Morphine-Induced Locomotion Increases following Viral Transfection of M5 Muscarinic Receptor Genes in the Ventromedial Hypothalamus of Wild-Type Mice

by

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Department of Psychology

University of Toronto

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Abstract

Excitatory M5 muscarinic acetylcholine receptors are expressed by mesencephalic dopamine (DA) neurons and, at the highest level in the brain, in the ventromedial hypothalamus (VMH). M5 knockout (KO) mice emit fewer ultrasonic vocalizations (USVs) during mating, and show 40-50% lesser morphine-induced locomotion as compared to wild-type (WT) mice. Following viral transfection of M5 muscarinic receptors, KO mice depict restored mating-induced USVs and enhanced morphine-induced locomotion and stereotypy which is consistent with DA neuron activation. The VMH is important for motivational processes, such as, feeding and producing USVs in rats. With a Herpes simplex virus (HSV), the M5 receptor gene was transfected into the VMH of WT mice along with a green fluorescent protein (GFP) marker gene. M5 transfection into neurons of the VMH increased locomotion in mice injected with 10mg/kg morphine by 6 times at peak, without affecting saline-induced locomotion. When haloperidol, a D2-selective dopamine blocker was injected into the same mice, locomotion was reduced by half. There were no significant differences in the amount of USVs produced. VMH may exert its effects on morphine through a DA dependent VTA pathway where M5 receptors also increase morphine-induced locomotion.
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<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>BSR</td>
<td>brain stimulation reward</td>
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<td>ChAT</td>
<td>choline acetyltransferase</td>
</tr>
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<td>CPP</td>
<td>conditioned place preference</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>EM1-LI</td>
<td>endomorphin 1</td>
</tr>
<tr>
<td>EM2-LI</td>
<td>endomorphin 2</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>LDT</td>
<td>laterodorsal tegmental nucleus</td>
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<tr>
<td>LH</td>
<td>lateral hypothalamus</td>
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<tr>
<td>MOR</td>
<td>morphine</td>
</tr>
<tr>
<td>MFB</td>
<td>medial forebrain bundle</td>
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<tr>
<td>NAc</td>
<td>nucleus accumbens</td>
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<tr>
<td>PPT</td>
<td>pedunculopontine tegmental nucleus</td>
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<tr>
<td>SAL</td>
<td>saline</td>
</tr>
<tr>
<td>SN</td>
<td>substantia nigra</td>
</tr>
<tr>
<td>SNC</td>
<td>substantia nigra—pars compacta</td>
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<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>VTA</td>
<td>ventral tegmental area</td>
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<td>WT</td>
<td>wildtype</td>
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Introduction

Opiates and Substance Abuse

Opiates are narcotic opioid alkaloids derived from the constituent components of the opium poppy plant, botanically known as *Papaver somniferum*. Opiates include morphine and its semi-synthetically derived component heroin. Since these drugs are soporific and induce feelings of intense pleasure and analgesia; morphine is aptly named after the Greek god of dreams, *Morpheus* (Brownstein, 1993).

The euphoriant use of opium has been a centuries-old tradition. Archaeological data pinpoints Iraq as the geographical location where opium was first isolated from a large cultivation of poppies as early as 3000 B.C. The therapeutic effects of morphine were recognized by the Sumerians as well as early dynastic Egyptians. By the eighth century A.D. opium had been brought by Arabs to India and China from where it made its way to all parts of Europe. Along with the healing and beneficial effects of opium use in medicines and during surgery as an analgesic, the highly rewarding euphoric properties of the drug caused it to be a strong reinforcer for subsequent use and abuse leading to the development of drug addiction. This issue is still prevalent and ever-increasing in today’s society (Brownstein, 1993).

Substance abuse takes a huge economic as well as emotional toll on humanity. Not only does it negatively impact the lives of those taking the drug but it also affects family and friends close to them. In Canada alone, the cost of illegal drugs to the country is $8.2 billion (Rehm et al., 2006). Substance abuse can lead to school failure, limited career options, dependence on public assistance, legal difficulties, and expensive hospitalizations as well as suicide. According to the Canadian Centre on Substance Abuse there are 125,000 injection drug users in Canada (Rehm et al., 2006) and morphine and heroin are among the top 3 most commonly injected drugs (Public Health Agency of Canada. 2006). In order to remediate this pressing concern, it is
important to understand the mechanisms of opiate drug action and reward, and its biological underpinnings; which is the purpose of my thesis.

**Opioid Receptors and Functions**

167 years after the German chemist, Friedrich Serttner first isolated the active principle of opium alkaloids in 1805, the pharmacology of morphine was defined at the receptor level (Pert & Snyder, 1973). Opioid receptors are included in the class A (Rhodopsin) family of G protein-coupled receptors that are instrumental in mediating the functions of many neurotransmitters and hormones in the body. These receptors can be activated either by endogenously produced opioid peptides such as enkephalins or beta-endorphins, or by exogenously administered opioid drugs such as morphine and heroin (Waldhoer, Bartlett & Whistler, 2004).

There are four opioid receptor subtypes: µ (mu), κ (kappa), δ (delta), and the more recently discovered nociceptin/orphanin FQ receptor. Opioid receptors have 7 transmembrane helices arranged in a counter clockwise fashion forming a tight helical bundle, providing a dynamic interface for the binding of various opioid ligands (Waldhoer, Bartlett & Whistler, 2004).

When the selective µ agonist DAMGO was unilaterally injected into the VTA, a dose-dependent preference for the drug-associated place was observed (Devine & Wise, 1994). In contrast, microinjections of the κ agonist U50-488H and the dynorphin derivative E-2078 into the VTA produced place aversions (Bals-Kubik et al, 1992; Mucha & Herz, 1985). This finding suggests that the µ opioid receptor is of particular relevance to the euphoric and pleasurable behavioural consequences observed after drug intake. Furthermore, in an operant behavioural study using a self-injection technique, it was found that after inducing physical dependence on morphine, self-injection varied inversely with the dose. This establishes that morphine is a
powerful reinforcer and produces immediate gratification (Weeks, 1972). Taken together, the results suggest that morphine acts on \( \mu \) opioid receptors to produce pleasurable and rewarding feelings.

\( \mu \) receptors are distributed throughout the fore-, mid- and hindbrain including areas such as the accessory olfactory bulb, caudate putamen, nucleus accumbens, endopiriform nucleus, fundus striati, habenula, amygdaloid nuclei, thalamus, hypothalamus, zona incerta, ventral tegmental area, interpeduncular nucleus, central gray, ventral hippocampal subiculum and dentate gyrus, substantia nigra and the superior and inferior colliculi (Sharif & Hughes, 1989; Kitchen et al, 1992).

Recent evidence has suggested a role for the periaqueductal gray matter (PAG) in the mediation of morphine-induced analgesia. The evidence implicating the PAG arises from several different experimental approaches: intracerebral microinjection studies, focal electrical stimulation studies, biochemical studies and electrophysiologic studies (Lewis & Gebhart, 1976). Analgesia can be induced by the direct injection of as little as 3 \( \mu \)g of morphine into the PAG. Antinociceptive effects have also been reported after injection of morphine into other brain areas, including the hypothalamus, periventricular gray and 4th ventricle (Sharpe, Garnett, & Cicero, 1974; Tsou & Jang, 1964); the most efficacious region with respect to morphine induced analgesia, however, appears to be the ventrocaudal PAG. Electrical stimulation of the PAG has also been found to produce profound increases in the response thresholds of animals in various analgesiometric tests (Balagura & Ralph, 1973; Mayer & Liebeskind, 1974). Although the animals in these studies were unresponsive to a noxious stimulus, they were not immobilized by the electrical stimulation and reportedly responded normally to touch, visual and auditory stimuli. Regional analysis for stereospecific opiate agonist and antagonist binding has further implicated the PAG as a potential central locus of opiate effects since significant amounts of
opiate binding sites have been demonstrated there. Initial work by Goldstein et al. (1971) and subsequent work by many other workers (Hiller, Pearson & Simon, 1973, Pert & Snyder, 1975) have demonstrated that there is an uneven distribution of high affinity stereospecific binding sites for opiates in various regions of the brain. The PAG, while not possessing the highest levels of opiate binding sites within the brain, appears to have perhaps the most behaviourally relevant opiate binding sites. Marked changes in behaviour do not result following morphine administration into other brain areas containing larger amounts of opiate binding sites, such as the caudate nucleus and amygdala (Broekkamp & Rossum, 1957; Wei, Segal, & Way, 1975).

It is suggested that the PAG exerts its analgesic and locomotory effect in response to morphine through the hypothalamus. Stimulation of the PAG activates enkephalin-releasing neurons which bind to µ opioid receptors. The activation of the µ opioid receptor inhibits the release of substance P from incoming first-order neurons in the spinal cord and, in turn, inhibits the activation of the second-order neuron that is responsible for transmitting the pain signal up the spinothalamic tract to the ventroposterolateral nucleus (VPL) of the thalamus. The nociceptive signal is inhibited before it is able to reach the cortical areas such as the anterior cingulate that interpret the signal as pain (Weitzman & Ross, 1962).

