Pergamon

Pharmacology Biochemistry and Behavior, Vol. 51, No. 4, pp. 675-680, 1995

0091-3057/95 $9.50 + .00

0091-3057(94)00436-6

Analgesia Produced by Immobilization Stress and an Enkephalinase Inhibitor in Amphibians

CRAIG W. STEVENS,[1] SONI SANGHA AND BRIAN G. OGG

Department of Pharmacology and Physiology, Oklahoma State University, College of Osteopathic Medicine, Tulsa, OK

Received 3 June 1994

STEVENS, C. W., S. SANGHA AND B. G. OGG. *Analgesia produced by immobilization stress and an enkephalinase inhibitor in amphibians.* PHARMACOL BIOCHEM BEHAV 51(4) 675-680, 1995.—The role of endogenous opioids in modulating pain transmission in amphibians was examined by two methods known to activate endogenous opioids in mammals. Analgesia was assessed using the acetic acid test in the Northern grass frog, *Rana pipiens*. One or 2 h of immobilization produced a significant analgesia lasting for at least 90 min. Systemic, but not spinal, administration of naloxone before immobilization prevented the analgesic effects seen in saline-pretreated controls. Spinal administration of the enkephalinase inhibitor, thiorphan, but not bestatin (both at 100 nmol/frog), produced significant analgesia. The analgesic effect of thiorphan was blocked by coadministration of intraspinal naloxone. These data are the first to suggest a role for endogenous opioid modulation of noxious stimuli in lower vertebrates by examination of stress-induced analgesia and the action of agents that inhibit enkephalin degradation.

Amphibians Acetic acid test Stress-induced analgesia Thiorphan Bestatin Alternative model Enkephalin Naloxone

THE ROLE of endogenous opioid peptides in modulating pain transmission is amenable to investigation by using various experimental methods that release or prolong the activity of endogenous opioids in the central nervous system. Stress-induced analgesia is mediated by opioid or nonopioid mechanisms; however, restraint or immobilization stress in mammals stimulates the release of endogenous opioids from brain and peripheral stores (11) and induces a mild analgesia as measured on the hot plate or by tail flick (2,4,5,12). Pharmacological studies confirm the activation of the endogenous opioid system as pretreatment with the opioid antagonist naloxone blocks the analgesia produced by immobilization stress in mice (1,13).

Another method to elucidate the role of endogenous opioid peptides in pain transmission is by the administration of agents that inhibit the enzymes responsible for the breakdown of the endogenous opioid peptides. The two predominant enzymes localized in mammalian brain tissue that perform this function, aminopeptidase N and enkephalinase (a.k.a. neutral endopeptidase), have been fully characterized and a variety of pharmacological agents have been developed to inhibit their activity (22). The first of the enkephalinase inhibitors synthesized was thiorphan, which blocks the cleavage of the Gly^3-Phe^4 of Met- and Leu-enkephalin and produces naloxone-sensitive analgesia on the hot plate jump test after central and peripheral administration to mice (21). A second agent, bestatin, inhibits the aminopeptidase and thus blocks the cleavage of the enkephalins at the terminus between the Tyr^1-Gly^2 bond (10). Bestatin has also been shown to produce naloxone-sensitive analgesia after intraventricular administration in rodents (6,7).

We have developed an alternative, nonmammalian model for pain and analgesia research based upon the behavioral response of the Northern grass frog, *Rana pipiens*, to the application of dilute acetic acid (27). Previous results using the acetic acid test demonstrated that amphibians exhibit dose-dependent and naloxone-sensitive analgesia following systemic administration of morphine and other opioids (16, 19,29,32) and that opioid tolerance occurs after daily morphine administration (28). Additionally, direct administration of opioids to the frog spinal cord by intraspinal injection produced a potent and dose-dependent analgesia as shown following administration of morphine and other alkaloids (30,31), Met-enkephalin, β-endorphin, and dynorphin (33), and highly

