In Vivo Activity of Norhydrocodone: An Active Metabolite of Hydrocodone

Dipesh M. Navani and Byron C. Yoburn

Department of Pharmaceutical Sciences, College of Pharmacy and Health Sciences, St. John’s University, Queens, New York

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ABSTRACT

Hydrocodone is primarily metabolized to hydromorphone and norhydrocodone. Although hydromorphone is a known active metabolite of hydrocodone, the in vivo activity of norhydrocodone is not well documented. In the current study, the pharmacodynamics of norhydrocodone were evaluated and compared with hydrocodone and hydromorphone. Binding studies established that norhydrocodone, similar to hydrocodone and hydromorphone, is a μ-selective opioid ligand. In vivo analgesia studies (tail flick) demonstrated that, following subcutaneous, intrathecal, and intracerebroventricular administration, norhydrocodone produced analgesia. Following subcutaneous administration, norhydrocodone was ∼70-fold less potent, and hydromorphone was ∼4.4-fold more potent than hydrocodone in producing analgesia. Following intrathecal administration, norhydrocodone produced a shallow analgesia dose-response curve and maximal effect of 15–45%, whereas hydrocodone and hydromorphone produced dose-dependent analgesia. Intrathecal hydromorphone was ∼174-fold more potent than intrathecal hydrocodone. Following intracerebroventricular administration, norhydrocodone had similar potency to hydrocodone in producing analgesia, while hydromorphone was ∼96-fold more potent than hydrocodone. Analgesia induced by the three drugs following subcutaneous, intrathecal, and intracerebroventricular administration was antagonized by subcutaneous naltrexone, confirming that it is opioid receptor-mediated. Subcutaneous norhydrocodone-induced analgesia was completely blocked by intracerebroventricular naltrexone, indicating that norhydrocodone-induced analgesia is likely a supraspinal effect. Seizure activity was observed following intrathecal administration of all three drugs. Norhydrocodone and hydromorphone were ∼3.7 to 4.6-fold more potent than hydrocodone in inducing seizure activity. Naltrexone did not antagonize opioid-induced seizure activity, suggesting that seizures were not opioid receptor-mediated. Taken together, norhydrocodone is an active metabolite of hydrocodone and may contribute to therapeutic and toxic effects following hydrocodone administration.

INTRODUCTION

Hydrocodone, a semisynthetic opioid, is metabolized primarily by CYP-mediated pathways (Otton et al., 1993; Hutchinson et al., 2004). In humans, rodents, and dogs, hydromorphone and norhydrocodone are the primary metabolites of hydrocodone (Cone et al., 1978; Li et al., 2013). In humans, more than 50% of total hydrocodone clearance is via CYP2D6- and CYP3A4-mediated pathways leading to formation of hydromorphone and norhydrocodone, respectively (Hutchinson et al., 2004). Other metabolites of hydrocodone found in lower concentrations in humans include 6α- and 6β-hydrocodol, which are formed via non-CYP-mediated pathways (Cone et al., 1978; Li et al., 2013). The metabolites of hydrocodone, except norhydrocodone, have been examined and demonstrated to possess some analgesic activity (Small et al., 1938). It has been suggested that hydrocodone is a prodrug and requires metabolism to hydromorphone, which is an active opioid agonist (Lurcott, 1998). However, hydromorphone may not play a critical role in the pharmacologic effect of hydrocodone, since inhibition of conversion of hydrocodone to hydromorphone had no significant influence on the analgesic potency of hydrocodone in rats (Tomkins et al., 1997) or abuse liability of hydrocodone in humans (Kaplan et al., 1997). At present, the in vivo contribution of the second major metabolite, norhydrocodone, to the pharmacodynamics of hydrocodone is not well documented.

The reported elimination half-life for hydrocodone following oral administration in humans is 3–9 hours (Otton et al., 1993; Hao et al., 2011) and for hydromorphone following intravenous and oral administration in humans, the t1/2 is 2–4 hours (Vallner et al., 1981; Parab et al., 1988). The reported half-life for norhydrocodone following oral hydrocodone administration is 8 hours (Hao et al., 2011). Studies analyzing human plasma and urine following oral hydrocodone indicated that norhydrocodone concentration exceeds that of hydromorphone, and in some cases, that of the parent compound (Hao et al., 2011; Valtier and Beharta, 2012). In addition, the examination of urine specimens revealed that norhydrocodone is detected for a significantly longer period compared to both hydrocodone and hydromorphone (Valtier and Beharta, 2012). Overall, this suggests that chronic administration of hydrocodone might lead to accumulation of the nor-metabolite.

