Pergamon

Pharmacology Biochemistry and Behavior, Vol. 50, No. 4, pp. 655-660, 1995

0091-3057/95 $9.50 + .00

0091-3057(94)00334-3

BRIEF COMMUNICATION

Chronic Social Stress Increases Levels of Preprogalanin mRNA in the Rat Locus Coeruleus

PHILIP V. HOLMES,*[1] D. CAROLINE BLANCHARD,† ROBERT J. BLANCHARD,‡ LINDA S. BRADY§ AND JACQUELINE N. CRAWLEY*

*Section on Behavioral Neuropharmacology, Experimental Therapeutics Branch, and
§Section on Functional Neuroanatomy, Clinical Neuroendocrinology Branch,
National Institute of Mental Health, Bethesda, MD 20892
†Pacific Biomedical Research Center and Department of Anatomy and Reproductive Biology,
J. A. Burns School of Medicine, University of Hawaii 96822
‡Department of Psychology, University of Hawaii 96822

Received 6 April 1994

HOLMES, P. V., D. C. BLANCHARD, R. J. BLANCHARD, L. S. BRADY AND J. N. CRAWLEY. *Chronic social stress increases levels of preprogalanin mRNA in the rat locus coeruleus.* PHARMACOL BIOCHEM BEHAV **50**(4) 655-660, 1995.—Galanin is a 29 amino acid neuropeptide that coexists with norepinephrine in approximately 80% of locus coeruleus (LC) neurons in the rat. The effects of chronic, naturalistic stress on preprogalanin mRNA in the LC was studied. The visible burrow system (VBS) served as the stress paradigm. Long-Evans rats (three males and two females) were housed together in the VBS for 13 days. The males established dominance hierarchies during this period. On day 14, preprogalanin mRNA in the LC was significantly elevated in subordinate rats compared to dominant and control rats, as measured by quantitative in situ hybridization. Levels of mRNA were positively correlated with the number of wounds by day 7 and day 14 and negatively correlated with body weight gain by day 14. These results suggest that the neuropeptide galanin may be overexpressed during chronic stress in rats.

Galanin In situ hybridization Visible burrow system Dominant Subordinate

THE RAT locus coeruleus (LC), which is the primary noradrenergic nucleus innervating the central nervous system (12,25), contains high levels of galanin, a 29 amino acid bioactive peptide (17,22,24,29). Approximately 80% of tyrosine hydroxylase-like immunoreactive neurons in the LC are immunoreactive for galanin (17). In contrast, the extensive coexistence of galanin and norepinephrine observed in LC neurons is not present in other brain stem noradrenergic neurons, such as the A1 and A2 cell groups (22).

Alterations in the activity of LC neurons have been implicated in adaptations to acute and chronic stress [see (15,20,30) for review]. The firing rate of LC neurons immediately increases following foot shock (9), hypovolemic stress (11,30), and morphine withdrawal (1). Tail shock and swim stress cause depletion of brain norepinephrine (15), and morphine withdrawal induces norepinephrine release in terminal fields of LC neurons (10,13). The involvement of galanin in the responses of LC neurons to stress has not been previously demonstrated. Neither osmotic/hypovolemic stress (14) nor swim stress (2) alter preprogalanin mRNA levels in the rat LC. Preprogalanin mRNA levels in the LC are also unchanged after chronic morphine treatment and withdrawal (18). These

[1] Requests for reprints should be addressed to Philip V. Holmes, Section on Behavioral Neuropharmacology, NIMH, Building 10, Room 4N214, Bethesda, MD 20892.

experiments suggest that experimentally induced increases in the firing rate of LC neurons are not sufficient to induce galanin gene expression. However, preprogalanin mRNA levels are increased by reserpine treatment (2,16,21), suggesting that long-term depletion of norepinephrine may induce galanin gene expression.

