

Reversal of Morphine-Induced Locomotion in M5
Muscarinic Receptor Knockout Mice with Food Deprivation
but not Bilateral Infusions of VTA BDNF

by

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Abstract

Cholinergic inputs from mesopontine tegmentum activate midbrain dopamine (DA) neurons via M5 muscarinic receptors. The M5 receptor is important for mesopontine stimulation-induced accumbal or striatal DA efflux, brain stimulation reward or morphine-induced conditioned place preference (CPP). M5 receptor knockout (KO) mice show 40-50% less morphine-induced locomotion. Pedunculopontine tegmental nucleus (PPT) lesions in rodents block morphine CPP, but are ineffective after 18 hours food deprivation, opiate dependence, or intra-VTA BDNF. Based on these findings, we investigated whether acute food deprivation or intra-VTA BDNF alters morphine-induced locomotion (3 and 10 mg/kg, i.p.) in C57BL/6 M5 KO mice. Non-deprived M5 KOs showed reduced morphine-induced locomotion, suggesting M5 receptors partly mediate morphine-induced locomotion. Morphine-induced locomotion was reversed in food-deprived mice, suggesting the stimulant effects of morphine were altered to bypass the PPT. Unexpectedly, intra-VTA BDNF infusions were ineffective in altering morphine-induced locomotion. Additionally, M5 KOs receiving intra-VTA saline showed no deficits in morphine-induced locomotion.

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List of Abbreviations

6-OHDA	6-hydroxydopamine
ACh	acetylcholine
BDNF	brain-derived neurotrophic factor
BSR	brain stimulation reward
ChAT	choline acetyltransferase
CPP	conditioned place preference
DA	dopamine
FD	food-deprived
GABA	gamma-aminobutyric acid
KO	knockout
LDT	laterodorsal tegmental nucleus
MOR	morphine
NAc	nucleus accumbens
PPT	pedunculopontine tegmental nucleus
SAL	saline
SN	substantia nigra
SNC	substantia nigra—pars compacta
TH	tyrosine hydroxylase
VTA	ventral tegmental area
WT	wildtype

Introduction

1. Overview

Drug addiction creates a huge burden on our society's economy through costs in health care, law enforcement, employment loss, prevention and research. In Canada alone, the estimated costs of drug addiction totalled approximately \$40 billion in 2002 (Rehm et al., 2006). The individual repercussions of drug addiction are just as concerning. For an addicted individual, the drug of abuse is persistently desired and taken compulsively. Addicts are so preoccupied with obtaining and using the abused drug that they knowingly forego other interests and responsibilities, with devastating consequences in the workplace, schools and homes.

Opiates, narcotic alkaloids isolated from the opium poppy (*Papaver somniferum*), are known for producing both analgesic *and* rewarding effects through their action on opiate receptors. For centuries, opiates have been used as effective pain killers (Brownstein, 1993). However, opiates such as morphine and heroin also produce euphoric effects that reinforce continued drug use and can ultimately lead to the development of drug addiction. Most recent data from Health Canada estimates there are 125,000 Canadians injecting drugs, and among the most commonly injected drugs is heroin (Health Canada, 2001). To combat this problem it is first necessary to develop an understanding of how opiates mediate their effects, both acutely and chronically. Therefore, the goal of my thesis is to contribute to a better understanding of how opiates mediate reward in the brain by examining opiate-induced behaviours in mice.

2. Dopamine

Dopamine (DA) is an important catecholamine neurotransmitter that is implicated in the rewarding effects of a variety of drugs and natural rewards (Wise & Rompre, 1989) as well as a number of neurological and psychiatric diseases (Mardsen, 2009).

Dopaminergic cell groups A8-A14 of the midbrain and diencephalon comprise a complex efferent system which originates in the retrorubal field (A8), subparafascicular thalamus nucleus

(A11), arcuate and caudal periventricular nucleus of the hypothalamus (A12), medial zona incerta of the subthalamus (A13) and rostral periventricular hypothalamus (A14) (Björklund & Nobin, 1973; Deutch, Goldstein, Baldino, & Roth, 1988; Fuxe & Hökfelt, 1969; Selemon & Sladek, 1981; Takada, Li, & Hattori, 1988). The two largest dopaminergic cell groups (A9 and A10) originate predominantly from two areas, the substantia nigra pars compacta (SNC) (A9) and ventral tegmental area (VTA) (A10) (Fallon & Moore, 1978). DA neurons in the SNC that project to the striatum (caudate nucleus and putamen) form the nigrostriatal system (Fallon & Moore, 1978) are important for motor function. Rats with 6-hydroxydopamine (6-OHDA) lesions to the SN show increased latencies for initiation of simple head movements (Carli, Evenden, & Robbins, 1985). MPTP-induced striatal DA loss leads to deficits in initiation and execution of simple arm movement (Schultz, Studer, Romo, Sundstrom, Johnsson, & Scarnati, 1989). Animal models such as this have closely linked the loss of these DA neurons in the SNC with the neurodegenerative disorder Parkinson's disease in humans (Deumans, Blokland, & Prickaerts, 2002). Consequently, patients with Parkinson's disease exhibit deficits in motor control, tremors and rigidity.

The striatum is also importantly involved in stereotypy, i.e. drug-induced repetitive behaviours such as grooming, jumping and gnawing that are normally seen in an animal's daily behaviour but occur at abnormally high frequencies (Mason, 1991; Canales & Graybiel, 2000). For example, cocaine induces stereotypy in monkeys (Saka, Goodrich, Harlan, Madras, & Graybiel, 2004) and 6-OHDA lesions of the dorsal striatum reduce amphetamine-induced stereotypy in rats (Joyce & Iverson, 1984).

VTA DA neurons of the mesolimbic system project to multiple areas including nucleus accumbens (NAc), amygdala, hippocampus and olfactory bulb (Fallon & Moore, 1978) to exert control over emotional states, motivated behaviours and reward (Alcaro, Huber, & Panksepp, 2007; Wise & Rompre, 1989). Accumbal dopamine increases in response to food (Schilström, Svensson, Svensson, & Nomikos, 1998), electrical stimulation to the VTA (Fiorino, Coury, Fibiger, & Phillips, 1993) and a variety of intra-VTA injected drugs (Vezina, 1993). Lesions of the VTA attenuate cocaine self-administration (Roberts & Koob, 1982) and self-stimulation (Fibiger, LePiane, Jakubovic, & Phillips, 1987). Additionally, accumbal DA blockade reduces reward associated with food (Wise & Colle, 1984; Wise & Schwartz, 1981), self-stimulation

(Fouriezios, Hansson, & Wise, 1978) and drugs including cocaine and heroin (Caine & Koob, 1994; Spyraiki, Fibiger, & Phillips, 1983). Finally, systemic DA antagonists block the locomotor stimulating effects of acute intra-VTA morphine (Vezina & Stewart, 1989). It is important to note, however, that van der Kooy and his group have found there are DA-independent contributions associated with the acute effects of opiates (see section 4.1).

2.1 GABAergic Control of Dopamine

Gamma-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the brain that exerts afferent control over the midbrain DA system. *In situ* hybridization methods using riboprobes for the enzyme glutamic acid decarboxylase (GAD) have shown there are populations of GABAergic neurons in both the VTA and SN (Nair-Roberts, Chatelain-Badie, Benson, White-Cooper, Bolam, & Ungless, 2008). In VTA, about 35% of cells were positively labelled for GAD and were indiscriminately mixed with the TH-labelled cell population. In SN, however, more than 60% are GAD-positive and located primarily in the pars reticulata region. GABA neurons in the VTA and SN are also known to synapse with DA neurons, as well as PPT and LDT neurons (Bayer & Pickel, 1991; Semba & Fibiger, 1992; Steininger & Rye, 1992; van den Pol, Smith, & Powell, 1985). GABA neurons are thought to be mainly associated with opioid receptors, as anatomical studies find μ -opioid receptors are located largely on non-dopaminergic neurons (Dilts & Kalivas, 1989; Garzon & Pickel, 2001). Furthermore, opioids hyperpolarize local GABA neurons, which results in excitation of DA neurons in the VTA (Johnson & North, 1992). Therefore, GABA neurons are proposed to be importantly involved in the effects of opiates, through disinhibition of DA neurons in VTA (Vanderschuren & Kalivas, 2000).

3. Acetylcholine

Acetylcholine (ACh) is another principal neurotransmitter which serves many functions in both the peripheral and central nervous system (Wess, 2004). Immunohistochemistry studies have defined eight cholinergic cell groups, Ch1-8 in the brain (Mesulam, Mufson, Wainer, & Levey, 1983; Schafer, Eiden, & Weihe, 1998). Of particular interest for this thesis are cell groups Ch5 and Ch6, found in the pedunculopontine tegmental nucleus (PPT, Ch5) and the laterodorsal tegmental nucleus (LDT, Ch6) and will be discussed in more detail below.

3.1 PPT and LDT

The PPT (Ch5) extends caudally from the posterior end of the substantia nigra and is bordered laterally by the superior cerebellar peduncle, dorsally by the cuneiform and deep mesencephalic nuclei, and ventrally by the pontine reticular nucleus (Inglis & Winn, 1995; Rye, Saper, Lee, & Wainer, 1987). The LDT is located medially in the pontine central gray and bordered anteriorly by the dorsal raphe and laterally by the posterior end of the PPT (Paxinos & Watson, 1998). Additionally, in-situ hybridization work suggests that there are distinct bundles of cholinergic, GABAergic and glutamatergic neurons comprising 20%, 58% and 22% of total neuron populations in the LDT, respectively (Winn, 2006).

Retrograde tract-tracing methods and choline acetyltransferase (ChAT) immunohistochemistry indicate that ChAT-positive cells are most densely located in the PPT and LDT. Labelled cells projecting to the substantia nigra (SN) originated ipsilaterally from the rostroventral pole of the PPT. In contrast, projections to VTA were bilaterally distributed from both the PPT and LDT. Therefore the LDT and PPT provide organized, selective cholinergic innervations to the VTA and SN (Oakman, Faris, Kerr, Cozzari, & Hartman, 1995).

3.2 Cholinergic Receptors

The various actions of ACh in the brain are mediated by fast, ionotropic nicotinic and slow, metabotropic muscarinic receptors (Wess, 2004). Nicotinic receptors are ligand-gated ion channels that are comprised of several different α - and β -subunits (Salamone & Zhou, 2000; Sargent, 1993).

Currently, five muscarinic acetylcholine receptor subtypes, M1-M5 have been identified (Bonner, Young, Brann, & Buckley, 1987; Bonner, Young, Brann, & Buckley, 1988; Kubo, et al., 1986). These muscarinic receptors belong to a family of seven transmembrane G-protein coupled receptors. The odd-numbered muscarinic receptor subtypes M1, M3 and M5 couple with subunits of the $G_{q/11}$ protein family. Activation of these receptors activates hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) to form inositol 1,4,5-trisphosphate (InsP₃) and diacylglycerol (DAG), leading to increases in intracellular stores of Ca²⁺ and cell excitability (Eglen & Nahorski, 2000). The muscarinic receptor subtypes M2 and M4 are coupled to G_i and

G_o subunits and have an inhibitory effect (Caulfield & Birdsall, 1998). Activation of these receptors inhibits adenylyl cyclase activity, leading to decreases in cAMP and cell excitability (Felder, 1995).

Immunoprecipitation methods indicate that the M5 receptor subtype represents less than 2% of the total muscarinic receptor family distribution in the brain (Yasuda et al., 1993). M5 mRNA has been detected in the SN, VTA, striatum, hippocampus and ventromedial hypothalamus (Levey, Kitt, Simonds, Price, & Brann, 1991; Vilaro, Palacios, & Mengod, 1990; Weiner, Levey, & Brann, 1990; Wang, et al., 2004). Furthermore, M5 and D2 mRNA are co-expressed in DA neurons in the SN and VTA (Wang et al., 2004; Weiner, Levey, & Brann, 1990). Based on this, and other work to be discussed below (section 3.3), it has been proposed that the M5 receptor is important in excitation of midbrain DA cells (Weiner, Levey, & Brann, 1990).

3.3 Cholinergic Activation of Dopamine

The PPT and the LDT, which provide the only known cholinergic inputs to the SN and VTA, play an important role in DA activation. Electrical stimulation of PPT or LDT produces a tri-phasic pattern of DA efflux in rats. The first phase of striatal (PPT-induced) and accumbal (LDT-induced) DA release lasts only 2-3 minutes and is mediated by activation of fast-acting ionotropic nicotinic and glutamatergic receptors. Administration of nicotinic and glutamatergic receptor antagonists into VTA (Forster & Blaha, 2000) or SN (Forster & Blaha, 2003) blocks this first phase in rats. During the second phase, striatal and accumbal DA release declines to below baseline levels for approximately 8 minutes. Muscarinic antagonism with scopolamine into PPT and LDT reduces this second phase in rats (Forster & Blaha, 2000; Forster & Blaha, 2003). Additionally, selective M2 methoctramine fully blocks the second phase, suggesting that this phase is induced by activation of inhibitory M2 receptors on cholinergic neurons of the PPT and LDT (Buckley, Bonner, & Brann, 1988; Levey, Kitt, Simonds, Price, & Brann, 1991; Vilaro, Palacios, & Mengod, 1990; Vilaro, Palacios, & Mengod, 1994). The third phase has a delayed onset at 8-9 minutes, followed by prolonged DA efflux lasting approximately 40 minutes. In rats, this final phase of DA efflux is mediated by muscarinic receptors in VTA and SN, as VTA or SN infusions of the muscarinic antagonist scopolamine blocks the third phase (Forster & Blaha,

2000; Forster & Blaha, 2003). In mice of the CD1x129 strain, the third phase of LDT-induced accumbal DA release is blocked by systemic scopolamine and is completely absent in homozygous mutants for the M5 muscarinic receptor (Forster, Yeomans, Takeuchi, & Blaha, 2001). This suggests that the third phase of DA efflux is solely mediated by the M5 muscarinic receptor subtype.

3.4 Muscarinic Receptors and Reward

VTA muscarinic receptors, particularly the M5 subtype, play an important role in many rewards for both rats and mice (Yeomans, Forster, & Blaha, 2001). Intra-VTA infusions of carbachol are sufficient to produce CPP (Yeomans, Kofman, & McFarlane, 1985). Rats also self-administer carbachol into the posterior VTA and this effect was reduced with scopolamine or the DA antagonist SCH 23390 (Ikemoto & Wise, 2002). Additionally, intra-VTA muscarinic antagonist atropine reduces brain-stimulation reward (BSR) to a greater extent than nicotinic antagonists (Yeomans & Baptista, 1997). Finally, infusion of M5 antisense oligonucleotides reduces sensitivity to BSR, as measured rightward shifts of rate-frequency curves, which were reversed by removal of the M5-specific oligonucleotides (Yeomans et al., 2000).

Since no successfully tested M5 selective ligands currently exist, the development of M5 knockout (KO) mice has been essential in studying the role of M5 receptors in DA-related reward functioning (Forster, Yeomans, Takeuchi, & Blaha, 2001). M5 KO mice also showed reduced cocaine self-administration (Fink-Jensen, et al., 2010; Thomsen, Woldbye, Wörtwein, Fink-Jensen, Wess, & Caine, 2005), reduced morphine-induced conditioned place preference (CPP) (Basile et al., 2002) and emit fewer ultrasonic vocalizations (USVs) in response to natural rewards such as sex (Wang, Liang, Burgdorf, Wess, & Yeomans, 2008).

4. Opiates

4.1 Opiate Reward

Opiate reward relies in large part on μ -opioid receptor activation in the VTA. Intracranially administered morphine into the VTA sustains reliable self-administration in rats (Bozarth & Wise, 1981). Intra-VTA morphine also produces conditioned place preference (CPP)

in rats (Nader & van der Kooy, 1997; Phillips & LePiane, 1980). Additionally, morphine-induced place preference is blocked with naloxone (Phillips & LePiane, 1980) or in mice lacking the μ -opioid receptor (Matthes et al., 1996), indicating a critical role of the μ -opioid receptor.

Many studies suggest there are DA-dependent and DA-independent pathways for opiate reward, depending on the “state” of the animal (Bechara & van der Kooy, 1992; Nader & van der Kooy, 1994; Nader & van der Kooy, 1997). In food-deprived or drug-dependent rats, CPP for a morphine-paired environment was blocked by alpha-flupenthixol, but not by lesions of the PPT. In contrast, lesions of the PPT, but not alpha-flupenthixol, completely abolished preferences for the morphine-paired environment in non-deprived or drug-naïve rats. These results suggest that opiate reward is transmitted largely through DA-independent pathways involving VTA descending inputs to the PPT in non-deprived or drug-naïve rats (Nader & van der Kooy, 1997). Conversely, opiate reward in food-deprived or drug-dependent rats is mediated largely through DA-dependent pathways in the VTA which bypass the PPT. Along this line of evidence, drug-naïve D₂ receptor KO mice showed reliable morphine-induced CPP, but did not show morphine CPP after drug-dependence and withdrawal (Dockstader, Rubinstein, Grandy, Low, & van der Kooy, 2001).

Most recently, Vargas-Perez et al. (2009) found that BDNF is capable of inducing a similar switch from DA-independent to DA-dependent opiate reward pathways in rats. In rats with intra-VTA BDNF infusions, systemic alpha-flupenthixol, but not PPT lesions, abolished morphine-induced preferences. They propose that BDNF produces these effects by inducing a drug dependent-like state similar to that seen previously in rats that were morphine-dependent and withdrawn (Bechara & van der Kooy, 1992; Nader & van der Kooy, 1997).

