FOOD, LEPTIN, AND THE HIPPOCAMPUS: THE ANXIOLYTIC POTENTIAL OF EATING

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ABSTRACT

A common feature of mood disorders is disturbances of circadian rhythm and fluctuations in body weight. These are also common side effects of clinical anxiolytics. Likewise, those with eating disorders usually have comorbid anxiety disorders. This suggests that there may be some link between eating, mood, and circadian rhythmicity. The hippocampus is involved in the control of anxiety; and circadian periodicity in the firing rates of pyramidal cells in area CA1 of the hippocampus has been linked to a food-entrainable oscillator. As this evidence links the hippocampus to anxiety, food, and circadian rhythmicity, this thesis examined whether reticular-elicited hippocampal RSA, a reliable assay of anxiolytic action, is modulated diurnally and/or by food. The effects of food on mood could be due to leptin – a hormone secreted in response to fat ingestion, which also shows a diurnal cycle. Leptin has been shown to be an effective anxiolytic in some behavioural tests of anxiety, but not others. So, this thesis also examined the effect of leptin on two test of anxiolytic potential linked to hippocampal function. Leptin was tested both on reticular-elicited hippocampal RSA and on an FI60 task. Both of these tests are reliable indicators of anxiolytic potential. We predicted that our hippocampal RSA test of anxiety would show a food-modulated circadian pattern of oscillation; and that leptin would have an anxiolytic-like effect on both reticular-stimulated hippocampal RSA and behaviour in the FI60. Six rats were implanted with bipolar hippocampal recording electrodes and bipolar reticular stimulating electrodes. RSA was elicited every hour for either 48 hours (food as normal at 1800 hours) or 58 hours (food 2, 26 and 50 hours after recording started). Starting times were staggered throughout the day. Circadian oscillation of hippocampal RSA was unclear, however
unexpected feeding decreased overall RSA frequency in an anxiolytic manner. Two animals were then administered leptin at doses 0mg/kg (control), 0.5mg/kg, and 1mg/kg (i.p.) and produced a 30 – 90 minute dose-dependent increase in the slope of the stimulation/frequency function of but did not change overall frequency. In the FI60 task 0.5mg/kg of leptin produced an anxiolytic-like effect similar to that shown by the active control chlordiazepoxide and the 1.0mg/kg dose produced an overall depression in responding similar to buspirone and fluoxetine. These results are largely consistent with previous effects of serotonergic anxiolytics and suggest that leptin may have some serotonergic anxiolytic action coupled with serotonergic release of corticosterone.
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The surgical and experimental procedures described in this thesis complied with the University of Otago’s Code of Ethical Conduct and The Committee on Ethics in the Care and Use of Laboratory Animals granted ethical approval for this study (reference numbers D44/11 (Electrophysiology) and D18/12 (Fixed Interval)).
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>5-HT</td>
<td>5 Hydroxytryptamine</td>
</tr>
<tr>
<td>5-HTP</td>
<td>5 Hydroxytryptophan</td>
</tr>
<tr>
<td>8-OH-DPAT</td>
<td>8-Hydroxy-2-(di-n-propylamino)tetralin</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>BDZs</td>
<td>Benzodiazepines</td>
</tr>
<tr>
<td>BUS</td>
<td>Buspirone</td>
</tr>
<tr>
<td>CA1/CA3</td>
<td>Cornu Ammonis 1/3</td>
</tr>
<tr>
<td>CDP</td>
<td>Chlordiazepoxide</td>
</tr>
<tr>
<td>Cl-</td>
<td>Chloride Ion</td>
</tr>
<tr>
<td>CORT</td>
<td>Corticosterone</td>
</tr>
<tr>
<td>CRF</td>
<td>Continuous Reinforcement</td>
</tr>
<tr>
<td>cub</td>
<td>Cubic</td>
</tr>
<tr>
<td>dev</td>
<td>Deviation</td>
</tr>
<tr>
<td>DRD2</td>
<td>Dopamine Receptor D2</td>
</tr>
<tr>
<td>DSMIV</td>
<td>Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition</td>
</tr>
<tr>
<td>EPM</td>
<td>Elevated Plus Maze</td>
</tr>
<tr>
<td>FET</td>
<td>Field Effect Transistor</td>
</tr>
<tr>
<td>FI60</td>
<td>Fixed Interval 60 seconds</td>
</tr>
<tr>
<td>FLU</td>
<td>Fluoxetine</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-Amino Butyric Acid</td>
</tr>
<tr>
<td>GAD</td>
<td>Generalised Anxiety Disorder</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloride</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>IBM</td>
<td>International Business Machines</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>LEP</td>
<td>Leptin</td>
</tr>
<tr>
<td>lin</td>
<td>Linear</td>
</tr>
<tr>
<td>LTP</td>
<td>Long Term Potentiation</td>
</tr>
<tr>
<td>n.s.</td>
<td>Not significant</td>
</tr>
<tr>
<td>OCD</td>
<td>Obsessive Compulsive Disorder</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>quad</td>
<td>Quadratic</td>
</tr>
<tr>
<td>RSA</td>
<td>Rythmic Slow-Wave Activity (also called Theta)</td>
</tr>
<tr>
<td>RT30</td>
<td>Random Time 30 seconds</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous injection</td>
</tr>
<tr>
<td>SSRIs</td>
<td>Specific Serotonin Reuptake Inhibitors</td>
</tr>
<tr>
<td>Stimulation Slope</td>
<td>The slope of the stimulation/frequency function</td>
</tr>
<tr>
<td>Stimulation Intercept</td>
<td>The intercept of the stimulation/frequency function</td>
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CHAPTER ONE

General Introduction

1.1 Preamble

Mood disorders are often combined with fluctuations in weight as well as disturbed sleeping patterns. Weight and sleep are also often changed by drugs used to treat these mood disorders. In particular, around two thirds of patients with eating disorders also suffer from some form of anxiety disorder (Kaye, Bulik, Thornton, Barbarich, & Masters, 2004). Likewise, pharmacological treatments for anxiety disorders have been shown to cause sleep disturbances as well as causing fluctuations (in both directions) in weight (Masand, 2000).

The link between anxiety and eating may depend on internal biological control mechanisms rather than being the result of shared simple external situational factors. Patients undergoing pharmacological treatment for anxiety have been shown to go through fluctuating weight changes and have also been reported to have disturbed circadian rhythms. The hippocampus has been linked to the control of anxiety (Gray & McNaughton, 2000) and there is circadian periodicity in the firing rates of pyramidal cells in area CA1 of the hippocampus (Munn & Bilkey, 2012) that is diurnal, but not entrained to light. It is possible that this circadian periodicity is related to a food-entrainable oscillator. This food-entrainable circadian oscillation seen in the hippocampus may also be responsible for the disruption of circadian rhythms seen in patients with anxiety disorder, as well as explaining fluctuations in weight.

Effects of food on anxiety are likely to be mediated by eating-related hormones. The hormone leptin is secreted by adipocytes in response to intake of
dietary fat. It is also released by adipocytes on a rhythmical basis over circadian-length timescales. There is preliminary, anecdotal evidence for affect-increasing effects of the consumption of a high-fat meal, which would result in the release of leptin among other compounds. Leptin is of interest as a supposed source of anxiolytic effects of food because it has been previously been demonstrated to display anxiolytic-like effects in several animal tests of anxiety.

Food could, then, impact on anxiety by releasing leptin, which could then alter hippocampal electrophysiology. Therefore the goals of the present research project were to examine 1) whether there is any circadian variation in anxiety-related aspects of hippocampal function and to examine the role of food in such variation; and, 2) whether the hormone leptin affects hippocampal-sensitive behaviour and hippocampal electrophysiology like established anxiolytic drugs.

In this introduction I will outline:

(1) the rationale for undertaking the study and clarify the link between anxiety disorders and leptin;

(2) the neurophysiological and behavioural assays I will use to assess anxiolysis.

1.2 Anxiety Disorders

Anxiety is a common and adaptive response to stressors of any kind. Many everyday experiences can trigger it, for example, talking to a group or being confronted with an unfamiliar dog (provided the dog is not too aggressive). Anxiety disorders, however, are clinically significant failings of the anxiety system, and are marked by inappropriately large or intrusive response to threat. There are many different kinds of anxiety disorder as defined by the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) (Pull, 1995) such
as generalized anxiety disorder (GAD), social phobia, specific phobia, post-traumatic stress disorder, and so forth. GAD being one of the most prevalent of these anxiety disorders (Ballenger et al., 2001) occurring in up to 10% of the population (Lieb, Becker, & Altamura, 2005). These disorders, however, all share an excessive response to perceived threat.

1.3 **Pharmacological Treatment of Anxiety**

The drugs commonly used to treat anxiety disorders can be broken down into two main groups: drugs that increase output from the Gamma-Amino Butyric Acid-A (GABA-A) receptor; and drugs that increase output from the 5-hydroxytryptamine (5-HT)\textsubscript{1A} receptor.

1.3.1 **The GABA Agonists**

The two most commonly used classes of drug that increase output from the GABA-A receptor as their main clinical action are the barbiturates and the benzodiazepines (Haefely et al., 1975). Both classes of drug bind to specific sites on the GABA-A receptor and, directly or indirectly, promote the passage of Cl\textsuperscript{-} ions into the neuron, causing hyperpolarization. The barbiturates essentially “force” the channel open in the same manner as GABA: allowing Cl\textsuperscript{-} ions to flow into the cell immediately. In contrast, the benzodiazepines, when bound, do not affect the channel directly but change the conformation of the site at which GABA acts. They, thus, facilitate the opening of the receptor in response to activation by endogenous GABA. They allow higher than baseline levels of Cl\textsuperscript{-} into the postsynaptic cell when GABA binds to its site; but they have no effect on Cl\textsuperscript{-} in the absence of GABA. This indirect mode of action makes the benzodiazepines the drugs of choice in the treatment of anxiety if the GABA-A receptor is the desired target. Because they do not open the Cl\textsuperscript{-} ionophore directly they are safe at very
high doses and so have a very broad therapeutic window. The barbiturates, on the other hand, have a relatively narrow therapeutic window, and are hazardous in even moderate overdose. These two major classes of GABA agonists present a similar profile of action: both are anxiolytic, muscle relaxant, sedative, addictive, and anticonvulsant.

1.3.2 The 5-HT1A Receptor Agonists

Another major class of drug commonly used to treat anxiety disorders is the serotonergic agonists which specifically work as agonists at 5-HT1A. Buspirone is a drug that acts as a direct partial agonist at the 5-HT1A receptor (Riblet, Taylor, Eison, & Stanton, 1982) and is clinically anxiolytic (Feighner, Merideth, & Hendrickson, 1982; Goa & Ward, 1986; Pecknold et al., 1985). It has a variety of other effects on other receptor systems such as the cholinergic and dopaminergic systems, but appears to act independently of the GABA receptor (Eison & Temple, 1986). It is not addictive and shares none of the other (e.g., sedative, muscle relaxant) properties of the GABA agonists (Balster & Woolverton, 1982). Also, unlike the GABA agonists, it is antidepressant. It has its own range of side effects including: initial dysphoria, headache, nervousness, and gastrointestinal upset (Goa & Ward, 1986).

1.3.3 Specific Serotonin Reuptake Inhibitors

The Specific Serotonin Reuptake Inhibitors (SSRIs) are another major type of serotonin agonist that have proved to be clinically effective anxiolytics as well as antidepressants (Ball, Kuhn, Wall, Shekhar, & Goddard, 2005; Nutt, 2004). These drugs exert their effect by blockade of the serotonin reuptake channel (thereby increasing levels of 5HT at 5HT1A, and other 5HT, receptors), and
possibly by causing desensitization of the 5-HT autoreceptor (Castro, Diaz, del Olmo, & Pazos, 2003; Hervás & Artigas, 1998; Lemberger et al., 1985). Like buspirone they share none of the side effects of the GABA agonists and have a range of side effects: sexual dysfunction, sleepiness and weight gain being ranked as the most bothersome (Cascade, Kalali, & Kennedy, 2009). Because of their relatively benign side effects SSRIs are the current drugs of choice for the treatment of anxiety. They are much better tolerated than buspirone, lacking its dysphoric effects, while, like buspirone, they lack the addictive and sedative effects of the GABA agonists and are antidepressant. In addition, and unlike buspirone, they also reduce panic and obsession.

