Published in final edited form as:

Am J Vet Res. 2017 July ; 78(7): 785–795. doi:10.2460/ajvr.78.7.785.

Antinociceptive and respiratory effects following application of transdermal fentanyl patches and assessment of brain μ -opioid receptor mRNA expression in ball pythons

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Abstract

OBJECTIVE—To quantify plasma fentanyl concentrations (*PFCs*) and evaluate antinociceptive and respiratory effects following application of transdermal fentanyl patches (*TFPs*) and assess cerebrospinal μ -opioid receptor mRNA expression in ball pythons (compared with findings in turtles).

ANIMALS—44 ball pythons (*Python regius*) and 10 turtles (*Trachemys scripta elegans*).

PROCEDURES—To administer 3 or 12 μ g of fentanyl/h, a quarter or whole TFP (TFP-3 and TFP-12, respectively) was used. At intervals after TFP-12 application in snakes, *PFCs* were measured by reverse-phase high-pressure liquid chromatography. Infrared heat stimuli were applied to the rostral surface of snakes to determine thermal withdrawal latencies after treatments with no TFP (control [*n* = 16]) and TFP-3 (8) or TFP-12 (9). Breathing frequency was measured in unrestrained controls and TFP-12-treated snakes. μ -Opioid receptor mRNA expressions in brain and spinal tissue samples from snakes and turtles (which are responsive to μ -opioid agonist drugs) were quantified with a reverse-transcription PCR assay.

RESULTS—Mean *PFCs* were 79, 238, and 111 ng/mL at 6, 24, and 48 hours after TFP-12 application, respectively. At 3 to 48 hours after TFP-3 or TFP-12 application, thermal withdrawal latencies did not differ from pretreatment values or control treatment findings. For TFP-12-treated snakes, mean breathing frequency significantly decreased from the pretreatment value by 23% and 41% at the 24- and 48-hour time points, respectively. Brain and spinal tissue μ -opioid receptor mRNA expressions in snakes and turtles did not differ.

CONCLUSIONS AND CLINICAL RELEVANCE—In ball pythons, TFP-12 application resulted in high *PFCs* but there was no change in thermal antinociception indicating resistance to μ -opioid-dependent antinociception in this species.

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Presented in abstract form at the 21st Annual Association of Reptilian and Amphibian Veterinarians, Orlando, Florida, 2014.

Veterinarians seek to minimize pain in all vertebrate species under their care. However, there is limited knowledge of pain perception and control in non-mammalian vertebrates.^{1,2} For reptiles in particular, there are several challenges to effectively detecting and assessing nociception and there is a general lack of knowledge regarding effective antinociception in these species.^{3,4,5} Reptiles have the necessary neuroanatomic structures to detect pain,^{5,6,7} and conditions considered painful in humans and domestic pets are assumed to be painful in reptiles.³ Unfortunately, antinociceptive drugs that are effective in one vertebrate class are not necessarily effective in other vertebrate classes. Thus, effective antinociception in reptiles requires species-specific assessment of the efficacy and potential deleterious effects of analgesic drugs.^{1,5}

Opioid receptors are highly conserved across vertebrate species,^{8,9} but opioid drugs have a wide range of efficacy among different reptiles. For example, μ -opioid receptor agonist drugs increase noxious thermal withdrawal latencies in crocodiles,¹⁰ as well as in bearded dragons and red-eared slider turtles.^{11,12} However, buprenorphine (a partial μ -opioid receptor agonist) does not increase noxious thermal withdrawal latencies in red-eared slider turtles.¹³ Likewise, butorphanol (a κ -opioid receptor antagonist and partial μ -opioid receptor agonist-antagonist) does not provide thermal antinociception in bearded dragons,¹² red-eared slider turtles,¹¹ or green iguanas.¹⁴ Surprisingly, there are no reports of studies^{4,5,12} demonstrating robust analgesic efficacy for any drug in snakes to our knowledge. In a study¹⁵ of pain during surgery in ball pythons, neither meloxicam nor butorphanol decreased physiologic variables, compared with findings in control animals treated with saline (0.9% NaCl) solution. In corn snakes, morphine sulfate (1 to 40 mg/kg) did not alter thermal withdrawal latencies and butorphanol (20 mg/kg) modestly increased thermal withdrawal latencies.¹² However, relatively high doses of butorphanol may not be clinically appropriate in debilitated patients.^{4,12} Within the context of these data, we hypothesized that plasma concentrations of μ -opioid receptor agonist drugs would have to be sustained to obtain antinociception in snakes. The use of a transdermal, continuous drug delivery system for pain management in snakes is an attractive option, as it would decrease frequencies of handling and injections while potentially improving healing and recovery time. Because the plasma concentrations of fentanyl that are therapeutic in mammals do not necessarily provide effective antinociception in other species, it is necessary to assess the analgesic efficacy of TFPs in snakes.

