Ethanol intake increases galanin mRNA in the hypothalamus and withdrawal decreases it

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Abstract

Alcoholism can be viewed as a motivational disorder that results from alterations in brain systems for ingestive behavior. Therefore, it was hypothesized that alcohol intake might alter the expression of hypothalamic peptides that stimulate feeding. Earlier studies showed that hypothalamic injection of the feeding-stimulatory peptide, galanin (GAL), increases the release of dopamine (DA) in the nucleus accumbens (NAc), as does systemic alcohol, leading to a focus on GAL. Results of this study demonstrate the following: (1) Ethanol, injected daily (0.8 g/kg 10% v/v) for 7 days in male rats, markedly increased the expression of GAL but not of neuropeptide Y (NPY). This occurred in specific hypothalamic nuclei, namely the dorsomedial nucleus (DMN), paraventricular nucleus (PVN) and perifornical lateral hypothalamus (PLH). (2) Rats induced to drink ethanol ad libitum, by gradually increasing the concentration from 1% to 9% v/v without adding sugar or flavoring, exhibited a similar stimulation of GAL mRNA in the PVN and GAL immunoreactivity in the DMN and PVN. (3) Rats given increasing ethanol concentrations, with 12 h access starting 4 h into the dark cycle, had a mean blood alcohol concentration of 18 mg/dl and exhibited a similar increase in GAL expression in the DMN and PVN. (4) Withdrawal from the opioid effects of 9% ethanol, produced by injection of naloxone (3 mg/kg sc), reversed this ethanol effect by significantly reducing GAL expression in the DMN and PLH below baseline levels. These studies suggest a possible role for hypothalamic GAL in alcohol abuse.

Keywords: Alcohol intake; Ethanol; Galanin; NPY; mRNA; Hypothalamus; Naloxone; Opioid; Rat

1. Introduction

The study of neurochemicals involved in controlling ingestive behavior may provide important insights into the mechanisms underlying the consumption of alcohol. Hypothalamic neuropeptides are powerful controllers of the urge to eat and to keep eating [1,2]. In addition to modulating food intake, the synthesis of these peptides is strongly affected by the substances being ingested [3]. Thus, it is important to determine whether any of the appetite-controlling peptides exhibits a similar pattern in relation to ethanol intake, with ethanol consumption modulating their expression and production in the hypothalamus.

Hypothalamic injection of the peptide, galanin (GAL), has a stimulatory effect on feeding behavior [4]. Further, food ingestion, particularly fat intake, increases the expression and synthesis of endogenous GAL [5,6], suggesting a positive feedback loop. This peptide coexists with opioids in hypothalamic neurons [7], and it synergizes with these peptides in their antinociceptive action [8]. Moreover, both GAL and the opioids are linked to the consumption of palatable, fat-rich diets, which alcohol-preferring animals are known to naturally prefer [9–14].

Neuropeptides that influence the release of dopamine (DA) provide another logical starting point. Many parts of the limbic system and limbic cortex are important in drug abuse, with much attention focused on DA in the nucleus accumbens (NAc) [15–17]. Synaptic DA is increased in the NAc by most drugs of abuse given systemically [18] or locally [19], including ethanol [20]. Moreover, it is in this area where intracellular changes are known to mark an addicted brain [21]. Therefore, if a hypothalamic peptide has a role in controlling alcohol ingestion, one should be...
able to establish a link between this peptide and DA in the NAc. It has been shown that GAL is one such peptide that, when injected into the hypothalamus, can stimulate DA release in the NAc [22].

Given the positive feedback between hypothalamic GAL and food ingestion and the link of GAL to the opioids and DA, the present set of experiments was designed to investigate the relationship of alcohol intake to the expression and production of GAL in the hypothalamus. Experiments were performed with both injection and voluntary consumption of alcohol. In addition to studying GAL, another peptide, neuropeptide Y (NPY), that stimulates feeding [23–25] was also examined. This peptide has very different properties from GAL in terms of its relationship to fat and carbohydrate intake [26–28] and thus may have a different role in relation to alcohol intake. The evidence indicates that NPY synthesis is reduced by alcohol consumption [29] and that NPY injection can suppress or increase the ingestion of alcohol [30–33]. Unlike NPY, there appear to be no previous studies directly relating GAL to alcohol ingestion.

