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Preweanling exposure to selective catecholamine transporter inhibitors differentially effects morphine-induced antinociception in adulthood

Joseph Marco Valentine

PREWEANLING EXPOSURE TO SELECTIVE CATECHOLAMINE TRANSPORTER INHIBITORS DIFFERENTIALLY EFFECTS MORPHINE-INDUCED ANTINOCICEPTION IN ADULTHOOD

A Thesis

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by

Joseph Marco Valentine

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ABSTRACT

The most common treatment for attention deficit hyper activity-disorder in school age (7-12) and pre-school age children is the psychostimulant methylphenidate. The long-term effects of this drug have been well characterized in preadolescent human and rats, yet little research is available on the enduring changes from exposure during the pre-school age years. Exposure to methylphenidate during different developmental stages leads to unique patterns of long-lasting effects. More specifically, periadolescent exposure to methylphenidate increases anxiety and depressive like behaviors, as well as, decreases cocaine seeking, while increased cocaine and morphine sensitivity have been observed from preweanling exposure to methylphenidate in the rat. Methylphenidate binds to both the dopamine and norepinephrine transporters making it unclear which neurotransmitter system's increased activity during the preweanling period leads to the potentiation in morphine-induced antinociception in early adulthood seen in past research. To determine which transporter site is responsible for these past effects, the current thesis exposed rats to the norepinephrine transporter blocker

atomoxetine (0, 0.3, 1, and 3 mg/kg) and the dopamine transporter blocker GBR-12909 (0, 1.5, 5, and 15 mg/kg) during the late preweanling period (i.e., postnatal day (PD) 11-PD 20). Rats were then tested in early adulthood (PD 60) on two nociception assays (tail-flick and hot-plate) after a challenge injection of morphine (0, 2.5, 5, and 10 mg/kg). There was a potentiation in morphine-induced analgesia from preweanling exposure to atomoxetine (1 mg/kg) and attenuation in morphine-induced antinociception from exposure to atomoxetine (0.3 mg/kg). GBR-12909 early exposure had little effect on morphine-induced analgesia in adulthood; however increased basal antinociception was observed with pretreatment GBR-12909 (15 mg/kg) compared to saline controls. Long-lasting alterations in opioid sensitivity have now been observed from early exposure to atomoxetine and methylphenidate. Therefore, the non-psychostimulant atomoxetine may not be a safer choice than methylphenidate in the treatment of ADHD. Efforts should be focused on the discovery of novel compounds with higher affinity for the dopamine transporter because GBR-12909 had few long lasting effects.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

Attention deficit hyperactivity disorder (ADHD) is a commonly diagnosed pediatric disorder. Presently it is estimated that 7% of school-age children in the United States meet this diagnostic criterion (Cormier, 2008; Spencer, Beidermen, & Mick, 2007). Moreover, the clinical diagnosis of ADHD is on the rise, with a 22% increase in parent-reported cases in the United States from 2003-2007 (Visser et al., 2010). The majority of children diagnosed with ADHD are treated with methylphenidate or amphetamine (Visser, Lesesne, & Perou, 2007), with 4.3% of all school-age children receiving psychostimulant medication (Visser et al., 2007). While a large number of children are prescribed psychostimulant medication, the use of such drugs is supported by data which show that psychostimulants are effective treatments for ADHD in children ages seven and older (Findling, 2008; Murray, 2010).

A recent epidemiological trend is to diagnose preschool-age children (3 - 5 years-old) with ADHD (Cormier, 2008; Davis & Williams, 2011; Murray, 2010). It

has been estimated that 2% of preschool-age children meet the diagnostic criteria for ADHD (Lavigne et al., 1996). Alarmingly, there has also been an increase in the number of 3 - 5 year-old children prescribed psychostimulants, with methylphenidate being the most commonly used compound (Zito et al., 2000). While the FDA has not approved the use of methylphenidate in preschool-age children it is often prescribed by doctors off label for the treatment of preschool ADHD (Davis & Williams, 2011; Murray, 2010).

Interestingly, while methylphenidate and other psychostimulants have been extensively tested in 7 - 17 year-old children; very little research has been conducted in preschool-age children. This lack of research leaves many questions about the efficacy and safety of psychostimulant use in this specific population (Kratochvil et al., 2004; Murray, 2010; Zito et al., 2000). Recently, the Preschool ADHD Treatment Study (PATS) found symptom improvement with the use of methylphenidate in 3 - 5 year-old children; however, the effect size was significantly smaller than in school-age children (Davis & Williams, 2011). Moreover, other studies report greater adverse side-effects with

psychostimulant medications in preschool-age children (Wigal et al., 2006). The neural plasticity observed during development is cause for concern because early exposure to psychostimulants during this time may induce alterations in brain chemistry that have long-lasting effects (Johnston, 2004).

In order to assess the effects of methylphenidate use on brain function during development, investigations have been conducted in rats using ages roughly analogous to early and late childhood (Andersen, 2003). Curiously, exposure to methylphenidate at different times during early development leads to different behavioral effects that persist into adulthood. For example, adolescent rats exposed to methylphenidate showed an increase in cocaine-seeking behavior (Brandon et al., 2001), whereas exposure during periadolescence attenuates cocaine preference (Carlezon, Mague, & Anderson, 2003; Mague et al., 2005).

More recent research has focused on exposure to methylphenidate during the preweanling period in rats, which is roughly equivalent to preschool-age children (Andersen, 2003). Preweanling exposure to methylphenidate increased morphine-induced conditioned place preference

(CPP), sucrose reinforced lever pressing, and the analgesic effects of morphine (Crawford et al., 2007; Cyr & Morgan, 2009; Hallady et al., 2009). These results suggest that exposure to methylphenidate during the preweanling period may increase the sensitivity of opioid receptors.

Currently, it is not known how early methylphenidate treatment alters the sensitivity of opioid receptors in adulthood. In particular, methylphenidate has a high affinity for both the dopamine and noradrenergic transporters (Heal, Cheetham, & Smith, 2009) and it is uncertain which neurotransmitter system is responsible for the changes in opioid receptor functioning. Therefore, the goal of the present proposal is to determine how early methylphenidate exposure alters opioid receptor functioning in adulthood. To this end, we will administer selective monoamine transporter inhibitors for dopamine and norepinephrine (GBR-12904 and atomoxetine) during the preweanling period and assess opioid function in adult rats by testing morphine-induced antinociception using the hot-plate and tail-flick tasks.

CHAPTER TWO

OPIOID RECEPTORS

The opioid system has long been known to play a role in analgesia, because pharmacological agents like morphine bind to opioid receptors. Aside from exogenous opiates, there are a small number of endogenous opioids, including beta-endorphin, enkephalins, and dynorphins, which bind to opioid receptors. There are three major classes of receptors in the opioid system: the μ-opioid receptor (MOR), κ-opioid receptor (KOR), and δ-opioid receptor (DOR), with each having a specific role in brain functioning (Bodnar & Hadjimarkou, 2001; Pasternak, 2004; Satoh & Minami, 1995; Snyder & Pasternak, 2003). Epsilon and ORL-1 are additional classes of opioid receptors; however, there is far less research on the function of these two receptors (Bodnar & Hadjimarkou, 2001).

Opioid receptors are seven transmembrane protein metabotropic receptors (Pasternak, 2004; Satoh & Minami, 1995), which couple to inhibitory guanine nucleotide-binding regulatory proteins (G-proteins) G_i and G_o (Williams, Christie, & Manzoni, 2001). These G-proteins phosphorylate potassium channels causing a shorter length

constant for incoming excitatory postsynaptic potential (EPSPs) (Charles & Hales, 2004). Opioid receptor activation can also cause inhibition by decreasing adenylyl cyclase activity, which attenuates cyclic-AMP production (Charles & Hales, 2004). All of the opioid receptor subtypes are similar in molecular structure, with 70% identical amino acid sequencing between any two receptors (Terenius & Johansson, 2010).

Of the three major classes of opioid receptors, the MOR has been the most extensively studied and is implicated in addictive processes and antinociception. Knockout mice lacking MORs exhibit virtually no analgesic effects and attenuated tolerance to morphine when administered intracerebroventricularly (Sorah et al., 1997). The MOR antagonist naltrexone blocks alcohol consumption in rats, which supports the hypothesis that MORs are involved in the addictive process (Ji et al., 2008). Morphine-6-glucuronide, a metabolite of morphine, has a higher infinity for MORs than morphine, while the metabolite morphine-3-glucuronide has almost no affinity for the MOR (Ulens et al., 2001). Antinociception experiments utilizing endomorphine-1-2, DAMGO, and naloxonazine have suggested two separate subtypes of the

MOR (mu_1 and mu_2), which are both involved in analgesia (Sakurada et al., 1999); however, the existence of these MOR subtypes is still being debated.

MORs play a modulatory role in several important loci in the brain. For instance, MORs are expressed postsynaptically in the ventral tegmental area, where they modulate the excitation of dopaminergic neurons projecting to the nucleus accumbens and cortex (Svingos et al., 2001). Synaptic plasticity in the dentate gyrus can be modulated by MORs located on the axon terminals of glutamatergic neurons (Milner & Drake, 2001). Aside from modulatory effects, over stimulation of MORs in the locus coeruleus causes long lasting changes in noradrenergic projections (Valentino & Bockstaele, 2001). MORs are found in virtually every part of the diencephalon, mesencephalon, hind brain, and spinal cord, yet very few MORs are found in the cerebral cortex (Delfs et al., 1994).

Delta opioid receptors (DOR) are the least characterized of the three main opioid receptors, though they have been implicated in pain management. DORs like MORs have two proposed receptor subtypes (δ_1 and δ_2) (Portoghese et al., 1992). Portoghese and colleagues

(1992) reported that the δ_1 receptor has high affinity for DPDPE and DADLE; whereas, δ_2 receptors have a low affinity for DPDPE and DADLE, but high affinity for DSLET and naltriben. DSLET and DPDPE are selective DOR agonists that elicit antinociception when micro-injected into the periaqueductal gray and medullary reticular formation (Ossipov et al., 1995). Antagonism of DORs in the rostral ventromedial medulla attenuate descending periaqueductal gray MOR regulated inhibitory projections to the spinal cord (Hirakawa, Tershner, & Fields, 1999). These findings suggest that central nervous system DORs play a role in analgesia.

In situ hybridization studies have been used to detect the location of DORs in the central nervous system. DORs are found in high densities where MORs are in low densities, while DORs are in low densities where MORs are in high densities. For instance, high densities of DORs are found in the cortex and the deep nuclei of the telencephalon, whereas MORs are almost nonexistent in the cortex (Mansour et al., 1994). Also, very few structures in the mesencephalon and diencephalon express DORs, although MORs are abundant in these brain regions (Mansour et al., 1994). DORs are robustly distributed

throughout the hindbrain except for the cerebellum (Mansour et al., 1994). DORs are also expressed on the presynaptic terminals of neurons in the periaqueductal gray (Commons et al., 2001), which suggests they modulate the efflux of presynaptic neurotransmitters by increasing inhibition at the terminal.

There are high levels of DOR expression in the spinal cord, which is similar to the pattern of MOR expression (Mansour et al., 1994). Substance P containing neurons in the spinal cord have low expression of DOR mRNA; however, DORs appear to inhibit substance P release (Aimone & Yaksh, 1989), suggesting that DORs are involved in the modulation of pain at the spinal level. DORs are also located on terminals that synapse onto GABAergic cell bodies and dendrites, suggesting that DORs modulate the flow of information to GABA containing neurons, thus causing disinhibition (Commons et al., 2001).

Unlike MORs, the kappa opioid receptors (KOR) do not have a role in addiction, but KORs have strong antinociceptive effects when stimulated. Exogenous ligands like U50,488 and U69,593 act as agonists at the KOR and elicit aversive, not appetitive, responses in animals and humans (Terenius & Johansson, 2010). The

latter finding suggests that KOR agonists have a low abuse potential (Terenius & Johansson, 2010). Much like MORs and DORs, KORs are divided into two subtypes, labeled κ_1 and κ_2 (Zukin et al., 1988). The endogenous KOR agonist dynorphin A and the exogenous ligand U50,488 bind to both κ_1 and κ_2 receptors (Zukin et al., 1988). The only ligand that preferentially binds to κ_1 is U69,593 and there are no known ligands that have a high affinity for the κ_2 receptor subtype (Zukin et al., 1988).

KORs are found throughout most of the telencephalon, including dense populations of receptors in the fourth layer of the cortex, caudate and putamen, amygdala, and nucleus accumbens (Mansour et al., 1994). KORs are found throughout the thalamus and hypothalamus, as well as the substantia nigra and ventral tegmental area (Mansour et al., 1994). Most of the KOR expression in the midbrain is in areas with little expression of MORs and DORs (Mansour et al., 1994). Hindbrain KOR expression is predominant in the medulla and periaqueductal gray, two areas known to be involved in antinociception (Mansour et al., 1994). KORs are expressed widely throughout the spinal cord, including high concentrations in areas where nociceptive axons synapse onto spinal interneurons (Mansour et al.,

1994). These findings suggest that KORs are important for analgesia at the spinal level.

The mesocorticolimbic pathway popularly called the "reinforcement pathway" consists of dopamine releasing neurons with cell bodies projecting from the ventral tegmental area to the nucleus accumbens and cortex. Since KORs are found on dopaminergic neurons in the ventral tegmental area, the use of a KOR agonist like dynorphin A can inhibit the release of dopamine throughout the midbrain and cortex (Werling et al., 1988). KOR activation in the mesocorticolimbic pathway can attenuate the rewarding aspects of illicit drugs (Svingos et al., 2001) via the inhibition of dopamine release just mentioned.

Unlike MORs and DORs, KORs found on glutamatergic neurons tend to be excitatory, which can lead to neurotoxic effects (Malan et al., 2000). KOR agonists alter feeding behavior, temperature regulation, and vasopressin release in rats, presumably due to the high density of KORs in the hypothalamus (Leander, Zerbe, & Hart, 1985).

MORs, KORs, and DORs often interact with each other in antagonistic and agonistic fashions. For instance,

MORs typically have an antagonistic effect on KORs (Smith et al., 2009). Matthes and colleagues (1998) found that DOR functioning was altered in animals lacking MORs, however KOR functioning was unaffected in MOR-deficient animals. This lends support to the hypothesis that DORs and MORs have complementary actions, while KORs do not. Aside from the individual receptor subtypes, opioid receptors are often expressed as heterodimers and may have completely different actions than the individual receptors (Gomes et al., 2000). The pairings of opioid receptor subtypes used to form heterodimers, like MOR/DOR, typically occur when one receptor is sparsely populating an area, thus causing that receptor to bind with a more densely populated receptor (Gomes et al., 2000). These heterodimers can bind pharmacological agents that are specific to one receptor subtype or the other, hence leading to an increased likelihood of activation (Gomes et al., 2000).

The opioid system consists of three major receptor categories MORs, KORs, and DORs, which are expressed throughout the central nervous system. Each type of receptor has a specific role in analgesia and addiction, as well as different modulatory functions in the brain.

MORs, KORs, and DORs are G-protein coupled receptors with similar structures.

