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Behavioral reactivity to stress: Amplification of stress-induced noradrenergic activation elicits a galanin-mediated anxiolytic effect in central amygdala

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Abstract

Brain norepinephrine (NE) modulates many aspects of the stress response. The interaction between NE and neuropeptides such as galanin, with which it is closely associated and which may be released from noradrenergic terminals under conditions of high activity, has not been well studied. We therefore investigated the modulatory effects of galanin in the central nucleus of the amygdala (CeA) on behavioral responsivity to stress when activation of the noradrenergic system was amplified using the adrenergic autoreceptor antagonist yohimbine (2.5 mg/kg ip). Either immobilization stress or yohimbine alone had anxiogenic effects on rat behavior in the elevated plus maze. However, yohimbine pretreatment before stress produced a paradoxical anxiolytic response, which we hypothesized was attributable to galanin release in CeA. Microdialysis verified that yohimbine amplified NE release in CeA during immobilization stress, and also showed that whereas there was no detectable change in galanin release in CeA during stress alone, there was an increase during immobilization stress after yohimbine pretreatment. Bilateral administration of the galanin antagonist M40 into CeA before stress blocked the anxiolytic influence of yohimbine pretreatment. Exogenous galanin mimicked the anxiolytic effect of yohimbine pretreatment, and this too was blocked by M40. These results suggest that amplifying the noradrenergic response to stress can recruit galanin release in CeA, which buffers the anxiety-like behavioral response to acute stress. The balance between noradrenergic and peptidergic neurotransmission may be modified by prior stress, drug treatment or genetic variability, and may represent a novel target for treatment of stress-related neuropsychiatric disorders. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Stress; Anxiety; Galanin; Central amygdala; Norepinephrine; Neuropeptide; Yohimbine

1. Introduction

The central nucleus of the amygdala (CeA) is important in conditioned fear and modulating affective responses to stress (Davis and Shi, 1999; Gray, 1993; LeDoux, 1998), and neurons in the CeA are activated by acute immobilization stress (Henke and Ray, 1992; Honkaniemi et al., 1992). Activation of the amygdala in conscious animals elicits behavioral and autonomic effects indicative of anxiety-like responses (Feldman and Wiedenfeld, 1998; Goldstein et al., 1996; Moller et al., 1997), suggesting that the CeA represents a site of convergence for stress-responsive and anxiety-mediating neural systems. Thus, afferents to the amygdala that modulate its activity during stress may influence the expression of stress-induced anxiety-like behavior.

The ascending noradrenergic neurotransmitter system is activated by stress (Morilak et al., 1987a,b; Pacak et al., 1993), and provides a dense innervation of the extended amygdala (Moore and Bloom, 1979). Norepinephrine (NE) modulates many behavioral, autonomic and endocrine components of the stress response (Palkovits et al., 1999; Southwick et al., 1999) and may have a specific role in modulating anxiety and fear (Charney et al., 1987, 1992). Pharmacological agents which increase firing of noradrenergic neurons and release of NE in the limbic forebrain modulate fear-and anxiety-like behaviors associated with stress (Charney et al., 1992; Grant et al., 1988; Hatfield et al., 1999). Systemic administration of the adrenergic α_2 autoreceptor antagonist yohimbine induces release of NE in target regions such as hypothalamus and hippocampus (Tjurmina et al., 1999), and elicits behaviors consistent with

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anxiety and fear (Handley and Mithani, 1984; Johnston and File, 1989).

In addition to NE, noradrenergic neurons also co-localize and presumably co-release other neurotransmitters, one of which is the neuropeptide galanin (Levin et al., 1987). Galanin is found in a number of limbic brain regions important for emotionality. Immunohistochemical studies have demonstrated galanin immunoreactivity in noradrenergic neurons of the locus coeruleus (Hokfelt et al., 1998) and terminals in the amygdala (Skofitsch and Jacobowitz, 1985). It is possible that GAL has a role in the process of stress adaptation in the noradrenergic system, as expression of prepro-GAL mRNA in LC is increased in response to chronic social stress or reserpine administration (Austin et al., 1990; Holmes et al., 1995). In addition to providing afferents to the amygdala, galanin-synthesizing neurons are also present within CeA and other limbic forebrain regions (Melander et al., 1988), and these neurons may themselves be targets of noradrenergic innervation (Kozicz, 1999; Ryan and Gundlach, 1996). These local galaninergic neurons are also responsive to stress, as prepro-galanin mRNA expression is increased in CeA following restraint stress (Sweerts et al., 1999).

Release of neuropeptide transmitters appears to require higher levels of neuronal activity than small-molecule neurotransmitters with which they are co-localized. For example, NE release exhibits a relatively linear relationship to electrical activity, but a much higher firing rate, or a pattern of burst-firing, is necessary to recruit release of galanin (Consolo et al., 1994; Hokfelt et al., 1995; Lundberg and Hokfelt, 1983). Thus, it is possible that co-release of galanin from noradrenergic terminals may be recruited during stress to modulate the effects of NE in target structures such as CeA, but only when high levels of noradrenergic activation are induced.

