ENTRAINMENT OF CIRCADIAN RHYTHMS IN MICE BY DAILY BOUTS OF

FOOD AVAILABILITY: ANALYSIS OF NEURAL GENE EXPRESSION PATTERNS

by

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ABSTRACT

The studies presented in this thesis were designed to investigate the effects of daily restricted feeding schedules on behavior and gene expression patterns in the brains of mice (*Mus musculus*). Animals maintained on a restricted feeding schedule in which a single meal is provided at the same time each day will, within a few days, show increased activity and arousal during the hours immediately preceding mealtime. This increase in activity (known as food-anticipatory activity or FAA) will appear even if mealtime occurs at a time of day that the animal is normally at rest and normally does not consume food. Restricted daily access to food can entrain an endogenous, self-sustaining circadian pacemaker that is separate from the light-entrainable pacemaker in the rodent suprachiasmatic nucleus (SCN). The identity of this food-entrainable pacemaker and the nature of the afferent signals that affect it are not known, despite a large number of studies aimed at identifying the critical substrate and signal involved.

The intense activity associated with food anticipation is probably mediated by mechanisms that normally arouse animals in order to increase the probability of finding and eating food. At the same time, anticipation probably helps prepare the digestive system for the meal. Recently, a pair of neuropeptides (orexin A and B, also known as hypocretin 1 and 2) was described which have been shown to play a role in sleep, arousal and feeding. Because an animal exposed to a restricted feeding schedule needs to be awake, aroused and prepared to ingest food during the limited daily window of food availability, the functioning of the orexin system is likely altered under conditions of food entrainment.

A series of studies were designed in order to investigate this possibility. The first characterized the normal circadian pattern of feeding in C57BL/6J mice and assessed their ability to generate FAA under a restricted feeding schedule (Chapter 2). Results show that mice, that normally exhibit nocturnal feeding patterns, are able to adapt to a restricted feeding schedule in which food is presented daily during the middle of the light phase. The second study investigated strain differences in the distribution of cell bodies containing orexin A and B in the brains of mice (Chapter 3). Orexin immunoreactive cell bodies were found outside of the lateral hypothalamic/perifornical region in all three strains of mice. Although only slight strain differences were found, substantial differences in distribution pattern were found between orexin A and B. The third study included several experiments designed to assess the response of the lateral hypothalamic orexin system to scheduled restricted feeding (Chapter 4). Results show that immediate-early gene expression within the lateral hypothalamus and within orexin-containing cells is altered in animals exposed to chronic restricted feeding. Furthermore, orexin mRNA expression is up-regulated in animals anticipating the arrival of their daily meal. The fourth and final study assessed immediate-early gene expression within brain structures receiving orexin input under conditions of restricted feeding (Chapter 5). It was found that chronic exposure to a restricted feeding schedule not only alters an animal's behavior and physiology, but also substantially modifies patterns of immediate-early gene expression in various brain structures. Furthermore, like behavior and physiology, it appears that altered gene expression patterns emerge after only a few days of exposure to the restricted feeding schedule. The results, their implications and future directions are discussed in Chapter 6.

LIST OF ABBREVIATIONS AND SYMBOLS USED

3V/III	Third ventricle
4V	Fourth ventricle
6-OHDA	6-hydroxydopamine
α	Active phase (alpha)
τ	Period of the endogenous oscillator (tau)
AC	Anterior commissure
AH	Anterior hypothalamus
AL	*Ad Libitum*
AP	Area postrema
Arc/ARC	Arcuate nucleus
CCl_4	Carbon tetrachloride
CRH	Corticotropin-releasing hormone
Cry	Cryptochrome
D	Dark
D3V	Dorsal third ventricle
DAB	Diaminobenzidine
DD	Constant dark
DLG	Dorsolateral geniculate
DMH	Dorsomedial hypothalamus
DOPAC	Dihydroxyphenylacetic acid
F	Fornix
FAA	Food-anticipatory activity
FEP	Food-entrainable pacemaker
FF	Fasted-Fed
FN	Fasted-NotFed
GAL	Galanin
IGL	Intergeniculate leaflet
I.P.	Intraperitoneal
Ir	Immunoreactivity

IR	Immunoreactive
L	Light
LC	Locus coeruleus
LD	Light-dark
LH	Lateral hypothalamus
LL	Constant light
M	Moderate
ME	Median eminence
MPOA	Medial preoptic area
MSG	Monosodium glutamate
NMDA	N-methyl-D-Aspartate
NPAS2	Neuronal PAS domain protein 2
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
OC	Optic chiasm
OT	Optic tract
Ox1R	Orexin receptor 1
Ox2R	Orexin receptor 2
Ox A	Orexin A
Ox B	Orexin B
PB	Parabrachial nucleus
PBS	Phosphate-buffered saline
PBS-Azide	Phosphate-buffered saline with sodium azide
PBS-X	Phosphate-buffered saline with triton-X 100
Per	Period
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus of the hypothalamus
PVT	Paraventricular nucleus of the thalamus
RCH	Retrochiasmatic area
RF	Restricted-Fed

RF3	Restricted-Fed Day 3
RF6	Restricted-Fed Day 6
RN	Restricted-NotFed
RN3	Restricted-NotFed Day 3
RN6	Restricted-NotFed Day 6
SCN	Suprachiasmatic nucleus
SON	Supraoptic nucleus
SSC	Sodium chloride – sodium citrate
T	Period of the exogenous cycle or zeitgeber
TMN	Tuberomammillary nucleus
TU	Olfactory tubercle
VLGN	Ventrolateral geniculate nucleus
VMH	Ventromedial hypothalamus
ZT	Zeitgeber time

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CHAPTER 1

General Introduction

1.1 Food-Anticipatory Activity

As any zookeeper will likely tell you, the best time to visit most animal exhibits is around feeding time, a time at which the animals are usually awake and active. There is plenty of anecdotal evidence suggesting that animals 'know' when mealtime occurs. Family pets can become excited and spend time around their food dish just before their habitual feeding time. Dairy cows will spontaneously come in from the field prior to milking time, during which they are normally given supplementary food. They have also been known to arrive early on those days following the Fall time change when the farmer starts milking an hour later than on the previous day. These and other examples suggest that animals are aware of environmental temporal information tied to food availability. Whether in captivity or in the wild, many factors in an animal's surroundings exhibit daily periodicity. Most of these cyclical events in the environment are tied to the 24 h day-night cycle. Access to resources that are key to survival, such as food and water, can depend greatly on time of day. Animals need to be able to adapt to feeding opportunities no matter when or where they occur so that they can exploit these events fully. To this end, animals have developed various strategies in order to maximize survival in a cyclical environment.

Along with environmental cycles, many endogenous physiological and behavioral parameters exhibit rhythmicity with a period that approximates the solar day (Rusak and Zucker, 1979). These biological rhythms are referred to as circadian rhythms since they recur approximately every 24 h (Rusak and Zucker, 1979). The effects of timed daily food availability on the expression of these rhythms were first described in the scientific literature by Richter, who observed that rats fed only once per day displayed an increase in activity for several hours prior to the time that food became available (Richter, 1922). Since then, the effects of temporally restricted daily feeding on behavioral and

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Invertebrates	Ants	(Schatz et al., 1994)
	Bees	(Aschoff, 1986; Frisch and Aschoff, 1987; Moore et al., 1989; Moore, 2001)
Fish	Catfish	(Boujard, 1995; Bolliet et al., 2001)
	Golden shiners	(Reebs, 2000; Reebs and Lague, 2000; Lague and Reebs, 2000)
	Goldfish	(Sánchez-Vázquez et al., 1997; Sánchez-Vázquez et al., 2001; Aranda et al., 2001)
	Medaka	(Weber and Spieler, 1987)
	Rainbow trout	(Bolliet et al., 2001)
	Red drums	(Leiner et al., 2000)
Birds	Chickens	(Rovee-Collier et al., 1998)
	Garden warblers	(Biebach et al., 1989; Biebach et al., 1994)
	House sparrows	(Hau and Gwinner, 1992; Hau and Gwinner, 1996; Hau and Gwinner, 1997; Heigl and Gwinner, 1999)
	Oyster catchers	(Daan and Koene, 1981)
	Pigeons	(Abe and Sugimoto, 1987; Phillips et al., 1993; Rashotte et al., 1995; Rashotte and Stephan, 1996)
	Starlings	(Wenger et al., 1991)
	Svalbard ptarmigan	(Reierth and Stokkan, 1998)
	Zebra finches	(Johnson and Rashotte, 2002)
Mammals	Baboons	(Koerker et al., 1974)
	Hamsters	(Rusak et al., 1988; Mistlberger, 1991b; Abe and Rusak, 1992; Mistlberger, 1992b; Mistlberger, 1993a; Mistlberger, 1993b; Borer and Clover, 1994; Masuda and Oishi, 1995)
	Marsupials	(O'Reilly et al., 1986; Coleman et al., 1989; Kennedy et al., 1991; Kennedy et al., 1996)
	Mink	(Zielinski, 1986)
	Owl monkeys	(Erkert, 2000)
	Rabbits	(Jilge et al., 1987; Jilge, 1992; Jilge and Stähle, 1993)
	Rhesus monkeys	(Mattern et al., 1993)
	Sheep	(Sugino et al., 2002a; Sugino et al., 2002b)
	Squirrel monkeys	(Sulzman et al., 1977; Apelgren et al., 1985; Aschoff and van Goetz, 1986; Aschoff, 1986; Boulos et al., 1989)
	Weasels	(Zielinski, 1986)
Other Species	Bullfrogs	(Van Bergeijk, 1967)
	Clams	(Williams and Pilditch, 1997)
	Crayfish	(De Miguel and Arechiga, 1994)
	Iguanas	(Wikelski and Hau, 1995)

Table 1.1. Studies on the effects of timed daily restricted feeding on behavioral and physiological rhythms in various species (for review see Boulos and Terman, 1980; Mistlberger, 1994; Stephan, 2001).

physiological rhythms have been studied using a wide range of species (see Table 1.1; for review see Boulos and Terman, 1980; Mistlberger, 1994; Stephan, 2001). The vast majority of what is known about the effects of timed daily feeding, however, stems from

work done in rats, and, to a lesser extent, in mice, and therefore, discussion in this chapter will focus almost exclusively on studies done using these species.

1.1.1 Forms of Anticipation

Animals maintained on a restricted feeding schedule in which a single meal is presented at the same time each day will, within a few days, show increased activity and arousal during the hours immediately preceding mealtime (Mistlberger, 1994). This occurs even if food is presented during the animal's normal rest phase, a time of day that the animal would not ordinarily consume food during *ad libitum* conditions. This bout of increased activity is known as food-anticipatory activity, or FAA, and has been detected in a range of behaviors in rats, including: general cage activity (Richter, 1922; Honma et al., 1983b; Terman et al., 1984; Mistlberger and Rechtschaffen, 1984); revolving drum or wheel-running activity (Richter, 1922; Bolles and de Lorge, 1962; Bolles and Stokes, 1965; Bolles and Moot, 1973; Coleman et al., 1982; Honma et al., 1983b); unreinforced lever pressing (Bolles and Stokes, 1965; Boulos et al., 1980; Terman et al., 1984); feeding site visits or activity directed at the food source (Mistlberger and Rusak, 1988); increased drinking (Boulos et al., 1980; Coleman et al., 1982; Mori et al., 1983; Terman et al., 1984); and even yawning (Holmgren et al., 1991). In mice, wheel-running activity has been the FAA measure of choice (Abe et al., 1989; Marchant and Mistlberger, 1997; Challet et al., 1998a; Challet et al., 1998b; Sharma et al., 2000; Holmes and Mistlberger, 2000; Pitts et al., 2003; Dudley et al., 2003). Other studies in mice have used measures such as general cage activity (Aschoff, 1986; Wakamatsu et al., 2001), drinking (Marchant and Mistlberger, 1997; Holmes and Mistlberger, 2000) and food-bin activity (Holmes and Mistlberger, 2000). The type of FAA displayed can depend on such factors as the species under study, the housing or experimental conditions used, and/or the nature of the stimulus being anticipated (Mistlberger, 1994).

Anticipation is manifest not only behaviorally, but also physiologically. Rhythms in many physiological parameters have been synchronized by mealtime (for review see Boulos and Terman, 1980). In rats, rhythms in body temperature (Krieger and Hauser,

1978), blood pressure and heart rate (Van den Buuse, 1999), plasma corticosterone (Takahashi et al., 1977; Morimoto et al., 1977; Krieger and Hauser, 1978; Holloway et al., 1979; Honma et al., 1983a), plasma thyroid hormones (Cokelaere et al., 1996), and liver and intestinal enzyme activity (Hopkins et al., 1973; Stevenson and Fierstein, 1976; Saito et al., 1980) are all synchronized by mealtime. The pre-feeding plasma corticosterone peak found in rats under restricted feeding seems to be specific to stimuli associated with feeding, since this peak did not precede daily cage cleaning or vaginal swabbing (Honma et al., 1984). Not all physiological rhythms are synchronized to periodic feeding, however. Levels of melatonin in the pineal gland and in plasma were not affected by restricted daily feeding schedules in rats, and maintained a stable phase relationship with the light-dark (LD) cycle (Holloway et al., 1979).

The observation that intestinal enzyme levels increased in anticipation of daily feeding, and that this rhythm persisted during total food deprivation (Saito et al., 1980), suggested the possibility that other functional parameters of the gastrointestinal system could be altered under restricted feeding schedules. Electromyogram recording of the stomach and duodenum showed that the number of 'irregular contractions' of the duodenum increased gradually during the 3-5 h period preceding a scheduled daily mealtime (Comperatore and Stephan, 1987). This anticipatory peak of intestinal motility persisted on days when food was withheld, and shifted gradually over a number of days in response to a phase delay of feeding time. These contractions were not a direct response to a lack of food within the gastrointestinal tract, since they peaked and then declined during total food deprivation.

Restricted feeding paradigms have been shown to synchronize several physiological rhythms in mice, including: body temperature (Nelson et al., 1975; Lakatua et al., 1983; Nelson and Halberg, 1986; Hotz et al., 1987); serum corticosterone level (Nelson et al., 1975); blood eosinophil cell number (Pauly et al., 1975); liver glycogen content and glycogen phosphorylase activity (Vilchez et al., 1975; Nelson et al., 1975); and colon cell proliferation (Lakatua et al., 1983). The wide range of behavioral and

physiological parameters shifted by scheduled daily feeding times suggests an important functional advantage to this plasticity.

Since FAA is a laboratory phenomenon, its relevance to the behavior of animals in natural settings is uncertain. But a few field studies of animal behavior in relation to feeding have yielded evidence for similar phenomena. The observation that honeybees congregated around a patio on which breakfast was normally served led to investigations on the time sense or 'Zeitgedächtnis' in this species (reviewed in Mistlberger, 1994; Moore, 2001). Bees have been shown to adjust their daily activity to coincide with times of food availability and can anticipate mealtime by arriving at the feeding location 1-2 h early (Aschoff, 1986; Frisch and Aschoff, 1987; Moore et al., 1989; Moore, 2001).

Birds, marine iguanas and nursing rabbit pups have also been observed to coordinate their behavior with periodic cycles of food availability in naturalistic settings (Daan and Koene, 1981; Jilge, 1993; Wikelski and Hau, 1995; Wilkie et al., 1996; Allingham et al., 1998; Escobar et al., 2000; Jilge et al., 2000). Further studies have shown that animals of various species are able to form associations of temporal and spatial information to optimize foraging efforts (Moore et al., 1989; Biebach et al., 1989; Boulos and Logothetis, 1990; Schatz et al., 1994; Mistlberger et al., 1996; Budzynski and Bingman, 1999; Reebs, 2000; Lukoyanov et al., 2002; Aragona et al., 2002).

1.1.2 How Do Animals Anticipate Feeding Time?

There are a number of possible mechanisms that could account for FAA to a 24 h feeding schedule. Animals might respond to cues directly associated with meal delivery, but since FAA begins on average ~3 h prior to mealtime, this is generally not possible. In principle, other exogenous cues recurring every 24 h, such as the transitions of the LD cycle or the environmental temperature cycle could be used by animals to estimate the time at which food will become available. When all environmental stimuli were kept constant, however, animals were still able to anticipate food access (Richter, 1922; Takahashi et al., 1977; Boulos et al., 1980). Furthermore, animals trained to receive a meal every 23 or 25 h were also able to anticipate mealtime, making it unlikely that these

animals were using an environmental timing cue (Boulos et al., 1980). In fact, a study in which external cues were presented to the animals to signal mealtime found that cueing actually inhibited, but did not abolish, food-anticipatory lever pressing (Terman et al., 1984). Thus, it seems that animals are relying on endogenous timing information to generate FAA.

Endogenous timing cues could be generated by an hourglass or interval timer which requires resetting at the beginning of each cycle and is incapable of self-sustained oscillation. Food ingestion, for example, could serve to reset the timer each day, and the gradual increase in hunger over the next day could serve as a signal to increase activity prior to mealtime. In this way, FAA would be triggered when some threshold of energy depletion was reached. Conversely, the mechanism providing endogenous timing information could be a self-sustaining oscillator that does not require daily resetting.

Tests to distinguish these possible mechanisms involved omitting one or more daily meals altogether, thereby eliminating the potential resetting signal. The results showed persistence of FAA, indicating that the endogenous timing mechanism generating FAA does not require resetting, but is, instead, a self-sustaining oscillator (Boulos et al., 1980; Coleman et al., 1982). Furthermore, when a meal is skipped, FAA initially increases and then decreases as the feeding window is passed indicating that hunger alone does not drive this increase in activity.

Several models have been proposed to explain an animal's ability to generate FAA. These models can be distinguished by the emphasis that is placed on the role that learning plays in the generation of FAA, and by their ability to account for several properties of FAA under various conditions (Mistlberger, 1994). These models have been referred to as 'associative', 'computational' and 'entrainment' models (reviewed in Mistlberger, 1994). A vast body of evidence, described in detail below, has led to the interpretation that the FAA observed both in the wild and in the lab is mediated by an endogenous, self-sustaining circadian oscillator that functions as a pacemaker under restricted feeding, synchronizing (or entraining) physiological and behavioral rhythms to

periodic food availability (the food-entrainable pacemaker, FEP; reviewed in Boulos and Terman, 1980; Mistlberger, 1994; Stephan, 2001; Stephan, 2002).

1.1.3 Adaptive Significance of Anticipation

The capacity for organisms to generate circadian rhythms appears to be an evolutionarily old and well-conserved phenomenon. Circadian rhythmicity likely serves to 1) integrate and synchronize endogenous physiological rhythms thereby also temporally sequestering incompatible physiological functions and 2) to coordinate these rhythms with periodic fluctuations in the external environment (Aschoff, 1964; Rusak and Zucker, 1979; Rusak, 1981; Schibler et al., 2003). Circadian rhythmicity confers adaptive advantages by coordinating endogenous physiology to mirror the environment, thereby allowing the organism to prepare for predictable periodic environmental events (Aschoff, 1964; Rusak and Zucker, 1979). The behavioral and physiological anticipation of periodic fluctuations in food availability likely represents one mechanism by which the endogenous circadian system can generate appropriate responses to important cycles in the environment. FAA ensures that the animal is awake and aroused prior to mealtime, increasing the probability that the animal will find and ingest food (Mistlberger, 1990). In addition, the alterations in physiological parameters discussed above appear to prepare the gastrointestinal system for the anticipated arrival of food.

1.2 Entraining the Food-Entrainable Pacemaker

Many spontaneously occurring rhythms in behavior and physiology are endogenously generated (for review see Rusak and Zucker, 1979). They persist in constant conditions and show a free-running period that usually deviates somewhat from 24 h. These rhythms synchronize to external cycles like the environmental LD cycle because the endogenous circadian pacemaker driving them becomes entrained (Mistlberger and Rusak, 2000). The period of the endogenous oscillator (τ) usually differs from that of the exogenous periodic stimulus or zeitgeber (T); therefore, the endogenous oscillator must be adjusted with daily phase shifts to entrain to the exogenous cycle. These phase

shifts compensate for the difference between τ and T, and thus a stable phase relation is established and maintained between the endogenously generated rhythm and the exogenous entraining cycle. In order to show that an endogenous rhythm is entrained by the zeitgeber, it is necessary to demonstrate that the zeitgeber controls the phase, and thus the period of the endogenous rhythm. FAA begins reliably from ~1-3 h prior to each mealtime, and thus, it appears that the phase of the FAA is being entrained by periodic food availability. Confirmation that this represents true entrainment comes from a number of additional findings.

Entrainment of circadian rhythms is only possible if the period of the entraining cycle does not deviate too greatly from τ; i.e., there are limits on the range of entrainment for each oscillator (Mistlberger and Rusak, 2000). Entrainment of the endogenous oscillator will not occur if the period of the zeitgeber falls outside these limits. The limits of entrainment reflect the maximum that an oscillator is able to shift within a single cycle in response to a particular stimulus. Thus, if τ were 25 h, and the maximum amount the oscillator could shift (either advance or delay) to a particular stimulus each day were 2 h, then the limits of entrainment would be 23-27 h.

Some of the first studies on rats using periodic food availability determined that these animals were unable to anticipate feeding bouts scheduled every 19 or 29 h (Bolles and de Lorge, 1962). Even when rats were born and raised under a 19 or 29 h LD cycle, they were still unable to synchronize behavioral rhythms to these cycles of food availability (Bolles and Stokes, 1965). Animals in these studies had no difficulty anticipating a meal presented every 24 h, and actually showed ~24 h modulation of their activity when exposed to a 29 h feeding schedule. These results confirm that FAA is not merely a behavioral response to hunger or periods of fasting, but that it is controlled by a biological clock with circadian limits of entrainment.

In one study, rats were able to anticipate meals recurring every 23 or 25 h with increased unreinforced lever pressing; however, they did not show this anticipation prior to meals occurring every 18 or 30 h (Boulos et al., 1980). A periodicity of 23 h appears to

be near the lower entrainment limit, since another study showed that rats housed under LD or in constant dark (DD) did not consistently anticipate meals scheduled every 23 h with increased wheel running (Lax et al., 1999). Additional investigations showed that while rats were able to show robust wheel-running in anticipation of 23.5, 24, 25, 26 and 27 h feeding schedules, they were unable to anticipate meals recurring every 21 or 22 h (Aschoff, 1986; Mistlberger and Marchant, 1995). Thus, the oscillator mediating FAA in rats appears to have limits of entrainment ranging between 23 and 27 h.

In order to demonstrate entrainment of an endogenous rhythm by an exogenous cycle, the endogenous rhythm should be recorded in its free-running (unentrained) form before and after exposure to the zeitgeber, as well as during zeitgeber exposure (Boulos and Terman, 1980). If stable entrainment were achieved to the zeitgeber, then the phase of the free-run following entrainment should reflect the phase of the entrained rhythm and not that of the extrapolated free-run prior to exposure to the zeitgeber. Applying this test of entrainment to FAA has proven difficult, since FAA emerges only in the presence of its zeitgeber (periodic food availability), rendering the estimation of period and phase prior to entrainment impossible.

Once FAA has been established, however, it has been shown to persist under constant conditions (food deprivation) for a number of cycles (Stephan et al., 1979b). Although restoration of *ad libitum* feeding abolishes FAA in most cases, in a few rare cases, FAA was shown to persist during *ad libitum* feeding (Boulos et al., 1980). When FAA does persist, in either food deprivation or *ad libitum* feeding, the phase of the persisting rhythm reflects prior mealtime, indicating that stable entrainment of this rhythm by the exogenous feeding cycle was achieved. When food deprivation days were alternated with intervening periods of *ad libitum* feeding, animals that had previously been entrained to a restricted feeding schedule expressed increased activity at times corresponding to prior mealtime during each of these food deprivation tests (Coleman et al., 1982; Rosenwasser et al., 1984; Clarke and Coleman, 1986). Thus, although behavior may become uncoupled from the underlying pacemaker during periods of *ad libitum*

feeding, the pacemaker continues to oscillate.

Another characteristic of entrained circadian rhythms is that, following a shift of the entraining zeitgeber, the rhythm will shift to realign with the exogenous cycle (Mistlberger and Rusak, 2000). Depending upon the magnitude of the zeitgeber shift, reentrainment may require more than one circadian cycle. In this case, the rhythm being measured will show 'transient' cycles until reentrainment is achieved. Studies in which an advance or delay of mealtime occurred showed that FAA shifted with transient cycles to reestablish entrainment, thus further confirming the endogenous, oscillator-like properties of the FEP (Stephan et al., 1979b; Stephan, 1984; Stephan, 1986a; Stephan, 1986b).

1.3 Where is the Food-Entrainable Pacemaker?

1.3.1 The Master Circadian Pacemaker

FAA represents the behavioral output of a self-sustaining, endogenous circadian pacemaker that is entrained by periodic feeding schedules, in contrast to the output driven by the light-entrainable pacemaker, which can often be seen to run persistently through a feeding schedule that entrains the FEP. The light-entrainable circadian pacemaker has been localized to the suprachiasmatic nucleus (SCN), a pair of nuclei located just dorsal to the optic chiasm within the hypothalamus that control most mammalian circadian rhythms (Moore and Eichler, 1972; Stephan and Zucker, 1972; Rusak and Zucker, 1979; Morin, 1994).

A great deal of effort has been focused on identifying the anatomical substrate of the FEP, following rejection of the most obvious option; namely, that the FEP is also the light-entrained SCN pacemaker. Several lines of evidence support the conclusion that the FEP is not located in the SCN.

When restricted feeding was imposed in constant lighting conditions (LL, DD or dim red light), rats and mice expressed two distinct bouts of activity with different periods (Stephan et al., 1979a; Boulos et al., 1980; Honma et al., 1983b; Aschoff, 1986; Abe et al., 1989; Marchant and Mistlberger, 1997; Lax et al., 1999; Holmes and

Mistlberger, 2000). One of these rhythms had a period that matched that of the feeding cycle and the other free-ran with a different τ. Similarly, studies conducted under 24 h LD cycles using feeding schedules with periods differing from 24 h showed that animals can simultaneously express 24 h rhythms entrained by light along with FAA entrained to the feeding schedule (Aschoff, 1986), although sometimes these rhythms also interact or interfere with each other. Rats given food access during two time periods every day were able to anticipate both meals (Bolles and Moot, 1973). Since the meals each occurred every 24 h, it is possible that a single endogenous oscillator was driving the anticipation to both. In a second study in which two daily mealtimes occurred on different schedules (24 and 25 h), rats were able to anticipate both meals (Edmonds and Adler, 1977). Because it is unlikely that one pacemaker is capable of driving two rhythms with differing periods, the results of these studies suggested the presence of at least two pacemakers (Stephan, 1986a).

Lesion studies have shown conclusively that the SCN, the site of the master circadian pacemaker, is not required for food anticipation or entrainment in rats or mice (Krieger et al., 1977; Stephan et al., 1979a; Boulos et al., 1980; Clarke and Coleman, 1986; Marchant and Mistlberger, 1997). The behavioral and physiological variables that entrain to feeding in intact animals also do so in SCN-ablated animals. In one study, a number of SCN-ablated mice did not show increased wheel running in anticipation of the daily meal (Marchant and Mistlberger, 1997). All of these animals did, however, show increased activity associated with prior mealtime when food access was delayed by 13 h, indicating that the endogenous oscillator was entrained by mealtime, even though this entrainment was not manifest in anticipatory activity. The lack of FAA in these mice was not correlated with incidental damage to a specific brain structure near the SCN, although it appeared that lesion size was an important factor; i.e. mice with the largest lesions failed to show FAA (Marchant and Mistlberger, 1997).

SCN-ablated animals have been used extensively to further characterize food anticipation and entrainment since they lack the light-entrainable rhythm that may

obscure or alter the expression of FAA. Animals become completely arrhythmic following SCN ablation under free-feeding conditions, but start to anticipate mealtime within a few days of exposure to a circadian feeding schedule (Stephan et al., 1979a; Stephan et al., 1979b; Boulos et al., 1980). SCN-ablated rats exposed to a schedule of two food access times per day were able to anticipate both meals just as control animals did (Stephan et al., 1979a; Stephan, 1989a). Similarly, when meal schedules with different periods were used, SCN-ablated animals were able to simultaneously anticipate meals recurring every 23.75 and 24 h, 24 and 24.5 h, and 25 and 26 h (Stephan, 1983; Stephan, 1989b). SCN-ablated rats, however, were unable to show simultaneous anticipation to three meals occurring each day (Stephan, 1989a). Therefore, it is likely that the FEP is made up of two oscillators, enabling the animal to anticipate more than one daily feeding opportunity, even if these recur with different periods.

Once FAA is established in SCN-ablated animals, it will persist for several cycles under total food deprivation (Stephan et al., 1979b; Boulos et al., 1980), and will reappear at an appropriate phase during subsequent food deprivation tests following intervening periods of *ad libitum* feeding (Clarke and Coleman, 1986; Yoshihara et al., 1997). In addition, advances or delays of mealtime result in phase shifts of FAA with transient cycles when the shift required is greater than can be accomplished in one cycle, just as in SCN-intact animals (Stephan et al., 1979b; Stephan, 1984; Stephan, 1992b). There may, however, be some fundamental differences between phase shifting responses of the SCN and those of the FEP (Stephan, 1992a). Whereas the SCN can be reset by a single light pulse, more than one cycle of feeding at new mealtime is required before full reentrainment is attained by the FEP.

SCN-ablated animals were able to anticipate mealtimes that differ from 24 h (Stephan et al., 1979b), but the circadian limits of entrainment still applied. Entrainment limits appear to be somewhat greater in SCN-ablated animals (23-31 h) than in intact controls (Stephan, 1981), at least when extreme periods are approached gradually. Animals were not able to anticipate meals recurring every 31 h if they were exposed to

this schedule directly following a 24 h schedule. If, on the other hand, they were exposed to restricted feeding schedules with gradually increasing period lengths, animals were able to show FAA to schedules of 31 h, and some even anticipated mealtime under schedules of 33 h (Stephan, 1981). This finding suggests that features of the underlying pacemaker (specifically its τ and phase response curve) can be influenced by past entrainment schedules, which has also been shown to occur with the light-entrainable circadian pacemaker (Pittendrigh and Daan, 1976; Mistlberger and Rusak, 2000).

Studies in which SCN-ablated rats were maintained on feeding schedules outside the limits of entrainment produced some unexpected and interesting results (Stephan, 1981; Stephan, 1992b). When meals were presented every 22 or 23 h, rats failed to show FAA prior to mealtime; however, they did express a free-running rhythm of activity with a period greater than 24 h. The long-term persistence of this free-running component adds further support to the hypothesis that the mechanism generating FAA is a self-sustaining circadian pacemaker (Stephan, 2001).

In agreement with the SCN lesion studies, neural activity within the SCN is also not influenced by restricted daily feeding schedules (Inouye, 1982a; Shibata et al., 1983). In one study, multiple unit activity rhythms recorded from the SCN were observed to free-run with a non-24 h period through a 24 h feeding schedule in constant light (Inouye, 1982a). General cage activity rhythms, recorded simultaneously in this study, were altered during restricted feeding, such that a large portion of activity was concentrated around mealtime. Thus, a clear dissociation between behavior and SCN neuronal activity was found (Inouye, 1982a). The results of a second study in which single neuron activity within the SCN was recorded *in vitro* following entrainment to a restricted feeding schedule also showed no effect of this schedule on SCN neuronal firing rhythms despite a clear effect on behavioral rhythms (Shibata et al., 1983).

1.3.2 Coupling Between the SCN and the Food-Entrainable Pacemaker

All of the evidence presented above is consistent with the idea that the light-entrainment and the food-entrainment systems are both functionally and anatomically

distinct; however, some evidence suggests that these two systems do interact (Stephan, 1986a; Stephan, 1986b; Stephan, 1986c; Cambras et al., 1993). Under free-feeding and free-running conditions, the light-entrainable pacemaker assumes phase control of those rhythms that are driven by the FEP under food restriction. Rats that had previously been exposed to restricted feeding were fed *ad libitum* under constant lighting conditions and then underwent total food deprivation (Rosenwasser et al., 1984). FAA emerged at a phase that was similar to the phase at which it had previously occurred relative to the free-running activity rhythm, although this was no longer the same clock time. In a similar study, previously restricted rats were exposed to phase shifts of the LD cycle during their subsequent *ad libitum* feeding period (Ottenweller et al., 1990). They were then probed with food deprivation tests for the reappearance of FAA, and results showed that FAA was phase shifted in conjunction with the shifted activity rhythm. Mice that were released into DD immediately upon return to *ad libitum* feeding following restricted feeding continued to show a bout of activity that free-ran from previous feeding time in a phase-locked position to the free-running light-entrainable rhythms (Challet et al., 1998b). These results suggest that the FEP and SCN are phase-locked or coupled during *ad libitum* feeding conditions.

Species differences in the coupling between light- and food- entrained rhythms have been reported. Most studies on bees, birds and fish, for instance, do not distinguish between two separate oscillatory mechanisms, and the results suggest that a single circadian component becomes entrained to mealtime in at least some of these species (Mistlberger, 1994; Stephan, 2001). Studies on food entrainment in hamsters have shown that, although it appears this species does have a separate oscillator that is distinct from the SCN, the coupling between these two circadian mechanisms is quite strong (Rusak et al., 1988; Mistlberger, 1991b; Abe and Rusak, 1992; Mistlberger, 1992b; Mistlberger, 1993b). Hamsters are herbivorous hoarders that do not adapt well to temporally restricted access to food, whereas rats are opportunistic omnivores that adapt readily to restricted feeding schedules. Thus, species differences in coupling strength between these

two circadian mechanisms likely reflect differences in species specific feeding strategies and behaviors (Mistlberger, 1994; Stephan, 2001).

Effects of restricted feeding schedules on light-entrainable rhythms

Despite the relative independence of the food- and light-entrainable systems, in a few cases, clear entrainment of the light-entrainable pacemaker by restricted feeding has been demonstrated in rats and mice (Edmonds, 1977; Cambras et al., 1993; Marchant and Mistlberger, 1997; Sharma et al., 2000). Entrainment by feeding cycles of the light-entrainable rhythm was usually observed only when the free-running period was very similar to the periodicity of the feeding schedule (Stephan, 1986b; Stephan, 1986c; Marchant and Mistlberger, 1997; Sharma et al., 2000). A very small period difference is not sufficient, however, to ensure entrainment, since experiments in which the period of food availability was deliberately set close to τ resulted in apparent entrainment in only a minority of the animals studied (Stephan, 1986c).

Strain differences in the response of the light-entrainable rhythms to restricted feeding have also been reported in mice. C57BL/6J mice exposed to restricted feeding exhibited two distinct bouts of activity, one that was entrained by restricted feeding, and one that continued to free-run in constant dim red light (Abe et al., 1989). In contrast, CS mice initially showed two bouts of activity that both appeared to entrain to restricted feeding, indicating that the light-entrainable rhythm in this strain was entrained by restricted feeding and/or that the two oscillators are more strongly coupled than in C57BL/6J mice (Abe et al., 1989). Strain differences in coupling between the SCN and the FEP may also explain the difference in adaptation to restricted feeding schedules found between C57BL/6J and C3H/2Ibg mice (Hotz et al., 1987). While C57BL/6J mice were able to adapt to a schedule in which food was presented for the first 4 h of the light phase, C3H/2Ibg mice rapidly lost weight and died on the same schedule. When food was gradually shifted to the first 12 h of the light phase, mice of both strains survived, although it appeared that the body temperature rhythms of C3H/2Ibg mice were not entrained by restricted feeding, as in C57BL/6J mice (Hotz et al., 1987).

Although restricted food availability does not appear to entrain the light-entrainable rhythms under most circumstances, changes in the expression of these rhythms during and following restricted feeding have sometimes been observed in both rats and mice. Various characteristics of the nocturnal component of activity can be altered during restricted access to food. For instance, the length of the active period (α) can be shortened significantly due to a consolidation or concentration of nocturnal activity under restricted feeding (Holmes and Mistlberger, 2000). Similarly, the free-running period or τ can be lengthened or shortened (Gibbs, 1979; Abe et al., 1989; Sharma et al., 2000; Holmes and Mistlberger, 2000). These changes in τ are not always in the predicted direction; i.e., decreasing the difference between τ and T.

Changes in total daily activity levels have also been reported under scheduled food access, and these may be related to the energy deficit induced by restricted feeding. Rats restricted to 15 g of food per day showed significantly more wheel-running activity than those fed *ad libitum* (Morse et al., 1995). A second group of rats given access to food for only 90 min each day ran even more, resulting in excessive body weight loss and a rapid deterioration in the health of these rats (Morse et al., 1995). Honma and colleagues described a study in which two groups of rats were exposed to restricted feeding schedules that varied only in the duration of mealtime (2 h or 4 h) (Honma et al., 1983a). Daily levels of wheel-running activity were significantly elevated in the animals with the 2 h meal, but were not different from baseline levels in animals fed daily for 4 h. Activity levels were also shown to increase significantly in rats under a similar 2 h restricted food access period (Stephan et al., 1979a). Likewise, when both duration (2 h) and amount (6 g) of daily food access were controlled, rats showed a significant increase in wheel running over baseline levels (Yi et al., 1993). Duration of mealtime may not, however, be the only factor contributing to changes in daily activity levels. In some cases, rats showed significantly less activity while exposed to a 2 h restricted feeding opportunity (Edmonds, 1977). In a study using 4 h of daily food access, average numbers of wheel revolutions recorded per day actually decreased significantly from baseline levels during

which food was available *ad libitum*, and increased to baseline levels following the reintroduction of *ad libitum* feeding (Stephan, 1986c). The correlates of restricted feeding that contribute to the changes in activity levels remain unknown.

Rats fed 50%, and mice fed 66% of their normal caloric intake early in the light phase of a full LD cycle showed a phase advance of their light entrained rhythms (Challet et al., 1996; Challet et al., 1997a; Challet et al., 1997c; Challet et al., 1998b). Light entrained rhythms peaked several hours earlier in restricted animals than in *ad libitum* fed animals. Since these animals consumed their full ration of food rapidly after presentation, food access in these studies was limited not only in amount, but also in duration. Interestingly, animals given 100% of their normal daily intake at the same time of day did not show this phase advance of activity onset (Challet et al., 1997c; Challet et al., 1998b), indicating that an energy deficit is required for the advancing effects on light-entrained rhythms. Both groups of animals did show the meal-associated components of increased activity and body temperature, which indicates a dissociation between the required conditions for the expression of FAA and the altered expression of the light-entrainable rhythms.

Effects of light on food-anticipatory activity

Just as restricted feeding schedules can affect the expression of light-entrainable rhythms, light can have disruptive effects on anticipatory wheel-running activity in rats. Rats housed in LL for a long period of time showed disruption of their light-entrainable rhythms and a delay in the development of FAA under a restricted feeding schedule (Mistlberger et al., 1990a). FAA normally develops within 3-10 days of initiation of the feeding schedule, and may develop even faster in SCN-ablated rats (Mistlberger, 1994); however, rats housed in LL took two to four weeks to develop FAA. These rats also failed to show an increase in wheel-running activity at previous mealtime when exposed to a 4 day food deprivation test (Mistlberger et al., 1990a).

This phenomenon may be explained by the effects of light and the SCN on overt activity in nocturnal rodents. Light can inhibit the expression of wheel running in

nocturnal animals, a phenomenon known as 'masking' (Mrosovsky, 1999; Mistlberger and Rusak, 2000). Similarly, the circadian system pacemaker in the SCN appears to promote wheel-running activity in the dark, and suppress it during the light phase, thereby consolidating activity and rest phases (Mistlberger and Rusak, 2000). In SCN-intact animals housed under LL, wheel running before mealtime would be inhibited both by light and by the output of the SCN, thereby delaying the overt expression of FAA. In SCN-ablated animals, activity inhibition by the SCN is eliminated (and photic inhibition may also be compromised), thus promoting the earlier appearance of FAA in these animals. This interpretation implies that the output of the food-entrainable system can be affected by light, but the data do not permit any conclusion as to whether the development of anticipation is affected directly by light. These data also imply that LL-induced arrhythmicity is functionally different from arrhythmicity following SCN ablation, at least for the food-entrainment mechanism (Mistlberger et al., 1990a).

The evidence presented above suggests that the SCN and the FEP are coupled, but it does not address the level at which this coupling occurs. It does not appear that each pacemaker is directly entrainable by each zeitgeber (food restriction and lighting cycles) (Stephan, 1986a). Their interactions might be mediated instead by competition for a common set of effector mechanisms; thus, there is probably a limit on the amount of activity that an animal can express within one day, and effects on activity controlled by the FEP would have repercussions for activity controlled by the SCN. In addition, there may be direct pacemaker-to-pacemaker interactions. The results described above are thus most likely due to interactions between the two pacemaker systems and between the rhythmic outputs they drive.

In summary, studies using SCN ablation or electrophysiological recording showed conclusively that the pacemaker housed within the SCN was not required for food entrainment or anticipation (Krieger et al., 1977; Stephan et al., 1979a; Inouye, 1982a; Shibata et al., 1983). This conclusion has led to a number of studies using various techniques and strategies in order to identify the anatomical location of the pacemaker

mediating food entrainment and anticipation.

1.3.3 Entrainment Pathways

Because it was known that most circadian rhythms are entrained by the LD cycle, it is not surprising that the localization of the master circadian pacemaker to the hypothalamic SCN followed shortly after the discovery of a direct retinal input to these nuclei (Moore and Eichler, 1972; Stephan and Zucker, 1972; Moore and Lenn, 1972). A similar logic has been applied to the quest for the FEP by attempting to define more precisely and then trace the sensory inputs involved in entrainment of the FEP.

Preingestive cues

Auditory cues associated with mealtime likely do not play a significant role in food entrainment, since, as discussed above, when these cues are omitted altogether, animals have no difficulty in anticipating mealtime. Other sensory modalities have been disrupted to ascertain whether food entrainment would be impaired. Blind rats were just as capable of anticipating daily meals as sighted rats, suggesting that the sight of food is of no consequence (Gibbs, 1979; Stephan, 1986c; Mistlberger and Mumby, 1992). Rats made anosmic by intranasal zinc sulphate injections or complete olfactory bulbectomies were not impaired in the development, maintenance or persistence of FAA (Coleman and Hay, 1990; Davidson et al., 2001b). In summary, preingestive auditory, visual or olfactory cues do not seem to be necessary for the development or the expression of FAA; therefore, researchers have shifted their attention to postingestive cues.

Postingestive cues

The majority of studies on food entrainment have involved temporally restricted access to food with intervening periods of total food deprivation; however, the amount of anticipation shown prior to meals does not seem to be influenced by increasing food access duration from 4 h to 10 h (Stephan and Becker, 1989). A decrease in anticipation was found for meals of 12 h in length, thus suggesting that some temporal restriction is required. The story is, however, somewhat more complicated for mealtime-associated plasma corticosterone levels. Serum corticosterone levels peaked prior to meals of 0.5 or 2

h in length, but they did not do so when rats were given access to food for 6 h (Honma et al., 1983a). It is possible that varying degrees of deprivation are required to induce expression of different FEP outputs. In other words, the threshold for behavioral activation prior to mealtime may be lower than that for increased plasma corticosterone secretion.

Rats have been shown to anticipate access to a highly palatable mash despite having *ad libitum* access to normal rat chow (Mistlberger and Rusak, 1987). This finding suggests that an energy deficit is not required for the development or maintenance of food anticipation or entrainment. Rats in this study did not anticipate a palatable but non-nutritive mash, however, indicating that some type of nutrient signal is required. Signals associated with amount of mash consumed, such as stomach distension for instance, do not seem to be sufficient, since some rats given the non-nutritive mash consumed amounts similar to those given the nutritive mash, yet no anticipation occurred in these rats. Furthermore, these results support the notion that cues associated with the act of ingestion, such as chewing or swallowing, or other gustatory signals are of no importance. Periodic access to water also does not seem to entrain the FEP in rats with *ad libitum* access to food (Mistlberger and Rechtschaffen, 1985). In some cases rats did anticipate the drinking event, but this was likely due to the fact that they had shifted feeding to coincide with water access. Rats under restricted access to both food and water at different times of day did show some evidence of water anticipation, but water appeared to be a much weaker zeitgeber when compared to food (Mistlberger, 1992a). Therefore, the mechanism responsible for food entrainment may be selectively sensitive to some correlate of nutrient ingestion. The hypothesis that cues specifically related to ingestion alone are of importance is further supported by research showing that daily handling or high activity levels do not mimic the effects of restricted feeding (Honma et al., 1984; Mistlberger, 1991a; Marchant and Mistlberger, 1996).

Mistlberger and colleagues showed that rats were unable to anticipate access to single macronutrients (fat, carbohydrate or protein) that were otherwise missing from

their diet (Mistlberger et al., 1990b). These macronutrients only became effective zeitgebers when they were presented alone or in combination in two meals per day without supplementary access to food. A previous study had shown that total caloric restriction was not required for FAA, but a minimum amount of food consumption during restricted access was important (Mistlberger and Rusak, 1987). The results of these two studies indicate that it may be the size of the meal that is critical (Mistlberger and Rusak, 1987; Mistlberger et al., 1990b). In other words, FAA may be induced only by meals that constitute a significant proportion of the animal's total daily caloric intake regardless of macronutrient content. This conclusion was further supported by findings indicating that a minimum of approximately 7 g of nutritive chow (~ 25.2 kcal) is required before FAA will shift to a new food access time (Stephan, 1997). This study also showed that gastric distension was not sufficient to phase shift the FEP, since FAA was not shifted in rats provided non-nutritive meals. Stephan and Davidson showed that glucose plus saccharin, but not saccharin alone was capable of phase shifting FAA in response to an 8 h delay of mealtime (Stephan and Davidson, 1998). Furthermore, 5.5 g of vegetable oil, which provided approximately 1.5 to 2 times the calories of the glucose meal, was unable to phase shift FAA in wheel running or food-bin approaches. These results suggest that the effectiveness of the zeitgeber depends on multiple factors including the amount ingested, the calorie content and the type of macronutrient provided.

The gastrointestinal system receives the zeitgeber (food); therefore, there must be some communication between the gut and the brain in order for food entrainment and anticipation to occur regardless of whether the FEP is located centrally or peripherally. If the FEP were located within the central nervous system, some feeding-associated signal would have to be transmitted from the digestive system to the brain. Conversely, if the FEP were located in a peripheral organ, signals would have to be sent to the brain for the initiation of FAA prior to each meal. Visceral information could be sent to the brain via neural or humoral pathways. Vagus nerve transection did not abolish the anticipatory increase in wheel running activity or serum corticosterone peak in rats maintained on

restricted feeding schedules (Moreira and Krieger, 1982; Comperatore and Stephan, 1990). Furthermore, non-vagal visceral deafferentation by capsaicin administration did not block the acquisition or expression of FAA during restricted feeding or subsequent food deprivation days (Davidson and Stephan, 1998) suggesting that the signals transmitting zeitgeber information from the gut to the central nervous system are likely humoral.

There are a variety of metabolic signals that may act as zeitgebers for food entrainment and anticipation. Plasma levels of metabolic and digestive elements including free fatty acids, glucose, triacylglycerides, ketone bodies, glucagon, insulin, motilin and leptin appear to be associated with mealtime (Escobar et al., 1998; Xu et al., 1999; Tanaka et al., 1999; Davidson and Stephan, 1999b; Díaz-Muñoz et al., 2000). Levels of various metabolic enzymes and peptides in the liver and digestive tract (e.g., glycogen, disaccharidases and motilin) also show rhythmicity coordinated with restricted feeding schedules (Hopkins et al., 1973; Vilchez et al., 1975; Nelson et al., 1975; Stevenson and Fierstein, 1976; Saito et al., 1980; Díaz-Muñoz et al., 2000). The levels of several metabolic and digestive factors in peripheral tissues and the circulatory system correlate with food anticipation; however, whether any or all of these factors play a significant role in food entrainment remains to be determined.

1.3.4 Lesion Studies

The endocrine system is intimately involved in energy metabolism, and may be involved in food entrainment. Because the corticosterone rhythm entrains easily to restricted feeding schedules it is possible that the adrenal glands could function as the FEP. Adrenalectomy, however, failed to prevent rats from expressing FAA in either wheel-running or unreinforced lever pressing to meals recurring every 23, 24 or 25 h, indicating that these glands are not required for food entrainment (Stephan et al., 1979a; Boulos et al., 1980).

A second endocrine organ, the pituitary gland, was hypothesized to play a role in food entrainment because of its involvement in regulating corticosterone release and its functional link to the SCN (Davidson and Stephan, 1999a). Despite its effect on body

weight regulation, hypophysectomy did not abolish anticipatory food-bin activity in rats. The anticipatory rise in body temperature present in sham-lesioned animals was not found in hypophysectomized animals; however, the authors stated that this was likely due to downstream effects stemming from the fact that energy maintenance and storage processes are impaired in these animals (Davidson and Stephan, 1999a).

Lesions within the central nervous system have contributed mostly negative results to the search for the FEP. Initial studies on the effects of ventromedial hypothalamic nucleus (VMH) lesions reported that rats were no longer able to anticipate regularly scheduled meals (Inouye, 1982b). In addition, colchicine injections into the VMH prevented the restricted feeding induced shift of peak plasma corticosterone from late in the light phase to early in the light phase (Choi et al., 1998). Another study showed, however, that FAA recovers following VMH ablation in rats (Mistlberger and Rechtschaffen, 1984). Furthermore, an intact VMH was not necessary for the development of the anticipatory serum corticosterone peak in response to restricted feeding (Honma et al., 1987), although a more restrictive feeding schedule was required during the weeks immediately following surgery. It is likely that the lack of FAA shortly after surgery is in some way related to the hyperphagia and the obesity that develop following VMH ablation. Rats exhibiting diet-induced obesity showed little to no FAA, but this effect was reversed once they lost weight on a low calorie diet (Persons et al., 1993). Thus, the effects of VMH lesions on food entrainment and FAA likely reflect an uncoupling of the behavioral output from the pacemaker.

Lesions of the VMH were shown to block the effects of calorie-restricted feeding on the light-entrainable rhythms of activity and body temperature in both rats and mice (Challet et al., 1997a; Challet et al., 1998a). Both electrolytic and neurotoxic (ibotenic acid) lesions were used in the study on rats, and animals from both conditions failed to display the phase advance in body temperature or wheel-running activity shown by sham-lesioned controls during restricted feeding (Challet et al., 1997a). These effects suggest that the VMH may play a role in the coupling between the FEP and the SCN.

Similar effects were found following lesions of the serotonergic terminals within the SCN, suggesting that serotonin may be involved in this coupling as well (Challet et al., 1997b). In mice, gold-thioglucose was injected peripherally, which resulted in selective damage to glucose-responsive neurons within the VMH (Challet et al., 1998a). Although this treatment selectively abolished the phase advance of light-entrainable rhythms in response to caloric restriction, it did not impair the increase in wheel running that occurred prior to mealtime. These results further strengthen the notion that although the VMH may play a role in the coupling between the FEP and the SCN, the FEP is not located within the VMH.

Ibotenic acid injections aimed at the lateral hypothalamus (LH) produced extensive damage but failed to inhibit FAA in rats exposed to a restricted feeding schedule (Mistlberger and Rusak, 1988), suggesting that this structure is not required for the generation of FAA. Other hypothalamic structures sustaining complete damage in one or more rats exhibiting normal FAA included the anterior hypothalamus, the zona incerta, the periventricular nucleus and the medial preoptic area (MPOA). Structures that were partially damaged included the dorsomedial hypothalamus (DMH), the VMH, the medial amygdala and the subthalamic nucleus.

Three animals sustaining radiofrequency induced lesions of the paraventricular nucleus of the hypothalamus (PVN) anticipated the arrival of a daily meal with increased visits to the food bin; however, they did not engage in increased general cage activity as measured by tilt floors (Mistlberger and Rusak, 1988). In contrast, animals sustaining combined lesions of the PVN and SCN did show FAA in both general cage and food-bin activity. The fornix was destroyed bilaterally in two of the three rats that did not show increased cage activity, and unilaterally in the other rat. Whether this is of importance remains unknown, but six other rats sustaining unilateral damage to the fornix showed no impairment in FAA. The findings of this study point to one critical caveat: the behavioral measure used to assess the effects of a lesion on the expression of FAA could determine whether or not a deficit is found (Mistlberger and Rusak, 1988).

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The role of the PVN in the generation of the prefeeding plasma corticosterone peak was assessed by Honma and colleagues (Honma et al., 1992). Injection of a catecholaminergic neurotoxin (6-hydroxydopamine or 6-OHDA) into the PVN reduced levels of norepinephrine in this structure and selectively inhibited the prefeeding plasma corticosterone peak without disrupting the light entrained corticosterone rhythm. Although the results of this study (Honma et al., 1992) combined with those of the one discussed above (Mistlberger and Rusak, 1988) suggest a role for the PVN in the generation of food anticipation and entrainment, further experimentation is required to determine the extent of PVN involvement in this phenomenon.

Neonatal monosodium glutamate (MSG) treatment was used to ablate the hypothalamic arcuate nucleus (Arc) to assess this structure's role in the expression of biological rhythms, including food entrainment (Mistlberger and Antle, 1999). Although this treatment resulted in a significant reduction in the number of neuropeptide Y (NPY) immunoreactive cell bodies within the Arc, food entrainment as measured by anticipatory food-bin activity was not impaired in these animals. In fact, FAA in MSG treated rats showed a higher amplitude, a longer duration and stronger persistence during food deprivation and *ad libitum* feeding following restricted food access. Thus, the authors concluded that the Arc likely plays more of a modulating, rather than a mediating role in food entrainment (Mistlberger and Antle, 1999).

The role of the limbic system in food entrainment was investigated because this system has many characteristics that would make it ideally suited to mediate this function (Mistlberger and Mumby, 1992). Large lesions damaging or destroying the hippocampus, the amygdala, the nucleus accumbens and various surrounding structures failed to abolish food anticipation or entrainment in rats, indicating that these structures are dispensable in generating FAA.

The intergeniculate leaflet (IGL) is a retinorecipient thalamic structure that is reciprocally connected to the SCN via the geniculohypothalamic tract (Morin, 1994). Lesions of the IGL did not prevent feeding anticipatory peaks in wheel-running activity

or body temperature in rats (Challet et al., 1996). Destruction of this nucleus did, however, block the phase advance of the light entrained peaks in activity and body temperature usually found in rats fed 50% of their normal caloric intake early in the light phase (Challet et al., 1996).

Although the hypothalamus is generally thought of as the predominant brain region involved in the regulation of feeding and energy homeostasis, there are several sites outside of the hypothalamus, specifically in the hindbrain or caudal brainstem, that are also important in the regulation of these functions (Grill and Kaplan, 2001; Grill and Kaplan, 2002). These sites include the nucleus of the solitary tract (NTS), the area postrema (AP), and the parabrachial nucleus (PB). The PB receives inputs from various sources, including both the NTS and the AP, and functions in the integration of visceral, humoral and gustatory information (Reilly, 1999). The PB is, therefore, a reasonable candidate for playing a role in food entrainment and anticipation. Rats sustaining ibotenic acid or electrolytic lesions of the bilateral PB showed little to no food-bin activity prior to mealtime, and a greatly reduced premeal rise in core body temperature (Davidson et al., 2000). The decrease in FAA seemed to be related to lesion size, since the animal with the largest lesion showed the least amount of FAA. These results could be interpreted in one of three ways: 1) the FEP is located peripherally and sends output signals to the brain via the PB; 2) the FEP is located centrally, and peripheral zeitgeber signals travel via the PB to the clock; or 3) the FEP is located in the PB. Further studies are required to distinguish these possible interpretations of the role of the PB.

In order to assess the role of the AP in transmitting peripheral signals to the PB for food entrainment, rats with lesions of this nucleus that displayed impaired conditioned taste aversion were exposed to a restricted feeding schedule (Davidson et al., 2001a). FAA as measured by food-bin approaches was not attenuated in these rats, and did not differ from sham-operated controls. In addition to the damage sustained by the AP, the NTS was moderately damaged in 5/8 lesioned animals. These results suggest that these structures are likely not involved in the transmission of restricted feeding cues from

the periphery to the brain, and further suggest that the PB must receive temporal feeding-related signals from other, yet to be identified structures (Davidson et al., 2001a).

1.3.5 Electrophysiological studies

Electrical activity within the SCN is rhythmic: neural activity in this structure reaches its peak during the light phase or subjective day whereas the rhythm peaks in antiphase in most other brain structures (Inouye and Kawamura, 1979). It seems likely that the proposed FEP would show rhythmic activity that is synchronized or entrained by mealtime. Thus, electrophysiological recording techniques like those used to record SCN neural function could be applied to various brain structures to determine whether neural activity correlates with restricted feeding schedules (Inouye, 1982a; Shibata et al., 1983). To date, these techniques have only been applied to the LH and the VMH (Inouye, 1983; Kurumiya and Kawamura, 1991). Both of these structures showed rhythmicity associated with mealtime; however, multiple unit activity within the LH was of greater amplitude, and persisted longer during food deprivation tests than that recorded from the VMH. The fact that the rhythmicity of neural activity persisted in constant conditions suggests that, at the very least, the LH and possibly the VMH may be linked in some way with the food entrainment system. The evidence from lesion studies that they were not required for food anticipation or entrainment suggests that if they play a role, it is redundant with that of another system (Mistlberger and Rechtschaffen, 1984; Honma et al., 1987; Mistlberger and Rusak, 1988).

1.3.6 Pharmacological Studies

Several studies have investigated the effect of food restriction schedules on levels of various neurochemicals. Restricted daily feeding significantly altered the levels of serotonin within the microdissected median eminence (ME), PVN, hippocampus and amygdala (Krieger et al., 1980). Changes in ME dopamine levels were also found during food restriction, but levels of norepinephrine were not affected in any of these brain regions (Krieger et al., 1980). Neurotransmitter levels in several brain areas were compared between animals actively anticipating their daily meal (0.5 h prior to mealtime)

and those that were not (6.5 h prior to mealtime) (Aragona et al., 2002). No differences between groups were found in levels of norepinephrine, epinephrine, serotonin, dopamine and the dopamine breakdown product dihydroxyphenylacetic acid (DOPAC) within the cortex, nucleus accumbens, PVN or LH.

The studies cited above determined total levels of these neurochemicals in microdissected brain regions (Krieger et al., 1980; Aragona et al., 2002) and, therefore, it is possible that these techniques do not adequately measure extracellular neurotransmitter levels. A study using *in vivo* microdialysis techniques did find that PVN norepinephrine levels were altered by restricted daytime feeding (Mitome et al., 1994). A significant norepinephrine peak preceded daily mealtime, and this rhythm persisted during three subsequent days of total food deprivation. A second microdialysis study found that acetylcholine levels began to rise 20-40 min prior to mealtime within the hippocampus and prefrontal cortex in food restricted rats, and that this anticipatory rise in acetylcholine was selectively blocked by the anxioselective benzodiazepine receptor ligand abecarnil (Ghiani et al., 1998). This drug did not block the increase in acetylcholine that appeared following the ingestion of food, demonstrating a dissociation between acetylcholine levels associated with anticipation and with ingestion (Ghiani et al., 1998).

Researchers have used other selective neurotransmitter antagonists in attempts to block the acquisition or expression of food entrainment. Daily injections of haloperidol (a dopamine antagonist) scheduled prior to mealtime during restricted feeding was unsuccessful in abolishing FAA despite substantial inhibition of general activity and food intake (Mistlberger and Mumby, 1992). Daily injections of the non-competitive *N*-Methyl-D-Aspartate (NMDA) receptor antagonist, MK-801, did block the appearance of the prefeeding increase in general cage activity (Ono et al., 1996). FAA was blocked in animals injected at the start of mealtime, at the end of mealtime and 2 h following the end of the meal, but not when the injection was given 16 h after the meal. Furthermore, mealtime-associated activity during total food deprivation following the six days of restricted feeding was absent in animals treated with MK-801, indicating that an NMDA

receptor mechanism may be involved in the learning processes required for food anticipation (Ono et al., 1996).

1.3.7 Gene Expression

Gene expression in the central nervous system

Over the past decade, research has revealed the complex and integrated involvement of several hypothalamic neuropeptide systems in the control of ingestive behaviors (Kalra et al., 1999; Schwartz et al., 2000; Schwartz, 2001; Druce and Bloom, 2003). Investigations of the expression of genes encoding hypothalamic neuropeptides have shown that several of these genes respond to restricted food availability. NPY mRNA was significantly up-regulated prior to mealtime in the Arc of rats maintained on a restricted feeding schedule, whereas pro-opiomelanocortin (POMC) and galanin (GAL) mRNA levels were significantly down-regulated in this structure (Brady et al., 1990). In the same rats, corticotropin-releasing hormone (CRH) mRNA was significantly down-regulated in the PVN. The results of a second study investigating medial basal hypothalamic (encompassing the Arc) levels of NPY, POMC and GAL mRNA found that these transcripts did not show rhythmicity associated with mealtime (Xu et al., 1999). The reasons for these discrepant results remain to be determined.

A subsequent study showed that the circadian CRH rhythm in the PVN and the ME, which peaked in the light phase under *ad libitum* conditions, peaked in the dark phase during diurnal restricted feeding (Honma et al., 1992). The lowest levels of CRH in both the PVN and the ME occurred just before mealtime. In contrast, extracellular NPY in the PVN was found to increase prior to mealtime and decrease following food access (Kalra et al., 1991; Yoshihara et al., 1996a). This rhythm persisted under food deprivation either immediately after the restricted feeding schedule (Kalra et al., 1991; Yoshihara et al., 1996a) or following intervening periods of *ad libitum* feeding (Yoshihara et al., 1996a). These extracellular NPY levels likely reflect NPY input from the brainstem, since NPY mRNA levels in the Arc increased under both restricted feeding and acute food deprivation, whereas NPY mRNA in the NTS seemed to be selectively responsive to

restricted feeding conditions (Yoshihara et al., 1996a; Ishizaki et al., 2003). This hypothesis was supported by the results of a follow-up study in which the ascending noradrenergic bundle was selectively lesioned using 6-OHDA (Yoshihara et al., 1996b). These lesions resulted in a loss of the premeal rise in extracellular NPY levels in the PVN, but did not abolish the increase in NPY following acute food deprivation. Thus, it appears that acute food deprivation leads to an increase in the PVN NPY levels stemming from the Arc, and that the meal-associated rise in PVN NPY stems from the ascending noradrenergic bundle originating in the brainstem (Yoshihara et al., 1996b).

In recent years, great advances have been made in identifying the molecular components of the mammalian circadian clock (Lowrey and Takahashi, 2000). The molecular mechanisms generating circadian oscillations appear to consist of autoregulatory transcription/translation feedback loops with both positive and negative regulatory elements. Within the mammalian SCN, the CLOCK and BMAL1 heterodimer regulates the transcription of the *Period* (*Per1*, *Per2* and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*) genes. The protein products of these genes then down-regulate their own transcription by interacting directly with the CLOCK:BMAL1 complex. Thus, CLOCK and BMAL1 act as positive regulatory elements, and the PERs and CRYs act as negative regulatory elements. These molecular events, along with delays associated with transcription/translation, post-translational modifications and dimerization, can account for the approximately 24 h periodicity shown by the circadian clock. In addition, the administration of light pulses affects the expression of these genes within the SCN, permitting a plausible account of how phase shifts and entrainment of the clock by light are achieved (Lowrey and Takahashi, 2000). It seems reasonable to assume that molecular mechanisms like these also underlie the functioning of the FEP. Since the FEP is known to exist outside the SCN, it is encouraging that these and other genes with similar properties are expressed rhythmically in brain regions outside the SCN (Zhou et al., 1997; Sakamoto et al., 1998; Oishi et al., 1998; Miyamoto and Sancar, 1999).

Several studies have assessed the expression of circadian clock genes in the brains

of mice under restricted feeding conditions (Wakamatsu et al., 2001; Hara et al., 2001b; Dudley et al., 2003). Results showed that the rhythmic expression of these genes within the SCN did not become associated with mealtime (Wakamatsu et al., 2001; Hara et al., 2001b). Expression patterns in brain areas outside of the SCN, however, did synchronize to restricted feeding schedules (Wakamatsu et al., 2001; Dudley et al., 2003). Furthermore, these rhythms of gene expression associated with restricted feeding were unaffected by lesions of the SCN (Wakamatsu et al., 2001). One study showed that gene expression rhythms outside of the SCN likely reflect direct, or masking effects of restricted feeding, since they were not shown to persist at the meal-associated phase following the cessation of the restricted feeding schedule (Wakamatsu et al., 2001). The persistence of these rhythms in gene expression was not assessed in the other study (Dudley et al., 2003); therefore, whether these altered patterns of expression represent activity of the FEP, or are simply due to masking effects of some correlate of the restricted feeding schedule remains to be determined.

Gene expression in peripheral tissues

Expression of so-called clock genes has been described not only in brain regions outside of the SCN, but also in various peripheral tissues (Zhou et al., 1997; Oishi et al., 1998; Zylka et al., 1998; Balsalobre, 2002). The expression of a number of these genes is cyclical and appears to be driven by the SCN (Sakamoto et al., 1998; Zylka et al., 1998; Miyamoto and Sancar, 1999; Yamazaki et al., 2000). These results raised the possibility that the FEP is, in fact, localized within one or more peripheral organs. Lesion studies had already eliminated the adrenal and pituitary glands as possible candidates (Stephan et al., 1979a; Boulos et al., 1980; Davidson and Stephan, 1999a). Complete removal of most other organs is not possible in survival studies; therefore, the assessment of clock or other gene expression patterns in these tissues offers a viable option for identifying those structures that express rhythmicity in concert with periodic daily feeding bouts (Stephan, 2002).

Rats maintained under different schedules of parenteral nutrition in which a

sustaining solution was continuously infused into the jugular vein for 12 h during either the light or dark phase showed gene expression patterns in the liver that were related to the phase of the infusion (Ogawa et al., 1997; Miki et al., 2003). Results also showed that gene expression within the SCN was phase shifted by parenteral nutrition infused exclusively during the light phase (Miki et al., 2003). As stated above, restricted feeding did not result in phase shifts in the expression of circadian genes within the SCN (Wakamatsu et al., 2001; Hara et al., 2001b). Why diurnal parenteral nutrient infusion resulted in the phase shift of SCN gene expression when diurnal restricted feeding did not remains to be determined.

Since the rats in the infusion studies were not actually ingesting food (Ogawa et al., 1997; Miki et al., 2003), it remained unclear whether similar alterations in peripheral gene expression patterns would follow mealtime in rats under restricted feeding. To address this issue, expression patterns of circadian genes in peripheral tissues (liver, kidney, heart and pancreas) and the SCN were assessed in mice exposed to restricted feeding (Damiola et al., 2000). Peripheral gene expression patterns were driven by food access time, whereas gene expression patterns in the SCN remained entrained to the LD cycle. Phase-resetting during restricted feeding occurred faster in the liver than in other tissues; however, the shift of gene expression patterns in the liver as a result of daytime feeding required a number of days of exposure to this feeding schedule to complete (Damiola et al., 2000).

When similar feeding paradigms were imposed on animals sustaining complete adrenalectomies, gene expression patterns in the liver and kidney were reset at a much faster rate than in sham-lesioned animals (Le Minh et al., 2001). Furthermore, animals lacking liver glucocorticoid receptors showed accelerated phase-resetting in the liver but not the kidney following exposure to daytime feeding. Based on these results, the authors concluded that glucocorticoid signaling functions to slow the phase-resetting of gene expression profiles in peripheral tissues driven by daytime feeding.

Animals in these studies were allowed access to food for 12 h, and no attempt was

made to determine when during this extended access period the mice were actually consuming the majority of their daily intake (Damiola et al., 2000; Le Minh et al., 2001). Previous studies have shown that FAA is related to the duration of the food access period, suggesting that food entrainment only occurs when meal durations are sufficiently restricted (Honma et al., 1983a; Stephan and Becker, 1989). Since neither FAA nor the persistence of these altered rhythms were assessed in these studies, it remains unknown whether these alterations in gene expression patterns are due specifically to entrainment of peripheral tissues by food access (Damiola et al., 2000; Le Minh et al., 2001).

Gene expression patterns in the liver were also phase shifted in mice exposed to restricted feeding conditions in which food was available for only 6 h in the middle of the light phase (Hara et al., 2001b). SCN ablation abolished the rhythmic expression of *Per1* and *Per2* in the liver under *ad libitum* feeding conditions, but did not block the induction of rhythmic expression of these genes in the livers of mice exposed to restricted feeding schedules, reinforcing the notion that although the SCN drives these rhythms in *ad libitum* conditions, it is not involved in the rhythmic expression of these genes induced by periodic feeding (Hara et al., 2001b).

When previously restricted animals were fasted following an intervening 7 days of *ad libitum* feeding, the peak of expression of clock genes in the liver did not occur at previous mealtime (Hara et al., 2001b). Furthermore, in contrast to the length of time required for peripheral gene expression patterns to adopt a new stable phase in mice fed only during the daytime (approximately 5 days), phase resetting is rapid (within 2-3 days) when feeding time is shifted from daytime to nighttime access (Le Minh et al., 2001). These results suggest that the rhythms of gene expression in the liver are rapidly reentrained by the LD cycle via the SCN following the cessation of restricted feeding, and that they do not persist in parallel with FAA, which has been shown to reemerge under food deprivation even after months of intervening *ad libitum* feeding (Rosenwasser et al., 1984; Clarke and Coleman, 1986).

1.3.8 Genetic Strategies (Mutants, Knockouts and Transgenics)

The use of animal models in which the expression of one or more genes is specifically altered has proven invaluable in the search for genetic determinants of behavior (Bolivar et al., 2000; Hamilton and Frankel, 2001). Although a few of these animal models have now been studied using restricted feeding paradigms, the potential of genetic strategies such as mutation, knockout or transgenic techniques has by no means been exploited fully.