CHAPTER THREE

CHATACHOLAMINE RECEPTORS

Dopaminergic Receptors

Dopamine receptors are one of the most studied receptor systems in the brain, because of their role in addiction and motor function. There are a number of behaviors influenced by dopamine receptor functioning, including: reward, reinforcement, movement, feeding, hormone regulation, learning, motivation, and sexual activity (Iversen & Iversen, 2006; Wise, 2008, 2009). In addition, dysregulation of dopamine receptor systems plays a role in disorders such as schizophrenia, Parkinson's disease, and Huntington's disease (Glickstein & Schmauss, 2001; Iversen, 1975). There are two different families of dopamine receptors: the D_1-like family, which consists of the D_1 and D_5 receptor subtypes, and the D_2-like family, which consists of D_2, D_3, and D_4 receptor subtypes (Garau et al., 1978; Glickstein & Schmauss, 2001; Iversen & Iversen, 2006; Vallone, Picetti, & Borrelli, 2000).

Dopamine receptors are seven transmembrane domain metabotropic receptors (Vallone et al., 2000) and the

D_1-like receptors bind to excitatory G_s and G_{olf} G-proteins, while the D_2-like receptors bind to inhibitory G_i and G_o G-proteins (Olianas & Onali, 1987). Stimulation of D_1-like receptors increases cAMP, whereas stimulation of D_2-like receptors attenuates the formation of cAMP (Spano, Govoni, & Trabucchi, 1978). Typically, D_1-like receptors are located postsynaptically and stimulate dendrites, while D_2-like receptors can be either postsynaptic or presynaptic autoreceptors (Glickstein & Schmauss, 2001).

The molecular structures of the dopamine receptors in each family are very similar, however when compared to the other family they are dissimilar in structure (Missale et al., 1998), which explains why exogenous dopamine agonists and antagonists preferentially bind to a particular family. The five dopamine receptors were elucidated by molecular cloning techniques and the utilization of knockout mice lacking certain dopamine receptors. After the identification of the five individual receptor subtypes, drugs were developed with high selectivity for each subtype. For instance, PD-168,077 is a D_4 agonist, L-741,626 is a D_2 antagonist,

SB277011A and FAUC 365 are D_3 antagonists, and L-745,870 is a D_4 antagonist (Sanna et al., 2011).

Radiolabeled agonists and antagonists are useful tools for determining which brain areas have high expression of the dopamine receptor families. There are three major pathways which contain high densities of dopamine receptors in the brain: the nigrostriatal, tuberoinfundibular, and the mesocorticolimbic systems (Iversen & Iversen, 2006; Valloneet al., 2000; Verheij & Cools, 2008; Wise, 2009). The mesocorticolimbic system consists of the ventral tegmental area projecting to the nucleus accumbens, amygdala, and frontal cortex; this pathway is known to be involved in reinforcement, reward, learning, and motivation (Iversen & Iversen, 2006; Vallone et al., 2000; Wise, 2009). D_1-like receptors are found in high densities on glutamatergic and GABAergic neurons in the ventral tegmental area and when activated cause an increase in motivation to seek rewarding stimuli (Perreault et al., 2012).

The nigrostriatal pathway consists of the substantia nigra projecting to the caudate and putamen; this system is involved in motor movement, habitual behaviors, and basic associations (Da Cunha et al., 2009; Wise, 2009).

Interestingly, D_1-like receptors are also located on glutamatergic and GABAergic neurons in the substantia nigra and when stimulated by a direct agonist cause an increase in reward seeking behaviors (Perreault et al., 2012). This suggests that D_1-like receptors are important for modulating dopamine neuron activation in the nigrostriatal and mesocorticolimbic pathways.

D_2-like receptors are also expressed in the nigrostriatal and mesocorticolimbic pathways and are found pre and postsynaptically. Presynaptic D_2-like receptors located on dopaminergic neurons function as regulators of dopamine synthesis and efflux (Hoffmann & Cubeddu, 1984). Postsynaptic D_2-like receptors located on dendrites are regulators of incoming EPSPs because they phosphorylate potassium channels causing increased potassium efflux and smaller EPSPs (Hyttel, 1984).

The tuberoinfundibular pathway consists of dopaminergic neurons in the hypothalamus projecting to the anterior pituitary and is involved in hormone regulation (Missale et al., 1998). D_2-like receptors are expressed in high concentration in the anterior pituitary and are involved in the modulation of prolactin release (Gudelsky, Annunziato, & Moore, 1978). More specifically,

increased activation of D_2-like receptors attenuates prolactin release, whereas decreased D_2-like activity increases prolactin release (Chang, Shin, & Pang, 1997).

Localization of the dopaminergic receptor subtypes (D_1-D_5) has been unraveled using autoradiography and *in situ* hybridization. The D_1 receptor is the most abundant dopamine receptor in the brain and it is expressed in the olfactory tubercle, thalamus, ventral tegmental area, nucleus accumbens, striatum, substantia nigra, and endopeducular nucleus (Boyson, McGonigle, & Molinoff, 1986; Lazarov et al., 1998). The D_5 receptor is predominantly expressed in areas where the D_1 receptor is not. For instance, D_5 receptor mRNA is highly expressed in the hippocampus, cerebral cortex, and mammillary bodies, with little or no expression in the olfactory tubercle, striatum, and nucleus accumbens (Woodruff et al., 1992).

D_1 and D_5 receptors are expressed more frequently on postsynaptic neurons, but they are also expressed presynaptically on GABAergic medium spiny neurons in the striatum and pyramidal neurons in the prefrontal cortex (Bergson et al., 1995; Huang et al., 1992).

Autoradiography has shown that high densities of D_2 receptors are located in the striatum, ventral tegmental

area, prefrontal cortex, cingulate gyrus, nucleus accumbens, and substantia nigra (Boyson et al., 1986). Interestingly, D_2 receptors found on GABAergic neurons in the nucleus accumbens coexist with opioid receptors (Perreault et al., 2012). Autoradiography has shown poor expression of D_3 receptors in the telencephalon, diencephalon, striatum, ventral tegmental area, and substantia nigra; however it has shown high expression of D_3 receptors in the accumbens olfactory tubercle and amygdala (Ochoa et al., 1995). There is high expression of D_3 receptors on substance P- and enkephalin-containing neurons in the nucleus accumbens (Surmeier, Song, & Yang, 1996). Using the radioligands [^{3}H]YM-09151-2 and [^{3}H]raclopride it was reported that D_4 receptors are highly expressed throughout the midbrain, hippocampus, cerebral cortex, diencephalon, and amygdala (Defagot & Antonelli, 1997).

In summary, the D_1-like and D_2-like families of receptors are expressed widely throughout the central and peripheral nervous systems and are involved in a wide range of behaviors, including learning, motivation, reinforcement, reward, feeding, and sexual activity. D_1-like receptors are excitatory and bind to G_s and G_{olf}

G-protein coupled receptors, whereas the D_2-like receptors are inhibitory and bind to G_i and G_o G-proteins. A dysregulation of dopaminergic functioning can lead to disorders like schizophrenia and Parkinson's disease; often pharmacological agents with primary actions at dopamine receptors are utilized to help treat these disorders.

Noradrenergic Receptors

Noradrenergic receptors are found throughout the central and peripheral nervous systems where they play a critical role in several different functions and behaviors, including cardiovascular regulation, analgesia, motor movement, cognition, reinforcement, stress response, and neural excitation (Ono & Fukuda, 1995; Stanford, 1995; Stone et al., 2011). Aside from normal functioning, noradrenergic receptor dysfunction has been associated with several physiological and psychopathological disorders, including high blood pressure, anxiety, depression, and ADHD (Gamo et al., 2010; Nicholas et al., 1996; Stanford, 1995).

Noradrenergic receptors are divided into two families α-adrenergic and β-adrenergic receptors, which

are further subdivided into α_1-adrenergic, α_2-adrenergic, β_1-adrenergic, β_2-adrenergic, and β_3-adrenergic receptor subtypes (Langer & Pemoule, 1982; Nicholas et al., 1996; Stone et al., 2011). cDNA cloning and *in situ* hybridization techniques have been used to discover additional noradrenergic receptor subtypes, including α_{1A}-adrenergic, α_{1B}-adrenergic, α_{1D}-adrenergic, α_{2A}-adrenergic, α_{2B}-adrenergic, α_{2C}-adrenergic (Duda, Chalberg, & Sharma, 1990; Lomasney et al., 1991), β_{3A}-adrenergic, and β_{3B}-adrenergic receptors (Lenard et al., 2006).

Noradrenergic receptors are found both post and presynaptically, they can be inhibitory or excitatory, and they are all metabotropic receptors with seven transmembrane domains (Nicholas et al., 1996). More specifically, β_2-adrenergic receptors are excitatory and couple to the G_s G-proteins, which increase cAMP levels and intracellular calcium concentrations (Daaka, Luttrell, & Lefkowitz, 1997). β_2-adrenergic receptors also couple to inhibitory G_i G-proteins, which reduce cAMP levels, decrease intracellular calcium concentrations, and activate potassium channels (Daaka, Luttrell, & Lefkowitz, 1997). Recent evidence has shown that β_1, β_{3A},

and β_{3B} adrenergic receptors couple to the excitatory G_s G-protein; however, β_{3A} and β_{3B} also couple to G_i G-protein though less prominently (Lenard et al., 2006; Mason et al., 1999). α_2-Adrenergic receptors are inhibitory and couple to G_i G-proteins, although α_2-adrenergic receptors can simultaneously couple to G_s G-proteins as well (Eason et al., 1992). α_1-Adrenergic receptors are excitatory and couple to a G_q G-protein (Yamamoto et al., 2009). G_q G-proteins activate phospholipase C which cleaves phosphatidylinositol biphosphate (PIP_2) into inositol trisphosphate (IP_3) and diacylglycerol (DAG), thus causing increased protein kinases C functioning and release of sequestered calcium stores (Yamamoto et al., 2009). Interestingly, individual receptor subtypes can be expressed as monomeric or heterotrimeric G-protein coupled receptors, meaning more than one receptor can be coupled to the same G-protein (e.g., α_{1A}/α_{1B} receptor or α_{1D}/β_2 receptor) (Hague et al., 2004). Heterotrimeric receptors allow for increased activation of the noradrenergic system from exogenous ligands, because ligand binding at either receptor can activate the associated G-protein.

Noradrenergic receptors are widely dispersed throughout the central and peripheral nervous system; however, certain receptor subtypes are localized in specific brain regions. Unfortunately, *in situ* hybridization studies using α_{1A}-adrenergic receptor mRNA have had difficulty pinpointing the brain regions these receptors are localized in, but recent research has shown the α_{1A}-adrenergic receptor exists in the cerebral cortex (Segura et al., 2010). α_{1B}-Adrenergic receptor mRNA is highly expressed in the raphe nuclei, cortex, thalamus, and pineal gland (Day et al., 1997; Segura et al., 2010). α_{1D}-Adrenergic receptors are located in the cortex, inferior olive, olfactory bulbs, motor neurons, and hippocampus (Day et al., 1997; Segura et al., 2010). α_{2A}-Adrenergic receptors are localized in several brain areas known to be involved in analgesia, including the locus coeruleus, periaqueductal gray, paraventricular nucleus, medulla, and spinal interneurons. α_{2A}-Adrenergic are also found in the pons and amygdala (Nicholas, Pieribone, & Hokfelt, 1993). In contrast, *in situ* hybridization studies have shown that α_{2B}-adrenergic receptors are only expressed in the thalamus (Nicholas et al., 1993). α_{2C}-Adrenergic receptors are found in the same

brain regions as the α_1-adrenergic family, including the cortex, hippocampus, olfactory bulb, cerebellum, globus pallidus, striatum, and dorsal root ganglia (Nicholas et al., 1993).

β_1-adrenergic receptors are expressed in the thalamus, cortex, septal nucleus, vestibular labyrinth, and throughout the entire myencephalon, metencephalon, and spinal cord (Nicholas et al., 1993). β_2-adrenergic receptors are expressed far less than β_1-adrenergic receptors; however, β_2-adrenergic are expressed in the hippocampus, thalamus, cerebellum, and olfactory bulbs (Nicholas et al., 1993). β_3-adrenergic receptors are expressed in the cortex, striatum, and limbic structures, but they are only expressed at low levels in the midbrain and hindbrain (Summers et al., 1995).

Noradrenergic receptors in the central nervous system play a role in learning and memory. More specifically, over activation of noradrenergic receptors in the locus ceoruleus potentiates hippocampal modulation of conditioned responding in an operant paradigm (Segal & Bloom, 1976). Gamo and colleagues (2010) found that antagonizing α_2-adrenergic receptors in the prefrontal cortex attenuated performance in a spatial memory task,

however stimulation of these receptors led to increased firing of neurons associated with the goal behavior and overall enhanced responding.

The central nervous system noradrenergic receptors also play a critical role in behavioral inhibition. For instance, administration of yohimbine a selective α_2-adrenergic receptor antagonist into the locus coeruleus leads to exacerbated inactivity in a forced swim task (Weiss et al., 1986). Similarly, a microinjection of salmeterol, a β-adrenergic agonist, or cirazolinean, an α_1-adrenergic agonist, into the lateral ventricle also increase immobility in the forced swim task (Weiss et al., 1986).

Increased activation of noradrenergic receptors and subsequent norepinephrine depletion in the locus coeruleus has been observed in rats exposed to a number of stressors (e.g., tail pinch, foot shock, and light flash, even decreased blood pressure) (Anisman, Pizzino, & Sklar, 1980). Also, there is an increase in norepinephrine efflux in the medial-frontal cortex during stress (Cenci et al., 1992). While noradrenergic receptor activation is increased during several different tonic and phasic stressors, the receptors themselves typically

remain unaltered. More specifically, increased activation of α_1, α_2, and β-adrenergic receptors in response to stressful stimuli did not enhance binding in an autoradiography assay (Lynch et al., 1983).

The noradrenergic system modulates nociception in the peripheral and central nervous system. Administration of α-adrenergic receptor agonists in the peripheral nervous system increased the sensitivity of capsaicin receptors and elicited hyperalgesia in patients who had dermal injuries (Kinnman, Nygards, & Hansson, 1997). Similarly, administration of an α_2-adrenergic receptor antagonist caused a reduction in pain for patients with skin injuries. These same analgesic effects were not observed after α_1-adrenergic receptor antagonist or agonist treatments (Banik et al., 2001).

Noradrenergic receptors also effect the modulation of pain at the spinal level. For instance, administration of α_{2A}- or α_{2C}-adrenergic receptor agonists into the spinal cord causes analgesia (Duflo et al., 2002; Graham, Hammond, & Proudfit, 2000). Activation of inhibitory α_{2A}-adrenergic receptors located presynaptically on primary nociceptive neurons also leads to antinociception (Fleetwood et al., 1985). α_{2C}-Adrenergic receptors have

similar inhibitory action as α_{2A}-adrenergic receptors and are located on spinal interneurons that activate ascending pain pathways (Olave & Maxwell, 2002). The excitatory α_1-adrenergic receptors also play a role in spinal analgesia because they are expressed on dorsal horn GABAergic interneurons, which inhibit pain pathways (Baba et al., 2000).

