M₅ Muscarinic Receptor Knockout Mice Show Reduced Morphine-Induced Locomotion but Increased Locomotion after Cholinergic Antagonism in the Ventral Tegmental Area

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ABSTRACT

M₅ muscarinic receptors are the only muscarinic receptor subtype expressed by mesencephalic dopamine neurons and provide an important excitatory input to mesolimbic and nigrostriatal dopamine systems. Here, we studied locomotion induced by systemic morphine (3, 10, and 30 mg/kg i.p.) in M₅ knockout mice of the C57BL/6 (B6) and CD1 × 129SvJ background strains. M₅ knockout mice of both strains showed reduced locomotion in response to 30 mg/kg morphine. B6 M₅ knockout mice were less sensitive to naltrexone in either the antagonism of morphine-induced locomotion or in the reduction of locomotion by naltrexone alone. This suggests that M₅ knockout mice are less sensitive to the effects of either exogenous or endogenous opiates on locomotion and that spontaneous locomotion in B6 mice is sustained by endogenous opiates. In B6 wild-type mice, ventral tegmental area (VTA) pretreatment with the muscarinic receptor antagonist atropine (3 μg bilateral), but not the nicotinic receptor antagonist mecamylamine (5 μg bilateral), reduced locomotion in response to 30 mg/kg morphine to a similar extent as systemic M₅ knockout, suggesting that reduced morphine-induced locomotion in M₅ knockout mice is due to the loss of M₅ receptors on VTA dopamine neurons. In contrast, in M₅ knockout mice, but not in wild-type mice, either intra-VTA atropine or mecamylamine alone increased locomotion by almost 3 times relative to saline and potentiated morphine-induced locomotion. Therefore, in M₅ knockout mice, blockade of either VTA muscarinic or nicotinic receptors increases locomotion, suggesting that in the absence of VTA M₅ receptors, VTA cholinergic inputs inhibit locomotion.

Opiates increase locomotion in rats through both dopamine-dependent and -independent mechanisms (Kalivas et al., 1983; Amalaric and Koob, 1985). Ventral tegmental area (VTA) infusions of the enkephalin analog, d-Ala²-Met⁵-enkephalinamide, induce locomotion that is reduced by nucleus accumbens fluphenazine pretreatment. Conversely, nucleus accumbens infusions of d-Ala²-Met⁵-enkephalinamide induce locomotion that is not reduced by either 6-hydroxydopamine lesions or fluphenazine pretreatment (Kelley et al., 1980; Kalivas et al., 1983).

In rats, neither 6-hydroxydopamine lesions of mesolimbic terminals nor systemic α-flupenthixol reduce systemic heroin-induced locomotion (Vaccarino et al., 1986). In mice, however, systemic haloperidol dose-dependently reduces systemic morphine-induced locomotion (Ito et al., 2008). Furthermore, dopamine-deficient mice, which have two inactive alleles of the tyrosine hydroxylase gene (Zhou and Palmiter, 1995), show reduced morphine-induced locomotion (Hnasko et al., 2005). These data suggest that opiate-induced locomotion depends more on dopamine in mice than in rats.

Cholinergic neurons of the pedunculopontine tegmental nucleus (PPT) and the laterodorsal tegmental nucleus (LDT) provide a major source of excitatory cholinergic and glutamatergic input to the VTA and substantia nigra (Oakman et al., 1995; Omelchenko and Sesack, 2006). Cholinergic activation of mesolimbic dopamine neurons in rats and mice is mediated through both muscarinic and nicotinic acetylcholine receptors in the VTA (Forster and Blaha, 2000; Forster et al., 2001). M₅ muscarinic receptors are critical for prolonged increases in nucleus accumbens and striatal dopamine in mice after electrical stimulation of the LDT or PPT, respectively (Forster et al., 2001; Miller et al., 2006).

In rats, infusions of the μ-opioid receptor agonist [d-Ala²,
N-Me-Phe⁴,Gly⁵-ol-enkephalin into the PPT also increase nucleus accumbens dopamine concentrations and induce locomotion that is blocked by systemic haloperidol, suggesting a dependence on mesolimbic dopamine activity (Klitenstein and Kalivas, 1994). Thus, the effects of systemic opiates on locomotion are mediated, in part, through dopamine-dependent mechanisms involving the VTA and PPT. In contrast, dopamine-independent mechanisms involving the nucleus accumbens, ventral pallidum, and mediodorsal thalamus also contribute (Austin and Kalivas, 1990; Churchill et al., 1992; Klitenstein and Kalivas, 1994). Forebrain opioid receptors are particularly critical for dopamine-independent locomotion induced by systemic heroin in rats (Amalric and Koob, 1985).

Cholinergic inputs to the VTA are important for the activating effects of morphine on dopamine. Lesions of either the LDT (Forster et al., 2002) or PPT (Miller et al., 2002) reduce either accumbal or striatal dopamine efflux in response to i.v. morphine in rats. VTA scopolamine pretreatment reduces accumbal dopamine efflux in response to systemic morphine (Miller et al., 2005). Furthermore, PPT lesions block the expression of morphine-conditioned place preference by systemic morphine in rats (Rezayof et al., 2007). Cholinergic activation of dopamine neurons in the VTA is also critical for the rewarding effects of nicotine (Corrigall et al., 1994), of brain stimulation reward (Yeomans and Baptist, 1997; Yeomans et al., 2000), or of morphine (Basile et al., 2002). Of the five muscarinic receptor subtypes, M₅ receptors are the only subtype whose mRNA is expressed by midbrain dopamine neurons (Vilaró et al., 1990; Weiner et al., 1990). Here, we tested the role of M₅ muscarinic receptors in the VTA on morphine-induced locomotion in mice. We first compared morphine-induced locomotion across a range of doses in wild-type and homozygous M₅ receptor knockout mice. Second, we tested the effects of muscarinic and nicotinic VTA receptor blockade on morphine-induced locomotion in wild-type mice and M₅ knockout mice.

