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Hypothalamic injection of non-opioid peptides increases gene expression of the opioid enkephalin in hypothalamic and mesolimbic nuclei: Possible mechanism underlying their behavioral effects

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Abstract

The peptides galanin (GAL) and orexin (OX) share common features with the opioid enkephalin (ENK) in their relationship to ingestive behavior, stimulating consumption of a fat-rich diet and ethanol when injected into the hypothalamus. Since receptors for GAL and OX are dense in areas where ENK-expressing neurons are concentrated, these non-opioid peptides may exert their effects, in part, through the stimulation of endogenous ENK. This study was conducted to determine whether injection of GAL or OX affects the expression of ENK in hypothalamic and mesolimbic nuclei involved in consummatory behavior. Rats were injected with GAL (1 μg), OX-A (1 μg), or saline vehicle just dorsal to the hypothalamic paraventricular nucleus (PVN). They were sacrificed one hour later for analysis of ENK mRNA levels in the PVN, ventral tegmental area (VTA), central nucleus of the amygdala (CeA), and nucleus accumbens (NAc). Both GAL and OX had similar effects, significantly increasing ENK mRNA expression in each of these areas, except for the NAc. This enhanced ENK expression in the PVN, VTA and CeA was demonstrated with real-time quantitative polymerase chain reaction and confirmed in separate groups using radiolabeled and digoxigenin-labeled *in situ* hybridization. These findings demonstrate that the non-opioid peptides, GAL or OX, which have similar effects on consummatory behavior, are also similar in their effect on endogenous ENK. In light of published findings showing an opioid antagonist to block GAL- and OX-induced feeding, these results provide additional evidence that ENK is involved in mediating the common behavioral effects of these peptides.

Keywords

orexin; galanin; enkephalin; rat; mRNA

1. Introduction

Certain peptides expressed in the hypothalamus share common features in their relationship with ingestive behavior. For example, injection of galanin (GAL) into the hypothalamic paraventricular nucleus (PVN) is found to stimulate the consumption of ethanol [66,75] as well as ingestion of a diet rich in fat [85]. Similar effects have been described with injection of

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orexin (OX) into the PVN [21,75]. This raises the possibility that GAL and OX may act through some common mechanism to produce these ingestive behaviors. Evidence suggests that this mechanism may involve the opioid peptide enkephalin (ENK). As with GAL and ORX, PVN injection of ENK analogues causes an increase in the consumption of ethanol [5] as well as a high-fat diet [56,79]. Furthermore, the feeding response induced by central injection of GAL or OX is greatly attenuated by administration of an opioid receptor antagonist [20,24,83-84]. Thus, the non-opioid peptides GAL and OX may affect consummatory behavior by stimulating the opioid ENK, perhaps within the PVN where it has an important role in ingestive behavior [87] but also in mesolimbic nuclei where it is involved in reward [19,42].

Neuroanatomical evidence suggests that GAL and OX may exert their effects through these different brain areas. Neurons expressing GAL, which are heavily concentrated in the PVN as well as other areas of the brain [30], send projections locally and to mesolimbic nuclei, such as the ventral tegmental area (VTA), central nucleus of the amygdala (CeA), and nucleus accumbens (NAc), where its receptors (GALR1 and GALR2) are expressed at moderate to high levels [33,59]. Similarly, neurons expressing OX, found exclusively in the perifornical and lateral area (PFLH) of the hypothalamus [16,73], project to the PVN and mesolimbic areas where the OX receptors (OX1R and OX2R) are densely expressed [48,51]. In these different areas, ENK-expressing neurons are known to be densely concentrated [6,15,25,72], providing the neuroanatomical basis for an interaction between the non-opioid and opioid peptides. Thus, both GAL and OX have the potential for far-reaching and possibly similar effects on ENK throughout the brain, in nuclei where this opioid may be involved in mediating different behavioral functions related to consummatory behavior.

This study was designed to test this possibility by examining the effects of GAL and OX injection on the expression of the opioid peptide ENK in different brain areas. Animals were injected with GAL, OX, or vehicle just dorsal to the PVN, and their effects on mRNA expression of ENK were examined in the PVN, VTA, CeA, and NAc. The results revealed a stimulatory effect of these non-opioid peptides, GAL and OX, on the opioid peptide ENK in these different brain areas.

2. Materials and methods

2.1. Subjects

Adult, male Sprague–Dawley rats (200-250 g, Charles River Laboratories International, Inc., Wilmington, MA) were housed individually, on a 12-hour reversed light/dark cycle. All animals were allowed 1 week to acclimate to their individual housing conditions, during which time they received *ad libitum* access to standard rodent chow (LabDiet Rodent Chow 5001, St. Louis, MO) and water. Separate groups of animals were used for each experiment. The housing facility was fully accredited by AAALAC, and the behavioral protocols were approved by the Rockefeller University Animal Care Committee.

2.2. Drugs

The drugs injected were orexin-A (human, rat, mouse) (1 μg) and galanin (rat) (1 μg), both from Sigma-Aldrich Inc. (St. Louis, MO, USA). They were dissolved in saline and injected in a volume of 1 μl. Saline vehicle was used as a control injection, also injected in a volume of 1 μl.

2.3. Surgery

Subjects were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), supplemented with ketamine as needed. Guide shafts, made of 21-gauge stainless steel, 12 mm in length, were implanted perpendicularly and unilaterally just dorsal to the PVN. The

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coordinates were A-P -1.8 (relative to bregma), L 0.4 (relative to midsaggital sinus), and D-V 5.0 (relative to level skull surface), with half on the left side and half on the right side. Injectors protruded 3 mm beyond the guide shafts. One week of recovery was provided after surgery before testing. Between procedures, stainless steel obdurators were left in the guide shafts to prevent occlusion.

