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Orexin in Rostral Hotspot of Nucleus Accumbens Enhances Sucrose 'Liking' and Intake but Scopolamine in Caudal Shell Shifts 'Liking' Toward 'Disgust' and 'Fear'

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The nucleus accumbens (NAc) contains a hedonic hotspot in the rostral half of medial shell, where opioid agonist microinjections are known to enhance positive hedonic orofacial reactions to the taste of sucrose ('liking' reactions). Within NAc shell, orexin/hypocretin also has been reported to stimulate food intake and is implicated in reward, whereas blockade of muscarinic acetylcholine receptors by scopolamine suppresses intake and may have anti-reward effects. Here, we show that NAc microinjection of orexin-A in medial shell amplifies the hedonic impact of sucrose taste, but only within the same anatomically rostral site, identical to the opioid hotspot. By comparison, at all sites throughout medial shell, orexin microinjections stimulated 'wanting' to eat, as reflected by increases in intake of palatable sweet chocolates. At NAc shell sites outside the hotspot, orexin selectively enhanced 'wanting' to eat without enhancing sweetness 'liking' reactions. In contrast, microinjections of the antagonist scopolamine at all sites in NAc shell suppressed sucrose 'liking' reactions as well as suppressing intake of palatable food. Conversely, scopolamine increased aversive 'disgust' reactions elicited by bitter quinine at all NAc shell sites. Finally, scopolamine microinjections localized to the caudal half of medial shell additionally generated a fear-related anti-predator reaction of defensive treading and burying directed toward the corners of the transparent chamber. Together, these results confirm a rostral hotspot in NAc medial shell as a unique site for orexin induction of hedonic 'liking' enhancement, similar to opioid enhancement. They also reveal distinct roles for orexin and acetylcholine signals in NAc shell for hedonic reactions and motivated behaviors.

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INTRODUCTION

Pleasure 'liking', or hedonic impact, is a fundamental aspect of sensory reward, and pathological dysfunction of hedonic brain circuitry may contribute to addiction, mood disorders, eating disorders, and obesity. To better understand and map the neural mechanisms underlying hedonic impact, several affective neuroscience studies have used the taste reactivity test to measure orofacial 'liking' reactions to sweet tastes (Berridge and Kringelbach, 2015). These affective facial expressions to taste are homologous in human infants, non-human primates, and even rodents (Berridge, 2000; Steiner *et al*, 2001), and hedonic brain mechanisms can be mapped by their ability to cause changes in such 'liking' reactions.

One example of this hedonic localization involves the nucleus accumbens (NAc), which contains a roughly cubic millimeter-sized 'hedonic hotspot' in the rostral half of medial shell in rats. In that rostral hotspot of NAc shell opioid agonist microinjections can double or triple the number of positive hedonic orofacial reactions (ie, 'liking' reactions) elicited by the taste of sucrose (Castro and Berridge, 2014; Pecina and Berridge, 2005). Conversely, in caudal shell, agonist microinjections reveal a 'hedonic coldspot', where opioid stimulation suppresses sucrose hedonic impact (Castro and Berridge, 2014; Pecina and Berridge, 2005). By contrast to the localized hotspot for sweetness 'liking', mu-opioid stimulations increase motivation to eat much more widely and homogeneously throughout the entire NAc shell (and in related structures), measured as increases in cue-triggered 'wanting' to obtain food rewards (eg, in instrumental breakpoint and pavlovian-instrumental transfer tests), as well as in food consumption (Castro and Berridge, 2014; Covelo *et al*, 2014; Maldonado-Irizarry *et al*, 1995; Pecina and Berridge, 2005; Pecina and Berridge, 2013; Smith and Berridge, 2005; Smith *et al*, 2011; Zhang and Kelley, 2000).

Other neurotransmitter systems in NAc also modulate food consumption, motivation, and hedonic impact of food rewards including, endocannabinoids, and amino acids (Maldonado-Irizarry *et al*, 1995; Shinohara *et al*, 2009; Soria-Gomez *et al*, 2007), some of which might interact with opioid signals in NAc shell (Faure *et al*, 2010; Mahler *et al*, 2007). Here we extended our analyses to orexin and acetylcholine (ACh) systems in NAc that modulate intake

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and food-motivated behaviors (Pratt and Kelley, 2004; Pratt *et al*, 2007; Thorpe and Kotz, 2005).

Orexin-A (hypocretin) is a hypothalamic peptide implicated in reward (Barson *et al*, 2015; Berthoud and Munzberg, 2011; Harris *et al*, 2005; Sharf *et al*, 2010), as well as in arousal (Espana *et al*, 2003; Rolls *et al*, 2011; Sutcliffe and de Lecea, 2002). Orexin can also amplify hedonic 'liking' reactions to sucrose taste, comparably to mu-opioid stimulation, if microinjected into another opioid hedonic hotspot located in posterior ventral pallidum (Ho and Berridge, 2013; Smith and Berridge, 2005). Anatomically, hypothalamic orexin neurons send projections throughout the brain, including to NAc shell (but not core) (Baldo *et al*, 2003; Peyron *et al*, 1998). This projection pattern raises the question of whether orexin might also enhance hedonic reactions within the NAc-opioid hotspot, similarly to the ventral pallidum hotspot. Orexin-induced increases in food intake can be prevented by opioid blockade (Sweet *et al*, 2004), and orexin in NAc can modulate phasic dopamine release, which may be related to incentive motivation to eat (Patyal *et al*, 2012; Thorpe and Kotz, 2005).

ACh in NAc has also been implicated in food reward (Perry *et al*, 2009; Perry *et al*, 2014; Pratt *et al*, 2007), and this role has been suggested to involve interactions with NAc-opioid and dopamine systems (Perry *et al*, 2014; Pratt and Kelley, 2005; Stouffer *et al*, 2015). Although early studies suggested that ACh might primarily suppress intake via satiety or aversion, since ACh levels rise gradually during food intake and after exposure to aversive tastes (Avena *et al*, 2008; Mark *et al*, 1992; Mark *et al*, 1995), more recent studies have implicated endogenous ACh in NAc and striatum in the appetitive motivation for food rewards (Perry *et al*, 2009; Perry *et al*, 2014; Pratt *et al*, 2007). For example, blockade of endogenous ACh in NAc by microinjections of the muscarinic antagonist scopolamine suppresses food intake, and establishes learned taste or place avoidances. Further, the ability of mu-opioid agonist microinjections in NAc to stimulate food intake is also blocked by simultaneous blockade of ACh muscarinic receptors (Perry *et al*, 2014), which alters striatal preproenkephalin mRNA levels (Pratt and Kelley, 2005), suggesting the possibility of an ACh-opioid interaction in NAc in food reward. Therefore, it is of interest to examine the roles of endogenous ACh in NAc on the hedonic impact of palatable foods as well as on the motivation to eat.