Materials and Methods

Mice. M₅ knockout mice were homozygous mutants for the M₅ muscarinic acetylcholine receptor gene. Mice were created using recombinant DNA methods on a mixed 129 SvJ × CD1 background as described by Takeuchi et al. (2002). Homozygous and wild-type mice were used to maintain a breeding colony based on homozygous M₅ knockout mice. CD1 background. Homozygous and wild-type mice were age-matched (2–4 months at the time of testing). Subsequent to obtaining M₅ knockout mice on a mixed 129 SvJ × CD1 background, homozygous knockouts were backcrossed to C57BL/6 mice over six generations to achieve a C57BL/6 background (Gerlai, 1996). A colony of these mice was maintained through breeding of homozygous M₅ knockout and wild-type controls. CD1 × 129SvJ mice will subsequently be referred to as 129 mice, whereas C57BL/6 mice will be referred to as B6. A total of 201 mice were used (21 129 wild-type, 21 129 M₅ knockout, 80 B6 wild-type, and 79 B6 M₅ knockout mice). Mice were housed in groups of two to five in opaque cages, with food and water available ad libitum on a 12/12-h light/dark cycle (lights on at 7:00 AM). Locomotor testing always took place between the hours of 9:00 AM and 6:00 PM (i.e., the inactive period of the cycle). A minimum of 1 week before initiation of the experiment, mice were removed from the breeding colony and brought to a small housing room adjacent to the testing room. Both rooms had controlled temperature (20 ± 1°C) and humidity (approximately 55–60%). This study was approved by the University of Toronto Animal Care Committee.

Locomotion Testing Apparatus. Mice were tested individually in nine black chambers (31 × 31 × 31 cm; University of Toronto, Department of Cell and Systems Biology Workshop). Mice were videotaped by a camera (model WV-CF484; Panasonic, Osaka, Japan) mounted approximately 2.1 m above the testing chambers. The testing room was illuminated by two 40-W red light bulbs (approximately 2.5 lux) mounted on the ceiling approximately 2.8 m above the testing chambers. Wild-type and M₅ knockout mice were run together in groups consisting of four to five wild-type and four to five M₅ knockout mice, each randomly assigned to a testing chamber.

Locomotion Testing Procedure. Locomotion testing took place across 3 consecutive days. Each day, mice were taken from the housing area in their home cages, weighed, and then placed into the testing room 20 min before initiation of locomotion testing. On the 1st day, spontaneous locomotion was tested in all mice for 2 h. For this, each mouse was removed from its cage and placed in the center of the assigned testing chamber without receiving any injection. Saline and morphine administration was counterbalanced at all doses tested (3, 10, or 30 mg/kg i.p.), such that half of the mice received morphine on day 2 and then saline on day 3, whereas the other half received saline on day 2 and then morphine on day 3.

Effects of Naltrexone Pretreatment on Morphine-induced Locomotion in B6 Mice. Locomotion testing took place across 2 consecutive days. On the 1st day, spontaneous locomotion was tested in all mice as described above. On day 2, each group of B6 wild-type and M₅ knockout mice was given two consecutive injections that involve combinations of the noncompetitive opioid receptor antagonist naltrexone (1 or 10 mg/kg i.p.), saline (10 ml/kg i.p.), or morphine (30 mg/kg i.p.). B6 wild-type and M₅ knockout mice were randomly assigned to the following groups (n = 6 per group): 1) saline then saline, 2) 1 mg/kg naltrexone then morphine, 3) 10 mg/kg naltrexone then morphine, and 4) saline then morphine. The two injections were given 5 min apart, during which mice were returned to their home cages. To minimize interference between drugs given at the same injection site, the two injections were made on opposite sides of the i.p. cavity. After the second injection, each mouse was immediately placed into one of the nine testing chambers, and locomotion was measured for 2 h. To test the effects of blocking endogenous opioids on basal locomotion levels in wild-type and M₅ knockout mice, two additional groups received: 1) 1 mg/kg naltrexone then saline and 2) 10 mg/kg naltrexone then saline.

Surgery. Eight B6 wild-type and seven B6 M₅ knockout mice were anesthetized with isoflurane (3% for induction and 1.5–2% for maintenance; O₂ flow rate, 1 L/min) and placed in a stereotaxic device. Mice were treated locally with lidocaine before scalp incisions. Each mouse was implanted with 26-gauge bilateral guide cannulae (Plastic Products, Roanoke, VA) with the tip of the guide cannula aimed 1 mm above the VTA. To avoid puncture of the sagittal sinus, cannulae were angled 10° medially. The stereotaxic coordinates used were: A/P, 0.50; M/L, +0.3; and D/V, +1.5 ( Paxinos and Franklin, 2004). Guide cannulae were fixed to the skull with dental acrylic cement anchored to three stainless steel skull screws. Patency of guide cannulae was maintained by insertion of a dummy cannula. Mice were allowed 10 days of recovery before initiation of the experiment.