Little is known regarding the role of galanin in modulating fear and anxiety. Central administration of galanin has been shown to decrease anxiety in rats, measured as an increase in punished drinking (Bing et al., 1993). However, increased anxiety has also been reported by the same group (Moller et al., 1999). Thus, given the importance of the noradrenergic system in modulating acute behavioral reactivity to stress, and the convergent innervation of CeA by these two neurotransmitters, it would be informative to address the possible involvement of galanin in modulating behavioral responses to acute stress when activation of the noradrenergic system is sufficient to recruit galanin release. Thus, in the present study, we investigated the potential role of galanin neurotransmission in the CeA in modulating acute behavioral reactivity to stress, after amplifying the stress-induced activation of the noradrenergic system by systemic pretreatment with yohimbine. Behavioral reactivity in this study was defined as the induction of anxiety-like responses, measured as a proportional reduction in open-arm exploration on the elevated plus maze following acute immobilization stress. Portions of this work have been presented in abstract form (Khoshbouei et al., 2000).

2. Materials and methods

2.1. Animals

One hundred ninety-two adult male Sprague–Dawley rats (Harlan, Indianapolis), weighing approximately 225–250 g upon arrival, were housed in groups of three in a room adjacent to the testing rooms. They were maintained on 12/12-h light/dark cycle (lights on at 07:00 h). Experiments were conducted between 09:00 and 14:00 h, during the light portion of the cycle. Food and water were available ad libitum. Rats were handled 5 min daily for one week prior to intiation of experiments. 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.

2.2. Experiment 1: Modulation of behavioral reactivity to acute immobilization stress by selective amplification of the noradrenergic stress response

Sixty rats were randomly assigned to four groups, comprising two drug pretreatment conditions and two stress conditions: vehicle/unstressed controls, vehicle/stressed, yohimbine/unstressed and yohimbine/stressed. Each rat was given an intraperitoneal injection of either saline vehicle or yohimbine (2.5 mg/kg in a volume of 1.0 ml/kg), and returned to the home cage for 20 min. They were then transported in a cage containing bedding from their home cage to an adjacent laboratory room where unstressed animals remained undisturbed, and stressed animals were subjected to 5 min immobilization stress.

2.2.1. Immobilization stress

Immobilization stress was applied according to published procedures (Mamalaki et al., 1992; Pacak et al., 1993, 1995) with slight modification. Briefly, animals were placed supine on top of a flat, plastic rack large enough to securely support the entire body (26×13 cm). The limbs were taped gently but securely to the rack with medical adhesive tape, and strips of tape were placed across the animals neck and back of the head to prevent excessive head movements. Care was taken to avoid applying undue pressure on the limbs with the tape, and the animals were monitored constantly throughout the procedure. After 5 min of immobilization, the animals were removed and returned to the holding cage, with home bedding, for 15 min. Unstressed animals were left undisturbed in the cage for an equivalent amount of time. Immediately following the post-stress recovery period (and 40 min after systemic yohimbine or

vehicle pretreatment), animals were tested on the elevated plus-maze.

2.2.2. Elevated plus-maze

Stress-induced anxiety-like behavioral reactivity was measured using the elevated plus-maze according to published procedures (Handley and Mithani, 1984; Pellow et al., 1985), with minor modification as indicated. The maze (AccuScan Instruments) consisted of four white plastic arms, 10×50 cm, oriented in the shape of a cross, intersecting at a 10×10 cm central platform. Two arms situated opposite each other were enclosed by walls 48 cm high (''closed arms''). The remaining two ''open arms'' had no walls, but were fitted with a 0.5-cm clear plastic rim around the edge to prevent animals falling off (Fernandes and File, 1996). The elevation of the maze was 75 cm from the floor. Dual infrared sensor beams positioned at the entry to each arm interfaced with a PC card controlled by Plus-Maze Software (AccuScan). To start the 5-min trial, rats were placed onto the center platform facing the junction of an open and closed arm. An arm entry was counted only when the first sensor beam was broken, then the second beam, positioned further in the arm, was also broken, and finally the first beam was released. Thus, an animal had to enter completely into an arm to trigger the required sequence. After 5 min, the program was terminated, the animal was removed, and the maze was washed and dried thoroughly.

Data collected from each trial included the number of entries and time spent in the central platform, number of open arm entries, number of closed arm entries, total time spent in open arms and total time spent in closed arms. From these data, the open/total ratios (OTR) for both Time and Entries, defined as the proportion of open arm exploration relative to total exploration in all arms (open/open + closed) were calculated as indicators of anxiety-like responses. Lower OTR values indicate reduced open-arm exploration, interpreted as an increase in anxiety, and higher OTR indicates a reduction in anxiety-like behavior. To monitor nonspecific locomotor effects, the number of entries into closed arms was taken as an indicator of general locomotor activity independent of anxiety (File et al., 1993). Any animal falling or jumping from the maze during the 5-min test was excluded from analysis.