Here we compared orexin-A microinjections and scopolamine microinjections at various sites in NAc medial shell for their effects on (1) intake of a palatable sweet food (chocolate candies), (2) positive 'liking' taste reactions elicited by oral infusions of sucrose solution, and (3) negative 'disgust' reactions elicited by infusions of bitter quinine solution. Our results suggest the existence of a localized hedonic hotspot for orexin enhancement of hedonic impact in rostral NAc shell (similar to opioid enhancement). The results also suggest a more widespread anatomical substrate, distributed throughout entire NAc shell, for orexin stimulation of food intake. A similar distributed NAc network is suggested for endogenous ACh contributions to positive hedonic impact and to food intake, with an additional motivational role for the caudal half of NAc, where ACh blockade additionally releases a fear-related anti-predator reaction of defensive treading.

METHODS

Subjects

Sprague Dawley rats (~3 months old) weighing 250–450 g at surgery (total $n = 25$ (female = 14, weight 250–300 g; male = 11, weight 350–450 g)) were housed in same-sex pairs at ~21 °C on a reverse 12 h light/dark cycle and used in the microinjection-behavior tests. An additional, separate group of four rats were used solely for histological analysis of Fos plume diameters (to assess diameters of a 'first drug microinjection'). All rats had *ad libitum* access to food and water in their home cage. All experimental procedures were followed and approved by the University of Michigan Committee on the Use and Care of Animals.

Surgery

Rats were implanted with oral and cranial cannulas as described previously (Castro and Berridge, 2014). Briefly, bilateral oral cannulas entered the mouth in the upper cheek pouch lateral to the first maxillary molar, ascended beneath the zygomatic arch, and then exited through the skin at the dorsal head cap (Grill and Norgren, 1978). In the same surgery, permanent microinjection guide cannulas were bilaterally implanted. Bilateral coordinates were identical for a given rat, but sites were staggered across rats to fill the entire NAc medial shell for the group as a whole. Rostral shell placements ($n = 15$) averaged around +3.1 anteroposterior (AP) from Bregma, bilateral ± 0.9 mm mediolateral (ML), and − 5.7 mm dorsoventral (DV). Caudal placements ($n = 10$) averaged between +2.6 and +2.8 mm AP, ± 0.9 mm ML, and − 5.7 and − 6.0 mm DV. After surgery, each rat received subcutaneous injections of carprofen (5 mg kg^{-1}) for pain relief, as well as topical antibiotic around the perimeter of the head cap. Rats received another dose of carprofen 24 h later, and reapplication of topical antibiotic, and were allowed to recover for 1 week before behavioral testing began.

Drug Microinjections

Rats were hand-held in the lap of the experimenter during NAc microinjections. Polyethylene PE-20 tubing was connected to a stainless steel microinjection cannula injector, which had a tip (16 mm, 29 gauge) extending 2 mm beyond the ventral end of guide cannulas to reach the NAc target site. On test days, solutions were brought to room temperature (~21 °C) before bilateral microinjection. Drugs were dissolved in a vehicle of artificial cerebrospinal fluid (ACSF). Microinjection solutions contained one of the following (drug order was counter-balanced across rats used for behavioral tests): (1) orexin-A, a hypothalamic neuropeptide (500 pmol per 0.2 µl; also known as hypocretin-1); (2) scopolamine, a muscarinic antagonist (10 µg per 0.5 µl); or (3) ACSF vehicle alone in a volume of 0.2 µl per side (vehicle control condition). Drug doses and volumes were chosen based on most behaviorally effective dose/volume from Thorpe and Kotz (2005) for orexin, and from Pratt *et al* (2007) for scopolamine. Drugs were prepared fresh at the beginning of each test group, and then either frozen (ACSF, orexin) or refrigerated (scopolamine) in solution for testing

later that week. Each 0.2 µl microinjection was delivered during a 1 min period at a speed of 0.2 µl min^{-1} by syringe pump. After bilateral microinjections, injectors were left in place for 1 min to allow for drug diffusion, after which obturators were replaced and rats were immediately placed in the taste reactivity testing chamber. Each rat received bilateral microinjections of only one drug or vehicle solution per test day.

Taste Reactivity Testing

Before testing, rats were each extensively handled to familiarize them with experimenters. They were then habituated to the test chamber for 25 min for 4 consecutive days, and received a mock injection of vehicle ACSF on the final day of habituation.

The taste reactivity test (Grill and Norgren, 1978; Steiner, 1973; Steiner *et al*, 2001) was used to measure a rat's affective orofacial reactions to either a sucrose solution (1.0%, 0.029 M) or a quinine solution (3×10^{-3} M). A 1 ml volume of each solution was infused over a 1 min period via syringe pump through plastic tubing connected to the rat's oral cannula (PE-50 connected to a PE-10 delivery nozzle). On each test day, the sucrose solution was infused 25 min after a NAc microinjection of vehicle, orexin, or scopolamine. After a 5 min delay, a 1-min infusion of quinine followed for a second taste reactivity test. This order was used because if quinine were first, the bitterness disgust could easily carry over and suppress positive reactions to subsequent sucrose. However, sweet tastes do not appear to disrupt negative 'disgust' reactions to subsequent bitterness in our experience, and so a sucrose-quinine order of testing was used (Pecina and Berridge, 2005). Orofacial taste reactivity responses to both solutions were video recorded via closeup lens for subsequent slow-motion video-analysis as described previously (Castro and Berridge, 2014).

Males and females were run in separate same-sex cohorts on different days to prevent any lingering opposite-sex odors from affecting behavior. Test chambers were cleaned with soap and water at the end of each test day. Male and female Fos groups were also run separately for microinjections and perfusions to prevent any pheromone modulation of neuronal gene expression.