1.3.4 Anxiolytics and the Hippocampus – Tests of Anxiolysis

The hippocampus has been implicated as a key structure in anxiety and in the inhibition of behaviour (Gray & McNaughton, 2000), there is also evidence that clinical anxiolytics impair hippocampal function (McNaughton, Kocsis, & Hajos, 2007). An effective neurophysiological assay of anxiolytic potential has been found to be the frequency of hippocampal Rhythmic Slow-Wave Activity (RSA) that is produced by stimulation of the reticular formation. Hippocampal RSA frequency has been shown to be reduced by all anxiolytics so far tested and is not reduced by drugs that are not anxiolytic.

RSA is a major type of oscillatory activity that occurs endogenously in the hippocampus. It is easily recognised by its regular sinusoidal appearance at a frequency that ranges between 4-9Hz. It can be reliably elicited by electrical stimulation of the reticular formation (Green & Arduini, 1954). Increasing stimulus intensities produces a linear increase in the frequency of hippocampal RSA. Hippocampal RSA may be the neural substrate behind anxiolysis and the
behavioural effects caused by hippocampal dysfunction (Gray, 1982; Gray & McNaughton, 2000).

All drugs that are clinically anxiolytic that have so far been tested decrease the intercept of the stimulation/frequency function produced by reticular driving of hippocampal RSA (McNaughton et al., 2007). These clinical anxiolytics are pharmacologically diverse; they include the benzodiazepines (GABA-A agonists) (McNaughton & Sedgwick, 1978), buspirone (5-HT1A agonist) (Coop & McNaughton, 1991), imipramine (monoamine agonist) (Zhu & McNaughton, 1991), and fluoxetine (5-HT agonist) (Munn & McNaughton, 2008). These drugs, as mentioned in section 1.3, have little in common apart from their effect on both anxiety and RSA, suggesting that these are functionally linked. Buspirone (BUS) and the classical anxiolytics both produce their maximal reduction in hippocampal RSA frequency 15 to 30 minutes post administration (Coop & McNaughton, 1991). Unlike benzodiazepines (BDZs) however, BUS does not produce a decrease in the slope of the stimulation/frequency function of RSA, and has been shown to produce a slight increase in the slope of the stimulation/frequency function (Coop & McNaughton, 1991).

The effect of anxiolytics on theta appears to be more than a simple assay of clinical action. Anxiolytics produce reductions in behavioural inhibition in tasks involving frustrative non-reward. The fixed interval 60 seconds (FI60) task is of particular note as effects of anxiolytics on the FI60 have been directly linked to changes in hippocampal RSA (Woodnorth & McNaughton, 2002), suggesting that drugs that affect the electrophysiological test of hippocampal RSA will also affect the behaviour elicited in the FI60 task, a result which has been seen with all anxiolytics tested across both of these tests of anxiolysis so far. The FI60 uses a
reinforcement schedule in which reinforcement is elicited only for the first response made by the subject after a fixed time period has elapsed since the most recent rewarded response (inter-reward period). Animals, having previously learned that lever pressing produces a reward regardless of the time lapse since the last reinforcement, then learn to suppress responding in the FI60 task until times when responding will elicit reinforcement. This behavioural inhibition is shown by animals making fewer responses directly after being presented with a reward, and showing an increase in responding towards the end of the inter-reward periods. This gradual decreasing occurrence of a learned behaviour is generally taken as an indication that the animals are inhibiting the previously learned behaviour of lever pressing. This pattern of learning can be affected by administering drugs to the animals; anxiolytics produce a unique effect on responding in the FI60 task.

When given BDZs, animals produce a different pattern of behaviour in the FI60 task compared to drug-free animals (Panickar & McNaughton, 1991). Animals given BDZs show an inability to inhibit behaviour, they not only show the characteristic response release early in the inter-reward period, but also show a sizeable increase in responses throughout the FI60 schedule (Woodnorth & McNaughton, 2002). Likewise, when given at low doses BUS has been shown to produce an increase in response rate in the FI60 task, particularly from around the middle to the end of the inter-reward periods (Zhu & McNaughton, 1995). However, higher doses (over 1.1mg/kg) have been shown to produce an overall decrease in responding throughout the FI60 schedule (Panickar & McNaughton, 1991) an effect which has also been produced by fluoxetine (FLU) (Munn & McNaughton, 2008). This is of interest because BUS and FLU are both serotonergic
anxiolytics, and are therefore pharmacologically dissimilar to the BDZs. These tests show that the hippocampus is a key structure in anxiety and anxious behaviours.

1.4 **Anxiety, the Hippocampus and Circadian Rhythmicity**

There is evidence that anxiety is linked to the circadian timing system. For example structural polymorphisms in some genes (such as DRD2 and PAWR) controlling the circadian timing system are linked to the incidence of several anxiety disorders (Sipila et al., 2010). Circadian phase has been linked to the differential expression of anxiety- or depression-typical behavior in a variety of tests in a sexually dimorphic fashion in rats, with female animals showing an increase (larger and across more behavioural tests in females than males) in anxiety-like behaviour when tested during their ‘dark phase’ compared to their ‘light phase’ (Verma, Hellemans, Choi, Yu, & Weinberg, 2010).

There is evidence that effects mediated by the 5-HT<sub>1A</sub> receptor show diurnality. 8-OH-DPAT, an anxiolytic 5-HT<sub>1A</sub>-specific agonist, has been shown to have different effects on behavioural tests of anxiety at different points in the diurnal cycle, with larger effects half way through the animals normal ‘dark phase’ (Lu & Nagayama, 1997). Corticosterone (CORT), also, has been shown to have major diurnal variations peaking towards the beginning of the active period (Keller-Wood, Shinsako, & Dallman, 1983).

Alterations in the circadian rhythmicity of the sleep/wake cycle were noted in patients suffering from anxiety disorders, causing their wake/sleep times to occur earlier (Limpido, Russo, & Bersani, 2008). It is clear, at least to a first approximation, that stress, 5-HT, and anxiety disorders may be linked via circadian rhythmicity.
The hippocampus also shows circadian rhythmicity in a variety of ways; there is a circadian modulation of gene expression that is believed to play a key role in circadian timing (Dolci et al., 2003). The magnitude of long-term potentiation (LTP) in the hippocampus has also been shown to be subject to a circadian-like periodicity (Chaudhury, Wang, & Colwell, 2005). In addition to reflecting circadian timing, there is also evidence of an independent circadian clock within hippocampal cells, which is involved in the regulation of LTP via the expression of genes that are fundamental to its expression (Wang et al., 2009).

On a coarser level than the expression of genes, neurons in area CA1 of the hippocampus, which are involved in encoding spatial information regarding an animal’s environment (Leutgeb et al., 2005), show a circadian-like modulation of their rate of firing which may be related to a food-entrainable oscillator (Munn & Bilkey, 2012). These examples of hippocampal diurnality/circadian rhythmicity suggest that a circadian-like rhythmicity underlies several functions within the hippocampus, including anxiety, and that this oscillation is elicited in response to food intake.

1.5 The Biological Role of Leptin, a Putative Anxiolytic

A variety of hormones are released into the bloodstream by various tissues in response to the intake of specific types of food. Intake of fats will promote the release of the hormone leptin from adipocytes. The exact function of leptin is not clear. It was supposed, initially, to be a satiety hormone; research in transgenic animals showed that leptin-receptor deficient animals rapidly become obese, while animals with normal leptin function remain a healthy weight. Treatment with leptin, though, produces no anorexic effect in normal animals (while
producing such an effect in obese, genetically leptin-receptor deficient, animals), and so it became clear that the role of leptin was far beyond that of a simple messenger of satiety.

### 1.5.1 Is Leptin Anxiolytic?

A number of studies have looked into the link between leptin and behavioural anxiety. Asakawa et al. (2003) demonstrated that obese mice displayed much less anxiety-stereotyped behaviour in an elevated plus maze (EPM) when administered systemic leptin than when administered physiological saline. There is evidence that abnormally low leptin levels are correlated with comorbid Obsessive Compulsive Disorder (OCD)/depression in humans (Atmaca, Tezcan, Kuloglu, & Ustundag, 2005). Animals with induced neonatal hyperleptinaemia display markedly increased levels of behavioural markers of anxiety (Finger, Dinan, & Cryan, 2010; Fraga-Marques et al., 2009; Levay et al., 2008). In a comparison with the established anxiolytic fluoxetine, leptin was equally effective at reducing anxiety-typical behaviours in a variety of tests such as the EPM and open field (Liu et al., 2010).

However there have also been studies that failed to provide evidence for leptin as an anxiolytic. Specifically Suomalainen & Mannisto (1998) found that leptin produced null effects on behavioural tests of anxiety and depression in mice. Leptin has also been shown to produce null effects on behavioural tests of anxiety in rats (Buyse, Bado, & Daugè, 2001; Thorsell, Caberlotto, Rimondini, & Heilig, 2002). With this conflicting evidence it is important to clarify whether or not leptin has potential anxiolytic properties, and which neurotransmitter systems mediate this anxiolytic activity.
Pharmacologically speaking, there is evidence that leptin modulates and is modulated by the activity of 5-HT, (Garcia-Alcocer et al., 2010; Morrison, 2004) which is involved in the anxiolytic function of serotonergic anxiolytics such as buspirone and fluoxetine. Administration of leptin increases brain serotonin (5-HT) metabolism in mice (Calapai et al., 1999). There is also evidence that administration of 5-HT precursor 5 hydroxytryptophan significantly increases serum leptin levels (Yamada et al., 1999), also serotonergic neurons are targets for leptin in the monkey (Finn et al., 2001), suggesting that leptin may be mediating 5-HT activity.

Furthermore, the 5-HT transporter, which is targeted by the SSRIs, is linked to obesity (Uceyler et al., 2010). There is also evidence that polymorphisms of the 5-HT receptor are linked to two eating disorders: anorexia and bulimia (Collier et al., 1997; Nacmias et al., 1999). This evidence suggests that these disorders of eating are linked to the 5-HT system. Therefore it may be the case that these eating disorders are affected by leptin activity at the 5-HT system.

1.6 Aim of Present Study

Circadian variation has been seen in CORT expression and actions mediated by the 5-HT\textsubscript{1A} receptor. There is also compelling evidence that the hippocampus shows circadian rhythmicity that is not entrained to light. These pieces of evidence all suggest circadian rhythmicity is involved in anxiety and anxious behaviours.

All of the drugs so far tested that are anxiolytic produce a reliable decrease in the frequency of reticularily-elicited hippocampal RSA, while none of the drugs so far tested that lack anxiolytic potential have been demonstrated to do so. Given
the circadian rhythmicity that has been shown in the hippocampus as well as those linked to anxiety, this leads to the initial hypothesis that:

• reticularly-elicited hippocampal RSA will show circadian rhythmicity

Considering that the hippocampal circadian variation found previously was not entrained to light, some other variable must be responsible. Munn and Bilkey (2012) speculate that this variable could be food. This leads to a modified form of our first hypothesis:

• Reticularly elicited hippocampal RSA will show a circadian oscillation that is entrained to food.

Leptin is a hormone released in response to consumption of food; it shows circadian variation. There is preliminary evidence that the hormone leptin is anxiolytic. It is therefore of interest to examine the effect of leptin on a reliable neurophysiological assay to test for a possible role for leptin in the diurnal and food-related changes in anxiety. This leads to the hypothesis:

• leptin will produce a dose-dependent decrease in the frequency of reticularly-elicited hippocampal RSA in the same way as other anxiolytics so far tested.

As with our neurophysiological test of anxiolysis, all drugs that have so far been shown to be anxiolytic also produce a reliable release of responding in tasks of frustrative non-reward such as the fixed interval 60 seconds task; and this test has been particularly strongly linked to changes in theta frequency (Woodnorth & McNaughton, 2002). This leads to our final hypothesis:
• leptin will produce a release of responding – an inappropriately high number of responses early in the inter-reward period in the FI60 task in the same way as other anxiolytics so far tested.

