Benzodiazepine receptors and the control of ingestive behaviour in the rat

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Declaration

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Suzanne Higgs

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Abstract

When administered systemically, benzodiazepine receptor agonists have been shown to increase food intake in a number of species. Conversely, benzodiazepine receptor inverse agonists bring about reliable decreases in feeding. The aim of the experiments reported in this thesis was to investigate the brain and behavioural mechanisms involved in the effects of benzodiazepines on ingestion.

The effect on food intake of microinjection of the benzodiazepine receptor agonist midazolam into the brainstem of the rat was investigated. A reliable hyperphagic response was elicited following injection of midazolam into both the IVth ventricle and the parabrachial nucleus (PBN). This increase in intake was reversed by pretreatment with the selective benzodiazepine receptor antagonist flumazenil. These results suggest that benzodiazepine receptors located in the brainstem, specifically in the PBN, may be responsible for the effects of benzodiazepines on ingestion.

In further experiments, a microstructural approach was adopted which involved analyzing the effects of benzodiazepine ligands on the detailed pattern of licking for both a carbohydrate and a fat in the rat. The effects of midazolam were similar to the effects of increasing concentration. The effects of the benzodiazepine receptor inverse agonist Ro 15-4513 were similar to the effects of decreasing concentration. These results suggest that benzodiazepines influence ingestive behaviour by modulating palatability.

The proposal that benzodiazepines may interact with opioids to influence feeding behaviour was examined in Chapters 7 and 8. Although the effects of the opioid agonist morphine and the opioid antagonist naloxone on licking behaviour were not the same as the effects of benzodiazepine ligands, naloxone blocked the effects of midazolam. These results suggest that the effects of benzodiazepine on palatability may depend on release of endogenous opioid peptides.

This work has implications for understanding the neural control of ingestive behaviour and may help in developing new therapies for clinical disorders such as anorexia and bulimia.

Dedication

I dedicate this thesis to my Mum and Dad with love and thanks.

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Contents

The pharmacology of benzodiazepines and their role in the control of ingestion

1.1 Chapter Overview

The potent stimulation of appetite by drugs which act as agonists at benzodiazepine receptors is now a well-known phenomenon (Cooper, 1980a). However, despite an abundance of data documenting the hyperphagic effect of benzodiazepine receptor agonists, there has been little investigation of the brain mechanisms involved in these effects. One of the aims of this thesis is to identify potential neural substrates mediating benzodiazepine-induced changes in food intake. Such studies are essential for increasing our understanding the effects of benzodiazepines, but may also shed light more generally on the neural control of ingestive behaviour.

Although many data have been gathered concerning the effects of benzodiazepines on ingestive responding, the behavioural mechanisms responsible for these effects have yet to be fully worked out. Such information could provide important insights into the contribution of specific processes to the control of food intake. Microstructural analysis of ingestive behaviour may be a useful tool in this respect. This technique involves the characterization of individual components of feeding, and is thought to provide information concerning the behavioural mechanisms involved in the control of food and fluid intake. However, microstructural analysis has not been widely used to study the effects of pharmacological manipulations on ingestion. Therefore, the second aim of this thesis is to use microstructural techniques to investigate in more detail the behavioural mechanisms responsible for the effects of benzodiazepines on ingestive behaviour.

As an introduction to the experiments reported in this thesis, the pharmacology of benzodiazepine receptor ligands will first be discussed. Second, experiments concerning the effects of benzodiazepine receptor ligands on ingestion will be reviewed. Finally, research conducted to date into the potential brain mechanisms involved in the effects of benzodiazepines on ingestive behaviour will be presented.

1.2 Benzodiazepine pharmacology and molecular biology

An understanding of the pharmacology of benzodiazepines is critical if these drugs are to be useful tools with which to investigate behaviour. Pharmacological studies are important because they may enable the development of more selective drugs with which to probe behaviour more precisely. However, behavioural studies may also have implications for pharmacology, and so it is essential that advances in the appreciation of benzodiazepine pharmacology proceeds in tandem with investigations of the behavioural effects of these drugs. Recent advances in the field of molecular biology have led to many novel pharmacological developments which may have implications for the mechanism of action of benzodiazepines. Therefore, the experiments reported in this thesis serve to complement progress in pharmacology and molecular biology by increasing our understanding of the behavioural and neural processes involved in the effects of benzodiazepines on ingestive behaviour.

The following section is concerned with reviewing advances in benzodiazepine pharmacology and is divided into several broad areas. First, the literature concerning the discovery of benzodiazepines and the identification of specific binding sites for these drugs in the central nervous system (CNS) will be reviewed. Second, the pharmacological effects of different classes of benzodiazepine receptor ligand will be discussed. Third, the relationship between benzodiazepine receptors and GABA function will be assessed. Finally, exciting developments in the field of molecular biology which have revealed unanticipated diversity in the $GABA_A$ receptor will be discussed, along with the implications of these results for the behavioural effects of benzodiazepines.

1.2.1 The discovery and development of benzodiazepines

The first benzodiazepine to be synthesised was chlordiazepoxide (CDP). Like many other drug developments, the discovery of the benzodiazepines owed more to serendipity than to rational drug design (Tinklenberg, 1977). Working at the pharmaceutical company Hoffman La Roche, the medicinal chemist Leo Sternbach discovered that a series of compounds he had synthesised were not benzheptoxdiazines

as he had thought, but instead were quinazolone 3-oxides. He tested the compounds for pharmacological activity and found that all but one of them was inert. This active compound was given to Lowell Randall who showed that it had distinctive pharmacological properties. In behavioural tests, it exhibited sedative, muscle relaxant and anticonvulsant activity (Randall et al., 1960), and was introduced to the market soon after under the trade name of Librium. CDP and other benzodiazepines with the same basic chemical structure are now used widely as therapeutic agents for many conditions including anxiety and insomnia.

Since the discovery of benzodiazepines specific binding sites for these compounds have been identified, both in the brain and in the periphery. Additionally, many new compounds have been developed which differ in both affinity and efficacy at these sites. In the following sections, literature concerning the structure and function of the benzodiazepine receptor, and the nature of ligand/receptor interactions will be reviewed to provide a basis for understanding the involvement of benzodiazepines in the control of ingestive behaviour.

1.2.2 The identification of specific benzodiazepine receptors

A major breakthrough in understanding the pharmacology of benzodiazepines was the identification of specific, high affinity binding sites in rat brain using the technique of radioligand binding assays (Braestrup and Squires, 1977; Mohler and Okada, 1977). This discovery generated great interest in the benzodiazepines. Within the space of a few years many papers were published reporting the results of benzodiazepine binding studies. It has been demonstrated that the binding is stereospecific, reversible and saturable. Additionally, a good correlation has also been shown to exist between the clinical potency of benzodiazepines and their ability to displace ^{3}[H] diazepam from the membrane binding sites (Mohler and Okada, 1977). These criteria had to be satisfied before it could be suggested that the binding sites constitute physiological receptors responsible for the pharmacological and behavioural effects of benzodiazepine ligands. Importantly, specific benzodiazepine binding has also been demonstrated in vivo

following injection of ^{3}[H] diazepam in the intact animal (Williamson, Paul and Skolnick, 1978).

The location of benzodiazepine receptors in the CNS has been determined using autoradiographic binding studies. Benzodiazepine receptors have been shown to be widely distributed throughout the central nervous system and periphery. In the rat brain they are found in particularly high density in the cerebral cortex, cerebellum, hypothalamus and limbic structures (Young and Kuhar, 1980).

Although the majority of early binding studies made use of membranes from rat brain, some experiments have compared the binding characteristics in both rat and human brain tissue. These investigations have revealed a great deal of similarity in the pharmacological properties of binding sites in rat and human brain (Sieghart, Eichinger, Riederer and Jellinger, 1985).

1.2.3 Central versus peripheral binding sites

Benzodiazepine receptors in the CNS are not the only binding sites which have been identified. Benzodiazepines also bind to receptors located in the periphery, and to micromolar sites. The peripheral-type binding sites are found in variety of tissues and are normally located in the mitochondrial membrane (Parola, Yamamura and Laird, 1993). No correlation between the affinity of benzodiazepine ligands for the peripheral site and their clinical effect has been shown. This suggests that peripheral benzodiazepine receptors are not responsible for the behavioural effects of benzodiazepines. The properties of the micromolar binding sites have yet to be fully characterised, but they have been shown to differ from both the peripheral and central types in that they exhibit very low affinity for benzodiazepines (Bowling and DeLorenzo, 1982).

1.2.4 Benzodiazepine ligands

The identification of specific benzodiazepine binding sites encouraged the development of many novel compounds with which to probe the behavioural effects of these compounds. Drugs that interact with benzodiazepine receptors (benzodiazepine ligands) have been categorized based on the cellular response which is elicited upon binding. There are three main classes of ligand: agonists, antagonists, and inverse agonists. Within the category of agonists and inverse agonists there also exists a subdivision of compounds referred to as partial agonists. These different classes of ligand will be considered in turn in the following section.

Benzodiazepine receptor agonists

The term 'benzodiazepine' properly refers to a class of drugs with a particular chemical structure. It has also been taken to refer to drugs from the benzodiazepine class that have an agonistic action at benzodiazepine receptors (i.e. elicit a cellular response). However, a number of exceptions to this terminology are worth noting. First, some ligands, such as the β-carbolines and pyrazoloquinolines, have a non-benzodiazepine structure and yet bind with high affinity to benzodiazepine binding sites (Haefely, Kyburz, Gereke and Mohler, 1985). Second, not all drugs with a benzodiazepine chemical structure act via benzodiazepine receptors. For example, the benzodiazepine tifluadom does not bind to benzodiazepine receptors and instead interacts specifically with the κ opiate receptor (Kley, Scheidemantel, Bering and Muller, 1983). Therefore, the term benzodiazepine receptor agonist is used preferentially to refer to compounds of any chemical structure that interact with the benzodiazepine receptor with agonist activity.

Benzodiazepine receptor antagonists

The first selective benzodiazepine antagonist to be described was the imidazobenzodiazepine derivative flumazenil (Ro 15-1788) (Hunkeler et al., 1981). This compound was found to have a high affinity for benzodiazepine receptor sites but possessed little or no intrinsic pharmacological activity (Bonetti et al., 1982). Flumazenil

blocks the effects of benzodiazepine receptor agonists by competing for the receptor sites. The development of selective antagonists like flumazenil was crucial for demonstrating the specificity of the benzodiazepine effects and supported the existence of specific pharmacological receptors mediating the behavioural effects of benzodiazepines. This is because the ability of a selective benzodiazepine receptor antagonist to block the effects of an agonist suggests that the effects of that agonist are mediated by the benzodiazepine receptor.

Benzodiazepine receptor inverse agonists

A novel class of benzodiazepine receptor ligands has been identified. These compounds act as agonists at the benzodiazepine receptor site (i.e. they possess intrinsic pharmacologic activity), but do not conform to the classical pharmacological profile of the benzodiazepines (for review see Sarter, Nutt, and Lister, 1995). One such compound is the ß-carboline ethyl-ß-carboline 3 carboxylate (ß-CCE) (Braestrup, Nielson, Honore, Jensen and Peterson, 1980). When tested in vivo this compound displays properties contrary to those of the benzodiazepines. ß-CCE binds with high affinity to benzodiazepine-like receptor sites, but has intrinsic anxiogenic (Dorow, Horowski, Paschelke, and Amin, 1983) and proconvulsant effects (Rossier et al., 1983). This contrasts with the anxiolytic and anticonvulsant actions of benzodiazepine agonists. To explain the unusual effects of ß-CCE and other related compounds, such as the ß-carboline DMCM, the term inverse agonist was introduced.

Until recently it was thought that the benzodiazepine receptor provided the only example of compounds with negative intrinsic activity. This was despite that fact that the concept of inverse agonism could have been predicted from traditional receptor theory. However, it is now recognised that ligands displaying inverse agonist properties may be relatively commonplace (Milligan, Bond and Lee, 1995). Additionally, it has been suggested that benzodiazepine inverse agonists may act at a binding site adjacent to that for agonists. For example, β-carbolines may not occupy the same site as benzodiazepines, but instead bind to a similar but overlapping site (Luddens and Wisden,

1991). The significance of inverse agonism has yet to be fully appreciated, but the increased range these compounds provide for receptor regulation could prove to be of enormous therapeutic benefit.

Benzodiazepine receptor partial agonists

Several benzodiazepine ligands have been described which exhibit activity intermediate to the actions of agonists and antagonists. An example of an agonist with intermediate activity is the β-carboline, ZK 91296 (Stephens et al., 1987). This compound differs from other agonists such as diazepam, because it exhibits a narrower pharmacological profile. It has been shown to be effective in anti-conflict tests but, unlike diazepam, produces little or no sedation or muscle relaxation. Another β-carboline, FG 7142 is an inverse agonist (Braestrup, Schmeichen, Neef, Nielson and Peterson, 1982), but it differs from other compounds in this class because it does not normally induce convulsions (Jensen et al., 1983).

The concept of partial agonism was initially evoked to explain the different profiles of activity of ZK 91296 and FG 7142 (Haefely, Martin and Polc, 1990). Partial agonists are low efficacy compounds that produce smaller responses than full agonists at the same level of receptor occupancy (Haefely et al., 1985). This theory can account for the selective effects of ZK 91296 and FG 7142 if it is also assumed that the target cells responsible for different effects vary in receptor density. For example, in an area with a high receptor density (large receptor reserve) both partial and full agonists will achieve a maximal response. However, in a receptor population with a low receptor reserve (low density), partial agonists will not occupy enough receptors to produce a pharmacological effect. One prediction from this hypothesis is that neurons mediating the anxiolytic effects of benzodiazepine agonists should have a large receptor reserve, whereas neurons mediating the sedative effects should have a low receptor reserve (Martin, 1988). Partial agonists are important therapeutically because they do not cause undesirable side effects such as sedation but still possess anxiolytic properties.

1.2.4 Summary

Benzodiazepines are therapeutically useful drugs that were synthesised serendipitously by the drug company Hoffmann La Roche in the 1950s. Specific binding sites for these drugs have been shown to exist in the brain, and it is now known that the benzodiazepine receptor not only recognizes benzodiazepines, but also drugs from other chemical classes (e.g. β-carbolines and pyrazoloquinolines), and ligands with different affinities and intrinsic efficacies. There are three types of benzodiazepine ligand: agonists, antagonist, and inverse agonists. Agonists bind to the benzodiazepine receptor and cause a cellular response. Antagonists bind to the receptor but do not display any intrinsic activity and competitively block the effects of agonists. Inverse agonists bind to the receptor and yet cause the opposite response to agonists. Additionally, the benzodiazepine receptor also binds compounds with efficacy that is either midway between antagonists and agonists (partial agonists), or between antagonists and inverse agonists (partial inverse agonists). As shown in Figure 1.1, the ß-carbolines provide an interesting example of functional activity spanning the whole range, from full agonist to full inverse agonist.

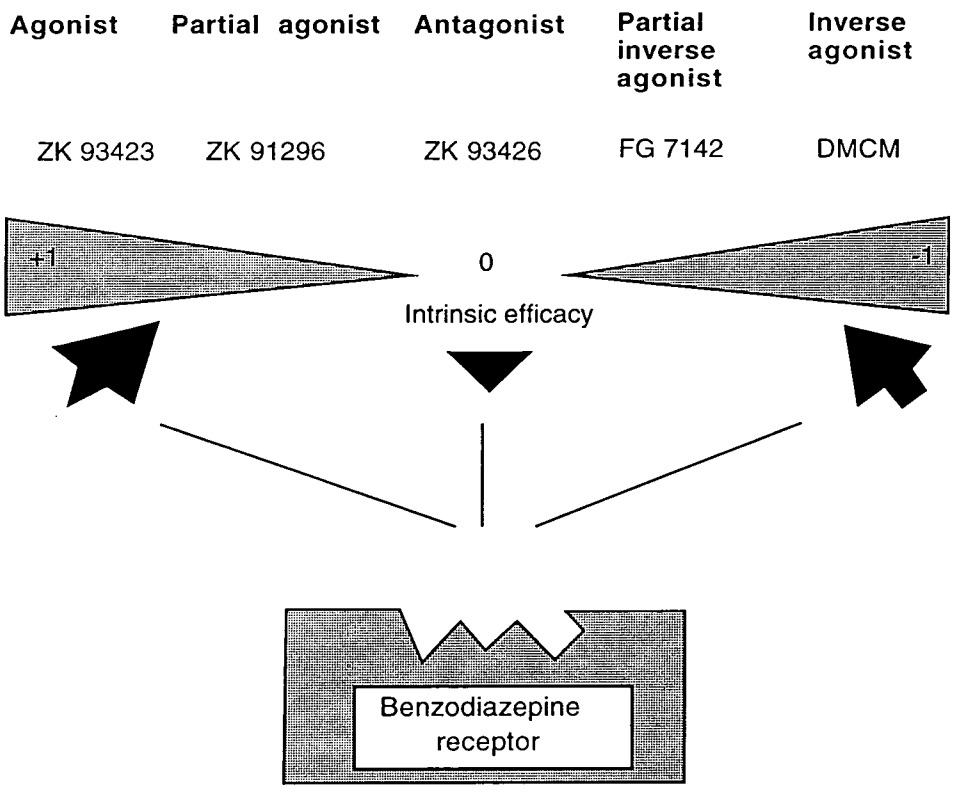


Figure 1.1 Spectrum of β-carboline ligands with different intrinsic efficacies at the benzodiazepine receptor from full agonist to full inverse agonist. Adapted from Haefely (1994).

1.2.5 GABAergic transmission

There is evidence to suggest a close association between the action of benzodiazepines and the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Haefely, Kulcsar, Mohler, Pieri and Polc, 1975). Therefore, to fully understand the effects exerted by benzodiazepines, it is necessary to understand the basic features of GABAergic neurotransmission.

GABA is the major inhibitory neurotransmitter in the vertebrate central nervous system. It is generally accepted that there are three classes of GABA receptor: the $GABA_A$, $GABA_B$ and $GABA_C$ receptor respectively. A distinction was first drawn between the $GABA_A$ receptor and the $GABA_B$ receptor by Hill and Bowery (1981).

These receptors were characterised by the different cellular effects observed upon binding of a ligand, and their affinities for specific agonists and antagonists. The $GABA_A$ receptor is sensitive to the GABA antagonist bicuculline, and is a ligand-gated chloride (Cl^-) ion channel. In contrast, the $GABA_B$ receptor is one of a family of receptors which are coupled either to calcium or potassium channels (Bormann, 1988). The $GABA_B$ receptor is activated selectively by the $GABA_B$ agonist baclofen, and is not affected by drugs that modulate $GABA_A$ transmission. A third class of GABA receptor was identified as a result of further binding studies. The $GABA_C$ receptor is insensitive to both bicuculline and baclofen and has been localized to a subpopulation of retinal neurons (Feigenspan, Wassle and Bormann, 1993).

The $GABA_A$ receptor is part of a superfamily of ligand-gated ion channels which also includes the nicotinic acetylcholine receptor, the glycine receptor and the $5\text{-}HT_3$ receptor. The structure of these receptors remains elusive since none of them have been studied using X-ray crystallography. However, structural models derived from electron microscopy (EM) suggest that these receptors are formed from the assembly of five distinct subunits. Each subunit is thought to span the membrane 4 times (Unwin, 1989, 1995). These models are based on the nicotinic acetylcholine receptor but may be typical of other members of the superfamily.

Synaptic inhibition occurs at the $GABA_A$ receptor when GABA binds and increases Cl- conductance through the receptor ionophore. This leads to hyperpolarization of the neuronal membrane which makes it less likely that excitatory currents will reach the threshold required for the induction of an action potential. GABA acts mainly at local interneurons to prevent over-excitation of neurons. However, the overall effect of GABA need not necessarily be inhibitory, because an arrangement of two GABAergic neurons in series could lead to disinhibition of a target neuron.

Benzodiazepines and the $GABA_A$ receptor complex

It has been proposed that benzodiazepines bring about their behavioural effects by acting at GABAergic synapses in the brain (Haefely et al., 1975). In support of this, benzodiazepines have been shown to enhance GABA binding (Costa, Guidotti and Toffano, 1978). The specific nature of this interaction has been confirmed by the results of receptor purification studies. A protein complex was isolated which binds both benzodiazepines and $GABA_A$ ligands (Schoch et al., 1985). This evidence indicates that the benzodiazepine receptor is an allosteric site on the $GABA_A$ receptor complex. Allosteric modulators act at sites different and distant from the primary ligand binding site. Such modulators can function either by altering the kinetics of the channel (e.g. the probability of channel opening), or by altering the affinity of the primary ligand for its binding site. Benzodiazepine agonists affect GABAergic transmission by increasing the frequency of $GABA_A$ receptor Cl^- channel opening (Haefely et al., 1985; Polc, 1988). The potency of GABA at the $GABA_A$ receptor complex is therefore increased. This effect can be seen when plotting a dose response curve for GABA induced Cl^- conductance. Agonists induce a shift to the left in this curve (less GABA is required to obtain the same Cl^- conductance), but do not increase the maximal response (Choi, Farb and Fischbach, 1981). Studies using benzodiazepine antagonists have shown that these compounds do not affect Cl^- conductance (Polc, Ropert and Snyder, 1981). Benzodiazepine inverse agonists reduce the effect of GABA by shifting the dose response curve to the right. These compounds also decrease the maximum Cl^- conductance (Polc, Laurent, Scherschlicht and Haefely, 1981). The effect of benzodiazepine ligands on GABA conductance is shown in Figure 1.2. Benzodiazepine receptor agonists act as positive modulators of GABA function, inverse agonists are negative modulators and antagonists are characterised by a lack of effect on GABA receptor function. However, the benzodiazepine receptor is not the only modulatory site on the $GABA_A$ receptor complex. Other sites include those for barbiturates, the convulsants picrotoxin and TBPS, neurosteroids, and ethanol (Sieghart, 1992).

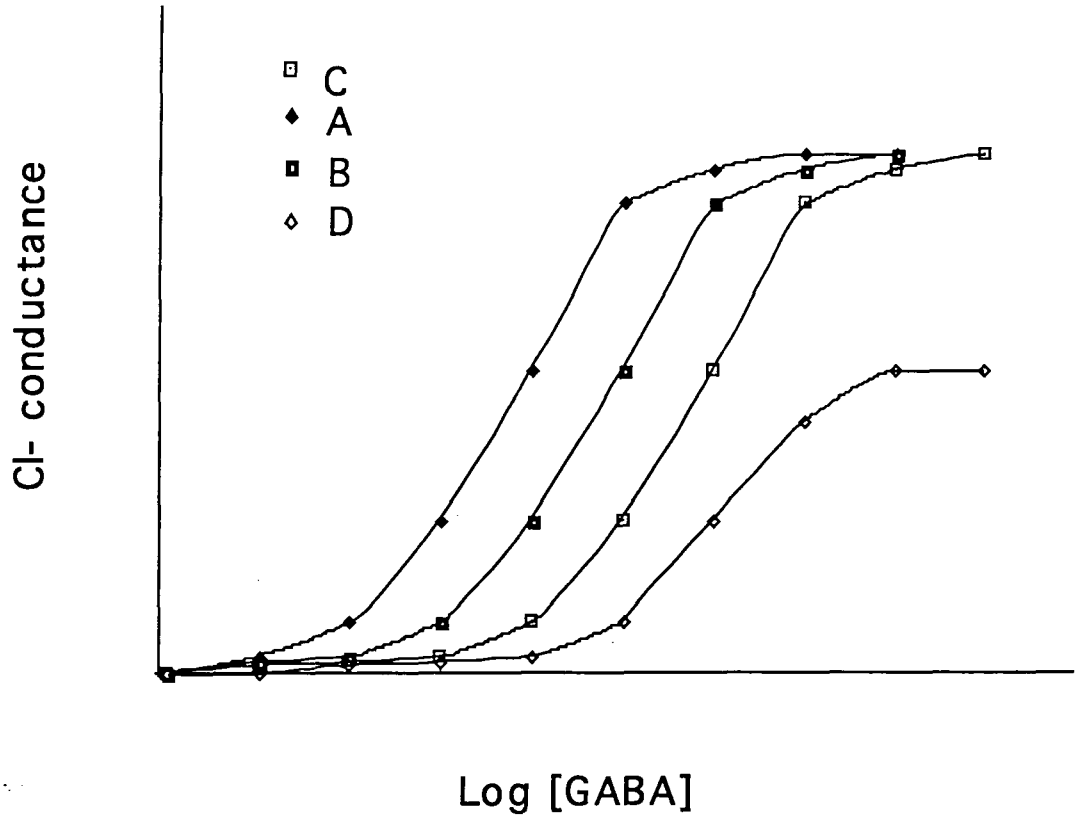


Figure 1.2 Theoretical dose-response curves for the effect of benzodiazepine ligands on GABA-induced chloride conductance. C is the control curve. A is in the presence of a full agonist, B is in the presence of a partial agonist and D is in the presence of an inverse agonist.

1.2.6 Summary

Benzodiazepine ligands exert their pharmacological effects through allosteric modulation of the $GABA_A$ receptor protein. This is achieved by modulating the frequency of Cl^- channel opening in the $GABA_A$ receptor. Benzodiazepine receptor agonists act as positive modulators and facilitate GABAergic transmission by increasing the frequency of channel opening. Inverse agonists depress GABA function by decreasing the probability of Cl^- channel opening and so act as negative modulators. Antagonists have no intrinsic activity and do not alter GABA function when they bind to the benzodiazepine receptor.

1.2.7 $GABA_A$ receptor subtypes

The $GABA_A$ complex is probably one of the best studied neurotransmitter receptors. As a result, the structure and function of this receptor is sufficiently advanced to explain how some benzodiazepine ligands modulate $GABA_A$ receptor function.

Although it was initially thought that the $GABA_A$ receptor was a single molecular species (Haring et al., 1985), it is now accepted that $GABA_A$ receptors constitute a heterogeneous population. The first evidence suggesting $GABA_A$ receptor multiplicity came from biochemical studies. First, polyacryamide gel electrophoresis (PAGE) revealed two major protein bands: a 53 kDa band (the α subunit) and a 57 kDa band (the β subunit). These were thought to combine to form a pentameric complex of total molecular weight of between 220 and 355 kDa. Second, selective photoaffinity labelling studies showed the α subunit was irreversibly labelled by [^{3}H] flunitrazepam, and the β subunit was labelled by [^{3}H] muscimol (Sigel, Stephens, Mamlaki and Barnard, 1983). Improvements in the resolution of gel electrophoresis techniques then revealed that these subunits consisted of several different proteins (Fuchs and Seighart, 1989).

Molecular biological cloning techniques have since allowed the identification of 15 different receptor subunits. These subunits have been categorized into 5 groups according to amino acid sequence homology (α, β, γ, δ, and ρ subunits). Each subunit is encoded on separate genes that can be differentially spliced to produce multiple isoforms. In the rodent brain, six α subunits have currently been identified (α1- α6), four β subunits (β1- β4), three γ subunits (γ1- γ3), a δ subunit and two ρ subunits located in the retina (Luddens and Wisden, 1991; Olsen and Tobin, 1990). This diversity is increased further by RNA editing which leads to the formation of phenotypic variants (Whiting, McKernan, and Iversen, 1990). For example, the γ subunit can exist in either a long or short form depending on the presence or absence of an eight amino acid insert (Wafford et al., 1991). The designation of ρ subunits as $GABA_A$ subunits has been challenged recently by Darlison and Albrecht (1995). These authors argue that ρ subunits,

originally found in the human retina (Cutting et al., 1991), do not assemble with α and β subunits to form $GABA_A$ chloride channels, but instead form part of the $GABA_C$ receptor.

Assembly of varying combinations of these 15 different subunits probably results in the creation of functionally diverse $GABA_A$ receptor subtypes. It is likely that several different subpopulations exist which differ pharmacologically and in their distribution in the brain. There is evidence that benzodiazepine pharmacology is determined by substitution of different subunits in the $GABA_A$ receptor complex (Luddens, Korpi and Seeburg, 1995). Therefore, an examination of the function of $GABA_A$ receptor subunits may be important for understanding the behavioural effects of benzodiazepine ligands. For example, $GABA_A$ receptor heterogeneity may provide a pharmacological explanation for the wide range of effects which result from modulation of GABAergic transmission by benzodiazepines, including anxiolytic, anticonvulsant, muscle relaxant and hyperphagic activity.

In summary, the $GABA_A$ receptor complex is formed from the assembly of five subunits. These subunits can be selected from an array of 15 different types that have been grouped into 5 classes (α,β,γ,δ and ρ). This means that it is possible to form many different $GABA_A$ receptors, depending on the subunit composition. Benzodiazepine receptors coupled to different $GABA_A$ receptor subtypes located in different brain areas could explain the diversity of benzodiazepine effects on behaviour.

Subunit functions

The identification of multiple receptor subunits does not allow the composition of functional receptors to be identified. To establish which subunits are assembled in the native receptor, and what the specific functional contribution of each subunit might be, recombinant $GABA_A$ receptors have been artificially expressed in Xenopus oocyte cells. Using an expression system like Xenopus means that the properties of the receptors can be studied in isolation from other receptors which are normally expressed in the neuronal

membrane. A combination of α and β subunits expressed in Xenopus has been found to successfully mimic GABA-induced chloride ion flux. However, receptors composed only of α and β subunits are not pharmacologically identical to the native $GABA_A$ receptor, because benzodiazepine potentiation is not observed (Levitan, Blair, Dione and Barnard, 1988). This result implies that other subunits are required for benzodiazepine activity.

The γ subunit, isolated by Pritchett and co-workers (1989), has been shown to be necessary for benzodiazepine function. Most studies have reported expression of the γ2 subunit because it was the first to be cloned. However, limited studies with the γ1 subunit (Ymer et al., 1990) suggest that it produces unusual responses. When the γ1 subunit is combined with α1 or α5, and β1 subunits in oocytes, a reduced potentiation is observed following flunitrazepam administration. In addition, the response to inverse agonists such as DMCM is either reduced, or unexpectedly potentiated (von Blankenfeld et al., 1990). Consequently, it has been suggested that this receptor subunit combination may not occur in vivo (Ymer et al., 1990). The novel γ3-subunit, recently isolated by Herb and colleagues (1991), also appears to confer benzodiazepine responsivity and has been shown to functionally replace the γ2 subunit (Knoflach et al., 1991).

Experiments in which receptors have been artificially produced in vitro, have shown that $GABA_A$ receptor subunits differ in function. The γ subunit is required to elicit a benzodiazepine response, but the α subunit appears to determine benzodiazepine pharmacology. For example, the type of α subunit affects both the efficacy and affinity of benzodiazepine binding (Sigel, Baur, Trube, Mohler and Malherbe, 1990; von Blankenfeld, 1990). Substitution of a δ subunit for a γ subunit in recombinant receptors results in a loss of benzodiazepine sensitivity (Shivers et al., 1989). This suggests that receptors containing a δ subunit may constitute $GABA_A$ receptors that are not modulated by benzodiazepines.

Multiple benzodiazepine receptor subtypes?

Early benzodiazepine binding studies identified compounds with differential binding characteristics. A distinction was drawn between Type I and Type II benzodiazepine receptors (Klepner et al., 1979). Some receptors displayed a greater affinity for benzodiazepines such as CL 218 872 and β-carbolines. These are the Type I receptors. They are distributed ubiquitously in the brain but are particularly abundant in the cerebellum. Type II receptors have a high affinity for benzodiazepines but a reduced affinity for other compounds. These receptors are more selectively located in the hippocampus, striatum and spinal cord.

The molecular basis for this potential receptor heterogeneity has been examined by expression of recombinant receptors in cell lines. Receptors composed from the α1, β1 and γ2 subunits have high affinity for CL 218 872 (Pritchett et al., 1989). The distribution of α1 subunits is also high in the cerebellum (Luddens et al., 1990), suggesting that this subunit confers Type I receptor pharmacology. Evidence indicates that several α subunits may be responsible for Type II receptor pharmacology. Receptors expressing the α2 and α3 subunits show lower affinity for benzodiazepine Type I selective ligands (Pritchett, Luddens and Seeburg, 1989; Pritchett and Seeburg 1990). Substitution of the α5 subunit results in Type II-like pharmacology but with a reduced affinity for the benzodiazepine ligand zolpidem (Pritchett and Seeburg, 1990).

The inverse agonist Ro 15-4513 has been shown to bind to cerebellar granule sites that are not labelled by [^{3}H] flunitrazepam. These sites, first described by Turner, Sapp and Olsen (1991) have been termed diazepam insensitive (DI) sites, because diazepam fails to displace a significant proportion of bound [^{3}H] Ro 15-4513 at these receptors. Several other inverse agonists including CGS 8216 bind to DI sites. However, DI sites do not uniquely bind inverse agonists since they also bind the partial agonist ZK 91216. The properties of DI sites are probably due to the inclusion of the α6 subunit. Luddens et al. (1990) found that DI like properties were exhibited by human embryonic kidney cells transfected with α6, β2, and γ2 subunit cDNAs (but not other subunits). Further evidence to support this conclusion is that the distribution of the α6 subunit

conveniently corresponds to the distribution of DI sites, because it is expressed almost exclusively in the cerebellum (Luddens et al., 1990). The insensitivity of the α6 subunit to diazepam is probably conferred by the presence of a single amino acid residue. Substitution of an arginine amino acid residue for a histidine in an α6 variant leads to diazepam sensitivity (Wieland, Luddens and Seeburg, 1992). Conversely, replacing a histidine residue with an arginine in the α1 subunit confers diazepam insensitivity (Kleingor, Wieland, Korpi, Seeburg and Kettenman, 1993). The α4 subunit probably also confers insensitivity to diazepam (Wisden et al., 1991).

Rather than two classes of benzodiazepine receptors, division into four classes may provide a better description of the data: Type I receptors (α1βγ2), Type II receptors (α2/3βγ2), Type III receptors (α5βγ2) which have a low affinity for zolpidem, and Type IV receptors (α6βγ2) which have low affinity for benzodiazepine agonists but high affinity for Ro 15-4513.

Heterogeneity of benzodiazepine receptors could explain the selective profile of benzodiazepine partial agonists. Partial agonists could differ in their affinity for specific receptor subtype populations located in different brain areas. This would mean that targeting one receptor population would lead to a highly selective behavioural outcome. However, because there are no selective antagonists for benzodiazepine receptor subtypes at present, this theory is difficult to test. In addition, the situation is made slightly more complicated by the fact that recent studies have shown that subunit composition may not only affect the affinity, but also the intrinsic activity of a compound (Puia, Vicini, Seeburg and Costa, 1991; Wafford, Whiting and Kemp, 1993). What this probably means is that for any receptor subtype it is necessary to consider the intrinsic efficacy of a ligand as well as its affinity. This suggests that compounds with a selective efficacy could be developed which affect GABA neurotransmission at some but not all receptor subpopulations. The first potential subtype selective antagonist for $GABA_A$ receptors has recently been reported. Furosemide has been shown to antagonize GABA evoked currents in cerebellar granule cells comprising the α6 subunit but not the α1 subunit (Korpi, Kuner, Seeburg and Luddens, 1995). Additionally, U-90042, a novel hypnotic

with specificity for receptors containing the α6 subunit has also been recently described (Tang, Smith, Carter, Im and Vonvoigtlander, 1995). These compounds and those like it will be important for investigating the functional significance of different receptor subunits.

Which combinations occur in vivo?

The actual composition of $GABA_A$ receptors in vivo, and the rules for subunit assembly are not precisely known. This is because although recombinant receptors can be artificially expressed in Xenopus and other systems, it does not necessarily follow that these receptor combinations will also occur in vivo. Some indication as to the most plausible subunit combinations in vivo has been gathered from studies examining the pattern of gene expression using in situ hybridization. This technique allows a map to be drawn of messenger RNA (mRNA) encoding in the brain. It is assumed that the levels of mRNA reflect levels of protein (receptor expression) in that area. The distribution of mRNA in the CNS varies for different subunits. Some subunits such as the α1 and β2 subunits are often co-localized (Wisden, Laurie, Monyer and Seeburg, 1992). Other evidence suggests that ρ subunit is only expressed in the retina (Cutting et al., 1991).

Other investigators have made use of antibodies to label specific subunits to identify subunit combinations. These antibodies precipitate varying amounts of receptor which can be detected by immunoprecipitation and immunoaffinity chromatography. Most receptors precipitated contain a single α subunit type (Duggan and Stephenson, 1990). Antibodies recognising all β subunits are able to precipitate 100% of purified benzodiazepine receptors, whereas antibodies raised to the γ2 subunit precipitate only 75% of receptors (Stephenson, Duggan and Pollard, 1990). For the δ receptor, specific antibodies precipitate only about 20-30% of purified benzodiazepine receptors (Benke, Mertens, Trzeciak, Gillessen and Mohler 1991). This suggests that most $GABA_A$ receptors associated with benzodiazepine binding sites contain α, β and γ subunits.

Evidence from both mRNA studies and antibody labelling suggests the existence of several likely combinations of $GABA_A$ receptor subunits in vivo (McKernan and Whiting, 1996). First, a combination of $\alpha 1\beta 2\gamma 2$ subunits, which probably corresponds to the Type I benzodiazepine receptor. Second, an $\alpha 2\beta 2/3\gamma 2$ subunit combination which may be equivalent to the Type II benzodiazepine receptor. Another likely combination of subunits is $\alpha 6\beta x\gamma 2$ which may correspond to the diazepam insensitive site, and is located preferentially on cerebellar granule cells. Other likely combinations include the $\alpha 2\beta x\gamma 1$, $\alpha 5\beta 3\gamma x$ and $\alpha 4\beta\delta$. Therefore, although the exact stoichiometry and subunit composition of naturally occurring $GABA_A$ receptors remains to be determined, some receptor combinations are probably more plausible than others. Further work is required to isolate specific subunit combinations from the brain.

1.2.8 Summary

The specific combination of $GABA_A$ receptor subunits may determine the resultant pharmacology of benzodiazepine receptor ligands. Theoretically, since it is possible that many different populations of $GABA_A$ receptors exist in vivo, stimulation of a particular population could result in selective behavioural effects. The availability of ligands for these different receptor populations would mean that behaviour could be affected more precisely. For example, it might be possible to elicit the hyperphagic effects of benzodiazepine receptor agonists without inducing anxiolytic or sedative activity.

It has been shown that the γ subunit is necessary for benzodiazepine receptor activation and that substitution of different γ subunit results in unusual responses to inverse agonists. Additionally, the type of α subunit is important for determining the binding of benzodiazepine ligands. The $\alpha 1$ subunit confers Type I receptor pharmacology, whereas $\alpha 2$ and $\alpha 3$ subunit inclusion leads to Type II receptor responses. Substitution of the $\alpha 5$ subunit also results in reduced affinity for the benzodiazepine ligand zolpidem. The $\alpha 6$ subunit confers insensitivity to diazepam and a selective affinity for the inverse agonist Ro 15-4513.

Four possible benzodiazepine receptor subtypes are shown in Fig 1.3. The availability of more selective drugs, specific for particular receptor subunit combinations, would allow behaviour to be affected much more discretely.

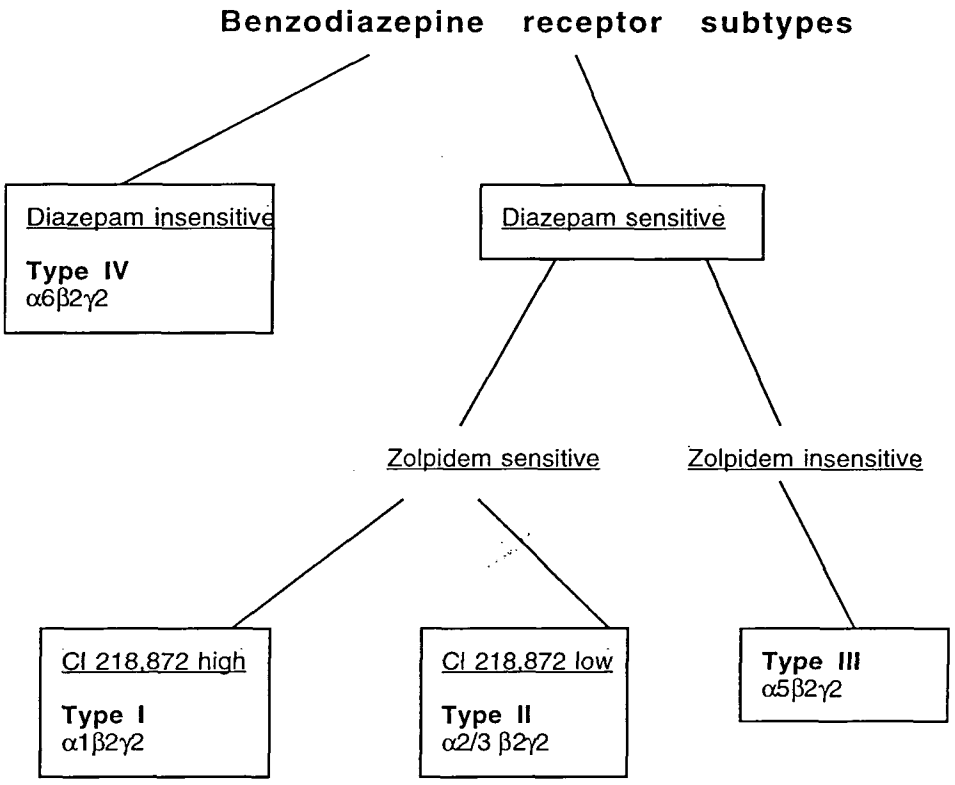


Figure 1.3 Diagram to show putative benzodiazepine receptor subtypes. Benzodiazepine receptor subtypes are represented by different combinations of $GABA_A$ receptor subunits and are differentiated in terms of ligand specificity. Adapted from Luddens et al. (1995).

1.2.9 Endogenous ligands

The presence of specific binding sites for benzodiazepines in the CNS suggested to many researchers the possibility that endogenous ligand could be found. The search for a naturally occurring ligand was encouraged by fact that the behavioural effects of the opioid antagonist naloxone had been explained by its blockade of endogenous opioid compounds (Hughes et al., 1975).

The first potential endogenous modulator of the benzodiazepine receptor complex to be identified was the β-carboline β–CCE. This compound was obtained from human urine but was later found to be an artifact of the extraction procedure (Braestrup and Nielson, 1980). Another possible candidate was purified from brain extracts by Guidotti and colleagues (Guidotti, Toffano and Costa, 1978). This compound was named diazepam-binding inhibitor (DBI) because it displaced ^{3}[H]-Diazepam from specific binding sites (Guidotti et al., 1983). DBI has been shown to possess inverse agonist like properties (Bormann, Ferrero, Guidotti and Costa, 1985). It has proconflict effects (Corda, Baker, Mendelson, Guidotti and Costa, 1983) and acts as an anxiogenic (Guidotti et al., 1983). Cleavage of DBI produces several peptide products that also inhibit diazepam binding. One product of cleavage is the neuropeptide octadecaneuropeptide (ODN). It has been proposed that DBI is a precursor of ODN and other smaller peptides which bind to benzodiazepine receptors (Ferrero, Guidotti, Conti-Tronconi and Costa, 1984). DBI immunoreactivity has been detected in the brain (Ferrero, Costa, Conti-Tronconi and Guidotti, 1986) and it is possible that ODN, derived from DBI, acts as a negative modulator in vivo. Naturally occurring positive allosteric modulators of benzodiazepine receptors have also been located in the brain. These compounds have been called endozepines and have been purified from both rat and human brain (Rothstein et al., 1992a,b).

The presence of an endogenous ligand has also been inferred indirectly from experiments that have demonstrated behavioural effects of the specific benzodiazepine antagonist flumazenil. It is assumed that flumazenil has no intrinsic efficacy and so can

only affect behaviour by blocking the action of an endogenous ligand. Flumazenil has been shown to provoke panic attacks in some studies (Nutt, Glue, Lawson and Wilson, 1990). However, it is controversial whether this effect reflects an intrinsic pharmacologic action of the drug, or is due to blockade of an endogenous ligand. It is true to say that although research aimed at elucidating an endogenous benzodiazepine ligand has yielded several candidates, there is no general consensus on the presence and activity of such compounds.

1.2.10 Interim summary

Over the past 25 years a range of ligands from full agonist to full inverse agonist have been developed which bind with high affinity to benzodiazepine receptors in the CNS. These ligands are useful tools which can be used to further our understanding of the neural and behavioural basis of ingestive behaviour.

It has been shown that benzodiazepine receptors are associated with the $GABA_A$ receptor complex and that benzodiazepines ligands modulate GABA function by altering the frequency of $GABA_A$ receptor Cl- channel opening. Recent developments in the field of molecular biology have revealed unexpected heterogeneity in the composition of the $GABA_A$/benzodiazepine receptor complex. This multiplicity is due to the differential assembly of at least 15 receptor subunits. Combining different subunits is proposed to result in the formation of different receptor subtypes possessing unique physiological properties and pharmacological profiles. This raises the possibility that the diverse effects of benzodiazepines on behaviour are due to stimulation of specific receptor subtypes in different brain areas. Functional analysis of $GABA_A$ receptor subtypes of known subunit composition could aid the development of subtype-selective drugs with which to probe behaviour more precisely. These pharmacological studies provide a basis for understanding how benzodiazepines may bring about the changes ingestive behaviour which are reviewed in the next section.

1.3 Benzodiazepines and food intake

The aim of this section is to review the literature concerning the specific effects of benzodiazepines on ingestive behaviour. The relationship between the appetite stimulating effects of benzodiazepine receptor agonists and their anxiolytic effects will first be discussed. The effect of benzodiazepine ligands on food intake will then be reviewed. Finally, experiments that have examined the behavioural mechanisms involved in benzodiazepine effects on ingestion will be addressed.

The first report to appear documenting the effects of benzodiazepines on food intake was the finding by Randall and colleagues (1960) that the benzodiazepine agonist chlordiazepoxide (CDP) increased food consumption in rats and dogs. This result was largely ignored at the time. However, since this original observation, benzodiazepine-induced hyperphagia has been reported for a whole range of agonists (Cooper, 1989). Moreover, the effect has also been reported across many different species, including the cat (Fratta, Mereu, Gessa, Pagletti and Gessa 1976; Mereu, Fratta, Chessa and Gessa, 1976), rabbit (Mansbach, Stanley, and Barret 1984), primate (Foltin, Ellis and Schuster, 1985), wolf (Kreeger, Levine, Seal, Callahan and Beckel, 1991), and pigeon (Cooper and Posados-Andrews, 1979). This suggests that the hyperphagic effect is not species-specific and does not depend upon a particular type of feeding strategy or diet.

1.3.1 Benzodiazepine-induced hyperphagia and anxiety

It has been argued that the hyperphagic effects of benzodiazepines can be explained by appealing to the anxiolytic action of these drugs (Poschel, 1971). This hypothesis suggests that benzodiazepine agonists increase food intake via a decrease in the anxiety associated with exposure to a novel test situation. Appealing though this explanation may be, it cannot account for all the data. For example, benzodiazepine receptor agonists have been shown to stimulate food intake in animals that have been fed to satiety and are fully familiarized with the test procedure (Cooper, Barber, Gilbert and Moores, 1985). In addition, Cooper has demonstrated that when a food-deprived animal

is given a choice between a novel and familiar food, CDP (5-10 mg/kg) increases the consumption of the familiar food, but not that of the novel food (Cooper and Crummy, 1978). This evidence suggests that instead of overcoming any neophobia towards the novel food, CDP may affect appetite directly. Such an interpretation is complicated however, by the results of an additional study in which increasing the dose of CDP to 15 mg/kg led to an increase in the consumption of the novel food (Cooper and McClelland, 1980). This result was explained in terms of a dual effect of CDP on both food neophobia and appetite. Increased intake of the familiar food at low doses was assumed to reflect a specific appetite-enhancing effect of this compound, while increased choice of the novel food at higher doses was thought to be due to an anti-neophobic action.

This hypothesis was examined further by testing for differences in the development of tolerance to the two effects (Cooper, Burnett and Brown, 1981). Tolerance has been shown to develop to the antianxiety action of benzodiazepines (File, 1980) but not to the hyperphagic effects of these compounds (Cooper and Francis, 1979a). Following acute injection of CDP, choice of the novel food was increased, whereas following chronic treatment, choice of the familiar food was enhanced. Novelty was assumed to be a critical factor in determining the effects of CDP since no tolerance was observed in a group of animals that had been previously familiarized with all the test foods. The explanation for these data was that tolerance developed rapidly to the anti-neophobic effects of CDP, leaving the appetite-enhancing effects of the drug intact. In the above experiment, an anti-neophobic action of CDP was detected, but this effect could nevertheless be dissociated from the additional hyperphagic effects of this compound (Cooper et al., 1981). Therefore, the increase in food intake caused by benzodiazepines is probably due to a specific effect on appetite because the effect cannot be fully explained by the action these drugs also have on anxiety induced by a novel test situation.

1.3.2 Benzodiazepine ligands and food intake

The following section deals with the effect of benzodiazepine receptor agonists, partial agonists, antagonists and inverse agonists on ingestive behaviour. Comparison across different classes of benzodiazepines ligands is important for demonstrating pharmacological specificity, because if a behavioural effect is mediated by benzodiazepine receptors then agonists, antagonists and inverse agonists should elicit predictable effects on behaviour.

Benzodiazepine receptor agonists

When administered peripherally, benzodiazepine agonists have been shown to reliably produce hyperphagia in rats and numerous other species (Cooper, 1980a, 1989). Non-benzodiazepines such as zopiclone which exhibit agonist properties at benzodiazepine receptors also induce increases in food intake (Cooper and Moores, 1985a). The increase in food intake can be blocked by administration of selective benzodiazepine receptor antagonists (Cooper and Moores, 1985b; Cooper et al., 1985), and the effect is stereospecific (Cooper and Yerbury, 1986a). Therefore, the hyperphagic effect of these compounds is probably due to a specific action at benzodiazepine receptors.

Benzodiazepine receptors are located in the central nervous system and at peripheral sites. The benzodiazepine receptor agonist clonazepam shows selectivity for central type benzodiazepine receptors, whereas Ro 5-4864 binds preferentially to peripheral type receptors. It has been shown that clonazepam, but not Ro 5-4864, stimulates an increase in the food consumption in rats (Cooper and Gilbert, 1985). This result implies that stimulation of central rather than peripheral type receptors accounts for the hyperphagic effects of benzodiazepine agonists.

The only benzodiazepine receptor agonist which apparently failed to enhance ingestive behaviour reliably was the novel sedative-hypnotic, zolpidem. Zolpidem is a full agonist which displays some anxiolytic activity, but also has pronounced sedative effects at low doses (Depoortere et al., 1986; Sanger and Zivkovic, 1988). Cooper and

others have provided data to suggest that when administered alone zolpidem has no effect on eating and drinking responses (Sanger and Zivkovic, 1988; Yerbury and Cooper, 1989). It was suggested that the unusual profile of zolpidem could be explained by its selectivity for benzodiazepine Type I receptor subtypes. According to this line of argument, Type I receptors are involved in regulating anxiolytic and sedative behaviour, but not ingestional responses. This result is of potential interest since it may provide evidence for mapping of subtype selective behaviours. More recently though, Stanhope and colleagues have shown that under certain circumstances a hyperphagic effect of zolpidem can be demonstrated (Stanhope, Roe, Dawson, Draper, and Jackson, 1993). The reason for this discrepancy may be that the experimental protocol used by Stanhope and associates minimised the sedative effects of the drug and so allowed an unmasking of its effects on food intake. In summary, a comprehensive range of benzodiazepine receptor full agonists have been tested in ingestive behaviour experiments and all have been shown to increase food intake under certain circumstances.

Benzodiazepine receptor partial agonists

Partial agonists are low efficacy compounds that display anxiolytic and anticonvulsant activity, but do not produce either ataxia or sedation (Haefely et al., 1990). A number of benzodiazepine receptor partial agonists such as bretazenil (Ro 16-6028) and the β-carboline ZK 91296 have been shown to increase in food intake in rats (Cooper, Yerbury and Desa, 1987; Yerbury and Cooper, 1987). However, not all partial agonists reliably induce increases in ingestive behaviour. Cooper and colleagues found that the pyrazoloquinolines CGS 9896 and CGS 9895 did not stimulate an increase in food consumption in non-deprived animals (Cooper and Gilbert, 1985; Cooper and Yerbury, 1986a). Several other authors have also failed to observe an effect of CGS 9896 and CGS 9895 on food intake in hungry rats (Sanger, Joly and Zivkovic, 1985). This is despite the fact that these drugs were able to antagonize the effect of benzodiazepine receptor full agonists (Cooper and Yerbury, 1986). The lack of effect of CGS 9896 and CGS 9895 on ingestive behaviour may be due to the low efficacy of

these compounds. Alternatively, it could be that CGS 9896 and CGS 9895 are subtype selective agonists which bind with reduced affinity to a population of benzodiazepine receptors specific for ingestional responses (Cooper et al., 1987). This conclusion is not supported by recent data gathered by Chen, Davis and Loew (1995). These authors found that in both deprived and non-deprived animals, CGS 9896 did induce an increase in intake of a palatable diet. Therefore, although it appears that in most cases benzodiazepine partial agonists increase ingestive behaviour, for compounds with very weak agonistic properties, such as CGS 9896 and CGS 9895, the degree of hyperphagia observed depends upon the specific test conditions.

Benzodiazepine receptor antagonists

In feeding tests, the benzodiazepine flumazenil and the β-carboline ZK 93426 behave as benzodiazepine receptor antagonists. When administered alone these compounds do not significantly affect feeding behaviour (Cooper and Gilbert, 1985; Estall and Cooper, 1986). However, flumazenil has been shown to block the increase in intake induced by clonazepam (Cooper and Gilbert, 1985).

Benzodiazepine receptor inverse agonists

If benzodiazepine receptor agonists increase food intake then it is predicted that inverse agonists should cause anorexia. This has been confirmed in many studies. The inverse agonists FG 7142, DMCM, and the weak inverse agonist CGS 8216 all produce dose-dependent decreases in the consumption of a palatable diet in non-deprived male rats (Cooper et al., 1985). The effect of FG 7142 can be reversed by administration of the antagonist ZK 93426 (Cooper, 1986b). In addition, midazolam, clonazepam and triazolam have all been found to block the reduction in intake produced by FG 7142 (Cooper, 1985a; Cooper et al., 1985). This suggests that the anorectic effect of inverse agonists is mediated by central benzodiazepine receptors.

As shown in Fig 1.5 control over eating behaviour can be exerted bidirectionally through drug action at benzodiazepine receptors. Agonists enhance food consumption, whereas inverse agonists reduce intake.

