Title: Chronic methadone treatment shows a better cost:benefit ratio than chronic morphine in mice

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Running title: Methadone cause less side effects than morphine

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Non-standard abbreviations: DMOR degrading mu opiate receptor, MOR mu opiate receptor, DOR delta opiate receptor

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Abstract

Chronic treatment of pain with opiate drugs can lead to analgesic tolerance and drug dependence. While all opiate drugs can promote tolerance and dependence, in practice, the severity of these unwanted side effects differs depending on the drug used. While each opiate drug has its own unique set of pharmacological profiles, methadone is the only clinically utilized opioid drug that produces substantial receptor endocytosis at analgesic doses. Here, we examined whether moderate doses of methadone carry any benefits over chronic use of equi-analgesic morphine, the prototypical opioid. Our data show that chronic administration of methadone produces significantly less analgesic tolerance than morphine. Furthermore, we found significantly reduced precipitated withdrawal symptoms after chronic methadone treatment than after chronic morphine treatment. Finally, using a novel animal model with a degrading mu opioid receptor (DMOR) we showed that, while endocytosis appears to protect against tolerance development, endocytosis followed by receptor degradation produces a rapid onset of analgesic tolerance to methadone. Together, these data indicated that opioid drugs that promote receptor endocytosis and recycling, such as methadone may be a better choice for chronic pain treatment than morphine and its derivatives which do not.
Introduction

Opiate drugs are the mainstay for the treatment of severe pain, but the utility of these drugs for chronic pain conditions is curtailed by the development of tolerance to the analgesic effects of drug. Importantly, the dose escalation necessary to overcome tolerance in chronic pain conditions not only puts patients at a greater risk for severe side effects, such as respiratory depression, but also increases the liability for dependence. Although there have been significant efforts designed to improve the utility of opiates, there has been little progress at identifying approaches to treatment that maintain analgesic efficacy with reduced side effects of tolerance and dependence. This is primarily because the mechanisms underlying development of tolerance and dependence as a consequence of chronic opioids use remain unresolved and are, thus, vigorously debated (Christie, 2008). This debate centers on the role of receptor desensitization, arrestin recruitment and endocytosis, versus the role of homeostatic adaptations in signal transduction as the primary mediators of analgesic tolerance and dependence (Raehal and Bohn, 2005; Martini and Whistler, 2007; Christie, 2008; Ingram and Traynor, 2009; Ueda and Ueda, 2009).

Two opiate drugs used for the management of chronic pain are methadone and morphine. Although these two drugs differ in their chemical structure, the primary target for their actions is the mu opioid receptor (MOR) (Eddy and May, 1973; Raynor et al., 1994). Beyond their structural differences, these two drugs also differ in a number of aspects regarding MOR pharmacology (Eddy and May, 1973). For example, methadone but not morphine promotes substantial arrestin recruitment (Whistler and von Zastrow, 1998;
Bohn et al., 2004). In addition, while morphine fails to drive significant endocytosis of the MOR, methadone more closely mimics the endogenous opiates and promotes substantial endocytosis (Keith et al., 1996; Sternini et al., 1996; Keith et al., 1998; Borgland et al., 2003; Milan-Lobo and Whistler, 2011). Hence, these two drugs provide an opportunity to help dissect the role of receptor recruitment of arrestin and receptor endocytosis in tolerance and dependence.

Following receptor recruitment of arrestin and receptor endocytosis, G protein coupled receptors can be either recycled back to the plasma membrane or targeted for degradation and “downregulated” (Hanyaloglu and von Zastrow, 2008). Although many studies have shown that the MOR is primarily recycled after endocytosis (Finn and Whistler, 2001; Whistler et al., 2002; Minnis et al., 2003; Yu et al., 2010), the hypothesis persists that receptor downregulation also contributes to opioid tolerance (Ueda and Ueda, 2009). We have generated a knock-in mouse that expresses a mutant version of the MOR, DMOR, for degrading MOR, that recruits arrestin, endocytoses, and is sorted for degradation in response to both morphine and methadone (Finn and Whistler, 2001; Whistler et al., 2002; Enquist et al., 2011). Thus, the DMOR animal model allowed us to investigate the impact of MOR downregulation on tolerance to methadone and morphine.