2.4. Test procedures

For each experiment, food was removed 90 minutes prior to injection. Injections were given 2 hours prior to the end of the light cycle. The OX, GAL, or saline vehicle (n=5/group) was delivered through concentric microinjectors made of 26-gauge stainless steel outside and fused-silica tubing inside (74 μm ID, 154 μm OD, Polymicro Technologies, Phoenix AZ) that protruded 3 mm beyond the guide shaft to reach just dorsal to the PVN (V 8.0). A volume of 1.0 μl was delivered during 1 min, and the microinjector remained in place for another 1 min to allow diffusion into the injection site. Animals were then sacrificed by rapid decapitation 1 hour after injection.

In Experiment 1, the PVN, VTA, CeA, and NAc were microdissected for measurements of ENK mRNA using quantitative real-time polymerase chain reaction (qRT-PCR). In Experiment 2, the whole brain was removed and placed in a 4% paraformaldehyde solution for ENK measurements using radiolabeled *in situ* hybridization histochemistry (ISH). In Experiment 3, the whole brain was removed and placed in a 4% paraformaldehyde solution for ENK measurements using digoxigenin-labeled *in situ* hybridization (DIG). This procedure of injecting and analyzing gene expression in the same hypothalamic nucleus has recently been used with leptin injection in the ventromedial hypothalamus [2].

2.5. Brain dissection

Immediately after sacrifice, the brain was placed in a matrix slicing guide with the ventral surface facing up. A total of three coronal cuts, yielding two slices, were made rostrally. The first cut was made in the anterior middle optic chiasm (Bregma −0.8 mm), according to the atlas of Paxinos and Watson [63]. The second cut was 1.5 mm rostral to this (Bregma −0.8 to 0.7 mm), yielding a first slice, which was discarded. Then, one 1.0 mm cut was made (Bregma 0.7 to 1.7 mm) rostral to this first slice, yielding a slice that was used for microdissection of the NAc (Bregma 0.7 to 1.7 mm). Caudal to the original slice, two additional 1.0 mm slices (Bregma −0.8 to −2.8 mm) were made, with the first used for microdissection of the PVN (Bregma −0.8 to −1.8 mm) and the second for the CeA (Bregma −1.8 to −2.8 mm). Further caudally, one 0.5 mm slice was made by cutting between the caudal boundary of the mamillary bodies and the rostral boundary of the pons, which was used for microdissection of the VTA (Bregma −5.6 to −6.1 mm).

These sections were placed on a glass slide and rapidly dissected under a microscope. The NAc was dissected bilaterally in an egg shape, with the dorsal tip beginning at the lateral ventricle, the medial aspect at the semilunar nucleus, the lateral aspect medial to the lateral stripe of the striatum, and the ventral edge along the ventral pallidum. The PVN was dissected as a reversed isosceles triangle, 1.0 mm bilateral to the third ventricle and between the fornix structures [11]. The CeA was dissected bilaterally as a circle, immediately dorsomedial to the basolateral amygdala and 0.2 mm dorsolateral to the optic tract. The VTA was also dissected bilaterally as a circle, lateral to the interfascicular nuleus, medial to the medial lemniscus, ventral to the red nucleus and dorsal to the paranigral nucleus.

2.6. Histology

Cannula placement was visually confirmed during brain dissection. Also, a cannulated rat not sacrificed for analysis of ENK mRNA was examined for microscopic verification of probe

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placement. This rat was injected with 0.25 μl methylene blue dye (Sigma, St. Louis, MO) and its brain kept in formalin for one week prior to slicing. The brain was then cut in 40 μm sections on a freezing microtome and slide-mounted for microscopic examination.

2.7. Quantitative real-time PCR

As previously described [11], total RNA from pooled microdissected samples was extracted with TRIzol reagent. RNA was treated with RNase-free DNase I before RT. For qRT-PCR, cDNA and minus RT were synthesized using an oligo-dT primer with or without SuperScript II Reverse Transcriptase. The SYBR Green PCR core reagents kit (Applied Biosystems, Foster City, CA) was used, with cyclophilin as an endogenous control. qRT-PCR was performed in MicroAmp Optic 96-well Reaction Plates (Applied Biosystems). This was done on an ABI PRISM 7900 Sequence Detection system (Applied Biosystems), under the condition of 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 1 minute at 60° C. Each study consisted of 4 independent runs of qRT-PCR in triplicate, and each run included a standard curve, a nontemplate control, and a negative RT control. The levels of target gene expression were quantified relative to the level of cyclophilin, using the comparative Ct method. The primers, designed with ABI Primer Express V.1.5a software from published sequences, were: (1) cyclophilin: 5′- GTGTTCTTCGACATCACGGCT -3′ (forward) and 5′-CTGTCTTTGGAACTTTGTCTGCA -3′ (reverse); and (2) E N K: 5′-GGACTGCGCTAAATGCAGCTA-3′ (forward) and 5′-GTGTGCATGCCAGGAAGTTG-3′ (reverse). The concentrations of primers were 100 nM. All reagents, unless indicated, were from Invitrogen (Carlsbad, CA).

2.8. Radiolabeled in situ hybridization histochemistry

Besides qRT-PCR, mRNA levels of ENK were measured with radiolabeled ISH. This technique allows for more anatomically precise measurements of gene expression than qRT-PCR. Antisense and sense RNA probes were labeled with 35S-UTP (Amersham Biosciences, Piscataway, NJ), as previously described [7,49]. Alternate free-floating coronal sections were consecutively processed as follows: 10 minutes in 0.001% proteinase K, 5 minutes in 4% paraformaldehyde, and 10 minutes each in 0.2 N HCl and acetylation solution, with a 10-minute wash in PB between each step. After the wash, the sections were hybridized with a ^{35}S-labeled probe (10^3 cpm/mL) at 55°C for 18 hours. Following hybridization, the sections were washed in 5X sodium chloride and sodium citrate (SSC), and the nonspecifically bound probe was removed by RNase (Sigma) treatment for 30 minutes at 37°C. Sections were then run through further stringency washes with 0.1 M dithiothreitol (Sigma) in 2X SSC and 1X SSC and 0.1X SSC at 55°C. Sections were finally mounted, air-dried, and exposed to a Kodak BioMax MR film for 18 to 24 hours at −80°C, when films were developed and microscopically analyzed. The sense probe control was performed in the same tissue, and no signal was found.