Food Intake Testing

A 1 h free intake test was administered immediately following the taste reactivity test on each test day. Rats previously had been habituated to the food intake chamber during the 4 habituation days. Each intake chamber ($23 \times 20 \times 45$ cm) contained a large pile of palatable chocolate (M&Ms), and an *ad libitum* water spout, and the floor was covered with 1 cm depth of corncob bedding. The amount of M&M candies (~20 g) was weighed before and after testing to calculate amount of food intake, and water consumption was measured. All behavior was video recorded and later scored for eating behavior (duration in seconds), water drinking behavior (in seconds), grooming behavior (in seconds), treading (in seconds), and for number of bouts of food sniffs, food carrying or burying, cage crosses, and rears.

Histology and Fos-like Protein Immunohistochemistry

After the last day of behavioral testing, rats were deeply anesthetized with an overdose of sodium pentobarbital. Rats were decapitated and the brains were extracted and fixed in 10% paraformaldehyde solution for 1–2 days followed by a 25% sucrose solution in 0.1 M NaPb for 2–3 days before slicing. 60-µm slices through the NAc were taken from each rat on a cryostat, mounted, dried, and stained with cresyl violet. Microinjection center was determined for each bilateral injection site and slides were compared with the stereotaxic atlas (Paxinos and Watson, 2007) to determine placement in the NAc.

Fos immunohistochemistry and plume analysis was performed on four naive rats, so that plume diameters would be maximal, and not shrunken due to gliosis/necrosis from any previous microinjection. Fos analysis was also performed on 10 rats from the microinjection-behavior test groups for comparison (as previously described by Castro and Berridge (2014)). In brief, rats received a microinjection of vehicle, orexin, or scopolamine 90 min before being killed and perfused. Brains were extracted, left in 4% paraformaldehyde for 24 h, and switched to a 25% sucrose solution the following day. Forty µm thick slices were taken on a cryostat through NAc and processed for Fos-like immunoreactivity using normal donkey serum, goat anti-c-fos (Santa Cruz Biotechnology), and donkey anti-goat AlexaFluor 488 (Invitrogen). Sections were mounted, air-dried, and coverslipped with ProLong Gold antifade reagent (Invitrogen).

The mapping of site effects for localization of function was constructed in a sagittal plane to allow representation of the entire rostrocaudal and DV extent of NAc medial shell. Symbols were color coded to express the intensity of taste reactivity or food intake behaviors relative to vehicle. Symbols were sized to match the mean maximal diameter of measured Fos plumes. For statistical contrasts, sites between +2.4 and +1.5 mm anterior to Bregma were classified as in rostral half of medial shell (ie, rostral to +1.5), and sites between +0.4 and +1.5 mm were classified as caudal placements (ie, caudal to +1.5).

Statistical Analysis

Statistical analyses were performed using non-parametric tests for within-subject (Friedman, Wilcoxon) and between-subject comparisons, and effect sizes and confidence intervals were included when appropriate.

RESULTS

Fos Plumes

Without drug, vehicle microinjections by themselves mildly increased local Fos by 125% over levels found in normal NAc tissue of intact brains, within a 0.38 mm radius of the injector tip (volume = 0.23 mm^3). Orexin microinjections produced more intense local inner plumes of Fos elevation, which were >200% over those vehicle levels as well as over normal tissue levels (radius = 0.14 mm for >200% over vehicle levels; volume = 0.011 mm^3). This inner orexin-induced plume was surrounded by a larger plume of moderate

>125% Fos elevation over vehicle levels (radius = 0.24 mm; volume = 0.058 mm^3), and that outer plume radius was similar to the orexin radius of 200% elevation over normal tissue levels (radius = 0.26 mm; volume = 0.074 mm^3). Thus, the total diameter of a Fos plume induced by orexin was ~0.5 mm, which was assigned to be the size of orexin site symbols in functional maps. Scopolamine microinjections produced a less intense inner excitatory Fos plume of >125% elevation over vehicle with a (radius = 0.20 mm; volume = 0.034 mm^3), surrounded by a larger inhibitory anti-plume where Fos expression was actually decreased by 25% below vehicle levels (radius = 0.23 mm; volume = 0.051 mm^3). This total diameter of 0.46 mm was assigned to scopolamine symbols in functional maps. The scopolamine-induced halo of inhibition could reflect either lateral interactions between medium spiny neurons, or opposing drug effects at different drug concentrations as the drug diffuses away from the microinjection center. Collectively, these data indicate that even vehicle microinjections produce a local plateau of mild elevation in Fos immunoreactivity, while scopolamine adds a 125% greater inner peak plus an outer surround inhibition, and orexin produces an even greater 200% inner peak with a broader base of 125% local elevation.

No Sex Differences in Drug Effects

Females and males were first compared for behavioral sex differences in taste reactivity or food intake measures after vehicle or drug microinjections. At baseline, female rats emitted more taste-elicited orofacial reactions than males after vehicle microinjections (vehicle, $\chi^2 = 6.945$, $p = 0.008$; sucrose-positive reactions, $Z = 2.635$, $p = 0.008$; sucrose-negative reactions, $Z = 2.70$, $p = 0.007$; and quinine-negative reactions: $Z = 2.097$, $p = 0.035$). These results are consistent with earlier reports that females generally display higher orofacial reactivity to taste palatability than males (Clarke and Ossenkopp, 1998; Flynn *et al*, 1993). Despite these baseline differences, males and females did not differ for orexin/scopolamine drug effects, expressed as percent change from vehicle baselines in taste reactivity (orexin, $\chi^2 = 2.865$, $p = 0.091$; scopolamine, $\chi^2 = 1.277$, $p = 0.258$), suggesting that the drugs similarly altered palatability in females and males. Since males and females did not differ in drug effects on hedonic impact, their data was pooled together for subsequent analyses of drug effects expressed as percent change from vehicle baselines. For food intake, no sex differences were observed in vehicle baseline, or in orexin or scopolamine conditions (vehicle, $Z = 0.408$, $p = 0.689$; orexin, $Z = 4.633$, $p = 0.110$; and scopolamine, $Z = 0.245$, $p = 0.810$). Female and male data for food intake were therefore similarly pooled in subsequent analyses. All main effects described below applied to both sexes, unless noted.