Additionally, drug-naïve mice lacking the M5 receptor show reduced morphine-induced CPP (Basile et al., 2002). This suggests that, particularly in mice, cholinergic activation of VTA DA neurons may also contribute to the effects of opiates in drug-naïve animals.

4.2 Opiates and Dopamine

On the basis of the evidence provided above, acutely administered opiates are thought to disinhibit midbrain DA neurons via inhibition of local GABA neurons, leading to enhanced DA

release in the NAc (Vanderschuren & Kalivas, 2000). GABAergic neurons of VTA and SN provide strong tonic inhibition to cholinergic neurons of the PPT and LDT (Saitoh, Hattori, Song, Isa, & Takakusaki, 2003). Activation of inhibitory μ -opioid receptors on VTA and SN GABA neurons leads to inhibition of these cells (Johnson & North, 1992) and therefore presumably a strong disinhibition of cholinergic PPT and LDT neurons.

Subsequently, cholinergic projections from PPT and LDT can activate midbrain DA neurons through the M5 receptor. For example, striatal and accumbal DA efflux is reduced in rats with lesions to either the PPT (Miller, Forster, Metcalf, & Blaha, 2002) or LDT (Forster, Falcon, Miller, Heruc, & Blaha, 2002), respectively. Muscarinic antagonism or lack of M5 receptors also blocks prolonged accumbal DA release (Forster & Blaha, 2000; Forster, Yeomans, Takeuchi, & Blaha, 2001).

Additionally, morphine-induced striatal (Miller, Forster, Yeomans, & Blaha, 2005) and accumbal DA efflux is blocked by intra-VTA scopolamine in rats and mice, or in mice lacking the M5 muscarinic receptor (Steidl, 2008). Therefore, morphine works preferentially through inhibitory μ -opioid receptors near GABA neurons in the VTA and SN to disinhibit PPT and LDT cholinergic neurons that work through M5 receptors (Figure 1).

5. Drug-Induced Locomotion

A variety of DA activating drugs including psychostimulants, opiates, ethanol and nicotine increase locomotion in both rats and mice. Drug-induced locomotion is used as a measure of reward under the presumption that the same neural circuits mediating drug-seeking or goal-directed approach behaviours are being activated (Wise & Bozarth, 1987). Undeniably, approach behaviours are crucial to survival, as any animal searching for food, water or a mate must move forward towards the objective (Steidl, 2008). Likewise, animals in the laboratory show motivated forward movement towards an environment associated with a drug or to self-administer drugs. Since the amount of locomotion is thought to reflect activation of the midbrain DA activation, drug-induced locomotion is used as a behavioural index of DA activation in rodents (Tzschentke, 2001).

5.1 Opiate-Induced Locomotion

In mice, opiates dose-dependently increase locomotion characterized as a “running fit,” and produce an effect called “Straub tail,” in which the tail is elevated in an arched and rigid position (Lee & Fennessy, 1976; Oliverio, 1976). Mice of the C57BL/6 strain are more sensitive than mice of the DBA/2j or 129Sv strain to the locomotor stimulating effects of systemic morphine (between 3 and 160 mg/kg, s.c.) (Brase, Loh, & Way, 1977; Murphy, Lam, & Maidment, 2001). Moreover, DBA/2j mice show no morphine-induced locomotor response at all.

Subcutaneous administration of irreversible opioid receptor antagonists in C57BL/6 mice dose-dependently blocks morphine-induced locomotion (Frischknecht, Siegfried, Riggio, & Waser, 1983). Similarly, mice lacking the μ -opioid receptor show no locomotion in response to systemic morphine (3 and 10 mg/kg, i.p.) (Hall et al., 2003). Also, systemic DA D1-receptor antagonist SCH 23390 dose-dependently reduced morphine-induced locomotion (Longoni, Spina, & Di Chiara, 1987). Recently, Steidl and Yeomans (2009) found that mice lacking the M5 receptor showed reduced morphine-induced locomotion by 40-50% relative to wild-type (WT) controls, particularly in C57BL/6 mice at a 10 mg/kg morphine dose. Furthermore, injections of the muscarinic receptor antagonist atropine into the VTA, prior to systemic morphine, resulted in similarly reduced locomotion in WT mice (Steidl & Yeomans, 2009). All these data support the idea that morphine-induced locomotion is attributed, at least in part, to μ -opioid receptor mediated disinhibition of PPT-M5 cholinergic inputs to VTA DA neurons.

6. Objectives

Activation of the midbrain DA system relies on PPT cholinergic inputs to VTA and SN. As such, striatal and accumbal DA efflux is attenuated after lesions of the PPT (Miller, Forster, Metcalf, & Blaha, 2002) or LDT (Forster, Falcon, Miller, Heruc, & Blaha, 2002) in rats. Muscarinic receptors, especially the M5 subtype, have been shown to be crucial for the activation of mesolimbic DA in mice, as M5 KO mice lack the third phase of prolonged accumbal DA release (Forster, Yeomans, Takeuchi, & Blaha, 2001; Steidl, 2008). Additionally, M5 KO mice show reduced accumbal DA and conditioned place preference after systemic morphine (Basile et al., 2002). In regards to drug-induced locomotion, M5 receptors appear to

mediate only half of morphine-induced locomotion in C57BL/6 mice (Steidl, 2008). Therefore, the effects of acutely administered opiates in mice appear to be mediated, at least in part, by PPT cholinergic activation of the VTA.

One theory proposes that the PPT, but not DA, is important for morphine-reward only in drug-naïve animals (Bechara & van der Kooy, 1992; Nader & van der Kooy, 1994; Nader & van der Kooy, 1997). Alternatively, morphine-reward becomes DA-dependent, through bypassing the PPT, in food-deprived, drug-dependent or BDNF infused rats. These data suggest that PPT contributions to opiate reward signalling can be bypassed during deprivation or dependence states, including dependence-like changes induced by intra-VTA BDNF. Whether such manipulations can modulate the mesolimbic DA system and change behavioural responses to opiates in mice is not clear.

The objective of this thesis was to determine the role of the M5 receptor in these various states and ultimately the involvement of the M5 receptor in drug dependence. The first goal was to test if food deprivation, like in rats, could induce a PPT- and M5-independent pathway for morphine reward. To do this, morphine-induced locomotion was measured in mice of the C57BL/6 strain after 18 hours food deprivation. If indeed, the PPT and M5 receptors are being bypassed, then morphine-induced locomotion deficits in M5 KO mice should be rescued. Secondly, BDNF was infused into the VTA of M5 KO mice to determine if the same changes could be elicited. Again, if intra-VTA BDNF induces a bypassing of the PPT and M5 receptors, a rescue or morphine-induced locomotion would be expected (Figure 1).

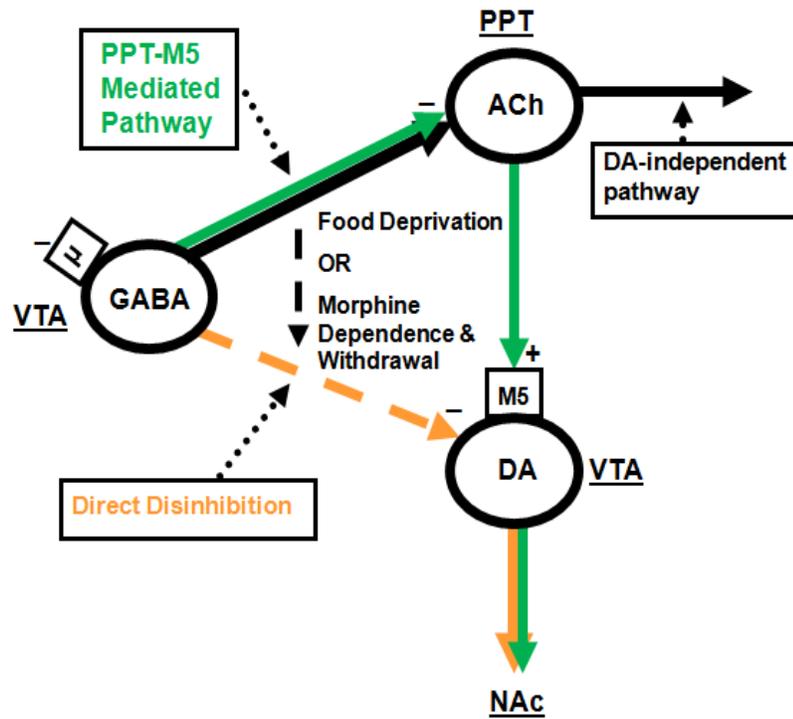


Figure 1. Model of neural circuits involved in opiate reward. Acutely administered morphine activates inhibitory μ -opioid receptors on GABA neurons in VTA, leading to disinhibition of PPT-M5 receptor mediated (green, Steidl, 2008) and DA-independent (black, Nader et al., 1997) reward pathways in drug-naïve or non-deprived animals. After food deprivation or morphine dependence and withdrawal, GABA neurons directly disinhibit VTA DA neurons (orange, Nader et al., 1997), bypassing PPT and M5 receptors to restore morphine-induced locomotion in M5 KO mice.

Experiment 1: Morphine-Induced Open-Field Locomotion Deficits in M5 Muscarinic Receptor Knockout Mice Reversed after 18 Hours Food Deprivation

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Abstract

Cholinergic inputs from the mesopontine tegmentum activate midbrain dopamine (DA) neurons via M5 muscarinic receptors. The M5 muscarinic receptor is important for accumbal or striatal DA efflux induced by mesopontine stimulation, for brain stimulation reward or for morphine-induced conditioned place preference (CPP). Mice lacking the M5 muscarinic receptor show a 40-50% reduction in morphine-induced locomotion (Steidl & Yeomans, 2009). Additionally, many studies show that pedunculo-pontine tegmental nucleus (PPT) lesions in rats and mice block morphine CPP (Nader, Bechara, & van der Kooy, 1997; Vargas-Perez et al., 2009), but PPT lesions are ineffective after 18 hours food deprivation. On the basis of these findings, we investigated whether acute food deprivation alters open-field locomotion induced by systemic morphine at 10 mg/kg (i.p.) in M5 knockout (KO) mice of the C57BL/6 strain. Food deprivation significantly increased saline-induced locomotion regardless of genotype, suggesting that food deprivation itself has a small locomotor stimulating effect in mice. Non-deprived M5 KO mice showed a reduction in morphine-induced locomotion similar to that seen previously, supporting the idea that M5 receptors mediate, in part, opiate-induced locomotion. Finally, food deprivation caused a reversal in morphine-induced locomotion, as open-field locomotion was rescued in M5 KO mice but reduced in WT mice. This suggests that the locomotor stimulant effects of morphine were altered by 18 hours food deprivation to bypass the PPT and reverse morphine-induced locomotion deficits in M5 KO mice.

Introduction

Opiate-induced locomotion in rats is mediated through both dopamine (DA)-dependent and -independent pathways. Infusions of the enkephalin analog, D-Ala²-Met⁵-enkephalinamide (DALA) into ventral tegmental area (VTA) increase locomotion which is blocked by nucleus accumbens (NAc) fluphenazine pre-treatment (Kalivas, Widerlöv, Stanley, Breese, & Prange, 1983). However, locomotion induced by NAc DALA is not blocked by either fluphenazine treatment or 6-hydroxydopamine (6-OHDA) lesions. Furthermore, neither systemic α -flupenthixol nor 6-OHDA lesions affects locomotion induced by systemic heroin (Vaccarino, Amalric, Swerdlow, & Koob, 1986).

In contrast, opiate-induced locomotion in mice appears to depend more on DA. For example, morphine-induced locomotion is reduced after systemic haloperidol (Ito, Mori, & Sawaguchi, 2008) or in DA-deficient mice (Hnasko, Sotak, & Palmiter, 2005). Additionally, concomitant measurement of accumbal DA efflux using microdialysis and locomotion after systemic morphine (3mg/kg) show that out of three strains tested (C57BL/6, 129Sv, DBA2), C57BL/6 mice showed the most morphine-induced accumbal DA release and locomotion (Murphy, Lam, & Maidment, 2001).

The pedunculopontine tegmental nucleus (PPT) and laterodorsal tegmental nucleus (LDT) provide cholinergic and glutamatergic inputs that activate DA neurons in the VTA and substantia nigra (SN) (Oakman, Faris, Kerr, Cozzari, & Hartman, 1995; Omelchenko & Sesack, 2005). Electrical stimulation of the LDT or PPT increases accumbal and striatal DA efflux, respectively (Forster, Yeomans, Takeuchi, & Blaha, 2001; Miller, Forster, Yeomans, & Blaha, 2005). In rats, prolonged accumbal or striatal DA efflux is blocked by scopolamine into VTA or SN (Forster & Blaha, 2000; Forster & Blaha, 2003). This line of evidence indicates that PPT and LDT neurons activate midbrain DA systems, mainly via muscarinic receptors in VTA and SN.

The M5 receptor is the only muscarinic subtype importantly involved in midbrain DA activation (Steidl, 2008). Midbrain DA neurons only show mRNA expression for one of the five muscarinic receptor subtypes—the M5 muscarinic receptor (Reever, Ferrari-DiLeo & Flynn, 1997; Vilaro, Palacios & Mengod, 1990; Weiner, Levey & Brann, 1990) Moreover, in mice of the CD1x129 strain, prolonged LDT-evoked accumbal dopamine release is blocked by systemic

scopolamine or in mice lacking the M5 receptor (Forster, Yeomans, Takeuchi & Blaha, 2001), suggesting that long-lasting accumbal DA efflux is solely mediated by the M5 receptor subtype.

Cholinergic innervations of VTA are also necessary for mediating the effects of opiates in rats and mice. Lesions of the PPT (Miller, Forster, Metcalf, & Blaha, 2002) or LDT (Forster, Falcon, Miller, Heruc, & Blaha, 2002) in rats attenuate striatal or accumbal DA release, respectively, after intravenous morphine. Intra-VTA morphine increases in accumbal DA are blocked after VTA scopolamine or in mice lacking the M5 muscarinic receptor (Steidl, 2008). Basile et al. (2002) found that M5 knockout (KO) mice show less accumbal DA release. Importantly, C57BL/6 mice given VTA muscarinic, but not nicotinic, antagonists or mice lacking the M5 receptor show 40-50% reductions in systemic morphine-induced locomotion (Steidl & Yeomans, 2009). Additionally, M5 KO mice show reduced cocaine self-administration (Fink-Jensen et al., 2010; Thomsen, Woldbye, Wörtwein, Fink-Jensen, Wess, & Caine, 2005), emit less ultrasonic vocalizations (USVs) in response to mating or female urine (Wang, Liang, Burgdorf, Wess, & Yeomans, 2008) and show reduced morphine CPP (Basile et al., 2002). This suggests that while morphine-induced DA activation depends entirely on the M5 receptor, morphine-induced behaviours such as locomotion and mating-induced USVs are mediated only in part by the M5 receptor.

Pathways for opiate reward can be altered, depending on the motivational state of the animal. In food-sated rats, PPT lesions, but not α -flupenthixol, block CPP to systemic morphine (Nader & van der Kooy, 1994). However, in rats food-deprived for 18 hours, α -flupenthixol, but not lesions of the PPT block morphine CPP. This work suggests that morphine reward is mediated through PPT, in non-deprived (food-sated) animals, but PPT becomes less important for morphine reward after acute food deprivation. Both CPP and the acute locomotor activating effects of morphine in non-deprived rats and mice critically depend on the PPT. Therefore, we want to determine whether food-deprivation effects that alter sensitivity of morphine CPP can similarly alter the locomotor-stimulating effects of morphine.

The aim of this study was to investigate whether sensitivity to morphine-induced locomotion can be altered by food deprivation, consistent with past work on morphine CPP (Nader, Bechara, & van der Kooy, 1997). To do this, both wildtype (WT) and M5 KO mice of the C57BL/6 strain were food-deprived for 18 hours and subsequently tested for morphine-

induced locomotion. According to Nader et al. (1997), after food deprivation, PPT should become less important for the locomotor stimulating effects of opiates. Therefore, the M5 receptor that mediates the output of PPT neurons to DA neurons should also become less crucial in mediating opiate-induced locomotion.

Methods

Mice

M5 muscarinic receptor mutant mice were created by methods described by Takeuchi et al., 2002. Using DNA for the rat M5 receptor gene, a search of mouse cDNA was completed to find a gene candidate for the M5 gene in mice. The coding region for the third intracellular loop (0.5 kb) of the protein was deleted, resulting in a loss of amino acids 251-531. Targeting vectors were created by placing a neomycin resistance gene in the same location as the deletion. Using electroporation, the targeting vector was then inserted into embryonic stem cells from mice of the 129SvJ strain. Cells showing homologous recombination, which were selected using G418 and GANC and tested with Southern blotting, were inserted into blastocysts from CD-1 mice. These blastocysts were then placed in pseudopregnant females to produce chimeric progeny. Using chimeric males, heterozygous F1 offspring were produced. Genotypes of F2 progeny were confirmed using the Southern blot method.

M5 muscarinic receptor mutant mice used in the current study were created on a mixed SvJ x CD1 background. Mice were maintained in homozygous WT and M5 KO breeding colonies and backcrossed for 6 generations to the C57BL/6 strain to create a C57BL/6 background (Gerlai, 1996). All mice were obtained from litters aged matched at 2-4 months at the start of testing.

