ROLE OF THE DORSOMEDIAL HYPOTHALAMUS IN RESPONSES EVOKED FROM THE PREOPTIC AREA AND BY SYSTEMIC ADMINISTRATION OF INTERLEUKIN-1β

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ABSTRACT

Joseph L. Hunt

ROLE OF THE DORSOMEDIAL HYPOTHALAMUS IN RESPONSES EVOKED FROM THE PREOPTIC AREA AND BY SYSTEMIC ADMINISTRATION OF INTERLEUKIN-1β

Recent studies in anesthetized rats suggest that autonomic effects relating to thermoregulation that are evoked from the preoptic area (POA) may be mediated through activation of neurons in the dorsomedial hypothalamus (DMH). Disinhibition of neurons in the DMH produces not only cardiovascular changes but also increases in plasma adrenocorticotropic hormone (ACTH) and locomotor activity mimicking those evoked by microinjection of muscimol, a $GABA_A$ receptor agonist and neuronal inhibitor, into the POA. Therefore, I tested the hypothesis that all of these effects evoked from the POA are mediated through neurons in the DMH by assessing the effect of bilateral microinjection of muscimol into the DMH on the changes evoked by microinjection of muscimol into the POA in conscious rats. In addition, I tested the hypothesis that neurons in the DMH mediate a specific response that is thought to signal through the POA, the activation of the HPA axis evoked by systemic administration of the inflammatory cytokine IL-1β. After injection of vehicle into the DMH, injection of muscimol into the POA elicited marked increases in heart rate, arterial pressure, body temperature, plasma ACTH and locomotor activity and also increased Fos expression in the hypothalamic paraventricular nucleus (PVN), a region known to control the release of ACTH from the

adenohypophysis, and the raphe pallidus, a medullary region known to mediate POA-evoked sympathetic responses. Prior microinjection of muscimol into the DMH produced a modest depression of baseline heart rate, arterial pressure, and body temperature but completely abolished all changes evoked from the POA. Microinjection of muscimol just anterior to the DMH had no effect on POA-evoked autonomic and neuroendocrine changes. Inhibition of neuronal activity in the DMH only partially attenuated the increased activity of the HPA axis following systemic injections of IL-1β. Thus, neurons in the DMH mediate a diverse array of physiological and behavioral responses elicited from the POA, suggesting that the POA represents an important source of inhibitory tone to key neurons in the DMH. However, it is clear that the inflammatory cytokine IL-1β must employ other pathways that are DMH-, and possibly POA-, independent to activate the HPA axis.

Joseph A. DiMicco, Ph.D., Chair

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1.0 INTRODUCTION

The preoptic area (POA) of the mammalian ventral forebrain continues to be a major focus of neurobiological research because of well-described contributions made by neurons in this region to critical homeostatic functions. Thus, the POA is thought to participate in the central neural regulation of a variety of physiological processes including osmotic balance (Almli & Weiss, 1974; McGowan et al., 1988), maternal behavior (for review, see Numan, 2007), sexual behavior (for review, see Paredes, 2003; Dominguez & Hull, 2005), sleep and arousal (for review, see McGinty et al., 2004; Saper et al., 2005; Jones, 2005), and feeding (Stanley et al., 1985; Kim et al., 2000; Szentirmai et al., 2006; Patterson et al., 2006). In addition to all of these, the POA has long been known to play a primary role in the regulation of body temperature (for review, see Boulant, 2000) and may be a key brain area that is responsive to circulating inflammatory or infectious agents.

Invasion of the body with infectious agents elicits a coordinated pattern of centrally-mediated autonomic and neuroendocrine responses known as the acute phase response. Pro-inflammatory cytokines, such as interleukin-1β (IL-1β), released into the circulation in response to infection are thought to be endogenous mediators that act on the brain to evoke these responses. Clinically, the acute phase response is characterized by fever, tachycardia, and activation of the hypothalamic-pituitary-adrenal (HPA) axis with subsequent increase in circulating glucocorticoids (McCance & Huether, 2002; Kumar et al., 2005). However, the exact mechanism by which these inflammatory cytokines evoke centrally-mediated response is still largely unclear.

As noted above, an area thought to be important to the generation of fever and responses to infectious or inflammatory agents is the POA. Interestingly, perfusion or microinjection of the POA with muscimol, a $GABA_A$ receptor agonist thought to be inhibitory to virtually all adult mammalian neurons (Johnston, 1996), elicits changes in diverse systems in anesthetized and conscious rats that may reflect its many roles. These changes include increases in body temperature, heart rate, and increases in plasma levels of adrenocorticotrophic hormone (ACTH), reflecting activation of the HPA axis, (Osborne et al., 1994; Osborne & Kurosawa, 1994; Osborne et al., 1994; Ishiwata et al., 2005; Zaretsky et al., 2006). These studies support the idea that neurons in the POA exert tonic inhibitory tone on the downstream effector mechanisms involved in these responses.