ABBREVIATIONS: CL, confidence limit; CYP, cytochrome P450; DAMGO, [D-Ala2,N-MePhe4,Gly-ol5]enkephalin; DPDPE, [D-Pen2,D-Pen5]enkephalin; HYC, hydrocodone; HYM, hydromorphone; ICV, intracerebroventricular; IT, intrathecal; NHYC, norhydrocodone; NTX, naltrexone; SC, subcutaneous.
While norhydrocodone has been suggested to be an inactive metabolite (Smith, 2009), one in vitro study examining G-protein activation by N-demethylated codeine congeners compared to the parent molecules demonstrated that norhydrocodone had lower potency but similar efficacy as hydrocodone for μ- and δ-opioid receptor–mediated G-protein activation (Thompson et al., 2004). Since these data indicate that norhydrocodone activates opioid receptor signaling and, as noted above, is a major metabolite of hydrocodone, we hypothesized that norhydrocodone may possess in vivo activity and might contribute to the pharmacodynamics of hydrocodone. In addition, based on the reports of other opioid non-metabolites (normeperidine, noroxycodone) having toxic properties in rodents and humans (Szeto et al., 1977; Umans and Inturrisi, 1982; Leow and Smith, 1994), we could not rule out that norhydrocodone may also have untoward characteristics.

In the current study, hydrocodone, hydromorphone, and norhydrocodone were evaluated for analgesic and neurotoxic activity in a mouse model. Subcutaneous, intrathecal, and intracerebroventricular routes of administration were used to examine each opioid agonist. Overall, the results indicate that, like hydrocodone and hydromorphone, norhydrocodone is a μ-selective opioid ligand and that the in vivo effects of norhydrocodone include both opioid receptor–mediated analgesia and non-opioid receptor–mediated neuroexcitatory effects.

Materials and Methods

Subjects

Male Swiss Webster mice, weighing 28 ± 2 g (mean ± S.D.), obtained from Taconic Farms (Germantown, NY), were used throughout. Animals were housed 10 per cage with food and water available ad libitum. Mice were allowed to acclimatize to the home cage for at least 24 hours prior to experimentation. All protocols were approved by the St. John's University Institutional Animal Care and Use Committee.

Receptor Binding Studies

Binding studies were performed as previously described (Yoburn et al., 1995; Sirohi et al., 2007). Mice (10–12/group) were killed, and spinal cords were removed and homogenized at 20,000 rpm (Brinkmann Polytron Homogenizer, Westbury, NY) in 15 ml of ice-cold 50 mM Tris buffer, pH 7.4. Homogenates were centrifuged at 15,000 rpm for 15 minutes at 4°C, and the pellet was resuspended and cold 50 mM Tris buffer, pH 7.4. Homogenates were centrifuged again and resuspended in 50 mM potassium phosphate buffer, pH 7.2. Protein was determined (Bradford, 1976) using reagent from Bio-Rad (Hercules, CA). For saturation studies, spinal cord homogenate was assayed in triplicate in tubes containing 0.08–10 nM [3H]DAMGO, 0.08–10 nM [3H]DPDPE, or 0.16–10 nM [3H]U69,593 (PerkinElmer Life and Analytical Sciences, Boston, MA). For competition studies, non-labeled levorphanol ([3H]DAMGO, [3H]DPDPE), or 10 nM naltrexone ([3H]U69,593). All tubes were incubated for 90 minutes at 25°C, then incubation was terminated by the addition of ice-cold 50 mM potassium phosphate buffer and samples were filtered over GF/B filters (Brandel, Gaithersburg, MD). Tubes were washed three times with phosphate buffer, and filters were counted. For [3H]U69,593 binding, filters were soaked in 0.5% w/v polyethylenimine (Sigma-Aldrich, St. Louis, MO) for 90 minutes before use. Binding data were analyzed by Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA) using nonlinear regression. Kᵢ and B_max values were calculated for saturation studies. Kᵢ values were determined for competition studies (Cheng and Prusoff, 1973). All binding data were best fit by a one-site model.

In Vivo Studies

General Procedure. Three routes of administration were used: subcutaneous, intrathecal, and intracerebroventricular. Mice were injected with hydrocodone, hydromorphone, or norhydrocodone based on doses determined in preliminary experiments. The following procedures were used for all three routes.