Noradrenergic receptors are expressed in brain regions critical for analgesia, including the periaqueductal gray, medulla, thalamus, locus coeruleus, and cortex (Pertovaara, 2006). As mentioned above, *in situ* hybridization studies have found high expression of α_2-adrenergic receptors in the midbrain and hindbrain. Antagonism of α_2-adrenergic receptors in the periaqueductal gray has no effect on antinociception (Ossipov & Gebhart, 1983), even though this brain region plays an important role in analgesia. Pertovaara (2006) deduced that α-adrenergic receptors in the medial portion of the medulla modulate antinociception when rats are stressed; however, these receptors do not play a significant role when animals are not stressed. There is also evidence that mesencephalic opioidergic receptors are influenced by α_1-adrenergic receptors in the medulla

(Bie et al., 2003). For example, administration of a α_2-adrenergic receptor agonist in the lateral medulla causes hyperalgesia (Ossipov & Gebhart, 1986). Infusion of norepinephrine into the locus coeruleus also causes analgesia, which suggests that this brain region may affect both afferent pain pathways to the spine and efferent pain projections to the somatosensory cortex (Mokha, McMillian, & Iggo, 1986; Voisin, Guy, & Dallel, 2005).

In summary, noradrenergic receptors are found throughout the peripheral and central nervous systems where they play a critical role in learning and memory, stress, and nociception. There are several noradrenergic receptor subtypes, including α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2, β_{3A}, and β_{3B}-adrenergic receptors, receptor subtypes tend to be localized in specific brain and spinal regions. β-Adrenergic receptors couple to both inhibitory and excitatory G-proteins and are typically found in the peripheral nervous system, where they regulate vasoconstriction and vasodilation. α_1-Adrenergic receptors couple to excitatory G-proteins, while α_2-adrenergic receptors couple to inhibitory G-proteins allowing them

to regulate behaviors such as behavioral inhibition, arousal, and analgesia.

CHAPTER FOUR

ONTOGENY OF OPIOID AND CHATACHOLAMINE RECEPTORS

Opioidergic Receptors

Three main types of opioid receptors exist, MOR, KOR, and DOR, each of which has a unique developmental pattern. For instance, Kivell and colleagues (2004) used autoradiography and *in situ* hybridization techniques to show that MOR receptors are expressed before birth (in utero), they increase in density from postnatal day 3 (PD 3) to PD 9, and are pruned from PD 9 until adulthood. MORs are expressed earlier in the brainstem which contains nuclei involved in analgesia (e.g., the periaqueductal gray), than in the cortex (Kivell et al., 2004). While expressed MOR proteins are found in the developing rat brain, it is unclear whether MORs are coupled to the G_i and G_o G-proteins at this stage (Thornton et al., 1998). Interestingly, MOR expression in the peripheral nervous system of newborn rats is higher than in adults (Nandi et al., 2004).

In situ hybridization studies show that adult like levels of KOR expression are evident by PD 3 in the rat brain (McLaughlin et al., 1995) and KOR can be detected

in different parts of the rat brain by embryonic day 13-15 (Georges et al., 1998). KORs are coexpressed with GABAergic receptors during development and are often present without the coexpression of MORs and DORs (Chen et al., 1999). More recently, KORs were found to increase dendritic spine densities by increasing the release of growth factors during development (Tsai et al., 2010).

DORs are expressed in high concentrations at birth, followed by pruning through adolescence into adulthood (Kivell et al., 2004). Some report that functional DORs (e.g., DORs that expand across the phospholipid bilayer and have ligand binding domains) are present after PD 7 (Kivell et al., 2004), whereas others have shown functional DORs at birth (Spain, Roth, & Coscia, 1985).

Even though each opioid receptor subtype (μ, κ, and δ) has a unique developmental profile, all of the opioidergic receptors are sufficiently functional during early stages of ontogeny to elicit analgesic responses under pharmacological challenge. The MOR and KOR are fully functional and expressed at adult levels almost immediately after birth and the DOR is in high concentrations at birth then is pruned through adolescents.

Dopaminergic Receptors

The utilization of autoradiography, *in situ* hybridization, and cDNA cloning techniques have led to a greater understanding of dopamine receptor development. However, the research is not always consistent when it comes to developmental patterns of the five dopamine receptor subtypes (Tarazi & Baldessarini, 2000).

D_1-like (D_1 and D_5) receptor mRNA expression is evident in mice as early as gestation day 12 and there is a strong correlation between mRNA expression and protein expression (Araki, Sims, & Bhide, 2007). D_1-like receptor expression increases progressively from gestation day 12 until adulthood (Araki et al., 2007). In the rat, radiolabeled SCH-23390, a D_1-like receptor antagonist, increases in binding from PD 7 to PD 28, then steadily decreases in binding from PD 29 until adulthood (Tarazi, Tomasini, & Baldessarini, 1999). In contrast, Teicher, Andersen, and Hostetter (1995) found higher D_1 receptor binding in adolescent rats than in adult rats using [^{3}H] SCH-23390. Dopamine is critical for the development of the D_1 receptor, because normal expression of D_1 receptors in rats with 6-hydroxydopamine lesions will only occur if

exogenous dopamine receptor agonists such as SKF-38393 are used (Sivam & Cox, 2006).

The dopamine D_2 receptor also has inconsistent reports concerning its development (Tarazi & Baldessarini, 2000). For instance, D_2 receptors were found to increase in density until PD 28 and were pruned in adolescence (Tarazi, Tomasini, & Baldessarini, 1998); while others report expression of D_2 receptors increases until PD 40 then are pruned until PD 60 (Teicher et al., 1995). D_3 receptors are expressed in high levels on PD 3 and show a consistent increase in radiolabeled ligand binding through adulthood (Stanwood et al., 1997).

Little information exists on the development of D_4 receptors due to the lack of selective agonists and antagonists for this receptor (Tarazi & Baldessarini, 2000). D_4 mRNA is present on PD 1 and rises to peak levels on PD 3 before being reduced to adult levels by early adolescence (Nair & Mishra, 1995). D_4 receptor binding increases from birth to PD 28, then binding decrease from PD 29 until adulthood (Tarazi et al., 1998). These discrepancies may be due to the different techniques used to characterize the development of the D_4 receptor.

The dopamine transporter (DAT) plays a critical role during development because of its ability to regulate dopamine levels in the synapse. DAT is expressed as early as the 18th day of gestation (Galineau et al., 2004) and increases at a steady rate from birth until PD 28 where they reach adult levels.

Quantification of dopamine receptors during ontogeny is difficult using the primary techniques, autoradiography and *in situ* hybridization because they yield different results. Interestingly, it is well documented that dopamine itself is critical for the normal growth of these receptors. Alterations in endogenous dopamine levels during early development can attenuate receptor expression, which may play a role in disorders like schizophrenia and ADHD. Normal dopamine levels and receptor expression during development is critical for reinforcement and motor movement.

Noradrenergic Receptors

Noradrenergic receptors are important for locomotion, arousal, visceral regulation, and nociception (Happe et al., 2004). During development, noradrenergic receptors regulate norepinephrine release, which can

affect normal brain growth by altering the migration of neurons, neuronal plasticity, and synaptogenesis (Happe et al., 2004). There are nine different noradrenergic receptors in total, three main families (α_1, α_2, and β) and each family is divided into three receptor subtypes (Murrin, Sanders, & Bylund, 2007). Each of these receptors has a unique developmental pattern and plays an important role in the brain.

Little information exists on the ontogeny of α_1-adrenergic receptor subtypes (α_{1a} α_{1b} α_{1c}); however there is a well-defined pattern of growth for the α_1-adrenergic receptors family. α_1-Adrenergic receptors in rats are present at birth, increase in expression from PD 1-21, and then are pruned to adult levels (Jones et al., 1985). Ten times more excitation of noradrenergic neurons occurs on PD 14 when mice are administered an α_1-adrenergic receptor agonist compared to PD 1 administration (Selvaratnam, Parkis, & Funk, 1998). A similar pattern of increased α_1-adrenergic receptor expression over the first two to three weeks of life arises in both rats and mice, which is a time period known for high levels of synapse formation (Happe et al., 2004).

α_2-Adrenergic receptors are involved in the normal regulation of brain development (Happe et al., 2004). α_{2A}-Adrenergic receptors are expressed at near adult levels by as early as gestation day 14 (Serhan et al., 1997). Also, there is a short-term rise in α_{2A}- and α_{2B}-adrenergic receptors during cell migration in several areas of the developing rat brain (Winzer & Leslie, 1997). *In situ* hybridization shows that unlike the α_{2A} the α_{2B}-adrenergic receptor is not expressed in the developing rat brain until PD 21, except for small amounts in the olfactory system (Winzer & Leslie, 1997). α_{2C}-Adrenergic receptor expression is near adult levels at birth and remains relatively stable throughout ontogeny (Winzer et al., 1997). Also, there is short-term α_{2C}-adrenergic receptor expression during cell differentiation in the cerebellum from PD 1-14 (Winzer et al., 1997).

β-adrenergic receptors have been detected as early as gestation day 13 in the rat and they increase in expression to adult levels around the third postnatal week (Ernste, Feenstra, & Boer, 1991). During embryogenesis there is a relatively greater concentration of β_2-adrenergic receptors than β_1; however, throughout the first postnatal week the β_2-adrenergic receptor is

pruned to adult levels (Ernste et al., 1991). The β_3-adrenergic receptor is less understood than other β-adrenergic receptors with little research to characterize its ontogeny.

The noradrenergic transporter (NET) plays an important role in development because of its ability to regulate norepinephrine levels in the synapse (Murrin et al., 2007). For instance, a norepinephrine transporter blocker administered during early ontogenogenic stages leads to a large increase in α_2-adrenergic receptor growth throughout the brain (Sanders et al., 2011). NET levels in the rat midbrain and forebrain are relatively low before PD 10; however, by PD 15 NET expression is much higher, and by PD 25 NET levels are greater than in adulthood (Sanders et al., 2005). NET levels in the hindbrain exist in very high concentrations at birth and are subsequently pruned into adulthood (Sanders et al., 2005).

In summary, noradrenergic receptors are implicated in normal development of the central nervous system. They also play a role in arousal, visceral innervation, nociception, and locomotion. During development, noradrenergic receptors modulate the release of

norepinephrine, while NET regulates the amount of norepinephrine in the synapse. These components of the noradrenergic system are targets for second generation antidepressants, which are frequently prescribed to pediatric populations (Murrin et al., 2007).

CHAPTER FIVE

PAIN

Pain has been described as an aversive emotional and sensory response to damaged tissue with a threshold that is very individualized (D'Mello & Dickinson, 2008; Loeser & Melzack, 1999; Riedel & Neeck, 2001). There are three main categories of pain that can be chronic, or more commonly, acute: neuropathic pain, physiological pain, and inflammatory pain (Riedel & Neeck, 2001). Pain from tissue damage tends to be acute because peripheral pain pathways and endogenous pain modulators are activated simultaneously (Devillers et al., 1995). Inflammatory and neuropathic pains can be characterized as chronic or acute when there is deteriorating tissue (e.g., arthritis or neuropathy); however, pain can be experienced without degeneration of tissue (e.g., pain caused by infections and the introduction of foreign matter (Djouhri et al., 2006). Interestingly, disorders like phantom limb pain suggest that peripheral input from nociceptors is not necessary for the perception of pain (Kew et al., 1997). Peripheral input is a critical part in the normal

physiological makeup of our pain circuitry and plays a role in non-pathological or acute nociception.

Nociceptors (A_δ and c fibers) are modified neurons which detect tissue damage through the use of transient receptor potential (TRP) channels (nonselective cation channels) that respond to harsh chemicals, changes in extreme temperature, and pressure (Kwan et al., 2006). A_δ nociceptors are myelinated and, therefore, transduce pain signals fast (Stein et al., 2009). These receptors are responsible for sharp initial pain (Stein et al., 2009). c Fibers are not myelinated and transduce pain signals significantly slower, thus they are responsible for dull pain after injury (Stein et al., 2009). Peripheral nociceptors are activated by tissue damage and they send pain signals to the spinal cord through the dorsal root ganglia where their cell bodies are located (Riedel & Neeck, 2001). Nociceptors synapse onto a spinal interneuron in the dorsal horn that, in turn, transmits pain information to neurons in the brainstem (Loeser & Melzack, 1999). Pain information then travels through the thalamus before reaching the primary somatosensory cortex (D'Mello & Dickenson, 2008). Once pain signals reach the

cortex the emotional aspects of pain can be experienced (Stein et al., 2009).

Glutamatergic N-methyl-D-aspartate (NMDA) receptors and nitric oxide (NO) are found in high concentrations in the dorsal horn and play a key role in nociception. More specifically, glutamate binds to NMDA receptors found on postsynaptic nociceptors causing an influx of Ca^{2+}, which activates nitric oxide synthase (NOS) (Kawamata & Omote, 1999). NOS converts L-arginine into NO, which can then easily diffuse to surrounding presynaptic neurons where it activates guanylyl-cyclase (Kawamata & Omote, 1999). Guanylyl-cyclase increases the production of cyclic guanosine monophosphate (cGMP) that activates phospholipase C (PLC). This enzyme cleaves phosphatidylinositol biphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG) (Kawamata & Omote, 1999). DAG can cause the release of sequestered calcium stores, as well as travel along the membrane phosphorylating potassium channels leading to excitation and hyperalgesia (Pantaleo et al., 1987). Interestingly, opioidergic receptors are colocalized with glutamatergic receptors on nociceptive neurons, where they act as modulators of glutamatergic activity (Munoz et al.,

2012). When stimulated, inhibitory MORs indirectly reduce the activity of NMDA receptors in the cortex (Munoz et al., 2012). Activation of KORs colocalized on NMDA receptors in the prefrontal cortex has a different inhibitory mechanism (direct antagonism) (Munoz et al., 2012; Svingos & Colago, 2002)

Analgesic systems (e.g., the opioid system) are simultaneously activated as pain signals are sent to the brain. Endorphins, dynorphins, and enkephalins are released by leukocytes (immune cells) in damaged peripheral tissue (Cabot et al., 2001). In the spinal cord, opioid receptor stimulation inhibits substance P and calcitonin gene related peptide (CGRP) neurons (Belanger et al., 2002). Opioid receptors couple to guanine nucleotide-binding regulatory proteins (G-proteins) G_i and G_o (Belanger et al., 2002). G_i and G_o G-proteins disassociate from activated opioidergic receptors with the binding of guanosine triphosphate allowing them to phosphorylate postsynaptic potassium channels (Charles & Hales, 2004).

When activated, substance P and CGRP neurons increase inflammation and contribute to hyperalgesia at areas of injury (Belanger et al., 2002; Cabot et al.,

2001). Intracellular signaling enzymes like cGMP dependent protein kinase G (PKG), protein kinase C (PKC), and cAMP dependent protein kinase A (PKA) can phosphorylate MORs and KORs leading to increased binding of β-arrestin (Groer et al., 2007). β-arrestin can inhibit G-protein coupling causing internalization and desensitization of the opioid receptors (Groer et al., 2007). MOR and KOR stimulation reduces hyperalgesia by inhibiting the actions of NMDA receptors located on nociceptive neurons in various brain regions (Jolas & Aghajanian, 1997). An injection of morphine into the nucleus raphe magnus or the left lateral ventricle causes potent analgesia on nociceptive paradigms in the rat (Cecchi et al., 2008; Duale, Sierralita, & Dallel, 2007; Hallady et al., 2009; Loyd, Morgan, & Murphy, 2007; Manning & Mayer, 1995).

