Published in final edited form as:

Neuroscience. 2017 May 14; 350: 124–132. doi:10.1016/j.neuroscience.2017.03.021.

Upregulation of orexin/hypocretin expression in aged rats: effects on feeding latency and neurotransmission in the insular cortex

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Abstract

Aging is associated with changes in numerous homeostatic functions, such as food intake, that are thought to be mediated by the hypothalamus. Orexin/hypocretin neurons of the hypothalamus regulate several physiological functions, including feeding, sleep and wakefulness. Evidence from both clinical and animal studies supports the notion that aging is associated with loss or dysregulation of the orexin system. Here, we used virus-mediated gene transfer to manipulate expression of orexin peptides in young and aged rats and examined behavioral and neurochemical correlates of food intake in these animals. Aged rats showed slower feeding latencies when presented with palatable food compared to young control rats, and these deficits were ameliorated by upregulation of orexin expression. Similarly, young animals treated with a virus designed to decrease preproorexin expression showed longer feeding latencies reminiscent of aged control rats. Feeding was also associated with increased acetylcholine, glutamate and GABA efflux in insular cortex of young control animals. Orexin upregulation did not restore deficits in feeding-elicited release of these neurotransmitters in aged rats, but did enhance basal neurotransmitter levels which may have contributed to the behavioral correlates of these genetic manipulations. These studies demonstrate that age-related deficits in behavioral and neurochemical measures of feeding are likely to be mediated, in part, by the orexin system. Because these same neurotransmitter systems have been shown to underlie orexin effects on cognition, treatments which increase orexin function may have potential for improving both physiological and cognitive manifestations of certain age-related disorders.

Keywords

orexin; hypocretin; insular cortex; microdialysis; feeding; aging

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Introduction

Aging is associated with changes in physiological regulatory functions such as food intake, energy balance and sleep patterns (Bonnet and Arand, 1989, Morley, 1997, Espiritu, 2008, Manini, 2010). Although all of these functions are affected by both peripheral and central mechanisms, they are presumed to result, at least in part, from alterations in brain regions that underlie appropriate endocrine, behavioral and cognitive responses to whole-organism homeostatic challenges. Additionally, translational and clinical studies have demonstrated a relationship between homeostatic dysfunction in aging and subsequent cognitive decline, suggesting the possibility of common neurobiological mediators of these outcomes (Buchman et al., 2005, Johnson et al., 2006, Altena et al., 2010, Fadel et al., 2013).

The hypothalamus is the primary CNS node for homeostatic regulation and animal studies support a role for hypothalamic dysfunction in age-related alterations in metabolism and food intake (Wolden-Hanson, 2006). The hypothalamus is also highly heterogeneous, containing dozens of named nuclei and even more distinct neuronal populations as characterized by neurotransmitter or neuropeptide expression. The orexin (hypocretin) neuropeptide system, found within the lateral hypothalamus and perifornical area, has received much interest since its discovery in the late 1990's for its role in regulating sleep-wake cycles, food intake, and stress and reward functions (de Lecea et al., 1998, Sakurai et al., 1998). Orexin neurons are lost in human narcolepsy with cataplexy (Siegel, 1999, Nishino et al., 2000) and are responsive to fluctuating levels of peripheral cues related to energy balance such as glucose (Burdakov et al., 2006) and leptin (Leinninger et al., 2011). An extensive literature has also implicated the orexin system in reward properties of abused drugs and natural reinforcers (Harris and Aston-Jones, 2006, Martin-Fardon et al., 2016) which may, in part, reflect orexin interactions with other neural signaling pathways classically associated with reward, such as dopamine or opioids (Espana, 2012, Muschamp et al., 2014). This diverse array of orexin-mediated functions has led to their description as "physiological regulators" that engage arousal and motivated behavior in response to whole organism homeostatic status (de Lecea et al., 2002, Li et al., 2014, Mahler et al., 2014). The potential significance of this neuropeptide system for age-related alterations in physiological regulation is further bolstered by accumulating literature demonstrating reductions in orexin neuron number, peptide expression, and receptor expression in aged animal models (Terao et al., 2002, Porkka-Heiskanen et al., 2004, Kessler et al., 2011). Clinical data suggests that human aging is also associated with alterations in orexin function that might be particularly exacerbated in disease states that comprise dysfunctions in both physiological regulation and cognition (Thannickal et al., 2007, Fronczek et al., 2012).

Orexin neurons project to a diverse array of forebrain targets that mediate the roles of these peptides in motivated behavior and arousal (Peyron et al., 1998). Several cortical areas are included in these projections, including the insular cortex (Hollander et al., 2008). The insular cortex is a site of integration of visceral and emotional processing, which has led to its description in primates as "interoceptive cortex"—a brain region that plays a key role in the conscious awareness of physiological status(Craig, 2002). This description is also consistent with demonstrated roles of the insular cortex in olfaction and gustation, which are also important for food intake (Augustine, 1996, Rolls, 2005, 2015).

