Molecular Neurobiology of Ingestive Behavior

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The concepts and tools of molecular biology may be applied to almost any component of the animal involved in ingestion, but two categories of model system are particularly relevant for molecular analysis: homeostatic regulation of neuropeptide expression in the hypothalamus and neuronal plasticity underlying persistent changes in ingestive behavior. Molecular approaches to these models are reviewed, focusing on our strategy for analyzing conditioned taste aversion learning. Three questions must be answered: Where do the long-term changes occur within the distributed neural network that mediates feeding? This answer reveals the site of neuronal restructuring mediated by gene expression. When does the transition occur from short-term expression to long-term persistence of the change in behavior? This transition reveals the critical time of gene expression. What genes are expressed during the change in behavior? The expression of thousands of genes in discrete subpopulations of cells is likely to be required during critical periods of neuronal restructuring. The identification of these genes is a general challenge for molecular neurobiology. The analysis of ingestive behavior can profit from molecular tools, but ingestion also provides informative models that elucidate the principles of time- and neuron-specific gene expression mediating complex behaviors. *Nutrition* 2000;16:827–836. ©Elsevier Science Inc. 2000

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INTRODUCTION

As a discipline, molecular biology is the study of gene expression, and hence the structure of genes and the regulation of protein synthesis. In a strict sense, molecular biology is the analysis of the transcriptional control of gene promoters, structure and variation in the coding regions of genes, and protein synthesis from mRNA transcription and translation. Molecular neurobiology is the study of the unique characteristics of gene expression in neurons (i.e., the neural-specific expression of genes required for connectivity, transmission, and excitability).

Molecular biology can be extended to the molecule-by-molecule analysis of all the intracellular processes preceding gene expression and subsequent to protein synthesis. Molecular events play a critical physiological role in all life processes, including ingestive behavior: in the synthesis and maintenance of all enzymes, receptors, transporters, peptide neurotransmitters, and so on. Molecular biologists have generated many potent technologies to isolate and define molecules within cells and to determine genetic differences between individuals. The tools of molecular biology may be profitably applied to the analysis of any component of the animal involved in ingestion. The path of reduction can be followed in numerous experimental models from behavioral phenomenon to physiological events to intracellular processes and finally to the genetic blueprints of the requisite proteins. For example, the analysis of cholecystokinin in satiety has proceeded from the behavioral effects of exogenous peptide[1] to mutational analysis of receptor sequence in vitro[2] and in vivo.[3]

Two categories of molecular models are of particular relevance to the study of ingestive behavior: 1) systems in which the understanding of homeostatically regulated gene expression elucidates long-term regulation of ingestive behavior and metabolic state (e.g., maintaining long-term caloric balance), and 2) systems in which understanding of the critical periods of gene expression is essential for understanding long-term adaptive changes in ingestive behavior (e.g., learning or other plastic responses to acute changes in the environment or physiological state). This review provides an outline of the salient applications and contributions of molecular biology to the analysis of ingestive behavior, and in particular describes an experimental approach to the molecular mechanisms of ingestive plasticity in conditioned taste aversion (CTA) learning.

NEUROPEPTIDES AS A HOMEOSTATIC MODEL

The analysis of hypothalamic neuropeptides that regulate ingestion exemplifies the first category. Because the molecular biology of the hypothalamus has been reviewed extensively,[4] the salient characteristics of the neuropeptides will only be outlined here.

Most hypothalamic neuropeptides are expressed constitutively and released from presynaptic terminals in a tonic fashion. When perturbed, however, by food deprivation (caloric deficit) or forced overfeeding (caloric surplus), neurotransmission is altered pre- and postsynaptically; concurrently, neuropeptide genes may be induced (e.g., NPY and its receptors after deprivation),[5] or suppressed (e.g., NPY after overfeeding induced by exogenous NPY).[6] Normal regulation of neuropeptide gene expression is essential for normal behavior and physiology, as revealed by the obesity of spontaneous mutants with altered peptides or peptide receptor function (e.g., ob/ob mice, which lack functional leptin, or db/db mice, which lack functional leptin receptors, show elevated NPY expression and hyperphagia). Because metabolic homeostasis requires continuous regulation at the level of neuropeptide transmission (on a time scale of minutes to hours), neuropeptide gene expression also requires continuous regulation (on a time scale of hours to days) to maintain appropriate neuropeptide levels.

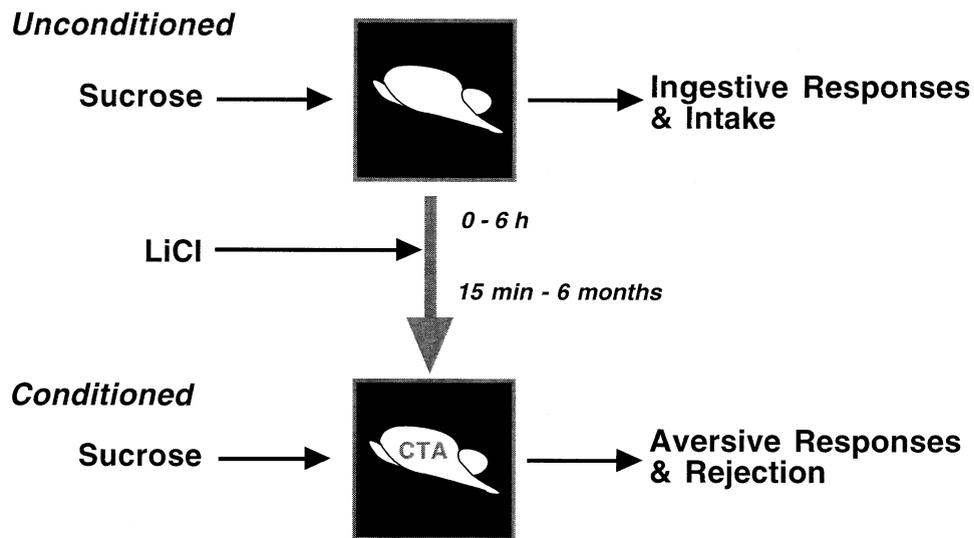The hypothalamic neuropeptides present several characteristics

FIG. 1. Schematic of the change in behavior response to the taste of a food before and after conditioned taste aversion (CTA) learning. Central processing of sweet taste results in ingestion prior to CTA acquisition. CTA acquisition persistently alters the central processing such that sweet taste now elicits a rejection response. A similar persistent change in behavioral response to taste or postingestive stimuli is seen in other ingestive behaviors with clear molecular mechanisms, for example, after sodium depletion or after estrogen treatment.