Computer-assisted microdensitometry of autoradiographic images was determined as described [49,70] on the MCID image analysis system (Image Research Inc., St. Catherines, ON, Canada). Microscale ^{14}C standards (Amersham Biosciences) were exposed on the same Kodak film with the sections and digitized. Gray-level/optical density calibrations were performed with a calibrated film-strip ladder (Imaging Research Inc.) for optical density. This was plotted as a function of microscale calibration values. All subsequent optical density values of digitized autoradiographic images fell within the linear range of the function. The values obtained represent the average of measurements taken from 10 sections per animal. Within each section, the optical density for the nucleus was recorded, from which the background optical density from a same-size area in the corpus callosum was subtracted. The mean value of the ethanol-drinking group in each experiment was reported as a percentage of the water-drinking control group.

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2.9. Digoxigenin-labeled in situ hybridization histochemistry

In situ hybridization histochemistry with digoxigenin-labeled probes was used to quantify ENK gene expression. This technique measures specifically the density of neurons expressing the peptide gene above threshold levels but not the level of mRNA expressed per cell. Brains were cut into 30 μm thick sections with a cryostat. DIG-labeled cRNA probes of ENK were synthesized by *in vitro* transcription as previously described [7,49]. Free-floating coronal sections were processed for DIG-ISH as for radiolabeled ISH until the high stringency wash with the exception of replacement with the DIG-labeled probe. After the high stringency wash, the sections were blocked and incubated in AP-conjugated sheep anti-digoxigenin antibody (Sheep Anti-DIG, Fab fragments, 1:1000; Boehringer Mannheim) overnight. After washing in Tris buffer (0.1 M, pH 9.5), the signal was revealed with NBT/BCIP. Then, the sections were mounted, dehydrated, and coverslipped as previously described [7].

Gene expression level was measured by semiquantification with Image-Pro Plus software (Version 4.5, Media Cybernetics Inc., Silver Spring, MD) as previously described [45]. It is expressed as the density of mRNA-containing cells, "cells/mm^2".

2.10. Data analysis

The data in the figures and table are expressed as mean ± SEM. Statistical analyses of these data were performed using unpaired, two-tailed *t*-tests.

3. Results

3.1. Effect of GAL and OX injection on ENK mRNA measured by qRT-PCR

This experiment used qRT-PCR to determine whether hypothalamic injection of the non-opioid peptides can modulate ENK gene expression in different brain sites. Rats with implanted cannulae aimed just dorsal to the PVN were given a single injection of GAL (n = 5), OX (n = 5), or saline vehicle (n = 5) and were examined for the expression of ENK mRNA, both locally and in different mesolimbic structures. Compared to saline vehicle, injection of GAL produced a significant increase in the expression of ENK mRNA in the PVN ($p < 0.05$), just below the area of the injection (Fig. 1). A similar effect was also detected in the VTA ($p < 0.01$) and CeA ($p < 0.01$), but not in the NAc (*ns*) (Fig. 1). Interestingly, a very similar change in ENK gene expression was observed with PVN injection of OX. As with GAL, this peptide increased ENK mRNA levels in the PVN ($p < 0.05$), VTA ($p < 0.001$) and CeA ($p < 0.05$), but not the NAc (*ns*) (Fig. 2). Thus, as shown by qRT-PCR, PVN injections of GAL and OX produce the same pattern of enhanced ENK expression in the different hypothalamic and mesolimbic nuclei examined, with the exception of the NAc where ENK was unresponsive to these peptides.

3.2. Effect of GAL and OX injection on ENK mRNA measured radiolabeled ISH

This experiment was conducted to confirm, with a different technique, the changes in gene expression observed in Experiment 1. The animals were, once again, implanted with cannulae aimed just dorsal to the PVN, injected with GAL (n = 5), OX (n = 5), or saline vehicle (n = 5), and were then examined for ENK mRNA levels using radiolabeled ISH. The results obtained in the PVN and extra-hypothalamic areas revealed a similar pattern to that shown with qRT-PCR. Compared to rats injected with saline vehicle, GAL injection again increased ENK mRNA levels in the PVN ($p < 0.001$), VTA ($p < 0.001$) and CeA ($p < 0.001$) but not the NAc (*ns*) (Fig. 3). The same pattern, of enhanced ENK mRNA in the PVN ($p < 0.001$), VTA ($p < 0.05$) and CeA ($p < 0.001$), but not the NAc (*ns)*, was also produced by injection of OX (Fig. 4). These results obtained with radiolabeled ISH are illustrated in photomicrographs showing ENK expression in the PVN and CeA of rats injected with GAL compared to saline (Fig. 5).

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Thus, the stimulatory effect of PVN injection of GAL or OX on ENK mRNA in different brain areas is robust and confirmed using ISH.

