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Excitatory and inhibitory effects of opioid agonists on respiratory motor output produced by isolated brainstems from adult turtles (*Trachemys*)

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Abstract

To determine how central opioid receptor activation alters turtle breathing, respiratory-related hypoglossal (XII) motor bursts were recorded from isolated adult turtle brainstems during 60-min bath-applications of agonists for delta- (DOR), kappa- (KOR), or nociceptin/orphanin (NOR) receptors. DADLE (DOR agonist) abolished XII burst frequency at 0.3-0.5 μ M. DPDPE (DOR agonist) increased frequency by 40-44% at 0.01-0.1 μ M and decreased frequency by $88 \pm 8\%$ at 1.0 μ M. U-50488 and U-59693 (KOR agonists) decreased frequency by 65-68% at 100 μ M and 50 μ M, respectively. Orphanin (NOR agonist) decreased frequency by 31-51% at 1.0-2.0 μ M during the first 30-min period. Orphanin (0.5 and 2.0 μ M) increased bursts/episode. Although morphine (10 μ M) abolished frequency in nearly all brainstems, subsequent co-application of phenylephrine (α_1 -adrenergic agonist, 20-100 μ M) with morphine restored activity to 16-78% of baseline frequency. Thus, DOR, KOR, and NOR activation regulates frequency and NOR activation regulates episodicity, while α_1 -adrenergic receptor activation reverses opioid-induced respiratory depression in turtles.

Keywords

reptile; chelonian; respiratory depression; episodic breathing

1. Introduction

Opioid drugs are clinically administered to relieve pain, as well as reduce coughing, diarrhea, and anxiety. However, opioid drugs have deleterious side effects, such as respiratory depression, nausea and vomiting, drowsiness, dry mouth, hypotension, and constipation (Petti and Arndt, 1993; Inturrisi, 2002). In mammals, opioid-dependent respiratory depression is due to decreased respiratory frequency, tidal volume, and chemoreceptor drive, as well as increased upper airway resistance and altered pulmonary mechanics (Santiago and Edelman, 1985; Shook et al., 1990; Babenco and Gross, 1993). Opioid-dependent changes in breathing are primarily due to opioid receptor activation on brainstem respiratory neurons (Denavit-Saubie et al., 1978; Rondouin et al., 1981; Takita et al., 1997; Gray et al., 1999; Takeda et al., 2001; Lalley, 2003, 2006). However, systemic opioid administration also activates peripheral opioid receptors in the lungs that modulate pulmonary reflexes and alter cardiorespiratory function (Willette and Sapru, 1982a,b;

Willette et al., 1982). Likewise, opioid receptor activation in the carotid body inhibits chemoreceptor afferent discharge (McQueen and Ribeiro, 1980; Kirby and McQueen, 1986).

In contrast, far less is known about the opioid drug effects on breathing in non-mammalian ectothermic vertebrates, such as jawless fish, amphibians, and reptiles. Mu-opioid (MOR) and delta-opioid (DOR), but not kappa-opioid (KOR), activation reduces respiratory burst frequency without altering burst pattern in isolated lamprey brainstems (Mutolo et al., 2007). In intact frogs, morphine (primarily MOR agonist with DOR and KOR activity) reduces lung ventilation frequency, lung episode frequency, and the number of lung breaths/episode (Vasilakos et al., 2005, 2006). In reptiles, such as red-eared slider turtles (*Trachemys scripta*), morphine and butorphanol (mixed KOR agonist and MOR agonist/antagonist) depresses ventilation due to a 60-80% reduction in breathing frequency (Sladky et al., 2007). This respiratory depression appears to be due to MOR and DOR activation since systemic administration of specific MOR or DOR agonists decrease ventilation by decreasing breath frequency (Johnson et al., 2008). On the other hand, KOR activation produces highly variable effects on breath frequency (some turtles stop breathing for min to h) and increases tidal volume in time-dependent manner (Johnson et al., 2008). Given that systemic drug administration in intact animals may also alter the function of peripheral chemo- and mechanoreceptors, it is important to test how central opioid receptor activation alters respiratory motor output. In isolated turtle brainstems (Johnson et al., 2002) and hemibrainstems (Majewski et al., 2008), MOR activation abolishes respiratory motor output, but the effects DOR or KOR activation *in vitro* are not known. Furthermore, only one study showed that NOR activation in neonatal rat brainstem-spinal cord preparations decreased respiratory frequency with little effect on spinal respiratory motor output amplitude (Takita et al., 2003). Thus, the role of NOR activation in adult vertebrate respiratory motor control is not known.