As their name implies, obese Zucker rats are significantly heavier than their lean littermates. This increased adipocity is due to a point mutation in the leptin receptor gene, which leads to impaired leptin signaling (Wang et al., 1998b). Leptin is produced by adipose tissue, and likely acts as a lipostatic feedback signal to the brain (Friedman, 2002). Previous work has shown that serum leptin levels are up-regulated following a meal in rats on a restricted feeding schedule (Xu et al., 1999). Leptin acts within the Arc by inhibiting the synthesis and release of NPY, and thereby inhibiting feeding (Friedman, 2002). In the Zucker rat, leptin insensitivity results in enhanced Arc NPY activity (Beck et al., 2001). It was hypothesized that this system could play a role in food entrainment (Mistlberger and Marchant, 1999).

Decreased leptin levels prior to mealtime resulting in increased Arc NPY release could function as a mechanism driving FAA. Conversely, increased leptin levels following a meal, during *ad libitum* feeding or in diet-induced obese animals may serve to inhibit FAA by inhibiting Arc NPY function. In either case, impaired leptin signaling in the Zucker rat, with the concomitant increased NPY levels in the Arc could serve to enhance FAA in these rats. Obese and lean Zucker rats were tested for food entrainment using a standard restricted feeding paradigm, and the results showed that obese Zucker rats exhibited enhanced FAA compared to their lean counterparts (Mistlberger and Marchant, 1999). In both the obese Zucker and the neonatal MSG rat models, leptin signaling within the Arc is impaired (Tang-Christensen et al., 1999), and both of these animal models exhibit enhanced FAA (Mistlberger and Marchant, 1999; Mistlberger and Antle, 1999). In

diet-induced obese rats, in which leptin signaling is presumably not impaired, FAA is attenuated (Persons et al., 1993). These results suggest that increased adipocyte leptin signals serve to inhibit FAA via the hypothalamic Arc NPY system (Mistlberger and Antle, 1999; Mistlberger and Marchant, 1999). The results also show, however, that intact leptin signaling is not required for the development or expression of FAA, and therefore likely does not function directly in driving the expression of food entrained rhythms.

Along with the discovery of the genetic components of the mammalian circadian pacemaker comes the exciting possibility of generating and testing genetic mouse models in which the expression of these specific clock genes is altered. The first of these models is the mutant mouse that led to the discovery of the mammalian *Clock* gene (Vitaterna et al., 1994; Antoch et al., 1997; King et al., 1997). As stated above, CLOCK protein forms part of the positive feedback regulatory element of the mammalian circadian clock within the SCN (Lowrey and Takahashi, 2000). *Clock* mutant mice show a significantly greater τ in DD and eventually become arrhythmic (Vitaterna et al., 1994). Initial studies of the effects of novelty-induced wheel running or calorie restriction in heterozygote mutant mice showed that these mice responded differently to non-photic stimuli, although it appeared from the actogram included in the manuscript that these mice were still able to anticipate daily feeding time (Challet et al., 2000).

A more comprehensive study of food entrainment in homozygous Clock mutant mice showed that food entrainment was not impaired as a result of this mutation (Pitts et al., 2003). Homozygous *Clock* mutants expressed FAA both in LD and DD, and FAA persisted even when these mice had become otherwise arrhythmic after extended periods in DD. These results led the authors to conclude that the FEP is likely based on a molecular mechanism that differs from the one found in the SCN (Pitts et al., 2003). Additional studies using *Clock* mutant mice further support this hypothesis (Minami et al., 2002; Oishi et al., 2002). Restricted daily feeding entrained circadian gene expression in the heart of both *Clock* mutant and control mice, indicating that entrainment of gene

expression rhythms in peripheral tissues by restricted feeding does not depend on the molecular clock mechanisms found in the SCN.

CLOCK is not the only circadian gene that forms a heterodimer with BMAL1. A paralog of CLOCK, neuronal PAS domain protein 2 (NPAS2), which shares a similar amino acid sequence, also heterodimerizes with BMAL1 in order to bind DNA (Reick et al., 2001). Furthermore, this heterodimer is also regulated by the CRY protein products. Interestingly, *Clock* and *Npas2* are not expressed in overlapping brain structures: *Clock*, but not *Npas2*, is expressed in the SCN, while *Npas2* is found in forebrain cortical and limbic regions, and in the basal ganglia (Zhou et al., 1997; Antoch et al., 1997; King et al., 1997).

Restricted daily feeding schedules with 4 h of food availability were used to assess the capacity of NPAS2 knockout mice to exhibit FAA (Dudley et al., 2003). Although control C57BL/6J mice appeared to have little difficulty adapting to the schedule used in this study, knockout animals became ill and some died shortly after the initiation of restricted feeding. When the duration of food access was increased from 4 h to 6 h, and food was placed directly on the cage floor, knockout animals faired somewhat better. Despite these modifications, however, knockout animals consumed less food than wild-type mice and lost significantly more weight during exposure to restricted food access.

The results also indicated that the development of FAA was delayed in NPAS2 knockout mice, although by Day 11 of the restricted feeding schedule FAA was similar between knockout and control mice. Furthermore, there was no difference in food intake or body weight between the two groups of mice when food access was restricted to the middle of the dark phase. These results suggest that although NPAS2 knockouts are impaired, they are not incapable of entraining to restricted feeding schedules. It should be noted, however, that based on the actogram of wheel running from a representative knockout mouse that was included with the published results, it is possible that what was interpreted as FAA may actually be the light-entrainable rhythm free-running through food access time (Dudley et al., 2003). Insufficient data were included in this record to

rule out this possibility. In summary, although these initial results suggest that NPAS2 may be involved in the adaptation to restricted feeding schedules, it remains unclear whether this gene is part of the FEP mechanism.

A transgenic rat model has been constructed in which the mouse *Per1* promoter is linked to a luciferase reporter gene (Yamazaki et al., 2000). Rhythmic gene expression can thus be monitored by measuring light emission directly in cultured excised tissues. Various tissues collected from transgenic rats (including the liver, lung and sections of the digestive tract) expressed luciferase activity rhythms that were synchronized by restricted daily feeding (Stokkan et al., 2001; Davidson et al., 2003). Luciferase activity within the SCN, and surprisingly, the stomach, was not synchronized to mealtime. The altered phase of luciferase activity in the liver was maintained for 2 days of fasting immediately following the restricted feeding schedule, indicating that this oscillation was self-sustained (Stokkan et al., 2001). During *ad libitum* feeding following entrainment to the restricted feeding schedule, peak luciferase activity in all tissues shifted back to the dark phase (Davidson et al., 2003). Under total food deprivation, neither stomach nor colon luciferase peaks shifted towards to prior mealtime. Although liver luciferase activity did return to a diurnal phase in previously food restricted rats, it did so also in rats acutely food deprived that had never before been exposed to diurnal restricted feeding. These results suggest that the liver, although responsive to food deprivation, does not appear to be able to sustain food-associated rhythmicity following the cessation of the restricted feeding cycles. When rats were exposed to two meals per day, robust FAA occurred before each meal, however none of the tissues collected (liver, esophagus, stomach, duodenum and colon) showed bimodal *Per1*-luciferase activity.

Similar *Per1*-luciferase results were found in a study using transgenic rats rendered diabetic with a single injection of streptozotocine indicating that insulin is not required for the phase-resetting of liver *Per1*-luciferase activity (Davidson et al., 2002). Furthermore, diabetic rats were able to anticipate mealtime, suggesting that insulin is not required for the expression of FAA.

In summary, although it appears that gene expression patterns in peripheral organs can be altered significantly by periodic access to food, no results presented to date show conclusively that peripheral organs mediate food entrainment and anticipation. In fact, several findings suggest the opposite. First, gene expression patterns, when tested for persistence under constant conditions, rarely if ever sustain rhythmicity associated with mealtime (Hara et al., 2001b; Davidson et al., 2003). Second, two meals scheduled each day do not result in bimodal gene expression patterns in peripheral tissues, although this schedule induces two daily peaks of FAA (Davidson et al., 2003). In addition, rats made cirrhotic by chronic injections of carbon tetrachloride (CCl_4) were no less able to anticipate mealtime with increases in drinking behavior than control rats (Escobar et al., 2002), suggesting that intact liver function is not required for food entrainment.

Thus, it appears that the peripheral tissues tested thus far (specifically the liver, kidneys, lung, heart and gastrointestinal tract) do not appear to be the site of the FEP. It has previously been suggested that the FEP may not be localized to one specific anatomically defined neural structure, but may instead be comprised of a collection of scattered neuronal populations within various brain regions (Mistlberger and Rusak, 1988). It may even be the case that the FEP is made up of both central and peripheral subsystems.

As stated above, FAA likely functions to ensure that an animal is awake and aroused prior to mealtime, increasing the probability that the animal will find and ingest food. In addition, the synchronization of various physiological parameters by mealtime likely functions to prepare the gastrointestinal system for the anticipated arrival of food. The system mediating food anticipation and entrainment, therefore, likely involves structures that regulate sleep-wake cyclicity, circadian rhythmicity, arousal and ingestion. Many brain structures and neuropeptide systems play a role in these functions, and it is likely that the activity of these is altered under restricted feeding conditions. One of these neuropeptide systems (the orexin system) was described recently.

1.4 Orexin

In 1998, a pair of novel neuropeptides were discovered and described almost simultaneously by two groups (De Lecea et al., 1998; Sakurai et al., 1998). These neuropeptides were named hypocretin 1 and 2 (De Lecea et al., 1998) and orexin A and B (Sakurai et al., 1998) but for this thesis, the terms orexin A and B will be used. Orexin A is a 33 amino acid peptide (Sakurai et al., 1998). Orexin B shares 13 of its 28 amino acid sequence with orexin A. The precursor polypeptide, termed preproorexin, is 130 amino acids in length. Two orexin receptors have been identified, named orexin receptor 1 (Ox1R) and 2 (Ox2R), and both belong to the G protein-coupled receptor family (Sakurai et al., 1998). Ox1R binds orexin A with higher affinity than orexin B, however Ox2R binds both orexin A and B with equal affinity (Sakurai et al., 1998).

The orexins were initially described as being produced exclusively by neurons within the perifornical LH (Sakurai et al., 1998; De Lecea et al., 1998). The LH has many efferent projections extending throughout the neuraxis, and studies have revealed that orexin neurons form a part of these projections. Orexin-containing projections are found in numerous brain regions ranging from the olfactory bulbs to the spinal cord (Peyron et al., 1998; Cutler et al., 1999; Van den Pol, 1999; Nambu et al., 1999; Date et al., 1999; Date et al., 2000a; Moore et al., 2001; Caillol et al., 2003). Based on neuroanatomical studies, the orexin system sends projections to brain regions involved in various functions, including: feeding (Arc, PVN, DMH, VMH, supraoptic nucleus, and PB); circadian rhythmicity (IGL and the shell of the SCN); and sleep and arousal (locus coeruleus (LC), tuberomammillary nucleus (TMN), raphe nuclei, paraventricular nucleus of the thalamus (PVT), PVN, preoptic area, laterodorsotegmental nucleus and pontine reticular formation) (Peyron et al., 1998; Cutler et al., 1999; Nambu et al., 1999; Date et al., 1999). Although the two orexin receptors differ substantially in their distribution patterns, they are also found to be widely distributed throughout the neuraxis (Trivedi et al., 1998; Lu et al., 2000; Greco and Shiromani, 2001; Hervieu et al., 2001; Marcus et al., 2001; Backberg et al., 2002; Cluderay et al., 2002).

Initial studies suggested that the orexin system was exclusively found within the brain (with the exception of the testis) (De Lecea et al., 1998; Sakurai et al., 1998; Mondal et al., 1999b); however, subsequent studies have discovered that both orexin A and B and their receptors are found in various peripheral tissues (López et al., 1999; Kirchgessner and Liu, 1999; Johren et al., 2001; Sanchez de Miguel and Burrell, 2002; Naslund et al., 2002; Nakabayashi et al., 2003). Tissues in which preproorexin, orexin A, orexin B, Ox1R and/or Ox2R were found include the pituitary gland, adrenal gland, testes, ovaries, kidneys, pancreas, heart, lung, thyroid and the gastrointestinal tract (Nakabayashi et al., 2003; Voisin et al., 2003). Furthermore, orexin A has been found to cross the blood-brain barrier readily, whereas orexin B is quickly degraded following peripheral administration and does not appear to cross from the circulatory system to the brain (Kastin and Akerstrom, 1999). The widespread projection pattern of the orexin system suggests that this neuropeptide likely plays a role in various functions.

1.4.1 Orexin and Feeding

Since their discovery in the LH, a structure known to be involved in regulating feeding (Kalra et al., 1999), the orexins have been thought to play a role in this function (Sakurai, 1999). Lesions of the LH result in an initial post-operative syndrome characterized by hypophagia and weight loss that varies in severity and rate of recovery depending on the location, type and size of lesion (Kalra et al., 1999). Furthermore, stimulation of the LH increases food intake (Kalra et al., 1999).

The initial report describing orexin also showed that central administration of the orexins, especially orexin A, to the 3rd ventricle increased food consumption in a dose-dependent manner (Sakurai et al., 1998). Since this initial report, the orexigenic effects of acute central injections of orexin have been investigated by various groups, producing mixed results (Lubkin and Stricker-Krongrad, 1998; Dube et al., 1999; Sweet et al., 1999; Edwards et al., 1999; Ida et al., 1999; Dube et al., 2000; Ida et al., 2000; Yamanaka et al., 2000; Jain et al., 2000; Sunter et al., 2001; Monda et al., 2003; Thorpe et al., 2003). Chronic central administration of orexin A increased daytime feeding, but did not increase

total daily food intake, since nocturnal feeding was decreased (Haynes et al., 1999; Yamanaka et al., 1999; Russell et al., 2002). Thus, although central injections of orexin can increase feeding under some conditions, it appears that effects on food ingestion depend on factors such as dose, time of day and site of the injection. Central injections of orexin have also been shown to increase c-Fos immunoreactivity in several hypothalamic areas involved in feeding (Date et al., 1999; Edwards et al., 1999; Yamanaka et al., 2000; Mullett et al., 2000) suggesting that the orexin system forms a part of the hypothalamic feeding-control network.

Central (intracisternal) injections of orexin A, but not orexin B, increased gastric acid secretion in a dose-dependent fashion, while peripheral injections were ineffective (Takahashi et al., 1999). Vagotomy blocked the effect of orexin A, suggesting that this gastric response is vagally mediated. This hypothesis was supported by evidence that orexin A elicited gastric motor responses when injected into the dorsal motor nucleus of the vagus (Krowicki et al., 2002). Furthermore, intracisternal injections of orexin A increased the relaxation of the proximal stomach and enhanced motility of the distal stomach (Kobashi et al., 2002). These effects of orexin A were blocked by vagotomy further supporting the notion that gastric effects of orexin are mediated by the vagus.

Acute fasting led to an up-regulation of preproorexin mRNA (Sakurai et al., 1998; Cai et al., 1999; López et al., 2000) but not orexin peptide levels within the LH as measured by radioimmunoassay (Mondal et al., 1999b). Other studies have not shown an increase in preproorexin mRNA, even after extended periods of food deprivation (Taheri et al., 1999; Tritos et al., 2001). The reasons for these discrepant results remain unclear. Central injections of antibodies raised against orexin A inhibited feeding behavior in acutely fasted rats (Ida et al., 2000; Yamada et al., 2000), indicating that endogenous orexin A plays a role in the normal feeding response following fasting.

Anatomical studies have shown that NPY, agouti-related peptide and α-melanocyte-stimulating hormone containing fibers are in close proximity to LH orexin neurons (Broberger et al., 1998; Elias et al., 1998; Horvath et al., 1999), and that orexin-

containing fibers make synaptic contacts with NPY- and POMC-containing neurons of the Arc (Horvath et al., 1999; Guan et al., 2001). Furthermore, Arc NPY and POMC neurons both express Ox1R (Funahashi et al., 2003). Thus, orexin neurons are reciprocally connected to hypothalamic peptidergic systems related to feeding. Further investigation revealed that the LH orexin and Arc NPY systems interact to control food intake in rats (Sahu, 2002). Insulin-induced hypoglycemia caused a greater number of LH orexin cells to express c-Fos immunoreactivity (Moriguchi et al., 1999; Cai et al., 2001), and to up-regulate preproorexin mRNA (Cai et al., 1999; Griffond et al., 1999). These results suggest that these neurons are themselves responsive to nutritional status signals. This hypothesis was confirmed by electrophysiological studies showing that isolated LH orexin neurons responded to glucose levels (Yamanaka et al., 2003). An additional study suggesting that the orexin system forms an integral part of the hypothalamic network regulating body weight and energy balance showed that LH/perifornical orexin-containing neurons project polysynaptically to brown adipose tissue (Oldfield et al., 2002).

Lateral hypothalamic orexin neurons appear to be involved in responding to circulating levels of leptin produced by adipocytes. Orexin neurons come in close contact with neurons expressing the leptin receptor (Funahashi et al., 2000), express the leptin receptor themselves (Hakansson et al., 1999; Horvath et al., 1999), and show electrophysiological responses to leptin *in vitro* (Yamanaka et al., 2003). Chronic peripheral leptin injections decrease orexin A peptide content in the LH of rats as determined by radioimmunoassay (Beck and Richy, 1999). Furthermore, exogenous leptin administration blocks the up-regulation of preproorexin mRNA following acute food deprivation (López et al., 2000).

Preproorexin mRNA levels within the LH were significantly lower in genetically obese (*ob/ob* and *db/db*) mice than in lean controls (Yamamoto et al., 1999) and these mice also showed a decrease in orexin A peptide levels [(Stricker-Krongrad et al., 2002) but see (Mondal et al., 2002)]. Both types of mice have a leptin signaling deficiency, the *ob/ob* mice because of a mutation of the *leptin* (or *obese*) gene, and the *db/db* mice because of a

mutation of the leptin receptor gene (Friedman, 2002). Restricting the amount of food to ~60% of *ad libitum* levels per day for 2 weeks significantly up-regulated preproorexin mRNA in obese, but in not lean control mice (Yamamoto et al., 2000). Chronic central administration of leptin to wild-type, *ad libitum* fed mice significantly decreased hypothalamic preproorexin mRNA expression levels (Yamanaka et al., 2003). Conversely, the same treatment significantly increased preproorexin mRNA in *ob/ob* mice. These apparently opposing results can be explained in terms of blood glucose levels. Orexin neurons are activated or inhibited under hypoglycemic or hyperglycemic conditions, respectively (Yamanaka et al., 2003). Thus, the hyperglycemia found in *ob/ob* mice correlates with decreased orexin levels in these mice, and treatments that normalize blood glucose levels, such as exogenous leptin or food restriction, also normalize orexin levels (Yamanaka et al., 2003).

Genetically obese Zucker rats also showed significantly lower preproorexin mRNA levels within the LH than their lean counterparts (Cai et al., 2000; Beck et al., 2001). This decrease was accompanied by the significant up-regulation of both *Ox1R* and *Ox2R* mRNA levels within the hypothalamus of obese rats compared to their lean controls (Beck et al., 2001). Furthermore, plasma orexin A levels were significantly decreased in obese rats (Beck et al., 2001). In contrast, orexin A and B peptide levels were significantly up-regulated in brain regions outside the LH in obese as compared to lean rats (Mondal et al., 1999a). For reasons that are not clear, other investigators showed no difference in orexin A content in the brains of obese rats compared to lean control rats (Taheri et al., 1999).

Ghrelin is a recently discovered peptide produced by the stomach that appears to act as an orexigenic agent when administered peripherally (Olszewski et al., 2003; Kalra et al., 2003). Ghrelin is up-regulated under conditions of negative energy balance and levels rise just prior to mealtime in both humans and sheep (Cummings et al., 2001; Cummings et al., 2002; Sugino et al., 2002a; Sugino et al., 2002b). Furthermore, chronic ghrelin administration results in hyperphagia and obesity in rodents (Tschop et al., 2000; Kalra

et al., 2003). The effects of ghrelin on food intake are likely mediated by the Arc NPY projection to the PVN and the LH orexin system (Seoane et al., 2003; Toshinai et al., 2003). Electrophysiological studies in isolated orexin neurons showed that they are depolarized following ghrelin application (Yamanaka et al., 2003). Ghrelin-containing terminals were shown to make direct contact with LH orexin neurons (Yamanaka et al., 2003). Central and peripheral ghrelin injections resulted in increased c-Fos expression within hypothalamic structures related to feeding, and specifically within orexin-containing neurons of the LH (Hewson and Dickson, 2000; Lawrence et al., 2002; Olszewski et al., 2003; Toshinai et al., 2003). Furthermore, pretreatment with orexin antibodies attenuated ghrelin-induced feeding, and ghrelin's orexigenic effects were diminished in orexin knockout mice (Toshinai et al., 2003).

In combination, the results of these studies attest to the fact that the LH orexin system plays a central role in the regulation of feeding and metabolism. A growing body of evidence suggests, however, that orexin is also implicated in various other regulatory functions.

1.4.2 Orexin and Sleep

Shortly after it was first described, it became clear that the orexin system plays a critical role in the control of sleep (Chicurel, 2000), and more specifically in the neuropathology associated with narcolepsy (Kilduff and Peyron, 2000; Hungs and Mignot, 2001; Scammell, 2003). Narcolepsy is a debilitating disorder characterized by uncontrollable sleep urges and bouts of sudden muscle weakness (cataplexy), affecting humans, dogs and other species (Overeem et al., 2001). Positional cloning of the mutant gene associated with canine narcolepsy revealed that it encoded the Ox2R (Lin et al., 1999). Further support for the role of orexin in narcolepsy is the finding that human narcoleptics have decreased levels of orexin A in the cerebrospinal fluid (Nishino et al., 2000) and a significant decrease in the number of orexin-containing neurons within the LH (Thannickal et al., 2000; Peyron et al., 2000). When LH orexin cells were ablated using an orexin B-conjugated neurotoxin (saporin), rats displayed symptoms consistent with

narcolepsy (Gerashchenko et al., 2001; Gerashchenko et al., 2003).

At approximately the same time that the discovery of the receptor mutation leading to canine narcolepsy was published, a report describing the effects of a lack of orexin ligand was also released (Chemelli et al., 1999). Remarkably, orexin knockout mice exhibited periods of behavioral arrest that resembled the cataplectic attacks of narcolepsy (Chemelli et al., 1999). Similarly, mice lacking the Ox2R also showed signs of narcolepsy, although some differences in symptoms were expressed by the ligand and Ox2R knockout mice (Willie et al., 2003).

Because orexin knockout mice lack the peptide ligand, but retain the cells that normally produce orexin, they may not be the best animal model for human narcolepsy, since it appears that in human narcolepsy, these cells are lost (Scammell, 2001). To address this issue, transgenic mice were produced that have an acquired loss of orexin-producing neurons resulting from the selective expression of a form of ataxin-3 that leads to apoptosis (Hara et al., 2001a). Like the orexin ligand knockout mice, these mice exhibited a behavioral phenotype consistent with narcolepsy.

Virtually all of the brain regions involved in the control of sleep (including the LC, the TMN, the dorsal raphe, the pontine reticular formation and the basal forebrain) receive direct orexin inputs (Peyron et al., 1998; Chemelli et al., 1999; Salin-Pascual et al., 2001). These regions also contain receptors for these ligands (Greco and Shiromani, 2001; Kilduff and De Lecea, 2001; Marcus et al., 2001). The functioning of orexin neurons appears to be related to the animal's sleep/wake state, and the levels of both preproorexin mRNA and orexin A in various brain regions have been shown to vary according to time of day (Taheri et al., 2000; Yoshida et al., 2001; Kiyashchenko et al., 2002). Furthermore, significantly more orexin-containing cells expressed c-Fos immunoreactivity at times when either nocturnal or diurnal animals were awake than when they were asleep (Estabrooke et al., 2001; Martinez et al., 2002).

Consistent with a proposed role for orexin in promoting wakefulness, administration of orexin A, but not B, to the LC suppressed REM sleep in a dose-

dependent manner (Bourgin et al., 2000). Orexin A administered to the lateral preoptic area 4 h into the light phase increased wakefulness and decreased slow-wave and REM sleep (Methippara et al., 2000). Orexin A also increased wakefulness when injected into the basal forebrain in a dose-dependent manner (Thakkar et al., 2001). Similarly, animals remained awake for several hours following lateral ventricular administration of orexin, even when these injections occurred during the animals' normal rest phase (Hagan et al., 1999; Piper et al., 2000; Espana et al., 2001). Increased daytime wakefulness was also observed in animals receiving a chronic infusion of orexin A (Yamanaka et al., 1999). Conversely, Ox2R antisense infusion into the pontine reticular formation led to an increase in REM sleep and the appearance of cataplexy in rats (Thakkar et al., 1999).

Histaminergic neurons in the TMN appear to be involved in promoting wakefulness by releasing histamine at their efferent targets (Huang et al., 2001; Eriksson et al., 2001; Yamanaka et al., 2002). Like other regions implicated in the control of sleep, the TMN receives inputs from LH orexin-containing cells (Peyron et al., 1998; Torrealba et al., 2003). Neurons within the TMN express Ox2R and, to a lesser extent, Ox1R (Eriksson et al., 2001; Willie et al., 2003). Electrophysiological recording showed that both orexin A and B increase the spontaneous firing rates of TMN neurons *in vitro* (Bayer et al., 2001; Eriksson et al., 2001; Yamanaka et al., 2002). Furthermore, orexin and histamine neurons appear to be reciprocally connected, indicating a close functional relationship between these two cell populations (Eriksson et al., 2001; Yamanaka et al., 2002). Modafinil, a wake-promoting drug used in the treatment of narcolepsy, induced increased c-Fos immunoreactivity within both perifornical orexin-containing neurons and neurons of the TMN (Chemelli et al., 1999; Scammell et al., 2000). Administration of orexin A into the TMN resulted in increased wakefulness correlated with increased histamine release in the MPOA and frontal cortex in rats (Huang et al., 2001). The effects of orexin B on histamine release were found to be weaker than those of orexin A (Ishizuka et al., 2002). Increased orexin-induced wakefulness was inhibited by the histamine 1 receptor antagonist pyrilamine in rats (Yamanaka et al., 2002).

Further support for the involvement of histamine in the wake-promoting effects of orexin A comes from studies of histamine 1 receptor knockout mice. These mice did not exhibit increased wakefulness following orexin A administration whereas wild-type control mice did (Huang et al., 2001). Mutation of the Ox2R is related to canine narcolepsy (Lin et al., 1999), and this receptor is expressed within the TMN (Marcus et al., 2001), suggesting that altered histaminergic function could contribute to the narcoleptic phenotype. Consistent with this hypothesis, histamine content in the cortex and thalamus was significantly decreased in narcoleptic Dobermans compared to age-matched control animals (Nishino et al., 2001a). In summary, the functioning of the orexin system is critical to the maintenance of normal sleep-wake patterns.

1.4.3 Orexin and Arousal

In addition to the wake-promoting functions of orexin, this neuropeptide may further be involved in behavioral activation. The results of several studies suggest that the orexins may have psychostimulatory effects. Central injections of orexin caused an increase in several behaviors including locomotor activity, stereotyped movements, grooming and burrowing, which may be mediated by downstream serotonergic and dopaminergic systems (Hagan et al., 1999; Ida et al., 1999; Nakamura et al., 2000; Espana et al., 2001; Yoshimichi et al., 2001; Sunter et al., 2001; Duxon et al., 2001; Matsuzaki et al., 2002). Chronic administration of orexin A also increased the occurrence of these behaviors in rats (Yamanaka et al., 1999). Similar to their effects on feeding, orexin A appeared to be more potent than orexin B (Ida et al., 1999; Espana et al., 2001; Jones et al., 2001) and the effects on behavior were dependent on the time-of-day of administration (Espana et al., 2002). Orexin A injected into the LH increased locomotor activity (wheel running) and food intake during the light phase, but increased locomotor activity alone during the dark phase in rats (Kotz et al., 2002). These results indicate that the effects of orexin on behavioral activation or arousal can be partially dissociated from effects on feeding (Kotz et al., 2002). Further support for the role of the orexin system in mediating behavioral activation stem from a report on the effects of systemic

administration of caffeine on c-Fos expression within orexin-containing cells (Murphy et al., 2003). Results showed that locomotor-inducing doses of caffeine selectively up-regulated c-Fos-immunoreactivity specifically within orexin-containing neurons.

Recent investigation suggests that the orexin system may be involved primarily in regulating behavioral activation or arousal. A significant positive relationship was found between increased levels of activity and levels of orexin A in the cerebrospinal fluid of dogs (Wu et al., 2002). This study also found that increased levels of orexin A following sleep deprivation were not related to sleep loss, but rather to activity levels during the sleep deprivation procedure (Wu et al., 2002). Similarly, the number of LH orexin neurons in cats that were also c-Fos immunoreactive was related to the amount of activity shown by the cats, not their state of wakefulness (Torterolo et al., 2003). Thus, increased c-Fos immunoreactivity in orexin neurons was found in cats that were alert, awake and moving, but not in cats that were alert and awake without movement, quietly awake, or quietly asleep. These results suggest that the orexin system is not involved with the maintenance of wakefulness per se, but rather is involved in promoting motor activation (Torterolo et al., 2003).

The complex interactions between feeding, sleep and arousal, and the involvement of orexin in each of these functions can be observed under conditions in which orexin signaling is disrupted. In addition to their narcoleptic phenotype, orexin/ataxin-3 transgenic mice also showed increased bodyweight paradoxically accompanied by decreased food intake. Increased bodyweight in these mice was likely related to an overall decrease in spontaneous locomotor activity compared to wild-type littermates (Hara et al., 2001a). Orexin ligand knockout mice also showed decreased food consumption under *ad libitum* feeding conditions, but maintained a normal bodyweight (Willie et al., 2001). These phenotypic differences may be explained by the fact that orexin neurons also contain other neuropeptides [e.g., galanin (Hakansson et al., 1999) and dynorphin (Chou et al., 2001)], and therefore, a loss of orexin-producing neurons results in a loss of these peptides as well. It is of interest to note that narcoleptic patients also show increased

bodyweight (Schuld et al., 2000; Dahmen et al., 2001; Nishino et al., 2001b). Narcoleptic dogs, on the other hand, tended to weigh less than their normal counterparts, and tended to show increased levels of activity, although this latter effect did not reach statistical significance (Wu et al., 2002).

In wild-type mice, food deprivation resulted in decreased non-REM sleep, increased REM latency, increased arousal and increased locomotor activity in a novel environment (Yamanaka et al., 2003). The effects of food deprivation are disrupted in orexin/ataxin-3 transgenic mice in which orexin-producing neurons are lost over development. Transgenic mice did not show altered sleep/wake patterns in response to food deprivation, although both types of mice did show a suppression of total REM sleep duration. Furthermore, orexin/ataxin-3 transgenic mice failed to show the increase in locomotor activity expressed by wild-type mice during the light phase under total food deprivation. The authors suggested that the increase in locomotor activity with the concomitant decrease in resting and sleep could represent increased foraging behavior brought about by the negative energy balance resulting from food deprivation (Yamanaka et al., 2003).

The lack of these behavioral alterations in orexin/ataxin-3 transgenic mice points to an essential role for orexin in behavioral adaptation to food restriction. Thus, in normal animals, food deprivation decreases plasma glucose and leptin levels and increases plasma ghrelin levels. Together, these effects result in increased orexin signaling, leading to increased wakefulness and arousal, which promote increased foraging behavior. The orexin system could then provide a link between peripheral energy balance signals and central mechanisms controlling sleep, arousal and feeding behaviors (Yamanaka et al., 2003).

1.5 Is Orexin Involved in Food Entrainment and Anticipation?

Food entrainment and anticipation ensure that an organism is awake, aroused and prepared to ingest food when it becomes available, even if food access occurs at a time of day when the animal would not customarily eat. Because orexin cells send projections to

brain regions involved in feeding, circadian rhythmicity, sleep and arousal, and appear to participate in the control of these biological functions, they are ideally suited to play a role in coordinating these functions during food entrainment. The premeal rise in circulating orexigenic factors such as ghrelin, combined with low levels of circulating leptin and insulin, would lead to an increase in hypothalamic NPY and orexin signaling. Increased LH orexin production would ensure that the animal is awake and would heighten behavioral activation to facilitate foraging behaviors and the likelihood of finding food. At the same time, the increase in central levels of NPY and orexin would increase the likelihood of food ingestion. Orexin signaling via vagal pathways would also increase gastric acid secretion and motility, which would serve to prepare the digestive system to receive a meal.

This cascade of events could function autonomously as a form of timing mechanism that is reset each day by the ingestion of a meal. Following food ingestion, leptin and insulin levels increase and ghrelin levels decrease, thereby shutting down the hypothalamic orexigenic system including NPY and orexin. We know, however, that food entrainment and anticipation do not require daily resetting to maintain rhythmicity (i.e., FAA persists during several days of fasting); therefore, this model requires an additional (circadian) component that is independent of postingestive consequences. Nevertheless, the involvement of the orexin system in regulating feeding, sleep and arousal strongly suggests that it would play a role in food entrainment and that its functioning would be altered by restricted food access during the normal rest phase.

To date, only two studies have looked specifically at the activity of the LH orexin system during food entrainment and anticipation. Rats exposed to a restricted daily feeding schedule for 3 weeks showed a significant up-regulation of c-Fos expression prior to scheduled mealtime within orexin-containing neurons compared to *ad libitum* fed controls (Kurose et al., 2002). Only 5% of orexin cells were c-Fos immunoreactive in *ad libitum* fed animals, while 22% of such cells were immunoreactive in animals on restricted feeding. In addition, food restriction significantly decreased Ox2R mRNA within the

PVN. These results suggest that the orexin system does respond to chronic food restriction procedures in rats.

A second study assessed the capacity for rats to entrain to restricted feeding schedules after neurotoxic lesions of LH orexin cells (Mistlberger et al., 2003). Rats were given bilateral injections of the ribosome inactivating neurotoxin saporin conjugated to orexin B. Because orexin neurons themselves express orexin receptors, they are vulnerable to the effects of this neurotoxin. Despite a significant decrease in the number of orexin-containing cells within the LH following the lesion, all rats exhibited either anticipatory drinking or anticipatory food-bin activity prior to mealtime under restricted feeding conditions.

The authors reported that only one animal had a complete lack of orexin-containing neurons. Figures included in the publication indicate that an animal without orexin-immunoreactive cells showed virtually no anticipatory drinking before mealtime, and that an animal lacking orexin (presumably the same rat) showed a significant increase in food-bin activity in the hours preceding mealtime. On the assumption that these two measures were from the same rat, these data suggest that a complete loss of LH orexin neurons does not prevent food anticipation or entrainment, as assessed by one behavioral measure (Mistlberger et al., 2003). Thus, although results of the first study suggest that the functioning of the orexin system is altered during restricted feeding (Kurose et al., 2002), results of the second study suggest that the orexin system is not required for food entrainment or anticipation (Mistlberger et al., 2003).

The experiments described within this thesis were designed to investigate further the functioning of the LH orexin system during food entrainment and anticipation in mice. The study described in Chapter 2 was designed to reliably obtain FAA in mice using a standard restricted feeding paradigm in two different housing conditions normally used in our laboratory. An additional experiment investigating the normal feeding patterns in C57BL/6J mice under *ad libitum* conditions is also included in this chapter. Chapter 3 describes an investigation of the distribution of orexin-containing cell bodies in three

different strains of *Mus musculus* (C57BL/6J, DBA and CD1). The experiments discussed in Chapter 4 investigated the effects of different feeding manipulations on behavior (wheel-running and food consumption) and immediate-early gene expression within the LH. Additional experiments in this chapter investigated the effects of different feeding conditions on immediate-early gene expression within orexin-containing neurons, and on hypothalamic preproorexin mRNA levels. The first experiment described in Chapter 5 was designed to investigate patterns of immediate-early gene expression in several brain regions receiving orexin efferent projections in mice under different feeding conditions. A second experiment included in Chapter 5 assessed immediate-early gene expression in the brains of mice as FAA developed over the first few days of exposure to a restricted feeding schedule. A general discussion of the combined results of these experiments is included in Chapter 6.

CHAPTER 2

Housing Conditions Influence the Expression of Food-Anticipatory Wheel-Running Activity in Mice (*Mus musculus*)

2.1 Introduction

When animals are allowed to consume food only during a limited daily interval under a restricted feeding schedule, they will come to anticipate feeding time (Mistlberger, 1994; Stephan, 2001; Stephan, 2002). This anticipation is manifest as increases in various behavioral and physiological measures such as body temperature, plasma corticosterone or activity level in the hours preceding scheduled feeding. The increased activity shown is usually referred to as food-anticipatory activity or FAA (Mistlberger, 1994; Stephan, 2001; Stephan, 2002).

Previous research has shown that anticipation of mealtime is mediated by entrainment of an endogenous circadian pacemaker (Mistlberger, 1994; Stephan, 2001; Stephan, 2002). Among the principal pieces of evidence supporting this conclusion are the following: FAA can be established only in anticipation of meals that are scheduled every 23 - 31 h (Stephan, 1981), indicating that this pacemaker has circadian limits of entrainment; and once established, FAA persists on a circadian basis in conditions devoid of any temporal information (Stephan et al., 1979b; Boulos et al., 1980).

Two lines of evidence indicate that the master circadian pacemaker, housed in the hypothalamic suprachiasmatic nucleus (SCN), is not involved in the development or expression of FAA (Mistlberger, 1994). Animals exposed to restricted feeding under constant lighting conditions show free-running (non-24 h) circadian rhythms (driven by the SCN) in addition to FAA, which is entrained to the 24 h meal schedule (Stephan et al., 1979a; Boulos et al., 1980; Honma et al., 1983b). Furthermore, rats sustaining complete lesions of the SCN are still able to entrain to restricted feeding schedules (Stephan et al., 1979a; Boulos et al., 1980; Clarke and Coleman, 1986).

Rats are the species of choice for most studies of food entrainment, since they are

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opportunistic feeders, are able to consume their entire daily food requirement in a relatively short period of time, and entrain readily to restricted feeding schedules (Mistlberger, 1994; Stephan, 2002). Mice, however, are being used increasingly in neuroscience research because of the vast amount of genetic information available for them and because they are more pragmatic from an animal care perspective (Hamilton and Frankel, 2001). Because of their small size and fast metabolism, standard laboratory mice (e.g., C57BL/6J), however, are not ideally suited for food entrainment research. They are less able to adapt to a restricted feeding schedule than rats are, and the standard test for food entrainment - complete food deprivation for 2 or more circadian cycles - cannot readily be performed in these mice. Despite these limitations, several studies have been done on the effects of restricted daily feeding schedules on behavioral rhythms in mice (Aschoff, 1986; Abe et al., 1989; Marchant and Mistlberger, 1997; Challet et al., 1998b; Sharma et al., 2000; Holmes and Mistlberger, 2000; Wakamatsu et al., 2001; Dudley et al., 2003; Pitts et al., 2003).

Studies of restricted feeding in mice have involved housing mice under standard light-dark (LD) cycles (Aschoff, 1986; Challet et al., 1998b; Sharma et al., 2000; Holmes and Mistlberger, 2000; Wakamatsu et al., 2001; Dudley et al., 2003; Pitts et al., 2003), in constant darkness (DD) (Aschoff, 1986; Sharma et al., 2000; Holmes and Mistlberger, 2000; Wakamatsu et al., 2001; Dudley et al., 2003; Pitts et al., 2003), or in constant dim red light (Abe et al., 1989; Marchant and Mistlberger, 1997). When feeding opportunities are restricted to the light phase of a standard LD cycle, mice display two distinct components of activity: one in the dark phase and the other in the light phase, associated with mealtime (Aschoff, 1986; Holmes and Mistlberger, 2000). When mice are exposed to restricted feeding under constant lighting conditions, there is usually clear evidence of two separate components of activity, one free-running, and one entrained to mealtime (Aschoff, 1986; Abe et al., 1989; Marchant and Mistlberger, 1997; Sharma et al., 2000); however, in some cases the main free-running or light entrained component alters its expression (Challet et al., 1998b; Holmes and Mistlberger, 2000) or becomes entrained by

the restricted feeding schedule (Abe et al., 1989). These different effects are thought to reflect differences among strains of mice in the coupling strength between the underlying light-entrainable and food-entrainable pacemakers (Abe et al., 1989). Mice sustaining complete lesions of the SCN retain the ability to show FAA under restricted feeding schedules (Marchant and Mistlberger, 1997), indicating that like rats, mice have two functionally and anatomically distinct circadian pacemakers: one entrainable by light and one entrainable by food availability.

Despite the difficulties associated with studying food entrainment in mice, the use of this behavioral paradigm in these animals is very important to progress in the analysis of this phenomenon. Currently, there exist large numbers of genetically modified mouse strains, and the number of such strains is increasing rapidly (Bolivar et al., 2000; Hamilton and Frankel, 2001). The use of restricted feeding paradigms in these various mouse models could not only illuminate the underlying mechanisms involved in food entrainment, but could also increase our understanding of ingestive behaviors in general.

For this thesis, experiments were conducted on C57BL/6J mice, which are a standard mouse strain used in many laboratories. We first assessed the normal circadian pattern of feeding in C57BL/6J mice under *ad libitum* food access and then assessed their ability to generate FAA. Because results obtained by different laboratories using standard behavioral tests can vary significantly, even under strictly controlled experimental conditions (Crabbe et al., 1999; Wahlsten et al., 2003), a preliminary experiment was conducted in order to generate FAA in mice using a previously described restricted feeding paradigm (Marchant and Mistlberger, 1997). We applied this paradigm under two different housing conditions that are used routinely in our laboratory, in order to determine the best experimental conditions for obtaining FAA in these mice. The results of this experiment show that the expression of anticipatory wheel running in mice depends on housing condition.

2.2 Methods

2.2.1 Animals and Housing

Nine adult male C57BL/6J mice obtained from Charles River Canada (St. Constant, Québec, Canada) were used to determine normal *ad libitum* feeding patterns. These mice were housed individually in standard mouse bin cages (27.5 cm X 16.5 cm X 12.5 cm) placed inside isolation cabinets (52 cm X 38 cm X 48 cm) under an LD cycle consisting of 12 h of light and 12 h of dark (LD 12:12), with lights on from 9:00 to 21:00.

Twenty additional adult male C57BL/6J mice obtained from Charles River were housed individually in cages (44.5 cm X 23 cm X 20 cm) under an LD 12:12 cycle. Each cage was equipped with a running wheel (17 cm in diameter) connected to a microswitch. The cages also contained a 7.5 cm long piece of PVC pipe (4.5 cm in diameter) wired to the grid floor on which the mice could climb to access food (during *ad libitum* conditions, see below) and the water spout on the cage top. For half of the experimental animals (n=10) the cages were placed on open shelves within the experimental room. For these animals, the room lights were on from 07:00 to 19:00 (~100-300 lux at the bottom of the cages). The cages housing the other half of the animals (n=10) were placed individually inside ventilated isolation cabinets (52 cm X 38 cm X 48 cm) containing a light source (cool white fluorescent tubes, Sylvania, ~300 lux). Lights were on inside the cabinets each day from 11:00 to 23:00.

Microswitch closures corresponding to wheel revolutions were recorded throughout the experiment and were stored in 5 min time bins using the Activity Counting System (Simon Fraser University, British Columbia, Canada). The data were dumped to disk every 24 h, and were sorted, visualized and analyzed using Circadia (Simon Fraser University, British Columbia, Canada).

Water was available *ad libitum* throughout the experiment and Rodent Laboratory Chow pellets (Agribrands, Ontario, Canada) were available *ad libitum* until the experimental procedures were initiated. Water bottles, bin cages and waste trays were changed once per week at random times of day. Experimental room temperatures were

maintained at ~23° C. All experiments were conducted according to Canadian Council on Animal Care guidelines and were approved by the Dalhousie University Committee on Laboratory Animals.

2.2.2 Procedures

The nine animals housed in bin cages were allowed to adapt to the isolation cabinets for six days. On the seventh day at 21:00 (at lights off, zeitgeber time [ZT] 12, by convention), all food was removed from the cage tops and was replaced with fresh food pellets. The food was then weighed at 4 h intervals (at ZT16, ZT20, ZT0, ZT4, ZT8 and ZT12) in order to determine the amount of food consumed by each mouse during the previous 4 h time bin.

Twenty animals were housed in cages with running wheels until they were all stably entrained to the LD cycle (see Figure 2.1). For the animals in isolation cabinets, all food was removed on Day 17 at ZT22 (i.e., 2 h before lights on) with the aid of an infrared viewer (Figure 2.1A). Food was removed from the cage tops of animals housed on open shelves at ZT0 (time of lights on) on Day 7 of recording (Figure 2.1B). Four or 6 h later (at ZT4), a glass specimen bottle containing 15.5 g of ProLab RMH 3000 powdered lab diet (ProLab, Ontario, Canada) moistened with vegetable oil was placed on each animal's cage floor. The food bottles were removed 12 h later at ZT16. On the following day, the food bottles were again placed on each animal's cage floor at ZT4, but were removed 10 h later at ZT14. Food removal on these days was done with the aid of an infrared viewer, since this occurred during the dark phase. For the next two days, food was again introduced at ZT4, but was removed 8 h and 6 h later, respectively (or at ZT12 and ZT10). For the next twelve or thirteen days, food was made available for only 4 h from ZT4 to ZT8. The experiment was terminated on the 17th day (for animals on open shelves) or on the 18th day (for animals in isolation cabinets) of restricted feeding, and mice were removed from the wheel cages.
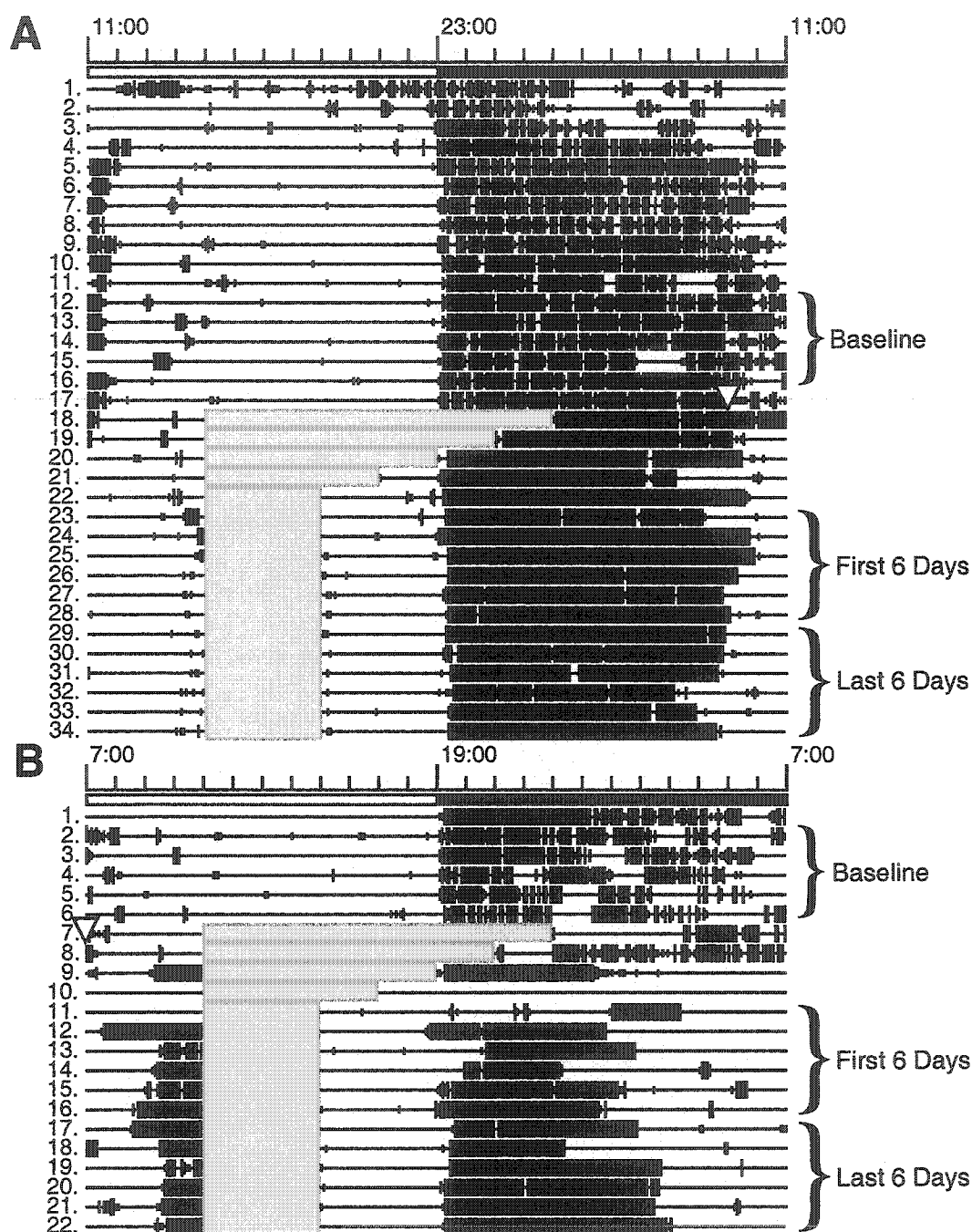
Figure 2.1. Actograms illustrating the experimental procedures for a representative mouse housed inside an isolation cabinet (A) and for one housed on an open shelf (B). Horizontal lines represent 24 h, as shown at top of graph. Days are plotted sequentially on the vertical axis. Vertical deflections on the horizontal lines represent microswitch closures corresponding to wheel revolutions occurring in each 5 min time bin, with larger deflections representing higher numbers of wheel revolutions. Animals were housed under an LD 12:12 cycle represented by the bar at the top of the graph (white = light phase, black = dark phase). Downward–pointing triangles indicate the time that all food was removed from the cage tops. The shaded bars correspond to the times of food availability during the restricted feeding schedule. The } indicates the intervals used for further analyses (see Results section).

2.2.3 Analyses

Consumption analyses

The pattern of food consumption shown by mice during *ad libitum* feeding conditions was determined by calculating the mean amount consumed by the nine mice (in g ± SEM) for each of the six 4 h time bins, and for the 12 h light and 12 h dark phases.

Behavioral analyses

The actograms generated for each of the twenty animals in the two housing conditions were initially assessed visually for the presence of patterns of wheel-running activity. Wheel-running data were then pooled by housing condition and were plotted using Circadia as average waveforms corresponding to three time intervals. The first interval consisted of five days of baseline recording occurring immediately prior to the initiation of the restricted feeding paradigm (Days 12-16 from 11:00 to 10:55 for animals in isolation cabinets, Figure 2.1A; Days 2-6 from 7:00 to 6:55 for animals on open shelves, Figure 2.1B). The second interval included six days starting from the first or second day with only 4 h of daily food availability (Days 23-28 from 11:00 to 10:55 for animals in isolation cabinets, Figure 2.1A; Days 11-16 from 7:00 to 6:55 for animals on open shelves, Figure 2.1B). The third interval consisted of the last six days of the restricted feeding paradigm (Days 29-34 from 11:00 to 10:55 for animals in isolation cabinets, Figure 2.1A; Days 17-22 from 7:00 to 6:55 for animals on open shelves, Figure 2.1B). Thus, for each of these three intervals, average numbers of wheel revolutions occurring during each 5 min time bin were collapsed over days and plotted as a standard average waveform.

Average amount of activity recorded per 5 min time bin over the 24 h day during each of the three time intervals was compared statistically between groups of mice in the two housing conditions. For more detailed comparisons between conditions of nocturnal activity and FAA, average numbers of wheel revolutions recorded during specific time bins were calculated using Circadia and were analyzed statistically. For an initial analysis of nocturnal activity, amount of wheel running recorded during the entire dark phase was

compared among the three recording intervals for animals in each of the two housing conditions. For the comparisons of nocturnal activity between groups of animals in the two conditions, nocturnal activity was divided into four 3 h time bins. To analyze the pattern and extent of any FAA shown, comparisons were made of the amount of activity recorded during each of two 2 h time bins beginning at the times of lights on and extending until feeding time. Average numbers of wheel revolutions (/5 min) recorded during each of these time bins were compared statistically among recording intervals and between animals in the two housing conditions.

Statistical analyses

Statistical comparisons involved a one-way ANOVA using StatView (SAS Institute, Inc., USA) followed by Student-Newman-Keuls post-hoc tests. Group differences with $P < 0.05$ were considered statistically significant. Group means are reported with SEM. In the text, all activity levels are reported as mean number of wheel revolutions/5 min time bin (± SEM).

2.3 Results

2.3.1 Food Consumption Patterns

Under *ad libitum* feeding conditions, mice consumed an average of 4.7 g of standard lab chow pellets per day. They consumed significantly more food during the dark phase (ZT12-0, 4.0 ± 0.24 g) than they did during the light phase (ZT0-12, 0.7 ± 0.17 g; $F_{(1,16)} = 133.3$, $P < 0.0001$). When consumption was broken down into six 4 h time bins, statistical comparisons also revealed significant differences (Figure 2.2; $F_{(5,48)} = 14.8$, $P < 0.0001$). Mice consumed significantly more food during the 4 h between ZT12-16 (1.78 ± 0.28 g) than during the 4 h intervals between ZT16-20 (1.00 ± 0.17 g; $P < 0.05$) and ZT20-0 (1.22 ± 0.15 g; $P < 0.05$). The food intake during all three nocturnal intervals was also significantly higher than during any of the diurnal 4 h intervals (ZT0-4, 0.11 ± 0.11 g; ZT4-8, 0.11 ± 0.11 g; ZT8-12, 0.44 ± 0.18 g; $P < 0.05$ for each comparison). No difference was found in the amount of food consumed among the three 4

Food Consumed by House Mice in 4 h Time Bins
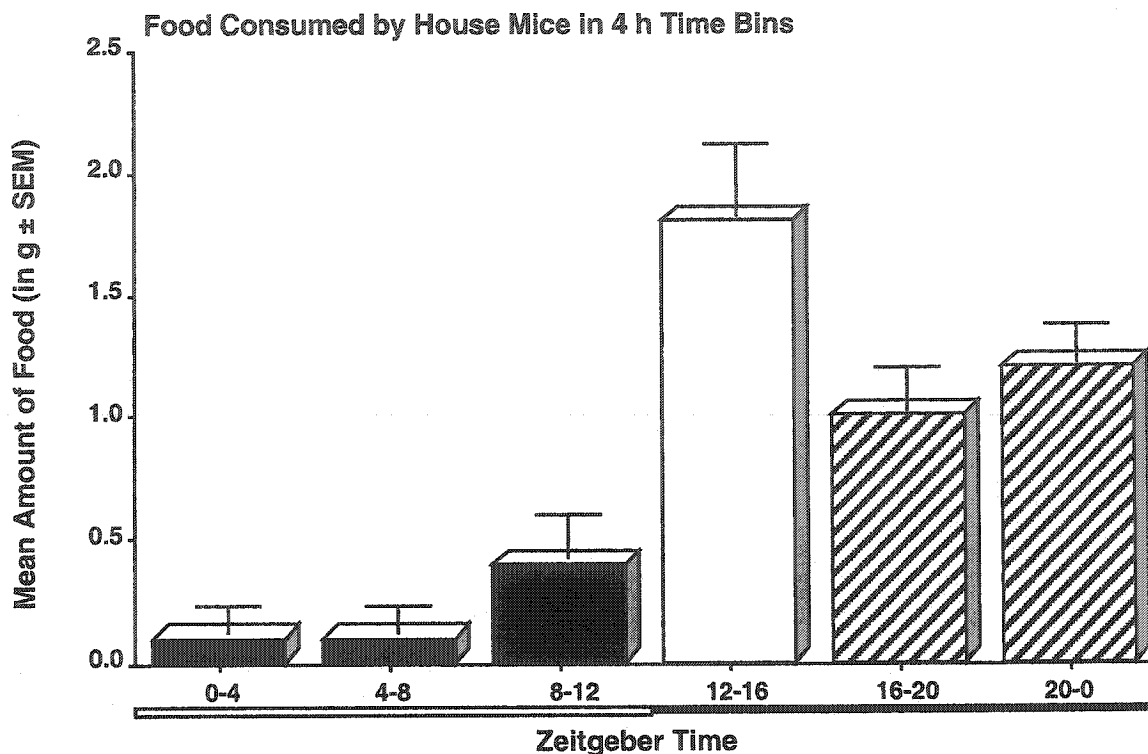
Figure 2.2. Amount of food consumed during each of six 4 h time bins. Time bins corresponding to zeitgeber times are plotted along the abscissa and average amount of food (in g ± SEM) is plotted along the ordinate. The LD 12:12 cycle is depicted in the bar shown along the bottom of the graph (white=light phase, black=dark phase). The open bar is significantly greater than the hatched bars and black bars ($P < 0.05$). The hatched bars are significantly greater than the black bars ($P < 0.05$).

h time bins in the light phase (Figure 2.2).

2.3.2 Effects of Restricted Feeding

All twenty animals were able to adapt to the restricted feeding schedule. Although body weight was not measured in this study, none of the animals appeared to lose weight during the course of the study, and all were able to consume their daily requirement of food during the 4 h mealtime. All animals were awake at the start of the meal, and within the first few days of the restricted feeding schedule, they all began eating as soon as the food was presented.

Visual inspection of the actograms of wheel-running activity recorded for animals in the two conditions indicated that animals housed in isolation cabinets did not show substantial increases in wheel-running activity in anticipation of mealtime, whereas

animals housed on open shelves did (see Figure 2.1). All animals housed on open shelves showed increased wheel-running activity prior to mealtime on at least seven of the days during which food was available for 4 h per day, whereas none of the animals housed in isolation cabinets did.

Three time periods of activity recording were chosen to further analyze the behavioral patterns shown by animals in the two conditions. The average waveforms for data recorded during the five days of baseline show a normal daily pattern of wheel-running activity for mice in both housing conditions, with high levels occurring during the dark phase and low levels occurring during the light phase of the LD 12:12 cycle (Figure 2.3).

Average activity levels were calculated for animals in the two conditions for the first six days during which food was available for only 4 h daily (Figure 2.4). The average
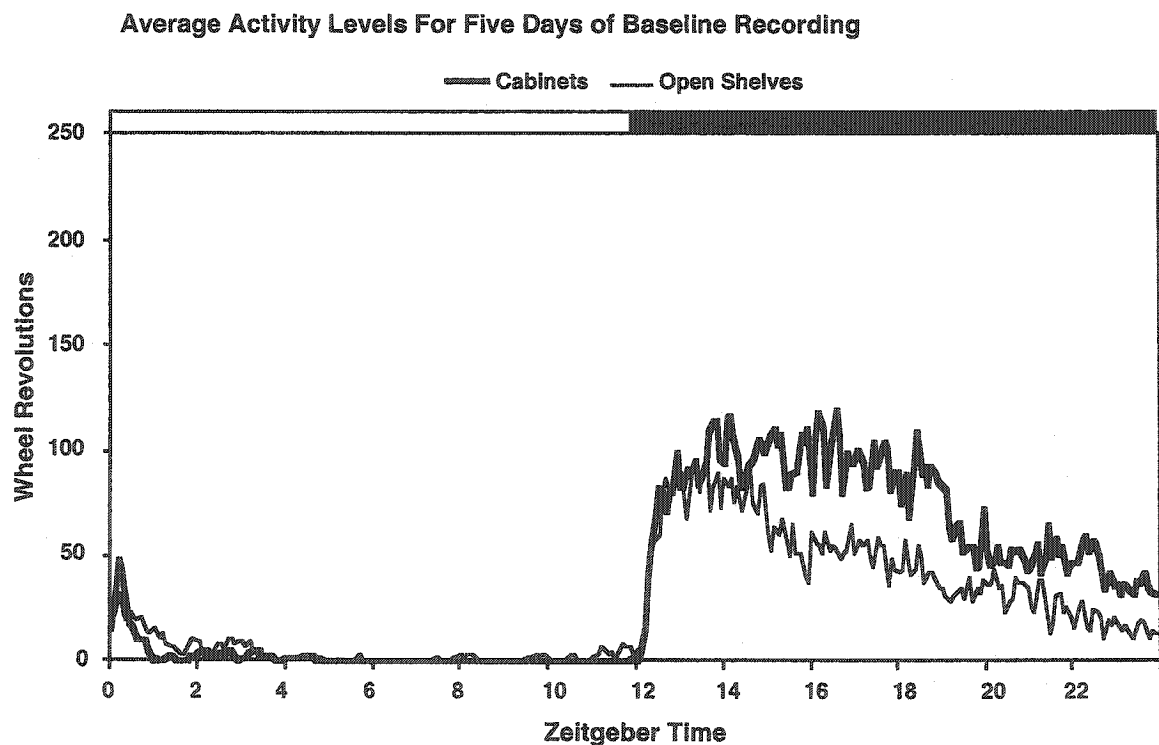
Figure 2.3. Average waveforms for the five days of baseline recording for animals housed in isolation cabinets (thick line; n=10) or on open shelves (thin line; n=10). Zeitgeber time is plotted on the abscissa and average activity levels (in wheel revolutions/5 min time bin) are plotted on the ordinate. The LD 12:12 cycle is shown in the bar across the top (white=light phase, black=dark phase).

waveforms still showed a normal daily pattern of wheel-running activity for mice housed under both conditions, with high levels occurring during the dark phase and low levels occurring during the light phase of the LD 12:12 cycle. Mice housed on open shelves also showed an increase in activity during the 2 to 4 h immediately preceding mealtime (Figure 2.4, thin line). Animals housed in isolation cabinets (Figure 2.4, thick line) did not show this anticipatory increase in activity.

Average waveforms for the last six days of the restricted feeding paradigm are shown in Figure 2.5. As was the case for the first days with 4 h of daily food availability (Figure 2.4), the animals continued to show a characteristic nocturnal increase in wheel running (Figure 2.5). During these six days of recording, mice housed on open shelves also showed an increase in wheel-running activity during the 2 h immediately preceding

Average Activity Levels For the First Six Days of the 4 h Restricted Feeding Schedule
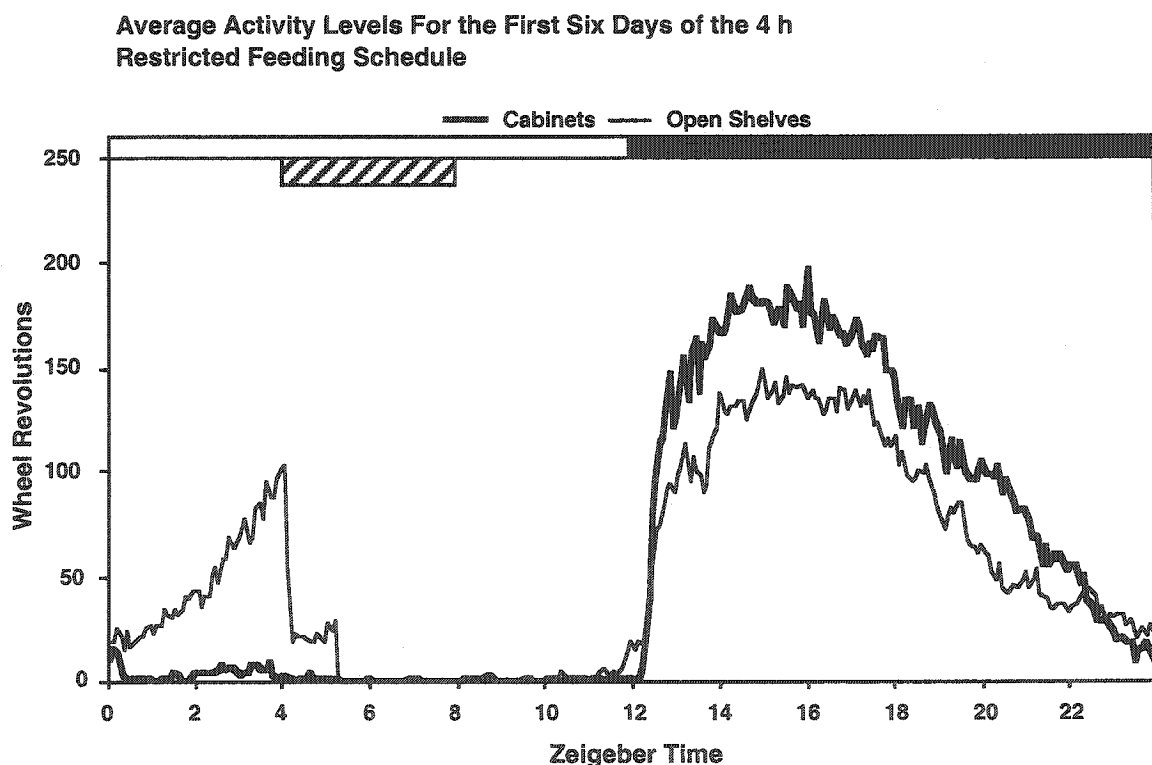
Figure 2.4. Average waveforms for the first six days of recording under the restricted feeding schedule for animals housed in isolation cabinets (thick line; n=10) or on open shelves (thin line; n=10). Zeitgeber time is plotted on the abscissa and average activity levels (in wheel revolutions/5 min time bin) are plotted on the ordinate. The LD 12:12 cycle is shown in the bar across the top (white=light phase, black=dark phase) and the time of daily food availability is indicated by the hatched bar.

mealtime (Figure 2.5, thin line), which was not shown by animals housed in isolation cabinets (Figure 2.5, thick line).

Both groups of animals showed a significant increase in total daily wheel-running activity during the restricted feeding schedule when compared to baseline levels (Figure 2.6; cabinets: $F_{(2,27)}$ = 4.2, P = 0.0259; open shelves: $F_{(2,27)}$ = 13.9, P < 0.0001). Average numbers of wheel revolutions per 5 min bin recorded over the full 24 h day for animals in cabinets during the first six days (57.3 ± 6.25) and the last six days (59.5 ± 7.07) of restricted feeding were significantly higher than those recorded during the five days of baseline (37.4 ± 4.11; P < 0.05). A similar increase in activity level was observed for animals housed on open shelves. Average numbers of wheel revolutions per 5 min bin over 24 h periods for animals on shelves during the first six days (52.4 ± 4.64) and the

Average Activity Levels For the Last Six Days of the 4 h Restricted Feeding Schedule
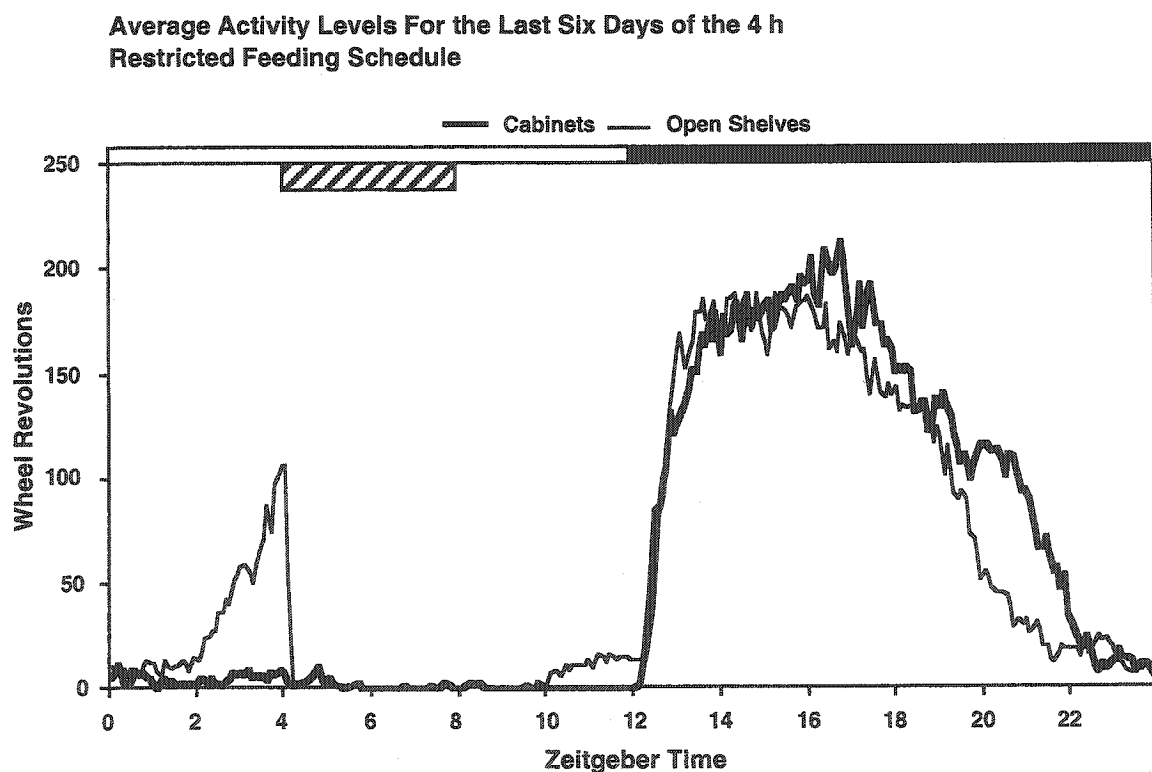
Figure 2.5. Average waveforms for the last six days of recording under the restricted feeding schedule for animals housed in isolation cabinets (thick line; n=10) or on open shelves (thin line; n=10). Zeitgeber time is plotted on the abscissa and average activity levels (in wheel revolutions/5 min time bin) are plotted on the ordinate. The LD 12:12 cycle is shown in the bar across the top (white=light phase, black=dark phase) and the time of daily food availability is indicated by the hatched bar.

last six days (57.7 ± 5.84) of restricted feeding were significantly higher than those recorded during the five days of baseline (25.1 ± 3.24; P < 0.05). Activity levels differed between groups in the two housing conditions only during baseline recording (Figure 2.6). Animals housed in isolation cabinets (37.4 ± 4.11) ran significantly more than animals housed on open shelves (25.1 ± 3.24) during this period of recording (Figure 2.6; $F_{(1,18)}$ = 5.5, P = 0.0301).