Pain resulting from inflammation or nerve damage leads to upregulation of MOR and KOR mRNA expression in dorsal root ganglia (DRG) neurons (Puehler et al., 2004, 2006). More specifically, bradykinin and other inflammatory agents increase the trafficking of opioid receptors to the plasma membrane in DRG (Patwardhan et al., 2005). Pain due to nerve damage differs from

inflammation because the upregulation of MORs and KORs appears at the damaged nerve and surrounding tissue not the DRG neurons (Walczak et al., 2005).

One of the main modulatory systems of pain perception in the central nervous system is the periaqueductal gray - rostral ventromedial medulla pathway (PAG - RVM), which indirectly controls the perception of noxious but not innocuous stimuli (Yaksh, Yeung, & Rudy, 1976). The PAG receives innervation from the anterior cingulate gyrus, prefrontal cortex, and amygdala, which are areas known for their role in the emotion, memory, and perception of pain (Calejesan, Kim, & Zhuo, 2000; Helmstetter & Tershner, 1994). RVM axons extend to the dorsal horn, where they terminate at the A_δ and c fiber interneuron juncture. Microinjections of morphine into the RVM or PAG produce analgesia in the rat (Tortorici, Morgan, & Vanegas, 2001). Aside from the PAG - RVM circuit, the dorsal reticular nucleus (DRt) and the ventral lateral medulla (VLM) are also involved in the modulation of pain perception (Almeida et al., 1999, 2006; Tavares, Lima, & Coimbra, 2002). For instance, electrical stimulation of the DRt produces hyperalgesia on the tail-flick and hot-plate tasks; whereas,

stimulation of the VLM can produce analgesia or hyperalgesia depending on the type of pain (Almedia et al., 1996; Lovick, 1990). More specifically, short-term inflammation or nerve damage leads to activation of the VLM by neurons in the dorsal horn and causes hyperalgesia; whereas, long-term inflammation leads to activation of the VLM and causes analgesia (Pinto, Limas, & Tavares, 2007). Short term activation of neurons in the DRt and VLM down regulate MOR and DOR expression leading to less nociceptive inhibition (Neto et al., 2008; Pinto et al., 2008).

The noradrenergic system is also involved in nociceptive functioning. α_1-, α_{2a}-, and α_{2c}-Adrenergic receptor mRNA is expressed in the dorsal root ganglia (Nicholes et al., 1993; Xie et al., 2001). Noradrenergic receptor activation has little role in nociceptive functioning of healthy tissue in the periphery; however, areas with damaged tissue are considerably affected by norepinephrine efflux and receptor stimulation (Ali et al., 2000; Fuchs, Meyer, & Raja, 2001). Subcutaneous injection of norepinephrine into undamaged skin elicits an inert response (Schattschneider et al., 2006). In contrast, injections of norepinephrine into A_δ and c

fibers of inflamed tissue or in sites of neuropathic pain lead to an increased nociceptive response (Bossut & Perl, 1995, 1996). During tissue damage, active recruitment of α_2-adrenergic receptors to the plasma membrane leads to increased nociception by receptor alterations in Ca^{2+} and K^+ channels via G-protein coupled receptors (Birder & Perl, 1999). α_1-Adrenergic receptors have also been implicated in hyperalgesia induced by peripheral tissue damage. For example, administration of an α_1-adrenergic receptor antagonist into the damaged area will attenuate pain perception (Hong & Abott, 1996). Intrathecal injections of an α_2-adrenergic receptor agonist leads to delayed latencies on the hot-plate and tail-flick tasks (Takano & Yaksh, 1992). Activated α_1-adrenergic receptors stimulate GABAergic interneurons in the dorsal laminae (Baba et al., 2000), which is an area implicated in pain perception and is a main target of analgesics.

The PAG innervates the pons and locus coeruleus, which are the major descending noradrenergic modulators of pain perception. Infusion of noradrenergic agonists into the locus coeruleus elicits an analgesic response (Janss, Jones, & Gebhart, 1987). Conversely, an injection of a noradrenergic antagonist leads to an attenuated

analgesic response (Janss et al., 1987). Injections of an α_2-adrenergic receptor antagonist into the pons elicits a hyperalgesic response (Aimone, Jones, & Gebhart, 1987). Interestingly, opioidergic-induced analgesia in the PAG - RVM pathway is attenuated by micro-injecting a noradrenergic antagonist into the locus coeruleus and pons (Bie et al., 2003), which shows a clear role for norepinephrine in pain modulation.

While opioidergic and noradrenergic pathways are implicated in the regulation of pain, the role of dopaminergic pathways in nociception is less understood. Disruption of dopaminergic functioning in the PAG reduces morphine-induced analgesia in the rat (Flores et al., 2004). An intrathecal injection of a dopamine D_2 receptor agonist induces antinociception on the tail-flick and hot-plate tasks (Gao et al., 1998). Psychostimulants (e.g., cocaine or methamphetamine) induce analgesia in tonic pain paradigms (Yamamotova et al., 2011). Moreover, attenuated analgesia in the formalin test is observed if raclopride (D_2 antagonist) is administered into the nucleus accumbens prior to nitrous oxide exposure (Koyanagi et al., 2008).

In summary, there are three major types of pain: neuropathic pain, inflammatory pain, and physiological pain. It is evident from phantom limb pain that input from the periphery is not necessary to experience pain. Nociceptors transmit information about peripheral tissue damage to the dorsal horn. Nociceptors synapse onto interneurons that decussate and send information to the hindbrain. Pain information is then relayed to the midbrain and then to the thalamus before being projected to the cortex. Once pain information reaches the cortex the emotional aspects of pain are perceived. There are many neurotransmitter systems involved in the transmission and perception of pain, including opioidergic, glutamatergic, GABAergic, noradrenergic, dopaminergic, and nitric oxide systems. Some enzymes and molecules, like nitric oxide, glutamate, arachidonic acid, and prostagladins, facilitate pain transmission, inflammation, and even induce hyperalgesia.

CHAPTER SIX

EARLY METHYLPHENIDATE EXPOSURE

Methylphenidate is an indirect dopamine and norepinephrine agonist, commonly prescribed to treat developmental disorders including ADHD (Marco et al., 2011). Administering methylphenidate to rodents during critical periods in development can have profound effects on the central and peripheral nervous system, including alterations to cell metabolism, changes in sensitivity to morphine, modifications in gene transcription in the striatum, and neurochemical changes that persist into adulthood (Marco et al., 2011).

Metabolic alterations resulting from early methylphenidate treatment include increases in oxidative stress, Na^+/K^+ ATPase activity, and mitochondrial function. Rats chronically treated (28 days) with methylphenidate (1, 2, or 10 mg/kg) from PD 25 to PD 53 had an increase in oxidative stress when compared to saline controls (Martins et al., 2006). More specifically, methylphenidate increased the production of the free radical, thiobarbituric acid reactive species (TARS), which lead to two negative outcomes associated

with an increase in oxidative stress: greater peroxidation of the phospholipid bilayer and increased protein carbonyl formation (Martins et al., 2006). Rats injected with methylphenidate (1, 2, or 10 mg/kg) from PD 25-53 also have increased Na^+/K^+ ATPase activity (Scherer et al., 2009). Pretreatment with methylphenidate (1, 2, 5, 10, or 20 mg/kg) from PD 25-53 increases mitochondrial enzyme functioning critical for ATP synthesis (Fagundes et al., 2007).

Early exposure to methylphenidate alters more than 700 genes found in the striatum (Adriani et al., 2006). The genes MPP3, shank2, and homer1 are altered by early methylphenidate exposure and they code for proteins which traffic glutamatergic NMDA and metabotropic GluR receptors to the membrane (Marco et al., 2011; Sala et al., 2001).

Aside from metabolic and genetic alterations, exposure to methylphenidate during adolescence can also cause behavioral changes in response to drug challenge. Attenuated responses to illicit drugs in adulthood are observed in animals pretreated with methylphenidate from PD 20-35. More specifically, rats exposed to methylphenidate during this period in ontogeny have a

decrease in responding for brain stimulation even with a challenge injection of cocaine (Mague, Anderson, & Carlezon, 2005). Mice receiving methylphenidate from PD 26-32 exhibit a decreased preference in cocaine-induced conditioned place preference (Mendes, Anderson, & Itzhak, 2003). Consistent with these findings, cocaine-induced conditioned place preference was also reduced in rats treated with methylphenidate from PD 20-35, thus showing the enduring methylphenidate-induced behavioral alterations to illicit drugs across species (Carlezon, Mague, & Anderson, 2003).

Exposure to methylphenidate from PD 20-36 in rats leads to an attenuated response to sex, novelty seeking, and sucrose, as well as increasing sensitivity to high stress situations (Bolanos et al., 2003). Rats exposed to methylphenidate from PD 27-56 had greater anxiety-like behaviors (increased time spent in the elevated plus mazes closed arm and faster acquisition of fear conditioning) than did rats exposed to methylphenidate from PD 27-76 (Britton & Bethancourt, 2009). Along with exacerbated anxiety, rats treated with methylphenidate from PD 20-35 showed an increase in depressive-like behaviors (Carlezon et al., 2003). Similarly, decreased

sucrose preference, increased anxiety (elevated plus maze), and stress (forced swim) are exhibited from early methylphenidate exposure (Wiley et al., 2009). Interestingly, chronic treatment with fluoxetine in adulthood could alleviate the depressive symptoms expressed by rats exposed to methylphenidate from PD 20-35 (Bolanos et al., 2008).

Methylphenidate treatment during the preweanling period results in different behavioral alterations than adolescents and periadolescents exposure. For example, pretreatment with methylphenidate during the preweanling period (e.g., PD 11-20) leads to increased preference for the morphine-paired room (Crawford et al., 2007). Interestingly, methylphenidate does not affect the magnitude of cocaine-induced CPP (Crawford et al., 2011). These data suggest that early methylphenidate treatment may have a direct effect on opioid receptors as opposed to modulating reward circuitry. In support of this hypothesis, early methylphenidate exposure enhanced morphine-induced antinociception and sucrose preference, behaviors known to be modulated by opioid receptors (Cyr & Morgan, 2009; Halladay et al., 2009).

Methylphenidate is commonly prescribed to pediatric populations with ADHD. This psychostimulant is an indirect catecholamine agonist that blocks the dopamine and norepinephrine transporters. Increased catecholamine activity can have detrimental effects during development, including decreased sensitivity to natural reward and an increased sensitivity to anxiety and depressive-like symptoms. Chronic administration of methylphenidate during the adolescent period, and the preweanling period can also alter cell metabolism and genetic expression.

CHAPTER SEVEN

THESIS STATEMENT AND PROPOSAL

Psychostimulants (methylphenidate, L-amphetamine, and D-amphetamine) are commonly prescribed to pediatric populations for ADHD (Murray, 2010). There is extensive research showing that methylphenidate is effective in treating the symptoms of ADHD in school age children, however much less is known about the efficacy of the drug when prescribed to children ages 3 - 5 (Murray, 2010). Studies in rodents suggest that pre-exposure to methylphenidate in early development increases opioid sensitivity in adulthood (Crawford et al., 2007; Cyr & Morgan, 2009; Halladay et al., 2009; Wiley et al., 2009).

Because methylphenidate has a high affinity for both dopamine and norepinephrine transporters (Fagundes et al., 2007), it is unknown which neurotransmitter system is responsible for the increased opioid sensitivity found in adulthood. Therefore, the goal of the present thesis is to determine whether dopamine or noradrenergic transporters are responsible for the increased sensitivity of opioid receptors after methylphenidate treatment.

We propose to administer the selective noradrenergic transporter blocker atomoxetine (Swanson et al., 2006) and the selective dopamine transporter blocker GBR-12909 (Szasz, Vizi, & Kiss, 2007) during the preweanling period (i.e., PD 11-20) to differentiate the effects of dopamine and noradrenergic transporter blockade. To assess the sensitivity of opioid receptors in adulthood, we will measure morphine-induced antinociception using the hot-plate and tail-flick tasks on PD 60. We hypothesize that GBR-12909 pre-exposure will not produce the same increases in opioid system sensitivity shown by Crawford and colleagues (2007, 2009). In support of this hypothesis, chronic administration of cocaine (a dopamine, serotonin, and norepinephrine reuptake blocker) to adult rats causes an increase in KOR density and prodynorphin gene expression in the striatum; however, chronic GBR-12909 administration did not have these effects (Collins et al., 2002; Romualdi et al., 2001). Furthermore, we hypothesize that atomoxetine pre-exposure during the preweanling period will enhance the analgesic effects of morphine, thus suggesting that alterations in the noradrenergic system during the preweanling period are sufficient for the persistent changes in opioid

system functioning. Consistent with this hypothesis, morphine-induced antinociception is potentiated in norepinephrine knockout mice and in wild-type mice co-administered a noradrenergic transporter blocker (Bohn et al., 2000). If neither GBR-12909 nor atomoxetine are sufficient to potentiate morphine-induced analgesia, we will conduct a third experiment where GBR-12909 and atomoxetine will be co-administered.

CHAPTER EIGHT

METHODS

Subjects

A total of 480 male Sprague-Dawley rats (n = 10 per group) reared at California State University, San Bernardino were used. Rat pups were culled to litters of 10 on PD 3 and weaned on PD 25. Subjects were housed in the colony room with food and water freely available. The colony room temperature was kept at 21-23°C with lights on from 6:00 A.M. to 8:00 P.M. The research protocol was approved by the Institutional Animal Care and Use Committee of California State University, San Bernardino and all subjects were cared for according to the "Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research" (National Research Council, 2010).

Drugs

Atomoxetine hydrochloride was purchased from Toronto Research Chemical (Toronto), while GBR-12909 and morphine were obtained from Sigma Chemicals (St. Louis, MO). Atomoxetine and GBR-12904 were dissolved in vehicle (50% DMSO in distilled water) and injected intraperitoneally (IP) at a volume of 5 ml/kg. Morphine was dissolved in

saline and injected subcutaneously (SC) at a volume of 1 ml/kg.

In Vivo Drug Treatment

Starting on PD 11, rats were weighed and injected with atomoxetine (0, 0.3, 1, or 3 mg/kg), GBR (0, 1.5, 5, or 15 mg/kg), a combined injection of atomoxetine (3 mg/kg) plus GBR (15 mg/kg) or vehicle (IP) for 10 consecutive days. After drug pretreatment, rats were left undisturbed until behavioral testing.

Apparatus

The tail-flick apparatus (UGO Basile North America, Collegeville, PA) and hot-plate analgesia meter (HTC Life Science Inc, Woodland Hills, CA) were used to assess antinociception in the present experiment. An adjustable laser beam was used to apply radiant heat in the tail-flick test. The hot-plate was heated and maintained at 54°C (±0.1°C).