Intracranial Injections and Morphine-Induced Locomotion. On the 1st day, spontaneous exploration was measured for 2 h. On the following days, mice were given combinations of intra-VTA muscarinic or nicotinic antagonists, followed by systemic morphine or saline. Mice were restrained, and injector cannulae (33 gauge; Plastic Products) were bilaterally inserted to protrude 1 mm beyond the tip of the guide cannulae. Injections were made using a 2-μl Hamilton microsyringe connected to Tygon tubing (0.0075 inches i.d., 0.080 inches o.d.; Cole-Parmer Instrument Co., Vernon Hills, IL) and a syringe pump (Harvard Apparatus model 975; Harvard Appa-
M5 Knockout Mice and Morphine-Induced Locomotion

Experiment 1: Morphine-Induced Locomotion in Wild-Type and M5 Muscarinic Receptor Knockout Mice

B6 Wild-Type Mice Show Greater Spontaneous Exploration Than 129 Wild-Type Mice. Spontaneous exploration, as measured by the distance traveled during first exposure to the testing chambers, was significantly greater in B6 than in 129 wild-type mice [F(1, 48) = 15.84, p < 0.001; Fig. 1A]. Throughout the 2-h period, locomotion levels were lower in 129 than B6 wild-type mice (Fig. 1B), with the strain difference most evident in the 2nd h of testing. In fact, B6 wild-type mice showed as much locomotion in the 1st h as 129 wild-type mice during the entire 2-h period (13,123 ± 954 versus 12,507 ± 934 cm; Fig. 1A). Therefore, B6 wild-type mice habituated to the locomotion chamber more slowly than 129 wild-type mice.

Importantly, total spontaneous exploration did not differ between wild-type and M5 knockout mice of either strain [t(39) = 1.41, p > 0.1 and t(70) = 0.23, p > 0.1 for 129 and B6 strains, respectively] at any time point in the 2-h testing period (Fig. 1, C and D). Furthermore, total saline-induced locomotion did not differ between wild-type and M5 knockout mice of either strain [t(39) = 0.63, p > 0.1 and t(70) = 0.73, p > 0.1 for 129 and B6 strains, respectively] at any time point in the 2-h testing period (Fig. 1, E and F). Thus, wild-type and M5 knockout mice of either strain also did not differ in how they responded to the handling associated with an injection.

Morphine-Induced Locomotion Is Reduced in M5 Muscarinic Receptor Knockout Mice. 30 mg/kg Morphine-induced locomotion. While 30 mg/kg i.p. morphine increased locomotion relative to saline in all mice tested, B6 wild-type mice showed slightly greater locomotion compared with 129 mice. Therefore, the B6 strain not only showed greater spontaneous exploration but was also more sensitive to the stimulant effect of morphine than the 129 strain.

For the 129 strain, morphine-induced locomotion was consistently lower in M5 knockout than wild-type mice (Fig. 2A). There was a significant main effect of genotype [F(1, 17) = 10.08, p < 0.01] and a significant interaction between genotype and treatment [F(1, 17) = 13.15, p < 0.01]. Post hoc analysis using Fisher’s LSD test showed that locomotion in response to saline did not differ with genotype (all p > 0.8), but morphine increased locomotion relative to saline in both genotypes (p < 0.00001 in wild-type mice and p < 0.0001 in...
M₅ knockout mice). Especially, morphine-induced locomotion was significantly lower in M₅ knockout than wild-type mice at all 10-min bins across the 2-h testing period (all \( p \) values < 0.001).

For the B6 strain, a significant three-way interaction between genotype, treatment, and time \( [F(2.75,71.65) = 2.90, p < 0.05] \) indicated that the temporal profile of morphine-induced locomotion was not equal across the 2-h period in wild-type and M₅ knockout mice (Fig. 2B). Post hoc comparisons of groups at individual time points using Fisher’s LSD test showed that in each time bin, 30 mg/kg morphine increased locomotion significantly relative to saline in both wild-type and M₅ knockout mice (all \( p \) values < 0.000001 for both wild-type and M₅ knockout mice). Most importantly, morphine-induced locomotion was significantly lower in M₅ knockout mice than wild-type mice for the first 90 min (\( p < 0.05 \) at 10 and 70–90 min, \( p < 0.001 \) at 20–40 min, and \( p < 0.01 \) at 50–60 min).

For 129 mice (Fig. 2C), 10 mg/kg morphine-induced locomotion only slightly. The main effect of time \( [F(2.5,29.5) = 4.39, p < 0.05] \) was modified by a significant interaction with
Fig. 2. Morphine-induced locomotion after saline (10 ml/kg i.p.) and three doses of morphine (3, 10, and 30 mg/kg i.p.) in 129 (left) and B6 (right) wild-type (+/+) and M₅ knockout (-/-) mice. Open squares, locomotion after saline in wild-type mice; open circles, locomotion after saline in M₅ knockout mice. Closed squares, locomotion after morphine in wild-type mice; closed circles, locomotion after morphine in M₅ knockout mice. A, 30 mg/kg morphine-induced locomotion in 129 wild-type (n = 9) and M₅ knockout (n = 11) mice; *, p < 0.001 wild-type morphine versus M₅ knockout morphine. B, 30 mg/kg morphine-induced locomotion in B6 wild-type (n = 14) and M₅ knockout (n = 14) mice; *, p < 0.05 at 10 to 90 min, wild-type morphine versus M₅ knockout morphine. C, 10 mg/kg morphine-induced locomotion in 129 wild-type (n = 7) and M₅ knockout (n = 6) mice. D, 10 mg/kg i.p. morphine-induced locomotion in B6 wild-type (n = 10) and M₅ knockout (n = 10) mice; *, p < 0.05 wild-type morphine versus M₅ knockout morphine. E, 3 mg/kg morphine-induced locomotion in 129 (n = 5) and B6 (n = 6) wild-type and M₅ knockout mice. F, 3 mg/kg morphine-induced locomotion in B6 wild-type (n = 12) and M₅ knockout (n = 12) mice.
treatment \[F(3.1,34.1) = 4.29, p < 0.05\]. Post hoc analyses using Fisher's LSD test showed that morphine overall (across both genotypes) increased locomotion significantly relative to saline between 30 and 110 min \((p < 0.05\) at 30, 60, and 110 min; \(p < 0.01\) at 50, 90, and 100 min; and \(p < 0.001\) at 40, 70, and 80 min).

