THE EFFECTS OF THE INTEGRATION OF ORAL AND GASTRIC STIMULATION ON FOS-LIKE IMMUNOREACTIVITY IN THE HINDBRAIN AND BEHAVIOURAL OUTCOME

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ABSTRACT

Conceptual and empirical analyses of the controls of feeding have emphasized the role of peripheral factors in the control of ingestive behaviour. Currently, it is understood that feeding is under the control of multiple peripheral factors. It follows that if eating is the result of multiple signals, it is necessary to design experiments that examine the ways in which these signals interact and combine to influence eating.

The experiments in this thesis accommodate the acknowledged multifactorial control of eating by examining the interaction of two classes of peripheral signals - oral (from the mouth) and gastric (from the stomach). The aim of the experiments is to determine how these two classes of signals interact to influence activity in the CNS (central nervous system) Fos immunohistochemistry was used as the marker of neural activity because of its practicality in determining brain areas activated by experimental treatments and since the technique provides a means to quantify the number of neurons activated.

The first experiment was designed to confirm that Fos immunoreactivity could be used as a marker of

neural activity induced under "physiological" conditions of feeding. The second experiment used sham feeding and real feeding to compare the patterns of neural activity stimulated by oral stimulation in isolation (sham feeding) or oral and gastric stimulation in combination (real feeding). The third experiment specifically compared FLI patterns induced by oral and gastric stimulation, each presented alone, or in combination under conditions of strict experimenter control. The fourth experiment continued this line of inquiry by addressing whether the order of the two stimulations, one appropriate (oral followed by gastric) and the other reversed (gastric followed by oral), influenced the Fos-like immunoreactivity (FLI) patterns seen in previous experiments. The final experiment examined whether the effect of order also had functional consequences with regard to the control of eating.

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GLOSSARY

AP-1	activator protein 1
cAMP	cyclic adenosine monophosphate
CCK	cholecystokinin
CNS	central nervous system
DMN	dorsal motor nucleus of the vagus
GABA	*gamma*-aminobutyric acid
HG	hypoglossal nucleus
FLI	Fos-like immunoreactivity
mRNA	messenger ribo nucleic acid
NMDA	N-methyl D-aspartate
NST	nucleus of the solitary tract

CHAPTER 1. GENERAL INTRODUCTION

Conceptual and empirical analysis of the controls of feeding have emphasized the role of peripheral factors in the control of ingestive behaviour (Smith and Gibbs, 1979). Much of the work in this area has sought to identify the peripheral signals that control the initiation or termination of meals. In the context of satiety or meal termination, discrete signals such as gastrointestinal distention (Smith and Gibbs, 1979), cholecystokinin (CCK) (Smith et. al, 1988) and intestinal osmolarity (Kissilef and Van Itallie, 1982), have been identified as playing some role in meal termination. Although it is currently understood that feeding is under the control of multiple peripheral factors, there have been relatively few experiments that attempt to determine how the various peripheral feeding signals interact to result in the termination of feeding. It follows that if eating is the result of multiple signals, it is necessary to design experiments that examine the ways in which these signals interact and combine to influence eating. It is only by carrying out these types of combination studies, that a clear

understanding of the multifactorial control of feeding can be achieved.

The experiments in this thesis examine the interaction of two classes peripheral signals - oral (from the mouth) and gastric (from the stomach). The aim of the experiments is to determine how these two classes of signals interact to influence activity in the CNS (central nervous system) and the feeding behaviour of the animal.

REVIEW OF SYNERGY EXPERIMENTS

Peripheral signals have been envisioned to interact in two ways to affect eating. The first are simple additive models where the independent effects of two signals simply sum algebraically when presented in combination. The more intriguing alternative, and one that demonstrates the difficulty of examining combinations of signals, are those few examples where peripheral signals combined synergistically, that is multiplicatively, when presented in an appropriate combination (Schwartz et al. 1991; McHugh and Moran, 1986; Forsyth et al. 1985).

Even before these synergistic types of integrative studies were being performed, there was

evidence that peripherial signals worked in concert with each other to produce their maximal satiety effects. The inability of oral and gastric stimulation alone to produce the same degree of satiety as when they are presented concurrently has been well documented, although the mechanisms controlling this phenomenon remain unclear. Numerous nutrient-loading experiments, that isolate gastric and post gastric stimulation, have demonstrated that food eaten normally, activating both oral and gastric mechanisms, produces greater satiating effects than food delivered directly into the stomach (ie. activating gastric mechanisms only) (Berkun, Kessen & Miller, 1952; Kisselef & Van Itallie, 1982; Miller, 1957). In preparations that isolate oral stimulation, such as sham feeding, rats will often feed three times the amount they would normally feed (Weingarten, 1990). Taken together, these experiments demonstrate that oral and gastric stimulation must be presented in the temporal context of specific peripheral feeding signals if they are to have their full effect in decreasing food intake. The experiments show that while these stimuli in isolation can effect feeding they have increased effect when they act together. However, the findings from these experiments do not indicate if the

stimuli combined effects are additive or multiplicative.

Findings like those above led to the design of experiments that were used to investigate the effect of the interaction of peripheral signals on satiety and determine if these signals act synergistically to reduce feeding. For example, McHugh and Moran (1986) demonstrated that a dose of exogenous CCK, that by itself was unable to reduce food intake, resulted in a significant suppression of eating if the subthreshold dose was combined with stomach distension. Neither the dose of CCK, nor the level of gastric distention produced by intragastric infusion of saline, in isolation, produced a reduction in feeding. But when they were presented in combination feeding was significantly reduced. The synergistic satiating effects of CCK and gastric distention has also been demonstrated at the electrophysiological level. Schwartz et al.(1993) showed that the combination of a gastric distention, produced by intragastric loads of 2 ml saline, and CCK administration stimulated rat gastric vagal afferent activity to a greater degree than could be predicted by examining the neural firing patterns elicited when either of these stimuli was presented in isolation. In a similar vein, Forsyth et

al (1985) showed that oral stimulation potentiated the satiating effects of CCK administration. In their experiment they demonstrated that a CCK administration that was subthreshold for suppressing eating, could decrease eating if it was accompanied by the oropharyngeal stimulation produced by sham feeding, a treatment that, by itself, was also incapable of reducing intake.

Synergy between peripheral signals has also been shown between oral signals and intraperitoneal (i.p.) glucose injections. Bedard and Weingarten, (1989) showed that oral stimulation, provided in an appropriate temporal context with i.p. glucose injections, suppressed sham feeding by up to 42%. Disrupting the temporal relationship between the oral stimulation and the i.p. injection reduced the observed degree of synergy, as reflected by an attenuated degree of food intake suppression.

A key early study demonstrating the synergistic effects of two peripheral feeding signals was done by Antin et al. (1977). In that paper, the authors examined the effects of an intra-duodenal (i.d.) perfusion of liquid food on sham feeding depending on whether the i.d. infusion preceded, coincided with or followed the initiation of eating.

The study showed clearly that the duodenal perfusion suppressed eating maximally if it was presented in the correct temporal context 12 min after the rat has begun to eat but the identical i.d. load was completely ineffective in suppressing eating if it was presented 12 min before sham feeding began. This result suggests, once again, that the temporal relationship between peripheral signals is critical to their final effects. A similar finding, reported by Baile et al.(1971), was that gastrointestinal glucose loads suppressed meal size only if they were accompanied by oral feeding. Similar infusions that were dissociated from oral stimulation were ineffective in supressing meal size.

While these experiments demonstrate the functional importance of synergistic interaction between oral and gastric/post gastric factors, they do not determine the neurophysiological correlate or site of these interactions. To explore how these peripheral afferent feeding signals are integrated in the CNS, the experiments reported here were designed to show both the differential neural activation and behavioural response of two feeding inputs, oral and gastric stimulation specifically, when they were presented alone or in combination.

WHY ORAL AND GASTRIC STIMULATION?

During ingestion of a meal, two of the primary sensations experienced are oral and gastric stimulation. Both of these peripheral signals play a key role in influencing the animal's subsequent feeding behaviour (Smith and Gibbs, 1979). The taste of the meal (i.e. palatability) can determine the amount an animal will eat (Sclafani, 1991). Prior associations of this taste to positive or negative consequences can lead to the preference of a food, as in the case of conditioned taste preferences (Sclafani, 1991), or rejection of a meal conditioned taste aversions (Chang, 1984). As well, the internal state of an animal can determine the hedonics of a specific taste at any given time (Capaldi, 1991). In general, experiments have shown that a taste that has been previously associated with nutrients will be perceived as more favourable to the animal than a taste that has no such association. Gastric stimulation from ingestion of a meal results in multiple changes in an animal's internal state which in turn lead to meal termination (e.g. stomach distention and release of hormones like CCK and gastrin). Food exiting the stomach then enters the intestinal tract where digestion continues and results in further

internal changes (eg. rise in blood glucose and insulin levels) as a result of nutrient absorption.

My thesis focuses on the oral and gastric signals at both the neuronal and the behavioural levels of analysis. There are three main reasons for the decision to focus on oral and gastric signal interaction. First, both classes of signals are known to be main components of the feeding act and play an influential role in determining meal size. Second, both classes of stimulation can be isolated and independently manipulated by the experimenter - oral via sham feeding; gastric via intragastric loads. Third, the afferent signal pathways of both oral and gastric stimulation are identified and, thus, provide a starting point at which to begin the investigation of how these signals integrate in the CNS.

C-FOS THE IMMEDIATE EARLY GENE

To study the interaction of two inputs at the neural level, one needs a marker for neural activity. Ideally the marker should indicate not only the sites of activity but also provide some index of the amount of activity. An effective neural marker for

accomplishing these goals was Fos immunohistochemistry. Below, I review the conceptual and technical basis for Fos immunohistochemistry and why the labelling of Fos-like proteins is an effective marker for neural activity.

The *c-fos* gene belongs to a subset of genes called proto-oncogenes, so named because, in their viral state, they become oncogenes with the potential to initiate tumours (Curran and Morgan, 1995). Proto-oncogenes encode a variety of proteins that share in common the property of being involved in the transmission of information between and within cells (Reddy *et al*, 1988). The *fos* proto-oncogene was originally discovered as the oncogene responsible for induction of bone tumours by the **F**inkle-Biskis-Jinkins murine **o**steogenic **s**arcoma virus (Curran and Teich, 1982) and was also carried by the Finkel-Biskis-Reilly murine retrovirus (Curran, 1988). The transformation of the normal *c-fos* gene into a cancer causing *v-fos* gene is thought to involve a breakdown in the controls that regulate the expression of the gene, so that the gene is turned continually "on"(Robertson, 1991).

DESCRIPTION OF FOS PROTEIN

In the vast majority of cell types, the basal levels of *c-fos* mRNA and Fos protein remain relatively low, the only exception being those cell types which are being continually acted upon by extracellular stimulation (Curran, 1988). Fos expression can be induced by a variety of stimuli associated with the process of differentiation or neuronal excitation. Early *in vitro* experiments used non-dividing neuronally differentiated PC 12 cells to form a series of induction and time course studies of the *c-fos* gene. These studies led to the discovery that Fos expression can be induced by voltage-gated calcium influxes (Morgan and Curran, 1986), neurotransmitters (Greenberg et al, 1986), barium ions (Curran and Morgan, 1986), and NGF (nerve growth factor) (Greenberg et al. 1986). The signalling pathways that link the cell surface stimuli to activation of *c-fos* expression are complex and not fully understood (Sharp, 1995). In general, it is thought that Ca^{+} entry through voltage gated calcium channels and activation of cAMP, leads to the phosphorylation and activation of CREB (c-AMP responsive element protein) which in turn binds to the

CRE (calcium response element) in the *c-fos* promoter. Another activation pathway involves Ca^{+} entry via the NMDA receptor which leads to activation of the protein kinase C pathway which in turn activates the SRE (serum response element) in the promoter region of the *c-fos* gene and induces transcription of the gene (Sheng and Greenberg, 1990; Sharp, 1995).

Despite the variety of stimuli that can generate Fos expression, the time-course of induction of *c-fos* mRNA remains relatively constant regardless of the stimulus that initiates Fos expression. mRNA transcription is rapid and transient, occurring within 5 minutes of stimulation and continuing for 15-20 min (Greenberg and Ziff, 1984; Greenberg *et al*, 1985). The level of mRNA protein in the cell reaches peak values 30-45 min following stimulation (Muller *et al*, 1984), and then declines rapidly. The half-life of the mRNA is short, roughly 12 min. Levels of the Fos protein usually peak 60 min following cellular stimulation and return to basal levels after 4 hours (Angel *et al*, 1988).

The *c-fos* gene encodes a 62 kDa nuclear protein that undergoes extensive post-translational modifications (Curran *et al*, 1984). The observation

that Fos is a nuclear protein found only in the nucleus of cells led to the suggestion that Fos might play a role as a transcriptional factor to promote the transcription of other genes. It is now known that Fos interacts with another nuclear protein Jun (from *c-jun* gene) to form a heterodimeric transcriptional factor called AP-1 (activator protein 1). AP-1 plays a key role in controlling both basal and inducible transcription levels of several genes such as interleukin 2, polyoma, stromelysin, human collagenase (Angel *et al*, 1987), and proenkephalin (Sonnenberg *et al*, 1989).