To test these hypotheses we will observe the circadian rhythmicity of reticulatively-stimulated hippocampal RSA as a neurophysiological assay of changes in anxiety control systems, and test whether or not it is the availability of food that produces this rhythmicity. Reticulatively-stimulated hippocampal RSA will be recorded continuously over a timeframe of several days, and food will be systematically manipulated. If this neurophysiological assay of anxiety is affected by food, it is of interest to examine in greater detail the biofeedback mechanisms that may be responsible. To examine whether leptin might be key in the regulation of anxiety, leptin will be tested to examine its effects on reticulatively-stimulated hippocampal RSA. A behavioural test of anxiety will also be used to confirm the anxiolytic potential of leptin. The FI60 is a reliable, reproducible behavioural assay of anxiolytic potential.
CHAPTER TWO

Circadian Variation in Rhythmic Slow-Wave Activity

2.1 Introduction

As discussed in Chapter 1, various lines of evidence suggest that the hippocampus reflects circadian variation in a variety of ways; there is a circadian modulation of gene expression that is believed to play a key role in circadian timing (Dolci et al., 2003). Neurons in area CA1 of the hippocampus, which are involved in encoding spatial information regarding an animal's environment (Leutgeb et al., 2005), show a circadian-like modulation of their rate of firing which is not entrained by light, but may be driven by some sort of food-entrainable oscillator (Munn & Bilkey, 2012). These pieces of evidence suggest a food-entrained circadian-like rhythmicity underlies several functions within the hippocampus. There is also evidence that anxiety is linked to the circadian timing system, disruption in circadian rhythmicity has been shown in patients with anxiety disorders (Limpido et al., 2008). It is clear, at least as a first approximation, that circadian oscillations, eating, and anxiety disorders may be linked.

The present experiment explored the possibility of hippocampal Rhythmic Slow-Wave Activity (RSA) showing a circadian cycle, as well as looking into what may be entraining this possible circadian cycle. The observations that RSA is implicated in anxiety, and that anxiety is modulated on a circadian timescale, made it of interest to examine whether RSA itself was modulated in a similar way.

The aim of the present experiment was to determine whether reticularly elicited hippocampal RSA has a circadian cycle/rhythmicity, and whether this might be elicited in response to food. In the present experiments we tested the
circadian rhythmicity of RSA first over a 48-hour and then over a 58-hour time period. It was hypothesised that the hippocampal RSA produced by reticular stimulation would show a circadian variation over a 24-hour cycle. We looked at animals who were being given unexpected food (irregular feeding time) and expected food (regular feeding time), in order to see if food was affecting any potential variation in hippocampal RSA.

2.2 General Method

2.2.1 Subjects

Five male Sprague-Dawley rats were obtained from the University of Otago Department of Laboratory Animal Sciences. Animals were aged 6-7 weeks upon arrival. At the time of their surgeries animals were aged between 7 and 14 weeks, and weighed between 392 and 475 grams.

Before they received surgery animals were housed in groups of three or four. They were housed in a 49cm X 31cm X 26.5cm cage with a plastic base and a wire mesh lid with a food hopper and a water bottle frame on the left side of the hopper. The floor of the cage was covered with 1-2cm of aspen bed shavings and a handful of shredded paper.

After surgery the animals were housed in individual cages similar to the cages described above, with a plastic base and a wire mesh lid with a food hopper and water bottle frame. These cages were smaller (33cm X 19cm X 26.5cm).

Animals were kept on a regular 12hour/12hour light/dark cycle, 0600hours – 1800hours lights on. Natural light was blocked out and the room temperature was maintained between 20-22° Celsius. All animals had ad libitum access to water and were kept on a restricted diet of Reliance Stock food pellets,
the amount of which was varied daily in order to maintain the animal's weights at 80% of their free feeding weight. Feeding was at 1800 hours each day.

The surgical and experimental procedures described in this thesis complied with the University of Otago's Code of Ethical Conduct and The Committee on Ethics in the Care and Use of Laboratory Animals granted ethical approval for this study (reference number D44/11).

2.2.2 Surgical apparatus and procedure

2.2.2.1 Electrodes

Surgeries were carried out by Robert Munn. Two pairs of electrodes were surgically implanted under stereotaxic guidance; a bipolar pair of recording electrodes aimed at the dorsomedial subiculum on one side of the brain, and a bipolar pair of stimulating electrodes aimed at nucleus reticularis pontis oralis on the other side of the brain. During the surgery an uninsulated silver earth wire was fixed to one of the screws in the skull. The bipolar electrodes consisted of two strands of Teflon-coated wire (0.0070” Teflon-coated, and 0.005” bare; A-M Systems Inc.) twisted together. At the end of each electrode the wires were untwisted a few turns and approximately 50mm of the Teflon coating was removed from the tip of the wire. This was done to enable a male Amphenol gold pin to be soldered onto the end of each strand of wire, with the use of solder and phosphoric acid flux. The tips of the wires at the other end of the electrodes were separated vertically. The tips of the recording electrodes were separated by 1.5mm and the tips of the stimulating electrodes were separated by 1mm. A piece of 0.25mm diameter uninsulated silver wire with a male gold pin soldered onto one end was used as the earth wire. Each of the gold pins of the electrodes were inserted into a plastic headcap. The screws used to secure the electrode to the
skull were stainless steel jeweller’s screws, which were screwed into the surface of the skull; six of these screws were used for each animal. The earth electrode was secured during surgery by winding it around several suitable skull screws.

2.2.2.2 Drug administration

Anaesthesia during surgery was achieved by intra-peritoneal injection of Ketamine HCl (0.75 ml/kg, 100mg/ml, i.p., Parnell Laboratories, NZ) with Medetomidine HCl (Domitor, 0.5 ml/kg, 1 mg/ml, i.p., Novartis Animal Health Australia). The Ketamine and Medetomidine were pre-prepared injectable solutions, and were delivered in the same syringe 15 to 30 minutes prior to surgery. Toe pinch and eye blink responses were used to assess the level of consciousness of the animals. Adequate anaesthesia was maintained throughout the operation by using top up doses of 0.2ml equal parts Ketamine and Medetomidine when deemed necessary based on the toe-pinch reflex. After the surgery was completed, animals were administered Atipamezole (Antisedan; 0.2 ml, 5 mg/ml, s.c., Novartis Animal Health Australia ; a specific antagonist of Medetomidine) to reverse the Medetomidine anaesthesia at a dose equal to the total amount of Domitor administered, including any top-up doses. This reversal of the anaesthesia usually showed a reasonable regaining of consciousness, with animals showing a pronounced toe-pinch response as well as voluntary head and jaw movements, within ten minutes of administrating the Antisedan. Before surgery animals were administered prophylactic antibiotic trimethoprim-sulphadimethylypyrimidine (Amphoprim, 0.2 ml, 60 mg/ml). Local anaesthesia was achieved by subcutaneous infiltration of Lignocaine HCl (0.1ml, 20mg/ml, s.c., Ethical Agents) into the scalp where the incision was going to be. Animals also received post-surgery analgesia by Carprofen (Norocarp, 0.1 ml, 50 mg/ml,
Norbrook NZ) as well as directly after surgery by Buprenorphine HCl (Temgesic, 0.1 ml, 0.3 mg/ml, Reckitt Benckiser NZ).

2.2.2.3 Surgery

In order to maintain the animal's body heat during surgery the surgery room was kept at around 20-25°C Celsius. This was achieved using a thermostatically controlled heater, which was mounted on one wall of the surgery room. There was also a heating pad placed on the surgical platform which was covered with an incontinence sheet. The animal was placed on the incontinence sheet, and the heating pad maintained the body temperature of the animal during the anaesthesia. There was a Kopf stereotaxic frame mounted on the surgical platform. Before surgery both the platform and the frame were swabbed with an 80% ethanol/20% water solution to guarantee sterility. This 80% ethanol solution was also used to sterilise all areas likely to be touched throughout the surgery as well as all the surgical instruments that were likely to be used. Throughout the surgery the surgical instruments remained on a fabric field which had been sterilized in an autoclave. During the surgery both the recording and the stimulating electrodes, together with the earth, the plastic head cap, and the skull screws were immersed in a bath of the 80% ethanol solution until they were required. The surgeon wore a laboratory coat and surgical gloves at all times throughout the surgery. Gloves were sterilised by repeated dipping in a bath of the 80% ethanol solution and were changed as needed.

Once the anaesthesia was complete and animals showed no reflex response to a toe-pincher, the animal’s head was shaved. Then the animal was carefully positioned on the stereotaxic frame on the surgery platform; held in position by insertion of ear bars as well as a nose and incisor bar. Using the antiseptic
Betadine (Faulding Pharmaceuticals), the surgeon swabbed the shaved area on the animal's head. Application of Tricin (Jurox Pty, Australia) prevented the animals' eyes from drying out during surgery. Once the animal was secured in the stereotaxic frame, the animal was covered with a plastic field that had been sterilized by immersion in an 80% ethanol bath. A sagittal incision from around 5mm rostral to Bregma to around 5mm caudal to Lamda was made in the scalp through the plastic field, keeping as close as was possible to the midline of the skull. The lateral muscle and skin tissue, in addition to the sterile plastic field, was pushed away from the midline and was held by four vascular clamps; two on each side of the incision, one posterior, one anterior. The connective tissue on the surface of the skull was scraped clear using a scalpel, and the surface of the skull was dried using cotton swabs. The lateral bone ridges of the skull served as the lateral boundaries from which the surgeon retracted the skin and muscle. The anterior and posterior boundaries of the incision were 5mm posterior to lambda and 5mm anterior from bregma.

In order to ensure the correct placement of the electrodes it was necessary for the surface of the skull to be flat. This was determined by attaching an electrode to a stereotaxic arm, lowering it until the tip of the electrode wire was touching the surface of the skull over lambda and bregma, taking relative depression measurements at both landmarks. If the measurements showed that the surface of the skull was not flat, the height of the incisor bar was altered to adjust the elevation of the animal's head. Using the same method, the relative elevation of lambda and bregma were measured again, and this was done until the measurements showed that the surface of the skull was flat between the two landmarks.
Using a hand-held battery-powered drill, six holes were drilled into the skull at convenient points along the lateral boundaries of the incision, with three holes drilled on each side of the skull. The stainless steel skull crews were then screwed into these six holes. Using bregma and the skull midline as reference points, the stereotaxic frame was used to establish the correct locations for the two holes the electrodes were to be lowered through. The recording electrode coordinates were 6.0mm posterior to and 2.0mm lateral from bregma; it was lowered to a depth of 5.0mm below the surface of the skull. The coordinates for the stimulating electrode were 7.0mm posterior to and 1.6mm lateral from bregma; it was then lowered to a depth of 8.5mm below the surface of the skull. These coordinates placed the recording electrode into the dorsomedial subiculum of the hippocampus, and the stimulating electrode in the region of the Nucleus Reticularis Pontis Oralis. The earth electrode was secured by winding it around several suitable skull screws. Once the stereotaxic frame had been used to determine the placements of the electrodes the handheld battery-powered drill was used to drill the holes for them to be lowered through.

Once the electrodes were lowered to the correct depth, they were held in place using a stereotaxic arm while they were secured to the skull surface and to the skull screws using Jet Acrylic dental cement. Once the dental cement had hardened the electrode was removed from the stereotaxic frame. It was ensured that the earth electrode had made contact with the skull before the dental cement had hardened. Then the headcap was secured and all of the exposed wires were covered using dental cement. Once the dental cement had hardened, the clamps holding the skin were removed, and the front and back ends of the incision were sutured closed so that the remaining skin held securely around the mound of
dental cement. Animals were monitored closely during their recovery. During their recovery the animals were given a mash containing sugar, water, and ground up food pellets, in addition to post-operative analgesia as described in section 2.2.2.2. All animals were allowed to recover for a minimum of ten days before they began testing for the experiment.

2.2.3 Reticular Testing

2.2.3.1 Apparatus

During testing and the experimental runs, the animal was placed in a square plastic bin (measuring 35cmx35cmx35cm), with 1-2cm of aspen wood shavings covering the floor of the bin. This bin was placed on top of a plastic box, which raised it, 35cm off the floor. One animal was placed in an identical bin, which had shredded paper covering the bottom instead of wood shavings, as this animal had an allergy to aspen wood shavings.

A cable with female gold connectors extruding from one end was plugged into the plastic headplug and screwed in to secure it. The cable also included a dual FET immediately after the headplug, to which the two recording channels were connected. Several rubberbands were used to reduce the slack in the cable so that the animals could not chew on it.

The output waveforms were amplified by a Grass P511K series pre-amplifier with a 1-30Hz band-pass filter; it was digitised at 128Hz by a Cambridge Instruments model 1401 Data acquisition device, and the digitised waveforms were then passed to the PC and displayed in real time using Spike 2 software (Cambridge Instruments). The analog waveforms were also concurrently displayed in real time on a Hitachi V-134 storage oscilloscope.
A computer-controlled programmable stimulator was used to stimulate the Nucleus Reticularis Pontis Oralis. The stimulator was controlled using a custom-written piece of software which allowed the stimulation intensity to be altered via an arbitrary scale factor, it also allowed the experimenter to manually trigger the stimulation. The digital stimulator was set to deliver a 500ms burst of 100Hz monophasic 0.1ms pulses. The onset of data acquisition from the 1401 was triggered by the output of the digital stimulator.

