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Neuroanatomical Specificity of Prolactin-Induced Hyperphagia and Expression of Fos-like Immunoreactivity following Central Administration of Prolactin

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ABSTRACT

Neuroanatomical Specificity of Prolactin-Induced Hyperphagia and Expression of Fos-like Immunoreactivity following Central Administration of Prolactin

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Concordia University, 1999

The present experiments extend our understanding of the orexigenic properties of prolactin (PRL) in female rats. PRL is known to increase food intake in virgin female rats when injected intracerebroventricularly (icv) but it is not known where in the brain PRL acts to promote feeding behavior. The first series of studies investigated the role of the paraventricular nucleus (PVN), ventromedial nucleus (VMH), and medial preoptic nucleus (MPOA) in the hyperphagic actions of PRL. *Ad libitum* fed virgin female rats received twice daily site-specific injections of PRL (800 ng) over a period of 10 days. Only subjects demonstrating regular vaginal cyclicity were included in the study. Food intake, body weight, and vaginal cyclicity were measured daily. Results showed that PRL significantly increased food intake when injected in the PVN. A nonsignificant trend towards a hyperphagic response in the last 5 days of testing was observed in rats receiving intra-VMH injections of PRL, and the MPOA was not responsive to the feeding stimulating properties of PRL. None of the manipulations affected body weight or vaginal cyclicity as demonstrated by vaginal smears. In sum, although it is possible that PRL action may occur at other sites, the present results reveal that PRL acts through the PVN to induce feeding in virgin female rats.

A second experiment investigated the pattern of cellular activation, as revealed by Fos-like immunoreactivity (Fos-lir), in the PVN, VMH, and MPOA of female rats

following treatments of varying lengths of icv administration of PRL. *Ad libitum* fed virgin female rats were given 1, 7 or 15 icv injections of PRL twice daily at a dose known to promote hyperphagia (2 μg/0.5 μl). The rats were sacrificed one hour after their last injection and their brains processed for Fos-lir. Significant changes in expression of Fos-lir were observed only in the PVN of rats having received 15 injections of PRL. Therefore the PVN is the hypothalamic site most responsive to the effect of PRL on feeding behavior, and the one in which most cellular activation was observed after chronic administration of PRL. Given that PRL can upregulate its own receptors, and that PRL effects in the brain occur by PRL binding to its receptors, it is plausible to suggest that significant increases in the expression of Fos-lir in the PVN of rats after prolonged icv administration of PRL may reflect an upregulation of PRL receptors. Suggestions for future studies that would elaborate on these results are proposed.

These are the first tests of the site-specificity of the central effect of PRL on feeding in rats, and of the expression of Fos-lir in selected hypothalamic sites after a varying number of icv injections of PRL. It is concluded that PRL plays a significant role in the PVN to promote feeding and cellular activation as evidenced by both behavioral and immunohistochemical data.

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INTRODUCTION

Prolactin (PRL) is a pituitary hormone (Riddle & Braucher, 1931) which is present in all vertebrates. It is involved in a wide range of physiological functions (for a review, see Nicoll, 1974) including osmoregulation (Bole-Feysot, Goffin, Edery, Binart, & Kelly, 1998; Ensor, Edmondson, & Phillips, 1972; Richardson, 1973), immunoregulation (Ben-Jonathan, Mershon, Allen, & Steinmetz, 1996; Russell, 1989), cellular proliferation and differentiation (Bole-Feysot et al., 1998; Kelly, Djiane, Postel-Vinay, & Edery, 1991), promotion of growth (Bole-Feysot et al., 1998; Clark & Bern, 1980), metabolism of neurotransmitters and neuropeptides (Tong & Pelletier, 1992), firing rate of hypothalamic neurons (e.g., Chan, Dudley, & Moss, 1983; Haskins & Moss, 1983), stimulation of tuberoinfundibular dopaminergic (TIDA) neurons (Moore, 1987), and the regulation of the sleep-wake cycle (Roky, Valatx, Paut-Pagano, & Jouvet, 1994).

PRL also mediates various behaviors such as migration (see Nicoll, 1974), thermoregulation (Drago & Amir, 1984), grooming and yawning in rats (Drago, 1988; Laping & Ramirez, 1988, respectively), sexual behavior (Harlan, Shivers, & Pfaff, 1983), parental behavior (Bridges, DiBiase, Loundes, & Doherty, 1985; Bridges, Numan, Ronsheim, Mann, & Lupini, 1990; Bridges & Mann, 1994; Riddle, Bates, & Lahr, 1935), and feeding behavior (Buntin & Figge, 1989; Buntin & Tesch, 1985; Byatt, Staten, Salsgiver, Kostelc, & Collier, 1993; Moore, Gerardo-Gettens, Horwitz, & Stern, 1986; Noël & Woodside, 1993; Sauvé & Woodside, 1996). The role PRL plays in the development of mammary glands and lactogenesis (see Nicoll, 1974), and hence the survival of the young, however, is probably the most well documented of its functions and has attracted the attention of both reproductive endocrinologists and animal scientists.

Lactation represents a great energetic challenge to mammalian females. To meet

the energetically costly demands of this reproductive state, female rats exhibit a marked hyperphagia during lactation (Fleming, 1976). The amount of food consumed by lactating females has been reported to be 300 % higher than that consumed prior to mating (e.g., Ota & Yokayama, 1967). Researchers reasoned that this elevated feeding response could be stimulated either by a direct response to the energy requirements of milk synthesis and delivery, the suckling stimulation provided by the pups, the hormonal correlates of lactation, or some combination of all three.

The most compelling findings suggesting that suckling stimulation and its hormonal consequences play a role in stimulating the hyperphagia of lactation were provided by investigators such as Cotes and Cross (1954), Fleming (1976), and Woodside (Millelire & Woodside, 1989; Woodside & Popeski, in press). They showed that nursing mother rats who suckled, but whose milk delivery was prevented by cutting the ducts carrying milk from the mammary gland to the nipple (i.e., the galactophores), were less hyperphagic than intact lactating mothers (Millelire & Woodside, 1989; Woodside & Popeski, in press), but were more hyperphagic than intact dams that were not suckled (Fleming, 1976). Moreover, the increase in food intake that occurs between the first and second week of lactation in intact lactating suckled females is also found to occur in galactophore-cut suckled females (Cotes & Cross, 1954; Woodside & Popeski, in press), although to a lesser degree. Together, these findings supported the thesis that the hormonal milieu created in the dam by suckling stimulation is a factor stimulating increased feeding. What remained to be determined was which hormonal events are critical for the induction of hyperphagia of suckled dams.

Suckling stimulation elicits the release of hormones from the pituitary, in particular adrenocorticotropic hormone (ACTH) (Voogt, Sar, & Meites, 1969), oxytocin, beta-endorphins (ß-endorphins) (Riskind, Millard, & Martin, 1984), and PRL (Mena & Grosvenor, 1971), all of which are necessary for the maintenance and continuation of

lactation. Many of these hormones have been shown to affect both metabolism and food intake (e.g., Arletti, Benelli, & Bertolini, 1989; Bjorkstrand & Uvnas-Moberg, 1996; Egawa, Yoshimatsu, & Bray, 1993; Gosnell, Morley, & Levine, 1986), but the potential role of PRL in the mediation of the hyperphagia of lactation has been actively investigated not only because PRL is the major lactogenic hormone and has diverse behavioral and physiological effects, but also because a direct proportional relationship exists among PRL levels, food intake, and litter size (Leon & Woodside, 1983), with greater increases in food consumption occurring in dams nursing large litters (Ota & Yokayama, 1967; Woodside & Popeski, in press), and hence, secreting larger amounts of PRL (Amenomori, Chen, & Meites, 1970).

Indeed significant increases in feeding behavior and levels of PRL are behavioral and hormonal hallmarks of lactation. The possibility of a causal relationship between PRL levels and the dramatic increase in feeding seen during lactation leads to the question of whether PRL itself has intrinsic orexigenic properties. There are many reports indicating that systemic administration of PRL produces significant increases in food intake in rats. For example, both subcutaneous (sc) injections of PRL (Gerardo-Gettens, Moore, Stern, & Horwitz, 1989a, 1989b; Leon, 1974; Noël & Woodside, 1993), and ectopic pituitary transplants secreting PRL into the systemic circulation of virgin female rats produce significant increases in food intake (Moore et al., 1986). Interestingly, studies in which comparable doses of PRL have been used, but that did not yield parallel results, are those in which the hormone administration period lasted for fewer than 9 days (Adler & Krieg, 1991; Fleming, 1976). Taken together, the findings from this line of research have shown clearly that PRL can affect feeding behavior, and that the duration of treatment is an important factor in the capacity of exogenous administration of PRL to produce orexigenic effects.

In addition to the work on the hyperphagic effects of systemic administration of

PRL in rats, there are reports that PRL acts centrally to promote feeding behavior in other species. The most elaborate body of work addressing this issue is that of Buntin and collaborators. Buntin and Tesch (1985) showed that treating male and female ring doves with twice daily intracerebroventricular (icv) injections of 1.0 μg of PRL induced a consistent 50-100 % elevation in daily food intake in both sexes. In another study, they showed that icv injections of PRL at doses ranging from 0.5 μg to 2 μg, and administered daily for 5 consecutive days to physiologically intact male ring doves, caused significant dose-dependent increases in food intake. These increases were up to about 120 % over baseline measures for the largest dose (Buntin & Figge, 1989). Further results determined that a single icv injection of PRL (44 pmoles) to male doves significantly increased total time spent feeding and average feeding bout duration (Buntin, 1989). This work has revealed that in the ring dove, PRL acts centrally to induce food intake, and that the response latency to sustained feeding is longer (6-10 hours) than that of other orexigenic agents such as neuropeptide Y (NPY) which, in the rat, elevates feeding within 2 hours of icv administration (Levine & Morley, 1984).

The first demonstration that centrally administered PRL increased food intake in mammals, however, was made more recently (Noël & Woodside, 1993). Noël and Woodside (1993) showed that in free feeding, intact female rats treated with twice daily icv injections of 2 μg of PRL for 10 days, daily average food intake increased by 5 grams above baseline measures. Although this increase in feeding is not as dramatic as that typically seen in intact nursing dams, it is noteworthy that it is similar to that observed in postparturient females suckling young, but not delivering milk due to galactophore ligation (Millelire & Woodside, 1989). This latter observation lends support to the notion that the hormonal environment created in the female as a result of the suckling stimulation plays a role in the hyperphagia of lactation.

Since the initial report by Noël and Woodside (1993), the central effects of PRL on feeding in rats have been replicated and extended. It is now known that PRL

promotes feeding in a dose-dependent manner, and that PRL does not selectively potentiate feeding during the light or dark phase of the day/night cycle (Sauvé & Woodside, 1996). These authors also showed that the feeding response produced by icv administration of PRL takes 2 days of twice daily injections before it manifests itself, and that this latency is shortened when female rats are placed under a food restriction regimen (Sauvé & Woodside, 1996) . In both cases, however, the latency of onset to PRL-induced increases in feeding is longer than that of central PRL administration in doves, as well as that of other agents that potentiate feeding in rats (e.g., NPY; Levine & Morley, 1984). Moreover, it has been noted that whereas systemic injections of PRL disrupt the estrous cycle of female rats (Noël & Woodside, 1993), central injections of the hormone do not (Noël & Woodside, 1993; Sauvé & Woodside, 1996), indicating that the latter route of administration for PRL produces a hyperphagic response which is not confounded by the hormonal consequences that an acyclic state incurs.

The latter result is important because in intact cycling females, PRL is known to stimulate the corpora lutea of the ovary which in turn, increases circulating levels of progesterone and renders females acyclic (Rothchild, 1960). Given that progesterone can stimulate feeding behavior (Wade, 1976), presumably through its anti-estrogenic effects (Wade & Gray, 1979), and that a state of acyclicity is accompanied by increases in food intake (Tarttelin & Gorski, 1971), a treatment that produces hyperphagia while disrupting estrous cyclicity becomes difficult to interpret. Enhanced feeding could result either from the effects of progesterone, or from the absence of the reduction in feeding which occurs on the day of proestrus (Tarttelin & Gorski, 1973), and not be due to a direct effect of PRL itself. Thus, a procedure in which PRL is injected centrally to female mammals provides a way to assess the effect of PRL on feeding without the confounding problems induced by acyclicity. Although these studies demonstrated PRL-stimulated feeding independent of changes in cyclicity, they did not address the

potential contributions of circulating hormones to the hyperphagic actions of PRL.

This question was addressed in a study by Sauvé and Woodside (1996) using ovariectomized female rats as subjects. The data revealed that *ad libitum* fed ovariectomized animals treated with icv injections of PRL (2 µg/0.5 µl/injection twice daily for 10 days) ate significantly more than saline-injected controls, indicating that the presence of ovarian hormones was not required for the central effect of PRL on feeding to occur. This contrasts with the effect of icv administration of PRL on maternal behavior in ovariectomized nulliparous female rats (Bridges et al., 1990), a model in which the presence of estrogens is necessary for PRL to stimulate maternal responses. Thus it is clear that when PRL is administered directly into the brain, the presence of steroid hormones is necessary for some PRL-induced behaviors to manifest themselves, but not others: PRL-induced maternal behavior is steroid-dependent, whereas PRL-induced feeding is not.

Taken together, results from these studies have given further support to the notion that PRL is a significant stimulator of feeding behavior. PRL can be added to a group of hormones and peptides whose centrally-mediated potentiating effect on feeding has already been established. Some of these agents include growth-hormone releasing factor (GRF) (Dickson & Vaccarino, 1990; Okada, Ishii, Minami, Sugihara, Shibasaki, & Wakabayashi, 1996; Tanaka, Egawa, Inoue, & Takamura, 1991; Vaccarino & Hayward, 1988), NPY (e.g., Stanley, Chin, & Leibowitz, 1985a; Stanley & Leibowitz, 1984; Stanley & Leibowitz, 1985), peptide YY (PYY) (e.g., Morley, Levine, Grace, & Kneip, 1985; Stanley, Daniel, Chin, & Leibowitz, 1985b), progesterone (Jankowiak & Stern, 1974; Wade, 1976), and ß-endorphin (Egawa et al., 1993; Gosnell et al., 1986).

In addition to showing that these agents have effects on feeding behavior, research has determined the central source of these substances and their sites of action

within the brain. For example, GRF is a peptide known to be produced by neurons originating in the arcuate nucleus of the hypothalamus (Merchenthaler, Thomas, & Arimura, 1984) with many fibers extending into the median eminence, dorsomedial nucleus, periventricular system, medial preoptic area (MPOA), suprachiasmatic nucleus (SCN), and the bed nucleus of the stria terminalis (BNST) (Merchenthaler et al., 1984; Sawchenko, Swanson, Rivier, & Vale, 1985). Studies have shown that GRF stimulates feeding optimally when injected into the MPOA/SCN region (Vaccarino & Hayward, 1988), and into the ventromedial nucleus of the hypothalamus (VMH) (Tanaka et al., 1991).

NPY is another orexigenic peptide whose source is the arcuate nucleus (Bai, Yamano, Shiotani, Emson, Smith, Powell, Tohyama, 1985; Danger, Tonon, Jenks, Saint-Pierre, Martel, Fasolo, Breton, Quirion, Pelletier, & Vaudry, 1990). NPY-containing cell bodies in the arcuate nucleus have fibers projecting to hypothalamic nuclei (Allen, Adrian, Allen, Tatemoto, Crow, Bloom, & Pollak, 1983). The paraventricular nucleus (PVN) which is particularly dense with NPY terminals and NPY receptor binding sites (Allen et al., 1983; Danger et al., 1990; White, Kershaw, Sanacora, Olchovsky, Finkelstein, & Berelowitz, 1990), has been shown to be a neural site mediating the hyperphagic actions of NPY (Jhanwar-Uniyal, Beck, Jhanwar, Burlet, & Leibowitz, 1993; Stanley & Leibowitz, 1984; Stanley & Leibowitz, 1985; Stanley et al., 1985a, 1985b). More recent studies have revealed that the perifornical hypothalamus, at the level of the caudal PVN, was the most sensitive hypothalamic site for NPY-induced eating in rats (Stanley, Magdalin, Seirafi, Thomas, & Leibowitz, 1993).

Studies on PRL have shown that the source of PRL in the bloodstream is the anterior pituitary (e.g., Ben-Jonathan, Arbogast & Hyde, 1989; Ben-Jonathan et al., 1996; Neill, 1988). For decades, the general consensus was that PRL of pituitary origin circulating in the periphery could not cross the blood brain barrier (Walsh, Posner, Kopriwa, & Brawer, 1978). In 1987, however, Walsh and colleagues characterized the

process by which PRL from the peripheral circulation could gain access to the brain by a receptor-mediated transport mechanism thought to be localized in the choroid plexus. Since the publication of this exciting finding, the presence and distribution of PRL-like immunoreactivity (PRL-lir) in the brain have been described by many researchers (DeVito, 1988; Emanuele, Metcalfe, Wallock, Tentler, Hagen, Beer, Martinson, Gout, Kirsteins, & Lawrence, 1986; Fuxe, Hökfelt, Eneroth, Gustafsson, & Skett, 1977; Griffond, Deray, Jacquemard, Fellmann, & Bugnon, 1994b; Hansen, Hansen, & Hagen, 1986; Harlan, Shivers, Fox, Kaplove, Schachter, & Pfaff, 1989; Nishizuka, Shivers, Leranth, & Pfaff, 1990; Siaud, Manzoni, Balmefrezol, Barbanel, Assenmacher, & Alonso, 1989; Toubeau, Desclin, Parmentier, & Pasteels, 1979).