Finally, respiratory depression is the most significant adverse side effect of opioid drugs in human and veterinary clinical medicine (Pattinson, 2008). Consequently, several pharmacological methods for reversing opioid-induced respiratory depression have been developed in mammalian experimental preparations. For example, opioid-induced respiratory depression is reversed while maintaining analgesia by co-administering opioid drugs with a serotonin 5-HT₄ agonist (Manzke et al., 2003), dopamine D₁ agonist (Lalley, 2005), cholinesterase inhibitor (Weinstock et al., 1980), ampakine (Ren et al., 2006), or DOR agonist drug (Su et al., 1998). For reptiles and other non-mammalian vertebrates, however, there are no studies testing whether opioid-induced respiratory depression can be reversed. Since α_1 -adrenergic receptor activation augments respiratory frequency (Johnson et al., 1998) and induces long-lasting frequency increases in isolated turtle brainstems (Wilkerson et al., 2003b), α_1 -adrenergic agonists are candidate drugs for reversing opioid-induced respiratory depression in reptiles.

To address these questions, adult turtle brainstems were isolated under *in vitro* conditions, spontaneous respiratory motor output on hypoglossal (XII) nerve roots was recorded, and brainstems were exposed to specific opioid receptor agonists. *In vitro* turtle brainstems are advantageous because inspiratory- and expiratory-related motor output is produced that is qualitatively similar to that produced by intact turtles (Douse and Mitchell, 1990; Johnson et al., 1998). Also, turtle brainstems are extremely resistant to hypoxia (Johnson et al., 1998; Jackson, 2000), which means that respiratory-related motor output can be produced by fully mature turtle brainstems for several days at physiologically relevant temperatures (Wilkerson et al., 2003a). We hypothesized that brainstem DOR and NOR activation would decrease respiratory burst frequency without altering burst amplitude, while brainstem KOR activation would have no effects. Surprisingly, we found that DOR had mixed, dose-dependent excitatory and inhibitory effects on respiratory burst frequency, KOR decreased

burst frequency, and NOR activation increased the number of bursts/episode (episode = two or more respiratory motor bursts occurring sequentially followed by a pause). Finally, we found that α_1 -adrenergic receptor activation reversed morphine-induced respiratory depression in isolated turtle brainstems.

2. Methods

All procedures were approved by the Animal Care and Use Committee at the University of Wisconsin-Madison School of Veterinary Medicine. Adult red-eared slider turtles (*Trachemys scripta*, $n = 155$; 750 ± 11 g) were obtained from commercial suppliers and kept in large open tanks where they had access to water for swimming, and heat lamps and dry areas for basking. Room temperature was set to 27–28°C with light 14 hr/day. Turtles were fed ReptoMin® floating food sticks (Tetra, Blacksburg, VA) 3–4 times per week.

2.1 Turtle brainstem preparations

Turtles were intubated and anesthetized with 5% isoflurane (balance oxygen) until head and limb withdrawal reflexes were eliminated, upon which the turtles were decapitated. The brainstem was removed and pinned onto Sylgard® in a recording chamber (5 ml volume). The tissue was superfused (4–5 ml/min) with artificial cerebrospinal fluid (aCSF) containing HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid]) buffer as follows (mM): 100 NaCl, 23 NaCHO₃, 10 glucose, 5 HEPES (sodium salt), 5 HEPES (free acid), 2.5 CaCl₂, 2.5 MgCl₂, 1.0 K₂PO₄, 1.0 KCl. The HEPES solution was bubbled with 5% CO₂-95 % O₂ to maintain pH 7.34 ± 0.01 , as measured periodically with a calomel glass pH electrode (Cole-Parmer Inst. Co., Vernon Hills, IL, USA). The low pH of the aCSF (normal turtle arterial pH = 7.6) is necessary to “drive” turtle brainstems to produce respiratory motor output that has an amplitude and frequency that permits experiments to be performed in a timely manner (see Johnson et al., 1998).

2.2 Electrophysiological recordings

Glass suction electrodes were attached to XII nerve rootlets (Fig. 1). Signals were amplified (10,000x) and band-pass filtered (1.0–500 Hz) using a differential AC amplifier (model 1700, A-M Systems, Everett, WA, USA) before being rectified and integrated (time constant = 200 ms) using a moving averager (MA-281/RSP, CWE, Inc., Ardmore, PA, USA). The signals were digitized (50 Hz) and analyzed using Axoscope software (Axon Instruments, Foster City, CA, USA) and MiniAnalysis software (Synaptosoft, Inc., Decatur, GA, USA).

2.3 Drugs

The following drugs were obtained from Sigma/RBI Aldrich (St. Louis, MO): DADLE (DOR agonist; [D-Ala², D-Leu⁵]-Enkephalin acetate salt), DPDPE (DOR agonist; [D-Pen^{2,5}]-Enkephalin hydrate), U-50488 (KOR agonist; (–)-trans-(1S,2S)-U-50488 hydrochloride hydrate), U-69593 (KOR agonist; (+)-(5 α ,7 α ,8 β)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide), orphanin (NOR agonist; Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln); and phenylephrine (α_1 -adrenergic receptor agonist). Morphine sulfate was obtained from Baxter Healthcare Corporation.

