Neurochemical phenotype of hypothalamic neurons showing Fos expression 23 h after intracranial AgRP

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Agouti-related protein (AgRP) is coexpressed with neuropeptide Y (NPY) in a population of neurons in the arcuate nucleus (ARC) of the hypothalamus and stimulates food intake for up to 7 days if injected intracerebroventricularly. The prolonged food intake stimulation does not seem to depend on continued competition at the melanocortin-4 receptor (MC4R), because the relatively specific MC4R agonist MTII regains its ability to suppress food intake 24 h after AgRP injection. Intracerebroventricular AgRP also stimulates c-fos expression 24 h after injection in several brain areas, so the neurons exhibiting delayed Fos expression might be particularly important in feeding behavior. Thus we aimed to identify the neurochemical phenotype of some of these neurons in select hypothalamic areas, using double-label immunohistochemistry. AgRP-injected rats ingested significantly more chow (10.2 ± 0.6 g) vs. saline controls (3.4 ± 0.7 g) in the first 9 h (light phase) after injection. In the lateral hypothalamus (particularly the perifornical area) 23 h after injection, AgRP induced significantly more Fos vs. saline in orexin-A (OXA) neurons (25.6 ± 4.9 vs. 4.8 ± 3.1%), but not in melanin-concentrating hormone (MCH) or cocaine- and amphetamine-regulated transcript (CART) neurons. In the ARC, AgRP induced significantly more Fos in CART (40.6 ± 5.9 vs. 13.4 ± 1.8%) but not NPY neurons. In the paraventricular nucleus, there was no significant difference in Fos expression induced by AgRP vs. saline in oxytocin and CART neurons. We conclude that the long-lasting hyperphagia induced by AgRP is correlated with and possibly partially mediated by hyperactive OXA neurons in the lateral hypothalamus and CART neurons in the ARC, but not by NPY and MCH neurons. The substantial increase in light-phase food intake by AgRP supports a role for the arousing effects of OXA. Activation of CART neurons in the ARC (which likely coexpress proopiocortin) could indicate attempts to activate counterregulatory decreases in food intake. AgRP competes with α-melanocyte-stimulating hormone (α-MSH) for the melanocortin-4 receptor (MC4R) and thus acts as an endogenous antagonist of MC4R (38, 49, 69). Stimulation of MC4R in the hypothalamus with the natural ligand α-MSH (63) or with pharmacological agonists such as MTII (46, 61) suppresses food intake. In contrast, intracerebroventricular injection of AgRP induces robust food intake (54) that lasts for several days (29). In addition, overexpression of AgRP (27) and chronic facilitation of AgRP receptor binding via overexpression of syndecan (51) lead to obesity in transgenic mice.

The long-lasting effect of AgRP on food intake (29) is unique among orexigenic and anorexigenic peptides, and the mechanism of the protracted action is not known. The two main possibilities for the protracted action of AgRP are that it 1) is slowly degraded and continuously interacts with specific receptors or 2) produces downstream signaling changes distal to its initial site of receptor interaction. Hagan and co-workers (29) have tested the first possibility by injecting AgRP and the MC4R agonist MTII either simultaneously or with a 24-h interval. When MTII was injected simultaneously with AgRP, it lost its ability to suppress food intake, suggesting direct competition for the MC4R (29). However, when injected 24 h after AgRP, MTII regained its ability to suppress food intake, suggesting that AgRP was no longer competing for the MC4R at that time (29). Although it is possible that AgRP continued to act on another, not yet identified receptor, this outcome pointed to the possibility that a single AgRP injection produced changes in activity of the neural network for feeding distal to the initial site of stimulation. This interpretation was reinforced by the subsequent observation (28) that parallel to increased food intake, expression of the immediate-early gene...
c-fos was increased in the lateral hypothalamic area, central nucleus of the amygdala, nucleus accumbens, and nucleus of solitary tract. Each of these brain areas has been implicated in the neural control of food intake in numerous reports, and it has been speculated that these heavily interconnected key areas are part of a central circuit making information available to several parallel processing loops (5). AgRP-induced Fos expression could thus be used as a probe to identify specific neuron populations involved in feeding behavior.

