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Glycyl-Glutamine Reduces Ethanol Intake at Three Reward Sites in P Rats

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Abstract

β -endorphin, implicated in modulation of ethyl alcohol reward, has neuron terminals in several reward sites. Alcohol consumption was reduced after ventricular or site specific injections into the nucleus accumbens of an opioid-derived dipeptide, glycyl-glutamine. The current study examined the effects of this dipeptide after site specific injections into additional reward sites. Alcohol preferring (P) rats, stereotactically implanted with bilateral guide cannulae into the nucleus accumbens, ventral tegmental area, and the central nucleus of the amygdala were given 30% alcohol and water in a 24 h voluntary 2-bottle choice paradigm. Upon achieving stable baseline intakes, glycyl-glutamine (GQ) doses were injected bilaterally, and the alcohol and water intakes and body weight recorded for the response and recovery. The data show reduced alcohol intake by 32 to 49.5% after 100 pmol glycyl-glutamine into reward sites (nucleus accumbens, ventral tegmental area, and central nucleus of the amygdala), but not after injections into control sites dorsal to reward sites. The order of sensitivity to the 1 fmol dose was amygdala $\geq$ ventral tegmental area $>$ accumbens. GQ was effective in reducing ethanol intake at reported β -endorphin terminal regions in each of the three reward sites tested. The effective doses were similar to reported endogenous GQ levels, consistent with the notion that it may function as part of an endogenous counter regulatory mechanism and represent a “stop drinking” signal in the high drinking, alcohol preferring P rats at these three reward sites.

Keywords

alcohol consumption; glycyl-glutamine; β -endorphin; P rats; brain reward sites

Introduction

Reward behaviors associated with alcohol (ethanol) drinking are correlated with discrete brain locations (Koob, 1999). Alcohol-stimulated reward behavior is partially defined by the drinking response to intracranial site specific application of pharmacological agonists and antagonists (Ikemoto and Wise, 2004; Koob 1992; Wise and Bozarth 1981). Such pharmacological experiments implicate several neuronal systems that operate in these reward sites to potentiate or inhibit the effects of alcohol. Two of these are the mesolimbic dopamine system and the opioid system. Dopaminergic neurons are thought to mediate reinforcement of ethanol drinking and opioid neurons to modulate that response (Herz, 1998; McBride, 2002;

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Spanagel et al., 1991; Spanagel et al., 1992). Several studies demonstrate that extracellular dopamine levels increase significantly in the nucleus accumbens (NAC), when animals are injected with ethanol (Marinelli et al., 2003), allowed to drink freely (Imperato and Di Chiara, 1985) or ingest ethanol in an operant paradigm (Weiss et al., 1993). Specific dopamine receptor antagonists demonstrate an opposite effect, reducing ethanol drinking (Samson and Hodge, 1993) when injected at the same brain sites. Ethanol not only increases dialysate dopamine, but also β -endorphin (deWaele et al., 1992; Gianoulakis, 1990; Marinelli et al., 2003; Olive et al., 2001). Opioid antagonists reduce, but do not abolish, the alcohol induced rise in dopamine, consistent with a modulatory function (Gonzales and Weiss, 1998). The pharmacology suggest ethanol increases dopamine release, that opioids further enhance its release, and that endogenous opioid-derived opioid antagonist may attenuate that release (Spanagel et al., 1991).

In addition to pharmacologic evidence, neuroanatomy supports a role for opioid modulation of ethanol consumption. A population of opioid neurons are the proopiomelanocortin (POMC)/ β -endorphin cells of the arcuate nucleus that project axons into several of the reward system nuclei, for example in the NAC, where they are co-localized with mesolimbic dopamine projection fields (Bloom et al., 1978; Khachaturian et al., 1985; Koob, 1999). Selection of the sites tested in the present study is based in part on reports of immuno-stained β -endorphin fibers and terminals in the NAC, the ventral tegmental area (VTA), the amygdala (CeM) and the arcuate nucleus (Arc N), which is complemented by opiate receptor localization and lesioning data (Akil et al., 1984; Barna et al., 1997; Finley et al., 1981; Gray et al., 1984; Khachaturian et al., 1984; Mansour et al., 1988; Mezey et al., 1985; Stengaard-Pedersen and Larsson, 1981; Watson et al., 1989). These β -endorphin projection fields should identify locations where β -endorphin and its derivatives are released and could act to modulate dopamine and subsequently alcohol drinking behavior.

