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Diminished feeding responsiveness to orexin A (hypocretin 1) in aged rats is accompanied by decreased neuronal activation

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Kotz, Catherine M., Mary A. Mullett, and ChuanFeng Wang. Diminished feeding responsiveness to orexin A (hypocretin 1) in aged rats is accompanied by decreased neuronal activation. Am J Physiol Regul Integr Comp Physiol 289: R359–R366, 2005. First published May 5, 2005; doi:10.1152/ajpregu.00717.2004.—Orexin A is produced in caudal lateral, posterior, perifornical, and dorsomedial hypothalamic areas. Orexin A neurons project throughout the hypothalamic area (rLHa) stimulates feeding and activates several feeding-regulatory brain areas. We hypothesized that aging diminishes feeding and c-Fos-ir in hypothalamic, limbic, and hindbrain regions was measured in two additional sets of rLHa-orexin A injected rats. In a separate study, orexin A effects on feeding and c-Fos-ir were measured in 6-mo-old rats. Orexin A significantly elevated feeding in rats aged 3, 6, and 12 mo in the 0–1 and 1–2-h time intervals, whereas in old rats this was significant in the 1–2-h time interval only. At 1 h, 6–8 (of 14) brain areas showed elevated c-Fos-ir in response to orexin A in 3- and 6-mo-old rats, but 24-mo-old rats exhibited attenuated or absent c-Fos-ir response in all brain regions except the hypothalamic paraventricular nucleus (PVN) and rostral nucleus of the solitary tract (rNTS). Orexin A did not elevate c-Fos-ir in 3-mo-old rats at 2 h after injection, whereas the PVN and mediodorsal thalamic nucleus (MD) showed elevated c-Fos-ir at 2 h in 24-mo-old rats. These data suggest that delayed and diminished feeding responses in old animals may be due to ineffective neural signaling and implicate the orexin A network as one feeding system affected by aging.

feeding behavior; sleep; lateral hypothalamus; aging; c-Fos-immunoreactivity

OREXIN A (ALSO KNOWN AS HYPOCRETIN 1) is a recently characterized neuropeptide that influences feeding behavior (10, 11, 33, 34) and the sleep-wake cycle (41). The lateral hypothalamic area (LHa) has historically been recognized as a central feeding center because of the observation that electrical stimulation of the LHa increases feeding behavior, whereas chemical lesions in this region result in anorexia and death (2). Orexin A neurons are predominant in the caudal LHa, the posterior hypothalamic area (PH), the perifornical area (PeF) and the dorsomedial hypothalamic area (10, 31, 33). Orexin A injected into the rLHa dose-dependently stimulates feeding (11, 34). Orexin A containing neurons project throughout the hypothalamic regions, including the PVN, the arcuate nucleus (Arc), the LHa, the PeF and the ventromedial hypothalamus (VMH), as well as to several extrahypothalamic sites (31). Many of these sites have well-established roles in feeding regulation, and mature orexin A peptide is abundant in several of these regions (27). The two orexin receptors, OX1R and OX2R, are widely distributed throughout the brain (1, 21, 33, 40). We demonstrated that rLHa-injected orexin A activates neurons [as measured by c-Fos-immunoreactivity (c-Fos-ir)] in several feeding-regulatory brain areas (26).

Appetite disorders are common among the elderly and may contribute to the high prevalence of anorexia in this population (24). Studies of age-related changes in physiological mechanisms influencing appetite indicate a decreased desire to eat, reduced meal size, slowing of gastric emptying, and autonomic nerve dysfunction (4, 6, 25). The mechanics of taste appear to remain intact in the elderly, because the density and function of taste buds are not altered with age (22, 23). This suggests that processes beyond the taste bud, or the perception of taste and/or caloric stimuli are dissociated from normal eating behavior. Decreased appetite may be associated with a decrease in the hedonic or pleasant aspects of food, which is indicative of inappropriate neural signaling within food-reward pathways. Age-induced alterations in nitric oxide, dynorphin, neuropeptide Y (NPY), cholecystokinin, and cytokines have all been implicated in the early satiation observed in older animals (25). Naloxone, an opioid receptor antagonist, has been shown to be less effective in decreasing feeding in old rats (12), and a recent small study in humans indicated that naloxone inhibited feeding in young subjects to a slightly greater (but insignificant) degree than that in the old subjects (20). A recent study also demonstrated that old (25–27 mo) Wistar rats do not display a feeding response to intracerebroventricular injections of orexin A (36).

