.33
9.1 1.87
10.7 2.07
3.6 0.57
Trial 5
8.2 1.70
3.1 1.34
10.3 1.73
13.9 1.33
3.1 1.21
9.3 1.04
5.2 0.80
C8
C9
C10
C11
C12
C13
C14
C15
Trial 3
4.0 1.49
5.3 2.51
7.4 2.14
7.4 1.31
3.5 0.59
7.5 0.37
3.6 1.02
13.0 1.86
Trial 4
5.0 2.31
5.4 2.19
8.3 1.95
5.6 1.40
3.4 0.86
7.0 0.61
11.1 1.03
8.6 1.53
Trial 5
2.8 1.61
2.2 1.58
8.0 1.83
2.2 1.14
1.4 0.35
6.1 0.48
7.4 1.01
9.3 1.52


E
Rat 5 (422) Day 3
Baseline Saline Day
C1
C2
C3
C4
C5
C6
C7
C8
9.8
13.2 1.04
6.3
3.4 1.6
4.8 2.3
17.0 1.10
12.1 1.32
11.4 0.82
Trial 3
10.7 1.45
10.7 1.09
4.2 2.33
11.8 2.29
9.8 2.68
13.0 1.35
13.5 1.24
11.8 0.82
Trial 4
8.9 1.33
7.5 0.88
8.4 2.53
14.7 2.83
6.0 2.36
12.3 1.18
10.7 1.43
12.8 1.03
Trial 5
C9
C10
C11
C12
C13
C14
C15
C16
5.9
6.6 0.7
7.2 2.6
14.1 0.90
22.1 1.06
14.6 1.06
6.3 2.16
13.9 0.42
Trial 3
8.1 1.96
12.7 1.08
6.5 2.42
17.7 0.89
21.1 1.33
15.2 1.34
18.8 2.47
11.8 0.42
Trial 4
8.0 1.20
6.7 1.09
5.5 2.20
14.8 0.74
19.1 1.35
12.8 1.44
16.7 1.83
14.3 0.68
Trial 5


F
Rat 5 (422) Day 4
Pregabalin Day
C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12
Trial 3
6.0 0.84 6.8 1.37 8.4 1.24 21.5 0.88 4.2 2.46 5.1 2.08 9.2 1.56 5.7 3.33 11.6 3.54 2.6 0.40 9.6 1.64 4.0 1.3
Trial 4
6.1 1.14 6.6 1.08 5.8 1.48 15.0 0.84 7.4 2.65 8.5 1.78 13.2 1.95 13.0 2.63 13.8 3.55 2.8 0.36 4.6 1.24 10.8 1.54
Trial 5
4.1 1.13 5.1 0.86 6.5 1.35 12.8 0.63 6.2 3.25 9.3 1.11 8.3 1.52 7.3 2.46 18.2 3.32 2.4 0.39 7.7 1.66 11.6 1.02
C13 C14 C15 C16 C17 C18 C19 C20 C21 C22 C23
Trial 3
6.3 0.36 9.0 0.31 10.2 1.02 1.9 1.28 2.7 0.54 5.6 0.27 3.3 0.65 7.6 1.42 4.5 1.76 5.1 1.50 24.9 0.28
Trial 4
7.3 0.59 7.7 0.30 11.4 1.39 2.8 1.18 3.0 0.60 6.3 0.21 3.7 0.49 6.5 1.53 3.6 2.40 4.8 2.14 26.6 0.21
Trial 5
5.6 0.55 7.2 0.31 6.9 1.34 1.5 1.53 2.5 0.61 4.3 0.31 2.3 0.67 6.4 1.48 3.8 2.49 8.5 1.72 24.0 0.20

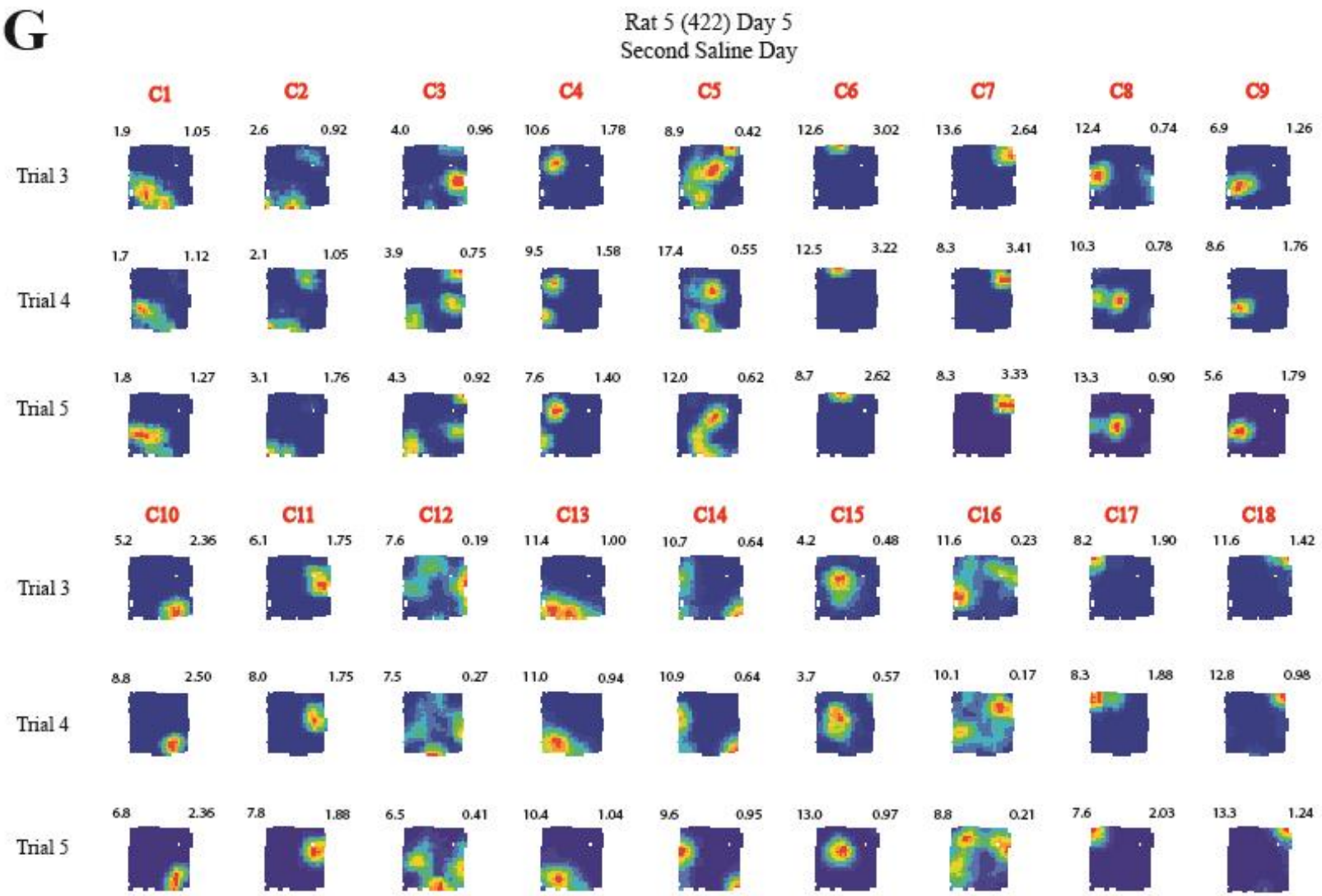

G
Rat 5 (422) Day 5
Second Saline Day
C1
C2
C3
C4
C5
C6
C7
C8
C9
Trial 3
1.9 1.05
2.6 0.92
4.0 0.96
10.6 1.78
8.9 0.42
12.6 3.02
13.6 2.64
12.4 0.74
6.9 1.26
Trial 4
1.7 1.12
2.1 1.05
3.9 0.75
9.5 1.58
17.4 0.55
12.5 3.22
8.3 3.41
10.3 0.78
8.6 1.76
Trial 5
1.8 1.27
3.1 1.76
4.3 0.92
7.6 1.40
12.0 0.62
8.7 2.62
8.3 3.33
13.3 0.90
5.6 1.79
C10
C11
C12
C13
C14
C15
C16
C17
C18
Trial 3
5.2 2.36
6.1 1.75
7.6 0.19
11.4 1.00
10.7 0.64
4.2 0.48
11.6 0.23
8.2 1.90
11.6 1.42
Trial 4
8.8 2.50
8.0 1.75
7.5 0.27
11.0 0.94
10.9 0.64
3.7 0.57
10.1 0.17
8.3 1.88
12.8 0.98
Trial 5
6.8 2.36
7.8 1.88
6.5 0.41
10.4 1.04
9.6 0.95
13.0 0.97
8.8 0.21
7.6 2.03
13.3 1.24

Figure 6.16 Key firing characteristics of place cells on Saline and Pregabalin Days; Rat 3 and Rat 5. A) Shows a rate map (Rat 3, pregabalin day, cell 1). The purple arrow leading from the value above the top left corner is indicating the locational peak rate (Hz) within the rate map. The value above the top right corner is Skaggs spatial information (bits/spike). **B**) Shows the cells recorded on the Baseline Saline Day for Rat 3 (n = 8), trials 3-5. The cell numbers are shown in red and the corresponding values of the locational peak rate and Skaggs spatial information are on the top left and top right corners of each rate map, respectively. **C**) Shows the cells recorded on the Drug Day for Rat 3 (n = 17), trials 3-5. The cell numbers are shown in red and the corresponding values of the locational peak rate and Skaggs spatial information are on the top left and top right corners of each rate map, respectively. **D**) Shows the cells recorded on the Second Saline Day for Rat 3 (n = 15), trials 3-5. The cell numbers are shown in red and the corresponding values of the locational peak rate and Skaggs spatial information are on the top left and top right corners of each rate map, respectively. **E**) Shows the cells recorded on the Baseline Saline Day for Rat 5 (n = 16), trials 3-5. The cell numbers are shown in red and the corresponding values of the locational peak rate and Skaggs spatial information are on the top left and top right corners of each rate map, respectively. **F**) Shows the cells recorded on the Drug Day for Rat 5 (n = 23), trials 3-5. The cell numbers are shown in red and the corresponding values of the locational peak rate and Skaggs spatial information are on the top left and top right corners of each rate map, respectively. **G**) Shows the cells recorded on the Second Saline Day for Rat 5 (n = 18), trials 3-5. The cell numbers are shown in red and the corresponding values of the locational peak rate and Skaggs spatial information are on the top left and top right corners of each rate map, respectively.

6.6 Discussion

6.6.1 Summary

Neurochemically dissimilar anxiolytic drugs have been shown to reduce hippocampal theta frequency (Gray & McNaughton, 2003), with a large proportion of those studies examining the stimulation-induced reduction of hippocampal theta. The current study examined the effect of a novel anxiolytic drug, pregabalin, on hippocampal theta frequency in freely-moving rats. This was accomplished by adapting the methodology used in the Wells and colleagues (Wells et al., 2013) study, which utilised the Burgess (2008) model. The Burgess model takes the well-documented positive relationship between theta frequency and running speed (Jeewajee, Barry, et al., 2008) and suggests that theta frequency has two components; one corresponding to the slope component of the theta-frequency-to-speed relationship and the other corresponding to the intercept component of the theta-frequency-to-speed relationship. This provides a framework for certain testable predictions set out in the Introduction chapters 1 and 3 above; one of which predicts that anxiolytic drugs will reduce the intercept component of the theta-frequency-to-speed relationship whilst having no effect on the slope component. Study 1A demonstrates that the anxiolytic drug pregabalin significantly reduced the intercept whilst having no significant effect on slope. The finding that pregabalin reduced intercept without affecting slope supports the notion that anxiolytic drugs specifically affect an emotional component of theta, or 'Type 2' theta. This notion was further supported with the finding that pregabalin had no significant effect on hippocampal place cells, which would be more associated with 'Type 1' theta.

6.6.2 Intercept component; pregabalin's effect on hippocampal theta

It has been well established that a range of anxiolytic drugs can reduce hippocampal theta frequency; which include benzodiazepines, 5-HT_{1A} agonist, and SSRIs (McNaughton et al., 2007). These demonstrations of hippocampal theta frequency reduction have been mostly accomplished through the stimulation of the reticular formation or septal driving (Coop &

McNaughton, 1991; McNaughton & Coop, 1991; McNaughton et al., 2007). Presently, there have been two studies that have examined anxiolytic reduction of theta frequency in freely-moving rats. In the first study, Woodnorth & McNaughton (2005) found that CDP reduced theta frequency, however their experimental methodology did not account for the effect of running speed on hippocampal theta frequency. In the second study, Wells and colleagues (Wells et al., 2013) found that CDP, buspirone and 0-2545 (a CB_1 agonist) reduced hippocampal theta frequency when controlling for running speed. In fact, they found a particular type of theta frequency reduction. Wells and colleagues built upon the Burgess (2008) model which dissociated hippocampal theta into the two components; the intercept and slope of the theta-frequency-to-speed relationship. They demonstrated this dissociation by having each animal forage for food in a square-walled environment for 10 minutes over a 19-day period. Over the 19-day period, the animals were injected with the 3 aforementioned anxiolytic drugs at low and high dosages, with each drug having a 4-day drug phase followed by a rest day. Saline injections were given directly after the 3rd trial on day 1 and day 3, whilst drug injections were given directly after the 3rd trial on day 2 and day 4. To account for running speed the authors analysed samples of locomotion in 10 small bins and applied both upper (30 cm/s) and lower (5 cm/s) limits of speed. This experimental and analytical methodological model clearly demonstrated theta reduction independent to the effects of running speed.

Study 1A built upon the Wells and colleagues' study with a modified experimental procedure (5 trials over 5 testing days). As with the Wells and colleagues' study, the results of Study 1A was able to demonstrate pregabalin's anxiolytic efficacy through the reduction of the intercept component of the theta-frequency-to-speed relationship without affecting the slope component. One key difference in Study 1A compared to the Wells and colleagues' study is that hippocampal theta frequency was recorded from both the dorsal and intermediate

hippocampus rather than solely from the dorsal hippocampus. This was done to explore the proposed duality of the hippocampus. Based on hippocampal theta being found throughout the dorso-ventral axis and its appearance to travel along this axis in a single wave (Patel et al., 2012), it was predicted that there would be no difference of intercept reduction between dorsal and intermediate hippocampal theta frequency. Initial examination of pregabalin's effect on dorsal and intermediate hippocampal theta frequency was performed across all subjects (high and low dose combined). As predicted, pregabalin significantly reduced the intercept component of the theta-frequency-to-speed relationship without affect the slope component from both the dorsal and intermediate hippocampus. However, further analysis, which solely examined the effects of low dose (17.5 mg/kg) pregabalin, found a significant reduction of the intercept component from the intermediate hippocampus but not from the dorsal hippocampus. This finding was especially interesting as previous research of interest to this thesis that have examined anxiolytic effects of hippocampal theta has been recorded solely in the dorsal hippocampus (Siok et al., 2009; Wells et al., 2013). Interesting still is that the effect size calculations note this analysis was performed with the appropriate sample size (n = 3). This result suggests that the intermediate hippocampus may be more sensitive to anxiolytics and anxiety modulation than the dorsal. Research into the role of the intermediate hippocampus has shown that it plays a role in rapid place learning (Bast et al., 2009) and anticipatory behaviour (Burton et al., 2009), suggesting that it shares modulation of behaviour characteristics with both the dorsal (spatial cognition) and ventral (anxiety modulation) hippocampus. Although it was noted previously that hippocampal theta travels along the dorso-ventral axis in a single wave, the results presented in Study 1A suggests that there are distinct regional differences.

6.6.3 Pregabalin's effect on aural temperature

Another aspect of theta frequency is its relationship to brain temperature. Temperature readings were taken for all subjects involved in Experiment 1. This was done to expand upon Wells et al, (2013) original experiment. In their study, they examined the temperature readings for all animals tested in their anxiolytic paradigm and also examined the correlation between the intercept component and slope component of the theta-frequency-to-speed relationship to the temperature readings during the pre-injection trials of two of the animals tested within their study. They noted that buspirone mildly reduced aural temperature and along with 0-2545 (a cannabinoid CB_1 receptor agonist; a putative anxiolytic), whilst CDP did not reduce aural temperature. This was an interesting effect due to anxiolytic drugs reportedly having a hypothermic (temperature-reducing) effect. One might argue that with the temperature readings of only two animals, CDP not reducing temperature may be due to the lack of subjects Wells and colleagues analysed. Within the current thesis, aural temperature readings for all the animals tested in Study 1A, showed no evidence of temperature reduction by pregabalin.

As with the Wells et al. (2013) study, a correlational analysis was undertaken to examine the relationship between aural temperature with the intercept and slope components. Unlike what was found in the Wells and colleagues' study, which found a positive linear relationship between aural temperature and the slope component, the current study found no relationship between aural temperature and the slope component in any of the seven animals tested in Experiment 1. However, there was one animal in which there was a positive relationship between aural temperature and the average, dorsal and intermediate intercept component (rat 5). This effect was surprising as the intercept component has been shown to be influenced by administration of anxiolytic drugs rather than movement-related influences of hippocampal

theta frequency. One explanation is simply that this result is an outlier rather than a significant finding in it of itself.

6.6.4 Pregabalin's effect on hippocampal place cells

The findings in Study 1A regarding hippocampal place cells were as predicted. One of the main topics of this thesis surrounds the dissociation between the slope component's relation to spatial cognition and the intercept component's relation to anxiety modulation. Hippocampal place cells fall into the category of spatial cognition and with the results of Study 1A showing that pregabalin had no effect upon the slope component of the theta-frequency-to-speed relationship, a logical prediction would be that pregabalin would also not affect hippocampal place cells. This prediction was validated as there was no significant difference in the characteristics of hippocampal place cells recorded between saline and pregabalin days.

6.6.5 Conclusions

McNaughton and colleagues (McNaughton et al., 2007) have suggested that the reduction of theta frequency could be seen as a reflection of the final common pathway of anxiolytic drugs, and that observing theta reduction could be used as a screening tool of anxiolytic compounds. The findings presented in Study 1A, however, demonstrate that the relationship between theta and anxiety is more complex in nature. The central aim of this study was influenced by the combination of the septo-hippocampal theory and the Burgess (2008) model. Gray & McNaughton's (2003) theory of the septo-hippocampal system draws on the parallel between lesions to the septo-hippocampus and the behavioural effects of anxiolytic drugs. This theory also points to the finding that all tested anxiolytic drugs, despite their different chemical structures, reduce hippocampal theta frequency. The main features of the Burgess' model which pertain to Study 1A concern the intercept and slope components of the broadly linear relationship of the theta-frequency-to-speed relationship; meaning as speed

increases, so too does theta frequency. In the Burgess' model, 'Type 1' theta corresponds to the slope component, whilst 'Type 2' corresponds to the intercept component; from this there are certain testable predictions. Taken together, Gray & McNaughton's theory of the septo-hippocampal system, and the intercept component corresponding to 'Type 2'/arousal-related theta, one prediction that can be made is that anxiolytic drugs will selectively reduce the intercept of the theta-frequency-to-speed relationship without effecting the slope component. The main result in Study 1A, in which pregabalin selectively reducing the intercept-component, was in line with both the prediction and previous research performed in the Wells and colleagues' study (Wells et al., 2013). However, the finding that there is a difference in how anxiolytic drugs effect hippocampal theta frequency between dorsal and intermediate hippocampus was not predicted. Hippocampal theta can be found throughout the dorso-ventral axis and appears to travel along that axis in a single wave. Meaning, other than amplitude, theta frequency remains consistent throughout the dorso-ventral axis (Patel et al., 2012). The intermediate hippocampus' seemingly hyper-sensitive reaction to an anxiolytic offers a more fractured view of the hippocampal formation rather than the classic view of the hippocampus' dichotomous nature, in which the dorsal hippocampus modulates spatial cognition and the ventral hippocampus modulates emotional processing. Previous research indicated that the intermediate hippocampus does play role in both spatial processing (Bast et al., 2009) and anxiety modulation (Burton et al., 2009). However, the Bast and colleagues' study demonstrated that the intermediate hippocampus is *required* in the translation of accurate spatial representation into behavioural actions, which is crucial for survival (Bast, 2007; Fanselow & Dong, 2010; Vann & Albasser, 2011); whilst the Burton and colleagues' study demonstrated bilateral lesions to both the intermediate and ventral hippocampus *together* largely abolishes anticipatory activity. These studies indicate that the importance of the intermediate hippocampus to spatial cognition versus its importance to anxiety

modulation would appear to favour its role in the former over the latter. The results in Study 1A, however, suggests that the intermediate hippocampus may play its own role in anxiety modulation. In summary, the results presented in this study appear to support the notion theta frequency reduction is a positive predictor of anxiolysis.

7 Environmental Familiarity

7.1 Environmental familiarity: background

This chapter examines aspects of Experiment 1 (Study 1B) which focuses on the effects of environmental familiarisation on hippocampal place cells, slope and intercept.

The hippocampal formation is thought to be involved in the detection of novelty and familiarity. Research has demonstrated the hippocampal formation's role in spatial contextual input and detector of mismatch (Gothard, Skaggs, Moore, & Mcnaughton, 1996; Lee, Hunsaker, & Kesner, 2005; Maasberg, Shelley, & Gilbert, 2012; Vago & Kesner, 2008; Vinogradova, 2001). This is thought to be in line with the hippocampal formation's role in context-dependent memory (O'Keefe & Nadel, 1978).

In relation to hippocampal theta frequency, Jeewajee and colleagues (Jeewajee, Lever, et al., 2008) found that environmental novelty reduced average theta frequency. Specifically, the reduction in frequency was most pronounced during unanticipated changes in a familiar environment rather than when introduced to a purely novel environment. Nevertheless, average theta frequency increased over repeat exposure or familiarization with the environment. One testable prediction from the Burgess' (2008) model suggests that theta frequency reduction in a novel environment is due to the reduction in the slope of the theta-frequency-to-speed relationship. Wells and colleagues (Wells et al., 2013) not only found that novelty reduced the slope component of the theta-frequency-to-speed relationship whilst having no significant effect on theta intercept, they also found that repeated exposure to a novel environment increased slope as that environment became familiar whilst having no significant effect on intercept component of the theta-frequency-to-speed relationship.

In relation to hippocampal place cells, studies have shown that environmental novelty causes entorhinal grid cells to expand (Barry et al., 2012). Entorhinal grid cells form an important

input to place cells (Barry et al., 2012; Wells et al., 2013), thus it stands to reason that any grid cell expansion would increase the size of hippocampal place field. Therefore, another prediction from the Burgess (2008) model is that a change in slope would predict a change in place field size. Based off this prediction Wells and colleagues found that the reduction of slope predicted the remapping of CA1 place cells and the size of place fields.

While the Wells study's experimental results were focused to the dorsal portion of the hippocampus and on environmental novelty specifically, the experimental results within Study 1B, whilst utilising the same predictive model, examined how environmental familiarisation effected the slope component of both the dorsal and intermediate portion of the hippocampus; along with how environmental familiarisation (exposure to a novel environment over time) effected hippocampal place cells. The prediction, however, remained the same as in Wells and colleagues' study; environmental familiarisation would steadily increase the slope component of the theta-frequency-to-speed relationship whilst having no significant effect on the intercept component, and that environmental familiarisation would significantly affect characteristics of hippocampal place fields.

7.2 Summary of analytical aims

The analysis in this chapter was performed to examine the effects of environmental familiarisation on hippocampal place cells, dorsal slope and intercept, and intermediate slope and intercept. Study 1B was analysed using the pre-injection trials (trials 1 – 4) across day (days 1 – 3) to examine environmental familiarisation without the introduction of the drug (pregabalin). Additionally, trial across days 1 – 5 were analysed using the slope and intercept data to examine how or if the drug affected hippocampal theta as it relates to the Burgess (2008) model. Repeated measure ANOVA and multiple regression were used to analyse the slope and intercept data, whilst repeated measure ANOVA, multiple regression and correlational analysis were used to analyse hippocampal place cell data.

7.3 Electrophysiology experiment

7.3.1 Experimental design

The basic design of Experiment 1 (see Chapter 5 for experimental timeline) was 5 x 10-minute trials per day for 5 days. Each rat experienced the square-walled environment a total of 25 trials.

7.3.2 Statistical analysis of intercept and slope data

To analyse the effect of environmental familiarisation upon slope and intercept, the average slope and intercept of each pre-injection trial (1 – 4) of all subjects (n = 7) were used. The analysis was divided into dorsal slope and intercept (n = 6) and intermediate slope and intercept (n = 6); the sample size for dorsal and intermediate are different from the total number of animals in Experiment 1 due to two animals being implanted in the dorsal or intermediate portion of the hippocampus in both hemispheres rather than each hemisphere supplying both dorsal and intermediate EEG (see Chapter 6, Table 6.1). Using repeated measure ANOVAs, the dorsal and intermediate data was initially analysed during the pre-injection trials across day (days 1 – 3) to examine how environmental familiarisation effected slope without the potential influence of pregabalin being in the subjects' system. The dorsal and intermediate data was then analysed across all experimental days (days 1 – 5) to further test the prediction in which environmental familiarisation would solely affect the slope component of the theta frequency-to-speed relationship whilst having no significant effect on the intercept component; further solidifying the prediction of a dissociative effect between anxiolytics (intercept) and familiarisation (slope) on hippocampal theta frequency. Both days 1 – 3 and days 1 – 5 were re-analysed to examine the robustness of the effect. This was done to account for the novelty of the experiment itself and to account for any potential novelty outliers which could account for a significant effect. Effect sizes were determined using G*Power (3.1.9.2) calculations of Cohen's *f*.

As mentioned in Chapter 5 (section 5.10), a technical error resulted in the missing data of trial 1-rat 1 on the first day of Experiment 1. To account for the missing value, the slope and intercept values for trial 1-rat 1 was interpolated. This was accomplished by taking the average of trials 2-4 for each animal on the first day (e.g., Rat 2, Day 1, trials 2 – 4, etc), and subtracting that value from trial 1 data for each animal. The subtracted values for all subjects were then averaged and that mean was subtracted by the average of trials 2 – 4 for rat 1 to obtain the trial 1-rat 1 value for Day 1 (see Table 7.1 for an example).

Table 7.1 The interpolation method used to obtain the value of trial 1-rat 1, with the resulting interpolated value highlighted red for this example.

Subject	T1	T2	T3	T4	Average of T2-T4	Average-T1	Rat 1 T1 Value
Rat 1	**X**	1.4	1.5	1.8	1.6	*(1.6 – 0.5)*	**= 1.1**
Rat 2	1.3	1.2	1.6	1.8	1.5	0.2	
Rat 3	1.5	1.7	1.7	2.1	1.8	0.3	
Rat 4	1.3	1.2	1.4	1.8	1.5	0.2	
Rat 5	1.0	1.6	1.9	2.0	1.8	0.8	
Rat 7	0.5	0.9	1.7	1.9	1.5	1.0	
Mean						**0.5**	

7.3.3 Statistical analysis of hippocampal place cells

To analyse the effect of environmental familiarisation on hippocampal place cells, repeated measure ANOVAs were used to examine the locational peak rate, global mean rate and Skaggs spatial information. The average distance between cell positions was also analysed by taking a cell's peak spike from one trial and subtracting it from the previous trial (trial 2 – trial 1, trial 3 – trial 2, trial 4 – trial 3). The distances between the first pre-injection trial and the last pre-injection trial (trial 4 – trial 1) were also analysed. The average place field size was also analysed to examine the effect of environmental familiarisation on hippocampal place cells. A correlational analysis was performed to examine any potential relationship

between slope and changes in field size. As with intercept and slope analysis, the missing place cell values from trial 1-rat 1 were interpolated (see Table 7.1 for a working example).

7.4 Slope Results

7.4.1 Dorsal slope increases across trial, no effect of day

A repeated measure ANOVA was used to examine the effects of environmental familiarisation on slope recorded from the dorsal hippocampus. As illustrated in Figures 7.1 – 7.3, there was a main effect of Trial (1 – 4); $F_{3,15} = 9.395$, $p = 0.001$. There was a significant increase of dorsal slope between trial 1 and trial 2 ($p = 0.006$, 0.326 ± 0.071 Hz/cm/s), trial 1 and trial 3 ($p = 0.009$, 0.349 ± 0.083 Hz/cm/s), and between trial 1 and trial 4 ($p = 0.008$, 0.528 ± 0.128). There was no main effect of Day upon dorsal slope; $F_{2,10} = 0.000236$, $p = 1.000$. Nor was there a significant interaction between Trial and Day; $F_{6,30} = 0.728$, $p = 0.631$. The effect size of dorsal slope increase across ***Trials*** was 1.372, with observed power of 0.983. The effect size of dorsal slope increase across ***Days*** was 0.007, with observed power of 0.05. The sample size used in this analysis was n = 6, with the appropriate sample size for dorsal slope increase for Trial with minimum power of 0.8 being n = 5, and the appropriate sample size for slope increase for Day is n = 102,495. This could suggest that the recency of environment exposure (time between trials vs time between days) influences the increase of slope whilst an animal becomes familiar with an environment.

Complementary to the above ANOVA, a multiple regression analysis for the effect of Trial and Day upon dorsal slope was performed to check for any overinfluential effect from interpolation for the missing value for rat 1-trial on day 1. *With* the interpolated value, a significant model emerged ($F_{2,69,}$ $p = 0.011$, adjusted $r^2 = 0.097$), whereby Trial positively predicted slope ($\beta = 0.35$, $p = 0.003$), but Day did not ($\beta = -0.002$, $p = 0.988$). Similarly, *without* the interpolated value, a significant model emerged ($F_{2,68}$, $p = 0.019$, adjusted r^2 =

0.083), whereby Trial positively predicted slope (β = 0.33, p = 0.005), but Day did not (β = -0.022, p = 0.850).

In summary, trial was a significant predictor of dorsal slope with slope increasing across trials throughout each day, with the effect of the interpolated value being minimal (β = 0.33 instead of β = 0.35).

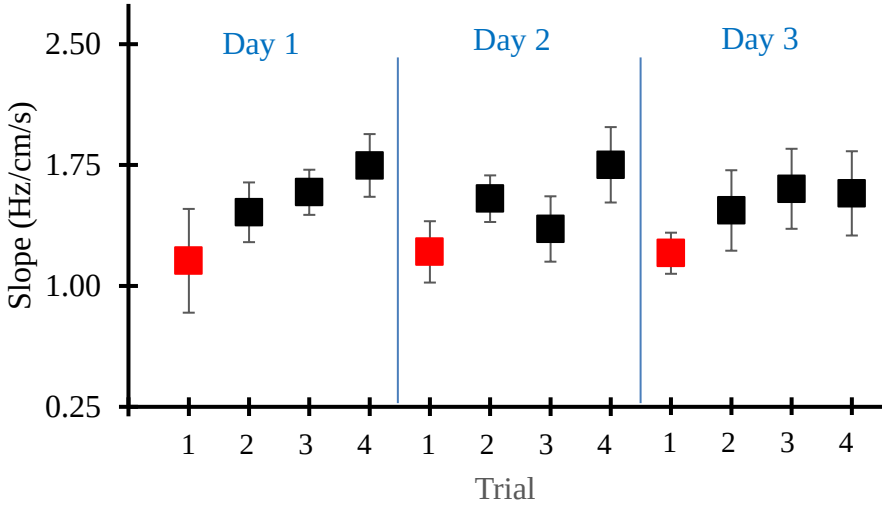


Figure 7.1 Dorsal slope increased across trial, within day. Each data point indicates the mean (± SEM) of rats 1 – 5 and 7. The red data points represent the first trial of each day. The black data points represent the 2nd – 4th trial of each day.

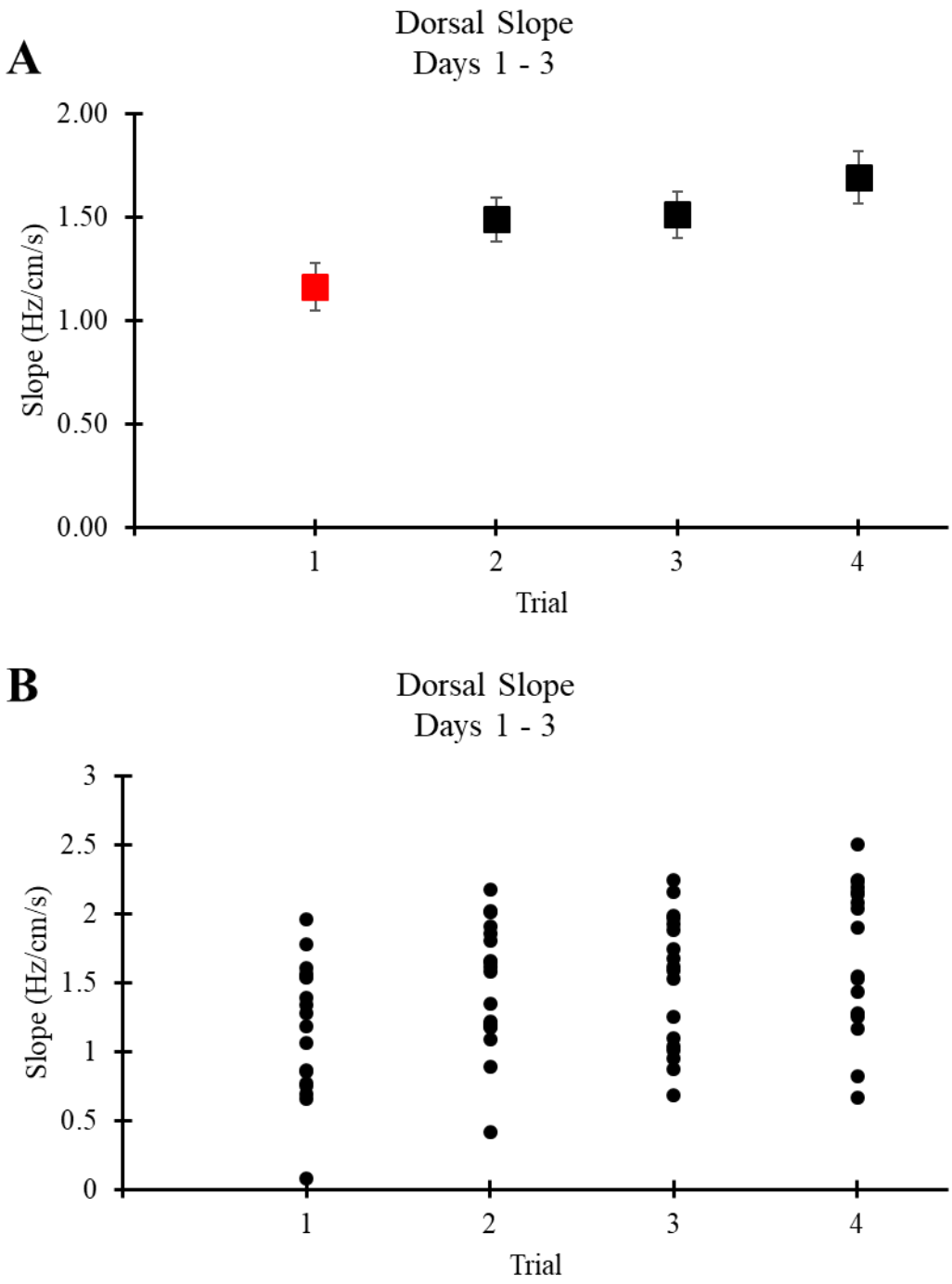


Figure 7.2 Average dorsal slope steadily increases across trial. A) Dorsal slope averaged for each trial shows increase. The largest slope increase occurred between trial 1 and trial 2. Each data point indicates the mean (±SEM) of all the trials (1 – 4) from rats 1 – 5 and rat 7. The red data point represents the first trial and the black data points represent trials 2 through 4. **B)** Dorsal Slope steadily increases across trials. Each data point represents the individual recorded slope within trial for days 1 – 3.