that play to the strength of molecular tools. Many of the neuropeptides are expressed exclusively in a small number of discrete cells either clustered (e.g., agouti-gene-related peptide [AGRP]-expressed only in NPYergic cells of the arcuate nucleus,[7] or scattered in the hypothalamus, e.g., orexinergic cells throughout the lateral hypothalamus).[8] Because they are in discrete cells, anatomical precision is required to examine gene expression; in situ hybridization and immunohistochemistry provide single-cell resolution of mRNA and protein expression, and peptidergic cells are an appropriate target for single-cell expression profiling. Because neuropeptides are themselves gene products, the presence and amount of the neurotransmitter can be monitored directly at the level of mRNA transcription and protein translation; this contrasts with the analysis, for example, of monoamine systems in which the expression of transmitter-processing genes such as tyrosine hydroxylase only indirectly suggests levels of monoamine synthesis.[9]

At the whole animal level, the neuropeptides have provided several models of how alterations in single-gene structure and function affect behavior. Obesity results after mutations in the promoter regions or coding regions of some peptides or their receptors. For example, the agouti-gene promoter is replaced with a different promoter that drives overexpression in the obese agouti mutant[10]; likewise, coding region mutations in either leptin[11] or its receptor[12] causes obesity, because leptin can no longer inhibit appetitive systems of the hypothalamus (i.e., NPY is overexpressed causing hyperphagia). Using polymerase chain reaction (PCR), mutants can now be genotyped before the emergence of an obvious physiological or behavioral phenotype, so that critical branch points in the development of the mutant obesity syndrome can be detected during ontogeny. Zucker rat pups with mutant leptin receptors do not become hyperphagic until postnatal day 12, and thus cannot be distinguished from lean siblings based on their ingestive behavior.[13] When the mutant pups were identified prior to hyperphagia by PCR detection of the genomic mutation, it was found that NPY mRNA overexpression occurred as early as postnatal day 2.[14] Finally, because neuropeptide genes are susceptible to direct genomic manipulation, and ingestion is a clear behavior for assessing the effects of mutation, several artificial transgenic mutants have been generated that manipulate neuropeptide gene expression in the hypothalamus and elsewhere (e.g., transgenic

knock-out of the NPY gene in ob/ob mutant mice attenuated the obesity caused by a lack of leptin).[15]

MODELS OF BEHAVIORAL PLASTICITY

In addition to maintaining homeostasis, animals can also adapt their behavior to long-term changes in the environment or their own physiological state. This behavioral plasticity typifies the second category of molecular models of ingestion. If the change in behavior is to persist (on a time scale of hours to months), inducible gene expression is required in addition to ongoing constitutive expression. The transient and tightly regulated inducible gene expression may, for example, mediate long-lasting changes in synaptic structure and function to alter behavioral responses. In these cases, the change in behavior can be correlated tightly to molecular events; indeed, the in vivo analysis of the behavioral time course and neural sites of plasticity focus the molecular analysis on particular cells at specific times.

There are several plastic models of ingestive behaviors that illustrate these principles: CTA, sodium appetite, and estrogen inhibition of ingestion (Fig. 1). The common features of these models are:

1. A radical, long-term change in the behavioral response to taste (CTA or sodium appetite) or postingestive sensory stimuli (estrogen). In CTA, animals learn to change their behavioral response to the taste or flavor of a food after they learn that the food is toxic, even if the food in innately palatable. This constitutes an adaptive change to a novel feature of environmental food sources. In sodium appetite, a change in internal state (e.g., hyponatremia leading to elevated angiotensin and aldosterone) changes the response of animals to the taste of high concentration sodium from rejection to acceptance, and sodium sources are avidly sought by the animal until the sodium deficit is reversed.[16] In many species, females decrease their food and water intake after another change in internal state: the increase in estrogen during ovarian cycles. Recent work has suggested that the estrogen-induced decrease in food intake is mediated by increased sensitivity to postingestive stimuli that induce satiety,[17] and thus the estrogen acts to decrease meal size.[18]

These changes in ingestive responses to taste and postingestive

stimuli can last for days to months after learning or steroid treatment. Radical changes that persist for days to months require restructuring of the underlying circuitry, and such restructuring requires alterations of synapses, changes in receptors or second messengers, or changes in transmitter levels—all of which require protein synthesis and hence gene expression.

2. A discrete time course during which the behavior undergoes a persistent change. The time course of the change in behavior response provides important clues as to the timing of underlying molecular events. Whereas initial observation may suggest only a sudden shift to a different behavior (e.g., rats acquire a CTA within minutes to hours after the pairing of taste and toxin), more detailed analysis may reveal discontinuities in the time course of the behavioral change. In particular, gene expression and protein synthesis are slow events that are completed only hours to days after intracellular signaling cascades are activated. Thus it is possible to dissect the time course of behavioral change into an immediate period that precedes protein synthesis (i.e., protein-synthesis independent) and a delayed period that persists because protein-synthetic events have induced long-term cellular changes.

Of course, "radical changes in behavioral response" are common to many instances of ingestion. A food-deprived rat will avidly consume a glucose solution, for example, but a sated rat will reject an intraoral infusion of glucose. This altered behavioral response to the taste of glucose, however, is transient, and decays within minutes to hours.[19] Thus the changes in behavior brought about by short-term satiety occur too rapidly, and disappear too soon, to be mediated by gene expression and protein synthesis.