The primary objective of the study reported here was to quantify plasma fentanyl concentrations and evaluate antinociceptive and respiratory effects following application of TFPs in ball pythons. Results of a previous study¹⁶ indicated that TFP application to ball pythons maintained therapeutic concentrations (> 1 ng of fentanyl/mL of plasma) for 7 days, but only 2 snakes were tested and the patches were attached with staples, which may have altered drug delivery. To test for fentanyl-induced analgesic effects in the present study, withdrawal latencies were measured in response to noxious thermal stimuli applied to the rostral portion of each snake's body. Because respiratory depression is an unwanted μ -opioid drug-associated effect in reptiles,¹¹ breathing frequency was measured noninvasively following TFP application in the ball pythons. Finally, little is known regarding the relative expression of μ -opioid receptors in the reptilian CNS. Accordingly, μ -opioid receptor mRNA

expression in samples of the brain and spinal cord of ball pythons were measured and compared with findings in red-eared slider turtles, which are highly sensitive to μ -opioid agonist drugs.^{11,13,17–21}

Materials and Methods

Animals

Ball pythons (*Python regius*; n = 44) of unknown sex were obtained from commercial suppliers.^{a, b} The mean $\pm$ SEM weight of the snakes was 196 ± 39 g (range, 46 to 1,038 g). Snakes were housed individually in standard rodent cages (approximately 26 cm in width, 48 cm in depth, and 20 cm in height) with free access to drinking water and a darkened hide chamber. Room temperature was maintained at 26° to 30°C, and a broad-spectrum UV light source provided light for 12 hours each day. Pythons were fed once each week with thawed frozen juvenile or adult mice, as appropriate for their body size. All snakes were considered healthy at the time of their participation in any given phase of the study.

Healthy adult red-eared slider turtles (*Trachemys scripta elegans*; n = 10) of either sex were obtained from commercial suppliers^c for quantification of μ -opioid mRNA expressions in brain and spinal cord samples. The mean $\pm$ SEM weight of the turtles was 594 ± 37 g (range, 423 to 772 g) were. Turtles were maintained in a large, open tank in which they had access to water for swimming and were provided with heat lamps and dry areas for basking. Room temperature was maintained at 27° to 28°C with fluorescent light provided for 14 hours each day. Turtles were fed floating food sticks^d 3 to 4 times each week.

Drug treatments

According to the manufacturer, a quarter of a TFP and a whole TFP deliver 3 μ g of fentanyl/h (12 to 67 μ g/kg/h) and 12 μ g of fentanyl/h (49 to 218 μ g/kg/h), respectively. These patch treatments were designated as TFP-3 and TFP-12, respectively. To obtain a TFP-3, a TFP-12 was cut into 4 equivalent pieces for individual use.

Assessment of plasma fentanyl concentration after TFP-12 application

TFPs that released 12 μ g of fentanyl/h were used. For each of 6 ball pythons, a TFP-12 was applied to the skin overlying the epaxial muscles just lateral to the vertebral column, approximately 10 to 13 cm from the tip of the nose. A blood sample (approx 0.4 mL) was obtained via cardiocentesis from each snake immediately prior to TFP-12 application (0 hours) and at 6, 24, and 48 hours after TFP-12 application. Blood samples were stored in microtainers containing heparin, placed on ice, and centrifuged ($2000 \times g$) for 10 minutes. Plasma was placed in fresh plastic tubes and frozen on dry ice. Analysis of fentanyl in the plasma samples was conducted with reverse phase high pressure liquid chromatography. The chromatography system consisted of a 2695 separation module and a single-quadrupole mass detector^e. Separation was achieved on a guard column^f followed by a 3.5- μ m C18

^aBackwater Reptiles, Sacramento, CA.

^bReptile Rapture, Monona, WI.

^cNiles Biological Supply, Sacramento, CA.

^dReptoMin floating food sticks, Tetra, Blacksburg, VA.

column (2.1 × 50 mm)^g. The mobile phase was a mixture of water with 0.1% formic acid and acetonitrile with 0.1% formic acid (90:10 ratio). The mobile phase was a mixture of: (solution A) water with 0.1% formic acid, and (solution B) acetonitrile with 0.1% formic acid (90:10 ratio). The mixture was pumped at a starting gradient of 90% solution A and 10% solution B and was adjusted to 10% solution A and 90% solution B over 4 min, and back to initial conditions over 4 min. The flow rate was 0.60 mL/min, and the column temperature was 30°C. The compounds were detected by positive selected ion recording (scan rate, 2 points/second; gain, 1×; capillary voltage, 0.8 kV; cone voltage, 15 V; ion source temperature, 150°C; and probe temperature, 600°C). Nitrogen was used as the nebulizing gas. Fentanyl was detected at 337.34 m/z (mass/charge ratio) and flurazepam (internal standard) was detected at 388.24 m/z. Fentanyl was extracted from plasma samples by liquid extraction with acetonitrile. Previously frozen plasma samples were thawed and vortexed; for each sample, 100 µL of plasma was transferred to a 7-mL glass screw top tube with 25 µL of internal standard (flurazepam, 0.1 µg/mL). One milliliter of acetonitrile was added and tubes were capped, vortexed (for 60 seconds), and centrifuged at 1,020 × *g* for 20 minutes. The supernatant was placed in a clean glass tube and evaporated to dryness with nitrogen gas. Each sample was reconstituted in 200 µL of mobile phase and 50 µL was injected into the high-pressure liquid chromatography system. Standard curves for plasma were prepared by spiking untreated plasma samples with fentanyl to produce a linear concentration range of 5 to 1,000 ng/mL. Spiked standards were processed exactly as were plasma samples. The mean recovery was 100% for fentanyl. With regard to fentanyl concentration, intra-assay variability ranged from 3% to 7% and inter-assay variability ranged from 2.6% to 8.2%. The lower limit of quantification was 0.1 ng/mL.

