Diet-derived nutrients mediate the inhibition of hypothalamic NPY neurons in the arcuate nucleus of mice during refeeding

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Submitted 15 December 2008; accepted in final form 24 April 2009

Becskei C, Lutz TA, Riediger T. Diet-derived nutrients mediate the inhibition of hypothalamic NPY neurons in the arcuate nucleus of mice during refeeding. Am J Physiol Regul Integr Comp Physiol 297: R100–R110, 2009. First published April 29, 2009; doi:10.1152/ajpregu.91014.2008.—Fasting activates orexigenic neuropeptide Y neurons in the hypothalamic arcuate nucleus (ARC) of mice, which is reversed by 2 h refeeding with standard chow. Here, we investigated the contribution of diet-derived macronutrients and anorectic hormones to the reversal of the fasting-induced ARC activation during 2 h refeeding. Refeeding of 12-h-fasted mice with a cellulose-based, noncaloric mash induced only a small reduction in c-Fos expression. Refeeding with diets, containing carbohydrates, protein, or fat alone reversed it similar to chow; however, this effect depended on the amount of intake. The fasting-induced ARC activation was unchanged by subcutaneously injected amylin, CCK (both 20 μg/kg), insulin (0.2 U/kg and 0.05 U/kg) or leptin (2.6 mg/kg). Insulin and leptin had no effect on c-Fos expression in neuropeptide Y or proopiomelanocortin-containing ARC neurons. Interestingly, CCK but not amylin reduced the ghrelin-induced c-Fos expression in the ARC in ad libitum-fed mice, suggesting that CCK may inhibit orexigenic ARC neurons when acting together with other feeding-related signals. We conclude that all three macronutrients and also non-nutritive, ingestion-dependent signals contribute to an inhibition of orexigenic ARC neurons after refeeding. Similar to the previously demonstrated inhibitory in vivo action of peptide YY, CCK may be a postprandial mediator of ARC inhibition.

c-Fos; insulin; leptin; amylin; cholecystokinin

THE HYPOTHALAMIC ARCUATE NUCLEUS (ARC) plays a key role in the control of energy homeostasis. The activity of ARC neurons correlates with the nutritional status in mice. Food-deprivation induces c-Fos expression, a widely used marker of neuronal activation (12), specifically in the orexigenic neuropeptide Y (NPY) expressing ARC neurons (5). This activation is rapidly reversed by 2 h refeeding with chow (29, 36). However, which feeding-related factors might signal the transition from the fasted to the fed state to the ARC and thus contribute to the reversal of its activation during refeeding is not clear yet. As we have shown, peripherally applied peptide YY (PYY) and glucose reversed the fasting induced c-Fos expression in the ARC similar to chow (3, 29). Therefore, these factors may be among the signals, which attune hypothalamic activity to the status of energy balance. Nevertheless, there is accumulating evidence that the ARC monitors other meal-associated metabolic and hormonal signals that might be relevant mediators as well. There is also increased research interest in a hypothalamic signaling function of nutrients. A role of glucose to modulate neuronal activity, particularly in the ARC, is well established (9). Several studies imply that intermediates of lipid metabolism might have a similarly large impact (15, 40). An increase of free fatty acid (FFA) availability following intracerebroventricular administration of oleic acid to fasted rats and inhibition of hypothalamic lipid oxidation, reduced food intake and NPY expression (21, 24, 25). Furthermore, oleic acid modulated the firing rate of ARC neurons of unknown phenotype (39). On the other hand, only very few studies investigated the potential role of amino acids (AA)s in the hypothalamic control of food intake. Dietary supplementation of arginine selectively reduced adipose tissue mass in obese Zucker rats (10), and leucine activated the mammalian target of rapamycin pathway in the hypothalamus and thereby specifically decreased the expression of the orexigenic Agouti-related peptide in the ARC (6, 22).

