Hypothalamic mapping of orexigenic action and Fos-like immunoreactivity following relaxin-3 administration in male Wistar rats

B. M. McGowan, S. A. Stanley, N. E. White, A. Spangeus, M. Patterson, E. L. Thompson, K. L. Smith, J. Donovan, J. V. Gardiner, M. A. Ghatei, and S. R. Bloom

Department of Metabolic Medicine, Imperial College London, Hammersmith Hospital, London, United Kingdom

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McGowan BM, Stanley SA, White NE, Spangeus A, Patterson M, Thompson EL, Smith KL, Donovan J, Gardiner JV, Ghatei MA, Bloom SR. Hypothalamic mapping of orexigenic action and Fos-like immunoreactivity following relaxin-3 administration in male Wistar rats. Am J Physiol Endocrinol Metab 292: E913–E919, 2007. First published November 28, 2006; doi:10.1152/ajpendo.00346.2006.—The insulin superfamily of peptides is characterized by a common structure typified by one intrachain and two interchain disulphide bridges (20). In addition to insulin and insulin-like growth factors, the family also includes several insulin-like peptides: relaxin-1, relaxin-3, and insulin-like peptide-3, -4, -5, and -6 (5). Classical relaxin (H2 in humans, M1 in mice, and R1 in rats, referred to as relaxin-1 for clarity) is highly expressed in the nervous system and primarily in the nucleus incertus (NI) of the brainstem (9, 44). This area has extensive projections to the forebrain, in particular to the hypothalamus, including the lateral hypothalamic and preoptic areas (18, 44). In addition, relaxin-3 immunoreactivity has been described in both the arcuate nucleus (ARC) and paraventricular nucleus (PVN) (5, 44).

Relaxin-1 and relaxin-3 receptors are highly expressed in the central nervous system. Unlike insulin, which signals via tyrosine kinase receptors, relaxin-1 interacts with the G protein-coupled receptors (GPCRs) relaxin family peptide receptor 1 (formerly LGR7) and RXFP2 (formerly LGR8) to increase intracellular cAMP (19). Autoradiography studies using [32P]relaxin-1 have shown high-affinity relaxin-1 binding sites in several hypothalamic areas, particularly the PVN, ARC, and supraoptic nucleus (SON) (32). RXFP1 mRNA and RXFP1-like immunoreactivity have been detected in the SON, PVN, and ARC (10). RXFP2 mRNA has been detected in the brain, mainly in the thalamus (35).

Relaxin-3 also binds and activates several GPCRs: RXFP1 (in common with relaxin-1) (40) and the previously orphaned GPCRs RXFP3 (formerly GPCR135) and RXFP4 (formerly GPCR142). In contrast to relaxin-1 signaling, relaxin-3 binding to RXFP3 and RXFP4 inhibit intracellular cAMP production (25, 26). Although RXFP4 is a pseudogene and absent in the rat (11), RXFP3 is expressed within the rat hypothalamus (10, 11, 26). In situ hybridization for RXFP3 demonstrates expression in both the PVN and SON with additional expression in preoptic and lateral hypothalamic areas (42, 43). In agreement with this, the RXFP3-specific agonist relaxin-3-insulin-like peptide-5 chimeric peptide binds in the PVN (24, 43).

Relaxin-3 has recently been shown (29) to stimulate food intake when administered into the third ventricle or PVN of male rats. This effect may be mediated by RXFP3. However, there are several additional hypothalamic areas where relaxin immunoreactivity is present and/or relaxin-3 binding sites have been demonstrated (44). Many of these nuclei are known to influence energy balance. We sought to map the hypothalamic areas where relaxin-3 influences energy intake by injection of relaxin-3 into regions demonstrating RXFP3 expression or relaxin binding and which have previously been implicated in regulation of food intake. To investigate which hypothalamic areas are activated in response to relaxin-3, immunohistochemistry for the early gene c-fos was examined following administration of relaxin-3 into the lateral ventricle.

* These authors contributed equally to the studies.