The present results show that ethanol injection and intake stimulate the expression and synthesis of GAL in the hypothalamus. Moreover, they demonstrate that naloxone reverses this effect of ethanol on GAL, suggesting the involvement of endogenous opioids. Some of these results have been presented in conference reports [34].

2. Methods

2.1. Subjects

Adult, male (275–498 g) and female (220–300 g) Sprague–Dawley rats (Taconic Farms, Germantown, NY) were housed individually on a 12 h reversed light/dark cycle. In all experiments, rodent chow and water were available ad libitum, and water was provided by an automatic watering system or in graduate cylinders when measurements were needed. All animals were given at least 1 week to acclimate to laboratory conditions, and all protocols were approved by the Institutional Animal Care and Use Committee.

At the end of each experiment, food was removed 1 h before sacrifice, unless otherwise noted, and the animals were killed by rapid decapitation. Brains were rapidly removed and were placed in chilled paraformaldehyde until prepared for analysis of hypothalamic GAL and NPY. Measurements of peptide mRNA and peptide immunoreactivity (ir) were made using in situ hybridization and immunocytochemistry. In the specified experiments, trunk blood was collected. All analyses were performed blind to the treatment condition.

2.2. Experimental procedures

In Experiment 1, male rats (n=10 per group) were injected intraperitoneally (ip) for 7 days with 0.8 g/kg of a 10% v/v solution of ethanol in saline or with saline alone as a control. Body weights were recorded daily. After the last injection, rats were deprived of food for 3 h and then sacrificed 5 h before the onset of the dark cycle. The brains were perfused and prepared for hypothalamic peptide assays.

In Experiment 2, two sets of male and female rats (n=20) were used. For the experimental groups (n=5 males, n=5 females), the rats were given ad libitum access to two graduated bottles of fluid, one with water and one with ethanol. For the control groups (n=5 males, n=5 females), both cylinders contained water. The right–left position of the two bottles was rotated each day to prevent place preference. In this experiment only, both water and ethanol solutions were adjusted to pH 3 in an attempt to make the alcohol taste less aversive. The ethanol concentration was increased stepwise from 1% to 9% v/v over the course of 20 days in the following manner: Days 1–4, 1%; Days 5–8, 2%; Days 9–12, 4%; Days 13–16, 7% and Days 17–20, 9%. Then, the animals were sacrificed and prepared for hypothalamic peptide assays.

In Experiment 3, in addition to water and chow, male Sprague–Dawley rats (n=10) were given access to ethanol in their home cages for 12 h/day for 20 days, with daily access starting 4 h after lights out and with ethanol concentrations increasing stepwise as described in Experiment 2. These ethanol rats were compared with control subjects (n=10), which were given ad libitum access to water. Body weights were recorded every 4 days (last day of each ethanol concentration). On Day 21, after 1 h of access to 9% ethanol, rats were sacrificed. Blood plasma samples were frozen and saved to measure blood alcohol levels, and the brains were prepared for the hypothalamic peptide assays. Blood alcohol concentrations were determined using an Analox GM7 Fast Enzymatic Metabolic Analyser (Lunenburg, MA).

In Experiment 4, similar to Experiment 3, male rats (n=8), in addition to water, were offered ethanol in their home cages for 12 h/day for 20 days, while control rats (n=8) were given ad libitum access to water. On Day 21, when the rats would normally be receiving their 9% ethanol, opioid withdrawal was instigated with the opioid antagonist, naloxone. Half of each group (control and ethanol) received naloxone (3 mg/kg sc), while the other half received saline injections. Thirty minutes after injection, the rats were sacrificed for hypothalamic peptide assays.

2.3. In situ hybridization

In situ hybridization with digoxigenin-labeled probes was used to quantify GAL gene expression. This technique measures specifically the density of neurons expressing the GAL gene above threshold levels but not the level of mRNA expressed per cell. This technique was chosen because it is sensitive, due in part to our procedure of using free-floating sections. It also has a high signal-to-
noise ratio, with almost no background, making quantification possible [5,35]. It is time efficient, allowing analyses of large groups of animals, and it maintains high-quality morphology, permitting examination and quantification of cells in specific hypothalamic nuclei. It also avoids problems of isotope contamination and decay. Results obtained with this technique are generally consistent with those obtained, in this and other labs, using such techniques as immunocytochemistry, radioimmunoassay, ribonuclease protection assay and isotopic in situ hybridization.