At the time, it was thought that immunoreactive PRL found in the brain originated from the pituitary and was transported into CSF (Login & Macleod, 1977; Rubin & Bridges, 1989) by its active transport through the choroid plexus (Walsh, Slaby, & Posner, 1987). Later reports, however, showed that PRL-lir in the hypothalamus of rats was maintained after hypophysectomy (DeVito, 1988; Emanuele, Metcalfe, Wallock, Tentler, Hagen, Beer, Martinson, Gout, Kirsteins, & Lawrence, 1987; Fuxe et al., 1977; Paut-Pagano, Roky, Valatx, Kitahama, & Jouvet, 1993; Toubeau et al., 1979), indicating that the pituitary may not be the only source of PRL, and that the brain itself might produce PRL and PRL-related peptides.

More recently, the detection of PRL mRNA by many investigators (Clapp, Torner, Gutierrez-Ospina, Alcantara, Lopez-Gomez, Nagano, Kelly, Mejia, Morales, Martinez de la Escalera, 1994; DeVito, Avakian, Stone, & Ace, 1992; Emanuele, Jurgens, Halloran, Tentler, Lawrence, & Kelley, 1992; Schachter, Durgerian, Harlan, Pfaff, & Shivers, 1984) has provided a strong argument in favor of PRL synthesis by brain cells, some of which have been located in the hypothalamus (Clapp et al., 1994; DeVito et al., 1992; Emanuele et al., 1992; Schachter et al., 1984). Presently, it appears

that two PRL-generating systems co-exist, one is the well known peripheral pituitary system (Ben-Jonathan et al., 1989; Neill, 1988), the other is a PRL system within the brain (e.g., Paut-Pagano et al., 1993). The latter system consists of PRL cell bodies originating exclusively in the lateral hypothalamic area surrounding the fornix, with dense networks of fibers in the hypothalamus, midline thalamic nuclei, BNST, raphe dorsalis, and locus coeruleus (Paut-Pagano et al., 1993). Regardless of its origin, however, PRL is thought to produce behavioral effects by acting on PRL receptors in the brain (e.g., Bakowska & Morrell, 1997; Kelly et al., 1991; Nicoll, 1974).

The localization of PRL receptors within the brain has been an active area of investigation. Initial studies have implied the existence of PRL receptors with the use of biochemical binding assays using membrane preparations and autoradiography to localize radioactive ligand. This technique has been employed in many species including rabbits (Di Carlo & Muccioli, 1981; Di Carlo, Muccioli, Lando, & Bellussi, 1985), pigs and sheep (Posner, van Houten, Patel, & Walsh, 1983), horse, ewe and pigeon (Muccioli, Bellusi, Ghè, Pagnini, & Di Carlo, 1988), toads (Lüthy, Segura, & Lüthy, 1985), and in ring doves by Buntin and collaborators (Buntin & Walsh, 1988). The purpose of one of the latter studies was to map the distribution of PRL binding sites within the CNS which could conceivably mediate the behavioral and/or physiological effects produced by intracranial administration of PRL (Fechner & Buntin, 1989). Using quantitative autoradiography, specific binding was detected in choroid plexus, medial habenula, lateral mesencephalic nucleus, hippocampus, parahippocampal area, preoptic area, and 4 hypothalamic sites: PVN, VMH, SCN, and tuberal region (TU).

Hnasko and Buntin (1993) then determined the specific neural sites mediating the hyperphagic action of PRL in doves. Doves received twice daily micro-injections of PRL, at doses below those required to induce hyperphagia when injected into the ventricles (i.e., 50 ng/ml), in a variety of diencephalic sites. Results revealed that intra-

VMH injections of PRL produced a robust hyperphagic response characterized by increases in food intake of 58 % over that recorded during a vehicle-injection period, whereas PRL infused into the TU, and the MPOA caused significant elevations in feeding behavior of 32 % and 26 %, respectively. It was concluded that, in doves, the VMH was a primary site of PRL action in enhancing feeding, and that other sites may also contribute to the orexigenic action of this hormone.

The identification of PRL binding sites within the rat brain has also been an area of active research. Twenty years ago, Walsh and colleagues (1978), also using the principles of competitive binding assay in conjunction with light microscope autoradiography demonstrated, for the first time, specific PRL binding sites localized on the ependyma of the choroid plexus of male and female rats. Since then, using these assays, binding has been detected consistently in the choroid plexus (Barton, Lahti, Piercey, & Moore, 1989; Walsh, Mangurian, & Posner, 1990), by one investigator in the median eminence (using iodine-125-labeled ovine PRL) (^{125}I-oPRL) (Barton et al., 1989), and by another group of researchers in the hypothalamus and substantia nigra (using iodine-125-labeled rat PRL) (^{125}I-rPRL) (Muccioli, Ghè, & Di Carlo, 1991).

At the time, the issue of whether the identification of PRL binding sites using radioactive ligands and autoradiography represented high affinity binding to a specific PRL receptor remained to be determined. The lack of evidence for PRL binding sites outside the choroid plexus, and specifically in the hypothalamus, contrasted with the large body of physiological evidence suggesting that several brain regions that did not exhibit any detectable PRL binding were likely to contain receptors to mediate the effects of PRL on behavior (e.g., PRL-induced maternal behavior after intra-MPOA injections of PRL; Bridges et al., 1990). Difficulties in demonstrating specific PRL binding sites within the rat hypothalamus using autoradiography was thought to be related to a low density of receptors in discrete nuclei within this region (Barton et al.,

1989). Aspects of autoradiography such as the lack of purity of the ligands used (e.g., oPRL, or human growth hormone) in binding assays, and the incubation conditions necessary for the visualization of good autoradiographic images following *in vitro* incubation (Crumeyrolle-Arias, Latouche, Jammes, Djiane, Kelly, Reymond, & Haour, 1993), have also been considered obstacles to the identification of hypothalamic PRL binding sites. For example, studies have revealed that PRL binds non-specifically in the brain in the presence of magnesium in the incubation medium (Barton et al., 1989; Mangurian, Walsh, & Posner, 1989).

In 1993, Crumeyrolle-Arias and collaborators optimized the incubation parameters, and achieved a hypothalamic cartography of PRL binding sites in the female rat brain using *in vitro* autoradiography and ^{125}I-monoclonal PRL receptor antibody (^{125}I-U5). Their data revealed maximal densities in the preoptic suprachiasmatic and arcuate nuclei, and minimal densities in the median eminence (contrary to previous findings), and infundibulum. With the advent of more sophisticated techniques, however, Chiu, Koos and Wise (1992) provided the first demonstration of PRL receptor gene expression in the hypothalamus. Using reverse-transcription polymerase chain reaction (RT-PCR), these authors found PRL receptor mRNA in the anterior hypothalamus, medial basal hypothalamus, and anterior pituitary gland of estradiol-treated ovariectomized rats. Shortly after the publication of these findings, Chiu and Wise (1994) modified several steps in the *in situ* hybridization method to increase sensitivity, and reported the presence of PRL receptor gene expression in cells of the MPOA, SON, arcuate nucleus, VMH, and choroid plexus. These findings were later extended and corroborated by another group of researchers with immunohistochemical techniques using monoclonal antibodies raised against PRL receptor purified from rat liver (Roky, Paut-Pagano, Goffin, Kitahama, Valatx, Kelly, & Jouvet, 1996).

More recently still, Bakowska and Morrell (1997) produced a neuroanatomical

map of individual neurons that express the long form of the PRL receptor using *in situ* hybridization (with [^{33}P] labelled cRNA probe) in female rats at different stages of pregnancy. PRL receptors exist in two forms, the long form (Shirota, Banville, Ali, Jolicoeur, Boutin, Edery, Djiane, & Kelly, 1990) and the short form (Boutin, Jolicoeur, Okamura, Gagnon, Edery, Shirota, Banville, Dusanter-Fourt, Djiane, & Kelly, 1988). Bakowska and Morrell (1997) focussed on the long form of the receptor because it is documented to be the main form in brain regions that play a key role in the mediation of the hormonal onset of maternal behavior (Nagano & Kelly, 1994). In addition to replicating the results of Chiu and Wise (1994), they determined the presence of neurons expressing the mRNA for the long form of the PRL receptor in the anteroventral periventricular nucleus, the PVN, as well as in various limbic system structures (Bakowska & Morrell, 1997) .

Finally, Pi and Grattan (1998a), using immunohistochemistry and estrogen-treated, ovariectomized female rats as subjects, published data which replicated those of previous investigators, and added the arcuate nucleus, and the SCN as hypothalamic sites where scattered immunostaining for PRL receptors was localized on neuronal cell bodies as well as neuronal processes. The only inconsistency between their data and those of previous studies (i.e., Bakowska & Morrell, 1997; Chiu & Wise, 1994), is that no significant immunostaining was observed in the PVN or VMH. More recently, the same authors (Pi & Grattan, 1998b) investigated the presence of both the short- and long-forms of PRL receptor mRNA in the hypothalamus of lactating rats (days 7-10 postpartum), and found that compared to diestrous rats, expression of both forms of PRL receptor mRNA was significantly increased in the choroid plexus, SON, and PVN, whereas only the long form was expressed at significantly higher levels in the VMH of lactating dams. Taken together, it is likely that methodological differences (e.g., reproductive state of subjects, sex of subjects, different techniques used, different

secondary antibody, duration or temperature of incubations) may account for the differences in results. Nevertheless, as a whole, this active area of research lends supportive evidence for direct action of PRL on the brain through binding to PRL receptors.

The growing evidence on the localization of PRL receptors, as well as that on the central production of PRL provide the opportunity to form increasingly accurate hypotheses concerning the role of specific brain regions in PRL-regulated neuroendocrine and behavioral events. The investigation of the central action of PRL on feeding behavior in mammals is a relatively recent area of research. It is presently well established that icv administration of PRL produces a stimulatory effect on feeding in rats (Noël & Woodside, 1993; Sauvé & Woodside, 1996). One question that remains unanswered is the site(s) of action of PRL on food intake. Another question unanswered is the mechanism(s) underlying the relatively long latency of onset to feeding produced by icv administration of PRL (Sauvé & Woodside, 1996).

The goal of the present thesis is to investigate these two issues using behavioral and molecular approaches. The first experiment was performed to expand the existing knowledge of the central effect of PRL on eating, and examine the anatomical specificity of PRL-induced hyperphagia in intact, virgin female rats. The second experiment was performed to investigate the relation between duration of treatment with icv injections of PRL and latency of activation of the neuronal mechanisms that regulate PRL-induced hyperphagia.

To address the first question, cycling, virgin female rats were implanted with a unilateral cannula aimed at either the PVN, VMH, or MPOA, and the investigation of the effect of PRL infused in each of these sites on food intake was undertaken. The PVN was chosen as a possible brain area involved in the orexigenic action of PRL, not only because it contains PRL receptors (Bakowska & Morrell, 1997; Roky et al., 1996)

and is thought to be a site of PRL synthesis (Clapp et al., 1994), but also because it has a long-standing history as a feeding-relevant structure in the hypothalamus (Anand & Brobeck, 1951; Leibowitz, 1978). The PVN mediates the orexigenic effect of many agents including norepinephrine (e.g., Hajnal, Mark, Rada, Lenard, & Hoebel, 1997; Leibowitz, 1978; Leibowitz, Hammer, & Chang, 1983; Leibowitz & Hor, 1982), opiates (e.g., Gosnell et al., 1986; Leibowitz & Hor, 1982; McLean & Hoebel, 1983), NPY (e.g., Leibowitz, Akabayashi, Alexander, & Wang, 1998; Morley, Levine, Gosnell, Kneip, & Grace, 1987; Stanley & Leibowitz, 1984; Stanley & Leibowitz, 1985), and galanin (e.g., Leibowitz et al., 1998; Tempel, Leibowitz, & Leibowitz, 1988). The PVN is also considered to be critical for the regulation of feeding as demonstrated by ablation studies (e.g., Gold, 1970; Leibowitz et al., 1983; Sclafani & Aravich, 1983).

Lesions studies have also revealed the importance of the VMH as a neural site involved in eating behavior (e.g., Hetherington & Ranson, 1940; Sclafani & Aravich, 1983). As with the PVN, PRL receptors (Bakowska & Morrell, 1997), and PRL-lir (Fuxe et al., 1977; Hansen et al., 1982; Toubeau, et al., 1979) have been located within the VMH suggesting that PRL-induced behavioral effects may be mediated at this site. The VMH is also known to mediate the hyperphagic properties of agents such as GRF (Tanaka et al., 1991), dynorphin (Gosnell et al., 1986), and NPY (Morley et al., 1987; Stanley et al., 1985a). The VMH has also been identified as a key target (Jacob, Dziura, Medwick, Leone, Caprio, During, Shulman, & Sherwin, 1997) for the anorectic properties of estrogen (Wade & Zucker, 1970a; Wade & Zucker, 1970b), and those of leptin (Halaas, Gajiwala, Maffei, Cohen, Chait, Rabinowitz, Lallone, Burley, & Friedman, 1995), the protein product of the ob gene (Campfield, Smith, Guisez, Devos, & Burn, 1995).

More relevant still to this thesis, are data revealing that intra-VMH PRL injections induce dramatic increases in food intake in ring doves (Hnasko & Buntin, 1993) by binding to PRL receptors located within this nucleus (Li, Kelly, & Buntin,

1995). Moreover, the VMH has been shown to be an important neural substrate regulating PRL-related neuroendocrine events in rats such as the semicircadian secretion of PRL characteristic of the first half of pregnancy (Butcher, Fugo, & Collins, 1972; Gunnet & Freeman, 1985; Lee, Arbogast, & Voogt, 1998) and pseudopregnancy (Gunnet, Mick, & Freeman, 1981), and to be involved in the rapid PRL-mediated induction of maternal behavior seen at parturition (Bridges & Mann, 1994). Taken together, these findings clearly implicate the VMH as a site critical for the regulation of feeding, body weight, energy balance, PRL secretion, and maternal behavior.

The MPOA was the third site chosen to investigate the anatomical specificity of PRL-induced hyperphagia. The MPOA has been identified as a key central site regulating the expression of maternal behavior (e.g., Fleming, Suh, Korsmit, & Rusak, 1994; Fleming & Walsh, 1994; Numan, 1988), and the stimulatory action of PRL on maternal responsiveness (e.g., Bridges et al., 1990). Several PRL-related endocrine events have also been documented to be mediated at the level of the MPOA. For example, the preovulatory PRL surge occurring in cycling female rats (Ben-Jonathan et al., 1989) is either reduced (Dyer, ter Haar, & Saphier, 1987) or eliminated (Kimura & Kawakami, 1978) by MPOA lesions. MPOA lesion studies (Freeman & Banks, 1980) have also revealed the role of this structure in the semicircadian pattern of PRL secretion observed during pseudopregnancy induced by stimulation of the uterine cervix (Gunnet & Freeman, 1983).

Additionally, there is evidence that a feeding function is subserved by the MPOA. Using radioimmunoassay and immunohistochemistry, both galanin and NPY levels within the MPOA (and within the PVN, incidentally) have been found to increase on the day of proestrus in female rats with greater body fat and a preference for fat-rich diets. In these animals, the peak in peptide levels has been accompanied by an increase in caloric intake, suggesting that these two peptides in the MPOA may contribute to

overeating and weight gain (Leibowitz et al., 1998). Moreover, some reports have shown that intra-MPOA injections of GRF (Dickson & Vaccarino, 1990; Vaccarino & Hayward, 1988), and MPOA lesions (Dagnault & Richard, 1997; Mascarenhas, 1986), stimulate feeding behavior, whereas others have documented the involvement of the MPOA in the anorectic action of estrogens (Dagnault & Richard, 1997; Mascarenhas, 1986).

Dagnault and Richard (1997) found that injections of estradiol directly into the medial preoptic nucleus, an area where estrogen receptors have been identified (e.g., Shughrue, Lane, & Merchenthaler, 1997), induced a dose-dependent reduction in food intake. These results corroborated previously published data (Mascarenhas, 1986) which revealed that ovariectomy-induced increases in food intake were decreased following intra-MPOA administration of estrogen. Finally, in ring doves, the MPOA has been identified as a site mediating the hyperphagic properties of PRL (Hnasko & Buntin, 1993). PRL injections into the MPOA resulted in a 26 % increase in food intake above baseline measures (Hnasko & Buntin, 1993), clearly indicating that the MPOA is implicated in the orexigenic action of PRL in this species.