The aim of the present study was to identify the neurochemical phenotype of some of the neurons showing c-Fos expression 23 h after a single intraventricular injection of AgRP. The lateral hypothalamic area exhibited the highest number of Fos-positive neurons in the study by Hagan et al. (28), and it has been demonstrated to harbor separate populations of neurons expressing melanin-concentrating hormone (MCH) (4, 9), orexin A (OXA) (48), and cocaine- and amphetamine-regulated transcript (CART) (12, 22), peptides that affect food intake when injected in the brain. Thus, using double-label immunohistochemistry, we looked at expression of these peptides in neurons of the rat lateral hypothalamus, as well as other areas of the hypothalamus, that exhibited Fos expression 23 h after a single intracerebroventricular injection of AgRP.

MATERIALS AND METHODS

Animals and housing. Twelve adult male Sprague-Dawley rats (Harlan Industries, Indianapolis, IN), weighing 280–320 g at the time of surgery, were housed individually in hanging wire mesh cages in a climate-controlled room (22 °C) on a 12:12-h light-dark cycle with lights on at 0700 and lights off at 1900. Food and water were available ad libitum except as specified below.

Intracerebroventricular injections of AgRP. Animals were anesthetized with ketamine, acepromazine, and xylazine (80/16/5.4 mg/kg sc) and given atropine (1 mg/kg ip). A 24-gauge stainless steel guide cannula (Plastics One) was aimed at the lateral ventricle (0.9 mm posterior to bregma, 1.6 mm lateral to midline, and 3.5 mm below the skull) or the third ventricle (2.8 mm posterior to bregma, on midline, and 8.1 mm below the skull). Fifteen days were allowed for recovery from surgery, at which time the animals went through repeated mock surgery, followed by AgRP injections at a volume of 3 μl/20 g. Consistent with this assumption, all rats had consumed 15 g of food at the time of euthanasia. Thus rats were most likely food deprived for ~6 h, comparable to the period of food deprivation in the study by Hagan et al. (28). Complete overnight food deprivation was not considered, because it may have caused stress, increased locomotion, and nutrient depletion, which are known to activate Fos expression in the hypothalamus (11).

Tissue processing and immunohistochemistry. Rats were deeply anesthetized with pentobarbital sodium (120 mg/kg) and transcardially perfused with heparinized saline (20 U/ml) followed by ice-cold, 4% phosphate-buffered (pH 7.4) paraformaldehyde. The brains were extracted, blocked, and postfixed in the same fixative overnight. Tissue was immersed for 24 h in 25% sucrose in 4% paraformaldehyde before cryosectioning. Frozen sections of 30 μm were cut in a cryostat, separated into five series, and either processed immediately or stored in cryoprotectant solution at –20°C.

One complete set of sections (10–15 sections) of one in five sections stretching the entire rostrocaudal dimension of the hypothalamus was processed for c-Fos immunohistochemistry using the avidin-biotin complex (ABC)–3,3′-diaminobenzidine tetrahydrochloride (DAB) method. All processing was done on free-floating sections. Briefly, the tissue was pretreated with a solution of 1% sodium borohydride in PBS. Appropriate washes in PBS followed this and subsequent incubations. For quenching endogenous peroxidase, sections were treated with 3% hydrogen peroxide-methanol (1:4) before blocking for 1 h in a solution of PBS with 0.5% Triton X-100 (PBST) containing 5% normal goat serum (NGS) and 1% BSA. Incubation in c-Fos primary antibody (Table 1) was for 20 h at room temperature, followed by 2-h incubation in biotinylated goat anti-rabbit secondary antibody (1:500; Jackson Immunoresearch, West Grove, PA). The sections were then incubated for 1 h in ABC (1:500; Vectastain ABC Elite kit, Vector Labs, Burlingame, CA). The blue-black nuclear Fos was visualized using a metal-enhanced DAB substrate kit (Pierce Chemical, Rockford, IL).