The aim of this study was to use moderate, equi-analgesic doses of methadone and morphine and directly compare whether these two drugs differed in their ability to promote analgesic tolerance and drug dependence. In addition, we used the DMOR
knock-in mice to determine if analgesic tolerance induction is altered as a consequence of receptor downregulation.
Material & Methods

Materials: Morphine sulfate pentahydrate was purchased from Mallinckrodt, (±)-methadone HCl and Naloxone HCl were purchased from Sigma-Aldrich, 0.9% sodium chloride was purchased from Hospira incorporated.

Animals: All mice included in the study were kept on a regular light dark cycle with *ad libitum* access to food and water. C57BL/6J mice were purchased from Jackson Laboratory and were allowed at least one week to acclimatize upon arrival. DMOR knock-in mice were generated as described (Enquist et al., 2011) and kept with wild type (WT) littermates. All animal experiments were performed in accordance with the Ernest Gallo Clinic & Research Center Institutional Animal Care and Use Committee guidelines.

Analgesic Response: Tail-flick Reflex to Heat Irradiation: Mice were tested for antinociception using the radiant heat tail-flick procedure. Briefly, mice were transferred to the test room one hour prior to tests to allow time to acclimatize. Each mouse was carefully wrapped in a small blanket and the tail was stimulated by light heat radiation. Each mouse was tested for tailflick reflex in response to radiant heat stimulation prior to test day. Mice with robust tail-flick reflexes and baseline latencies of 2.0 through 3.5 s were included in the study; a maximum latency of 10 seconds was set as the cutoff time to minimize damage to the tail. Maximum effect of drug on tail-flick was achieved between 20 and 30 minutes after sub-cutaneous (s.c.) injection of drug (see Figure 1B).
Dose response was measured by cumulative drug addition, and nociceptive assessment 20 minutes after each s.c. administered dose, three doses per animal. Data is presented as percentage of maximal possible effect = ((latency after drug - baseline) / (cutoff - baseline)) * 100.

**Tolerance induction:** C57BL/6J wild type mice were injected sub-cutaneously once daily at noon using ED$_{50}$ doses of methadone (3 mg/kg) or morphine (6 mg/kg) dissolved in sterile 0.9% saline solution. C57BL/6J:SV129 mice (knock-in DMOR and WT littermates) were injected sub-cutaneously (s.c.) twice daily with 10 mg/kg methadone dissolved in sterile 0.9% saline solution. The significance of tolerance was evaluated by comparing 95% confidence intervals (Figure 2, Table 1) or tested by repeated-measures ANOVA combined with Dunnett’s multiple comparison test (Figure 4).

**Precipitated withdrawal – drug dependence:** Six groups of animals were included in this study (2 groups for each drug: morphine n=9, methadone n=9, saline n=12, where n is number of animals per group). One group per drug (morphine, methadone or saline) received a single injection of the drug (Acute) followed by naloxone 30 minutes later, and one group per drug received 7 days of drug treatment prior to naloxone (Chronic). Each animal was injected with the drug on the test day (methadone 3 mg/kg, morphine 6 mg/kg or equal volume of saline) followed 30 minutes later by a naloxone injection (0.5 mg/kg) and was monitored for 15 minutes by three independent scorers blind to drug. Behaviors scored were paw tremor (PT, shakes of paws unrelated to grooming), wet dog shakes (WDS, full body shakes unrelated to grooming) and jumps (escape attempts, all
four paws off the floor). The total (global) score for each animal was calculated by calculating the sum of each independent behavior combined. The sessions were video recorded for record keeping. The significance of within drug-group effects on all behaviors, and between global score of all three groups, before and after chronic drug exposure was analyzed separately by two-way ANOVA and Bonferroni posttests.
Results

Methadone and morphine at the ED_{50} dose produce antinociception of equal duration in C57B6/J mice.