Rat 7, Dorsal Slope

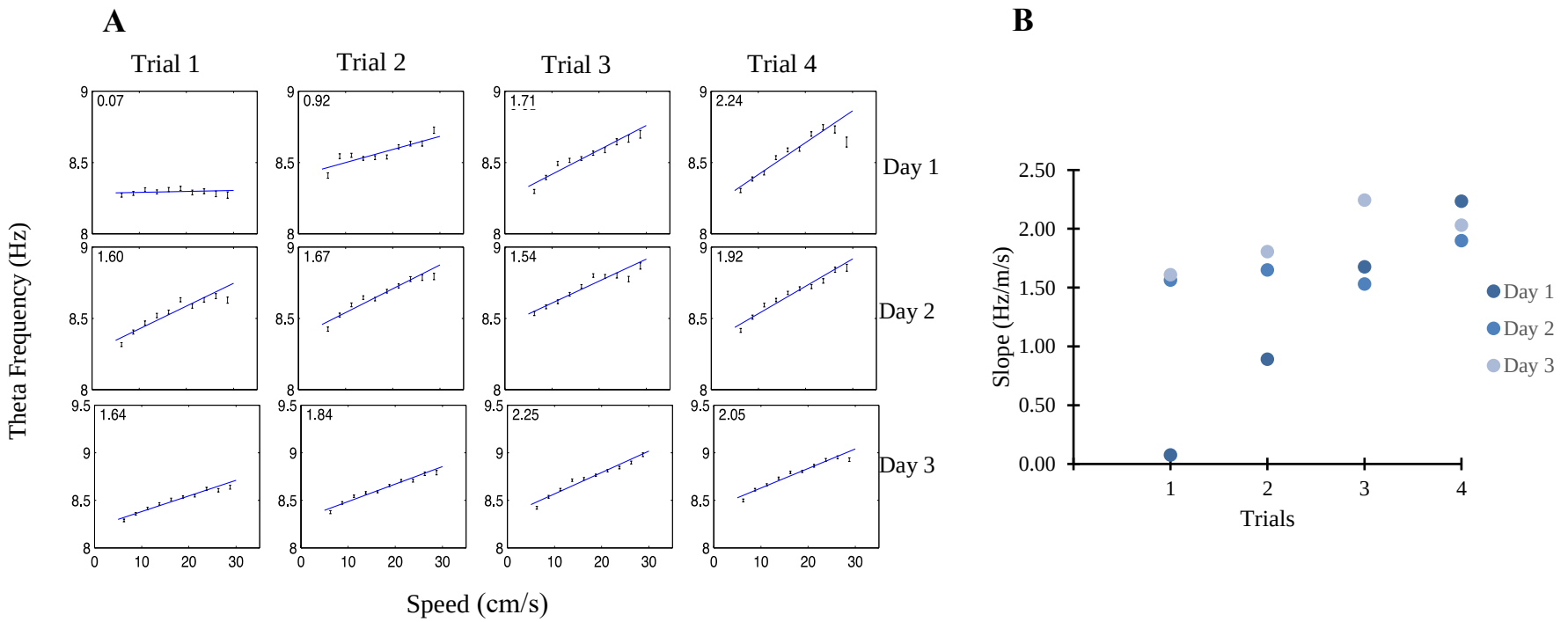


Figure 7.3 Rat 7; dorsal slope increase across trial, within day 1 – 3. A) Illustrates the linear increase of dorsal slope across trials within each day. From left to right, the individual dorsal slopes for trial 1 to trial 4 are shown linearly with each row representing a single day; with the top row being Day 1 and the bottom row being Day 3. The number in the top left corner of each box indicates the slope value for that trial. Specifically, this graph illustrates that environmental familiarisation increases slope in the dorsal hippocampus. B) Using a scatter plot, this graph illustrates the effect of familiarisation on the increase of dorsal slope within each trial, with the darkest to the lightest points representing Day 1 – Day 3 slope, respectively.

To examine the robustness of dorsal-slope increase across trial, a repeated measure ANOVA was performed excluding the data from day 1. This was performed to see if the phenomenon of the significant slope increase could only be attributed to the initial novelty of the environment (Day 1 exposure) rather than attributed to the across-trial familiarisation of the environment. The main effect of Trial (i.e. the across-trial increase of the slope component across pre-injection trials) remained statistically significant; $F_{3,15} = 5.887$, $p = 0.007$. There was a significant increase of slope between trial 1 and trial 2 ($p = 0.021$, 0.298 ± 0.090 Hz/cm/s), trial 1 and trial 3 ($p = 0.023$, 0.270 ± 0.083), and between trial 1 and trial 4 ($p = 0.007$, 0.455 ± 0.105). There was no main effect of Day (i.e. across-day environmental familiarisation, $F_{1,5} = 0.0004$, $p = 0.985$); nor was there a significant interaction between Trial and Day; $F_{3,15} = 1.357$, $p = 0.294$. The effect size of dorsal slope increase across Trials was 1.086, with observed power of 0.885. The effect size for dorsal slope increase across Days was 0.009, with observed power of 0.05. The sample size used in this analysis was n = 6, with appropriate sample size for dorsal slope increase across Trials with minimum power of 0.8 being n = 6, and the appropriate sample size for dorsal increase across Days 2 – 3 being n = 100,602. As with the previous analysis, this result would suggest that the continued increase of dorsal slope is influenced by the recency in which the subjects are exposed to the environment. Figure 7.4 illustrates the dorsal slope increases across trials within the day.

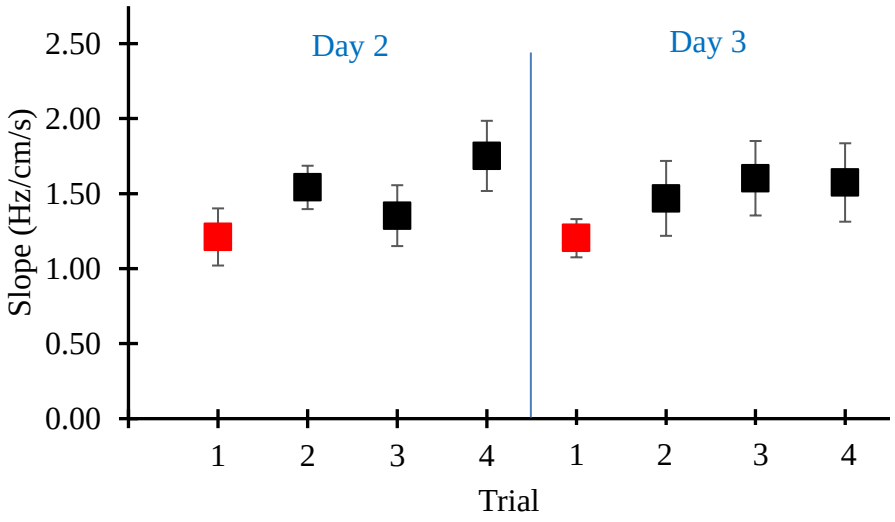


Figure 7.4 Dorsal slope increased across trial with Day 1 data excluded, days 2 – 3. Each data point indicates the mean (±SEM) of all the trials (1 – 4) from rats 1 – 5 and rat 7. The red data point represents the first trial and the black data points represent trials 2 through 4.

7.4.2 Dorsal slope increases across trial, no effect of days 1 – 5

Analysis into the effect of dorsal slope on environmental familiarisation was initially performed on the Days 1 – 3 to account for the potential pharmacodynamic effects of pregabalin. Additional analysis utilising all experimental days (days 1 – 5) was performed to further examine the effects of environmental familiarisation on the slope component of the theta-frequency-to-speed relationship; with the prediction that the significant increase in slope would remain with the addition of two more days.

As illustrated in Figures 7.5 to 7.7, a repeated measure ANOVA found a main effect of Trial; $F_{3,15} = 18.068$, $p = 0.00003$. There was a significant increase of dorsal slope between trial 1 and trial 2 ($p = 0.001$, 0.350 ± 0.051 Hz/cm/s), trial 1 and trial 3 ($p = 0.004$, 0.408 ±0.080 Hz/cm/s), trial 1 and trial 4 ($p = 0.003$, 0.623 ±0.112 Hz/cm/s), and between trial 3 and trial 4 ($p = 0.036$, 0.215 ±0.075 Hz/cm/s). There was no main effect of Day upon slope ($F_{4,20} = 0.255$, $p = 0.903$), nor was there a significant interaction between Trial and Day; $F_{12,60} = 0.797$, $p = 0.652$. The effect size of dorsal slope increase across Trials was 1.900, with

observed power of 0.999. The effect size of dorsal slope increase across Day was 0.225, with observed power of 0.093. The sample size used for this analysis was n = 6, with the appropriate sample size for dorsal slope increase for Trial with minimum power of 0.8 being n = 3, and the appropriate sample size for dorsal slope increase for Day being n = 62. This result further supports the notion that recency of exposure (time between trials versus time between days) to the environment contributes to significant the increase of the slope component across Trials.

A multiple regression analysis for the effect of trial and day upon dorsal slope was performed to check for any overinfluential effect from interpolation for missing value (rat 1-trial 1). With the interpolated value, a significant model emerged ($F_{2,117}$, $p = 0.00002$, adjusted $r^2 = 0.155$), whereby Trial positively predicted slope ($\beta = 0.40$, $p = 0.000006$), but Day did not ($\beta = 0.10$, $p = 0.26$). Without the interpolated value, a significant model emerged ($F_{2,116}$, $p = 0.00005$, adjusted $r^2 = 0.144$), whereby Trial positively predicted slope ($\beta = 0.39$, $p = 0.00001$), but Day did not ($\beta = 0.085$, $p = 0.322$). In summary, trial was a significant predictor of dorsal slope, with slope increasing over trials throughout each day, and the effect of the interpolated value was minimal ($\beta = 0.39$ instead of $\beta = 0.40$).

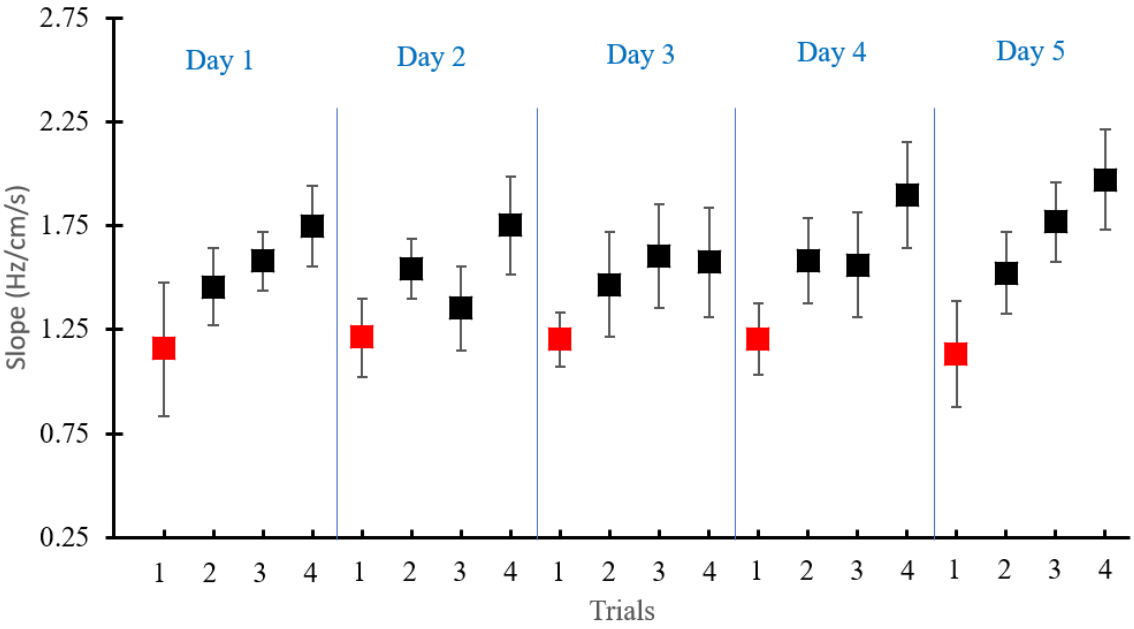


Figure 7.5 Dorsal slope increases across trial, with the lowest slope values on the first trial of each day (days 1 – 5). Each data point indicates the mean (± SEM) of rats 1 – 5 and 7. The red data points represent the first trial of each day. The black data points represent the 2nd – 4th trial of each day.

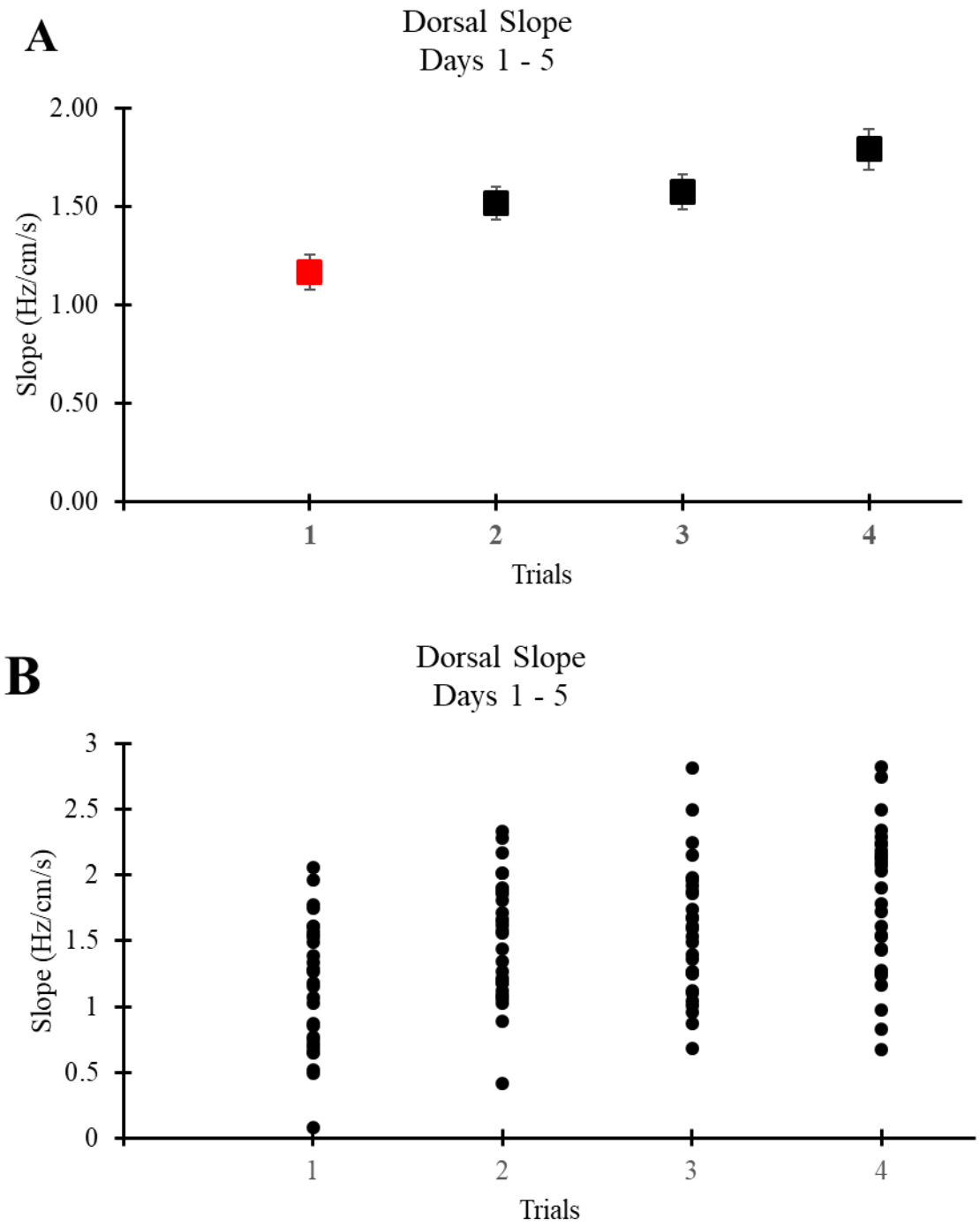


Figure 7.6 Average dorsal slope steadily increases across trial, days 1 – 5. A) Dorsal slope averaged for each trial shows increase. The largest slope increase occurred between trial 1 and trial 2. Each data point indicates the mean (±SEM) of all the trials (1 – 4) from rats 1 – 5 and rat 7. The red data point represents the first trial and the black data points represent trials 2 through 4. **B)** Dorsal slope steadily increases across trials. Each data point represents the individual recorded slope within trial for days 1 – 5.

Rat 7, Dorsal Slope

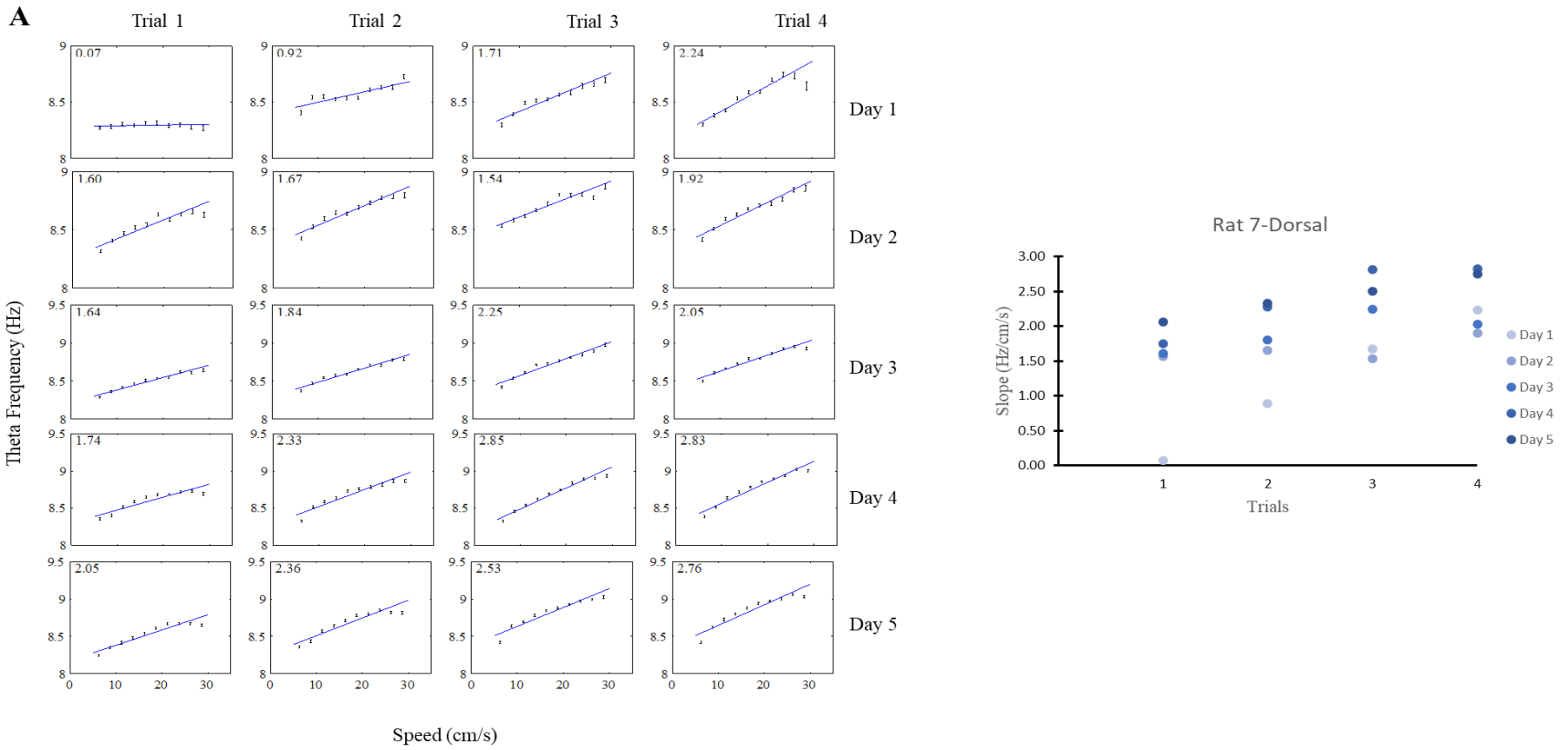


Figure 7.7 Rat 7; dorsal slope increases across trial, within days 1 – 5. A) Illustrates the linear increase of dorsal slope across trials within each day. From left to right, the individual dorsal slopes for trial 1 to trial 4 are shown linearly with each row representing a single day, with the top row being Day 1 and bottom row being Day 5. The number in the top left corner of each box indicates the slope value for that trial. Specifically, this graph illustrates that environmental familiarisation increases slope in the dorsal hippocampus across days 1 – 5. **B)** Using a scatter plot, this graph illustrates the effect of familiarisation on the increase of dorsal slope within each trial, with the darkest to the lightest points representing Day 1 – Day 5 slope, respectively.

The robustness of the dorsal slope increase was examined with a repeated measure ANOVA by excluding the slope data from Day 1. The increase of dorsal slope across Trials remained statistically significant with Day 1 data was excluded; $F_{3,15} = 22.395$, $p = 0.000009$). There was a significant increase of dorsal slope between trial 1 and trial 2 ($p = 0.001$, 0.342 ±0.049 Hz/cm/s), trial 1 and trial 3 ($p = 0.0004$, 0.383 ±0.046 Hz/cm/s), trial 1 and trial 4 ($p = 0.002$, 0.611 ± 0.097 Hz/cm/s), trial 2 and trial 4 ($p = 0.037$, 0.269 ± 0.095 Hz/cm/s), and between trial 3 and trial 4 ($p = 0.032$, 0.228 ± 0.077). There was no main effect of Day (days 2 – 5; $F_{3,15} = 0.340$, $p = 0.797$); nor was there a significant interaction between Trial and Day; $F_{9,45} = 1.110$, $p = 0.376$. The effect size of dorsal slope increase across Trials was 2.113, with observed power of 0.999. The effect size of slope across Days 2 – 5 was 0.261, with observed power of 0.241. The sample size used in this analysis was n = 6, with the appropriate sample size of dorsal slope increase for Trials being n = 3, and the appropriate sample size of dorsal slope increase for Days 2 – 5 being n = 56. Figure 7.8 illustrates the increase of dorsal slope across Trial.

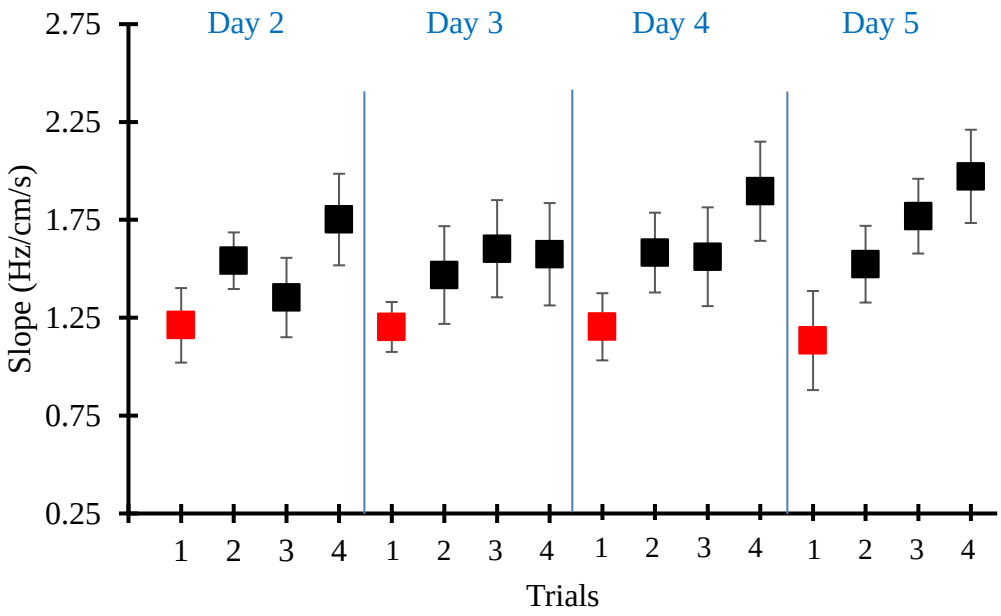


Figure 7.8 Dorsal slope increases across trial with Day 1 data excluded, days 2 – 5. Each data point indicates the mean (±SEM) of all the trials (1 – 4) from rats 1 – 5 and rat 7. The red data point represents the first trial and the black data points represent trials 2 through 4.

7.4.3 Intermediate slope increase across trial within days 2 – 3

A repeated measure ANOVA was used to examine the effects of environmental familiarisation on slope recorded from the intermediate hippocampus. Although Figures 7.9 - 7.10 illustrate a trend of increased slope across trials, there was no main effect of Trial; $F_{3,15} = 2.924$, $p = 0.068$. There was also no main effect of Day; $F_{2,10} = 0.861$, $p = 0.452$. Nor was there a significant interaction between Trial and Day; $F_{6,30} = 1.171$, $p = 0.348$. The effect size of intermediate slope increase across Trials was 0.765, with observed power of 0.574. The effect size of intermediate slope increase across Days was 0.415, with observed power of 0.159. The sample used in this analysis was n = 6, with the appropriate sample for intermediate slope increase for Trials with minimum power of 0.8 being n = 9, and the appropriate sample size for intermediate slope increase for Days being n = 31.

A multiple regression analysis for the effect of Trial and Day upon intermediate slope was performed to check for any overinfluential effect from interpolation for the missing value,

Rat 1-Trial 1-Day 1. With the interpolated value, there was no significant model ($F_{2,69}$, p = 0.141, adjusted R^2 = 0.028). Neither Trial (β = 0.20, p = 0.087) nor Day (β = 0.12, p = 0.317) significantly correlated with intermediate slope. Similarly, without the interpolated value there was no significant model ($F_{2,68}$, p = 0.240, adjusted R^2 = 0.013). Neither Trial (β = 0.18, p = 0.133) nor Day (β = 0.10, p = 0.418) significantly correlated with intermediate slope.

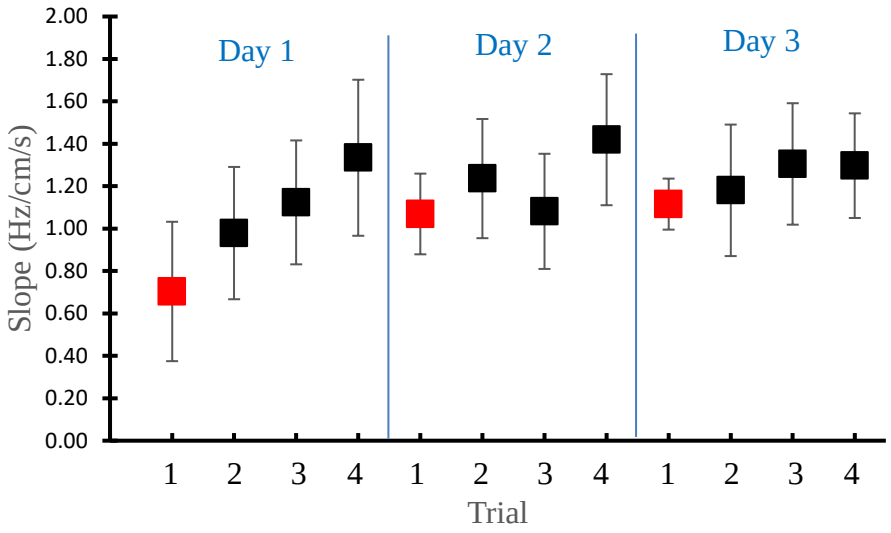


Figure 7.9 No effect of environmental familiarisation upon intermediate slope. Each data point indicates the mean (± SEM) of rats 1 – 2 and 4 – 7. The red data points represent the first trial of each day. The black data points represent the 2nd - 4th trial of each day.

Rat 7, Intermediate Slope

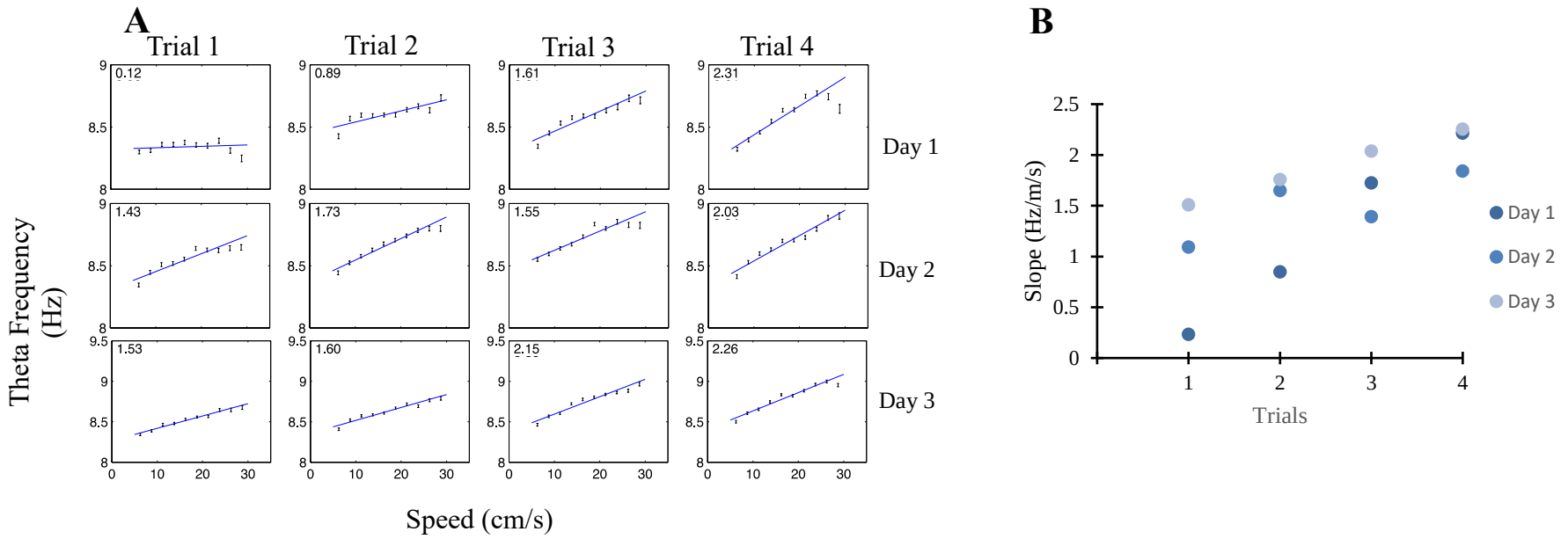


Figure 7.10 Rat 7; intermediate slope did not significantly increase across trial or day. A) Illustrates the linear increase of intermediate slope across trials within each day. From left to right, the individual intermediate slopes for trial 1-to-trial 4 are shown linearly with each row representing a single day, with the top row being Day 1 and the bottom row being Day 3. The number in the top left corner of each linear block are the individual slopes for that trial. B) Using a scatter plot, this graph illustrates the effect of familiarisation on the increase of intermediate slope within each trial, with the darkest to the lightest points representing Day 1 – Day 3 slope, respectively.

Although there was no effect of Trial nor Day upon intermediate slope, a repeated measure ANOVA was performed excluding the data from Day 1 to examine the robustness of the non-result. Conversely, there was a main effect of Trial; $F_{3,15} = 3.771$, $p = 0.034$. Pairwise comparison showed that intermediate slope significantly increased between trial 1 and trial 4 ($p = 0.016$, 0.078 ± 0.022). There was no main effect of Days 2 – 3 ($F_{1,5} = 0.040$, $p = 0.850$); nor was there a significant interaction between Trial and Day; $F_{3,15} = 2.188$, $p = 0.132$. The effect size of intermediate slope increase across Trial was 0.869, with observed power of 0.70. The effect size for intermediate slope increase across Days was 0.09, with observed power 0.053. The sample size used in this analysis was n = 6, with the appropriate sample size for intermediate slope increase for Trial with minimum power of 0.8 being n = 7, and the appropriate sample size for intermediate slope increase for Day being n = 977. Figure 7.11 illustrates the increase of intermediate slope across Trial.

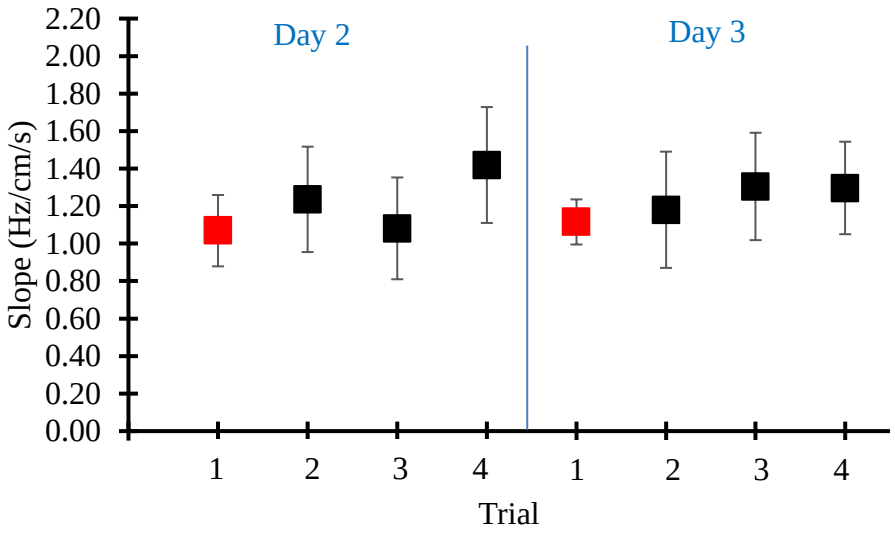


Figure 7.11 Intermediate slope increases across trial with Day 1 data excluded, days 2 – 3. Each data point indicates the mean (± SEM) of rats 1 – 2 and 4 – 7. The red data point represents the first trial and the black data points represent trials 2 through 4.

