Published in final edited form as:
Vet Clin North Am Exot Anim Pract. 2011 January ; 14(1): 33–44. doi:10.1016/j.cvex.2010.09.007.

Analgesia in Amphibians: Preclinical Studies and Clinical Applications

Craig W. Stevens, Ph.D.
Professor of Pharmacology, Department of Pharmacology & Physiology, Oklahoma State University-Center for Health Sciences, Tulsa, Oklahoma, U.S.A.

SYNOPSIS

Preclinical studies of analgesia in amphibians or recommendations for clinical use of analgesics in amphibian species are extremely limited. This article briefly reviews the issues surrounding the use of analgesics in amphibians starting with common definitions of pain and analgesia when applied to non-human animals. Nociceptive and endogenous opioid systems in amphibians are reviewed and results of preclinical research on opioid and non-opioid analgesics summarized. Recommended opioid and non-opioid analgesics are summarized and practical recommendations made for their clinical use.

Keywords

analgesia; amphibians; opioid; acetic acid test; *Rana pipiens*

INTRODUCTION

Analgesia is the selective loss of pain sensation leaving other cognitive, sensory and motor functions intact and ideally, unaltered. General anesthesia is the loss of all sensory and motor function, and a total lack of consciousness and awareness. Both analgesia and anesthesia are defined by description of these states in humans; it is at best an educated guess whether these definitions can also be applied accurately to non-human animals, or amphibians. Comparative neurology suggests that the potential for pain is less substantial in amphibians and other earlier-evolved vertebrates compared to humans.[1–4]

There are very limited studies on either analgesia or anesthesia in amphibian species and only a few agents are recommended for clinical use. Anesthesia studies can be done to determine appropriate pharmacological agents and dosages by assessing simple measures like ability of an animal to right itself or to withdrawal its limb from a strong forceps pinch [5–9] but such studies will not be considered further here. In contrast, studies of analgesia are more difficult in amphibians as a validated assay is needed in which the animal exhibits a specific behavior in response to a potentially painful stimulus and in which that response is

Corresponding author: Craig W. Stevens, Ph.D., OSU-Center for Health Sciences, 1111 W. 17th Street, Tulsa, OK 74107, 918.561.8234 (ph.), 918.561.8276 (fax), cw.stevens@okstate.edu.

The author has nothing to disclose.

reduced by a potential analgesic agent. A well-validated alternative pain and analgesia model using *Rana pipiens* has been in use for over twenty-five years,[10,11] however, in general, studies of analgesia in any non-mammalian species are surprisingly limited.[12,13] As discussed elsewhere, there are a number of reasons for the limited research on pain and analgesics using non-mammalian pain models, including the biased view of alternative models by funding agencies, dismissive regulations of non-mammalian species, and lack of a scientific and zoological perspective by some scientists, veterinarians, and animal welfare experts.[14] This article will review the nociceptive pathways and related systems in amphibians, summarize the results of preclinical studies of analgesics in amphibian pain models, and provide clinical guidelines and rationale for the use of analgesics in amphibians.

NOCICEPTION AND PAIN IN AMPHIBIANS

Nociception is used to describe the transmission of noxious stimuli and subsequent processing up to the point in the brain whereby the pain experience, if present, is mediated. However, for ease of use and to make research findings accessible to a general audience, the term pain is generally used instead of *nociception*, and analgesia is used instead of *antinociception* in non-human animal studies. As our attitudes about animals are reflected by the language we use describing them and their possible attributes, word usage in this case is not a trivial point.[15] Most pain researchers accept this less precise usage of the words pain and analgesia (*nociception* and *antinociception* being too cumbersome and pedantic) but this does not necessarily imply that these researchers believe that the animals they treat or use for research experience pain *as we do*. It also does not mean that researchers using non-human animals believe that animals do not feel pain. In this regard, we must remain agnostic, using the best available science to guide our judgment. To think we know what an animal *experiences* or is *feeling* is not science but science fiction. The terms pain and analgesia are used here for convenience and in doing so make no assumptions as to the capacity of non-mammalian animals to experience pain.

The nociceptive pathways of amphibians were recently reviewed in detail.[16] In brief, there are no major differences between amphibian nociceptive afferents and those primary afferents of other vertebrates including mammals. Early electrophysiological work suggested that the detection of all noxious sensory stimuli, including chemical, was mediated exclusively by the same free nerve endings associated with thermal sensitivity in amphibians.17 Amphibians, like mammals, possess both myelinated and unmyelinated afferent fibers running concurrently in mixed-fiber peripheral sensory nerves. These afferent fibers have been delineated into three general classes based on morphology, conduction velocities, latency of response, and action potential characteristics: large, heavily myelinated A fibers; small, thinly myelinated B fibers; and small, unmyelinated C fibers. Their characteristics correlate well with those observed in corresponding classes of somatic sensory afferents in mammals: Aβ, Aδ and C fibers, respectively.16 In studies that separated fibers both by conduction velocity and fiber diameter in amphibians, small slowly-conducting fibers transmitted the majority of all impulses induced by noxious heat, pinching, pin pricks and the application of dilute acetic acid to the skin. [17, 18] A recent study closely examined the nociceptive primary afferent fibers excited in the acetic acid test (see below) and found that concentrations producing the behavioral response to the cutaneously applied acid solution evoked both Aβ and C-fibers to an equal extent.[19]