3. Specific neural circuitry within the distributed peripheral-central network mediating ingestive behavior that is differentially activated before and after the change in behavior. The neural network mediating ingestive behavior is known in outline: orosensory and visceral afferents relay sensory stimuli of food to the nucleus of the solitary tract (NST) and other brain stem sites, which in turn interconnect with the pontine parabrachial nucleus, the thalamus, the hypothalamus, the amygdala, insular cortex, and so on. The response to food stimuli is mediated by the same network both before and after learning or steroid-induced changes. Because the behavioral outputs of the network are altered in these models, however, the neural activity of the network in response to ingestive stimuli (e.g., the taste of the toxic food, or the taste of salt in the sodium-depleted animal) must be altered. In fact, altered patterns of electrophysiological activity or c-Fos expression have been reported in all three models.[20–23]

Furthermore, these persistent changes probably require specific circuits optimized for associative or steroid-modulated changes. The plastic circuits may overlap with innate circuits, or they may be segregated anatomically. For example, studies in decerebrate rats revealed that innate responses to tastes (e.g., ingestion of sweet sucrose) could be mediated by just the pons and medulla. Decerebrate rats cannot learn or remember a CTA against sucrose, however, so forebrain circuits are necessary both to acquire the CTA and subsequently to reverse the response of the innate hindbrain circuits to the conditioned taste.[24]

4. The change in behavior requires gene expression. To be a model for molecular neurobiology, gene expression must be an essential mediator of the change in ingestive behavior. There is prima facia evidence that gene expression is essential in these models. Gene expression is implicated in many forms of learning and memory because long-term memory is attenuated or blocked when protein synthesis is inhibited[25]; this is true in CTA learning as well.[26] The link of aldosterone-induced sodium appetite and estrogen-inhibited ingestion with gene expression is even clearer, because the classical action of steroids is to directly bind cytoplasmic receptors that translocate to the cell nucleus to bind DNA and regulate gene expression.

The goal of molecular neurobiology in these models of plasticity is to define the cell-specific gene expression underlying the long-term changes in ingestive behavior. In particular, three questions must be answered:

1. Where do changes in the neural network occur? There are probably specific neurons or circuits of neurons that respond differentially to taste or postingestive stimuli before and after the change in behavior. These circuits are likely to be the sites where long-term plasticity mediated by gene expression occurs after learning or steroid treatment.
2. When do the changes in behavior occur? In particular, is there a short-term (protein-synthesis-independent) phase, and a long-term phase of behavioral change? The transition from short-term to long-term responses demarcates the critical period of gene expression.
3. What genes are expressed at the transition between short-term and long-term phases of behavioral response? It is likely that numerous genes (>1000) are necessarily expressed at the time of plasticity to induce neuronal restructuring. The expression of this multitude of genes, however, may be restricted to a very small number of critical neurons.

MOLECULAR NEUROBIOLOGY OF CTA LEARNING

Here we review our approach to answering these questions for CTA learning. CTA is a form of associate learning by which an animal rejects and avoids a food that has been previously paired with a toxic effect. CTA learning occurs after a single pairing of taste and toxin, and persistently changes the animal's behavioral response to the taste stimulus. For example, rats find sweet taste innately palatable. After the taste of sucrose is paired with a toxic injection of LiCl, rats reverse their response to sucrose from acceptance and ingestion to aversion and rejection. This radical change in behavioral response persists for months after only a small number of pairings.[27]

Changes in Neural Circuitry

Recently we and others have begun characterizing the changes in neural circuitry that accompany the persistent change in behavioral response after CTA learning. The approach we have taken is to define patterns of neuronal activity in the brain that correlate uniquely with the behavioral expression of a CTA, and thus reflect neuronal changes in response to taste that are dependent on contingent experience of taste and toxin. By defining sites of change in the networks governing ingestion, we hope to elucidate the sites at which restructuring after gene expression occurs at the time of learning.

The two key methodologies we have used are intraoral catheters to control the timing and extent of orosensory stimulation, and c-Fos-like immunohistochemistry (c-Fos) to track neuronal activity. The intraoral catheter allows an identical orosensory stimulus (i.e., 6.6 mL of 5% sucrose infused over 6 min) to be presented to the rat before and after conditioning, regardless of the behavioral response of the rat. The rat has control over consumption, because it may swallow the infusion (e.g., before CTA acquisition), passively reject the infusion by allowing it to drip from the mouth, or actively reject the infusion with vigorous mouth, head, and paw movements (e.g., during CTA expression). Unlike in single- or two-bottle intake tests, however, the rat that receives an intraoral infusion of sucrose receives a fixed amount or duration of orosensory stimulation. Thus, the intraoral catheter allows us to evaluate the change in response of the central neural networks to a standard stimulus before and after CTA acquisition.

Using intraoral catheters, rats were given a CTA by pairing a standard intraoral infusion of sucrose with a toxic LiCl injection.[22] Rats were implanted with intraoral catheters and received an intraoral infusion of 5% sucrose (6.6 mL over 6 min) followed by LiCl injection (0.15 M, 12 mL/kg intraperitoneally) 30 min later. Intake of the sucrose was measured by weighing rats immediately
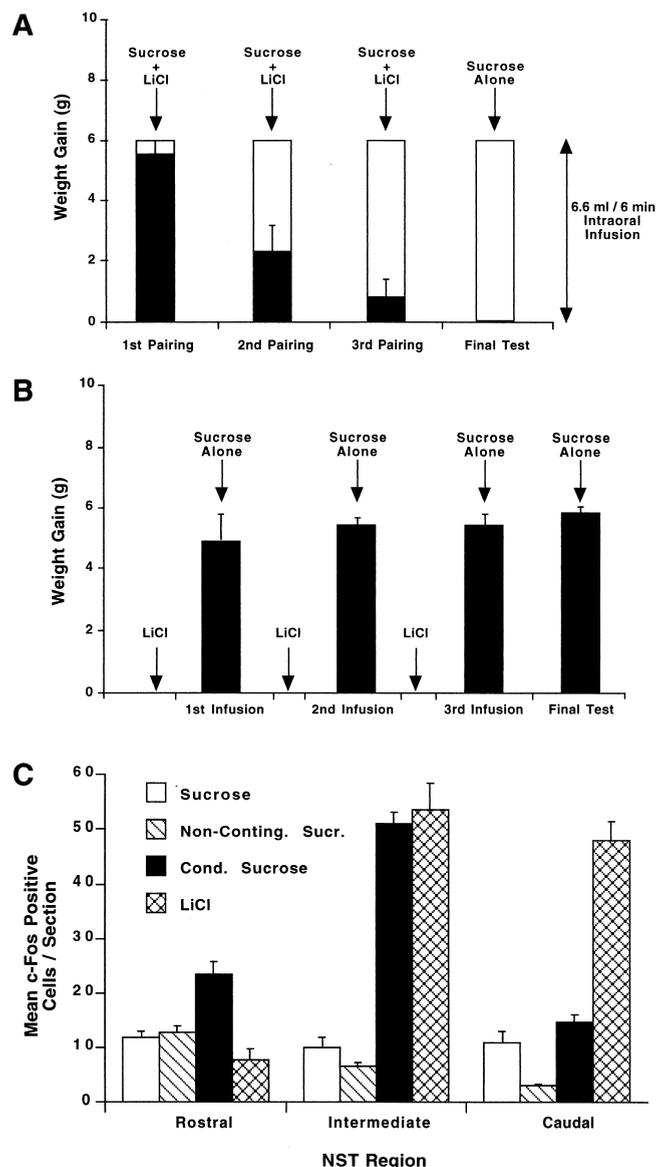
FIG. 2. (A) Change in intraoral intake of rats after pairing intraoral infusions of 5% sucrose with injections of 0.15 M LiCl (12 mL/kg). (B) Intraoral intake of rats after non-contingent experience of intraoral infusions of 5% sucrose separated by 24 h from injections of 0.15 M LiCl (12 mL/kg). (C) Quantification of c-Fos induction in three regions of the nucleus of the solitary tract (NST) induced by LiCl or by sucrose in unconditioned rats, after non-contingent pairing of sucrose and LiCl, or after contingent pairing of sucrose and LiCl. $n = 5$–8 in each group.