Following rapid decapitation, brains were removed, postfixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) overnight at 4 °C and then stored in a 25% sucrose–PB (pH 7.4) at 4 °C for 48 h. The brains were then frozen at −80 °C until day of use.Brains were cut into 30 μm thick sections with a cryostat. Antisense cRNA probes (final concentration, 5 μg/ml), labeled with digoxigenin, were prepared by in vitro transcription. Briefly, a 678 bp HindIII fragment of the rat GAL cDNA (a gift of Dr. Maria E. Vrontakis, University of Manitoba, Winnipeg, Canada) or a 511 bp EcoRI fragment of the rat NPY cDNA (a gift of Dr. Steven Sabol, NIH, Bethesda, MD) was subcloned into a modified plasmid containing a T7 promoter. The GAL plasmid linearized with HindIII or the NPY plasmid linearized with EcoRI was transcribed in the presence of Digoxigenin-11-UTP (Boehringer Mannheim), as previously described [36].

Free-floating coronal sections were treated with protease K (0.001% protease K in 50 mM Tris–HCl, 5 mM EDTA) for 10 min and fixed with 4% paraformaldehyde. After postfixation, sections were inactivated for endogenous alkaline phosphatase with 0.2 N HCl and acetylated with 0.25% acetic anhydride (Sigma) for 10 min each. After dehydration, sections were incubated in hybridization buffer (50% formamide, 1× Denhardt’s solution, 10% dextran sulfate) and digoxigenin-labeled GAL or NPY at 55 °C for 18 h. Between each step, sections were rinsed twice in 0.1 M PB (pH 7.2) for 5 min each.

After hybridization, sections were treated with the following: 5× SSC wash at 60 °C for 20 min, a stringent wash in 50% formamide for 30 min at 60 °C and a 30 min digestion of excess probe with RNase A (1 μg/ml) at 37 °C. Sections were then incubated with sheep antidigoxigenin antibody conjugated to AP (Antidig-AP, Fab fragments, 1:1000; Boehringer Mannheim) for 16–24 h at room temperature. The developer was freshly prepared by the addition of 50 μl 4-nitroblue tetrazolium chloride solution (NB; Boehringer Mannheim) and 37.5 μl 5-bromo-4-chloro-3-indolyl-phosphate-solution (BCIP, X-phosphate; Boehringer Mannheim) in 10 ml Tris buffer (100 mM Tris–HCl, 100 mM NaCl, 50 mM MgCl₂, pH 9.5) at room temperature in the dark for 4 h to 1 day. Lastly, the sections were mounted on slides, treated for 10 min with 4% paraformaldehyde, dehydrated using graded series of ethanol and xylene and coverslipped.

2.4. Immunocytochemistry

Brains were collected and stored as described above for in situ hybridization. The tissue was placed in an 80% methanol PBS solution containing 0.03% H₂O₂ for 30 min. Incubation was carried out with normal goat serum (dilution 1:10 in PBS with 0.5% Triton X-100) for 30 min. The tissues were then transferred into the GAL primary antibody (dilution 1:20,000, supplied by Dr. Steven M. Gabriel, Mt. Sinai School of Medicine, New York) or the NPY primary antibody (dilution 1:12,500, supplied by Dr. Marvin R. Brown, University of California, San Diego) at room temperature for 24–48 h. The tissues were then exposed to secondary antiserum, biotinylated antirabbit IgG (Vectastain Elite Kit, Vector) for 1 h. All sections were processed further using standard Vectastain ABC techniques. Staining then occurred by exposure to 0.05 M Tris–HCl buffer solution with 0.01% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) containing 0.03% H₂O₂. Between each step, the sections were rinsed twice with 0.01 M PBS (pH 7.4). Finally, the sections were mounted on slides, dehydrated with graded series of ethanol and xylene and coverslipped.

