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S-Methadone augments R-methadone induced respiratory depression in the neonatal guinea pig

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Abstract

Methadone is administered as a racemic mixture, although its analgesic and respiratory effects are attributed to *R*-isomer activity at the mu-opioid receptor (MOP). Recently, we observed a four-fold increase in inspiratory time in three-day old guinea pigs following an injection of racemic methadone. We hypothesized that this effect was due to augmentation of *R*-methadone induced respiratory depression by the *S*-methadone isomer. In the current longitudinal study, we injected three-, seven-, and fourteen-day old neonatal guinea pigs with saline, *R*-methadone, *S*-methadone, or *R*- plus *S*-methadone in order to characterize the roles of the individual isomers, as well as the synergistic effects of co-administration. Using plethysmography, we measured respiratory parameters while breathing room air and during a 5% CO₂ challenge. *S*-methadone alone had no respiratory effects. However, the *R*- plus *S*-methadone group showed greater respiratory depression and increased inspiratory time than the *R*-methadone group in the youngest animals, suggesting that the respiratory effects of *R*-methadone are augmented by *S*-methadone in early development.

1. Introduction

Methadone is a synthetic opioid analgesic used increasingly to treat acute and chronic pain in infants (Koren et al. 1989; Chana et al. 2001; Anand 2007). The benefits of using methadone as an analgesic are its potency and oral availability, combined with a prolonged half-life (Olsen et al. 1977) that allows for less frequent dosing. However, there are negative side effects as well, the most significant of which is respiratory depression. Respiratory depression is characterized by decreases in inspiratory minute volume, breathing frequency, and tidal volume (Olsen et al. 1981; Richardson et al. 1984; Nettleton et al. 2007) that can eventually lead to death from anoxia. This side effect is mediated predominantly via mu opioid receptors (MOP) located in the respiratory control areas of the brainstem (Mansour et al. 1988; Matsuda et al. 2001; Smith et al. 2004).

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While its analgesic and respiratory depressive properties are generally attributed to the *R*-isomer alone (Smits et al. 1974; Horng et al. 1976; Olsen et al. 1977), methadone is traditionally administered as a racemic mixture of the two enantiomeric isomers *R*-methadone and *S*-methadone. The magnitude of the respiratory effects resulting from treatment with a racemic mixture can be quite large for neonates. For example, during a recent study examining the effects of acute opioid treatment in neonatal guinea pigs, we observed a four-fold increase in inspiratory time after a single injection of racemic methadone in 3-day old pups but not in older animals, or after a morphine injection (Nettleton et al. 2007). Based on this observation, it seems possible that *R*-methadone and *S*-methadone have an interactive developmental effect on respiration. Therefore, in this project we attempted to isolate and characterize the effects of *R*-methadone and *S*-methadone with respect to neonatal development. We hypothesized that *S*-methadone acts synergistically with *R*-methadone to produce increased respiratory effects in early stages of development, and predicted that neonatal guinea pigs receiving a racemic mixture of methadone would have more respiratory depression than those receiving either isomer alone.

2. Methods

2.1. Animals

Dunkin-Hartley guinea pigs were bred using six-week old females (300-400 g) and five-month old males (Charles River, Wilmington, MA, USA). Pups and dams shared housing with 12 hour controlled light/dark cycles and ad libitum food and water. Dunkin-Hartley guinea pigs were chosen as a model for this study for a number of reasons: 1) The neonatal guinea pig is similar to a neonatal human in that it is born at term with a relatively mature central nervous system and is able to regulate its body temperature; 2) both humans and guinea pigs metabolize methadone into the same products, and pregnant adults eliminate methadone in time-frames that are comparable (Olsen et al. 1980; Pak et al. 1981); and 3) previous research in our laboratory using the neonatal guinea pig has supported that it is a good model for opioid effects on breathing. Using this model, we looked at respiratory parameters in guinea pigs that were given drug or saline injections on days 3, 7, and 14, post-partum. As this study was longitudinal, the same animals were studied on all three days. Experiments were approved by the Oregon Health & Science University Institutional Animal Care and Use Committee and studies were conducted according to the guidelines adopted by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

2.2. Treatment groups

24 study pups from 15 litters were assigned randomly to one of four treatment groups: 1) vehicle (0.9% saline), 2) *S*-methadone (5 mg/kg), 3) *R*-methadone (5 mg/kg), or 4) *R*- plus *S*-methadone (10 mg/kg, 5 mg/kg of each isomer). Each group consisted of 3 males and 3 females. The first two groups will be referred to as “control groups”, while the latter two will be called “active drug groups”. To reduce litter effects, only 1 male and 1 female from each litter were used. In case of an all female or male litter only one animal was studied. The pups were treated at day 3, 7, and 14 post-partum (day 1 was defined as the first day pups were observed in the tub). The scruff of the neck of each pup was shaved, and all injections were administered subcutaneously (s.c.) at this site. The methadone doses were chosen using previous studies analyzing methadone induced respiratory depression (Nettleton et al. 2007). Also, because both isomers are non-competitive NMDA antagonists, an additional 6 pups (3 males and 3 females) were given *R*-Methadone (5mg/kg) plus MK-801 (0.01 mg/kg), a known non-competitive NMDA antagonist in order to investigate whether NMDA receptor antagonism was involved in the respiratory effects we observed. Respiratory data for these animals were collected as described above.