As a whole, these findings suggest that the PVN, VMH, and MPOA may be hypothalamic areas of relevance to the study of the specificity of the feeding-stimulating properties of PRL in female rats. Chapter 1 presents an investigation of the role of the PVN, VMH, and MPOA in the hyperphagic action of PRL in virgin female rats. Chapter 2 examines the mechanism(s) underlying the relatively long latency of onset to feeding following icv injections of PRL (Sauvé & Woodside, 1996) using Fos-like immunoreactivity (Fos-lir) as a marker for cellular activation.

CHAPTER 1

Neuroanatomical Specificity of Prolactin-Induced Hyperphagia.

INTRODUCTION

The aim of the first experiment was to investigate the potential role of the PVN, VMH, and MPOA in the hyperphagic action of PRL. It was hypothesized that a dose of PRL that was ineffective when introduced into the ventricles (800 ng; see Sauvé & Woodside, 1996) would potentiate feeding when injected into brain areas known to be involved in feeding behavior and to contain PRL receptors (Bakowska & Morrell, 1997; Crumeyrolle-Arias et al., 1993; Roky et al., 1996). The rationale behind this hypothesis is based on several reports showing that the administration of agents directly into neuronal sites in which their cognate receptors are located elicits greater behavioral responses than the administration of equivalent doses into the ventricles, and similarly, administration of small doses to receptor-rich sites may produce behavioral changes identical to those resulting from the administration of larger doses into the ventricles.

For example, 1) GRF stimulates feeding behavior in rats more effectively when injected into the MPOA/SCN (Dickson & Vaccarino, 1990; Vaccarino & Hayward, 1988), and VMH (Tanaka et al., 1991) than when administered into the ventricles; similarly, 2) injections of NPY, and PYY into the PVN (Stanley et al., 1985b) elicit a much stronger feeding response in rats compared to that produced by icv administration (Clark, Kalra, Crowley, & Kalra, 1984; Levine & Morley, 1984); 3) smaller doses of NPY than those effective when administered into the ventricles are sufficient to evoke an equivalent food intake response when injected into the PVN (Kalra, Dubé, Fournier, & Kalra, 1991); 4) doses of leptin that do not suppress feeding when injected into the

ventricles, significantly reduce food intake when administered into the VMH (Jacob et al., 1997); and 5) doses of PRL that fail to produce a significant change in maternal behavior in rats when given intracerebroventricularly, are effective when injected directly into the MPOA (Bridges et al., 1990).

GENERAL MATERIALS AND METHODS

Subjects

Fifty female Wistar rats from Charles River Breeding Farms (St-Constant, Québec) were used in these studies. Upon arrival in the laboratory, animals were group-housed (3 or 4 per cage) in polypropylene cages (45 cm x 25 cm x 20 cm) for at least one week during which they were handled daily for several minutes. The rats were maintained on a 12 hour light/dark cycle with lights on at about 0700 hr and lights off at about 1900 hr, and kept at a room temperature of 20 ± 2° C. To be included in the study, female rats were required to demonstrate normal 4 or 5 day estrous cycles as determined by daily vaginal smears.

Surgeries

After a minimum of 7 days of acclimatization and handling, rats were anesthetized under sodium pentobarbital (60 mg/kg) and implanted with a unilateral stainless steel 22 gauge guide-cannula (Plastics Products). Four stainless steel screws were threaded into the cranium and dental acrylic was moulded around the screws and the cannula to secure the cannula to the skull. Dummy-cannulae which extended 1 mm beyond the tip of the guide-cannulae were inserted immediately following surgery. Rats were allowed a minimum of 5 days to recover from the surgery.

Habituation

The first estrous smear recorded at least five days following the surgery marked the beginning of the period of habituation to the injection procedure. Habituation proceeded for 4 or 5 days (i.e., the duration of one estrous cycle) and consisted of one mock injection given daily at about 0700 hr. During this time period, food intake, body weight, and vaginal smears were recorded and served as baseline measures.

On the last day of habituation, animals were assigned to one of two conditions (*control-saline* or *treatment-PRL*) based on their baseline food intake. Group assignment was performed so the groups had similar mean food intake and an evenly distributed range of food intake.

Procedures

All subjects had *ad lib* access to powdered rat chow (Lab Diet Meal # 5001, PMI Feeds, Inc.) and water throughout the habituation and treatment periods. The food was contained in glass jars (5 cm in diameter and 7 cm deep) which were glued with epoxy to aluminium sheets (1 mm x 8 cm x 17 cm) bent in an S-shape such that the top lip curved over to fasten onto the side wall of the cage under the cage lid. These jars were then placed in dishes (11 cm in diameter and 5 cm deep) to collect potential spillage (note: rats that consistently spilled food outside this dish and into the bedding were eliminated from the study).

Testing began on the day of estrous immediately after the period of habituation to the injection procedure and was carried out over a period of 10 days. A testing day consisted of twice daily injections (at 0700 hr and 1900 hr) of either 800 ng of oPRL (Sigma Chemicals, P.O. Box 14508 St-Louis, Missouri, 63178, U.S.A.) dissolved in saline and buffered to a pH of approximately 7 or saline. All injections were administered in a volume of 0.5 µl through a 28 gauge internal cannula which was

attached to polyethylene tubing connected to a 1.0 µl Hamilton syringe (model # 7001). Injections were given to rats in their home cages. Both PRL and saline were administered over a minimum of 45 seconds after which the injector was left in place for an additional 45 seconds and then replaced by the dummy-cannula. Vaginal smears, body weight and food intake measures were taken daily at the time of the morning injection.

Histology

Following completion of each experiment, rats were injected with an overdose of sodium pentobarbital (0.5 ml) and perfused with 0.9 % saline followed by 10 % Formol saline. Brains were stored in 10 % paraformaldehyde for several days, frozen and then sliced with a cryostat into 30 µm sections or with a vibratome into 50 µm slices. Cannula placements were verified with cresyl violet staining and determined by a minimum of two investigators with the aid of a stereotaxic atlas (Paxinos & Watson, 1986). Only data from animals identified as having placements directly within, or in close proximity to, the PVN, VMH and MPOA, were used in statistical analyses (as in e.g., Bridges et al, 1990; Bridges, Robertson, Shiu, Sturgis, Henriquez, & Mann, 1997; Stanley & Leibowitz, 1985).

To verify the accuracy of right lateral ventricle placements, rats were given one icv injection of 2 µl of Angiotensin II (25 µg/ml; Sigma Chemicals) a few days prior to the start of the habituation period. If subjects showed an immediate drinking response, cannulae placements were accepted as accurate (Hulsey & Martin, 1992). In addition to this measure, at the end of the study, subjects were sacrificed by asphyxiation with carbon dioxide, guillotined, and the obturators were removed and replaced by the injector cannula. Rats were injected with 1.0 µl of India ink over 60 seconds, and injectors were left in place for about one minute before the brains were removed. A coronal section was made at the point of implantation, and the tips of the injectors were located and recorded. A placement was judged accurate when the right lateral ventricle

was filled with ink.

Statistical Analyses

To determine the effect of intra-PVN administration of PRL on feeding behavior, each subject's daily food intake was subtracted from its own baseline and a three-factor analysis of variance (ANOVA) was performed with treatment condition (saline or PRL injections) and site of injection (right lateral ventricle or PVN) as the between group factors, and days of testing as the within group factor. In light of previous data showing that icv injections of 800 ng of PRL do not significantly increase ingestive behavior (Sauvé & Woodside, 1996), rats implanted with a cannula aimed at the right lateral ventricle served as additional controls, and planned comparisons were calculated when appropriate.

To determine whether PRL affected food intake when infused into the VMH or the MPOA, each subject's daily food intake was subtracted from its own baseline, and these data were subjected to a two-factor ANOVA with treatment (*saline* or *PRL*) as the between group factor and days of testing as the within group factor. Tukey's post-hoc test was used when necessary.

To determine whether each site-specific treatment with PRL affected body weight, the difference between each subject's average body weight on Day 10 was subtracted from its baseline measure and a one-way ANOVA was performed on these data with groups as the between factor.

EXPERIMENT 1a:

The Effect of Intra-PVN Injections of PRL on Food Intake in Virgin Female Rats.

Twenty-four female rats weighing an average of 275 at the start of the experiment were used as subjects. Cannula implantations aimed at either the PVN (N = 15) (coordinates AP: -1.8 mm; L: -0.4 mm; DV: -7.8 mm; Paxinos & Watson, 1986), or the right lateral ventricle (N = 9) (coordinates anterior-posterior (AP): -0.2 mm; lateral (L): -1.6 mm; dorsal-ventral (DV): -5.0 mm below the surface of the skull, incisor bar 5 mm above the interaural line; Pelligrino, Pelligrino, & Cushman, 1979), were carried out. The latter group of subjects served as additional controls to replicate that lack of effect of 800 ng of PRL on food intake (Sauvé & Woodside, 1996).

EXPERIMENT 1b:

The Effect of Intra-VMH Injections of PRL on Food Intake in Virgin Female Rats.

Thirteen female rats, weighing an average of 267 grams, were implanted with a unilateral guide-cannula aimed at the VMH using coordinates AP: -2.8 mm; L: -0.7 mm; DV: -9.7 mm (Paxinos & Watson, 1986).

EXPERIMENT 1c:

The Effect of Intra-MPOA Injections of PRL of Food Intake in Virgin Female Rats.

Thirteen female rats weighing an average of 257 grams were used for this experiment. Rats were implanted with a unilateral guide-cannula aimed at the MPOA using coordinates AP: -0.30 mm, L: -0.50 mm, DV: -8.5 mm (Paxinos & Watson, 1986).

RESULTS

EXPERIMENT 1a:

The Effect of Intra-PVN Injections of PRL on Food Intake in Virgin Female Rats.

Twice daily injections of PRL for 10 days significantly increased food intake in cycling virgin female rats ($F [1, 20] = 9.505$, $p = 0.0059$). Simple effects revealed that 800 ng of PRL produced a hyperphagic response when injected into the PVN ($F [1, 20] = 9.363$, $p = 0.006$). For the sake of clarity, only the data from the 2 groups receiving intra-PVN injections are shown and the data from the animals receiving icv injections are omitted from Figure 1. Interestingly, PRL exerted an orexigenic effect immediately on the first few days of testing. Figure 2 illustrates that, averaged over the 10 days of testing, intra-PVN injections of PRL produced an increase in food intake of 2.23 ± 0.81 grams over baseline measures. As expected, treating rats with icv injections of 800 ng of PRL did not produce an enhanced feeding response.

Results indicated that body weight was not affected by intra-PVN administration of PRL ($F [3, 20] = 1.429$, $p = 0.2640$). This finding is shown in Figure 3.

Consistent with previous findings (Noël & Woodside, 1993; Sauvé & Woodside, 1996), the hyperphagic effect of PRL was obtained without disrupting vaginal cyclicity. Two animals receiving intra-PVN injections of saline and one receiving intra-PVN injections of PRL became acyclic during testing.

Histological sections depicting cannula placements in the PVN (-1.40 mm to -1.80 mm Anterior-Posterior) can be seen in Figure 4.

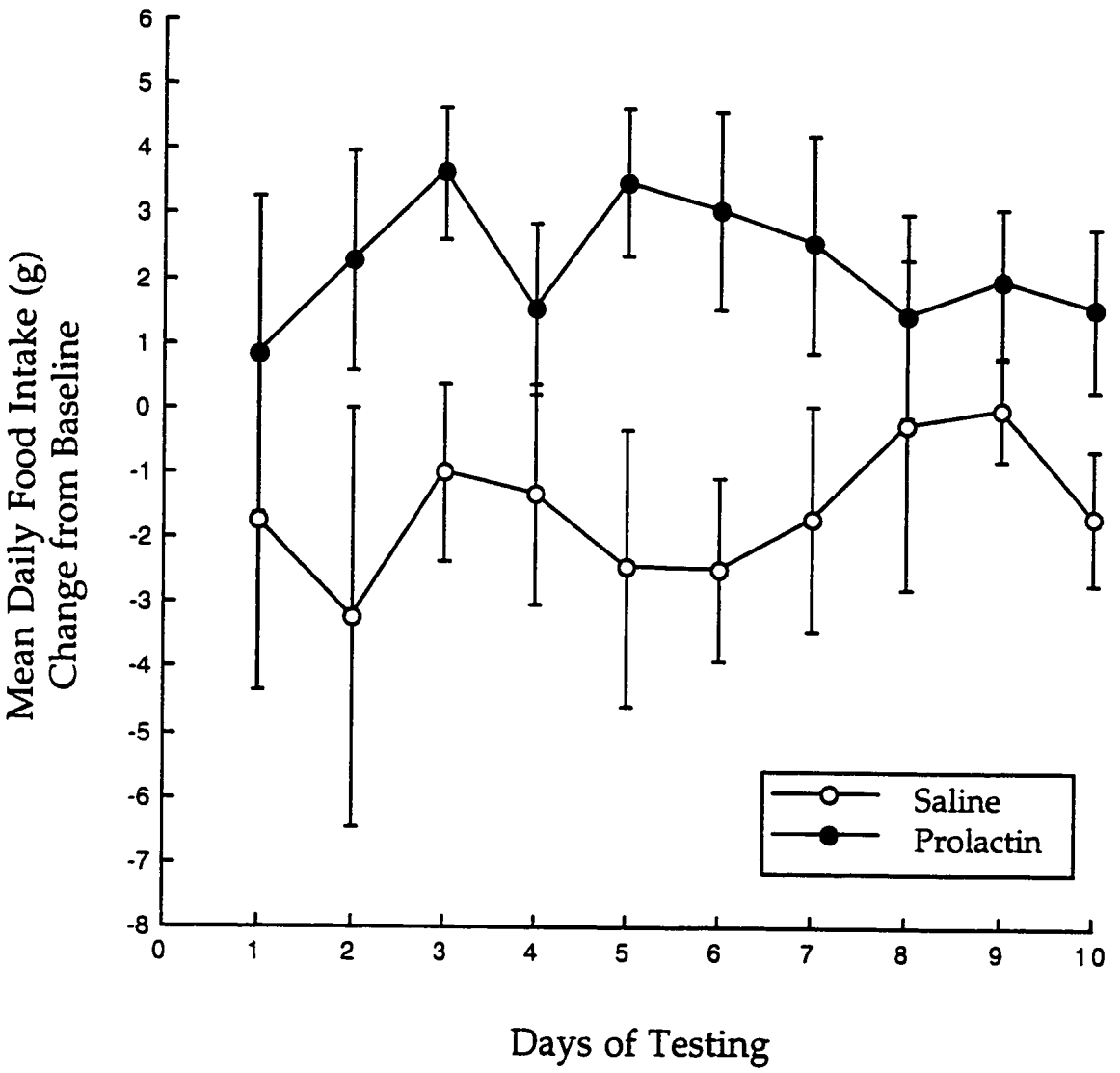

Figure 1: Mean daily food intake of virgin female rats treated with intra-PVN injections of saline or prolactin (800 ng).

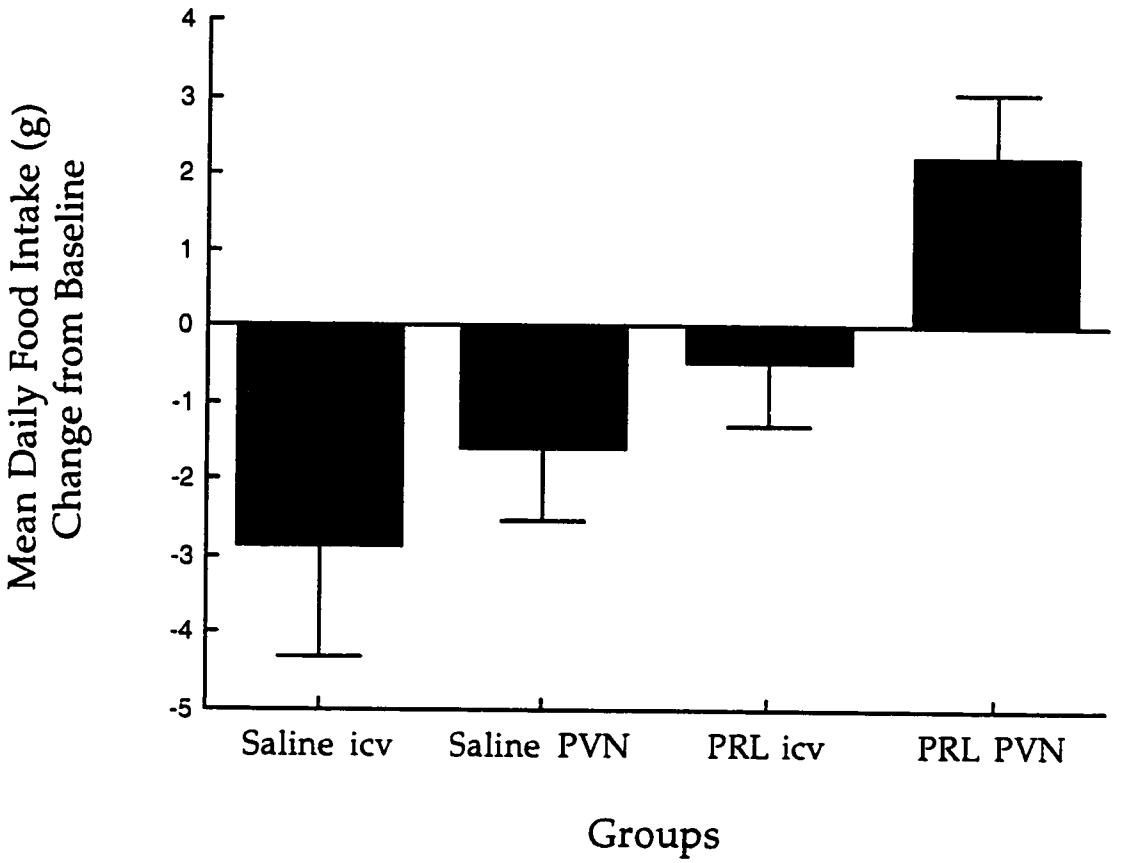

Figure 2: Mean daily food intake averaged over the 10 days of testing for all 4 groups.

