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DISTRIBUTION OF GALANIN MESSENGER RNA-EXPRESSING CELLS IN MURINE BRAIN AND THEIR REGULATION BY LEPTIN IN REGIONS OF THE HYPOTHALAMUS

C. C. CHEUNG,[a]* J. G. HOHMANN,[c]* D. K. CLIFTON[b] and R. A. STEINER[a,b,c]†

[a]Department of Physiology and Biophysics, School of Medicine, University of Washington, Seattle, WA 98195, USA

[b]Department of Obstetrics and Gynecology, School of Medicine, University of Washington, Seattle, WA 98195, USA

[c]Graduate Program in Neurobiology and Behavior, School of Medicine, University of Washington, Seattle, WA 98195, USA

Abstract—Galanin is widely distributed throughout the mammalian brain and has been implicated in the regulation of food intake, metabolism and reproduction—functions that are also thought to be under the control of leptin. To investigate the possible role of galanin in mediating the physiological effects of leptin in the mouse, we had three experimental objectives: first, to map the distribution of galanin messenger RNA-expressing cells in the brain of the mouse; second, to assess the effects of leptin on galanin gene expression in areas of the brain thought to be involved in the regulation of body weight and reproduction; and third, to determine whether galanin neurons in these regions express leptin receptor messenger RNA. We found the pattern of galanin messenger RNA expression in the mouse brain to be similar, but not identical, to that in the rat. Leptin treatment (2 μg/g for six days) significantly reduced cellular levels of galanin messenger RNA in the hypothalamic periventricular nucleus of leptin-deficient obese *(ob/ob)* mice ($P < 0.01$) by approximately 30%; however, leptin did not appear to influence the expression of galanin in the arcuate or dorsomedial nucleus of the hypothalamus. Galanin-producing neurons in the arcuate, dorsomedial and periventricular nuclei did not appear to express leptin receptor messenger RNA ($P > 0.05$).

These results demonstrate that galanin distribution patterns in the mouse brain are comparable to other species and, yet, possess unique features. In addition, galanin-expressing neurons in the hypothalamic periventricular nucleus are targets for regulation by leptin; however, the effect of leptin on galanin gene expression is likely to be mediated indirectly, perhaps through either proopiomelanocortin- or neuropeptide Y-expressing cells in the hypothalamus. © 2001 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: neuropeptide, gene regulation, neuroanatomy, *in situ* hybridization, leptin receptor.

Galanin is a 29 amino acid neuropeptide that was originally isolated from the porcine gut.[42] In mammals, galanin is widely distributed throughout the central and peripheral nervous systems.[26] One curious observation about galanin's expression in the brain of mammals is its species specificity, which is particularly evident in the rat and monkey. For example, in the rat there is little or no expression of galanin in the hippocampus but there are abundant galanin-containing cells in the dorsal raphe.[23,34] In the monkey, however, galanin producing cells are found throughout the hippocampus, yet are absent from the dorsal raphe.[17] To date, there has been no detailed mapping of the distribution of galanin in the mouse brain. Because of the growing importance of the mouse as a genetic model for understanding the brain, it is vital that we know the comparative anatomy of neuropeptide expression in the murine brain and use this information to shed light on the differences and similarities between species. The first objective of this study was to complete a detailed map of the distribution of galanin in the mouse brain and to highlight the common and unique features of galanin's pattern of expression in the mouse compared to that of other mammalian species.

Evidence suggests that galanin plays a role in physiological processes that are regulated by body fat reserves, such as feeding behaviors and reproduction.[3,7,36,45] Information about the status of fat reserves is communicated throughout the body by the adipocyte-derived hormone leptin, which is secreted into the general circulation. The CNS is a major target for leptin's action and long form leptin receptor (Ob-Rb) mRNA is expressed by neurons in distinct anatomical regions of the brain.[9] In the rat, both Ob-Rb and galanin are expressed in hypothalamic

*These authors contributed equally to this work.

†Corresponding author. Tel.: +1-206-543-8712; fax: +1-206-685-0619.

E-mail address: steiner@u.washington.edu (R. A. Steiner).

Abbreviations: Arc, arcuate nucleus; bp, basepair; CTP, cytosine 5′-triphosphate; DEPC, diethyl pyrocarbonate; DMN, dorsomedial nucleus; DMV, dorsal motor nucleus of vagus; Dnase, deoxyribonuclease; EDTA, ethylenediaminetetraacetic acid; GTP, guanosine 5′-triphosphate; LC, locus coeruleus; NTS, nucleus of solitary tract; Ob, obese; Ob-Rb, leptin receptor long form; PAGE, polyacrylamide gel electrophoresis; PeN, periventricular nucleus; POA, preoptic area; POMC, proopiomelanocortin; PVN, paraventricular nucleus; RNase, ribonuclease; SBRs, signal to background ratios; SSC, standard saline citrate; UTP, uridine 5′-triphosphate.

nuclei that are involved in regulating food intake and reproductive function, including the arcuate (Arc) and dorsomedial (DMN) nuclei.[9,24,34] These observations suggest the possibility that at least some of the effects of body fat stores on feeding and reproduction are linked to leptin's action on galanin-expressing neurons in the hypothalamus; however, hypothalamic interactions between leptin and galaninergic neurons have not yet been addressed in either the rat or mouse.

A second objective of this study was to examine whether galanin is involved in mediating the effects of leptin in the hypothalamus of the mouse. To explore this question, we tested the hypothesis that leptin regulates the activity of galaninergic neurons by evaluating the effects of leptin on galanin gene expression in the leptin-deficient *ob/ob* (obese) mouse. Next, having observed that leptin altered the expression of galanin mRNA in the hypothalamic periventricular nucleus (PeN), we tested the hypothesis that leptin acts directly on these cells by examining whether these and other galaninergic neurons in the hypothalamus express Ob-Rb mRNA.