Several opioid derivatives (Loh, 1992) implicated in modulation of ethanol drinking are cleavage products of β -endorphin-(1-31). β -endorphin undergoes processing in neurons to several shortened non-acetylated and acetylated forms, among which is β -endorphin-(1-27); and whenever a cleavage produces a shortened form, the dipeptide, glycyl-glutamine (β -endorphin-(30-31) (GQ)) is also formed (Smyth et al., 1983). The sum of the several shortened forms and GQ are produced in approximately equimolar amounts. Thus, even though GQ may not be as easily measured at concentrations in physiological samples, collectively the shortened β -endorphin peptides may serve as an index of the amounts of GQ formed. The importance of this phenomenon is in the opioid-derived peptide ratios which have been demonstrated to differ in high vs. low drinking strains of rats. Specifically, in vitro hypothalamic slice studies report that high drinking Alco alcoholic (AA) rats show reduced release of β -endorphin-(1-27) and proportionally higher β -endorphin into the tissue bath compared to low drinking Alco non-alcoholic (ANA) rats (de Waele et al. 1994). Deficits in hypothalamic β -endorphin soma would be reflected in the terminals of those neuron projections into reward sites. The de Waele report reasonably suggested an endogenous inhibitory role for β -endorphin-(1-27). That suggestion is supported by reduced alcohol intake following injection of β -endorphin-(1-27) into the NAC of P rats (Resch et al., 2005). Thus there are two shortened forms that reduce alcohol intake, the 27 amino acid peptide and GQ (Resch et al., 2005). Further, if GQ is deficient in reward sites then replacement would be expected to reduce alcohol intake, which it does in one reward site, NAC. The present report tests the hypothesis for two additional reward sites. GQ test site selection was thus based on the occurrence of β -endorphin projection fields in reward sites.

Additional studies indicate there is an important difference between β -endorphin-(1-27) and GQ. β -endorphin-(1-27), both reduces ethanol intake (Resch et al., 2005) and acts as a partial agonist reducing the β -endorphin-(1-31) induced release of dopamine in NAC (Spanagel et al., 1991). However, the co-produced dipeptide GQ, which also antagonizes ethanol intake

(Simpson et al., 1998) appears to utilize different mechanisms to exert its effects in brain. From a NOVA screen (NIMH/NovaScreen Drug Discovery and Development Program, unpublished data) it is clear that GQ does not significantly bind to opioid receptors or to other known neurotransmitter receptors or transporters. Supporting this finding, Unal et. al. (1994) has also shown that GQ will not displace tritiated naloxone from opiate receptors. However, GQ may operate by a membrane mechanism as indicated by its hyperpolarization of neurons in the brain stem following iontophoretic application (Parish et al., 1983). Thus the 27 residue peptide and classical opioid antagonists block opioid mu and delta receptors, but the co-produced GQ acts through a unique, and as yet undefined, mechanism.

Despite their differences in mechanism of action, both of these shortened forms of β -endorphin are implicated in the control of ethanol intake. However, the distribution of the shortened forms of β -endorphin is not uniform (Berglund et al., 1989; Dennis et al., 1983). Also, much of the β -endorphin-(1-27) in the ventral striatum, including the NAC, is in the form of N-acetyl- β -endorphin-(1-27) (Dennis et al., 1983), which is inactive in reducing alcohol drinking (Resch, 2005). Thus the dipeptide fragment, GQ, at some sites could be a principal endogenous peptide that reduces ethanol consumption. GQ action, which has not been tested in VTA and CeM, are both innervated by β -endorphin similar to NAC. Thus in this series of experiments we extend previous findings in P rats to investigate the hypothesis that GQ reduces ethanol consumption when injected into three reward sites, i.e. NAC, VTA, and CeM.

Materials And Methods

Animals

For these experiments male alcohol preferring (P) rats (Center for Alcohol Studies, Indiana University Medical Center, Indianapolis, IN), weighing 300-350 g, were maintained on a 12:12 photoperiod (lights on: 0700 h), with food and water *ad libitum*. Each rat was handled and weighed daily and maintained in individual cages in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (publication No. 80-23, revised 1996), with all experimental protocols approved by the UMKC Animal Care and Use Committee.