Sequential double labeling for various peptides, including CART, MCH, NPY, oxytocin (OT), and OXA, followed the Fos staining (Table 1). With the exception of the OXA antibody, the protocol was for simple immunofluorescence. Sections were incubated in the blocking solution described above once more before incubation in the second primary antiserum for 40 h at 7°C. Alexa 594 goat anti-rabbit IgG (1:2,000; Molecular Probes, Eugene, OR) was applied for 2 h at room temperature in the dark, and after 1 h in 70% glycerol; the sections were mounted in 100% glycerol with the anti-fade agent 5% n-propyl gallate added.

Table 1. Source and dilution of antibodies used

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Host</th>
<th>Dilution</th>
</tr>
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<tbody>
<tr>
<td>c-Fos (AB-5)</td>
<td>Oncogene, Cambridge, MA</td>
<td>Rabbit</td>
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<tr>
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<td>Phoenix, Belmont, CA</td>
<td>Rabbit</td>
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<td>MCH (H07047)</td>
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<td>Rabbit</td>
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</tr>
<tr>
<td>NPY (22940)</td>
<td>DiaSorin, Stillwater, MN</td>
<td>Rabbit</td>
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</tr>
<tr>
<td>OXA (PC345)</td>
<td>Oncogene, Cambridge, MA</td>
<td>Rabbit</td>
<td>1:2,500*</td>
</tr>
<tr>
<td>OT (20063)</td>
<td>DiaSorin, Stillwater, MN</td>
<td>Rabbit</td>
<td>1:1,500</td>
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</tbody>
</table>

CART, cocaine- and amphetamine-regulated transcript; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; OXA, orexin A; OT, oxytocin. *With biotinylated tyramine amplification.
The OXA antibody was stained using biotinylated tyramine amplification. After Fos staining, the sections were again treated with hydrogen peroxide-methanol and blocked with the NGS-BSA-PBST solution. Orexin primary antibody incubation followed for 20 h at room temperature and the biotinylated goat anti-rabbit secondary antibody (1:500; Jackson) incubation for 2 h at room temperature. ABC (1:500; Vector) was applied for 1 h, biotinylated tyramine (1:200 in PBS-0.02% H$_2$O$_2$) for 15 min, and Texas Red streptavidin (500; Vector) was applied for 1 h, biotinylated goat anti-rabbit secondary antibody (1:500; Vector) for 2 h at 7°C, covered. After final washing in PBS, the tissue was cleared in 70% glycerol before mounting in the n-propyl gallate solution.

**Counting procedures and statistical analysis.** For the quantitative assessment of Fos expression, sections from one series containing the lateral hypothalamic area and the ARC (from just caudal to the PVN (−2.4 mm) to the prehymalicular nucleus (−4.2 mm)) or the PVN (−1.6 to −2.4 mm) were selected. For the ARC and PVN, the area of interest was circled, and for the lateral hypothalamic area, a rectangular box was placed over an area centered around the fornix, using an interactive image analysis system (KS400, Carl Zeiss, Thornwood, NY).

A macro with several processing steps was developed for automatic field counts of Fos-stained cell nuclei. First, images were normalized over the entire intensity range of 0–255 and corrected for differences in local illumination intensity by the addition of inverted low-pass complements. Next, dark-stained elements (Fos-labeled nuclei) were identified by a thresholding step. After filtering through a size and shape criterion, erosion followed by dilation was used to separate labeled cell nuclei that partially occluded one another. The macro was tailored for observation with a ×5 objective at a specified illumination level and calibrated for each batch of sections by comparing manual counts made by two observers.

Counts were performed separately for both hemispheres. Damaged sections (or hemispheres) and sections showing Fos expression induced by the nearby injector cannula track were excluded from analysis. Thus counts from 4 to 12 hemispheres on two to six 30-µm-thick sections were obtained, and an average was calculated for each area and rat. One-way ANOVA was used to test for statistical differences.