For B6 mice (Fig. 2D), a significant three-way interaction between genotype, treatment, and time \([F(2.5,45.5) = 3.31, p = 0.05]\) was obtained. Post hoc comparisons using Fisher's LSD test showed that morphine increased locomotion significantly relative to saline between 20 and 120 min \((p = 0.01\) at 20 and 90–120 min and \(p < 0.001\) at 30–80 min) in wild-type mice while not increasing locomotion significantly in the 1st h of testing in M5 knockout mice \((p = 0.05\) at 10–60 and 120 min and \(p = 0.05\) at 70–110 min). For most of the 2nd h, there were significant differences of morphine compared with saline, but the amount of morphine-induced locomotion in M5 knockouts within 10-min time bins was similar across most of the testing period. At the time points where significant differences were obtained, saline-induced locomotion decreased (i.e., declining in knockouts across the 2nd h) rather than morphine-induced locomotion increasing. Most importantly, morphine-induced locomotion was significantly lower in M5 knockout mice compared with wild-type mice for most of the 2-h period \((p = 0.05\) at 30, 40, 70, and 80 min; \(p < 0.01\) at 50 and 60 min, with a nonsignificant trend at 90 min, \(p = 0.052\); stars in Fig. 2D).

3 mg/kg Morphine-induced locomotion. Morphine (3 mg/kg) morphine did not increase locomotion overall relative to saline in 129 mice but did in B6 mice. For 129 mice, 3 mg/kg morphine had little effect in either wild-type or M5 knockout mice (Fig. 2E). The significant main effect of time \([F(4.8,43.2) = 11.3, p < 0.00001]\) was modified by a significant interaction with treatment \([F(3.7,33.5) = 3.6, p < 0.05]\). Post hoc analyses using Fisher's LSD test showed that morphine overall (across both genotypes) increased locomotion significantly compared with saline at only 110 and 120 min \((p < 0.05\).

For B6 mice (Fig. 2F), the main effect of time \([F(5.1,112.3) = 12.3, p < 0.00001]\) was modified by a significant interaction with treatment \([F(5.1,111.4) = 13.8, p < 0.00001]\). Post hoc analyses using Fisher's LSD test showed that morphine overall (across both genotypes) increased locomotion significantly compared with saline at 50 and 70 to 120 min \((p < 0.01\). Thus, similar to spontaneous exploration (Fig. 1B), B6 mice showed sustained increases in locomotion after low doses of morphine.

**Naltrexone Antagonism of Morphine-Induced Locomotion Is Reduced in B6 M5 Knockout Mice.** Locomotion induced by 30 mg/kg morphine in B6 wild-type and M5 knockout mice was blocked by naltrexone (Fig. 3A). A signif-
Significant interaction was observed between genotype and treatment \( F(33,40) = 3.42, p < 0.05 \). Post hoc analyses showed that total morphine-induced locomotion was reduced in \( M_5 \) knockout relative to B6 wild-type mice \( (p < 0.01) \), consistent with the previous experiment (Fig. 2B). In both wild-type and \( M_5 \) knockout mice, both the 1 and 10 mg/kg doses of naltrexone significantly reduced total morphine-induced locomotion \( (p < 0.0001) \).

Although 10 mg/kg naltrexone was equally effective in blocking morphine-induced locomotion in wild-type and \( M_5 \) knockout mice across the entire 2-h testing period, 1 mg/kg naltrexone was less effective in blocking morphine locomotion in \( M_5 \) knockout mice (Fig. 3A). Specifically, after 1 mg/kg naltrexone, mice showed a gradual increase over the 2nd h of testing, but this increase was greater in \( M_5 \) knockout mice than in wild-type mice. This was supported by a significant interaction between genotype, treatment, and time \( F(33,440) = 2.24, p < 0.001 \). Post hoc analyses showed that 1 mg/kg naltrexone did not significantly reduce morphine-induced locomotion in the final 30 min of testing in \( M_5 \) knockout mice, unlike in wild-type mice.

\( M_5 \) Knockout Mice Are Less Sensitive to the Inhibitory Effects of Endogenous Opioid Blockade on Saline-Induced Locomotion. To test the effects of naltrexone on basal locomotor levels, two additional groups of wild-type and \( M_5 \) knockout mice were tested for their response to 1 and 10 mg/kg naltrexone followed by saline and compared with the saline-saline-treated group (Fig. 3B). A significant interaction was found among genotype, treatment, and time \( F(22,330) = 2.24, p < 0.01 \).

In B6 wild-type mice 1 mg/kg naltrexone reduced locomotion relative to saline at most time points during the 1st h \( (p < 0.05) \) and especially at all time points in the 2nd h of testing \( (p < 0.0001) \). This indicates that saline-induced locomotion in B6 wild-type mice depends on endogenous opiates, especially in the 2nd h of testing.