The present experiments assessed the involvement of galaninergic neurons of the LC in adaptations to chronic stress using visible burrow systems (VBS), a naturalistic, social stress paradigm. When mixed-sex rat groups are housed in chambers with connecting tunnels designed after those constructed by rats in natural environments, the males quickly develop strong dominance hierarchies (3). Subordinate males show strongly increased defensive behaviors (6), weight loss (3), increased levels of basal plasma corticosterone and decreased corticosterone binding globulin (5), reduced mineralocorticoid and glucocorticoid receptor binding in hippocampus (8), and widespread changes in serotonin systems (4,23). These findings indicate that subordinate status is a powerful stressor.

METHOD

Subjects

Subjects were 24 adult male rats of the Long-Evans strain maintained by the University of Hawaii Laboratory Animal Services. Animals were housed in mixed-sex groups (three males and two females per burrow) in the VBS. Control males of the same strain and age were singly housed in standard wire-mesh cages in the same rooms as those in which the VBS were located.

Visible Burrow System

Apparatus. The VBS were constructed of Plexiglas, with black opaque side panels and clear top panels to permit viewing. Bedding material was provided. The VBS consisted of a surface area, with no ceiling, surrounded by a light baffle 125 cm in height. A 15 watt incandescent light was mounted on the inside of the light baffle 56 cm above the surface area, and provided illumination only for the surface area. The chambers, 18 cm in height, had removable lids and were connected to the surface area by tunnels made of clear Plexiglas tubing 8 cm in diameter. The incandescent light was maintained on a 12 L : 12 D cycle and provided the only illumination for the entire apparatus. The dark cycle (lights out) began at 1600 h.

Activity within the VBS was recorded using a videocamera and infrared light source mounted above the apparatus. Thus, activity could be monitored in complete darkness during lights-out peroids. Food and water were freely available in the surface area of the VBS. Food was kept in a feeder that made it difficult for the animals to transfer pellets into the chambers.

Procedure. The subjects were housed in the VBS for 13 days, in two cohorts of four colonies each. The first cohort was established 48 h prior to the second, with day 1 signifying the day the colony was established. On days 1, 3, 7, and 9, a 6 h videorecording was made for each VBS starting at lights out. To ensure adequate access to food and water, all male subjects were removed from the VBS, weighed, and singly housed with free access to food and water in their original home cage for 8 h during the light cycle on days 7, 8, 9, and 10. Any animal less than 80% of initial body weight was given wet mash during these vacations.

In situ hybridization. On day 14, rats were sacrificed, brains were removed, frozen in dry ice, and stored at −70°C. Fifteen micron sections through the LC were cut with a cryostat and thaw-mounted on microscope slides. Approximately every fifth section was stained with 0.1% thionin to determine anatomical location. The region of LC used for in situ hybrid-

TABLE 1

MEASURES FOR DOMINANT AND SUBORDINATE RATS OF EIGHT VISIBLE BURROW SYSTEM COLONY GROUPS (MEAN + SEM)

Measure	Dominants	Subordinates
% Initial weight		
Day 7	97.68 ± 1.07	81.45 ± 1.06*
Day 14	100.34 ± 2.43	76.88 ± 2.06*
% Surface time		
Day 3	75.41 ± 12.03	6.00 ± 2.46*
Day 7	92.46 ± 3.98	3.45 ± 1.82*
Day 9	93.79 ± 3.4	4.29 ± 2.51*
Total wounds/scars		
Day 7	1.29 ± 0.61	17.90 ± 1.12*
Day 14	0.71 ± 0.56	14.70 ± 1.95*
Total offense frequency		
Days 3,7,9	12.86 ± 4.87	2.00 ± 1.89†
Total bite frequency		
Days 3,7,9	7.28 ± 1.46	1.00 ± 0.61*
Total defense frequency		
Days 3,7,9	0.57 ± 0.43	3.00 ± 1.10

*$p < 0.001$; †$p < 0.05$.