Address for reprint requests and other correspondence: S. Bloom, Dept. of Metabolic Medicine, Imperial College London, 6th Floor Commonwealth Bldg., Hammersmith Hospital, Du Cane Road, London W12 0NN, UK (e-mail: s.bloom@imperial.ac.uk).

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MATERIALS AND METHODS

Materials. Human relaxin-3 (H3) was purchased from Phoenix Pharmaceuticals (Belmont, CA) and synthesized by Phoenix using solid phase synthesis. H3 was dissolved in vehicle (10% acetonitrile in 0.9% saline) for all studies.

Animals. Male Wistar rats (Specific Pathogen Free; Charles River) weighing 200–250 g were maintained in individual cages for all studies. Animals were housed in conditions of controlled light (12:12-h light-dark, lights on at 0700) and temperature (21°C) with ad libitum access to water and food (RM1 diet; Special Diet Service). All studies undertaken were approved under the British Home Office Animals (Scientific Procedures) Act of 1986 (project license 70/5516).

Intranuclear cannulation and injection. Surgical procedures and handling were carried out as previously described (29). Briefly, animals were implanted with permanent 26-gauge stainless steel guide cannulae (Plastics One, Roanoke, VA) projecting into the anterior preoptic area (APOA), medial preoptic area (MPOA), SON, dorsomedial hypothalamic nucleus (DMH), ARC, and lateral hypothalamic area (LHA) according to the coordinates based on the atlas of Paxinos and Watson (Table 1 and Fig. 1) (33). Animals were allowed to recover for 7 days before studies commenced and were habituated to handling and the injection procedure.

Intranuclear injections of H3 (180 pmol) or vehicle were administered in 1 μl for 1 min by a 33-gauge stainless steel injector placed in and projecting 1 mm below the end of the guide cannula. The dose of H3 used results in a submaximal stimulation of food intake in intraparaventricular (i)PVN dose-response studies (28). An injection of 1 μl is reported to diffuse 1 mm³ in the hypothalamic PVN (47). H3 was dissolved in vehicle (as described above), and studies were performed in the early light phase (0900–1000) in ad libitum-fed rats. Animals were returned to their home cage following injection with preweighed chow, and food was weighed at 1, 2, 4, 8, and 24 h after injection.

Cannula position was verified histologically at the end of the study, as previously described (48). Briefly, immediately following death, 1 μl of india ink was injected intranuclearly and guide cannulae removed. The brains were rapidly dissected, fixed in 4% paraformaldehyde, and sliced into 40-μm-thick sections. Sections were mounted on gelatin-coated slides and stained with thionine to visualize the cannulae. The positions of the cannulae were determined by superimposing the sections on high-resolution photographs of the rat brain (Paxinos and Watson, 1997). The coordinates of the injection sites were calculated using the stereotaxic coordinates from the atlas of Paxinos and Watson (33). Coordinates of the injection sites are presented in Table 1.

Table 1. Coordinates of intranuclear injection

<table>
<thead>
<tr>
<th>Hypothalamic Nucleus</th>
<th>Distance From Bregma</th>
<th>Lateral to Midline</th>
<th>Depth</th>
</tr>
</thead>
<tbody>
<tr>
<td>APOA</td>
<td>+0.4</td>
<td>0.5</td>
<td>8.2</td>
</tr>
<tr>
<td>MPOA</td>
<td>−0.8</td>
<td>0.3</td>
<td>8.8</td>
</tr>
<tr>
<td>SON</td>
<td>−1.3</td>
<td>1.8</td>
<td>9.3</td>
</tr>
<tr>
<td>DMH</td>
<td>−3.1</td>
<td>0.5</td>
<td>8.8</td>
</tr>
<tr>
<td>ARC</td>
<td>−3.3</td>
<td>0.3</td>
<td>10.0</td>
</tr>
<tr>
<td>LHA</td>
<td>−3.6</td>
<td>1.7</td>
<td>8.8</td>
</tr>
</tbody>
</table>

Coordinates of hypothalamic nuclei used for cannulation (in mm). APOA, anterior preoptic area; MPOA, medial preoptic area; SON, supraoptic nucleus; DMH, dorsomedial hypothalamic nucleus; ARC, arcuate nucleus; LHA, lateral hypothalamic area (33). +Rostral distance from bregma; −caudal distance from bregma.