2.3. Drug preparation

Both isomers of methadone-HCl (Research Triangle Institute, Research Triangle Park, NC, USA) were obtained in pure crystalline form and diluted separately in sterile 0.9% saline solution (Abbott Laboratories, Chicago, IL, USA) to a final concentration of 5 mg/ml. Dilutions were stored in 500 μ l aliquots at -80°C , and thawed when needed. Racemic methadone was prepared by separately diluting both *R*- and *S*-isomers in sterile 0.9% saline solution to a final concentration of 10 mg/ml each. Then, equal volumes of these solutions were mixed to obtain a final concentration of 10 mg/ml (5 mg/ml *R*-methadone plus 5 mg/ml *S*-methadone). Racemic methadone was formulated in this manner in order to ensure that all methadone used was from the same lot. MK-801 (Sigma-Aldrich, MO, USA) was dissolved at 1 mg/ml in 0.9% saline and stored at 4°C . Solutions of *R*-methadone plus MK-801 were made immediately prior to the injection from the aforementioned stock solutions.

2.4. Locomotor activity

Locomotor activity was measured for 15 minutes prior to respiratory measurements and drug treatment and again for 15 minutes at 45 minutes after drug treatment. These data were used as a measure of sedation. On each test day (3, 7, or 14) individual pups were placed into a Plexiglas cage that was housed between the optical sensors of the Opto-Varimax mini activity monitor (Columbus Instruments, Columbus, OH, USA). Locomotor activity was measured by counting every beam break resulting from ambulatory movements as well as non-horizontal movements such as digging, scratching and grooming.

2.5. Ventilatory and metabolic measurements

All ventilation data was collected using a non-invasive dual chamber plethysmograph and Biosystem XA software version 2.7.9 (Buxco Electronics, Sharon, CT, USA). Metabolic data was measured using Oxymax CO_2 and O_2 analyzers and data was acquired with Oxymax software 2.4.2 (Columbus Instruments, Columbus, OH, USA). Detailed methods have previously been outlined (Nettleton et al. 2007).

Each study gas was administered using a separate head chamber. The two gases used were room air (RA; 21% O_2 , balance N_2) or a 5% CO_2 mixture (5% CO_2 , 30% O_2 , balance N_2). To ensure a purely hypercapnic response, 30% O_2 was included in the 5% CO_2 gas mixture to prevent drug induced hypoxia; all hyperoxic effects were considered negligible when compared with the increase in respiration caused by 5% CO_2 . The flow rate through the head chamber for RA was 1 L/min, while for 5% CO_2 it was 1.7 L/min.

The following parameters were measured: Breathing frequency (f_R ; breaths $\cdot\text{min}^{-1}$), air flow ($\text{ml}\cdot\text{sec}^{-1}$), inspiratory time (T_I ; sec), expiratory time (T_E ; sec), oxygen consumption ($\dot{V} \text{O}_2$; $\text{ml}\cdot\text{min}^{-1}\cdot 100\text{g}^{-1}$; sample rate = 1 ml/min), and carbon dioxide production ($\dot{V} \text{CO}_2$; $\text{ml}\cdot\text{min}^{-1}\cdot 100\text{g}^{-1}$; sample rate = 1 ml/min). From these data the following were derived and normalized for pup body weight: Tidal volume (V_T ; $\text{ml}\cdot 100\text{g}^{-1}$), inspiratory minute ventilation ($\dot{V} I$; $\text{ml}\cdot\text{min}^{-1}\cdot 100\text{g}^{-1}$), inspiratory effort (V_T/T_I ; $\text{ml}\cdot\text{sec}^{-1}\cdot 100\text{g}^{-1}$).

2.6. Experimental procedure

Prior to each study session, pups were weighed individually and then placed into the Opto-Varimax mini activity monitor Plexiglas cage. Activity was measured for 15 minutes, after which pups were placed into the plethysmograph chamber with a steady flow of RA for a 10 min acclimation period. Baseline measurements (time zero) were collected using a flow of RA for an additional 10 min, then the gas was switched to 5% CO_2 . The CO_2 was discontinued after 5 min, and the pup was given either vehicle (saline) or drug via subcutaneous injection. Then RA and CO_2 were administered and measurements were taken

as previously described. Over the 2 hours following the injection, breathing measurements were taken at 0.25 hr, 0.5 hr, 1 hr, 1.5 hr, and 2 hr time points. At 0.75 hr after drug administration, total and ambulatory activities were measured a second time, and then the pups were placed back into the plethysmograph chamber to complete breathing measurements. This protocol was repeated for every pup in each group on days 3, 7, and 14.

2.7. Data acquisition and statistical analysis

Locomotor activity was analyzed using a two-way repeated measures analysis of variance (ANOVA) with factors of age and treatment (Systat Software version 3.1, Inc. Richmond, CA, USA). Immediately following activity data collection and the acclimation period, respiratory data was collected during a 10 min period of RA exposure and then during 5 min of 5% CO₂ (CO₂ challenge). Metabolic and ventilatory parameters were assessed during RA breathing over the first 6 and last 4 min, respectively. Only ventilation was measured during CO₂ challenge. All reported data was derived from the average of the last 2 min of each measurement period when a steady-state had been achieved.

The point of maximum respiratory depression for each animal was defined as the lowest recorded value of $\dot{V}_I$ while breathing 5% CO₂. Values corresponding to these time points were selected for all other parameters, and the averages were calculated. These data are presented as mean $\pm$ standard error (SE).

