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Comparison Of Affective Analgesia And Conditioned Place Preference Following Cholinergic Activation Of

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COMPARISON OF AFFECTIVE ANALGESIA AND CONDITIONED PLACE PREFERENCE FOLLOWING CHOLINERGIC ACTIVATION OF VENTRAL TEGMENTAL AREA SUBREGIONS

by

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DEDICATION

To my parents, Maria and Constantin Schifirneț

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CHAPTER 1. INTRODUCTION

> *Nature has placed mankind under the governance of two sovereign masters,* ***pain*** *and* ***pleasure****. It is for them alone to point out what we ought to do, as well as to determine what we shall do. […]. They govern us in all we do, in all we say, in all we think: every effort we can make to throw off our subjection, will serve but to demonstrate and confirm it. In words a man may pretend to abjure their empire: but in reality he will remain subject to it all the while*.
>
> Jeremy Bentham, 1789[1]

Pain is a conscious, subjective, and unpleasant experience that consists of sensory-discriminative, affective-motivational and cognitive-evaluative components (Melzack & Casey, 1967). The sensory-discriminative component of pain provides information about the temporal occurrence, the spatial localization, the physical qualification, and the intensity quantification of the noxious stimulus and elicits rapid responses (i.e., withdrawal reflexes) designed to prevent further or potential injury. The affective-motivational dimension can be distinguished from its discriminative sensory aspects (Craig & Sorkin, 2001) and renders the noxious stimulus with a distinctly unpleasant character that ultimately motivates behaviors such as avoidance and recuperation (Borszcz, Johnson, & Fahey, 1994). The cognitive-evaluative component is responsible for the appraisal of the meanings, consequences, and predictability of the painful sensations and injury. Each of these components is mediated and modulated through different forebrain mechanisms (Casey, 1999).

The affective and cognitive components of pain interact and attribute the negative emotional coloring to the pain experience (Almeida, Roizenblatt, & Tufik, 2004), thus generating

[1] The first sentence of this paragraph has been used as a *motto* also by Leknes & Tracey (2008).

emotional disturbances such as fear, anger, frustration, stress, anxiety, and depression (Price, 2000, 2002). Affective reactions to pain can generate "fear-avoidance" beliefs (Asmundson, Norton, & Allerdings, 1997; Waddell, Newton, Henderson, Somerville, & Main, 1993) and "catastrophizing" thoughts (Carleton, Abrams, Asmundson, Antony, & McCabe, 2009; Sullivan, Stanish, Waite, Sullivan, & Tripp, 1998) that further increase the distress of the patient in pain, and thus leading to a vicious circle characterized by development and maintenance of pain behaviors (McCracken, Zayfert, & Gross, 1992) such as decreased self-efficacy (Arnstein, Caudill, Mandle, Norris, & Beasley, 1999), avoidance behavior (Asmundson, Norton, & Norton, 1999; Vlaeyen & Linton, 2000), work loss, and general disability in activities of daily living (Fordyce, Shelton, & Dundore, 1982; Philips, 1987; Waddell et al., 1993). Furthermore, the development and maintenance of these secondary emotional disturbances exacerbate the suffering of patients in pain, and thereby motivate individuals to seek medical attention. Indeed, "it is suffering, not pain, that brings patients into doctor's offices in hopes of finding relief" (Loeser, 2000). Supporting this observation, many researchers found that fear of pain may be more disabling than pain itself (Crombez, Vlaeyen, Heuts, & Lysens, 1999; Fordyce et al., 1982; McCracken et al., 1992; Philips, 1987; Waddell et al., 1993; Wade, Dougherty, Hart, Rafii, & Price, 1992) and that fear of pain can be used as a reliable predictor for the perceived intensity of acute pain (George, Dannecker, & Robinson, 2006; Hirsh, George, Bialosky, & Robinson, 2008).

As a consequence, the need for better medical treatment of pain and better pain management therapies makes understanding the mechanisms through which the emotional aspect of pain is generated, maintained and finally suppressed of paramount importance. Whereas considerable progress has been made in understanding the neural mechanisms that subserve the

sensory-discriminative component of the pain, comparatively little is known about the neural circuits underlying the generation and suppression of the affective aspect of the pain experience.

More than a century ago, it was observed that strong analgesics, like morphine, heroin, amphetamine, and cocaine, have a high abuse potential and are self-administered by both humans and animals (Himmelsbach, 1943; May, 1953; Morphine and Heroin Addiction: Departmental Committee's Report, 1926; Spender, 1887), suggesting that the neural substrates of reward and antinociception overlap (Oberst, 1943). Franklin (1989, 1998) proposed that the ability of these drugs (i.e. opioids and psychostimulants) to induce a positive affect underlies both their addictive liability and their analgesic action. The positive affective state generated by these drugs should reduce the level of distress that normally accompanies noxious stimulation. This phenomenon is referred to as "affective analgesia" and reflects preferential suppression of the emotional reaction to pain. The affective analgesia hypothesis proposes that neural substrates underlying reward contribute to suppression of the affective response to pain. The proposed research provides the first systematic investigation into the capacity of brain reward circuit activation to suppress the affective response of rats to noxious stimulation.

1.1. Ventral Tegmental Area (VTA) in Reward and Reinforcement

Although the terms reward and reinforcement are more often than not used interchangeably, there is a clear distinction between an internal positive subjective state (reward) and the capacity of that state to support appetitive conditioning (reinforcement) (Wise, 1996). The ability of natural (i.e., food, water, sex) or artificial (i.e., drugs of abuse, intracranial self-stimulation - ICSS) rewards to induce a positive affective state relies, at least partially, on the integrity of dopaminergic projections from the VTA to nucleus accumbens (NAc) of the striatum (for reviews, see Bardo, 1998; Di Chiara et al., 1999; Di Chiara, 2002; Ikemoto, 2007; Kiyatkin,

2002; Koob & Volkow, 2010; Schultz, 2000; Wise, 2004). This idea is supported by a plethora of data derived from microinjection, microdialysis, electrophysiological, and neuroimaging studies (Table 1). For example, in vivo microdialysis experiments in rats revealed that chemical (Westerink, Kwint, & deVries, 1996) or electrical (Fiorino et al., 1993) activation of the VTA results in an increase of dopamine (DA) release in NAc. This release is associated with reward, as rats that electrically self-stimulate the VTA display an increase in accumbal DA up to almost 200% (Fiorino et al., 1993). Likewise, drugs that are abused in humans (e.g. amphetamine, cocaine, opiates, nicotine, ethanol[2]) increase DA availability in the rat NAc, drugs that induce a negative affective state in humans (e.g. κ-opioid agonists, tifluadom, bremazocine) decrease DA availability in the rat NAc, and drugs that do not induce any affective state in humans (e.g. imipramine, atropine, antihistamines) do not change the DA levels in the rat NAc (Di Chiara & Imperato, 1988). These findings are in agreement with the data provided by imaging studies in humans with DA tracers (e.g. raclopride isotopes), which showed that drugs of abuse like amphetamine or cocaine increase DA availability in the striatum and this increase is associated with self-reports of "high" and "euphoria", in a directly proportional fashion (for a review of these studies, see Volkow, Fowler, Wang, Baler, & Telang, 2009). Conversely, the levels of striatal DA release were unchanged in the subjects in which the psychostimulants did not induce euphoric effects. Not only have directly rewarding stimuli elicited mesolimbic DA release, but also the stimuli that were previously associated with a reward (Schultz, Dayan, & Montague, 1997).

[2] These drugs of abuse act as DA agonists as they increase the DA availability in the brain either by acting directly on the VTA neurons (e.g. opioids disinhibit the DA cells, Johnson & North, 1992; nicotine activates the DA neurons, Mereu et al, 1987; ethanol lifts the GABA inhibition from the DA neurons, Xiao & Ye, 2008) or by blocking the DA transporter or the DA D_2 autoreceptor (e.g. for amphetamine, see Jones, Gainetdinov, Wightman, & Caron, 1998; for cocaine, see Beuming et al., 2008).

Besides mediating the positive affective state that characterizes a reward, the role of DA, especially accumbal DA, in reinforcement is also well-substantiated by the literature. For instance, a rat will learn to press a lever in order to receive food, water, sexual contact, or drugs of abuse and during learning of these appetitive instrumental tasks the DA release in NAc is increased (Cheng & Feenstra, 2006; Hernandez & Hoebel, 1988), as well as during the performance of the appetitive tasks (Phillips, Stuber, Heien, Wightman, & Carelli, 2003). Also, rats readily self-administer DA agonists like heroin and cocaine if the DA system is intact, but they cease to do so if the mesoaccumbal DA system is depleted (Wise & Rompre, 1989). The involvement of the DA system in reinforcement is further supported by the finding that disruption of DAergic transmission either by systemically blocking the DA receptors or by activating the DA autoreceptors results in impairments in appetitive conditioning in both rodents (Gerber, Sing, & Wise, 1981; Ikemoto & Wise, 2004; Wise & Schwartz, 1981) and humans (Pizzagalli et al., 2008; Santesso et al., 2009). Likewise, even if animals successfully learned an appetitive conditioning task, they do not perform normally if the mesoaccumbal DAergic system is impaired (Parkinson et al., 2002). Furthermore, extinguished lever pressing for cocaine is reinstated by microinjections of cocaine or other DA agonists into NAc (Schmidt, Anderson, & Pierce, 2006), behavior that is blocked by pretreatment with DA antagonists into the same region (Anderson, Schmidt, & Pierce, 2006). Taken together, these findings point to the paramount role of DA release in NAc for both reward and reinforcement processes.

1.2. VTA in Pain and Analgesia

Involvement of the VTA in antinociception is supported by the finding that electrical stimulation of the VTA abolishes escape responding produced by stimulation of the nucleus reticularis gigantocellularis (Anderson, Diotte, & Miliaressis, 1995). Nucleus reticularis

gigantocelluaris (nRGC) is a medullary link of the spinoreticulothalamic pathway that transmits noxious information from the spinal cord to the medial thalamus and related forebrain structures (Almeida et al., 2004). Similarly, electrical stimulation of lateral hypothalamic sites that support self-stimulation, an effect dependent on VTA activation (Wise, 1996), also attenuates escape responding to nRGC stimulation (Simson & Coons, 1989). Stimulation of the lateral hypothalamus produces antinociception in the footshock test (Lopez & Cox, 1992), tail-flick test (Aimone, Bauer, & Gebhart, 1988; Franco & Prado, 1996) and hot-plate test (Carstens, Fraunhoffer, & Suberg, 1983). Simulation of the lateral hypothalamus also reduced the amplitude of evoked potentials to noxious peripheral stimulation in medial thalamic targets (centromedian–parafascicular complex) of nRGC and suppressed escape responding to the same noxious stimulus (Butkevich & Kassil, 1999). Nociceptive processing by these medial thalamic sites contributes to production of affective responses to pain in both humans and animals (Delacour, 1971; Harte, Kender & Borszcz, 2005; Harte, Lagman & Borszcz, 2000; Kaelber et al., 1975; Mark, Ervin, & Yakovlev, 1962; Weigel & Krauss, 2004; Whittle & Jenkinson, 1995; Young et al., 1995).

Release of DA into NAc from axon terminals of DAergic neurons in VTA contributes to the antinociceptive action of VTA stimulation (see Table 3). Opioids administered systemically or into the VTA increase DA metabolism (Kalivas & Richardson-Carlson, 1986; Latimer, Duffy & Kalivas, 1987) and extracellular levels of DA (Pontieri, Tanda & Di Chiara, 1995) in the NAc. Similarly, systemic administration of amphetamine or cocaine elevates extracellular levels of DA in the NAc (Pontieri, Tanda, & Di Chiara, 1995). Microinfusions of morphine into the VTA or amphetamine into NAc suppress paw-licking in the formalin test but do not alter withdrawal latencies in the tail flick test (Altier & Stewart, 1996; Manning, Morgan, & Franklin, 1994).

These antinociceptive effects in the formalin test were blocked by pretreatment of NAc with the DA receptor antagonist raclopride (Altier & Stewart, 1998). Likewise, neurotoxic lesions of DA neurons in VTA block the suppression of paw-licking in the formalin test produced by systemic administration of morphine or amphetamine, but do not alter the increase in tail flick latencies generated by these drug treatments (Clarke & Franklin, 1992; Morgan & Franklin, 1990). Alternately, intra-NAc microinjections of the D_2 agonist quinpirole reduced nociceptive responding during the formalin test in a dose-dependant manner and this effect was blocked by administration of the D_2 antagonist raclopride into NAc (Taylor, Joshi, & Uppal, 2003). As tail flicks and paw-licking are respectively organized at spinal and supraspinal levels of the neuraxis (for a review, see Le Bars, Gozariu, & Cadden, 2001) these findings indicate that activation of mesoaccumbal DA projections selectively suppresses or masks nociceptive processing at supraspinal levels of the neuraxis.

1.3. Analgesia and Reward: Motivational Continuum Hypothesis

Opioids and psychostimulants elicit mesolimbic DA release and this activation induces a positive affect (see Table 1) and, as stated above, Franklin's affective analgesia hypothesis (1989, 1998) postulates that this positive affect suppresses the emotional distress associated with the pain experience without reducing the actual sensory experience. Altier and Stewart (1999a) postulated that the relation between the rewarding and antinociceptive actions of analgesic drugs such as opiates and psychostimulants can be understood from the perspective of a motivational continuum. This motivational continuum has poles of extreme negative and positive affect with normal affect located in the middle. When administered in the normal affective state, opiates and psychostimulants shift the continuum from normal affect to positive affect through activation of the brain reward circuitry. The shift to the positive pole of the continuum may underlie the

addictive liability of opiates or psychostimulants. In contrast, when noxious stimulation generates a negative affective state, opioids and psychostimulants suppress pain affect through their activation of brain reward circuitry. This activation shifts the negative affective state to the middle of the motivational continuum producing affective analgesia. It is important to stress that while the same circuitry is activated in both cases, the baseline is changed in the latter example; these drugs administered in the negative state do not induce a positive state (Fig. 1).

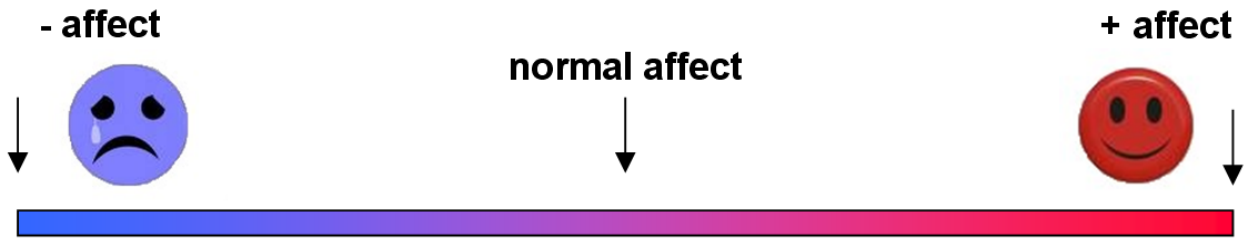


Figure 1. **The motivational continuum.** If the organism is in a negative affective state (e.g. such as that generated by noxious stimulation), the opiates and psychostimulants shift the motivational continuum towards a neutral affective state, producing affective analgesia by activating the brain reward circuitry. If the organism is in a neutral affective state, the opiates and psychostimulants shift the motivational continuum towards a positive affective state that underlies their addictive liability, by activating the brain reward circuitry.

The motivational continuum hypothesis is supported by the observation that patients who are given morphine for pain relief, and hence in a strong negative affective state, rarely become addicted or develop withdrawal symptoms (Melzack, 1990). Alternately, addiction, tolerance, and withdrawal commonly follow morphine administration during neutral or positive affective states. Thus, when opioids are used for pain management, they may help the patient to achieve an affective state normally experienced when free of pain, but not the extreme positive affect that might support addiction. Supporting this view are reports that the capacity of morphine to serve as a reinforcer in a conditioned place-paradigm (CPP) is attenuated when given to rats that are in a chronic/tonic pain state (Narita et al., 2005; Ozaki et al., 2002; Suzuki, Kishimoto, & Misawa, 1996; Suzuki, Kishimoto, Misawa, Nagase, & Takeda, 1999) and that morphine fails to induce

dependence in rats when administered during a pain state (Abbott, Franklin, Ludwick, & Melzack, 1981; Colpaert, 1996; Vaccarino & Couret, 1993; Vaccarino et al., 1993).

In summary, the DA release from the VTA into the NAc underlies both the analgesia and the rewarding effects induced by drugs of abuse. Clearly, neural circuits that underlie affective analgesia and reinforcement overlap, but the extent of the overlap is currently unknown. The present study systematically investigates the extent of the overlap between affective analgesia and reinforcement.

1.4. Acetylcholine in VTA: The Common Mediator

In addition to opioids and psychostimulants, the availability of mesoaccumbal DA can be also increased by endogenous substances like acetylcholine. VTA neurons display a variety of nicotinic receptors subtypes (Adell & Artigas, 2004; Azam, Winzer-Serhan, Chen, & Leslie, 2002; Klink, de Kerchove d'Exaerde, Zoli, & Changeux, 2001) and at least the muscarinic receptor M_5 (Vilaro, Palacios, & Mengod, 1990; Yeomans, Forster, & Blaha, 2001) and M_2 subtypes (Garzon & Pickel, 2006). Microinjecting nicotinic and muscarinic receptor agonists into the VTA excites DAergic neurons in the VTA via activation of local cholinergic receptors (Calabresi, Lacey & North, 1989; Lacey, Calabresi, & North, 1990; Niijima & Yoshida, 1988), and increases the release of DA in NAc (Gronier & Rasmussen, 1998; Nisell, Nomikos, & Svensson, 1994). Continuous infusion of the non-specific cholinergic agonist carbachol[3] into the VTA causes an up to 140% elevation in extracellular DA levels in the ipsilateral NAc (Westerink et al., 1996), and DA efflux in the NAc is increased by intra-VTA injection of the prototypical

[3] Carbachol induces excitation of the DA neurons within VTA by activating both muscarinic and nicotinic receptors via a L-type Ca^{2+} channel facilitation mechanism (Zhang, Liu & Chen, 2005)

muscarinic agonist muscarine. Alternately, baseline levels of accumbal DA are reduced by intra-VTA administration of the muscarinic antagonist scopolamine (Miller & Blaha, 2005).

Cholinergic projections to the VTA arise bilaterally mostly from the laterodorsal tegmental (LDTg) and less from the adjacently located caudal pedunculopontine (PPTg) nuclei (Blaha et al., 1996; Oakman, Faris, Kerr, Cozzari, & Hartman, 1995; Omelchenko & Sesack, 2005). Approximately 50% of LDTg neurons make synaptic contact (presumably excitatory) with DA neurons in VTA that project to NAc or prefrontal cortex (PFC), the strength of excitatory input being greater for DA neurons providing mesoaccumbal versus mesoprefontal projections. These LDTg neurons also provide moderate excitatory inputs to GABAergic neurons in VTA that project to NAc. The remaining 50% of LTDg/PPTg neurons make synapses (presumably inhibitory) more with the mesoprefrontal than mesoaccumbens neurons (Omelchenko & Sesack, 2005, 2006; Fig. 2). Besides projecting to mesolimbic and mesocortical structures, the GABA cells within VTA also send collaterals to the DA neurons, making mostly inhibitory synaptic contacts (Bayer & Pickel, 1991; Johnson & North, 1992; Omelchenko & Sesack, 2009; Sugita, Johnson, & North, 1992). Thus, acetylcholine is likely to depolarize the DA neurons in VTA either directly or indirectly by inhibiting the GABA interneurons and thus lifting the inhibition from the DAergic cells.

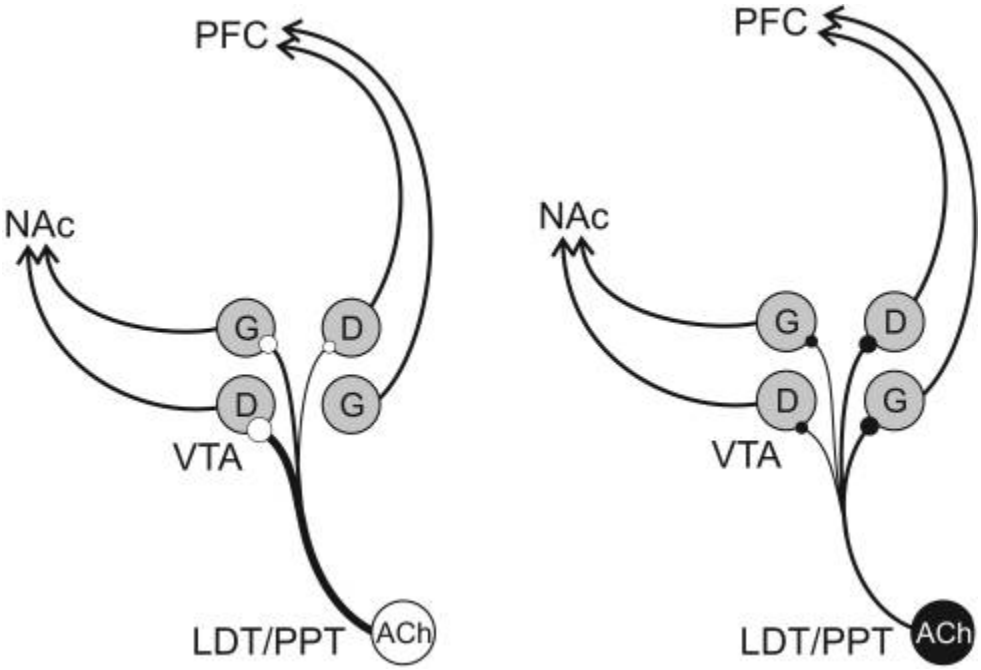


Figure 2. **Schematic drawing of cholinergic synapses within the VTA**. The drawing depicts the excitatory (white) and inhibitory (black) synapses made by cholinergic cells with the VTA DAergic (D) and GABAergic (G) neurons. The thickness of cholinergic axons depicts the approximate number of the connections. From Omelchenko & Sesack (2006).

Lesions of acetylcholine-producing neurons in LDTg block the DA release in NAc induced by intra-VTA neostigmine (cholinesterase inhibitor, Blaha et al., 1996). The accumbal efflux of DA that accompanies electrical stimulation of the LDTg is also attenuated following injection of muscarinic or nicotinic antagonists into the VTA (Forster & Blaha, 2000; Lester, Miller, & Blaha, 2010). LDTg-induced accumbal efflux of DA is also reduced in mutant mice with deletion of the M_5 receptor (Forster, Yeomans, Takeuchi, & Blaha, 2002; Yeomans et al, 2001).