3.3. Effect of GAL and OX injection on ENK cell density measured by DIG

To provide a better visualization of the ENK-expressing neurons affected by GAL or OX injection, this experiment, using the same test paradigm, employed DIG, which allows ENK-expressing neurons to be individually counted. With a cannula aimed just dorsal to the PVN, rats were injected with GAL (n = 5), OX (n = 5), or saline vehicle (n = 5). In this experiment, the NAc was not analyzed, as it failed in both Experiments 1 and 2 to exhibit any change in response to GAL or OX. Examination of the saline-injected animals revealed ENK-expressing neurons in the different areas under study. In the PVN, a dense concentration was detected in the medial parvicellular area at all anterior-posterior levels and in the lateral magnocellular area of the posterior PVN. In the VTA, ENK-expressing cells were scattered throughout the nucleus, and in the CeA, they were densely expressed in the lateral and capsular parts and moderately or sparsely expressed in the medial part. Consistent with Experiments 1 and 2, the injection of GAL or OX in the PVN was found to have a stimulatory effect on these different populations of ENK neurons. Compared to rats injected with saline vehicle, GAL and OX both increased the density of ENK-expressing neurons in the PVN ($p < 0.001$), VTA ($p < 0.001$), and CeA (GAL: $p < 0.001$, OX: $p < 0.01$) (Table 1). These results obtained with DIG are illustrated in photomicrographs of the PVN and CeA of rats injected with GAL compared to saline (Fig. 6). Thus, the finding that PVN peptide injections stimulate ENK gene expression throughout different brain regions was once again confirmed.

3.4. Histology

Based on visual inspection at the time of brain dissection and on microscopic verification, injections sites were found to be located just dorsal to the PVN. No injector tip was more than 0.7 mm above the dorsal aspect of the PVN, nor were any observed within the borders of the PVN itself. The location of the injections is illustrated in Fig. 7.

4. Discussion

Building on the shared features of GAL, OX and ENK with respect to food and ethanol consumption as described in Section 1, we wanted to determine whether injection of the non-opioid peptides have effects on ENK mRNA expression, within or outside the hypothalamus, that may contribute to their common, stimulatory effect on consummatory behavior. The results obtained with qRT-PCR demonstrated a similar effect of GAL and OX injection on ENK expression in the PVN, VTA and CeA, with no change with either peptide observed in the NAc. With a single injection just dorsal to the PVN, both of these non-opioid peptides increased ENK mRNA levels by up to 50%. These findings were confirmed with the more anatomically precise, radiolabeled ISH technique, which revealed a 20-40% increase in mRNA expression, and also with DIG, which showed a 40% increase in the density of ENK-expressing neurons in these 3 areas. This is the first study showing hypothalamic injection of an orexigenic peptide, such as GAL and OX, to influence mRNA expression of an opioid peptide that is known to promote consummatory behavior.

Injection of GAL or OX immediately dorsal to the PVN may alter gene expression by acting on different receptor subtypes and peptide systems within the PVN. The distribution of GAL and OX terminals within this nucleus is found to be similar [16,74]. Whereas there are no anatomical studies showing the existence of GAL and OX receptors specifically on ENK-expressing neurons, the PVN is known to have moderate-to-high levels of the GAL receptors, GALR1 and GALR2 [29,53-54], and the OX receptors, OX1R and OX2R [48,96], and these receptors are found to be located on PVN neurons that express other peptides, e.g., vasopressin,

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oxytocin, or corticotrophin-releasing factor [3,29]. Electrophysiological studies demonstrate that the GALR2 and both OX receptors are neuronally excitatory [18,90], although GALR1 is inhibitory [90]. Thus, cellular mechanisms exist within the PVN that may underlie the similar behavioral and neurochemical effects produced by injection of GAL or OX.

The finding that these non-opioid peptides have a local stimulatory effect on ENK expression suggests that this opioid in the PVN may be involved in mediating their behavioral effects. This is consistent with evidence that administration of ENK in the PVN acts similarly to GAL and OX, in stimulating the ingestion of food [52,91], particularly a high-fat diet [56,79], and of ethanol [5]. Also, PVN ENK mRNA is similar to GAL and OX mRNA in being increased by the ingestion of a high-fat diet [10] or ethanol [8,61] and it is similar to OX mRNA in being stimulated by nicotine [38,47]. Direct support for the idea that ENK mediates the actions of GAL or OX in promoting consummatory behavior is provided by the finding that an opioid antagonist blocks the feeding response induced by injection of these non-opioid peptides [12, 20]. Recent evidence showing that PVN injection of an ENK analogue can stimulate the release of dopamine (DA) in the NAc [67], similar to PVN injection of GAL [68], suggests the additional involvement of this catecholamine, which is known to mediate the motivational aspects of feeding behavior [62].

The finding that ENK mRNA in the VTA, similar to the PVN, is stimulated by hypothalamic injection of GAL and OX suggests a role for this area in mediating the behavioral effects induced by these non-opioid, orexigenic peptides. The PVN and VTA are connected via a bi-directional, mu-receptor mediated pathway [65], and the PVN sends projections to the VTA that contain corticotrophin-releasing factor [71]. The VTA is known to have a predominant role in reward function. Neurons containing DA are concentrated in the VTA [82], and injection studies in this area show enhanced activity of DA cells in response to GAL [22] and drug-seeking in response to OX [31]. A function in reward specifically for ENK in the VTA is supported by evidence obtained with local administration of opioid agonists and antagonists. Injections of ENK analogues into this region are found to stimulate food intake [50,55,58] and establish place preferences [4,64], while opioid antagonists suppress food intake [69] and ethanol consumption [37]. Moreover, ENK mRNA in the VTA, like the PVN, is found to be stimulated by chronic consumption of ethanol [9]. Therefore, the enhanced ENK expression in the VTA induced by hypothalamic GAL or OX injection may contribute to the stimulation of food and ethanol intake based on its rewarding characteristics.