There is some uncertainty concerning the central terminations of primary afferent fibers within the amphibian spinal gray matter;[20] most, but not all studies indicate that sensory afferents from the skin terminate in areas of the dorsal field of the frog spinal cord that corresponds to mammalian laminae I-IV. It appears that in more recently evolved vertebrates, primary afferent fibers retract their ventral horn connections and become more

restricted to the superficial laminae of the spinal dorsal horn.[21] This may reflect an overall vertebrate evolutionary vector to put more neurons in between reflex arcs to allow for finer control of responses and greater response modulation.

The primary afferent nerve fibers in mammals terminate in the dorsal horn of the spinal cord and release pain-signaling neurotransmitters such as substance P, calcitonin gene-related peptide and glutamate. Using immunohistochemical techniques, these algogenic substances are readily identified in abundance in the spinal dorsal horn of amphibians. [22,23] In mammals, substance P and glutamate excite second-order neurons which have their cell bodies in the dorsal horn and send long fibers upward to form the ascending pain pathway.[24] Such second-order neurons within the dorsal horn that receive direct primary afferent input have not been identified in amphibians.[25] Also present in the mammalian spinal cord are endorphinergic neurons which release met-enkephalin, an endogenous opioid peptide, to act presynaptically to inhibit the release of substance P and postsynaptically to decrease the firing of the second-order pain neurons. Likewise, met-enkephalin, as well as opioid receptors, are abundant in the amphibian spinal cord.[10,16] Additionally, using *in situ* hybridization to identify neurons expressing mRNA for met-enkephalin, endorphinergic neurons were identified throughout the brain and spinal cord of *Rana pipiens*.[26] Thus while the most detailed studies have only been performed in mammals, the existence of these key pain neurotransmitters and endogenous opioid peptides suggests that basic mechanisms of pain transmission and endogenous opioid peptides within the spinal cord are common in both amphibians and mammals. An important caveat to remember, however, is that the presence of opioid receptors and endorphins (met-enkephalin or β-endorphin) in the CNS of any vertebrate is *not* by itself supporting evidence that these animals feel pain, as some authors suggest.[27–29] Endorphins and opioid binding sites are located in a number of organisms that do not have the capacity for anything like *human* pain and have very limited nervous systems such as the cockroach, crab, and snail.[30–33]

Ascending nociceptive pathways in amphibians are unclear as the location of second-order neurons in the spinal cord and the route of their fibers that carry this information to the brain are unknown.[34] Functional studies of evoked responses in supraspinal sites after specific noxious stimulation of peripheral fibers are lacking. A single study found that electrical stimulation of the sciatic nerve produces evoked potentials in posterior thalamic nuclei and primordial hippocampal structures in frogs.[35] However, the general sensory tracts from spinal neurons make direct connections with neuron groups farther towards the front of the brain as the phylogeny of vertebrates is ascended.[36] Throughout phylogeny, all the target sites of spinal tracts in the brain increase in complexity, specialization, and number of neurons, suggesting that even nociceptive messages to the thalamus in an amphibian and mammal may not be similar.

Cortical tissue, whether in limbic or cerebral regions, is a highly complex and laminated structure which is a relatively recent development in the evolution of the vertebrate nervous system. We know from human experience, that decreasing the activity of cortical neurons by anesthesia or surgical lesion results in a loss of the full appreciation of pain; the patient reports an awareness of pain “but it no longer bothers them”.[37] Recent studies using positron imaging techniques also show specific areas of the cortex activated by noxious stimuli in awake humans.[38] For these reasons, there is agreement among various scientific organizations that an intact cortex assists in the appreciation of pain.[39–41] It is likely that amphibians, without either a cerebral or limbic cortex, have a diminished potential for the appreciation of pain. Even the most rudimentary cerebral and limbic cortex does not appear until class *Reptilia*. In this sense, the use of amphibians may represent a ‘purer’ model system for the study of nociception, possibly without the additional factors of learning and conditioning which can interfere with the accurate measurement of analgesia in mammals.[42]

PRECLINICAL STUDIES OF AMPHIBIAN ANALGESIA

Pain Models in Amphibians

The first successful algesiometric model developed in the amphibian was described by Pezalla, who used dilute concentrations of cutaneously applied acetic acid and watched for a wiping response.[43] Called the acetic acid test (AAT), a determination of the nociceptive threshold (NT), as signaled by the wiping response, 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 and proceeded with increasing concentrations until the NT was reached. The NT was defined as 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 five seconds if the animal failed to respond. If the animal failed to respond, testing continued with the next highest concentration of acetic acid on the opposite hindlimb. The wiping response in frogs, like the tail-flick in rodents, remains intact after a high spinal transection, demonstrating sufficient circuitry in the spinal cord to mediate this behavior.[44] The acetic acid stimulus excites primary afferent fibers consistent with nociceptive stimulation.19›45 This wiping response has not been observed in the laboratory in the absence of noxious stimuli and appears specific for assessing nociception. The wiping response is also the basis for much research on the motor systems of the amphibian spinal cord.46