Certain properties of Fos and Jun differentiate these proteins from each other. Jun, but not Fos, can form homodimers and activate transcription. The Fos:Jun heterodimer has a much higher binding affinity for the AP-1 promoter site than does the Jun:Jun homodimer; this higher affinity allows the heterodimer to increase transcription to a greater degree than the Jun:Jun complex. Moreover, in contrast to the relatively short periods that Fos is detectable after cellular stimulation (4 hrs), Jun is still detectable 12 hours after stimulation(Angel *et al*,

1988).

The fact that Fos is rapidly degraded is consistent with the interpretation that Fos induction is associated with the initiation of the synthesis of proteins in response to cellular stimulation, while Jun is responsible for sustaining the response. The slow degradation of Jun also makes it a less sensitive marker of cellular activation than Fos. The levels of Jun that are measured following an experimental treatment may have been induced by a stimulus encountered prior to the one under investigation. In contrast, the rapid transcription of *c-fos*, and the rapid degradation of the Fos protein, make it an ideal protein to use as a punctate marker of neural activity.

FOS AS A NEURAL MARKER

Fos immunohistochemistry, the technique that labels Fos proteins in cells, is now being widely used as a method for identifying activated cells in the brain. Before it was possible to use Fos as a neural marker, a series of experiments were done to show that increases in Fos levels were correlated with cellular activation (Hunt *et al*, 1987; Morgan *et al*, 1987; Sagar

et al, 1988).

As discussed previously, early experiments by Greenberg et al. (1986) demonstrated directly *in vitro* that various neurotransmitters as well as Ca^{+} could induce transcription of the *c-fos* gene in post-mitotic cells. The first *c-fos* induction studies in *vivo* examined the ability of the convulsant Metrazole to induce *c-fos* expression in mice. C-*fos* expression was measured by probing for mRNA through Northern analyses (Morgan *et al.*, 1987). Analysis showed that mRNA from *c-fos* reached a maximum level approximately 60 min after i.p. Metrazole (pentylenetetrazole) injection, and returned to baseline levels after 180 minutes. Another important finding was that all Fos-containing cells were neurons, and Fos was confined to the nuclei of the neurons (Morgan *et al*, 1987; Mugnaini *et al*, 1989).

Morgan *et al.* (1987) was also the first study to employ Fos immunohistochemistry, the technique that localizes cells expressing Fos protein. After the Metrazole injections, Fos was observed in the nuclei of neurons of the dentate gyrus, pyriform and cingulate cortices. The specificity of the immunostaining for Fos was verified by demonstrating that the same labelling

pattern was produced with three different Fos antisera but not by an irrelevant antiserum. Specificity was further established by showing that labelling could be blocked by adding a synthetic Fos protein to the Fos antiserum prior to the immunostaining.

Another discovery from this key study was that unstimulated neurons display FLI (Fos-like immunoreactivity), indicating Fos is expressed under normal physiological conditions. As well, those areas in the CNS that exhibited the highest level of Fos expression are the same areas that have high basal levels of FLI.

It should be noted that there are four members of the Fos family (c-Fos, FosB, Fra-1, and Fra-2) all of which share certain sequence homologies (Piechaczyk and Blanchard, 1994) and whose proteins can be labelled during Fos immunohistochemistry. Since the Morgan study showed the other members of the Fos family were produced after injection of the convulsant it is important to use the term Fos-like immunoreactivity (FLI) when describing the labelling patterns seen after Fos immunohistochemistry, and it is this convention that is followed in this thesis.

It is important to take into consideration that FLI does not necessarily identify all neurons that

are active. Hunt *et al.* (1987) used noxious stimulation to induce FLI in neurons in the dorsal horn of the spinal cord, but remarked that many more neurons must be involved in the response to the peripheral stimulation than the number that were Fos labelled. This suggests that not all activated cells are labelled with Fos. Also, since the role Fos plays after cellular activation is still unclear, it is uncertain how closely FLI is correlated with different types of cellular activation (see Morgan and Curran, 1989 for a review) and caution must be taken in overinterpreting the presence or absence of FLI after an experimental treatment.

ADVANTAGES OVER OTHER NEURAL MARKERS

Fos immunohistochemistry has some advantages over other neurophysiological mapping techniques, such as 2-DG autoradiography and electrical recording.

Control studies performed by Sharp *et al.* (1988), confirmed that Fos labelling, when present, was localized to sites of neural activation by demonstrating a correlation between FLI and (^{14}C)-2-deoxyglucose (2-DG) uptake in rat motor/sensory cortex

as a result of electric stimulation of the cortex. An increased sensitivity of FLI as a marker of neural activity, relative to 2-DG autoradiography, is demonstrated in experiments involving water deprivation showing increased Fos expression in the paraventricular and supraoptic nuclei of the hypothalamus (areas known to be activated during water deprivation) even though no increase in glucose metabolism in those areas, measured by 2-DG autoradiography, could be observed.

A second advantage of Fos immunohistochemistry is that Fos labelling localizes to the nuclei of individual neurons thus allowing quantification of the number of activated cells, a feature not possible with 2-DG methods which stain whole areas, rather than individual cells. The capacity to identify individual cells cannot be achieved with the more direct measure of neural activity, electrical recording (except for unit recordings). While recording studies provide a temporal resolution of the effects of a stimulus on a set of neurons that is finer than that possible with Fos immunohistochemistry, it cannot provide a clear measure of the number of neurons activated in a given brain region.

FOS IMMUNOHISTOCHEMISTRY AND FEEDING RESEARCH

Recently, Fos immunohistochemistry has been used to identify neural systems, particularly in the hindbrain, that participate in the control of feeding behaviour (Olsen et al, 1993; Fraser and Davison, 1993; Emond and Weingarten 1995). These studies were important, in the first instance, in establishing that the "physiological" stimulus of eating was sufficient to elicit Fos expression in the rat brain. Fraser et al (1995) attempted to ascertain the contribution of oral and gastric afferent signals on FLI in the hindbrain using sham feeding and inflation of gastric balloon preparations to isolate oral stimulation and gastric distention respectively. They found that both of these treatments in isolation increased FLI throughout the NST. The effects of these two treatments in combination on FLI were never examined. The effects of a gastric load on FLI were examined by Kobashi et al (1993) who showed intragastric injection of hypertonic saline increased Fos expression in the NST, area postrema, and parabranchial nucleus. Yamamoto et al (1994) did studies investigating the effect of taste on FLI. However, because the flavoured liquids were ingested, the contribution of oral stimulation in isolation was

incomplete. While this previous research has attempted to determined the FLI patterns elicited by isolated oral or gastric stimulation, the effects of these stimulations in combination on FLI have yet to be examined outside the context of a normal meal using preparations that can isolate oral and gastric stimulation.

THE NUCLEUS OF THE SOLITARY TRACT AND THE DORSAL MOTOR NUCLEUS OF THE VAGUS

To study oral-gastric integration of afferent signals the most likely site where this integration could occur had to be chosen, to focus the investigation. The nucleus of the solitary tract (NST) represents one of the first areas where oral and gastric afferent signals could be integrated.

The NST is where the gustatory and visceral afferents of the facial (VII), glossopharyngeal (IX), and vagus (X) nerves synapse. Specifically, the primary fibres from the seventh, ninth and tenth nerves terminate caudally in the solitary tract (Norgren, 1984). Gustatory afferents terminate primarily in the rostral end of the NST (Blomquist, 1964; Hamilton, 1984; Norgren, 1978) and the majority of gut vagal

afferent fibres terminate in the caudal region of the NST. The medial NST contains both visceral and gustatory afferent terminals (Hamilton, 1984; Shapiro, 1985).

Because of the topographical configuration of afferents in the NST, this area is most often analyzed in rostral, medial and caudal levels. Rostrally, the NST's area is small and more poorly defined but it expands rapidly caudally eventually reaching and merging with the commissural nucleus of Cajal. In the rostral level the majority of gustatory primary afferent fibres of the seventh, ninth and tenth nerves terminate, while some continue caudally as far as the commissural nucleus of Cajal. The cell bodies for the gustatory fibres are in the petrosal or nodose ganglion (Crosby et al., 1962). Stimulation studies of the chorda tympani, the nerve which contains gustatory afferent axons from the anterior two-thirds of the tongue, produced short-latency responses in the rostral tip of the NST (Blomquist and Antem, 1965). This, combined with horseradish peroxidase mapping studies, indicates that the rostral NST receives the majority of gustatory afferent signals from the tongue. It should be noted, however, that this compartmentalization is not complete as gustatory afferents also extend into

the caudal NST. The medial NST contains afferent nerve endings whose axons distribute in the chorda tympani, glossopharyngeal, and vagus nerves (Contreras, et al, 1977). The caudal third of the NST has been identified as the main site where the visceral afferent nerves, and therefore where sensory signals from the stomach, terminate (Norgren, 1984). However, work by Kalia and Sullivan (1982) shows that the sensory fibres in the rat vagus terminate throughout the NST. They demonstrated that there are considerable arborizations of vagal afferents in the NST and processing of this afferent input occurs at various levels rostrocaudally.

Cells in the NST project rostrally to the parabrachial gustatory areas which in turn send fibres that ascend into the forebrain via the ipsilateral central tegmental tract to terminate eventually in the thalamic gustatory area and the central nucleus of the amygdala (Norgren, 1976). Tracing studies indicate that efferents from NST also project rostrally to parabrachial nuclei which act as a relay for the visceral efferent system whose primary nerves initially terminate in the caudal portion of the NST (Norgren, 1984).

A key mapping study by Kalia and Sullivan (1982) used horseradish peroxidase neurohistochemistry

to outline the sensory and motor connections of the vagal projections. They found that the vagus, which transmits afferent sensory signals from the viscera, enters the medulla dorsolaterally and the axons travel to the solitary tract to terminate in both ipsilateral and contralateral NST and dorsal motor nucleus of the vagus (DMN). The cell bodies for the visceral afferents are in the nodose ganglion (inferior ganglion). While the majority of axons terminated in the medial and caudal regions of the NST, there was also a significant population of fibres that terminated in the rostral NST. The DMN, although it may contain some sensory terminals, is the primary site of descending vagal motoneurons to the stomach and other viscera, (Kalia and Sullivan, 1982; Berthoud et al, 1991). The motoneurons in the DMN possess numerous dendritic connections with regions of the overlying NST (Shapiro and Miselis, 1985). This sets up the possibility that sensory information from the stomach can be relayed directly to appropriate visceral effectors which in turn could affect changes in the stomach's secretory-motor activities.

THESIS FOCUS AND OUTLINE OF EXPERIMENTATION

The fundamental task of physiological psychology is to explain the behaviour of the organism in terms of its biological underpinnings. Prior research has begun to map out the brain areas activated by specific peripheral signals believed to be involved in the control of eating, when these signals are presented alone. The present research accommodates the acknowledged multifactorial control of eating by designing experiments to determine how these signals are processed when presented together. In the series of experiments that constitute this thesis the focus was to understand some of the properties by which two classes of peripheral feeding-related signals may be integrated at the neuronal and functional levels. Oral and gastric stimulation were the two afferent signals that were chosen to be examined, for reasons outlined before. Fos immunohistochemistry was used as the marker of neural activity because of its practicality in determining brain areas activated by experimental treatments and since the technique provides a means to quantify the number of neurons activated.

The first experiment was designed to confirm that Fos immunoreactivity could be used as a marker of

neural activity induced under "physiological" conditions of feeding. The experiment also served to reinforce the inference that the NST and DMN were profitable areas of the rat's brain to investigate and pursue in future experiments. The second experiment used sham feeding and real feeding to compare the patterns of neural activity stimulated by oral stimulation in isolation (sham feeding) or oral and gastric stimulation in combination (real feeding). The third experiment specifically compared FLI patterns induced by oral and gastric stimulation, each presented alone, or in combination under conditions of strict experimenter control. The fourth experiment continued this line of inquiry by addressing whether the order of the two stimulations, one appropriate (oral followed by gastric) and the other reversed (gastric followed by oral), influenced the FLI patterns seen in previous experiments. The final experiment examined whether the effect of order also had functional consequences with regard to the control of eating.

CHAPTER II. GENERAL METHODS & MATERIALS

Subjects

Subjects were male Long-Evans rats (300-400 g) purchased from Charles River Inc.(St. Constant, Quebec). Rats were housed individually in stainless steel hanging cages in a colony room maintained on a 12:12 light/dark cycle and at 21°C.

Brain Fixation and Fos Staining

Rats were sacrificed by a 1 ml intraperitoneal (ip) injection of 350 mg chloral hydrate or 1 ml Somnotol (Sodium pentobarbitol) (6.5 mg/ml). Rats were perfused transcardially, via a 16 gauge needle placed in the left ventricle, with approximately 200 ml of 0.15 M saline followed by approximately 150 ml of 4% (w/v) paraformaldehyde in KPBS (potassium phosphate buffer solution). Brains were removed and stored overnight in 4% paraformaldehyde with 25% (w/v) sucrose.