Thermal antinociception experiments

An incomplete crossover experimental design was used to evaluate TFP-dependent changes in thermal antinociception. The incomplete crossover experimental design was influenced solely by the data collected during this phase of the study. Sixteen snakes were used in control experiments (no TFP application). Of those 16 snakes, 8 were each subsequently treated with a TFP-3 (delivering 3 µg of fentanyl/h) and 9 were each subsequently treated with a TFP-12 (delivering 12 µg of fentanyl/h); 5 snakes received both treatments on separate occasions. The rate of fentanyl delivery in reptiles is not precisely known, although in prehensile-tailed skinks, fentanyl is effectively absorbed across the skin following transdermal administration, attaining detectable plasma concentrations within 4 to 6 hours after application.²²

There was a minimum 12-day period between control experiments and drug administrations, and between drug administrations. The washout duration was not based on pharmacokinetic data because no such data were available. However, the 12-day washout period was considered sufficient because there were no changes in baseline respiratory data between drug treatment experiments for each of the 5 snakes. Snakes were monitored daily for any deleterious effects of the fentanyl patches, but none were observed. The observer (R.J.K.) in

^eAcuity QDa mass detector, Waters, Milford, MA.

^fXBridge C18 guard column, Waters, Milford, MA.

^gXBridge C18 column (2.1 × 50 mm, 3.5 µm), Waters, Milford, MA.

the antinociception experiments was unable to be blinded because of the visible nature of fentanyl patches. Sham experiments were planned as controls, but we were unable to obtain identical patches that lacked fentanyl from the manufacturer or any other source. Use of any other type of adhesive patch would be evident to the observer during the antinociception experiments, thereby preventing performance of true sham experiments.

The degree of antinociception was assessed by measuring the latency of the withdrawal reflex in response to a noxious heat stimulus by use of a standard apparatus^{h,23} and established methods.^{11,12,20,21} The testing chamber comprised clear, plastic boxes (17 × 13 × 14 cm) placed atop an elevated glass surface. Barriers between boxes and all sides of the chamber were opaque except for the top and front to provide visual access for the observer. Smaller snakes were placed within a tinted, transparent, plastic hide boxⁱ (15 × 18 × 11 cm) to limit movement and exploratory activity. An infrared heat source was applied directly beneath the glass surface under the ventral surface of the snake's body 10 to 17 cm from the tip of the nose. The stimuli were applied in approximately the same location on the rostral third of the snake's body regardless of the snake's length.

The intensity setting on the device was 70 for most of the experiments. In our experience, the setting is established empirically based on the animal's response. The goal was to obtain withdrawal latencies of 5 to 10 seconds to allow a dynamic range sufficient to detect increases or decreases in the latencies. The heat source delivered a stimulus that constantly increased in temperature, starting at approximately 25°C. With an electronic temperature probe, we measured temperatures of 40° to 41°C at the time when it was painful for our fingers and likely causing discomfort for the snakes.

Prior to testing, snakes were conditioned for at least 3 days to acclimate them to the chamber, infrared heat stimuli, and random movements of the observer's hand and infrared heat source underneath the glass. The apparatus automatically measured latencies via a motion-sensitive timer, which stopped counting when the animal moved or withdrew its body from the heat stimulus (at 39° to 40°C). As a precaution to prevent tissue damage, the apparatus allowed for only a maximum exposure of 32.6 seconds. Preliminary experiments were performed to determine the most consistent and reproducible body location for application of the heat stimulus, the appropriate number of trials, time between trials to minimize learned responses, and the optimal intensity to achieve baseline withdrawal latencies of 5 to 15 seconds. Mean withdrawal latency for each time point was the mean of 2 latencies obtained via individual stimulus applications 15 minutes apart. All antinociception experiments were conducted at an ambient temperature of 26° to 28°C.

On each testing day, snakes were allowed a 3-hour acclimatization period to adjust to the chamber prior to data collection. Control treatments, during which no TFP was administered, were completed to assess potential time-dependent changes in withdrawal latency. After the 12-day period, snakes were randomly assigned to receive either the TFP-3 (delivery of 3 µg of fentanyl/h) or TFP-12 (delivery of 12 µg of fentanyl/h) treatment.^j

^hUgo Basile Plantar Analgesia Instrument, model 37370, Ugo Basile Company, Comerio, Italy.

ⁱSuper Pet "mini igloo", Petsmart, Phoenix, Ariz.

^jFentanyl Transdermal System, Mylan Pharmaceuticals, Morgantown, WV.

Patches were adhered to the skin overlying the epaxial muscles just lateral to the vertebral column approximately 10 to 13 cm from the tip of the nose (the stimulus location was approximately equivalent relative to the length of the snake's body) and at the same relative location on each snake. Snakes were inspected throughout the duration of the experiment to ensure that patches remained firmly and entirely adhered. For 5 of the snakes, a minimum 12-day washout period was allowed before the experiment with the other TFP treatment was performed. Thermal withdrawal latencies were obtained immediately before (0 hours [baseline]) and at 3, 6, 9, 24, and 48 hours after TFP-3 or TFP-12 placement or start of the control experiment. Snakes remained in the chamber during the 0- to 9-hour data collection time points. Snakes were constantly monitored by visual inspection.

Respiratory experiments

A complete crossover experimental design with a minimum 7-day washout period between control (no TFP) and TFP-12 experiments was used to evaluate TFP-dependent changes in respiration in ball pythons. The 7-day washout period was considered adequate because there were no systematic changes in baseline breathing frequency among the snakes during the course of the respiratory experiments. Snakes were monitored daily for any deleterious effects of the fentanyl patches, but none were observed. Breathing frequency (breaths/min) was measured in 10 conscious, unrestrained ball pythons. Of these 10 snakes, 3 were also used in the plasma fentanyl concentration assessment experiments and 7 were used in the thermal antinociception experiments. Control experiments were performed prior to drug administration experiments. Snakes were placed in opaque, air-tight chambers (inner dimensions, 10 × 20 cm) with constant air flow supplied at approximately 0.2 L/min. To detect breathing movements, the inflow and outflow ports were closed and a pressure transducer^k attached to a separate port converted pressure changes to voltage signals. Upward deflections indicated increased pressure (expiration) and downward deflections indicated decreased pressure (inspiration). These signals were digitized and analyzed with a data acquisition system^l and analyzed offline with computer software.^m Snakes were conditioned to the chamber (with airflow) for 60 to 90 min/d for 2 to 4 days prior to testing. On the day of testing, snakes were placed in the chamber (with airflow) and allowed a 60- to 90-minute period of acclimation. Breathing frequency data were collected immediately prior to (0 hours [baseline]) and at 6, 24, and 48 hours after application of no patch or TFP-12 application. All respiratory experiments were conducted at an ambient temperature of 26° to 28°C.