On the basis of these findings we hypothesized that each individual macronutrient plays an important role in the modulation of feeding-related ARC activity. To test this hypothesis, we refed fasted mice with different diets containing only a single macronutrient. First, this allowed us to identify which nutrient is mainly responsible for the reversal of the fasting-induced c-Fos expression after chow refeeding. Second, by varying the amount of the individual nutrients in these diets, we could associate the magnitude of an effect on ARC activity with the amount of intake. These experimental diets were based on a vanilla-flavored noncaloric mash of cellulose (NCM) that is readily eaten by rodents despite its nutrient deficiency. Refeeding the mice with NCM alone without nutrient supplementation allowed us to dissociate the effects of feeding-related nutrient independent stimuli from the impact of macronutrients on the reversal of the fasting-induced ARC activation. To our knowledge, no study has yet investigated a potential role of preabsorptive signals in modulating ARC activity. In all these experiments, not only the metabolic, but also the hormonal profile of the animals was analyzed, because nutrient-stimulated hormone release may be an indirect mechanism in modulating ARC activity. In all these experiments, not only the metabolic, but also the hormonal profile of the animals was analyzed, because nutrient-stimulated hormone release may be an indirect mechanism contributing to meal-related modulation of ARC activity. To test this hypothesis, we also investigated the ability of CCK, amylin, insulin, and leptin to reverse the fasting–induced c-Fos expression in ARC neurons. Although leptin has been conceptualized as an adiposity signal, we tested this hormone because leptin plasma levels also parallel short-term changes in the nutritional state (13). Leptin and insulin are thought to activate anorectic proopiomelanocortin (POMC) and inhibit orexigenic NPY neurons (34). Therefore, we combined immunohistochemistry for c-Fos with immunohistochemistry for green fluorescent protein (GFP) in POMC neurons of transgenic mice and with in situ hybridization histochemistry for...
NPY mRNA in these experiments to distinguish the phenotype of the activated ARC neurons.

**MATERIALS AND METHODS**

*Animals.* Adult male C57BL/6N or transgenic mice expressing GFP in the POMC neurons (kind gift of B. B. Lowell, Beth Israel Deaconess Medical Center, Boston, MA) were used as indicated. All animals were housed individually in a temperature-controlled room (22°C) under a 12:12-h light-dark cycle (lights off 9 AM). Animals were handled daily for at least 3 wk before the experiments according to the procedure recommended by Ryabinin et al. (33) to reduce handling-induced c-Fos expression. All animal procedures were approved by the Veterinary Office of the Canton of Zurich.

The experimental protocol was similar in all experiments. Mice were fed ad libitum or were food deprived during 12 h of the light phase. At dark onset (9 AM), mice received the respective treatment (refeeding or injection) and were killed 2 h later. The fasted groups remained food deprived until this time point, that is, for a total duration of 14 h.

*Diets.* All animals received their respective test diets for 3 days in parallel with chow within a week before the experiments. The nutrient contents of the different diets are shown in Table 1. Rodent chow was from Kliba Nafag (#3430; Gossau, Switzerland). NCM consisted of 2.5% parts α-cellulose, 1 part mineral oil, and 10 parts of water containing 0.1% sodium saccharine and 0.2% vanilla extract (Altrinom, Lage, Germany). The carbohydrate-supplemented NCM (NCMC) contained d-glucose (Sigma, Buchs, Switzerland) and corn starch (Kliba Nafag), the protein supplemented NCM (NCMP) contained calcium caseinate (Emmy, Luzern, Switzerland), and the fat supplemented NCM (NCMF) contained lard (Kliba Nafag). The high-protein diet (NCMHP) contained whey protein (generous gift of Alpha-Laval, Switzerland) because the acceptence of a high amount of casein in the NCM diet was very low. Pure lard was used as a high-fat diet (LARD). The metabolizable energy content of the diets was calculated using an energy content of 16.7 kJ/g for carbohydrates and proteins and 37.7 kJ/g for fat.

**Effect of nutrients on the fasting-induced c-Fos expression in the ARC.** First, we quantified c-Fos expression in mice that were fasted and refeeding for 14 h (with chow for 2 h after 12 h of fasting). At dark onset (9 AM), mice received the respective treatment (refeeding or injection) and were killed 2 h later. The fasted groups remained food deprived until this time point, that is, for a total duration of 14 h.