Hui (2003) used fluorescent retrograde labeling combined with immunofluorescence histochemical staining for endomorphin 1(EM1-LI) and endomorphin 2 (EM2-LI) and to investigate whether endomorphin 1-immunoreactive and endomorphin 2-immunoreactive neurons in the hypothalamus and nucleus tractus solitarii project reciprocally between these two regions. They found that endomorphin 1/Fluoro-Gold or endomorphin 2/Fluoro-Gold double-labeled neuronal cell bodies were predominantly observed in the arcuate nucleus of the hypothalamus, a few of which were also observed in the posterior hypothalamic area and periventricular hypothalamic nucleus.
EM1-LI and EM2-LI have a high affinity and specificity for the μ-opioid receptor which mediates the reinforcing properties of opiates as selective μ antagonists are able to block the effects of opiates in a dose-dependent manner (Koob, 1998). Further, selectively bred μ-KO mice do not show reinforcement to morphine (Matthes et al., 1996). Opiates infused into the VTA produce dose-dependent responses in NAc DA levels. However, the μ opiate receptor is found predominantly on non-DA neurons (Di Chiara et al., 1988). While some of the rewarding effects of opiates are dependent on DA neurons and receptors, there remain strong DA-independent effects that are still rewarding in the absence of DA. Infusion of alpha-flupenthixol, a DA antagonist, into the NAc fails to block morphine conditioned place-preference in morphine-naïve rats or mice (Laviolette et al., 2002). Koob and Bloom (1988) demonstrated that even when all of the DA projections are destroyed, opiates maintain their rewarding properties at the NAc. Further, DA deficient mice, that possess two inactivated alleles for the TH gene, show reduced morphine-induced locomotion (Hnasko et al., 2003). Thus, both DA-dependent (VTA) and DA-independent (PAG or VMH) mechanisms may be involved in the rewarding properties of opiates.

**Dopamine Systems**

Early studies used mainly the conditioned place preference (CPP) paradigm to measure the rewarding properties of opiates (van der Kooy, Mucha, O’Shaughnessy, & Bucenieks, 1982). Place preference for animals with VTA morphine infusion can be blocked by systemic dopamine antagonists (Bozarth & Wise, 1981); thus indicating that opiate induced rewarding effects may be influenced by dopamine neurotransmission.

Dopamine (DA) is an important catecholamine neurotransmitter that is an important element in the brain reward system and is also involved in the mechanistic action of many drugs
of abuse such as cocaine, amphetamine and nicotine (DiChiara & Bossareo, 2007). DA is also influential in the neurobiology of several neurological and psychiatric disorders such as positive symptoms of schizophrenia, Parkinson’s disease, and attention deficit hyperactivity disorder (Wise & Rompre, 1989; DiChiara & Bossareo, 2007; Mardsen, 2009). Although DA neurons account for less than 1% of the total neuronal population, they have a profound effect on brain function (Bjorklund & Dunnett, 2007; Bjorklund & Lindvall, 1984; Arias-Carion & Poppel, 2007). The binding of DA to its metabotropic receptors induces synaptic plasticity which forms the basis for reward related incentive learning and memory (Kandel, 2000; Berridge & Robinson, 1993, Miller et al, 1990, Pessiglione et al., 2006).

Just as DA is released in response to natural stimuli, such as food and opportunities for mating, in order for them to be rewarding, DA is also required for addictive drugs to produce reward (Kelley & Berridge, 2002; Di Chiara, 1998; Koob & Bloom, 1988; Wise & Rompre, 1989). The most obvious difference between natural goal objects, such as food, and addictive drugs is that the latter have no intrinsic ability to serve a biological need. However, because both addictive drugs and natural rewards produce dopamine efflux, addictive drugs mimic the effects of natural rewards and can thus shape behavior (Montague, Hyman, & Cohen, 2004; Kelley, & Berridge, 1998; Berke, 2003).

The majority of DA-containing cells originate from a single embryological cell group that develops at the mesencephalic-diencephalic junction and projects to various forebrain locations (Hynes & Rosenthal, 1999). In the adult brain, DA neurons are most commonly found in the ventral part of the mesencephalon but are also localized in the diencephalon and olfactory bulb (Bjorklund & Dunnett, 2007; Bjorklund & Lindvall, 1984; Arias-Carion & Poppel, 2007). Mesodiencephalic DA neurons form a specific neuronal group that includes the ventral tegmental area (VTA), substantia nigra pars compacta (SNc), and the retrorubral field (RRF). These cell
groups give rise to the nigrostriatal, mesolimbic and mesocortical DA pathways (Fallon & Moore, 1978). DA neurons in the nigrostriatal system project from the SNc into the caudate-putamen nucleus. The nigrostriatal pathway plays an essential role in the control of voluntary movement and it is the degeneration of DA neurons in the SNc that causes the motor deficits observed in PD. Similar to patients with PD, animals with selective lesions of the nigrostriatal dopamine system also show deficits in the initiation of movement (Carli, Evenden, & Robbins, 1985). This system is also important for the expression of drug-induced stereotypy, the display of repetitive (stereotyped) movements following high doses of stimulant drug.

The mesolimbic and mesocortical DA systems originate in the VTA, and are implicated in emotional responses, such as motivation, and reward-related learning respectively (Mogenson et al., 1980). DA cells that project from the VTA to the nucleus accumbens, olfactory tubercle, septum, amygdala and hippocampus, constitute the mesolimbic pathway, whereas DA cells that project from the VTA and innervate fibres in the prefrontal, cingulate and perirhinal cortex, constitute the mesocortical DA pathway (Wise, 2004).

**Afferent Control of the Dopamine System**

The mesocorticolimbic and nigrostriatal dopamine systems are innervated by various neurotransmitter systems such as acetylcholine, GABA, and glutamate, which mediate the functions of the cell bodies in the VTA and SNc.

**GABAergic Control of Dopamine Systems**

Gamma-Aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the mammalian central nervous system. There is significant evidence that the VTA, SN and RRF contain significant populations of GABAergic neurons admixed with DA neurons (Nagai et al., 1983; Mugniani and Oertel, 1985; Kosaka et al., 1987). *In situ* hybridization for glutamic acid
decarboxylase (GAD) messenger RNA (mRNA) to identify GABAergic cells in midbrain revealed that about 35% of the cells in the VTA are positively labelled for GAD and are indiscriminately mixed with the tyrosine hydroxylase (TH) labelled cell populations (Nair-Roberts, Chatelain-Badie, Benson, White-Cooper, Bolam, & Ungless, 2008). In contrast the SN, contains more than 60% of GAD-positive cells that are located primarily in the pars reticulata region (Kalivas et al., 1992). GABA neurons in the VTA and SN are known to synapse with DA neurons (van den Pol, Smith, & Powell, 1985; Bayer & Pickel, 1991), mesencephalon neurons in the PPT and LDT (Bayer & Pickel, 1991; Semba & Fibiger, 1992; Steininger & Rye, 1992; van den Pol, Smith, & Powell, 1985), as well as areas outside the ventral mesencephalon such as forebrain regions including nucleus accumbens and prefrontal cortex (Steffensen, Svingos, Pickel, & Henriksen, 1998; Van Bockstaele & Pickel, 1995). GABA neurons are likely to be critically involved in the regulatory activity of dopaminergic neurons and the inhibitory output of these nuclei. (Johnson & North, 1992; Westerink, Kwint, & deVries, 1996).

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, VTA microdialysis studies in conscious rats reveal that the increase in DA levels elicited by morphine was completely blocked by peripheral injection of naloxone, an opioid receptor antagonist, whereas a dose-dependent decrease in somatodendritic extracellular DA was produced following the administration of a GABA agonist (Kliténwick, Dewitte & Kalivas, 1992). Similar to this line of evidence, it has been observed that opioids do not affect the principal DA-containing neurons but hyperpolarize secondary GABAergic interneurons which reduces the spontaneous GABA-mediated synaptic input to the DA cells (Johnson & North, 1992). Therefore, stimulation of μ-opioid inhibits the
activity of GABAergic afferents to dopamine neurons, eradicating tonic inhibitory regulation (Vanderschuren & Kalivas, 2000; Klitenwick, Dewitte & Kalivas, 1992).

**Acetylcholine**

Acetylcholine (ACh) is the neurotransmitter synthesized by cholinergic neurons from choline and acetyl coenzyme A through the action of the enzyme choline acetyltransferase (ChAT). It was the first neurotransmitter to be identified in the heart, and has since then been found to have many functions of both the CNS and PNS (Wess et al., 2007; Whittaker, 1990). Immunohistochemistry studies have identified eight different cholinergic cell groups (Ch1-8) in the brain (Mesulam, Mufson, Wainer, & Levey, 1983; Schafer, Eiden, & Weihe, 1998). Two of the cell groups, Ch5 and Ch6, are particularly important due to their involvement in arousing thalamic and DA function. Ch5 cell groups are clustered in the pedunculopontine tegmental nucleus (PPT), whereas Ch6 clusters are found in the laterodorsal tegmental nucleus (LDT).