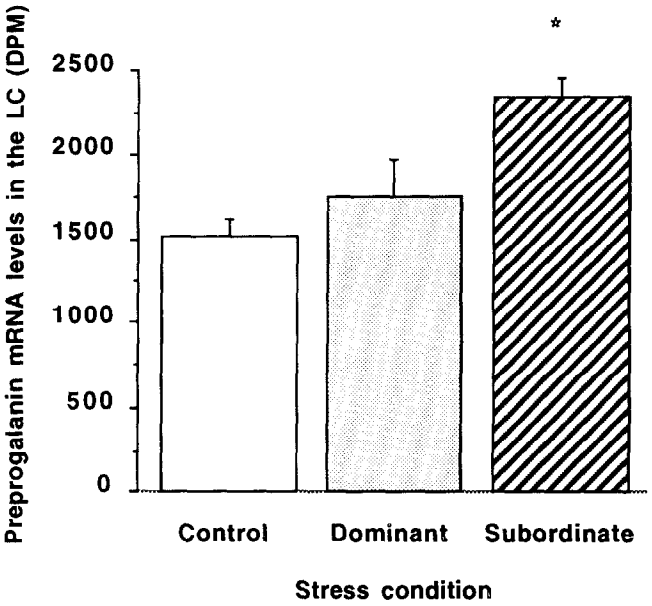

FIG. 1. Effect of chronic social stress on preprogalanin mRNA levels in the rat locus coeruleus. Adult male Long-Evans rats were housed in groups of three along with two females in the VBS for 13 days. Clear dominance relationships occurred during this period. Brains were removed at the end of the experiment and 15 micron sections through the locus coeruleus were hybridized with an [^{35}S]-labeled deoxynucleotide probe complementary to porcine preprogalanin. Data are expressed as mean disintegrations per minute (dpm) ± standard error. *Indicates significantly different from dominant rats and rats in the control group ($p < 0.01$).