The reinforcing properties of opioids, psychostimulants and ICSS are mediated, at least partly, by cholinergic activation of mesoaccumbal DA neurons. Acetylcholine release in the VTA is significantly elevated by rewarding events such as intravenous cocaine self-administration (You, Wang, Zitzman, & Wise, 2008), subcutaneous morphine injections

(Rezayof, Nazari-Serenjeh, Zarrindast, Sepehri, & Delphi, 2007), lateral hypothalamic self-stimulation, eating, and drinking (Rada, Mark, Yeomans, & Hoebel, 2000). Although both nicotinic and muscarinic acetylcholine receptors in the VTA mediate the rewarding effects of cocaine, morphine and lateral hypothalamic stimulation, it appears that the muscarinic receptors are more involved in reward processing than nicotinic receptors. Muscarinic receptors in the VTA contribute to the increase in accumbal DA generated by systemic administration of morphine and this increase is readily attenuated by the infusion of the non-selective muscarinic antagonist scopolamine into VTA (Miller, Forster, Yeomans & Blaha, 2005). Blockade of muscarinic rather than nicotinic receptors in VTA results in attenuation of the reinstatement of cocaine seeking and VTA DA levels induced by VTA perfusion of neostigmine (You et al., 2008), and in the attenuation of the rewarding effects of the lateral hypothalamic stimulation (Yeomans & Baptista, 1997). Correspondingly, infusion into the VTA of antisense oligonucleotides targeting muscarinic M_5 mRNA inhibited local M_5 receptor binding and reduced lateral hypothalamic self-stimulation (Yeomans et al., 2000). Mutant mice with deletion of the M_5 receptor exhibit reduced CPP learning with systemic injections of morphine or cocaine (Basile et al., 2002; Fink-Jensen et al., 2003), and show reduced cocaine self-administration (Thomsen et al., 2005). Similarly, muscarinic receptor blockade in VTA by scopolamine attenuates cocaine enhancement of LDTg stimulation-evoked NAc DA release in the mouse (Lester, Miller, & Blaha, 2010). Alternately, the rewarding effects of lateral hypothalamic stimulation are enhanced by infusion of acetylcholine into the VTA (Olds, Yuwiler, Olds, & Yun, 1964; Redgrave & Horrell, 1976), whereas the muscarinic antagonist atropine infused into the VTA completely blocks self-stimulation of the lateral hypothalamus (Rada et al., 2000).

Not surprisingly, acetylcholine receptor activation within the VTA is rewarding. Intra-VTA administration of carbachol supports development of CPP learning, and rats learn to self-administer carbachol into the VTA (Ikemoto & Wise, 2002; Yeomans, Kofman, & McFarlane, 1985). These reinforcing effects of carbachol were attenuated more effectively by pre-treating VTA with muscarinic versus nicotinic receptor antagonists.

1.5. VTA Heterogeneity

1.5.1. Reward

However, Ikemoto & Wise (2002) observed regional differences within the VTA in the ability of carbachol to activate DAergic reward circuitry. Specifically, administration of carbachol into the posterior VTA (pTVA), but not the anterior VTA (aVTA), supported development of CPP. Also, rats learned to self-administer carbachol into the pVTA, but not into the aVTA (Ikemoto & Wise, 2002). Similarly, rats learn to self-administer opiates (Zangen, Ikemoto, Zadina, & Wise, 2002), cocaine (Rodd et al., 2005), nicotine (Ikemoto, Qin, & Liu, 2006), ethanol (Rodd-Henricks, McKinzie, Crile, Murphy, & McBride, 2000), tetrahydrocannabinol (cannabinoid agonist, Zangen, Solinas, Ikemoto, Goldberg, & Wise, 2006), muscimol ($GABA_A$ agonist, Ikemoto, Murphy, & McBride, 1998), and CPBG ($5\text{-}HT_3$ agonist, Rodd et al., 2007), into the pVTA, but not the aVTA.

The functional differences observed by the self-administration and CPP studies may be explained by differences in the efferent projections of the aVTA versus the pVTA. Namely, Ikemoto (2007) conducted a series of comprehensive double immunostaining studies (with tyrosine hydroxylase and fluorogold) and found that the cathecholaminergic neurons in pVTA predominantly projects to the medial part of the NAc shell and the medial olfactory tubercle, structures critical for mediating the rewarding effects of drugs of abuse as revealed by ICSS and

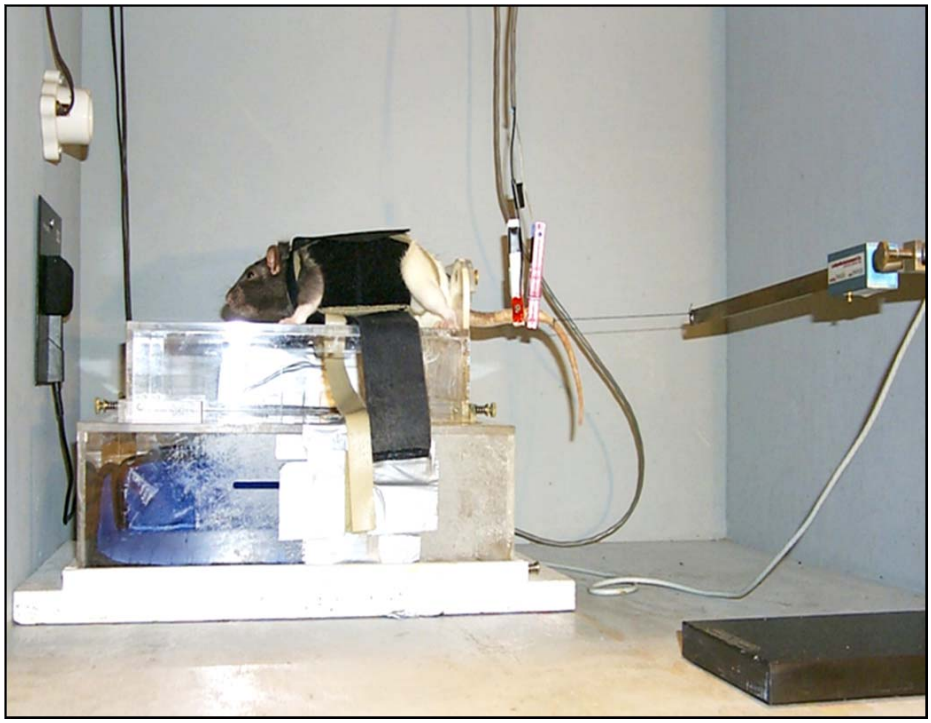
CPP studies (Ikemoto, 2007, 2010; Ikemoto & Donahue, 2005; Ikemoto, Qin, & Liu, 2005; Sellings, Bahamouri, McQuade, & Clarke, 2008; Sellings, McQuade, & Clarke, 2006a,b). Alternatively, the aVTA provides little or no projections to the medial NAc shell and medial olfactory tubercle, but instead projects to the NAc core, NAc ventral shell, lateral tubercle, and dorsal striatum, into which application of DAergic drugs is not reinforcing (Ikemoto, 2007).

1.5.2. Affective Analgesia

Given the aforementioned heterogeneity within the VTA of carbachol to support reinforcement, we evaluated regional differences within the VTA of carbachol to produce affective analgesia (Schifirneț & Borszcz, 2007; Schifirneț, Karim, Lucas, & Borszcz, 2008). According to Franklin's affective analgesia hypothesis (1989, 1998), it is reasonable to infer that if antinociception elicited by cholinergic activation of the VTA depends on activation of mesoaccumbal reward circuitry, then this antinociceptive action should exhibit regional differences within the VTA. Namely, the activation of the rewarding pVTA projections should be conducive to affective analgesia, whereas the activation of the non-rewarding aVTA projections should not produce affective analgesia.

Research in this laboratory has validated vocalization afterdischarges (VAD) as a model of pain affect in rats. These vocalizations occur following a brief (1 s) noxious tailshock and are spectrographically distinct from vocalizations that occur during tailshock (VDS). Systemically administered drugs that preferentially suppress the affective reactions to pain in humans, like morphine, fentanyl, and diazepam (Chapman & Feather, 1973; Gracely, McGrath, & Dubner, 1978; Price, Harkins, Rafii, & Price, 1986; Price, Von der Gruen, Miller, Rafii, & Price, 1985) also preferentially suppress production of VADs (Borszcz et al., 1994; Caroll & Lim, 1960; Hoffmeister, 1968; Levine, Feldmesser, Tecott, Gordon, & Izdebski, 1984). Damage to or drug

treatments into forebrain sites known to contribute to the affective responses of humans to clinical and experimental pain (e.g. amygdala, thalamus, hypothalamus, anterior cingulate gyrus – aCC; Ballantine, Cassidy, Flanagan, & Marino, 1967; Foltz & White, 1968; Hebben, Corkin, Eichenbaum, & Shedlack, 1985; Sano, Yoshioka, Ogashiwa, Ishijima, & Ohye, 1966; Sweet, 1980; Uematsu, Konigsmark, & Walker, 1974; Whittle & Jenkinson, 1995)) selectively suppress the generation of VADs (Borszcz, 1999, 2006; Borszcz & Leaton, 2003; Borszcz & Streltsov, 2000; Greer, 2007; Greer, Wronkowicz, Harte, & Borszcz, 2005; Harte et al., 2000, 2005; Harte, Hoot, & Borszcz, 2004; Harte, Spuz, Greer, & Borszcz, 2005; Hoffmeister, 1968; Kender, Harte, Munn, & Borszcz, 2008; Munn & Borszcz, 2002; Munn, Harte, Lagman, & Borszcz, 2009; Nandigama & Borszcz, 2003). Additionally, the capacity of tailshock to support fear conditioning in the rat relies on its capacity to elicit VADs (Borszcz, 1993, 1995, 2006, Borszcz & Leaton, 2003). Besides the VAD model, there are virtually no animal pain models that can directly quantify the innate, unconditional, affective reaction to pain.



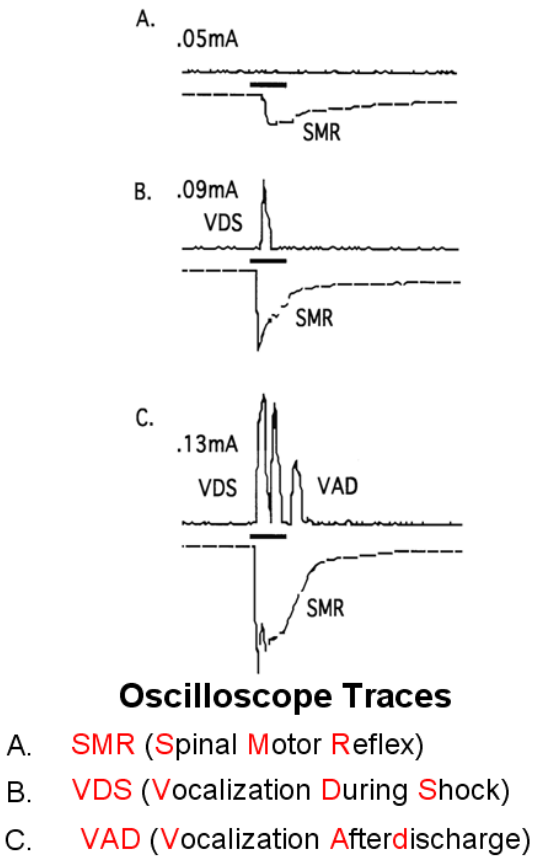


Figure 3. **Affective analgesia assessment**. *Top*. The animal is restrained on a Plexiglas pedestal in a custom made Velcro® body suit. The electric shock is delivered via two electrodes attached to the tail and the subsequent tail-flicks and vocalizations are recorded by a computer. *Bottom*. Oscilloscope traces depicting the recorded behaviors. The top line represents the output of the microphone, the middle line represents the duration of the shock, and the bottom line represents the output of the displacement transducer. Note the initiation of VADs immediately after the shock. For a complete description of the test, see Schifirneț (2009).

Consistent with our previous report (Kender et al., 2008), administration of carbachol into the pVTA produced dose-dependent elevation of the current thresholds for tailshock to elicit VAD and VDS with the effect greater for VAD threshold. Alternately, the current intensity to elicit spinal motor reflexes (SMR = tail flick and hindlimb flexion) was not altered by injection of carbachol into the pVTA. This affective analgesia was mediated by muscarinic receptors as it was effectively blocked by pretreating the VTA with the muscarinic receptor antagonist atropine but not with the nicotinic receptor antagonist mecamylamine. Administration of carbachol into

the aVTA also preferentially elevated VAD threshold that was blocked by administration of either atropine or mecamylamine (Schifirneț et. al, 2008). These results indicate that carbachol-induced affective analgesia relies on the activation of muscarinic receptors in pVTA and muscarinic and nicotinic activation in aVTA.

Furthermore, during the course of this study we observed that administration of carbachol in the area between aVTA and pVTA was ineffective in producing antinociception. This intermediate area of the VTA had previously not been identified as a functionally separate region of the VTA, and we labeled it as the midVTA (Fig. 4).

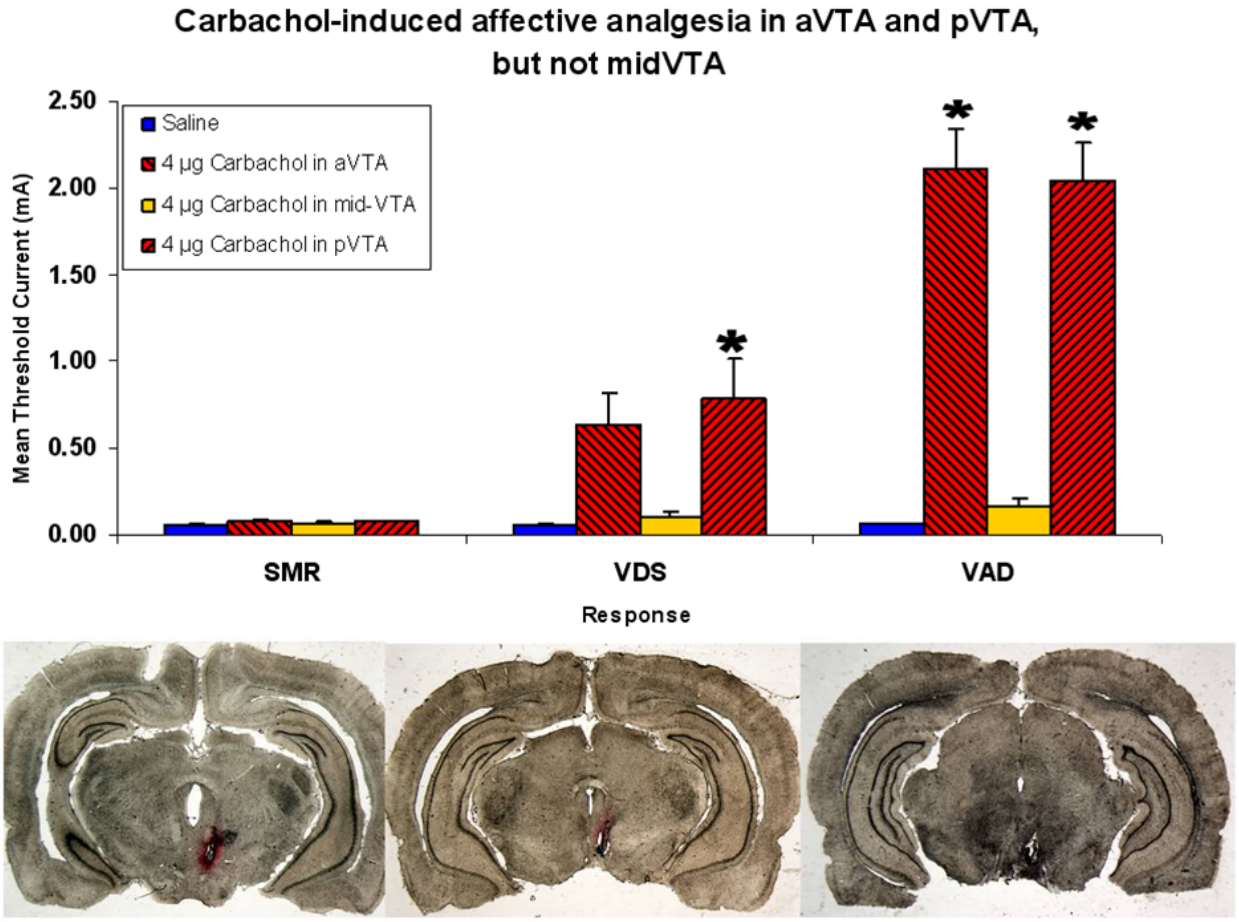


Figure 4. **Carbachol induces affective analgesia in aVTA and pVTA, but not midVTA.** ***Top.*** Unilateral administration of carbachol (4 µg) into the midVTA (yellow) failed to elevate VAD thresholds as compared with the same dose administered in aVTA and pVTA (red) or with

saline (blue). * Thresholds significantly elevated compared to saline, $p < .05$. *Bottom.* Coronal slices representing aVTA, midVTA, and pVTA, respectively.

Taken together, the aforementioned results point to a discrepancy between the ability of carbachol to produce reward and to induce affective analgesia, at least in the aVTA. Therefore, this study re-assessed the ability of carbachol to support CPP in the three subregions of the VTA using a carbachol dose that induces affective analgesia (i.e. 4 μg). Additionally, the carbachol-induced affective analgesia in the Schifirneț et al. (2008) study relied on the activation of muscarinic receptors in pVTA and muscarinic and nicotinic activation in a VTA as shown by the fact that administration of atropine in the pVTA and administration of both atropine and mecamylamine in the aVTA attenuated carbachol-induced affective analgesia. Thus, the present study also assessed the differential involvement of muscarinic and nicotinic receptors in activating the reward brain circuitry in the VTA subregions by pretreating the VTA with the same doses of atropine (i.e., 60 μg) and mecamylamine (i.e., 45 μg) that proved efficacious in attenuating the carbachol-induced affective analgesia.

Finally, the distinction between the three subregions of the VTA in the rostro-caudal axis relies on functional findings, and not on anatomical landmarks. From a stereological point of view, the main VTA zones rich in DA-producing neurons are paranigral nucleus (PN) and parabrachial pigmented nucleus (PBN; Ikemoto, 2007; Nair-Roberts et al., 2008). However, from an anatomical standpoint, the VTA is an area whose borders and components are still a matter of debate (for an extensive review regarding VTA nuclei nomenclature, division, and projections, see Ikemoto, 2007). For example, the 4th edition of Paxinos & Watson's rat brain atlas (1998) is markedly different than the 6th edition of the same atlas (2007). These differences are to be seen both at the level of aVTA and pVTA (Fig. 5), making difficult the identification of accurate stereotaxic coordinates. Therefore, a tyrosine hydroxylase immunohistochemistry (TH

IHC) study was conducted in order to identify the cells that produce cathecholamines and, thus, properly adjust the coordinates for the stereotaxic surgeries (see Methods).

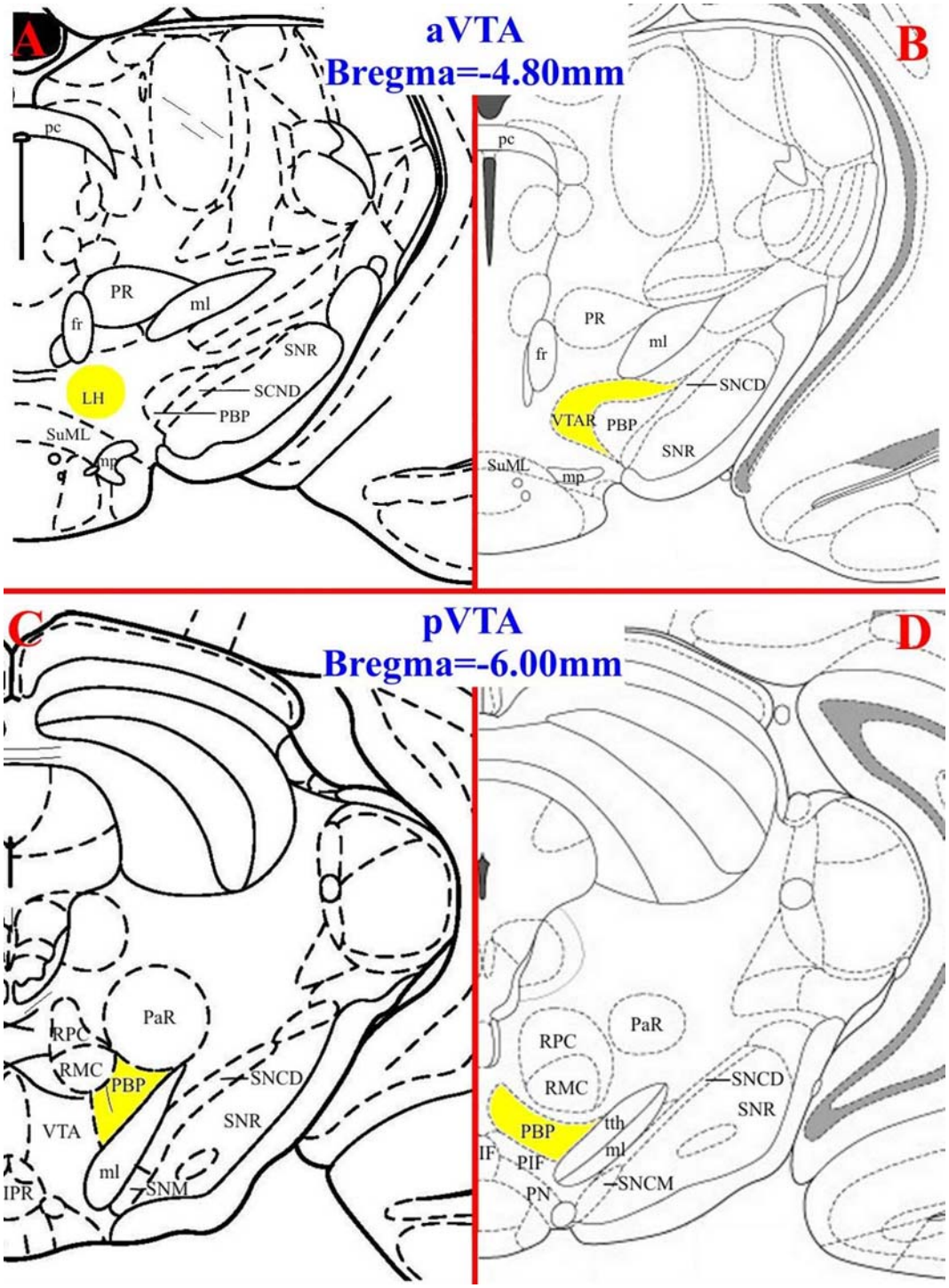


Figure 5. **VTA heterogeneity**. Excerpts from Paxinos and Watson 4th edition (left) and 6th edition (right) rat brain atlases showing coronal sections through the right midbrain of the rat.

Top. Section at - 4.80 mm posterior to Bregma from the 4th edition (**A**) and 6th edition (**B**), at the level of the putative aVTA. Note that at the same coordinates (DV=8.2mm, ML=1mm) the atlases point to two different structures, LH and VTAR, respectively. *Bottom*. Sections at approx. - 6.00 mm posterior to Bregma, from the 4th edition (**C**) and 6th edition (**D**) at the level of the putative pVTA. Note the differences in coordinates for the PBP. Abbreviations: fr, fasciculus retroflexus; IF, interfascicular nucleus; IPR, interpeduncular nucleus, rostral subnucleus; LH, lateral hypothalamic area; ml, medial lemniscus; mp, mammillary peduncle; PBP, parabrachial pigmented nucleus; pc, posterior commissure; PIF, parainterfascicular nucleus of the VTA; PN, paranigral nucleus of the VTA; pr, prerubral field; RMC, red nucleus, magnocellular part; RPC, red nucleus, parvocellular part; SNCD, substantia nigra, compact part, dorsal tier; SNCM (SNM), substantia nigra, compact part, medial tier; SNR, substantia nigra, reticular part; SuML, supramammillary nucleus (lateral part); tth, trigeminothalamic tract; VTA, ventral tegmental area; VTAR, ventral tegmental area, rostral part (aVTA).