2.2.3.2 Procedure

At the beginning the cable was plugged into the animal’s headcap, the animal was placed into the bin and allowed to settle into the testing environment. Once the animal had ceased all exploratory behaviour the animal was tested for the quality of the recording and effectiveness of stimulation. In order to be included in the experiment animals had to show clear RSA with no noise or interference problems, and the RSA frequency was required to increase in proportion to the stimulation current given. If any of the animals did not meet these two criteria they were excluded from the experiment.

Next the animals were tested further to ensure that the optimal stimulation voltages would be used so that reliable RSA could be elicited from each animal. In order to do this, animals were started at low stimulation intensities and the stimulation current was increased gradually until the animal showed any movement artefact in their RSA recordings, or showed pronounced head turning. During this process certain thresholds were noted; the minimum stimulation current required to produce reliable RSA, and the maximum current that either showed no movement artefacts, head turning, or produced 9Hz RSA. Using these thresholds (which varied between animals) five equally spaced voltage settings
ranging from minimum to maximum were established for each of the five animals. These stimulation ranges varied between animals to accommodate the varying stimulation thresholds in order to remain consistent with previous experiments carried out in the current lab (Munn & McNaughton, 2008).

2.2.3.2.1 Experiment One

Three experimentally naïve animals were used. Each experimental run involved 49 stimulation periods; one every hour, on the hour, over a 48 hour period. The starting times were staggered across animals throughout the 24 hours of the day (0500 hours, 1000 hours, 1200 hours, 1700 hours, 2200 hours, and 2400 hours). Each stimulation period lasted between 10 and 15 minutes, during which the selected stimulation intensities were run from low-high, then high-low, twice, resulting in four stimulations at each of the stimulation intensities. Animals were fed at their regular time of 1800 on each experimental day. At the end of each 48-hour run the animals were disconnected from the testing apparatus and returned to their home cage.

2.2.3.2.2 Experiment Two

In Experiment Two, one animal from Experiment One as well as two experimentally naïve animals were used. Each of the experimental runs lasted for 58 hours and the feeding times during testing were changed from the normal feeding time so that an animal received food two hours following the starting time of their run, then again at hour 26, and again at hour 50. The beginnings of the runs were staggered across the twenty-four hours of the day, as explained in Experiment One (0200 hours, 1000 hours, 1400 hours, 1400 hours, 1800 hours, and 2200 hours). Stimulations were run as in Experiment One for a total of 59 stimulation periods. Feeding time occurred at unexpected times throughout the
day, in order to see if any hippocampal rhythmicity was occurring in response to the feeding, or to some other daily activity/environmental change (for example light cycle). The recording time was also extended in order to acquire more data with which to analyse any potential circadian rhythm.

2.2.4 Analysis of Data

A mean RSA frequency value was produced from the four recording values for each of the hourly stimulation voltages for each of the 49 or 59 hour time points for each of the twelve runs carried out in these experiments. Since there is a linear relationship between stimulation voltage and the frequency of reticular-elicited RSA, the intercept and slope of the stimulation/frequency function (hereafter referred to as the ‘stimulation intercept’ and the ‘stimulation slope’) were derived from the mean RSA values at each voltage by means of a least-squares linear regression. In this way, each stimulation run yielded a single value for both the stimulation intercept and the stimulation slope. The stimulation intercept was calculated at 1.2 times the threshold voltage used for each animal, not zero voltage. The stimulation slope was then normalised across animals using the maximum value for an animal to normalise all data points for that animal. The stimulation intercept and stimulation slope data were then smoothed using a three point moving average across the hourly time points (thus losing the first and last values in the series) and these averages were then used for further analysis.

Analysis of variance (ANOVA) was performed on the stimulation slope and stimulation intercept data using the PASW software package. Individual polynomial components of the data were examined for possible linear, quadratic, or cubic trends in the data.
In order to compare the effect of food on the stimulation intercept and stimulation slope of hippocampal RSA the data from each of the four runs were lined up so that they began at the first time the animal was fed during the recording, (discarding data from before this point if necessary) and an ANOVA was performed analysing differences between the hours of recording, each of the runs individually, as well as comparing the initial runs (Run 1) with the replication runs (Run 2). This was also done in a way to look at the effect of entry into a novel environment with (Experiment Two) or without (Experiment One) unexpected food, for this the data from the first runs of each experiment (both Run 1s) was analysed from the beginning of the recordings and an ANOVA was performed analysing differences between the hours of recording, and each of the runs, this was carried out for the stimulation slope data and the stimulation intercept data. The replicate runs were not used in this novel environment analysis as at the beginning of Run 2 the animals already had 48 hours experience in the recording environment.

A Post-Hoc restricted analysis was carried out on the stimulation intercept data comparing the difference between Runs 1 and 2 of Experiment One and Run 2 of Experiment Two (excluding Run 1 from Experiment Two) in which the data from the three runs were lined up so that they began at the first time the animal was fed during the recording, (discarding data from before this point if necessary) and an ANOVA was performed analysing differences between the hours of recording and each of the runs.
2.3 Results

2.3.1 Intercept

2.3.1.1 Arrival of food

Figure 2.1 shows the average frequency stimulation intercept of hippocampal RSA at 1.2 times the threshold voltage for each of the runs for both Experiments One and Two. Data are lined up relative to feeding time. Run 1 of Experiment One shows the frequency stimulation intercept of animals that were fed at the regular time of 1800 hours, and as it is the first run the recording environment was still a relatively novel environment. Run 2 only differed from Run 1 in Experiment One in that the animal had previously spent 48 hours in the recording environment. Run 1 in Experiment Two shows the stimulation intercept of animals straight after entering the novel recording environment where they then received unexpected food. Run 2 only differed from Run 1 in Experiment Two in that the animals had already completed Run 1 so were familiar with the environment and may have associated entry into the environment with the presentation of food which followed.

An ANOVA exploring the relationship between each of the four runs of both Experiment One and Experiment Two showed that there was a significant difference between the first runs of each experiment and the second runs (hour x run; cub x dev, \( F(1,28) = 11.925, p=0.026 \)), suggesting that the replications of the experiment produced different results.
Figure 2.1. The mean frequency of reticular-elicited hippocampal RSA over a 28-hour period, in which animals receive food at Hour One. Experiment One runs are lined up on regular feeding time of 1800 hours, and the starting times occur randomly across the x-axis, these are coded as Not Start/Run1 and Not Start/Run2 (Not Start referring to the fact that the first data points do not show the start of recording – but the start of feeding). Experiment Two runs are lined up on feeding time which occurred two hours after the beginning of recording, these are coded as Start/Run1 and Start/Run2 (Start referring to the fact that the first data points show the start of recording).

2.3.1.1.1 Post-Hoc Restricted Analysis

An ANOVA was run exploring the relationship between the hourly frequency stimulation intercept of Runs 1 and 2 of Experiment One and Run 2 of Experiment Two. Results showed that these three runs were similar over the entire recording duration (hour x run; cub x dev, $F(1,28) = 0.825$ n.s.). As the previous analysis (including Run 1 of Experiment Two) showed a significant difference between runs and this one does not, this suggests that Run 1 of Experiment Two differed from the other three recording sessions and produced
the significant difference between the initial runs of each experiment and their replications. In comparison with the other three recording sessions, Run 1 of Experiment Two shows a sharp decrease following presentation of a novel environment as well as unexpected food showing a low point at hour 8, then increasing up to hour 17.

2.3.1.2 Entry into the environment

Figure 2.2. The mean frequency of reticular-elicited hippocampal RSA over a 28-hour period, in which Hour One represents the hour in which the animals were placed into the novel environment for the first time (only the initial runs as at the beginning of Run 2 the animals had already 48 hours experience in the recording environment). Run 1 of Experiment One is lined up on entry into the environment, which does not line up with feeding time; it is coded as Not Food (meaning that the start of recording – and therefore the first data points – do not align with feeding time). Run 1 of Experiment Two is lined up on entry into the environment which also lines up with the arrival of unexpected food two hours after entry into the environment, it is coded as Food (meaning that the start of recording – and therefore the first data points – align with feeding).
Figure 2.2 shows the average frequency stimulation intercept of hippocampal RSA at 1.2 times the threshold voltage for each of the runs for both Experiments One and Two. Data are lined up relative to the beginning of each recording. Results showed a significant difference in the mean frequency of hippocampal RSA across the hours of recording showing a cubic pattern (hour; cub, \( F(1,28) = 12.244 \), \( p=0.025 \)). There was also a significant difference between Run 1 of Experiment One (entry into a novel environment) and Run 1 of Experiment Two (entry into a novel environment and unexpected food) (run x hour; dev x cub, \( F(1,28) = 9.318 \), \( p=0.038 \)).

2.3.2 Slope

2.3.2.1 Arrival of food

Figure 2.3 shows the average stimulation slope of hippocampal RSA for each of the runs for both Experiments One and Two relative to feeding time.

![Graph showing average stimulation slope of hippocampal RSA](image)

**Figure 2.3.** The mean stimulation slope of reticular-elicited hippocampal RSA over a 28-hour period, in which animals receive food at Hour One. This graph used the same coding as Figure 2.1.
Runs 1 and 2 of Experiment One and Run 2 of Experiment Two show a general increase in stimulation slope for up to 7 hours after feeding. Run 2 of Experiment Two shows a larger but briefer increase in stimulation slope than that seen in both runs of Experiment One. Run 1 of Experiment Two shows a sharp decrease in stimulation slope following the arrival into a novel environment as well as the presentation of unexpected food, showing a low point at hour 4, before increasing up to hour 7. There was no significant difference between any of these runs (hour x run; cub x dev, $F(1.28) = 0.003$, n.s.).

2.3.2.2 Entry into a novel environment

Figure 2.4 shows the effect of entry into a novel environment on the average stimulation slope of hippocampal RSA in both Run 1 from Experiment One and Run 1 from Experiment Two.

Run 1 of Experiment One shows the stimulation slope of animals straight after entering the novel recording environment and for the following 27 hours, these animals were fed at their usual time of 1800 hours. Run 1 in Experiment Two shows the stimulation slope of animals straight after entering the novel recording environment where they then received unexpected food.

Animals in both experiments showed a decrease in slope following entry into the environment (hour x run; lin x cub, $F(1.28) = 0.51$, n.s.).
Figure 2.4. The mean stimulation slope of reticular-elicited hippocampal RSA over a 28-hour period, in which Hour One represents the hour in which the animals were placed into the novel environment for the first time (only the initial runs as at the beginning of Run 2 the animals had already 48 hours experience in the recording environment). Run 1 of Experiment One is lined up on entry into the environment, which does not line up with feeding time; it is coded as Not Food. Run 1 of Experiment Two is lined up on entry into the environment which also lines up with the arrival of unexpected food two hours after entry into the environment, it is coded as Food.

Figure 2.5 shows the effects of food itself estimated as the difference between the food and not food groups of Figure 2.4 on the stimulation slope of hippocampal RSA.

The ‘food effect’ shown in Figure 2.5 shows a tendency to increase the stimulation slope which peaks between six and eight hours after feeding. This effect appears similar to the effect of feeding on the stimulation intercept of hippocampal RSA that is illustrated in Figure 2.3.
The difference in the mean stimulation slope of reticular-elicited hippocampal RSA in animals that have just entered a novel environment either with or without unexpected food over a 28-hour period. This was determined by subtraction between the data sets shown in Figure 2.4.

2.4 Discussion

The results showed that expected food did not have any effect on the stimulation intercept of hippocampal RSA, but that unexpected food produced a significant decrease in the stimulation intercept of hippocampal RSA for up to 7 hours following arrival of unexpected food. It was also shown that food, both expected and unexpected, produced an increase in the stimulation slope of hippocampal RSA. Although in Figure 2.3 Run 1 in Experiment Two shows a sharp initial decrease in stimulation slope, further analysis looking at the effect on RSA of the first entry into the novel environment showed that this dip was present in the initial runs of each experiment, and not in the following runs (once the environment was no longer novel). One possible explanation for the difference between Runs 1 and Runs 2 is that the environment they are being placed in is no longer a novel environment, suggesting that entry into a new environment causes
a sharp decrease in the stimulation slope of hippocampal RSA, this decrease is smaller in animals that are presented with food upon entry into the novel environment. When looking at the change in slope starting at feeding time (Figure 2.3) there was no significant difference between each of the runs or experiments, the runs all showed the same cubic pattern across the recording. A cubic pattern was also seen when looking at the change in the stimulation intercept starting at feeding time (Figure 2.1), but only in the runs where food was expected (Runs 1 and 2 from Experiment One, and Run 2 from Experiment Two). There are definite cubic patterns of hippocampal RSA shown when feeding time is lined up, as well as when the data was lined up on entry into the novel environment, suggesting that there is some sort of periodic oscillation in hippocampal RSA.