Double-labeled neurons were counted manually with the help of a conventional microscope (Zeiss Axioscop). The red fluorescence in the cytoplasm was viewed through the epifluorescence mode simultaneously with the dark DAB-stained cell nuclei through the transmitted light mode. With the use of ×40 objective and ×10 ocular, we inspected each neuron exhibiting cytoplasmic immunofluorescence for the presence of a Fos-positive nucleus by focusing up and down and varying the intensity of transmitted light. Neurons were counted as double labeled if the fluorescent cytoplasm and dark nucleus were in the same focal plane and if both labels were clearly above background. Neurons with only labeled cytoplasm but without a Fos-stained nucleus were also counted. For counts of orexin, MCH, and CART in the lateral hypothalamus, the area was divided into four quadrants, with imaginary vertical and horizontal dividing lines crossing at the ventromedial border of the fornix or with the help of a reticle placed in one ocular. Initially, two independent observers blinded to the treatment condition of the animals counted double-labeled neurons in different areas and with labeling for different peptides. Because there was good agreement (±10% or less), only one person counted the rest of the sections.

Raw counts were averaged over two to six hemispheres from one to three sections, and the percentage of peptide-positive neurons expressing Fos was calculated. Individual t-tests were used for comparisons between AgRP- and saline-injected rats for the different peptides.

**RESULTS**

**Food intake.** AgRP-injected rats (n = 6) consumed significantly more chow than saline-injected control rats (n = 4) during the first 9 h (light phase) after injection (10.2 ± 0.6 g vs. 3.4 ± 0.7 g, P < 0.01) (Fig. 1). All rats consumed the 15 g of preweighed chow during the last 14 h before Fos assessment.

**AgRP-induced Fos expression.** Twenty-three hours after injection, AgRP-injected rats showed significantly higher numbers of Fos-expressing neurons than saline-injected control rats in the lateral (perifornical) hypothalamus (41.3 ± 6.7 vs. 14.5 ± 3.0 labeled nuclei per section and hemisphere, P < 0.01), ARC (60.4 ± 4.8 vs. 23.8 ± 2.4 labeled nuclei per section and hemisphere, P < 0.01), and PVN (48.3 ± 6.5 vs. 14.4 ± 2.6 labeled nuclei per section and hemisphere, P < 0.01) (Figs. 2 and 3).

**Fos expression in OXA neurons.** As reported by Nambu et al. (48), neurons expressing orexin immunoreactivity were widely distributed over most of the lateral hypothalamic area, with a concentration in the perifornical area. It was mainly, but not exclusively, in this area where a considerable number of Fos-positive neurons expressing Fos was significantly higher in AgRP-injected rats (4.8 ± 3.1% vs. 25.6 ± 4.9%, P < 0.01) (Fig. 5A). The total number of orexin neurons was not significantly different for the two treatment conditions (Fig. 5C).

**Fos expression in MCH neurons.** MCH-immunoreactive neurons were roughly codistributed with orexin neurons throughout the lateral hypothalamus as has previously been reported (6, 13). However, in contrast to orexin, many MCH neurons were found in the zona incerta, just dorsal to the lateral hypothalamic area, and in the far-lateral aspects of the lateral hypotha-
lamic area. Only the rare Fos+/MCH+ (double-labeled) neuron was found in either saline- or AgRP-injected rats (Fig. 4C). In a representative sample of a similar number of MCH neurons (Fig. 5D) from the perifornical area, there was no difference in the percentage of MCH neurons expressing Fos (saline, 2.6 ± 1.6%; AgRP, 3.7 ± 1.6%; not significant) (Fig. 5B).

Fos expression in CART neurons. As reported by Broberger (12), significant populations of CART-immunoreactive neurons were found in the ARC and lateral hypothalamus, with smaller populations in other areas of the hypothalamus. In the ARC, most of the CART neurons were located in the ventrolateral part. We found the largest number of Fos+/CART+ (double-labeled) neurons in this area (Fig. 4E). Analyzing the entire ARC, the percentage of CART neurons expressing Fos was significantly higher in AgRP-injected rats than in saline-injected rats (40.6 ± 5.9% vs. 13.4 ± 1.8%, \( P < 0.01 \)) (Fig. 6A).