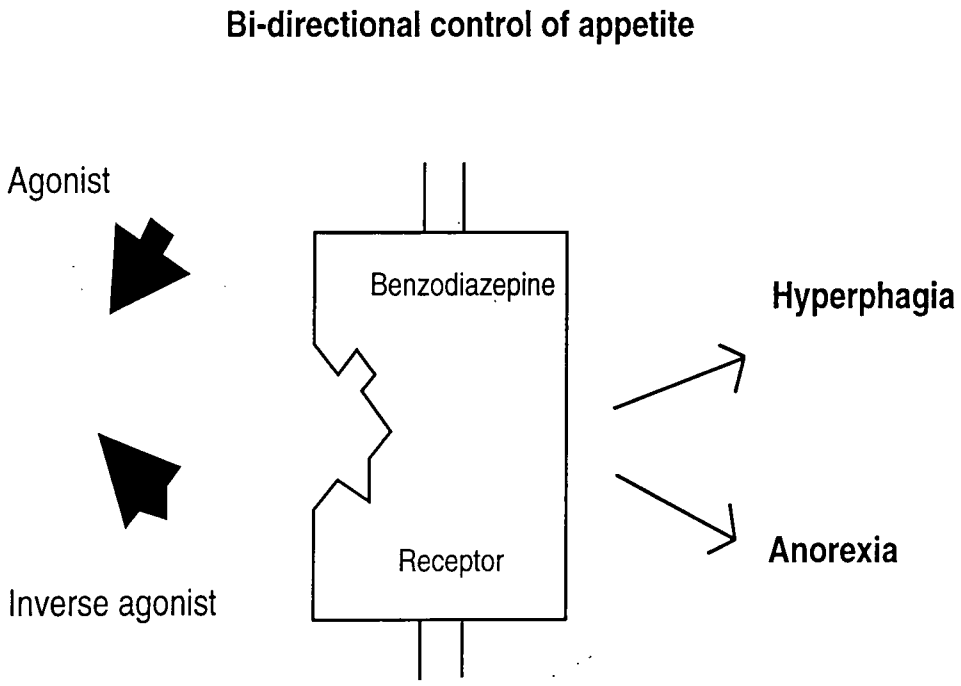


Figure 1.5 Diagram to show bidirectional control of food intake at the level of the benzodiazepine receptor. Agonists increase food intake and inverse agonists decrease food intake.

1.3.3 Benzodiazepine-induced hyperphagia and GABA function

Despite evidence of a close association between GABA and benzodiazepines there has been some debate concerning the relevance of this relationship for the effects of benzodiazepine ligands on food intake. If benzodiazepine receptor agonists exert their effects on ingestive behaviour via facilitation of GABAergic transmission then it is predicted first, that GABA agonists should increase food intake, and second, that GABA antagonists should block benzodiazepine-induced hyperphagia. This hypothesis has been tested with mixed results. The GABA agonist muscimol has been shown to increase

food intake in rats in some cases (Grandison and Guidotti, 1977), but not others (Sanger, 1984). Studies using GABA antagonists have provided similarly ambiguous findings. Birk and Noble (1982) found that the increase in ingestive behaviour induced by diazepam could be attenuated by pretreatment with both picrotoxin and bicuculline. However, this result has not been replicated by other authors (Sanger, 1984; Shepherd and Broadhurst, 1982). The lack of effect of muscimol on feeding behaviour observed by Sanger (1984) may have been due to the systemic route of administration used, because muscimol is reported to have poor penetration into the brain due to rapid metabolism (Snodgrass, 1978). One difference between GABA agonists and benzodiazepine agonists which might explain these results is that GABA agonists produce a global effect at $GABA_A$ receptors throughout the central nervous system, whereas benzodiazepine agonists require the presence of endogenous GABA for their effects.

However, more difficult to explain without abandoning the hypothesis that GABAergic transmission mediates the hyperphagic effect of benzodiazepine ligands, is the finding that GABA antagonists did not block the hyperphagic effect of CDP. An important point in this respect may be that not all $GABA_A$ receptors are coupled to benzodiazepine receptors. It could be that an action of GABA mimetics at sites not linked benzodiazepine receptors effectively masks any effect at the benzodiazepine $GABA_A$ receptor complex. Alternatively, because GABA antagonists block other behavioural effects of benzodiazepines such as their anti-conflict effects (Scheel-Kruger and Peterson, 1982) and sedative effects (Billingsley and Kubena, 1978), it could be that GABA mediates some but not all of the behavioural effects of these drugs. Because of problems in interpreting the results of the effect of GABA antagonists on feeding behaviour induced by benzodiazepine receptor agonists, a role for GABA in mediating the hyperphagic effect of these drugs has not been unequivocally demonstrated. However, the wealth of pharmacological and biochemical data suggesting an association between GABA and the behavioural effects of benzodiazepines means that GABA facilitation is the most likely mechanism of action.

1.3.4 Summary

Evidence suggests that benzodiazepine receptor ligands exert a specific effect on ingestive behaviour that is distinct from the additional action of these drugs on anxiety processes. It has been shown that benzodiazepine-induced changes in food intake are mediated by benzodiazepine receptors located in the central nervous system. It is possible to exert bidirectional control over feeding behaviour, depending on the nature of the benzodiazepine receptor ligand: agonists cause hyperphagia, inverse agonists have an anorectic action, and antagonists block the effects of these compounds. The effects of these drugs on ingestive behaviour are probably mediated via modulation of GABAergic transmission mediated by $GABA_A$ receptors.

1.3.5 Behavioural processes

The control of food intake involves the integration of many complex signals which may be metabolic, hormonal or neural in nature. Because of this, many opportunities exist for pharmacological intervention in the system. Therefore, although it is consequently often quite easy to demonstrate a drug-induced effect on appetite, the interpretation of such an effect may not be straightforward. For example, a drug could affect food intake in any of three major ways. First, it could affect satiety processes. A drug could influence the post-ingestive consequences of feeding such that the inhibition of hunger brought on by the food itself is either increased or decreased. Second, it could alter the sensory perception of the food (taste, smell and texture), or its palatability. Finally, the drug might not have a direct effect on the controls of ingestive behaviour at all but instead induce non-specific effects. For example, an animal may have a motoric or sensory deficit and not be able to contact the food properly or even detect its presence. Alternatively, the drug may induce sickness. Much of the behavioural pharmacology of appetite has been engaged in determining the specificity of drug-induced changes in ingestion. Some progress has been made in analyzing the behavioural mechanisms responsible for the effects of benzodiazepine ligands on food intake. The results of an extensive period of research into the behavioural mechanisms responsible for

benzodiazepine-induced changes in food intake are reviewed below. Early theory tended to emphasize the importance of hunger and satiety as important factors, whereas later theory has focused on the role of taste factors or palatability.

1.3.6 Hunger and satiety

It has been known since 1974 that diazepam will increase operant responding for food pellets in non-deprived rats (Wise and Dawson, 1974). One explanation for this effect is that the effects of benzodiazepines on feeding behaviour are similar to those produced by food deprivation. However, some authors have detected differences between the effects of food deprivation and hunger. This suggests that benzodiazepine receptor agonists do not produce their effects on food intake by mimicking hunger (Hunt, Poulos and Cappell, 1988; Shepherd and Broadhurst, 1982). Recent experiments using an incentive learning paradigm lend support to this conclusion (Balleine, Ball and Dickenson, 1994). Balleine and colleagues (1994) found that instead of producing a state analogous to hunger, midazolam influenced the incentive value ascribed to the food. Consumption of a non-nutritive saccharin solution was only enhanced in animals that had previously been given the opportunity to learn about the incentive value of the food under the midazolam condition. These authors argued that incentive value is determined by the hedonic response to the food, and that benzodiazepines increase incentive value through an increase in the perceived palatability of the stimulus. It would be expected that inverse agonists would reduce the incentive value of the instrumental outcome because of a decrease in palatability. This hypothesis has yet to be confirmed, but evidence from intra-cranial self stimulation (ICSS) suggests that a result to the contrary would be extremely surprising. Pellow and Herberg (1984) found that FG 7142 produced a dose related decrease in responding for rewarding stimuli that was effectively blocked by CDP.

An early explanation of benzodiazepine-induced increases in food intake placed emphasis on the potential anti-satiety effects of these drugs (Margules and Stein, 1967). The results of more recent experiments involving manipulation of within-meal satiety

factors do not provide evidence to support this interpretation. Midazolam has been shown to increase food consumption in animals that have been pre-fed to satiety, suggesting that it is probably not suppressing satiety signals (Cooper et al., 1985). This led investigators to examine the possibility that instead of mimicking hunger or inhibiting satiety, benzodiazepines may increase food intake by affecting palatability.

1.3.7 Palatability

If the usefulness of a scientific concept is defined by its ability to stimulate research then the concept of palatability has proved to be extremely useful indeed. Despite this, difficulties with the definition and misunderstanding of the term have led to many calls that it should be abandoned as an explanatory construct in the field of ingestive behaviour research. A full discussion of palatability is beyond the scope of this thesis. Nevertheless, given the pervasiveness of the term within the literature it is necessary to define what is meant by the term palatability in this thesis. Traditionally, palatability has been taken to refer to invariant properties of tastants which lead to them being either accepted or rejected by consumers. As Grill and Berridge (1985) have pointed out though, this definition constitutes a stimulus measure and has clear limitations. A tastant which is consumed readily by animals, such as sucrose, is often described as a 'palatable' stimulus. The problem with this definition is that the response to sucrose may not always be predictable. For example, a sucrose solution accepted by an animal on one occasion may be rejected on another. Acceptance of the sucrose may depend not only on some perceptual quality of stimulus itself, but also on the physiological state of the animal (e.g. sated versus non-sated). Conditioned associations may also have an influence, such as the previous pairing of the stimulus with illness (Garcia and Koelling, 1966). As an alternative to a stimulus measure, Grill and Berridge (1985) have suggested that palatability might be more properly defined as the outcome of the integration of taste signals with internal state and associative signals. A mathematical model of ingestive behaviour proposed by Davis and Levine (1977) suggests that palatability factors exert excitatory control over feeding while internal-state factors (e.g.

post-ingestive feedback) have an inhibitory effect. Despite this, the use of the term 'palatable' as a modifier for a taste stimulus may be justified in certain cases since the palatability of some tastes is relatively stable under a broad range of conditions.

The use of the terms palatable and palatability in this thesis reflect their usage in the literature. The terms will sometimes be used to describe a taste stimulus (as in 'palatable' mash) but will also be referred to in a more strictly defined sense to describe the integrative process occurring in the central nervous system elicited by taste stimuli.

Various attempts have been made to provide an operational definition of palatability. This has mainly involved the development of tests which measure the behavioural response to taste stimuli. Such tests include the taste preference test and sham feeding paradigm. There has been considerable debate about which of these paradigms constitutes the best measure of palatability. Each test has obvious drawbacks and limitations. This means that a single test is unlikely to provide privileged information concerning palatability. Therefore, the most useful exercise is probably to draw conclusions from the effects of a drug manipulation across a range of these paradigms, rather than merely relying on one. Gathering evidence from several experimental sources is the most reasonable way of asserting that a drug is preferentially affecting palatability. Benzodiazepine ligands provide a good example of a drugs which have been examined in many tests thought to provide a measure of palatability.

Palatable mash consumption

It has been argued that studies in which animals are offered a highly palatable diet enable conclusions to be drawn concerning the effect of a drug manipulation on hedonic factors involved in the control of eating behaviour (Blundell, 1987). Often the test food is a palatable sweet wet mash which is consumed in large quantities by non-deprived rats. Benzodiazepine receptor agonists dramatically increase the consumption of this diet (Cooper and Gilbert, 1985). These results provide evidence that benzodiazepine receptor agonists may increase food intake by modulating hedonic responses to foodstuffs.

Predictably, benzodiazepine receptor inverse agonists reduce intake of palatable

food. It has been reliably demonstrated that FG7142 (a ß-carboline) and CGS 8216 (a pyrazoloquinoline) and Ro 15-4513 (an imidazobenzodiazepine) all produce dose-dependent and significant decreases in palatable food intake (Cooper et al., 1985; Cooper, Bowyer and van der Hoek, 1989).

Sham feeding

The sham feeding paradigm provides a means of studying the effect of oropharyngeal factors on the consumption of palatable food. In this preparation, the aim is to eliminate postingestional factors by allowing ingested material to drain out of the stomach via a chronically implanted gastric fistula. Such fistulated animals display a pronounced satiety deficit, and it has been proposed therefore that the technique provides a measure of palatability (Weingarten and Watson, 1982). Midazolam and clonazepam have been shown to produce significant increases in the sham feeding of a palatable sucrose solution compared with a vehicle control condition (Cooper, van der Hoek and Kirkham, 1988). This suggests that the increase in consumption caused by benzodiazepine receptor agonists is not due to an effect on the rate of gastric emptying, because it is still observable when food material is prevented from accumulating in the gut. Postabsorbative factors are also less likely to be able to account for benzodiazepine-hyperphagia, although it has been argued that the sham feeding preparation may not totally eliminate absorption (Sclafani and Nissenbaum, 1985). Despite this, data from sham feeding experiments seems to suggest that benzodiazepines have an effect on oropharyngeal factors controlling intake rather than postingestional effects (Cooper et al., 1988).

The inverse agonists CGS 8216, Ro 15-3503 and FG 7124 all produce dose-related decreases in real and sham feeding of a palatable sucrose solution (Kirkham and Cooper, 1987; Cooper et al., 1988). CGS 8216 was found to cause a 50% reduction in sucrose consumption. The suppression of sham feeding caused by CGS 8216 was also antagonized by flumazenil suggesting the effect is mediated by central benzodiazepine receptors (Kirkham and Cooper, 1987). CGS 8216 has been shown to increase plasma

corticosterone levels, an effect that is also observed in response to stress (Pellow and File, 1985). Therefore, it is possible that CGS 8216 decreased intake because it induced a stress-like state. However, several pieces of evidence argue against this interpretation. First, sham drinking of water was unaffected by CGS 8216. This indicates that the decrease in sucrose consumption was not due to a non-specific deficit because of stress. Second, the effect of CGS 8216 on palatable food consumption was not abolished by adrenalectomy. This suggests that the effects of this drug on ingestive behaviour are not related to increased plasma corticosterone levels (Cooper and Kirkham, 1987). Third, the decrease in the rate of ingestion caused by this inverse agonist is similar to the effects observed when decreasing sucrose concentration in the sham feeding rat. Therefore, the effect of CGS 8216 and other inverse agonists on ingestive behaviour are probably better explained by a diminution in palatability.

Taste preference

It is possible that benzodiazepine-induced hyperphagia is a general effect, and that following administration of benzodiazepine receptor agonists the consumption of all ingestive material is enhanced indiscriminantly. Alternatively, benzodiazepine ligands may act on feeding responses related to the evaluation of taste stimuli. The taste preference paradigm allows this hypothesis to be tested directly. Instead of having access to a single bottle, animals are given a choice between two bottles. If benzodiazepine receptor agonists produce a non-specific increase in ingestive behaviour then an increase in the consumption of both solutions should be observed in this test. If the effect is specific for taste related behaviour then consumption of one solution should be enhanced selectively. Maickel and Maloney (1974) exposed water-deprived rats to water, or solutions of 0.2% saccharin or 0.5% tartartic acid. These authors noted that although benzodiazepine receptor agonists had a general hyperdipsic effect, this effect was more pronounced when animals had access to the sweet saccharin solution. One explanation for this result is that the agonists were enhancing sweet taste preference, an effect distinct from the general hyperdipsia also observed. This hypothesis was only explicitly tested

much later in 1988, by Cooper and Yerbury who used a two-bottle intake test in which rats were given a choice between a preferred (0.05%) saccharin solution and water. In this experiment, clonazepam increased the consumption of the saccharin solution without causing a concomitant increase in the consumption of water. Using the same preference test, this result has since been extended to include a range of benzodiazepine receptor full agonists such as CDP (Parker, 1991), the β-carboline abecarnil (Cooper and Greenwood, 1992), and partial agonists including bretazenil and Ro 17-1812 (Cooper and Green, 1993). These data indicate that benzodiazepine receptor agonists specifically affect ingestive behaviour since the results cannot be accounted for by arguing that there was general increase in arousal. In addition, saccharin is a non-nutritive stimulus and so this supports the conclusion that benzodiazepines do not significantly affect the postingestive consequences of feeding.

The prediction that inverse agonists produce the opposite effect to agonists and block sweet taste preferences has been tested in several studies. The inverse agonists FG 7142, CGS 8216, Ro 15-4513 all reduce saccharin intake in a two-choice test without affecting water consumption (Cooper, 1986a; Cooper et al., 1989; Kirkham and Cooper, 1986). This result contrasts with the effects of other well-known anorectic agents such as amphetamine which display a general hypodipsic effect and reduce consumption of both water and saccharin (Maickel and Webb, 1972).

To examine whether the effect of benzodiazepine ligands on hedonic responses to food extends to salty tastes, salt preference tests have been used. Isotonic saline is readily ingested by rats and benzodiazepine agonists have been shown to cause an increase in the consumption of a palatable 0.9% NaCl solution (Estall and Cooper, 1987; Turkish and Cooper, 1984). Abecarnil has also been shown to increase intake of a 0.9% NaCl solution, without affecting water consumption in a two-choice test (Cooper and Greenwood, 1992). The same result has been obtained using the partial agonists bretazenil and Ro 17-1812 (Cooper and Barber, 1993). Therefore, it appears that modulation of preferences by benzodiazepines is not limited only to sweet tastes.

The effect of inverse agonists on salt preference has been examined with

somewhat surprising results. Cooper and Barber (1993) did not observe any reduction in the preference for a 0.9% saline solution in a two-bottle test following administration of Ro 15-4513. In addition, this compound did not increase the relative aversion to a stronger 1.8% NaCl solution. This result implies that unlike sweet tastes there is no bidirectional control of preference for salt. However, Ro 15-4513 may have an atypical pharmacological action and so it will be necessary to examine the effects of other inverse agonists before any firm conclusions can be drawn. Alternatively, the mechanisms underlying salt and sweet preference may be different and so caution must be exercised in equating the two.

It could be argued that benzodiazepine agonists increase intake in the taste preference test by having an anti-aversive effect rather than acting directly on palatability. However, this explanation is not supported by data from experiments investigating quinine aversion. Adding quinine to fluids and foods causes them to have an aversive taste. Margules and Stein (1967) observed that oxazepam can overcome this aversion. Cooper and Green (1993) employed a two-bottle intake test in which rats were given a choice between water and 0.005% quinine HCl. In this case the animals display a preference for water. Following treatment with bretazenil and Ro 17-1812, consumption of water was unaffected, whereas an increase in the ingestion of quinine was observed. This evidence suggests that it is unlikely that benzodiazepine agonists merely enhance the choice of a more preferred fluid, because water intake was not affected in the test. It could still be argued that this result is due to an anti-aversive effect. However, this conclusion is not supported by the effect of benzodiazepine receptor inverse agonists on quinine aversion. In a two-bottle test, FG 7142 did not elicit an increase in the aversion to quinine, although a decrease in preference for saccharin was observed in the same study (Cooper, 1986a). This evidence implies that inverse agonists attenuate the positive reactions to sapid stimuli without affecting aversive reactions. In summary, the most parsimonious explanation of these taste preference data is to suggest that benzodiazepine ligands increase the palatability of flavoured solutions.

Taste reactivity

The taste reactivity test (Grill and Norgren, 1978a,b) is a method that enables the dissociation of the ingestion of fluids from the hedonic or aversive responses to those stimuli. In this paradigm, sapid solutions are infused directly into the oral cavity of rats. The orofacial responses and body movements elicited are then recorded. These responses are species-specific, reliably emitted, and are referred to as fixed action patterns (FAPs). The pattern of responding indicates whether the animal is reacting to the infused solution in a positive, ingestive manner, or with aversive reactions. It has been proposed that this test provides a relatively pure measure of palatability. Using a taste reactivity paradigm Berridge and Treit (1986) found that CDP increased the number of ingestive FAPs elicited in response to solutions of 0.03M sucrose, 0.01M HCl and 10^{-3}M QHCl, but had little or no effect on aversive or neutral FAPs. This evidence suggests that CDP enhances the positive palatability of ingested fluids, and has been replicated in several studies (Berridge, 1988; Treit and Berridge, 1990; Treit, Berridge and Schultz, 1987). Midazolam has also been shown to affect taste reactivity responses in rats which do not have an intra-oral cannula but instead are allowed to sample taste stimuli voluntarily. In this modification of the taste reactivity test, midazolam has been shown to increase the number of ingestive responses to a 3% sucrose solution (Gray and Cooper, 1995). These data are consistent with those reported for oral-cannulated animals.

The effect of inverse agonists in the taste reactivity paradigm has not been so meticulously investigated. Treit et al. (1987) found that the enhancement of positive palatability produced by CDP could be antagonised by flumazenil and the weak inverse agonist CGS 8216. However, CGS 8216 did not have any intrinsic inhibitory activity, even at doses found to produce reliable effects on sham feeding (10 mg/kg). This result may have been due to a floor effect, and further studies will be essential to characterize the effect of inverse agonists in the taste reactivity paradigm. It might be predicted that inverse agonists would only affect positive palatability and so would attenuate positive ingestive reactions while not affecting aversive reactions, but this hypothesis remains to be tested.

1.3.8 Summary

There is much evidence to suggest that the changes in food intake elicited by administration of benzodiazepine ligands is due to a specific effect of these drugs on palatability. In support of this view, midazolam has been shown to increase sucrose sham feeding and ingestive taste reactivity responses. Several benzodiazepine receptor agonists have also been shown to enhance the consumption of a preferred saccharin solution, without affecting water intake, in a two-bottle choice test. In addition to the tests described above such as sham feeding and taste reactivity, it has been suggested that analysis both of the time course and detailed structure of the components of ingestive behaviour may provide information concerning the behavioural mechanisms responsible for changes in intake. Studies in this area could provide important new information to elucidate further the mechanisms involved in the effects of benzodiazepine ligands on ingestive behaviour. The next section is concerned with reviewing the techniques available for analyzing the detailed structure of ingestive behaviour.

1.3.9 Temporal analysis of feeding behaviour

Feeding behaviour varies along two dimensions: the contextual and the temporal (Blundell, 1987). Context refers to the impact of environmental conditions on feeding behaviour. The temporal dimension reflects the pattern of feeding over time. Much research activity has been focused on analysis of the temporal pattern of feeding behaviour. The reason for this may have something to do with the relative ease of recording the occurrence of feeding events with the use of automatic recording equipment. For example, the availability of reasonably priced computers to log large numbers of data points has been a key factor in the development of this area (Clifton, 1987). It has been argued that examination of temporal patterns of feeding and drinking allows conclusions to be drawn concerning the behavioural mechanisms responsible for any changes in food intake. Blundell (1987) has suggested that analysis of the sequence of ingestive behaviours may be particularly useful for distinguishing between pharmacological manipulations which mimic naturally occurring physiological processes

as opposed to those which induce pathological or non-specific effects. Merely measuring intake would not yield enough information to allow such a distinction to be made.

Several problems may be encountered in attempting to describe the pattern of feeding behaviour over time. The first involves defining the most appropriate unit of classification to use. An important point in this respect is that the components of feeding behaviour are not distributed randomly over time. Thus, an animal engaged in feeding or drinking will chew or lick in runs of rapidly repeated stereotyped actions. These sequences of behaviours, which are often referred to as bouts, are then separated by pauses in which non-ingestive behaviour is displayed. Additionally, over longer periods bouts of ingestive behaviour also occur in clusters or meals. It may be reasonable to assert therefore, that feeding behaviour is organised into naturally occurring descriptive units. This acknowledgement means that discrete episodes of feeding (bouts and meals), can be treated as variables that may reflect specific changes in the control of ingestive behaviour. The study of bouts of behaviour has proved to be fruitful area of research, especially in the field of ethology (McFarland, 1970; Slater, 1974).

Having accepted the usefulness of analyzing ingestive behaviour in terms of bouts and meals, a second problem presents itself. This problem is concerned with choosing an appropriate bout or meal criterion (Clifton, 1987; Machlis, 1977). Bout structure is determined by the length of the intervals between ingestive acts rather than the acts themselves. Traditionally, bouts of behaviour have been defined by choosing an interval (x_t) to separate one bout from another. An interval less than x_t is classified as a within-bout interval, and an interval equal to or greater than x_t is classified as a between-bout interval. The setting of this criterion has often been done arbitrarily by merely observing the behaviour. Recently, to provide a more objective definition, bout criterions have been derived from log survivorship functions. A log survivorship analysis first involves back cumulation of interval frequency (starting with the longest not the shortest interval). This cumulative frequency is then plotted on a log scale against interval length. Events that are randomly distributed over time follow a negative exponential distribution. When plotted on a log survivor plot, this kind of distribution appears as a straight line. However,

intervals between feeding events do not conform to this simple model. It is not surprising therefore that a single negative exponential does not provide a good fit for ingestive behaviour data. Instead, a broken stick appearance describes the data quite well. The curve descends steeply at first and then subsequently declines more gradually. The sum of two negative exponential distributions generates a better fit for this data. This supports the assumption that it is possible to divide the intervals that separate feeding events into two categories generated by different processes: those which have a high probability of a further event following (within-bout interval) and those which have a low probability of another event occurring (between-bout intervals). It has been suggested that the simplest way of defining a bout would be to use a criterion just to the right of the most obvious break point in the log survivorship function (Slater, 1974). Errors of classification will always be made, since the distributions are overlapping and so classification into two mutually exclusive categories is impossible. However, log survivor plots generated in feeding experiments generally have sharp discontinuities making misclassification less likely. Choosing an error that is too short will have most impact on the data, and so the best strategy is to select a criterion that is slightly longer than the optimal to reduce this possibility. Once a suitable criterion has been defined, feeding data can be analyzed according to bout or meal size and frequency among other parameters. This technique has been applied to the study of long term changes in meal patterns over 24h in rats and to analyzing the structure of single meals, both of which are described below.

Meal pattern analysis

Analysis of meal patterns usually involves the monitoring of feeding in free feeding animals over a 24h period. Frequently, animals are tested in a specially adapted home cage that enables the time of removal of a food pellet from the hopper to be recorded (Clifton, 1987). This method has the advantage of allowing animals to be observed in a more normal feeding situation because deprivation or access to palatable food is not required to induce animals to feed.

The effect of bretazenil on meal patterning has been examined by Clifton and

Cooper (1996). Bretazenil increased the size of the first meal following drug treatment. However, no increase in food intake was observed over the 24h period. Therefore, the effect of the drug was short lived. One factor to allow for when interpreting these results is that bretazenil is a short acting drug and so may have ceased to be effective after 2h. Nevertheless, the results do imply that bretazenil does not induce long term changes in eating patterns since the size of further meals was not significantly different from controls. Importantly, these data confirm that benzodiazepine hyperphagia can be demonstrated in a situation where the animals are fully habituated to a quiet unchanging environment.

Microstructural analysis

Examination of the pattern of feeding over long periods, can often provide information concerning the mechanisms responsible for drug-induced changes in intake. However, sometimes it may be more revealing to focus on behaviour within a meal. Characterization of the individual components of ingestive behaviour is called microstructural analysis. This kind of analysis may be especially pertinent for the study of the effects of benzodiazepines ligands on ingestive behaviour. Meal pattern data suggests that benzodiazepine-induced hyperphagia may be rapid in onset and fairly short lived. Assuming this is the case, it might be expected that quantification of feeding behaviour at a fine grained level would be more appropriate than 24h analysis of feeding and drinking patterns.

Microstructural analysis has been applied to eating and drinking behaviour alike. Such analysis involves dividing feeding behaviour within a meal into bouts as described above. Examination of the effects of benzodiazepine receptor ligands on solid food intake has shown that midazolam increases intake via a lengthening the individual bouts of ingestion in rats consuming a palatable mash (Cooper and Yerbury, 1986b). Midazolam did not affect the frequency of bouts. Similar analysis conducted for the inverse agonist FG 7142 showed that this compound reduced the duration of bouts, but did not affect the number of bouts.

Microstructural analysis has been more frequently applied to the study of licking behaviour. This is probably a direct result of the availability of lickometers to record accurately the occurrence of individual licks. The effects of manipulating palatability on bout structure has been thoroughly examined (Davis and Perez, 1993; Davis and Smith, 1990; Davis and Smith, 1992). Davis and Smith (1992) found that for a range of carbohydrates, bout size increased monotonically with increasing concentration. Consequently, it has been suggested that bout duration may be sensitive to changes in palatability.

A microstructural approach may also be sensitive to non-specific motor effects on licking behaviour. Licking in rats is highly stereotyped in nature, consisting of fast rhythmic tongue protrusions. Stellar and Hill (1952) showed that rats lick at a reasonably constant rate of about 6-7 licks per second. This is the intrabout lick rat which is calculated by counting the number of intervals between licks in a bout and then dividing by the time spent drinking. Any disruption of this rate would suggest that a pharmacological treatment might be affecting sensorimotor coordination, as distinct from a specific effect on the controls of ingestion. Thus, Davis and Smith (1992) showed that intrabout lick rate is reduced by moving the drinking spout progressively further away from an animal, but not by altering the solution concentration. These data suggest that microstructural analysis provides a useful model for studying the effects of benzodiazepines on ingestive behaviour.

Rate analysis

It is important to distinguish between two types of rate measure referred to in the literature. The first type of measure is the rate of consumption within a bout, or local rate of eating which has been referred to previously. The second is the change in the rate of licking over test session (overall lick rate). The model for the control of ingestive behaviour proposed by Davis and Levine (1977) makes specific predictions concerning the effects of excitatory orosensory input and post-ingestive negative feedback on the rate of ingestion. These predictions have been confirmed experimentally in a number of

studies (Davis and Levine, 1977; Davis, Kung and Rosenak, 1995; Davis and Smith, 1990). This suggests that analysis of overall lick rate provides important information concerning the relative contribution of these variables to the control of food intake. For example, it is found that initial lick rate increases with increasing carbohydrate concentration (Davis, 1973; Davis and Smith 1988), but decreases as a result of quinine administration (Davis and Levine, 1977). Therefore, the initial lick rate has been taken as a measure of palatability. The rate of clearance of ingested substances from the stomach can be manipulated by adding mannitol to the test meal. Mannitol is absorbed from the intestine very slowly and is found to have a selective effect on the decline in the rate of licking within a test period. The decrease in the rate of licking has thus been taken to be a measure of post-ingestive negative feedback (Davis, Collins and Levine, 1975; Davis and Levine, 1977). An advantage of examining the change in the rate of ingestion over a meal is that it enables the interaction between orosensory stimulation and post-ingestive negative feedback to be studied rather than studying these factors in isolation.

Satiety sequence

Changes in feeding behaviour over time can also be analyzed in terms of the transition from feeding to non-feeding components. It has been noted that if animals are deprived overnight and then allowed to feed to satiety the next day they exhibit a characteristic sequence of behaviours. Initially, feeding predominates but then this gives way to other behaviours which occur in a reproducible sequence as follows: Feeding - activity - grooming - sleep/resting (Antin et al., 1975). If a drug is having a specific effect on satiety then it should induce a shift in behaviour on the temporal plane. If on the other hand, a drug is having a non-specific effect on behaviour then this would be reflected in a disruption of component parts of the sequence rather then a temporal shift. Clifton and Cooper (1996) have recently examined the effect of bretazenil on the satiety sequence. Despite a high baseline intake of palatable mash in this experiment, bretazenil was found to increase consumption in a 30 min test. A decrease in the time spent engaged in active behaviours and grooming was also observed, and there was a significant increase in

resting. Bretazenil appeared to speed the transition from feeding to activity and grooming, but also enhanced resting. Bretazenil did not significantly extend feeding behaviour in the temporal plane, but counter-intuitively displayed a profile similar to anorectic drugs such as the indirect 5-HT agonist fenfluramine. One interpretation of the results is to say that bretazenil did not increase food intake by suppressing satiety but merely increased consumption only in the early part of the test. This conclusion is consistent with other data suggesting that the action of benzodiazepines on food intake is related to the initial hedonic evaluation of ingested materials. The results also indicate that enhanced progression from feeding to resting in the satiety sequence may not be a reliable indicator of an effect on satiety mechanisms.

1.3.10 Summary

Detailed examination of the components of feeding in short term tests (analysis of bout structure) and over 24h periods (meal pattern analysis) can provide information concerning the behavioural mechanisms responsible for changes in food intake. Analysis of the change in the rate of ingestion within a meal can also allow the contribution of factors such as satiety and palatability in determining meal size to be assessed. No data have been published to date concerning the effect of benzodiazepine ligands on the rate and microstructure of licking behaviour. Therefore, studies of this kind may be important in determining the behavioural mechanisms responsible for benzodiazepine-induced changes in intake.

1.4 Neural substrates

Since the hyperphagic effects of benzodiazepine receptor agonists were first reported in the 1960s, progress has been made in defining the behavioural mechanisms of action for the effect these drugs on ingestion. Concurrent with progress in understanding the behavioural mechanisms involved in benzodiazepine-induced changes in food intake, there has also been an increase in understanding the pharmacology and molecular biology of benzodiazepine receptors. Consequently, there is now a comprehensive profile of

compounds from full agonists through to partial agonists, antagonists, partial inverse agonists and full inverse agonists with which it is possible to exert bidirectional control at the receptor level.

Despite such behavioural and pharmacological advances, little evidence has been gathered to date concerning the neural substrate for the effect benzodiazepine ligands on ingestive behaviour. The focus for the few studies that have been carried out has been on brainstem structures. Given the fairly sparse distribution of benzodiazepine receptors in this area may seem somewhat surprising. Nevertheless, there is evidence to suggest that brainstem sites, although traditionally neglected in the study of ingestive behaviour, may have an important role to play in the control of feeding. Berridge (1988) has shown that the increase in positive ingestive responses occurring following administration of CDP can still be observed in a decerebrate rat preparation. In this preparation a transection is made at the level of the midbrain effectively isolating communication of hindbrain structures with forebrain areas. This result is important because it indicates that the neural circuitry necessary for the effects of benzodiazepine receptor agonists in the taste reactivity test may be located within the lower brainstem. Since taste is an important determinant of ingestive behaviour it is possible that benzodiazepine receptor agonists might increase food intake by acting on neural systems involved in the processing of taste information. Following this argument, if benzodiazepine-induced hyperphagia is due to a change in the taste properties of ingested materials, then brainstem mechanisms might be expected to be involved not only in the effects of benzodiazepines in the taste reactivity paradigm but also in intake tests.

1.5 Interactions with opioids

It has been suggested that benzodiazepine and opioid effects on behaviour may be interdependent. In support of this hypothesis, opiate antagonists have been shown to block the effects of benzodiazepines in a range of behavioural tests. For example, naloxone has been shown to block the anti-conflict effects of diazepam in the Geller-Seifter test (Soubrie, Joubert and Thiebot, 1980). In addition, some biochemical evidence

also suggests that the effect of benzodiazepines may be related to endogenous opioid action. Duka and her colleagues have demonstrated that benzodiazepines modulate enkephalin release (Duka, Wuster and Herz, 1979).

The possibility that the effects of benzodiazepines on ingestive behaviour are mediated in some way by opioids has also been investigated (Cooper, 1983a; Reid, 1985). Examination of the effects of both opioids and benzodiazepine ligands on food intake reveals some striking similarities. For example, morphine has been shown to cause a significant increase in food consumption when administered systemically (Kavaliers and Hirst, 1985; Sanger and McCarthy, 1980). Various other opioid agonists cause reliable increases in feeding comparable to benzodiazepine-induced stimulation of ingestion (Cooper, Moores, Jackson and Barber, 1985; Gosnell, Levine and Morley, 1983, 1986; Morley, Levine, Kneip, and Grace, 1982). Opioid agonists, like benzodiazepine receptor agonists, increase the preference for saccharin in water deprived rats (Calcagnetti and Reid, 1983). In addition, several authors have shown that morphine also enhances positive taste reactions in a manner similar to benzodiazepine agonists (Doyle, Berridge and Gosnell, 1993; Rideout and Parker, 1996). Conversely, opioid antagonists, which block the action of endogenous opioids, have been shown to inhibit food intake (Cooper, 1980; Holtzman, 1974, 1975; Margules, Moisset, Lewis, Shibuya and Pert, 1978), attenuate sweet taste preference (Cooper, 1983; Le Magnen, Marfaing-Jallat and Devos, 1980), and reduce sucrose sham feeding (Kirkham and Cooper, 1988a; Rockwood and Reid, 1982). The effect of the opioid antagonist naloxone on sham feeding sucrose is reversed by increasing sucrose concentration (Kirkham and Cooper, 1988b). This suggests that opioids, like benzodiazepines, may be involved in palatability (for review see Cooper and Kirkham, 1993).

It has been suggested that hedonic evaluation of foodstuffs may be mediated by benzodiazepine receptors which then leads to the release of endogenous opioid peptides (Cooper, 1983a). This hypothesis predicts that opioid antagonists should block benzodiazepine-induced effects on ingestion. Opioid antagonists have been shown to block benzodiazepine-induced hyperphagia (Birk and Noble, 1981; Britton, Britton,

Dalton and Vale, 1983; Jackson and Sewell, 1985; Naruse, Asami and Koizumi, 1989; Stapleton, Lind, Merriman and Reid, 1979). However, opiate antagonists can also reduce food intake in the absence of any other treatment (Apfelbaum and Mandenoff, 1981; Cooper and Turkish, 1989). In some of the above experiments, the effect of naloxone when administered alone was not thoroughly examined and so this makes it difficult to interpret the results of any potential interaction. An attenuation of benzodiazepine-induced increases in food intake by naloxone may have been the result of an additive action of both these drugs. Further work is required to investigate the role of the opioid system in benzodiazepine-induced effects on feeding.

1.3.13 Summary

No systematic attempt has been made to date to investigate the neural substrate for benzodiazepine-induced changes in feeding behaviour. Evidence from the taste reactivity paradigm suggests that benzodiazepines may affect the early processing of taste stimuli. Berridge found that increases in ingestive responding in the taste reactivity test, which is characteristic of benzodiazepine agonists, could still be observed in a decerebrate rat preparation. If the effects of benzodiazepine ligands on food intake are mediated by the same receptor populations responsible for the enhancement of hedonic responding, then examination of brainstem sites may prove to be fruitful in this area.

It has been reported that the effects of benzodiazepines can be blocked by administration of opiate antagonists. This suggests a role for endogenous opioids in benzodiazepine-induced effects on ingestive behaviour. The similarity between the effects of benzodiazepine agonists and opioid agonists on food intake supports this hypothesis, but the precise nature of any interaction remains to be thoroughly investigated.

1.6 Aims

The main aims of this thesis are first, to investigate the neural substrate for benzodiazepine-induced changes in food intake, and second, to further examine the behavioural processes responsible for the effect of benzodiazepine ligands on ingestive behaviour. This work forms the basis of a longer term goal which is to explain how the molecular effects of benzodiazepines translate into neuronal activity within particular brain areas to bring about specific changes in ingestive behaviour. This kind of investigation could help to increase understanding generally of the mechanisms involved in the control of ingestion.

The experiments reported in Chapters 2 and 3 used microinjection techniques to examine the neural basis of benzodiazepine-induced hyperphagia. There is some evidence to suggest that brainstem structures may be involved in the effects of benzodiazepine ligands on food intake (see section 1.4). The aim of the experiments in Chapter 2 was to determine whether hindbrain structures play an important role in the hyperphagic effect of benzodiazepines. In Experiment 1, midazolam was injected directly into the IVth ventricle, and its effect on palatable food intake was measured. In Experiment 2, the selective benzodiazepine antagonist flumazenil was used to establish specific benzodiazepine receptor-mediation of an enhanced feeding effect.

These initial experiments were followed up in Chapter 3 with injections of midazolam into an area proximal to the IVth ventricle. The parabrachial nucleus (PBN) of the pons was identified as a possible candidate for mediation of the effects observed following injection into the IVth ventricle. The effect of injection of midazolam into the PBN on consumption of a palatable mash was investigated (Experiment 3). Experiment 4 then checked if the response to midazolam is mediated by specific benzodiazepine receptors by pre-treating the animals with flumazenil. The aim of Experiment 5 was to determine whether the effect of midazolam injected directly into the PBN increased the consumption of palatable food whether solid or liquid. Non-deprived rats were trained to consume a palatable 3% sucrose solution and the potential hyperphagic effect of intra-PBN midazolam was investigated (Experiment 5). The behavioural specificity of the

effects of intra-PBN midazolam was also examined by investigating the effect of injections of midazolam on locomotor activity (Experiment 6).

It has been suggested that analysis of the rate and microstructure of ingestion may provide information concerning the behavioural mechanisms responsible for changes in intake. However, this kind of analysis has been limited to the study of carbohydrate intake. The aim of the experiments reported in Chapter 4 was to extend these observations to the consumption of fats as well as carbohydrates. A microstructural approach was adopted to compare the licking responses to a carbohydrate (sucrose) and a lipid emulsion (Intra-lipid) in both a 20-min test (Experiment 7) and a brief contact test (Experiment 8). The rationale was that this would provide a starting point for interpretation of the effects of drugs on the consumption of both lipid and carbohydrates.

There have been no reports of the effects of benzodiazepine ligands on the microstructure of licking for either carbohydrates or fats. Chapter 5 investigated the effect of midazolam on licking responses for sucrose and Intra-lipid. The aim of Experiment 9 was to examine in detail the effect of midazolam on the temporal and microstructural characteristics of licking for Intra-lipid in a 20-min test. Licking for sucrose was also measured under the same conditions. In Experiment 10, the aim was to focus on potential oropharyngeal determinants of ingestion in more detail by limiting access to the liquids to an initial 60s period.

In Chapter 6 the effect of the benzodiazepine receptor inverse agonist Ro 15-4513 on the microstructure of sucrose and lipid drinking was analyzed using a brief contact test. The aim of Experiment 11 was to test the hypothesis of bidirectional control at the level of the benzodiazepine receptor.

The experiments reported in Chapter 7 were carried out to investigate further the suggestion that endogenous opioids are involved in benzodiazepine-induced effects on ingestive behaviour. First, a microstructural analysis of the effect of the opioid agonist morphine and the antagonist naloxone on lipid consumption was performed (Experiments 12 and 13). The aim of these experiments was to examine whether the effects of benzodiazepines and opioids on microstructural components of licking behaviour are

similar. In Chapter 8, a microstructural analysis of the effect of naloxone on midazolam induced increases in intake was carried out using a brief contact test (Experiment 14). Previous reports of an interaction between benzodiazepines and opioids in the control of food intake had yielded inconsistent results. The aim of Experiment 14 was to improve on previous designs and use a dose of naloxone shown to have no intrinsic effect on intake.

The general aim of this set of experiments was to provide new information concerning the potential neural and behavioural mechanisms involved in the effects of benzodiazepine ligands on ingestive behaviour.

The effects of direct administration of midazolam into the IVth ventricle on ingestive behaviour

2.1 Introduction

Following systemic administration, benzodiazepine receptor agonists such as chlordiazepoxide (CDP) and midazolam reliably produce hyperphagia in numerous species including the rat (Cooper, 1980a, 1989). For example, benzodiazepine receptor agonists have been shown to stimulate a substantial increase in food consumption in non-deprived rats trained to eat a palatable diet (Cooper et al., 1985). The hyperphagic effect can be blocked by a specific benzodiazepine receptor antagonist such as flumazenil (Cooper et al., 1985; Cooper and Moores 1985b).

It is unlikely that the increase in food intake brought about by benzodiazepine agonists is due to an inhibition of satiety. Instead, it has been proposed that benzodiazepine-induced hyperphagia is due to an increase in the palatability or hedonic value of ingested materials (Berridge and Pecina, 1995). In support of this view, midazolam has been shown to increase sucrose sham feeding (Cooper, et al., 1988), and several benzodiazepine agonists have been shown to selectively enhance the consumption of a preferred saccharin solution, without affecting water intake, in a two-bottle choice test (Cooper and Green, 1993; Cooper and Yerbury, 1988; Roache and Zabik, 1986). Furthermore, CDP has also been shown to enhance positive ingestive responses in the taste reactivity paradigm (Berridge and Treit, 1986; Treit et al., 1987).

Despite much progress in analyzing the behavioural mechanisms underlying the effects of benzodiazepines on ingestion, little is known about the neural substrate for benzodiazepine-induced hyperphagia. An important result relevant to possible central sites of action of benzodiazepine agonists, is that CDP retains its effect to enhance positive taste reactions in the chronic decerebrate rat (Berridge, 1988). These data appear to indicate that the lower brain-stem may contain the site(s) of action for the effects of CDP in the taste reactivity paradigm. In an extension of this work, Berridge and Pecina

(1995) have shown recently that direct administration of the benzodiazepine receptor agonist diazepam into the IVth ventricle in the hindbrain of rats was sufficient to enhance the positive hedonic reactions elicited by a 7% sucrose solution. The conclusion from these data is that structures close to the IVth ventricle may be involved in mediating the effects of benzodiazepine receptor agonists in the taste reactivity test. Since the influence of postingestive factors are eliminated in the taste reactivity test, the increase in ingestive responding brought about by benzodiazepine agonists is probably due to an effect on the processing of taste stimuli.

The proposal that the hyperphagic effect of benzodiazepine agonists might be due to the influence these drugs have on taste processing is supported by the effectiveness of these compounds in the sham feeding, and taste preference tests. Benzodiazepine-induced increases in food consumption may also depend on brainstem taste systems. The present experiments were designed to investigate the possibility that hindbrain structures may be important for the hyperphagic effects of benzodiazepine agonists. In Experiment 1, the water soluble benzodiazepine receptor agonist midazolam was injected directly into the IVth ventricle of the hindbrain, and its effect on food intake was determined. It was predicted that if brainstem structures are important for the hyperphagic effects of benzodiazepine agonists then injection of midazolam into the IVth ventricle should mimic the effects of systemic administration. Blockade of the effect by the selective benzodiazepine receptor antagonist flumazenil was used as a test of the specific benzodiazepine receptor mediation of the enhanced feeding effect (Experiment 2).

2.2 Method

2.2.1 Animals

Twenty three non-deprived adult male hooded rats (General strain bred in the School of Psychology, University of Birmingham) weighing 300-500 g were used. Rats were housed individually in plastic cages in a room with a constant temperature of 22± 2 °C, and were maintained on a 12h light:dark cycle (lights on at 8.00). Standard laboratory food pellets (Pilsbury 41B, Heygate and Sons, U.K.) and water were available at all times, except during testing. Behavioural testing was conducted in the light phase.

2.2.2 Drugs

The benzodiazepine receptor agonist midazolam maleate (Roche, Basel, Switzerland) was prepared for injection by dissolving in isotonic saline. Midazolam was used preferentially to other agonists because it is soluble in water. The doses used, were 3 and 30 μg/μl of midazolam which had been determined to be effective in previous pilot experiments. The vehicle used in control injections was isotonic saline.

The selective benzodiazepine antagonist flumazenil (Ro 15-1788) (Roche, Basel, Switzerland) was prepared for injection by ultrasonic dispersion in distilled water to which Tween 80 (BDH Chemicals Ltd, Poole, England) had been added. The dose used in these experiments was 20 mg/kg or vehicle (distilled water to which Tween 80 had been added). This dose was used because it had been shown in previous experiments to be effective in blocking the hyperphagia induced by systemic midazolam (Cooper et al., 1985; Cooper and Moores, 1985b).

2.2.3 Surgery

For implantation of stainless-steel guide cannulae, rats were anesthetized and placed in a stereotaxic frame. The anesthetic used was pentobarbital at a dose of 60 mg/kg i.p. A single stainless steel guide cannulae (21 gauge, 16 mm length) was implanted 1 mm dorsal to the IVth ventricle (coordinates L 0, A-P, -10.5 V -6). Bregma was used as a reference point and the coordinates were taken from the atlas of Paxinos and

Watson (1982). The cannulae were fixed to the skull using three screws and dental acrylic. Stylets were placed in the guide cannulae to prevent occlusion, and the rats were allowed seven days to recover before behavioural testing occurred. Postoperative care involved weighing the rats daily and applying an antibiotic wound powder to the headmount if necessary.

2.2.4 Injection procedure

Central microinjection of midazolam was performed using an injection cannula connected by a polyethylene tube to a micrometer-driven 10 µl Hamilton syringe. The injection needles protruded 1 mm beyond the tip of the guide cannula, and accuracy of injections was ensured by observing the progress of an air bubble in the tubing. The volume infused was 3 µl, injected over a period of 1-min. Each rat was then placed immediately in the test cage and food intake over a 30-min test period was measured. In the case of pretreatment with flumazenil, the antagonist was administered via the i.p. route 15-min prior to central injection of midazolam. The volume injected was 1 ml/kg. Two days prior to testing each rat received a sham injection of isotonic saline to familiarize it with the microinjection procedure.

2.2.5 Test meal

The sweetened mash meal was made up daily according to the following formula: 100 ml sweetened condensed milk, 400 ml ground maintenance diet (Special Diet Services Ltd., Essex, UK), and 200 ml distilled water. The constituents were mixed to produce a soft mash. This recipe has been previously shown to be readily consumed by rats (Cooper et al., 1985).

2.2.6 Procedure

Following recovery from surgery, the rats were adapted to eating the palatable sweetened mash. Familiarization continued until a steady baseline intake of mash was observed which was achieved after a period of approximately 10 days. Each rat was given 30-min access to 50 g portions of the diet placed in a clear plastic dish inside an individual stainless steel test cage. The consumption of the sweetened mash was measured to the nearest 0.1 g with corrections made for any spillage. During testing, rats did not have access to water or maintenance diet. All testing was carried out during the light cycle to insure relatively low baseline levels of consumption.

2.2.7 Histology

At the end of each experiment rats were deeply anaesthetized with pentobarbital and a small quantity of methylene blue dye was injected through the cannula. Each rat was then perfused transcardially with isotonic saline followed by a 10% formalin solution. After decapitation, the excised brains were fixed in a 10% formalin solution for one week. The fixed brains were then frozen and sectioned sagitally on a freezing microtome and the correct placement of the cannulae was verified histologically. The histological work was conducted blind with respect to the behavioural results.

2.2.8 Statistical design and analysis

A repeated-measures design was used throughout in which each rat served as its own control and 24h elapsed between successive injections. A 24h wash-out period was considered sufficient since midazolam is a short acting drug with a half life of 27 $\pm$ 1 min when injected peripherally in rats. The data were analyzed using a one-way analysis of variance (ANOVA) for repeated-measures. Post hoc comparisons between means were carried out using Dunnett's t-test or a Newman-Keuls multiple comparisons test. Statistical tests were performed using Statview SE+graphics (Abacus Concepts Inc., Berkeley, CA, 1987) and a result was considered statistically significant if $p < 0.05$.

2.3 Results

Experiment 1: The effect of IVth ventricular midazolam on ingestion of sweet mash.

Of the fifteen rats used in Experiment 1, three rats did not undergo behavioural testing due to problems following surgery. Histological analysis revealed that all cannulae were correctly targeted in the remaining animals and therefore all of the remaining 12 rats were included in the data analysis (Figure 2.1). Midazolam produced a dose-dependent increase in food consumption ($F_{2,22} = 5.61$, $p < 0.01$). The baseline intake of 13.8 g was increased to 18 g after 30-min (Figure 2.2). Individual comparisons with a Dunnett's t-test revealed that a significant increase occurred at the 30 µg/µl dose ($p < 0.01$). Midazolam had an effect early in the test session and a significant increase in intake was evident after 10-min ($F_{2,22} = 7.25$, $p < 0.01$). The difference between treatment groups did not reach significance at 20-min ($F_{2,22} = 0.23$, n.s.), but was evident at the end of the 30-min session. A summary of the results is shown in Table 2.1.

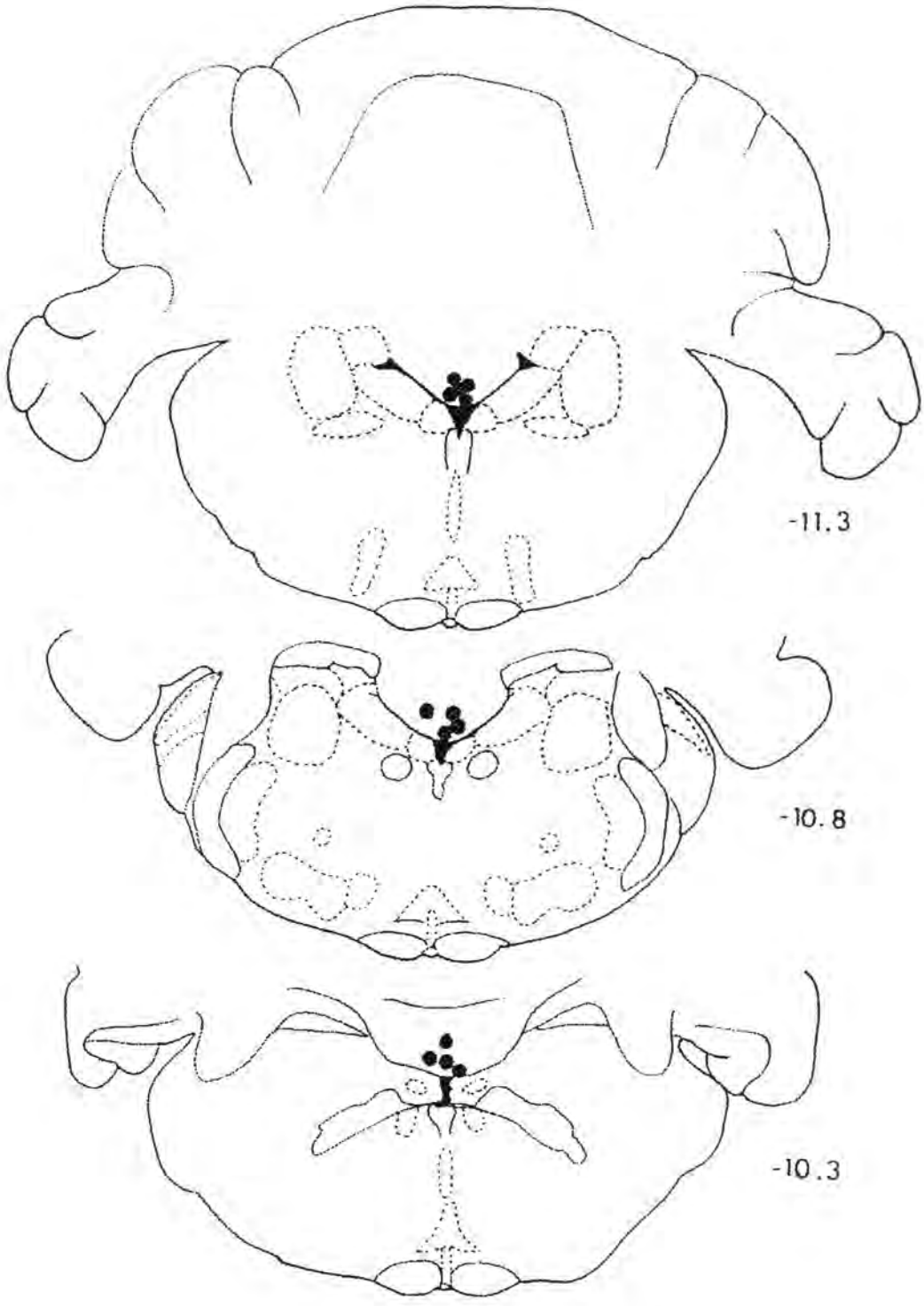


Figure 2.1 The distribution of injection sites in the IVth ventricle for rats used in Experiment 1. Sections are redrawn from Paxinos and Watson (1982). Each shows the location of the injection cannula tip for 1 rat. Section numbers refer to mm from bregma.