1.6. Behavioral Assessment

Introduced in the 1940s and then refined almost two decades later, the conditioned place preference (CPP) paradigm is a validated experimental protocol for measuring drug reward (for reviews, see Bardo and Bevins, 2000; McBride, Murphy, & Ikemoto, 1999; Tzschentke, 1998, 2000). "Animals, just like humans, prefer and approach environments that have been repeatedly paired to stimuli with positive motivational properties" (DiChiara, 2000, p. 299). The conceptual framework of the CPP paradigm is based on classical (Pavlovian) conditioning learning theory. Namely, "the primary motivational properties of a drug or non-drug treatment serve as an unconditioned stimulus (US) that is repeatedly paired with a previously neutral set of environmental stimuli which acquire, in the course of conditioning, secondary motivational properties such that they can act as conditioned stimuli (CS) which can elicit approach (or withdrawal, if the primary motivational properties of the treatment were aversive) when the animal is subsequently exposed to these stimuli" (Tzschentke, 1998, p. 616). Although there are ardent debates regarding the extent of the isomorphism between the reward processes underlying CPP and the reinforcement processes underlying self-administration, the majority of researchers agree that, as compared with the self-administration paradigm, the CPP is more sensitive to the

natural state of the organism, because the testing session in the latter protocol occurs when the animal is in a drug-free state (Bardo & Bevins, 2000).

As mentioned earlier, several classes of drugs of abuse act as DA agonists as they increase the DA availability in the brain either by acting directly on the VTA neurons (e.g. opioids, nicotine, ethanol) or by blocking the DA transporter or the DA D_2 autoreceptor (e.g. amphetamine, cocaine). Most DA agonists strongly support the formation of CPP, effect that is readily blocked by administering DA antagonists (for a cross-indexed bibliography of these and other related studies that used CPP from 1957 to 1996, see Schechter & Calcagnetti, 1993, 1998). Consequently, it is of no surprise that CPP is robustly induced by drugs of abuse like morphine (Mueller, Perdikaris, & Stewart, 2002), heroin (Hand, Stinus, & Le Moal, 1989), amphetamine (Spyraki, Fibiger, & Phillips, 1982), cocaine (Spyraki, Nomikos, & Varonos, 1987), nicotine (Yararbas, Keser, Kanit, Pogun, 2010), and ethanol (Gremel & Cunningham, 2008), effects that are prevented by administration of DA antagonists (for morphine, Manzanedo, Aguilar, Rodríguez-Arias & Miñarro, 2001; for nicotine, Acquas, Carboni, Leone, & Di Chiara, 1989, for amphetamine, Liao, 2008, for heroin, Spyraki, Fibiger, & Phillips, 1983; for cocaine, Bilsky, Montegut, Nichols, & Reid, 1998; for ethanol, Walker & Ettenberg, 2007).

1.7. Specific Aims:

- Evaluate the ability of carbachol to support CPP learning in aVTA, midVTA and pVTA.
- Evaluate the pharmacological specificity of carbachol effects by challenging CPP acquisition with nicotinic and muscarinic antagonists.
- Identify the location of cathecholaminergic cells within VTA in the Long-Evans rat via TH staining.

- Compare across VTA subregions the ability of carbachol to induce CPP with our previous findings of its capacity to elicit affective analgesia.

CHAPTER 2. METHODS

2.1. Animals

Seventy-six naïve male Long-Evans rats were housed as pairs in plastic cages and given *ad libidum* access to food and water. Housing was provided in a climate-controlled vivarium maintained on a 12:12-hr circadian cycle with lights on at 0700 hrs. All testing was conducted between 0800 and 1700 hrs. Rats were handled every three days for at least one week before testing to minimize possible effects of stress from human contact. Also, upon arrival, rats were given 5-7 days of acclimatization within the new environment. All procedures in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Wayne State University.

2.2. Surgery

All surgeries were performed under aseptic conditions. Rats were anesthetized with sodium pentobarbital (50mg/kg, i.p.) following pretreatment with atropine sulfate (1 mg/kg, i.p.). For VTA implants, a stainless steel 33-gauge custom cannula was stereotaxically implanted unilaterally (right side), according to coordinates extrapolated from the rat brain atlas of Paxinos and Watson (1998) and to the immunohistochemistry data. The coordinates (in mm) relative to the Bregma suture and the top of the skull were for the pVTA: AP = - 4.5, ML = + 2.5, DV = -7.3, for the midVTA: AP = - 5.0, ML = + 2.5, DV = -7.3, and for the aVTA: AP = - 5.5, ML = + 2.5, DV = -7.3. Guides were affixed to the skull with 4 stainless steel bone and cranioplastic cement. Each guide cannula was fitted with a 28-gauge dummy cannula that extended the length of the guide to keep it clear and free of debris. Rats were given 7-10 days to recover before the initiation of testing.

2.3. Apparatus

The place conditioning apparatus consisted of two dimly-lit Plexiglas chambers (43 cm long X 21.5 cm wide X 30.5 cm high) separated by an opaque black wall with a guillotine door in the middle (8 cm wide). One chamber differed from the other by wall pattern (horizontal vs. vertical black and whites line, each 2.5 cm wide) and floor type (horizontal vs. vertical bars). Each chamber was equipped with four horizontal photobeam arrays: two arrays were mounted on the each of longer sides at a height of 4.5 cm and two arrays were mounted on the longer side opposed to the guillotine wall, at a height of 4.5 cm and 12 cm, respectively. Each photobeam array was spaced by 2.5 cm from one another (Fig. 6).

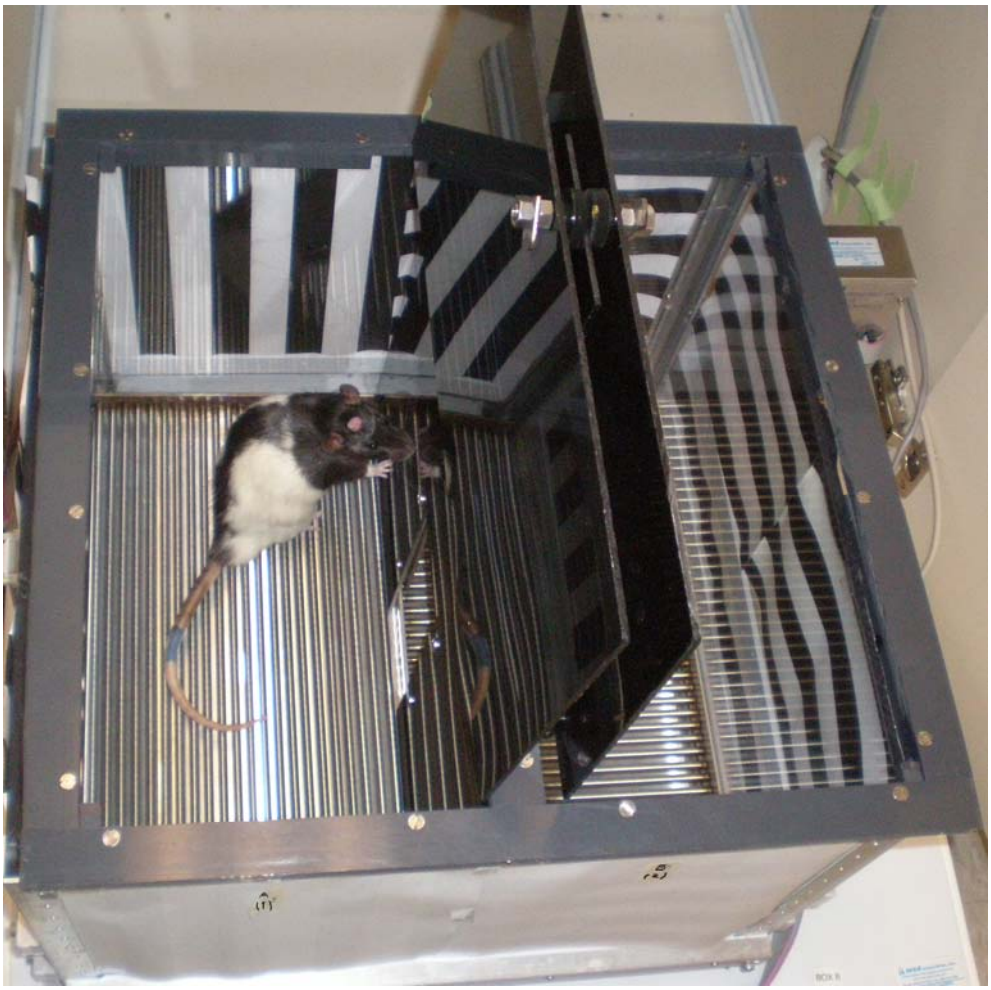


Figure 6. **The CPP apparatus.** One chamber differed from the other by wall pattern (horizontal vs. vertical black and white lines, each 2.5 cm wide) and floor type (horizontal vs. vertical bars).

2.4. Procedure

2.4.1. Conditioned Place Preference

Each experiment consisted of three sessions (Fig. 7): habituation (day 1), conditioning (days 2 to 7), and testing (day 8). On Day 1, the **Habituation** day, the guillotine door was open and the animals in a drug-free state were given free access to both chambers for 15 minutes. As our dependent measure, we recorded the amount of time each rat spent in each chamber. In order to minimize novelty effects and to ensure that rats had equal access to both chambers, each animal was placed in front of the opened guillotine door, facing the opposite chamber. On the first day of **Conditioning**, the rats received either saline or carbachol and were immediately confined to one chamber for 15 min. The guillotine door was closed. The next day, rats were administered the opposite drug and restricted to the opposite chamber for 15 minutes. This procedure was repeated for the remaining days of the conditioning session. Thus, each rat was exposed to each chamber three times, in an alternate fashion. On Day 8, the **Test** day, each rat was placed in the opposite chamber than on day 1, facing the opened guillotine door. Rats had access to both chambers for 15 min in a drug-free state and the time spent in each chamber was recorded.

	Habituation	Conditioning						Test
TREATMENT	Sham	Drug	Saline	Drug	Saline	Drug	Saline	Sham
CHAMBER	A or B	A	B	A	B	A	B	A or B
TIME	*Day 1*	*Day 2*	*Day 3*	*Day 4*	*Day 5*	*Day 6*	*Day 7*	*Day 8*

Figure 7. **The CPP timeline.** The experimental design permitted the alternation of the chamber (half of the rats received the drug in chamber A and the other half in chamber B) and order (half of the rats received the drug first and the other half received the saline first).

After each rat exposure, the chambers were cleaned with 0.75 % Alconox (VWR) and then aerated with Nilotron (Nilodor, Inc.) to eliminate odors from other rats. The temporal data and other behavioral variables (e.g. locomotion, velocity, distance travelled, etc.) were recorded each day with the aid of the Activity Monitor software, version 5 (MED Associates, Inc.).

2.4.2. Experiment 1: Carbachol-Induced CPP Learning

During CPP conditioning sessions, three groups of rats with cannulation targeting aVTA, midVTA, or pVTA received unilateral (right) microinjections of 4 µg/.25 µl carbachol or vehicle solution (normal saline - Sal) into the VTA on alternate days and were then immediately placed into the CPP apparatus. Each group contained 7-8 rats.

2.4.3. Experiment 2: Antagonism Analyses

To evaluate the pharmacological specificity of carbachol, four groups of rats with cannulations targeting aVTA and pVTA, respectively, received unilateral (right) microinjections of either a muscarinic or nicotinic antagonist 7-10 min prior to carbachol administration. Every animal in the muscarinic antagonism group received unilateral injections of Sal + Sal or 60 µg atropine + 4 µg carbachol on alternate conditioning days; whereas, every animal in the nicotinic antagonism group received unilateral injections of Sal + Sal or 45 µg mecamylamine + 4µg carbachol on alternate conditioning days. All injections were made in a constant volume of .25 µl. Each group of animals contained 6-9 rats. As midVTA injections of carbachol failed to support CPP, an antagonism analysis could not be conducted.

2.4.4. Drug Injections

Intracerebral injectors targeted at the VTA extended 1.7 mm beyond the end of the cannula. All injections were administered in a constant volume of 0.25 µl via a 33-gauge injector. All injections were made at a constant rate over 1 min, via an infusion pump (Harvard Model PHD 2000), and injectors were left in place for 2 min after the completion of injections to aid in the diffusion of drugs into the tissue. Carbachol, atropine, and mecamylamine were dissolved in normal sterile saline solution. Carbamoylcholine chloride (carbachol), Atropine sulfate (atropine), and Mecamylamine hydrochloride (mecamylamine) were purchased from a local branch of Sigma-Aldrich.

2.4.5. TH Immunocytochemistry and Histology

Unless otherwise specified, all chemicals were purchased from a local branch of Sigma-Aldrich or Fisher. TH immunoreactivity was conducted in order to localize cathecholaminergic cells. Under deep anesthesia (150 mg/kg pentrobarbital), the animals (n = 8) were transcardially perfused with saline solution followed by a solution of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. After perfusion, the brains were removed from the skulls and post-fixed in the same solution at room temperature for 3 h. Brains were then placed in sugar buffered formalin and stored at 4°C until sectioned. For each brain, serial coronal slices (45 µm) were obtained on a freezing microtome (Leica SM2000R) and placed in 24-free floating well plates containing 1 ml 0.1M phosphate buffered saline (PBS) at pH 7.4. The following steps were similar with the Xavier et al. (2005) protocol and were performed at room temperature with medium agitation, unless otherwise noted. The free-floating sections were pretreated with 1 ml of 0.3% H_2O_2 in 0.1M PBS for 30 min, washed three times with 1 ml of 0.1M PBS, and blocked with 500 µl of Blocking Buffer (BB) for 60 min, containing 1% Normal Goat Serum, 1% bovine serum albumin, 0.3% Triton X-100, in 0.1M PBS solution. The sections were then incubated over night

with 200 µl of monoclonal tyrosine hydroxylase primary antibody raised in mice (Sigma), diluted 1:750 in BB. After washing three times with 1 ml of 0.01M PBST (PBS mixed with 0.02% Triton X-100), the sections were incubated for 120 min. in 200 µl secondary antibody, prediluted, biotynilated, raised in goats (Chemicon). Sections were washed again three times with PBST and incubated for 60 min. with 200 µl Avidin-Biotin complex (Vector laboratories). Slices then were rinsed three times in 1 ml of PBST and two times in 1 ml of 0.01M Tris-HCl solution to bring the pH of the tissue from 7.4 to 7.6. The immunoreaction was developed by incubating each section for 5 min in a 100ul diaminobenzene (DAB, Sigma) medium with nickel intensification (1 pellet DAB dissolved in 5 ml of distilled H20 containing 60 mg Nickel Ammonium Sulfate). The last step of the immunoreaction was completed by adding 100 µl of peroxidated DAB (1 µl of 30% H_2O_2 to 2.5 ml DAB medium) to the sections and then quickly removing the solution and stopping the reaction with 1 ml 0.05 Tris-HCl. Finally, the sections were rinsed in distilled H_2O, mounted on microscope gelatin-coated glass slides out of 0.01M PBS, dehydrated in ethanol (70%, 95%, 100%, 100%; 2 min each), cleared with CitriSolv™ (Fisher) and xylene and then covered with Permount® (Fisher) and coverslips.

Rats that did not undergo TH immunohistochemistry were euthanized by carbon dioxide asphyxiation. The injection sites were marked by an injection of 0.25 µl of safrin-O dye (EM Science). The safranin-O injection was performed in the same fashion as the drug injections. Brains were extracted and placed in 20% (w/v) sucrose formalin solution for 48-72 hours. Brains were then sectioned in slices of 45 µm thickness on a freezing microtome, and injection sites were localized with the aid of the Paxinos and Watson (1998, 2007) brain atlases and of the TH data by two experimenters, one of whom was unaware of the behavioral outcomes.

2.5. Data analyses

Rats that did not complete the experiment due to unforeseen circumstances (illness, blocked cannulae, n = 3) were excluded from the data analyses. Statistically, CPP was defined as significantly more time spent in the drug-paired compartment following conditioning sessions (Test Day) compared to prior to conditioning (Habituation Day). Accordingly, significant effects of treatment were determined by paired-sample Student's *t*-tests for each VTA region. The significance threshold alpha was set at .05. Statistical analyses were performed using SPSS.

CHAPTER 3. RESULTS

3.1. Behavioral Profile

In most of the rats, carbachol injections did not change the observable immediate behavior. However, a minority of the animals displayed either hyperactivity (e.g., ipsilateral rotation, increased grooming hyperlocomotion, increased exploration and rearing) or hypoactivity (very calm, almost immobile, with eyes half-closed) both during carbachol injection and diffusion. These behavioral effects were not consistent in the same animal (i.e. did not exhibit the behavioral profile following every injection) and a particular profile was not restricted to a particular VTA subregion.

3.2. Initial Chamber Preference

Rats were tested using an unbiased procedure in a two chamber CPP apparatus (see Methods for details of training and microinjection procedures). Because there are reports that rats may have an initial tendency to prefer one of the two chambers (for a discussion on this methodological issue, see Bardo & Bevins, 2000, p. 38), a comparison of the amount of time spent in each chamber on the Habituation Day was conducted. Collapsing data across all groups, rats initially spent an equal amount of time in each chamber ($t(56) = .39$, $p > .05$), indicating that an initial chamber preference did not confound the results of CPP training (Fig. 8).

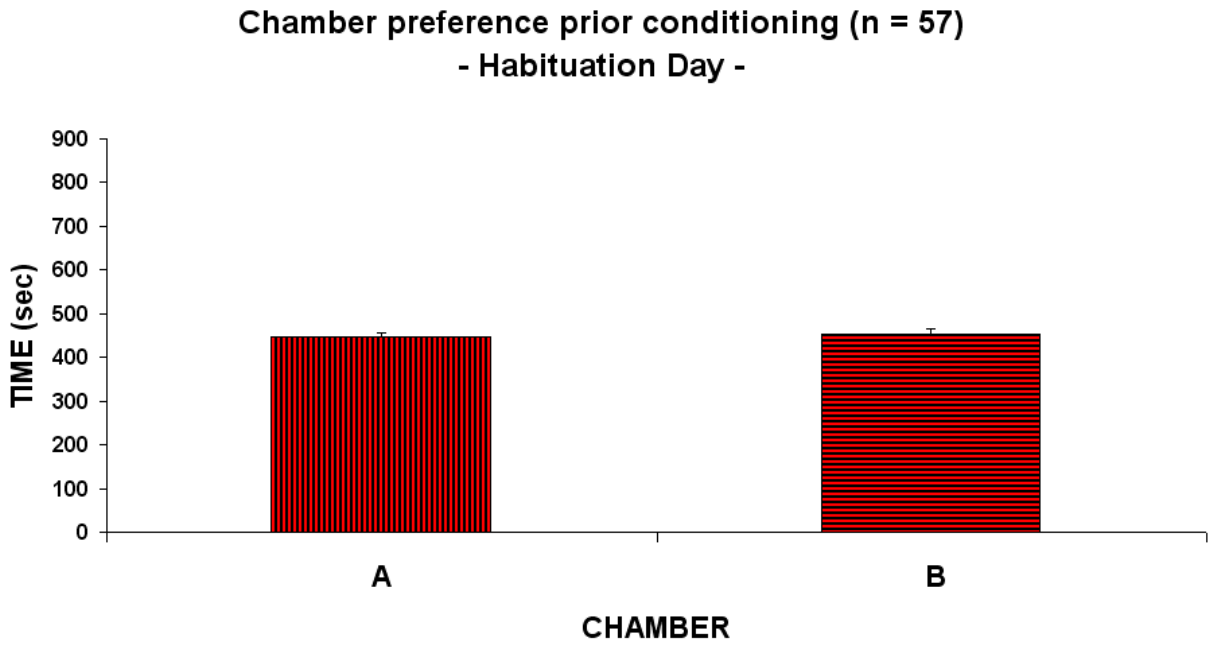


Figure 8. **Animals did not exhibit any tendency to prefer one chamber vs. another prior to conditioning**. The rats spent almost exactly equal amount of time in each chamber.

3.3. Experiment 1: Carbachol-Induced CPP Learning

Unilateral administration of carbachol (4µg in .25µl) into either the pVTA (Fig. 9) or aVTA (Fig. 10) was effective in supporting CPP learning. The amount of time spent in the carbachol-paired chamber was directly compared before and after the conditioning took place. Rats spent significantly more time in the carbachol-paired compartment after conditioning (pVTA: $t(6) = 3.98$, $p < .01$; aVTA: $t(5) = 4.04$, $p = .01$).

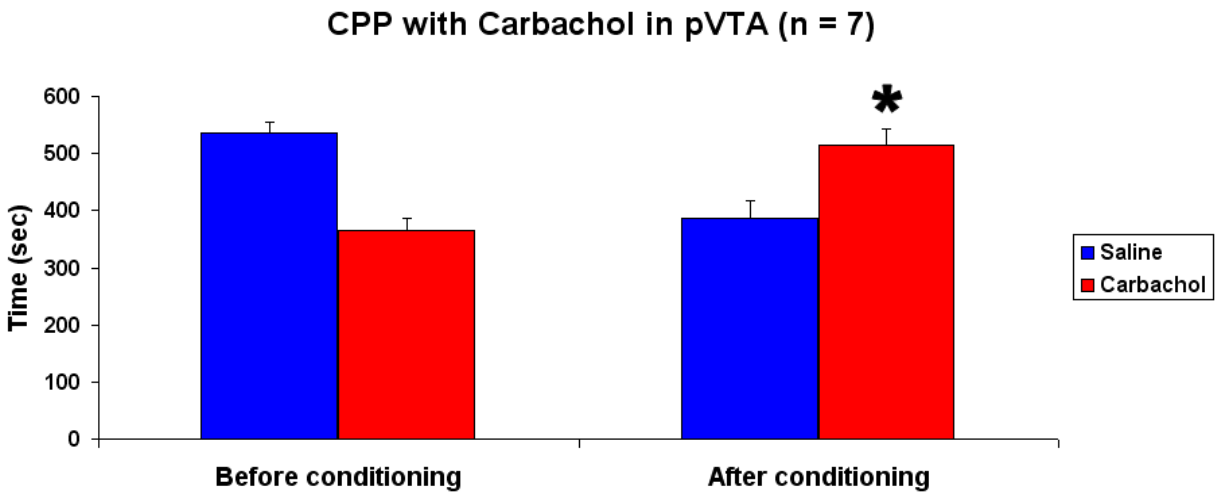


Figure 9. **Unilateral administration of carbachol into pVTA produces CPP.** *Significantly more time spent in the carbachol paired compartment after vs. before conditioning, $p < .01$.

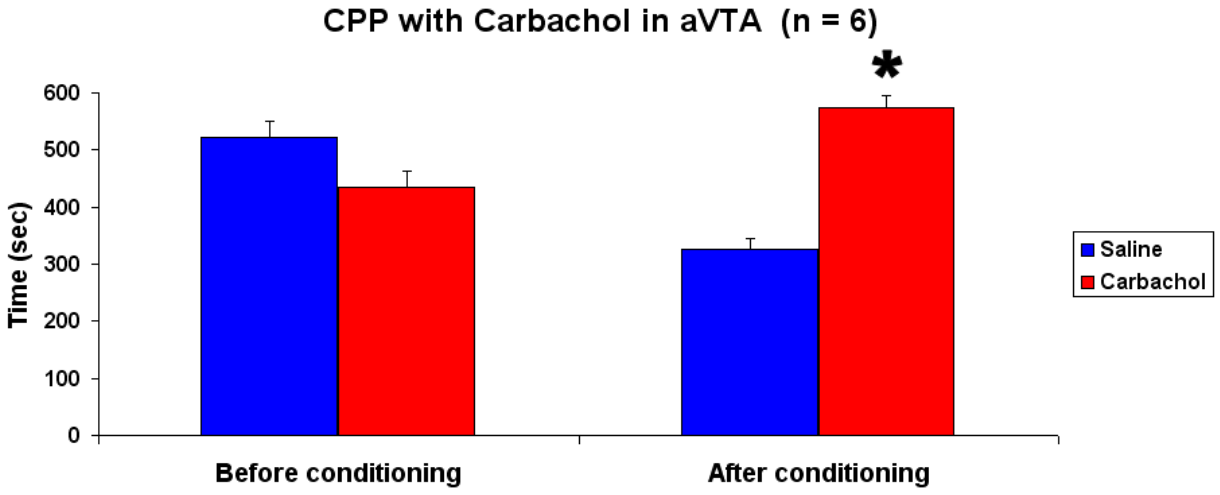


Figure 10. **Unilateral administration of carbachol into aVTA produces CPP.** *Significantly more time spent in the carbachol paired compartment after vs. before conditioning, $p = .01$.