While the downstream neural circuitry involved in POA-evoked responses is not entirely clear, recent evidence supports a role for the dorsomedial hypothalamus (DMH) in at least some of these responses. Disinhibition of neurons in the DMH by local microinjection of the $GABA_A$ receptor antagonist bicuculline methiodide (BMI) evokes hyperthermia, tachycardia, and increases in plasma ACTH (Soltis & DiMicco, 1991a,b; DeNovellis et al., 1995; Bailey & DiMicco, 2001; Zaretskaia et al., 2002), responses similar to those evoked by microinjection of muscimol into the POA (Zaretsky et al., 2006). The former studies indicate that responses evoked from the DMH are under tonic inhibition. However, the source of this inhibitory tone remains unknown. Considering that the POA is the single greatest source of afferents to the DMH (Thompson & Swanson, 1998), it seems likely that the POA represents a source of the tonic inhibition whose existence was first implied by the dramatic effects of microinjection of BMI into

the DMH (DiMicco and Abshire, 1987). Furthermore, recent studies in anesthetized rats show that inhibition of neuronal activity in the DMH attenuates thermogenesis and/or tachycardia evoked by intrapreoptic microinjection of another substance, prostaglandin E_2 (PGE_2) (Zaretskaia et al., 2002; Madden & Morrison, 2004; Nakamura et al., 2005). These studies support the importance of neuronal activity in the DMH in responses evoked from the POA. However, to date no studies have specifically investigated the role of the DMH in responses evoked by microinjection of muscimol into the POA. In addition, if the DMH is critical to responses evoked from the POA, then investigation of the role of the DMH in systemic inflammatory responses is also warranted at this time.

Thus, in the current thesis I explored the hypothesis that neuronal activity in the DMH mediates (1) physiological and behavioral responses evoked from the POA and (2) activation of the HPA axis elicited by systemic administration of a specific inflammatory cytokine, IL-1β.

1.1 Role of the POA in thermoregulation and the febrile response

Much of our understanding of the role of the POA in thermoregulation derives from the study of the response to microinjection of PGE_2 into this region, an established model for the acute febrile response seen in inflammatory and infectious states. Fever is thought to result in large part from the local generation of prostaglandins in the POA (Milton, 1998; Aronoff & Neilson, 2001). Accordingly, intrapreoptic microinjection of PGE_2 in rats elicits hyperthermia, sympathetically mediated tachycardia, and increased circulating levels of ACTH (Murakami & Watanabe, 1989; Nakamura et al., 2002; Zaretskaia et al., 2003; Madden & Morrison, 2004; Nakamura et al., 2005; Zaretsky et

al., 2006). However, these studies do not indicate the mechanisms by which PGE_2 acts in the POA to elicit these physiologic changes.

One possibility is that PGE_2 inhibits the activity of critical neurons in the POA to provoke some or all of these changes. Direct application of PGE_2 has been shown to suppress the tonic activity of warm-sensitive neurons in the POA (Ranels & Griffin, 2003; 2005). It is neuronal activity in the POA that is thought to exert inhibitory tone on various downstream autonomic and neuroendocrine brain regions (Morrison, 2004; Zaretsky et al., 2006). Inhibition of neurons in the POA with the neuronal inhibitor muscimol elicits tachycardia, hypertension, hyperthermia, and activation of the HPA axis (Osborne & Kurosawa, 1994; Osborne et al., 1994; Ishiwata et al., 2005; Zaretsky et al., 2006). This pattern of responses is identical to that evoked by PGE_2 in POA (Zaretsky et al., 2006). Inhibition of the subpopulation of warm-sensitive neurons in the POA likely explains the similarities in responses evoked by injections of PGE_2 and muscimol into this region. Interestingly, the application of muscimol to the POA in conscious rats increases locomotor activity, a response not seen with PGE_2 (Osborne et al., 1993; Ishiwata et al., 2005; Zaretsky et al., 2006). This effect may be due to the inhibition of warm-insensitive neurons in the POA by muscimol, neurons that are activated by PGE_2 (Ranels & Griffin, 2005). While these studies suggest a mechanism by which substances such as PGE_2 might act in the POA to produce physiologic changes, they do not address the potential efferent pathways that mediate these responses.