At each time point, respiratory data were recorded during a 10- to 12-minute session (closed chamber) before resuming airflow through the chamber for 30 to 40 minutes. A second set of respiratory data were recorded during another 10- to 12-minute session. Pilot experiments had demonstrated that breathing frequency was unchanged when the chamber was closed for 20 minutes (data not shown). Thus, it was unlikely that there was marked CO₂ accumulation or hypoxia during a 10- to 12-minute recording session. If quiet, steady breathing over a period of 10 minutes was recorded in each of the 2 recording sessions, the mean of the

^kModel DP45-14, Validyne Engineering Corporation, Northridge, CA.

^lDigiData 1200, Axon Instruments, Sunnyvale, CA.

^mpClamp software, Axon Instruments, Sunnyvale, CA.

breathing frequencies from those sessions was calculated for that time point. If quiet, steady breathing was not recorded in one of the 2 recording sessions, a third 10- to 12-minute session was repeated 30 to 40 minutes later. Data were always averaged between 2 recording sessions. Breathing frequency was measured by counting downward inspiratory deflections on the recordings. Obvious irregular movement artifacts were not counted. Snakes were monitored daily following and in between respiratory experiments.

Quantification of μ -opioid receptor mRNA expression in cerebrospinal tissue samples

To gain reliable quantitative information regarding cerebrospinal tissue mRNA expressions, it was important to isolate and freeze the collected tissue samples as soon as possible to prevent mRNA degradation. The goal was to render the snakes insensitive to painful stimuli as rapidly as possible and allow for rapid decapitation and harvesting of tissue samples from the cortex, midbrain (region containing the colliculi), brainstem (pons and medulla), and spinal cord (C3–C4 region). To this end, a high dose of pentobarbital (400 to 750 mg/kg) was administered intracoelomically or IM to each of 15 snakes. There were no obvious differences in snake responses to pentobarbital following intracoelomic or IM administration. Of these 15 snakes, 2 were also used in the plasma fentanyl concentration assessment experiments. After injection of pentobarbital, the snakes were relatively insensitive to gentle sharp poking within 1 to 2 minutes, and completely insensitive to noxious pinching within 2 to 4 minutes. There was also no movement or response to decapitation. Pithing prior to decapitation was not feasible because pithing would destroy the tissues that were needed for harvesting. Decapitation abolishes blood flow to the brain, which contributes to depression of brain function in this hypoxia-sensitive species and prevents washout of pentobarbital from the cerebrospinal tissues. Within 1 to 2 minutes after decapitation, the cortex, midbrain, brainstem, and spinal cord (C3–C4 region) were removed (thereby completely disrupting all nociception) and frozen to preserve μ -opioid mRNA. By use of these methods, the snakes were exposed to brief noxious stimuli (pentobarbital injection) before being rapidly made insensitive to any further noxious stimuli. Turtles ($n = 10$) were intubated and anesthetized with 5% isoflurane (balance oxygen) until the head and limb withdrawal reflexes were eliminated, after which the turtles were decapitated. Tissue samples were removed from the cortex, midbrain (region containing the colliculi), brainstem (pons and medulla), and spinal cord (C3–C4 region) and frozen at -80°C until analysis. Tissue samples from snakes and turtles were homogenized in *RNA isolation reagent*,ⁿ and total RNA was harvested according to the manufacturer's protocol. An RT-PCR assay was performed with 1.0 μg of total RNA as a template for the reverse transcription reaction and a mixture of oligo dT and random hexamers with Moloney murine leukemia virus reverse transcriptase^o according to the manufacturer's instructions. The quantitative PCR procedure was conducted by monitoring, in real-time, the increase in fluorescence of the SYBR green dye^p with a sequence detection system.^q Primer specificity was assessed^r prior to use, and all dissociation curves had a single peak with an observed melting temperature consistent

ⁿTri-Reagent, Sigma-Aldrich, St. Louis, MO.

^oM-MLV RT, Life Technologies, Grand Island, NY.

PSYBR-GREEN Master Mix Dye, Life Technologies, Grand Island, NY.

^qTaqMan 7300 Sequence Detection System, Applied Biosystems, Carlsbad, CA.

^rAvailable at: blast.ncbi.nlm.nih.gov/. Accessed June 27, 2014.

with the intended amplicon sequences. Primer sequences for μ -opioid receptors were obtained by use of the gene sequences for the Burmese python (*Python bivittatus* [Accession No. NW_006533509.1]) and painted turtles (*Trachemys scripta elegans* [Accession No. NC_024220.1]). Multiple gene sequence alignment analysis^s was used to identify regions conserved between both species. The PCR primers for μ -opioid receptors and β -actin in snakes and turtles were designed in the most conserved regions of the respective genes. The forward and reverse sequences were used for β -actin analysis in both the ball python and red-eared slider tissues were 5'-CAT GTT TGA GAC CTT CAA CAC C-3' and 5'-CTT CAT AGA TGG GCA CAG TGT-3', respectively. The forward and reverse sequences were used for μ -opioid receptor analysis in both the ball python and red-eared slider tissues were 5'-CTC TGC ACC ATG AGT GTG GAT CGC-3' and 5'-CCA GTT GCA GAC ATT GAC AAT TTT G-3', respectively. μ -Opioid receptor gene expression data were analyzed by means of the delta C_T method, as previously described.²⁴ All samples were run in duplicate and values were normalized against β -actin gene expression. If the normalized gene expression data for an individual sample was > 2 SDs from the mean, the sample was excluded as an outlier by use of the Grubb's test. Turtle cortical μ -opioid mRNA was normalized to 1.0 and all other turtle and snake tissue mRNA samples were expressed relative to turtle cortical μ -opioid mRNA levels.