It was hypothesised that the hippocampal RSA produced by reticular stimulation would show a circadian variation over a 24-hour cycle, the activity recorded in these experiments did show periodic oscillations, however it is not strictly circadian, as it does not appear to be modulated on a 24-hour cycle. It is possible that the variation in stimulation slope and frequency shown in these experiments may be tied to a behavioural stimulus, such as feeding or entry into the environment, rather than a strict circadian timing mechanism. The hippocampus (particularly area CA3) has been shown to respond to novelty (Hunsaker, Rosenberg, & Kesner, 2008; Villarreal, Gross, & Derrick, 2007). Animal responses to novelty have been shown to be linked with anxiety-like behaviour, animals who showed persistently low exploratory activity were then shown to display more anxiety-like behaviour in a number of behavioural tests of anxiety (Mällo et al., 2007). It is possible that the effect shown in response to entry into the environment was caused by novelty-related anxiety.
Unexpected food produced a decrease in the stimulation intercept of hippocampal RSA, previous studies have shown that anxiolytics produce a decrease in the stimulation intercept of artificially stimulated hippocampal RSA, usually in a dose-dependent pattern (Coop & McNaughton, 1991; McNaughton & Sedgwick, 1978; Munn & McNaughton, 2008; Zhu & McNaughton, 1991). As all anxiolytics so far tested produce this characteristic response, which has now been shown to be produced by unexpected food, it is possible that the unexpected presentation of food may hold anxiolytic potential. This potential anxiolytic effect of unexpected food may be caused by any one of the chemicals released in response to unexpected food.

Future studies looking at the circadian oscillation of hippocampal RSA should look at longer experimental runs in order to see if any of the cubic oscillations we observed occur repeatedly and on what sort of time-frame the oscillation occurs. They could also get a clearer view of the effect of food on hippocampal RSA by extending the recording time for several days, in order to see how the effect lasts over time and if it is repeated with every meal.

2.5 Conclusion

It was found that food causes an increase in the stimulation slope of hippocampal RSA. It was also shown that being placed into a novel environment produced a decrease in the stimulation slope of hippocampal RSA, and this decrease is smaller when animals were presented with unexpected food shortly after entry into the novel environment. Unexpected food causes a decrease in the stimulation intercept of hippocampal RSA, an effect that is also produced by anxiolytics.
CHAPTER THREE

The Effect of Leptin on Rhythmic Slow-Wave Activity

3.1 Introduction

In Chapter 2 it was shown that unexpected food intake resulted in a decrease in the frequency of reticularly-stimulated hippocampal Rhythmic Slow-Wave Activity (RSA). This is an effect that is also produced by clinical anxiolytics, through a variety of possible mechanisms as it has been shown to be produced by both GABAergic (McNaughton & Sedgwick, 1978) and serotonergic (Coop & McNaughton, 1991) anxiolytics. The effect seen in Chapter 2 was produced following the consumption of an unexpected meal, it is possible that the effect was caused by a response to food intake.

Intake of fats will promote the release of the hormone leptin from adipocytes (Dallongeville et al., 1998). There is evidence that the serotonin precursor 5-hydroxytryptophan (5-HTP) dose-dependently increases serum leptin in mice (Yamada, Sugimoto, & Ujikawa, 1999). Furthermore, the 5 hydroxytryptamine (5-HT) transporter, which is targeted by the specific serotonin reuptake inhibitors (SSRIs), is linked to obesity, as male 5-HT knock-out mice developed obesity (Uceyler et al., 2010). Leptin has been shown to produce an anxiolytic effect equal to that of the serotonin reuptake inhibitor fluoxetine in behavioural tests of anxiety (Liu et al., 2010).

There is a lot of evidence suggesting the anxiolytic potential of leptin, as all anxiolytics so far tested affect hippocampal RSA, and the hippocampus is thought to be crucial in the regulation of anxiety, it is of interest to investigate the effect of leptin on hippocampal RSA.
The aim of the current experiment is to examine the effect of leptin on reticular-elicited hippocampal RSA. Two experimental doses of 0.5mg/kg and 1.0mg/kg of leptin were tested. 1.0mg/kg (i.p.) doses have been shown to have an anxiolytic effect on behaviour in a comparative study with fluoxetine by (Liu et al., 2010) and 0.5mg/kg was tested to observe whether or not there was a dose-dependent effect of leptin on RSA. Since all the anxiolytics so far tested reduce the frequency of hippocampal RSA (most often in a dose-related manner) and leptin has shown anxiolytic potential, it was hypothesised that leptin would reduce the frequency of hippocampal RSA, and that the 1.0mg/kg dose would produce a greater decrease in RSA frequency than the 0.5mg/kg dose.

3.2 General Method

3.2.1 Subjects

There were two subjects in the leptin Reticular Stimulation experiment. Both had previously been used in Experiment One, which was described in Chapter 2. The rats began this leptin experiment within two weeks of finishing their final run in the first experiment carried out in Chapter 2. They were two male Sprague-Dawley rats were obtained from University of Otago Department of Laboratory Animal Sciences. The animals were 6-7 weeks on arrival, and 11 – 14 weeks at time of surgery, weighing between 452 and 475 grams at surgery. These animals were kept under the same housing, temperature, and dietary conditions as described in Chapter 2. These animals had undergone surgery and electrode implantation before taking part in the previous RSA experiment described in Chapter 2. Details of the electrodes, anaesthesia, and surgery can be found in Chapter 2 (sections 2.2.2.1, 2.2.2.2, and 2.2.2.3 respectively).
The surgical and experimental procedures described in this thesis complied with the University of Otago’s Code of Ethical Conduct and The Committee on Ethics in the Care and Use of Laboratory Animals granted ethical approval for this study (reference number D44/11).

3.2.2 Reticular Testing

3.2.2.1 Apparatus

A detailed description of the equipment used in this experiment can be found in Chapter 2 (Section 2.2.3.1), as animals were tested with the same equipment for both experiments.

3.2.2.2 Procedure

The cable was plugged into the animal’s headcap and the animal was allowed to settle into the testing environment as described in Chapter 2. The animal was initially tested to ensure that the five stimulation intensities used in the experiment in Chapter 2 were still effective at producing reliable RSA. Three different doses of leptin were used; these were 1mg/kg, 0.5mg/kg, and 0mg/kg (control). Animals received each of these doses twice, with two experimental days per dose. One animal was administered these doses in an ABCCBA fashion and the other animal was administered the doses in a CBAABC fashion to counterbalance any effect of order. Each testing session lasted for 4 hours, and involved 17 stimulation runs. The first stimulation run was carried out 15 minutes prior to the drug administration, there was then another stimulation run directly post-injection, and then another run 15 minutes post-injection. Stimulation runs then occurred every 15 minutes until the final stimulation at 225 minutes post injection. Each stimulation period lasted for five to ten minutes, over which time the stimulation was run as described in Chapter 2, running the chosen voltage span
four times (low to high, high to low, repeat). The experiment lasted for 16 days, including 6 experimental days with a rest period of two days before the next experimental day to ensure complete elimination of the leptin from the animal before the next dose was administered.

3.2.2.3 Drug Preparation and Administration

Over the six experimental days of this experiment each rat was delivered each of the drug concentrations twice. The three leptin concentrations were 1mg/kg LEP, 0.5mg/kg LEP, and 0mg/kg LEP (in which the animal was administered a matched equivalent volume of Phosphate Buffered Saline (PBS) vehicle). All PBS used was pH adjusted to 8.0. Injectable leptin solution was prepared by diluting 1mg of recombinant mouse leptin (supplied by NIDDK's National Hormone and Peptide Program and A.F. Parlow) in 1ml of PBS. The LEP doses were prepared freshly on the day of injection and were administered via intra-peritoneal (i.p.) injection using a 1ml syringe and a 26g needle at a volume of 1 ml/kg of body weight. This yielded a dose of 1mg/kg in the undiluted solution. In order to produce the 0.5mg/kg dose of LEP, the original 1mg vial recombinant mouse leptin was diluted in 2ml PBS, which yielded a dose of 0.5mg/kg in the diluted solution. This dilution was carried out to deliver the same ratio of solution to body weight for each animal.

3.2.3 Analysis of Data

A mean RSA frequency value was produced from the four recording values for each of the five stimulation voltages for each of the quarter-hourly time points investigated in the current experiment. Since there is a linear relationship between stimulation voltage and the frequency of reticular-elicited RSA, the intercept and slope of the stimulation/frequency function (hereafter referred to as
‘stimulation intercept’ and ‘stimulation slope’) were derived from the mean RSA values at each voltage by means of a least-squares linear regression. In this way, each stimulation run yielded a single value for both stimulation intercept and stimulation slope. The stimulation intercept was calculated at 1.2 times the threshold pre-drug voltage used for each animal, not zero voltage in order to remain consistent with previous experiments carried out in the current laboratory (Munn & McNaughton, 2008). The stimulation slope was then normalised across animals using the maximum value for each animal to normalise each data point. The stimulation intercept and stimulation slope data were then used for further separate analyses.

Analysis of variance (ANOVA) was performed on the stimulation slope and stimulation intercept data using the GENSTAT software package. Subjects, Experimental days, dose, and time were examined for possible linear, quadratic, or cubic trends in the data.

3.3 Results

3.3.1 Intercept

Figure 3.1 shows the effect of 0, 0.5, and 1.0mg/kg of leptin on the stimulation intercept of hippocampal RSA averaged across both repetitions of these doses in both animals at 1.2 times the threshold pre-drug voltage over the 225 minutes following the time of injection. Overall there was no change across time (time, quad $F(1,42)=0.32$, n.s.) and no significant variation between the three leptin doses (dose x time, lin x quad $F(1,42)=0.10$, n.s.).
Figure 3.1. The effect of 0mg/kg LEP, 0.5mg/kg LEP, and 1.0mg/kg LEP on the mean frequency of reticular-elicited hippocampal RSA at 30 minute intervals for 225 minutes post-injection.

3.3.2 Slope

Figure 3.2 illustrates the effect of 0mg/kg LEP, 0.5mg/kg LEP, and 1.0mg/kg LEP on the mean normalised stimulation slope of hippocampal RSA for both animals over the 225 minutes following the time of injection. 1.0mg/kg produced a clear increase in the mean stimulation slope starting at 30 minutes post-injection and decreasing back to a similar range as the other two doses at 90-150 minutes post-injection and 0.5mg/kg had an intermediate effect between the 1.0 and 0.0mg/kg doses, producing a linear dose response curve (dose x time, lin x quad $F(1,42)=4.74$. p= 0.035).
Figure 3.2. The effect of 0mg/kg LEP, 0.5mg/kg LEP, and 1.0mg/kg LEP on the stimulation slope of reticular-elicited hippocampal RSA at 30 minute intervals for 225 minutes post-injection.

3.4 Discussion

The results of this experiment showed that leptin had no effect on the stimulation intercept of hippocampal RSA at either 0.5mg/kg or 1.0mg/kg. This result does not support the hypothesis that leptin would reduce the frequency of hippocampal RSA in a dose related manner. There was a significant dose-dependent increase in the stimulation slope. This effect is similar to that of 5HT_{1A} agonist buspirone (BUS) which produced a slight increase on the stimulation slope (Coop & McNaughton, 1991), but differs in that BUS also reduced the stimulation intercept.

This change in the stimulation slope suggests that leptin produces a decrease in the stimulation intercept at low RSA frequency and an increase in the...
stimulation intercept at high frequency RSA, which presents itself as producing no overall effect on the stimulation intercept of hippocampal RSA. This effect presents the possibility that, at least in tasks in which hippocampal RSA frequency is typically low (up to around 5 or 6Hz), leptin may be anxiolytic. The reasons behind this frequency-specific effect of leptin are unclear, however corticosterone has also been shown to produce an increase in the stimulation slope of hippocampal RSA (McNaughton & Coop, 1991).

