

# Administration of the galanin antagonist M40 into lateral septum attenuates shock probe defensive burying behavior in rats

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## Abstract

Galanin (GAL) has been implicated in modulating anxiety, although a precise role remains unclear. Previous studies revealed anxiolytic effects, anxiogenic effects, or no effect, depending on the test, brain region, route of drug administration and context. We have shown previously that microinjection of the GAL antagonist M40 into central amygdala blocked an anxiolytic response to acute stress on the elevated plus maze when rats were pretreated with yohimbine, suggesting an anxiolytic effect of GAL. By contrast, we also showed that microinjection of M40 into the lateral bed nucleus of the stria terminalis attenuated anxiety-like behavioral responses to stress on the plus maze and social interaction tests, implying an anxiogenic effect for GAL. The behavioral response to stress on both these tests is a reduction of an ongoing behavior (open-arm exploration or social interaction, respectively). To better understand the anxiety-modulating role of GAL, it is also important to ascertain its effect on a response that represents an activation rather than suppression of behavior. Thus, in this study, we investigated an active behavioral response to acute stress in rats, the shock-probe defensive burying response. Bilateral microinjections of M40 into lateral septum (LS), a region important to this response and innervated by GAL, dose-dependently decreased burying without affecting immobility. No change was seen in hindpaw withdrawal latency on a thermosensitivity assay, suggesting that the reduction in burying behavior was not attributable to changes in cutaneous pain sensitivity. These results indicate that in LS, GAL facilitates the active anxiety-like behavioral response on the defensive burying test, similar to its facilitatory effect on anxiety-like stress-induced suppression of behavior in the lateral bed nucleus. These results highlight the fact that, rather than a unified system-like role in modulating anxiety, the effects of GAL can be either facilitating or attenuating, and are region-specific, context-specific and response-specific.

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## 1. Introduction

The brain neuropeptide galanin (GAL) has been implicated in the modulation of a variety of behavioral and cognitive processes, including somatic sensation, feeding, sexual behavior, learning and memory in rats (Crawley, 1999; Millan, 1999; Park and Baum, 1999). In particular, GAL has also been shown to be involved

in anxiety-like behavioral responses to acute stress exposure, although a precise role for GAL in anxiety remains unclear (Wrenn and Crawley, 2001; Holmes et al., 2003; Morilak et al., 2003). Previous studies have shown anxiolytic effects, anxiogenic effects, or no effect, depending on the test, brain region, route of administration (i.e., local or intraventricular), and the context (i.e., effects on basal behavior or on stress-induced behavioral responses, see Khoshbouei et al., 2002a,b). Previously, we have shown that bilateral microinjection of the GAL antagonist M40 into the central amygdala blocked the paradoxical

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anxiolytic response to stress seen when rats were pretreated with yohimbine, suggesting an anxiolytic effect of endogenous GAL release in this brain region (Khoshbouei et al., 2002a). By contrast, we have also shown that bilateral microinjection of M40 into the lateral bed nucleus of the stria terminalis attenuated anxiety-like behavioral responses to acute immobilization stress on the elevated plus maze and social interaction tests, implying an anxiogenic effect for GAL in this region (Khoshbouei et al., 2002b). Some of the ambiguity regarding GALs influence may derive from the fact that many of its effects in the central nervous system result from modulation of the actions of other neurotransmitters (Bartfai et al., 1993a,b; Bartfai, 1995). However, the direction of the modulatory effect (facilitatory or inhibitory) that GAL exerts on behavior may also depend in part on the nature of the response being measured.

For instance, the behavioral response to stress on both of the tests used in the above-mentioned studies (elevated plus-maze and social interaction test) involved the reduction of a specific ongoing behavior (open-arm exploration or social behavior, respectively). To better understand the nature of the modulatory role of GAL in the behavioral response to acute stress, then, it is important also to ascertain its effect on a response that represents an activation of behavior. The shock-probe defensive burying test (SPDB) evokes such a response (Pinel and Treit, 1978; Treit et al., 1981). After receiving a shock from a stationary electrified probe, rats typically exhibit a varying period of inactivity and immobility (a passive response), followed by bouts of burying behavior (an active response). This behavior consists of vigorous burrowing, shoveling and flicking movements with the forepaws and head to displace the cage bedding material toward the probe. This defensive burying behavior is never observed in the absence of shock, and has been validated as an ethologically relevant, active response elicited by and directed specifically at the probe (Pinel and Treit, 1978; Treit et al., 1981).