On the basis of the demonstrated role of orexin A in feeding regulation and evidence suggesting that signaling in appetite regulatory brain pathways in aged animals may be altered, we hypothesized that old age diminishes the feeding response to rLHa-injected orexin A and that these differences are accompanied by differences in level of activation in central nuclei with roles in feeding behavior. To test this, we measured feeding and brain c-Fos-ir in response to rLHa-injected orexin A in several age groups of Fischer 344 rats.

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**MATERIALS AND METHODS**

**Animals.** Male Fischer 344 rats (National Institute of Aging derived, Harlan, Indianapolis, IN) were individually housed in conventional hanging cages with a 12:12-h light-dark photoperiod (lights on at 0700) in a temperature-controlled room (21–22°C). Teklad-verified Lab Chow (8604), and water was allowed ad libitum, except where noted. For the feeding studies, mean body weight in each group was as follows: 3 mo: 297 ± 7 g; 6 mo: 349 ± 6 g; 12 mo: 335 ± 7 g and 24 mo: 362 ± 6 g. Body weight was significantly different between 3- and 24-mo-old rats (P = 0.0011). Mean body weight of the 6-mo-old rats was significantly different from rats aged 3 mo (P < 0.05) but not significantly different from the 12 or 24-mo-old rats. Mean body weight of the 12 mo-old rats was not significantly different from rats aged 3 mo, 6, or 24 mo (P > 0.1). For the c-Fos-ir studies, mean body weight for each treatment group was as follows: study 1—3 mo, aCSF: 162 ± 3 g; 24 mo, orexin A: 157 ± 4 g; 24 mo, aCSF: 363 ± 8 g; 24 mo, orexin A: 355 ± 14 g and for study 2—3 mo, aCSF: 179 ± 21 g; 3 mo, orexin A: 182 ± 18 g; 24 mo, aCSF: 494 ± 12 g; 24 mo, orexin A: 496 ± 28 g. For the follow-up study in 6-mo-old rats, mean body weights were as follows: aCSF: 333 ± 10 g and orexin A: 348 ± 9 g. In all studies, there were significant differences in body weight between the young and old rats (P < 0.0001), but there were no significant difference in body weight among treatment groups within each age group (P > 1).

**Experimental Design**

**Feeding studies.** rLHa-cannulated rats at ages 3, 12, and 24 mo (n = 12–18 per group) were given graded doses of orexin A (0, .5, 1, and 2 nmol in 0.5 μl) in a repeated-measures, counterbalanced design. The 0.5-nmol and 1-nmol doses were chosen based on what has previously been shown to be effective in eliciting feeding in young Sprague-Dawley rats (20, 22). The 2-nmol dose was included to allow for the possibility that aged animals may require a higher dose of orexin A to elicit feeding responses similar to that induced by lower doses in young rats. Food intake was measured at 1 h, 2 h, and 4 h after injection. Injections were carried out between 1300 and 1500 h. This injection time was chosen based on our previous work demonstrating that orexin A in the rLHa significantly stimulates feeding during this time of day (38). There were at least 72 h between injections. Three-month-old rats are undergoing rapid growth and are of significantly lower body weight than 12- or 24-mo-old rats. To determine whether these metabolic differences influence orexin A-induced feeding responses, a second study in 6-mo rLHa-cannulated rats (n = 9) of similar body weight as 12- and 24-mo-old rats was performed. The same feeding study design listed above was used.

**c-Fos studies.** rLHa-cannulated rats aged 2–3 mo and 24 mo were injected with either artificial cerebrospinal fluid (aCSF) or orexin A (1 nmol/0.5 μl) (study 1: n = 7–9 per treatment group; study 2: n = 5 per treatment group). At 1 h (c-Fos study 1) and 2 h (c-Fos study 2) after injection, the rats were perfused and the brains processed for c-Fos immunohistochemistry. No food was allowed after injection. Injections were carried out between 1300 and 1500 h. A set of 6-mo-old rLHa-cannulated rats was tested for c-Fos-ir response 1 h after injection of orexin A. These rats (n = 9) were the same rats used for the feeding study. Ten days elapsed between studies. Brains were processed as indicated above. All experiments were reviewed by and received approval from the Minneapolis Veterans Affairs Medical Center Institutional Animal Care and Use Committee.