Table 1. Nutrient content of the diets (in % wt/wt) and the amount of intake during 2 h refeeding of mice fasted for 12 h.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Chow</th>
<th>NCM</th>
<th>NCMP</th>
<th>NCMF</th>
<th>NCMHP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch Content</td>
<td>35</td>
<td>14.9</td>
<td>0.31±0.06</td>
<td>0.24±0.01</td>
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</tr>
<tr>
<td>Simple sugars Content</td>
<td>6.7</td>
<td>28</td>
<td>0.06±0.01</td>
<td>0.05±0.00</td>
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<tr>
<td>Protein Content</td>
<td>18.5</td>
<td>10.26</td>
<td>0.04±0.01</td>
<td>0.06±0.00</td>
<td></td>
</tr>
<tr>
<td>Fat ME</td>
<td>10.26</td>
<td>4.81</td>
<td>2.08</td>
<td>2.26</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. The amount of total intake of single nutrients (g) and the energy intake (ME, metabolizable energy, kJ) is shown. *Significant difference in protein intake vs. both chow and NCMP; †Significant difference in fat intake vs. both chow and NCMF (P < 0.05).
PB) for 48 h, and snap frozen in CO₂. Coronal sections (20 μm) were cut in a cryostat (CM 3050 Leica, Nussloch, Germany). The hypothalamic sections were collected in three separate groups and were thaw mounted on microscopic glass slides (SuperFrost Plus, Faust, Schaffhausen, Switzerland). After air-drying at room temperature and rehydrating in PBS, sections were incubated in blocking solution for 2 h (1.5% rabbit normal serum + avidin; Vector Laboratories, Burlingame, CA). The primary antibody (polyclonal goat anti-c-Fos, Santa Cruz; 1:10,000 + biotin, Vector Laboratories) was applied for 48 h at 4°C. The unbound antibody was removed by washing in PBS before the sections were incubated with the secondary antibody (biotinylated rabbit-anti-goat, Vectastain-Elite ABC Kit, Vector Laboratories; 1:200) for 2 h at room temperature. After incubation in ABC solution (Vectastain-Elite ABC Kit, Vector Laboratories), diaminobenzidine (DAB) was used as a chromogen [0.04% in PBS with 0.02% H₂O₂ and for color enhancement 0.08% NiCl₂ (×6 H₂O), 0.01% CoCl₂ (×6 H₂O)]. Finally, the sections were dehydrated in graded alcohols, cleared in xylene, and coverslipped with Entellan (Merck, Darmstadt, Germany).

One set of hypothalamic sections of the POMC-GFP mice was double stained for GFP and c-Fos. Immunostaining for c-Fos was conducted first as described above. GFP was visualized by the same immunoperoxidase procedure using anti-GFP antibodies (1:5,000; Stratagene, Santa Clara, CA) and aminoethyl-carbazole as chromogen (Zymed, San Francisco, CA). All brain sections were treated under the same conditions.

The localization of c-Fos-expressing neurons was identified according to the mouse brain atlas by Hof et al. (11). In all animals, the most rostral section was located first on which the infundibular stem appeared attached. Starting from here, advancing rostrally, six consecutive sections were counted bilaterally in a blind fashion for c-Fos-positive cells in the ARC (Br −1.6 to −1.1), in an area within 150 μm from the wall of the third ventricle. The mean value of the cell counts/section of an individual animal was used for statistical analyses. Representative photomicrographs were taken with a digital camera (AxioCam, Carl Zeiss AG, Feldbach, Switzerland).

**In situ hybridization and immunohistochemistry.** In transgenic POMC-GFP mice, one set of hypothalamic brain sections was processed for in situ hybridization histochemistry for NPY mRNA and c-Fos immunohistochemistry. This was done with the sole purpose to phenotype neurons, not to quantify changes in NPY expression. The antisense (5′-CAGGGCCACCCAGGCGAGCCGCGCTCGAGCGACTGGCCCGCCCGAGCGTAGTACAACCAGGGATTGGCTGTGGAGACTCCCTCGCTCTATCTGCTCTGTTGTTGCTCTGAGGGGCGTACCCCAGACCGCGGACAAATCCG) and sense riboprobes (kind gift of H. Herzog, Garvan Institute of Medical Research, Sydney, Australia) were labeled with digoxigenin (DIG), according to the manufacturers’ instructions (Roche, Rotkreuz, Switzerland). The in situ hybridization was conducted first, using RNase-free solutions and glassware. The sections were air dried at room temperature, rehydrated in PBS, treated with glycine (0.1 M in PBS) for 5 min, acetylated (in 0.25% acetic anhydride for 10 min), rinsed in PBS, and hybridized in a hybridization buffer containing 200ng/ml DIG-labeled riboprobes (18 h at 45°C). Following the posthybridization washes and digestion with RNase A, alkaline-phosphatase conjugated anti-DIG antibody (1:500, Roche) was applied overnight and detected in NBT/BCIP solution (Roche). After rinsing in PBS, immunostaining for c-Fos was conducted as described previously (2, 3, 29), except that the DAB solution did not contain nickel and cobalt, which resulted in a brown precipitate.

**Blood glucose measurements.** The effect of NCM refeeding and 12-h ad libitum access to NCM on blood glucose levels was monitored for 1h in C57BL/6N mice (n = 8/group) by tail bleeding, as reported previously (3). Animals were food deprived or had ad libitum access to NCM for 12 h during the light phase. At dark onset, a drop of blood was obtained by tail tip amputation from unanesthetized mice and analyzed by a portable glucometer (Glucometer Elite, Bayer). Following blood collection, the animals were returned to their home cages, and half of the fasted animals were given access to NCM. The blood sampling was repeated 10, 20, 30, 45, and 60 min later.