Opioid Analgesia in Amphibians

Using the AAT, initial and ongoing studies of the analgesic effects of opioid administration in amphibians was conducted using non-selective opioid agonists, endogenous opioid peptides, and antagonists. [47–52] These studies showed that both exogenous opioid agonists and endogenous opioid peptides could raise the nociceptive threshold in amphibians by an action at an opioid receptor. Tolerance to the analgesic effects of daily morphine administration was documented[53] and stress-induced release of endogenous opioids was shown to produce analgesia in amphibians which was potentiated by enkephalinase inhibitors.[54] Other behavioral studies include an investigation of the effects of opioids on noxious and non-noxious sensory modalities[55,56], an examination of agents acting on alpha$_2$ adrenergic receptors after systemic and spinal administration,[57,58] and studies aimed at determining the selectivity of opioid receptors in amphibians.[59–63]

Later results of systematic studies examining the analgesia of selective *mu*, *delta*, or *kappa* opioid agonists administered by different routes yielded an important finding: The relative analgesic potency of *mu*, *delta*, or *kappa* opioid agonists after systemic, intraspinal, or intracerebroventricular administration in amphibians was highly correlated to that observed in typical mammalian models and to the relative analgesic potency of opioid analgesics in human clinical studies.[64–67] This data established the amphibian model using the AAT as a robust and predictive adjunct or alternative non-mammalian model for the testing of opioid analgesics.

Two other studies also used the AAT to examine the potential analgesic effects of opioids in ranid frogs. Using *Rana pipiens*, Suckow and colleagues demonstrated a dose-dependent analgesic effect of butorphanol, a mixed *mu-kappa* opioid receptor agent.[27]. Butorphanol has the logistical advantage that unlike the more potent opioids such as morphine, it is not a Schedule II agent and therefore easier to obtain for veterinary use and to keep in animal facilities. Using the common European water frog, *Rana esculenta*, Benyhe and colleagues confirmed the potency of morphine on the AAT and also showed that oxymorphazone, an analog of oxymorphone that binds irreversibly to *mu* opioid receptors, produced a long-

lasting analgesia up to 48 h.[68] Table 1 provides a summary of the dosage range and ED_{50} values for the analgesic effect of opioid agonists used in behavioral studies of in *Rana pipiens* following systemic administration.

A recent study examined the potential analgesic effects of buprenorphine and butorphanol in another amphibian species, the Eastern red-spotted newt (*Notophthalmus viridescens*). [69] In this study, the AAT was not used but rather animals underwent bilateral forelimb amputation and 'analgesia' was assayed by changes in food consumption, spontaneous movement, and other non-specific measures. There was a significant shortening of the time to resume normal behaviors following post-operative systemic administration of buprenorphine or bath application of butorphanol compared to animals receiving no post-operative agents. Finally, using the Japanese fire-belly newt (*Cynops pyrrhogaster*), administration of the neuropeptide RFamide produced an increase in the latency to withdrawal the newt's tail from a hot lamp (like the common tail-flick test used in rodents) which was blocked by the opioid antagonist, naloxone.[70]

Non-Opioid Analgesia in Amphibians

The acetic acid test (AAT) applied in studies using *Rana pipiens* clearly demonstrated dose-dependent and receptor-mediated opioid analgesia in amphibians. There were a number of other analgesic agents shown to be analgesic in typical rodent models that were also effective on the AAT. Potential non-opioid analgesics tested included antipsychotic, benzodiazepine, barbiturate, antihistamine, non-steroidal anti-inflammatory (NSAID), and partial opioid agents. Specifically, chlorpromazine and haloperidol (antipsychotics), chlordiazepoxide (a benzodiazepine), buprenorphine (partial opioid agonist) and diphenhydramine (histamine antagonist) produced moderate to strong analgesic effects.[71] Indomethacin and ketorolac (NSAIDs), butorphanol (partial opioid agonist), and pentobarbital (a barbiturate) produced weaker but still had significant analgesic effects. Peak analgesic effects for the highest, non-lethal doses of the potent agents showed a relative analgesic potency of morphine > chlorpromazine > chlordiazepoxide > buprenorphine > diphenhydramine > haloperidol. It should be noted, however, that full dose-response curves were not generated thus ED_{50} values were not calculated.

Studies by Brenner and colleagues and Suckow's group also demonstrated the analgesic efficacy of $alpha_2$ adrenergic agonists, such as clonidine, dexmedotomidine, and xylazine at specific adrenergic receptor sites.[27,57,58] An additional NSAID agent, flunixin meglumine, was also an effective analgesic after intracelomic injection in *Rana pipiens*.[27] In a model of anesthesia using adult bullfrogs (*Rana catesbeiana*) it was noted that responses to strong forcep pinching of the hindlimb remained intact in animals anesthetized with thiopental, suggesting that this barbiturate is not a high potency analgesic agent in amphibians.[5] Table 2 provides a summary of the doses tested and maximum percent effect (M.P.E.) for non-opioid and partial opioids in behavioral studies in *Rana pipiens* following systemic administration.