EXPERIMENTAL PROCEDURES

Animals

In Experiment 1, adult male C57BL6/J × 129 SV mixed strain mice ($n = 3$) bred in the animal colony at the University of Washington were used. In Experiment 2, five-month-old male *ob/ob* ($n = 10$) and age-matched lean wild-type C57BL/6J mice ($n = 5$) were purchased from Jackson Laboratories (Bar Harbor, ME). In Experiment 3, 90-day-old male C57BL/6J wild-type mice ($n = 5$) were obtained from B&K Universal (Kent, WA). All animals were housed in groups of two to five, unless otherwise stated, and maintained on a 12-h light/dark cycle with lights off at 18.00 h in the animal care facilities of the University of Washington Department of Comparative Medicine. The University of Washington's Animal Care Committee approved all animal procedures in accordance with the NIH Guide to Care and Use of Laboratory Animals. All efforts were made to minimize both the suffering and the number of animals used.

Experimental design

In Experiments 1 and 3, animals were killed by CO_2 asphyxiation, and brains were removed, frozen on dry ice and stored at −80°C. All brains were cryosectioned at 20 μm. In Experiment 2, animals were housed singly prior to the start of the experiment. *Ob/ob* animals were divided into two groups: one group received a daily i.p. injection of 2 μg/g leptin and was fed *ad libitum*, the other group received a daily i.p. injection of an equivalent volume of physiological saline and was pair-fed to the leptin-treated animals. Wild-type animals received daily i.p. injections of physiological saline and were fed *ad libitum*. All injections were given within 1 h before lights off. Food intake and body weight were measured daily. After six days of injections, animals were killed in the afternoon (12.30–15.00 h) by CO_2 asphyxiation, blood was collected by cardiac puncture and the animals were decapitated. Brains were rapidly removed, frozen on dry ice and stored at −80°C. Brains collected for Experiment 1 were sectioned from the olfactory bulb to the caudal medulla; brains collected for Experiments 2 and 3 were sectioned from the medial preoptic area to the mammillary body.

Leptin

Recombinant full-length human leptin protein, generously provided by ZymoGenetics, was produced in *Saccharomyces cerevisiae*, purified to near homogeneity (>95%) by analytical high-pressure liquid chromatography, and quantified by mass spectroscopy. The control solution for leptin was 50 mM sodium borate (pH 8.0) in 0.9% sodium chloride.

Riboprobe preparation

^{35}S-labeled mouse galanin probe (Experiment 1). The plasmid vector pGemT-Easy containing a 493 bp cDNA corresponding to the entire coding region of preprogalanin was kindly provided by Dr James Hyde of University of Kentucky. The plasmid was linearized with SacII and transcribed with SP6 to generate a cRNA antisense probe complementary to mouse galanin mRNA. A sense probe was generated by linearizing the plasmid with PstI, and transcribed with T7. The isotopically labeled mouse galanin antisense riboprobe was generated in an *in vitro* transcription reaction with the following reagents: 25 μl of 12.5 mCi/ml ^{35}S-labeled uridine 5′-triphosphate (UTP), which represents 25% of UTP, and 2 μl cold thio-labeled UTP in a final concentration of 50 mM α-thio-UTP; 2 μl mixture of 50 μM each of ATP, cytosine 5′-triphosphate (CTP), and guanosine 5′-triphosphate (GTP); 2 μl RNase block; 3 μl SP6; 2 μg linearized mouse galanin plasmid vector DNA; 2 μl 10× transcription buffer (Boehringer Mannheim, Indianapolis, IN); and 10 μl diethl pyrocarbonate (DEPC)-treated water. The transcription reaction was incubated at 37°C for 1.5 h. The reaction mixture was then mixed with 1 μl DNase I for 15 min before the reaction was terminated with the addition of 4 μl EDTA and 1 μl tRNA. The probe mixture was brought to a final volume of 50 μl with DEPC-treated water, purified with a Quickspin G-50 Sephadex spin column (Boehringer Mannheim, Indianapolis, IN) and run on a polyacrylamide gel to check for probe integrity.

^{35}S-labeled rat galanin probe (Experiment 2). The plasmid vector Bluescript-13 containing a 680-bp cDNA to rat galanin mRNA was kindly provided by Dr Maria Vrontakis of University of Manitoba at Winnipeg. The fragment consists of 372 bp of coding sequence, flanked by 124 bp of 5′ non-coding sequence and 184 bp of 3′ non-coding sequence. The plasmid was linearized with HindIII for the generation of an antisense probe and with SmaI for the generation of a sense control probe. The isotopically labeled rat galanin riboprobe was generated in an *in vitro* transcription reaction with the following reagents: 32.4 μl of 12.5 mCi/ml ^{35}S-labeled UTP, which represents 25% of UTP, and 2.68 μl cold thio-labeled UTP in a final concentration of 50 mM α-thio-UTP; 1.5 μl of 50 μM each of ATP, CTP, and GTP; 1.5 μl RNase block; 3 μl T7; 1.5 μg linearized rat galanin plasmid vector DNA; 1.5 μl DL-dithiothreitol; 3 μl 10× Reaction buffer (Ambion, Austin, TX); and 16.5 μl DEPC-treated water. The reaction mixture was incubated at 37°C for 1 h and for an additional 35 min after another 3 μl of T7 was added. The reaction mixture was then mixed with DNase I for 15 min before the reaction was terminated with the addition of 6 μl EDTA and 1 μl tRNA. The probe mixture was purified with a Quickspin G-50 Sephadex spin column and run on a polyacrylamide gel to check for probe integrity.

Digoxigenin-labeled rat galanin probe (Experiment 3). A protocol similar to that for digoxigenin-labeled proopiomelanocortin (POMC) probe[4] was used in the preparation of the digoxigenin-labeled rat galanin probe. The reaction mixture consisted of: 2 μl digoxigenin mix; 1× reaction buffer; 2 μl RNase blocker; 1 μg DNA; 2 μl T7 polymerase; and 11 μl DEPC-treated water. All ingredients were incubated at 37°C for 1 h, treated with 1 μl of 20U DNase for 30 min, and the reaction was terminated with 4 μl EDTA. The probe was purified with a Quickspin G-50 Sephadex spin column and checked with agarose gel electrophoresis. An *in situ* hybridization assay was performed to determine the optimal working concentration for this digoxigenin-labeled rat galanin riboprobe.