A total of 48 mice were used (24 WT, and 24 M5 KO). For this experiment, mice were group-housed (2-3 per cage) and maintained on a 12 hour light/dark cycle (lights on 7:00 AM-PM). Food and water was provided *ad libitum* except during behavioural testing. Prior to experimentation, mice were relocated from the main colony to a housing room adjoining the testing room for one week. Temperature ($20 \pm 1^\circ\text{C}$) and humidity (55-60%) was regulated for

both the housing and experimental rooms. This study was approved by the University of Toronto Animal Care Committee.

Locomotion Testing Apparatus, Protocol and Data Acquisition

The open-field apparatus used for measuring locomotion is as described by Steidl and Yeomans (2009). Behavioural testing occurred during the light cycle, between 10:00 AM and 5:00 PM. Each mouse was randomly assigned to a 31x31x31 cm black chamber (University of Toronto, Department of Cell & Systems Biology Workshop). For all experiments, WT and KO mice were tested together with drug treatments counterbalanced. Mice were recorded with a video camera (Panasonic, Model #WV-CP484, Osaka, Japan) placed approximately 2.1 m above the testing apparatus. The testing room was lit by two 40-watt light bulbs located approximately 2.7 m above testing chambers.

Locomotor testing occurred over 3 consecutive days. At the start of each testing day, mice were removed from the housing room and weighed in an adjacent preparation room. Next, all mice, in their home cages, were placed in the experimental room for 20 minutes to habituate to the experimental area. The first day of testing served to habituate mice to the testing chambers used in the subsequent locomotion tests. Mice were weighed and placed in the experimental room for 20 minutes, then placed in the testing chambers for 2 hours without receiving any injections. On the next two days, M5 KO and WT mice were randomly assigned to two groups: 1) non-deprived and saline/ morphine drug treatment, 2) food-deprived (18 hours food deprivation) and saline/ morphine drug treatment. Administration of saline and morphine (10 ml/kg and 10 mg/kg, i.p., respectively) was counterbalanced so half the mice were exposed to saline on day 2 and morphine on day 3, and the other half were exposed to saline and morphine in the reverse order. After mice were injected, they were carefully placed in the center of their appropriate testing chambers and locomotion was recorded for 2 hours. All locomotion data was analyzed with Noldus Ethovision tracking software (Wageningen, Netherlands).

Drugs

Morphine sulphate pentahydrate was acquired from Sigma-Aldrich (St.Louis, MO). Morphine was dissolved in 0.9% sterile saline. Intraperitoneal injections of morphine were given at a dose of 10 mg/kg and at a volume of 10 ml/kg.

Statistical Analysis

For locomotion testing, time course data was analyzed separately in food-deprived and non-deprived C57BL/6 M5 KO and WT mice using a repeated measures ANOVA with distance moved as the dependent variable, drug treatment (saline or morphine) and time (12, 10-minute time bins from 0-120 minutes) as repeated within-subject factors and genotype as the between-subject factor. Assumed sphericity was tested for each analysis and when violated, degrees of freedom for within-subject factors and interactions involving within-subject factors were adjusted with Greenhouse-Geisser adjustments (for all cases, epsilon was less than 0.75). Post hoc tests were not possible because there were less than 3 groups for the between-subject factor (genotype). Therefore, to further analyze possible interactions at individual time bins, time course data was analyzed using a univariate ANOVA, with genotype and drug treatment as the between-subject factors and distance moved per 10-minute time bin as dependent factors.

Total morphine-induced locomotion was analyzed separately for food-deprived and non-deprived M5 KO and WT mice using a 2-way univariate ANOVA, with distance moved as the dependent variable and genotype and drug treatment (saline or morphine) as fixed factors. Post hoc test results were not obtained because between-subject factors contained less than 3 groups. Further analysis of drug treatment effects on locomotion in non-deprived and food-deprived groups was conducted using student's t-test with Bonferonni corrections.

To investigate the effects of deprivation state on total saline- and morphine-induced locomotion in M5 KO and WT mice, data were analyzed using a univariate ANOVA, with total distance moved (after saline or morphine) as the dependent variable and genotype and deprivation state (food-deprived, or non-deprived) as fixed factors. Again, since post hoc tests were not possible, specific interactions within genotype (M5 KO or WT) across treatment (food-deprived vs. non-deprived) were investigated using student's t-tests with Bonferonni corrections.

Results

Saline-Induced Locomotion: Non-deprived vs. Food-deprived Mice

Total saline-induced locomotion increased 32% for WT mice and 40% for M5 KO mice after food deprivation (Figure 2). Univariate ANOVA indicates that there was no significant effect of genotype, but there was a significant main effect of deprivation state on saline-induced locomotion ($F(1, 24) = 10.634, p < 0.01$). Therefore, while mice of both genotypes showed more saline-induced locomotion after food deprivation, M5 KO and WT mice did not respond differently to handling and subsequent saline-injection.

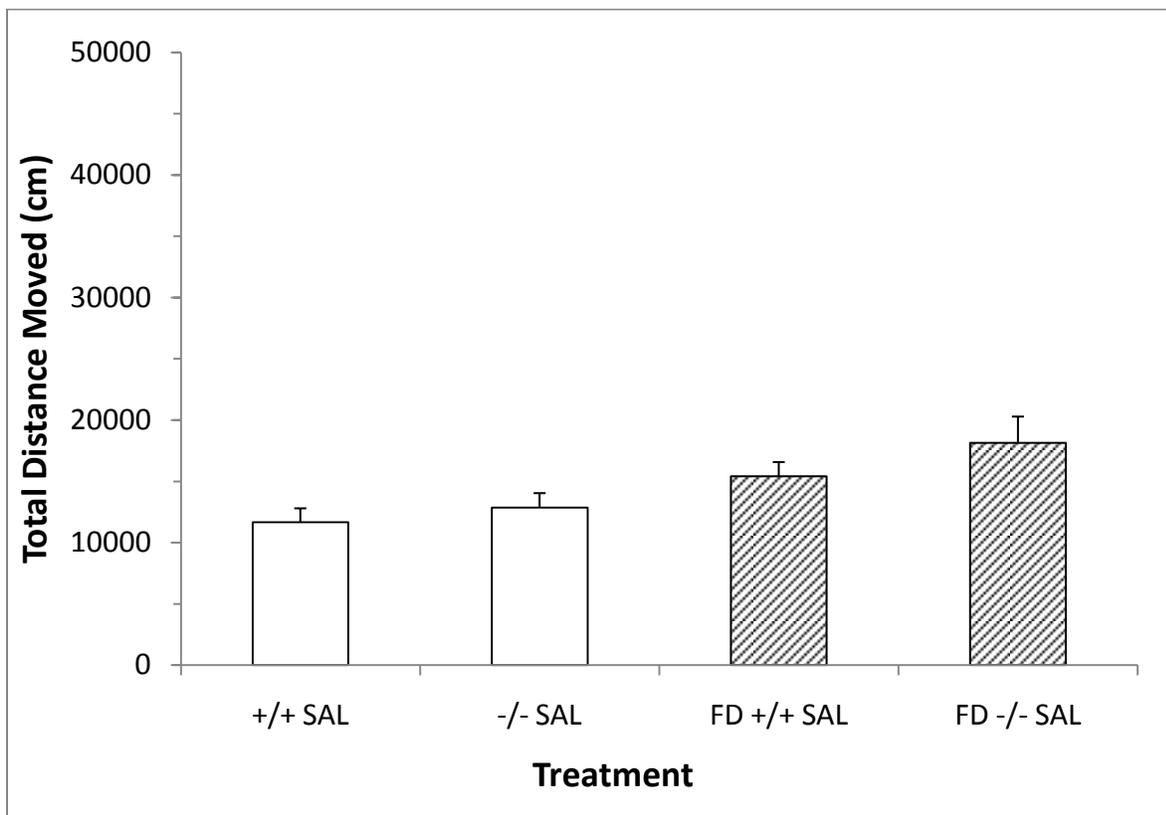


Figure 2. Total saline-induced locomotion in non-deprived M5 KO (-/-, n=7) and WT (+/+, n=8) mice, and food-deprived M5 KO (-/-, n=8) and WT (+/+, n=7) mice. Error bars represent standard errors of the mean. SAL= saline, FD=food-deprived.

Total Morphine-induced Locomotion: Non-deprived vs. Food-deprived Mice

Relative to non-deprived saline controls, non-deprived M5 KO mice showed an 86% increase and non-deprived WT mice showed a 272% increase in morphine-induced locomotion (Figure 3A). Univariate ANOVA shows there was a significant main effect of drug treatment ($F(1, 25)= 43.888, p<0.000001$), genotype ($F(1, 25)= 7.940, p<0.01$) and a significant interaction between drug treatment and genotype ($F(1, 25)= 10.187, p<0.01$). Student's t-test with Bonferonni corrections indicates that both M5 KO and WT mice showed greater locomotion after systemic morphine than after saline ($t(11)= 2.999, p=0.024$; $t(14)= 6.390, p=0.00004$, respectively).

Saline- and morphine-induced locomotion totals in food-deprived mice are shown in Figure 3B. M5 KO mice showed a 126% increase and WT mice showed a 73% increase in morphine-induced locomotion, compared to saline controls. Univariate ANOVA shows a significant main effect of drug treatment ($F(1, 25)= 25.977, p<0.0001$) and genotype ($F(1, 25)= 6.579, p=0.017$), but no significant interaction between drug and genotype. Again, both M5 KO and WT mice showed significantly more locomotion in response to morphine compared to saline ($t(13)= 3.914, p=0.004$; $t(12)= 3.826, p=0.004$).

Additionally, there was a significant interaction between genotype and deprivation state on morphine-induced locomotion (univariate ANOVA: $F(1, 24)=13.823, p<0.01$). Importantly, food-deprived M5 KO mice showed a 71% increase in total morphine-induced locomotion relative to non-deprived M5 KO mice and a 54% increase relative to food-deprived WT mice, but these changes were not significant (Figure 3C; $t(11)= 2.371, p=0.074$, $t(12)= 2.264, p=0.086$, respectively).

In contrast, non-deprived M5 KO mice showed 45% less total morphine-induced locomotion than non-deprived WT mice (Figure 3C; $t(12)= 2.986, p= 0.022$). Furthermore, food deprivation induced a reversal of locomotor responses, as food-deprived WT mice showed 39% decrease in total morphine-induced locomotion compared to non-deprived WT mice (Figure 3C; $t(13)= 2.919, p=0.024$).

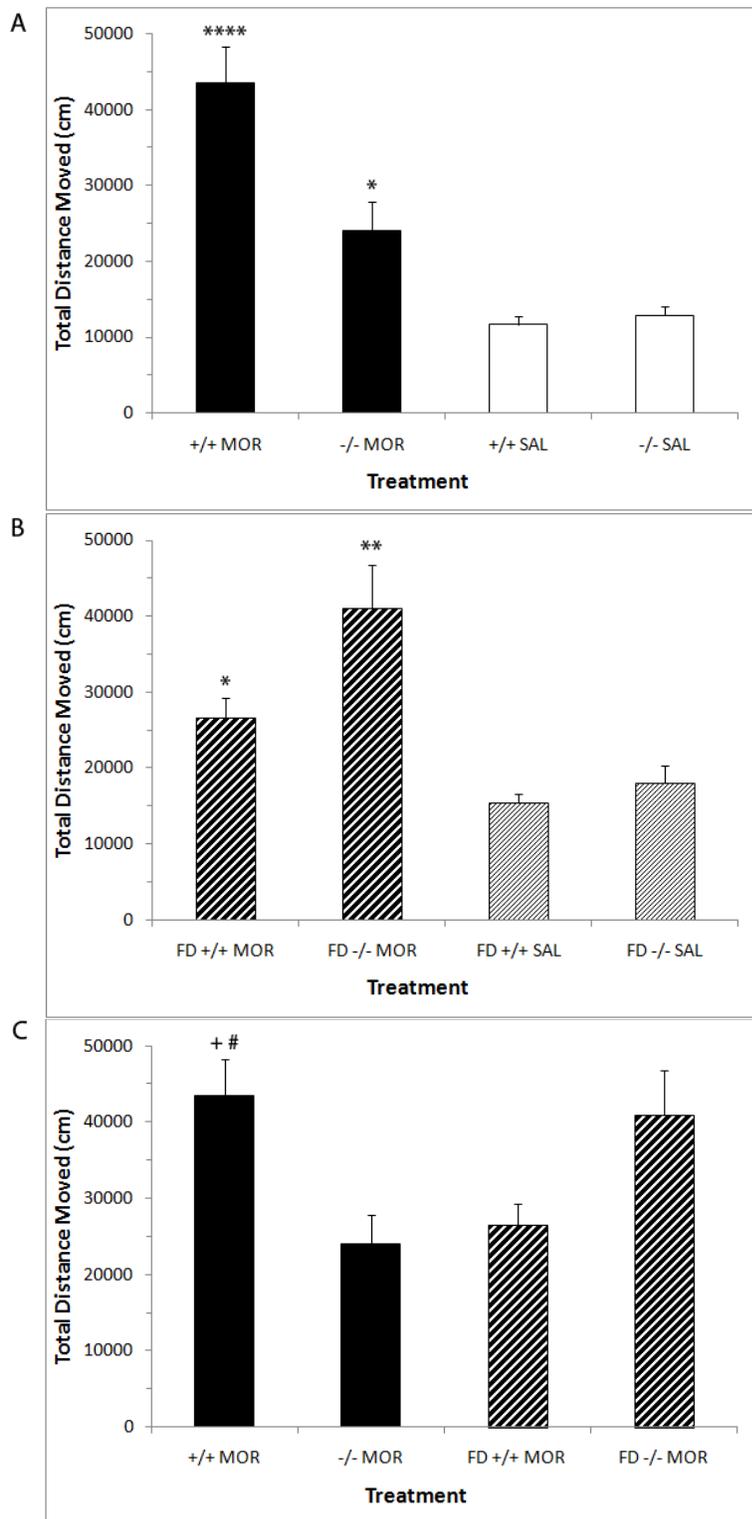


Figure 3. Reversal of morphine-induced locomotion (10 mg/kg, i.p.) after food deprivation. Total saline- and morphine-induced locomotion in (A) non-deprived M5 KO (-/-, $n_{SAL}=7$; $n_{MOR}=6$) and WT (+/+, $n_{SAL}=8$; $n_{MOR}=8$), and (B) food-deprived M5 KO (-/-, $n_{SAL}=8$; $n_{MOR}=7$) and WT (+/+, $n_{SAL}=7$; $n_{MOR}=7$) mice. (C) shows a comparison of total morphine-induced locomotion in non-deprived and food-deprived M5 KO and WT mice. * indicates significant difference between morphine- vs. saline-induced

locomotion ($p < 0.05$), ** ($p < 0.01$), **** ($p < 0.0001$). + indicates significant difference in morphine-induced locomotion between non-deprived and food-deprived WT mice ($p < 0.05$), # indicates significant difference in morphine-induced locomotion between non-deprived WT and non-deprived M5 KO mice ($p < 0.05$). Error bars represent standard errors of the mean. SAL= saline, MOR= morphine, FD= food-deprived.

Time courses for Morphine-Induced Locomotion: Non-deprived vs. Food-deprived Mice

Time courses for morphine-induced locomotion in non-deprived mice are shown in Figure 4A. Repeated measures ANOVA shows there was a significant main effect of drug treatment ($F(1, 12) = 36.016$, $p < 0.0001$), time ($F(3.965, 47.574) = 8.277$, $p < 0.0001$), genotype ($F(1, 12) = 7.785$, $p = 0.016$) and significant three way-interaction between drug treatment, time and genotype ($F(4.587, 55.040) = 2.914$, $p = 0.024$). Further analysis of individual time bins using univariate ANOVA indicates that WT mice showed more morphine-induced locomotion than M5 KO mice for all 10-minute time bins between minutes 10-90 (10-20, $F(1, 12) = 7.011$, $p = 0.021$; 20-30, $F(1, 12) = 9.699$, $p < 0.01$; 30-40, $F(1, 12) = 13.546$, $p < 0.01$; 40-50, $F(1, 12) = 19.034$, $p < 0.001$; 50-60, $F(1, 12) = 10.937$, $p < 0.01$; 60-70, $F(1, 12) = 11.541$, $p < 0.01$; 70-80, $F(1, 12) = 7.633$, $p = 0.017$; 80-90, $F(1, 12) = 5.353$, $p = 0.039$).

Time courses for morphine-induced locomotion in food-deprived mice are shown in Figure 4B. As in non-deprived animals, there was a significant main effect of drug treatment ($F(1, 12) = 21.126$, $p < 0.001$), time ($F(2.481, 29.775) = 6.908$, $p < 0.01$) and genotype ($F(1, 12) = 8.046$, $p = 0.015$), but only a two-way interaction between drug treatment and time ($F(3.138, 37.654) = 8.590$, $p < 0.0001$). Using univariate ANOVA to analyze individual time bins, a significant effect of genotype was obtained at minutes 50-60 ($F(1, 12) = 6.307$, $p = 0.027$) and 60-70 ($F(1, 12) = 5.364$, $p = 0.039$). That is, M5 KO mice showed significantly increased morphine-induced locomotion relative to WT mice after food deprivation between 50-70 minutes.