**Drugs.** Orexin A was purchased from Phoenix Pharmaceuticals (Mountain View, CA) and was dissolved in aCSF, aliquoted and kept at 4°C until needed for a maximum of 2 wk. The orexin A used in the first feeding study and the first two c-Fos-ir studies were from the same lot. The orexin A used in the follow-up feeding and c-Fos-ir studies in 6-mo-old rats was by necessity from a different lot.

**Cannulation.** Rats were anesthetized with Nembutal (40 mg/kg) and fitted with a 28-gauge stainless steel cannula placed just above the rLHa region. Stereotaxic coordinates were determined from the rat brain atlas of Paxinos and Watson (30) and are as follows: −2.0 mm lateral and −2.1 mm posterior to bregma, and 7.3 mm below the skull surface. The injector extended 1 mm beyond the end of the guide cannula. For all cannulations, the incisor bar was set at 3.3 mm below the ear bars. These coordinates were chosen to target the rLHa region that we previously found produces the most robust feeding response to injected orexin A (38). At least 10 days elapsed after surgery before experimental trials.

**Injections.** Injection cannulae (33 gauge) were purchased from Plastics One, (Roanoke, VA). They are fabricated such that when inserted to their maximum depth, they protrude 1.0 mm beyond the tip of the guide cannula. Volume of 0.5 μl was injected slowly over 30 s, with injector left in place an additional 15 s to ensure extrusion from the tip and to minimize distribution of drug upward on the cannula tract. After injection, this cannula was withdrawn, the stylet replaced, and the rat returned to its home cage.

**Verification of injection.** Cross sections of the rLHa region were stained (with antibody to c-Fos in the c-Fos studies or thionin for the feeding study) and examined under a dissecting scope to determine placement based on the rat brain atlas of Paxinos and Watson (30). In determining correct placement, we measured a circle with a 0.25-mm radius from the observed injection site. If the correct site lay within this radius, we judged the placement to be correct. If the correct site did not lie within this radius, the placement was judged to be incorrect. This method is based upon diffusion coefficients for brain-injected substances, assuming limited uptake at the site of injection (8, 28). For the first feeding study, there were nine misplaced cannulae (out of 57): one from the 3-mo-old group, five from the 12-mo-old group and three from the 24-mo-old group. For the first two c-Fos studies, all cannulae were deemed correctly placed. For the follow-up feeding study and c-Fos-ir study in 6-mo-old rats, there was one misplaced cannula. Data from animals with incorrectly placed cannulae were excluded from the statistical analyses. A representative histological photo of a typical injection site is depicted in Fig. 1.

**Tissue preparation for immunohistochemistry.** Animals were anesthetized with pentobarbital sodium (60 mg/kg), killed by cardiac puncture, and then rapidly perfused through the aorta with 100 ml 0.9% sodium chloride. Animals were then slowly perfused with 500 ml 4% paraformaldehyde/phosphate-buffered saline solution. Brains

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**Fig. 1.** Representative brain slice demonstrating a typical injection site. PVN: paraventricular nucleus; opt: optic tract. The dark vertical line is the cannula tract. Magnification is ×2.
were removed, blocked, and stored in 10% sucrose/4% paraformaldehyde/phosphate-buffered saline overnight at room temperature. Brains were transferred into 10% sucrose/Sorenson’s solution and stored at 4°C until sectioning. Frozen sections were cut on a sliding microtome in 40-μm sections through the areas of interest. Every third section was selected for immunohistochemical processing, and thus the first, fourth, and seventh sections (rostro to caudal) were counted. Immediately after slicing, brain sections were placed in cryoprotectant (30% ethylene glycol-10% sucrose in phosphate buffer) and stored at −20°C until use (within 90 d).

c-Fos immunohistochemistry. c-Fos is an intermediate early gene within cell nuclei and elevation of c-Fos protein indicates that a particular neuron has been activated (14). Staining for c-Fos-ir in specific brain sites allows determination of the number of neurons within a particular brain region that were activated in response to a stimulus, and “number of c-Fos-ir cells” indicate the number of cells that are immunoreactive for c-Fos, or “activated” within a specific brain region.