Surgery

Rats were anesthetized with isoflurane (2%) for stereotaxic surgery (Kopf, Tujunga, CA). Stainless steel guide tubes (Plastics One, Roanoke, VA) were stereotaxically implanted 1 mm above the intended injection site at the following coordinates: NAC: AP = 1.7 mm from bregma, L = ± 1.0 mm, H₀ = -5.5 mm (below) dura; VTA: AP = -4.8 mm from bregma, L = ± 1.0 mm, H₀ = -6 mm from dura; and CeM: AP = -2.2 mm from bregma, L = ± 4.0 mm, H₀ = -7.0 mm from dura (Paxinos and Watson, 1998). Guide tubes were secured to the skull with anchor screws and acrylic dental cement. Incisions were closed and animals allowed to recover from surgery for five days before re-establishing alcohol intake baseline values and starting experiments.

Ethanol Preference Determination

Ethanol was introduced to P rats in their home cages using a series of increasing concentrations from 3% to 30% as previously reported (Myers, 1971; Resch et al., 2005; Simpson et al., 1998). Briefly, rats were provided continuous access (24 h/day) free-choice of 2 bottles, one containing water and the other the graded sequence of ethanol concentrations. In this group of rats, the preferred concentration was 30%, and therefore the concentration used in experiments. The volume of ethanol intake was measured and calculated as grams of ethanol/kg of body weight/day (g/kg/day). Only rats drinking 30% ethanol with intakes > 4 g/kg/day were used in this study. In the present study, all of the rats met the foregoing criteria. Water and ethanol

intakes were measured daily using graduated drinking tubes (Dyets, New Brunswick, NJ). The position of drinking bottles were switched randomly to avoid position habits by the rats. Body weight was also measured daily. Experiments proceeded after approximately two weeks when animals' intakes demonstrated stable daily values ($\pm 10\text{-}15\%$).

Injections

Injections were delivered to each of the three brain sites in separate experiments using 28 ga. stainless steel injectors passed through 22 ga. bilateral guide cannulae (Plastics One, Roanoke VA). GQ (100 pmol in 1 μl each side) or saline (vehicle) treatments were pressure injected bilaterally over a period of 30 seconds using a Hamilton microliter syringe. Injectors remained in the injection site for an additional 15 seconds and were then removed and replaced by styletes. Rats were then returned to their home cages for the remainder of the experiment.

Experimental Procedure

Experiments were done as previously reported using unrestrained P rats adapted to the 2-bottle free-choice, 24 h continuous access paradigm (Resch et al., 2005). Daily body weights and intakes of water and ethanol were recorded for 3 days before, to assure a stable baseline, and 3 days after central bilateral injections of GQ (100 pmol/ μl /side). A one time injection was done on Day 3 directly after the morning weighing and recording the measurements of ethanol and water. Measurements were repeated at subsequent 24 h intervals through Day 6. In a few animals, injections of biotinylated dextran amine (BDA) were done at the GQ application site two days prior to sacrifice. At sacrifice, brains were collected, blocked and sectioned. All cannula placement and injection sites were verified by inspection and in a few animals by histologic staining. Coronal sections through the injection sites for NAC and CeM were counterstained with cresyl violet and for VTA immunostained for tyrosine hydroxylase (TH). Immunostaining employed a primary mouse anti-tyrosine hydroxylase antibody (Chemicon) and a secondary goat anti-mouse antibody tagged with Alexa Fluor 488 (Molecular Probes) in floating sections and visualized with a Olympus FlowView 300, laser scanning confocal microscope. The TH staining for dopamine soma in the A10 region (German and Manaye, 1993) was used to add an additional confirming parameter to the landmarks identifying the injection site in the anterior VTA.

Statistical Evaluation of Data

Data are reported as the mean percent difference $\pm$ S.E.M. of ethanol consumption from a saline vehicle control group vs. a drug treatment group. Unless specified, statistical testing evaluated data with an ANOVA, followed by a post hoc Bonferroni test where appropriate. Where appropriate, comparison of means were evaluated with one-tail independent or dependent t-tests (Minitab or Instat). Statistical significance was accepted at P values < 0.05 .