Data analysis

Commercially available software^t was used to analyze data with a repeated-measures, 2-way ANOVA (data from the thermal antinociception and respiratory experiments) and 2-way ANOVA (data from the RT-PCR experiments). Normality and equal variance assumptions were satisfied for the thermal antinociception, respiratory, and RT-PCR data. Post-hoc comparisons were completed with the Student-Newman-Keul test. Linear regression analysis was performed with the same software.^t All data are expressed as mean $\pm$ SEM with significance inferred at a value of $P < 0.05$.

Results

Plasma fentanyl concentrations

The 6 snakes used for this assessment weighed 286 ± 97 g (range, 70 to 709 g); the weight range spanned the range of weights for snakes used in the thermal antinociception and respiratory experiments. Fentanyl was not detected in plasma samples obtained from the snakes prior (0 hours) to the application of a TFP-12. After TFP-12 application, plasma fentanyl concentration was 79 ± 45 ng/mL at the 6-hour time point, 238 ± 71 ng/mL (range, 12 to 501 ng/mL) at the 24-hour time point, and 111 ± 46 ng/mL at the 48-hour time point. Smaller snakes generally had higher plasma fentanyl concentrations, compared with those for larger snakes, but this was a weak correlation (eg, $r^2 = 0.56$ for comparison of calculated mean ng/mL/kg versus mass at the 24-hour time point; data not shown). These data indicated that the TFPs used (providing 12 μ g of fentanyl/h) were highly effective in delivering fentanyl into the bloodstream of ball pythons.

^sMULAN; Available at mulan.decode.org/. Accessed June 27, 2014.

^tSigma Stat, Jandel Scientific Software, San Rafael, Calif.

Thermal antinociception experiments

In the control experiments, the 16 snakes were untreated (no TFP application) and mean baseline (0-hour) withdrawal latency was 9.7 ± 0.6 seconds. Latencies did not change ($P > 0.05$) from baseline in untreated snakes, with mean latencies of 9.3 to 10.0 seconds at 3 to 48 hours (Figure 1). For the snakes treated with a TFP-3 ($n = 8$) or a TFP-12 (9), mean baseline withdrawal latencies were 10.0 ± 0.6 seconds and 9.8 ± 1.1 seconds, respectively; both baseline withdrawal latencies were similar to the value determined in the control experiments. For both drug treatments, mean withdrawal latencies at 3 to 48 hours after TFP application were not changed from baseline ($P > 0.05$).

Respiratory experiments

After acclimating to the breathing chamber, each of the 10 snakes had rhythmic increased and decreased pressure changes corresponding to expiration and inspiration, respectively (Figure 2). Results of control experiments in which snakes had no TFP application indicated that there were no time-dependent changes ($P > 0.05$) in breathing frequency from baseline (0-hour) value (3.5 ± 0.4 breaths/min) to that at the 48-hour time point (3.3 ± 0.5 breaths/min; Figure 3). In contrast, breathing frequency decreased ($P = 0.013$ for drug effect) from a baseline value of 3.2 ± 0.3 breaths/min to 2.3 ± 0.3 breaths/min and 1.9 ± 0.2 breaths/min at 24 and 48 hours after TFP-12 application, respectively. Compared with control experiment findings, this represented a decrease in breathing frequency of 28% and 41%, respectively, when snakes received the TFP-12 treatment. Although tidal volume could not be accurately quantified with this technique, inspiratory traces generally increased in amplitude when breathing frequency was depressed, suggesting that tidal volume may have increased during depression of breathing frequency.

Quantification of μ -opioid receptor mRNA expression in brain and spinal tissue samples

Tissue samples from the cortex, midbrain (collicular level), brainstem (pons and medulla), and spinal cord (C3–C4 region) were collected from 15 snakes and 10 turtles for comparison of μ -opioid receptor mRNA expression. The PCR cycle numbers for snake β -actin mRNA and μ -opioid receptor mRNA were 19.8 ± 0.5 (range, 15.8 to 23.6) and 26.9 ± 0.5 (range, 21.0 to 30.0), respectively. The PCR cycle numbers for turtle β -actin mRNA and μ -opioid receptor mRNA were 18.4 ± 0.2 (range, 15.8 to 22.8) and 26.0 ± 0.3 (range, 21.8 to 28.6), respectively. Relative to turtle cortex (set to 1.0), overall μ -opioid receptor mRNA expressions in the cerebrospinal tissue samples from turtles and snakes did not differ ($P = 0.53$). For turtles, midbrain μ -opioid receptor mRNA expression was greater ($P = 0.001$) than that in cortical tissues; however, turtle brainstem and spinal cord expressions were similar to that in cortical tissues (Figure 4). For snakes, midbrain and brainstem μ -opioid receptor mRNA expressions were greater ($P = 0.001$ and $P = 0.009$, respectively) than that in cortical tissues; spinal cord expression was similar to that in cortical tissues.