Based on the results from a preceding study, data was collapsed for sex, and two-way repeated measures analyses of variance (ANOVA) (Systat Software version 3.1, Inc. Richmond, CA, USA) with factors of age and treatment, as well as time and treatment, were performed (Nettleton et al. 2007). For comparison of RA and CO₂ breathing at a baseline one-way repeated measures ANOVA was used. We considered *p* values less than 0.05 significant, and the Holm-Sidak method was used to perform all post-hoc comparisons. All parameters were analyzed in this manner. In addition, the Grubbs test for detecting outliers was performed on each data set. This test was chosen since, due to the small population of each group, outliers have a major effect on the statistical analysis. Out of 72 tests (24 pups times 3 days), data for only one pup on day 3 was excluded as an outlier.

3. Results

3.1. Gestation length, litter size, and pup weights

The average gestation length for the pregnant dams was 69.2 ± 0.3 days with an average litter size of 2.9 ± 0.2 pups. There was no significant difference in pup weights at any age for either males or females, or the test groups. The mean weight of the pups (*n*=24) on each day of testing was as follows: Day 3 = 103.1 ± 1.4 g, day 7 = 130.5 ± 3.3 g, day 14 = 189.6 ± 4.3 g.

3.2. Locomotor activity

Treatment groups were not different from each other prior to drug administration, but there was a main effect of age (*p*<0.05) with older animals being generally more active than younger pups. All animals were active, but showed great variability in their activity (120 – 740 beam breaks) (Table 1). Following drug administration, there was a main effect of drug (*p*<0.001), and an age-drug interaction (*p*<0.05). Both active drug groups were significantly less exploratory than control groups showing significantly reduced locomotor activity (*p*<0.001 for all comparisons, 0 – 101 beam breaks). Active drug groups were not statistically different from each other, suggesting an equivalent level of sedation between the *R*-methadone and the *R*- plus *S*-methadone groups. Likewise, there were no main differences

between the saline and *S*-methadone control groups following drug administration, while animals tended to be less active overall (72 – 507 beam breaks).

3.3. Baseline ventilatory measurements

Baseline respiratory measurements were taken prior to drug treatment and after a 10 minute period that allowed each animal to become acclimated to the plethysmograph chamber (Table 2). $\dot{V}_I$, V_T , and V_T/T_I decreased with age during both RA breathing and CO₂ challenge ($p < 0.001$ for all comparisons). In addition, f_R decreased with age only during the CO₂ challenge ($p < 0.001$). T_I and T_E , however, increased with age during CO₂ breathing ($p < 0.001$). When comparing the RA and CO₂ baseline measurements, $\dot{V}_I$, f_R , V_T , and V_T/T_I increased from RA to CO₂ challenge ($p < 0.001$ for all comparisons), while T_I and T_E decreased ($p < 0.001$). In summary, these data show that the animals reacted to the increase in CO₂ by taking deeper, slower breaths.

3.4. Time action curves for inspiratory minute ventilation ($\dot{V}_I$), frequency (f_R), and tidal volume (V_T)

Respiratory depression on day 3—Figures 1A-1C compare RA breathing for parameters $\dot{V}_I$, f_R , and V_T on day 3. During RA breathing, a main effect of time was observed for $\dot{V}_I$ ($p < 0.001$) and f_R ($p < 0.001$). Only trends for drug effects were found for $\dot{V}_I$ ($p = 0.06$) and f_R ($p = 0.08$). No interaction occurred during RA breathing for either $\dot{V}_I$ or V_T , although some significant differences were found between the *R*- plus *S*-methadone group and other groups for $\dot{V}_I$ (Fig. 1A). However, a drug-time interaction was observed for f_R during RA breathing indicating that the *R*- plus *S*-methadone group experienced more profound and longer-lasting depression of frequency compared to control groups ($p < 0.01$).

Figures 1D-1F compare breathing during CO₂ challenge for parameters $\dot{V}_I$, f_R , and V_T on day 3. During CO₂ challenge, main effects of time were observed for $\dot{V}_I$ ($p < 0.001$) and f_R ($p < 0.001$), though this effect was not observed for V_T (Fig. 1F). Unlike during RA breathing, significant main effects of drug were found for all three parameters indicating that active drug groups experienced overall respiratory depression compared to control groups ($p < 0.001$ for all comparisons). Main effects were also qualified by significant drug-time interactions for all parameters indicating that active drug groups experienced large and long-lasting respiratory depression compared to control groups ($p < 0.001$ for all comparisons). The *R*- plus *S*-methadone group showed significant respiratory depression compared to both control groups for all time points after drug injection ($p < 0.001$ for all comparisons), whereas the *R*-methadone group was different for only the first three time points after injection ($p < 0.01$ for all comparisons). Importantly, the *R*- plus *S*-methadone group was also significantly more depressed than the *R*-methadone group for $\dot{V}_I$ ($p < 0.01$, Fig. 1D) and f_R ($p < 0.001$, Fig. 1E).