2.4 Experimental protocols

After the brainstems were allowed to equilibrate for 4–6 h, baseline data were obtained by recording 30 min of spontaneous respiratory motor activity before adding drugs to the reservoir. After a 60-min drug exposure, the bath was washed with drug-free aCSF for 120 min. For the reversal of morphine-induced depression experiments, baseline recordings (30

min) were obtained, morphine (10 μ M) was bath-applied (60 min), and then morphine (10 μ M) plus phenylephrine (at different concentrations) was applied for 60 min.

2.5 Data Analysis

Respiratory burst frequency was defined as the number of bursts/min. Percent baseline frequency was calculated by setting baseline frequency to 100% and all other frequencies relative to baseline frequency. Burst amplitude was defined as the maximum height of the burst relative to baseline burst amplitude. Two or more bursts separated by less than the average duration of a single burst were defined as an episode. All measurements were averaged into 30-min bins and reported as the mean $\pm$ SEM. A two-way ANOVA with repeated measures design (Sigma Stat, Jandel Scientific Software, San Rafael, CA, USA) were used to determine if data were significantly different ($p < 0.05$) from baseline and time controls. If normality or equal variance assumptions were violated, data were ranked, and the ANOVAs recalculated. Multiple comparisons were made using Student-Newman-Keul's test.

3. Results

3.1 DOR-dependent excitatory and inhibitory effects on burst frequency

For brainstems ($n=12$) not exposed to drug, there were no changes in XII burst frequency during the 180-min period (Figs. 2B, 2C) and a time-dependent decrease in burst amplitude of $23 \pm 5\%$ after 180 min ($p=0.007$; Fig. 2D). When DADLE (0.1 μ M; DOR agonist) was bath-applied to turtle brainstems ($n=5$), there were no changes in XII burst frequency (Figs. 2B, 2C) or amplitude (Fig. 2D). In contrast, bath-applied DADLE at 0.3 μ M ($n=6$) or 0.5 μ M ($n=6$) abolished nearly all respiratory motor output within 60 min (burst frequency was very low at 0.02 bursts/min in one brainstem at 0.5 μ M) ($p=0.001$ for drug effect; Figs. 2A, 2B, 2C). DADLE-dependent frequency depression was reversible with frequency returning to 78–127% of baseline after a 2.0-h washout (Fig. 2C). Since most brainstems exposed to 0.3 or 0.5 μ M DADLE were silent, we could not determine whether DADLE altered burst amplitude. However, after a 30-min exposure to 0.3 μ M DADLE, burst amplitude was $0.85 \pm 0.04\%$ of baseline ($p=0.028$; Fig. 2D), suggesting that DADLE also decreased burst amplitude. Following the 2.0-h washout, burst amplitude was decreased by $16 \pm 6\%$ and $30 \pm 8\%$ for the 0.3 μ M and 0.5 μ M DADLE exposures, respectively ($p=0.009$ and $p=0.013$; Fig. 2D). In brainstems ($n=3$) exposed to 20 μ M DADLE, XII motor activity was rapidly abolished in three brainstems and did not recover after a 2.0-h washout (data not shown).

In contrast to the DADLE exposures, bath-applied DPDPE (DOR agonist) produced dose-dependent, biphasic effects on XII burst frequency. Brainstems exposed to low DPDPE concentrations at 0.01 μ M ($n=7$) or 0.1 μ M ($n=6$) increased XII burst frequency by $40 \pm 15\%$ ($p=0.023$) and $44 \pm 13\%$ ($p < 0.001$) after 60 min, respectively (Figs. 3A₁, 3B, 3C). For brainstems exposed to 1.0 μ M DPDPE ($n=11$), XII burst frequency decreased by $88 \pm 8\%$ after 60 min ($p=0.026$; Figs. 3A₂, 3B, 3C). Nearly all of the DPDPE-dependent frequency effects were reversed following a 2.0-h washout (Figs. 3B, 3C). DPDPE at 0.01–1.0 μ M had no effect on burst amplitude during drug application (Fig. 3D).

3.2 KOR activation decreases burst frequency and amplitude

Bath-applied U-50488 (KOR agonist) at 20 μ M ($n=5$) and 50 μ M ($n=8$) did not alter XII burst frequency (Figs. 4B, 4C) and amplitude (Fig. 4D). U-50488 at 100 μ M ($n=7$) decreased burst frequency by $65 \pm 13\%$ ($p=0.001$ for drug effect) during the 60-min exposure, an effect that persisted after a 2.0-h washout (Figs. 4A, 4C). This concentration of U-50488 also decreased burst amplitude by $23 \pm 13\%$ after the 60-min exposure ($p=0.015$; Fig. 4A, 4D). Similarly, lower concentrations of a different KOR agonist, U-69593, at 10

μM ($n=4$) and $20\ \mu\text{M}$ ($n=3$) did not change XII burst frequency (Figs. 5B, 5C) or amplitude (Fig. 5D). At $50\ \mu\text{M}$ ($n=5$), U-69593 decreased burst frequency by $68 \pm 21\%$ after the 60-min exposure ($p<0.001$ for drug effect) that did not return to baseline levels after a 2.0-h washout (Figs. 5A, 5C). U-69593 at $100\ \mu\text{M}$ did not alter burst amplitude (Fig. 5D).