Like the PVN and VTA, the CeA responds to injection of GAL and ORX with a significant increase in ENK expression. Whereas the PVN does not appear to project directly to the CeA, it may communicate indirectly via the VTA, which sends DA projections to the CeA [32,60], and the CeA, in turn, may communicate with the PVN via mu-mediated projections [27] and GABA projections [88-89] that return back to the PVN. The CeA has been implicated in reward and addiction [76] and may also play a role in states of anxiety [43]. Thus, the increased expression of ENK in the CeA suggests a possible role for this opioid in mediating both ingestive behavior and anxiety. Food intake is stimulated by injection of GAL into the amygdala [13,44,80] or ENK analogues in the CeA [28,41,46], and opioid antagonists in the CeA suppress operant responding for ethanol [23,35]. Further, endogenous ENK expression in the CeA is enhanced by intake of ethanol [14] as well as nicotine [47]. This enhanced expression of CeA ENK may reflect an increase in anxiety, as ENK levels in the amygdala are stimulated by immobilization stress [36] or predator odor [34] and reduced by adrenalectomy [1]. This opioid in the CeA is suggested to provide a coping mechanism, since mice with high anxiety, when faced with a predator odor, show a blunted elevation of ENK compared to those with low anxiety [34]. Thus, the increase in ENK mRNA in this nucleus produced by hypothalamic GAL or OX injection may have a function in attenuating the impact of stressors or enhancing processes involved in addiction.

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Of the four areas examined in the rats injected with GAL and OX, the NAc was the only one that failed to show any change in ENK mRNA. The basis for this is unclear, as ENK analogues injected into this nucleus, particularly in the border between the shell and core subregions, produce similar effects to those seen in the PVN, VTA and CeA, namely, an increase in consumption of food [55], fat-rich diet [94], and ethanol [95]. Also, endogenous ENK in the NAc, similar to the PVN, is enhanced by consumption of ethanol [17]. However, there are clear differences between the NAc and PVN that should be noted. In the NAc, GAL or OX injection fails to enhance ethanol intake [75], in contrast to the stimulatory effect in the PVN [75], and ENK mRNA in the NAc is suppressed by morphine [26,86] and hedonic foods, such as sucrose [81] and chocolate Ensure [40], once again in contrast to the PVN [10]. It is noteworthy that these investigations, as in the present study, did not separately examine the shell and core of this nucleus, subregions with differential functions, respectively, in motivational valence or goal-directed behavior from conditioned learning [39,78]. Thus, the lack of effect in the present report and divergent effects observed in the literature may reflect the degree to which these subregions of the NAc were involved.

Behavioral studies examining the relationship between GAL and ENK or between OX and ENK generally suggest that these peptides may interact positively. This is demonstrated by studies of food intake, showing GAL-induced feeding to be blocked by pretreatment with icv injection of the general opioid antagonist naloxone [20] or co-injection of a mu-receptor antagonist [84]. Thus, endogenous ENK, when stimulated by GAL, may contribute to the overconsumption of fat or ethanol [8,10]. Studies of avoidance behavior also suggest a positive relationship between GAL and ENK, with the GAL-induced decrease in active behavioral avoidance blocked by peripheral administration of naloxone [77]. Further, the finding that peripheral administration of a GAL agonist diminishes morphine withdrawal [92] suggests that GAL can substitute for morphine, although other evidence suggests that it may block morphine-induced place preference [93]. Similar to GAL, there is evidence that OX may also interact positively with ENK. The increase in food intake induced by central OX injection is blocked by pretreatment with naloxone [24,83], suggesting that OX exerts its effects through endogenous ENK. The reverse possibility, that ENK acts through enhanced functioning of OX, is supported by the findings that feeding induced by the ENK-analogue, DAMGO, in the NAc is blocked by an OX antagonist in the VTA [97] and that morphine-induced place preference is diminished by VTA injection of an OX antagonist and is lost in mice lacking the gene for prepro-orexin [57]. Together, these behavioral findings support the possibility that both GAL and OX exert at least some of their effects by enhancing the functioning of the ENK system.

4.2. Conclusions

In summary, injection of GAL and OX into the PVN leads to a similar pattern of ENK expression in hypothalamic and extra-hypothalamic nuclei. These results suggest that these non-opioid peptides acting within the hypothalamus may have similar behavioral effects, in part, through their common, stimulatory effect on opioid expression specifically in the PVN, VTA and CeA. Through this positive relationship, GAL and OX may function together with ENK to promote excess consummatory behavior.

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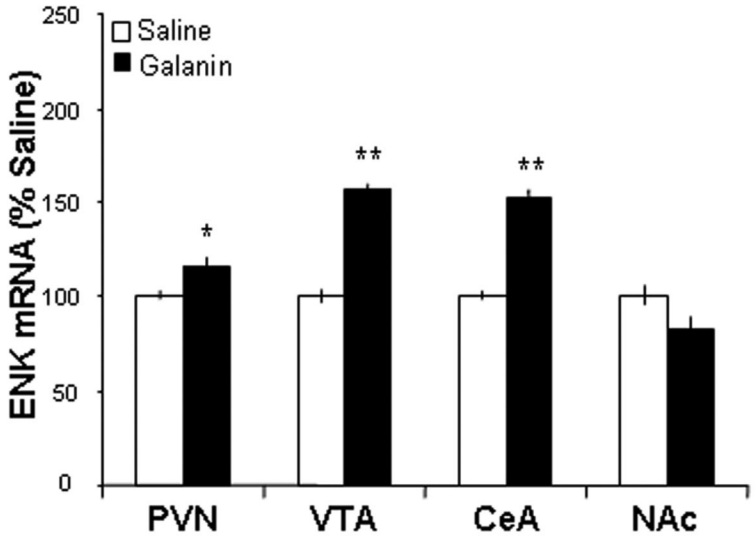

Fig. 1.

Injection of galanin into the hypothalamic paraventricular nucleus (PVN) enhances enkephalin mRNA expression in the PVN, ventral tegmental area (VTA), and central nucleus of the amygdala (CeA), but not in the nucleus accumbens (NAc) as assessed by quantitative real-time polymerase chain reaction (n = 5/group). Values are mean ± S.E.M., $^{**}p < 0.01$, $^{*}p < 0.05$ vs. saline injection.