The lateral septum (LS) is important in the behavioral expression of anxiety (Thomas, 1988; Menard and Treit, 1996; Sheehan et al., 2004), and has been shown to modulate performance on several behavioral tasks designed to measure anxiety-like responding, including the social interaction test, elevated plus-maze, and SPDB test (Cheta et al., 2000; De Boer and Koolhaas, 2003; Sheehan et al., 2004). Electrolytic lesions to the LS completely abolished burying behavior on the SPDB task (Menard and Treit, 1996). Moreover, the septal area is richly innervated by galanin-containing fibers (Melander et al., 1986a,b; Jacobowitz et al., 2004). Some of these represent co-localization of GAL with norepinephrine in fibers originating from stress-reactive noradrenergic neurons in the locus coeruleus and caudal brain stem (Moore, 1978). Thus, for these reasons we investigated the effects of microinjecting the galanin antagonist M40

bilaterally into the lateral septum on shock-probe defensive burying behavior. Portions of this work have been presented in abstract form (Echevarria et al., 2004).

## 2. Methods

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio, and were consistent with NIH guidelines for the care and use of laboratory animals. All efforts were made to minimize animal pain, discomfort and suffering, and to minimize the number of rats used.

### 2.1. *Animals and stereotaxic surgery*

A total of 75 adult male Sprague–Dawley rats (Harlan, Indianapolis, IN), 250–275 g, were used. Rats were housed in groups of 3 upon arrival in the animal facility and maintained on a 12-h light cycle (lights on at 07:00 h). After at least 1-week acclimatization, rats were anesthetized with a cocktail of ketamine 43 mg/ml, acepromazine 1.4 mg/ml, xylazine 8.6 mg/ml, 1.0 ml/kg, i.m., with a 25% supplement administered as needed. They were placed in a stereotaxic frame and implanted bilaterally with 22 ga stainless steel guide cannulae aimed at the LS with a 22° lateral approach (coordinates from bregma, with the angle: AP +0.7 mm, ML  $\pm$ 2.4 mm, DV –4.0 mm), placing the tips 1 mm above the target sites, corresponding to plate 16 in the atlas of Paxinos and Watson (1998). Obdurators were inserted into the guide cannulae to maintain patency. After surgery, rats were treated prophylactically with antibiotic (penicillin G, 300,000 IU/ml, 1.0 ml/kg, s.c.), and allowed at least 1 week of recovery before behavioral testing, during which they were housed singly, with food and water available ad libitum.

### 2.2. *Local drug microinjections in LS*

The GAL antagonist M40 (American Peptide, Sunnyvale, CA) was dissolved in saline vehicle at a concentration of 9.9 mg/ml and GAL (American Peptide, Sunnyvale, CA) was dissolved in saline at 7.9 mg/ml. Aliquots of each were stored at –80 °C until use. An aliquot was used only once after thawing and any unused portion was discarded.

On the testing day, rats were transported in their home cage to the behavioral testing room. The obdurators were replaced with 30 ga stainless steel microinjection cannulae, which extended 1 mm beyond the tips of the guides, placing them in the LS. The microinjection cannulae were connected by PE-10 tubing to a Hamilton syringe mounted on a syringe pump (Instech, Plymouth Meeting, PA). After inserting the injectors, rats were al-

lowed one hour to acclimatize in their home cage before drug injections. Bilateral microinjections (0.4  $\mu$ l/side) of saline vehicle, M40 (0.02–2.0 nmol), GAL (1.0 nmol), or a combination of M40 + GAL (2.0 nmol + 1.0 nmol, respectively), were then made into LS at a rate of 0.1  $\mu$ l/min. After the injections were complete, injectors remained in place for 5 min to allow for diffusion before withdrawing slowly. Behavioral testing took place 15 min after cannulae removal.