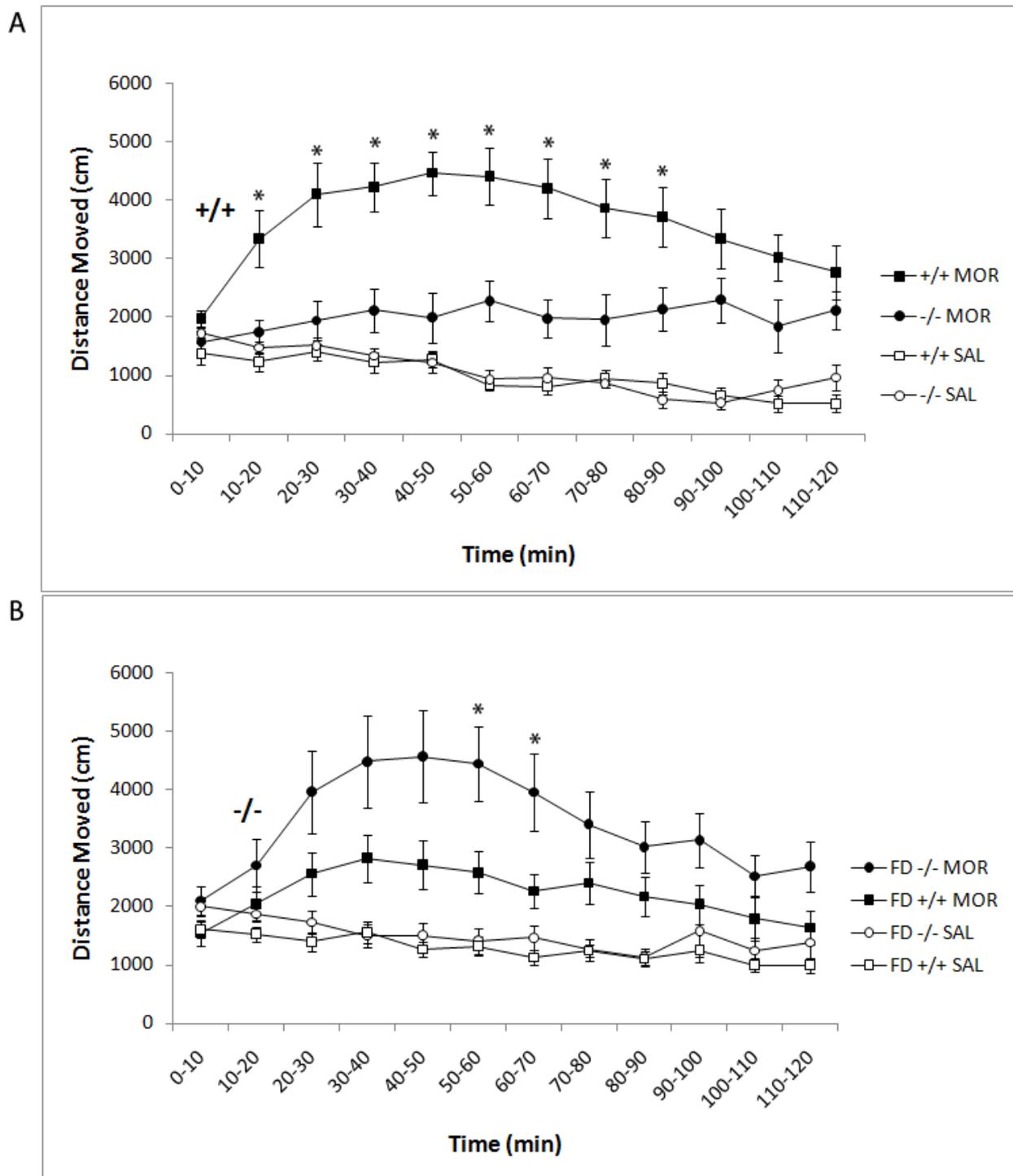


Figure 4. Time courses of saline- and morphine-induced locomotion (10 mg/kg, i.p.) in (A) non-deprived M5 KO ($-/-$, $n_{SAL}=7$; $n_{MOR}=6$) and WT ($+/+$, $n_{SAL}=8$; $n_{MOR}=8$) and (B) food-deprived M5 KO ($-/-$, $n_{SAL}=8$; $n_{MOR}=7$) and WT ($+/+$, $n_{SAL}=7$; $n_{MOR}=7$) mice. * indicates significant effect of genotype on morphine-induced locomotion ($p<0.05$). Error bars represent standard errors of the mean. SAL= saline, MOR= morphine, FD= food-deprived.

Discussion

In this study, the effects of 18 hours food deprivation on morphine-induced locomotion in M5 KO mice of the C57BL/6 strain were investigated. Non-deprived M5 KO mice show a 45% reduction in total morphine-induced locomotion compared to non-deprived WT mice. Importantly, 18 hours food deprivation elicits a reversal in morphine-induced locomotion, rescuing deficits in M5 KO mice and reducing morphine-induced locomotion in WT mice. These results show that food deprivation reduces the contribution of the M5 receptor to morphine-induced locomotion. This is consistent with previous evidence that PPT contributions to morphine CPP are reduced by food deprivation.

Food Deprivation Increases Saline-Induced Locomotion in M5 KO and WT Mice

Food-deprived M5 KO and WT mice showed greater saline-induced locomotion compared to non-deprived mice (Figure 2). Others have reported that food-restriction in conjunction with environmental novelty increases locomotion or exploratory activity in rats (Adlerstein & Fehrer, 1955; Campbell & Sheffield, 1953; Fehrer, 1956). Though all mice were handled and habituated to the testing chambers on day 1, they received no injection on habituation day. On subsequent testing days, the saline injection itself could serve as a novel stimulus to slightly increase locomotion induced by systemic saline.

Additionally, stressors such as tail shock, foot shock or restraint stress have been shown to increase accumbal DA (Abercrombie, Keefe, DiFrischia, & Zigmond, 1989; Imperato, Angelucci, Casolini, Zocchi, & Puglisi-Allegra, 1992; Kalivas & Duffy, 1995), though Pothos et al. (1995) have reported that food restriction with 20% weight loss, in fact, reduces basal extracellular DA concentrations by up to 50% in the NAc. Homegenate assays showed that underweight rats had more total extracellular and intracellular DA content in NAc. This suggests that DA is synthesized but not released from presynaptic terminals, resulting in large intracellular pools of DA in VTA. The animals used in the current study did not exceed 17% weight loss over the course of testing. According to Pothos et al. (1995), weight loss with reduced accumbal DA levels could lead to intensified food- or drug-seeking in attempts to restore extracellular DA

levels. Certainly, forward locomotion would be necessary to approach food or a drug (Steidl, 2008) and would be reflected by increased saline-induced locomotion in food-deprived mice.

Non-Deprived M5 KO Mice Show Reduced Morphine-Induced Locomotion

In agreement with recent work from our lab (Steidl & Yeomans, 2009), non-deprived M5 KO mice showed a 45% reduction in total morphine-induced locomotion relative to non-deprived WT mice (Figure 3A). Steidl & Yeomans also showed that VTA pretreatment with the muscarinic antagonist atropine, but not the nicotinic antagonist mecamylamine, reduced morphine-induced locomotion (30 mg/kg, i.p.) in WT mice of the C57BL/6 strain by 47%, similar to the reduction observed in morphine-induced locomotion for mice lacking the M5 receptor. They also found VTA atropine did not reduce morphine-induced locomotion in M5 KO mice. Furthermore, systemic haloperidol has been shown to reduce morphine-induced locomotion in mice of the ddY strain by up to 74% (Ito, Mori, & Sawaguchi, 2008). These findings with the present data suggest that VTA M5 muscarinic receptors, and not nicotinic receptors, are critically important in mediating half of locomotion induced by systemic morphine and that this component of morphine-induced locomotion is DA-dependent.

Food Deprivation Rescues Morphine-Induced Locomotion in M5 KO Mice

Importantly, 18 hours food deprivation completely rescued morphine-induced locomotion in M5 KO mice (Figure 3C), suggesting that the PPT-M5 mediated pathway for morphine-induced locomotion was bypassed. After food deprivation, morphine may activate mesolimbic DA neurons and locomotion through direct disinhibition by local GABA neurons (Johnson & North, 1992; Kalivas, Duffy, & Eberhardt, 1990). This result is in agreement with previous findings, in which food deprivation, morphine dependence and withdrawal, or VTA BDNF infusions induced a switch from a PPT-dependent to a DA-dependent opiate-reward pathway that does not require the PPT (Bechara & van der Kooy, 1992; Dockstader, Rubinstein, Grandy, Low, & van der Kooy, 2001; Nader & van der Kooy, 1997; Nader & van der Kooy, 1994). They found that these changes were transient, as alleviating doses of morphine in withdrawn rats restored the blocking effects of PPT lesions for morphine place preference (Bechara and van der Kooy, 1992). Thus, they proposed that the DA-dependent reward circuit reflected an overshadowing of an unaltered PPT-mediated pathway underlying acute opiate reward. Laviolette and van der Kooy

(2004) found that CPP induced by VTA infusions of GABA_A receptor antagonist bicuculline were blocked by PPT lesions in opiate-naïve, but not opiate dependent and withdrawn, rats. Others have reported that PPT lesions also block the rewarding effects of amphetamine, opiates and sex in drug-naïve animals (Kippin & van der Kooy, 2003; Olmstead & Franklin, 1994; Olmstead, Munn, Franklin, & Wise, 1998). More recently, dopamine blockade with α -fluenthixol was found to block the rewarding effects of VTA bicuculline in rats that previously received infusions of BDNF into the VTA (Vargas-Perez et al., 2009). These findings suggest that the switch from a PPT-dependent to a DA-dependent pathway for opiate reward is mediated by GABA_A receptors on local VTA GABA neurons.

Food Deprivation Reverses Morphine-Induced Locomotion in WT Mice

The effects of food deprivation in the current study are not one directional, but rather induce a reversal of morphine-induced locomotion. Here, food-deprived WT mice showed a 39% decrease in morphine-induced locomotion compared to non-deprived WT mice (Figure 3C). In non-deprived mice, Steidl (2008) found that accumbal DA release induced by VTA morphine was blocked either by pretreatment with VTA scopolamine in WT mice or in M5 KO mice. This suggests that under normal circumstances, where mice have not experienced food deprivation or weight loss, the PPT-M5 pathway solely mediates morphine-induced DA activation. In comparison with the current results, it appears that food deprivation leads to the activation of a PPT-independent, but DA-dependent, pathway for morphine-induced locomotion as seen with the rescue of food-deprived M5 KO locomotion, but is not sufficient to compensate for the loss of the main PPT-M5 pathway mediating DA activation.

Since homozygous mutants lack the M5 receptor from birth, these mice may experience chronic understimulation of PPT-M5-mediated DA activation and therefore, show a variety of behavioural drug- and reward-related deficits (Basile, et al., 2002; Steidl & Yeomans, 2009; Wang, Liang, Burgdorf, Wess, & Yeomans, 2008). This chronic understimulation could lead to the development of a hypersensitive mesolimbic DA system. For example, DA deficient mice are 3-13 times more sensitive to the activating effects of L-dopa on spontaneous locomotion and 3-6 times more sensitive to the locomotor stimulating effects of SKF 81297 (D1 receptor agonist) or quinpirole (D2 receptor agonist) (Kim, Szczypka, & Palmiter, 2000; Zhou & Palmiter, 1995). Similarly, food deprivation could induce a switch to activate a hypersensitive PPT-M5-

independent pathway for VTA DA activation in M5 KO mice, leading to rescued morphine-induced locomotion. Therefore, the M5-independent pathway for VTA DA activation may provide a more important contribution to morphine-induced locomotion for M5 KO than WT mice. Based on the findings discussed above and the current results, the balance of cholinergic input to VTA DA neurons, which is mediated by the M5 receptor, appears to be shifted by the same motivational manipulations used by van der Kooy and his group to produce a reversal in morphine-induced locomotion. Moreover, the current results extend previous findings to establish a link between DA-independent pathways (Nader et al., 1997) and PPT-M5 mediated pathways for DA activation and morphine-induced locomotion (Steidl, 2008; Steidl & Yeomans, 2009). Figure 5 summarizes discussed and proposed pathways for morphine-induced locomotion and reward.

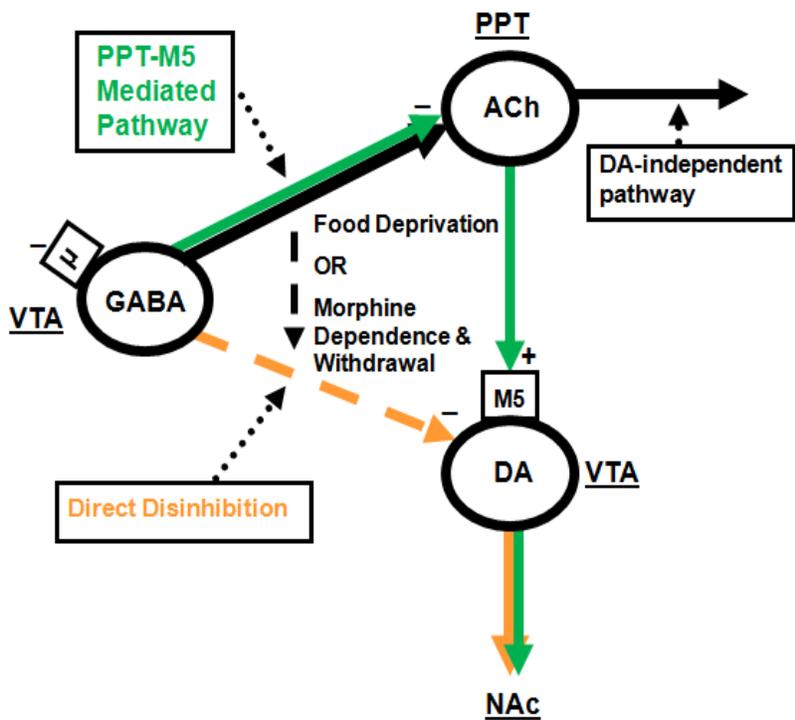


Figure 5. Model of neural circuits involved in opiate reward. Acutely administered morphine activates inhibitory μ -opioid receptors on GABA neurons in VTA, leading to disinhibition of PPT-M5 receptor mediated (green, Steidl, 2008) and DA-independent (black, Nader et al., 1997) reward pathways in drug-naïve or non-deprived animals. After food deprivation or morphine dependence and withdrawal, GABA neurons directly disinhibit VTA DA neurons (orange, Nader et al., 1997), bypassing PPT and M5 receptors to restore morphine-induced locomotion in M5 KO mice.

Time Courses for M5 KO Mice May Reveal PPT-mediated, DA-independent Contributions to Morphine-induced Locomotion

Steidl & Yeomans (2009) found that M5 KO C57BL/6 mice showed peak levels of morphine-induced locomotion (10 mg/kg, i.p.) between 40 and 50 minutes that reduced almost back to baseline by the end of the second hour of testing. In contrast, they found that M5 KO mice of the same strain showed delayed increases in locomotion induced by systemic morphine at the highest dose tested (30 mg/kg). Furthermore, this rise in locomotion did not peak or fall back to near baseline by the end of the two hour test.

In the present study, non-deprived WT mice showed increases in morphine-induced locomotion (10 mg/kg, i.p.), which peaked at approximately 45 minutes and steadily decreased, albeit not to baseline levels, for the remainder of the test (Figure 3A). Consistent with previous data for morphine-induced locomotion at 30, but not 10, mg/kg (Steidl & Yeomans, 2009), non-deprived M5 KO mice showed delayed increases in morphine-induced locomotion that did not peak, but remained steady for the 2 two hours of testing (Figure 3A). In contrast to non-deprived M5 KO mice, food-deprived M5 KO mice showed faster increases in morphine-induced locomotion, to levels similar to non-deprived WT mice (Figure 3B). However, morphine-induced locomotion in food-deprived M5 KO mice dropped more rapidly than in non-deprived WT mice, after peaking at approximately 45 minutes. Considering that morphine-induced accumbal DA efflux is blocked in M5 KO mice (Steidl, 2008) but presumably rescued after food deprivation, the current data suggests that the small, delayed increase in morphine-induced locomotion in non-deprived M5 KO mice and the greater reduction in morphine-induced locomotion after 45 minutes in food-deprived M5 KO mice may be due to a DA-independent mechanism.

Nader and van der Kooy (1994) proposed that in non-deprived rats, opiate reward transmission occurs through a PPT-mediated pathway which is independent of DA. This DA-independent pathway, as discussed earlier, can be bypassed by 18 hours food deprivation. Comparison with the present results further suggests that the delayed increase in morphine-induced locomotion in non-deprived M5 KO mice may be both DA-independent and mediated by the PPT.