Mice were tested for analgesia using the tail-flick assay at 5–240 minutes post-injection (see “Analgesia Studies” to determine the time of peak analgesic effect. Dose-response studies were conducted for each drug at the time of peak analgesic effect. Finally, the effect of naltrexone on opioid-induced analgesia was determined. Quantal data were used for dose-response functions and to determine ED₅₀,ₘ for analgesia, and graded data were collected for time-action and naltrexone-antagonism studies. The naltrexone-antagonism studies were designed to test analgesia at the time of peak effect for both the opioid agonists (hydrocodone, hydromorphone, norhydrocodone) and the opioid antagonist naltrexone. The time of peak effect for subcutaneous naltrexone has been shown to be 45 minutes (Sirohi et al., 2007).

Mice injected intrathecally with hydrocodone, hydromorphone, and norhydrocodone were observed for seizure activity for up to 30 minutes (see “Seizure Activity Assay”). The effect of subcutaneous and intrathecal naltrexone on intrathecal hydrocodone, hydromorphone, and norhydrocodone-induced seizures was studied. In these studies, agonists were injected intrathecally at the time of peak effect for subcutaneous naltrexone. Quantal data were collected for seizure dose-response functions to determine ED₅₀,ₘ and to examine the effect of naltrexone on opioid-induced seizures.

Subcutaneous Analgesia Studies. Mice (N = 5–6/group) were injected with hydrocodone (1.25 mg/kg), hydromorphone (0.3 mg/kg), or norhydrocodone (4–200 mg/kg) and tested for analgesia at 5–240 minutes post-injection to determine the time of peak analgesic effect. Next, mice (N = 5–6/dose) were injected with hydrocodone (0.625–3 mg/kg), hydromorphone (0.1–0.4 mg/kg), or norhydrocodone (4–200 mg/kg) and tested for analgesia at the time of peak effect and ED₅₀,ₘ estimated. Lastly, mice (N = 6–11/group) were injected subcutaneously with either saline or naltrexone (1 mg/kg) and then injected immediately with hydrophormone (0.3 mg/kg) or norhydrocodone (100 mg/kg); or 30 minutes later injected with hydrocodone (2 mg/kg). Forty-five minutes after saline or naltrexone injections at the time of peak effect of each opioid and naltrexone, mice were tested for analgesia.

Intrathecal Analgesia Studies. Mice (N = 5–9/group) were injected with hydrocodone (1–5 μg/mouse), hydromorphone (0.005–0.5 μg/mouse), or norhydrocodone (0.01–1 mg/mouse) and tested for analgesia at 5–240 minutes post-injection to determine the time of peak analgesic effect. Next, mice (N = 6–11/dose) were injected with hydrocodone (0.1–5 μg/mouse), hydromorphone (0.001–0.05 μg/mouse), or norhydrocodone (0.001–1 μg/mouse) and tested for analgesia at the time of peak effect and ED₅₀,ₘ estimated. Lastly, mice (N = 6–14/group) were injected subcutaneously with either saline or naltrexone (1 mg/kg or 10 mg/kg) and then 35 minutes later injected with hydrocodone (5 μg/mouse) or hydromorphone (0.05 μg/mouse), or 40 minutes later injected with norhydrocodone (0.1 μg/mouse). Forty-five minutes after saline or naltrexone injections at the time of peak effect of each opioid and naltrexone, mice were tested for analgesia. In the intrathecal analgesia studies, it was noted that high doses of norhydrocodone (>1 μg/mouse) induced seizure activity in mice.
Intracerebroventricular Analgesia Studies. Mice (N = 5–6/group) were injected with hydrocodone (1–20 μg/mouse), hydromorphone (0.01–0.1 μg/mouse), or norhydrocodone (1–10 μg/mouse) and tested for analgesia at 10–180 minutes post-injection to determine the time of peak analgesic effect. Next, mice (N = 6–12/dose) were injected with hydrocodone (0.5–20 μg/mouse), hydromorphone (0.01–0.1 μg/mouse), or norhydrocodone (0.25–4 μg/mouse) and tested for analgesia at the time of peak effect and ED50s estimated. In addition, mice (N = 5–11/group) were injected subcutaneously with either saline or naltrexone (1 mg/kg) and then 15 minutes later injected with hydromorphone (0.1 μg/mouse); or 30 minutes later injected with hydrocodone (10 μg/kg) or norhydrocodone (4 μg/mouse). Forty-five minutes after saline or naltrexone injections at the time of peak effect of each opioid and naltrexone, mice were tested for analgesia.

It was hypothesized that subcutaneous norhydrocodone-induced analgesia was predominantly a supraspinal effect, and therefore, we examined the effect of intracerebroventricular naltrexone on subcutaneous norhydrocodone. Mice (N = 5/group) were injected subcutaneously with norhydrocodone (200 mg/kg) and then 30 minutes later injected intracerebroventricularly with either saline or naltrexone (10 μg/mouse). Forty-five minutes after subcutaneous norhydrocodone injections mice were tested for analgesia.