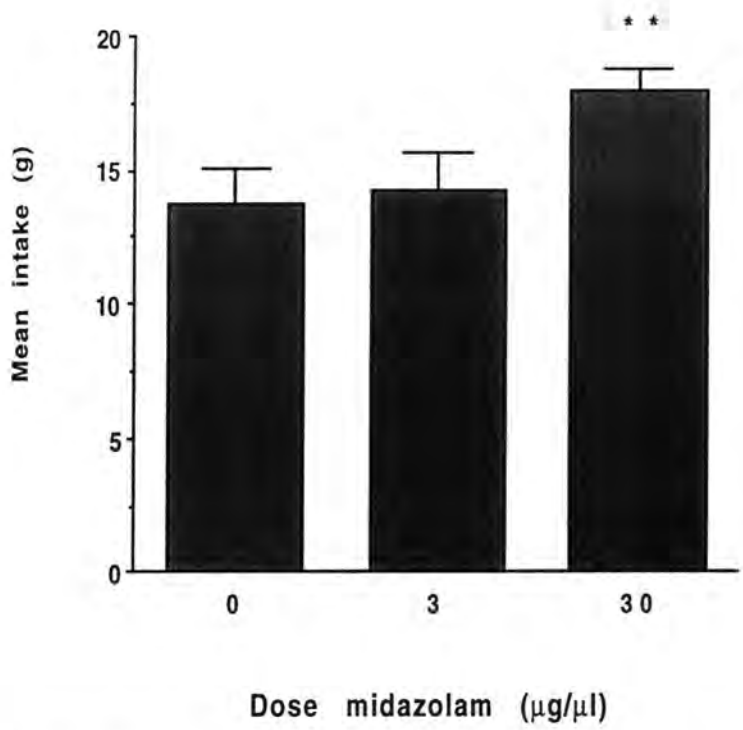


Figure 2.2 The effect of direct injection of midazolam (3 and 30µg/µl) into the IVth ventricle on intake of sweet wet mash after 30-min + S.E.M. n = 12. Asterisk indicates significantly different from vehicle condition ** $p < 0.01$ (Dunnett's t-test).

Table 2.1 The effects of IVth ventricular midazolam on the intake of sweet wet mash.

Treatment	Mash consumed (g)		
	10-min	20-min	30-min
Vehicle	5.9 ± 0.8	10.6 ± 1.1	13.8 ± 1.3
midazolam 3 µg/µl	7.0 ± 0.7	11.7 ± 1.1	14.3 ± 1.3
midazolam 30µg/µl	9.6** ± 0.6	11.7 ± 1.3	18.00** ± 0.8

Results are shown mean intake (g) + S.E.M. n = 12. Levels of significance ** $p < 0.01$ (Dunnett's t-test).

Experiment 2: The effect of flumazenil on hyperphagia induced by IVth ventricular midazolam

Of the eight rats used in Experiment 2, one rat did not undergo behavioural testing due to problems following surgery. Histological analysis revealed that all cannulae were targeted correctly in the remaining seven animals (Figure 2.3). A one-way repeated-measures ANOVA revealed a significant effect of drug treatment ($F_{3, 18} = 7.66$, $p < 0.01$). A Newman-Keuls multiple comparison test showed that the dose of 30 µg/µl of midazolam significantly increased consumption of the mash compared with the control condition ($p < 0.01$). The baseline intake of 11.7 g was increased by 50% to 16.8 g (Figure 2.4). This increase was almost completely blocked by pretreatment with flumazenil (20 mg/kg). When administered alone, flumazenil had no significant effect on the ingestion of sweet wet mash. The effect of midazolam (30 µg/µl) was observed within the first 10-min of the test. A significant increase in food intake was already evident after 10-min ($F_{3, 18} = 9.93$, $p < 0.001$). This early increase was also effectively blocked by pretreatment with flumazenil. The hyperphagic effect of midazolam was maintained after 20-min ($F_{3, 18} = 8.06$ $p < 0.01$). The results are shown in Table 2.2.

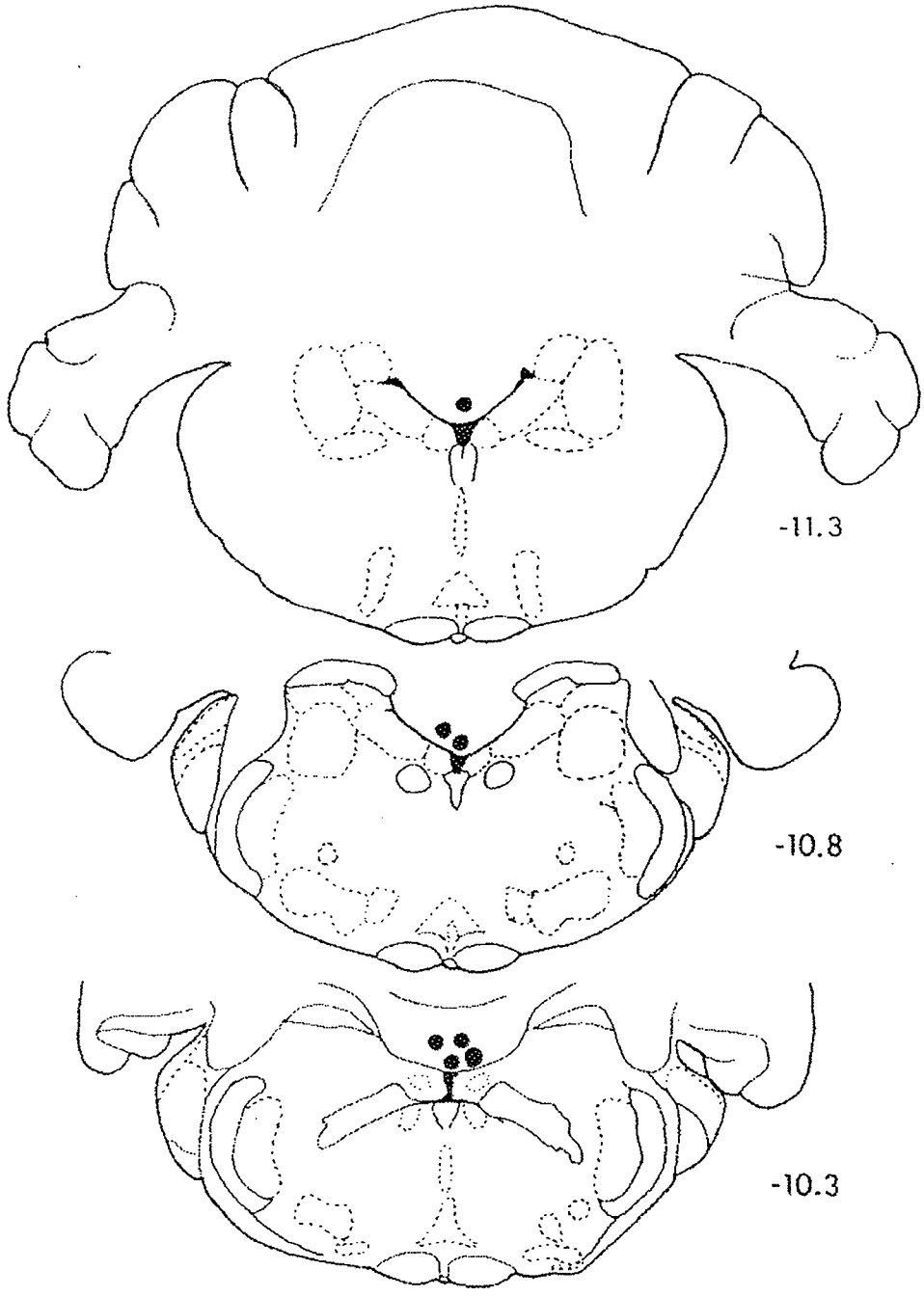


Figure 2.3 The distribution of injection sites in the IVth ventricle for rats used in Experiment 2. Sections are redrawn from Paxinos and Watson (1982). Each shows the location of the injection cannula tip for 1 rat. Section numbers refer to mm from bregma.

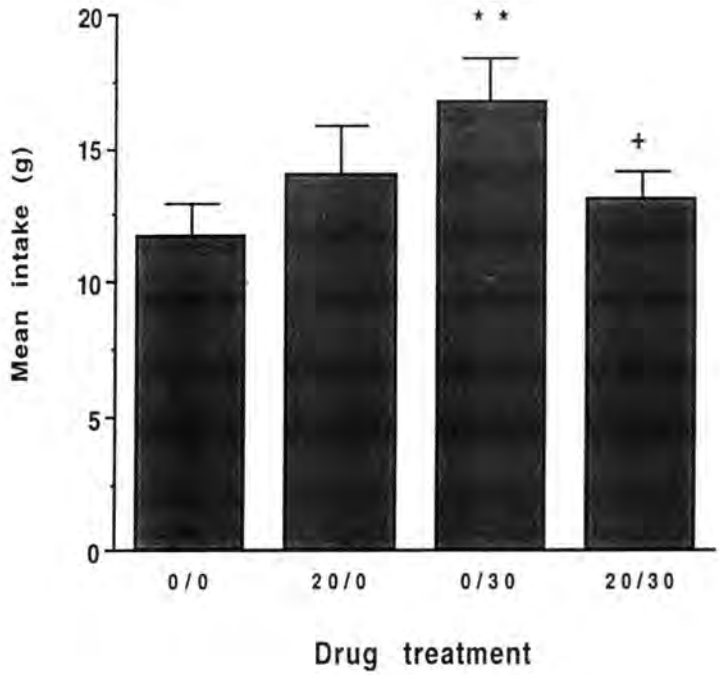


Figure 2.4 The effect of flumazenil (20 mg/kg) on hyperphagia induced by injection of midazolam (30 µg/µl) into the IVth ventricle after 30-min + S.E.M. n = 7. Asterisk indicates significantly different from vehicle condition ** $p < 0.01$ + indicates significantly different from vehicle/midazolam condition $p < 0.05$.

Table 2.2 The effect of flumazenil on hyperphagia induced by IVth ventricular midazolam

Treatment	Mash consumed(g) 10-min	20-min	30-min
Vehicle Vehicle	7.4 ± 1.4	10.8 ± 1.1	11.7 ± 1.2
Flumazenil Vehicle	7.6 ± 1.1	12.8 ± 1.7	14.1 ± 1.8
Vehicle Midazolam	12.4** ± 1.0	15.3** ± 1.5	16.7 ** ± 1.6
Flumazenil Midazolam	8.6+ ± 1.2	11.4+ ± 1.1	13.1 + ± 1.0

Results are shown mean intake (g) ± S.E.M. n = 7 rats per condition. Levels of significance: ** $p < 0.01$, different from veh/veh condition. + $p < 0.05$, different from veh/mdz condition (Newman-Keuls multiple comparisons test)

2.4 Discussion

The results of Experiments 1 and 2 show that direct injection of a benzodiazepine receptor agonist into the IVth ventricle is sufficient to induce a significant hyperphagic response in non-deprived adult male rats. The increase in food intake observed by this route of administration is comparable to that obtained following systemic administration of benzodiazepine receptor agonists (Cooper et al., 1985). Antagonism of the hyperphagic effect by flumazenil is an indication that the effect is mediated by specific benzodiazepine receptors. These results argue for central mediation of benzodiazepine-induced hyperphagia, and point to lower brainstem structures as possible locations of the site of action of the drug.

Berridge (1988) has shown that the effects of benzodiazepine receptor agonists in the taste reactivity test remain intact in midbrain-transected rats. More recently, Berridge and Pecina (1995) have shown that direct administration of diazepam into the IVth ventricle of the rat enhances hedonic reactions to a 7% sucrose solution. The present data are consistent with these results in showing that the lower brainstem may be important for the effects of benzodiazepines on ingestive responses.

The precise location of the brainstem receptors remains uncertain but the parabrachial nucleus (PBN) in the pons may be a possible candidate for mediation. Data from an autoradiographic binding study has highlighted a population of benzodiazepine receptors in or near the PBN (Higgs, Gilbert, Barnes, and Cooper, 1993). Hence, benzodiazepine receptors in this area may be involved in the effects of benzodiazepines on ingestive behaviour. There are several reasons for asserting that the PBN may be an important central site of action for the effects of benzodiazepines on food intake. First, the neuroanatomy of the gustatory system has been carefully mapped, and it is well established that the PBN contains the second central relay for the taste pathway (Norgren, 1976; Norgren and Leonard, 1973; Norgren and Pfaffmann, 1975). It has been established that the PBN makes extensive connections to many areas of the brain which are involved in the control of ingestive behaviour, such as the hypothalamus (Krukoff, Harris and Jhamandas, 1993; Norgren 1976; Saper and Loewy, 1980) and amygdala

(Norgren 1976), and this prompts the notion that the PBN itself may contribute to the execution of such behaviours. It is possible that benzodiazepine receptor agonist modulation of feeding may occur even earlier on the taste pathway in the nucleus of the solitary tract (NTS), which is the first relay in the gustatory pathway (Norgren, 1978). This hypothesis remains to be tested, but a role for the NTS seems less likely given that the autoradiographic binding study failed to reveal any benzodiazepine receptors in this nucleus (Higgs et al., 1993).

Presently, it is not possible to rule out the possibility that structures rostral to the IVth ventricle are also involved in benzodiazepine-induced hyperphagia. Direct injection of benzodiazepine receptor agonists into the forebrain would help to address this issue, but the present results do serve to draw attention to brainstem participation in the control of ingestive behaviour, particularly in relation to benzodiazepine effects. Evidence for this focus is provided by Berridge and Pecina (1995) who have compared the relative efficacy of IVth and lateral ventricle microinjections of diazepam in increasing ingestive responding in the taste reactivity paradigm. The dose threshold required for lateral ventricular administration to enhance hedonic responses, was significantly greater than for IVth ventricular injections. These data suggest that the continued investigation of brainstem mechanisms of ingestion would prove to be of great value.

An important question arising from the present studies is whether the effects of benzodiazepines in the taste reactivity test are mediated by the same receptor populations underlying benzodiazepine-induced hyperphagia. It is possible that the increase in hedonic reactions following benzodiazepine administration may be dissociable from the effect of these drugs on food consumption. This dissociation could occur either in terms of distinguishable sites of action, or in terms of specific receptor subtypes within a specific location, or both. Alternatively, it may be that the hyperphagic effect depends upon and is secondary to the enhancement of hedonic reactions to taste stimuli. This would suggest that whenever an increase in hedonic reactions is achieved (e.g. by benzodiazepine treatment) an increase in food consumption would follow. This idea is consistent with the possibility that benzodiazepines enhance food palatability. Integrating

the results of the present study with the data of Berridge and Pecina (1995) provides some support for this view since IVth ventricular administration of a benzodiazepine agonist has been shown to be increase both hedonic reactions to taste stimuli and food consumption. This conclusion is reinforced by the observation that the increase in consumption of the palatable mash caused by midazolam was observed after just 10-min of the test session had elapsed. This suggests that the hyperphagic effect was probably not caused by a reduction in satiety occurring at the end of the feeding period.

In summary, the present studies indicate that the neural substrate for benzodiazepine-induced hyperphagia may be located in the brainstem although precisely which structures are involved remains to be elucidated.

The effects of direct administration of midazolam into the parabrachial nucleus on ingestive behaviour

3.1 Introduction

In the previous chapter it was shown that a reliable hyperphagia could be elicited following microinjection of midazolam into the IVth ventricle of non-deprived rats. It was then shown this effect is receptor specific because it was completely abolished by the selective benzodiazepine receptor antagonist flumazenil. It was proposed that at least one of the populations of receptors responsible for the effects of benzodiazepines on food intake may be located in the brainstem, in the proximity of the IVth ventricle. The aim of the experiments in this chapter was to further assess the contribution of brainstem structures to benzodiazepine-induced increases in food intake.

The major target for Experiments 3, 4, 5, and 6 was the parabrachial nucleus (PBN) of the pons. One reason why the PBN was selected as a potential site of action is because this nucleus contains the second relay for the taste projection system (Norgren and Leonard, 1973; Norgren and Pfaffman 1975). Taste is an important determinant of food intake (Pfaffmann, 1982), and so the PBN is well-placed to mediate the effects of benzodiazepines on ingestive behaviour. Another reason to suggest the involvement of the PBN in benzodiazepine-induced hyperphagia is that an autoradiographic binding study has revealed a population of benzodiazepine receptors in the region of the PBN (Higgs et al., 1993). Additionally, it is well established that the PBN makes extensive connections to many other areas of the brain involved in the control of ingestive behaviour, such as the hypothalamus (Krukoff et al., 1993; Norgren, 1976; Saper and Loewy, 1980) and amygdala (Norgren 1976).

The aim of Experiment 3 was to establish whether an injection of midazolam into the PBN can elicit a hyperphagic response. Experiment 4 was designed to check if the response to midazolam is mediated by specific benzodiazepine receptors. The PBN can be subdivided up into a number of distinct regions (Krukoff et al., 1993; Saper and

Loewy 1980). In the experiments reported in this chapter, guide cannulae were targeted either to medial or lateral areas of the PBN to examine whether a functional dissociation between these two areas could be demonstrated.

Systemic administration of benzodiazepine receptor agonists enhances the consumption of both liquid and solid foods (Cooper and Greenwood 1992; Cooper et al., 1987). The aim of Experiment 5 was to determine whether the effects of midazolam injected directly into the PBN mimics the effects of systemically administered benzodiazepine receptor agonists in increasing the consumption of both solid and liquid foods. Non-deprived rats were trained to consume a 3% sucrose solution and the potential hyperphagic effect of intra-PBN midazolam was investigated.

Besides their effects on food intake, benzodiazepine receptor agonists have anxiolytic, sedative, and muscle relaxant properties. It has been suggested that the wide ranging effects of these drugs may be due to stimulation of receptor subpopulations specific for one type of behaviour, located in different brain areas. The aim of Experiment 6 was to examine the possibility that benzodiazepine receptors in the PBN constitute a subpopulation of receptors specific for ingestional responses. An activity monitor was used to measure locomotion following administration of intra-PBN midazolam. As a control experiment, the intake of a sweetened wet mash was also measured in the same rats but on a separate occasion, to ensure that any result obtained was not a false positive due to failure of the drug to reach the receptors.

Virtually all previous work on the PBN and ingestive responses has involved lesion techniques which have obvious drawbacks and limitations (Spector, 1995). The aim of these studies was to employ a pharmacological approach with the specific purpose of determining if the PBN is a principle site of action for benzodiazepine-induced hyperphagia.

3.2 Method

3.2.1 Animals

Thirty eight non-deprived adult male hooded rats (General strain bred in the School of Psychology, University of Birmingham) weighing 300-400 g at the beginning of experimentation were used. Rats were housed individually in plastic cages in a room with a constant temperature of 22± 2 °C, and were maintained on a 12h light:dark cycle (lights on at 8.00). Standard laboratory food pellets (Pilsbury 41B, Heygate and Sons, U.K.) and water were available at all times, except during testing. Behavioural testing was conducted in the light phase.

3.2.2 Drugs

The water soluble benzodiazepine receptor agonist midazolam maleate (Roche, Basel, Switzerland) was prepared for injection by dissolving in isotonic saline. The doses used, were 3, 10 and 30 µg/µl of midazolam. This dose range was chosen because in Chapter 2 it was shown that 3 and 30 µg/µl of midazolam increased intake of mash following injection into the IVth ventricle of rats. The vehicle used in control injections was isotonic saline. The selective benzodiazepine receptor antagonist flumazenil (Ro 15-1788) (Roche, Basel, Switzerland) was prepared for injection by ultrasonic dispersion in distilled water to which Tween 80 (BDH Chemicals Ltd, Poole, England) had been added. The dose used, was 20 mg/kg which has been shown previously to block the hyperphagic effect of benzodiazepine agonists (Cooper and Moores, 1985b).

3.2.3 Surgery

For implantation of stainless steel guide cannulae, rats were anesthetized with medetomidine, 250 µg/kg (Domitor) and ketamine, 60 mg/kg (Vetelar) combination anesthetic. Effects of the medetomidine were reversed using atipmazole (Antisedan) at a dose of 1 mg/kg. The analgesic buprenorphine (Temgesic) was also administered prior to recovery at a dose of 0.03 mg/kg. Guide cannulae were targeted bilaterally to either the medial or lateral parabrachial nucleus (coordinates for medial parabrachial: L + 1.4 A-P

-9.4 V -5.8, coordinates for lateral parabrachial: L +2.2 A-P -9.4 V -6.2). The cannulae tips were implanted 2 mm dorsal to the intended injection site. Bregma was used as a reference point and the coordinates were taken from the atlas of Paxinos and Watson (1982). Dental acrylic and three screws were used to fix the cannulae to the skull. Stylets were placed in the cannulae to prevent occlusion, and the rats were allowed seven days to recover before behavioural testing occurred. Postoperative care involved weighing the rats daily and applying an antibiotic wound powder to the headmount if necessary.

3.2.4 Injection procedure

Central microinjection of midazolam was performed bilaterally using injection cannulae connected by a polyethylene tube to a micrometer-driven 10 µl Hamilton syringe. The injection needles protruded 2 mm beyond the tip of the guide cannula and accuracy of injections was ensured by observing the progress of an air bubble in the tubing. The volume infused was 0.5 µl each side, injected over a period of 30s. Each rat was then placed immediately in the test cage and food intake over a 30-min test period was measured. Flumazenil was administered via the i.p. route 15-min prior to central injection of midazolam. The volume injected was 1 ml/kg. Two days prior to testing each rat received a sham injection of isotonic saline to familiarize it with the microinjection procedure.

3.2.5 Food intake measurement

Mash intake Rats were first adapted to eating a sweetened wet mash for a period of approximately 10 days. The meal was made up daily according to the following formula: 100 ml sweetened condensed milk, 400 ml ground maintenance diet (Special Diet Services Ltd., Essex, U.K.) and 200 ml distilled water. It has been shown that this mash is readily consumed by non-deprived rats (Cooper et al., 1985). Each rat was given 30-min access to 50 g portions of the diet placed in a clear plastic dish inside an individual stainless steel test cage. The consumption of the mash was measured to the nearest 0.1 g with corrections made for any spillage.

Sucrose intake Familiarisation with a 3% sucrose solution occurred over a period of approximately seven days. Rats were transferred from their home cages to testing cages in which they had access to a single drinking spout attached to a 50 ml graduated cylinder. Sucrose intake was recorded volumetrically to the nearest ml after the 20-min test period.

3.2.6 Locomotor activity

A photo-cell activity monitor was used to measure locomotor activity. This consisted of a black plastic cylindrical container 30 cm in height. An outer circle of diameter 42 cm enclosed an inner circle of diameter 18 cm. The arrangement created a 12 cm wide corridor which allowed the rats to move in a circular path. Three photobeams, positioned near to the floor, monitored the activity of the rat. When a beam was interrupted this registered as one count. Each rat was familiarised with the test procedure over a period of five days. Individual rats were exposed to a 20-min session in the photocell activity monitor.

3.2.7 Histology

At the end of each experiment rats were deeply anaesthetized with pentobarbital and a small quantity of methylene blue dye was injected through the cannula. Each rat was then perfused transcardially with isotonic saline followed by a 10% formalin solution. After decapitation, the excised brains were fixed in a 10% formalin solution for one week. The fixed brains were then frozen and sectioned sagitally on a freezing microtome and the correct placement of the cannulae was verified visually. The histological work was conducted blind with respect to the behavioural results. Rats with cannulae placements further than 1 mm from the intended injection site were judged to be non-PBN placements and so were analyzed separately from PBN placements. This criterion was chosen based on previous studies showing that an injection volume of 0.5 µl diffuses to spread over a 1 mm area (Myers, 1966).

3.2.8 Statistical analysis

All data were analyzed using a one-way analysis of variance (ANOVA) for repeated-measures. Comparisons between means were carried out using Dunnett's t-test or a Newman-Keuls multiple comparisons test. Statistical tests were performed using Statview SE+graphics (Abacus Concepts Inc., Berkeley, CA, 1987) and a result was considered statistically significant if $p < 0.05$.

3.3 Results

Experiment 3: The effect of direct injection of midazolam into the PBN on intake of a palatable mash

Of the twelve animals used in Experiment 3, one did not undergo behavioural testing due to problems following surgery. Histological analysis of the remaining eleven animals showed that not all the placements were targeted at the PBN and so the data for those rats where the cannulae were located more than 1 mm outside the PBN (n = 5) were analyzed separately from the PBN placements (n = 6) (Figure 3.1). In Experiment 3, no difference was observed between the responses of rats receiving lateral as opposed to medial injections of midazolam into the PBN, and so their data were pooled. The results are summarized in Table 3.1.

PBN placements. A one-factor repeated-measures ANOVA revealed a significant effect of the drug after 30-min ($F_{3,15} = 9.78$, $p < 0.001$). Midazolam (3-30 µg/µl in 0.5 µl) significantly increased consumption of the mash relative to the control condition. The baseline intake of 8.6 g was almost doubled to 15.5 g (Figure 3.2a). Post hoc tests indicated that the 10 µg/µl and 30µg/µl conditions differed significantly from the vehicle condition ($p < 0.01$).

Non-PBN placements. A one-factor repeated-measures ANOVA revealed that injections which were not within 1 mm of the PBN had no significant effects on intake ($F_{3,12} = 0.17$, n.s.). The baseline intake of 13.3 g was not affected by administration of midazolam into areas around the PBN (Figure 3.2b).

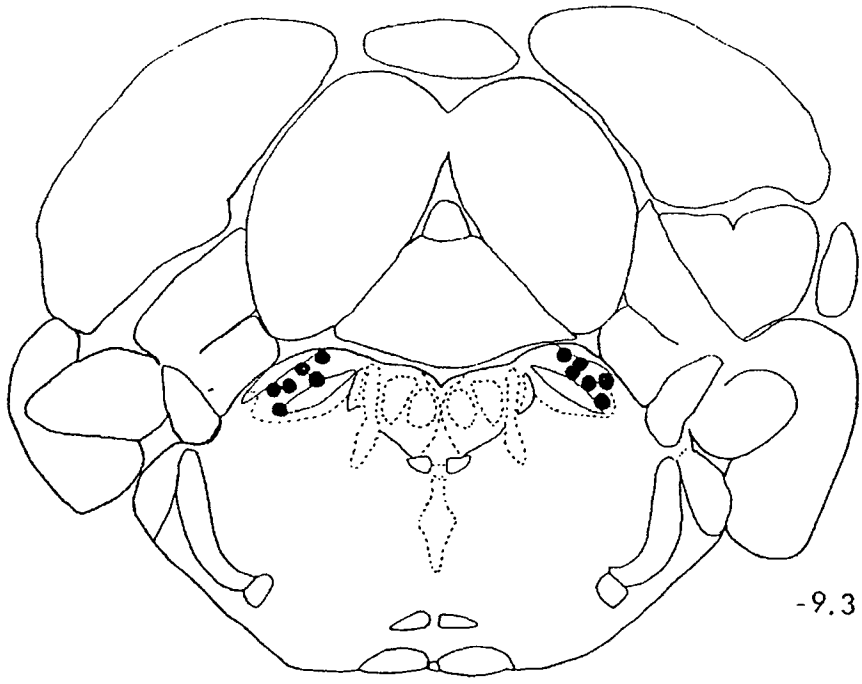


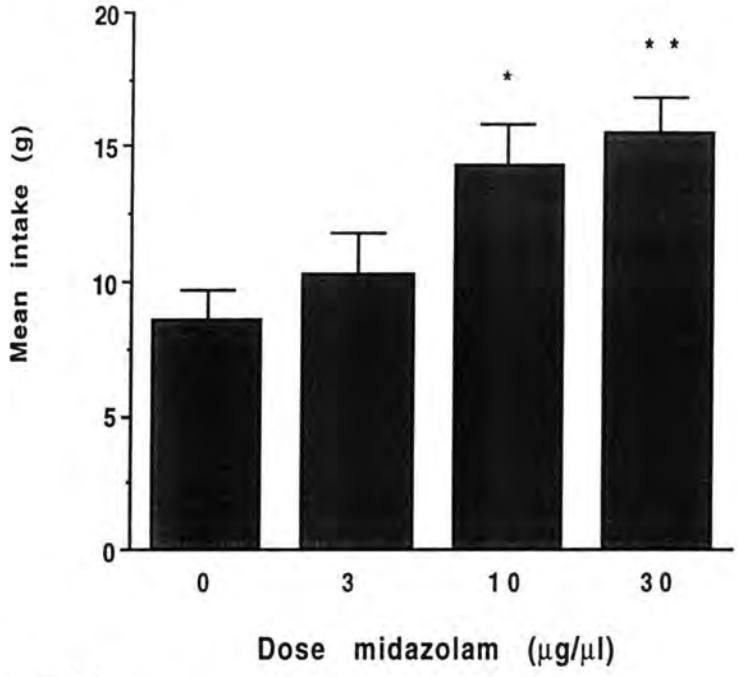
Figure 3.1 The distribution of injection sites in the parabrachial nucleus for rats used in Experiment 3. Sites are shown bilaterally. Sections are redrawn from Paxinos and Watson (1982). Section numbers refer to mm from bregma.

Table 3.1 The effect of intra-parabrachial midazolam on the intake of sweet wet mash.

Treatment	Mash consumed (g) PBN	Non-PBN
Vehicle	8.6 + 1.1	13.3 ± 2.4
3 µg/µl midazolam	10.3 ± 1.5	12.3 ±1.4
10 µg/µl midazolam	14.2 * ± 1.5	14.2 ± 2.5
30 µg/µl midazolam	15.6 ** ± 1.3	13.3 ± 0.9

Results are shown mean intake (g) ± S.E.M. PBN (n = 6) non-PBN (n = 5). Intake recorded over 30-min. Levels of significance: * $p < 0.05$ ** $p < 0.01$ (Dunnett's t-test)

a) PBN



b) Non-PBN

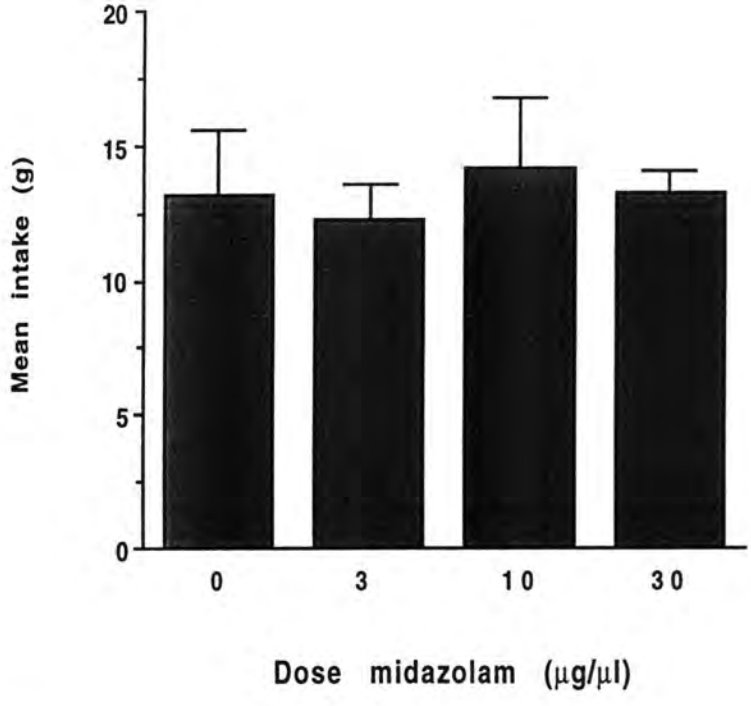


Figure 3.3 Intake of a sweet wet mash (g) after 30-min as a function of increasing dose of midazolam (3-30 µg/µl) for a) PBN (n = 6) and b) Non-PBN placements (n = 5) + S.E.M. Asterisk indicates significantly different from vehicle condition * $p < 0.05$ ** $p < 0.01$ (Dunnett's t-test).

Experiment 4: The effect of pretreatment with flumazenil on hyperphagia induced by injection of midazolam into the PBN

Following histological examination it was found that of the ten animals used in Experiment 4, in four cases the guide cannulae were located further than 1 mm away from the intended area and so the data for these rats were analyzed separately from the PBN placements (n = 4) (Figure 3.3). The histology for two rats showed extensive tissue damage such that it was impossible to determine the placement site, and so the data for these rats were discarded. No difference was observed in the results for rats receiving lateral as opposed to medial injections of midazolam into the PBN, and so the data were pooled for analysis. A summary of the results is shown in Table 3.2.

PBN placements. A one-factor repeated-measures ANOVA revealed a significant effect of treatment after 10-min ($F_{3,9} = 8.16$, $p < 0.01$). Intra-PBN injection of midazolam dose-dependently enhanced mash consumption relative to the control saline injection. Pairwise comparison with a Newman-Keuls multiple comparison test revealed that the 30 μg/μl dose significantly increased ingestion of the mash after 10-min ($p < 0.01$) (Figure 3.4a). Flumazenil (20 mg/kg) administered alone had no significant effect on mash intake after 10-min. However, flumazenil did significantly attenuate the increase in ingestion caused by midazolam as indicated by the significant pretreatment x drug interaction ($F_{1,1} = 10.96$, $p < 0.05$). No significant effect of drug treatment was observed in the PBN placements after 30-min. This was despite the fact that the baseline intake of 12.8 g was nearly doubled following administration of midazolam alone to 21.8 g.

Non-PBN placements. Analysis of the data from rats with guide cannulae more than 1 mm away from the PBN did not reveal a significant effect of drug treatment after 10-min ($F_{3,9} = 0.57$, n.s.). The baseline intake of 9 g was not significantly affected (Figure 3.4b). There was still no drug effect evident after 30-min ($F_{3,9} = 1.1$, n.s.).

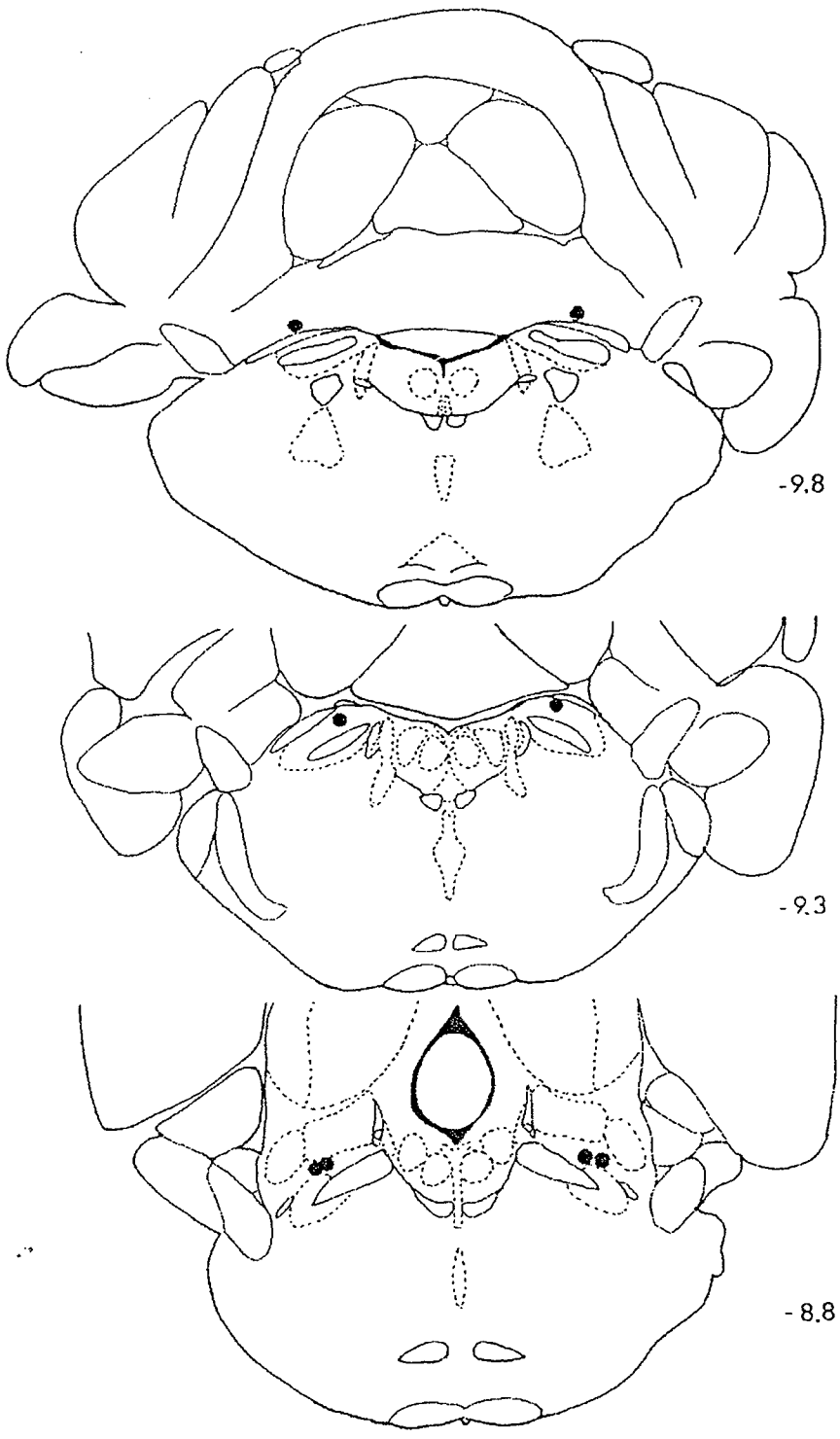
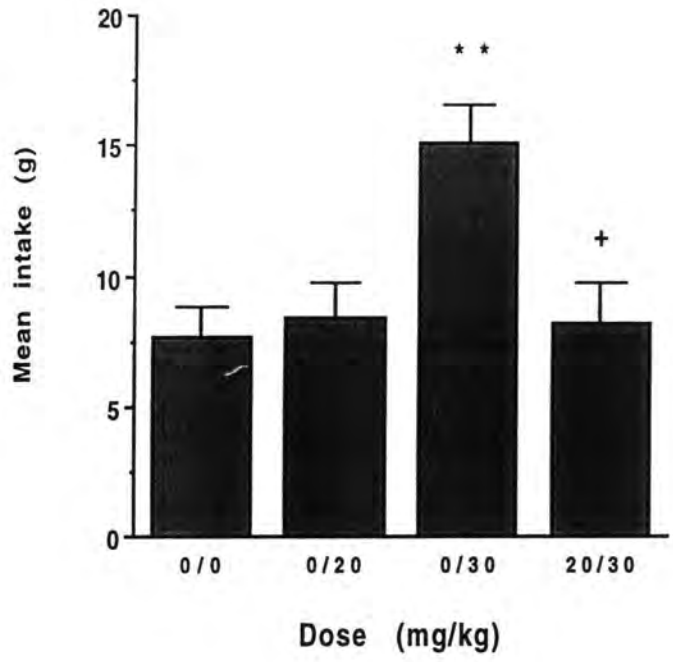


Figure 3.3 The distribution of injection sites in the parabrachial nucleus for rats used in Experiment 4. Sites are shown bilaterally. Sections are redrawn from Paxinos and Watson (1982). Section numbers refer to mm from bregma.

a) PBN



b) Non-PBN

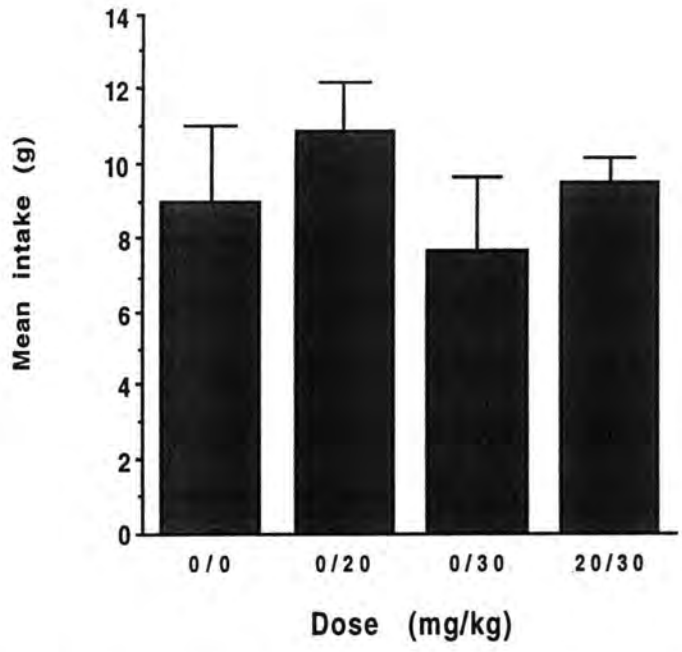


Figure 3.4 Effect of flumazenil (20 mg/kg) on hyperphagia induced by intra-PBN midazolam (30 µg/µl) for a) PBN (n = 4) and b) Non-PBN placements (n = 4) after 10-min + S.E.M. Asterisk indicates significantly different from vehicle condition ** $p < 0.01$ + significantly different from vehicle/midazolam condition $p < 0.05$.

Table 3.2 The effect of flumazenil on hyperphagia induced by intra-parabrachial midazolam.

Treatment	Mash consumed (g)	
	PBN	Non-PBN
Vehicle Vehicle	7.7 ± 1.1	8.9 ± 2.0
Flumazenil Vehicle	8.4 ± 1.4	10.8 ± 1.3
Vehicle Midazolam	15.1 ** ± 1.5	7.6 ± 2.0
Flumazenil Midazolam	8.2 + ± 1.6	9.5 ± 0.7

Results are shown mean intake (g) ± S.E.M. PBN (n = 4) Non-PBN (n = 4). Intake recorded over 10-min. Levels of significance: ** $p < 0.01$, different from Veh/Veh condition. + $p < 0.05$, different from Veh/Midazolam condition (Newman-Keuls multiple comparisons test).

Experiment 5: The effect of direct injection of midazolam into the PBN on intake of a 3% sucrose solution

Of the eight rats used in Experiment 5, two did not undergo behavioural testing due to problems following surgery. Histological analysis showed that in the remaining six rats, not all the placements were correctly targeted. Five rats had cannulae that were correctly targeted. In one rat the cannulae missed the target area by more than 1 mm and so the data from this rat was discarded. Although cannulae were targeted at both the lateral and medial PBN, no difference was observed between the results for these groups and so the data were pooled. The results are summarized in Table 3.3.

PBN placements. A one-factor repeated-measures ANOVA revealed a significant effect of drug in the PBN group ($F_{3,12} = 12.81$, $p < 0.001$). Midazolam (3-30 μg/μl in 0.5 μl) increased consumption of the 3% sucrose solution relative to the control condition (Figure 3.5). The high baseline intake of 21.4 ml was increased to 25.6 ml after 20-min (Figure 3.6). Post hoc tests indicated that the 10 μg/μl ($p < 0.05$) and the 30 μg/μl ($p < 0.01$) condition differed significantly from the vehicle condition.

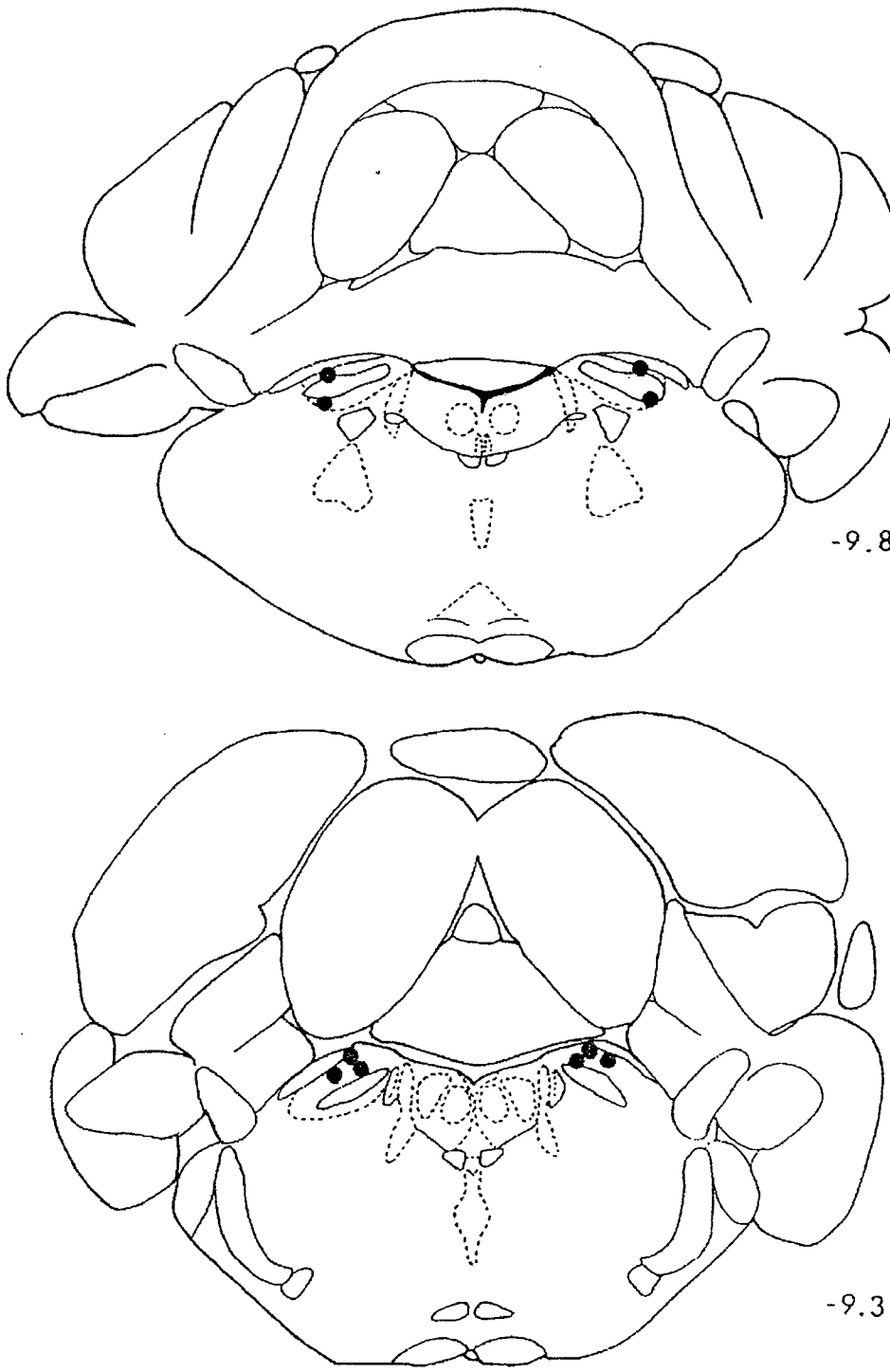


Figure 3.5 The distribution of injection sites in the parabrachial nucleus for rats used in Experiment 5. Sites are shown bilaterally. Sections are redrawn from Paxinos and Watson (1982). Section numbers refer to mm from bregma.

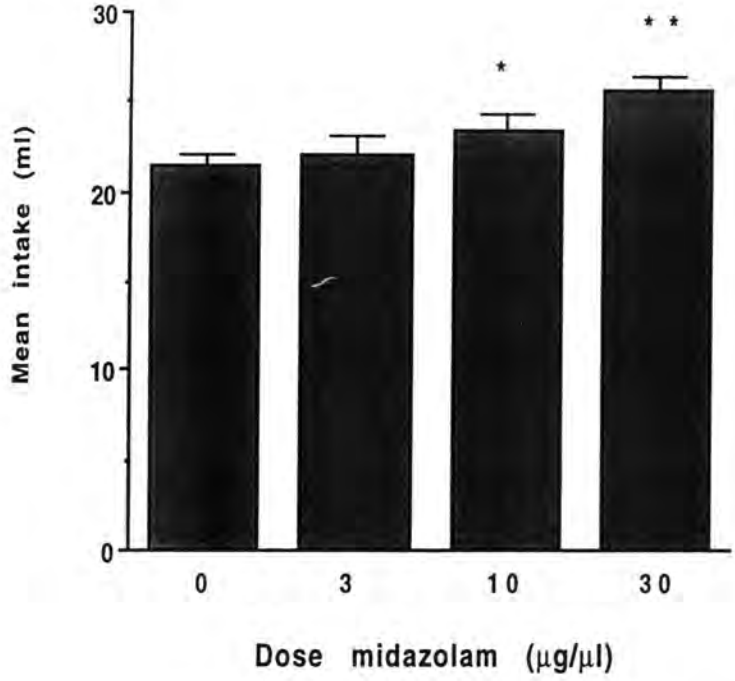


Figure 3.6 Intake of a 3% sucrose solution (ml) after 20-min as a function of increasing dose of midazolam (3-30 µg/µl) (n = 5) + S.E.M. Asterisk indicates significantly different from vehicle condition * $p < 0.05$ ** $p < 0.01$ (Dunnett's t-test).

Table 3.3 The effect of intra-parabrachial midazolam on the intake of a 3% sucrose solution.

Treatment	Sucrose consumed (ml)
Vehicle	21.4 ± 0.6
3 µg/µl midazolam	22 ± 2.3
10 µg/µl midazolam	23.4 * ± 0.9
30 µg/µl midazolam	25.6 ** ± 0.9

Results are shown mean intake (ml) ± S.E.M. n = 5. Intake recorded over 20-min. Levels of significance: * $p < 0.05$ ** $p < 0.01$ (Dunnett's t-test).

Experiment 6: The effect of direct injection of midazolam into the PBN on locomotor activity

Locomotor activity

Of the eight rats used in Experiment 6, the data from one rat had to be discarded from the analysis due to a fault in the equipment. In six cases the cannulae were correctly targeted (Figure 3.7). There was one case where the guide cannula missed the intended target by more than 1 mm and so this rat was not included in the statistical analysis.

PBN placements. Results are shown in Figure 3.8a. A one-factor repeated-measures ANOVA revealed no significant effect of treatment after 20-min ($F_{3,15} = 2.19$, n.s.). The mean count for the vehicle treated group was 224.6 in 20-min and this was not significantly affected by drug administration.

Mash intake

A paired t-test revealed that there was a significant effect of the drug ($p < 0.05$). Midazolam (30 μg/μl in 0.5 μl) significantly increased consumption of the mash after 30-min. The baseline intake of 7.6 g was substantially increased to 12.1 g (Figure 3.8b). A summary of the results is shown in Table 3.4.

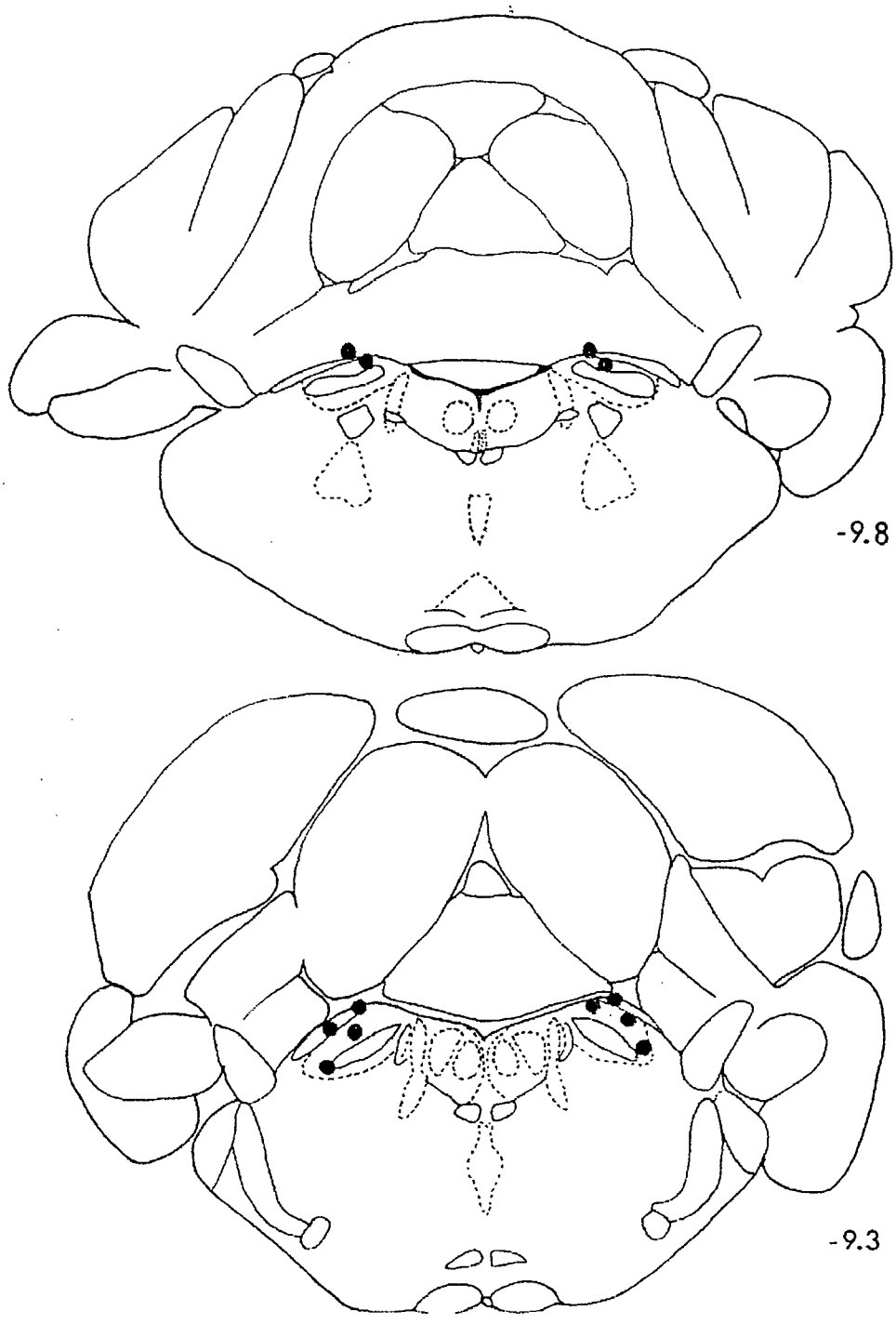
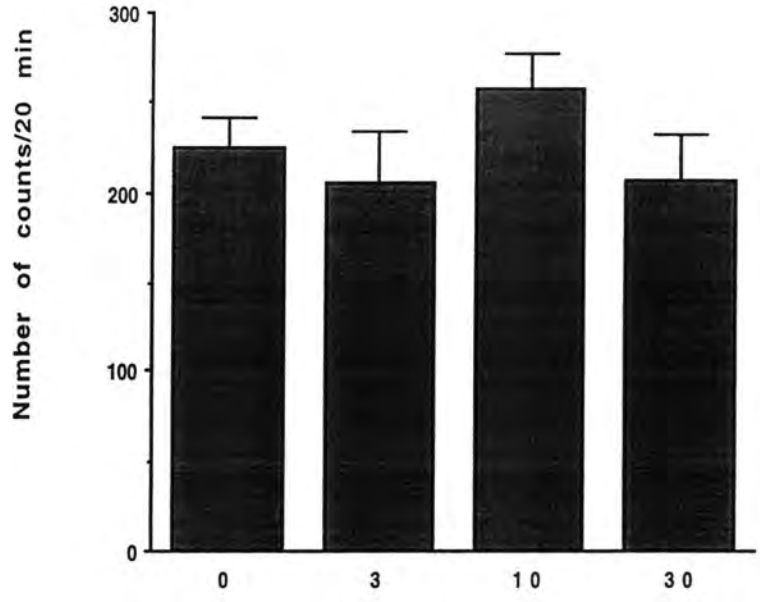


Figure 3.7 The distribution of injection sites in the parabrachial nucleus for rats used in Experiment 6. Sites are shown bilaterally. Sections are redrawn from Paxinos and Watson (1982). Section numbers refer to mm from bregma.

a) Locomotor activity



b) Mash intake

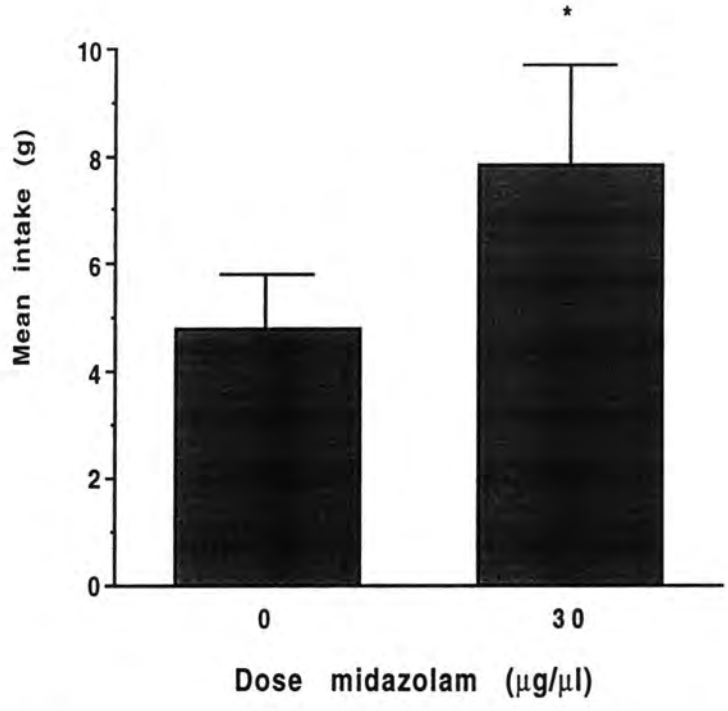


Figure 3.8 Effect of intra-PBN midazolam on a) locomotor activity and b) Intake of a sweet wet mash (n = 6) + S.E.M. Asterisk indicates significantly different from vehicle condition * $p < 0.05$.