Results

The initial experiments measured ethanol intake before and after 100 pmol GQ injections into three brain sites of P rats. The data (Fig. 1) show mean percent difference $\pm$ SEM for ethanol intake following GQ and saline vehicle treatments in different groups of rats.

Comparisons used a 3 site by 2 drug ANOVA. Main effect for sites had F value = 0.37 (df 2,50) $P < 0.694$. The drug difference (GQ and saline) had an F value = 27.48 (df 1,50) $P < 0.001$. The Bonferroni for saline vs. GQ is $P < 0.0001$, in NAC, CeM, and VTA. The data also show the mean % change in drinking after saline vehicle control injections were not significant, and in this respect, were similar to aCSF vehicle controls (Simpson et al., 1998) and saline vehicle controls (Resch et al., 2005) in previous work.

The next set of experiments measured ethanol intake responses following GQ injections at target sites and a second injection dorsal to the intended target sites as shown in the schematics. Fig 2A-C shows both histologic evidence and schematic representations of injection sites. Histologic evidence for the location of injections into effective target sites as identified by stereotaxic coordinates and tissue landmarks. Figure 2A shows a coronal section with an injector damage tract ending in NAC lateral to the midline and medial to the anterior commissure (aca). Further lateral is the caudate putamen. Figure 2B shows the injector tract and injection site in the central nucleus of the amygdala. The site lies lateral to the third ventricle between the lateral hypothalamic area and dorsal to the piriform cortex. Figure 2C shows the injector tract and injection site in the anterior VTA lateral to the midline, dorso-lateral to a portion of the supramammillary nucleus and medial to the substantia nigra pars reticular.

Figure 2A-C also shows schematic diagrams for effective (target) and ineffective (control) injection sites in three separate experiments, one for each of the reward sites. The diagrams illustrate the AP stereotaxic coordinates from bregma for each of the injection sites: Two injections were done in each animal: one in the dorsal control site and the other in the target site. Table 1 shows the mean 24 hr alcohol intakes in g/kg/day and in mean percent change. The table also shows the comparison using a dependent t-test of intakes the day before versus the day after GQ injection (100 pmol). It is clear from the data that at dorsal control sites there were no differences in alcohol intake following injection of GQ. Conversely at the targeted reward sites, in NAC, CeM and VTA, injection of GQ resulted in significantly reduced 24 hr ethanol intake ($P < 0.001$). The effectiveness of GQ is limited to the targeted reward sites.

Figure 3 shows the results of three doses of GQ and vehicle controls at each of the three effective target sites. Using an ANOVA based on the General Linear Model (MINITAB), the overall F test for NAC shows $P < 0.006$ and a post hoc Bonferonni of $P < 0.012$ for 100 pmol GQ vs. saline. The 1 pmol and 1 fmol doses were not significant. For CeM the overall F test shows $P < 0.003$ and the post hoc Bonferonni of $P < 0.02$ for saline vs 100 pmol GQ; $P < 0.003$ for saline vs. 1 pmol GQ; and $P < 0.014$ for saline vs. 1 fmol GQ. For VTA the overall F test shows $P < 0.001$ and the post hoc Bonferonni of $P < 0.001$ for saline vs. 100 pmol GQ; $P < 0.007$ for saline vs. 1 pmol GQ; and $P < 0.004$ for saline vs. 1 fmol GQ.

A threshold dose was established for NAC at 1 pmol. A 1 pmol injection of GQ was marginal but not significant. A 1 fmol dose, which was not significant in NAC, was significantly different from saline in the CeM and VTA and failed to identify a threshold in the later two sites.

Discussion

The principal findings in this report are that 24 h voluntary ethanol intake was significantly reduced after injection of GQ into each of three brain sites recognized as important for ethanol reward. The data also show that the required dose is significantly lower than the 100 nmol dose used in our previous reports (Simpson et al., 1998; Resch et al., 2005). Further, the data showed that GQ elicited no response from dorsal control sites. Finally, comparisons of site sensitivity data showed that the CeM and the VTA are a 1000 times more sensitive to GQ than is NAC.