CLINICAL APPLICATIONS OF AMPHIBIAN ANALGESIA

The clinical judgment of the attending veterinarian will ultimately determine the appropriate instances when an analgesic agent should be administered in an amphibian species. Cases of trauma to limbs or surgical interventions are possible scenarios when analgesics would be given. As pointed out in a recent opinion paper, the use the postoperative analgesics in African clawed frogs (*Xenopus leavis*) after surgery for removal of oocytes used in biomedical research is currently an unresolved clinical issue.[72] Frog oocytes are often used for electrophysiological and molecular pharmacology studies due to their large size and ability to express transfected mRNA into receptor or channel proteins. There are established

guidelines now for many university researchers on oocyte harvesting by repeated laparotomy (cf. Google search for 'oocyte harvesting guidelines'). Most institutions copy the guidelines from the National Institutes of Health-Office of Animal Care and Use (NIH-OACU) in their most recent revision.[73] These NIH guidelines do not mention any use of analgesics for postoperative care, but do suggest that animals be monitored daily for "appetite as well as for any complications such as dehiscence or infection. Such adverse effects would be reasons for immediate euthanasia."

A minority of other institutional animal care and use committees (IACUC) guidelines for oocyte harvesting from *Xenopus* do explicitly state that analgesics should be given and provide doses, as this example from University of North Carolina: "Post-operative care should be provided as with any species. Suggested analgesics for *Xenopus* are butorphanol, 25 mg/kg intracelomic, or xylazine, 10 mg/kg intracelomic. Both butorphanol and xylazine have a calming effect." Other sets of guidelines, as in this example from University of Buffalo-SUNY, are even more explicit and outright state that analgesia should be addressed: "All frogs should receive at least one dose of post-operative analgesia. Acceptable analgesics include: flunixin meglumine (25 mg/kg intracoelomic once) and xylazine (10 mg/kg intracoelomic, every 12–24 h." Both of these institutions are recommending agents and doses based on the single study by Suckow and colleagues.[27] Further studies are needed to determine the relative potency of other opioid and non-opioid analgesics shown to be active in preclinical studies (see Tables 1,2).

The issue of hypothermia for amphibian anesthesia or analgesia is also inadequately addressed in scientific studies. The guidelines mentioned above for oocyte removal all state that hypothermia is not an adequate method for inducing analgesia or for carrying out surgical procedures. There was one elegant study that examined hypothermia-induced analgesia in *Rana pipiens* using the acetic acid test.74 These investigators found that placement of a tourniqueted limb in icewater did produce analgesia in the acetic acid test compared to the untreated contralateral limb. Furthermore, this analgesic effect was blocked by administration of the general opioid antagonist, naltrexone, suggesting the involvement of endogenous opioid peptide release. This finding has to be interpreted in light of other studies which show: 1) amphibians in cold-adaptation show a decrease in pain thresholds75,[76] and 2) amphibians that are immobilized or in stressful situations produce opioid-mediated stress analgesia.[54,77]

A major concern is the risk-benefit ratio of using experimental drugs on zoological companion animals like amphibians. Most clinical treatments in non-mammalian species are by definition experimental as little research has or is being done using these earlier-evolved vertebrates. It is knotimesus, the use of clinical analgesics in amphibians should be undertaken by those veterinarians who are willing to explore the non-mammalian 'pain' literature and come to their own evidence-based conclusions. Practical recommendations for amphibian analgesia should follow the edict "first do no harm."

SUMMARY

Preclinical and clinical studies of analgesia in amphibians are extremely limited. Almost all preclinical studies were done using *Rana pipiens* and the acetic acid test while the greatest clinical need is for treatment of *Xenopus laevis* with post-operative analgesics after harvesting of oocytes in academic research settings.[72] There are also a number of zoos and herpetological collections that may also benefit from clinical information in recognizing and treating potential pain states in amphibians. Besides the inherent need of pain and analgesia studies in amphibians to guide the clinical treatment of these zoological companion animals, preclinical research using non-mammalian models has yielded a surprising wealth of

information on the vertebrate evolution of endogenous opioid peptides[78–80] and opioid receptor systems.[81–84] There is a great need for further research that can be realized by increased awareness and funding of amphibian models by alternative animal model foundations, increased preclinical and clinical research using *Xenopus* species, and increased involvement of exotic companion animal veterinarians in the research process.

Acknowledgments

This work was supported in part by Grant No. DA012248 from the National Institutes of Health.