[1] Requests for reprints should be addressed to Craig W. Stevens, Associate Professor of Pharmacology, OSU-COM, 1111 West 17th Street, Tulsa, OK 74107-1898. Email: stevens@vms.ocom.okstate.edu

selective opioid agonists (26). Finally, amphibians appear to possess a well-developed endogenous opioid system, replete with high concentrations of opioid binding sites and endogenous opioid peptides (25). Although the above studies suggest that the general mechanism of opioid action in amphibians may be similar to that observed in humans and other mammalian species, there has not been a systematic examination of stress-induced analgesia and the effect of the inhibition of enkephalin-degrading enzymes in any nonmammalian model. In a previous study of stress-induced analgesia in amphibians, Pezalla showed that immobilization produced a significant rise in the pain threshold, which was blocked by naloxone, and that tolerance developed to the daily immobilization sessions (18).

In the present study, we sought to confirm and extend investigations of endogenous opioid peptides and pain processing in amphibians by two methods. Firstly, we assessed the time course and peak effects of immobilization-induced analgesia on the acetic acid test and the effect of pretreatment with the opioid antagonist naloxone after systemic and spinal administration. Secondly, we examined the analgesic effects of spinally administered thiorphan or bestatin and the effect of opioid antagonism by coadministration of naloxone and thiorphan. The present results confirm the opioid nature of immobilization stress in amphibians and show, for the first time in a nonmammalian species, the analgesic effects of the enkephalinase inhibitor, thiorphan.

METHOD

Animals

Northern grass frogs, *Rana pipiens* (a.k.a. leopard frogs, mean weight, 32.0 g), were obtained from a commercial supplier (NASCO, Fort Atkinson, WI) during the months of March through July. Frogs were held in flow-through, stainless steel aquaria in groups of 48 immediately after arrival and fed live crickets three times a week. At least 2 days prior to their use in any experiment animals were transferred to the laboratory and placed in individual plastic cages (22 L × 16 W × 14 cm H) with mesh tops and sufficient tapwater. On experimental days, water was first adjusted to a depth of 0.5 cm (80 ml) to expose the dorsum of the thigh for acetic acid testing (see below) and 2 h later baseline pain thresholds (PT) were obtained.

The Acetic Acid Test

The acetic acid test to estimate the pain threshold (PT) in frogs was recently elaborated (27). Briefly, 10 solutions of acetic acid were serially diluted (1 : 2, water : acid) from glacial acetic acid (17.5 M) and given a code number from 0 to 10 with the lowest code number equal to the lowest concentration of acetic acid. Code numbers were related to the actual concentration of acetic acid by the expression: log M = 0.1716 (code number) − 0.5186. Testing was done by placing, with a Pasteur pipette, a single drop of acid on the dorsal surface of the frog's thigh. Testing began with the lowest concentration of acid and proceeded with increasing concentrations until the PT was reached. The PT was defined as the code number of the lowest concentration of acid that caused the frog to vigorously wipe the treated leg with either hindlimb. To prevent tissue damage, the acetic acid was immediately wiped off with a gentle stream of distilled water once the animal responded or after 5 s if the animal failed to respond. In the latter case, testing continued on the opposite hindlimb with the next higher concentration. An animal that failed to respond to the highest concentration (#10, glacial acetic acid) was assigned the cutoff value of 11. The baseline PT did not differ among the different treatment groups ($p > 0.05$, one-way ANOVA) and saline-treated controls did not show a significant increase in PT from baseline values at any time after treatment ($p > 0.05$, t-test, data not shown).

Immobilization Studies

Animals were randomly assigned to stress and control cohorts in groups of six. After baseline PT determination, immobilized animals were wrapped tightly in rubber sink mesh (fashioned into bags with wire closures) and placed back into individual cages for 1, 2, or 3 h. Controls were handled for the same period of time that it took to place an animal into the mesh bag and were placed back into individual cages. After the elapsed time of immobilization, experimental animals were freed from the mesh bags and control animals were again handled, and posttreatment PT was determined at 5, 30, and 90 min.