Table 3.4 The effect of intra-parabrachial midazolam on locomotor activity

Treatment	Counts in 20-min
Vehicle	221.8 ± 21.5
3 μg/μl midazolam	198.4 ± 33.2
10 μg/μl midazolam	260.4 ± 23.6
30 μg/μl midazolam	212 ± 30.6

Results are shown mean counts in 20-min ± S.E.M. n = 6.

3.4 Discussion

In the present experiments, direct administration of midazolam (10 and 30 μg/μl) into the PBN caused a significant increase in the consumption of a palatable diet in non-deprived rats. This increase in intake was blocked by adminstration of the selective benzodiazepine receptor antagonist flumazenil. Injection of midazolam directly into the PBN (3-30 μg/μl) also significantly increased the consumption of a 3% sucrose solution, despite the fact that the rats were consuming at high baseline levels. These data are in close agreement with the effects of peripherally-administered benzodiazepines on ingestive behaviour (Cooper et al., 1985). Injection of midazolam (3-30 μg/μl) into the PBN did not affect locomotor activity, although in the same rats, the highest dose of 30 μg/μl significantly increased the consumption of a palatable mash.

In the previous chapter, it was shown that injection of midazolam into the IVth ventricle induced a significant increase in food consumption. This increase was attenuated by pretreatment with flumazenil. This suggests that the effects of midazolam when injected into the PBN were due to an action at benzodiazepine receptors. The blockade of the effects of midazolam by flumazenil also argues against the effects of midazolam being due to any artifact of pH, osmolarity or hydraulic pressure. The data from Experiments 3 and 4 suggest that this effect may have been due to rapid diffusion of the drug from the

IVth ventricle to benzodiazepine receptors in the PBN. An action at benzodiazepine receptors in the PBN, rather than diffusion to other brain areas provides the most likely explanation of the data for several reasons. First, analysis of data from rats where the guide cannulae were more than 1 mm away from the PBN revealed that in these cases there was no increase in consumption following direct administration of midazolam. This is wholly consistent with the finding that benzodiazepine receptors are present in the PBN but not in adjacent areas (Higgs et al., 1993). Second, the injection volume used was 0.5 µl which has been reported to spread 1 mm from the injection site (Myers, 1966). This means that diffusion from the PBN injection site to the IVth ventricle is unlikely to be able to account for the results. Nevertheless, it might be predicted that the behaviourally-effective dose for PBN injection compared with IVth ventricle injection should be lower if receptors in the PBN were responsible for the IVth ventricle effect. We found no difference in the doses effective in increasing food intake for both the previously reported IVth ventricle experiments and the current experiments. However, the volumes injected in both cases were different. The volume injected into the IVth ventricle was 3 µl, compared with a volume of 0.5 µl which was injected into the PBN. If the results obtained when injecting into the PBN were due to a ventricular site of action, then it might be expected that when injecting a much larger volume into the IVth ventricle (3 µl), a lower dose would be effective. Since this was not so, the IVth ventricle results might be better explained by diffusion from the ventricle to receptors in the PBN. Taken together, this evidence indicates that the hyperphagic effect depends upon benzodiazepine receptors located in the PBN.

No difference was found in the current experiments between injections into the medial versus the lateral regions of the PBN. Therefore, the data from both sets of rats were combined. The reason for the failure to distinguish between medial compared with lateral injection sites, despite the differential connections of these two areas, may have been due to spread of the injection volume through the entire PBN. Future experiments investigating the contribution of sub-regions within the PBN would involve injecting

smaller volumes of drug to allow more accurate distinctions between the functioning of different regions to be made.

It should be noted that in Experiment 1, the average intake (g) in the vehicle condition for the non-PBN group was higher than for the PBN group. A difference in the baseline measure was therefore observed, and it could be argued that any real effect on feeding was consequently being masked in the non-PBN group. However, this argument can be counteracted by the observation that the rats were not consuming at ceiling levels, and that the difference in baseline intake was not observed for the non-PBN rats in Experiment 2. This suggests that the discrepancy was due to an absolute difference in the baseline consumption between the two groups of rats, and that a possible drug effect was not being obscured.

In Experiment 5, injection of midazolam into the PBN increased consumption of a palatable sucrose solution. This result suggests that the effect of intra-PBN midazolam on ingestive behaviour applies not only to solid foods but can also be extended to liquid foods. This suggests that the effects of Intra-PBN midazolam on ingestive behaviour are similar to the effects of peripheral administration.

In small doses benzodiazepine receptor agonists cause hyperlocomotion and in large doses produce sedation (File, 1981). The hyperlocomotion and sedative effects of these drugs following systemic administration were completely absent over the range of doses used in the present experiments. The absence of any effect on locomotion cannot be accounted for in terms of a general lack of drug effect in since in the same rats, a significant increase in mash intake was observed. The results of Experiment 6 indicate that benzodiazepine receptors in the PBN may be specific for ingestional behaviour.

Benzodiazepine receptors are associated with the $GABA_A$ receptor complex. This is supported by evidence that they co-immunoprecipitate (Schoch et al., 1985). The existence of specific benzodiazepine receptor subtypes is supported by the observation that the $GABA_A$ receptor complex comprises subunits which have multiple isoforms (see Chapter 1 for discussion). Varying the combination of $GABA_A$ receptor subunits has been shown to determine the resultant pharmacology of benzodiazepine receptor ligands

(Luddens et al., 1995). It is possible that different populations of $GABA_A$ receptors exist in vivo, and that stimulation of a particular population results in selective behavioural effects. The benzodiazepine receptor population identified in the PBN may constitute a subpopulation specific for the effects of these compounds on ingestive behaviour. The development of subtype specific ligands would help to resolve this issue.

The effect of lesioning the PBN on the behavioural response to taste stimuli has been widely studied by several investigators. The results of these studies have shown that an intact PBN is important for the execution of a variety of taste-guided behaviours. For example, PBN lesioned rats are unable to form conditioned taste aversions (CTAs) (Reilly, Grigson and Norgren, 1993), have blunted responses to taste stimuli, and do not reliably express a depletion-induced sodium appetite (for review see Spector, 1995). Since the PBN is contains third order gustatory neurons this is perhaps not surprising. However, PBN lesions do not simply render rats ageusic or unable to detect taste stimuli. Despite blunted responses to unconditioned tastants, PBN lesioned rats can discriminate between taste stimuli and show concentration dependent responses (Flynn et al., 1991a,b; Spector, Grill and Norgren, 1993). It is unlikely that the decreased responsiveness to taste stimuli in PBN lesioned rats results from a reduction in the perceived intensity of the stimulus. Spector and colleagues have shown that PBN lesioned rats which fail to learn a CTA can nevertheless use taste stimuli as signals for other reinforcing events. For example, such rats perform competently in a taste-signalled shock avoidance task (Spector, Scalera, Grill and Norgren, 1995). This suggests that PBN lesions may result in an impairment of the affective response to taste stimuli rather than a purely sensory deficit. Therefore, the PBN may be involved in mediating the affective response to taste stimuli. Integrating the data gathered from lesion studies with the present results suggests that the increase in food intake caused by midazolam injected into the PBN results from an increase in hedonic responding mediated by receptors in this area. This conclusion is consistent with evidence from systemic injections of benzodiazepine agonists which suggest that these drugs increase ingestive behaviour by increasing palatability (Berridge and Pecina, 1995). Further experiments examining the

effect of intra-PBN midazolam in sham feeding and taste preference tests are required to examine this hypothesis.

The PBN may not be the only important structure for the effects of benzodiazepines on ingestive behaviour and it is possible that benzodiazepine receptor agonist modulation of feeding may occur even earlier on the taste pathway in the nucleus of the solitary tract (NTS) which is the first relay in the gustatory pathway (Norgren, 1978). This possibility remains to be tested, but a role for the NTS seems less likely given that an autoradiographic binding study failed to reveal any benzodiazepine receptors in this nucleus (Higgs et al., 1993). In addition, other brain sites such as the amygdala and hypothalamus which are known to be involved in the control of ingestive behaviour may also be important for the effects of benzodiazepines on food intake. This possibility could be examined by performing a cannula-mapping study.

In conclusion, the studies reported in this chapter indicate that the neural substrate for benzodiazepine-induced hyperphagia may be located in the brainstem, specifically in the PBN. Stimulation of receptors located in the PBN may affect ingestive behaviour without affecting locomotor behaviour.

Analysis of lick rate and microstructure of licking for sucrose and Intra-lipid

4.1 Introduction

A large body of evidence suggests that under real-feeding conditions, carbohydrate intake is determined by at least two important variables which have been modelled using principles from control theory by Davis and Levine (1977). The first variable is orosensory information, which stimulates ingestion via a positive feedback mechanism. The second is postingestive information which counteracts this stimulation, and reduces intake via a negative feedback signal. The model makes very specific predictions about the way in which manipulation of these two control variables should affect the rate of ingestion. One prediction is that the initial rate of ingestion of a solution should reflect the hedonic evaluation, or palatability of that stimulus. This hypothesis is well supported by data from experiments that have examined the effect of manipulating the concentration of carbohydrate solutions. Increasing carbohydrate concentration is assumed to increase orosensory stimulation and so alter the hedonic response to the solution being ingested. Many studies have shown that initial lick rate increases as a function of increasing carbohydrate concentration (Davis, 1973; Davis and Levine, 1977; Davis and Smith, 1988). This confirms the prediction that initial lick rate reflects changes in the underlying assessment of palatability. A second prediction is that the rate of decline of licking over time should reflect the development of a negative feedback signal arising from the postingestional consequences of feeding (Davis, et al., 1975; Davis and Levine, 1977). This prediction has been tested by adding mannitol to a carbohydrate test meal. Mannitol is absorbed from the intestine very slowly and has the effect of slowing the rate of clearance of ingested substances from the stomach. The effect of mannitol on the rate of ingestion is consistent with that predicted by the model. Mannitol does not decrease the initial rate of licking, but enhances the decline in the rate of licking within a test session (Davis and Levine, 1977). The model has proved to be successful because it can account

for a variety of experimental results, and because it allows investigators to separate out the effects of palatability and postingestional feedback in determining meal size.

In an extension of this work, Davis and Smith (1992) have suggested that microstructural analysis of the licking behaviour of rats may also provide information concerning the role of orosensory and postingestive factors in influencing ingestive behaviour. Microstructural analysis involves describing in detail the pattern of individual components of feeding behaviour (e.g. licks). The duration of bouts of licking has been shown to increase as a function of increasing concentration (Davis and Smith, 1992; Smith et al., 1992). This contrasts with the relationship between intake and concentration which varies non-monotonically according to an inverted U-shaped function, probably reflecting increased negative feedback associated with accumulation of ingested material in the gastrointestinal (GI) tract (Davis and Levine, 1977; Spector and Smith, 1984). Thus, it has been suggested that the duration of bouts may reflect changes in orosensory input which are uncontaminated by postingestive influences. Microstructural analysis may also provide information concerning non-specific motor effects on licking behaviour. Stellar and Hill (1952) showed that rats lick at a reasonably constant rate of about 6-7 licks per second. Any disruption of this rate might suggest that a treatment is affecting sensorimotor coordination, as distinct from an effect on the controls of ingestion. Thus, Davis and Smith (1992) showed that the intrabout lick rate is affected by moving the drinking spout progressively further away from an animal, but not by altering the solution concentration.

The Davis and Levine model could provide useful information concerning the mechanisms responsible for drug- or lesion-induced changes in ingestive behaviour. For example, if a drug were reducing food intake by decreasing palatability then it would be expected to decrease the initial rate of licking and reduce mean bout duration. Such information could not be gathered from merely measuring intake. However, one potential problem with the model is that experimental evaluation of it has been limited so far to the effect of manipulating the concentration of carbohydrates. If the model is to provide an adequate description of ingestive behaviour, then it must also account for the ingestion of

other macronutrients such as fats. Additionally, such information is crucial if unambiguous interpretations of lesions or pharmacological manipulations are to be made when these are linked to macronutrient variables. The aim of the present experiments was to investigate whether the predictions made by the Davis and Levine model also apply when the test fluid is a fat emulsion. The emulsion used, was Intra-lipid (Pharmacia Ltd, Milton Keynes, U.K.). This is a commercially-prepared lipid emulsion consisting of fractionated soya bean oil, fractionated egg phospholipids and glycerol (200 kcal per 100 ml). It is well suited for use in lickometer studies because it emulsifies well, is not viscous, and is readily consumed by rats.

The aim of Experiment 7 was to examine in detail the temporal and microstructural characteristics of licking for Intra-lipid in a 20-min test. Licking for sucrose was also measured under the same conditions. Rats were given 20-min access to various concentrations of either Intra-lipid (1%, 3% and 10%) or sucrose (1%, 3%, 10% and 30%). In Experiment 8, the aim was to minimize longer-term negative feedback from the GI tract by limiting access to the test fluids to an initial 60s period of licking. Several concentrations of sucrose or Intra-lipid were presented within the test session in a random order, and the licking behaviour analyzed. A short interval (10s) intervened between subsequent presentations because it had been shown previously that such rapid evaluation does not yield significantly different results from those obtained using longer inter-trial intervals (Smith, Davis and O' Keefe, 1992). This brief contact method has the advantage that licking responses to a range of concentrations can be evaluated rapidly within a single session.

4.2 Method

4.2.1 Animals

Forty naive non-deprived adult male hooded Lister rats (Charles River, U.K.) weighing 300-350 g at the beginning of training were used. They were housed in pairs in plastic cages in a room with a constant room temperature of 21±2 °C, and were maintained under a 12h light:dark cycle (lights on at 08.00). Rats were allowed ad lib

access to food pellets, (SDS RMI (E), Essex, U.K.) and water, except during testing. All testing was carried out in the light phase between 09.00 and 13.00h.

4.2.2 Test meal

Rats had access to various concentrations of sucrose (granulated cane sugar) or Intra-lipid emulsions which were made up freshly each day. The Intra-lipid emulsions were made up by diluting a 20% commercial preparation with tap water and the sucrose solution was made up to volume each day using tap water.

4.2.3 Apparatus

Testing was carried out using an MS80 multistation lick analysis system (Dilog Instruments, Tallahassee, FL). Rats were placed in a perspex chamber which was approximately 16 cm wide, 30 cm long and 20 cm tall. A perspex lid with four small holes in the top secured the chamber. The floor was made of stainless steel rods which were spaced 1 cm apart. An opening in the centre of the front wall of the chamber allowed rats access to a drinking spout which was located 85 mm from the floor of the chamber. This portal could be covered by a motorized shutter so that drinking from the spout was carefully controlled. The drinking spout was located 3 mm behind the wall so that to lick from the spout the rat was required to protrude its tongue through a small slot. This was to ensure that only licks were recorded and not contact made by the paws or snout, etc. The time of each lick to the nearest millisecond was recorded for later data analysis. Every time the rat contacted the drinking tube this completed a computer-monitored electronic circuit and was counted as a lick. Bottles containing the fluids were mounted in a line on a metal platform that could be moved backwards and forward by a reversible motor. This allowed any bottle to be stationed in front of the drinking slot for a given presentation. The opening of the shutter and positioning of the bottles was computer-controlled. The number of presentations, duration of each presentation, and the duration of the interpresentation interval were all determined by the experimenter.

4.2.4 Procedure

Experiment: 7: Microstructural analysis of drinking for sucrose and Intra-Lipid over a 20-min session

Training Twenty rats were divided into two groups (n = 10 per group), and were well familiarized with the test apparatus and procedure. Each group had access to one training fluid which was either a 30% concentration sucrose solution (granulated cane sugar) or a 10% concentration Intra-lipid emulsion. The presentation time was 20-min. Presentation began with the shutter opening to allow access to the drinking spout, but the 20-min test session did not start until the rat had started licking. Testing began once the amount ingested within a session showed no significant increase over three consecutive days. This was achieved after a period of approximately 10 days.

Testing In the test phase rats were given access to four different concentrations of sucrose (1, 3, 10 and 30%), or three different concentrations of Intra-lipid (1, 3 and 10%). The test fluids were offered in ascending order of concentration with the same concentration being offered on two consecutive days. This allowed the rats to adapt to a new concentration to avoid novelty effects. Only data from the second day were analyzed. The rats were tested for 20-min on each concentration. The total amount ingested after this period was recorded by successive weighings of the bottles.

Experiment 8: Microstructural analysis of drinking for sucrose and Intra-Lipid in a brief contact test

Training Twenty rats were divided into two groups (n = 10) and were well familiarized with the test apparatus and procedure. This involved placing each rat in the test chamber were they had access either to a 3% concentration sucrose solution or a 3% concentration Intra-lipid emulsion. Each rat received four or three presentations of 60s with an interpresentation interval (IPI) of 10s. For each presentation the bottle contained either 3% sucrose or 3% Intra-lipid. This procedure continued until the rats were

consuming steady baseline levels across presentations and across days (approximately 10 days).

Testing The test phase was identical to the training phase in that rats had four presentations of 60s with an IPI of 10s. However, in the test phase, instead of only having access to one concentration of fluid, the rats had access to all concentrations of either sucrose (1, 3, 10 and 30 %) or Intra-lipid (1, 3 and 10%). Each concentration was presented for a total duration of 60s. The order of presentation was randomised. Testing occurred on two consecutive days and only data from the second test day were analyzed. The order of presentation of fluid concentrations was counterbalanced across rats and across days.

4.2.5 Data analysis

The data collection programme recorded the time of each lick contact to the nearest millisecond. These lick time data were then analyzed using Dilog software (Ross Henderson, Dilog Instruments Tallahassee, Florida), followed by further processing using a Microsoft Exel spreadsheet programme. The change in lick rate across the test session was determined by calculating the number of licks in successive time bins for individual rats and then averaging across rats. In Experiment 7, an exponential decay function of the form $y = Ae^{(-Bt)}$ was fitted by a least squares method to the rate of licking data using the Sigma Plot graphics programme (Jandel Corp. Rafael, CA, 1986). This function was chosen because it provides a good description of the decline in the rate of licking which occurs, and is of theoretical importance (Davis and Levine, 1977). The results are expressed as the value of the intercept (A) and the rate constant of the decay function (B). A Spearman correlation coefficient was then calculated for the actual values and those predicted by the fit, to give a measure of how well the fit described the data. In Experiment 8, the main effect of concentration on the rate of licking in 60s was analyzed using a one-way repeated-measures ANOVA.

Microstructural analysis of the rats licking behaviour was also performed. Licking in rats is a highly stereotyped activity that occurs in sustained periods of licking or

'bouts' which are separated by pauses or inter-bout intervals (ILIs). The lick data were first grouped into bouts by specifying an upper ILI of 400 ms. This definition of a bout was used because it had been established in previous studies that an interval of 400 ms was just longer than the break point in a log survivor plot of ILIs (Morris, 1993). The data analysis programme gave the number of bouts and the duration of each bout within a presentation. The mean bout duration was calculated by summing over the presentation and taking an average value for each rat. Another parameter examined was the intrabout lick rate. This was calculated as the number of licks in a bout minus one, divided by the total duration of the bout. The latency to engage in drinking (time from shutter opening to first lick) was also examined. All data were analyzed using a one-way repeated-measures analysis of variance (ANOVA). Post hoc comparisons were made using a Dunnett's t-test. Statistical tests were performed using Statview SE+graphics (Abacus Concepts Inc., Berkeley, CA, 1987) and a result was considered statistically significant if $p < 0.05$.

4.3 Results

Experiment 7: Microstructural analysis of drinking for sucrose and Intra-Lipid over a 20-min session.

Intake

Figure 4.1 shows the intake of both sucrose and Intra-lipid as a function of concentration. In both cases, the volume consumed varied significantly with concentration: sucrose ($F_{3, 27} = 13.69$, $p < 0.001$); Intra-lipid ($F_{2, 18} = 5.72$, $p < 0.01$). The relationship between concentration and intake in both cases was non-monotonic. In the sucrose group, there was a marked increase in consumption when the concentration was increased from the 1% to the 3% concentration ($p < 0.01$). When the concentration was raised from the 10% to the 30% concentration, there was a decrease in consumption.

Increasing Intra-lipid concentration from 1% to 3% stimulated a modest increase in the volume ingested, although this was not significant in a post hoc test. An increase in the concentration from 3% to 10% produced a significant reduction in intake ($p < 0.05$).

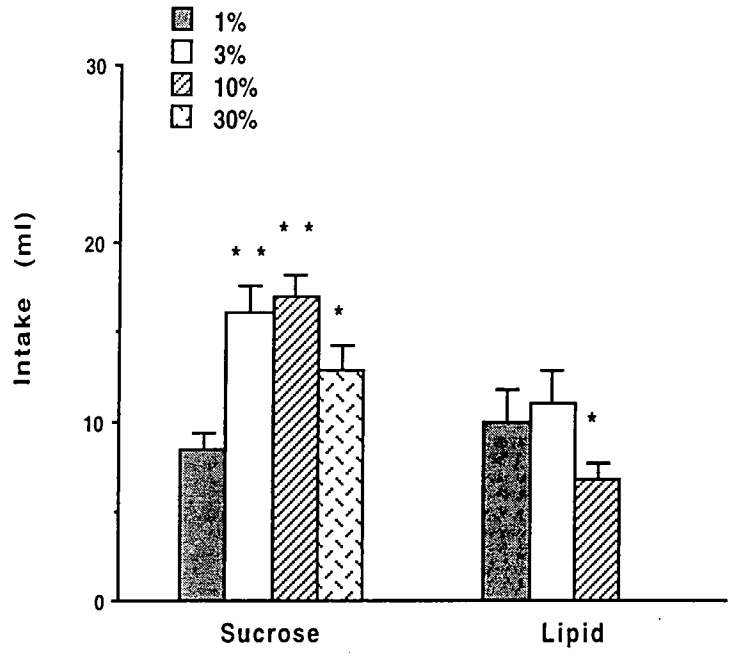
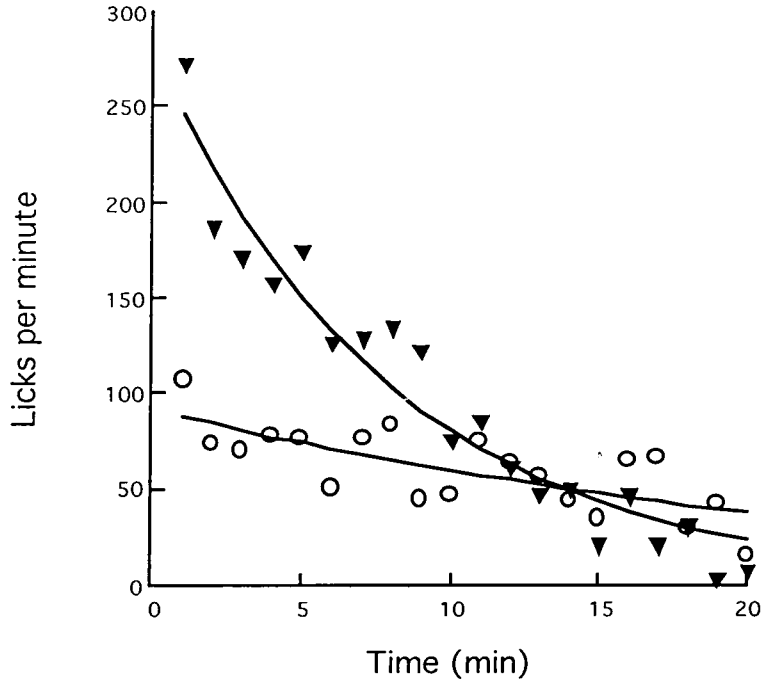


Figure 4.1. Intake of sucrose and Intra-lipid in a 20-min test as a function of concentration (%) + S.E.M. Asterisk indicates significantly different from 1% * $p < 0.05$ ** $p < 0.01$ (Dunnett's t-test).

Rate of licking

The data for the rate of licking at 1-min intervals for both sucrose and Intra-lipid are presented in Figure 4.2 which shows the effect of 1% and 30% sucrose and the effect of 1% and 10% Intra-lipid. An exponential decay function was fitted to the data and the parameters derived from the curve-fitting procedure are shown in Table 4.1. Increasing the concentration of either sucrose or Intra-lipid affected both the initial rate of licking and the rate constant. Increasing concentration resulted in an increase in the initial rate of licking (A). This was true for both sucrose and Intra-lipid. Increasing concentration of sucrose and Intra-lipid also increased the estimate of the slope function (B).

a) Sucrose



b) Intra-lipid

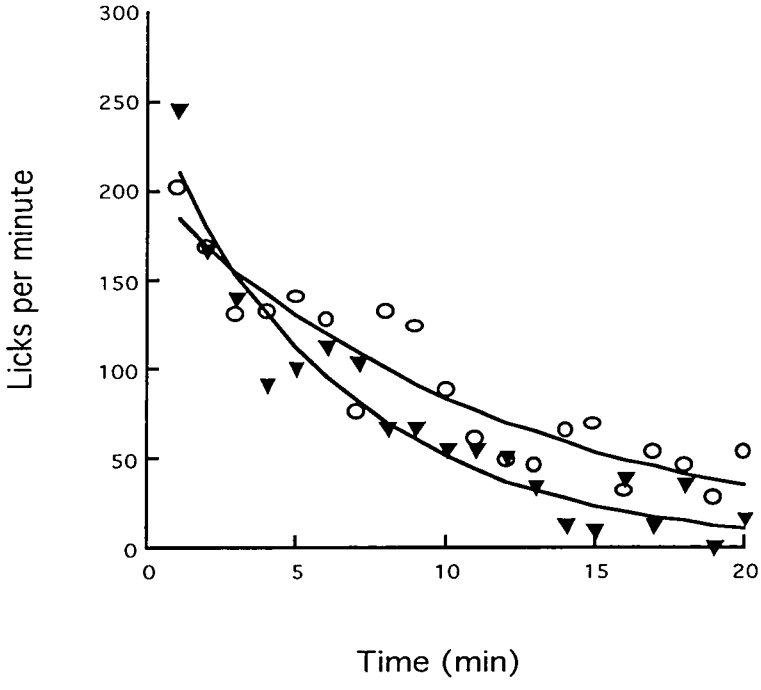


Figure 4.2 The effect of concentration on the rate of licking (licks per min) for a) sucrose and b) Intra-lipid. The lines are the least squares fit of the function $y = Ae^{(-Bt)}$ to the data. Open circles (O) indicate the 1% condition, filled triangles (▲) indicate the 10% (Intra-lipid) or 30% (sucrose) condition.

Table 4.1 Parameter estimates and standard errors and for the least squares fit to the rate of licking of the function $y = Ae^{(-Bt)}$.

Concentration (%)	A	B	r
	Sucrose		
1			
Estimate	92.1	0.04	0.74
S.E.	8.82	0.01	
30			
Estimate	279.3	0.12	0.98
S.E.	16.14	0.01	
	Intra-Lipid		
Concentration (%)	A	B	r
1			
Estimate	201.8	0.09	0.9
S.E.	13.6	0.01	
10			
Estimate	245.9	0.16	0.93
S.E.	17.6	0.01	

Parameters A and B describe the intercept and rate constant respectively. r is the correlation coefficient for the actual and predicted values calculated using a Spearman correlation coefficient.

Microstructural analysis.

Number of licks Figure 4.3 shows the data for the total number of licks generated in the test period for sucrose and Intra-lipid. There were concentration-dependent effects on licking for both sucrose ($F_{3, 27} = 10.14$, $p < 0.001$) and Intra-lipid drinking ($F_{2, 18} = 8.47$, $p < 0.01$). In each case the relationship was non-monotonic. For sucrose drinking, there was a large increase in the number of licks when the sucrose concentration was raised from the 1% to 3% or 10% concentration. At 30% sucrose concentration, the total number of licks declined from the peak level. Similarly, there was a slight enhancement in licking for Intra-lipid when the concentration was raised from the 1% to the 3% concentration, with an absolute decrease in the number of licks (compared with 1%) when the concentration reached 10%. These data parallel the intake data shown in Figure 4.1.

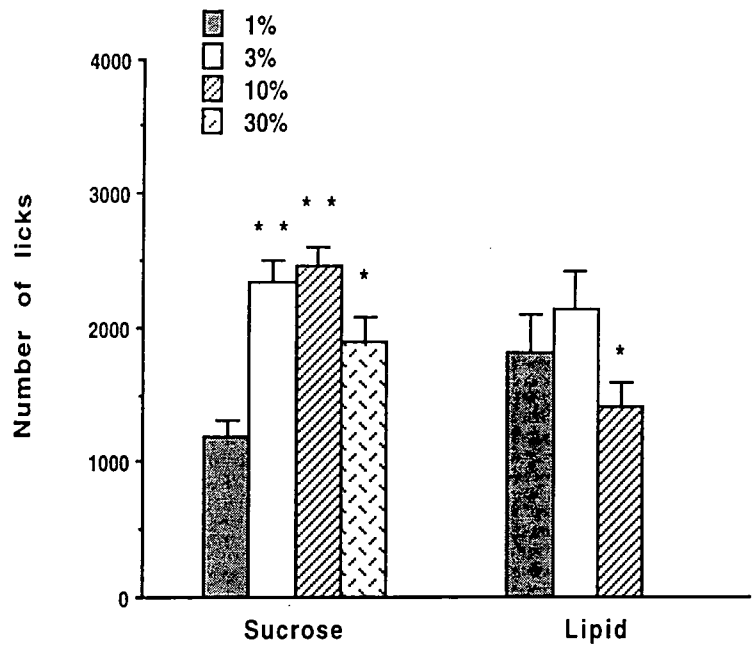


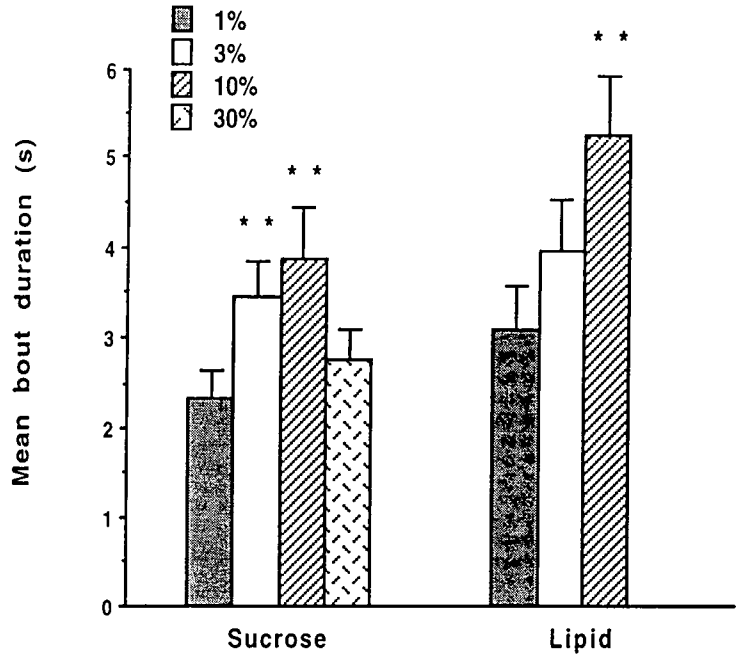
Figure 4.3. The number of licks for sucrose and Intra-lipid drinking in a 20-min test as a function of concentration (%) + S.E.M. Asterisk indicates significantly different from 1% * $p < 0.05$ ** $p < 0.01$ (Dunnett's t-test).

Mean bout duration For both sucrose ($F_{3, 27} = 10.98$, $p < 0.001$) and Intra-lipid drinking ($F_{2, 18} = 10.76$, $p < 0.001$), an increase in concentration led to an increase in the mean bout duration, although for sucrose drinking this relationship was non-monotonic because there was a decrease in mean bout duration at 30% sucrose (Figure 4.4a).

Bout number As shown in Figure 4.4b a trend was observed towards increasing bout number with increasing sucrose concentration but this did not reach significance ($F_{3, 27} = 2.42$, n.s.). Manipulating Intra-lipid concentration significantly affected the number of bouts in the test session ($F_{2, 18} = 8.112$ $p < 0.01$). Increasing Intra-lipid concentration led to a decrease in the number of bouts (Figure 4.4b).

In summary, an increase in sucrose concentration increased the size of bouts but did not greatly affect the number of bouts; in contrast, increasing Intra-lipid concentration led to an increase in the size of bouts, but decreased their number.

a) Mean bout duration



b) Number of bouts

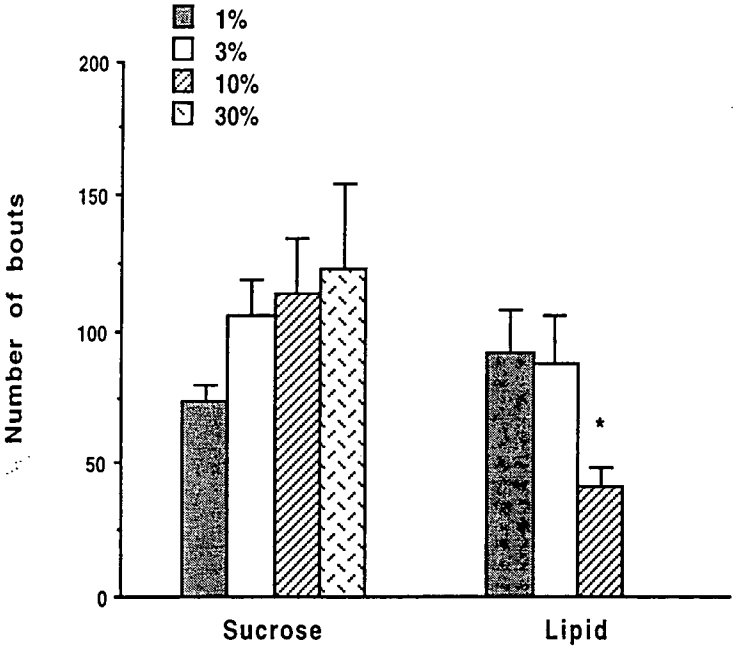
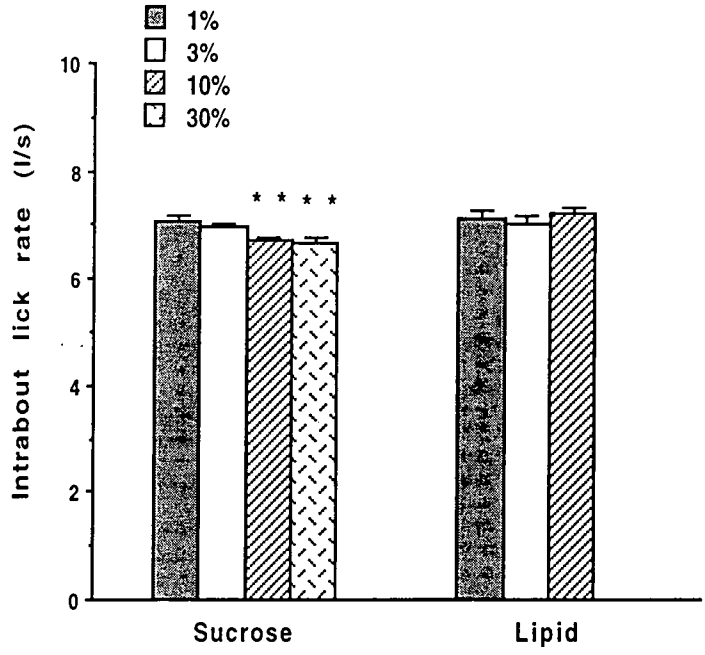


Figure 4.4 a) Mean bout duration and b) number of bouts for sucrose and Intra-lipid drinking in a 20-min test as a function of concentration (%) + S.E.M. Asterisk indicates significantly different from 1% * $p < 0.05$ ** $p < 0.01$ (Dunnett's t-test).

Intrabout lick rate The rate of licking within bouts (intrabout lick rate) was significantly affected by manipulating the concentration of both sucrose ($F_{3, 27} = 8.23$, $p < 0.001$) and Intra-lipid ($F_{2, 18} = 5.14$, $p < 0.05$). An increase in the concentration of sucrose led to a significant decrease in the intrabout lick rate (Figure 4.5a). In the case of Intra-lipid drinking, increasing the concentration increased the rate of licking although this effect was not concentration dependent. At the 10% concentration of Intra-lipid, the rate of licking within bouts was significantly greater than that at the 3% concentration ($p < 0.01$), but the 1% condition did not differ significantly from the 10% condition (Figure 4.5a).

Latency The latency to start drinking was significantly affected by manipulating concentration for sucrose drinking ($F_{3, 27} = 3.62$, $p < 0.05$) (Figure 4.5b). Post hoc analysis showed that the 10% ($p < 0.05$) and 30% ($p < 0.01$) concentration of sucrose differed significantly from the 1% concentration condition. As shown in Figure 4.5b, increasing Intra-lipid concentration did not affect the latency to engage in drinking ($F_{2, 18} = 0.82$, n.s.). However, there was a trend towards increasing latency with increasing Intra-lipid concentration, although this did not reach significance.

a) Intrabout lick rate



b) Latency

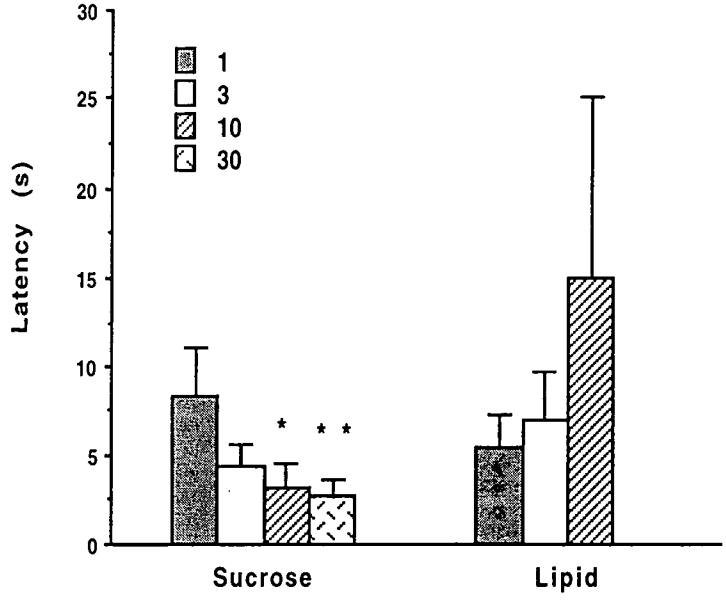


Figure 4.5 a) Intrabout lick rate and b) latency for sucrose and Intra-lipid drinking in a 20-min test as a function of concentration (%) + S.E.M. Asterisk indicates significantly different from 1% * $p < 0.05$ ** $p < 0.01$ (Dunnett's t-test).

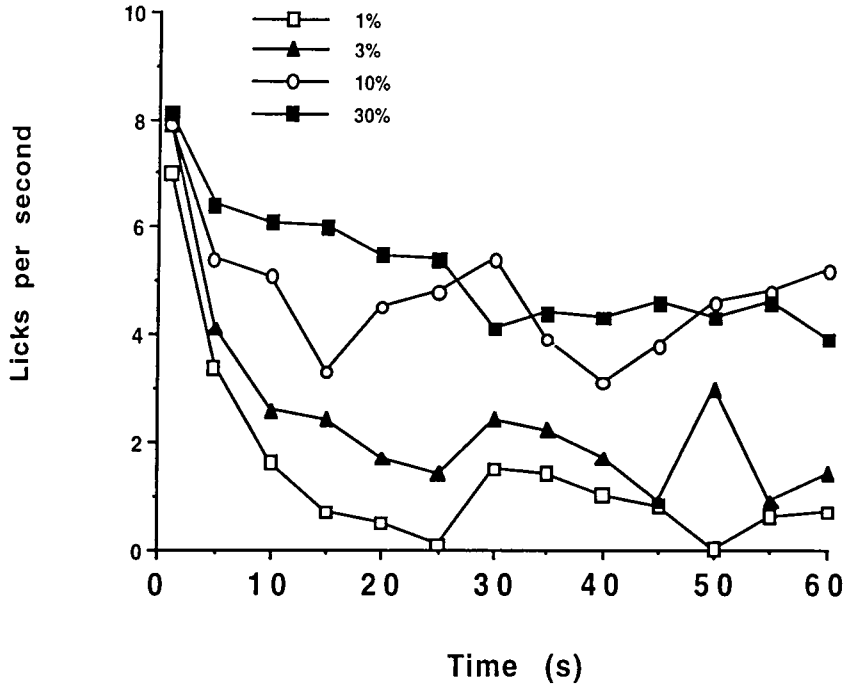
Experiment 8: Microstructural analysis of drinking for sucrose and Intra-lipid in a brief contact test

Rate of licking

A one-way repeated-measures ANOVA revealed that for sucrose there was a significant effect of concentration on the rate of licking over 60s ($F_{3, 27} = 18.6$, $p < 0.01$). The effect of sucrose concentration on the rate of licking is shown in Figure 4.6a. An increase in the initial rate of licking was observed with increasing concentration of sucrose. There was then a gradual decline in the number of licks per second over the 60s for each concentration (Figure 4.6a). This decline was inversely proportional to concentration. For the 30% concentration of sucrose, the rate of licking was maintained at a relatively high level over the 60s session. At lower concentrations, the rate of licking declined rapidly leading to a separation of the concentration curves after 60s.

The effects of Intra-lipid concentration on the rate of licking were similar to the patterns obtained for sucrose (Figure 4.6b). There was a significant effect of Intra-lipid concentration on the rate of licking ($F_{2, 18} = 20.02$, $p < 0.01$). The highest concentration of Intra-lipid (10%) stimulated a higher initial rate of licking than 1% concentration. The rate of licking for Intra-lipid also declined over the test session. As can be seen in Figure 4.6b the rate of decline was similar for each concentration of Intra-lipid. Therefore, the curves show a parallel decrease over the test session.

a) Sucrose



b) Intra-Lipid

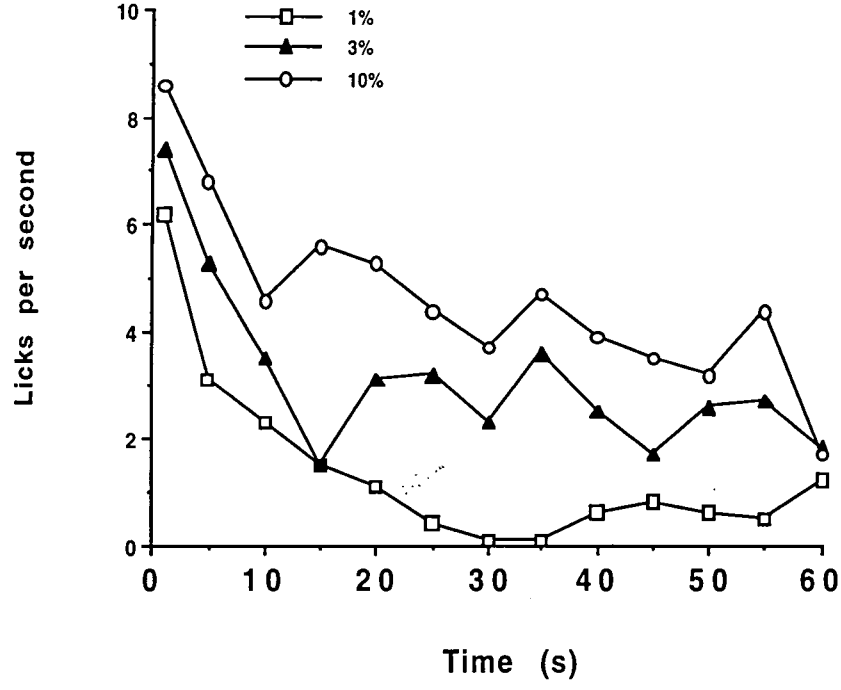


Figure 4.6 The average number of licks per second at 1s intervals as a function of concentration (%) for a) sucrose and b) Intra-lipid in a brief contact test. Different concentrations are plotted separately

Microstructural analysis

Number of licks An increase in fluid concentration led to a significant and monotonic increase in the total number of licks in the test session for both sucrose ($F_{3,27} = 21.9$, $p < 0.001$) and Intra-lipid drinking ($F_{2,18} = 20.0$, $p < 0.001$) (Figure 4.7).

Mean bout duration Mean bout duration varied significantly as a function of fluid concentration. Increasing the concentration led to a monotonic increase in the mean bout duration for both sucrose ($F_{3,27} = 5.5$, $p < 0.01$) and Intra-lipid drinking ($F_{2,18} = 8.38$, $p < 0.01$) (Figure 4.8a).

Number of bouts There was a significant effect of concentration on bout number for sucrose drinking, although post hoc comparisons revealed that this effect was not concentration-dependent ($F_{3,27} = 3.7$, $p < 0.05$) (Figure 4.8b). Manipulating the concentration of Intra-lipid did not significantly affect the number of bouts in the 60s test period ($F_{2,18} = 2.01$, n.s.) (Figure 4.8b).

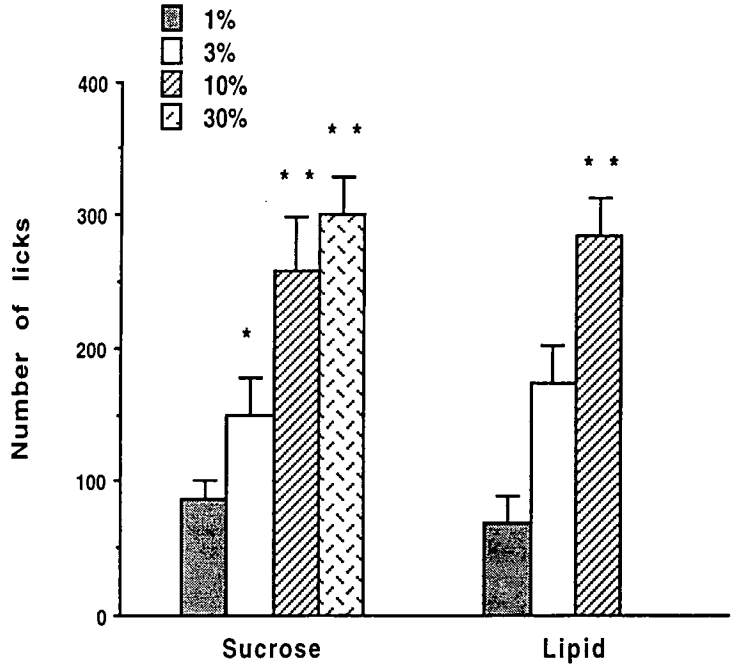
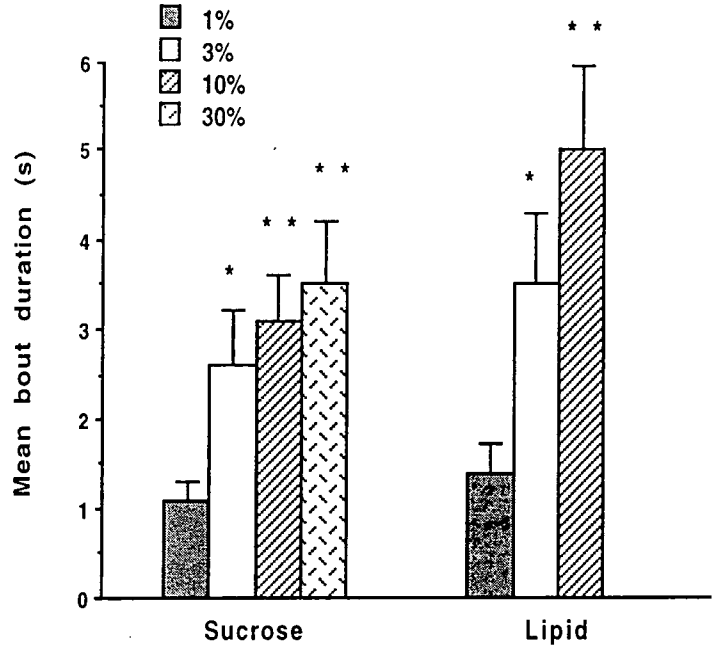


Figure 4.7 The number of licks for sucrose and Intra-lipid drinking in a brief contact test as a function of concentration (%) + S.E.M. Asterisk indicates significantly different from 1% * $p < 0.05$ ** $p < 0.01$ (Dunnett's t-test).

a) Mean bout duration



b) Bout number

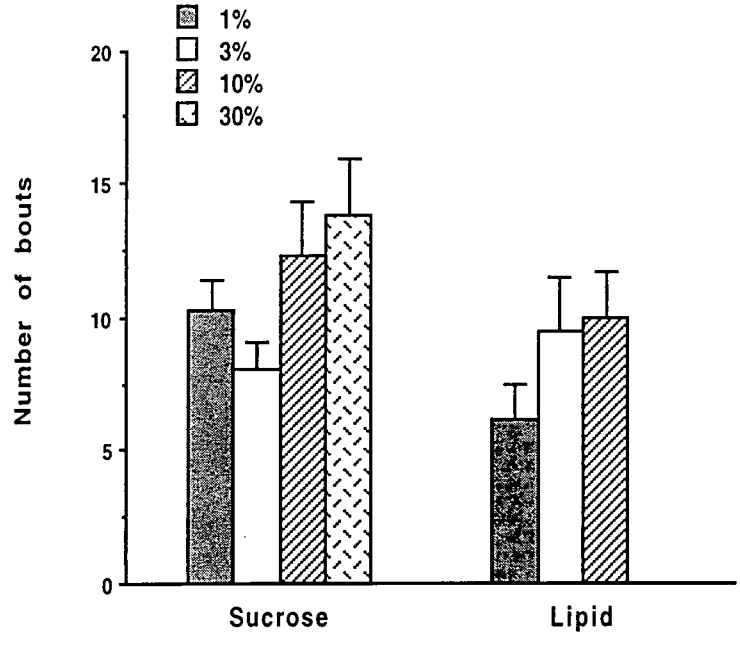
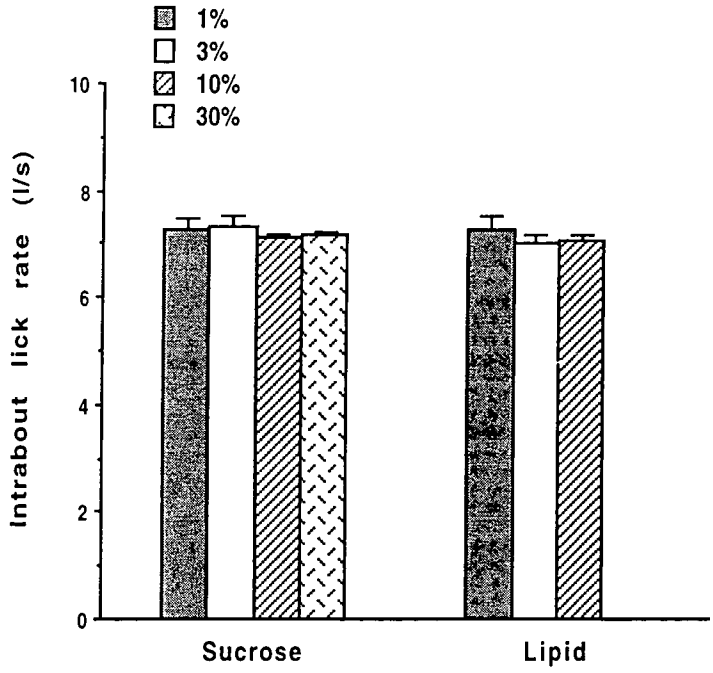


Figure 4.8 a) Mean bout duration b) number of bouts for sucrose and Intra-lipid drinking in a brief contact test as a function of concentration (%) + S.E.M. Asterisk indicates significantly different from 1% * $p < 0.05$ ** $p < 0.01$ (Dunnett's t-test).

Intrabout lick rate The rate of licking within bouts was not affected by manipulating fluid concentration. This was the case for both sucrose ($F_{3,27} = 0.6$, n.s.) and Intra-lipid drinking ($F_{2,18} = 0.18$, n.s.). An increase in the concentration of both sucrose and Intra-lipid did lead to a slight decrease in the intrabout lick rate, although this did not reach significance (Figure 4.9a).

Latency There was no effect of manipulating concentration on the latency to engage in drinking for either the sucrose ($F_{3,27} = 2.8$, n.s.) or Intra-lipid groups ($F_{2,18} = 0.9$, n.s.). As can been seen in Figure 4.9b, there was a trend towards an increase in latency with increasing concentration, but this was not significant.

a) Intrabout lick rate



b) Latency

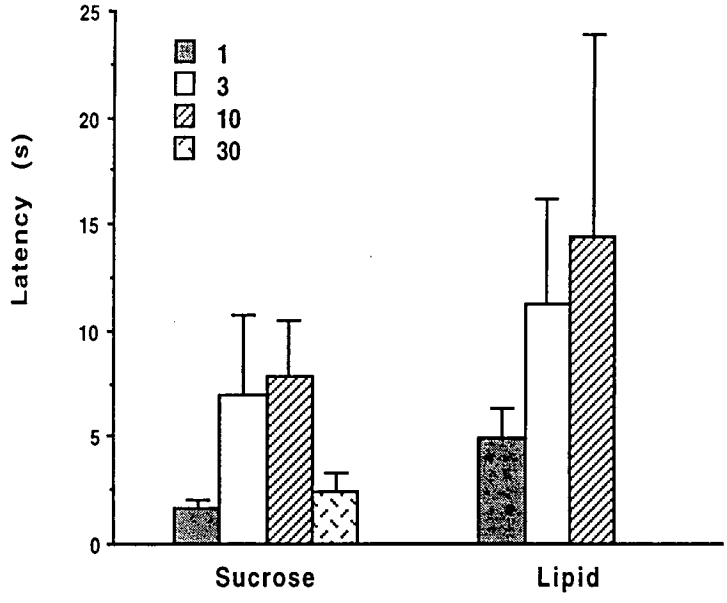


Figure 4.9 a) Intrabout lick rate and b) latency for sucrose and Intra-lipid drinking in a brief contact test as a function of increasing concentration (%) + S.E.M.

