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# Regulation of Feeding-Associated Peptides and Receptors by Nicotine

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## Abstract

Although numerous epidemiological studies have provided convincing evidence for the inverse association between tobacco smoking and body weight, the molecular mechanisms underlying this relationship are not well-understood. Nicotine, as a potent secretagogue, could be expected to influence the levels and expression of many classes of neurotransmitters, as well as of cell-membrane constituents linked to neurotransmission, including signal transducers and related effectors. A potentially major group of candidate molecules that could be involved in feeding-related actions of nicotine are the numerous neuropeptides and peptide hormones shown in the past two decades to regulate food intake and energy expenditure. These could include neuropeptide Y (NPY), orexins, leptins, and uncoupling proteins (UCPs). Some of these peptides were already shown to respond to nicotine treatment in terms of regulation of levels and of activity at the level of cell-membrane receptors. The primary objective of this review is to summarize our current understanding of the regulatory effects of nicotine on the food intake and energy expenditure as related to the expression levels of leptin, NPY, orexin, uncoupling proteins, and of NPY and orexin receptors.

**Index Entries:** Body weight; feeding; leptin; nicotine; NPY; orexin; receptor; regulation; UCP.

## Introduction

Nicotine has been consumed by humans in various forms for many centuries. Several epidemiological and pharmacological studies have established a relation between cigarette smoking and human diseases, such as coronary heart disease (CHD), peripheral vascular disease, and cancer *(1–4)*. On the other hand, some epidemiological studies suggest that nicotine may have protective effects against degenerative processes such as Alzheimer's disease (AD), Parkinson's disease (PD), and Tourette's syndrome *(5–7)*. The mechanisms relating to these diverse associations are largely unknown. A strong association between cigarette smoking

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and major depression and schizophrenia has also been established *(8–13).* Despite widespread knowledge of the health consequence of cigarette smoking and a large number of public health campaigns directed at both smoking cessation and prevention of the onset of smoking, a large fraction of human population worldwide continue smoking (e.g., approx 27% of all adults in the U.S.A. *[14]*). The failure to quit smoking is primarily attributed to the addictive properties of nicotine. The fear of weight gain after cessation of smoking is another reason, particularly among women *(15).* A good understanding of this inverse association at the molecular level may provide clues to limiting these weight gains, as well as pharmacological basis for identifying novel targets within the central nervous system (CNS) that can be used to control weight through the development of appropriate medicines.

## An Overview of Nicotine and Nicotinic Acetylcholine Receptor

### *Nicotine*

Nicotine, as a general neurotransmitter-releasing agent, mainly acts by activation of ligand-gated ion channels. The direct activity of this alkaloid is thought to occur through nicotinic acetylcholine receptors, many subtypes of which were identified in the vertebrate (*see* section on Nicotinic Acetylcholine Receptors). In the brain, nicotine is transformed into a series of metabolites, including nornicotine and cotinine. Nornicotine is degraded slowly, appears to accumulate to levels sufficient to produce significant stimulation of brain nicotinic receptors *(16),* and could be the principal active form of nicotine in the brain. The stable nicotine-processing product cotinine may not be as effective, due to its low affinity at brain nicotinic sites *(17).* Metabolism of nicotine in visceral tissues can proceed along largely similar routes *(18).*

Nicotine is known to acutely increase the release of neurotransmitters, especially the aminergic species, including norepinephrine (NE) *(19),* dopamine (DA) *(20),* and 5-hydroxytryptamine (5-HT) *(21),* usually with an acceleration of catecholamine turnover. A direct action of nicotine (e.g., in the release of dopamine) can occur through the recently demonstrated post-synaptic nicotinic receptors *(22).* Long-term repeated or continuous treatment with nicotine could, however, result in desensitization of neurotransmitter responses to the alkaloid *(23,24).* Stimulation of catecholamine release or turnover by nicotine can be abolished by ganglionic blockers, e.g., chlorisondamine *(25).*

The monoaminergic transmitters affected by nicotine in brain are known to co-transmit as the fast component with the variety of signal-reinforcing or stimulus-maintaining neuropeptides, many of which are also known to be released through depolarization of excitable membranes by agents such as potassium ion or *Veratrum* alkaloids *(26,27).* Stimulation by nicotine could thus complement, augment, or substitute for the activity of sites that are highly voltage-sensitive and operate mainly via depolarization. Acute stimulation by nicotine is known to increase the secretion and circulating levels of several neuropeptides, which include proopiomelanocortin (POMC)-derived α-MSH *(28)* and ACTH *(29).* Stimulation of vasopressin and oxytocin production by nicotine can be expected from the known induction of c-Fos in the paraventricular (PVN) and supraoptic nucleus (SON) by nicotine *(30);* the presence of α7-receptor was recently documented in both the PVN and the SON of the hypothalamus *(31).* There is evidence for direct stimulation of vasopressin *(32)* as well as oxytocin *(33)* secretion by nicotine, which probably would vary considerably depending on dosage of nicotine *(30)* and the application regimen. The release of vasoactive intestinal peptide in taenia caecum of the guinea pig *(34)* and in frontal cortex of the rat could be regulated by nicotine *(35).* The secretion of insulin and glucagon apparently could be regulated by pancreatic and extrapancreatic ganglia, and is sensitive to nicotine and other nicotinic agonists *(36).*

Nicotine also induces an acute stimulation of the release of pituitary gonadotropins luteinizing hormone (LH), follicle-stimulating hormone (FSH), and prolactin, which could be connected to an increased rate of turnover of catecholamines *(37)* and to discharge of aminergic neurotransmitters by nicotine *(38).* This could be followed by a decrease in the levels of these transmitters over chronic treatment, resulting in long-term desensitization of the secretion of gonadotropins, and reduction of their plasma levels *(39).*

### *Nicotinic Acetylcholine Receptors*

There are two general types of nicotinic acetylcholine receptors (nAChRs). One type is found exclusively at the neuromuscular juncture and the other is distributed throughout the central and peripheral nervous systems (CNS/PNS). Both types of the receptor may be responsible for mediating the effects of nicotine in brain as well as in periphery. However, the brain nAChRs are more likely to mediate the main effects of nicotine in this organ. Muscle nAChRs are made of four different subunits (termed alpha, beta, delta, and epsilon), with a fixed stoichiometric ratio of 2:1:1:1 in adult muscle *(40).* In contrast, the neuronal nAChRs have thus far been shown to contain only α and β subunits hybridized in differing combinations. While there is indeed a fundamental similarity between these two types of receptors, there exist a few prominent differences *(41,42).* The foremost difference is the subunit make-up and diversity of the subunit combinations able to produce the neuronal nAChR. The diversity of neuronal nAChRs probably provides the ability to differentiate the variable signaling environments throughout the brain.