^{33}P-labeled mouse long-form leptin receptor probe (Experiment 3). The plasmid vector containing a cDNA to mouse Ob-Rb mRNA was kindly provided by Joseph Kuijper at ZymoGenetics. A 1111 bp fragment was cloned into the vector pCR-script

Amp SK(+). The plasmid was linearized with SpeI and transcribed with T3 to generate a 537-bp fragment that maps to the intracellular domain of the mouse leptin receptor. The Ob-Rb riboprobe was generated in an *in vitro* transcription reaction with the following reagents: 0.25 mCi of ^{33}P-labeled UTP that was dried down; 2 μl each of 0.5 mM ATP, CTP and GTP; 2 μl 10× transcription buffer; 1.5 μg linearized plasmid DNA; 40 U T3; 80 U RNase blocker; and 8.5 μl DEPC-treated water to bring the total volume to 20 μl. After 2 h of incubation at 37°C, 1 μl tRNA and 4 μl EDTA were added to terminate the reaction. The total volume of the reaction was brought to 45 μl with DEPC-treated water before it was purified with a Quickspin G-50 Sephadex spin column. The probe yield was calculated based on the scintillation counter readings, and the integrity of the probe was confirmed by acrylamide gel electrophoresis followed by autoradiographic development of photographic film.

Single- and double-label in situ *hybridization*

Experiments 1 *and* 2. Tissue sections were processed according to a previously described protocol, with some modifications.[10] In brief, tissue was fixed in 4% paraformaldehyde, acetylated, delipidated in chloroform, rehydrated in 95% ethanol and air-dried. Tissue sections were then hybridized overnight at 60°C with ^{35}S-labeled mouse (Experiment 1) or rat (Experiment 2) galanin riboprobe at a concentration of 0.25 μg/ml/kb. All slides were treated with RNase and washed with standard saline citrate (SSC) at increasing stringencies, which included two washes in 0.1×SSC at 65°C. Sections were then dehydrated in an ethanol series and air-dried. All slides were dipped in diluted NTB2 emulsion (Kodak, Rochester, New York) and were exposed for 22 days (Experiment 1) or nine days (Experiment 2), before development with Kodak D-19 developer (Kodak, Rochester, New York).

Experiment 3. Slides were processed for double-label *in situ* hybridization as previously described, with some modifications.[21] Slides were processed initially in a similar fashion to those in experiments 1 and 2. They were then hybridized overnight at 60°C with the ^{33}P-labeled mouse Ob-Rb riboprobe, which was used at 2.5 pmol/ml, and the digoxigenin-labeled rat galanin riboprobe, which was used at 1:300 dilution. After the slides were washed twice in 0.1×SSC at 65°C, they were incubated with alkaline phosphatase-conjugated digoxigenin antibody (Boehringer Mannheim, Indianapolis, IN) at 1:1000. Next, they underwent a chromagen reaction with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate at 37°C for approximately 2 h, until optimal color was achieved. After drying, slides were dipped in 3% parlodion, followed by undiluted Kodak NTB-3 emulsion, and then exposed at 4°C for five days. Galanin mRNA-expressing neurons were identified as cells with purple–blue precipitate.

Image analysis

For all three experiments, the atlas of Franklin and Paxinos[12] was used to identify specific regions of the mouse brain. The image analyses in Experiments 2 and 3 were performed blindly by a reader who was unaware of the assignment of slides (and sections) to either particular treatment groups or genotypes.

Experiment 1. The entire mouse brain, from the olfactory bulb to the caudal medulla, was analysed by reflected-light, dark-field microscopy for the presence of silver grain clusters (indicating the presence of galanin mRNA-containing cells) in defined brain regions. Sections were cut at 20 μm thickness, and approximately 150 sections were examined for each animal. Each brain region with galanin-expressing cells was qualitatively scored for both the number of cells and relative level of galanin mRNA expression.

Experiment 2. Coronal brain sections were matched across animals. The following areas with their respective coordinates relative to Bregma were included in the analysis: Arc (−1.34 mm to −2.06 mm), DMN (−1.46 mm to −2.06 mm) and PeN (0.14 mm to −0.22 mm). These areas were chosen because of their possible involvement in food intake and reproduction. The number of grains per cell, which is a semiquantitative indicator of the amount of mRNA present within a cell, was measured with an automated grain-counting system, as previously described.[5] Only the left half of each brain section was analysed.

Experiment 3. The following areas were included in the analysis: Arc, DMN and PeN. Sections containing digoxigenin-labeled neurons were analysed by an automated image processing system to estimate the percentage of galanin neurons that express Ob-Rb mRNA. The image processing system consisted of a Pixel Grabber video acquisition board (Perceptics Corp., Knoxville, TN) attached to a Macintosh IIfx computer (Apple Computer Corp., Cupertino, CA). The sections were viewed under a Zeiss Axioscope microscope (Zeiss, New York, NY) equipped with a 40× epi-illumination dark-field objective. Video images were obtained by a Dage model 65 camera (Dage-MIT, Michigan City, IN) attached to the microscope. Digoxigenin-labeled neurons (representing galanin mRNA-containing cells) were first visualized under bright-field illumination, and on the corresponding video image, the boundaries of the digoxigenin-labeled cell bodies were outlined. Next, the illumination was switched to dark-field, and silver grains (representing Ob-Rb mRNA containing cells) were counted over the outlined neurons as well as over the surrounding area, as previously described.[4]

Control experiments

In separate experiments, the identity and integrity of the radioactively labeled galanin and Ob-Rb cRNA probes were verified by polyacrylamide gel electrophoresis (PAGE) against known standards. Control experiments conducted with sense riboprobes revealed no specific labeling. In a separate experiment, the identity and integrity of the digoxigenin-labeled galanin cRNA probe was verified by agarose gel electrophoresis against a known standard and was shown to label cells in virtually identical anatomical regions of the brain as did the ^{35}S-labeled probe.