In contrast, in \( M_5 \) knockout mice, 1 mg/kg naltrexone did not reduce locomotion relative to saline at any time point \( (p > 0.1) \). Likewise, 10 mg/kg naltrexone reduced locomotion relative to saline for the first 50 min in wild-type mice \( (p < 0.05) \) while not affecting locomotion in \( M_5 \) knockout mice \( (p < 0.1) \). This suggests that \( M_5 \) knockout mice were less sensitive to the effects of endogenous opioid blockade on saline-induced activity levels.

Experiment 2: Effects of VTA Atropine and Mecamylamine on Morphine-Induced Locomotion in B6 Wild-Type and \( M_5 \) Knockout Mice

**Histology.** Figure 4A shows VTA injection sites in B6 wild-type and \( M_5 \) knockout mice. Injector cannulae tips were determined to be within the anatomical boundaries of VTA, and only mice that met this criterion were used for subsequent data analysis. Injection sites in both wild-type and \( M_5 \) knockout mice were found along the rostrocaudal extent of the VTA, with a slight tendency for the injection sites to be more caudal in knockout mice. In two wild-type mice, the
injection sites were dorsal to the VTA within the red nucleus. The entire rostrocaudal extent of the VTA in mice is only 0.9 mm. A 0.3-μl injection volume produces a fluid sphere with an ~0.68-mm diameter, which would encompass most of the VTA, even for injection sites in the most rostral or most caudal portions of the VTA. Figure 4B shows two representative sections illustrating the extent of damage at injection sites after up to six injections.

**VTA Atropine Reduces Systemic Morphine-Induced Locomotion in Wild-Type, but Not in M₅ Knockout, Mice.** Total locomotion induced by 30 mg/kg i.p. morphine across the 2-h testing period in both wild-type mice [74,035 ± 9700 versus 68,444 ± 10,088 cm, t(18) = 0.333, p = 0.7] and M₅ knockout mice [48,582 ± 4175 versus 44,487 ± 6736 cm, respectively, t(18) = 0.379, p = 0.7] tested in Experiment 2 was similar to that observed in Experiment 1. Analysis of total locomotion revealed a significant interaction between genotype and treatment [F(3,27) = 11.14, p < 0.0001]. Post hoc analyses showed that total morphine-induced locomotion in M₅ knockout mice was lower than wild-type mice (p < 0.05), consistent with Experiment 1. Second, VTA atropine significantly reduced total morphine locomotion in B6 wild-type mice (p < 0.0001) but significantly increased total morphine locomotion in M₅ knockout mice (p < 0.01). Third, VTA atropine on its own did not affect total locomotion relative to saline in wild-type mice (p > 0.6) but significantly increased total locomotion relative to saline in M₅ knockout mice (p < 0.0001).

Figure 5, A and B, show the time course of locomotion in B6 wild-type and M₅ knockout mice, respectively. In wild-type mice, repeated-measures ANOVA revealed a significant interaction between treatment and time [F(33,132) = 5.38, p < 0.000001]. Post hoc analyses showed that locomotion after the saline/morphine treatment was significantly higher than the saline/saline treatment across the entire 2-h testing period (all p values < 0.00001). Second, the atropine/morphine treatment significantly reduced locomotion relative to saline/morphine treatment at all times (p < 0.01 at 10, 40, 50, 60, 80, 90, and 100 min and p < 0.00001 at 20, 30, 70, 110, and 120 min). In fact, VTA atropine pretreatment completely blocked morphine-induced locomotion for the first 40 min, during which locomotion was not significantly different from saline levels (p > 0.05). Subsequently, locomotion gradually increased over the next 70 min and remained above saline levels for the rest of the testing session (p < 0.05 at 40–70 min and p < 0.00001 at 80–120 min). Finally, the atropine/saline treatment slightly increased locomotion relative to saline levels between 50 and 70 min in wild-type mice. However, statistically, the two conditions differed significantly only at 60 min (p < 0.05) and approached significance at 50

![Fig. 5](https://www.aspetjournals.org/doi/abs/10.1124/jpet.270.7.270)

*Fig. 5.* Effects of 3 μg of bilateral VTA atropine on 30 mg/kg i.p. morphine-induced locomotion in B6 wild-type (+/+) (A, n = 6) and M₅ knockout (−/−) mice (B, n = 6). Solid circles, VTA saline followed by systemic morphine; open circles, VTA atropine followed by systemic morphine; open triangles, VTA atropine followed by systemic saline; open diamonds, VTA saline followed by systemic saline (Sal). *, atropine/morphine versus saline/morphine, p < 0.05.


...intra-VTA atropine on its own in wild-type mice was slight. The atropine effect on morphine-induced locomotion observed in wild-type mice was anatomically specific to the VTA. Injection sites that were dorsal to the VTA (Fig. 4, inverted triangles) did not reduce morphine-induced locomotion (data not shown because this group contained only two mice).

Figure 5B shows the time course of locomotion in M5 knockout mice. Repeated-measures ANOVA revealed a significant interaction between treatment and time [$F(33,165) = 7.67$, $p < 0.000001$]. Post hoc analyses comparing the four treatment conditions separately showed that locomotion after the saline/morphine treatment was significantly higher relative to the saline/saline treatment in most time bins ($p < 0.05$ at 10, 20, 50, 60, and 70 min and $p < 0.0001$ between 80 and 120 min). In M5 knockout mice, unlike in wild-type mice, the atropine/morphine treatment reduced locomotion relative to the saline/morphine treatment for only the first 20 min of testing ($p < 0.05$ at 10 and 20 min). In fact, thereafter the atropine/morphine treatment increased locomotion significantly relative to saline/saline treatment in M5 knockout mice between 20 and 70 min ($p < 0.05$ at 40, 50, 60, and 70 min). The atropine/morphine treatment increased locomotion significantly relative to saline/saline treatment in most time bins ($p < 0.05$ at 40 min and $p < 0.00001$ between 50 and 120 min). The atropine/saline treatment increased locomotion significantly in M5 knockout mice, in all time bins, except for 10 min, compared with the saline/saline treatment ($p < 0.05$ at 20, 30, and 110 min and $p < 0.00001$ between 40 and 100 and at 120 min). Thus, unlike wild-type mice, VTA atropine induced high levels of locomotion in M5 knockout mice. The locomotor-activating effect of VTA atropine in M5 knockout mice was as high as the effect of morphine, occurred at shorter time bins and did not add with the morphine effect.