Immunohistochemistry for c-Fos-ir was performed as previously described (26, 29, 32). Briefly, tissue sections were removed from the cryoprotectant, and free-floating sections were rinsed in PBS 4 × 5 min. Endogenous peroxidase was quenched with a 1:1:8 solution of methanol, 30% H2O2, and PBS. After a 4 × 5 min rinse with PBS, sections were incubated in blocking serum (2% normal goat serum, 0.1% BSA, 0.2% Triton X-100 in PBS) for 20 min, and rinsed again in PBS 3 × 5 min. Sections were then incubated in rabbit anti-c-Fos IgG (Oncogene Science, Uniondale, NY) at a dilution of 1:50,000 at 4°C for 48 h. Sections were washed 6–8 × 5 min in PBS and then incubated in biotinylated goat anti-rabbit IgG (1:400, Vector Laboratory, Burlingame, CA) for 1 h at room temperature. Sections were rinsed 4 × 5 min in PBS and then incubated in Vectastain Elite ABC reagent (1:800, Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Sections were rinsed 2 × 5 min in PBS and 3 × 5 min in Trizma buffer (60.06 g HCl and 1.39 g base/l; Sigma, St. Louis, MO). Sections were then incubated with DAB (5 mg)-nickel sulfate in Trizma buffer (60.06 g HCl and 1.39 g base/l; Sigma, St. Louis, MO) for 2.5 min. The DAB reaction was stopped with distilled water after ~10 min, and the sections were rinsed 3 × 5 min with distilled water. Sections were then mounted for analysis.

Tissue analysis and quantification. Stained and mounted sections were viewed via light microscopy with an Optronics (Goleta, CA) TEC-470 cooled CCD camera mounted on a Leitz Orthoplan 2 microscope. For each area, three sections per animal most representative of the site under study were selected, and the images were fed into a Power Macintosh G3 computer and analyzed using National Institutes of Health Image 1.51 software. This allows quantification of c-Fos-ir positive nuclei within a measured area. The output is the number of c-Fos-ir-positive nuclei per 0.1 mm². Data from three slices per animal were averaged and represents the value for that animal. For the rLHa, there were occasionally sections that contained distortion due to the injection. On the basis of the idea that there could be artifactual c-Fos-ir staining due to damage at the injection site, these values were removed from the analysis. In the first c-Fos-ir study, 19 brain regions were selected for analysis. These sites included hypothalamic and thalamic regions (Arc, MD, rLHa, PeF, PH, PVN, and VMH), limbic regions (bed nucleus of the stria terminalis, BNST; central nucleus of the amygdala, CeA; nucleus accumbens, core, NaccC and shell, NaccSs), and hindbrain regions (nucleus of the solitary tract, rostral, nNTS, caudal, cNTS and medial, mNTS extents). In the second c-Fos-ir study, five of the hypothalamic/thalamic brain regions were analyzed: MD, rLHa, PeF, PH, and PVN. In the third c-Fos-ir study (6-mo-old rats), 12 brain regions were analyzed: MD, rLHa, PeF, PH, PVN, BNST, CeA, NaccC, NaccSs, rNTS, cNTS, and mNTS.

Statistics

Feeding studies. For the initial analysis, a three-factor ANOVA, with time, treatment, and age as independent variables, and food intake as the dependent variable, was performed. Food intake values within each time-point were then analyzed by two-factor repeated-measures ANOVA, with treatment and age as the independent variables. The endpoint was food intake, with time intervals compacted (0–1 h, 1–2 h, and 2–4 h). When main effects due to treatment and age were observed, each time-interval and age group were analyzed by one-factor repeated-measures ANOVA, followed by paired t-tests to determine differences among treatment means.

c-Fos studies. In the first two c-Fos-ir studies, the mean number of c-Fos-ir positive nuclei for each brain site examined within each experimental group was analyzed by two-factor ANOVA, with treatment and age representing the independent variables. When main effects were observed, the effect of orexin A on c-Fos-ir in each brain site and in each age group was analyzed by one-factor ANOVA followed by paired t-tests to determine differences among treatment means. For the follow-up c-Fos-ir study in 6 mo-old rats, data were analyzed by one-factor ANOVA, with treatment as the independent variable and c-Fos-ir in each brain region the independent variable.