Statistical analysis

In Experiment 2, the number of galanin mRNA-expressing cells and the mean number of grains/cell in each examined region were calculated for every animal. For each area, the cell counts and mean number of grains/cell were analysed by ANOVA, followed by Fisher's PLSD test, when the ANOVA indicated a significant difference between groups.

In Experiment 3, signal-to-background ratios (SBRs) were calculated for all of the galanin mRNA-containing neurons that were measured. For each area, a one-sample sign test was used to determine whether the SBRs for the cells in that area were significantly greater than one (one is the value that would be expected if there were no specific signal).[39] Results of statistical tests were considered significant at $P < 0.05$.

RESULTS

Galanin messenger RNA distribution in the brain (Experiment 1*)*

Galanin mRNA-containing cells were identified as clusters of silver grains in anatomically distinct nuclei throughout the CNS of the mouse (Fig. 1; Table 1). In the olfactory bulb, moderate numbers of labeled cells were found throughout the glomerular layer, but few cells were seen elsewhere. The prosencephalon contained numerous galaninergic cells, from the diagonal band of Broca to the supramammillary nucleus. Labeling was generally moderate in the telencephalon, with the highest numbers of cells found in the bed nucleus of

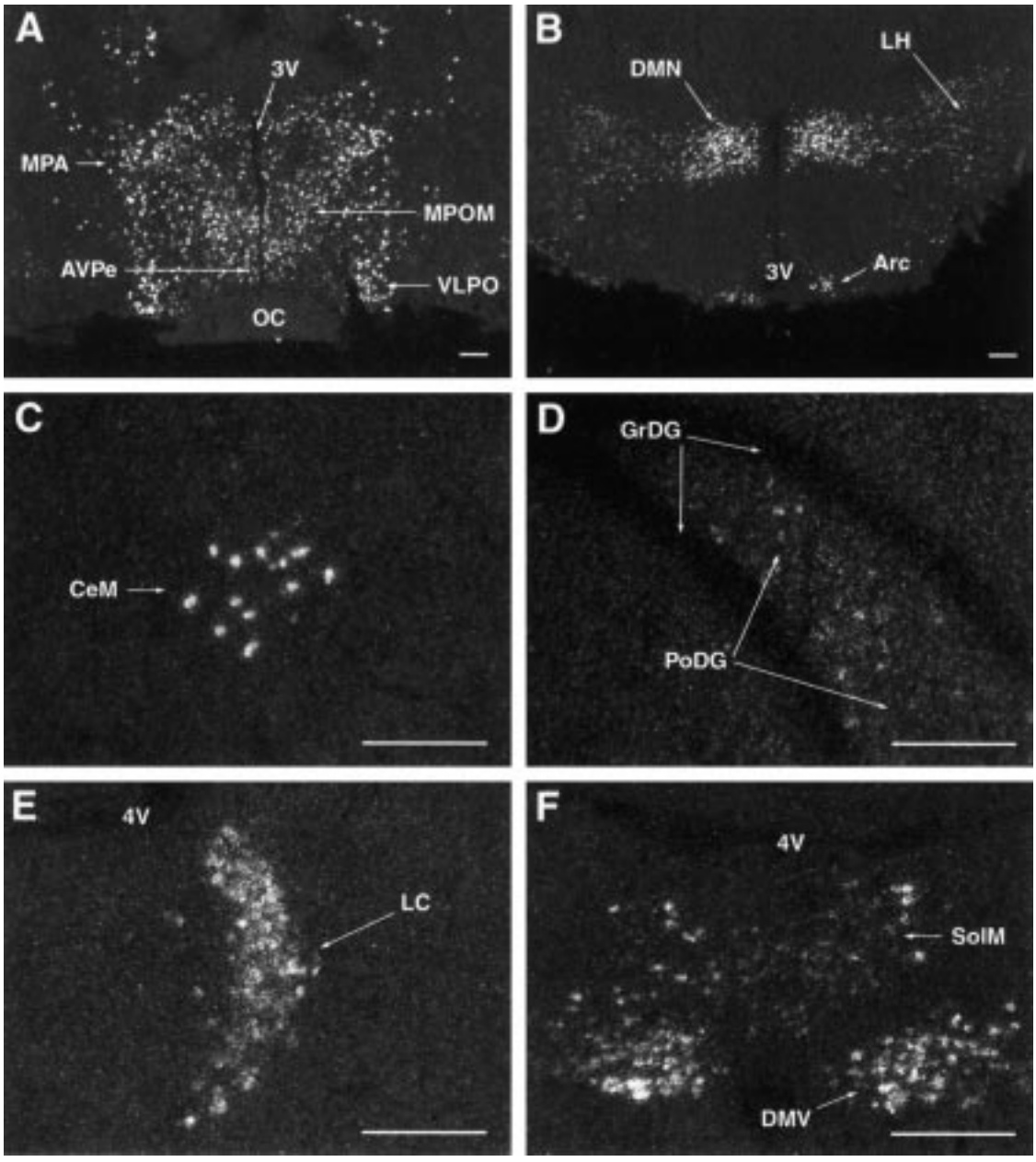

Fig. 1. Photomicrographs of galanin mRNA-containing cells in the (A) preoptic area, (B) mid-hypothalamus, (C) central nucleus of the amygdala, (D) dentate gyrus, (E) locus coeruleus, and (F) dorsal vagal complex. AVPe, anteroventral periventricular nucleus; CeM, central nucleus of the amygdala; DMN, dorsomedial hypothalamic nucleus; GrDG, granular layer of the dentate gyrus; LH, lateral hypothalamus; MPA, medial preoptic area; MPOM, medial preoptic nucleus (median division); OC, optic chiasm; PoDG, polymorphic layer of the dentate gyrus; SolM, solitary tract nucleus medial; VLPO, ventrolateral preoptic nucleus; 3V, third ventricle; 4V, fourth ventricle. Scale bars = 200 μm.

the stria terminalis, the horizontal limb of the diagonal band of Broca and the subfornical organ. The dentate gyrus had moderate labeling, primarily in the caudal polymorphic layer (Fig. 1D). Several divisions of the amygdala had only modest cell counts; however, these few cells were intensely labeled (Fig. 1C). Virtually no labeling was seen in the cortex, nucleus accumbens or striatum.