For opioid antagonist experiments, naloxone hydrochloride (a generous gift from Dupont-Merck, NC) was given by systemic (100 nmol/g in a volume of 10 μl/g) or intraspinal (100 nmol/frog in a volume of 5 μl) administration after baseline PT determination and 1 h before treatment on immobilized or handled-only control groups. Corresponding saline-administered controls were also included to evaluate the effects of the injection and naloxone on the acetic acid test (N = 6–12 for all treatment groups and animals were used only once). Systemic administration was done by SC injection into the dorsal lymph sac with a tuberculin syringe. Details of the technique of intraspinal administration in the frog have been previously published (30).

Enzyme Inhibitor Studies

Thiorphan and bestatin were obtained from commercial sources (Sigma Chemical Co., St. Louis, MO) and mixed in sterile 0.9% saline to yield nanomolar doses (both given by intraspinal administration at 100 nmol/frog). For opioid antagonist experiments, naloxone was given at a dose of 100 nmol/frog concurrent with the enkephalinase inhibitor. Treatment began after determination of baseline PT, and posttreatment measures were obtained at 30, 120, and 240 min after administration. Saline control and naloxone alone groups were corun (N = 6–12 for all treatment groups and animals were used only once).

Data Analysis and Statistics

Data was converted from the raw code numbers (PT) to maximum percent effect (MPE) by the following formula:

$$\text{MPE} = \frac{\text{Posttreatment PT} - \text{Baseline PT}}{\text{Cut-off value (11)} - \text{Baseline PT}} \times 100$$

Data for the time course curves were plotted as the mean of each animal's individual MPE at each time point. Peak MPE across the time course data for each individual animal was pooled for each treatment and plotted as bar graphs for statistical comparisons. MPE data were entered into a microcomputer program (PHARM/PCS v.4, MicroComputer Specialists, Philadelphia, PA) for statistical comparisons using the Student's t-test, one-way ANOVA, and Newman-Keuls post hoc test. Significant effects were considered at the $p <$ 0.05 level.

RESULTS

Immobilization Stress

As shown in Fig. 1, immobilization of animals for 1 or 2 h produced a significant and long-lasting analgesia that persisted for at least 90 min after release from restraints. The peak analgesic effects (inset graph, Fig. 1) of both treatment groups were significantly different from the control group, with peak MPE values of 7.2 ± 3.0, 25.6 ± 4.4, and 50.6 ± 13.0 for control, 1-h, and 2-h restraint groups, respectively (mean + SEM, see legend for statistical tests). Immobilization for 2 h produced a greater analgesic effect than 1-h immobilization, but immobilization for 3 h did not produce a greater effect than that produced by 2 h of immobilization (data not shown).

Saline or naloxone given by systemic or spinal routes of administration did not produce any change in the PT in handled-only control groups (Fig. 2, open bars). Systemic naloxone pretreatment (100 nmol/g) significantly blocked the analgesia produced by 2 h of immobilization compared to saline-pretreated immobilized animals (SAL-SC, 41.9 ± 11.4 vs. NAL-SC, 11.6 ± 2.9). In contrast, naloxone pretreatment by spinal administration (100 nmol/frog) did not block the analgesic effects of immobilization seen in the saline-pretreated immobilized animals (SAL-IS, 33.6 ± 8.1 vs. NAL-IS, 30.2 ± 16.5).

Administration of Enzyme Inhibitors

As shown in Fig. 3, the spinal administration of thiorphan (100 nmol/frog), but not bestatin (100 nmol/frog), produced a long-lasting but mild analgesic effect that persisted for at least 240 min. The peak analgesic effect of the thiorphan group was significantly greater than that observed in the saline-administered controls (THIOR, 33.5 ± 7.7 vs. SAL, 6.7 ± 4.9) whereas the bestatin group showed a trend towards increased pain thresholds but did not reach significance (15.4 ± 4.4, see inset graph, Fig. 3). In separate groups of animals, the spinal administration of saline or naloxone (100 nmol/frog) did not produce any change from baseline PT (Fig. 4, open bars). The administration of thiorphan produced a significant analgesic effect compared to saline controls (THIOR, 35.1 ± 10.9 vs. SAL, 10.1 ± 4.5). The coadministration of