RESULTS

Feeding studies. Three-factor ANOVA, with time, treatment, and age as independent variables, and food intake as the dependent variable indicated significant main effects of each of these factors on the feeding response (F2,528 = 3.444, P = 0.0327; F3,528 = 7.160, P = 0.0001; F5,528 = 4.921, P = 0.0076, respectively). Separate two-factor ANOVAs (age and treatment as the independent variables) within each time interval indicate that in the 0–1 h time interval, food intake was significantly increased in the 3-mo-old animals (F3,51 = 7.666, P = 0.0003, Fig. 2A) and the 12-mo-old animals (F3,30 = 4.857, P = 0.0072, Fig. 2A) but not in the 24-mo-old animals (F3,51 = 0.321, P = 0.8103, Fig. 2A). Post hoc analysis of the 0–1 h time interval in the 3- and 12-mo-old rats indicates that all doses of orexin A significantly stimulated feeding (P < 0.04, Fig. 2A). In the 1- to 2-h time interval, food intake was significantly stimulated in all three age groups (3 mo: F3,51 = 11.525, P < 0.0001; 12 mo: F2,33 = 2.753, P = 0.0581; 24 mo: F3,51 = 100.022, P < 0.0001, Fig. 2B). Post hoc analysis of the 1–2 h time interval indicates that in the 3-mo- and 24-mo-old rats, all doses of orexin A significantly stimulated feeding (P < 0.02, Fig. 2B). In the 12-mo-old rats, only the highest dose of orexin A significantly stimulated feeding (P = 0.0304, Fig. 2B). To determine whether baseline feeding response was different among age groups, we ran an analysis of the feeding response in aCSF-treated rats only in each time interval (age was the independent factor and food intake in the aCSF-treated rats only was the dependent variable) and found no statistically significant differences at either time point: 0–1 h: F2,44 = 1.944, P = 0.1552; 1–2 h: F4,44 = 0.376, P = 0.6890). In the 6-mo-old rats, food intake was significantly increased by 0.5 nmol orexin in the 0–1 h interval and 2-nmol dose of orexin in the 1–2 h interval (P = 0.0267 and P = 0.0230, respectively, Fig. 2, A and B). Although this study was performed and the data analyzed separately from that of the 3, 12, and 24 mo rats, the data have been included in the same figure for ease of comparison across groups. Data from the 2- to 4-h time interval in all studies were analyzed, and rats that ate in the first 2 h (mostly orexin-treated rats) showed decreased intake in the 2–4 h time interval (data not shown), as has been reported.
previously (38) and likely reflects a compensatory response to previously elevated feeding rather than to orexin A itself. However, complete compensation does not occur, as 0–4 h intake in orexin A-treated rats in all age groups was significantly higher than that in the control groups (data not shown).

c-Fos Studies

Study 1. Brain sites chosen for examination were sites involved in feeding regulation that had been previously identified as showing elevated c-Fos-ir after rLHa injection of orexin A in 3-mo-old Sprague-Dawley rats (26). Our previous experience with Sprague-Dawley rats suggested that the use of this strain may be problematic because of its relatively early and unexpected mortality. F344 rats were used in the current study to represent a more feasible and widely used aging model. In hypothalamic and thalamic regions, two-factor ANOVA indicated that there were significant main effects of treatment and/or age in the rLHa, MD, PeF, PH, and PVN (*P < 0.05, Fig. 3). In the rLHa, MD, PeF and PH, orexin A injection significantly elevated c-Fos-ir in the young animals but not in the old animals (Fig. 3). In the PVN, orexin A treatment significantly elevated c-Fos-ir in both age groups (*P < 0.05, Fig. 3). There were no main effects of treatment or age in the Arc or VMH (*P > 0.05, Fig. 3). In the limbic regions, there were significant main effects of treatment and/or age in the BNST and the NAccC (*P < 0.04, Fig. 4). Post hoc analysis indicates that orexin A treatment significantly stimulated c-Fos-ir in the BNST and the NAccC in young (*P < 0.05, Fig. 4) but not old animals and that orexin A treatment did not significantly influence c-Fos-ir in the CeA or NAccSh in either age group (*P > 0.05, Fig. 4). In the hindbrain, there were main effects of orexin A treatment on c-Fos-ir in the rNTS and in the mNTS (*P < 0.05, Fig. 5). Post hoc analysis indicates that

Fig. 2. Effect of orexin A injection (0.5, 1, and 2 nmol) on feeding behavior in the (A) 0–1 h and (B) 1–2 h postinjection intervals in rats aged 3, 6, 12, and 24 mo. **P < 0.006 and *P < 0.04 compared with artificial cerebrospinal fluid (aCSF)-treated animals within each time interval and each age group.