Nocturnal activity levels (ZT12-0) were compared statistically among recording intervals (i.e., five days of baseline, six days at the beginning, and six days at the end of the 4 h daily restricted feeding schedule). Both groups of animals showed a substantial increase in nocturnal levels of wheel-running activity during both of the restricted feeding schedule intervals when compared to baseline levels (cabinets: $F_{(2,27)}$ = 4.1, P = 0.0274;
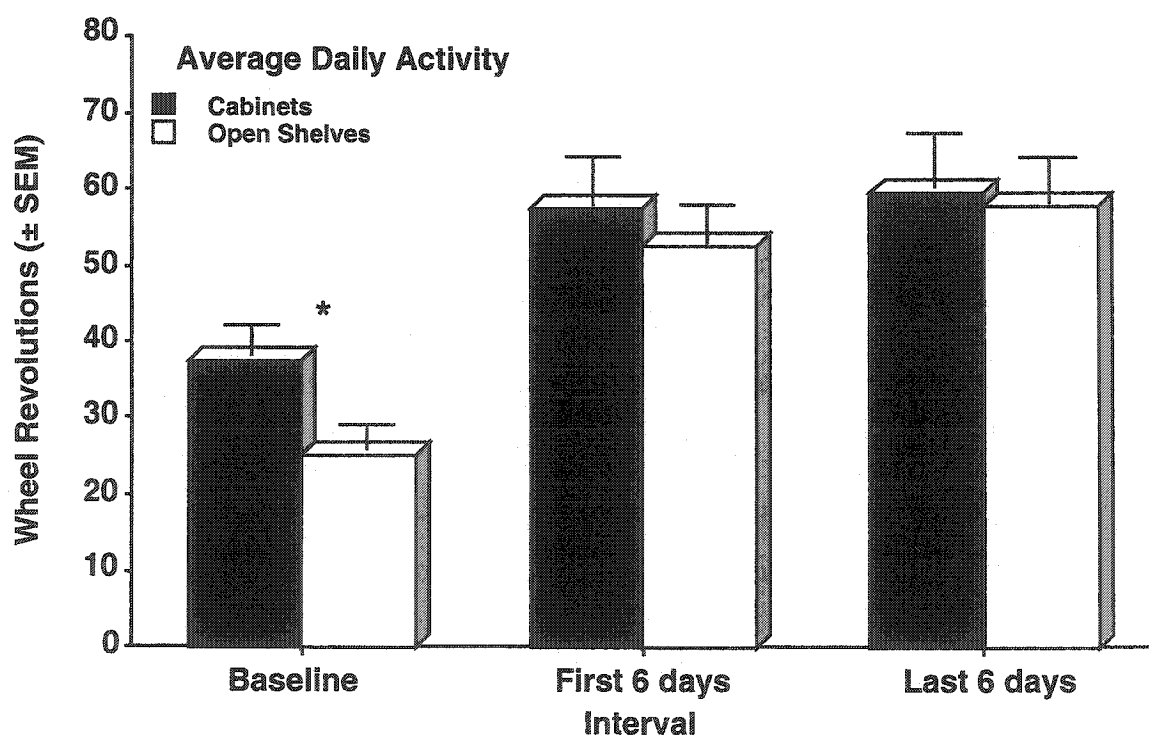
Figure 2.6. Daily activity levels recorded during each of three intervals for animals housed in isolation cabinets (black bars; n=10) or on open shelves (white bars; n=10). Recording intervals are plotted along the abscissa and average numbers of wheel revolutions/5min time bin (± SEM) are plotted along the ordinate. The * indicates a significant difference between groups (P < 0.05).

open shelves: $F_{(2,27)}$ = 9.3, P = 0.0009). Average numbers of wheel-revolutions per 5 min bin recorded in the dark phase for animals in cabinets during the first six days (112.9 ± 12.78) and the last six days (117.0 ± 14.48) of restricted feeding were significantly higher than those recorded during the five days of baseline (72.7 ± 8.01; P < 0.05). A similar increase in nocturnal activity levels was observed for animals housed on open shelves. Average numbers of wheel revolutions per 5 min bin recorded in the dark phase for animals on shelves during the first six days (85.2 ± 9.00) and the last six days (101.8 ± 12.05) of restricted feeding were significantly higher than those recorded during the five days of baseline (46.2 ± 6.12; P < 0.05).

The patterns of nocturnal activity were also compared statistically between animals in the two housing conditions (Figure 2.7). For this comparison, nocturnal activity was divided into four 3 h time bins. During baseline recording, animals housed in isolation cabinets showed significantly more wheel-running activity than those on open shelves during the later part of the dark phase (Figure 2.7A; ZT15-18: 97.2 ± 12.44 and 53.8 ± 8.35, respectively, $F_{(1,18)}$ = 8.4, P = 0.0096; ZT18-21: 66.0 ± 10.34 and 37.3 ± 7.12, respectively, $F_{(1,18)}$ = 5.2, P = 0.0349; ZT21-0: 44.0 ± 6.30 and 20.5 ± 5.37, respectively, $F_{(1,18)}$ = 8.1, P = 0.0107). Animals housed in isolation cabinets ran significantly more than animals housed on open shelves for the first 3 h of the dark phase in the first six days of the 4 h restricted feeding schedule (Figure 2.7B; ZT12-15: 134.5 ± 13.45 and 98.4 ± 8.50, respectively; $F_{(1,18)}$ = 5.1, P = 0.0358). Nocturnal activity levels did not differ significantly between housing conditions during the last six days of the 4 h restricted feeding schedule (Figure 2.7C).

Activity levels that occurred in anticipation of the daily meal, or during the 4 h from lights on to scheduled mealtime, were compared statistically among the three recording intervals. Activity levels recorded during ZT0-2 did not differ among recording intervals for animals housed on open shelves. A significant difference in activity levels among the three recording intervals was found for animals housed in isolation cabinets ($F_{(2,27)}$ = 3.7, P = 0.0386). They ran significantly more in baseline recording during ZT0-2

Figure 2.7. Nocturnal activity levels recorded during each of three intervals for animals housed in isolation cabinets (black bars; n=10) or on open shelves (white bars; n=10). The intervals were: five days of baseline (A), six days at the beginning of the 4 h restricted feeding schedule (B) and six days at the end of the 4 h restricted feeding schedule (C). Zeitgeber time is plotted along the abscissa and average numbers of wheel revolutions/5 min time bin ($\pm$ SEM) are plotted along the ordinate. The * indicates a significant difference between groups ($P < 0.05$).
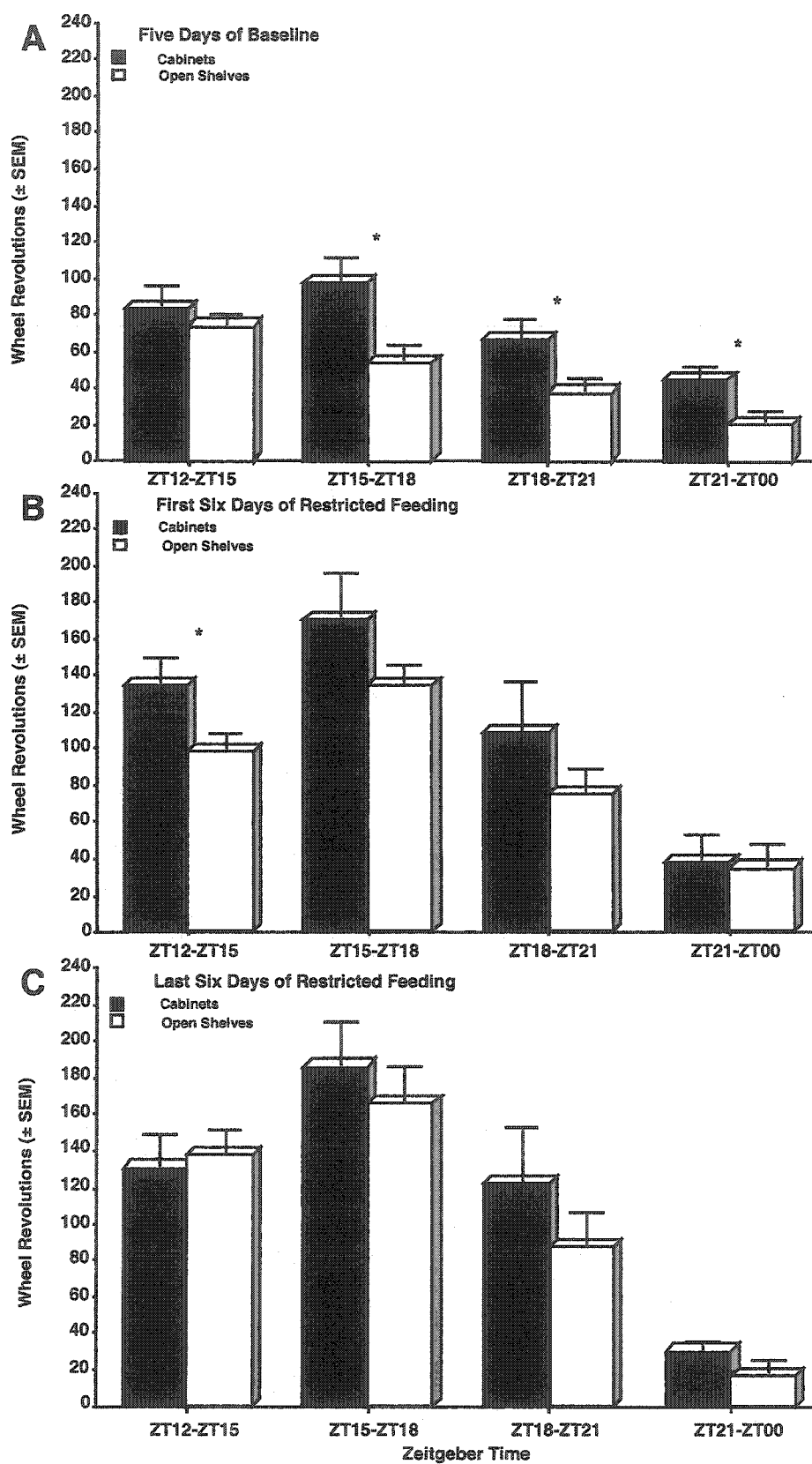
Figure 2.7

(10.0 ± 2.73) than they did during the last six days of the restricted feeding schedule (3.8 ± 1.88; $P < 0.05$). Animals housed on open shelves showed significantly more wheel-running activity during ZT2-4 under restricted feeding than they did during baseline recording ($F_{(2,27)} = 19.3$, $P < 0.0001$; baseline: 5.3 ± 1.70; first six days: 67.6 ± 7.78; last six days: 53.1 ± 10.11; $P < 0.05$). For animals housed in isolation cabinets, however, activity levels in the 2 h immediately preceding mealtime did not differ significantly among the recording intervals. The average number of wheel revolutions per 5 min bin recorded during baseline (2.2 ± 1.21) did not differ significantly from those recorded during the first six days (5.1 ± 1.04) or the last six days (4.9 ± 1.70) of restricted feeding.

There were no statistically significant differences between mice in the two housing conditions in baseline activity levels during ZT0-2 or ZT2-4 (Figure 2.8A). During the first six days of restricted feeding, animals housed on open shelves showed significantly higher levels of activity than those housed in isolation cabinets (Figure 2.8B; ZT0-2: 26.9 ± 6.86 and 3.0 ± 1.04, $F_{(1,18)} = 11.9$, $P = 0.0029$; ZT2-4: 67.6 ± 7.78 and 5.1 ± 1.04, $F_{(1,18)} = 63.2$, $P < 0.0001$). During the last six days of restricted feeding, animals housed on open shelves ran significantly more during ZT2-4 (53.1 ± 10.11) than did those in isolation cabinets (Figure 2.8C; 4.9 ± 1.70, $F_{(1,18)} = 22.1$, $P = 0.0002$). There was no significant group difference during ZT0-2 during the last recording interval.

2.4 Discussion

The results of the present study show that C57BL/6J mice, which normally consume the majority of their daily food requirement during the dark phase (see Figure 2.2), were able to adapt to a restricted feeding schedule with food available for only 4 h in the middle of the light phase. All twenty animals consumed enough food each day to sustain themselves for the duration of the experiment. Within the first few days of the restricted feeding schedule, all mice started eating almost immediately after food was made available.

Restricted feeding increased the amount of daily activity expressed by mice in

Figure 2.8. Activity levels recorded during ZT0-2 and ZT2-4 during each of three intervals for animals housed in isolation cabinets (black bars; n=10) or on open shelves (white bars; n=10). The intervals were: five days of baseline (A), six days at the beginning of the 4 h restricted feeding schedule (B) and six days at the end of the 4 h restricted feeding schedule (C). Zeitgeber time is plotted along the abscissa and average numbers of wheel revolutions/5 min time bin ($\pm$ SEM) are plotted along the ordinate. The * indicates a significant difference between groups ($P < 0.05$).
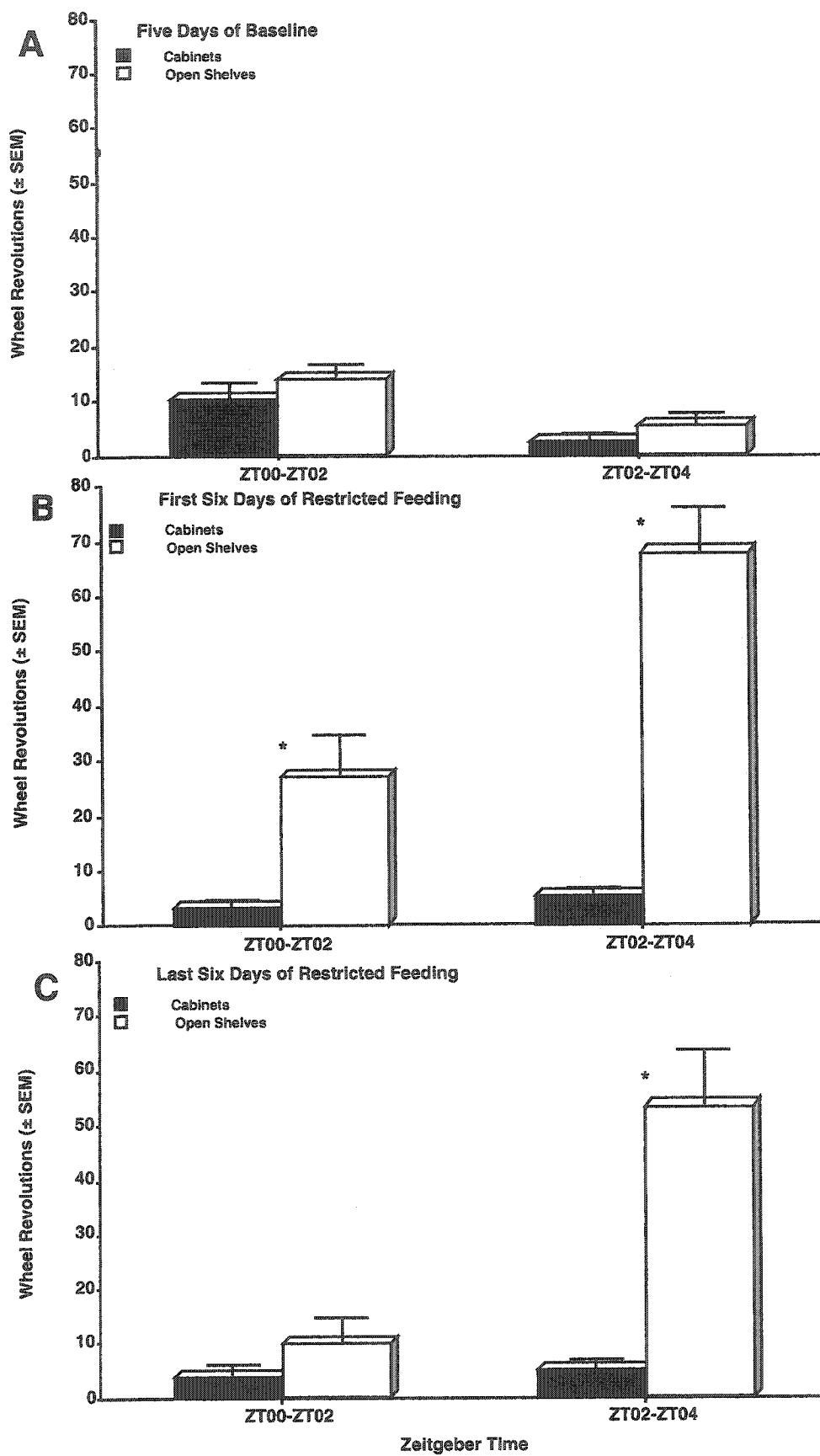
Figure 2.8

both housing conditions (Figure 2.6). The restricted feeding schedule also altered the normal pattern of nocturnal wheel running in all twenty mice (Figure 2.7). Activity levels in the dark phase were significantly elevated during both the early and late stages of the restricted feeding schedule when compared to levels recorded during baseline (Figures 2.3, 2.4 and 2.5). In addition, nocturnal activity during restricted feeding was more consolidated and was concentrated towards the beginning of the dark phase (Figures 2.1, 2.4, 2.5 and 2.7).

This consolidation of nocturnal activity might be attributable to either the restriction in feeding time or to a potential reduction of total intake resulting from this restriction. Previous studies suggest that it is the restriction of feeding time that is critical. Restricted daily feeding early in the light phase (ZT2) of a standard LD cycle causes a phase advance of the nocturnal activity bout in mice and rats such that the onset of activity preceded the time of lights off (Challet et al., 1997a; Challet et al., 1997b; Challet et al., 1997c; Challet et al., 1998a; Challet et al., 1998b). In these studies, animals were fed 50-66% of their *ad libitum* daily intake; therefore, feeding was restricted not only in duration, but also in amount. Animals fed 100% of their normal intake at ZT2 did not show this phase advance of the nocturnal bout of activity (Challet et al., 1997c; Challet et al., 1998b). Mice fed either 66% or 100% of their normal intake at ZT2 did show a consolidation of their nocturnal activity bout (Challet et al., 1998b).

Based on these results, the authors concluded that an energy deficit, or hypometabolic state, due to chronic calorie restriction is required for the phase advance of the nocturnal bout of activity in response to restricted feeding at ZT2 (Challet et al., 1998b). The consolidation of the nocturnal activity bout, however, seems to occur in response to restricted temporal access to food and does not seem to require a significant metabolic deficit. Thus, the alteration of the pattern of nocturnal activity shown by animals in the present study (a consolidation of activity without a concomitant phase advance in onset) resembles that shown by animals given access to 100% of their normal intake early in the light phase. A similar consolidation and contraction of the nocturnal

bout of activity was also reported for Balb/C mice exposed to restricted feeding in an LD cycle (Holmes and Mistlberger, 2000).

A surprising result of this study is that housing condition influenced the expression of FAA in mice (Figure 2.8). Animals housed in isolation cabinets did not show a significant increase in wheel running in the hours immediately preceding mealtime, while those on open shelves did (Figures 2.1, 2.3, 2.4, 2.5 and 2.8). This lack of FAA cannot be explained by a general reduction of wheel running by isolated mice, since those in cabinets ran as much as those on open shelves over the whole day, and actually ran more during baseline recordings when food was available *ad libitum* (Figures 2.3, 2.6 and 2.7). The lack of anticipatory wheel running in these animals is also unlikely to reflect a lack of entrainment of the food-entrainable pacemaker, since all of these animals were awake and aroused at mealtime, when mice are normally asleep or inactive, suggesting that they were actively anticipating a meal.

Previous studies have shown that animals may fail to demonstrate anticipation of scheduled feeding times using one measure of activity while showing robust anticipation as assessed by another. For example, rats with lesions of the paraventricular nucleus of the hypothalamus did not show anticipatory tilt-cage activity, but did show robust FAA directed at the food bin (Mistlberger and Rusak, 1988). Mice exposed to a restricted feeding schedule showed robust wheel-running in anticipation of mealtime, but did not engage in anticipatory drinking (Marchant and Mistlberger, 1997; Holmes and Mistlberger, 2000). Squirrel monkeys placed on a restricted daily feeding schedule anticipated mealtime with a decrease, rather than increase, in activity, measured in this case as amount of tree climbing (Aschoff, 1986; Aschoff and van Goetz, 1986). The monkeys reduced their activity in anticipation of mealtimes because they were sitting in front of the food cup during the interval preceding food availability, not because they had failed to acquire information about the timing of food availability. Assessment of levels of 'food-cup activity' (measured as numbers of tugs at the food cup) revealed that these animals were, in fact, anticipating the time of food availability. Had tree-climbing activity

been the only measure recorded, one might have concluded that the monkeys failed to anticipate mealtime.

All of the animals in cabinet housing started eating immediately after food was presented, and all were able to consume enough food within the 4 h feeding window to survive for the duration of the experiment. These observations suggest that they had adapted physiologically to the feeding schedule. During the course of the experiment, we also observed that the majority of these mice were hanging on their cage tops close to the cabinet door when food was being delivered. Cage-top climbing has been shown to be an activity that mice regularly engage in, especially when they do not have access to a wheel (Harri et al., 1999). Wheel running and cage-top climbing in mice may be functionally equivalent (Harri et al., 1999); thus, these mice may have been expressing anticipation through cage-top climbing rather than wheel running. Since cage-top climbing was not recorded in this study, this interpretation remains hypothetical.

A previous study of food entrainment in rats showed that when mealtime was preceded by an auditory cue, food-anticipatory lever pressing was attenuated, although not abolished (Terman et al., 1984). It is possible that, for animals housed in isolation cabinets, the opening of the cabinet door immediately prior to mealtime functioned as a salient exogenous cue signaling mealtime, and that the presence of this cue abolished the expression of anticipatory wheel running. For animals housed on open shelves, the cues associated with mealtime (e.g., the opening of the experimental room door) may have been less salient, since these animals were more frequently exposed to non-specific environmental disturbances than those housed in isolation cabinets.

Since exogenous cueing only attenuated FAA in rats, it is unclear why it should have resulted in the complete loss of anticipatory activity observed in this study in mice. It may be that other factors contributed to this result. Light inhibits activity in mice (Mrosovsky et al., 1999) and it is possible that the light intensity was slightly higher for animals in the isolation cabinets than for those on open shelves. The temperature during the light phase was also slightly higher within the cabinets than in the open experimental

room. Thus, it is possible that higher light intensity and/or temperature within the cabinets could have decreased the amount of anticipatory wheel running expressed by these animals. Other evidence described above indicates that these mice had adapted to the timing of food availability. These observations emphasize that FAA is an output of the food-entrainable pacemaker and that a lack of FAA as assessed by one measure does not necessarily imply that the pacemaker is not entrained by the feeding schedule.

In summary, the results of the present study show that C57BL/6J mice are normally strongly nocturnal in their feeding, but are able to entrain to, and anticipate, a meal during the light phase when subjected to a restricted daily feeding schedule. The expression of the output of the food-entrainable pacemaker in specific behaviors may be sensitive to features of the laboratory environment, including housing conditions.

CHAPTER 3

Distribution of Orexin-Containing Cell Bodies in Three Strains of Mice (*Mus musculus*)

3.1 Introduction

In 1998, two groups independently identified a set of two novel neuropeptides, orexin A and B, also known as hypocretin 1 and 2 (Sakurai et al., 1998; De Lecea et al., 1998). Orexin was originally thought to play a role in feeding because it is produced exclusively by neurons in the lateral hypothalamic and perifornical regions (Sakurai et al., 1998). The lateral hypothalamus (LH) has historically been known as a feeding center because lesions of this structure result in hypophagia, and stimulation of this region leads to hyperphagia (Kalra et al., 1999). Central administration of these peptides resulted in an increase in feeding in unfasted rats (Sakurai et al., 1998; Edwards et al., 1999; Jain et al., 2000). Furthermore, preproorexin mRNA was shown to be up-regulated following fasting (Sakurai et al., 1998; Cai et al., 1999; López et al., 2000), although orexin levels did not change in response to fasting in other studies (Taheri et al., 1999; Mondal et al., 1999b; Tritos et al., 2001).

It was later discovered that these neuropeptides also play a significant role in sleep (Kilduff and Peyron, 2000). Orexin knockout mice were found to display many of the characteristics of the sleep disorder narcolepsy (Chemelli et al., 1999). Almost at the same time this discovery was reported, it was found that narcoleptic dogs had a mutant form of orexin receptor 2 (Lin et al., 1999). Subsequent studies have reported that human narcoleptics have low to undetectable levels of orexin A within the cerebrospinal fluid (Nishino et al., 2000), and that postmortem analyses show a significant reduction in brain orexin levels in narcoleptic patients (Thannickal et al., 2000; Peyron et al., 2000).

The orexin-producing cells send projections widely within the brain and spinal cord (Peyron et al., 1998; Van den Pol, 1999; Nambu et al., 1999; Date et al., 1999). Efferent projections are found within the hypothalamus, as well as extra-hypothalamic

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sites such as the paraventricular nucleus of the thalamus (PVT), the locus coeruleus (LC) and the olfactory bulbs. The wide central projection patterns suggest that orexin plays a role in numerous hypothalamic regulatory functions including arousal, energy homeostasis and food intake, as well as in the control of cardiovascular, neuroendocrine and temperature control systems (Kukkonen et al., 2002; Smart and Jerman, 2002).

Previous studies have shown slight species differences in the distribution of orexin-containing cell bodies or fibers within the brain (Wagner et al., 2000; McGranaghan and Piggins, 2001), as well as differences between the distribution of orexin A and B within species (Cutler et al., 1999; Date et al., 2000b). To our knowledge, the distribution patterns of orexin A and B within the brains of different strains of mice have not been studied. Strain differences have been reported in behavioral and physiological measures such as circadian rhythmicity, ingestive behaviors and performance on behavioral tests (Schwartz and Zimmerman, 1990; Kotlus and Blizard, 1998; Crabbe et al., 1999; Smith et al., 2000; Wahlsten et al., 2003). For these reasons, we assessed the distribution patterns of orexin A and orexin B-immunoreactive (IR) cell bodies in the brains of mice from three different strains (CD1, DBA and C57BL/6J). Differences were found in the distribution of orexin A-IR and orexin B-IR cell bodies both within and among animals of the three strains studied.

3.2 Methods

3.2.1 Animals and Housing

Six adult male C57BL/6J (referred to as C57) mice were obtained from Charles River Canada (St. Constant, Québec, Canada). Five adult male CD1 and five adult male DBA mice were obtained from an in-house colony. All animals were group-housed (2-3 per cage) by strain in mouse bin cages (27.5 cm X 16.5 cm X 12.5 cm) in separate colony rooms under standard light-dark (LD) 12:12 cycles. Before the start of this experiment, the CD1 and DBA mice were used in other experiments unrelated to this study. Food (Rodent Laboratory Chow, Agribrands, Ontario, Canada) and water were available *ad*

libitum. Two additional C57 mice housed in identical conditions were used for immunocytochemical control procedures (see below). All experiments were conducted according to Canadian Council on Animal Care guidelines and were approved by the Dalhousie University Committee on Laboratory Animals.

3.2.2 Immunocytochemistry

All animals were killed with a barbiturate overdose (Sodium pentobarbitol, 0.5 cc of 65 mg/ml, I.P.) and were perfused transcardially with 0.9% physiological saline followed by 4.0% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) immediately following injections. Brains were removed and allowed to post-fix in 4.0% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for ~48 h at 4° C. The brains were then transferred to 0.1M phosphate buffer (pH 7.4) containing 20% sucrose for ~48 h, also at 4° C. Coronal sections (30 μm thick) were cut using a cryostat and were collected into 0.01M phosphate-buffered saline with sodium azide (PBS-azide, pH 7.4). Tissue was collected from the start of the anterior commissure through to the end of the LC and every fourth section was processed for a given antibody. Anatomical localization of each of these structures was determined using a mouse brain atlas (Franklin and Paxinos, 1997).

Immunocytochemical detection of orexin A and orexin B was done using standard procedures. Briefly, collected sections were rinsed in 0.01M phosphate-buffered saline (PBS) for 5 min and then in 0.3% hydrogen peroxide in 0.01M PBS for 10 min to block endogenous peroxidase activity. They were then rinsed twice in 0.01M PBS for 5 min, and were rinsed three times for 5 min in 0.01M PBS between each of the following steps. Sections were incubated in 0.01M PBS with triton-X 100 (PBS-X) containing 3% normal rabbit serum (Vector Laboratories Canada, Inc., Ontario, Canada) for a minimum of 60 min at 4° C. They were then incubated for a minimum of 48 h in an affinity-purified goat polyclonal primary antibody raised against the carboxy terminus of orexin A or orexin B peptide (sc-8070 or sc-8071; Santa Cruz Biotechnology, Inc., USA) at 4° C. These antibodies were shown to recognize orexin A or B, respectively, of mouse origin

specifically by Western blotting (Santa Cruz Biotechnology, Inc.). Primary antibodies were diluted to 1:10,000 in PBS-X containing 3% normal rabbit serum.

Following incubation in the primary antibody, sections were placed in a PBS-X solution containing biotinylated anti-goat IgG secondary antibody raised in rabbit (1:200; Vector Labs) in 3% normal rabbit serum for a minimum of 1 h at 4° C. Sections were transferred to a solution of Avidin/Biotin Complex (Elite ABC Kit; 1:100; Vector Labs) in PBS-X for 1 h also at 4° C. Immunoreactivity was visualized using diaminobenzidine (DAB; 0.2 mg/ml of 0.1M phosphate buffer (pH 7.4); Sigma Aldrich Canada, Ltd., Ontario, Canada) as the chromogen. Hydrogen peroxide (0.35 µl/ml) was used as the catalyst. Once the desired depth of color was reached, sections were mounted on gelatin-coated slides. These were allowed to dry overnight and were dehydrated in serial dilutions of ethanol. Slides were cleared using Histoclear (Diamed Lab Supplies, Ltd., Ontario, Canada) and coverslipped using Entellan (BDH, Darmstadt, Germany) as the mounting medium.

3.2.3 Control Procedures

Brain sections from two C57 mice not used in the conditions described above were reacted for detection of orexin A and B in exactly the same way as described above. Alternate sections from these brains were processed for immunocytochemistry following preincubation of the primary antibodies with two concentrations of the orexin A and B blocking peptides (peptide:primary antibody, 10:1 and 20:1; Santa Cruz) for 2 h at room temperature. Additional sections from a C57 mouse were also reacted as described, but in this case, the primary antibodies were omitted altogether.

3.2.4 Analyses

All sections were inspected visually for distribution, number and staining intensity of immunopositive cell bodies using a microscope (Olympus BH-2, Olympus Corporation, N.Y., USA). Representative sections of various brain regions were photographed using an Olympus Camedia digital camera (Olympus America Inc., USA) mounted on the microscope. Photographs were imported into Adobe Photoshop (version

4.0.1) to assemble the figures.

3.3 Results

Robust immunoreactivity was found in the control tissue reacted for orexin A and B (Figures 3.1A and C, respectively), but no immunoreactivity was found in sections for which the primary antibodies were omitted (Figure 3.1E). A reduction in immunoreactivity was found in sections reacted following preincubation of the primary antibody with blocking peptide at the lowest concentration (10:1; orexin A and B; Figures
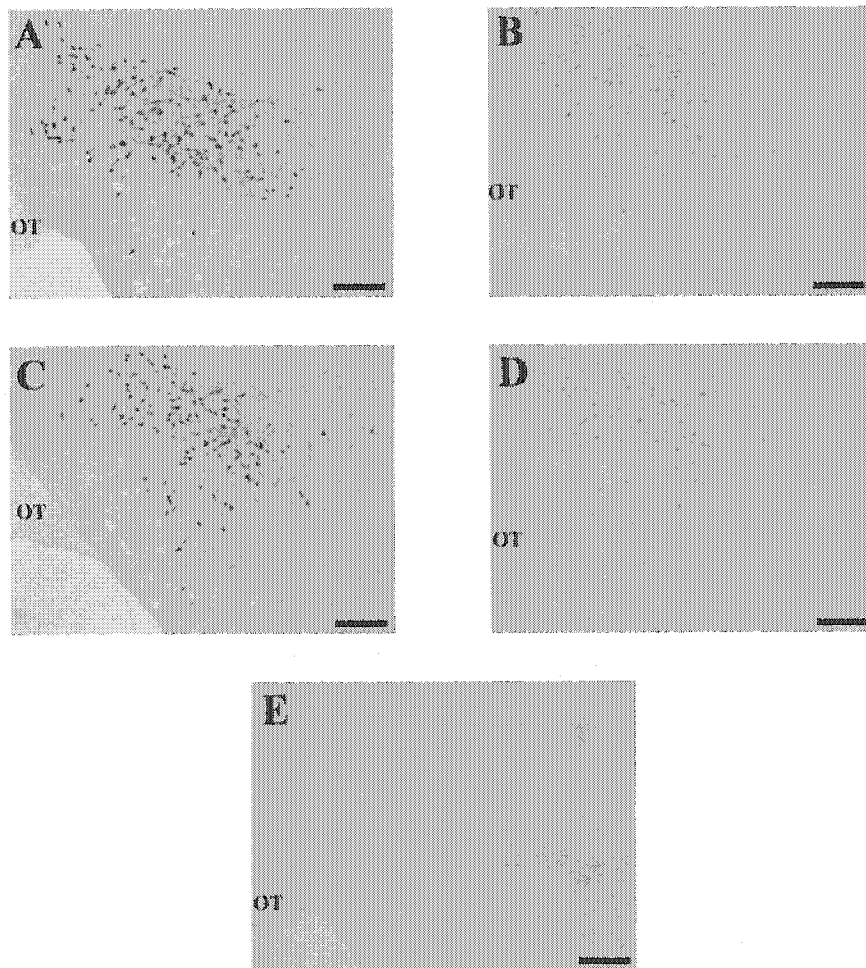
Figure 3.1. Immunoreactivity for orexin A and B in the LH/perifornical region with or without primary antibody preincubation with blocking peptide or with primary antibody omitted. A: orexin A; B: orexin A following preincubation with 10X blocking peptide; C: orexin B; D: orexin B following preincubation with 10X blocking peptide; D: primary antibody omitted. OT = optic tract. Scale bar = 200 µm.

3.1B and D, respectively). A slightly larger reduction in the intensity of immunostaining resulted following primary antibody preincubation with the higher concentration of blocking peptides (20:1; data not shown), but immunoreactivity was not blocked entirely in any section by preincubation with the blocking peptides.

A large number of neurons within the LH/perifornical region of mice from all three strains showed immunoreactivity for both orexin A and B (Figure 3.2). Cells in this region had large somata (approximately 20-30 μm) and were darkly stained. Virtually all of these cells showed processes emerging from the perikarya. This group of neurons extended into

Figure 3.2. Immunoreactivity for orexin A (left column) and B (right column) in the LH/perifornical region of mice of different strains (CD1: top row; DBA: middle row; C57: bottom row). F = fornix. Scale bar = 100 μm.

the surrounding regions [i.e., the anterior hypothalamus (AH) and the dorsomedial hypothalamus (DMH)]. Cell bodies found in the AH were also large and darkly stained with distinct processes emerging from the cell bodies (Figure 3.3), but those found within the DMH tended to be somewhat smaller (approximately 10-20 μm; Figure 3.4) and showed fewer processes. Examples of large and small somata with dark, moderate or light immunostaining are shown in Figure 3.5. The cell bodies found in the AH and DMH showed immunoreactivity for both orexin A and B (Figures 3.3 and 3.4). Animals of all three strains had comparable numbers of orexin A-IR and orexin B-IR perikarya within

Figure 3.3. Immunoreactivity for orexin A (left column) and B (right column) in the AH/RCH of mice of different strains (CD1: top row; DBA: middle row; C57: bottom row). Scale bar = 100 μm.

the LH/perifornical region and the AH (see Table 3.1). It appeared, however, that CD1 animals had slightly more orexin A-IR cell bodies within the DMH than animals of the other two strains (Table 3.1). An additional group of small cell bodies with light to moderate immunoreactivity for both orexin A and B was found within the retrochiasmatic area (RCH) of animals of all three strains (Figure 3.3). This group of cell bodies was dispersed throughout the ventral RCH along the supraoptic decussation. All three groups of animals had comparable numbers of the small orexin A-IR cells within the RCH; however, there appeared to be a difference among strains in the number of orexin B-IR
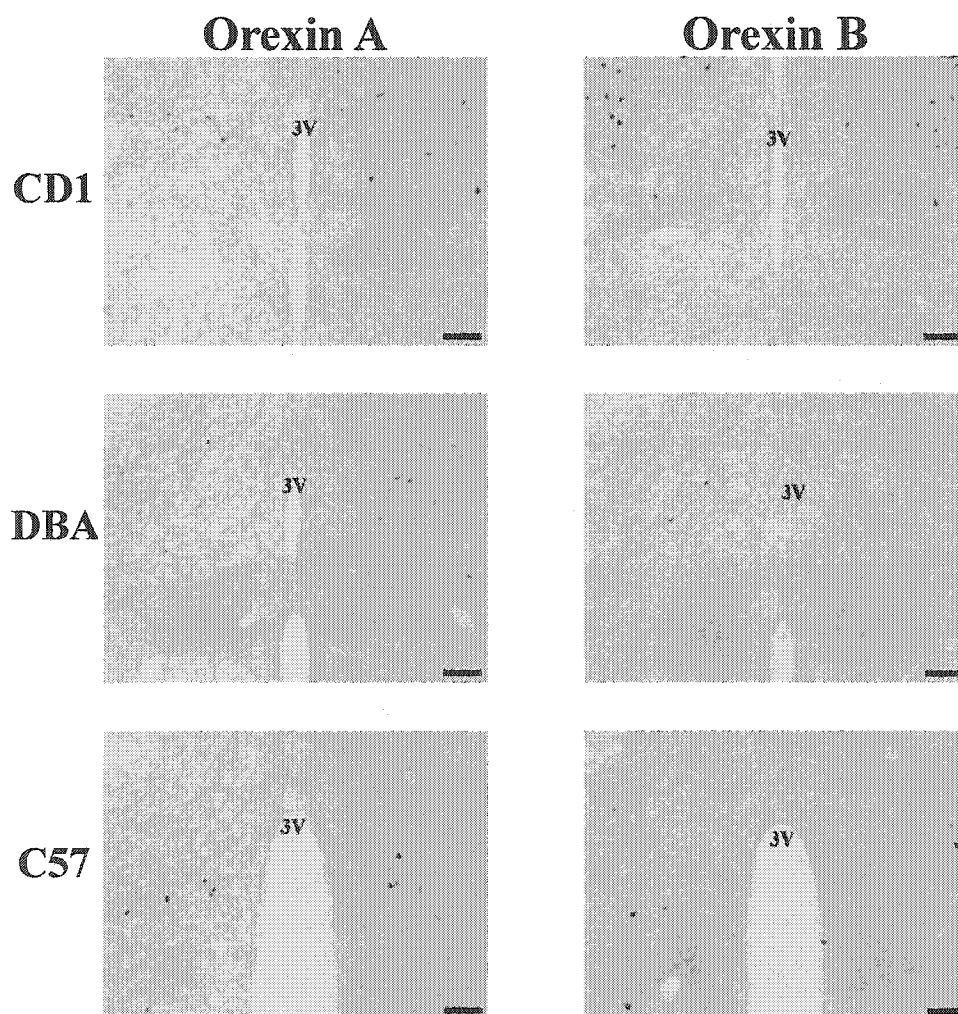
Figure 3.4. Immunoreactivity for orexin A (left column) and B (right column) in the DMH of mice of different strains (CD1: top row; DBA: middle row; C57: bottom row). 3V = 3rd ventricle. Scale bar = 100 μm.

Large-Dark

Small-Dark

Small-Moderate

Small-Light
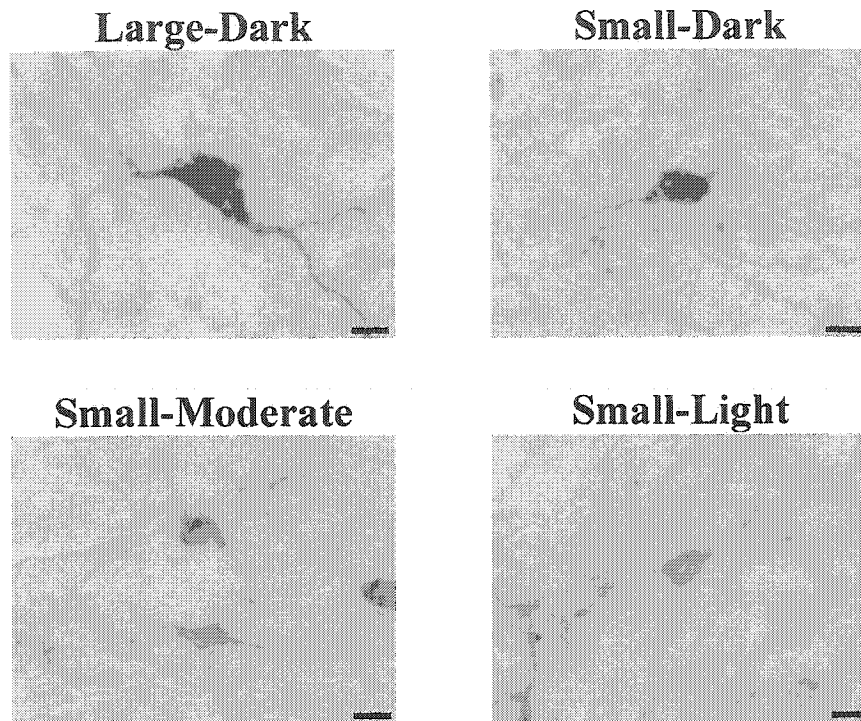
Figure 3.5. Large and small somata showing immunoreactivity for orexin A. An example of a large cell body is shown at top left. Examples of small cell bodies with dark, moderate or light staining are shown in the other three pictures. Scale bar = 10 μm.

perikarya in this region (Table 3.1). Five of the six C57 animals had approximately 15-30 cells within this area, whereas four of the five DBA mice had approximately 1-15 cells. Three animals of the CD1 strain had 1-15 cells and the remaining two animals had 15-30 cells in this region. Thus, there appear to be differences in orexin B-IR cell numbers both within and among strains in this brain region.

Cell bodies immunopositive for orexin A were also found in hypothalamic structures outside of the regions described above. As shown in Figure 3.6, animals in all three strains showed orexin A-IR cell bodies within the paraventricular nucleus of the hypothalamus (PVN). These cell bodies were small, and the staining was light but discernible. In some cases, these orexin-IR cell bodies were also found extending ventrally along the 3rd ventricle into the subparaventricular zone. Figure 3.7 shows an example of a

CD1 mouse with the darkest immunostained perikarya within the PVN. No animal of any strain had orexin B-IR somata within the PVN. CD1 and DBA animals all showed more than 30 orexin A-IR cell bodies within the PVN; however, only half of the C57 animals showed more than 30 immunopositive cells within this structure (see Table 3.1). The remaining C57 animals showed 15-30 orexin A-IR cells in the PVN.

		CD1	DBA	C57
LH/perifornical	Ox A	+++ [D]	+++ [D]	+++ [D]
(large somata)	Ox B	+++ [D]	+++ [D]	+++ [D]
AH	Ox A	++ [D]	++ [D]	++ [D]
(large somata)	Ox B	++ [D]	++ [D]	++ [D]
DMH	Ox A	(++) [D]	+ [D]	+ [D]
(small somata)	Ox B	+ [D]	+ [D]	+ [D]
RCH	Ox A	++ [L/M]	++ [M]	++ [M]
(small somata)	Ox B	(++) [L]	+ [L]	++ [L/M]
PVN	Ox A	+++ [L]	+++ [L]	(+++) [L]
(small somata)	Ox B	0	0	0
SON	Ox A	(+) [L]	+ [L]	(+) [L]
(small somata)	Ox B	0	0	0

+: 1-15 cells; ++: 15-30 cells; +++: >30 cells; (): ~ half in the group had fewer cell bodies

Table 3.1. Number of orexin A-IR (Ox A) and orexin B-IR (Ox B) cell bodies found in the brains of mice from three different strains. The symbols indicate the maximum number of cells observed among animals of this strain. Parentheses indicate that approximately half of the animals in that group had fewer than this maximum number of cell bodies in that region. Intensity of the staining (light [L], moderate [M] or dark [D] is indicated (0: no immunopositive cell bodies). LH: lateral hypothalamus; AH: anterior hypothalamus; DMH: dorsomedial hypothalamus; RCH: retrochiasmatic area; PVN: paraventricular nucleus of the hypothalamus; SON: supraoptic nucleus.
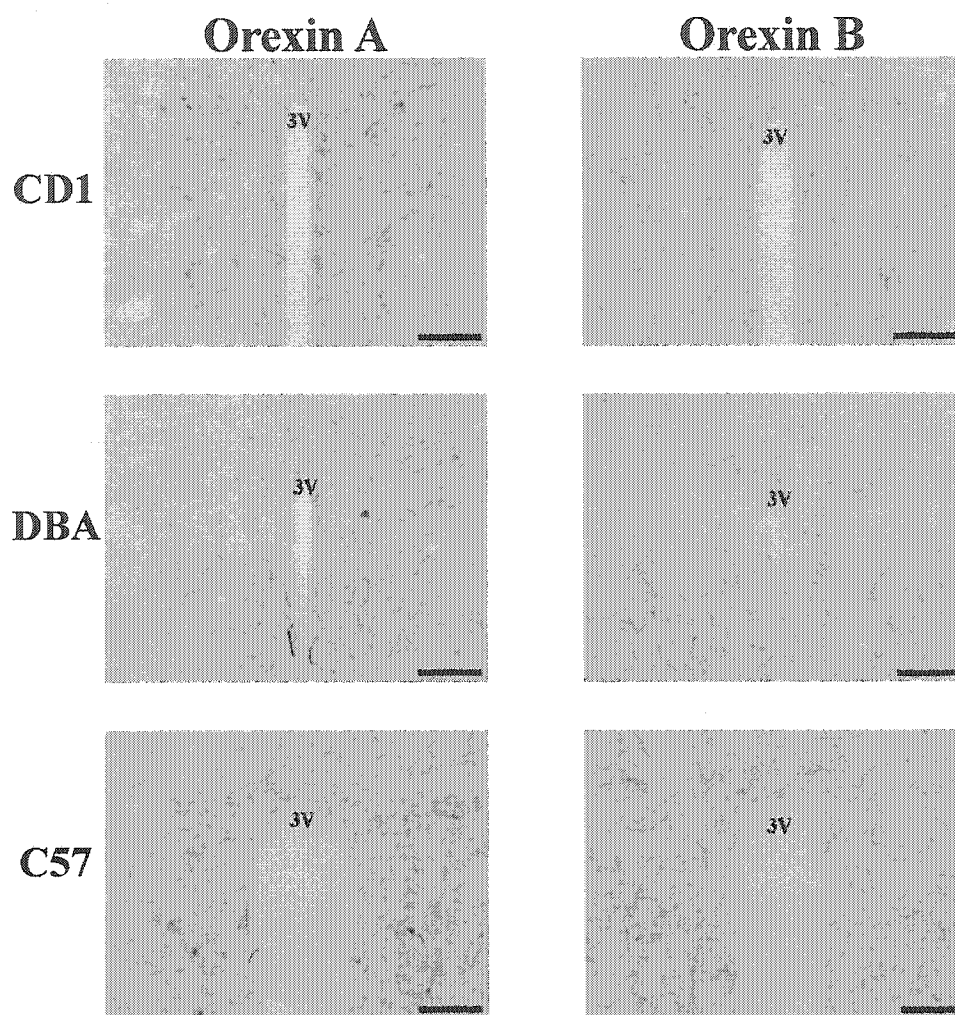
Figure 3.6. Immunoreactivity for orexin A (left column) and B (right column) in the PVN of mice of different strains (CD1: top row; DBA: middle row; C57: bottom row). 3V = 3rd ventricle. Scale bar = 100 μm.
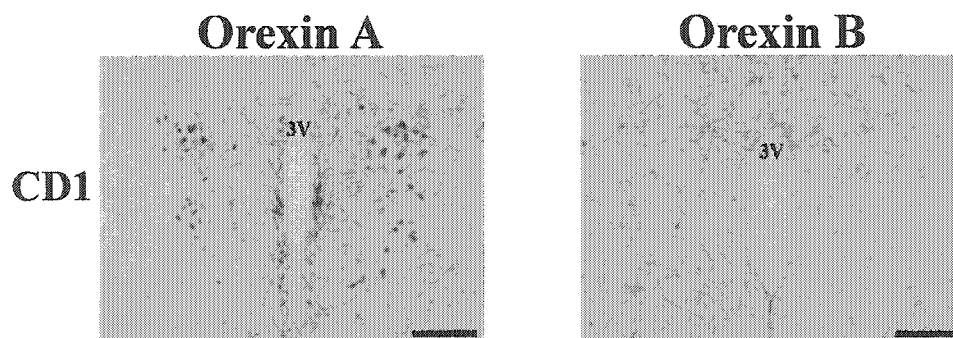
Figure 3.7. Immunoreactivity for orexin A (left column) and B (right column) in the PVN of a CD1 mouse. 3V = 3rd ventricle. Scale bar = 100 μm.

Animals of all three strains showed a small number of very lightly stained cell bodies within the supraoptic nucleus (SON) following immunocytochemistry for orexin A, but not for orexin B (Table 3.1; Figure 3.8). These somata resembled those found within the PVN, but because there were many immunopositive fibers within the SON, these cell bodies were more difficult to distinguish than those found within the PVN (Figures 3.6 and 3.7). All DBA mice showed 1-15 orexin A-IR cell bodies in the SON, whereas some CD1 and C57 mice did not show any orexin A-IR somata in this structure.
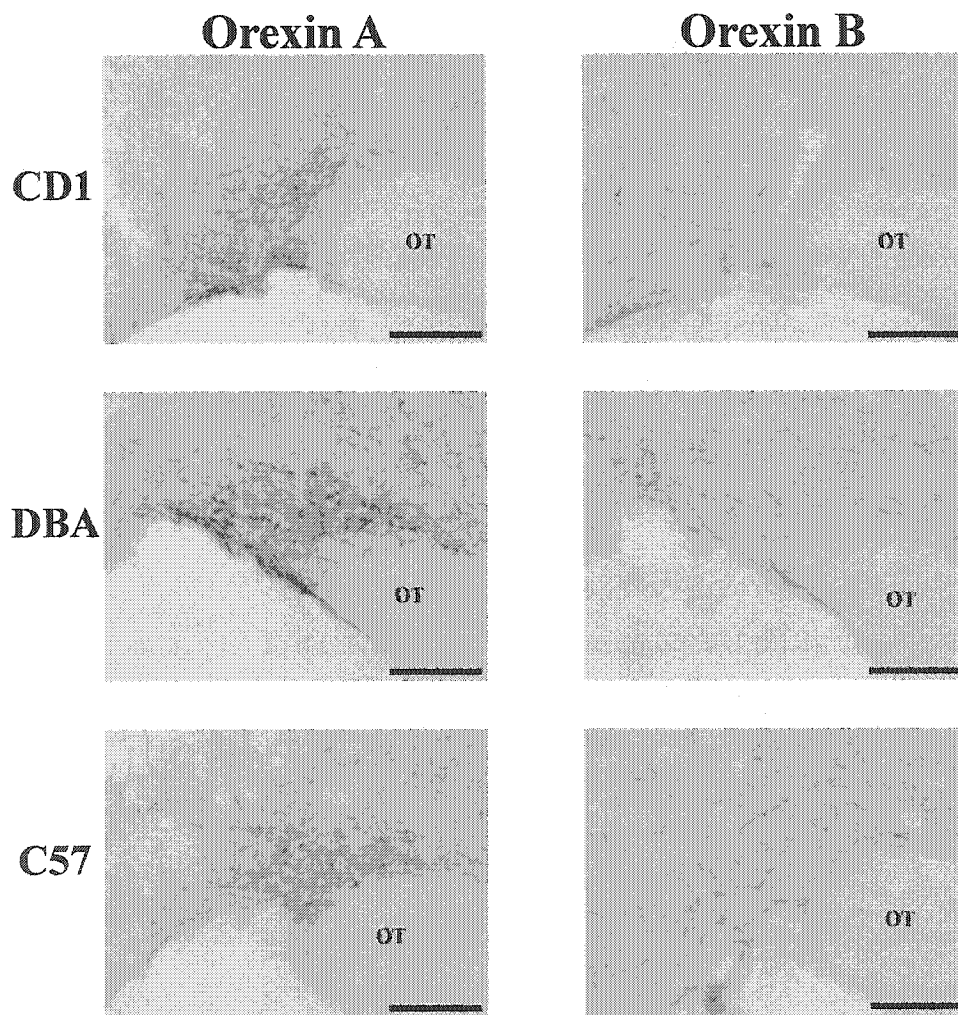
Figure 3.8. Immunoreactivity for orexin A (left column) and B (right column) in the SON of mice of different strains (CD1: top row; DBA: middle row; C57: bottom row). OT = optic tract. Scale bar = 100 μm.

As shown in Figure 3.9, a band of fibers stained for orexin A but not B was found throughout the internal lamina of the median eminence (ME). This differential pattern of staining between orexin A and B was found in all animals of all three strains (Figure 3.9). In addition to this dark band of orexin A fiber staining, small immunopositive structures were found in both the orexin A and B stained ME (Figure 3.9; e.g., top left panel and bottom right panel). Because the morphology and size of these bodies were irregular, it is unclear whether or not these were cell bodies.
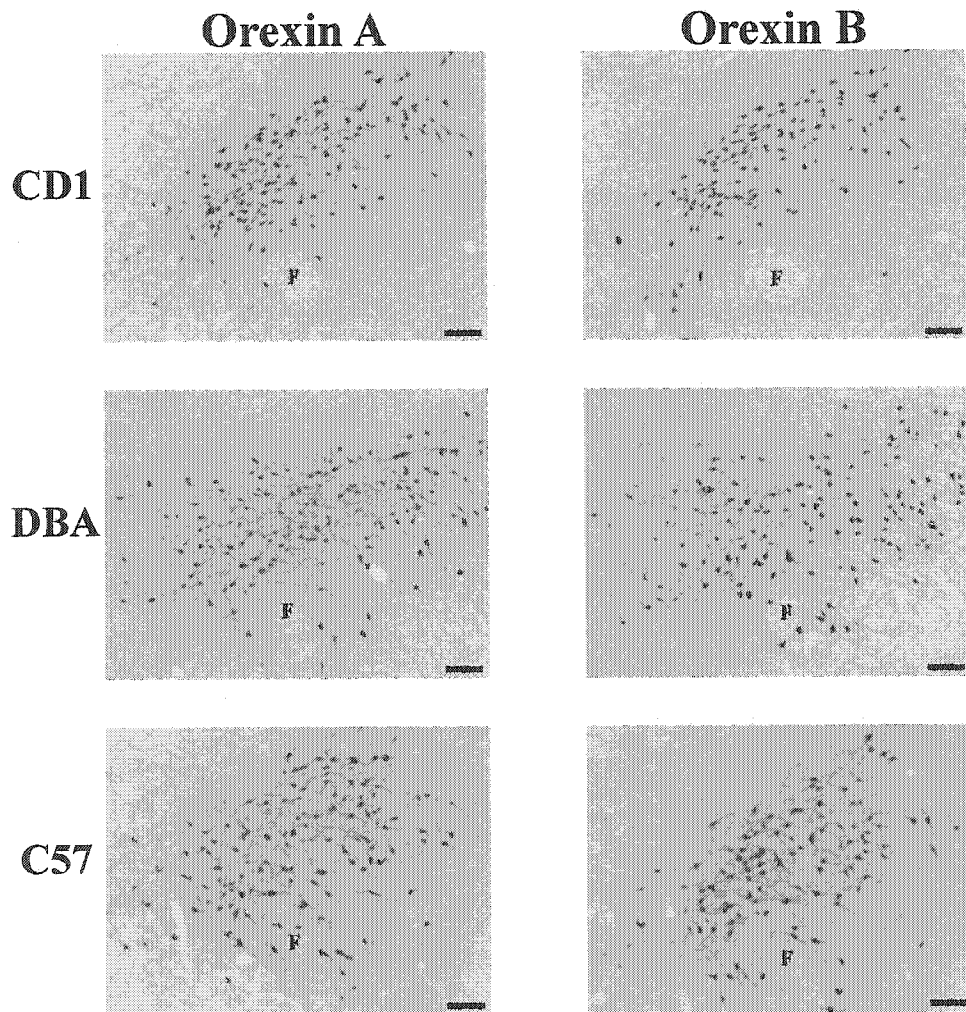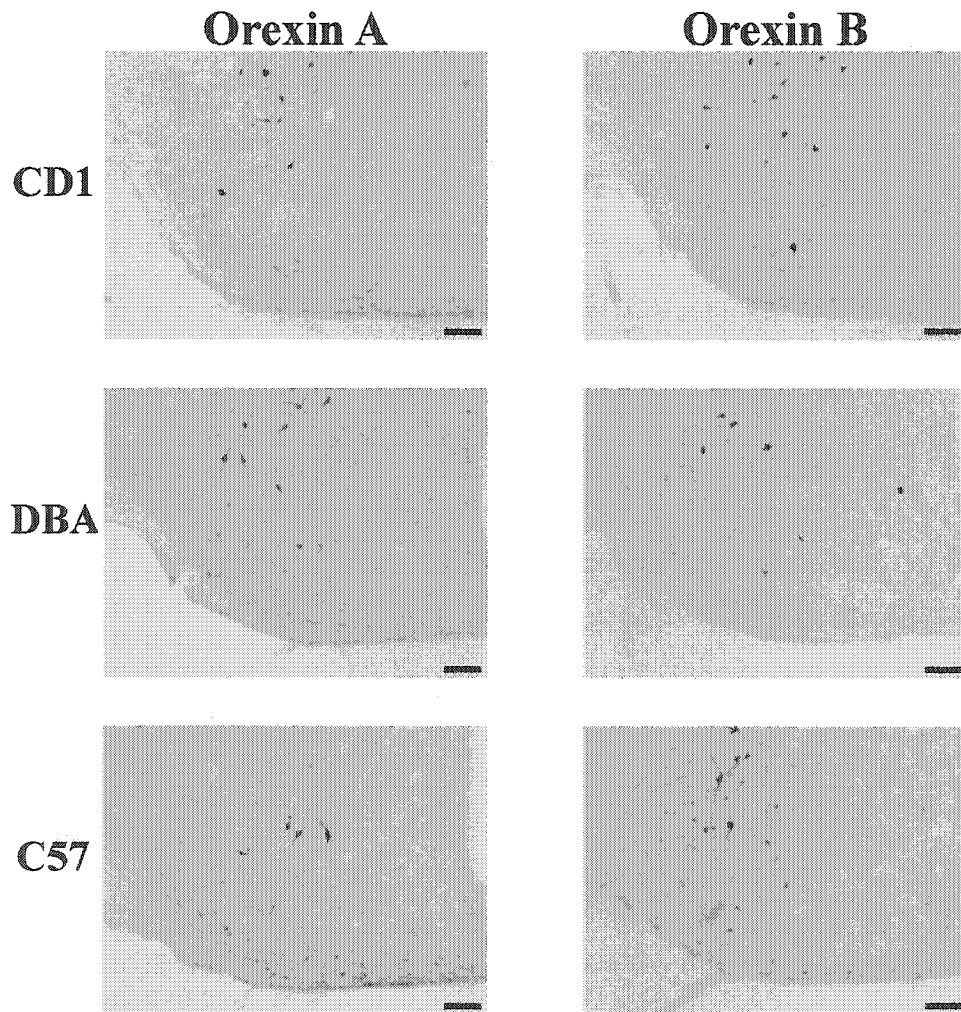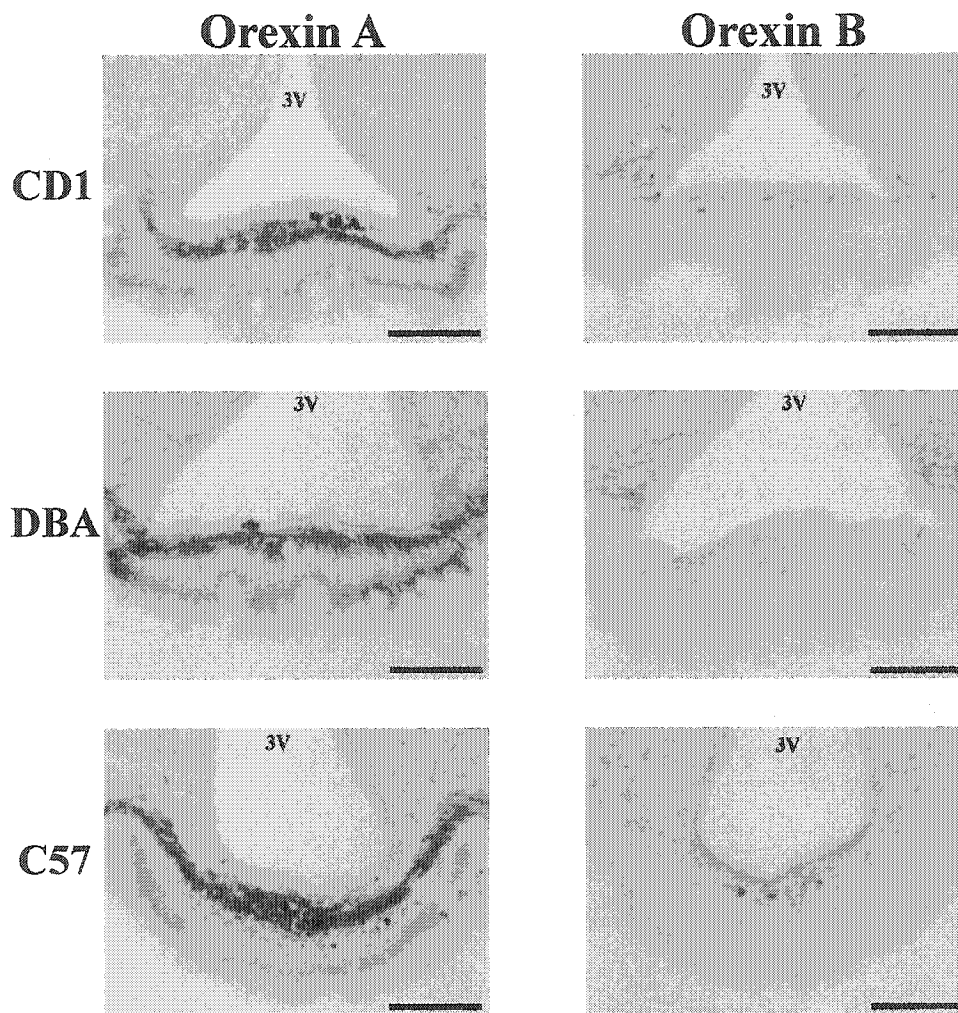

Figure 3.9. Immunoreactivity for orexin A (left column) and B (right column) in the ME of mice of different strains (CD1: top row; DBA: middle row; C57: bottom row). 3V = 3rd ventricle. Scale bar = 100 μm.

A small population of orexin-IR cell bodies was found outside of the hypothalamus in the brains of all mice, specifically within the hindbrain. These perikarya resembled the large, darkly stained cells found within the LH/perifornical region (e.g., top left panel of Figure 3.5). Examples of hindbrain cell bodies containing orexin A immunoreactivity are shown in Figure 3.10, and examples of somata immunostained for orexin B are shown in Figure 3.11. Some of these cells show clear processes emerging from the perikarya (e.g., the top right panels in Figure 3.10 and 3.11). The immunoreactivity in these cells appears to be cytoplasmic since the nucleus of at least
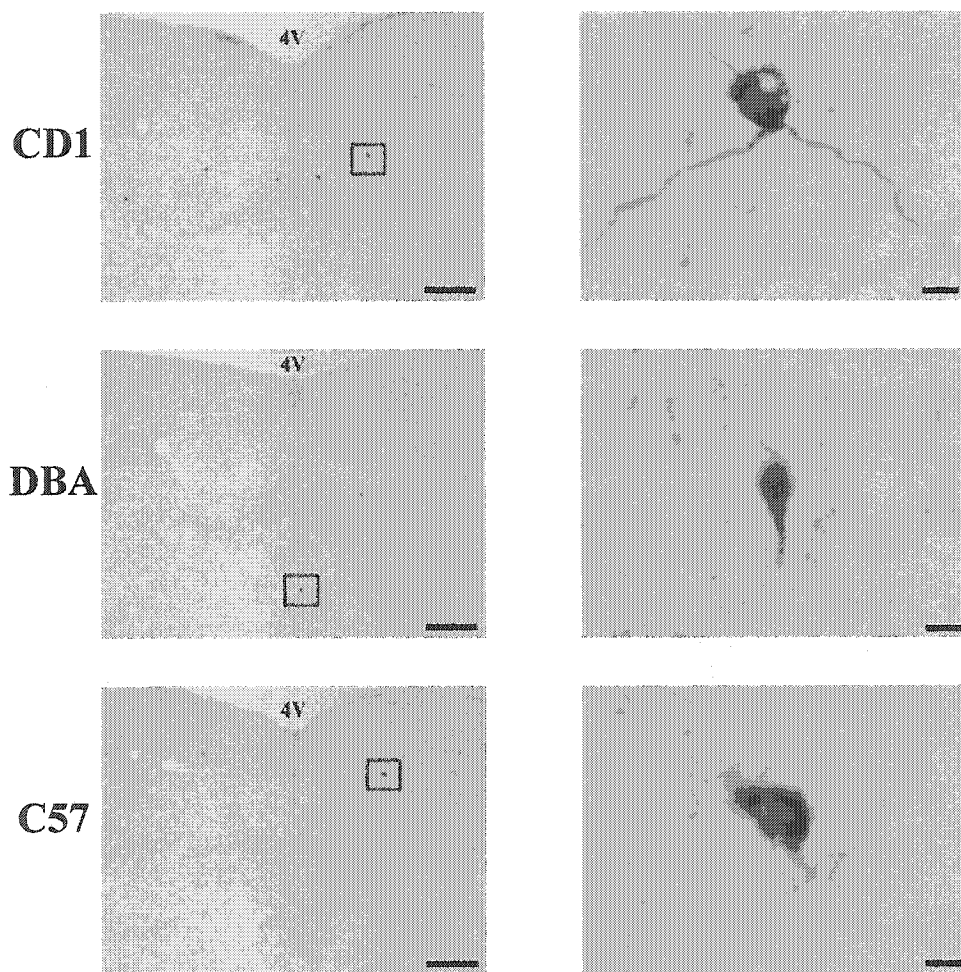
Figure 3.10. Immunoreactivity for orexin A in the hindbrain of mice of different strains (CD1: top row; DBA: middle row; C57: bottom row). Photographs in left column are shown at low magnification (4V = 4th ventricle; scale bar = 200 µm). Cell bodies outlined in the photographs on the left are shown at higher magnification in the right column (scale bar = 10 µm).

some of these cells does not contain immunostaining (e.g., the top right panel in Figure 3.10). These somata were not localized to one hindbrain structure, but were dispersed throughout the periaqueductal grey.

Counts of these hindbrain cells indicated that there were few in all strains (Figure 3.12), although CD1 animals had slightly more orexin-IR cell bodies than animals of the other two strains (CD1: 2-14; DBA: 2; and C57: 0-3 orexin A-IR somata in hindbrain regions). The number of orexin B-IR cell bodies ranged from 1 to 6 for CD1 mice, and from 0 to 2 for DBA and C57 mice. The number of immunopositive cell bodies found
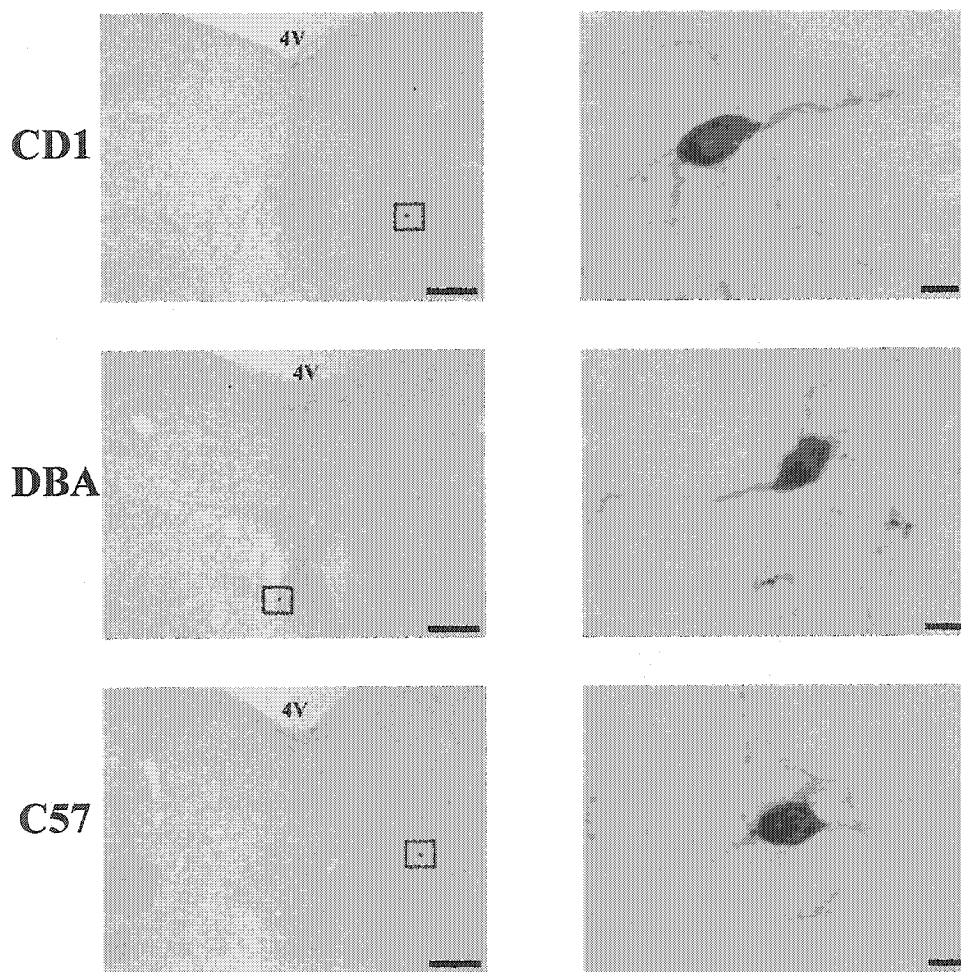
Figure 3.11. Immunoreactivity for orexin B in the hindbrain of mice of different strains (CD1: top row; DBA: middle row; C57: bottom row). Photographs in left column are shown at low magnification (4V = 4th ventricle; scale bar = 200 μm). Cell bodies outlined in the photographs on the left are shown at higher magnification in the right column (scale bar = 10 μm).