1.2 Efferent pathways mediating POA-evoked responses

One region that appears to be involved in autonomic responses evoked from the POA is the raphe pallidus, a midline medullary region that comprises the location of sympathetic premotor neurons regulating heart rate and thermogenesis (Morrison et al., 1999). Direct projections have been identified from the raphe pallidus to the intermediolateral column of the spinal cord where preganglionic neurons are located that mediate sympathetic drive to the heart and brown adipose tissue, the major thermogenic organ in rats (Loewy, 1981; Miura et al., 1983; Jansen et al., 1995; Nakamura et al., 2004). Disinhibition of neurons in the raphe pallidus with BMI elicits thermogenic and cardiovascular responses that are similar to those evoked by PGE_2 or muscimol in the POA. It is known that the POA sends direct projections to the raphe pallidus (Nakamura et al., 2002). In addition, the injection of PGE_2 into the POA stimulates thermogenesis in brown adipose tissue in rats (Amir & Schiavetto, 1990), an effect that is blocked by inactivation of neurons in the raphe pallidus (Madden & Morrison, 2003). The sympathetic thermogenesis evoked by PGE_2 in the POA is thought to be mediated by these direct pathway that exists from the POA to the raphe pallidus (Nakamura et al., 2002), however the participation of neurons in other brain regions has not been excluded.

Another downstream brain center thought to mediate POA-evoked responses is the hypothalamic paraventricular nucleus (PVN). The PVN is the location of neurons that are thought to represent the final common pathway for activating the HPA axis, the neuroendocrine hallmark of the acute phase response (Webster & Sternberg, 2004). As described above, microinjection of either PGE_2 or muscimol into the POA produce robust activation of the HPA axis (Zaretsky et al., 2006), presumably through the activation of

neurons in the PVN. Upon stimulation, cells of the PVN release corticotrophin-releasing factor (CRF) into the hypothalamic-hypophyseal portal blood to stimulate the release of ACTH into the systemic circulation from the anterior pituitary gland (Webster & Sternberg, 2004). In response to increased circulating levels of ACTH, the adrenal glands synthesize and release glucocorticoids (Webster & Sternberg, 2004). A major function of glucocorticoids is to serve as an endogenous anti-inflammatory and anti-pyretic molecule to suppress the production and activity of inflammatory cytokines (Munck et al., 1984; Tatro, 2000). Glucocorticoids bind to steroid receptors that then act as nuclear transcription factors to inhibit the production and release of cytokines from macrophages, limiting the inflammatory response (Munck et al., 1984; Webster & Sternberg, 2004; Bhattacharyya et al., 2007). This is evident by the fact that adrenalectomy prevents the increase in plasma glucocorticoids and results in an exaggerated febrile response to lipopolysaccharide (LPS), a glycolipid component of the bacterial cell wall (Coelho et al., 1992). The exaggerated fever seen after adrenalectomy is reduced by replacement therapy with corticosterone, the primary glucocorticoid in rats (Morrow et al., 1993).

Systemic administration of bacterial endotoxin or inflammatory cytokines increases circulating levels of ACTH and glucocorticoids through the activation of neurons in the PVN. Systemic administration of either LPS or IL-1β evokes increases in neuronal activity in the PVN as indicated by dose-dependent increases in the expression of the protein Fos in this region (Ericsson et al., 1994; Elmquist et al., 1996). Central microinjection of PGE_2 directly into the POA also elicits an increase in the expression of Fos in the region of the PVN (Scammell et al., 1996), suggesting that the responses

elicited by systemic administration of LPS or IL1-β involve the generation of endogenous PGE_2 in the POA. The immunohistochemical detection of Fos, the protein product of the early immediate gene c-fos, has been widely utilized as a marker for neuronal activation (Morgan & Curran, 1991; Herrera & Robertson, 1996). While the sensitivity and specificity of the expression of Fos as a marker of neuronal activity has been a matter of debate since its inception (Dragunow & Faull, 1989), this technique remains an invaluable research technique for the identification of putative neural pathways involved in complex central mediated responses. However, the absence of detectable Fos does not exclude the participation of neuronal population in a physiological response since inhibition of neurons does not appear to be associated with the expression of Fos (Chan et al., 1993).

Given the identification of neurons in the POA that project to the PVN (Swanson & Sawchenko, 1983), activation of the HPA axis evoked from the POA has been widely assumed to be mediated through these direct projections (Johnson & Gross; 1993; Elmquist et al., 1997). However, more recent evidence points instead to a role for projections from the POA to the DMH, a brain center involved in autonomic and neuroendocrine regulation (DiMicco et al., 2002).