2.2.3. Measurement of NE release in CeA by microdialysis

To verify that systemic pretreatment with yohimbine amplified activation of the noradrenergic system by immobilization stress, a separate group of 12 animals were used for NE microdialysis. Rats were anesthetized (cocktail of ketamine 43 mg/ml, acepromazine 1 mg/ml, xylazine 8.6 mg/ml, given in a dose of 0.8 ml/kg im, with 25% supplement administered as needed) and placed in a stereotaxic frame. A guide cannula (CMA/12) was implanted with the tip positioned above the CeA using the following coordinates: AP − 2.5 mm, ML + 3.9 mm, DV − 7.0 mm relative to bregma. The cannula was anchored to the skull with four jeweler screws and acrylic dental cement. After the surgery the animals were housed singly. Microdialysis was performed 5–7 days after the surgery. On the day of the experiment, a microdialysis probe (CMA/12) with 2 mm of active membrane was inserted into the guide cannula. The probe extended 2 mm beyond the tip of the guide cannula, placing it in the CeA. The probe was perfused with artificial cerebrospinal fluid (aCSF; 147 mM NaCl, 2.5 mM KCl, 1.3 mM $CaCl_2$, 0.9 mM $MgCl_2$, pH 7.4) at a flow rate of 2 μl/min. After a 2-h equilibration period, four baseline samples were collected. Sample collection time was 30 min, resulting in sample volume of 60 μl. Animals were then subjected to 30-min immobilization stress, during which the stress sample was collected. The rats were then released and returned to their cages, and four recovery samples were collected. For 6 of the rats, yohimbine was administered systemically (2.5 mg/kg ip) 30 min prior to the immobilization period. Following all experiments, placement of the dialysis probe within the CeA was verified histologically.

Concentration of NE in the dialysate samples was measured by HPLC with coulometric detection (ESA Coulochem 2 detector) using Waters Millenium software. Dihydroxybenzoic acid was used as an internal standard, and NE was quantified against a calibration curve ranging from 0.5 to 50 pg. Detection limit for NE, defined as a signal-to-noise ration of 3:1, was approximately 1.5 pg/sample.

2.2.4. Measurement of galanin release in CeA by microdialysis

To determine whether systemic pretreatment with yohimbine prior to immobilization stress induced galanin release in CeA, a separate group of 18 animals were used for galanin microdialysis. The procedures were identical to those described above for NE microdialysis, with minor modification as follows. Two guide cannulae (CMA/12) were implanted bilaterally above the CeA. On the day of the experiment, a microdialysis probe (CMA/12) with 2 mm of active membrane was inserted into each of the guide cannulae, extending 2 mm beyond the tips of the guides, placing them in the CeA. The probes were perfused with aCSF containing 2% BSA and 0.5% Bacitracin (Consolo et al., 1994) at a flow rate of 1.8 μl/min. Sample collection time was 30 min, and dialysate collected from both probes was pooled into a single sample for each time point. All samples were frozen immediately and stored at − 80 °C until assayed. After a 2-h equilibration period, 4 baseline samples were collected, then animals were subjected to 30-min immobilization stress, during which one stress sample was collected. The rats were then released and returned to their cages, and three recovery samples were collected. For 12 of the rats, yohimbine was administered systemically (2.5 mg/kg ip) 30 min prior to the immobilization period, while the remaining rats received systemic vehicle injections. Following all experiments, placement of the dialysis probes within the CeA was verified histologically. Concentration of galanin in the

dialysate samples was measured by radioimmunoassay using a commercially available kit (Peninsula, Belmont, CA). Samples were measured in two separate assays. Detection limits for galanin in the two assays, defined at $\leq 98\%$ of total binding in each respective standard curve, were 0.7 and 1.2 pg/sample. Intra- and interassay coefficients of variation were less than 7% and 8%, respectively. In vitro recovery of galanin through the dialysis probes, determined by collecting samples as described above from probes immersed in a 1-nM solution of GAL, was approximately 2.1%.

2.3. Experiment 2: The role of galanin in the CeA in modulating the behavioral response to stress after amplification of noradrenergic activation by yohimbine

To assess the behavioral effects of local drug microinjection into CeA prior to stress, 102 rats were randomly assigned to 7 groups, defined by the systemic drug pretreatment condition (vehicle or yohimbine), the drugs administered by local microinjection into CeA prior to stress exposure (vehicle, the galanin antagonist M40 [1.0 or 4.0 nmol], galanin alone [1.0 nmol] or M40 followed by galanin), and the stress condition (5 min immobilization stress or unstressed). The doses of drugs administered into the amygdala were initially determined from previously published experiments (Crawley et al., 1993; McDonald and Crawley, 1996).

Rats weighing 275–280 g at the time of surgery were anesthetized and placed in a stereotaxic apparatus as above. Guide cannulae, consisting of two lengths of 23 gauge stainless steel tubing (Small Parts), were implanted bilaterally, so as to position the tips 1.0 mm above the CeA (coordinates from bregma: AP − 2.5 mm, ML ± 3.9 mm, DV − 7.2 mm). The guide cannulae were fitted with 30 gauge obdurators, and anchored to the skull with four jewelers screws and acrylic dental cement. Following all surgical preparations, animals were housed individually for 5–7 days prior to testing.

On the day of the experiment, the obdurators were removed and replaced with 30 gauge stainless steel injectors (Small Parts) extending 1.0 mm beyond the tip of the guide cannulae, placing them in the CeA. Each rat was given an intraperitoneal injection of either vehicle (1.0 ml/kg) or yohimbine (2.5 mg/kg in 1.0 ml/kg), then returned to the home cage for 20 min. The microinjection cannulae were connected by PE-10 polyethylene tubing to a Hamilton syringe mounted on a syringe pump. Twenty min after systemic pretreatment with vehicle or yohimbine, bilateral microinjections were made into CeA of vehicle (sterile distilled water), the galanin antagonist M40 (1 or 4 nmol, American Peptide), galanin (1 nmol, American Peptide) or M40 followed 3 min later by galanin. All drugs were injected in a volume of 0.5 μl at a rate of 0.5 μl/min. Following completion of the microinjections, cannulae were left in place for 3 min before withdrawing.