2.5. Quantification of in situ hybridization and immunocytochemistry

A digital imaging system, with the help of a rat brain atlas [37], was used for quantification of cell density (density of cells per mm²) in a specific area of the brain. The hypothalamic areas, dorsomedial nucleus (DMN), paraventricular nucleus (PVN), perifornical hypothalamus (PLH) and arcuate nucleus (ARC), were examined. To obtain a total of six separate readings per subject at a given level, the density of cells in three to four sections at the same level was routinely analyzed, with a second section read if an area was unreadable due to tissue damage. A Leitz microscope was used with a 4× illumination objective, and a Leitz voltage monitor (Model 050260) kept the light voltage at 12 V to maintain light intensity. The digital image was collected by a video camera connected to an IBM computer with WScan Array Software (Galai Lat./Migdal Haemek, Israel). The area of interest was outlined using the Drawing Tool available in the software and the rat brain atlas as a reference. The defined area was always at the same level on all sections chosen for control and experimental animals. To count the number of black pixels above background level within the areas of interest, a threshold value was first established, as described in the instruction manual. Briefly, using 10 randomly selected sections from the experiment, this threshold was set by matching the number of objects counted by the software in a defined area of each section with the number of objects counted manually in that same area. When these numbers of objects were found to match in all 10 sections, this threshold value was kept constant.
and used for all sections counted within a given experiment. By setting the threshold, the software then permitted the experimenter to count the objects above background level.

2.6. Data analysis

Alcohol intake was calculated as g ethanol/kg body weight. Hypotheses regarding treatment groups, brain areas and behavioral measures were tested using a two-way ANOVA followed by unpaired t tests, as specified in Section 3. Blood alcohol concentration and body weights were analyzed by Student’s t tests.

3. Results

3.1. Experiment 1: alcohol injections increased GAL in specific hypothalamic nuclei

This experiment tested the effect of ethanol injection on the expression and production of GAL and NPY in various hypothalamic nuclei. The data revealed a marked increase in GAL mRNA \( [F(3,79) = 3.46, P < .03] \) in rats injected with ethanol compared with saline \( (n = 10 \) per group) (Fig. 1). This effect was statistically significant \( (P < .05) \) in three hypothalamic areas, the DMN, the anterior portion of the PVN and the PLH, as illustrated in the photomicrographs of Fig. 2. This change in mRNA was coupled with a reliable increase in GAL peptide-ir (GAL-ir) \( [F(3,79) = 5.01, P < .001] \), which was significant in each of the three areas (Fig. 1).

In contrast to these three sites, ethanol injection had no effect on GAL in the posterior region of the PVN (mRNA: \( 715 \pm 42 \) vs. \( 734 \pm 97 \) cells per mm\(^2\), peptide-ir: \( 1323 \pm 56 \) vs. \( 1192 \pm 47 \) objects per mm\(^2\)) or the ARC (mRNA: \( 1753 \pm 133 \) vs. \( 1757 \pm 112 \) cells per mm\(^2\), peptide-ir: \( 1160 \pm 42 \) vs. \( 1136 \pm 32 \) objects per mm\(^2\)). The body weights of the age-matched saline and ethanol groups were also not significantly different.

In addition to this anatomical specificity, the effect of alcohol administration was peptide specific. The measurements of NPY mRNA in the ARC revealed no change in ethanol-treated rats compared with controls (2045 \( \pm 161 \) vs. 2140 \( \pm 205 \) cells per mm\(^2\)). Further, NPY peptide-ir in ethanol rats showed a small overall decrease \( [F(1,59) = 4.11, P < .05] \), which was statistically significant in the ARC \( (1030 \pm 7 \) vs. \( 1112 \pm 36 \) objects per mm\(^2\), \( P < .02) \) but not the PVN \( (1070 \pm 30 \) vs. 1084 \( \pm 42 \) objects per mm\(^2\)) or DMN \( (448 \pm 13 \) vs. 470 \( \pm 12 \) objects per mm\(^2\)).

3.2. Experiment 2: ad libitum voluntary alcohol consumption stimulates GAL expression

Although the injection procedure used in Experiment 1 delivers a controlled amount of ethanol to the rat, it has distinct disadvantages, in that it provides no information about spontaneous behavior, and the stress of the injections may have lasting effects [38]. Therefore, the analyses of

Fig. 2. Photomicrographs illustrating the stimulatory effect of ethanol on GAL mRNA in the DMN, PVN and PLH, as graphed in Fig. 1 (Experiment 1). Abbreviations = f: fornix, opt: optic tract, sox: supraoptic decussation.
hypothalamic GAL in relation to ethanol ingestion were examined in rats, both males and females, allowed to consume ethanol voluntarily. After 20 days of ethanol access at escalating concentrations (see Section 2), the male rats increased their consumption on 9% ethanol to an average of 5.3 g/kg/day and the females consumed 5.7 g/kg/day.