7.4.4 Intermediate slope increases across trial, no effect of days 1 – 5

A repeated measure ANOVA was used to examine the effect of Trial and Day upon intermediate slope by examining the trials for days 1 – 5. There was a main effect of Trial; $F_{3,15} = 6.876$, $p = 0.004$ (Figures 7.12 to 7.14). There was a significant increase of intermediate slope between trial 1 and trial 3 ($p = 0.026$, 0.464 ± 0.148 Hz/cm/s), trial 2 and trial 4 ($p = 0.0018$, 0.280 ± 0.081 Hz/cm/s), and between trial 3 and trial 4 ($p = 0.022$, 0.175 ± 0.053 Hz/cm/s). There was no main effect of Day ($F_{4,20} = 1.367$, $p = 0.281$), nor was there a significant interaction between Trial and Day; $F_{12,60} = 1.147$, $p = 0.022$. The effect size of intermediate slope increase across Trial was 1.173, with observed power of 0.931. The effect size of intermediate slope increase across Day was 0.523, with an observed power of 0.348. The sample size used for this analysis was n = 6, with the appropriate sample size for intermediate slope increase for Trial with minimum power of 0.8 being n = 5, and the appropriate sample size for intermediate slope increase for Day being n = 14.

A multiple regression analysis for the effect of Trial and Day upon intermediate slope was performed to check for any overinfluential effect from interpolation for the missing values of Rat 1-Trial 1-Day 1. *With* the interpolated value, a significant model emerged ($F_{2,117}$, $p = 0.001$, adjusted $R^2 = 0.093$), whereby Trial ($\beta = 0.26$, $p = 0.004$) and Day ($\beta = 0.21$, $p = 0.018$) predicted slope. Similarly, *without* the interpolated value, a significant model emerged ($F_{2,116}$, $p = 0.003$, adjusted $R^2 = 0.080$), whereby Trial ($\beta = 0.24$, $p = 0.028$) and Day ($\beta = 0.20$, $p = 0.007$) positively predicted slope.

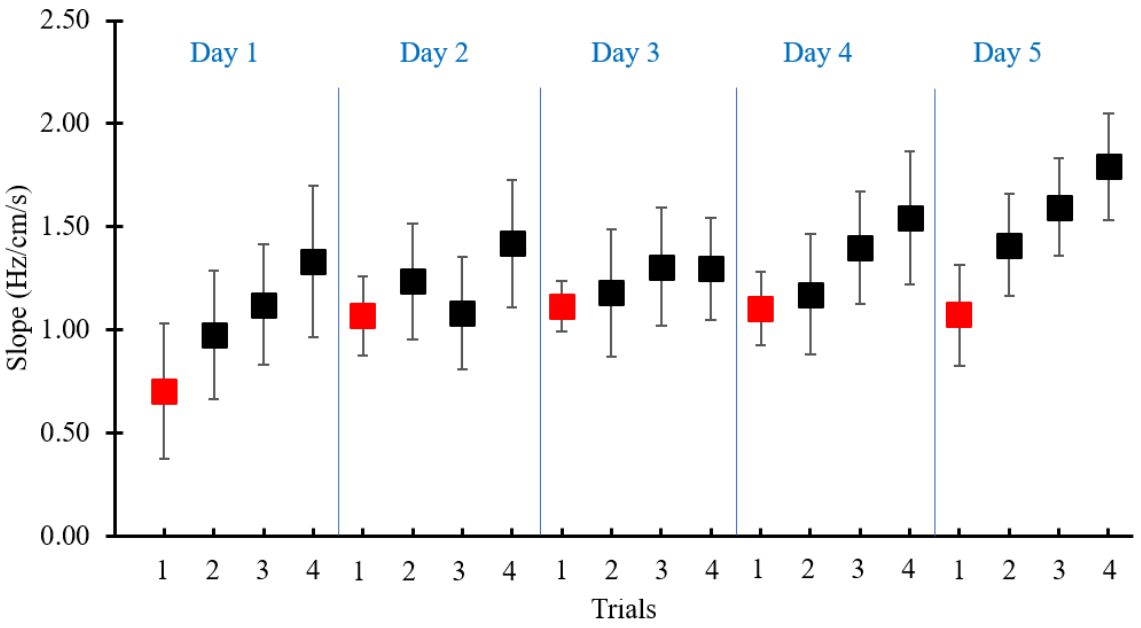


Figure 7.12 Intermediate slope increases across trial, with the lowest slope values on the first trial of each day. Each data point indicates the mean (± SEM) of rats 1 – 2 and 4 – 7. The red data points represent the first trial of each day. The black data points represent the 2nd – 4th trial of each day.

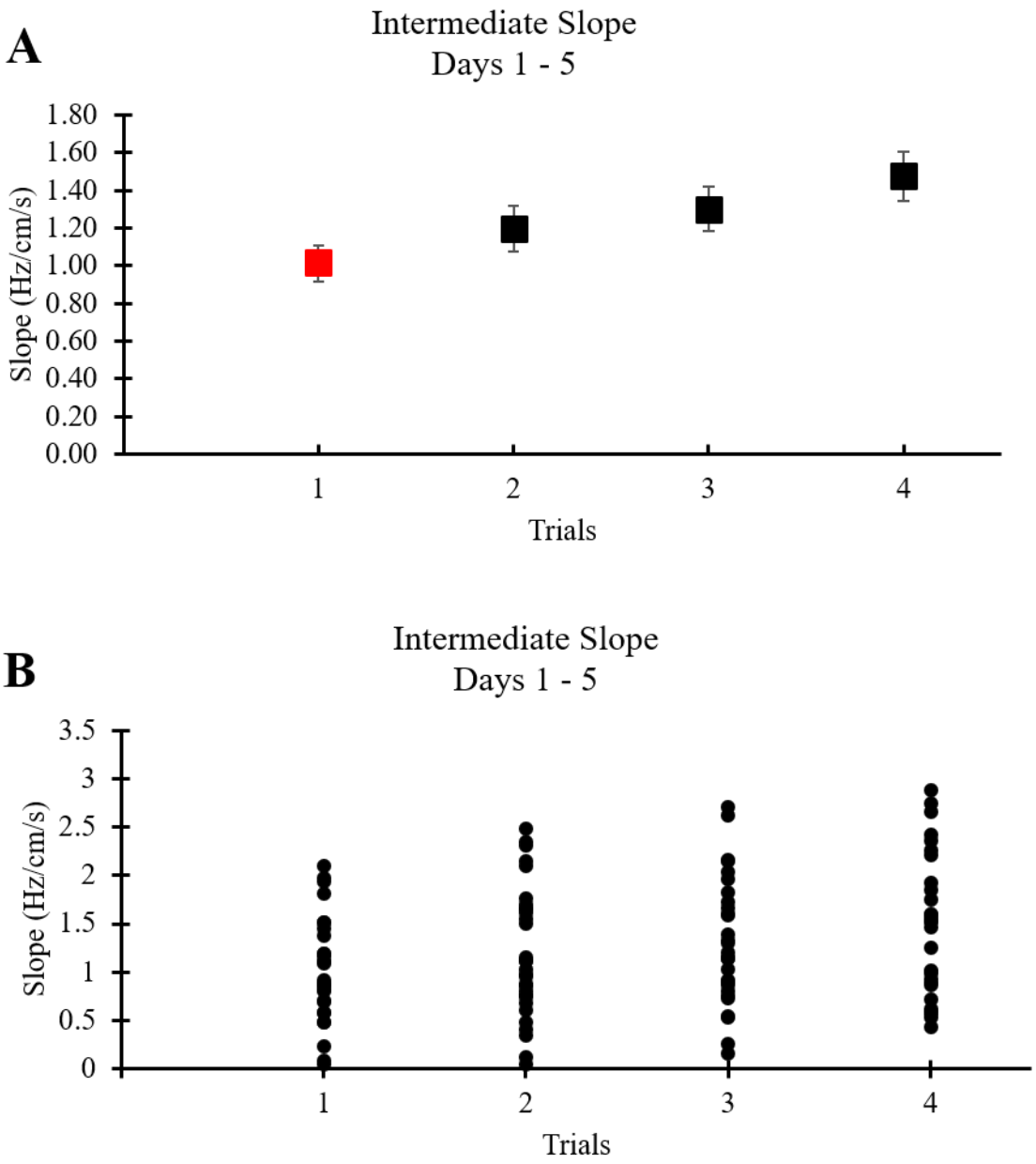


Figure 7.13 Intermediate slope steadily increases across trial, days 1 – 5. A) Intermediate slope averaged for each trial shows increase. The largest slope increase occurred between trial 3 and trial 4. Each data point indicates the mean (±SEM) of all the trials (1 – 4) from rats 1 – 2 and 4 – 7 for days 1 – 5. The red data point represents the first trial and the black data points represent trials 2 through 4. **B)** Intermediate slope steadily increases across trials. Each data point represents the individual recorded slope within trial for days 1 – 5. Each data point represents the individual recorded slope for days 1 – 5 within trial.

Rat 7, Intermediate Slope

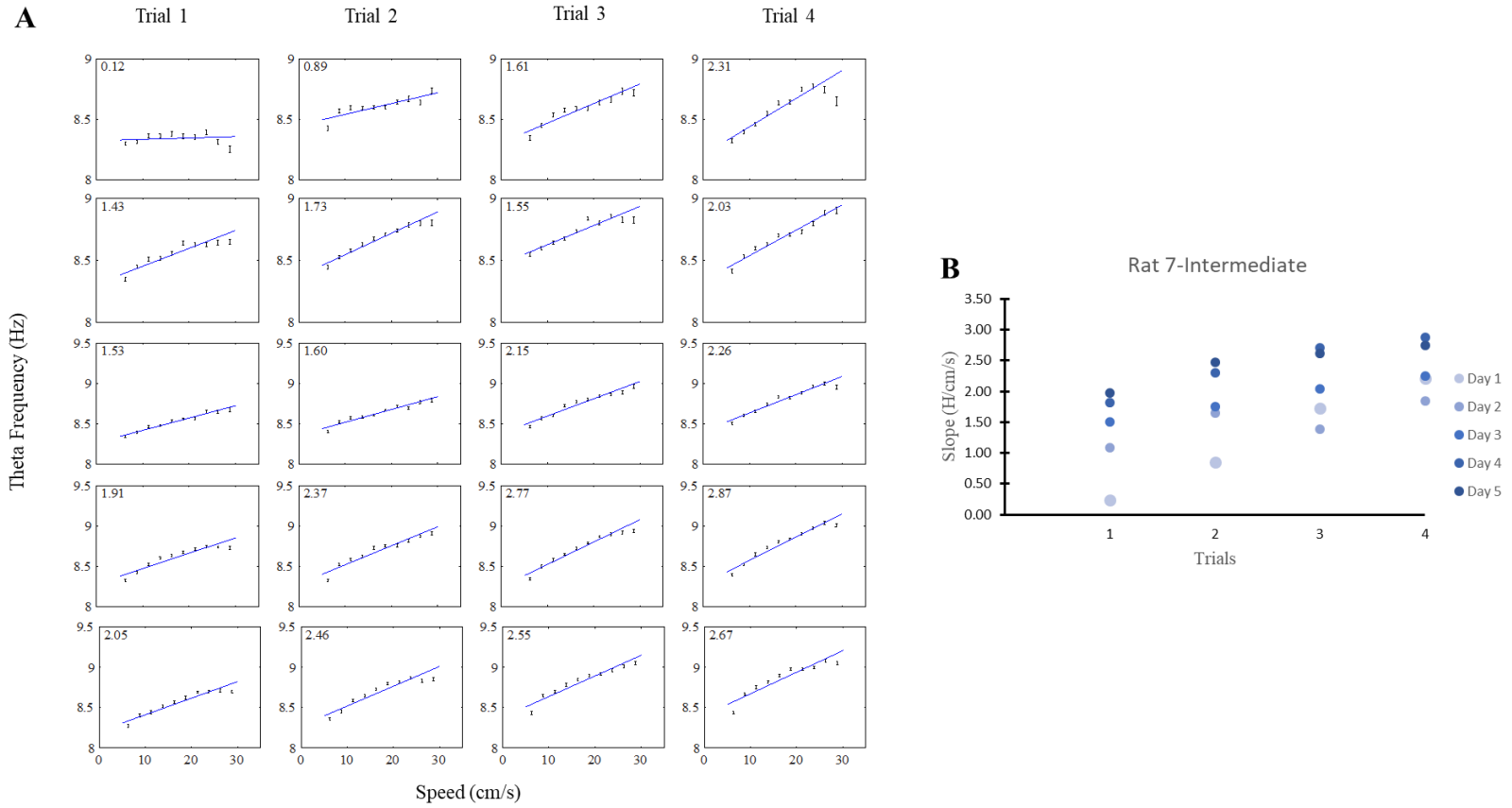


Figure 7.14 Rat 7; intermediate slope increases across trials, within days. A) Illustrates the linear increase of intermediate slope across trials within each day. From left to right, the individual intermediate slopes for trial 1-to-trial 4 are shown linearly with each row representing a single day, with the top row being Day 1 and the bottom row being Day 5. The number in the top left corner of each linear block are the individual slopes for that trial. Specifically, this graph illustrates the importance of 'Day 1' slope in significantly increases intermediate slope across days 1 – 5. B) Using a scatter plot, this graph illustrates the effect of familiarisation on the increase of intermediate slope within each trial, with the darkest to the lightest points representing Day 1 – Day 5 slope, respectively.

To examine the robustness of intermediate slope increase across Trial, a repeated measure ANOVA was performed excluding the data from Day 1. There was a main effect of Trial with the data from Day 1 was excluded; $F_{3,15} = 7.822$, $p = 0.002$. There was a significant increase of intermediate slope between trial 1 and trial 3 ($p = 0.035$, 0.256 ± 0.089 Hz/cm/s), trials 1 and trial 4 ($p = 0.0015$, 0.422 ±0.116 Hz/cm/s), trial 2 and trial 4 ($p = 0.011$, 0.261 ±0.067 Hz/cm/s), and between trial 3 and trial 4 ($p = 0.031$, 0.167 ± 0.056). There was no main effect of Days 2 – 5 ($F_{3,15} = 0.932$, $p = 0.450$), nor was there was a significant interaction between Trial and Day; $F_{9,45} = 1.439$, $p = 0.200$. The effect size of intermediate slope increase across Trial was 1.259, with observed power of 0.960. The effect size of intermediate slope increase across Day was 0.311. The sample size used in this analysis was n = 6, with the appropriate sample size for intermediate slope increase for Trial with minimum power of 0.8 being n = 5, and the appropriate sample size for intermediate slope increase for Day being n = 40. Figure 7.15 illustrates the increase of intermediate slope across Trial.

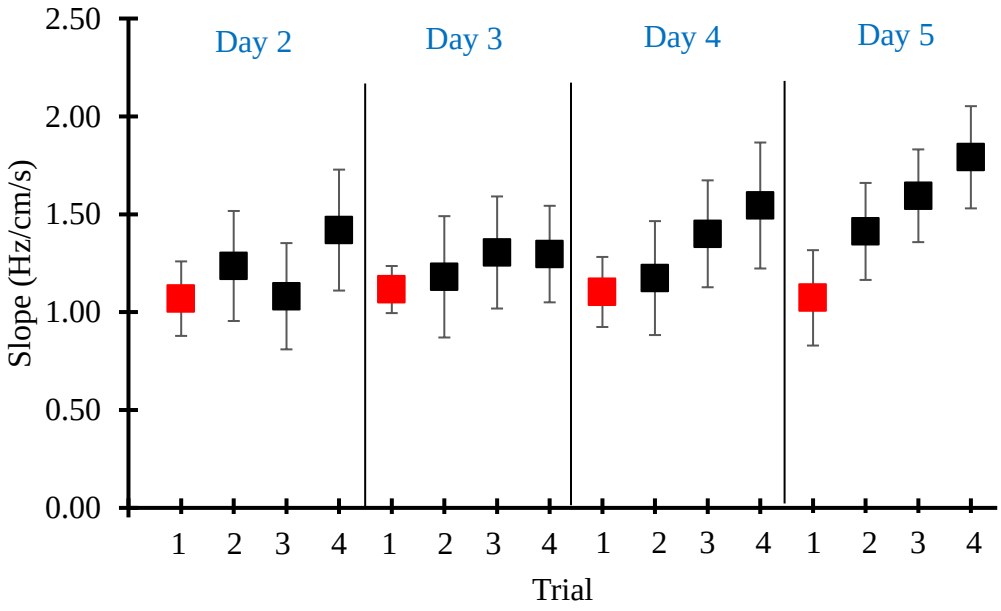


Figure 7.15 Intermediate slope increases across trial with Day 1 data excluded, days 2 – 5. Each data point indicates the mean (± SEM) of rats 1 – 2 and 4 – 7. The red data point represents the first trial and the black data points represent trials 2 through 4.

7.5 Intercept Results

7.5.1 Dorsal intercept, no effect of trial or day

A repeated measure ANOVA showed there was no main effect of Trial; $F_{3,15} = 2.101$, $p = 0.143$. There was also no main effect of Day; $F_{2,10} = 0.370$, $p = 0.700$, nor was there a significant interaction between Trials and Day; $F_{6.30} = 1.758$, $p = 0.142$. The effect size of dorsal intercept across Trial was 0.648, with observed power of 0.431. The effect size of dorsal intercept across Day was 0.272, with observed power of 0.094. The sample size used in this analysis was n = 6, with the appropriate sample size of dorsal intercept for Trial being n = 11, and the appropriate sample size for dorsal intercept for Day being n = 68. Figure 7.16 illustrates the non-effect of environmental familiarisation on dorsal intercept.

A multiple regression analysis for the effect of Trial and Day upon dorsal intercept was performed to check for any overinfluential effect from interpolation for the missing values from Rat 1-Trial 1-Day 1. With the interpolated value, there was no significant model ($F_{2,69}$, $p = 0.811$, adjusted $R^2 = -0.023$. Neither Trial ($\beta = 0.06$, $p = 0.627$) nor Day ($\beta = 0.05$, $p = 0.670$) significantly correlated with dorsal intercept. Similarly, without the interpolated value, there was no significant model ($F_{2,68}$, $p = 0.833$, adjusted $R^2 = -0.024$). Neither Trial ($\beta = 0.06$, $p = 0.646$) nor Day ($\beta = 0.05$, $p = 0.687$) significantly correlated with dorsal intercept.

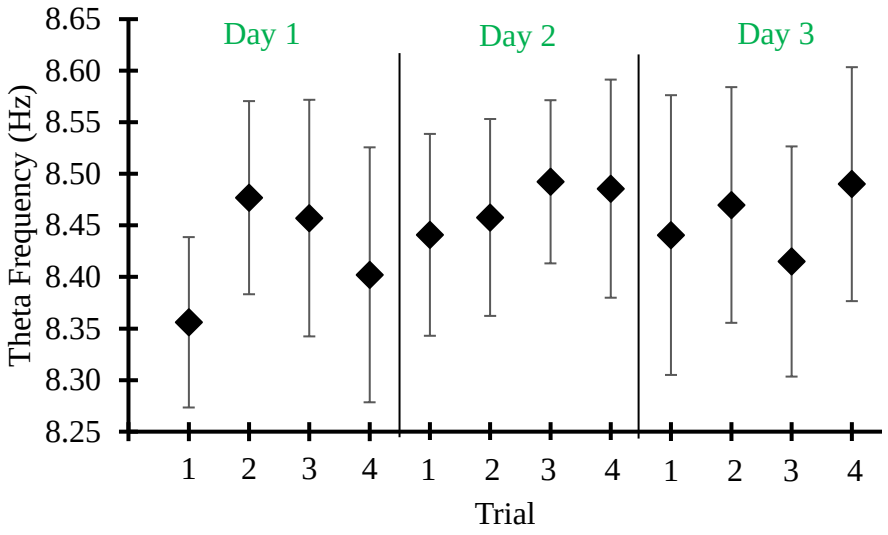


Figure 7.16 No significant effect of environmental familiarisation upon dorsal intercept, days 1 – 3. Each data point indicates the mean (± SEM) of rats 1 – 5 and rat 7.

To examine the robustness of this non-effect, a repeated measure ANOVA was conducted without the data from Day 1. There was still no main effect of Trial ($F_{3,15} = 0.803$, $p = 0.511$), no main effect of Day 2 – 3 ($F_{1,5} = 0.133$, $p = 0.730$), nor was there a significant interaction between Trial and Day ($F_{3,15} = 1.112$, $p = 0.375$). The effect size of dorsal intercept across Trial was 0.400, with observed power of 0.182. The effect size of dorsal intercept across Days 2 – 3 was 0.163, with observed power of 0.06. The sample size used in this analysis was n = 6, with the appropriate sample size for dorsal intercept for Trial with minimum power of 0.8 being n = 25, and the appropriate sample size for dorsal intercept for Day being n = 297. Figure 7.17 illustrates the effect of Trial upon environmental familiarisation.

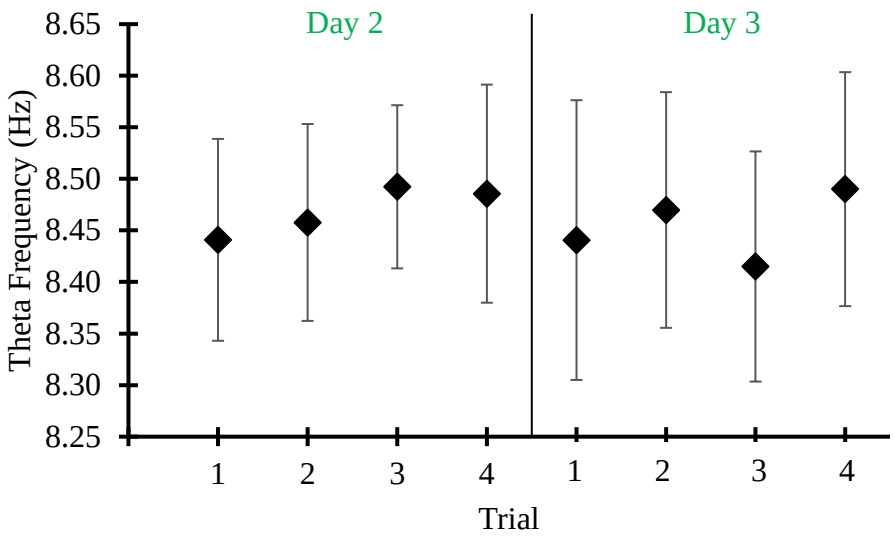


Figure 7.17 No significant effect of environmental familiarisation upon dorsal intercept with Day 1 data excluded, days 2 – 3. Each data point indicates the mean (± SEM) of rats 1 – 5 and rat 7.

7.5.2 Dorsal intercept, no effect of trial or days 1 – 5

As with the slope data, an analysis was performed to examine the effect of Trial and Day (1 – 5) upon environmental familiarisation. A repeated measure ANOVA was performed and found that there was no main effect of Trial ($F_{3,15} = 0.569$, $p = 0.644$), no main effect of Day ($F_{4,20} = 0.324$, $p = 0.859$, nor was there a significant interaction between Trial and Day; $F_{12,60} = 0.722$, $p = 0.724$. The effect size of dorsal intercept across Trial was 0.337, with observed power of 0.140. The effect size of dorsal intercept across Day was 0.255, with observed power of 0.108. The sample size used in this analysis was n = 6, with the appropriate sample size for dorsal intercept for Trial with minimum power of 0.8 being n = 35, and the appropriate sample size for dorsal intercept for Day being n = 49. Figure 7.18 illustrates the non-significant effect of environmental familiarisation on dorsal intercept.

A multiple regression analysis for the effect for the effect of Trial and Day upon dorsal intercept was performed to check for any overinfluential effect from interpolation for the missing value for Rat 1-Trial 1-Day 1. *With* the interpolated value, there was no significant

model ($F_{2,117}$, p = 0.723, adjusted R^2 = -0.011). Neither Trial (β = 0.02, p = 0.868) nor Day (β = 0.07, p = 0.431) significantly correlated with dorsal intercept. Similarly, *without* the interpolated value, there was no significant model ($F_{2,116}$, p = 0.743). Neither Trial (β = 0.01, p = 0.884) nor Day (β = 0.07, p = 0.488) significantly correlated with dorsal intercept. In summary, neither Trial nor Day predicted dorsal intercept, and the effect of the interpolated value was minimal (β = 0.01 instead of β = 0.02; β = 0.07 instead of β = 0.07).

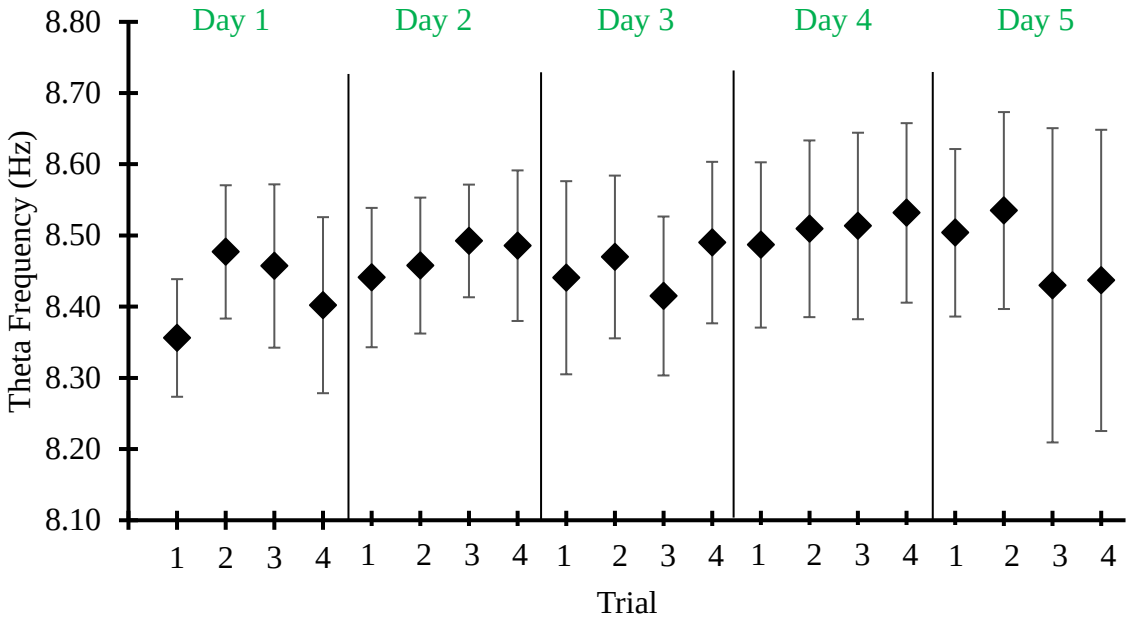


Figure 7.18 No significant effect of environmental familiarisation upon dorsal intercept, days 1 – 5. Each data point indicates the mean (± SEM) of rats 1 – 5 and rat 7. The red data points represent the first trial of each day. The black data points represent the 2nd – 4th trial of each day.

Analysis of dorsal intercept across Day (1 – 5) with the exclusion of Day 1 data found that there was no main effect of Trial ($F_{3,15}$ = 0.569, p = 0.644), no main effect of Days 2 – 5 ($F_{4,20}$ = 0.324, p = 0.859), nor was there a significant interaction between Trial and Day ($F_{12,60}$ = 0.722, p = 0.724). The effect size of dorsal intercept across Trial was 0.220, with observed power of 0.085. The effect size of dorsal intercept across Day was 0.201, with observed

power of 0.080. The sample size used in this analysis was n = 6, with the appropriate sample size for dorsal intercept for Trial with minimum power of 0.8 being n = 78, and the appropriate sample size for dorsal intercept for Day being n = 92. Figure 7.19 illustrates the non-significant effect of environmental familiarisation on dorsal intercept.

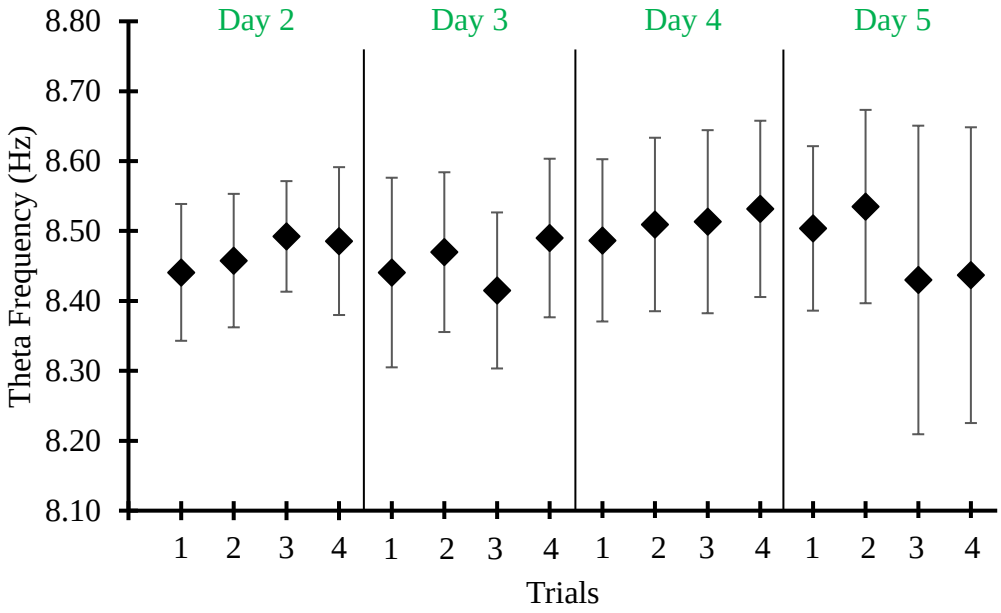


Figure 7.19 No significant effect of environmental familiarisation upon dorsal intercept with Day 1 data excluded, days 2 – 5. Each data point indicates the mean (± SEM) of rats 1 – 5 and rat 7.

7.5.3 Intermediate intercept increases across trial, no effect of day

A repeated measure ANOVA was performed to examine the effect of environmental familiarisation on intermediate intercept. There was a main effect of Trial; $F_{3,15} = 4.942$, $p = 0.014$. There was a significant increase of intermediate intercept between trial 1 and trial 2 ($p = 0.036$, 0.060 ± 0.021), and between trial 1 and trial 4 ($p = 0.019$, 0.056 ±0.016). There was no main effect of Day ($F_{2,10} = 0.490$, $p = 0.627$), nor was there a significant interaction between Trial and Day; $F_{6,30} = 1.839$, $p = 0.125$. The effect size of intermediate intercept across Trial was 0.994, with observed power of 0.819. The effect size of intermediate intercept across Day was 0.313, with observed power of 0.109. The sample size used in this

analysis was n = 6, with the appropriate sample size for intermediate intercept for Trial with minimum power of 0.80 being n = 6, and the appropriate sample size for intermediate intercept for Day being n = 52. Figure 7.20 illustrates the significant increase of intermediate intercept across Trial.

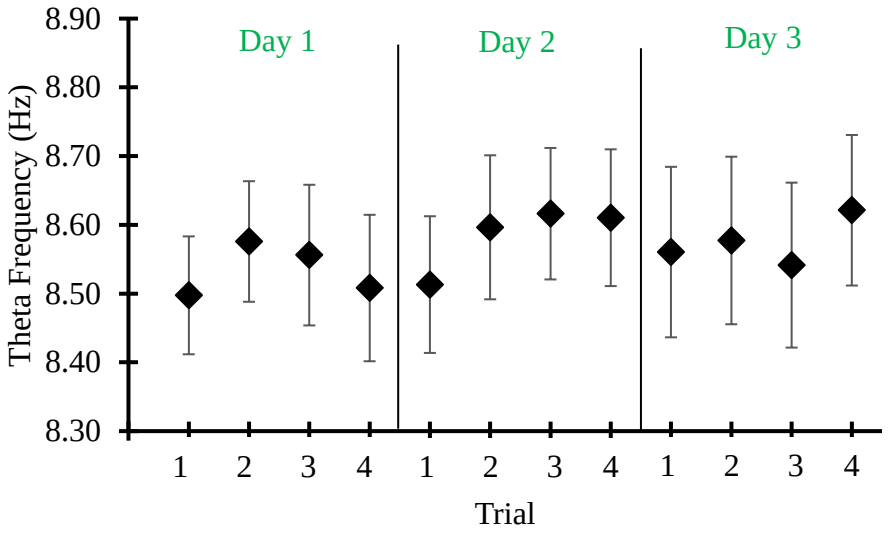


Figure 7.20 Intermediate intercept increases across trial, within day. Each data point indicates the mean (± SEM) of rats 1 – 2 and 4 – 7.

The effect main effect of Trial remained significant after a repeated measure ANOVA was performed excluding the data from Day 1; $F_{3,15} = 3.771$, $p = 0.034$. Pairwise comparison showed that there was a significant increase in intermediate intercept between trial 1 and trial 4 ($p = 0.016$, 0.078 ±0.022 Hz). There was no main effect of Days 2 – 3 ($F_{1,5} = 0.040$, $p = 0.850$), nor was there a significant interaction between Trial and Day; $F_{3,15} = 2.188$, $p = 0.132$. The effect size of intermediate intercept for Trial was 0.869, with observed power of 0.70. The effect size of intermediate intercept for Day was 0.09, with observed power of 0.053. The sample size used in this analysis was n = 6, with the appropriate sample size for intermediate intercept for Trial being n = 7, and the appropriate sample size for intermediate

intercept for Day being n = 977. Although the repeated measure ANOVA reported a main effect of Trial, that effect was slightly underpowered. Figure 7.21 illustrates the increase of intermediate intercept across Trail.