Reference List

1. Stevens CW. Alternatives to the use of mammals for pain research. Life Sci 1992;50:901–912. [PubMed: 1548975]
2. Stevens CW. An amphibian model for pain research. Lab Animal 1995;24:32–36.
3. Stevens, CW. An alternative model for testing opioid analgesics and pain research using amphibians. In: van Zutphen, LFM.; Balls, M., editors. Animal Alternatives, Welfare and Ethics. Amsterdam: Elsevier; 1997. p. 247-251.
4. Rose JD. The neurobehavioral nature of fishes and the question of awareness and pain. Rev Fish Sci 2002;10:1–38.
5. Downes H, Koop DR, Klopfenstein B, Lessov N. Retention of nociceptor responses during deep barbituate anesthesia in frogs. Comp Biochem Physiol 1999;124:203–210.
6. Lafortune M, Mitchell MA, Smith JA. Evaluation of medetomidine, clove oil and propofol for anesthesia of leopard frogs, *Rana pipiens*. J Herpe Med Surg 2001;11:13–18.
7. Barter LS, Antognini JF. Kinetics and potency of halothane, isoflurane, and desflurane in the northern leopard frog *Rana pipiens*. Vet Res Commun 2008;32:357–365. [PubMed: 18351438]
8. Guénette SA, Hélie P, Beaudry F, Vachon P. Eugenol for anesthesia of African clawed frogs (*Xenopus laevis*). Vet Anesth Analgesia 2007;34:164–170.
9. Guénette SA, Beaudry F, Vachon P. Anesthetic properties of propofol in African clawed frogs (*Xenopus laevis*). J Amer Assoc Lab Anim Sci 2008;47:35–38.
10. Stevens CW. Opioid antinociception in amphibians. Brain Res Bull 1988;21:959–962. [PubMed: 3066446]
11. Stevens, CW. Non-mammalian models for the study of pain. In: Conn, PM., editor. Sourcebook of models for biomedical research. Totowa: Humana Press; 2008. p. 341-352.
12. Greenacre C, Paul-Murphy J, Sladky KK, Storms T. Reptile and amphibian analgesia. J Herpe Med Surg 2005;15:24–30.
13. Paul-Murphy J, Ludders JW, Robertson SA, Gaynor JS, Hellyer PW, Wong PL. The need for a cross-species approach to the study of pain in animals. J Am Vet Med Assoc 2004;224:692–697. [PubMed: 15002806]
14. Stevens, CW. Alternative models for pain research: A translational, non-mammalian model with an ethical advantage. In: Warnick, JE.; Kalueff, AV., editors. Translational Neuroscience in Animal Research: Advancement, Challenges, and Research Ethics. Hauppauge: Nova Science; 2010. p. 3-27.
15. Beer, C. Motives and metaphors in considerations of animal nature. In: Pfaff, DW., editor. onsiderations of Animal Nature. New York: Springer-Verlag; 1983. p. 125-151.
16. Stevens CW. Opioid research in amphibians: an alternative pain model yielding insights on the evolution of opioid receptors. Brain Res Rev 2004;46:204–215. [PubMed: 15464208]
17. Adrian ED, Cattell M, Hoagland H. Sensory discharges in single cutaneous nerve fibres. J Physiol 1931;72:377–391. [PubMed: 16994216]
18. Maruhashi J, Mizuguchi K, Tasaki I. Action currents in single afferent nerve fibres elicited by stimulation of the skin of the toad and the cat. J Physiol 1952;117:129–151. [PubMed: 14955805]