Orexin in Rostral Shell Enhances Hedonic Reactions to Sucrose

Orexin-A microinjections altered orofacial reactions to sucrose, but only at particular rostrocaudal locations in NAc shell (Kruskal–Wallis, rostral *vs* caudal sites, $\chi^2 = 5.867$, $p = 0.015$) (Figure 1). At sites located in the rostral half of medial shell of NAc (ie, overlapping with the previously identified opioid hotspot), orexin microinjection caused a 200–400% increase in the number of positive hedonic reactions elicited by sucrose taste, compared with vehicle control trials for the same rats (Friedman's ANOVA, $\chi^2 = 17.868$, $p = 0.000132$; Wilcoxon, $Z = -2.559$, $p = 0.010$; Rostral NAc, $\chi^2 = 21.571$, $p = 0.000021$; $Z = -3.413$, $p = 0.001$; $r = 0.88$; and 96.5% CI (3, 6)). By contrast, orexin stimulation in caudal NAc shell produced no change in hedonic reactions to sucrose ($\chi^2 = 6.914$, $p = 0.032$; $Z = -1.193$, $p = 0.233$). Thus, hedonic enhancements appeared to be restricted to a subregion in the rostral half of medial shell.

Within the rostral half of medial shell, the magnitude of enhancement of sucrose hedonic impact did not differ between dorsal and ventral portions of the rostral zone (entire shell: $\chi^2 = 0.153$, $p = 0.395$; rostral, $\chi^2 = 1.233$, $p = 0.267$) (Figure 2). However, our ventral sites in this study did not extend into the most ventral 25% (~0.5 mm) portion of rostral shell, making it difficult to know if the orexin hotspot filled the entire rostral half of medial shell or merely the dorsal two-thirds of the rostral half. The latter would be most similar to the original map of opioid hotspot (Pecina and Berridge, 2005). Microinjections of orexin in rostral shell did not alter aversive reactions to either sucrose (which always remained near zero ($Z = -0.813$, $p = 0.416$)) or to quinine ($Z = -1.630$, $p = 0.103$). Similarly, orexin microinjections even at rostral sites failed to alter positive hedonic reactions to quinine (which always remained near zero; $Z = -1.414$, $p = 0.157$).

Using the 0.5 mm-diameter outer Fos plume measurements to estimate extent of drug-impact spread from effective sites, the rostral boundary of the orexin hedonic hotspot extended to where the corpus callosum joins hemispheres (AP +2.52). It is difficult to know if the boundary extends any more anteriorly, because we did not have any sites further rostral than +2.28 mm to Bregma. The caudal boundary was well mapped by posterior silent sites, which revealed the orexin hotspot extended caudally to the edge of the paralamboid septal nucleus (AP+1.44), beyond which orexin sites no longer had hedonic effects. The medial boundary reached approximately the lateral septum dorsally, the rostral ventral pallidum caudally, and the islands of Calleja at some mid-AP sites ventrally (ML ±0.51). The lateral boundary was always the border between NAc shell and core (ML ±1.44). The hotspot extended dorsally to the lateral septum and lateral ventricle (DV −6.06), and ventrally at least to the bottom one-fourth of medial shell (DV −8.34).

We calculated the volume of the orexin enhancement hotspot to be ~1.34 mm^3 using the inner 200% Fos plumes volumes. This volume is similar to the opioid hotspot previously reported in the same rostrodorsal region of medial shell (Castro and Berridge, 2014; Pecina and Berridge, 2005), though slightly larger (112% compared with opioid hotspot). This similarity suggests that orexin and opioid signals share nearly the same anatomical hotspot within medial shell for hedonic enhancement of sucrose 'liking' reactions.

Orexin Enhances Food Intake Throughout Entire Shell

Orexin microinjection at virtually all sites throughout the entire medial shell of NAc increased palatable food intake by ~150% (chocolate M&M candies) compared with vehicle trials in the same rats ($\chi^2 = 30.333$, $p = 0.0000001$; $Z = -2.001$,