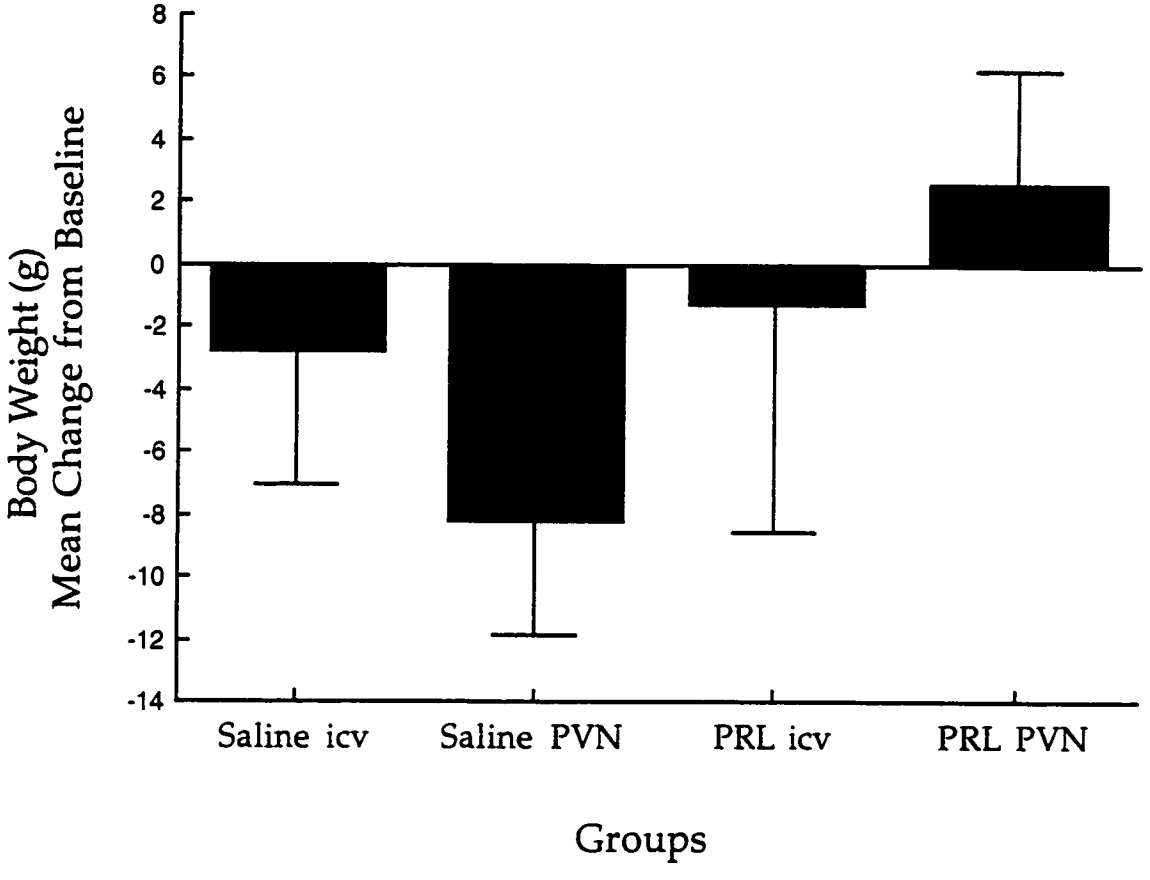

Figure 3: Mean difference in body weight between the last day of testing and baseline measures.

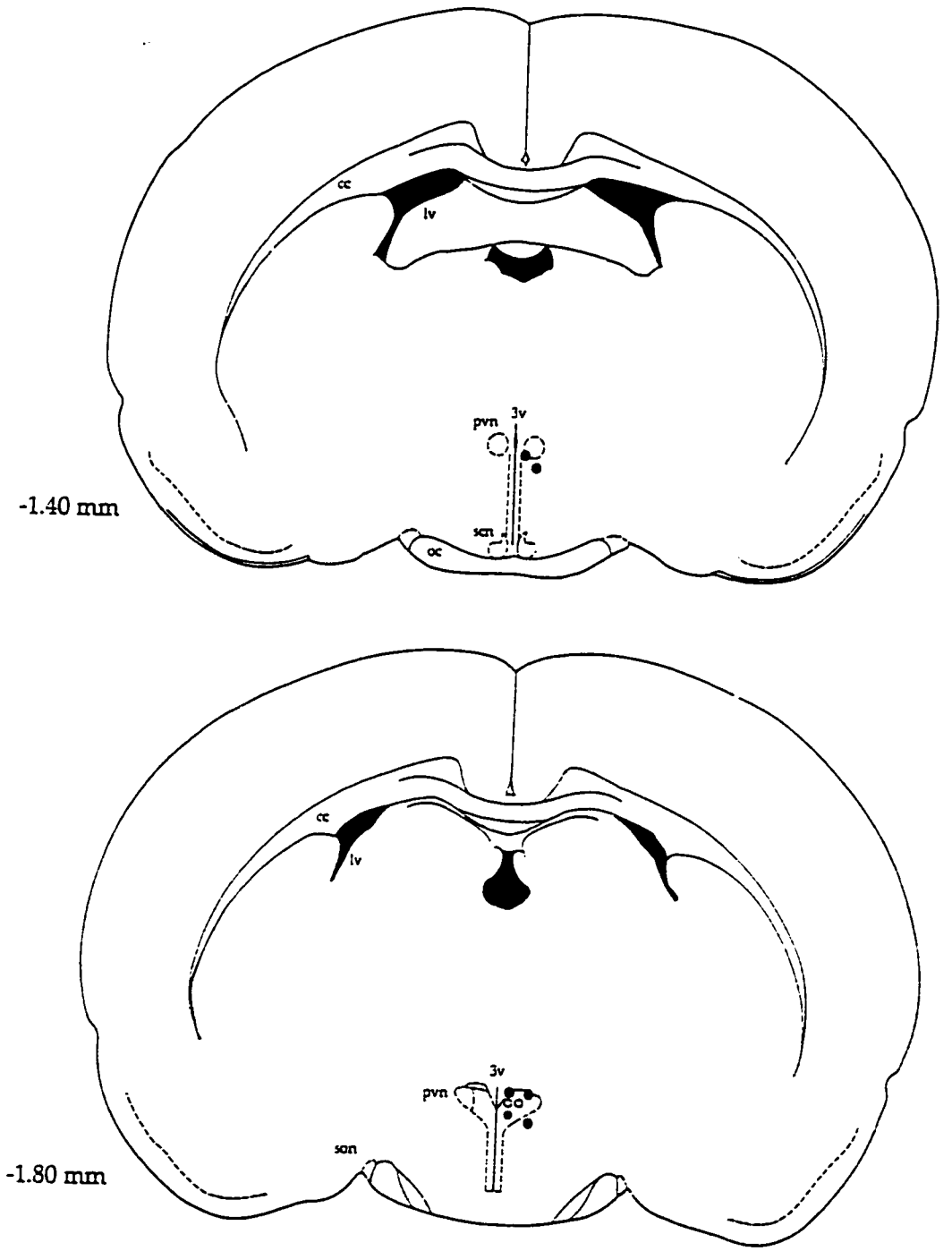

Figure 4: Histological drawings showing examples of saline (○) and prolactin (●) infusion sites in the PVN (Paxinos & Watson Atlas; -1.40 mm to -1.80 mm).

3v, third ventricle; *cc*, corpus callosum; *lv*, lateral ventricle; *oc*, optic chiasm; *scn*, suprachiasmatic nucleus; *son*, supraoptic nucleus.

EXPERIMENT 1b:

The Effect of Intra-VMH Injections of PRL on Food Intake in Virgin Female Rats.

Data from two subjects in the experimental group were eliminated from analyses because proper baseline measures were not obtained (animals spilled food outside the glass dishes). A two-way ANOVA revealed that intra-VMH injections of PRL failed to potentiate feeding in virgin female rats ($F [1, 9] = 1.311$, $p = 0.2817$). Interestingly, as seen in Figure 5, a trend towards a hyperphagic response was observed towards the end of the 10-day hormone administration period, but there was no group by day interaction ($F [9, 81] = 1.711$, $p = 0.0998$). To explore further this trend, the data were submitted to a three-way ANOVA (group x bloc x day) to determine whether PRL-injected female rats increased significantly their food intake during the second half of testing (i.e., Days 6 to 10). Results showed that there was no group by bloc interaction. The original ANOVA did reveal a main effect for days of testing ($F [9, 81] = 2.383$, $p = 0.0190$), however. A post-hoc analysis (Tukey's HSD) indicated that there existed significant differences between the mean daily food intake measured on Day 1 and that on Days 3, 7 and 9 ($p < 0.05$).

As in the previous experiment, the average body weight of rats injected with PRL in the VMH was not significantly different from baseline measures ($F [1, 9] = 0.260$, $p = 0.6224$) (data not shown). Similarly, vaginal cyclicity was not disrupted by the treatment with PRL. One animal from each group showed vaginal cytology indicative of persistent estrous in the second half of testing.

Figure 6 shows histological sections depicting cannula placements in the VMH (-2.30 mm to -2.80 mm Anterior-Posterior).

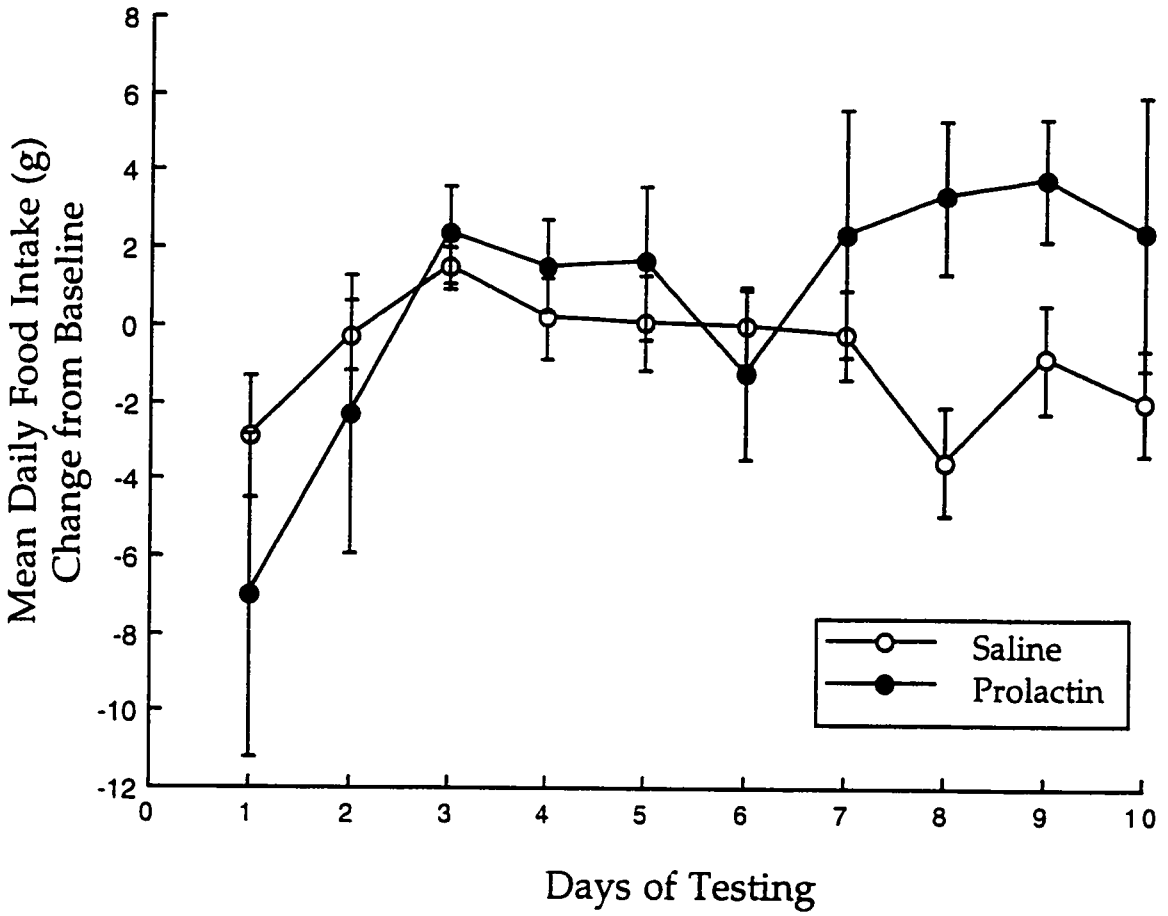

Figure 5: Mean daily food intake of virgin female rats treated with intra-VMH injections of saline or prolactin (800 ng).

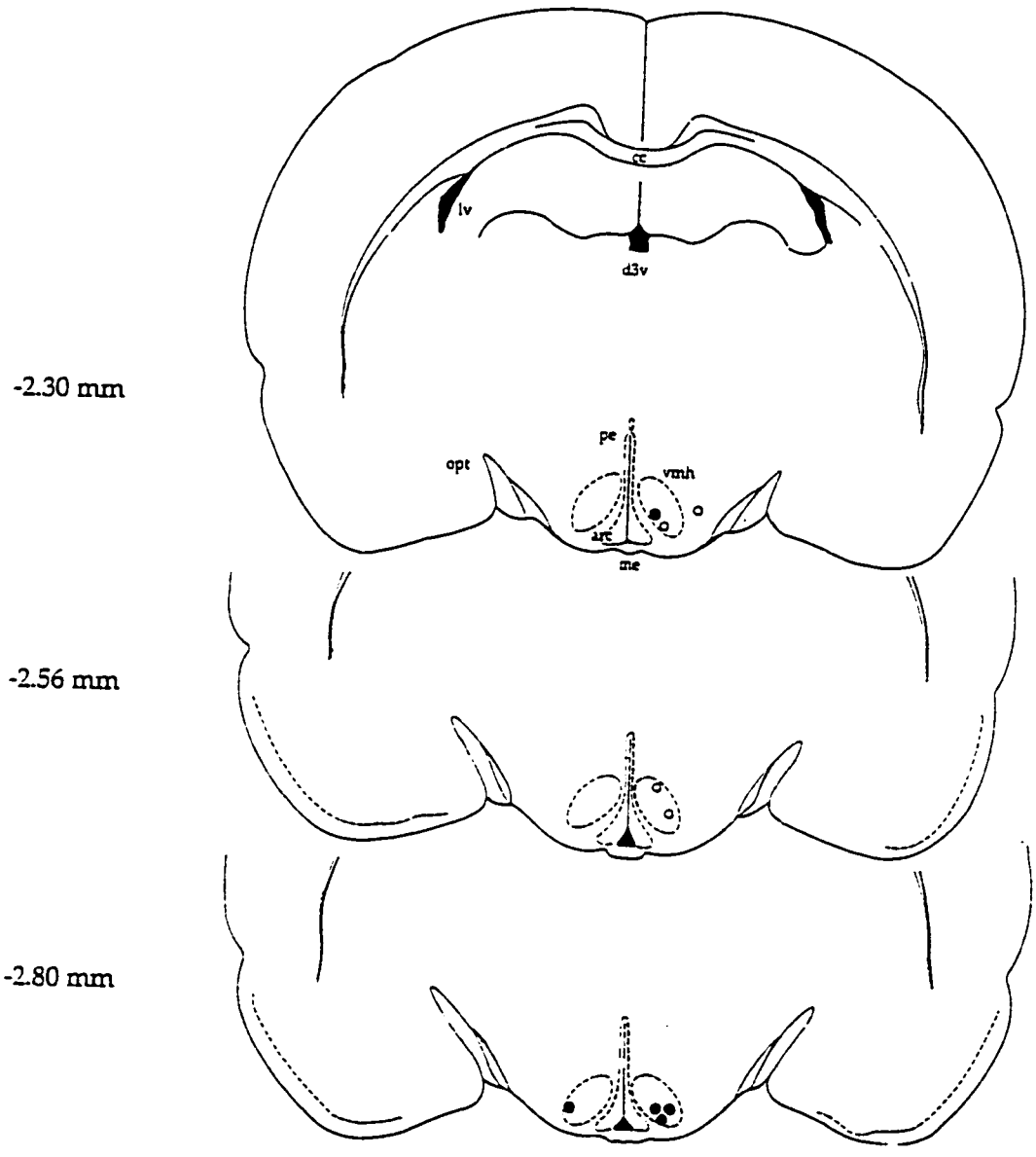

Figure 6: Histological drawings showing examples of saline (o) and prolactin (●) infusion sites in the VMH (Paxinos & Watson Atlas; -2.30 mm to -2.80 mm).

arc, arcuate nucleus; *cc*, corpus callosum; *d3v*, dorsal third ventricle; *lv*, lateral ventricle; *me*, median eminence; *opt*, optic tract; *pe*, periventricular nucleus; *vmh*, ventromedial nucleus.