Unstressed rats were returned to their cages immediately after microinjections, and stressed rats were subjected to 5 min immobilization stress exactly as described above. After termination of the stress, the stressed animals were returned to their cages for 15 min, then tested as described above on the elevated plus-maze. Unstressed animals remained undisturbed in their cages for an equivalent time after microinjection before testing.

After each experiment, bilateral microinjections (0.5 μl) of 1% Evans Blue dye were made through the cannulae. Anatomical localization of the injection sites were determined histologically after counterstaining the sections with Cresyl violet. Cases in which one or both injections sites were located outside the CeA were eliminated from analyses.

2.4. Data analyses

For all experiments, data were analyzed by ANOVA, with significance determined at $P < .05$. For the microdialysis experiments, two-way ANOVA (Group × Time) were used, with repeated measures over time. Where ANOVA indicated significant main effects or interactions, post-hoc comparisons were made using Fisher's PLSD test. Any rats that fell or jumped from the plus-maze during the 5-min behavioral test, or any rats in which histological examination determined that injection sites or probe placement fell outside the CeA, were eliminated from further analysis a priori.

3. Results

3.1. Experiment 1: Modulation of behavioral reactivity to acute immobilization stress by selective amplification of the noradrenergic stress response

In all experiments with the elevated plus-maze, we found that open-arm exploratory behavior as indicated by both OTR for time and OTR for entries always varied in the same direction and in response to the same manipulations. However, we also found that OTR for time was generally the more sensitive, consistent and reliable measure. While in all cases the OTR for entries showed the same pattern of response as time, the variability also tended to be greater for entries, and some effects that clearly achieved significance for time failed to reach statistical significance for entries ($P < .06–.07$). Thus, we have presented only the analyses of OTR for time.

3.1.1. Effects of stress and yohimbine on anxiety-like behavior on the elevated plus-maze

In the first experiment testing the effects of stress and yohimbine, applied independently and in combination, ANOVA revealed a significant treatment effect on OTR for Time ($F_{3,37} = 3.793$, $P < .05$). Subsequent post hoc analyses indicated that 5-min immobilization stress induced

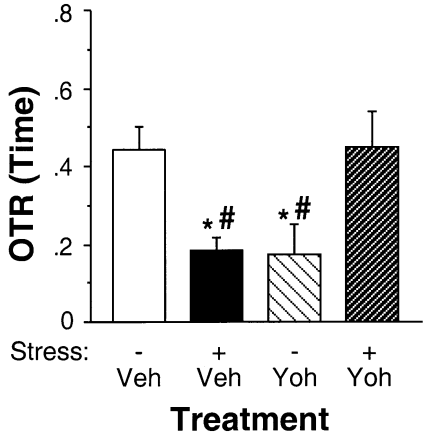

Fig. 1. Interactive effects of stress and yohimbine on behavioral reactivity measured on the elevated plus-maze. Exploratory activity on the open arms was measured as OTR for time (ratio of time spent in open arms relative to total time spent in all four arms). Values represent the mean ± S.E.M. ($n = 7–18$ per group). * $P < .05$ compared to the control group. # $P < .05$ compared to the yohimbine-stress group.

a significant decrease in OTR for time, indicative of the expected anxiogenic effect of a mild acute stress exposure (Fig. 1). An anxiogenic effect similar to that induced by acute stress was also seen following systemic yohimbine administration (Fig. 1). However, when these two anxiogenic stimuli were combined, there was a significant increase in OTR to a level that was not different from that of untreated, unstressed control rats (Fig. 1). This indicates that yohimbine pretreatment reversed the anxiogenic response to acute stress. In no case was there any significant change in locomotor activity, measured by the number of closed arm entries ($F_{3,37} = 1.816$, n.s., Fig. 2).

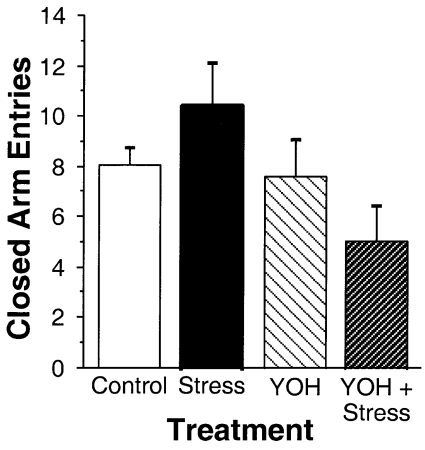

Fig. 2. Lack of effect of stress or yohimbine on locomotor activity measured on the elevated plus-maze. Locomotor activity was measured as the total number of closed arm entries. No significant effects were observed for any of the treatment conditions.

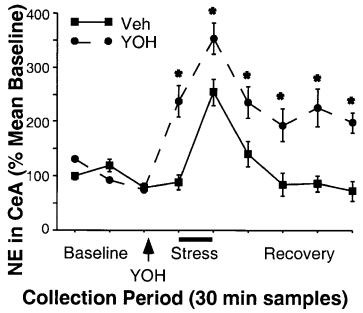

Fig. 3. NE release in CeA. Yohimbine pretreatment amplifies the immobilization stress-induced release of NE in CeA as measured by microdialysis. Sampling intervals were 30 min. Immobilization stress was applied during the fourth collection interval (bar). Values expressed as mean percent baseline ± S.E.M. ($n = 6–8$ per group). Mean baseline NE levels for control and yohimbine-pretreated groups were 2.9 ± 0.4 and 3.5 ± 0.6 pg/sample, respectively. * $P < .05$ compared to vehicle-treated control group.