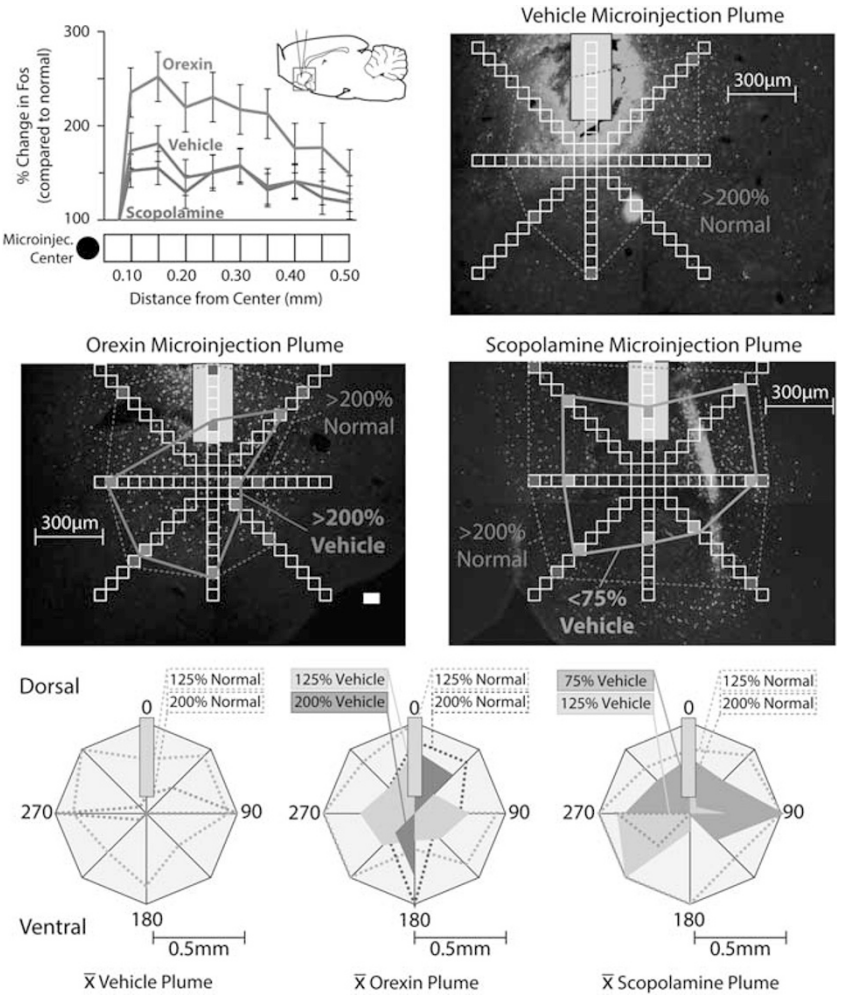

Figure 1 Orexin and scopolamine drug microinjection Fos plumes. Local Fos expression surrounding vehicle, orexin, or scopolamine microinjections (top-left). Photomicrographs show Fos expression after vehicle (top-right), orexin (middle-left), or scopolamine (middle-right) microinjections in NAc shell for individual rats, compared with levels in normal NAc tissue. Plumes 200% elevated over normal tissue levels are outlined with a dashed red line, whereas 200% elevations over slightly higher vehicle microinjection levels are outlined by red solid lines (blue solid lines = 25% decrease below vehicle-induced levels). Mean plume radius shown for vehicle (bottom-left), orexin (bottom-middle), and scopolamine (bottom-right) microinjections relative to normal control brains (dashed lines) or vehicle brains (filled lines). NAc, nucleus accumbens. A full color version of this figure is available at the *Neuropsychopharmacology* journal online.

$p = 0.045$; $r = 0.28$; 95.7% CI (2.8, 4.9)). Sites in the caudal half of shell were as effective as sites in the rostral half at supporting increases in eating (caudal mean = 7.41, S.E. = 1.09; rostral mean = 6.69, S.E. = 0.93; $\chi^2 = 0.077$, $p = 0.781$). Similarly, there was no difference between dorsal *vs* ventral sites in medial shell for orexin-induced increases in intake ($\chi^2 = 0.013$, $p = 0.909$). Thus, orexin increased eating equally throughout virtually the entire medial shell (Figure 4), consistent with previous reports by Thorpe and Kotz (2005). Widespread distribution of sites throughout NAc for orexin-induced increase in intake is also similar to mu-opioid stimulation of eating throughout the entire NAc shell (despite the localization of hedonic hotspots for both in rostral shell, and not caudal shell) (Castro and Berridge, 2014; Zhang and Kelley, 2000).

Scopolamine at All Sites Suppresses Sucrose Hedonic Impact and Elevates Quinine 'Disgust'

Microinjections of the muscarinic ACh antagonist scopolamine in medial shell suppressed positive hedonic orofacial reactions elicited by the taste of sucrose by ~30% below vehicle control levels in the same rats (Figure 2) ($\chi^2 = 17.868$, $p = 0.000132$; $Z = -2.585$, $p = 0.010$; $r = 0.37$; 95.7% CI (−3, 0)). Essentially all sites throughout medial shell generated similar suppressions of 'liking' reactions to sucrose, with no difference in magnitude between rostral *vs* caudal sites ($\chi^2 = 0.946$, $p = 0.397$), or dorsal *vs* ventral sites ($\chi^2 = 2.432$, $p = 0.119$). However, despite suppressing hedonic reactions to sucrose, scopolamine microinjections never actually induced aversive gapes or other 'disgust' reactions to sucrose ($\chi^2 = 4.617$, $p = 0.099$; $Z = -1.451$, $p = 0.147$).

By contrast, aversive 'disgust' reactions to bitter quinine, which were already robust on control trials after vehicle microinjections, were nearly doubled in number after scopolamine microinjections at essentially all sites throughout medial shell ($\chi^2 = 21.273$, $p = 0.000024$; $Z = -3.311$, $p = 0.001$; $r = 0.47$; 95.7% CI (8, 23)). The elevation of quinine 'disgust' reactions was equally robust at sites, whether in rostral halves or caudal halves of medial shell ($\chi^2 = 1.294$, $p = 0.255$). Positive hedonic reactions to quinine,