### 2.3. Shock-probe defensive burying test

A total of 57 rats were used in the shock-probe defensive burying test, adapted from [Pinel and Treit \(1978\)](#). Testing was conducted in a novel polystyrene cage, 26  $\times$  48  $\times$  21 cm, identical to the rats home cage, but with the lid modified to allow the rats behavior to be videotaped from above for off-line scoring. Testing was carried out under normal overhead ambient lighting (220 lux measured in the test chamber). Fresh bedding (Teklad Sani-Chips, Harlan, Indianapolis, IN) was placed in the cage to a depth of 5 cm prior to each test. The shock probe was a glass rod, 1.0 cm dia, wrapped with two alternating, non-touching 18 ga copper wires and spaced 5 loops/cm. The probe protruded 6 cm into one end of the cage, 2.0 cm above the surface of the bedding. The wires were attached to a shock generator (model H13-15, Coulbourn Instruments, Allentown, PA) set to deliver 2 mA DC current when the probe was touched. To begin a test session, a rat was introduced into the cage at the end opposite the shock probe, facing away from the probe. Rats typically approached the probe to investigate within 10–15 s, making contact with the paw or snout. Upon contacting the probe, the current was turned off so only a single shock was delivered and the 15-min test period began. After withdrawing from the probe, rats typically showed a variable period of inactivity before beginning to burying, usually within about 6–8 min (latency to onset of burying). “Burying” consisted of burrowing into the bedding with their snout and upper body, then “plowing” the bedding toward the probe, and also flicking bedding toward the probe with the dorsal surface of the forepaws. After each test, the cage was washed with a wet sponge and the bedding replaced with 5 cm fresh bedding before testing the next rat.

Behavioral scoring, modified from [De Boer and Koo-lhaas \(2003\)](#), was performed from video by an experimenter blind to the drug condition of the test animal. In addition to total burying time, other measures analyzed included latency to contact the probe (an indicator of potential changes in locomotion or exploratory activity), latency to onset of burying, total immobility time, and number of probe returns, rearings, exploratory stretches and defecations. All measures were analyzed by ANOVA, followed by post hoc analyses using the

Newmann–Keuls test where ANOVA indicated significant main effects or interactions. Because a number of rats in several of the drug-treated groups failed to exhibit burying behavior, total burying time was also analyzed non-parametrically using the Kruskal–Wallis test, followed post hoc by Dunn’s multiple comparison test where significant main effects were indicated. Significance was determined in all analyses at  $p < 0.05$ .

After behavioral testing, rats were sacrificed and brains processed histologically for determination of microinjection cannulae placement. Any case in which one or both cannulae tips were located outside the LS was eliminated from further analyses a priori, and was not included in the total number of rats reported to have been used.

### 2.4. Control experiment to test for changes in cutaneous pain sensitivity

A control experiment was conducted on a separate group of 18 rats to determine whether changes in defensive burying behavior in response to contact with the shock probe following microinjections into LS may have been attributable in part to changes in cutaneous pain sensitivity, using the radiant heat method ([Hargreaves et al., 1988](#)). Animals were prepared surgically with microinjection cannulae as above. After bilateral microinjections into LS of saline, M40 (0.2 nmol), or GAL (1.0 nmol), rats were placed in the thermal sensitivity test chamber (manufactured by Dr. Tony Yaksh, University of California, San Diego, CA), consisting of a clear acrylic box set atop a glass platform. After a 20-min habituation period to prevent excessive movement and escape behavior, a beam of radiant heat was applied through the platform to the  $\lambda 50$  target on the plantar surface of one hindpaw. A photocell detected withdrawal of the hindpaw and the latency was recorded. Both hindpaws were tested alternately, with 5 min between trials. The average of 4 trials was used to calculate mean hindpaw withdrawal latency for each rat. Data were analyzed by ANOVA, with significance determined at  $p < 0.05$ .

## 3. Results

### 3.1. Effects of GAL manipulations in the LS on behavioral activity on the SPDB task

One-way ANOVA indicated a significant treatment effect of drug microinjections into LS on Bury Time ( $F_{5,51} = 4.151$ ,  $n = 7$ –15 per group,  $p < 0.01$ ). Subsequent post hoc comparisons using the Newmann–Keuls test revealed that M40 microinjection into LS dose-dependently reduced defensive burying behavior, with a significant decrease seen in rats receiving injections