The same protocol was used to test the effect of insulin (0.05 and 0.2 U/kg sc) on blood glucose in the wild-type littermates of the

![In situ hybridization and immunohistochemistry.](http://ajpregu.physiology.org/)

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**Fig. 1.** Refeeding with chow completely reversed the fasting-induced c-Fos expression in the arcuate nucleus (ARC). Representative ARC sections immunostained for c-Fos of 14-h fasted (A), chow-refed (B) and ad libitum chow-fed (C) mice. Bar charts show the quantitative results of c-Fos expression (D), plasma insulin (E), leptin (F), ghrelin (G), free fatty acid (FFA) (H) and beta-hydroxy-butyrate (BHB) (I) levels in the different groups. a,bDifferent letters indicate significant differences between groups (P < 0.05). 3V: 3rd ventricle. Scale bar: 100 μm.
POMC-transgenic mice (n = 6/group) that were fasted for 12 h. Saline-injected animals served as controls. The experiments were repeated using a cross-over design with 3 days between trials.

Hormone and metabolite measurements. Before transcardial perfusion in the immunohistological experiments, blood was taken from the anesthetized mice by puncturing the right ventricle. Blood glucose (Glucometer Elite), plasma insulin and leptin (mouse endocrine lin-complex kit; Linco Research, St. Charles, MO), ghrelin (ghrelin total RIA kit; Linco Research), free fatty acid (FFA), beta-hydroxy butyrate (BHB), and triglyceride (TG) levels (Cobas Mira analyzer, Roche) were determined.

Statistics. Group means were compared by Student’s t-test or by one-way ANOVA, followed by Student-Newman-Keuls post hoc test. P < 0.05 was considered significant. Results are presented as means ± SE.

RESULTS

Refeeding with chow. Two-hour chow intake was 0.88 g in the refed group (the amount of single nutrients and the energy content is shown in Table 1). Mice with ad libitum access to chow and refed animals had very low c-Fos expression in the ARC (Fig. 1, B and C). Food deprivation for 14 h significantly increased the number of c-Fos-positive cells (Fig. 1, A and D). In refed and in ad libitum-fed mice, plasma insulin and leptin levels were significantly higher, and ghrelin, FFA, and BHB concentrations were lower than in the fasted mice (Fig. 1, E–I). Previously, we had shown that fasting blood glucose levels are restored to ad libitum-fed values already after 1 h refeeding with chow; thus, in these experiments, blood glucose measurements were not repeated (3).

Refeeding with nutrient-free diet. During 2-h refeeding, mice ate 1.94 g NCM. Mice with ad libitum access to NCM for 14 h and NCM-refed mice had significantly less c-Fos-positive neurons in the ARC than fasted mice (Fig. 2, A–D). However, compared with the fasted group, the reduction in c-Fos expression was markedly less in the NCM-refed group than in the chow-refed group. Further, c-Fos expression in the ARC of mice with ad libitum access to NCM was markedly higher than in mice with ad libitum access to chow (P = 0.016; compare with Fig. 1). There was no significant difference in the blood hormone and metabolite levels between the fasted, NCM-refed,
and NCM ad libitum-fed groups (Fig. 2, E–J). At the time of perfusion (2 h after refeeding), blood glucose levels were similar in fasted, NCM refed, and NCM ad libitum-fed groups (Fig. 2J). When blood glucose levels were monitored for 1 h after refeeding, no difference was found in the blood glucose levels at any time point between these three groups (data not shown).

Refeeding with nutrient-supplemented NCM matched to 2-h chow intake. The mean intake of NCMC, NCMP, and NCMF was 1.62 g, 1.55 g and 1.80 g, respectively (the amount of single nutrients and the energy content is shown in Table 1). There was no significant difference in the individual nutrient intake between any of these diets compared with the intake from chow (Table 1). Refeeding mice with any nutrient containing NCM significantly reduced c-Fos expression in the ARC compared with refeeding with nonsupplemented NCM (Fig. 3, A–E). In NCMC-refed mice, a similarly low number of c-Fos-positive neurons was detected as after refeeding with chow ($P = 0.4$). Insulin levels increased significantly only in the NCMC group (Fig. 3F). Plasma leptin and ghrelin levels did not differ significantly between the NCM, NCMF, NCMP, and NCMC-refed mice (Fig. 3, G and H). FFA levels only decreased following NCMC refeeding (Fig. 3I). In the NCMC and NCMP-refed mice, BHB concentrations were significantly lower (Fig. 3J), while blood glucose levels were higher compared with the other two groups (Fig. 3K). TG levels were only measured in NCM and NCMF-refed mice, and no difference was found between these groups (0.43 ± 0.03 and 0.47 ± 0.03 mmol/ml, respectively).