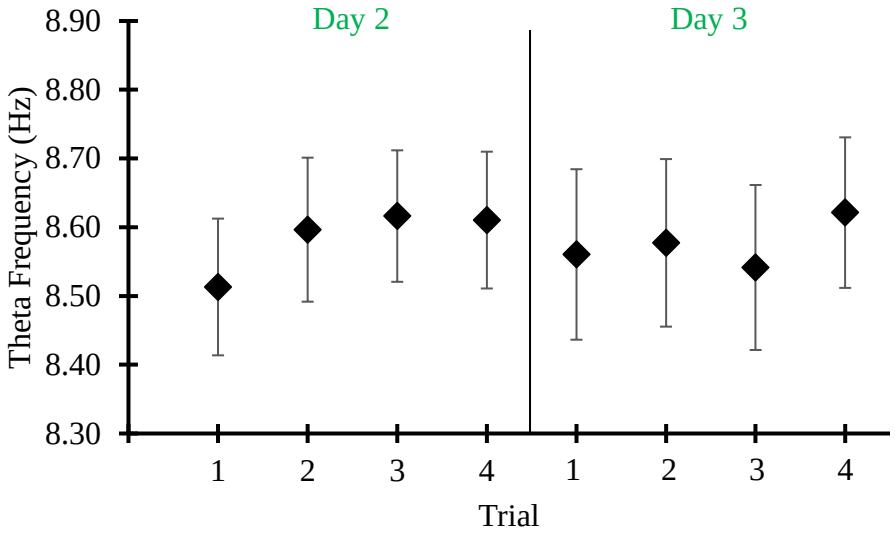


Figure 7.21 Intermediate intercept increases across trial with Day 1 data excluded, days 2 – 3. Each data point indicates the mean (± SEM) of rats 1 – 2 and 4 – 7.

7.5.4 Intermediate intercept increases across trial, no effect of days 1 – 5

A repeated measure ANOVA was used to examine the effect of environmental familiarisation on intermediate intercept across Day (1 – 5). There was a main effect of Trial; $F_{3,15} = 11.245$, $p = 0.0004$. Intermediate intercept increased between trial 1 and trial 2 ($p = 0.017$, 0.071 ±0.020 Hz), trial 1 and trial 3 ($p = 0.012$, 0.059 ± 0.015), and between trial 1 and trial 4 ($p = 0.003$, 0.075 ±0.014 Hz). There was no main effect of Day ($F_{4,20} = 0.865$, $p = 0.502$), nor was there a significant interaction Trial and Day; $F_{12,60} = 1.048$, $p = 0.419$. The effect size of intermediate intercept across Trial was 1.499, with observed power of 0.994. The effect size of intermediate intercept across Day was 0.417, with observed power of 0.227. The sample size used in this analysis was n = 6, with the appropriate sample size for intermediate

intercept for Trial being n = 4, and the appropriate sample size for intermediate intercept for Day being n = 20. Figure 7.22 illustrates the intermediate intercept increase across Trial.

A multiple regression analysis for the effect of Trial and Day upon intermediate intercept was performed to check for any overinfluential effect from interpolation for the missing value for Rat 1-Trial 1-Day 1. *With* the interpolated value, there was no significant model ($F_{2,117}$, p = 0.302, adjusted R^2 = 0.004). Neither Trial (β = 0.09, p = 0.338) nor Day (β = 0.11, p = 0.224) significantly correlated with intermediate intercept. Similarly, *without* the interpolated value, there was no significant model ($F_{2,116}$, p = 0.331, adjusted R^2 = 0.002). Neither Trial (β = 0.09, p = 0.355) nor Day (β = 0.11, p = 0.238) significantly correlated with intermediate intercept. In summary, neither Trial nor Day predicted intercept, and the effect of interpolated value was virtually non-existent in this analysis.

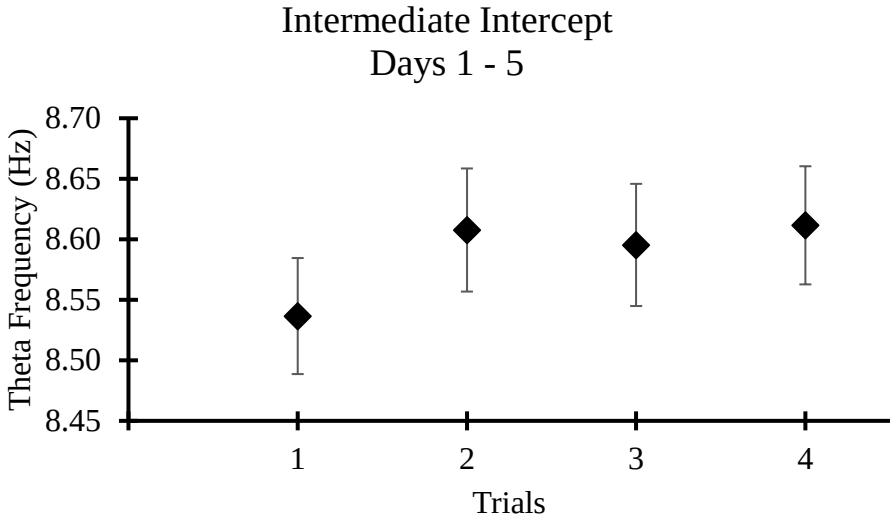


Figure 7.22 Intermediate intercept increases across trial within day, days 1 – 5. Each data point indicates the mean (± SEM) of rats 1 – 2 and 4 – 7.

To test the robustness of this effect, a repeated measure ANOVA was performed excluding the data from Day 1. There was a main effect of Trial; $F_{3,15}$ = 7.962, p = 0.002. There was a significant increase of intermediate intercept between trial 1 and trial 2 (p = 0.046, 0.069 ±

0.026), trial 1 and trial 3 (p = 0.044, 0.059 ± 0.022), and between trial 1 and trial 4 (p = 0.006, 0.090 ± 0.020). There was no main effect of Day ($F_{3,15}$ = 0.481, p = 0.701), nor was there a significant interaction between Trial and Day; $F_{9,45}$ = 0.823, p = 0.591. The effect size of intermediate intercept across Trial was 1.259, with observed power of 0.960. The effect size of intermediate intercept across Day was 0.311, with observed power of 0.125. The sample size used in this analysis was n = 6, with the appropriate sample size for intermediate intercept for Trial with minimum power of 0.80 being n = 5, and the appropriate sample size for intermediate intercept for Day being n = 40. Figure 7.23 illustrates the increase of intermediate intercept across Trial.

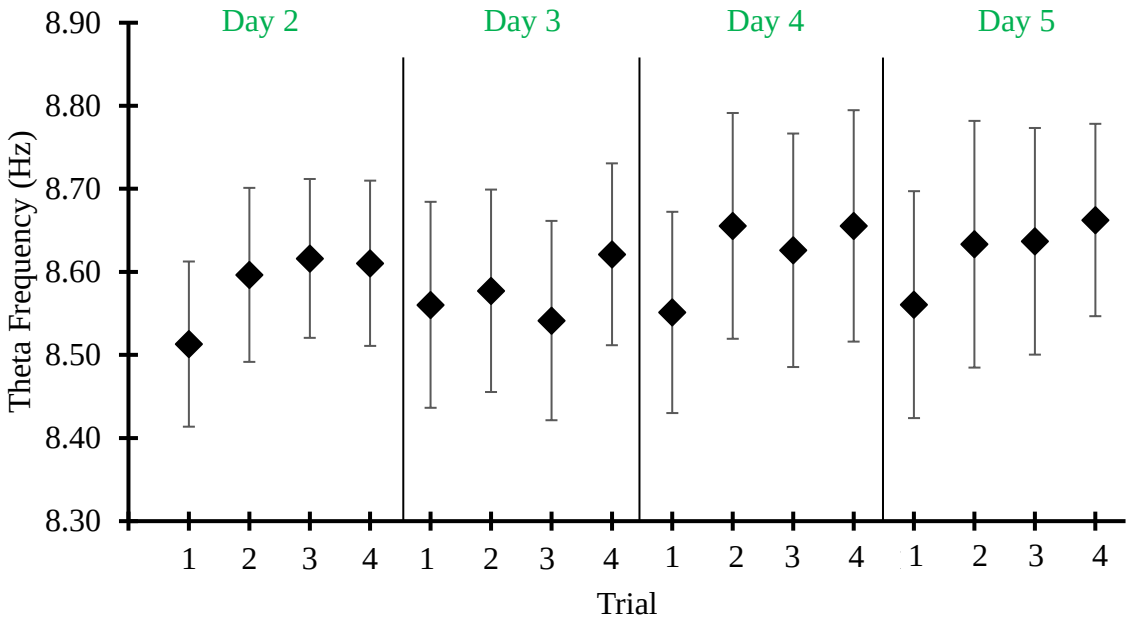


Figure 7.23 Intermediate intercept increases across trial with Day 1 data excluded, days 2 – 5. Each data point indicates the mean (±SEM) of the trial (1 – 4) from rats 1 -2 and 4 – 7.

7.6 Environmental Novelty and Familiarisation's effect on hippocampal place cells

As stated in chapter 6 (section 6.5) out of the seven rats test, five rats yielded pyramidal cells.

To examine the effect of environmental familiarisation on hippocampal place cells, mixed

model ANOVAs were performed on global mean rate, locational peak rate, spatial information, the distance of place cells between pre-injection trials, and place cell field size. The cells used to examine the effects of environmental novelty and familiarisations were those found during the first 3 days of exposure to the experimental apparatus. Days 1 – 3 yielded 95 pyramidal cells (Table 7.1). The distance values were calculated by taking the pixel coordinates (x, y) from a cell's peak spikes and converting those values into centimetres. Using the distance formula ($d = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}$), the difference from each cell between each trial was calculated (trials (trial 2 – trial 1), (trial 3 – trial 2), and (trial 4 – trial 3)) and those values were used in the analysis. Additionally, a repeated measures analysis was performed on the difference in distance between the last pre-injection trial (trial 4) and the initial pre-injection trial (trial 1). Place field size was calculated using the same method described in Chapter 6 (section 6.5.1.4) where the field size in bins was converted to cm^2 with the change index calculated after that.

Table 7.2 Number of cells recorded for each animal, days 1 – 3.

Rat ID	Number of Cells	Total
Rat 1	23	95
Rat 2	13	
Rat 3	15	
Rat 4	9	
Rat 5	35	

7.6.1 Environmental familiarisation effects upon hippocampal place cells (global mean rate, locational peak rate and Skaggs spatial information)

7.6.1.1 Global mean rate

As stated in Chapter 6 (section 6.5.1.1), global mean rate was calculated by dividing the number of spikes hippocampal pyramidal cells fired by trial time (600 seconds). A repeated

measure ANOVA was performed to examine the effect of environmental familiarisation on global mean rate. There was a main effect of Trial; $F_{3,12} = 3.814$, $p = 0.039$. Global mean rate significantly increased between trial 1 and trial 2 ($p = 0.006$, 0.256 ± 0.047), and between trial 1 and trial 3 ($p = 0.021$, 0.327 ± 0.088). There was no main effect of Day ($F_{2,8} = 0.182$, $p = 0.837$), but there was a significant interaction between Trial and Day; $F_{6,24} = 2.863$, $p = 0.030$. The effect size of global mean rate across Trial was 0.976, with an observed power of 0.670. The effect size of global mean rate across Day was 0.212, with an observed power of 0.069. The sample size used in this analysis was n = 5, with the appropriate sample size for Trial with minimum power of 0.8 being n = 6, and the appropriate sample size for Day being n = 110. Although a significant effect was reported, the effect of global mean rate increasing across Trial was underpowered with having only 5 subjects, suggesting that a stronger effect would be detected with a larger sample size. Figure 7.24 illustrates the steady increase of global mean rate across Trial.

As with slope and intercept analysis, a multiple regression analysis for the effect of Trial and Day upon global mean rate was performed to check for any overinfluential effect from interpolation for the missing value of Rat 1-Trial 1-Day 1. With the interpolated value, there was no significant model ($F_{2,57}$, $p = 0.182$, adjusted $R^2 = 0.025$). Neither Trial ($\beta = 0.22$, $p = 0.089$) nor Day ($\beta = -0.09$, $p = 0.474$) significantly correlated with global mean rate. Similarly, without the interpolated value there was no significant model ($F_{2,56}$, $p = 0.201$, adjusted $R^2 = 0.022$). Neither Trial ($\beta = 0.21$, $p = 0.109$) nor Day ($\beta = -0.10$, $p = 0.449$) significantly correlated with global mean rate. In summary, neither Trial nor Day predicted global mean rate, which further supports the notion that the analysis is under powered. The effect of the interpolated value was minimal (trial: $\beta = 0.21$ instead of $\beta = 0.22$; day: $\beta = -0.10$ instead of $\beta = -0.09$).

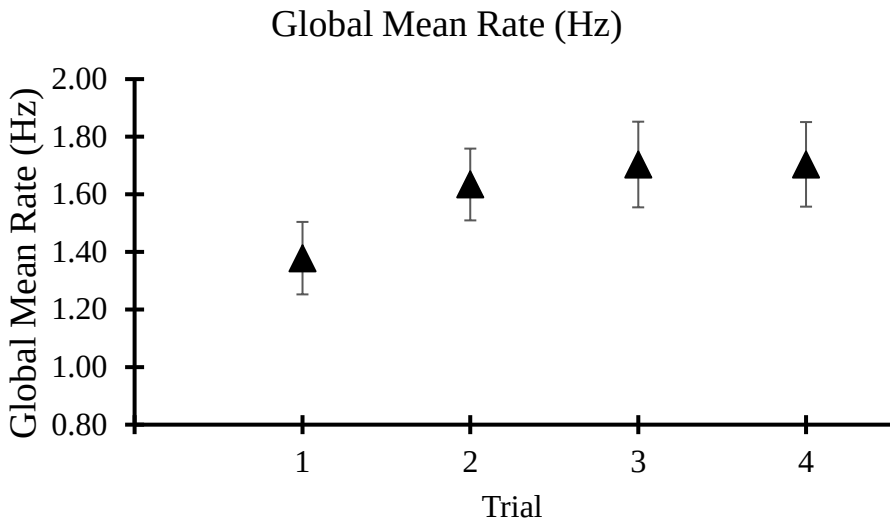


Figure 7.24 Global mean rate increases across trials within day. Each data point indicates the mean (±SEM) of trial (1 – 4) from rats 1 – 5, days 1 – 3.

7.6.1.2 Locational peak rate

A repeated measure ANOVA was performed to examine environmental familiarisation on locational peak rate and found that there was no main effect of Day; $F_{2,8} = 0.053$, $p = 0.949$. Although there was no main effect of Trial ($F_{3,12} = 3.814$, $p = 0.059$), pairwise comparison found that locational peak rate significantly increases between trial 1 and trial 2 ($p = 0.045$, 1.089 ± 0.377). There was no significant interaction between Trial and Day; $F_{6,24} = 0.960$, $p = 0.472$. The effect size of locational peak rate across Trial was 0.905, with observed power of 0.600. The effect size of locational peak rate across Day was 0.115, with observed power of 0.056. The sample size used for this analysis was n = 5, with the appropriate sample size with minimum power of 0.8 for Trial being n = 7, and the appropriate sample size for Day being n = 369. As with global mean rate, the results for locational peak rate suggests that the analysis was underpowered. Although there was no main effect of Trial, it was approaching significance in addition to the pairwise comparison showing an effect between trial 1 and trial 2 in locational peak rate increase. Figure 7.25 illustrates that increase of locational peak rate across Trial.

A multiple regression analysis for the effect of Trial and Day upon locational peak rate was performed to check for any overinfluential effect from interpolation for the missing value for Rat 1-Trial 1-Day 1. *With* the interpolated value, there was no significant model ($F_{2,57}$, p = 0.177, adjusted R^2 = 0.026). Neither Trial (β = 0.24, p = 0.064) nor Day (β = -0.01, p = 924) significantly correlated with locational peak rate. Similarly, *without* the interpolated value there was no significant model ($F_{2,56}$, p = 0.163, adjusted R^2 = 0.029). Neither Trial (β = 0.25, p = 0.058) no Day (β = -0.001, p = 0.991) significantly correlated with locational peak rate. In summary, neither Trial nor Day predicted locational peak rate, and the effect of interpolated value was slight (trial: β = 0.25 instead of β = 0.24; day: β = -0.001 instead of β = -0.01).

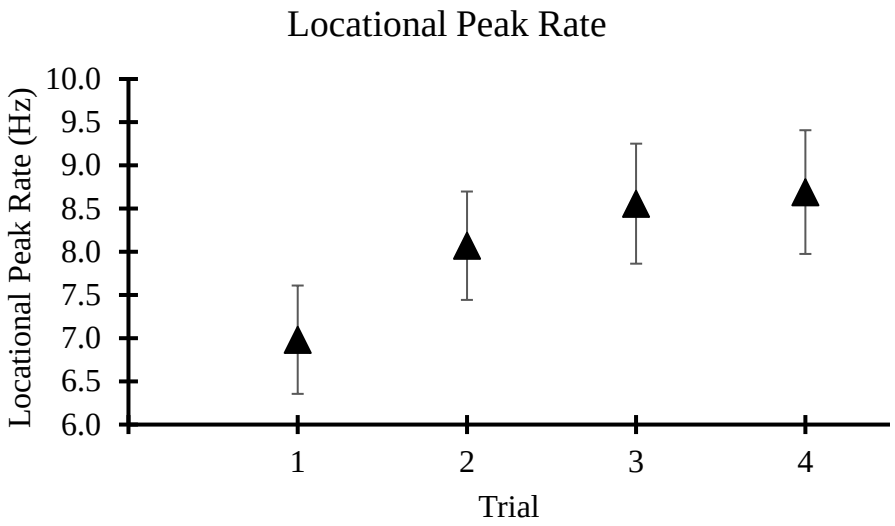


Figure 7.25 Locational peak rate did not significantly increase across trial. Pairwise comparison found a significant increase between trial 1 and trial 2. Each data point indicates the mean (±SEM) of trial (1 – 4) from rats 1 – 5.

7.6.1.3 Skaggs spatial information

A repeated measure ANOVA was used to examine environmental familiarisation on Skaggs spatial information. There was a main effect of Day; $F_{2,8}$ = 9.172, p = 0.009 (Figure 7.26). Skaggs spatial information significantly increased between day 1 and day 3 (p = 0.030, 0.327

± 0.099), and between day 2 and day 3 (p = 0.022, 0.256 ± 0.070). There was no main effect of Trial ($F_{3,12}$ = 1.764, p = 0.207), nor was there a significant interaction between Trial and Day; $F_{6,24}$ = 2.271, p 0.071. The effect size of Skaggs spatial information across Trial was 0.664, with observed power of 0.347. The effect size of Skaggs spatial information across Day was 1.513, with observed power of 0.889. The sample size used in this analysis was n = 5, with the appropriate sample size with minimum power of 0.80 for Skaggs spatial information for Trial was n = 11, and the appropriate sample size for Skaggs spatial information for Day was n = 5. Figure 7.26 illustrates the significant increase of Skaggs spatial information across Day.

A multiple regression analysis for the effect of Trial and Day upon Skaggs spatial information was performed to check for any overinfluential effect from interpolation for the missing value from Rat 1-Trial 1-Day 1. *With* the interpolated value, a significant model emerged ($F_{2,57}$, p = 0.0002, adjusted R^2 = 0.236), whereby Day positively predicted Skaggs spatial information (β = 0.48, p = 0.001), but Trial did not (β = 0.19, p = 0.098). Similarly, *without* the interpolated value, a significant model emerged ($F_{2,56}$, p = 0.0002, adjusted R^2 = 0.238), whereby Day positively predicted Skaggs spatial information (β = 0.48, p = 0.0001), but Trial did not (β = 0.20, p = 0.086). In summary, Day was a significant predictor of Skaggs spatial information, with Skaggs increasing across Day, and the effect of the interpolated value was minimal (β = 0.48 for both).

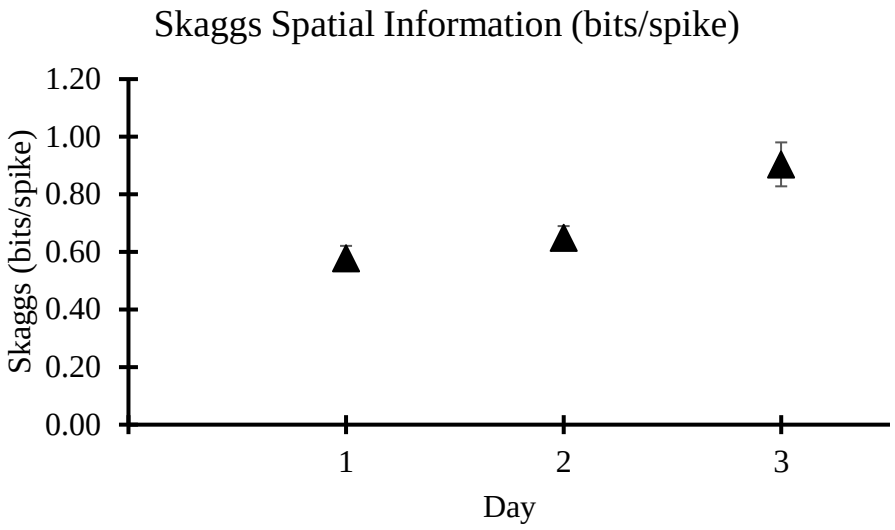
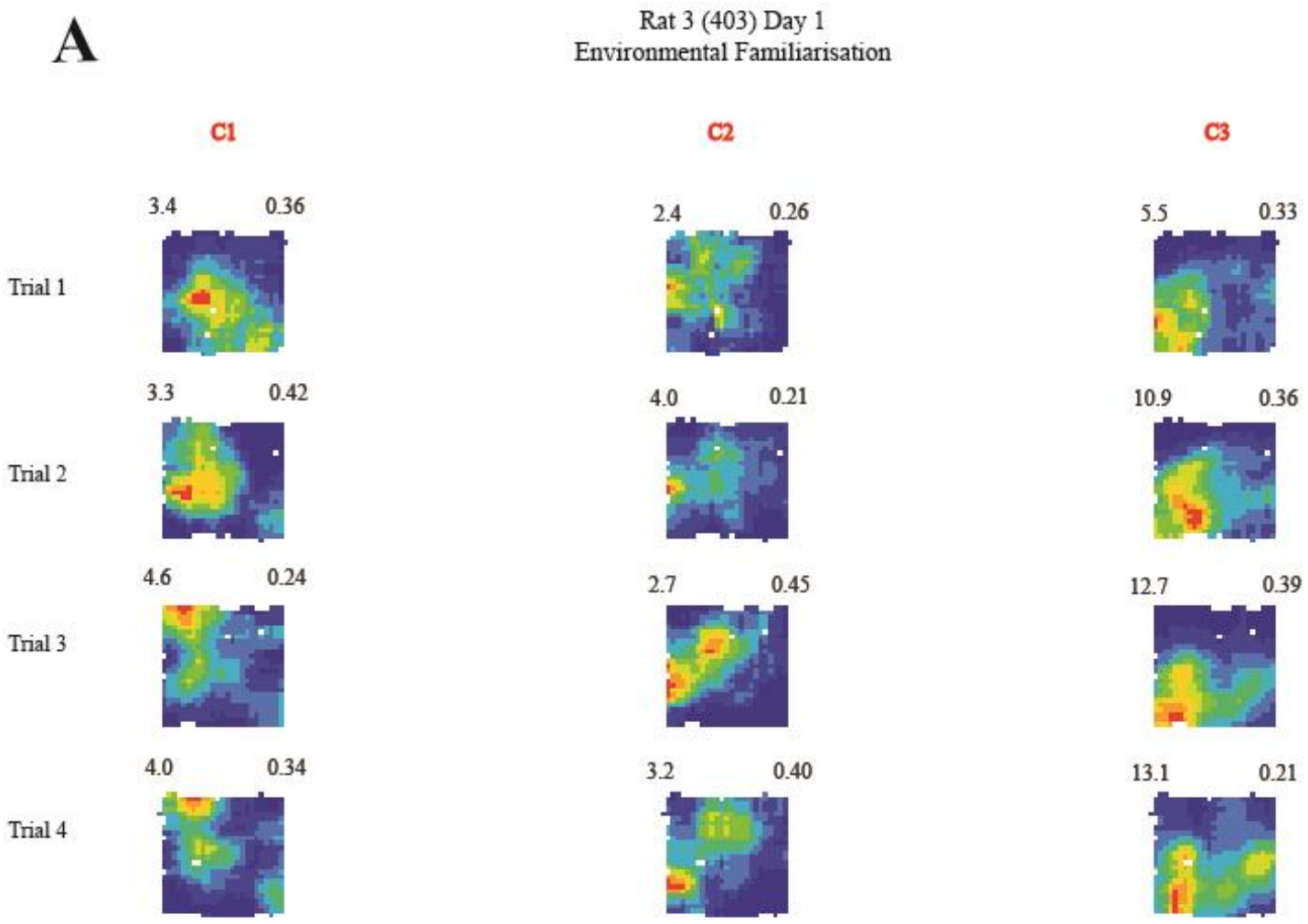
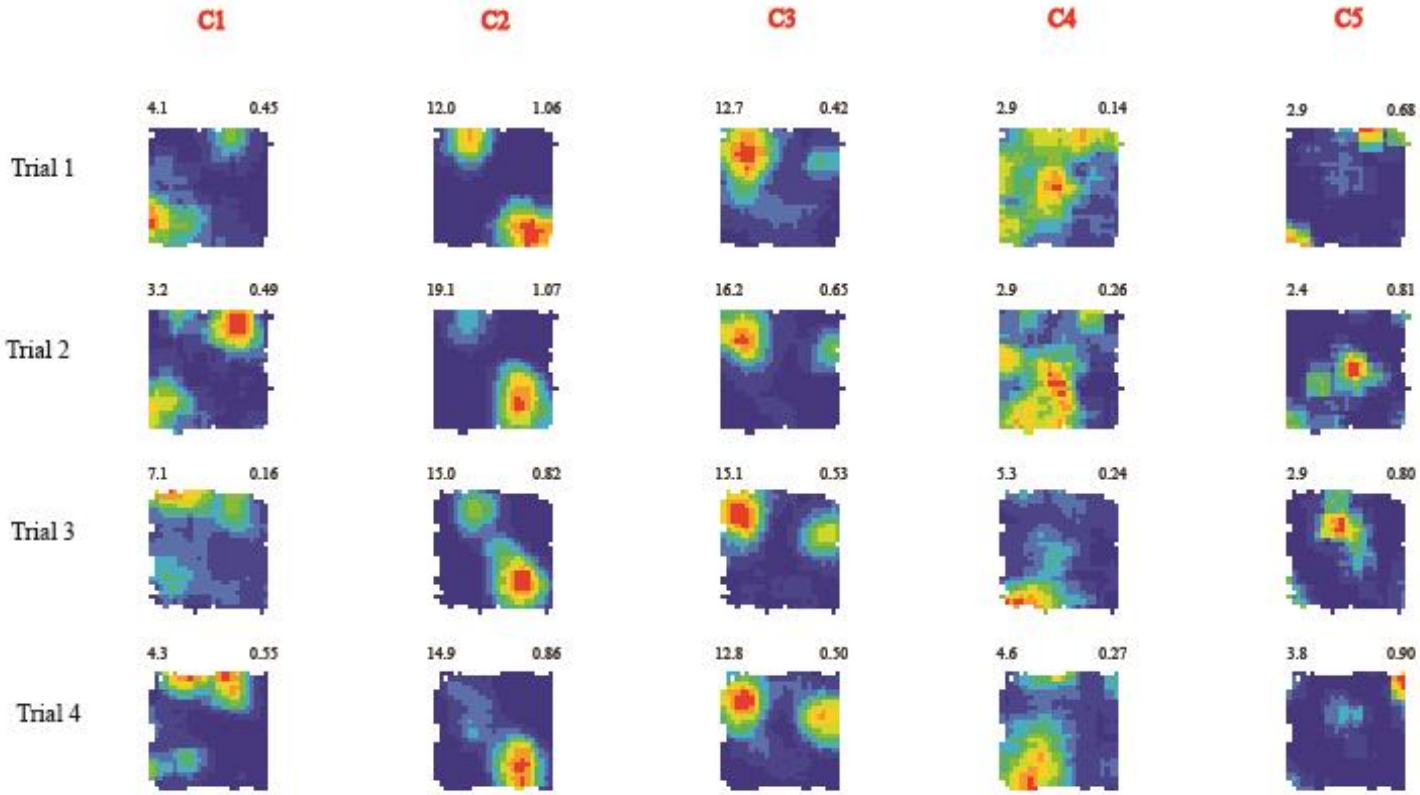


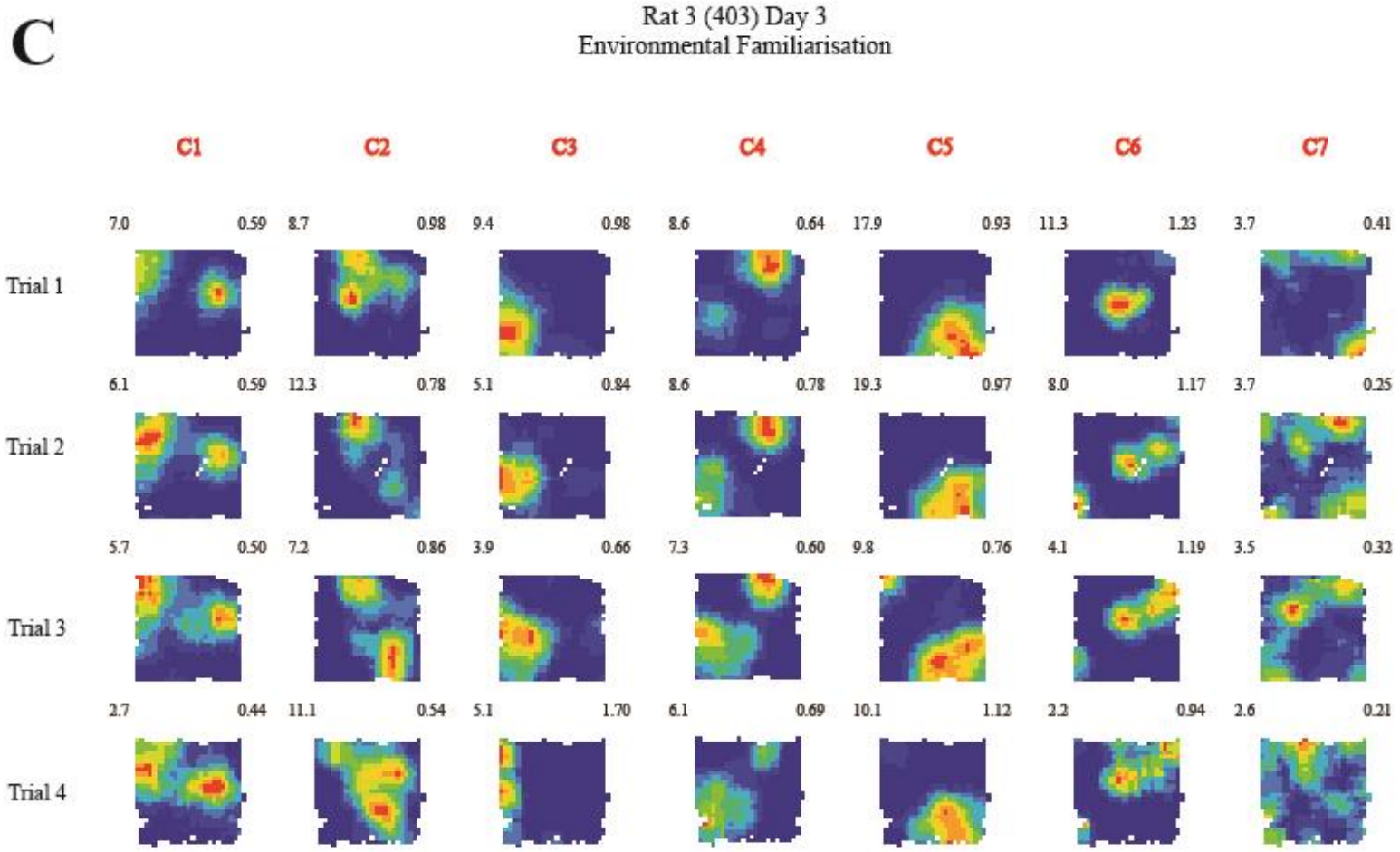
Figure 7.26 Skaggs spatial information increased across day. Each data point indicates the mean (±SEM) of trial (1-4) from rats 1-5.