19. Hamamoto DT, Simone DA. Characterization of cutaneous primary afferent fibers excited by acetic acid in a model of nociception in frogs. J Neurophysiol 2003;90:566–577. [PubMed: 12750420]
20. Nikundiwe AM, De Boer-van Huizen R, Ten Donkelaar HJ. Dorsal root projections in the clawed toad (*Xenopus laevis*) as demonstrated by anterograde labeling with horseradish peroxidase. Neuroscience 1982;7:2089–2103. [PubMed: 7145089]
21. Joseph BS, Whitlock DG. The morphology of spinal afferent-efferent relationships in vertebrates. Brain Behav Evol 1968;1:2–18.
22. Lorez HP, Kemali M. Substance P, met-enkephalin and somatostatin-like immunoreactivity distribution in the frog spinal cord. Neurosci Lett 1981;26:119–124. [PubMed: 6170916]
23. Inagaki S, Senba E, Shiosaka S, Takagi H, Kawai Y, Takatsuki K, et al. Regional distribution of substance P-like immunoreactivity in the frog brain and spinal cord: immunohistochemical analysis. J Comp Neurol 1981;201:243–254. [PubMed: 6169747]
24. Yaksh, TL.; Stevens, CW. Properties of the modulation of spinal nociceptive transmission by receptor-selective agents. In: Dubner, R.; Gebhart, GF.; Bond, MR., editors. Proceedings of the 5th World Congress on Pain. Amsterdam: Elsevier; 1988. p. 417-435.
25. Simpson, JI. Functional synaptology of the spinal cord. In: Llinas, R.; Precht, W., editors. Frog Neurobiology: A Handbook. Berlin: Springer-Verlag; 1976. p. 728-749.
26. Rothe-Skinner KS, Stevens CW. Distribution of opioid-expressing neurons in the frog: an *in situ* hybridization study. Analgesia 1995;1:683–686.
27. Terril-Robb L, Suckow M, Grigdesby C. Evaluation of the analgesic effects of butorphanol tartarate, xylazine hydrochloride, and flunixin meglumine in leopard frogs (*Rana pipiens*). Cont Topics Lab Anim Sci 1996;35:54–56.
28. Machin KL. Fish, amphibian, and reptile analgesia. Vet Clin North Am Exot Anim Pract 2001;4:19–33. [PubMed: 11217460]
29. Machin KL. Amphibian pain and analgesia. J Zoo Wild Med 1999;30:2–10.
30. Verhaert P, De Loof A. Immunocytochemical localization of a methionine-enkephalin-resembling neuropeptide in the central nervous system of the american cockroach, *Periplaneta americana* L. J Comp Neurol 1985;239:54–61. [PubMed: 3900153]
31. Hanke J, Jaros PP, Willig A. Autoradiographic localization of opioid binding sites combined with immunogold detection of Leu-enkephalin, crustacean hyperglycaemic hormone and moult inhibiting hormone at the electron microscopic level in the sinus gland of the shore crab, *Carcinus maenas*. Histochemistry 1993;99:405–410. [PubMed: 8392990]
32. Kavaliers M, Hirst M, Teskey GC. A functional role for an opiate system in snail thermal behavior. Science 1983;220:99–101. [PubMed: 6298941]
33. Hanke J, Willig A, Jaros PP. Opioid receptor types for endogenous enkephalin in the thoracic ganglion of the crab, *Carcinus maenas*. Peptides 1996;17:965–972. [PubMed: 8899815]
34. Spray, DC. Cutaneous receptors: pain and temperature receptors of anurans. In: Llinas, R.; Precht, W., editors. Frog Neurobiology: a handbook. Berlin: Springer-Verlag; 1976. p. 607-628.
35. Vesselkin NP, Agayan AL, Nomokonova LM. A study of thalamo-telencephalic afferent systems in frogs. Brain Behav Evol 1971;4:295–306. [PubMed: 5315770]
36. Munoz A, Munoz M, Gonzalez A, Ten Donkelaar HJ. Spinal ascending pathways in amphibians: cells of origin and main targets. J Comp Neurol 1997;378:205–228. [PubMed: 9120061]
37. White, JC.; Sweet, WH. Pain and the Neurosurgeon. Springfield: Thomas; 1969.
38. Talbot JD, Marrett S, Evans AC, Meyer E, Bushnell MC, Duncan GH. Multiple representations of pain in human cerebral cortex. Science 1991;251:1355–1358. [PubMed: 2003220]
39. Andrews EJ, Bennett BT, Clark JD, Houpt KA, Pascoe PJ, Robinson GW, et al. 1993 report of the AVMA panel on euthanasia. J Amer Vet Med Assoc 1993;202:229–249. [PubMed: 8428829]
40. Smith, JA.; Boyd, KM. Lives in the Balance. Oxford: Oxford University Press; 1991. p. 1-295.
41. Van Sluyters, RC. Handbook for the Use of Animals in Neuroscience Research. Washington D.C.: Society for Neuroscience; 1991.