Alternately, unilateral administration of carbachol (4µg in .25µl) into the midVTA failed to support CPP learning (Fig. 11). There was no significant difference between the time spent in the carbachol-paired compartment before and after conditioning ($t(7) = .22$, $p > .05$).

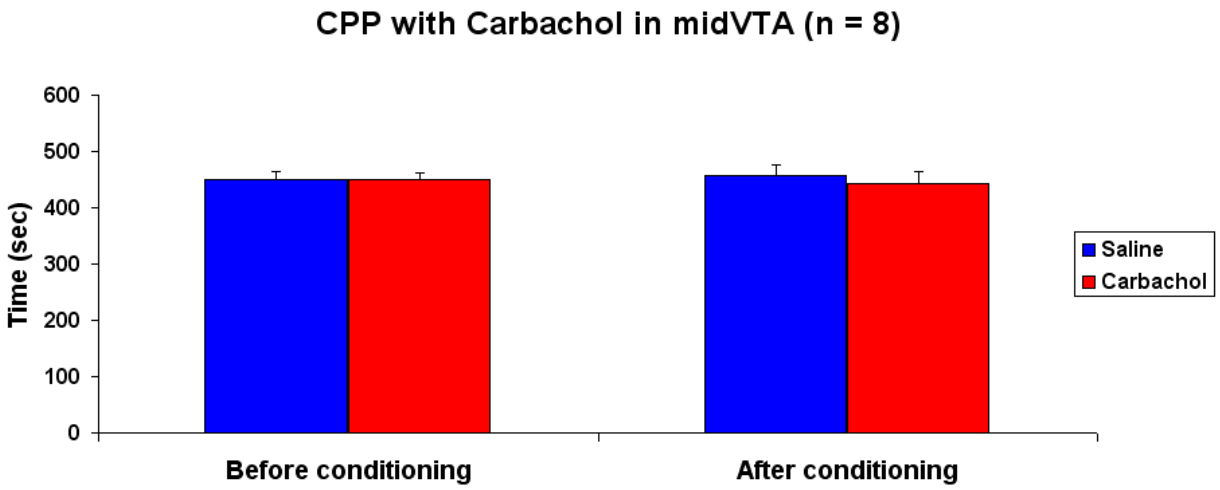


Figure 11. **Unilateral administration of carbachol into the midVTA does not produce CPP.** Note that the rats spent almost the exact amount of time in both chambers after conditioning.

3.4. Locomotion

The behavioral effects observed in some rats following injection of carbachol were not observed on conditioning days when rats received vehicle injections or on the Test Day when no injections were administered. Nevertheless, possible confounding effects of carbachol-induced locomotion during the Test Day were assessed (Fig. 12). The level of locomotor activity (defined as number of photobeam breaks) prior to carbachol treatment (Habituation Day) was compared to that observed during the Test Day. No difference in locomotion was observed in groups administered carbachol into the aVTA or midVTA ($t(5) = .50$, $p > .05$ and $t(7) = 1.14$, $p > .05$, respectively). However, rats that received carbachol in the pVTA during conditioning exhibited elevated locomotion during the Testing Day ($t(6) = 3.36$, $p < .05$).

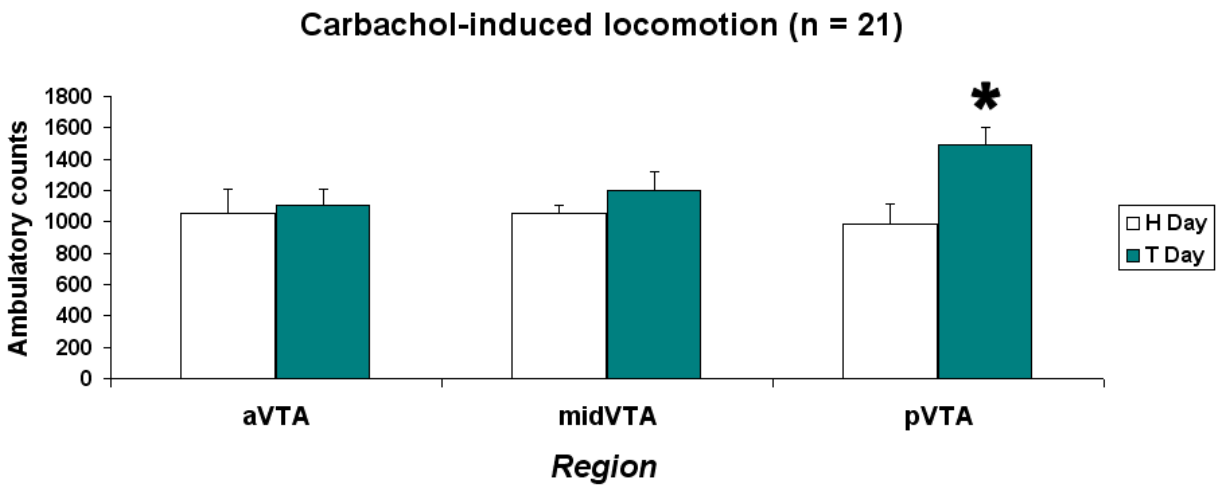


Figure 12. **Carbachol-induced locomotor activity.** * Significantly more photobeam breaks during the Testing (T) Day compared with the Habituation (H) Day, $p < .05$.

As depicted in Fig. 13, rats that received carbachol in the pVTA also exhibited an increased number of chamber crossing during the Test Day as compared to the Habituation Day ($t(6) = 2.49$, $p = .05$). No difference in the number of chamber crossing was observed in rats that were administered carbachol into aVTA or midVTA ($t(5) = 1.65$, $p > .05$ and midVTA, $t(7) = 1.87$, $p > .05$, respectively).

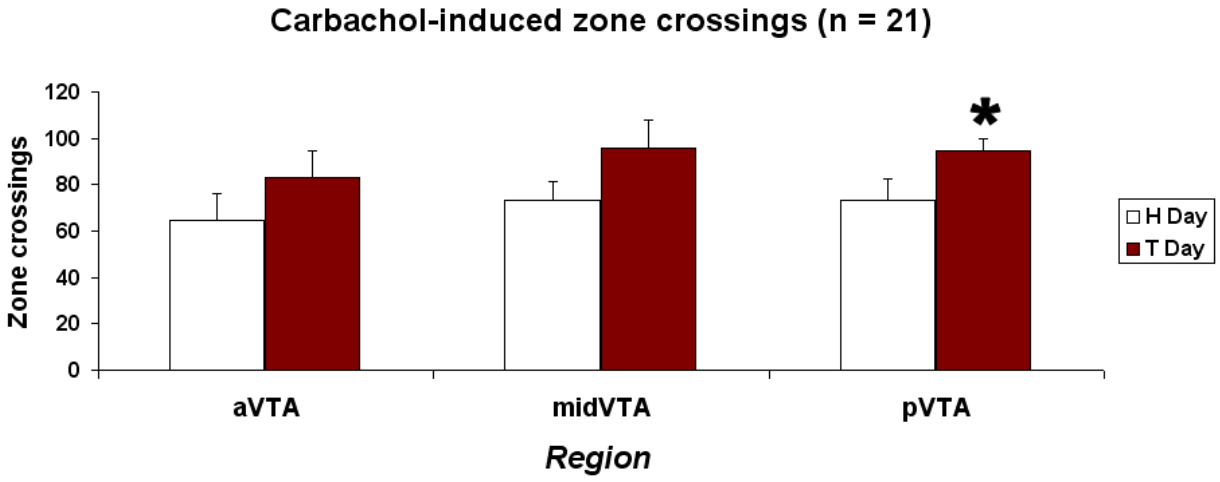


Figure 13. **Carbachol-induced zone crossings.** * Significantly more chamber crossings during the Testing (T) Day compared with the Habituation (H) Day, $p = .05$.

It is unlikely that the CPP learning observed by the group administered carbachol into pVTA is confounded by the increased locomotor activity and chamber crossing observed during the Test Day. Despite increased sampling of both chambers this group spent significantly more time in the carbachol-paired chamber. Typically, it is a decrease in locomotor activity and chamber crossings that is suspected as confounding chamber preference during the Test Day, i.e. "the higher the rate of locomotion in one compartment relative to the other, the greater is the probability that the rat will leave that compartment" (Martin-Iverson, Reimer, & Sharma, 1997, p. 328).

3.5. Experiment 2: Antagonism Analysis

The pharmacological specificity of carbachol-induced CPP was evaluated by pretreating the VTA with muscarinic (atropine) or nicotinic (mecamylamine) antagonists prior to carbachol administration.

3.5.1. Muscarinic Receptors

Unilateral administration of atropine (60 µg in .25µl) prior to carbachol (4µg in .25µl) injections into either the pVTA (Fig. 14) or aVTA (Fig. 15) prevented the development of carbachol-induced CPP learning. The amount of time spent in the drug-paired chamber was directly compared before (Habituation Day) and after (Test Day) the conditioning took place. There was no significant difference between the amount of time the animals spent in the atropine + carbachol paired chamber and the saline + saline compartment after conditioning when the drugs were administered in pVTA ($t(5) = .22$, $p > .05$). The rats that received the atropine + carbachol treatment in aVTA spent less time in the drug-paired compartment than in the vehicle-paired compartment after conditioning ($t(8) = 2.94$, $p < .05$).

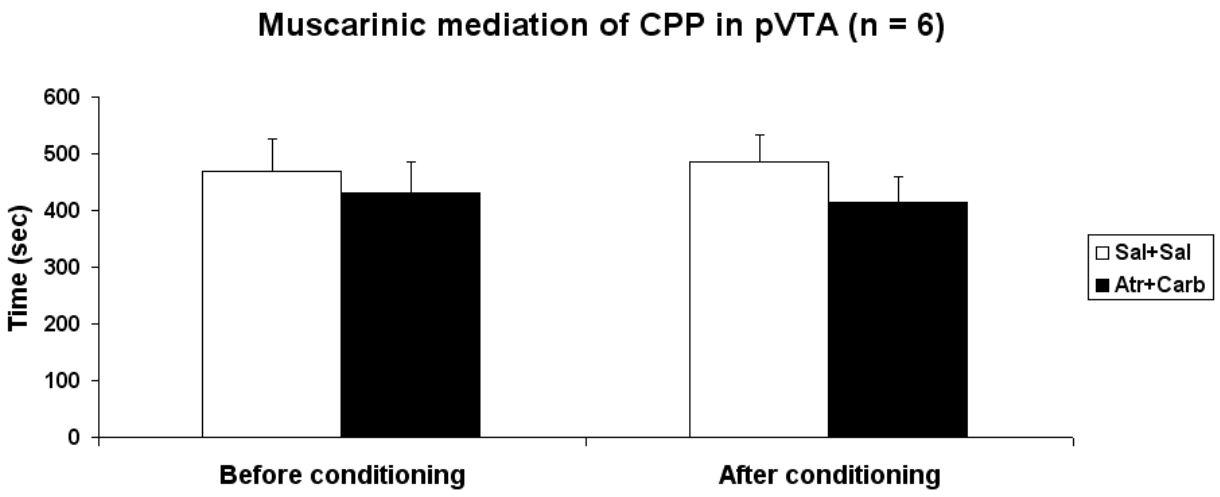


Figure 14. **Muscarinic mediation of CPP in pVTA.** Atropine pretreatment prevented the formation of carbachol-induced CPP**.** *Legend: Atr = atropine, Carb = carbachol, Sal = saline.*

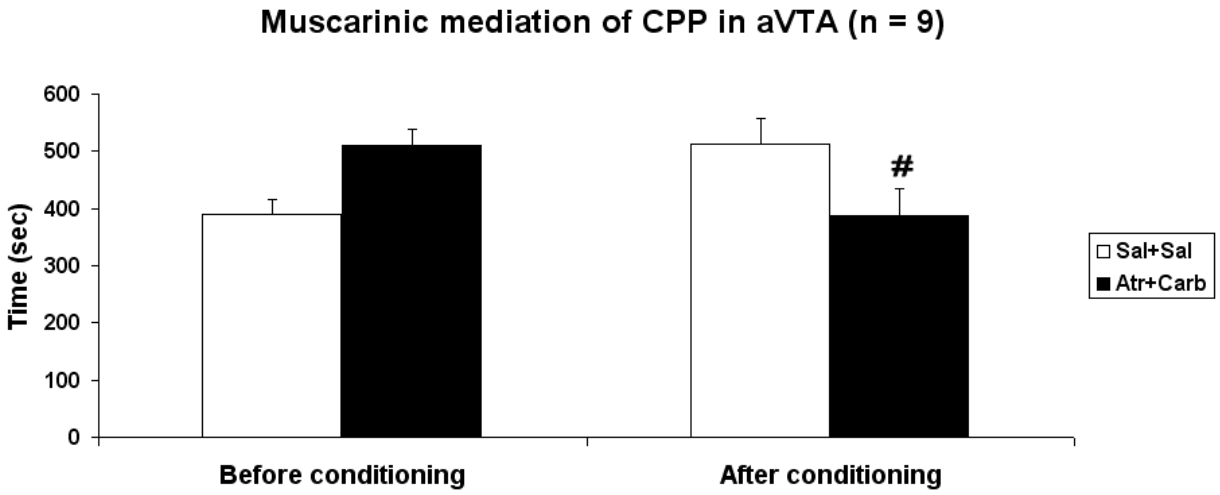


Figure 15. **Muscarinic mediation of CPP in aVTA.** Atropine pretreatment prevented the formation of carbachol-induced CPP**.** # Significantly less time spent in the drug-paired compartment after vs. before conditioning, $p < .05$. *Legend: Atr = atropine, Carb = carbachol, Sal = saline.*

3.5.2. Nicotinic Receptors

Unilateral administration of mecamylamine (45 µg in .25µl) prior to carbachol (4 µg in .25 µl) injections into either the pVTA (Fig. 16) or aVTA (Fig. 17) prevented the development of carbachol-induced CPP learning. The amount of time spent in the drug-paired chamber was

directly compared before (Habituation Day) and after (Test Day) the conditioning took place. There was no significant difference between the amount of time the animals spent in the mecamylamine + carbachol paired chamber and the saline + saline compartment after conditioning (pVTA: $t(5) = 1.77$, $p > .05$; aVTA: $t(6) = .99$, $p > .05$).

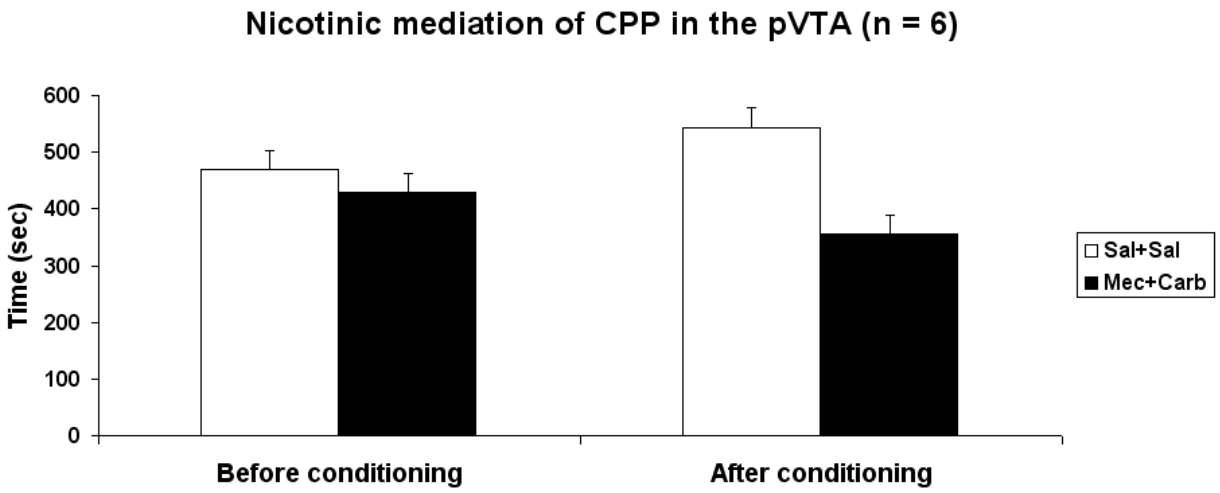


Figure 16. **Nicotinic mediation of CPP in pVTA.** Mecamylamine pretreatment in pVTA prevented the formation of carbachol-induced CPP**.** *Legend: Carb = carbachol, Mec = mecamylamine, Sal = saline.*

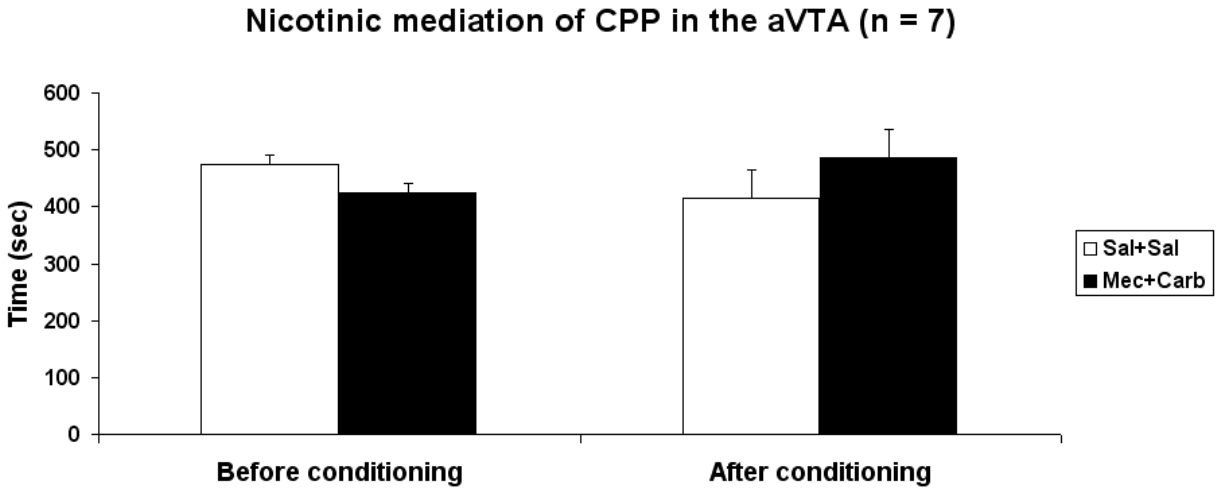


Figure 17. **Nicotinic mediation of CPP in aVTA.** Mecamylamine pretreatment in pVTA prevented the formation of carbachol-induced CPP**.** *Legend: Carb = carbachol, Mec = mecamylamine, Sal = saline.*

3.6. Tyrosine Hydroxylase Immunohistochemistry

Figure 18 shows the localization of catecholamine producing neurons within aVTA (top), midVTA (middle) and pVTA (bottom) of the intact adult male Long-Evans rat. Surgery coordinates for CPP studies were adjusted accordingly. See Methods for experimental details.

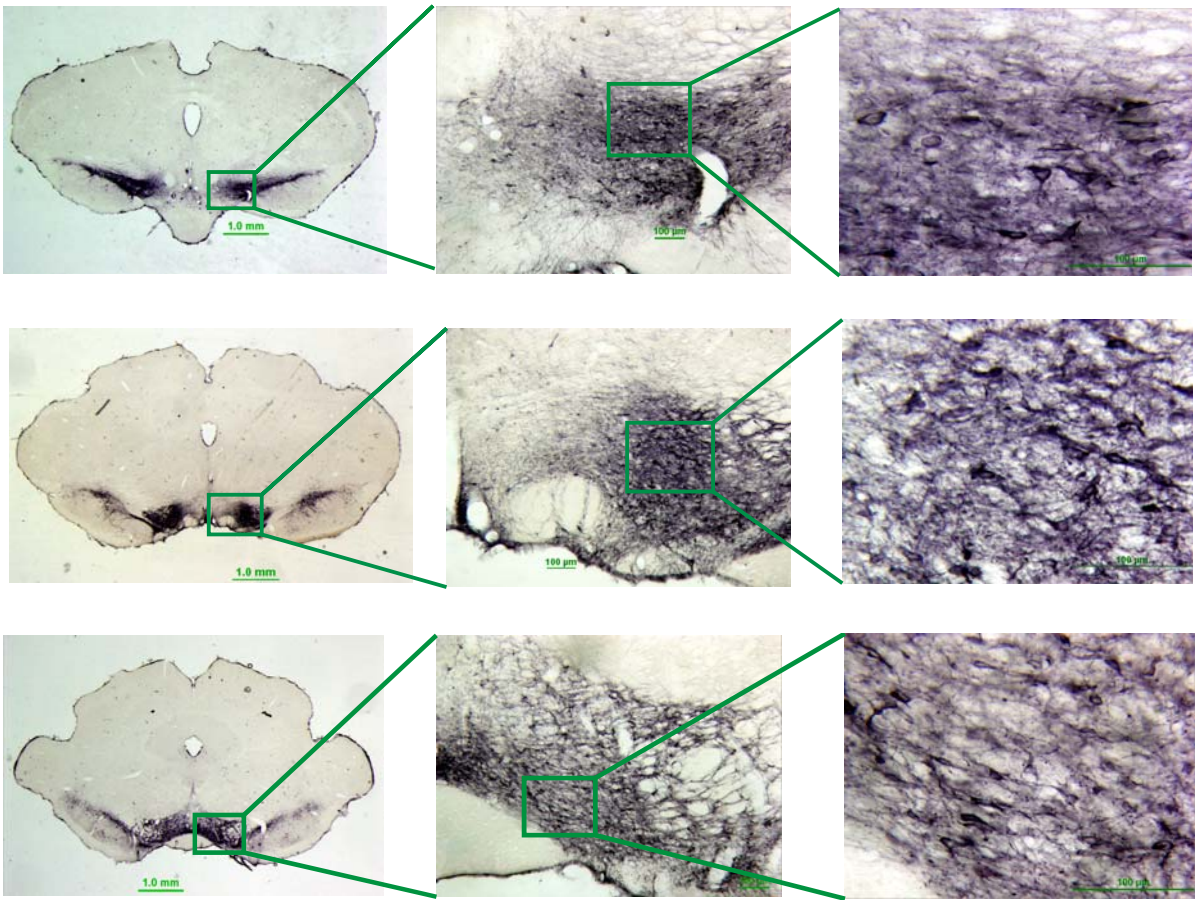


Figure 18. **Catecholamine-producing neurons within VTA.** *Top*. aVTA. *Middle.* midVTA. *Bottom*. pVTA. The staining was obtained via TH IHC.

The exact location of the injection sites performed in aVTA, midVTA, and pVTA is shown in Fig. 19, Fig. 20, and Fig. 21, respectively.