Motivational Considerations and Alternative Explanations for Reduced Morphine-Induced Locomotion in Food-deprived WT Mice

From a motivational perspective, it is unclear why activation of a direct disinhibition pathway for DA activation would lead to less morphine-induced locomotion in WT mice. One might expect that a food-deprived animal would be in a motivational state conducive to exploration and forward movement towards food. Feeding behaviours are closely linked with DA, as accumbal DA increases in response to food (Schilström, Svensson, Svensson, & Nomikos, 1998), DA antagonists decrease feeding in rats (Wise & Raptis, 1986) and DA deficient mice show severe deficits in food intake (Szczycka, et al., 2001; Szczycka, et al., 1999). Food deprivation with 20% weight loss is known to enhance the rewarding or stimulant effects of a variety of drugs including cocaine (Bell, Stewart, Thompson, & Meisch, 1997), amphetamine (Cabeza de Vaca & Carr, 1998) and morphine (Deroche, Piazza, Casolini, Le Moal, & Simon, 1993). Additionally, a variety of drugs that modulate the mesolimbic DA system are known to dose-dependently affect feeding in rats. For example, low doses of DA agonist amphetamine increase food-intake (0.25 mg/kg, i.p.) but do not increase locomotion, while higher doses (1-2 mg/kg, i.p.) have anorexic effects in rats and stimulate amphetamine-induced locomotion (Evans & Vaccarino, 1986, 1990). Similarly, low doses of morphine (2.0 mg/kg) have been shown to increase food intake, while higher exposures dose-dependently decrease feeding (10-30 mg/kg) in rats (Marks-Kaufman & Kanarek, 1980). Evans and Vaccarino suggest that drugs such as amphetamine somehow selectively potentiate feeding or locomotor behaviours depending on the dose of the drug. In the present study, the morphine dose used (10 mg/kg, i.p.) is associated with the anorexic properties of the drug in rats. Perhaps food-deprived WT mice responded to the anorexic effects rather than the locomotor inducing effects of morphine at the 10 mg/kg dose. Furthermore, Nader and van der Kooy (1994) observed that after 18 hours food deprivation rats show CPP for morphine, suggesting that morphine can substitute for the motivational aspects of food. If systemic exposure to morphine in food-deprived WT mice alleviated the motivational properties of food, then a decreased motivation for food could be reflected in the reduction of morphine-induced locomotion seen in WT mice. In other words, an animal with no motivation to find food does not have the impetus for approach behaviours or forward movement.

An alternative explanation is that reduced locomotion in food-deprived WT mice may reflect energy conservation in response to starvation. Weight-restricted rats have shown reduced basal levels of locomotion (Pothos, Creese, & Hoebel, 1995) and severe food deprivation results in reduced energy expenditure in part due to decreased spontaneous activity in rats (Even & Nicolaïdis, 1993). This explanation is unlikely, however, for two reasons. First, food-deprivation was found to significantly increase saline-induced locomotion for mice of both genotypes. Secondly, food-deprived M5 KO mice showed a rescue of morphine-induced locomotion, to levels similar for non-deprived WT mice.

Other Neurochemical Systems Affect the Interaction between Food and Drugs

The interaction between food deprivation and drug effects is complex. Thus, it is important to take into consideration the involvement of other neurochemical systems. Food restriction in rats has been shown to increase plasma corticosterone by 200-400% (Broocks, Schweiger, & Pirke, 1990). Corticosterone has also been shown to be important in the effects of food restriction or deprivation on drug-induced locomotion and reinstatement of drug-seeking. Food restriction to 80% of initial body-weight sensitizes rats to the psychomotor effects of both amphetamine and morphine, but not in animals with blocked corticosterone secretion by adrenalectomy (Deroche, Piazza, Casolini, Le Moal, & Simon, 1993). Moreover, reinstatement of cocaine seeking induced by acute food deprivation (21 hours) is blocked in rats with bilateral adrenalectomy (Shalev, Marinelli, Baumann, Piazza, & Shaham, 2003). There is increasing evidence that other hormones including insulin and leptin regulate feeding behaviours through direct modulation of VTA DA neurons (reviewed by Palmiter, 2007). Both leptin and insulin receptors are found on VTA DA neurons and intracerebroventricular (i.c.v.) insulin increases mRNA for DA transporter (DAT) in VTA and SN, which would presumably facilitate removal of DA at the synapse to reduce DA activation (Figlewicz, Evans, Murphy, Hoen, & Baskin, 2003; Figlewicz, Szot, Chavez, Woods, & Veith, 1994). I.c.v. administration of insulin or leptin also reverses CPP for sucrose or high fat foods in rats (Figlewicz, Bennett, Evans, Kaiyala, Sipols, & Benoit, 2004; Figlewicz, Higgins, Ng-Evans, & Havel, 2001), thereby modulating the motivational aspects of food reward. Finally, food deprivation-induced reinstatement of heroine

seeking is reduced after i.c.v. insulin (Shalev, Yap, & Shaman, 2001), providing evidence that hormones involved in feeding may also be involved in the motivational and rewarding effects of drugs. Therefore, the interactions between DA and other hormonal systems must be examined before the relationship between food deprivation and drug-induced behaviours can be fully understood.

Conclusions

The data presented here suggest that the M5 muscarinic receptor, as shown previously (Steidl & Yeomans, 2009), is important for approximately half of the locomotor stimulating effects of acutely administered morphine in non-deprived mice of the C57BL/6 strain. Additionally, food deprivation effects on opiate reward generalize to morphine-induced locomotion and produce a reversal in morphine-induced locomotion in M5 KO and WT mice of the C57BL/6 strain. Finally, a component of morphine-induced locomotion in non-deprived M5 KO mice may be mediated by a PPT-dependent pathway that is independent of DA, as seen for opiate reward in non-deprived rats (Nader and van der Kooy, 1992).

Acknowledgements

We thank Dr. Derek van der Kooy for suggesting acute food deprivation as a novel way to test morphine-induced locomotion in mice and for providing technical assistance.

Experiment 2: Effects of VTA BDNF on Open-Field Morphine-Induced Locomotion in Wild-type and M5 Muscarinic Receptor Knockout Mice

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Abstract

Cholinergic inputs from the mesopontine tegmentum mediate midbrain dopamine (DA) activation via M5 muscarinic receptors. M5 receptors are crucial for mesopontine stimulation-induced accumbal or striatal DA release, brain stimulation reward and conditioned place preference (CPP) to opiates. M5 muscarinic receptor knockout (KO) mice show a 40-50% reduction in systemic morphine-induced locomotion (Steidl & Yeomans, 2009). Furthermore, many studies show that pedunclopontine tegmental nucleus (PPT) lesions block morphine CPP in rats, but not after 18 hours food deprivation or VTA infusions of brain-derived neurotrophic factor (BDNF) (Nader, Bechara, & van der Kooy, 1997; Vargas-Perez et al., 2009). The aim of this study was to determine whether VTA BDNF infusions alter the locomotor stimulant effects of opiates. Open-field locomotion induced by systemic morphine (3 and 10 mg/kg, i.p.) was measured in M5 KO mice of the C57BL/6 strain. Mice that received control infusions to VTA showed no genotype differences for morphine-induced locomotion at either the 3 or 10 mg/kg dose. This result does not agree with previous findings that the M5 receptor mediates up to 50% of morphine-induced locomotion (10 mg/kg) in mice. Additionally, at both the 3 and 10 mg/kg dose, neither VTA BDNF infusions nor genotype had an effect on spontaneous locomotion, saline-induced locomotion or morphine-induced locomotion. These results are also in disagreement with previous studies showing opiate reward pathways do not depend on the PPT following bilateral VTA infusions of BDNF.

Introduction

Opiate-induced locomotion in rats is mediated through both dopamine (DA)-dependent and -independent pathways. Infusions of enkephalin analog, D-Ala2-Met5-enkephalinamide (DALA) into ventral tegmental area (VTA) increase locomotion which is blocked by nucleus accumbens (NAc) fluphenazine pre-treatment (Kalivas, Widerlöv, Stanley, Breese, & Prange, 1983). However, locomotion induced by NAc DALA is not blocked by either fluphenazine treatment or 6-hydroxydopamine (6-OHDA) lesions. Furthermore, neither systemic α -flupenthixol nor 6-OHDA lesions affects locomotion induced by systemic heroin (Vaccarino, Amalric, Swerdlow, & Koob, 1986).

In contrast, opiate-induced locomotion in mice appears to depend more on DA. For example, morphine-induced locomotion is reduced after systemic haloperidol (Ito, Mori, & Sawaguchi, 2008) or in DA-deficient mice (Hnasko, Sotak, & Palmiter, 2005). Additionally, concomitant measurement of accumbal DA efflux using microdialysis and locomotion after systemic morphine (3mg/kg) show that out of three strains tested (C57BL/6, 129Sv, DBA2), C57BL/6 mice showed the most morphine-induced DA release and locomotion (Murphy, Lam, & Maidment, 2001).

The pedunculo-pontine tegmental nucleus (PPT) and laterodorsal tegmental nucleus (LDT) provide cholinergic and glutamatergic inputs crucial for DA activation in the VTA and substantia nigra (Oakman, Faris, Kerr, Cozzari, & Hartman, 1995; Omelchenko & Sesack, 2005). Electrical stimulation of the LDT or PPT increases accumbal and striatal DA efflux, respectively (Forster, Yeomans, Takeuchi, & Blaha, 2001; Miller, Forster, Yeomans, & Blaha, 2005). In rats, prolonged accumbal or striatal DA efflux is blocked by scopolamine into VTA or SN (Forster & Blaha, 2000; Forster & Blaha, 2003). This line of evidence indicates that PPT and LDT are critical for activation of midbrain DA systems, mainly via muscarinic receptors in VTA and SN.

The M5 receptor is the only muscarinic subtype importantly involved in midbrain DA activation (Steidl, 2008). Midbrain DA neurons only show mRNA expression for one of the five muscarinic receptor subtypes—the M5 muscarinic receptor (Reever, Ferrari-DiLeo & Flynn, 1997; Vilaro, Palacios & Mengod, 1990; Weiner, Levey & Brann, 1990). Moreover, in mice of the CD1x129 strain, prolonged LDT-evoked accumbal dopamine release is blocked by systemic

scopolamine or in mice lacking the M5 receptor (Forster, Yeomans, Takeuchi, & Blaha, 2001), suggesting that long-lasting accumbal DA efflux is solely mediated by the M5 receptor subtype.

Cholinergic innervations of VTA are also necessary for mediating the effects of opiates in rats and mice. Lesions of the PPT (Miller, Forster, Metcalf, & Blaha, 2002) or LDT (Forster, Falcon, Miller, Heruc, & Blaha, 2002) in rats attenuate striatal or accumbal DA release, respectively, after intravenous morphine. Intra-VTA morphine increases in accumbal DA are blocked after VTA scopolamine or in mice lacking the M5 muscarinic receptor (Steidl, 2008). Basile et al. (2002) found that M5 knockout (KO) mice show less accumbal DA release after systemic morphine. Importantly, C57BL/6 mice given VTA muscarinic, but not nicotinic, antagonists or mice lacking the M5 receptor show 40-50% reductions in systemic morphine-induced locomotion (Steidl & Yeomans, 2009). Additionally, M5 KO mice show reduced cocaine self-administration (Fink-Jensen et al., 2010; Thomsen, Woldbye, Wörtwein, Fink-Jensen, Wess, & Caine, 2005), emit less ultrasonic vocalizations (USVs) in response to mating and female urine (Wang, Liang, Burgdorf, Wess, & Yeomans, 2008) and show reduced morphine CPP (Basile et al., 2002). This suggests that while morphine-induced DA activation depends entirely on the M5 receptor, morphine-induced behaviours such as locomotion are mediated only in part by the M5 receptor.

Pathways for opiate reward can be altered, depending on the animal's prior drug experience. In drug-naïve rats, PPT lesions, but not α -flupenthixol, block CPP to systemic morphine (Bechara & van der Kooy, 1992; Nader & van der Kooy, 1997). However, in morphine or heroin dependent and withdrawn rats, α -flupenthixol, but not lesions of the PPT block systemic or VTA morphine CPP (Bechara & van der Kooy, 1992; Nader & van der Kooy, 1997). Recently, VTA BDNF has been shown to induce motivational changes similar to that of opiate dependence and withdrawal. While in vehicle infused rats, PPT lesions block morphine CPP, PPT lesions do not block morphine CPP in VTA BDNF infused animals (Vargas-Perez, et al., 2009). This work suggests that morphine reward is mediated through PPT in drug-naïve or vehicle infused animals, but PPT is not necessary for morphine reward after opiate dependence with withdrawal or infusions of BDNF into the VTA. Both CPP and the acute locomotor activating effects of morphine in non-deprived rats and mice critically depend on the PPT.

Therefore, we want to determine whether food-deprivation effects that alter sensitivity of morphine CPP can similarly alter the locomotor-stimulating effects of morphine.

The aim of this study was to investigate whether cholinergic input to VTA DA can be shifted by VTA BDNF, consistent with past work on morphine CPP (Nader, Bechara, & van der Kooy, 1997). To do this, we infused BDNF into the VTA of both wildtype (WT) and M5 KO mice of the C57BL/6 strain and subsequently tested for morphine-induced locomotion. According to Nader et al. (1997), VTA BDNF should alter pathways mediating the locomotor stimulating effects of opiates to bypass the PPT. Therefore, the M5 receptor should also become less important in mediating opiate-induced locomotion.

Methods

Mice

M5 muscarinic receptor mutant mice were created on a mixed SvJ x CD1 background by methods described by Takeuchi et al., 2002. Mice were maintained in homozygous WT and M5 KO breeding colonies and backcrossed on the C57BL/6 strain for 6 generations to create a C57BL/6 background (Gerlai, 1996). In all experiments, litters were aged matched at 2-4 months at the start of testing.

A total of 49 mice were used (24 WT, and 25 M5 KO). For this experiment, mice were group housed (2-6 per cage) and maintained on a 12 hour light/dark cycle (lights on 7:00 AM-PM). Locomotion testing occurred between 10:00 AM and 5:00 PM. Food and water was provided *ad libitum* except during behavioural testing. Prior to experimentation, mice were relocated from the main colony to a housing room adjoining the testing room for one week. Temperature ($20 \pm 1^\circ\text{C}$) and humidity (55-60%) was regulated for both the housing and experimental rooms. This study was approved by the University of Toronto Animal Care Committee.

Surgery

Mice were anesthetized with inhaled isoflurane (4% to induce and 2-3% to maintain anesthesia) and mounted in a stereotaxic frame (MyNeuroLab, St. Louis, MO). Body

temperature was maintained at 37° C with a temperature-regulated heating pad (TC-1000; CWE Inc., New York, NY). For BDNF microinjections, 10µl Hamilton syringes (Hamilton, Reno, NV) were used. Tygon tubing (0.1905 mm diameter) was attached to 30 gauge injectors which were fed through 23 gauge guide cannulae. BDNF was reconstituted in sterile 0.9% saline (0.5µg/µl) and was infused bilaterally into the VTA at 0.5µl per side using the following coordinates relative to bregma at 10° angles: AP -3.54 mm, ML ±1.18 mm and DV -4.15 mm from the dura. Following surgery, mice were individually housed and allowed one week for recovery prior to behavioural testing.

Locomotion Testing Apparatus, Protocol and Data Acquisition

The open-field apparatus used, for measuring locomotion, was as described by Steidl and Yeomans (2009). Locomotion testing occurred between 10:00 AM and 5:00 PM. Each mouse was randomly assigned to a 31x31x31 cm black chamber (University of Toronto, Department of Cell & Systems Biology Workshop). For all experiments, WT and KO mice were tested together and counterbalanced for drug treatment. Mice were recorded with a video camera (Panasonic, Model #WV-CP484, Osaka, Japan) placed approximately 2.1 m above the testing apparatus. The testing room was lit by two 40-watt light bulbs located approximately 2.7 m above testing chambers.

Locomotor testing occurred over 3 consecutive days. On day 1 (habituation day) mice were weighed and placed in the testing room for 20 minutes, then put in the testing chambers for 3 hours without receiving any injections. For the next two days, M5 KO and WT mice were randomly assigned to two groups: 1) VTA-vehicle and saline/ morphine drug treatment, 2) VTA-BDNF and saline/morphine drug treatment. Administration of saline and morphine (10 ml/kg and 3 or 10 mg/kg, i.p., respectively) was counterbalanced on day 2 and 3 so half the mice were exposed to saline on day 2 and morphine on day 3, and vice versa. After mice were injected, they were placed in the center of their randomly assigned testing chambers and locomotion was recorded for 3 hours. All locomotion data was analyzed with Noldus Ethovision tracking software (Wageningen, Netherlands).

It should be noted that morphine-induced locomotion for 30 mg/kg (i.p.) was also tested before and after VTA BDNF. However, this high dose of morphine elicited stereotypy (e.g.

circling) in some mice, making locomotion data difficult to interpret. Therefore, locomotion data for 30 mg/kg morphine (i.p.) is not presented here.

Histology

Immediately after testing, animals were deeply anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused with 20 ml 0.9% saline and 20 ml of 4% paraformaldehyde solution. Brains were removed and stored in a 30% sucrose/10% formalin solution overnight prior to sectioning using a Cryostat. Sections were mounted and examined by light microscopy following cresyl violet staining.

Drugs

For these experiments, brain-derived neurotrophic factor (BDNF) and morphine sulphate pentahydrate were obtained from Sigma-Aldrich (St. Louis, MO). All drugs were dissolved in sterile 0.9% saline. Intraperitoneal injections of morphine were given at a dose of 3 or 10 mg/kg and at a volume of 10 ml/kg.

Statistical Analysis

For time courses and total locomotion, only data acquired from mice with bilateral VTA placements were used for statistical analysis.

For locomotion testing, time course data was analyzed separately for VTA saline (SAL) and VTA BDNF-infused C57BL/6 M5 KO and WT mice using a repeated measures ANOVA with distance moved as the dependent variable, drug (saline or morphine) and time (9, 20-minute time bins from 0-180 minutes) as repeated within-subject factors and genotype as the between-subject factor. Assumed sphericity was tested for each analysis and when violated, degrees of freedom for within-subject factors and interactions involving within-subject factors were adjusted with Greenhouse-Geisser adjustments (for all cases, epsilon was less than 0.75). Post hoc tests were not possible because there were less than 3 groups for the between-subject factor (genotype).