**PPT and LDT**

In several mammalian species the PPT (Ch5) has been shown to extend caudally from the posterior pole of the substantia nigra to the lateral tip of the superior cerebellar peduncle and rostral parabrachial nucleus (Rye, Saper, Lee, & Wainer, 1987; Steininger et al., 1992) It dorsally borders the cuneiform and deep mesencephalic nuclei, and ventrally borders the pontine reticular nucleus (Inglis & Winn, 1995; Rye, Saper, Lee, & Wainer, 1987). The cells of the LDT are located more medially in the pontine central gray, bound anteriorly by the dorsal raphe and laterally by the posterior end of the PPT (Paxinos & Watson, 1998). Furthermore, in-situ hybridization studies reveal distinct populations of cholinergic (20%), glutamatergic (22%), and GABAergic (58%) neurons in LDT (Wang, Tagliferro, & Morales, 2007; Winn, 2006).
In order to map cholinergic projections from PPT and LDT to midbrain dopaminergic nuclei, stereotaxic microinjections of Fluoro-Gold- or rhodamine-labeled microspheres were made to either SN or VTA, with the aim of immunohistochemically visualizing ChAT (Oakman, Faris, Kerr, Cozzari, & Hartman, 1995). It was revealed that ChAT-positive cells are most densely located in the PPT and LDT. Retrograde labelled cells projecting to the SN originated ipsilaterally from the rostroventral pole of the PPT. In contrast, VTA-projecting neurons were equally distributed on both sides of the PPT and LDT. These findings indicate that cholinergic inputs from LDT and PPT are functionally organized to innervate both the SN and more densely VTA dopaminergic nuclei (Oakman, Faris, Kerr, Cozzari, & Hartman, 1995).

**Cholinergic Receptors**

There are two distinct families of receptors through which ACh exerts its mediating actions on mesocorticolimbic and nigrostriatal DA neurons through — the nicotinic ACh receptors (nAChRs) and the muscarinic ACh receptors (mAChRs). The nAChRs function as fast ACh-gated cation channels comprised of pentamers of several different α- and β-subunits (Salamone & Zhou, 2000; Sargent, 1993), whereas the mAChRs are members of the superfamily of slower G-protein-coupled receptors (Wess, Eglin & Gautam, 2007). Molecular-cloning studies have distinguished between five mAChR subtypes, M1–M5 (Bonner, Young, Brann, & Buckley, 1987; Bonner, Young, Brann, & Buckley, 1988; Kubo, et al., 1986). Activation of the muscarinic receptor subtypes M1, M3 and M5 lead to increases in intracellular stores of Ca2+ and cell excitability by binding to pertussis toxin-insensitive G proteins (Gq/G11 family) (Eglen & Nahorski, 2000). The muscarinic receptor subtypes M2 and M4 are coupled to pertussis toxin-sensitive G proteins (Gi/Go) the activation of which leads to the inhibition of adenylyl cyclase,
the enzyme that synthesizes cyclic AMP, thus decreasing cell excitability (Harteneck, Plant, & Schultz, 2000; Felder, 1995).

Due to the lack of specific mAChR subtype-selective small-molecule ligands and the fact that most tissues or cell types express multiple mAChRs in a complex pattern, it took many years to determine which specific mAChR subtypes are involved in mediating the actions of Ach on DA neurons in the brain. To overcome these hinderances, researchers have developed gene-targeting techniques to generate mutant mice deficient in one or more mAChR subtype (Wess, Eglin & Gautam, 2007). Studies have revealed that M1–M5 mAChR-knock out (KO) mice can be produced that are viable and fertile, and do not show any major anomalies (Wess, 2004; Matsui et al., 2000). Each muscarinic receptor deficient mouse strain displays distinct pharmacological, behavioural, biochemical, neurochemical and electrophysiological deficits or changes (Wess, 2004; Matsui, M. et al., 2004) highlighting the importance of each receptor in various bodily functions.

As discussed previously most drugs of abuse, including morphine and heroin, trigger an increase in dopamine release in the nAcc which in turn reinforces repeated use of the drug due to the pleasurable effects induced by DA efflux. Forster et al. (2002) demonstrated that electrical stimulation of the LDT triggers a three-phasic pattern of changes in dopamine efflux in the nAcc of wild-type mice. The first phase of PPT and LDT-induced DA release lasts 2-3 minutes and is blocked by the administration of nicotinic and glutamatergic receptor antagonists into VTA (Forster & Blaha, 2000) or SN (Forster & Blaha, 2003). Thus, it is mediated by the activation of fast-acting ionotropic nicotinic and glutamatergic receptors. During the second phase, DA release declines to below baseline levels for approximately 8 minutes. Selective M2 methoctramine fully blocks the second phase. This suggests 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). Muscarinic antagonism with scopolamine into PPT and LDT also reduces this second phase in rats (Forster & Blaha, 2000; Forster & Blaha, 2003). Approximately 8-9 minutes later, the third phase begins which is characterized by prolonged DA efflux lasting approximately 40 minutes. The final phase of DA efflux is also mediated by muscarinic receptors in VTA and SN, as infusions of the antimuscarinic drug scopolamine blocks the third phase (Forster & Blaha, 2000; 2003). This phase is completely abolished though in M5KO mice (Forster, G. L., Yeomans, J. S., Takeuchi, J. & Blaha, C. D., 2002) indicating the importance of the excitatory M5 receptors located on the dopamine-containing VTA and SN neurons in mediating DA efflux.

Consistent with these studies, researchers have also demonstrated reduced rewarding effects of morphine and cocaine in CPP tests (Basile et al., 2003; Fink-Jensen et al, 2003, Thomsen et al., 2005), a decrease in morphine-stimulated dopamine efflux and Fos B expression in the nAcc (Basile et al., 2003), significantly less severe morphine dependent withdrawal symptoms when treated with the opioid receptor antagonist naloxone (Basile et al., 2003), and reduced acute cocaine self-administration (Wess, Eglin, Gautam, 2007), in M5 receptor deficient mice as compared to wild-type control mice. Finally infusions of M5 antisense oligonucleotides reduce sensitivity to brain stimulation reward (BSR), an effect reversed by the removal of the M5-specific oligonucleotides (Yeomans et al., 2000). These findings further stress a role of M5 receptors in mediating the effects of opiate drug addiction.

M5 receptor subtype represents less than 2% of the total muscarinic receptor family distribution in the brain (Yasuda et al., 1993). Various areas that M5 mRNA has been detected include the SN, VTA, striatum, hippocampus and ventromedial hypothalamus (Levey, Kitt,

**Dopamine and Opiate Reward**

According to the dual systems model for opiate reward, dopamine mediates opiate motivation depending on the deprivation state of the animal. When an animal is opiate dependent and in withdrawal, or food-deprived, dopamine mediates the motivational properties of stimuli, however when an animal is drug-naive or food-sated, dopamine does not (Nader et al., 1997). Many studies have been done in order to show the presence of DA-independent versus DA dependent opiate reward pathways. D2 receptor KO mice demonstrate acquisition of morphine CPP in drug-naive state but fail to acquire place preference when conditioned in the deprived state (Dockstader, Rubinstein, Grandy, Low, & van der Kooy, 2001). Furthermore, infusion of alpha-flupenthixol, a DA antagonist, into the NAc fails to block morphine conditioned place-preference in morphine-naïve animals as compared to wild types, suggesting that accumbal dopamine receptors are required for opiate reward signaling only in drug-deprived motivational states (Laviolette, Nader, van der kooy, 2002). Further, in food-deprived or drug-dependent rats, CPP for a morphine-paired environment is blocked by alpha-flupenthixol, but not by lesions of the PPT. In contrast, lesions of the PPT, but not alpha-flupenthixol, completely abolish 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 animals (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 and nucleus accumbens but which bypass the PPT.

**The Combined Effects of GABA and Acetylcholine on Dopamine Efflux**

Based on the findings presented, it is hypothesised that opiates exert their rewarding effects through inhibition of GABA neurons leading to the disinhibition of mesopontine DA neurons and hence increased DA efflux 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 μ-opioid receptors on VTA and SN GABA neurons leads to inhibition of GABA (Johnson & North, 1992) and hence a strong disinhibition of cholinergic PPT and LDT neurons is predicted. PPT and LDT activate midbrain DA neurons through M5 receptors located on these neurons. This is so because muscarinic antagonism or lack of M5 receptors blocks prolonged accumbal DA release (Forster & Blaha, 2000; Forster, Yeomans, Takeuchi, & Blaha, 2001). Furthermore, 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). Along the same line of evidence, morphine-induced striatal (Miller, Forster, Yeomans, & Blaha, 2005) and accumbal DA efflux is blocked by intra-VTA scopolamine, a muscarinic antagonist, in rats and mice, or in mice lacking the M5 muscarinic receptor (Steidl, 2008). Therefore, morphine, like other opiate drugs works specifically through inhibitory μ-opioid receptors on GABA neurons in the VTA and SN to disinhibit PPT and LDT cholinergic neurons, which exert their effects through M5 receptors to induce DA release.

**Drug-Induced Locomotion**
According to the psychomotor stimulant theory of addiction, it is believed that a common underlying measure of all addictive substances is psychomotor activation (Wise & Bozarth, 1987). This view elaborates on the theory that all positive reinforcers activate a common biological mechanism associated with approach behaviours. This biological mechanism includes dopaminergic fibers that project from midbrain dopamine systems to limbic and higher cortical regions to induce motion. The locomotor effects of these drugs seem to derive from increased concentrations of dopamine in synapses of nucleus accumbens, and the stereotypy seems to derive from increased concentrations of dopamine in synapses of the caudate nucleus (Creese & Iversen, 1975; Kelly et al., 1975). We know from the studies reviewed above that NAc is activated by afferent cholinergic projections from the VTA in order to elicit pleasurable feelings upon drug administration. Studies have also shown that 6-hydroxydopamine lesions of the region of the nucleus accumbens block the locomotor activation and attenuate the reinforcing properties of amphetamine and cocaine as measured by self-administration (Roberts et al., 1980, Lyness et al., 1979).