Mammalian-brain nAChRs can be grouped into four classes *(43,44).* Type I nAChRs bind α-bungarotoxin, and appear to predominantly or exclusively contain the α7 subunit *(45),* highly permeable to $Ca^{2+}$ ion *(46).* Type II nAChRs contain the β2 subunit, bind nicotinic agonists with high affinity (but not α-bungarotoxin), have a much larger electrophysiological potency for nicotine over cytisine, and should have the α4 and β2 composition in most brain regions, with other α subunits contributing in specific areas. Type III nAChRs (constituted mainly of α3 and β4 subunits) attach epibatidine with high affinity, and electrophysiologically respond to cytisine as well as to nicotine. Type IV nAChRs bind cytisine and epibatidine with high affinity, and electrophysiologically respond to cytisine similar or better than to nicotine. This type also exhibits faster desensitization at high doses of nicotine than type III.

At present, there are only a few combinations of subunits proven to actually exist in the brain ganglia. The combinations of subunits to these nAChRs include: a receptor with two α4's and three β2's was found using immunopurification, α4 or α3 together with α5, and α7 together with α8 *(47).* Using in vitro hybridization with mRNA specific to the neuronal nAChR subunit, a wide distribution of the separate subunits was found throughout the brain. Co-localizations of α2 and α4 have been observed throughout the brain. Co-localization of α2/β2, α3/β4, and homo-oligomer α7 expression were found to be restricted to certain areas of the brain *(48).*

## Effects of Tobacco on Body Weight

### *Human Studies*

The evidence toward an inverse association between cigarette smoking and body weight is convincing from many cross-sectional and prospective studies in humans. Most but not all surveys indicate that smokers weigh less than nonsmokers, and that those who stop smoking gain weight and those who start smoking lose weight. A comparison of male monozygotic twins with their identical siblings revealed that smokers had 5.3–10 lbs lower weight than nonsmokers *(14).* Persons who quit smoking have been found to gain more weight than nonsmokers *(49),* with increments in both central and peripheral adiposity *(50),* and regardless of sex *(51).* The weight gain is

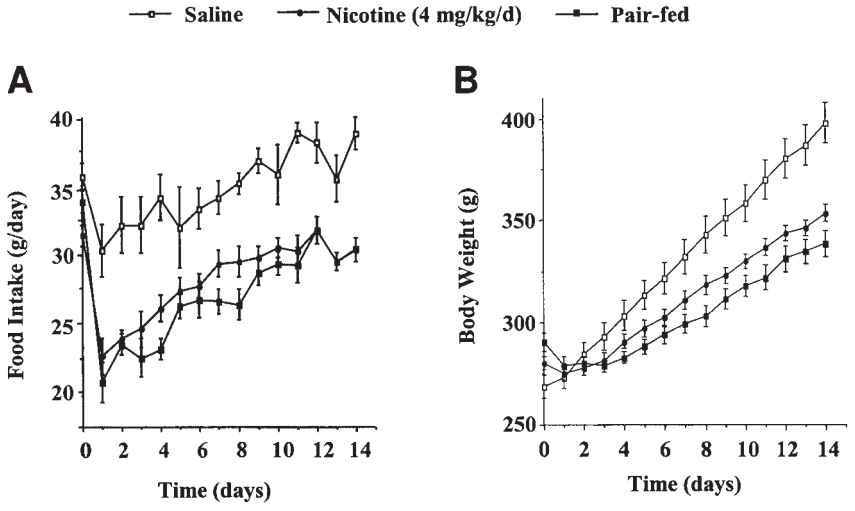


Fig. 1. Effects of nicotine on food intake and body weight. Daily food intake **(A)** and body weight **(B)** were determined throughout the 14 d of saline or nicotine (4 mg/kg/d) treatment. A pair-fed group that received saline injections and the same amount of food consumption by paired nicotine-injected rats is also shown. Values are means ± SEM. Reprinted from ref. *(57)* by permission of Elsevier Science B.V.

particularly high in recent quitters *(52–54)*, but continues over 16 yr of follow-up *(55)*. Most smokers perceive cigarette smoking as an effective strategy of weight control, and would not tolerate significant weight gain upon quitting; this represents one of the major motives for continuing to smoke, particular among women *(15)*.

## *Animal Studies*

Studies of nicotine's effects on body weight in rodents support the inverse association between tobacco smoking and body weight observed in human studies, showing that nicotine is responsible for the effects of smoking on body weight *(56)*. This conclusion, however, was largely derived from studies using quite high doses of nicotine (i.e., 6–12 mg/kg/d). We therefore re-investigated the above relationship with lower doses of nicotine that are more similar to those taken by human smokers. At the dose of 4 mg/kg/d, nicotine-treated rats gained weight consistently slower than control rats, and weighed 11% less than the saline controls after 14 d of treatment ($p < 0.01$). The average daily food intake was reduced by 19.5% ($p < 0.01$) in the free-feeding nicotine-treated rats (28.1 ± 0.36 g/d) compared to saline controls (35 ± 0.49 g/d) (Fig. 1; *57*). Effects of nicotine were the greatest in the first few days, with a reduction of food intake by approx 30% in nicotine-treated rats. These results confirmed other reports showing an inverse association between nicotine and body weight, suggesting that the reduction in body weight can be explained by reduced food intake at doses of nicotine characteristic of chronic human smoking.

However, the mechanisms underlying the alterations in body weight related to smoking are not well-understood. Several possible mechanisms have been proposed for the effects of nicotine on body weight *(14,56)*. Some human and animal studies suggest that nicotine suppresses food intake *(57–60)*, while others argue that nicotine increases the metabolic rate *(61–63)*. In any case, the effects of nicotine on appetite and body weight cannot be completely explained by either mechanism exclusively.

## Effects of Nicotine on Genes that Regulate Food Intake and Body Weight

The identified neurotransmitter or hormone genes that regulate appetite and body weight include those coding for leptin *(64)*, NPY *(65)*, orexin or hypocretin *(66,67)*, melanocortin 4 receptors for melanocyte-stimulating hormone (MC4-Rs) *(68,69)*, cocaine and amphetamine-regulated transcript (CART) *(70,71)*, cholecystokinin (CCK) *(72)*, corticotropin-releasing hormone (CRH) *(73)*, and uncoupling proteins (i.e., UCP-1, -2, and -3) *(74,75)*. Among these peptides, NPY and orexin stimulate food intake, while the others are inhibitors of appetite and food consumption (for reviews, *see 76,77*) except for UCPs 1–3, which have been implicated in the regulation of body temperature and body weight *(74,75)*. Although many regulatory neurotransmitter/hormone genes on appetite and food intake have been identified and characterized, whether the effects of nicotine on food intake and body weight can be explained by changes in the expression levels of these genes remains largely unknown. Therefore in following sections we will focus on genes whose expression levels have been reported to be regulated by nicotine administration.

### *Leptin*

Leptin, a species-specific circulating hormone produced by white adipose cells, is a negative regulator of food consumption. Leptin appears to exert its effects on CNS through the long-form leptin receptor (Ob-Rb) *(78)*, which is highly expressed in the hypothalamus, a critical region for regulation of food intake and body weight *(79)*. Leptin has regulatory effects on several feeding-inducing peptide genes. In ob/ob mice and fasted rats, exogenous treatment with leptin suppresses NPY overexpression *(80–82)*. In contrast, other food intake-suppressor genes such as CART, CCK, MC4-R, and CRH are up-regulated by leptin (for a review, *see 76*). Whether orexin (*see* section on Orexins/Hyprocretins) is regulated by leptin remains to be determined. Based on the facts that leptin is a fundamental link between peripheral metabolic signals and the brain, and that there are leptin receptors in the lateral hypothalamus *(76,83)*, it is predicted that orexin might be regulated by leptin as well.