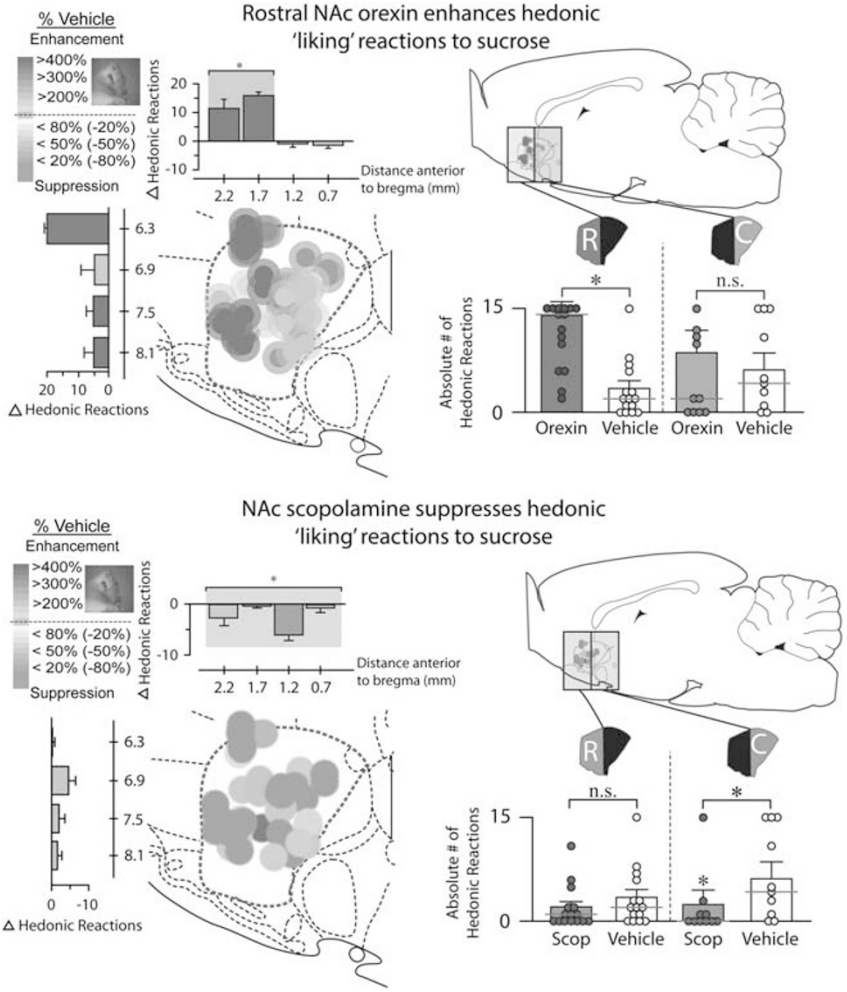

Figure 2 Orexin and scopolamine oppositely modulate hedonic reactions to sucrose. Sagittal causation maps for localization of function in NAc medial shell, showing changes in orofacial hedonic (liking) reactions elicited by sucrose taste after microinjections of either orexin or scopolamine (bottom; both compared with vehicle microinjection tests in the same rat). Each symbol placement indicates a microinjection site, the symbol size reflects the size of Fos plumes produced by that drug, and symbol color reflects the behavioral effects of the drug microinjection, shown as percentage change from vehicle control levels (enhancements: yellow–orange–red; suppressions: blue). Bars above and to the left of sagittal maps show mean absolute change in number of orofacial reactions induced by drug microinjection at that anterior–posterior or dorsal–ventral level. Total numbers of hedonic reactions are depicted in the bars graphs to the right of the sagittal maps, showing means with SEMs as bars (median as pink line), and individual data points as scatterplot circles. A full color version of this figure is available at the *Neuropsychopharmacology* journal online.

which were already nearly zero after vehicle microinjections, remained near zero and unchanged after scopolamine microinjections ($\chi^2 = 2.8$, $p = 0.247$).

Scopolamine Suppresses Food Intake

Scopolamine microinjections throughout medial shell similarly caused a 50% suppression of intake of palatable M&M chocolate candies ($\chi^2 = 30.333$, $p = 0.0000001$; $Z = 3.760$, $p = 0.00017$; $r = 0.53$; 95.7% CI (−2.9, −2.5)). This intake suppression did not differ between rostral and caudal sites (Figure 3) ($\chi^2 = 1.632$, $p = 0.201$), again consistent with previous reports of intake suppression at various NAc sites by Pratt and Kelley (2005). ACh blockade also decreased time spent drinking water ($\chi^2 = 12.194$, $p = 0.002$; $Z = 2.898$, $p = 0.004$) and time spent rearing ($\chi^2 = 11.810$, $p = 0.003$; $Z = 3.00$, $p = 0.003$).

Scopolamine Increases Fearful/Defensive Treading

Scopolamine microinjections, especially in the caudal half of medial shell, also caused a fivefold increase in emission of defensive treading compared with vehicle days ($\chi^2 = 12.194$, $p = 0.002$; $Z = 2.898$, $p = 0.004$; $r = 0.41$; 95.7% CI (0, 20)) (Figure 4). Defensive treading was elicited more intensely at microinjection sites in the caudal half of shell than in the rostral half of shell ($\chi^2 = 4.963$, $p = 0.026$; Rostral: $Z = 1.718$, $p = 0.086$; Caudal: $Z = 2.293$, $p = 0.022$). Defensive treading or burying is a natural anti-predator response of rodents, which is used to throw debris forward toward a localized threat, sometimes actually burying the object (ie, rattlesnake or shock prod) (Coss and Owings, 1978; Reynolds and Berridge, 2001,2008; Treit *et al*, 1981). Defensive treading was not emitted randomly within the chamber (indicating it was not simply a motor reaction), but rather was directionally

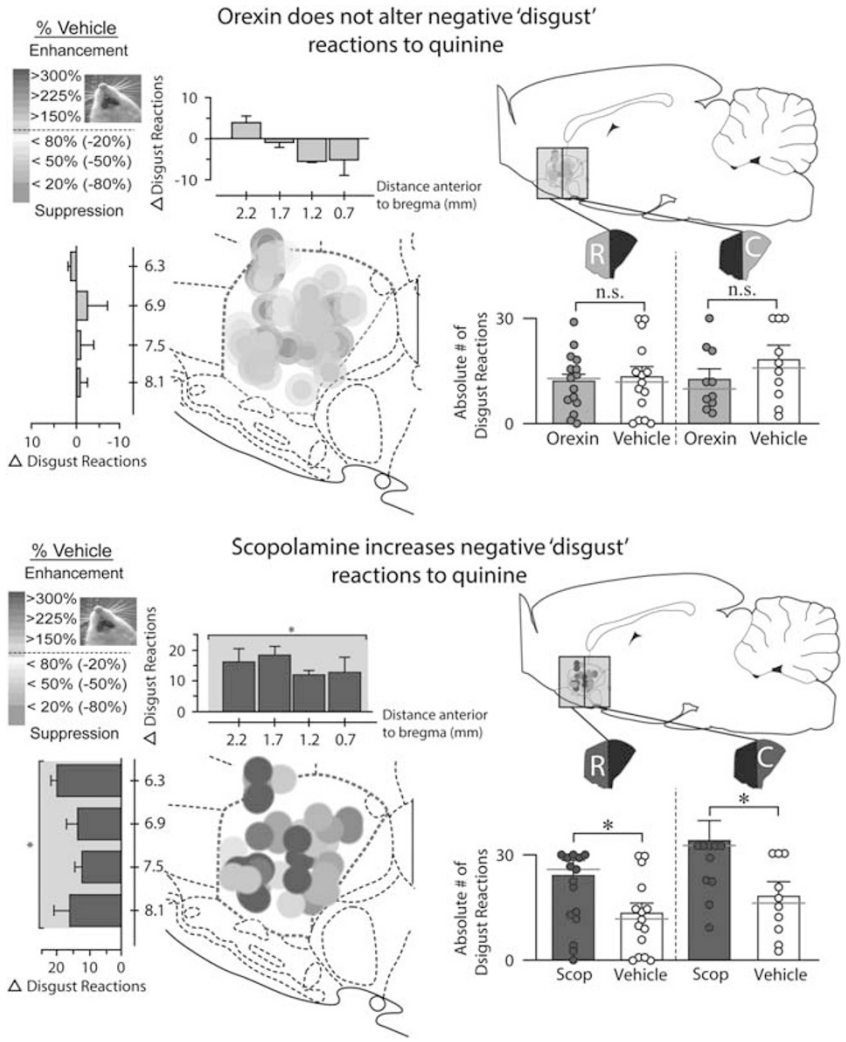

Figure 3 Scopolamine increases aversive 'disgust' reactions to bitter quinine. Sagittal maps showing changes in aversive (disgust) orofacial reactions to bitter quinine taste after microinjections of orexin or scopolamine (bottom). Maps and symbols as in Figure 2, but with increases in 'disgust' reactions reflected by shades of purple. A full color version of this figure is available at the *Neuropsychopharmacology* journal online.

focused toward the four corners of the transparent plastic chambers, which may have reflected light in a slightly glittering fashion.