Brains were frozen and sectioned at 40 µ in a cryostat at -20°C. Relevant sections of hindbrain were

placed in individual wells of a tissue culture plate (24 wells/plate). Three to four sections were placed in each well; each well contained 2 ml of PBS (phosphate buffer solution)(pH 7.3). The PBS was removed, sections were incubated in 0.3% H_2O_2 for 1 hour, then washed in PBS, and then incubated for 30 minutes at room temperature in 1.5% normal goat serum in PBS and 0.3% Triton X-100. Sections were then incubated for 39-44 hours in 200 µl of Fos primary antibody (Oncogene, rabbit polyclonal - batch #3922201) diluted 1:1000 (experiment 1) or 1:3000 (experiments 2-4)in 2%(w/v) BSA in PBS. Sections were maintained at 4°C and gently agitated throughout the entire period.

The primary antibody was removed and sections were washed three times (10 min/wash) with PBS/0.1% Triton X-100. They were then placed for 45 min in a 200 µl mixture of 0.5% (v/v) biotinylated secondary antibody, 1.5% (v/v) normal goat serum and 0.3% (v/v) Triton X-100. Sections were then washed three times in PBS and incubated for 45 min in 200 µl of Vectastain ABC reagent (Dimension Lab). After three additional PBS washes, sections were incubated in a diaminobenzidine tetrahydrochloride (DAB) chromagen for 8 to 10 min to stain Fos-like products black. The DAB reaction was halted with excess 0.5 M sodium acetate

and the sections were mounted on gelatin-coated slides. After drying overnight, sections were cleared in an ascending series (70%, 95%, 100%) of ethanol baths (10 min/bath) followed by a 45 min bath in Histoclear (Diamed).

Two immunochemistry controls were run in every experiment to verify the specificity of the Fos immunohistochemistry. First, some sections were processed following the normal protocol but the sections were not incubated in the primary Fos antibody. Second, other sections were processed according to the protocol above but were not incubated in secondary antibody. In addition, to control for staining variability between immunohistochemistry runs, each immunochemistry run contained matched sections from both experimental and control brains.

Fos Quantitative Analysis

The MCID image analyzer (Imaging Research Inc, St. Catherines, Ontario) was used for quantitative analysis. Sections were magnified using a Zeiss microscope and the image was relayed to the MCID system via a Dage 72 series (Sony) video camera.

The grain count feature of the BRS software was used to count labelled cells. The target definition option of the BRS programme was used to define parameters of cells to be counted. By setting the BRS grain count feature to minimum and maximum settings that would ignore areas too small or large to be cells the number of false positives was minimized. By establishing and setting the mean cell size the programme could then estimate the number of labelled cells in a dark region containing a clump of labelled cells. These parameters were adjusted for each of the brain regions analyzed.

The minimum and maximum values were determined by using the programme's individual cell count feature to determine the size of the smallest and largest cells in a particular brain region. The mean cell size was determined by taking a count of the entire brain region being analyzed and using the average grain size as the mean cell size. The mean cell size was used by the programme to estimate the number of cells contained in a clump of cells. Importantly, once these criteria were defined for a particular region, they were held constant when performing the quantitative analysis of brains for the different groups in the experiment. This ensured that whatever error might have been

imposed by the selection of these parameters was applied equally to both the experimental and control groups.

Two brain regions were analyzed: the dorsal motor nucleus of the vagus and the nucleus of the solitary tract. These areas were differentiated on the basis of well-defined anatomical descriptions, particularly issues of cell size and density. Three planes of section through these brain areas were examined: caudal to the area postrema (Bregma -14.1 mm), medial to the area postrema (Bregma -13.8 mm) and rostral to the area postrema (Bregma -13.5 mm)(coordinates derived from the Paxinos and Watson Atlas, 1982).

Surgery

Gastric Cannulation

To permit sham feeding (experiments 2 and 5), rats were implanted with chronic, indwelling gastric cannulae. Each gastric cannula consisted of an 11 mm stainless steel tube (8.5 mm outer diameter [OD], 7.9 mm inner diameter [ID]), flanged at both ends. Marlex mesh (2.5 cm x 2.5 cm) was secured to the middle of the cannula shaft with dental cement 24 hr prior to surgery

and helped stabilize the cannula once it was *in situ*. A set screw threaded into the cannula shaft allowed the gastric cannula to be occluded and, when in place maintained the continuity of the gastrointestinal tract for real feeding.

Rats were deprived of food for 24 hr prior to gastric cannulation. Sodium pentobarbitol (Somnotol, loading dose:65 mg/kg, intraperitoneal injection was used to anaesthetize the animals.

To implant a gastric cannula, a laparotomy, approximately 2.5 cm long was made in the abdominal skin and peritoneal wall. The stomach was exposed and two concentric purse-string silk (5-0) sutures were sewn in the wall of the forestomach. A stab incision, in the centre of the purse string sutures, permitted one end of the cannula to be inserted into the stomach. The cannula was secured by tightening the purse-string stitch. The free end of the cannula was brought through a 1 cm stab wound in the left abdominal wall and Marlex mesh (2.5 cm x 2.5 cm) was applied over the free end of the cannula between the abdominal wall and skin. The cannula was exteriorized with a 1 cm stab wound in the skin. A single silk (3-0) purse string surrounding the stab wound in the skin secured the exteriorized

cannula. The cannula was closed with a set screw. The abdominal wall was closed with interrupted catgut (3-0) sutures and the skin was closed with stainless steel wound clips. Immediately following surgery, wounds were treated with a topical antibacterial gel (Furacin, Austin Laboratories Ltd., Canada). Animals were allowed a minimum of 14 days to recover from surgery and were maintained on *ad lib* food during this week.

Statistical Analysis

A MANOVA was used to analyze differences between treatment groups. A separate analysis was conducted for each of the two brain sites and the three levels of the hindbrain. A Neuman-Keuls adjustment was used to correct the α level for the multiple analyses.

CHAPTER III. EXPERIMENTS

EXPERIMENT 1

Real Feeding Elicits Fos Expression in the Rat Hindbrain

The first experiment was designed to demonstrate that Fos immunoreactivity could be used as a marker of neural activity induced under "physiological" conditions of feeding by comparing FLI expression in a rat which had eaten a meal to a control rat which had not eaten.

Fos immunohistochemistry had been applied previously to identify hindbrain neural systems that participate in the control of feeding. However, the results obtained were inconsistent. In a comprehensive study, Olsen et al (1993) examined the effects of various manipulations, that either enhanced or suppressed food intake, on Fos expression in hindbrain nuclei. In general, treatments that decreased food intake (eg. high dose CCK, lithium chloride) were correlated with increased Fos-like immunoreactivity (FLI) in the NST; treatments that stimulated feeding (eg. food deprivation, insulin-induced hypoglycaemia)

were associated with increased FLI in the DMN. In contrast, other studies that used metabolic inhibitors that typically increase food intake (mercaptoacetate and 2-DG), reported enhanced FLI in both the DMN and NST (Ritter,S., 1991). Treatments that typically suppress food intake,(eg. intraintestinal nutrient infusions and CCK-8), elicited Fos-like expression in both NST and area postrema (Ritter,R., 1991). Fos-like expression in the hindbrain has also been studied in response to more "physiological" stimuli, specifically the act of eating itself. Fraser and Davison (1993) found that simple ingestion of a meal induced FLI in both NST and DMN. However, others reported that meal ingestion increased FLI in NST only, and not in DMN (Olsen et al, 1993).

The hypoglossal nucleus, which contains the motoneurons that direct tongue and facial movement, was also examined in this experiment. The hypoglossal nucleus receives direct cortical input from the cerebral cortex and contains the cell bodies of the motoneurons of the IXth and Xth cranial nerves (Wan et al., 1982). Since tongue and facial movement occurs during the feeding process it is an ideal region to examine to verify that FLI was correlated with neural activation.

This experiment had three aims. At the time I conducted this experiment, neither the Fraser and Davidson (1993) or Olsen (1993) studies had been published, thus leaving it unclear whether the simple act of eating was sufficient to elicit Fos expression. At the time this study was conducted, Fos expression had been revealed only following severe experimental treatments, such as kindling or i.p. injection of hypotonic saline. Thus, our first aim was to show that physiological stimulation provided by feeding could produce Fos expression in the rat brain.

Once experiments similar to the one reported here had begun to be published, the literature revealed no consistent pattern of FLI in response to eating. Thus, the second aim of this experiment was to confirm a profile of Fos-like activation in hindbrain nuclei in response to feeding. Finally, the third aim of this research was to address a continuing limitation in the use of Fos immunohistochemistry as a mapping tool; specifically the problem of quantification. For this experiment I describe and verify the utility of an efficient, sensitive and reproducible method, for quantifying the amount of FLI using image analysis software.

METHOD

For two weeks, rats were adapted to a schedule in which they had access to food, Purina powdered chow, for only two hours a day, at a fixed time (11:00 am) during their light cycle. After training, "Experimental" rats were provided with food as usual and were sacrificed 90 min after food presentation. "Control" rats were sacrificed at the normal feeding time but without being allowed to eat. Preliminary experiments verified that the one and a half hour interval itself (11 am vs 12:30 pm) (ie. the interval that separated the times at which the control and experimental rats were sacrificed) produced no differential Fos expression in these hindbrain regions.

RESULTS

Experimental animals weighed the same as their controls on the first day of training (E: 321 ± 12.9 gm; C: 317 ± 13.2 gm) and at sacrifice (E: 311 ± 14.3 gm; C: 308 ± 13.4 gm). Experimental rats ate a meal of 15.6 ± 0.7 gm on the day of sacrifice.

The group mean number of labelled cells in the

experimental and control conditions in the three hindbrain nuclei analysed is shown in Figure 1. Experimental rats (ie. those that had taken a meal) expressed significantly more FLI compared to the control rats (ie. those that had not eaten) in the hypoglossal, $t(7) = 2.47$, $p<0.05$ and dorsal motor, $t(7) = 2.81$, $p<0.05$ nuclei. No group differences in the amount of Fos-like labelling were seen in the NST, $t(7) = 0.46$, NS. Photomicrographs of representative sections showing FLI are shown in Figure 2.

Figure 1: Effects of a solid meal on Fos-like immunoreactivity (FLI) in the hindbrain: comparison of the number of Fos-labelled cells in the hypoglossal nucleus (HG), the dorsal nucleus (DMN) and the nucleus of the solitary tract (NST) in rats that have eaten a meal (n = 8) compared to control rats that have not eaten a meal (n = 8). Data shown are group means ± 1 SEM.