A
Rat 3 (403) Day 1
Environmental Familiarisation
C1
C2
C3
Trial 1
Trial 2
Trial 3
Trial 4
3.4
0.36
2.4
0.26
5.5
0.33
3.3
0.42
4.0
0.21
10.9
0.36
4.6
0.24
2.7
0.45
12.7
0.39
4.0
0.34
3.2
0.40
13.1
0.21

B

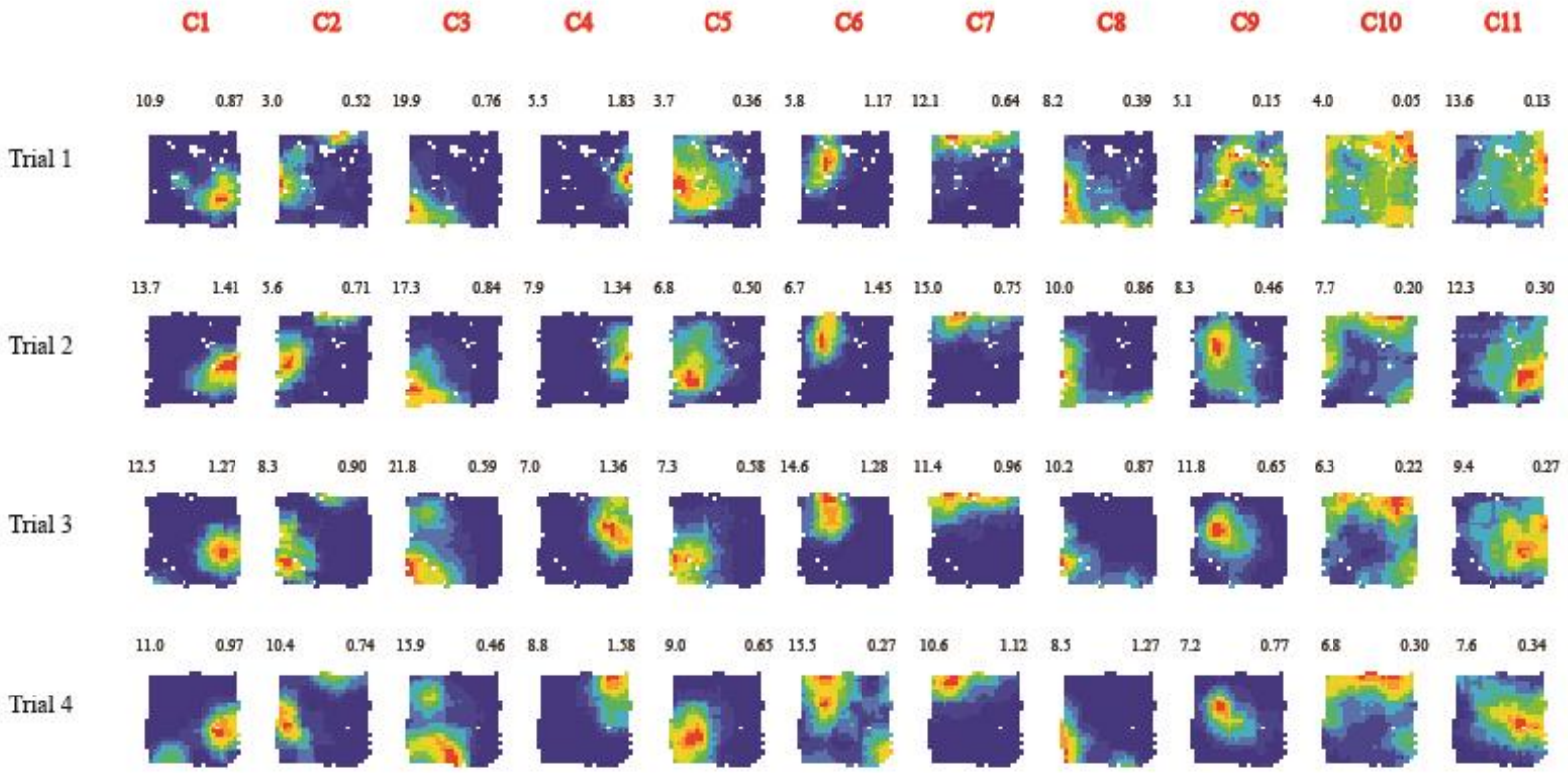
Rat 3 (403) Day 2
Environmental Familiarisation




C
Rat 3 (403) Day 3
Environmental Familiarisation
C1
C2
C3
C4
C5
C6
C7
Trial 1
7.0 0.59
8.7 0.98
9.4 0.98
8.6 0.64
17.9 0.93
11.3 1.23
3.7 0.41
Trial 2
6.1 0.59
12.3 0.78
5.1 0.84
8.6 0.78
19.3 0.97
8.0 1.17
3.7 0.25
Trial 3
5.7 0.50
7.2 0.86
3.9 0.66
7.3 0.60
9.8 0.76
4.1 1.19
3.5 0.32
Trial 4
2.7 0.44
11.1 0.54
5.1 1.70
6.1 0.69
10.1 1.12
2.2 0.94
2.6 0.21

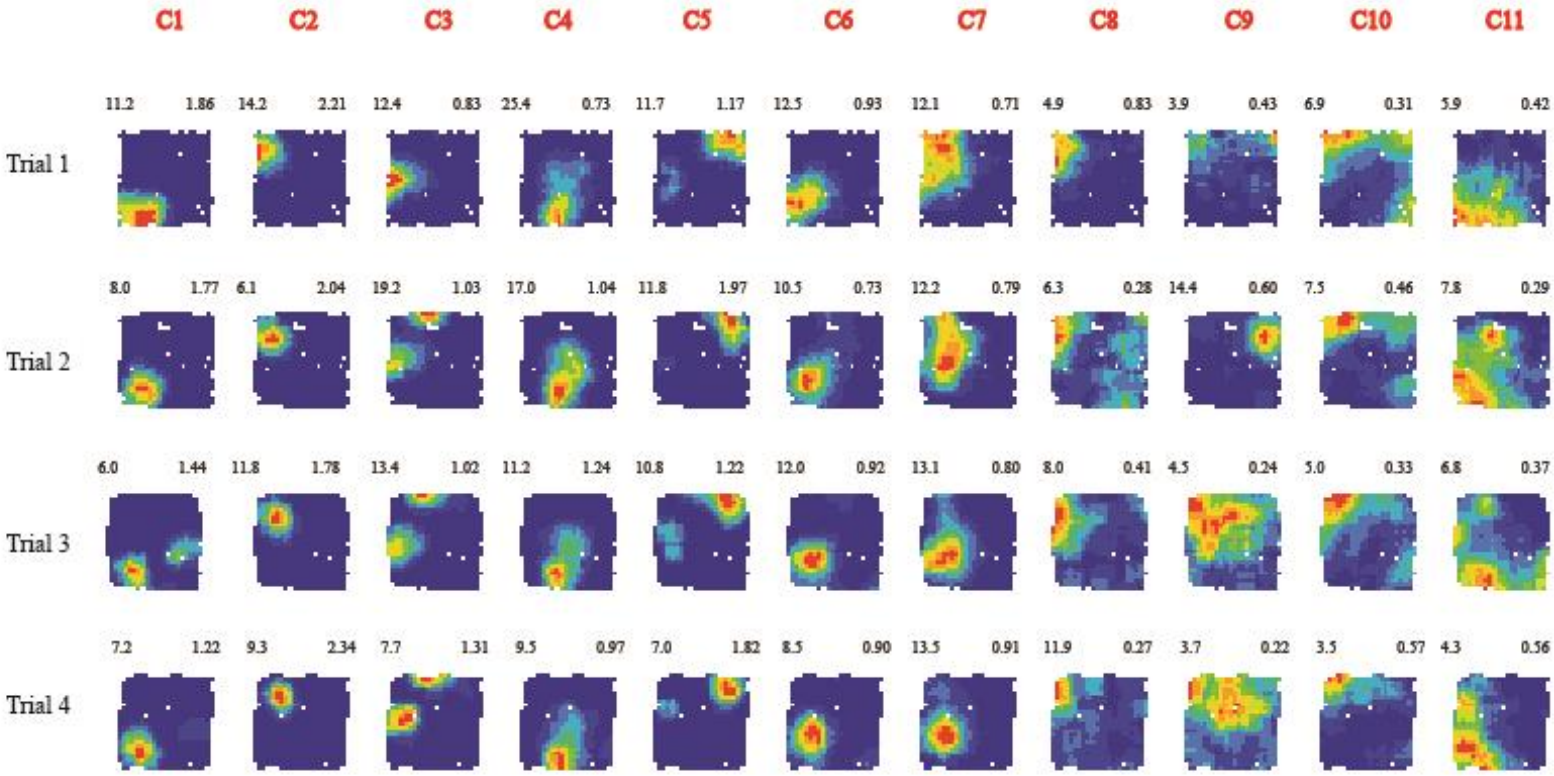
D

Rat 5 (422) Day 1
Environmental Familiarisation



E

Rat 5 (422) Day 2
Environmental Familiarisation



F

Rat 5 (422) Day 3
Environmental Familiarisation

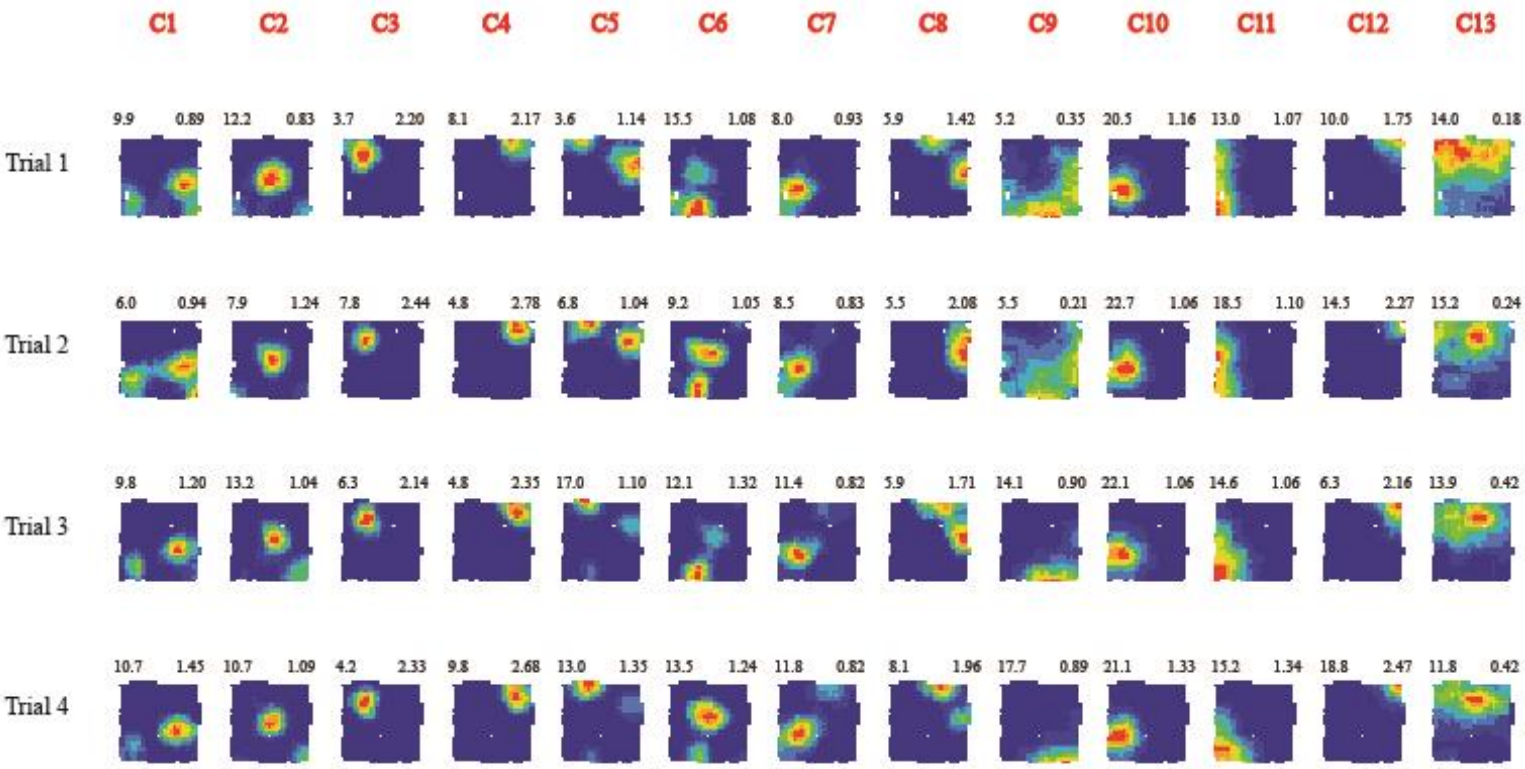


Figure 7.27 Key firing characteristics of place cells days 1 – 3; Rat 3 and Rat 5. A) Shows the cells recorded on Day 1 for Rat 3 (n = 3), pre-injection trials 1-4. The cell numbers are shown in red and the corresponding values of the locational peak rate and Skaggs spatial information are on the top left and top right corners of each rate map, respectively. **B**) Shows the cells recorded on Day 2 for Rat 3 (n = 5), pre-injection trials 1-4. The cell numbers are shown in red and the corresponding values of the locational peak rate and Skaggs spatial information are on the top left and top right corners of each rate map, respectively. **C**) Shows the cells recorded on Day 3 for Rat 3 (n = 7), pre-injection trials 1-4. The cell numbers are shown in red and the corresponding values of the locational peak rate and Skaggs spatial information are on the top left and top right corners of each rate map, respectively. **D**) Shows the cells recorded on Day 1 for Rat 5 (n = 11), pre-injection trials 1-4. The cell numbers are shown in red and the corresponding values of the locational peak rate and Skaggs spatial information are on the top left and top right corners of each rate map, respectively. **E**) Shows the cells recorded on Day 2 for Rat 5 (n = 11), pre-injection trials 1-4. The cell numbers are shown in red and the corresponding values of the locational peak rate and Skaggs spatial information are on the top left and top right corners of each rate map, respectively. **F**) Shows the cells recorded on Day 3 for Rat 5 (n = 13), pre-injection trials 1-4. The cell numbers are shown in red and the corresponding values of the locational peak rate and Skaggs spatial information are on the top left and top right corners of each rate map, respectively.

7.6.2 Environmental familiarisation effect on place field stability

To further examine environmental familiarisation's effect upon hippocampal place cells, the difference in distance between each cell's peak rate bin (aka pixel) on adjacent trials was explored, in order to get a measure of place field stability. In every trial, there is a spatial bin, typically near the centre of a place field, where a given place cell fires at its highest rate (after smoothing). The idea of the analysis is that if place fields are stable from trial to trial, the location of this peak rate bin, for a given place cell, should be similar from trial to trial. It might be predicted that when the environment is unfamiliar, for a given place cell, across-trial peak bin locations are more unstable (i.e. further distant) and when the environment is familiar, they are closer to each other. In this manner, an examination of inter-trial peak location distance was used a measure of stability. This value should be higher when fields are less stable, and smaller when fields are more stable. As mentioned previously (Chapter 6), the distance values were calculated by taking the coordinates (x, y) from the cell's peak rate bin and converting those values into centimetres. Using the distance formula ($d = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}$), the difference in the location of the peak bin across each pre-injection trial was calculated for each cell ((trial 2 – trial 1), (trial 3 – trial 2), and (trial 4 – trial 3)). Each cell's difference in location of the peak bin was averaged into a single value of day and trial difference (i.e., average difference in location of Rat 1 cells for trial 2 – trial 1, Day 1, etc.). The single averaged values were used in the subsequent analysis subjects.

A repeated measure ANOVA was performed and showed that there was no main effect of Trial changes; $F_{2,8} = 0.431$, $p = 0.664$. There was also no main effect of Day ($F_{2,8} = 0.737$, p = 0.509), nor was there a significant interaction between Trial and Day; $F_{4,16} = 1.147$, p = 0.370. The effect size of place cell distance comparison across Trial was 0.328, with observed power of 0.098. The effect size of place cell distance comparison across Day was 0.430, with observed power of 0.135. The sample size used in this analysis was 5, with the appropriate

sample size with minimum power of 0.80 for Trial being n = 48, and the appropriate sample size for Day being n = 29. Although the analysis did not find a significant effect, Figure 7.28 illustrates a decrease in place cell distance comparison across trials, which is in the expected direction; as the environment becomes familiar, the place cells become more stable.

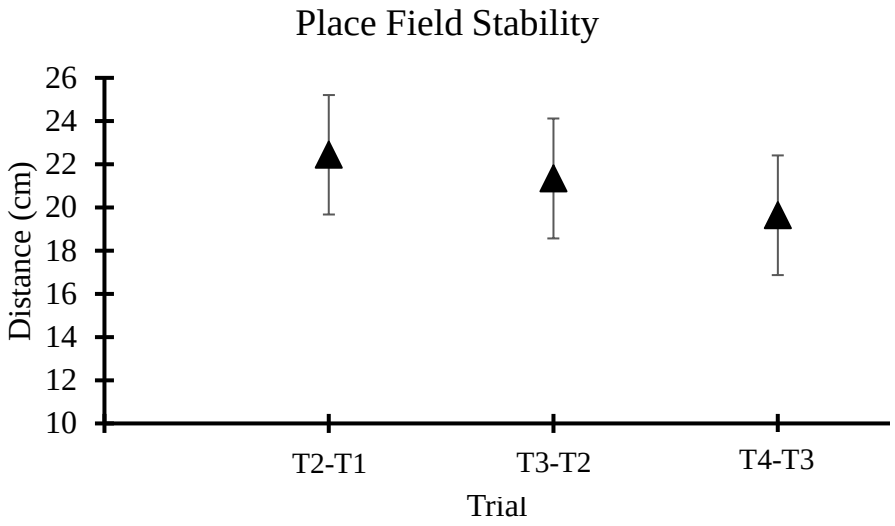


Figure 7.28 Place field stability. This graph illustrates how the distance between peak rate bins decreases (stabilise) from trial-to-trial, implying place field stabilises as the environment becomes familiar. Each data point indicates the mean difference (±SEM) of trial (trial 2 – trial 1; trial 3 – trial 2; trial 4 – trial 3) from rats 1 – 5.

7.6.3 Environmental familiarisation effect on hippocampal place field area

To examine the effects of environmental familiarisation on hippocampal place field size, the field size for each cell was converted to cm^2 (area). Using the field size *area* values, a repeated measure was performed and found that there was no main effect of Trial ($F_{3,12}$ = 0.466, p = 0.711), no main effect of Day ($F_{2,8}$ = 0.632, p = 0.556), nor was there a significant interaction between Trial and Day ($F_{6,24}$ = 0.728, p = 0.632). The effect size of place field size area across Trial was 0.341, with observed power of 0.118. The effect size of place field

size area across Day was 0.397, with observed power of 0.122. The sample size used in this analysis was n = 5, with the appropriate sample size with minimum power of 0.8 for place field size area for Trial being n = 34, and the appropriate sample size for Day being n = 34. Although there was no significant effect of environmental familiarisation on field size area, Figure 7.29 illustrates that field size area was decreasing across Day. This trend supports the notion that as the environment becomes familiar, hippocampal place fields become more stable which would result in a decrease in field size area.

A multiple regression analysis for the effect of Trial and Day upon average field size area was performed to check for any overinfluential effect from interpolation for the missing value from Rat 1-Trial 1-Day 1. *With* the interpolated value, there was no significant model ($F_{2,57}$, p = 0.315, adjusted R^2 = 0.006). Neither Trial (β = 0.007, p = 0.957) nor Day (β = -0.20, p = 0.130) significantly correlated with average field size area. Similarly, *without* the interpolated value there was no significant model ($F_{2,56}$, p = 0.284, adjusted R^2 = 0.010). Neither Trial (β = -0.007, p = 0.956) nor Day (β = -0.21, p = 0.114) significantly correlated with average field size area.

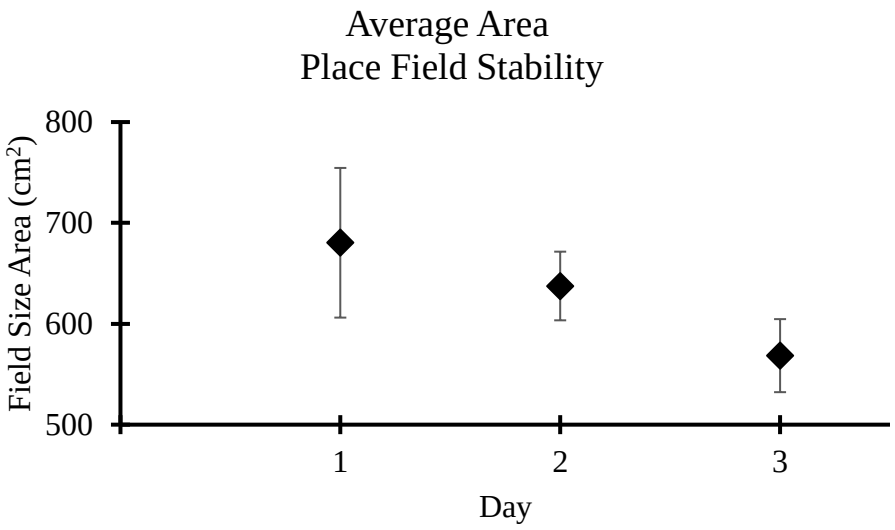


Figure 7.29 Average area, place field stability. This graph illustrates the average place field area decreases (stabilise) from day-to-day, implying place field stabilises as the environment becomes familiar. Each data point indicates the average area (±SEM) of trial (trial 1 to trial 4) from rats 1 – 5.

7.6.3.1 Slope component and hippocampal place fields

Additionally, correlational analyses were performed to examine environmental familiarisation's effect on the slope component correlated with the size/field area of hippocampal place cells. Using the same method in Chapter 6 to obtain field size change index to normalise the area of the place field size, a correlation was performed to compare the average of the normalised area for each trial to the average slope for each trial (e.g., Rat 1, day 1, trial 1 average normalised area compared to average slope, etc). The correlational analysis found no significant relationship between average slope and normalised field size area; r = 0.171, p = 0.195 (Figure 7.30).

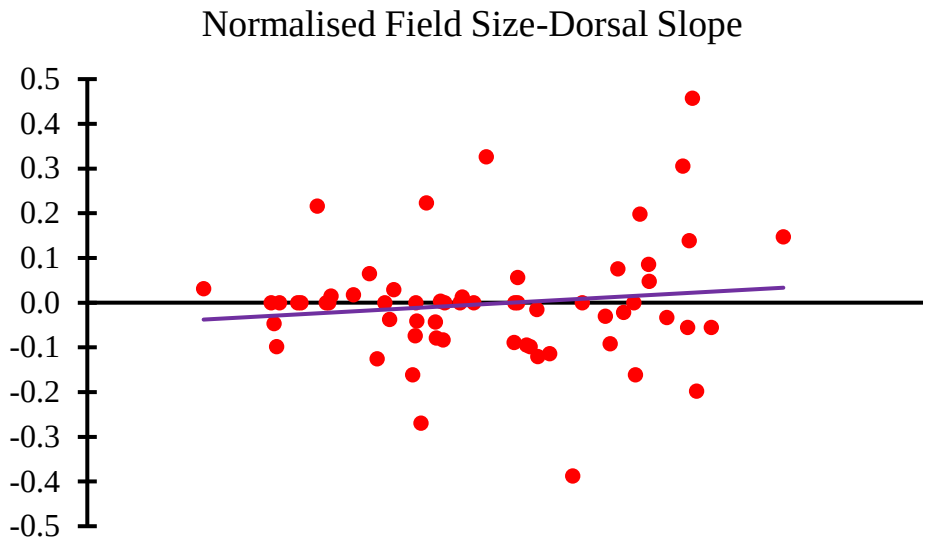


Figure 7.30 No correlation between average slope and normalised field size. Each data point represents the normalised field size (along the x-axis) compared to the average slope (along the y-axis).

7.7 Discussion

7.7.1 Summary of Study 1B

Study 1B examined the effect of environmental familiarisation on the slope and intercept component of the theta-frequency-to-speed relationship in the dorsal and intermediate portion of the hippocampus. As predicted, environmental familiarisation increased the dorsal slope component. This effect was seen across pre-injection trials (trials 1 – 4) within day (1 – 5) and within days 1 – 5. Intermediate hippocampus, on the other hand, had no effect of environmental familiarisation across trial nor across pre-drug /familiarisation days. An effect of trial was present when the analysis was performed excluding the data from Day 1 and when the analysis was performed including days 1 – 5. Environmental familiarisation did not affect the dorsal intercept component, as predicted. However, intermediate intercept increased across trial within days and within days 1 – 5.

Study 1B also examined the effects of environmental familiarisation on characteristics of hippocampal place cells. Although there was no main effect of environmental familiarisation

on locational peak rate (Hz), post-hoc analysis found that there was a significant increase between trial 1 and trial 2. There was an effect of environmental familiarisation on Skaggs spatial information, which increased across day. Global mean rate increased across pre-injection trials with an interaction of day. Environmental familiarisation had no effect on average area of hippocampal place fields nor an effect on change in distance between hippocampal place cells. Correlational analysis found that the average area of a place field for each day did not correlate with the average of slope of each day. A normalised data set of hippocampal area was also analysed using a correlational analysis and found that there was no correlation between normalised area and the slope component of the theta-frequency-to-speed relationship.

Overall, hippocampal theta recorded in the dorsal hippocampus behaved as predicted; an effect of environmental familiarisation on dorsal slope without affecting dorsal intercept. Whilst, hippocampal theta recorded in the intermediate hippocampus produced unpredicted results. The effect of environmental familiarisation on characteristics of hippocampal place cells produced results one might expect, with locational peak rate, global mean rate, and Skaggs spatial information all increasing as the animal became familiar with the environment. Although place field stability and average area analysis did not produce an effect, both results were along a trend that was expected; as the environment became familiar the hippocampal place cells stabilised with decreases in average area and distance between cells.

7.7.2 Environmental familiarisation's effect on the slope component

Previous research into the effects of environmental familiarisation noted an increase of the slope component of the theta-frequency-to-speed relationship without effecting the intercept component (Wells et al., 2013). In Study 1B, this effect was elicited in the dorsal hippocampus. Examination of dorsal slope across trial for both days 1 – 3 and days 1 – 5

found a strong effect. However, when examining the effect of environmental familiarisation, intermediate slope did not initially elicit a significant increase when examined across the days. Effect size analysis suggests that the non-significant result could be accounted for by the need of a larger sample size, more so than environmental familiarisation having no effect on intermediate slope. In fact, when analysed excluding the data from Day 1 and analysed across days 2 – 5, environmental familiarisation significantly increased intermediate slope across trial. With the results from both the dorsal and intermediate slope, the effects of environmental familiarisation appear to be more sensitive in the dorsal hippocampus more so than the intermediate hippocampus. Although not predicted, this dichotomy in the results indicates that there is a separation, albeit minimal, between the dorsal and intermediate hippocampus in modulating spatial cognitive processes. Previous research has noted a clear distinction in animal behaviour between the dorsal and ventral hippocampus, with the former being related to spatial cognitive processes and the latter related to the modulation of fear/anxiety (Bannerman et al., 2004; Fanselow & Dong, 2010). The intermediate hippocampus, which lies between the dorsal and ventral hippocampus, has overlapping characteristics of the dorsal and ventral hippocampus (Fanselow & Dong, 2010), with research indicating the intermediate hippocampus' importance in the translation of new and rapid learning into efficient behavioural performance (Bast et al., 2009). The 'stutter step' in the initial result of environmental familiarisation's effect on intermediate slope further implies that there is some overlap in functionality.

The results of environmental familiarisation's effect on the slope component also highlight the effect of exposure recency. For both the dorsal and intermediate hippocampus, the slope was flattest on the first trial of each day. The recency in which the animal was exposed to the environment from the last trial of the previous day to the first trial of the next day (~19 hours) compared to exposure of the environment in the successive trials (30-minute inter-trial-

intervals) suggests that trial 1 slope from Day 2 onwards could implicate the re-familiarisation of the environment. This notion is supported by the maintaining/subsequent significance of the slope increase across trial within day when excluding day 1 for both dorsal and intermediate slope.

Overall, the results of environmental familiarisation's effects on dorsal and intermediate slope are as predicted. The slope component from both areas of the hippocampus increased as the environment became familiar. The role the intermediate hippocampus plays in spatial cognitive processes requires more exploration, along with the time spent between environment exposure's effect on slope.

7.7.3 Environmental familiarisation's effect on the intercept component

In the current thesis one of the key attributes of the prediction formulated from the Burgess (2008) model is that if one component of the theta-frequency-to-speed relationship is effected, the other would not be; i.e., anxiolytic drugs effect the intercept component, but not the slope component. In Study 1B, analysis of environmental familiarisation's effect on the dorsal intercept component was as predicted; there was no effect. Conversely, intermediate intercept significantly increased across trials. Importantly, the increase of the intercept component could reflect the anticipation of receiving an intraperitoneal injection after trial 4, i.e. an anxiogenic anticipation, rather than an effect of environmental familiarisation. Potentially consistent with this interpretation is that intercept on trial 4 of Day 1 does not increase relative to the other trials, but if anything, decreases. On this Day 1 of course, unlike the other days, the rat has *not* experienced any i.p. injection. On Day 2, the trial 4 value is similar to the trial 3 value, and on Day 3, the trial 4 value, if anything increases. None of these changes are major, but they are consistent with an increasing anticipation, based on learning, that an injection, an aversive event, takes place on trial 4. When it is anticipated that future events which are aversive are likely to occur, this is often experienced as 'anticipatory

anxiety'. 'Anticipatory anxiety' is a concept and phrase used by Gray and McNaughton (2003) to refer to anxiety, which is conditioned, involving memory, as opposed to anxiety which is unconditioned and species-specific (e.g. the rat's immediate response to cat odor). Then applying the newer anatomical perspective on anxiety, whereby the dorsal hippocampus is not linked to anxiety, but the ventral hippocampus is linked to anxiety, it seems plausible that it is only the intermediate hippocampus, not the dorsal, which has some ventral-like anatomical connections supporting anxiety, that shows the theta intercept increase, as might be expected if anxiety increases over the trials within a day. It is notable that the Pentkowski and colleagues' study showing effects of ventral but not dorsal hippocampal lesions on conditioned and unconditioned anxiety in rats used lesion sites that were centered upon the 'intermediate' hippocampus (Pentkowski, Blanchard, Lever, Litvin, & Blanchard, 2006). In that study, minimal lesions involved the intermediate hippocampus more than the ventral hippocampus (see Figure 3 of Pentkowski et al., 2006). In other words, if the interpretation of hippocampally-driven anticipatory anxiety for a likely impending painful injection is reasonable, it is reasonable to suppose that such anxiety would be driven by intermediate rather than dorsal hippocampus. Naturally, this anxiety-based interpretation would be consistent with the long-standing view that dorsal hippocampus is not crucial for anxiety, while the intermediate hippocampus is associated with anticipatory behaviour (Burton et al., 2009), presumably including anticipatory anxiety (see discussion above in Introductory section 2.8). Thus, with the experimental procedure for Experiment 1, as environmental familiarity increases, so would anticipation of injection (anxiogenic). In other experimental procedures, (Hinman et al., 2011; Long et al., 2014) as environmental familiarity increases, one would expect that anxiety would be reduced, since no further 'harms' are introduced into a once novel environment. Arguably, then, the fact that Hinman et al., 2011 and Long et al., 2014 observed that repeated exposure of an environment decreased theta power much more

in the temporal portion of the hippocampal formation could be interpreted as primarily due to a reduction in anxiety over time rather than due to environmental habituation *per se*.

Previous research on the effects of anxiogenic drugs have shown no effect on hippocampal theta frequency (Yeung, Lu, Hughes, Treit, & Dickson, 2013). Specifically, Wells and colleagues found that the anxiogenic drug FG7142 had no effect on the slope nor the intercept component of the theta-frequency-to-speed relationship (Wells et al., 2013). In their study, Wells and colleagues implanted exclusively in the dorsal hippocampus. As detailed above, the intermediate hippocampus is associated with anticipatory behaviour and anticipatory anxiety, implying that the intermediate hippocampus is more so than the dorsal hippocampus would be more sensitive to anticipatory stimuli (anxiogenic). If the anticipation of an injection is accurately interpreted as an anxiogenic stimuli, then the results from Study 1B provide initial evidence that anxiogenic stimuli can significantly affect hippocampal theta frequency, localised to the intermediate hippocampus.

7.7.4 Environmental familiarisation effects on hippocampal place cells

Within the confines of the Burgess model, the main prediction as it relates to hippocampal place cells, proposes that changes in response to the theta-frequency-to-speed relationship in changes to grid scale are in response to environmental (and possibly pharmacological) manipulations (Burgess, 2008). For example, environmental novelty reduced theta frequency/reduced the slope component of the theta-frequency-to-speed relationship (Jeewajee, Lever, et al., 2008; Wells et al., 2013). Additionally, grid scale firing has shown an increase in spatial scale when a rat is in a novel environment (Fyhn, Hafting, Treves, Moser, & Moser, 2007). From this, predictions of place cell remapping can be made given the interruption of place cell inputs due to changes in grid scale (Burgess, 2008). Thus, Study 1B aims to demonstrate the stabilisation of hippocampal place cell characteristics as an environment becomes familiar. Five characteristics were taken into consideration when

assessing the effects of environmental familiarisation on hippocampal place cells; global mean rate, locational peak rate, Skaggs spatial information, field size area, and spatial stability (peak spike distance comparison). In terms of locational peak rate, global mean rate and Skaggs spatial information, the prediction would expect these characteristics to increase as the environment becomes more familiar. To reiterate, Skaggs spatial information (bits/spike) is based on a formula used to determine the average rate of change in neuron firing in response to potential manipulations and other changes. Skaggs spatial information saw a steady increase, indicating that as the animals became more familiar with the environment the localisation of firing for each place cell increased, thus stabilising. This was also reflected in global mean rate (Hz) increasing across trial. Although locational peak rate was not significant, the data responded in a trend that was expected; an increase across trial (Figure 7.25). In terms of average area and spatial stability (peak rate comparison), the prediction would expect these characteristics to be an inverse of the slope component; meaning as slope increases, area and peak rate comparison decrease. Neither characteristic was shown to be statistically significant, however, as with locational peak rate, both did decrease as the rat became more familiar with the environment (Figure 7.28 and Figure 7.29). Additionally, correlational analysis was performed to determine if there was a detectable relationship between the slope component and average area. The prediction was that as slope increased, place field area would decrease. The correlational analysis of average area of each day compared to slope average of each day found no significant relationship. The correlational analysis was underpowered with having only 15 values (3 days with 5 animals; 15 values), therefore not finding a relationship between the slope and area was unsurprising. Future examinations between slope the component and area of place field size would need a larger sample size (more days) to discern any relationship. Additional comparisons between dorsal and intermediate place field areas with average slope would be interesting as place

cells in the dorsal hippocampus are smaller than those found in the intermediate hippocampus (Burgess, 2008; Hartley et al., 2014).

Overall, the results of environmental familiarisation's effect on hippocampal place cells imply that as the environment became familiar hippocampal place cells stabilised. Future research into the effects of environmental familiarisations on hippocampal place cells would need to ensure a hefty sample size to detect significant changes in place cell characteristics.

7.7.5 Conclusion

The main results of the chapters are as follows:

1) The slope component of the theta-frequency-to-speed relationship increased over trials within day, even after the exclusion of Day 1, and this was especially present in the ***dorsal*** hippocampus.

This would suggest that the dorsal portion of the hippocampus may be more sensitive to environmental changes than the intermediate portion. This result is consistent with the Wells et al, 2013, in which their study demonstrated that after an animal repeatedly exposed to a novel environment, the slope component of the theta-frequency-to-speed relationship increase in the dorsal portion of the hippocampus.

2) The intercept component of the theta-frequency-to-speed relationship increased over trials within day, even after the exclusion of Day 1, in the ***intermediate*** hippocampus.

The interpretation of this suggests the intermediate hippocampal is more involved in the modulation of anxiety. This is a speculative interpretation that presumes that the anticipation of an injection is anxiogenic, and that this anticipation increases with experience of the daily injection after trial 4.