Potential Independence of Changes in Taste Reactivity, Food Intake, and Defensive Treading

Food intake and 'liking' reaction enhancement by orexin microinjections in the NAc rostral shell hotspot were not highly correlated (Spearman's R; $I = -0.148$, $p = 0.598$, $R^2 = 0.0219$). That appeared to be because orexin microinjections produced roughly 250% increases in sucrose-elicited 'liking' reactions, regardless of whether the intake increase was small (110–149% of vehicle levels) or large (>150%). Although scopolamine microinjections in NAc shell tended to suppress sucrose 'liking' reactions, increase bitterness 'disgust' reactions, suppress food intake, and in caudal shell increase defensive treading, there was not a close statistical association among these effects. Similarly, the degree of scopolamine suppression of food intake was not correlated to the degree of suppression of hedonic reactions to sucrose or enhancement of quinine aversion (sucrose suppression: $\rho = -0.027$, $p = 0.90$, $R^2 = 0.05$; quinine enhancement: $\rho = 0.224$, $p = 0.293$, $R^2 = 0.014$). Finally, the increase in fearful/defensive treading caused by scopolamine microinjections was also statistically independent of the increase in 'disgust' reactions to quinine caused by those same microinjections ($\rho = 0.137$, $p = 0.0.672$, $R^2 = 0.019$). However, since taste reactivity and intake/treading behaviors were tested at different times after microinjections, their temporal separation might have promoted a degree of uncoupling. For sites outside the hotspot, orexin-induced increases in intake were not accompanied by any hedonic enhancement, making the stimulation of eating even more independent.

The hedonic suppression caused by scopolamine microinjections also was not tightly correlated to the elevation of quinine 'disgust' reactions ($\rho = -0.174$, $p = 0.417$, $R^2 = 0.03$). This result appears to be because scopolamine microinjections roughly doubled the number of quinine 'disgust' reactions regardless of whether it only slightly reduced sucrose 'liking' reactions (25% reduction; ie, 75% of vehicle levels) or produced a greater hedonic suppression.

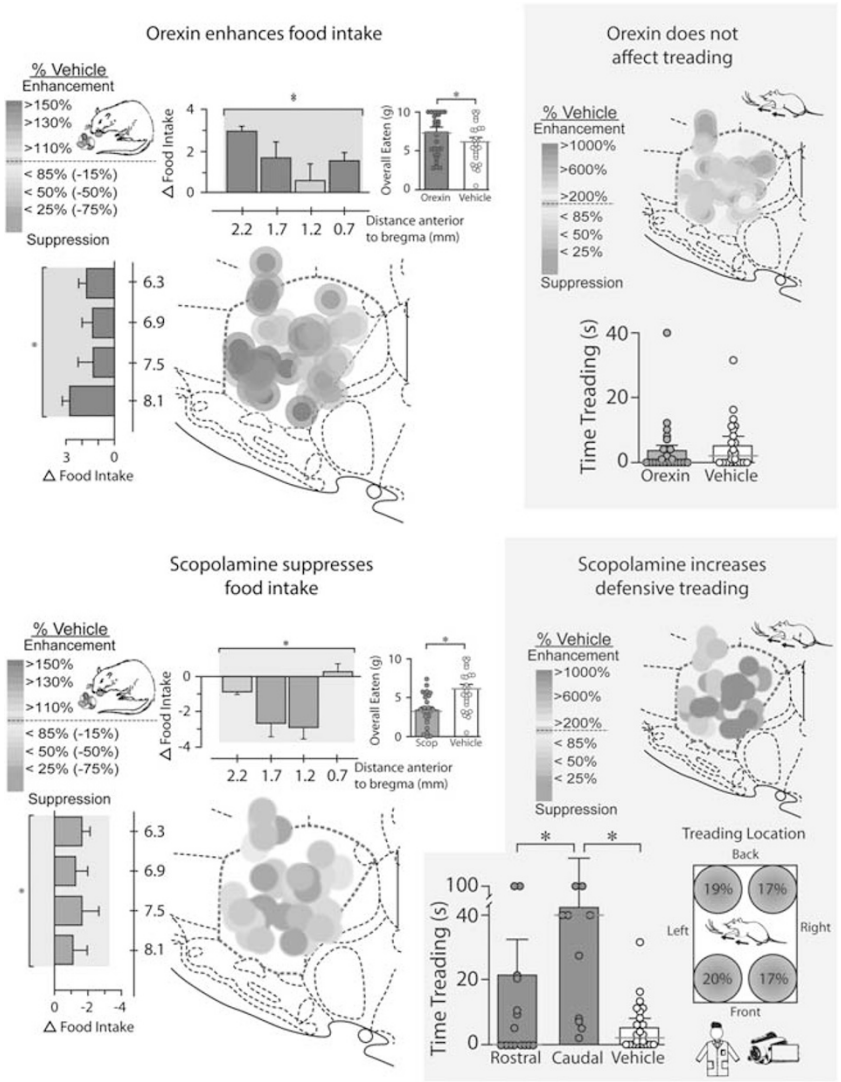

Figure 4 Orexin and scopolamine oppositely affect food intake. Sagittal causation maps for changes in food intake (palatable chocolate M&M candies) and treading after either orexin (top) or scopolamine (bottom) microinjections. Intake effects are displayed as percentage changes from vehicle levels (enhancements: green; suppressions: blue) and treading effects are displayed as percentage changes from vehicle levels (enhancements: red; suppressions: blue). Maps and symbols otherwise as in Figure 2. The bottom-right panel displays the amount of time spent treading at each corner of the chamber. Location of experimenter and video camera designate the front of the chamber. A full color version of this figure is available at the *Neuropsychopharmacology* journal online.