before and after the intraoral infusion. The conditioned rats received a total of three pairings of an intraoral infusion of sucrose and LiCl at 48-h intervals. Three days after the third pairing of sucrose and LiCl, rats received a final unpaired intraoral infusion of 5% sucrose. Because rats had received prior contingent experience of sucrose and toxic LiCl, they rejected all of the final intraoral infusion of sucrose (Fig. 2A). One hour after the intraoral infusion, the rats were perfused and the brain stems processed for c-Fos immunohistochemistry.

The control group received three intraoral infusions of sucrose at 48-h intervals, and three injections of LiCl, but sucrose and LiCl were administered non-contingently: LiCl injections were administered 24 h before each intraoral infusion of sucrose. Three days after the third intraoral infusion of sucrose and LiCl injection, rats

received a final unpaired intraoral infusion of 5% sucrose. Because rats had received only non-contingent experience of sucrose and toxic LiCl, they did not acquire a CTA against sucrose and thus consumed all of the final intraoral infusion of sucrose (Fig. 2B). One hour after the intraoral infusion, the non-contingent controls were perfused also and the brain stems processed for c-Fos immunohistochemistry. Additional controls included unconditioned rats perfused 1 h after their first intraoral infusion of sucrose, or rats perfused 1 h after LiCl injection.

In parallel with the change in behavioral response to the intraoral infusion of sucrose, we observed a marked change in the induction of c-Fos in the brain stem NST. Because c-Fos mRNA and protein are synthesized rapidly in response to transsynaptic activity in many cells of the brain, c-Fos can be used as a marker of neuronal responses to sensory stimuli. By visualizing the c-Fos expression on tissue sections using in situ hybridization (for mRNA) or immunohistochemistry (for protein), we can map out central relays and sites of integration and association after stimulation and behavioral responses. Compared with other activity-monitoring techniques such as electrophysiological recording, c-Fos analysis has a number of advantages. Using c-Fos allows simultaneous visualization of neuronal activity with cellular resolution in multiple sites across the brain. The cellular response is quantifiable by counting the number of c-Fos-positive nuclei or the density of the c-Fos in situ hybridization signal. The phenotype of the activated cells can be determined by double-labeling with other histochemical probes. (There are several important caveats to using c-Fos, however: first, not all cells express c-Fos after stimulation, so not all activity is revealed; second, a~1-h delay is required for c-Fos to be synthesized in the cells, so the temporal resolution is poor; and third, c-Fos histochemistry is a postmortem technique!)

An intraoral infusion of sucrose induced little or no c-Fos in the NTS in either naïve rats or rats with only non-contingent experience of sucrose and LiCl (Fig. 2A). In conditioned rats that rejected the intraoral infusion of sucrose, the sucrose induced c-Fos specifically in the medial, intermediate NST (iNST) abutting the fourth ventricle just rostral to the area postrema (AP) (Fig. 3).[22] The pattern of c-Fos induced by conditioned sucrose overlapped with (but was not identical to) the pattern of c-Fos induced by LiCl itself in the iNST and subpostremal NST. Thus, there was a population of cells (presumed to be neurons) within the iNST that was not activated by 5% sucrose unless the rat had acquired a CTA against sucrose. Concurrent with our studies, Swank and Bernstein[28] observed the same increased c-Fos expression in response to an intraoral infusion of saccharin in the iNST after saccharin was paired with LiCl.

A series of experiments was then undertaken to establish that the increased c-Fos induction in the iNST was correlated with the prior contingent experience of taste and toxin (i.e., the learning per se) and not secondary to a behavioral or physiological aspect of CTA expression. For example, the expression of a CTA against an intraoral infusion of sucrose is the very vigorous orofacial and somatic behaviors of rejection (e.g., gaping, headshakes, forearm flailing) the expel the sucrose from the mouth. Is the c-Fos induction in the iNST in response to conditioned sucrose secondary to these rejection behaviors? This hypothesis was tested easily using intraoral infusions of quinine, a bitter tastant that is innately unpalatable and actively rejected during intraoral infusions by unconditioned rats.[29]

We examined the induction of c-Fos in the iNST after an intraoral infusion of quinine sulfate (0.3 mM, 6.6 mL/6 min) in unconditioned rats and in rats that received non-contingent pairing of quinine and LiCl injection; in both these groups, the intraoral infusion of quinine failed to induce c-Fos above background levels despite complete and active behavioral rejection of the intraoral infusion.[30,31] In rats that had received three contingent pairings of an intraoral infusion of quinine with LiCl injection, however, an unpaired intraoral infusion of quinine induced c-Fos in the iNST[30];
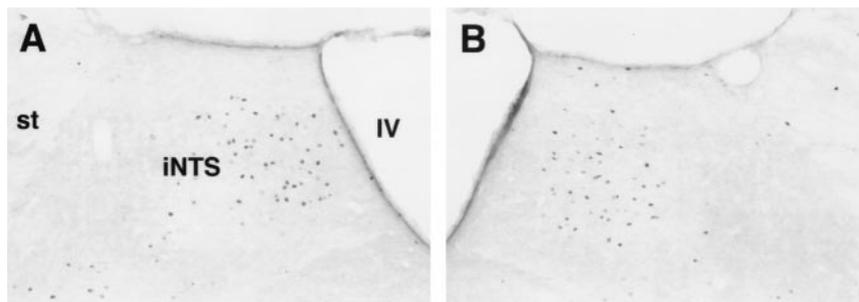
FIG. 3. Coronal photomicrograph of c-Fos immunoreactivity induced in the medial, intermediate nucleus of the solitary tract (iNST) by conditioned sucrose (A) or conditioned quinine (B) IV, fourth ventricle; st, solitary tract.

furthermore, the pattern of c-Fos induced by conditioned quinine was identical to the pattern of c-Fos induced by conditioned sucrose (Figs. 3 and 4). Thus, the increased c-Fos in the iNST was not correlated with rejection of an intraoral infusion per se, but with prior contingent experience of the taste and toxic consequences.