In the diencephalon, the thalamus contained relatively few galanin cells, but a continuous ring of weakly labeled cells was seen extending from the central lateral to the central medial thalamic nuclei. The hypothalamus contained the largest number and densest labeling of any forebrain region. Most divisions of the preoptic area (POA) were intensely labeled, particularly in the medial and ventromedial divisions (Fig. 1A). The area surrounding the rostral third ventricle had many highly expressing cells, most notably in the anteroventral PeN and throughout the rostral PeN (Fig. 2). The supraoptic, perifornical, paraventricular (PVN) and Arc nuclei all contained moderate numbers of cells, while the lateral hypothalamic area had scattered cells throughout its

Table 1. Relative distribution of galanin messenger RNA-containing cells (cell count) and their levels of galanin expression (intensity) in the mouse brain

Area	Cell count	Intensity
Rhinencephalon		
Olfactory bulb		
External plexiform layer	+	+
Glomerular layer	++	+
Telencephalon		
Medial septal nucleus	+	+
Lateral septal nucleus	+	+
Bed nucleus of the stria terminalis	++	++
Bed nucleus of the anterior commissure	++	++
Diagonal band of Broca		
Vertical limb	+	+
Horizontal limb	++	++
Dentate gyrus	++	+
Amygdala		
Central nucleus (medial division)	++	+++
Central nucleus (lateral division)	+	+++
Medial nucleus (posterodorsal division)	++	+
Basal nucleus of Meynert	++	++
Diencephalon		
Thalamus		
Paraventricular thalamic nucleus	+	+
Central medial thalamic nucleus	+	+
Central lateral thalamic nucleus	++	+
Paracentral thalamic nucleus	+	+
Hypothalamus		
Ventromedial preoptic nucleus	++	+++
Anteroventral periventricular nucleus	+++	+++
Median preoptic nucleus	+	+
Medial preoptic area	+++	+++
Medial preoptic nucleus (median division)	+++	+++
Ventrolateral preoptic nucleus	++	+++
Anterodorsal preoptic nucleus	++	++
Lateral preoptic area	++	++
Periventricular nucleus	+++	+++
Lateral hypothalamic area	++	+
Supraoptic nucleus	++	++
Paraventricular nucleus	++	+
Anterior hypothalamic area (posterior division)	+	++
Dorsomedial hypothalamic nucleus	+++	+++
Perifornical area	++	++
Arcuate nucleus	++	++
Posterior hypothalamic area	+	+
Dorsal tuberomammillary nucleus	+	+
Supramammillary nucleus	+	+
Mesencephalon		
Olivary pretectal nucleus	+	+
Edinger–Westphal nucleus	+	++
Oculomotor nucleus	+	++
Rostral linear nucleus (raphe)	+	+
Subbrachial nucleus	+	+
Dorsal lateral periaqueductal gray	+	++
Rhombencephalon		
Kolliker–Fuse nucleus (A7 region)	+	+
Subcoeruleus nucleus	+	+
Laterodorsal tegmental nucleus	+	+
Lateral parabrachial nucleus	+	+
Locus coeruleus	+++	+++
A5 area	+	++
Inferior salivatory nucleus	++	++
Lateral paragigantocellular nucleus	++	++
Dorsal cochlear nucleus	+	+
Inferior olive nucleus	++	+
Medial vestibular nucleus	+	+
Solitary tract nucleus	++	++
Dorsal motor nucleus of vagus	+++	+++
Raphe pallidus nucleus	+	+

Table 1 (*continued*)

Area	Cell count	Intensity
A1 area	+	++
A2 area	+	+
Lateral reticular nucleus	++	++
Circumventricular		
Subfornical organ	++	+
Vascular organ of the lamina terminalis	+	++
Area postrema	+	+

Adult male C57BL6/J × 129SV mice ($n = 3$) brains were examined by using ^{35}S-labeled riboprobes complementary to mouse galanin mRNA. *In situ* hybridization assays were performed from the olfactory bulb through the brain to the caudal extent of the medulla, and all sections were analysed for the presence of silver grain clusters representing galanin-expressing cells. All anatomical areas listed are according to the atlas of Franklin and Paxinos.[12]

rostrocaudal extent (Fig. 1B). The most densely labeled nucleus of the hypothalamus was the DMN, with intensely labeled, tightly packed cells found in all divisions, most notably in the rostral portions (Fig. 1B).

The mesencephalon contained the fewest number of galanin-producing cells in the brain, with scattered cells noted in the Edinger–Westphal and oculomotor nuclei, and only occasional-labeled cells observed elsewhere. In the rhombencephalon, most noradrenergic cell groups contained galanin mRNA, including A7, A5, A1 and A2. The locus coeruleus (LC) contained large numbers of tightly clustered, intensely labeled cell bodies, throughout its rostrocaudal extent (Fig. 1E). Many densely labeled cells were also found in the vagal complex, most obviously in the solitary tract nucleus (NTS) and the dorsal motor nucleus of vagus (DMV; Fig. 1F). Distinct, small groups of cells were noted in the inferior salivatory, paragigantocellular and lateral reticular nuclei. No specific labeling was detected in the cerebellum.