In addition to receiving orexin afferents from the hypothalamus, the insular cortex receives input from several neuromodulatory transmitters, including the Ch4 subdivision of the basal forebrain cholinergic system (BFCS). Although Ch4 projections to the cortex are diffuse, reciprocal inputs to the BFCS from cortex are not, deriving disproportionately from a few areas which include the agranular insular cortex (Mesulam and Mufson, 1984, Zaborszky et al., 1997). Given the role of the BFCS in attentional processing, this circuitry may be important for biasing attentional resources toward external stimuli related to underlying physiological status. In addition to acetylcholine, however, effects of feeding-related stimuli on the insular cortex are likely to involve other neurotransmitter systems, including the primary fast-acting amino acid neurotransmitters, glutamate and GABA. Orexins modulate release of all three of these neurotransmitters in a variety brain regions and experimental contexts (John et al., 2003, Fadel et al., 2005, Thorpe et al., 2006, Stanley and Fadel, 2011).

Here, we altered expression of prepro-orexin in young and old rats to test the hypothesis that age-related alterations in behavioral and neurochemical correlates of basic feeding behavior may have an orexinergic basis. We further examined how acetylcholine, glutamate and GABA release in the insular cortex are modulated as a function of aging or alterations in orexin expression.

Experimental Procedures

Animals

Young (3 months old upon arrival) and aged (27 months old upon arrival) male Fisher 344/Brown Norway F1 hybrid rats obtained from the National Institute on Aging colony (Baltimore, MD) were single-housed on a 12–12 hr. light-dark cycle (lights on at 07:00 hr) with standard rat chow and water available *ad libitum* for at least one week prior to surgery or other experimental procedures. All *in vivo* experiments were initiated at least one hour after lights on and were concluded at least two hours prior to lights off. All animal care and use procedures were carried out in accordance with protocols written under the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of South Carolina.

All animals were handled daily during the first week. During the second week, daily handling continued and food and water intake were recorded. Throughout the third week, the animals were mildly food restricted to achieve 95% of their free-fed body weight and habituated to microdialysis testing bowls for 2–3 hours/day (parabolic clear plastic bowls; Bioanalytical Systems, Inc., West Lafayette, IN). During habituation, all animals were trained to receive a single palatable treat (Bacon Softies; BioServe, Fleming, NJ) concurrent with 20 minutes of sudden darkness. This was done at the same time every day with half of the animals training at 13:30 hours and the other half at 14:30 hours. The time to start consuming the treat was recorded as the latency to feed. This or similar manipulations have been shown previously to produce robust increases in prefrontal cortical acetylcholine release concurrent with rapid approach and consumption of the palatable food (Fadel et al., 1996, Frederick-Duus et al., 2007). During the fourth week, animals continued with training and underwent stereotaxic surgery for hypothalamic virus injection and insular cortex guide

cannula placement. Training did not occur on surgery day or one day post-op to allow for recovery. Following surgery, the animals were food restricted to achieve 85–90% of their original free-fed body weight. During weeks five and six, all rats received two microdialysis sessions, separated by an “off” day, concurrent with the feeding/darkness paradigm. After all experiments were completed, animals were deeply anesthetized with isoflurane and sacrificed via transcardial perfusion. Brains were removed, post-fixed in 4% paraformaldehyde for 48 hours, and then cryoprotected in 0.1 M phosphate buffer with 30% sucrose.

Surgery

Under sodium pentobarbital (60–65 mg/kg) or ketamine (80 mg/kg)/xylazine (8 mg/kg anesthesia, animals received bilateral intrahypothalamic injections of 0.2 μL (5 × 10E6 tu/μL) of preproorexin (PPOX) sense or antisense lentivirus or 0.2μL of control virus (GFP only) with the following stereotaxic coordinates relative to bregma (Paxinos and Watson, 2007): AP −2.5 mm, L+1.2 mm, DV −9.0 mm (young); AP −2.9 mm, L +1.6 mm, DV −9.4 mm (aged). Lentiviruses containing the rat PPOX cDNA inserted in either sense or antisense orientation and control transgene expression cassettes under a phosphoglycerate kinase-1(*pgk*-1) promoter were constructed at the University of South Carolina School of Medicine Viral Vector Core Facility. Fourteen aged animals received sense PPOX sense (AS group) and twelve received control GFP virus (AC group). Ten young animals received PPOX antisense (YAS group) designed to knockdown PPOX expression and ten received control GFP virus (YC group). At the same time a guide cannula (Bioanalytical Systems, Inc., West Lafayette, IN) was placed in the left agranular insular cortex (AIC) at the following stereotaxic coordinates relative to bregma (Paxinos and Watson, 2007): AP +2.0 mm, L +4.6 mm, DV −5.0 mm (young); AP +2.0 mm, L +5.0 mm, DV −5.4 mm (aged). Guide cannulas were affixed to the skull by stainless steel screws and dental cement. At the conclusion of surgery all animals received a subcutaneous injection of the analgesic buprenorphine (0.02 mg/kg) to ease post-operative pain.