Additional evidence has accumulated that c-Fos induction in the iNST is a specific neuronal correlate of CTA expression. The increased c-Fos induced by a conditioned taste occurs after conditioning with LiCl, amphetamine,[31] or ethanol.[32] The altered c-Fos response requires orosensory conditioning[33] and does not correlate with a fear or stress response.[34] The activation of the iNST is not secondary to vagally mediated visceral responses during CTA expression (e.g., diarrhea), because subdiaphragmatic vagotomy does not attenuate the c-Fos response to conditioned sucrose.[35] The change in responsiveness of the iNST is persistent: an intraoral infusion of sucrose is rejected and induces c-Fos in the iNST 6 months after three pairings of sucrose and LiCl.[27] The conditioned c-Fos response can be extinguished, however, if the behavioral CTA is extinguished with repeated non-contingent intraoral infusions given to conditioned rats.[22,30] Lesions that block behavioral expression of a CTA also attenuate the c-Fos response in the iNST (description to follow).

As a result of these studies, c-Fos induction in the iNST stands as the best neuronal correlate of CTA acquisition and expression. Much research remains to be conducted on the identity, connectivity, and function of the c-Fos-expressing cells in the iNST. The anatomical site is suggestive: the NST is the first central relay of taste and visceral sensory input, and is adjacent to the chemoreceptive AP, which is thought to detect toxic Li in the blood.[36] The NST has many reciprocal connections with other brain regions implicated in CTA learning, such as the parabrachial nucleus, gustatory cortex, and amygdala.[37] The iNST in particular receives a dense input from the central nucleus of the amygdala.[38] Bernstein and colleagues[39–41] have demonstrated that decerebration at the midbrain,[39] or more specific forebrain lesions of the amygdala[40] and gustatory cortex,[41] block or attenuate the c-Fos response of the iNST to an intraoral infusion of conditioned saccharin.

These c-Fos studies have identified novel sites of plasticity (e.g., iNST) and confirmed the critical role of sites implicated previously by lesion studies (e.g., amygdala). Furthermore, an advantage of combining c-Fos with lesions is that neuronal activity can be assessed in other brain sites upstream or downstream of the lesion site. Thus, c-Fos studies can extend earlier lesion studies by mapping the functional dependencies among multiple nodes of the distributed network. More important for the molecular analysis,
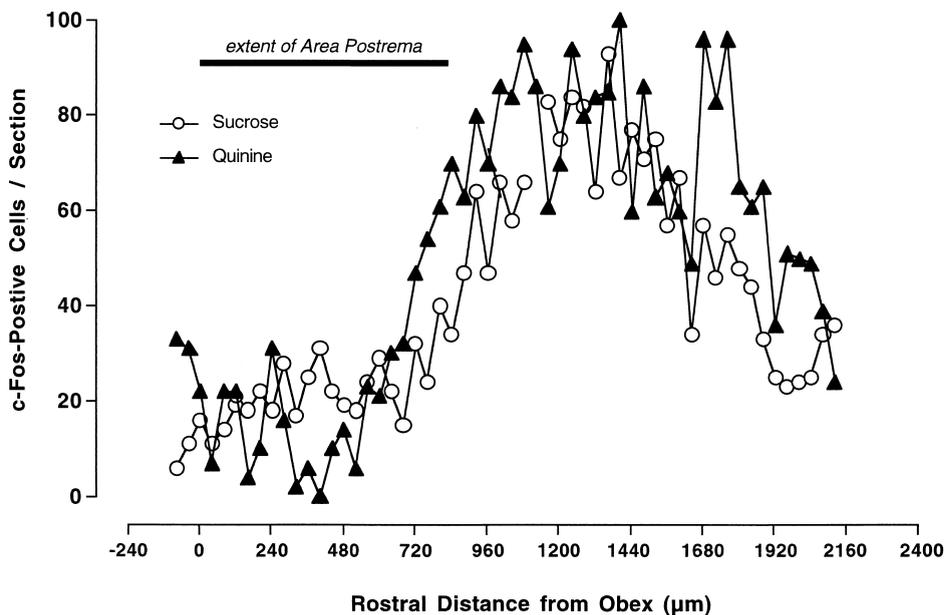

FIG. 4. Caudal-to-rostral distribution of c-Fos-positive cells within the nucleus of the solitary tract (NST) of consecutive coronal 40-μm sections induced by an intraoral infusion of sucrose or quinine in a sucrose- or quinine-conditioned rat. After pairing with LiCl, intraoral infusions of sucrose or quinine induced a nearly identical number and pattern of c-Fos-positive cells in the intermediate NST rostral to the area postrema.

the c-Fos-expressing cells in the iNST may be part of a specific forebrain–hindbrain circuit from amygdala and gustatory cortex to iNST that persistently changes its response to a taste stimulus after the taste has been paired with a toxin. Thus we may focus the molecular analysis on the cells and synapses of this circuit at the time of learning, much as molecular analysis of spatial learning has focused on plastic circuits within the hippocampus (i.e., mediating long-term potentiation), or the molecular analysis of reproductive behavior has focused on the estrogen-receptive cells of the ventromedial hypothalamus (i.e., modulating lordosis circuits in midbrain and spinal cord).

Timing of Behavioral Change

Functional anatomical studies can determine the neural sites of change that accompany a long-term change in behavior. In addition, the time course of the change in behavior must be defined, so that the critical time of protein synthesis and gene expression within the neural network can be pinpointed. In recent models of learning and memory, short-term and long-term phases of memory have been distinguished operationally by the contribution of gene expression.[42] Learning, for example, classical conditioning, involves a change in behavioral response to the conditioned stimulus. This change often occurs immediately after learning, and thus there is an immediate short-term memory that may not persist for more than minutes or hours. Because it is rapid and labile, the short-term memory does not involve gene expression and protein synthesis: there is not enough time for gene expression to occur, and no permanent restructuring of neural circuits occurs in the short term. If consolidation occurs, however, the memory may persist for days or months; this is the long-term phase of memory. Persistent changes are hypothesized to require long-lasting structural changes in neural circuits, and hence require protein synthesis. The timing of the transition from rapid, labile, and protein-synthesis-independent short-term memory to persistent, protein-synthesis-dependent long-term memory brackets the critical period of behavioral consolidation and underlying gene expression.