1.3 Role of the DMH in autonomic and neuroendocrine regulation

An area known to be important in the regulation of autonomic and neuroendocrine responses seen in experimental stress in rats is the DMH. Chemical stimulation or disinhibition of neurons in the DMH results in tachycardia, hypertension, hyperthermia, and activation of the HPA axis (Soltis & DiMicco, 1991a,b; DeNovellis et al., 1995;

Bailey & DiMicco, 2001; Zaretskaia et al., 2002), a pattern of responses identical to those evoked by microinjection of PGE_2 or muscimol into the POA (Zaretsky et al., 2006). Microinjection of BMI or excitatory amino acids (EAA) into the DMH elicits dose-dependent increases in heart rate and arterial pressure (Soltis & DiMicco, 1991a.) These responses appear to be independent of the PVN, a brain center commonly thought to be the principal hypothalamic site for autonomic integration. Doses of BMI or EAAs capable of evoking marked increases in heart rate and modest increases in arterial pressure when injected into the DMH failed to produce similar increases when injected into the PVN or into an intermediate area between the two nuclei (DeNovellis et al., 1995). Furthermore, the latency to onset of tachycardia was significantly less in animals receiving microinjections of BMI into the DMH as compared to injections into the PVN (DeNovellis et al., 1995). These results suggest that hypothalamic neurons responsible for autonomic cardiovascular responses are located in the DMH. Previous reports that examined the effects of microinjection of relatively large doses of EAAs into the PVN have suggested a role for this region in sympathetically mediated tachycardia and hypertension (Jin & Rockhold, 1989; Martin et al., 1991; Martin & Haywood, 1993). However, none of these studies controlled for the spread of drug to nearby brain areas, such as the DMH. Furthermore, the injection of BMI or EAAs into the DMH of conscious rats also elicits increases in locomotor activity, a response not seen with similar injections into the PVN (Bailey & DiMicco, 2001). Interestingly, microinjection of muscimol into the DMH abolishes air stress-induced tachycardia (Lisa et al., 1989), an effect again shown not to be the result of drug spreading to the PVN (Stotz-Potter et al., 1996a). Microinjection of muscimol into of the DMH also prevents stress-induced

increases in plasma levels of ACTH (Stotz-Potter et al., 1996b) and expression of Fos in the PVN (Morin et al., 2001). Together, these studies indicate that activation or disinhibition of neurons in the DMH elicits a specific and reproducible pattern of physiological and behavioral responses. This pattern is identical to that evoked by the injection of muscimol into the POA, suggesting a possible role for the DMH in POA-evoked responses.

1.4 Role of the DMH in POA-evoked responses

Anatomical connections have been identified that support a role for the DMH in responses evoked from the POA. As mentioned earlier, the POA represents the single greatest source of afferents to the DMH (Thompson & Swanson, 1998). The DMH in turn projects heavily to the parvocellular PVN where the majority of CRF-releasing neurons are located (Ter Horst & Luiten, 1986; 1987). The DMH also sends projections to the raphe pallidus (Ter Horst & Luiten 1986; Thompson et al., 1996; Samuels et al., 2002), the location of neurons whose activity appears to be responsible for DMH-induced tachycardia and thermogenesis (Samuels et al., 2002; Cao et al., 2004). As mentioned above, the raphe pallidus is the location of neurons responsible for the tachycardia and thermogenesis induced by intrapreoptic microinjection of PGE_2 (Nakamura et al., 2002; Morrison, 2003). Zaretskaia and colleagues demonstrated that the unilateral microinjection of muscimol into the DMH causes modest but significant reductions in the tachycardia and hyperthermia evoked by intrapreoptic injection of PGE_2 (Zaretskaia et al., 2002). This represents the first direct evidence indicating a role for the DMH in of POA-evoked responses. Later, bilateral inhibition of the DMH was shown to produce

nearly complete suppression of the cardiovascular and thermogenic responses evoked by injection of PGE_2 into the POA (Madden & Morrison, 2004; Nakamura et al., 2005). Together these results suggest that inhibition of neurons in the POA may elicit sympathetically-mediated increases in heart rate and body temperature through disinhibition of neurons in the DMH. However, all of these studies used anesthetized rats, and to date no studies examining the role of the DMH in POA-evoked responses in conscious animals have appeared. Also, while the DMH has been shown to play a critical role in the activation of the HPA axis in experimental models for stress (for review, see DiMicco et al., 2002), no evidence exists implicating the DMH in the increases in plasma ACTH or locomotor activity evoked from the POA. In fact, recent studies fail to consider the DMH in this regard and instead emphasize projections to other regions (see Uschakov et al., 2006; 2007). In light of the evidence that supports the role of the DMH in responses evoked from the POA, it is possible to infer a role for the DMH in the diverse changes signaled through this region, including those evoked by systemic infection or inflammation.