In vivo microdialysis

Following two days of recovery, animals were habituated to microdialysis bowls for five days prior to the first microdialysis experiment. During microdialysis, stylets were removed from the guide cannulae and replaced with semipermeable probes (BAS, 30kDa cutoff) that extended 2.0 mm past the end of the cannula. Probes were perfused with artificial cerebral spinal fluid (aCSF; 150mM NaCl, 3.0 mM KCl, 1.7 mM CaCl2, 0.9 mM MgCl2, 4.9 mM D-glucose, plus 50 nM neostigmine bromide to promote reliable recovery of ACh) at a rate of 2.0 μL/minute. Collection of microdialysates in 15 min (30 μL) intervals began 2.5 to 3.0 hours after probe insertion. The microdialysis session consisted of four baseline collections, one stimulus collection (Bacon Softie with darkness), and four post-stimulus collections. Microdialysates were stored at −80 degrees C until analysis by HPLC with electrochemical detection as previously described with 10 μL of each dialysate used for as previously described for glutamate and GABA (Reznikov et al., 2007, Stanley and Fadel, 2011) and 20 μL used for ACh analysis (Fadel et al., 2005).

Histology

Cryoprotected brains were coronally sectioned (44 μm) using a freezing microtome. Representative sections through the rostrocaudal extent of the AIC were mounted and stained for acetylcholinesterase to verify probe placement (Figure 3D). All tissue for immunohistochemical analysis processed was processed according to previously described protocols with minor modifications (Frederick-Duus et al., 2007, Kessler et al., 2011). Hypothalamic sections were processed for GFP to verify virus expression. Briefly, free-floating sections were incubated in mouse anti-GFP antibody (1:1,000; Millipore, Inc., Temecula, CA; cat# MAB3580) for 48 hr. Tissue was then incubated with biotinylated donkey anti-mouse secondary antibody (1:1,000; Jackson ImmunoResearch Laboratories, West Grove, PA; cat# 715-065-151) for 1.5 hr followed by horseradish peroxidase-conjugated streptavidin (SHRP; 1:1,600; Jackson; cat# 016-030-084) for 1 hour. GFP labeling was visualized by adding hydrogen peroxide to the tissue sections in the presence of diaminobenzidine, generating a light brown stain in GFP-immunoreactive areas (Fig. 1A).

The efficacy of the lentivirus to modulate orexin expression was further verified by incubating rostral cortical sections (containing the AIC) in goat anti-OXA antibody (1:1,000; Santa Cruz Biotech, Santa Cruz, CA; cat# SC-8070) or rabbit anti-OXA (1:1,000; EMD Millipore; cat# PC362) for 48 hr followed by biotinylated donkey anti-goat (1:1,000 Jackson; cat# 705-005-003) or biotinylated donkey anti-rabbit (1:1,000 Jackson; cat# 711-065-152) for 1.5 hr followed by an incubation in SHRP as described above. Tissue was developed using nickel/cobalt-enhanced diaminobenzidine, generating a blue-black immunoprecipitate in orexin-positive fibers.

After dehydration and coverslipping, tissue sections were examined by light microscopy using Neurolucida (version 11.01.1) software (MicroBrightField; Wiliston, VT). Two serial sections per animal at approximately 1.6 mm rostral to bregma were analyzed in a 400,000 μm^2 area within the AIC. All fibers were traced in a three dimensional plane and the total length per box was calculated by the software. An average was then calculated for each animal based on those numbers.

Statistical analysis

Baseline neurotransmitter levels in dialysates were calculated as the mean concentration of the four baseline collections for each animal. Data were then expressed as percent-of-baseline for each animal at each time point, then averaged within each treatment group. Group differences for each neurotransmitter analyte (Glutamate, GABA, and ACh) were revealed by repeated measures two-way analysis of variance (ANOVA) followed by Bonferroni planned comparisons. Differences in baseline neurotransmitter levels, total orexin-immunoreactive fiber length, and latency to feed were tested by one-way ANOVA with Tukey's Multiple Comparison Test post-hoc analysis. In addition, a Pearson's correlation was performed to on total orexin-immunoreactive fiber length and latency to feed. All values are expressed as the mean ± SEM. All statistical tests were done using GraphPad Prism version 5.02 (GraphPad Software Inc.; San Diego, CA).

Results

Virus expression

All virus treatment conditions (Control; PPOX Sense; PPOX Antisense) produced robust GFP expression in the hypothalamus surrounding the zone of infusion (Fig. 1A). Typically, bilateral GFP expression was observed in the medial hypothalamus, extending into the adjacent perifornical and lateral hypothalamus. In some cases, GFP expression extended dorsally along the infusion needle track to include the overlying zona incerta (Fig. 1A, left side). Modulation of orexin expression was further analyzed by quantifying OXA-immunopositive fiber length in the AIC (Fig. 1B,C), where one-way ANOVA yielded a significant effect of GROUP, $F(3,32) = 5.789$, $p < 0.01$. Post-hoc analysis revealed the AS group had significantly more OXA immunoreactivity in the AIC than AC animals, consistent with a virus-mediated enhancement of orexin expression in this target cortical area ($p < 0.01$). Furthermore, YC rats had significantly greater OXA immunoreactivity than AC animals ($p < 0.05$), consistent with previously-reported age-related reductions in orexin expression (Kessler et al., 2011). The YAS animals showed a trend for decreased OXA immunoreactivity relative to YC rats, but no significant differences were observed. Neither PPOX sense nor antisense expression altered food intake or weight relative to age-matched control animals.