Each time two different drugs are used, several pharmacological parameters are altered including affinity, potency and in vivo pharmacokinetics and dynamics. Thus, to directly compare methadone and morphine as antinociceptives, we first established the ED_{50} of each drug in the radiant heat tailflick paradigm by a cumulative dose response regimen (see methods, n=20 animals per drug). Methadone had an ED_{50} of 2.9 mg/kg (confidence interval 1.7-4.1mg/kg) whereas morphine had an ED_{50} of 5.8 mg/kg (confidence interval 3.9-7.6 mg/kg) (Figure 1A, Table1). Since the molecular weight of morphine is twice that of methadone (for the formulations used in this study morphine pentahydrate is 758 g/mole, methadone 346 g/mole) these data suggest that, per mole, these two drugs are equally potent.

We next examined the duration of antinociception produced by morphine (8 mg/kg) and methadone (4 mg/kg). These drug doses were carefully picked to avoid ceiling effects while eliciting a high analgesic response (90% maximum possible effect). An investigator blind to the drug administered monitored the analgesic effect (Figure 1B, n=11 animals per drug). The drugs showed near identical duration of effect, producing significant antinociception for 120 minutes. Likewise, the areas under curve for morphine and methadone were not significantly different (Figure 1C).
Chronic morphine produces significantly more antinociceptive tolerance than chronic equi-analgesic methadone.

We next assessed the development of antinociceptive tolerance to repeated morphine and methadone exposure. On day 1, antinociception was assessed by cumulative dose response as in Fig. 1. Mice then received drug once per day for 4 days (ED$_{50}$ dose: methadone 3 mg/kg or morphine 6 mg/kg). On day 6, antinociception was again assessed by cumulative dose response. Both treatments produced antinociceptive tolerance (Figure 2, Table 1); however, the degree of tolerance produced by methadone was significantly less than that produced by morphine. The ED$_{50}$ of methadone after chronic treatment was 4.3 (confidence interval 2.9-5.8mg/kg), versus 2.9 in naïve mice (a 1.5 fold shift in potency, Figure 2A, Table 1). The ED$_{50}$ of morphine after chronic treatment was 13.5 (confidence interval 9.6-16.9) compared to 5.8 in naïve animals (a 2.5 fold shift in potency, Figure 2B, Table 1). In addition, the effect of the maximal dose of methadone (6 mg/kg) was decreased by only 22% on day 6 compared to day 1, while the effect of the maximal dose of morphine was reduced by 63%.

Morphine induces significantly greater drug dependence than methadone.
We next assessed the degree of dependence induced by chronic treatment with equi-antinociceptive morphine and methadone. Mice (n=9 animals per group), were treated once per day with an EC\textsubscript{50} dose of morphine (6 mg/kg), or methadone (3 mg/kg). On day 7, mice were given their usual drug dose followed 30 minutes later by naloxone (0.5 mg/ml, s.c.) (indicated as Chronic in Fig.3). As a control for acute withdrawal, opioid naïve mice were given a single injection of morphine (6 mg/kg) or methadone (3 mg/kg) followed 30 minutes later by naloxone (0.5 mg/kg) (indicated as Acute in Fig. 3). As a control we included groups that received only acute or chronic saline followed by naloxone (Fig. 3C). Naloxone-precipitated withdrawal behaviors (paw tremor, wet dog shakes and jumps) were scored for 15 minutes by an investigator blind to treatment in these four groups.