Total spontaneous locomotion during habituation and morphine-induced locomotion was analyzed separately for VTA SAL and VTA BDNF-infused M5 KO and WT mice using a 2-way univariate ANOVA, with distance moved as the dependent variable and genotype and drug

(saline or morphine) as fixed factors. Post hoc test results were not obtained because between-subject factors contained less than 3 groups.

Results

Histology

Bilateral VTA injection sites in M5 KO and WT mice for the 3 and 10 mg/kg morphine groups are shown in Figure 6. Cannulae tip placements were considered hits if bilaterally located within the anatomical boundaries of the VTA. Some placements that lay outside the boundaries of the VTA were considered hits if tips were located less than 0.5 mm from VTA, since intercranial BDNF microinjections do not diffuse more than approximately 0.5 mm from the infusion site (Shirayama, Chen, Nakagawa, Russell, & Duman, 2002). Unilateral hits and bilateral misses were excluded from data analysis.

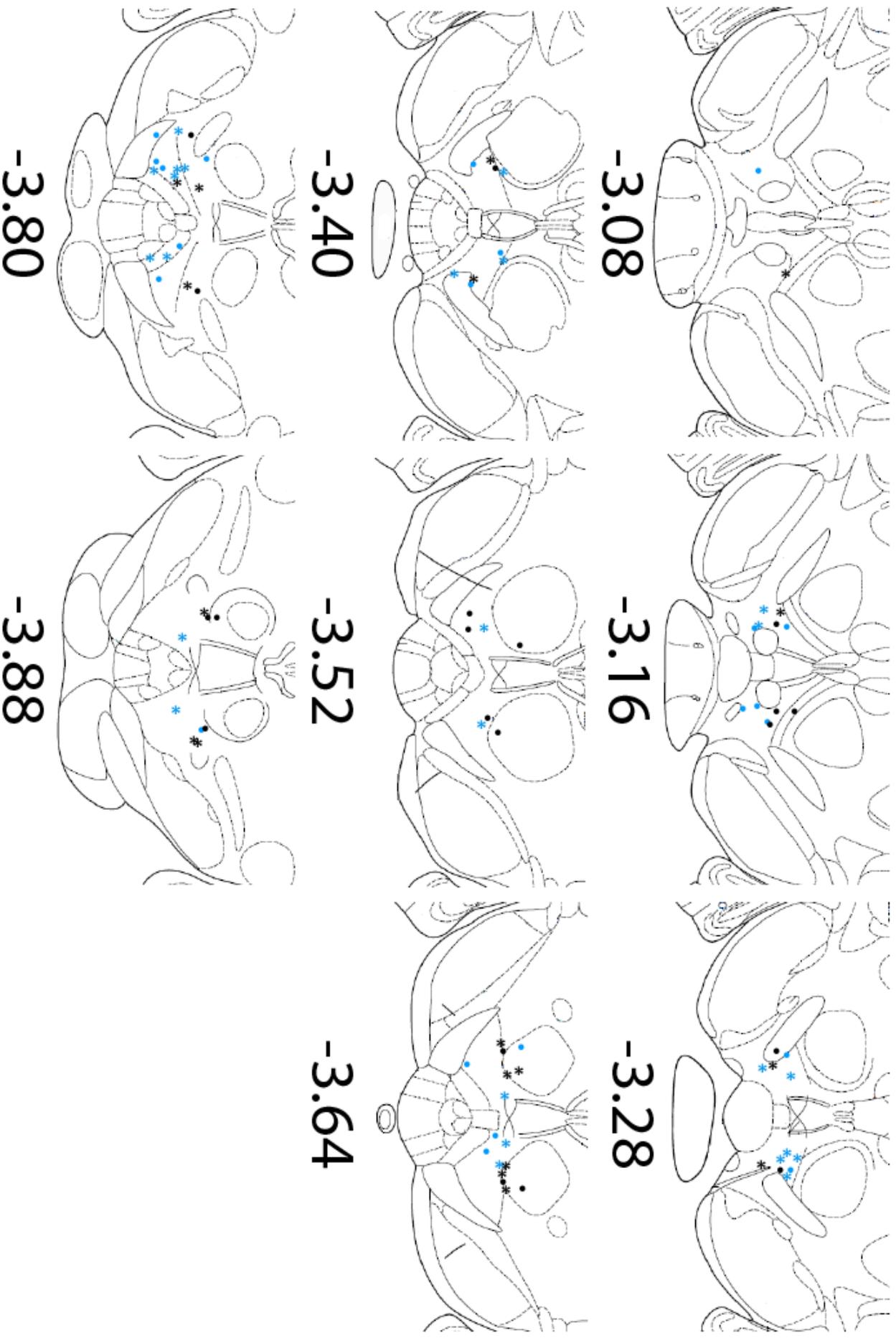


Figure 6. VTa cannula placements in M5 KO (black asterisks, n=9) and WT mice (black circles, n=10) in the 3 mg/kg morphine group and M5 KO (blue asterisks, n=13) and WT mice (blue circles, n=11) in the 10 mg/kg group. Numbers below sections denote rostro-caudal distances from bregma (adapted from mouse brain atlas by Paxinos and Franklin, 2001).

3 mg/kg Morphine

Spontaneous Locomotion

Time courses for spontaneous locomotion are shown in Figure 7. Univariate ANOVA indicates there was a significant effect of time ($F(3.060, 48.956) = 59.695, p < 0.00001$), but not of genotype or treatment. This result indicates that both M5 KO and WT mice after both VTA SAL and BDNF infusions did not respond differently to a novel environment.

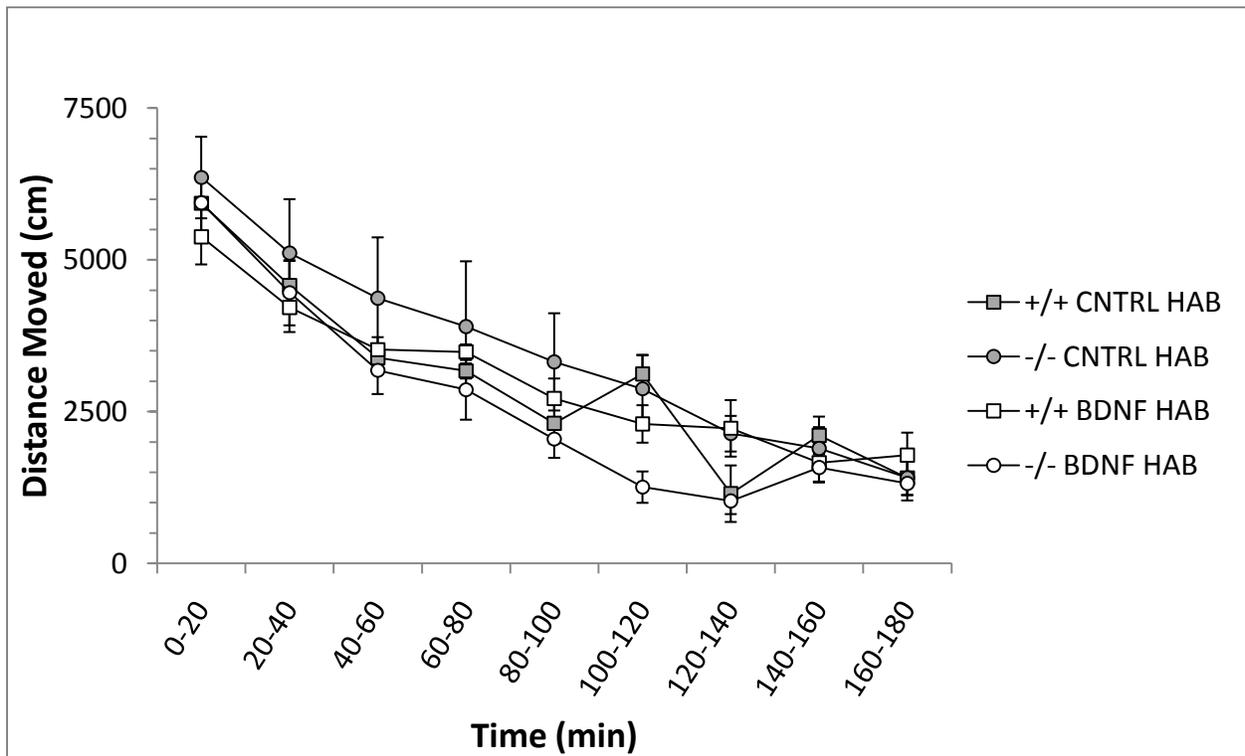


Figure 7. Time courses of spontaneous locomotion in VTA BDNF-infused M5 KO (-/-, n=6) and WT (+/+, n=6) mice as well as VTA SAL-infused M5 KO (-/-, n=3) and WT mice (+/+, n=3). There was a significant main effect of time on spontaneous locomotion ($p < 0.00001$), but no significant effect of genotype or treatment. CNTRL=control (VTA SAL), SL=spontaneous locomotion. Bars represent standard errors of the mean.

Saline- and Morphine-Induced Locomotion

Time courses for saline- and morphine-induced locomotion in M5 KO and WT mice after VTA SAL and BDNF are shown in Figure 8A and B. Repeated measures ANOVA found for both groups that there was a significant effect of drug (for VTA SAL: $F(1,5) = 35.405, p < 0.01$;

for VTA BDNF: $F(1,8)= 20.012, p<0.01$) and time (for VTA SAL: $F(8,40)= 8.787, p<0.00001$; for VTA BDNF: $F(8,64)= 17.590, p<0.00001$), but no effect of genotype.

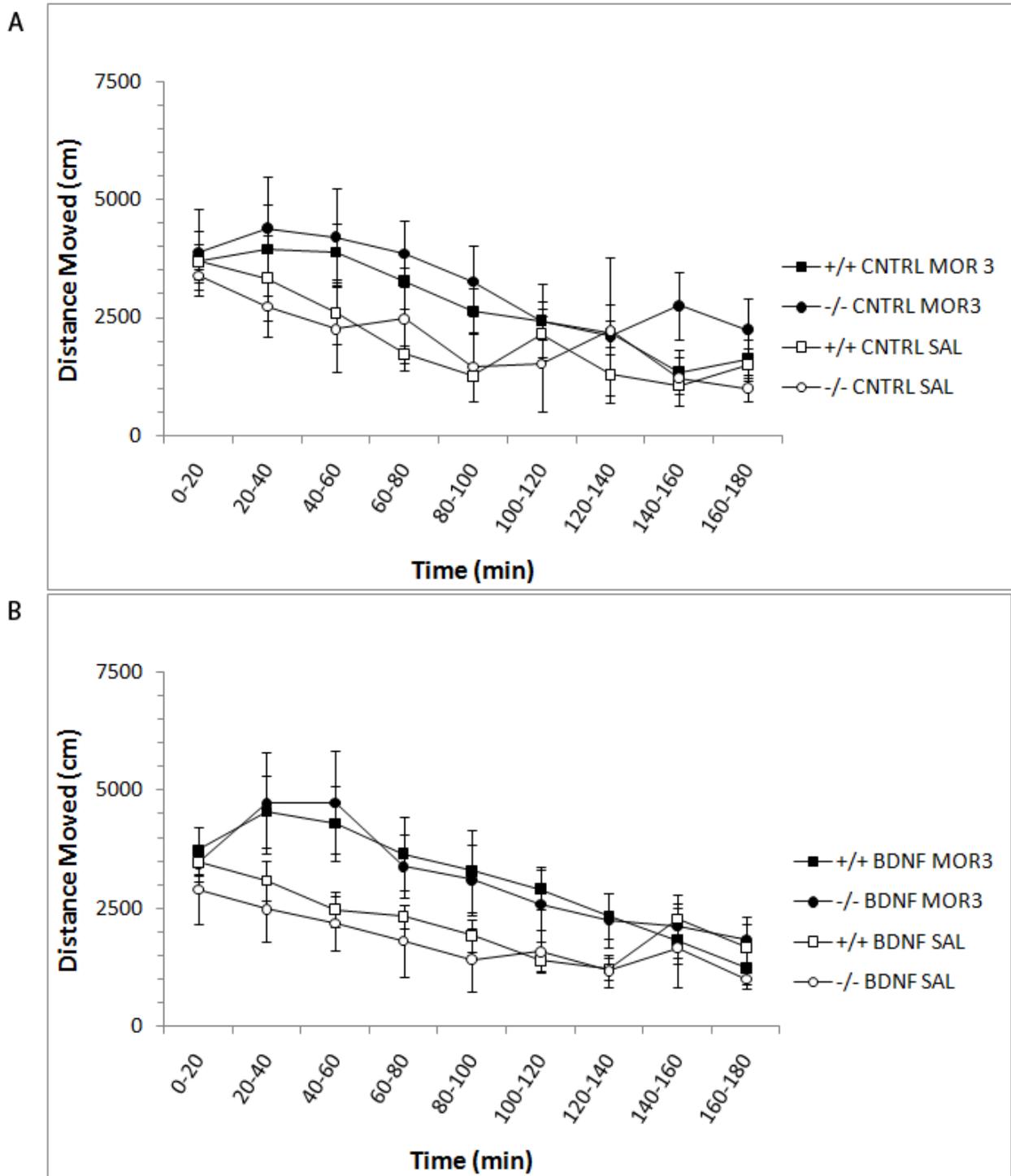


Figure 8. Time courses of saline- and morphine-induced locomotion (3 mg/kg, i.p.) in (A) VTA SAL-infused M5 KO (-/-, n=3) and WT (+/+, n=3) mice and (B) VTA BDNF-infused M5 KO (-/-, n=6) and WT (+/+, n=6) mice. There was a significant effect of drug (saline vs. morphine) and time ($p<0.01$ for all

comparisons) as well as an interaction between drug and time. There was no significant effect of genotype on saline- or morphine-induced locomotion. SAL=saline, MOR10= morphine (10 mg/kg, i.p.). Bars represent standard errors of the mean.

Total morphine-induced locomotion for both VTA BDNF and VTA SAL-infused M5 KO and WT mice was analyzed using univariate ANOVA and is shown in Figure 9. There was no effect of either genotype or treatment (VTA SAL vs VTA BDNF) on morphine-induced locomotion.

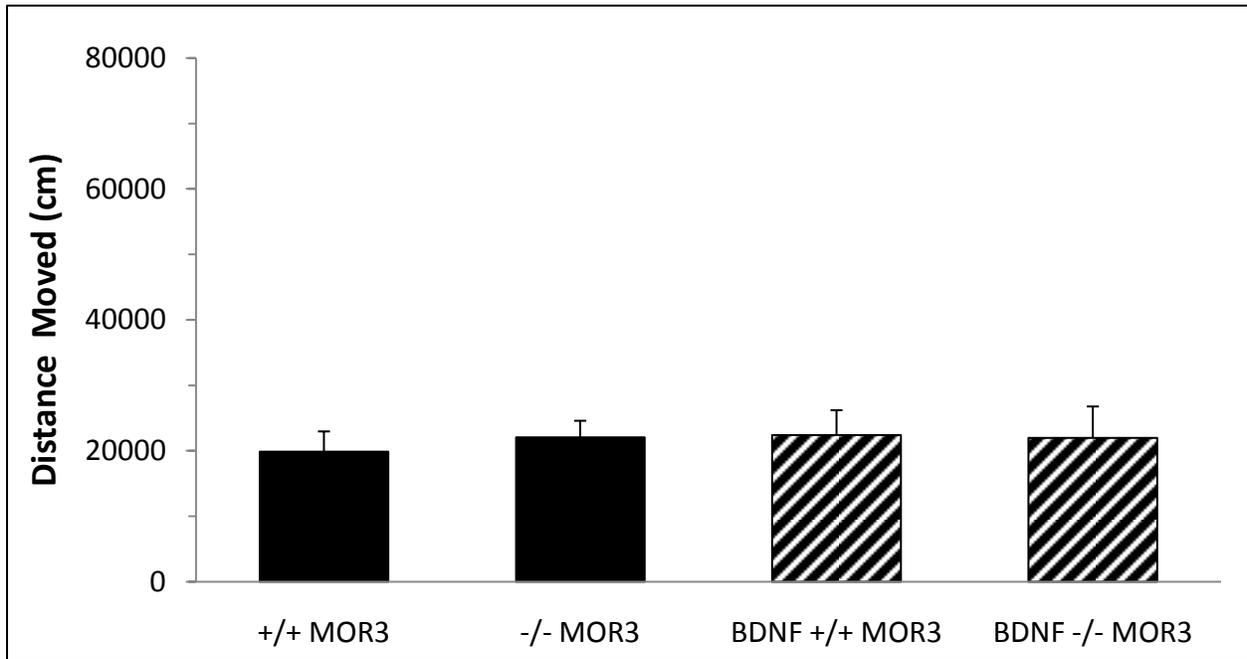


Figure 9. Total morphine-induced locomotion (3 mg/kg, i.p.) in VTA SAL M5 KO (-/-, n=3) and WT (+/+, n=3), and VTA BDNF-infused M5 KO (-/-, n=6) and WT (+/+, n=6) mice. There was no significant effect of either genotype or treatment on total morphine-induced locomotion. MOR10=morphine (10 mg/kg, i.p.). Bars represent standard errors of the mean.

10 mg/kg Morphine

Spontaneous Locomotion

Time courses for spontaneous locomotion are shown in Figure 10. Univariate ANOVA indicates there was a significant effect of time ($F(2.820, 42.306) = 58.962, p < 0.00001$), but not of genotype or treatment. This indicates that both M5 KO and WT mice did not respond differently to a novel environment.