Regulation of galanin messenger RNA by leptin (Experiment 2)

In the PeN, cellular levels of galanin mRNA were significantly reduced in *ob/ob* mice given leptin compared to vehicle-treated, pair-fed controls (leptin-treated *ob/ob*: 18.8 ± 1.9 vs pair-fed *ob/ob*: 27.8 ± 3.5; $P < 0.01$; Figs 2, 3). Levels of galanin mRNA in the PeN of lean controls were similar to those in the leptin-treated *ob/ob* animals (lean controls: 18.7 ± 0.4 vs leptin-treated *ob/ob*: 18.8 ± 1.9; Figs 2, 3). There were no differences in the total number of galanin mRNA-expressing cells among the experimental groups (data not shown). Leptin treatment did not significantly alter levels of galanin mRNA in either the Arc or DMN of *ob/ob* mice, and there were no significant differences in cellular levels of galanin mRNA between *ob/ob* mice and lean controls in either of these areas (Fig. 3).

Co-localization of galanin and ob-Rb messenger RNAs (Experiment 3)

The distribution of Ob-Rb mRNA that we observed in the hypothalamus was consistent with earlier reports in the mouse,[24] with abundant expression being evident in the Arc and DMN, where there are numerous galaninergic cells (Fig. 4).[34] However, visual inspection of these hypothalamic nuclei revealed only the rare galanin mRNA-expressing cell in either the Arc or DMN that appeared to coexpress Ob-Rb mRNA. Based on a conservative threshold of SBRs = 6, less than 5% of digoxigenin-labeled galaninergic neurons in the Arc, DMN or PeN could be said to coexpress Ob-Rb and statistical analysis failed to provide any clear evidence for co-labeling in any of these hypothalamic nuclei.

DISCUSSION

Comparative distribution and intensity of galanin expression among species

Results of the distribution analysis for galanin mRNA-expressing cells in the brain of the mouse reveal a pattern of galanin expression similar, but with several important differences, to that previously described for the rat.[6,14,34] As in the rat, the highest numbers of galanin mRNA-expressing cells were observed in the diencephalon and rhombencephalon, with moderate labeling in the telencephalon and mesencephalon. In the forebrain, the presence of galanin mRNA within all of the major basal forebrain areas that contain cholinergic neurons suggests that galanin plays a similar role in modulating acetylcholine synthesis and release in the mouse as has been postulated for the rat and primate (reviewed in Refs 27 and 29). A notable and curious finding in the mouse is the apparent lack of galanin message in the cortex and striatum—areas that have been reported to contain both galanin mRNA and peptide in primates (Cebus monkeys, baboons and humans)[17] and rats.[6,23,41] One major difference between several of these studies and ours is that the mice used in our study did not receive colchicine pretreatment, whereas the majority of rat studies have used colchicine to enhance galanin peptide levels. Colchicine has been shown to induce the expression of neuropeptide genes, including galanin,[6] and since the studies in the rat where galanin has been reported to be expressed in the cortex and striatum entailed colchicine as a pretreatment, any direct comparison between the results we have obtained in the mouse and these earlier

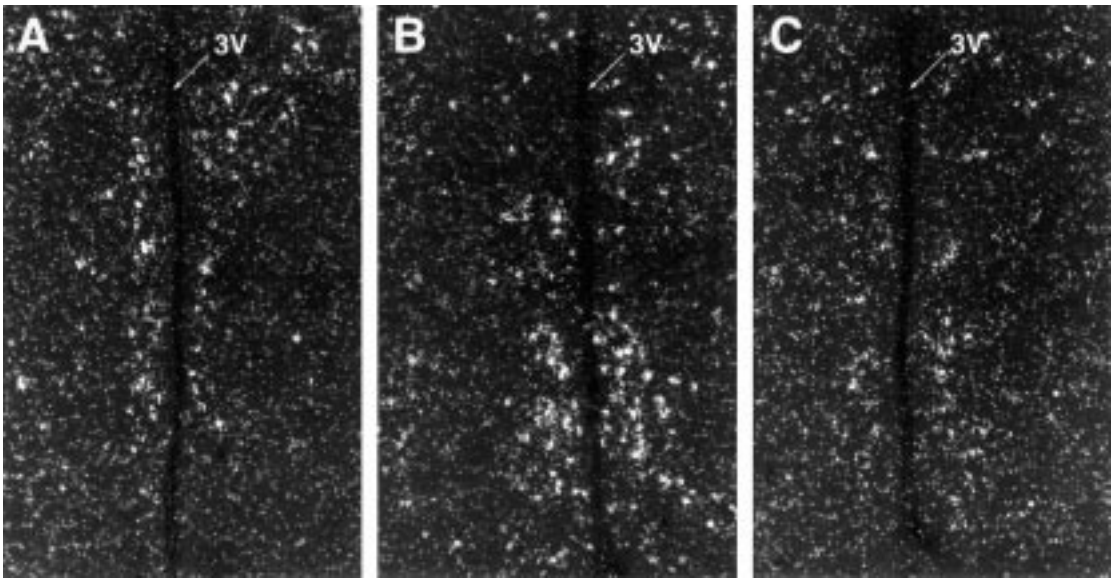

Fig. 2. Photomicrographs of galanin-mRNA containing cells in the periventricular nucleus of (A) an *ob/ob* mouse treated with leptin, (B) a pair-fed *ob/ob* mouse treated with saline, and (C) a lean control mouse. 3V, third ventricle.