Taken together, these findings are consistent with a view that the dorsal hippocampus is specialised for space and memory, and the more temporal hippocampus is specialised for anxiety modulation.

8 Behavioural Effects of Pregabalin: Elevated Plus Maze and Open Field

8.1 Background: anxiolytic behavioural effect

Pregabalin's efficacy as an anxiolytic has been demonstrated in both animal models of anxiety and within clinical studies, as reviewed in Chapter 4. As discussed previously (section 6.6.4), McNaughton and colleagues. (2007), have argued that a more sensitive test of a drug's anxiolytic efficacy is by whether the drug can reduce hippocampal theta frequency. As detailed in Chapter 6, pregabalin significantly reduced the intercept component of the theta frequency-to-speed-relationship. Another important aspect in testing pregabalin's efficacy as in anxiolytic drug is in practical behavioural models of anxiety. Behavioural models used to assay anxiolytic efficacy are often categorised as conditioned responses or unconditioned responses to stimuli (Rodgers et al., 1997). Conditioned fear and anxiety animal models use fearful responses associated with a specific cue (conditioned stimuli), such as the Vogel conflict test and foot-shock. These learned associations can be unlearned or actively repressed and are a highly-relevant form of testing for certain types of anxiety disorders, such as phobias and post-traumatic stress disorder (PTSD; Steimer, 2011). Unconditioned fear and anxiety models are tasks of aversion and do not require training or the use of a learned stimuli, such as the elevated plus maze and the open field. These types of models are highly-relevant for testing anxiety states induced by unconditioned stimuli, such as generalised anxiety disorder (GAD; Carobrez & Bertoglio, 2005). Although conditioned behaviour models allow for experimenter control over behavioural baseline, these behavioural models often require the training of subjects, water or food deprivation, and the use of electric shock. In contrast, behavioural models of unconditioned response may be more prone to variable baselines, but they have a higher degree of ecological/ethological validity

with less susceptibility to confounds from learning/memory or hunger/thirst (Rodgers et al., 1997a; Steimer, 2011). Taking these points into consideration, the experiments (Experiment 2 and Experiment 3) presented in this chapter examine pregabalin's anxiolytic efficacy using unconditioned models of behaviour.

As mentioned previously (Chapter 4), there have been a wide number of studies that have examined the pharmacological action of pregabalin. These studies suggest that pregabalin's high affinity binding to $\alpha_2\delta$ predicts anxiety-like activity (Lotarski et al., 2011). Currently, there a small number of studies that have examined the efficacy of pregabalin using animal models of conditioned (Evenden et al., 2006; Field et al., 2001; Lotarski et al., 2011) and unconditioned anxiety (Baastrup et al., 2011; Field et al., 2001; Nicolas et al., 2007; Zohar et al., 2008) with the overall result of these studies showing a reduction in anxiety-like behaviours. Accordingly, this chapter asks if pregabalin reduces anxiety-like behaviours in the unconditioned behavioural models the elevated plus maze (Experiment 2) and open field (Experiment 3). As has been the practice in previous studies of examining anxiolytic efficacy, Chlordiazepoxide (CDP), a classic benzodiazepine agonist, was used as a positive control to examine pregabalin's anxiolytic efficacy (Field et al., 2001; Griebel et al., 2000). Based on previous research and the results described in Chapter 6, both pregabalin and CDP should significantly reduce anxiety-like behaviours in both the elevated plus maze and the open field.

8.2 Experiment 2: Elevated Plus Maze

8.2.1 Experimental aims

The aim of this experiment was to examine the anxiolytic effects of pregabalin using the elevated plus maze. In using the elevated plus maze (EPM) paradigm, one generally operates under two assumptions. First, as mentioned previously (section 8.1), the EPM is generally classified as in unconditioned model of behaviour, which implies that the model itself is

aversive and is useful in testing unconditioned aversion along with predicting behaviour linked to unconditioned anxiety (A P Carobrez & Bertoglio, 2005). Second, the EPM is an ethological animal model of anxiety, which implies that the elicited behaviours have an ecological meaning to the animal tested. Quantifying the ethological behaviours observed in the EPM include spatiotemporal measures (% open and closed arm entries and % open and closed arm time), risk assessment behaviours such as the frequency of stretched attenuated posture (SAP) and defaecation (faecal boli), vertical activity such as rearing frequency, and exploration behaviours (head-dip and SAP frequency; (Rodgers et al., 1997).

Experiment 2 examines the above ethological measures to assess pregabalin's anxiolytic efficacy (behavioural analysis described below, section 8.2.3). The animals were randomly allocated to one of three experimental groups; (a) vehicle control (n = 8), (b) chlordiazepoxide (CDP; n = 8), and (c) pregabalin (n = 10); methods described below.

8.2.2 Methods

8.2.2.1 Subjects

Subjects were male Lister Hooded rats (n = 26, Envigo, Wyton, UK), weighing between 315-475g. They were housed in groups of 5 under a 12h light cycle (lights off at 1900h). Food and water were available ad libitum. Thirty-one animals were originally tested in both Experiment 2 (EPM) and Experiment 3 (OF). The animals included in the statistical analysis for Experiment 2 was n = 26, and the animals included for Experiment 3 was n = 29. Further explanation for removal from analytical criteria is described below (see section 8.2.4 and section 8.3.4).

8.2.2.2 Drugs

Drugs were dissolved in 0.9% saline. Injections were given intraperitoneally in a volume of 1ml/kg of body weight. Animals received a single injection of one of the following drugs: chlordiazepoxide (CDP, 2.5 mg/kg), pregabalin (17.5 mg/kg), or saline. Injections were given

30 minutes prior to testing. Each group was given one injection only, being one of the two drugs or saline (control) injections. Drugs were obtained from Sigma-Aldrich Company Ltd., Poole, UK.

8.2.2.3 Apparatus

The elevated plus maze (EPM) used in this experiment was a wooden plus shape box that stood 50 cm above the floor with two open arms (50 x 10 cm) and two enclosed arms (50 x 10 x 50 cm), with the open and closed arms connected by a centre arm (10 x 10cm). The outside of the wooden box was painted black, whilst the inside of the closed arm and the surface of the open arms were painted grey. The apparatus was placed inside of a dry Morris Water Maze, surrounded by a curtain (Appendix A). The illumination in the open arms was bright (1160 lux) whilst the illumination in the closed arm was dim (2 lux). Figure 8.1 shows a bird's eye view of the test environment and holding area.

8.2.2.4 Experimental design

Thirty minutes prior to the start of the experiment, each animal received an injected (i.p). Subjects were counterbalanced into one of 3 groups (saline = 8, CDP = 8, pregabalin = 10). After thirty-minutes, each subject was placed in the EPM for a single trial, lasting 5 minutes. Each group experienced the apparatus for the same amount of time, following the same experimental procedure.

8.2.2.5 Experimental procedure

8.2.2.5.1 Habituation

The day prior to testing, each animal was removed from their home cage and weighed. After an hour, each animal was removed from their home cage, placed in a holding cage (fully bedded, with access to water) and taken to the procedure room. There, each animal was scruffed (approximately 3 times), placed back in the holding cage and taken to the testing room and placed on a platform. After 5 minutes, each animal was removed from the holding

cage and held for approximately 2 minutes. The animal was then placed back into the holding cage and transported to a secondary lab to be tested in an Open Field Experiment (n = 21) or were transported back to its home cage (n = 5).

8.2.2.5.2 Testing procedure

Each animal received an injection of either saline (1 kg/ml), CDP (2.5 mg/kg) or pregabalin (17.5 mg/kg) and then placed in the holding cage. Thirty minutes after injection, the DVD recorded was started and each animal was placed in the centre of the apparatus, facing the north open arm, for 5 minutes. Each experiment was observed and recorded through a video link. After the completion of the trial, the experimenter would remove the subject from the EPM, transport the subject to the holding area and the environment was cleaned.

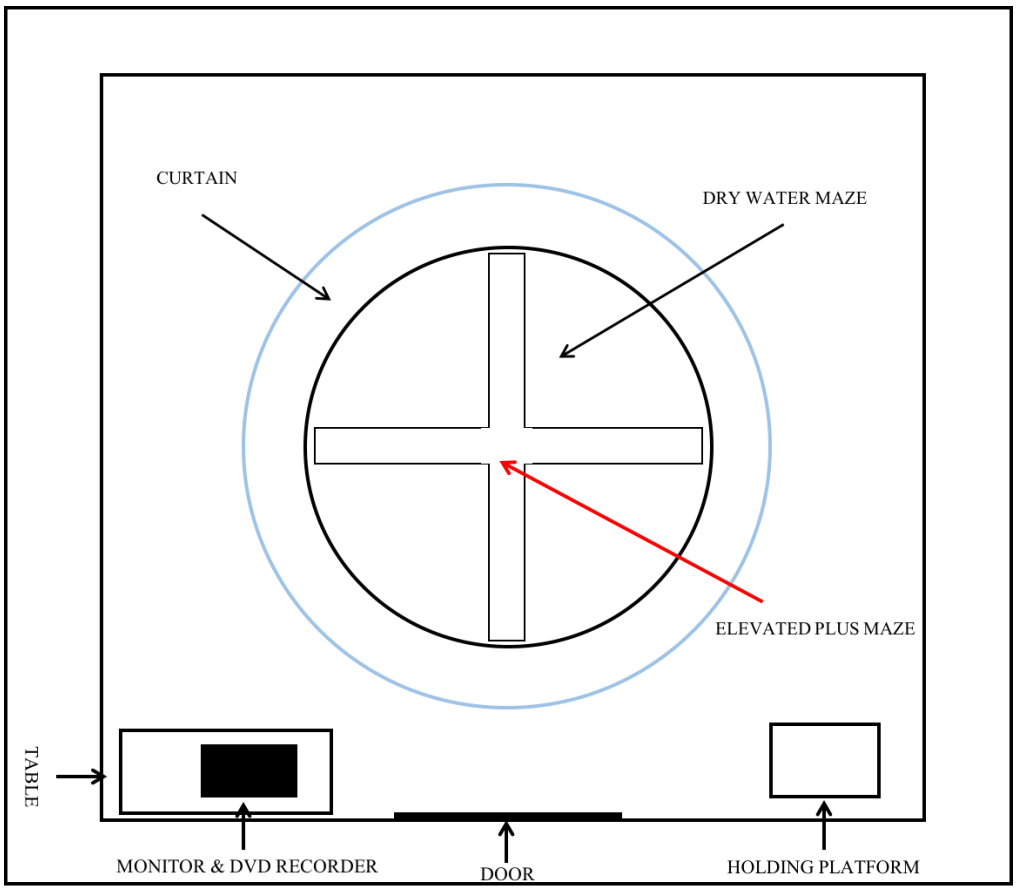


Figure 8.1 Bird's eye view of the Elevated Plus Maze laboratory setup.

8.2.3 Behavioural analysis

Spatiotemporal behaviour was scored from the DVDs using EthoVision 3.0 (Noldus, 2001), ethological analysis software which enabled the collection of duration and frequency data for centre zone, open and closed arm visits. Subjects were only considered to be in one section at a time where most of their body is located. To measure 'positive thigmotaxis' or the aversion to open spaces (Carobrez & Bertoglio, 2005; Cruz et al., 1994; Treit et al., 1993) the frequency of open and closed arm entries and the amount of time spent in the open and closed arms of the EPM was taken.

Although the primary indication of anxiety in the EPM has been the spatiotemporal measures, research has shown that the conventional measures of the EPM are highly sensitive to benzodiazepines/$GABA_A$ receptor-related drugs, with other drugs, like buspirone, having more varied results (Griebel, 1995; Rodgers et al., 1997). This has led to some researchers to question the reliability of the EPM (Dawson & Tricklebank, 1995; Moser, 1989). It has been argued, however, that the validity and the reliability of the EPM might be improved by implementing a more ethological approach to data collection (Moser, 1989; Rodgers et al., 1997). Therefore, additional to the spatiotemporal measures, risk assessment, vertical activity, and exploration behaviours were scored by the experimenter using DVD recordings of each subject's exploration in the EPM. These behaviours were defined as follows:

- Stretched attenuated posture (SAP) – defined by an exploration posture in which the animal stretches forward towards the open arm and then retracts to its position (Moraes et al., 2008). The frequency of SAP was recorded for analysis.
- Head-dipping – defined as protruding head over open arm and dipping the head down towards the floor (Griebel et al., 2000). The frequency of head-dips was recorded for analysis.
- Faecal boli – the number of faecal boli was recorded for analysis.

- Rearing – defined as the rising on hind legs (Setem et al., 1999). The frequency of rears was recorded for analysis.

8.2.4 Statistical analysis

Originally, thirty-one male Lister Hooded rats were tested across both behavioural paradigms. However, 10 animals were excluded from the EPM test for either falling off one of the open arms during testing or for being given an incorrect dose of CDP (5 mg/kg rather than 2.5 mg/kg). Twenty-one animals remained in the EPM original data set, and 10 additional animals were tested solely in the EPM paradigm. Of those additional 10 animals, 5 remained on the EPM apparatus for the duration of the experiment, and were included in the analysis, bringing the total number of animals in Experiment 2 is n = 26. To examine the amount of time spent in the open and closed arms and the frequency of entries into the open and closed arms of the EPM, the following ratio equations were used:

$$\%\ time\ in\ open\ arms = \left(\frac{time\ spent\ in\ open\ arms}{time\ spent\ in\ open\ arms\ +\ time\ spent\ in\ closed\ arms}\right) x\ 100$$

$$\%\ of\ open\ arm\ entry = \left(\frac{number\ of\ open\ arm\ entries}{number\ of\ open\ arm\ entries\ +\ number\ of\ closed\ arm\ entries}\right) x\ 100$$

Frequency of SAP, head-dips, rearing and faecal boli were recorded by the experimenter. One-way between-subjects ANOVAs were performed to analyse the spatiotemporal and ethological measures.

8.2.5 EPM Results

8.2.5.1 Spatiotemporal analysis

One-way between-subjects ANOVAs performed to examine the percent time spent in the open arms and percent number of open arm entries. There was no significant effect of percent time spent in open arm ($F_{2,23} = 0.449$, $p = 0.644$), nor was there a significant effect of percent number of open arm entries ($F_{2,23} = 0.521$, $p = 0.601$). The effect size of percent time spent in open arms was 0.190, with observed power of 0.117. The effect size of percent number of open entries was 0.204, with observed power of 0.129. The sample size used in this analysis was n = 26, with the appropriate sample size of percent time in open arms being n = 270, and the appropriate sample size of percent number of open entries being n = 237. Figures 8.2 and 8.3 both illustrate the difference in percent time and entry into the open arms of the EPM.

Although pregabalin did not significantly reduce anxiety-like behaviours in the EPM, using CDP as the positive control should have seen a significant increase in open arm exploration for that drug. As mentioned previously, research has suggested that the elevated plus maze is a more sensitive test of the anxiolytic effects of benzodiazepines (Rodgers et al., 1997), meaning one would expect to see a significant result. Thus, the non-effect of both pregabalin and CDP could suggest that the conditions of the test itself may have been highly anxiogenic rather than neither drug possessing any anxiolytic properties.

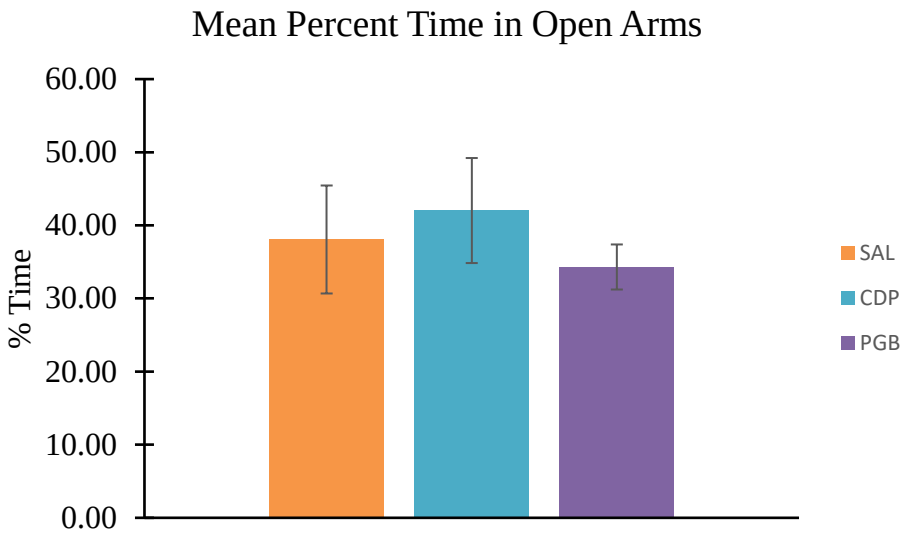


Figure 8.2 **Mean percent time in open arms.** Each bar shows the mean (±SEM) of time spent in the open arms for each group. There was no significant difference of mean time spent in open arms between groups. Abbreviations: *SAL*, saline; *CDP*, chlordiazepoxide; *PGB*, pregabalin.

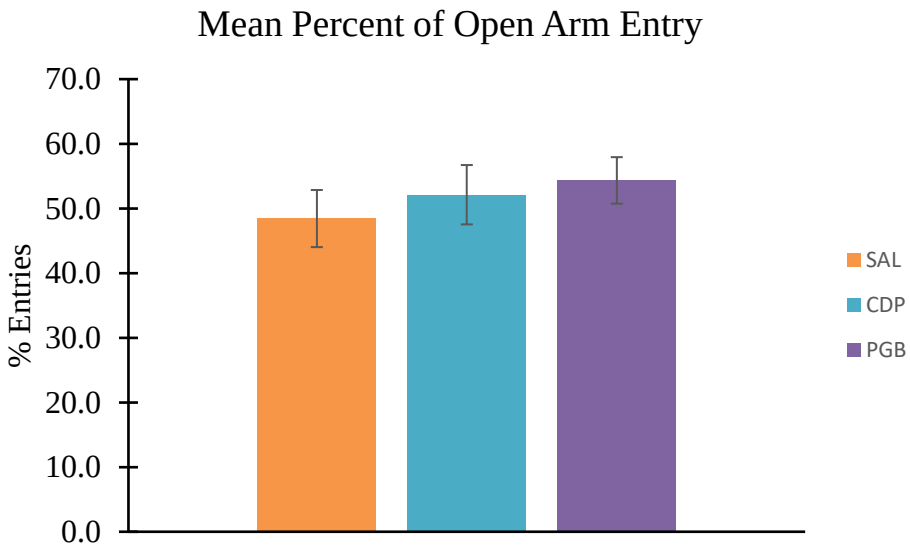


Figure 8.3 **Mean percent of open arm entry.** Each bar shows the mean (±SEM) of entries into the open arms for each group. There was no significant difference of mean open arm entries between groups. Abbreviations: *SAL*, saline*; CDP*, chlordiazepoxide*; PGB*, pregabalin.

8.2.5.2 Ethological results

Although the primary indices of EPM anxiety is based on the spatiotemporal measures of open arm avoidance, risk assessment behaviours are significant behavioural indicators, which are more closely related to fear and anxiety (Carobrez & Bertoglio, 2005). In that respect, the subsequent analysis examines risk assessment, vertical activity, and exploratory behaviour exhibited during the EPM, with Table 8.1 providing an overview of the results.

Table 8.1 Elevated plus maze, ethological results

Ethological measure	F stats	SAL – PGB (*p* value)	SAL – CDP (*p* value)	PGB – CDP (*p* value)
Head dipping	$F_{2,23} = 0.150$, $p = 0.862$	$p = 0.996$	$p = 0.871$	$p = 0.896$
SAP	$F_{2,23} = 1.899$, $p = 0.172$	$p = 0.837$	$p = 0.491$	$p = 0.155$
Rears	$F_{2,23} = 2.881$, $p = 0.076$	$p = 0.178$	$p = 0.079$	$p = 0.843$
Faecal boli	Chi-square = 6.590, $p = 0.037$, df = 2			

Abbreviations: *CDP*, chlordiazepoxide; *PGB pregabalin; SAL*, saline.

8.2.5.3.1 Head dips

One-way between-subjects ANOVA was performed to examine the amount of head-dips between each group. There was no significant main effect of the number of head-dips between the saline, CDP and pregabalin group; $F_{2,23} = 0.150$, $p = 0.862$. The effect size of head-dips in the EPM was 0.111, with observed power of 0.072. The sample size used in this analysis was n = 26. The appropriate sample size for head-dip with minimum power of 0.8 being n = 782. Figure 8.4 illustrates the difference in *mean* head-dips between each experimental group.

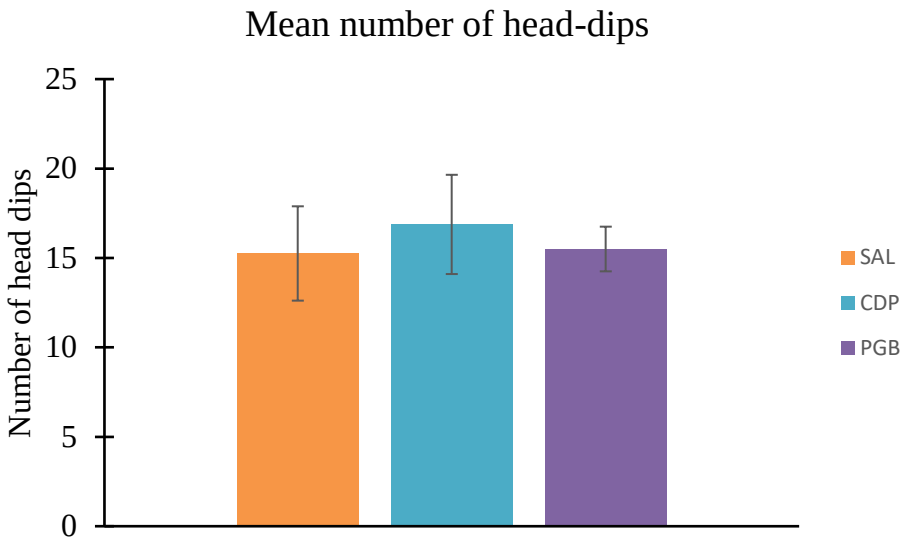


Figure 8.4 Mean number of head-dips. Each bar shows the mean (±SEM) number of head-dipping. There was no significant difference of mean head-dips between groups.

8.2.5.3.2 Stretch attenuated posture

One-way between-subjects ANOVA was performed to examine the number of stretch attenuated postures (SAP) exhibited between each group. There was no significant main effect of number of SAP between the saline, CDP and pregabalin group; $F_{2,23} = 1.899$, $p = 0.172$. The effect size of SAP was 0.369, with observed power of 0.332. The sample size used for this analysis was n = 26, with the appropriate sample size for SAP with minimum power of 0.8 being n = 75. Figure 8.5 illustrates the difference in mean SAP between each experimental group.

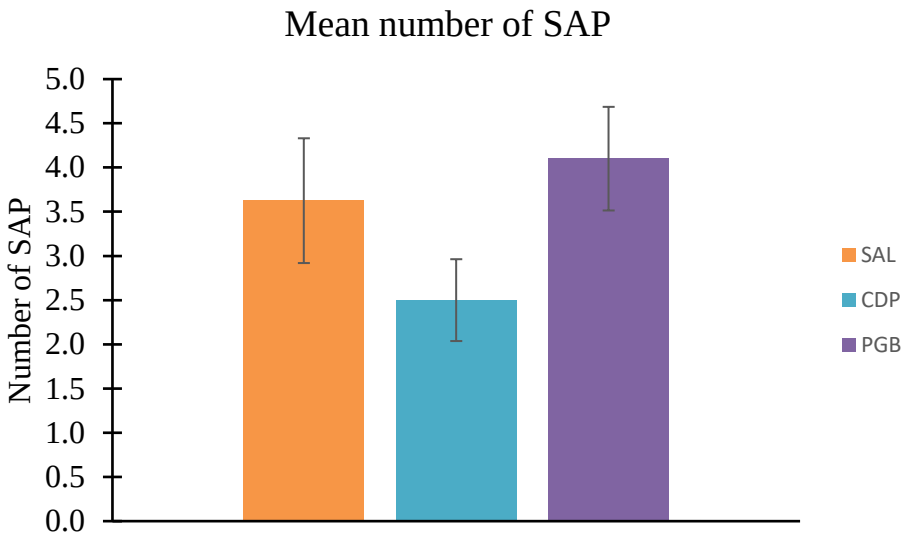


Figure 8.5 Mean number of SAP. Each bar shows the mean (±SEM) number of stretch attenuated posture (SAP) for each group. There was no significant difference of mean SAP between groups.

8.2.5.3.3 Number of rears

One-way between-subjects ANOVA was performed to examine the amount of rears between each group. There was no significant difference between the number of rears between the saline, CDP and pregabalin group; $F_{2,23} = 2.881$, $p = 0.076$. The effect size of number of rears was 0.439, with observed power of 0.451. The sample size used in this analysis was n = 26, with the appropriate sample of number of rears with minimum power of 0.8 being n = 54. Figure 8.6 illustrates the difference in mean number of rears between each experimental group.

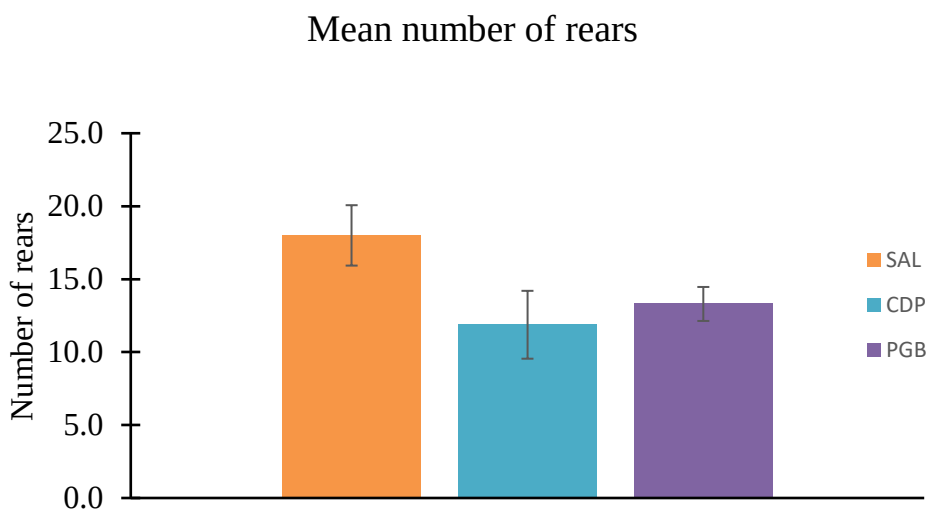


Figure 8.6 Mean number of rears (Elevated Plus Maze). Each bar shows the mean (±SEM) number of rears for each group. There was no significant difference of mean rears between groups.

8.2.5.3.4 Faecal bolli

A Kruskal-Wallis test was performed to examine the difference in the amount of faecal boli between each group. A Kruskal-Wallis test, which is based on ranks, was used instead of ANOV because of boli was strongly non-normal, and indeed there were quite a lot of zero values in the data. The Kruskal-Wallis test does not assume normality and is less sensitive to outliers than ANOVA. There was a significant difference between the number of faecal bolli between groups; Chi-square = 6.590, p = 0.037, df = 2. Animals in the saline group defecated significantly more than animals in the pregabalin group (U = 15.000, n_1 = 8, n_2 = 10, p = 0.027). The effect size of faecal boli between saline and pregabalin was 1.33, with observed power of 0.73; the effect size between CDP and saline was 0.124, with observed power of 0.06; and the effect size between pregabalin and CDP was 1.078, with observed power of 0.55. The sample size between saline and pregabalin was n = 18, with the appropriate sample size with minimum power of 0.8 being n = 22. The sample size between CDP and saline was n = 16, with the appropriate sample size being n = 2,136. The sample size between pregabalin and CDP n = 18, with the appropriate sample size being n = 32. Although a significant effect

was reported, the effect of animals in the pregabalin group having fewer faecal boli than those in the saline group was slightly underpowered with only 18 subjects. Figure 8.7 illustrates that the animals in the pregabalin group had fewer faecal boli present during the EPM experiment.

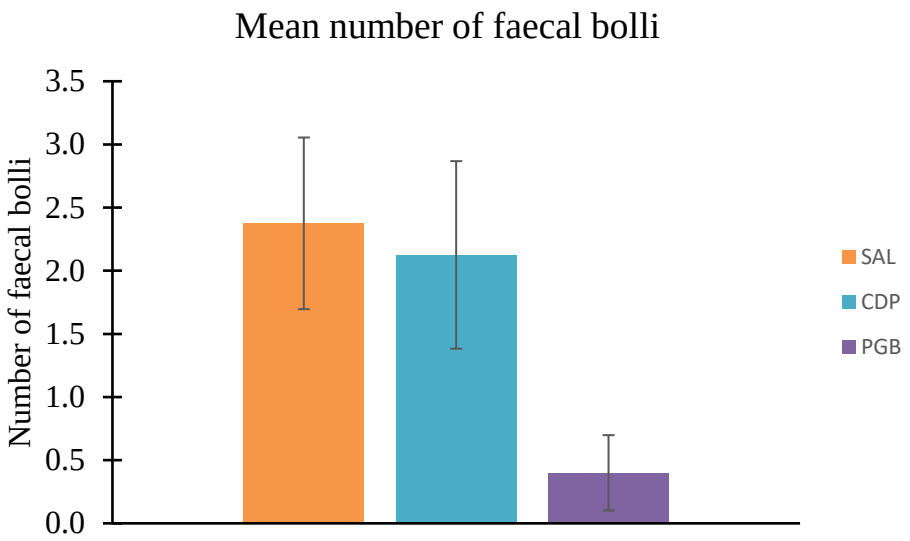


Figure 8.7 Mean number of faecal bolli (Elevated Plus Maze). Each bar shows the mean (±SEM) number of faecal boli for each group. There was a significant difference of mean faecal boli between groups with pregabalin group defecated significantly less than the saline group.

8.2.5.3.5 EPM discussion

In examining the ethological measures, pregabalin was the only drug to produce a significant result. An increase of defaecation in the EPM has been associated with anxiety behaviours in rats (Broadhurst, 1957; Pellow et al., 1985). This effect was present in both the saline and CDP groups, whilst the animals in the pregabalin group demonstrated a reduction in anxiety by less defaecation. Although this result demonstrates an anxiolytic effect of pregabalin, it should be noted that the other behavioural measures examined are more likely to demonstrate a drug's anxiolytic effect. This is especially true with stretch attenuated posture (SAP), as it is considered to be a prominent risk assessment measure due to its sensitivity to classic anxiolytics like CDP (Griebel, Rodgers, Perrault, & Sanger, 1997). The non-effect of CDP

and pregabalin on these ethological measures could, once again, suggest the conditions of the experiment may have been highly anxiogenic.

8.3 Experiment 3: Open Field

8.3.1 Experimental aims

The aim of this experiment was to examine the anxiolytic effects of pregabalin on thigmotaxic behaviour and ethological measures using a circular open field. The open field (OF) was originally used to describe 'emotionality' in rodents (Hall, 1934). Hall (1943) used a circular OF similar to the one in used in Experiment 3 (see section 8.3.2.3) to examine how animals behaved in a 1.2 m circular arena and found that 'emotional' animals compared to 'non-emotional' animals entered the centre part of the arena less. Since then, the open field has become a popular paradigm to test the effects of anxiolytics in rodents (Prut & Belzung, 2003). As with the EPM, the OF is an unconditioned fear and anxiety model with increased time spent in the centre part of the arena as the main measure of reduced anxiety in rodents. Additionally, the OF often measures the vertical activity of rearing, defensive behaviours of defaecation and the amount of time grooming as measures of anxiety (Prut & Belzung, 2003).

Experiment 3 examines the time spent in the centre, the frequency of faecal boli, rearing and time spent grooming to measure pregabalin's anxiolytic efficacy (behavioural analysis described below; section 8.3.3). The animals were randomly allocated to one of three experimental groups; (a) vehicle control (n = 10), (b) CDP (n = 9), and (c) pregabalin (n = 10); methods described below.

8.3.2 Methods

8.3.2.1 Subjects

Subjects were male Lister Hooded rats (n = 29, Envigo, Wyton, UK), weighing between 315-475g. They were housed in groups of 5 under a 12h light cycle (lights off at 1900h). Food and

water were available ad libitum. Description of subject analytical criteria is described below (section 8.3.4).

8.3.2.2 Drugs

Drugs were dissolved in 0.9% saline. Injections were given intraperitoneally in a volume of 1ml/kg of body weight. Animals received a single injection of one of the following drugs: chlordiazepoxide or CDP (2.5 mg/kg), pregabalin or pregabalin (17.5 mg/kg), or saline. Injections were given 30-40 minutes prior to testing. A group of 10 were each given one of the two drug doses and a group of 10 were control (saline injection). Drugs were obtained from Sigma-Aldrich Company Ltd., Poole, UK.

8.3.2.3 Apparatus

The open field (OF) used in this experiment was a wooden circular shape. A square board (2 x 2 m) was placed on top of hexagonal platform (20 cm in height), with the circular wooden frame placed on top forming the OF. This made the floor of the environment 121 cm in diameter, while the walls surrounding the OF 38 cm in height, and the inside of the OF was grey (Appendix B). The arena was lit by the ceiling lights within the testing room (2000 lux). Figure 8.8 shows a bird's eye view of the test environment and holding area.