VTA Mecamylamine Does Not Reduce Morphine-Induced Locomotion in B6 Wild-Type, but Potentiates Morphine-Induced Locomotion in M5 Knockout, Mice. Analysis of total locomotion revealed a significant interaction between genotype and treatment [$F(3,27) = 5.47$, $p < 0.01$]. The saline/saline and saline/morphine data were the same as for the atropine data above. VTA mecaminamine did not affect total morphine locomotion in wild-type mice while significantly increasing morphine locomotion in M5 knockout mice ($p < 0.01$). VTA mecaminamine on its own significantly increased locomotion relative to saline in M5 knockout ($p < 0.001$) but not wild-type ($p > 0.1$) mice.

Figure 6, A and B, show the time course of locomotion in B6 wild-type and M5 knockout mice, respectively. In wild-type mice, repeated-measures ANOVA revealed a significant in-

![Graph](image-url)

**Fig. 6.** Effects of 5 μg of bilateral VTA mecaminamine on 30 mg/kg i.p. morphine-induced locomotion in B6 wild-type (+/+) (A, $n = 5$) and M5 knockout (-/-) (B, $n = 6$) mice. For both wild-type and knockout mice, the saline/saline and saline/morphine data are the same as shown in Fig. 5. Solid circles, VTA saline followed by systemic morphine; open circles, VTA mecaminamine followed by systemic morphine; open triangles, VTA mecaminamine followed by systemic saline; open diamonds, VTA saline followed by systemic saline. *, mecaminamine/morphine versus saline/morphine, $p < 0.05$. 

$p = 0.079$) and 70 ($p = 0.085$) min. Thus, the effect of intra-VTA atropine on its own in wild-type mice was slight. The atropine effect on morphine-induced locomotion observed in wild-type mice was anatomically specific to the VTA. Injection sites that were dorsal to the VTA (Fig. 4, inverted triangles) did not reduce morphine-induced locomotion (data not shown because this group contained only two mice).
teraction between treatment and time [$F(33,132) = 1.84, p < 0.01$]. Post hoc analyses comparing the four treatment groups separately in each time bin showed that in all bins, locomotion after the saline/morphine treatment was significantly higher relative to the saline/saline treatment (all $p < 0.00001$). Furthermore, the mecamylamine/morphine treatment significantly reduced locomotion relative to saline/morphine only at 110 and 120 min ($p < 0.05$) and, thus, was essentially ineffective in reducing morphine-induced locomotion. Consistent with this, morphine-induced locomotion after VTA pretreatment with mecamylamine was increased relative to saline in all time bins ($p < 0.01$ at 10 and 40 min and $p < 0.00001$ at 20, 30, and 50–120 min). Finally, the mecamylamine/saline treatment also slightly increased locomotion above saline levels. However, statistically, the two conditions differed significantly only at 30 min ($p < 0.05$) while showing nonsignificant trends at 60 ($p = 0.058$), 70 ($p = 0.056$), and 80 ($p = 0.069$) min.

Figure 6B shows the time course of locomotion in $M_5$ knockout mice. Repeated-measures ANOVA revealed a significant main effect of treatment [$F(3,15) = 16.63, p < 0.00001$] that was modified by a significant interaction with time [$F(3,89,19.46) = 3.99, p < 0.05$]. Post hoc analysis comparing the four treatment groups separately showed that in most time bins, locomotion after the saline/morphine treatment was significantly higher relative to the saline/saline treatment ($p < 0.05$ at 10, 20, 50, and 60 min and $p < 0.0001$ between 70 and 120 min). Furthermore, unlike in wild-type mice, the mecamylamine/morphine treatment increased locomotion significantly relative to the saline/morphine treatment in most time bins ($p < 0.05$ at 20 and 60–120 min and $p < 0.00001$ at 30–50 min). Consistent with this, the mecamylamine/morphine treatment also increased locomotion significantly relative to the saline/saline treatment ($p < 0.0001$ at 20 and 60 min and $p < 0.000001$ at 30–50 and 70–120 min). Finally, unlike wild-type mice, VTA mecamylamine on its own increased locomotion significantly relative to saline across the entire 2-h testing period ($p < 0.01$ at 10, 30, 40, 50, 110, and 120 min and $p < 0.00001$ at 20 and between 60 and 100 min). The locomotor-activating effect of VTA mecamylamine in $M_5$ knockout mice added to the morphine effect, so that the combination of mecamylamine and morphine induced more locomotion than morphine alone in all time bins where the morphine effect was evident (~50–120 min).

**VTA Atropine and Mecamylamine Increase Locomotion in $M_5$ Knockout Mice, More Than in Wild-Type Mice.** $M_5$ knockout mice showed more locomotion than wild-type mice after VTA treatment with atropine or mecamylamine relative to saline (Fig. 7). The atropine effect peaked at 40 to 50 min in $M_5$ knockout mice. In comparison, locomotion induced by VTA mecamylamine in $M_5$ knockout mice peaked at 20 min, earlier than for atropine.