Number of Cell Bodies Immunoreactive For Orexin A or B in the Hindbrain of Mice
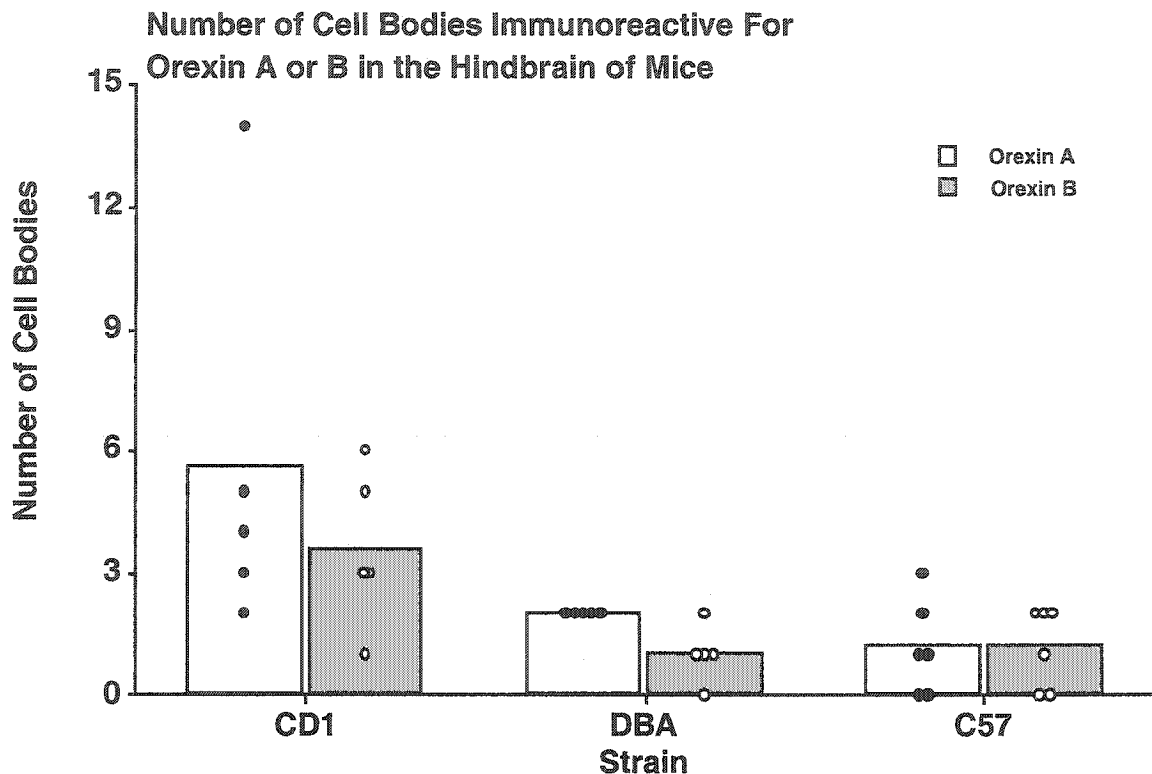
Figure 3.12. Numbers of cell bodies immunoreactivity for orexin A (open bars) and B (hatched bars) found in the hindbrain of mice of different strains. The strains are plotted along the abscissa. Average numbers of cell bodies for each strain are plotted along the ordinate. Dots represent individual data points.

within one brain section ranged from 0 to 6 (the brain section with 6 orexin A-IR cell bodies is shown in the top left panel of Figure 3.10). All animals had at least one cell body within the hindbrain stained for either orexin A or B. These numbers likely underestimate the actual number of perikarya containing orexin A or B within the hindbrain since only every fourth brain section was used for orexin A or B immunocytochemistry.

3.4 Discussion

All animals in this study showed a distinct population of orexin A-IR and orexin B-IR cells within the LH/perifornical region (Figure 3.2). In agreement with results described previously for various species [rat (Peyron et al., 1998; Elias et al., 1998; Broberger et al., 1998; Cutler et al., 1999; Horvath et al., 1999; Nambu et al., 1999; Date

et al., 1999; Wagner et al., 2000), mouse (Elias et al., 1998; Broberger et al., 1998; Wagner et al., 2000), hamster (Mintz et al., 2001; McGranaghan and Piggins, 2001), Nile grass rat (Novak and Albers, 2002), cat (Wagner et al., 2000; Zhang et al., 2001), sheep (Archer et al., 2002) and primate (Elias et al., 1998; Horvath et al., 1999; Moore et al., 2001)], this group of cells extended into the AH/RCH (Figure 3.3) and DMH (Figure 3.4). This study also found that orexin immunoreactivity occurred in cell bodies outside of the originally described LH/perifornical region. Perikarya within the PVN (Figures 3.6 and 3.7) and SON (Figure 3.8) contained immunostaining for orexin, as did perikarya outside of hypothalamus, specifically within hindbrain regions (Figures 3.10, 3.11 and 3.12).

Only minor strain differences in the distributions of cell bodies containing orexin A and B were found in the present study. CD1 mice tended to have slightly more orexin A-IR cell bodies within the DMH than C57 and DBA animals (Table 3.1; Figure 3.4). Within the RCH, DBA mice had the smallest number of orexin B-IR perikarya (Table 3.1; Figure 3.3). Significant strain differences among four mouse strains were found when total brain content of orexin A and orexin B was measured using an enzyme immunoassay technique (Lin et al., 2002). CD1 mice were not included in this study, but results showed that C57 mice tended to have lower brain orexin content than DBA mice (Lin et al., 2002). It is unclear whether these modest strain differences in both total brain content and orexin cell number are related in any way to strain differences in physiology and/or behavior.

Orexin A and B stem from a single precursor polypeptide, preproorexin (Sakurai et al., 1998); therefore, one would anticipate that these neuropeptides would invariably be colocalized, unless the two peptides were subsequently processed differently (see below). The majority of studies looking at the distribution of orexin within the brain have used antibodies recognizing only one of the two neuropeptides and/or the preproorexin peptide, on the assumption that the distribution of the two neuropeptides would be identical (Broberger et al., 1998; Elias et al., 1998; Peyron et al., 1998; Horvath et al., 1999; Van den Pol, 1999; Nambu et al., 1999; Wagner et al., 2000; Moore et al., 2001). Some studies using antibodies for both orexin A and B reported that their distributions

were indistinguishable (Date et al., 1999; Zhang et al., 2002), although a few reports describe differences in patterns of immunoreactivity between orexin A and B (Cutler et al., 1999; Date et al., 2000b; Sanchez de Miguel and Burrell, 2002; Ciriello et al., 2003; Caillol et al., 2003). Studies using radioimmunoassay procedures in microdissected brain regions found significant differences in total peptide content between orexin A and B (Mondal et al., 1999b). Differences in total brain content between orexin A and B were also reported in a study using enzyme immunoassay (Lin et al., 2002). We also observed several differences between orexin A and B immunostaining. For instance, the number of orexin A-IR cell bodies within the RCH, and to some extent within the DMH, tended to be higher than the number of orexin B-IR cell bodies (Table 3.1). In addition, we found differences in immunoreactivity between these two neuropeptides in the ME, PVN and SON (discussed below).

The internal lamina of the ME of animals of all three strains contained dark orexin A fiber staining that was absent in sections stained with orexin B antibody (Figure 3.9). This difference in fiber staining within the ME was not observed in a study on rats (Date et al., 2000b); however, a study using radioimmunoassay did find significantly higher orexin A content in the ME compared to orexin B (Mondal et al., 1999a). Further study is required to assess whether differences in staining intensity between orexin A and B within the ME in this study reflects differences in neuropeptide processing or transport. Interestingly, a report of a study also using rats but staining only for orexin A stated that orexin A-IR neurons were occasionally found within the ME (Chen et al., 1999).

We found orexin A-IR perikarya within the PVN and SON, whereas no orexin B-IR cell bodies were found in these two brain areas (Figures 3.6, 3.7 and 3.8). To our knowledge, orexin-IR somata have never been reported in these brain areas in mice. The intensity of staining within these cells was light; therefore, it is possible that if background staining is high, these cell bodies would not be easily discernible. This is not the first report of orexin-IR cell bodies occurring outside of the perifornical/LH area, however. A recent report described orexin B-containing perikarya in the bed nucleus of

the stria terminalis and the amygdala of the rat (Ciriello et al., 2003). Some studies have reported orexin-IR cell bodies in the periventricular area of the hypothalamus or within the subparaventricular zone (Broberger et al., 1998; Moore et al., 2001). In cats, for instance, the distribution of the LH orexin cells extends further medially (to the 3rd ventricle) and ventrally (to the arcuate nucleus) than has been described for rodents (Wagner et al., 2000). Another study done in cats found scattered orexin-IR perikarya in the PVN, the periventricular nucleus, the suprachiasmatic nucleus, and the infundibular nucleus (or arcuate nucleus) (Zhang et al., 2001). In humans, cell bodies are sometimes found within the periventricular zone (Moore et al., 2001). Immunopositive somata were found within the RCH in hamsters (Mintz et al., 2001). In the diurnal Nile grass rat, a small group of orexin B-IR perikarya were found within the AH and the RCH extending ventrally into the optic tract (Novak and Albers, 2002). In the chicken, orexin B-containing cell bodies are found in two hypothalamic structures; the periventricular hypothalamic nucleus and the LH (Ohkubo et al., 2002). One study, presented in abstract form, described a large population of orexin A-IR and orexin B-IR cell bodies along the edges of the 3rd ventricle (in the periventricular hypothalamic nucleus) and in the ME of turtles (Eiland et al., 2001). Orexin-IR somata are found exclusively within the ventral hypothalamic nucleus of the toad (Shibahara et al., 1999), and are found within the suprachiasmatic nucleus, the anterior preoptic area, the magnocellular preoptic nucleus and the ventral hypothalamic nucleus in the frog (Galas et al., 2001). These results show significant species differences in the distribution of orexin-containing cell bodies. It is possible that particular populations of orexin cells play roles in distinct physiological functions, and that species differences in physiology are reflected in differences in orexin cell distributions.

Orexin A-IR perikarya found within the PVN and the SON (e.g., Figure 3.5, bottom right panel) were smaller and less darkly stained than those found within the LH/perifornical region (e.g., Figure 3.5, top left panel). It is possible that these cells produce orexin A; however this seems unlikely for several reasons. First, orexin B was

not colocalized within these cell bodies, and as stated earlier, one would generally expect to find both neuropeptides together. Second, these cell populations have never been reported to contain preproorexin mRNA, although preproorexin mRNA has been found within ependymal cells lining all ventricles of the rat brain (Kummer et al., 2001). An alternative interpretation is that the orexin A immunoreactivity reflects orexin A bound to post-synaptic receptors on the surface of the somata. This could explain the light staining intensity found in these brain regions. If this is the case, however, then one would expect similar results in other brain regions receiving dense orexin projections, such as the PVT or LC. We did not find orexin-IR perikarya within any other brain region receiving dense orexin projections.

Finally, it is possible that these cells are selectively taking up orexin A from other sources. We know that orexin A and B are processed differently, and that orexin B is less stable than orexin A (Kastin and Akerstrom, 1999; Mondal et al., 1999b; Yoshida et al., 2003). Orexin A, but not B, readily crosses the blood-brain barrier from the periphery (Kastin and Akerstrom, 1999), and orexin B appears to be rapidly degraded within the cerebrospinal fluid (Yoshida et al., 2003). Cells within the PVN and SON may be taking up orexin A directly from terminal fibers originating in the LH/perifornical region, indirectly from the cerebrospinal fluid, or from an as yet unknown source. Further experiments are needed to investigate the origin of the orexin A found within the somata of these cells. For example, radio-labeled orexin A could be injected peripherally to assess whether cells within the PVN and SON selectively take-up peripheral orexin. Tracer studies coupled with double-label immunocytochemistry could be performed to assess whether these cells specifically receive projections from orexin-producing cells within the LH.

A small number of orexin A-IR and orexin B-IR perikarya were found in hindbrain regions of all mice in this study (Figures 3.10, 3.11 and 3.12). These cell bodies, and those found within the PVN and SON, were not found within control sections in which the primary antibodies were omitted, indicating that this immunoreactivity was specific for

orexin. The presence of orexin immunopositive perikarya in hindbrain regions was surprising since orexin is thought to be produced exclusively within cells of the lateral hypothalamus; however, orexin and/or its receptors have also been reported to be produced by cells of the olfactory epithelium, and in the periphery within the testes, the adrenal glands and the gut (Sakurai et al., 1998; López et al., 1999; Kirchgessner and Liu, 1999; Johren et al., 2001; Sanchez de Miguel and Burrell, 2002; Naslund et al., 2002; Caillol et al., 2003). One study reported that orexin-IR somata were rarely observed in brain areas outside of the hypothalamus, but the authors did not further localize them (Chen et al., 1999). To our knowledge, these hindbrain cell bodies have never before been described in any species, perhaps because the small number of cells labeled have previously been over-looked. It is also unlikely that they would be detected with conventional *in situ* hybridization methods. It is unknown what the function of these hindbrain orexin cells might be, and whether or not they form a network distinct from the hypothalamic orexin system. Because of their small number and scattered distribution, it may be difficult to assess their function directly.

In summary, the results of this study show that there are small differences in orexin immunoreactivity among three strains of mice. More importantly, we report substantial differences in distribution between orexin A- and orexin B-containing cell bodies. In addition, we show the presence of cell bodies outside of the hypothalamus that contain immunoreactivity for both orexin A and B. The functions of these distinct cell populations, and of the orexin they contain, remain to be determined.

CHAPTER 4

The Response of the Lateral Hypothalamic Orexin System of Mice During Food Anticipation and Entrainment

4.1 Introduction

Light is the best-studied environmental cue involved in synchronizing (entraining) endogenously generated circadian rhythms (Mistlberger and Rusak, 2000). Its effects are mediated by known components of the mammalian circadian system (e.g., the retina, retinohypothalamic tract, suprachiasmatic nucleus (SCN), and intergeniculate leaflet) (Morin, 1994). Many studies have shown, however, that restricted daily access to food can also act as an entraining agent (Mistlberger, 1994; Stephan, 2001; Stephan, 2002). Repeated cycles of restricted daily access to food trigger arousal and increased activity in anticipation of the daily feeding time, which is referred to as food-anticipatory activity, or FAA. Because there is no environmental signal indicating that food will soon be available in the standard restricted feeding paradigm (in the absence of any external cycles indicating time of day), the onset of activity 1-3 h before feeding must be generated by an endogenous mechanism. From a host of studies conducted by a number of labs, we know this mechanism is a self-sustaining circadian pacemaker (Mistlberger, 1994; Stephan, 2001; Stephan, 2002). The identity of this food-entrainable pacemaker and the nature of the afferent signals that affect it are not known, despite a large number of studies aimed at identifying the critical substrate and signal involved. What is known, however, is that restricted daily access to food entrains a circadian pacemaker that is both anatomically and functionally separate from the light-entrainable pacemaker in the rodent SCN (Mistlberger, 1994; Stephan, 2001; Stephan, 2002).

The function of food entrainment and/or anticipation is not yet fully understood; however, the intense activity associated with food anticipation is probably mediated by mechanisms that normally awaken and arouse animals in order to increase the probability of finding and eating food. At the same time, anticipation probably helps prepare the

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digestive system for the meal. The system responsible for anticipation of feeding times, therefore, likely involves mechanisms that integrate the responses of a variety of brain regions with functions related to sleep/wake, arousal and eating.

In 1998, two novel neuropeptides were discovered and described independently by two research groups (Sakurai et al., 1998; De Lecea et al., 1998). Although the pair of neuropeptides (which stem from a single precursor) were given different names by the two groups, [orexin A and B (Sakurai et al., 1998) or hypocretin 1 and 2 (De Lecea et al., 1998), respectively] they turned out to be identical peptides. Orexin A and B were originally thought to play a role in the regulation of feeding because central orexin injections increased food consumption in both fasted and free-feeding animals (Sakurai et al., 1998; Dube et al., 1999; Sweet et al., 1999; Edwards et al., 1999; Ida et al., 1999; Dube et al., 2000; Yamanaka et al., 2000; Jain et al., 2000). Endogenous levels of orexin were also up-regulated following fasting (Sakurai et al., 1998; Cai et al., 1999; López et al., 2000). In addition, orexin-producing cells in the brain seem to be present only in the lateral hypothalamus (LH) and surrounding regions [(Peyron et al., 1998; Sakurai et al., 1998; De Lecea et al., 1998); but see results described in Chapter 3]. They receive inputs from, and interact with, cells containing other peptides (e.g., leptin, ghrelin, neuropeptide Y, melanin-concentrating hormone, and agouti-related peptide) that play key roles in the regulation of feeding (Broberger et al., 1998; Elias et al., 1998; Horvath et al., 1999; Funahashi et al., 2000; Guan et al., 2001; Yamanaka et al., 2003). Furthermore, the LH has historically been known as a feeding center (Kalra et al., 1999). LH lesions result in hypophagia and decreased bodyweight, whereas stimulation of the LH results in increased food consumption (Devenport and Balagura, 1971; Berger et al., 1971; Zigmond and Stricker, 1973; Ball, 1974; Kalra et al., 1999). Multiple-unit recordings showed increased electrical activity in the LH that anticipated mealtime in rats on a restricted feeding schedule (Kurumiya and Kawamura, 1991), suggesting a possible role in mediating FAA; however, another study showed that LH-ablated animals were still able to anticipate daily feeding opportunities (Mistlberger and Rusak, 1988).

Recent work has shown that orexin plays a significant role in the regulation of sleep (Kilduff and Peyron, 2000; Chicurel, 2000; Hungs and Mignot, 2001; Scammell, 2003). Orexin knockout mice show signs of cataplexy (Chemelli et al., 1999; Hara et al., 2001a), and narcoleptic dogs were found to have a mutation in orexin receptor 2 (Lin et al., 1999). Similarly, a large number of narcoleptic human patients have virtually undetectable levels of orexin A in their cerebrospinal fluid and show a reduction in the number of orexin cells within the LH (Thannickal et al., 2000; Peyron et al., 2000; Nishino et al., 2000). Other studies suggest that the increase in feeding after central injections of orexin may be partially explained by a general increase in arousal and activity (Yamanaka et al., 1999; Ida et al., 1999; Sunter et al., 2001). It may be that the orexin system is related more to behavioral activation and arousal than to the specific regulation of either sleep or feeding (Wu et al., 2002; Torterolo et al., 2003).

Orexin-containing cells in the LH project to many targets in the brain, including some implicated in the regulation of circadian rhythms, arousal and food intake (Peyron et al., 1998; Cutler et al., 1999; Nambu et al., 1999; Date et al., 1999). The LH orexin cells are, therefore, well-positioned to serve as a mechanism mediating food anticipation by linking feeding centers with those involved in behavioral activation. It is possible that the orexin system functions in the signaling pathway that establishes or maintains food entrainment and anticipation. Pilot data from this lab presented in abstract form indicated that the levels of orexin peptide in the LH, measured by immunocytochemistry, did not appear to change following feeding manipulations (de Groot et al., 2000a; de Groot et al., 2000b). This is likely because the constitutive levels of the peptide are already high in these cells and any changes would likely not be detected using standard immunocytochemical techniques. Orexin peptide levels also did not change following a period of acute food deprivation as measured by radioimmunoassay (Mondal et al., 1999b). Levels of the immediate-early gene c-Fos in the LH, however, were found to differ among various feeding conditions in pilot studies (de Groot et al., 2000a; de Groot et al., 2000b).

Because the orexin system could potentially play a role in food anticipation and entrainment, we investigated whether the orexin-containing cells within the LH respond to temporally restricted food access. Levels of the immediate-early gene products c-Fos and Jun-B in the LH were compared among groups of mice exposed to three conditions: entrainment to cycles of restricted food availability, acute food deprivation and *ad libitum* feeding. C-Fos expression within orexin-immunoreactive (IR) cells and preproorexin mRNA levels were also compared among animals in these different feeding conditions. The results indicate that the activity of orexin-containing cells in the LH is altered by manipulations of food availability.

4.2 Methods

4.2.1 Animals and Housing

Sixty-five adult male C57BL/6J mice obtained from Charles River Canada (St. Constant, Québec, Canada) were used in the experiments described in this chapter. Housing and data recording procedures for 53 of these animals were identical to those of animals housed on open shelves described in Chapter 2. The twelve remaining mice were housed individually (n=8) or in a group of four in standard mouse bin cages (27.5 cm X 16.5 cm X 12.5 cm) placed inside isolation cabinets (52 cm X 38 cm X 48 cm). All experiments were conducted according to Canadian Council on Animal Care guidelines and were approved by the Dalhousie University Committee on Laboratory Animals.

4.2.2 Procedures

The 53 animals housed in wheels were assigned randomly to one of two feeding conditions after a minimum of six days of acclimatization to the running wheel and housing conditions, once stable entrainment to the light-dark (LD) cycle was attained. Twenty-seven animals remained on *ad libitum* feeding (*Ad Libitum*; Figure 4.1A, B and C). For the other 26 animals, all food was removed from the cage tops at the time the lights came on (designated as zeitgeber time 0 [ZT0] by convention), and a restricted feeding paradigm identical to that described in Chapter 2 was initiated (Restricted; Figure

Figure 4.1. Actograms illustrating the experimental procedures for a representative mouse in each of the five conditions: *Ad Libitum* (A); Fasted-NotFed (B); Fasted-Fed (C); Restricted-NotFed (D) and Restricted-Fed (E). Horizontal lines represent 24 h, as shown at the top of the graph. Days are plotted sequentially on the vertical axis. Vertical deflections on the horizontal lines represent microswitch closures corresponding to wheel revolutions occurring in each 5 min time bin, with larger deflections representing higher numbers of wheel revolutions. Animals were housed under an LD 12:12 cycle represented by the bar at the top of the graph (white = light phase, black = dark phase). Downward–pointing triangles indicate the time at which all food was removed from the cage tops. Shaded bars correspond to times of food availability during the restricted feeding schedule. Upward pointing arrows indicate the time of refeeding. Circles indicate the time at which the animal was killed. The } indicates the interval used for further analyses (see Results section).
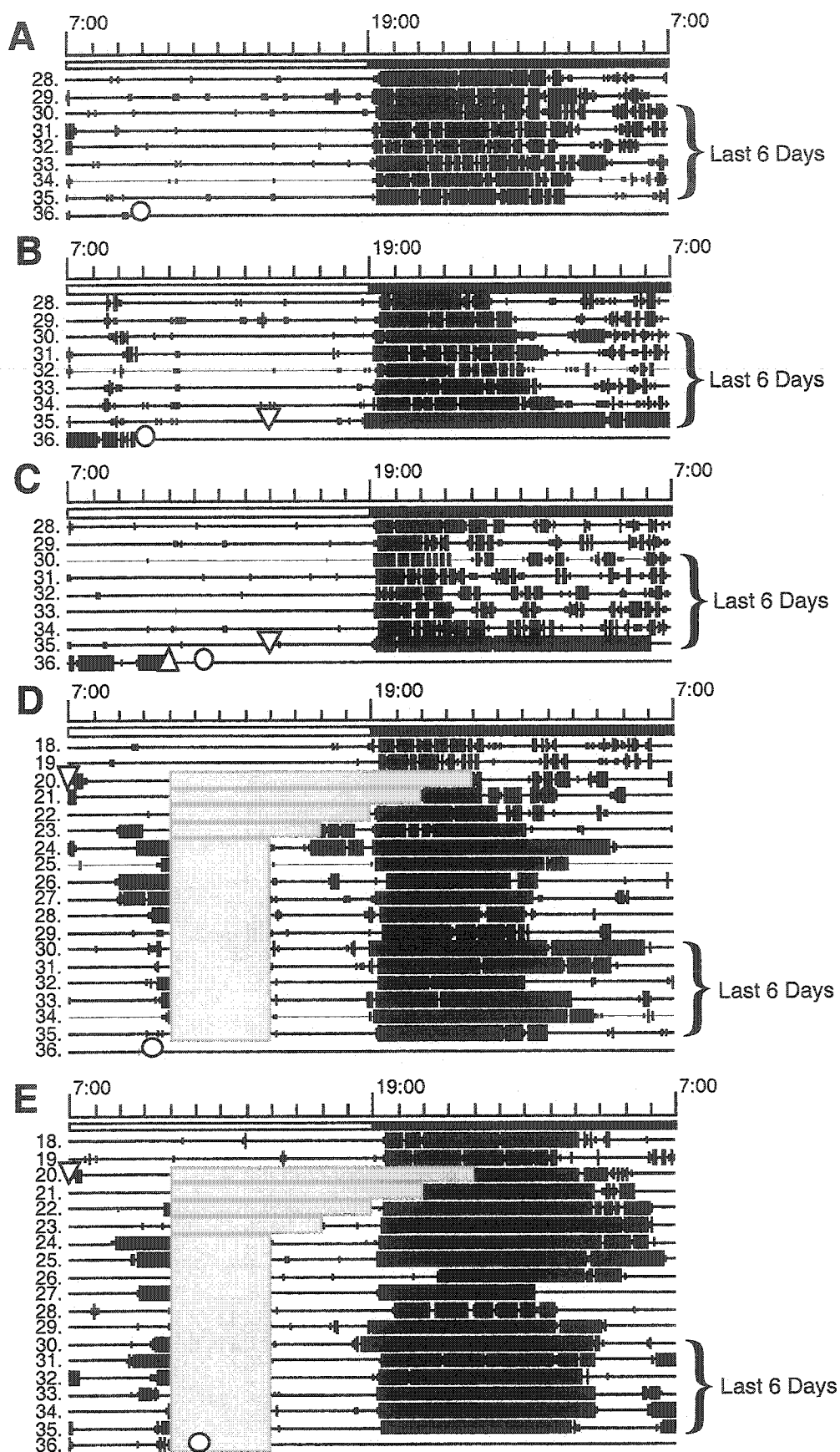
Figure 4.1

4.1D and E).

On the 17th day of restricted feeding, the 26 animals were divided into two groups: Restricted-NotFed (RN) or Restricted-Fed (RF). Half of these animal were killed (see below) approximately 30 min prior to normal mealtime (at ZT3.5; RN; Figure 4.1D) and half were given their normal meal at ZT4 and were killed approximately 1 h later at ZT5 (RF; Figure 4.1E). The 27 animals that had been fed *ad libitum* were divided randomly into three groups: *Ad Libitum* (AL), Fasted-NotFed (FN) or Fasted-Fed (FF). For 18 of these animals, all food was removed from their cages at ZT8 (FN and FF; Figure 4.1B and C: downward pointing arrow on Day 35). On the following day, nine of these animals were killed at ~ZT3.5 (FN; Figure 4.1B). Nine additional *ad libitum* fed animals were killed at ~ZT3.5 (AL; Figure 4.1A). At ZT4, a specimen bottle containing approximately 15.5 g of powdered lab chow and sugar moistened with vegetable oil was placed on the cage floor of the remaining nine animals (FF; Figure 4.1C: upward pointing arrow on Day 36). These animals were then killed approximately 1 h later at ZT5.

The twelve animals housed in standard bin cages were allowed to adapt to the LD cycle for eleven days. On the 12th day at ZT8, food was removed from the cage tops of eight animals that were housed individually (FN and FF). On the following day, four of these animals were killed at ~ZT3.5 (FN). The four group-housed animals were killed at ~ZT3.5 without having their food disturbed (AL). The remaining four animals were given a specimen bottle containing approximately 15.5 g of powdered lab chow mixed with sugar and moistened with vegetable oil at ZT4 (FF). These animals were killed approximately 1 h later at ZT5.

Forty-five animals were killed at the times described above using a barbiturate overdose (0.5 cc of 65 mg/ml Sodium pentobarbitol, I.P.). These animals were perfused and brains were extracted and processed in preparation for immunocytochemistry as described in Chapter 3. After 20 animals were killed (n=4 per feeding condition), their stomachs and their contents were removed, weighed and photographed. Average stomach/content weights for each feeding condition were calculated and compared

statistically.

The remaining 20 animals (8 housed in running-wheel cages and 12 housed in bin cages) were killed by decapitation. Brains were dissected out and were frozen rapidly on dry ice. They were stored at $-80°$ C until they were sectioned coronally (14 μm thick) from the start of the anterior commissure to the end of the LH using a cryostat. The level of these anatomical landmarks was determined using a mouse brain atlas (Franklin and Paxinos, 1997). Sections were mounted on glass specimen slides which were stored at $-80°$ C. Every sixth section was processed for detection of preproorexin mRNA expression using *in situ* hybridization (see below).

4.2.3 Immunocytochemistry

Brain sections of 25 animals were processed for immunocytochemical detection of the immediate-early gene products c-Fos and Jun-B in the LH (n=5 per feeding condition). Every fourth section was reacted to detect c-Fos and adjacent sections were reacted for Jun-B. The standard avidin-biotin technique for immunocytochemistry described in Chapter 3 was used. Both primary antibodies were raised in rabbit (Jun-B, generously donated by Dr. Rodrigo Bravo; C-Fos, Ab-5, Oncogene Research Products, USA), and were used at a dilution of 1:20,000 in phosphate-buffered saline with Triton-X 100 (PBS-X) containing 3% normal goat serum. The secondary antibody used for both reactions was biotinylated anti-rabbit IgG secondary antibody raised in goat (1:200; Vector Labs), which was also diluted in PBS-X containing 3% normal goat serum. For Jun-B, nickel chloride (0.03%) was added to the diaminobenzidine (DAB) solution to enhance the visibility of the IR product. Following immunocytochemical reactions, the sections were mounted on slides and were prepared for analyses as described in Chapter 3. Sections were inspected using a microscope (Olympus BH-2, Olympus Corporation, N.Y., USA) and immunoreactivity for c-Fos and Jun-B was assessed and quantified as described below.

Brain sections of 20 animals were used to assess colocalization of c-Fos immunoreactivity with orexin A immunoreactivity (n=4 per feeding condition). Every

fourth section was processed for immunocytochemistry as described above, with the following modifications. Sections were preincubated in 3% normal donkey serum (Sigma Aldrich, Canada) diluted in PBS-X at 4° C for 1 h followed by a 48 h incubation at 4° C in primary antibody against c-Fos raised in rabbit (Ab-5, Oncogene). The primary antibody was diluted to 1:20,000 in PBS-X containing 3% normal donkey serum. Sections were then incubated for 1 h in a PBS-X solution containing 3% normal donkey serum and biotinylated anti-rabbit IgG secondary antibody raised in donkey (1:200; Jackson ImmunoResearch Laboratories Inc., USA). The tissue was developed as described above for Jun-B (i.e., with the addition of 0.03% nickel chloride). Sections were then incubated for 48 h at 4° C in affinity-purified goat polyclonal primary antibody raised against the carboxy terminus of orexin A peptide (sc-8070; Santa Cruz) diluted to 1:50,000 in PBS-X containing 3% normal donkey serum. Tissue was then incubated at 4° C for 1 h in a PBS-X solution containing 3% normal donkey serum and biotinylated anti-goat IgG secondary antibody raised in donkey (1:200; Jackson ImmunoResearch Laboratories). The reaction was completed as described above for c-Fos (i.e., without the addition of nickel chloride).

4.2.4 *In situ* Hybridization

Brains of 20 animals (n=4 per feeding condition) killed by decapitation were used to detect the presence of preproorexin mRNA by *in situ* hybridization. Synthetic oligonucleotide probes were obtained from Sigma Genosys (Ontario, Canada). The preproorexin antisense oligonucleotide (5' CGT CTT TAT TGC CAT TTA CCA AGA GAC TGA CAG 3') was complementary to nucleotides 533-565 (5' CTG TCA GTC TCT TGG TAA ATG GCA ATA AAG ACG 3') of *Mus musculus* preproorexin mRNA (Genbank Accession Number AF041242). The oligonucleotide probe (10 μM) was radio-labeled at the 3' end with [α-^{33}P]dATP (2000 Ci/mmol; Mandel Scientific) using reagents from the 3' end-labeling kit (Amersham Pharmacia Biotech, USA) and terminal deoxynucleotidyl transferase (Promega Corp., USA). The probe was centrifuged through a Sephadex G-25 spin column (Pharmacia) to remove unincorporated radionucleotides.

Specimen slides were allowed to thaw at room temperature and the tissue was fixed for 5 min in phosphate-buffered saline (PBS) containing 4% paraformaldehyde. Specimen slides were then rinsed twice in PBS for 3 min followed by one rinse in 2 x sodium chloride - sodium citrate (2xSSC; pH 7.0) for 20 min. They were then dried at room temperature for at least 1 h, after which labeled oligonucleotide probe in hybridization buffer (200 µl) was added to each specimen slide. The hybridization buffer contained 50% formamide, 5xSSC, 10% dextran sulfate, 1xDenhardt's reagent, 20mM sodium phosphate (6.8 pH), 0.2% SDS, 5mM EDTA, 10 mg/ml poly A, 50 mg/ml sheared salmon sperm DNA, and 50 mg/ml yeast RNA. The slides were covered with parafilm and were placed inside a 40° C hybridization incubator overnight. The next day, the parafilm covers were removed from each slide with 1xSSC. The slides were washed 4 times in 1xSSC for 15 min each at 56° C, 4 times for 15 min in 0.5xSSC at 56° C and twice for 30 min in 0.25xSSC at room temperature. The slides were rinsed briefly in double distilled water and were left out at room temperature to dry overnight. The sections were exposed to Kodak Hypermax film for three consecutive periods (first for 7 days, then 5 days, and then 3 days) at room temperature. The film from the five-day exposure was chosen for further analysis (see below). After exposure to film, the brain sections were counterstained for Nissl substance using cresyl violet, dehydrated in serial dilutions of ethanol, cleared in Histoclear and coverslipped using Entellan.

4.2.5 Analyses

Behavior

Wheel-running activity was recorded continuously for 53 animals in five feeding conditions (AL: n=9, Figure 4.1A; FN: n=9, Figure 4.1B; FF: n=9, Figure 4.1C; RN: n=13, Figure 4.1D; RF: n=13, Figure 4.1E). Data were plotted as actograms using Circadia and these were inspected visually to assess activity patterns. Wheel-running activity recorded during the last six days of the restricted feeding schedule, or during the corresponding six days for those animals not on restricted feeding, was analyzed further by plotting the data as average waveforms using Circadia. Data from all mice with *ad*

libitum food access were combined into an average waveform for this interval (including those in the AL, FN and FF conditions; n=27; Figures 4.1A, B and C). [Note that during the interval analyzed, animals in the acutely fasted conditions (FN and FF) had not yet been exposed to this treatment and had been treated identically to the AL animals.] Similarly, the data from all mice that had been on restricted daily food access were combined into another average waveform (including those in the RN and RF conditions; n=26; Figures 4.1D and E).

For statistical comparisons among feeding conditions, average numbers of wheel revolutions recorded during specific time bins were calculated using Circadia. Average amount of activity recorded over the 24 h day during the last six days of restricted feeding was compared statistically between the two groups of mice (*ad libitum* or restricted). For more detailed comparisons of nocturnal activity, wheel running recorded during the dark phase was divided into four 3 h time bins (ZT12-15, ZT15-18, ZT18-21 and ZT21-0). To analyze the pattern and extent of any FAA shown, comparisons were made of the amount of activity recorded during each of two 2 h time bins beginning at the times of lights on and extending until feeding time (ZT0-2 and ZT2-4).

A similar analysis was performed of activity recorded on the last experimental day. For this analysis, animals were divided into three groups: *Ad libitum* (AL: n=9; Figure 4.1A), Fasted (including FN and FF: n=18; Figures 4.1B and C) and Restricted (including RN and RF: n=26; Figures 4.1D and E). An average waveform for each of these conditions was generated for data recorded from ZT8 to ZT8. For statistical comparisons, average numbers of wheel revolutions recorded on the last experimental day during the specific time bins described above were calculated using Circadia. Throughout the text, all activity levels are reported as mean number of wheel revolutions/5 min time bin (± SEM).

Immediate-early gene expression in the LH

The distribution and density of c-Fos- and Jun-B-IR cell nuclei in the LH were analyzed visually using a microscope (Olympus BH-2, Olympus Corporation, N.Y., USA). Following initial visual inspection, one section containing the LH with the highest

number of positively stained nuclei for each animal was chosen for further analysis and quantification. This section corresponded roughly to Figures 43 or 44 (approximately 1.46 mm and 1.58 mm from bregma) in the mouse brain atlas (Franklin and Paxinos, 1997). Two pictures, each containing one unilateral LH, were captured using a COHU High Performance CCD camera mounted on the microscope, and IR cell nuclei were counted using ScionImage (Version 1.62c, National Institutes of Health, USA; modified by Scion Corporation). LH sections stained for c-Fos or Jun-B for each animal were counted bilaterally and counts were summed and averaged for each feeding condition. An investigator blind to experimental condition performed all of the quantification.

c-Fos expression in orexin-containing cells

For each animal, two representative sections containing the LH were chosen for quantification and analysis, as described above. Pictures were taken with an Olympus Camedia digital camera (Olympus America Inc., USA) mounted on an Olympus BH-2 microscope. The LH on each side of the two sections was photographed to yield four photographs of the LH for each of the 20 animals. Pictures were analyzed using ScionImage as described above. Three independent investigators, blind to experimental condition, counted all cell bodies containing orexin A in each picture. c-Fos-IR cell nuclei identified within cell bodies containing orexin A immunoreactivity were also counted. A Pearson product-moment correlation coefficient among the three counts for total orexin A cell bodies was calculated as $r = 0.874$ and among the three counts for total double-labeled cells was calculated as $r = 0.911$. The average was calculated of the three counts of orexin-IR cells, and of the three counts of c-Fos-IR orexin cells for each LH section. The percentage of orexin A-IR cells containing c-Fos immunoreactivity in each of the four representative LH sections for each animal was calculated. These values were then grouped according to experimental condition and were compared statistically as described below.

Preproorexin mRNA expression

For the analysis of preproorexin mRNA expression, two representative sections

containing the LH were chosen as described above for each animal (n=20) using the cresyl violet counterstained sections. The film containing the chosen sections was photographed using an Olympus Camedia digital camera and analyzed using ScionImage, as described above. A square outline was drawn that encompassed the entire unilateral LH. This template was centered over the LH to determine optical density values for each unilateral LH, resulting in four optical density values per animal. An additional reading of optical density using the same template was taken for each picture in an area just dorsal to the third ventricle, with the template centered at midline. This reading of background optical density for each section was subtracted from the two values of optical density taken from the bilateral LH to get a relative optical density reading for each unilateral LH section. An investigator blind to experimental conditions obtained all optical density readings. The four relative optical density values for each animal were averaged and data were grouped by experimental condition and analyzed statistically.

Statistical analyses

All statistical comparisons were made by one-way ANOVA using StatView (SAS Institute Inc., USA) followed by the Student-Newman-Keuls post-hoc tests. Group differences with $P < 0.05$ were considered statistically significant. Group means are reported with SEM.

4.3 Results

4.3.1 Wheel-Running Activity

Visual inspection of the actograms recorded for the 26 animals exposed to the restricted feeding schedule indicated that all animals anticipated the arrival of their daily meal with increased activity (see Figures 4.1D and E). There was, however, day-to-day variability in the amount and the duration of activity occurring before mealtime for all of the animals recorded. None of the mice showed increased wheel running (or FAA) prior to mealtime on all twelve days when food was available for only 4 h. Twenty-five of the 26 mice anticipated mealtime on more than half of these twelve days, with the majority

anticipating ten or eleven meal times. The remaining mouse showed FAA on only five days.

The average waveforms of data recorded during the last 6 days of the restricted feeding schedule clearly show that animals exposed to this schedule on average increased their wheel-running activity level before mealtime, as well as during the dark phase (Figure 4.2). Average daily wheel-running levels recorded over the last 6 days were significantly higher for animals that were meal fed (n=26; 65.4 ± 3.92) compared to levels for animals fed *ad libitum* (n=27; 36.6 ± 2.42, $F_{(1,51)}$ = 39.6, P < 0.0001).

As is shown in Figure 4.3, animals exposed to restricted feeding ran significantly more than animals fed *ad libitum* during the first three 3 h time bins of the dark phase (ZT12-15, 148.9 ± 8.19 and 101.0 ± 6.12, respectively, $F_{(1,51)}$ = 22.2, P < 0.0001; ZT15-

Average Activity Levels For the Last Six Days of the 4 h Restricted Feeding Schedule
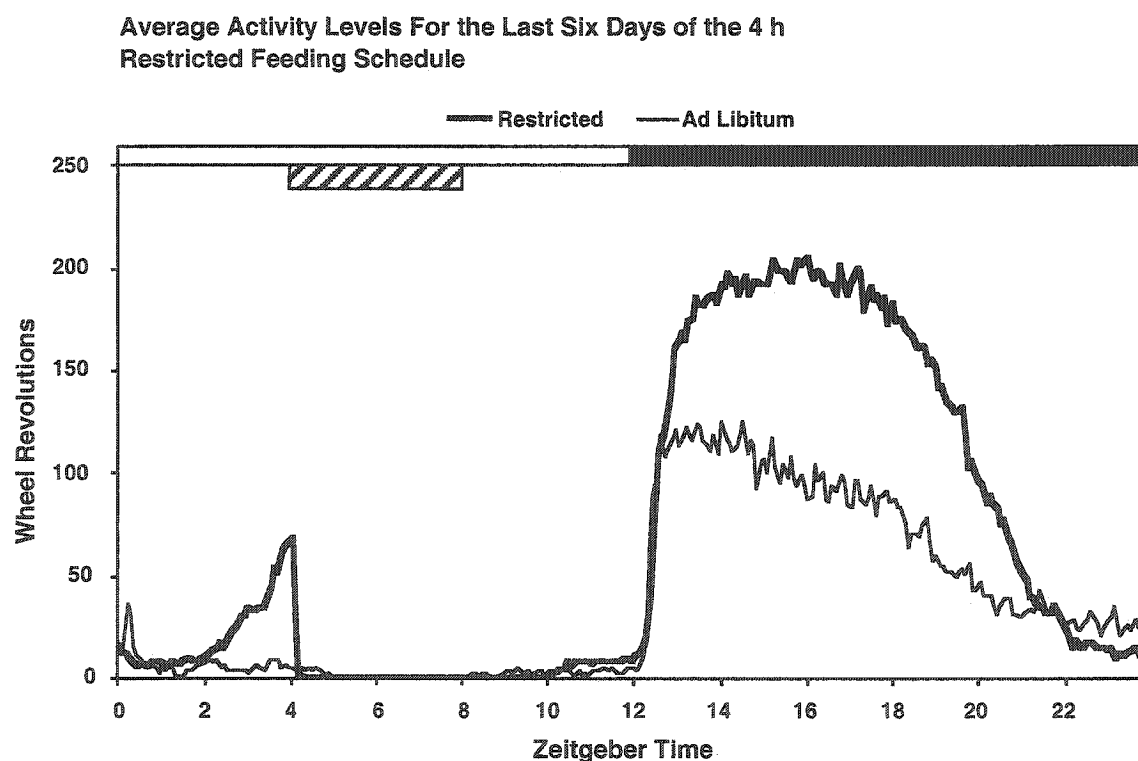
Figure 4.2. Average waveforms for the last six days of recording for animals exposed to the restricted feeding schedule (thick line; n=26) or fed *ad libitum* (thin line; n=27). Zeitgeber time is plotted on the abscissa and average activity levels (in wheel revolutions/5 min time bin) are plotted on the ordinate. The LD 12:12 cycle is shown in the bar across the top (white=light phase, black=dark phase) and time of food availability is indicated by the hatched bar.

18, 193.4 ± 12.10 and 94.3 ± 7.27, respectively, $F_{(1,51)}$ = 50.0, P < 0.0001; ZT18-21,

123.4 ± 13.44 and 53.7 ± 5.52, respectively, $F_{(1,51)}$ = 23.6, P < 0.0001). In addition,

activity levels recorded during the 2 h time bin immediately preceding mealtime were

significantly higher for animals habitually fed meals at that time than for those fed *ad*

libitum (Figure 4.4; 32.8 ± 7.06 and 5.8 ± 2.28, respectively, $F_{(1,51)}$ = 13.6, P = 0.0005).

Activity levels did not differ between groups for the last 3 h of the dark phase (Figure

4.3), or the first 2 h of the light phase (Figure 4.4).
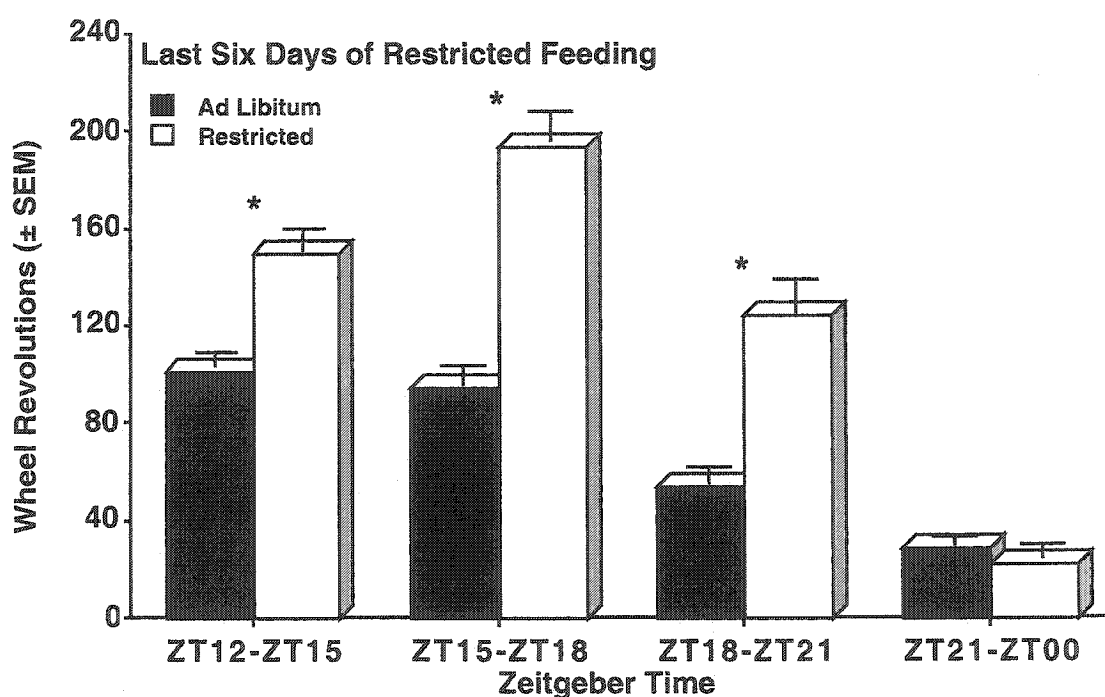
Figure 4.3. Nocturnal activity levels recorded during the last six days of the restricted feeding schedule for animals fed *ad libitum* (black bars; n=27) or on restricted feeding (open bars; n=26). Time bins corresponding to zeitgeber times are plotted along the abscissa and average numbers of wheel revolutions/5 min time bin (± SEM) are plotted along the ordinate. The * indicates a significant difference between groups (P < 0.05).
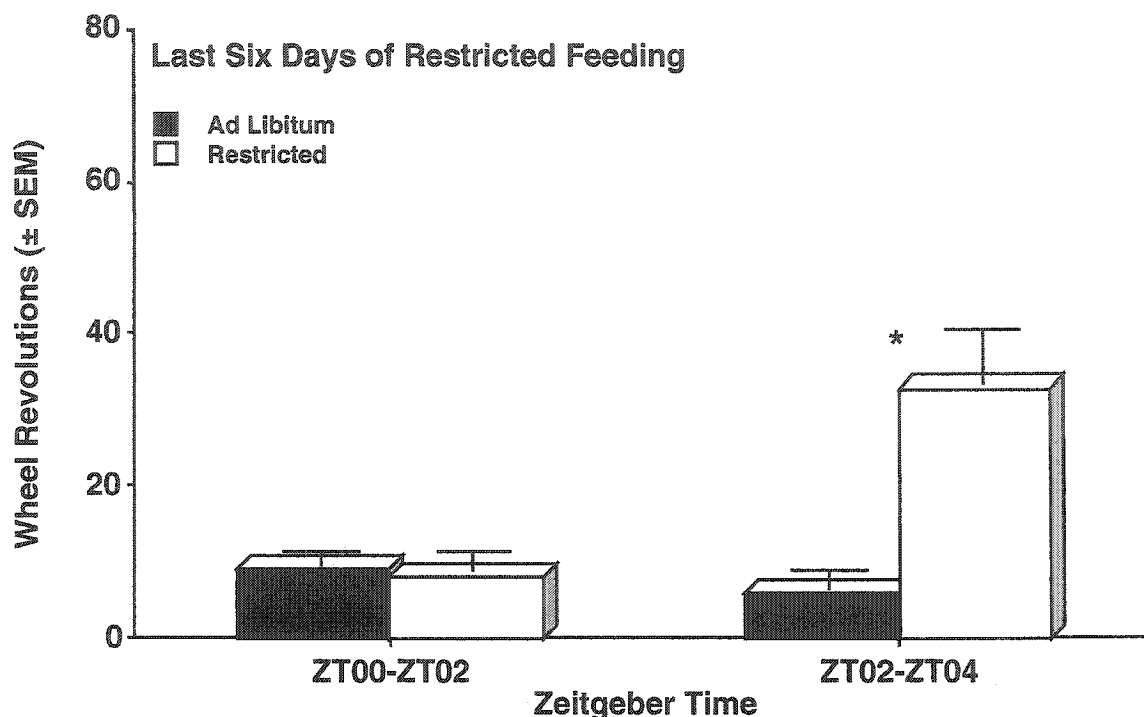
Figure 4.4. Activity levels recorded during ZT0-2 and ZT2-4 during the last six days of the restricted feeding schedule for animals fed *ad libitum* (black bars; n=27) or on restricted feeding (open bars; n=26). Time bins corresponding to zeitgeber times are plotted along the abscissa and average numbers of wheel revolutions/5 min time bin (± SEM) are plotted along the ordinate. The * indicates a significant difference between groups (P < 0.05).

Figure 4.5 shows the average waveform of activity recorded on the last day of the experiment (from ZT8-8) for animals entrained to restricted feeding (thick line), acutely fasted (shaded line) and fed *ad libitum* (thin line). Nocturnal activity levels were highest for animals on restricted feeding, but activity levels during the first 4 h of the light phase were highest for acutely fasted animals (Figure 4.5). Statistical analyses revealed a significant difference in total activity level among groups ($F_{(2,50)}$ = 8.5, P = 0.0007). Average numbers of wheel-revolutions over the last experimental day for animals that were acutely fasted (n=18, 75.5 ± 6.55) and animals entrained to restricted feeding (n=26, 60.7 ± 4.63) were significantly higher than for animals fed *ad libitum* (n=9, 35.8 ± 3.43; P < 0.05).

Average Activity Levels For the Last Experimental Day
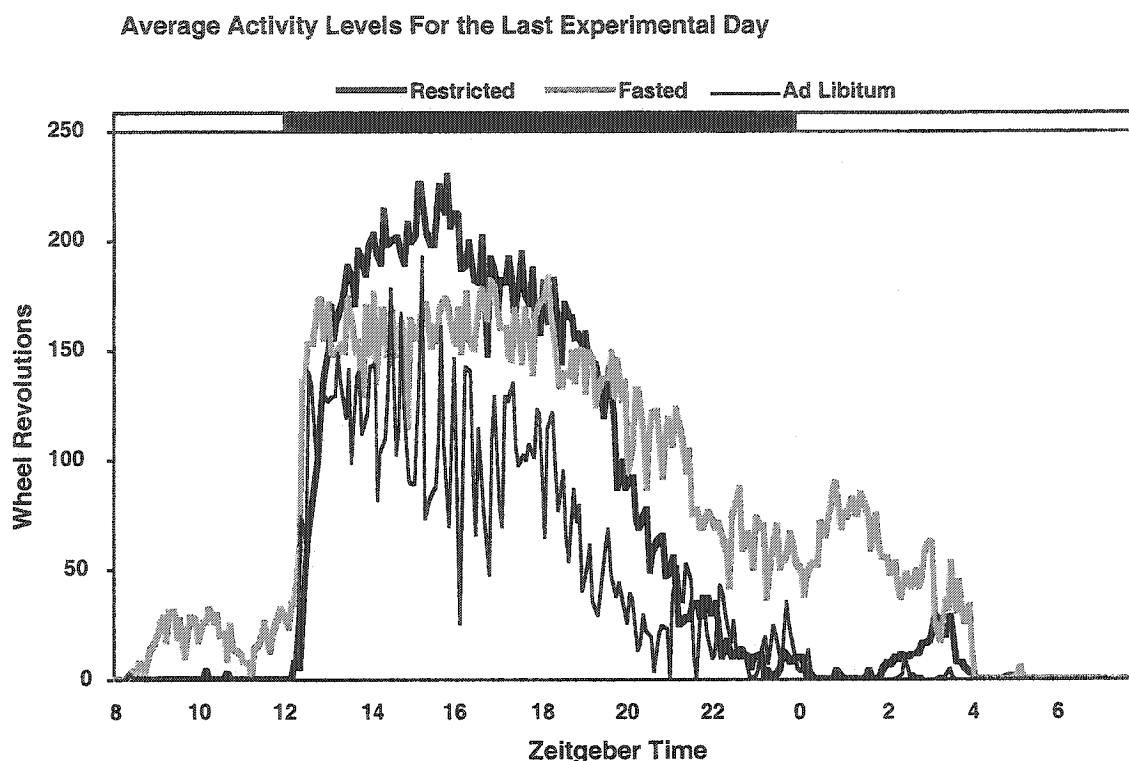
Figure 4.5. Average waveforms for the last day of recording for animals exposed to the restricted feeding schedule (thick line; n=26), acutely fasted (shaded line; n=18) or fed *ad libitum* (thin line; n=9). Zeitgeber time is plotted on the abscissa and average activity levels (in wheel revolutions/5 min time bin) are plotted on the ordinate. The LD 12:12 cycle is shown in the bar across the top (white=light phase, black=dark phase).

Figure 4.6 shows the nocturnal activity levels for each of the three groups on the last day of the experiment. Statistically significant differences in activity levels among feeding conditions were found during ZT15-18 ($F_{(2,50)}$ = 7.0, P = 0.0021), ZT18-21 ($F_{(2,50)}$ = 4.0, P = 0.0238) and ZT21-0 ($F_{(2,50)}$ = 7.9, P = 0.0010). Both acutely fasted and restricted animals expressed significantly higher levels of activity than *ad libitum* fed animals during ZT15-18 (fasted: 161.7 ± 11.92, restricted: 192.0 ± 14.21, *ad libitum*: 105.3 ± 10.82; P < 0.05) and ZT18-21 (fasted: 133.0 ± 12.02, restricted: 118.4 ± 18.34, *ad libitum*: 48.8 ± 14.76; P < 0.05). Acutely fasted animals ran significantly more than animals in the other two conditions during ZT21-0 (Figure 4.6; fasted: 72.0 ± 13.83, restricted: 18.1 ± 8.33, *ad libitum*: 21.5 ± 5.72; P < 0.05).
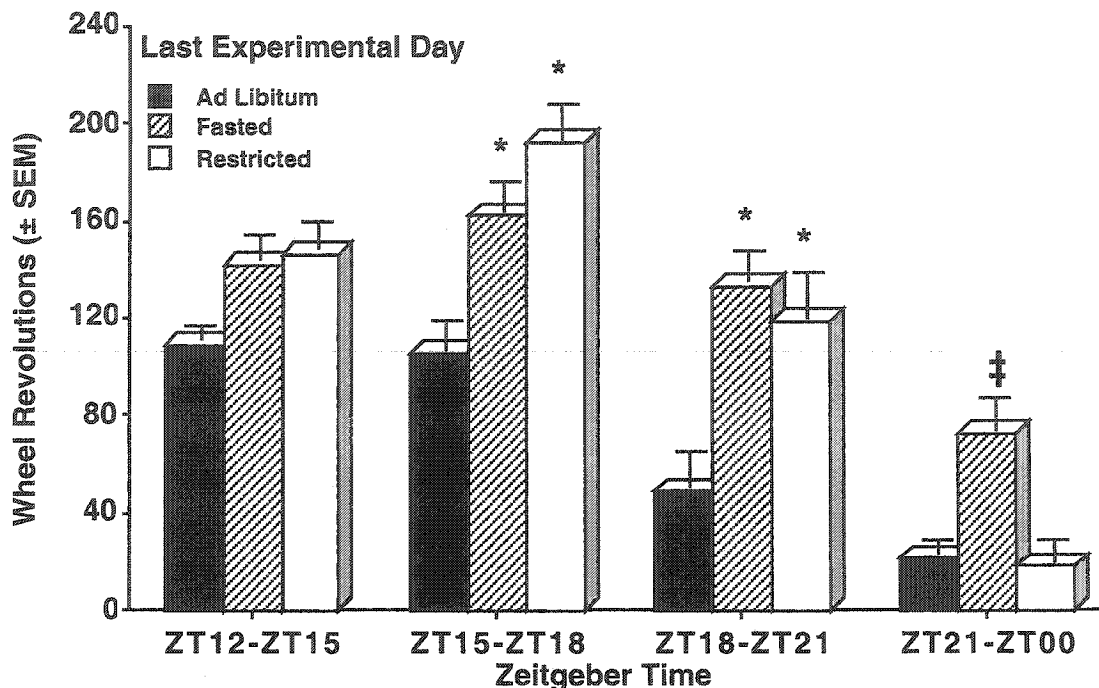
Figure 4.6. Nocturnal activity levels recorded on the last day of the experiment for animals fed *ad libitum* (black bars; n=9), acutely fasted (hatched bars; n=18) or on restricted feeding (open bars; n=26). Time bins corresponding to zeitgeber times are plotted along the abscissa and average numbers of wheel revolutions/5 min time bin (± SEM) are plotted along the ordinate. The * indicates a significant difference from the *ad libitum* condition (P < 0.05). The ‡ indicates a significant difference from the *ad libitum* and restricted conditions (P < 0.05).

Activity levels recorded during the beginning of the light phase on the last experimental day are shown in Figure 4.7. Statistical analyses revealed a significant difference in activity level among feeding conditions during ZT0-2 ($F_{(2,50)}$ = 16.2, P < 0.0001) and ZT2-4 ($F_{(2,50)}$ = 4.0, P = 0.0246). Acutely fasted animals ran significantly more than animals in the other two conditions during ZT0-2 (Figure 4.7; fasted: 67.3 ± 16.14, restricted: 1.4 ± 0.56, *ad libitum*: 1.2 ± 0.41; P < 0.05). During ZT2-4, acutely fasted animals ran significantly more than animals in the *ad libitum* condition (Figure 4.7; fasted: 42.1 ± 11.71, *ad libitum*: 1.1 ± 0.59; P < 0.05). Wheel-running levels recorded for animals in the restricted condition (13.9 ± 7.70) did not differ significantly from those recorded for animals in either of the other two conditions during ZT2-4 (Figure 4.7).
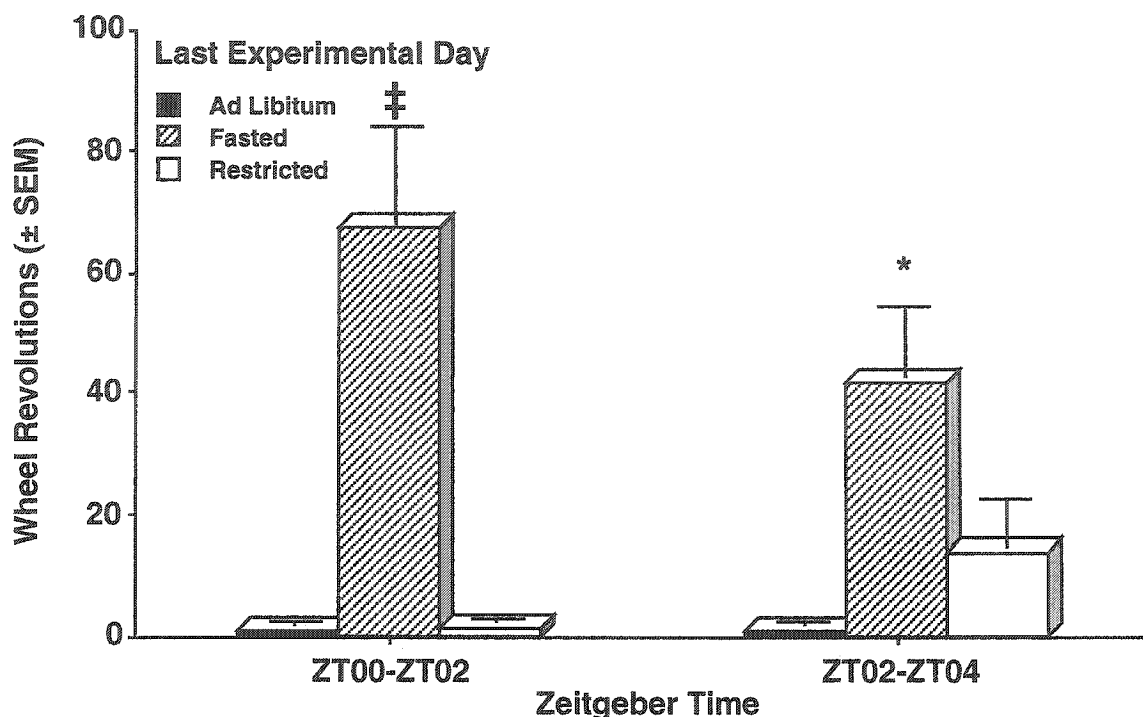
Figure 4.7. Activity levels recorded during ZT0-2 and ZT2-4 on the last day of the experiment for animals fed *ad libitum* (black bars; n=9), acutely fasted (hatched bars; n=18) or on restricted feeding (open bars; n=26). Time bins corresponding to zeitgeber times are plotted along the abscissa and average numbers of wheel revolutions/5 min time bin (± SEM) are plotted along the ordinate. The * indicates a significant difference from the *ad libitum* condition (P < 0.05). The ‡ indicates a significant difference from the *ad libitum* and restricted conditions (P < 0.05).

4.3.2 Stomach Weights

Stomachs of 4 animals from each of the 5 feeding conditions (AL, FN, FF, RN and RF) were removed immediately following perfusion. A significant difference in weight was found among stomachs of animals in the different feeding conditions (Figure 4.8; $F_{(4,15)}$ = 290.9, P < 0.0001). FN and RN animals had had no access to food for 19.5 h prior to time of barbiturate injection. Stomachs of animals in these conditions were completely empty (Figure 4.8; FN: 0.3 ± 0.04 g; RN: 0.4 ± 0.01 g). Animals of group AL had a small amount of food in their stomachs (Figure 4.8; 0.7 ± 0.06 g), as did animals of group FF (Figure 4.8; 1.0 ± 0.09 g). These stomachs were significantly heavier than those of the FN and RN animals (P < 0.05). Animals in the RF condition had stomachs that weighed the most (Figure 4.8; 4.1 ± 0.17 g; P < 0.05).

Figure 4.8. Stomach weights of animals in the five feeding conditions: AL = *Ad Libitum*; FN = Fasted-NotFed; FF = Fasted-Fed; RN = Restricted-NotFed and RF = Restricted-Fed (n=4/group). Experimental condition is plotted along the abscissa and average stomach weight (in g ± SEM) is plotted along the ordinate. An open bar indicates an average that is significantly greater than both the hatched bars and the black bars ($P < 0.05$). The hatched bars are significantly greater than the black bars ($P < 0.05$). Pictures of representative stomachs from animals in each feeding condition are shown.

4.3.3 Immediate-Early Gene Expression in the LH

The number of c-Fos-IR cell nuclei within one section containing the bilateral LH was determined for each of 25 animals in five different feeding conditions (n=5/group; AL, FN, FF, RN and RF). Figure 4.9 shows the average number of c-Fos-IR cell nuclei per experimental condition; photographs of the LH of representative animals in each
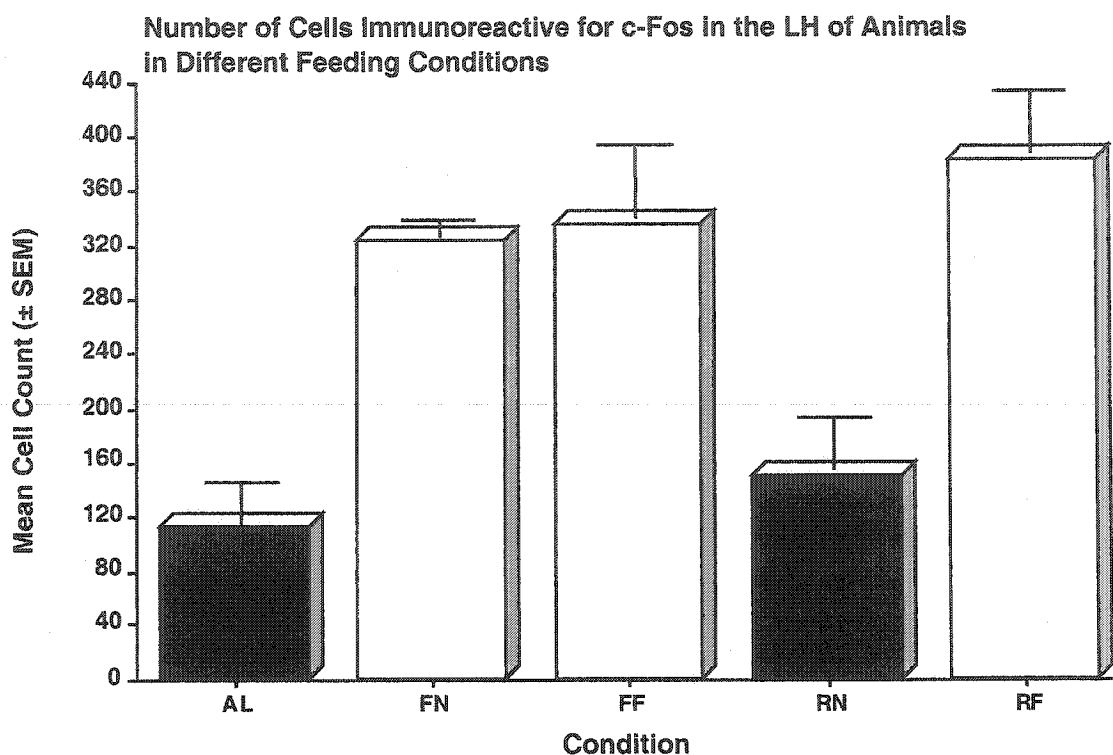
Figure 4.9. Average number of c-Fos-IR cell nuclei in the LH of animals in the five feeding conditions (AL = *Ad Libitum*, FN = Fasted-NotFed, FF = Fasted-Fed, RN = Restricted-NotFed and RF = Restricted-Fed; n=5/group). Experimental condition is plotted along the abscissa and average cell count (± SEM) is plotted along the ordinate. The open bars are significantly greater than the black bars (P < 0.05).

condition are shown in Figure 4.10. A significant difference in number of c-Fos-IR cell nuclei was found among the five feeding conditions ($F_{(4,20)}$ = 10.3, P = 0.0001). Animals in the FN (322.6 ± 11.09), FF (335.6 ± 53.59) and RF (383.8 ± 44.98) conditions had significantly higher numbers of c-Fos-IR cell nuclei than did those in the AL (112.4 ± 28.51) and RN (150.6 ± 35.93) conditions (Figure 4.9; P < 0.05).

Immunoreactivity for Jun-B was assessed for five animals in each of the five feeding conditions by counting Jun-B-IR cell nuclei in one representative LH section bilaterally (Figure 4.11). Although the pattern of results resembles those obtained for c-Fos immunoreactivity, overall numbers of Jun-B-IR cell nuclei were considerably lower (Figure 4.11; AL: 4.4 ± 1.25; FN: 15.6 ± 10.54; FF: 14.2 ± 8.15; RN: 2.2 ± 0.80; RF: 26.8 ± 6.23). The results did not differ significantly among groups ($F_{(4,20)}$ = 2.2, P = 0.1029).

Figure 4.10. c-Fos immunoreactivity in the LH of representative animals from each of the five feeding conditions (AL = *Ad Libitum*, FN = Fasted-NotFed, FF = Fasted-Fed, RN = Restricted-NotFed and RF = Restricted-Fed). III = 3rd ventricle, OT = optic tract, ARC = arcuate nucleus.