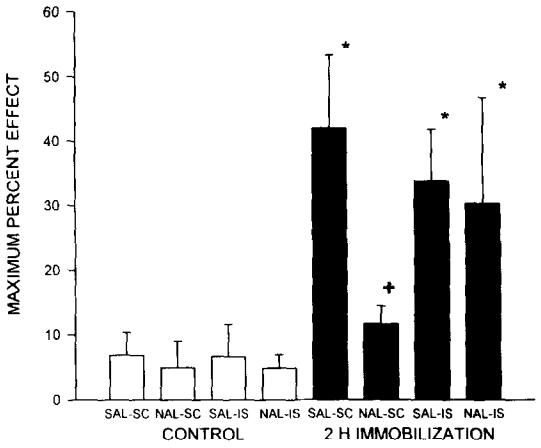

FIG. 2. Peak analgesia following 2h immobilization with antecedent administration of saline or naloxone (100 nmol/frog, intraspinal; 100 nmol/g, subcutaneous). Naloxone or saline pretreatment by either route was given after baseline pain thresholds were obtained and 1 h before immobilization or handling. Data plotted as mean + SEM MPE for peak analgesic responses from individual animals across the time course presented in Fig. 1. Open bars are handled-only controls, closed bars 2h immobilized groups. Group identification given under bars whereby SAL = saline, NAL = naloxone pretreatment, and SC = subcutaneous and IS = intraspinal administration. N = 4–6 animals per treatment group. *Significant difference from corresponding handled-only control group; + significant difference from 1h immobilized group (one-way ANOVA followed by Newman-Keuls, $p < 0.05$).

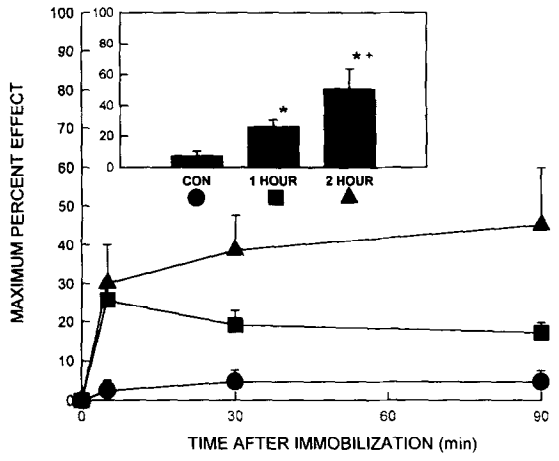

FIG. 1. Time course of analgesia following 1- or 2-h immobilization stress in amphibians. Animals were restrained in rubber sink mesh as described and assessed for pain thresholds on the acetic acid test at 5, 30, and 90 min after release. Control animals were handled only but not restrained. Data plotted as mean + SEM MPE for N = 6–8 animals per treatment group. Inset graph shows the mean + SEM MPE for each group across the time course curve. *Significant difference from control group; + significant difference from 1h immobilized group (one-way ANOVA followed by Newman-Keuls, $p < 0.05$).

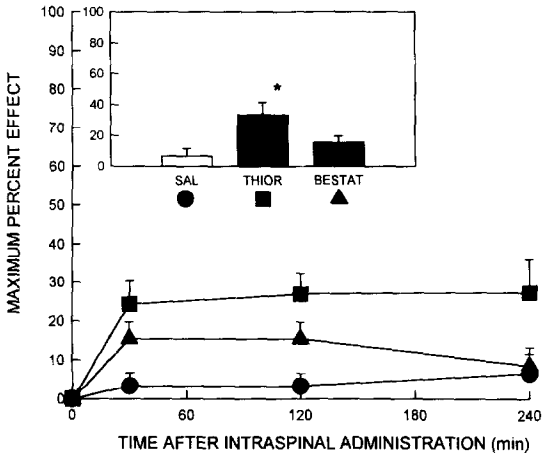

FIG. 3. Time course of the analgesic effects following intraspinal administration of thiorphan (100 nmol/frog), bestatin (100 nmol/frog), or saline. Data plotted as plotted as mean + SEM MPE for N = 6–8 animals per treatment group. Inset graph shows the mean MPE for each group across the time course. *Significant difference from the saline-injected control group (one-way ANOVA followed by Newman-Keuls, $p < 0.05$).