**Discussion**

In B6 $M_5$ knockout mice, total morphine-induced locomotion across the 2-h testing period was reduced by 47% at the 30 mg/kg dose and 45% at the 10 mg/kg dose. In 129 $M_5$ knockout mice, total morphine-induced locomotion was reduced by 48% at the 30 mg/kg dose. Thus, a mechanism involving $M_5$ receptors is necessary for almost half of morphine-induced locomotion. Haloperidol pretreatment (0.01–0.1 mg/kg i.p.) in ddY mice dose-dependently reduces 20 mg/kg s.c. morphine-induced locomotion between 20 and 75% (Ito et al., 2008). A comparison with the present data suggests that most dopamine-dependent morphine-induced locomotion in mice is mediated through $M_5$ muscarinic receptors.

$M_5$ receptors and mRNA have been found, albeit at very low levels, throughout the brain (Wei et al., 1994; Wang et al., 2004), and their loss could affect dopamine transmission independent of the VTA. For example, $M_5$ knockout mice show reduced striatal dopamine release due to the loss of $M_5$ receptors on striatal terminals (Yamada et al., 2001). Experiment 2 showed that bilateral VTA atropine pretreatment (3 μg/side) reduced locomotion induced by 30 mg/kg i.p. morphine in B6 wild-type mice by 47% on average, very similar to the reduction in morphine-induced locomotion observed in $M_5$ knockout mice. In contrast, bilateral VTA mecamylamine pretreatment (5 μg/side) did not alter the locomotion induced by 30 mg/kg i.p. morphine in wild-type mice. Therefore, excitatory muscarinic input to the VTA, important in mediating morphine-induced dopamine release in rats (Miller et al., 2005), is also important for morphine-induced locomotion in mice and critically depends on $M_5$ mescarinic, but not nicotinic, receptors. In support of this, morphine-induced locomotion in $M_5$ knockout mice, which are missing the target receptor, was not reduced by VTA atropine.

Previous studies in $M_5$ knockout mice have found reduced amphetamine-induced locomotion (Wang et al., 2004), reduced cocaine-conditioned place preference (Fink-Jensen et al., 2003), reduced cocaine self-administration (Thomsen et al., 2005), reduced morphine-conditioned place preference, and reduced naloxone-precipitated physical withdrawal symptoms (Basile et al., 2002). The present results show that excitatory muscarinic-mediated input to VTA dopamine neurons is important in mediating the acute stimulant effects of morphine.

Electrophysiological recordings from midbrain dopamine neurons in rat brain slices suggest that opiates disinhibit VTA dopamine neurons by inhibiting local GABA neurons (Johnson and North, 1992). The present data suggest either that the disinhibition of dopamine neurons by opiates requires the presence of somatic $M_5$ receptors or that an additional mechanism exists that involves disinhibition of PPT cholinergic neurons, resulting in the activation of ascending, $M_5$-mediated cholinergic input to VTA dopamine neurons (Fig. 8). Laviolette et al. (2004) suggest that two separate mechanisms in VTA can mediate the acute rewarding effects of opiates, depending on the prior drug history of the animal. In drug-naive animals, VTA opiates activate descending GABA projections to the PPT, whereas in dependent/withdrawn animals, VTA opiates act through the disinhibition of dopamine neurons. All the mice tested in the current locomotion experiments were opiate naive, so VTA opiates should have activated descending GABAergic inputs to the PPT. Thus, this pathway could, in turn, disinhibit ascending cholinergic inputs to the VTA to produce dopamine activation through $M_5$ muscarinic receptors and consequently increase locomotion. Consistent with this hypothesis, we found that increases in nucleus accumbens dopamine efflux induced by VTA infusions of morphine (50 ng) are absent in $M_5$ knockout mice and are blocked by VTA pretreatment with the musca-
nic receptor antagonist scopolamine (50 μg) in wild-type mice (Steidl et al., 2008).

In M₅ knockout mice, the potentiation of morphine-induced locomotion by VTA mecamyline was stronger and longer lasting (between 10 and 120 min) relative to VTA atropine (between 40 and 70 min). In fact, pretreatment with VTA mecamyline in M₅ knockout mice increased morphine-induced locomotion to levels comparable with wild-type mice, suggesting that nicotinic antagonism in the VTA excited locomotion by a mechanism that added with the effect of morphine on locomotion. The fact that the effect of mecamyline added with the effect of morphine in M₅ knockout mice suggests that the effect of nicotinic antagonism is mediated through mechanisms independent of morphine (Fig. 8, right). In contrast, the effect of VTA atropine on locomotion in M₅ knockout mice, which did not add with the effect of morphine, is probably mediated through non-M₅ muscarinic receptors working via mechanisms shared with morphine (Fig. 8, left).

Experiment 2 allowed study of the role of non-M₅ VTA muscarinic and nicotinic receptors in locomotion. Either VTA atropine or mecamyline had only a slight effect on locomotion in wild-type mice but greatly increased locomotion in M₅ knockout mice. Previously, we found that scopolamine-induced locomotion (3 mg/kg i.p.) is greater in 129 M₅ knockout mice than wild-type controls (Chintoh et al., 2003). The current data in B6 M₅ knockout mice are consistent with this finding and suggest that the effects of systemic scopolamine in M₅ knockout mice were, at least in part, due to effects of scopolamine on non-M₅ muscarinic receptors in the VTA. This difference between wild-type and M₅ knockout mice suggests that the inhibitory effects of non-M₅ VTA muscarinic receptor subtypes and nicotinic receptors on locomotion become apparent only when the M₅ receptor is removed. In accordance, in M₅ knockout mice, either VTA atropine or mecamyline inhibited what under normal circumstances would be a cholinergic receptor-mediated inhibition of locomotion (i.e., disinhibition led to increased locomotion).