1.5 Clinical relevance of systemic inflammation

The prevalence of systemic inflammation in hospitalized patients is a major concern for clinicians. Recently, the systemic inflammatory response syndrome (SIRS) was defined as a clinical syndrome manifested by the presence of two of the following four clinical signs; (1) hyperthermia, (2) tachycardia, (3) hyperventilation, or (4) leukocytosis (ACCP/SCCM, 1992). Approximately one-third of hospitalized patients meet the criteria for SIRS, and an even greater incidence of SIRS is seen in intensive care

patients (Brun-Buisson, 2000). The early recognition of SIRS is important because these patients experiencing acute systemic inflammation have a significantly higher risk of morbidity and mortality than patients without SIRS. Approximately one-third of patients with SIRS have or will progress to a septic condition (Rangel-Frausto et al., 1995), and the presence of SIRS upon admission is shown to carry a greater risk for mortality in emergency patients (Sun & Aikawa, 1999). A major dilemma in treating patients meeting the criteria for SIRS is that the defining clinical signs do not assist the physician in identifying the underlying cause of the inflammatory response, thus preventing appropriate treatment. A better understanding of the central mechanisms involved in the generation of systemic inflammatory responses may promote the development of better triage and treatments for patients with SIRS or active inflammation without an identified cause.

1.6 Characterizing the acute inflammatory response

Before examining the role of the DMH in the inflammatory response, a distinction must be made between peripherally- and centrally-mediated acute inflammatory responses. A wide variety of exogenous insults that threaten the integrity of cells or tissues evoke a set of well-defined inflammatory reactions. While infectious agents are the most commonly acknowledged and arguably the most researched, other insults known to evoke acute inflammatory responses include trauma, chemical or physical agents, and any other insult causing cellular necrosis (Kumar et al., 2005). The similarity and defining characteristic of these insults is the generation of endogenous inflammatory substances such as cytokines (Kumar et al., 2005). The primary goal of the acute

inflammatory response is to alter normal homeostasis in order to minimize tissue damage, and the vast majority of the physiologic changes that characterize the acute inflammatory response serve this protective function, at least initially (Nakano et al., 1987; Baumann & Gauldia, 1994; Kapcala et al., 1995).

The acute inflammatory response is frequently described in terms of its peripherally-mediated components. The peripheral response to an inflammatory agent is typically localized to the site of the insult. Nearly two millennia ago, Celsus described the four cardinal signs of acute inflammation as rubor, tumor, calor, and dolor (redness, swelling, heat, and pain, respectively; Spencer, 1935). Much later, microscopic examination of acutely inflamed tissue described the local changes in blood flow, vascular permeability, and exudate formation with accompanied leukocytosis that are ultimately responsible for the clinical features of local inflammation (Cohnheim, 1889). These changes in vascular hemodynamics and chemotaxic regulation of cellular migration are the key processes of the local inflammatory response serving to minimize damage from invading insults (Kumar et al., 2005). While inflammatory substances such as cytokines appear to be the endogenous mediators of these peripheral or local responses, it is unlikely that the central nervous system contributes significantly.

Invasion of the body with infectious or inflammatory agents also elicits a coordinated pattern of centrally-mediated processes classically referred to as the acute phase response (Saper & Breder, 1994). The acute phase response is typified by autonomic and neuroendocrine changes that function to establish an environment that protects the host from further damage (McCance & Huether, 2002; Kumar et al., 2005). These responses include hyperthermia, tachycardia, and activation of the HPA axis (Saper & Breder,

1994). The invading insult is thought to stimulate the release of inflammatory cytokines from host immune cells (Cohen, 2002), and these endogenous substances act on the central nervous system to elicit these effects (Saper & Breder, 1994; Watkins et al., 1995). However, the mechanisms and neural pathways by which circulating inflammatory cytokines elicit centrally-mediated responses remain a matter of speculation (Watkins et al., 1995; Blatteis & Sehic, 1998; Conti et al., 2004).