Respiratory depression on day 7—Figures 1G-1I compare RA breathing for parameters $\dot{V}_I$, f_R , and V_T on day 7. During RA breathing, a main effect of time was observed for $\dot{V}_I$ ($p < 0.01$) and f_R ($p < 0.001$) similar to day 3, while a trend was observed for V_T ($p = 0.08$). While no main effects of drug emerged for any parameters, the drug-time interaction which occurred for f_R on day 3 was maintained ($p < 0.001$). Figures 1J-1L compare breathing during CO₂ challenge for parameters $\dot{V}_I$, f_R , and V_T on day 7. During CO₂ challenge, main effects of time and drug were found for all parameters as well as drug-time interactions, similar to those found for day 3 ($p < 0.01$ for all comparisons). Active drug groups showed significant respiratory depression compared to both control groups for most time points for $\dot{V}_I$ ($p < 0.01$) and f_R ($p < 0.001$) similar to day 3, although differences for V_T

were less profound (Fig. 1L). Notably, the *R*- plus *S*-methadone group was still significantly more depressed than the *R*-methadone group for $\dot{V}_I$ ($p=0.01$, Fig. 1J) and f_R ($p<0.01$, Fig. 1K), but to a lesser extent than on day 3.

Respiratory depression on day 14—Figures 1M-1O compare RA breathing for parameters $\dot{V}_I$, f_R , and V_T on day 14. During RA breathing, the main effect of time seen for these parameters on days 3 and 7 was maintained ($p<0.01$ for all comparisons). While no main effects of drug emerged, significant drug-time interactions occurred for all parameters ($p<0.05$ for all comparisons). Figures 1P-1R compare breathing during CO_2 challenge for parameters $\dot{V}_I$, f_R , and V_T on day 14. During CO_2 challenge, main effects of time and drug were found for all parameters as well as drug-time interactions, similar to those found for days 3 and 7 ($p<0.001$ for all comparisons except drug-time interaction for V_T , $p<0.05$). Active drug groups still showed significant respiratory depression compared to both control groups for $\dot{V}_I$ ($p<0.001$) and V_T ($p<0.01$), and for f_R during the first hour ($p\leq 0.01$). However, the *R*- plus *S*-methadone group was no longer significantly different from the *R*-methadone group for any parameters.

3.5. Ventilation at maximum depression

Maximum depression was defined for each animal as the time point during CO_2 challenge at which $\dot{V}_I$ had the lowest recorded value following drug treatment. Table 3 illustrates the time to maximum depression for all drug groups containing *R*-methadone. Maximum depression was reached between 15 – 60 min for all animals. There were no statistical differences among these treatment groups or with age. All other parameters, including RA data at maximum depression, were selected matching these time points (Fig. 2).

Inspiratory effort—Inspiratory effort at maximum depression during RA breathing showed a main effect of drug ($p<0.001$, Fig. 2A). Both active drug groups had a reduced inspiratory effort compared to controls ($p<0.01$, except *R*-methadone vs. *S*-methadone $p<0.05$). The *R*- plus *S*-methadone group showed a decreased inspiratory effort compared to controls and the *R*-methadone group on day 3. No differences were seen in older animals.

During CO_2 breathing, there were main effects for drug and age ($p<0.001$) and an agedrug interaction ($p<0.05$). Active drug groups had significantly depressed inspiratory effort when compared to both controls ($p<0.001$). The effect of age was due to the oldest pups (day 14) having a reduced inspiratory effort compared to days 3 and 7 ($p<0.01$). In addition, *R*- plus *S*-methadone caused greater depression of inspiratory effort than *R*-methadone overall ($p<0.05$, Fig. 2B). The *R*- plus *S*-methadone group showed a significantly greater depression compared to the *R*-methadone group on day 3. On day 7, there was continued greater depression for the *R*- plus *S*-methadone group, although it was not statistically significant ($p=0.04$) due to multiple comparisons.

Inspiratory time—Inspiratory time at maximum depression showed main effects for drug ($p<0.001$) and age ($p<0.05$) during both RA breathing and CO_2 challenge. During RA breathing, only the *R*- plus *S*-methadone group had a statistically increased T_I compared to control groups ($p<0.001$, Fig. 2C). T_I for the *R*-methadone group was elevated compared to saline and *S*-methadone controls ($p=0.01$ and $p=0.03$, respectively), but did not reach statistical significance due to multiple comparisons. T_I was significantly increased in the *R*- plus *S*-methadone group when compared to controls on all 3 days, while the *R*-methadone group was significantly elevated only on day 14, but not on day 3 ($p=0.04$), and day 7 ($p=0.03$) due to multiple comparisons.

In contrast, both active drug groups had an increased T_I compared to controls during CO_2 challenge ($p < 0.001$, except *R*-methadone vs. Saline $p < 0.01$). The increase in T_I for the *R*- plus *S*-methadone group during CO_2 challenge was also greater than for the *R*-methadone group ($p < 0.01$, Fig. 2D). T_I of the *R*- plus *S*-methadone group was higher compared to controls on all days and higher than the *R*-methadone group in the first week. For *R*-methadone, T_I increased with age and was different from control on days 7 and 14.

Expiratory time—Expiratory time at maximum depression showed a main effect of age ($p < 0.05$) during RA breathing, but no effects of drug (Fig 2E). T_E increased with age and was overall longer on day 14 compared to day 3 ($p < 0.01$). During CO_2 challenge, there were main effects for drug and age ($p < 0.001$). T_E was greatest in 14 day old animals compared to younger animals ($p < 0.01$). T_E of the *R*- plus *S*-methadone group was longer compared to all other treatment groups ($p < 0.001$ vs. controls, $p < 0.01$ vs. *R*-methadone). *R*-methadone was not different from saline ($p = 0.03$) or *S*-methadone ($p = 0.06$) due to multiple comparisons (Fig 2F). T_E was significantly increased in the *R*- plus *S*-methadone group compared to controls (all days), and compared to the *R*-methadone group on days 3 and 7. The *R*-methadone group was different from controls only on day 14.