In contrast to the ARC, few Fos+/CART+ double-labeled neurons were present throughout the lateral hypothalamus and zona incerta. A representative sample from the perifornical area showed no difference in AgRP- and saline-injected rats (0.9 ± 0.05% vs. 0.4 ± 0.3%, not significant). Similarly, only a few CART/Fos double-labeled neurons were found in the PVN (Fig. 4F), and the percentage was not significantly higher in AgRP-injected rats.

Fos expression in NPY neurons. While some neurons scattered in the basal ganglia and cortex exhibited intense NPY immunoreactivity, neurons in the dorsomedial part of the ARC were only weakly stained, and analysis for double labeling was also difficult because of the abundance of brightly stained axon

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**Fig. 2.** Examples of c-Fos expression in the perifornical area of the lateral hypothalamus (A and B), arcuate nucleus (C and D), and paraventricular nucleus of the hypothalamus (E and F), as assessed 23 h after ICV injection of AgRP (1 nmol in 3 \( \mu l \) of sterile saline) or saline. fx, Fornix; 3V, third ventricle.

**Fig. 3.** Quantitative assessment of Fos expression in perifornical area of the lateral hypothalamus (PeF/LH), arcuate nucleus (arcuate n.), and paraventricular nucleus of the hypothalamus (paraventricular n.) induced by AgRP (1 nmol in 3 \( \mu l \) of sterile saline, \( n = 6 \)) or saline (\( n = 4 \)) injected ICV 23 h earlier. c-Fos counts correspond to areas shown in Fig. 2 and reflect average per section. *\( P < 0.01 \), based on separate \( t \)-tests for each area.

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**Fig. 4.** Examples of c-Fos expression in the perifornical area of the lateral hypothalamus (A and B), arcuate nucleus (C and D), and paraventricular nucleus of the hypothalamus (E and F), as assessed 23 h after ICV injection of AgRP (1 nmol in 3 \( \mu l \) of sterile saline) or saline. In a representative sample of a similar number of MCH neurons (Fig. 5D) from the perifornical area, there was no difference in the percentage of MCH neurons expressing Fos (saline, 2.6 ± 1.6%; AgRP, 3.7 ± 1.6%; not significant) (Fig. 5B).

**Fig. 5.** Quantitative assessment of Fos expression in perifornical area of the lateral hypothalamus (PeF/LH), arcuate nucleus (arcuate n.), and paraventricular nucleus of the hypothalamus (paraventricular n.) induced by AgRP (1 nmol in 3 \( \mu l \) of sterile saline, \( n = 6 \)) or saline (\( n = 4 \)) injected ICV 23 h earlier. c-Fos counts correspond to areas shown in Fig. 2 and reflect average per section. *\( P < 0.01 \), based on separate \( t \)-tests for each area.

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**Fig. 6.** Examples of c-Fos expression in the perifornical area of the lateral hypothalamus (A and B), arcuate nucleus (C and D), and paraventricular nucleus of the hypothalamus (E and F), as assessed 23 h after ICV injection of AgRP (1 nmol in 3 \( \mu l \) of sterile saline) or saline. In a representative sample of a similar number of MCH neurons (Fig. 5D) from the perifornical area, there was no difference in the percentage of MCH neurons expressing Fos (saline, 2.6 ± 1.6%; AgRP, 3.7 ± 1.6%; not significant) (Fig. 5B).
terminals in this area (Fig. 4D). In a relatively small sample, we found no difference between AgRP-injected rats (43 neurons/rat, \( n = 4 \)) and saline-injected rats (44 neurons/rat, \( n = 3 \)) in the percentage of NPY neurons expressing Fos (0.5 ± 0.4% vs. 1.5 ± 0.7%; Fig. 6, B and D).

Fos expression in OT neurons. No Fos+/OT+ double-labeled neurons were present in the PVN (Fig. 4G).

DISCUSSION

In this study, we confirm the observation made previously by Hagan et al. (28) that intracerebrally delivered AgRP results in increased Fos expression after 23 h in specific hypothalamic and other brain areas. This is in contrast to the typically transient expression of c-Fos induced by a variety of stimuli, lasting 1–8 h (10, 20, 26, 44, 65). Although the selective MC4R agonist MTII was unable to suppress food intake immediately after AgRP injection, it was readily able to do so when given 24 h after AgRP injection (29). Therefore, the delayed expression of Fos does not seem to depend on a continued action of AgRP on the MC4R, but rather on signaling changes distal to the original site of action, possibly delineating the neural substrate causally
related to the continued increased food intake also observed after AgRP (28).