3.1.2. Measurement of NE release in CeA by microdialysis

Pre-drug levels of NE measured in baseline dialysate samples collected from the CeA of control and yohimbine groups were 2.9 ± 0.4 and 3.5 ± 0.6 pg/sample, respectively. ANOVA and subsequent post hoc analyses indicated that 30-min immobilization stress significantly elevated the release of NE in CeA, reflected by an increase in the concentration of NE in microdialysate samples ($F_{1,8} = 17.35$, $P < .003$; Fig. 3). Systemic administration of yohimbine itself increased the pre-stress baseline levels of NE in CeA, and yohimbine pretreatment also significantly amplified the increase in NE release elicited in CeA by acute immobilization stress (Fig. 3).

3.1.3. Measurement of galanin release in CeA by microdialysis

Of 18 rats subjected to bilateral GAL microdialysis, two were eliminated from analysis a priori because histological examination determined that one of the probes was not located in the CeA. Two additional animals were removed from analysis because of a lack of detectable GAL in samples collected from those rats. For the remaining 14 subjects ($n = 7$ per group), any individual samples in which GAL levels fell below the detection limits of the assays were substituted with the detection limit values for that assay for purposes of further analysis. This occurred in 15 of 112 total samples, collected from 9 of 14 rats, and all of these were either baseline or recovery samples (i.e., none were collected during stress). With this caveat in place, a two-way ANOVA with repeated measures over time showed a significant Time × Treatment interaction ($F_{1,7} = 2.389$, $P < .05$). Post hoc analyses revealed an increase in galanin content of the dialysate samples collected in CeA during acute immobilization stress, but only in the rats that were pretreated with systemic yohimbine ($P < .05$, Fig. 4). No such change was observed in the stress-only group. However, because of the low basal values, and the occurrence of nondetectable GAL values in approximately 10% of the

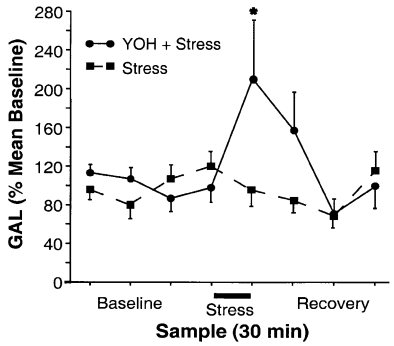

Fig. 4. Galanin release in CeA. A detectable increase in release of galanin in CeA was induced by immobilization stress only after yohimbine pretreatment. Sampling intervals were 30 min. Immobilization stress was applied during the fourth collection interval (bar). Values expressed as mean percent baseline ± S.E.M. of pooled bilateral samples ($n = 7$ per group). Mean baseline GAL levels for control and yohimbine-pretreated groups, determined as described in the text, were 4.6 ± 0.4 and 3.3 ± 0.6 pg/sample, respectively. * $P < .05$ by repeated measures over time.

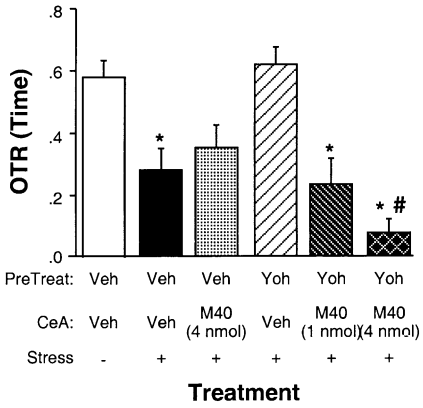

Fig. 5. Galanin antagonist in the CeA prevents the anxiolytic influence of yohimbine pretreatment on behavioral stress reactivity. Local microinjection of galanin antagonist M40 (1.0 or 4.0 nmol/0.5 μl) into CeA blocked the anxiolytic effect of the combined yohimbine + stress treatment in a dose-dependent fashion. OTR values expressed as mean ± S.E.M. ($n = 5–11$ per group). * $P < .05$ compared to both the unstressed vehicle-pretreated control group as well as to the YOH-pretreated + stress group; # $P < .05$ compared to the vehicle-pretreated stress-only group.

baseline samples, it is not possible for us to state with certainty that there was no change in galanin release in the stress-only group. Nonetheless, it is clear from these results that galanin release in the CeA did increase significantly during immobilization stress in the rats pretreated with yohimbine, and that the increase in galanin release observed in this group was significantly greater than any increase in release that may have occurred during stress in the vehicle-treated rats.

3.2. Experiment 2: The role of galanin in the CeA in modulating the behavioral response to stress after amplification of noradrenergic activation by yohimbine

In this experiment testing, the effect of local GAL antagonist administration into CeA on the effects of YOH and stress on exploratory behavior in the plus maze, there was a significant overall main treatment effect ($F_{5,35} = 6.641$, $P < .001$; Fig. 5). In replication of Experiment 1, vehicle microinjections into CeA did not alter the anxiogenic effect of acute immobilization stress, seen as a significant decrease in OTR relative to unstressed controls ($P < .01$, Fig. 5). Nor did vehicle microinjections bilaterally into CeA alter the anxiolytic effect of yohimbine pretreatment prior to acute immobilization stress (Fig. 5).