It is hypothesised that drug-elicited goal-directed behaviour such as running is analogous to an adaptive survival mechanism which causes an animal to explore and indulge in risk taking behaviour. Due to fear of predation, animals such as rodents do not usually venture from their nests unless there is a strong motivational need to eat, drink or mate (Wasserman, 2008). These natural reinforcers have all acquired rewarding properties presumably due to the efflux of DA upon their acquisition, and the urge to seek them represents a need to seek their rewarding effects disregarding the possibility of predation. The motivation to obtain these rewards is so strong that the anticipation of them alone may be enough to induce DA release which may elicit goal-directed locomotor behaviour.

*Morphine-induced Locomotion*
Morphine has also been shown to induce locomotion in a dose dependent fashion. This effect is strongly mediated through M5 receptors. This can be seen through the observation that M5 knockout mice show a 50% reduction in total morphine-induced locomotion as compared to wild-types (Steidl and Yeomans, 2009). It was also found that M5 knockout mice are less sensitive to the locomotor inhibiting effects of naltrexone, either alone or in combination with morphine (Steidl and Yeomans, 2009). Furthermore, M5 deficient mice display reduced locomotion in response to atropine or mecamylamine (non selective muscarinic agonists) administration into the VTA (Steidl and Yeomans, 2009). This indicates that non-M5 receptors in VTA strongly inhibit locomotion whereas M5 receptors induce it.

Opioid receptor mediated effects on locomotion also exist as opioid receptor antagonists reduce the amount of morphine-induced locomotion observed in mice in a dose dependent fashion (Frischknecht, Siegfried, Riggio, & Waser, 1983). Furthermore, μ-opioid receptor deficient mice show no locomotion in response to systemic morphine at 3 and 10 mg/kg doses (Hall et al., 2003). Morphine-induced locomotion is heavily dependent on dopamine as systemic DA D1-receptor antagonist SCH 23390 dose-dependently reduced morphine-induced locomotion (Longoni, Spina, & Di Chiara, 1987) and pre-treating dopamine deficient mice with a single 30mg/kg dose of L-DOPA one hour prior to testing morphine-induced locomotion restored locomotor responses (Hnasko, Sotak, & Palmiter, 2005). Therefore, all these results indicate that morphine-induced locomotion is elicited through μ-opioid receptor mediated disinhibition of PPT and LDT cholinergic inputs to VTA DA neurons through M5 receptors and in part through non-DA systems.

_Ultrasonic Vocalizations as Measures of Reward_
Important information about the motivational state of an animal can be obtained by measuring its vocalization, most of which occur in the ultrasonic range. Such ultrasonic vocalizations (USVs) are used to communicate information regarding their identity (individual or group), their group status or mood (dominance, fear, or aggression), their next likely behaviour (approach, play, groom, or mount), environmental conditions (presence of predators or location of food) and to facilitate mother–infant interactions (Portfors, 2007). Rodents have often been used to study basic mechanisms underlying emotional and motivational functions through studying their USVs.

**Types of Calls**

Rats emit 2 distinctly different types of USVs. The first is in the 18-25-kHz range (22-kHz) and the second in the 30-100-kHz range (50-kHz). Each type depends on the physical and psychological demands of the environment suggesting that these vocalizations represent different affective states of the animal (Portfors, 2007). Juvenile and adult rats emit 22-kHz vocalizations in a number of aversive and distressing behavioral situations including exposure to predators, inescapable pain, startling noises, and during male–male aggression and social defeat. The behaviors the rats exhibit in these types of situations and during production of the 22-kHz vocalizations include enhanced breathing and tense crouching. When these 22-kHz vocalizations are played back to rats, they display freezing and avoidance behaviors. Thus, 22-kHz vocalizations may function primarily to indicate negative affective state in rats and secondarily as alarm calls to warn conspecifics of predators or other potential threats (Portfors, 2007).

Rats emit short, chirping 30-100-kHz vocalization during sexual behaviors, male agonistic behaviors during fighting, juvenile play, and manual tactile stimulation (‘tickling’) by experimenters. During the emission of 50-kHz vocalizations, rats are engaged in locomotor activities such as approaching another rat, rearing, and exploring. These vocalizations have not
been recorded during freezing behaviors as occurs with 22-kHz vocalizations. Consequently, since 50-kHz vocalizations occur in contexts involving potential rewards, emission of this vocalization by a rat may indicate a positive affect in the same manner as do the excitement and heightened arousal elicited by anticipation of rewards (Portfors, 2007). VMH lesions in male rats abolish sociosexual behaviours such as scent marking and 50 kHz USVs (Harding & McGinnis, 2003).

Squeaks and USVs are used by mice in different contexts to convey affect (Panksepp et al., 2007). Mouse USVs can be induced by female mouse urine and pheromones and are often observed during sexual behaviour, a rewarding context. Hence, squeaks in mice are believed to express negative affect while USVs often denote positive affect. The negative squeaks of a mouse can be compared with the 22 kHz calls of a rat, and the positive 50 kHz rat vocalizations can be compared with the mouse USVs (Portfors, 2007).

**Mating-induced USVs**

Male mice reliably produce 50 kHz USVs in the anticipation of sexual encounters (Whitney & Nyby, 1979). Furthermore, female mice are attracted to male USVs (Guo and Holy, 2007) and hence they may facilitate courtship and mating representing an evolutionarily adaptable response. The presence of a female, however, is not required in order for a male to produce USVs as female urine and/or pheromones are sufficient enough to elicit them. In addition, the elicitation of USVs is strain, but not species, dependent in that female urine from other mouse strains can elicit USVs but neither female rat nor human urine is able to do so (Wang et al., 2008). Urine from immatures of either sex however does not elicit ultrasounds from socially experienced adult males indicating that it is the anticipation of sexual reward that elicits the vocalizations (Whitney & Nyby, 1979). Like pup USVs that have evolved to assist in mother-
infant bonding, male USVs might enhance physical proximity and enable successful courtship and mating (McIntosh et al., 1978).

**USVs and Dopamine**

There is significant evidence to suggest that 50-kHz frequency modulated calls represent activation of the DA system. For example, microinjections of amphetamine in and around the nucleus accumbens in the rat brain induced the emission of short 50-kHz calls and increased motor and locomotor activity (Burgdorf, Knutson, Panksepp & Ikemoto, 2001; Thompson, Leonard & Brudzynski, 2006). The amphetamine-induced increases in 50-kHz calls were dose-dependent and strongest when the shell of the accumbens was activated. The calls were also reversed by local application of D1 or D2 dopamine antagonists (Thompson, Leonard & Brudzynski, 2006). Glutamate injections in the hypothalamic pre-optic area also caused the production of 50 kHz calls and this was due to the release of dopamine as the emission calls was reversed by application of dopamine antagonist, haloperidol (Wintink & Brudzynski, 2001). Similarly, male M5 deficient mice show an 80% reduction in USVs as compared to wild-types when paired with a female or injected with amphetamine (Wang, Liang, Burgdorf, Wess, & Yeomans, 2008).

**HSV Viral Transfection**

Viral vectors have gained widespread use as vehicles for studying gene function. With this technique, the effect of a single gene can be studied by using viruses to splice a particular sequence of DNA into a living host. The most widely used defective viral vector is the Herpes Simplex Virus (HSV)-1 (Slacks and Miller, 1996). There are many advantages to using this particular virus. Firstly, it can efficiently infect neurons by entering them in a latent state. This allows them to maintain their transcription activity without interrupting neural functioning
(Simonato et al., 2000). A further advantage to using the HSV is that it has a very large genome, 152 kilobases (kb) of double stranded DNA coding for 75 genes, which has been fully mapped out and it is relatively easy to manipulate also allowing for large inserts (Simonato et al., 2000). An additional advantage of using this virus is that gene expression is observed promptly after the HSV is infused into the brain, reaching maximal expression within 24-72 hours. Expression reduces by 50% 5 days after viral transfection and drops another 50% after 6 days. By the 10th day of viral transfection, the virus is completely removed from the brain (Simonato et al., 2000).

A modification commonly made to the HSV is the inclusion of a gene for Green Fluorescent Protein (GFP). GFP is a 238 amino acid bioluminescent protein that was originally isolated from the jellyfish, Aequora victoria (Aboody-Guterman et al., 1997). GFP has become an integral part of many viral vectors as it only requires light to activate (Desai and Person, 1998) and conveniently allows the ability to visualize successful viral transfections (Aboody-Guterman et al., 1997). GFPs can fluoresce in different colours, such as red, yellow, or blue, in response to different wavelengths of light (Lichtman & Conchello, 2005). A further modification to the virus is to make it replication-defective so that it cannot lead to infections. The HSV virus has been used in many important studies measuring adaptations in brain reward systems. Repeated exposure to morphine or cocaine increases expression of the AMPA (glutamate) receptor subunit GluR1 within the VTA (Fitzgerald, Ortiz, Hamedani & Nestler, 1996). To directly investigate whether a drug-induced molecular adaptation also causes drug-induced behavioral adaptations, such as sensitization, Neve et al., (2005) used the HSV vector to elevate GluR1 levels within the VTA of drug naive rats. Rats with viral-mediated elevations in GluR1 expression within the VTA showed dramatic increases in sensitivity to the rewarding effects of morphine (Carlezon et al., 2000). These data suggest that the behavioral consequences of repeated exposure to morphine are mimicked by gene transfer of GluR1 into the VTA and
support a causal relation between previously disparate drug-induced molecular and behavioral adaptations.