3.3 NOR activation

Bath-applied orphanin (NOR agonist) at $0.5\ \mu\text{M}$ ($n=4$) did not alter XII burst frequency or amplitude (Figs. 6B, 6C, 6D). At concentrations of $1.0\ \mu\text{M}$ ($n=8$) and $2.0\ \mu\text{M}$ ($n=6$), burst frequency decreased by $31 \pm 13\%$ ($p=0.027$) and $51 \pm 10\%$ ($p=0.015$), respectively, during the first 30 min of drug application, but returned to baseline levels during the next 30 min and 2.0-h washout period (Fig. 6C). Burst amplitude was not altered by orphanin at 0.5 , 1.0 , and $2.0\ \mu\text{M}$ (Fig. 6D). However, orphanin at $0.5\ \mu\text{M}$ increased episodic discharge from 1.6 ± 0.4 to a peak of 2.1 ± 0.5 after 30 min. Orphanin at $2.0\ \mu\text{M}$ increased episodic discharge from 1.6 ± 0.1 bursts/episode to 2.0 ± 0.2 bursts/episode after 60-min. When plotted as the change in bursts/episode, both $0.5\ \mu\text{M}$ and $2.0\ \mu\text{M}$ produced significant drug effects ($p=0.014$ and $p=0.007$, respectively; Figs. 6A, 6E). Orphanin at $1.0\ \mu\text{M}$ increased bursts/episode in 5/8 brainstems, but decreased in 2/8 brainstems such that there was no effect ($p=0.20$; Fig. 6E).

3.4 α_1 -adrenergic receptor activation reverses morphine-induced respiratory depression

To determine the dose-dependent effects of morphine on turtle respiratory motor output, turtle brainstems were exposed to a 60-min bath-application of morphine at $0.01\ \mu\text{M}$ ($n=7$), $0.1\ \mu\text{M}$ ($n=5$), $1.0\ \mu\text{M}$ ($n=7$), or $10\ \mu\text{M}$ ($n=4$). Morphine reversibly decreased XII burst frequency in a dose-dependent manner with no effect on amplitude (Fig. 7A). When graphed as raw frequency, morphine at all four concentrations had significant drug effects ($p<0.001$), but there was high variability in baseline frequency (Fig. 7B). When graphed as percent of baseline frequency, morphine applied at 0.1 - $10\ \mu\text{M}$ decreased frequency within 30 min and depressed frequency by at least 79% at 60 min ($p<0.016$ for drug effect; Fig. 7C). All morphine effects on frequency were reversed during washout except for the data at $1.0\ \mu\text{M}$, which was still decreased by 58%. Morphine did not alter amplitude at all concentrations; there was only a small time dependent decrease for all data at 90 and 210 min ($p<0.41$; Fig. 7D).

To test whether α_1 -adrenergic receptor activation reverses morphine-induced respiratory depression, morphine ($10\ \mu\text{M}$) was bath-applied for 60 min before co-application of morphine ($10\ \mu\text{M}$) and phenylephrine for 60 min ($20\ \mu\text{M}$, $n=7$; $50\ \mu\text{M}$, $n=10$; $100\ \mu\text{M}$, $n=7$). Morphine abolished XII motor output in nearly all brainstems and phenylephrine restored burst frequency to within 33%, 78%, and 16% of baseline frequency for the 20, 50, and $100\ \mu\text{M}$ phenylephrine concentrations, respectively (Figs. 8A, 8B). Burst frequency during co-application of morphine and $50\ \mu\text{M}$ phenylephrine (0.35 ± 0.12 bursts/min) was not different from baseline frequency (0.45 ± 0.07 bursts/min; $p>0.05$; Fig. 8B). During morphine and phenylephrine co-application, amplitude was restored to 49%, 65%, and 32% of baseline with respect to the 20, 50, and $100\ \mu\text{M}$ phenylephrine concentrations, respectively (Fig. 8C).

4. Discussion

This is the first study to examine the effects of central opioid receptor activation on respiratory motor output in isolated brainstems from an adult reptile. The main finding was that DOR and KOR activation decreased respiratory burst frequency with only one KOR agonist (at relatively high concentrations) decreasing burst amplitude. NOR activation increased the number of XII bursts/episode, which is the first demonstration of NOR-

dependent modulation of episodic respiratory motor discharge under *in vitro* conditions. Finally, this study was the first to show that opioid-induced respiratory depression can be reversed in ectothermic vertebrates via an α_1 -adrenergic mechanism.