Thus, memory consolidation provides a useful heuristic for the analysis of gene expression mediating persistent changes in behavior. Importantly, it is the behavioral analysis that reveals and defines the timing of the critical underlying molecular events. Because CTA is a form of associative learning, the concept of consolidation may be easily applied to determine when gene expression occurs after the pairing of taste and toxin. However, most work on CTA has focused on its unique long-term temporal properties: CTA learning tolerates a long delay between taste and toxin (up to 12 h),[43] it uses toxins that can linger in the body long after the pairing with taste (e.g., Li), and it persists for months after only a few pairings.[27] As a result, there have been few studies of CTA memory in the short-term (minutes to hours) after the pairing of taste and toxin.

Recently, we have formally demonstrated a short-term phase of CTA memory that is rapid, labile, and protein-synthesis independent.[26] Rats implanted with intraoral catheters received an intraoral infusion of 5% sucrose; 30 min later they were injected with LiCl (0.15 M, 12 mL/kg). Using the intraoral catheter, the orosensory stimulus could be readministered at arbitrary times after the pairing. Rats received an intraoral infusion of sucrose at 15 min, 1 h, 6 h, or 48 h after the LiCl injection. At all time points, rats rejected almost all the sucrose. The rapid rejection of sucrose at 15 min or 1 h after the LiCl injection was not due to the toxic effects of the LiCl, because rats consumed almost all of an intraoral infusion of sucrose 15 min or 1 h after a non-contingent injection of LiCl.

Furthermore, this rapid short-term CTA is labile and quickly forgotten at lower doses of LiCl. When rats received intraoral infusions of sucrose paired with LiCl at three doses (76, 38, and 19 mg/kg), they all rejected subsequent intraoral infusions of sucrose
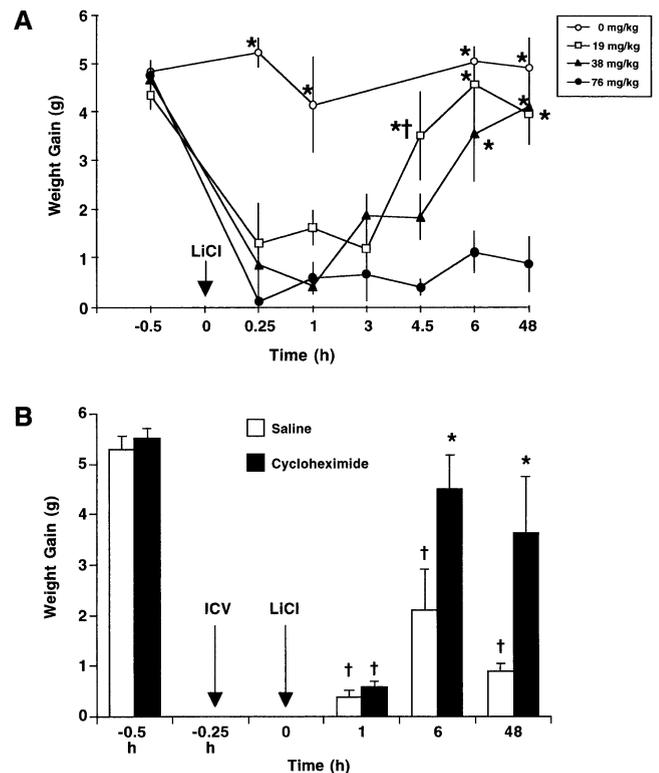

FIG. 5. (A) Dose-dependent expression of a labile short-term conditioned taste aversion (CTA). Intraoral intake of 5% sucrose after contingent pairing of an intraoral infusion of 5% sucrose (6.6 mL over 6 min) with different doses of LiCl (19, 38, or 76 mg/kg) or 0.15 M NaCl (12 mL/kg). Individual rats were tested only once at one time point ($n = 4$–9). $*P < 0.005$ versus intake after 76 mg/kg, $†P < 0.05$ versus intake after 38 mg/kg. (B) Protein synthesis-independent and -dependent expression of CTA. Rats received an intraoral infusion of 5% sucrose, followed 15 min later by an intracerebroventricular injection of 0.15 M NaCl (10 μL; open bars) or cycloheximide (250 μg/10 μL; black bars); 15 min after the intracerebroventricular injection, all rats received LiCl (76 mg/kg, intraperitoneally). $*P < 0.05$ versus NaCl-treated rats, $†P < 0.05$ versus sucrose intake during pairing.

at 15 min, 1 h, and 3 h after the LiCl injections. Beginning around 4.5 h after the LiCl injection, however, rats that had received sucrose paired with the lower doses of LiCl began to consume intraoral infusions of sucrose again. Thus, rats conditioned with sucrose paired with 38 or 19 mg/kg LiCl did not express a CTA by 6 h after the pairing (Fig. 5A). This finding suggested that at low doses of LiCl, rats express a short-term CTA that does not consolidate to a long-term, persistent CTA.

Finally, the short-term expression of a CTA is not blocked by a protein synthesis inhibitor, whereas the long-term CTA appears to require protein synthesis. Rats implanted with intraoral catheters and intracerebroventricular cannulas received an intraoral infusion of 5% sucrose; 15 min later they were injected with the protein synthesis inhibitor cycloheximide (250 μg intracerebroventricularly) or saline; 15 min later they were injected with LiCl (0.15 M, 12 mL/kg). When given an intraoral infusion of sucrose 1 h after the LiCl, both cycloheximide- and saline-treated rats rejected almost all the sucrose; thus both groups expressed a short-term CTA. When given an intraoral infusion of sucrose 6 or 48 h after the LiCl, only the saline-treated rats rejected the sucrose; cycloheximide-treated rats consumed most of the sucrose, and thus did not express a long-term CTA (Fig. 5B).

These results are consistent with earlier reports of rapid CTA expression using aversive taste reactivity to track the behavioral

response after the pairing of sucrose with LiCl,[44,45] and with several reports that long-term CTA expression can be blocked with ventricular or site-specific protein synthesis inhibitors.[46–48] Our experiments have bracketed the time of consolidation from the rapid short-term phase of CTA to the protein-synthesis-dependent long-term phase to sometime within the first 6 h after the pairing of taste and toxin. This 6-h span is the target time period in which to examine gene expression that modulates the long-term changes in behavior and neural circuitry.