EXPERIMENT 1c:

The Effect of Intra-MPOA Injections of PRL of Food Intake in Virgin Female Rats.

A two-way ANOVA performed with treatment (*saline* or *PRL*) as the between group factor and days as the within group factor revealed a main effect for days ($F [9, 99] = 2.521$, $p = 0.0121$), and no main effect for treatment ($F [1, 11] = 1.748$, $p = 0.2129$) indicating that 800 ng of PRL injected twice daily in the MPOA does not affect eating. In fact, as shown in Figure 7, the daily average food intake of PRL-treated rats remained consistently below baseline levels.

Results of analyses performed on body weight measures, and observations of vaginal cyclicity paralleled those of the two previous studies. PRL did not affect body weight ($F [1, 11] = 2.726$, $p = 0.1270$) (data not shown), nor vaginal cyclicity as determined by examination of daily vaginal smears. One saline-injected animal showed vaginal cytology indicative of persistent estrous throughout testing, whereas one PRL-injected animal showed vaginal cytology indicative of persistent estrous during the second half of testing.

Figure 8 illustrates histological sections showing cannula placements in the MPOA (-0.26 mm to -0.92 mm Anterior-Posterior).

DISCUSSION

The results of Experiment 1a, b, and c showed that, of the three sites studied, the PVN was the most sensitive to the orexigenic properties of PRL. Many injections of PRL were necessary before an increase in feeding was observed in the VMH, and PRL did not stimulate feeding when infused in the MPOA. This suggests that the PVN is a primary

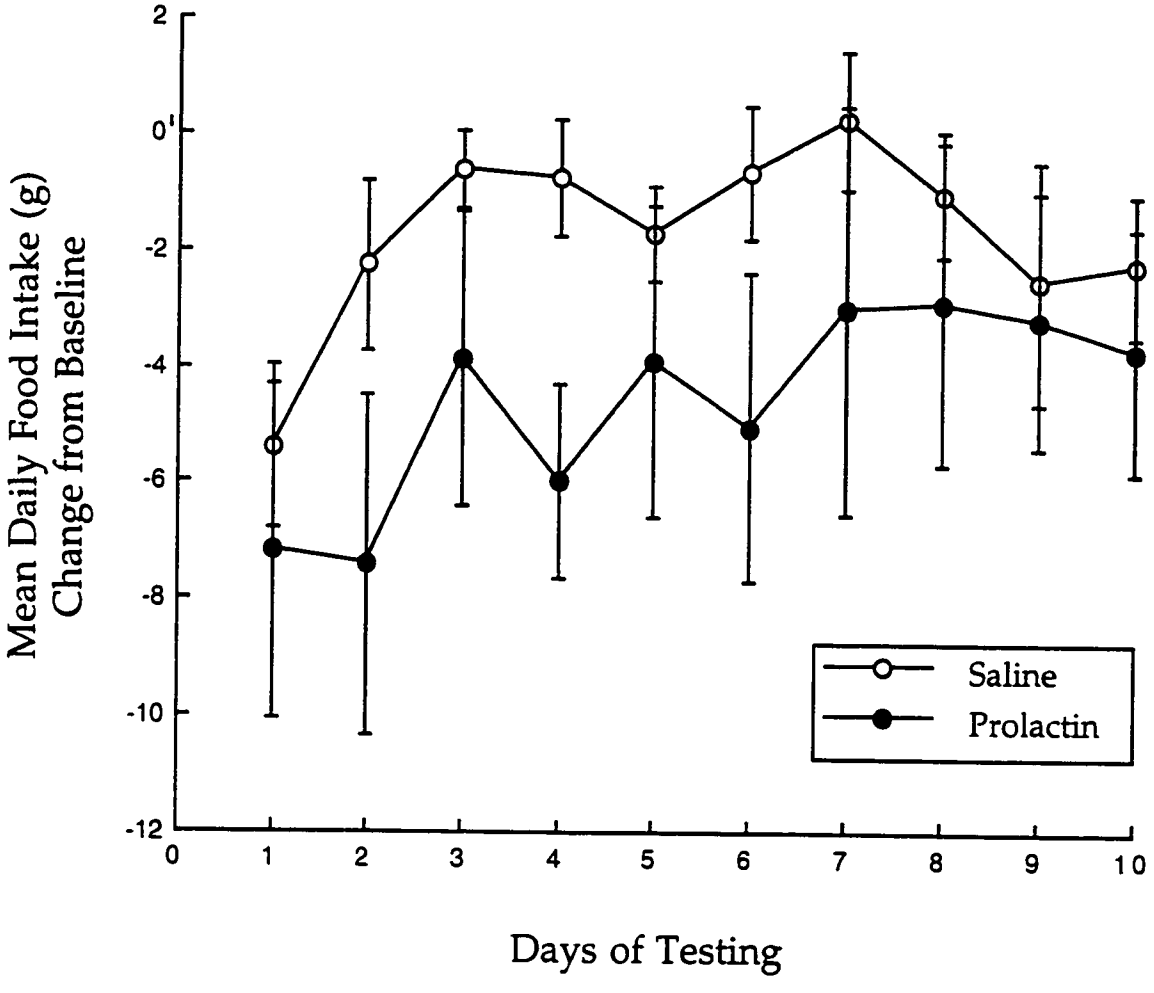

Figure 7: Mean daily food intake of virgin female rats treated with intra-MPOA injections of saline or prolactin (800 ng).

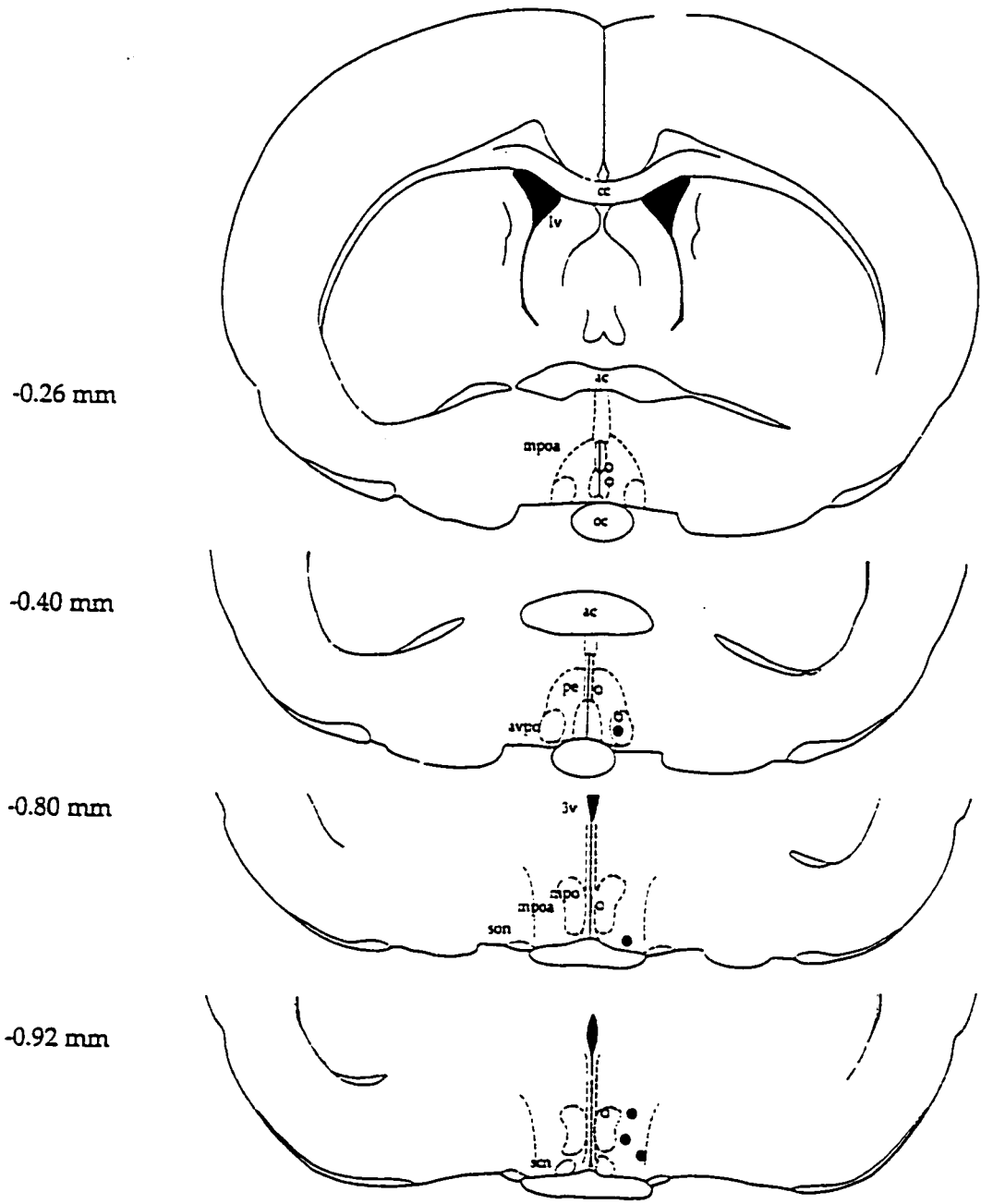

Figure 8: Histological drawings showing examples of saline (o) and prolactin (●) infusion sites in the MPOA (Paxinos & Watson Atlas; -0.26 mm to -0.92 mm).

3v, third ventricle; *ac*, anterior commissure; *avpo*, anteroventral preoptic nucleus; *cc*, corpus callosum; *mpo*, medial preoptic nucleus; *mpoa*, medial preoptic area; *lv*, lateral ventricle; *oc*, optic chiasm; *scn*, suprachiasmatic nucleus; *son*, supraoptic nucleus.

site of action for PRL-stimulated hyperphagia in virgin female rats, and that the VMH and the MPOA are not directly involved in PRL-induced feeding.

The present results also revealed that both body weight and vaginal cyclicity remained unchanged by the treatment with PRL. Future studies should extend these results by investigating the potential role of other hypothalamic sites in PRL-induced feeding, as well as measuring other behaviors and physiological effects which might be induced by hypothalamic injections of PRL.

CHAPTER 2

Expression of Fos-like Immunoreactivity in Selected Hypothalamic Sites of Virgin Female Rat Brain Following Central Administration of PRL.

INTRODUCTION

Previous studies from this laboratory showed that when PRL was injected twice daily into the ventricles, it took at least 3 days to see enhanced food intake (Sauvé & Woodside, 1996). The data presented in this thesis indicate clearly that the magnitude of the feeding response, and the latency of onset to feeding produced by PRL varies as a function of the site in which it is infused: intra-PVN injections of PRL significantly increased food intake in virgin female rats as of the second day of testing (i.e., after 3 injections), whereas a non-statistically significant trend towards a PRL-induced hyperphagic response manifested itself in the second half of testing in rats receiving intra-VMH injections of PRL. One question that arises from these data is what accounts for the varying latencies of onset to the feeding response? Possible events that may contribute to the varying latencies of onset to feeding include the site of application (local versus icv) of PRL, or spreading of the hormone to other sites. Corollary questions are what is the relation between duration of treatment with PRL and the latency of activation of the neuronal mechanisms that regulate PRL-induced food intake? And what is the pattern of neuronal activity that emerges from repeated injections of PRL in the PVN, VMH, and MPOA of virgin female rats? The following study was carried out to investigate these issues by evaluating Fos-lir, a marker for cellular activation, after a varying number of icv injections of PRL.

Fos is the protein product of the protooncogene c-*fos* which is one of a class of

immediate early genes (IEGs) (Lau & Nathans, 1987). It is rapidly and transiently expressed (Curran, 1988) in a wide range of neurons after various stimuli such as sensory (e.g., Hunt, Pini, & Evan, 1987; Pfaus & Heeb, 1997), electrical (e.g., Sagar, Sharp, & Curran, 1988), behavioral (e.g., Baum & Everitt, 1992; Ceccatelli, Villar, Goldstein, & Hokfelt, 1989), and hormonal (e.g., Cattaneo & Maggi, 1990; Hoffman, Smith, & Verbalis, 1993). The role of Fos in nuclear signal transduction can be described as follows. Extracellular stimuli or cell surface signals bring about changes in the cytoplasm of cells, more specifically in second messenger levels, which lead to an induction of both c-*fos* and c-*Jun*. The latter is a member of the Jun family which consists of other oncogene products (e.g., Jun B, Jun D). The activation involves distinct regulatory elements in the 5′ flanking region of the two genes. Following translation in the cytoplasm, the protein products of c-*fos* and c-*Jun*, Fos and Jun, are rapidly translocated to the nucleus where they associate via a structural motif called a leucine zipper, to form a heterodimeric protein complex (Morgan & Curran, 1989). This heterodimer then binds with high affinity to the activator-protein-1 (AP-1) DNA consensus recognition sequence, TGACTCA (Curran & Franza, 1988), to regulate gene expression. Given that most neurons express little or no c-*fos* under baseline conditions (Morgan & Curran, 1989), the localization of the expression of the Fos protein in the nucleus of cells by immunocytochemistry reveals stimulus-specific neuronal activation (Hunt et al., 1987; Sagar et al., 1998) in several areas in a single brain.

Numerous reports have documented the expression of Fos-lir in the MPOA, PVN, and VMH after a variety of stimuli related to feeding, metabolism, and PRL-regulated behavioral and neuroendocrine effects. For example, activation of Fos-lir has been detected in the MPOA of 1) female rats during early pregnancy (Lee et al., 1998) in a manner that paralleled the bi-daily surges of PRL secretion characteristic of this time of gestation, 2) rat dams during the expression of maternal behavior (Fleming et al.,

1994; Fleming & Walsh, 1994), and 3) rats after the ingestion of a palatable meal (Park & Carr, 1998). Fos-lir is also expressed in the PVN after the administration of feeding stimulants like NPY (icv) (Li, Xu, Rowland, & Kalra, 1994; Yokosuka, Xu, Pu, Kalra, & Kalra, 1998), inhibitors of fatty acid oxidation (e.g., methyl palmoxirate; Horn & Friedman, 1998a), fructose analogues (e.g., 2,5-anhydro-D-mannitol; Horn & Friedman, 1998b), as well as after central or peripheral administration of food intake suppressants such as leptin (icv) (Van Dijk, Thiele, Donahey, Campfield, Smith, Burn, Bernstein, Woods, & Seeley, 1996; Yokosuka et al., 1998), cholecystokinin (CCK) (intraperitoneal (ip)) (Wang, Martinez, Barrachina, & Tache, 1998), and bombesin (Li & Rowland, 1996). Incidentally, Yokosuka and colleagues (1998) also found a significant number of Fos-lir-positive neurons in the VMH of rats after icv injections of NPY, and leptin. Together these data clearly show that neurons within these 3 hypothalamic nuclei respond to, and are sensitive to hormonally-related, and feeding-altering stimuli, at a cellular level, as measured by Fos-lir immunohistochemistry.

In the present study, Fos-lir in the MPOA, PVN, and VMH was evaluated after 1, 7, and 15 icv injections of PRL, a number of injections which corresponds to 1, 4 and 8 days of treatment, respectively. Comparisons between Fos-lir and length of PRL treatment are described. The dose of 2 μg/0.5 μl of PRL was chosen for this study because it has shown to produce consistently significant increases in food intake in *ad lib* fed virgin female rats (Noël & Woodside, 1993; Sauvé & Woodside, 1996). Given the pattern of behavioral data obtained in the previous series of experiments, it was hypothesized that Fos-lir in the PVN and VMH might increase as a function of the number of PRL injections. It also seemed plausible to suggest that given the greater effect of PRL on food intake in the PVN, that more expression of Fos-lir might be detected in this nucleus, and this, after fewer injections compared to that in both the VMH, and the MPOA.

EXPERIMENT 2:

Expression of Fos-like Immunoreactivity in the MPOA, PVN, and VMH of Virgin Female Rat Brain Following Central Administration of PRL.

Materials, Methods, and Procedures

Twenty-two female Wistar rats were implanted with a permanent 22 gauge guide-cannula aimed at the right lateral ventricle (see Experiment 1a) and given a 7 day post-surgery recovery period after which they were habituated for 4 days to the injection period to minimize stress-related effects on expression of Fos-lir. Rats were handled daily for several minutes before and after the surgery, as well as throughout the experiment. As in the previous experiments, central injections of either saline (N = 10) or PRL (N = 12) were carried out twice daily at approximately 0700 and 1900 for either 5 or 8 days, and only once at 0700 for subjects in the "1 injection" condition.