Fig. 1. Schematic representation of coronal sections through the rat hypothalamus [Paxinos G and Watson C (33)] depicting the injection site and position from bregma with respect to each hypothalamic nucleus cannulated in the present study. The injection site is magnified on the right of each coronal section. APOA, anterior preoptic area; MPOA, medial preoptic area; SON, supraoptic nucleus; DMH, dorsomedial hypothalamic nucleus; ARC, arcuate nucleus; LHA, lateral hypothalamic area. +Rostral distance from bregma; −caudal distance from bregma. Reprinted from The Rat Brain in Stereotaxic Coordinates (4th ed.) (Figs. 16, 21, 23, 32, 33, and 34), with permission from Elsevier.
hyde, dehydrated in 40% sucrose, and frozen in liquid nitrogen. A freezing sled microtome (Shandon Southern Products, Cheshire, UK) was used to take 20-μm coronal sections. Sections were compared with the corresponding section from the rat brain atlas (33). Data from animals were excluded if the injection site extended >0.2 mm outside the intended injection site or if any ink was detected in the cerebral ventricular system. The final numbers of animals with correct cannula placement, and therefore included in the analysis, were APOA (n = 9–10/group), MPOA (n = 8–10/group), SON (n = 7/group), DMH (n = 8–10/group), ARC (n = 7/group), and LHA (n = 9–11/group).

** lateral ventricle cannulation and injection.** Surgical procedures and handling were carried out as previously described (29). Briefly, animals were implanted with 22-gauge stainless steel cannulae (Plastics One) projecting into the lateral ventricle (LV). Animals were allowed to recover for 7 days before studies commenced and were habituated to handling and the injection procedure. LV injections of 5 μl were administered for 1 min via a 28-gauge stainless steel injector. Correct cannula position was verified by a positive orexigenic response to neuropeptide Y (NPY; 5 nmol) given in the early light phase. Animals consuming <2 g of food in the 2 h following NPY administration were excluded from the study. Administration of vehicle or H3 to determine Fos-like immunoreactivity was performed in the early light phase (0900–1000) in ad libitum-fed rats.

**Immunohistochemical measurement of Fos-like immunoreactivity following relaxin-3 administration.** Fos-like immunoreactivity (FLI), as a marker of neuronal activation, was determined in response to H3, as previously described (31). Briefly, LV-cannulated rats received either vehicle or H3 (540 pmol; n = 5/group) into the LV. They were returned to their home cage with food removed. The LV dose of H3 administered was three times higher than the dose used for the intranuclear studies and based on pilot data (not shown). Ninety minutes after injection, animals were terminally anesthetized and transcardially perfused with phosphate buffered saline (PBS; 0.1 M, pH 7.4) followed by 4% paraformaldehyde in PBS. Brains were immediately removed and placed in 4% paraformaldehyde in 0.1 M PBS and transferred to 40% sucrose 24 h later. They were frozen at −80°C and cut into 40-μm sections. For each brain, sections ∼120 μm apart, covering a region extending from the rostral aspect of the APOA to the caudal aspect of the LH, were stained and examined as described below.

Sections were immersed in 0.6% hydrogen peroxide in methanol (vol/vol) for 30 min to inactivate endogenous peroxidase activity, followed by incubation with 3% normal goat serum for 2 h to block nonspecific binding. The primary antibody, rabbit anti-c-fos (Ab-5; Calbiochem, San Diego, CA), was applied in a 1:20,000 dilution overnight at room temperature. Slides were then incubated for 2 h with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA), dilution 1:400, followed by avidin-biotin-peroxidase complex (1:220) for 1 h (Dako Cytomation, Glostrup, Denmark). The antigen-antibody complex was visualized with 3,3'-diaminobenzidine in 0.01% hydrogen peroxide. Sections were then mounted. Quantification of FLI was performed manually directly from the microscope image by an observer blinded to the treatment group, using a Nikon Eclipse E800 microscope. The number of matched sections examined per animal for the reported nuclei are as follows: SON 3–4 sections, PVN 2–3 sections, and ARC 5–7 sections.