Expression of Multiple Genes

Having identified potential sites of neuronal plasticity in CTA learning (c-Fos-positive cells), and the period of time when gene expression is necessary for CTA consolidation (within 6 h of acquisition), the tools of molecular biology must then be applied to determine what genes are expressed, and whether their expression is necessary and sufficient for the long-term change in ingestive behavior.

Evidence suggests that specific nodes in the neural network are activated at the transcriptional level during CTA acquisition. Following intraperitoneal injection of LiCl, for example, c-Fos and other immediate-early genes are expressed in the hypothalamus, amygdala, lateral parabrachial nucleus, and NTS.[49,50] c-Fos is itself a transcription factor that regulates other target genes by dimerizing with AP-1 family members and binding the AP-1 promoter element.[51] Thus, c-Fos expression at the time of CTA acquisition is not only a marker of activity, but c-Fos may also serve a functional role in CTA learning. Indeed, a necessary role for c-Fos gene expression is supported by several antisense studies, in which the administration of c-Fos antisense (or antisense to CREB, an upstream inducer of c-Fos expression) into the amygdala[52,53] or fourth ventricle[54] attenuated c-Fos protein expression and long-term CTA learning.

Although c-Fos expression may be necessary for long-term CTA expression, it is clearly not sufficient. c-Fos protein is synthesized in visceral and associative sites of the brain after LiCl injection, but the rat only learns a CTA if the LiCl has been paired with a taste stimulus. The induction of c-Fos by LiCl identifies a candidate set of neurons that may be involved in CTA learning, but additional gene expression required for learning must be present in these cells after contingent taste and toxin pairing. In fact, the number of required, additional genes may be vast, including a complex network of transcription factors (e.g., Fos-family members, Jun family members, and others) and a variety of potential target genes mediating structural changes (e.g., synaptic proteins and neurotransmission genes).

Furthermore, the expression of multiple genes is likely to occur in only a small subset of cells within any brain region. For example, LiCl induces c-Fos in less than 5% of the neurons within the subpostremal NST. Thus, the ultimate challenge is to describe the expression of thousands of genes within single cells of the network.

Two recent techniques of molecular biology have the potential to provide cellular resolution while monitoring expression of the entire genome: aRNA amplification from single cells, and gene expression profiling with cDNA microarrays (Fig. 6). Multiple genes can be amplified from small pieces of brain tissue, or even single cells, using the antisense RNA (aRNA) amplification protocol developed by Eberwine and colleagues.[55] As in reverse transcription (RT)-PCR, mRNA is reverse-transcribed into cDNA in the first step of aRNA amplification. In PCR amplification, particular species of cDNA are then amplified using short DNA primers that are specific to a limited number of genes. In contrast, aRNA amplification non-specifically amplifies all cDNA species by attaching a T7 RNA polymerase promoter to the 5′ poly-dT end of the cDNAs. By attaching a promoter to one end of every cDNA, the aRNA protocol in effect makes every cDNA into an artificial
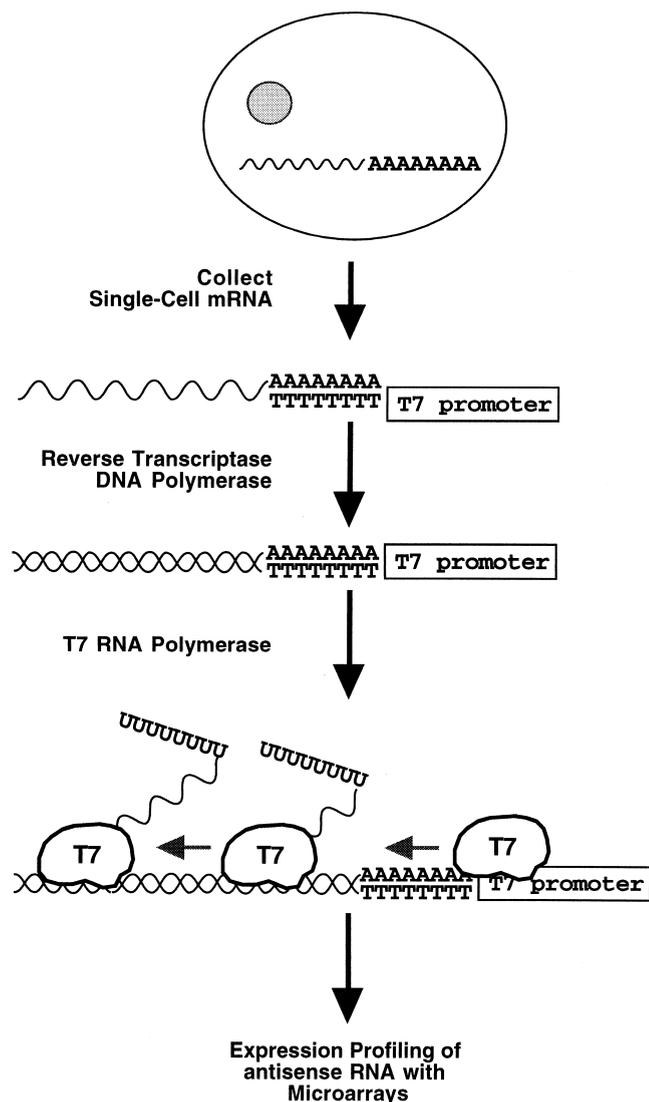

FIG. 6. Schematic of the antisense RNA (aRNA) amplification procedure and probing with cDNA microarrays. By attaching a T7 promoter sequence to the polyA tail of mRNA extracted from single cells, T7 polymerase can generate thousands of copies of the mRNA sequence. The copies are antisense to the original mRNA, however (note the polyU head on the antisense RNA, as opposed to the polyA tail on the original mRNA). If the antisense RNA is generated with labeled nucleotides, the aRNA can be hybridized against DNA microarrays to identify gene expression.

minigene, consisting of a promoter and a coding region: when the T7 RNA polymerase enzyme is added to the test tube, it transcribes RNA from each of the artificial minigenes, just as mammalian RNA polymerase transcribes RNA from endogenous genes. Because the promoter is put on the tail-end of the cDNA, the minigene is backwards (oriented with the coding region upstream of the promoter) compared with the endogenous gene (oriented with the promoter upstream of coding region)—hence the amplified RNA is antisense to endogenous mRNA.