In addition to being a negative regulator of food intake, leptin is also a positive regulator of energy expenditure. Most of evidence for that is derived from studies on mice with mutations in the leptin gene, or in leptin-signaling pathways. Two studies have demonstrated that the reduced food intake associated with leptin administration to ob/ob mice does not fully account for the leptin-induced weight loss *(84,85)*. More direct evidence was provided by Pelleymounter et al. *(86)*, who reported that leptin administration increased oxygen consumption in ob/ob mice but not in their lean counterparts. Furthermore, Collines et al. *(87)* have shown that leptin increased norepinephrine turnover in brown adipose tissue (BAT) of ob/ob mice, suggesting that the mechanism of the leptin-induced increase in energy expenditure is related to heightened thermogenesis in BAT.

The effects of nicotine on plasma leptin concentrations have been contradictory in human studies. Two epidemiological studies *(88,89)* in different ethnic groups showed that plasma leptin concentrations are significantly lower in smokers than in nonsmokers, even after adjusting for body mass index (BMI), an estimate of adiposity. Significant decreases in plasma leptin concentrations independent of adiposity have also been detected in both full-term and pre-term newborns born to mothers who had been smoking during pregnancy compared to those born to nonsmoking parents *(90)*. Furthermore, the same group reported a significant dose-response relationship between the number of cigarettes smoked during pregnancy and leptin concentrations in the term newborns' umbilical cord blood. On the basis of these observations, it was suggested that smoking, via nicotine mechanisms,

may modulate leptin biosynthesis and consequently reduce body weight. If smoking increases the sensitivity to leptin in a system controlling fat deposition, negative feedback may lower leptin levels. Controversially, Eliasson and Smith *(91)* reported that plasma leptin concentrations were higher in smokers and other long-term users of nicotine than in nonsmokers. In another recent study, Oeser et al. *(92)* found no changes in plasma leptin concentrations after 7 d of nicotine abstinence under controlled dietary conditions, suggesting that alterations in leptin expression may not contribute to post-cessation weight gain. The discrepancies in human studies could be caused by differences in the investigated population samples, degree of obesity, prevalence of smoking, and occurrence of diabetes.

Consistent with most human RIA data *(88–90)*, significantly lower leptin RNA expression level in perirenal fat depots and plasma concentrations were obtained in nicotine-treated rats (RNA: 0.764 ± .019; protein: 1.55 ± 0.17 ng/mL) compared to saline controls (RNA: 0.861 ± 0.019; protein: 3.03 ± 0.37 ng/mL; $p < 0.01$; [*93*]). Importantly, a dose-dependent relationship between plasma leptin concentrations and the concentration of nicotine administered in rodent has also been detected in our study. Smoking has previously been shown to increase plasma catecholamines and free fatty acid (FFA) concentrations *(94,95).* Catecholamines, in turn, increase lipolysis in humans *(94)* and have been shown to decrease leptin concentrations. However, it remains to be determined whether the observed reduction in plasma leptin concentrations in humans and rodents was due to direct effects of nicotine on leptin biosynthesis in adipocytes, or to indirect effects through other regulatory pathways of neurotransmitters in the CNS.

### *Uncoupling Proteins*

UCPs, unique proteins located in the inner mitochondrial membrane, are thought to transport fatty acid anions from the inner surface to the outer surface of the inner mitochondrial membrane *(96–98).* At the outer surface, the anions are neutralized by protonation, and could be vectorially returned via the membrane lipid. The net result is the exothermic movement of protons to the inside of the inner mitochondrial membrane, down the proton-concentration gradient, and uncoupled from ATP synthesis. In an alternate model, UCPs are proton transporters, using fatty acids as activating cofactors. UCP-1, the first identified uncoupling protein, is encoded by a nuclear gene, which is expressed only in BAT *(99,100).* Transcription of UCP-1 is induced by cold exposure, β-adrenergic stimulation, and thyroid hormones *(101).* Recently, two additional uncoupling proteins, UCP-2 and UCP-3, have been identified *(102–104).* These two uncoupling proteins have 59 and 57% homology, respectively, with UCP-1, and 73% homology with each other. In common with UCP-1, both UCP-2 and UCP-3 can partially uncouple mitochondrial respiration. UCP-2 is expressed in many tissues, including epididymal and perirenal white adipose tissue (EWAT, PWAT), heart and muscle, while UCP-3 is expressed mainly in BAT and skeletal muscle. It has been shown by Scarpace et al. *(74,75)* that leptin-induced increase in energy expenditure occurs through increased thermogenesis in BAT, including increases in oxygen consumption and in UCP-1 and UCP-3 gene expression in BAT (UCP-2 mRNA also increasing in EWAT). These results suggest a role for UCP-1, UCP-2, and UCP-3 as part of the mechanism by which leptin contributes to energy expenditure. Indeed, all such proteins, via their properties of generating heat, may be implicated in the regulation of body temperature and body weight. Furthermore, the presence of UCP-2 in tissues other than the rodent BAT (e.g., WAT, lung, kidney, and liver) and the presence of UCP-3 in BAT as well as in skeletal muscles, makes UCP-2 and UCP-3 potential candidates for regulating body weight in large mammals (which usually lack UCP-1 in BAT). The leptin receptor has been identified on adipocytes *(105),* and a strong correlation between the decrease in leptin mRNA and the increase in

UCP-2 in EWAT suggests that both of these processes are mediated by the same pathway, possibly a leptin-signaling pathway.

It has long been known that nicotine may increase thermogenesis in rats *(106)* but the mechanism underlying this pharmacological effect is largely unknown. As UCP-1 is one of the primary molecules involved in thermogenesis in brown fat, it has been hypothesized that it may play a role in this effect of nicotine. Indeed, recently, Yoshida et al. *(107)* found that nicotine increases UCP-1 mRNA and protein in both brown fat and subcutaneous and retroperitoneal white adipose tissues in mice. A similar pharmacological effect of nicotine on the expression of UCP-1 in the rat was found in our laboratory. By using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and Western-blot analysis, we examined the UCP-1 mRNA and protein levels in BAT samples that were isolated from rats received saline, nicotine, or saline plus limited food (to match the saline rats) by ip injection 5 times per day for 14 d. We found that UCP-1 mRNA and protein level in BAT were significantly higher in nicotine-treated animals relative to the saline control or pair-fed rats. Whether nicotine has any effects on the expression of UCP-2 and UCP-3, and how important the UCPs induced by nicotine could be in regulating thermogenesis, remains to be determined. Based on the facts that leptin administration increases the expression of UCP-1 in BAT and UCP-2 in EWAT, and that nicotine decreases leptin biosynthesis, we suspect that nicotine may affect the expression of UCP-1 and UCP-3 in BAT, and UCP-2 in WAT as well.