4.4 Discussion

In Experiments 7 and 8, a comparison was made between the licking responses for sucrose and Intra-lipid to determine if the model of ingestive behaviour proposed by Davis and Levine (1977) can account for the effects of manipulating the concentration of fats as well as carbohydrates. The results show that the effect of manipulating fluid concentration on the general pattern of licking responses was the same for both sucrose and Intra-lipid. The initial lick rate for sucrose and Intra-lipid in a 20-min test was found to increase as a function of increasing concentration. Additionally, in a brief exposure test, mean bout duration was found to vary monotonically with concentration.

In Experiment 7, the rate of licking over a 20-min test session was examined for several concentrations of sucrose and Intra-lipid. The initial rate of licking for these nutrients was found to increase with increasing concentration. The monotonic relationship between the initial rate of licking and increasing concentration contrasted with the relationship between concentration and intake in the same test which varied non-monotonically according to an inverted U-shaped function. The decrease in intake observed at higher concentrations of sucrose and Intra-lipid probably reflected the emergence of an inhibitory feedback signal due to the accumulation of fluid in the gut. These results support the notion that the rate of licking during the initial period of licking for both sucrose and Intra-lipid reflects the stimulating effectiveness of the solution uncontaminated by negative feedback and so provides a measure of palatability.

Increasing concentration also increased the slope of the rate function for sucrose and Intra-lipid drinking. This is consistent with the hypothesis that the rate of decay of licking provides a measure of postingestive negative feedback and can be explained as follows: increasing concentration led to an increase in the initial rate of ingestion. Consequently, the rate of accumulation of fluid in the gut was also increased, thus leading to an increase in the strength of a postingestive negative feedback signal. It is interesting that although the intake of 1% sucrose and 1% Intra-lipid were quite similar, rate analysis revealed differences between the magnitude of the initial rate of licking and slope function. The initial rate of licking for 1% sucrose was lower than that for 1%

Intra-lipid. However, the overall intake was roughly the same after 20-min because the rate of decline of 1% Intra-lipid drinking was much greater than that for 1% sucrose drinking. Such a difference between sucrose and Intra-lipid drinking could not have been identified from simple measures of intake and would suggest that even at low concentrations Intra-lipid is highly palatable.

Microstructural analysis showed that manipulating concentration also produced changes in bout structure. Davis and Smith (1992) found that in a 30-min intake test, the only parameter to vary monotonically with increasing sucrose concentration was the bout duration. These authors suggested that this measure, like the initial rate of ingestion, could provide information concerning changes in palatability. The effect of manipulating sucrose and Intra-lipid concentration over 20-min in Experiment 7 did not produce such clear-cut effects on bout structure. Mean bout duration did significantly increase with increasing concentration of Intra-lipid. For sucrose drinking however, mean bout duration increased when concentration was raised form 1% to 10%, but there was then a decrease in mean bout duration when concentration was raised to 30%. The non-monotonic effect of sucrose concentration on mean bout duration may have been due to changes in mean bout duration over time. Early in the test session drinking may have been characterized by few bouts of long duration due to the influence of palatability factors. However, towards the end of the test session, when postingestive factors predominate, bouts may have been more frequent but shorter. This interpretation is supported by the findings of Morris (1993), who showed that for concentrated sucrose solutions, bout duration was not constant across a 20-min test session. Bout duration was longer at the beginning of the test, but much shorter during the latter part of the test, possibly due to the influence of postingestive factors (Morris, 1993). The conclusion to be drawn from this is that changes in mean bout duration may only reflect changes in palatability when the test session is kept short to avoid the influence of postingestive factors. This problem is addressed later in Experiment 8 where the test session was limited to 60s.

There were no significant effects of concentration on the number of bouts for sucrose drinking, although there was a trend towards increasing bout number with increasing concentration. For Intra-lipid drinking, there was a significant decrease in the number of bouts at 10% which probably occurred as a consequence of the increase in mean bout duration at this concentration. The lack of effect of concentration on bout number is consistent with previous reports (Davis and Smith, 1992) and suggests that changes in bout number do not provide information concerning changes in palatability.

In Experiment 7, the rate of licking within bouts was affected by manipulating the concentration of sucrose and Intra-lipid. Increasing sucrose concentration led to a decrease in the intrabout lick rate. This result is similar to the findings of Davis and Smith (1992) who also found a decrease in the rate of licking for sucrose with increasing concentration. These authors suggested that this effect could not account for any variability in intake and may have been an artifact of the test situation. One possible explanation for the results obtained in Experiment 7 is that at higher concentrations the tongue remained in contact with the lick tube for longer. A prolonging of the lick cycle at higher concentrations may have been due to increased viscosity when concentration is increased, or may be related to the increase in the stimulating effectiveness of higher concentrations. In the case of Intra-lipid drinking, there was an increase in the rate of licking within bouts at 10% compared with 1%. This result is harder to explain, and it is clear that the interpretation of intrabout lick rate is more complicated than first suggested by Davis and Smith (1992). Further work is required to examine the effects of viscosity and palatability on the rate of licking within bouts.

The latency to engage in drinking was affected by manipulating sucrose concentration but not by manipulating Intra-lipid concentration. There was a decrease in latency with increasing sucrose concentration. It has been suggested that latency may provide a measure of motor incapacitation. However, the effect of sucrose concentration on latency suggests that this measure may also reflect changes in other variables. It is possible that changes in latency reflect changes in the motivation to start drinking. Such an explanation would suggest that increasing sucrose concentration in Experiment 7

resulted in increased motivation drink, possibly because of the increased palatability of higher concentrations of sucrose. The lack of effect of manipulating Intra-lipid concentration on latency may suggest that increasing Intra-lipid concentration does not affect motivation in the same way as manipulating sucrose concentration. It is clear that interpreting changes in latency may be problematic because several factors may lead to differences in the time to engage in drinking. Therefore, the conclusion from this is that caution must be exercised in explaining these differences.

Microstructural analysis of sucrose and Intra-lipid drinking over a 60s presentation period revealed great similarity in the pattern of licking for these nutrients. The results for sucrose and Intra-lipid were comparable for all the variables examined, suggesting that there are no significant differences in the way in which rats respond to Intra-lipid and sucrose over the initial 60s period of drinking. The results obtained for sucrose drinking in the present experiments are consistent with previously published data for a range of other carbohydrates (Davis, 1973; Davis et al., 1995; Davis and Smith, 1992).

The rate of licking curves for sucrose showed that over the 60s test there was a gradual decline in the change in the rate of licking which was inversely proportional to concentration. These data are consistent with the findings of Smith et al. (1992) who found that the rate of licking for various carbohydrates including sucrose in a 30s test declined steadily, with lower concentrations showing a greater rate of decline. In Experiment 8, the rate of licking for sucrose was similar to the rate of licking for Intra-lipid. With Intra-lipid there was also a decline in the rate of licking over the test sessions which was affected by manipulating concentration. There was an effect of Intra-lipid concentration after the first second of licking with higher concentration stimulating much higher rates of licking than lower concentrations. These results suggest that the increase in the initial rate of licking observed in Experiment 7 can be detected even within the first minute of presentation. As suggested previously (Smith et al., 1992), the decline in the rate of licking for both sucrose and Intra-lipid is most probably not due to inhibitory postingestive feedback since the test session is too short for a large enough volume of

fluid to have accumulated in the GI tract. Instead, the effect is better explained by the emergence of adaptation to chemical or mechanical stimulation (Smith et al., 1992). Since this adaptation is sensitive to varying concentration, further examination of this effect may be important in determining the mechanisms responsible for the differential stimulating effectiveness of palatable solutions.

Analysis of the total number of licks in 60s showed that this measure varied monotonically with concentration for both sucrose and Intra-lipid. Increasing concentration significantly increased the total number of licks in the session. In the brief contact test, the influence of postingestive feedback is minimized and so the relationship between the number of licks and concentration probably reflects orosensory determinants of ingestion.

Microstructural analysis revealed that for both sucrose and Intra-lipid the increase in the number of licks was due to an increase in the mean bout duration rather than an increase in the number of bouts. Mean bout duration increased significantly with increasing concentration of sucrose and Intra-lipid. This contrasts with the relationship between sucrose concentration and mean bout duration in the 20-min presentation used in Experiment 7 which was non-monotonic. Davis and Smith (1992) have suggested that a monotonic relationship between mean bout duration and carbohydrate concentration indicates that this measures reflects the hedonic potency of ingested fluids, and so provides a robust measure of palatability. The differential effect of sucrose concentration on mean bout duration obtained in Experiments 7 and 8 suggests that this measure may only provide information concerning the palatability in a brief contact test where the effect of postingestional negative feedback has been minimized.

The finding that mean bout duration increases with increasing Intra-lipid concentration in a brief contact test contrasts with more recent findings of Davis et al. (1995) who examined licking responses to the ingestion of corn oil. These authors found that in the first minute of licking for this fat, the mean bout duration did not vary as a function of increasing concentration. It was suggested that the lack of effect of corn oil concentration on mean bout duration may have been due to the fact that changes in this

parameter reflect gustatory, rather than somatosensory stimulation. It could be that the initial response to corn oil is determined by texture rather than taste. In support of this, human psychophysical studies have shown that texture is important in determining perceived fattiness (Mela, 1988). It has been suggested by Mindell, Smith and Greenberg (1990) that fat texture is processed by oral tactile mechanisms of the trigeminal system. This system, rather than the gustatory system may be responsible for the differential stimulating properties of fats. The discrepancy between the present results and those of Davis et al. (1995) could be explained by the fact that corn oil and Intra-lipid have different viscosities and textures. Further work is required to examine the role of viscosity and texture in determining the ingestion of fats. Additionally, Intra-lipid and corn oil may differ in their ability to stimulate the gustatory system. Although there has been little examination of the role of flavour in fat consumption to date, it could be that flavour is also an important determinant of fat palatability.

In summary, the results of the Experiment 8 showed that mean bout duration varied monotonically with both sucrose and Intra-lipid concentration. This suggests that mean bout duration is sensitive to the palatability associated with sucrose and Intra-lipid. However, the results of Davis et al. (1995) indicate that mean bout duration may not be sensitive to the changes in oropharyngeal stimulation provided by all fats. It remains to be fully determined whether the effect of manipulating concentration on bout duration is related specifically to gustatory as opposed to other somatosensory stimulation.

Manipulating concentration did not have any effect on the rate of licking within bouts for either sucrose or Intra-lipid drinking in the brief contact test used in Experiment 8. This differs from the finding in Experiment 7 where intrabout lick rate varied with increasing fluid concentration. Therefore, the effects of concentration on this measure may be dependent on the presentation time. The intrabout lick rate was affected by concentration in the 20-min test, but not in the brief contact test. This may have been due to an effect of habituation apparent during a 20-min test but absent during a 60s presentation. The conclusion from this is that caution must be exercised in interpreting any changes in the intrabout lick rate because this measure may not be so robust a

measure of motoric disruption as suggested by Davis and Smith (1992).

The latency to engage in drinking was not significantly affected by manipulating concentration of sucrose or Intra-lipid. This contrasts with the effect of sucrose concentration on latency which was obtained in Experiment 7. This suggests that further work is required to examine the effect of factors such as concentration on latency before firm conclusions can be drawn concerning the processes which are important in determining changes in latency.

In summary, these data confirm the usefulness of analyzing the rate of ingestion and microstructure of licking behaviour in providing information concerning the processes responsible for controlling intake of carbohydrates and extends these conclusions to the control of Intra-lipid intake. The initial lick rate increased with increasing concentration suggesting that this measure may provide information concerning the palatability of both sucrose and Intra-lipid. It was shown that for both sucrose and Intra-lipid drinking, the effect of concentration on the initial rate of licking occurred within the first few seconds of the test. Mean bout duration increased monotonically as a function of concentration in a 60s test, but there was no significant effect of concentration on bout number. This suggests that in a brief contact test, mean bout duration may provide a measure of palatability which applies to both sucrose and Intra-lipid.

An important aim of the experiments reported in this chapter was to establish control patterns of ingestion for sucrose and Intra-lipid before going on to investigate the effects of pharmacological manipulations on licking behaviour. In Experiments 7 and 8, it was shown that manipulating fluid concentration had specific effects on the microstructure of licking for both sucrose and Intra-lipid. From this it was suggested that microstructural analysis can provide information concerning changes in palatability. In subsequent chapters, the effects of drug adminstration on licking patterns will be compared with the effects of manipulating concentration to help elucidate the factors responsible for the effects of benzodiazepine ligands on intake.

The effects of midazolam on the microstructure of licking for sucrose and Intra-lipid

5.1 Introduction

It has recently been suggested that the increase in food intake brought about by administration of benzodiazepines is due to an effect on palatability (Berridge and Pecina, 1995). Evidence to support this argument derives from the effects of benzodiazepine receptor agonists in tests which are thought to provide a measure of palatability. Such tests include the taste reactivity paradigm, the taste preference test and sham feeding preparation. In the taste reactivity test, benzodiazepine receptor agonists have been shown to increase ingestive reactions to sapid solutions (Berridge and Treit, 1986; Gray and Cooper, 1995; Treit and Berridge, 1990). This suggests that they might be enhancing the hedonic value of ingested substances. Additionally, benzodiazepine receptor agonists exert selective effects in taste preference tests (Cooper and Green, 1993; Cooper and Yerbury, 1988; Roache and Zabik, 1986), and increase sucrose sham feeding in the gastric fistulated rat (Cooper, et al., 1988).

It has been appreciated for some time that analysis of the rate and microstructure of ingestive behaviour may provide information concerning the factors responsible for controlling food and fluid intake (Davis and Levine, 1977; Davis and Smith, 1992). However, these techniques have not been systematically applied to the study of the effects of benzodiazepines on ingestive responses. Cooper and Yerbury (1986b), examined changes in the microstructure of solid food consumption and found that midazolam enhanced intake by increasing the duration of bouts as opposed to their number. However, there has been no investigation of the effects of benzodiazepines on the microstructure on licking behaviour. Microstructural analysis of licking patterns is often preferred to analysis of solid food consumption because the use of automated lickometers to measure licking for fluids in rats provides a more accurate way of determining the timing of ingestive events.

Studies have shown that manipulation of orosensory and postingestive factors achieved by manipulating the concentration of ingested fluids produces quite specific effects on the rate and microstructure of licking for carbohydrates. The initial rate of licking and duration of bouts have been shown to vary monotonically with increasing concentration of carbohydrates (Davis, 1973; Davis and Levine, 1977; Davis and Smith, 1992). Hence, it has been suggested that these parameters provide the means to measure palatability. It has been also been proposed that the rate of decline of licking provides an estimate of the increasing influence of inhibitory postingestive factors as ingestive behaviour proceeds (Davis, et al., 1975; Davis and Levine, 1977). The results of the previous chapter suggest that these measures also provide a measure of palatability and postingestive feedback associated with at least one source of fat, Intra-lipid. In Experiment 7, increasing the concentration of either sucrose or Intra-lipid increased the initial lick rate and led to a steeper rate of decline of licking. In a brief contact test (Experiment 8), mean bout duration increased with increasing concentration of both sucrose and Intra-lipid. Comparison of the effects of benzodiazepines with the effects of manipulating concentration on licking patterns could provide new information concerning the behavioural mechanisms involved in benzodiazepine-induced hyperphagia.

The aim of the present experiments was to use the licking microstructural approach to further investigate the behavioural mechanisms responsible for benzodiazepine-induced changes in ingestive behaviour. The effects of midazolam on the microstructure of licking were compared with the effects obtained when manipulating fluid concentration.

An additional aim was to compare and contrast the effects of benzodiazepine receptor agonist administration on the consumption of carbohydrates versus fats. There are no previous reports of the effect of benzodiazepines on the consumption of specific macronutrients. Therefore, such information would be of great interest. I f benzodiazepine agonists increase ingestive behaviour through an enhancement of palatability, then this should operate as much on the hedonic evaluation of fats as on that of carbohydrates.

The data from two experiments are reported in this chapter. In Experiment 9, the effects of midazolam on the rate and microstructure of licking for both a 3% sucrose solution and a 1% Intra-lipid emulsion in a 20-min test were examined. This experiment demonstrated that midazolam increased the rate of licking in the initial part of the test. To examine this effect in greater detail, Experiment 10 was designed to investigate the responses to a range of concentrations of sucrose and Intra-lipid in a brief contact test. The microstructural variables examined were the total number of licks, mean bout duration, number of bouts, intrabout lick rate and the latency to engage in drinking.

5.2 Method

5.2.1 Animals

Forty naive non-deprived adult male hooded Lister rats (Charles River, U.K.) weighing 300-350 g at the beginning of training were used. They were housed in pairs in plastic cages in a room with a constant room temperature of 21±2 °C, and were maintained under a 12h light:dark cycle (lights on at 08.00). Rats were allowed ad lib access to food pellets, (SDS RMI (E), Essex, U.K.) and water, except during testing. All testing was carried out in the light phase between 09.00 and 13.00h.

5.2.2 Drugs

The benzodiazepine receptor agonist midazolam maleate (Roche, Basel, Switzerland) was prepared for injection by dissolving in distilled water. The doses used in these experiments were 0.3, 1 and 3 mg/kg. It has been shown previously that midazolam elicits a hyperphagia when injected i.p. within this dose range (Cooper et al., 1985). The vehicle used in control injections was distilled water.

5.2.3 Test meal

Rats had access to various concentrations of sucrose solutions (granulated cane sugar) or Intra-lipid emulsions (Pharmacia Ltd, Milton Keynes, U.K.) which were made up freshly each day. The Intra-lipid emulsions were made up by diluting a 20% commercial preparation with tap water, and the sucrose solutions were made up to volume each day using tap water.

5.2.4 Apparatus

Testing was carried out using the MS80 multistation lickometer described in detail in the Chapter 4 (Section 4.2.3).

5.2.5 Procedure

Experiment 9: Microstructural analysis of the effect of midazolam on drinking for a 3% sucrose solution and a 1% Intra-lipid solution over a 20-min session

Training Twenty rats were divided into two groups (n = 10 per group), and were well familiarized with the test apparatus and procedure. Each group had access to one training fluid which was either a 3% sucrose solution or a 1% Intra-lipid emulsion. These concentrations were chosen to give low baseline levels of consumption. Training involved placing each animal in the lickometer chamber for a period of 20-min during which they had access to the appropriate training fluid. This procedure continued until steady baselines of consumption were observed across familiarization days (approximately 10 days). Two days prior to testing each animal received a sham injection of distilled water to familiarize it with the injection procedure.

Testing Midazolam was administered at doses of 0.3, 1 and 3 mg/kg 20-min prior to testing. 20-min after injection the rats were placed in the lickometer chamber where they had access to either the 3% sucrose solution or the 1% Intra-lipid emulsion for 20-min. A within-subjects design was used in which each animal acted as its own control. The order of injections was counterbalanced and a period of 48h elapsed between each series of injections to avoid carry-over effects.

Experiment 10: Microstructural analysis of the effect of midazolam on drinking for sucrose and Intra-lipid in a brief contact test

Training Twenty rats were divided into two groups (n = 10 per group). Each group was well familiarized with the test apparatus and procedure. This involved placing each rat in the test chamber were they had access to a range of sucrose (1, 3, 10 and 30%) or Intra-lipid (1, 3 and 10%) concentrations in a random order. Each concentration was presented for 60s and a 10s interval intervened between subsequent presentations. This procedure continued until steady baseline levels of licking were observed across days (approximately 10 days). Two days prior to testing each animal received a sham injection of distilled water to familiarize it with the injection procedure.

Testing Following training, rats received i.p. injections of midazolam (0.3, 1 and 3 mg/kg) or vehicle. 20-min after injection the rats were placed in the lickometer chamber where they had access to all concentrations of either sucrose (1, 3, 10 and 30%) or Intra-lipid (1, 3 and 10%). Each concentration was presented for a total duration of 60s. The order of presentation was randomised. A repeated-measures design was used in which each animal was tested at each dose. Injections were counterbalanced and 48h elapsed between treatments to avoid carry-over effects.

5.2.5 Data analysis

The lick time data were analyzed as described in Chapter 4 (Section 4.2.5) using Dilog software written by Ross Henderson followed by further processing using a Microsoft Excel spreadsheet. The number of licks during successive minutes was calculated for each animal and then these minute-by-minute rates were averaged over all rats in each condition. In Experiment 9, an exponential decay function of the form $y = Ae^{(-Bt)}$ was fitted by a least squares method to the rate of licking data using the Sigma Plot graphics programme (Jandel Corp. Rafael, CA, 1986). In Experiment 10, the effect of midazolam on the rate of licking at each concentration of sucrose and Intra-lipid was analyzed using a one-way repeated-measures ANOVA.

Various microstructural variables were also examined: the total number of licks, the mean bout duration, the number of bouts, the intrabout lick rate (licks per second within bouts), and latency to engage in drinking (time from shutter opening to first lick).

In Experiment 9, the microstructural data were analyzed using a one-way repeated-measures analysis of variance (ANOVA). In Experiment 10, the microstructural data were analyzed using a two-way repeated-measures ANOVA, with drug dose and concentration of fluid as factors. Where there was no significant interaction between the two main factors, the main effect of drug collapsed across fluid concentration was considered. Post hoc comparisons to determine any significant differences between doses were made using a Dunnett's t-test. Statistical tests were performed using Super Anova and Statview SE+graphics (Abacus Concepts Inc., Berkeley, CA). A result was considered statistically significant if $p < 0.05$.

5.3 Results

Experiment: 9: Microstructural analysis of the effect of midazolam on drinking for a 3% sucrose solution and a 1% Intra-lipid emulsion over a 20-min session

Intake

Figure 5.1 illustrates the effect of midazolam on intake (ml) of both sucrose and Intra-lipid in the 20-min test. A one-factor repeated-measures ANOVA revealed a significant effect of the drug in the sucrose group ($F_{3,27} = 3.17$, $p < 0.05$). However, post hoc comparisons indicated that only the 3 mg/kg condition differed significantly from the vehicle condition ($p < 0.05$). For Intra-lipid drinking, midazolam did not significantly affect the volume consumed in the 20-min test ($F_{3, 27} = 0.4$, n.s.). The baseline intake of 12 ml was not altered by drug administration.

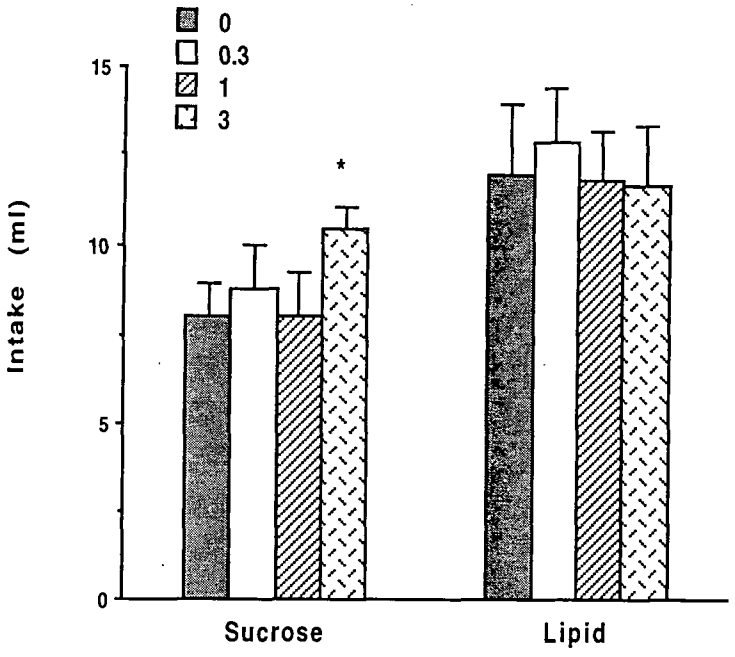


Figure 5.1 Intake of sucrose and Intra-lipid in a 20-min test as a function of increasing dose of midazolam (0.3-3 mg/kg) + S.E.M. Asterisk indicates significantly different from vehicle condition * $p < 0.05$ (Dunnett's t-test).

Rate of licking

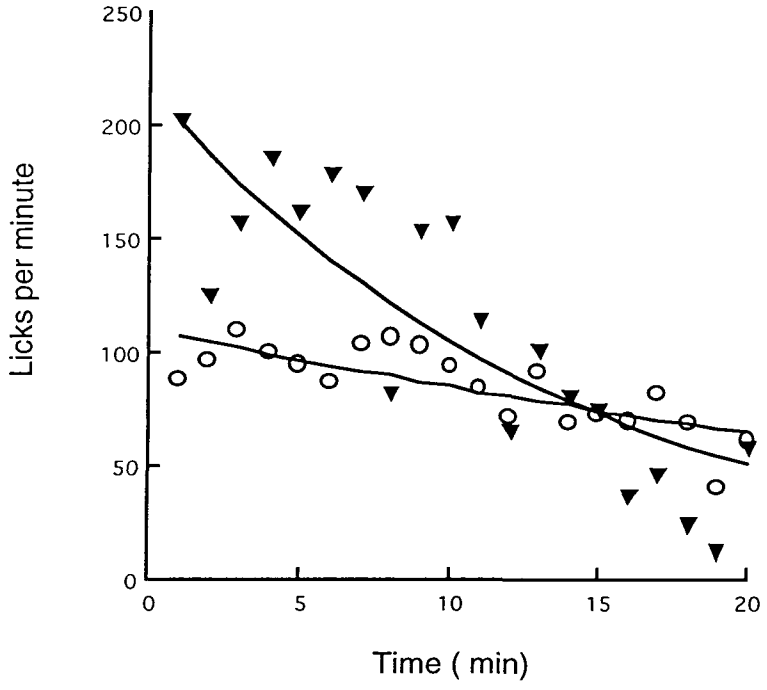
The effect of midazolam on the rate of licking for sucrose drinking at 1-min intervals is shown in Figure 5.2a. The rate of licking under the vehicle condition is compared with the rate of licking following administration of 3 mg/kg of midazolam. Figure 5.2b shows the rate of licking for Intra-lipid under the vehicle condition compared with the 3 mg/kg of midazolam condition. For both sucrose and Intra-lipid, an exponential decay function of the form $y = Ae^{(-Bt)}$ was fitted to the data. The parameters derived from the curve-fitting procedure are shown in Table 5.1. A Spearman correlation revealed that the function $y= Ae^{(-Bt)}$ provided a good description of the data. Midazolam affected both the initial rate of licking (A) and the rate constant (B). The 3 mg/kg dose of midazolam dramatically increased the initial rate of licking and increased the estimate of the slope function for both sucrose and Intra-lipid.

Table 5.1 Parameter estimates and standard errors and for the least squares fit to the rate of licking of the function $y = Ae^{(-Bt)}$.

	Sucrose		
Midazolam (mg/kg)	A	B	r
0			
Estimate	110.17	.0.02	0.97
S.E.	6.29	0.005	
3			
Estimate	216.2	0.07	0.89
S.E.	21.0	0.01	
	Intra-lipid		
Midazolam (mg/kg)	A	B	r
0			
Estimate	144.62	0.02	0.68
S.E.	21.1	0.008	
3			
Estimate	275.02	0.1	0.91
S.E.	15.7	0.009	

Parameters A and B describe the intercept and rate constant respectively. r is the correlation coefficient for the actual and predicted values calculated using a Spearman correlation coefficient.

a) Sucrose



b) Intra-lipid

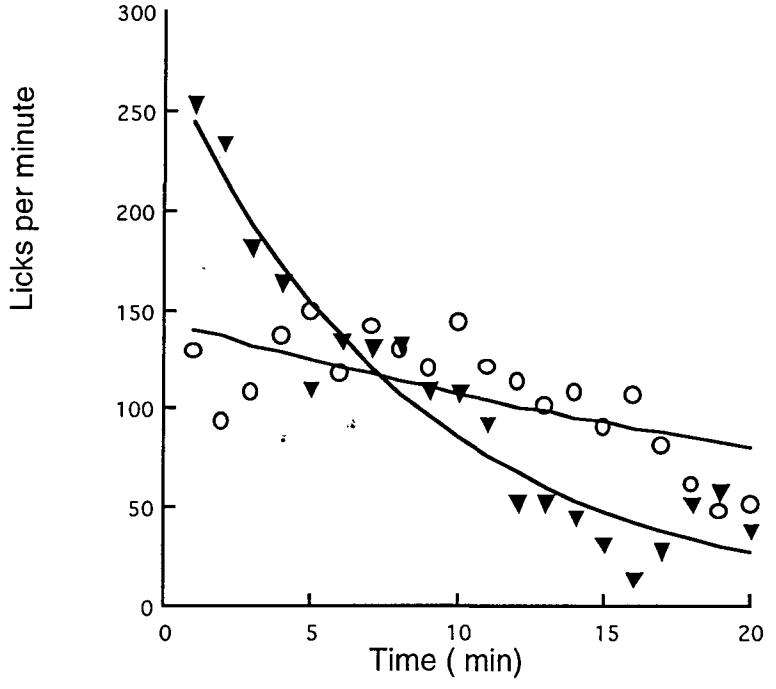


Figure 5.2 The effect of midazolam (3 mg/kg) on the rate of licking (licks per min) for a) sucrose and b) Intra-lipid. The lines are the least squares fit of the function $y = Ae^{(-Bt)}$ to the data. Open circles (○) indicate the vehicle condition, filled triangles (▲) indicate the 3 mg/kg midazolam condition.

Number of licks

The number of licks in the test session closely reflected the data for intake, so that for sucrose drinking, midazolam increased the overall number of licks during the test session ($F_{3,27} = 4.59$, $p < 0.01$). Post hoc comparisons showed that only the 3 mg/kg dose produced a significant increase in the number of licks ($p < 0.05$). In the case of Intra-lipid drinking, there was no effect of the drug on the total number of licks in the 20-min session ($F_{3,27} = 0.9$, n.s.) (Figure 5.3).

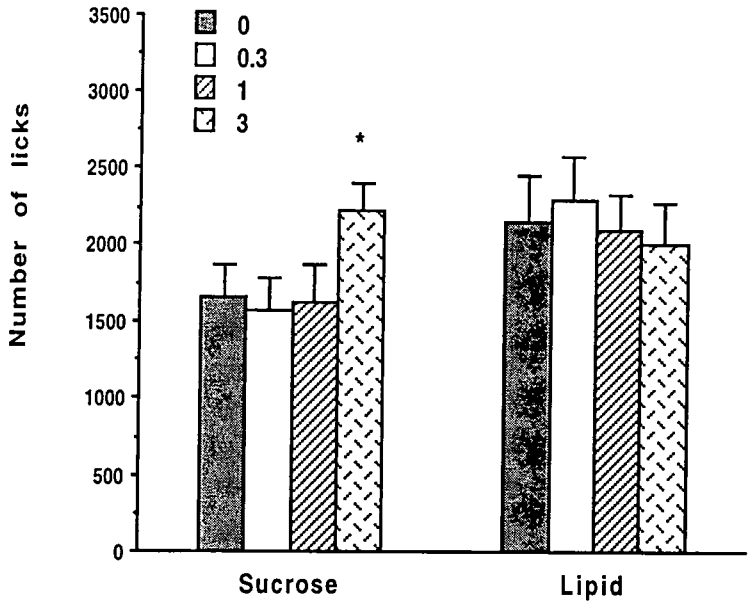


Figure 5.3 Total number of licks for sucrose and Intra-lipid drinking in a 20-min test as a function of increasing dose of midazolam (0.3-3 mg/kg) + S.E.M. Asterisk indicates significantly different from vehicle condition * $p < 0.05$ (Dunnett's t-test).

Microstructural analysis

Mean bout duration Figure 5.4 indicates that for sucrose drinking, an increase in the dose of midazolam led to a slight increase in the mean bout duration, but this did not reach statistical significance ($F_{3,27} = 1.26$, n.s.). There was no effect of drug treatment on the mean bout duration for Intra-lipid drinking ($F_{3,27} = 0.3$, n.s.) (Figure 5.4).

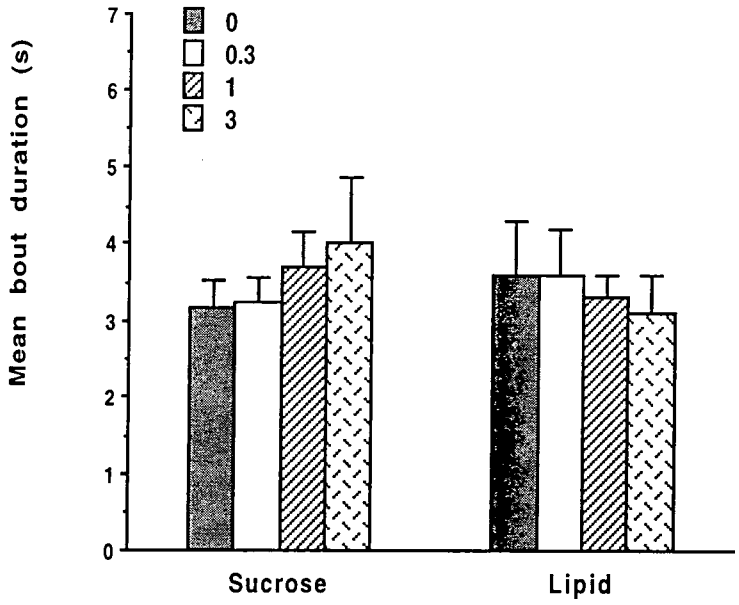


Figure 5.4 Mean bout duration for sucrose and Intra-lipid drinking in a 20-min test as a function of increasing dose of midazolam (0.3-3 mg/kg) + S.E.M. Asterisk indicates significantly different from vehicle condition * $p < 0.05$ (Dunnett's t-test).

Number of bouts Figure 5.5 shows that for sucrose drinking there was a significant effect of midazolam on the number of bouts elicited ($F_{3,27} = 3.07$, $p < 0.05$). Post hoc comparisons revealed that the 3 mg/kg dose of midazolam significantly increased bout number. No significant effects of midazolam on bout number were obtained for the Intra-lipid group, although there was a trend observed towards an increase in bout number with increasing dose of midazolam ($F_{3,27} = 1.2$, n.s.) (Figure 5.5).

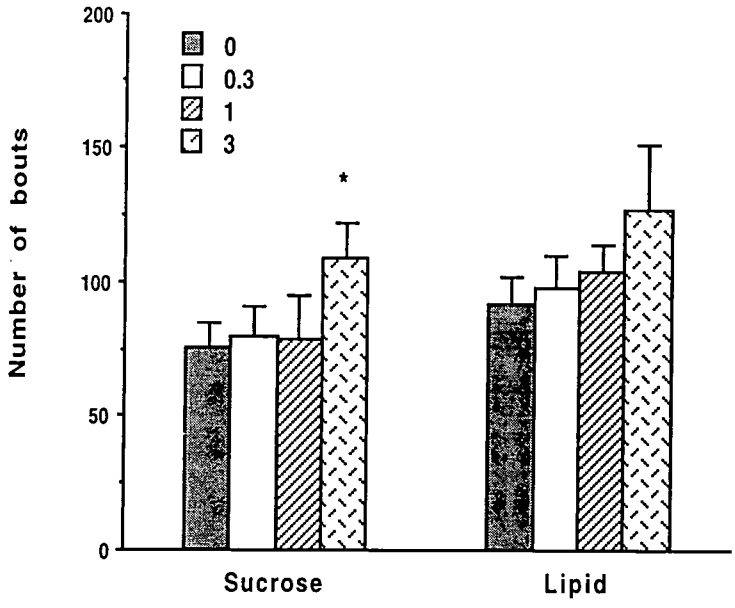


Figure 5.5 Number of bouts for sucrose and Intra-lipid drinking in a 20-min test as a function of increasing dose of midazolam (0.3-3 mg/kg) + S.E.M. Asterisk indicates significantly different from vehicle condition * $p < 0.05$ (Dunnett's t-test).

Intrabout lick rate The rate of licking within bouts (intrabout lick rate) for both the sucrose ($F_{3,27} = 9.98$, $p < 0.001$) and Intra-lipid groups ($F_{3,27} = 44.5$, $p < 0.001$) was significantly affected by administration of midazolam. Increasing the dose of midazolam led to a dose-dependent decrease in the intrabout lick rate. Post hoc comparisons showed that this effect was significant at 1 mg/kg and 3 mg/kg of midazolam for both sucrose and Intra-lipid drinking (Figure 5.6).

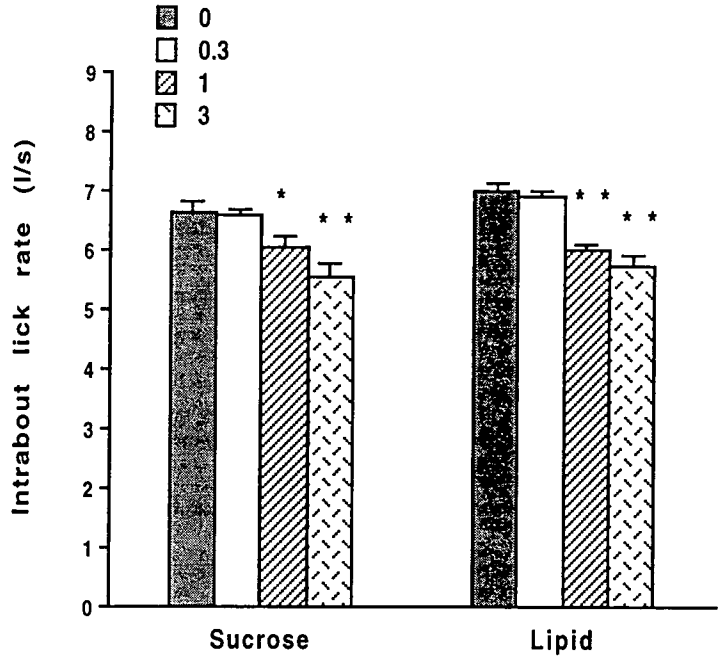


Figure 5.6 Intrabout lick rate for sucrose and Intra-lipid drinking in a 20-min test as a function of increasing dose of midazolam (0.3-3 mg/kg) + S.E.M. Asterisk indicates significantly different from vehicle condition * $p < 0.05$ ** $p < 0.01$ (Dunnett's t-test).

Latency Figure 5.7 shows the effect of midazolam on the latency to engage in drinking. Drug administration had no significant effect on this parameter for either sucrose ($F_{3,27} = 0.37$, n.s.), or Intra-lipid drinking ($F_{3,27} = 0.91$, n.s.). However, there was a tendency for latency to decrease with increasing dose of midazolam for both sucrose and Intra-lipid groups.

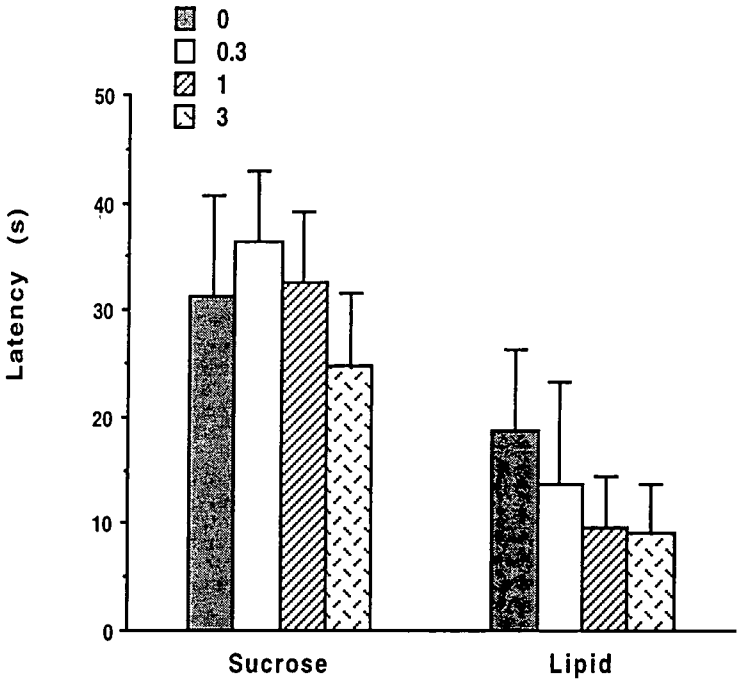


Figure 5.7 Latency for sucrose and Intra-lipid drinking in a 20-min test as a function of increasing dose of midazolam (0.3-3 mg/kg) + S.E.M.

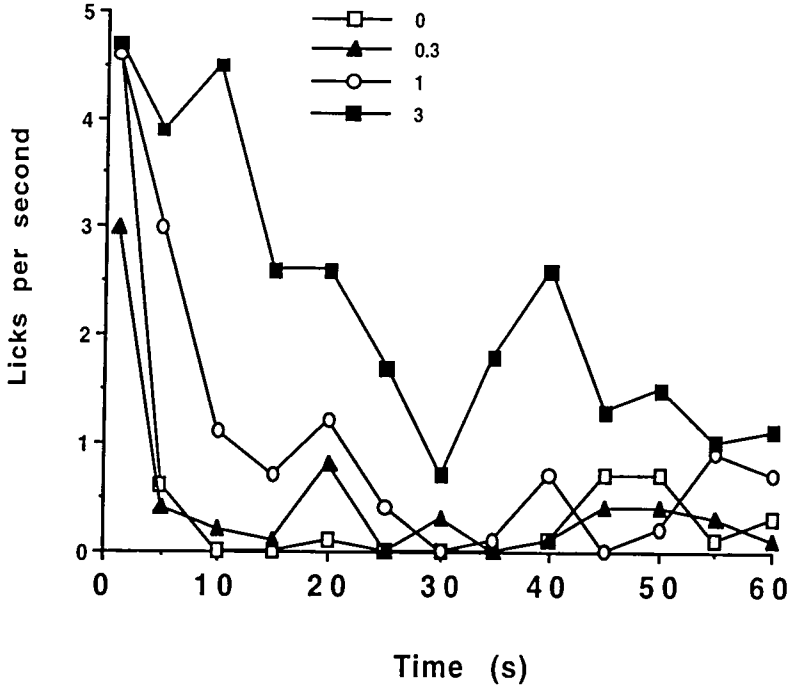
Experiment 10: Microstructural analysis of the effect of midazolam on drinking for sucrose and Intra-lipid solutions in a brief contact test

Rate of licking

Midazolam had a significant effect on the rate of licking at the 1% concentration of sucrose ($F_{3, 27} = 8.29$, $p < 0.001$). There were no significant effects of midazolam at either the 3% ($F_{3, 27} = 1.84$, n.s.), 10% ($F_{3, 27} = 1.85$, n.s.) or 30% concentrations of sucrose ($F_{3, 27} = 1.53$, n.s.). Figure 5.8a shows the effect of midazolam (0.3-3 mg/kg) on the rate of licking for the 1% sucrose concentration. Midazolam increased the rate of licking after the first few seconds. At the 3 mg/kg dose of midazolam, there was a higher rate of licking compared with the control vehicle condition which was maintained throughout the test session

The results for Intra-lipid drinking were similar for those obtained for sucrose drinking. There was a significant effect of midazolam on the rate of licking for Intra-lipid at the 1% concentration ($F_{2, 18} = 9.19$, $p < 0.001$). However, there was no effect of midazolam on the rate of licking at either the 3% ($F_{2, 18} = 1.05$, n.s.) or 10% concentrations of Intra-lipid ($F_{2, 18} = 0.16$, n.s.) The results for the 1% concentration of Intra-lipid are shown in Figure 5.8b. Midazolam stimulated an increase in the rate of licking for Intra-lipid after the first second of drinking, and led to a higher rate of licking compared with the vehicle condition for the rest of the test session. For both sucrose and Intra-lipid drinking there was a decline in the rate of licking across the test session which was evident for all conditions. This decline in the rate of licking was less marked following midazolam adminstration.

a) 1% sucrose



b) 1% Intra-lipid

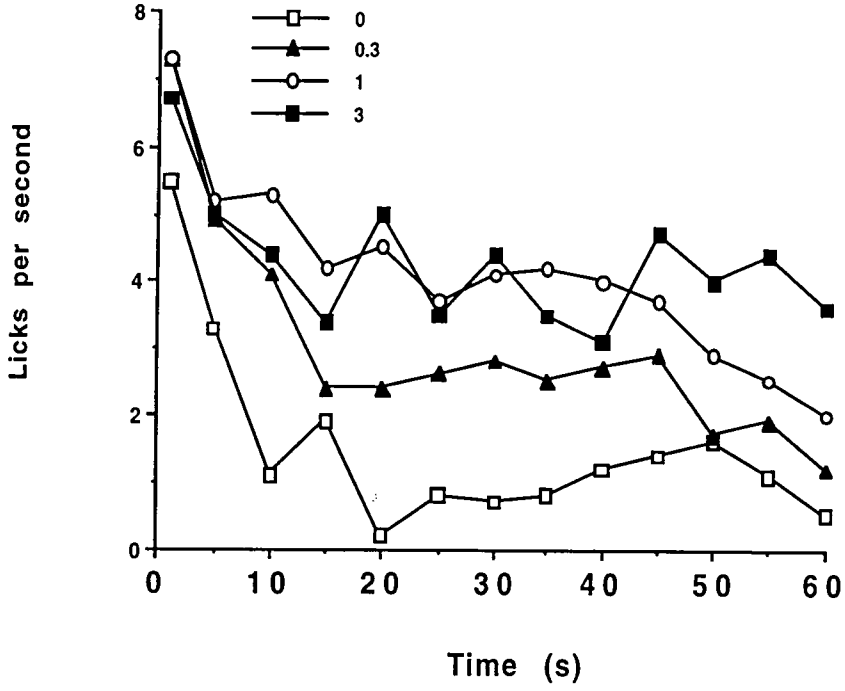


Figure 5.8 The average number of licks per second at 1s intervals as a function of increasing dose of midazolam (0.3-3 m/kg) for a) 3% sucrose and b) 1% Intra-lipid in a brief contact test. Each dose is plotted separately.

Number of licks

A two-way repeated-measures ANOVA revealed that for sucrose drinking there was a significant main effect for both drug dose ($F_{3,27} = 4.5$, $p < 0.01$) and fluid concentration ($F_{3,27} = 135.23$, $p < 0.001$) on the total number of licks. The main effect of drug on number of licks for sucrose is shown in Figure 5.9. Increasing the dose of midazolam led to a monotonic increase in the total number of licks. Table 5.2 shows that the total number of licks also increased monotonically as a function of increasing sucrose concentration. There was no significant drug-concentration interaction ($F_{9,81} = 1.7$ n.s.).

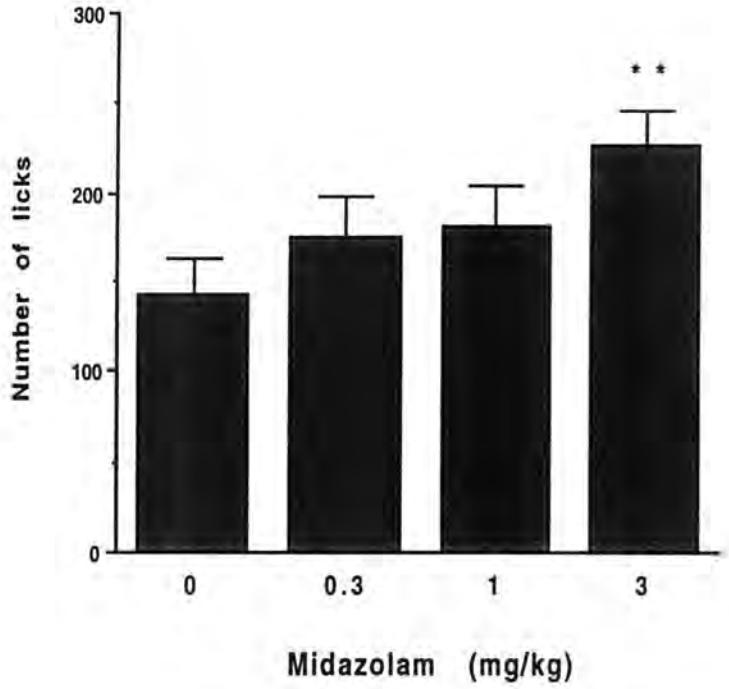


Figure 5.9 Total number of licks for sucrose drinking in a brief contact test as a function of increasing dose of midazolam (0.3-3 mg/kg) + S.E.M. Asterisk indicates significantly different from vehicle condition ** $p < 0.01$ (Dunnett's t-test).

Midazolam significantly increased the number of licks for Intra-lipid drinking ($F_{3,27} = 4.35$, $p < 0.01$). There was also a main effect of concentration for Intra-lipid drinking ($F_{2,18} = 38.8$, $p < 0.001$) and a drug-concentration interaction ($F_{6,54} = 3.5$, $p < 0.01$). Figure 5.10 shows that this interaction probably arose because the effect of midazolam was more pronounced at 1% Intra-lipid. Midazolam did not increase the number of licks at 10% Intra-lipid perhaps due to ceiling effects. The effect of Intra-lipid concentration on the number of licks is shown in Table 5.2. Increasing Intra-lipid concentration led to an increase in the number of licks.

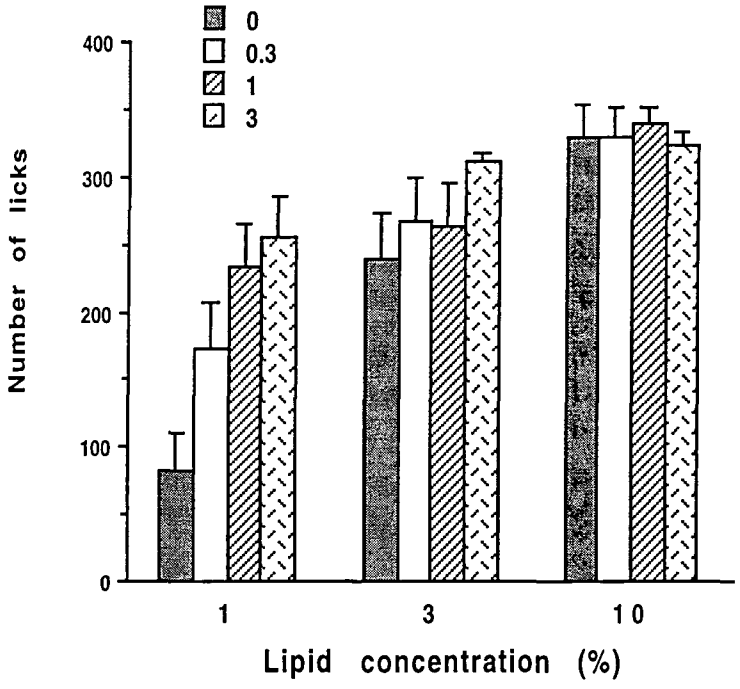


Figure 5.10 Total number of licks for Intra-lipid drinking in a brief contact test as a function of increasing dose of midazolam (0.3-3 mg/kg) + S.E.M.

Table 5.2 Main effect of fluid concentration on total number of licks

Total licks ± S.E.M.

Fluid	1%	3%	10%	30%
Sucrose	57.8± 12.7	100.6± 15.7	243.2± 18.5	320.7± 8.0
Intra-lipid	186.2± 18.4	270.5± 14.4	331.3± 8.8	

n = 10 animals per group

Microstructural analysis

Mean bout duration An increase in mean bout duration was observed as a function of increasing dose of midazolam ($F_{3,27} = 5.6$, $p < 0.01$) (Figure 5.11). Mean bout duration was also an increasing function of sucrose concentration ($F_{3,27} = 10.32$, $p < 0.001$) (Table 5.3). There was no main effect of drug on mean bout duration for Intra-lipid drinking, but Figure 5.11 shows that there was a trend towards an increase in mean bout duration with increasing dose of midazolam ($F_{3,27} = 1.9$, n.s.). Mean bout duration increased with increasing concentration of Intra-lipid ($F_{2,18} = 13.16$, $p < 0.001$) (Table 5.3). There were no significant drug-concentration interactions for either sucrose ($F_{9,81} = 0.6$, n.s.) or Intra-lipid drinking ($F_{6,54} = 0.9$, n.s.).

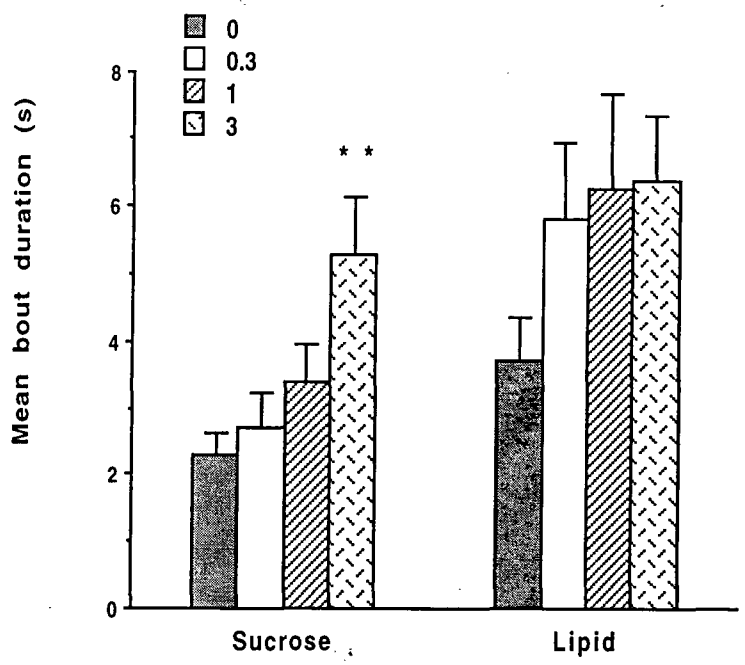


Figure 5.11 Mean bout duration for sucrose and Intra-lipid drinking in a brief contact test as a function of increasing dose of midazolam (0.3-3 mg/kg) + S.E.M. Asterisk indicates significantly different from vehicle condition ** $p < 0.01$ (Dunnett's t-test).

Table 5.3 Main effect of fluid concentration on mean bout duration

	Mean bout duration (s) ± S.E.M.			
Fluid	1%	3%	10%	30%
Sucrose	1.5± 0.3	2.2± 0.3	4.2± 0.5	5.9± 0.8
Intra-lipid	2.9± 0.3	5.4± 0.8	8.3± 1.2	

n = 10 animals per group

Number of bouts For sucrose drinking, a two-way repeated-measures ANOVA did not reveal a main effect of drug ($F_{3,27} = 0.28$, n.s.). The lack of effect of midazolam on the number of bouts for sucrose drinking is shown in Figure 5.12. However, there was a main effect of sucrose concentration on the number of bouts ($F_{3,27} = 27$, $p < 0.001$) (Table 5.4), but no interaction with drug dose ($F_{9,81} = 1.4$, n.s.). An increase in sucrose concentration led to an increase in the number of bouts.