These data demonstrate that respiratory depression caused by *S*- plus *R*-methadone compared to *R*-methadone alone is more severe in 3 day old pups. Differences are still seen in 7 day olds, but are resolved by day 14.

3.6. Involvement of NMDA antagonism on maximum depression

To test if the increased respiratory depressive effects of *R*- plus *S*-methadone group are a result of the antagonistic effects of *S*-methadone at the NMDA receptor, we treated a group of pups with *R*-methadone plus MK-801, a non-competitive antagonist at the NMDA receptor. MK-801 had no effect on the time to reach maximum depression (Table 3). Figure 3 compares $\dot{V}_I$, f_R and V_T for all *R*-methadone containing groups at maximum depression during CO_2 challenge. Data during RA breathing paralleled these findings and is not shown. For inspiratory volume, there were main effects of drug and age ($p < 0.01$). *R*- plus *S*-methadone causes more severe overall reduction of $\dot{V}_I$ at maximum depression than *R*-methadone or *R*-methadone plus MK-801 ($p < 0.01$), showing statistical significance on days 3 and 7 (Fig 3A). Similarly, there were main effects for drug ($p < 0.05$) and age ($p < 0.001$) for frequency. *R*- plus *S*-methadone shows a greater decrease in f_R than the other two treatments; this effect vanished as the pups aged (Fig. 3B). The greater reduction is significant in 3 day old pups, while in 7 day old pups only a trend remains ($p = 0.03$, not significant due to multiple comparisons). For tidal volume there was a main effect of age ($p = 0.01$), but no main effect of drug (Fig. 3C). On day 3, there is a trend showing a reduced V_T for the *R*- plus *S*-methadone group compared to the two other groups ($p = 0.04$ and $p = 0.02$, respectively, not statistically significant due to multiple comparisons).

Importantly, the *R*-methadone and *R*-methadone plus MK801 groups were not different from each other for any respiratory parameters. These data show that MK-801 does not have the same effects as *S*-methadone on respiration, when combined with *R*-methadone, suggesting the effects of *S*-methadone are independent of its NMDA antagonism.

3.7. Metabolic data during maximum depression

Fig. 4 shows oxygen consumption ($\dot{V}_{\text{O}_2}$) and CO_2 production ($\dot{V}_{\text{CO}_2}$), as well as $\dot{V}_I / \dot{V}_{\text{O}_2}$ and $\dot{V}_I / \dot{V}_{\text{CO}_2}$ at maximum depression after drug administration while breathing RA. At time points corresponding to maximum depression of $\dot{V}_I$, both $\dot{V}_{\text{O}_2}$ and $\dot{V}_{\text{CO}_2}$ had main effects of drug and age, as well as an age-drug interaction ($p < 0.05$ for all comparisons,

Fig. 4 A,B). Overall, the *S*- plus *R*-methadone group was significantly different from all other groups and for both measurements. On day 3, the *R*- plus *S*-methadone treatment group had a significantly lower $\dot{V} O_2$ and $\dot{V} CO_2$ than the other groups. There was no difference between controls and the *R*-methadone group. Similarly, there were no statistical effects for $\dot{V} I/ \dot{V} O_2$ and $\dot{V} I/ \dot{V} CO_2$ for any treatment groups (Fig. 4 C,D), suggesting neither treatment causes hyper- nor hypoventilation.

These data show that the consumption of oxygen and production of carbon dioxide is not changed in the *R*-methadone group when compared with controls, while being significantly reduced in the youngest animals in the *R*- plus *S*-methadone group.

4. Discussion

The purpose of this study was to determine if the respiratory depressive effects of *R*-methadone were augmented by *S*-methadone in neonatal guinea pigs, when given as a racemic mixture. We developed this hypothesis based on our recent findings (Nettleton et al. 2007), where we observed bradypnea in 3-pday old pups after single acute doses of methadone. If this hypothesis is valid, the group given *R*- plus *S*-methadone would have greater and longer lasting respiratory depression than the group given *R*-methadone alone.

The major finding of this study was that *R*-methadone caused significantly reduced respiratory depression compared to that of racemic methadone. Racemic methadone, like most opioids, characteristically decreases and blunts the ventilatory response to hypercapnic conditions (Nettleton et al. 2007), as we have shown here by measuring $\dot{V} I$, f_R , and V_T . In the current study, however, we established that these effects were significantly smaller for animals given *R*-methadone alone during CO_2 challenge, especially in the youngest animals. Even during RA breathing, the *R*- plus *S*-methadone group showed some significant differences from control groups (as seen in Nettleton et al. 2007) on days 3 and 7, while the *R*-methadone group was indistinguishable from controls across all three parameters under this condition. Additionally, while the *R*- plus *S*-methadone group experienced significant increases in inspiratory and expiratory time during both RA breathing and CO_2 challenge as compared to controls, the *R*-methadone group showed significantly smaller increases in these parameters, and was not statistically different from controls overall during RA breathing.