Fig. 3. Effect of orexin A injection (1 nmol) on c-Fos-immunoreactivity (c-Fos-ir) (mean number of c-Fos-positive cell nuclei per area ± SE) in hypothalamic and thalamic brain regions in young and old animals. *Orexin A injection resulted in a significant (*P < 0.05) elevation of c-Fos-ir compared with aCSF-treated animals, within the respective age category. Arc, hypothalamic arcuate nucleus; rLHa, rostral lateral hypothalamic area; MD, mediodorsal thalamic nuclei; PeF, perifornical hypothalamus; PH, posterior hypothalamus; PVN, hypothalamic paraventricular nucleus; VMH, ventromedial hypothalamus.

Fig. 4. Effect of orexin A injection (1 nmol) on c-Fos-ir (mean number of c-Fos-positive cell nuclei per area ± SE) in limbic brain regions in young and old animals. *Orexin A injection resulted in a significant (*P < 0.05) elevation of c-Fos-ir compared with aCSF-treated animals, within the respective age category. BNST, bed nucleus of the stria terminalis; CeA, central nucleus of the amygdala; NAccC, nucleus accumbens, core; NAccSh, nucleus accumbens, shell. n = 6–9 per treatment group.
c-Fos-ir in the rNTS was significantly elevated by orexin A treatment in both age groups (P < 0.04, Fig. 5) but that c-Fos-ir in the mNTS was not significantly influenced by treatment in either age group (P > 0.1, Fig. 5). There was no significant main effect of treatment or age on c-Fos-ir in the cNTS (P > 0.6, Fig. 5). To determine whether basal expression of c-Fos-ir was different between young and old animals, data from the aCSF-treated rats were analyzed by one-factor ANOVA, with age as the independent variable and c-Fos-ir as the dependent variable. Although in many brain regions there was reduced basal c-Fos-ir in the old rats compared with that in the young rats, these differences were not significant (P > 0.08).

Study 2. Because aged rats demonstrated a delayed feeding response to orexin A compared with young rats (feeding occurred 2 h after injection in aged rats and 1 h after injection in young rats), activation of some brain sites may be coincidentally delayed. Thus, in this study, we killed animals at 2 h after injections, instead of at 1 h after injection as in study 1. Brain sites chosen for examination were hypothalamic/thalamic sites involved in feeding regulation that were identified as showing elevated c-Fos-ir after rLHa injection of orexin A in young rats in the first c-Fos-ir study. Two-factor ANOVA indicated that there were significant main effects of treatment and/or age in all brain regions analyzed (P < 0.05, Fig. 6). For the MD and PVN, orexin A injection significantly elevated c-Fos-ir in the old animals (P = 0.0167 and P = 0.0115, respectively, Fig. 6) but not in the young animals (P = 0.2963 and P = 0.8528, respectively, Fig. 6). For the rLHa, PeF and PH, orexin A injection did not significantly alter c-Fos-ir in either age group. To determine whether basal expression of c-Fos-ir was different between young and old animals, data from the aCSF-treated rats were analyzed by one-factor ANOVA, with age as the independent variable and c-Fos-ir as the dependent variable. Although in all brain regions, there was reduced basal c-Fos-ir in the old rats compared with that in the young rats, this was only statistically significant for the rLHa, PVN, and PeF (P < 0.04).

Study 3 (6-mo-old rats). The purpose of this study was to determine whether 3-mo-old and 6-mo-old rats differed in their c-Fos-ir response to orexin A. Brain sites chosen for analysis were those in which c-Fos-ir was significantly affected or showing a trend toward an effect due to orexin A in the 3-mo-old rats in study 1. Brain regions showing a significant elevation of c-Fos-ir in response to an injection of 1 nmol orexin in 6-mo-old rats include the PVN (P = 0.0051, Fig. 7), the MD (P = 0.0395, Fig. 7), the rLHa (P = 0.0341, Fig. 7), the PeF (P = 0.0312, Fig. 7) the rNTS (P = 0.0136, Fig. 7), and the mNTS (P = 0.0162, Fig. 7). Brain areas not significantly affected (P > 0.05) were the PH, the BNST, the CeA, the NAccC, and the NAccSh (Fig. 7).