DISCUSSION

Overview

Microinjections of orexin-A in the rostral half of NAc medial shell caused a 300% increase in the number of positive orofacial 'liking' reactions elicited by sucrose taste, whereas sites in the caudal half of shell failed to increase sucrose 'liking', revealing an anatomical orexin hotspot for hedonic enhancement. This hedonic hotspot was anatomically similar to the opioid hotspot in rostral shell previously mapped for mu-, delta-, and kappa-opioid stimulations (and overlapped with an endocannabinoid hedonic hotspot in dorsal shell previously mapped for anandamide enhancements (Castro and Berridge, 2014; Mahler *et al*, 2007)). However, unlike opioid stimulation in NAc, orexin stimulation never suppressed 'liking' reactions at posterior sites in the caudal half of medial shell, which were merely hedonically silent. By contrast, the motivation to eat, expressed as higher food consumption, was increased by orexin at all sites throughout the entire medial shell. Widespread anatomical NAc stimulation of intake is also similar to mu-opioid stimulation, which increases eating at all sites throughout shell and core, as well as in dorsal and ventrolateral regions of neostriatum, in central nucleus of amygdala, and in medial prefrontal cortex (Castro and Berridge, 2014; DiFeliceantonio *et al*, 2012; Mahler and Berridge, 2009; Mena *et al*, 2011; Pecina and Berridge, 2005; Ragnauth *et al*, 2000; Richard *et al*, 2013; Thorpe and Kotz, 2005; Zhang and Kelley, 2000).

Regarding ACh in hedonic impact and motivation, scopolamine blockade of NAc ACh muscarinic receptors typically suppressed both sucrose 'liking' reactions to below 50% normal levels and reduced palatable food intake to

50–70% of normal levels at most sites throughout medial shell. Scopolamine microinjections throughout nearly the entire NAc medial shell also doubled the number of 'disgust' reactions elicited by bitter quinine. However, scopolamine's induction of negative affect was never strong enough to actually create 'disgust' reactions to the sweet taste of sucrose (unlike GABA stimulations in caudal shell, which can reverse sucrose reactions from 'liking' to 'disgust' (Faure *et al*, 2010; Ho and Berridge, 2014; Reynolds and Berridge, 2002)). Finally, ACh blockade by scopolamine specifically in the caudal half of medial shell additionally elicited fear-related defensive treading behavior, which was directed toward locations in the chamber that may have been perceived as more threatening than others (eg, light-reflecting corners). These results suggest that endogenous muscarinic ACh signals, when present, help maintain the overall positive hedonic impact of the taste of food, as well as amplifying the motivation to eat, and in the caudal shell also potentially exerting an anxiolytic action.

Orexin Rostral Hotspot

Why the rostral half of NAc medial shell contains an anatomical hotspot for orexin and opioid hedonic enhancements needs further explanation, but it is known that rostral shell has several unique anatomical features that differentiate it from caudal shell, and which could be relevant. For example, the NAc rostrodorsal quadrant of medial shell has distinct inputs from a region in infralimbic cortex and outputs to ventral pallidum and hypothalamus that are different from other medial shell quadrants (Thompson and Swanson, 2010), resulting in a closed-circuit corticolimbic-thalamocortical loop that runs parallel to loops passing through other regions of NAc shell. In addition, the rostral half of shell also has septal-like anatomical features that distinguish it from the extended amygdala-like features of caudal shell (Thompson and Swanson, 2010; Zahm *et al*, 2013). Neurons in rostrodorsal medial shell also have distinct morphological features, such as fewer spiny dendrites and smaller medium spiny neuronal cell bodies than other areas of NAc (Meredith *et al*, 2008; Zahm *et al*, 2013).

Neurochemically, the cellular mechanism for orexin enhancement of sucrose 'liking' also remains unclear. Orexin is typically thought to have excitatory depolarization effects on neurons (Korotkova *et al*, 2003; Marcus *et al*, 2001; Sakurai *et al*, 1998; Trivedi *et al*, 1998; van den Pol *et al*, 2002; Zhu *et al*, 2003). However, neurons in NAc may exclusively contain orexin-2 receptors (OX_r2) (Ch'ng and Lawrence, 2015; Trivedi *et al*, 1998), which can be coupled with either Gi or Gq subunits, and may inhibit neurons via the augmentation of inhibitory GABA signals (Martin *et al*, 2002; Zhu *et al*, 2003). If so, neuronal inhibition by orexin could be more similar to GABAergic hyperpolarizations or Gi-coupled opioid or endocannabinoid signaling. Future work could clarify the role of NAc neuronal inhibition *vs* excitation for hedonic enhancement.

Scopolamine Causes a Shift Toward Negative Affect and Motivation

The ability of scopolamine microinjections throughout medial shell to suppress hedonic reactions to sucrose, increase aversive 'disgust' reactions to bitter quinine, and suppress intake of palatable food is consistent with the hypothesis by Kelley *et al* (2005) that endogenous ACh signals in NAc shell promote food intake (potentially by enhancing palatability). Their ACh-appetite hypothesis arose from the original demonstrations that scopolamine microinjections into NAc suppressed food intake, and that NAc scopolamine microinjections also could serve as an unconditioned stimulus to induce conditioned avoidance of either a paired taste or a paired place (Pratt and Kelley, 2004; Pratt *et al*, 2007). ACh also appears to interact with mu-opioid signals in NAc shell, as indicated by reports that NAc scopolamine reduces preproenkephalin mRNA levels, and blocks the ability of mu-opioid agonist microinjection in NAc to stimulate eating (Perry *et al*, 2014; Pratt and Kelley, 2005). A role for NAc ACh in incentive motivation and reward also seems consistent with reports that spontaneous firing in NAc neurons (including ACh interneurons) is evoked by reward events (Morris *et al*, 2004), interactions between insulin and ACh interneurons can directly modulate dopamine release in response to food rewards (Stouffer *et al*, 2015), and that optogenetic inhibition of ACh interneurons in NAc prevents the establishment of a cocaine conditioned place preference (Witten *et al*, 2010).

CONCLUSION

These results reveal an anatomically localized hedonic hotspot in NAc rostral half of medial shell for orexin enhancement of sweetness 'liking', but more distributed orexin mechanisms in NAc shell for stimulating motivation or 'wanting' to eat palatable food. They also support a positive role for endogenous NAc ACh signals in both hedonic impact and appetitive motivation. Collectively, these data help elucidate how orexin and ACh neurochemical signals in NAc contribute to sensory hedonic impact and the motivation to eat.

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