The hormone administration period began on different days for different subgroups of subjects such that on a day of testing, one rat from each condition received its last icv injection. One hour after injection, subjects were deeply anesthetized with an ip injection of sodium pentobarbital (0.5 ml) and perfused transcardially with 300 ml of cold saline (0.9 %) followed by 300 ml of cold 4 % paraformaldehyde (pH 7.3 - 7.4) (Sigma) in a 0.1 M phosphate buffer (pH 7.4). The brains were removed and refrigerated over night in individual vials containing 4 % paraformaldehyde. The following day, coronal sections 50 μm in thickness were cut with a vibratome. Sections were taken throughout the rostral-caudal extent from the MPOA to the VMH. Every second section was kept to be processed for Fos immunohistochemistry.

The immunohistochemical procedure consisted of incubating the sections for 30 minutes in 0.9 % Trizma Buffered saline (TBS) and 30 % w/w hydrogen Peroxide (Sigma, #H-1009), after which they were rinsed three times for five minutes with fresh

TBS, then transferred to a solution of 0.30 % Triton TBS and 3 % Normal Goat Serum (Vector Labs, 3 % NGS, #s-1000), and refrigerated for 90 minutes. After this time period, tissues were directly placed in the primary antibody to Fos (Ab-5, PC-38, Oncogene Research Products, Calbiochem), along with 0.30 % Triton TBS and 3 % Normal Goat Serum, and incubated for 48 hours in the refrigerator.

Sections were then rinsed three times for five minutes in fresh TBS before they were immersed in the secondary antibody (ABC Kit - Anti-Rabbit made in Goat, #BA-1000, Vector Labs). Tissues remained refrigerated in this solution which also contained 0.30 % Triton TBS and 3 % Normal Goat Serum, for one hour. The second to last step required that sections be rinsed three times for five minutes in fresh TBS, and processed with a standard vectastain ABC kit (Vector Labs). Sections were then incubated in the refrigerator for two hours. After the usual three rinses in TBS, peroxidase activity was revealed using the buffer solution containing 0.03 % diaminobenzidine, 0.0016 % nickel ammonium sulfate, 0.002 % cobalt chloride, and 0.001 % hydrogen Peroxide (DAB kit). Slices were then mounted onto gelatinised slides, left to dry overnight, and dehydrated in each of the following solution consecutively for three minutes: distilled water, 70 % alcohol, 95 % alcohol, 100 % alcohol, Xylene, and fresh Xylene again. The final step involved coverslipping the slides using Permount (# SP15-500, Fisher Scientific).

Sections were visualized using a Sony XC77 camera mounted on a light microscope (Labolux Lertz GMBH). Images were captured using the NIH image 1-59 analysis system installed on a Power Macintosh 6100 computer. Labelled cells were counted on all rostral-to-caudal hemisections obtained through each of the three hypothalamic areas of interest, and the average number of immunostained cells per area, per animal, was computed.

Statistical Analyses

To determine the effect of varying number of icv injections of PRL on Fos-lir in the MPOA, PVN, and VMH of virgin female rats, 3 two-factor ANOVAs were conducted with treatment (*saline* or *PRL*) and number of icv injections (1, 7 or 15) as the between group factors.

RESULTS

Figure 9 illustrates that Fos-lir in the MPOA did not vary as a function of treatment, nor as a function of the number of icv injections of PRL. Figure 10, however, shows that in the PVN, a different profile of expression of Fos-lir emerged. PRL-injected animals tended to show a positive relationship between number of injections of PRL and number of cells expressing Fos-lir. Whereas there was a marginally close main effect for treatment (F [1, 15] = 3.717, p = 0.0730), a one-way ANOVA performed on the data obtained from females receiving a total of 15 icv injections revealed a significant difference in Fos-lir (F [1, 5] = 9.604, p = 0.0363) indicating that PRL significantly increased neuronal activity in the PVN of these animals. Finally, as seen in Figure 11, no differences in Fos-lir were noted in the VMH. Figure 12 shows clear examples of the differences in Fos-lir in the PVN and VMH of animals having received 15 icv injections of PRL.

DISCUSSION

The findings from this study showed that a significant difference in Fos-lir was observed in the PVN and not in the MPOA or VMH of female rats that had been exposed to chronic administration of PRL (15 icv injections). It is noteworthy that Fos-lir within the PVN tended to increase as a function of the number of PRL injections received, a trend which was observed neither in the MPOA nor the VMH. Given that there is growing evidence that PRL upregulates its own receptors (Bakowska & Morrell, 1997; Fujikawa, Soya, Yoshizato, Sakaguchi, Doh-Ura, Tanaka, & Nakashima, 1995; Kelly et al., 1991; Muccioli & Di Carlo, 1994; Sakaguchi, Tanaka, Ohkubo, Fujikawa,

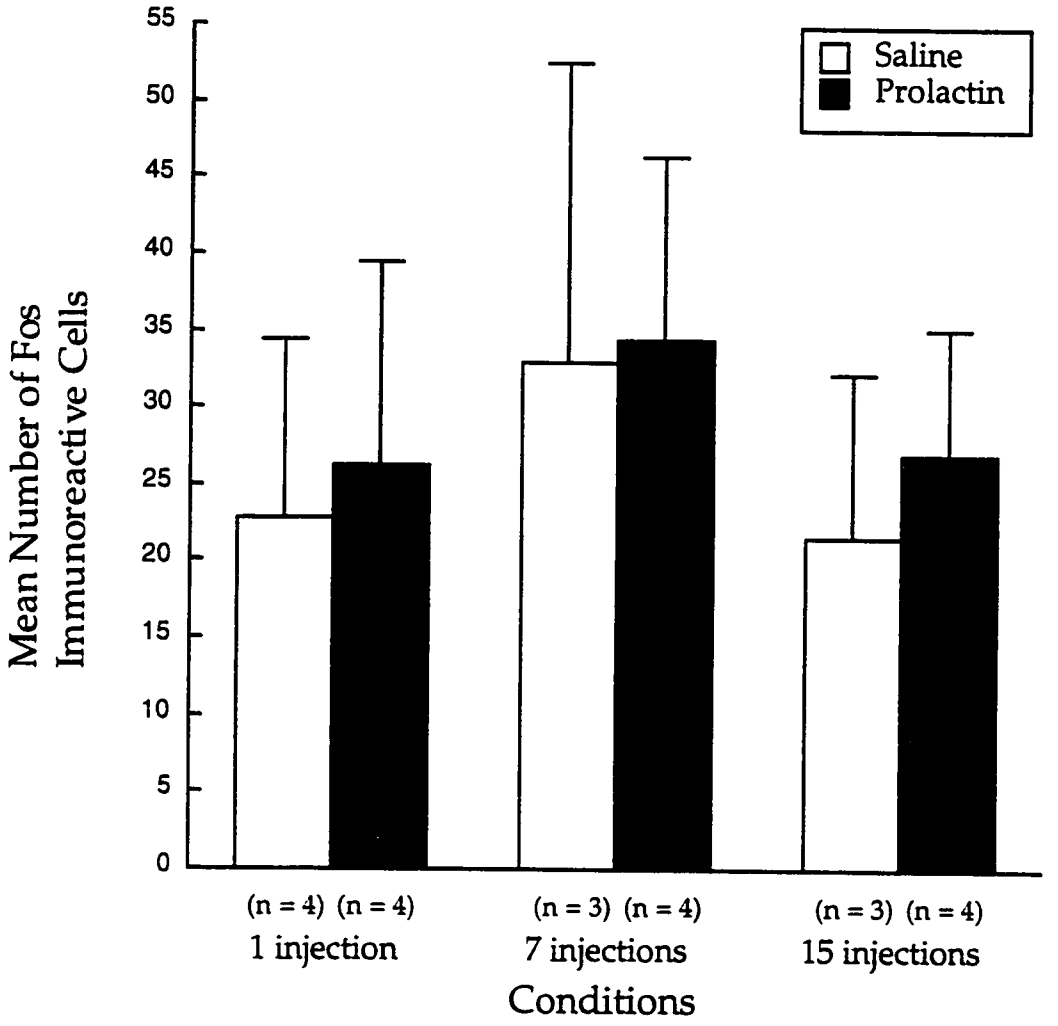

Figure 9: Mean number of Fos immunoreactive cells in the MPOA of virgin female rats that received icv injections of saline or prolactin (2 μg).

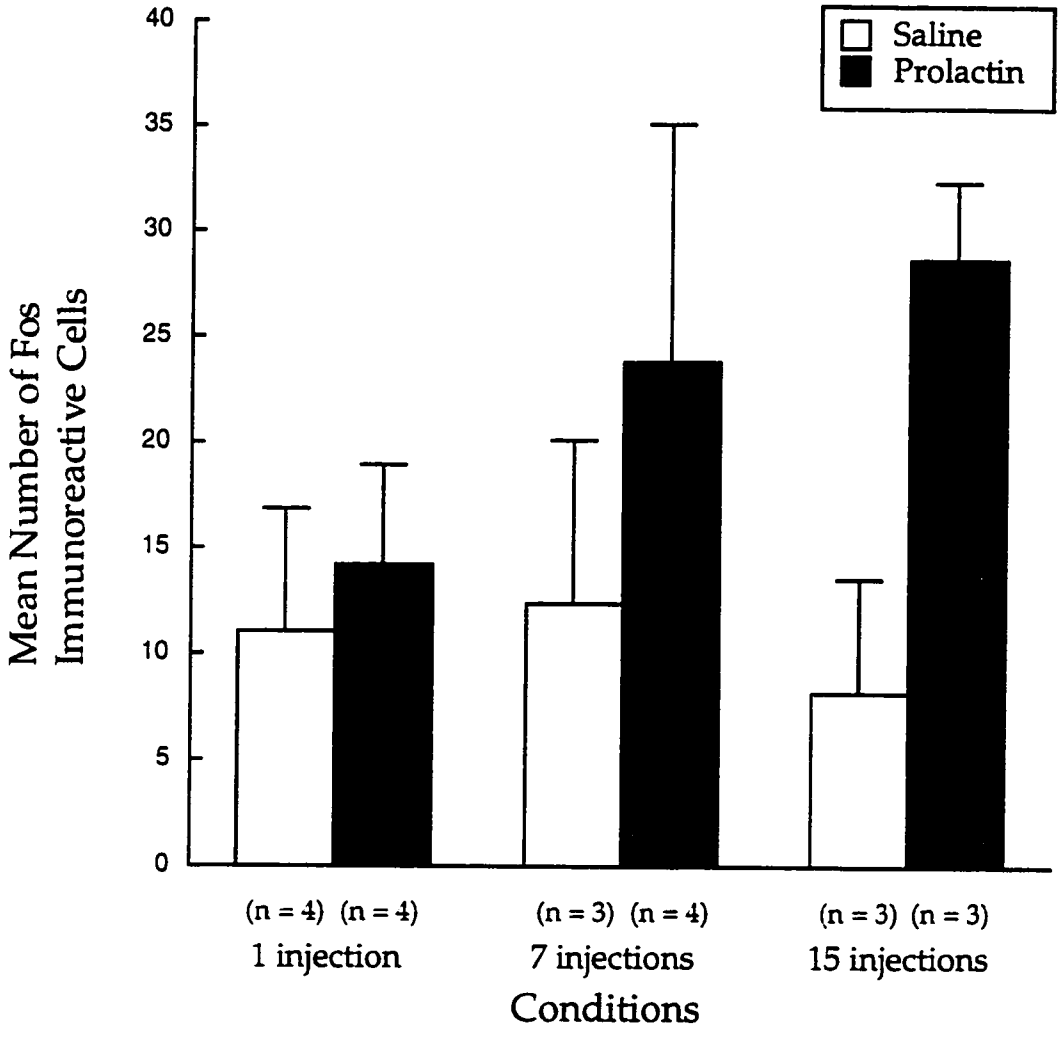

Figure 10: Mean number of Fos immunoreactive cells in the PVN of virgin female rats that received icv injections of saline or prolactin (2 μg).

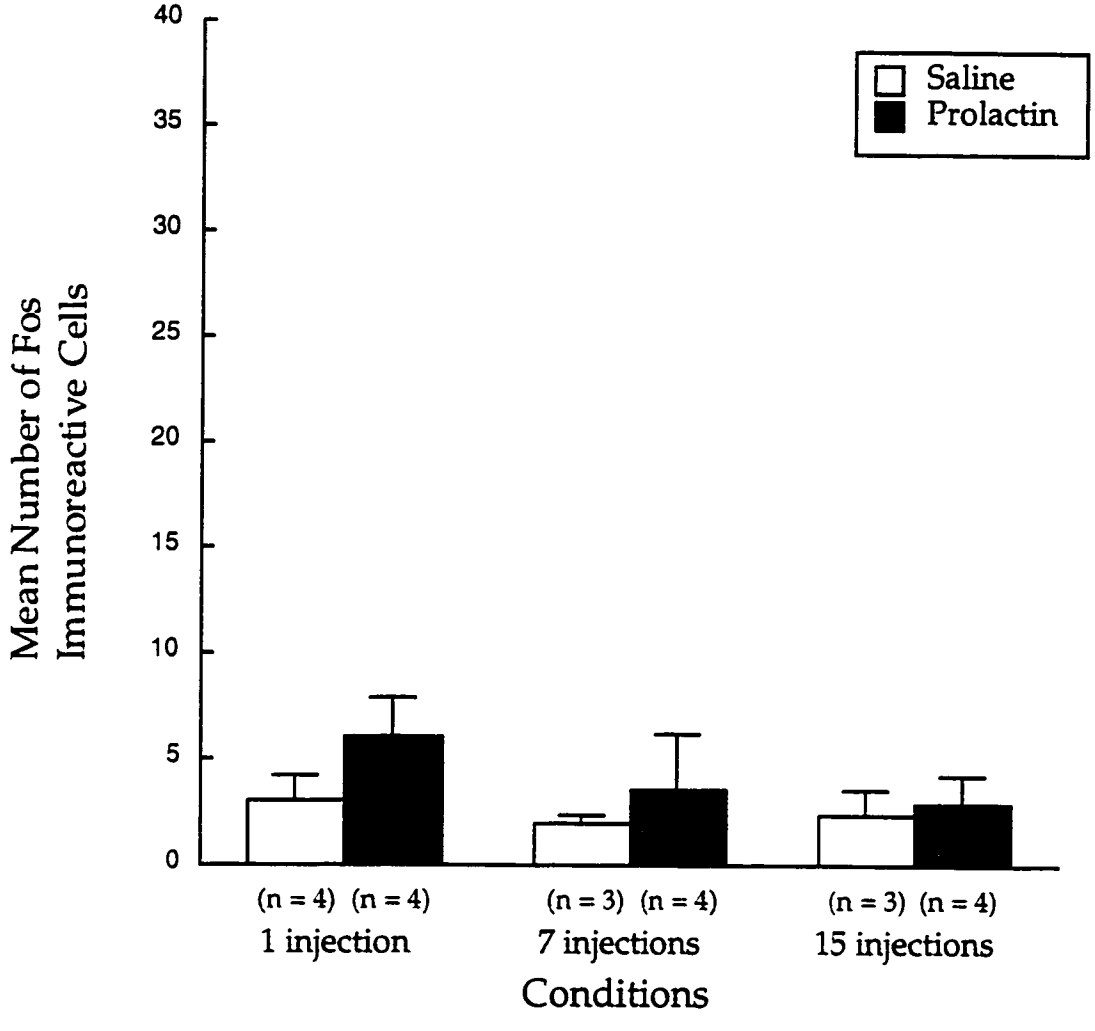

Figure 11: Mean number of Fos immunoreactive cells in the VMH of virgin female rats that received icv injections of saline or prolactin (2 μg)

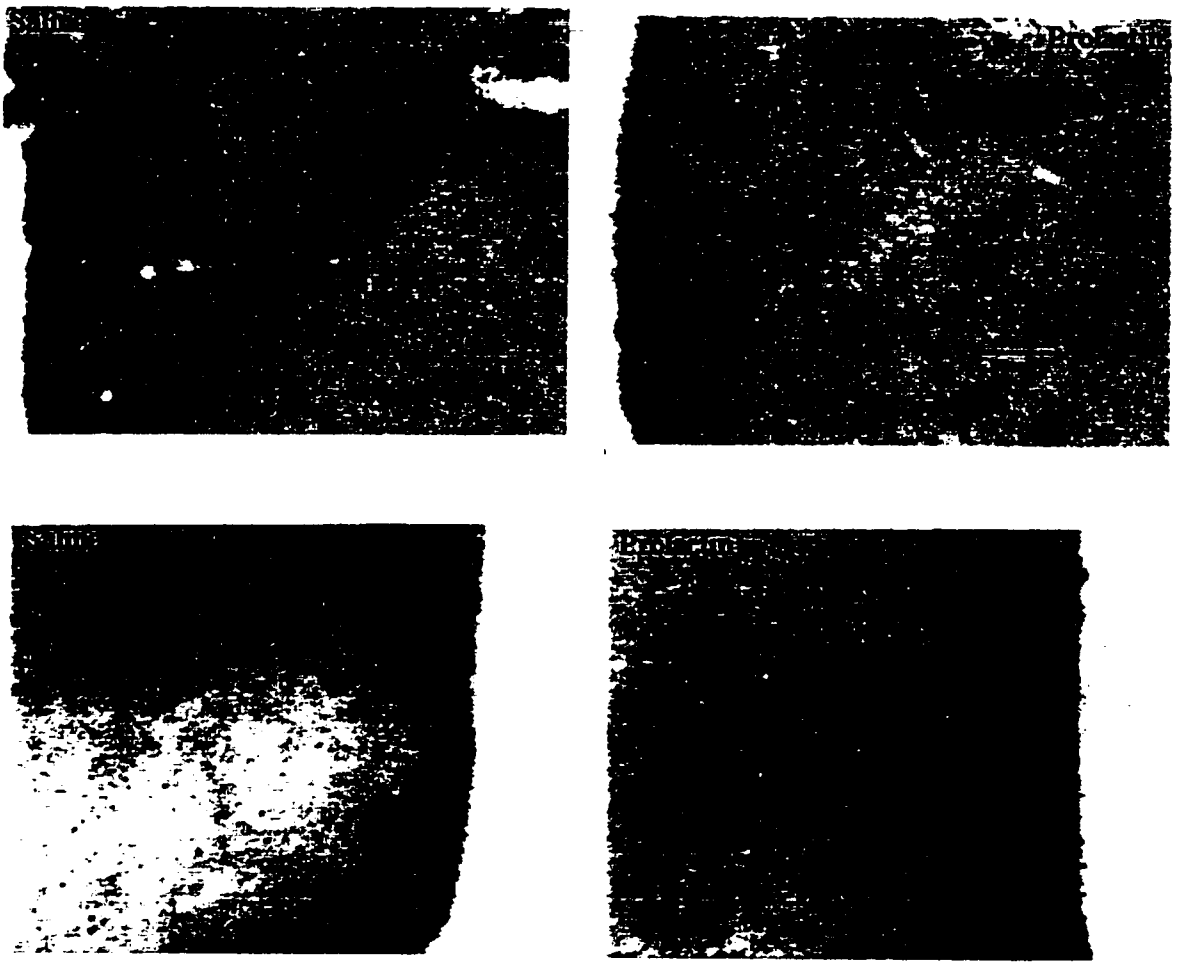

Figure 12: Examples of Fos-lir in the PVN (top panels) and VMH (bottom panels) of animals sacrificed one hour after having received a total of 15 icv injections of saline or PRL (2 μg).