Naloxone produced few signs of withdrawal in mice who received only a single dose of morphine (Figure 3A, morphine Acute), primarily paw tremors, with a global score of 21 ± 7. In contrast, mice treated chronically with morphine showed substantial signs of withdrawal (Figure 3A, morphine Chronic), including jumps, with a global score of 50 ± 6 (interaction significant for treatment and behavior; \( F[3,64]=3.124, p<0.05 \), and post test revealed significant increases in jumps \( p<0.05 \) and global withdrawal \( p<0.001 \) scores after chronic versus acute treatment with morphine). In contrast, there was no difference in the degree of withdrawal in mice treated with acute methadone (global score 24 ± 4) versus those treated with chronic methadone (global score 25 ± 5) (Figure 3B). Likewise, chronic saline injections did not lead to an increase in precipitated withdrawal symptoms compared to acute saline injections (chronic saline global score 6.8 ± 3.8 versus saline acute 6.1 ± 3.0) (Figure 3C). Finally, statistical comparison of the global
score after acute or chronic treatment with each drug showed a clear interaction between drug and treatment (drug x treatment, $F[2,54] = 6.651, p<0.01$) and posttest showed that only chronic morphine treatment led to a significant increase in withdrawal symptoms ($p<0.001$) which was significantly greater than the chronic global score of methadone ($p<0.001$).

**MOR down-regulation does not contribute to analgesic tolerance to methadone**

These data suggest that endocytosis *per se* is not responsible for opioid tolerance, since methadone produces greater endocytosis (Keith et al., 1998; Celver et al., 2004) but less tolerance than morphine (Figure 1). We hypothesized that the lack of substantial tolerance to methadone, despite its ability to drive significant receptor desensitization (Arttamangkul et al., 2008; Quillinan et al., 2011), arrestin recruitment (Bohn et al., 2004) and endocytosis (Sternini et al., 1996; Keith et al., 1998; Celver et al., 2004; He and Whistler, 2005), reflects that the MOR is recycled and resensitized after endocytosis. If this were the case, we would expect methadone to produce substantial tolerance if the MOR could not be recycled. To examine this possibility we utilized mice expressing a mutant form of the MOR, DMOR (for degrading MOR) that is targeted for degradation after endocytosis (Finn and Whistler, 2001; Enquist et al., 2011).

The antinociceptive effect of methadone in both genotypes, wild type (WT) and DMOR was indistinguishable (Fig. 4A). To examine the rate of tolerance development, mice of both genotypes were treated with methadone (10 mg/kg) twice per day and antinociception was measured 30 minutes after the first dose every other day for 7 days.
(Fig. 4B). Unlike WT mice, DMOR mice showed significant antinociceptive tolerance to methadone by day 3 (each group analyzed separately by repeated-measures ANOVA, no significant change for WT, $F(3,24)=9186$, $p<0.0001$ for overall treatment x days in DMOR, Dunnett’s multiple comparison test $p<0.01$ each for day1 versus day 3, day 5 and day 7) which was exacerbated by additional days of treatment (Figure 4B, DMOR grey bars, WT black bars). Thus, endocytosis can promote tolerance under conditions where MOR is targeted for degradation (Fig. 4A), but under normal conditions where MOR is recycled, endocytosis appears to protect against the development of tolerance (Fig. 2).
Discussion

Here we show that chronic methadone treatment causes substantially reduced antinociceptive tolerance and physical dependence than chronic morphine treatment, under conditions of equivalent pain relief. Furthermore, we show that, while receptor endocytosis protects against antinociceptive tolerance in wild type mice, it enhances tolerance in an animal model in which the MOR is mutated to enhance receptor degradation after endocytosis.

We carefully titrated drug levels to ensure equivalent pain relief in this study. However, a recent study, that use four times the molar concentration of methadone versus morphine (and a 32x higher concentration of methadone than that used here), also showed that the potency shift was much greater with chronic morphine than with chronic methadone (Raehal and Bohn, 2010). Specifically, Raehal and Bohn showed that chronic methadone produced a 1.8x potency shift compared to the 1.5x shift we show here, while morphine produced a 2.9x shift compared to the 2.3x shift we show here. This would indicate that methadone could maintain its beneficial side effect profile of reduced tolerance even across a wide concentration window.