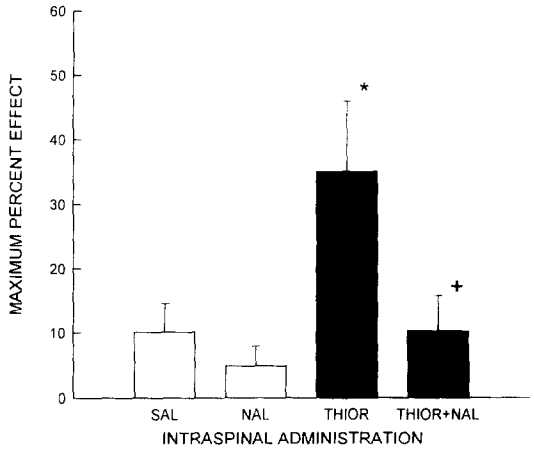

FIG. 4. Peak analgesia following spinal administration of saline, naloxone (100 nmol/frog), thiorphan (100 nmol/frog), or thiorphan plus naloxone (both at 100 nmol/frog). Data are plotted as mean + SEM MPE for peak analgesic responses from individual animals across the time course presented in Fig. 3. Open bars are the saline and naloxone controls and closed bars are the treatment groups. Group identification given under bars whereby SAL = saline, NAL = naloxone, THIOR = thiorphan, and THIOR + NAL = thiorphan plus naloxone. N = 4–6 animals per treatment group. *Significant difference from saline-injected control group; + Significant difference from thiorphan alone group (one-way ANOVA followed by Newman-Keuls, $p < 0.05$).

thiorphan and naloxone significantly blocked the analgesic effects of thiorphan administered alone (THIOR + NAL, 10.2 ± 5.6).

DISCUSSION

The present study is the first to examine the role of endogenous opioids in a nonmammalian species by the spinal administration of inhibitors of enkephalin degradation and confirms and extends previous studies of stress-analgesia produced by immobilization in amphibians (18). The acetic acid test reliably estimates the pain threshold in amphibians and has been used previously for studies of exogenously administered opioids and morphine tolerance in *Rana pipiens* (see Introduction).

Immobilization Stress-Induced Analgesia

In mammals, restraint or immobilization evokes a stress response characterized by the release of catecholamine and hormones from peripheral and central stores (11,20,34). Studies of immobilization or restraint in rodent models showed that this form of stress induces an analgesia that is mediated by endogenous opioids, as analgesic effects were blocked by pretreatment with opioid antagonists (2,4,5,12). The first study of stress-induced analgesia in amphibians suggested a role for endogenous opioids in immobilization stress (18). In this earlier study, immobilization stress induced a mild analgesia, which was blocked but not reversed by naloxone, and displayed a decremental effect (i.e., tolerance) after daily immobilization. In the present study, the observed stress-induced analgesia was "dose dependent" in that 2 h of immobilization produced a greater degree of analgesia than 1 h of restraint. However, 3 h of immobilization did not produce greater degree of analgesia, suggesting a saturating mechanism that might be expected if the analgesic effects of immobilization stress were mediated by the release of endogenous opioid peptides. Naloxone administered systemically, but not after spinal administration, significantly blocked the analgesic effects of immobilization stress. It is unlikely that the intraspinal dose of naloxone was too low to block opioid receptors as intraspinal naloxone doses of the same magnitude completely block the analgesic effects of exogenous opioids given by the spinal route (26,30). However, further studies are clearly needed to more precisely determine the mechanisms of restraint stress in frogs.