4.1 Comparing *in vitro* studies on opioid-induced changes in respiration

The *in vitro* experimental approach offers control over drug application (*e.g.*, concentration, duration, and location) and stability for long-term extra- and intracellular recordings. Thus, the effects of opioid receptor activation on respiratory motor output have been examined in a wide range of *in vitro* vertebrate preparations from lamprey (Mutolo et al., 2007), frogs (Vasilakos et al., 2005, 2006), and neonatal rodents (Murakoshi et al., 1985; Greer et al., 1995; Johnson et al., 1996; Takita et al., 1997, 1998, 2000, 2003; Gray et al., 1999; Takeda et al., 2001; Janczewski et al., 2002; Mellen et al., 2003; Kobayashi et al., 2005; Tanabe et al., 2005; Onimaru et al., 2006; Ren et al., 2006). When comparing findings from these studies, different results are likely due to differences between experimental approaches, such as species (mammalian vs. reptile vs. amphibian) and animal age (mature adult vs. immature neonate). More subtle differences include the duration of drug application (short- vs. long-term periods; see discussion in Takita et al., 1997), function of motor output on the recorded nerve (pump air vs. maintain airway patency), and the amount of brain tissue in the preparation (intact brainstem vs. thin medullary slice). For example, MOR activation decreases inspiratory burst frequency in highly reduced neonatal rat medullary slices (*e.g.*, Greer et al., 1995; Johnson et al., 1996), but increases inspiratory burst frequency in neonatal rat pons-medulla-spinal cord preparations (Tanabe et al., 2005).

4.2 DOR activation and respiratory control

In mammals, systemic or central administration of DOR agonists reduces ventilation by decreasing breath frequency (Pazos and Florez, 1983, 1984; Ward and Takemori, 1983; Haddad et al., 1984; Hassen et al., 1984; Schaeffer and Haddad, 1985; Chen et al., 1996). DOR activation in the ventral respiratory group of anesthetized rats decreases phrenic nerve amplitude with little change in respiratory frequency (Lonergan et al., 2003). In neonatal rat *in vitro* slice or brainstem-spinal cord preparations, however, DPDPE has little or no effect on respiratory burst frequency (Greer et al., 1995; Takita et al., 1997, 1998) or respiratory-related neurons (Takeda et al., 2001). Thus, DOR activation appears to alter respiratory rhythm and pattern formation primarily in adult, rather than neonatal, mammals.

In ectothermic vertebrates, such as lamprey, DPDPE application to isolated brainstems *in vitro* decreases respiratory burst frequency by 30% with no change in burst shape or amplitude (Mutolo et al., 2007). For intact red-eared slider turtles, systemic DOR agonist administration depressed ventilation by decreasing breath frequency (Johnson et al., 2008). Since DORs are found in abundance within the turtle CNS (Xia and Haddad, 2001), we hypothesized that DOR activation depressed breathing by acting directly on rhythm generating neurons in the brainstem. In the present study, this hypothesis was supported by the finding that both DADLE and DPDPE decreased XII burst frequency with little change in amplitude.

There were, however, several differences in the responses to DADLE and DPDPE in this study. For example, DADLE was more potent (no respiratory activity in all brainstems at 0.3 and 0.5 μ M) compared to DPDPE (no respiratory activity in 7 of 11 brainstems at 1.0 μ M). This may be due to DADLE crossing over to activate MOR (Goldstein and Naidu, 1989) since MOR activation severely depresses XII respiratory burst frequency in isolated adult turtle brainstems (Johnson et al., 2002). The apparent increased potency of 0.3 μ M DADLE compared to 0.5 μ M DADLE on frequency and amplitude at 30 min post-drug administration (*e.g.*, Figs. 2C, 2D) was likely due to variability in drug responsiveness and

the relatively low sample size. In addition, there was a biphasic response to DPDPE in which low DPDPE concentrations (0.01, 0.1 μM) increased XII burst frequency while a high DPDPE concentration (1.0 μM) nearly abolished XII respiratory motor activity (Fig. 3C). One explanation may be that DPDPE activated different intracellular signaling pathways in a concentration-dependent manner. In cultured neurons and hybrid neuroblastoma cells, bath-applied DPDPE at low concentrations (<1.0 nM) decreased a K^+ conductance via a cholera toxin-sensitive mechanism, which is consistent with signaling through G_s proteins (Fan & Crain, 1995). However, DPDPE applied at higher concentrations (>1.0 nM) increased a K^+ conductance via a pertussis toxin-sensitive mechanism, which is consistent with signaling through $\text{G}_{i/o}$ proteins (Fan & Crain, 1995). In isolated turtle brainstems, DPDPE at lower concentrations may have decreased a K^+ conductance on respiratory rhythm generating (or modulatory neurons) and increased XII burst frequency. At the higher concentrations, DPDPE may have increased a K^+ conductance on the same neurons and decreased XII burst frequency. Alternatively, higher concentrations of DPDPE may have acted non-specifically and crossed over to activate other neurotransmitter receptors. We hypothesize that DADLE did not produce a biphasic response similar to DPDPE because: a) DADLE co-activated MOR, b) DADLE and DPDPE differentially activated specific DOR subtypes (δ_1 , δ_2), or c) DADLE and DPDPE differentially altered excitability of other neurons projecting to brainstem rhythm generating neurons.