The peptide changes observed with ad libitum ethanol ingestion compared with water intake were similar to those obtained in the injection study of Experiment 1, with more consistent effects observed in the females. For the males (n = 5 per group), analysis of the brain areas separately with t tests showed that GAL mRNA approached statistical significance in the DMN (P = .08), and GAL-ir showed a similar trend in the PLH (P = .08). Analysis by ANOVA in the females (n = 5 per group) showed significantly elevated GAL mRNA [F(2,29) = 4.00, P < .03] across the four brain areas (Fig. 3). Post hoc analyses showed a significant increase (P < .05) in the anterior region of the PVN in the ethanol-drinking subjects compared with the control rats, with no change in the DMN (Fig. 3) or PLH. There was also a significant difference in GAL-ir between ethanol-drinking and control rats [F(1,29) = 10.2, P < .001], with a reliable effect observed in the DMN (P < .03) and anterior part of the PVN (P < .02), as illustrated in the photomicrographs of Fig. 4. This experiment therefore confirms the basic finding that alcohol enhances hypothalamic GAL in specific hypothalamic nuclei and extends it to include rats that voluntarily drink alcohol.

3.3. Experiment 3: exposure to alcohol on a 12 h intermittent schedule stimulates GAL

Two groups of rats, water control and ethanol (n = 10 per group), were maintained for 20 days with water available ad libitum, and in the latter group, ethanol was available on a 12 h schedule (see Section 2). The ethanol group consumed an average of 3.9 ± 0.7 g/kg/day on 7% ethanol and 5.3 ± 0.6 g/kg/day on 9% ethanol. When sacrificed on the final day after 1 h of ethanol access, the rats had a blood alcohol level of 18 ± 0.3 mg/dl, which was significantly different from that of the control rats [t(18) = 56.18, P < .001]. There was no difference observed in body weight between water-drinking and ethanol-drinking rats.
Analyses of hypothalamic GAL revealed a significant increase in GAL mRNA in the ethanol-drinking rats \((F(1,55)= 14.4, P < .01)\) across the four areas examined. This change in mRNA was observed specifically in the DMN and PVN \((P < .05)\) (Fig. 5). The measures of GAL peptide revealed a similar change \((F(1,53)= 3.97, P < .05)\), with a reliable effect in the DMN \((P < .001)\) but not the PVN (Fig. 5). No change in GAL mRNA or peptide-ir was detected in the PLH or ARC.

3.4. Experiment 4: naloxone reverses alcohol effects on GAL expression

After 20 days on the 12 h schedule of ethanol access, naloxone \((3 \text{ mg/kg sc})\) or saline was injected to assess the effect of opioid withdrawal on hypothalamic GAL. A total of four groups \((n = 4 \text{ per group})\) were tested, with two groups (saline or naloxone) maintained on water and two groups (saline or naloxone) maintained on ethanol. The rats drinking ethanol consumed an average of 4.77 ± 0.8 g/kg/day of 7% ethanol and 5.2 ± 0.7 g/kg/day of 9% ethanol. There were no differences in body weight between any of these groups.

Analyses of the four groups of rats (water-saline vs. water-naloxone and ethanol-saline vs. ethanol-naloxone) revealed significant effects (Fig. 6). In the saline-injected rats, comparisons between water-drinking and ethanol-drinking rats across the four hypothalamic areas revealed a tendency for a stimulatory effect of alcohol on GAL mRNA \((F(1,31)= 3.43, P < .08)\).

In the ethanol-drinking rats, comparisons between saline-injected and naloxone-injected rats showed a clear effect of this opioid antagonist. Opioid withdrawal after naloxone significantly reduced GAL mRNA \((F(1,31)= 14.9, P < .001)\) across the four areas (Fig. 6). A reliable suppression of 15–25% in these ethanol-drinking rats was observed specifically in the DMN \((P < .04)\) and PLH \((P < .05)\), with little effect in the PVN (Fig. 6) and no change in the ARC. This is in contrast to naloxone in water-drinking control rats, which compared with saline had no effect on GAL expression when examined across the four areas \((F(1,31)= 1.14, P > .10)\). When the areas were analyzed individually, a small increase in GAL mRNA after naloxone was detected in the DMN \((P < .05)\) (Fig. 6) but not in the other areas. Analyses of GAL peptide-ir data revealed no significant differences between the various groups.