Number of Cells Immunoreactive for Jun-B in the LH of Animals in Different Feeding Conditions
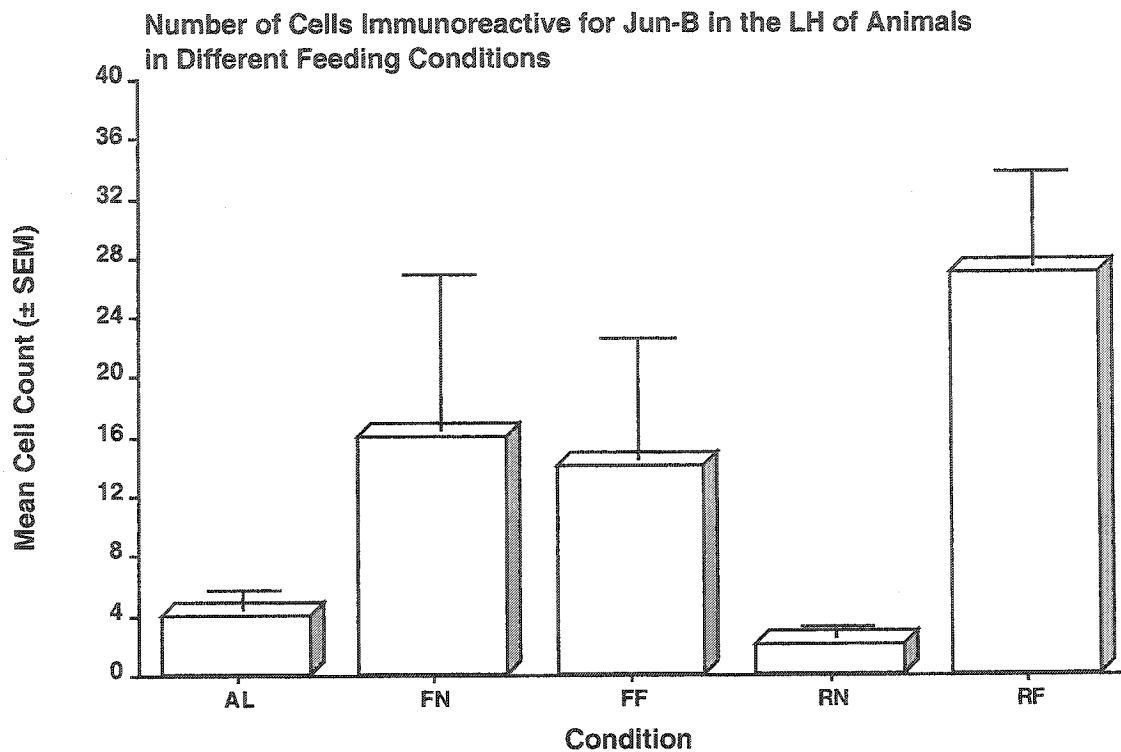
Figure 4.11. Average number of Jun-B-IR cell nuclei in the LH of animals in the five feeding conditions (AL = *Ad Libitum*, FN = Fasted-NotFed, FF = Fasted-Fed, RN = Restricted-NotFed and RF = Restricted-Fed; n=5/group). Experimental condition is plotted along the abscissa and average cell count (± SEM) is plotted along the ordinate.

4.3.4 c-Fos Immunoreactivity in Orexin A-Containing Cells

The percentage of cells containing orexin A that were immunopositive for c-Fos in the LH/perifornical area was calculated for four animals in each of the five feeding conditions (Figure 4.12). Photographs of the LH of representative animals from each condition are shown in Figure 4.13. Statistical analysis revealed a significant difference in percentage of double-labeled cells among the five feeding conditions ($F_{(4,75)}$ = 45.3, P < 0.0001). Animals in the FN condition had the highest percentage of orexin cells that contained c-Fos-IR cell nuclei in the LH/perifornical area (42.6 ± 2.27; P < 0.05). Animals in the FF and RF conditions had a significantly higher percentage of double-labeled cells than did those in the AL and RN conditions (Figure 4.12; AL: 11.0 ± 1.34, FF: 23.5 ± 1.90, RN: 10.3 ± 0.55, RF: 28.0 ± 2.97).
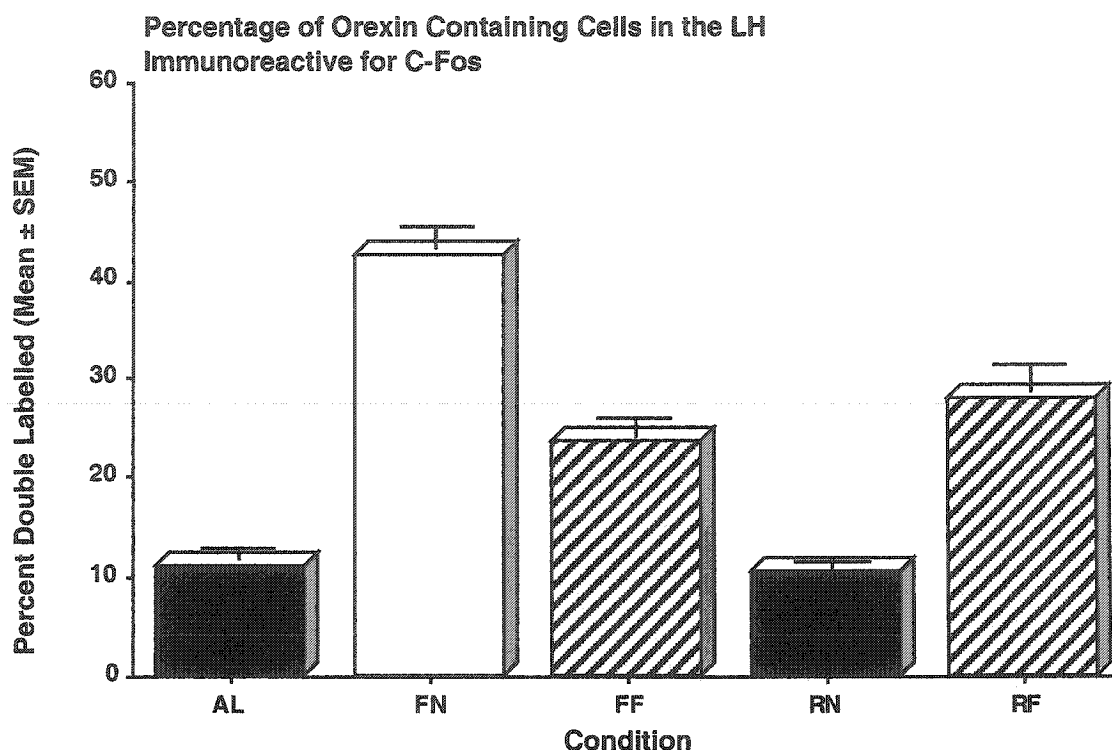
Figure 4.12. Percentage of orexin A-immunopositive cell bodies containing c-Fos-IR cell nuclei for animals in the five feeding conditions (AL = *Ad Libitum*, FN = Fasted-NotFed, FF = Fasted-Fed, RN = Restricted-NotFed and RF = Restricted-Fed; n=4/group). Experimental condition is plotted along the abscissa and percentage of double-labeled cells (± SEM) is plotted along the ordinate. The open bar is significantly greater than the hatched bars and the black bars (P < 0.05). The hatched bars are significantly greater than the black bars (P < 0.05).

4.3.5 *Preproorexin* mRNA in the LH

Relative optical density measures of the LH of mice in five feeding conditions (n=4/group; AL, FN, FF, RN and RF) were taken from film exposed for five days to slides that had been hybridized with a radiolabeled oligonucleotide probe to preproorexin mRNA. As is shown in Figure 4.14, statistical analysis revealed a significant difference among the feeding conditions ($F_{(4,75)}$ = 4.6, P = 0.0021). The film of the section containing the LH is illustrated in Figure 4.15, along with the corresponding cresyl violet counterstained section for representative animals from each condition. Relative optical density was significantly higher for animals anticipating a daily meal (RN: 15.8 ± 2.30)

than for those in the other conditions (AL: 8.7 ± 1.56, FN: 11.2 ± 0.99, FF: 7.3 ± 1.15, RF: 9.8 ± 1.19; $P < 0.05$).
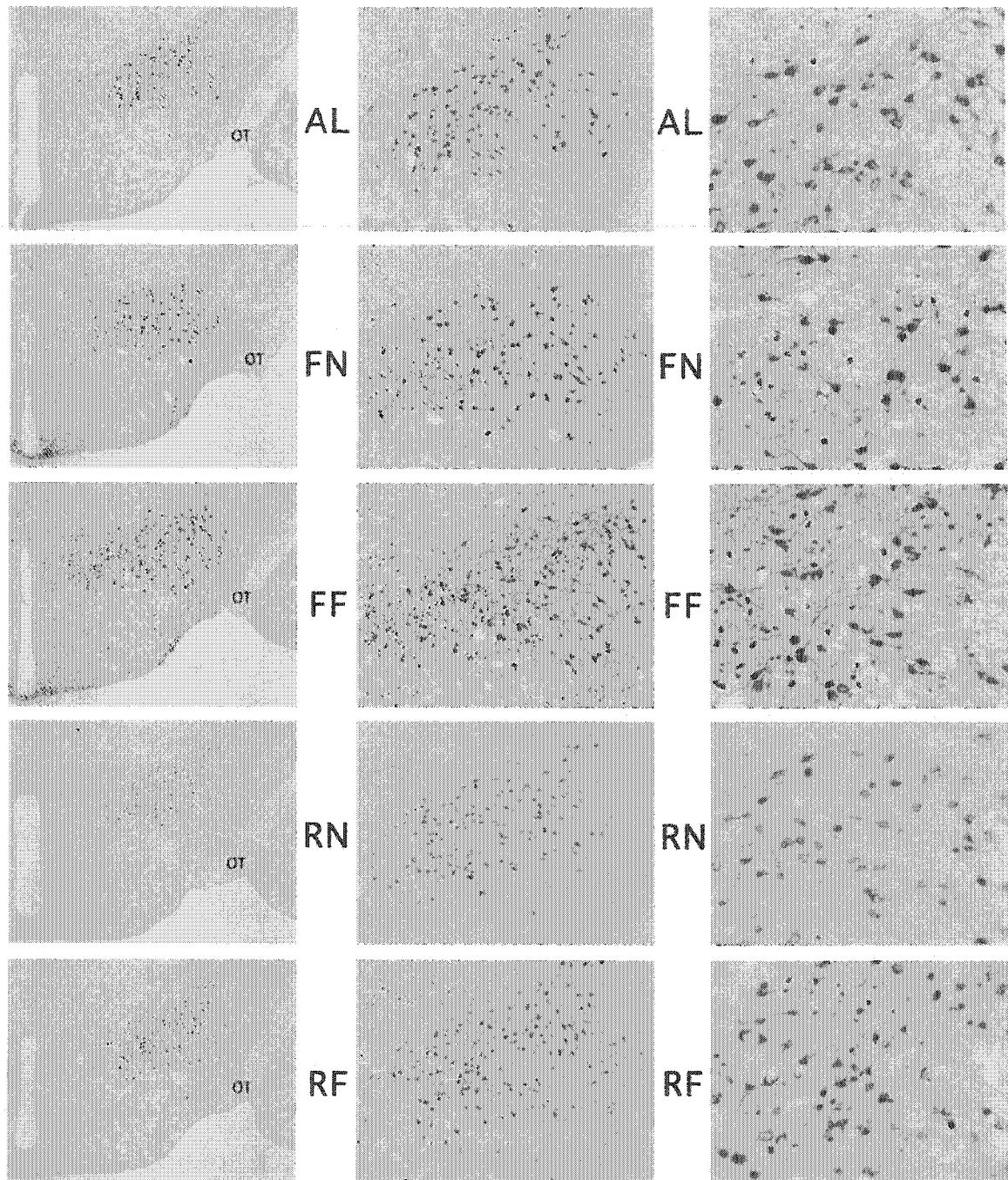
Figure 4.13. c-Fos immunoreactivity in orexin A-IR cells of the LH of representative animals from each of the five feeding conditions (AL = *Ad Libitum*, FN = Fasted-NotFed, FF = Fasted-Fed, RN = Restricted-NotFed and RF = Restricted-Fed). Photographs illustrate these cells at low, medium and high magnification from left to right. OT = optic tract.
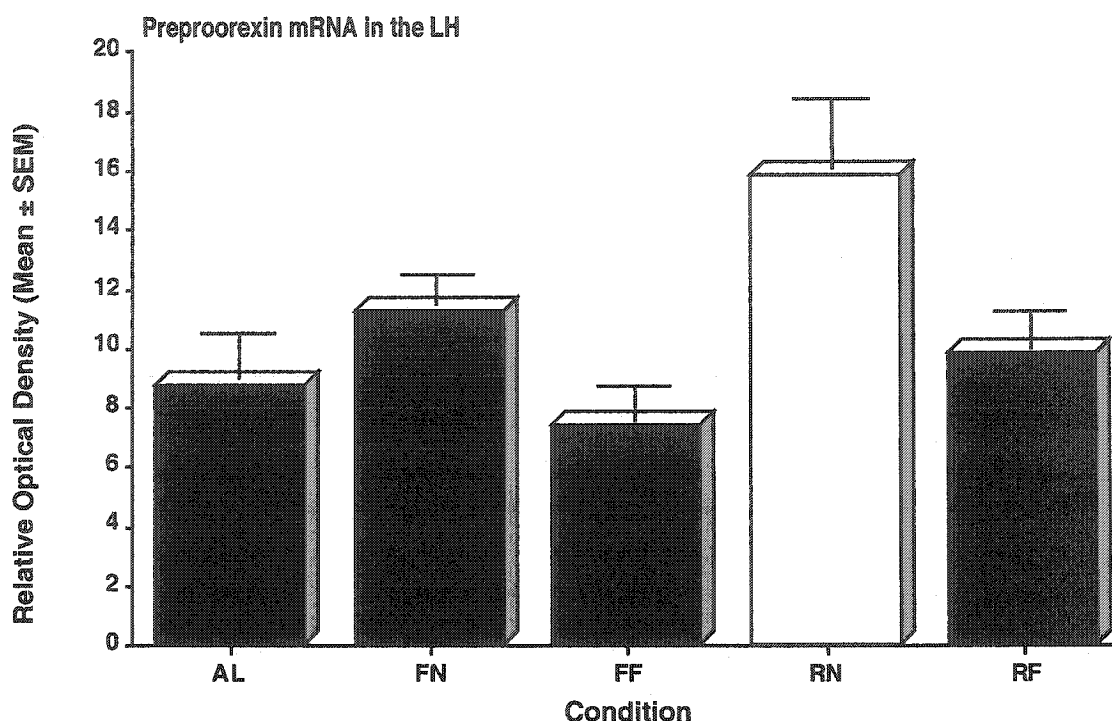
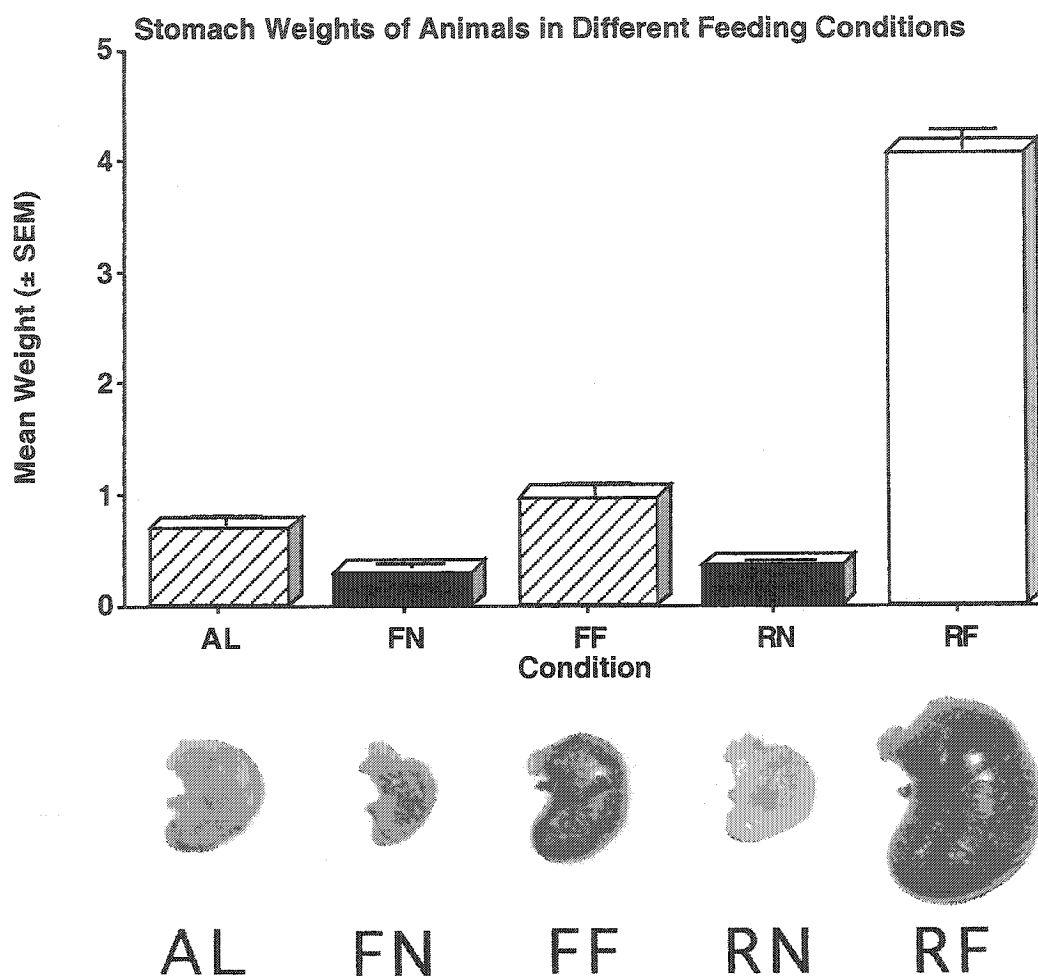Figure 4.14. Relative optical density of preproorexin mRNA for animals in the five feeding conditions (AL = *Ad Libitum*, FN = Fasted-NotFed, FF = Fasted-Fed, RN = Restricted-NotFed and RF = Restricted-Fed; n=4/group). Experimental condition is plotted along the abscissa and relative optical density (± SEM) is plotted along the ordinate. The open bar is significantly greater than the black bars (P < 0.05).

4.4 Discussion

Consistent with previous reports, exposure to a scheduled daily meal during the light phase modified wheel-running activity patterns in mice (Abe et al., 1989; Marchant and Mistlberger, 1997; Challet et al., 1998b; Holmes and Mistlberger, 2000; Sharma et al., 2000). Mice on a temporally restricted feeding schedule showed not only the characteristic FAA prior to meal time, but also a significant increase in activity during the dark phase over that shown by *ad libitum* fed animals (see Figures 4.1-4.4). Nocturnal activity in restricted animals also appeared more consolidated and concentrated within the earlier part of the dark phase (see Figures 4.1-4.3). This observation is consistent with previous reports on alterations of nocturnal activity patterns during restricted feeding and the phase advance of nocturnal activity found in mice restricted to a single meal early in
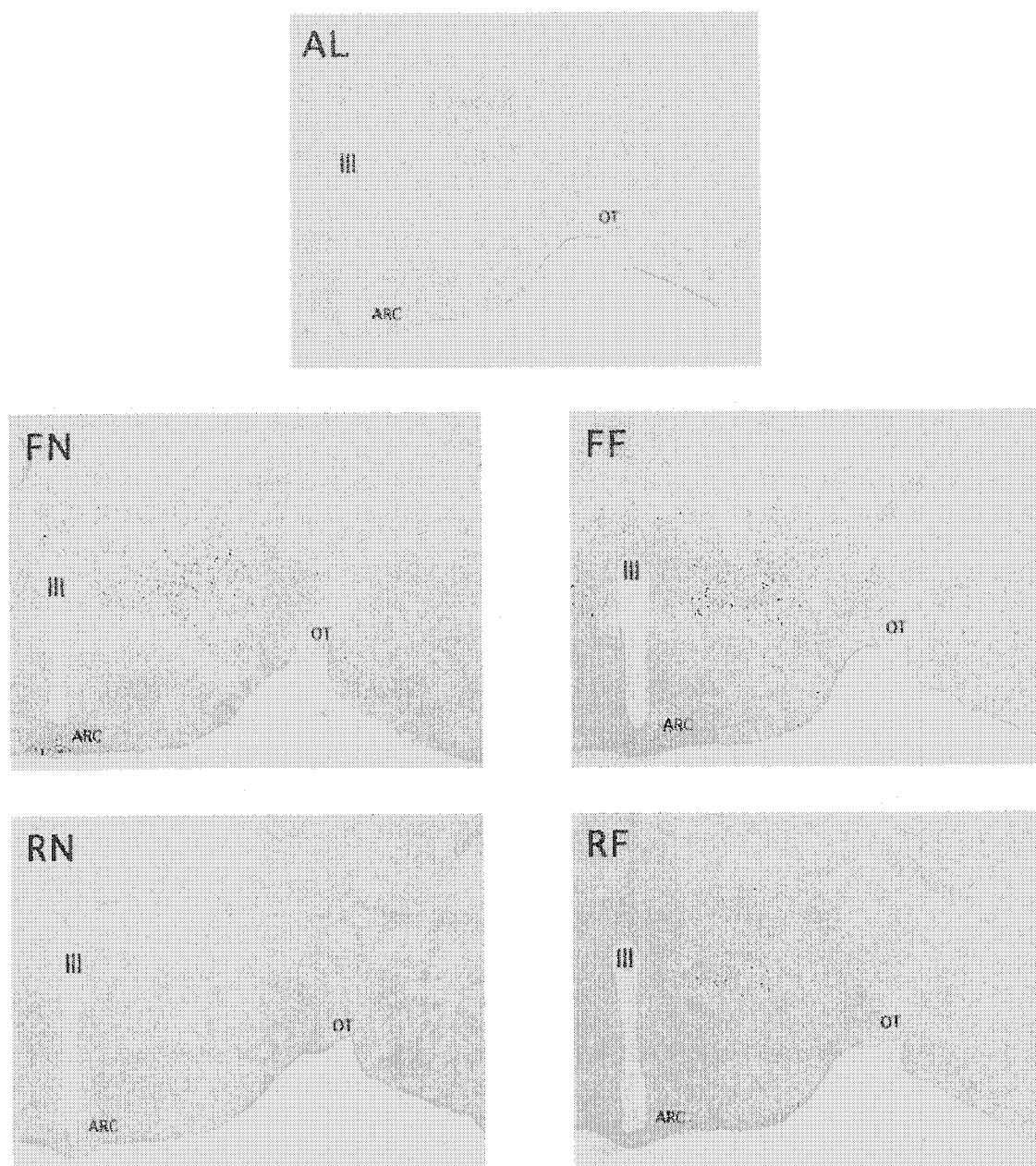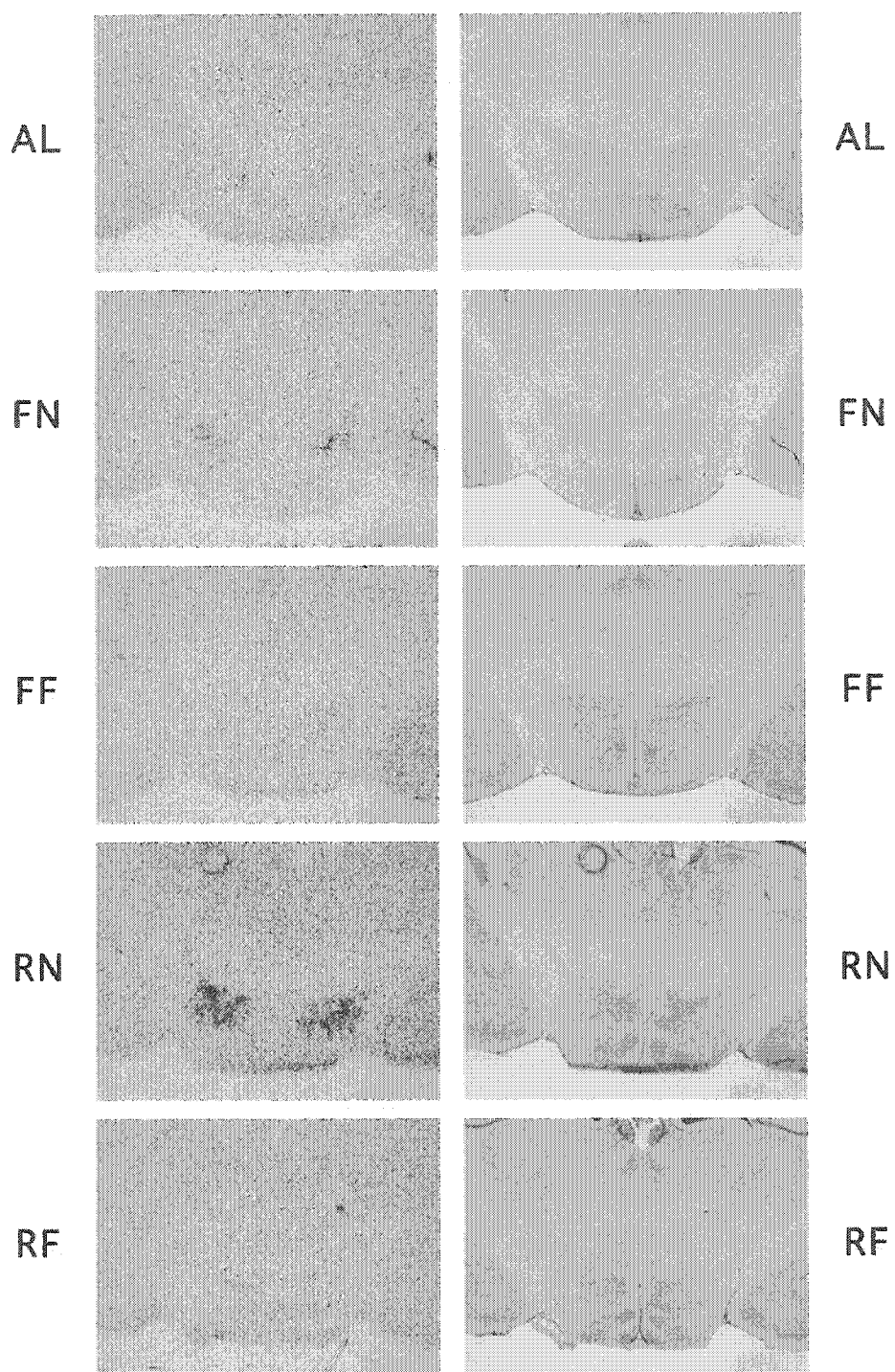
Figure 4.15. Preproorexin mRNA in the LH of representative animals from each of the five feeding conditions (AL = *Ad Libitum*, FN = Fasted-NotFed, FF = Fasted-Fed, RN = Restricted-NotFed and RF = Restricted-Fed) is shown on the left. The right panel shows the same sections counterstained with cresyl violet.

the light phase (Challet et al., 1998a; Challet et al., 1998b; Holmes and Mistlberger, 2000).

Both the group of mice that had been on a restricted meal schedule and the group that was acutely fasted ran significantly more than the group fed *ad libitum* on the last day of the experiment (Figures 4.1, 4.5-4.7). The 24 h activity profiles of restricted animals, however, differed substantially from those of acutely fasted mice (see Figures 4.5, 4.6 and 4.7), despite the fact that acutely fasted and chronically food-restricted mice were deprived of food for the same length of time on the final day. All groups showed vigorous activity early in the dark phase (Figure 4.6), but their patterns differed late in the dark phase and during the beginning of the next light phase (Figures 4.6 and 4.7). Fasted animals showed high activity levels that declined somewhat during the latter half of the dark phase and remained close to that level into the light phase up to the time they were killed (Figures 4.5, 4.6 and 4.7). Restricted animals showed high activity in the dark phase that declined to very low levels in the late dark and early light phase. These mice then showed a distinct increase in activity immediately before the scheduled time of food availability (see Figures 4.1, 4.5, 4.6 and 4.7). Thus, animals that had been exposed to a scheduled daily meal responded quite differently to a 20 h fast than those that had not had that experience.

Animals exposed previously to a feeding schedule were also able to consume significantly more during a 1 h feeding opportunity than those that had not been trained (Figure 4.8). This difference may reflect the adaptive significance of the mechanisms responsible for food entrainment and anticipation. An animal that is able to take full advantage of a meal opportunity, despite the fact that it occurs at a time of day at which this species does not normally eat, is more likely to survive when food sources are scarce or otherwise limited. The selective increase in activity before the scheduled meal may reflect an arousal process that contributes to the capacity to consume a large amount of food in a short period of time. Other metabolic changes must also contribute to this capacity, since fasted mice were at least as active at the time of meal feeding as the

restricted mice, but consumed far less food (Figure 4.8).

Immediate-early gene expression was altered in the LH of animals that were either acutely fasted or entrained to a restricted feeding schedule (Figures 4.9-4.11). Levels of Jun-B expression in this structure were low, and no significant differences were found among feeding conditions, although the pattern of results resembled those found for c-Fos (see Figures 4.9 and 4.11). The pattern of results for c-Fos expression showed that acute food deprivation results in an up-regulation of this protein in the LH, regardless of whether the animals were refed (Figures 4.9 and 4.10). For restricted animals, however, only those that were fed at mealtime showed a comparable up-regulation of c-Fos expression (Figures 4.9 and 4.10). This result demonstrates that the LH of animals on a restricted feeding schedule responds differently to a ~19.5 h period of food deprivation than does the LH of naïve animals.

Because the LH contains a heterogeneous population of cell types (Broberger et al., 1998; Elias et al., 1998; Peyron et al., 1998; Elias et al., 2001), we investigated whether the orexin-producing cells in the LH were those that exhibited altered gene expression in response to food deprivation following experience with a feeding schedule. We, therefore, measured c-Fos expression within orexin A-IR cells (see Figures 4.12 and 4.13), as well as the levels of transcription of preproorexin mRNA under these feeding conditions.

Acute food deprivation led to an increase in c-Fos expression within orexin-IR cells (Figures 4.12 and 4.13). Approximately 42% of orexin cells expressed c-Fos following 19.5 h of fasting, and this decreased to approximately 23% following an hour of refeeding. In these fasted animals, overall levels of c-Fos in the LH did not decrease following refeeding (FF; Figures 4.9 and 4.10), despite a decrease in the proportion of orexin-IR cells that contained c-Fos immunoreactivity (FF; Figures 4.12 and 4.13). Thus, other cell types continued to show increased c-Fos levels even after refeeding.

In contrast to these results, restricted mice that were anticipating a daily meal did not show increased levels of c-Fos within the LH (nor specifically within orexin cells)

despite a duration of food deprivation equal to that of the acutely fasting mice (RN; Figures 4.9, 4.10, 4.12 and 4.13). These results are in disagreement with a recent report showing significantly increased c-Fos expression within LH orexin neurons in rats exposed to a daily restricted feeding schedule (Kurose et al., 2002). In this study, rats were killed just prior to mealtime, and c-Fos expression levels in orexin neurons in restricted rats were compared to those in *ad libitum* fed rats. In restricted rats, 22% of orexin-IR cells expressed c-Fos whereas only 5% of these neurons contained c-Fos in *ad libitum* fed rats (Kurose et al., 2002). Acutely fasted animals were not included in the rat study; therefore it is unclear whether these increased c-Fos levels in restricted rats were due to chronic exposure to restricted feeding or to acute effects of the 22 h fasting period in rats. In the present study, both *ad libitum* fed mice (AL) and chronically restricted mice (RN) showed c-Fos expression in approximately 10% of orexin-IR cells (RN; Figures 4.12 and 4.13). Further study is required to assess whether these different results represent a species difference in orexin neuron response to chronic exposure to restricted feeding.

Restricted mice did show a significant increase in preproorexin mRNA levels, which was not shown by animals that were food deprived without previous training (Figures 4.14 and 4.15). The lack of an increase in preproorexin mRNA levels in acutely fasted animals was somewhat surprising since other groups have reported an increase following fasting for a longer duration (Sakurai et al., 1998; Cai et al., 1999; López et al., 2000). The period of fasting in our paradigm may not have been sufficient to observe a statistically significant increase but mice in the FN group did show a non-significant tendency toward higher preproorexin mRNA levels (see Figures 4.14 and 4.15). Conversely, other groups have reported no increase in orexin mRNA, even after significantly long periods of total food deprivation (Taheri et al., 1999; Tritos et al., 2001).

The increase in preproorexin mRNA and low levels of c-Fos within the LH of restricted animals anticipating a daily meal were both completely reversed in restricted

animals once they had consumed a 1 h meal (see Figures 4.9 and 4.14). In these RF mice, the levels of both preproorexin mRNA and c-Fos immunoreactivity within the LH were indistinguishable from those of animals acutely fasted and then refed.

It is clear from these results that the activity of orexin-IR cells of the LH is altered by the experience of repeated daily cycles of restricted food availability. Whether this modification in the physiology of orexin neurons is related to the development of food entrainment and anticipation was not assessed in this study. Mistlberger et al. concluded that the orexin neurons of the LH are not, in fact, required for food entrainment or anticipation (Mistlberger et al., 2003). They injected rats centrally with the ribosome inactivating protein saporin conjugated to orexin B (or hypocretin 2). This treatment results in a selective loss of neurons expressing receptors for the orexin B ligand. Since orexin neurons have autoreceptors, they are among those targeted by injection of this neurotoxin into the LH area (Gerashchenko et al., 2001). Animals in which all, or virtually all, of the orexin-producing neurons of the LH were lost were still able to anticipate the arrival of a daily meal (Mistlberger et al., 2003). Thus, the distinctive physiological activity shown by orexin neurons in the LH after cycles of food restriction and availability does not appear to be essential to the development of FAA in rats.

Recent reports have suggested that the orexin system may be primarily responsible for regulating general behavioral activation and somatomotor activity rather than sleep or feeding specifically (Yamanaka et al., 1999; Hagan et al., 1999; Ida et al., 1999; Nakamura et al., 2000; Espana et al., 2001; Yoshimichi et al., 2001; Sunter et al., 2001; Duxon et al., 2001; Wu et al., 2002; Matsuzaki et al., 2002; Torterolo et al., 2003). Since most animals used in this study were monitored in activity wheels, we had a direct measure of behavioral activation to correlate with levels of activation of orexin neurons in the LH. On the last experimental day, both acutely fasted and chronically restricted animals showed increased wheel-running activity as compared to *ad libitum* fed animals (Figures 4.5 and 4.6). All animals in the fasted conditions, and most of the animals in the restricted conditions, had engaged in high levels of wheel running immediately before they

were killed (Figures 4.1 and 4.7). Despite high activity levels in both restricted and fasted animals, c-Fos levels in the LH in general, and specifically within the orexin-IR cells, differed among these conditions (see Figures 4.9 and 4.12). Animals in the RN condition did not show increased levels of c-Fos in the LH (within or outside of the orexin cells). They did, however, show increased levels of preproorexin mRNA, unlike mice in other treatment conditions (Figure 4.14). These results indicate that behavioral activation or increased arousal levels alone are not sufficient to account for levels of either c-Fos or preproorexin mRNA within orexin cells of the LH.

In summary, animals anticipating a daily meal showed no increase in c-Fos levels within the LH, but did show an increase in the transcription of preproorexin. After they were fed for 1 h at their habitual meal time, there was an up-regulation of c-Fos within the LH and in approximately 28% of orexin producing neurons, accompanied by a decrease in the amount of preproorexin mRNA. This pattern of results cannot be attributed solely to the transition from being hungry to being relatively sated, since acutely fasted animals should then have shown a similar pattern of results both before and after refeeding. Thus, the results of the present study show that restricted daily feeding alters not only an animal's behavior and physiology, but also substantially alters the patterns of gene expression in LH neurons. One caution with this interpretation is that restricted animals ate more than acutely fasted animals when refed (Figure 4.8). One may expect that significant differences in total food consumption during the 1 h food access period might contribute to differences in gene expression. Surprisingly, no differences in gene expression were found between these two conditions (FF and RF) in this study. In addition, animals in three conditions (AL, FN and RN) were killed at ZT3.5, whereas animal in the other two conditions (FF and RF) were killed at ZT5. Although it is possible that this slight difference in the time of day that these animals were killed could have influenced the results of the present study, we did not find a pattern of immediate-early or orexin gene expression that would indicate that this was the case.

Although these experiments focused only on gene expression within the LH, an

astute observer might have noticed the striking differential pattern of c-Fos immunoreactivity in other hypothalamic structures shown in Figures 4.10 and 4.13, most notably in the arcuate nucleus. These salient changes suggest that a number of other brain regions may express altered patterns of gene expression after exposure to food-restriction schedules. Experiments designed to test this hypothesis are described in Chapter 5.

CHAPTER 5

Immediate-Early Gene Expression in Brain Regions Receiving Orexin Input in Mice Entrained to Periodic Daily Feeding Bouts

5.1 Introduction

An animal that is fed at only one scheduled time each day will, over time, become aroused and active prior to meal onset (Mistlberger, 1994; Stephan, 2001; Stephan, 2002). This increase in activity is known as food-anticipatory activity or FAA. Available evidence indicates that FAA is likely the result of the entrainment of a self-sustaining circadian pacemaker, the location of which remains unknown (Mistlberger, 1994; Stephan, 2001; Stephan, 2002). Entrainment to mealtime ensures a host of adaptive responses to cyclic food availability. In order to take full advantage of a meal when it occurs, the animal needs to be awake and the digestive system needs to be prepared to receive food. Increased arousal would also be beneficial, as it would increase the likelihood of finding food. Thus, food entrainment ensures that the animal is awake and aroused, and that the digestive system is prepared, maximizing survival in times when food may only be available periodically.

Researchers have used numerous techniques and strategies in order to find the circadian pacemaker involved in food entrainment, but none has been successful (Mistlberger, 1994; Stephan, 2002). In 1998, a pair of novel neuropeptides, orexin A and B (also known as hypocretin 1 and 2), was first described (Sakurai et al., 1998; De Lecea et al., 1998). These neuropeptides have been shown to play a role in regulating feeding, sleep and arousal (Willie et al., 2001; Smart and Jerman, 2002; Rodgers et al., 2002; Sakurai, 2003). The orexins are produced by cells in the lateral hypothalamus (LH) and perifornical region that project widely throughout the neuraxis (Peyron et al., 1998; Cutler et al., 1999; Nambu et al., 1999; Date et al., 1999), and likely act to integrate the activity of various regulatory systems (Mondal et al., 2000; Kilduff and Peyron, 2000). For these reasons, the lateral hypothalamic orexin system is ideally positioned to play a role in food

entrainment and anticipation.

Immediate-early genes encode proteins that are up-regulated rapidly and transiently within cells in response to a variety of stimuli (Morgan and Curran, 1989; Sheng and Greenberg, 1990; Morgan and Curran, 1991; Hughes and Dragunow, 1995; Herrera and Robertson, 1996; Herdegen and Leah, 1998). The induction of these genes occurs in the absence of *de novo* protein synthesis in response to the activation of second messenger cascades. The protein products of immediate-early genes (also referred to as inducible transcription factors or protooncogenes) function as transcription factors, regulating the expression of other, late-response genes by binding to specific recognition sites or response elements in their promoter regions (Herdegen and Leah, 1998). Thus, the expression of immediate-early genes reflects a change in function associated with subsequent altered gene expression. Therefore, immediate-early gene expression may not be closely tied to ongoing neuronal activation, but the induction of these proteins can be used as a reliable index of other aspects of neuronal responses to a stimulus.

The goal of this study was to identify brain regions that are involved in food entrainment by characterizing immediate-early gene expression in brain regions receiving orexin input in mice undergoing food entrainment. In addition, we assessed immediate-early gene expression in the brains of mice as they were adapting to a standard restricted feeding paradigm, i.e., as food entrainment and anticipation were developing. Patterns of immediate-early gene expression in animals exposed to a daily restricted feeding schedule were compared to those in animals fed *ad libitum* or fasted acutely in order to identify those brain regions that responded differentially to chronic food restriction.

5.2 Methods

5.2.1 Animals and Housing

Forty adult male C57BL/6J mice were obtained from Charles River Canada (St. Constant, Québec) and were used in two experiments. Housing and data recording conditions were identical to those described for animals housed on open shelves in

Chapter 2. All experiments were conducted according to Canadian Council on Animal Care guidelines and were approved by the Dalhousie University Committee on Laboratory Animals.

5.2.2 Procedures

Experiment 1

After six days of acclimatization to the running wheel, when stable entrainment to the light-dark (LD) cycle was achieved, animals were randomly assigned to each feeding condition: *Ad Libitum* (n=12; Figure 5.1A, B and C) or Restricted (n=8; Figure 5.1D and E). For those animals in the restricted condition, procedures were identical to those described in Chapter 2.

On the 16th day of restricted feeding, the eight restricted animals were divided into two groups: Restricted-NotFed (RN) or Restricted-Fed (RF). Half of these animal were killed with a barbiturate overdose (0.5 cc of 65 mg/ml Sodium pentobarbitol, I.P.) approximately 30 min prior to normal mealtime (at zeitgeber time [ZT] 3.5; RN; Figure 5.1D) and half were given their normal meal at ZT4 and were killed approximately 1 h later at ZT5 (RF; Figure 5.1E). The time of light onset is designated as ZT0, by convention. The twelve animals that had been fed *ad libitum* were divided equally into three groups: *Ad Libitum* (AL), Fasted-NotFed (FN) or Fasted-Fed (FF). For eight of these animals, all food was removed from their cages at ZT8 (FN and FF; Figure 5.1B and C: downward pointing arrow on Day 21). On the following day, four of these animals were killed at ~ZT3.5 (FN; Figure 5.1B). The four *ad libitum* fed animals were also killed at ~ZT3.5 (AL; Figure 5.1A). At ZT4, a specimen bottle containing approximately 15.5 g of powdered lab chow and sugar moistened with vegetable oil was placed on the cage floor of the remaining four animals (FF; Figure 5.1C: upward pointing arrow on Day 36). These animals were then killed approximately 1 h later at ZT5.

Experiment 2

Animals were allowed to acclimatize to the housing conditions to ensure stable entrainment to the LD cycle. At ZT0 on Day 29 of recording, all food was removed from

Figure 5.1. Actograms illustrating the experimental procedures for a representative mouse in each of the five conditions: *Ad Libitum* (A); Fasted-NotFed (B); Fasted-Fed (C); Restricted-NotFed (D) and Restricted-Fed (E). Horizontal lines represent 24 h, as shown at the top of the graph. Days are plotted sequentially on the vertical axis. Vertical deflections on the horizontal lines represent microswitch closures corresponding to wheel revolutions occurring in each 5 min time bin, with larger deflections representing higher numbers of wheel revolutions. Animals were housed under an LD 12:12 cycle represented by the bar at the top of the graph (white = light phase, black = dark phase). Downward–pointing triangles depict the time that all food was removed from the cage tops. Shaded bars correspond to the times of food availability during the restricted feeding schedule. Upward pointing arrows indicate the time of refeeding. Circles indicate the time the animal was killed. The } indicates the interval used for further analyses (see Results section).
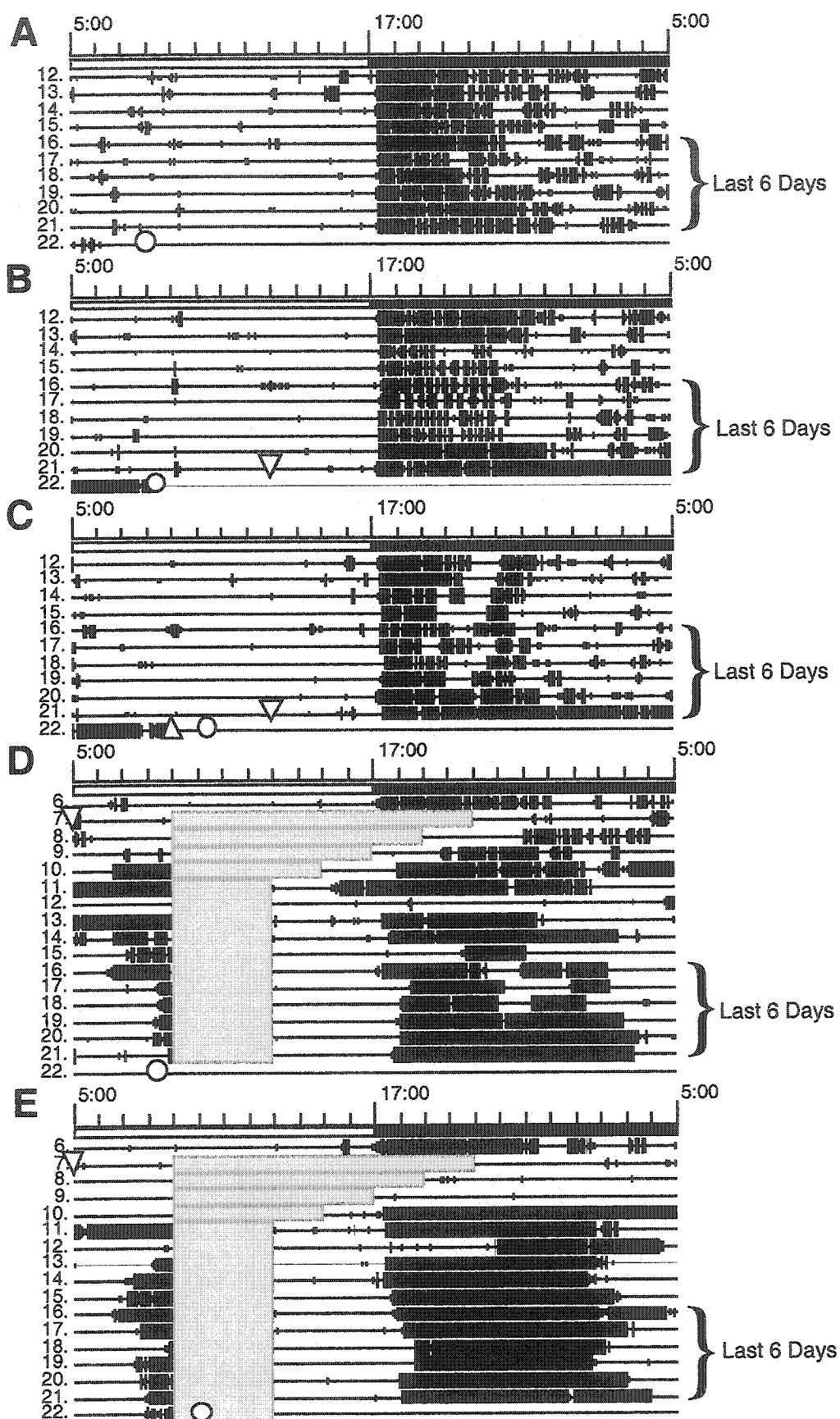
Figure 5.1

the cage tops (downward pointing arrows in Figure 5.2). A restricted feeding paradigm was then introduced as described in Chapter 2. Animals were randomly assigned to one of four conditions (n=5/group): Restricted-NotFed Day 3 (RN3; Figure 5.2A), Restricted-Fed Day 3 (RF3; Figure 5.2B), Restricted-NotFed Day 6 (RN6; Figure 5.2C) and Restricted-Fed Day 6 (RF6; Figure 5.2D). On the third day of the restricted feeding schedule, five animals were killed at ~ZT3.5 (~ 30 min before mealtime; Figure 5.2A; RN3). For the remaining animals, the meal was provided at the regularly scheduled time. One h later (at ZT5) an additional five animals were killed (Figure 5.2B; RF3). On the sixth day of the restricted feeding schedule, five animals were killed at ~ZT3.5 (Figure 5.2C; RN6). The remaining animals were fed at normal mealtime and were killed one h later at ZT5 (Figure 5.2D; RF6).

Perfusion and tissue collection

The animals were perfused and brains were removed, post-fixed, cryoprotected and sectioned as described in Chapter 3. Brain sections (divided into 4 equal parts) were stored in 0.01M phosphate-buffered saline with sodium azide (PBS-azide) until processed for immunocytochemical detection of immediate-early gene expression (see below). Every fourth section was processed for a given antibody. Stomachs and their contents were also removed and weighed following perfusion. Average stomach/contents-weights for each feeding condition were calculated and were compared statistically.

5.2.3 Immunocytochemistry

Experiment 1

Every fourth brain section was processed for immunocytochemical detection of the protein products of one of these genes: *c-fos*, *jun-b*, or *orexin*, as described in Chapters 3 and 4. The remaining set of sections was stored improperly, and therefore the integrity of the tissue was compromised. For this reason, brain tissue from 20 animals used in a previous study with identical housing and experimental procedures (n=4 per feeding condition; see chapter 4) were used for immunocytochemical detection of NGFI-A protein.
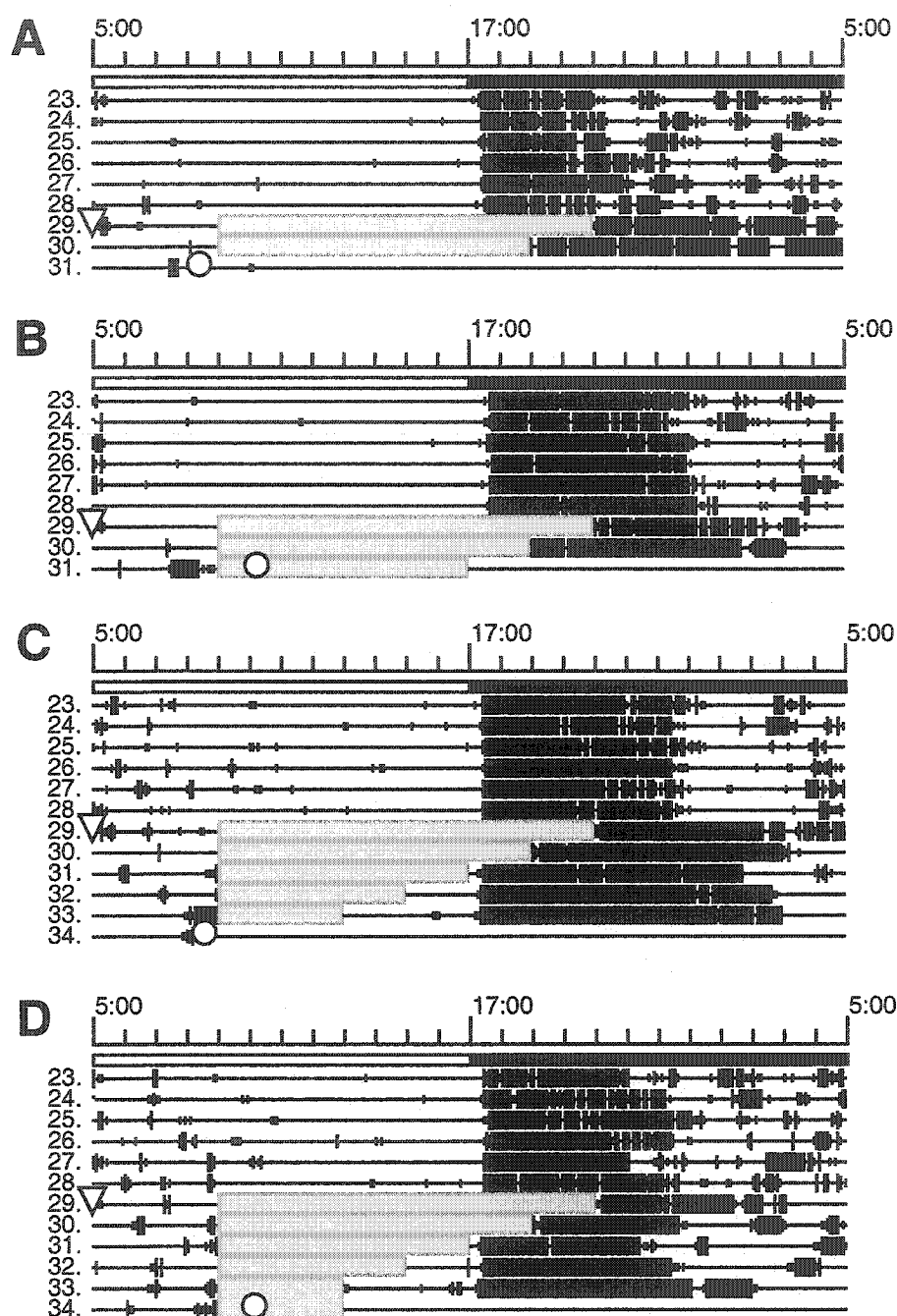
Figure 5.2. Actograms illustrating the experimental procedures for a representative mouse in each of the four conditions: Restricted-NotFed Day 3 (A), Restricted-Fed Day 3 (B), Restricted-NotFed Day 6 (C) and Restricted-Fed Day 6 (D). See Figure 5.1 for details.

For immunocytochemical detection of orexin A, primary antibody raised in goat (1:10,000; C-19; Santa Cruz Biotechnology, Inc., USA) and biotinylated anti-goat IgG secondary antibody raised in rabbit (1:200; Vector Laboratories Canada, Inc., Ontario,

Canada) were used. For detection of the immediate-early genes, primary antibodies raised in rabbit (C-Fos, 1:20,000, Ab-5, Oncogene Research Products, USA; Jun-B, 1:20,000, generously donated by Dr. Rodrigo Bravo; NGFI-A, 1:5000, Santa Cruz) were used. The secondary antibody was biotinylated anti-rabbit IgG secondary antibody raised in goat (1:200; Vector Labs). Each antibody was diluted in phosphate-buffered saline with triton-X 100 (PBS-X) and 3% normal rabbit serum (Vector Labs). For Jun-B, nickel chloride (0.03%) was added to the diaminobenzidine (DAB) solution to enhance the visibility of the immunoreactive product. Sections were mounted on slides and prepared for analyses as described in Chapter 3.

Experiment 2

Immunocytochemistry for the immediate-early genes c-Fos and NGFI-A was performed exactly as described above and in Chapter 4.

5.2.4 Analyses

Behavior

Wheel-running activity data were plotted as actograms using Circadia and these were inspected visually to assess wheel-running activity patterns (Figures 5.1 and 5.2). All further analyses of behavior were performed on data recorded for animals in Experiment 1 only. Activity during the last six days of the restricted feeding schedule, or during the corresponding six days for those animals not on restricted feeding, was analyzed further by plotting the data as 24 h average waveforms using Circadia. Data from all mice with *ad libitum* food access were combined into an average waveform for this six-day interval (including those in the AL, FN and FF conditions; n=12; Figures 5.1A, B and C) since these animals had been treated identically during this time period. Similarly, the data from all mice that had been on restricted daily food access were combined into another average waveform (including those in the RN and RF conditions; n=8; Figures 5.1D and E).

For statistical comparisons among feeding conditions, average numbers of wheel revolutions recorded during specific time bins were calculated using Circadia. Average

amount of activity recorded over the 24 h day during the last six days of restricted feeding was compared statistically between the two groups of mice (*ad libitum* or restricted). For more detailed comparisons of nocturnal activity, wheel running recorded during the dark phase was divided into four 3 h time bins (ZT12-15, ZT15-18, ZT18-21 and ZT21-0). To analyze the pattern and extent of any FAA shown, comparisons were made of the amount of activity recorded during each of two 2 h time bins beginning at the times of lights on and extending until feeding time (ZT0-2 and ZT2-4).

A similar analysis of activity recorded on the last experimental day was also performed. For this analysis, data from all animals were divided into three groups: *Ad libitum* (AL: n=4), Fasted (including FN and FF: n=8) and Restricted (including RN and RF: n=8). An average waveform for each of these conditions was generated for the 24 h period ending at ZT8 on the last experimental day. Data were divided into specific time bins for statistical analyses as described above. Throughout the text, activity is expressed as the average number of wheel revolutions/5 min time bin (± SEM).

Orexin A immunoreactivity

The distribution of orexin A-immunoreactivity (Ir) in both cell soma and efferent fibers was analyzed visually using a microscope (Olympus BH-2). Representative sections of various brain regions (see below) were photographed using an Olympus Camedia digital camera (Olympus America Inc., USA) mounted on the microscope. Photographs were imported into Adobe Photoshop (version 4.0.1) to assemble the figures.

Immediate-early gene expression – Experiment 1

The distribution and density of cell nuclei containing c-Fos-Ir, Jun-B-Ir and NGFI-A-Ir in the brains of the twenty mice were analyzed visually using a microscope (Olympus BH-2). Following initial visual inspection, thirteen brain structures were chosen for further analysis and quantification: the olfactory tubercle (TU), the paraventricular nucleus of the thalamus (PVT), the suprachiasmatic nucleus (SCN), the paraventricular nucleus of the hypothalamus (PVN), the supraoptic nucleus (SON), the

intergeniculate leaflet (IGL), the arcuate nucleus (Arc), the lateral hypothalamus (LH), the dorsomedial nucleus of the hypothalamus (DMH), the ventromedial hypothalamic nucleus (VMN), the tuberomammillary nucleus (TMN), the parabrachial nucleus (PB) and the locus coeruleus (LC). These structures were chosen because they received orexin input (see Results below), they have been implicated previously in the functions of feeding, arousal, sleep or circadian rhythmicity, and/or they appeared to show differential immediate-early gene expression patterns upon initial visual inspection. Identification of the various brain structures was made using a mouse brain atlas (Franklin and Paxinos, 1997).

For the quantification of immediate-early gene expression, one or two representative sections from each animal that contained a specific anatomical level of the selected brain areas were chosen. The same anatomical level of each structure was counted for each animal. Pictures of the chosen sections were captured using a COHU High Performance CCD camera mounted on the microscope, and immunopositive cell nuclei were counted using ScionImage (version 1.62c, National Institutes of Health, USA; modified by Scion Corporation). For the PVN, the DMH, the LH, the PB, the TMN and the VMH, one representative section was counted bilaterally. For the Arc, the SON, the SCN, the TU and the LC, two representative sections were counted unilaterally. Two representative sections of the midline PVT, and two representative sections of the bilateral IGL were counted. Outlines that encompassed each anatomical structure were drawn using ScionImage and these were used as templates so that only those immunoreactive cell nuclei falling within these structures were counted. Anatomical landmarks were used to align template placement in an identical manner for all animals. In this way, the same area of each structure was counted for animals from all conditions. Counts were summed and averaged for each feeding condition. As a control procedure, sections of several brain structures were also counted at different anatomical levels and these counts were compared to the counts obtained at the original anatomical level. The second counts yielded results similar to the original counts, and therefore, only the first

counts were used in further analyses. An experimenter blind to experimental condition performed all of the quantification. Representative sections of each brain region were photographed using a digital camera as described above and figures were made using Adobe Photoshop.

Immediate-early gene expression – Experiment 2

The distribution and density of cell nuclei containing c-Fos-Ir and NGFI-A-Ir in various brain regions were analyzed visually using a microscope (Olympus BH-2). Several brain structures were chosen for further analysis and quantification. These brain structures were chosen based on the results of Experiment 1 in order to evaluate the development of changes in gene expression patterns associated with chronic exposure to the restricted feeding schedule (see Results section). The criteria for inclusion were: 1) that the immediate-early gene cell count in group RN or RF differed significantly from group AL; 2) that the immediate-early gene cell counts differed significantly between group RN and RF; and 3) that the pattern of results were not the same as that found for the FN and FF groups. Identification of the various brain structures was made using a mouse brain atlas (Franklin and Paxinos, 1997). The quantification of immediate-early gene expression in each brain structure was done exactly as described for Experiment 1 above, and the same anatomical level of each structure was counted for each animal. An experimenter blind to experimental condition performed all of the quantification. Representative sections of each brain region were photographed using a digital camera as described above and figures were made using Adobe Photoshop.

Statistical analyses

All statistical comparisons were made by one-way ANOVA using StatView (SAS Institute, Inc., USA) followed by the Student-Newman-Keuls post-hoc tests. Group differences with $P < 0.05$ were considered statistically significant. Group means are reported with SEM.

5.3 Results

5.3.1 Wheel-Running Behavior

Experiment 1

Visual inspection of the actograms recorded for the eight animals exposed to the restricted feeding schedule indicated that all animals anticipated the arrival of their daily meal; however, there was day-to-day variability in the amount and duration of the anticipatory activity (see Figure 5.1D and E). Seven of the eight animals showed increased wheel-running activity (or FAA) on at least six of the eleven days with 4 h of food availability. The remaining animal showed increased activity on only four days.

The average waveforms of data recorded during the last six days of the restricted feeding schedule clearly show that, on average, animals exposed to this schedule increased
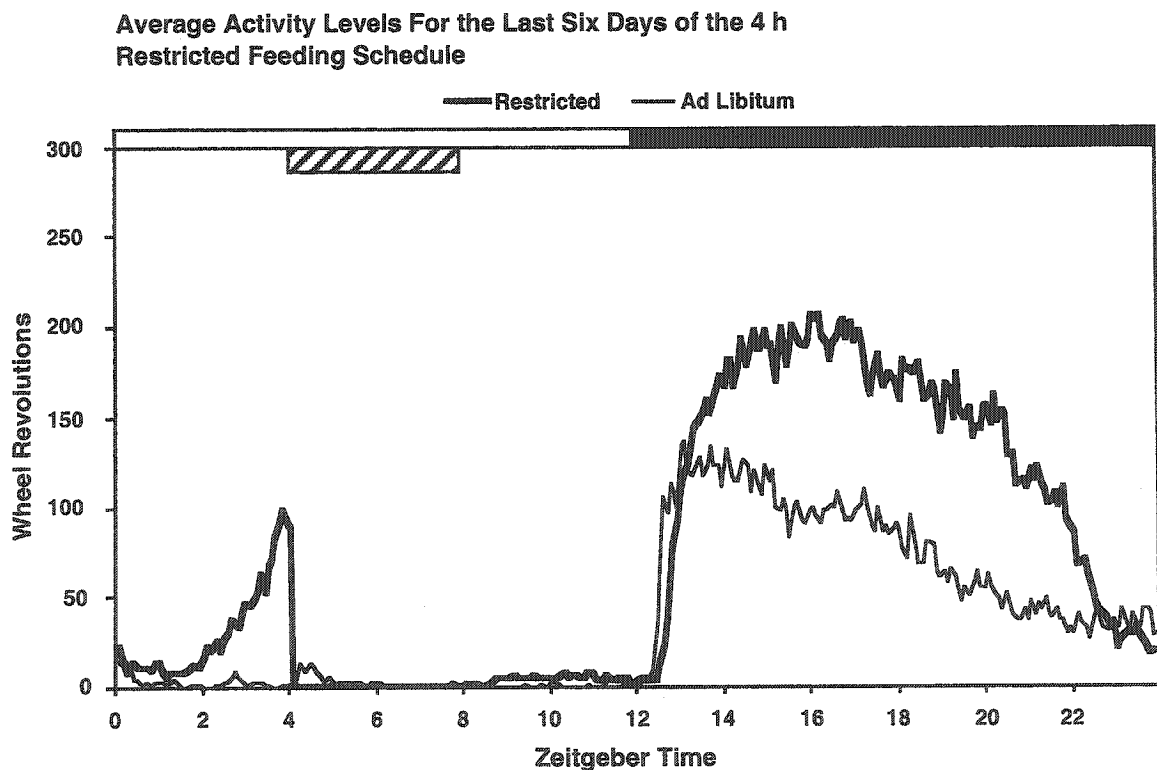
Figure 5.3. Average waveforms for the last six days of recording for animals exposed to the restricted feeding schedule (thick line; n=8) or fed *ad libitum* (thin line; n=12). Zeitgeber time is plotted on the abscissa and average activity levels (in wheel revolutions/5 min time bin) are plotted on the ordinate. The LD 12:12 cycle is shown in the bar across the top (white=light phase, black=dark phase) and the time of food availability is indicated by the hatched bar.

their wheel-running behavior before mealtime, as well as during the dark phase (Figure 5.3). Average daily wheel-running levels recorded over the last six days were significantly higher for animals that were meal fed (n=8; 70.7 ± 6.87) compared to those for animals fed *ad libitum* (n=12; 37.3 ± 3.98, $F_{(1,18)}$ = 20.3, P = 0.0003).

As shown in Figure 5.4, statistically significant differences in nocturnal activity levels were found between feeding conditions. Animals exposed to restricted feeding ran significantly more than animals fed *ad libitum* during the last three 3 h time bins of the dark phase (Figure 5.4; ZT15-18, 187.8 ± 16.05 and 96.6 ± 12.94, respectively, $F_{(1,18)}$ = 19.7, P = 0.0003; ZT18-21, 152.0 ± 26.39 and 59.2 ± 9.72, respectively, $F_{(1,18)}$ = 14.5, P = 0.0013; ZT21-0, 61.0 ± 11.93 and 36.6 ± 3.94, respectively, $F_{(1,18)}$ = 5.1, P = 0.0358). Figure 5.5 shows that activity levels recorded during the 2 h time bin immediately
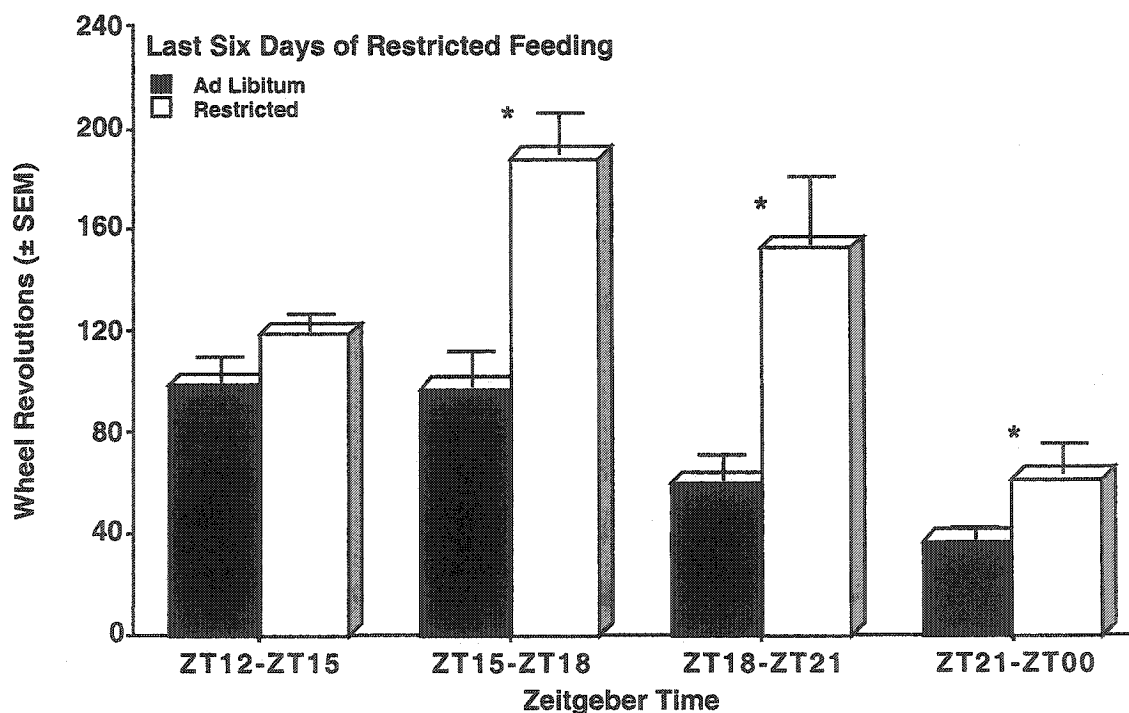
Figure 5.4. Nocturnal activity levels recorded during the last six days of the restricted feeding schedule for animals fed *ad libitum* (black bars; n=12) or on restricted feeding (open bars; n=8). Time bins corresponding to zeitgeber times are plotted along the abscissa and average numbers of wheel revolutions/5 min time bin (± SEM) are plotted along the ordinate. The * indicates a significant difference between groups (P < 0.05).
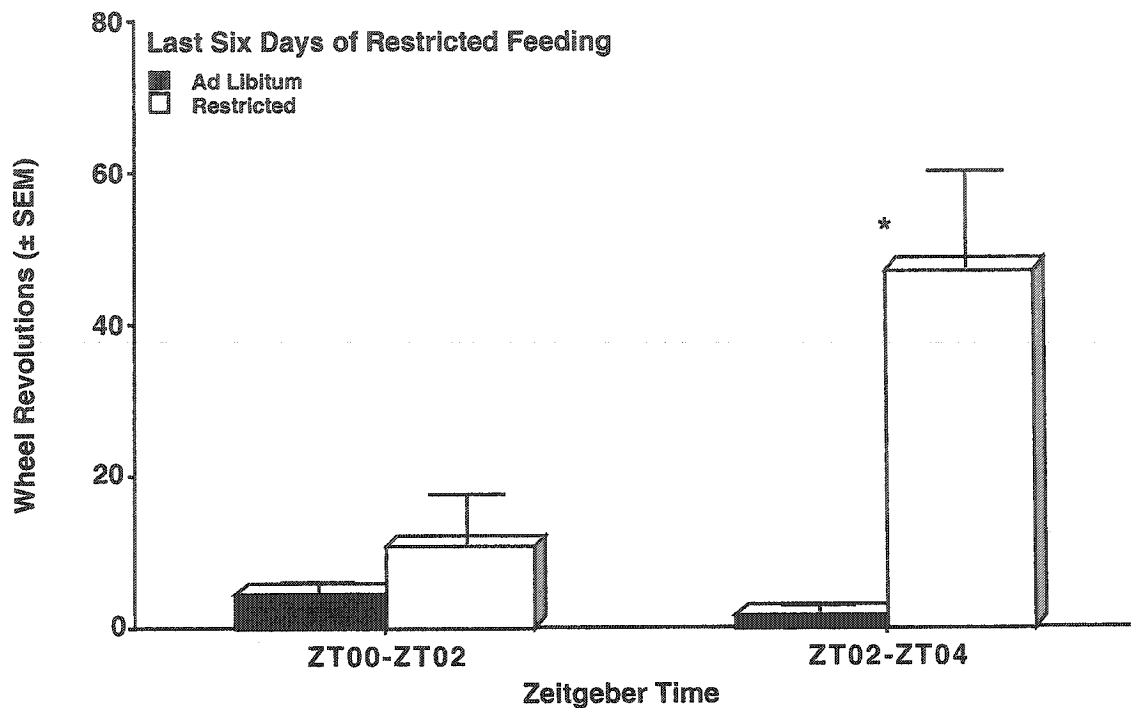
Figure 5.5. Activity levels recorded during ZT0-2 and ZT2-4 during the last six days of the restricted feeding schedule for animals fed *ad libitum* (black bars; n=12) or on restricted feeding (open bars; n=8). Time bins corresponding to zeitgeber times are plotted along the abscissa and average numbers of wheel revolutions/5 min time bin (± SEM) are plotted along the ordinate. The * indicates a significant difference between groups (P < 0.05).

preceding mealtime were significantly higher for animals anticipating the meal than for those fed *ad libitum* (47.0 ± 12.58 and 1.5 ± 0.69, respectively, $F_{(1,18)}$ = 20.0, P = 0.0003). No significant differences in activity levels were found in the first 3 h of the dark phase (Figure 5.4), or the first 2 h of the light phase (Figure 5.5).