![Fig. 5. Effect of orexin A injection (1 nmol) on c-Fos-ir (mean number of c-Fos positive cell nuclei per area ± SE) in hindbrain regions in young and old animals. *Indicates that orexin A injection resulted in a significant (P < 0.05) elevation of c-Fos-ir compared with aCSF-treated animals, within the respective age category. cNTS, nucleus of the solitary tract, caudal extent; mNTS, nucleus of the solitary tract, medial extent; rNTS, nucleus of the solitary tract, rostral extent. n = 6–9 per treatment group.](http://ajpregu.physiology.org/)

![Fig. 6. Effect of orexin A injection (1 nmol) on c-Fos-ir (mean number of c-Fos-positive cell nuclei per area ± SE) in five hypothalamic regions in young and old animals. *Orexin A injection resulted in a significant (P < 0.05) elevation of c-Fos-ir compared with aCSF-treated animals, within the respective age category. n = 5 per treatment group.](http://ajpregu.physiology.org/)
DISCUSSION

The current study shows that aged animals have delayed and diminished feeding (Fig. 2, A and B) and absent or diminished neuronal activation (as measured by c-Fos-ir) in several brain regions important to feeding behavior (Figs. 3–5), in response to orexin A given into the rLHa. Increasing the length of time after injection before analyzing for c-Fos-ir resulted in elevated c-Fos-ir in the MD of old rats (Fig. 6), which was not observed at 1 h after injection, suggesting delayed activation of this region. Elevated c-Fos-ir in the PVN observed in young and old rats killed 1 h after injection was also present in old animals, but not in young animals, 2 h after stimulation with orexin A, which could reflect prolonged activation in old animals. A comparable study showed that aged (25–27 mo) Wistar rats do not display a feeding response to ventricular injections of orexin A (36). Taken together, these data suggest that diminished feeding responses after orexin A administration in older animals may be due to ineffective or delayed neural signaling in feeding-regulatory brain regions.

The mechanism underlying decreased responsiveness to orexin A is currently unknown. Studies in mice show no significant changes in brain orexin mRNA with age (37), but in the same study, it was shown that OX1R and OX2R levels (as measured by receptor mRNA) were diminished in several brain regions (37). If this is true for aged rats, one potential mechanism for delayed feeding and absent or diminished c-Fos-ir response to orexin A injections may be diminished receptor levels, although concurrent measures of receptor binding capacity would more definitively determine this possibility. A study in cats indicates that brainstem orexin A containing terminals are decreased with age (45), whereas an analysis of cerebrospinal fluid in patients ranging from 0 to 79 years indicate no significant differences in CSF orexin A levels due to age (15).

Fasting-induced feeding and elevation in gene expression of several orexigenic neuropeptides, including neuropeptide Y, AGRP, or their receptors, are blunted in aged animals, suggesting that interoceptive cues mediating hunger during fasting may not elicit responses seen in young animals (9, 43). Feeding response to exogenous AGRP remains intact in old animals (42), whereas feeding response to other orexigenic neuropeptides, including neuropeptide Y and opioids are diminished (3, 12). Opioids are associated with meal maintenance (19), and age-related declines in food intake are associated with shorter duration of meals (4), suggesting that diminished opioid signaling during aging may result in shorter meal duration. These findings, together with our current data and that of others showing decreased feeding responsiveness to orexin A (36) suggest that functionality of several orexigenic systems decline with age.