Feeding latency

As previously reported (Frederick-Duus et al., 2007), young food-restricted control rats rapidly approached and consumed the palatable food stimulus (Fig. 2A). One way ANOVA revealed a main effect of GROUP ($F(3,33) = 5.708$, $p = 0.0029$) on feeding latency. Post hoc analyses indicated that the latency was significantly greater for AC and YAS rats compared to YC animals ($p < 0.05$ for both comparisons). Similarly, upregulation of PPOX expression in the aged rats significantly decreased latency to feed relative to age-matched controls ($p < 0.05$) and eliminated age-related differences with the young control animals ($p > 0.05$). In addition, Pearson correlation analysis revealed a highly significant negative correlation ($r = -0.46$; $p = 0.005$) between OXA fiber length within the insular cortex and latency to approach and consume the palatable food stimulus (Fig. 2B).

Microdialysis

For insular cortical ACh efflux, two-way ANOVA revealed a significant interaction between TIME and GROUP ($F(3,23) = 1.775$, $p = 0.019$) as well as a main effect of TIME ($F(3,23) = 8.447$, $p < 0.0001$). As we have previously reported in the prefrontal cortex of control animals (Frederick-Duus et al., 2007), presentation of the darkness/food stimulus in young, food-restricted rats produced a robust increase in insular cortex ACh levels that peaked during the second post-stimulus collection (Fig. 3A), confirmed by post hoc Bonferroni analysis ($p < 0.001$). Although aged rats that received the PPOX sense virus showed a trend toward increased ACh efflux during the first post-stimulus collection relative to age-matched controls, this did not reach significance ($p = 0.102$). Basal AIC ACh efflux did not differ as a function of age or virus condition ($p > 0.05$; Fig. 3A, right).

Presentation of the palatable food stimulus also increased insular glutamate levels in YC rats relative to YAS animals, although this increase was delayed and reached significance only at the final time point following the stimulus ($p < 0.01$; Fig. 3B). There were significant differences in basal glutamate levels in the insular cortex as well ($F(3,29) = 11.15$, $p < 0.001$), with the AS group showing elevated glutamate relative to each of the other groups (all $p < 0.05$; Fig. 3B, right).

Similar to glutamate, only the YC rats showed stimulus-elicited increases in insular cortex GABA efflux, with a delayed peak effect that was significantly greater than both YAS ($p < 0.05$) and AC ($p < 0.01$) groups at the eighth collection. Also similar to glutamate, there were significant group differences in basal GABA efflux ($F(3,29) = 14.95$, $p < 0.001$) with the AS group of rats showing significantly elevated basal levels of GABA in the insular cortex relative to each of the other groups (all $p < 0.05$; Fig. 3C, right).

Post-mortem acetylcholinesterase staining revealed that successfully targeted microdialysis probes were predominantly located in the deeper layers of the agranular insular cortex (AIC), with some dorsal extension into dysgranular and granular subregions of the insula (Fig. 3D).

Discussion

These experiments demonstrate that aged, food restricted rats have altered behavioral and neurochemical responses to a compound palatable food stimulus that may, in part, be mediated by deficits in orexin neurotransmission.

Virus-mediated modulation of orexin expression and feeding behavior

We used lentivirus-mediated gene transfer to increase preproorexin expression in aged rats and decrease preoproorexin expression in young rats. The primary measures of viral efficacy we utilized were expression of the reporter protein, GFP as well as orexin fiber length—a measure of orexin fiber density—in the primary orexin projection region of interest for our study, the insular cortex. By both measures, the preproorexin sense virus restored orexin expression in the insular cortex of aged rats to levels comparable to young control animals (Fig. 1). It is important to note that preproorexin expression in our animals was under the control of a non-specific (*pgk*) promoter. Thus, while our viral deposits were limited to the hypothalamic areas where orexin neurons are of greatest density, it is likely that there was some degree of ectopic expression. One candidate neuronal population for such expression might be MCH neurons, which are also distributed in the LH and dorsally-contiguous zona incerta and play a role in feeding (Qu et al., 1996, Hervieu, 2006). Orexin and MCH neurons share projections to areas that modulate reward and motivated behavior although their effects on these circuits may differ. In the nucleus accumbens, for example, both orexin and MCH promote food intake, but whereas intra-accumbens orexin increases activity and energy expenditure (Thorpe and Kotz, 2005, Teske et al., 2010), intra-accumbens MCH has no or negative effects on these measures (Georgescu et al., 2005, Guesdon et al., 2009). There are additional considerations with ectopic expression, including the potential decoupling of orexin co-release with co-expressed neurotransmitters such as glutamate (Torrealba et al., 2003, Henny et al., 2010) or dynorphin, which has been shown to be important in the context

of orexin effects on reward (Muschamp et al., 2014). However, while ectopic orexin expression is an important caveat, an advantage to this approach is that it may have allowed us to overcome the limitation of a decreased number of orexin neurons in our aged rats, which we have previously shown have a naturally-occurring loss of 40–50% of this cell population (Kessler et al., 2011). In addition, we did not observe increased orexin fiber labeling in normally orexin-sparse brain regions, such as the caudate-putamen or cerebellum. Furthermore, it is likely that any functional effects of ectopic orexin expression would be limited to areas that are orexin-receptive under normal conditions, as there is little evidence for orexin acting on noncognate receptors.