Discussion

To our knowledge, this is the first study to examine the analgesic and respiratory effects of an opioid drug administered transdermally in any reptile species. Also, this is the first study to quantify μ -opioid receptor mRNA expression in snakes and another reptile species (ie,

turtles) to assess whether differences in μ -opioid receptor mRNA expression is associated with biologically different responses to μ -opioid receptor agonist drugs. The study findings indicated that fentanyl administered transdermally, even at relatively high doses (as indicated by quantification of plasma fentanyl concentrations), was not effective in increasing thermal withdrawal latencies, but decreased breathing frequency at 24 and 48 hours after application of a TFP-12 in ball pythons. The similar expressions of central μ -opioid receptor mRNA in snakes and turtles suggested that snakes do not have comparatively lower μ -opioid receptor expression in the CNS. Thus, ball pythons likely express functional μ -opioid receptors, but μ -opioid receptor signaling appears to be altered such that fentanyl administration only depresses breathing frequency with minimal antinociceptive effects (as determined by the noxious thermal stimulus experiments in the present study).

The pharmacokinetics of fentanyl in 2 reptile species have been previously evaluated in 2 studies.^{16,22} In ball pythons, plasma fentanyl concentrations reached approximately 1 ng/mL within 4 hours after application of a TFP that delivered 12.5 μ g of drug/h and remained at approximately 4 to 12 ng/mL at 168 hours after TFP application.¹⁶ In prehensile-tailed skinks, fentanyl was applied as a 10% exposure of total surface area of a TFP delivering 25 μ g of drug/h for 72 hr. Fentanyl was effectively absorbed across the skin, reaching detectable plasma concentrations within 4 to 6 hours and lasting for > 72 hours.²² Results of the present study indicated that TFPs remain attached to snakes for > 48 hours and effectively deliver fentanyl to the blood. Plasma fentanyl concentrations in this study were relatively high (50 to 200 ng/mL), compared with findings for the ball pythons in the previous study,¹⁶ perhaps because the snakes we used were much smaller (weight range was 46 to 1,038 g, compared with a range of 2.2 to 2.9 kg in the previous study¹⁶). When TFPs are applied to dogs, plasma fentanyl concentrations > 0.6 ng/mL are considered analgesic.²⁵ In the human medical literature, plasma fentanyl concentrations between approximately 0.5 and 2.0 ng/mL are considered analgesic.²⁶ The plasma concentrations of fentanyl that provide analgesia in reptiles are not known (even in μ -opioid receptor agonist-sensitive species, such as turtles), but the plasma fentanyl concentrations in ball pythons exceeded concentrations considered therapeutic in many domestic veterinary species by at least 2 orders of magnitude. Thus, TFPs appear to be effective for delivering fentanyl to the bloodstream in snakes at what is generally considered to be biologically and clinically meaningful concentrations in other species.

In the present study, there are 2 factors to note regarding the plasma fentanyl concentrations in ball pythons. First, given the impressively high plasma fentanyl concentration at 24 hours after TFP-12 application (mean concentration of 238 ng/mL with therapeutic concentrations of approximately 1.0 ng/mL), it was likely that application of a TFP-3 delivered fentanyl to achieve a plasma concentration exceeding the 1.0 ng/mL threshold. Second, despite generating high plasma fentanyl concentrations with TFP-12 application, the 12-day washout was sufficient for the thermal antinociception experiments studies because baseline withdrawal latencies were not altered from those obtained in snakes under control conditions.

Several clinically relevant drugs that activate μ -opioid receptors appear to induce antinociception in reptiles. Morphine and meperidine increase thermal withdrawal latencies

in crocodiles,¹⁰ whereas morphine increases thermal withdrawal latencies in bearded dragons and red-eared slider turtles.^{11,12} In addition, hydromorphone increases thermal withdrawal latencies in red-eared slider turtles, but buprenorphine surprisingly has no effect on withdrawal latencies in this species.¹³ Oral administration of tramadol (a μ -opioid receptor agonist and serotonin- and norepinephrine-reuptake inhibitor) also increases thermal withdrawal latencies in red-eared slider turtles.²⁰ For snakes, there are no studies demonstrating analgesic efficacy for any drug,⁴ to our knowledge. In a study of postsurgical pain in ball pythons, administration of meloxicam and butorphanol 3 hours prior to surgery did not alter physiologic variables measured at 1.5 and 72.5 hours after surgery, compared with findings in control snakes injected preoperatively with saline solution.¹⁵ In our laboratory, morphine sulfate (1 to 40 mg/kg) administered SC did not alter thermal withdrawal latencies in corn snakes.¹²

The present study was conducted to determine whether snakes would respond to a μ -opioid receptor agonist drug that was delivered via TFPs over an extended period. In mammals, transdermal delivery of fentanyl provides effective long-lasting antinociception in the treatment of perioperative and chronic pain.^{27–31} After TFP-12 application, the high plasma fentanyl concentrations measured in ball pythons, in addition to the respiratory data indicating that breathing frequency was decreased from the pretreatment value at 24 and 48 hours, suggested that TFPs on ball pythons resulted in fentanyl plasma concentrations sufficient to activate central μ -opioid receptors and depress breathing frequency.