Our metabolic data also showed differences between racemic methadone and *R*-methadone. Only the *R*- plus *S*-methadone group showed decreased $\dot{V} O_2$ and $\dot{V} CO_2$ compared to control and *R*-methadone groups for day 3. These differences did not affect $\dot{V} I/ \dot{V} O_2$ or $\dot{V} I/ \dot{V} CO_2$, suggesting that decreased oxygen metabolism for these animals resulted in decreased ventilation as well for the doses we used in this study. However, metabolic parameters were only measured during RA breathing in order to maintain a steady state, so metabolism during CO_2 challenge is not known.

Interestingly, our results as a whole suggest that the synergistic effects of *S*-methadone on *R*-methadone regarding respiratory depression are developmental in nature, and have the greatest impact on the youngest animals. For all ventilatory parameters, differences between *R*-methadone and *R*- plus *S*-methadone groups are largest for day 3. Differences can still be observed to a lesser extent on day 7, but seem to resolve largely by day 14. Taken together, the results of this study suggest that administration of only the active *R*-methadone isomer would decrease the unwanted side effect of respiratory depression caused by racemic methadone in the neonate. In addition, it has been suggested that *S*-methadone causes an increase in the QTc interval, which can lead to cardiac arrhythmias and sudden death

(Skjervold et al. 2006; Eap et al. 2007). Therefore, we conclude that replacing the use of racemic methadone in the neonate with the active *R*-methadone isomer only may lower the risk of severe respiratory depression as well as cardiac arrhythmias.

Although the findings of this study clearly show that racemic methadone causes a statistically greater degree and duration of respiratory depression than *R*-methadone alone, it is unknown why this effect is observed. At least four possible interactions may be causing the observed increase in respiratory depression: 1) spontaneous isomerization of the *S*-methadone isomer to the *R*-methadone isomer, 2) noncompetitive antagonism of the NMDA (*N*-methyl-D-aspartate) receptor by *S*-methadone, 3) DOP (delta opioid receptor) agonism by one or both isomers, and 4) developmental and stereoselective effects of CYP 450 enzymes on methadone metabolism and clearance.

4.1. Spontaneous isomerization

The increased effects observed within the *R*- plus *S*-methadone treatment group could have been a result of spontaneous isomerization of the *S*-methadone enantiomer into the *R*-methadone isomer. However, this hypothesis was discounted since there was no difference in respiratory effects between the control groups given saline and *S*-methadone. In addition, we found no data to support that spontaneous isomerization occurs *in vivo* for these isomers.

4.2. NMDA antagonism

Several studies have investigated the respiratory effects of NMDA receptor blockade in neonates and adults, as well as intact or vagotomized test subjects, and have implicated the NMDA receptor both in inspiratory off-switching and apneusis (Foutz et al. 1989; Schweitzer et al. 1990; Morin-Surun et al. 1995; Cassus-Soulanis et al. 1995; Borday et al. 1998; Waters et al. 2005). For example, in adult guinea pigs, blockage of the NMDA receptors combined with decreased vagal signal transmission has resulted in apneusis, a pattern of atypical breathing defined by a prolonged inspiratory phase (Morin-Surun et al. 1995). These studies raise several questions regarding the involvement of the NMDA receptor in respiratory rhythmogenesis and control. Additionally, while there is a large difference between the binding affinities of *R*- and *S*-methadone at the MOP (Kristensen et al. 1995; Wallisch et al. 2007), both isomers are equipotent non-competitive antagonists of the NMDA receptor (Gorman et al. 1997). To test this hypothesis, we performed a series of trials using MK-801, a noncompetitive NMDA receptor antagonist (Wong et al. 1986; Woodruff et al. 1987; Souverbie et al. 1996), as well as MK-801 co-administered with *R*-methadone. These data revealed a greater level of respiratory depression in the *R*- plus *S*-methadone treated group than in either the *R*-methadone alone or the *R*-methadone plus MK-801 groups, suggesting that there is minimal or no involvement of the NMDA receptor in the increased respiratory depressive effects observed after administration of *R*- plus *S*-methadone.

4.3. DOP agonism

In addition to the MOP receptor, both isomers of methadone are also partial agonists at the DOP receptor (Kristensen et al. 1995; Liu et al. 1999). Several published studies have suggested that both the MOP and DOP receptors play a role in respiratory depression (Pazos et al. 1983; Pazos et al. 1984; Chen et al. 1996; Johnson et al. 2008). Furthermore, a study involving DOP ligands, given concomitantly with the opioid alfentanil, showed that paradoxically both agonists and antagonists attenuated respiratory depression without affecting analgesia (Su et al. 1998). These results suggest that the DOP receptor affects respiration, but not necessarily analgesia, via an interaction with the MOP receptor. In whole brain studies with neonatal rats, DOP receptor expression is minimal and increases with age

(Kent et al. 1981; Petrillo et al. 1987); however, the opposite occurs in the brainstem (Kivell et al. 2004), the brain region most often associated with respiration.

If neonatal guinea pig developmental DOP expression is similar to that of the rat, it is possible that DOP agonism in conjunction with MOP agonism by methadone leads to an increase in respiratory depressive effects above and beyond what is seen from MOP agonism alone, which would resolve with age as DOP expression in the brainstem decreases. While one study found the IC₅₀ for *S*-methadone to be about 25 times higher than for *R*-methadone at the DOP *in vitro*, suggesting that *S*-methadone is ineffective at this receptor alone (Kristensen et al. 1995), little is known about stereoselective binding *in vivo* at the DOP in the presence of the MOP. Additionally, these experiments used the bovine caudate nucleus rather than the guinea pig brainstem. At present, DOP agonism offers an interesting potential explanation of the effects observed in this study. However, much further study is needed.