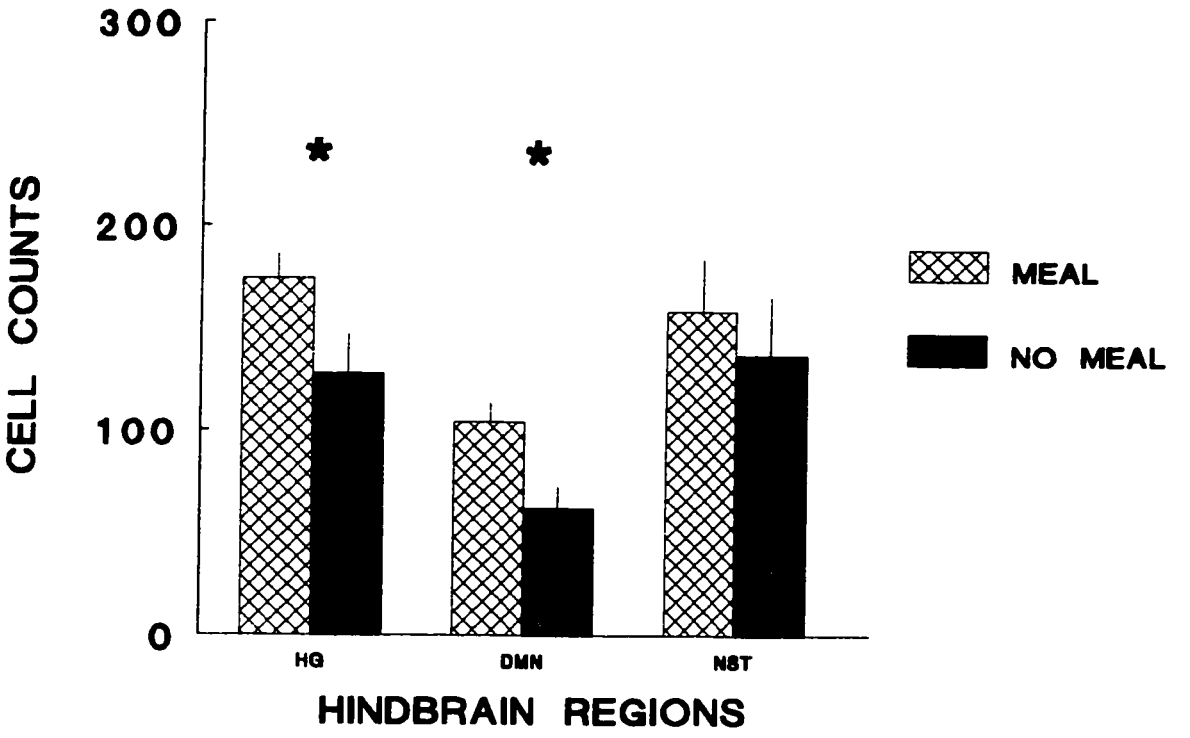
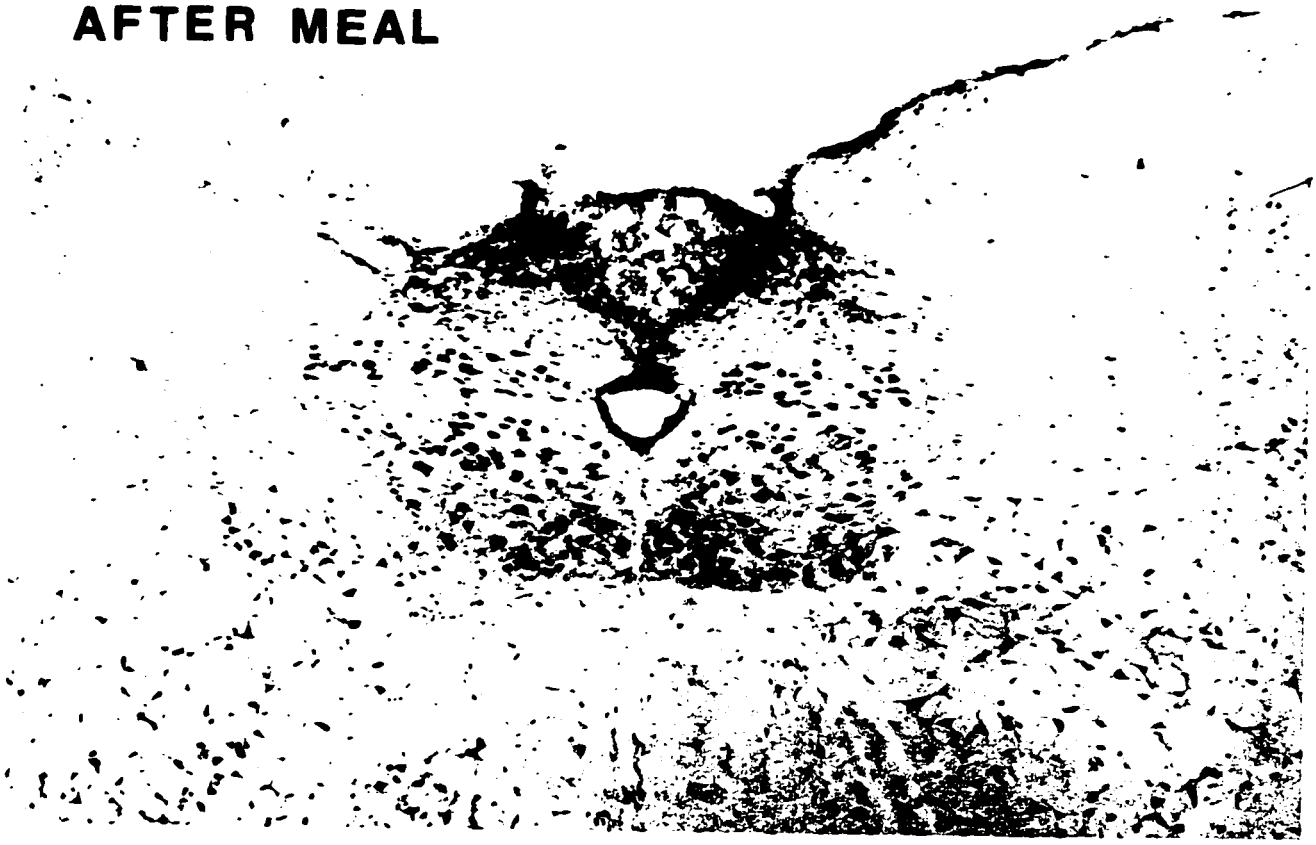
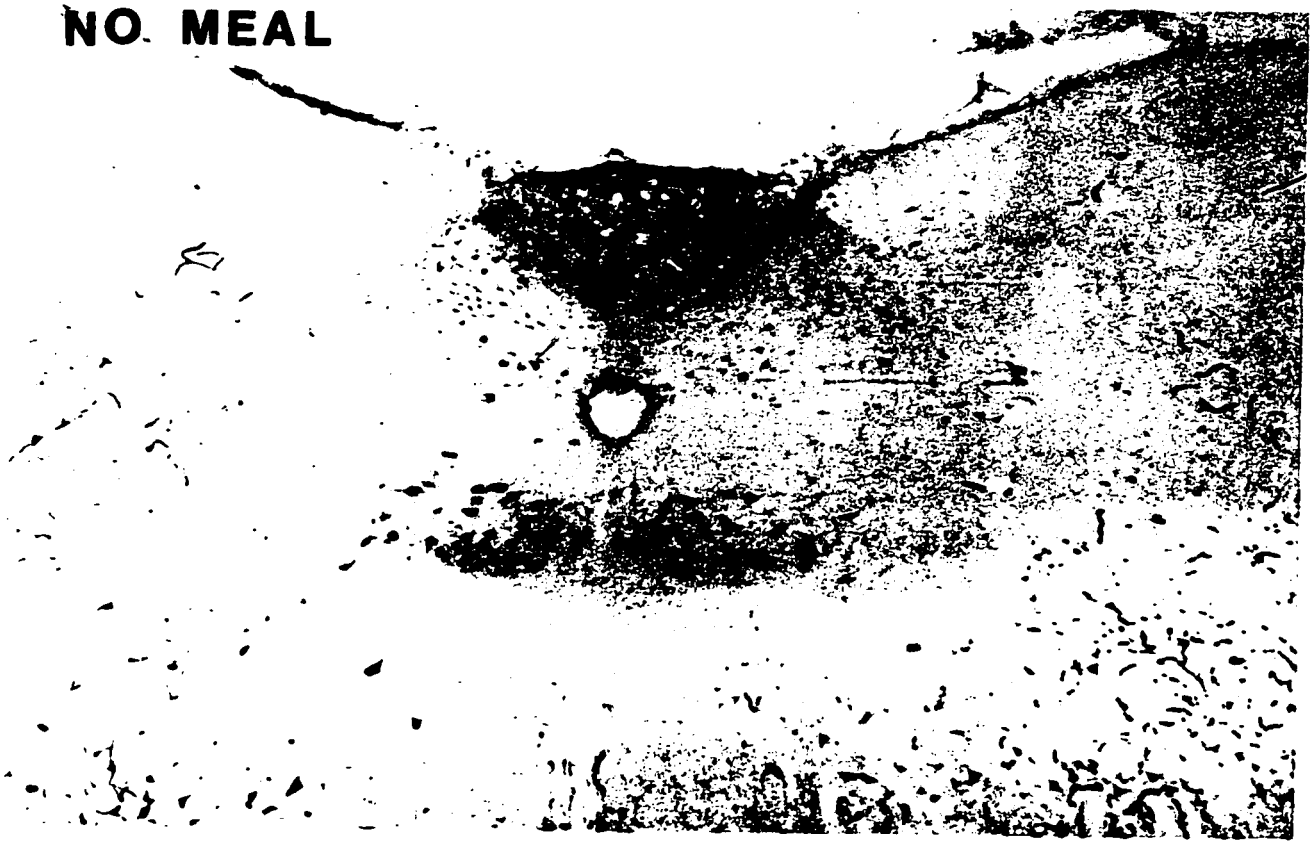

300
200
100
0
CELL COUNTS
*
*
HG
DMN
NST
HINDBRAIN REGIONS
MEAL
NO MEAL

Figure 2: Photomicrographs of representative sections processed according to immunohistochemistry protocol described in text illustrating effects of a meal on FLI in hindbrain. a) after meal. b) no meal.

AFTER MEAL



NO MEAL



DISCUSSION

The aims of this experiment were to determine if eating was sufficient to induce FLI in the rat brain, to identify the FLI pattern that was produced by eating, and to establish a procedure for quantifying the amount of Fos-like activation.

This study shows that ingestion of a meal significantly increased Fos-like activation in selective brain sites, thus confirming the utility of FLI in more detailed analysis in later experiments. The particular result I obtained is partially consistent with a previous study (Fraser and Davison, 1993) reporting meal-induced FLI in both DMN and NST but is inconsistent with the findings of Olsen et al (1994) who demonstrated increased FLI in NST only after eating. It is unclear why different experiments lead to varying results. However, the variability in the reports of FLI distribution in response to a meal underscores our lack of knowledge concerning the variables that influence Fos-like activation following feeding. Variables such as the deprivation state of the animal, type and/or amount of food eaten, that are

not consistent across these studies, may affect the amount and distribution of FLI in hindbrain as a result of eating.

Within the present study, however, the profile of FLI results across brain areas is consistent and rational. Eating is associated with tongue movements and activation of vagally-influenced events such as gastric acid secretion and peristalsis (Shapiro and Miselis, 1985). Thus, it is not surprising that eating compared to a control condition where no food is eaten, increased FLI in the HG, the site of cell bodies of neurons controlling the tongue movements (Wan et al., 1982) and DMN, the location of cell bodies of the efferent vagus (Shapiro and Miselis, 1985). Meal termination, or satiety, is associated, presumably, with activation of vagal afferents. Thus, our inability to detect FLI in the NST, a termination site of vagal sensory afferents (Norgren, 1984), after eating is anomalous. However, this result is better understood in the light of experimental results reported in later studies described in this thesis.

The quantification method we used to assess the degree of Fos labelling employed an automated image analysis program to count cells. It had several advantages over manual counting. A threshold darkness

level was set so that only Fos labelled cells would be counted. While this threshold was set at a subjective level by the experimenter (ie. some labelled cells may not have been counted, and/or some nonlabelled cells may have been counted) it ensured that both control and experimental sections were counted using the same criteria. Using an automated programme to perform the cell counts helped eliminate the possibility of experimenter bias during the counts. One weakness of using a cell counting programme is that it will count a tight grouping of cells as one cell. To correct for this, the programme allows a mean cell size to be entered in its criteria (determined ahead of time by the experimenter) so the programme will estimate the number of cells that make up the cluster of cells and add this to the count. Again, though, this procedure is applied equally to experimental and control brains. Overall, the programme's ability to perform objective cell counts rapidly, with full reproducibility, makes it a useful tool for quantification of Fos labelling.

This study achieved its three aims. It demonstrated that ingestion of a meal could elicit Fos expression, that the FLI patterns observed in the hindbrain nuclei were similar to those reported in other studies and it verified the utility of the

automated image analysis system for FLI in hindbrain.

EXPERIMENT 2

Real Feeding versus Sham Feeding

The previous experiment identified the FLI patterns induced by a meal but did not provide insight into which specific perpheral signal (e.g. oral, gastric, etc.) was responsible for the Fos-like activation observed during feeding. To answer this question requires procedures that isolate the contribution of specific peripheral signals.

Experiments revealing Fos-like activation in response to feeding (Fraser and Davison, 1993 and Olsen et al, 1994) did not identify the component of eating responsible for the Fos activation. In this experiment, a behavioural preparation, sham feeding was used, that activates oral stimulation specifically and compared the FLI activated by it to that elicited by real feeding in which both oral and gastric (and postgastric) signals are activated.

The purpose of this experiment was to compare the distribution of FLI resulting from oral stimulation alone or oral plus gastric plus postgastric stimulation during a meal. This was achieved by using an experimental preparation, sham feeding, that isolates oral factors since, in this preparation, a gastric

cannula is implanted that allows the meal to drain out of the stomach before it can activate distension or postgastric stimulation. While some nutrient may actually be absorbed even with this "sham feeding" preparation the amount is small (Sclafani and Nissenbaum, 1985) and, based on other studies, its impact has no functional consequence, at least with respect to eating (Gowans and Weingarten, 1992; Young et al., 1974).

METHOD

A chronically-indwelling gastric cannula was implanted into each of the eighteen rats according to procedures described in my methods section. After one week recovery, rats were adapted to a daily sham feeding schedule. Specifically, after an overnight deprivation, rats were moved from their home cages to a test room. Their stomachs were cleaned of food by flushing warm water into the stomach via the open cannula. Then, a 15 cm long collecting tube was screwed into the cannula and rats were placed in individual Plexiglas cages onto which a graduated cylinder containing 1 M sucrose was suspended. Rats sham fed for 30 minutes; liquid diet flowed freely out

of the stomach when the cannula was opened. After sham feeding, the cannula was closed and rats were returned to their home cages and permitted to eat Purina rat chow pellets *ad lib* for the rest of the day until the imposition of their overnight fast.

On test days, rats were prepared for sham feeding according to the usual procedure. On this day, however, "Real" rats were allowed to real feed by replacing the screw in the cannula. These rats real fed 1 M sucrose for 30 minutes. "Sham" rats were prepared according to the normal protocol and were allowed to sham feed except that their intake was limited to the amount that had been real fed by a matched "Real" rat. Pairs of "Real" and "Sham" rats were sacrificed 90 minutes after the initiation of real or sham feeding.

