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SELECTIVE FOS INDUCTION IN HYPOTHALAMIC OREXIN/HYPOCRETIN, BUT NOT MELANIN-CONCENTRATING HORMONE NEURONS, BY A LEARNED FOOD-CUE THAT STIMULATES FEEDING IN SATED RATS

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Abstract—Associative learning can enable cues from the environment to stimulate feeding in the absence of physiological hunger. How learned cues are integrated with the homeostatic regulatory system is unknown. Here we examined whether the underlying mechanism involves the hypothalamic orexigenic neuropeptide regulators orexin/hypocretin (ORX) and melanin-concentrating hormone (MCH). We used a Pavlovian conditioning procedure to train food-restricted rats to associate a discrete cue, a tone, with food pellets distinct from their regular lab chow diet. Rats in the conditioned group (Paired) received presentations of a tone immediately prior to food delivery, while the rats in the control group (Unpaired) received random presentations of the same number of tones and food pellets. After conditioning rats were allowed *ad libitum* access to lab chow for at least 10 days before testing. At test sated rats were presented with the tones in their home cages, and then one group was allowed to consume food pellets, while another group was left undisturbed until sacrifice for Fos induction analysis. The tone cue stimulated food consumption in this setting; rats in the Paired group consumed larger amounts of food pellets than rats in the Unpaired group. To examine Fos induction we processed the brain tissue using fluorescent immunohistochemistry methods for combined detection of Fos and characterization of ORX and MCH neurons. We found a greater percentage of ORX and Fos double-labeled neurons in the Paired compared to the Unpaired condition, specifically in the perifornical area. In contrast, there were very few MCH neurons with Fos induction in both the Paired and Unpaired conditions. Thus, the food-cue selectively induced Fos in ORX but not in MCH neurons. These results suggest a role for ORX in cue-induced feeding that occurs in the absence of physiological hunger.

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Abbreviations: ANOVA, analysis of variance; CR, conditioned response; CS, conditioned stimulus; DAPI, 4′,6-diamidino-2-phenylindole; IEG, immediate early gene; KPBS, potassium phosphate-buffered saline; MCH, melanin-concentrating hormone; MCH-1R, MCH receptor1; NPY, neuropeptide Y; ORX, orexin/hypocretin; US, unconditioned stimulus.

Key words: appetite, conditioning, non-homeostatic feeding, motivation, obesity, orexin.

INTRODUCTION

Learning plays an important role in the control of food consumption. Through associative learning, arbitrary cues from the environment can become signals for food (Pavlov, 1927). Food signals, and associated anticipatory mechanisms, function to prepare an organism for the signaled meal and as such aid in the physiological control of eating (e.g., Woods, 1991; Berridge, 2004). Learned food-cues also acquire an ability to stimulate eating in the absence of physiological hunger in animals and humans (e.g., Weingarten, 1983; Birch et al., 1989, for review see Petrovich, 2011).

This ability of food-cues to stimulate eating on demand, in conditions where calories and nutrients are available, may have been adaptive when food availability was uncertain. However, it is becoming maladaptive in contemporary environments that are rich in readily available, palatable, high-calorie foods. In our world, food-cues, which are plentiful in the form of food advertisements and other food reminders, are relentless appetite stimulants that ultimately encourage overeating and contribute to obesity (for reviews see Hill et al., 2003; Volkow and Wise, 2005; Small, 2009; Berthoud, 2011). Nevertheless, how learned food-cues are integrated with physiological signals to control food intake is currently unknown.

To begin to delineate the critical brain systems involved in cue-driven feeding, here we examined whether the underlying mechanism involves recruitment of lateral hypothalamic neurons that express orexigenic neuropeptide regulators. Prior evidence showed that the lateral hypothalamus is a necessary node in the cue-induced feeding network (Petrovich et al., 2002; for review see Petrovich, 2011); however, specific neuronal mediators are unknown. Two orexigenic neuropeptides are expressed in the lateral hypothalamus: orexin/hypocretin (ORX) and melanin-concentrating hormone (MCH) (Nahon et al., 1989; Broberger et al., 1998; Elias et al., 1998; Sakurai et al., 1998; Swanson et al., 2005; Morton et al., 2006; Hahn, 2010). Interestingly, ORX and MCH are expressed in separate neuronal populations (Broberger et al., 1998; Elias et al., 1998; Peyron et al., 1998; Swanson et al., 2005; Hahn,

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2010), and their functions in the control of food intake are also distinct.

Converging evidence has established a critical regulatory function for MCH in energy homeostasis (e.g., Qu et al., 1996; Shimada et al., 1998; Ludwig et al., 2001), and as such MCH would be an effective substrate for cue-induced feeding. In contrast, ORX's role in feeding is more complex. ORX stimulates feeding (e.g., Sakurai et al., 1998; Rodgers, 2000; Clegg et al., 2002), but it is also important for other motivated behaviors driven by food and drug rewards (e.g., Harris et al., 2005; Thorpe et al., 2005; Nair et al., 2008; Borgland et al., 2009; Choi et al., 2010; Sharf et al., 2010a; for reviews see Cason et al., 2010; Sharf et al., 2010b), and it is critical for wakefulness and arousal (e.g., de Lecea et al., 1998; Chemelli et al., 1999; Mieda et al., 2004; Berridge et al., 2010; Boutrel et al., 2010). These diverse functions have been conceptualized to reflect the role of ORX in coordinating the current motivational state with adaptive physiological and behavioral responses (e.g., Yamanaka et al., 2003; for reviews see Willie et al., 2001; Saper, 2006; Tsujino and Sakurai, 2009), a role which is supported by its widespread connectional network and extensive receptor distribution (e.g., Peyron et al., 1998; Marcus et al., 2001; Baldo et al., 2003; Yoshida et al., 2006). There is also evidence that distinct ORX subsystems might mediate arousal and reward-seeking functions (e.g., Harris and Aston-Jones, 2006). As such, ORX might be an important motivational and appetite substrate for cue-driven feeding.