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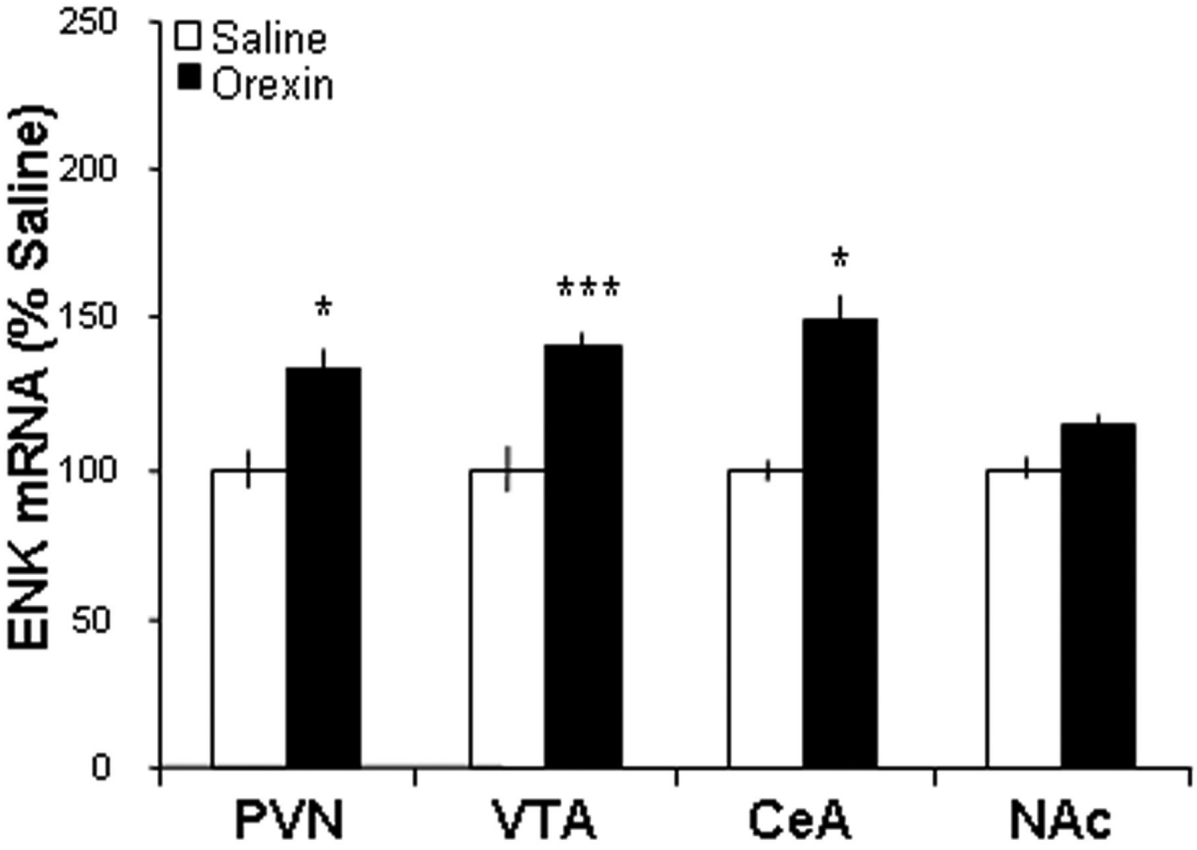

Fig. 2.

Injection of orexin into the hypothalamic paraventricular nucleus (PVN) enhances enkephalin mRNA expression in the PVN, ventral tegmental area (VTA), and central nucleus of the amygdala (CeA), but not in the nucleus accumbens (NAc) as assessed by quantitative real-time polymerase chain reaction (n = 5/group). Values are mean ± S.E.M., ***$p < 0.001$, *$p < 0.05$ vs. saline injection.

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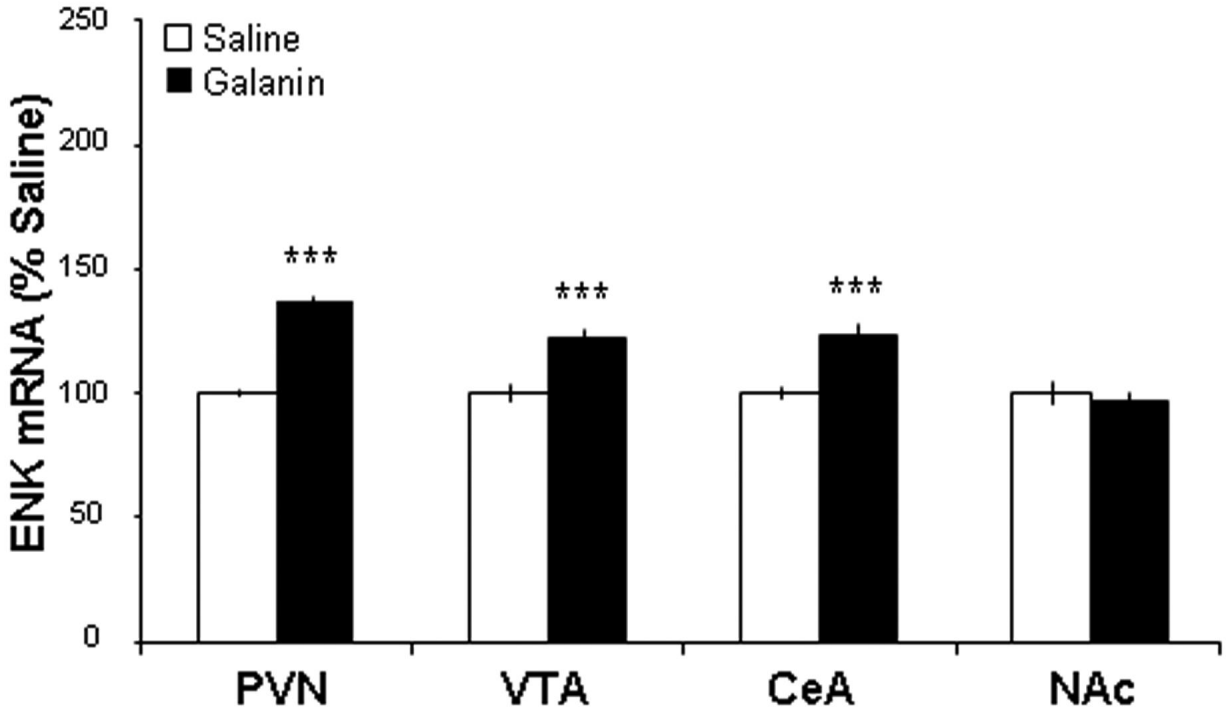