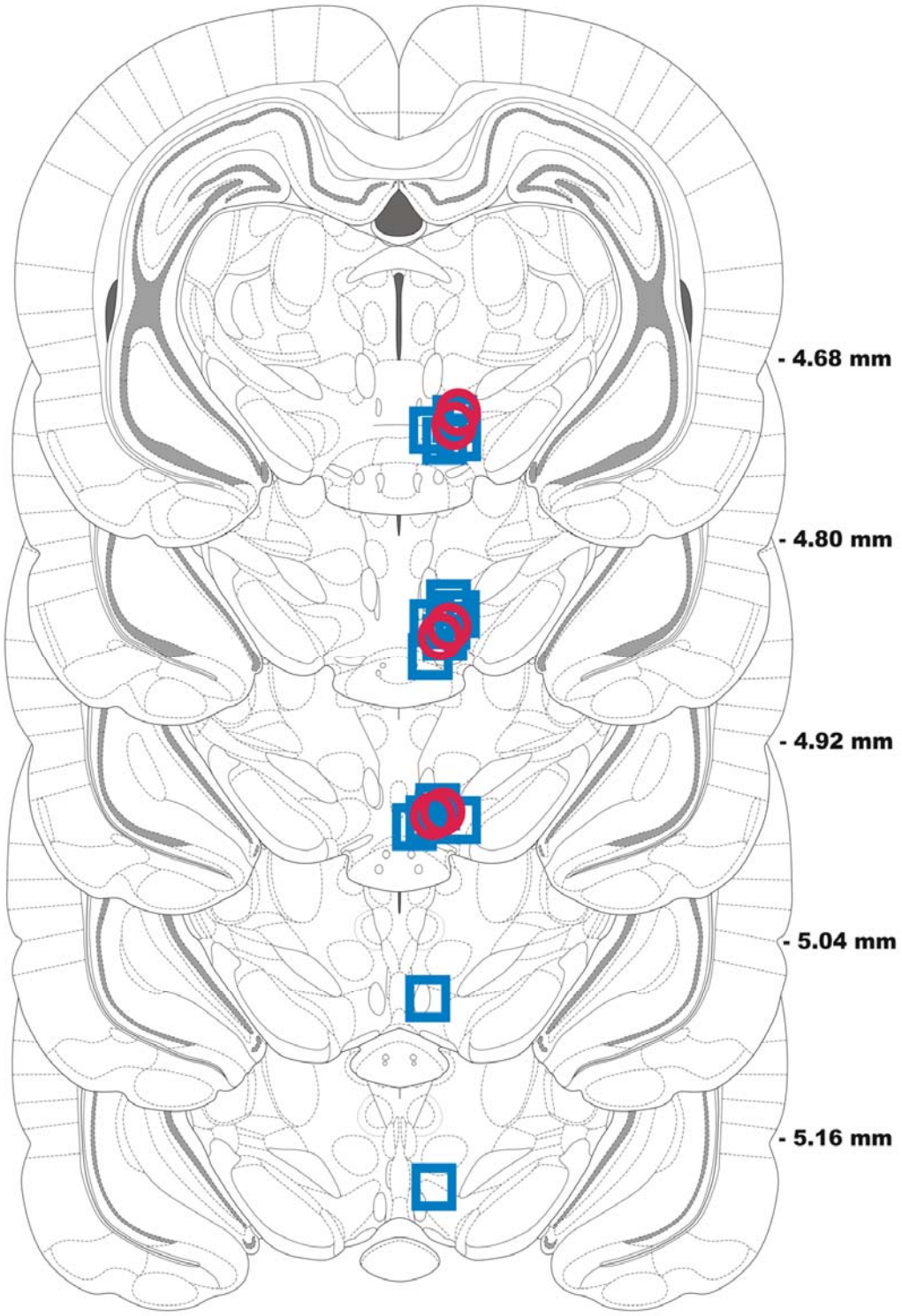


Figure 19. **Coronal diagrams extracted from Paxinos and Watson (2007) representing the cannula placements in aVTA.** The red circles represent the location of the 4 μg carbachol microinjections and the blue squares represent the location of either 60 μg atropine + 4 μg

carbachol or 45 mecamylamine + 4 μg carbachol microinjections. Numbers on the right side of diagrams represent coordinates in millimeters posterior to Bregma.

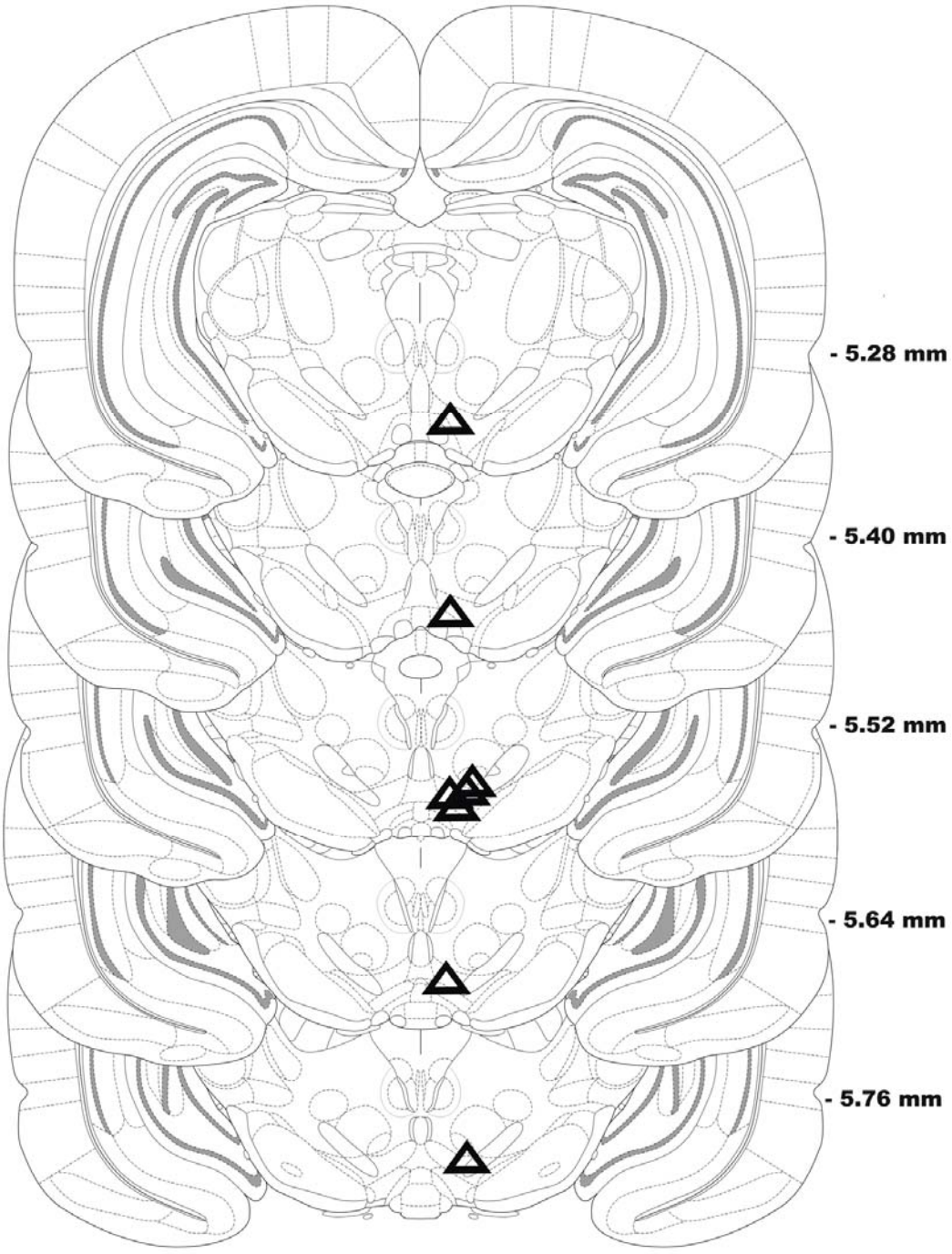


Figure 20. **Coronal diagrams extracted from Paxinos and Watson (2007) representing the cannula placements in midVTA.** The black triangles represent the location of the 4 μg carbachol microinjections. Numbers on the right side of diagrams represent coordinates in millimeters posterior to Bregma.

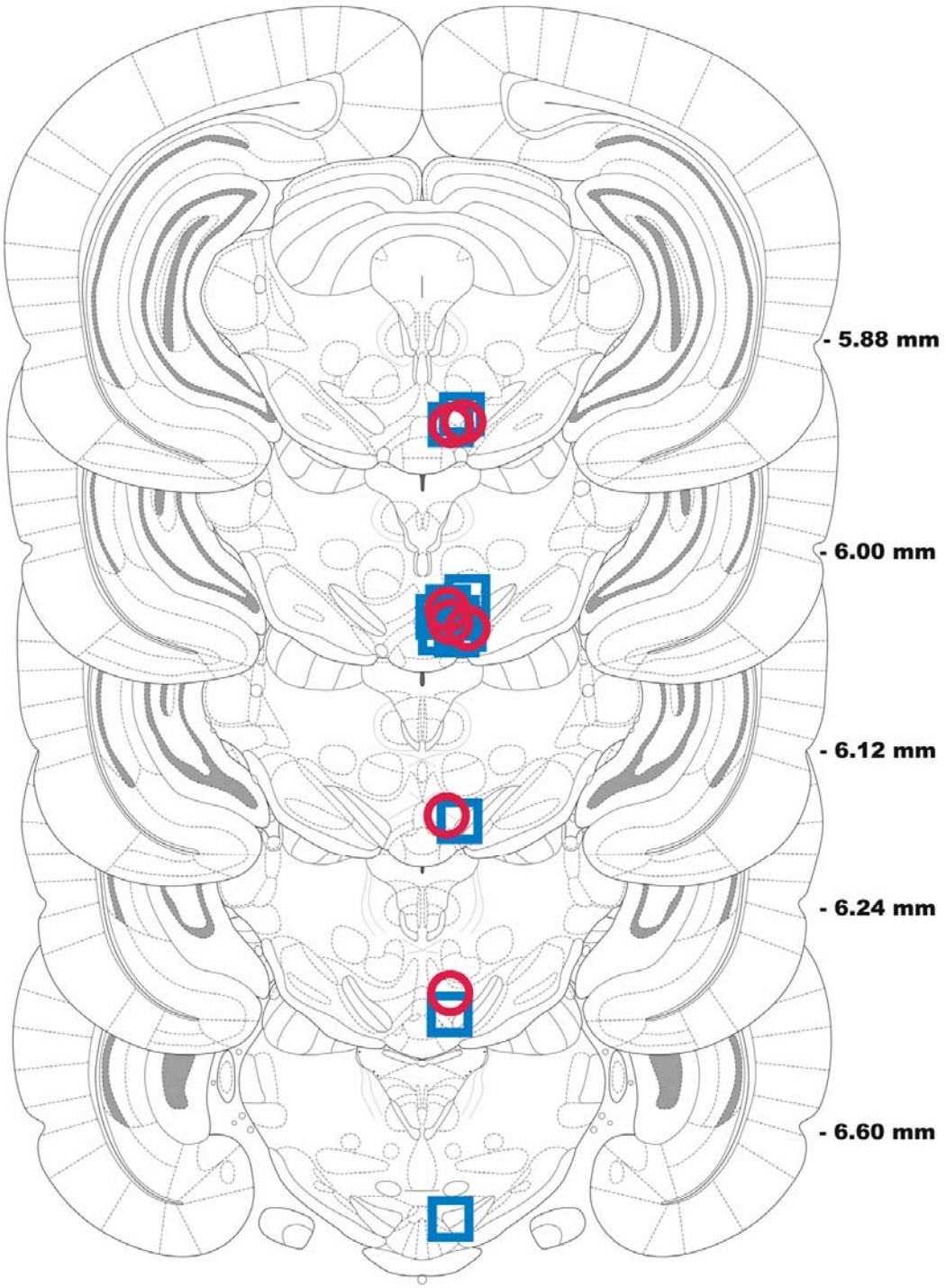


Figure 21. **Coronal diagrams extracted from Paxinos and Watson (2007) representing the cannula placements in pVTA.** The red circles represent the location of the 4 µg carbachol micro injections and the blue squares represent the location of either 60 µg atropine + 4 µg carbachol or 45 mecamylamine + 4 µg carbachol microinjections. Numbers on the right side of diagrams represent coordinates in millimeters posterior to Bregma.

3.7. Comparison between Carbachol-Induced Affective Analgesia and Reward

Figure 22 compares the capacity of 4 µg carbachol administered into subregions of the VTA to support CPP learning (present study) and generate increases in VAD threshold (Schifirneț & Borszcz, 2007). The capacity of carbachol to elevate VAD threshold depended on the subregion of the VTA into which carbachol was injected. Comparison of VAD thresholds following injection of carbachol into the aVTA, midVTA and pVTA revealed significant differences ($F(2,40) = 46.4$, $p < .001$). Planned pairwise comparisons revealed that the mean threshold current intensity necessary to elicit VAD was significantly lower when the drug was delivered into the midVTA compared with aVTA ($t(23) = 8.91$, $p < .001$) and pVTA ($t(25) = 10.08$, $p < .01$). Comparison between 4 µg carbachol treatment in aVTA and pVTA indicated no difference in the VAD threshold ($t(32) = .65$, $p = .52$).

Similarly, analysis of the groups of animals that received unilateral 4 µg carbachol injections in the aVTA, midVTA, and pVTA in the present study revealed that the CPP score is significantly affected by the region into which the drug is delivered ($F(2,18) = 7.32$, $p = .005$). The CPP score is defined as time spent in the carbachol-paired chamber *after* conditioning minus time spent in the carbachol-paired chamber *before* conditioning. Specifically, direct planned comparisons revealed that the animals spent significantly less amount of time in the drug-paired chamber when the drug was delivered into the midVTA compared with aVTA ($t(12) = 3.36$, $p < .01$) and pVTA ($t(13) = 3.31$, $p < .01$). Comparisons between 4 µg carbachol treatment in aVTA and pVTA indicated no difference in the CPP score ($t(11) = .03$, $p = .98$).

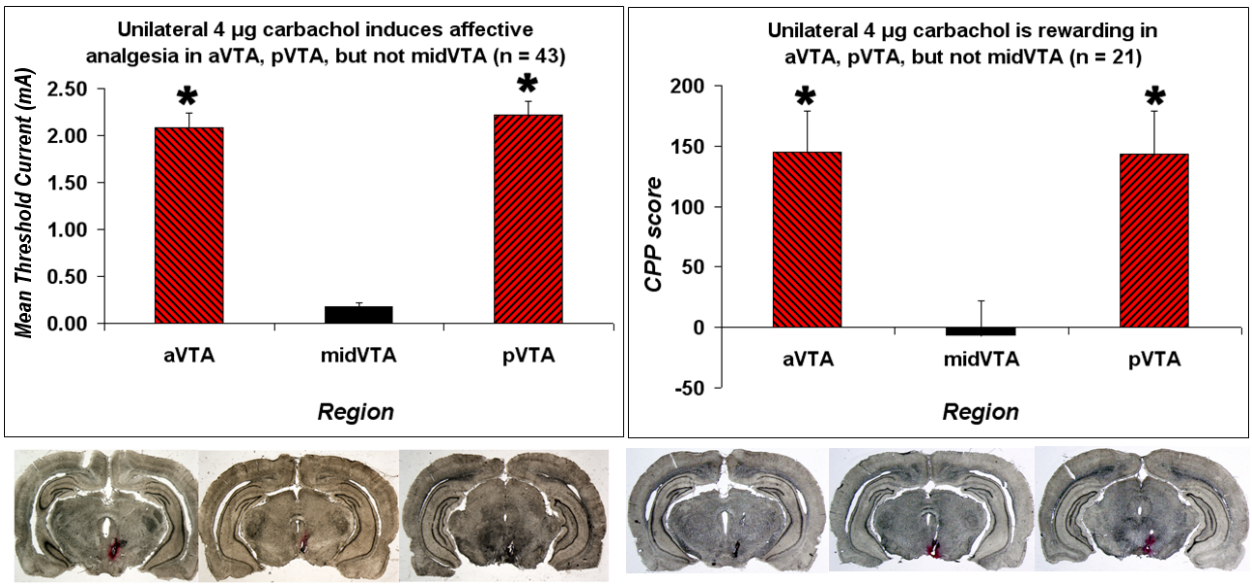


Figure 22. **Unilateral 4 µg carbachol injections support the development of CPP learning and affective analgesia in both the aVTA and pVTA, but not in the midVTA.** *Top left.* Carbachol (4 µg) administered in mid-VTA (black) failed to elevate VAD thresholds as compared with the same dose administered in aVTA and pVTA (red). ***** Thresholds significantly elevated compared to midVTA, $p < .01$. *Top right.* Carbachol (4 µg) administered aVTA and pVTA (red), but not in mid-VTA (black) supported the acquisition of CPP learning in mid-VTA (black). ***** CPP score significantly elevated compared to midVTA, $p < .01$. *Bottom.* Coronal slices representing the aVTA, midVTA, and pVTA, respectively, from the Schifirneț & Borszcz (2007) study (left) and from the present study (right).

CHAPTER 4. DISCUSSION

This study is the first to directly compare the extent of overlap between cholinergically mediated reward and affective analgesia within different VTA regions. We tested Franklin's (1989, 1998) analgesia hypothesis that postulated that activation of the brain reward circuit should be conducive to affective analgesia Our data indicate that unilateral 4 µg carbachol injections support both the development of CPP learning and affective analgesia in both the aVTA and pVTA, but not in the midVTA (Fig. 22), supporting the affective analgesia hypothesis. However, the extent of overlap between the neural circuits underlying affective analgesia and reward is only partial, as different cholinergic receptors are responsible for these effects in different subregions of the VTA. Whereas both nicotinic and muscarinic receptors contribute to carbachol-induced affective analgesia aVTA, as shown by the ability of both atropine and mecamylamine to reduce the carbachol-induced increases in VAD threshold, only the muscarinic receptors are mediating the analgesic action of carbachol in the pVTA, because mecamylamine was ineffective in attenuating the carbachol analgesia, but atropine reliably blocked this effect. On the other hand, the rewarding effects of carbachol are mediated by the activation of both nicotinic and muscarinic receptors in both aVTA and pVTA, as indicated by the fact that both atropine and mecamylamine prevented the development of CPP in both VTA subregions.

4.1. Differential Cholinergic Activation of the VTA in Reward and Analgesia

Two previous studies addressed the rewarding properties of intra-VTA carbachol administration by employing the CPP paradigm. The first study observed that carbachol (0.5 µg and 2 µg/side) is reinforcing in the VTA (Yeomans et al., 1985). Yet, in this study, different VTA subregions were not assessed, as the cannulae placements ranged from - 4.8 mm to - 6.3

mm from Bregma, covering the majority of the VTA. The second study conducted by Ikemoto & Wise (2002) evaluated the capacity of carbachol to support CPP in the aVTA and pVTA and found that carbachol supports CPP learning only in the pVTA. Nevertheless, this study used significantly lower doses of carbachol (ranging from 0.00546 µg to 0.091 µg) as compared with our doses that proved efficacious in inducing affective analgesia (2 and 4 µg). At the lowest dose (1 µg) used in the Schifirneț & Borszcz (2007) study, carbachol failed to induce affective analgesia in any VTA subregion. Therefore, it is possible that low doses (0.09 µg) of carbachol are not rewarding, but the high dose (4 µg) used in this study are able to induce CPP in the aVTA.

When 4 µg carbachol was administered in the midVTA, as opposed to the aVTA or pVTA, it failed to induce either affective analgesia or reward, suggesting that carbachol-induced CPP is anatomically specific to the aVTA and pVTA. To the best of my knowledge, there is only one study published to date that found an anterior-posterior bimodal activation within the VTA. Marcangione & Rompré (2008) trained rats to self-administer electrical stimulation to the posterior mesenchephalon and assessed the subsequent c-Fos expression within VTA. The authors found that in both the aVTA and pVTA there was an increase in the c-fos expression following posterior mesenchephalon stimulation, but the lateral midVTA exhibited the lowest number of Fos-positive cells. The fact that the midVTA is not involved in the rewarding effects of posterior mesenchephalon self-stimulation (Marcangione & Rompré, 2008) or in the locomotor (Museo & Wise, 1995) and rewarding effects of cytisine[4] (R. A. Wise, personal communication, 6/17/2010) indicates that unlike the aVTA and pVTA, the activation of the midVTA is not reinforcing. Taken together, and concordant with the affective analgesia

[4] Cytisine is a nicotinic agonist that supports the development of CPP when delivered into the aVTA (Museo & Wise, 1994)

hypothesis, these results suggest that there is a similar regional heterogeneity within the VTA mediating analgesia and reward: wherever carbachol is reinforcing within the VTA, it also produces affective analgesia; conversely, wherever in the VTA carbachol injections are not reinforcing, this drug also fails to induce affective analgesia.

The carbachol-induced affective analgesia and reward in the aVTA and pVTA is thought to be mediated by its binding to the muscarinic and nicotinic receptors located on the dopaminergic neurons within the VTA. Thus, carbachol activation of the dopaminergic neurons mimics the actions of acetylcholine release from the LTDg and PPTg nuclei[5], resulting in subsequent dopamine release into the terminal VTA efferent sites. As stated above, cholinergic activation of the VTA following electrical stimulation of LTDg (Forster & Blaha, 2000; Forster et al., 2002; Yeomans et al., 2001) or intra-VTA administration of cholinergic agonists, like carbachol (Westerink et al., 1996), oxotremorine M (muscarinic agonist, Gronier & Rasmussen, 2000), or nicotine (Blaha et al., 1996) results in increased accumbal DA efflux. This cholinergically mediated DA release into the NAc is reinforcing as it is associated with the rewarding effects of morphine (Rezayof et al., 2007), cocaine (You et al., 2008), and lateral hypothalamic self-stimulation (Rada et al., 2000).

Activation of muscarinic rather than nicotinic receptors seems to be more involved in reward processing (Yeomans & Baptista, 1997, You et al., 2008). Both the increase in accumbal DA release and the rewarding effects associated with morphine injections (Miller et al., 2005), cocaine self-administration (You et al., 2008), lateral hypothalamic stimulation (Rada et al., 2000; Yeomans & Baptista, 1997; Yeomans et al., 2000) are blocked more by pretreating the

[5] These cholinergic structures that project to the VTA are also involved in noxious (LDTg, Kayama & Ogawa, 1987; PPTg, Carlson et al., 2004; Iwamoto, 1991; Kayalioglu & Balkan, 2004) and reward – related (LDTg, Nakahara, Ishida, Nakamura, Furuno, & Nishimori, 2001; PPTg, Okada, Toyama, Inoue, Isa, & Kobayashi, 2009; Olmstead, Munn, Franklin, & Wise, 1998) information processing.

VTA with muscarinic rather than nicotinic antagonists. In concordance with this, our study found that the VTA muscarinic receptors mediate the rewarding and analgesic effects of carbachol, as atropine blocked the carbachol-induced CPP and affective analgesia in both the aVTA and pVTA.