Experiment 1: Atomoxetine Pre-exposure

In Experiment 1 rats were exposed to atomoxetine (0.3, 1, or 3 mg/kg) or vehicle during the preweanling period. On PD 58-59 subjects were habituated to the

tail-flick and hot-plate procedures with the machines turned off. Habituation consisted of individual rats being held down in the proper position on the tail-flick machine for 2 min, then being placed in the hot-plate apparatus for 2 min. This procedure occurred twice daily. Morphine-induced antinociception testing began on PD 60. This assay consisted of three baseline trials spaced 20 min apart, in which the rats' tails were laid across the tail-flick laser and their latency to tail-flick were recorded. Immediately after the tail-flick assay, rats were tested for paw lick latency on the hot plate. After the third baseline trial, rats were injected with morphine (0, 2.5, 5, or 10 mg/kg, SC) and placed back in their home cages for 20 min. Three additional test trials were completed, with a 20 min interval between trials. To avoid tissue damage, rats were removed from the tail-flick apparatus after 15 s or hot-plate after 30 s if an incorrect response was made.

Experiment 2: GBR-12909 Pre-exposure

Experiment 2 utilized the same procedure as described in Experiment 1; however, animals were

pre-exposed to GBR-12909 (1.5, 5, or 15 mg/kg) or vehicle during the preweanling period.

Experiment 3: Combined GBR/Atomoxetine Pre-exposure

If neither selective transporter blocker produces effects similar to methylphenidate, Experiment 3 would have been conducted using the same procedures for habituation and testing as described for Experiment 1. However, animals would have been exposed to vehicle or atomoxetine (3 mg/kg) plus GBR (15 mg/kg) during the preweanling period.

Data and Statistical Analysis

Prior to statistical analysis, data was screened for outliers, homoscedasticity, and normality.

Antinociception was measured as the latency to tail-flick in the tail-flick task or latency to paw-lick or jump in the hot-plate task. Baseline latencies were analyzed by separate one-way ANOVAs (pre-exposure drug).

The three baseline trials were averaged to form a single baseline score for each subject in the analyses. A maximal analgesic effect, defined as [(test latency - baseline latency) / (cut-off time - baseline latency)] ×

100 was utilized as the dependent variable. The tail-flick and hot-plate assays are known for high variability in baseline-trials. The maximal analgesic effect score was used because it adjusts for possible differences in baseline latencies between treatment groups, making it easier to discriminate true differences in drug-induced antinociception. The maximal analgesic effect score also allows for easy comparison to other journal articles using the same measure (Cyr & Morgan, 2009; Hall et al., 2011; Halladay et al., 2009).

Independent variables for Experiment 1 included pre-exposure to atomoxetine (0, 0.3, 1, or 3 mg/kg) and challenge injection of morphine (0, 2.5, 5, or 10 mg/kg). Manipulations for Experiment 2 consisted of pre-exposure to GBR (0, 1.5, 5, or 15 mg/kg) and test injection of morphine at the same doses as Experiment 1. Experiment 3's independent variables would have been pre-exposure to atomoxetine (3 mg/kg) plus GBR (15 mg/kg), or vehicle treatment and challenge injection of morphine at the same doses as the first two experiments. Separate ANOVAs with Bonferroni post-hoc comparisons were used to assess group difference in the three experiments. More specifically, a 4 x 4 factorial ANOVA was used for Experiment 1

(atomoxetine dose × morphine dose), a 4 × 4 factorial ANOVA (GBR dose × morphine dose) was used for Experiment 2, and a 2 × 4 factorial ANOVA GBR/atomoxetine dose × morphine) would have been used for Experiment 3 to assess group differences.

Table 1. Methodology

	Experimental Design		
	Exp. 1 Atomoxetine	Exp. 2 GBR-12909	Exp. 3 Ato./GBR
IV 1 Pretreatment	(0, 0.3, 1, or 3 mg/kg)	(0, 1.5, 5, or 15 mg/kg)	(0 or 3mg/kg & 15 mg/kg ato/GBR)
IV 2 Challenge-injection	(0, 2.5, 5, 10 mg/kg) morphine	(0, 2.5, 5, 10 mg/kg) morphine	(0, 2.5, 5, 10 mg/kg) morphine
DV	% max. analgesic effect	% max. analgesic effect	% max. analgesic effect

Note: Exp. 1 & 2 are 4×4 and *Exp.* 3 is a 2×4 factorial ANOVA's

CHAPTER NINE

RESULTS

Statistical Screening

Frequency distributions of the baseline trials, testing trials, and percent maximal analgesic effect (MAE) were normal, yet one animal in the atomoxetine study was removed for discontinuity from the distribution on hot-plate latencies. No animals were removed from the GBR-12909 study.

Significant differences were observed between test-trial latencies in the atomoxetine study. Therefore, separate maximal analgesic effect scores were calculated for each of the three test-trials in both studies. Each test trial was 20 min apart and morphine's efficacy increased during each trial due to slow absorption typical after subcutaneous injections. Separate MAE scores allowed for visualization of subtle pretreatment differences at different morphine efficacies.

Experiment 1

As expected, steady increases in body weight were observed during pretreatment atomoxetine days [$F_{(2,221)} = 3302.37$, $p < 0.05$]. Two atomoxetine litters

with significantly smaller mean body weights were removed from the final analysis. Body weight was not affected by atomoxetine pretreatment on antinociception test day.

There was a trend towards differences in tail-flick latencies between baseline trials in the atomoxetine experiment; however, differences did not reach statistical significance [$F_{(2,247)} = 2.74$, $p = 0.07$]. Atomoxetine pretreatment did not alter latencies on the tail-flick task for the three baseline trials [$F_{(3,130)} = 0.33$, $p > 0.05$] (Figure 1). In contrast to the tail-flick, there was a significant difference between baseline trials on the hot-plate assay for the atomoxetine experiment [$F_{(2,260)} = 6.73$, $p < 0.05$]. Specifically, baseline one was significantly different from baselines two and three; however, baseline two and three did not differ amongst each other (Bonferroni; $p < 0.05$). Atomoxetine pretreatment did not have an effect on baseline trials for the hot-plate paradigm [$F_{(3,130)} = 0.25$, $p > 0.05$] (Figure 1).

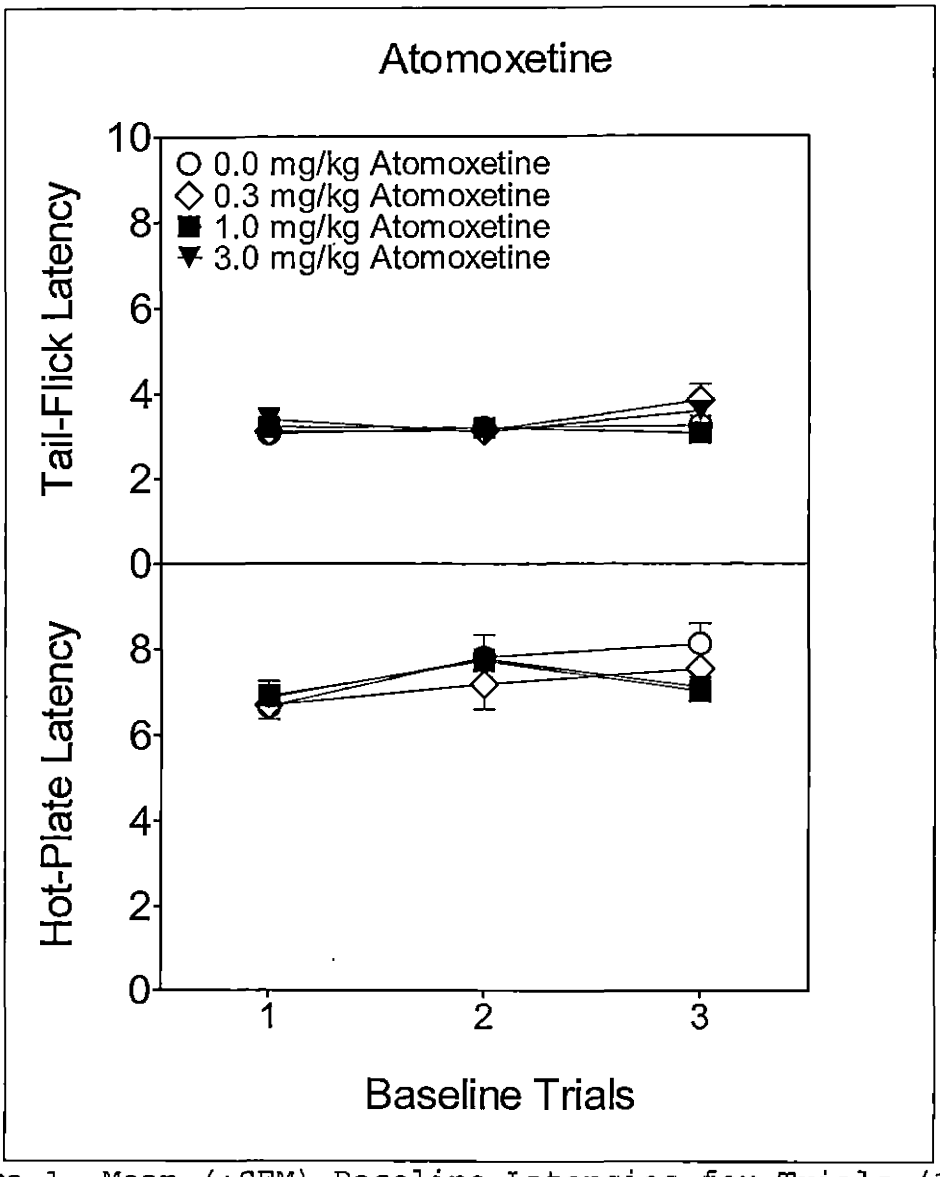


Figure 1. Mean (±SEM) Baseline Latencies for Trials (1-3) on the Tail-flick (Top Graph) and Hot-plate (Bottom Graph) Assays in PD 60 Rats. Animals Received Pretreatment with Atomoxetine (0, 0.3, 1, or 3 mg/kg) from PD 11-20

Atomoxetine: Tail-flick

Pretreatment with atomoxetine did not affect opioid sensitivity in adulthood on the tail-flick task. There were no differences between atomoxetine doses in MAE scores on test trial 1 [$F_{(3,132)} = 0.28$, $p > 0.05$] (Figure 3). As expected, there was a significant main effect for morphine dose on test trial 1 [$F_{(3,132)} = 29.85$, $p < 0.05$]. More specifically, saline controls had significantly lower MAE scores than all three morphine doses, the 2.5 and 5 mg/kg dose groups did not significantly differ from each other; however their scores were significantly lower than the 10 mg/kg group (Bonferroni; $p < 0.05$) (Figure 2).

Similar results were observed for test trial 2, which had no differences between MAE scores between pretreatment atomoxetine groups [$F_{(3,132)} = 0.83$, $p > 0.05$] and test trial 3 [$F_{(3,132)} = 0.83$, $p > 0.05$] (Figure 3). There was a significant morphine dose main effect on test trial 2 [$F_{(3,132)} = 65.29$, $p < 0.05$] and test trial 3 [$F_{(3,132)} = 71.09$, $p < 0.05$] (Figure 2). Each morphine dose was significantly different from the other three doses (Bonferroni, $p < 0.05$).

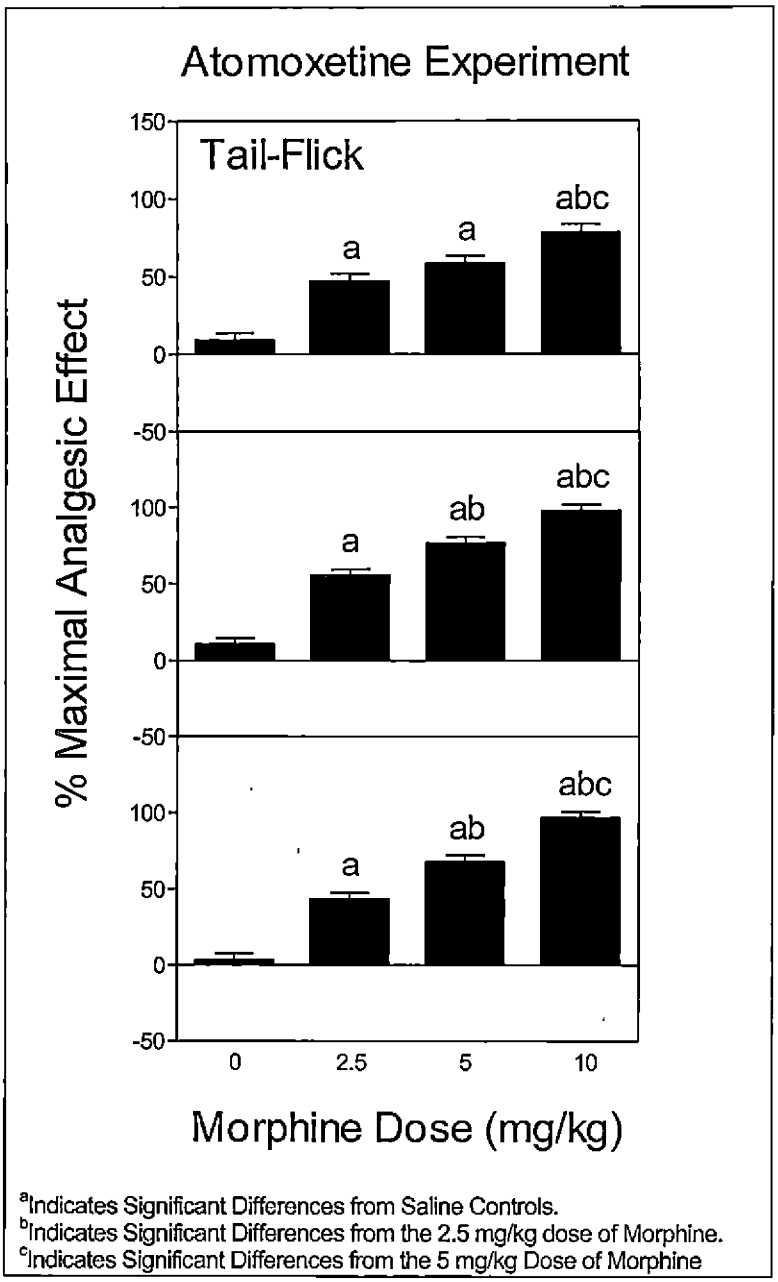


Figure 2. Mean Maximal Analgesic Effect Scores (MAE) on Tail-flick Assays for Test-trial 1 (Top Graph), Test-trial 2 (Middle Graph), and Test-trial 3 (Bottom Graph) in PD 60 rats. MAE Scores have been Collapsed over Atomoxetine Pretreatment Condition