Fig. 3.

Injection of galanin into the hypothalamic paraventricular nucleus (PVN) enhances enkephalin mRNA expression in the PVN, ventral tegmental area (VTA), and central nucleus of the amygdala (CeA), but not in the nucleus accumbens (NAc) as assessed by radiolabeled *in situ* hybridization histochemistry (n = 5/group). Values are mean ± S.E.M., ***$p < 0.001$vs. saline injection.

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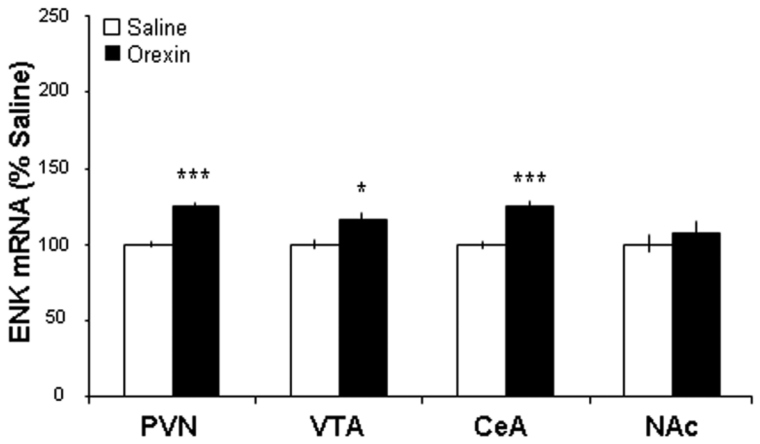

Fig. 4.

Injection of orexin into the hypothalamic paraventricular nucleus (PVN) enhances enkephalin mRNA expression in the PVN, ventral tegmental area (VTA), and central nucleus of the amygdala (CeA), but not in the nucleus accumbens (NAc) as assessed by radiolabeled *in situ* hybridization histochemistry (n = 5/group). Values are mean ± S.E.M., ***$p < 0.001$, *$p < 0.05$ vs. saline injection.

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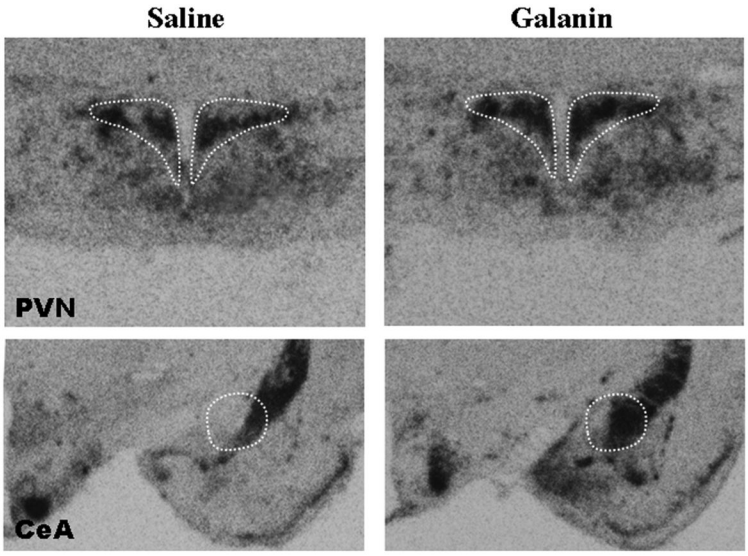

Fig. 5.

Photomicrographs showing radiolabeled *in situ* hybridization histochemistry analysis of enkephalin (ENK) mRNA in the paraventricular nucleus (PVN) and central nucleus of the amygdala (CeA) of rats injected in the PVN with galanin or saline vehicle (4X magnification). The anatomical pattern of expression was the same in response to injection of orexin. The ventral tegmental area is not included here due to its low levels of ENK expression.