42. Bardo MT, Hughes RA. Exposure to a nonfunctional hot plate as a factor in the assessment of morphine-induced analgesia and analgesic tolerance in rats. Pharmacol Biochem Behav 1978;10:481–485. [PubMed: 461477]
43. Pezalla PD. Morphine-induced analgesia and explosive motor behavior in an amphibian. Brain Res 1983;273:297–305. [PubMed: 6604562]
44. Fukson OI, Berkinblit MB, Feldman AG. The spinal frog takes into account the scheme of its body during the wiping reflex. Science 1980;209:1261–1263. [PubMed: 7403886]
45. Hamamoto DT, Forkey MW, Davis WL, Kajander KC. The role of pH and osmolarity in evoking the acetic acid-induced wiping response in a model of nociception in frogs. Brain Res 2000;862:217–229. [PubMed: 10799688]
46. Giszter SF, McIntyre J, Bizzi E. Kinematic strategies and sensorimotor transformations in the wiping movements of frogs. J Neurophysiol 1989;62:750–767. [PubMed: 2788719]
47. Johnson SM, Duggan AW. Dependence in the absence of tolerance to morphine. Eur J Pharmacol 1984;97:305–308. [PubMed: 6538507]
48. Price DD, Hu JW, Dubner R, Gracely RH. Peripheral supression of first pain and central summation of second pain evoked by noxious heat pulses. Pain 1977;5:76–78.
49. Crain SM, Crain B, Peterson ER. Development of cross-tolerance to 5-hydroxytryptamine in organotypic cultures of mouse spinal cord-ganglia during chronic exposure to morphine. Life Sci 1982;31:241–247. [PubMed: 7121205]
50. Sullivan AF, Dickenson AH. Electrophysiologic studies on the spinal antinociceptive action of *kappa* opioid agonists in the adult and 21-day-old rat. J Pharmacol Exp Ther 1991;256:1119–1125. [PubMed: 2005577]
51. Stevens CW, Toth G, Borsodi A, Benyhe S. Xendorphin B1, a novel opioid-like peptide determined from a *Xenopus laevis* brain cDNA library, produces opioid antinociception after spinal administration in amphibians. Brain Res Bull 2007;71:628–632. [PubMed: 17292806]
52. Stevens CW, Martin KK, Stahlheber BW. Nociceptin produces antinociception after spinal administration in amphibians. Pharmacol Biochem Behav 2008;91:436–440. [PubMed: 18804120]
53. Stevens CW, Kirkendall K. Time course and magnitude of tolerance to the analgesic effects of systemic morphine in amphibians. Life Sci 1993;52:PL111–PL116. [PubMed: 8464324]
54. Stevens CW, Sangha S, Ogg BG. Analgesia produced by immobilization stress and an enkephalinase-inhibitor in amphibians. Pharmacol Biochem Behav 1995;51:675–680. [PubMed: 7675842]
55. Willenbring S, Stevens CW. Thermal, mechanical and chemical peripheral sensation in amphibians: opioid and adrenergic effects. Life Sci 1996;58:125–133. [PubMed: 8606621]
56. Willenbring S, Stevens CW. Spinal *mu, delta* and *kappa* opioids alter chemical, mechanical and thermal sensitivities in amphibians. Life Sci 1997;61:2167–2176. [PubMed: 9393936]
57. Brenner GM, Klopp AJ, Deason LL, Stevens CW. Analgesic potency of alpha-adrenergic agents after systemic administration in amphibians. J Pharmacol Exp Ther 1994;270:540–545. [PubMed: 7915313]
58. Stevens CW, Brenner GM. Spinal administration of adrenergic agents produces analgesia in amphibians. Eur J Pharmacol 1996;316:205–210. [PubMed: 8982687]
59. Newman LC, Wallace DR, Stevens CW. Characterization of 3H–diprenorphine binding in *Rana pipiens:* Observations of filter binding enhanced by naltrexone. J Pharmacol Toxicol Meth 1999;41:43–48.
60. Stevens CW, Newman LC. Spinal administration of selective opioid antagonists in amphibians: evidence for an opioid unireceptor. Life Sci 1999;64:L125–PL130.
61. Newman LC, Wallace DR, Stevens CW. Selective opioid agonist and antagonist displacement of [^{3}H]-naloxone binding in amphibian brain. Eur J Pharmacol 2000;397:255–262. [PubMed: 10844122]
62. Newman LC, Wallace DR, Stevens CW. Selective opioid receptor agonist and antagonist displacement of [^{3}H]-naloxone binding in amphibian spinal cord. Brain Res 2000;884:184–191. [PubMed: 11082500]