Microinjections of the galanin antagonist M40 into CeA dose dependently reversed the anxiolytic influence of yohimbine pretreatment. Administration of 1.0 nmol M40 into the CeA of animals pretreated with yohimbine before stress blocked the anxiolytic effect of yohimbine pretreatment and resulted in an OTR that was not different from animals exposed to stress alone. Microinjection of 4.0 nmol M40 into CeA of stressed animals pretreated with yohimbine produced an even greater reduction in OTR, unmasking an additional anxiogenic influence of the combined stress + yohimbine treatment in the presence of galanin receptor blockade (Fig. 5).

In a separate experiment to determine if exogenous galanin could mimic the anxiolytic effect of yohimbine, galanin was microinjected bilaterally into CeA prior to stress in the absence of yohimbine pretreatment. ANOVA and post hoc comparisons showed a significant increase in OTR produced by galanin microinjection into CeA prior to stress ($F_{3,28} = 7.791$, $P < .001$). Thus, the anxiolytic influence of yohimbine pretreatment observed in the previous experiment was mimicked by exogenous galanin administration into CeA (Fig. 6). This anxiolytic effect of galanin was blocked by co-administration of M40 (4.0 nmol), which

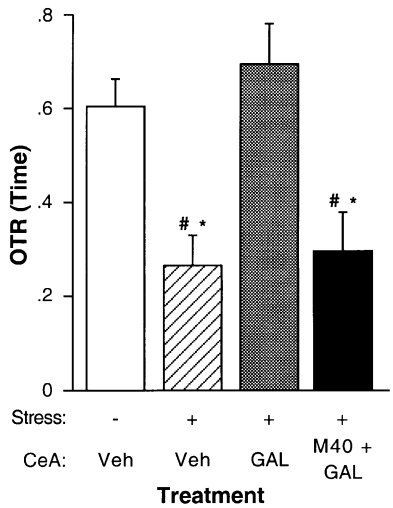

Fig. 6. Exogenous galanin in CeA mimics the anxiolytic effect of yohimbine administration prior to stress. Exogenous galanin (1 nmol/0.5 μl) in CeA during stress mimicked the anxiolytic effect of yohimbine pretreatment. The anxiolytic effect of exogenous galanin was blocked by co-administration of M40 (4 nmol/0.5 μl). OTR values expressed as mean ± S.E.M. ($n = 5–11$ per group). * $P < .05$ compared to unstressed vehicle controls. # $P < .05$ compared to the stress-galanin group.

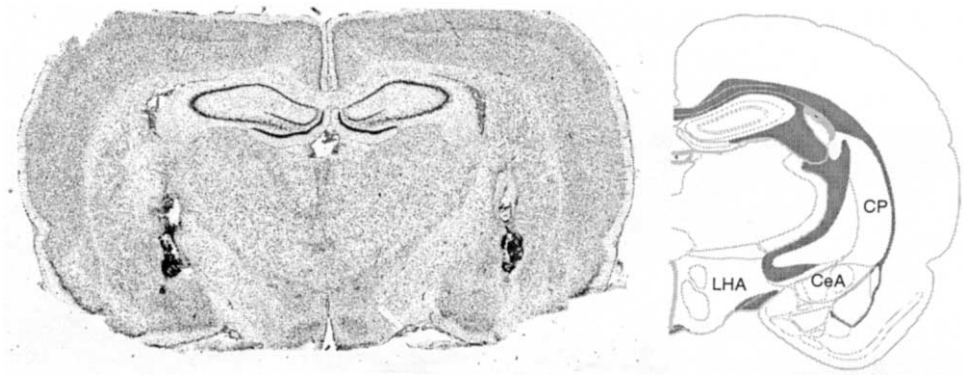

Fig. 7. Histological verification of injection site localization in CeA. At left is a representative section through the amygdala, stained with Cresyl violet, showing the extent of distribution of a bilateral microinjection (0.5 μl) of 1% Evans Blue dye made after the completion of an experiment through the same cannulae used for drug administration. The injections were both centered in the CeA, as portrayed in the schematic illustration at right, adapted from plate 27 of the atlas of Swanson (1992). CeA: central amygdala; LHA: lateral hypothalamic area; CP: caudate–putamen.

restored the post-stress OTR to a level that was no different from that after stress with vehicle injection in CeA (Fig. 6). Administration of M40 alone into CeA in the absence of yohimbine pretreatment had no effect on the anxiogenic effect of stress (Fig. 6), suggesting an absence of an endogenous galanin-mediated anxiolytic effect when stress-induced activation of the noradrenergic system was not amplified by yohimbine.

None of the drug treatments in either of these microinjection experiments produced any change in general locomotor activity as measured by number of closed arm entries in the plus-maze ($F_{5,35}=1.696$ and $F_{3,28}=1.414$, respectively, both n.s., data not shown). Histological analyses verified that the injection sites were centered bilaterally in the CeA (Fig. 7). Any cases in which one or both injections sites were located outside the CeA were eliminated from analyses. This resulted in elimination of eight cases (not included in the total animal count).