Brain Site Selection

Injection sites used in the present report (Fig. 2A-C) were selected in part from those reported by others to influence ethanol drinking behaviors (Koob, G.F., 1992). Additionally, we used the criteria of sites that have a representation of β -endorphin fibers. Similar to what other investigators have found in these same sites, we demonstrated ineffective injection sites in the area dorsal to the effective target sites. Comparison of histologic landmarks of injection sites and stereotaxic coordinates from those reports and used in the present report indicate comparable injection sites were used for GQ administration.

Specifically, a few reports show results of site-specific injections of opioid antagonist into the NAC, VTA, or CeM (Bechtholt and Cunningham, 2005; Devine and Wise, 1994; Heyser et al., 1999). Bechtholt and Cunningham injected methylnaloxonium, an opioid receptor antagonist, into VTA and NAC. Ethanol conditioned place preference was blocked by the antagonist in VTA, but not NAC. Devine and Wise (1994) administered opioid agonists into VTA showing mu- and delta-opioid reinforcement of opiate self-administration. Selection of the VTA injection sites used in this study was based on the structural evidence of dopaminergic soma in the injection site regions as provided by immunostaining (German and Manaye, 1993; Swanson LW, 1982; Finley et al., 1981) and the functional evidence of opiate responsiveness as reported by Bozarth (1987). The injection site selection used in the present report took into account the functional segregation of anterior and posterior VTA (Bozarth, 1987; Zangen A et al., 2002; Olsen et al., 2005). Specifically, Olson defines anterior VTA from -4.9, to -5.4mm from bregma and the posterior VTA from -5.5 to -6.2mm. Based in part on the brain landmarks illustrated in their histology, Rodd (Rodd et al., 2004, 2005a, 2005b) and Bozarth appear to concur. It will be interesting in future experiments to investigate sites in the posterior VTA to see if similar reductions in ethanol intake will occur following GQ injections.

Clearly, the data (Fig. 1) show that injection of GQ into VTA, NAC and CeM, reduces ethanol intake, superficially similar to opioid antagonism of drinking. However, even though the effects are similar there is one essential difference. GQ, an opioid-derived peptide, does not bind to opiate receptors, as shown by a NOVA screen. Further, displacement data shows GQ did not displace ^3H -naloxone from its receptors (Unal et al., 1994). Thus, GQ suppresses alcohol intake by a different mechanism than opiate receptor antagonists.

Characteristics of GQ Action

There are two known shortened forms of β -endorphin that reduce voluntary alcohol intake, opening the question of their relative importance. One might expect β -endorphin-(1-27) to function in this role. However, the mere occurrence of opiate receptors in a reward site may not by itself be a sufficient indicator of an endogenous opiate receptor antagonist role limiting drinking. Even though it is clear that β -endorphin-(1-27) (partial agonist) reduces voluntary alcohol drinking following injections into NAC (Resch et al., 2005), the situation may be more complicated. β -endorphin-(1-27) also appears as N-acetyl- β -endorphin-(1-27), which does not reduce drinking (Resch, 2005). More importantly, the inactive N-acetylated form appears to predominate in the NAC (Dennis et al., 1983). That seems to remove one candidate counter-regulatory peptide, leaving GQ, produced and co-released with other shortened forms of β -endorphin (Smyth et al., 1983), with a possible role as an effective drinking antagonist in NAC.

Much of the evidence supporting an endogenous role for GQ as an inhibitor of alcohol intake has been summarized in the introduction. For GQ to function in a regulatory role it should fulfill several criteria, including but not limited to the following. First, it should be produced by neurons that innervate the reward sites. Second, exogenous GQ should reduce drinking, implying endogenous GQ would do the same. And third, endogenous GQ should be released in response to alcohol administration. The first criterion is met in the literature summarized in the introduction; the second by the present report; and the third is the subject of a future experiment. However, preliminary indicators suggest the third criterion may also be satisfied indirectly by dialysis experiments showing the release of β -endorphin from NAC, VTA and CeM following an ethanol stimulus (Jarjour and Gianoulakis, Abstr. 2006; Lam et al., Abstr. 2006; Marinelli et al., 2003). β -endorphin release strengthens the hypothesis that GQ could normally be concurrently released in these reward sites; however, GQ has not yet been measured in similar experiments.