4.3 KOR activation and respiratory control

The effects of KOR activation on breathing are not consistent. On one hand, KOR agonists have little effect on breathing when administered (intracerebroventricularly, intravenously, or subcutaneously) in conscious rodents and monkeys (Freye et al., 1983; Castillo et al., 1986; Howell et al., 1988; Dosaka-Akita et al., 1993; Fujibayashi et al., 1996). Likewise, in isolated lamprey brainstem-spinal cord preparations, KOR had no effect on lamprey respiratory motor output (Mutolo et al., 2007). On the other hand, systemic administration of KOR agonists in cats decreases phrenic nerve discharge amplitude and duration, and shortens inspiratory and expiratory duration (Haji and Takeda, 2001). Similarly, KOR agonist injections into the ventral respiratory group decrease tidal volume and respiratory rate in anesthetized rats (Hassen et al., 1984). In isolated neonatal rat brainstem-spinal cord preparations, U-50488 decreases respiratory burst frequency and amplitude, and hyperpolarizes inspiratory neurons in the brainstem (Takita et al., 1997; Takeda et al., 2001).

In awake, freely swimming turtles, systemic administration of U-50488 produces highly variable effects on breath frequency and increases tidal volume in a time-dependent manner (Johnson et al., 2008). Some turtles increased breath frequency while other turtles stopped breathing for minutes to hours (Johnson et al., 2008). Since very little is known about KOR activation and respiratory control, it's possible that KOR activation altered the function of rhythm generating, chemosensory, mechanosensory, or modulatory neurons. As a first step towards understanding KOR-dependent changes in turtle respiratory function, KOR agonists were found to reduce XII burst frequency (and amplitude for one drug) in isolated turtle brainstems in this study. This suggests that KOR activation alters rhythm generation by either acting directly on rhythm generating neurons or modulatory neurons projecting to the rhythm generator. These results are similar to what was found in anesthetized rats (Hassen et al., 1984) and neonatal rat brainstem-spinal cord preparations (Takita et al., 1997; Takeda et al., 2001), but no effects on respiration were observed in lamprey brainstem-spinal cords (Mutolo et al., 2007) and conscious mammals (Freye et al., 1983; Castillo et al., 1986; Howell et al., 1988; Dosaka-Akita et al., 1993; Fujibayashi et al., 1996). The contrasting results may be due to differences in experimental approaches (species, anesthesia, animal

age, drugs, *etc.*), or KOR-dependent modulation of breathing may not be as well conserved in vertebrates as MOR-dependent respiratory depression.

4.4 NOR-dependent regulation of episodic discharge

In mammals, NOR are similar to the other opioid receptors with respect to distribution in the brain and activation of specific G-protein signaling pathways (Harrison and Grandy, 2000). NOR activation alters many physiological functions (*e.g.*, nociception, feeding, locomotion, reproduction) and drugs acting at NOR may be used to treat several diseases in humans (Harrison and Grandy, 2000; Chiou et al., 2007). With respect to respiratory control, bath-application of orphanin (10-100 nM, 20 min) to isolated neonatal rat brainstem-spinal cord preparations decreased inspiratory burst frequency in a dose-dependent manner (Takita et al., 2003). In this study on adult turtle brainstems, similar results were obtained since orphanin decreased respiratory bursts transiently during the first 30 min of drug exposure in a dose-dependent manner. However, burst frequency in turtle brainstems returned to baseline levels during the next 30-60 min. It will be necessary to test longer orphanin exposures in *in vitro* neonatal rat preparations to determine if orphanin-dependent respiratory depression is transient in rats as it was in turtles. Also, it will be important to determine under what physiological role that NOR activation plays in regulating respiration in intact turtles.

Episodic breathing, in which clusters of breaths are separated by non-ventilatory periods, is considered to be on one end of a periodic breathing pattern continuum (Milsom, 1991; Reid et al., 2003). Episodic breathing is expressed in fish, amphibians, reptiles, and some mammals under certain conditions (Milsom, 1991). Factors that regulate or modulate episodic breathing include chemosensory and mechanosensory inputs (Kinkead and Milsom, 1997; Reid et al., 2003) and central structures rostral to the brainstem (Kinkead et al., 1997; Milsom et al., 1997; Reid et al., 2000; Gargaglioni et al., 2007). In ectothermic vertebrates, the capacity for episodic breathing is contained within the isolated brainstems of tadpoles (Straus et al., 2000), bullfrogs (Kinkead et al., 1994; Reid and Milsom, 1998), and turtles (Douse and Mitchell, 1990; Johnson et al., 1998). In turtles, episodic breathing can also be observed in turtle brainstems that were completely cut along the midline into hemibrainstems (Majewski et al., 2008). This suggests that episodic breathing in ectothermic vertebrates may be produced by the pontomedullary respiratory network contained on one side of the brainstem.