4.4. Developmental and stereoselective effects of CYP 450 enzymes

Several of the human CYP 450 enzymes have been implicated in methadone metabolism: 1A2, 3A4, 2B6, 2C19, 2C9, 2C8, and 2D6 (Boulton et al. 2001; Gerber et al. 2004; Totah et al. 2007; Rollason et al. 2008; Weschules et al. 2008; Shiran et al. 2009). The human newborn has little methadone metabolizing CYP 450 enzyme expression (Blake et al. 2005); however, expression levels in the neonatal guinea pig are unknown. The lack of significant levels of these enzymes that are vital to the metabolism and clearance of methadone, as well as other drugs, could explain why we observed an increase in respiratory depression in the 3-day old neonatal guinea pig, but not the 14-day old, if the enzyme expression is similar to the human neonate. Prior to significant development of CYP 450 enzymes, the neonate would rely more heavily on renal clearance of methadone. Since in our study we used test groups given either 5 mg/kg of *R*-methadone alone, 5 mg/kg of *S*-methadone alone, or 5 mg/kg of *R*-methadone plus 5 mg/kg of *S*-methadone, the group given racemic methadone had twice as much drug to compete for limited enzyme concentrations than the other groups. This difference in dose could explain why the duration and degree of respiratory depression were increased, as it would take longer for the neonate to clear 10 mg/kg of racemic methadone, than only 5 mg/kg of either isomer. Once CYP 450 enzyme activity begins to increase as the neonate develops, methadone metabolism and clearance may increase such that the 14-day old guinea pig readily metabolized the doses we chose without significant enantiomer competition. However, this hypothesis needs to be examined further using multiple doses of racemic methadone and its enantiomers.

Stereoselectivity of CYP 450 enzymes could also contribute to the differences in respiratory depressive effects we observed for *R*-, *S*-, and *R*- plus *S*-methadone groups. However, there is controversy as to which CYP 450 enzymes are primarily responsible for the stereoselective biotransformation of *R*- and *S*-methadone (Wang et al. 2003; Collier et al. 2007; Rollason et al. 2008; Kharasch et al. 2008a; Kharasch et al. 2008b). While these studies together suggest CYP 450 enzymes 2B6 and 2C19 may have the most influence on stereoselective methadone metabolism in the human, the specific CYP 450 enzymes required for metabolism of methadone in the guinea pig have not yet been identified. Further investigation is required to determine the contributions of CYP 450 enzymes to the metabolism of methadone in the neonatal guinea pig.

5. Conclusion

This study has produced novel findings that indicate that the respiratory depressive effects observed in methadone treated neonates are not attributable entirely to the *R*-methadone isomer, but that the supposedly inactive *S*-methadone isomer is synergistically contributing as well. Furthermore, our findings suggest that the increased respiratory depressive effects

of racemic methadone are probably not a result of the antagonistic activity of *R*- and *S*-methadone at the NMDA receptor. This discovery has clinical relevance for the administration of methadone as an analgesic for infants in that it may be beneficial to treat patients using only the *R*-methadone isomer as opposed to a racemic mixture. *R*-methadone treatment may reduce not only respiratory effects, but also the cardiac effects that have been suggested by the medical literature.

However, it is important not only to establish why neonates experience a greater degree of respiratory depression after opioid administration, but also to determine whether *R*-methadone alone causes an equal level of antinociception as the racemic mixture. In this regard, we noticed no overall difference in locomotor depression between the active drug groups, suggesting that there is an equivalent level of sedation when compared to the *R*- plus *S*-methadone group. However, sedation is not equivalent to analgesia. Though there are studies suggesting that the analgesic effects of *R*-methadone are equivalent to those of racemic methadone in humans (Lemberg et al. 2006), more studies are still needed to definitively determine the levels of analgesia in both groups.

Future research should include a detailed examination of the mechanism of action for *S*-methadone, including action at the DOP receptor, CYP 450 metabolism and clearance, and CYP 450 development in the neonate, in order to determine how this previously presumed ineffectual opioid is augmenting the respiratory depression that was believed to be caused solely by *R*-methadone.

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Fig. 1. Time action curves for ventilatory response of neonatal guinea pigs during RA breathing and CO_2 challenge for $\dot{V}_I$, f_R , and V_T on days 3 (A-F), 7 (G-L), and 14 (M-R). Data presented as mean $\pm$ SE ($n=6$)

* $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs both control groups

† $p<0.05$, †† $p<0.01$, ††† $p<0.001$ vs R-methadone

Fig. 2. Maximum respiratory depression in neonatal guinea pigs for V_T/T_I (A-B), T_I (C-D), and T_E (E-F) during RA breathing and CO_2 challenge. Data presented as mean $\pm$ SE (n=6)

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs both control groups

† $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs R-methadone

^a $p < 0.05$ vs day 7 same treatment

^b $p < 0.05$ vs day 14 same treatment

Fig. 3. Maximum respiratory depression for neonatal guinea pig treatment groups containing *R*-methadone for $\dot{V}_I$ (A), f_R (B), and V_T (C) during CO₂ challenge and NMDA antagonism. Data presented as mean $\pm$ SE (n=6)
 * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs both control groups
 † $p < 0.05$, †† $p < 0.01$ vs *R*-methadone
^a $p < 0.05$ vs day 7 same treatment
^b $p < 0.05$ vs day 14 same treatment

Fig. 4.