### *Neuropeptide Y (NPY)*

NPY, a 36 amino acid peptide, is one of the most abundant neuropeptides in the mammalian brain and is highly concentrated in the hypothalamus *(108).* Within the hypothalamus, NPY is synthesized largely in neurons of the arcuate nucleus, which send projections into surrounding hypothalamic structure. Comprehensive reviews on the topics concerning biochemistry, cell biology, physiology, and pharmacology of this peptide have been presented by Wahlestedt and Reis in 1993 *(109)* and Gehlert in 1998 *(110).* NPY is also produced in peripheral locations, such as the adrenal gland *(111).* The peptide passes the brain-blood barrier (BBB) *(112),* and can therefore be distributed to locations that do not possess synaptic connections. It also appears that NPY is quite efficiently distributed by cerebrospinal fluid (CSF), which would explain its presence in locales not possessing any NPY-producing cell bodies, or significant neuronal projections, such as the lateral hypothalamus.

While NPY is firmly established as a noradrenergic co-transmitter *(113),* it could also interact with dopamine-operated pathways *(114).* It is less involved with opioid pathways. Endorphins and most of natural enkephalins differ from NPY-like peptides by the lack of C-terminal amidation, which precludes significant cross-reactivity of the peptides at the respective receptors. The role of NPY in the release of sympathetic transmitters is probably mainly inhibitory *(115).* In many cell types, NPY inhibits adenylyl cyclase activity (e.g., *116,117*). A potentially large role of NPY-related peptides could be expected in phosphatidylinositol-specific phospholipase C (PtdIns-PLC) activity, since $Y_1$, $Y_2$, $Y_4$ and $Y_5$ receptors are all sensitive to PtdIns-PLC inhibitors in attachment of subtype-selective ligands *(118,119).*

In most cases, exogenous NPY administration consistently stimulates feeding; NPY is considered to be of fundamental importance as a component of the hypothalamic control of appetite and body weight. Evidence supporting this implication comes from experiments showing that an increase in NPY secretion in the PVN at the beginning of the dark period is associated with food intake. Expression of NPY mRNA in the arcuate nucleus is increased in response to fasting (when leptin levels rapidly falls) and in chronic moderate food restriction *(120).* Treatment with exogenous leptin suppresses NPY overexpression *(80,82).* Because there is an inverse association

between nicotine use and food intake, and NPY is a stimulator of feeding, one could predict a decrease of NPY expression by nicotine. However, such prediction is in contrast to the results reported by several laboratories, including ours *(57,59,121).* Frankish et al. *(59)* reported that NPY peptide levels were significantly decreased after 1 or 12 d of nicotine treatment, while NPY mRNA levels were decreased by 40% after 1 d, but increased by 40% after 12 d of the treatment. We found significantly higher NPY mRNA (20–50%) and peptide (24–69%) in the nicotine-treated rats compared to saline and pair-fed control animals *(57).* The discrepancy between the aforementioned studies could be due to dosaging and/or method of administration of nicotine. The study in *(59)* used a higher dose of nicotine (12 mg/kg/d) than our study (2–6 mg/kg/d). This interpretation is corroborated by our finding that rats receiving nicotine 6 mg/kg/d had a 30% reduction in their NPY mRNA levels compared to rats receiving 4 mg/kg/d. The two studies also differed in the way of nicotine administration (continuous infusion via osmotic minipumps [*59*] vs five daily ip injections [*57*]). Similar to our results, Hiremagalur and Sabban *(121)* found that the NPY mRNA levels are elevated in the adrenal only when nicotine is injected subcutaneously and not if infused by osmotic minipumps.

### NPY Receptors

NPY acts by binding to G protein-linked receptors. At least six receptors that respond to peptides of the NPY family, designated $Y_1$ through $Y_6$, have been identified. These molecules all are receptors of rhodopsin family, possessing seven transmembrane domains. The $Y_1$, $Y_4$, and $Y_6$ subtypes are more than 40% identical in structure, share sensitivity to a number of agents and usually are considered as the $Y_1$-like NPY receptor group. Subtype NPY-$Y_5$ is considered to be the primary mediator of NPY-induced feeding, and the $Y_1$ receptor was also amply documented to support feeding *(122–127).*

The $Y_1$ subtype is the post-synaptic neuronal receptor especially involved with adenylyl cyclase inhibition and $Ca^{2+}$ entry, strongly dependent on interaction with Gα-subunits for an appropriate ligand binding and activation. The $Y_1$ receptor is probably subject to extensive internalization in most systems, as it shares C-terminal features with rhodopsin family G-protein coupling receptors (GPCRs) known to possess large membrane dynamics (angiotensin $AT_{1A}$, bradykinin $Bk_2$, and $\beta_1$-adrenergic receptor). The $Y_2$ receptor is presumably mainly presynaptic receptor *(110).* Studies to date do not support a major role for this receptor in the regulation of feeding *(128).* The $Y_5$ receptor is similar to the $Y_1$ and the $Y_4$ receptor in sensitivity to ion transport inhibitors, and also responds to $Y_1$-selective, $Y_2$-selective and $Y_4$-selective peptides. Levels of $Y_5$ mRNA in human forebrain are larger than those of any other known NPY receptor subtype *(129).* However, the receptor is very difficult to appropriately differentiate from other subtypes by ligand binding, and thus far no reliable estimates exist on its organismic levels. No important role in the regulation of feeding was thus far documented for the $Y_3$, $Y_4$, and $Y_6$ subtypes.

Treatment with nicotine at 4 mg/kg/d by five ip injections (0900–1700 h; the dose found to increase hypothalamic NPY in the rat [*57*]) induced a decrease in NPY $Y_1$ receptors in both the anterior hypothalamic area (AHA) and the posterior hypothalamus (PHA) at 14 d (Fig. 2), without a parallel decline in $Y_1$ receptor density in the piriform cortex. No significant change in the density of NPY $Y_2$ receptors was found in any area examined at 14 d of nicotine treatment. The decline of AHA and PHA $Y_1$ was found at three discrete inputs of up to 125 pM of competing agonist, indicating absence of major changes in receptor affinity with nicotine administration (Fig. 2). There also were no significant density or affinity changes for NPY $Y_1$ sites in the piriform cortex (Fig. 2C), or for $Y_2$ sites in any of the areas examined (Fig. 2D–F).