Similarly, psychostimulant drugs such as amphetamine or cocaine regulate the transcription factor CREB in the NAc (Cole, Konradi, Douglass & Hyman, 1995). In rats, HSV mediated elevations of CREB in the NAc make low doses of cocaine aversive, and higher doses of cocaine less rewarding (Carlezon, 1997). These types of behavioural studies demonstrate how viral-mediated gene transfer offers a unique strategy with which to examine how temporally specific alterations in the expression of a single, localized gene product can lead to changes in behaviors that reflect complex motivational states. Moreover, such studies help to identify intracellular pathways and alterations in gene expression that are potential targets for new generations of pharmacotherapies for addiction, depression, and perhaps other neuropsychiatric disorders (Carlezon, 1997).

**Hypothalamic Involvement in Dopamine Systems**

In females the VMH plays a role in the regulation of sexual behavior. Lesion, stimulation, and hormone implant studies in female rats have revealed that estradiol and progesterone act at the level of the ventrolateral hypothalamus to modulate the lordosis circuit (Blaustein & Erskine, 2002; Pfaff, Schwartz-Giblin, McCarthy, & Kow, 1994). The VMH contains a high concentration of androgen receptors (Chamness, King, & Sheridan, 1979; McGinnis, Davis, Meaney, Singer, & McEwen, 1983; Simerly, Chang, Muramatsu, & Swanson, 1990), and male reproductive behaviors are known to depend on circulating levels of testosterone (Hull, Meisel, & Sachs, 2002). It has been shown that blockade of androgen receptors in the VMH results in fewer males copulating (McGinnis, Williams, & Lumia, 1996); androgen receptor antagonist administered to the VMH impairs ultrasonic vocalizations and sexual
motivation (Harding & McGinnis, 2004) and that testosterone implanted into this region is sufficient to restore sexual motivation in castrated animals (Harding & McGinnis, 2003).

Dopamine’s role in the hypothalamus is very different from its role in the NAc. DA in the lateral hypothalamus (LH) is involved in the anorexigenic effect of amphetamine. This can be observed through the finding that a local injection in LH of sulpiride, a D2 receptor antagonist is sufficient to induce feeding and drinking (Leibowitz, 1975) and with prolonged injections, obesity (Baptista, 1999). Microdialysis studies reveal that eating induces DA efflux in the LH. Hence, DA in the hypothalamus may also be important in locomotion related to food and water seeking (Parada et al., 1990). From the VMH GABAergic projections are sent to the LH which may inhibit feeding (Beverley & Martin, 1989). The VMH also receives DAergic inputs which have an opposing effect as that observed in the LH causing a decrease in DA efflux during a meal (Yang et al., 1997). The source of DA could be from local DA clusters as well as mesolimbic system (Fuxe & Ungerstedt, 1968).

As the hypothalamus is an important site for many motivating behaviours such as hunger, thirst, sex and analgesia (Valenstein, Cox, & Kakolewski, 1968); is close to the arcuate nucleus which is the hub of endomorphin production and is connected to the PAG, it would be interesting to see the behavioural drug induced effects in this region through the activation of M5 receptors.

**Objectives of Current Thesis**

As discussed above, the PPT and LDT exert their physiological effects of inducing DA release in the NAc through activation of M5 receptors located on midbrain DA neurons. This makes the study of M5 receptors an imperative and essential task in understanding opiate drug action and drug addiction. Most studies however have focused on studying M5 receptors in the
VTA and SN. It has been observed however that a large cluster of M5 receptors also exist in the ventromedial portion of the hypothalamus (VMH; Levey, Kitt, Simonds, Price, & Brann, 1991; Vilaro, Palacios, & Mengod, 1990; Weiner, Levey, & Brann, 1990; Wang, et al., 2004). It can be seen in the Allen Brain Atlas that M5 receptors indicate moderate to high expression levels in the ventromedial hypothalamus (Figure 1).

In this thesis, HSV was utilized to transfect the M5 mAChR and GFP genes into the VMH of CD1-129 mice in order to activate M5 receptors. The technique was borrowed from Neve et al. (2005) to see the effects of activating these receptors above and beyond normal levels. As discussed, M5 KO mice show several deficits in their responses to rewarding stimuli. In response to morphine, these mice show 40-50% less locomotor activation. In response to a female in a mating paradigm, they also emit 70-80% fewer USVs. By reintroducing the M5 gene into the VTA, these deficits were able to be rescued (Wasserman, 2009), providing evidence for the importance of the M5 receptor in the VTA for reward-seeking and the stimulant effect of morphine. The VMH however is still under studied in terms of cholinergic DA activation and of DA-related behaviours although it is also rich in muscarinic receptors, which is why it is the chosen site of activation for this project.

In order to test whether there was an involvement of DA in inducing morphine-induced locomotion according to a VMH-VTA circuit, haloperidol injections were given prior to morphine and saline administration in a separate group of mice to see the effects on motility.

Haloperidol is a potent neuroleptic that has a high affinity for binding with and blocking D2 receptors; it is thus a DA antagonist (Costall, & Olley, 1971). Previous lesion studies have exemplified that DA receptors in the striatum are involved in neuroleptic–induced catalepsy (Creese, Usdin & Snyder, 1979), therefore a smaller dose of Haloperidol was used (0.6mg/kg) as compared to morphine (10mg/kg).
Figure 1. Allen Brain Atlas (a) Reference Picture for VMH (b) Expression of M5 receptors (c) Expression of M5 receptors in the VMH depicting moderate to high expression as indicated by the green and red clusters.

**Materials and Methods**

*Mice*

Twelve male CD1x129 WT mice were used in this experiment. They were all 3 months old and weighed approximately 30-40 g each. Six randomly chosen mice underwent surgery for HSV-M5-GFP transfection targeting the VMH whereas the other six mice underwent surgery using the control vector.

*Housing*
Mice were housed in groups of 2-4 in opaque cages. Throughout the duration of the experiment mice were provided with food and water ad libitum, except during behavioural testing. The mice were maintained on a 12 hour light/dark cycle (lights on 7:00 AM-PM). Testing always took place between the hours of 9am to 6pm (i.e. the inactive period of the cycle). A minimum of one week prior to initiation of the experiment, mice were relocated from the main breeding colony to a small housing room adjacent to the testing room. All housing and experimental rooms had controlled temperature (20 ± 1 °C) and humidity (approximately 55-60%). All experimental protocols using animals were performed in accordance with the *Guide to the Care and Use of Experimental Animals* (Canada), and approved by the Animal Care Committee of the University of Toronto.

*Time Course of Experiment*

All experiments were carried out over the course of 5 days with 2-4 mice infected and tested weekly. On Day 1, surgery was performed and the HSV-M5-GFP or Control virus was infused bilaterally into the VMH of anaesthetised mice. The following day (Day 2), animals received post-operative care and were allowed to recover. Testing began on Day 3, on which mating induced USVs were collected and analysed. On day 4, locomotion tests were performed on animals after saline (10 mL/kg) followed 2 hours later by 10 mg/kg morphine injections. On day 5, the mice were perfused, and their brains extracted. The following week, the brains were sliced, stained, and sections prepared for histological analysis.
Table 1: Time course of the experiment

| DAY 1 | Surgery  
<table>
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<tbody>
<tr>
<td></td>
<td>HSV –M5 or HSV-Control</td>
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<tr>
<td>DAY 2</td>
<td>Post-operative care</td>
</tr>
<tr>
<td>DAY 3</td>
<td>Mating-induced USV collection</td>
</tr>
<tr>
<td>DAY 4</td>
<td>Saline and 10mg/kg morphine locomotion</td>
</tr>
<tr>
<td>DAY 5</td>
<td>Perfusion and Histology</td>
</tr>
</tbody>
</table>

**Surgery**

Mice were anesthetised in a plexiglass box infused with isoflurane gas. A concentration of 3% isoflurane was used to induce analgesia followed by a decrease to 1.5-2% isofluorane for the duration of the surgery. O₂ flow rate was maintained at 1 L/min. Once anesthetised, mice were transferred to a mouth piece and given sub cutaneous (s.c.) injections of 10 mL/kg of 10 mg/mL Anafen (Merial Canada Inc, Baie d’Urfé, QU), a nonsteroidal anti-inflammatory ketoprofen solution, 10 mL/kg of 50 mg/mL Baytril (Bayer Health Care, Toronto, ON), an antibacterial and antifungal solution, and 10 mL/kg sterile saline to restore hydration. The mice were then shaven from between the eyes to between the ears so that the skin overlying the skull may be reached for incision. The mice were then placed in a Benchmark Stereotaxic frame (Leica Microsystems, Bannockburn, IL) with body temperature maintained at 38 ± 0.5 ºC using a TC-1000 Temperature Controller heating pad (CWE INC). Before scalp incision, the scalp was disinfected with 70% ethanol and treated locally with an iodine solution. The skull was then slit open using a blade and bregma and lambda were identified. Holes were then drilled in the skull at coordinates for VMH: M/L ± 1.22; A/P -2.70 (Paxinos and Franklin, 2004) to allow access to the brain. Tygon tubing (0.1905 mm diameter) was attached to 30 gauge injectors, which were
fed through 23 gauge guide cannulae and inserted through the stereotaxic arms to insert into the brain. To avoid puncture of the sagittal sinus, cannula were angled 10° medially and lowered to coordinates D/V, -5.58 (Paxinos and Franklin, 2004). Bilateral microinjections (0.3 μl) of the HSVM5-GFP vector or Control virus were delivered manually over 5 min through the injector cannula and were left in place for an additional 10 min to ensure diffusion. To verify the delivery of each intracranial injection, an air bubble was observed moving a calibrated distance through the Tygon tubing. After the period allowed for diffusion, cannula were carefully retracted, the animal’s wound was sutured using a continuous blanket stitch using a silk thread and Polysporin was applied to the wound. The animals were given pure O₂ until recovery from anaesthetic was observed. Following surgery, each mouse was placed in a clean individual cage and given food moistened with water, topped with Nutri-cal (Vetoquinol), a palatable high-calorie dietary supplement.