Sudo, & Nakashima, 1996; Sugiyama, Minoura, Kawabe, Tanaka, & Nakashima, 1994; Sugiyama, Minoura, Toyoda, Sakaguchi, Tanaka, Sudo, & Nakashima, 1996), it is possible that the significant increase in Fos-lir observed in the PVN of rats sacrificed after 8 days of treatment reflects an upregulation of PRL receptors within this nucleus, which in turn, may serve to regulate PRL-induced feeding behavior. This exciting hypothesis is worthy of empirical attention.

GENERAL DISCUSSION

The goal of this thesis was to investigate the neuroanatomical specificity of the stimulatory action of PRL on feeding behavior in the female rat, and the pattern of cellular activation produced by central administration of PRL following varying lengths of treatment. The studies in Chapter 1 investigated the involvement of the PVN, VMH, and MPOA in PRL-induced hyperphagia, and the results revealed that the PVN was the most sensitive of these sites. The stimulatory effect of PRL on food intake was not observed when rats received intra-MPOA injections, and took many injections to induce when intra-VMH injections of the hormone were administered. This suggests that the MPOA and the VMH are not directly involved in PRL-induced feeding, and that effects seen after long-term treatment, may be the result of diffusion of PRL to other sites, such as the PVN. Thus, although PRL effects at other neural sites may also contribute to the orexigenic property of PRL, the present findings suggest that of the three sites examined, the PVN is a primary site of action in promoting hyperphagia in virgin female rats.

The aim of the second study was to investigate the pattern of cellular activation, as measured by Fos-lir, after an increasing number of icv injections of PRL. Significant differences in Fos-lir were found only in the PVN of rats that received 15 icv injections of PRL. One exciting parallel that can be drawn from these independent data sets is that, of the sites studied, the PVN is the nucleus most responsive to the hyperphagic action of PRL (Experiment 1a), and the nucleus in which most cellular activation was recorded (Experiment 2). PRL, therefore, seems to exert significant action in the PVN, a site previously implicated in the control of food intake (Anand & Brobeck, 1951; Leibowitz, 1978). These data are the first reports documenting the hypothalamic nuclei through which PRL exerts its feeding-enhancing properties in rats. They are also the first

to examine the pattern of cellular activation resulting from icv administration of PRL. Together, the present findings make a valuable contribution to the relatively new study of the effects of central infusions of PRL on behavior and physiology, in general, and specifically, on food intake.

The findings of Experiment 1a revealed that PRL produced significant increases in feeding behavior when infused into the PVN. This effect is remarkable considering that subjects were not energetically challenged by either food restriction or deprivation, and had free access to food. Interestingly, the increase in food intake observed as a result of the treatment (800 ng of PRL) was comparable to that produced by icv injections of PRL at a higher dose (i.e., 2 μg injected intracerebroventricularly; Sauvé & Woodside, 1996). The fact that a dose of PRL that was ineffective when administered into the ventricular system (Sauvé & Woodside, 1996) promoted feeding when infused directly into the PVN (Experiment 1a), provides strong support for the notion that PRL acts, at least in part, at this site to mediate feeding behavior.

The findings of Experiment 1a revealed two additional interesting observations. One is an inter-species difference in the hyperphagic properties of PRL, and the other is an intra-species comparison. Firstly, the hyperphagic response obtained after intra-PVN injections of PRL to female rats contrasted with that obtained in doves, a species in which the PVN is implicated in the regulation of feeding (Kuenzel, 1982; Kuenzel & McMurtry, 1988), but is not responsive to the orexigenic properties of PRL (Hnasko & Buntin, 1993). Secondly, whereas the feeding response produced by intra-PVN administration of PRL in virgin female rats was not as dramatic as that observed in intact lactating females, it was similar to that observed in galactophore-cut suckled dams (Cotes & Cross, 1954; Millelire & Woodside, 1989; Woodside & Popeski, in press). One obvious reason for this discrepancy is that the subjects in Experiment 1a were not subjected to the high metabolic demands that suckling young impose. Another

reason for this discrepancy might be related to the hormone regimen used. That is, there may exist another combination of dose, frequency, and duration of treatment with PRL that would potentiate feeding maximally.

The hormone treatment used in the studies of Chapter 1 entailed two daily injections of PRL for 10 days. This hormone regimen does not replicate the hormonal profile of lactation. Lactating dams experience high and sustained endogenous titers of PRL, as well as dramatic, rapid rises in PRL secretion stimulated by suckling (Amenomori et al., 1970). The hormone administration regimen used in the present experiments, however, replicates more closely the bi-daily surge of PRL secretion characteristic of the first 10 days of pregnancy (Butcher et al., 1972; Gunnet & Freeman, 1985; Lee et al., 1998). The point of interest is that the magnitude of the feeding response produced by the bi-daily administration of PRL in the PVN of virgin female rats was similar to that observed in pregnant rats during the first week of gestation (e.g., Slonaker, 1924). Thus, in light of the fact that there exists a positive relationship between PRL levels and amounts of food consumed (Amenomori et al., 1970; Leon & Woodside, 1983; Ota & Yokayama, 1967; Woodside & Popeski, in press), and that both ingestive behavior and PRL levels are at their highest during lactation especially, and to a lesser extent during the second week of pregnancy, future studies should manipulate the daily exposure to central PRL (e.g., with the use of minipumps) for varying durations (i.e., more than 10 days) to monitor how they interact to affect feeding.

Direct actions of PRL on the brain are thought to occur through binding to PRL receptors (Bakowska & Morrell, 1997). Although the results of Experiment 1a are consistent with this idea, they do not exclude other possibilities such as the binding of PRL to growth-hormone (GH) receptors which resemble PRL receptors in structure (Kelly et al., 1991). To test whether the hyperphagic effect of intra-PVN administration of

PRL was mediated through the interaction of PRL with PRL receptors within this site (Bakowska & Morrell, 1997), one could emulate the elegant study conducted by Li et al. (1995) in ring doves. Their procedure involved injecting anti-PRL receptor antibodies to inhibit the binding of PRL to PRL receptors in the VMH, a site highly sensitive to PRL-induced hyperphagia in doves (Foreman, Lea, & Buntin, 1990; Hnasko & Buntin, 1993), prior to treating subjects with intra-VMH administration of PRL. They observed that indeed, this treatment inhibited the expected hyperphagic response indicating that, in the avian brain, PRL-induced increases in feeding are mediated by interactions with PRL receptors. Obtaining the same pattern of results in the rat using a similar protocol targeting the PVN, and using antibodies to PRL receptors (e.g., U5 or T6; see Pi & Grattan, 1998a) or antisense oligodeoxynucleotides to PRL receptors (e.g., McCarthy, Kleopoulos, Mobbs, & Pfaff, 1994), would confirm that, in the rat brain, PRL-induced hyperphagia is also a receptor-mediated effect in the PVN.

The findings of Experiment 1b indicated that intra-VMH injections of PRL did not affect the average daily food intake over the 10 days of testing. In the rat, the VMH plays a clear role in feeding behavior (Hetherington & Ranson, 1940). Electrical stimulation of the VMH curtails eating behavior (Brobeck, Tepperman, & Long, 1943), whereas destroying the VMH leads to extensive overeating (Hetherington & Ranson, 1940). Studies of the effect of PRL on neuronal activity in the rat VMH have shown that iontophoretically applied PRL in this site increases the activity of neurons in the dorsal and rostral portions of the VMH (Chan, et al., 1983; Haskins & Moss, 1983; Moss, Chan, & Dudley, 1985).

In light of these data, one would expect that PRL injected in the VMH might stimulate neuronal activity, and in turn, inhibit feeding behavior. The results of Experiment 1b, however, show that intra-VMH administration of PRL does not inhibit feeding. As a matter of fact, a trend towards an increase in food intake was observed in the second half of the 10-day period of testing. Whereas the interpretation of this

finding remains speculative, it could reflect the result of diffusion of PRL to other sites, such as the PVN, or the result of hormonal priming. Moreover, the fact that PRL did not affect feeding when injected in the VMH does not rule out the possibility that PRL influences other behaviors known to be mediated at this this site. For example, studies have shown that in rats, the VMH is critical for the occurrence of mating-related behavior such as lordosis (Pfaff & Sakuma, 1979; Phelps, Nance, & Saporta, 1980), and is involved in the stimulation of maternal behavior (Bridges & Mann, 1994).

The findings of Experiment 1c revealed that intra-MPOA injections of PRL did not affect feeding. These data, together with those indicating no effect of PRL on food intake in the VMH, contrast with results of studies conducted with doves. In doves, PRL administration in these nuclei (Foreman et al., 1990; Hnasko & Buntin, 1993) produced a robust hyperphagic response indicating that in the avian brain, both the VMH and MPOA play a role in PRL-induced feeding.

Although the goal of the studies of Chapter 1 was to investigate the effect of site-specific injections of PRL on feeding, their effect on vaginal cyclicity and body weight were also of interest. Results showed that PRL did not affect estrous cyclicity, as determined by vaginal smears, in any of the sites in which it was infused. The finding that intra-MPOA injections of PRL did not disrupt vaginal cyclicity is especially interesting and puzzling because of the established role of the MPOA-anterior hypothalamic (AH) region of the female rat in estrous cyclicity (Jakubowski & Terkel, 1986). Data supporting the hypothesis that PRL injected in the MPOA might render females acyclic include anatomical evidence demonstrating an overlap of the region containing gonadotropin-releasing hormone (GnRH)-producing neurons (Malik, Silverman, & Morrell, 1991) with PRL receptors (Bakowska & Morrell, 1997; Pi & Grattan, 1998a, 1998b), and data suggesting that, *in vitro*, PRL inhibits the release and synthesis of GnRH in an immortalized cell line of GnRH-producing neurons (Milenkovic,

D'Angelo, Kelly, & Weiner, 1994), which in turn, could inhibit luteinizing hormone secretion, ovarian function, and female fecundity (Smith, 1978). In spite of this evidence, the results of Experiment 1c indicated that PRL did not affect estrous cyclicity when injected in the MPOA.

Several considerations may help to explain the failure of intra-MPOA injections of PRL to disrupt estrous cyclicity. It is possible that one of the components of the hormone administration regimen used (i.e., dose, frequency of injections, and/or duration of treatment) produced central levels of PRL which were below those necessary to disrupt estrous cyclicity through one of the mechanisms previously mentioned. Alternatively, the location of the cannulae in the MPOA of female rats in Experiment 1c may not have corresponded perfectly with the region where the principal population of GnRH-producing neurons in rats reside, that is, in the anteroventral periventricular region of the MPOA (Malik et al., 1991), where PRL might have affected estrous cyclicity. Lastly, the lack of PRL-induced disruption of estrous cyclicity from intra-MPOA injections might be attributed to the marked functional heterogeneity of the MPOA-AH continuum. Jakubowski and Terkel (1986) showed that only specific lesions of the MPOA-AH affected the estrous cycle of female rats. Lesions located most rostrally and dorsally, sparing the SCN, resulted in a series of pseudopregnancies, whereas those located more caudally and ventrally, still sparing the SCN, conserved regular estrous cycles while causing only occasional pseudopregnancies. Finally, MPOA-AH lesions which damaged the SCN, resulted in persistent vaginal cornification (Jakubowski & Terkel, 1986).

Like vaginal cyclicity, body weight remained unchanged after the administration of PRL in the PVN, VMH, and MPOA. The lack of effect of PRL on body weight in spite of a concomitant PRL-induced increase in food intake (see Experiment 1a) is a phenomenon which has been documented by other researchers (Pfaff, 1969), and

previously by our laboratory (Noël & Woodside, 1993; Sauvé & Woodside, 1996). Possible explanations for the fact that PRL increased food intake without increasing body weight include the fact that PRL may work through the direct elevation of metabolic levels (Hanwell & Linzell, 1973). It is also possible that PRL treatment decreased adipose tissue lipoprotein lipase activity as well as fatty acid synthesis indirectly by reducing circulating levels of insulin as during lactation (Wade & Schneider, 1992). Insulin is a hormone that stimulates increases in food intake, body weight, and body fat content by increasing tissue uptake of glucose and fatty acids, promoting lipogenesis and glycogen synthesis, and inhibiting lipolysis (Wade & Schneider, 1992). The absence of weight gain following intra-PVN administration of PRL may have been due to PRL-induced hypoinsulinemia and its metabolic consequences.

Finally, it has been noted that the route of administration of PRL predicts whether weight gain will accompany increases in food intake. Reports have shown that whereas sc injections of PRL produced both a hyperphagic response and an increase in body weight (Noël & Woodside, 1993), icv administration of PRL increased feeding without affecting weight gain (Noël & Woodside, 1993; Sauvé & Woodside, 1996). The former finding could be attributed to PRL-induced increases in progesterone levels (Rothchild, 1976) and consequently, progesterone-induced increases in feeding and body weight (Jankowiak & Stern, 1974; Tarttelin & Gorski, 1971; Tarttelin & Gorski, 1973; Wade, 1976).

Taken together, the results of the studies in Chapter 1 suggest that future studies should monitor a wider range of behaviors and physiological parameters that could be sensitive to central injections of PRL to determine their relative sensitivity to PRL and their interactions. Some of the interesting behavioral effects known to be induced by hyperprolactinemia include excessive grooming (Drago, 1988), regulation of sleep (Roky, Obal, Valatx, Bredow, Fang Pagano, & Krueger, 1995), and decreases in motor activity (Gonzales-Mora, Guadalupe, & Mas, 1990; Slonaker, 1924), to name a few. The

behavioral and neurochemical consequences of the relationship between PRL and dopamine are also pertinent to the understanding of PRL-induced feeding. Whereas PRL stimulates the release of dopamine from TIDA neurons (Gonzales-Mora et al., 1990; Moore, 1987), it has antagonistic actions on dopaminergic transmission in the mesolimbic dopaminergic system which in turn produce decreases in motor behavior (Gonzales et al., 1990). Studies investigating the role of dopamine in food intake, however, have shown that both feeding itself (e.g., Martel & Fantino, 1996), and injections of opioids in the ventral tegmental area activate the mesolimbic dopaminergic system (Noël & Wise, 1995). In conclusion, the simultaneous measurement of a wider band of responses would provide a richer understanding of the relationship between PRL-produced hyperphagia and other PRL-produced effects.

To extend the findings of the experiments in Chapter 1 on the neuroanatomical mapping of PRL-produced feeding, future studies must also investigate the role of other hypothalamic sites through which PRL could mediate its orexigenic properties. Sites of particular interest include the perifornical area, lateral to the PVN, and the lateral hypothalamus (LH). The perifornical area stimulates interest not only because it is known to mediate NPY-induced feeding (Stanley et al., 1993), but also because it has been identified as a site containing PRL-lir neurons (Paut-Pagano et al., 1993). The LH is another nearby site of potential significance in PRL-induced hyperphagia given that it has been shown to mediate excitatory amino acid-elicited eating (e.g., glutamate) (Stanley, Willett, Donias, Ha, & Spears, 1993), and because PRL-immunoreactive (ir) perikarya have been detected in the lateral hypothalamic areas of the median and posterior hypothalamus of colchicine treated rats (Paut-Pagano et al., 1993; Siaud et al., 1989).