RESULTS

On the day of sacrifice, the mean weights of "Real" rats and "Sham" rats were similar (E: 364 $\pm$ 5.3 gm; C: 367 $\pm$ 10.5). "Real" rats ate an average of 15.8 $\pm$ 0.9 ml of 1 M sucrose on the sacrifice day; the intake of "Sham" rats was clamped at this level.

"Sham" rats demonstrated significantly increased in FLI in the DMN, $t(9) = 4.33$, $p<0.005$ and the NST, $t(9) = 5.88$, $p<0.0005$, compared to "real" feeding rats. There were no significant differences in cell counts between the two groups in the HG $t(9) = 1.74$, NS. The group mean cell counts are shown in Figure 3. Figure 4 shows the photomicrographs for all 10 pairs of rats used in this study; the actual cell counts for each pair are presented in Table 1.

Figure 3: Comparison of the effects of real feeding and sham feeding on FLI in the hindbrain: the number of Fos-labelled cells in the HG, DMN, and NST in rats that real fed a 1 M sucrose meal (n = 10) and rats that sham fed an equal amount of 1 M sucrose (n = 10). Data shown are group means ± 1 SEM.

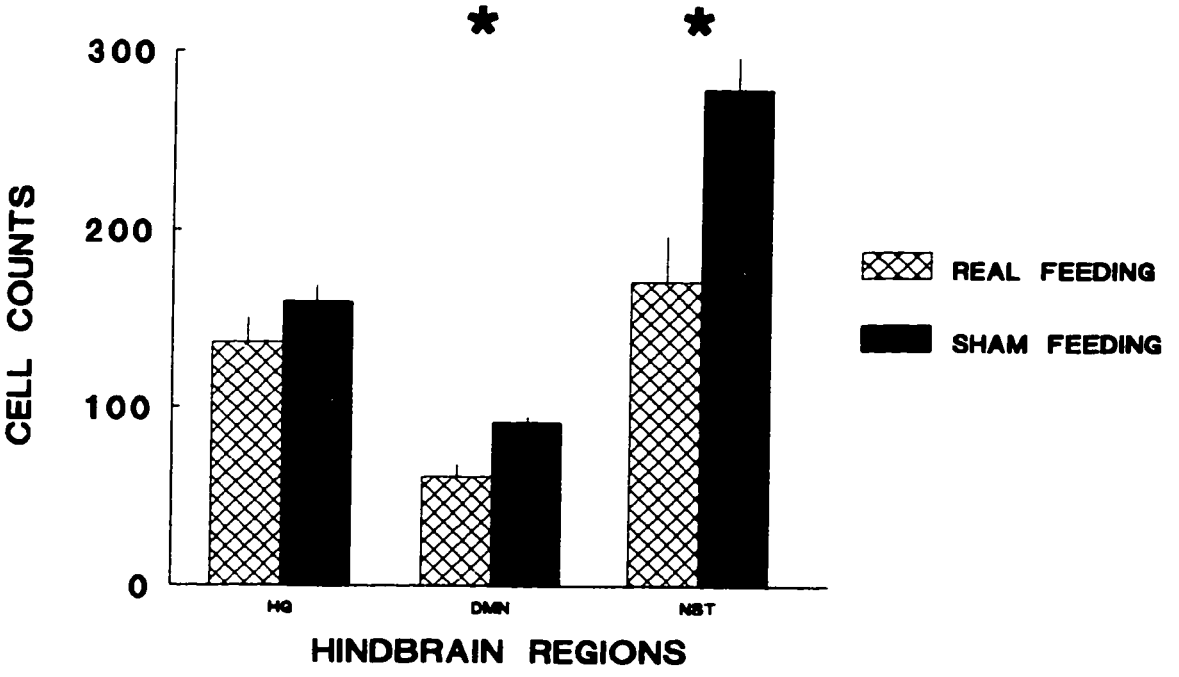

300
200
100
0
CELL COUNTS
*
*
HQ
DMN
NST
HINDBRAIN REGIONS
REAL FEEDING
SHAM FEEDING

Figure 4: Photomicrographs of representative sections illustrating differences in FLI labelling between yoked pairs of rats that (a) real fed or (b) sham fed. Individual rows represent yoked pairs.

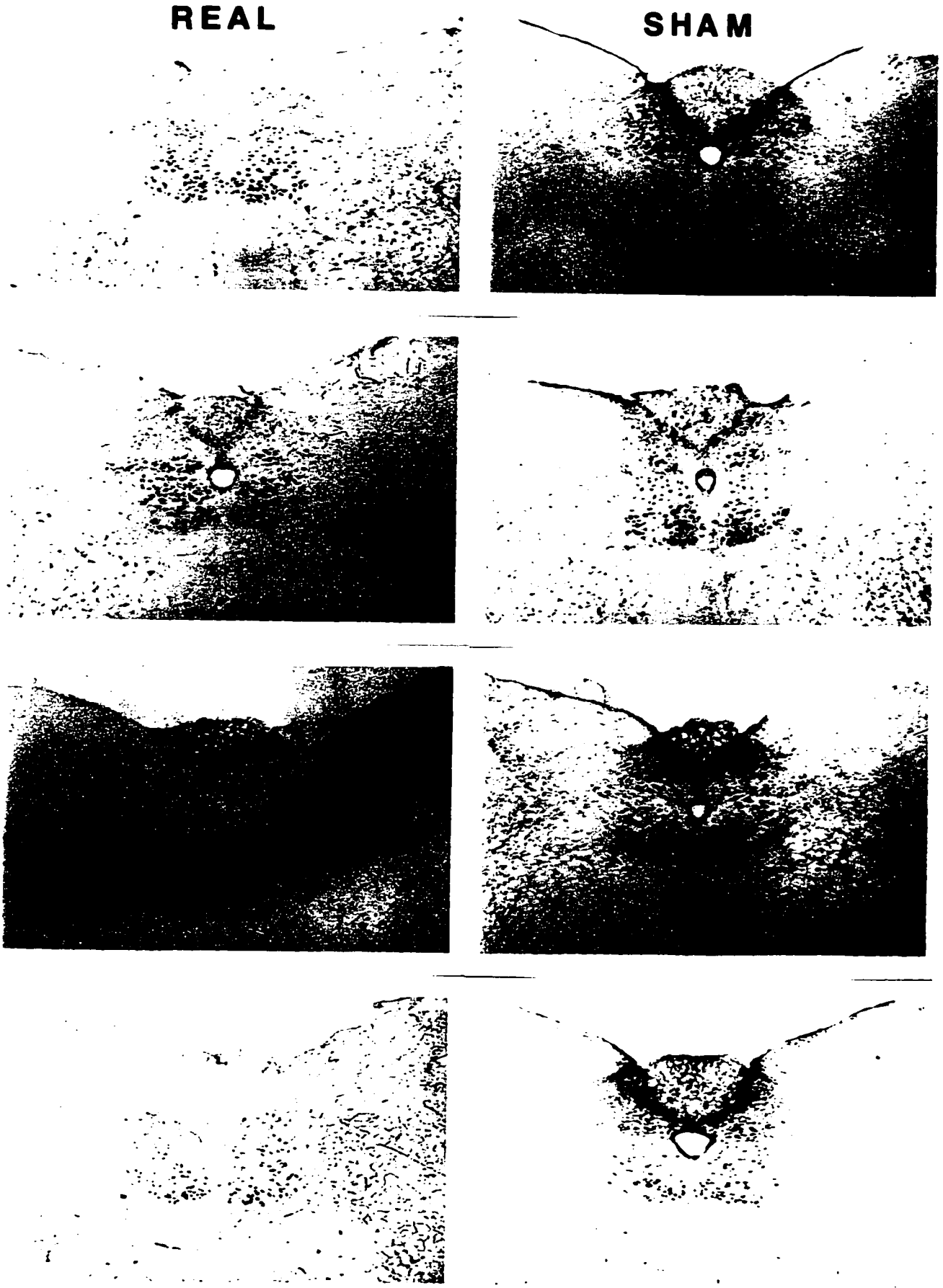

REAL
SHAM

Table 1: Effects of "Real" and "Sham" Feeding on Fos-Like Imunnoreactivity in the Rat Hindbrain

Pair #	HG		DMN		NST	
	RF	SF	RF	SF	RF	SF
1	146	124	51	88	26	218
2	106	150	51	91	61	213
3	129	163	50	109	108	306
4	202	169	102	106	134	189
5	179	172	74	103	228	276
6	58	101	30	76	244	283
7	172	208	40	85	192	318
8	132	184	74	92	240	326
9	81	186	41	81	195	268
10	155	138	93	78	274	383

Data shown are actual cell counts. (DMN = dorsal motor nucleus of vagus; HG = hypoglossal nucleus; NST = nucleus of the solitary tract; RF = real feed; SF = sham feed).

DISCUSSION

It was quite unexpected that there would be increased FLI in DMN and NST under sham-feeding, compared to real-feeding, conditions. The individual data presented in Fig 3 and Table 1 indicate, however, the robustness of this finding. We observed increased FLI in sham-feeding, compared to real-feeding, rats in 9 of the 10 comparisons in the case of the DMN and in 10 out of 10 comparisons in the case of the NST. One would presume, a priori, that real feeding would result in greater Fos-activation because, in the instances of real feeding, both the oral and the gastric/postgastric afferent stimulation should stimulate FLI whereas, in sham feeding, only the oral component should be activated. There is one other report indicating increased FLI in paraventricular nuclei under sham feeding conditions compared to real feeding (D. Richard, personal communication). One possible explanation is that, under certain circumstances, gastric and/or postgastric stimulation may actually decrease FLI in NST and DMN. Before that conclusion is drawn, however, an independent assessment of the role

of gastric and/or postgastric stimulation on FLI in the hindbrain vagal nuclei is required.

Based upon absolute cell counts alone, it appears that the oral stimulation provided by sham feeding may be the most potent stimulus for FLI in the NST. The results from this experiment suggest that the effect of peripheral feeding signals acting together, such as oral and gastric in the real feed rats, may not be predictable by observing the effects of these signals in isolation. The next experiment verifies this suggestion by examining, under strict experimenter control, the effects of oral and gastric stimulation, presented in isolation or in combination, on FLI.

EXPERIMENT 3

The Effects of Oral and Gastric Stimulation, in Isolation or in Combination, on Fos Expression in the Rat Brain

This experiment was designed to verify the suggestion of the previous experiment that combined oral and gastric stimulation results in less FLI in the NST than oral stimulation alone. This experiment expanded the scope of the investigations by adding a treatment that isolated gastric stimulation, along with a treatment that isolated oral stimulation, and compared the resultant FLI to animals receiving both stimulations in combination.

This experiment expanded on the previous studies in two important ways. First, it used anaesthetized rats. Second, it analysed FLI in three different levels of the NST and DMN. Anaesthetized animals were used to reduce the variability in Fos expression that may have resulted from variables such as anticipation of a meal (Emond and Weingarten, 1995) or movement. More importantly though, anaesthetized preparations allowed oral and gastric stimulations to be delivered under full experimenter control.

This experiment also addresses the issue,

noted in the introduction, that oral and visceral afferents terminate at different levels of the hindbrain nuclei. As noted in the introduction, the gustatory afferents terminate primarily in the medial and rostral levels and the visceral afferents terminate primarily in the caudal level. Although these divisions are not absolute, additional information about the origin of the peripheral signals leading to the FLI activation can be gained by performing cell counts at three levels of the hindbrain nuclei.

METHOD

This experiment was designed to compare the distribution and extent of FLI in the vagal nuclei in response to oral and gastric stimulation presented alone or in combination. We tested four groups of Charles River Long Evans male rats (300-450 g) that were maintained *ad lib* on dry rat chow pellets. For two weeks prior to the test day, rats were trained to drink 15 ml of an evaporated milk-based liquid diet every morning. Twenty four hrs before the test, the chow was removed and rats were given one last 15 ml meal of liquid diet. This ensured that all rats had ingested a

final meal of equal size at the same time before the imposition of a 24 hr fast. After the deprivation period, rats were anaesthetized with urethane (1.2 g/kg) and remained anaesthetized for the duration of the experiment.

The four treatment groups were: "Oral"; "Gastric"; "Oral/Gastric"; and "Control". "Oral" animals received restricted oral stimulation accomplished by infusing 1 M sucrose over the tongue at a constant rate of 0.63 ml/min for 19 min. "Gastric" rats received isolated gastric stimulation delivered by a direct intragastric infusion, via a plastic feeding tube, at the same perfusion parameters as "Oral" rats. The third group, "Oral/Gastric" rats, received simultaneous oral and gastric infusions at the above infusion parameters. "Control" rats received neither gastric nor oral stimulation but were treated the same as rats in the other conditions in all other respects. Ninety minutes after treatment, rats were killed with a 1 ml chloral hydrate dose. The brains were removed and processed for FLI using the protocol described before.