**Statistical analysis.** Food intake data are shown as means ± SE. The effect of relaxin-3 on food intake following administration into a hypothalamic nucleus was compared with vehicle by unpaired Student’s t-test at each time point. Fos-like immunoreactivity data, expressed as the mean number of FLI-positive neurons per section, was nonparametric and compared using Kruskal-Wallis one-way ANOVA on ranks with post hoc Student-Newman-Keuls method. In all cases, P < 0.05 was considered statistically significant.

**Results**

Localization of the hypothalamic sites of the orexigenic action of relaxin-3. Animals received either vehicle or H3 (180 pmol) in one of six different hypothalamic nuclei in the early light phase. H3 significantly increased food intake in the first hour following injection into the SON, ARC, and the APOA [0- to 1-h food intake: SON 0.4 ± 0.2 (vehicle) vs. 2.9 ± 0.5 g (H3), P < 0.001; ARC 0.7 ± 0.3 (vehicle) vs. 2.7 ± 0.2 g (H3), P < 0.05; and APOA 0.8 ± 0.1 (vehicle) vs. 2.2 ± 0.2 g (H3), P < 0.05] (Fig. 2A). There was no significant difference in interval food intake between control and treated groups at later time points.

The greatest orexigenic response in the first hour was seen in the SON, where cumulative food intake was also significantly increased ≥8 h following peptide administration [0- to 8-h food intake: SON 1.8 ± 0.6 (vehicle) vs. 4.5 ± 0.6 g (H3), P < 0.01]. (Fig. 3C). A significant increase in cumulative food intake was also observed in the APOA ≥4 h following administration [0- to 4-h food intake: APOA 1.1 ± 0.2 (vehicle) vs. 3.2 ± 0.3 g (H3), P < 0.05] (Fig. 3A). In the ARC, food intake was significantly increased in the first hour following administration, with no significant difference in cumulative food intake at later time points (Fig. 3E).

There was no significant difference in food intake following injection of H3 in the MPOA (Fig. 3B), DMH (Fig. 3D), or LHA (Fig. 3F) at any time point. Twenty-four-hour food intake was not significantly altered following H3 injection into any area (Fig. 2B).
FLI in the hypothalamus in response to relaxin-3. Hypothalamic FLI was examined following intracerebroventricular (ICV) administration of H3. A 130% increase in FLI was observed in the SON in response to H3 compared with vehicle-treated controls [FLI-positive neurons per section (SON): vehicle 8.1 ± 2.0 vs. H3 18.6 ± 3.2, P < 0.05; Fig. 4, A and B]. However, there were no significant differences in FLI between the control and treatment groups in the PVN [FLI-positive neurons per section (PVN): vehicle 120.4 ± 22.0 vs. H3 101.0 ± 5.5] or ARC [FLI-positive neurons per section (ARC): vehicle 56.9 ± 3.0 vs. H3 52.6 ± 6.5; Fig. 4, C and D]. Although not formally quantified, there was no apparent difference in FLI in the MPOA, APOA, DMH, and LHA between vehicle and H3-treated animals.

DISCUSSION

Relaxin-3 is a recently described member of the insulin superfamily, and its roles and sites of action are largely unknown. Our previous work (28, 29) demonstrated a significant orexigenic effect of H3 following ICV and PVN administration. These studies extend previous data to demonstrate that H3 is a potent orexigen following administration into several hypothalamic nuclei.

Relaxin-3 binds to and activates at least two hypothalamic GPCRs: RXFP1 and RXFP3. Evidence from binding studies (with nonspecific ligands such as relaxin-1 and more specific ligands such as relaxin-3/insulin-like peptide-5 chimeric peptide) and in situ hybridization suggests that these receptors are
widespread in the hypothalamus, PVN, SON, ARC, LHA, and preoptic areas (32, 42, 43).