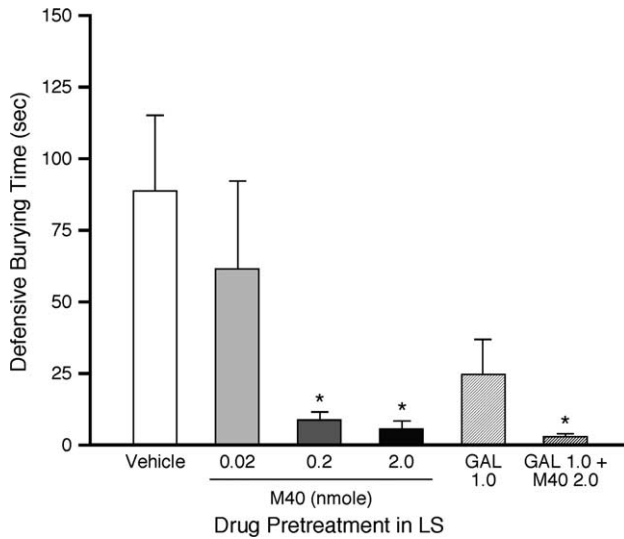


Fig. 1. Effects of M40 (0.02–2.0 nmol), GAL (1.0 nmol), or M40 + GAL on shock-probe defensive burying time (Mean  $\pm$  SEM,  $n = 7$ –15 per group). M40 produced a dose-dependent suppression of burying behavior. GAL also produced a moderate, but non-significant decrease in burying, and did not alter the suppression of burying behavior seen after M40 alone (2.0 nmol). \* $p < 0.05$  compared to vehicle controls.

of 0.2 or 2.0 nmol/side compared to vehicle-injected controls (Fig. 1). Microinjection of GAL itself also moderately reduced bury time, although this was not a significant decrease. Moreover, this dose of GAL (1.0 nmol) failed to affect the inhibition of bury time induced by the highest dose of M40 (Fig. 1).

A test of normality indicated that the burying time data in the vehicle-control group exhibited a normal distribution. However, because a proportion of animals in the drug-treated groups failed to exhibit any burying behavior (defined as  $< 15$  s over the entire 900 s test), data in some of these groups were not distributed normally. Thus, effects on burying time were also analyzed non-parametrically, with exactly the same results as obtained using ANOVA. A Kruskal–Wallis test indicated a significant main treatment effect ( $H_5 = 21.54$ ,  $p < 0.001$ ). As above, post hoc comparisons using Dunn's test revealed a significant decrease in bury time in groups receiving the two highest doses of M40, 0.2 and 2.0 nmol, compared to vehicle, with no attenuation of the inhibition of burying behavior seen after administering GAL together with the highest dose of M40.

In parametric analyses of the other behavioral measures scored on this test, there was no effect on total Immobility Time ( $F_{5,51} = 1.544$ ,  $p > 0.19$ , Fig. 2). There was a significant increase in latency to onset of burying behavior ( $F_{5,51} = 2.643$ ,  $p < 0.05$ ), but only in the group treated with both GAL and the highest dose of M40 together (data not shown). There were no significant changes in any other behavioral measures, including other measures of active responding (latency to contact

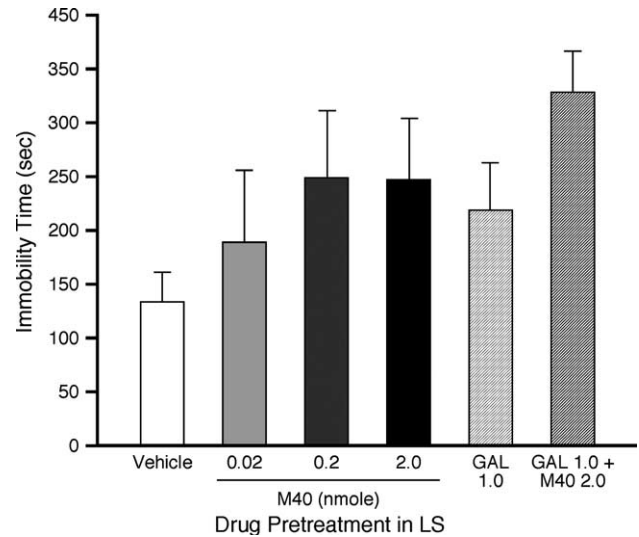


Fig. 2. Lack of effect of M40 (0.02–2.0 nmol), GAL (1.0 nmol), or M40 + GAL on immobility time measured during the shock-probe defensive burying test (Mean  $\pm$  SEM,  $n = 7$ –15 per group). No significant effects were observed for any treatment condition.

the probe, number of probe returns, rearings, exploratory stretches and defecations, all  $p > 0.05$ , data not shown).