Thus, here we examined whether a learned food-cue functionally activates ORX- or MCH-producing neurons. We identified immediate early gene (IEG) *c-fos* protein product (Fos) induction in ORX- and MCH-identified neurons using a fluorescent double-label immunohistochemistry method. We used our recently developed behavioral preparation, which allowed us to map food-cue Fos induction independent of the training context, and in the absence of food (Reppucci and Petrovich, 2012a). This is important because the training context, as well as food presentation and consumption, could stimulate Fos induction in the brain areas of interest.

In brief, here we trained rats to associate a discrete cue, a tone (conditioned stimulus, CS) with food pellets (unconditioned stimulus, US) distinct from their regular chow diet. Then during testing sated rats were given tone (CS) presentations in their home cages. Following CSs, one group of rats was left undisturbed until sacrifice to examine cue-induced Fos expression in ORX and MCH neurons (brain analysis group), while another group of rats was given access to food pellets to assess the cue's effects on feeding (food consumption group).

EXPERIMENTAL PROCEDURES

Subjects

Forty experimentally naïve, male Long–Evans rats approximately 2 months of age (Charles River Laboratories; Raleigh, NC, USA), were individually housed and maintained on a 12-h light/dark cycle (lights on at 6:00). Upon arrival, subjects were allowed 1 week to acclimate to the colony room, during which time they had *ad libitum* access to standard laboratory chow and water and were handled daily. All housing and testing procedures were in compliance with the National Institutes of Health *Guidelines for Care and Use of Laboratory Animals*, and approved by the John Hopkins University and Boston College Institutional Animal Care and Use Committees.

Apparatus

The behavioral training was conducted in two sets of behavioral chambers (Set 1: $30 \times 24 \times 30$ cm, Set 2: $30 \times 28 \times 30$ cm; Coulbourn Instruments, Allentown, PA, USA) located in rooms that were different from the colony housing rooms, and different from the rooms used for testing (see Behavioral procedure section). All chambers had aluminum tops and sides, a transparent Plexiglas back and front, a recessed receptacle for food ("food cup", 3.2×4.2 cm), and grid floors. One set of chambers had a black Plexiglas panel placed on top of the grid floor, and were enclosed in isolation cubicles ($79 \times 53 \times 53$ cm; Coulbourn Instruments, Allentown, PA, USA) composed of monolithic rigid foam walls, which isolate chambers from ambient sound and light. All chambers were dimly illuminated, and a ventilation fan provided masking noise (55–60 dB) during training sessions. Stimulus presentation was controlled by software (LabView: National Instruments, Austin, TX, USA or GraphicState 3.0: Coulbourn Instruments, Allentown, PA, USA). Video cameras recorded behavior during training.

Behavioral procedure

Experimental design is outlined in Fig. 1. Before behavioral training, rats were gradually reduced to 85% of their *ad libitum* weight. Additionally, prior to the start of the conditioning protocol all rats received one magazine training session in which they learned to eat from the food cup. During this session rats received 16 trials of food pellet delivery (45-mg pellets, formula 5TUL; Test Diets, Richmond, IN, USA); no other stimuli were presented. After the magazine session, rats received 10 training sessions (one session per day, excluding weekends) each approximately 32 min in length. For half of the rats (conditioned group, Paired), these sessions each consisted of eight presentations of the CS, a 10-s tone (1.5–2 kHz, 75 dB), immediately followed by delivery of the US, two food pellets, into the food cup. For the other half of the rats (control group, Unpaired), the sessions consisted of the same number of tone and food presentations as the Paired group, but delivered in a non-conditional random order. After the last training session, rats had *ad libitum* access to standard laboratory chow for 10–16 days to allow rats to reach at least 110% of their pre-training body weight. During this time, rats were habituated to a new testing room on two occasions. For the first habituation rats were brought to the testing room and left undisturbed in their home cages for 15 min. The second habituation was the same except that lab chow was removed immediately prior to transporting the rats to the testing room to acclimate them to the food removal that would occur prior to testing. Additionally, a group of rats that would be given food after testing was habituated to the glass dish ($107 \times 87 \times 70$ mm) that would be used for food pellet presentation. For these rats, on three occasions that were separate from testing room habituation, the empty glass dishes were left for one hour in the rats' home cages in the colony room.

On test day rats were transported to the testing room, and all food was removed from the cage just prior to transport. During the test, rats remained in their home cages and were given 10 presentations of the CS (10-s tone) over 5 min. After the test one group of rats ($n = 24$; brain analysis group) was