On postoperative Day 2, mice were briefly anaesthetized once again using isofluorane (3% for induction and 1.5 - 2% for maintenance; O₂ flow rate, 1 L/min) and treated for postoperative care. Mice were given s.c. 10 mL/kg of Anafen in 10 mL/kg 0.9% sterile saline to help reduce pain and assist with hydration. The wound was lightly cleaned with sterile saline and fresh Polysporin was again applied to the stitches. The animal was given new moist food with Nutri-cal and returned to the housing area.

Drugs

Morphine sulfate pentahydrate (Sigma, St. Louis, MO) was dissolved in 0.9% sterile saline and intraperitoneal injections were given at a dose of 10mg/kg and at a volume of 10 ml/kg. Morphine doses were calculated according to an average weight of 30-50g/mouse.

Haloperidol was also dissolved in 0.9% sterile saline and intraperitoneal injections were given at a dose of 0.6mg/kg and at a volume of 10 ml/kg. Haloperidol doses were calculated
according to an average weight of 30-50g/mouse. Previous research suggests that a dose of 1mg/kg of haloperidol is sufficient to induce cataleptic seizures (Sanberg, 1980). For this reason a dose of 0.6 mg/kg was chosen to be consistent with the literature and in order to prevent any extreme neuroleptic effects.

**Mating-Induced USVs**

*Equipment*

USVs were recorded using an Ultrasound Detector D1000X (Pettersson Elektronik AB, Uppsala, Sweden). The sampling frequency was set to a maximum 300 kHz to pick up all calls between the frequency ranges of 1 to 300 kHz. On Day 3 group housed WT female CD1-129 mice were placed in separate polyethylene cages with fresh bedding and brought into the testing room. During testing, the top of the cages were removed and a condenser microphone of the D1000X was suspended 12 cm above the bottom of the cage using an arm.

*Procedure*

Each male transfected with HSV-M5 or Control Virus was placed successively in the USV chamber with a female. Immediately prior to the pairing, audio and video acquisition was turned on and continued for 4 minutes. The females were randomly chosen from a group and were not repeated twice. The digitized audio and video recordings were coded and blindly scored by trained scorers.

*Analysis*

The ultrasonic audio recordings were analysed using Avisoft SASLab Pro software (Avisoft Bioacoustics, Berlin, Germany). This program performs a continuous fast-Fourier analysis and displays the results on a sonogram. Blind scorers reviewed each 4 minute trial and noted the number of times each ultrasonic vocalization was emitted.

*Statistics*
Total USV counts each minute were taken in a repeated measure design and analyzed with a repeated measures multivariate and univariate analysis of variance (ANOVA). The within-subject repeated factor was Time whereas the between subject factor was Group. The dependent variable was the total number of calls produced each minute. Separate univariate ANOVAs were also conducted at each minute with Group as the between-subject factor.

**Locomotion Testing**

**Apparatus**

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). Mice were recorded with a video camera (Panasonic, Model #WV-CP484, Osaka, Japan) placed approximately 2 m above the testing apparatus. The testing room was lit by two 40-watt light bulbs located approximately 3 m above testing chambers.

**Locomotion Paradigm**

At the start of 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. They were then placed in the testing chambers for 1 hour without receiving any injections to acclimatize to the boxes. Mice were then briefly removed from their boxes for an i.p. saline injection and were immediately placed back in the centre of their box. Video recordings occurred over the next 2 hours to track saline-induced locomotion. After the culmination of 2 hours, mice were again briefly removed from their boxes to be injected i.p. with 10 mg/kg morphine, dissolved in 0.9% saline, before being returned to the centre of their respective boxes (Figure 2). Again, video acquisition occurred for 2 hours to track
the 10 mg/kg morphine-induced locomotion. Following the morphine run, mice were returned to their home cages and returned to the housing room.

In a second experiment done to measure the effects of haloperidol injections on saline- and morphine-induced locomotion, the same paradigm was used, however, haloperidol was injected 20 minutes prior to either the saline or morphine injections. This delay in injecting was given because peak plasma concentrations of haloperidol occur in 15 to 20 minutes. Therefore in order to confirm that all DA receptors have been blocked before the injection of morphine, this time lag between injections was necessary.

Figure 2. Open-Field Apparatus. Mice are placed in the centre of a black box. Their psychomotor locomotion is being tracked by a computerized tracking program calculates the distance travelled. Saline- and 10 mg/kg morphine-induced locomotion was scored for a 2 hour period in each trial.

**Analysis**

Video records were analyzed using Noldus Ethovision (Groningen, Netherlands), which provided a measure of total forward locomotion in cm for each 10 minute time bin. Data obtained for saline and morphine was analyzed separately.

**Statistics**
Locomotor activity was analyzed with a Mixed Model ANOVA design with the repeated within-subjects factors set as Time (each of the 12, 10 minute bins) and Dose (0, 10 mg/kg morphine), the fixed between-subjects factor as Group, and the dependent measure as ‘distance travelled over a 10 min period’. Main effects of Time, Dose, and Group were calculated as well as their interactions. At each individual dose, a Mixed Model ANOVA was also performed to further investigate the main effects of Group and Time and their interaction. 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.

Histology

On the last day of the experiment (Day 5) mice were 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 then extracted from the skull and stored in 10% formalin 30% sucrose solution for 2 days prior to sectioning (50 microns) using a Cryostat. Sections were mounted and examined by light microscopy following cresyl violet staining.

Results

Histology

Estimated coordinates of the cannula tips in the VMH for WT mice are shown in Table 2. Cannulae tip placements were considered hits if bilaterally located within the anatomical boundaries of the VMH. Figure 3 shows the injection sites on para-sagittal atlas sections. Six mice (M2-HSV, M3-HSV, M4-HSV, M5-HSV, M1-CNT, M5-CNT) showed bilateral “hits” in
and around the VMH. Six mice (M1-HSV, M6-HSV, M2-CNT, M3-CNT, M4-CNT, M6-CNT) had unilateral VMH “hits”. See Plate I for GFP fluorescence.
<table>
<thead>
<tr>
<th>Mouse/Batch</th>
<th>Left Hemisphere</th>
<th>Right Hemisphere</th>
<th>Hit</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Bregma</td>
<td>D/V</td>
<td>M/L</td>
</tr>
<tr>
<td>M1 (HSV)</td>
<td>-1.70</td>
<td>5.60</td>
<td>0.25</td>
</tr>
<tr>
<td>M2 (HSV)</td>
<td>-1.70</td>
<td>5.70</td>
<td>0.25</td>
</tr>
<tr>
<td>M3 (HSV)</td>
<td>-1.70</td>
<td>5.70</td>
<td>0.25</td>
</tr>
<tr>
<td>M4 (HSV)</td>
<td>-1.70</td>
<td>5.70</td>
<td>0.25</td>
</tr>
<tr>
<td>M5 (HSV)</td>
<td>-1.70</td>
<td>5.60</td>
<td>0.50</td>
</tr>
<tr>
<td>M6 (HSV)</td>
<td>-1.94</td>
<td>5.70</td>
<td>0.80</td>
</tr>
<tr>
<td>M1 (CNT)</td>
<td>-1.70</td>
<td>5.70</td>
<td>0.25</td>
</tr>
<tr>
<td>M2 (CNT)</td>
<td>-1.75</td>
<td>5.25</td>
<td>0.25</td>
</tr>
<tr>
<td>M3 (CNT)</td>
<td>-1.70</td>
<td>5.90</td>
<td>0.60</td>
</tr>
<tr>
<td>M4 (CNT)</td>
<td>-1.70</td>
<td>5.25</td>
<td>1.00</td>
</tr>
<tr>
<td>M5 (CNT)</td>
<td>-1.58</td>
<td>5.60</td>
<td>0.75</td>
</tr>
<tr>
<td>M6 (CNT)</td>
<td>-1.70</td>
<td>5.50</td>
<td>0.60</td>
</tr>
</tbody>
</table>

Table 2. Estimated co-ordinates of cannula tip placements within VMH
Figure 3. VMH cannula placements in WT mice. Numbers indicate rostro-caudal distances from bregma (adapted from mouse brain atlas by Paxinos & Franklin, 2001)
**Saline- and Morphine-Induced Locomotion**

A Univariate Analysis of Variance (ANOVA) indicates that there was a significant difference between the mean locomotion of Control animals in the saline and morphine conditions \( F(1,22)=5.231, p<0.05 \), as well as a significant difference between the mean locomotion of HSV-M5 mice in both the saline and morphine conditions. \( F(1,22)=7.522, p<0.05 \). Between the two conditions (HSV-M5 and Control), there was also a significant difference between the mean locomotion in the morphine trial \( F(1,22)=57.174, p<0.05 \), but not in the saline trial \( F(1,22)=.459, p>0.05 \). This indicates that saline did not induce any locomotion above and beyond base line in both conditions, as expected. However, due to the transfection of the M5 virus, a significant difference in the amount of morphine locomotion between the two groups was observed.