Intrathecal Seizure Studies. As noted above (see “Intrathecal Analgesia Studies”), mice injected intrathecally with doses of norhydrocodone >1 μg/mouse demonstrated seizure activity. To explore this informal observation, dose-response studies for intrathecal opioid agonist–induced seizures were conducted (see “Seizure Activity Assay”). Mice (N = 5–10/dose) were injected intrathecally with hydrocodone (5–50 μg/mouse), hydromorphone (0.5–7.5 μg/mouse), or norhydrocodone (1–10 μg/mouse) and observed for seizure activity and ED50 estimated. Next, mice (N = 6–15/group) were injected subcutaneously with either saline or naltrexone (10 or 30 mg/kg) and then 45 minutes later injected intrathecally with hydrocodone (50 μg/mouse), hydromorphone (5 μg/mouse), or norhydrocodone (10 μg/mouse) and observed for seizure activity. In addition, mice (N = 5–6/group) were injected with intrathecal naltrexone (10 μg/mouse) alone or intrathecal naltrexone (10 μg/mouse) and intrathecal hydrocodone (50 μg/mouse), hydromorphone (5 μg/mouse), or norhydrocodone (10 μg/mouse) and observed for seizure activity.

Intrathecal Injection

Mice were injected intrathecally using a modification of the method of Hylden and Wilcox (1980). Mice were briefly (~2 minutes) anesthetized with isoﬂurane/oxygen (4:96) and injected (2–4 μl) in the lumbar intrathecal space using a 30-gauge needle attached to a 10-μl glass syringe (Hamilton, Reno, NV). The needle was held in place for 10 seconds following each injection.

Intracerebroventricular Injection

Mice were injected intracerebroventricularly using previously described methods (Pedigo et al., 1975; Yoburn et al., 1988). Mice were briefly (~2 minutes) anesthetized with isoﬂurane/oxygen (4:96), and a small scalp incision was made to expose the skull. The incision was closed following injection with a surgical staple. Drug solutions (4 μl) were injected 2 mm lateral and 2 mm posterior to bregma using a 2.5-mm long 27-gauge needle attached to a 25-μl glass syringe (Hamilton). The needle was held in place for 30 seconds following each injection.

Analgesia Assay

Analgesia (antinociception) was determined using the tail-flick assay (Tailliff Model 37360; UGO Basile, Comerio, Varese, Italy). The ventral surface of the tail was exposed to heat approximately 2 cm from the tip of the tail. The heat stimulus was adjusted so that the baseline tail-flick latency would typically be 2–4 seconds (actual mean ± S.D. baseline latency = 2.4 ± 1.1 seconds). Baseline testing was performed for each animal before drug administration. During baseline testing, if the animal did not remove its tail from the heat source by 10 seconds, the test was terminated and the animal was excluded from further study. Following drug treatment, if a mouse did not remove its tail from the heat source by 10 seconds, the test was terminated, a latency of 10 seconds was recorded, and the mouse was defined as analgesic. All testing was conducted by a tester who was unaware of the treatment of an individual mouse.

Seizure Activity Assay

Mice were observed for seizure activity for up to 30 minutes following intrathecal injection of opioid agonists. The effects included: agitation, hind limb extension, and rotatory movements, followed by development of tonic-clonic seizures and in some cases loss of righting reflex (Lutfy et al., 1994). The onset of seizure activity was always within 15 minutes of intrathecal injection. The duration of seizures was not determined. A tester unaware of individual mouse treatment observed the animals and identified a mouse as positive for seizure activity. Seizure activity was collected as quantal data.

Drugs

Hydromorphone bitartrate, hydromorphone hydrochloride, and naltrexone hydrochloride were obtained from Spectrum Chemicals and Laboratory Products (Gardena, CA). Norhydrocodone hydrochloride was obtained from Lipomed Inc. (Cambridge, MA). Drugs for all the injections (subcutaneous, intrathecal, intracerebroventricular) were dissolved in 0.9% saline, and doses are expressed as the free base.

Data Analysis

Receptor binding studies were analyzed using one-way analysis of variance (ANOVA) and post-hoc Student-Newman-Keuls tests (Pﬁzer version 5.0; GraphPad Software, Inc.). Quantal dose-response data were analyzed using the BLISS-21 computer program (Department of Statistics, University of Edinburgh, Edinburgh, UK). This program uses Probit analysis (Finney, 1973) to calculate ED50, standard errors, 95% conﬁdence intervals, and relative potency estimates (ratio of respective ED50). The effect of naltrexone on subcutaneous, intrathecal, and intracerebroventricular opioid-induced analgesia data was analyzed using the Student’s t test or one-way ANOVA and post-hoc Student-Newman-Keuls tests (Pﬁzer version 5.0; GraphPad Software, Inc., La Jolla, CA). The effect of naltrexone on intrathecal opioid-induced seizures was analyzed using χ² (Pﬁzer version 5.0; GraphPad Software, Inc.). In all cases, the level of signiﬁcance was P < 0.05.