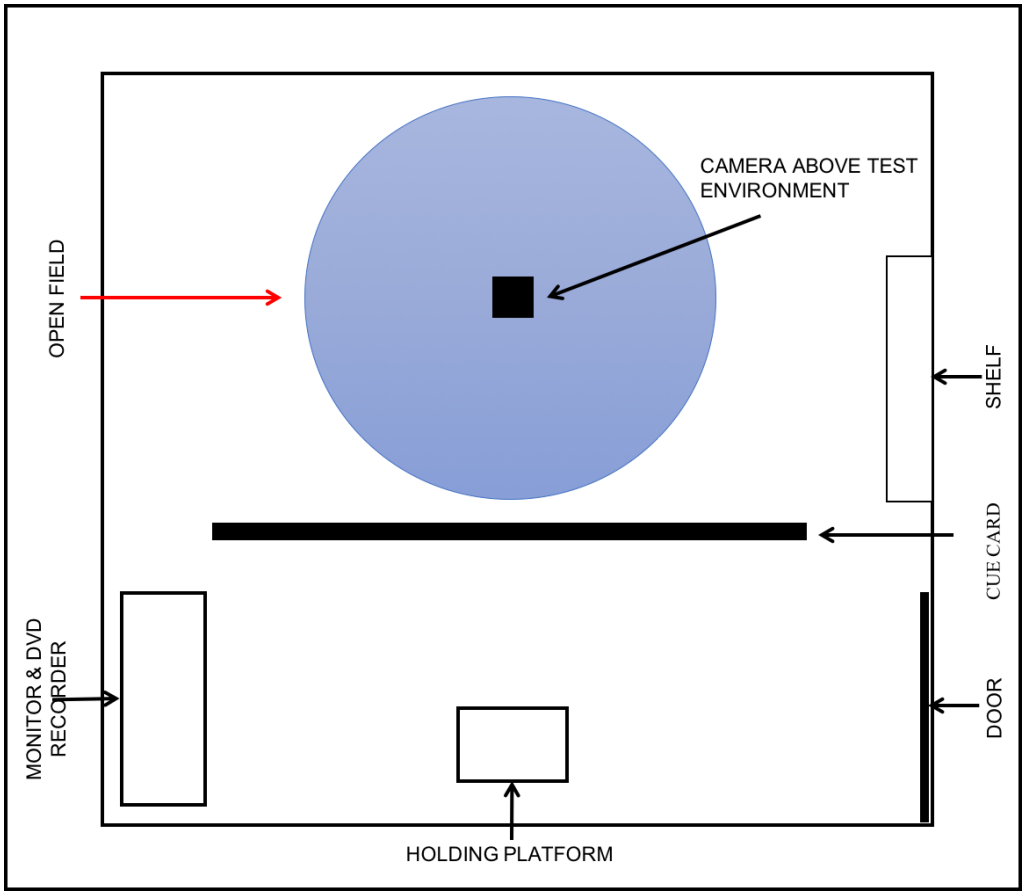


Figure 8.8 Bird's eye view of the Open Field laboratory setup.

8.3.2.4 Experimental Design

Forty-minute prior to the start of the experiment, each animal received an injection (i.p). Each subject that was tested in the OF was tested in the EPM. There was no counterbalancing between tests to ensure that each animal used in the OF paradigm had no deviation of experimental procedure/exposure. Subjects were counterbalanced into one of 3 groups (saline = 10, CDP = 9, pregabalin = 10). After forty-minutes, each subject was placed in the OF for a single trial, lasting 10 minutes. Each group experienced the apparatus for the same amount of time, following the same experimental procedure.

8.3.2.5 Experimental Procedure

8.3.2.5.1 Habituation

Prior to testing, after each animal is taken through the habituation process for the Elevated Plus Maze experiment, each animal is placed back in the holding cage and transported to another lab. There, the animal's holding cage is placed on a holding platform. After 5 minutes, each animal is held for approximately 2 minutes before being placed back into the holding cage. Each animal is then transported back to its home cage

8.3.2.5.2 Testing Procedure

After completion of the EPM experiment, each animal is transported to a secondary lab location. Approximately 40-minutes post-injection, each animal is placed in the centre of the OF, facing the north wall, for 10 minutes. Each experiment was observed and recorded through a video link. After completion of the trial, each animal was removed from the arena, placed in the holding area and the environment was cleaned.

8.3.3 Behavioural Analysis

Thigmotaxic behaviour (the tendency for a rodent to remain close to walls) was scored from the DVDs using EthoVision 3.0 (Noldus, 2001), ethological analysis software which enabled the collection of duration data for central and peripheral visits. To measure frequency of faecal boli, rearing and time spent grooming, DVDs of each animals OF experiment were used to score the data. Rearing was defined as standing on the animal's hind-legs, whilst grooming was defined as protracted washing of the animal's coat (Prut & Belzung, 2003)

8.3.4 Statistical Analysis

Originally, thirty-one male Lister Hooded rats were tested across both behavioural paradigms. However, 2 animals were excluded from the OF test for being given the incorrect CDP dose (5mg/kg rather than 2.5 mg/kg). The total number of animals analysed for the OF was 29. Additionally, each animal was recorded exploring the OF for 10 minutes, however, each animal ceased exploring the OF after approximately 4 minutes. For the purpose of

analysis, only the first 5 minutes of exploration was used to examine the anxiolytic effects of each group.

The examination of thigmotaxis using the circular OF was achieved by measuring the percentage of time each animal spent exploring the more central or inner zone of the arena. For the purpose of this experiment, the inner zone was defined by 80% of the arena's surface. Therefore, the inner zone was 96.8cm in diameter, whilst the outer zone was 24.2cm in diameter (Figure 8.10)

The percentage of time spent in the inner zone of the OF was calculated using the same ratio equation used to analyse the percent time in the EPM:

$$\%\ time\ in\ inner\ zone = \left(\frac{time\ spent\ in\ inner\ zone}{time\ spent\ in\ inner + time\ spent\ in\ outer}\right) \times 100$$

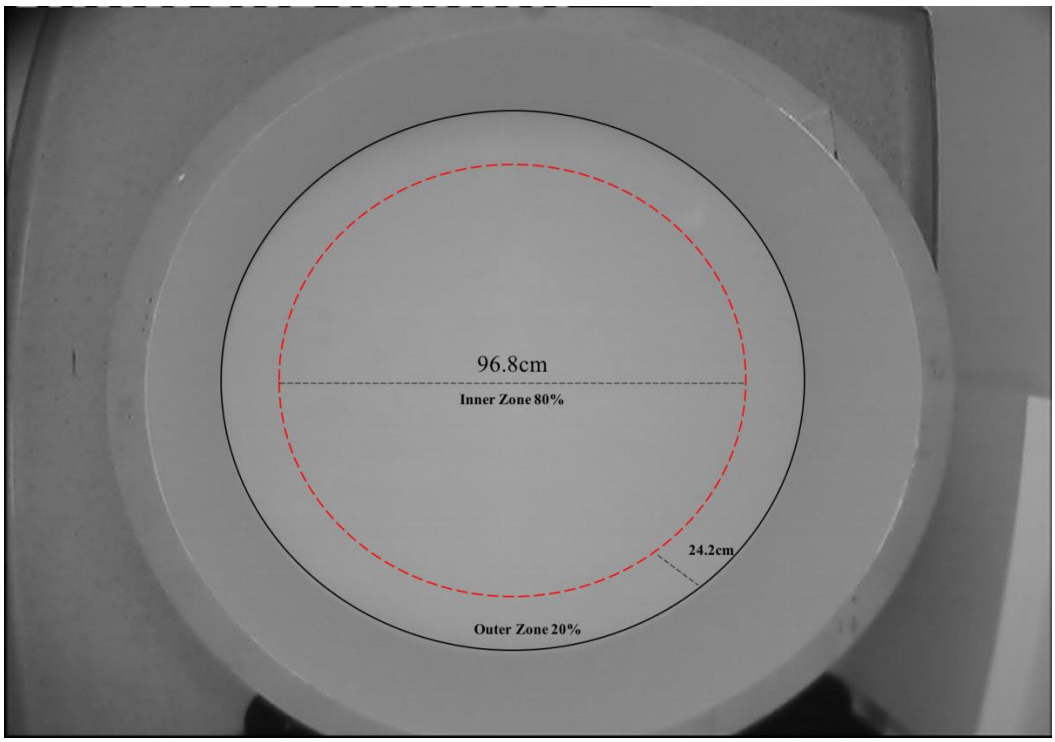


Figure 8.9 Defined inner and outer zone of the open field.

8.3.5 Open Field Results

8.3.5.1 Percent time in inner zone

A one-way between-subjects ANOVA was performed to examine the difference in the amount of time spent in the inner zone of the OF between each experimental group. There was a significant main effect of percent time in the inner zone; $F_{2,26} = 6.221$, $p = 0.006$. Animals in the CDP group explored the inner zone more than both the animals in the saline group ($p = 0.012$, 12.53 ± 3.9%) the pregabalin group ($p = 0.017$, 12.02 ± 3.9%). The effect size of mean percent time spent in the inner zone was 0.559, with observed power of 0.721. The sample size used in this analysis was n = 29, with the appropriate sample size for mean percent time spent in the inner zone with minimum power of 0.8 being n = 36. Therefore, although there was a significant effect with animals in the CDP group spending more time in the inner zone, the study is slightly underpowered. Figure 8.10 illustrates the animals in the CDP group spent significantly more time in the inner zone than animals in both the saline and pregabalin group.

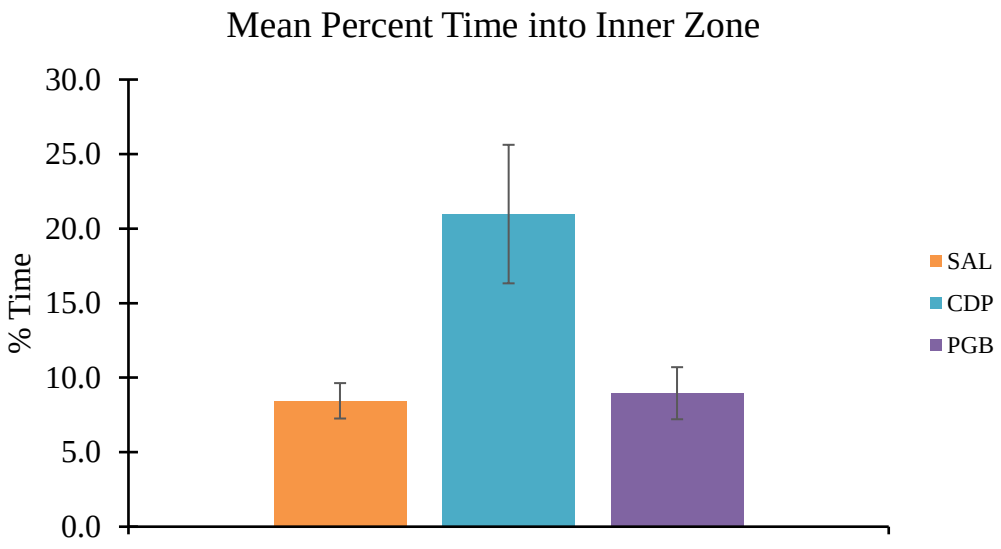


Figure 8.10 Mean percent time in Open Field Inner Zone. CDP animals spent more time exploring the inner zone than pregabalin or saline animals. Each bar represents the mean (±SEM) amount of time animals spent within the inner zone.

8.3.5.2 Percent of inner zone entry

A one-way between-subjects ANOVA was performed to examine the percent of inner zone entries between each experimental group. There was no significant main effect of percent inner zone entries between experimental groups; $F_{2,26} = 0.736$, $p = 0.489$. The effect size of mean percent entry into the inner zone was n = 0.227, with observed power of 0.192. The sample size used in this analysis was n = 29, with the appropriate sample for mean percent entry into the inner zone with minimum power of 0.8 being n = 192. This would suggest that the number of times an animal enters the inner zone is not as effective of a measurement of anxiety as the amount of time an animal spends in the inner zone. Figure 8.11 illustrates there was no difference in entry into the inner zone of the OF between groups.

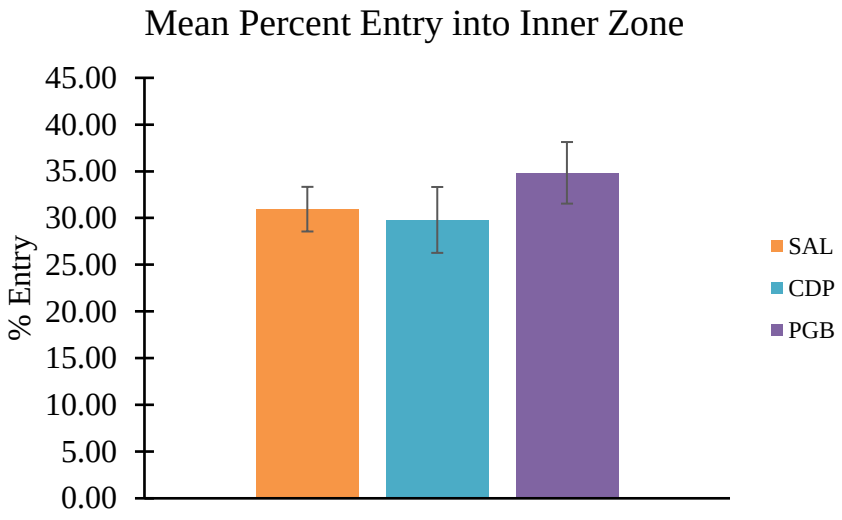


Figure 8.11 Mean percent entry into Open Field Inner Zone. There was no significant difference between experimental groups. Each bar represents the mean (±SEM) number of entries into the inner zone.

8.3.5.3 Ethological results

The ethological measures examined in the OF were the frequency of faecal boli, the frequency of rearing and time spent grooming. Table 8.2 provides an overview of the results.

Table 8.2 Open field, ethological results

Ethological measures	**Statistics**	**SAL – PGB (*p* value)**	**SAL – CDP (*p* value)**	**PGB – CDP (*p* value)**
Faecal boli	Chi-square = 2.514, *p* = 0.284, df = 2			
Rears	$F_{2,26}$ = 6.629, *p* = 0.005	*p* = 0.037	*p* = 0.875	*p* = 0.014
Grooming	$F_{2,26}$ = 14.926, p = 0.00005	*p* = 0.000047	*p* = 0.003	*p* = 0.533

Abbreviations: CDP, chlordiazepoxide; PGB, pregabalin; SAL, saline.

8.3.5.3.1 Faecal bolli

A Kruskal-Wallis test was performed, for reasons given in section 8.2.5.3.4, to examine the difference in the number of faecal bolli between each group. There was no significant difference found in the number of faecal boli between each group; Chi square = 2.514, *p* = 0.284, df = 2. The effect size of faecal boli between saline and CDP was 0.681, with observed power of 0.28; the effect size of pregabalin and saline was 0.388, with observed power of 0.12; and the effect size of CDP and pregabalin was 0.271, with observed power of 0.08. The sample size between saline and CDP was n = 19, with the appropriate sample with minimum power of 0.8 being n = 74. The sample size between pregabalin and saline was n = 20, with the appropriate sample size being n = 222. The sample size between CDP and pregabalin was n = 19, with the appropriate sample size being n = 454. In summary, in order to find an effect of anxiolytic drugs on the quantity of faecal boli in the OF, the study would need ~3.9X the number of subjects between saline and CDP and ~11.1X the number of subjects between

saline and pregabalin. Figure 8.12 illustrates the difference in the number of faecal boli between each group.

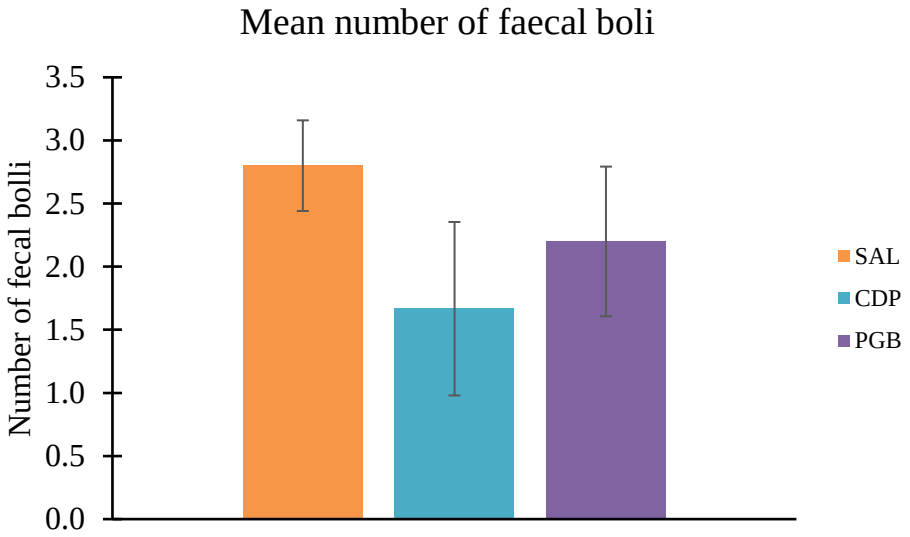


Figure 8.12 Mean number of faecal boli (Open Field). Each bar shows the mean (±SEM) number of faecal boli for each group. There was no significant difference of mean faecal boli between groups. Abbreviations: *SAL,* saline*; CDP,* chlordiazepoxide*; PGB,* pregabalin.

8.3.5.3.2 Number of rears

One-way between-subjects ANOVA was performed to examine the number of rears between each group (Figure 8.14). The main effect of group was statistically significant; $F_{2,26} = 6.629$, $p = 0.005$. Animals in the pregabalin group reared significantly less than those in both the CDP $p = 0.003$, 7.056 ± 2.11 rears) and the saline group ($p = 0.007$, 6.00 ± 2.1 rears). The effect size of mean number of rears was 0.571, with observed power of 0.74. The sample size used in this analysis was n = 29, with the appropriate sample size for mean number of rears with minimum power of 0.8 being n = 33. Thus, though the ANOVA implicated a significant effect of animals in the pregabalin group rearing less than animals in both the CDP and saline group, the study is slightly underpowered. Figure 8.13 illustrates the difference in mean number of rears between groups.

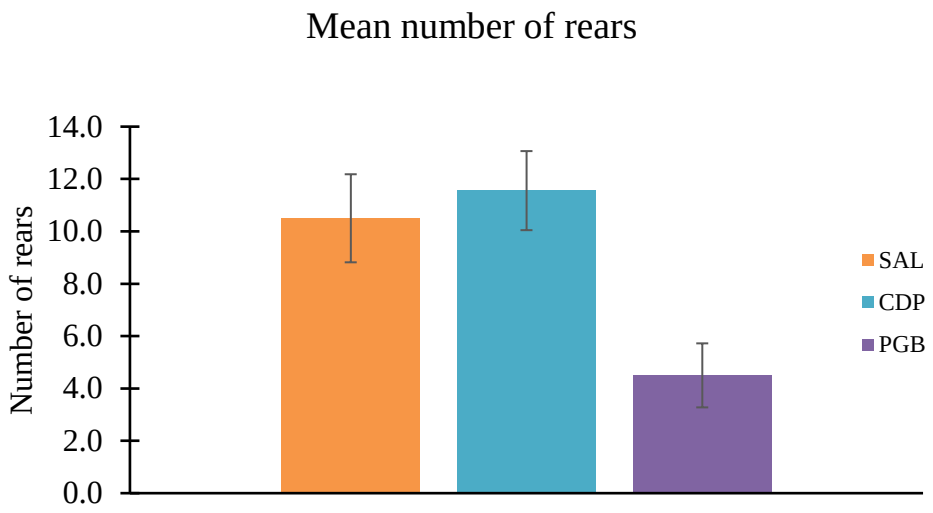


Figure 8.13 Mean number of rears (Open Field). Each bar shows the mean (±SEM) number of rears for each group. There was a significant difference of mean rears between groups, with animals in the pregabalin group rearing less than both the saline and CDP groups. Abbreviations: *SAL*, saline*; CDP*, chlordiazepoxide*; PGB*, pregabalin.

8.3.5.3.3 Grooming

One-way between-subjects ANOVA was performed to examine the time spent grooming between each group (Figure 8.15). The main effect of group was statistically significant; $F_{2,26} = 14.926$, $p = 0.000048$. Animals in the saline group spent significantly more time grooming than animals in both the pregabalin group ($p = 0.00005$, 29.78 ± 5.63 s) and CDP group ($p = 0.002$, 21.76 ± 5.78 s). The effect size of mean time spent grooming was 0.718, with observed power of 0.914. The sample size used in this analysis was n = 29, with the appropriate sample size for mean time spent grooming with minimum power of 0.8 being n = 24. This would suggest that the amount of time an animal spends grooming in the OF is a powerful ethological measure of anxiolysis in a circular OF arena.

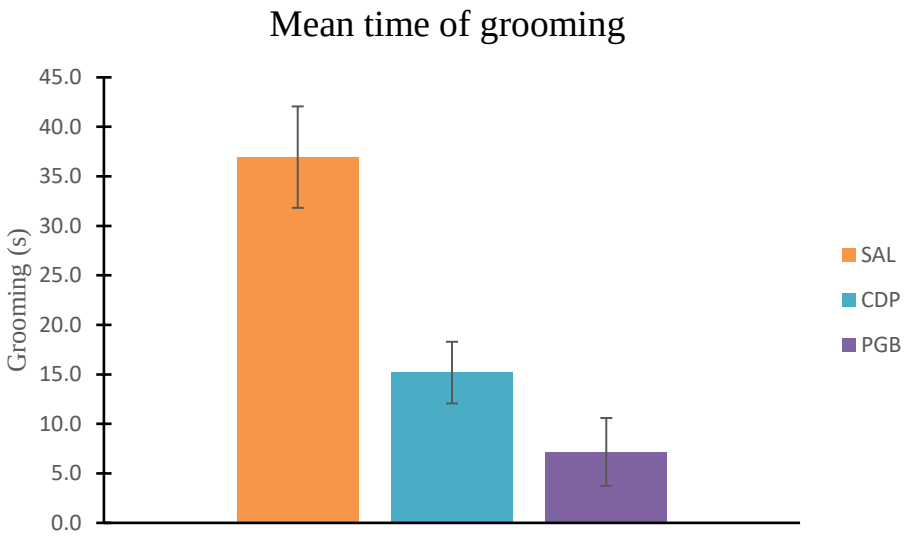


Figure 8.14 Mean time spent grooming. Each bar shows the mean time spent grooming for each group. There was a significant difference of mean time spent grooming between groups, with the animals in the saline group spending more time grooming than animals in either the pregabalin or CDP groups. Abbreviations: *SAL*, saline*; CDP*, chlordiazepoxide*; PGB*, pregabalin.

8.3.5.3.4 OF discussion

Unlike what was observed in the EPM paradigm, the OF paradigm demonstrated the anxiolytic efficacy of pregabalin. The most prominent example was in the decrease of time spent grooming. Previous experiments using the OF have shown benzodiazepines reduce grooming behaviours in the OF (Barros, Tannhauser, Tannhauser, & Tannhauser, 1994; Depaulis & Vergnes, 1986; Yerbury & Cooper, 1987). The reduction in the number of rears, on the other hand, is less clearly an index of anxiolysis. Research has shown that anxiolytics have both decreased (Gai & Grimm, 1982; Miller, Galpern, Greenblatt, Lumpkin, & Shader, 1990; Novas, Wolfman, Medina, & de Robertis, 1988) and increased (Bruhwyler, Chleide, Houbeau, & Mercier, 1991; Yerbury & Cooper, 1987) the frequency of rearing in the OF. The relationship of rearing to anxiety is discussed in detail in Lever et al. (2006). These authors note Gray and McNaughton's (2000) argument that rearing can be thought of as a risk assessment behaviour whose frequency can be described by an inverse U curve according to perceived threat intensity. Thus, they argue anxiolytics typically reduce rearing,

but that this depends upon how aversive the test setting is. For instance, in a high-stress open field setting buspirone increased rearing, while in a less aversive setting buspirone decreased rearing (see discussion in Lever et al, 2006). The frequency of faecal boli has also been shown to decrease and increase when an animal has been administrated an anxiolytic in the OF paradigm (see review in Prut & Belzung, 2003), but reduction in boli is typically considered to reflect anxiolysis. Altogether, the results of the OF test can arguably be interpreted in terms of pregabalin's anxiolytic efficacy in an animal model of behaviour8.4 Discussion

8.4.1 Summary of results

Both the EPM and OF experiments were undertaken to examine the anxiolytic effects of pregabalin on rodent behaviour. CDP was used in these experiments as a comparative measure of pregabalin's effect on anxiety reduction. The studies that have examined the effects of pregabalin on elevated plus maze have been able to show its anxiolytic effects. However, Experiment 2 (EPM), the experiment yielded a negative result for both pregabalin and CDP except the frequency of defaecation. As previously stated, this result was surprising especially when considering that the EPM is thought to be sensitive to benzodiazepines (Rodgers, 1997).

The results from the open field paradigm yielded results are what one would except. CDP reduced thigmotaxic behaviour with more time spent in the centre zone of the arena, whilst pregabalin and saline did not reduce thigmotaxic behaviour. Both pregabalin and CDP reduced grooming behaviour, whilst pregabalin solely reduced the number of rears in the OF. The thigmotaxic result was somewhat expected given that the studies examining pregabalin's efficacy in animal behaviour models may be majorly positive, but there are only a handful of examples to pull from. Be that as it may, the decrease in grooming behaviour appears to be

the most significant result as a decrease in grooming is behaviour associated with a decrease in anxiety (Prut & Belzung, 2003).

8.4.2 Experiment 2 Discussion

The results from Experiment 2 did not produce the data that was expected; with only the animals in the pregabalin group possessing the singular effect of producing fewer faecal boli than animals in both the saline and CDP groups. This small effect can be attributed to many factors. One factor to explain finding a singular effect in Experiment 2 relates to the notion that research has shown that the elevated plus maze task may be more suited for testing anxiety-like behaviour in mice more so than in rats (Rodgers & Dalvi, 1997). Another factor to consider is the decision to inject pregabalin 30 minutes before the animals were placed into the EPM. Previous research has shown the injections of classic anxiolytics (benzodiazepines and 5-HT_{1A} partial agonists) 30-minutes before being placed in the EPM has resulted in animals spending more time in the open arms than animals in a control group (Bertogilo & Carobrez, 2004; Bertoglio & Carobrez, 2003; Cruz et al., 1994; Dunn, Corbett, & Fielding, 1989; Moser, 1989). However, the studies that have examined pregabalin's anxiolytic efficacy in the EPM began the experiment at least 40 minutes post-injection (Baastrup et al., 2011; Field et al., 2001; Zohar et al., 2008); with those animals spending more time exploring the open arms than animals in the control group. One could infer that the decision to place the animals in the EPM 30-minutes rather than 40-minutes post-injection could account for the lack of results in Experiment 2, with the time to maximal plasma concentration being ~1 h in one study (Ben-Menachem, 2004).

Another factor to consider is that there is a small number of publications in which pregabalin successfully reduces anxiety-like behaviours in rats using the EPM task. A review published in 2013 revealed that between 1960 – 2012 there have been over 2,500 tests of anxiolytic drugs using the elevated plus maze (Griebel & Holmes, 2013). Of those 2,500 articles, only

three report a positive result of pregabalin reducing anxiety-like behaviour in the EPM (Baastrup et al., 2011; Field et al., 2001; Zohar et al., 2008). In those studies, the doses that were effective ranged from as low as 10 mg/kg to as high as 300 mg/kg. It is certainly plausible that the lack of research examining pregabalin's anxiolytic efficacy using the elevated plus maze may reflect a 'file drawer problem' where non-significant results are unpublished. Thus, the dose chosen for Experiment 2 was not based on the previous research examining pregabalin's anxiolytic efficacy in the EPM, but rather its effectiveness in Experiment 1, Study 1A (Chapter 6). It stands to reason that the logical prediction would be that 17.5 mg/kg would be an adequate dose to elicit a significant behavioural effect in the EPM. Having the singular effect being that the animals in the pregabalin group had fewer faecal boli than those in the saline and CDP groups does support the notion that the dose used in Experiment 2 was adequate. However, studies have shown that the EPM task is better suited for detecting benzodiazepines (Bertoglio & Carobrez, 2003; Carobrez, 2003; Carobrez, Teixeira, & Graeff, 2001; Menard & Treit, 1999; Rodgers et al., 1997) with inconsistent results when testing serotoninergic drugs and certain drugs from the neuropeptide systems (Menard & Treit, 1999). That said, the non-significant effect of CDP suggests that the EPM task was not a particularly useful task in our hands.

8.4.3 Experiment 3 Discussion

In contrast to Experiment 2, the results from Experiment 3 show both CDP and pregabalin reduce anxiety-like behaviour in the OF. In terms of the spatiotemporal analysis, animals in the CDP group explored the inner zone of the OF for a significantly longer amount of time than the animals in the saline and pregabalin groups. Using the spatiotemporal measure to assess anxiolysis is a more 'traditional' means to ascertaining the efficacy of an anxiolytic drug. CDP being the only one of the two anxiolytic drugs to affect the spatiotemporal measure speaks to the notion that like with the EPM, the OF is best suited for testing the

anxiolytic efficacy of classical benzodiazepines (Prut & Belzung, 2003). However, both CDP and pregabalin elicited a reduced grooming response; animals in both groups spent less time grooming than animals in the saline group. The reduction of this ethological measure in the OF has been previously demonstrated with benzodiazepines (Barros et al., 1994; De Souza Spinosa, Gerenutti, & Bernardi, 2000; Depaulis & Vergnes, 1986; Yerbury & Cooper, 1987), making chlordiazepoxide's reduction in the amount of time spent grooming unsurprising. Pregabalin eliciting a similar reduction in the OF could thus suggest pregabalin's anxiolytic efficacy. However, attributing a decrease in grooming as demonstrable anxiolytic efficacy is somewhat tenuous. For one, grooming behaviour in rats can be attributed to factors apart from anxiolysis. One factor is in response to temperature; for instance, when a rat runs around the OF, its body temperature rises leading the rat to spread saliva on the surface of its body (grooming), effectively 'sweating' to reduce its body temperature (Hainsworth & Stricker, 1971; Poole & Stephenson, 1977; Stricker & Hainsworth, 1971; Yanase, Kanosue, Yasuda, & Tanaka, 1991). It is possible that the decrease in grooming behaviour is a response to body temperatures being lowered from an anxiolytic, mitigating the need to 'sweat,' and resulting in a decrease in grooming. In early studies, chlordiazepoxide was shown to reduce body temperatures (Locker & Koffer, 1962; Schmidt, Kappey, & Albers, 1961). However, there are studies that demonstrate lower doses of CDP do not decrease body temperature (Froger-Colléaux et al., 2011; Mead, Li, & Kapur, 2008). Mead and colleagues found rats given 10 mg/kg of CDP over the course of 7 days did not experience a significant change in body temperature (Mead et al., 2008). It is possible that even though CDP has been shown to decrease body temperature, the results from Experiment 3 likely demonstrate the anxiolytic effects of CDP because the dose used was not high enough to elicit a temperature response. Clinical research has shown that the use of gabapentinoids in perioperative pain management has no effect on body temperature (Helenius, Puhakka, Manner, Pajulo, & Helenius, 2018),

whilst an animal study shows that pregabalin results in dose-dependent (200 mg/kg i.v.) hypothermia (Sharma et al., 2011). As with CDP, a reduction in body temperature to account for pregabalin's effect on decrease grooming would suggest that a higher dose need than that used Experiment 3. Thus, interpretation of the results lends to the conclusion that CDP and pregabalin's decrease in time spent grooming is a result of their anxiolytic properties, specifically.

In addition to a decrease in time spent grooming, animals in the pregabalin group saw a decrease in the number of rears in the open field. The behaviour of rearing is linked not only in response to fear/anxiety, but also to information-gathering, learning and memory, and environmental novelty (Lever et al., 2006). Lever and colleagues suggest that in the open field, rearing behaviour in rodents implicates a motivation for information-gathering by using their hind-legs to gain a 'vantage point' to survey the area; appearing to use distal more so than proximal cues. The height from rearing provides advantages to certain sensory systems such as spatial mapping and defensive behaviours (Dielenberg & McGregor, 2001; Lever et al., 2006; Sheppe, 1966). The height from rearing also promotes learning about a spatial environment by providing information from distal cues and additional information from environmental boundaries (Lever et al., 2006). When placed in novel environment, there is an increase rearing. This behaviour seems to be related to hippocampal functions. For example, in a mismatch task, rearing levels are low in a familiar environment, then increase in a novel environment which is accompanied with place cell remapping in the CA1 (Lever et al., 2006). In terms of anxiety and fear modulation, Gray & McNaughton (2003) propose that the level of rearing (increased vs decreased) can be described as an inverse U curve, where rearing increases during low levels of fear and high levels of anxiety, and decreases during high levels of fear and low levels of anxiety. They note that anxiolytic drugs generally reduce rearing.

The reduction in rearing could be due to cognitive as well as emotional changes, however. The hippocampal formation also appears to have strong implications in rearing behaviour in response to anxiety. Lesions to the medial septum/diagonal band of Broca (MS/DBB) elicits a reduction in rearing (Bengelloun, Finklestein, Burright, & Donovick, 1977; Bengelloun, Nelson, Zent, & Beatty, 1976; Poplawsky & Isaacson, 1983; Ricceri, Calamandrei, & Berger-Sweeney, 1997). Rearing also appears to be involve in both types of hippocampal theta, with observations of theta amplitude and rearing being heavily correlated (Cerbone & Sadile, 1994) and rearing being described a 'classic behaviour' that elicits hippocampal theta responses (Robinson & Vanderwolf, 1978);implicating the septo-hippocampal system in modulating rearing behaviour. Modifying hippocampal physiology could thus affect hippocampally-mediated cognition as well as hippocampally-mediated emotionality. Overall, it is conceivable that pregabalin's reduction of rearing behaviour is linked to its anxiolytic efficacy but studies examining pregabalin's anxiolytic effects in the open field would need to be undertaken to support the validity of this finding.

8.4.4 Conclusion

The results from Experiment 2 and Experiment 3 provide partial support of pregabalin's anxiolytic efficacy in animal models of anxiolysis, with Experiment 3 being more indicative. Although both experiments were partially successful in demonstrating pregabalin's efficacy, the data supports the notion that the theta-frequency reduction assay may be more sensitive than both the elevated plus maze and open field in detecting anxiolytic efficacy. Apart from the spatiotemporal analysis in the open field, the data obtain from these two experiments can be attributed to different behavioural responses separate from anxiety modulation. To this point, McNaughton and colleagues have argued, "The extent to which the reticular-elicitation tests lacks false positives and false negatives is in strong contrast to the bulk of current animal models used in anxiolytic detection. It seems likely that it involves a critical neural

pathway for the action of the drugs, (McNaughton et al., 2007, p. 334).” McNaughton and colleagues also stated “overall, then, we believe the data we have reviewed suggest that reticular-elicited theta is an excellent screen for anxiolytic action – capable of detecting drugs with novel modes of action…” (McNaughton et al., 2007, p. 343). Taken together, the results of Study 1A, and Experiment 2 and Experiment 3 are consistent with a revised version of McNaughton and colleagues’ claim that anxiolytic efficacy is more aptly tested with theta-frequency reduction, here the theta frequency intercept reduction using the theta-frequency-to-speed assay, than by traditional animal tests of anxiety-like behaviour.