One brain region thought to participate in the generation of acute phase responses is the hypothalamus. The hypothalamus is a collection of functionally and anatomically discrete nuclei near the ventral surface of the brain that is traditionally recognized as an important area in the maintenance of homeostasis (Hess, 1954). In a classic study, Hess found that electrical stimulation of the hypothalamus produced a set of responses termed the 'defense reaction' that was typified by marked activation of the sympathetic nervous system (Hess, 1954). Since the acute phase response to infection and inflammation is also characterized by sympathetic activation, it seems likely that areas of the hypothalamus are involved in these changes in homeostasis. However, the mechanisms by which infectious or inflammatory agents evoke acute phase responses remain unclear.

1.7 Peripheral induction of the acute phase response

Bacterial infection is a well-recognized activator of the acute phase response. Systemic administration of LPS elicits an increase in body temperature in humans (Sundy et al., 2006) and animals (Roth & Souza, 2001) and has been widely used as an experimental model of fever (Blatteis & Sehic, 1998; Roth & Souza, 2001). Other components of the acute phase response elicited by subseptic doses of LPS include

tachycardia, hypertension, and increases in plasma levels of ACTH and glucocorticoids (Dunn, 1992; Givalois et al., 1994; Turnbull et al., 1998; Nalivaiko et al., 2005). Peripheral inhibition of β_1-adrenergic receptors suppresses LPS-induced tachycardia suggesting that sympathetic activation is a component of LPS-induced acute phase responses (Nalivaiko et al., 2005).

The production of endogenous inflammatory cytokines is thought to mediate acute inflammatory responses evoked by bacterial infections (Saper & Breder, 1994; Watkins et al., 1995). Administration of LPS results in an increase in circulating levels of several pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α in humans (Sauermann et al., 2007) and animals (Givalois et al., 1994; Turnbull et al., 1998; Johnson et al., 2002). These cytokines are released from activated macrophages and circulating monocytes (Cohen, 2002) after bacterial antigenic motifs known as pathogen-associated molecular patterns (PAMPs) are recognized (Medzhitov & Janeway, 1998). The binding of LPS to Toll-like receptors (TLRs), specifically TLR4, on macrophages leads to the activation of gene promoters and ultimately increases in the release of inflammatory cytokines (Takeuchi et al., 1999). Furthermore, substances that block the action of inflammatory cytokines attenuate the febrile response evoked by LPS. Administration of neutralizing soluble receptors for TNF-α significantly attenuates the fever evoked by intra-arterial administration of LPS (Roth et al., 1998). Systemic administration of an IL-1 receptor antagonist also attenuates fever (Roth & Souza, 2001) and activation of the HPA axis (Kakucska et al., 1993) evoked by LPS.

Systemic administration of inflammatory cytokines such as IL-1β elicits physiological responses similar to those evoked by LPS. Administration of IL-1β in rats

evokes sympathetically mediated tachycardia and hypertension, marked hyperthermia, and substantial increases in plasma levels of ACTH and corticosterone (Besedovsky et al., 1986; Sapolsky et al., 1987; Berkenbosch et al., 1987; Dascombe et al., 1988; Murakami et al., 1990; Gwosdow et al., 1990; Bataillard & Sassard, 1994; Kannan et al., 1996; Turnbull et al., 1998). In addition, the administration of IL-1β to humans in doses capable of eliciting fever results in significant increases in heart rate and arterial pressure (Haefeli et al., 1993). While these findings support the hypothesis that circulating cytokines act as endogenous mediators for acute phase responses to bacterial challenges, they do not indicate the mechanism or central pathways through which inflammatory cytokines initiate these responses.

1.8 Central site of action of cytokines: Role of the preoptic area

Peripheral cytokines are thought to act on the central nervous system to evoke acute phase responses (Saper & Breder, 1994; Watkins et al., 1995). However, the blood-brain barrier (BBB) prevents the passage of relatively large, hydrophilic molecules such as cytokines from the systemic circulation into the brain (Blatteis & Sehic, 1998; Turnbull & Rivier, 1999). Therefore, the exact mechanism by which cytokines elicit centrally-mediated responses remains unclear. One hypothesis is that inflammatory cytokines such as IL-1β evoke the production of the lipid PGE_2 as a secondary messenger to act on neurons in the POA (Stitt, 1986), an area critical to the induction of fever (Saper & Breder, 1994). Supporting this idea, the level of PGE_2 is increased in the POA in animals treated with LPS, an effect that is blocked by inhibiting its local synthesis (Sehic et al., 1996; Blatteis & Sehic, 1998). In the midline of the POA is the organum