Results

Receptor Binding Studies. Opioid receptor (μ, δ, κ) saturation binding studies were performed in mouse spinal cord tissue homogenate. The mean (±S.D.) Bmax and KD from three independent experiments were: [3H]DAMGO (μ): 164 (±39) fm/mg protein, 1.4 (±0.4) nM; [3H]DPDPE (δ): 31 (±8) fm/mg protein, 1.2 (±0.2) nM; [3H]U69,593 (κ): 28 (±1) fm/mg protein, 3.3 (±0.4) nM. Competition binding studies for hydrocodone, hydromorphone, and norhydrocodone indicated that each opioid was relatively μ-selective, with lowest afﬁnity for κ-receptors (Table 1). At μ- and κ-opioid receptors, there was a signiﬁcant difference (P < 0.05) in potency among all three opioids with potency for hydromorphone > hydrocodone > norhydrocodone (μ: FP(2,6) = 31.11; κ: FP(2,6) = 16.31). At δ-opioid receptor, the order of potency was the same, although the difference was just outside the signiﬁcance level (FP(2,6) = 4.58, P = 0.06).
Subcutaneous Analgesia Studies. The time of peak effect for analgesia following subcutaneously administered hydrocodone, hydromorphone, and norhydrocodone was estimated as 15, 45, and 45 minutes, respectively (Fig. 1A). Dose-response studies for subcutaneous opioid-induced analgesia were conducted at the time of peak effect for each opioid, and ED50s were determined (Fig. 1B; Table 2). The order of potency for subcutaneous opioid-induced analgesia was hydromorphone > hydrocodone > norhydrocodone. Hydromorphone was significantly more potent (~5.4-fold), and norhydrocodone was significantly less potent (~70-fold) than the parent compound in producing analgesia following subcutaneous administration (P < 0.05, Probit analysis). Mice were injected with naltrexone (1 mg/kg s.c.) and hydrocodone (2 mg/kg s.c.), hydromorphone (0.3 mg/kg s.c.), or norhydrocodone (100 mg/kg s.c.) and tested for analgesia at the time of peak effect for both antagonist and agonist (see Materials and Methods). Naltrexone significantly antagonized subcutaneous hydrocodone, hydromorphone, and norhydrocodone-induced analgesia, confirming that systemic opioid-induced analgesia is opioid receptor–mediated (Fig. 1C).

Intrathecal Analgesia Studies. The time of peak effect for analgesia following intrathecally administered hydrocodone, hydromorphone, and norhydrocodone was estimated as 10, 10, and 5 minutes, respectively (Fig. 2A). To examine the possible role of the intrathecal injection protocol itself on tail-flick latency, mice (N = 5) were injected with saline (intrathecal) and tested for analgesia at 5, 10, 15, and 30 minutes. Following injection, there was no significant change from baseline tail-flick latency (F4,20 = 1.21, P > 0.05), suggesting that the intrathecal injection protocol did not alter nociception. Dose-response studies for intrathecal opioid-induced analgesia were conducted at the time of peak effect for each opioid, and ED50s were estimated (Fig. 2B; Table 2). Hydromorphone was significantly more potent (~174-fold) than hydrocodone in producing analgesia following intrathecal administration (P < 0.05, Probit analysis). Intrathecal norhydrocodone produced a shallow dose-response function.

### Table 1

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mean Kᵢ (95% CI)</th>
<th>Selectivity Ratios</th>
</tr>
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<tr>
<td></td>
<td>μ nM</td>
<td>δ nM</td>
</tr>
<tr>
<td>HYC</td>
<td>56 (40–80)</td>
<td>1429 (974–2097)</td>
</tr>
<tr>
<td>HYM</td>
<td>1.3 (0.9–2.0)</td>
<td>35 (23–55)</td>
</tr>
<tr>
<td>NHYC</td>
<td>142 (96–210)</td>
<td>2166 (1472–3188)</td>
</tr>
</tbody>
</table>

*a Data are means of three independent experiments.

*b Selectivity ratios were determined by dividing the respective Kᵢ values. See Materials and Methods for details.