The average waveform shown in Figure 5.6 depicts wheel-running data recorded during the last 24 h, ending at ZT8, for animals entrained to mealtime (n=8; thick line), acutely fasted (n=8; shaded line) or fed *ad libitum* (n=4; thin line). Average numbers of wheel-revolutions recorded on the last experimental day differed significantly among the experimental groups ($F_{(2,17)}$ = 13.0, P = 0.0004). Animals that were acutely fasted (n=8, 84.7 ± 2.43) and animals entrained to restricted feeding (n=8, 76.8 ± 8.03) ran

Average Activity Levels For the Last Experimental Day
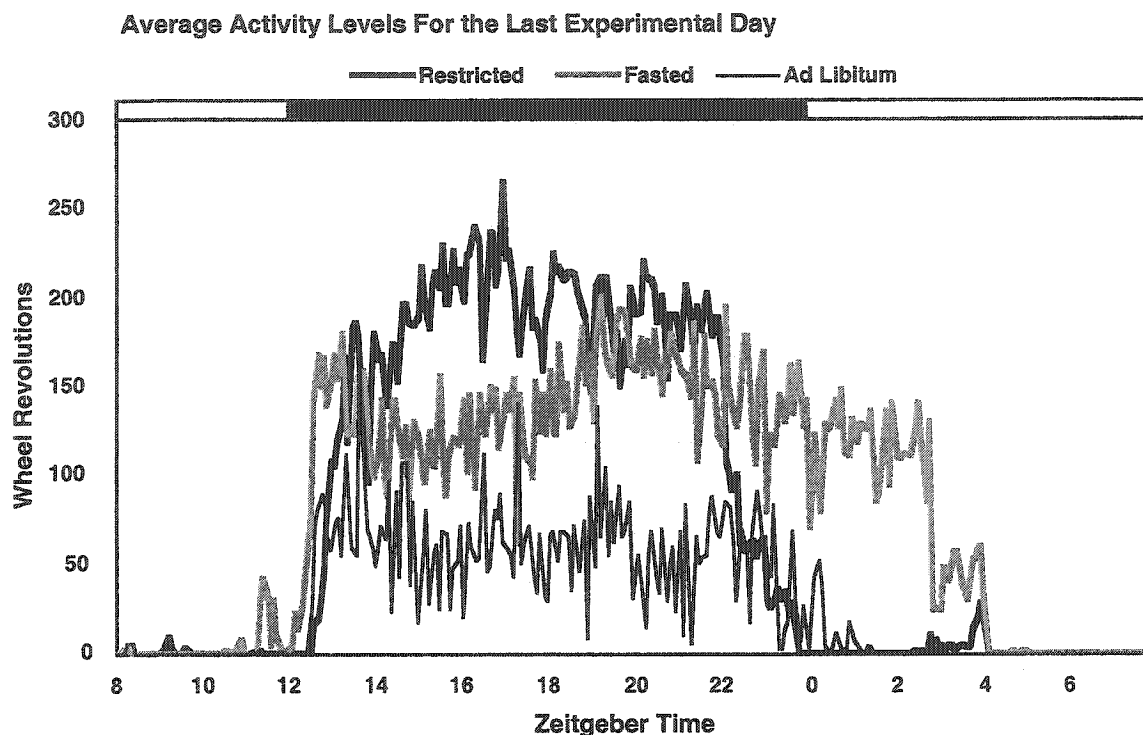
Figure 5.6. Average waveforms for the last day of recording for animals exposed to the restricted feeding schedule (thick line; n=8), acutely fasted (shaded line; n=8) or fed *ad libitum* (thin line; n=4). Zeitgeber time is plotted on the abscissa and average activity levels (in wheel revolutions/5 min time bin) are plotted on the ordinate. The LD 12:12 cycle is shown in the bar across the top (white=light phase, black=dark phase).

significantly more than did animals fed *ad libitum* (n=4, 29.6 ± 11.93; P < 0.05).

Significant differences in the number of wheel revolutions recorded during the dark phase during ZT15-18 ($F_{(2,17)}$ = 17.1, P < 0.0001), ZT18-21 ($F_{(2,17)}$ = 5.1, P = 0.0185) and ZT21-0 ($F_{(2,17)}$ = 4.6, P = 0.0252) were found among groups (Figure 5.7). Specifically, during ZT15-18, activity counts for animals in the restricted condition (206.5 ± 18.79) were significantly higher than counts for animals in the fasted condition (122.8 ± 9.29; P < 0.05), and both of these groups expressed higher activity levels than those fed *ad libitum* (57.9 ± 24.86; P < 0.05). During ZT18-21, animals in both the restricted (193.7 ± 33.46) and fasted (162.6 ± 8.11) conditions ran significantly more than animals that were fed *ad libitum* (59.7 ± 35.49; P < 0.05). For the last 3 h of the dark phase, only fasted animals (143.5 ± 9.50) ran significantly more than *ad libitum* fed animals (50.2 ± 24.35; P < 0.05). During this 3 h interval, the activity levels recorded for
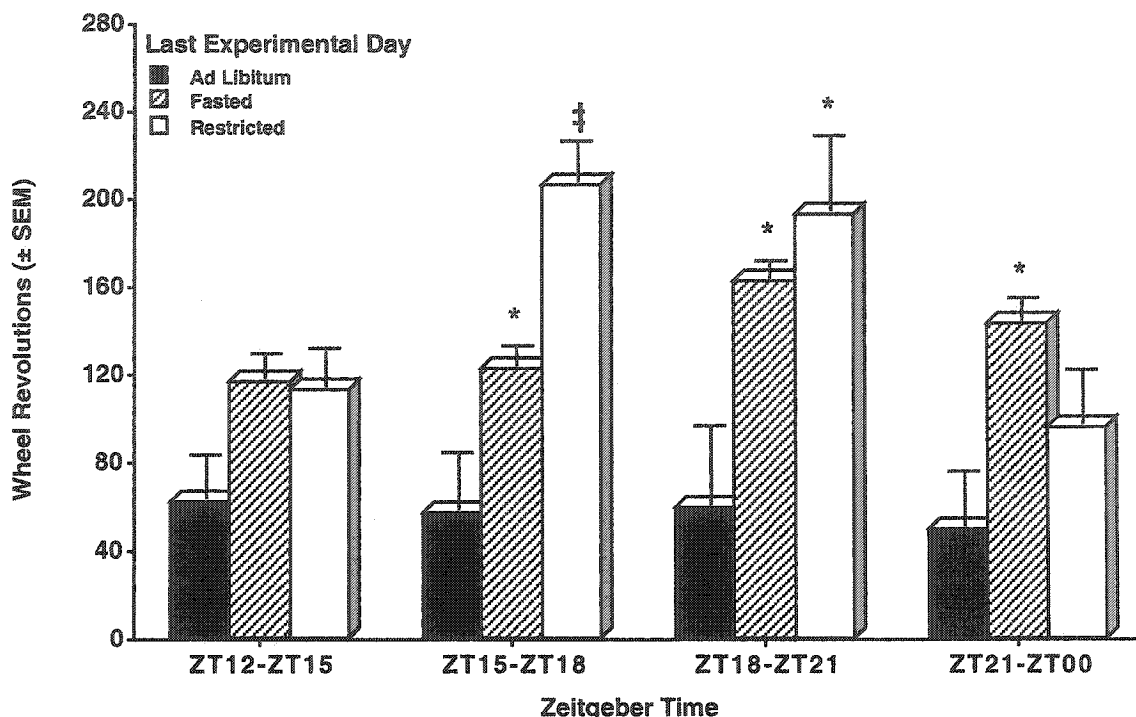
Figure 5.7. Nocturnal activity levels recorded on the last day of the experiment for animals fed *ad libitum* (black bars; n=4), acutely fasted (hatched bars; n=8) or on restricted feeding (open bars; n=8). Time bins corresponding to zeitgeber times are plotted along the abscissa and average numbers of wheel revolutions/5 min time bin ($\pm$ SEM) are plotted along the ordinate. The * indicates a significant difference from the *ad libitum* condition ($P < 0.05$). The ‡ indicates a significant difference from the *ad libitum* and restricted conditions ($P < 0.05$).

restricted animals (96.7 $\pm$ 24.21) did not differ from those recorded for animals in the other conditions (Figure 5.7).

Significant differences in activity levels among feeding conditions were found for the two 2 h time bins occurring during the light phase on the last day of the experiment (Figure 5.8; ZT0-2, $F_{(2,17)} = 32.8$, $P < 0.0001$; ZT2-4, $F_{(2,17)} = 44.1$, $P < 0.0001$). For both of these time periods, fasted animals (117.4 $\pm$ 16.77 and 72.8 $\pm$ 8.75, respectively) ran significantly more than animals in the restricted (0.1 $\pm$ 0.13 and 4.5 $\pm$ 1.89, respectively) and *ad libitum* conditions (8.8 $\pm$ 6.96 and 0.5 $\pm$ 0.51, respectively; $P < 0.05$ for all comparison). Amount of wheel running did not differ significantly between the restricted and *ad libitum* conditions for either of these time periods (Figure 5.8).
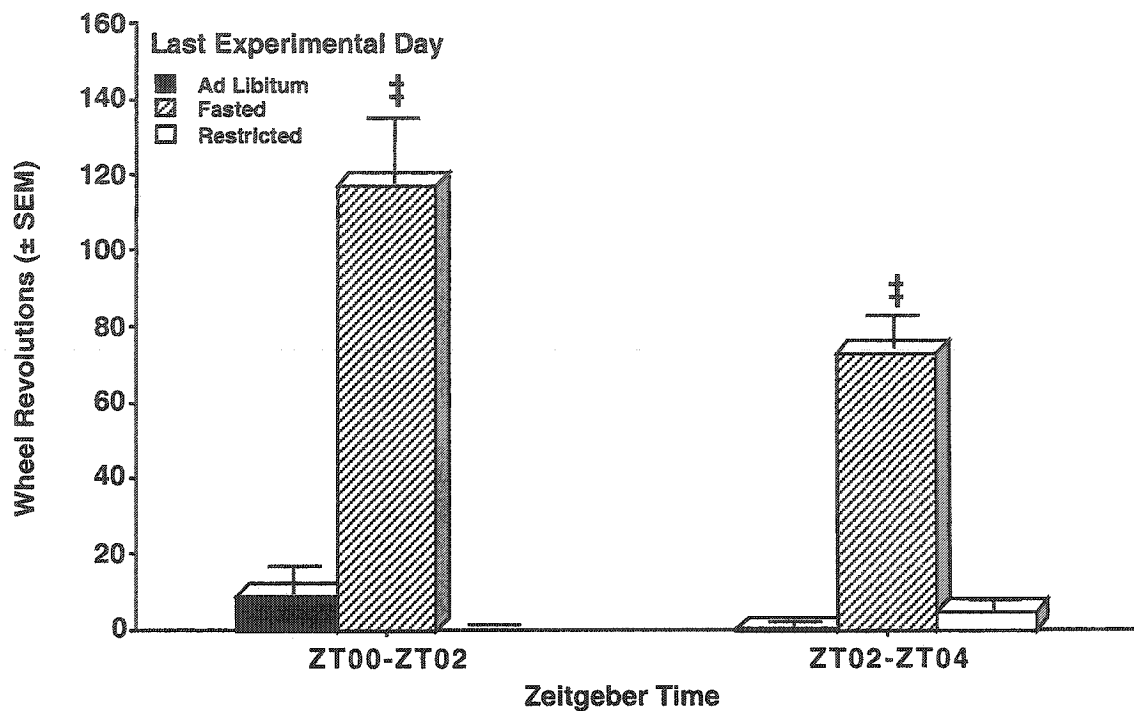
Figure 5.8. Activity levels recorded during ZT0-2 and ZT2-4 on the last day of the experiment for animals fed *ad libitum* (black bars; n=4), acutely fasted (hatched bars; n=8) or on restricted feeding (open bars; n=8). Time bins corresponding to zeitgeber times are plotted along the abscissa and average numbers of wheel revolutions/5 min time bin (± SEM) are plotted along the ordinate. The ‡ indicates a significant difference from the *ad libitum* and restricted conditions (P < 0.05).

Experiment 2

The actograms of wheel-running activity for animals in Experiment 2 were inspected for the presence of a distinct bout of activity preceding mealtime (FAA). On Day 3 of restricted feeding, 12/20 animals showed a clear bout of FAA. On Day 4, 8/10 remaining animals showed anticipatory wheel-running. Seven animals showed FAA on Day 5, and on the final day of restricted feeding, only 5/10 animals anticipated mealtime with increased wheel running.

5.3.2 Stomach Weights

Stomachs of animals from each of the five feeding conditions in Experiment 1 (n=4/group) and the four conditions in Experiment 2 (n=5/group) were removed

immediately following perfusion. A statistically significant difference in weight was found among stomachs of animals in the different feeding conditions (Figure 5.9; $F_{(8,31)} = 62.1$, P < 0.0001). The stomachs of animals in the AL (0.5 ± 0.10 g), FN (0.3 ± 0.01 g), RN3 (0.3 ± 0.02 g), RN6 (0.3 ± 0.01 g) and RN (0.3 ± 0.03 g) conditions did not differ significantly from each other, but weighed significantly less than the stomachs of animals in the four remaining conditions (P < 0.05 for all comparisons). The stomachs of animals in the FF condition (1.2 ± 0.10 g) weighed significantly less than those of animals in the three remaining conditions (P < 0.05 for all comparisons). The stomachs of animals in the RF3 condition (1.7 ± 0.10) weighed significantly less than those of animals in both the RF6 (2.8 ± 0.25 g) and RF (4.0 ± 0.43 g) conditions (P < 0.05 for both comparisons). Animals in the RF condition had stomachs that weighed the most (P < 0.05).
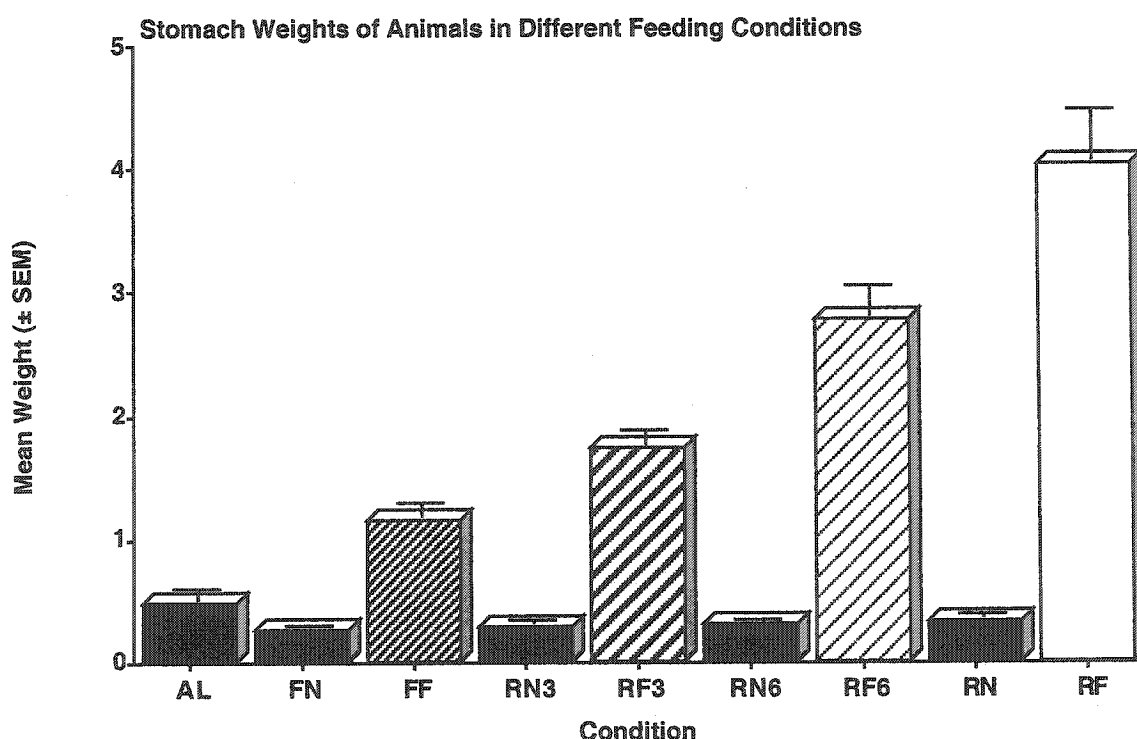
Figure 5.9. Stomach weights of animals in Experiment 1 (AL = *Ad Libitum*; FN = Fasted-NotFed; FF = Fasted-Fed; RN = Restricted-NotFed and RF = Restricted-Fed; n=4/group) and Experiment 2 (RN3 = Restricted-NotFed Day 3; RF3 = Restricted-Fed Day 3; RN6 = Restricted-NotFed Day 6 and RF6 = Restricted-Fed Day 6; n=5/group). Experimental condition is plotted along the abscissa and average stomach weight (in g ± SEM) is plotted along the ordinate. The bars that do not share the same shading differ significantly from each other (P < 0.05).

5.3.3 Orexin Efferent Distribution

The distribution of orexin A-Ir in the brains of the twenty mice from Experiment 1 was identical to that described in Chapter 3. Briefly, a dense population of immunopositive somata was found within the LH/perifornical area of the hypothalamus extending into the DMH and the anterior hypothalamus (AH). Smaller immunopositive cell bodies were found within the retrochiasmatic area (RCH), the PVN and the SON. Fibers containing orexin A-Ir were found in brain regions throughout the neuraxis. Photographs of orexin A-Ir within the brain structures chosen for immediate-early gene quantification are shown in Figures 5.10, 5.11, 5.12, 5.13 and 5.14. Photographs were taken at the approximate anatomical levels used for quantification. No appreciable difference in orexin A-Ir was found among animals of the five feeding conditions.

Figures 5.10 and 5.11 show photographs of hypothalamic structures containing orexin A-Ir. The LH and perifornical regions contained a large number of densely labeled perikarya with immunoreactive processes (Figures 5.10A and B). The Arc contained no immunopositive somata, but did contain immunoreactive fibers, as did the median eminence (Figures 5.10C and D; similar to that described in Chapter 3). The DMH, PVN and SON all contained immunoreactive fibers and small immunoreactive perikarya (Figures 5.10E - H and 5.11A and B). Orexin immunoreactive fibers were found within the VMH and the TMN, representative sections of which are shown in Figures 5.11 C to F. Figures 5.11 C and D also show immunoreactive cell bodies within the AH and the RCH.
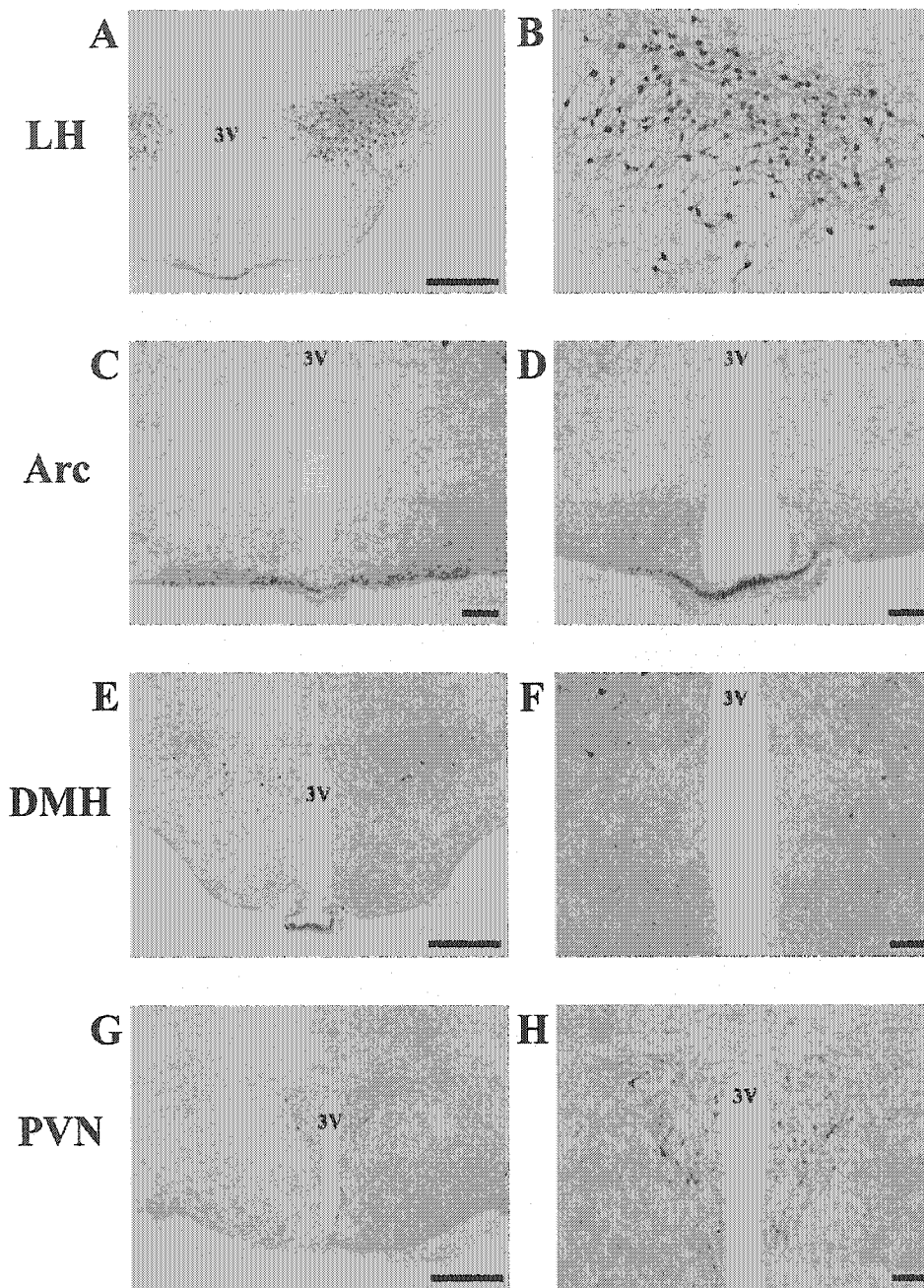
Figure 5.10. Orexin A-Ir in brain structures within the hypothalamus. A and B: lateral hypothalamus (LH); C and D: arcuate nucleus (Arc); E and F: dorsomedial hypothalamic nucleus (DMH); G and H: paraventricular nucleus of the hypothalamus (PVN). Photographs of the LH, DMH and PVN are shown at low (left column) and high (right column) magnification. Photographs of the Arc are shown at two anatomical levels. The scale bars in A, E and G = 500 μm. The scale bars in B, C, D, F and H = 100 μm. 3V = 3rd ventricle.
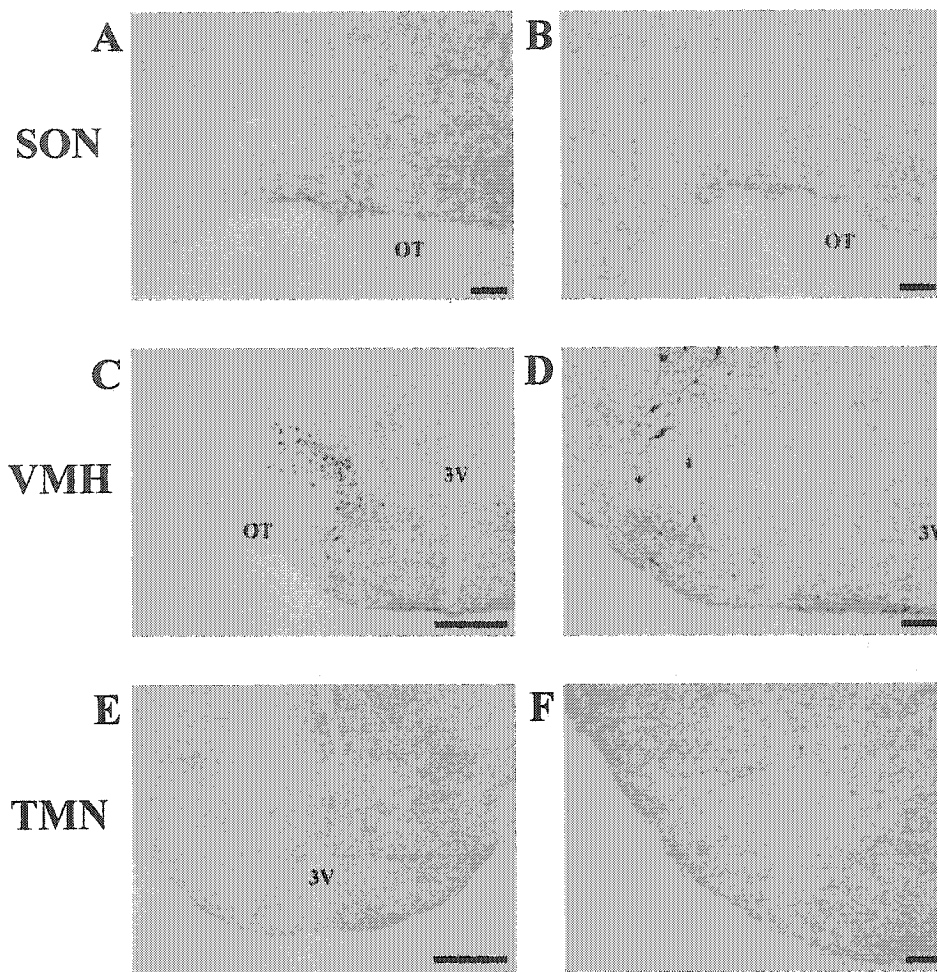
Figure 5.11. Orexin A-Ir in brain structures within the hypothalamus. A and B: supraoptic nucleus (SON); C and D: ventromedial hypothalamic nucleus (VMH); E and F: tuberomammillary nucleus (TMN). Photographs of the VMH and TMN are shown at low (left column) and high (right column) magnification. Photographs of the SON are shown at two anatomical levels. The scale bars in C and E = 500 μm. The scale bars in A, B, D and F = 100 μm. OT = optic tract, 3V = 3rd ventricle.

Two forebrain structures outside of the hypothalamus selected for immediate-early gene quantification also contained orexin A immunoreactive fibers (the PVT and TU; Figure 5.12). The two anatomical levels of the PVT and one level of the TU used for quantification of immediate-early genes are shown (Figure 5.12).
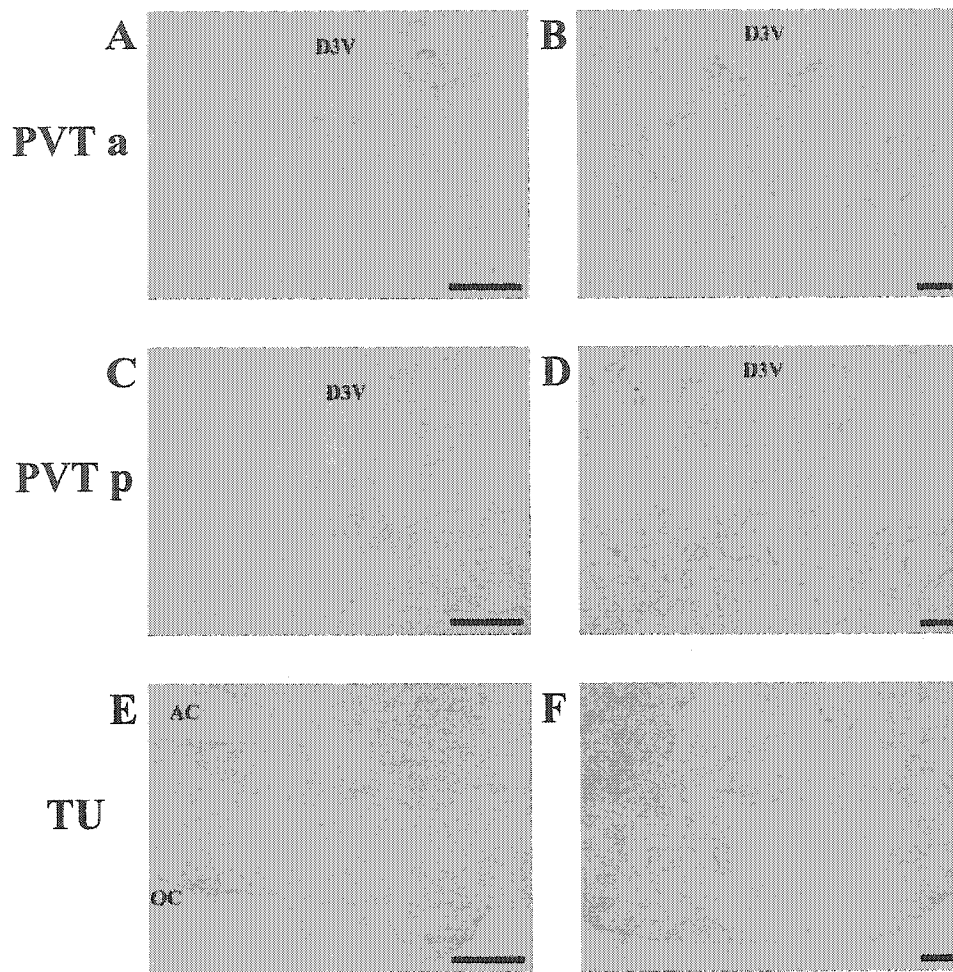
Figure 5.12. Orexin A-Ir in the paraventricular nucleus of the thalamus (PVT) and the olfactory tubercle (TU). The PVT is shown at two anatomical levels (anterior = a; posterior = p). A and B: anterior PVT; C and D: posterior PVT; E and F: TU. Photographs are shown at low (left column) and high (right column) magnification. The scale bars in A, C and E = 500 μm. The scale bars in B, D and F = 100 μm. D3V = dorsal 3rd ventricle, AC = anterior commissure, OC = optic chiasm.

Two brain structures that play a significant role in circadian rhythmicity were selected for immediate-early gene quantification (the SCN and IGL; Figure 5.13). Very few fibers containing orexin A-Ir were found within the SCN itself, although immunoreactive fibers surrounding this structure were evident (Figures 5.13A and B). One orexin A immunoreactive cell body was found in the subparaventricular zone just dorsal to the SCN (Figure 5.13A). A population of fibers containing orexin A-Ir was found extending throughout the IGL (Figures 5.13C and D).
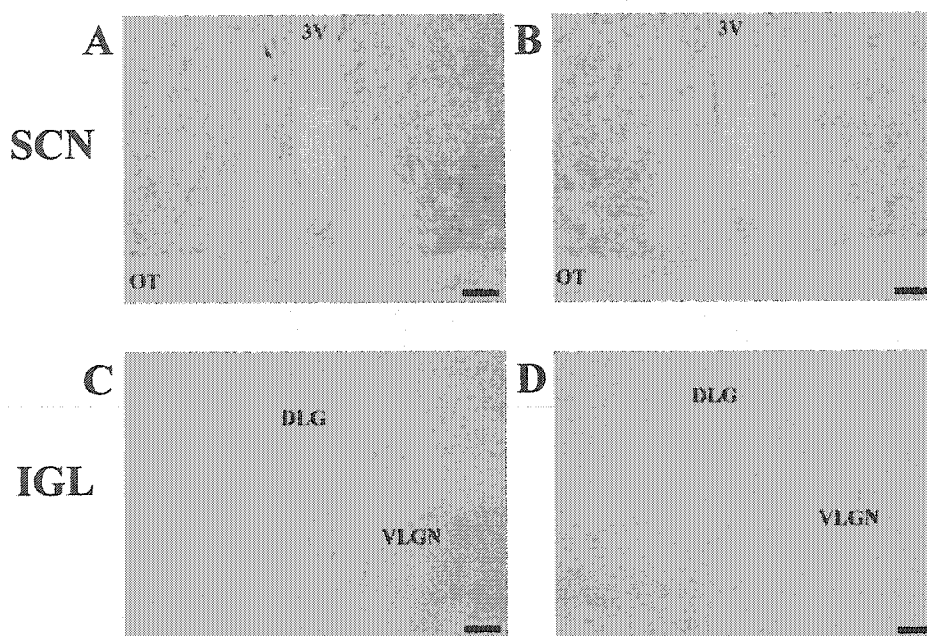
Figure 5.13. Orexin A-Ir in the suprachiasmatic nuclues (SCN) and the intergeniculate leaflet (IGL). Photographs are shown at two anatomical levels. A and B: SCN; C and D: IGL. The scale bars = 100 μm. 3V = 3rd ventricle, OT = optic tract, DLG = dorsolateral geniculate nucleus, VLGN = ventrolateral geniculate nucleus.

Two hindbrain structures were selected for immediate-early gene analyses: the PB and the LC (Figure 5.14). Both of these structures contained orexin A immunoreactive fibers (Figure 5.14): the LC contained the densest orexinergic input of all hindbrain structures. The two anatomical levels of the LC and the one level of the PB used for immediate-early gene quantification are shown (Figure 5.14).
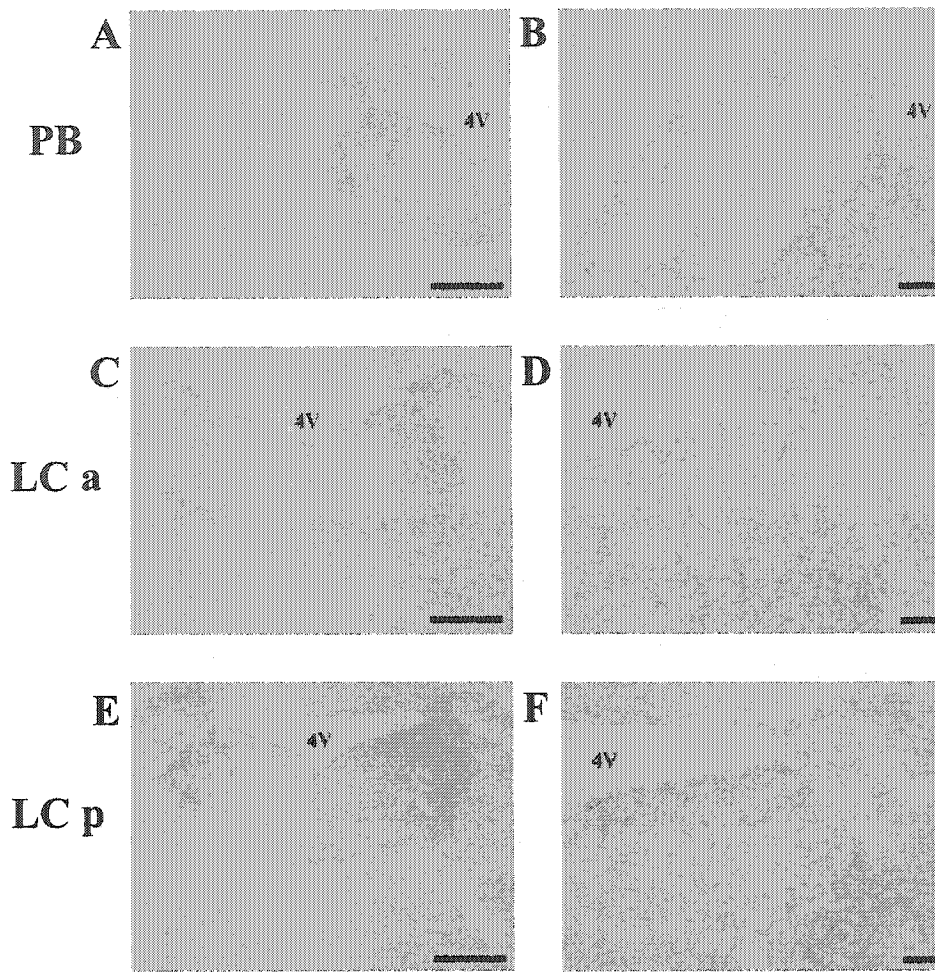
Figure 5.14. Orexin A-Ir in the parabrachial nucleus (PB) and the locus coeruleus (LC). Photographs of the LC are shown at two anatomical levels (anterior = a; posterior = p). A and B: PB; C and D: anterior LC; E and F: posterior LC. Photographs are shown at low (left column) and high (right column) magnification. The scale bars in A, C and E = 500 μm. The scale bars in B, D and F = 100 μm. 4V = 4th ventricle.

5.3.4 Immediate-Early Gene Expression – Experiment 1

In order to assess whether acute or habitual fasting alters gene expression within the brains of mice before or after access to food, levels of the three immediate-early genes in the brains of animals from the four feeding conditions (FN, FF, RN and RF) were compared to those of animals in the AL condition.

c-Fos expression

Table 5.1 shows the average number of cell nuclei containing c-Fos-Ir in the brains

of animals in each of the feeding conditions. Significant differences in c-Fos-Ir were found among the five feeding conditions in all thirteen structures selected for quantification (Table 5.1).

c-Fos	AL	FN	FF	RN	RF	
PB	10.2±2.72 a,b	45.7±12.18 b	98.0±16.74 c	11.2±7.74 a	92.5±6.89 c	$F_{(4,15)}$ = 16.6; P < 0.0001
SON	16.2±10.99 a	50.5±22.21 a,b	81.7±19.65 b	19.5±8.53 a	86.7±12.85 b	$F_{(4,15)}$ = 4.5; P = 0.0142
Arc	1.7±1.11 a	85.0±9.75 b	122.0±6.14 c	24.5±22.23 a	25.2±6.34 a	$F_{(4,15)}$ = 18.7; P < 0.0001
PVN	4.0±1.58 a	209.0±25.49 b	154.0±43.16 b	15.2±13.92 a	50.7±13.50 a	$F_{(4,15)}$ = 14.1; P < 0.0001
DMH	17.0±3.70 a	79.0±10.93 b	164.0±12.84 c	22.5±11.32 a	98.2±3.12 b	$F_{(4,15)}$ = 41.8; P < 0.0001
LH	59.2±7.55 a	232.0±21.44 b	290.5±64.62 b	67.5±39.18 a	220.7±7.81 b	$F_{(4,15)}$ = 8.7; P = 0.0008
TU	5.5±2.90 a	45.5±4.63 b	84.7±13.95 c	11.7±7.32 a	72.5±7.93 c	$F_{(4,15)}$ = 18.3; P < 0.0001
PVT	112.0±12.32 a	291.5±23.72 b	344.5±23.07 b	145.2±54.92 a	258.7±11.23 b	$F_{(4,15)}$ = 11.1; P = 0.0002
VMH	8.5±2.06 a	18.5±6.93 a,b	47.2±11.70 b	9.7±6.49 a	32.7±9.48 a,b	$F_{(4,15)}$ = 4.2; P = 0.0172
LC	13.0±2.41 a	68.0±8.07 a,b	102.0±23.46 b	28.2±22.37 a	57.2±4.25 a,b	$F_{(4,15)}$ = 5.3; P = 0.0071
SCN	146.5±18.44 a	239.5±11.20 b	217.2±32.39 a,b	133.0±22.74 a	191.5±14.45 a,b	$F_{(4,15)}$ = 4.6; P = 0.0127
IGL	22.7±7.81 a	103.2±23.82 b	57.0±10.75 a,b	11.5±4.57 a	75.0±9.70 b	$F_{(4,15)}$ = 8.2; P = 0.0010
TMN	23.2±8.11 a	49.7±15.50 a,b	50.5±11.64 a	31.7±10.16 a	86.7±11.81 b	$F_{(4,15)}$ = 4.4; P = 0.0155

Table 5.1. c-Fos immunoreactive cell counts in brain structures of mice in the five different feeding conditions (AL = *Ad Libitum*; FN = Fasted-NotFed; FF = Fasted-Fed; RN = Restricted-NotFed and RF = Restricted-Fed; n=4/group). Group averages are shown ± SEM. Results of statistical comparison among conditions for each structure are shown in the column on the right. Shaded boxes indicate group means that differed significantly from the AL group mean (P < 0.05). For each row, values not sharing a letter (a, b or c) differ significantly (P < 0.05). PB = parabrachial nucleus; SON = supraoptic nucleus; Arc = arcuate nucleus; PVN = paraventricular nucleus of the hypothalamus; DMH = dorsomedial hypothalamic nucleus; LH = lateral hypothalamus; TU = olfactory tubercle; PVT = paraventricular nucleus of the thalamus; VMH = ventromedial hypothalamic nucleus; LC = locus coeruleus; SCN = suprachiasmatic nucleus; IGL = intergeniculate leaflet; TMN = tuberomammillary nucleus.

The four groups that had undergone manipulations of food availability (FN, FF, RN and RF) showed distinctive patterns of c-Fos-Ir compared to the AL control group (see Figures 5.15 and 5.16 and Table 5.1). Within the PB and the SON, c-Fos-Ir was significantly higher in the FF and RF groups than in the AL group (Figure 5.15A: SON). Animals in both of the fasted conditions (FN and FF) showed increased c-Fos-Ir compared to AL controls in the Arc and the PVN (Figure 5.15B: Arc). The DMH, LH, TU and PVT, expressed significantly more c-Fos-Ir in both fasted conditions (FN and FF) and the RF condition than in the AL condition (Figure 5.15C: DMH). Within the VMH and the LC, only the FF condition had significantly more c-Fos-Ir than the AL condition. The FN group showed significantly higher c-Fos-Ir counts than the AL condition in the SCN and the IGL (Figure 5.16A: SCN). In the IGL, RF animals also showed greater c-Fos-Ir than AL controls. The TMN was the only structure in which the RF group alone showed significantly more c-Fos-Ir than the AL animals (Figure 5.16B: TMN). In none of the structures in which c-Fos-Ir was quantified was c-Fos-Ir up-regulated in the RN group when compared to the AL group (Table 5.1).

Figure 5.15. Average number of c-Fos immunoreactive cell nuclei in three brain structures of animals in the five feeding conditions (AL = *Ad Libitum*, FN = Fasted-NotFed, FF = Fasted-Fed, RN = Restricted-NotFed and RF = Restricted-Fed; n=4/group). Results for the supraoptic nucleus (A), the arcuate nucleus (B) and the dorsomedial hypothalamic nucleus (C) are shown. Experimental condition is plotted along the abscissa and average cell count (± SEM) is plotted along the ordinate. For each structure, the group averages shown as open bars are significantly greater than the AL group average (P < 0.05).

A c-Fos in the Supraoptic Nucleus

B c-Fos in the Arcuate Nucleus

C c-Fos in the Dorsomedial Hypothalamic Nucleus

Figure 5.15

Figure 5.16. Average number of c-Fos immunoreactive cell nuclei in two brain structures of animals in the five feeding conditions (AL = *Ad Libitum*, FN = Fasted-NotFed, FF = Fasted-Fed, RN = Restricted-NotFed and RF = Restricted-Fed; n=4/group). Results for the suprachiasmatic nucleus (A) and the tuberomammillary nucleus (B) are shown. Experimental condition is plotted along the abscissa and average cell count ($\pm$ SEM) is plotted along the ordinate. For each structure, the group averages shown as open bars are significantly greater than the AL group average (P < 0.05).

Jun-B expression

Table 5.2 shows the results of Jun-B-Ir quantification. Although the overall numbers of immunoreactive cell nuclei were low in most of the structures, 10/13 structures chosen for quantification showed significant differences in Jun-B-Ir among the

five feeding conditions, with all differences from the AL group appearing in acutely fasted animals. No significant differences in Jun-B-Ir were found among groups within the PB, the SON or the TMN (Table 5.2).

Jun-B	AL	FN	FF	RN	RF	
PB	0.5±0.29 a	3.2±2.02 a	5.2±1.70 a	1.7±1.03 a	4.2±0.85 a	$F_{(4,15)} = 2.0$; N.S.
SON	1.0±0.71 a	8.2±1.70 a	8.2±2.84 a	3.2±1.89 a	4.7±1.25 a	$F_{(4,15)} = 3.0$; N.S.
Arc	0.2±0.25 a	60.0±6.74 b	59.7±12.43 b	15.5±15.50 a	3.0±1.35 a	$F_{(4,15)} = 10.1$; $P = 0.0004$
PVN	0.0±0.00 a	51.7±8.58 c	34.0±9.01 b	2.7±2.75 a	1.2±0.63 a	$F_{(4,15)} = 17.1$; $P < 0.0001$
DMH	0.5±0.29 a	10.7±5.22 a	78.2±9.95 b	0.5±0.50 a	15.5±6.22 a	$F_{(4,15)} = 32.1$; $P < 0.0001$
LH	0.2±0.25 a	9.0±2.04 a	23.5±5.78 b	4.2±2.95 a	3.0±0.82 a	$F_{(4,15)} = 9.0$; $P = 0.0006$
TU	19.5±8.81 a	117.7±24.26 a,b	171.2±47.86 b	72.2±26.15 a,b	120.0±27.74 a,b	$F_{(4,15)} = 3.7$; $P = 0.0275$
PVT	0.5±0.50 a	1.2±1.25 a	11.0±2.48 b	0.2±0.25 a	0.7±0.25 a	$F_{(4,15)} = 13.2$; $P < 0.0001$
VMH	0.2±0.25 a	3.0±1.78 a	12.5±4.59 b	1.5±0.96 a	2.0±0.71 a	$F_{(4,15)} = 4.7$; $P = 0.0114$
LC	0.0±0.00 a	0.7±0.48 a	5.0±1.29 b	0.5±0.29 a	0.7±0.48 a	$F_{(4,15)} = 9.4$; $P = 0.0005$
SCN	5.2±2.02 a	107.2±25.38 b	37.5±12.71 a	15.7±12.83 a	5.5±2.63 a	$F_{(4,15)} = 9.3$; $P = 0.0005$
IGL	0.0±0.00 a	3.7±1.55 b	0.2±0.25 a	0.0±0.00 a	1.0±0.41 a	$F_{(4,15)} = 4.8$; $P = 0.0106$
TMN	5.2±2.53 a	2.2±0.95 a	9.7±3.54 a	2.2±0.95 a	6.5±1.66 a	$F_{(4,15)} = 2.1$; N.S.

Table 5.2. Jun-B immunoreactive cell counts in brain structures of mice in the five different feeding conditions (AL = *Ad Libitum*; FN = Fasted-NotFed; FF = Fasted-Fed; RN = Restricted-NotFed and RF = Restricted-Fed; n=4/group). Group averages are shown ± SEM. Results of statistical comparison among conditions for each structure are shown in the column on the right (N.S. = not statistically significant). Shaded boxes indicate group means that differed significantly from the AL group mean ($P < 0.05$). For each row, values not sharing a letter (a, b or c) differ significantly ($P < 0.05$). PB = parabrachial nucleus; SON = supraoptic nucleus; Arc = arcuate nucleus; PVN = paraventricular nucleus of the hypothalamus; DMH = dorsomedial hypothalamic nucleus; LH = lateral hypothalamus; TU = olfactory tubercle; PVT = paraventricular nucleus of the thalamus; VMH = ventromedial hypothalamic nucleus; LC = locus coeruleus; SCN = suprachiasmatic nucleus; IGL = intergeniculate leaflet; TMN = tuberomammillary nucleus.

Figure 5.17. Average number of Jun-B immunoreactive cell nuclei in three brain structures of animals in the five feeding conditions (AL = *Ad Libitum*, FN = Fasted-NotFed, FF = Fasted-Fed, RN = Restricted-NotFed and RF = Restricted-Fed; n=4/group). Results for the arcuate nucleus (A), the dorsomedial hypothalamic nucleus (B) and the suprachiasmatic nucleus (C) are shown. Experimental condition is plotted along the abscissa and average cell count ($\pm$ SEM) is plotted along the ordinate. For each structure, the group averages shown as open bars are significantly greater than the AL group average ($P < 0.05$).
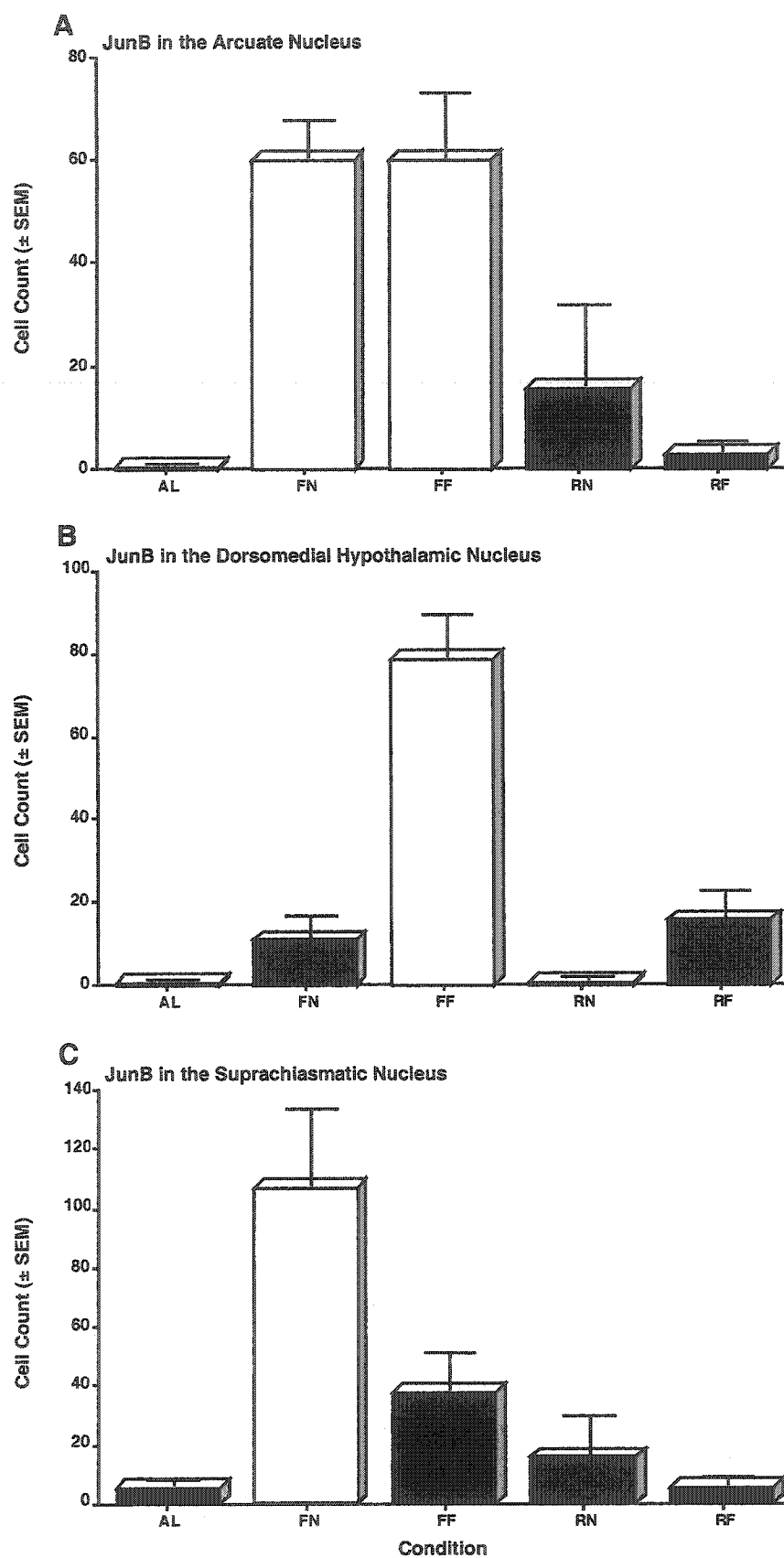
Figure 5.17

Significant differences in Jun-B-Ir were found when animals that had been exposed to feeding manipulations (FN, FF, RN and RF) were compared to control animals (AL; see Figure 5.17 and Table 5.2). In the Arc and the PVN, Jun-B-Ir was up-regulated in both groups of acutely fasted animals (FN and FF) when compared to the AL group (Figure 5.17A: Arc). The DMH, LH, TU, PVT, VMH and LC of mice in the FF condition showed increased Jun-B-Ir compared to those of mice in the AL condition (Figure 5.17B: DMH). The FN group had significantly more Jun-B-Ir in the SCN and IGL than the AL group (Figure 5.17C: SCN). Jun-B-Ir was not significantly altered in either of the chronically restricted groups (RN and RF) compared to the AL animals in any of the thirteen brain structures analyzed (Table 5.2).

NGFI-A expression

Table 5.3 shows the results of the NGFI-A-Ir analyses. Significant differences in NGFI-A-Ir were found among feeding conditions in 10/13 structures. No significant differences in NGFI-A-Ir were found among groups within the TU, the LC and the IGL (Table 5.3).

Comparisons of NGFI-A-Ir were made between the four experimentally manipulated conditions (FN, FF, RN and RF) and the control condition (AL), and statistically significant differences were found (see Figures 5.18 and 5.19 and Table 5.3). Within the PB and the SON, NGFI-A-Ir was up-regulated in the FF and RF conditions when compared to the AL condition (Figure 5.18A: SON). Both of the acutely fasted conditions (FN and FF) showed increased NGFI-A-Ir within the Arc and PVT (Figure 5.18B: Arc). The FN, FF and RF animals expressed significantly more NGFI-A-Ir in the PVN, the DMH and the LH than the AL animals (Figure 5.18C: DMH). Within the VMH, NGFI-A-Ir was significantly up-regulated in the FF group when compared to the AL group. The FN group showed significantly more NGFI-A-Ir within the SCN than the AL group (Figure 5.19A: SCN). NGFI-A-Ir was up-regulated from AL group levels within the RN condition in only one structure, the TMN (Figure 5.19B: TMN).

NGFI-A	AL	FN	FF	RN	RF	
PB	14.0±1.29 a	58.0±15.81 a,b	54.7±15.34 b	61.7±20.54 a,b	138.7±2.39 c	$F_{(4,15)}$ = 13.6; P < 0.0001
SON	5.5±1.55 a	8.5±2.40 a	20.0±4.74 b	3.0±1.58 a	18.0±2.27 b	$F_{(4,15)}$ = 7.5; P = 0.0016
Arc	0.7±0.25 a	45.5±18.80 b	51.7±14.76 b	2.2±1.65 a	14.7±5.25 a,b	$F_{(4,15)}$ = 4.8; P = 0.0105
PVN	5.7±0.63 a	100.7±18.91 b	106.5±17.96 b	25.2±7.11 a	130.2±10.73 b	$F_{(4,15)}$ = 17.7; P < 0.0001
DMH	9.5±3.43 a	114.0±28.00 b	235.2±7.11 c	20.0±10.23 a	132.0±19.79 b	$F_{(4,15)}$ = 31.7; P < 0.0001
LH	81.7±7.19 a	141.7±16.71 b	208.7±18.91 c	57.2±5.57 a	156.2±16.41 b	$F_{(4,15)}$ = 18.5; P = 0.0008
TU	213.2±43.45 a	292.7±60.11 a	228.0±16.32 a	239.7±48.41 a	235.5±9.35 a	$F_{(4,15)}$ = 0.6; N.S.
PVT	26.0±10.51 a,b	67.5±16.07 c	144.7±13.03 d	8.2±1.65 a	48.2±6.28 b,c	$F_{(4,15)}$ = 24.1; P < 0.0001
VMH	4.2±1.65 a	2.7±1.11 a	49.5±10.55 b	9.5±5.06 a	17.7±6.02 a	$F_{(4,15)}$ = 10.4; P = 0.0003
LC	18.0±4.14 a	20.2±9.19 a	35.7±5.53 a	10.7±4.33 a	22.5±6.12 a	$F_{(4,15)}$ = 2.0; N.S.
SCN	6.7±2.95 a	21.5±6.86 b	3.0±0.41 a	5.7±2.29 a	2.2±0.25 a	$F_{(4,15)}$ = 5.0; P = 0.0089
IGL	74.7±9.88 a	84.2±10.66 a	56.7±9.39 a	63.5±9.21 a	79.0±10.13 a	$F_{(4,15)}$ = 1.3; N.S.
TMN	2.5±1.50 a	4.7±2.02 a	5.7±1.03 a	12.7±1.49 b	7.5±0.96 a	$F_{(4,15)}$ = 7.1; P = 0.0021

Table 5.3. NGFI-A immunoreactive cell counts in brain structures of mice in the five different feeding conditions (AL = *Ad Libitum*; FN = Fasted-NotFed; FF = Fasted-Fed; RN = Restricted-NotFed and RF = Restricted-Fed; n=4/group). Group averages are shown ± SEM. Results of statistical comparison among conditions for each structure are shown in the column on the right (N.S. = not statistically significant). Shaded boxes indicate group means that differed significantly from the AL group mean (P < 0.05). For each row, values not sharing a letter (a, b, c or d) differ significantly (P < 0.05). PB = parabrachial nucleus; SON = supraoptic nucleus; Arc = arcuate nucleus; PVN = paraventricular nucleus of the hypothalamus; DMH = dorsomedial hypothalamic nucleus; LH = lateral hypothalamus; TU = olfactory tubercle; PVT = paraventricular nucleus of the thalamus; VMH = ventromedial hypothalamic nucleus; LC = locus coeruleus; SCN = suprachiasmatic nucleus; IGL = intergeniculate leaflet; TMN = tuberomammillary nucleus.
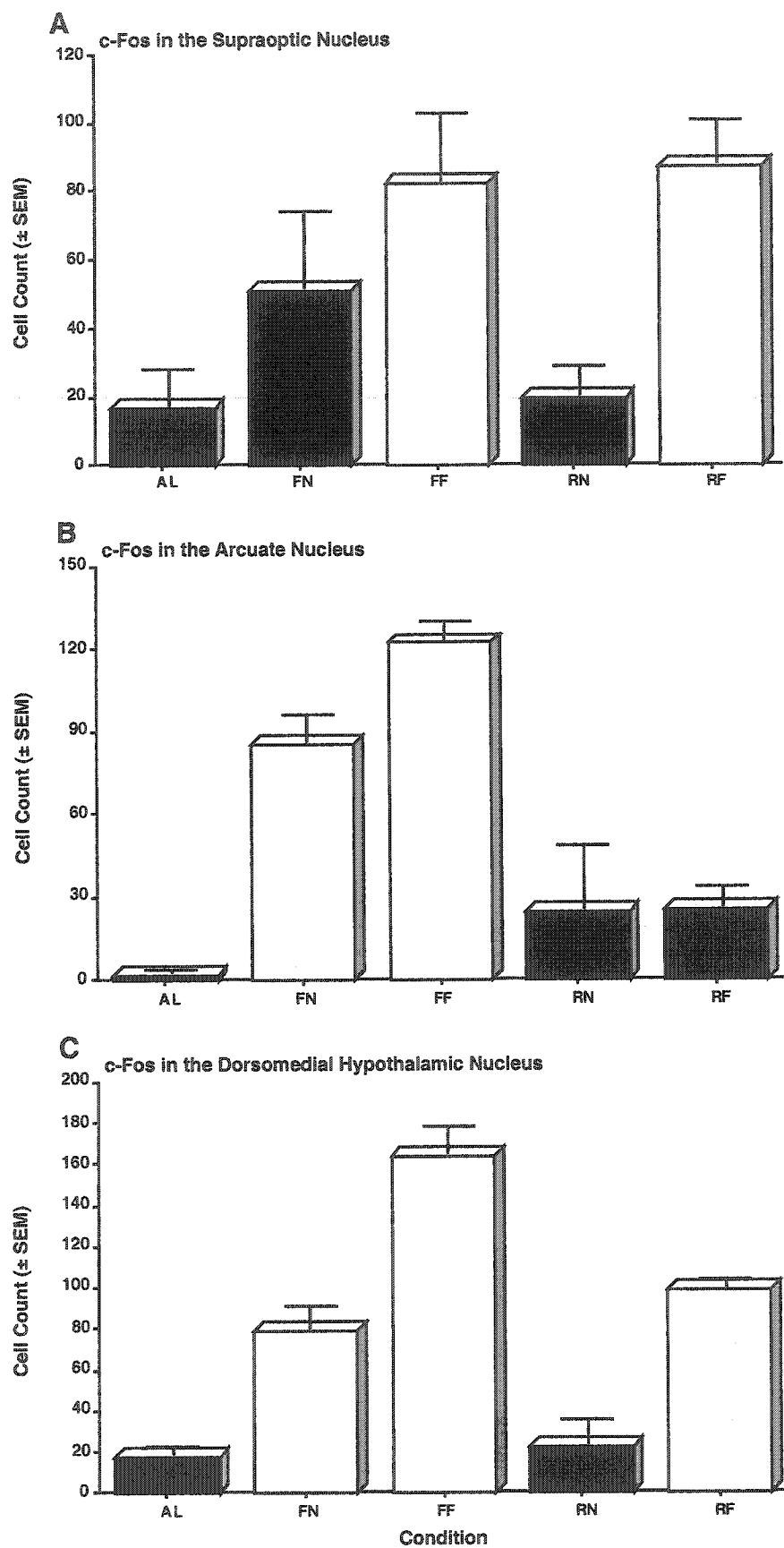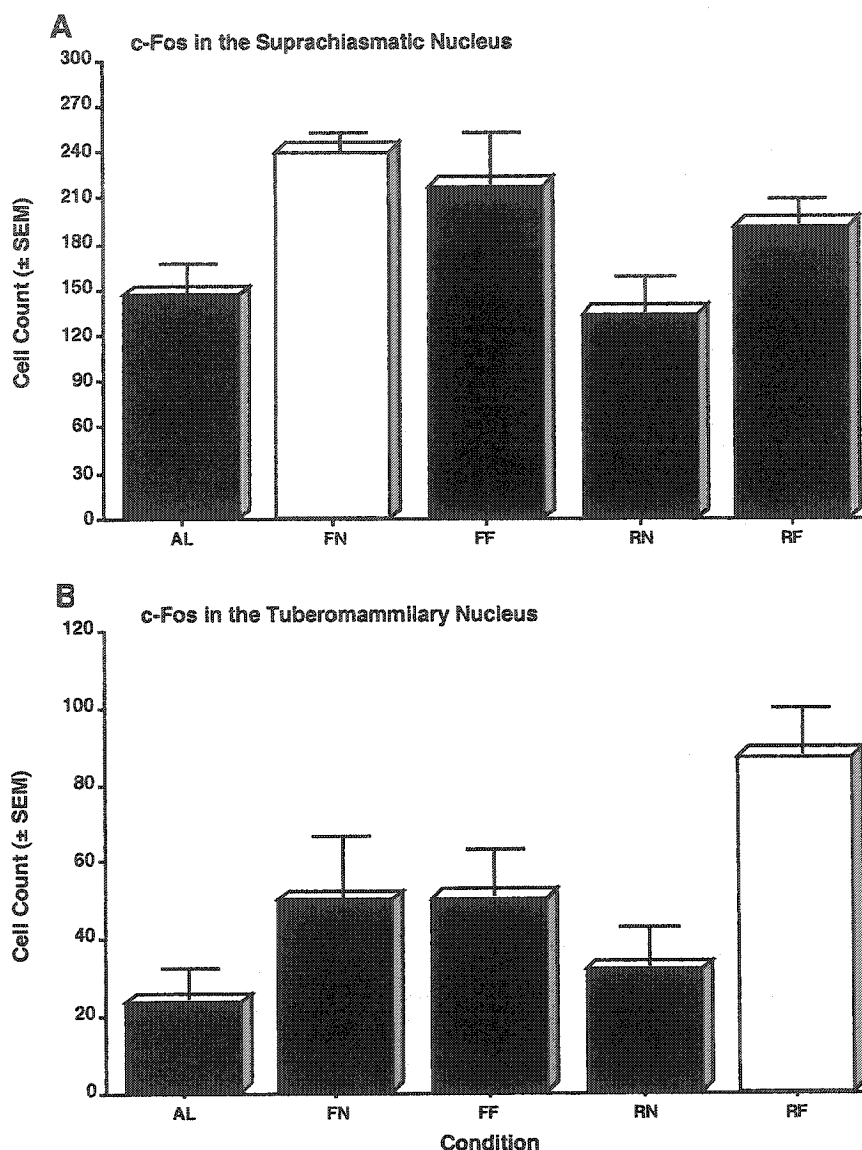
Figure 5.18. Average number of NGFI-A immunoreactive cell nuclei in three brain structures of animals in the five feeding conditions (AL = *Ad Libitum*, FN = Fasted-NotFed, FF = Fasted-Fed, RN = Restricted-NotFed and RF = Restricted-Fed; n=4/group). Results for the supraoptic nucleus (A), the arcuate nucleus (B) and the dorsomedial hypothalamic nucleus (C) are shown. Experimental condition is plotted along the abscissa and average cell count (± SEM) is plotted along the ordinate. For each structure, the group averages shown as open bars are significantly greater than the AL group average ($P < 0.05$).

A NGFI-A in the Supraoptic Nucleus

B NGFI-A in the Arcuate Nucleus

C NGFI-A in the Dorsomedial Hypothalamic Nucleus
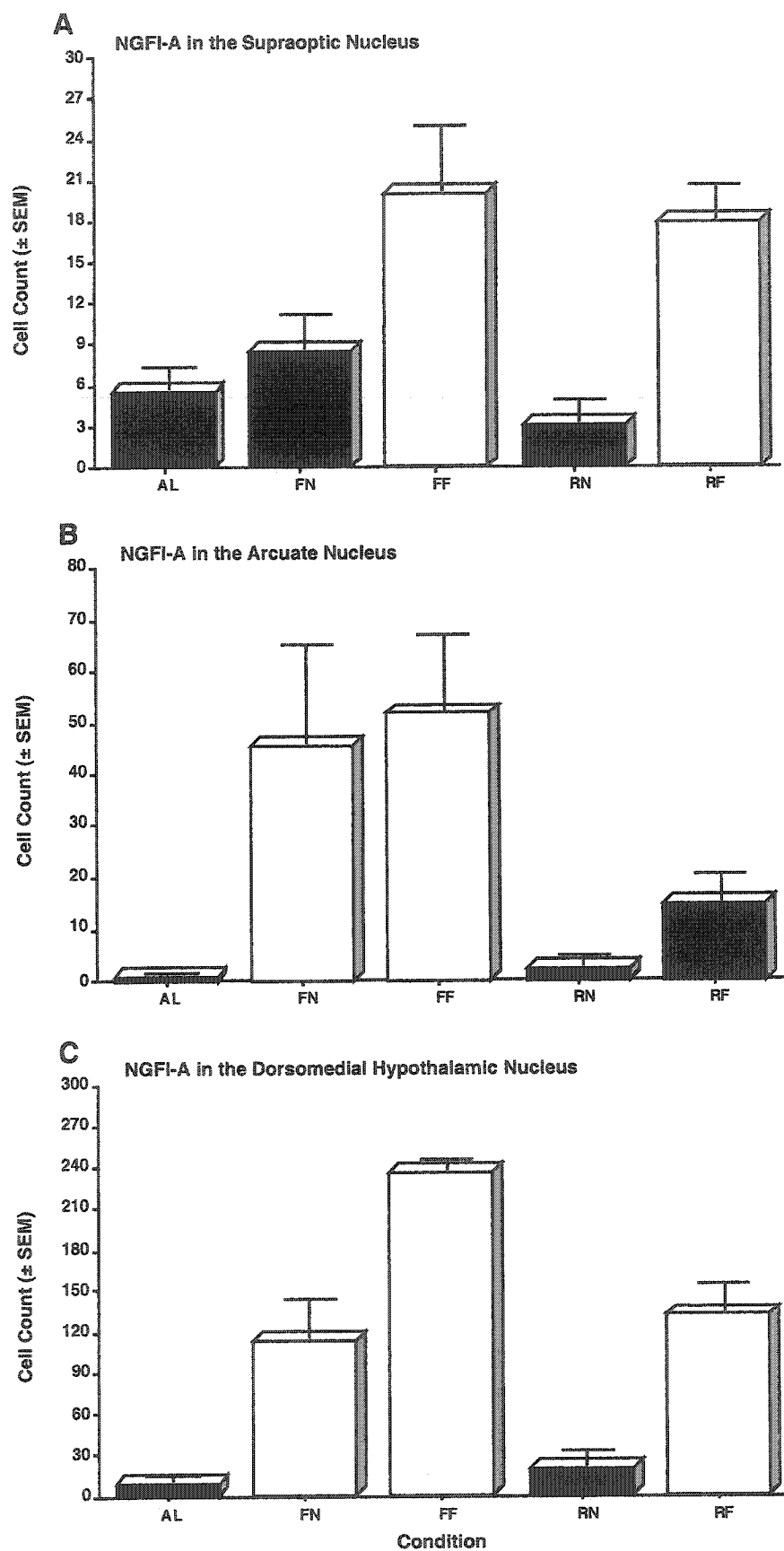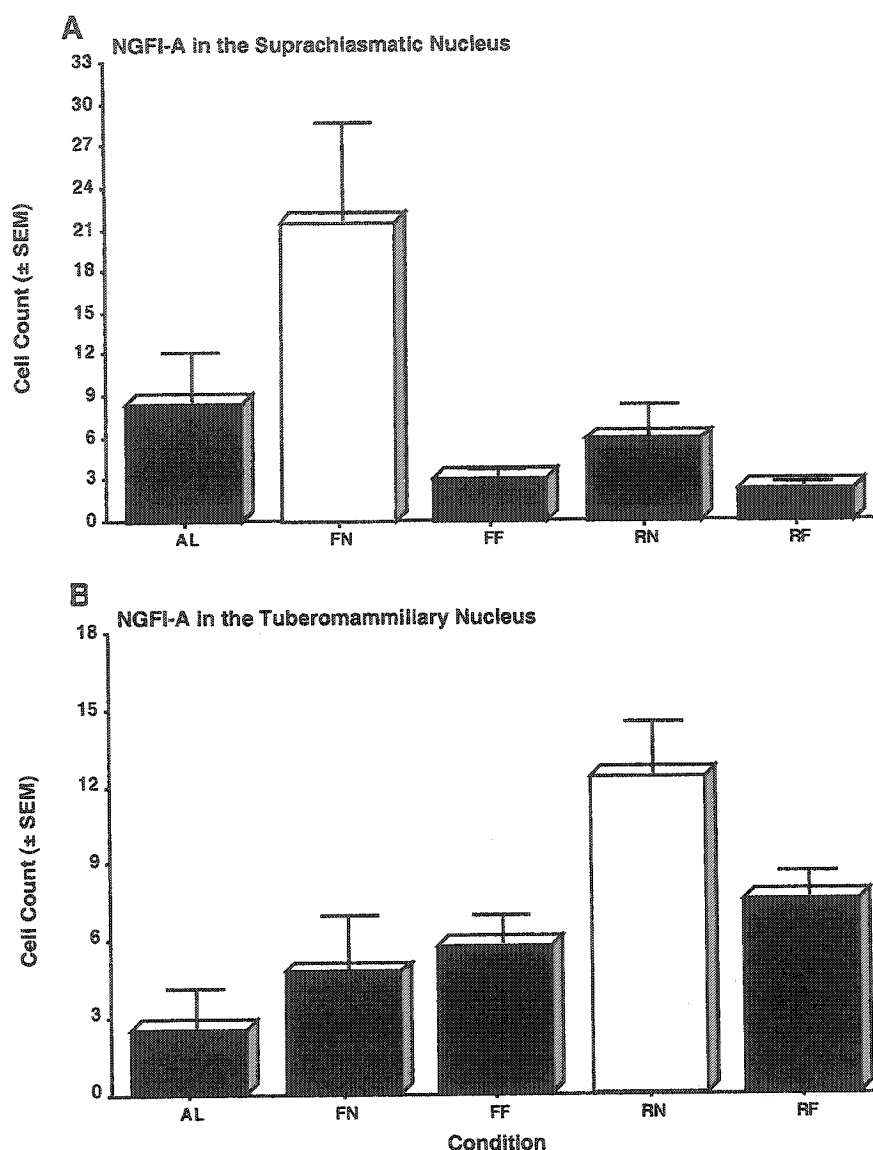
Figure 5.18

Figure 5.19. Average number of NGFI-A immunoreactive cell nuclei in two brain structures of animals in the five feeding conditions (AL = *Ad Libitum*, FN = Fasted-NotFed, FF = Fasted-Fed, RN = Restricted-NotFed and RF = Restricted-Fed; n=4/group). Results for the suprachiasmatic nucleus (A) and the tuberomammillary nucleus (B) are shown. Experimental condition is plotted along the abscissa and average cell count (± SEM) is plotted along the ordinate. For each structure, the group averages shown as open bars are significantly greater than the AL group average (P < 0.05).