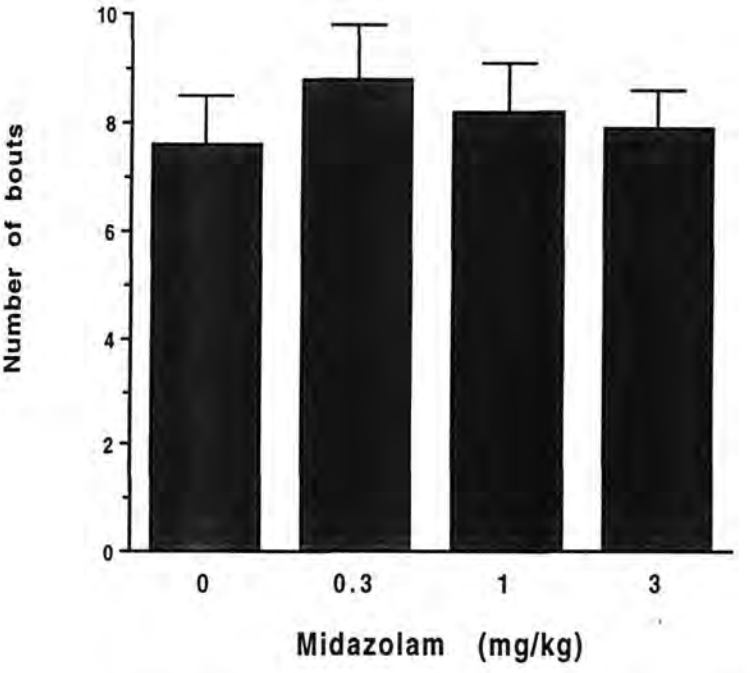


Figure 5.12 Number of bouts for sucrose drinking in a brief contact test as a function of increasing dose of midazolam (0.3-3 mg/kg) + S.E.M.

In the case of Intra-lipid drinking, there was no main effect of either drug ($F_{3,27} = 1.4$, n.s.) or concentration ($F_{2,18} = 0.3$, n.s.) on bout number. However, there was a significant interaction between these two factors ($F_{6,54} = 2.63$, $p < 0.05$). Figure 5.13 shows that this interaction arose because midazolam had differential effects at different concentrations of Intra-lipid. At the 1% concentration of Intra-lipid, increasing the dose of midazolam increased the number of bouts. At the 3% and 10% concentration conditions midazolam did not have any effect on bout number.

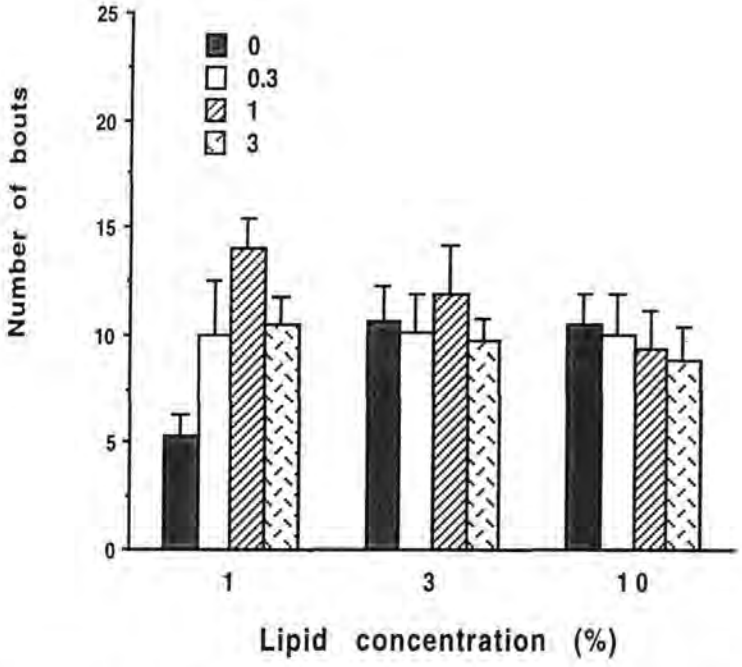


Figure 5.13 Number of bouts at different concentrations of Intra-lipid in a brief contact test as a function of increasing dose of midazolam (0.3-3 mg/kg)+ S.E.M.

Table 5.4 Main effect of fluid concentration on number of bouts

Number of bouts ± S.E.M.

Fluid	1%	3%	10%	30%
Sucrose	4.0± 0.4	6.3± 0.5	10.2± 1.0	12.2± 0.9
Intra-lipid	9.9± 0.9	10.6± 0.8	9.6± 0.8	

n = 10 animals per group

Intrabout lick rate There were significant main effects of drug dose on the intrabout lick rate for both sucrose ($F_{3,27} = 6.58$, $p < 0.001$) and Intra-lipid ($F_{3,27} = 24.4$, $p < 0.001$) drinking, but no significant interactions: sucrose ($F_{9,81} = 1.2$, n.s.); Intra-lipid ($F_{6,54} = 0.9$, n.s.). As shown in Figure 5.14, midazolam dose-dependently decreased the intrabout lick rate for both sucrose and Intra-lipid drinking. For sucrose drinking there was no main effect of concentration on intrabout lick rate ($F_{3,27} = 0.6$, n.s.). For Intra-lipid drinking, increasing the concentration led to a decrease in the intrabout lick rate ($F_{2,18} = 6.2$, $p < 0.01$) (Table 5.5.).

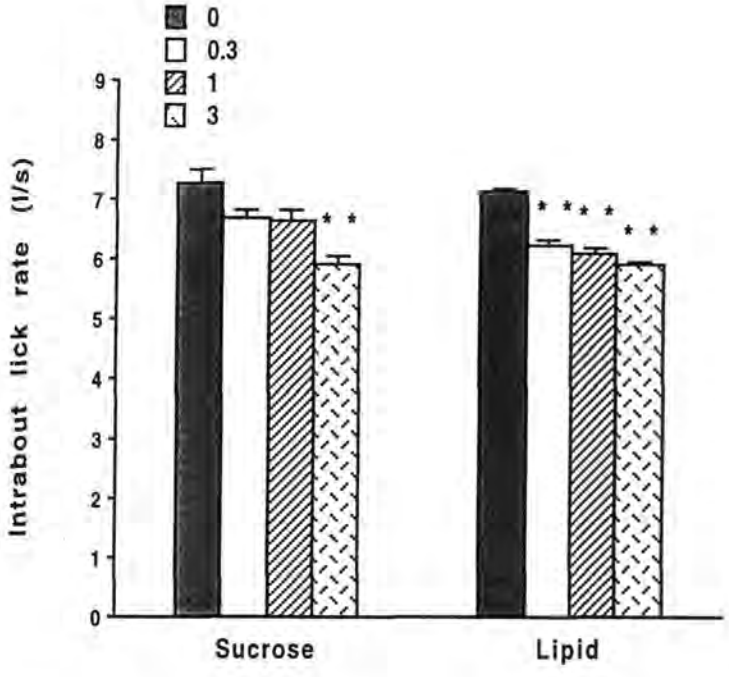


Figure 5.14 Intrabout lick rate for sucrose and Intra-lipid drinking in a brief contact test as a function of increasing dose of midazolam (0.3-3 mg/kg) + S.E.M. Asterisk indicates significantly different from vehicle condition ** $p < 0.01$ (Dunnett's t-test).

Table 5.5 Main effect of fluid concentration on intrabout lick rate

	Intrabout lick rate (l/s) ± S.E.M.			
Fluid	1%	3%	10%	30%
Sucrose	6.4± 0.3	6.8± 0.2	6.7± 0.1	6.6± 0.1
Intra-lipid	6.5± 0.1	6.3± 0.1	6.3± 0.1	

n = 10 animals per group

Latency No significant effects of drug administration on latency were observed for either sucrose ($F_{3,27} = 0.14$, n.s.) or Intra-lipid ($F_{3,27} = 0.28$, n.s.) drinking (Figure 5.15). The effect on the latency to engage in drinking of increasing the concentration of sucrose and Intra-lipid is shown in Table 5.6. There were no main effects of concentration for either sucrose ($F_{3,27} = 1.4$, n.s.) or Intra-lipid ($F_{2,18} = 0.02$, n.s.) or significant interactions.

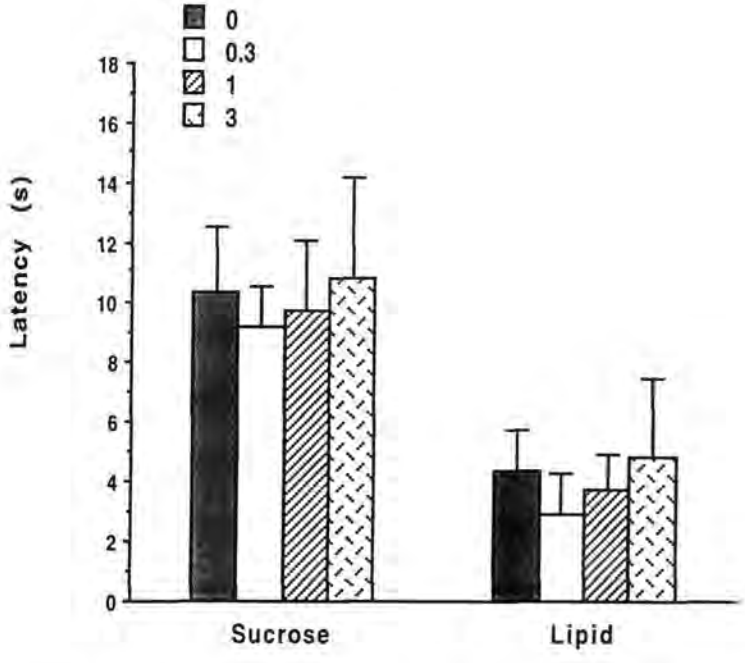


Figure 5.15 Latency for sucrose and Intra-lipid drinking in a brief contact test as a function of increasing dose of midazolam (0.3-3 mg/kg) + S.E.M. Asterisk indicates significantly different from vehicle condition ** $p < 0.01$ (Dunnett's t-test).

Table 5.6 Main effect of fluid concentration on latency

	Latency (s) ± S.E.M.			
Fluid	1%	3%	10%	30%
Sucrose	12.2± 2.7	10.0± 1.9	6.1± 1.0	11.8± 3.2
Intra-lipid	3.8± 1.1	4.2±1.4	3.8±2.0	

n = 10 animals per group

5.4 Discussion

In this chapter a microstructural approach was employed to investigate the behavioural mechanisms responsible for the effects of the benzodiazepine agonist midazolam on ingestive behaviour. The effects of midazolam on the rate of licking and bout structure were compared with the effects of manipulating the concentration of sucrose and Intra-lipid. In a 20-min test session, (Experiment 9) midazolam affected the rate of licking in a manner similar to that observed when increasing concentration. In a brief contact test, (Experiment 10) midazolam increased mean bout duration, an effect which was also observed when increasing concentration. A second aim was to compare the effect of midazolam on the consumption of sucrose versus Intra-lipid. Despite differences in overall intake, the effects of this drug on the rate and microstructure of licking for Intra-lipid was quite similar to the effects observed for sucrose drinking.

In Experiment 9, midazolam significantly increased the consumption of a 3% sucrose solution in a 20-min test. This result is consistent with previous findings which have demonstrated a hyperphagic effect of midazolam (Cooper et al., 1985) However, in Experiment 9, there was no significant effect of midazolam on intake of 1% Intra-lipid. This is the first report of the effect of a benzodiazepine agonist on fat consumption. The lack of effect of midazolam on Intra-lipid consumption cannot be explained by the fact that the rats were consuming at ceiling levels because the intake of Intra-lipid was approximately 15 ml, and rats have been shown to drink up to 25 ml of fluid in a 20-min test (Cooper and Green, 1993). From this, it might be concluded that midazolam-induced hyperphagia is limited to consumption of carbohydrate solutions. However, rate analysis showed that although midazolam did not increase intake of Intra-lipid, it did alter the pattern of ingestive responding for this fat in the early part of the test session, and this effect was similar to that observed for sucrose drinking. Midazolam increased the initial rate of licking for both sucrose and Intra-lipid drinking. There is evidence to suggest that the initial rate of licking is an appropriate measure of palatability. Davis and colleagues have shown that the initial lick rate increases as a function of increasing carbohydrate concentration (Davis and Levine, 1977; Davis and Smith, 1988). In Chapter 4, it was

shown that this measure also reflects changes in the palatability of Intra-lipid emulsions. The increase in the initial lick rate caused by midazolam suggests that it might be enhancing the palatability of both sucrose and Intra-lipid. It is possible that the effects of midazolam on palatability are responsible for the well-known hyperphagic effects of this drug. However, the data also suggest that changes in palatability may not always lead to an increase in intake, because despite similar effects of midazolam on the initial lick rate for sucrose and Intra-lipid, a hyperphagia was only observed for sucrose drinking. Possible reasons for this discrepancy will be discussed below. The results from Experiment 9 demonstrate the advantage of using rate analysis. The effect of midazolam on the pattern of ingestion for Intra-lipid would have been overlooked if intake had been the only measure.

Midazolam also altered the rate of decline of licking across the test session. Increasing the dose of midazolam led to an increase in the rate constant for both sucrose and Intra-lipid drinking. This parameter probably reflects the emergence of an inhibitory negative feedback signal due to the accumulation of ingested material in the GI tract and consequent post-absorptive processes (Davis and Levine, 1977). For example, it has been shown that the more concentrated a sugar is, the steeper the slope of the rate function, due to more rapid accumulation in the stomach (Davis and Smith, 1988). The increase in the rate constant following midazolam adminstration may indicate that this drug affects the postingestive consequences of licking. However, the increase in the rate of decay of licking may also have occurred as a consequence of the increase in the initial rate of licking. A large increase in the initial rate would lead to faster accumulation of the fluid in the GI tract and so result in a greater rate of decline in licking. This may provide an explanation for the lack of effect of midazolam on Intra-lipid Intra-lipid intake, because the increase in the initial rate of licking may have lead to a very steep decline in the rate of licking thus opposing the initial stimulatory effect. This is supported by the fact that the slope estimate for Intra-lipid drinking was greater than that for sucrose drinking.

From the microstructural analysis over the 20-min session it is not clear how the increase in lick rate caused by midazolam was achieved. For sucrose drinking, no

significant effect on mean bout duration was observed although there was a small trend towards an increase in mean bout duration with increasing dose of midazolam. Midazolam did significantly increase the number of bouts of sucrose drinking in the 20-min session. For Intra-lipid drinking, there were no significant effects of midazolam on either mean bout duration or number of bouts. In the previous chapter it was shown that manipulating the concentration of sucrose and Intra-lipid did not have reliable effects on mean bout duration over a 20-min test. However, marked effects of concentration on this parameter were revealed when the test session was limited to 60s. A specific effect of midazolam on bout duration may have been limited to the early part of the test session. This possibility is discussed later with respect to the results of Experiment 10.

In Experiment 9 midazolam had a dramatic effect on the intrabout lick rate for both sucrose and Intra-lipid in the 20-min session. There was a dose related decrease in the intrabout lick rate following midazolam administration. It has been shown previously that the benzodiazepine receptor agonist CDP also decreased the rate of eating for food pellets and powder (Cooper and Francis, 1979b). These results are consistent with the motor effects of benzodiazepine agonists when administered peripherally. Midazolam has muscle relaxant effects which probably interfered with the execution of the lick cycle and thus led to a reduction in the rate of licking within bouts. This possibility could be tested by using an electromyograph to measure muscle movements under midazolam treatment. The decrease in intrabout lick rate caused by midazolam may seem counter-intuitive because this drug also increases the initial rate of licking when integrated over time. One explanation is that midazolam may have dual effects on ingestive behaviour. The effect of midazolam on the initial rate of licking may be related to changes in palatability, whereas the effect on the intrabout lick rate may be due to the muscle relaxant effects of the drug. It is possible that the mechanisms involved in these two effects may be different and further work is required to examine their neurochemical and anatomical bases. However, caution must be exercised in interpreting the effects of midazolam on intrabout lick rate because in the previous chapter it was shown that this parameter may also be affected by manipulating the concentration of the test solution.

There was no effect of midazolam on the latency to engage in drinking. This is the time from the shutter opening to the time that the animal makes the first lick. Latency could provide a measure of malaise or sedation, because if the rat was prevented from approaching the lick spout, this would be reflected in a longer latency. The lack of effect of midazolam on latency suggests that any sedation induced by the drug did not impair locomotor behaviour to the extent that it made it difficult for the animal to approach the lick spout. However, it has also been suggested that latency may also reflect the motivation to feed. Increased motivation to feed might result in a shorter latency. Therefore, the results of experiment 9 may indicate that midazolam was not altering motivation to engage in licking. This result contrasts with previous reports of the effect of benzodiazepine receptor agonists on latency to feed. Cooper and Francis (1979b) found that CDP reduced latency to consume food pellets and powder suggesting that it may increase the motivation to feed. This discrepancy may have been due to the difference between the food types used. The conclusion to be drawn from this is that many different factors may influence the latency to feed, and so interpreting changes in this measure may be problematic.

The results of Experiment 9 showed that the effects of midazolam were evident in the initial period of ingestion, and so Experiment 10 was designed to examine the microstructure of licking in the first 60s of drinking for both sucrose and Intra-lipid. An increase in the rate of licking for both sucrose and Intra-lipid was evident after the first few seconds of licking for these nutrients. For 1% sucrose and 1% Intra-lipid, the rate of licking under the vehicle condition declined over the 60s. The decline in the rate of licking was attenuated by treatment with midazolam, and this led to an increase in the total number of licks during the test session. The effect of midazolam on the rate of licking in 60s was similar to that obtained when manipulating the concentration of sucrose and Intra-lipid in Experiment 8. This suggests that midazolam may have an effect on the immediate hedonic response to sucrose and Intra-lipid. This evidence is consistent with taste reactivity studies which have shown that midazolam has a rapid effect on the ingestive responding to fluids infused directly into the mouth, and provides further

evidence that benzodiazepines increase ingestion by enhancing palatability rather than affecting satiety processes.

Midazolam increased the number of licks for both sucrose and Intra-lipid in the brief contact test, although for Intra-lipid, no increase in number of licks was observed at the 10% concentration due to ceiling effects. The increase in the total number of licks caused by midazolam was due to an effect on mean bout duration rather than the number of bouts. Midazolam caused a monotonic increase in the size of bouts for sucrose and Intra-lipid drinking, but did not systematically affect their number. Midazolam did increase bout number for Intra-lipid drinking, but this effect was only evident at the lowest concentration and there was no main effect of drug administration on bout number. Midazolam has been shown previously to increase the mean bout duration (Cooper and Yerbury, 1986b) in rats consuming solid food. Increasing the concentration of sucrose and Intra-lipid in Experiment 8 also increased bout duration. This suggests that midazolam might be affecting ingestive behaviour by increasing palatability.

However, the effect of midazolam in Experiment 10 was not the same as the effect of manipulating concentration. Midazolam only increased mean bout duration, whereas increasing concentration affected both mean bout duration and bout number. This was not so in Experiment 8 when increasing concentration increased mean bout duration but did not affect bout number. The discrepancy between the effects of manipulating concentration in Experiment 10 (increase in mean bout duration and bout number) and the results obtained in Experiment 8 (selective increase in mean bout duration) may have been due to differences in the protocol. In Experiment 10, the rats were trained to consume all concentrations of sucrose and Intra-lipid over a period of approximately 10 days, whereas in Experiment 8 rats were trained on one concentration and then had access to the other concentrations for 2 days only. The effect of manipulating concentration on bout number in Experiment 10 may have been due to the experience the rats had with the test fluids. Repeated exposure to the test fluids may have affected an underlying process separate from palatability, which was then reflected in changes in bout number. It is possible that changes in bout number may be indicative of

changes in incentive salience. An increase in bout frequency means that an animal is returning to a stimulus more often and so may be indicative of an increase in the salience of that stimulus. Testing of this hypothesis requires investigation of the effects of manipulations which are thought to affect salience attribution on licking microstructure. The possibility that bout frequency may reflect changes in incentive salience is discussed further in Chapters 7 and 8 and in the General Discussion.

The intrabout lick rate was reduced following administration of midazolam in the 60s test. This is consistent with the effect of midazolam observed in Experiment 9. However, manipulating the concentration of Intra-lipid also decreased the intra-bout lick rate. This was not so for sucrose. In the previous chapter it was shown that manipulating fluid concentration can affect the rate of licking within bouts for both sucrose and Intra-lipid. Taken together, this evidence suggests that the rate of licking within bouts may be affected by properties of the ingested solution such as viscosity and so may not provide a pure measure of motoric deficits.

In Experiment 10 the latency to engage in drinking was not affected by midazolam. This is consistent with the lack of effect of midazolam on latency observed in Experiment 9.

In summary, midazolam increased the initial rate of licking for both sucrose and Intra-lipid in a 20-min test, and increased mean bout duration over a range of concentrations in a brief contact test. The effect of midazolam on sucrose and Intra-lipid consumption was similar to the effects obtained when manipulating concentration. This is consistent with the possibility that benzodiazepine agonists enhance palatability. The data also demonstrate that the increase in palatability caused by midazolam is not limited to prototypic taste stimuli but also applies to Intra-lipid drinking.

The effects of the imidazobenzodiazepine Ro 15-4513 on the microstructure of licking for either sucrose or Intra-lipid

6.1 Introduction

In addition to the category of benzodiazepine receptor agonists, there is another class of drugs which also bind with high affinity to benzodiazepine receptors, but produce effects that are opposite to those associated with classical agonists (Braestrup et al., 1982). Members of this latter category are referred to as inverse agonists. Instead of inducing hyperphagia, inverse agonists exhibit anorectic properties. Dose-related reductions in food intake in rats following administration of a number of benzodiazepine receptor inverse agonists have been shown in several studies (Cooper, et al., 1985; Cooper et al., 1989). A consequence of these opposing actions is that control over eating behaviour can be exerted bidirectionally through drug actions at benzodiazepine receptors. Agonists enhance food consumption, whereas inverse agonists reduce intake. In both cases, the effects of these two types of drugs can be blocked by selective benzodiazepine receptor antagonists (Cooper, 1986b; Cooper and Moores, 1985b).

There has been a considerable amount of research into the behavioural and pharmacological characteristics of benzodiazepine-induced hyperphagia. Evidence suggests that benzodiazepine agonists may increase food intake by enhancing palatability (Berridge & Pecina, 1995). This hypothesis is supported by the results reported in Chapter 5 of this thesis. Investigation of the change in the rate of licking for sucrose and Intra-lipid solutions over a 20-min test (Experiment 9) showed that midazolam may increase food intake by increasing the initial rate of licking. Microstructural analysis in a brief contact test then showed that this initial stimulation of licking is due to an increase mean bout duration as opposed an increase in bout number (Experiment 10). The effects of midazolam were found to be similar to the effects obtained when manipulating concentration. This is consistent with the possibility that this drug alters palatability.

In contrast, less is known about the behavioural characteristics of the anorectic effects of inverse agonists. The aim of Experiment 11 was to provide information concerning the behavioural mechanisms responsible for the anorectic action of benzodiazepine receptor inverse agonists. The effect of the inverse agonist Ro 15-4513 on licking for sucrose and Intra-lipid in a brief contact test was examined and compared with the effects of manipulating concentration, and with the effects of midazolam. A brief contact test was used because it had been shown previously that this procedure allows rapid evaluation of the effects of drug manipulations on the licking responses to ingested fluids. It was predicted that the effects of Ro 15-4513 on the rate of licking and bout structure would be the inverse of the effects of increasing concentration.

Experiment 11: Microstructural analysis of the effect of Ro 15-4513 on drinking for sucrose and Intra-lipid in a brief contact test

6.2 Method

6.2.1 Animals

Twenty naive non-deprived adult male hooded Lister rats (Charles River, U.K.) weighing 300-350 g at the beginning of training were used. They were housed in pairs in plastic cages in a room with a constant room temperature of 21±2 °C, and were maintained under a 12h light:dark cycle (lights on at 08.00). Rats were allowed ad lib access to food pellets, (SDS RMI (E), Cambridge, U.K.) and water, except during testing. All testing was carried out in the light phase between 09.00 and 13.00h.

6.2.2 Drugs

The benzodiazepine partial inverse agonist Ro 15-4513 (ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5a][1,4]benzodiazepine-carboxylate) (Roche, Basel, Switzerland) was ultrasonically dispersed in distilled water to which Tween 80 (BDH Chemicals Ltd. Poole, England) had been added. It was injected i.p. in a volume of 1 ml/kg, 20-min prior to experimentation. The doses used, were 0.3, 1 and 3 mg/kg of

Ro 15-4513, or its vehicle. These doses were chosen because it had been previously shown that 1-10 mg/kg of Ro 15-4513 is sufficient to induce a significant reduction in food intake (Cooper et al., 1989).

6.2.3 Test meal

Rats had access to various concentrations of sucrose solutions (granulated cane sugar) or Intra-lipid emulsions (Pharmacia Ltd, Milton Keynes, U.K.) which were made up freshly each day. The Intra-lipid emulsions were made up by diluting a 20% commercial preparation with tap water and the sucrose solution was made up to volume each day using tap water.

6.2.4 Apparatus

Testing was carried out using the MS80 multistation lickometer described in detail in the Chapter 4 (Section 4.2.3).

6.2.5 Procedure

Training Twenty rats were divided into two groups (n = 10 per group). Each group was well familiarized with the test apparatus and procedure. This involved placing each rat in the test chamber where they had access either to sucrose (1, 3-10 and 30%) or to Intra-lipid (1, 3 and 10%) solutions in a random order. Each concentration was presented for 60s, and a 10s interval intervened between subsequent presentations. This procedure was followed until steady baseline levels of licking were observed across days (approximately 10 days). Two days prior to testing each animal received a sham injection of distilled water to familiarize it with the injection procedure.

Testing Following the training period rats received i.p. injections of Ro 15-4513 (0.3, 1 and 3 mg/kg) or vehicle. 20-min after injection of Ro 15-4513 the rats were placed in the lickometer chamber where they had access to all concentrations of either sucrose (1, 3, 10 and 30%) or Intra-lipid (1, 3 and 10%). Each concentration was presented for a total duration of 60s. The order of presentation was randomised. A repeated-measures design was used in which each animal was tested at each dose. Injections were counterbalanced and 48h elapsed between treatments to avoid carry-over effects.

6.2.6 Data analysis

The lick time data were analyzed as described in the Chapter 4 (section 4.2.5) using Dilog software written by Ross Henderson followed by further processing using a Microsoft Excel spreadsheet. The effect of Ro 15-4513 on the rate of licking at each concentration of sucrose and Intra-lipid was analyzed using a one-way repeated-measures ANOVA.

Various microstructural variables were also examined: the total number of licks, mean bout duration, number of bouts, intrabout lick rate (licks per second within bouts) and latency to engage in drinking (time from shutter opening to first lick). The microstructural data were analyzed using a two-way repeated-measures ANOVA, with drug dose and concentration of fluid as factors. Where there was no significant interaction between the two main factors, the main effect of drug treatment collapsed across fluid concentration was considered. Post hoc comparisons to determine any significant differences between doses were made using a Dunnett's t-test. Statistical tests were performed using Super Anova and Statview SE+graphics (Abacus Concepts Inc., Berkeley, CA). A result was considered statistically significant if $p < 0.05$.

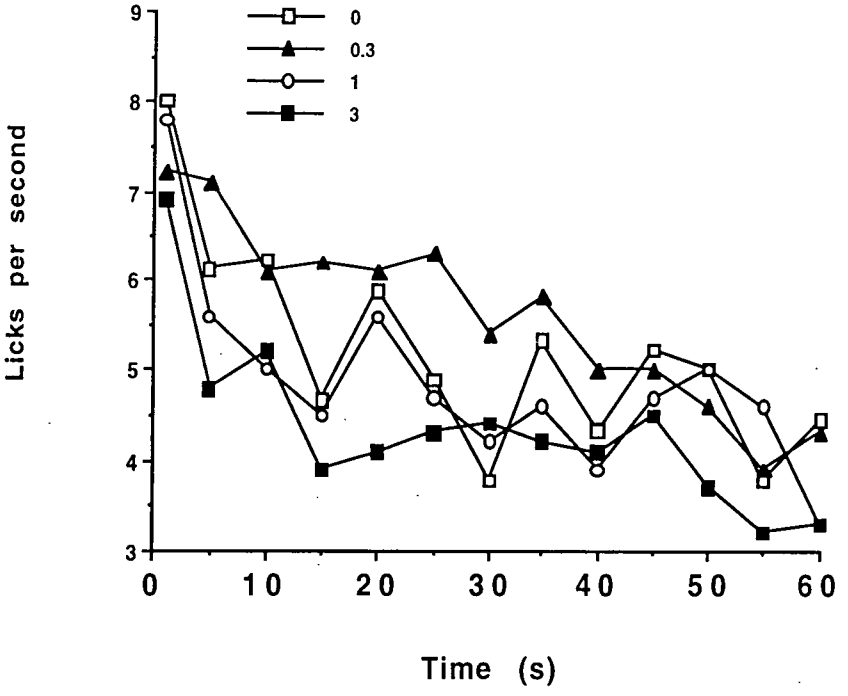
6.3 Results

Rate of licking

Ro 15-4513 had a significant effect on the rate of licking for the 1% ($F_{3,27} = 3.61$, $p < 0.05$) and 30% sucrose concentrations ($F_{3,27} = 7.77$, $p < 0.001$). There was no effect of Ro 15-4513 on the rate of licking at the 3% ($F_{3,27} = 1.4$, n.s.) or the 10% concentrations of sucrose ($F_{3,27} = 1.83$, n.s.). Fig 6.1a shows the effect of Ro 15-4513 (0.3-3 mg/kg) on the rate of licking for the 30% concentration of sucrose. Ro 15-4513 decreased the rate of licking after the first second. Ro 15-4513 caused a steeper rate of decline of licking compared with the vehicle control condition.

The results for Intra-lipid drinking were similar to those obtained for sucrose drinking. There was a significant effect of Ro 15-4513 on the rate of licking for the 3% ($F_{2,18} = 3.38$, $p < 0.05$) and 10% concentrations of Intra-lipid ($F_{2,18} = 4.71$, $p < 0.01$). There was no effect of Ro 15-4513 on the rate of licking for the 1% Intra-lipid concentration ($F_{2,18} = 1.8$, n.s.). The results for the 10% concentration of Intra-lipid drinking for comparison with sucrose are shown in Figure 6.1b. The graph shows that Ro 15-4513 caused a significant decrease in the rate of licking after the first second of drinking and led to a steeper decline in the rate of licking over the 60s.

a) 30% Sucrose



b) 10% Intra-lipid

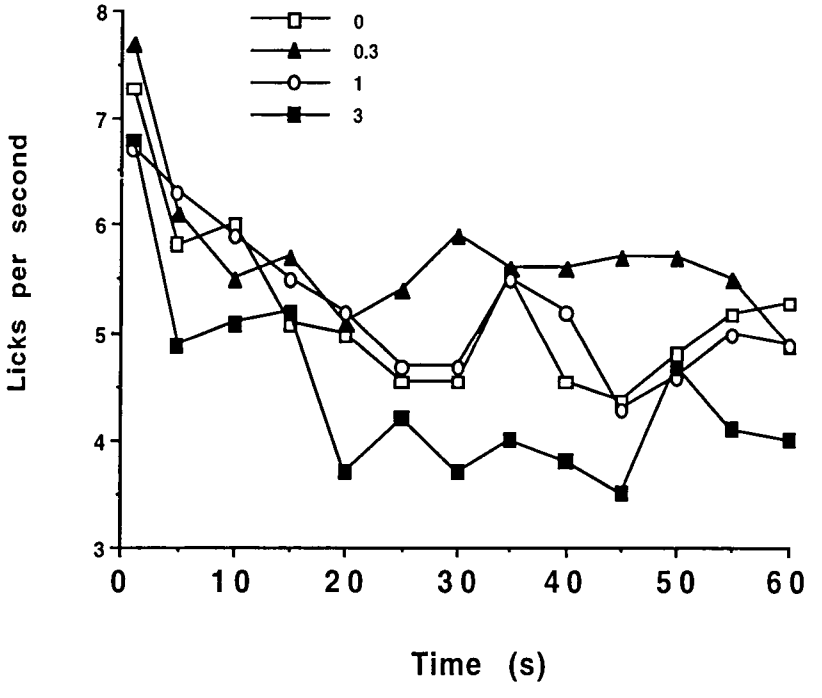


Figure 6.1 The average number of licks per second at 1 s intervals as a function of increasing dose of Ro 15-4513 (0.3-3 mg/kg) for a) 30% sucrose and b) 10% Intra-lipid in a brief contact test. Each dose is plotted separately.

Number of licks

A two-way repeated-measures ANOVA revealed that for sucrose drinking there was a significant main effect of both drug ($F_{3,27} = 4.23$, $p < 0.01$) and concentration ($F_{3,27} = 138.58$, $p < 0.001$) on the total number of licks. The main effect of drug on the number of licks for sucrose is shown in Figure 6.2. Ro 15-4513 decreased the number of licks in the test session. As shown in Table 6.1 increasing sucrose concentration had the opposite effect to increasing dose of Ro 15-4513, leading to an increase in the number of licks. Ro 15-4513 also significantly decreased the number of licks for Intra-lipid ($F_{3,27} = 6.35$, $p < 0.01$) (Figure 6.2). An increase in the concentration of Intra-lipid led to an increase in the number of licks ($F_{2,18} = 84.13$, $p < 0.001$) (Table 6.1). There were no significant interactions between drug dose and concentration, for either sucrose ($F_{9,81} = 1.1$, n.s.) or Intra-lipid ($F_{6,54} = 1.7$, n.s.). The total number of licks for sucrose and Intra-lipid decreased as a function of increasing dose of Ro 15-4513. In contrast, increasing the concentration of both sucrose and Intra-lipid led to a monotonic increase in the number of licks.

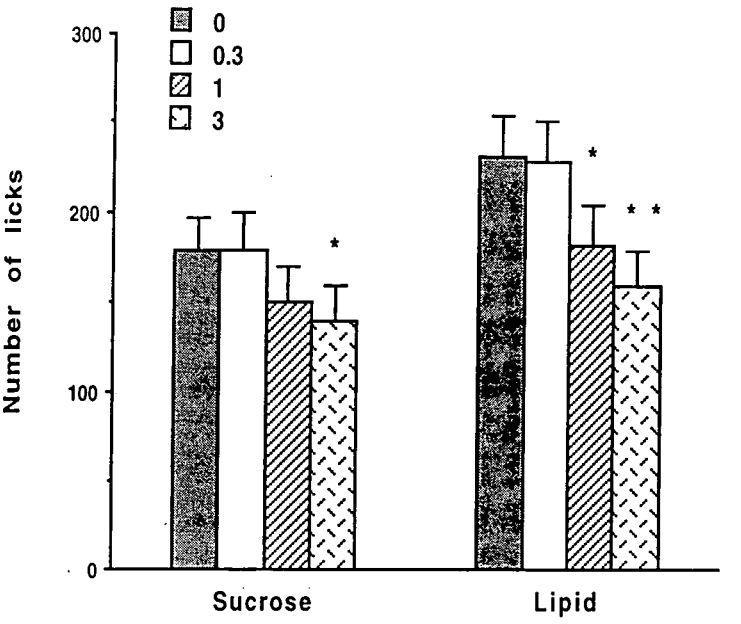


Figure 6.2. Total number of licks for sucrose and Intra-lipid drinking in a brief contact test as a function of increasing dose of Ro-154513 (0.3-3 mg/kg) + S.E.M. Asterisk indicates significantly different from vehicle * $p < 0.05$ ** $p < 0.01$ (Dunnett's t-test).

Table 6.1 Main effect of fluid concentration on total number of licks

	Total licks ± S.E.M.			
Fluid	1%	3%	10%	30%
Sucrose	25.4± 5.9	85.6± 10.2	239.6± 11.1	295.1± 10.3
Intra-lipid	91.9± 10.2	196.2± 9.6	311.5± 9.5	

n = 10 animals per group

Microstructural analysis

Mean bout duration Ro 15-4513 significantly decreased mean bout duration for sucrose drinking ($F_{3,27} = 3.2$, $p < 0.05$) (Figure 6.3a). Post hoc analysis showed that the 1 mg/kg dose of Ro 15-4513 significantly decreased mean bout duration. The effect of Ro 15-4513 on Intra-lipid drinking was similar to that observed for sucrose drinking. An increase in the dose of Ro 15-4513 led to a significant reduction in mean bout duration for Intra-lipid drinking ($F_{3,27} = 7.8$, $p < 0.001$). In addition to a main effect of drug there was also a significant main effect of concentration on mean bout duration for both sucrose and Intra-lipid drinking (Table 6.2). Mean bout duration was an increasing function of concentration for both sucrose ($F_{3,27} = 43.1$, $p < 0.001$) and Intra-lipid ($F_{2,18} = 8.0$, $p < 0.01$) drinking. There was no significant drug-concentration interaction for sucrose drinking ($F_{9,81} = 0.8$, n.s.), but there was a significant interaction for Intra-lipid drinking ($F_{6,54} = 3.1$, $p < 0.05$) (Figure 6.3b). This interaction probably occurred because the effect of Ro 15-4513 was less marked at the 1% concentration, perhaps due to floor effects. There was a trend towards decreasing mean bout duration following Ro 15-4513 administration at 1% Intra-lipid, but because the mean bout duration under the vehicle condition was only 2s on average, any further reduction may have been precluded.

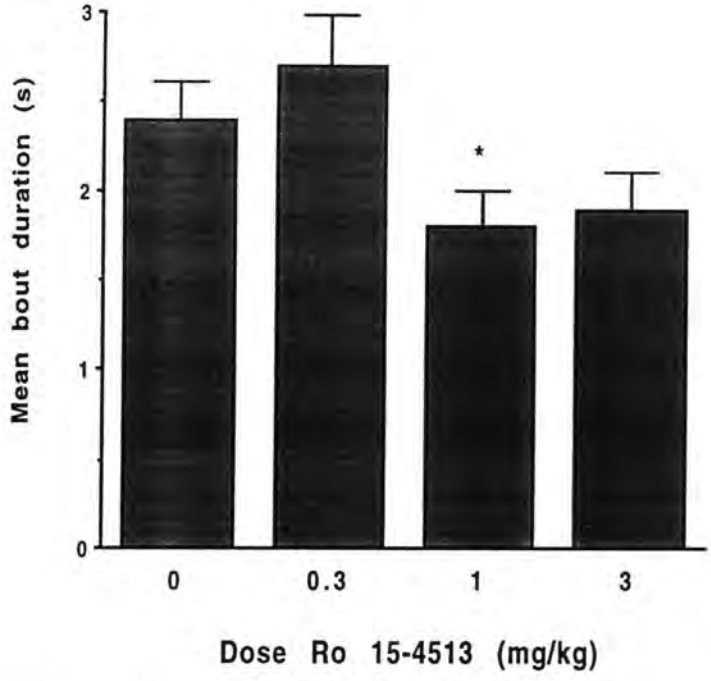
Table 6.2 Main effect of fluid concentration on mean bout duration

Mean bout duration (s) ± S.E.M.

Fluid	1%	3%	10%	30%
Sucrose	0.9± 0.2	1.5± 0.1	3.2+ 0.3	3.3± 0.2
Intra-lipid	2.1± 0.2	3.5± 0.4	9.2± 1.7	

n = 10 animals per group

a) Sucrose



b) Intra-lipid

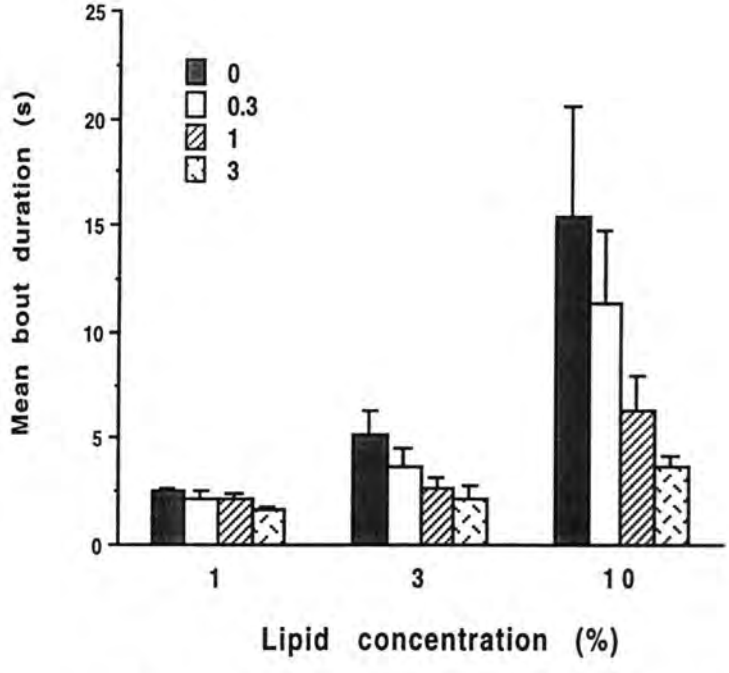


Figure 6.3 Mean bout duration for a) for sucrose and b) Intra-lipid drinking in a brief contact test as a function of increasing dose of Ro 15-4513 (0.3-3 mg/kg) + S.E.M. Asterisk indicates significantly different from vehicle * $p < 0.05$ (Dunnett's t-test).

Number of bouts Ro 15-4513 did not significantly affect the number of bouts for sucrose drinking ($F_{3,27} = 0.23$, n.s.) (Figure 6.4). In contrast, Table 6.3 shows that an increase in sucrose concentration led to a significant increase in bout number ($F_{3,27} = 36$, $p < 0.001$). There was no significant drug-concentration interaction ($F_{9,81} = 1.4$, n.s.). The number of bouts for Intra-lipid drinking was significantly affected by manipulating both drug dose ($F_{3,27} = 3.3$, $p < 0.05$) and concentration ($F_{2,18} = 4$, $p < 0.05$), although there was no significant interaction between these two factors ($F_{6,54} = 1.7$, n.s.). Ro 15-4513 increased the number of bouts for Intra-lipid drinking (Figure 6.4). An increase in the Intra-lipid concentration also led to an increase in bout number (Table 6.3).

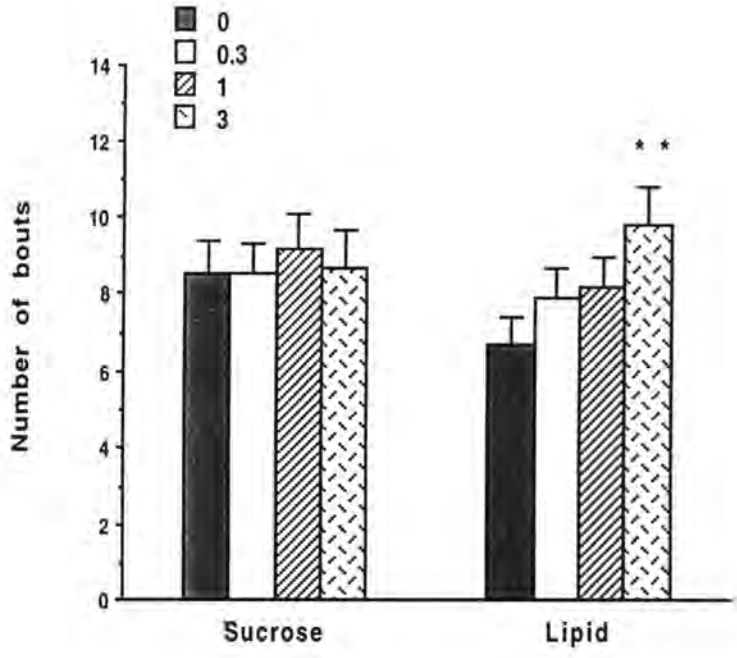


Figure 6.4 Number of bouts for sucrose and Intra-lipid drinking in a brief contact test as a function of increasing dose of Ro 15-4513 (0.3-3 mg/kg) + S.E.M. Asterisk indicates significantly different from vehicle ** $p < 0.01$ (Dunnett's t-test).

Table 6.3 Main effect of fluid concentration on number of bouts

	Number of bouts ± S.E.M.			
Fluid	1%	3%	10%	30%
Sucrose	2.6± 0.5	6.6± 0.7	10.7± 0.8	13.9± 0.6
Intra-lipid	6.4± 0.5	9.3± 0.9	9.0+ 0.8	

n = 10 animals per group

Intrabout lick rate There was no effect of Ro 15-4513 on intrabout lick rate for sucrose drinking ($F_{3,27} = 1.7$, n.s.) However, an increase in sucrose concentration led to a significant decrease in intrabout lick rate ($F_{3,27} = 7.8$, $p < 0.001$) (Table 6.4.). The rate of licking within bouts for Intra-lipid was significantly decreased by Ro 15-4513 ($F_{3,27} = 6.0$, $p < 0.01$) (Figure 6.4). Decreasing Intra-lipid concentration also decreased the rate of licking within bouts ($F_{2,18} = 5.4$, $p < 0.01$) (Table 6.4). There were no significant drug-concentration interactions observed for either sucrose ($F_{8,81} = 1.5$, n.s.) or Intra-lipid drinking ($F_{6,54} = 2.1$, n.s.)

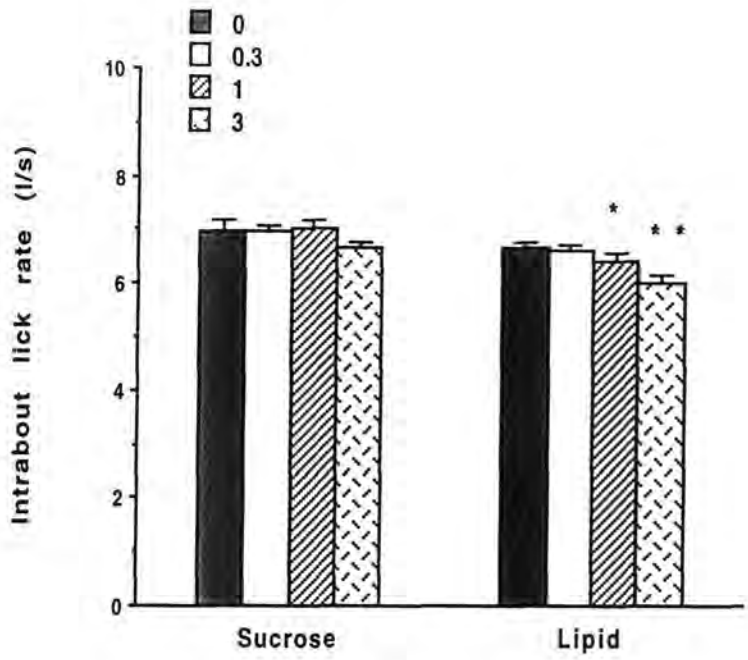


Figure 6.6 Intrabout lick rate for sucrose and Intra-lipid drinking in a brief contact test as a function of increasing dose of Ro 15-4513 (0.3-3 mg/kg) + S.E.M. Asterisk indicates significantly different from vehicle * $p < 0.05$ ** $p < 0.01$ (Dunnett's t-test).

Table 6.4 Main effect of fluid concentration on intrabout lick rate

	Intrabout lick rate (l/s) ± S.E.M.			
Fluid	1%	3%	10%	30%
Sucrose	7.0± 0.2	7.3± 0.1	6.8± 0.1	6.6± 0.06
Intra-lipid	6.6± 0.1	6.3± 0.1	6.2± 0.1	

n = 10 animals per group

Latency The latency to engage in sucrose drinking was significantly affected by both drug dose ($F_{3,27} = 3.0$, $p < 0.05$) and concentration ($F_{3,27} = 4.2$, $p < 0.01$). Figure 6.7 shows that increasing the dose of Ro 15-4513 led to an increase in the latency. In contrast, an increase sucrose concentration led to a decrease in latency (Table 6.5). There was no main effect of drug on latency for Intra-lipid drinking ($F_{3,27} = 1.6$, n.s.), although there was a trend for the latency to increase with increasing dose of Ro 15-4513 (Figure 6.7). There was a significant decrease in latency when Intra-lipid concentration was increased ($F_{2,18} = 7.02$, $p < 0.01$) (Table 6.5). There were no significant drug-concentration interactions for either sucrose ($F_{9,81} = 0.7$, n.s.) or Intra-lipid drinking ($F_{6,54} = 0.9$, n.s.).

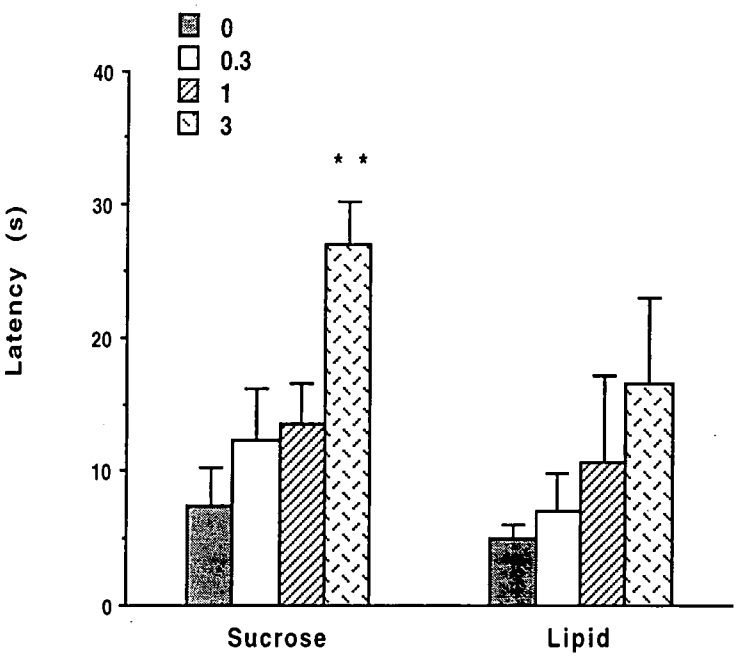


Figure 6.7 Latency for sucrose and Intra-lipid drinking in a brief contact test as a function of increasing dose of Ro 15-4513 (0.3-3 mg/kg) + S.E.M. Asterisk indicates significantly different from vehicle ** $p < 0.01$ (Dunnett's t-test).

Table 6.5 Main effect of fluid concentration on latency

	Latency (s) ± S.E.M.			
Fluid	1%	3%	10%	30%
Sucrose	25.2± 5.6	23.6± 8.4	6.4± 1.6	5.0± 0.7
Intra-lipid	20.4± 5.9	5.8 ±1.7	3.1± 0.9	

n = 10 animals per group

6.4 Discussion

Experiment 11 showed that the benzodiazepine receptor inverse agonist Ro 15-4513 reduced the total number of licks for a range of concentrations of both sucrose and Intra-lipid in a brief contact test. This result is consistent with earlier findings which have demonstrated an anorectic effect of this compound (Cooper et al., 1985; Cooper et al., 1989). Rate analysis showed that the effect of Ro 15-4513 was evident within the first few seconds of licking. Additionally, microstructural analysis indicated that the decrease in the number of licks brought about by Ro 15-4513 was due to a reduction in mean bout duration as opposed to reduction in bout number. The effect of Ro 15-4513 on licking was the converse to that obtained when concentration was increased.

In the present experiment, Ro 15-4513 decreased the rate of licking over the 60s second test session and this effect was evident after the first second. Increasing the dose of Ro 15-4513 also led to a more rapid decline in the rate of licking over 60s. In Chapter 4 it was shown that an increase in the concentration of sucrose and Intra-lipid led to an increase in the rate of licking after the first few seconds. Therefore, the effect of increasing the dose of Ro 15-4513 was opposite to that observed when increasing concentration. This suggests that Ro 15-4513 may have acted to reduce the palatability of the ingested fluids.

There is further evidence which supports this interpretation, based on the effects of Ro 15-4513 on the microstructure of licking for sucrose and Intra-lipid. The decrease in the number of licks was due to a decrease in the duration of bouts rather than a

decrease in their number. Ro 15-4513 significantly decreased mean bout duration for sucrose drinking but did not affect the number of bouts. Therefore, following Ro 15-4513 administration rats took the same number of bouts but these bouts were shorter. For Intra-lipid drinking, Ro 15-4513 also decreased mean bout duration although this effect was more pronounced at higher concentrations due to floor effects at lower concentrations of Intra-lipid. Ro 15-4513 actually increased the number of bouts for Intra-lipid. However, the increase in bout number was not sufficient to overcome the decrease in mean bout duration because there was an overall decrease in the total number of licks. The reason for this increase is not clear, but one possibility is that there was a compensatory increase in bout number which occurred as a result of the decrease in bout duration. The results from previous experiments suggest that in brief contact test mean bout duration provides a measure of palatability (See Chapter 4; Davis and Smith 1992). Therefore the data from Experiment 11 suggest that Ro 15-4513 may reduce palatability.

It is also possible that the decrease in the number of licks caused by Ro 15-4513 occurred as a result of this drug affecting the ability of the rats to perform the necessary motor patterns to lick properly. This possibility was examined by measuring the rate of licking within bouts. The intrabout lick rate has been shown to be affected by moving the drinking spout further away from an animal but not by manipulating concentration (Davis and Smith, 1992). Therefore, intrabout lick rate has been suggested to provide a measure of motor incapacitation. The effect of Ro 15-4513 administration in Experiment 11 on this measure was dependent on the type of test fluid. There was no significant effect of Ro 15-4513 on intrabout lick rate for sucrose drinking. However, Ro 15-4513 dose-dependently reduced the rate of licking within bouts for Intra-lipid drinking. This suggests that Ro 15-4513 may be having a deleterious effect on the motor control of licking and that licking for Intra-lipid may be more sensitive to these effects. However, the results also suggest that the possible motor impairing effects of Ro 15-4513 cannot account for the anorectic effect of this compound because Ro 15-4513 reduced the number of licks for both sucrose and Intra-lipid but only affected the intrabout lick rate for Intra-lipid. One problem with interpreting the effect of Ro 15-4513 on intrabout lick

rate is that it was also shown in Experiment 11 that manipulating concentration affected this variable. Increasing the concentration of sucrose and Intra-lipid led to a decrease in the intrabout lick rate. This suggests that intrabout lick rate may not only reflect changes in motor capacity but also other factors related to changes in concentration. Therefore, the effects of Ro 15-4513 on intrabout lick rate should be interpreted with caution.

The latency to engage in sucrose drinking was significantly increased by Ro 15-4513 administration. There was a trend towards an increase in latency for Intra-lipid drinking although this did not reach significance. This suggests that Ro 15-4513 may have affected either the rat's motor capacity, or motivation to drink. However, there was also a significant effect of fluid concentration on latency. Increasing concentration led to a decrease in the latency to engage in drinking. It has been suggested that olfactory cues may be responsible for effects of concentration on latency. Using a brief contact test, Rhinehart-Doty and colleagues (Rhinehart-Doty, Schumm, Smith and Smith, 1994) found an inverse relationship between sucrose concentration and latency in a brief contact test. They went on to demonstrate that if olfactory cues were masked, this relationship was no longer evident. This suggests that the animals were using olfactory cues to decide when to engage in drinking. It is possible that the effect of Ro 15-4513 on latency was due to an effect on these olfactory cues. The rats may have been discriminating between the test fluids on the basis of olfaction and Ro 15-4513 may have affected this discrimination.