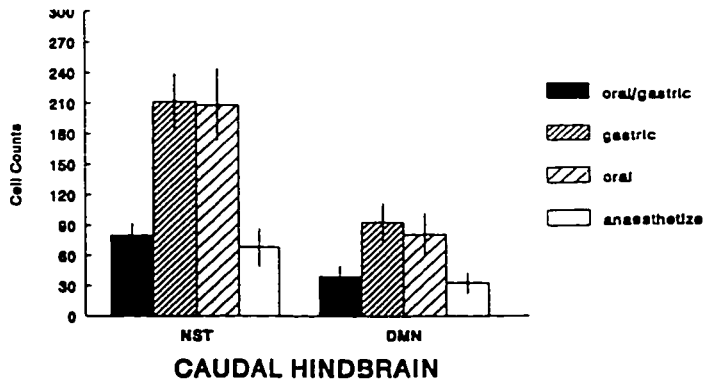
RESULTS

The group mean number of labelled cells in the four groups in the two hindbrain nuclei analysed is shown in Fig 5. Representative photomicrographs of sections are shown in Fig. 6. The results from this experiment demonstrated that oral and gastric stimulation in combination resulted in significantly lower FLI compared to either oral or gastric stimulation provided in isolation. Rats that have been given oral or gastric infusions in isolation expressed significantly more FLI compared to both the rats who had been given these infusions in combination and the anaesthetized "Control's". The number of FLI labelled cells in the oral/gastric group did not differ from the baseline Fos levels observed in "Control's". The differences between the Oral/Gastric group and the Gastric and Oral groups were reflected in significant group differences in FLI counts at all three levels of the NST (Caudal: $F(3,40) = 8.33$, $p<0.0005$; Medial: $F(3,40) = 7.20$, $p<0.001$; Rostral: $F(3,40) = 5.78$, $p<0.01$).

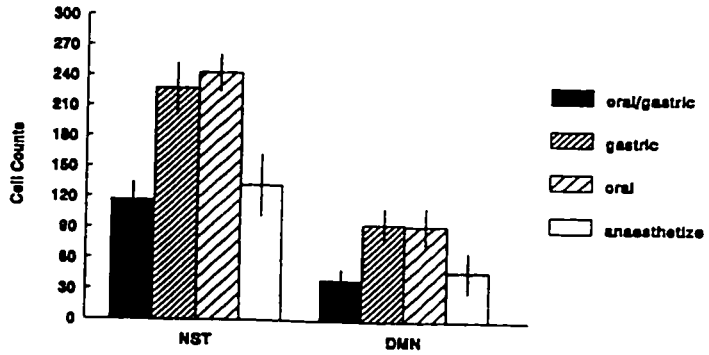
Rats that had been given oral or gastric stimulations in isolation expressed significantly more FLI compared to rats which had been given these infusions in combination and anaesthetized Controls, in

the medial and rostral levels of the DMN (Medial: $F(3,40)=3.37$, $p<0.05$; Rostral: $F(3,40)=5.17$, $p<0.005$).

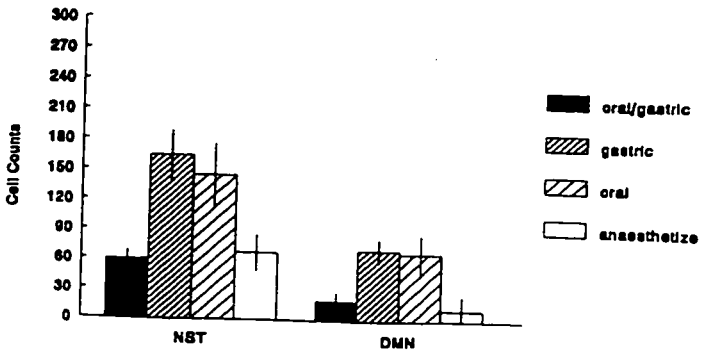
Figure 5: Comparison of the effects of oral and gastric stimulation, alone and in combination, on FLI in the hindbrain of anaesthetized rats: the number of Fos-labelled cells in the DMN and NST in rats that received oral stimulation (12 ml of 1 M sucrose) in isolation (n = 9), gastric stimulation (12 ml of 1 M sucrose) in isolation (n = 9), combined oral and gastric stimulation (n = 9), and control rats who received no stimulation (n = 9). Cell counts of caudal, medial, and rostral levels of the NST are shown. Data shown are group means ± 1 SEM.



CAUDAL HINDBRAIN

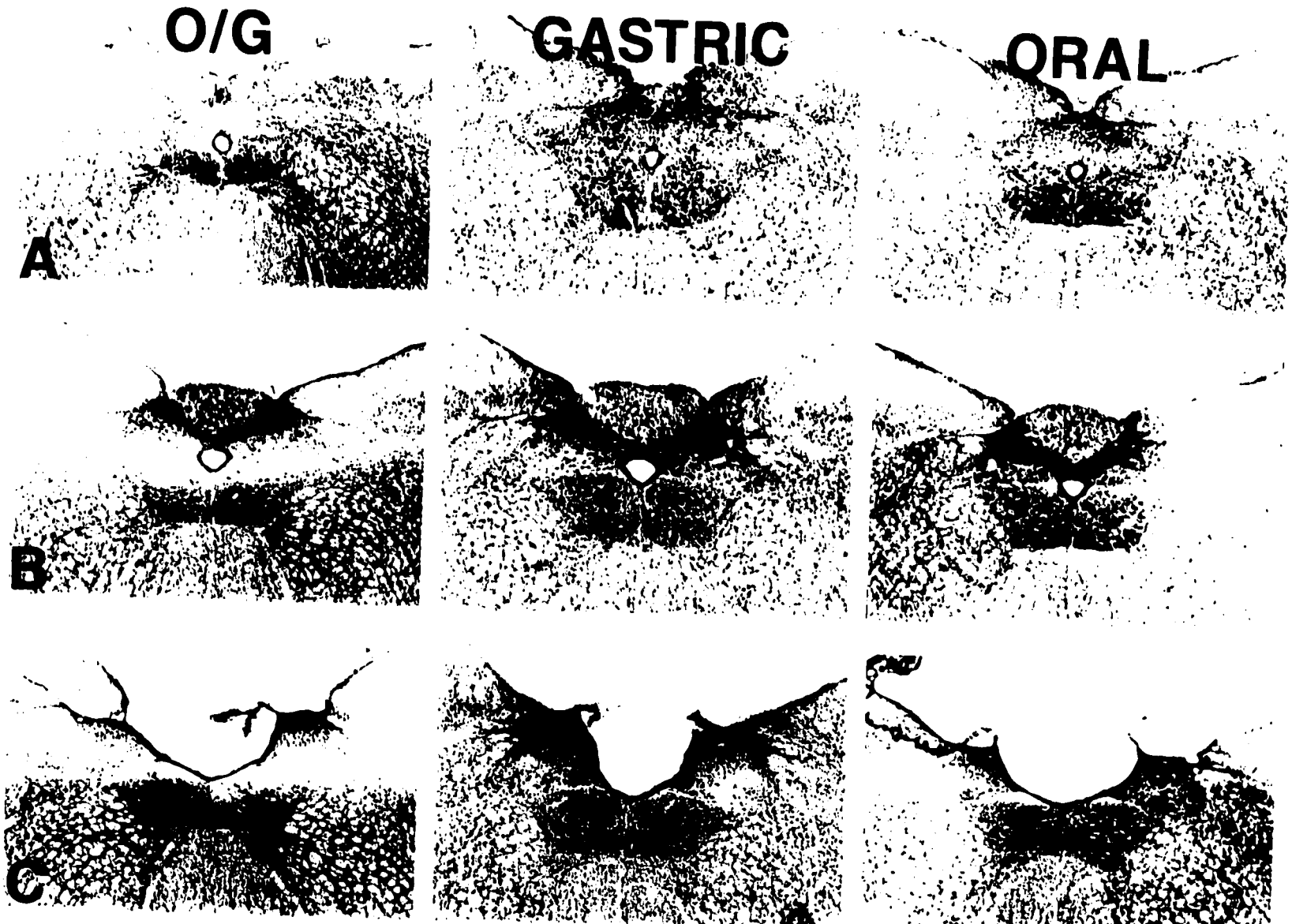


MEDIAL HINDBRAIN



ROSTRAL HINDBRAIN

Figure 6: Photomicrographs of representative sections illustrating differences in FLI between rats who received i) oral/gastric **(O/G)** ii) gastric or iii) oral stimulation: in a) caudal b) medial c) rostral levels of the NST.


O/G
GASTRIC
ORAL
A
B
C

DISCUSSION

This study demonstrates that the Fos expression elicited when both oral and gastric signals are presented concurrently cannot be predicted by the FLI seen when they are presented in isolation. That is, the FLI patterns produced by oral and gastric stimulation in combination are not an additive function of the FLI patterns induced by these two stimulations in isolation.

This experiment examined FLI after oral and gastric stimulation, alone and in combination. In comparison to anaesthetized controls, both the isolated oral and gastric stimulation increased levels of Fos expression in the NST and DMN. This result replicates the findings from previous work that showed isolated oral stimulation (Fraser et al, 1995; Yamamoto et al, 1994) and isolated gastric stimulation (Fraser et al, 1995; Kobashi et al, 1993) produced increased FLI in the NST in comparison to controls. This result is also consistent with our previous finding in freely behaving rats (Emond and Weingarten, 1995; and previous experiments in this thesis) showing that combined oral and gastric stimulation results in significantly less FLI in the NST than oral stimulation alone.

A comparison of FLI levels in the isolated oral and gastric stimulation conditions and the anaesthetized Control group reveals that the two "isolated" treatments represent elevation of FLI levels above baseline. But presentation of the two signals in combination results in levels of FLI at basal levels. The demonstration that the neural activation produced by combining two distinct afferent peripheral signals cannot be predicted from the neural activation produced by either signal presented alone, suggests the conclusion that preparations used frequently to isolate the roles of oral (sham feeding) or gastric (gastric load) afferent activity may not adequately capture the contribution of these signals when they are embedded in the context that approximates normal eating, ie. the signals in combination.

EXPERIMENT 4

The Effect of Presentation Order of Oral and Gastric Stimulation on Fos Expression

The previous experiment established that isolated oral or gastric stimulation elevated Fos expression compared to simultaneous presentation of these two inputs. This finding raises the question of whether the reduced FLI seen in the combined treatment condition is dependent simply on the two stimulations being presented concurrently or some necessity that they are presented in a sequence that mimics the presentation order seen during real feeding. In the previous experiment, I used simultaneous presentation of oral and gastric stimulation. In this experiment, the effect of varying temporal order of the two stimulations is identified.

Comparing two groups of rats that receive identical amounts of oral and gastric stimulation, differing only in their presentation order, also allows an assessment of whether the quantity of stimulation in the previous experiment contributed to the lowered FLI in oral and gastric conditions compared to oral and

gastric alone.

METHODS

The protocol of this experiment was similar to the previous one except that the Oral/Gastric group was replaced by two test conditions, Oral-->Gastric and Gastric-->Oral, that differed by the order in which they received that gastric and oral infusions. As in the previous experiment, rats ate a 15 ml liquid diet meal 24 hr before they were anaesthetized with urethane (1.2 g/kg). Animals in the "Oral" treatment condition had 12 ml of 1 M sucrose perfused over their tongue at a constant rate of 0.80 ml/min for 15 min; "Gastric" animals received direct intragastric infusions at identical parameters. Animals in the "Oral-->Gastric" group received oral stimulation, a 15 min wait, followed by gastric stimulation. "Gastric-->Oral" animals received gastric stimulation, a 15 min wait followed by oral stimulation. Ninety minutes after the termination of the treatments, rats were killed and their brains were processed for FLI using the protocols described before.

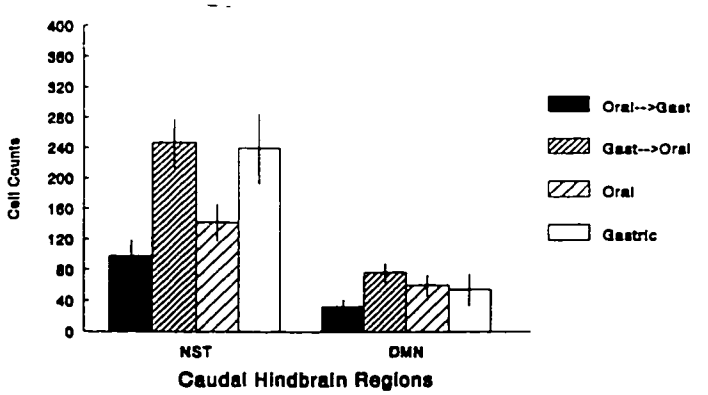
RESULTS

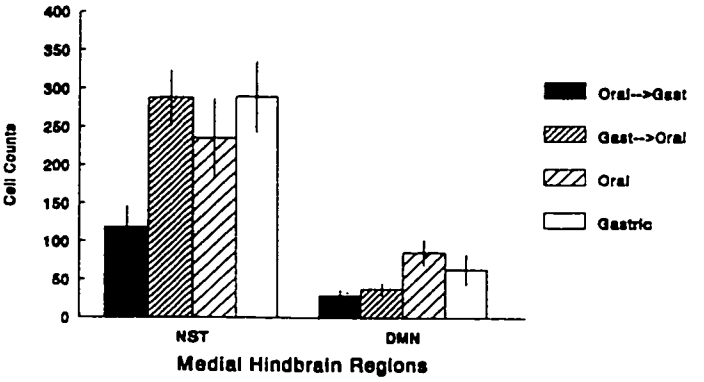
The group mean cell counts are shown in Fig. 7 and a representative photomicrograph from each group is presented in Fig. 8. The results from this experiment showed that the order of oral and gastric stimulation affected Fos expression in the rat hindbrain.