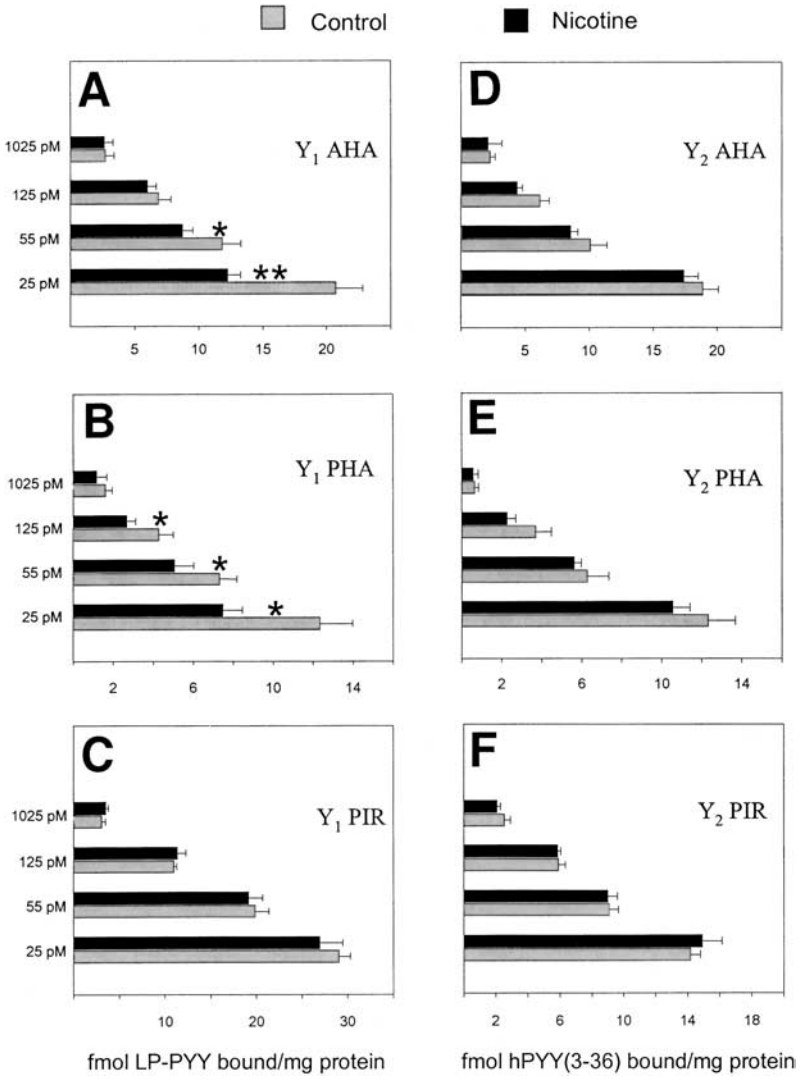


Fig. 2. Binding of subtype-selective NPY receptor agonists (Leu$^{31}$, Pro$^{34}$) human PYY and human PYY(3–36) to particulates from brain areas of rats treated for 14 d with nicotine at 4 mg/kg/d, and the corresponding control rats. Left, Competition of the binding of [$^{125}$I] (Leu$^{31}$, Pro$^{34}$)hPYY (selective for $Y_1$ subtype as assayed at 2 nM of human pancreatic polypeptide [*118*]) by unlabeled (Leu$^{31}$, Pro$^{34}$)hPYY at particulates from brain areas of nicotine-treated rats and of solvent-injected control rats. The binding of 25 pM of [$^{125}$I] (Leu$^{31}$, Pro$^{34}$)hPYY to anterior/medial hypothalamic **(A),** posterior hypothalamic **(B)** and piriform cortical **(C)** particulates was competed by 0, 30, 100, and 1000 pM of cold (Leu$^{31}$, Pro$^{34}$)hPYY in the presence of 2 nM unlabeled hPP, defining the nonspecific binding at 100 nM of unlabeled (Leu$^{31}$, Pro$^{34}$)hPYY. The data represent averages of binding to particulates from six individual animals in each group. Right, Competition of the binding of [$^{125}$I]hPYY(3–36) (selective for $Y_2$ subtype of brain or kidney NPY receptor, and not sensitive to pancreatic polypeptides at up to 10 nM *[118]*) by unlabeled hPYY (3–36) at particulates from brain areas of nicotine-treated rats and of solvent-treated control rats. The binding of 25 pM of [$^{125}$I]hPYY(3–36) to anterior/medial hypothalamic **(D),** posterior hypothalamic **(E),** and piriform cortical **(F)** particulates was competed by 0,30, 100, and 1000 pM of cold hPYY (3–36), defining the nonspecific binding at 100 nM of unlabeled hPYY (3–36). The data represent averages of binding to particulates from six individual animals in each group. The ANOVA testing indicated no significant variation for the $Y_2$ binding, while there was a positive ANOVA among $Y_1$ groups.

The large excess in NPY levels that we observed in forebrain areas of rats chronically treated by nicotine is accompanied by, and could be largely responsible for, the downregulation of hypothalamic receptors that bind a common $Y_1/Y_4/Y_5$ site ligand, ($Leu^{31}$, $Pro^{34}$) human peptide YY. Based on our pharmacological evidence, these sites largely represent the $Y_1$ receptors, but could also include some $Y_5$ receptors, which are difficult to differentiate from the $Y_1$ subtype by the binding of peptidic ligands currently available. Our findings indicate a long-term, chronic decrease in a population of receptors that could be specifically involved in the maintenance and reinforcement of feeding behaviors. This decrease could have contributed significantly to the observed reduction of food intake and body weight in nicotine-treated animals.

The long-term increase in NPY receptor-agonist signaling could also induce a downregulation of specific G-protein α-subunit subtypes or populations, analogous to activity of β-adrenergic and prostanoid *(130)* and $\alpha_2$-adrenergic agonists *(131).* The hypothalamic $Y_1$ sites downregulated by chronic nicotine could also be associated with a specific Gα subtype, or present at specific neuronal locations. We found (Fig. 3A) that the sensitivity of $Y_1$ binding in anterior hypothalamus to both an agonist and an antagonist of the Gα nucleotide site is strongly decreased in chronic nicotine treatment. On the other hand, the binding parameters of hypothalamic Gα nucleotide sites, as checked by competition of [$^{35}$S]GTP-γ-S by GTP-γ-S, were not changed by chronic nicotine (Fig. 3B). These findings indicate that the nicotine-resistant population of the $Y_1$ receptors is associated with Gα subunits that have a lower average sensitivity to guanine nucleotides, such as Gqα, with more rigid nucleotide 'switches' *(132),* and a stronger interaction with phospholipase(s) C (*see* e.g., *133*). We have indeed observed (Parker, Kane, and Li, in preparation) that the sensitivity of hypothalamic $Y_1$ binding to U73122, an irreversible inhibitor of phosphatidylinositol-specific phospholipase C, is also reduced in chronic nicotine treatment.