vaculosum laminae terminalis (OVLT), one of the few brain areas unprotected by the BBB where peripheral cytokines can access the central nervous system to elicit physiological responses (Saper & Breder, 1994; Turnbull & Rivier, 1999). Cytokines are thought act in the OVLT to elicit the production of PGE_2 that can then act on neurons in the POA to evoke fever and possibly other components of the acute phase response (Saper & Breder, 1994). Using perfusion microdialysis, Komaki and colleagues demonstrated that intravenous administration of IL-1β elicits increased levels of PGE_2 in the OVLT and POA (Komaki et al., 1992). Electrolytic ablation of an area that included the OVLT prevented LPS-induced fever (Blatteis et al., 1983). While the cellular source of PGE_2 in the OVLT and POA evoked by cytokines is unclear, most evidence indicates a prominent role for the cerebral vasculature. Peripheral administration of IL-1β induces cyclooxygenase (COX), the rate limiting enzyme in the synthesis of PGE_2, in the blood vessels of the brain, suggesting that vascular or perivascular cells are responsible for the central production of PGE_2 in response to circulating IL-1β (Cao et al., 1996; Lacroix & Rivest, 1998). While mRNA for the IL-1 receptor has previously been demonstrated in the vasculature throughout the brain (Yabuuchi et al., 1994; Ericsson et al., 1995), Konsman and colleagues were the first to report the presence of functional IL-1 receptors specifically on the vasculature of the OVLT, supporting their role in cytokine-induced PGE_2 signaling (Konsman et al., 2004). Other possible sources of central PGE_2 include microglia, macrophages, astrocytes, and neurons (Turnbull & Rivier, 1999).

If PGE_2 does act in the POA as the endogenous secondary mediator in cytokine-evoked responses, then three conditions must be demonstrated. First, specific binding sites for PGE_2 should be apparent on cells in the POA. Receptors selective for PGE_2,

more specifically the EP_3-receptor, have been identified on the somatodendritic portion of neurons in the POA (Nakamura et al., 1999; Oka et al., 2000; Nakamura et al., 2005). Binding of PGE_2 to EP_3-receptors is thought to produce a change in the firing rate of critical neurons in the POA. As mentioned above, the application of PGE_2 decreases the firing rate of warm-sensitive neurons in the POA (Ranels & Griffin, 2003; 2005). This change in the pattern of neuronal activity is thought to initiate the mechanism for the induction of fever and possibly other acute responses evoked from the POA (Boulant, 2000). Second, the application of PGE_2 directly to the POA should produce physiological and neuroendocrine responses that mimic those seen with systemic administration of inflammatory cytokines. Intrapreoptic microinjection of PGE_2 elicits dramatic increases in body temperature and is now a commonly used experimental model of fever (Zaretskaia et al., 2003; Madden and Morrison, 2004; Nakamura, 2005). As seen with the systemic administration of inflammatory cytokines, the microinjection of PGE_2 into the POA in conscious rats also evokes tachycardia, hypertension, and the activation of the HPA axis (Zaretsky et al., 2006). Third, blocking the action of PGE_2 in the POA should prevent responses evoked by peripheral administration of inflammatory cytokines. Microinjections of a PGE_2-antagonist or COX-inhibitor into the OVLT or POA prevent the increase in plasma levels of ACTH evoked by IL-1β (Katsuura et al., 1990). Together, these findings support the hypothesis that PGE_2 in the region of the POA acts as a secondary mediator for circulating inflammatory cytokines such as IL-1β.

If inflammatory agents do in fact evoke physiologic responses through the POA, then these responses likely employ the same neural pathways as those used with direct chemical manipulation of this region. As indicated previously, stimulation of neurons in

the DMH elicits cardiovascular, neuroendocrine, and thermogenic responses that closely resemble the responses to infection or systemic inflammation (DiMicco et al., 2002; DiMicco et al., 2007). However, the role of the DMH in mediating physiological responses evoked by specific inflammatory agents such as IL-1β has never been explicitly studied.

1.9 Specific Aims

The hypothesis of this thesis is that neuronal activity in the DMH mediates (1) physiological and behavioral responses evoked from the POA and (2) activation of the HPA axis elicited by systemic administration of IL-1β. The first aim of my thesis is to establish the role of the DMH in a variety of responses evoked from the POA. The second aim is to determine the role of the DMH in a specific change thought to be mediated by the POA, the activation of the HPA axis evoked by IL-1β

1.9.1 Specific Aim 1: Determine the role of neuronal activity in the DMH in responses evoked by the microinjection of muscimol into the POA