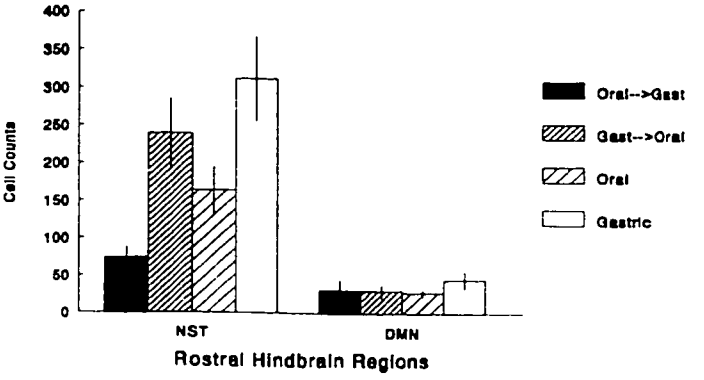
In all three levels of the NST, the "Oral-->Gastric" treatment resulted in significantly less FLI than the other three treatments (Oral; Gastric; and Gastric-->Oral) (Caudal: $F(3,34)=6.12$, $p<0.005$; Medial: $F(3,34)=5.17$, $p<0.05$; Rostral: $F(3,34)=7.51$, $p<0.005$). The only exception to this finding was that the "Oral-->Gastric" and "Oral" groups had similar levels of FLI in the caudal region of the NST. Animals receiving identical oral and gastric stimulation, but in the reverse order ("Gastric-->Oral"), had similar cell counts to isolated "Oral" and "Gastric" groups at all levels of the NST.

In the DMN, cell counts were similar for all four conditions at all three levels, the sole exception being significantly higher FLI in the medial section of the Oral condition in comparison to the Oral-->Gastric and Gastric-->Oral condition ($F(3,34)=3.96$, $p<0.05$).

Figure 7: Comparison of the effects of the order of presentation of oral and gastric stimulation on FLI in the hindbrain of anaesthetized rats: the number of Fos-labelled cells in the DMN and NST in rats that received oral stimulation (12 ml of 1 M sucrose) prior to gastric stimulation (12 ml of 1 M sucrose)(n = 9), gastric stimulation prior to oral stimulation (n = 9), oral stimulation in isolation(n = 9), and gastric stimulation in isolation (n = 9). Cell counts of caudal, medial, and rostral levels of the NST are shown. Data shown are group means ± 1 SEM.


400
360
320
280
240
200
160
120
80
40
0
Cell Counts
NST
DMN
Caudal Hindbrain Regions
Oral-->Gast
Gast-->Oral
Oral
Gastric


400
350
300
250
200
150
100
50
0
Cell Counts
NST
DMN
Medial Hindbrain Regions
Oral-->Gast
Gast-->Oral
Oral
Gastric


400
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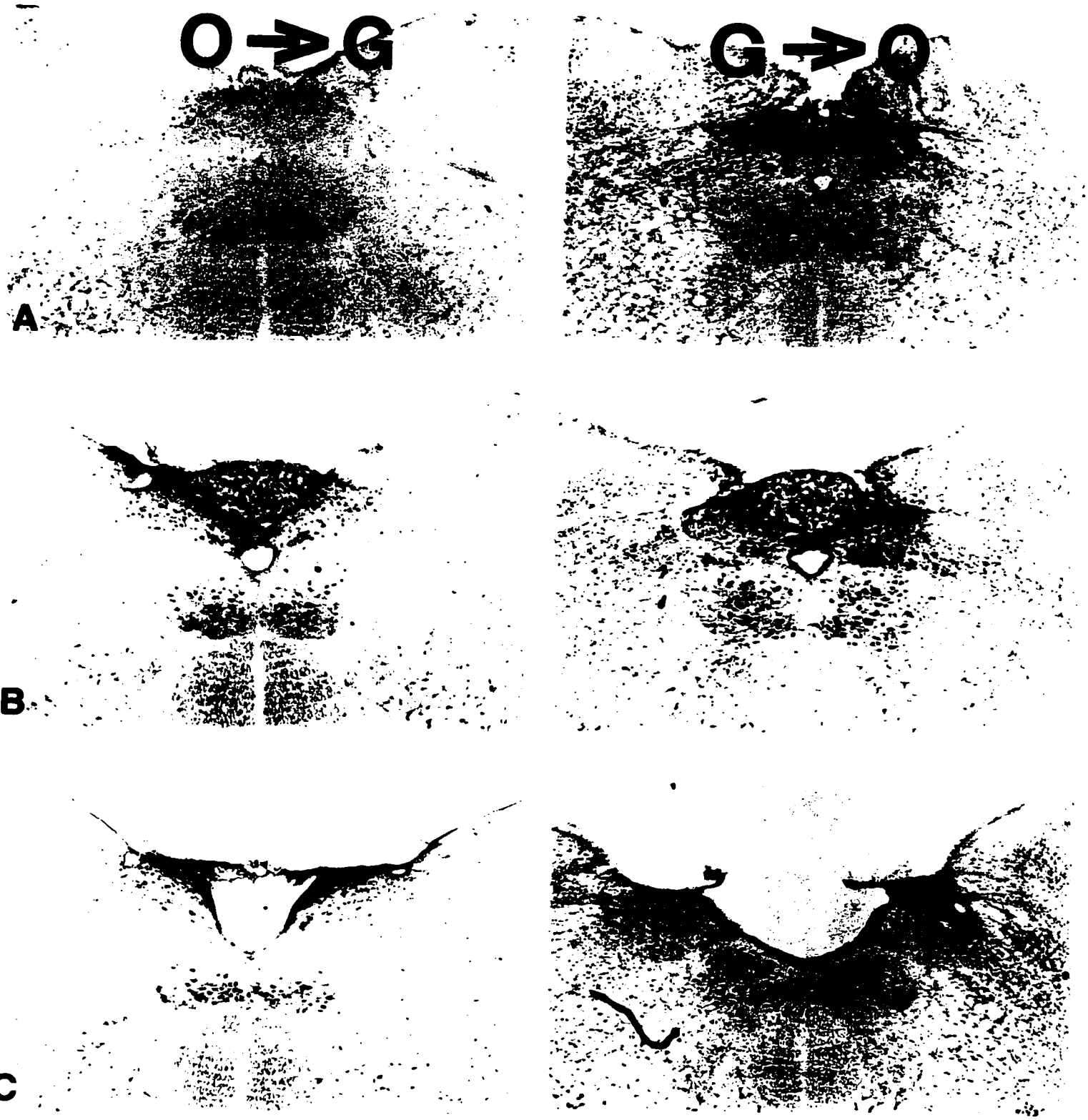
Cell Counts
NST
DMN
Rostral Hindbrain Regions
Oral-->Gast
Gast-->Oral
Oral
Gastric

Figure 8: Photomicrographs of representative sections illustrating differences in FLI between rats who received i) oral before gastric **(O->G)** or ii) gastric before oral **(G->O)** stimulation: in a) caudal b) medial c) rostral levels of the NST.


O→G
G→O
A
B
C

DISCUSSION

The results from this experiment show that the order of oral and gastric stimulation affected FLI elicited in the hindbrain. The condition that most closely mimics real feeding (ie. Oral-->Gastric) resulted in less Fos expression in the NST than the three other conditions (Oral alone; Gastric alone; Gastric-->Oral). As in the previous experiment, decreased FLI in the NST is observed when both stimulations are presented together. This experiment demonstrates, though, that separating the two stimulations by 15 minutes maintains this supression. Thus, the two stimulations do not have to be presented concurrently for the suppression of Fos expression to occur. This is, perhaps, not surprising given that, during normal eating, oral stimulation normally precedes gastric stimulation during a meal.

Unlike the previous experiment, there was very little difference between the FLI in the DMN between the four conditions. It is possible that oral and gastric stimulation need to be presented concurrently to produce decreased FLI (and inhibit signal transmission) in the DMN in comparison to oral or gastric stimulation alone.

EXPERIMENT 5

The Effect of Presentation Order of Oral and Gastric Stimulation on Behavioural Outcome

The previous experiment showed that the presentation order of oral and gastric stimulation influenced expression of FLI in the rat brain. The following experiment was designed to examine the relationship between the patterns of FLI to functional aspects of ingestive behaviour. This study, therefore, allows some insight into the functional significance of different Fos patterns. To accomplish this, this study used the treatment conditions from the previous experiment, where differential Fos patterns were identified, as experimental conditions in a preload-satiety paradigm.

With the exception of Robertson (1991), there are few studies that have investigated the relationship between Fos patterns and behaviour. Thus it is unclear whether differential patterns of Fos expression signal any behavioural, or functional, differences. This experiment was designed to determine how the order of oral and gastric stimulation, that resulted in differential FLI in the previous experiment, correlated

with behavioural effects.

METHODS

This experiment was designed to replicate, as closely as possible, the four experimental treatments used in the previous experiment.

Fourteen rats were implanted with chronically indwelling gastric cannulas according to procedures described elsewhere (Weingarten and Powley; 1980). After two weeks recovery, rats were adapted to an overnight deprivation schedule. Each day, rats were removed from their home cages to a test room. Their stomachs were cleaned of food by flushing warm water into the stomach via the open cannula. Then, a 15 cm long collecting tube was screwed into the cannula and rats were placed in individual Plexiglas cages onto which a drinking spout was suspended. 1 M sucrose was delivered into the spout at a constant rate of 0.80 ml/min for 15 min. Over a period of one week, with daily sessions, rats were trained to sham feed this food. The sham feed period was 15 min long. After the 15 min sham feeding period, the cannula was closed and rats were returned to their home cages and permitted to feed rat chow pellets *ad lib* for the rest of the day

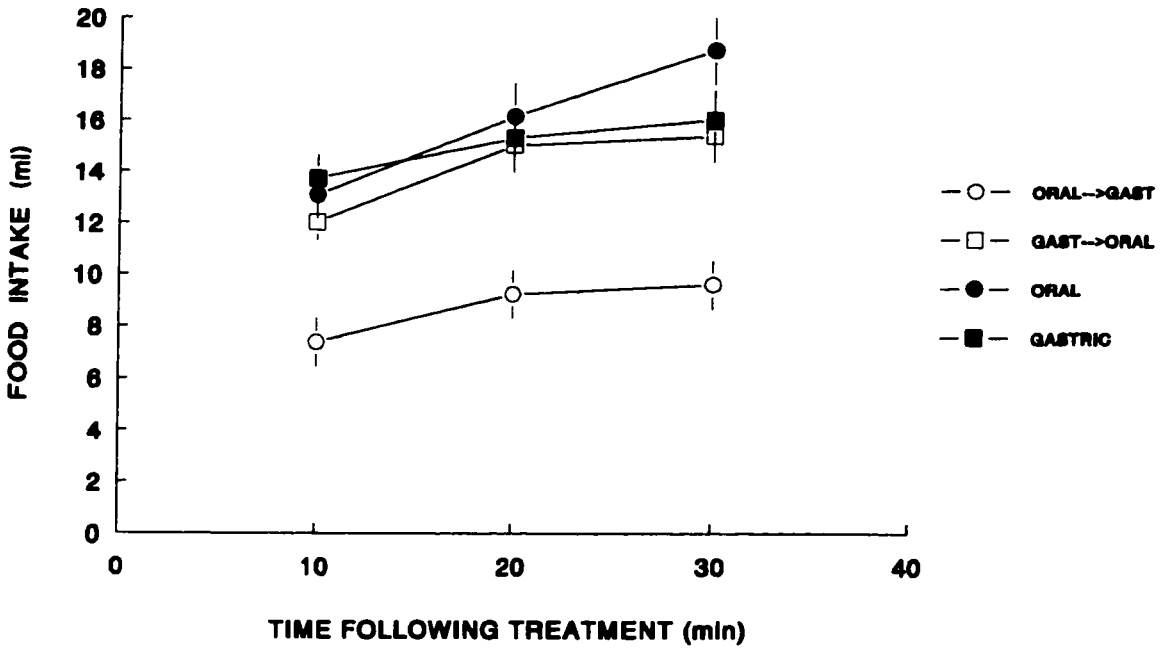
until the imposition of their overnight fast.