Although not to the same extent as muscarinic receptors, the activation of the nicotinic receptors from the VTA is also reinforcing. Knock-out mice with deletion of nicotinic receptor genes that underwent viral restoration of the nicotinic receptors α4β2 and α6β2 selectively in the VTA self-administer i.v. nicotine, but not if these receptors are missing from the VTA or are restored elsewhere in the brain (Pons et al., 2008), suggesting that these particular nicotinic subtypes expressed onto the DA neurons are necessary and sufficient for nicotine self-administration. Nicotinic activation of the VTA DA neurons results in increased DA accumbal efflux (Cadoni, Muto, Di Chiara, 2009; Yoshida et al., 1993; Zhang et al., 2009) and this efflux is blocked by intra-VTA application of nicotinic antagonists (Gotti et al., 2010; Nissel et al., 1994). The reinforcing properties of nicotine depend on the integrity of mesoaccumbal DA terminals, as shown by the fact that nicotine self-administration is reduced in rats with intra-NAc 6-OHDA lesions[6] (Corrigall, Franklin, Coen, & Clarke, 1992). Alternately, intra-VTA microinjection of the nicotinic antagonist DHβE attenuates i.v. nicotine self-administration behavior (Corrigall, Coen, & Adamson, 1994). Nicotine also supports the development of CPP (Vastola, Douglas, Varlinskaya, & Spear, 2002; Yararbas et al., 2010) and the acquisition of this learning is blocked by intra-NAc shell D_1 antagonists (Spina, Fenu, Longoni, Rivas & Di Chiara, 2006). Consistent with these findings, the results of our study revealed blockade of nicotinic receptors by mecamylamine into either aVTA or pVTA prevents the development of carbachol-

[6] When administered into the catecholaminergic terminal sites, like NAc, this neurotoxin is taken up by the terminal buttons of DA axons and transmitted via retrograde transport to cell bodies in VTA where it destroys these cells by inducing apoptosis (for a review, see Blum et al., 2001).

induced CPP learning. On the other hand, mecamylamine reduced the carbachol-induced affective analgesia only when administered into the aVTA, but not in the pVTA.

It is important to mention that administration of an antagonist prior to a non-specific agonist is not identical to administration of the other agonist alone. In other words, administration of mecamylamine + carbachol does not equal administration of muscarine. If this were true, then administration of atropine prior carbachol in our study, for example, would not have prevented the development of CPP learning, as the activation of the nicotinic receptors within the VTA is reinforcing. Therefore, the present study suggests that there is a subpopulation of muscarinically activated AND nicotinically inhibited neurons within the pVTA that is involved in affective analgesia, but not in reward (Fig. 23). Conceivably, in order to produce a compound that when administered into the pVTA has an analgesic, but not a rewarding effect, this compound must be a combination of a muscarinic agonist and a nicotinic antagonist. Thus, at least at the level of the pVTA, the neural circuits contributing to affective analgesia and reward overlap only partially. Further studies are needed to characterize the potential analgesic and/or rewarding effects of direct nicotinic or muscarinic activation of the VTA by injecting nicotine or muscarine alone, without actively inhibiting the other receptors type.

	AA			CPP		
	aVTA	mid VTA	pVTA	aVTA	mid VTA	pVTA
Carbachol	+	-	+	+	-	+
Atropine + Carbachol	-	N/A	-	-	N/A	-
Mecamylamine + Carbachol	-	N/A	+	-	N/A	-

Figure 23. **The differential involvement of nicotinic and muscarinic receptors in aVTA, midVTA, and pVTA in affective analgesia (AA) and conditioned place preference (CPP).** Note that in the pVTA there is a subpopulation of muscarinically activated AND nicotinically inhibited neurons that is involved in affective analgesia, but not in reward.

It is possible that the differential involvement of cholinergic receptors in the three VTA subregions in reward and analgesia relies on the different densities of nicotinic and muscarinic receptors in these areas. Unfortunately, there is no extant evidence of the distribution of the cholinergic receptors in these VTA subregions. Thus, future studies that perform ultrastructural localization of the cholinergic receptors in the VTA would provide useful information that help assess whether cholinergic receptors display a regional heterogeneity within VTA.

Another possibility is that the cholinergic agents used in this study bound to receptors located on non-dopaminergic neurons. From a quantitative point of view, the most recent unbiased stereological estimate of the rat VTA reports that GABA neurons constitute about 35% of the VTA, with glutamate neurons constituting about 2-3%, and the remaining 63% (approx. 40,000 cells) being DA neurons (Nair-Roberts et al., 2008). It is important to stress that the distribution of these three cell types is not uniform across the VTA, with more glutamate and less GABA neurons in the aVTA and more GABA cells and almost no glutamate neurons in the pVTA (Nair-Roberts et al., 2008; Yamaguchi, Sheen, & Morales, 2007). In addition to the VTA neurons, *en passant* axons and most of the VTA terminals possess muscarinic and nicotinic receptors (Adell & Artigas, 2004) and binding to cholinergic agents to these receptors can result in either depolarization or hyperpolarization of VTA neurons. For example, the nicotinic receptor α4β2 is expressed by GABA afferents and the nicotinic receptor α7 by glutamate inputs (Keath, Iacoviello, Barrett, Mansvelder, & McGehee, 2007). These cytoarchitectonic differences may result in differential modulation of VTA subregions by cholinergics and, as a consequence,

differential involvement of the VTA subregions in reward and analgesia. Further studies designed to identify the muscarinic and nicotinic receptor subtypes responsible for the results obtained in this study, along with their exact location, would provide further insight into the mechanisms of cholinergically mediated analgesia and reward.

4.2. Downstream Effects

4.2.1. Nucleus accumbens (NAc)

As mentioned earlier, previous studies suggested that the activation of the pVTA, but not the aVTA, mediates the reinforcing effects of opiates (Zangen et al., 2002), cocaine (Rodd et al., 2005), nicotine (Ikemoto, et al., 2006), ethanol (Rodd-Henricks et al., 2000), tetrahydrocannabinol (Zangen et al., 2006), muscimol ($GABA_A$ agonist, Ikemoto et al., 1998), CPBG (5-HT_3 agonist, Rodd et al., 2007), and low doses of carbachol (Ikemoto & Wise, 2002). These functional differences are thought to rely on the differences in efferent projections of these two subregions: pVTA projects predominantly to limbic structures critical for reinforcement (e.g., medial part of the NAc shell and the medial olfactory tubercle), whereas aVTA projects to limbic regions less involved in reinforcement processes (e.g., NAc core, NAc ventral shell, lateral tubercle, and dorsal striatum (Ikemoto, 2007, 2010).

However, besides striatum and the olfactory tubercle, the VTA sends efferents to other subcortical structures, such as the habenula, bed nucleus of stria terminalis, amygdala, hippocampus, and septum (for reviews, see Deniau, Thierry, & Feger, 1980; Moore & Bloom, 1978; Oades & Halliday, 1987; Swanson, 1982). Moreover, the mesocortical dopaminergic system, comprising of the VTA's efferents to prefrontal, insular and cingulate cortices (Fluxe et al., 1974, Ohara et al., 2003; Williams & Goldman-Rakic, 1998), is well characterized and heavily investigated by cognitive neuroscientists (Wise, 2004). In addition to the mesolimbic

and mesocortical projections, VTA neurons send several sparse efferents to the adjacent substantia nigra (Ferreira, Del-Fava, Hasue, & Shammah-Lagnado, 2008), thalamus (Beckstead, Domesick, & Nauta, 1979), hypothalamus (Phillipson, 1979), locus coeruleus (Oades & Halliday, 1987), dorsal raphe (Kalen, Skagerberg, & Lindvall, 1988), and periaqueductal grey (PAG, Kirouac, Li, & Mabrouk, 2004).

As mentioned above, Ikemoto (2007) found that the pVTA predominantly projects to the medial part of the NAc shell and the medial olfactory tubercle; in contrast, the aVTA projects mostly to the NAc core, NAc ventral shell, lateral tubercle, and dorsal striatum. Somewhat inconsistent findings were reported by Lammel et al. (2008) who found by retrograde tracing that the medial VTA projects to mPFC, amygdala, NAc core and medial shell, whereas the lateral VTA (whether posterior or anterior) projects to the NAc lateral shell (Lammel et al, 2008). Yet other groups maintain that the medial VTA projects to the lateral habenula, locus coeruleus and parabrachial nucleus, the dorsal VTA projects to the pregenual aCC, the ventral VTA projects to NAc, septum, amygdala, and supragenual aCC, and the ventroanterior VTA projects to the hippocampus and entorhinal cortex (Swanson, 1982). Also, the anterodorsal VTA is the predominant origin for mesocortical projections, whereas the posteroventral portion of the VTA gives rise to the mesolimbic projections (Fluxe, et al., 1974, Oades & Halliday, 1987). With respect to the mesocortical system, the pregenual cortex receives projections from the medial VTA, the supragenual cortex from the ventrolateral VTA and the perirhinal cortex from the dorsolateral VTA (Lindvall, Bjorklund, & Divac, 1978). Also, it is important to mention that the midVTA is a region that has not yet been investigated as a separate functional subunit of the VTA and its afferent and efferent projections are unknown.

Inasmuch as some of the divergences in these tracing studies can be accounted for by species differences or tracing methods, more research needs to be conducted to asses the anatomical organization of the mesolimbic and mesocortical neurons within the VTA. Also, there is virtually no evidence of cell distribution or afferents/efferents labeling in the midVTA. Nevertheless, these findings indicate that 1) the subpopulations of the neurons that project to a particular region are distributed throughout the VTA on rostro-caudal, ventro-dorsal, and medio-lateral axes, and 2) the mesolimbic efferents tend to cluster more in the posterior than in the anterior VTA, more medially than laterally, whereas the mesocortical efferents display the opposite pattern.

Although the carbachol microinjections in both the present study and the Schifirneț & Borszcz (2007) study were performed in the center of the aVTA and the pVTA, and thus not allowing a finer distinction between medial and lateral aspects of the VTA, it can be speculated that carbachol activated most of the reward-processing clusters of mesoaccumbal neurons within the pVTA and the aVTA, albeit there are less mesoaccumbal rewarding cell populations in the aVTA. If the reinforcing and analgesic neural substrates within the VTA were entirely shared, then the amplitude of the VAD thresholds increases and the magnitude of CPP score following carbachol administration would have varied according to the distribution of the mesoaccumbal reward populations in the pVTA and the aVTA (i.e., a slight decrease in the VAD thresholds and CPP score in the aVTA compared with pVTA). Since there was virtually no difference between the carbachol-induced increase in VAD thresholds and CPP score in the aVTA and the pVTA (Fig. 20) it is concluded that the carbachol-induced affective analgesia and reward when delivered in to the pVTA is mediated primarily by DA release in NAc. On the other hand, the carbachol-induced affective analgesia and reward in the aVTA may rely on DA release in other

terminal structures, presumably mesocortical. Thus, while the rewarding and analgesic effect obtained by cholinergic activation of the pVTA may rely on activation of the mesoaccumbens dopaminergic system, the same effects obtained in the aVTA may rely on the DA release into structures other than NAc, like aCC, insula, or amygdala.

4.2.2. Anterior cingulate cortex (aCC)

The aCC is paramount for the generation of the affective-motivational aspect of the pain experience, but it is not involved in the processing of the sensory-discriminative aspect (Cao et al., 2009; Gao, et al., 2004; Johansen & Fields, 2004; Johansen, Fields, & Manning, 2001; Lei, Sun, Gao, Zhao, & Zhang, 2004; Li et al., 2009; Ren et al., 2006; Sewards & Sewards, 2002; Sun et al., 2008; Treede, Kenshalo, Gracely, & Jones, 1999; Vogt, 2005; Xie, Huo, & Tang, 2009), and its activation is required for the generation of pain unpleasantness (Kulkarni et al., 2005; Rainville, Duncan, Price, Carrier, & Bushnell, 1997; Vogt & Sikes, 2000). Moreover, surgical cingulotomy in humans results in a pain relief that is associated with the attenuation of the pain affect (Hassenbusch, Pillay, & Barnett, 1990; Hurt & Ballantine, 1974; Pillay & Hassenbusch, 1992; Wilson & Chang, 1974). In point of fact, in a striking similarity with the patients that receive morphine for severe pain, the patients with cingulotomies report that the pain is still present, but it does not bother them. The aCC receives DAergic (Oades & Halliday, 1987), GABAergic (Carr & Sesack, 2000) and possibly glutamatergic input from the VTA (Sulzer & Rayport, 2000; Trudeau, 2004). Stimulation of the VTA also inhibits the aCC neurons that respond to noxious stimuli (Mantz, Milla, Glowinski, & Thierry, 1988; Pirot, Glowinski, & Thierry, 1996). Correspondingly, microinjections of DA or DA agonists into the aCC suppress pain behaviors associated with long-term nociception elicited by sciatic denervation in the rat and these antinociceptive effects are blocked by microinjecting DA antagonists into the aCC

(Lopez-Avila, Coffeen, Ortega-Legaspi, del Angel, & Pellicer, 2004). Taken together, these findings suggest that the DAergic projection from the VTA to the aCC is essential for analgesia. Thus, it is possible that the increase in the VAD thresholds following carbachol administration into the aVTA observed in the Schifirneț & Borszcz (2007) study is partially dependent on the DA release into the aCC.

Converging evidence suggests that the aCC is a structure critical also for reward and reinforcement. To the extent that in rat the aCC is part of the PFC (for an extensive review, see, Uylings, Groenewegen, & Kolb, 2003), DA agonists like amphetamine and cocaine increase extracellular DA in rat PFC and cocaine facilitates both PFC and medial forebrain bundle (MFB) self-stimulation (Moody & Frank, 1990); this facilitation of PFC self-stimulation is completely blocked by DA antagonists (McGregor, Atrens, & Jackson, 1992). Similarly, cocaine (Goeders & Smith, 1983) and DA (Goeders & Smith, 1986) self-administrations into the aCC are abolished by microinjecting D_1 DA antagonists or lesioning the DA terminals in this region. In addition, DA antagonists microinjected into the prelimbic portion of medial PFC (mPFC) block the reinforcing effects of i.v. cocaine (McGregor & Roberts, 1995), whereas VTA lesions block cocaine self-administration (Roberts & Koob, 1982). Normally, rats will work harder if the reward is bigger, but not if they are given systemic D2 antagonists or if their DA terminals within aCC are lesioned (Walton et al., 2009). Taken together, these finding suggest that the DA input to aCC is required for drug self-administration and reward processing. Thus, the development of CPP following carbachol administration into the aVTA can be explained, at least partially, by the reinforcing effects of DA release into the aCC.

4.2.3. Insula

Another of the target structures of the VTA involved in pain processing, analgesia, and reward is the insula. Many imaging studies have shown that insular activation is correlated with the conscious, subjective feeling of craving or urge of taking drugs of abuse such as cocaine, heroin, alcohol, and nicotine (for a review of these studies, see Naqvi & Bechara, 2009). Conversely, damage to the insular cortex promotes a "disruption of smoking addiction" in humans (Naqvi, Rudrauf, Damasio, & Bechara, 2007, p. 531), suggesting that this region plays a central role in the initiation of drug urges. Likewise, lidocaine inactivation of the insula abolished the ability of amphetamine to induce CPP learning (Contreras, Ceric, & Torrealba, 2007), bringing further evidence that the "insular cortex is a key structure in the perception of bodily needs that provides direction to motivated behaviors" (p. 655). With respect to reinforcement, both D_1 receptor activation and inactivation in the rat dorsal agranular insular cortex reduce the lever-pressing for cocaine, but only the D_1 blockade results in a significantly reduced amount of cocaine intake in the addicted rat (Di Pietro, Mashhoon, Heaney, Yager, & Kantak, 2008). Interestingly, D_1 receptor blockade also disrupted the food-maintained responding and consumption in the normal rat, suggesting that the DAergic afferents to the insular cortex are important not only for reward processing in a dependent organism, but also for the initiation and maintenance of the motivation to seek natural reinforcers.

Based on the observation that the insula receives ample information from the body and thalamus about interoceptive sensations (e.g., pain, inflammation, temperature, taste, itch, sensual touch, tickle, air hunger, visceral and muscular sensation), Craig (2002) proposed that the primary role of the insula is to create a map of the bodily states that, together with other structures, might constitute the "basis for human awareness of the physical self as a feeling entity" (p. 663). According to this view, the insula is a crucial part of a system that not only

updates the interoceptive maps based on signals from the body when something is changed, but also makes these maps available to awareness (i.e. consciousness). These neural maps are not devoid of meaning, since their continuous updates are necessary for the brain to maintain homeostasis. As such, the insula, along with the amygdala, aCC, and ventromedial PFC, might make available to consciousness emotions associated with imbalances in the bodily states, like "urges", "wantings", "desires", or what Damasio (1994) calls "somatic markers" (also, for a discussion of the somatic-marker hypothesis, see Bechara, Damasio, & Damasio, 2000). With respect to drugs of abuse, the insula is probably a central player in representing the interoceptive effects of these drugs in the form of a neural map (Naqvi & Bechara, 2009). Therefore, when the body is experiencing withdrawal symptoms, the insula translates these interoceptive signals into a conscious, subjective feeling of "urge" to address the imbalance by taking the drug that restores the homeostasis. Naqvi & Bechara (2009) proposed that DA release from the VTA into the insula might contribute to the updates of the neural map from the insula about drug-induced interoceptive changes by bringing information about the hedonic value of the drug use.

The homeostatic role of the insular cortex is also supported by pain research. Pain can be viewed as a homeostatic emotion destined to signal the violation of the integrity of the body (Craig, 2003), and, not surprisingly, insular activation during pain experience is the most frequently reported finding in the imaging studies on pain processing (Apkarian, Bushnell, Treede, & Zubieta, 2005). Moreover, electrical stimulation of the human posterior insula produces pain sensations like stinging, burning or disabling pain in a somatotopic fashion (Ostrowsky et al., 2002) and the subjective intensity of pain is correlated with activation of the insula (Coghill, Sang, Maisog, & Iadarola, 1999; Koyama, McHaffie, Laurienti, & Coghill, 2005). Alternately, patients with insular damage show absent or inadequate emotional responses

to painful stimuli (i.e. pain asymbolia; Berthier, Starkstein, & Leiguarda, 1987; Greenspan, Lee, & Lenz, 1999), underscoring the importance of the insula in processing the affective-motivational aspect of pain.

Of note, the rostral agranular insular cortex (RAIC) is a region that is heavily innervated by DAergic fibers (Jones, Kilpatrick, & Phillipson, 1986; Ohara, et al., 2003) and DA release in the insula is essential for antinociception. Injections of the DA reuptake inhibitor GBR-12935 into the RAIC result in dose-dependant inhibition of the pain behaviors induced by formalin inflammation that is reduced by administration of the selective D_1 receptor antagonist SCH-23390 into the RAIC (Burkey, Carstens, & Jasmin, 1999). Consistent with these results, DAergic stimulation of the insula by intra-RAIC GBR-12935 injections reduced the noxious stimulus-induced *c-fos* expression in nociceptive spinal dorsal horn neurons, as well as the firing of these neurons in response to noxious heat application to the paw, suggesting that the role of DA release into RAIC is to inhibit nociception (Burkey et al., 1999). However, it appears that different DA receptors play different roles in the insula in different pain conditions: the activation of D_2 and the blockade of D_1 elicit antinociception in a neuropathic rat model as measured by a decrease in the autotomy behavior, but the opposite pattern is without effects (Coffeen et al., 2008). These data suggest that the activation of D_1 receptor is pronociceptive in a chronic pain condition (Coffeen et al., 2008), but antinociceptive in acute pain (Burkey et al., 1999), whereas D_2 activation is antinociceptive in the chronic neuropathic pain condition. As opposed to the subcortical D_1 and D_2 receptors (Uchimura, Higashi, & Nishi, 1986, but see Greif, Lin, Liu, & Freedman, 1995), the cortical D_1 and D_2 receptors usually result in an opposite pattern of postsynaptic membrane polarization (i.e., depolarization vs. hyperpolarization; Godbout, Mantz, Pirot, Glowinski, & Thierry, 1991; Parfitt, Gratton, & Bickford-Wimer, 1990);

therefore, it is conceivable that inhibition of insula neurons by DA D_2 is conducive to analgesia, whereas activation by DA D_1 is pronociceptive.

Whereas it is evident that intra-insular DA is required both for reward processing and analgesia, more research is necessary to disentangle the roles of different DA receptors in the insula, the downstream synaptic events, and the extent of the involvement of insular DA in all these processes.

4.2.4. Amygdala

The amygdala is an almond-shaped structure in the medial temporal lobe containing at least twelve subdivisions (LeDoux, 2000) and has bidirectional connections with the VTA (for amygdalar efferents, see Fudge & Haber, 2000; for VTA efferents, see Swanson, 1982). DAergic afferents to the amygdala arise from the substantia nigra and VTA and project via the medial forebrain bundle (de la Mora, Gallegos-Cari, Arizmendi-Garcia, Marcellino, & Fuxe, 2009). Since the 1950s, a considerable amount of research has been published on the involvement of the amygdaloid complex in negative affect, particularly fear and aggression (e.g. Fernandez De Molina & Hunsperger, 1959; LeDoux, 2000).

Exposure to a painful stimulus is obviously a threatening event, and therefore it is not surprising that the amygdala is involved in the processing and modulation of pain[7]. Bilateral lesions of the rat amygdala reduce emotional pain reactions such as ultrasonic vocalizations to an

[7] It is important to stress that the amygdalar involvement in aversive and noxious processing is complex and not uniformly distributed across all the amygdalar nuclei (for a review of each amygdalar nucleus involvement in pain, see Neugebauer, Li, Bird, & Han, 2004). Moreover, even within a particular amygdalar nucleus there are regional differences with respect to aversive processing. For example, microinjecting DA or the DA agonist bromocriptine into the posterior, but not anterior, BLA "dose-dependently attenuated cold restraint stress (3 h at 4°C)-induced gastric ulcer formation in rats" (Ray & Henke, 1991, p.786). There are also sex and lateralization differences in the amygdalar involvement in aversive and noxious processing, with females, but not males, showing increased DA release in BLA in a restraint stress paradigm (Mitsushima, Yamada, Takase, Funabashi, & Kimura, 2006), and with right amygdala being more involved in an chronic arthritic pain paradigm in rats that the left amygdala (Ji & Neugebauer, 2009).

electric shock (Goldstein, Rasmusson, Bunney, & Roth, 1996) or shock-induced hyperalgesia (Crown, King, Meagher, & Grau, 2000). Furthermore, bilateral lesions of the amygdala reduce the antinociceptive action of morphine in the rat and cannabinoids in rhesus monkeys in a warm-water tail-withdrawal assay (Manning, Merin, Meng, & Amaral, 2001) and dramatically increase the vocalization thresholds in the rat tail-flick test, but leave the tail withdrawal latencies unaltered (Calvino, Levesque, & Besson, 1982). In agreement with this, Borszcz & Leaton (2003) found that electrolytic lesions of the central amygdala (CeA) of rats preferentially increased the tailshock threshold to elicit VADs, leaving VDS and SMR thresholds intact. These investigators also reported the CeA lesions blocked the capacity of tailshock to support Pavlovian fear conditioning. To the extent that the VAD threshold elevation reflects suppression of the affective dimension of pain, microinjections of morphine into the basolateral amygdala (BLA) result in a dose-dependent preferential increase in VAD threshold and this effect is reversed by the administration of the opiate receptor antagonist methylnaloxonium into the BLA (Nandigama & Borszcz, 2003).

The involvement of amygdala in pain affect is further confirmed by imaging studies in humans, where it has been found that the amygdala activation corresponds with the subjective perception of thermal painful stimuli, but not with intensity of non-painful thermal stimuli (Bornhovd et al., 2002). In line with this, several imaging experiments with humans involving placebo analgesia paradigms have shown that amygdalar activation is correlated with placebo responses (Bingel, Lorenz, Schoell, Weiller, & Büchel, 2006; Craggs, Price, Perlstein, Verne, & Robinson, 2008; Wager, Scott, & Zubieta, 2007). To the extent that the placebo effect engages the neural circuitry that subserves the affective-motivational dimension of pain (for a review, see Zubieta & Stohler, 2009), amygdalar activation in placebo conditions is preferentially correlated

with suppression of the affective–motivational and not the sensory-discriminative dimensions of pain.