The results presented above show that acute food deprivation for 19.5 h alters immediate-early gene expression in several brain regions, and that refeeding following a period of acute fasting further alters gene expression patterns. Repeated cycles of exposure to a schedule of food restriction eliminate the changes in gene expression

observed in response to a ~20 h period of food deprivation. In contrast to this finding, food intake after a ~20 h period of food deprivation results in gene expression patterns in habitually food-restricted animals that overlap, but are not identical to, those found in animals that are acutely fasted.

In summary, there is a pattern of gene expression changes in the brain that appears when an animal has recently been eating. This pattern differs depending upon whether the meal was regularly scheduled (and therefore anticipated) or whether it occurred at an unpredictable phase. There is also a pattern of gene expression changes in the brain that is associated with acute food deprivation. This pattern of change in gene expression is not expressed in animals once they have experienced repeated, regular cycles of deprivation and refeeding. To further explore these changes in gene expression patterns, posthoc comparisons were made between specific experimental conditions.

5.3.5 Effects of Refeeding Following Fasting

In order to assess the effects of refeeding following a 20 h fast in either the acute or chronic condition, immediate-early gene quantification results for the FF group were compared to those for the FN group, and those for the RF group were compared to those for the RN group (Table 5.4).

c-Fos expression

Refeeding following a 20 h fast altered c-Fos-Ir within some of the thirteen brain structures in both acutely fasted animals and chronically restricted animals (Table 5.4). For acutely fasted animals, a 1 h feeding opportunity increased c-Fos-Ir within the PB, the Arc, the DMH and the TU (c-Fos - Acute; Table 5.4). c-Fos-Ir was up-regulated following a 1 h meal in chronically restricted animals within the PB, the SON, the DMH, the LH, the TU, the PVT, the IGL, and the TMN (c-Fos - Chronic; Table 5.4). Photographs of c-Fos-Ir within the PB and the SON of representative animals from the RN and RF conditions are shown in Figure 5.20.

Effects of 1 h of food access following acute or chronic fasting.

	c-Fos		Jun-B		NGFI-A	
	Acute	Chronic	Acute	Chronic	Acute	Chronic
PB	+	+				+
SON		+			+	+
Arc	+					
PVN			−			+
DMH	+	+	+		+	+
LH		+	+		+	+
TU	+	+				
PVT		+	+		+	+
VMH			+		+	
LC			+			
SCN			−		−	
IGL		+	−			
TMN		+				−

Table 5.4. Effects of refeeding following a 20 h fast on immediate-early gene expression in 13 brain structures. For the Acute condition, results of the Fasted-Fed (FF) group were compared to those of the Fasted-NotFed (FN) group. For the Chronic condition, results of the Restricted-Fed (RF) group were compared to those of the Restricted-NotFed (RN) group. The + indicates that the fed group had higher immunoreactive cell counts and the − indicates that the fed group had lower immunoreactive cell counts than their unfed controls. Differences indicated are significant ($P < 0.05$). Blanks = no significant difference between groups.

In summary, refeeding following a 20 h fast increased c-Fos-Ir in the PB, DMH and TU of both acutely and chronically fasted animals. c-Fos-Ir was up-regulated within the Arc of acutely fasted but not chronically restricted animals following refeeding, and was up-regulated within the SON, LH, PVT, IGL and TMN of chronically restricted but not acutely fasted animals following food consumption.

Jun-B expression

In acutely fasted animals, a 1 h opportunity to feed resulted in a significant up-regulation of Jun-B-Ir within the DMH, the LH, the PVT, the VMH, and the LC compared to those not allowed to consume food (Jun-B - Acute; Table 5.4). Within the PVN, the SCN and the IGL, Jun-B-Ir was significantly down-regulated in animals allowed
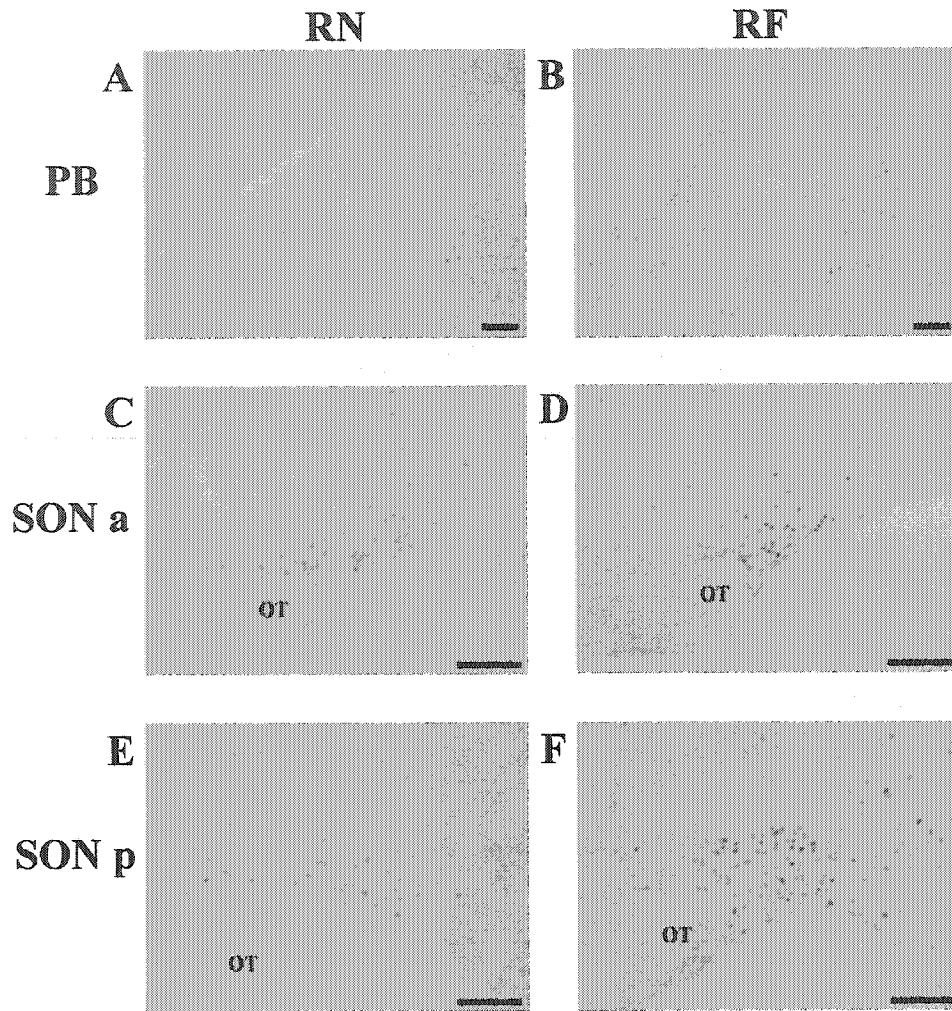
Figure 5.20. c-Fos-Ir in the parabrachial nucleus (PB) and the supraoptic nucleus (SON) of representative animals from the Restricted-NotFed (RN) and Restricted-Fed (RF) conditions. Photographs of the SON are shown at two anatomical levels (anterior = a; posterior = p). The scale bars = 100 μm. OT = optic tract.

access to food for 1 h following a 20 h fast when compared to those not refed (Jun-B - Acute; Table 5.4). In contrast, consuming a meal after an equivalent deprivation period at the regularly scheduled mealtime in chronically restricted animals had no effect on Jun-B-Ir in any of the brain regions analyzed. That is, no significant differences in Jun-B-Ir were found between the RN and RF groups in any brain structure (Jun-B - Chronic; Table 5.4). Thus, the changes in Jun-B-Ir found in the brains of animals acutely fasted and refed were absent in animals that had been exposed to repeated daily cycles of fasting and refeeding.

NGFI-A expression

Food access for 1 h following a 20 h fast increased NGFI-A-Ir significantly within

the SON, the DMH, the LH, the PVT and the VMH of acutely fasted animals (NGFI-A -

Acute; Table 5.4). Access to food significantly decreased NGFI-A-Ir within the SCN of

animals acutely fasted when compared to those not receiving food (NGFI-A - Acute;

Table 5.4). In animals entrained by the restricted feeding paradigm, access for 1 h to the

normal daily meal increased NGFI-A-Ir within the PB, the SON, the PVN, the DMH, the
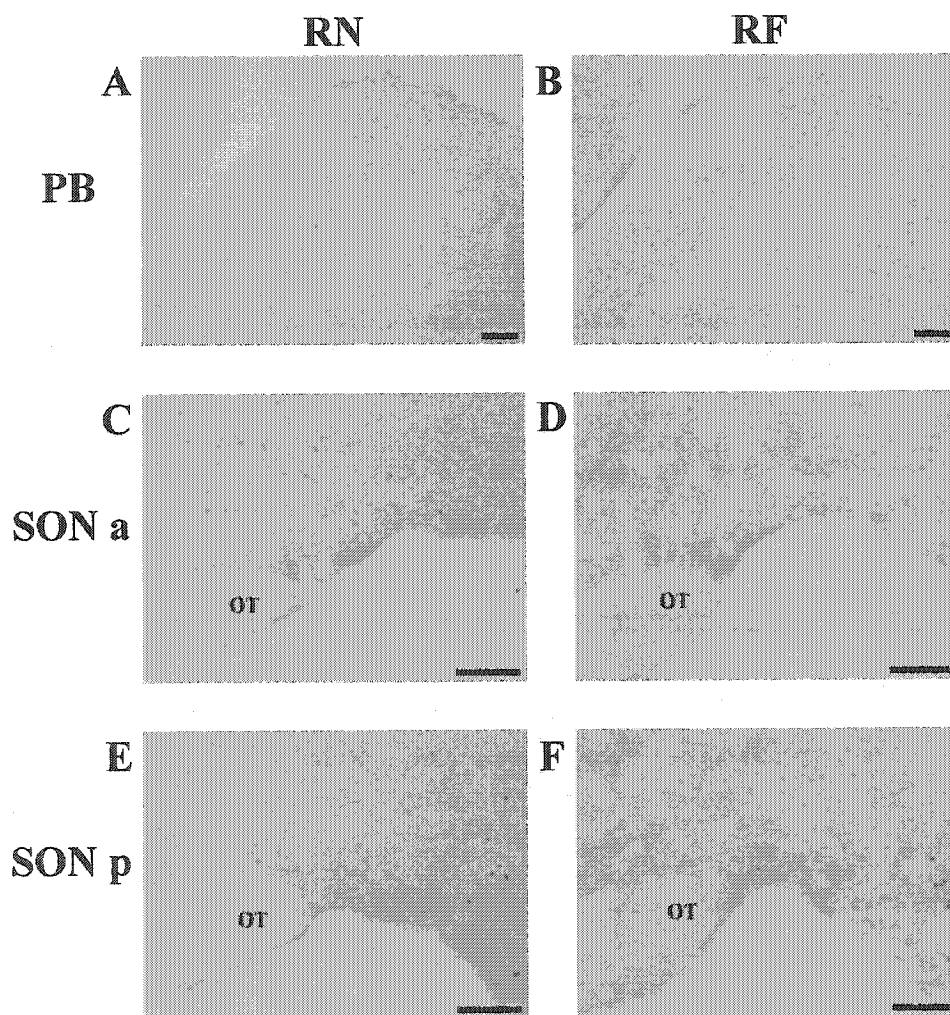
Figure 5.21. NGFI-A-Ir in the parabrachial nucleus (PB) and the supraoptic nucleus (SON) of representative animals from the Restricted-NotFed (RN) and Restricted-Fed (RF) conditions. Photographs of the SON are shown at two anatomical levels (anterior = a; posterior = p). The scale bars = 100 μm. OT = optic tract.

LH and the PVT (NGFI-A - Chronic; Table 5.4; Figure 5.21: PB and SON). NGFI-A-Ir within the TMN was decreased in chronically restricted animals following access to a 1 h meal at the regular mealtime (NGFI-A - Chronic; Table 5.4; Figure 5.22).

To summarize, access to food following 20 h of food deprivation up-regulated NGFI-A-Ir within the SON, DMH, LH and PVT of both acutely and chronically fasted animals. NGFI-A-Ir was up-regulated after food access in the VMH of acutely fasted but not chronically restricted animals, and in the PB and PVN of chronically restricted but not acutely fasted animals. In contrast, NGFI-A was down-regulated after food access in the SCN of acutely fasted but not chronically restricted animals, and in the TMN of chronically restricted but not acutely fasted animals.
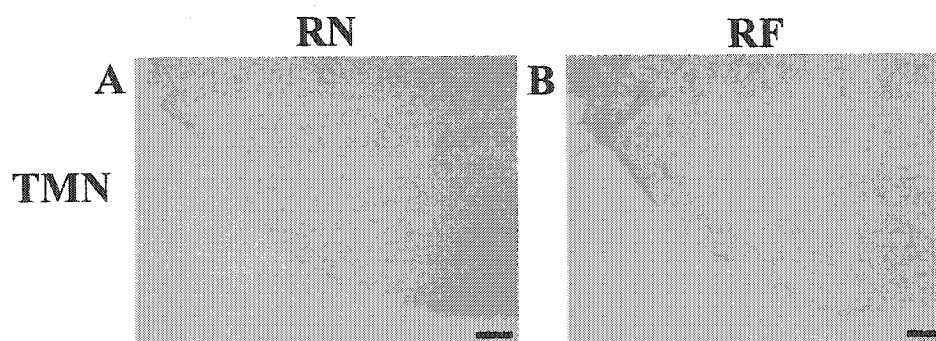
Figure 5.22. NGFI-A-Ir in the tuberomammillary nucleus (TMN) of representative animals from the Restricted-NotFed (RN) and Restricted-Fed (RF) conditions. The scale bars = 100 μm.

5.3.6 Effects of Chronic Exposure to Daily Restricted Feeding

In order to assess the effects of chronic food restriction on immediate-early gene expression within the brain, immediate-early gene quantification results for the RN group were compared to those for the FN group, and those for the RF group were compared to those for the FF group (Table 5.5).

c-Fos expression

c-Fos-Ir was significantly down-regulated within the PB, the Arc, the PVN, the DMH, the LH, the TU, the PVT, the SCN and the IGL in chronically restricted animals

Effects of chronic restriction before or after 1 h of food access.

	c-Fos		Jun-B		NGFI-A	
	Not Fed	Fed	Not Fed	Fed	Not Fed	Fed
PB	−					+
SON						
Arc	−	−	−	−	−	
PVN	−	−	−		−	
DMH	−	−			−	−
LH	−				−	−
TU	−					
PVT				−		−
VMH				−		
LC				−		
SCN	−		−		−	
IGL	−		−			
TMN		+			+	

Table 5.5. Effects of chronic restriction on immediate-early gene expression in 13 brain structures. For the Not Fed condition, results of the Restricted-NotFed (RN) group were compared to those of the Fasted-NotFed (FN) group. For the Fed condition, results of the Restricted-Fed (RF) group were compared to those of the Fasted-Fed (FF) group. The + indicates that the restricted group had higher immunoreactive cell counts and the − indicates that the restricted group had lower immunoreactive cell counts than their acutely fasted controls. Differences indicated are significant (P < 0.05). Blanks = no significant difference between groups.

anticipating a daily meal (RN) when compared to acutely fasted animals (FN) (c-Fos − Not Fed; Table 5.5; Figure 5.23: Arc and PVN; Figure 5.24: DMH, LH, TU and PVT; Figure 5.25: SCN and IGL). Chronically restricted animals allowed a 1 h meal at their habitual mealtime showed significant differences in c-Fos-Ir in several brain structures when compared to acutely fasted animals that were refed at the same time (c-Fos - Fed; Table 5.5). The RF group showed significantly less c-Fos-Ir within the Arc, the PVN and the DMH when compared to the FF group. In contrast, c-Fos-Ir was up-regulated in the TMN of chronically restricted animals compared to acutely fasted animals following a 1 h feeding opportunity (c-Fos - Fed; Table 5.5; Figure 5.26).

Thus, chronic exposure to a restricted feeding schedule altered c-Fos-Ir in the Arc, PVN, and DMH both before and after habitual mealtime. Furthermore, c-Fos-Ir was altered by chronic restriction before mealtime in the PB, LH, TU, PVT, SCN and IGL, and after mealtime in the TMN.

Figure 5.23. c-Fos-Ir in the arcuate nucleus (Arc) and the paraventricular nucleus of the hypothalamus (PVN) of representative animals from the Fasted-NotFed (FN) and Restricted-NotFed (RN) conditions. Photographs of the Arc are shown at two anatomical levels (anterior = a; posterior = p). The scale bars = 100 μm. 3V = 3rd ventricle.

Figure 5.24. c-Fos-Ir in the dorsomedial hypothalamic nucleus (DMH), the lateral hypothalamus (LH), the olfactory tubercle (TU) and the paraventricular nucleus of the thalamus (PVT) of representative animals from the Fasted-NotFed (FN) and Restricted-NotFed (RN) conditions. The scale bars = 100 μm. 3V = 3rd ventricle, D3V = dorsal 3rd ventricle.
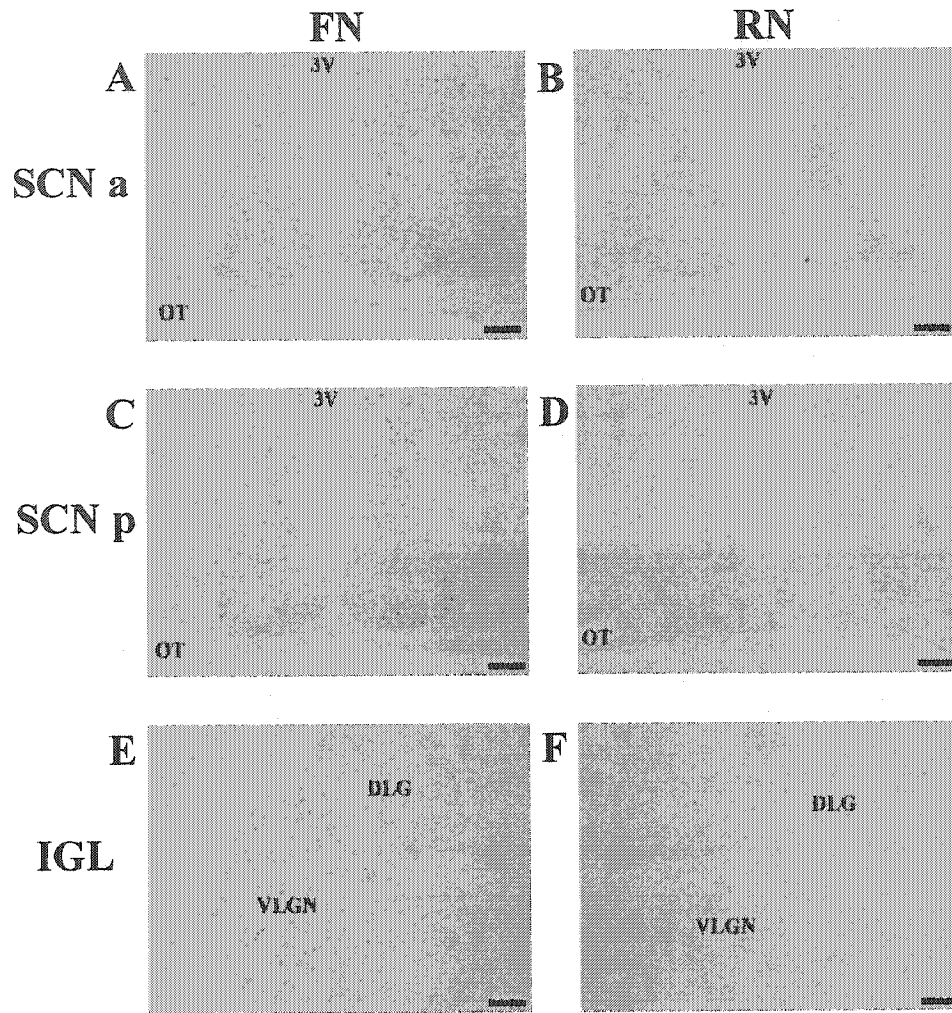
Figure 5.25. c-Fos-Ir in the suprachiasmatic nucleus (SCN) and the intergeniculate leaflet (IGL) of representative animals from the Fasted-NotFed (FN) and Restricted-NotFed (RN) conditions. Photographs of the SCN are shown at two anatomical levels (anterior = a; posterior = p). The scale bars = 100 μm. 3V = 3rd ventricle, OT = optic tract, DLG = dorsolateral geniculate nucleus, VLGN = ventrolateral geniculate nucleus.
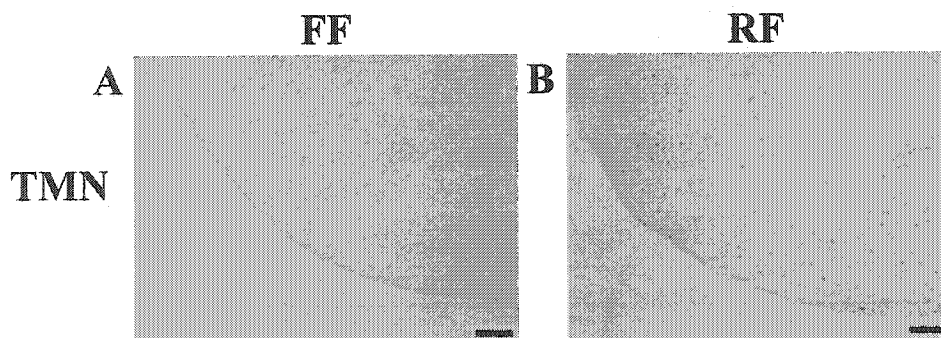
Figure 5.26. c-Fos-Ir in the tuberomammillary nucleus (TMN) of representative animals from the Fasted-Fed (FF) and Restricted-Fed (RF) conditions. The scale bars = 100 μm.

Jun-B expression

In animals anticipating their daily meal, Jun-B-Ir was significantly down-regulated in the Arc, the PVN, the SCN and the IGL when compared to animals that were acutely fasted (Jun-B – Not Fed; Table 5.5; Figure 5.27: Arc and PVN; Figure 5.28: SCN and IGL). As shown in Figures 5.28 E and F, overall Jun-B-Ir within the IGL was very low, although a statistically significant up-regulation of Jun-B-Ir was found for animals in the FN condition when compared to all other conditions. When chronically restricted animals were given food for 1 h at the regular time, Jun-B-Ir was significantly decreased in the
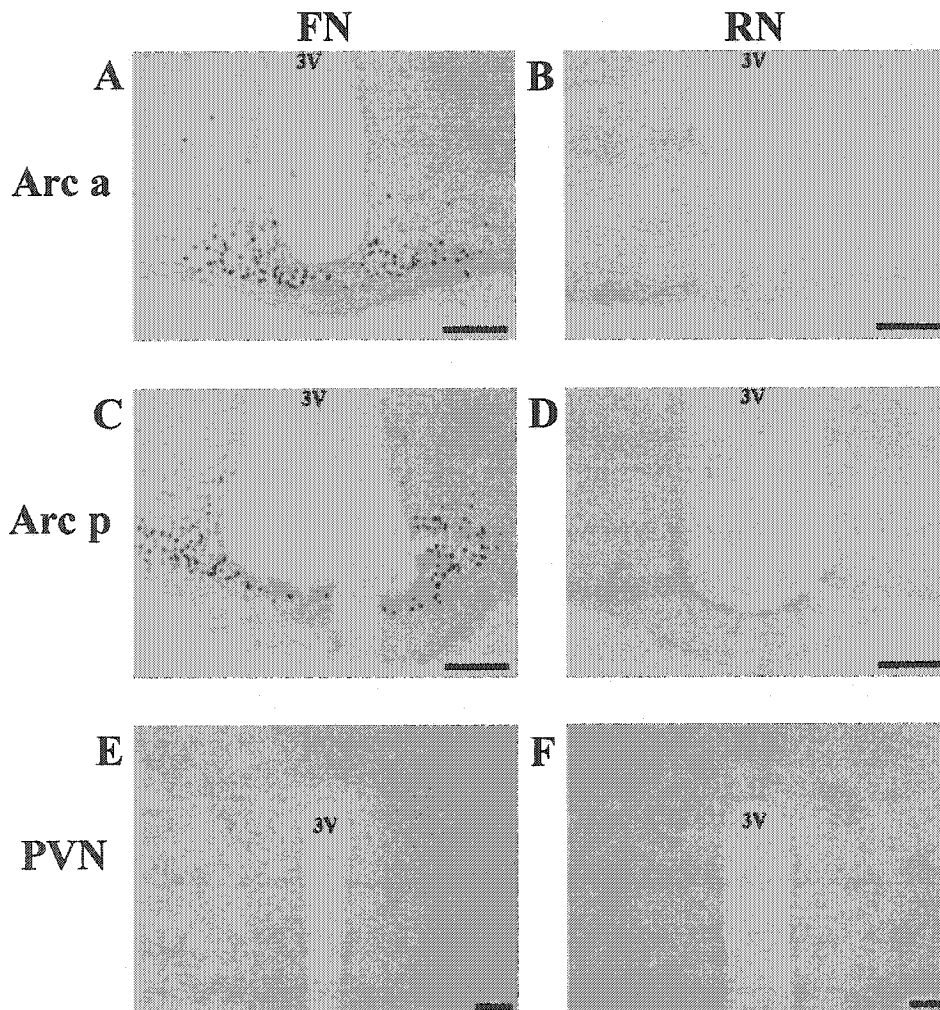
Figure 5.27. Jun-B-Ir in the arcuate nucleus (Arc) and the paraventricular nucleus of the hypothalamus (PVN) of representative animals from the Fasted-NotFed (FN) and Restricted-NotFed (RN) conditions. Photographs of the Arc are shown at two anatomical levels (anterior = a; posterior = p). The scale bars = 100 μm. 3V = 3rd ventricle.

Arc, the PVN, the DMH, the LH, the PVT, the VMH and the LC when compared to acutely fasted animals refed for 1 h (Jun-B - Fed; Table 5.5; Figure 5.29: DMH, LH, TU and PVT). There was a high degree of within-group variability for Jun-B-Ir within the TU; therefore, although the representative photographs shown in Figures 5.29E and F suggest a difference between conditions, there was no statistically significant difference between these groups (Table 5.2). Jun-B-Ir counts were very low in the LH (Figures 5.29C and D) and the PVT (Figures 5.29G and H), but statistically significant differences among groups were found for these structures (Table 5.2).
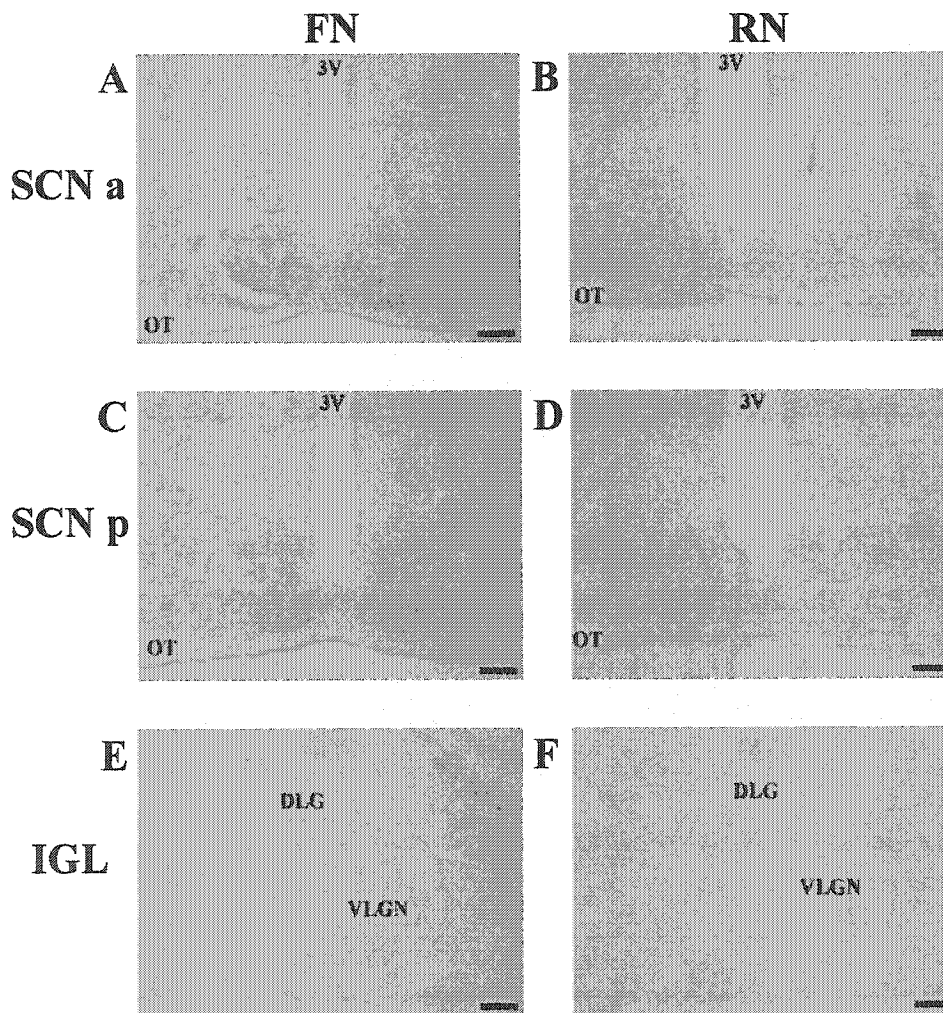
Figure 5.28. Jun-B-Ir in the suprachiasmatic nucleus (SCN) and the intergeniculate leaflet (IGL) of representative animals from the Fasted-NotFed (FN) and Restricted-NotFed (RN) conditions. Photographs of the SCN are shown at two anatomical levels (anterior = a; posterior = p). The scale bars = 100 μm. 3V = 3rd ventricle, OT = optic tract, DLG = dorsolateral geniculate nucleus, VLGN = ventrolateral geniculate nucleus.

Figure 5.29. Jun-B-Ir in the dorsomedial hypothalamic nucleus (DMH), the lateral hypothalamus (LH), the olfactory tubercle (TU) and the paraventricular nucleus of the thalamus (PVT) of representative animals from the Fasted-Fed (FF) and Restricted-Fed (RF) conditions. The scale bars = 100 μm. 3V = 3rd ventricle, D3V = dorsal 3rd ventricle.

In summary, chronic exposure to a restricted feeding schedule altered Jun-B-Ir in the Arc and PVN both before and after food access at habitual feeding time. In addition,

Jun-B-Ir was altered by chronic restricted feeding before mealtime in the SCN and IGL, and after mealtime in the DMH, LH, PVT, VMH and LC.

NGFI-A expression

Animals that were anticipating their daily feeding opportunity expressed significantly lower levels of NGFI-A-Ir within the Arc, the PVN, the DMH, the LH, the PVT and the SCN when compared to animals that were acutely fasted (NGFI-A – Not Fed; Table 5.5; Figure 5.30: Arc and PVN; Figure 5.31: DMH, LH and PVT; Figure 5.32: SCN). In contrast, animals anticipating the daily meal showed significantly more NGFI-
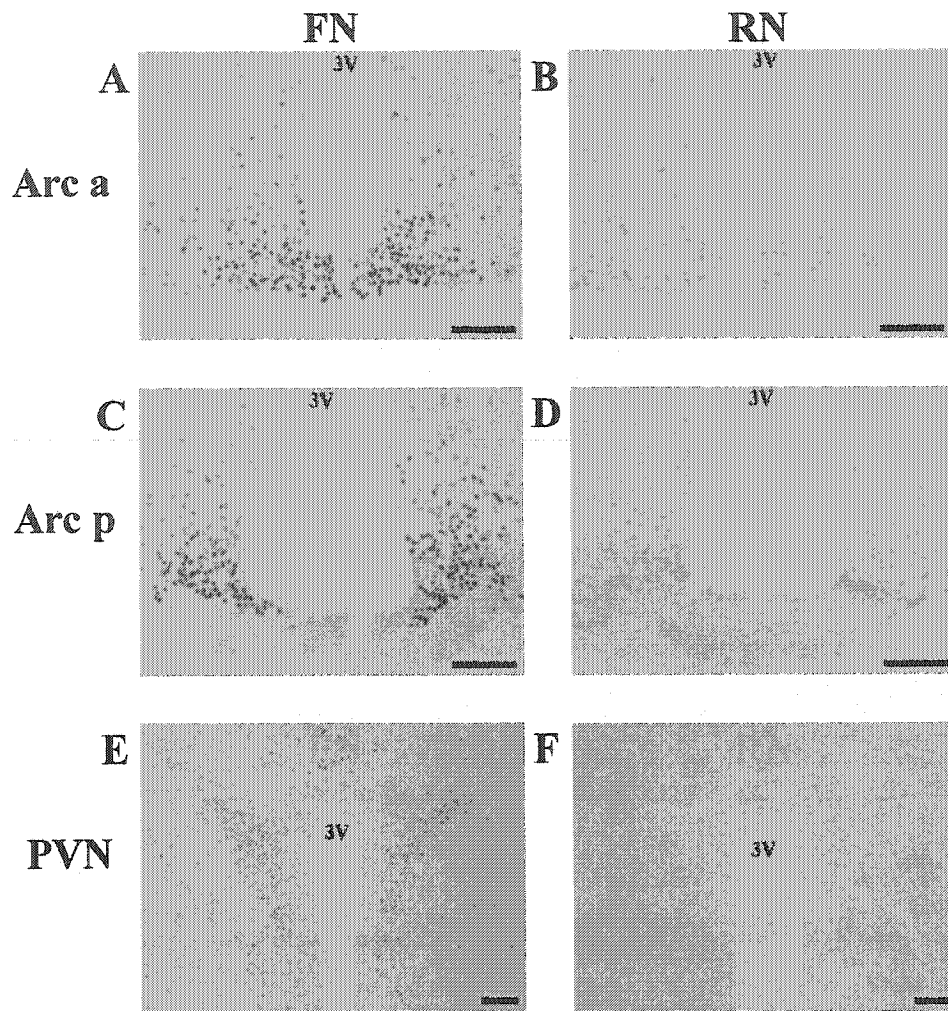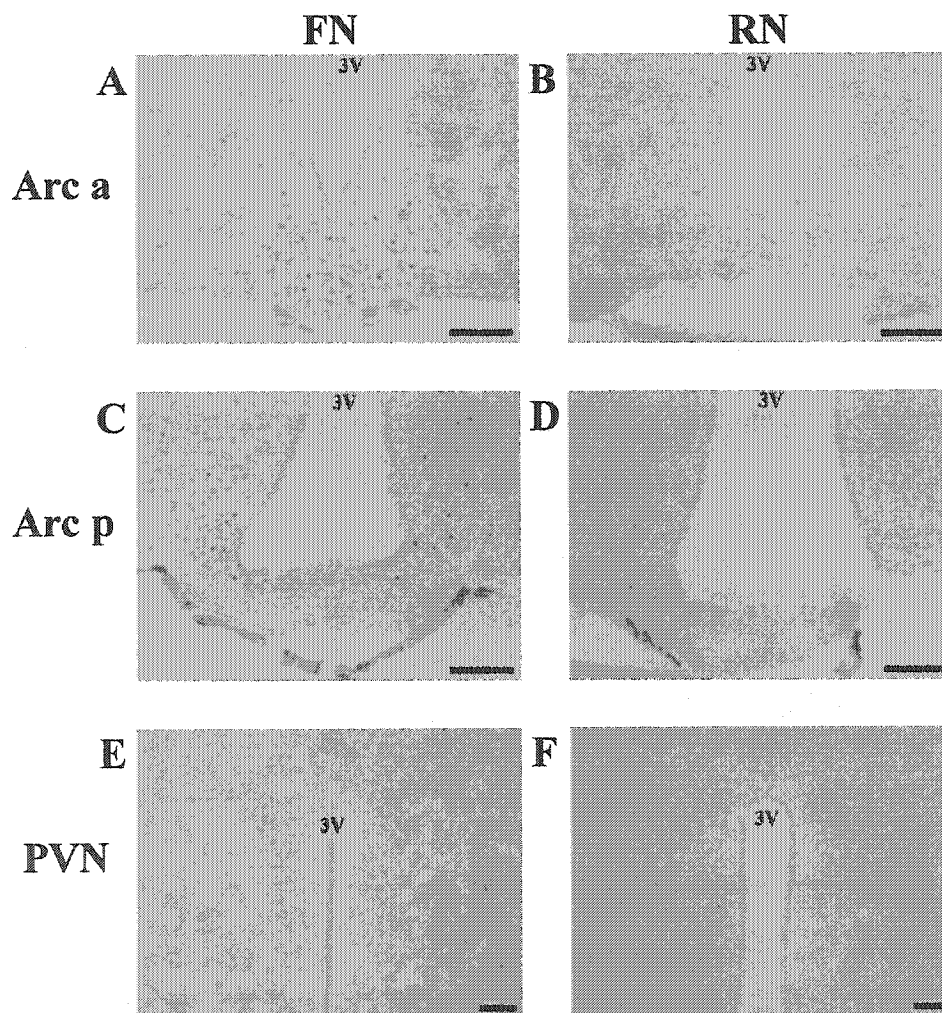

Figure 5.30. NGFI-A-Ir in the arcuate nucleus (Arc) and the paraventricular nucleus of the hypothalamus (PVN) of representative animals from the Fasted-NotFed (FN) and Restricted-NotFed (RN) conditions. Photographs of the Arc are shown at two anatomical levels (anterior = a; posterior = p). The scale bars = 100 μm. 3V = 3rd ventricle.

A-Ir within the TMN than did animals that were acutely fasted (NGFI-A – Not Fed; Table 5.5). Chronically restricted animals allowed access to their normal daily meal showed significantly more NGFI-A-Ir within the PB than did animals acutely fasted and refed (NGFI-A - Fed; Table 5.5). In contrast, restricted animals allowed to feed showed significantly less NGFI-A-Ir within the DMH, the LH, the PVT and the VMH than those acutely fasted and refed (NGFI-A - Fed; Table 5.5; Figure 5.33: VMH).

To summarize, NGFI-A-Ir was altered by chronic exposure to a restricted feeding
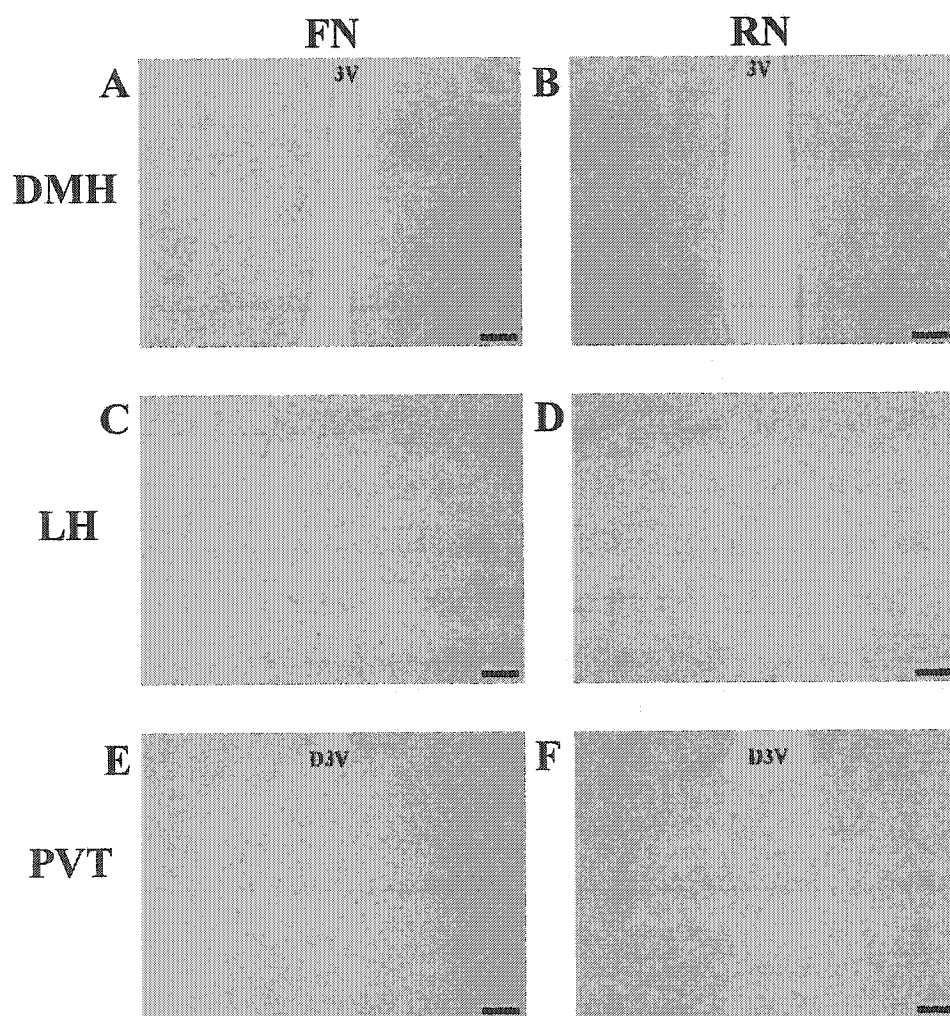
Figure 5.31. NGFI-A-Ir in the dorsomedial hypothalamic nucleus (DMH), the lateral hypothalamus (LH) and the paraventricular nucleus of the thalamus (PVT) of representative animals from the Fasted-NotFed (FN) and Restricted-NotFed (RN) conditions. The scale bars = 100 µm. 3V = 3rd ventricle, D3V = dorsal 3rd ventricle.

schedule in the DMH, LH and PVT both before and after the regularly scheduled mealtime. Furthermore, NGFI-A-Ir was altered in chronically restricted animals before mealtime in the Arc, PVN, SCN and TMN, and after mealtime in the PB and VMH.
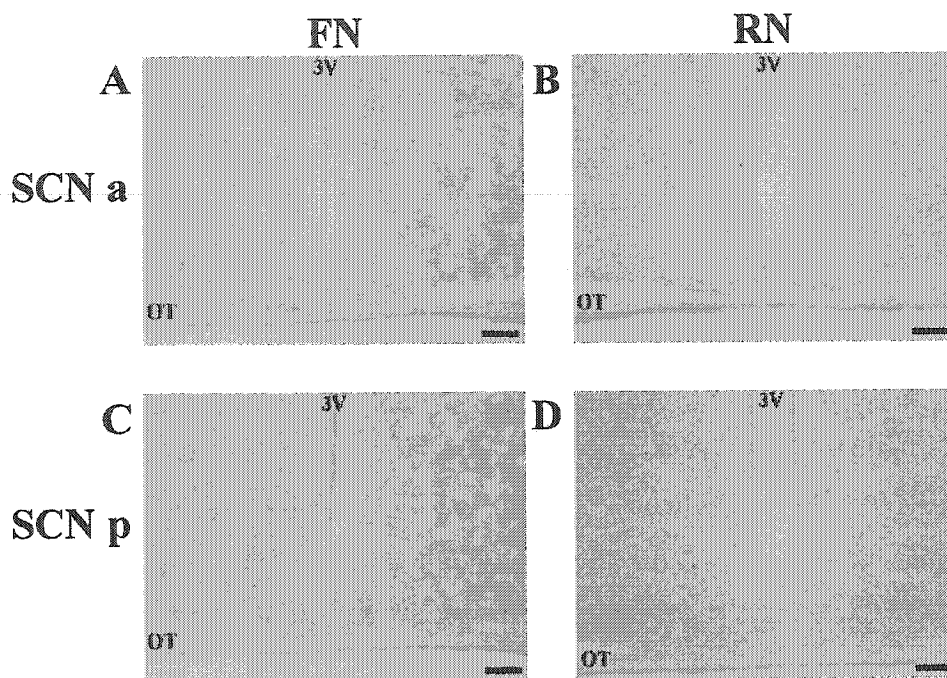
Figure 5.32. NGFI-A-Ir in the suprachiasmatic nucleus (SCN) of representative animals from the Fasted-NotFed (FN) and Restricted-NotFed (RN) conditions. Photographs of the SCN are shown at two anatomical levels (anterior = a; posterior = p). The scale bars = 100 μm. 3V = 3rd ventricle, OT = optic tract.
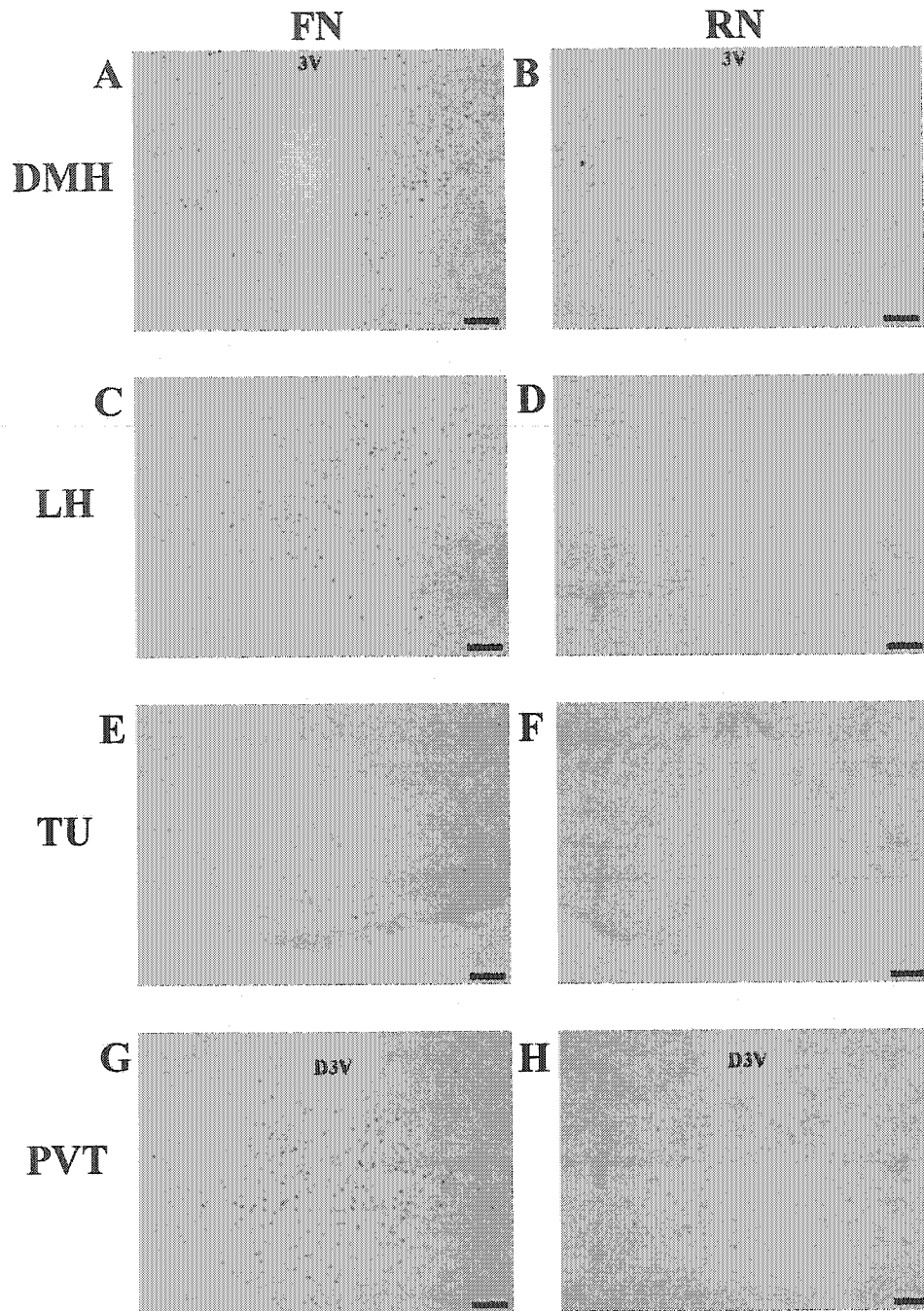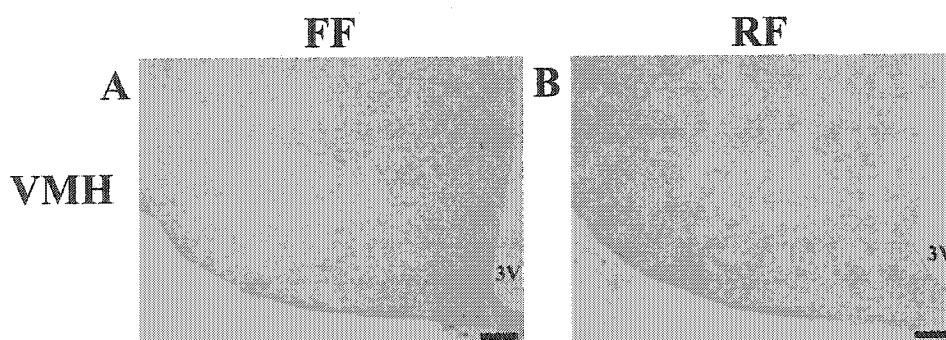

Figure 5.33. NGFI-A-Ir in the ventromedial hypothalamic nucleus (VMH) of representative animals from the Fasted-Fed (FF) and Restricted-Fed (RF) conditions. The scale bars = 100 μm. 3V = 3rd ventricle.

5.3.7 Immediate-Early Gene Expression – Experiment 2

Six brain structures processed for detection of c-Fos-Ir and four brain structures processed for detection of NGFI-A-Ir in Experiment 1 met the three criteria for inclusion in Experiment 2 (see Methods section). None of the brain structures showing Jun-B-Ir met the inclusion criteria. For c-Fos analyses, the DMH, the LH, the IGL, the PVT, the TMN and the TU were included. For NGFI-A, the PVN, the DMH, the LH and the TMN were included.

c-Fos expression

Statistical analyses revealed significant differences in c-Fos-Ir among the four feeding conditions in all six structures included in the analyses (Table 5.6).

Two patterns of c-Fos-Ir results were found among the six structures analyzed. Examples of these are shown in Figure 5.34. c-Fos-Ir was significantly up-regulated in the RF6 condition when compared to the other three conditions within the DMH, the LH,

c-Fos	RN3	RF3	RN6	RF6	
DMH	7.2±3.68 a	9.2±3.17 a	1.2±0.58 a	42.4±11.46 b	$F_{(3,16)}$ = 8.9; P = 0.0011
LH	49.6±18.24 a	62.4±15.45 a	10.4±5.94 a	111.2±20.09 b	$F_{(3,16)}$ = 6.8; P = 0.0035
TU	1.8±0.49 a	3.8±1.36 a	0.6±0.40 a	7.8±1.50 b	$F_{(3,16)}$ = 8.9; P = 0.0011
PVT	23.6±8.79 a	33.4±9.90 a	7.4±3.06 a	60.0±11.19 b	$F_{(3,16)}$ = 6.3; P = 0.0051
IGL	61.4±12.50 b	60.0±5.33 b	18.0±3.08 a	65.2±5.75 b	$F_{(3,16)}$ = 8.7; P = 0.0012
TMN	7.2±1.11 b	5.6±0.98 b	1.2±0.58 a	8.8±1.32 b	$F_{(3,16)}$ = 10.0; P = 0.0006

Table 5.6. c-Fos immunoreactive cell counts in brain structures of mice in the four different feeding conditions (RN3 = Restricted-NotFed Day3; RF3 = Restricted-Fed Day 3; RN6 = Restricted-NotFed Day 6; RF6 = Restricted-Fed Day 6; n=5/group). Group averages are shown ± SEM. Results of statistical comparison among conditions for each structure are shown in the column on the right. For each row, values not sharing a letter (a or b) differ significantly (P < 0.05). DMH = dorsomedial hypothalamic nucleus; LH = lateral hypothalamus; TU = olfactory tubercle; PVT = paraventricular nucleus of the thalamus; IGL = intergeniculate leaflet; TMN = tuberomammillary nucleus.

Figure 5.34. Average number of c-Fos immunoreactive cell nuclei in three brain structures of animals in the four feeding conditions (RN3 = Restricted-NotFed Day3; RF3 = Restricted-Fed Day 3; RN6 = Restricted-NotFed Day 6; RF6 = Restricted-Fed Day 6; n=5/group). Results for the dorsomedial hypothalamic nucleus (A), the intergeniculate leaflet (B) and the tuberomammillary nucleus (C) are shown. Experimental condition is plotted along the abscissa and average cell count ($\pm$ SEM) is plotted along the ordinate. For each structure, the group averages shown as open bars are significantly greater than group averages shown as black bars ($P < 0.05$).

A c-Fos in the Dorsomedial Hypothalamic Nucleus

B c-Fos in the Intergeniculate Leaflet

C c-Fos in the Tuberomammillary Nucleus
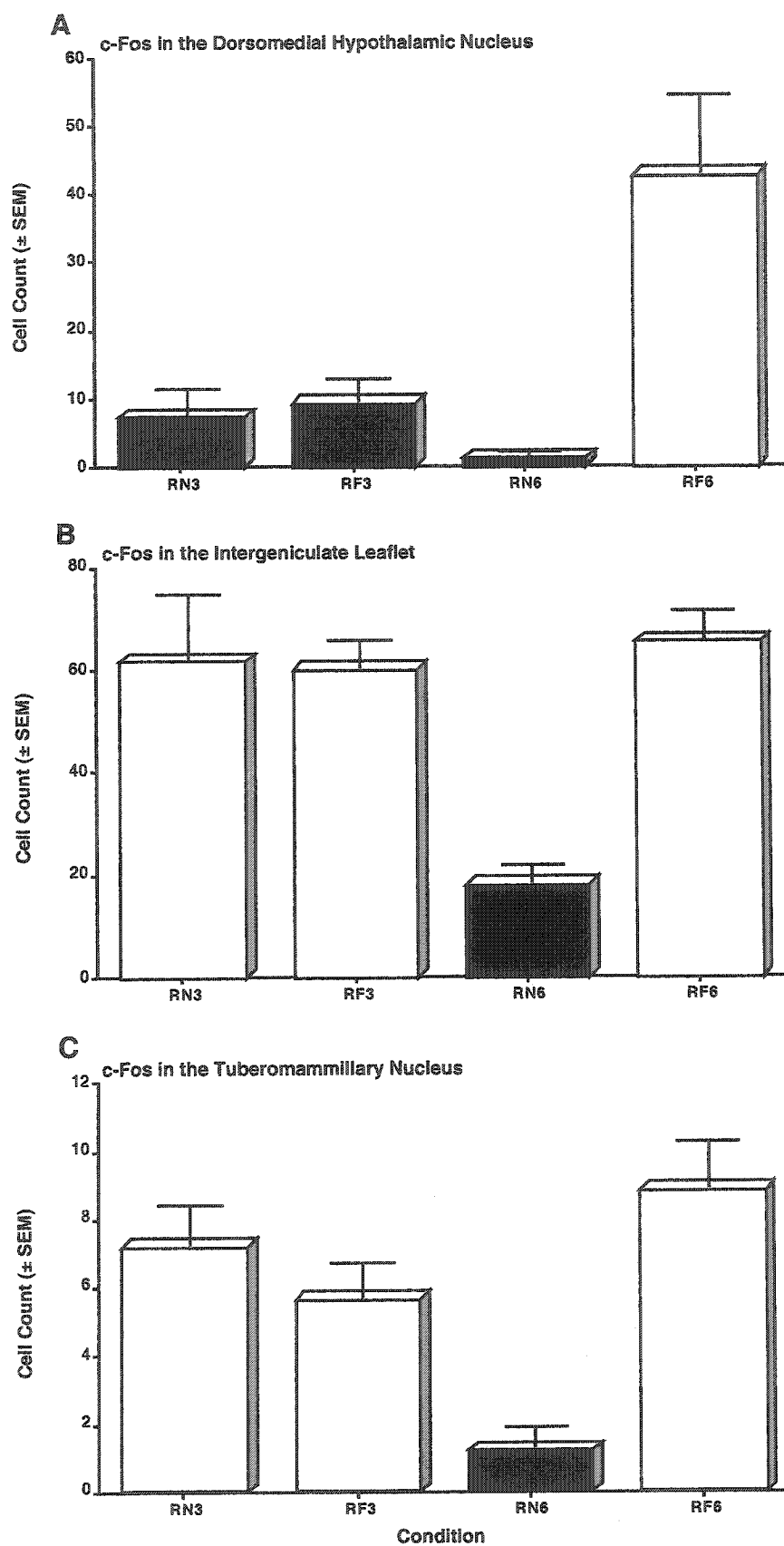
Figure 5.34

the TU and the PVT (see Figure 5.34A). Within the IGL and the TMN, significant down-regulation of c-Fos-Ir in the RN6 condition was found (see Figures 5.34B and C).

Access to a 1 h meal did not alter c-Fos-Ir in any of the six brain structures for animals killed on Day 3 of restricted feeding (RN3 and RF3; Table 5.6). On Day 6, however, 1 h of food access significantly up-regulated c-Fos-Ir within all six brain structures (RN6 and RF6; Table 5.6; Figure 5.35: DMH, LH, TU, and PVT; Figure 5.36: IGL; Figure 5.37: TMN). The numbers of c-Fos immunoreactive cell nuclei within the TU and the TMN were extremely low (Table 5.6); however, significant differences among conditions were found (see Figures 5.35E and F and 5.37A and B).

c-Fos-Ir in these structures changed significantly as exposure to the restricted feeding schedule continued from Day 3 to Day 6 (Table 5.6). c-Fos-Ir was significantly down-regulated within the IGL and the TMN in animals anticipating their daily meal on Day 6 compared to those anticipating a meal on Day 3 (RN3 and RN6; Table 5.6). Within the DMH, the LH, the PVT and the TU, animals refed on Day 6 expressed significantly more c-Fos-Ir than did those refed on Day 3 (RF3 and RF6; Table 5.6).
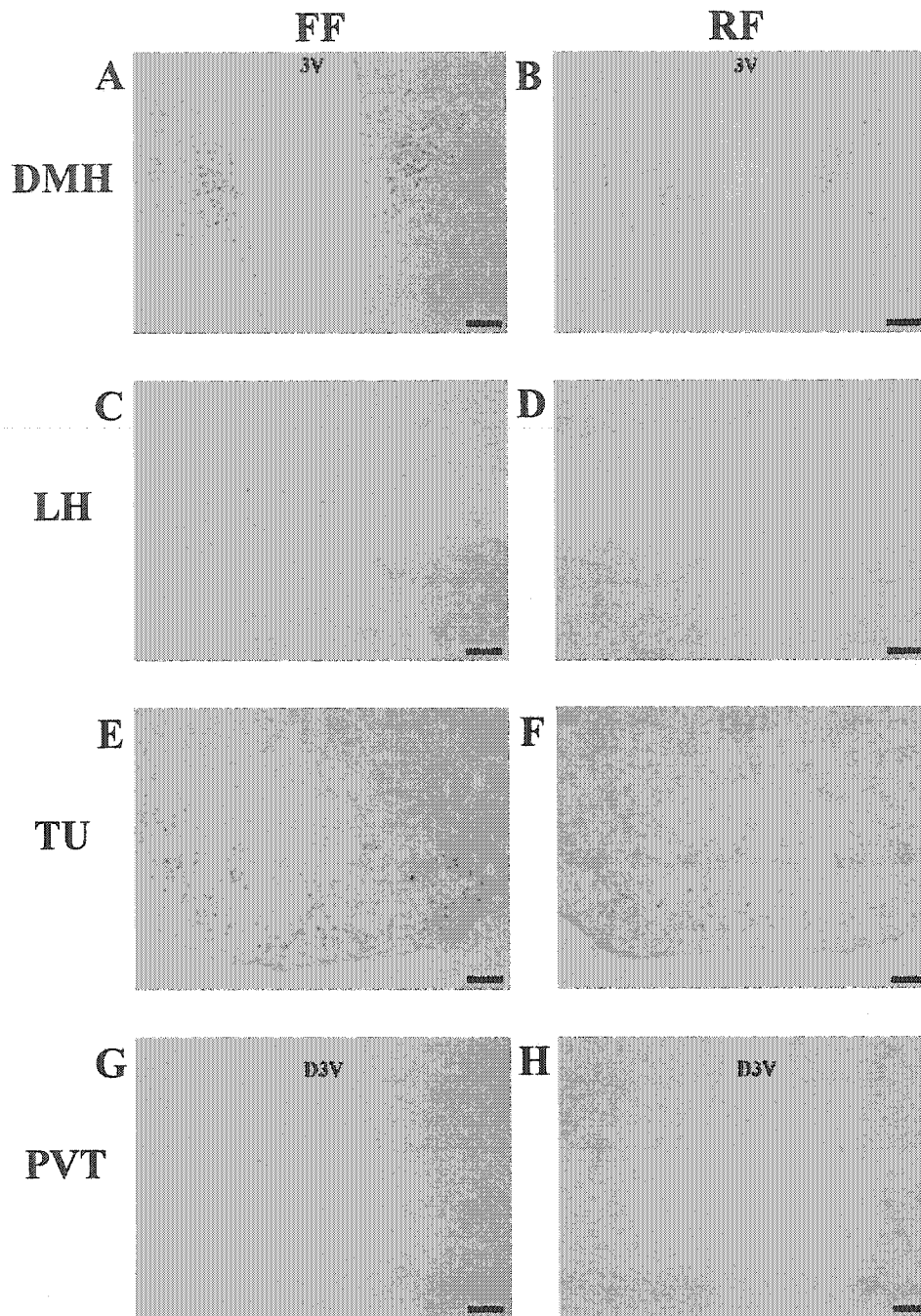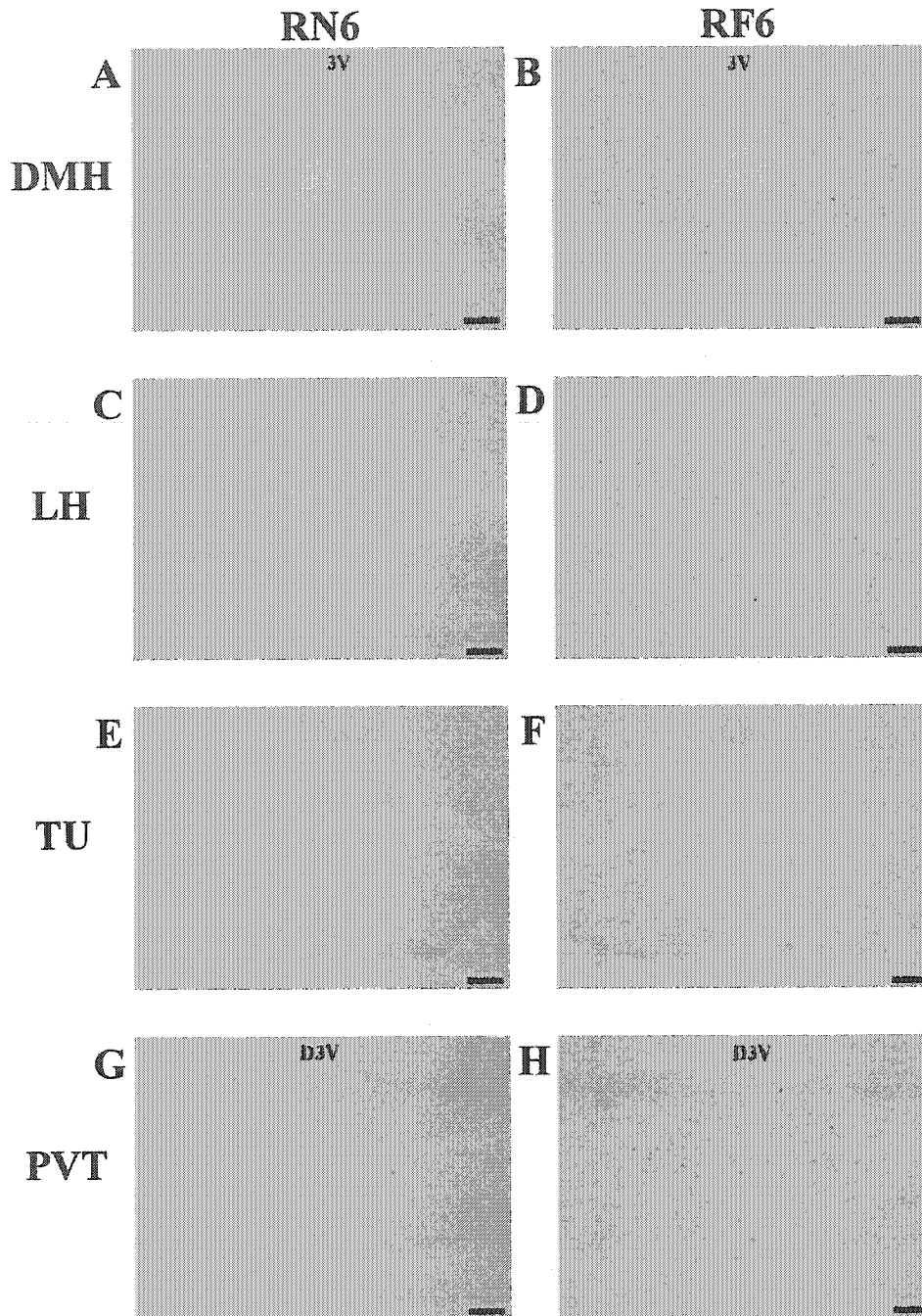
Figure 5.35. c-Fos-Ir in the dorsomedial hypothalamic nucleus (DMH), the lateral hypothalamus (LH), the olfactory tubercle (TU) and the paraventricular nucleus of the thalamus (PVT) of representative animals from the Restricted-NotFed Day 6(RN6) and Restricted-Fed Day 6 (RF6) conditions. The scale bars = 100 μm. 3V = 3rd ventricle, D3V = dorsal 3rd ventricle.
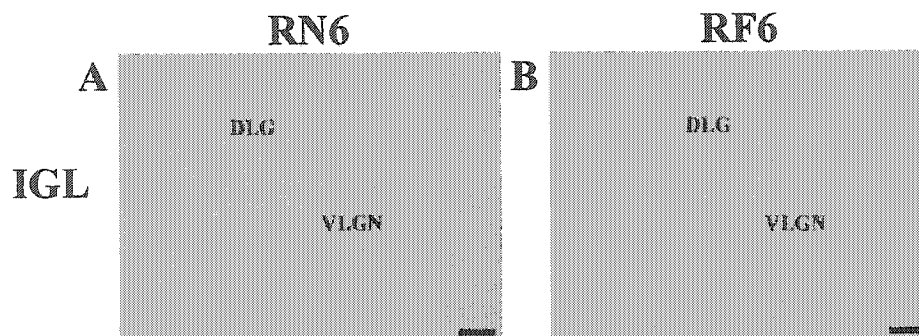
Figure 5.36. c-Fos-Ir in the intergeniculate leaflet (IGL) of representative animals from the Restricted-NotFed Day 6(RN6) and Restricted-Fed Day 6 (RF6) conditions. The scale bars = 100 μm. DLG = dorsolateral geniculate nucleus, VLGN = ventrolateral geniculate nucleus.
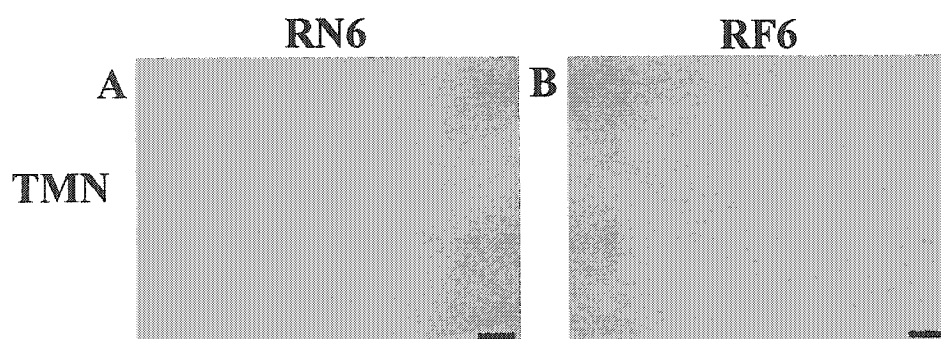
Figure 5.37. c-Fos-Ir in the tuberomammillary nucleus (TMN) of representative animals from the Restricted-NotFed Day 6(RN6) and Restricted-Fed Day 6 (RF6) conditions. The scale bars = 100 μm.