Refeeding with LARD and NCMHP. The 2-h intake of the NCMHP and LARD diet was 1.51 g and 0.28 g, respectively (the amount of single nutrients and the energy content is shown in Table 1). The intake of protein and fat was significantly higher during the 2-h refeeding than the intake after chow, or NCMP and NCMF refeeding, respectively. Both LARD and NCMHP refeeding reversed the fasting-induced c-Fos expression in the ARC (Fig. 4, A–D). The number of c-Fos-positive cells in these groups was similarly low as after NCMC or chow refeeding, respectively ($P = 0.1$ for lard vs. chow; $P = 0.2$ for LARD vs. NCMC; $P = 0.4$ for NCMHP vs. Chow; $P = 0.5$ for NCMHP vs. NCMC). Insulin levels were significantly higher in the NCMHP-refed mice compared with the LARD and

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**Fig. 3.** Refeeding with NCM supplemented with any macronutrients reduced the fasting-induced c-Fos expression in the ARC more than NCM refeeding alone. Representative ARC sections immunostained for c-Fos of mice refed with unsupplemented NCM (A), with NCM+fat (NCMF) (B), NCM+protein (NCMP) (C) or NCM+carbohydrate (NCMC) (D). Bar charts show the quantitative results of c-Fos expression (E), plasma insulin (F), leptin (G), ghrelin (H), free fatty acid (FFA) (I), BHB (J), and blood glucose levels (K) in the different groups. *a,b* Different letters indicate significant differences between groups ($P < 0.05$). Scale bar: 100 μm.
fasted groups (Fig. 4). Leptin levels seemed to be higher after NCMHP refeeding, but the differences did not reach statistical significance (P = 0.09; Fig. 4F). In the LARD-refed group, none of these parameters differed from the fasted mice. Ghrelin levels tended to be lower in the NCMHP and LARD groups compared with the fasted animals (P = 0.063; Fig. 4G). FFA and BHB plasma concentrations were significantly lower in the NCMHP-refed mice compared with the other groups (Fig. 4H, I). Blood glucose levels were similar in all three groups (P = 0.05, Fig. 4J). TG levels did not differ between the fasted (0.35 ± 0.01 mmol/ml) and LARD-refed (0.40 ± 0.03 mmol/ml) groups.

Effect of anorectic hormones on the fasting-induced c-Fos expression in the ARC. Amylin and CCK had no effect on the number of c-Fos-positive neurons in the ARC of fasted mice (72.2 ± 5.9 and 89.4 ± 5.8 cells/section) compared with saline (79.9 ± 10.2 cells/section). As a positive control for amylin and CCK treatment, we confirmed a strong c-Fos expression in the area postrema and nucleus of the solitary tract (not shown), which are known to be activated by peripheral amylin and CCK, respectively (30, 32).

The higher dose of insulin (0.2 U/kg) had no effect on the total number of c-Fos-positive cells in the ARC of fasted POMC-GFP mice (143.0 ± 22.2 cells/section) compared with saline (133.1 ± 25.2 cells/section). Of all the c-Fos-positive neurons 95.2 ± 1.5% and 96.7 ± 1.7% contained NPY mRNA, while only 0.2 ± 0.2% and 0.6 ± 0.2% contained POMC in the saline and insulin-treated mice, respectively. There was no difference in the total number of NPY (155.9 ± 6.8 vs. 148.6 ± 6.4 cells/section) neurons between the two groups. Although the number of POMC-positive neurons seemed to be higher in the insulin-treated group (40.8 ± 4.8 cells/section) compared with saline (26.1 ± 5.5 cells/section), this difference did not reach significance. Similar to the higher dose, the lower dose of insulin (0.05 U/kg) had no effect on any of these parameters in the ARC. Representative photomicrographs of the ARC of saline, and insulin-treated animals are shown in Fig. 5, A–F. The higher dose of insulin significantly reduced blood glucose levels 20, 30, 45, and 60 min after injection in
12-h fasted mice compared with saline injection. The lower insulin dose had no effect on the blood glucose levels during the 60 min of measurement (Fig. 5G).