To test whether reduction of preproorexin expression in young rats replicated the feeding behavior phenotype of aged rats, we employed a lentivirus containing the rat preproorexin cDNA inserted in antisense orientation relative to the *pgk* promoter. Transcription of long antisense RNAs effectively reduce gene expression in vivo, presumably by transcriptional repression or RNA interference (Davidkova et al., 1998, Weiss et al., 1999, Yeomans et al., 2005). However, in young rats treated with preproorexin antisense virus, the reduction in orexin fiber density in insular cortex did not reach statistical significance. This may reflect the limitations of immunohistochemistry for quantifying protein expression, as lightly immunoreactive fibers and darkly immunoreactive fibers of the same length would be assigned an equal measurement in our analysis. Additional factors in the failure to observe reduced orexin fiber density include 1) the fact that not all relevant, orexin-positive neurons in the tissue sections likely are transduced by the virus, limiting our ability to demonstrate knockdown across all fibers and 2) the possibility that the strength of preproorexin expression driven by the *pgk* promoter may be low relative to native expression. However, the dramatic effects of antisense expression on feeding latency and insular neurochemistry strongly argue for the effectiveness of our lentiviral approach to decrease functional correlates of orexin activity.

All animals in our feeding experiments were mildly food-restricted and received multiple exposures to the darkness/food stimulus. Latency to feed in this paradigm can be considered an index of motivated behavior (Wise and Raptis, 1985, Barbano and Cador, 2006), driven by a combination of homeostatic (energy balance deficit resulting from mild food restriction) and hedonic (highly palatable “Bacon Softie”) factors. Because all animals received multiple sessions with presentation of the food, there may also be elements of feeding as an entrained, learned behavior reflected in the latency measure. Several studies have demonstrated a convincing role for the orexin system in meditating cue-conditioned feeding (Petrovich et al., 2012, Cason and Aston-Jones, 2013, Cole et al., 2015, Keefer et al., 2016). Dissecting out the contributions of each of these—including how they are altered in aging—is beyond the scope of the current experiments but may be a fruitful avenue for future studies (Kmiec, 2006). Of greatest interest, however, is the age-related increase in latency and the ability of increased orexin expression to reduce latency in aged animals or orexin antisense to increase latency in young rats. The results of these bidirectional manipulations argue strongly that the age-related behavioral deficits we observed have an orexinergic basis, rather than resulting from some other factor such as motor impairment. Consistent with this hypothesis are previous demonstrations that orexins play important roles in food entrainment anticipatory behavior (Akiyama et al., 2004, Mieda et al., 2004).

Aging, orexin and neurochemical correlates of feeding in the insular cortex

Orexin neurons send robust projections to the basal forebrain, including its cholinergic components (Eggermann et al., 2001, Espana et al., 2001, Fadel et al., 2005). We have previously shown that orexin lesions or orexin antagonists attenuate behavioral and prefrontal cortical neurochemical responses to food-related cues in young rats (Frederick-Duus et al., 2007). Consistent with these observations, young animals treated with the preproorexin antisense virus in these experiments failed to show increased ACh release in the insular cortex upon food presentation. Prior work has shown that ACh in the insular cortex plays an important role in conditioned taste aversion and experiences of novel tastes (Miranda et al., 2003, Bermudez-Rattoni et al., 2004). However, we saw robust increases in insular ACh efflux in our young animals even though the palatable food stimulus was neither novel nor aversive. This may have been driven, in part, by the mild food restriction paradigm to which our animals were subjected. Thus, ACh release in the insular cortex is likely to reflect not just novelty, but motivational salience of a tastant or food-paired stimulus.

We observed no effect of orexin knockdown on basal ACh release in the insular cortex. This may reflect that orexin inputs to the BFCS are only acutely activated in response to food-paired stimuli. However, the physiological factors (e.g., decreased glucose levels) that mediate hunger-elicited activation of orexin neurons are likely to be present prior to food presentation (Yamanaka et al., 2003, Burdakov and Gonzalez, 2009). Thus, it seems more likely that orexin inputs to the BFCS do not drive basal cortical ACh release but only potentiate cholinergic activation in combination with other excitatory basal forebrain inputs associated specifically with food cues. The source of these other inputs is unclear but may include glutamatergic inputs from areas such as the prefrontal cortex or the insular cortex itself (Carnes et al., 1990, Gaykema et al., 1991, Zaborszky, 2002).