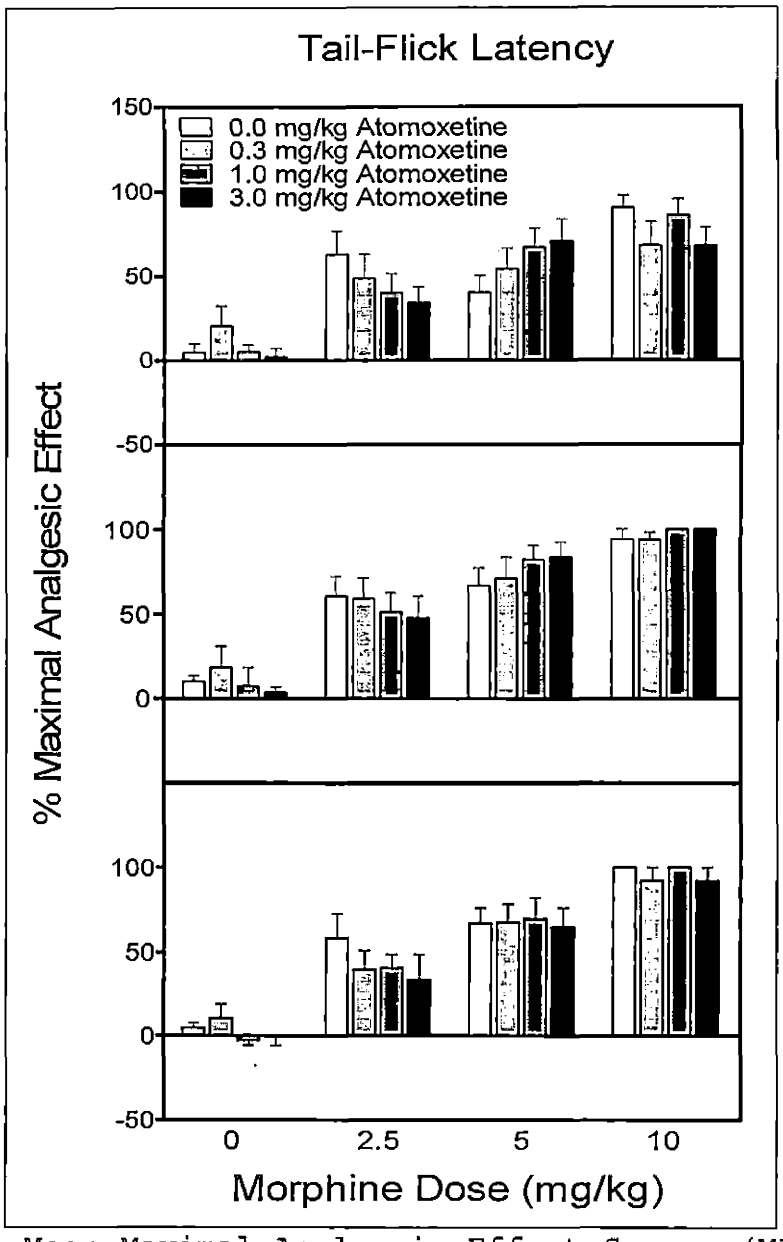


Figure 3. Mean Maximal Analgesic Effect Scores (MAE) on the Tail-flick Assay for Test-trial 1 (Top Two Graphs), Test-trial 2 (Middle Two Graphs), and Test-trial 3 (Bottom Two Graphs) in PD 60 Rats. Animals were Pretreated with Atomoxetine (0, 0.3, 1, or 3 mg/kg) from PD 11-20

Atomoxetine Experiment: Hot-plate

In contrast to the tail-flick assay, pre-exposure to atomoxetine did alter opioid sensitivity in adulthood on the hot-plate procedure. This effect, however, was not apparent on test-trial 1 [$F_{(3,132)} = 0.57$, $p > 0.05$] (Figure 5). There was a significant main effect of morphine for test-trial 1 [$F_{(3,132)} = 21.20$, $p < 0.05$]. More specifically, saline controls had significantly lower MAE scores than animals given 5 or 10 mg/kg dose of morphine, but saline controls did not differ from animals given the 2.5 mg/kg dose (Bonferroni; $p < 0.05$) (Figure 4). Also MAE scores did not significantly differ when given either the 5 or 10 mg/kg doses (Bonferroni; $p < 0.05$) (Figure 4).

There was a significant main effect for pretreatment atomoxetine on MAE scores for test trial 2 [$F_{(3,132)} = 6.60$, $p < 0.05$] (Figure 5), as well as, a significant main effect for morphine dose [$F_{(3,132)} = 71.66$, $p < 0.05$] (Figure 4). Test day administration of saline yielded significantly lower MAE scores on test trial 2 compared to rats injected with morphine (Figure 4). The 5 mg/kg dose produced higher MAE scores than animals administered the 2.5 mg/kg, and the

10 mg/kg dose produced the highest levels of analgesia (Bonferroni; $p < 0.05$) (Figure 4).

Rats receiving atomoxetine (0.3 mg/kg) had significantly lower MAE scores compared to animals given a 0, 1.0, or 3.0 mg/kg dose on test trial 2 (Figure 5). However, the three latter doses did not significantly differ from each other. Separate one-way ANOVA's were conducted to assess a priori differences between atomoxetine groups at different doses of morphine. Administration of morphine (10 mg/kg) to animals pretreated with atomoxetine (0.3 mg/kg) had significantly lower MAE scores than saline controls, 1.0, or 3.0 mg/kg dose of atomoxetine [$F_{(3,31)} = 6.98$, $p < 0.05$] (Figure 5).

Similar to test trial 2, there was significant differences between atomoxetine groups MAE scores on test trial 3 [$F_{(3,132)} = 3.43$, $p < 0.05$] (Figure 5) and a significant main effect for morphine dose [$F_{(3,132)} = 177.44$, $p < 0.05$] (Figure 4). Pretreatment with atomoxetine (1.0 mg/kg) produced significantly higher MAE scores than saline controls (Bonferroni; $p < 0.05$) (Figure 5). Preweanling exposure to atomoxetine (0.3 mg/kg) resulted in significantly lower MAE scores than subjects administered the 1 or 3 mg/kg doses

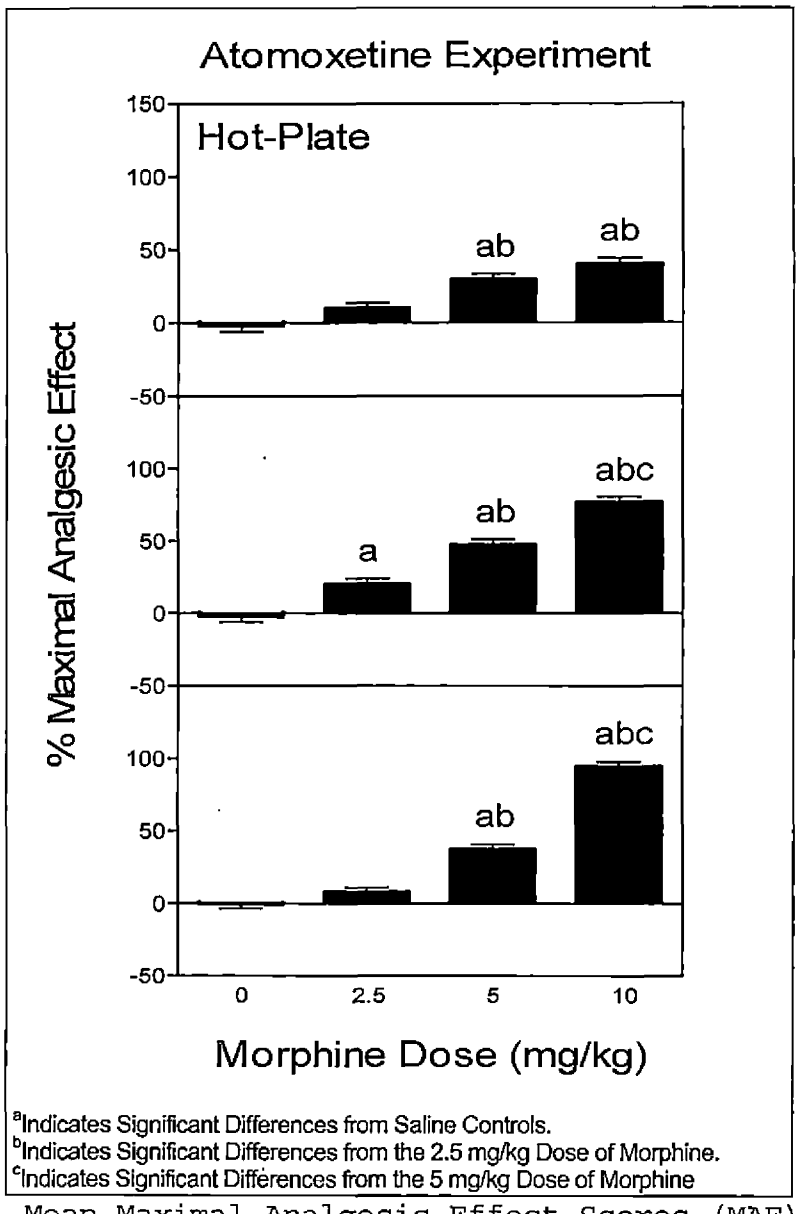
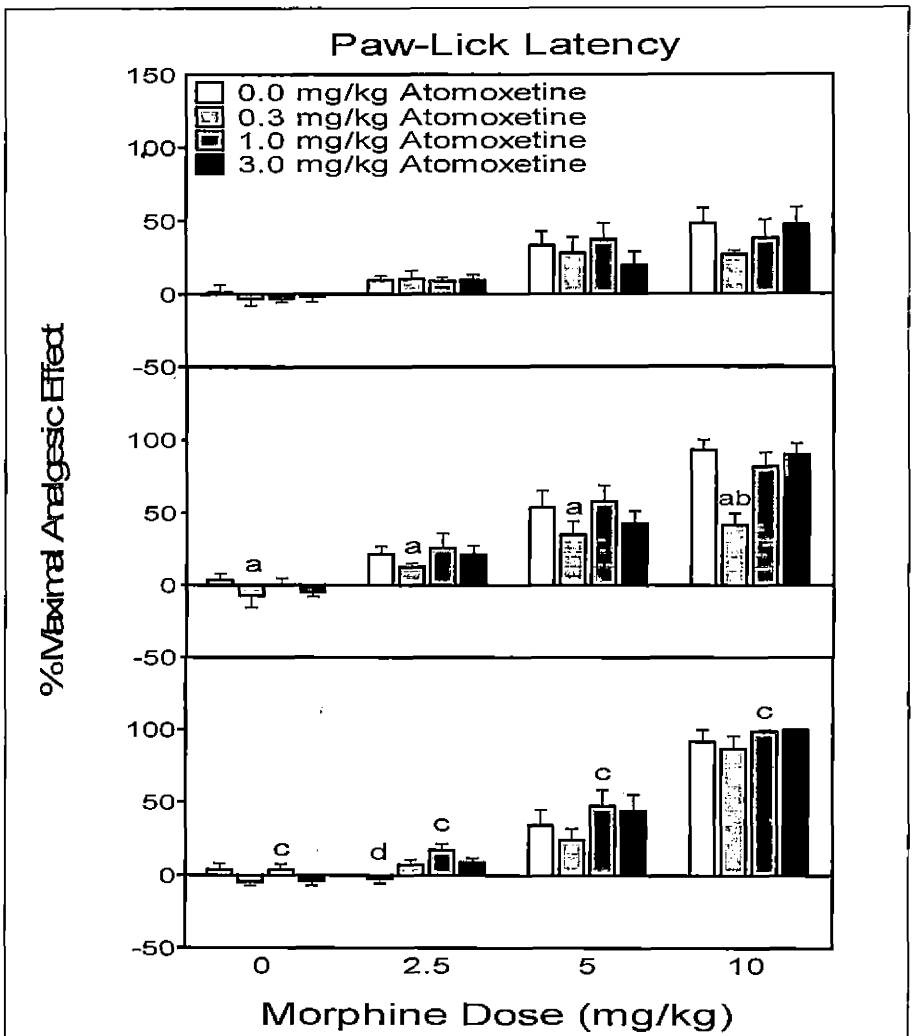


Figure 4. Mean Maximal Analgesic Effect Scores (MAE) Hot-plate Assays for Test-trial 1 (Top Graph), Test-trial 2 (Middle Graph), and Test-trial 3 (Bottom Graph) in PD 60 Rats. MAE Scores have been Collapsed over Atomoxetine Pretreatment Condition



[a]Indicates Significantly Lower MAE Scores at the 0.3 mg/kg Atomoxetine Compared to the Other Three Doses in Test-trial 2.
[b]Indicates Significantly Lower MAE Scores at the 0.3 mg/kg Dose of Atomoxetine Compared to the Other Three Doses Found from a One-way ANOVA Analysis of the 10 mg/kg Dose of Morphine on Test-trial 2.
[c]Indicates Significantly Higher MAE Scores at the 1.0 mg/kg Dose of Atomoxetine Compared to Saline Controls on Test trial 3.
[d]Indicates Significantly Lower MAE Scores for Saline Controls Compared the 1 mg/kg Dose of Atomoxetine in a Separate One-way ANOVA Analysis of the 2.5 mg/kg Dose of Morphine

Figure 5. Mean Maximal Analgesic Effect Scores (MAE) on the Hot-plate Assay for Test-trial 1 (Top Two Graphs), Test-trial 2 (Middle Two Graphs), and Test-trial 3 (Bottom Two Graphs) in PD 60 Rats. Animals were Pretreated with Atomoxetine (0, 0.3, 1, or 3 mg/kg) from PD 11-20. Test-trial 2 and Test-trial 3 had Significant Atomoxetine Dose Main Effects

(Bonferroni; $p < 0.05$) (Figure 5). Separate one-way ANOVA's for a priori assessment revealed that animals injected on test day with morphine (2.5 mg/kg), and pretreated with atomoxetine (1.0 mg/kg) had significantly higher MAE scores compared to saline controls [$F_{(3,33)} = 5.45$, $p < 0.05$] (Figure 5).

Saline controls and morphine (2.5 mg/kg) did not differ on test trial 3; however, injections with morphine (5 mg/kg) had significantly different MAE scores compared to the other three doses (Bonferroni; $p < 0.05$) (Figure 4). Subjects receiving morphine (10 mg/kg) had significantly higher MAE scores than all three other morphine doses as well (Figure 4).

Experiment 2

Steady increases in body weight were observed during pretreatment GBR-12909 days [$F_{(2,319)} = 4438.96$, $p < 0.05$]. Body weight was not affected by GBR-12909 pretreatment on antinociception test day.

Although there was no differences in latency to tail-flick between GBR-12909 drug doses on any baseline trial (Figure 6), there was a significant GBR-12909 dose by baseline trial interaction on the hot-plate task

[$F_{(6,278)} = 3.10$, $p < 0.05$]. Animals receiving GBR-12909 (15 mg/kg) had significantly higher latencies on baseline-trials 1 and 2 but not 3 for the hot-plate compared to the saline controls (Figure 6).

GBR-12909: Tail-flick

Preweanling exposure to GBR-12909 did not alter opioid sensitivity in adulthood. No differences in MAE scores were observed between GBR-12909 groups on test trial 1 [$F_{(3,146)} = 0.13$, $p > 0.05$]; test trial 2 [$F_{(3,146)} = 0.14$, $p > 0.05$]; or test trial 3 [$F_{(3,146)} = 0.86$, $p > 0.05$] (Figure 8).