4. Discussion

4.1. Impact of alcohol on hypothalamic GAL

Cells that express GAL are concentrated in several hypothalamic areas, including the DMN, PVN, PLH and ARC. They send out dense projections throughout the hypothalamus as well as to other parts of the forebrain [39–41]. The results of the different experiments in the present study agree in showing that alcohol increases the expression of the GAL gene. This change in GAL mRNA is accompanied by a significant enhancement in GAL peptide, suggesting that the increase in message produces additional peptide, which is then transported to the hypothalamic release sites for functional purposes.

The results additionally reveal anatomical specificity in this effect of ethanol on GAL. Specifically, they identify two hypothalamic nuclei, the DMN and PVN, as being consistently responsive to the stimulatory effect of ethanol on GAL and the PLH as showing a less consistent response. The increased expression and production of GAL in the DMN and PVN were produced by ethanol administered via
systemic injections, ad libitum voluntary intake or intermittent voluntary intake. This site specificity is underscored by the finding in Experiment 1 that ethanol injection affected GAL in cell bodies in the anterior parvocellular region of the PVN but not in the posterior magnocellular region, less than 0.5 mm away. In the PLH, immediately lateral to the DMN, the stimulatory effect of ethanol on GAL was evident after injection but not in response to ethanol intake, while in the ARC no effect was evident under any condition. This anatomical specificity may explain an earlier report that failed to detect a significant correlation between amount of alcohol consumed and GAL levels in cerebral spinal fluid of alcoholic men [42].

4.2. Mechanisms underlying alcohol-stimulated GAL expression

Measurements in Experiment 3 revealed a significant increase in blood alcohol concentration to 18 mg/dl. This level is in the range reported in a study that used the sucrose-fading technique to induce alcohol intake and that also observed a significant change in behavior [43]. This suggests that it is circulating alcohol, or some parameter related to alcohol and its metabolism, that may be stimulating hypothalamic GAL.

There are other studies indicating that GAL is also increased by dietary fat. In normal-weight rats given a choice of macronutrient diets, GAL levels are positively correlated with the intake of fat but not of carbohydrate or protein [44]. Moreover, when fed a single diet rich in fat, GAL expression is elevated relative to that of rats on a moderate- or low-fat diet [1]. The area identified in this relationship between GAL and fat intake is the PVN, specifically the anterior parvocellular region of this nucleus. This is one of the sites where GAL is affected by alcohol intake (Fig. 2), suggesting that these phenomena may have similar underlying mechanisms. This suggestion is consistent with the finding that GAL neurons in the ARC and the posterior region of the PVN, which are unresponsive to alcohol ingestion, are also unaffected by fat ingestion [1]. This leaves unexplained, however, why the DMN is responsive to alcohol intake but not to fat ingestion [1,44].

GAL in the PVN is also consistently elevated in genetic and dietary obesity, suggesting that it may respond to a rise in body fat [5,28]. In the present experiments involving ethanol, the age-matched control groups in each of the experiments exhibited no difference in body weight. Thus, such factors as weight gain or adiposity are unlikely to be involved in the stimulatory effect of ethanol injection or consumption on GAL gene expression.

4.3. Relationship of GAL to the opioids and DA

Extensive evidence associates GAL with the opioids, which may imply a connection to addictive processes. An opioid antagonist blocks GAL-induced feeding [9,45], and it reduces alcohol intake in various species [46–48]. Ethanol is like the opiates and psychostimulants, in that it has the ability to increase extracellular DA in the NAc [20]. During naloxone-induced withdrawal in ethanol-treated rats, accumbens DA is lowered while acetylcholine (ACh) is released (Rada and Hoebel, unpublished data). This suggests that endogenous opioid peptides control the balance between DA and ACh in rats addicted to alcohol. This DA/ACh reversal has also been seen during withdrawal from morphine and nicotine [49–51]. Thus, naloxone causes neurochemical signs of withdrawal in alcohol-treated animals. A role for the opioids as one of several neurotransmitter systems in alcohol dependency is well known, with levels of endogenous opioids positively related to the ingestion of alcohol and opioid antagonists reducing the reinforcing effects of alcohol [52–54].