The food stimulus in young rats was associated with increased insular glutamate release relative to control animals, although, this effect did not reach statistical significance until the final collection period (Fig. 3B). The slow-developing nature of this response suggests that, under normal conditions, glutamate release in the insular cortex may reflect post-ingestive aspects of food-related stimuli (Oliveira-Maia et al., 2012). Interestingly, aged rats did not show acute increases in glutamate release in response to food presentation, nor was this response restored by increased orexin expression. Aged rats treated with the preproorexin virus did, however, show marked elevations in basal insular cortex glutamate levels relative to the other age and virus treatment conditions. It is tempting to speculate that basal elevations in insular glutamate animals may have facilitated processing of interoceptive cues related to hunger, thus promoting the lower feeding latency response in these animals. If so, this suggests that different presynaptic neurochemical mechanisms in the insula may converge on postsynaptic targets that facilitate appropriate behavioral responses to homeostatically-relevant stimuli. Consistent with this possibility, insular cortex ACh signaling has been shown to facilitate unconditioned taste-related signaling mediated in part by glutamatergic transmission (Bermudez-Rattoni et al., 2004). Whether such an interaction mediates the feeding latency responses in our animal model remains to be clearly demonstrated.

Similar to glutamate, insular cortex GABA levels rose slowly following food presentation in young control rats and only achieved significance well after the food stimulus (Fig. 3C). Although orexin knockdown in young rats abolished this effect, orexin upregulation in aged rats did not restore an acute GABAergic response. Again however, as with glutamate, aged rats treated with the preproorexin virus did show elevations in basal GABA levels in the insular cortex.

Future studies will be required to more definitively characterize the role of insular cortical orexin signaling, including the receptor mechanisms involved, in age-related alterations in feeding and cognition. Prior work has demonstrated that insular orexin transmission regulates nicotine reward (Hollander et al., 2008) and our work is consistent with a similar role in neurochemical and behavioral responses to food stimuli in food-restricted animals. In addition, it is likely that age-related reductions in orexin signaling in other brain regions underlying motivated behavior and cognition may also play a functionally significant role in these processes. Such candidates include the prefrontal cortex and ventral tegmental area (Fadel and Deutch, 2002, Lambe et al., 2005, Vittoz and Berridge, 2006, Cole et al., 2015).

In humans, aging is associated with alterations in several physiological functions, including experiencing of food reward (Morley, 2001) and subjective awareness of hunger state and other interoceptive phenomena thought to be mediated by the insula (De Castro, 1993). Multiple factors are likely to contribute to these deficits, including dysfunction of brain areas and circuits that underlie these important functions. However, the significance of age-related alterations in food intake and other aspects of physiological regulation is illustrated by clinical reports of an association between these deficits and subsequent likelihood of cognitive decline, including Alzheimer's disease (Buchman et al., 2005, Johnson et al., 2006, Buchman et al., 2014). This link could be attributed to the fact that in addition to its role in regulation of physiological functions such as feeding and sleep-wake stability, the orexin system also promotes cognitive functions such as attention (Lambe et al., 2005, Deadwyler et al., 2007, Boschen et al., 2009, Fadel and Burk, 2010). The current studies further demonstrate that age-related alterations in feeding behavior and neurotransmission have an orexinergic basis, and suggest that orexin-based therapies may have utility in treating conditions which have both homeostatic and cognitive components. While virus-mediated upregulation of orexin expression is unlikely to find clinical application in the near future, additional means of enhancing central orexin transmission in aging may be feasible, including intranasal orexin delivery (Deadwyler et al., 2007, Baier et al., 2011, Dhuria et al., 2016) or development of systemically-deliverable small molecule orexin agonists (Scammell and Winrow, 2011).

Acknowledgments

This work was supported by the National Institutes of Health (R01AG050518; R01AG030646).

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Highlights

- Aged rats show deficits in orexin/hypocretin expression, feeding latency and insular cortex neurotransmitter release
- Upregulated orexin expression in old rats partly restored behavioral and neurochemical correlates of food intake
- Orexin antisense in young rats produced an “old” phenotype in measures of feeding latency and insular cortex neurochemistry
- Orexins may integrate behavioral, neurochemical and cognitive responses to homeostatic deficits that manifest in aging