In this study, the SON is the hypothalamic area most sensitive to the orexigenic actions of H3. Food intake increased more than sevenfold compared with control following injection in this area. In keeping with previous studies (29), the orexigenic response does not extend beyond the first hour, although cumulative food intake is increased for \( t/11349 \) h. Administration of several neuropeptides into the SON has been demonstrated to influence food intake in previous studies. Ghrelin (48) and cocaine- and amphetamine-regulated transcript (CART) (2) significantly stimulate food intake, whereas cholecystokinin (6) inhibits food intake after SON administration. Numerous neuropeptides are expressed within the SON, including those known to modulate food intake, such as NPY (23), galanin (17), and CART (15). In addition, the neurons in the SON respond to peripheral signals such as leptin (21). However, no leptin receptors have been demonstrated in this region (16), and therefore, these actions may be indirect.

In keeping with the orexigenic action of H3 seen in the SON, central administration of H3 induced a small but significant increase in FLI in this area. Relaxin-3 immunoreactive fibers (44) and RXFP-3 receptors are both present in the SON, and thus increased FLI may be a consequence of H3 binding to RXFP-3. RXFP-1 is also expressed in the SON, and although it is the cognate receptor for relaxin-1, it also binds relaxin-3 with lower affinity. Previous work (30, 41) has demonstrated peripheral and ICV administration of relaxin-1 to stimulate water intake and induce FLI in the magnocellular neurons of the SON. It has been shown recently (4) that central administration of relaxin-3 results in a significant increase in water intake. Thus FLI in the SON may be in keeping with binding and stimulation of RXFP-1 receptors by H3. It would be interesting to determine whether relaxin-3-induced FLI and relaxin-3 receptors are colocalized with vasopressin neurons, indicating a role in water homeostasis or with an alternative cell population that may act to modulate food intake. However, relaxin-3 in the SON may also be part of a stress “circuit.” Relaxin-3-expressing neurons in the NI exhibit FLI in response to stress (44), and therefore, projections from the NI to the SON may possibly play a role in stress-related vasopressin release. Relaxin-3 immunoreactive fibers in the SON also raise the possibility of an autocrine role for relaxin-3 in this nucleus (9). Whether relaxin-3 receptors are localized to these immunoreactive fibers, and if so, whether they play an inhibitory or excitatory role, would also be of great interest.

Binding to either RXFP-3 or RXFP-1 may result in FLI as a direct effect of H3 within the SON. However, an indirect induction of FLI by H3 may also be possible. The major inputs into the SON are from extrahypothalamic areas, the subfornical organ, medial and lateral septum, and the median preoptic nuclei within the forebrain and from the A1 region and nucleus of the solitary tract within the brain stem (46). However, the NI projects to the ventral tegmental area and the zona incerta (18) that provide inputs to the SON (46) and may provide an indirect pathway for relaxin-3 induced activation of the SON.

ARC administration of H3 led to a fourfold increase in food intake in the first hour following administration. The ARC has long been known to influence energy homeostasis. Lesions of the ARC by neonatal monosodium glutamate administration produce obesity (8). Intra-arcuate administration of many peptides alters feeding, including NPY (7), melanin-concentrating hormone (1), neuromedin U (49), and ghrelin (48). The ARC also expresses several neuropeptides that regulate energy balance (38) and are modulated by peripheral signals such as leptin (12).

We have also shown that H3 administration into the APOA significantly increases food intake. Although the preoptic area
is not traditionally associated with regulation of energy balance, leptin and estrogen administration in this area modulate energy balance (3, 13), and lesioning of preoptic areas alters food intake and body weight (27). However, neither RXFP3 nor RXFP1 receptor expression or binding have been reported in the preoptic area in rats, although RXFP3 expression is present in the preoptic area of the mouse (42).