Morphine-induced locomotion (30 mg/kg) depended on opioid receptors in both B6 wild-type and M5 knockout mice because both 1 and 10 mg/kg naltrexone reduced locomotion. The 1 mg/kg naltrexone dose was less effective in blocking

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**Fig. 7.** Effects of VTA treatment with 3 μg of bilateral atropine, 5 μg of bilateral mecamyline, or 0.3 μl of saline in B6 (n = 6) and M₅ knockout (n = 6) mice. Open diamonds, VTA saline followed by systemic saline; open squares, VTA mecamyline followed by systemic saline; solid squares, VTA atropine followed by systemic morphine. VTA atropine increases locomotion slightly in wild-type mice (A) while increasing locomotion strongly in M₅ knockout mice (C). VTA mecamyline increases locomotion slightly in wild-type mice (B) while increasing locomotion strongly in M₅ knockout mice (D).
VTA postsynaptically. Non-M₅ muscarinic and nicotinic (N) receptors were sufficient to reduce basal locomotion of endogenous enkephalin peptides, increases open-field locomotion in wild-type mice, particularly in the 2nd h of testing (Fig. 1B). The 3 mg/kg dose of morphine increased locomotion dependent on GABA disinhibition of PPT cholinergic and dopamine functions. Furthermore, M₅ knockout mice are less sensitive to the locomotor inhibiting effects of naltrexone, either alone or in combination with morphine. This suggests that the effects of both exogenous and endogenous opiates on locomotion depend on M₅ muscarinic receptors. Finally, greater sustained spontaneous locomotion in the B6 strain of mice appears to be due to the effects of endogenous opioids.

In conclusion, M₅ muscarinic receptors are needed for approximately half of locomotion elicited by morphine via opiate receptors, probably via M₅-mediated excitatory input to VTA dopamine neurons. In M₅ knockout mice, locomotion is strongly elicited by VTA atropine or mecamylamine, indicating that non-M₅ receptors in VTA strongly inhibit locomotion. Additionally, M₅ knockout mice are less sensitive to the locomotor inhibiting effects of naltrexone, either alone or in combination with morphine. This suggests that in M₅ knockout mice, the inhibitory effect of 1 mg/kg naltrexone on locomotion on morphine was lost in the final 30 min.

In wild-type mice, both the 1 and 10 mg/kg naltrexone doses on their own reduced locomotion to below saline levels during the 1st h of testing. This finding is consistent with previous mouse data showing that systemic administration of the irreversible opioid antagonist β-chloralnaltrexone reduces activity (Kozak et al., 1985), whereas systemic administration of RB 101 in mice, which reduces enzymatic degradation of endogenous enkephalin peptides, increases open-field locomotion (Nieto et al., 2005). Together, these data suggest that endogenous opioid activity contributes to maintaining open-field locomotion and that the doses of naltrexone used in the current study were sufficient to reduce basal locomotor levels. In contrast, in M₅ knockout mice, neither 1 nor 10 mg/kg naltrexone on its own reduced locomotion to below saline levels.

B6 wild-type mice showed greater spontaneous locomotion than 129 wild-type mice, particularly in the 2nd h of testing (Fig. 1B). The 3 mg/kg dose of morphine increased locomotion only in the B6 mice again in the 2nd h (Fig. 2E). The 1 mg/kg naltrexone dose blocked saline-induced locomotion also in the 2nd h, again only in the B6 wild-type mice (Fig. 3B). These three results together suggest that greater sustained spontaneous locomotion in the B6 strain of mice is due to the stimulant effects of endogenous opioids.

M₅ knockout mice showed less sensitivity to naltrexone in two ways. Naltrexone (1 mg/kg) reduced spontaneous locomotion in wild-type but not M₅ knockout mice. Naltrexone (1 mg/kg) reduced morphine-induced locomotion less in M₅ knockout than wild-type mice. Previously, we found that naltrexone (0.33 and 1.0 mg/kg) was less effective in increasing ultrasonic separation calls in M₅ knockout than wild-type mouse pups (Podgorski et al., 2008). This raises the possibility that endogenous opioids act via M₅ receptors to affect dopamine functions.

Fig. 8. Model of mechanisms by which opiates can activate dopamine-dependent locomotion via GABA disinhibition of PPT cholinergic and VTA dopamine neurons. M₅ receptors excite dopamine neurons in the VTA postsynaptically. Non-M₅ muscarinic and nicotinic (N) receptors are on GABA neurons and on presynaptic terminals resulting in net inhibition of dopamine neurons, especially in M₅ knockout mice.

References


Kelley AE, Stinus L, and Iversen SD (1980) Interactions between D-Ala-Met-lys-phenylalanine and D-Ala-2-Met-lys-phenylalanine on GABA neurons and on presynaptic terminals resulting in net inhibitory effects that endogenous opioids act via M₅ receptors to affect dopamine functions.

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Steidl S, Miller AD, Blaha CD, and Yeomans JS (2008) M5 muscarinic receptor knockout mice show no dopamine release following morphine, but increased locomotion following cholinergic antagonists, in the ventral tegmental area, in Program No. 197.14; 2008 Neuroscience Meeting Planner; Washington, DC. Society for Neuroscience, Washington, DC.


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