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**Fig. 1.** Hydrocodone-, hydromorphone-, and norhydrocodone-induced analgesia following subcutaneous administration. (A) Mice were injected subcutaneously with HYC, HYM, or NHYC and tested for analgesia at 5–240 minutes post-injection. HYC and HYM time-action studies are results of a single experiment. NHYC time-action was examined with a range of doses (see Materials and Methods) and a representative dose is presented. B = baseline tail-flick latency. (B) For dose-response studies, mice were injected with HYC, HYM, or NHYC and tested for analgesia at the time of peak effect. HYC, HYM, and NHYC dose-response curves are results of single experiment (see Table 2 for ED50s). (C) Mice were injected subcutaneously with either saline or NTX and then injected subcutaneously with HYC, HYM, or NHYC. Mice were tested for analgesia at the time of peak effect of each opioid and NTX. Data presented for antagonism of analgesia are the results of one (HYC, NHYC) or two combined (HYM) independent experiments. *Significantly different (P < 0.05) compared with saline injected animals.
for spinal analgesia, and the ED$_{50}$ could not be estimated, although 15–45% of mice were analgesic at intrathecal doses ≥0.001 μg/mouse. Norhydrocodone (>1 μg/mouse i.t.) induced seizure activity in mice that precluded the examination of analgesic effects at higher doses (see “Intrathecal Seizure Studies”). Next, mice were injected with naltrexone (1 or 10 mg/kg s.c.) and hydrocodone (5 μg/mouse i.t.), hydromorphone (0.05 μg/mouse i.t.), or norhydrocodone (0.1 μg/mouse i.t.) and tested for analgesia at the time of peak effect for both antagonist and agonist. Naltrexone (1 mg/kg s.c.) significantly (P < 0.05) antagonized intrathecal hydrocodone and intrathecal hydromorphone-induced analgesia. A higher dose of naltrexone (10 mg/kg s.c.) was required to significantly antagonize intrathecal norhydrocodone-induced analgesia (F$_{2,29}$ = 3.63, P < 0.05). Antagonism by naltrexone confirms that intrathecal opioid-induced analgesia is opioid receptor-mediated (Fig. 2C).

**Intracerebroventricular Analgesia Studies.** The time of peak effect for analgesia following intracerebroventricularly administered hydrocodone, hydromorphone, and norhydrocodone was estimated as 15, 30, and 15 minutes, respectively (Fig. 3A). To examine the possible role of the intracerebroventricular injection protocol on tail-flick latency, mice (N = 13) were injected with saline (intracerebroventricular) and tested for analgesia at 10, 15, and 30 minutes. Following injection, there was no significant change from the baseline tail-flick latency (F$_{3,48}$ = 1.71, P > 0.05). Dose-response studies for intracerebroventricular opioid-induced analgesia were conducted at the time of peak effect for each opioid agonist, and ED$_{50}$s were determined (Fig. 3B; Table 2). The order of potency for intracerebroventricular opioid-induced analgesia was hydromorphone > norhydrocodone ≥ hydrocodone. Hydromorphone was significantly more (∼96-fold) potent than hydrocodone in producing analgesia following intracerebroventricular administration (P < 0.05, Probit analysis). There was no significant difference in the potency of intracerebroventricular norhydrocodone and intracerebroventricular hydrocodone for induction of analgesia (P > 0.05, Probit analysis). Mice were injected with naltrexone (1 mg/kg s.c.) and hydrocodone (10 μg/mouse i.c.v.), hydromorphone (0.1 μg/mouse i.c.v.), or norhydrocodone was estimated as 15, 30, and 15 minutes, respectively (Fig. 3A).

**Fig. 2.** Hydrocodone-, hydromorphone-, and norhydrocodone-induced analgesia following intrathecal administration. (A) Mice were injected intrathecally with HYC, HYM, and NHYC and tested for analgesia at 5–240 minutes post-injection. Time-action studies were conducted with a range of doses for each opioid (see Materials and Methods) with similar results. Representative data from a single dose are presented. B = Baseline tail-flick latency. (B) For dose-response studies, mice were injected with HYC, HYM, or NHYC and tested for analgesia at the time of peak effect. HYC and HYM dose-response curves are results of a single experiment and the NHYC dose-response curve is the mean result of three independent experiments (see Table 2 for ED$_{50}$s). (C) Mice were injected s.c. with either saline or NTX and then injected intrathecally with HYC, HYM, or NHYC. Data presented for antagonism of analgesia are the results of one (HYC, HYM) or two combined (NHYC) independent experiments. *Significantly different (P < 0.05) compared with saline injected animals.
and tested for analgesia at the time of peak effect for both antagonist and agonist. Naltrexone significantly antagonized intracerebroventricular hydrocodone-, hydromorphone-, and norhydrocodone-induced analgesia, confirming that supraspinal opioid-induced analgesia was opioid receptor-mediated (Fig. 3C).