The unexpected finding in this study was that orphanin increased episodic discharge without altering overall XII burst frequency in isolated turtle brainstems (see 90-min time points in Figs. 6C and 6E). This is one of the first examples of a drug increasing episodic respiratory discharge in an *in vitro* turtle brainstem preparation. To our knowledge, only baclofen (GABA_B receptor agonist) and nitric oxide change episodic bursts to singlet bursts without changing ventilatory drive during drug application in isolated tadpole brainstems (Straus et al., 2000; Harris et al., 2002). Although the effects of altered GABA_B or nitric oxide modulation on episodic turtle respiratory output are not known, drugs such as orphanin are powerful experimental tools that can be used to study the mechanisms underlying episodic breathing in reduced *in vitro* preparations.

4.5 Opioid-dependent regulation of respiration and nociception in turtles

Endogenous ligands for opioid receptors regulate nociception, stress responses, learning and memory, eating and drinking, locomotion, and cardiorespiratory function (Bodnar, 2008). The precise role of endogenous opioid ligand release in turtles, especially with respect to breathing, is poorly understood. Based on this *in vitro* study and a previous study in intact turtles (Johnson et al., 2008), MOR and DOR activation have the greatest impact on turtle

breathing by depressing breath frequency. Since DOR are found in greater abundance compared to MOR in red-eared slider turtles (Xia and Haddad, 2001), we hypothesize that endogenous ligands for DOR activation (*e.g.*, enkephalins) will play the largest role in modulating respiration. With respect to antinociception in turtles, it appears that only MOR activation is effective (Sladky et al., 2009). This is problematic because MOR activation also causes profound respiratory depression in intact turtles (Sladky et al., 2007; Johnson et al., 2008).

Discovering and testing drug combinations that reverse opioid-induced respiratory depression in ectothermic vertebrates, such as reptiles, is interesting from a scientific point of view. However, providing analgesia to reptiles in a veterinary clinical setting is also important because pain relief in all vertebrates is considered ethically obligatory (Paul-Murphy et al., 2004). Also, providing analgesia during and following surgical procedures may facilitate recovery and healing, and a more rapid return to normal behaviors (Hardie et al., 1997; Lang 1999). Currently, little is known about drugs that produce analgesia with little or no respiratory depression in reptiles. Isolated turtle brainstems provide a valuable tool for rapidly screening clinically-relevant analgesic opioid drugs for respiratory depression as well as drug combinations that can reverse opioid-induced respiratory depression.

4.6 Reversal of opioid-induced respiratory depression via α_1 -adrenergic receptor activation

Pharmacological strategies for reversing opioid-induced respiratory depression while preserving analgesia include augmentation of central glutamatergic excitatory synaptic transmission with ampakine drugs (Ren et al., 2006), or augmentation of peripheral and central chemosensory inputs with doxapram (Yost, 2006). The best experimental models for reversing opioid-induced respiratory depression while maintaining analgesia involve activation of serotonergic 5-HT₄ or dopaminergic D₁ receptor subtypes in mammals (Manzke et al., 2003; Lalley, 2005). Activation of signaling pathways for these two receptor subtypes increases and restores intracellular cAMP levels which are depressed following opioid receptor activation generation (Ballanyi et al., 1997).

It is not clear yet whether phenylephrine-dependent reversal of morphine-induced respiratory depression in isolated turtle brainstems represents a new molecular mechanism for reversing opioid-induced respiratory depression. α_1 -adrenergic receptors are primarily coupled to the G_{q/11} family of G-proteins, which activates the phosphoinositol pathway to cause release of Ca²⁺ ions from intracellular stores and downstream activation of protein kinase C (Hein and Michel, 2007). However, α_1 -adrenergic receptor activation is also known to couple with other G-proteins (G_i, G_o, G_s, G_{12/13}), as well as modulate K⁺, Na⁺, and Ca²⁺ channels (Hein and Michel, 2007). Furthermore, α_1 -adrenergic receptor activation increases intracellular cAMP levels in rat liver cells (Morgan et al., 1984) and may act to increase cAMP levels similar to 5-HT₄ and D₁ receptor activation in respiratory neurons. Thus, we hypothesize that α_1 -adrenergic receptor activation acts via several mechanisms to increase excitability within the turtle respiratory network and restore breathing.

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Fig. 1. Isolated turtle brainstem produces spontaneous respiratory motor output. (A) Drawing of turtle brainstem with cranial nerves marked as shown on the left. A suction electrode attached to the hypoglossal (XII) nerve records respiratory motor output. (B) Trace of integrated and rectified respiratory motor output demonstrating both singlet and doublet XII motor bursts produced by the same brainstem.

Fig. 2.