Inhibitors of Enkephalin Degradation

Central administration of the enkephalinase inhibitor, thiorphan, and the aminopeptidase inhibitor, bestatin, produce analgesia following intraventricular administration in rodents (6,7,21). After spinal administration, thiorphan produced a significant analgesia as measured on the hot plate test, but not on the tail flick test (35). As suggested by these authors and elsewhere (7,24), the inhibition of enkephalinase and/or aminopeptidase enzymes is most likely to result in significant analgesia on behavioral tests that use a mild, prolonged noxious stimulus rather than an intense, short stimulus. In the present study, intraspinal administration of a high dose of thiorphan, but not that of bestatin, produced significant analgesia that was blocked by coadministration of naloxone. Newer enzyme-inhibiting agents, such as kelatorphan (9) and the prodrug RB101 (15), inhibit both enzymes responsible for enkephalin degradation and result in a greater degree of analgesia than either thiorphan or bestatin given alone. In this case, the nature of the nociceptive test used to measure the analgesic effects of the mixed peptidase inhibitors does not seem to be as critical as with thiorphan or bestatin alone, and the activity profile mimics morphine in all behavioral tests of analgesia (22,23). Clearly studies with these newer inhibitors are needed to further examine the efficacy of enzyme inhibition in amphibians.

Endogenous Opioid Peptides Mediating Stress and Thiorphan Analgesia in Amphibians

With regard to the opioid peptide potentiated by the spinal administration of thiorphan, it is likely that it is the action of Met-enkephalin or possibly the extended heptapeptide Met-enkephalin-Arg-Phe producing the observed analgesia. Although spinal administration of Met-enkephalin, dynorphin A(1-13), or β-endorphin produce potent and dose-dependent analgesia in amphibians (33), the efficiency of enkephalinase in cleaving the Gly^3-Phe^4 bond is greatly diminished with carboxy-extended sequences as found in dynorphin or endorphin sequences (22). Leu-enkephalin and Met-enkephalin are both encoded on the proenkephalin gene in mammals, but the proenkephalin gene sequence in the African clawed frog (*Xenopus laevis*) contains only sequences for the Met-enkephalin and Met-enkephalin-extended products (14). This finding, along with consistent radioimmunoassay and immunohistochemical data from other amphibian species, suggests that anurans such as *Rana pipiens* do not express Leu-enkephalin per se, but only as part of dynorphin products (8). Thus, thiorphan effects after spinal administration in amphibians are most likely mediated solely by Met-enkephalin and not by both Met- and Leu-enkephalin, as might be expected in mammalian species.

Finally, it should be noted that an early study using the same amphibian model also showed that amphibians, like

mammals, can be exposed to a stressor that produces analgesic effects that are not blocked by naloxone (17). It is possible that descending serotonergic or adrenergic pathways terminating in the spinal cord of amphibians may play a role in stress analgesia that is not opioid in nature. In this regard, we have also observed that systemic and spinal administration of alpha-adrenergic agents in amphibians produces a potent and dose-dependent analgesia, which is blocked by selective alpha$_2$-adrenergic antagonists [(3); unpublished observations]. Thus, both opioid and nonopioid systems in the amphibian brain and spinal cord appear to be involved in pain modulation and, as demonstrated in mammalian models, may be differentially activated depending upon the particular stressor.

In conclusion, the present results suggest that studies of stress-induced analgesia may not be limited to mammals and that amphibians, and perhaps all vertebrates [see (25,27)], may share common mechanisms for the activation of endogenous opioid systems. The finding that the enkephalinase inhibitor thiorphan produces naloxone-sensitive analgesia after spinal administration in amphibians also confirms and extends previous results using mammalian species. In general, these data further support the development of nonmammalian models for opioid and pain research with a particular emphasis on the amphibian model described herein.

ACKNOWLEDGEMENTS

Much appreciation to the technical assistance of Ms. Lisa Deason. Brian Ogg was supported by a Medical Student Research Award from the Oklahoma Osteopathic Association, and Soni Sangha was a visiting researcher from the University of Tulsa. This research was supported by NIH grant (R29 DA07326) and a research grant from the Whitehall Foundation.