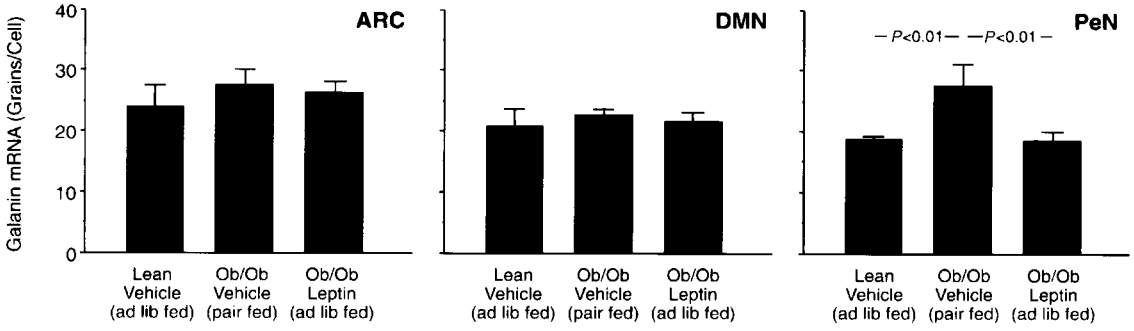

Fig. 3. Cellular galanin mRNA levels (as indicated by the number of grains/cell) in the Arc, DMN and PeN of three groups of mice. The groups consisted of normal mice (lean, vehicle-treated and *ad libitum* fed; $n = 4$) and leptin-deficient mice (*ob/ob*) either treated with leptin and allowed to feed *ad libitum* ($n = 5$), or treated with vehicle and given the same amount of food (pair-fed) as consumed by the leptin-treated group ($n = 5$). There were no significant differences among groups in any of the areas except the PeN where vehicle-treated *ob/ob* mice had significantly higher levels of galanin mRNA. Bars represent means ± S.E.M.

published studies in the rat must be viewed cautiously. Studies in the rat examining the distribution of galanin mRNA in non-colchicine-treated animals report little if any galanin gene expression in these areas.[14,34] However, in the primate studies, where colchicine was not used, galanin appears to be expressed robustly in the striatum,[17] suggesting an unequivocal difference in distribution of galanin mRNA-containing cells between primate and mouse. Another notable observation in the mouse is the modest, yet highly reproducible, presence of galanin mRNA within the dentate gyrus of the hippocampus. This contrasts with studies of this region in the rat (without colchicine pretreatment), where galanin mRNA is not apparently expressed and the peptide is detected only with the use of certain antibodies.[6,14,22,34,41] In primate studies, galanin-containing neurons are found throughout the hippocampal formation, in contrast to the discrete localization in the dentate gyrus found in our study of the mouse.[17]

Within the hypothalamus, most nuclei exhibited at least moderate labeling, with notable exceptions being the ventromedial and suprachiasmatic nuclei, which had no detectable signal for galanin mRNA. In the mouse, relatively few cells with modest intensity of labeling were observed in both the parvocellular and magnocellular regions of the PVN, which contrasts with the reports of high levels of galanin mRNA and peptide seen in the PVN of the rat.[23,34,41] However, in complete accord with observations in the rat, we observed very high numbers of labeled cells in the DMN, PeN and POA, underscoring the general importance of these nuclei as major sources of hypothalamic galanin. As a step towards understanding the functional significance of these galaninergic cells, it would be interesting to learn whether these cells coexpress other peptides. Rajendren *et al*. have recently shown in the mouse that gonadotropin-releasing hormone neurons within the POA contain significant amounts of galanin and that this co-localization phenomenon is sexually dimorphic and sex-steroid dependent,[31] corroborating earlier findings in the rat.[25] The results of our analysis

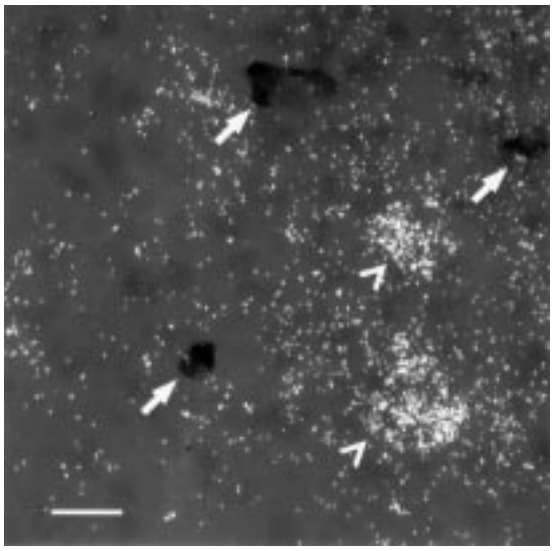

Fig. 4. Photomicrograph illustrating the lack of Ob-Rb mRNA expression in hypothalamic galaninergic neurons. White silver grains mark the presence of Ob-Rb mRNA (arrowheads) and digoxigenin-labeled galanin mRNA-expressing neurons appear as darkly stained cells (arrows). A neuron that expresses both Ob-Rb mRNA and galanin mRNA is identified as having silver grain clusters superimposed on a darkly stained cell. Scale bar = 20 μm.

of galanin mRNA expression in the hypothalamus of the mouse are in good agreement with a recent report of galanin immunoreactivity in the murine basal forebrain,[32] although the relative abundance of galanin peptide reported is at slight variance with our mRNA findings in various regions. It is conceivable that this variance results from methodological differences between the two studies; alternatively, it is possible that it simply reflects the fact that levels of mRNA and peptide are regulated differentially in some brain regions, as has been reported in the hypothalamus for cells that are actively secreting peptides.[40]

The midbrain of the mouse contained very little galanin mRNA, which is consistent with findings in primates where no galanin-containing cells are observed.[17] Our results in the mouse also concur with those in the rat, except in the dorsal raphe, where in the rat (but not the mouse), there are abundant galanin-producing cells,[23,34,41] suggesting that the putative interactions between galanin and the serotonergic system might be highly species specific (reviewed in Ref. 13).

In the brainstem of the mouse, galanin mRNA was particularly abundant in two areas, the LC and dorsal vagal complex. In the LC, galanin mRNA was nearly as abundant as in the DMN and POA of the hypothalamus, in agreement with previous studies in the rat. Many scattered cell groups were found in other parts of the brainstem, but the only other large population of galanin mRNA-expressing cells was in the vagal complex, notably in the dorsal NTS and DMV. The distribution of galanin mRNA-containing cells in the NTS of the mouse accords well with previously published reports in other species.[17,41] However, the robust population of galanin mRNA-expressing cells we observed in the DMV is apparently unique to the mouse, since studies in the rat and primates report only low numbers of galanin-containing cell bodies in this region, despite the appearance of abundant fiber tracts.[17,23,34,41] Although the functional significance of this observation is unknown, one plausible hypothesis would be that in the mouse, galanin modulates preganglionic parasympathetic neurons.