Interestingly, no FLI was observed in either the ARC or preoptic area despite significant increases in food intake following relaxin-3 administration. Although statistically significant, the increased FLI in the SON following relaxin-3 administration is small, and since it is absent in both the ARC and preoptic area, it is possible that c-fos expression is not a marker of neurons activated by relaxin-3 feeding pathways. Dissociation between c-fos expression and neuronal depolarization has been described previously (22). One of the principal pathways for c-fos induction is via increased intracellular cAMP. The second messenger pathway of RXFP-3, the cognate receptor for relaxin-3, is not well understood. However, in contrast to RXFP-1, binding and stimulation of RXFP-3 decreases intracellular cAMP. Other immediate early gene products have also been used to monitor neuronal activity, although these tend to be less reliable markers than c-fos (22). It may be interesting to examine the expression of these markers, but electrophysiological recording from RXFP-3-expressing cells would provide more information regarding neuronal firing in response to relaxin-3. Both electrophysiological (39) and tracing studies (14) have demonstrated that extensive connectivity exists between hypothalamic nuclei, including pathways that are leptin-responsive. For example, reciprocal connections exist between the ARC and suprachiasmatic nucleus and between the suprachiasmatic nucleus and the SON (34). Thus relaxin-3-induced neuronal firing in one hypothalamic region, such as the SON, may influence activity in several additional hypothalamic nuclei.

The receptor mediating the actions of H3 on food intake is unknown. Previous work (29) has shown that relaxin-1, which binds RXFP-1, does not significantly increase food intake, suggesting that the orexigenic actions of H3 may be mediated by RXFP-3. H3 significantly increased food intake in both the ARC and APOA, but in the rat, the RXFP-3-specific ligand relaxin-3/insulin-like peptide-5 chimeric peptide does not bind in these regions, and no RXFP-3 expression has been reported (24, 32, 43). Similarly, administration of H3 into the LHA had no effect on food intake, yet low-level RXFP-3 expression has been described here (43). Therefore, it is possible that the orexigenic actions of relaxin-3 may be mediated by a novel receptor. It would be interesting to examine the effect of the RXFP-3-specific ligand relaxin-3/insulin-like peptide-5 chimeric peptide administration on energy intake.

Relaxin-3 immunoreactivity has been demonstrated within fibers in the LH, ARC, and sparsely in the PVN (44). This raises the possibility of an autocrine role for relaxin-3. However, as described above, the cognate receptor for relaxin-3, RXFP-3, is not found within the ARC (11, 26, 42, 43), although RXFP-1 is expressed here (10). Therefore, in the ARC, relaxin-3 may have an autocrine role on RXFP-1 receptors. Both RXFP-3 receptors and relaxin-3 immunopositive fibers are expressed in the PVN, and here it may act in an autocrine fashion to modulate either feeding or endocrine function (29). Dual in situ hybridization for RXFP-3 and immunohistochemistry for relaxin-3 would be needed to confirm whether RXFP-3 mRNA and relaxin-3 immunoreactivity are localized to the same neurons.

The dose of H3 administered in this study was submaximal to minimize diffusion between hypothalamic nuclei, but this possibility cannot be excluded. If diffusion from the administration site to a responsive area, such as the PVN, were responsible for the orexigenic effects seen, one might expect an increase in food intake following injection into those areas closest to the PVN. However, there was no acute orexigenic effect following either MPOA or LHA administration of H3 even though these areas are anatomically closer to the responsive PVN than the SON, APOA, or ARC. In addition, diffusion might be expected to result in a delayed feeding response, but relaxin-3 administration in the SON, APOA, and ARC results in a prompt increase in food intake. The acute results obtained here are therefore unlikely to be due to diffusion.

In summary, we have shown that relaxin-3 significantly stimulates feeding in several hypothalamic nuclei, the SON, ARC, and APOA, in addition to its known orexigenic effects when administered into the PVN. The orexigenic actions in the ARC, in the absence of reported RXFP-3 receptor expression, raise the possibility of an additional relaxin receptor. We have also demonstrated a small increase in FLI in the SON in response to relaxin-3 but no change in FLI with relaxin-3 in the ARC or preoptic area despite significant orexigenic actions. It would be interesting to examine the electrophysiological effects of relaxin-3 on RXFP-3-expressing cells and characterize the responsive neurons in these areas.

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RELAXIN-3 AND FEEDING IN THE HYPOTHALAMUS