The proposal that the anorectic effect of Ro 15-4513 results from a change in palatability fits in with the notion of bidirectional control of ingestive behaviour mediated by specific benzodiazepine receptors, as first proposed by Cooper (1985b). According to this theory, the effects of benzodiazepine receptor inverse agonists on feeding should be opposite to that of classical agonists. It has recently been argued that benzodiazepine receptor agonists may exert their effects on feeding behaviour by modulating palatability (Berridge and Pecina, 1995). This conclusion is supported by the effect of midazolam on the microstructure of licking for sucrose and Intra-lipid reported earlier in Chapter 5. In Experiment 10, midazolam increased the number of licks by increasing the mean bout

duration. This effect is consistent with the proposal that midazolam enhances palatability. The effect of Ro 15-4513 on sucrose and Intra-lipid consumption was the opposite to that observed as a result of midazolam administration. In the experiment reported in this chapter, Ro 15-4513 decreased the total number of licks by decreasing the mean bout duration. This result consistent with the proposed bidirectional control of feeding, because it suggests that benzodiazepine receptor inverse agonists decrease consumption as a consequence of a reduction in the palatability of ingested foods.

In summary, Ro 15-4513 decreased the number of licks for sucrose and Intra-lipid in a brief contact test. The decrease in total licks was due to a decrease in mean bout duration. This suggests that Ro 15-4513 may reduce intake by reducing palatability. These data support the proposal that ingestive behaviour can be affected bidirectionally at the level of the benzodiazepine receptor. Ro 15-4513 may induce some motor impairments because the rate of licking for Intra-lipid was slowed following drug treatment. However, this effect cannot account for the decrease in number of licks also observed. Ro 15-4513 reduced the latency to engage in drinking and this may have been to an effect on olfactory cues related to the test fluids.

The effects of morphine and naloxone on the microstructure of licking for Intra-lipid

7.1 Introduction

There is much evidence to suggest a role for endogenous opioids in the control of food intake. Opioid agonists have been shown to increase food intake under a variety of experimental conditions (Martin, Wikler, Eades and Pescor, 1963; Gosnell et al., 1983; Morley et al., 1982; Sanger and McCarthy, 1980). Conversely, many studies have shown that opioid antagonists inhibit food intake (Cooper, 1980b; Holtzman, 1974, 1975; Margules et al., 1978). The effectiveness of intracerebroventricular injections of opioid agonists in increasing food intake suggests a central site of action for these drugs on ingestive behaviour (Gosnell et al., 1986; Levine, Grace and Billington, 1991; Morley and Levine, 1983). This is supported by the finding that the quaternary form of naloxone, which does not pass the blood brain barrier, is ineffective in tests of ingestive behaviour (Brown and Holtzman, 1981; Cooper and Turkish, 1983).

Research into the behavioural mechanisms underlying the effects of opioids on ingestive behaviour suggests that they alter ingestive behaviour by modulating reward or palatability. For example, opioid agonists have been shown to increase preference for saccharin over water in water-deprived rats (Calcagnetti and Reid, 1983). Opioid antagonists on the other hand reduce saccharin preference (Cooper, 1983b; Le Magnen et al., 1980; Siviy and Reid, 1983). Additionally, opioid antagonists decrease sucrose sham feeding in rats (Kirkham, 1990; Kirkham and Cooper, 1988a,b; Rockwood and Reid, 1982). The effects of morphine in the taste reactivity test also supports the hypothesis that opioid agonists increase food intake by altering palatability. Morphine has been shown to enhance positive hedonic responding to sucrose in rats (Doyle et al., 1993; Rideout and Parker, 1996). The results of these studies suggest that the determination of palatability may depend on endogenous opioid activity.

The explanations which have been put forward to account for the effects of opioids on ingestive behaviour are similar to those developed to explain the effects of benzodiazepines. Opioid receptor agonists and benzodiazepines have similar effects in taste preference, sham feeding and taste reactivity tests. This suggests that both classes of drug may affect palatability mechanisms. The similarities between the effects of opioids and benzodiazepines on ingestive behaviour makes the suggestion of a putative interaction between the two appealing. It has been proposed that the hedonic response to food-stuffs may be affected by benzodiazepine receptor ligands, which then leads to the release of endogenous opioid peptides (Cooper, 1983a).

The aim of the experiments reported in this chapter was to investigate this hypothesis further by comparing the effects of benzodiazepine receptor ligands on the microstructure of licking with those of the opioid agonist morphine and the opioid antagonist naloxone. The effects of benzodiazepine receptor ligands on the pattern of licking for sucrose and Intra-lipid have been reported in previous chapters. In Experiment 10, it was shown that the benzodiazepine receptor agonist midazolam increased the number of licks for sucrose and Intra-lipid in a brief contact test by increasing the mean bout duration. The benzodiazepine receptor inverse agonist Ro 15-4513 had the opposite effect in Experiment 11, reducing the total number of licks by decreasing mean bout duration. These results are consistent with the hypothesis that benzodiazepines alter food intake by enhancing palatability.

If the effects of benzodiazepines on palatability are mediated by changes in the release of endogenous opioids then we should expect the effects of morphine on responding for Intra-lipid to be similar to that of midazolam, and the effects of naloxone should be similar to the effects of Ro 15-4513. Such similarities would also provide further evidence to support a possible interrelation between opioids and benzodiazepines in the control of ingestion. Experiment 12 was designed to examine the effect of morphine (0.3-3 mg/kg) administration on the licking responses for three concentrations of Intra-lipid in a brief contact test. The effect of naloxone (0.3-3 mg/kg) was then investigated under the same conditions (Experiment 13).

7.2 Method

7.2.1 Animals

Twenty one naive non-deprived adult male hooded Lister rats (Charles River, U.K.) weighing 300-350 g at the beginning of training were used. They were housed in pairs in plastic cages in a room with a constant room temperature of 21±2 °C, and were maintained under a 12h light:dark cycle (lights on at 08.00). Rats were allowed ad lib access to food pellets, (SDS RMI (E), Cambridge, U.K.) and water, except during testing. All testing was carried out in the light phase between 09.00 and 13.00h.

7.2.2 Drugs

Morphine sulphate (Macfarland Smith, Edinburgh, U.K.) was dissolved in distilled water and injected subcutaneously (s.c.) in a volume of 1 ml/kg, 60-min prior to experimentation. The doses were 0.3, 1 and 3 mg/kg of morphine or its vehicle. These doses were chosen because it had been previously shown that morphine can stimulate intake in non-deprived rats over this dose range (Sanger and McCarthy, 1980). An injection-test period of 60-min was used because morphine has been shown to decrease food intake up to 60-min following injection effect, probably due to the sedative effects of this drug (Lesham, 1988). Increases in intake are then observed once the initial sedative effect has worn off. Naloxone hydrochloride, at doses of 0.3, 1 and 3 m/kg (Sterling Winthrop, U.K.) was dissolved in distilled water and injected i.p. in a volume of 1 ml/kg, 20-min prior to testing. This dose range was used because previous studies have demonstrated a decrease in food intake in non-deprived rats using similar doses (Cooper, 1980).

7.2.3 Test meal

Rats had access to various concentrations of Intra-lipid emulsions (Pharmacia Ltd, Milton Keynes, U.K.) which were made up freshly each day. The Intra-lipid emulsions were made up by diluting a 20% commercial preparation with tap water.

7.2.4 Apparatus

Testing was carried out using the MS80 multistation lickometer described in detail in the Chapter 4 (Section 4.2.3).

7.2.5 Procedure

Experiment 12: Microstructural analysis of the effect of morphine on drinking for Intra-lipid in a brief contact test

Training Ten rats were first well familiarized with the test apparatus and procedure. This involved placing each rat in the test chamber were they had access to a range of Intra-lipid emulsions (1, 3 and 10%) in a random order. Each concentration was presented for 60s and a 10s interval intervened between subsequent presentations. This procedure continued until steady baseline levels of licking were observed across days (approximately 10 days). Two days prior to testing each rat received a sham injection of distilled water to familiarize it with the injection procedure.

Testing Following the familiarization period rats received s.c. injections of morphine (0.3, 1 and 3 mg/kg) or vehicle. 60-min after injection of morphine the rats were placed in the lickometer chamber where they had access to all concentrations of Intra-lipid (1, 3 and 10%). Each concentration was presented for a total duration of 60s. The order of presentation was randomised. A repeated-measures design was used in which each rat was tested at every dose. Injections were counterbalanced and 48h elapsed between treatment to avoid carry-over effects.

Experiment: 13: Microstructural analysis of the effect of naloxone on drinking for sucrose and Intra-lipid in a brief contact test

Training Eleven rats were familiarized with the test procedure and apparatus according to the procedure described for Experiment 12.

Testing Following the familiarization period rats received i.p. injections of naloxone (0.3, 1 and 3 mg/kg) or vehicle. 20-min after injection of naloxone the rats were placed in the lickometer chamber where they had access to all concentrations of Intra-lipid (1, 3 and 10%). Each concentration was presented for a total duration of 60s. The order of presentation was randomised. A repeated-measures design was used in which each rat was tested at every dose. Injections were counterbalanced and 48h elapsed between treatment.

7.2.6 Data analysis

The lick time data were analyzed as described in the Chapter 4 (Section 4.2.5) using Dilog software written by Ross Henderson followed by further processing using a Microsoft Excel spreadsheet. The main effect of drug treatment on the rate of licking at each concentration of Intra-lipid was analyzed using a one-way repeated-measures ANOVA.

Various microstructural variables were also examined: the total number of licks, mean bout duration, number of bouts, intrabout lick rate (licks per second within bouts) and latency to engage in drinking (time from shutter opening to first lick). The microstructural data were analyzed using a two-way repeated-measures ANOVA, with drug dose and fluid concentration as factors. Where there was no significant interaction between the two main factors, the effect of drug collapsed across concentration was considered. Post hoc comparisons to determine any significant differences between doses were made using a Dunnett's t-test. Statistical tests were performed using Super Anova and Statview SE+graphics (Abacus Concepts Inc., Berkeley, CA). A result was considered statistically significant if $p < 0.05$.

7.3 Results

Experiment: 12: Microstructural analysis of the effect of morphine on drinking for Intra-lipid in a brief contact test

Rate of licking

A one-way repeated-measures ANOVA showed that there was a significant effect of morphine on the rate of licking at the 1% Intra-lipid concentration ($F_{3,30} = 3.48$, $p < 0.05$). There was no effect of morphine administration on the rate of licking at the 3% ($F_{3,30} = 1.36$, n.s.) or the 10% concentration of Intra-lipid ($F_{3,30} = 0.85$, n.s.). The effect of morphine on the rate of licking for the 1% concentration of Intra-lipid is shown in Figure 7.1. There was a decrease in the initial rate of licking as a result of treatment with morphine (0.3-3 mg/kg). After 20s of licking, morphine (3 mg/kg) stimulated a higher rate of licking relative to the control vehicle condition. This higher rate of licking was then maintained for the remainder of the test session.

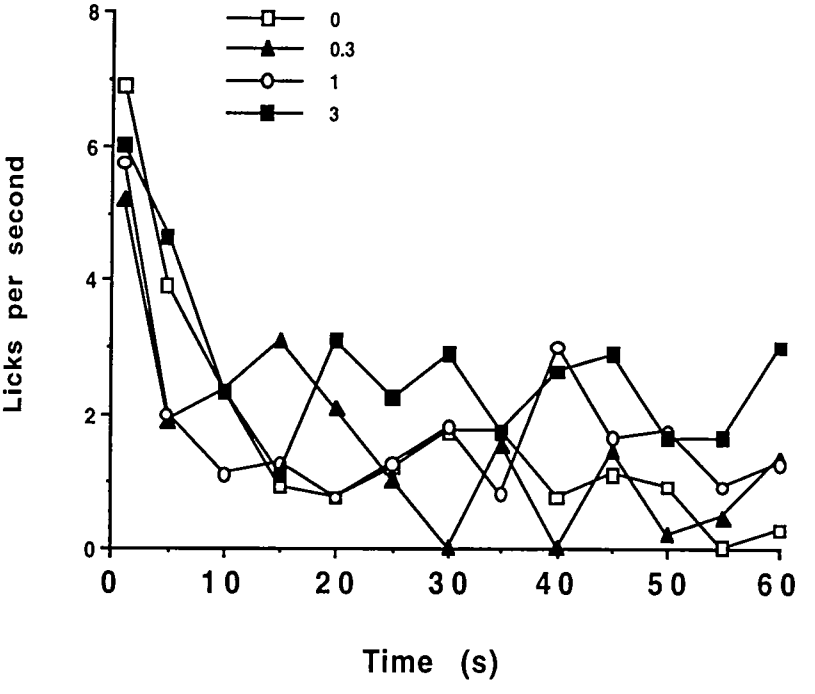


Figure 7.1 The average number of licks per second at 1 s intervals as a function of increasing dose of morphine (0.3-3 m/kg) for 1% Intra-lipid in a brief contact test. Each dose is plotted separately

Number of licks

There was a significant main effect of pretreatment with morphine on the total number of licks for Intra-lipid ($F_{3,30} = 3.2$, $p < 0.05$). Figure 7.2 shows the effect of morphine on the number of licks. Increasing the dose of morphine led to an increase in the total number of licks. There was also a main effect of concentration ($F_{2,20} = 56.2$, $p < 0.001$), with total licks increasing as a function of increasing concentration (Table 7.1). However, there was no significant drug-concentration interaction ($F_{6,60} = 1.8$, n.s.).

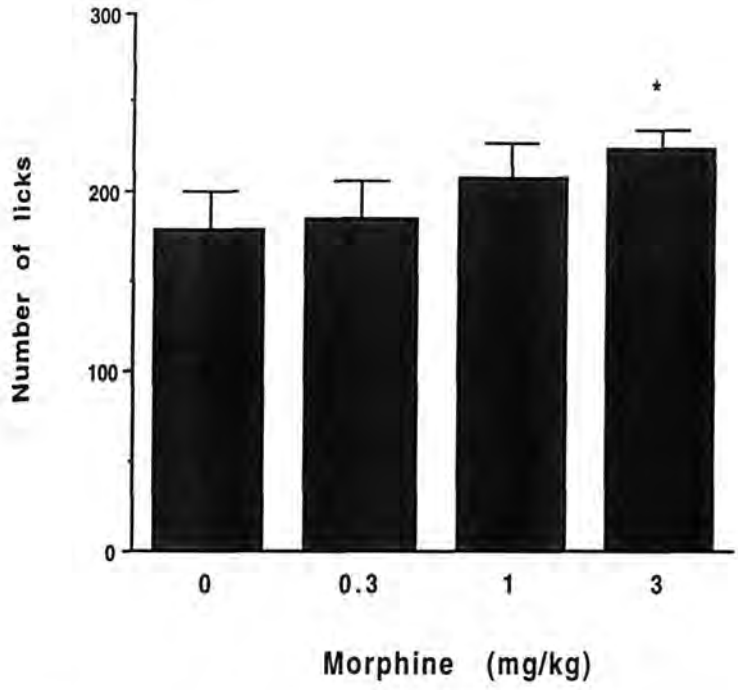


Figure 7.2 The number of licks for Intra-lipid drinking in a brief contact test as a function of increasing dose of morphine (0.3-3 mg/kg) + S.E.M. Asterisk indicates significantly different from vehicle * $p < 0.05$ (Dunnett's t-test).

Table 7.1 Main effect of Intra-lipid concentration on total number of licks

	Total licks ± S.E.M.		
Concentration	1%	3%	10%
Intra-lipid	114.7± 11.5	198.5± 13.7	282.9± 11.1

n = 10 animals per group

Microstructural analysis

Mean bout duration There was a significant main effect of drug on mean bout duration ($F_{3,30} = 4.7$, $p < 0.01$) (Figure 7.3). However, the effect of morphine on this parameter was opposite to the effect on total number of licks shown in Figure 7.2. Increasing the dose of morphine led to a significant decrease in the mean bout duration. Table 7.2 shows that an increase in concentration led to an increase the duration of bouts ($F_{2,20} = 9.7$, $p < 0.001$). There was no significant interaction between drug and concentration on mean bout duration ($F_{6,60} = 1$, n.s.).

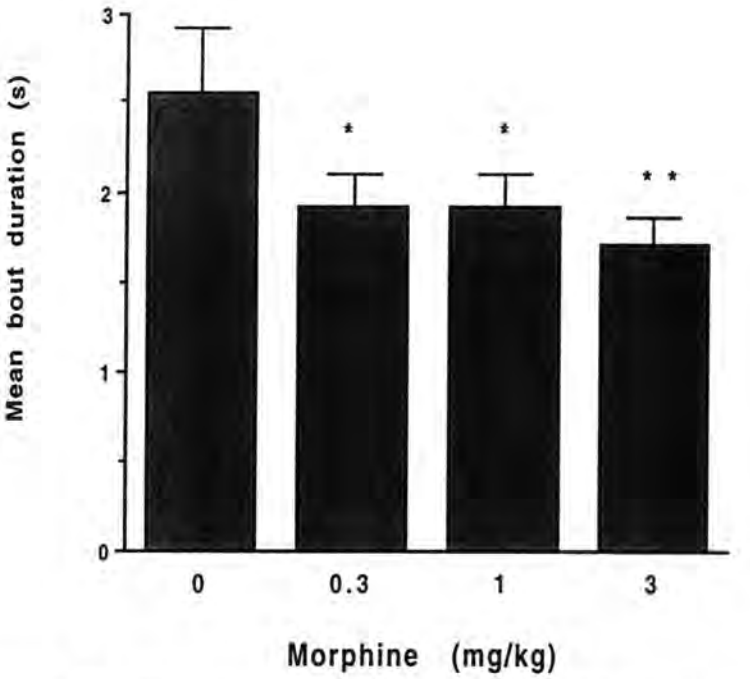


Figure 7.3 Mean bout duration for Intra-lipid drinking in a brief contact test as a function of increasing dose of morphine (0.3-3 mg/kg) + S.E.M. Asterisk indicates significantly different from vehicle * $p < 0.05$ ** $p < 0.01$ (Dunnett's t-test).

Table 7.2 Main effect of Intra-lipid concentration on mean bout duration

	Mean bout duration (s) ± S.E.M.		
Concentration	1%	3%	10%
Intra-lipid	1.5± 0.1	1.9± 0.2	2.6± 0.3

n = 10 animals per group

Bout number The increase in the total number of licks was probably due to an effect on bout number rather than bout duration. Morphine dose-dependently increased bout number ($F_{3,30} = 26.9$, $p < 0.001$) (Figure 7.4). A post hoc test revealed that the 1 mg/kg and 3 mg/kg doses of morphine produced a significant increase in bout number ($p < 0.01$). Manipulating the concentration of Intra-lipid also had significant effects on bout number ($F_{2,20} = 37.9$, $p < 0.001$). An increase in Intra-lipid concentration led to an increase in the number of bouts (Table 7.3). No significant interactions were observed between drug dose and concentration on bout number ($F_{6,60} = 1.5$, n.s.).

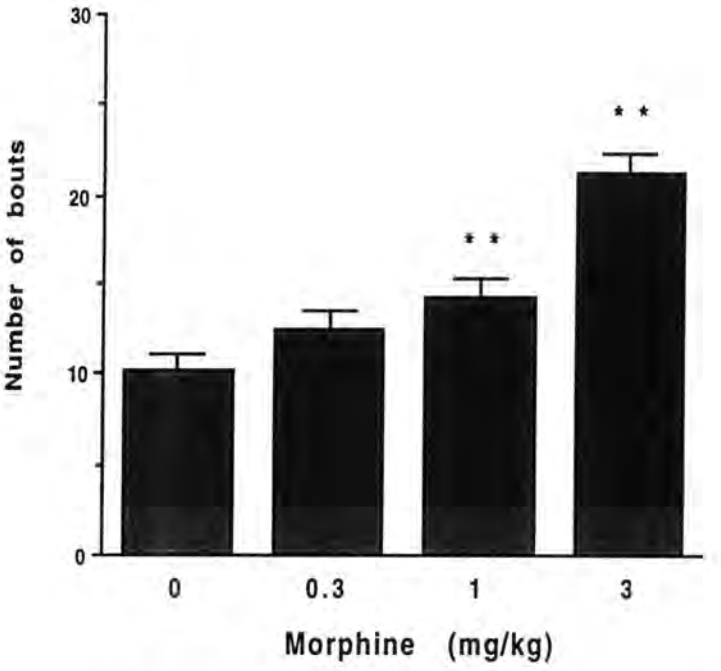


Figure 7.4 Number of bouts for Intra-lipid drinking in a brief contact test as a function of increasing dose of morphine (0.3-3 mg/kg) + S.E.M. Asterisk indicates significantly different from vehicle * $p < 0.05$ ** $p < 0.01$ (Dunnett's t-test).

Table 7.3 Main effect of Intra-lipid concentration on bout number

	Number of bouts ± S.E.M.		
Concentration	1%	3%	10%
Intra-lipid	9.9± 0.8	16.0± 1.2	17.6± 0.9

n = 10 animals per group

Intrabout lick rate As shown in Figure 7.5, there was a significant main effect of drug on the rate of licking within bouts ($F_{3,30} = 22.3$, $p < 0.001$), although a Dunnett's t-test showed that only the 3 mg/kg dose differed significantly from the vehicle condition ($p < 0.01$). Manipulating concentration also significantly affected the intrabout lick rate ($F_{2,20} = 32.8$, $p < 0.001$). Increasing concentration led to a decrease in the intrabout lick rate (Table 7.4). The lack of a significant interaction term showed that the effect of morphine was constant at all levels of concentration ($F_{6,60} = 1.7$, n.s.).

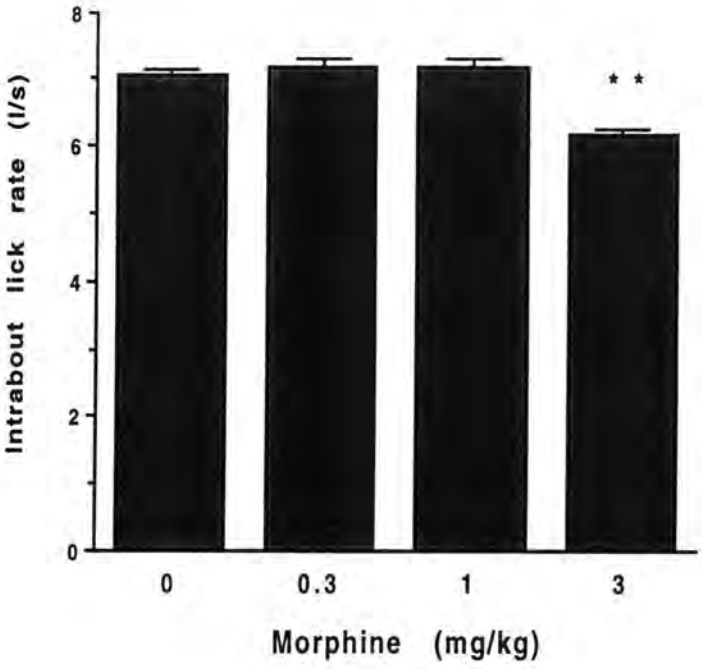


Figure 7.5 Intrabout lick rate for Intra-lipid drinking in a brief contact test as a function of increasing dose of morphine (0.3-3 mg/kg) + S.E.M. Asterisk indicates significantly different from vehicle ** $p < 0.01$ (Dunnett's t-test).

Table 7.4 Main effect of Intra-lipid concentration on intrabout lick rate

	Intrabout lick rate (l/s)± S.E.M.		
Concentration	1%	3%	10%
Intra-lipid	7.2± 0.1	6.8± 0.1	6.6± 0.07

n = 10 animals per group

Latency Morphine did not significantly affect the latency to engage in drinking ($F_{3,30} = 1.2$, n.s.). However, there was a trend towards a decrease in latency with increasing dose of morphine (Figure 7.6). There was a significant main effect of concentration on latency ($F_{2,20} = 3.9$, $p < 0.05$). Table 7.5 shows that there was an inverse relationship between latency to engage in drinking and concentration. There was no significant drug-concentration interaction observed for this parameter ($F_{6,60} = 0.8$, n.s.).

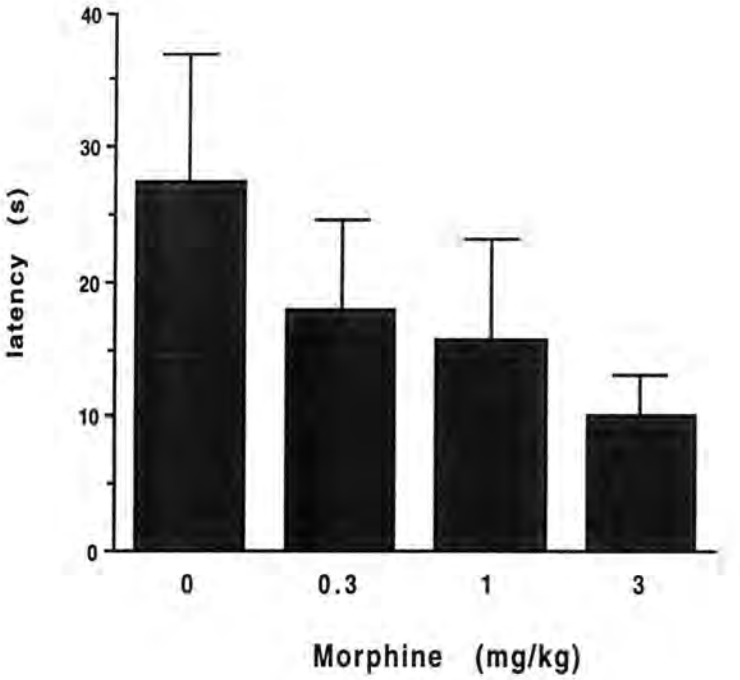


Figure 7.6 Latency for Intra-lipid drinking in a brief contact test as a function of increasing dose of morphine (0.3-3 mg/kg) + S.E.M.

Table 7.5 Main effect of Intra-lipid concentration on latency

	Latency (s) ± S.E.M.		
Concentration	1%	3%	10%
Intra-lipid	32.4± 8.1	15.2± 6	5.9± 1.4

n = 10 animals per group

Experiment: 13: Microstructural analysis of the effect of naloxone on drinking for sucrose and Intra-lipid in a brief contact test

Rate of licking

There were no significant effects of naloxone on the rate of licking over 60s for either the 1% ($F_{3,30} = 1.65$, n.s.), 3% ($F_{3,30} = 1.8$, n.s.), or 10% concentration of Intra-lipid ($F_{3,30} = 1.47$, n.s.). Figure 7.7 shows the effect of naloxone on 1% Intra-lipid for comparison with the effects of morphine.

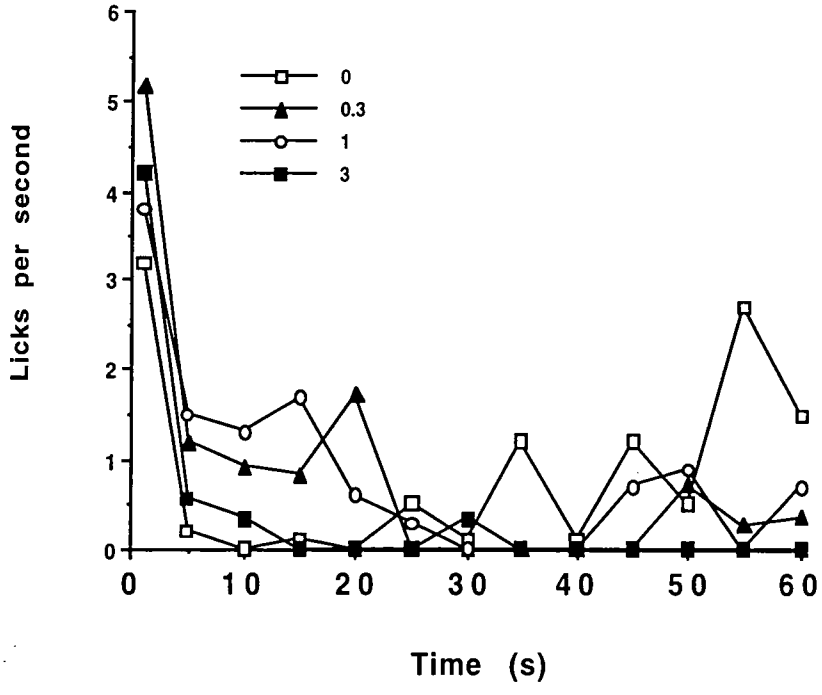


Figure 7.7 The average number of licks per second at 1 s intervals as a function of increasing dose of morphine (0.3-3 m/kg) for 1% Intra-lipid in a brief contact test. Each dose is plotted separately.

Number of licks

As shown in Figure 7.8, naloxone brought about a decrease in the total number of licks ($F_{3,30} = 5.2$, $p < 0.01$). Post hoc analysis showed that only the 3 mg/kg dose of naloxone significantly decreased the number of licks ($p < 0.01$). There was a main effect of manipulating Intra-lipid concentration on the total number of licks ($F_{2,20} = 83.7$, $p < 0.001$). An increase in concentration led to an increase in the total number of licks (Table 7.6). There was no significant drug-concentration interaction ($F_{6,60} = 0.8$, n.s.).

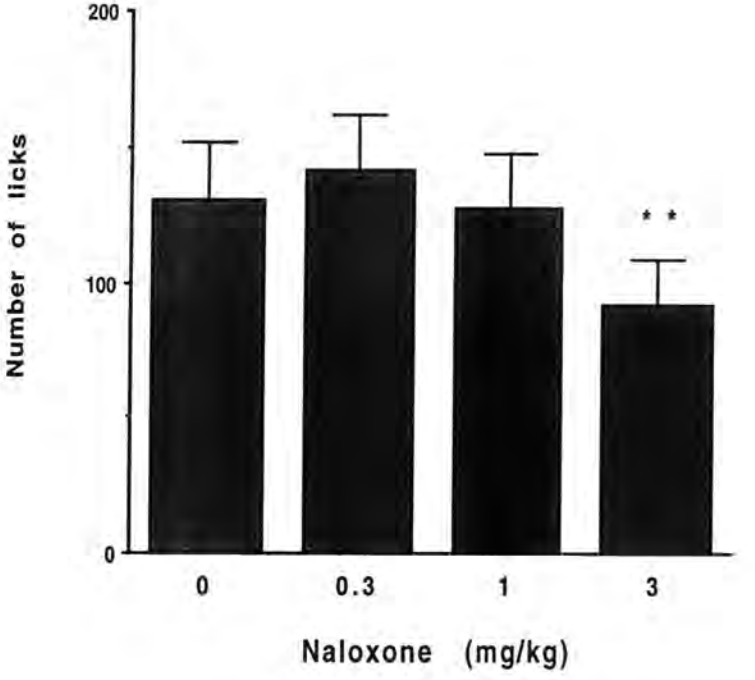


Figure 7.8 The number of licks for Intra-lipid drinking in a brief contact test as a function of increasing dose of naloxone (0.3 - 3 mg/kg) + S.E.M. Asterisk indicates significantly different from vehicle ** $p < 0.01$ (Dunnett's t-test).

Table 7.6 Main effect of Intra-lipid concentration on total licks

	Total licks ± S.E.M.		
Concentration	1%	3%	10%
Intra-lipid	32.6± 6.1	97.6± 13.6	238.7± 11.8

n = 11 animals per group

Microstructural analysis

Mean bout duration Figure 7.9 shows the effect of naloxone on the mean bout duration. Naloxone did not significantly affect mean bout duration ($F_{3,30} = 0.4$, n.s.). There was a significant main effect of concentration on mean bout duration ($F_{2,20} = 42.2$, $p < 0.001$). An increase in concentration of Intra-lipid led to a monotonic increase in the mean bout duration (Table 7.7). There was no interaction between drug and concentration on mean bout duration ($F_{6,60} = 0.6$, n.s.).

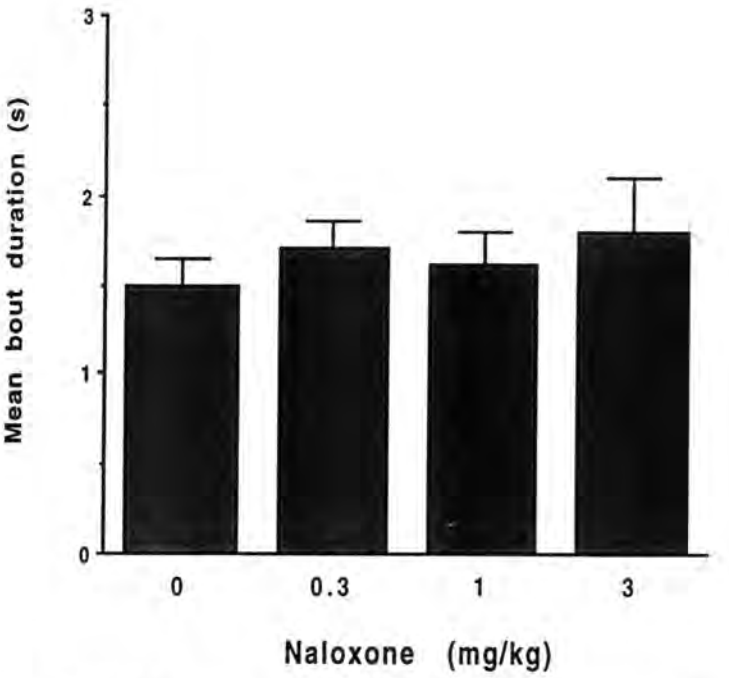


Figure 7.9 Mean bout duration for Intra-lipid drinking in a brief contact test as a function of increasing dose of naloxone (0.3-3 mg/kg) + S.E.M.

Table 7.7 Main effect of Intra-lipid concentration on mean bout duration

	Mean bout duration (s) ± S.E.M.		
Concentration	1%	3%	10%
Intra-lipid	1.0± 0.09	1.4± 0.1	2.5± 0.2

n = 11 animals per group

Bout number Naloxone significantly deceased the number of bouts in the test session ($F_{3,30} = 7.9$, $p < 0.001$) (Figure 7.10). Post hoc analysis showed that the 3 mg/kg dose significantly reduced bout number ($p < 0.01$). Therefore, the decrease in the number of licks was due to a decrease in the number of bouts as opposed to their duration. The main effect of concentration ($F_{2,20} = 44.3$, $p < 0.001$) is presented in Table 7.8. An increase in concentration led to an increase in bout number. There was no significant drug-concentration interaction ($F_{6,60} = 1$, n.s.).

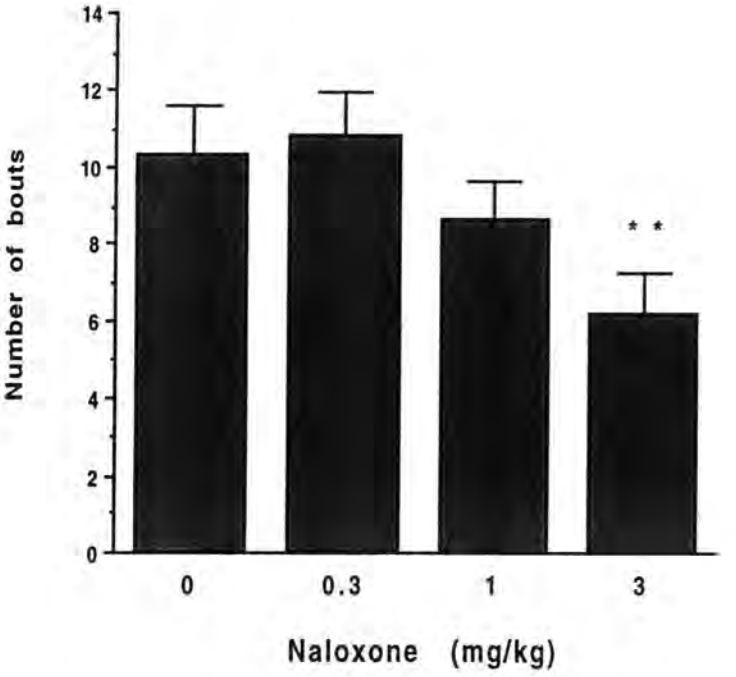


Figure 7.10 Number of bouts for Intra-lipid drinking in a brief contact test as a function of increasing dose of naloxone (0.3-3 mg/kg) + S.E.M. Asterisk indicates significantly different from vehicle ** $p < 0.01$ (Dunnett's t-test).

Table 7.8 Main effect of Intra-lipid concentration on bout number

	Number of bouts ± S.E.M.		
Concentration	1%	3%	10%
Intra-lipid	4.2± 0.5	8.5± 1.0	14.3± 0.8

n = 11 animals per group

Intrabout lick rate Figure 7.11 shows the effect of naloxone on the intrabout lick rate. For this parameter, there was a significant main effect of both drug ($F_{3,30} = 8.3$, $p < 0.001$) and concentration ($F_{2,20} = 8.6$, $p < 0.01$) but no drug-concentration interaction ($F_{6,60} = 1.4$, n.s.). At all doses, naloxone significantly decreased the rate of licking within bouts. Increasing concentration also led to a decrease in the intrabout lick rate (Table 7.9).

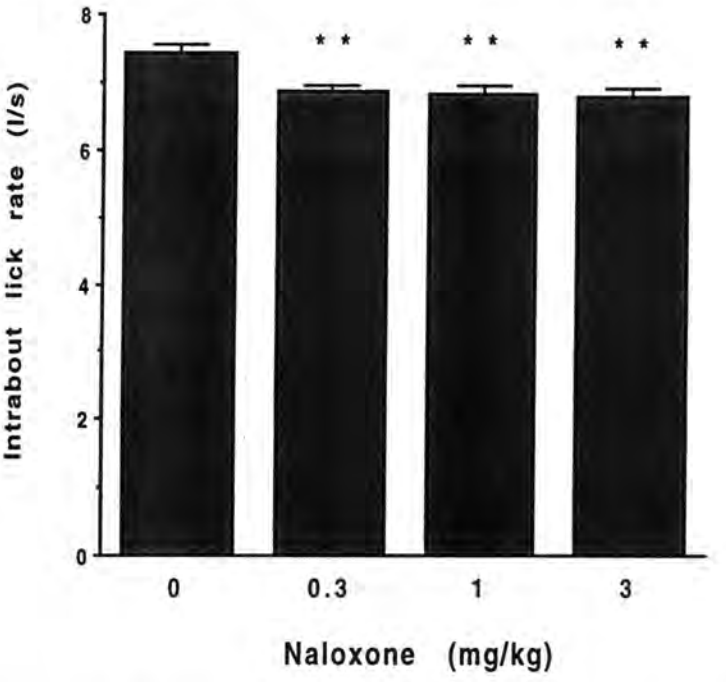


Figure 7.11 Intrabout lick rate for Intra-lipid drinking in a brief contact test as a function of increasing dose of naloxone (0.3-3 mg/kg) + S.E.M. Asterisk indicates significantly different from vehicle ** $p < 0.01$ (Dunnett's t-test).

Table 7.9 Main effect of Intra-lipid concentration on intrabout lick rate

	Intrabout lick rate (l/s)± S.E.M.		
Concentration	1%	3%	10%
Intra-lipid	7.2± 0.1	7.0 ± 0.1	6.8± 0.04

n = 11 animals per group

Latency Figure 7.12 shows that the latency to start drinking was not significantly affected by pretreatment with naloxone ($F_{3,30} = 2.1$, n.s.). However, there was a main effect of concentration on this parameter ($F_{2,20} = 3.5$, $p < 0.05$), although this effect was only marginally significant (Table 7.10). An increase in concentration led to a decrease in the latency.

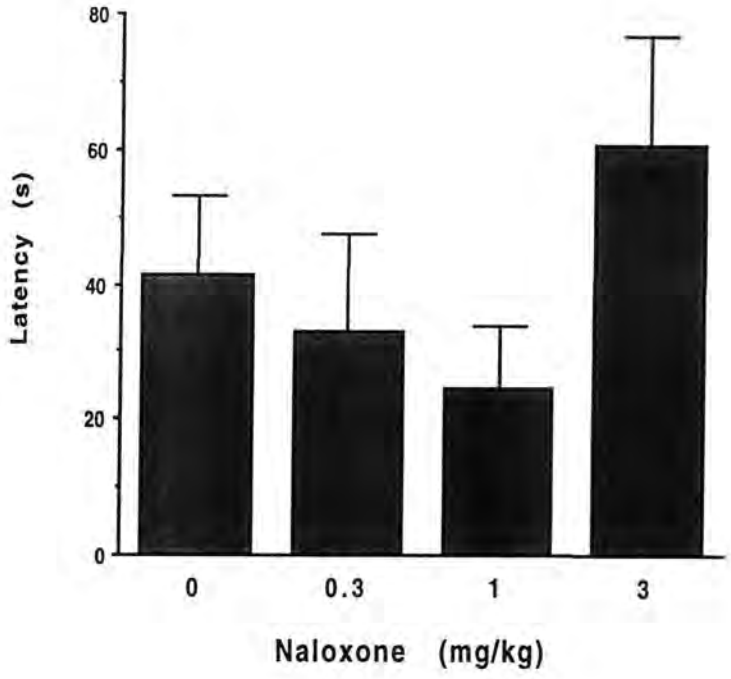


Figure 7.12 Latency for Intra-lipid drinking in a brief contact test as a function of increasing dose of naloxone (0.3-3 mg/kg) + S.E.M.

Table 7.10 Main effect of Intra-lipid concentration on latency

	Latency (s) ± S.E.M.		
Concentration	1%	3%	10%
Intra-lipid	50.3± 10.1	50.9± 13.9	18.3± 7.7

n = 10 animals per group

7.4 Discussion

The results of Experiment 12 showed that morphine increased the total number of licks for Intra-lipid in a brief contact test. These data are consistent with previous findings showing that morphine can increase the consumption of fats (Gosnell, Krahn and Majchrzak, 1990). The number of bouts for Intra-lipid increased following morphine administration. There was a compensatory reduction in mean bout duration. Therefore, the increase in the number of licks observed following treatment with morphine was due to an increase in bout number rather than bout duration. The results of Experiment 13 showed that the effects of naloxone on responding for Intra-lipid were the opposite to that observed for morphine. Naloxone decreased the number of licks for Intra-lipid by decreasing bout number. There was no effect of naloxone on mean bout duration.

The effects of morphine and naloxone on the microstructure of licking for Intra-lipid contrast with the effects of benzodiazepine ligands reported in Chapters 5 and 6. Morphine and midazolam both increased the total number of licks for Intra-lipid in a brief contact test but did so in different ways. Midazolam increased the mean bout duration, whereas morphine increased the number of bouts. Conversely, Ro 15-4513 and naloxone both decreased the number of licks. Ro 15-4513 did so by decreasing mean bout duration, whereas naloxone decreased the number of bouts. Therefore, despite similarities in the taste preference, sham feeding and taste reactivity test, the effects of benzodiazepines and opioids can be dissociated using microstructural analysis. This suggests that contrary to previous assertions, the effects of benzodiazepine receptor ligands and opioid receptor ligands on ingestive behaviour are not exactly the same. Although these results do not rule out the possibility of an interaction between benzodiazepines and opioids in the control of ingestive behaviour, they do indicate that the specific nature of any interaction requires further investigation.

The lack of effect of morphine and naloxone on mean bout duration does not provide evidence to support the hypothesis that these drugs affect palatability mechanisms. Mean bout duration has been shown to be sensitive to changes in the hedonic properties of ingested fluids (Davis and Smith, 1992), but this measure was not

affected by opioid administration. It is unlikely that the effects of morphine and naloxone can be explained by appealing to satiety mechanisms because the effects of these drugs were obtained in a brief contact test where the influence of postingestive factors is minimal. In addition, the ability of opioids to increase the consumption of preferred foods and influence sham feeding suggests that reward mechanisms may be important. In Chapter 5, it was suggested that changes in bout number may be indicative of changes in incentive salience. Following this argument, the effect of morphine and naloxone on bout number may mean that these drugs are not affecting palatability but incentive salience attribution. Morphine may have increased bout number by increasing the ability of the test fluid to maintain the rat's interest, whereas naloxone may have reduced bout number by decreasing the salience of the test fluid, thus making the rats more distractible. Although there is no direct evidence for this hypothesis, such a conclusion is supported by the results of a recent study by Badiani and colleagues (Badiani, Leone, Noel and Stewart, 1995). The effects of injection of the μ opioid agonist DAMGO into the ventral tegmental area (VTA) on feeding were found to be similar to the effects of morphine observed in Experiment 12. DAMGO increased the amount of time spent eating food pellets due to an increase in the number of feeding bouts. In a further experiment, DAMGO also increased the time spent gnawing when non-food objects (e.g. balsa wood) were available in addition to food items. This led Badiani and colleagues (1995) to conclude that DAMGO may increase the incentive salience directed towards stimuli. It is possible that the effects of systemic administration of morphine and naloxone on Intra-lipid drinking obtained in Experiments 12 and 13 were due to effects on incentive salience as a result of binding at opioid receptors in the VTA. In support of this interpretation, some investigators have found that the ability of opioid agonists to enhance feeding behaviour is more reliable following repeated injection (Levine and Billington, 1989). The effects of concentration on bout number were similarly only observed following repeated exposure to the test fluids. This observation may add to the argument that opioids affect incentive salience since it has been argued that the attribution of salience depends on previous exposure to the effects of a reinforcer.

The mechanisms involved in the effects of morphine and naloxone on licking patterns are not clear, but some speculations can be made. It has been suggested that opioids interact with the dopaminergic system at the level of the VTA. In support of this proposal, opioid receptors have been identified in close proximity to dopaminergic cell bodies (Sesack and Pickel, 1992). Electrophysiological studies have also shown that morphine and other opioid agonists can increase the rate of dopaminergic cell firing (Gysling and Wang, 1983; Mathews and German, 1984). In vivo dialysis studies have confirmed that administration of opioids can modulate the release of dopamine into the nucleus accumbens (Di Chiara and Imperato, 1988; Spanangel et al., 1990). An interaction between opioids and dopamine in the control of feeding behaviour is suggested by the finding that injection of opioid agonists into the VTA stimulates feeding (Badiani et al., 1995; Mucha and Iversen, 1986; Noel and Wise, 1995). Predictably, injection of naloxone into the VTA has been found to decrease consumption of a palatable apple juice (Segall and Margules, 1989). The effects of injection of opioids into the nucleus accumbens has also been examined. Injection of morphine into the nucleus accumbens has been shown to increase food consumption in rats (Majeed et al., 1986; Mucha and Iversen, 1986). Taken together, these data suggest that binding of opioids in the VTA may influence feeding behaviour by interacting with mesolimbic dopamine projections. Significantly, such an interaction would be consistent with the proposal that opioids may alter incentive salience attribution. It has recently been suggested that dopamine may mediate the incentive motivation effects of reinforcers (Robinson and Berridge, 1993; Berridge, Vernier and Robinson, 1989). An obvious prediction from this is that drugs which influence dopamine transmission should affect bout number. This possibility remains to be fully tested, but evidence gathered to date appears to suggest that this may be the case. Schneider, Davis, Watson, and Smith (1990) have shown that the dopamine D_2 receptor antagonist raclopride reduces the number of bouts for rats sham drinking sucrose. Al Nasar and Cooper (1994) have also shown that the selective D_1 agonist A-68930 reduces feeding by decreasing bout frequency without affecting bout duration. If the effects of morphine are due to release of dopamine then it would also be

predicted that administration of dopamine antagonists should block increases in bout number. This possibility has not been tested, but a functional interaction between dopamine and opioids in the control of ingestive behaviour has been suggested by the results of a study conducted by Evans and Vaccarino (1990). These authors found that the feeding induced by morphine could be blocked by pretreatment with the dopamine antagonist flupenthixol.

An alternative explanation for the results obtained in the present experiments is that administration of morphine and naloxone led to non-specific motor effects which then affected the pattern of licking for Intra-lipid. This possibility was examined in the present experiments by measuring changes in both the intrabout lick rate and the latency to start drinking. The highest dose of morphine (3 mg/kg) significantly reduced the intrabout lick rate. This may suggest that at this dose, morphine was disrupting the rat's ability to perform the necessary movements to lick normally. Such an effect on intrabout lick rate may have consequently interfered with the pattern of licking causing the rats to break up runs of licks which may have resulted in an increase in bout number. However, this explanation is unsatisfactory because the effect on intrabout lick was not dose-related. Conversely, the effect of morphine on bout number was dose-related, with increasing doses of morphine leading to a monotonic increase in the number of bouts. This suggests that the effect of morphine on intrabout lick rate cannot account for the changes also observed in bout number.

Naloxone also reduced the intrabout lick rate. This effect was not dose-related because significant decreases in the rate of licking within bouts were observed at all doses used. It is feasible that the decrease in intrabout lick rate caused by naloxone resulted in a decrease in the number of licks and subsequently the number of bouts. Arguing against this conclusion is the observation that a significant effect on number of licks and bout number was only observed under the highest dose of naloxone (3 mg/kg), whereas the intrabout lick rate was reduced by the same amount at all doses. This suggests that although naloxone may induce non-specific effects, these cannot explain the effects on bout structure which were also observed.

There were no significant effects of morphine on the latency to engage in drinking, but there were certain trends observed in the data. There was a tendency for morphine to dose-dependently reduce the latency to start drinking. This result is similar to the effect of DAMGO injected into the VTA reported by Badiani and colleagues (1995). These authors found that DAMGO significantly decreased the latency to start feeding. As mentioned previously in this thesis, effects on latency are difficult to interpret. However, the tendency of morphine to decrease this measure may be indicative of an increase in the motivation to feed.

There was no effect of naloxone on latency. An increase in latency at the highest dose of naloxone was observed, but this did not reach significance. The lack of effect of naloxone on latency is consistent with previous reports that naloxone does not affect meal initiation (Kirkham and Blundell, 1984). Although not significant, the increased latency seen following administration of the 3 mg/kg dose of naloxone may have reflected an avoidance of the test fluid at this dose. It has been shown that when paired with the presence of a fluid or place a rat prefers, naloxone causes subsequent avoidance of that food or place (Frenk and Rogers, 1979; Stolerman and D'Mello, 1978). Therefore, the putative aversive properties of naloxone could have caused the changes in the number of licks and bout number obtained in Experiment 14. However, Lesham (1984) has suggested that the aversive effects of naloxone cannot totally account for its anorectic effect and so it is unlikely that the effect of naloxone on latency can be explained by the aversive properties of naloxone.

The results of the present studies do not allow firm conclusions to be drawn concerning the receptor subtypes involved in the effects of opioids on licking behaviour. The μ receptor subtype may be implicated in the effects of morphine, but the lack of selectivity of naloxone means that this drug does not provide any information about which receptor subtype is mediating its effects. Studies using specific opioid receptor ligands suggest the involvement of μ, δ and κ subtypes in the control of feeding behaviour (Gosnell et al., 1983; Jackson and Cooper, 1985; Morley and Levine, 1983). However, the results of such studies have also highlighted some interesting differences in

the involvement of opioid receptor subtypes across different experimental situations which may have some bearing on the interpretation of the results from Experiments 13 and 14. Noel and Wise (1995) have shown that injection of the selective μ opioid agonist DAMGO and the selective δ opioid agonist DPDE into the VTA enhanced feeding responses. However, these authors have also shown that intra-VTA injection of κ selective agonist U-50 488H did not significantly affect feeding responses (Noel and Wise, 1993). The lack of effect of U-50 488H is compatible with the observation that injection of this compound into the VTA does not alter dopamine release into the nucleus accumbens (Di Chiara and Imperato, 1988). This evidence suggests that the effects of μ and δ receptor agonists, but not κ agonists, may be mediated by the mesolimbic dopamine system. If the effects of morphine and naloxone on licking behaviour are due to alterations on dopamine transmission then it would be predicted that these effects would also be observed following μ and δ agonist administration but not κ agonist treatment.

In summary, a microstructural analysis of the effects of morphine on Intra-lipid drinking in brief contact test showed that this drug increased the number of licks for Intra-lipid by increasing bout number. Naloxone had the opposite effect and decreased the number of licks by decreasing bout number. A comparison of the effects of morphine and naloxone on licking patterns with the effects of benzodiazepine ligands revealed that these drugs had different effects on licking patterns. Therefore, although benzodiazepine and opioids may have similar effects in taste preference, sham feeding and taste reactivity tests, they can be differentiated in terms of their effects on licking behaviour. These results suggest that further investigation of a putative interaction between benzodiazepines and opioids in the control of food intake is warranted.

Interactions between naloxone and midazolam determined using microstructural analysis of licking for Intra-lipid

8.1 Introduction

It has been proposed that benzodiazepine-induced hyperphagia may be related to endogenous opioid activity (Britton et al., 1981; Cooper, 1983a). Evidence from biochemical studies would appear to support this suggestion. For example, benzodiazepine agonists have been shown to modulate the release of enkephalins in the brain (Duka et al., 1979; Wuster et al., 1980). The increase in enkephalin release caused by benzodiazepine receptor agonists has also been blocked by naloxone (Duka et al., 1979).

Potential interactions between the effects of benzodiazepines and opioids specifically on ingestive behaviour has been examined by investigating the effects of opioid antagonists on increases in food intake caused by benzodiazepine agonists. In several studies it has been shown that the opioid antagonist naloxone can block benzodiazepine-induced hyperphagia (Birk and Noble, 1981; Britton et al., 1981; Naruse et al., 1989; Stapleton et al., 1979). This evidence suggests that benzodiazepines may affect ingestive behaviour by causing the release of endogenous opioid peptides which then act at opioid receptors. Importantly for this hypothesis it has also been shown that opioid agonists can increase food intake under a variety of experimental conditions (Martin et al., 1963; Morley et al., 1982; Sanger and McCarthy, 1980). However, in the previous chapter it was shown that the effects of opioid receptor ligands on the microstructure of licking for Intra-lipid are not the same as the effects of benzodiazepine receptor ligands. Hence, some additional investigation of potential interactions between benzodiazepines and opioids is warranted. The aim of Experiment 14 was to use a microstructural approach to examine in greater detail potential benzodiazepine/opioid interactions in the control of ingestion.

One problem with previous studies in this area has been that opioid antagonists decrease food intake when administered alone, and this has not been rigorously controlled for. In some studies, attenuation of the hyperphagic effects of benzodiazepines may have been due to subtraction of drug effects, rather than a specific interaction. To overcome this problem, the opioid antagonist naloxone was administered at doses which are ineffective when administered alone.

Another criticism of previous studies has been that the effect of opioid antagonists on feeding behaviour induced by benzodiazepine agonists has only been assessed using intake measures. This raises the possibility that any attenuation of benzodiazepine effects on ingestive behaviour may have been due to a non-specific interaction resulting from effects on motor capacity, rather than a specific effect on appetite. A microstructural approach was adopted in Experiment 14 to allow the specificity of any interaction between opioids and benzodiazepines to be investigated.