9 General Discussion

9.1 Overview of research question

The Burgess (2008) model presents a two component model of theta frequency. The model suggests that the slope and intercept of the broadly linear relationship between theta frequency and running speed can be thought of as reflecting the two components of theta, Type 1 and Type 2 respectively. These two components are represented by the intercept of the theta-frequency-to speed relationship at 0 cm/s and the slope of the theta-frequency-to speed relationship. Burgess' (2008) model suggested that slope reflects Type 1 or transitional movement theta (O'Keefe, 2007) and that the intercept reflects Type 2 or arousal-related theta (Kramis et al., 1975). The main contribution of the present thesis has been to advance our knowledge of the different functional associations of two frequency components of hippocampal theta; broadly speaking, as discussed further below, anxiolytic drug effects act on the intercept component, environmental familiarity acts upon the slope component.

9.2 Study 1A

The aim of Study 1A presented in this thesis was performed to examine the effect of pregabalin on hippocampal theta in freely moving rats. As discussed in Chapter 3, there is an abundance of research that implies that there is a relationship between hippocampal theta and anxiety. All drugs that have been clinically effective in treating anxiety in humans (barbiturates, benzodiazepines, SSRIs and 5-HT_{1A} receptor agonists), and have been tested on the theta frequency reduction assay, reduce the frequency of reticular-elicited theta (McNaughton et al., 2007), despite their neurochemical dissimilarity. Pregabalin, the sole drug used in Study 1A, has also been shown to reduce theta frequency of elicited hippocampal theta (Siok et al., 2009). Siok and colleagues implanted rats in the dorsal hippocampus (CA1) for recording and the nucleus pontis oralis (nPO) for stimulation; intensities ranging from 0.06-0.16 mA. The rats were either injected with diazepam (0.32

mg/kg, 1.0 mg/kg) or they were injected with pregabalin (10mg/kg, 17.8mg/kg, 32 mg/kg). Comparison of theta frequency was averaged between stimulation intensities over a 30-minute pre-drug period and the 30-90-minute post-drug and vehicle administration period. They found that diazepam significantly reduced theta frequency at both doses, whilst there was a dose-dependent decrease of theta intercept with pregabalin (32 mg/kg).

The study presented by Siok et al (2009) could be considered as a key factor for the present thesis. However, it was not obvious that pregabalin would have reduced theta frequency at all. Assuming that pregabalin were to reduce theta frequency, it was not obvious how theta frequency reductions in the anaesthetised-based-intensity-to-theta frequency model mapped onto theta frequency reduction in the freely moving rat. Furthermore, there is no particular consistency about the effects of anxiolytic drugs within the anaesthetised-based intensity-to-theta-frequency model. For instance, CDP has been shown to solely reduce slope and Buspirone to solely reduce intercept of the intensity-to-theta-frequency function (Coop & McNaughton, 1991; McNaughton, Richardson, & Gore, 1986). These two findings taken together would suggest that anxiolytic drugs do not have a homogeneous effect on theta frequency, which is opposed to the hypothesis presented within this thesis. Yet, as stated previously, the Wells et al (2013) findings showed that both CDP and buspirone had the homogeneous effect of reducing the intercept of the theta-frequency-to-speed function in freely moving rats. The main difference between the findings reported by McNaughton and colleagues (1986) and Coop & McNaughton (1991), with the findings reported by Wells and colleagues' is the use of reticular stimulation. Much of what has been established regarding the effects of anxiolytic drugs and hippocampal theta has been learned through *in vivo* studies involving reticular stimulation (Neil McNaughton et al., 2007). Whether the animal was anaesthetised or freely-moving during reticular stimulation, the outcome of the reduction of theta frequency was observed (John, Kiss, Lever, & Erdi, 2014, Figure 9.1). This reintroduces

the problem of the heterogenous effect of theta frequency reduction (CDP reducing slope, buspirone reducing intercept). This problem was seemingly addressed within the Wells study.

The use of the Burgess (2008) model, which dissociates the change of theta frequency using a linear model, within the Wells study was able to produce a homogeneous effect of anxiolytic drugs; anxiolytics reduced the intercept of the theta-frequency-to-speed relationship in freely-moving rats. From this, one could assume that the model used within the Wells study could be applied to the testing of pregabalin. The Wells study was able to build upon such works as McNaughton and colleagues (1986), and Coop & McNaughton (1991) with the crucial difference of sampling theta from the locomotion of freely-moving rats. Thus, building upon the Siok and colleagues' study and utilising the paradigm outlined in the Wells study, Study 1A was able to demonstrate that pregabalin reduced the intercept of the theta-frequency-to-speed-relationship whilst having no effect of slope in freely-moving rats. Different from the Siok study, there wasn't a strong dose-dependent difference in the reduction of intercept of the theta-frequency-to-speed relationship. Considering the results of the Siok study, in which the lower dosages of pregabalin (10mg/kg and 17.8mg/kg) did not significantly reduce theta frequency, one would assume that the lower dose of pregabalin used in my Study 1A (17.5mg/kg) would not produce a significant reduction in theta frequency. However, the results from my Study 1A demonstrated that the lower dose used (17.5 mg/kg) effectively reduced the intercept of the theta-frequency-to-speed relationship, whilst the higher dose approached significance. The higher-dose experiment used only 3 rats: it is very likely this result would have reached statistical significance with more rats.

The results from Study 1A also point to a distinct difference between the dorsal and intermediate hippocampus in anxiety modulation. As stated previously, both the Wells and colleagues (2013) and Siok and colleagues' (2009) studies found anxiolytic drugs reduced hippocampal theta recorded from the dorsal hippocampus. In Study 1A, the results pregabalin

affected intermediate hippocampal theta more than dorsal hippocampal theta. It's important to note that hippocampal theta can be found throughout the dorso-ventral axis and has been described as a traveling wave. Meaning, other than amplitude, theta frequency remains consistent throughout the dorso-ventral axis (Patel et al., 2012). The results hint that the traveling wave idea may be a very useful approximation to hippocampal theta, but is not a literal description of hippocampal theta, because the present results do suggest some frequency differences in the dorsal vs intermediate hippocampus. It has been suggested that the dorsal hippocampus specifically modulates spatial cognition and the ventral hippocampus specifically modulates emotional processing. Previous research has indicated that the intermediate hippocampus does play a role in both spatial processing (Bast et al., 2009) and anxiety modulation (Burton et al., 2009). The intermediate hippocampus seems to be required in the translation of accurate spatial representation into behavioural actions, (Bast, 2007; Bast et al., 2009; Fanselow & Dong, 2010; Vann & Albasser, 2011). The function of the intermediate hippocampus considered alone is not clear. Certainly, bilateral lesions to both the intermediate and ventral hippocampus *together* largely abolished anticipatory activity (Burton et al., 2009). The results in Study 1A, suggest that the intermediate hippocampus, independent of the ventral hippocampus, could potentially play a role in anxiety modulation in addition to its role in spatial cognitive processing.

In summary, the results from Study 1A suggests that not only is the theta-frequency reduction assay a more sensitive test of anxiolysis (McNaughton et al., 2007), but that the paradigm initially established by Wells and colleagues is a sensitive method in testing anxiolytic effects of different types of drugs. It remains to be seen if the freely-moving theta approach is more sensitive than the reticular-formation assay. At present, four different drugs (CDP, Buspirone, putative anxiolytic 0-2545 CB1 and pregabalin) have been shown to reduce the intercept of the theta frequency-to-speed-relationship. This is significant because each drug that has

shown this effect is acting upon different primary targets (Kavoussi, 2006; Martin et al., 2006; Neil McNaughton et al., 2007; Wells et al., 2013). This could suggest that there is a common mechanism of anxiolytic drugs that involves the hippocampal formation.

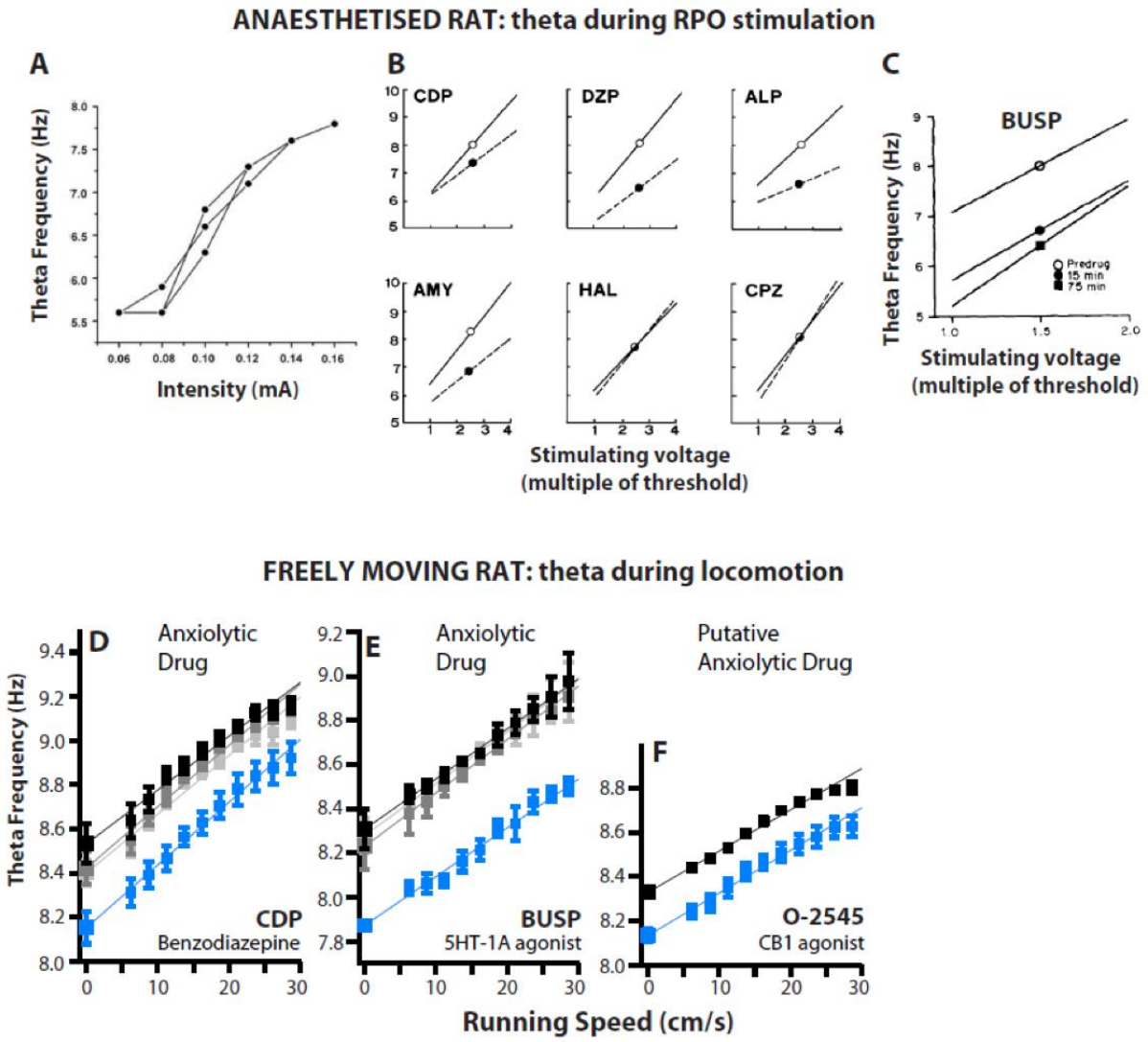


Figure 9.1 Anxiolytic drugs reduced frequency in reticular-stimulated rats. A) Broadly linear relationship between reticular formation stimulation intensity and theta frequency of anaesthetised rats. **B)** Anxiolytic drugs reduce theta frequency in reticular-elicited theta. Open circles indicate baseline, closed circles indicate drugs. Notably, in this example, CDP reduces the slope in the intensity-to-theta-frequency function. **C)** Buspirone reduces theta frequency in the intensity-to-theta-frequency function. **D-F)** Three neurochemically dissimilar drugs reduce intercept of the theta-frequency-to-speed relationship (D – CDP, benzodiazepine agonist; E – Buspirone – 5-HT_{1A} agonist; F – 0-2545, putative anxiolytic, CB1 agonist). Adapted from (John et al., 2014).

9.3 Study 1B

Although Experiment 1 was primarily designed with the purpose of testing the anxiolytic efficacy of pregabalin (Study 1A), the paradigm used for Experiment 1 provided the opportunity to perform Study 1B, which involved the examination of the effects of environmental familiarity upon the slope component of the theta-frequency-to-speed relationship. The results from Study 1B provided further evidence that the slope component of the theta-frequency-to-speed relationship is affected by environmental familiarity. The paradigm used in Study 1B was based on a study in the Wells et al., (2013) experiment. In that study, Wells and colleagues, recording from the dorsal hippocampus, examined if the slope reduction observed when a rat was exposed to a novel environment after repeated exposure to a separate familiar environment was intrinsically based on the novelty of the environment and not based on the differences between the two environments themselves. They examined the slope component of the familiar environment from the time the rat was first introduced, until it became familiar. They found that the slope component increased as the novel environment became familiar. Study 1B examined the effect of environmental familiarisation across pre-injection trials for days 1 – 3 and for days 1 – 5. Each animal was introduced to the environment (see Chapter 5 procedural details) across four trials before receiving an injection and a being placed back in the environment for a fifth trial. The results of Study 1B not only examined the effect of environmental familiarisation upon the slope component, it also examined the differences between the slope and intercept components of dorsal and intermediate hippocampal theta frequency. As predicted, repeated exposure to a novel environment increased the slope of the theta-frequency-to-speed relationship as the environment became familiar. Dorsal slope increased across trial within each day (days 1 – 3 and days 1 – 5). Intermediate slope did not produce a significant increase when examined across days 1 – 3 but did when examined across days 1 – 5. The slope result implies that dorsal slope is more sensitive at detecting environmental familiarisation than intermediate

slope despite both dorsal and intermediate slope increasing across trials, whether significantly or not.

Additional research into the function of slope as it relates to environmental novelty and familiarity have produced similar results. Newman and colleagues (Newman et al, 2013) examined the relationship between theta frequency and running speed. Noting that the rate of theta frequency increases as running speed increases has been shown to depend on environmental familiarity (Wells et al., 2013), Newman and colleagues' found not as definitive increase in the slope component of the theta-frequency-to-speed relationship compared to baseline trials. Although Newman et al, (2013) found that slope increased over repeated exposure of the testing environment, the methods in which this accomplished were not performed as formally as it was in this thesis. That is to say, Newman and colleagues had varied degrees of when the animals were re-exposed to the environment (3-6 hr recovery period). In Experiment 1, each animal was exposed to the environment at the same time, over the same ITI (30 minutes) for five consecutive days. Nevertheless, the Newman et al (2013) were able to demonstrate an increase in slope of the theta-frequency-to-speed in relation to environmental familiarity. The present thesis results confirms and extends the findings of Wells et al. (2013) and Newman et al. (2013). This was accomplished by a having a consistent methodological protocol across animals and repeating the exposure of the environment over consecutive days for each animal. This was demonstrated with the trial 1 slope being lower after cumulative experience (Chapter 7, Figures 7.1). This effect would appear to be a reflection on recency (thirty-minute inter-trial interval vs ~21-hr interval between days). The robustness of this effect was demonstrated when dorsal slope was reanalysed excluding Day 1 slope values for days 1 – 3 and days 1 – 5.

9.3.1 Study 1B intercept component

A key aspect of both Study 1A and Study 1B is that the prediction formulated from the Burgess (2008) model dictates that each component is affected separately; i.e., intercept is reduced by anxiolytics, but not slope; and slope is increased by environmental familiarisation, not intercept. In Study 1B, however, intermediate intercept had a significant response to environmental novelty: intercept increased across trial within day and within days 1 – 5. This was speculatively interpreted as a possible anxiogenic anticipation response of the injection following the fourth trial of each day. Evidence to support this comes from Day 1 data. The fourth trial on the first day does not increase relative to other trials, but if anything, decreases. Of course, on Day 1 as opposed to any other experimental day, the rat has not experienced any i.p. injection. On Day 2, the fourth trial value is similar to the third trial value, and on Day 3, trial 4 value increases, with Day 4 trial 4 valued increasing higher than the previous day's trial 4 value, and Day 5 trial 4 value increasing higher than the previous day's trial 4 value. Along with the intermediate hippocampus' role in anticipatory behaviour (Burton et al., 2009), and anticipatory anxiety (Pentkowski et al., 2006), the effect of intermediate intercept increase in Study 1B is potentially a novel effect finding a positive modulation of theta intercept by anxiogenic stimuli. This is especially apparent with previous research showing that anxiogenic drugs do not significant increase hippocampal theta frequency (Wells et al., 2013; Yeung et al., 2013). It's important to note that in both the Wells and colleagues, and Yeung and colleagues' studies, hippocampal theta frequency was recorded in the dorsal hippocampus, further implicating that the intermediate hippocampus is involved in the modulation of anxiogenic stimuli.

9.4 Hippocampal place cells

9.4.1 Anxiolytic negative effect on hippocampal place cells

Study 1A demonstrated that hippocampal place cells did not change as a result of pregabalin. These negative results are important as, assuming that place cells are linked to cognition, they

further support the notion that modulation of spatial cognition is to the slope component, and anxiety-modulation is tied to the intercept component.

9.4.2 Testing environmental familiarisation's effect on theta slope and hippocampal place cells

The results of Study 1B demonstrated that environmental familiarisation plays a role in certain hippocampal place cell characteristics. In terms of the Burgess (2008) model, the prediction, as it relates to hippocampal place cells, proposes that changes in response to the theta-frequency-to-speed relationship's change in grid scale are in response to environmental manipulation. In other words, a rat being placed in a novel environment and becoming familiar with it over time, (environmental manipulation) impacts the theta-frequency-to-speed relationship and thus grid scale. The impact in grid scale translates to place cells' change and stabilisation due to the disruption of place cell inputs from the changes in grid scale. Study 1B investigated the possibility that theta slope could be linked to experience-dependent place cell changes. This did presuppose that theta slope would be flatter when the environment was novel and less familiar, and that appreciable place cell changes could be measured as the environment became increasingly familiar. The assumption of the Burgess model (2008) that environmental familiarisation increases slope was borne out in so much as slope increased over trials within a day, and this was not simply because of effects of Day 1, when the environment was completely novel. As it turned out, place cell changes with experience were relatively subtle. Five characteristics were taken into consideration when examining the effects of environmental familiarisation on hippocampal place cells. Skaggs spatial information and global mean rate were found to increase across trial. Locational peak rate, although not significantly, also increased across trial. The average area of place cells did not significantly change, however the size of the average field decreased across day. Peak rate comparison/distance between cells also did not significantly change, however the distance between average place cells decreased across trial. The lack of appreciable effects of place

cell change in Study 1B could perhaps be attributed to sample size. The number of cells recorded was n = 95. Effect size calculations performed post hoc (see Chapter 7) support the need for a larger number of place cell recordings to see an effect of environmental familiarisation. The aim of Study 1B was to examine the effects of environmental manipulations on hippocampal theta and hippocampal place cells over time (familiarisation). Future research would need to ensure a substantial sample size, with many cells sampled per rat, to more accurately examine whether effects of environmental familiarisation and novelty upon hippocampal theta and spatial signalling can be explained by the Burgess (2008) model as it pertains to the slope component and grid scale.

In summary, environmental familiarisation increased slope across the trials within a day, but this was not systematically related to place cell characteristics.

9.5 Experiment 2 and Experiment 3

Although the results presented in this thesis show that the novel anxiolytic pregabalin had no effect of hippocampal place cells, research into the effect of anxiolytics on the hippocampus and spatial learning has produced varying results. For instance, Pan & McNaughton, (1997) observed that CDP affected spatial learning in a water maze task whilst reducing theta frequency. Benzodiazepines (e.g., CDP) are GABAergic anxiolytics and research into GABA receptor's influence on spatial navigation has shown that it may play a crucial role (Deng et al., 2009). Buspirone, which acts on serotonin, has also been shown to impair spatial navigation (McNaughton & Morris, 1992; Rowan, Cullen, & Moulton, 1990). Both of these findings suggest that there should be a common behavioural correlate that demonstrates anxiolysis. However, classic animal models of anxiety, such as elevated plus maze, have been inconsistent when testing non-GABAergic anxiolytics (Rodgers, 1997).

Experiment 2 involved testing pregabalin's anxiolytic efficacy, with CDP as a positive control, in the elevated plus maze. In this experiment, the animals were injected with either

pregabalin, CDP, or saline 30 minutes prior to entering the arena. Surprisingly, neither anxiolytic drug resulted increased time spent in the open arm. Pregabalin, however, did elicit a reduction in anxiety-like behaviour in so much as it resulted in fewer faecal boli than CDP and saline.

One potential explanation for the minimal decrease in anxiety-like behaviour in the EPM is related to the test itself. Animal models of anxiety, like the elevated plus maze task, have produced inconsistent results for non-GABAergic modulators (Rodgers, 1997). Buspirone is an example of a false-negative result in animal models of anxiety. Buspirone anxiolytic effects were not recognised until it was used in clinical testing as an antipsychotic (Goa & Ward, 1986). Potential explanations of this notion is that the false positives and negatives of animal models of anxiety could be related to the fact that those models (EPM, open field, light-dark box) work best with mice more than rats (Rodgers & Dalvi, 1997) and are mostly sensitive to benzodiazepines . And yet, CDP did not increase open arm behaviours in the EPM experiment in the current thesis. Other reasons for the lack of anxiety-like behaviour reduction in Experiment 2 could relate to placing of the elevated plus maze inside of a dry Morris Water Maze. The decision to place an elevated plus inside of a Morris Water Maze was purely a matter of space and procedural constraints in ensuring that the animals did not see either testing apparatus. In order to reach the EPM, the experimenter needed to climb a step ladder, remove a curtain encasing the apparatus and fully step into the Morris Water Maze (dry, of course) to place the animal in the EPM. This was somewhat cumbersome. The additional locomotion and effort involved in moving up and down to reach the EPM, and the extended drawing open then closing of the curtains (which could not be done prior to the experiment without exposing the animal to the apparatus) might have been somewhat unusual aspects of our EPM setup. It is simply not clear how these changes to the classic EPM set up

might have affected the rats, but it is possible that they rendered the rats less sensitive to the effects of CDP.

Experiment 3, on the other hand, produced results that were more consistent with typical results when examining anxiolytic efficacy in animal models of behaviour. As expected, CDP animals spent more time exploring the centre zone of the OF than saline animals, thus validating the task as implanted. Pregabalin did not increase centre zone time, but both pregabalin and CDP animals spent less time grooming than animals in the saline group, and pregabalin animals spent less time rearing than animals in the CDP and saline group. Benzodiazepines have previously demonstrated both a reduction in grooming and a reduction in rearing in behaviour in the OF(Barros et al., 1994; De Souza Spinosa et al., 2000; Depaulis & Vergnes, 1986; Fahey et al., 2001; Gai & Grimm, 1982; Hughes, 1993; Novas et al., 1988; Yerbury & Cooper, 1987). Pregabalin eliciting the same behaviour suggests that the OF results could be behavioural demonstrations of pregabalin's anxiolytic efficacy. However, as described in Chapter 8 (section 8.4.3), both these ethological measures offer less standard behavioural indications of anxiolysis. A decrease in grooming behaviour could in principle be attributed to body temperature; rat grooming is akin to sweating (Hainsworth & Stricker, 1971; Poole & Stephenson, 1977). If body temperature is lowered than there is less of a need to groom or 'sweat'. Research has shown that CDP can reduce body temperature (Locker & Koffer, 1962; Schmidt et al., 1961). However, subsequent studies have shown that lower doses of CDP do not reduce body temperature (Froger-Colléaux et al., 2011; Mead et al., 2008), and with the dose used in Experiment 2 and Experiment 3 being 2.5 mg/kg, it is unlikely that a reduction in body temperature accounts for a reduction in grooming. Clinical research into the effects of gabapentinoids in perioperative pain management has shown no effect on body temperature (Helenius et al., 2018), whilst an animal study has demonstrated dose-dependent (200 mg/kg i.v.) hypothermia (Sharma et al., 2011). As with CDP, the dose-

dependent hypothermia would occur for a much higher dose than those used in Experiment 2 and Experiment 3 (17.5 mg/kg). Thus, the interpretation of the results lends to the conclusion that CDP and pregabalin reduction of time spent grooming is in response to their anxiolytic properties, exclusively.

Pregabalin's decrease in the number rears in the OF can be attributed to other factors aside from anxiolysis. Rearing can be associated with information-gathering, learning and memory, environmental novelty, along with fear/anxiety (Lever et al., 2006). Briefly, rearing in rodents can be seen as information-gathering due to the 'vantage point' gained using their hind-legs. This height advantage provides information for certain sensory systems such as spatial mapping and defensive behaviours (Dielenberg & McGregor, 2001; Sheppe, 1966). Rearing behaviours also change in response to novelty and familiarity of an environment. For instance, mismatch tasks show that rearing decreases in familiarity and increases in novelty compounded with place cell remapping in said novel environment (Lever et al., 2006). As it relates to fear/anxiety, Gray and McNaughton (2003) proposed an inverse U curve in the level of rearing (increased vs decreased). Increase rearing occurs during low levels of fear and high levels of anxiety, and decrease rearing occurs during high levels of fear and low of levels of anxiety (Gray & McNaughton, 2003). They also note that anxiolytic drugs generally reduce rearing. Hippocampal theta also has strong implications in modulating rearing behaviour, with lesions to the medial septum/diagonal band of Broca (MS/DBB) eliciting a reduction in rearing (Bengelloun et al., 1977, 1976; Poplawsky & Isaacson, 1983; Ricceri et al., 1997). In all, it could be argued that pregabalin's reduction in rearing behaviour, together with the reduction in grooming, demonstrates its anxiolytic efficacy.

In summary, the results from Experiment 2 were not really effective in demonstrating pregabalin's anxiolytic efficacy, whilst Experiment 3 could potentially be seen to demonstrate pregabalin's anxiolytic efficacy, though not using the standard measure of

thigmotaxis reduction. At least in the case of the EPM, the results support the notion of the theta-frequency reduction assay as a more reliable test of an anxiolytic (McNaughton et al., 2007), with the OF being a positive behavioural measure of anxiolysis.

9.6 Summary

In summary, the findings in this thesis are mostly consistent with the double dissociation in which anxiolytic drugs act upon the intercept component and environmental familiarity acts upon the slope component. The somewhat partial results from both behavioural models could arguably indicate that the electrophysiological method of examining hippocampal theta frequency as it relates to running speed is more sensitive and reliable assay in determining the anxiolytic effects of a drug.

However, the findings within this thesis with regards to slope and intercept did offer one unexpected result. Intermediate ***intercept*** showed an effect of environmental familiarity, increasing in later trials (dorsal intercept did not). One argument for the effect could be that that increase in intercept reflected an increase in anticipatory anxiety, i.e. anticipation of painful albeit relatively brief injection after trial 4. This possibility should be investigated further. In general, the bidirectional modulation of theta intercept by anxiety has been under-explored.

9.6.1 Future research

As stated above, the finding that intermediate intercept in Study 1B, preliminary indicating an anxiogenic response, needs to be investigated further. Lesion studies examining the behavioural correlates in dorsal vs ventral lesions have demonstrated behavioural differences. Dorsal hippocampal lesions have disrupted spatial learning in the water maze, radial arm maze and T-maze, whilst ventral hippocampal lesions have little to no effect (Moser et al., 1993). Lesions to the ventral hippocampus, on the other hand, reduce neophagia (fear of novel food), increase social interactions and increase exploration in open arms in the elevated

plus maze (Bannerman et al., 2004; McHugh, Deacon, Rawlins, & Bannerman, 2004); linking the ventral hippocampus to anxiety modulation. Lesions to the intermediate hippocampus have shown the intermediate hippocampus plays a role in both spatial cognition by impairing rapid place learning (Bast et al., 2009), and to play a role in anxiety modulation with intermediate hippocampal lesions abolishing anticipatory behaviour (Burton et al., 2009). With previous research examining anxiogenic drug effect on hippocampal theta localised to the dorsal hippocampus, future research should focus on the intermediate and possibly the ventral hippocampus.

Future research into environmental familiarisation's effect on hippocampal place cells should incorporate recording both theta and ample place cells from the dorsal and intermediate hippocampus and associated entorhinal cortex. This would help to examining if environmental familiarisation's effect on grid scale could provide further support for the Burgess (2008) model.

One intriguing hint that should be followed up was the suggestion that there are differential sensitivities in the dorsal and intermediate hippocampus, with dorsal slope more sensitive than intermediate slope to environmental familiarisation, and intermediate intercept more sensitive than dorsal intercept to anxiety modulation.

9.7 Overall summary

9.7.1 Main findings

1. Study 1A replicated the broad result that anxiolytic drugs reduce theta frequency (Coop & McNaughton, 1991; McNaughton & Coop, 1991; McNaughton et al., 2007; Wells et al., 2013). In particular, Study 1A showed that the anxiolytic pregabalin reduces theta frequency in naturally occurring locomotion-based theta in freely moving rats.

2. More specifically, in line with the Wells et al (2013) interpretation of the Burgess (2008) model, Study 1A confirmed the prediction that pregabalin reduces the intercept of the theta-frequency-to-speed relationship, without affecting slope. Consistent with the Burgess (2008) model's identification of slope with cognitive spatial variables, and intercept with non-spatial variables, pregabalin did not affect the firing characteristics of dorsal hippocampal place cells.

3. Again, consistent with the Wells et al (2013) interpretation of the Burgess (2008) model, Study 1B showed that the slope component of hippocampal theta frequency increased with environmental familiarity (i.e. trial within day), and this result was most robust in the dorsal hippocampus.

4. Broadly speaking, Experiment 1 demonstrated a double dissociation of two components of hippocampal theta frequency: the slope component increased with environmental familiarity, while the intercept component decreased upon administration of an anxiolytic drug.

5. A possible exception to the Burgess (2008) model, in which slope solely corresponds with environmental familiarisation, and intercept solely corresponds with anxiolysis is intermediate intercept increasing across trials within a day for Study 1B. However, arguably, this increase would reflect anxiogenic anticipation of an aversive stimulus rather than environmental familiarity *per se*.

6. The results from Experiment 2 and Experiment 3 provided only partial behavioural evidence for pregabalin as an anxiolytic. Arguably, the results from Experiment 2 and Experiment 3 appear to give credence to the notion that theta frequency reduction is a

more accurate and reliable assay in testing the anxiolytic effectiveness of anxiolytic drugs than behavioural assays (McNaughton et al., 2007).

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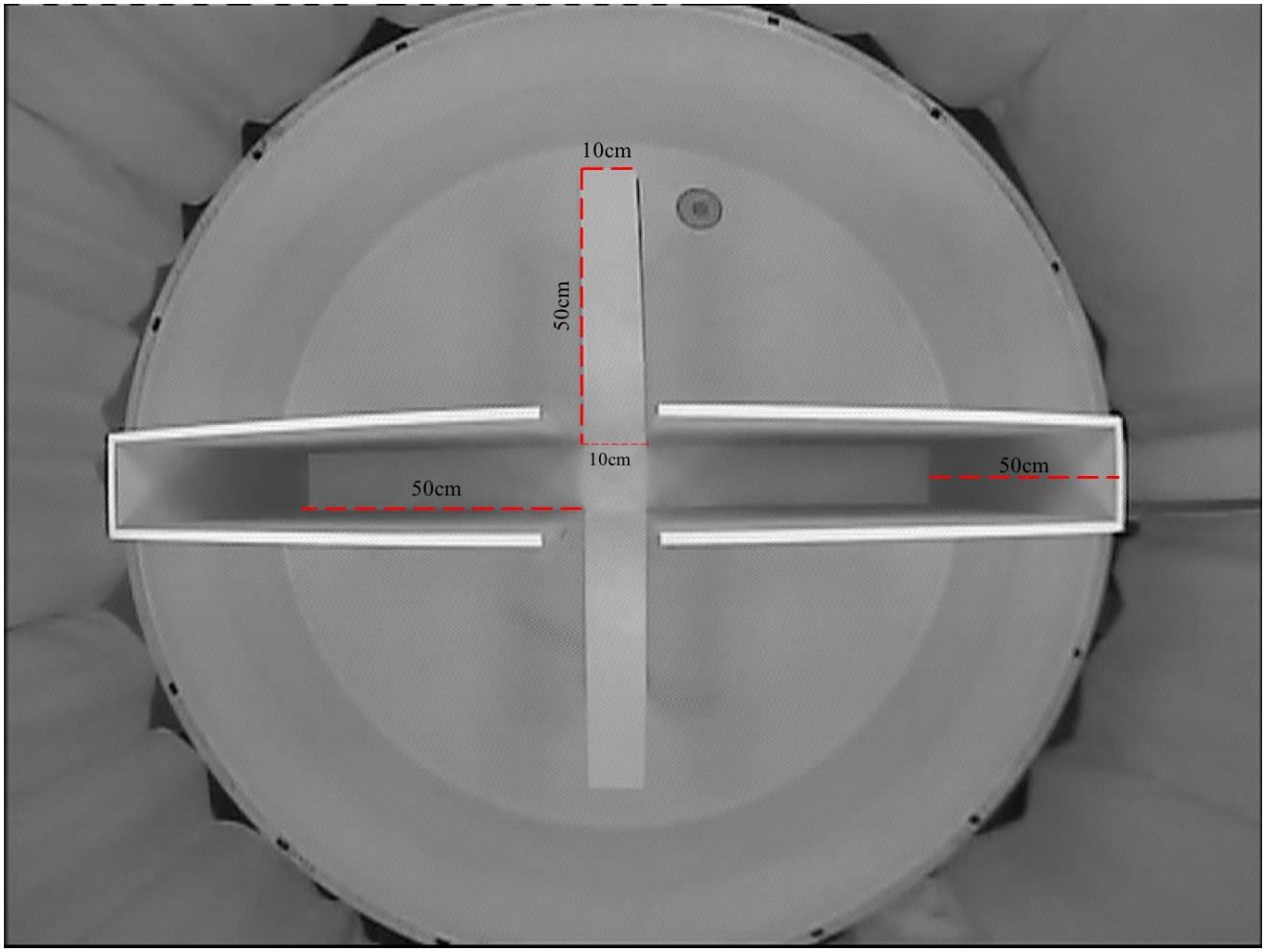
Appendix

Appendix A



Appendix B