In addition, here we found no signs of dependence in mice after chronic methadone treatment compared to those produced by acute withdrawal (Fig. 3). In the human literature there is ample evidence of withdrawal signs and dependence among methadone maintenance patients (Bakstad et al., 2009; Awgu et al., 2010; Lobmaier et al., 2010).
However, it is important to keep in mind that these patients were first dependent on heroin, which like morphine does not promote receptor endocytosis. Thus, the methadone “dependence” in these patients likely reflects their underlying heroin dependence that cannot be reversed simply by promoting receptor endocytosis. Indeed, methadone is almost never given as a first line analgesic in human medicine, so there is no clear data showing whether methadone would have reduced liability to produce tolerance and dependence compared to morphine (or oxycontin, Dilaudid, or fentanyl, none of which produce endocytosis at analgesic doses), when given as a first line therapeutic. There are also reports of methadone dependence in preclinical animal models (see e.g. Raehal and Bohn, 2010). However, these reports used significantly higher doses of methadone, and did not examine whether chronic treatment produced a greater degree of dependence that acute withdrawal from these single high doses of methadone. We did not examine here whether chronic administration of continuous methadone, more comparable to that used in patients, produced a different endocytic pattern than intermittent dosing. However, previously we have shown that chronic continuous administration of opioid cocktails that drive endocytosis continue to do so after at least seven days of treatment (He et al., 2002). Furthermore, our previous studies have shown that the morphine remains unable to drive substantial endocytosis even after chronic continuous administration (He et al., 2002).

It has been suggested that methadone may produce reduced tolerance and dependence, not because it promotes receptor endocytosis, but because of its ability to antagonize NMDA receptors and thereby counteract maladaptive changes in glutamate transmission.
(Davis and Inturrisi, 1999; Stringer et al., 2000). Of course, these two mechanisms are not mutually exclusive. Indeed, methadone may be a better therapeutic choice than morphine both because of its ability to drive endocytosis and its ability to antagonize NMDA receptors. Previously we found that sub-analgesic doses of methadone, mixed with morphine reduce the development of both tolerance and dependence (He and Whistler, 2005). However, the beneficial effects of methadone in this study could not be achieved when only the methadone enantiomer with high NMDA receptor affinity but no ability to drive receptor endocytosis was used (He and Whistler, 2005). Thus, while NMDA antagonism may add to the benefits of methadone, it may be less important than the ability of this drug to promote MOR endocytosis. In support of this hypothesis, enhanced endocytosis of the MOR in response to morphine as a consequence of pharmacological cocktails that do not contain methadone (He et al., 2002) reduces the severity of morphine tolerance and dependence. Likewise enhanced morphine induced endocytosis through release of endogenous opioids (Zollner et al., 2008) and in mice with mutant MORs that endocytose and recycle (Kim et al., 2008; Madhavan et al., 2010), also reduces morphine tolerance and dependence independent of any effect on NMDA receptors. Instead, we propose that the failure of morphine to induce substantial arrestin recruitment and endocytosis promotes homeostatic adaptations in MOR expressing neurons. These adaptations are likely diverse and could include changes in channel activity, second messenger activity, and gene expression (reviewed by e.g. Nestler, 1997; Nestler, 2004; Berger and Whistler, 2010). We are also particularly interested in examining whether endocytosis of the MOR can prevent the upregulation of the delta opioid receptor (DOR) associated with chronic morphine (Rothman et al., 1986; Cahill et
Indeed, upregulation of the DOR has been implicated in morphine tolerance (Abdelhamid et al., 1991) and this redistribution is dependent on arrestin at least in some circuits (Hack et al., 2005). In fact, recent studies indicate that there is a direct interaction between DOR and MOR, that these dimer interactions increase with morphine tolerance (Gupta et al., 2010), and that the heteromer has a different pharmacological profile than MOR alone (Rozenfeld and Devi, 2007; Gupta et al., 2010; Milan-Lobo and Whistler, 2011).