In addition to the foregoing three criteria, several additional characteristics of GQ are of interest for its therapeutic potential. The P rat has been suggested as an animal model of human

alcoholism (Lankford et al., 1991), in which the opiate systems involvement in alcoholism is well demonstrated. GQ, as a post-translational product from an opiate, appears to have possibilities as an extension of that work. Following are measures of some characteristics of GQ action in the P rat which may be important for its therapeutic potential.

One characteristic of GQ is its actions in related reward behaviors. The data show GQ specifically reduces alcohol drinking but does not generalize to related reward behaviors, having no effect on body weight or water intake (Resch et al., 2005; Simpson et al., 1998). Additionally, GQ inhibited nicotine (Goktalay et al., 2006) and morphine (Cavun et al., 2005) reward in a place preference test, and similarly had no effect on food intake. Also, in addition to selective behavioral effects, anatomically, GQ shows reduced alcohol drinking following injections into the reward sites tested in the present report but not when injected into dorsal control sites (Fig. 2A-C; Table I). Thus GQ appears to be selective for some reward behaviors and not others, and in specific anatomic sites.

Another characteristic of GQ action relates to the effectiveness of different doses at different reward sites. Previous reports indicate reward sites show differential sensitivity to opioid antagonists (Heyser et al., 1999) and data in the present report show a similar pattern for GQ. Our site sensitivity comparisons using our lowest dose (1 fmol) showed a clear difference between CeM (39%) and VTA (32%) vs. NAC (16%) or saline (12%). NAC showing responses in the pmol range (Fig. 3) was at least 1000 times less sensitive than either the CeM or VTA at 1 fmol. The patterns of site sensitivity between NAC and CeM are similar to those reported for methylnaloxonium (Heyser et al., 1999). These findings correlate with similar sensitivity differences reported for a different neurotransmitter receptor system in the central amygdala, i.e. that of the GABA_A receptor antagonist SR95531. This antagonist in the amygdala reduced ethanol consumption 8 times more than it did in NAC (Hyytia and Koob, 1995). As with other antagonists, the salient finding of the GQ comparison is that the amygdala and VTA appear to be significantly more sensitive than NAC.

The dose story, however, is incomplete. The data indicate that the 1 fmol dose is not the bottom of a dose-effect relationship in two of the sites. Additional studies will be required to determine the lowest effective doses. Further, the 1 fmol to 1 pmol doses imply that responsiveness to GQ may be in a physiological range, and is consistent with levels from tissue extracts which contained 2.23 pmol GQ per gram of tissue (Parish et al., 1983; Smyth et al., 1983). The sensitivity of the CeM and VTA suggest the possibility that these sites may be a normal endogenous target for a GQ modulator attenuating drinking.

A final characteristic of GQ action is the interval during which alcohol drinking is suppressed. Three sets of data support the observed duration of GQ effect. First, H³-GQ injected icv shows no evidence of breakdown in a sample taken 60 min later and analyzed in an HPLC (pers. Communication, WR Millington). Second, this finding supports our initial observation that GQ reduced drinking over 48 h following an injection protocol of GQ twice-a-day for 3 days icv (Simpson, et al., 1998). Finally, third, the data (Fig. 3) show that only fmol amounts of GQ are required to elicit the reduction of drinking, indicating that very little of an injected dose would have to remain in tissue to be effective. Thus a 24 h GQ effect to reduce drinking in this report is consistent with previous data.

From the foregoing observations we propose the following hypothesis: The genetic high drinking strains may be deficient in inhibitory peptides. GQ is not the first small endogenous peptide reported to be involved with reducing drinking. Pasard (2001) using alcohol naïve mice from a genetic high drinking line (C57) shows they have lower cyclo-(His-Pro) levels than controls and also that exogenous cyclo-(His-Pro) reduces drinking. This finding is similar to reports showing that the high drinking AA rat has low shortened forms of β -endorphin vs.

higher levels in low drinking ANA rats (deWeale et al., 1994). These shortened forms increased with alcohol treatment but much less so in AA compared with ANA rats. Both of these models show lower levels of peptides in high drinking strains. The data in the present report support the possibility that GQ may well fit a pattern in the P rat similar to the one shown in the C57 mice and in the AA/ANA rats. If so, injection of GQ into reward sites represents replacement of the dipeptide that may be genetically low in the alcohol preferring P rat.