4. Discussion

In this study, we investigated a possible role for galanin in modulating a behavioral–affective component of the stress response in the CeA when stress-induced activation of the noradrenergic system had been amplified by prior administration of systemic yohimbine. In the first experiment, we demonstrated that acute immobilization stress and systemic administration of yohimbine both induced anxiety-like reductions in open-arm exploration on the elevated plus maze when administered by themselves. The anxiogenic effect of yohimbine is consistent with theories suggesting a role for NE in stress, arousal and anxiety (Aston-Jones et al., 1991; Charney et al., 1987). However, when these two stimuli were combined, i.e., when the noradrenergic response to immobilization stress was amplified by pretreating with yohimbine, the anxiogenic effect of the acute stressor was attenuated. It may seem paradoxical at first that combining two anxiogenic stimuli induced an anxiolytic response, and we attempted to resolve this paradox by hypothesizing that the combination of anxiogenic stimuli recruited a new anxiolytic mechanism in the CeA that was not evoked by either stimulus alone, namely the release of galanin. Using microdialysis, we verified that yohimbine pretreatment not only amplified stress-induced release of NE in the CeA, but release of the neuropeptide galanin was also evoked in the CeA in this condition.

In the second experiment, we microinjected the galanin antagonist M40 into CeA to test the hypothesis that galanin participated in the anxiolytic effect observed when animals were given yohimbine prior to stress. Local bilateral administration of M40 into the CeA prior to stress completely blocked, in a dose-dependent fashion, the anxiolytic influence of yohimbine pretreatment on the behavioral response to acute stress. Administration of exogenous galanin into the CeA was itself capable of inducing an anxiolytic effect during stress that mimicked yohimbine pretreatment, and this too was blocked by M40. Therefore, we concluded that the release of galanin in CeA was responsible in large part for the anxiety-attenuating effects of yohimbine treatment given prior to stress.

The galanin-mediated attenuation of stress-induced anxiety-like behavioral responses in the CeA was not evident when immobilization stress was applied alone, as M40 administration had no effect in this condition. Rather, the anxiolytic influence of galanin was only manifest when the impact of the stressor, and subsequent activation of the noradrenergic system, was amplified by yohimbine pretreatment. One possible source of this galanin release could have been from the noradrenergic terminals innervating the CeA, i.e., GAL that is co-localized with NE. More than 80% of noradrenergic neurons in the locus coeruleus also contain galanin (Melander et al., 1986). It has been shown both in vitro and in vivo that co-localized neuropeptides are released preferentially at higher levels of neuronal activity than those required for classical small-molecule neurotransmitters such as NE (Bartfai et al., 1988; Consolo et al., 1994; Hokfelt, 1991). Thus, whereas NE may be preferen-

tially released from noradrenergic afferent terminals innervating the amygdala with low-to-moderate levels of activation, yohimbine pretreatment may have amplified the stress-induced activation of the NE system sufficiently to recruit co-release of galanin from the noradrenergic terminals, resulting in the anxiolytic effect observed.

This suggestion was supported by the results of our microdialysis experiment, which showed the stress-induced release of galanin in CeA to be greater after yohimbine pretreatment. It is necessary to exercise caution in concluding that there was no galanin released in the absence of yohimbine pretreatment, as the galanin levels measured in baseline samples were very close to the sensitivity limits of the radioimmunoassay for GAL. Thus, even though we did not detect any increase in galanin in the dialysate samples collected during stress without yohimbine pretreatment, it is possible that a slight increase could have occurred and remained below detection. Nonetheless, we can conclude with confidence that stress-induced release of galanin in CeA was significantly greater following yohimbine pretreatment than it was in the absence of yohimbine.

Alternatively, it is possible that some or all of the galanin released in CeA may not have originated from noradrenergic terminals. The amygdala itself contains a number of galanin-synthesizing neurons, as well as galanin receptors (Melander et al., 1988; Ryan and Gundlach, 1996; Skofitsch and Jacobowitz, 1985; Waters and Krause, 2000). Both chronic and acute restraint stress have been shown to increase pre-pro-galanin mRNA expression in the CeA (Sweerts et al., 2000), indicating that galaninergic neurons within CeA are stress-responsive. Thus, a second possible source of galanin released in CeA by the combination of yohimbine and stress may have been the galanin-containing neurons intrinsic to the CeA. Noradrenergic afferent terminals have been shown to contact galanin-positive targets in the bed nucleus of the stria terminalis (Kozicz, 1999), a component of the extended amygdala closely related to CeA. It is thus possible that noradrenergic afferents also contact galaninergic targets in CeA, and that NE activates these intrinsic galanin-synthesizing neurons in response to stress only when a certain level of noradrenergic transmission has been reached, such as that occurring when the stress is preceded by yohimbine pretreatment.

Regardless of the source of galanin release in CeA, it reduced the magnitude of the anxiogenic response to stress, but only when a sufficient level of activity had been achieved in the noradrenergic system innervating the CeA. Moreover, when the anxiolytic influence of galanin was blocked by the highest dose of M40, an additive anxiogenic effect of the combined yohimbine plus stress treatment was revealed. As might be expected from the combination of two anxiogenic stimuli, the OTR in this condition was significantly lower than that following stress alone, suggesting that galanin release in the CeA had masked the additional anxiogenic effect of yohimbine that was evident when the drug was given alone.