The aRNA transcription is the amplification step, because the RNA polymerase makes thousands of copies of RNA from each of the cDNA minigenes. Furthermore, all the cDNAs recovered from the tissue or single cell are amplified, because a T7 promoter has been added to the end of every cDNA. The aRNA technique has been estimated to amplify at least 50% of all species of mRNA from fresh cells, and at least 30% of all species of mRNA from

fixed, histochemically processed tissue. Because mammalian neurons may express ~90,000 genes, even 30% of all genes is a very large number. If the aRNA amplification is repeated twice on the same sample, million-fold amplification can be achieved (with less error than after PCR, because the error propagates through only two rounds of aRNA amplification, rather than 30 cycles of PCR). The aRNA amplification has been applied to single cultured cells,[55] and even subcellular compartments such as dendrites.[56] It has also been applied to single cells identified by immunohistochemistry in fixed tissue sections.[57] Thus it may be possible to amplify multiple genes from single cells identified in feeding-related areas of the brain, for example, by c-Fos immunohistochemistry.

Complementary to the ability to amplify multiple genes from single cells is the use of cDNA microarrays to screen for the expression of specific genes. As the sequencing of the genomes of humans and other species is completed, all genes will become known: the focus of molecular neurobiology will then shift from the discovery of genes to the identification of the particular genes that are expressed in particular cells at critical times. If the genotype of the cell is defined as the specific DNA code of all the genes within the nucleus of the cell, the molecular phenotype of the cell can be defined as the combination of the mRNAs (representing a subset of the total genotype) being expressed by the cell. This is called the "gene expression profile" of the cell.

Expression profiling can now be accomplished for sets of genes ranging from dozens to thousands, using gene arrays.[58] Sequence-specific cDNAs or shorter DNA oligomers are arrayed onto nylon membranes or glass slides; using robotic "microspotters," dots of DNA can be arrayed at densities of >10 000 genes on a single glass microscope slide. The total pool of mRNA species is then extracted from a tissue, converted to labeled cDNA, and incubated with the DNA microarray. If a particular gene is expressed in the tissue, then the cDNA of that gene from the tissue will hybridize with the cDNA immobilized on the microarray. By labeling the mRNA (e.g., with fluorescent or radioactive tags), the hybridized dot of cDNA on the microarray can be detected. Thus, the set of hybridized cDNAs represents the set of genes transcribed into mRNA by the tissue. Because a significant fraction of the mammalian genome can be fit onto one microarray, a nearly complete gene expression profile can be established very rapidly.

Gene expression profiling, along with automated sequencing, is one of the most significant spin-offs of the large-scale genome projects revolutionizing molecular neurobiology. Although microarrays are currently expensive (ranging in price from hundreds to thousands of dollars per replicate), there is no doubt that the price will fall; within a decade, microarray technology will be as accessible to the individual investigator as PCR is now. As in the early days of PCR, there are significant issues that need to be resolved of sensitivity, quantification, and the analysis of thousands of data points across different cells and treatments. Nonetheless, gene expression profiling is already being applied to ingestive models: Kisley et al.,[59] for example, analyzed changes in the expression of multiple receptors in single hypothalamus after estrogen treatment using aRNA amplification and small gene arrays. Also recently, the expression profile of genes in skeletal muscle after prolonged food restriction was visualized with a 6000 gene array.[60] More than 100 genes underwent at least a two-fold change in expression level with aging; 30 months of 76% food restriction attenuated the change in expression of more than 60% of these genes.[60] The role of these genes in aging is largely unknown.

CONCLUSIONS

The scheme presented for the analysis of CTA as a model of molecular neurobiology of ingestive behavior can be comfortably

mapped onto the models of sodium depletion and estrogen reduction of intake. In sodium depletion, a forebrain–hindbrain circuit has been outlined from subfornical organ, hypothalamus, and amygdala to parabrachial nucleus and NST[61,62]; this circuit integrates peripheral depletion signals (aldosterone, angiotensin detected in the anterior hypothalamus) and modulates gustatory function (enhanced preference for sodium). In the estrogen model, the circuitry is less clear. The enhanced behavioral sensitivity of the estrogen-treated rat to peripheral satiety signals within a meal (e.g., food, CCK, and bombesinlike peptides) suggests modulation of the sensitivity of the visceral neuraxis[63]; the localization of estrogen receptors by in situ hybridization or immunohistochemistry within the visceral neuraxis will provide candidate sites.

Both sodium depletion and estrogen treatment require steroid actions to cause long-term changes in behavior. Because steroids (including aldosterone and estrogen) typically have both short-term, non-genomic effects and long-term, genomic effects,[64] the behavioral effects of steroids can be dissected into short-term and long-term phases. An inherent advantage of the steroid models in the analysis of gene expression is the direct transcriptional regulation of target genes by the steroid receptors. For example, rather than generic protein synthesis inhibitors, steroid-induced gene expression can be modulated with specific receptor antagonists[65] or receptor antisense[66]; with these treatments, only steroid-induced protein synthesis will be compromised.

As with CTA, the biggest challenge will be to enumerate the multitude of genes that must be expressed after sodium depletion or estrogen treatment in a small number of cells that mediate changes in ingestive behavior. The paradigms of stress and reproduction provide precedents for the analysis of steroid-induced structural changes and gene expression in neurons. In the case of estrogen, changes in neural and synaptic architecture have been described in the hippocampus and hypothalamus.[67,68] Furthermore, the transcriptional effects of estrogen on some genes have been well characterized (e.g., progesterone receptor and enkephalin),[69] although the induction of many more genes remains to be analyzed.

SUMMARY

Ingestive behavior, like other complex behaviors, allows the animal to maintain homeostasis over short-term fluctuations in the environment, or to make more radical, long-term adaptations in response to changes in the environment (e.g., diet toxicity) or the animal's own requirements (e.g., for reproduction). Because feeding has an essential role in the life of animals, the physiological substrate for feeding has evolved features that are advantageous for the application of molecular biology: a discrete set of sensory inputs, a robust central neural network, and an extended time span of behavioral and metabolic actions. Not only can the analysis of ingestion be advanced using the tools of molecular biology, but ingestion in turn provides informative models for understanding how specific gene expression mediates complex behaviors.

ACKNOWLEDGMENTS

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