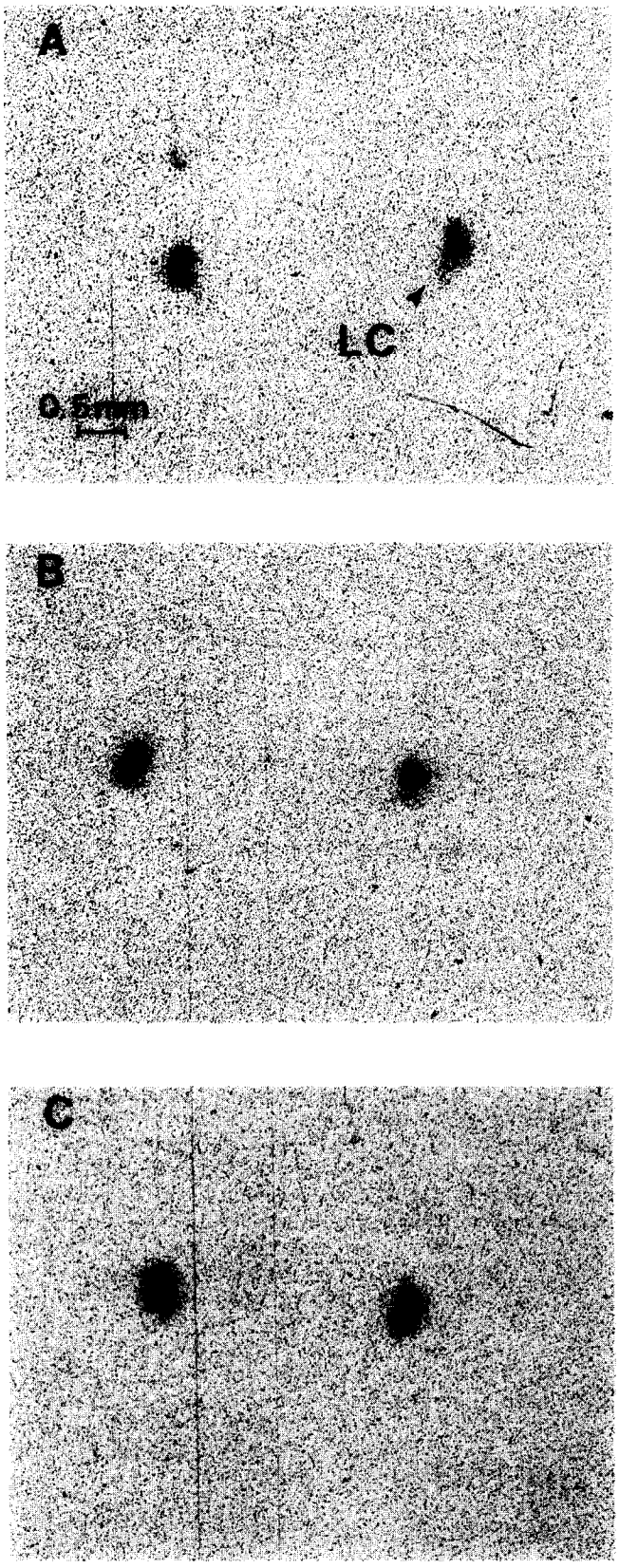

FIG. 2. Respresentative autoradiographs of rat brain sections hybridized with a [^{35}S]-labeled deoxynucleotide probe complementary to porcine preprogalanin. (A) Control; (B) dominant; (C) subordinate.

ization corresponded to 9.7 to 10.00 mm posterior to bregma in the rat brain atlas of Paxinos and Watson (26). The procedures for pretreatment, hybridization, and quantitation were similar to those previously described (2,7). Briefly, sections were fixed in 4% formaldehyde in 0.12 M sodium phosphate-buffered saline for 10 min (PBS, pH 7.4), dehydrated in 70%, 80%, 90%, and 100% ethanol washes for 1 min each, delipidated in chloroform for 5 min, and treated with 0.25% acetic anhydride in 0.1 M triethanolamine/0.9% saline buffer for 10 min to reduce nonspecific binding of probe. The preprogalanin oligonucleotide probe was complementary to bases 115–153 of porcine preprogalanin mRNA (2,27) and was labeled at the 3′ end using terminal deoxynucleotidyl transferase (25 units/ml; Bethesda Research Lab, Gaithersburg, MD), ^{35}S-dATP (1000–1500 mCi/mmol; New England Nuclear, Boston, MA), and tailing buffer. Sections were hybridized with 0.5 $\times$ 10^6 cpm of radiolabeled probe in buffer containing 50% formamide, 4 $\times$ SSC (0.15 M NaCl/0.015 M sodium citrate, pH = 7.2), 500 μg/ml salmon sperm DNA, 250 μg yeast tRNA, 1$\times$ Denhardts solution, and 10% dextran sulfate. Sections were incubated for 20 h in a humid chamber at 37°C. Sections were then washed in 2 $\times$ SSC/50% formamide at 40°C and 1 $\times$ SSC at 20°C. Hybridized sections were exposed to x-ray film (β-max Hyperfilm, Amersham, Arlington Heights, IL) in autoradiographic cassettes for periods of 2, 3, and 5 days. Brain paste standards with known quantities of radioactivity were coexposed with the samples on each film to convert transmittance values to DPM values.

Data Analysis

Determining dominance. Agonistic behaviors were scored from the videotapes. These included offensive behaviors (chase, lateral attack, on top of, and biting), defensive behaviors (flight, on the back), and biting. Additionally, the time spent on the surface area for all male subjects during videotaped periods was also scored. On days 7 and 14, the number and location of all visible wounds was recorded by gently handling the animal and brushing aside the fur with a small brush. The following measurements for all males within each colony were compared: number of wounds, total surface time on days 2 and 5, and percent weight loss (weight prior to sacrifice vs. weight prior to colony formation). If a single subject within a given colony had the highest scores in all three measurements, it was designated the dominant male for that colony. Otherwise, dyadic interactions between colony members were examined, and the subject initiating the most attacks and showing the least defensiveness in dyadic interactions with each of the other colony males was designated the dominant.

Each of the eight colonies showed clear dominance relationships, with one male in each colony meeting all of the above criteria. However, the dominant male of colony 1 died on day 11. A subordinate male had previously died in this colony, and the remaining subordinate showed no change in avoidance of the surface area or weight loss over the next 2 days. This male was treated as a subordinate, albeit in a colony without a dominant.