Twenty hours before testing, rats were given a last 15 ml liquid diet meal and then deprived of food overnight. During testing, each rat was tested under one of four preload conditions corresponding to the oral and gastric stimulations used in the Fos study. Oral stimulation was accomplished through sham feeding (as described above). Gastric stimulation was accomplished via a 12 ml infusion of 1 M sucrose delivered directly intragastrically through the cannula at the same rate as the Oral condition. The Oral-->Gastric condition had Oral stimulation followed by a 15 min rest, followed by 15 min of gastric stimulation. The Gastric-->Oral condition had the stimulation order reversed. These four treatments served as the preload conditions, that were followed, in all test conditions, by the opportunity to eat a liquid diet meal. The amount of liquid diet eaten after the preload condition was measured at 10 min intervals. All 14 rats were subjected to each of the 4 preload conditions once, with 3 days of *ad lib* feeding in their home cages in between test days.

RESULTS

The cumulative food intake of the rats at 10 min intervals is presented in Fig. 9. The Oral-->Gastric preload treatment resulted in significantly less test meal intakes reflecting greater satiety resulting from the preloads than any of the other three test conditions when examined at 10, 20 or 30 min following the preload treatment (10 min: $F(3,39) = 20.59$, $p<0.0005$; 20 min: $F(3,39) = 16.16$, $p<0.0005$; 30 min: $F(3,39) = 15.61$, $p<0.0005$).

Figure 9: Mean intakes of liquid diet meal (± 1 SEM) following four preload treatments. The cumulative 30 min liquid diet intake was measured, at 10 min intervals, after rats were exposed to four preload treatments: oral stimulation (sham feeding 12 ml of 1 M sucrose) prior to gastric stimulation (gastric load of 12 ml 1 M sucrose) (n = 14), gastric stimulation prior to oral stimulation (n = 14), oral stimulation alone (n = 14), and gastric stimulation alone (n = 14).


FOOD INTAKE (ml)
20
18
16
14
12
10
8
6
4
2
0
0
10
20
30
40
TIME FOLLOWING TREATMENT (min)
ORAL-->GAST
GAST-->ORAL
ORAL
GASTRIC

DISCUSSION

This experiment demonstrates that the differential Fos expression seen in the previous experiment correlates with a differential functional effect in a preload feeding experiment. The four conditions from the Fos experiment were replicated as closely as possible in the behavioural study so the results from both studies could be compared. In the behavioural experiment, the Oral-->Gastric preload treatment produced a larger reduction in food intake than that seen in all other preload treatments, while the Gastric-->Oral preload treatment had no greater meal reduction effects than oral or gastric stimulation alone. This result, coupled with the results of previous experiments, indicate that reduced Fos expression in the NST following Oral/Gastric stimulation correlates with differential functional aspects of eating, in the case of this experiment, increased satiety.

CHAPTER IV. GENERAL DISCUSSION

Using Fos immunohistochemistry, I was able to add to the current understanding of how afferent feeding signals are integrated together and processed in the rat hindbrain.

In my first experiment, I verified the finding, reported by other labs (Olsen, 1993; Fraser, 1993), that Fos expression could be induced by the behavioral act of feeding. Earlier *in vivo* Fos experiments had used extreme experimental treatments to induce Fos expression and, thus, left the question of whether Fos immunohistochemistry was a useful marker for neural activation precipitated by behavioral acts, unanswered. The results of my first experiment showed that simple ingestion of a meal stimulated FLI expression and revealed the pattern of FLI in hindbrain nuclei as a result of this behaviour.

The next series of experiments investigated which components of eating led to the Fos expression produced by ingesting a meal. The act of feeding is composed of several components (eg. anticipation of the

meal, oral stimulation, gastric stimulation, intestinal stimulation, etc.). By using the sham feeding preparation to isolate the oral stimulation, I was able to demonstrate the effect of oral afferent signals in the activation of the hindbrain induced by a meal. From this experiment, it was discovered that isolated oral stimulation resulted in greater Fos expression in the NST in freely feeding rats in comparison to rats which "real" fed a meal and which experienced, therefore, oral, gastric and postgastric stimulation. My subsequent experiments, in anaesthetized rats, went on to show that both isolated oral and gastric stimulation produced significantly more FLI in the NST than these stimulations presented in combination. By comparison to control groups that received no stimulation, I concluded that the increased Fos expression seen when gastric and oral stimulations are presented in isolation is inhibited by presenting the stimuli in combination.

The last two experiments investigated the role presentation order played in the FLI patterns and feeding behaviour consequences. These experiments demonstrated that it was necessary to receive the oral and gastric stimulation in the order they would be experienced during a meal if the suppression of FLI in

the NST was to be observed. Rats that received identical oral and gastric stimulation, but in reverse order, showed similar FLI levels to those animals that received either stimulation in isolation.

A behavioural study was designed to see whether the changes in FLI correlated with behaviour. A comparison of the results from the final two experiments reveals that lower FLI in the NST was associated with increased satiety in a preload experiment. This is one of the few demonstrations that differential patterns of Fos expression can be related to behavioral outcomes.

Another general finding that ties these experiments together is that experimental treatments, that provide oral and gastric stimulation in a way that mimics real feeding, result in baseline levels of FLI in the NST. This was seen in the very first experiment in which ingestion of a meal resulted in no significant change in Fos expression in the NST of the experimental rats. It was also seen in all subsequent experiments in the real feed, Oral/Gastric, and Oral--->Gastric groups, respectively.

One interpretation of these findings is that the Fos expression we observed is the neural correlate or consequence of the "satiety" induced by the

treatments and conditions we used. The increased FLI seen after isolated gastric and oral stimulation is due to increased neural activity in the afferent visceral and gustatory pathways. The baseline FLI levels seen after treatments that mimic real feeding indicates there is reduced neural activation of the visceral and gustatory pathways after a meal. The behavioural experiment shows this reduced neural activity corresponds to a reduction in food intake, but the functional linking between FLI expression and behaviour remains unclear. It is possible that the increased neural activation seen when oral and gastric stimulation are presented in isolation reflect a continued propensity of the rat to continue feeding. This may also account for the increased FLI seen in the DMN in these conditions since this FLI may be evidence of efferent signals to stomach and viscera to increase secretory-motor events (e.g., gastric acid secretion and stomach contractions) required for digestion. The "satiety" signals produced by a meal, however, inhibit signal transmission along the visceral and gustatory afferents, thus inhibiting oral and gastric stimuli from prolonging a meal, and allowing meal termination to occur.

Another possible explanation is that the

increased FLI seen when the rat is exposed to a novel stimulus (ie. Gastric-->Oral) reveals the presence of long term changes, due to learning, that are taking place in the neural circuits, changes that would not take place when a familiar stimulus is presented. An animal would be learning about the unfamiliar stimulations (ie. Oral alone, Gastric alone, Gastric-->Oral) and possibly associating them with their post ingestive consequences. Expression of the Fos protein would be the first step in initiating the transcription of other genes involved in inducing long term changes (e.g. ones that lead to long-term potentiation) that lead to memory formation. To accept this explanation, one would have to assume the sham feeding training was not sufficient to prevent this "memory formation" from occurring in the oral alone condition.

One implication which arises from this series of experiments is that it is important to determine how the various peripheral feeding signals affect ingestive behaviour in the context of a meal. While important discoveries can be made by examining these signals in isolation, it is clear that their effects may vary greatly when presented in conjunction with other feeding stimuli. Experiments must be designed to examine the effects of peripheral feeding signals on

other peripheral feeding signals, as well as feeding itself.

There are two main questions that arise from this series of studies. First, is it the ability to reduce food intake that leads to the decreased FLI seen in the hindbrain after exposure to conditions that mimic real feeding? To test this one would have to determine if the FLI pattern, elicited by a stimulus, is altered as a stimulus' ability to induce meal termination (ie. cause satiety) is altered through experience. If an animal were trained, through multiple exposures, to associate satiety with a stimulation not normally associated with satiety (e.g. gastric load) we would predict that exposure to that stimulation would elicit less FLI in a trained rat than an untrained rat.

Second, and more importantly, an attempt to identify which sensory neurons are primarily involved in this "inhibition" of FLI during conditions that model real feeding could be carried out. Do the visceral and gustatory afferents inhibit each other's neural transmission or are they acting on a third set of neurons that integrate the information provided by these two afferent pathways? One way to investigate this is through double labelling studies that stain the

two afferent pathways and label for Fos after oral or gastric stimulation. Another approach that could more directly determine if inhibition is taking place and where it is occurring would be staining for receptors of inhibitory neurotransmitters (eg. GABA, glycine). Also, by using a paired-electrical stimuli test, an electro-physiological technique that can measure inhibitory processes using paired pulses, an extracellular estimate of recurrent inhibition in the afferent pathways under investigation could be investigated. This procedure could be carried out using a chronic preparation that allows multiple conditions (ie. oral, gastric, oral/gastric) to be examined.

Through these studies, and others like them, we will begin to form better understanding of where and how afferent feeding signals integrate to produce feeding behaviour.

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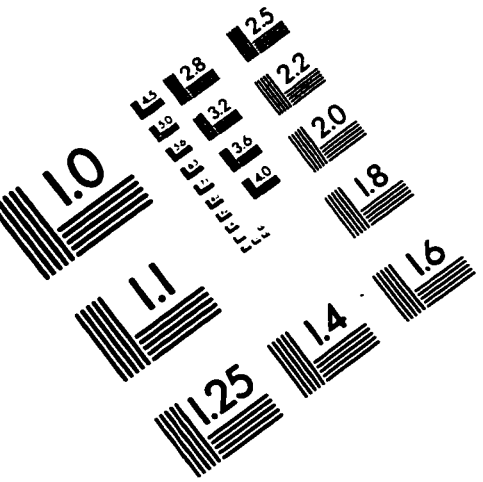
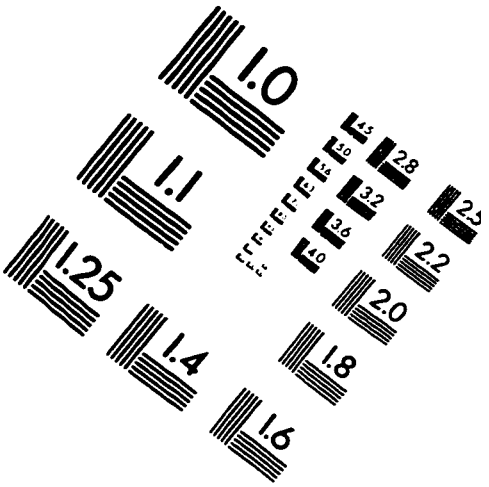
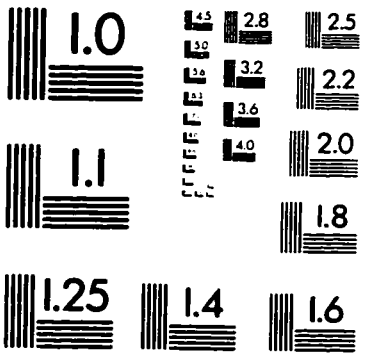
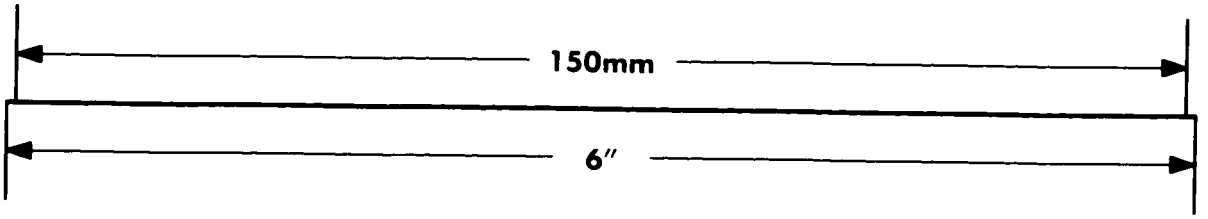
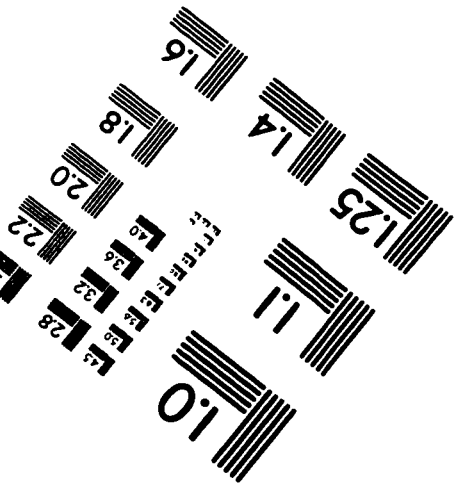
IMAGE EVALUATION TEST TARGET (QA-3)

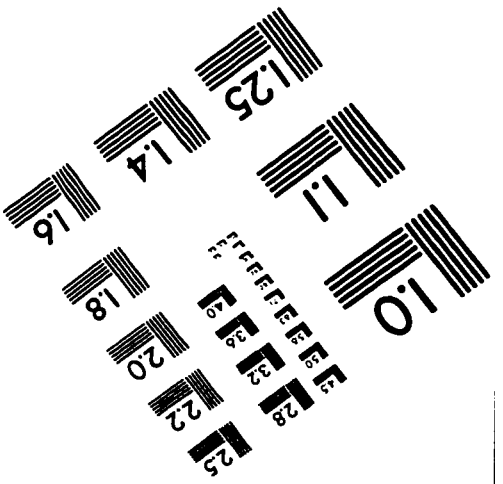












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