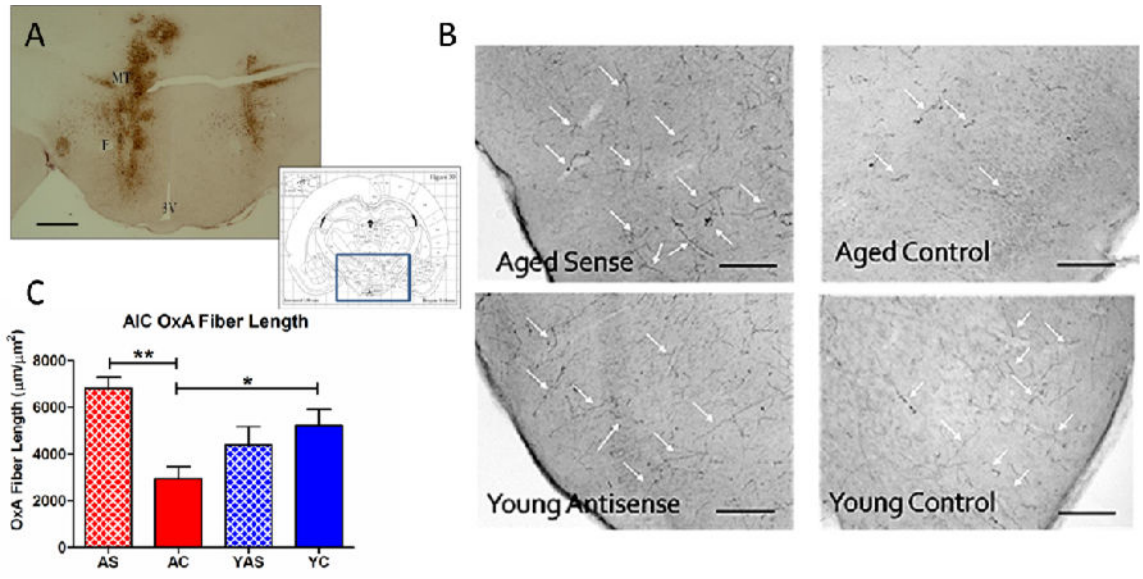

Figure 1.

Modulation of orexin expression by lentivirus-mediated gene transfer. Bilateral infusions of lentivirus encoding preproorexin sense or antisense were made into the lateral hypothalamus and perifornical area. **A.** Immunohistochemical detection of GFP expression (brown staining) in hypothalamus following lentivirus infusions. The area encompassed in the photomicrograph, corresponding to roughly Bregma – 3.1 mm, is indicated by the blue box (inset; (Paxinos and Watson, 1998)). **B.** Immunohistochemical detection of orexin fiber labeling in insular cortex. Age/treatment are indicated in the lower left portion of each photomicrograph. White arrows indicate examples of orexin-immunoreactive fibers. **C.** Quantitation of orexin fiber expression following lentivirus-mediated modulation of orexin expression. Aged control rats had significantly lower levels of orexin expression (as measure by sum total fiber length within a defined region of insular cortex) than their young control counterparts. Treatment with the preproorexin sense virus significantly increased total orexin-positive fiber length to levels similar to young control animals. Abbreviations: F, *fornix*; MT, *mammillothalamic tract*; 3V, *third ventricle*; AS, *aged preproorexin sense*; AC, *aged control*; YAS, *young preproorexin antisense*; YC, *young control*. Scale bars, approximately 1 mm (A) and 500 μm (B). *$p < 0.05$. **$p < 0.01$. Group sizes: N = 12 (AC), N = 7 (AS), N = 10 (YC), N = 8 (YAS).

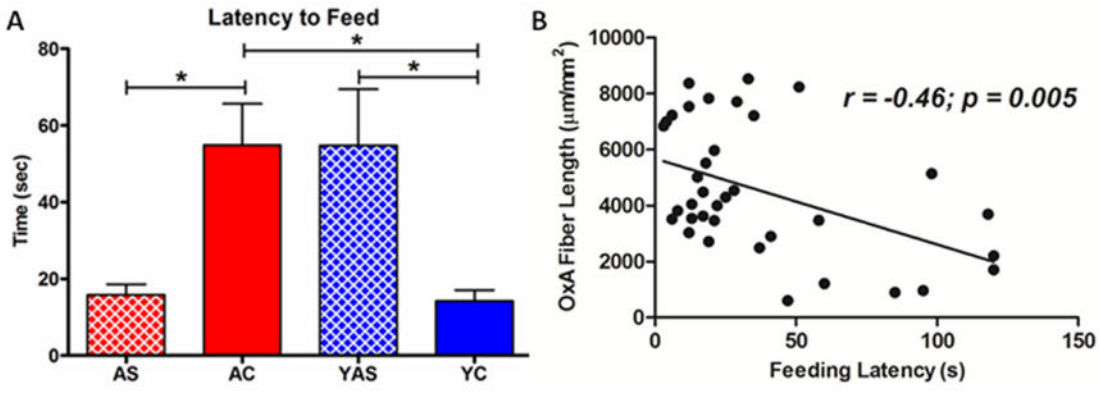

Figure 2.

Latency (seconds ± SEM) to approach and consume a palatable food reward. **A.** Aged control (AC) rats showed significantly greater latency to approach and consume a palatable food reward (Bacon Softie) than their young control (YC) counterparts. Similarly, young rats treated with preproorexin antisense virus (YAS) showed significantly longer latency to approach and consume the food reward than YC rats. Treatment with preproorexin sense virus significantly reduced feeding latency in old rats to levels comparable to YC animals. *$p < 0.05$. **B.** Correlational analysis of all subjects revealed a highly significant negative correlation between insular cortex OxA fiber length and feeding latency. Group sizes: N = 12 (AC), N = 7 (AS), N = 10 (YC), N = 8 (YAS).