REFERENCES

1. Amir, S.; Amit, Z. Endogenous opioid ligands may mediate stress-induced changes in the affective properties of pain related behavior in rats. Life Sci. 23:1143–1152; 1978.
2. Appelbaum, B. D.; Holtzman, S. G. Characterization of stress-induced potentiation of opioid effects. J. Pharmacol. Exp. Ther. 231:555–565; 1984.
3. Brenner, G. M.; Deason, L. L.; Klopp, A. J.; Stevens, C. W. Alpha2 analgesia after systemic administration in amphibians. J. Pharmacol. Exp. Ther. 270:540–545; 1994.
4. Calcagnetti, D. J.; Holtzman, S. G. Factors affecting restraint stress-induced potentiation of morphine analgesia. Brain Res. 537:157–162; 1990.
5. Calcagnetti, D. J.; Holtzman, S. G. Potentiation of morphine analgesia in rats given a single exposure to restraint stress immobilization. Pharmacol. Biochem. Behav. 41:449–453; 1992.
6. Chaillet, P.; Marcais-Collado, H.; Costentin, J.; Yi, C. C.; De La Baume, S.; Schwartz, J. C. Inhibition of enkephalin metabolism by, and antinociception of, bestatin, an aminopeptidase inhibitor. Eur. J. Pharmacol. 86:329–336; 1983.
7. Costentin, J.; Vlaiculescu, A.; Chaillet, P.; Natan, L. B.; Aveaux, D.; Schwartz, J. C. Disassociated effects of inhibitors of enkephalin-metabolising peptidases or naloxone on various nociceptive responses. Eur. J. Pharmacol. 123:37–44; 1986.
8. Dores, R. M.; McDonald, L. K.; Steveson, T. C.; Sei, C. A. The molecular evolution of neuropeptides: Prospects for the 90's. Brain Behav. Evol. 36:80–99; 1990.
9. Fournie-Zaluski, M.-C.; Chaillet, P.; Bouboutou, R.; Coulaud, A.; Cherot, P.; Waksman, G.; Costentin, J.; Roques, B. P. Analgesic effects of kelatorphan, a new highly potent inhibitor of multiple enkephalin degrading enzymes. Eur. J. Pharmacol. 102: 525–528; 1984.
10. Hui, K. S.; Gioannini, T.; Hui, M.; Simon, J.; Lajtha, A. An opiate receptor-associated aminopeptidase that degrades enkephalins. Neurochem. Res. 10:1047–1058; 1985.
11. Kalin, N. H.; Carnes, M.; Barksdale, C. M.; Shelton, S. E.; Stewart, R. D.; Risch, S. C. Effects of acute behavioral stress on plasma and cerebrospinal fluid ACTH and beta endorphin in rhesus monkeys. Neuroendocrinology 40:97–101; 1985.
12. Keim, K. L.; Bigg, E. B. Physiological and biochemical concomitants of restraint stress in rats. Pharmacol. Biochem. Behav. 4: 289–297; 1976.
13. Kulkarni, S. K. Heat and other physiological stress-induced analgesia: Catecholamine mediated and naloxone reversible response. Life Sci. 27:185–188; 1980.
14. Martens, G. J. M.; Herbert, E. Polymorphism and absence of leu-enkephalin sequences in proenkephalin genes in *Xenopus laevis*. Nature 310:251–254; 1984.
15. Noble, F.; Soleilhac, J. M.; Soroca-Lucas, E.; Turcaud, S.; Fournie-Zaluski, M.-C.; Roques, B. P. Inhibition of the enkephalin-metabolizing enzymes by the first systemically active inhibitor, prodrug RB 101, induces potent analgesic responses in mice and rats. J. Pharmacol. Exp. Ther. 261:181–190; 1992.
16. Pezalla, P. D. Morphine-induced analgesia and explosive motor behavior in an amphibian. Brain Res. 273:297–305; 1983.