DADLE (DOR agonist) reduces XII burst frequency. (A) Integrated XII respiratory output is shown prior to (*left*), during bath application of 0.5 μM DADLE (*middle*), and after 120 min of washout (*right*). (B, C) DADLE reversibly reduced respiratory burst frequency in a dose- and time-dependent manner. (D) DADLE reduced XII burst amplitude after 30 min at 0.3 μM. After 60 min of DADLE exposure, respiratory discharge occurred in none of the brainstems at 0.3 μM and only 1/6 brainstems at 0.5 μM. * $p < 0.05$ compared time controls at that time point; # $p < 0.05$ compared to baseline for that drug dose; † $p < 0.05$ for drug effect.

Fig. 3.

DPDPE (DOR agonist) has excitatory and inhibitory effects on XII burst frequency. (A₁) Integrated XII respiratory motor output is shown prior to drug application (*left*), during bath application of 0.1 μM DPDPE (*middle*), and after 90 min of washout (*right*). At this lower dose of DPDPE, XII burst frequency was increased during drug application. (A₂) Similar traces for bath application of 1.0 μM DPDPE. At this higher dose of DPDPE, burst frequency was abolished during drug application. (B, C) DPDPE increased XII burst frequency at 0.01 – 0.1 μM DPDPE, but decreased frequency to nearly zero at 1.0 μM DPDPE. (D) Burst amplitude was not altered with bath applied DPDPE at 0.01 – 1.0 μM. **p*<0.05 compared time controls at that time point; #*p*<0.05 compared to baseline for that drug dose; &*p*<0.05 for time-dependent effect for all data at that time point; †*p*<0.05 for drug effect.

Fig. 4.

U-50488 (KOR agonist) reduces XII burst frequency. (A) Integrated XII respiratory output is shown prior to drug application (*left*), during bath application of 100 μ M U-50488 (*middle*), and after 120 min of washout (*right*). U-50488 at 100 μ M reduced XII burst frequency (B, C) and amplitude (D); the other concentrations did not alter respiratory discharge. * $p < 0.05$ compared time controls at that time point; # $p < 0.05$ compared to baseline for that drug dose; † $p < 0.05$ for drug effect.

Fig. 5.

U-69593 (KOR agonist) reduces XII burst frequency. (A) Integrated XII respiratory output is shown prior to drug application (*left*), during bath application of 50 μ M U-50488 (*middle*), and after 90 min of washout (*right*). U-69593 at 50 μ M reduced XII burst frequency (B, C) and amplitude (D); the other concentrations did not alter respiratory discharge. * $p<0.05$ compared time controls at that time point; # $p<0.05$ compared to baseline for that drug dose; & $p<0.05$ for time-dependent effect for all data at that time point; † $p<0.05$ for drug effect.

Fig. 6.

Orphanin (NOR agonist) transiently reduces XII burst frequency but increases episodic discharge. (A) Integrated XII respiratory output is shown prior to drug application (*left*), during bath application of 2.0 μ M orphanin (*middle*), and after 120 min of washout (*right*). (B, C) Orphanin decreased burst frequency after 30 min, but frequency returned to baseline levels after 60 min. (D) XII burst amplitude was not altered by orphanin at 0.5- 2.0 μ M. (E) When graphed as the change in bursts/episode, orphanin at 0.5 and 2.0 μ M (but not 1.0 μ M) significantly increased bursts/episode. * $p < 0.05$ compared time controls at that time point; # $p < 0.05$ compared to baseline for that drug dose; & $p < 0.05$ for time-dependent effect for all data at that time point; † $p < 0.05$ for drug effect.

Fig. 7.

Morphine reduces respiratory burst frequency. (A) Integrated XII respiratory motor output is shown prior to (*left*), during bath-application of 0.1 μ M morphine (*middle*), and after 90 min of washout (*right*). (B,C) Morphine reversibly reduces respiratory burst frequency in a dose- and time-dependent manner at concentrations ≥ 0.1 μ M. (D) Morphine had little or no effect on respiratory burst amplitude. * $p < 0.05$ compared time controls at that time point; # $p < 0.05$ compared to baseline for that drug dose; & $p < 0.05$ for time-dependent effect for all data at that time point; † $p < 0.05$ for drug effect.

Fig. 8.

α_1 -adrenergic activation reverses morphine-induced respiratory depression. (A) Integrated XII respiratory motor output is shown during baseline (*left*), bath-application of morphine (*middle*), and co-application of morphine (10 μ M), and phenylephrine (50 μ M; *right*). (B) Respiratory burst frequency of turtle brainstems during baseline ("B"), morphine (10 μ M) application ("M"), and co-application of morphine (10 μ M) and phenylephrine at different concentrations ("M/PE"). Phenylephrine was co-applied at 20 μ M (white bars), 50 μ M (black bars), 100 μ M (gray bars). (C) Respiratory burst amplitude is shown normalized to baseline amplitude. * $p < 0.05$ compared to baseline.