The results observed in Experiment 4 provide further evidence linking endogenous GAL to opioids that underlie aspects of alcohol dependency. A functional significance of GAL in alcoholism is implied by the finding that opioid withdrawal, induced by an opiate antagonist, reversed the ethanol effect. Compared with saline injection, naloxone produced a significant decrease in GAL mRNA level in ethanol-drinking rats while having little or no effect in water-drinking rats. Opioid withdrawal caused a significant GAL suppression in the DMN as well as the PLH, with an insignificant trend in the PVN and no change in the ARC. Thus, GAL neurons may have a specific role in controlling alcohol intake that may in part involve opioid connections to forebrain DA.

The injection of GAL in the PVN causes the release of DA in the NAc [22]. Of particular interest is the finding that this effect is seen only in rats that, on separate tests, exhibit a significant GAL-induced increase in feeding behavior. This links GAL-induced overeating to DA release. The same treatment with GAL also decreases extracellular ACh in the NAc, a reciprocal response that is believed to disinhibit eating [15]. This is in contrast to PVN injection of NPY, which has little effect on accumbens DA/ACh balance. This evidence suggests that appetite for food is initiated in part by hypothalamic GAL, which selectively controls the DA/ACh balance in the NAc, a site where instrumental behavior is gated. The published results, demonstrating a stimulatory effect of fat ingestion on endogenous GAL, which in turn stimulates further feeding, suggest the existence of a positive feedback loop that may contribute to overeating until the cycle is interrupted by postingestional satiety signals [28,55]. The discovery in the present study, that alcohol increases the expression of GAL, raises an interesting possibility that GAL mediates alcohol’s stimulatory effect on accumbens DA as well as its inhibitory effect on ACh and that, through this mechanism, GAL may have some impact on the consumption of alcohol.
4.4. Role of NPY in alcohol intake

Another feeding-stimulatory peptide, NPY, provides a valuable control in showing the specificity of the stimulatory effect of ethanol on GAL. As demonstrated in Experiment 1, this peptide, expressed in neurons of the ARC, is actually decreased by prolonged ethanol treatment. Further, this effect occurs in the same rats that show a stimulation of GAL, underscoring the difference between these two peptides. This finding is consistent with published studies showing that NPY levels in the ARC are reduced by alcohol intake and inversely related to alcohol ingestion in transgenic mice, and that alcohol consumption is increased in mutant mice lacking the NPY Y1 receptor gene [29,56–58].

It is notable that NPY differs from GAL in several respects, one being its association with dietary carbohydrate rather than fat [59]. For example, the consumption of a high-carbohydrate diet stimulates the expression of NPY while fat intake reduces it [59]. Also, the injection of NPY produces a stronger stimulatory effect on feeding in the presence of carbohydrate [60] in contrast to GAL [61]. The possibility that NPY has a role in controlling ethanol consumption needs to be further defined in light of evidence that NPY injection can suppress or increase alcohol ingestion depending in part upon site of injection [30–33].

5. Conclusions

There is prior evidence that GAL in the PVN and possibly nearby sites is involved in a positive feedback process that contributes to the overconsumption of food. This peptide also releases DA and lowers ACh in the NAc motivational system, and it is closely related to opioid systems. The finding that ethanol increases GAL expression in specific hypothalamic sites, and opioid withdrawal decreases it, raises new and interesting questions concerning the role of hypothalamic GAL as well as the opioids in the consumption of alcohol.

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References


Kanter SN, Sławekci CJ, Ehlers CL. Neuropeptide Y administration into the third ventricle does not increase sucrose or ethanol self-administration but does affect the cortical EEG and increases food intake. Psychopharmacology (Berl) 2002;160:146–54.

Badia-Elder NE, Stewart RB, Powrozek TA, Roy KF, Murphy JM, Li TK. Effect of neuropeptide Y (NPY) on oral ethanol intake in Wistar, alcohol-prefering (P), and -nonpreferring (NP) rats. Alcohol Clin Exp Res 2001;25:386–90.