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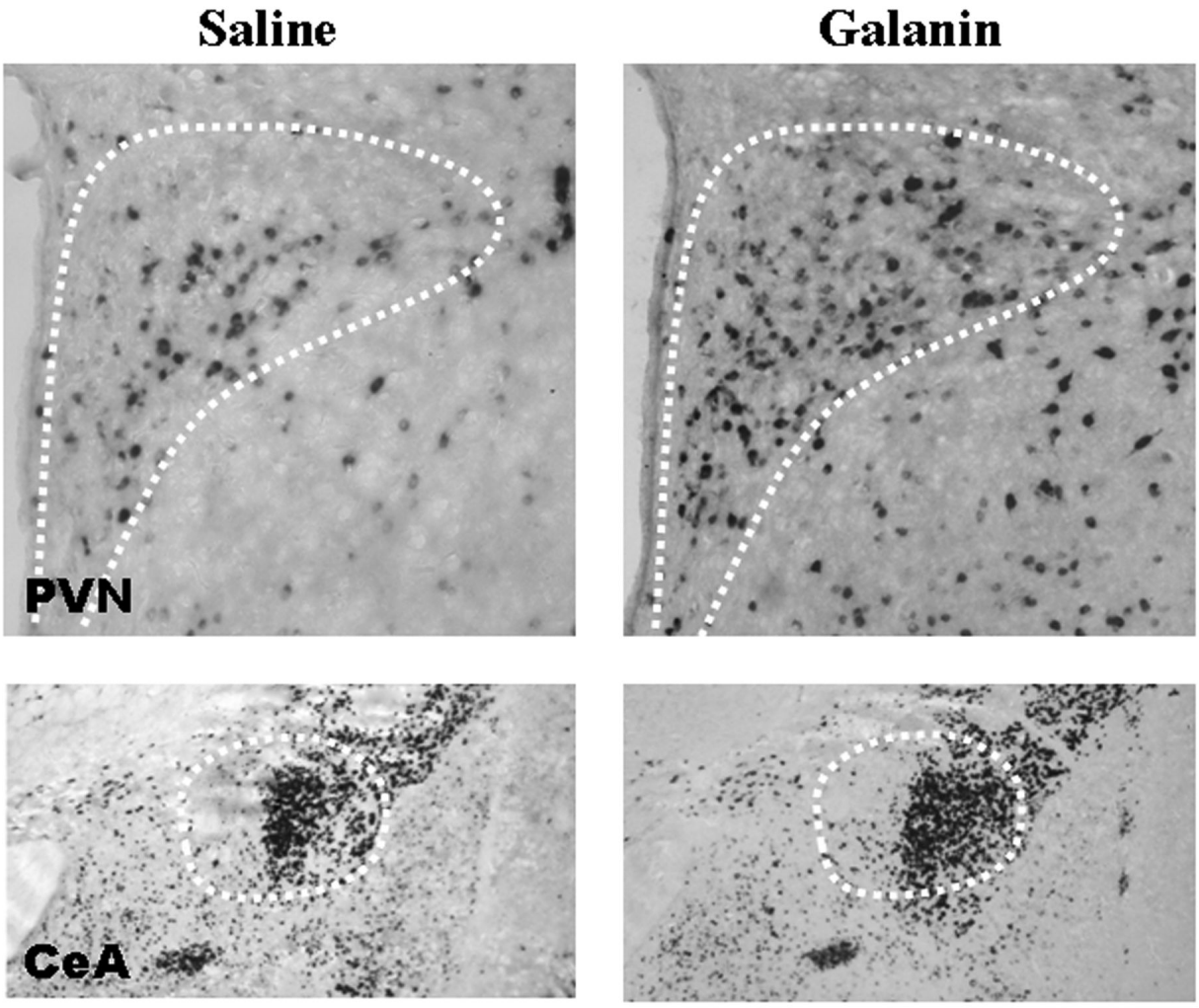

Fig. 6.

Photomicrographs showing digoxigenin-labeled *in situ* hybridization histochemistry analysis of cells expressing enkephalin (ENK) in the paraventricular nucleus (PVN) (10X magnification) and central nucleus of the amygdala (CeA) (4X magnification) of rats injected in the PVN with galanin or saline vehicle. The anatomical pattern of expression was the same in response to injection of orexin. The ventral tegmental area is not included here due to its low levels of ENK expression.

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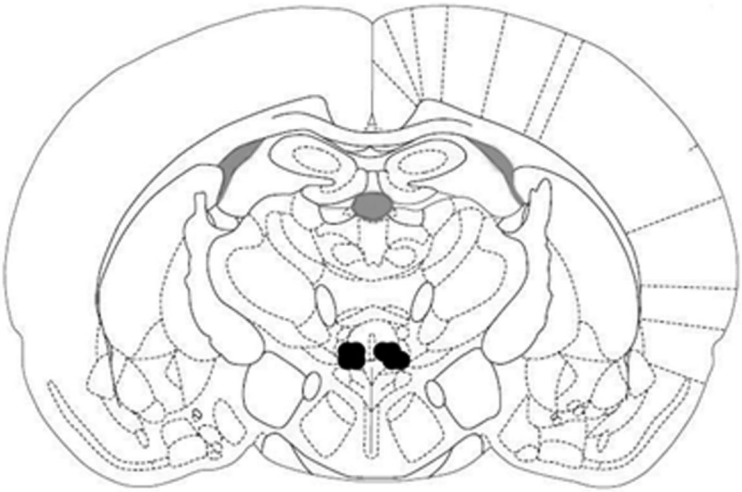

Fig. 7.

Histological verification of injector sites. Black dots indicate injector tip placement. Injections were unilateral and counterbalanced, left and right. Coronal section is −1.8 mm caudal to Bregma. Adapted from *The Rat Brain*, compact 3rd edition, G. Paxinos and C. Watson, Copyright 1997, with permission from Elsevier.

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Table 1

Cell density of hypothalamic and extra-hypothalamic enkephalin is elevated in rats injected in the paraventricular nucleus (PVN) with galanin (n = 5) or orexin (n = 5) versus saline (n = 5) as assessed by digoxigenin-labeled *in situ* hybridization. Values are mean ± S.E.M.

	PVN cells/μm × 10 $^{-4}$	**VTA cells/μm × 10 $^{-5}$**	**CeA cells/μm × 10 $^{-3}$**
Saline	2.47 ± 0.18	1.32 ± 0.04	0.97 ± 0.02
Galanin	3.41 ± 0.06***	1.78 ± 0.02***	1.40 ± 0.04***
Orexin	3.37 ± 0.03***	1.76 ± 0.02***	1.28 ± 0.11**

*** $p < 0.001$

** $p < 0.01$ vs. saline.