A surprising finding of the present study was that TFP application did not alter noxious thermal withdrawal latencies in snakes. We hypothesized that μ -opioid receptor protein concentration regulating nociception are decreased in snakes, compared to findings in other reptiles; however, results of the present study indicated that μ -opioid receptor mRNA expressions in 4 cerebrospinal regions in ball pythons and red-eared slider turtles (a species that is sensitive to μ -opioid agonist drugs) are similar. It is possible that μ -opioid receptor protein concentration is not correlated with mRNA expression, such that μ -opioid receptor protein concentration in central nociceptive neurons is less than that in respiratory-related neurons despite similarities in μ -opioid receptor expression. Alternatively, μ -opioid receptors in snake central nociceptive neurons might be less responsive to μ -opioid agonist drugs because of morphine-resistant polymorphisms (as expressed in human μ -opioid receptors³²) or differences in posttranslational mRNA modifications. This seems unlikely, however, because even if there was a large right shift in the dose-response curve for opioid drugs in snakes, one would expect at least some small increase in thermal withdrawal latencies at high plasma fentanyl concentrations. Finally, there may be regional differences in the CNS such that μ -opioid receptors in central nociceptive neurons are either uncoupled from their normal signaling pathways or some local factor is produced that interferes with opioid agonist drugs binding to or activating μ -opioid receptors. The fact that opioid-resistant nociception is observed in corn snakes¹² and ball pythons suggests that this may be a widespread phenomenon in snakes that will should be investigated in future studies.

Application of a noxious thermal stimulus to an animal's limb is an established method for assessment of nociception and analgesic efficacy in mammals,²³ birds,^{33,34} and reptiles.^{11,12,14,20,21} For our experiments in ball pythons, experimental conditions were

carefully designed to minimize spontaneous movement and optimize stimulus sensitivity, conditioning, and body location for stimulus application. However, the thermal withdrawal model is only one method for assessing nociception. There may be distinct differences in thermal nociceptive pathways as opposed to other types of somatic nociceptive sensation, and these differences may vary across species. The response of snakes to a noxious thermal stimulus may differ from responses to other nociceptive stimuli,¹² or the observed response in snakes to noxious thermal stimuli may have more to do with temperature or touch perception as opposed to nociception. We acknowledge that a positive control (ie, a drug with established analgesic properties that increases withdrawal latencies) has not been identified yet for use in snake noxious thermal antinociception studies, and that identification of such a positive control will be an important step for validating this experimental approach. On the other hand, the hypothesis that snake responses to noxious thermal stimuli are unique compared to other reptiles seems unlikely because in response to the noxious thermal stimulus, 100% of the snakes in the present study displayed very evident avoidance behaviors (eg, visibly flinching or moving away) in a manner similar to that of rodents or other reptiles. Although perioperative behavioral and physiologic experiments are useful and important, we suggest that the noxious thermal withdrawal test is valuable for screening potential analgesic drugs. The technical improvements in the experimental approach in our study may be useful for future thermal antinociception experiments in snake species because we were able to obtain consistent, reproducible withdrawal latencies, which has been difficult to achieve in previous studies involving the thermal withdrawal method in snakes.¹²

Another potential caveat to the antinociception experiments in the present study was the possibility that the presence and activity of the observer may alter withdrawal latencies. To address this potential concern, snakes were acclimated to the apparatus for several days prior to the experiments. The noxious thermal device was moved repeatedly and randomly underneath the glass surface to get the snakes used to the investigator moving around the apparatus. Use of ball pythons in this type of experiment is advantageous because they prefer to ball up and remain relatively immobile for extended periods. In the present study, smaller snakes were placed in translucent hide boxes that encouraged them to ball up within the apparatus. On the basis of our extensive experience with this species, there were no obvious indications that the snake behavior was affected by the presence of an observer. If this were the case, there would be considerable variability in the withdrawal latency data.

Respiratory depression during opioid drug administration remains an important problem in human and veterinary medicine. Nevertheless, little is known regarding how opioid drugs modulate breathing in reptiles. In intact red-eared slider turtles, injection of either a μ -opioid or δ -opioid receptor agonist drug decreases ventilation owing to a decrease in breath frequency with little change in tidal volume.¹⁸ Similarly, bath application of either a μ -opioid or δ -opioid receptor agonist drug to isolated turtle brainstems spontaneously results in a respiratory motor pattern with a decrease in respiratory burst frequency in a concentration-dependent manner.^{17,19} With respect to clinically relevant drugs, breathing frequency decreases following administration of morphine and butorphanol¹¹ and tramadol.²⁰ Because μ opioid receptor activation decreases the frequency of respiratory-

related motor output in brainstems of frogs³⁵ and lampreys,⁸ opioid-dependent depression of respiratory frequency appears to be evolutionarily conserved in amphibians and jawless fish.

For snakes, however, little is known with respect to opioid-induced changes in breathing. In 1 study,¹² corn snakes appeared to tolerate morphine doses as high as 40 mg/kg without obvious deleterious effects (ie, death), but breathing frequency was not quantified. This dose of morphine was 40 times the dose that causes significant respiratory depression in turtles,¹¹ suggesting that the respiratory control system in snakes is resistant to μ -opioid receptor agonist drugs. In the present study, whole-body, closed chamber plethysmography was used because this allowed the ball pythons to coil up within the chamber and breathe quietly. Although this noninvasive technique does not allow accurate quantification of tidal volume, breathing frequency was easily measured. This is important because most previous studies have revealed that μ -opioid receptor activation decreases ventilation in ectothermic vertebrates primarily by decreasing breathing frequency. Accordingly, breathing frequency in ball pythons was significantly decreased from the pretreatment value at 24 and 48 hours following TFP application. This has suggested that ball pythons have functional μ -opioid receptors within the respiratory control system that modulate breathing frequency in a manner similar to that in other ectothermic vertebrates.