Kramis, Vanderwolf, and Bland (1975) showed that there is a distinction between high and low frequency hippocampal RSA, in that 0-7Hz RSA is seen in immobile animals, whereas 7-12Hz RSA is produced during movement. Sainsbury (1998) also distinguish between two kinds of hippocampal RSA (also called theta), Type 1 theta (6-12Hz) and Type 2 theta (4-9 Hz). Sainsbury, Heynen, and Montoya (1987) showed that Type 2 theta occurred in rats when they were in the presence of a threatening animal (cats or ferrets were used in the experiment). This suggests that hippocampal RSA can be divided into two types and that Type 2 (atropine sensitive) theta may be responsible for reaction to threat while Type 1 theta serves some other function. The data in the current experiments suggest that leptin may be showing an anxiolytic effect on hippocampal RSA, but only at lower frequencies. It is possible that leptin may only be decreasing lower-frequency elicitation and, in fact, increasing the higher-frequency elicitation.

While it would appear that there is no effect of leptin on the stimulation intercept, the baseline produced by the PBS control was not consistent over the experimental sessions. This suggests the number of runs may have been too small and a great deal of error is present in the data. It would be useful to rerun this
experiment increasing the number of runs per animal, in order to provide a consistent baseline from which to compare drug effects.

Another important finding of this experiment was that the strongest effects of leptin on the stimulation slope of hippocampal RSA occurred between 30 minutes and 90 minutes post injection. This is important because it gives a good indication of how much time should pass between drug administration and testing using leptin. This result should be taken into account when designing future experiments testing the effect of leptin on behavioural measures of anxiety. Future experiments should investigate the effect of leptin on tests of behavioural anxiety that produce a range of different hippocampal RSA frequencies, to see if leptin produces an anxiolytic effect in tasks that produce low frequency RSA, and what effects it has on behavioural tasks that produce medium frequency RSA and high frequency RSA. This would help explore the frequency specific effect of leptin, whether anxiety produced at low frequencies is markedly different from anxiety produced at high frequencies, and why leptin affects one as an anxiolytic and the other as an anxiogenic.

3.5 Conclusion

Leptin was shown to dose-dependently increase the stimulation slope of hippocampal RSA suggesting that leptin may reduce the stimulation intercept at low hippocampal RSA frequencies but increased it at high frequencies. It was also found that i.p. administration of leptin produces the largest changes on the stimulation slope of hippocampal RSA between 30 minutes and 90 minutes post-injection. This result suggests that future tests using leptin should time their experiments to occur within that time frame to ensure that leptin is producing its maximum effect.
CHAPTER FOUR

The Effects of Leptin on Fixed Interval Responding

4.1 Introduction

Chapter 3 showed that leptin changes the slope of the function relating stimulation intensity to hippocampal Rhythmic Slow-Wave Activity (RSA) frequency while not changing the intercept. This suggests that leptin can preferentially reduce lower RSA frequencies while increasing higher RSA frequencies. So, at least in behavioural tasks in which RSA frequency is predominantly low, leptin may be anxiolytic. In order to test this hypothesis behaviourally, we screened leptin for a typical anxiolytic profile in the fixed interval 60 second (FI60) task. Although there are other behavioural tests which could be used to determine leptin's anxiolytic potential, such as the open field or the elevated plus maze, the FI60 task produces a more uniform response to anxiolytics (Woodnorth & McNaughton, 2002). It was therefore of interest to examine the effect of leptin on responding in the FI60 schedule.

Animals, having previously learned that lever pressing produces a reward regardless of the time lapse since the last reinforcement, then learn the FI60 task in which they generally learn to inhibit their behavioural response of lever pressing until times when responding will elicit reinforcement. This behavioural inhibition is characterised by animals making fewer responses directly after being presented with a reward, and showing an increase in responding towards the end of the inter-reward periods. This gradual decreasing occurrence of a learned behaviour is generally taken as an indication that the animals are inhibiting the previously learned behaviour of lever pressing. This pattern of learning can be
affected by administering drugs to the animals; anxiolytics produce a unique effect on responding in the FI60 task. Animals administered anxiolytics generally show a release of responding in the early part of the inter-reward period, showing a lack of the behavioural inhibition of responding which is seen in drug-free animals (Panickar & McNaughton, 1991).

In order to assess the effects of leptin on FI60 response, two doses of leptin (LEP) were used: 0.5mg/kg LEP and 1.0mg/kg. Two control groups were also included in this experiment; one inactive control group, which was administered the phosphate buffered saline (PBS) vehicle solution and one active control group, which was administered 5mg/kg of chlordiazepoxide (CDP). A dose of 5mg/kg CDP was chosen as this dose has previously been shown to produce an anxiolytic effect in FI60 task (Woodnorth & McNaughton, 2002). It was hypothesised that leptin administration would produce a characteristic anxiolytic-like release of responding early in the FI60 schedule, with the 1.0mg/kg dose producing a more pronounced effect showing a dose-dependent increase.

4.2 **General Method**

4.2.1 **Subjects**

Twenty-four male Sprague-Dawley rats were obtained from the University of Otago Department of Laboratory Animal Sciences. Animals were aged 7 weeks upon arrival. The animals were experimentally naive before the experiment. At the beginning of the pre-training sessions the animals were 10 weeks old and weighed between 243 and 323 grams.

The animals were housed in regular group cages, as described in Chapter 2, in groups of four. Once animals were allocated into different drug groups they were rehoused so that one animal from each of the four drug groups was in each
cage. This was to ensure that all the animals from a single drug group were not housed together. Twelve days before the pre-training sessions began the animals were taken off ad libitum access to food and placed on a restricted diet of Reliance Stock food pellets the amount of which was varied daily in order to maintain the animals’ weights at 80% of their free feeding weight, they were given ad libitum access to water throughout the experiment. Animals were kept in the same temperature and lighting conditions as described in Chapter 2.

The surgical and experimental procedures described in this thesis complied with the University of Otago’s Code of Ethical Conduct and The Committee on Ethics in the Care and Use of Laboratory Animals granted ethical approval for this study (reference number D18/12).

4.2.2 Apparatus

Six standard operant chambers (Camden instruments, U.K.) were used throughout the training sessions as well as all the experimental trials. The dimensions of the operant chambers were 57.5cm by 34.5cm by 39cm. The operant chambers contained a smaller housing chamber, inside of which the animal would carry out the training/experimental trials; the dimensions of the housing chamber were 24cm by 24cm by 26cm. A food delivery system distributed individual 45mg Dustless Precision Pellets (Campden Instruments LTD., UK) as reinforcers to a food hopper in the housing chamber through a plastic tube. The front-facing wall of the operant chamber was hinged from the bottom in order to allow access to the interior. The front-facing wall also had a circular tinted window 16.6cm in diameter, which allowed a view of the interior of the chamber.
The interior chamber of the operant chamber had three metal walls, a metal ceiling, and a horizontal grid floor. The front-facing wall of the housing chamber was a transparent Perspex wall that could be unlatched from the top of the box and pulled down to allow access to the chamber. One of the walls had a recessed 5cm by 6.5cm food hopper with a Perspex door hinged from the top, the food hopper was set in the middle and at the bottom of the wall. Two retractable metal levers were set into the wall, one on either side of, and equidistant from, the hopper. Directly above each lever there were two 2.8W stimulus lights set into the wall. The other two metal walls of the housing chamber were bare. In the centre of the metal ceiling of the housing chamber there was a 2.8W house light, which was on throughout the training and experimental trials. There was also an electric fan, which provided ventilation for the animals, and also produced a constant level of background noise.

Six operant chambers were used concurrently for the experimental trials. They were set up three across with a second row of chambers resting on top of the first, on a bench 75.5cm from the floor. Three IBM compatible computers, which were running Visual Basic 6 software with LABJACK control components, each controlled two operant chambers. Custom Visual Basic 6 programs were used to deliver the three training schedules. This meant that the computers controlled the timing and delivery of the reinforcements, as well as recording the number of lever presses the animal made in the Continuous Reinforcement (CRF) and Fixed Interval 60 seconds (FI60) tasks, and the number of nose pokes into the food hopper in the Random Time 30 seconds (RT30) task. The computers also recorded the time each lever press was made during the FI60 task, these responses were added into one of twelve five-second bins, according to the interval since the last
reward that they were made. Responses made between zero and five seconds since the last reward were allocated to bin one; responses made between five and ten seconds since the last reward were allocated to bin two and so on.

4.2.3 Procedure

4.2.3.1 Drug assignment and Preparation

The animals were each assigned to one of the 1mg/kg LEP, 0.5mg/kg LEP, PBS (control), or 5mg/kg CDP (active control) groups in a counterbalanced fashion so that the average CRF response rate of the four groups immediately before the experiment was as similar as possible. The mean CRF response rates of the groups differed by not more than 5% overall. Then animals were assigned to group cages of four animals and rehoused so that each cage contained one animal from each of the four drug groups. This was to avoid biases that may have arisen from housing multiple animals from the same drug group together.

The two doses of LEP were prepared as detailed in Chapter 3. Injectable CDP solution was prepared by diluting 5mg of Chlordiazepoxide Hydrochloride (CDP; Sigma Chemical Co., Louis, USA) in 1ml of 0.9% saline. The CDP solutions were prepared freshly on the day of injection and were administered via intraperitoneal injection using a 1ml syringe and a 26g needle at a volume of 1ml/kg of body weight. This yielded a 5mg/kg dose for animals in this drug group.

Animals in the 0mg/kg, 0.5mg/kg and 1.0mg/kg leptin drug groups were administered LEP 35 minutes before they were scheduled to begin their FI60 training session. This was to ensure that the maximum effect of leptin would be occurring during their testing session, specifically to peak half way through the thirty-minute FI60 session. The time interval was chosen based on the electrophysiological experiments in Chapter 3 that suggest that 50 minutes is the
time of maximum inflection in the slope reduction caused by 1.0mg/kg leptin. Animals in the 5mg/kg CDP drug group were administered CDP 10 minutes before they were schedules to begin their FI60 training session to ensure that the maximum effect of CDP would be occurring during the session, this time interval was based on previous data from electrophysiological and FI60 experiments using CDP (Woodnorth & McNaughton, 2002).

4.2.3.2 Autoshaping

Each of the animals participating in the FI60 behavioural experiment received preliminary training to ensure that the animals could reliably insert their head into the hopper in order to retrieve a food pellet. Each animal was run on an RT30 schedule once a day. In each RT30 training session, a food pellet was delivered at pseudo-random intervals with a range of 0-60 seconds and a mean reinforcement interval of thirty seconds, the distribution of these pellets was determined by the computer. Both levers were retracted for the entire session, as no response was required from the animal to elicit food pellets. The hopper light shone for one second to indicate the delivery of a food pellet, the noise of the delivery system that delivered the pellet also served as an indicator. Each of these RT30 training sessions lasted for thirty minutes and each of the animals were run once a day at the same time each day. In order to encourage the animals to investigate the food hopper and put its head inside, a small pellet of the animal's regular food (Reliance Stock Pellets) was placed in the hopper so that it wedged open the Perspex hopper door. Once each animal was reliably responding to the light and sound cues of reinforcement by putting its head in the hopper, they were moved onto the next stage of training. The animals all required five sessions of RT30 training before they were reliably responding to reinforcement cues.
4.2.3.3 Continuous Reinforcement

Having completed the RT30 training schedule, all animals started on a CRF schedule. Each CRF training schedule lasted for thirty minutes and each of the animals ran once a day, at the same time each day, just as described in the RT30 schedule. The lever to the left of the hopper door was extruded throughout the CRF training sessions, and every press of the lever triggered the delivery of a food pellet, regardless of the time passed since the last food pellet. To encourage the rats to approach the lever in their first training session, the protruding lever was coated in wet mash made from the animals’ regular food (Reliance Stock Pellets), sucrose and water. Once the animals were consistently pressing the lever and receiving a food pellet, another two drug-free CRF sessions were run to ensure the rate of responding was stable. Four training days were required to establish reliable lever pressing responses in the animals. Prior to the final CRF training session, animals were assigned to four separate drug groups based on their CRF responses to ensure that the average CRF response rates were consistent across the four groups. This rehousing was carried out in order to remain consistent with previous experiments carried out in the current lab (Munn & McNaughton, 2008). They then ran a final CRF training session after having been administered the drug corresponding to the group to which they were assigned. The animals began their Fixed Interval training the day following this final CRF training session.