Painful stimuli result in DAergic activation of the amygdala as microdialysis studies showed that electric footshock or stimuli paired with electrical shocks increases DA release in the rat amygdala (Herman et al., 1982; Young & Rees, 1998). Also, chronic inflammatory pain in rodents induces increase in DA in the amygdala, as detected with high-performance liquid chromatography (HPLC) (Neugebauer, Galhardo, Maione, & Mackey, 2009). Additionally, aversive electrical stimulation of the inferior colliculus increases the DA and serotonin release in BLA, but not CeA (Macedo, Martinez, de Souza Silva, & Brandao, 2005). These findings suggest that aversive stimulation, whether painful or not, conditioned or unconditioned, results in DA release in amygdala.

Unfortunately, the role of amygdalar DA with respect to pain affect and analgesia is somewhat unclear, given the paucity of studies that conducted direct DAergic manipulations within the amygdala coupled with pain paradigms. More research into DA involvement in pain affect in amygdala is clearly warranted.

Nevertheless, there is indirect evidence that might shed a little light on the role of DA in amygdala. From a physiological point of view, opioid administration results in decreased amygdalar excitation (for a review of opioid receptor function, see Simonds, 1988). As opposed to morphine, DA has mainly excitatory effects in the amygdala[8]. Thus, if inactivation of the amygdala by lesions or opioids results in affective analgesia, as suggested by the findings

[8] Similar to the cingulate and insular cortices, DA in amygdala can have complex actions: the total output of amygdala can be decreased on increased by DA, depending whether it excites directly the projection neurons, or it inhibits them indirectly via GABAergic interneuron activation, as revealed by in vitro patch-clamp recordings in rodents (Bissiere, Humeau, & Luthi, 2003; Kroner, Rosenkranz, Grace, & Barrionuevo, 2005). Moreover, the DA local action in amygdala can be excitatory or inhibitory, depending whether it binds to the D1 receptors or D2 autoreceptors, respectively. Furthermore, DA can excite or inhibit local GABA interneurons in BLA (Marowsky, Yanagawa, Obata, & Vogt, 2005).

presented above, it would be plausible to assume that amygdalar excitation by DA will augment pain behaviors, or, at least, would not result in analgesia. If this were the case, then less DA into the amygdala would correlate with higher pain thresholds. But, some evidence suggests that rodents with amygdalar DA depletion produced either by intra-amygdala 6-OHDA injections (which results in selective catecholamine depletion, Ashford & Jones, 1976) or by a COMT knockout (Kambur et al., 2008) have the same baseline nociceptive thresholds as controls, as measured by response latencies in pain tests such as tail-flick, hot-plate or foot-shock. These inconsistencies might be reconciled if the role of amygdala in processing pain in general is considered.

With the caveat that there are differences in pain paradigms, experimental conditions and behavioral variables which still need to be addressed, it is generally believed that the lateral and basolateral nuclei attach emotional significance to noxious sensory information (i.e. pain affect), which is then transmitted to the CeA, which, in turn, can send projections to the descending pain control structures in the brainstem (e.g. PAG, rostroventral medulla - RVM) and thus modulates pain behavior (Neugebauer, et al., 2009)[9]. The BLA is under tight regulatory control from mPFC and DA in BLA has dual action: lifts the inhibition from the amygdalar projecting neurons that are under tight mPFC inhibition and augments the sensory signal from cortical areas (Grace & Rosenkranz, 2002; Rosenkranz & Grace, 1999, 2001, 2002a, b). Since DA enhances the sensory inputs to BLA, it is proposed here that DA in the BLA also augments the processing of a noxious stimulus by increasing the firing rate of the BLA neurons that project to CeA.

[9] This view is consistent with the fear and anxiety research that suggests that the lateral and basolateral nuclei together are a sensory interface where CS-US associations are made during aversive conditioning, whereas CeA is the effector system that initiates the autonomic, endocrine and behavioral reactions to the aversive stimulus (LeDoux, 2000)

The CeA, which also receives DAergic projections that acts as modulator of the local synapses (de la Mora et al., 2009), has dual action with respect to pain; it has been shown that CeA manipulations (lesions or electrical stimulations) can enhance pain and can inhibit pain, by activating or inhibiting, respectively, the neurons from the PAG that are part of the endogenous descending pain modulatory system, depending on the negative affective state of the organism (Neugebauer, et al., 2004). In other words, affective states like anxiety (that can be elicited by the threat of an electric shock) do not engage the endogenous descending pain modulatory system, but fear (elicited by exposure to three brief shocks) does so (Rhudy & Meagher, 2000), presumably via differential CeA regulation of the PAG neurons. Also, stress induced analgesia or hyperalgesia are well documented phenomena (for reviews of stress-induced analgesia, see Butler & Finn, 2009, of stress-induced hyperalgesia, see Imbe, Iwai-Liao, & Senba, 2006). Without going into detail of how fear and anxiety are produced, the main difference between these states is the subjective emotional intensity. Therefore, it is conceivable that there is a threshold after which the CeA initiates the endogenous pain suppression and DA is modulating this threshold. By increasing firing rate in the BLA and potentiating the noxious sensory signals, DA "forces" the CeA to initiate or not the endogenous opioid system, depending on the affective encoding done in the BLA. Whether or not the CeA would initiate the opioid system depends on the affective coloration assigned by the BLA to the noxious stimulus, on the affective state of the organism prior to the noxious stimulation, and on the type of pain. Indeed, not all types of pain trigger the endogenous opioid system; endogenous opioid system comes into play with prolong, but not acute stimuli (Watkins & Mayer, 1986).

In terms of pain affect, if DA increase in the BLA results in amygdalar disinhibition, then this would facilitate affective behaviors. The finding that intra-amygdalar DA microinjections

attenuates morphine analgesia in a foot-shock test (Rodgers, 1977) appears contradictory with the DAergic role proposed here, but it is not, as this test measured pain behaviors that are organized at the spinal level. Also, this author injected DA into the cortico-medial amygdala, and therefore there are regional differences between amygdalar nuclei to be considered. To the extent that BLA attaches emotional significance to the painful stimulus, then it is conceivable that DA manipulations in this region would not change the reflex latencies, but it would change the emotional coloration of the stimulus. In other words, intra-amygdalar DA would not affect the sensory dimension of pain, but the affective aspect, the latter of which is not being captured by measures like reflex latencies. The same rationale would apply to the lack of changes in nociceptive threshold found with intra-amygdalar DA depletion described above. In summary, the evidence reviewed above suggests that DA in the amygdala serves a modulatory role, facilitating adaptive behavioral responses to painful stimuli. Thus, the carbachol-induced affective analgesia obtained in the Schifirneț & Borszcz (2007) study in the aVTA could be partially mediated by DA release in the BLA or CeA. To test this hypothesis, further studies should challenge directly the intra-VTA carbachol induced affective analgesia by microinjecting DA antagonists in BLA and CeA, respectively.

Amygdalar nuclei are also involved in reward processing, as electrophysiological recordings from monkey amygdala revealed that at least 35% of amygdalar neurons respond to food reward consumption exclusively, with the other neurons responding to either bar pressing for food or to the tone or light that had been associated with the reward (Nakano et al., 1987). It appears that the involvement of the amygdala in reward processing relates to reward-related learning, as lesions of the rat the BLA block the ability of cocaine-associated cues to lower the threshold for ICSS (Hayes & Gardner, 2004) and impair different aspects of instrumental

(Balleine, Killcross, & Dickinson, 2003) and Pavlovian (Hatfield, Han, Conley, Gallagher, & Holland, 1996) conditioning.

In agreement with this, DA release in the amygdala is required for the formation of different types of reward-related learning, as rats who received DA receptor antagonists into the BLA fail to engage in cocaine seeking behavior under a second-order schedule of reinforcement (Di Ciano & Everitt, 2004). Of note, DA D_1 receptor blockade in the BLA significantly disrupts the conditioned reinstatement of cocaine self-administration (Alleweireldt, Hobbs, Taylor, & Neisewander, 2006), but it does not affect cocaine self-administration itself (See, Kruzich, & Grimm, 2001), suggesting that the role of DA in the BLA is to regulate reward-related associative learning, and not the basic incentive value of the reward (for an alternative view, see Hitchcott & Phillips, 1998a, 1998c). In addition, blockade of D_3 receptors in the amygdala results in impaired Pavlovian conditioning to both natural (sucrose) and drug rewards (amphetamine) (Hitchcott & Phillips, 1998b). Therefore, it is apparent that DA in the amygdala, at least in the BLA, is required for reward-related learning, whether Pavlovian or operant, and the ability of carbachol to support development of CPP in the present study when delivered into the aVTA might be mediated also by DA release in amygdalar nuclei.

4.2.5. Other Terminal Sites

The involvement of VTA DA release in analgesia and reward processing within in NAc, aCC, insula, and amygdala might explain the carbachol-induced affective analgesia and reward in aVTA and pVTA, but does not fully account for the fact that there is a subpopulation of muscarinically activated AND nicotinically inhibited neurons within the pVTA that is involved in affective analgesia, but not in reward. One possible explanation is that these neurons project to terminal sites that process analgesic, but not reward-related information.

One of these putative sites is the PAG, to which the VTA sends both DAergic and GABAergic projections (Kirouac et al., 2004). Whereas there are no reports that the PAG plays a significant role in reward processing, there is ample evidence for the involvement of the PAG in pain modulation and pain affect (Dostrovsky & Deakin, 1977; Guimarães, Guimarães, & Prado, 2000; Heinricher, Cheng, & Fields, 1987; Vaccarino, Clemmons, Mader, & Magnusson, 1997). The PAG contributes to analgesia by activating both ascending projections to the forebrain and thalamic sites essential for the production of the affective dimension of pain and descending projections to the rostral ventromedial medulla that result in the suppression of pain transmission at the level of spinal cord (Behbehani, 1995; Borszcz, 1995, 1999). For example, the affective analgesia obtained after morphine microinjections into the nucleus parafascicularis thalami (nPf) is blocked by muscimol ($GABA_A$ agonist) injections in the ventrolateral PAG (Munn et al., 2009). Alternately, the analgesia induced by morphine injections in the ventral PAG is dose-dependently reduced by administration of methysergide (5-HT antagonist) in the CeA or nPf (Borszcz & Streltsov, 2000).

These results suggest that there is a functional interaction between PAG, CeA and nPf in modulating the affective dimension of pain. Thus, it is conceivable that the analgesic, but not-rewarding effect of muscarinically activated and nicotinically inhibited neurons within the pVTA is possibly mediated by their projections to PAG, a site paramount for antinociception, but not for reward-related processing.

4.3. The Role of the VTA DA in Analgesia and Reward

4.3.1. Reinforcement and Reward: ***The Masking Hypothesis***

The data presented above emphasizes the role of cholinergically-induced DA release from the VTA in affective analgesia and reward. But the question of how is DA exactly

producing analgesia and reward in each of the structures discussed above is still unanswered. Namely, is DA producing analgesia by suppressing the pain transduction and thus disrupting the pain circuitry? Or is the activation of the VTA DA inducing a positive affective state that is superimposed on the negative affective state produced by pain and thus attenuating the pain experience? In an attempt to answer these questions, in the following section I will discuss some of the most prominent hypotheses regarding the role of DA in general and then, based on the available evidence, provide a framework for understanding the role of DA in pain, analgesia, and reward in the context of DA function.

4.3.1.1. Nucleus Accumbens Evidence

Since its discovery as a neurotransmitter and not just a precursor of norepinephrine and epinephrine in 1957 (Carlsson, Lindqvist, & Magnusson, 1957) for which Carlsson received the Nobel prize, DA is incontestably the molecule for which more hypotheses have beet put forward than for any other neurotransmitter. There has been so much research of DA and DA function(s) in addiction, for example, that this neurotransmitter achieved the status of celebrity both in the scientific world and in the popular media as the 'pleasure molecule' (Marsden, 2006). As DA has definite roles in Parkinson disease, schizophrenia, pair-bonding, cardiovascular regulation, kidney function and others, only a few of the hypotheses - regarding reward and reinforcement - are briefly discussed in the following pages, as they are the most relevant to pain and analgesia. Out of these, the most known hypothesis (and oldest) is usually referred to as the hedonic hypothesis of DA or simply the reward hypothesis[10].

[10] Proposed initially by Wise (Wise, 1982; Wise, Spindler, deWit, & Gerberg, 1978), the anhedonia hypothesis has been refined and enlarged to include as separate subdivisions the DA involvement in reinforcement, reward, incentive motivation, and hedonia (see Wise, 2004, 2008). Here are highlighted only the major concepts that received the most empirical support.

According to the reward hypothesis, the role of DA in appetitive reinforcement is thought to convey the reward signal itself (Wise, 1996) and when this signal is blocked (e.g. by DA antagonists), the "goodness" of the stimuli is blunted:

> *In introspective language we would say that neuroleptics [that disrupt the midbrain dopamine system] appear to take the pleasure out of normally rewarding brain stimulation, take the euphoria out of normally rewarding amphetamine, and take the "goodness" out of normally rewarding food* (Wise, Spindler, deWit, & Gerberg, 1978, p. 263).

As seen in Chapter 1.2., this hypothesis received substantial support from studies that assessed both the reinforcing and the rewarding effects of DA agonists. Drugs of abuse like opioids and psychostimulants act as DA agonists as they increase the DA availability in the brain. Such drugs have high abuse potential, are self-administered in both animals and humans, and as outlined in Chapter 1.2., they are also potent analgesics. Based on this observation, as described in Chapter 1.3., Altier and Stewart (1999a) proposed that these drugs act as analgesics because they shift the motivational state from a negative affective state, such as that produced by a painful stimulus, to a normal affective state by promoting the DA availability into the NAc. On the other hand, these drugs achieve addictive liability when administered in a normal affective state, because they shift the motivational continuum from a neutral affective state towards a positive affective state, by promoting DA availability into the same site, i.e. NAc (Fig. 1).

Of course, one can find many caveats with the accumbal DA hypothesis of reward. For instance, Ikemoto (2007) pointed out that the pleasure felt during anticipation of reward is different (and thus probably subserved by different neural mechanisms) from the sensory

pleasure of the consumption of reward. Whether DA is necessary or is merely modulating these two types of pleasure it is still unclear. Another problem stems from the techniques employed to asses the role of DA. To mention only one example, the CPP paradigm is often used for measuring reinforcement of a particular drug, even though CPP is a form of Pavlovian learning, and not operant, thus being more suitable for reward measurement (Wise, 1996; Wise & Rompre, 1989).

Nonetheless, the reward hypothesis has dominated DA research for the past 25 years and the fact the DA release in NAc is associated with at least some form of a positive state is a well-documented phenomenon. Corroborating this with the finding that DA release/availability in NAc is also associated with analgesia, it seems plausible that the way in which mesoaccumbal DA mediates affective analgesia in the present study is by producing a positive affective state that shifts the motivational continuum towards the middle, as Altier & Stewart (1999a) suggested. In doing so, accumbal DA effectively *masks* the negative affect produced by pain and shifts the motivational continuum to a more positive affective state that would allow the organism to more effectively ignore the pain and engage in adaptive behaviors destined to escape the noxious stimulus and/or to avoid further injury.

4.3.2. Salience: ***The Pain Transmission Suppression/Facilitation Hypothesis***

However, the DA theory of reward is incomplete, as it cannot account for the fact that reward without DA is possible. In a series of experiments with DA-deficient mice created by inactivating the tyrosine hydroxylase gene, the Palmiter group (Zhou & Palmiter, 1995) found that these mice will die of starvation if DA is not restored to the striatum because they do not approach food placed literally in front of their noses (Hnasko, Szczypka, Alaynick, During, & Palmiter, 2004) or they eat such insignificant amounts of food that are not enough to keep them

alive (Szczypka et al., 1999), but they still prefer sucrose over water (Cannon & Palmiter, 2003). Additionally, neurochemical 6-OHDA lesions of the NAc do not disrupt the 'liking' of sweet solutions, as assessed by the taste reactivity test (i.e., observing the evolutionarily conserved affective reactions of rats to sucrose, Berridge & Robinson, 1998). With respect to drugs of abuse, the DA-depleted mice display robust CPP with morphine (Hnasko, Sotak, & Palmiter, 2005) and cocaine (Hnasko, Sotak, & Palmiter, 2007), suggesting that the ability of these drugs to induce a positive affect must either 1) not rely on DA or 2) relies on DA, but in the absence of it, a compensatory mechanism mimics the DA action. Because fluoxetine, a serotonin transporter blocker, induces CPP in the DA-depleted animals, but not in the control animals (Hnasko et al. 2007), it seems that the second explanation is more plausible.

Moreover, the reward hypothesis of DA function predicts that the A10 neurons should be inhibited, or, at least, should not fire in the presence of an aversive stimulus. If DA signals to the NAc carry a positive affect and fire only during presentation or expectation of rewards, then one would expect the DA neurons to be silent during aversive stimulation. Indeed, an experiment employing *in vivo* electrophysiological recordings has shown that DA neurons within VTA are uniformly inhibited during foot pinch of anaesthetized rats whereas the non-DAergic neurons are activated (Ungless et al., 2004). However, as shown in Table 1, there is considerable evidence that VTA DA neurons are active during aversive stimulation like tail pinch (Smith et al., 1997) and this activation is associated with increased accumbal DA (Young, 2004). In light of this evidence, the same group that found that DA neurons are uniformly inhibitive during noxious stimulation, repeated their previous experiments and found that dorsal VTA (PBN) does not respond to noxious stimulation (consistent with the reward hypothesis), but ventral VTA (PN) shows phasic activation by electric footshocks (Brischoux, Chakraborty, Brierley, & Ungless,

2009). Thus, there are at least two different populations of neurons within the VTA that have different firing patterns to negatively or positively valenced stimuli.

As a consequence of these and similar findings, the incentive salience hypothesis of DA has been put forward, initially by Berridge and Robinson (Berridge & Robinson, 1998; Robinson & Berridge, 1993, 2003), and then refined and expanded by others. According to this hypothesis, the DA signal does not carry the hedonic value of a stimulus (the 'liking'), rather it carries a different component of reward, the 'wanting'. In other words, during the process of Pavlovian learning, DA mediates the transformation of the neutral representation of the conditioned stimulus into an attractive and 'wanted' incentive that 'grabs attention', thus the reinforcing stimuli acquire incentive motivational properties. For example, both a light that will predict an electric shock and the sight of palatable food will increase DA release, because both stimuli need to 'grab the attention' of the animal as they are salient events for the organism. However, additional data suggest that the incentive motivational role of DA extends beyond conditioned stimuli. Supporting this idea, data from electrophysiology and microdialysis studies have shown that a wide range of salient unconditioned stimuli like pain, loud tones, bright lights, and novel environments increase the firing of DA neurons (for a review of these studies, see Horvitz, 2000).

The incentive-salience theory and its derivatives shift the focus of DA neurons as reward detectors to a broader role as high relevance for behavior or salient stimuli detectors (Salamone, Correa, Mingote, & Weber, 2005). With respect to aversive stimulation, this theory implies that VTA neurons are involved in processing both aversive and appetitive stimuli, as long as they display incentive salience properties.

Thus, the VTA DA signals a *change* in the environment that is of importance for the animal. This signal is not devoid of meaning (of which the animal might be aware of or not) because a salient stimulus or event has motivational properties; it requires the organism to be ready for a potential change in the environment and therefore be able to engage in a *goal-directed behavior* that is responsive to that environmental change. Therefore is not surprising that a painful stimulus, which is a change in the environment that has incentive-motivational properties, results in massive DA release in various brain structures.

4.3.2.1. Anterior Cingulate Cortex Evidence.

Pain is a complex experience that captures attention, it is a salient event, and requires "alerting and orienting to the potentially threatening stimulus, evaluating and anticipating the threat and executing an appropriate escape response, as well as learning and memory to avoid future encounters" (Hutchison, Davis, Lozano, Tasker, & Dostrovsky, 1999, p. 404).

The aCC is a structure implicated in performance monitoring and error detection (Bechtereva, Shemyakina, Starchenko, Danko, & Medvedev, 2005) and attentional processes (for a review, see Raz & Buhle, 2006), and its role in attention is to "focus greater attention on behaviorally relevant stimuli to limit the processing of distracting events" (Weissman, Gopalakrishnan, Hazlett, & Woldorff, 2005, p. 229). In addition, the evidence presented in Chapter 4.2.2. outlines 1) the role of aCC in generating the affective dimension of pain, 2) the role of DA release in aCC in producing affective analgesia, and 3) the role of DA release in aCC in reward and drug self-administration.

Therefore, it is proposed here, and in concordance with the motivational-incentive hypothesis of DA function, that the role of DA in aCC is to *prevent* the generation of the affective dimension of pain in order to shift attention to goal-directed behaviors that would result

in an escape from the painful stimulus. Thus, the saliency signal is effectively transformed into a pain suppression signal in the aCC enabling generation of behaviors more important for the organism, like escaping, attending to the injury, or preventing further injury. Pain is unquestionably a salient event, but perhaps the need, the 'want' to escape pain and its causes is more salient and it is possible that this information is what VTA conveys to the aCC.

Likewise, during drug-self administration, the DA signal of the aCC could carry incentive-motivational information relevant for orienting/shifting attention, error detection, and/or performance monitoring destined to prepare the organism for goal-directed behavior. Indeed, one line of evidence links the VTA DA neuronal firing more to the expectation of rewards rather than the hedonic value of the stimulus, particularly in the neocortical regions (Schultz, 2002). Therefore, the fact that intra-aCC DA antagonists block diverse aspects of self-administration and reinforcing properties of drugs of abuse might not necessarily reflect a reduction of the hedonic value of the stimulus carried by the DA release in the aCC, but rather a reward expectation signal that is disrupted (i.e. a salient event is about to happen).

4.3.2.2. Insula Evidence.

It is plausible that a saliency signal is generated not only in response to relevant external events, but also to the events that change the internal equilibrium of the organism. As both external events like a painful stimulus and drugs of abuse result in interoceptive changes, the insular cortex plays a central role in creating and updating a neural map of these bodily changes that serve the purpose of maintaining homeostasis. It is believed that the insular cortex, along with other structures, is a part of a circuit that attaches emotional valence to these interoceptive changes. Indeed, there is evidence that the activity of the insula is strongly associated with some

of the feelings associated with interoceptive changes like disgust (Wicker et al., 2003), craving (Contreras et al., 2007), and the unpleasantness of pain (Greenspan et al., 1999).

As outlined in Chapter 4.2.3, the DA input to the insular cortex is essential for both analgesia and reward processing. Integrating the data presented above and taking into account the general homeostatic role of insula in reward and analgesia, it is hypothesized here that the DA signal to the insular cortex brings information vital to the updating the neural map of the current state of the organism with the purpose of restoring homeostasis. This hypothesis is consistent with the incentive-motivational role of DA.