Leptin had no significant effect on the number of c-Fos-positive cells in the ARC of fasted POMC-GFP mice (132.8 ± 36.8 cells/section) compared with saline-treated mice (104.1 ± 26.8 cells/section). Of all the c-Fos-positive neurons, 97.2 ± 0.8% and 98.0 ± 0.2% contained NPY mRNA, while only 0.8 ± 0.3% and 0.5 ± 0.2% contained POMC in the saline- and leptin-treated mice, respectively. There was no difference in the total number of NPY (141.0 ± 8.1 vs. 159.2 ± 11.5 cells/section) or POMC (23.4 ± 1.4 vs. 20.3 ± 2.9 cells/section) neurons between the two groups. Representative photomicrographs of the ARC of saline and leptin-treated animals are shown in Fig. 6, A–D. In an additional group of nontransgenic C57BL/6 mice (n = 6/group), similar results were found as in POMC-GFP mice, with no difference in the number of c-Fos-positive cells in the ARC between saline (119.4 ± 17.3 cells/section) and leptin (129.0 ± 11.4 cells/section)-injected fasted mice. In these leptin injected mice, leptin levels were above the detection level of the assay.

**Effect of amylin and CCK in the ARC of ad libitum-fed mice.** In ad libitum-fed saline-injected mice, only few c-Fos-positive neurons were found in the ARC. CCK and amylin administration alone had no effect (Fig. 7, A and B). Ghrelin significantly increased c-Fos expression in the ARC, although this response was lower than the fasting-induced activation (compare with Fig. 1). c-Fos expression in the ARC was significantly lower in mice that were pretreated with CCK before ghrelin injection, compared with ghrelin alone, but the number of c-Fos-positive cells was significantly higher than in the saline-injected controls (Fig. 7A). There seemed to be fewer c-Fos-expressing neurons in the amylin/ghrelin coinjected group (by 20%) compared with the ghrelin-injected group, but the difference just failed to reach significance (P = 0.061; Fig. 7B). As before, all amylin- and CCK-injected mice showed a strong c-Fos expression in the area postrema and in the nucleus of the solitary tract (not shown). In contrast to the ghrelin-treated mice, there were
hardly any c-Fos-expressing neurons detectable in the ARC of the PYY/ghrelin coinjected group (Fig. 7C). The number of c-Fos-positive cells was similar to the saline-injected animals. In these studies, the 2-h food intake of mice that received PYY (0.48 ± 0.09 g) and PYY + ghrelin (0.30 ± 0.06 g) was significantly less than the intake of mice that received ghrelin only (0.8 ± 0.04 g).

DISCUSSION

The ARC is a receptive brain region for numerous peripheral feeding-related signals and plays a key role in the control of food intake and energy homeostasis. The current study identified some of these feeding-related signals to be involved in the modulation of the activity of the orexigenic ARC neurons during the transition from the fasted to the fed state.

To our knowledge, this is the first study to suggest that the ARC responds not only to nutrient-dependent factors but also integrates signals associated with the ingestion of food per se, as refeeding with a nutrient-free diet reduced ARC activity. These signals might include visual and olfactory stimuli or ingestion-induced gustatory and mechanical inputs. Nevertheless, only the nutrient-containing diets reversed the fasting-induced ARC activation completely, indicating that nutrient-independent stimuli play the predominant role in the suppression of orexigenic ARC neurons during refeeding.

The inhibitory effect of chow refeeding seems to be mainly due to its carbohydrate content, because the intake of a similar amount of carbohydrates (NCMC) alone was sufficient to completely reverse the ARC activation. The other nutrients probably also contribute to the reversal of the c-Fos expression by chow refeeding, because both NCMF and NCMP diets reduced the ARC activation more than NCM alone.

The mechanisms underlying the effects of nutrients are unknown, but likely involve a direct neuronal effect. In the

Fig. 7. CCK partially blocked the ghrelin-induced ARC activation in ad libitum-fed mice (A), while amylin had only little inhibitory effect (B). PYY blocked the ghrelin induced c-Fos expression in the ARC completely (C). Amylin, CCK, or PYY alone had no effect on the basal c-Fos expression in the ARC. a,b Different letters indicate significant differences between groups (P < 0.05).
ARC, the NPYergic neurons that increase c-Fos expression during fasting (5, current study) are directly inhibited by glucose (9, 23), and intraperitoneal glucose reverses the fasting induced c-Fos expression in the ARC (3). Hence, the inhibitory effect of chow or NCMC refeeding in this study may be largely due to a direct effect of absorbed glucose on ARC neurons. An increased availability of glucose might have contributed to the neuronal effect of the protein-supplemented diets as well, because dietary AAs are gluconeogenic substrates (19).

An interesting finding of our studies was that both a diet containing only protein (NCMHP) and only fat (LARD) reversed the fasting-induced neuronal activation in the ARC, similar to chow refeeding. These results imply a similarly important role for these nutrients in modulating hypothalamic activity as carbohydrates. Whether, similar to glucose, these nutrients directly affect neuronal activity remains to be elucidated. AAs and metabolites arising from fat utilization (FFA, BHB) or ingestion (TG) are also potential candidates to modulate neuronal activity. Similar to glucose, they reduce food intake or body weight following intracerebroventricular administration (8, 9, 24, 27). Furthermore, FFAs modulate the firing rate of ARC neurons, and AAs and FFAs both activate intracellular signaling pathways in ARC neurons (6, 22, 39). Nevertheless, to date, there is no evidence for a direct effect of AAs on neuronal activity in the ARC.