### 3.2. Cutaneous pain sensitivity

In this control experiment, ANOVA revealed no significant effects of M40 (0.2 nmol) or GAL (1.0 nmol) microinjections into LS on hindpaw withdrawal latency

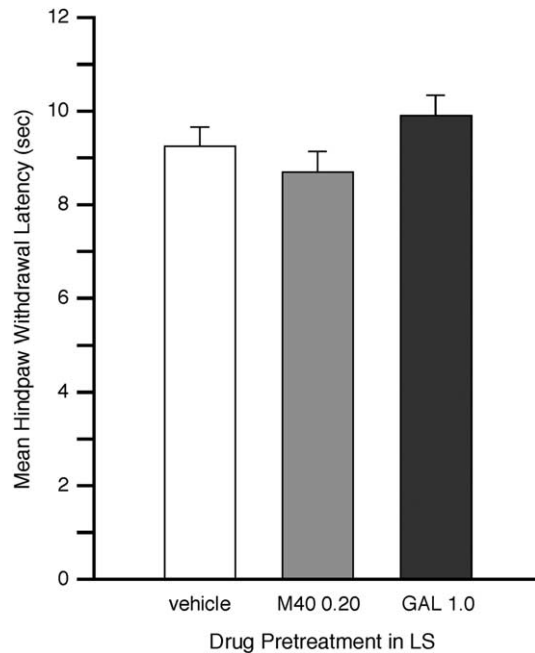


Fig. 3. Lack of effect of M40 (0.2 nmol, an effective dose in reducing burying time, see Fig. 1), or GAL (1.0 nmol) on hindpaw withdrawal latency on the cutaneous pain thermosensitivity test (Mean  $\pm$  SEM,  $n = 6$  per group). No significant effects were observed for any treatment condition.

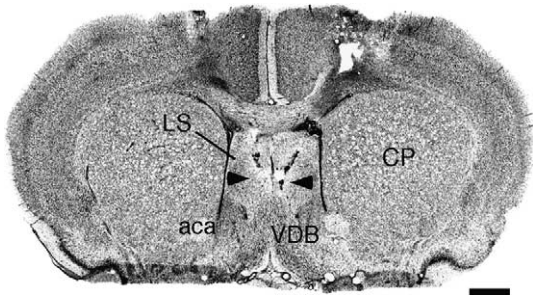


Fig. 4. Representative brain section stained with Cresyl violet, corresponding to plate 16 in the atlas of Paxinos and Watson (1998), showing histological localization of microinjection sites bilaterally in the lateral septum (arrowheads). Abbreviations: aca, anterior commissure; CP, Caudate-Putamen; LS, Lateral Septum; VDB, Diagonal Band nucleus, ventral limb. Scale bar = 1 mm.

in the thermal sensitivity test ( $F_{2,15} = 1.55$ ,  $n = 6$  per group,  $p = 0.24$ , Fig. 3). Thus, it is unlikely that the changes seen in burying behavior on the shock-probe test after M40 microinjections into LS can be attributed to changes in sensitivity to the shock stimulus.

### 3.3. Histological localization of microinjection sites

Out of a total of 88 rats used for microinjections in these experiments, 13 subjects were eliminated from analysis a priori because one or both injection sites were determined to be outside the LS, resulting in a total of 75 rats in which the microinjections were localized bilaterally to the LS (Fig. 4). In cases with misplaced injections, no consistent effects were observed on any behavioral measures, though these cases were too few in any given drug condition for a systematic analysis.

## 4. Discussion

In the present series of experiments, bilateral microinjections of the GAL antagonist M40 into the lateral septum dose-dependently attenuated burying behavior on the SPDB test compared to saline controls. The attenuation of burying behavior by M40 suggests that the endogenous release of GAL in the LS facilitates this active anxiety-like behavioral response to acute stress. It is unlikely that the decrease in burying behavior seen following M40 could be the result of a general motor impairment, as total immobility time was not altered, indicating no general reduction in locomotor activity, and there was also no change in a number of other behavioral measures scored, both passive and active, including the latency to contact the probe, number of post-shock probe returns, number of rearings, and exploratory stretches. Further, it has been shown recently that the central administration of exogenous GAL does not interfere with the fine motor skills required for operant responding (Brewer et al., 2004).

Thus, it is unlikely that the gross motor skills involved in burying were affected. In addition, it is also unlikely that the effects of M40 were attributable to a change in sensitivity to the shock as a noxious stimulus. In a control experiment assessing cutaneous thermal pain sensitivity, there was no change in hindpaw withdrawal latency following either M40 or GAL microinjections in LS. Thus, the effects of M40 administration appeared to be specific to the active defensive burying response elicited by shock-probe exposure.