However, there are alternative explanations for the 23-h Fos expression that we did not directly test in the present study and therefore cannot rule out. AgRP is likely to stimulate other targets in the hypothalamus. It has been demonstrated (40) that AgRP still increases food intake in MC4R-deficient mice.

From the present results and the study by Hagan et al. (28), it cannot be excluded that different populations of neurons expressed Fos immediately after AgRP and 1 day later. Consequently, we cannot rule out that Fos expression in a different population of neurons than the ones described in this study was responsible for the enhanced food intake immediately after AgRP compared with saline. The total numbers of CART (C) and NPY neurons (D) were not different for the 2 treatments. *P < 0.01 (t-test).

Fig. 6. Quantitative assessment of 23-h prolonged Fos expression induced by AgRP in CART and NPY neurons of the arcuate nucleus.

There was a significantly higher proportion of CART neurons (A) but not NPY neurons (B) expressing Fos induced by AgRP compared with saline. The total numbers of CART (C) and NPY neurons (D) were not different for the 2 treatments. *P < 0.01 (t-test).

We found that AgRP resulted in Fos expression 23 h after injection in orexin neurons, but not in MCH and CART neurons of the lateral hypothalamus. All these peptides have previously been implicated in the control of food intake. Orexin and MCH are expressed in separate but roughly codistributed populations of neurons in the lateral hypothalamus with similar projection patterns to most other hypothalamic nuclei as well as to areas in the forebrain, hindbrain, and spinal cord (7, 8, 13, 23, 48). The primary physiological function of hypothalamic orexin neurons seems to be the regulation of arousal state and sleep-wake cycle (16, 24, 31, 56). However, because intracerebral injections of orexin (21, 52, 59, 68) increase food intake and injections of orexin receptor antagonists (33, 52) or anti-orexin antibody (67) decrease food intake, orexin may play an additional physiological role in satiety and energy homeostasis that goes beyond its arousing effects (66). Because 95% of orexin neurons also produce dynorphin (18) and selective ablation of these neurons in mice (in contrast to knocking out only orexin) resulted in late-onset obesity (31), dynorphin could also play a role in the control of energy balance.

It is thus not surprising that some of the neurons activated by AgRP in the present study are of the orexin phenotype. Activation of orexin neurons would likely result in increased release of orexin and dynorphin at their terminals and thus lead to increased arousal and food intake, particularly during the light period when rats normally rest or sleep. This would explain the large increase in food intake during the initial 9-h light period after AgRP injection. When OXA was continuously infused intracerebroventricularly, food intake was only increased during the day (68). Furthermore, natural Fos expression in hypothalamic orexin neurons varied with behavioral state, being highest at night and lowest early in the day (24). Orexin neurons are also activated in various glucopenia models (11, 14, 45), a fuel-depletion signal that is known to stimulate food intake and arousal.

MCH may be more directly involved in the control of food intake and energy balance than orexin (62). MCH knockout mice are hypophagic and lean (57), whereas MCH overexpression causes hyperphagia, obesity, and insulin resistance (39) and intracerebroventricular MCH injections acutely increase food intake (53). On the basis of observations in models of estrogen-induced anorexia in male rats (47), it was suggested that under conditions of food restriction, estrogen acts to inhibit lateral hypothalamic MCH neurons receiving excitatory input from medial hypothalamic NPY neurons. Because ARC NPY neurons coexpress AgRP, it could have been expected that exogenous AgRP would stimulate lateral hypothalamic MCH neurons. Unexpectedly, AgRP injection did not increase Fos expression in MCH neurons in the present study, although this does
not completely rule out that their function was affected via non-Fos-dependent signaling pathways. This latter notion is supported by the observation that while insulin treatment significantly stimulated MCH mRNA expression it did not stimulate c-Fos expression in MCH neurons (4). It will be interesting to elucidate the signaling pathway for increased expression of MCH mRNA and protein.