The absence of detectable levels of galanin mRNA in areas such as the cerebellum, cortex and striatum of the mouse, which in other species have been reported to contain galanin, may reflect genuine species differences. However, it is also possible that differences between laboratories in the relative sensitivity in the methods used for detecting galanin mRNA account for the apparent differences in the distribution profile among species.

Regulation of galanin gene expression by leptin

Because both galanin and leptin are thought to play an important role in the regulation of feeding and reproduction, a primary goal of this study was to investigate whether galanin might be involved in mediating the effects of leptin, particularly in the hypothalamus. We found first that levels of galanin mRNA were approximately 50% higher in the PeN of leptin-deficient *ob/ob* mice compared to wild-type controls and second that treatment of *ob/ob* mice with leptin reduced levels of galanin mRNA in the PeN to that found in wild-type controls. Leptin did not appear to affect galanin gene expression in either of the two other hypothalamic areas studied (Arc and DMN). The apparent lack of regulation of galanin expression by leptin in these two nuclei parallels findings in the rat, which suggests that outside the anterior PVN/PeN, galanin does not play a significant role in the regulation of feeding, metabolism and adiposity (reviewed in Ref. 18).

The significance of leptin's regulation of galanin gene expression in the PeN is uncertain. In the rat, the anterior portion of the hypothalamic PVN is the site where galanin is thought to be most effective in the regulation of ingestive behaviors.[19] Specifically, galanin in the PVN preferentially increases fat ingestion.[20,43] In addition, leptin administration reduces galanin expression in the PVN of the rat.[35] Together, these findings suggest that leptin decreases food intake in the rat partially by reducing galanin-induced fat ingestion. Owing to the generally low abundance of galanin mRNA in the PVN of the mouse, we were unable to assess the possible effects of leptin on galanin expression in this region. However, it is conceivable that the population of leptin-responsive galaninergic neurons in the PeN of the mouse is homologous to those in the anterior PVN/PeN region of the rat.

Our observation that galanin gene expression is up-regulated in a subpopulation of neurons in the leptin-deficient *ob/ob* mouse is consistent with the notion that galanin neurons are involved in the regulation of body weight. Galanin has been shown to act centrally to increase feeding and reduce sympathetic activity—actions that would generally decrease metabolism.[2,28,36] Thus, it seems plausible that the up-regulation of galaninergic activity in the *ob/ob* mouse contributes to

the obese phenotype of these animals by stimulating hyperphagia and slowing metabolic activity. Furthermore, galanin has been shown to induce a natural preference for fat consumption (reviewed in Ref. 18), and indeed, the *ob/ob* mouse displays a distinct preference for fat in its diet.[33] Finally, leptin administration to *ob/ob* mice reduces food intake and increases sympathetic activity,[8,16,30] both of which could be partly attributable to the reduced expression of galanin. If some of the effects of leptin on body weight in the mouse are mediated by galanin, our results suggest that galaninergic neurons in the PeN are those most likely to be responsible for producing these effects.

Coexpression of galanin and the leptin receptor

In addition to investigating the effects of leptin on galanin gene expression, we sought to determine whether leptin acts directly on galanin neurons by searching for neurons that express both galanin and Ob-Rb mRNAs. We reasoned that the galanin-expressing neurons most likely to express Ob-Rb would be those residing in the PeN and found to be regulated by leptin. However, since it is plausible that leptin regulates other gene products in galanin neurons, we explored the possibility that galaninergic neurons in other hypothalamic nuclei might also express Ob-Rb. Again, we focused on the Arc and DMN in the hypothalamus because of their putative role in the regulation of food intake. Quantitative analysis of the hypothalamic nuclei failed to reveal the presence of Ob-Rb in galaninergic neurons, although subjective visual inspection suggested that an occasional cell appeared to coexpress both galanin and Ob-Rb mRNAs. This low level of co-expression is in concert with observations in the rat that only a few galanin-containing neurons in the Arc display leptin receptor immunoreactivity.[15] We cannot exclude the possibility that some galanin neurons do express Ob-Rb mRNA and that only a few Ob-Rb-expressing galanin neurons are needed to mediate leptin's action through galanin. However, galanin gene expression in the PeN may also be regulated indirectly by leptin through other leptin-sensitive signaling systems. For example, insulin has been shown to inhibit galanin expression in the hypothalamus;[46] therefore, it seems possible that the increased sensitivity to insulin, occurring as a result of leptin administration, facilitates insulin's inhibitory action on galanin gene expression.[1,37] Another possibility is that leptin's effects on galanin-expressing neurons in the PeN are mediated by other Ob-R-expressing neurons. In this scenario, POMC neurons in the Arc are plausible candidates, since they are regulated by leptin, express leptin receptor mRNA and heavily innervate the PeN.[4,11,38,44]

In summary, galanin is one of many neuropeptides implicated in the regulation of body weight and reproduction, and thus may mediate some of leptin's effects on these processes. Galanin-expressing neurons are widely distributed throughout the brain of the mouse, including the DMN and Arc, which are known to be involved in the regulation of body weight and reproduction. Nevertheless, we could adduce no evidence for a leptin effect on the expression of galanin in neurons in these two areas. Rather, our results point to the hypothalamic PeN as a potential primary target for leptin's actions. The observation that leptin inhibits the expression of galanin mRNA in the PeN suggests that neurons in this region play a role in mediating the effects of leptin on one or more basal regulatory processes. Although the identity of those processes cannot be determined from these results, the regulation of body weight and reproduction are possibilities. Since galanin-expressing neurons in the PeN do not appear to express Ob-Rb, information about circulating levels of leptin must reach these neurons indirectly, either by some leptin-regulated hormone(s) or through other hypothalamic neurons that do express Ob-Rb. The functional significance of leptin's interaction with galanin-expressing neurons in the PeN remains to be determined.

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