The galanin-mediated attenuation of behavioral reactivity to stress observed in this study may represent a form of negative feedback regulation of noradrenergic modulation of the stress response. Yohimbine pretreatment, in addition to inducing galanin release, also increased stress-induced activation of NE release, as would be expected following α_2 adrenergic autoreceptor blockade. NE has been strongly implicated in behavioral arousal and anxiety associated with stress (Aston-Jones et al., 1991; Charney et al., 1987; Redmond, 1987). Thus, it seems likely that the anxiogenic component of yohimbine pretreatment that was masked by galanin may be attributable to a noradrenergic mechanism. In this way, once the stress-induced activation of the noradrenergic system is elevated sufficiently, GAL release is recruited in CeA, which then acts in turn to buffer the behavioral reactivity and anxiogenic responses associated with the elevated noradrenergic activity.

Few previous studies have addressed a possible role for galanin in the modulation of anxiety-like behaviors, and these studies have generated inconsistent results (Bing et al., 1993; Moller et al., 1999). An anxiolytic effect, measured as an increase in punished drinking, was reported after intracerebroventricular administration of galanin (Bing et al., 1993). By contrast, in another experiment conducted by the same group, administration of galanin into the amygdala produced an apparent anxiogenic response on the same test, while no effect was observed on the elevated plus maze (Moller et al., 1999). A number of factors may account for the inconsistencies between these experiments, and also for the lack of effect on the plus maze compared to the clear effects on exploratory behavior in the plus maze that were observed in the present study. First, intracerebroventricular administration of drugs is likely to affect many brain regions, perhaps eliciting different or opposing responses. Also, there are many tests of anxiety-like behavior in rats, and depending on the nature of the experimental manipulations, they can generate very different results (File, 1995; Spear and File, 1996). Galanin has been implicated in a variety of appetitive, nociceptive and memory-related processes (Crawley, 1999; Hokfelt et al., 1999), and the punished drinking test involves aspects of all of these, including thirst, consummatory behavior, motivation, conditioning, and pain perception, as well as anxiety (Menard and Treit, 1999). Thus, the behavioral effects observed in this test after intracerebral administration of galanin may not have been related exclusively to changes in anxiety. In addition, the results obtained on any presumed measure of anxiety can be very sensitive to differences in testing, housing and handling conditions (File and Fluck, 1994). Such factors may have accounted for some differences between the results of these previous experiments and the present study. Finally, an important aspect of the present study is that we explicitly addressed a possible role for GAL in modulating stress-induced behavioral reactivity, measured as a change in exploratory behavior following an acute stress exposure, rather than examining possible effects of

GAL on baseline exploratory behavior, as was done in the previous experiments. In the present study, exogenous galanin administered into CeA was shown to buffer behavioral reactivity to acute stress, manifest as an attenuation of the stress-induced reduction in open-arm exploration in the plus maze. However, in the previously cited experiments, no stressor was applied. Thus, the effects tested in these experiments were on baseline behavior, and baseline levels of anxiety may have been sufficiently low that exogenously administered galanin had no effect. Nevertheless, in the current study, stress-induced release of endogenous galanin in the CeA following yohimbine pretreatment exerted a clear and consistent anxiolytic effect.

The interaction between NE activity and the neuropeptide GAL demonstrated in this experiment raises the possibility of noradrenergic interaction with other peptide neurotransmitters with which it is closely associated, such as NPY. NPY is also co-localized extensively with NE, though less in LC and more in medullary noradrenergic cell groups than GAL (Sawchenko et al., 1985; Zardetto-Smith and Gray, 1995). NPY has been shown to modulate behavioral responsivity to fear and stress (Heilig et al., 1994). Specifically, administration of NPY into CeA produced anxiolytic effects (Heilig et al., 1993), and selective blockade of NPY-Y1 receptors in CeA decreased open arm exploration on the elevated plus maz (Heilig et al., 1994; Wahlestedt et al., 1993). Consistent with a possible NPY-noradrenergic interaction, NPY attenuated the anxiety-like reduction in social interaction that was possibly attributable to noradrenergic supersensitivity following DSP4-induced denervation (Kask et al., 2000). These observations, together with the results of the present experiment, suggest that, depending on the nature of the stressor and the response elicited, the subset of noradrenergic neurons that are activated, and the degree to which this system is activated by specific stressors, a variety of potential modulatory interactions in the CeA could occur involving NE, NPY and GAL. Regulation or modification of these interactions may provide for considerable flexibility, specificity and plasticity in the modulatory effects exerted by activation of the noradrenergic system in response to stress.

In summary, the results of these experiments suggest that amplification of the noradrenergic response to stress can recruit the release of galanin in CeA, which then acts to buffer the anxiogenic effects of NE. Thus, the net behavioral response to stress, and the nature of the modulatory influence exerted by the noradrenergic system on behavioral stress reactivity will ultimately depend on the overall level of activation of this system, and the resulting balance between NE and peptide neurotransmission. This balance, in turn, may be subject to regulation or modification as a result of prior stress exposure, regulatory changes in the reactivity or sensitivity of the noradrenergic system, chronic drug treatment, or even genetic variability. Dysregulation of the normal interaction between NE and galanin in the amygdala may thereby contribute to stress-related neuropsychiatric disorders such as depression, post-traumatic stress disorder or other anxiety disorders. Further, the interaction between NE and galanin may represent a possible mechanism by which genetic predisposition may confer a differential vulnerability to stress, and may also represent a novel target for future therapeutic strategies aimed at treating stress-related psychiatric disorders.

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