Finally, our results from this study with the DMOR mice suggest that, while receptor endocytosis can protect against the development of tolerance, it can also produce tolerance if the receptor is not recycled. For example, etorphine at high doses has been shown to produce tolerance and accompanying downregulation of MOR in vivo (Duttaroy and Yoburn, 1995; Yabaluri and Medzihradsky, 1997). Thus, the post-endocytic fate of the MOR may be dependent on the agonist used. Ultimately, the combined effect of a drug’s ability to induce receptor endocytosis, and the postendocytic fate of the receptor will determine the physiological responsiveness to chronic drug treatment.
Authorship contributions

Participated in research design: Enquist, Ferwerda, Milan-Lobo, Whistler

Conducted experiments: Enquist, Ferwerda, Milan-Lobo

Contributed new reagents or analytic tools: Ferwerda

Performed data analysis: Enquist, Milan-Lobo

Wrote or contributed to the writing of the manuscript: Enquist, Whistler
References


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_Curr Biol_ **18**:129-135.


Stringer M, Makin MK, Miles J and Morley JS (2000) d-morphine, but not l-morphine, has low micromolar affinity for the non-competitive N-methyl-D-aspartate site in


Footnotes

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Figure legends

Figure 1. **ED$_{50}$ and effect duration of methadone and morphine.** Data shown are drug-induced increases in tailflick latency described as maximum possible effect. A) Antinociceptive dose response to morphine (grey triangle) and methadone (black square) using cumulative dosing (n=20 animals per drug). B) Drug effect duration after a single injection of methadone (4 mg/kg) or morphine (8 mg/kg) (n=11 animals per drug). C) Cumulative area under the curve for each drug from B.

Figure 2. **Cumulative dose response of morphine and methadone on tailflick latencies in drug naïve and drug tolerant animals.** Cumulative dose response of A) morphine and B) methadone on tailflick latencies in drug naïve mice (solid line) and mice treated with repeated morphine or methadone for 6 days (hatched line). N=20 animals per drug. See Table 1 for confidence intervals.

Figure 3. **Naloxone precipitated withdrawal signs after acute and chronic morphine and methadone treatment.** Mice (n= 9 per group) were treated with a single dose (Acute) or repeated doses (Chronic) of A) morphine  B) methadone or C) saline. 30 minutes later mice were injected with naloxone (0.5 mg/kg), and withdrawal signs were scored for fifteen minutes. Data is presented as number of events over the fifteen minutes period plus a summarized global score. *p<0.05, *** p<0.001, two-way ANOVA, Bonferronis posttests. PT paw tremor; WDS wet dog shakes.
Figure 4. Methadone antinociception and tolerance in WT and DMOR mice. Data shown are drug-induced increases in tailflick latency described as maximum possible effect. A) Cumulative dose response of methadone in WT and DMOR mice. B) WT and DMOR mice (n= 8 per group) were injected with methadone (10 mg/kg) twice per day. Maximum possible effect was measured every other day starting on day 1. ** p<0.01, result analyzed within each group by repeated-measures ANOVA followed by Dunnett’s multiple comparison test. Black bars represent WT and grey bars represent DMOR.
**Table 1. Analgesic ED$_{50}$ and confidence interval of methadone and morphine by tailflick**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Acute (mg/kg)</th>
<th>Treatment day 6 (mg/kg)</th>
<th>Rightshift (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methadone</td>
<td>2.9 (1.7-4.1)</td>
<td>4.3 (2.9-5.8)</td>
<td>1.5</td>
</tr>
<tr>
<td>Morphine</td>
<td>5.8 (3.9-7.6)</td>
<td>13.5 (9.6-16.9)</td>
<td>2.3</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2

A. Maximum possible effect (%) vs. Morphine (mg/kg) for Day 1 and Day 6.

B. Maximum possible effect (%) vs. Methadone (mg/kg) for Day 1 and Day 6.
Figure 3
Figure 4

A. Maximum possible effect (%) vs. Methadone (mg/kg)

B. Maximum possible effect (%) over Treatment day

Legend:
- WT MOR
- DMOR

** indicates statistical significance.