The delayed feeding response to orexin A in old animals could be indicative of several phenomena that may generalize to aging rather than to the orexin system per se, such as decreased receptors, synaptic activity, cell loss, and other components of neural signaling. These hypotheses were not tested in the current studies, but decreases in any of these parameters would be revealed as diminished neural activation after orexin A in the rLHa. If c-Fos protein itself declines with age, it’s possible that other neuropeptides not associated with feeding behavior may show the same diminished c-Fos-ir response as we observed with orexin A, but the finding that baseline values for c-Fos-ir were similar between age groups for most brain regions lessens this concern (Figs. 3–6). Elevated c-Fos-ir after AGRP has been reported in several brain areas (46), and the finding that responsiveness to AGRP is preserved in old animals (42), when responsiveness to other orexigenic neuropeptides is not, argues against nonspecific decreased c-Fos-ir responsiveness in old animals.
Changes in c-Fos expression and c-Fos-ir reflect neuronal stimulation (14), but there is an approximate 30- to 90-min gap in time between neuronal stimulation and observable c-Fos-ir. Increasing the time between orexin A injection and c-Fos-ir analysis allows increased accumulation of c-Fos protein. In cases in which neuronal stimulation is delayed, diminished, or prolonged, extending this lag time may allow for an observable response in c-Fos-ir. Allowing a longer time between injection and brain processing for c-Fos-ir in our study indicated continued neural responsiveness in the PVN and new activation of the MD, which was not evident in the young animals (Fig. 6). Thus increasing the lag time between stimulation and c-Fos-ir processing resulted in elevated neuronal signaling in the MD and revealed prolonged stimulation of the PVN, suggesting that these two regions are important to feeding in aged animals. In addition to the PVN, the hindbrain rNTS showed neural responsiveness to orexin A injection in old animals at the early time point. Although the phenotypes of the activated neurons are unknown, NPY in the PVN and opioids in the NTS are functionally connected in the regulation of feeding (16, 17). Orexin A-induced feeding requires functional NPY signaling (44) and thus the maintenance of signaling in both PVN and NTS after orexin A stimulation in aged rats may represent preservation of this pathway. It is possible that these two sites are fundamentally important for processing feeding signals in aged animals, although other brain areas not measured in this study may also be involved.

The chemical phenotypes of the neurons affected by orexin A injection into the rLHa are unknown, but the two orexin receptors, OX1R and OX2R, are expressed by neurons in the LHa containing melanin-concentrating hormone (MCH). MCH is an orexigenic neuropeptide (1, 7), and it is possible that orexin-feeding responses are mediated by MCH. In the limbic regions tested, there was no c-Fos-ir response to orexin A stimulation in aged animals, which may imply diminution of reward pathways due to age. Several studies show decreased expression of pro-opiomelanocortin, proenkephalin, and pro-dynorphin in aged animals (13, 18, 35), which may contribute to the lack of orexin A-induced c-Fos-ir in limbic regions in the present study. Future studies using double-labeling techniques will be necessary to assess the chemical phenotypes of neuronal populations affected by rLHa orexin A stimulation in aged animals.

On the basis of the possibility that the exponential growth curve of 3-mo-old rats renders them inappropriate controls for aged (24 mo) rats, we tested the effect of orexin A in the rLHa on feeding behavior and c-Fos-ir response in 6-mo-old adult rats to determine whether the responses were similar to that of 3-mo-old rats. As shown in Figs. 2 and 7, the feeding and c-Fos-ir responses were similar in 3-, 6-, and 12-mo-old rats, albeit with some differences. In the feeding study, there were fewer effective doses in the 6-mo-old rats than that observed in the 3- and the 12-mo-old rats. We suspect this was because the study of 6-mo-old rats was performed in a smaller group of animals, yielding less power to detect statistically significant differences. Likewise, differences in the c-Fos-ir results, though essentially very similar, may be due to decreased power. With the exception of the PH and the NAccC, all regions in which c-Fos-ir were significantly elevated in response to orexin A in 3-mo-old rats were comparably affected in the 6-mo-old rats. Together, these data indicate that the lower body weight and different metabolic profile of the 3-mo-old rats does not translate into different feeding and c-Fos-ir responses to orexin A compared with that in adult, 6-mo-old rats. Brain development of neural pathways underlying fundamental physiological processes such as feeding behavior is completed very early on, and thus central processing of orexigenic stimuli may be somewhat less affected by maturation from juvenile to adult.

Orexin A has multiple physiological roles (41), and c-Fos-ir results reported here likely reflect functions in addition to feeding, but several of the brain sites activated by rLHa-injected orexin A in this study and in Sprague-Dawley rats (26), including the mNTS, rLHa, NAccSh, PVN, CeA, and BNST, have also been shown to be activated during normal feeding or refeeding paradigms (29, 32, 39). Further, prepro-orexin is elevated in response to fasting (5, 33), which is the expected response of an orexigenic signal neuropeptide. In summary, the present data provide evidence of aging-induced decreases in functioning of orexin A neural signaling pathways and may have implications for dysregulation of energy intake observed in the elderly.

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