It is unclear whether under our conditions a direct hypothalamic effect of the absorbed lipids is plausible. Refeeding with none of the diets increased FFA and BHB levels, and the fat-containing diets had no effect on TG levels but potently inhibited ARC neurons. We cannot exclude that the lack of changes in the lipid metabolites after refeeding might be due to the timing of the measurement and the high rate of lipid utilization in the fasted state. In fasted animals, circulating FFA and BHB levels are already high, as a result of the increased fat metabolism. Hence, it is questionable whether and how the increased availability of fatty acids following fat ingestion might signal energy surfeit and inhibit orexigenic neurons. A possible mechanism is that the source of lipids (dietary chylomicrons vs. FFAs from adipose tissue) is distinguished by the brain, similar to peripheral tissues (4).

It appears plausible that the degree of energy repletion has an influence on the reversal of ARC activity during refeeding. The fact that a higher amount of energy intake during NCMHP vs. NCMP and LARD vs. NCMF refeeding was linked to a stronger inhibition of fasting-induced c-Fos expression clearly supports this assumption. There is evidence that the intracellular ATP:AMP ratio is an important signal reflecting the energy status. A potential mediator of a shift in the hypothalamic ATP:AMP ratio might be AMPK (41). Following overnight food deprivation, hypothalamic AMPK activity is increased in mice, and refeeding for 2 h with Chow reverses this activation (20). Furthermore, there is accumulating evidence that intermediates of carbohydrate and lipid metabolism, e.g., during a shift from fatty acid oxidation to fat storage, also signal a shift from energy depletion to repletion in hypothalamic neurons (40). Therefore, it will be interesting to elucidate the involvement of AMPK and metabolic intermediates in the nutrient-dependent inhibition of orexigenic ARC neurons.

Nutrients might also modulate neuronal activity indirectly by inducing peripheral hormone release. Such hormones may include insulin and leptin, for which the ARC is a well-characterized target site (34). Furthermore, CCK and amylin might also have an impact on ARC activity via projections from hindbrain structures (16, 17, 28, 32). Interestingly, none of these peptides reduced c-Fos expression in the ARC of fasted mice in the present study, arguing against their crucial role, at least under the current experimental conditions. Generally, we used supraphysiological doses of these hormones, so that the lack of effect was unlikely to be due to an ineffective dosage. Furthermore, amylin and CCK induced high c-Fos expression in their primary central target areas in the AP and in the NTS, respectively. Concerning insulin, neither a dose that slightly reduced blood glucose, nor a dose that had no effect on blood glucose levels had an effect in the ARC. Our interpretation of these data is that the postprandial increase in plasma insulin most likely does not play an important role in reducing fasting-induced c-Fos expression in the ARC. We cannot exclude that the lack of effect of the higher insulin dose may have been counteracted by the slight reduction in the glucose levels. However, low physiological glucose levels do not seem to prevent a hormone-dependent reversal of fasting-induced ARC activation in general. This can be inferred from the fact that PYY reverses fasting-induced c-Fos expression in fasted animals (29). In our experience, blood glucose levels in fasted animals are similar to the nadir of glucose levels after insulin treatment (6.47 ± 0.3 mmol/l vs. 5.97 ± 0.4 mmol/l; 3). Further, we cannot entirely exclude that the lower insulin dose, which had no effect on blood glucose levels, may, in fact, have been subthreshold.

There is strong evidence from in vitro electrophysiological studies that insulin and leptin inhibit the orexigenic NPYergic ARC neurons (7, 14). POMC neurons, on the other hand, seem to be excited by leptin and inhibited by insulin (14). On the basis of these studies, the lack of effect of insulin and leptin on