As expected, there was a morphine dose main effect for trial 1 [$F_{(3,146)} = 31.51$, $p < 0.05$] (Figure 7). More specifically, saline controls had lower MAE scores than subjects receiving any morphine dose (Bonferroni; $p < 0.05$) (Figure 7). MAE scores did not differ for animals receiving morphine (2.5 and 5 mg/kg), yet administration of the 10 mg/kg dose had significantly higher MAE scores than the other three doses (Bonferroni; $p < 0.05$) (Figure 7).

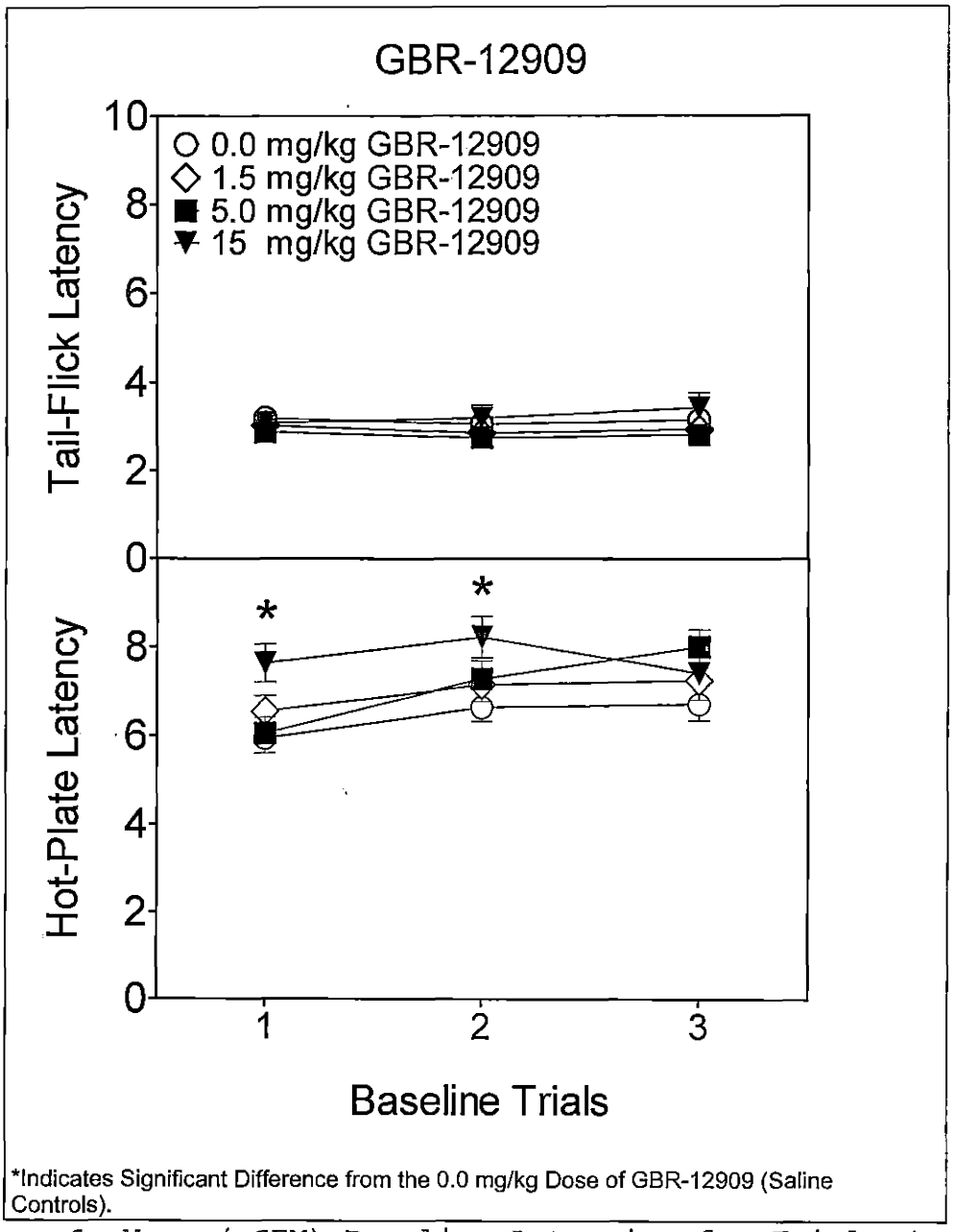


Figure 6. Mean (±SEM) Baseline Latencies for Trials (1-3) on the Tail-flick (Top Graph) and Hot-plate (Bottom Graph) Assays in PD 60 rats. Animals Received Pretreatment with GBR-12909 (0, 1.5, 5, or 15 mg/kg) from PD 11-20

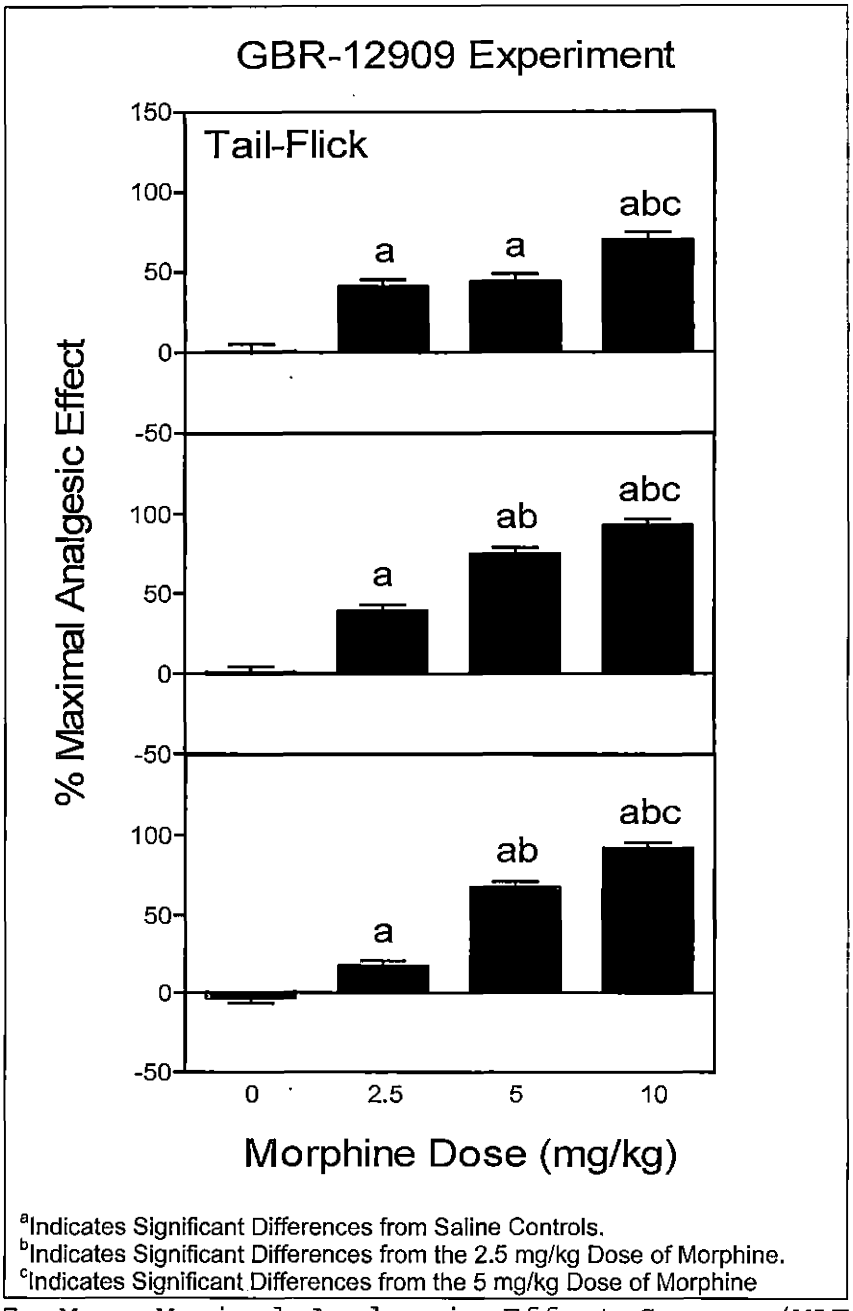


Figure 7. Mean Maximal Analgesic Effect Scores (MAE) on Tail-flick Assays for Test-trial 1 (Top Graph), Test-trial 2 (Middle Graph), and Test-trial 3 (Bottom Graph) in PD 60 Rats. MAE Scores have been Collapsed over GBR-12909 Pretreatment Condition

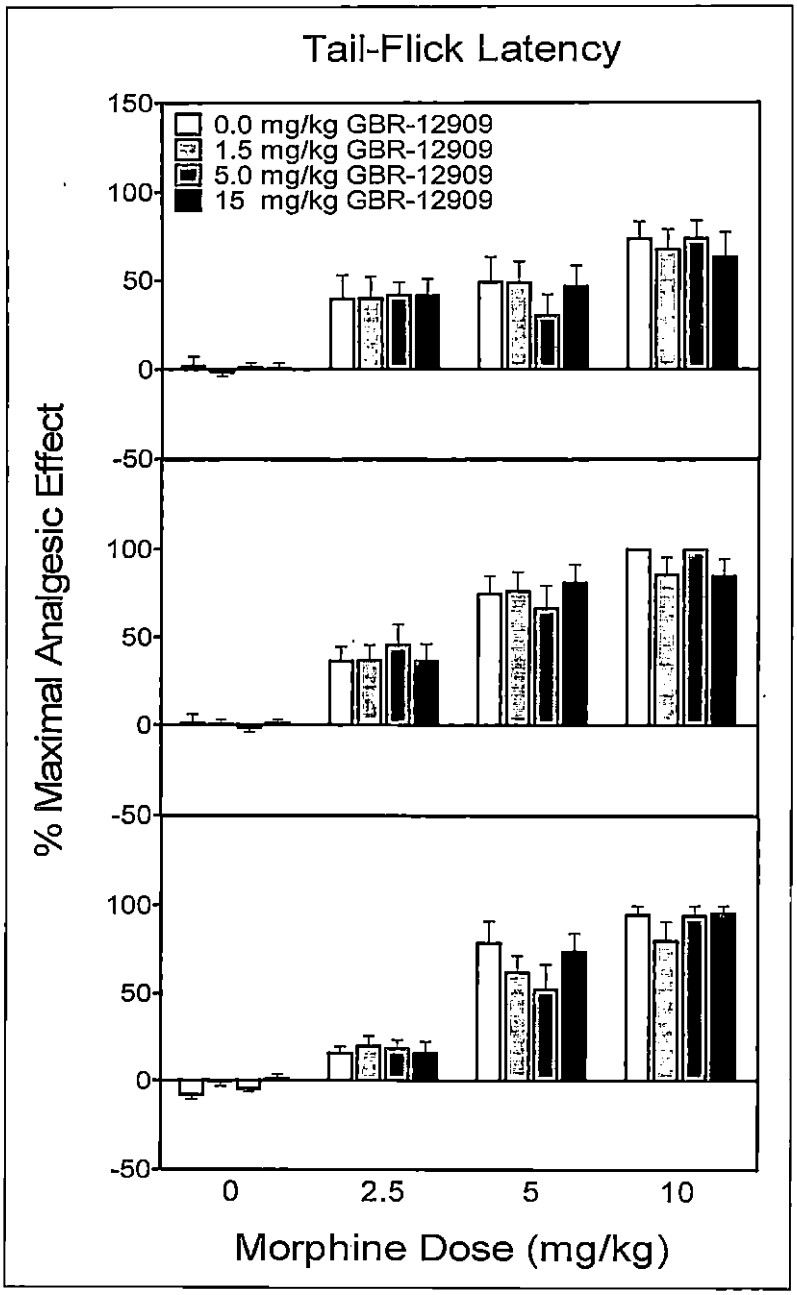


Figure 8. Mean Maximal Analgesic Effect Scores (MAE) on the Tail-flick Assay for Test-trial 1 (Top Two Graphs), Test-trial 2 (Middle Two Graphs), and Test-trial 3 (Bottom Two Graphs) in PD 60 Rats. Animals were Pretreated with GBR-12909 (0, 1.5, 5, or 15 mg/kg) from PD 11-20

Similar to test trial 1, there was a significant morphine dose main effect on test trial 2 [$F_{(3,146)} = 100.52$, $p < 0.05$] and test trial 3 [$F_{(3,146)} = 141.92$, $p < 0.05$]. Each dose of morphine was significantly different from the other three doses on test trial 2 and 3 (Bonferroni; $p < 0.05$) (Figure 7).

GBR-12909 Experiment: Hot-plate

Pre-exposure to GBR-12909 had little impact on opioid sensitivity in adulthood on the hot-plate procedure. MAE scores did not differ between GBR-12909 doses on test-trial 1 [$F_{(3,146)} = 0.42$, $p > 0.05$], test-trial 2 [$F_{(3,146)} = 1.22$, $p = 0.31$], or test-trial 3 [$F_{(3,146)} = 1.40$, $p = 0.25$] (Figure 10). Pretreatment with GBR-12909 (15 mg/kg) lead to a trend toward lower MAE scores compared to saline controls on test trial 2 and 3 though it did not reach statistical significant (Bonferroni; $p > 0.05$). Also, pretreatment with GBR-12909 (1.5 mg/kg) trended toward significantly lower MAE scores on test-trial 3.

There was a significant morphine dose main effect for test trial 1 [$F_{(3,146)} = 18.94$, $p < 0.05$], test trial 2 [$F_{(3,146)} = 119.70$, $p < 0.05$], and test trial 3 [$F_{(3,146)} = 212.35$, $p < 0.05$] (Figure 9). On test trial 1

saline control animals had significantly lower MAE scores than all three doses of morphine, injection of the 2.5 mg/kg dose was trending toward lower MAE scores compared to the 5 mg/kg dose, and administration of the 5 mg/kg produced scores that did not differ from the 10 mg/kg dose (Bonferroni; $p < 0.05$). On test trial 2 each dose of morphine was significantly different from the other three doses (Bonferroni; $p < 0.05$). On test trial 3 there was no difference between saline controls and animals administered morphine (2.5 mg/kg), while animals receiving the 5 mg/kg dose had significantly higher MAE scores than those given saline or 2.5 mg/kg doses (Bonferroni; $p < 0.05$). Animals given the 10 mg/kg had the highest MAE scores of any morphine dose (Bonferroni; $p < 0.05$).