NGFI-A expression

Significant differences in NGFI-A-Ir were found among the four conditions in two of the four structures (Table 5.7: DMH and LH). No significant differences in NGFI-A-Ir among experimental conditions were found within the PVN or the TMN.

The pattern of NGFI-A-Ir results found within the DMH is shown in Figure 5.38. Although the pattern of results for the LH resembled that for the DMH, the number of cell nuclei containing NGFI-A-Ir of animals in the RF6 condition did not differ significantly from those of animals in the other conditions (Table 5.7).

Food consumption up-regulated NGFI-A-Ir significantly within the DMH and the LH of animals on Day 3 (RN3 and RF3; Table 5.7; Figure 5.39), and within the DMH of animals on Day 6 (RN6 and RF6; Table 5.7).

NGFI-A	RN3	RF3	RN6	RF6	
PVN	0.6±0.40 a	16.4±6.14 a	14.4±12.70 a	5.8±1.65 a	$F_{(3,16)} = 1.1$; N.S.
DMH	8.8±2.11 a	148.0±13.58 b	33.4±21.45 a	140.8±6.48 b	$F_{(3,16)} = 30.0$; P < 0.0001
LH	45.8±7.05 a	233.4±61.19 b	79.8±26.78 a	143.8±18.06 a,b	$F_{(3,16)} = 5.6$; P = 0.0079
TMN	38.8±3.57 a	30.4±3.25 a	36.4±7.60 a	28.0±3.77 a	$F_{(3,16)} = 1.1$; N.S.

Table 5.7. NGFI-A immunoreactive cell counts in brain structures of mice in the four different feeding conditions (RN3 = Restricted-NotFed Day3; RF3 = Restricted-Fed Day 3; RN6 = Restricted-NotFed Day 6; RF6 = Restricted-Fed Day 6; n=5/group). Group averages are shown ± SEM. Results of statistical comparison among conditions for each structure are shown in the column on the right (N.S. = not statistically significant). For each row, values not sharing a letter (a or b) differ significantly (P < 0.05). PVN = paraventricular nucleus of the hypothalamus; DMH = dorsomedial hypothalamic nucleus; LH = lateral hypothalamus; TMN = tuberomammillary nucleus.
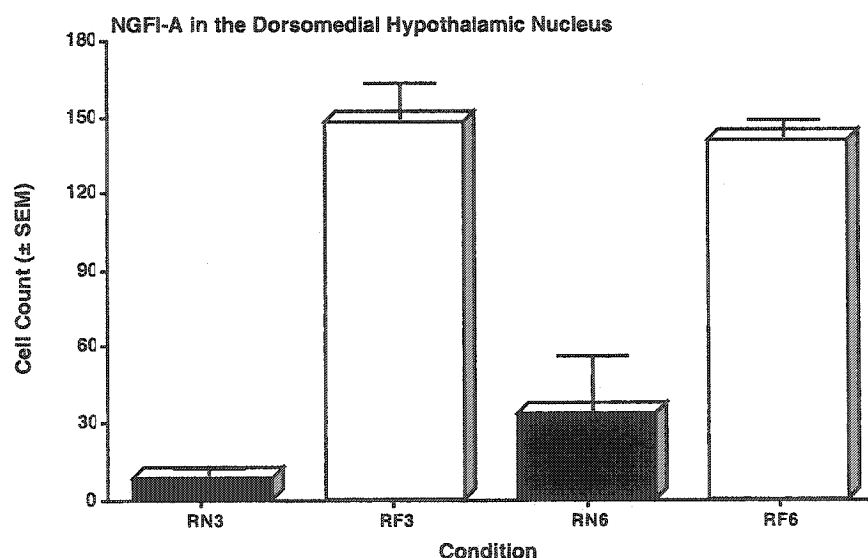
Figure 5.38. Average number of NGFI-A immunoreactive cell nuclei in the dorsomedial hypothalamic nucleus of animals in the four feeding conditions (RN3 = Restricted-NotFed Day3; RF3 = Restricted-Fed Day 3; RN6 = Restricted-NotFed Day 6; RF6 = Restricted-Fed Day 6; n=5/group). Experimental condition is plotted along the abscissa and average cell count (± SEM) is plotted along the ordinate. The group averages shown as open bars are significantly greater than group averages shown as black bars (P < 0.05).

Lengthened exposure to the restricted feeding schedule did not alter NGFI-A-Ir responses. No significant differences in NGFI-A-Ir were found in any brain structure
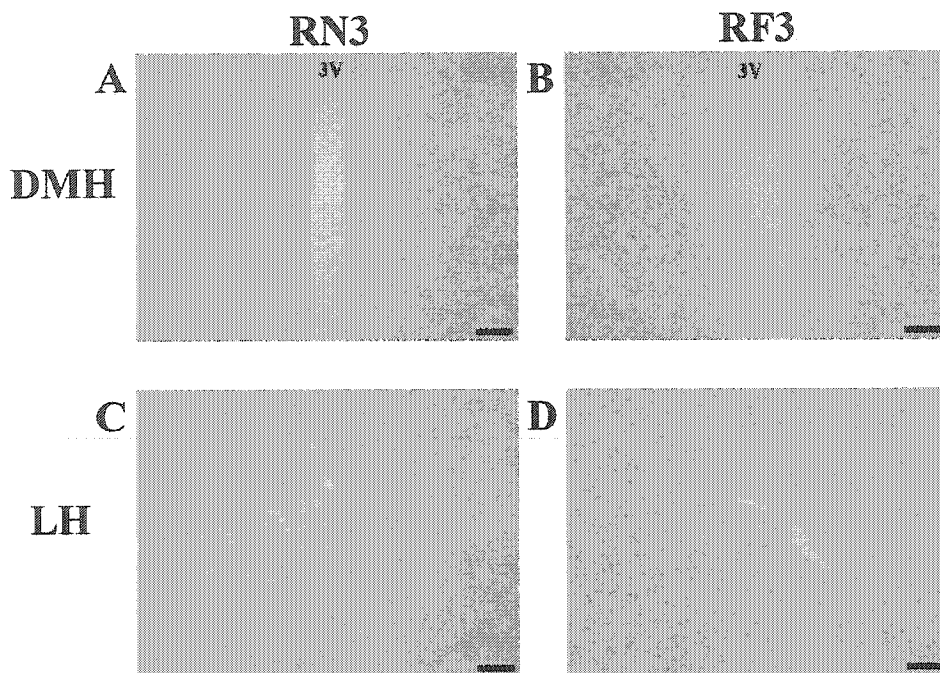
Figure 5.39. NGFI-A-Ir in the dorsomedial hypothalamic nucleus (DMH) and the lateral hypothalamus (LH) of representative animals from the Restricted-NotFed Day 3(RN3) and Restricted-Fed Day 3 (RF3) conditions. The scale bars = 100 μm. 3V = 3rd ventricle.

between the Day 3 and Day 6 conditions, regardless of feeding condition (RN3 and RN6; RF3 and RF6; Table 5.7).

5.4 Discussion

5.4.1 Effects of Restricted Feeding on Behavior and Food Consumption

Consistent with previous reports, and with the results described in Chapter 4, exposure to a restricted feeding schedule altered both nocturnal and diurnal activity patterns of animals in Experiments 1 and 2 (Abe et al., 1989; Marchant and Mistlberger, 1997; Challet et al., 1998b; Holmes and Mistlberger, 2000; Sharma et al., 2000). Animals in Experiment 1 showed the characteristic increase and consolidation of nocturnal activity and the diurnal bout of FAA prior to mealtime (Figures 5.1D and E, 5.3, 5.4 and 5.5). In Experiment 2, both FAA and the consolidation of nocturnal activity began to appear at Day 3 and were quite evident by Day 6 of the restricted feeding paradigm (Figure 5.2).

Acute fasting had a significant effect on wheel-running activity levels of animals in

the present study, as it did for acutely fasted animals in Chapter 4. Acutely fasted animals expressed significantly higher nocturnal activity levels that remained elevated well into the light phase on the last day of the experiment (Figures 5.6, 5.7 and 5.8). Despite these high levels of activity and arousal, acutely fasted animals were not able to consume as much food within a 1 h feeding opportunity as were animals that had experience with periodic feeding at this time of day (Figure 5.9). Exposure to the restricted feeding schedule for only 3 days significantly increased the amount of food that animals were able to consume during the 1 h of food access. Consumption increased further with increased length of exposure to the restricted feeding paradigm (Figure 5.9), thus confirming and extending the stomach weight results reported in Chapter 4.

These findings reinforce the notion that the mechanisms responsible for food entrainment and anticipation have adaptive significance for animals. Food entrainment not only ensures that the animal is awake and aroused at mealtime, but also serves to prepare the animal to ingest and (presumably) digest food. The results of this study suggest, therefore, that the increased capacity to ingest food during the early light phase accompanies the development and maintenance of FAA.

5.4.2 Effects of Restricted Feeding on Orexin A Expression

No significant differences in orexin A protein levels were found among feeding conditions in the present study. This is likely due to the fact that baseline orexin A levels within the brain are high, and any increase or decrease would be very difficult to detect using immunocytochemical techniques. In agreement with the present results, no change in orexin peptide levels (as measured by radioimmunoassay) was found following a period of acute food deprivation (Mondal et al., 1999b). The orexin A expression patterns found within the brains of mice in this study replicated those described in Chapter 3 (Figures 5.10, 5.11, 5.12, 5.13 and 5.14).

5.4.3 Effects of Acute Fasting on Immediate-Early Gene Expression

Fasting without refeeding (FN)

Fasting significantly altered the pattern of immediate-early gene expression as

compared to *ad libitum* feeding conditions. The levels of all three immediate-early genes were up-regulated within the Arc, the PVN and the SCN following an acute 19.5 h fast (AL and FN; Tables 5.1, 5.2 and 5.3). Within the DMH, the LH and the PVT, both c-Fos-Ir and NGFI-A-Ir levels were up-regulated (AL and FN; Tables 5.1 and 5.3). Within the IGL, c-Fos-Ir and Jun-B-Ir were up-regulated following acute food deprivation (AL and FN; Tables 5.1 and 5.2). C-Fos-Ir alone was up-regulated within the TU following acute fasting (AL and FN; Table 5.1). In summary, a 19.5 h fast significantly increased the expression of at least one immediate-early gene within the Arc, the PVN, the DMH, the LH, the TU, the PVT, the SCN and the IGL. Acute food deprivation did not alter immediate-early gene expression within the PB, the SON, the VMH, the LC or the TMN (AL and FN; Tables 5.1, 5.2 and 5.3).

A previous study of c-Fos expression in the brains of fasted mice following injections of cholecystokinin (CCK) or leptin also reported that c-Fos-Ir was up-regulated within the Arc, the PVN and the DMH of mice fasted for 24 h compared to those fed *ad libitum* (Wang et al., 1998a). This study found no effects of acute fasting alone on c-Fos expression in the other structures examined [the nucleus of the solitary tract, the area postrema, the central nucleus of the amygdala (Wang et al., 1998a)].

Fasting with refeeding (FF)

A 1 h opportunity to ingest food following a 20 h fast significantly increased the expression of all three immediate-early genes within the Arc, the PVN, the DMH, the LH, the PVT and the VMH compared to animals fed *ad libitum* (AL and FF; Tables 5.1, 5.2 and 5.3). Levels of c-Fos-Ir and NGFI-A-Ir were up-regulated within the PB and the SON (AL and FF; Tables 5.1 and 5.3), and levels of c-Fos-Ir and Jun-B-Ir were up-regulated within the TU and the LC following fasting with refeeding (AL and FF; Tables 5.1 and 5.2). Thus, at least one immediate-early gene product was up-regulated within the PB, the SON, the Arc, the PVN, the DMH, the LH, the TU, the PVT, the VMH, and the LC in animals acutely fasted and then refed when compared to animals fed *ad libitum*. Immediate-early gene expression levels within the SCN, the IGL and the TMN were not

altered within this group of animals (FF) when compared to the AL group (Tables 5.1, 5.2 and 5.3).

5.4.4 Effects of Restricted Feeding on Immediate-Early Gene Expression

Restriction without refeeding (RN)

Only one structure showed altered expression of one immediate-early gene in animals anticipating a daily meal compared to those fed *ad libitum*: NGFI-A-Ir was up-regulated within the TMN during the period of meal anticipation (AL and RN; Table 5.3). NGFI-A-Ir was not altered within any of the remaining twelve structures in animals anticipating their daily meal when compared to animals fed *ad libitum* (AL and RN; Table 5.3). Jun-B-Ir and c-Fos-Ir did not differ significantly between animals of the AL and RN conditions in any of the thirteen brain structures (Tables 5.1 and 5.2).

Restriction with refeeding (RF)

In animals allowed access to food at their scheduled mealtime, c-Fos-Ir and NGFI-A-Ir were up-regulated within the PB, the SON, the DMH and the LH (AL and RF; Tables 5.1 and 5.3). In addition, c-Fos-Ir was increased within the TU, the PVT, the IGL and the TMN (AL and RF; Table 5.1), and NGFI-A-Ir was increased within the PVN of these animals (AL and RF; Table 5.3). The remaining four structures (the Arc, the VMH, the LC and the SCN) did not show altered c-Fos or NGFI-A expression in RF animals compared to AL animals (AL and RF; Tables 5.1 and 5.3). As was the case for RN animals, Jun-B-Ir levels were not altered in animals fed at their scheduled mealtime compared to those fed *ad libitum* (AL and RF; Table 5.2).

5.4.5 Effects of Refeeding Following Fasting on Immediate-Early Gene Expression

In the present study, animals were deprived of food for ~20 h and were then killed with or without an opportunity to ingest food for 1 h. Animals that were deprived of food for the very first time on the last day of the experiment (FN) showed an up-regulation of immediate-early gene expression in the Arc, PVN, DMH, LH, TU, PVT, SCN and IGL compared to animals fed *ad libitum*. When acutely fasted animals were allowed access to food for 1 h (FF), immediate-early gene expression was up-regulated in

the PB, SON, VMH and LC in addition to the structures mentioned above, but was no longer up-regulated in the SCN and IGL when compared to *ad libitum* fed animals.

Animals that were deprived of food following repeated exposure to a daily restricted feeding schedule (RN) showed an up-regulation of only one immediate-early gene (NGFI-A) within only one structure (TMN) when compared to animals fed *ad libitum*. When chronically restricted animals were allowed access to food for 1 h at their regularly scheduled mealtime (RF), the expression of at least one immediate-early gene was up-regulated in the PB, SON, PVN, DMH, LH, TU, PVT, IGL and TMN when compared to *ad libitum* fed animals.

Refeeding following acute food deprivation (FF vs FN)

When acutely fasted animals were allowed access to food for 1 h, c-Fos-Ir was up-regulated within the PB, the Arc, the DMH and the TU compared to those fasted but not refed (c-Fos - Acute; Table 5.4). Jun-B-Ir was up-regulated within the DMH, the LH, the PVT, the VMH, and the LC; but was down-regulated within the PVN, the SCN and the IGL (Jun-B - Acute; Table 5.4). NGFI-A-Ir was up-regulated within the SON, the DMH, the LH, the PVT and the VMH; but was down-regulated in the SCN (NGFI-A - Acute; Table 5.4). In general then, refeeding following 20 h of acute food deprivation increased the expression of at least one immediate-early gene within the PB, the SON, the Arc, the DMH, the LH, the TU, the PVT, the VMH, and the LC. In contrast, refeeding following acute fasting decreased the expression of at least one immediate-early gene within the PVN, the SCN and the IGL. No immediate-early gene protein in the TMN was altered significantly by refeeding acutely fasted animals (Acute; Table 5.4).

Refeeding following chronic food deprivation (RF vs RN)

When animals expecting a meal were allowed to consume food at mealtime, c-Fos-Ir was up-regulated in the PB, the SON, the DMH, the LH, the TU, the PVT, the IGL and the TMN (c-Fos - Chronic; Table 5.4). Similarly, NGFI-A-Ir was up-regulated within the PB, the SON, the PVN, the DMH, the LH and the PVT; however, it was down-regulated within the TMN after food consumption (NGFI-A - Chronic; Table 5.4). No

significant difference in Jun-B-Ir was found between animals in the RN and RF conditions (Jun-B – Chronic; Table 5.4). To summarize, the level of expression of at least one immediate-early gene (either c-Fos or NGFI-A) was increased following feeding at mealtime in habitually restricted animals within the PB, the SON, the PVN, the DMH, the LH, the TU, the PVT, and the IGL. Immediate-early gene expression within the TMN was either increased (c-Fos) or decreased (NGFI-A) following food ingestion at the regularly scheduled mealtime. Immediate-early gene expression was not altered in the Arc, the VMH, the LC or the SCN after the consumption of food by chronically restricted animals (Chronic; Table 5.4).

There appears to be an overlapping core of gene expression changes that are seen in response to meal consumption, both in acutely fasted and chronically restricted animals. Furthermore, there are a few structures that differ somewhat in their gene expression responses to food consumption depending upon the history of exposure to the feeding schedule. In other words, some structures respond differently to the meal after 16 days of eating at that time of day. To explore this further, comparisons were made between animals that were acutely fasted and those that were chronically restricted.

5.4.6 Comparisons of Acute Fasting to Chronic Restriction

Both the FN and the RN groups were deprived of food for 19.5 h, while the FF and RF groups were food deprived for 20 h and then given 1 h of access to food before being killed. Despite these similarities in feeding conditions, animals that were chronically restricted showed significant differences in immediate-early gene expression patterns compared to those that were acutely fasted.

Acutely fasted animals showed up-regulated immediate-early gene expression within the Arc, the PVN, the DMH, the LH, the TU, the PVT, the SCN and the IGL when compared to *ad libitum* fed animals (FN and AL; Tables 5.1, 5.2 and 5.3). In contrast, chronically restricted animals showed up-regulated immediate-early gene expression only within the TMN when compared to *ad libitum* fed animals (RN and AL; Tables 5.1, 5.2 and 5.3).

Acutely fasted animals allowed 1 h of food access showed up-regulated immediate-early gene expression within the PB, the SON, the Arc, the PVN, the DMH, the LH, the TU, the PVT, the VMH, and the LC compared to animals fed *ad libitum* (FF and AL; Tables 5.1, 5.2 and 5.3). Chronically restricted animals fed at their regularly scheduled feeding time showed up-regulated immediate-early gene expression within the PB, the SON, the PVN, the DMH, the LH, the TU, the PVT, the IGL and the TMN compared to the AL group (RF and AL; Tables 5.1, 5.2 and 5.3). Thus, animals refed after an acute fast showed immediate-early gene up-regulation within the Arc, the VMH and the LC that was not shown by animals refed after repeated exposure to the same fasting-refeeding paradigm. In contrast, restricted animals that were refed after chronic scheduled feeding showed immediate-early gene up-regulation within the IGL and TMN that was not shown by acutely fasted and refed animals.

Before scheduled mealtime (RN vs FN)

While still fasting, habitually restricted animals showed lower levels of c-Fos-Ir than acutely fasted animals in nine brain structures (the PB, the Arc, the PVN, the DMH, the LH, the TU, the PVT, the SCN and the IGL; c-Fos – Not Fed; Table 5.5). Similarly, four brain structures (the Arc, the PVN, the SCN and the IGL) showed significantly lower levels of Jun-B-Ir in restricted animals compared to fasted animals (Jun-B – Not Fed; Table 5.5). Finally, NGFI-A-Ir was significantly down-regulated within the Arc, the PVN, the DMH, the LH, the PVT and the SCN of restricted animals compared to acutely fasted animals (NGFI-A – Not Fed; Table 5.5). In contrast, NGFI-A-Ir was greater within the TMN of restricted animals than in the TMN of acutely fasted animals (NGFI-A – Not Fed; Table 5.5).

In summary, the level of expression of at least one immediate-early gene was significantly decreased in nine brain structures (the PB, the Arc, the PVN, the DMH, the LH, the TU, the PVT, the SCN and the IGL), was significantly increased in one brain structure (the TMN), and was not altered in three structures (the SON, the VMH and the LC) in animals anticipating a meal compared to those acutely fasted. These results suggest

that there is a global suppression of immediate-early gene expression, usually evoked by acute food deprivation, within brain structures of animals entrained by, and anticipating daily bouts of food availability, with one exception: the TMN.

After scheduled mealtime (RF vs FF)

Expression levels of c-Fos-Ir within the Arc, the PVN and the DMH were significantly lower in animals given food access at their regularly scheduled mealtime than in animals that were acutely fasted and refed (c-Fos - Fed; Table 5.5). In contrast, animals fed their scheduled daily meal showed increased c-Fos-Ir within the TMN that was not found in animals acutely fasted and then refed (c-Fos - Fed; Table 5.5). Jun-B-Ir was significantly lower following 1 h of food access in animals chronically restricted compared to those that were acutely fasted in seven brain structures (the Arc, the PVN, the DMH, the LH, the PVT, the VMH and the LC; Jun-B - Fed; Table 5.5). Animals that were restricted and refed showed significantly lower levels of NGFI-A-Ir within the DMH, the LH, the PVT and the VMH than acutely fasted animals allowed access to food (NGFI-A - Fed; Table 5.5). In contrast, chronically restricted animals given food access showed significantly more NGFI-A-Ir within the PB than animals acutely fasted and refed (NGFI-A - Fed; Table 5.5).

Taken together, these results show that in animals given food access at expected mealtime, the expression of at least one immediate-early gene was decreased in seven brain structures (the Arc, the PVN, the DMH, the LH, the PVT, the VMH, and the LC), was increased in two brain structures (the TMN and the PB), and remained unchanged in four structures (the SON, the TU, the SCN and the IGL) when compared to those acutely fasted and refed.

5.4.7 Immediate-Early Gene Expression as FAA Develops

The results of Experiment 1 showed that animals anticipating their regularly scheduled meal showed a global suppression of immediate-early gene expression in several brain structures when compared to animals that were acutely fasted. This suppression of immediate-early gene expression was reversed following food access at habitual mealtime.

In contrast, acutely fasted animals tended to express elevated levels of immediate-early gene expression both before and after food access in these same areas. In order to investigate the development of this pattern of results, animals were killed following 3 or 6 days of exposure to the restricted feeding paradigm. c-Fos-Ir was assessed in the DMH, the LH, the TU, the PVT, the IGL and the TMN, and NGFI-A-Ir was assessed in the PVN, the DMH, the LH and the TMN.

On Day 3 of restricted feeding, c-Fos-Ir was low within the DMH, the LH, the TU and the PVT of animals both before and after mealtime (RN3 and RF3; Table 5.6). This result is in contrast to the high levels of c-Fos-Ir found in acutely fasted animals in Experiment 1 (Table 5.1). On Day 6, however, c-Fos-Ir was up-regulated following food access compared to levels found before mealtime (RN6 and RF6; Table 5.6). This pattern of c-Fos expression closely resembles that found in these brain regions of animals exposed to the restricted feeding paradigm for 16 days in Experiment 1 (Table 5.1). Thus, it appears that differences in c-Fos-Ir patterns between acutely fasted and chronically restricted animals found in Experiment 1 are present by the 6th day of restricted feeding. Similarly, c-Fos-Ir patterns within the IGL and the TMN found in animals on Day 6 of the restricted feeding schedule resemble those found in animals exposed to the schedule for 16 days (see Tables 5.1 and 5.6).

NGFI-A-Ir results for the DMH and the LH in Experiment 2 suggest that the reduced expression of this gene found in Experiment 1 animals anticipating a daily meal compared to those allowed access to food is already present by Day 3 of restricted feeding (see Tables 5.3 and 5.7). In contrast, the up-regulation of NGFI-A-Ir within the PVN following a meal in chronically restricted animals (Experiment 1; RN and RF; Table 5.3) is not present in animals killed on Day 3 or Day 6 (Table 5.7). Similarly, the up-regulation of NGFI-A-Ir within the TMN prior to meal access in chronically restricted animals was not found in animals killed on Day 3 or Day 6.

In summary, differences in immediate-early gene expression found in Experiment 1 between chronically restricted animals before and after meal access appear to be present

in the DMH and the LH by Day 3 (NGFI-A), and within the DMH, the LH, the TU, the PVT, the IGL and the TMN by Day 6 (c-Fos). Interpretation of these results, however, is limited by the fact that *ad libitum* fed and acutely fasted animals were not included in Experiment 2. Comparisons made between the results of Experiment 1 and 2 are hindered by the fact that the immunocytochemistry and analyses were not performed simultaneously. Thus, although it appears that several brain regions show altered gene expression by Day 6 and even by Day 3 of restricted feeding, these conclusions require confirmation from further studies. Furthermore, although the patterns of results found for acutely fasted animals in Experiment 1 appear to differ substantially from those found for animals killed on Day 3 in Experiment 2 (e.g., c-Fos-Ir within the DMH, the LH, the TU and PVT; Tables 5.1 and 5.6), a direct comparison in a single study would be required to demonstrate that this difference is entirely attributable to the experience of three days of a restricted feeding schedule.

5.4.8 Immediate-Early Gene Expression During Food Entrainment and Anticipation

In the present study, we assessed immediate-early gene expression patterns within structures that received orexin inputs and/or have been shown to play a role in sleep, arousal, circadian rhythmicity or feeding. When results of animals that were exposed to a daily restricted feeding schedule were compared to those of animals that were fed *ad libitum* or were acutely fasted, several distinct patterns of immediate-early gene expression responses emerged.

The parabrachial nucleus and the supraoptic nucleus

Within the PB and the SON, c-Fos-Ir and NGFI-A-Ir were up-regulated in animals given 1 h of food access following a 20 h food deprivation period regardless of whether animals were acutely or chronically restricted (see Tables 5.1 and 5.3). Jun-B-Ir within the PB and SON was not altered under any feeding condition when compared to *ad libitum* fed animals (Table 5.2). These results suggest that these brain structures respond to some correlate of feeding, and do not respond differentially with respect to chronic schedules of food restriction. It is also possible that the pattern of results found for these

two structures is in some way related to the slightly different times of day that the groups of animals were killed, rather than to some correlate of food ingestion. Animals in the AL, FN and RN conditions were killed at ZT3.5, whereas animals in the FF and RF conditions were killed at ZT5. Both c-Fos and NGFI-A were significantly greater in the PB and SON of animals killed at ZT5 compared to those killed at ZT3.5. To address this possibility, future studies could compare the results of animals in these conditions killed at ZT5 without refeeding.

A previous study done in rats showed that lesions of the PB significantly impaired food entrainment and anticipation (Davidson et al., 2000). For this reason, we expected to find some differences in immediate-early gene expression patterns between acutely and chronically restricted mice in this brain region specifically. Direct comparisons of immediate-early gene expression in the PB between acutely and chronically restricted animals revealed that c-Fos-Ir was down-regulated in animals anticipating their daily meal (RN) compared to those that were acutely fasted (FN; c-Fos, Not-Fed; Table 5.5). Furthermore, NGFI-A-Ir was up-regulated in the PB of chronically restricted animals following food consumption (RF) compared to those that were acutely fasted and refed (FF; NGFI-A, Fed; Table 5.5). Thus, although it appears that these structures respond selectively to refeeding, regardless of whether the meal was anticipated, some differences in patterns of immediate-early gene expression between the acute and chronic condition did emerge within the PB.

The arcuate nucleus and the paraventricular hypothalamic nucleus

Acute food deprivation resulted in a significant up-regulation of all three immediate-early genes within the Arc and PVN compared to levels found in *ad libitum* fed animals regardless of whether the animals were refed (see Tables 5.1, 5.2 and 5.3). This pattern of immediate-early gene responses was absent in animals that had been exposed to the chronic restriction schedule, although NGFI-A-Ir was up-regulated within the PVN in the RF condition (Table 5.3). In other words, acute food deprivation led to an up-regulation of immediate-early genes within the Arc and PVN, and this response was

reversed in animals that were entrained to the restricted feeding schedule. Direct comparisons between acutely and chronically restricted animals confirmed that immediate-early gene expression within the Arc and PVN was reduced in animals habitually food deprived regardless of whether or not they were refed (Table 5.5). The one exception was NGFI-A; it was not down-regulated in chronically restricted and refed animals within these two structures when compared to acutely fasted animals that were refed (Table 5.5). In contrast to the present results, c-Fos-Ir within the PVN was up-regulated following meal access in rats exposed to restricted feeding when compared to rats fed *ad libitum* (Angeles-Castellanos et al., 2004). At the present time, the reason(s) for these discrepant c-Fos-Ir results within the PVN remain unknown.

NGFI-A-Ir within the PVN was assessed in animals that were only exposed to the restricted feeding schedule for three or six days. In contrast to the high levels of NGFI-A expression found in animals that were acutely fasted, NGFI-A-Ir was low within the PVN of animals in all four conditions of experiment 2 (Table 5.7). These results suggest that the up-regulation of NGFI-A found following acute fasting is no longer present after just three days of experience with the restricted feeding schedule. Furthermore, the up-regulation of NGFI-A found in animals that had consumed their regular meal (RF) appears to require more than 6 days of experience with the feeding schedule to emerge.

The roles of the Arc and the PVN in the mediation of food entrainment and anticipation have been investigated previously using a number of techniques. Ablation of the Arc using neonatal monosodium glutamate (MSG) administration did not block the acquisition or expression of FAA (Mistlberger and Antle, 1999). Lesions of the PVN, however, abolished the prefeeding plasma corticosterone peak in all animals tested (Honma et al., 1992) and appeared to block the expression of increased cage activity in anticipation of the daily meal in a subset of animals (Mistlberger and Rusak, 1988). Restricted daily feeding has been associated with changes in the levels of serotonin and norepinephrine within the PVN (Krieger et al., 1980; Mitome et al., 1994). The levels of expression of various neuropeptides within the Arc and the PVN have also been shown to

be altered in response to chronic restricted feeding schedules (Brady et al., 1990; Kalra et al., 1991; Honma et al., 1992; Yoshihara et al., 1996a).

Extracellular levels of neuropeptide Y (NPY) within the PVN have been shown to increase prior to mealtime in food-restricted rats, and this rhythm in NPY concentration persisted under periods of food deprivation following the cessation of the restricted feeding schedule (Kalra et al., 1991; Yoshihara et al., 1996a). The NPY found within the PVN stems from two distinct sources, the Arc and the nucleus of the solitary tract (NTS). In an elegant series of studies, it was determined that the increase in PVN NPY associated with chronic restricted feeding originated in the brainstem (Yoshihara et al., 1996a; Yoshihara et al., 1996b; Ishizaki et al., 2003). Results showed that the Arc increases NPY production in response to food deprivation but that the NTS NPY is only up-regulated under conditions of chronic temporally restricted feeding. In other words, NPY mRNA was up-regulated within the Arc under conditions of both acute food deprivation and chronic food restriction, whereas NPY mRNA within the NTS appeared to be selectively responsive to restricted feeding conditions.

In summary, the immediate-early gene results presented in this chapter, along with the results of the prior studies outlined above, suggest that the PVN and the Arc may play a role in both the acquisition and expression of food entrainment and FAA. Further experiments are required to fully understand how these structures are implicated in the mediation of this function.

The dorsomedial hypothalamus and lateral hypothalamus

Within the DMH and LH, c-Fos-Ir and NGFI-A-Ir were up-regulated in animals that were acutely fasted regardless of whether they were refed, and in animals that were chronically restricted only once they had received their regular meal (Tables 5.1 and 5.3). Direct comparisons of c-Fos-Ir and NGFI-A-Ir between animals that were acutely fasted and those that were chronically restricted revealed that acutely fasted animals showed significantly more immunoreactive cell nuclei than did those that were anticipating their daily meal (Not Fed; Table 5.5). Comparisons of animals that were refed following acute

or chronic fasting showed that, within the DMH, both immediate-early genes were down-regulated in the chronically restricted group following food access (Fed; Table 5.5). Within the LH, only NGFI-A-Ir was significantly lower in the RF group compared to the FF group (Fed; Table 5.5). The pattern of results for Jun-B within these structures differed substantially from the results for c-Fos and NGFI-A. Jun-B-Ir was only up-regulated within these structures in animals that were acutely fasted and refed (Table 5.2). Direct comparisons between acutely and chronically fasted animals confirmed that levels of Jun-B-Ir did not differ between conditions before meal access, but were significantly lower in animals of the chronic condition following mealtime (Table 5.5).

The levels of c-Fos-Ir in the DMH and LH of animals killed on Day 3 and Day 6 of the restricted feeding schedule showed that the up-regulation of c-Fos-Ir found following acute fasting was no longer present on Day 3 of the schedule, and that the pattern found following sixteen days of exposure to the schedule emerged some time before Day 6 (Table 5.6). For NGFI-A-Ir, the pattern of results found in animals killed on Day 3 already resembled that found in animals exposed to the schedule for sixteen days (and differed from that of acutely fasted animals; Table 5.7). Thus, the pattern of results suggests that the gene expression response to acute fasting found within these structures is diminished some time before Day 3 of exposure to the restricted feeding schedule, and that the gene expression response to chronic temporal food restriction emerges some time before Day 3 (for NGFI-A) or Day 6 (for c-Fos).

In a study looking specifically at c-Fos expression patterns in multiple brain structures of rats entrained to restricted feeding and those fed *ad libitum*, c-Fos-Ir was found to be up-regulated following meal access within the DMH and the perifornical area of chronically restricted animals (Angeles-Castellanos et al., 2004). In animals anticipating their daily meal, c-Fos-Ir was not increased compared to *ad libitum* fed animals, although a non-significant trend towards up-regulation was observed in the DMH, LH and the perifornical area. In the present study, immediate-early gene quantification within the LH was done using a template that encompassed the perifornical area and no attempt was

made to quantify these regions separately. Despite this and other differences in quantification methods used, the results published for these brain regions in rats (Angeles-Castellanos et al., 2004) are in agreement with those described in the present study.

A second experiment investigated the persistence of altered patterns of c-Fos-Ir in the brains of animals that had previously been exposed to a restricted feeding schedule (Angeles-Castellanos et al., 2004). These animals were fed *ad libitum* for 5 days, and were then food deprived for 3 days before being killed. Results were compared to those from naïve animals killed after 3 days of food deprivation. c-Fos-Ir within the LH was up-regulated both before and after previous mealtime in animals that had had prior experience with the restricted feeding schedule. Within the DMH, c-Fos-Ir was up-regulated only after previous mealtime. Thus, the gene expression patterns within the LH and the DMH associated with prior mealtime appear to persist under constant feeding conditions after the cessation of the restricted feeding schedule.

This result is in agreement with electrophysiological results that showed persistence of neural activity patterns within the LH in association with mealtime (Kurumiya and Kawamura, 1991), and further suggests that gene expression within these structures, like neural activity, corresponds closely to food entrainment and FAA. Another study reported that neurons within the LH showed intense c-Fos-Ir in animals expecting food, but not allowed to consume it (Takase et al., 2000). Because this result was only mentioned within the discussion section, and the actual data were not included in the published manuscript, this should be interpreted with caution. It is of interest to note that gene expression within the LH and DMH was altered following only 3 days of exposure to the restricted feeding schedule in Experiment 2 of the present study. Taken together, these results suggest that the DMH and the LH may form an integral part of the network mediating food entrainment, although the results of a prior lesion study indicate that the LH is not necessary for the acquisition or expression of FAA in rats (Mistlberger and Rusak, 1988).

The olfactory tubercle and paraventricular thalamic nucleus

The patterns of c-Fos-Ir and Jun-B-Ir found within the TU and PVT resembled those found within the DMH and LH. c-Fos-Ir was up-regulated in animals that were acutely fasted with or without refeeding (FN and FF), and in animals chronically restricted following food access (RF; Table 5.1). Jun-B-Ir was only up-regulated in these structures in animals that were acutely fasted and refed (FF; Table 5.2). No significant difference in NGFI-A-Ir within the TU was found among animals of the five feeding conditions, but this protein was up-regulated within the PVT in animals that were acutely fasted (FN and FF; see Table 5.3). Post-hoc analyses of the c-Fos-Ir results revealed that this protein was significantly down-regulated in the TU and PVT of the RN group compared to the FN group (Table 5.5). NGFI-A was significantly down-regulated within the PVT of chronically restricted animals both before and after meal access (RN and RF), but Jun-B was only down-regulated within this structure in chronically restricted animals after food consumption (RF; see Table 5.5). No significant differences in NGFI-A-Ir and Jun-B-Ir within the TU were found between animals in the acute or chronic conditions.

The results of Experiment 2 showed that patterns of c-Fos-Ir within the TU and PVT again closely resembled those found in the DMH and LH. The up-regulation of c-Fos-Ir that occurred following acute fasting (FN and FF) within the TU and PVT was absent in animals killed on Day 3 of the restricted feeding schedule, and the pattern found in chronically restricted animals (RN and RF) was present in animals killed on Day 6 (Table 5.6).

In a study on rats given access to food early within the light phase, c-Fos-Ir was assessed within the PVT and was compared to that of animals fed *ad libitum* (Challet et al., 1997). No difference in c-Fos-Ir was found between restricted and *ad libitum* fed rats within the PVT prior to mealtime. This is in agreement with the results of the present study. C-Fos-Ir within the PVT was only up-regulated in chronically restricted mice following consumption of their regularly scheduled meal, and not while they were anticipating mealtime. Unfortunately, c-Fos-Ir was not assessed in rats following habitual

mealtime; therefore, direct comparisons of the results of this study and the rat study are somewhat limited.

The ventromedial hypothalamus and locus coeruleus

The pattern of results found within the VMH and LC was similar for all three immediate-early genes. Immediate-early gene proteins were significantly up-regulated within these structures only in animals of the FF condition (Tables 5.1, 5.2 and 5.3). Although the pattern of expression of NGFI-A within the LC resembled that found for the other two genes, results for this structure did not reach statistical significance. The results suggest, therefore, that these structures respond selectively to food consumption, but only when the meal was not anticipated. Post-hoc comparisons, however, suggested that the apparent differences between the chronic and acute conditions found for these structures were subtle. Significant differences between the RF and FF conditions were found for Jun-B (both structures) and NGFI-A (VMH only), but not for c-Fos (Table 5.5).

A study of rats entrained to two meals per day showed that c-Fos-Ir was significantly up-regulated within the laterodorsal tegmental nucleus and the LC in rats following regular morning meal access (Inzunza et al., 2000). We found a non-significant trend towards an increase of c-Fos-Ir in the LC of restricted and fed mice (RF) when compared to restricted mice that were not fed (RN). Acutely fasted or *ad libitum* fed animals were not included in the rat study, and the numbers of immunopositive cell nuclei reported for each structure were extremely low (i.e., within the LC - 0.5 ± 0.1 prior to the meal versus 1.6 ± 0.2 after the meal); however, the results appear to be in agreement with those reported for the present study (Inzunza et al., 2000).

Another study done in rats reported no significant difference in VMH c-Fos-Ir between animals entrained to a restricted feeding schedule and those fed *ad libitum* either before or after habitual mealtime (Angeles-Castellanos et al., 2004). Furthermore, c-Fos-Ir within the VMH assessed in fasted animals that had previously been entrained to a restricted feeding schedule did not differ from that in naïve fasted animals. Thus the

results of this and the present study suggest that the VMH does not respond differentially to temporally restricted daily feeding. Studies using restricted feeding schedules in animals sustaining lesions of the VMH have also shown that this structure does not appear to be required for food entrainment or the expression of FAA (Mistlberger and Rechtschaffen, 1984; Honma et al., 1987).

The suprachiasmatic nucleus and intergeniculate leaflet

Within the SCN, the expression of all three immediate-early genes was up-regulated in animals that were acutely fasted but not given access to food (Tables 5.1, 5.2 and 5.3). c-Fos-Ir and Jun-B-Ir were also up-regulated within the IGL of these animals. In addition, c-Fos-Ir was up-regulated within the IGL of animals in the RF condition (Table 5.1). Comparisons of immediate-early gene results within the SCN of animals acutely fasted (FN) with those exposed to a daily restricted feeding schedule (RN) showed that expression of these genes was down-regulated in chronically restricted animals (Table 5.5). The expression of c-Fos and Jun-B within the IGL was also significantly lower in chronically restricted animals (Table 5.5).

Because c-Fos-Ir within the IGL was found to differ significantly between animals of the RN and RF conditions in Experiment 1, expression of this gene within the IGL was assessed in animals on Day 3 and Day 6 of the restricted feeding schedule. c-Fos-Ir was up-regulated within the IGL of animals that were acutely fasted, although the up-regulation was not statistically significant for the FF condition (Table 5.1). In contrast to the results reported above for the DMH, LH, TU and PVT, exposure to the restricted feeding schedule for three days did not reverse this up-regulation of c-Fos expression within the IGL. Immunoreactivity counts for the IGL of animals killed on Day 3 resembled those of animals killed following acute fasting. The results for animals killed on Day 6 did resemble those found for animals killed after sixteen days of exposure to the restricted feeding schedule, which suggests that in this structure, as in the others, immediate-early gene expression patterns found following chronic exposure to restricted feeding emerge some time before Day 6.

In a previous study, the levels of c-Fos-Ir were assessed within the SCN and IGL of calorie-restricted rats fed early in the light phase (Challet et al., 1997). c-Fos-Ir was up-regulated in the IGL and was down-regulated in the SCN just prior to mealtime in restricted rats compared to *ad libitum* fed rats. We did not find any significant difference in c-Fos-Ir within the SCN or the IGL of mice anticipating their daily meal compared to those fed *ad libitum*, and only found an up-regulation of c-Fos-Ir within the IGL following food consumption. Unfortunately, c-Fos-Ir was not assessed immediately following mealtime in the rat study (Challet et al., 1997). The difference in results between our study and the one on rats may be due to timing of the daily meal, and/or the timing of tissue collection in relation to mealtime. Furthermore, there may be a species difference in the way that the circadian system responds to temporal food restriction. The latter possibility appears to be unlikely since a second study done on rats found no difference in c-Fos-Ir within the SCN between animals entrained to restricted feeding and those fed *ad libitum* either before or after food access at habitual mealtime (Angeles-Castellanos et al., 2004). Thus, it appears that in both mice and rats, c-Fos-Ir is not up-regulated within the SCN by chronic exposure to a restricted feeding schedule.

The tuberomammillary nucleus

The pattern of immediate-early gene expression found within the TMN in Experiment 1 did not resemble that of any other structure. c-Fos-Ir in this structure was up-regulated in chronically restricted animals following consumption of their regularly scheduled meal (RF), and NGFI-A-Ir was up-regulated in animals anticipating the arrival of their daily meal (RN; see Tables 5.1 and 5.3). Post-hoc comparisons revealed that c-Fos-Ir was significantly greater within the TMN of animals from the RF than the FF condition, and that NGFI-A-IR was significantly greater within the TMN of animals from the RN than the FN condition (Table 5.5). Jun-B-Ir within the TMN was not altered in any of the feeding conditions (Table 5.2).

A study looking at c-Fos-Ir in the brains of rats entrained to two daily meals reported a significant up-regulation of c-Fos-Ir within the TMN in rats anticipating the

morning meal (Inzunza et al., 2000). Control animals fed *ad libitum* or acutely fasted were not included in this study. As stated above, we found an up-regulation of c-Fos-Ir within the TMN only once restricted mice had consumed food at mealtime. A second study of rats also showed that c-Fos-Ir was up-regulated within the TMN only after animals had had access to food at habitual mealtime (Angeles-Castellanos et al., 2004).

In the first rat study, animals were killed on Day 5 of the restricted feeding paradigm (Inzunza et al., 2000), whereas the mice in Experiment 1 of the present study were killed on Day 16, and the rats in the second experiment were killed following three weeks of exposure to the restricted feeding schedule (Angeles-Castellanos et al., 2004). Half of the mice in Experiment 2 were killed on Day 6 of the restricted feeding paradigm. In the TMN of these mice, c-Fos-Ir was significantly down-regulated in animals anticipating a daily meal, compared to those that were fed at mealtime. On Day 3, however, c-Fos-Ir was high within the TMN of animals killed both before and after mealtime. It is possible that the timing of tissue collection in relation to mealtime and/or experience with the restricted feeding schedule could be related to the differences between the results reported here and those reported for rats in the first study (Inzunza et al., 2000). Despite these differences, it is intriguing that immediate-early gene expression patterns within the TMN appeared to be altered as a result of food entrainment in both studies using rats (Inzunza et al., 2000; Angeles-Castellanos et al., 2004) and in the present study on mice.

5.4.9 Concluding Remarks

In the present study, the levels of three different immediate-early genes were assessed in several brain regions that have been implicated previously in functions such as feeding, arousal, sleep/wake or circadian rhythmicity. Since animals exposed to restricted feeding must be awake, aroused and prepared to receive a meal, even if mealtime occurs at a time of day when the animal would normally not eat, it was hypothesized that gene expression patterns in at least a number of these structures would be altered as a result of food entrainment. Surprisingly, the levels of immediate-early gene expression within the

brains of animals anticipating a scheduled daily meal were found not to differ significantly from those of animals fed *ad libitum*. No brain structure included in the analysis, with the exception of the TMN, showed a pattern of immediate-early gene expression that would suggest a specific role in the mediation of food entrainment and anticipation. The patterns of results suggest instead that the functioning of numerous brain regions is altered under conditions of food entrainment and anticipation.

The single most striking finding in terms of patterns of changes in immediate-early gene expression was the reversal (by 16 cycles of exposure to the restricted feeding schedule) of the changes in gene expression resulting from a ~20 h period of food deprivation. Acute food deprivation resulted in a general increase in levels of immediate-early gene expression within the brain that was not observed in animals chronically exposed to daily periods of food deprivation. Therefore, the experience of a chronic food restriction schedule is related to a global decrease in immediate-early gene expression compared to that shown in the brains of acutely fasted mice.

This result implies one of two things: 1) the presence of a mechanism that actively suppresses immediate-early gene expression in response to food deprivation when the arrival of a meal can be predicted; or 2) that chronic exposure to a regular schedule of fasting and refeeding results in a set of stable physiological adaptations that include altered gene expression responses to periods of food deprivation. The results of Experiment 2, combined with those of Experiment 1, suggest that both of these things may occur as stable entrainment develops. Acute food deprivation led to an up-regulation of immediate-early genes in several brain regions that was no longer present in the brains of animals exposed to the restricted feeding schedule for three days. By Day 6 of the restricted feeding schedule, gene expression patterns closely resembled those found in the brains of animals exposed to the restricted feeding schedule for a full 16 days.

Thus, by Day 3, there is a loss of the acute gene expression response to fasting, but the pattern expressed by chronically adapted animals has not yet fully emerged. By Day 6, however, immediate-early gene expression patterns suggest a full emergence of the

adapted pattern seen on Day 16. In summary, it appears that the gene expression response to acute fasting becomes suppressed over the first three days of exposure to a restricted feeding schedule, and that the altered gene expression patterns associated with stable entrainment to the feeding schedule emerge some time between Day 3 and Day 6.

Based on the results of the present study, it appears that chronic exposure to a restricted feeding schedule substantially alters the functional activity of several brain regions such that they no longer respond to periods of food deprivation in the same manner as those of naïve animals do. It is possible that increased immediate-early gene expression is required only for processes through which food entrainment and anticipation develop, and that stable food entrainment is associated with cellular alterations that do not require the expression of immediate-early genes.

Immediate-early genes encode proteins that function as transcription factors regulating the expression of other, late-response genes by binding to DNA within promoter regions (Herdegen and Leah, 1998). The protein encoded by the *NGFI-A* gene (also known as *Egr-1*, *Krox24*, *zif268*, *Tis8* and *Zenk*) contains three zinc finger motifs in its DNA binding domain, and binds DNA at the Krox response element (Herdegen and Leah, 1998; Davis et al., 2003). Members of the Fos and Jun families form dimers (known as AP-1 complexes) that bind to the AP-1 recognition site in the promoter region of other genes (Herdegen and Leah, 1998). Although members of the Jun family can form dimers with other Jun family members (homodimers), members of the Fos family can only form dimers with members of the Jun family (heterodimers). The composition of the AP-1 complex, and its interaction with other transcription factors, determines whether the expression of the late-response gene is promoted or inhibited (Herdegen and Leah, 1998). Thus, by regulating the expression of other genes (e.g., those encoding structural proteins, neurotransmitters, neuropeptides, receptors or ion channels), transcription factors modify cellular function. This cellular modification could lead to changes in the cell's responses to subsequent stimulation. Therefore, it may not be surprising that increased immediate-early gene expression was not found in the brains of animals that were anticipating their

daily meal.

The decreased levels of immediate-early gene expression found in animals repeatedly exposed to the cycle of fasting and refeeding likely reflect the stable altered physiological function in these brain regions. Animals that have been exposed to a restricted feeding schedule for a sufficiently long duration to express significantly altered behavior and physiology will likely also show substantial cellular alterations in various brain regions known to be involved in sleep, arousal and food intake. These alterations could include changes in neurotransmitter/neuropeptide content, receptor expression levels, connectivity and/or dendritic branching. Further experimentation should reveal whether any or all of these neural alterations occur in concert with the development of stable food entrainment.

In conclusion, the results of the present study indicate that exposure to a restricted feeding schedule results in global changes in immediate-early gene expression patterns within the brains of mice in addition to alterations in behavior and physiology. Additional experiments are required in order to further clarify the significance of these altered immediate-early gene expression patterns, and to identify how these altered patterns are related to long-term alterations within the brain. Based on the present results, and those of the other studies mentioned, however, it seems likely that food entrainment is mediated by a network of brain regions.

CHAPTER 6

General Discussion

Four studies were presented in this thesis. The results of the first of these studies, described in Chapter 2, showed that mice, which are normally strictly nocturnal eaters, are able to adapt to a restricted feeding schedule in which food is only available in the middle of the light phase. Although all animals in this study appeared to be entrained by the restricted feeding schedule, the expression of anticipatory wheel running was influenced by housing conditions. Animals housed on open shelves showed robust wheel-running activity in the hours preceding mealtime; however, animals housed in isolation cabinets failed to show this anticipatory bout of activity.

In the second study, described in Chapter 3, we assessed the distribution of orexin A and B containing cell bodies in the brains of mice from three different strains. Although we expected to find some differences in expression patterns among strains, we found more pronounced differences between the expression of orexin A and B. Cell bodies immunoreactive for these neuropeptides were also found in brain regions not previously shown to contain these peptides. For instance, a small number of immunopositive cell bodies were found in the hindbrain of animals of all three strains.

We assessed the response of the lateral hypothalamic orexin system to restricted feeding in the study described in Chapter 4. To do this, we looked at immediate-early gene expression in the lateral hypothalamus (LH), c-Fos expression within orexin A-containing cells, and levels of preproorexin mRNA in the brains of mice in five feeding conditions. Animals that were anticipating their daily meal showed increased levels of preproorexin mRNA within the LH. These animals, however, did not express increased levels of immediate-early gene protein products within the LH, nor within the orexin-containing cells specifically. The results of this study also showed that animals that have had previous experience with a 20 h fasting – 4 h feeding procedure responded differently to a 20 h fast compared to those that were acutely fasted. These differences in response

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were manifest both behaviorally and in gene expression patterns within the brain.

Chapter 5 described a study looking at immediate-early gene expression in various brain regions of mice in five different feeding conditions, and in the brains of mice that were adapting to a restricted feeding paradigm. This study found that animals that have had previous experience with a restricted feeding paradigm showed different behavioral responses than those that were acutely fasted, and that these differences were evident after only a few days of exposure to daily restricted feeding. In addition, changes in immediate-early gene expression levels were found among groups in various brain regions. The most striking result of this study was a global reduction in immediate-early gene expression in the brains of animals anticipating their daily meal, indicating that animals trained on a restricted feeding schedule have global changes in neural function.

6.1 Food Entrainment and Anticipation in Mice

Acute total food deprivation exerted significant effects on behavior in mice. A total of 26 animals housed with access to activity wheels were completely deprived of food for 19.5-20 h beginning at the onset of the light phase as part of the studies described in Chapters 4 and 5. Acute food deprivation resulted in a significant increase in nocturnal wheel-running behavior that continued into the next light phase (see Figures 4.1, 4.5, 4.6, 4.7, 5.1, 5.6, 5.7 and 5.8). Activity levels of fasted animals remained elevated compared to those of animals fed *ad libitum* until the fasted animals were refed or were killed, although some fasted animals ran to exhaustion before this time. Increasing levels of activity under conditions of total food deprivation would appear on the surface to be counterproductive, since it should be in the animals' best interest to conserve energy under these conditions. On the other hand, increased activity could also represent an adaptive response to food shortage under natural conditions, if one assumes that increasing activity levels in the wild would increase the probability of finding food.

Animals exposed to a daily restricted feeding schedule also showed increased nocturnal activity levels when compared to animals fed *ad libitum* (see Figures 4.1, 4.2,

4.3, 5.1, 5.3, and 5.4). The pattern of increased wheel-running levels was, however, different from that of animals exposed to acute fasting (see Figures 4.5, 4.6, 5.6 and 5.7). Chronically restricted animals ran significantly more during the early part of the dark phase, but wheel-running levels decreased during the latter part of the dark phase, and remained low at the beginning of the light phase. Activity levels then increased again in the hours immediately preceding habitual mealtime.

In agreement with previous reports (Aschoff, 1986; Abe et al., 1989; Marchant and Mistlberger, 1997; Holmes and Mistlberger, 2000), the results of the studies described in Chapter 2, 4 and 5 show that mice, which normally consume the majority of their daily food during the dark phase (Figure 2.2), were able to adapt to a feeding schedule in which food was presented only during the middle of the light phase. Within a few days of exposure to this schedule, mice showed an increase in wheel-running activity in the hours immediately preceding mealtime (see Figures 4.1, 4.2, 4.4, 5.1, 5.2, 5.3 and 5.5). This increase in activity is referred to as food-anticipatory activity, or FAA. The results of the study described in Chapter 2 indicate that the expression of FAA can depend, at least in part, on the housing conditions used. The results of Experiment 2 described in Chapter 5 show that the changes in daily activity patterns (both increased nocturnal activity and FAA) are already present after only 3 days of exposure to the restricted feeding paradigm (see Figure 5.2). Although FAA showed great day-to-day variability in duration and intensity, the results clearly showed that mice, like rats, are able to anticipate mealtime (Mistlberger, 1994; Stephan, 2001; Stephan, 2002).

Stomachs and their contents were removed from animals immediately after they were killed in the studies described in Chapter 4 and 5. The weights of these stomachs were used as an index of how much the acutely fasted and restricted animals were able to consume during the 1 h of food access following a 20 h fast (see Figures 4.8 and 5.9). The results of this analysis in Chapter 4 showed that animals that were chronically exposed to food at this time of day were able to consume significantly more food during this access time than animals that had had no prior exposure to the restricted feeding schedule (Figure

4.8). This result was replicated in the study describe in Chapter 5 (Figure 5.9). Furthermore, exposure to the restricted feeding schedule for only 3 days significantly increased the amount of food that animals were able to consume during the 1 h food access period when compared to animals that were acutely fasted. The results showed that the capacity to rapidly ingest food increased in concert with repeated exposure to the restricted feeding paradigm. Whether this increased capacity to ingest food reflects an adaptation at the level of the digestive system, the central nervous system, or both, remains to be determined. It is likely that the functioning of both central and peripheral systems is altered during exposure to a restricted feeding schedule.

For the studies presented in this thesis, chronically restricted animals were not exposed to total food deprivation once FAA was established to ascertain whether the FAA was, in fact, driven by an endogenous, self-sustaining pacemaker. We, therefore, have no direct evidence that the FAA expressed by animals in the present studies reflects the output of an entrained FEP. Similarly, we do not know whether the changes in gene expression patterns, or in the increased capacity to ingest food rapidly are dependent on entrainment of the FEP. Total food deprivation for more than one circadian cycle is not tolerated well by mice, and it is likely for this reason that these types of tests of FEP entrainment have not been performed in most studies using mice. One study did perform this test (Pitts et al., 2003), and tests in which feeding time was delayed have also been performed in food-entrained mice (Marchant and Mistlberger, 1997; Holmes and Mistlberger, 2000). The results of these studies indicated that, as in rats, FAA in mice is driven by a mechanism that is self-sustaining.

6.2 The Role of the Orexin System in Food Entrainment and Anticipation

The LH has previously been described as a feeding center, since lesions of this structure lead to a syndrome characterized by hypophagia and weight loss, and stimulation within this region leads to increased food consumption (Kalra et al., 1999). Although food entrainment and anticipation were not impaired following lesions of the

LH (Mistlberger and Rusak, 1988), electrophysiological recording studies showed that increased neural activity within this structure anticipated mealtime in chronically restricted rats (Kurumiya and Kawamura, 1991). Furthermore, this rhythm of neural activity persisted in constant conditions of food deprivation following cessation of the restricted feeding schedule. These results imply that although the LH may not be necessary for food entrainment or anticipation, this structure may form part of the network mediating this function. We found that immediate-early gene (c-Fos) expression was elevated within the LH of animals acutely fasted (with or without refeeding), but was only elevated within the LH of chronically restricted animals following food access at habitual mealtime (see Figures 4.9 and 4.10). These results suggest that the functioning of the LH was altered in chronically restricted animals when compared to acutely fasted animals.

The LH is comprised of a number of different neuronal cell types containing various neuropeptides (Broberger et al., 1998; Elias et al., 1998; Peyron et al., 1998; Elias et al., 2001). A distinct population of cells within this region produces a set of neuropeptides called orexin A and B (also known as hypocretin 1 and 2; De Lecea et al., 1998; Sakurai et al., 1998). The orexins have previously been shown to play a role in diverse functions, including feeding, sleep and arousal (Sakurai, 1999; Sutcliffe and De Lecea, 2000; Mondal et al., 2000; Kilduff and Peyron, 2000; Willie et al., 2001; Beuckmann and Yanagisawa, 2002). Animals that are exposed to a restricted feeding schedule in which food is presented only during the normal rest phase must, in order to eat and survive, be awake and aroused at a time they normally are not, and must be prepared to ingest food at a time when they normally would not. Therefore, feeding, sleep and arousal, as well as the mechanisms that control each of these functions, must be altered under conditions of temporally restricted feeding. Because the orexin system has been shown to play a role in each of these functions, it appears to be ideally suited to play a role in food entrainment and anticipation.

In order to evaluate the functioning of the LH orexin system in food entrainment

and anticipation, we assessed orexin protein levels, as well as c-Fos immunoreactivity (Ir) within orexin-containing cells of the LH. In addition, we also measured preproorexin mRNA levels in this brain region. In agreement with pilot studies from this lab (de Groot et al., 2000a; de Groot et al., 2000b), as well as other published reports (Mondal et al., 1999b), the levels of orexin protein within the brains of mice did not differ among the five feeding conditions used in these studies. This is likely because the constitutive levels of these proteins are very high, and any changes would likely not be observed using standard immunocytochemical techniques.

The level of immediate-early gene expression within the LH, and specifically within orexin-containing cells, was not up-regulated in animals that were actively anticipating the arrival of their daily meal (see Figures 4.9, 4.10, 4.12 and 4.13). This is in contrast to levels recorded from animals acutely fasted for the same number of hours before being killed (see Figures 4.9, 4.10, 4.12 and 4.13). In animals that were acutely fasted, c-Fos-Ir within the LH and specifically within the LH orexin cells was significantly up-regulated compared to animals that were fed *ad libitum* (see Figures 4.9, 4.10, 4.12 and 4.13). In contrast, preproorexin mRNA levels were selectively up-regulated only within the LH of animals that were anticipating mealtime (see Figures 4.14 and 4.15). Both groups of animals were deprived of food for 19.5 h prior to being killed, and, therefore any correlate of this feeding manipulation should be comparable between groups. The only difference between groups was whether or not the animals had had previous experience with food presentation at that time of day. The results of these experiments suggest that the functioning of the LH orexin system is altered in animals that are exposed to a temporally restricted daily feeding schedule.

A study investigating the effects of restricted feeding on c-Fos expression within orexin-containing cells reported that c-Fos-Ir was up-regulated in orexin neurons of rats exposed to a restricted feeding schedule compared to levels found in animals fed *ad libitum* (Kurose et al., 2002). Only 5% of orexin neurons contained c-Fos-Ir in *ad libitum* fed rats, whereas 22% of orexin neurons contained c-Fos-Ir in rats anticipating the arrival

of their daily meal. Because acutely fasted rats were not included in this study for comparison, it is difficult to speculate about the reasons that c-Fos-Ir would be up-regulated in orexin cells in rats anticipating the meal but not in mice under similar conditions. These authors also reported a down-regulation of mRNA levels for the orexin receptor 2 within the paraventricular nucleus of the hypothalamus (PVN; Kurose et al., 2002).

Despite the differences between the two, the results of this study (Kurose et al., 2002), combined with those of the study described in Chapter 4, imply that the functioning of the orexin system is altered in animals exposed to daily restricted feeding schedules. These results further suggest that the orexin system may form an integral part of the mechanism mediating food entrainment and anticipation. The results of another study, however, indicate that the LH orexin system is not required for food entrainment or anticipation (Mistlberger et al., 2003). Ablation of LH orexin neurons by the neurotoxin saporin did not result in an impairment of food entrainment or anticipation in rats. Thus, although the orexin system is altered under schedules of daily restricted feeding and may participate in adaptation to the feeding schedule, it appears that this system is not essential for the expression of food entrainment.

6.3 Immediate-Early Gene Expression in Food Entrainment

Immediate-early genes encode proteins that are up-regulated rapidly and transiently within cells in response to a variety of stimuli (Morgan and Curran, 1989; Sheng and Greenberg, 1990; Morgan and Curran, 1991; Hughes and Dragunow, 1995; Herrera and Robertson, 1996; Herdegen and Leah, 1998; Davis et al., 2003). They have been used extensively as an assay of cellular activation, and although the lack of immediate-early gene expression does not indicate a lack of neuronal activity, the induction of these proteins can be, and has been, used as a reliable index of neuronal response to a stimulus. For instance, immediate-early gene expression has previously been used as a tool to assess changes in various brain regions in response to restricted

feeding in rats (Challet et al., 1997; Choi et al., 1998; Takase et al., 2000; Inzunza et al., 2000; Angeles-Castellanos et al., 2004). The results of these studies have shown that gene expression in various brain regions is altered during food entrainment and anticipation. Unfortunately, none of these studies have produced results that would implicate any one structure in the mediation of food entrainment, with the exception, perhaps, of the TMN. Instead, the results of these studies, in combination with the results reported in Chapter 5, indicate that chronic exposure to a restricted feeding schedule results in global alterations in gene expression patterns in the brain.

For the studies described in Chapters 4 and 5, immediate-early gene expression patterns in the brains of animals exposed to a restricted feeding schedule were compared to those of animals fed *ad libitum* or fasted acutely. Chronically restricted animals and acutely fasted animals were killed either before or after 1 h of food access. Thus, the effects of a period of ~20 h of total food deprivation could be compared between animals acutely exposed to this treatment and those repeatedly exposed to daily periods of fasting and refeeding. In addition, the effects of refeeding following a 20 h fast could be assessed in both chronically and acutely food restricted animals.

The results of these studies showed that there is a set of brain structures that show an up-regulation of immediate-early genes in response to a single period of food deprivation. Refeeding following this acute 20 h fast further alters immediate-early gene expression patterns in the brain. In contrast, a period of food deprivation in chronically restricted animals did not cause up-regulation of immediate-early genes similar to that found following acute fasting. Refeeding at habitual mealtime in chronically restricted animals led to up-regulation of immediate-early genes in some of the same structures showing up-regulated immediate-early gene expression in animals refed following acute fasting; however, some differences in patterns of gene expression were found between chronically restricted and acutely fasted animals following food access.

Only two daily time points were assayed in the studies included in Chapters 4 and 5. Gene expression patterns were assessed before and during habitual mealtime in

chronically restricted animals, and at the same time points in animals acutely fasted or fed *ad libitum*. The possibility that immediate-early genes were up-regulated in the brains of animals entrained to restricted feeding at some other time point over the circadian cycle, therefore, cannot be ruled out. The persistence of the altered gene expression patterns following the cessation of the restricted feeding schedule was also not assessed in the current studies. It is possible, therefore, that if previously restricted animals were to be food deprived following an intervening period of *ad libitum* feeding, patterns of immediate-early gene expression would be similar to those of animals acutely fasted without prior experience with a restricted feeding schedule. Although the experiment testing this possibility has not yet been performed in mice, we have reason to doubt that this would occur.

The persistence of altered immediate-early gene expression patterns was tested directly in a study on rats (Angeles-Castellanos et al., 2004). Animals were entrained to a restricted feeding schedule for three weeks and were then given *ad libitum* access to food for five days. They were then deprived of food for 3 days, along with a control group of animals that had never before been exposed to a restricted feeding schedule, and c-Fos-Ir was assessed in several brain structures. In previously restricted animals, c-Fos-Ir within the LH was significantly higher both before and after expected mealtime than in naïve animals. Within the dorsomedial hypothalamus (DMH), c-Fos-Ir was significantly higher in experienced than in naïve animals after expected mealtime (Angeles-Castellanos et al., 2004). These results show that prior experience with a restricted feeding schedule produces long-term neural modifications associated with altered gene expression responses to subsequent periods of food deprivation.

Animals that have had prior experience with a daily restricted feeding schedule show altered behavioral responses to subsequent periods of food deprivation than do animals that have not had that experience (Coleman et al., 1982; Rosenwasser et al., 1984; Clarke and Coleman, 1986). This finding suggests that prior feeding experiences can cause stable alterations in an animal's physiology and behavior. One study assessed the

responses of mice to a psychostimulant (amphetamine; Cabib et al., 2000). Before assessment of response to amphetamine, mice were first food restricted until they lost 20% of their body weight over 12 days, and then allowed to regain lost body weight during free feeding. They found that prior experience with food shortage abolished strain differences normally found in animals that had not had this experience. Not only do the results of this study call into question the notion of consistent and reliable phenotypic differences between inbred strains of mice, but they also demonstrate that food restriction can exert effects long after *ad libitum* food access has been restored (Cabib et al., 2000). Although the specific feeding regimen was not mentioned within the manuscript, and, therefore, it cannot be determined whether the mice in this study were possibly entrained to a temporally restricted feeding schedule, it is likely that the long-term effects found in this study would also be present in animals with prior exposure to a circadian restricted feeding schedule.

Acute food deprivation resulted in the up-regulation of immediate-early gene expression in various brain structures of mice in the experiments described in Chapters 4 and 5. Although the specific stimulus associated with food deprivation that results in the up-regulation of these genes in the brain is not known, animals chronically exposed to a restricted feeding schedule must be exposed repeatedly to this stimulus. Chronic or repeated exposure to various stimuli (e.g., narcotics and other pharmacological agents, stressors or electroconvulsive stimulation) has been shown to alter gene expression patterns in the brain (Herdegen and Leah, 1998; Nestler et al., 1999; Kelz and Nestler, 2000). The expression of several immediate-early genes, although initially up-regulated following acute exposure to these stimuli, is no longer up-regulated by the stimulus after repeated presentation (Herdegen and Leah, 1998; Nestler et al., 1999).

The expression of at least one set of immediate-early genes, however, appears to be associated selectively with chronic exposure to these stimuli. These chronic Fos-related antigens have been identified as novel, biochemically modified isoforms of ΔFosB, which is a product of the *fosB* gene (Nestler et al., 1999; Kelz and Nestler, 2000). ΔFosB

is not found after acute exposure to a stimulus, but is up-regulated only following chronic stimulus exposure. Once ΔFosB has been induced, it is remarkably stable and expression levels have been shown to remain elevated for weeks to months after the last stimulus exposure. It is possible that chronic exposure to daily periods of food deprivation alternating with periods of food access under a restricted feeding schedule results in the up-regulation of these stable forms of ΔFosB with the concomitant down-regulation of the expression of other immediate-early genes. Furthermore, it is possible that stable changes in expression of genes such as ΔFosB contribute to the altered behavioral and physiological responses to subsequent periods of food deprivation. Future studies will be designed to investigate these possibilities.

6.4 Conclusions and Future Directions

The results of the studies presented in this thesis confirmed that mice are able to adapt to a daily restricted feeding schedule in which food is presented during their normal rest phase. Furthermore, using immunocytochemical and in situ hybridization techniques, it was shown that the expression of orexin and immediate-early genes was altered within the brains of mice exposed to restricted feeding. Because exposure to a chronic restricted feeding schedule leads to global modifications in both behavior and physiology, it is likely that the altered gene-expression patterns reported in the present studies represent only a small portion of the neural adaptations that are occurring as the animal becomes stably entrained.

Food entrainment and anticipation ensure that an organism is awake, aroused and prepared to ingest food when it becomes available, even if food access occurs at a time of day when the animal would not customarily eat. Because orexin cells send projections to brain regions involved in feeding, circadian rhythmicity, sleep and arousal, and appear to participate in the control of these biological functions, it was hypothesized that they may play a role in coordinating these functions during food entrainment. There are numerous brain regions and neuropeptide systems that are involved in functions that are altered in

animals during food entrainment. Thus, although the results presented do suggest that the activity of the orexin system and of brain regions receiving orexin inputs is altered in animals exposed to a restricted feeding schedule, it remains unknown whether this is the only system that is altered during the acquisition and/or the expression of FAA. Furthermore, it remains unknown whether these changes in activity are due to entrainment of the FEP itself or to an unidentified correlate of adaptation to the restricted feeding schedule.

Future studies designed to investigate gene expression patterns in other brain regions may reveal additional structures that respond differentially to restricted feeding schedules. Furthermore, studies of other neuropeptide systems involved in functions such as sleep, arousal or feeding may implicate alternative structures in food entrainment and anticipation. As has been demonstrated by a number of recent studies, the use of genetic mouse models in food entrainment studies could prove invaluable in the investigation of the mechanisms underlying this phenomenon (Oishi et al., 2002; Minami et al., 2002; Dudley et al., 2003; Pitts et al., 2003). In addition, new techniques for the assessment of altered patterns of gene expression (e.g., microarray technology and proteomics) open up more avenues for the continued search for the elusive food-entrainable pacemaker.

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