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**Fig. 8.** Schematic model of pathways integrating the main findings of the current study. During refeeding, mainly nutrient-dependent signals are involved in the inhibition of NPYergic arcuate nucleus (ARC) neurons in the hypothalamus. Absorbed glucose is likely to have a direct neuronal effect; such an effect of amino acids (AAs) and free fatty acids (FFA) is also plausible. Additionally, the ARC neurons might sense the nutrient-dependent energy repletion via intracellular energy sensors, e.g., AMP-activated protein kinase (AMPK). Indirectly, nutrient-induced PYY and CCK release may also to be involved in the mediation of the effect of nutrients. Nutrient-independent preabsorptive signals also have an inhibitory effect in the arcuate nucleus; however, their impact is low.
c-Fos expression in the NPY and POMC neurons in our studies was unexpected. It has to be emphasized that our results under in vivo conditions cannot be directly compared with the results of in vitro electrophysiological studies, showing a prompt inhibitory effect of leptin in bath application. Further, our results seem to be in line with previous in vivo studies, showing no inhibitory effect of a single bolus leptin injection (≈4 mg/kg ip) on NPY mRNA levels in the ARC of 24 h fasted mice 2 and 6 h after application (35). Finally, the lack of effect of exogenous insulin and leptin administration on the fasting-induced c-Fos expression is in good concert with our refeeding studies, because most of the nutrient-supplemented NCM diets did not increase fasting insulin or leptin levels, while they reduced the fasting-induced ARC activation. Therefore, our results imply that leptin and insulin do not seem to have a major contribution to the short-term inhibition of ARC neurons during refeeding. Interestingly, our recent unpublished data indicate that repeated low dose injections of leptin (0.1 mg/kg sc every 3 h) during a 14-h food-deprivation period markedly reduced the fasting-induced c-Fos expression in the ARC. Hence, it seems plausible that leptin may have a delayed or tonic effect rather than a short-term episodic effect during refeeding.

On the basis of our findings, it seems unlikely that the feeding-related increase in peripheral amylase, CCK, insulin, or leptin levels alone provides a sufficient signal to reverse the fasting-induced ARC activation. However, the simultaneous release of these peptides and other postprandial factors together may contribute to the inhibition of ARC neurons during refeeding. Such feeding-related signals may enhance the effects of each other, as it has been shown, e.g., for insulin and leptin to potentiate the effects of CCK and amylase on c-Fos expression (CCK) or eating (CCK; amylase) (1, 18, 31, 37). Our observation that in ad libitum-fed mice, that is, in the presence of other feeding-related factors, CCK partly prevented the ghrelin-induced activation of putative NPY neurons supports this assumption.

Interestingly, PYY potently inhibited the fasting-induced c-Fos expression (29) and prevented the ghrelin-induced activation in the ARC (current study). Although in electrophysiological studies, PYY, leptin, and insulin all have a direct inhibitory effect on putative NPYergic neurons in the ARC, our current results suggest that their in vivo effects on fasting-induced c-Fos expression differ. Further studies are warranted to elucidate whether these differences may be due to different time courses of their effects.

In our study, ghrelin levels tended to decrease after nutrient intake, although except for chow, none of the diets restored the values to that measured in ad libitum-fed mice. While in our previous study, we showed that an increase in ghrelin levels is not a necessary signal to activate the ARC during fasting (2), a fall in ghrelin levels after refeeding might contribute to or at least facilitate the reversal of fasting-induced activation.

**Perspectives and Significance**

In summary, our studies identified several factors which attenuate ARC activity to the nutritional status (Fig. 8). We demonstrated that diet-derived nutrients mediate strong inhibitory effects on NPY neurons of the ARC, which appear to be dependent on the amount of intake. The nutrient-dependent effects could be dissociated from nutrient-independent signals related to the ingestion of noncaloric bulk, which also inhibits ARC neurons but to a much lesser extent. Moreover, we demonstrated acute inhibitory effects of PYY and CCK on ARC neurons, which may indirectly mediate the nutrient-dependent suppression of orexigenic ARC neurons. Nevertheless, the effect of CCK seems to depend on other feeding-related signals, which enhance its effect. Although leptin and insulin levels were restored within 2 h of refeeding, these peptides individually do not seem to have a major contribution to the reversal of the ARC activation in such a short term.

Our findings extend the concept that the ARC represents an important site in the short-term control of energy intake. However, further studies are required to unravel the underlying mechanisms, particularly a possible direct effect of nutrients on ARC neurons. In this respect, the involvement of energy sensors and other intracellular signaling pathways will be of major interest.

**ACKNOWLEDGMENTS**

We are grateful to B. B. Lowell for providing the POMC transgenic mice and N. Balthasar and E. Edelstein for their help with the GFP immunostaining protocol (Beth Israel Deaconess Medical Center, Boston, MA). We thank H. Herzog for providing the NPY riboprobes and N. Lee for the superb support with the in situ hybridization (Garvan Institute of Medical Research, Sydney, Australia). The technical help of B. Schneider and B. Grenacher is deeply acknowledged (University of Zurich).

**GRANTS**

This study was supported by the Research Committee and Young Academics Support Committee of the University of Zurich. C. Becskei was a recipient of a fellowship grant from the Zurich Centre of Integrative Human Physiology (University of Zurich).

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