4.2.3.4 Fixed Interval

Having completed the CRF training, the animals began a FI60 reinforcement schedule. A FI60 schedule requires 60 seconds to elapse since the previous delivery of a food pellet before a lever press will trigger the delivery of another food pellet. Once 60 seconds have passed following delivery of a food
pellet, one single lever press will produce another food pellet. Following this food pellet, another 60-second non-reward period elapsed, and so on until the thirty-minute training session was finished. The animals were run in the FI60 task once each day, at the same time each day (between 1200 and 1600). The FI60 training sessions ran every day for 14 consecutive days.

4.2.4 Analysis of Data

For the purposes of analysis and illustration, the twelve bins were compressed into six bins so that the responses from bins one and two were now placed into bin one, responses in bins three and four were now in bin two, etc. Raw data were transformed by a logarithmic \(\log_{10}(X+1.0)\) function in order to normalise the error variance (Zar, 2007).

The raw data were analysed by Analysis of Variance (ANOVA) using the Genstat package (VSN international). Within subject factors such as responding across bins and days were extracted, and the presence of linear, quadratic, and cubic polynomial components in the data were analysed to investigate the effect of each drug on the response rates in the beginning, middle, and end of the FI60 schedule as well as over experimental days.

The data were subjected to gross analysis in order to assess overall interactions in the data. Then a restricted analysis compared the 0mg/kg PBS control group with the 0.5mg LEP and the 1.0mg LEP groups to investigate trends in the effect of dose of leptin on the response rates throughout the FI60 schedule.

4.3 Results

Figure 4.1 shows the average number of bar press responses per bin for each drug group at the beginning, middle, and end of the 15 days of testing. At times immediately after reinforcement, animals displayed low responding.
Responses increased as time to reinforcement approached (bins, lin, $F(1,6) = 694.86. p<0.001$) reaching a maximum in close proximity to reinforcement. Immediately prior to reinforcement, the increase in response rate reached a plateau (bins, quad, $F(1,2) = 26.34. p<0.001$).

Figures 4.2A and 4.2C illustrate the number of responses made at the beginning of (bin 1) and at the end (bin 6), respectively, of each inter-reward period across the 15 experimental days. Animals showed a general decrease in responding across days in bin 1 and an increase in responding across days in bin 6 (bins x days, quad x lin $F(1,22) = 137.62. p<0.001$).

As well as the difference in response rate across bins animals also showed differences across the different drug groups. Animals in the 1.0mg/kg LEP group showed an overall reduced rate of responding compared to the control group and the 5.0mg/kg CDP, and the 0.5mg/kg group showed an increase in response rate compared to the 0mg/kg LEP control group (see Figure 4.1). This effect was small at the beginning of training; became more pronounced through the middle days of the training, before reducing in size toward the end of training (bins x drug x days, quad x drug x quad $F(1,22) = 6.06. p=0.014$).
**Figure 4.1.** The average number of responses per bin of the FI60 schedule by each of the 0mg/kg LEP, 0.5mg/kg LEP, 1.0mg/kg LEP, and 5.0mg/kg CDP groups. Each binpair contains the average data from all animals in each drug group averaged across Days 1-4 (A), Days 6-9 (B), and Days 11-15 (C). The non-linear axis is the result of the logarithmic transform used to normalise the data.
4.3.1 Post-Hoc Restricted Analysis

The presence of a significant effect of drug in the overall analysis (as shown in Figure 4.1C) led to a restricted analysis in order to explicitly examine the effect of different doses of leptin against control in the absence of the CDP active control. Animals in the 1.0mg/kg LEP group showed a significantly lower overall rate of response than both the 0.5mg/kg LEP and 0mg/kg LEP groups, and the 0.5mg/kg LEP group showed a higher response rate than both the 0mg/kg LEP control group and 1mg/kg LEP group. These differences were most pronounced in the middle bins (2, 3, and 4) compared to the earlier ones (1, 5, and 6), and the response rates between the dose groups was greatest during the middle days of training (Days 6-9 – Figure 4.1B) with the 0mg/kg LEP groups responding dropping to rate closer to that of the 1.0mg/kg LEP group in the final days of the training (Days 11-15, Figure 4.1C), (bins x dose x days, quad x quad x quad $F(1,1680) = 6.06. p=0.014$; cub x quad x quad $F(1,1680) = 7.01. p=0.008$).

The difference in dose changed over days of training, producing similar effects in Days 1-4 (Figure 4.1A), with the 1.0mg/kg LEP dose showing an overall decrease across Days 6-9 (Figure 4.1B), and the 0.5mg/kg LEP dose showing an increase in responding in the early bins across Days 11-15 (Figure 4.1C) (bins x dose x days; quad x cub x lin $F(1,1680) = 7.31. p=0.007$).
Figure 4.2. The average number of bar press responses made in Bin 1 (A), Bin 3 (B), and Bin 6 (C) of the FI60 schedule by the 0mg/kg LEP, 0.5mg/kg LEP, 1.0mg/kg LEP and 5.0mg/kg CDP drug groups over the 15 days of the experiment. The non-linear axis is the result of the logarithmic transform used to normalise the data.
4.4 **Discussion**

The present experiment demonstrates that a high dose (1.0mg/kg LEP) of leptin depresses responding in the FI60 schedule in bins towards the end of the inter-reward period, but that a low dose (0.5mg/kg LEP) releases responding in the early bins of the schedule. The low dose, therefore, is producing a characteristic anxiolytic-like effect. The 5.0mg/kg dose of CDP acted successfully as an active control, showing an overall increase in responding, particularly in the early bins as in previous experiments with this drug.

It was hypothesised that leptin would produce a characteristic anxiolytic-like release of responding early in the FI60 schedule in a dose-dependent fashion. This was not observed in the expected form. We did see a modest anxiolytic-like effect in the 0.5mg/kg LEP dose that was equivalent to CDP at the end of testing; but the higher dose of 1.0mg/kg showed a depression of responding. The results of the present experiment are similar to those produced by the serotonergic agonist buspirone (BUS) (Panickar & McNaughton, 1991), as well as results produced by the specific serotonin reuptake inhibitor (SSRI) fluoxetine (FLU) (Munn & McNaughton, 2008).

The current experiment showed that leptin produces a similar behavioural effect on the FI60 task to BUS, which suggests that leptin may be working in a pharmacologically similar way to BUS rather than the Gamma-Amino Butyric Acid-(GABA)ergic anxiolytics such as benzodiazepines (BDZs). BDZs and BUS are pharmacologically distinct, and this can explain the behavioural differences they produce in the FI60 task. Chlordiazepoxide (CDP), a BDZ, is a GABA-A agonist, and has been shown to block the release of the endogenous stress hormone corticosterone (CORT) as a response to stress (File, 1982). However, BUS, a direct
5 hydroxytryptamine (5-HT)$_{1A}$ agonist, does not block stress-induced CORT release (Urban, Van de Kar, Lorens, & Bethea, 1986), and in fact, at high doses (over 1mg/kg), triggers the release of CORT without the presence of an external stressor (De Boer, Slangen, & Van der Gugten, 1991; Matheson, Gage, White, Dixon, & Gipson, 1988). Animals can become tolerant to this CORT-releasing effect through long-term treatment with BUS (De Boer et al., 1991). Studies have shown that long-term exposure to BUS (i.e. once the animals have become tolerant to the CORT-releasing effect) produces a response release early on in the FI60 task, and a general increase in responding throughout the FI60 schedule, both of the behaviours seen in animals being treated with BDZs (Zhu & McNaughton, 1995). BUS and CDP also show similar effects on responding in the FI60 task when endogenous release of CORT has been blocked in the animals through metyrapone treatment (McNaughton, Panickar, & Logan, 1996).

These studies demonstrate that the CORT-releasing effect of BUS is to some extent responsible for the behavioural differences between it and BDZs in the FI60 task, and that once this effect is removed (either through tolerance or blocking it pharmacologically) BUS and BDZs show a similar pattern of responding in the FI60 task. This CORT-release effect produced by BUS is believed to be mediated through BUS’s activity as an agonist at 5-HT$_{1A}$ receptors (De Boer et al., 1991), as this effect is not seen with BDZs. This idea is supported by the fact that similar behavioural effects in the FI60 have been seen with the SSRI fluoxetine (Munn & McNaughton, 2008) a general 5-HT agonist (Malagié, Trillat, Jacquot, & Gardier, 1995).

The similarities between the results produced by leptin in the FI60 task and those previously shown by both BUS and FLU results suggests that leptin's
anxiolytic potential may be mediated through its relationship with 5-HT activity. If leptin is acting like BUS and FLU to produce these effects it would mean that leptin was working as a 5-HT agonist and that high doses of leptin may cause a release of CORT without an external stressor as was seen in BUS (De Boer et al., 1991; Matheson et al., 1988).

In order to find out if leptin is producing this anxiolytic-like effect at low doses through interaction with CORT, it would be of interest to run a FI60 schedule comparing the effects of leptin on FI60 response when endogenous release of CORT is blocked using metyrapone, and when CORT release is functioning normally. Another way to investigate how leptin is producing these behavioural effects would be to pair it with a 5-HT\textsubscript{1A} antagonist, and see if it still produces the effects shown in the current experiment, seeing if the effects produced in this experiment were mediated through activity at the 5-HT\textsubscript{1A} receptor. It would also be of interest to compare the effect of long-term exposure to BUS, FLU, and leptin on FI60 response. This would show if long-term exposure to leptin produces the same tolerance to the CORT releasing effect shown in BUS (De Boer et al., 1991).

Future studies on the effect of leptin on FI60 responding would benefit from including a range of different doses, in order to find the optimal dose for anxiolytic-like effects, as well as to find the cut-off dose where leptin starts to produce the depression in responding.

4.5 Conclusion

Leptin is successfully detected as an anxiolytic in this test of behavioural inhibition. A low dose of leptin produces an increased amount of responding in the early bins of the FI schedule; an effect which has been produced by all anxiolytics
so far tested, independent of pharmacology. High doses of leptin produce a depression of responding in the FI60 task, an effect which has also been produced by BUS and FLU. It seems likely that this depression is caused by indirect CORT agonism as has been shown to be the case with BUS; such an effect of high doses of drug on responding in the FI60 task appears to be characteristic of anxiolytics which agonise 5-HT\textsubscript{1A} receptors.
CHAPTER FIVE

General Discussion

5.1 Summary of findings

The major findings of this research are listed briefly below and then discussed in the same order.

1) It was shown that entry into a novel environment was followed by a decrease in the slope of the voltage/frequency function (hereafter referred to as ‘stimulation slope’) of hippocampal Rhythmic Slow-Wave Activity (RSA), and this decrease is smaller in animals that received unexpected food shortly after entry into the novel environment.

2) The arrival of food (both expected and unexpected) was followed by an increase in the stimulation slope of hippocampal RSA. The arrival of unexpected food was followed by a decrease in the intercept of the voltage/frequency function (hereafter referred to as ‘stimulation intercept’) of hippocampal RSA. Various cubic oscillation patterns in the hippocampal RSA were seen over recording times.

3) Leptin was shown to affect reticular-elicited hippocampal RSA, producing a dose-dependent increase in the stimulation slope of hippocampal RSA. However, unlike with food, no overall change in stimulation intercept (equivalent to mean frequency) was detected.

4) The maximum increase in stimulation slope occurred between 30 minutes post-injection until around 90 minutes post-injection. After 90 minutes the stimulation slope of the hippocampal RSA decreased back to within baseline range.
5) Leptin produced effects similar to a serotonergic anxiolytic in the fixed interval 60 (FI60) task. It released responding in the early bins at a dose of 0.5mg/kg to a similar extent to that shown by the clinical anxiolytic chlordiazepoxide (CDP, 5mg/kg). A higher dose of leptin (1.0mg/kg) depressed responding. This change in behavioural effect between low and high doses is unlike benzodiazepines but similar to that reported for serotonergic anxiolytics in the fixed interval task (Munn & McNaughton, 2008; Panickar & McNaughton, 1991).

5.2 The Effect of Entry Into a Novel Environment on Hippocampal RSA

Hippocampal RSA was affected by entry into a novel environment. When an animal entered the recording area for the very first run there was a large decrease in the stimulation slope of hippocampal RSA. There was no such decrease on entry to the recording area in the second run when the animal had previously spent 48 or more hours in the recording environment and so was likely to be familiar with the surroundings. It was also shown that this decrease in stimulation slope following entry into a novel environment was smaller in animals that received unexpected food shortly after entry into the novel environment. This may be due to the fact that food increases the stimulation slope, somewhat counteracting the ‘novel environment’ effect of decreasing the stimulation slope of hippocampal RSA.