A Multivariate Mixed Model ANOVA across both conditions revealed a significant between subjects effect \( F(1,10)=412.232, p<0.05 \). These results indicate that animals given HSV-M5 transfections and Control transfections differed significantly in the amount they locomoted during the 2 hours time period. There was also a significant effect of time \( F(1,10)=17.959, p<0.05 \) and the interaction between time and condition \( F(1,10)=5.326, p<0.05 \). This indicates that a change in locomotion occurred during each time bin and between saline and morphine trials during each time course period respectively. There was also a significant interaction between dose (saline or morphine) and condition (HSV-M5 and Control) indicating that the average locomotion differed between conditions for both the 0 and 10mg/kg morphine dose.

A Univariate ANOVA revealed that a significant difference exists between the mean locomotion of HSV-M5 animals in the morphine and haloperidol-morphine conditions \( F(1,22)=34.039, p<0.00 \). This indicates that after a haloperidol injection, morphine-induced
locomotion changed significantly from the condition in which haloperidol was not injected prior to a morphine-injection. There were no significant differences in the amount mice locomote in the saline and haloperidol-saline conditions for HSV-M5 injected mice. There were also no significant differences in the M5-Control conditions in terms of morphine and haloperidol-morphine or saline and haloperidol-saline conditions.

Figure 4. A comparison of the time course of saline-induced locomotion in HSV-M5 (n=6) and Control (n=6) mice. As expected, there is no significant difference in locomotion between HSV-M5 and Control mice. Bars represent standard error of the mean.
Figure 5. A comparison of the time course of morphine-induced locomotion (10mg/kg i.p) in HSV-M5 (n=6) and Control (n=6) mice. There is a 3x increase in locomotion in HSV-M5 mice as compared to Control mice. Bars represent standard error of the mean.
Figure 6. A comparison of the time course of morphine- and haloperidol-morphine induced locomotion in HSV-M5 (n=6) and Control (n=6) mice. There is a 3x increase in locomotion in HSV-M5 mice. Bars represent standard error of the mean.

**USVs**

A Univariate ANOVA showed no significant difference in type II or DA dependent calls between M5-HSV and Control-HSV groups of mice: minute 1 F(1, 9) = 0.464, p > 0.3; minute 2, F(1, 9) = 0.463, p > 0.5; minute 3 F(1, 9) = 0.046, p > 0.8 and minute 4 F(1, 9) = 0.034, p > .85. There were also no differences in the amount of type III calls produced by both conditions: minute 1 F(1, 9) = 1.92, p > 0.199; minute 2 F(1, 9) = 0.767, p > 0.4; minute 3 F(1, 9) = 0.773, p > 0.4 and minute 4 F(1, 9) = 1.475, p > 0.25. These results suggest that an increase in M5 receptor genes in the VMH do not affect the types of calls produced by mice. There was a significant difference between average type I USVs and type II and III calls F(1, 2) = 6.45, p < 0.01.
More importantly, a Multivariate ANOVA across both conditions, M5-HSV and Control-HSV revealed insignificant between subjects effects $F(1, 6) = 0.42, p > 0.8$. These results indicate that there was no difference in the total amount of calls (type I, II and III) produced over the four minutes (Figure 7).

![Figure 7](image_url)

**Discussion**

The main goal of this thesis was to investigate the effects of systemically injected morphine after transfecting an M5-HSV virus into the VMH of WT mice. The results indicate that adding M5 muscarinic receptors to the VMH increase the stimulant effects of systemic morphine.

**Saline and Morphine-Induced Locomotion**

Saline-induced locomotion in mice injected with M5-HSV and or Control virus were not significantly different from each other. This is consistent with both Steidl (2007) and Wasserman’s (2009) work in which both show similar distances across the 2 hour bins during...
saline locomotion trials. This is expected though as saline does not have any psychomotor effect on mice and the locomotion elicited by it represents just a baseline estimation of motion. In the current thesis, high locomotion is seen during the first 30 minutes as compared to both Steidl (2007) and Wasserman (2009). This however, is only due to a difference in habituation methods. I habituated my mice for 1 hour in the locomotion box only, whereas Wasserman (2009) and Steidl (2007) habituated their mice to the testing room for an hour prior to putting them in the test boxes for an additional hour of habituation. In future studies I will make it imperative to follow consistent protocols so as to ensure that my results may be more readily comparable.

In WT mice injected with the M5-HSV virus, morphine induced locomotion increased by 6 times 30-60 minutes after a systemic morphine injection, as compared to mice with transfections using the HSV-Control virus. According to Steidl & Yeomans (2009) a mechanism involving M5 receptors mediates approximately 45 to 48% of systemic morphine-induced locomotion. The peak locomotion in Wasserman’s (2010) work reached approximately 6000 cm in WT mice injected with the M5-HSV virus. This is 2 times the peak results of the locomotion observed in the current thesis (3500cm). This disparity may suggest that there may be more of a DA involvement in the VTA as compared to the VMH which may produce the difference observed in morphine-induced locomotion.

According to Steidl (2007) and Wasserman (2009), M5 is an excitatory muscarinic receptor that depolarizes DA neurons and can lead to DA efflux. M5 KO mice lack the gene and as a result, have reduced DA related behaviours. By transfecting the gene back into the VTA, the M5 mAChR was restored to DA neurons and the behavioural DA related deficits of the KO mice were rescued (Wasserman, 2009). Increased efflux in NAc and striatal DA leads to increased morphine-induced locomotion, reward seeking, and stereotypy. This suggests that although the M5 has a key role in the VMH to produce locomotion in response to exogenous opioids, the
effect is not as strong as that produced in the VTA. The reason for this may be lesser activation of DA neurons by VMH stimulation or a more limited involvement of non-DA neurons in this locomotion circuit. That is, there may be DA-independent effects that cause locomotion following VMH activation that produce this difference in results. The μ opiate receptor is found predominantly on non-DA neurons (Di Chiara et al., 1988). While some of the rewarding effects of opiates may be dependent on DA neurons of the arcuate nucleus and DA receptors in the VMH, there remain strong DA-independent effects that are still rewarding in the absence of DA.

These DA-independent effects may be mediated through the PAG. Recent evidence has suggested a role for the PAG in morphine-induced analgesia and has further implicated the PAG as a potential central locus of opiate effects since significant amounts of opiate binding sites have been demonstrated there (Hiller, Pearson, & Simon, 1973; Pert & Snyder, 1975). Stimulation of the PAG activates enkephalin-releasing neurons which bind to μ opioid receptors (Pert, Pasternak, & Snyder, 1975). These receptors may be present in the VMH and their activation may inhibit the release of pain signals up the spinothalamic tract to the thalamus. Therefore, the locomotion effect may be associated with analgesia, an overall sense of relief that may motivate the mice to move about. Further research in this area is required however to understand what the exact mechanisms are underlying morphine-induced locomotion in the VMH.

Increased DA in the NAc is believed to be a cause of goal-oriented locomotion (Tzschentke, 2001). Thus, the enhancement of locomotion after 10 mg/kg morphine may result from a cholinergic effect on VMH DA neurons through transfection of the M5 mAChR. Morphine exerts its rewarding effects primarily through activating the μ-opioid receptor on GABA neurons in the posterior VTA (Ford et al., 2006). Upon activation, GABA neurons are inhibited, which subsequently disinhibits the PPT/LDT and/or SN/VTA (Miller et al., 2002; Forster et al., 2002b). This disinhibition increases cholinergic release in the VTA which activates
mACHRs on DA neurons causing increased efflux in the NAc and striatum (Steidl and Yeomans, 2009). This increased DA efflux is thought responsible for morphine-induced locomotion. The fact that VMH HSV-M5-GFP transfection caused an increase in locomotion when injected with morphine as compared to saline, suggests that the VMH could be a part of this PPT/ LDT neuronal circuit that mediates drug induced rewarding behaviours.

Neural projections exist from limbic forebrain structures and hypothalamus along the medial forebrain bundle (MFB) to the VTA. This makes the VTA an important and perhaps an essential, contributor towards drug-induced rewarding effects (Krieger, Conrad, & Plaff, 1979). It also provides further evidence in support for the hypothesised circuit mentioned above. Similarly, when discrete lesions were placed at the tips of the electrodes in the MFB-LH, from which electrical stimulation elicited drinking and feeding, degenerated fibers were traced to the VTA (Huang & Mogenson, 1972). Also, lesions placed caudal to stimulation electrodes in the MFB-LH were observed to attenuate elicited feeding, presumably by disrupting the neural connections to the VTA (Bergquist, 1970).

Moreover, experiments have shown that discrete lesions of the neural connections between limbic forebrain structures and the VTA disrupt signals for the initiation of goal-directed behaviors and not merely the motor components of these behavioral responses. The VTA is the locus of mesolimbic dopaminergic neurons which project to nucleus accumbens as well. Electrical stimulation of the VTA was shown to alter the spontaneous firing rate of neurons recorded from the nucleus accumbens (Yim and Mogenson, 1979). Responses included long latencies of excitation and DA efflux leading to locomotion (Mogenson, Jones & Yim, 1980).