### *Orexins/Hypocretins*

Similar to NPY, orexins or hypocretins are positive regulators of food intake, discovered by two laboratories almost simultaneously *(66,67).* This newly identified hypothalamic neuropeptide family consists of two members, orexin-A and orexin-B, with about 50% identity at the amino acid level. Both neuropeptides are derived from a single 131-residue precursor, named prepro-orexin/hypocretin. The production of prepro-orexin/hypocretin and subsequent cleavage into orexin-A and B peptides is almost exclusively located in the area of lateral hypothalamus (LHA). The axon projections of orexin extend to many areas of the brain and spinal chord *(134–142).* As a result, the physiologic behaviors associated with orexins are diverse. While two reports showed involvement especially of orexin-A in ingestive behaviors in the rat *(66,143),* a study in mice attributed the orexigenic activity of orexin-A to stimulation of energy metabolism *(144).* Disregulation of the orexin pathway also induces narcolepsy *(145,146),* which implies that orexins possess a role in regulation of sleep and wakefulness *(147,148).* Many studies have henceforth implicated orexins in regulation of physiologic functions and responses such as pain *(149),* sleep, arousal, blood pressure *(140,150,151),* stress *(152,153),* and continued to show a role of orexins in ingestive behaviors *(154).*

Because orexins are known to be involved in the control of food intake, we looked for possible changes in orexin expression in the chronic nicotine paradigm, which is characterized by a highly reproducible reduction of food intake. Our hypothesis was that orexin levels would decrease upon nicotine administration. However, our results indicated a dose-dependent increase in prepro-orexin mRNA production upon chronic nicotine administration *(155).* A subsequent experiment involved a control group limited to the food intake of nicotine-treated animals (and also receiving injections of the saline-solvent; "yoked" controls). The yoked-saline group had orexin mRNA levels

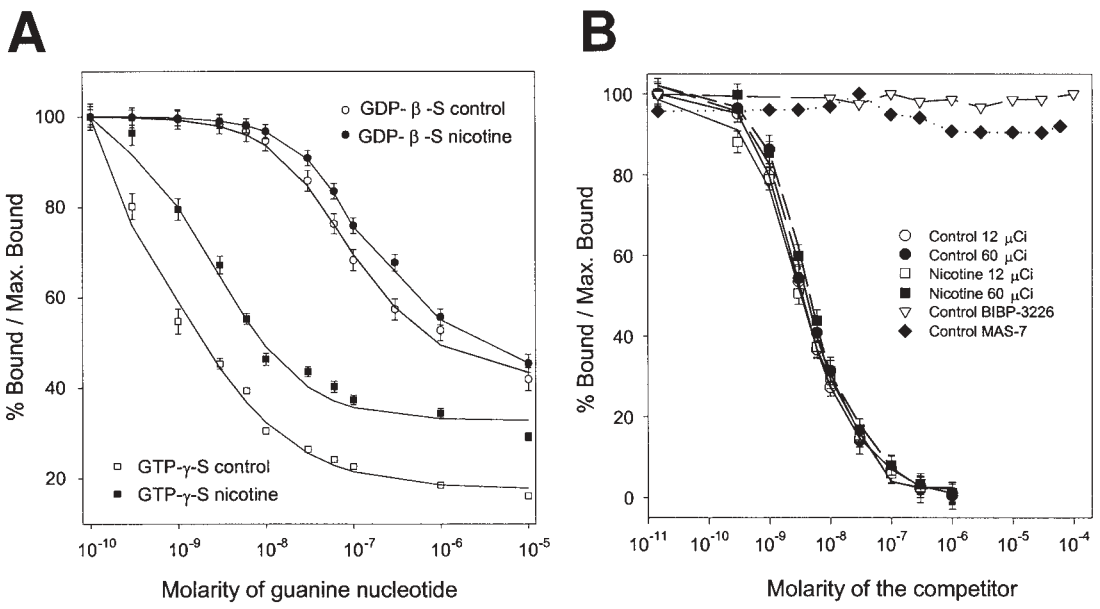


Fig. 3. Sensitivity to guanosine polyphosphates in the binding of [$^{125}$I](Leu$^{31}$, Pro$^{34}$) human PYY and of [$^{35}$S]GTP-γ-S to particulates from brain areas of nicotine-treated and control rats. **(A)** Competition by ligands of the G-protein α-subunit nucleotide binding site, agonist GTP-γ-S and antagonist GDP-β-S, of the binding of [$^{125}$I] LP-PYY to particulates from anterior hypothalamus of rats treated with nicotine (14 d), and the corresponding control rats. With GTP-γ-S, the $IC_{50}$ values, in nM, over the displacement range of 0.001–10 μ*M* (with percent specific binding displaced at 10 μ*M* of the nucleotide in parenthesis) were 7 ± 1.3 (84%) and 20.6 ± 5.6 (71%) with particulates from control and nicotine-treated animals, respectively (n = 5 for both; $p < 0.05$). With GDP-β-S (n = 2), the corresponding $IC_{50}$ values were 87 ± 11 (58%) and 176 ± 21 (54%; $p < 0.05$) for AHA, and 151 ± 20 (72%) vs 151 ± 13 (71%) for PIR particulates. The $Y_1$ binding was completely inhibited by the nonpeptidic $Y_1$-selective antagonist BIBP-3226, with $IC_{50}$ values of 3.7 ± 0.4 nM (92% inhibition) for control, and 6.5 ± 3 nM (94% inhibition) for nicotine-treated group ($p < 0.05$). The $Y_1$ binding was also largely inhibited by receptor mimic at the Gα subunit, mastoparan superanalog MAS-7 *(185);* the control data were best fit to two components of 0.195 ± 0.08 (19%) and 14.2 μ*M* (70%), while the nicotine-treated animal data fit a single component of 14.7 ± 2 μ*M* (89%). **(B)** Competition by nonlabeled GTP-γ-S, BIBP-3226 and MAS-7 of the binding of [$^{35}$S]GTP-γ-S to anterior hypothalamic particulates from control and nicotine-treated (14 d) rats. Two levels of tracer input (12 and 60 μCi/liter of assay) were tested against cold GTP-γ-S. The $K_D$ values in nM GTP-γ-S at 12 μCi/L were 4.6 ± 0.8 and 4.7 ± 0.48 for control and nicotine-treated animals' particulates, respectively; the corresponding $B_{max}$ values (in pmol/mg particle protein) were 86 ± 7.5 and 87.5 ± 6.8 (n = 2 for both). At the input of 60 μCi/l, the correspondent $K_D$ values (nM) were 3.9 ± 0.4 and 4.2 ± 0.3, and the respective $B_{max}$ values (pmol/mg protein) were 90 ± 9.4 and 91.2 ± 9.1 (n = 2 for both). At 1 μ*M,* nonlabeled GTP-γ-S displaced more than 98% of the total binding of [$^{35}$S]GTP-γ-S.

similar to free-feeding control animals, thus demonstrating that the increase in prepro-orexin mRNA found in nicotine-treated rats was due to the effects of nicotine administration, and not to factors such as differences in body weight and food intake. This increase in prepro-orexin mRNA led us to investigate orexin-A and B levels in regions of the hypothalamus. Using radioimmunoassays of orexins A and B, we measured the levels of these peptides in multiple areas of the hypothalamus. We discovered significant increases of orexin A in the dorsal medial hypothalamus (DMH) and of orexin B in DMH and hypothal-

amic paraventricular nucleus (PVN). Because the DMH was shown to be sensitive to the orexigenic effects of orexin A *(143),* we believe that the increased levels of orexin in this area may contribute to the hypophagic aspect of chronic nicotine treatment. Increasing levels of orexin A in the DMH would tend to increase the food intake. However, nicotine reduces food intake. We suspect the reason behind this phenomenon is the consequence of a decrease in orexin-A receptor ($OX_1$-R) affinity and density due to the excessive activation via the increased levels of orexin A. Reduction of $OX_1$-R receptors suggests a decrease in orexin signaling in the DMH (*see* section on Orexin Receptors).