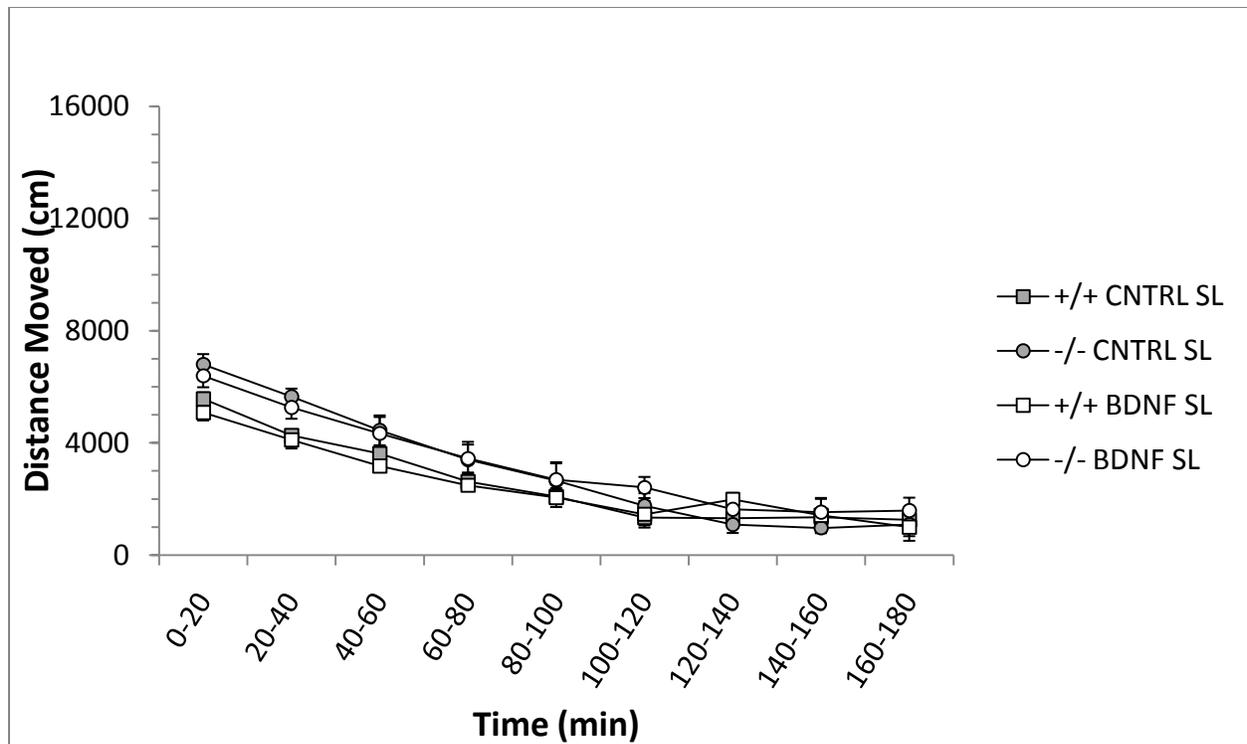


Figure 10. Time courses of spontaneous locomotion in VTA BDNF-infused M5 KO (-/-, n=6) and WT (+/+, n=5) mice as well as VTA SAL-infused M5 KO (-/-, n=7) and WT mice (+/+, n=6). There was a significant main effect of time on spontaneous locomotion, but no significant effect of genotype or treatment. CNTRL=control (VTA SAL), SL=spontaneous locomotion. Bars represent standard errors of the mean.

Saline- and Morphine-Induced Locomotion

Time courses for saline- and morphine-induced locomotion in M5 KO and WT mice after VTA SAL and BDNF are shown in Figure 11A and B. For both treatments, repeated measures ANOVA found there was a significant effect of drug (for VTA SAL: $F(1,7)= 42.411$, $p<0.001$; for VTA BDNF: $F(1,3)= 10.825$, $p<0.05$) and time (for VTA SAL: $F(8,56)= 17.833$, $p<0.00001$; for VTA BDNF: $F(8,24)= 8.072$, $p<0.0001$) and a significant interaction between drug and time (for VTA SAL: $F(8,56)= 16.108$, $p<0.00001$; for VTA BDNF: $F(8,24)= 3.287$, $p<0.05$), but no effect of genotype.

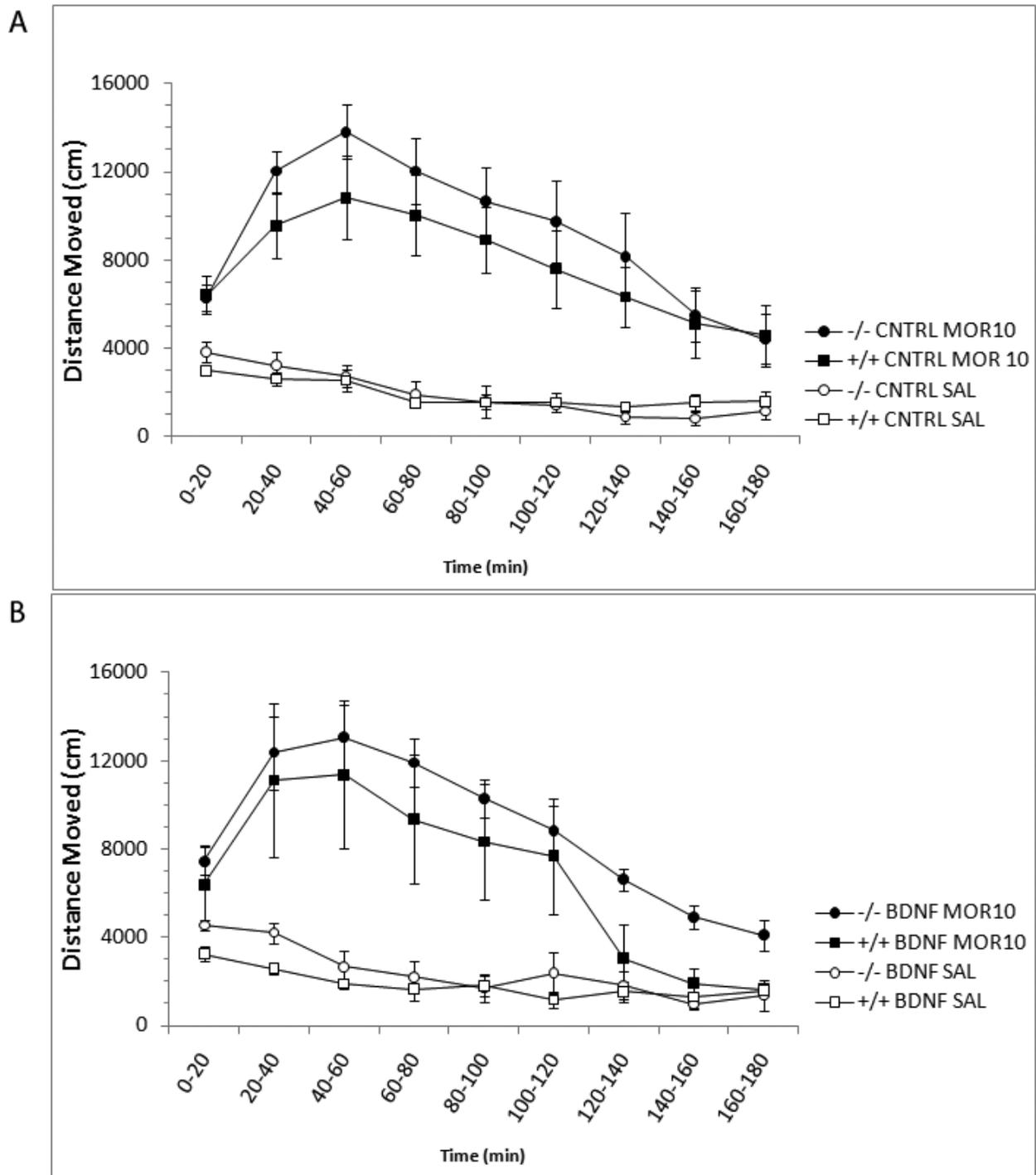


Figure 11. Time courses of saline- and morphine-induced locomotion (10 mg/kg, i.p.) in (A) VTA SAL-infused M5 KO (-/-, n=7) and WT (+/+, n=6) mice and (B) VTA BDNF-infused M5 KO (-/-, n=6) and WT (+/+, n=5) mice. There was a significant effect of drug (saline vs. morphine) and time as well as an interaction between drug and time. There was no significant effect of genotype on saline- or morphine-induced locomotion. SAL=saline, MOR10= morphine (10 mg/kg, i.p.). Bars represent standard errors of the mean.

Total morphine-induced locomotion for both VTA BDNF and VTA SAL-infused M5 KO and WT mice was analyzed using univariate ANOVA and is shown in Figure 12. There was no effect of either genotype or treatment (VTA SAL vs. VTA BDNF) on morphine-induced locomotion.

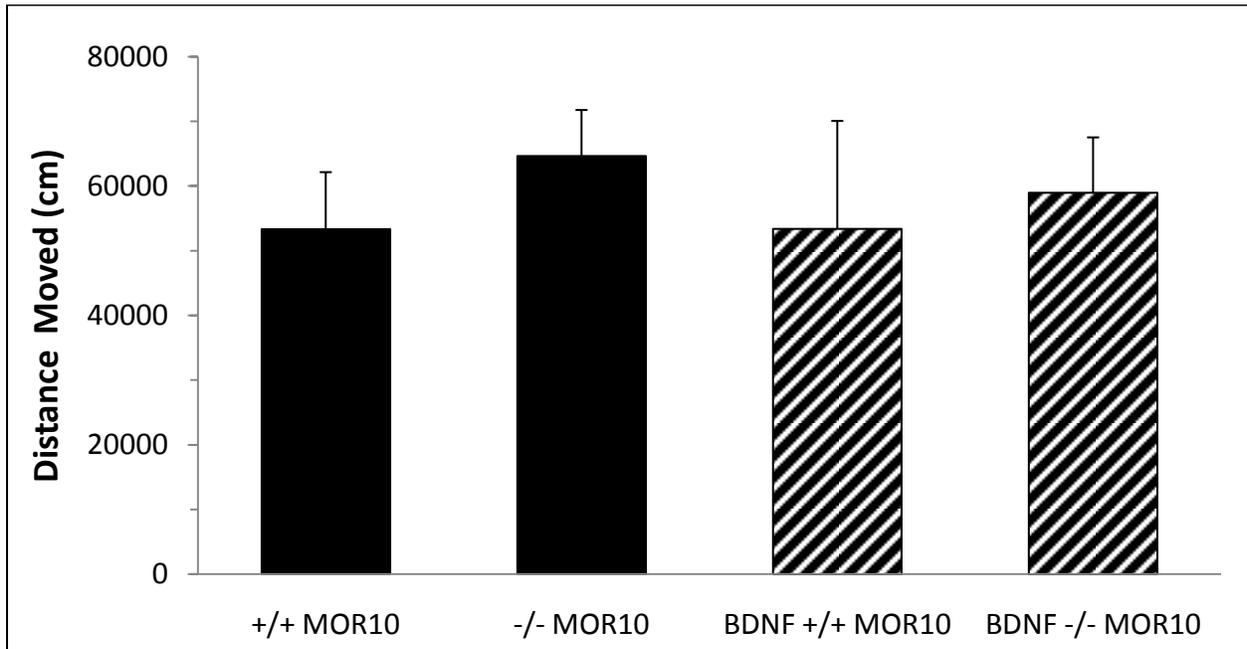


Figure 12. Total morphine-induced locomotion (10 mg/kg, i.p.) in VTA SAL M5 KO (-/-, n=7) and WT (+/+, n=6), and VTA BDNF-infused M5 KO (-/-, n=6) and WT (+/+, n=5) mice. There was no significant effect of either genotype or treatment on total morphine-induced locomotion. MOR10=morphine (10 mg/kg, i.p.). Bars represent standard errors of the mean.

Discussion

Morphine-Induced Locomotion in Control Mice: Mouse Colony Considerations

Here, we examined whether the effects of bilateral VTA infusions of BDNF on morphine CPP could similarly alter the locomotor stimulant properties of morphine in M5 KO and WT mice of the C57BL/6 strain. We did not find any differences in morphine-induced locomotion between M5 KO and WT mice bilaterally infused with saline (Figure 8 and 11). This conflicts with recent findings that M5 KO mice show a 40-50% deficit in morphine-induced locomotion (Steidl & Yeomans, 2009). These unexpected results may be attributable to recent changes within the C57BL/6 strain mouse colony. Prior to December, M5 KO mice showed reliable deficits in morphine-induced locomotion, ethanol drinking and mating-induced ultrasonic vocalizations (USVs) (Hastings, Euler, & Yeomans, 2009; Steidl & Yeomans, 2009; Wang, Liang, Burgdorf, Wess, & Yeomans, 2008). Though M5 KO mice in this colony showed a variety of behavioural deficits, individual variability existed such that some M5 KO mice do not show behavioural deficits. However, once averaged with many samples, the M5 KO deficits remained large. In December, the mouse colony was reduced in anticipation of decreased testing during the winter break. Experiments for the current study were then conducted January onward after colony numbers were increased. It is possible that the M5 KO mice randomly selected to maintain the colony through December were more sensitive to the stimulant effects of morphine. Re-building the colony on this subset of more sensitive animals could have reduced behavioural deficits for morphine-induced locomotion in M5 KO mice.

To test whether stress associated with surgery could have activated compensatory mechanisms to block behavioural deficits in M5 KO mice, unmanipulated (no surgery or systemic injections) M5 KO and WT mice were tested for mating-induced USVs. There were no significant differences between total calls or call type between M5 KO and WT mice, suggesting that surgical procedures did not alter behavioural deficits most commonly associated with loss of the M5 receptor (Figure 13). Additionally, a subset of randomly selected mice used in these studies was genotyped, confirming that no genetic contamination had occurred.

It should be noted that a new C56BL/6 colony has been bred in the Centre for Biological Timing and Cognition (CBTC) since March 2009. All mice in this colony are litter mates (i.e. M5 KO and WT mice are raised together by heterozygous mothers) and M5 KO mice have consistently shown reliable deficits in ethanol drinking (Euler & Yeomans, 2009, unpublished). All experiments on the C57BL/6 strain subsequent to the current study on VTA BDNF have been conducted exclusively on the new colony mice.

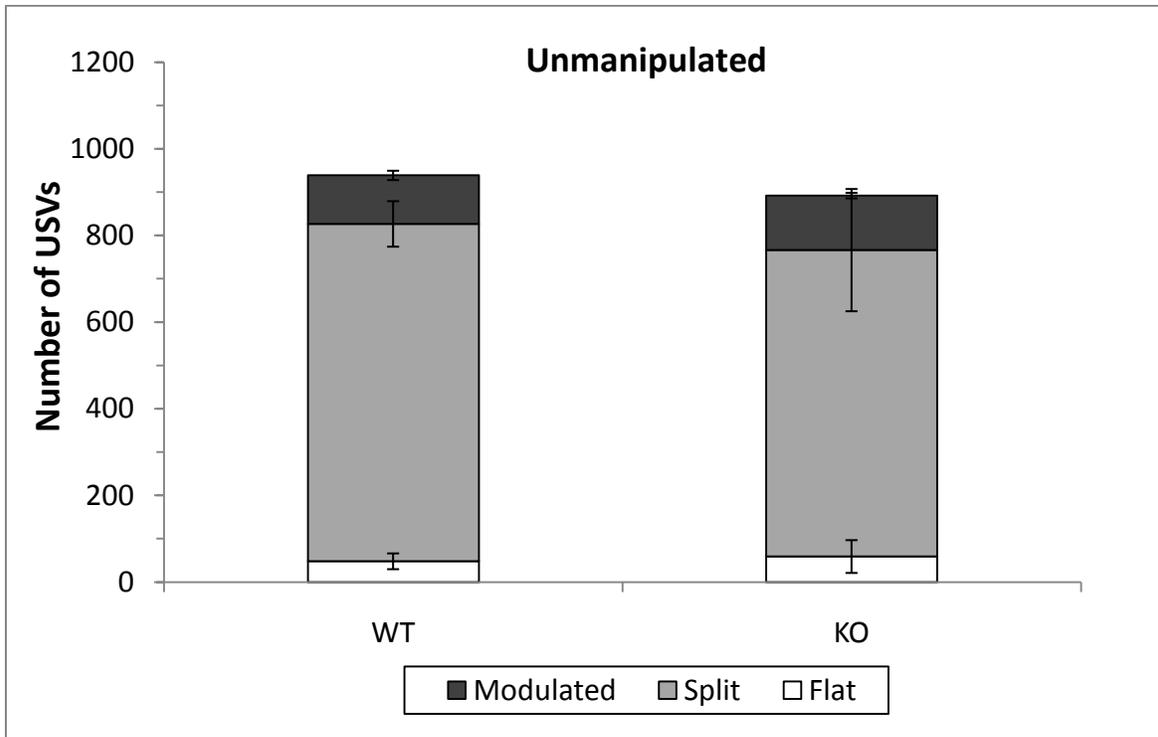


Figure 13. Mating-induced USVs in M5 KO (n=6) and WT (n=5) mice that did not receive surgery or systemic drugs (unmanipulated). There was no effect of genotype on either total calls or call type. Bars represent standard errors of the mean.