In sum, the experiments of Chapter 1 studied the role of the PVN, VMH, and MPOA in PRL-induced hyperphagia, and results showed that the PVN was the most

sensitive of these sites. These are the first findings to reveal the anatomical specificity of PRL action in promoting feeding activity through microinjections into hypothalamic sites within the female rat brain. Future research should extend these results, and explore the role of several other sites in the orexigenic properties of PRL.

The findings of Experiment 2 showed clearly that a significant number of cells expressed Fos-lir in the PVN of rats that were subjected to 7 and a half days of twice daily icv injections of PRL. These data suggest that neurons of the PVN are sensitive, at a cellular level, to prolonged exposure to PRL, and provide the opportunity to formulate interesting empirical questions about the meaning of this increased cellular activation. As mentioned, it is known that PRL stimulates an increase of its own receptors in the brain (Bakowska & Morrell, 1997; Fujikawa et al., 1995; Kelly et al., 1991; Muccioli & Di Carlo, 1994; Sakaguchi et al, 1996; Sugiyama et al., 1994; Sugiyama et al., 1996). Increases in the gene expression for PRL receptors have been measured in the brain of rats subjected to a variety of stimuli known to elevate PRL secretion, namely restraint stress in water (Fujikawa et al., 1995), pup contact-induced maternal behavior (Sakaguchi et al., 1996; Sugiyama et al., 1996), and during reproductive status characterized by elevated levels of PRL such as at proestrus, mid- and late gestation, and lactation (Sugiyama et al., 1994). A positive correlation between circulating concentration of PRL by treatment with PRL itself or drugs that stimulate PRL release, and number of PRL receptors in the hypothalamus of rats, has also been documented (Muccioli & Di Carlo, 1994).

In light of these findings, it is plausible to suggest that the detection of a significant number of cells expressing Fos-lir in the PVN of rats sacrificed after chronic icv administration of PRL may reflect an upregulation of PRL receptors in this neural site. The investigation of levels of expression of PRL receptor mRNA within the PVN of rats using either RT-PCR or *in situ* hybridization methods after varying lengths of icv

treatment with PRL would directly address this pertinent issue.

Another question raised when studying the effect of a stimulus on Fos immunoreactivity as a marker for cellular activation is the phenotype of the activated neurons. Given that the Fos protein is nuclear, the simultaneous detection of markers located within the cytoplasm becomes feasible with the use of standard double-labeling techniques (Hoffman et al., 1993). Both PRL-lir (DeVito, 1988; Emanuele et al., 1987; Fuxe et al., 1977; Griffond et al., 1994b; Hansen et al., 1986; Harlan et al., 1989; Nishizuka et al., 1990; Siaud et al., 1989; Toubeau et al., 1979) and PRL mRNA (DeVito et al., 1992; Emanuele et al., 1992; Schachter, et al., 1984) have been detected in the hypothalamus and PVN of rats (Clapp et al. 1994). Thus, it is possible that icv injections of PRL stimulate the synthesis of PRL within neurons in the hypothalamus. Double-staining for Fos-lir and PRL-lir with the use of an antibody to rat PRL after icv treatment with PRL would provide this information. More specific still would be to take slices throughout the PVN and separate consecutive sections into two sets: one to be treated with immunocytochemistry for Fos-lir, the other with *in situ* hybridization or RT-PCR to detect PRL mRNA, and compare the staining of the two sets of tissues for significant overlap (e.g., Kamegai, Minami, Sugihara, Higuchi, & Wakabayashi, 1994). A significant overlap would suggest that icv administration of PRL activated the cells in the PVN to stimulate PRL synthesis.

Alternatively, there are other systems within the PVN that PRL could stimulate and with which PRL might have a functional interaction to promote feeding behavior. The opioidergic system is a likely candidate given its involvement in feeding (e.g., Leibowitz & Hor, 1982). ß-endorphin is a 31 amino acid peptide derived from the precursor molecule proopiomelanocortin, which has feeding-stimulating properties. Studies have shown that injections of ß-endorphin into the PVN of satiated rats reliably increase food intake (Gosnell et al., 1986; Leibowitz & Hor, 1982), a response which is

reversed by local administration of the selective opiate antagonist naloxone (Leibowitz & Hor, 1982). To study the relationship between PRL and ß-endorphin in PRL-induced hyperphagia, one could administer an opiate antagonist prior to the administration of PRL, and note how this treatment affects PRL-induced feeding.

In addition to its effect on feeding behavior, the anatomical distribution of ß-endorphin corroborates a possible synergy or functional relationship between it and PRL. The arcuate nucleus is the main source of ß-endorphin neurons. Their projections extend to various sites in the brain (Cuello, 1983), including the median eminence (Dupont, Barden, Cusan, Mérand, Labrie, & Vaudry, 1980), an area associated intimately with the hypophyseal portal vessels leading to the anterior pituitary (Page, 1988) where the highest concentration of ß-endorphin is found (Eipper & Mains, 1980). In sum, both the behavioral action and the neuroanatomy of ß-endorphin lend support to the suggestion that this opioid shares a functional relationship with PRL, and thus may act in synergy with PRL to induce feeding. The empirical investigation of such a hypothesis could be undertaken with the use of dual-immunohistochemical techniques.

There is also compelling evidence supporting the idea that the endogenous opioid dynorphin could mediate PRL-induced behavior. Studies have shown that, like ß-endorphin, central administration of dynorphin stimulates marked increases in feeding behavior (Lambert, Wilding, al-Dokhayel, Bohuon, Comoy, Gilbey, & Bloom, 1993). More interesting still are the results of studies conducted by Griffond and collaborators. They performed immunocytochemical investigations and provided exciting data showing that dynorphin B-immunoreactivity, was co-localized in a population of PRL-ir neurons of the rat LH (Griffond et al., 1994b).

In another study, they coupled immunocytochemistry and *in situ* hybridization using oligonucleotide probes complimentary to dynorphin mRNA, and found that neurons containing the mRNA encoding preprodynorphin which synthesizes dynorphin B, were co-localized with a peptide related to PRL (Griffond, Colard, Deray, Fellmann,

& Bugnon, 1994a). Although the authors cautioned that the interactions of these immunoreactive neurons with other populations of neurons appeared complex, the data certainly supported the hypothesis that dynorphin B may act in synergy with PRL in discrete regions of the hypothalamus to promote feeding in rats. In addition to these exciting findings are data showing that PRL-ir neurons within the LH appear to correspond to a subpopulation of glucose-sensitive neurons as shown by the capacity of one single insulin injection to stimulate increases in Fos-lir in PRL-ir neurons within the LH (Bahjaoui-Bouhaddi, Fellmann, & Bugnon, 1994).

NPY is another orexigenic agent which may mediate PRL-induced hyperphagia at the level of the PVN. In addition to its widely recognized physiological role in the hypothalamic control of feeding (e.g., Clark et al., 1984; Morley & Levine, 1984), and its potent orexigenic response when infused in the PVN (Stanley & Leibowitz, 1985), reports have shown that icv administration of NPY induces Fos-lir in cells located in the PVN of rats, both in the absence of feeding, and in response to feeding (Lambert, Phillips, Wilding, Bloom, & Herbert, 1995; Li et al., 1994). More relevant to the questions posed in this thesis, are recent findings from the laboratory of Buntin and collaborators. They studied changes in NPY-immunoreactivity in the hypothalamus of *ad lib* fed ring doves injected with PRL (1 µg/2 µl/day icv for 5 days). Because results revealed that PRL injections produced a significantly higher number of NPY-ir cells in the infundibular region, the authors suggested that NPY may mediate PRL-induced hyperphagia in doves, and that this potential relationship may, in part, explain the long-latency to feeding produced by PRL (Peterson & Buntin, 1998) relative to that of other feeding stimulants (e.g., NPY; Levine & Morley, 1984).

The possible involvement of GH in PRL-induced hyperphagia is also of particular interest for several reasons. Previous studies in doves have demonstrated that, like PRL, turkey, ovine, and human GH are capable of producing significant

elevations in food intake (Buntin & Figge, 1989). Bridges and Millard, (1988) showed that ovine GH, like PRL, stimulates maternal behavior in inexperienced, hypophysectomized, ovariectomized, steroid-primed female rats. Moreover, given that PRL and GH are structurally related molecules (Bole-Feysot et al., 1998), that both their receptors belong to the same cytokine receptor superfamily (Bole-Feysot et al., 1998), that they are both elevated during pregnancy and lactation (Escalada, Sanchez-Franco, Velasco, & Cacicedo, 1997; Klindt, Robertson, & Friesen, 1981) when feeding behavior is also increased, and that GH has known hyperphagic effects of its own (Dickson & Vaccarino, 1990; Vaccarino & Hayward, 1988), the possibility that GH may contribute to the hyperphagia of lactation merits specific empirical attention.

Whereas the increased Fos-lir obtained in the PVN of rats after chronic icv treatment with PRL stimulates the formulation of exciting hypotheses, it is equally worthy to speculate about the meaning of negative findings. That is, the results of Experiment 2 revealed the absence of significant induction of expression of Fos-lir in the MPOA and VMH of female rats following central injections of PRL. Although it is possible that icv administration of PRL did not affect MPOA and VMH neurons at a cellular level, it certainly does not necessarily indicate this. It is equally plausible to consider that other immediate early genes (IEGs) in the cells of these two neural substrates were activated by the stimulus.

The expression of Fos-lir is stimulus-specific. Hoffman and colleagues (1993) have documented this fact by describing situations in which stimulus-produced cellular activation was undeniable, yet expression of Fos-lir was absent. In one study of luteinizing hormone-releasing hormone (LHRH) neuronal function (Lee, Abbud, Hoffman, & Smith, 1993), the administration of an excitatory amino acid (N-Methyl-D,L-aspartic acid) resulted in increased activity of LHRH neurons, as evidenced by increased LH secretion, without the concomitant expression of c-*fos* in LHRH neurons.

In contrast, another study revealed that both Fos and Jun proteins were expressed in LHRH neurons during the proestrous LH surge (Lee, Abbud, Smith, & Hoffman, 1992), reinforcing the notion that expression of Fos is stimulus-dependent. Although there is no doubt that the detection of c-*fos* as a marker of neuronal activation is an extremely powerful technique (Hunt et al., 1987; Sagar et al., 1988), it is also clear, and noteworthy, that neurons have the capacity to express a number of IEG products (Hoffman et al., 1993). What remains to be established is whether there is a different pattern of expression of IEG products following different stimuli.

Another similar, and perhaps more pertinent, example that substantiates this point is provided by data of a study conducted by Polston and Erskine (1995). These researchers first studied mating-induced increases in Fos-lir in animals receiving numbers of intromissions across a range relevant to the induction of the PRL surges of early pregnancy. They found that Fos-lir was expressed in the amygdala, preoptic area, BNST, and VMH. In a second study, a mating-stimulus known to induce Fos-lir in the areas mentioned, and hence probably also inducing bi-daily surges of PRL, was used to evaluate the expression of another IEG, egr-1 (Milbrandt, 1987). Results revealed that egr-1 was expressed in each of the same areas, as well as in another division of the BNST, and in the PVN, indicating that egr-1 may be a more sensitive marker for mating-induced (and possibly PRL-related) neural activation in these areas than is Fos. Indeed, as previously mentioned, Fos is one of a class of IEG protein products, and further investigations will determine whether one of these IEGs may be more sensitive to icv injections of PRL than is Fos.

In sum, both the investigation of the site-specificity of the hyperphagic properties of PRL in the rat, and the induction of the IEG c-*fos* in female rat brain following icv administration of PRL described in this thesis, are novel approaches to the study of the orexigenic properties of PRL. Of the three sites investigated in this thesis, the PVN was the nucleus most responsive to the hyperphagic action of PRL, and the nucleus in which

most cellular activation was recorded following chronic central administration of PRL. Taken together, the present data make a meaningful contribution to the understanding of the role of PRL in the control of food intake, and of the mechanisms through which it operates

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APPENDIX A:

Source Tables of Analyses of Variance: Experiments 1a, 1b, and 1c.

Table 1

Source table for the ANOVA performed on the mean daily food intake for the 10 days of drug administration for the two groups implanted with cannulae aimed at the right lateral ventricle and the two groups with cannulae aimed at the PVN in Experiment 1a

Source	SS	df	MS	F	P
Group	533.657	1	533.657	9.505	0.0059
Brain Site	216.872	1	216.872	3.863	0.0634
Group x Site	27.350	1	27.350	0.487	0.4933
Error	1122.950	20	56.148		
Days	200.307	9	22.256	1.203	0.2957
Group x Days	102.005	9	11.334	0.613	0.7854
Site x Days	116.278	9	125.920	0.698	0.7099
Grp. x Site x Days	203.895	9	22.655	1.225	0.2825
Error	3330.203	180	18.501		

Table 2

Source table for the ANOVA performed on the difference between the mean body weight on the last day of testing (Day 10) and that of baseline for the four groups in Experiment 1a

Source	SS	df	MS	F	P
Group	408.176	3	136.059	1.429	0.2640
Error	1904.836	20	95.242		

Table 3

Source table for the ANOVA performed on the mean daily food intake for the 10 days of drug administration for the two groups in Experiment 1b

Source	SS	df	MS	F	P
Group	60.982	1	60.982	1.311	0.2817
Error	418.641	9	46.516		
Day	383.842	9	42.649	2.383	0.0190
Group x Day	275.569	9	30.619	1.711	0.0998
Error	1449.697	81	17.897		

Table 4

Source table for the ANOVA performed on the mean daily food intake of the first and last 5 days of the drug administration period for the two groups in Experiment 1b

Source	SS	df	MS	F	P
Group	60.982	1	60.982	1.311	0.2817
Error	418.641	9	46.516		
Block	24.562	1	24.562	0.555	0.4754
Group x Block	106.381	1	106.381	2.403	0.1555
Error	398.504	9	44.278		
Day	225.670	4	56.417	3.058	0.0288
Group x Day	158.965	4	39.741	2.154	0.0941
Error	664.270	36	18.452		
Bloc x Day	133.611	4	33.403	3.108	0.0270
Group x Bloc x Day	10.223	4	2.556	0.238	0.9151
Error	386.923	36	10.748		

Table 5

Source table for the ANOVA performed on the difference between the mean body weight on the last day of testing (Day 10) and that of baseline for the two groups in Experiment 1b

Source	SS	df	MS	F	P
Group	22.947	1	22.947	0.260	0.6224
Error	794.252	9	88.250		

Table 6

Source table for the ANOVA performed on the mean daily food intake for the 10 days of drug administration for the two groups in Experiment 1c

Source	SS	df	MS	F	P
Group	274.544	1	274.544	1.748	0.2129
Error	1727.488	11	157.044		
Day	234.627	9	26.070	2.521	0.0121
Group x Day	275.569	9	30.619	1.711	0.0998
Error	1023.650	99	10.340		

Table 7

Source table for the ANOVA performed on the difference between the mean body weight on the last day of testing (Day 10) and that of baseline for the two groups in Experiment 1c

Source	SS	df	MS	F	P
Group	667.453	1	667.453	2.726	0.1270
Error	2693.270	11	244.843		

APPENDIX B:

Source Tables of Analyses of Variance: Experiment 2.

Table 1

Source table for the ANOVA performed on the difference in Fos-lir in the MPOA of animals receiving 1, 7, or 15 icv injections of saline or PRL in Experiment 2

Source	SS	df	MS	F	P
Group	64.740	1	64.740	0.112	0.7419
Condition	408.267	2	204.133	0.354	0.7072
Grp. x Condition	15.558	2	7.779	0.013	0.9866
Error	9224.108	16	576.507		

Table 2

Source table for the ANOVA performed on the difference in Fos-lir in the PVN of animals receiving 1, 7, or 15 icv injections of saline or PRL in Experiment 2

Source	SS	df	MS	F	P
Group	702.068	1	702.068	3.717	0.0730
Condition	145.483	2	72.741	0.385	0.6869
Grp. x Condition	255.392	2	127.696	0.676	0.5235
Error	2833.341	15	188.889		

Table 3

Source table for the ANOVA performed on the difference in Fos-lir in the PVN of animals receiving 15 icv injections of saline or PRL in Experiment 2

Source	SS	df	MS	F	P
Group	628.122	1	628.122	9.604	0.0363
Error	261.618	5	65.405		

Table 4

Source table for the ANOVA performed on the difference in Fos-lir in the VMH of animals receiving 1, 7, or 15 icv injections of saline or PRL in Experiment 2

Source	SS	df	MS	F	P
Group	16.485	1	16.485	1.588	0.2257
Condition	16.842	2	8.421	0.811	0.4618
Grp. x Condition	5.806	2	2.903	0.280	0.7597
Error	166.093	16	10.381		