To test whether there exists a role of DA in producing the morphine-induced locomotor effect observed, I subsequently tested mice with systemic injections of haloperidol (0.6mg/kg i.p), a D2-selective DA antagonist. M5-HSV mice in the haloperidol-morphine condition
displayed an almost 50% reduction in locomotion as compared to animals given solely morphine. Since Control mice injected with morphine had baseline levels of locomotion to begin with, injecting haloperidol in this group did not reduce their locomotor in such a dramatic way. Therefore, although after M5-HSV transfections in the VMH, the locomotion effect may not be as strong as that elicited in the VTA; it still has a DA component as it is reduced by systemic haloperidol injections. Ideally, two more control conditions would be necessary to validate the results: haloperidol-saline, to observe the inhibitory effects of this haloperidol dose on locomotion, and saline-morphine, to observe the effects of a previous injection on morphine-induced locomotion. Previous work with this dose of haloperidol however, suggests that these controls are unlikely to change the conclusion that DA receptors mediate part of the facilitating stimulatory effects of morphine.

This DA effect may be mediated through the DA neurons present in the VTA or elsewhere. According to studies carried out by Wasserman (2009) and Wang et al., (2003) the spread of HSV is approximately 1 mm in diameter and therefore does not likely spread to activate the VTA which is 2 mm posterior to the region. However, since the hypothalamus is a fairly small structure in the mouse brain, an HSV transfection in this area will likely spread to surrounding regions involving the arcuate nucleus activating EM1-LI and EM2-LI neurons as well as the dorsal edge of the VMH which is involved in beta-endorphin release. EM1-L1 and EM2-L1 neurons preferentially bind to the μ- opioid receptor which mediates the reinforcing properties of opiates. Endorphin neurons project to the NAc and thus DA neurons may be activated through this pathway as well to elicit locomotion.

The VTA is one of the most sensitive brain regions for the rewarding and locomotor effects of opioids. It contains mu-opioid receptors, which mediate GABA–dependent disinhibition of dopamine transmission to the nucleus accumbens. Hypothalamic EM-LI cells
project to the VTA, where they could play a natural role in this circuitry. Both EM1-LI and EM2-LI cells are present in the periventricular nucleus, between the dorsomedial and ventromedial hypothalamus and between the ventromedial and arcuate nuclei (Greenwell et al., 2002). Injections of endomorphin antibodies to the anterior and posterior VTA were both effective in producing double-labeled cells, and an anterior–posterior topographical organization between the VTA and hypothalamus (Greenwell et al., 2002). The results support the idea that some endomorphin-containing neurons in the hypothalamus project to the VTA, where they may modulate reward and locomotor circuitry. Endomorphins could serve as natural agonists in an opioid-mediated reward pathway (Greenwell et al., 2002). Further research however is required to gain a clearer picture of this hypothesis.

**USV Production**

There were no significant differences in total USV production between the M5-HSV or Control-HSV groups indicating that in the VMH, the M5 receptor may be more involved in producing opiate-induced locomotion rather than USV production, unlike the VTA. Wang et al. (2008) demonstrated that amphetamine injections exclusively increased frequency-modulated calls in males. Furthermore, M5 KO mice call less than a fifth as much as WT mice (Wang et al., 2008). These calls however, are fully restored through bilateral HSV-M5-GFP transfection of the VTA of M5 KO mice (Wasserman, 2009). The M5 mAChR is believed to increase USV production by causing tonic depolarization of DA neurons in the VTA through ACh from PPT/LDT neurons (Rada et al., 2000).

Interestingly, Wasserman (2010) also found that WT mice sing less after M5-HSV infusions in the VTA. The premise for this finding is that because WT mice already have a normal amount of M5 receptors and an HSV-M5 injection would only further enhance the
amount of M5 receptors, there is an abundance of DA in the mouse brain. Hence there is no motivation to sing and call for mating as they are already satisfied and therefore there is no functional purpose of producing calls (Wasserman, 2010). This theory has yet to be proven however and research is ongoing in this area using systemic haloperidol injections in order to investigate whether there is an increase in calling after a decrease in DA is induced. In the VMH the USV mediated circuit is unclear and further research is required in order to gain a better understanding of DA related calls in mice.

**Future Directions in Feeding and Limitations**

The VMH is most commonly associated with homeostasis and feeding behaviour. Since dopamine’s role in the hypothalamus is very different from its role in the NAc, it would be interesting to see what the effect of eating would be after M5-HSV transfections in WT mice. Measuring feeding behaviour was beyond the scope of this thesis but is something I am interested to investigate in the future. DA in the lateral hypothalamus (LH) is involved in the anorexigenic effect of amphetamine. This can be observed through the finding that a local injection in LH of sulpiride, a D2 receptor antagonist is sufficient to induce feeding and drinking (Leibowitz, 1975) and with prolonged injections, obesity (Baptista, 1999). Microdialysis studies reveal that eating induces DA efflux in the LH. Hence, DA in the hypothalamus may also be important in locomotion related to food and water seeking (Parada et al., 1990). From the VMH GABAergic projections are sent to the LH which may inhibit feeding (Beverley & Martin, 1989). The VMH also receives DAergic inputs which have an opposing effect as that observed in the LH causing a decrease in DA efflux during a meal (Yang et al., 1997). The source of DA could be from local DA clusters as well as the mesolimbic system (Fuxe & Ungerstedt, 1968).
The arcuate nucleus contains two populations of first-order, leptin-responsive neurons, specifically agouti-related protein/neuropeptide Y (NPY/AGRP) and pro-opiomelanocortin (POMC/CART) neurons (Schwartz and Porte, 2005). NPY/AGRP neurons promote weight gain and are inhibited by leptin (Cowley et al., 2001; van den Top et al., 2004), whereas POMC/CART neurons promote weight loss and are activated by leptin (Cowley et al., 2001; Elias et al., 1999). It may be possible that transfection of M5 receptor genes in the VMH triggers the inhibition of leptin receptors causing mice to move about with the motivation to look for food because they are hungry and this is the basis of their locomotion rather than DA induced drug related locomotion. Leptin is essential for normal body weight balance, but the exact mechanisms by which leptin activates hypothalamic neuronal circuitries is known to a limited extent. In order to find pharmaceutical approaches to treat eating disorders such as obesity, further studies will be needed to reveal the exact mechanisms by which leptin lowers body weight and which role leptin and leptin receptors have in the pathogenesis of human obesity.

A confusing issue that may arise with an M5-HSV transfection study, which takes into account feeding behaviour and leptin receptors, may be that because the hypothalamus is a fairly small area and the LH and arcuate have opposing effects on feeding, the chances of both areas getting infected are moderate to high unless a very small dose of virus is injected. This may result in opposing behaviours being activated resulting in unclear results. In order to counteract this problem, a more controlled and precise mechanism of injecting the virus must be deduced in order to prevent the spread from one area of the hypothalamus to the other.

Future research can also include labelling of neurons in the VMH with tyrosine hydroxylase (TH) and GAD along with GFP to confirm whether the same neurons that are being transfected with the M5 virus are also dopamine neurons, and whether there exist GABA neurons in the VMH that may mediate the cholinergic transmission directly from the VMH. This
would confirm the proposition that the morphine induced locomotion due to HSV-M5 transfection in the VMH is due to a similar DA dependent pathway as that observed after VTA transfections.

Another important consideration that may validate DAs role in producing morphine-induced locomotion in the VMH is the use of microdialysis studies. Microdialysis in the NAc DA neurons can be done during morphine-induced locomotion testing. The results of this experiment will validate whether NAc DA concentrations in vivo cause the drug-induced effects observed. It will also clarify the relationship between the VMH and PPT LDT-VTA pathway.

**Summary**

Morphine induced locomotion increased by 6 times at peak as compared to saline induced locomotion in M5-HSV transfected mice. Mice infected with the Control virus did not show locomotion above and beyond spontaneous locomotion even after being systemically injected with morphine. Furthermore, saline induced locomotion in both M5-HSV and Control animals was comparable and did not significantly differ from each other. Hence, the M5 receptor plays an important role in drug-induced locomotion in the VMH implicating a role for this area in the PPT-LDT dopamine pathway. Haloperidol decreased morphine-induced locomotion by 50% in M5-HSV mice while having no effect on control animals, suggesting that there exists a role of DA in mediating the stimulatory effects of morphine. The results of this experiment further add to the neurobiological understanding of the drug reward pathway. The VMH is not typically included in studies investigating drug-related reward but may now be an important brain area to take into consideration. Furthermore, pharmacological medication to combat drug addiction can now include the VMH in prospective brain areas to target.
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Appendices

Appendix I: Locomotor Responses of Each Mouse after Control Vector Transfection

M1-CNT

M2-CNT

M3-CNT
Appendix II: Locomotor Responses of Each Mouse after Bilateral HSV-M5 Transfection

M2-HSV M5

M3-HSV M5
Appendix III: Locomotor Responses of Each Mouse after Unilateral HSV-M5 Transfections

Appendix IV: Time course of saline- and morphine-induced locomotion (10mg/kg i.p) in Control mice
Figure 7. Time course of saline- and morphine-induced locomotion (10mg/kg i.p) in Control mice (n=6). There was a significant effect of drug (saline vs morphine). Bars represent standard error of the mean.
Appendix V: Time course of saline- and morphine-induced locomotion (10mg/kg i.p) in HSV-M5 mice

Figure 8. Time course of saline- and morphine-induced locomotion (10mg/kg i.p) in HSV-M5 mice (n=6). There was a significant effect of drug (saline vs. morphine). Bars represent standard error of the mean.
Plates

Plate I: GFP expression in VMH neurons