Conclusions

In summary, the data demonstrate that GQ is effective in reducing alcohol intake at reported β -endorphin terminal regions in each of the three reward sites tested, i.e. CeM, VTA and NAC. Findings in this report extend our previous results, which showed GQ reduced alcohol intake after icv injection and again after site-specific injection into NAC. The effective doses of GQ in the present report are in a physiological range and similar to reported endogenous GQ levels. Pharmacologically, CeM and VTA respond to lower doses of GQ than NAC to inhibit alcohol intake in high drinking P rats. These data are consistent with the notion that GQ may occur as part of an endogenous counter regulatory mechanism in normal rats that is reduced or missing in the high drinking, alcohol preferring P rats at these three reward sites.

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Figure 1.

Ethanol Intake after Injection of GQ into 3 Brain Sites of P rats. The data show the mean percent difference $\pm$ SEM following injection of GQ (100 pmol) or saline in different groups of rats. The ANOVA used is from MINITAB and is based on the General Linear Model. It shows a significant drug effect and no significant difference between sites. The post hoc Bonferonni for saline vs. GQ is ** = $P < 0.0001$, in NAC, CeM, and VTA.

Figure 2A

Figure 2B

Figure 2C **Figure 2A-C.**

Histology and Schematics of Injection Sites. The figure shows histology of the injector damage tract and schematics for target site (filled circles) and control injection site (filled triangles) at each of the three brain sites: Ethanol intake data are shown in Table 1. Stains: 2A shows a cresyl violet stained coronal section through NAC; 2B shows a cresyl violet stained coronal section through CeM; 2C shows a fluorescent stain for TH (Alexa 488, (Molecular Probes)) in a coronal section through VTA. Magnification: Histological sections were photographed through a 2X lens for all but VTA, which used a 4X lens. Abbreviations: (Fig 2A) anterior commissure (aca), caudate putamen (CPu), nucleus accumbens shell (NAC), (Fig 2B) central nucleus of the amygdala (CeM), lateral hypothalamus (LH), ventromedial hypothalamus (VMH), third ventricle (3V), piriform cortex (Pir); (Fig 2C) rostral ventral tegmental area (VTA), supra mammillary (SuM), substantia nigra reticular (SNR). Coordinates at the bottom of each figure = mm from bregma (Paxinos and Watson, 1998).

Figure 3.

Inhibition of Ethanol Intake Following Three Doses of GQ in Three Reward Sites. The data are expressed as the mean % decrease $\pm$ SEM for ethanol intake after 3 doses of GQ (1 fmol, 1 pmole, and 100 pmole) or saline, were injected into the NAC, CeM, or VTA. Using an ANOVA based on the General Linear Model (MINITAB), the overall F tests for all three sites was significant. The significant post hoc Bonferonni comparisons for saline vs. GQ doses were as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table I

Effective Target & Ineffective Dorsal Control Injection Sites

Brain Region/ Coordinates:	Ethanol Consumption (g ethanol/kg body wt./24 h)		
	Before GQ	After 100 pmol GQ	% Change
NAC			
Ineffective dorsal (-5.5 mm) N = 10/group	10.4 ± 0.7	10.2 ± 0.94	-2.5 ± 0.83
Effective target (-6.5 mm) N = 10/group	11.3 ± 1.04	5.8 ± 0.87 **	-49.5 ± 6.69
CeM			
Ineffective dorsal (-7.0 mm) N = 6	8.15 ± 0.65	8.76 ± 0.86	7.3 ± 0.5
Effective target (-8.0 mm) N = 6	9.95 ± 0.66	6.59 ± 0.75 **	-34.3 ± 4.8
VTA			
Ineffective dorsal (-6.0 mm) N = 11	9.14 ± 0.99	8.86 ± 1.10	-3.06 ± 0.39
Effective target (-7.0 mm) N = 12	9.26 ± 0.57	5.23 ± 0.57 **	-44.2 ± 3.38

Data are the mean ± SEM. P values represent comparison between ethanol consumption “before” vs. “after” GQ injections in a 1-tail dependent t test.

** = P < 0.001; values for all ineffective (control) injection sites = ns.