The mechanism through which nicotine is activating the increased production of orexin is unclear at present. An increase in dopamine (e.g., *156*) and serotonin (e.g., *157*) release in the LHA upon nicotine administration could result in activation of orexin cell bodies. As discussed in the monoamine section (*see* section on Effects of Nicotine on Monoamines), the LHA is a critical area for ingestive behaviors, and may prove to represent an interconnecting pathway that is altered by the chronic presence of nicotine. Furthermore, the increased activity within the LHA upon nicotine administration may lead to altered physiologic conditions beyond ingestive behavior.

### *Orexin Receptors*

Two G-protein-coupled receptors for orexins ($OX_1$-R and $OX_2$-R) with 64% identity at the amino acid level have been cloned and sequenced thus far *(66).* Orexin A is a specific and high-affinity agonist for $OX_1$-R, while $OX_2$-R is nonselective receptor for both orexin-A and orexin-B, but the binding affinity for orexin-B is higher than for orexin-A *(66).* Based on the binding of [$^{125}$I]-labeled orexin-A and Tyr0-extended orexin-B, the orexin-A sites appear to be present in much larger numbers than the orexin-B sites in most areas of the forebrain, and can be identified in hypothalamic areas by selective competition of [$^{125}$I]orexin-A by orexin peptides, secretin, and NPY *(158).* The orexin-A receptor is characterized by a relatively low affinity for its agonist peptides, along with a broad cross-reactivity including NPY, secretin and pituitary adenylate cyclase-stimulating peptide (PACAP) with affinities similar to, or even exceeding that of orexin-A *(158).*

The relatively low affinity of orexin-A for its specific binding sites is similar to that found for LHRH at its receptor *(159).* The low affinity of both receptors with physiological agonists could be related to an evolved constrainment of the signaling. In the case of orexin-A this could be connected to a co-regulation by NPY. The concentration of NPY in most forebrain areas exceeds that of orexin-A by at least two orders of magnitude (compare e.g., *108,138*). Also, NPY is known to be readily distributed by blood circulation as well as via the CSF *(112).* The prevailing much higher concentration of NPY might modulate the activity of orexin-A receptor over periods sufficiently separated in time from orexin discharges. The short dwelling times of agonist ligands could also be tied to economy of receptor recycling. Because the structure of C-terminal intracellular domain of the orexin-A receptor is comparable to that of the $AT_{1A}$ or $BK_2$ receptors (both of which are subject to internalization upon ligand binding [*160,161*]), a lower binding strength of physiological agonist peptides may aid conservation of active orexin-A receptors. This could be critical in view of the known metabolic stability of orexin-A peptide *(162),* as well as of the orexin-A receptor sensitivity to NPY.

Our recent finding of an upregulation of prepro-orexin mRNA, orexin peptides, and orexin receptor mRNAs by chronic nicotine treatment (*see* section on Orexins/Hypocretins and ref. *155*) pointed to the possibility of changes of orexin receptor levels in forebrain, especially in areas found to be enriched in these sites, in response to alterations of neurotransmitter release and balance created by chronic nicotine treatment. This is the more likely in terms of an essentially equal sensitivity of the binding of

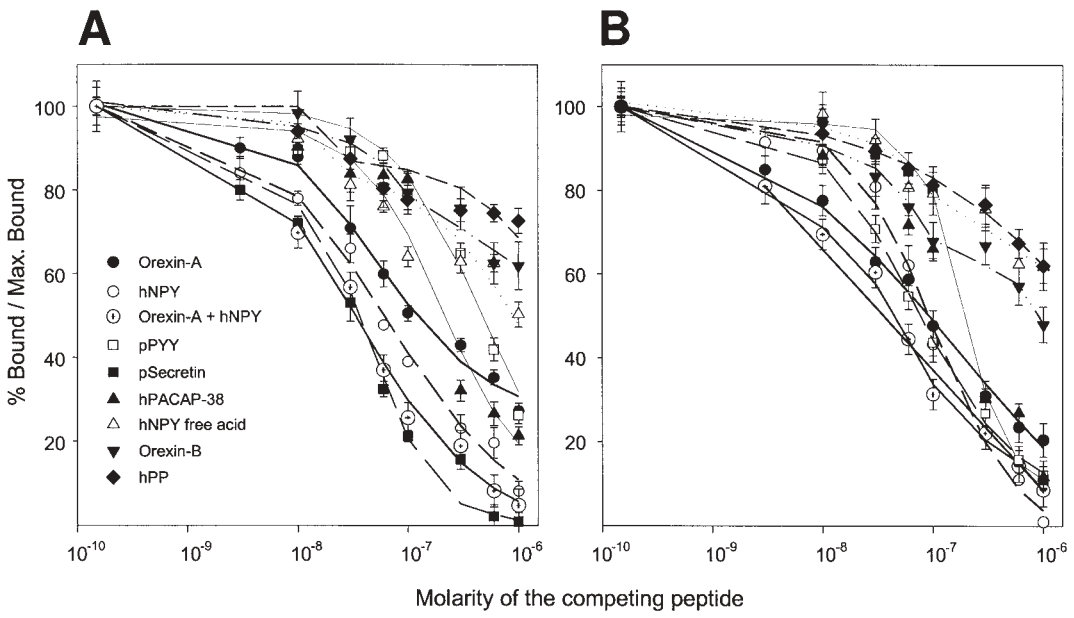


Fig. 4. Competition by various neuropeptides of [$^{125}$I] orexin-A binding to particulates from CHO cells expressing orexin-A receptor, or from rat anterior hypothalamus. At least two assays with nine discrete concentration points were done for each peptide in either paradigm. The $K_I$ value (nM) for the binding displaced at up to 1 μ*M* of the respective peptide is followed (in parenthesis) by percent inhibition at 1 μ*M* of the peptide. **(A)** Particulates from CHO cells expressing orexin-A receptor. The $K_I$ values for the displaced fraction were 52 ± 14 (73%) for orexin-A, 64 ± 19 (92%) for hNPY, 36.7 ± 8.1 (95%) for orexin-A and hNPY at hNPY at equimolar inputs, 176 ± 58 (74%) for porcine PYY, 38.5 ± 7.9 (99%) for porcine secretin, 129 ± 37 (79%) for human PACAP-38, 597 ± 72 (41%) for mouse/rat orexin-B, 417 ± 299 (35%) for hNPY free-acid form, and ~600 (30%) for hPP. **(B)** Particulates from rat anterior hypothalamus. The $K_I$ values for the displaced fraction were 82 ± 9.9 (79.5%) for orexin-A, 98 ± 22 (99%) for hNPY, 57 ± 9.1 (94%) for orexin-A and hNPY at equimolar inputs, 188 ± 23 (89%) for pPYY, 68 ± 5.8 (87%) for porcine secretin, 160 ± 64 (85%) for hPACAP-38, 592 ± 176 (52%) for mouse/rat orexin-B, 417 ± 149 (38%) for hNPY free-acid form, and ~ 700 (37%) for hPP. Adapted from ref. *(158)* by permission of Academic Press.

orexin-A to either CHO-cell expressed orexin-A receptors or to rat hypothalamic sites, to orexin-A, human/rat NPY, and porcine secretin *(158),* as seen in Fig. 4. Orexin-A sites are well-expressed in the hypothalamus and much less represented in cortical areas (Table 1; *158*).