Quantitation of relative mRNA levels in the locus coeruleus. Quantitation of autoradiographic films was performed using a computerized image analysis system (Image 1.38 software, W. Rasband, NIMH) as previously described (2). Transmittance values of brain paste standards with known radioactivity were fit with a third degree polynomial to convert transmittance values from sections of the LC to disintegrations per minute (DPM) units. These units are not absolute

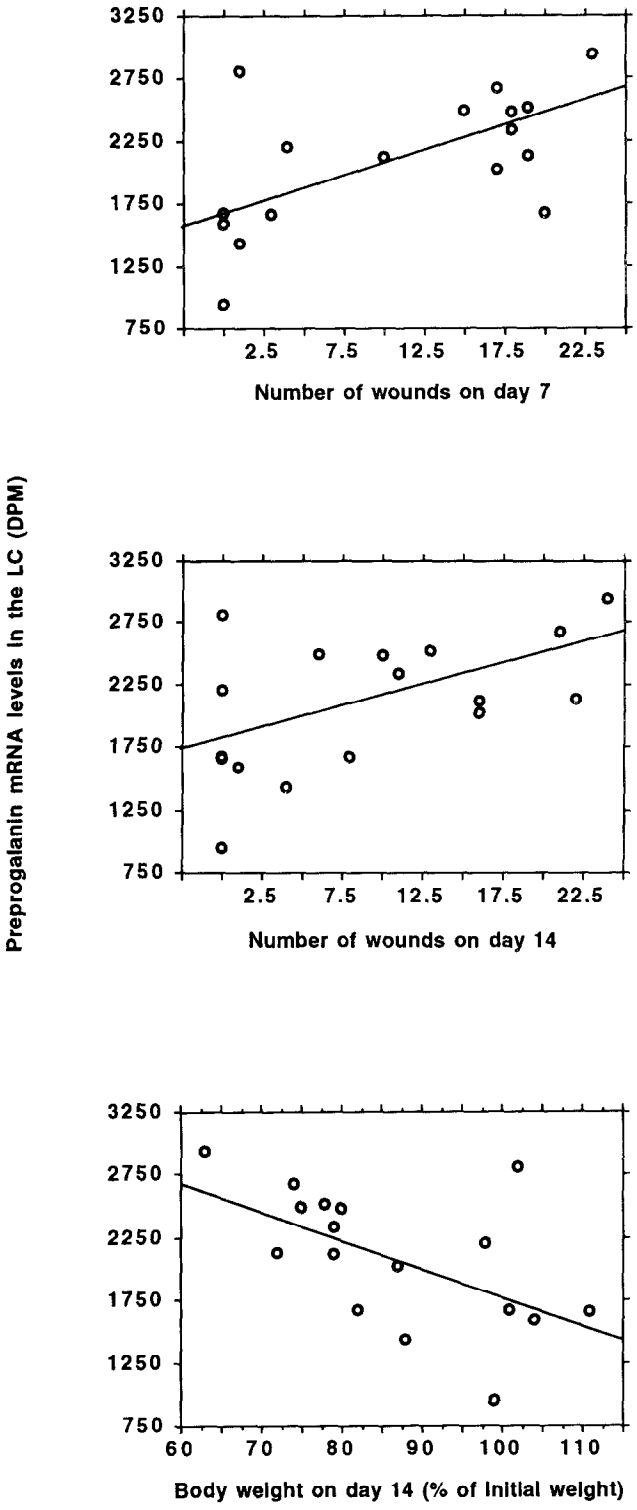

FIG. 3. Scatterplots illustrating the relationship between levels of preprogalanin mRNA in the LC and the number of wounds on day 7 and 14 and change in body weight on day 14 expressed as percentage of initial weight in dominant and subordinate rats. Significant positive correlations were observed between the number of wounds on days 7 and 14 and preprogalanin mRNA levels ($r = 0.586$ and 0.538, respectively; $p < 0.05$). A significant negative correlation was detected between change in body weight on day 14 (percent of initial weight) and preprogalanin mRNA levels ($r = -0.573, p < 0.05$).

measures of mRNA concentrations, but serve as the basis for the comparative analysis of relative mRNA levels between treatment groups.