It was hypothesized that systemic norhydrocodone-induced analgesia was predominantly a supraspinal effect. To test this hypothesis, mice were injected subcutaneously with norhydrocodone (200 mg/kg s.c.) and naltrexone (10 μg/mouse i.c.v.). Intracerebroventricular administration of naltrexone completely antagonized the analgesic effect of systemic norhydrocodone (Fig. 4), suggesting that subcutaneous norhydrocodone-induced analgesia is primarily mediated via supraspinal opioid receptors.

Intrathecal Seizure Studies. As noted above (see “Intrathecal Analgesia Studies”), norhydrocodone (>1 μg/mouse) was observed to produce seizure activity in mice after spinal injection. Therefore, dose-response studies of intrathecal opioid-induced seizures were conducted, and ED₅₀ were determined (Fig. 5A; Table 3). The order of potency for opioid-induced seizures following intrathecal administration was norhydrocodone ≈ hydromorphone > hydrocodone. Norhydrocodone and hydromorphone were significantly more potent (~4.6- and 3.7-fold, respectively) than the parent compound in induction of seizures (P < 0.05, Probit analysis). Next, the effect of subcutaneous and intrathecal naltrexone on opioid-induced seizures was examined. Naltrexone (10 mg/kg s.c. and 10 μg/mouse i.t.) did not antagonize seizures induced by intrathecal opioid agonists (Fig. 5B). In addition, a higher dose of naltrexone (30 mg/kg s.c.) was also unable to significantly antagonize seizures produced by norhydrocodone (10 μg/mouse i.t.). Naltrexone alone (10 μg/mouse i.t.) did not induce any seizure activity (data not shown). At the doses used, no seizure activity was observed following subcutaneous or intracerebroventricular administration of hydrocodone, hydromorphone, and norhydrocodone.
Discussion

In the current study, we examined the pharmacodynamics of hydrocodone and its major metabolites hydromorphone and norhydrocodone in the mouse. Hydrocodone and hydromorphone-induced pharmacological effects have been well studied in humans and rodents (Small et al., 1938; Murray and Hagen, 2005; Peckham and Traynor, 2006; Susce et al., 2006; Kumar et al., 2008; Madia et al., 2009). In addition, some reports suggest that hydromorphone, an active metabolite of hydrocodone, is primarily responsible for hydrocodone-induced in vivo effects (Otton et al., 1993; Lurcott, 1998). To date, the in vivo effects of norhydrocodone are not well documented. If norhydrocodone is active in vivo, it may contribute to the pharmacodynamic profile of hydrocodone since plasma concentrations of norhydrocodone in humans have been reported to be greater than that of hydromorphone (Hao et al., 2011). Furthermore, norhydrocodone is detected in urine for significantly longer than hydromorphone and hydrocodone (Valtier and Bebarta, 2012).

The present binding studies demonstrated that norhydrocodone, hydrocodone, and hydromorphone are \( \mu \)-selective opioid ligands, although norhydrocodone has lower affinity at \( \mu \)- and \( \kappa \)-opioid receptors compared with hydrocodone (Table 1). In vitro norhydrocodone has been shown to be moderately lower in potency, with similar efficacy, to hydrocodone at \( \mu \)- and \( \delta \)-opioid receptors (Thompson et al., 2004). While binding studies and in vitro pharmacodynamic data raise the possibility that norhydrocodone might possess in vivo activity and contribute to the opioid receptor-mediated effects of hydrocodone, to our knowledge, the in vivo activity of norhydrocodone has not been thoroughly examined.

Norhydrocodone was active in vivo following subcutaneous, intrathecal, and intracerebroventricular administration. The potency and maximal analgesic effect of norhydrocodone compared with the parent varied depending on the route of administration, with subcutaneous injection resulting in low potency (~70-fold less) but similar maximal analgesic effect (Fig. 1B; Table 2). Spinally, norhydrocodone produced reduced analgesic effect (15–45%) compared with hydrocodone (67%) and hydromorphone (100%), and its ED\(_{50}\) value could not be accurately determined (Fig. 2B; Table 2). Supraspinally, norhydrocodone has similar potency compared with hydrocodone and produced equal maximal analgesic effect (Fig. 3B; Table 2). Hydromorphone was the most potent opioid

<table>
<thead>
<tr>
<th>Route of Administration *</th>
<th>HYC</th>
<th>HYM</th>
<th>NHYC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intrathecal (( \mu )g/mouse)</td>
<td>19.43 (12.63–31.63)</td>
<td>5.25 (3.54–8.27)</td>
<td>4.27 (2.89–6.76)</td>
</tr>
</tbody>
</table>

* Mice were injected intrathecally with an opioid agonist and observed for seizure activity as described in Materials and Methods.