Metabolic parameters $\dot{V}O_2$ (A), $\dot{V}CO_2$ (B), $\dot{V}_I/\dot{V}O_2$ (C), and $\dot{V}_I/\dot{V}CO_2$ (D) for neonatal guinea pigs at the same time periods as the maximum respiratory depression during CO_2 challenge. Data presented as mean $\pm$ SE (n=6)

** $p < 0.01$, *** $p < 0.001$ vs both control groups

†† $p < 0.01$ vs R-methadone

^b $p < 0.05$ vs day 14 same treatment

Table 1

Locomotor activity (number of beam breaks) before and 45 min after drug administration (mean $\pm$ SE and range)

	Age	Before Drug Treatment	After Drug Treatment
Saline	Day 3	349 $\pm$ 59 (150-537)	193 $\pm$ 30 (132-335) ^a
	Day 7	302 $\pm$ 39 (207-472)	328 $\pm$ 53 (171-507)
	Day 14	384 $\pm$ 60 (229-640)	212 $\pm$ 34 (72-290)
<i>S</i> -Methadone	Day 3	323 $\pm$ 57 (124-452)	246 $\pm$ 50 (78-353)
	Day 7	264 $\pm$ 32 (184-404) ^b	162 $\pm$ 26 (80-257) ^{†††}
	Day 14	467 $\pm$ 29 (341-575)	299 $\pm$ 39 (170-475)
<i>R</i> -Methadone	Day 3	366 $\pm$ 83 (125-697)	14 $\pm$ 3 (3-22)***
	Day 7	396 $\pm$ 56 (151-601)	30 $\pm$ 14 (0-101)**
	Day 14	451 $\pm$ 51 (339-740)	15 $\pm$ 4 (0-29)***
<i>R</i> - plus <i>S</i> -Methadone	Day 3	247 $\pm$ 50 (94-442)	15 $\pm$ 5 (0-27)***
	Day 7	282 $\pm$ 49 (166-469)	33 $\pm$ 8 (3-57)**
	Day 14	305 $\pm$ 59 (120-503)	19 $\pm$ 6 (2-43)***

**
 $p < 0.01$

 $p < 0.001$ vs saline and *S*-methadone

†††
 $p < 0.001$ vs saline

^a
 $p < 0.05$ vs day 7 same treatment

^b
 $p < 0.05$ vs day 14 same treatment; n = 6 per group

Table 2

Baseline respiratory data for $\dot{V}_I$, f_R , V_T , V_T/T_I , T_I , and T_E (mean $\pm$ SE)

		$\dot{V}_I$ (ml·min ⁻¹ ·100g ⁻¹)	f_R (breaths·min ⁻¹)	V_T (ml·100g ⁻¹)	V_T/T_I (ml·sec ⁻¹ ·100g ⁻¹)	T_I (sec)	T_E (sec)
Day 3	RA	88 $\pm$ 6 ^{†††} ***	142 $\pm$ 5	0.62 $\pm$ 0.03 ^{†††} ***	3.6 $\pm$ 0.3 [†] ***	0.18 $\pm$ 0.007	0.26 $\pm$ 0.011
	CO ₂	324 $\pm$ 16 ^{†††} ***	188 $\pm$ 3 ***	1.74 $\pm$ 0.07 ^{†††} ***	12.0 $\pm$ 0.6 ^{†††} ***	0.15 $\pm$ 0.003 ***	0.18 $\pm$ 0.003 ^{††} ***
Day 7	RA	71 $\pm$ 4 *	150 $\pm$ 7	0.49 $\pm$ 0.02 *	3.0 $\pm$ 0.2 *	0.17 $\pm$ 0.008	0.26 $\pm$ 0.011
	CO ₂	272 $\pm$ 10 ***	182 $\pm$ 3 ***	1.51 $\pm$ 0.05 ***	10.5 $\pm$ 0.4 ***	0.14 $\pm$ 0.003 ***	0.19 $\pm$ 0.004 ***
Day 14	RA	57 $\pm$ 5	141 $\pm$ 7	0.42 $\pm$ 0.03	2.4 $\pm$ 0.3	0.19 $\pm$ 0.011	0.28 $\pm$ 0.015
	CO ₂	213 $\pm$ 9	162 $\pm$ 4	1.32 $\pm$ 0.04	8.1 $\pm$ 0.1	0.17 $\pm$ 0.005	0.21 $\pm$ 0.006

n = 24; RA, room air; CO₂, 5% CO₂ challenge[†] p<0.05^{††} p<0.01^{†††} p<0.001 vs. day 7

*

p<0.05

** p<0.01

*** p<0.001 vs. day 14

Table 3Time (min) to maximum depression for the *R*-methadone containing drug groups (mean $\pm$ SE and range)

	Day 3	Day 7	Day 14
<i>R</i> -Methadone	25 $\pm$ 3 (15-30)	30 $\pm$ 7 (15-60)	35 $\pm$ 8 (15-60)
<i>R</i> - plus <i>S</i> -Methadone	25 $\pm$ 3 (15-30)	23 $\pm$ 3 (15-30)	25 $\pm$ 3 (15-30)
<i>R</i> -Methadone plus MK801	28 $\pm$ 7 (15-60)	23 $\pm$ 3 (15-30)	25 $\pm$ 3 (15-30)