Statistical Analysis

Measures of weight change, surface time, wounds, offensive behavior, bite frequency, and defensive behavior for dominant and subordinate rats were analyzed with two-sample Student's *t*-tests. Data from the in situ hybridization experiments were analyzed by one-way analyses of variance (ANOVA) and post hoc Student-Newman-Keuls tests with a critical value set at 0.05. Correlation coefficients were calculated to assess the relationship between mRNA levels in the LC and a) change in body weight expressed as the percentage of initial body weight on days 7 and 14; b) the percentage of surface time on days 3, 7, and 9; c) the total number of wounds on days 7 and 14; and d) total offense, bite, and defense frequencies for dominant and subordinate rats.

RESULTS

Visible Burrow System Behavior

Table 1 presents data for weight change, surface times, wounds, and offensive and defensive behaviors for dominant and subordinate rats. As indicated in Table 1, total offense frequency ($p < 0.05$) and total biting ($p < 0.001$) were both reliably higher for dominants. Total defense approached, but failed to reach ($0.1 > p > 0.05$) statistical significance. Significant differences in weight loss ($p < 0.001$), number of wounds ($p < 0.001$), and surface time patterns ($p < 0.001$) were observed between dominants and subordinates.

In Situ Hybridization Analysis of Preprogalanin mRNA in the LC

Figure 1 illustrates the effects of chronic social stress in the VBS on relative levels of preprogalanin mRNA in the LC. One-way ANOVA revealed significant differences in levels of preprogalanin mRNA between dominant, subordinate, and naive rats, $F(2, 20) = 7.9, p < 0.01$. Post hoc Student-Newman-Keuls tests revealed that preprogalanin mRNA levels were significantly higher in subordinate rats than in dominant rats and rats from the control condition. Representative autoradiographs hybridized brain sections from a dominant, subordinate, and control rat are presented in Fig. 2.

Scatterplots for significantly correlated variables are presented in Fig. 3. The number of wounds on days 7 and 14 was significantly correlated with levels of preprogalanin mRNA in the LC in dominant and subordinate rats ($r = 0.586$ and 0.538, respectively; $n = 17, p < 0.05$). The percentage of initial body weight on day 14 was negatively correlated with preprogalanin mRNA levels ($r = -0.573$; $n = 17$, $p < 0.05$). No significant correlations were detected for the percentage of initial body weight on day 7 ($r = -0.118$); the percentage of surface time on day 3 ($r = -0.427$), 7 ($r = -0.054$), and 9 ($r = -0.158$); or the total offense ($r = -0.188$), bite ($r = 0.36$), and defense frequencies ($r = -0.142$) for dominant and subordinate rats.

DISCUSSION

Relative levels of preprogalanin mRNA in the LC were higher in subordinate rats than in dominant rats and controls. These results suggest that the chronic stress associated with subordinate status in the VBS causes increases in galanin gene

expression in the LC. The stress inherent in the VBS is multivariate and dynamic, and precise identification of the stress-related variables responsible for altering preprogalanin mRNA levels was not addressed in this preliminary investigation. However, correlation analyses revealed an association between alterations in mRNA levels and the amount of exposure to painful stimuli (i.e., the number of wounds) as well as an association between mRNA levels and weight loss. Though there are to date no previous studies that have directly examined the effects of either noxious stimulation or body weight loss on preprogalanin mRNA in the rat LC, one previous study has demonstrated that 14 days of food restriction reduces the levels of preprogalanin mRNA in the rat arcuate nucleus (7). The direction of the effect observed in LC neurons in the present study is, thus, opposite that previously observed in hypothalamic neurons.