It has been suggested that benzodiazepine receptor agonists increase food intake by enhancing palatability (Berridge and Pecina, 1995). In support of this, the benzodiazepine receptor agonist midazolam has been shown to increase the total number of licks in a brief contact test by increasing the duration of bouts for sucrose and Intra-lipid drinking (Chapter 5). If the palatability effects of midazolam are dependent on opioid peptide release, then naloxone should block the increase in mean bout duration induced by midazolam. This prediction was tested in Experiment 14 by examining the effect of pretreatment with naloxone (0.1 and 0.3 mg/kg) on the microstructure of licking for Intra-lipid under midazolam.

In Chapter 5, it was also shown that midazolam decreased the rate of licking within bouts, probably as a result of the muscle relaxant effects of this drug. An additional aim of Experiment 14 was to examine whether naloxone blocks both the palatability and muscle relaxant effects of midazolam. Therefore, the effect of pretreatment with naloxone on the decrease in intrabout lick rate caused by midazolam was also examined.

Experiment 14: Microstructural analysis of the effect of naloxone on drinking induced by midazolam in a brief contact test

8.2 Method

8.2.1 Animals

Ten non-deprived adult male hooded Lister rats (Charles River, U.K.) weighing 300-350 g at the beginning of training were used. They were housed in pairs in plastic cages in a room with a constant room temperature of 21±2 °C, and were maintained under a 12h light:dark cycle (lights on at 08.00). Rats were allowed ad lib access to food pellets, (SDS RMI (E), Cambridge, U.K.) and water throughout. All testing was carried out in the light phase between 09.00 and 13.00h.

8.2.2 Drugs

Naloxone hydrochloride at doses of 0.1 and 0.3 mg/kg (Sterling Winthrop, U.K.) was dissolved in distilled water and injected i.p. in a volume of 1 ml/kg 20-min prior to testing. These doses were selected to be non-effective when administered alone. In Experiment 12, it was shown that a dose of 0.3 mg/kg did not affect licking responses. Midazolam maleate (Roche, Basel, Switzerland) was prepared for injection by dissolving in distilled water. Midazolam was administered i.p. in a dose of 1.8 mg/kg, 15-min prior to testing. The vehicle used in control injections was distilled water.

8.2.3 Test meal

Rats had access to various concentrations of Intra-lipid emulsions (Pharmacia Ltd, Milton Keynes, U.K.) which were made up freshly each day. The Intra-lipid emulsions were made up by diluting a 20% commercial preparation with tap water.

8.2.4 Apparatus

Testing was carried out using the MS80 multistation lickometer described in detail in the Chapter 4 (Section 4.2.3).

8.2.5 Procedure

Training Ten rats were well familiarized with the test apparatus and procedure. This involved placing each rat in the test chamber were they had access to a range of Intra-lipid emulsions (1, 3 and 10%) in a random order. Each concentration was presented for 60s and a 10s interval intervened between subsequent presentations. This procedure continued until steady baseline levels of licking were observed across days (approximately 10 days). Two days prior to testing each rat received a sham injection of distilled water to familiarize it with the injection procedure.

Testing Following the initial familiarisation period the rats received i.p. injections of drugs. A repeated-measures design was used in which rats had injections of vehicle/vehicle, 0.1 mg/kg naloxone/vehicle, 0.3 mg/kg naloxone/vehicle, vehicle/midazolam, 0.1 mg/kg naloxone/midazolam, 0.3 mg/kg naloxone/midazolam. After injection of drugs, the rats were placed in the lickometer chamber where they had access to all concentrations of Intra-lipid (1, 3 and 10%). Each concentration was presented for a total duration of 60s. The order of presentation was randomised. A period of 48h elapsed between each series of injections to ensure dispersal of drugs and injections were counterbalanced across rats.

8.2.6 Data analysis

The lick time data were analyzed as described in the Chapter 4 (Section 4.2.5) using Dilog software written by Ross Henderson followed by further processing using a Microsoft Excel spreadsheet. Various microstructural variables were examined: the total number of licks, mean bout duration, number of bouts, intrabout licks rate (licks per second within bouts) and latency to engage in drinking (time from shutter opening to first lick).

The microstructural data were analyzed using a two-way repeated-measures ANOVA, with drug dose and fluid concentration as factors. Where there was no significant interaction between the two main factors, the effect of drug collapsed across concentration was considered. Post hoc comparisons to determine any significant

differences between doses were made using a Dunnett's t-test. Statistical tests were performed using Super Anova and Statview SE+graphics (Abacus Concepts Inc., Berkeley, CA). A result was considered statistically significant if $p < 0.05$.

8.3 Results

Number of licks

A two-way repeated-measures ANOVA showed that for the total number of licks there were significant main effects of both drug ($F_{5,45} = 5.2$, $p < 0.001$) and concentration ($F_{2,18} = 314.8$, $p < 0.001$), but no significant interaction ($F_{10,90} = 1.4$, n.s.). Post hoc analysis showed that only the vehicle/midazolam condition differed significantly from the control vehicle/vehicle condition ($p < 0.01$). As shown in Figure 8.1, midazolam increased the number of licks compared to the control vehicle/vehicle condition. When administered alone, naloxone did not significantly effect the number of licks. However, pretreatment with naloxone significantly attenuated the increase in the number of licks caused by midazolam (Figure 8.1). Post hoc analysis showed that the naloxone/midazolam conditions differed significantly from the vehicle/midazolam condition ($p < 0.05$). The effect of concentration on the total number of licks is shown in Table 8.1. An increase in concentration led to an increase in the total number of licks.

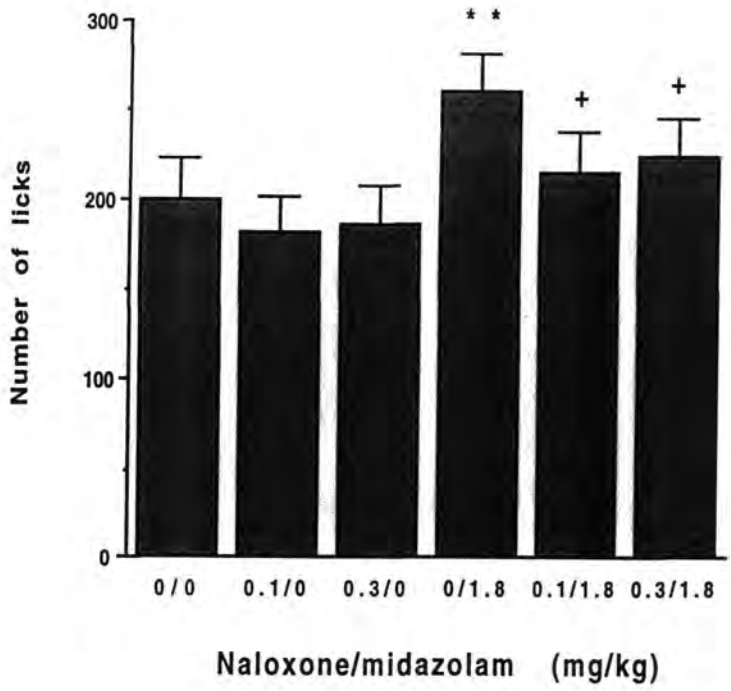


Figure 8.1 The effect of naloxone (0.1 and 0.3 mg/kg) and midazolam (1.8 mg/kg) on the number of licks for Intra-lipid drinking in a brief contact test + S.E.M. Asterisk indicates significantly different from vehicle/vehicle condition ** $p < 0.01$. + indicates significantly different from vehicle/midazolam condition $p < 0.05$ (Dunnett's t-test).

Table 8.1 Main effect of Intra-lipid concentration on total licks

	Total licks ± S.E.M.		
Concentration	1%	3%	10%
Intra-lipid	96.9± 11.8	217.9± 12.4	318.8± 5.2

n = 10 animals per group

Microstructural analysis

Mean bout duration There were significant effects of both drug ($F_{5,45} = 8.2$, $p < 0.001$) and concentration ($F_{2,18} = 25.9$, $p < 0.001$) on mean bout duration, but no interaction between these two factors ($F_{10,90} = 1.6$, n.s.). Administration of naloxone alone had no significant effect on the mean bout duration. Post hoc analysis showed that midazolam significantly increased mean bout duration ($p < 0.01$). Pretreatment with 0.3 mg/kg of naloxone blocked the increase in mean bout duration stimulated by midazolam ($p < 0.01$). Increasing the concentration of Intra-lipid led to an increase in mean bout duration (Table 8.2).

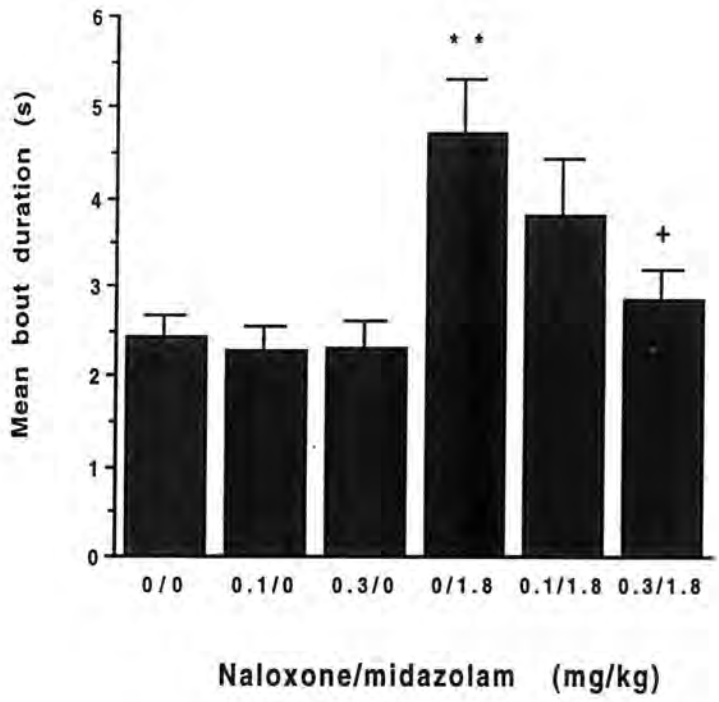


Figure 8.2 The effect of naloxone (0.1 and 0.3 mg/kg) and midazolam (1.8 mg/kg) on mean bout duration for Intra-lipid drinking in a brief contact test + S.E.M. Asterisk indicates significantly different from vehicle/vehicle condition ** $p < 0.01$. + indicates significantly different from vehicle/midazolam condition $p < 0.01$ (Dunnett's t-test).

Table 8.2 Main effect of Intra-lipid concentration on mean bout duration

	Mean bout duration (s) ± S.E.M.		
Concentration	1%	3%	10%
Intra-lipid	1.9± 0.3	2.9± 0.2	4.4± 0.4

n = 10 animals per group

Number of bouts As can be seen in Figure 8.3, the number of bouts was not significantly affected by drug treatment ($F_{5,45} = 0.8$, n.s.). However, there was a main effect of concentration on bout number which is shown in Table 8.3. Increasing concentration led to an increase in bout number ($F_{2,18} = 42.1$, $p < 0.001$). There was no interaction between drug treatment and concentration on the number of bouts ($F_{10,90} = 1.8$, n.s.).

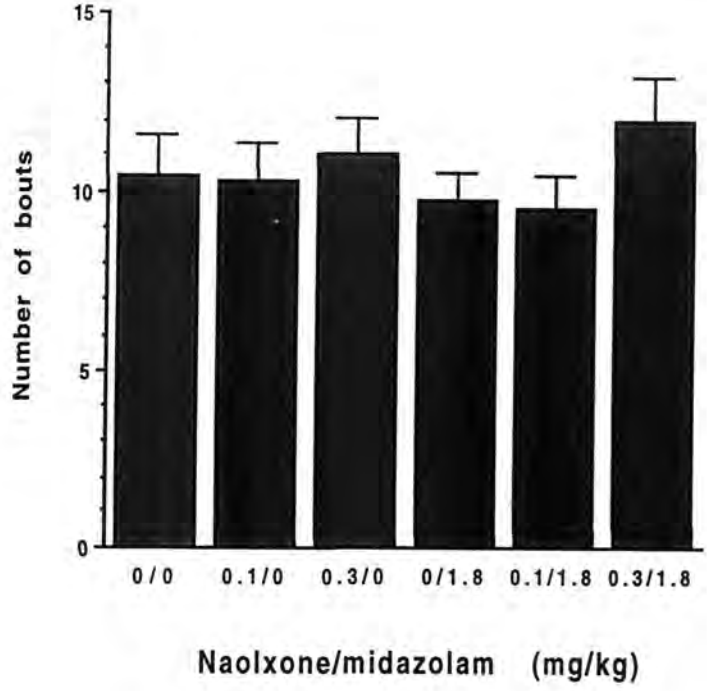


Figure 8.3 The effect of naloxone (0.1 and 0.3 mg/kg) and midazolam (1.8 mg/kg) on bout number for Intra-lipid drinking in a brief contact test + S.E.M.

Table 8.3 Main effect of Intra-lipid concentration on bout number

	Number of bouts ± S.E.M.		
Concentration	1%	3%	10%
Intra-lipid	6.9± 0.7	11.6± 0.7	13.3± 0.5

n = 10 animals per group

Intrabout lick rate Figure 8.4 shows the effect of drug administration on the rate of licking within bouts. A two-way repeated-measures ANOVA revealed a main effect of both drug ($F_{5,45} = 8.3$, $p < 0.001$) and concentration ($F_{2,18} = 13.8$, $p < 0.001$) on intrabout lick rate, although there was no significant interaction ($F_{10,90} = 1.7$, n.s.). Post hoc analysis showed that naloxone administered by itself did not significantly affect the intrabout lick rate. However, administration of midazolam caused a significant reduction in the rate of licking within bouts ($p < 0.01$). The reduction in intrabout lick rate brought about by midazolam was not reversed by pretreatment with naloxone. Post hoc tests showed that the naloxone/midazolam conditions did not differ significantly from the vehicle midazolam condition but did differ significantly from the control vehicle/vehicle condition ($p < 0.01$). Increasing concentration led to a decrease the intrabout lick rate (Table 8.4).

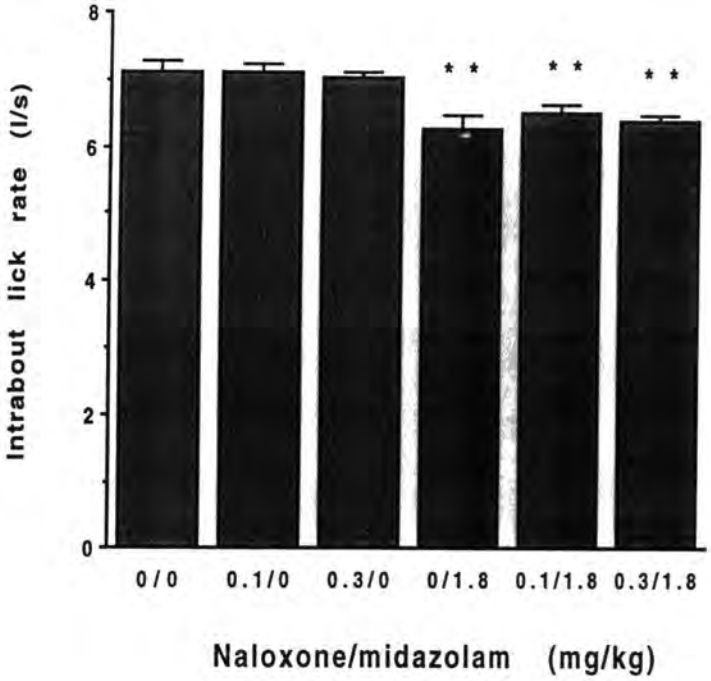


Figure 8.4 The effect of naloxone (0.1 and 0.3 mg/kg) and midazolam (1.8 mg/kg) on intrabout lick rate for Intra-lipid drinking in a brief contact test + S.E.M. Asterisk indicates significantly different from vehicle/vehicle condition ** $p < 0.01$ (Dunnett's t-test).

Table 8.4 Main effect of Intra-lipid concentration on intrabout lick rate

Intrabout lick rate (l/s) $\pm$ S.E.M.

Concentration	1%	3%	10%
Intra-lipid	7.1$\pm$ 0.1	6.7$\pm$ 0.1	6.5$\pm$ 0.05

n = 10 animals per group

Latency There was no main effect of drug on the latency to engage in drinking ($F_{5,45} = 1.3$, n.s.). However, as shown in Figure 8.5 there were certain trends in the data. There was a tendency towards an increase in the latency following administration of naloxone alone or in combination with midazolam, although this did not reach significance. A main effect of concentration on latency was obtained ($F_{2,18} = 8$, $p < 0.01$). There was an inverse relationship between concentration and latency, with higher concentrations leading to a shorter latency to drink. There were no significant drug-concentration interactions ($F_{10,90} = 1.5$, n.s.).

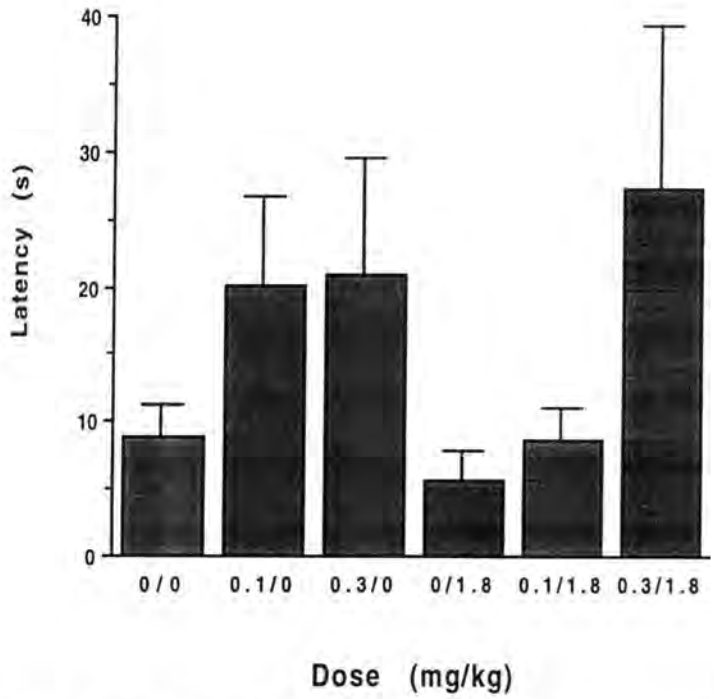


Figure 8.5 The effect of naloxone (0.1 and 0.3 mg/kg) and midazolam (1.8 mg/kg) on Latency for Intra-lipid drinking in a brief contact test + S.E.M.

Table 8.5 Main effect of Intra-lipid concentration on latency

	Latency (s) ± S.E.M.		
Concentration	1%	3%	10%
Intra-lipid	32.2± 7.4	5.4± 1.2	8.2± 3.3

n = 10 animals per group

8.4 Discussion

In Experiment 14, it was shown that midazolam increased the number of licks for Intra-lipid in a brief contact test and that this was due to an increase in the mean bout duration. This result confirms previous findings reported in Chapter 5. When administered alone, naloxone did not affect the number of licks for Intra-lipid. However, pretreatment with naloxone did block the increase in the number of licks caused by midazolam. This result is consistent with previous reports of the ability of naloxone to block the effects of benzodiazepine agonists on food intake in rats and hamsters (Birk and Noble, 1981; Britton et al., 1981; Naruse et al., 1989; Stapleton et al., 1979). Naloxone significantly attenuated the increase in total number of licks by selectively inhibiting the increase in mean bout duration produced by midazolam. Midazolam also decreased the rate of licking within bouts. At the doses of naloxone used in the present experiment, no effect on intrabout lick rate was obtained. When administered in conjunction with midazolam, naloxone did not reverse the effects of this drug on intrabout lick rate. There was no main effect of drug adminstration on the latency to engage in drinking, although there was a tendency for naloxone to increase the latency.

The increase in mean bout duration stimulated by midazolam is consistent with the proposal that benzodiazepines enhance palatability because mean bout duration has been shown to increase monotonically with increasing concentration of Intra-lipid in a brief contact test (Experiment 8). Adminstration of low doses of naloxone blocked the effect of midazolam on mean bout duration. This suggests that endogenous opioid peptides may be involved in the palatability effects of benzodiazepine agonists.

It has been suggested that naloxone may act as a $GABA_A$ antagonist, and so the effect of naloxone may have been due to an action of naloxone at the $GABA_A$ receptor complex. However, it is unlikely that this explanation can account for the effects of naloxone on mean bout duration because there is no evidence that naloxone binds to the $GABA_A$ receptor at the doses used in Experiment 14 (Goldinger, 1981; Gruol, Barker and Smith, 1980). A more plausible explanation is that the enhancement of palatability by

caused by midazolam is due to release of endogenous opioids and that this release is blocked by pretreatment with naloxone.

The results of Experiment 14 do not provide any information concerning the neural substrate for benzodiazepine/opioid interactions in the determination of palatability. However, the PBN may be a likely candidate. In Chapter 3 it was shown that direct administration of midazolam into the PBN resulted in a significant increase in the consumption of a palatable wet mash diet. This suggests that a population of benzodiazepines receptors in the PBN may be important for the effects of benzodiazepines agonists on feeding. In support of this, analysis of the distribution of the opioid receptor subtypes in that rat and human brain has revealed that μ and K receptors are also localised in the PBN (Mansour, Khachaturian, Lewis, Akil and Watson, 1988). In addition, the evoked responses of gustatory neurons in the PBN have been found to be sensitive to morphine (Hermann and Novin, 1980). It has also been shown that feeding elicited by electrical stimulation of the LH can be affected by opioid microinjection of naloxone into the PBN (Carr, Bak and Simon, 1990). This evidence suggests that the PBN may be well placed to mediate benzodiazepine/opioid interactions. There is some evidence which points to a connection between the PBN and the lateral hypothalamus (LH), maybe opioid-dependent, which may be important for interactions between benzodiazepines and opioids in the control of ingestion. For example, Touzani and colleagues (Touzani, Tramu, Nahon and Velley, 1993) have demonstrated that a projection from the lateral hypothalamus to the PBN is immunoreactive to alpha-neoendorphin antisera. Direct injection of morphine into the PBN has been shown to alter saccharin preference, which is in turn affected by lesions of the LH (Moufid-Bellancourt and Velley, 1994; Touzani and Velley, 1990). Although the possibility of the PBN being involved in benzodiazepine/opioid interactions remains to be thoroughly tested, further examination of the contribution of the PBN to the control of eating behaviour may prove to be useful in bringing together much of the neuropharmacological data.

It is not clear from Experiment 14 which opioid receptor subtype is involved in the effects of naloxone. However, evidence from previous studies indicates that activity at the δ subunit may be important. Jackson and Sewell (1985) showed that the hyperphagic effect of diazepam in rats was blocked by the selective δ antagonist ICI 154, 129.

The results from Experiment 13, reported in Chapter 7, showed that when administered alone, naloxone (0.3-3 mg/kg) decreased the number of licks for Intra-lipid by decreasing the number of bouts. It was suggested that this effect of naloxone may have occurred as a result of alterations in incentive salience. Consideration of the results of both Experiments 13 and 14, suggests that opioid peptides may play multiple roles in the control of feeding. The ability of naloxone to block the increase in mean bout duration induced by midazolam obtained in Experiment 14 suggests that opioids may be involved in the palatability effects of benzodiazepines. However, the decrease in bout number obtained at higher doses of naloxone in Experiment 13 also suggests that these drugs may have a role to play in incentive salience attribution. It is possible that the opioids affect both palatability and incentive salience and these effects depend on different receptor subtypes located in distinct brain areas. Further work is required to examine the neural and pharmacological basis of the effects of opioid peptides on food reward.

The results of Experiment 14 suggest that the inhibitory action of naloxone is specific to the palatability effects of midazolam. The decrease in intrabout lick rate produced by midazolam was not blocked by naloxone. This result is in agreement with the report that the muscle relaxant effects of chlordiazepoxide are not antagonized by naloxone (File, 1982). Agmo and colleagues (Agmo, Galvan, Heredia and Morales, 1995) also showed recently that naloxone did not block the motor impairment induced by chlordiazepoxide and diazepam in a rotorod test. Therefore, endogenous opioids may be important for the palatability effects of benzodiazepines but not for the motor effects of these drugs.

There was no effect of naloxone or midazolam on the latency to start drinking. These data agree with the results of Experiments 10 and 13 and have been discussed in previous chapters.

In summary, the results of Experiment 14 showed that the increase in total number of licks for Intra-lipid stimulated by midazolam in a brief contact test was blocked by pretreatment with naloxone. This effect of naloxone was due to a selective attenuation of mean bout duration. The decrease in intrabout lick rate brought about by midazolam was not reversed by naloxone. These results suggest that endogenous opioid peptides may be involved specifically in the palatability effects of benzodiazepine receptor agonists.

General discussion

9.1 Introduction

The aim of the experiments reported in this thesis was to provide new information concerning the neural and behavioural mechanisms involved in the effects of benzodiazepine receptor ligands on ingestion. The purpose of this chapter is to examine whether these aims have been fulfilled and discuss the implications of the results obtained. This discussion is divided into five sections. The first section deals with the findings reported in Chapters 2 and 3 and the conclusions which can be drawn concerning the potential neural substrate for benzodiazepine-induced hyperphagia. The second section discusses the effects of benzodiazepine ligands on the microstructure of licking behaviour (Chapters 5 and 6), and what these results reveal about the behavioural mechanisms underlying benzodiazepine effects on ingestion. The third section is concerned with evaluating the findings reported in Chapters 7 and 8 and what they signify for the hypothesis that the effects of benzodiazepines on ingestive behaviour are related to endogenous opioid activity. Proposals for future work which may help to elucidate further the neural and behavioural bases for benzodiazepine effects in ingestion are then presented in the fourth section. Finally, the clinical implications of this work will briefly be discussed in section five.

9.2 Neural mechanisms

The aim of the experiments reported in Chapters 2 and 3 was to identify potential brain sites important for the effects of benzodiazepines on ingestive behaviour. Systemic adminstration of drugs does not provide any information concerning the location of the receptor populations responsible for mediating any observed effect. Therefore, a microinjection procedure was employed in the experiments reported in Chapters 2 and 3. This involved injecting the benzodiazepine receptor agonist midazolam directly into the brain to determine potential sites of action of this drug on feeding behaviour.

9.2.1 Brainstem sites

Brainstem sites may be important for the effects of benzodiazepines on ingestive behaviour. For example, Berridge (1988) has shown that the increase in hedonic reactions observed following benzodiazepine adminstration is retained in the chronic decerebrate rat preparation. This suggests that the receptor population(s) responsible for the effects of benzodiazepines on the hedonic reaction to tastants may be located in the brainstem. It is possible that the effects of benzodiazepines in the taste reactivity test are related to the hyperphagic effects of these drugs. Hence, a brainstem receptor population may also be important for mediating the effects of benzodiazepine on food intake. This possibility was investigated in Experiments 1 and 2.

The results of Experiment 1 showed that direct administration of midazolam into the IVth ventricle significantly increased intake of a palatable wet mash diet in non-deprived rats. This increase was blocked by pretreatment with the selective benzodiazepine receptor antagonist flumazenil (Experiment 2). These data suggest that benzodiazepine receptors located in the vicinity of the IVth ventricle may be important for mediating the hyperphagic effects of benzodiazepines.

9.2.2 The role of the PBN

Microinjection of drugs into a ventricle does not provide specific information concerning the location the receptor population(s) responsible for mediating a behavioural effect. The aim of the Experiments reported in Chapter 2 was to assess the contribution of brainstem structures to benzodiazepine effects on food intake. The target for this work was the parabrachial nucleus (PBN) of the pons. The PBN was chosen because it is close to the IVth ventricle and contains a population of benzodiazepine receptors (Higgs et al., 1993). Additionally, this nucleus forms part of the taste projection system. It has been hypothesised that benzodiazepine-induced hyperphagia may be related to changes taste processing, and so it was thought that the PBN would be a prime candidate to mediate the effects of benzodiazepines on food intake.

In Experiment 3, direct injection of midazolam into the PBN resulted in a significant and dose-related increase in the consumption of a palatable wet mash diet and a 3% sucrose solution. This hyperphagic effect was blocked by pretreatment with flumazenil. However, in cases where midazolam was injected to sites outside the PBN, no effect on intake was observed. These results indicate that benzodiazepine receptors located in the PBN may constitute a population of receptors responsible for mediating the effects of benzodiazepine on ingestive behaviour.

Evidence from Experiment 6 suggests that the benzodiazepine receptor population in the PBN may be specific for the effects of benzodiazepine agonists on ingestive behaviour. Intra-PBN midazolam had no effect on locomotor activity. This result contrasts with the effects of systemically administered benzodiazepines which cause a hyperlocomotion at low doses and a hypolocomotion at high doses. Further work is required to test the hypothesis that benzodiazepine receptors in the PBN are specific for the effects of these drugs on ingestion (see section 9.6). However, a molecular basis for such a dissociation may be provided by the diversity observed in the $GABA_A$ receptor complex. Benzodiazepine receptors in the PBN may be coupled to a $GABA_A$ receptor subtype, and allosteric modulation of this subtype may lead to specific effects on ingestive behaviour. Testing of this hypothesis awaits the development of compounds specific for particular subunit combinations.

9.2.3 Implications for the neural control of ingestion

The results of the experiments reported in Chapters 2 and 3 have implications for traditional views of the neural control of ingestive behaviour. It has been suggested that the brain functions in a hierarchical fashion, whereby top level components exert control over low level structures (see Grill and Berridge, 1985, for discussion). Within this framework it is assumed that the extent to which neural integration occurs is greater in structures near or at the top of the hierarchy. This model rests on the premise that the hierarchy is constructed along anatomical lines, with the cortex exerting control over more caudal structures assumed to be much less sophisticated in their integrative

capacities. The results presented in Chapters 2 and 3, taken in conjunction with the findings of Grill and Norgren (1978a,b) and Berridge (1988), have important implications for this classical view of brain functioning. Thus, the effects of CDP on ingestive responding in the intact and decerebrate rat have been shown to be identical. Additionally, direct stimulation of the brain stem with benzodiazepine receptor agonists has been shown to result in normal taste reactivity and ingestive responses. Therefore, it appears that brainstem circuitry is capable of performing the complex integrations necessary for responding normally to taste stimuli. This is at odds with the assumption that the control of complex behaviours can only occur in more rostral brain structures.

The results from Chapters 2 and 3 suggest that the hierarchical view of the neural control of ingestion and other behaviours needs to be reevaluated. Alternative possibilities might include a two-way hierarchy involving reciprocal connections between higher and lower level components, or distributed processing of information. However, assessment of these alternative models requires the formation of testable hypotheses based on theoretical advances which unfortunately are some way off.

9.2.4 Conclusions

In summary, the results presented in Chapters 2 and 3 of this thesis suggest that benzodiazepine receptors located in the brainstem, possibly in the PBN, may constitute an important site of action for the effects of these drugs specifically on ingestive behaviour.

9.3 Behavioural mechanisms

Analysis of changes in the rate of licking for fluids and the examination of licking behaviour at a microstructural level provides information concerning the factors determining intake (Davis and Levine, 1977; Davis and Smith, 1992). The aim of the experiments in Chapters 5 and 6 was to use a microstructural approach to investigate the behavioural mechanisms involved in the effects of benzodiazepines on ingestive behaviour.

Manipulating the concentration of a solution is assumed to change its palatability which is then thought to be reflected in specific changes in the microstructure of ingestion. On the basis of evidence from taste preference, sham feeding and taste reactivity studies, it has been suggested that the effects of benzodiazepines on ingestive behaviour are due to changes in palatability (Berridge and Pecina, 1995). The aim of the experiments reported in Chapter 5 was to compare the effects of midazolam on licking behaviour with the effects of increasing in concentration. The rationale was that any similarity between the effects of midazolam and increasing concentration would be consistent with the view that midazolam enhances palatability.

Control over ingestive behaviour can be exerted bidirectionally at the level of the benzodiazepine receptor (Cooper, 1985b). The aim of the experiments reported in Chapter 6 was to examine this hypothesis by investigating whether the effects of the benzodiazepine inverse agonist Ro 15-4513 are opposite to those of midazolam at the microstructural level.

An additional aim of the Experiments reported in Chapter 5 and 6 was to examine the generality of the effects of benzodiazepine on ingestion by investigating the effects of midazolam and Ro 15-4513 on the ingestion of either fats or carbohydrates.

9.3.1 What can microstructural analysis tell us?

One problem with models of ingestive behaviour based on microstructural analysis has been that experimental validation of these models has been limited to the study of carbohydrates. Before any drug effects could be examined in this thesis, it was necessary to establish whether the effects of manipulating carbohydrate concentration on the microstructure of licking were the same as the effects observed when manipulating fat concentration. The effect of manipulating the concentration of a carbohydrate (sucrose) and a fat (Intra-lipid) on the rate and microstructure of licking are described below. The aim of these experiments was to determine if changes in various microstructural parameters can provide reliable information concerning the factors involved in influencing intake of both fats and carbohydrates.

Initial rate of licking

In Experiment 7, the initial rate of licking increased monotonically with increasing concentration of both sucrose and Intra-lipid. This contrasted with the relationship between concentration and intake which varied non-monotonically, probably due to an increase in the strength of an inhibitory negative feedback signal at higher concentrations. These results suggest that the initial rate of licking reflects changes in the palatability of both sucrose and Intra-lipid.

Mean bout duration

In the 20-min test employed in Experiment 7 there was no clear relationship between fluid concentration and mean bout duration. However, when the test session was limited to 60s, mean bout duration was found to vary monotonically with concentration of both sucrose and Intra-lipid. The effect of fluid concentration on mean bout duration was therefore dependent on the length of the test session. This suggests that it is important to consider the length of the test session when interpreting changes in the microstructure of licking. Providing the test session is kept short, mean bout duration provides a reliable measure of changes in the palatability of both sucrose and Intra-lipid. However, in longer term tests, mean bout duration may be affected by factors other than palatability.

Whether changes in mean bout duration in a brief contact test reflect changes in the palatability of all fats is not clear. Davis and colleagues (1995) did not find that mean bout duration increased with increasing the concentration of corn oil. One explanation for this finding is that mean bout duration may provide a measure of changes in taste palatability but not the palatability associated with other orosensory factors such as texture. Further investigation of the effect of manipulating the concentration of different fats on mean bout duration is required to elucidate more precisely what changes in this parameter indicate (see section 9.6).

Number of bouts

There were no systematic effects of concentration on the number of bouts for sucrose and Intra-lipid in the 20-min (Experiment 7) or 60s test sessions (Experiment 8). This suggests that changes in bout number do not provide information concerning changes in palatability.

Although manipulating concentration in Experiment 8 did not significantly affect bout number, in experiments reported in later chapters, in which the effects of manipulating both concentration and drug dose were measured, increasing concentration led to a monotonic increase in bout number. This discrepancy may have been due to differences in the protocols used in the different experiments. In Experiment 8, the rats were trained on one concentration of sucrose and Intra-lipid and then had access to all concentrations in a random order. In later experiments, the rats were trained to consume all concentrations of sucrose and Intra-lipid over a prolonged training period before the main effects of drug dose and concentration were investigated. Changes in bout number may have reflected changes in an underlying process dependent on the rats having previous experience of the test fluids. The question is: What could that process be?

It has already been suggested in this thesis that manipulating concentration alters the palatability of ingested fluids, which is then reflected in changes in mean bout duration. However, besides their affective properties, sucrose and Intra-lipid also act as incentives, i.e. they are targets for motivated behaviour (Toates, 1986). The effect of concentration on bout number may have been due to changes in incentive motivation. If a change in incentive motivation is to account for the effect of concentration on bout number then two important questions must be addressed. First, is it predicted that a change in incentive motivation should lead to an effect on bout number? Second, is the process of incentive motivation affected by previous experience with the stimuli? In answering both questions it is necessary to discuss the concept of incentive salience (Berridge, 1996; Toates, 1986).

Incentive salience refers to the attractiveness of a stimulus or its ability to direct approach behaviour. The attribution of incentive salience results from a three-stage

process that first involves activation of the neural substrate responsible for mediating pleasure. Second, pleasure is associated with the stimulus via associative learning. Third, salience is attributed to the representation associated with the stimulus. Incentive motivation theory predicts that because an increase in the concentration of sucrose and Intra-lipid increases positive affect, it should also result in an increase in incentive salience. An increase in incentive salience would result in an increase in instrumental behaviour directed towards the stimulus which might be manifested in an increase in bout number. For example, an increase in the attractiveness of the stimulus might mean that the animal returns to it more often, thus increasing the number of bouts. Importantly, this process would be dependent on previous exposure with the stimuli because experience would be necessary for the attribution of salience. Although there is no direct evidence to support this conclusion, it is reasonable that changes in incentive salience might be reflected in changes in bout number. To test this hypothesis it would be necessary to examine the effect of manipulations which are thought to affect incentive salience (e.g. changes in dopamine transmission), and establish if these manipulations affect bout number (see section 9.6).

Intrabout lick rate

There was a significant effect of fluid concentration on intrabout lick rate in Experiment 7. This result was unexpected because it had been hypothesised that the intrabout lick rate is relatively constant across a range of experimental conditions. For example, it has been shown that the rate of licking within bouts is not affected by manipulating concentration, but is altered by moving the drinking spout progressively further away from a rat (Davis and Smith, 1992). Consequently, it has been suggested that the intrabout lick rate may provide a measure of changes in motor capacity. The results of Experiment 7 do not support the suggestion that intrabout lick rate provides a pure measure of motor dysfunction, because this parameter was also influenced by manipulating concentration. It is conceivable that changes in viscosity at higher concentration disrupted the rat's ability to perform the necessary movements to lick

properly. The conclusions which can be drawn from these results are that changes in intrabout lick rate must be interpreted with caution and additional work is required to investigate the conditions under which this measure is disrupted.

Latency

Changes in latency may provide a measure of motor deficits induced by an experimental manipulation. The rationale for this is that if a treatment induces a motor impairment then this might prevent an animal from approaching the lick spout. From this it would be predicted that manipulating concentration would have no effect on the latency to start drinking. However, in Experiment 7, manipulating concentration did have an effect on latency. There was a decrease in latency with increasing sucrose concentration. This suggests that latency may also be sensitive to factors associated with changes in concentration, such as palatability. One explanation for the effect of concentration on latency is that changes in this parameter also reflect changes in the motivation to drink.

There is evidence to suggest that the basis for this effect may be olfactory cues. Using a brief contact test, Rhinehart-Doty and colleagues (1994) found an inverse relationship between concentration and latency and that this relationship was no longer evident when olfactory cues were masked. It is possible that the animals learned that certain olfactory cues were associated with highly palatable (more concentrated) solutions and that this influenced the time taken before drinking commenced. The conclusion from this is that the latency to engage in drinking may be influenced by palatability via olfactory learning. Therefore, changes in latency may not only reflect motor impairments but also changes in palatability. However, the results from Chapter 4 also suggest that the effect of concentration on latency is not reliable because no effect of manipulating concentration was observed in Experiment 8. The conclusion to be drawn from these results is that many factors may lead to a change in latency and so any effect on latency may be difficult to interpret unless considered in conjunction with changes in other parameters.

9.3.2 Conclusions

The results from Chapter 4 showed that manipulating concentration had similar effects on the pattern of licking for both sucrose and Intra-lipid. This suggests that the microstructural approach can provide information concerning the factors involved in controlling intake of carbohydrate and at least one source of fat, i.e. Intra-lipid. The data suggest that the initial lick rate can provide information concerning changes in palatability. In a brief contact test, changes in mean bout duration also reflect changes in palatability. The effect of manipulating concentration in naive versus experienced animals suggests that changes in bout number may be indicative of changes in incentive salience, although this hypothesis requires further validation. Intrabout lick rate was not found to be as resistant to changes in concentration as previously suggested. Although this measure may provide information concerning impairment of motor acts required in ingestion, some caution needs to be exercised in interpreting changes in intrabout lick rate. Similarly, the latency to engage in drinking may be influenced by many factors including motor impairment but also changes in motivation which may be related to olfactory learning. Therefore, changes in latency should be interpreted with respect to changes in other parameters.

9.3.3 Effects of benzodiazepine ligands on microstructural parameters

Midazolam had similar effects on the pattern of licking of both sucrose and Intra-lipid. This is the first detailed demonstration of the effects of benzodiazepine on the ingestion of a fat. The results suggest that the effects of benzodiazepines in ingestive behaviour are not limited to prototypic taste stimuli but may also extend to the consumption of fats. However, although microstructural analysis revealed similarities in the effects of midazolam on sucrose and Intra-lipid drinking, had intake been the only measure a different conclusion would have been reached because midazolam increased intake of sucrose but not Intra-lipid. This highlights the value of microstructural analysis and shows that benzodiazepine agonists may affect patterns of ingestion without actually

increasing intake. Therefore, it is not benzodiazepine-induced hyperphagia per se, but the processes which may, or may not, lead to increases in food intake which require study. Some evidence as to the mechanisms involved can be gleaned from the results of experiments in Chapter 5.

Midazolam increased the initial lick rate for sucrose and Intra-lipid in a 20-min test. This is consistent with the hypothesis that benzodiazepine agonists affect the hedonic evaluation of ingested foods, and is in agreement with the effect of midazolam in other tests of palatability, including the sham feeding preparation and taste reactivity paradigm. The increase in palatability caused by midazolam did not result in an increase in intake for Intra-lipid drinking because the increase in the initial lick rate led to faster accumulation of Intra-lipid in the GI tract. This may have then led to a stronger negative feedback signal which brought ingestion to a close more quickly. This suggests that changes in palatability can be dissociated from changes in intake. Increases in palatability need not lead to an increase in intake. Further evidence to support an effect of midazolam on palatability comes from the effect of this drug in the brief contact test employed in Experiment 10. Midazolam increased the total number of licks for sucrose and Intra-lipid by increasing the mean bout duration.

Midazolam also decreased the intrabout lick rate. This effect is consistent with the known muscle relaxant effects of midazolam. Midazolam did not affect latency to engage in drinking which may indicate that it was not affecting the rat's ability to access the spout or its motivation to drink.

The effects of the benzodiazepine receptor inverse agonist Ro 15-4513 on the pattern of licking in a brief contact test was found to be the opposite to the effects of increasing concentration. Ro 15-4513 decreased the number of licks for sucrose and Intra-lipid by decreasing mean bout duration. This suggests that Ro 15-4513 was decreasing palatability. The proposal that the effects of Ro 15-4513 results from a change in palatability fits in with the notion of bidirectional control of ingestive behaviour mediated by specific benzodiazepine receptors, as first proposed by Cooper (1985b). According to this theory the effects of benzodiazepine receptor inverse agonists on

feeding should be opposite to that of classical agonists. The results reported in Chapter 5 suggest that benzodiazepine agonists influence ingestive behaviour by increasing palatability. The effects of Ro 15-4513 on licking patterns is consistent with the proposed bidirectional control of feeding, since they confirm that benzodiazepine receptor inverse agonists decrease consumption as a consequence of a reduction in the palatability of ingested foods.

It might be expected that the receptor population mediating benzodiazepine-induced changes in palatability should be located in brain regions with associations with orosensory systems. In Chapter 3 it was suggested that the PBN may contain benzodiazepine receptors responsible for mediating the effects of benzodiazepines on ingestive behaviour. The PBN makes a connection with the pontomedullary parvocellular reticular formation (PCRt) (Herbert, Moga, and Saper, 1990). The PCRt may integrate viscerosensory and orosensory inputs for the modulation of oromotor systems involved in the control of licking behaviour (Ter Horst, Liem, and van Willigen, 1991). A connection between the PBN and PCRt might provide the neural basis for benzodiazepine-induced changes in licking behaviour although this remains to be thoroughly investigated.

9.3.4 Benzodiazepines and taste palatability?

The effects of benzodiazepines ligands on licking patterns suggests that these drugs exert bidirectional control over ingestive behaviour by modulating palatability. The possibility that benzodiazepine receptors in the PBN may mediate the effects of midazolam and Ro 15-4513 on licking behaviour suggests that these effects may be related specifically to taste palatability because the PBN forms part of the gustatory system. The stimulatory properties of fats may be related to oral tactile mechanisms rather than gustatory mechanisms. In support of this, it has been shown that in human psychophysical experiments, texture is the stimulus dimension which correlates most with fattiness (Mela, 1988). It is interesting therefore that midazolam and Ro 15-4513 affected ingestion of both sucrose and Intra-lipid. This result may suggest that the effects

of benzodiazepines on ingestion are related to the palatability associated with both taste and other orosensory factors. However, because there has been no systematic attempt to examine the taste stimulating properties of fats, it is not clear at this time whether gustatory mechanisms play a role in the control of Intra-lipid intake. The mechanisms involved in Intra-lipid consumption may be different from those involved in the intake of other fats such as corn oil. For example, Davis et al., (1995) did not find that mean bout duration varied as a function of concentration of corn oil concentration, whereas mean bout duration for Intra-lipid drinking in Experiment 8 did increase monotonically as a function of concentration. It could be that changes in mean bout duration reflect changes in gustatory rather than somatosensory stimulation. Additional work is required to examine whether the effect of manipulating concentration on bout duration is related specifically to gustatory stimulation, before any firm conclusions can be drawn concerning the specificity of benzodiazepine effects specifically on taste stimuli (see section 9.6).

9.3.5 Conclusions

A microstructural analysis of licking behaviour revealed that midazolam increased the initial rate of licking for both sucrose and Intra-lipid in a 20-min test. In a brief contact test, midazolam increased the number of licks for sucrose and Intra-lipid by increasing the duration of bouts. This evidence is consistent with the hypothesis that benzodiazepine agonists enhance palatability. The benzodiazepine receptor inverse agonist had the opposite effect to midazolam in a brief contact test. Ro 15-4513 decreased the number of licks for sucrose and Intra-lipid by decreasing mean bout duration. These data suggest that Ro 15-4513 reduces palatability, and are consistent with the notion of bidirectional control of ingestive behaviour at the level of the benzodiazepine receptor. The data also suggest that the effects of benzodiazepine on ingestive behaviour are not limited to prototypical taste stimuli but also extend to the consumption of at least one fat: Intra-lipid.

9.4 Benzodiazepine/opioid interactions

The effects of benzodiazepines on ingestive behaviour may be related to release of endogenous opioid peptides (Cooper, 1983a). This proposal is based on two lines of evidence. First, it has been noted that the effects of benzodiazepine receptor ligands on ingestive behaviour are similar to the effects of opioids (Cooper and Higgs, 1994). Second, it has been demonstrated that the effects of benzodiazepines on food intake can be blocked by pretreatment with opioid antagonists (Birk and Noble, 1981; Britton et al., 1983; Naruse et al., 1989). The aim of the experiments reported in Chapters 7 and 8 was to use a microstructural approach to examine the proposed interaction between benzodiazepines and opioids in the control of ingestive behaviour in more detail.

Microstructural analysis of the effects of morphine and naloxone on the licking patterns for Intra-lipid in a brief contact test was performed (Chapter 7). Morphine and naloxone affected the total number of licks for Intra-lipid by altering bout number. Morphine increased the number of licks by increasing bout number and naloxone decreased the number of licks by decreasing bout number. These effects contrasted with the effects of benzodiazepine ligands on licking behaviour obtained in Chapters 5 and 6.

The results do not support the argument that the effects of benzodiazepines and opioids on ingestive behaviour are similar, and therefore have implications for the proposed interaction between benzodiazepines and opioids. The lack of similarity between the effects of opioids and benzodiazepines on licking behaviour does not rule out an interaction, but indicates that any putative interaction requires further investigation. A detailed examination of benzodiazepine/opioid interactions on ingestion was carried out in Chapter 8.

The effect of pretreatment with the opioid antagonist naloxone on the licking patterns induced by midazolam was examined in Experiment 14. Midazolam increased the total number of licks by increasing mean bout duration which is consistent with an effect of this drug on palatability. Naloxone attenuated the increase in the total number of licks brought about by midazolam by selectively attenuating the increase in mean bout duration. The fact that naloxone blocked this increase in mean bout duration suggests that

opioid peptides may be involved in the effects of benzodiazepines agonists on ingestive responding.

Naloxone did not block all the effects of midazolam on licking microstructure because the decrease in intrabout lick rate caused by midazolam was not reversed by pretreatment with naloxone. This suggests that opioid peptides maybe involved selectively in mediating the effects of benzodiazepines on palatability.

The effects of morphine and naloxone on licking patterns deserve further attention because they do not support the hypothesis that opioid peptides influence palatability. Morphine has been shown to enhance ingestive responding in the taste reactivity test thus suggesting that it may influence the hedonic evaluation of food stuffs. However, closer examination of the literature indicates that this effect is not very robust, and is obtained in some studies (Doyle et al., 1993) but not others (Parker et al., 1992). An alternative hypothesis to explain the effects of opioids on licking behaviour is that the effect of these drugs reflects changes in incentive salience attribution. The results of Chapter 4 suggest that changes in bout number may be indicative of changes in incentive salience and so the effects of morphine and naloxone on bout number may be due to an effect on incentive salience attribution. However this hypothesis is tentative and requires further testing (see section 9.6)

The results presented in this thesis demonstrate that it is possible to pharmacologically affect bout number without affecting mean bout duration and vice versa. Such a double dissociation supports the notion that these measures reflect different underlying processes with different neural substrates. This is similar to the distinction drawn by Berridge (1996) who has also suggested that separate neural systems are responsible for mediating wanting (incentive salience) versus liking (palatability). It is possible that microstructural analysis of licking behaviour is a method which allows the neural basis of these two processes to be investigated and suggest that further use of this method may provide information concerning the neural controls of wanting and liking.

9.5 Summary

The experiments reported in this thesis suggest that receptors located in the brainstem, more specifically in the PBN may be responsible for mediating the effects of benzodiazepines on food intake. Location of benzodiazepine receptors in an area involved in taste processing is consistent with the subsequent finding that benzodiazepines alter ingestive behaviour by modulating palatability. The results also suggest these effects may be dependent to some degree at least on release of opioid peptides.

9.6 Future work

There are a number of experiments which arise from the current work which would help to elucidate further the brain and behavioural mechanisms involved in the effects of benzodiazepines on ingestive behaviour.

The results of experiments reported in Chapter 3 suggest that the PBN may be an important site of action for the effects of benzodiazepines on food intake. This possibility could be examined further by investigating the effects of lesions of the PBN on benzodiazepine-induced hyperphagia. If an intact PBN is necessary for the effects of benzodiazepines on ingestive behaviour then lesioning the PBN would be predicted to abolish the ability of systemically administered benzodiazepine agonists to stimulate a hyperphagia. However, if the PBN is merely sufficient for benzodiazepine effects on ingestion, then lesioning the PBN would not be expected to block all forms of benzodiazepine-induced enhancement of ingestive behaviour. This would suggest that other brain sites are involved which need to be identified. This possibility could be examined by performing a cannula-mapping study. Other brain sites known to be involved in feeding behaviour such as the NTS, hypothalamus and amygdala, are possible candidates for such a study.

It would also be of interest to extend the pharmacological and behavioural investigation of the role of the PBN in benzodiazepine-induced hyperphagia. First, by examining the effect of administration of other agonists and inverse agonists into this area. Second, it is important to determine if the hyperphagic effect of intra-PBN

midazolam is due to an enhancement of palatability by examining the effect of injection into the PBN in the sham feeding and taste reactivity paradigm.

The microstructural analysis of sucrose and Intra-lipid drinking carried out in Chapter 4 raises some interesting questions concerning the processes reflected in changes in microstructural parameters. Increasing Intra-lipid concentration in chapter 4 was found to increase mean bout duration suggesting that changes in mean bout duration may reflect changes in palatability associated with changes in concentration of this fat. However, Davis et al., (1995) did not find that changes in the concentration of corn oil led to changes in mean bout duration. To resolve this discrepancy it would be necessary to examine the effect of manipulating the concentration of other fats to determine if Intra-lipid is a special case. The effect of Intra-lipid concentration on mean bout duration may be related to the taste properties of this fat. This hypothesis could be tested by examining the response of gustatory neurones to different concentrations of this fat.

In Chapter 4 it was suggested that changes in bout number may reflect changes in incentive salience, but that this proposal needed further validation. There is evidence to suggest that dopamine is involved in incentive salience attribution (Berridge et al., 1989; Robinson and Berridge, 1993). Therefore, it would be useful to examine whether drugs which affect dopamine transmission alter bout number in a brief contact test. It would be predicted that dopamine receptor antagonists would decrease the number of licks in a brief contact test by decreasing bout number.

It was also shown in Chapter 4 that the intrabout lick rate was influenced by changes in the concentration of sucrose. In subsequent chapters it was shown that under some circumstances changes in intrabout lick rate are observed following increases in Intra-lipid concentration. This indicates that further experiments are required to clarify the factors involved in influencing intrabout lick rate. It is possible that viscosity influences this parameter. One way of examining this proposal would be to add an agent which thickened the fluids without affecting their taste properties, such as guar gum. It is also possible that palatability factors affect the intrabout lick rate. Therefore, it would be of

interest to look at how lick rate varies both within bouts of drinking and across test sessions.

Following on from microstructural investigation of the effects of systemically administered benzodiazepines it would be revealing to look at the effects of these drugs administered into brain sites such as the PBN. In Chapter 5, systemically administered midazolam increased mean bout duration, but also decreased the intrabout lick rate. In Chapter 3 it was hypothesised that the receptor population in the PBN might be specific for the effects of benzodiazepines on ingestive behaviour. Convergence of these two hypotheses would predict that intra-PBN midazolam would have a specific effect on mean bout duration but would not affect the intra-bout lick rate. However, this hypothesis remains to be tested.

The experiments presented in Chapter 8 provide evidence to suggest an interaction between benzodiazepines and opioids in the determination of palatability. Further studies could identify the specific opioid receptor subtypes responsible for mediating these effects. This could be done by using specific opioids receptor antagonists to block the effects of midazolam on mean bout duration. It would also be interesting to examine the possibility that the interaction between opioid and benzodiazepines may occur in the PBN by investigating the effects of direct injection of opioid antagonists into the PBN on the hyperphagic effects of benzodiazepines. It was suggested in Chapter 8 that opioids may have several effects on ingestive behaviour. Therefore, it would be of interest to compare the effects of receptor selective opioid ligands on the microstructure of licking to see if differences in the effects of these ligands can be highlighted.

9.7 Clinical implications

The experiments in this thesis suggest that the effects of benzodiazepines on ingestive behaviour may be due to changes in the palatability of ingested foods and this effect may occur early in the processing of taste stimuli. Drug action at specific benzodiazepines receptor populations in the brainstem may affect the processes involved in assigning hedonic value to food items and therefore modulate normal patterns of ingestion. This suggests that the acceptability of ingested foodstuffs may depend on drug action at benzodiazepine receptors.

Benzodiazepines may also have an important role to play in the understanding and treatment of eating disorders such as obesity and bulimia and anorexia. Changes in endogenous benzodiazepine levels may lead to alterations in benzodiazepine receptor function which then results in abnormal ingestive patterns. This suggests that the development of new drugs targeted at benzodiazepines receptor subtypes specific for ingestive behaviour could hold the key for therapeutic advances in the treatment of eating disorders. Selective inverse agonists may be especially useful in the treatment of obesity. One reason for the high incidence of obesity in Western cultures may be the easy access which people have to vast range of highly palatable food items which often leads to overconsumption. Selective attenuation of the palatability associated with these foods may aid people who for health reasons are required to reduce their weight.

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