17. Pezalla, P. D. Stress induced analgesia in frogs: A naloxone insensitive system. Brain Res. 278:354–358; 1983.
18. Pezalla, P. D.; Dicig, M. Stress-induced analgesia in frogs: Evidence for the involvement of an opioid system. Brain Res. 296: 356–360; 1984.
19. Pezalla, P. D.; Stevens, C. W. Behavioral effects of morphine, levorphanol, dextrorphan and naloxone in the frog *Rana pipiens*. Pharmacol. Biochem. Behav. 21:213–217; 1984.
20. Pol, O.; Campmany, L.; Gil, M.; Armario, A. Behavioral and neurochemical changes in response to acute stressors: influence of previous chronic exposure to immobilization. Pharmacol. Biochem. Behav. 42:407–412; 1992.
21. Roques, B. P.; Fournie-Zaluski, M.-C.; Soroca, E.; Lecomte, J. M.; Malfroy, B.; Llorens, C.; Schwartz, J. C. The enkephalinase inhibitor thiorphan shows antinociceptive activity in mice. Nature 288:286–288; 1980.
22. Roques, B. P.; Noble, F.; Dauge, V.; Fournie-Zaluski, M.-C.; Beaumont, A. Neural endopeptidase 24.11: Structure, inhibition, and experimental and clinical pharmacology. Pharmacol. Rev. 45:87–146; 1993.
23. Schmidt, C.; Peyroux, J.; Noble, F.; Fournie-Zaluski, M.-C.; Roques, B. P. Analgesic responses elicited by endogenous enkephalins (protected by mixed peptidase inhibitors) on a variety of morphine-sensitive noxious tests. Eur. J. Pharmacol. 192:253–262; 1991.
24. Schwartz, J. C.; Costentin, J.; Lecomte, J. M. Pharmacology of enkephalinase inhibitors. Trends Pharmacol. Sci. 472–476; 1985.
25. Stevens, C. W. Opioid antinociception in amphibians. Brain Res. Bull. 21:959–962; 1988.
26. Stevens, C. W. Spinal analgesia in frogs: Studies with highly selective opioid agents. Soc. Neurosci. Abstr. 17:110; 1991.
27. Stevens, C. W. Alternatives to the use of mammals for pain research. Life Sci. 50:901–912; 1992.
28. Stevens, C. W.; Kirkendall, K. Time course and magnitude of tolerance to the analgesic effects of systemic morphine in amphibians. Life Sci. 52:PL111–116; 1993.
29. Stevens, C. W.; Klopp, A. J.; Facello, J. A. Analgesic potency of mu and kappa opioids after systemic administration in amphibians. J. Pharmacol. Exp. Ther. 269:1086–1093; 1994.
30. Stevens, C. W.; Pezalla, P. D. A spinal site mediates opiate analgesia in frogs. Life Sci. 33:2097–2103; 1983.
31. Stevens, C. W.; Pezalla, P. D. Naloxone blocks the analgesic action of levorphanol but not of dextrorphan in the leopard frog. Brain Res. 301:171–174; 1984.

32. Stevens, C. W.; Pezalla, P. D. Opioid system downregulation during hibernation in amphibians. Brain Res. 494:227–231; 1989.
33. Stevens, C. W.; Pezalla, P. D.; Yaksh, T. L. Spinal antinociceptive action of three representative opioid peptides in frogs. Brain Res. 402:201–203; 1987.
34. Ushijima, I.; Mizuki, Y.; Hara, T.; Obara, N.; Minematsu, N.; Yamada, M. Effects of lithium and purinergic compounds on the behavioral and physiological aspects of restraint stress in rats. Pharmacol. Biochem. Behav. 42:431–435; 1992.
35. Yaksh, T. L.; Harty, G. J. Effects of thiorphan on the antinociceptive actions of intrathecal [D-Ala2,Met5] enkephalin. Eur. J. Pharmacol. 79:293–300; 1982.