Previous reports indicate that manipulations that increase the activity of locus coeruleus neurons do not necessarily induce galanin gene expression (2,14,18). Preprogalanin mRNA levels are increased following reserpine treatment (2,16,21) but are not affected by osmotic/hypovolemic stress (14), swim stress (2), or chronic morphine treatment and withdrawal (18). The mechanism for stress-induced alterations in preprogalanin mRNA levels may, therefore, be associated with the noradrenergic depletion reported to occur during chronic stress (15). Alternatively, galanin gene expression may be regulated hormonally. Galaninergic neurons in the LC project to the paraventricular nucleus of the hypothalamus (17,22,28). This anatomy suggests a link between galaninergic systems in the LC and regulation of the hypothalamo-pituitary-adrenal (HPA) axis. Infusions of galanin into the paraventricular nucleus attenuates stress-induced adrenocorticotropic hormone (ACTH) secretion (19). Treating anterior pituitary cells with galanin in vitro has no effect on corticotropin-stimulated ACTH secretion, indicating that the inhibitory action of galanin on ACTH release occurs at the level of the neuroendocrine hypothalamus (19). These findings suggest that galanin may function as an endogenous inhibitory modulator of hypothalamic corticotrophic activity. Increased levels of plasma corticosterone have previously been demonstrated in subordinate rats in the VBS paradigm (5). The increase in preprogalanin mRNA observed in subordinate rats may, thus, be a reflection of compensatory responses to stress-induced activation of the HPA axis.

REFERENCES

1. Aghajanian, G. K. Tolerance of locus coeruleus neurones to morphine and suppression of withdrawal response by clonidine. Nature 276:186–188; 1978.
2. Austin, M. C.; Cottingham, S. L.; Paul, S. M.; Crawley, J. N. Tyrosine hydroxylase and galanin mRNA levels in locus coeruleus neurons are increased following reserpine administration. Synapse 6:351–357; 1990.
3. Blanchard, D. C.; Blanchard, R. J. Behavioral correlates of chronic dominance-subordination relationships of male rats in a seminatural situation. Neurosci. Biobehav. Rev. 14:455–462; 1990.
4. Blanchard, D. C.; Cholvanich, P.; Blanchard, R. J.; Clow, D. W.; Hammer, R. P.; Rowlett, J. K.; Bardo, M. T. Serotonin, but not dopamine, metabolites are increased in selected brain regions of subordinate male rats in a colony environment. Brain Res. 568: 61–66; 1991.
5. Blanchard, D. C.; Spencer, R.; Weiss, S. M.; Blanchard, R. J.; McEwen, B. S.; Sakai, R. R. The visible burrow system as a model of chronic social stress: Behavioral and neuroendocrine correlates. Psychoneuroendocrinology 20:117–134; 1995.
6. Blanchard, R. J.; Blanchard, D. C. Anti-predator defensive behaviors in a visible burrow system. J. Comp. Psychol. 103:70–82; 1989.
7. Brady, L. S.; Smith, M. A.; Gold, P. W.; Herkenham, M. Altered expression of hypothalamic neuropeptide mRNAs in food-restricted and food-deprived rats. Neuroendocrinology 57:441–447; 1990.
8. Chao, H. M.; Blanchard, D. C.; Blanchard, R. J.; McEwen, B. S.; Sakai, R. M. The effect of social stress on hippocampal gene expression. Mol. Cell. Neurosci. 4:543–548; 1993.
9. Chiang, C.; Aston-Jones,G. Response of locus coeruleus neurons to foot shock stimulation is mediated by neurons in the rostral ventral medulla. Neuroscience 53:705–715; 1993.
10. Crawley, J. N.; Laverty, R.; Roth, R. H. Clonidine reversal of increased norepinephrine metabolite levels during morphine withdrawal. Eur. J. Pharmacol. 57:247–250; 1979.
11. Curtis, A. L.; Drolet, G.; Valentino, R. J. Hemodynamic stress activates LC neurons of unanesthetized rats. Brain Res Bull. 31: 737–744; 1993.
12. Dahlstrom, A.; Fuxe, K. Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies on brain stem neurons. Acta Physiol. Scand. 62(Suppl. 232):1–55; 1964.
13. Done, C.; Silverstone, P.; Sharp, T. Effect of naloxone-precipitated withdrawal on noradrenaline release in rat hippocampus in vivo. Eur. J. Pharmacol. 215:333–336; 1992.
14. Foster, O. J. F.; Chowdrey, H. S.; Larsen, P. J.; Lightman, S. L. Differential regulation of tyrosine hydroxylase, neuropeptide Y and galanin gene expression in the pons and medulla oblongata following chronic oral administration of 2% saline: A combined in situ hybridization and immunohistochemical study. Neuroendocrinology 55:544–551; 1992.
15. Glavin, G. B. Stress and brain noradrenaline: A review. Neurosci. Biobehav. Rev. 9:233–243; 1985.
16. Gundlach, A. L.; Wisden, W.; Morris, B. J.; Hunt, S. P. Localization of preprogalanin mRNA in the rat brain: In situ hybridization study with a synthetic oligonucleotide probe. Neurosci. Lett. 114:241–247; 1990.
17. Holets, V. R.; Hökfelt, T.; Rokaeus, Å.; Terenius, L.; Goldstein, M. Locus coeruleus neurons in the rat containing neuropeptide Y, tyrosine hydroxylase or galanin and their efferent projections to the spinal cord, cerebral cortex and hypothalamus. Neuroscience 24:893–906; 1988.
18. Holmes, P. V.; de Bartolomeis, A.; Koprivica, V.; Crawley, J. N. Lack of effect of chronic morphine treatment and naloxone-precipitated withdrawal on tyrosine hydroxylase, galanin, and neuropeptide Y mRNA levels in the rat locus coeruleus. Synapse (in press).
19. Hooi, S. C.; Maiter, D. M.; Martin, J. B.; Koenig, J. I. Galaninergic mechanisms are involved in the regulation of corticotropin and thyrotropin secretion in the rat. Endocrinology 127:2281–2288; 1990.
20. Jacobs, B. L. Single unit activity of locus coeruleus neurons in behaving animals. Prog. Neurobiol. 27:183–194; 1986.
21. Kadowaki, K.; Emson, P. C. Increase in galanin gene expression in locus coeruleus neurones of the rat following reserpine treatment. Mol. Brain Res. 15:156–160; 1992.
22. Levin, M. C.; Sawchenko, P. E.; Howe, P. R. C.; Bloom, S. R.; Polak, J. M. Organization of galanin-immunoreactive inputs to the paraventricular nucleus with special reference to their relationship to catecholaminergic afferents. J. Comp. Neurol. 261: 562–582; 1987.
23. McKittrick, C. R.; Blanchard, D. C.; Blanchard, R. J.; McEwen, B. S.; Sakai, R. R. Serotonin receptor binding in a colony model of chronic social stress. Biol. Psychiatry (in press).
24. Melander, T.; Hökfelt, T.; Rokaeus, Å. Distribution of galanin-