CART is another peptide produced by neurons throughout the hypothalamus and other brain areas (12, 22) implicated in the control of food intake (1, 35, 64). However, only female CART-deficient mice develop hyperphagia and obesity and only when fed a high-fat diet (2). There are also conflicting results concerning CART’s effects and specificity on food intake, with reports of both decreases (35) and increases (1).

In the lateral hypothalamus, CART is coexpressed in many MCH neurons (12, 22). The lack of AgRP-induced Fos expression in CART neurons in the present study is consistent with the assumption that most of the neurons identified as CART positive are also MCH positive.

In the ARC, CART is coexpressed in proopiomelanocortin (POMC) neurons (23, 64) and in a few neuropeptide-Y neurons (22), but not in NPY neurons. We found the highest percentage of AgRP-induced Fos expression in CART neurons of the ARC, particularly its ventrolateral portion. The fact that α-MSH, a peptide produced by POMC neurons, inhibits food intake seems to conflict with the orexigenic effect of intracerebroventricular injection of AgRP. However, activation of these neurons 23 h after AgRP could indicate a compensatory (or counterregulatory) mechanism by which the overreplete animal is trying to decrease food intake. Because AgRP stimulates food intake beyond 24 h and for up to 5 days (29), it is clear that if such a compensatory mechanism is activated in the ARC, it is ineffective in curbing appetite.

Since arcuate NPY neurons and their projections are considered the most orexigenic peptide system, they might have been expected to show protracted activation by AgRP. However, we did not find increased Fos expression in the limited sample of NPY neurons counted. If NPY and POMC neurons in the ARC are seen as acting in concert to increase or decrease food intake, lack of activation of NPY neurons may be part of the same counterregulatory mechanism mentioned above.

Finally, in the paraventricular hypothalamus, we were unable to associate the increased Fos expression with any neuronal phenotype. It is possible that some of the activated neurons express CART, but the staining intensity was not enough for a quantitative assessment. Antibodies for other peptides such as thyrotropin-releasing hormone (TRH), galanin, and arginine vasopressin and/or probes for in situ hybridization of mRNAs require testing. This also applies to the other examined areas, in which large numbers of Fos-activated neurons remain unaccounted for by their phenotype.

**Perspectives**

A true time course was assessed in only a few of the numerous reports on neural activation-induced Fos expression. Of those true time course studies, a majority found only transient Fos expression lasting no longer than a few hours to a variety of stimuli (e.g., 10, 26, 42, 43). However, prolonged (up to several days) and/or oscillating Fos expression was reported in some studies using hormonal (34, 55, 58) or ischemic (60) stimuli. In addition, long-lasting sensitization of Fos expression induced by certain stimuli was also demonstrated (32, 41). Assuming that the initial stimulus (AgRP acting through a specific receptor) is no longer present after one or more days, the question is what stimulus (stimuli) keeps Fos expression elevated or leads to delayed expression in a particular neuron. In the case of thyroidectomy-induced Fos expression in TRH neurons of the PVN 6 days after thyroidectomy, it was speculated that the decrease in circulating thyroid hormone was the stimulus (34). Interestingly, Fos expression was not increased 1 or 3 days after thyroidectomy (34), even though thyroid hormone levels must have been very low, suggesting more than a simple lack of circulating thyroid hormone as the stimulus for Fos expression.

In the case of AgRP, we know neither the exact physiological function of hypothalamic AgRP nor the whole spectrum of neural and behavioral effects that last longer than a few hours. All of these are potential candidate stimuli for Fos expression after 1 day. The only behavioral change that has been measured is food intake. Because food intake was matched between experimental and control rats and prevented for the last few hours before euthanasia, it is very unlikely to be the stimulus for increased Fos expression in our study. We suggest that intracerebral AgRP may cause lasting or delayed changes in neuronal activity, sensitivity to other stimuli, and/or even neuronal morphology, but these changes need to be identified in future studies.

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AgRP INDUCES Fos IN OREXIN BUT NOT MCH NEURONS