The ability to differentiate effects of a given experimental manipulation on active behavioral responses to acute stress as compared to passive behavioral responses, i.e., those that represent the elicitation of a distinct, stimulus-oriented behavior rather than an inhibition of ongoing behavior, is an important and informative feature of the shock-probe defensive burying test (De Boer and Koolhaas, 2003). The nature and relative balance with which the active (i.e., burying) and passive (i.e., immobility) components of the response elicited by continued exposure to this stimulus will therefore represent an integrated net behavioral output, dictated by activity in several brain circuits and in a number of stress-responsive neurotransmitter systems. The LS in particular appears to be essential for the expression of the active behavioral response elicited in this test, as lesions of this area specifically abolished defensive burying (Menard and Treit, 1996). The present results would further suggest that the endogenous release of GAL in the LS facilitated specifically the active behavioral component of the acute burying response to shock-probe exposure, and did not influence the passive immobility response.

However, we also found that the exogenous administration of GAL itself into LS produced a moderate but non-significant reduction in burying behavior as well. M40 has been shown to possess partial agonist properties in some preparations, although primarily in vitro and often at high concentration (e.g., Bartfai et al., 1993a,b). However, in other studies, when microinjected into regions of limbic forebrain in vivo, at doses similar to those used in the present study, only antagonist effects of M40 were evident (Corwin et al., 1993; Mazarati et al., 1998). Moreover, in the present study, a monophasic dose–response effect was obtained after M40, with only reductions in defensive burying behavior being observed, even at the lowest effective dose of M40, whereas a much higher dose of GAL was only marginally effective. Thus, it is unlikely that the effects of M40 are attributable to partial agonist effects. A more likely explanation for the slight reduction seen after GAL is that exogenous administration of the neuropeptide agonist may have activated a different population of GAL-responsive cells, or a different set of GAL receptors in LS than those that were activated specifically by the release of the endogenous neuropeptide, and that were

therefore blocked specifically by M40 administration during the defensive burying response elicited by shock probe exposure.

The source of endogenous GAL release in LS is currently unknown, although GAL-positive cell bodies, GAL receptors, and a moderate density of GAL terminal fibers have been identified in LS (Jacobowitz et al., 2004). GAL immunoreactivity has been observed in several brain areas involved in processing emotional stimuli and responding to acute stress, that may also provide a source of innervation to LS, including several hypothalamic subnuclei, regions of the amygdala, periaqueductal gray, and regions in the limbic basal forebrain (Melander et al., 1986a; Holmes et al., 2003). The LS also receives input from the locus coeruleus and dorsal raphe nucleus. In these two stress-responsive brainstem monoaminergic nuclei, GAL is co-expressed extensively with norepinephrine and serotonin, respectively, thus representing potential sources of ascending GAL innervation of LS (Melander et al., 1986b; Pieribone et al., 1998; Kehr et al., 2002). Finally, within LS itself, local GAL neurons may be a source of endogenous GAL release in addition to extrinsic GAL afferents to LS.

The anxiolytic effect seen in the present study after M40 application into LS is similar to the previous observations of an anxiolytic effect of M40 administration into the lateral bed nucleus of the stria terminalis, which attenuated anxiety-like behavioral responses to acute immobilization stress, including the stress-induced reduction of open arm exploration on the elevated plus maze and reduction of social behavior in the social interaction test (Khoshbouei et al., 2002b). By contrast, microinjection of GAL into the amygdala produced an anxiogenic response on the punished drinking test (Moller et al., 1999), with no effect on the elevated plus maze, whereas in another study, M40 administration blocked specifically an anxiolytic effect on the elevated plus maze exerted by release of endogenous GAL in the central amygdala (Khoshbouei et al., 2002a).

Taken together, the results of the present study, along with the previous studies discussed above, would suggest that the modulatory influence exerted by endogenous GAL on affective behavioral responses to acute stress, as well as the behavioral effects of systemic pharmacological manipulations that may alter endogenous GAL activity, will be dependent on the brain region involved in the specific response being measured, the nature of the response and the task used to assess it, and the physiological context in which the response is evoked. Thus, there does not appear to be an integrated, anxiety-modulating function that can be ascribed to GAL at a “systems” level in the brain. Rather, the region- and response-specificity of its modulatory influence on acute stress-induced behavioral responses will likely continue to present a considerable challenge to present and future efforts aimed at developing novel therapeutic strategies

targeting brain GAL for the treatment of stress-related affective and/or anxiety disorders (see also discussions in Branchek et al., 2000; Wrenn and Crawley, 2001; Barreda-Gomez et al., 2005; Barrera et al., 2005).

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