Changes in orexin-A sites in the hypothalamic locations could reflect the increase in both orexin and NPY peptides in the chronic nicotine paradigm *(57,155)* by mechanisms that could be similar to those involved in the down-regulation of NPY-$Y_1$ sites. The orexin-A receptor is apparently dependent on a link to phosphatidylinositol-specific phospholipase C in its ligand binding *(158).* The orexin-A receptors could, however, be more resistant to internalization by agonist peptides (which appear to also include NPY and secretin *[158]*) than the $Y_1$ receptor. A chronic elevation of NPY levels could increase the sequestration, and possibly even the internalization, of orexin-A sites.

## Effects of Nicotine on Monoamines

Small molecule neurotransmitters (i.e., glutamate, GABA, norepinephrine, dopamine,

Table 1
Specific Binding of [$^{125}$I] Orexin-A to Particulates from CHO Cells Expressing Cloned Orexin-A Receptor and From Rat Forebrain Area[a]

| Source | Mean ± SEM | Significance vs AHA |
|---|---|---|
| AHA | 12.2 ± 0.54 | NS |
| PHA | 11.8 ± 0.16 | NS |
| CHO-OX-AR cells | 9.92 ± 0.19 | * |
| HIPP | 5.75 ± 0.71 | ** |
| PIR | 4.77 ± 0.74 | ** |
| LAT | 4.77 ± 0.41 | ** |
| PAR | 4.05 ± 0.29 | ** |

[a]The results are in femtomoles orexin-A specifically bound per mg particulate protein. The significance at levels of 95% (*) and 99% (**) confidence refers to Tukey *t*-tests on individual means following a positive ANOVA.

[b]Adapted from ref. *(158)* by permission of Academic Press.

serotonin) initiate the majority of the neurotransmissions known to date. Taking into account the wide effects nicotine has within the CNS, it is not surprising that its administration alters the complex pathways regulating the ingestive behavior. Recently, there has been an exciting explosion of knowledge about peptides involved in ingestive behaviors. However, it must not be forgotten that these peptides often work in concert with smaller molecule neurotransmitters. An overview of the pertinent pathways, both peptidergic and monoaminergic neurotransmission, should provide a useful framework to better understand how the interplay of these pathways may be affected by nicotine. In this section, we will focus on the effects nicotine may have on these small molecule neurotransmitters in the context of their regulation upon appetite.

Over the past couple decades, a substantial evidence accumulated to show that norepinephrine stimulates, and dopamine and serotonin (5-HT) inhibit the ingestion of food *(163–165).* Dopamine is believed to decrease food intake through its actions within the hypothalamus. In particular dopamine acts on the lateral hypothalamus area. Infusion of dopamine into the perifornical lateral hypothalamus was shown to decrease food intake *(166,167).* Furthermore, amphetamine-driven anorexia is believed to be connected to the catecholamines dopamine and norepinephrine within the lateral hypothalamic area *(166–169).* Dopamine levels have been shown through in vivo microdialysis to increase in the perifornical lateral hypothalamus during the ingestion of food *(163).* Dopamine is also believed to be the catecholamine responsible for the reinforcing behavior in the ingestion of food *(163,170,171).*

Serotonin is strongly implicated in pathways responsible toward decreasing food intake *(167).* Using in vivo microdialysis, it was shown that extracellular serotonin levels increase in the lateral and medial hypothalamus in response to the anticipation and during food intake *(172,173).* Furthermore, it is believed that anorectic drugs such as fenfluramine and fluoxetine act through enhancing the transmission of 5-HT *(167).*

A significant amount of research into nicotine is focused upon its addictive properties as related to smoking cigarettes. The reward properties of nicotine are believed to result from its actions on the mesolimbic dopaminergic system through increasing levels of dopamine in the nucleus accumbens after activation of neurons located within the ventral tegmental area (VTA). Through a series of experiments, ingestive behavior has been linked to this same reward system via lateral hypothalamic stimulation of the dopaminergic neurons in the VTA, which result in increased levels of dopamine levels in the nucleus accumbens *(163,170,171,174–177).* Thus, drugs of abuse such as nicotine exploit the normal reinforcing pathways used by the body to provide a similar sense of pleasure from eating.

Two aspects of neuronal activity generated within the lateral hypothalamus could relate to effects of nicotine on ingestive behaviors. First, this area of the hypothalamus is known to provide inhibitory signaling in food intake, and second, it plays a role in activating the mesolimbic

system that drives the reinforcement of food intake behavior. Because nicotine is known to produce widespread activation of monoaminergic sites within the brain, it follows from the above considerations that the lateral hypothalamus may be a critical area for hypophagic activity of nicotine. In vivo microdialysis has shown that nicotine administration directly into the LHA results in an increase in the regional DA and 5-HT levels in the rat *(178,179).* Dopamine increased significantly during the infusion of nicotine but dropped to baseline immediately following the end of infusion. The 5-HT levels slowly decreased after the end of nicotine infusion, taking 80 min to reach the baseline.

## Summary and Remarks

The research areas of ingestive behaviors and nicotine actions are broad and diverse. A number of direct and indirect experiments provide a clear, yet not surprising, connection between these areas. Nicotine has multiple effects throughout the CNS and PNS, involving a number of possible regulatory sites known to be connected to ingestive behaviors. This review has attempted to summarize such molecular sites. Chronic treatment by nicotine could result in upregulation of neuropeptide Y and orexins in forebrain areas important in regulation of feeding, while the NPY $Y_1$ and orexin-A receptors could be simultaneously downregulated. In the periphery, leptin is downregulated while UCP1 is upregulated under nicotine administration. Finally, the monoamines serotonin and dopamine are upregulated in the LHA by nicotine.

It is evident then that there is a complex signal modulation occurring in the presence and absence (loss of nicotine exposure) of nicotine. At the level of peripheral signaling, leptin is produced in proportion to the lower amount of fat found in nicotine-treated animals. This should signal to the brain to produce higher amounts of the orexigenic molecules such as NPY and orexin. However, this signal is apparently attenuated through the downregulation of their receptors. At the same time, the monoamines are increased under nicotine treatment and act to inhibit food intake. These signals together comprise the physiologic actions that induce satiation below levels of food intake needed to cover the actual energy output, resulting in undernourishment.

It appears that the effects of nicotine on body weight cannot be explained by decrease in energy intake or increase in energy expenditure. At present we are just beginning to understand the molecular mechanisms behind these actions. This review will hopefully serve as a platform to integrate these mechanisms and provide future directions of research that will better define the molecular actions of nicotine on ingestive behaviors.

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