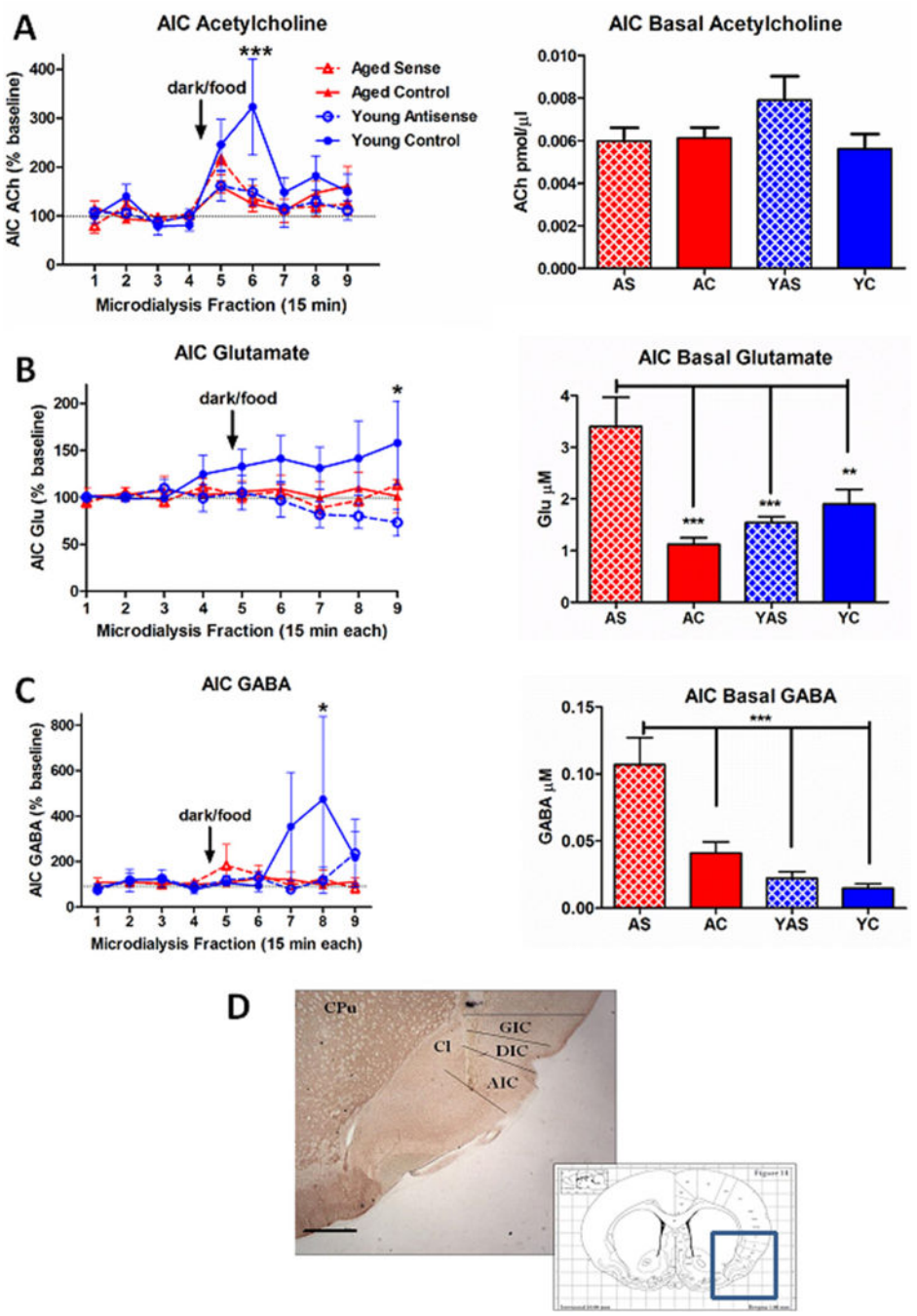

Figure 3.

Modulation of neurotransmitter efflux in insular cortex following virus-mediated gene transfer and presentation of an entrained palatable food stimulus (darkness/food). **A.** (*left*) Young control (YC) rats showed a robust cholinergic response to presentation of the food stimulus that peaked during the second post-stimulus collection. This effect was abolished in aged control (AC) rats and young animals treated with preproorexin antisense (YAS). While there was a trend for preproorexin sense treatment to restore a response to the food stimulus in aged rats (AS), this did not reach significance. (*right*) Neither age nor virus treatment

altered basal ACh levels insular cortex. ***$p < 0.001$ vs. baseline. **B.** (left) Following presentation of the food stimulus, YC rats showed a gradual increase in insular cortex glutamate levels that peaked and reached statistical significance during the final post-stimulus collection. (*right*) Treatment with preproorexin sense virus increased basal glutamate levels in insular cortex of AS rats relative to all other ages and treatment conditions. *$p < 0.05$ vs. baseline. **C.** (*left*) Presentation of the food stimulus was associated with a delayed increase in insular cortex GABA efflux in YC rats that reached significance only during the eighth collection (fourth post-stimulus collection). (*right*) Similar to glutamate, AS rats showed significant elevations in basal GABA levels in insular cortex relative to the other treatment conditions. *$p < 0.05$ vs. baseline. **D.** Representative histochemical verification of microdialysis probe placement. The probe tract typically extended through deeper layers of granular (GIC) and dysgranular (DIC) subdivisions of insular cortex at a level corresponding to roughly Bregma + 1.0 mm (inset; (Paxinos and Watson, 1998)) and had its greatest length in agranular insular cortex (AIC). Other abbreviations: CPu, *caudate-putamen*; Cl, *claustrum*. Scale bar, approximately 1.0 mm. Group sizes: N = 7–10 (AC), N = 6 (AS), N = 7–9 (YC), N = 7–8 (YAS).