To quantify μ -opioid receptor mRNA expression in snakes and turtles in the present study, a semi-quantitative RT-PCR procedure was chosen because it allowed quantification of specific sequences within the μ -opioid receptor genes for each species. Immunohistochemical analysis is a logical choice for localizing μ -opioid receptor protein but extensive work would be required to verify that commercially available polyclonal antibodies bind to reptile μ -opioid receptors. Likewise, autoradiography has been used to localize and quantify μ - and δ -opioid receptors in turtles and rats,³⁶ but the selectivity for radioactive μ - and δ -opioid receptor ligands in turtles has not been accurately determined. For example, presumably selective δ -opioid receptor agonist drugs are known to cross over and activate μ -opioid receptors.³⁷ Thus, an RT-PCR method offers a high degree of specificity for measuring μ -opioid receptor mRNA, with the acknowledged limitation that concentrations of μ -opioid receptor protein would need to be localized and quantified by other methods. Red-eared slider turtles were chosen for comparison with ball python snakes because these turtles are highly responsive to μ -opioid receptor agonist drugs. Although we found similar expressions of μ -opioid receptor mRNA in 4 cerebrospinal regions in ball pythons and turtles, reptiles may generally have lower amounts of μ -opioid receptors, compared with mammals such as rodents.³⁶ The PCR cycle numbers for the standard gene (β -actin) and μ -opioid receptors were relatively high in ball pythons and turtles, which suggested that there was low expression of μ -opioid receptors in these species. Despite the potentially low μ -opioid receptor mRNA expression in the tissues of the CNS, the difference in sensitivity to μ -opioid receptor agonist drugs between turtles and snakes is still surprising and requires further investigation to develop appropriate pharmacological treatments for antinociception in snakes.

Snakes, including venomous species, are routinely kept in zoological collections and are commonly examined in private veterinary practices. The use of a transdermal, continuous delivery system for pain management in snakes is an attractive treatment option as it would

decrease the frequencies of handling and injection administration while likely improving healing and recovery time. Although transdermal administration of fentanyl did not appear to be effective in the snakes in the present study, one should not rule out the potential for transdermal delivery systems to be used in reptile species. For long-lasting antinociception, transdermal administration of fentanyl may be useful in chelonians and lizards given that morphine increases thermal withdrawal latencies in red-eared slider turtles and bearded dragons.^{11,12} With respect to opioid drug responses, there are clearly major species differences within the class Reptilia, which pose unique problems in veterinary medicine.

Acknowledgments

Supported in part by the Merial Summer Scholars Program (Kharbush), Morris Animal Foundation Summer Scholars Program (Gutwillig); the Department of Comparative Biosciences, School of Veterinary Medicine, University of Wisconsin-Madison (Johnson); and NIH grant R01 HL111598 (Kimyon, Watters).

Abbreviations

TFP	Transdermal fentanyl patch
RT	Reverse transcription

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Figure 1.

Mean $\pm$ SEM thermal withdrawal latencies during a 48-hour period in ball pythons that were or were not treated transdermally with fentanyl. Sixteen snakes were used in control experiments (no TFP application [A]). Of those 16 snakes, 8 were each subsequently treated with a TFP-3 (delivering 3 µg of fentanyl/h [B]) and 9 were each subsequently treated with a TFP-12 (delivering 12 µg of fentanyl/h [C]); 5 snakes received both treatments on separate occasions. The degree of antinociception was assessed immediately before (0 hours) and at 3, 6, 9, 24, and 48 hours after TFP-3 or TFP-12 application (or start of the control experiment) by measuring the latency of the withdrawal reflex in response to a noxious heat stimulus in a standardized manner. There was a minimum 12-day period control experiments and drug administrations, and between drug administrations. Mean thermal withdrawal latency of ball pythons included in either drug treatment group did not differ ($P > 0.05$) from the value for snakes in the control experiment at 0 hours or at any other time point.

Figure 2.

Representative respiratory-related traces obtained by whole-body, closed-chamber plethysmography illustrating breathing movements in an untreated ball python (left traces) and a ball python following TFP-12 application (right traces). Breathing frequency was assessed before (0 hours) and at 3, 6, 9, 24, and 48 hours after TFP-12 application or start of the control experiment. Downward deflections represent inspiratory movements (Insp) and upward deflections represent expiratory movements (Exp). The patterns of the traces indicate that fentanyl decreased breathing frequency (e.g., note traces at 24-hour time point).

Figure 3.

Mean $\pm$ SEM breathing frequencies during a 48-hour period in 10 snakes that were assessed following no treatment (control; circles) or following application of a TFP-12 (triangles) in a crossover experiment. Breathing frequency was assessed by whole-body, closed-chamber plethysmography before (0 hours) and at 3, 6, 9, 24, and 48 hours after TFP-12 application or start of the control experiment. *For the TFP-12 treatment, the value is significantly ($P < 0.05$) different from that at 0 hours. †At this time point, value is significantly ($P < 0.05$) different from the control value. ‡ Indicates $P < 0.05$ for drug effect, which compares all the control data with the TFP-12 data.

Figure 4.

Mean $\pm$ relative μ -opioid receptor mRNA expressions (arbitrary units [AU]) in tissue samples obtained from the cortex, midbrain (region containing the colliculi), brainstem (pons and medulla), and spinal cord (C3–C4 region) in 15 ball pythons (black bars) and 10 turtles (white bars). Data were normalized to turtle cortex μ -opioid receptor mRNA expression (ie, turtle cortex mean expression = 1.0). Turtle midbrain μ -opioid receptor mRNA expression was greater than the turtle cortex expression, whereas snake midbrain and brainstem μ -opioid receptor mRNA expressions were greater than the snake cortex expression. *Within a bracket, value differs significantly $P < 0.05$ from that of the cortical tissue from the same species.