VTA BDNF Does Not Alter Morphine-Induced Locomotion in M5 KO or WT Mice

Unexpectedly, VTA infusions of BDNF did not alter morphine-induced locomotion in M5 KO or WT mice compared to saline infused control mice (Figure 9 and 12). A variety of motivational manipulations have been shown to alter pathways for opiate CPP. In food-sated or drug-naïve rats, PPT lesions, but not α -flupenthixol, block morphine CPP (Bechara & van der

Kooy, 1992; Nader & van der Kooy, 1994; Nader & van der Kooy, 1997). After food deprivation or opiate dependence and withdrawal, however, α -flupenthixol, but not PPT lesions, block morphine CPP (Bechara & van der Kooy, 1992; Nader & van der Kooy, 1994; Nader & van der Kooy, 1997). This work suggests that pathways for opiate reward can be shifted from a PPT-dependent to a DA-dependent pathway that does not require the PPT. Recently, Vargas-Perez et al. (2009) found that bilateral VTA infusions of BDNF induced similar alterations in pathways mediating morphine reward. They suggest that VTA BDNF induces a “morphine-dependent-like reward state” to bypass the PPT.

Indeed, BDNF is highly linked to drugs of abuse. Self-administration of psychostimulants such as cocaine produced transient increases in accumbal levels of BDNF (Graham, Edwards, Bachtell, DiLeone, Rios, & Self, 2007). BDNF levels in the VTA, NAc and amygdala progressively increase for up to 90 days of withdrawal from cocaine (Grimm, Lu, Hayashi, Hope, Su, & Shaham, 2003). In contrast, chronically administered opiates have been reported to both be ineffective in altering BDNF levels in the VTA (Numan, et al., 1998), or decrease the number of BDNF-positive labelled cells in the VTA (Chu, Zuo, Meng, Lee, Han, & Cui, 2007). Others have reported the potentiating effects of VTA BDNF infusions on cocaine seeking after withdrawal (Lu, Dempsey, Liu, Bossert, & Shaham, 2004), cocaine-induced locomotion and the development of cocaine sensitization (Horger, Lyasere, Berhow, Messer, Nestler, & Taylor, 1999). Heterozygous KOs for BDNF have shown delayed development of cocaine sensitization (Horger et al., 1999), reduced cocaine CPP and less cocaine-induced locomotion (Hall, Drgonova, Goeb, & Uhl, 2003). BDNF is also importantly involved in the survival and differentiation of neurons during development (Aid, Kazantseva, Piirsoo, Palm, & Timmusk, 2007) as well as learning and memory in adulthood (Tyler, Alonso, Bramham, & Pozzo-Miller, 2002; Yamada, Mizuno, & Nabeshima, 2002). Furthermore, changes in BDNF expression in the brain have been linked to a variety of neurological disorders including depression (Russo-Neustadt & Chen, 2005), Alzheimer’s disease and Parkinson disease (Murer, Yan, & Raisman-Vozari, 2001).

The current results are not in agreement with work by Vargas-Perez et al. (2009) or results obtained in experiment 1, as VTA BDNF did not alter the locomotor stimulant properties of morphine in M5 KO and WT mice (Figure 9 and 12). The findings here are also in

disagreement with the broader literature which establishes a strong relationship between BDNF and the effects of abused drugs.

Methodological Considerations

It is unclear why BDNF did not alter morphine-induced locomotion in either M5 KO or WT mice. Though BDNF is a large polypeptide (dimer of two identical 119 amino acid subunits), infusions were tracked with the movement of an air bubble separating molecular water and BDNF solutions in the Tygon tubing connected to injectors. Other methods were not used to confirm the presence of BDNF in the VTA of mice, however.

As mentioned in the methods section, morphine-induced locomotion was tested at the 30 mg/kg (i.p.) dose, but not included in data analysis due to the presence of stereotypy. Steidl and Yeomans (2009) did not observe stereotypy at this dose, but rather reported that WT mice of the C57BL/6 strain showed a ceiling effect for morphine-induced locomotion. Though there was no effect of VTA BDNF on morphine-induced locomotion in the present results, both M5 KO and WT mice showed the highest levels of morphine-induced locomotion (10 mg/kg, i.p.) ever reported for the C57BL/6 strain. It is possible then, that BDNF did not potentiate morphine-induced locomotion at this dose because mice were already running maximally. However, this does not explain why VTA BDNF did not alter morphine-induced locomotion at the 3 mg/kg dose.

Many studies suggest that the effects of a variety of drugs are altered by the rostro-caudal site of administration. Infusions of μ -opioid receptor selective peptide endomorphine-1 into caudal regions of the VTA of rats produce stronger self-administration, CPP and locomotion than infusions into rostral VTA (Zangen, Ikemoto, Zadina, & Wise, 2002). Furthermore, rats will self-administer or show increased locomotion induced by the GABA_A receptor agonist muscimol or Δ^9 tetrahydrocannabinol infused into caudal, but not rostral, VTA (Ikemoto, Murphy, & McBride, 1998; Zangen, Solinas, Ikemoto, Goldberg, & Wise, 2006). Here, there was a wide spread of rostro-caudal VTA cannulae placements (Figure 6). To determine whether there were differing effects of rostral vs. caudal VTA BDNF infusions on morphine-induced locomotion, data was also analyzed based on rostro-caudal infusion sites (data not shown). When sample size was large enough to warrant statistical analysis, there were no significant differences in morphine-

induced locomotion when comparing mice that received rostral vs. caudal VTA BDNF infusions. Previously, Steidl (2008) also found rostral vs. caudal VTA atropine infusions had no effect on morphine-induced locomotion. However, the current study and Steidl's work (2008) infused a volume of 0.5 μ l or 0.3 μ l, respectively, into the VTA of mice. Studies comparing the effects of rostro-caudal drug microinjections infused a volume of 0.1-0.5 μ l into the VTA of rats (Ikemoto et al., 1998; Zangen et al., 2002; Zangen et al., 2006). The VTA extends from -2.92 to -3.88 mm in mice (Paxinos and Franklin, 2001) and twice as long in rats from -4.68 to -6.84 mm, relative to bregma (Paxinos & Watson, 1998). Therefore, the method that Ikemoto et al. (1998) and Zangen et al. (1998, 2002) used produced more localized infusions into the VTA, allowing them to examine the effect of rostro-caudal placements on drug-related behaviours with better spatial resolution.

Conclusions

In conclusion, bilateral VTA BDNF infusions did not shift cholinergic input to DA neurons to bypass the PPT. Additionally, M5 KO animals with VTA saline infusions did not show a 40-50% reduction in morphine-induced locomotion as seen previously (Steidl & Yeomans, 2009). Finally, changes in the C57BL/6 mouse colony used for this study may have resulted in a blocking of the M5 KO deficit in morphine-induced locomotion.

General Discussion

The objective of this thesis was to determine if the same motivational manipulations (food deprivation and VTA BDNF) that change pathways for opiate reward in rats generalize to morphine-induced locomotion in mice of the C57BL/6 strain. Many studies show that PPT lesions, but not α -flupenthixol, block morphine CPP in drug-naïve rats, whereas α -flupenthixol, but not PPT lesions, block opiate reward in food-deprived, opiate dependent and withdrawn, or VTA BDNF-infused rats (Nader et al., 1997; Vargas-Perez et al., 2009). This body of work suggests that the motivational state of the animal can change the pathways mediating opiate reward.

In rats, PPT lesions have been shown to block striatal DA release induced by morphine (Miller, Forster, Metcalf, & Blaha, 2002) while LDT lesions or VTA scopolamine block morphine-induced accumbal DA efflux (Forster, Falcon, Miller, Heruc, & Blaha, 2002; Miller, Forster, Yeomans, & Blaha, 2005). More recently, Steidl (2008) showed that the M5 receptor is importantly involved in accumbal DA release by VTA morphine and locomotion induced by systemic morphine in mice of the CD1x129 and C57BL/6 strain. This suggests that accumbal DA efflux and morphine-induced locomotion in the C57BL/6 strain may be due, in part, to PPT-M5 mediated activation of VTA DA neurons. Similarly, M5 KO mice also show reduced cocaine self-administration (Thomsen, Woldbye, Wörtwein, Fink-Jensen, Wess, & Caine, 2005), cocaine CPP (Fink-Jensen, et al., 2003), morphine CPP (Basile, et al., 2002) and mating-induced USVs (Wang, Liang, Burgdorf, Wess, & Yeomans, 2008).

Furthermore, Steidl & Yeomans (2009) reported that M5 KO mice of the CD1x129 and C57BL/6 strains show deficits in locomotion induced by systemic morphine at three doses (3, 10 and 30 mg/kg). Therefore, in experiment 1, I first replicated previous findings that M5 KO mice show a 40-50% reduction in open-field locomotion induced by systemic morphine (10 mg/kg) (Steidl & Yeomans, 2009).

Secondly, I found that 18 hours food deprivation induced a reversal in morphine-induced locomotion, completely rescuing M5 KO morphine-induced locomotion (10 mg/kg, i.p.) (Figure 3C). This result supports the idea that acute food deprivation alters the stimulant effect of systemic morphine. This change results in a bypassing of the PPT and rescues morphine-induced

locomotion in M5 KO mice. Importantly, this also shows that PPT-M5 mediated pathways needed for the acute effects of morphine in mice and rats (Forster, Falcon, Miller, Heruc, & Blaha, 2002; Miller, Forster, Metcalf, & Blaha, 2002; Steidl, 2008; Steidl & Yeomans, 2009) are linked to the PPT- and DA-dependent pathways for opiate reward (Nader et al., 1997). Like morphine CPP, food deprivation appears to induce a double dissociation for morphine-induced locomotion. While M5 KO mice showed a complete rescue of morphine-induced locomotion, WT mice showed a 39% decrease in morphine-induced locomotion after 18 hours food deprivation. I suggest that this reversal may be due to supersensitivity of the DA neurons of M5 KO mice, as a result of chronic understimulation of mesolimbic DA.

In experiment 2, bilateral infusions of BDNF into the VTA did not alter morphine-induced locomotion in either M5 KO or WT mice. Additionally, control animals bilaterally infused with saline showed no genotype differences in morphine-induced locomotion. I propose that the absence of a genotype difference in morphine-induced locomotion is due to genetic instabilities in the mice tested, following reduction and re-building of the C57BL/6 colony on a small subset of mice. However, a new colony of C57BL/6 mice in the CBTC has shown reliable genotype differences for ethanol preference (Euler and Yeomans, 2010, unpublished abstract). Mice from this new CBTC colony have exclusively been used for all testing on the C57BL/6 strain, subsequent to studies conducted in this thesis.

Additionally, prior to BDNF testing, I also attempted to examine the effects of chronic morphine exposure and withdrawal (Bechara and van der Kooy, 1992) on morphine-induced locomotion in M5 KO mice of the C57BL/6 strain. Morphine was chronically administered via mini-osmotic pumps (Alzet Model 1007D, Durect Corporation, California, USA). The subcutaneously implanted pumps continuously administered either morphine solution (5 mg/kg/hr) or physiological saline (0.5 μ l/hr) for 7 days and were immediately removed afterwards (see Dockstader et al., 2001). The utmost care was taken to ensure that all surgical equipment, osmotic pumps and solutions were sterile. After removal of the osmotic pumps, mice chronically exposed to morphine were then withdrawn for 3 days, to allow sufficient recovery from minor pump removal surgeries, and tested for locomotion induced by systemic morphine (10 mg/kg, i.p.). This study did not progress past preliminary stages for 3 reasons, however. First, mice that received continuous infusions of morphine showed a 16% death rate (4 out of 25 mice

tested) which was not particular to genotype. Second, many of the mice, regardless of genotype, showed little grooming, minimal movement and weight loss on test days. Therefore, these mice were removed from the experiment as they were considered too unhealthy for testing. While approximately 50% of mice previously implanted with morphine pumps did show somatic withdrawal symptoms (almost exclusively wet-dog shakes), Dockstader et al. (2001) observed strong naloxone-precipitated somatic withdrawal symptoms (wet dog shakes, jumping, sniffing, tremors and teeth chattering) in mice that were made morphine dependent and withdrawn. Third, morphine dependent and withdrawn mice that were included in testing did not show significant increases in morphine-induced locomotion relative to saline controls. It is unlikely that mice were expressing the effects of morphine tolerance taking into account the observation of withdrawal symptoms in approximately half of chronically exposed and withdrawn mice. Additionally, using the same methods as Dockstader et al. (2001), previous work from our lab shows that both M5 KO and WT mice of the C57BL/6 strain show strong naloxone-precipitated withdrawal symptoms following 7 days of continuous morphine infusion at a rate of 5 mg/kg/hr (Paterson, Steidl, & Yeomans, 2006, unpublished).

One major criticism of this work could be that food deprivation effects on morphine-induced locomotion may not be relevant to morphine CPP. Though morphine-induced locomotion is an easy and useful way to measure the stimulant effects of drugs in animals, it is not a direct measure of preference. Previously, Steidl (2008) attempted to investigate the role of the M5 receptor for morphine CPP in an unbiased paradigm, but reliable morphine CPP could not be acquired in these mice.

More recently, work from our lab shows that M5 KO mice of the C57BL/6 strain drink approximately 30% less ethanol than WT mice in a two-bottle choice test (Hastings, Euler, & Yeomans, 2009). Systemic naltrexone reduces ethanol drinking in WT, but not M5 KO mice, suggesting that part of ethanol consumption may be dependent on DA activation mediated by endogenous opioids. Furthermore, ethanol drinking deficits in M5 KO mice are reversed after 18 hours food deprivation, similar to morphine-induced locomotion, albeit the effects are not as pronounced. Therefore, the effects of food deprivation on morphine CPP and morphine-induced locomotion seem to also generalize to ethanol reward to reverse ethanol preferences in M5 KO and WT mice. If food deprivation indeed rescues the DA-dependent component of ethanol

drinking that is mediated by endogenous opioids in M5 KO mice, then food deprivation should also rescue the inhibiting effects of naltrexone on ethanol drinking in these mice. This would further provide evidence that ethanol reward in food-deprived M5 KO mice is mediated by endogenous opioid activation of the mesolimbic DA system.

Future Directions

This thesis presented findings on the effects of acute food deprivation and bilateral VTA BDNF infusions on open-field locomotion induced by systemic morphine. I report that food deprivation, but not VTA BDNF, alters pathways mediating morphine-induced locomotion to bypass the PPT and M5 receptors. Considering studies investigating the effects of food deprivation on ethanol preference as well as morphine dependence and withdrawal on morphine-induced locomotion, this thesis is only part of a larger effort to test the generality of the effects of various motivational manipulations used by van der Kooy's group to alter morphine CPP. Additionally, this body of work extends Steidl's theory (2008) regarding pathways for acute morphine.

To provide direct evidence that the PPT is crucial for morphine-induced locomotion in non-deprived mice, but not food-deprived mice, PPT lesion studies can be conducted. If acute food deprivation bypasses the PPT as proposed by Nader et al. (1997), then PPT lesions should block morphine-induced locomotion in non-deprived mice, but should not affect the reversal of morphine-induced locomotion in food-deprived M5 KO and WT mice.

Bechara and van der Kooy (1992) proposed that changes in opiate reward from PPT-dependent to DA-dependent pathways after morphine dependence and withdrawal were not permanent. They found that alleviating doses of morphine restored the blocking effects of PPT lesions for morphine CPP, suggesting that the DA-dependent pathway induced by morphine dependence and withdrawal could be overcome. If food deprivation also induces transient overshadowing of unaltered PPT-dependent pathways mediating morphine-induced locomotion in non-deprived mice, alleviating doses of food should restore the PPT-dependent component of DA activation and reinstate M5 KO morphine-induced locomotion deficits.

The measurement of stimulant-induced locomotion is thought to reflect, in large part, the degree of midbrain DA activation (Tzschentke, 2001). In mice, systemic administration of haloperidol (Ito, Mori, & Sawaguchi, 2008) or DA-deficiency (Hnasko, Sotak, & Palmiter, 2005) reduces morphine-induced locomotion. Additionally, mice of the C57BL/6 show concomitant morphine-induced locomotion and accumbal DA release (Murphy, Lam, & Maidment, 2001). Using electrochemical methods, Steidl (2008) found that prolonged increases in accumbal DA, induced by 50 ng infusions of morphine into the VTA, were absent after intra-VTA muscarinic antagonism or in M5 KO mice. This suggests that the 40-50% reduction in morphine-induced locomotion in M5 KO mice is DA-dependent. To determine whether the reversal in morphine-induced locomotion observed after food deprivation is DA-dependent, accumbal DA levels should be measured. If food deprivation alters cholinergic input to DA neurons such that direct GABA inhibition activates supersensitive VTA DA neurons in M5 KO mice, it would be expected that food-deprived M5 KO mice would show more morphine-induced accumbal DA efflux than food-deprived WT mice.

As mentioned in the discussion for Experiment 1, there may be a PPT-dependent component of morphine-induced locomotion which is independent of DA. To confirm whether there is a time-dependent delayed activation of a DA-independent pathway for morphine-induced locomotion (Figure 4A and B), the effects of systemic haloperidol on morphine-induced locomotion can be examined. In non-deprived mice, haloperidol would be expected to reduce morphine-induced locomotion in WT mice for approximately the first 45 minutes of testing, to levels similar for M5 KO mice. Morphine-induced locomotion in non-deprived M5 KO mice would be predicted to remain unchanged. In contrast, if food deprivation induces a PPT-independent, but DA-dependent, pathway for morphine-induced locomotion, then haloperidol should completely block morphine-induced locomotion in both M5 KO and WT mice.

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