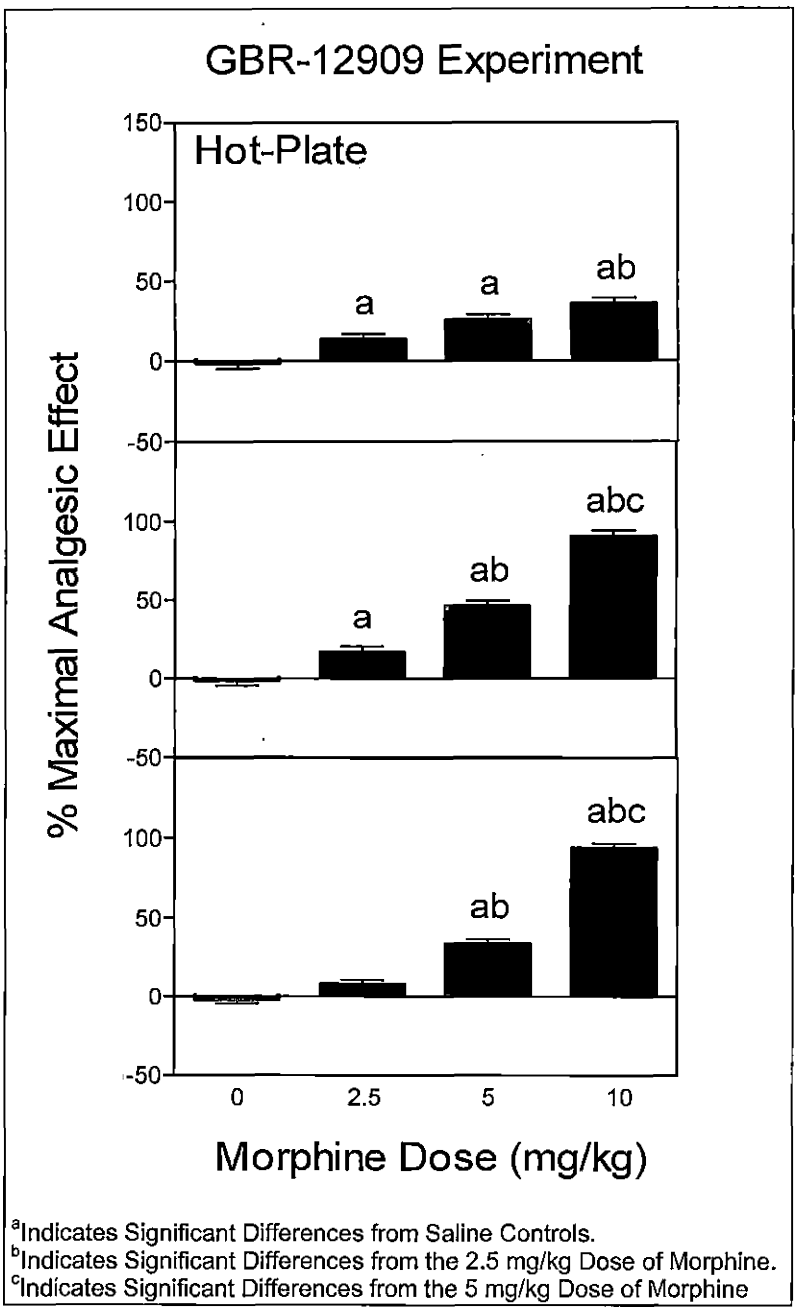


Figure 9. Mean Maximal Analgesic Effect Scores (MAE) on Hot-plate Assay for Test-trial 1 (Top Graph), Test-trial 2 (Middle Graph), and Test-trial 3 (Bottom Graph) in PD 60 Rats. MAE Scores have been Collapsed over GBR-12909 Pretreatment Condition

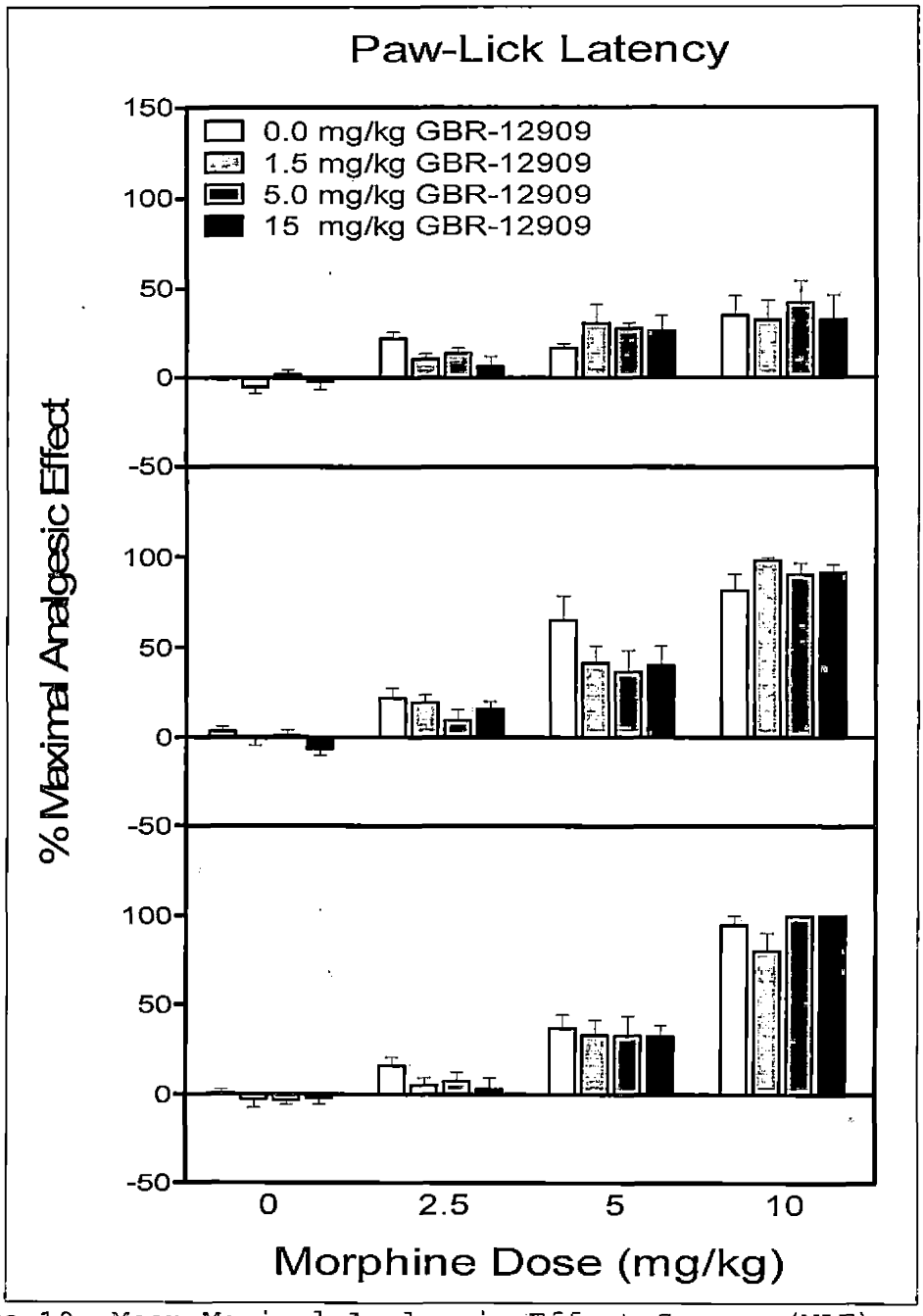


Figure 10. Mean Maximal Analgesic Effect Scores (MAE) on the Hot-plate Assay for Test-trial 1 (Top Two Graphs), Test-trial 2 (Middle Two Graphs), and Test-trial 3 (Bottom Two Graphs) in PD 60 Rats. Subjects were Pretreated with GBR-12909 (0, 1.5, 5, or 15 mg/kg) from PD 11-20

CHAPTER TEN

DISCUSSION

The purpose of this thesis was to elucidate which neurotransmitter system (e.g. norepinephrine or dopamine) was responsible for potentiated morphine-induced antinociception seen after preweanling exposure to methylphenidate (Cyr & Morgan, 2009; Halladay et al., 2009). It was hypothesized that increased activity in the noradrenergic system during the preweanling period would lead to a similar potentiation. Support for this hypothesis came from NET knockout mice which also show a potentiation in morphine-induced analgesia (Bohn et al., 2000).

It was also predicted that inhibition of the DAT by GBR-12909 would not alter opioid sensitivity in adulthood. Similar studies found that administration of cocaine but not GBR-12909 increased opioid receptor densities and release in the CNS (Collins et al., 2002; Romualdi et al., 2001), which suggests that GBR-12909 would not alter opioid-induced antinociception.

In support of our hypothesis, we found that increased activation of the noradrenergic system by

atomoxetine was capable of potentiating morphine-induced antinociception in adult rats. Specifically, pretreatment with a 1.0 mg/kg dose of atomoxetine leads to increased maximal analgesic effect scores compared to saline controls. As mentioned earlier, this increase in morphine-induced antinociception is similar to that observed in mice lacking the norepinephrine transporter (Bohn et al., 2000) but not in mice lacking the serotonin transporter (Hall et al., 2011).

Increases in opioid mRNA and opioid peptide release in the striatum was also observed after exposure to cocaine but not GBR-12909 (Collins et al., 2002; Romualdi et al., 2001). Since serotonin knockout mice and dopamine transporter inhibitor GBR-12909-treated animals did not affect opioid sensitivity but cocaine did, it is believed that the increased norepinephrine activity of cocaine lead to increased opioid activity which is similar to the current study's findings. Also, in another investigation administration of the norepinephrine reuptake inhibitor reboxetine produced an increase in DAMGO-induced analgesia (Romero, Guzzo, & Duarte, 2012). This increase in opioid sensitivity from over-activation of noradrenergic synapses may be linked to the upregulation

of α-adrenergic/opioidergic heterodimers in brain regions involved in regulating nociception (Jordan et al., 2003). Animals treated with atomoxetine in the current study also may have had increased activation of similar heterodimers which lead to the changes in opioid sensitivity seen in this morphine-induced antinociception paradigm.

Preweanling exposure to a 0.3 mg/kg dose of atomoxetine resulted in attenuated MAE scores compared to saline controls for the hot-plate task, which was an unpredicted and interesting finding of the current study. Rats tested in a morphine-induced antinociception paradigm 120 days after depletion of noradrenergic terminals in the CNS showed an attenuated response to morphine over saline controls (Jasmin, Boudah, & Ohara, 2003). Together these data demonstrate that alterations in the noradrenergic system have significant effects on opioid functioning.

α_1- and α_2-Adrenergic receptors are known to modulate the response to opioid analgesia (Bie et al., 2003). More specifically, activation of excitatory α_1-adrenergic receptors increases antinociception, while activation of inhibitory α_2-adrenergic receptor reduces morphine-induced

analgesia (Bie et al., 2003). Pretreatment with a low dose of atomoxetine (0.3 mg/kg) may stimulate the upregulation of inhibitory α_2-adrenergic receptors, while preweanling exposure to a higher dose of atomoxetine (1 mg/kg or 3 mg/kg) may increase excitatory α_1-adrenergic receptors expression. This hypothesis is partially supported by a studying showing that mice lacking the norepinephrine transporter have an increase in α_2-adrenergic receptors proteins in the CNS (Gilsbach et al., 2006).

It was proposed that preweanling exposure to GBR-12909 the dopamine reuptake blocker would have no effect on morphine-induced antinociception in adulthood. Although not statistically significant, there was a clear trend towards attenuated MAE scores from subjects given a 2.5 mg/kg dose of morphine and pretreated with the 15 mg/kg dose of GBR-12909 for the hot-plate task on test trial 1. Animals pretreated with the 15 mg/kg doses of GBR-12909 showed a trend toward reduced MAE scores on the hot-plate for test trial 2 and animals administered the 1.5 mg/kg or 15 mg/kg doses of GBR-12909 also showed a trend toward attenuated MAE scores on the hotplate-paradigm for test trial 3. Lesions of dopamine

neurons located in the periaqueductal gray matter cause a reduction in heroin- and morphine-induced antinociception (Flores et al., 2004). Similarly, mice unable to synthesize dopamine have attenuation in morphine-induced analgesia (Hnasko, Sotak, & Palmiter, 2005). It is clear that disruptions in the dopamine system can lead to attenuated responses in opioid analgesia similar to the results of the current study.

In a past investigation exposure to GBR-12909 caused a significant down regulation of surface level dopamine transporter proteins while cocaine did not (Kunko, Loeloff, & Izenwassar, 1997). This reduction in dopamine transporter proteins is not a complete absence of DAT as is observed in Flores and colleagues (2004) study where animals had dopamine neuron lesions in the PAG. The current experiment may have had a similar reduction in DAT levels from pre-exposure to GBR-12909, which lead to attenuated morphine-induced analgesia that would have been statistically significant if DAT proteins had been completely abolished as in (Flores et al., 2004).

Preweanling exposure to the high dose of GBR-12909 increased basal antinociception on the hot-plate procedure compared to saline controls. This increase in

antinociception is similar to the increase seen in mice lacking the dopamine transporter (Spielewoy et al., 2000). Dopamine D_3 receptor knockout mice also had an increased basal antinociceptive phenotype (Li et al., 2012). Dopamine transporter knockout mice have increased expression of DOR mRNA in adulthood (Moine, Fauchey, & Jaber, 2002). While rats lacking either D1 or D3 dopamine receptors have an upregulation of MOR mRNA in the CNS (Zhou et al., 2007). Taken together, alterations in dopaminergic functioning can lead to an upregulation of opioid receptor mRNA and increases in basal antinociception. It is possible that pretreatment with 15 mg/kg GBR-12909 during the preweanling period also induces an alteration of dopaminergic functioning sufficient to increase basal antinociception.

Increased activity in noradrenergic or dopaminergic systems during development can cause long lasting changes in opioid sensitivity. Noradrenergic, dopaminergic, and opioidergic receptors share a similar signaling transduction pathway which starts with the G_i and G_o G protein coupled receptors. The G_i and G_o G protein coupled receptors indirectly inhibit the action of cAMP response element binding protein and cAMP response element

modulator (Parlato et al., 2010; Vilardaga et al., 2008). Noradrenergic, D_2-like, and opioidergic receptors activity is modulated by regulator of G protein signaling 4 (RGS4) (Stratinaki et al., 2013), which regulates the G_i and G_o G protein coupled receptors. Opioid receptors can form heterooligomers with both dopamine and α-adrenergic receptors in brain areas known to be involved in nociception (Juhasz et al., 2008; Vilardaga et al., 2008). The alterations in opioid functioning from increased synaptic activity in the dopamine and norepinephrine systems during the preweanling period make sense, given that norepinephrine and dopamine receptors can form heterodimers with opioid receptors and increase activity in their shared signaling transduction pathway.

In conclusion, the current study found that preweanling exposure to a low dose of atomoxetine attenuated morphine-induced analgesia, while exposure to higher doses potentiated it. Interestingly, preweanling exposure to GBR-12909 increased basal antinociception. No significant differences in morphine-induced analgesia were found after pretreatment with GBR-12909 although there was a clear trend suggesting GBR-12909 exposure decreased morphine-induced analgesia.

Early exposure to methylphenidate causes long lasting alterations to opioid sensitivity in rats (Crawford et al., 2007; Crawford et al, 2009; Cyr & Morgan, 2009). These long lasting changes are concerning because children are being prescribed methylphenidate during an analogous time period. Similar alterations in the opioid system from pre-exposure to the non-psychostimulant atomoxetine were observed in this study. The similarities in results between (Crawford et al., 2007; 2009; Cyr & Morgan, 2009) and the current experiments would suggest that atomoxetine use during the pre-school years is not a safer choice than methylphenidate. Our research also suggests that drugs with higher specificity for the dopamine transporter may have fewer long-term side effects.

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