5.3 The Effect of Food (Both Expected and Unexpected) on Hippocampal RSA

Unexpected food caused a decrease in the stimulation intercept of hippocampal RSA, an effect which has been shown by all anxiolytics so far tested (Coop & McNaughton, 1991; McNaughton & Sedgwick, 1978; Munn &
McNaughton, 2008; Zhu & McNaughton, 1991), suggesting that unexpected food has an anxiolytic effect which is not seen in response to an expected meal. It was also apparent that food produced an increase in the stimulation slope of hippocampal RSA. The results were also suggestive that although there is a global decrease in RSA frequency (and hence lower stimulation intercept) the decrease occurs to a greater extent at lower frequencies, as was observed with administration of exogenous leptin in Chapter 3.

5.4 The Effect of Leptin on Hippocampal RSA

Administration of leptin produced a dose-related increase in the stimulation slope of hippocampal RSA, an effect produced by food in the Chapter 2 experiments. This suggests that it is possible that this increase in stimulation slope caused by food may be being caused by the leptin being released in response to the meal the animals have eaten. However, unlike food, there was no significant change in the stimulation intercept. This change in stimulation slope but not stimulation intercept is unique to leptin, with previous drugs either changing stimulation intercept and stimulation slope concurrently or changing stimulation intercept without changing stimulation slope (McNaughton & Coop, 1991). The selective change in stimulation slope means that leptin is increasing high frequency RSA while it is decreasing low frequency RSA. This suggests that in tasks in which RSA frequency is typically low (up to around 5 or 6Hz), leptin may reduce frequency and so be anxiolytic; but that in tasks in which RSA frequency is typically high (7-8Hz) it could be anxiogenic. The specific mechanisms behind this unusual stimulation slope-specific effect of leptin are unclear, however one hypothesis is discussed in section 5.7.
It is also important to note that as an overall decrease in stimulation intercept was not shown in the Chapter 3 leptin experiment it is likely that leptin is working in combination with another hormone or chemical, which is released in response to food intake. In Chapter 2 it was shown that food produces an overall decrease in the stimulation intercept of hippocampal RSA, leptin produces a decrease, but only at lower frequencies; perhaps a partner hormone is affecting the higher frequency RSA when an animal eats a meal. As to what this hormone is, ghrelin is reduced in response to feeding and produces an anxiogenic effect in the Elevated Plus Maze (EPM) (Carlini et al., 2002), it is possible that the anxiolytic effects caused by the reduction of ghrelin produced by feeding may combine with the anxiolytic effect of increasing leptin, resulting in an overall decrease in the frequency of hippocampal RSA.

5.5 The Importance of Timing in Testing the Anxiolytic Effects of Leptin

The results show that the maximal increase in the stimulation slope of hippocampal RSA from an intraperitoneal (i.p.) injection of leptin occurs from 30 minutes post-injection through to 90 minutes post-injection. For leptin to have an effect on anxiety, behavioural tests should be carried out at least 30 minutes after leptin administration. It is possible that this may be the cause of some of the conflicting results in previous studies, for example, Suomalainen and Mannisto (1998) found a null result for leptin acting as an anxiolytic in the EPM when they tested animals only 10-20 minutes post-injection, however Liu et al. (2010) found leptin to produce anxiolytic-like effects in the EPM equal to those shown by FLU when the tests were run 30 minutes post-injection of leptin. It is important to
determine the correct timing and dosage of leptin for an anxiolytic effect before it is possible to accurately interpret the results from previous behavioural studies on the anxiolytic effects of leptin.

5.6 The Effect of Leptin on the Fixed Interval Task

Leptin was detected as an anxiolytic in the FI60 task, showing a release of responding in the early bins of the FI60 schedule at a dose of 0.5mg/kg. This effect was qualitatively very similar to that produced by the active control CDP, which has been previously shown to produce a robust “anxiolytic-typical” effect on responding in the FI60 task (Woodnorth & McNaughton, 2002). Leptin at a higher dose of 1.0mg/kg produced an overall depression of responding throughout the schedule. This pattern of responding (release of responding at lower doses and depression of responding at higher doses) is typical of several serotonergic anxiolytics such as buspirone (BUS)(Panickar & McNaughton, 1991) and fluoxetine (FLU)(Munn & McNaughton, 2008). Other GABAergic anxiolytics such as benzodiazepines do not show this depression of responding at high (non-sedative) doses and instead show a dose dependent increase in release of responding (Zhu & McNaughton, 1995).

5.7 Is Leptin Behaving in a Similar Fashion to Buspirone?

BUS is known to release corticosterone (CORT) (De Boer et al., 1991), CORT produces an increase in the stimulation slope of hippocampal RSA (McNaughton & Coop, 1991) an effect also produced by leptin administration (Chapter 3). It is therefore possible that leptin triggers the release of CORT, in support of this idea there is evidence that stressed animals administered leptin have higher levels of serum CORT than stressed animals administered saline (Thorsell et al., 2002).
Such an effect, if greater than that of buspirone relative to the effect on stimulation intercept, may have distorted any underlying decrease in the frequency of hippocampal RSA which leptin would otherwise have produced.

Due to the FI60 results showing an overall depression at the higher dose of leptin, an effect which has only been shown in serotonergic anxiolytics, it is possible that the anxiolytic effect being produced by leptin is being produced via similar mechanisms to those caused by BUS and FLU, such as 5 hydroxytryptamine (5-HT) agonism, as BUS is a direct 5-HT\textsubscript{1A} agonist (Urban et al., 1986) and FLU is a general 5-HT agonist (Malagié et al., 1995). There is some evidence that leptin activity might be mediated through 5-HT (Calapi et al., 1999; Finn, Cunningham, Rickard, Clifton, & Steiner, 2001; Yamada et al., 1999).

As discussed in Chapter 4, BUS, at high doses (over 1mg/kg), triggers the release of CORT without the presence of an external stressor (De Boer et al., 1991; Matheson et al., 1988). Studies have demonstrated that this CORT-releasing effect of BUS is to some extent responsible for the behavioural differences between it and benzodiazepines (BDZs) in the FI60 task, and that once this effect is removed (either through tolerance (De Boer et al., 1991) or blocking it pharmacologically (McNaughton et al., 1996) BUS and BDZs show a similar pattern of responding in the FI60 task. This CORT-release effect produced by BUS is believed to be mediated through BUS's activity as an agonist at 5-HT\textsubscript{1A} receptors (De Boer et al., 1991), as this effect is not seen with BDZs, which actually block the release of CORT (McNaughton et al., 1996). This idea is supported by the fact that similar behavioural effects in the FI60 have been seen with the selective serotonin reuptake inhibitor (SSRI) fluoxetine (FLU) (Munn & McNaughton, 2008).
The similarities between the results produced by leptin in the FI task and those previously shown by both BUS and FLU suggests that leptin’s anxiolytic potential may be mediated through its relationship with 5-HT activity. This is in line with previous findings that link leptin with 5-HT activity (Calapi et al., 1999; Finn et al., 2001; Yamada et al., 1999). If leptin is acting like BUS and FLU to produce these effects it would mean that leptin was working as a 5-HT agonist and that high doses of leptin may cause a release of CORT without an external stressor as was seen in BUS (De Boer et al., 1991; Matheson et al., 1988).

BUS and FLU are both serotonergic anxiolytics and they are also clinical antidepressants. One of the characteristic symptoms of depression in the DSMIV is fluctuations in weight, suggesting that for some reason energy regulation is being altered. Another of the DSMIV symptoms of depression is disregulation of sleep patterns, which suggests some sort of disturbance in the circadian rhythms of patients suffering from depression. Leptin is a hormone involved in energy regulation and has been shown to fluctuate diurnally, it is possible that regulating leptin levels may help to normalise the energy intake and sleeping patterns of patients with depression, anxiety disorder, or both of these disorders occurring comorbidly.

5.8 **Future research**

Future studies looking at the circadian oscillation of hippocampal RSA should look at longer experimental runs in order to see if any of the cubic oscillations we observed occur repeatedly and on what sort of time-frame the oscillation occurs. They could also get a clearer view of the effect of food on hippocampal RSA by extending the recording time for several days, in order to see how the effect lasts over time and if it is repeated with every meal. Also
considering that unexpected food produced a decrease in stimulation intercept of hippocampal RSA it would be of interest to run the experiment tested in Chapter 3 again, but using unexpected food as the putative “anxiolytic” instead of an i.p. injection of leptin. The results of the present experiments suggest that food may well be detected by the standard anxiolytic test. There are, however, logistical problems with this proposal. Eating a meal takes longer and is of variable duration when compared to an injection, which is of known duration and timing. Consumption of food is entirely voluntary; some animals might not eat as much, or as fast etc. these would need to be worked out for the experiment to be valid.

Future experiments should investigate the effect of leptin on tests sensitive to anxiolytic drugs to see if leptin produces an anxiolytic effect in tasks that produce low frequency RSA (i.e. the water maze), and an anxiogenic effect in tasks that produce high frequency (i.e. the open field). This would help explore the frequency specific effect of leptin shown in Chapter 3. The change in stimulation slope may suggest that anxiety produced at low frequencies is somehow different from anxiety produced at high frequencies. Future research should look into why leptin affects one as an anxiolytic and does not do the same for the other.

In order to find out if leptin is producing some of its effects through interaction with CORT, it would be of interest to run both reticular stimulation and a FI60 schedule comparing the effects of leptin when endogenous release of CORT is blocked using the specific inhibitor metyrapone, to the response when CORT release is functioning normal, as has been done with BUS (McNaughton et al., 1996). If leptin produces the same result in both cases it would mean that the anxiolytic effect of leptin was not produced through an interaction with CORT, and therefore, by extension, is probably not mediated through 5-HT. Another way to
investigate how leptin is producing these behavioural effects would be to pair it with a 5-HT$_{1A}$ antagonist, such as pindolol (Coop, McNaughton, & Scott, 1992) and see if it still produces the effects shown in the current experiment. It would also be of interest to compare the effect of long-term exposure to BUS, FLU, and leptin on Fl60 response. This would show if long-term exposure to leptin produces the same tolerance to the CORT releasing effect shown in BUS (De Boer et al., 1991; Zhu & McNaughton, 1995).

5.9 Conclusion

It was found that both leptin (dose-dependently) and food cause an increase in the stimulation slope of hippocampal RSA. It was also shown that being placed into a novel environment produced a decrease in the stimulation slope of hippocampal RSA, and this decrease is smaller when animals were presented with unexpected food shortly after entry into the novel environment. Unexpected food causes a decrease in the stimulation intercept of hippocampal RSA, an effect that is also produced by anxiolytics. Leptin showed no overall effect on the stimulation intercept, this result has not yet been seen in any other agents tested in this way. The change in stimulation slope suggests that leptin affects hippocampal RSA differently at different stimulation intensities, decreasing the low frequencies of RSA produced by low levels of stimulation and increasing the higher-frequencies produced by high levels of stimulation. This suggests that leptin may be both anxiolytic and anxiogenic, depending on the theta frequency elicited by the anxious behaviours.

Leptin was successfully detected as an anxiolytic in the Fl60 test of behavioural inhibition. A low dose of leptin produced an increased amount of responding in the early bins of the Fl60 schedule; an effect which has been
produced by some doses of all anxiolytics so far tested, independent of pharmacology. A high dose of leptin produced a depression of responding in the FI60 task, an effect which has also been produced by the serotonergic anxiolytics BUS and FLU. It seems likely that this depression is caused by indirect CORT agonism as has been shown to be the case with BUS; such an effect of high doses of drug on responding in the FI60 task appears to be characteristic of anxiolytics which agonise 5-HT1A receptors. As there is evidence of leptin action via 5-HT it is possible that leptin produces this effect of CORT-agonism via 5-HT, this could be clarified by running the FI60 with leptin again and including a leptin group in which 5-HT action is blocked.

It was also found that i.p. administration of leptin produced the largest changes on the stimulation slope of hippocampal RSA between 30 minutes and 90 minutes post-injection. This suggests that future tests using leptin should time their experiments to occur within that time frame to ensure that leptin is producing its maximum effect and may account for previous discrepant reports.

As leptin plays a role in anxiety levels as well as energy-intake, it suggests that further exploration of leptin may help to develop a treatment for anxiety disorders. If leptin’s anxiolytic potential can be fully understood, it would be an important first step in explaining the link between mood and eating. Such a link could help explain not only how the anxiety system is regulated endogenously, but help explain why some individuals are susceptible to obesity and excess energy intake; this may, in fact, be related to a commensurate mood disorder. Such a discovery would open the door for potential novel treatments for both disorders of affect and disorders of metabolism.
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