Correspondingly, the analgesia that follows DAergic activation of the insula might be the result of a process of pain transmission suppression, a process similar to the prevention of the generation of the affective dimension of pain in the aCC. Indeed, both insula and aCC are involved in the generation of the affective dimension of pain, although the unpleasantness of pain serves different purposes in these two areas: whereas insula integrates different signals from the body, attaches emotional valence to them *and* makes these feeling available to consciousness, the aCC supplements the motor aspect of motivations to emotional stimuli (volition, agency) (Craig, 2009a, 2009b). Therefore, DA in insula IF it binds to D_2 receptors (see Chapter 4.1.2.) might prevent the pain unpleasantness from either being generated or from being made available to awareness. In either case, the behavioral result is affective analgesia. On the other hand, IF DA binds to the D_1 receptors, then the result could be a heightened signal regarding the saliency of the pain stimulus, thus facilitating the insular cortex in the generation of the conscious feeling of unpleasantness. It would be interesting to investigate the circumstances under which D_1 or D_2 receptors are activated.

4.3.2.3. Amygdala Evidence

The modulatory role of the DA saliency signal is even more apparent in the amygdala. As mentioned above, the BLA and the CeA nuclei have distinct roles in pain processing: the BLA attaches negative emotional valence to stimuli, whereas the CeA is the effector, it "acts" on the input provided by BLA. This type of connection parallels the relationship between insular and cingulate cortices. However, the DA input to amygdala, whereas it still brings a saliency signal, has a different role than the input to the two cortical areas. Namely, the DA release into the BLA augments the pain signal, consistent with the salience-motivational hypothesis of DA function. It is proposed that this augmentation facilitates adaptive responses to the painful stimuli.

Electrophysiological recordings from CeA during application of DA in a painful setting have not yet been conducted, but the available evidence summarized in Chapter 4.2.4. suggests that DA is capable to modulate the CeA output during pain. As a consequence, the CeA can either activate or inhibit the endogenous opioids release, as CeA projects to and modulates the descending pain modulatory system subserved by PAG and RVM (Neugebauer et al., 2009). At *prima facie*, it would seem paradoxical that organisms do not make use of the endogenous opioid system every time a painful stimulus occurs, regardless of type or situation. And yet, one can think of a situation when an augmentation of the painful stimulus (like chronic pain, inflammation) (read amygdalar activation and inhibition of the endogenous opioids) would be adaptive, because it forces the organism to pay attention and take action, thus avoiding further injury. On the other hand, "in life-threatening situations (actual or perceived), when survival demands 'fight or flight'-like decisions, the amygdala acts to suppress attention to pain as a less important but possibly distracting factor to guarantee survival" (Neugebauer, 2007, p. 2).

Perhaps DA in amygdala provides a gate for aversive stimuli enabling a switch between starting on and shutting off the endogenous opioid system.

With respect to reward processing within amygdala, it is possible that the disruption of different reward-related learning tasks by DA antagonists reflects the absence of a signal that conveys the incentive-motivational value of a rewarding stimulus. Amygdala would require such a signal to perform adequate reward-related learning with the purpose of adaptive goal-directed behavior. Indeed, the impairments seen in cocaine seeking behavior (Di Ciano & Everitt, 2004) or conditioned reinstatement of cocaine self-administration behavior (Alleweireldt et al., 2006) after intra-BLA microinjections of DA antagonists might reflect the disruption of a motivational signal destined to facilitate reward-related learning phenomena.

CHAPTER 5. CONCLUDING REMARKS

In summary, the present study is the first to systematically evaluate and compare the extent of the overlap between the neuronal circuits underlying reward and affective analgesia by investigating the participation of the cholinergically activated DA release from three VTA subregions in reward and affective analgesia. Additionally, by analyzing the two major theories of DA function – the reward theory and the salience theory -, it is hypothesized here that VTA DA plays different roles in reward and analgesia, depending on the terminal region.

In the NAc, VTA DA is not interfering directly with the pain processing system, but it induces affective analgesia by producing a positive affective state that is superimposed on the negative affective state produced by pain and thus masking pain affect. In other words, both the unpleasantness of pain and the pleasant feeling generated by the DA release in NAc are simultaneously processed in the brain, but they compete for what economists and computational theorists termed resource allocation. Competition between opposing motivational system has long been hypothesized by psychological theories such as the Opponent Process Theory proposed by Solomon & Corbit (1974) and supported by Koob & Le Moal (2008) or the Motivation-Decision Model proposed initially by Fields (2007) and refined later by Leknes & Tracey (2008). By themselves, both pain and reward are powerful motivational states that result in a learning signal to either avoid or approach, respectively, the environmental stimulus or situation that caused them. This "teaching signal" (Fields, 2004, p. 571) is used by other brain regions (presumably cortical) for decision-making processes and goal-directed behaviors destined to keep the actions of the organism adaptable and coherent. When both these powerful teaching signals occur together, pleasure and unpleasantness compete for processing resources, because cortical areas like the aCC and PFC must act based on one signal, but not the other,

since approach and avoidance are behavioral actions that cannot be performed simultaneously. The positive affect generated by DA release in NAc during a painful state competes with the negative affect generated by the noxious stimulus, thus masking the pain signal and shifting the motivational balance toward a more positive affective state. This idea is concordant both with the motivational continuum theory proposed by Altier & Stewart (1999a) and with the reward hypothesis of DA function.

On the other hand, DA release in other brain regions effectively suppresses or facilitates the pain signal, concordant with the incentive-motivational theory of DA function. Specifically, DA release in the aCC and in the insula (when binding to D_2 receptors) suppresses the pain transmission circuitry, but DA release in the amygdala (particularly the BLA) and in the insula (when binding to D_1 receptors) facilitates the pain signal. Presumably, DA release in these regions increases the saliency of either the pain signal or the need to escape pain, depending on which is more adaptive for the organism at a given time. Thus, the salient signal carried by DA can serve as 1) a suppressor of the generation of the affective dimension of pain in the aCC in order to shift attention to goal-directed behaviors designed to escape injury, 2) a suppressor or facilitator of the pain signal in insula in order to enable behaviors designed to achieve homeostasis, and 3) a modulator of the endogenous descending pain modulatory system in amygdala in order to enable adaptive responses to pain.

In summary, when DA reaches its terminal regions, it seems that it is differently utilized by these structures. Thus, the reward and salience hypotheses are not incongruent with each other; rather they are complementary theories.

In conclusion, major challenges remain, not the least of which is the understanding of the production and suppression of the affective dimension of pain. However, it is apparent that the

brain's 'pleasure molecule' plays a significant role in the modulation of the affective reaction to pain. The obvious importance of the pain affect to the pain experience and the necessity of finding a potent analgesic that lacks abuse potential clearly warrant further studies.

APPENDIX A. TABLES

Table 1

VTA-NAc pathway involvement in reward and reinforcement

Species	Behavioral paradigm	Method	Main Results	Reference
Humans	Oral intake of alcohol or orange juice 30 min prior to tracer injection	PET with [^{11}C] raclopride (D_2 radioligand), MRI	Radiotracer binding potential was reduced bilaterally in the NAc in the alcohol condition compared to the orange juice condition, indicative of increased extracellular DA. The magnitude of the change in radiotracer binding correlated with the alcohol-induced increase in heart rate, which is thought to be a marker of the psychostimulant effects of the drug.	Boileau et al., 2003
Rats	CPP	Intra-VTA morphine	Morphine supports CPP learning in the VTA, but not in the adjacent areas.	Bozarth, 1987
Humans	Smoking inside the scanner	PET with D2 radiotracer, genotyping	Smokers with genes associated with low resting DA tone have greater smoking-induced (phasic) DA release into ventral caudate/NAc during smoking than those with alternate genotypes.	Brody et al., 2006
Rats	ICSS of MFB	E-PHYS, FSCV	ICSS elicited DA release in the NAc and produced coincident time-locked changes (predominantly inhibitions) in the activity of a subset of NAc neurons. Similar responses were elicited with noncontingent stimulations. The changes in firing rate induced by noncontingent stimulations were reversed by the $GABA_A$ receptor antagonist bicuculline.	Cheer, Heien, Garris, Carelli, & Wightman, 2005
Rats	i.v. CB agonists and antagonists	FSCV	The CB agonist produced dose-dependently increases extracellular NAC DA and this is manifested as an increase in the frequency and amplitude of rapid DA transients in the NAc. These effects are reversed by a CB1 antagonist.	Cheer, Wassum, Heien, Phillips, Wightman, 2004
Starved rats	Instrumental learning for food pellets	microdialysis	The rats that learned the task showed significantly higher increases in NAc DA than rats that did not learn the task in the first session. The NAc DA increase was similar in both learning groups in the second session.	Cheng & Feenstra, 2006
Rats	i.p. or s.c. drug administration	microdialysis	Drugs abused by humans (e.g., opiates, ethanol, nicotine, amphetamine, and cocaine) increased extracellular DA in NAc. Drugs with aversive properties (e.g., agonists of K opioid receptors, U-50,488, tifluadom, and bremazocine) reduced NAc DA. Drugs not abused by humans [e.g., imipramine (an antidepressant), atropine (an antimuscarinic drug), and diphenhydramine (an antihistamine)] failed to	Di Chiara & Imperato, 1988

			modify synaptic DA concentrations.	
Humans	i.v. amphetamine	PET with [^{11}C] raclopride (D2 radioligand)	The magnitude of NAc DA release and binding correlates positively with the hedonic (euphoric) response to amphetamine.	Drevets et al., 2001
Rats	ICSS of VTA	microdialysis, HPLC	Increases in extracellular NAc DA were positively correlated with the rate of ICSS.	Fiorino et al., 1993
Humans	Instrumental tasks for amphetamine	NAc cannulation	Rats will perform different variants of instrumental tasks in order to receive direct intra-NAc injections of amphetamine.	Hoebel, Monaco, Hernandez, Aulisi, Stanley, et al, 1983
Rats	Cocaine self-administration	FSCV	NAc DA levels increased during perception of cocaine-associated cues, during lever-pressing for cocaine, and during consumption of cocaine. Remarkably, these behaviors could be elicited by electrically evoking NAc DA release from the VTA.	Phillips et al., 2003
Humans	i.v. cocaine	PET with [^{11}C] cocaine (DAT) radioligand	Cocaine at doses commonly abused by humans blocked between 60-77% of DAT in the dorsal striatum (whose DAT response to cocaine is similar to NAc). This occupancy was positively correlated with the subjective reports of high and rush. The DAT occupancy must be greater than 47% for cocaine users to subjectively perceive cocaine as rewarding.	Volkow et al., 1997
Rats	ICSS of VTA/MFB	E-PHYS	NAc neurons exhibit vigorous activation, both antidromically and orthodromically, in response to self-administered trains of stimulation in VTA/MFB.	Wolske, Rompre, Wise, & West, 1993
Rats	Heroin i.v. self-administration	FSCV	Heroin self-administration increased the extracellular NAc DA, in a dose-dependent and naloxone-reversible manner.	Xi, Fuller, & Stein, 1998
Mutant mice (TH -/-)	Feeding and locomotion recording	i.p. injections of L-DOPA, quinpirole and SKF 81297	Although the mutant mice behave and develop normally for the first postnatal week, they display symptoms of bradykinesia and hypophagia and they will die of starvation by 3 to 4 weeks without intervention. Restoration of DA function by L-DOPA induces near normal activity, feeding, and growth levels.	Zhou & Palmiter, 1995

Note: A query on the search engine Stanford HighWire (that includes PubMed) to contain the words "dopamine", "accumbens", and "reward", all in the same abstract, rendered 817 results on May 1st, 2010. Thus, only a handful of the studies that seemed most relevant to the topic described herein were included in this table, in alphabetical order.

Abbreviations. CB – cannabinoid; CPP – conditioned place preference; E-PHYS – electrophysiological recordings; FSCV – *in vivo* fast-scan cyclic voltammetry; ICSS – intra-cranial self-stimulation; HPLC – high performance liquid chromatography; fMRI – functional magnetic resonance imaging; PET – positron emission tomography; TH – tyrosine hydroxylase.

Table 2

VTA involvement in pain

Species	**Behavioral paradigm**	**Method**	**Main Results**	**VTA involvement**	**Cell type**	**Reference**
Humans	Noxious thermal stimuli (46°C)	fMRI	Noxious stimuli increased the signal several brain regions implicated in reward processing, such as the VTA/PAG region and extended amygdala, among other structures.	Activation	N/A	Becerra, Breiter, Wise, Gonzalez, & Borsook, 2001
Anesthetized rats	Footshock	E-PHYS, JCL, TH IHC	Dorsal VTA is inhibited by footshocks, the ventral VTA is phasically excited by footshock.	Excitation and inhibition	DA	Brischoux et al., 2009
Humans	Noxious electrical stimuli to either the midline lower abdomen or rectum	fMRI	Increases in signal were observed in the VTA/SN, PAG, parabrachial nuclei/nucleus coeruleus, and red nucleus bilaterally to both stimuli.	Activation	N/A	Dunckley et al., 2005
Humans	Noxious thermal stimuli to the hand accompanied by visual cues	fMRI	Increases in signal were observed in the VTA and other regions before and during pain. Activation of insula during pain was predicted by activity in both the entorhinal cortex and VTA during anticipation of pain.	Activation	N/A	Fairhurst, Wiech, Dunckley, & Tracey, 2007
Anesthetized rats	Noxious mechanical stimulation of the skin	E-PHYS	Following application of noxious stimuli, 37% (n=14) of the VTA cells were inhibited, 58% (n=22) showed no response, and 5% (n=2) were excited.	Mostly inhibition or unresponsive	N/A	Hentall, Kim, & Gollapudi, 1991
Rats	Formalin test	*c-fos* IHC	Tonic pain activates DAergic and CCKergic neurons from the VTA.	Activation	DA, CCK	Ma, Zhou, & Han, 1993

Anesthetized rats	Foot pinch, tail pinch, stimulation of the vaginal cervix	E-PHYS	For foot pinch and tail pinch tests, suppression of DA neurons occurred more frequently than activation (68% vs. 13%). For the same tests, the non-DA neurons half had decreased activity (43%) and half had increased activity (46%).	Mostly inhibition	DA, non-DA	Maeda & Mogenson, 1982
Anesthetized rats	Noxious tail-pinch	E-PHYS	The mesocortical DA neurons responded to tail pinch, either by an excitation (65%), or by an inhibition (25%). In contrast, most DA neurons projecting either to the NAc or the septum remained unaffected.	Mostly inhibition	DA	Mantz, Thierry, & Glowinski, 1989
Rats	Tail-shock	NAc microdialysis	Both tail-shock and intra-VTA capsaicin induce DA release in NAc, and this release is blocked by intra-VTA microinjection of the TRPV1 antagonist iodoresineferatoxin.	Excitation	DA	Marinelli, Pascucci, Bernardi, Puglisi-Allegra, & Mercuri, 2005
Rats	Formalin injections into the lumbar muscles and skin	*c-fos* IHC	Fos-immunoreactive neurons were observed in the VTA, spinal cord, NAc core, BLA, PAG and other regions	Activation	N/A	Ohtori et al., 2000
Young and old rats	Tail pinch	*c-fos* IHC	More Fos-immunoreactive neurons were observed in the VTA and other regions of the young rats than the middle-aged rats	Activation	N/A	Smith et al., 1997
Anesthetized rats	Foot pinch	E-PHYS	The VTA neurons that are excited by aversive stimuli are not DAergic; the DA neurons are uniformly inhibited.	Excitation and inhibition	DA, non-DA	Ungless, Magill, & Bolam, 2004
Rats	Footshock	NAc microdialysis	DA levels in NAc increased after each shock.	Excitation	DA	Young, 2004

Abbreviations. BLA – basolateral nucleus of the amygdala; IHC – immunohistochemistry; JCL - juxtacellular labeling.

Table 3

VTA involvement in analgesia

Species	**Behavioral paradigm**	**Method**	**Main Results**	**Reference**

Rats	Formalin test	Bilateral intra-VTA infusions of the Substance P analogue, DiMe-C7	DiMe-C7 induced analgesia	Altier & Stewart, 1993
		Bilateral infusions of amphetamine into the medial prefrontal cortex	Amphetamine failed to induce analgesia	
		Bilateral infusions of amphetamine into the NAc	Amphetamine induced analgesia	
	Tail flick test	Intra-VTA DiMe-C7 or intra-NAc amphetamine	No analgesia	
Rats	Formalin test	Intra-VTA infusions of the opioid antagonist naltrexone	Reduced stress-induced analgesia	Altier & Stewart, 1996
Rats	Formalin test	intra-VTA infusions of neuropeptide FF	Blocked analgesia induced by intro-VTA morphine or exposure to footshock stress	Altier & Stewart, 1997a
Rats	Formalin test, Tail flick test	Intra-VTA or intra-NAc infusions of tachykinin agonists	All injections produced analgesia in the formalin, but not in the tail-flick test	Altier & Stewart, 1997b
Rats	Formalin test	Intra-NAc infusions of DA antagonists	Blocked the analgesia induced by intra-VTA infusions of the substance P analog, DiMe-C7 or morphine and intra-NAc infusions of amphetamine	Altier & Stewart, 1998
Rats	Formalin test	Intra-VTA infusions of the tachykinin NK-l receptor antagonist, RP-67580	Blocked footshock stress-induced analgesia	Altier & Stewart, 1999b
Rats	Aversive electrical stimulation of the NRGi	ICSS of VTA	Long-lasting suppression of aversion produced by the NRGi following VTA brain stimulation	Anderson et al., 1995
Rats	Formalin test	6-OHDA lesion of the NAc	The lesion reduced the analgesic effect of amphetamine, but had no effect on morphine analgesia	Clarke & Franklin, 1992
Rats	Rhizotomy (section of dorsal roots C5 to Th1 included)	6-OHDA lesion of the VTA	The lesion induced an increase in the autotomy behavior	Gorea & Lombard, 1984
Rats	The VAD test	Intra-VTA microinjections of carbachol and atropine	Carbachol induced affective analgesia in a dose-dependant manner and this effect was blocked by atropine. Carbachol had no effect on the SMR thresholds	Kender et al., 2008
Rats	Tail or foot pinch tests	Intra-VTA infusions of the GABA-A agonist muscimol	Muscimol potentiates the analgesia induced by i.p. halothane or pentobarbital	Ma & Leung, 2006
Rats	Formalin test	Intra-VTA microinjections of the opioid antagonist naloxone, s.c. morphine	Morphine produced almost complete analgesia in the Phase 2 of the formalin test that was not reversed by 3 µg naloxone.	Manning & Franklin, 1998

Rats	Tail-flick test	Electrical stimulation of the VTA	Analgesia	Mayer, Wolfle, Akil, Carder, & Liebeskind, 1971
Rats	Formalin test, tail-flick test	6-OHDA lesion of the VTA	The lesion blocked the analgesic effect of amphetamine and morphine in the formalin, but not in the tail-flick test.	Morgan & Franklin, 1990
Rats	Hot plate test	Electrical lesion of the VTA	No effect on the analgesia induced by morphine or the κ-opioid agonist U-50,488H.	Ohno, Yamamoto, & Ueki, 1987
Rats	Acute and chronic pain tests	Selective chemical lesions with 6-OHDA or/and kainic acid of the VTA, SN and striatum	All lesions of DAergic terminals in the striatum decreased the latencies of all nociceptive reflexes and accelerated the time of onset of autotomy behavior. Kainic acid lesions of the SN-VTA did not produce significant changes in the latencies of nociceptive reflexes or in the autotomy criteria.	Saade, Atweh, Bahuth, & Jabbur, 1997
Rats	The VAD test	Intra-VTA microinjections of carbachol, mecamylamine, and atropine	Carbachol induced affective analgesia in a dose-dependant manner in both anterior and posterior VTA. This effect was blocked by atropine and mecamylamine in anterior VTA and by atropine in posterior VTA. Carbachol had no effect on the SMR thresholds.	Schifirneț, 2009
Rats	Carrageenan inflammation of the paw	Radiofrequency lesions or electrical stimulation of the VTA	VTA lesions enhanced the occurrence of autotomy behavior, whereas VTA stimulation facilitates analgesia	Sotres-Bayon, Torres-Lopez, Lopez-Avila, del Angel, & Pellicer, 2001
Rats	Formalin test	Intra-NAc D1 and D2 agonists and antagonists administration	Quinpirole dose-dependently inhibited the Phase 2 nociception in the formalin test, effect that was blocked by raclopride, suggesting that the NAc D2 receptors are involved in antinociception. The D1 agonist results were inconclusive.	Taylor et al., 2003
Mice	Formalin test	Systemic administration of DAergic agents	Both D1 and D2 receptors agonists and antagonists induced antinociception in different phases of the formalin test.	Zarrindast, Nassiri-Rad, & Pazouki, 1999

Abbreviations. 6-OHDA – 6-hydroxydopamine; NRGi - nucleus reticularis gigantocellularis.

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ABSTRACT

COMPARISON OF AFFECTIVE ANALGESIA AND CONDITIONED PLACE PREFERENCE FOLLOWING CHOLINERGIC ACTIVATION OF VENTRAL TEGMENTAL AREA SUBREGIONS

by

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Major: Psychology (Behavioral & Cognitive Neuroscience)

Degree: Doctor of Philosophy

Activation of the dopaminergic mesolimbic reward circuitry that originates in the ventral tegmental area (VTA) is postulated to preferentially suppress affective reactions to noxious stimuli (affective analgesia, AA). VTA dopamine neurons are activated via cholinergic inputs, and we have observed that microinjections of the acetylcholine agonist carbachol suppressed vocalizations of rats that occur following administration of brief (1 sec) tail-shocks (vocalization afterdischarges = VAD). VADs are a validated rodent model of pain affect. In addition, the capacity of carbachol to support reinforcement appears to be regionally dependent within VTA. Ikemoto & Wise (2002) reported that carbachol was self-administered in the posterior VTA (pVTA), but not the anterior VTA (aVTA). We have previously reported that carbachol preferentially increased the threshold current intensity for eliciting VADs in aVTA and pVTA, but not midVTA. This carbachol-induced AA is mediated by muscarinic receptors within the pVTA and by both muscarinic and nicotinic receptors within the aVTA. Using the conditioned place preference paradigm (CPP), the present study evaluated the muscarinic versus nicotinic involvement in intra-VTA carbachol-induced CPP learning by administering atropine

(muscarinic antagonist) and mecamylamine (nicotinic antagonist) into the VTA prior to carbachol treatment. The present study indicates that unilateral carbachol (4 µg/0.25 µl) supports the CPP learning in aVTA and pVTA, but not midVTA. Additionally, both atropine (60 µg/0.25 µl) and mecamylamine (45 µg/0.25 µl) reliably prevented the development of carbachol-induced CPP in the aVTA and pVTA. Thus, this study is the first to directly compare the extent of overlap between cholinergically mediated reward and affective analgesia within different VTA regions. The results are discussed in terms of anatomical and physiological properties of the VTA, with emphasis of cholinergically activated mesolimbic and mesocortical systems. Finally, based on two of the most prominent hypotheses regarding the role of DA in general, a framework is provided for understanding the role of DA in pain, analgesia, and reward in the context of DA function.

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AUTOBIOGRAPHICAL STATEMENT

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I was born in Bucharest, Romania, in 1977. After receiving my Bachelor of Psychology from the Babeş-Bolyai University of Cluj-Napoca (2002), I earned my Master's Diploma from the Bucharest University in Cognitive Sciences with perfect grades (2005). In 2005 I enrolled in the Behavioral and Cognitive Neuroscience Ph. D. program at Wayne State University, with Dr. George S. Borszcz as my advisor. Since then, I have been investigating the involvement of ventral tegmental area in the modulation of the emotional dimension of pain. After receiving my Ph. D., I will continue investigating the neurobiology of emotion as a Scientist Fellow in Dr. Satoshi Ikemoto's laboratory, at National Institute of Drug Abuse, National Institutes of Health.