like immunoreactivity in the rat central nervous system. J. Comp. Neurol. 248:475–517; 1986.
25. Moore, R. Y.; Bloom, F. E. Central catecholamine neuron systems: Anatomy and physiology of the norepinephrine and epinephrine systems. Annu. Rev. Neurosci. 2:113–168; 1979.
26. Paxinos, G.; Watson, C. The rat brain in stereotaxic coordinates. 2nd ed. San Diego: Academic Press; 1986.
27. Rökaeus, Å.; Brownstein, M. J. Construction of a porcine adrenal medullary cDNA library and nucleotide sequence analysis of two clones encoding a galanin precursor. Proc. Natl. Acad. Sci. USA 83:6287–6291; 1986.
28. Sawchenko, P. E.; Pfeiffer, S. W. Ultrastructural localization of neuropeptide Y and galanin immunoreactivity in the paraventricular nucleus of the hypothalamus in the rat. Brain Res. 474:231–245; 1988.
29. Skofitsch, G.; Jacobowitz, D. M. Immunocytochemical mapping of preprogalanin-like neurons in the rat central nervous system. Peptides 6:509–546; 1985.
30. Svensson, T. H. Peripheral, autonomic regulation of locus coeruleus noradrenergic neurons in brain: Putative implications for psychiatry and psychopharmacology. Psychopharmacology (Berlin) 92:1–7; 1987.