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**Table 3**
ED\(_{50}\) estimates for hydrocodone-, hydromorphone-, and norhydrocodone-induced seizure activity.
analgesic of the three drugs for all routes of administration (Table 2). Subcutaneous naltrexone significantly antagonized subcutaneous, intrathecal, and intracerebroventricular hydrocodone-, hydromorphone-, and norhydrocodone-induced analgesia, confirming that analgesia was opioid receptor-mediated (Figs. 1C, 2C, and 3C). The analgesic properties of subcutaneous norhydrocodone appear to be mostly mediated by supraspinal opioid receptors. First, as noted above, intrathecal norhydrocodone was associated with a reduced analgesic effect, whereas intracerebroventricular norhydrocodone produced full analgesia (Figs. 2B and 3B). Second, subcutaneous norhydrocodone-induced analgesia was blocked by intracerebroventricular naltrexone injection (Fig. 4). Taken together, these results suggest that systemic norhydrocodone induces opioid receptor–mediated analgesia, primarily via supraspinal opioid receptors. The low potency of subcutaneous norhydrocodone may be related to reduced access of the drug to the CNS, primarily supraspinal sites.

Spinal opioid-induced neuroexcitation has been reported in humans and rodents (Frenk et al., 1984; Parkinson et al., 1990). Studies have demonstrated that opioid nor-metabolites (e.g., normeperidine, noroxycodone) are more potent than parent compounds in inducing seizure activity in rodents (Umans and Inturrisi, 1982; Leow and Smith, 1994). In the present study, hydrocodone, hydromorphone, and norhydrocodone induced seizures following spinal administration. Intrathecal norhydrocodone and hydromorphone were roughly equipotent in inducing seizures. Intrathecal hydrocodone was less potent than intrathecal norhydrocodone and intrathecal hydromorphone in producing seizure activity (Fig. 5A; Table 3). No seizure activity was observed following subcutaneous or intracerebroventricular administration of these compounds, within the range of doses tested. Apparently, intrathecal seizure activity is not opioid receptor-mediated since it was not antagonized by subcutaneous or intrathecal naltrexone (Fig. 5B). These results agree with previous reports examining neuroexcitation following a range of opioid agonists (Umans and Inturrisi, 1982; Frenk et al., 1984; Yoburn et al., 1990; Leow and Smith, 1994).

The present results raise the possibility that neuroexcitatory effects may develop following hydrocodone administration. Although there is at least one report of neuroexcitation following hydrocodone treatment (Lauterbach, 1999), it is not known if this is related to the parent or the metabolites. In a study in which formation of hydromorphone is inhibited and norhydrocodone potentially increased, there was an increase in unpleasant effects of oral hydrocodone (e.g., dysphoria, physical unpleasantness; Otton et al., 1993). It is possible that the unpleasant effects might be a precursor to frank neuroexcitatory actions, although this is not known. Nevertheless, following acute oral hydrocodone, the plasma and urine levels of norhydrocodone invariably exceed that of the hydromorphone and sometimes that of hydrocodone (Hao et al., 2011; Valtier and Bebarta, 2012). Chronic hydrocodone administration might result in substantial levels of the nor-metabolite that may mediate unpleasant subjective effects and perhaps incipient neuroexcitatory action. Further studies are required to examine this possibility.

In summary, norhydrocodone is a μ-selective opioid ligand and produces analgesia, which appears to be primarily supraspinal. Following spinal injection, it induces non-opioid receptor–mediated neuroexcitation and is less effective in producing analgesia compared with hydrocodone and hydromorphone. Overall, the observation that norhydrocodone possesses in vivo activity raises the possibility that it might play a role in therapeutic as well as toxic effects following acute or chronic hydrocodone treatment. Further studies are needed to evaluate the concentration of norhydrocodone in CNS following hydrocodone administration to address these issues.

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Authorship Contributions

Participated in research design: Navani, Yoburn. Conducted experiments: Navani. Performed data analysis: Navani, Yoburn. Wrote or contributed to the writing of the manuscript: Navani, Yoburn.

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Norhydrocodone: An Active Metabolite of Hydrocodone

Address correspondence to: Dr. Byron C. Yoburn, Department of Pharmaceutical Sciences, College of Pharmacy and Health Sciences, St. John’s University, 8000 Utopia Parkway, Queens, NY 11439. E-mail: yoburnb@stjohns.edu