63. Newman LC, Sands SS, Wallace DR, Stevens CW. Characterization of *mu*, *kappa*, and *delta* opioid binding in amphibian whole brain tissue homogenates. J Pharmacol Exp Ther 2002;301:364–370. [PubMed: 11907194]
64. Stevens CW, Klopp AJ, Facello JA. Analgesic potency of *mu* and *kappa* opioids after systemic administration in amphibians. J Pharmacol Exp Ther 1994;269:1086–1093. [PubMed: 8014851]
65. Stevens CW. Relative analgesic potency of *mu*, *delta* and *kappa* opioids after spinal administration in amphibians. J Pharmacol Exp Ther 1996;276:440–448. [PubMed: 8632308]
66. Stevens CW, Rothe KS. Supraspinal administration of opioids with selectivity for *mu*, *kappa*, and *delta* opioid receptors produces analgesia in amphibians. Eur J Pharmacol 1997;331:15–21. [PubMed: 9274924]
67. Mohan S, Stevens CW. Systemic and spinal administration of the *mu* opioid, remifentanil, produces antinociception in amphibians. Eur J Pharmacol 2006;534:89–94. [PubMed: 16487509]
68. Benyhe S, Hoffmann Gy, Varga E, Hosztafi S, Toth G, Borsodi A, et al. Effects of oxymorphazone in frogs: long lasting antinociception *in vivo*, and apparently irreversible binding *in vitro*. Life Sci 1989;44:1847–1857. [PubMed: 2472540]
69. Koeller CA. Comparison of buprenorphine and butorphanol analgesia in the eastern red-spotted newt (*Notophthalmus viridescens*). J Am Assoc Lab Anim Sci 2009;48:171–175. [PubMed: 19383214]
70. Kanetoh T, Sugikawa T, Sasaki I, Muneoka Y, Minakata H, Takabatake I, et al. Identification of a novel frog RFamide and its effect on the latency of the tail-flick response of the newt. Comp Biochem Physiol C Toxicol Pharmacol 2003;134:259–266. [PubMed: 12600686]
71. Stevens CW, MacIver DN, Newman LC. Testing and comparison of non-opioid analgesics in amphibians. Contemp Top Lab Anim Sci 2001;40:23–27. [PubMed: 11451391]
72. Green SL. Postoperative analgesics in South African Clawed frogs (*Xenopus laevis*) after surgical harvest of oocytes. Comp Med 2003;53:244–247. [PubMed: 12868565]
73. Wyatt, R. National Institutes of Health, Office of Animal Care and Use. Washington DC: 2007. Guidelines for egg and oocyte harvesting in *Xenopus laevis*.
74. Suckow M, Terril L, Grigdesby C, March P. Evaluation of hypothermia-induced analgesia and influence of opioid antagonists in leopard frogs (*Rana pipiens*). Pharmacol Biochem Behav 1999;63:39–43. [PubMed: 10340522]
75. Stevens CW, Pezalla PD. Endogenous opioid system down-regulation during hibernation in amphibians. Brain Res 1989;494:227–231. [PubMed: 2570621]
76. Stevens, CW. Environmental factors influencing pain physiology in amphibians. In: Mallick, BN.; Singh, R., editors. Environment and Physiology: 38th Annual Conference of the Association of Physiologists and Pharmacologists of India; New Delhi: Narosa Publishing House; 1994. p. 54-61.
77. Pezalla PD, Dicig M. Stress-induced analgesia in frogs: evidence for the involvement of an opioid system. Brain Res 1984;296:356–360. [PubMed: 6322930]
78. Dores RM, Lecaude S, Bauer D, Danielson PB. Analyzing the evolution of the opioid/orphanin gene family. Mass Spectrom Rev 2002;21:220–243. [PubMed: 12533798]
79. Dores RM, Lecaude S. Trends in the evolution of the proopiomelanocortin gene. Gen Comp Endocrinol 2005;142:81–93. [PubMed: 15862552]
80. Alrubaian J, Lecaude S, Barba J, Szynskie L, Jacobs N, Bauer D, et al. Trends in the evolution of the preprodynorphin gene in teleosts: Cloning of eel and tilapia prodynorphin cDNAs. Peptides 2006;27:797–804. [PubMed: 16274850]
81. Stevens CW. Opioid research in amphibians: a unique perspective on mechanisms of opioid analgesia and the evolution of opioid receptors. Rev Analgesia 2003;7:122–136.
82. Stevens CW, Brasel CM, Mohan S. Cloning and bioinformatics of amphibian *mu*, *delta*, *kappa*, and nociceptin opioid receptors expressed in brain tissue: Evidence for opioid receptor divergence in mammals. Neurosci Lett 2007;419:189–194. [PubMed: 17452077]
83. Stevens CW. The evolution of vertebrate opioid receptors. Front Biosci 2009;14:1247–1269. [PubMed: 19273128]
84. Brasel CM, Sawyer GW, Stevens CW. A pharmacological comparison of the cloned frog and human *mu* opioid receptors reveals differences in opioid affinity and function. Eur J Pharmacol 2008;599:36–43. [PubMed: 18930720]

Table 1

Characteristics of opioid analgesics following systemic administration in *Rana pipiens*[64,67] The most potent drugs are listed first and in order of their calculated ED_{50} values.

Opioid Agonist	Receptor type	Dosage Range[a] nmol/g	ED_{50} value[b] nmol/g	ED_{50} value[c] µg/g
Fentanyl	*mu*	1–30	1.4	0.8
Enadoline	*kappa*	1–30	5.8	2.3
remifentanil	*mu*	1–30	7.1	2.8
levorphanol	*mu*	1–100	7.5	1.9
U50488H	*kappa*	1–100	8.5	3.1
methadone	*mu*	10–300	19.9	6.2
bremazocine	*kappa*	10–100	44.4	14.0
Morphine	*mu*	10–300	86.3	32.7
buprenorphine	*mu*	30–300	99.1	46.3
meperidine	*mu*	30–1000	128.1	31.6
Codeine	*mu*	10–1000	140.3	42.0
nalorphine	*kappa*	100–300	320.9	99.9

[a] effective dosage range in nmol/g body weight

[b] dose that gives 50% analgesic effects, in nmol/g body weight.

[c] equivalent ED_{50} in µg/g body weight dosing.

Table 2

Characteristics of non-opioid and partial opioid drugs following systemic administration in *Rana pipiens*[57,71] Morphine is included for comparison. The most efficacious drugs are listed first and drugs producing less than 40% effect (M.P.E.) are listed below the bold line.

Agent	Class	Tested dose[a] nmol/g	M.P.E.[b]	Equivalent µg/g
morphine	opioid analgesic	300	100	114
dexmedetomidine	alpha$_2$ adrenergic	3	78.0	0.6
chlorpromazine	antipsychotic	100	62.9	32
chlordiazepoxide	benzodiazepine	300	51.3	90
buprenorphine	partial opioid agonist	30	48.6	14
clonidine	alpha$_2$ adrenergic	300	45.0	69
diphenhydramine	H1 antagonist	200	43.2	51
Haloperidol	antipsychotic	30	41.1	11
Indomethacin	NSAID	300	39.6	107
Ketorolac	NSAID	100	39.1	26
Butorphanol	partial opioid agonist	100	36.9	33
Pentobarbital	barbiturate	30	27.7	8

[a] nmol/mg. The conversion of nmol/mg body weight doses to µg/g body weight doses is given in the last column.

[b] M.P.E. = maximum percent effect of analgesia observed at that dose in an average of 4–8 animals.