The POA has been identified as an important area through which peripheral inflammatory substances signal centrally-mediated responses caused by infection. Microinjection of muscimol, a $GABA_A$-agonist and neuronal inhibitor, into the POA produces physiologic responses that are similar to those seen following systemic administration of bacterial endotoxin or the inflammatory cytokine IL-1β, including the induction of fever, tachycardia, and activation of the HPA axis (Zaretsky et al., 2006). A change in neuronal firing in the POA is thought to evoke these responses through direct projections to downstream autonomic and neuroendocrine brain centers, such as the raphe

pallidus and the PVN. However, these effect may also involve neural projections from the POA to the DMH, a region containing neurons that project in turn to the PVN and raphe pallidus and where neuronal activity has been implicated in febrile and cardiovascular responses evoked from the POA (Zaretskaia et al., 2003; Madden & Morrison, 2004; Nakamura et al., 2005). Therefore, I hypothesized that the physiological and behavioral responses evoked by inhibiting neurons in the POA are mediated by the activity of neurons in the DMH. To test this hypothesis, I evaluated the effect of microinjections of muscimol into the DMH on the responses evoked by microinjection of muscimol into the POA. The results provided insights into the central pathways regulating responses evoked from the POA and suggested a mechanism for the central processing of systemic inflammatory signals.

1.9.2 Specific Aim 2: Determine the role of neuronal activity in the DMH in the activation of the HPA axis evoked by systemic injection of IL-1β and LPS

Systemically administrated IL-1β is a powerful activator of the HPA axis, an effect thought to be mediated by the POA (Katsuura et al., 1990). Results of studies addressing the first specific aim implicated the DMH in the regulation of a variety of responses evoked from the POA, including activation of the HPA axis, and so suggested a possible role for the DMH in responses evoked by peripheral inflammatory signals. Thus, neuronal activity in the DMH was hypothesized to mediate the activation of the HPA axis following systemic administration of IL-1β. To test this hypothesis, increases in plasma ACTH and/or corticosterone and the expression of Fos in the PVN, the site of neurons representing the final common pathway in the activation of the HPA axis, were evaluated after intra-arterial injection of IL-1β prior to and after the microinjection of

muscimol into the DMH. To further examine the role of the DMH in the activation of the HPA axis evoked by peripheral inflammatory signals, similar experiments were performed with the intra-arterial injection of LPS, a substance known to produce elevations in circulating IL-1β as well as other inflammatory cytokines. The results of these experiments were the first to address the role of the DMH in the regulation of the response to a specific inflammatory cytokine.

2.0 METHODS

In experiments described in this thesis, I examined the role that activity of neurons in the DMH plays in the physiological and behavioral responses evoked from the POA and in the activation of the HPA axis following systemic administration of IL-1β and LPS. The methods I employed to achieve this goal involved modifications to previously established techniques developed in our laboratory specifically for use in the experimentation on conscious, freely moving rats. All of the surgical and experimental protocols were approved by Indiana University's Institutional Animal Care and Use Committee.

2.1 Animals

Male Sprague-Dawley rats (Harlan; Indianapolis, IN) weighing 300 ± 20 gm were used in all experiments. Animals were housed singly with free access to rat chow and water in the university's Laboratory Animal Resource Center (LARC) under a 12 hour light-dark cycle with the lights turning on at 0700 hr. On days of surgery or experimentation, the rats were transferred to the laboratory and returned to LARC thereafter.

2.2 Experimental design

To investigate the role of the DMH in the regulation of acute inflammatory responses, I designed experiments that employed the technique for central microinjections in conscious animals developed in our laboratory. The first of these studies tested the role of neuronal activity in the DMH in physiological and behavioral

responses evoked from the POA, a brain area shown to be important in the transmission of peripheral inflammatory signals to central nuclei that control acute phase responses. I then designed experiments to investigate the role of neuronal activity in the DMH in the activation of the HPA axis caused by systemic administration of a specific pro-inflammatory cytokine, IL-1β, and LPS, a component of the bacterial cell wall known to induce the systemic production of this and other inflammatory cytokines. All experiments were performed on conscious, freely-moving rats between 10 am and 2 pm to minimize the effect of circadian variation.

2.2.1 Effect of microinjection of muscimol into the DMH on responses evoked from the POA

The DMH has never been implicated in the activation of the HPA axis evoked by manipulation of the POA. The inhibition of neurons in the DMH has been shown to attenuate the thermogenic and cardiovascular responses to the injection of PGE_2 into the POA of anesthetized rats (Nakamura et al., 2005; Madden & Morrison, 2004; Zaretskaia et al., 2003). The microinjection of muscimol into the POA produces increases in heart rate, arterial pressure, body temperature, and plasma ACTH that are similar to those caused by the microinjection of PGE_2 into identical sites, and also causes marked behavioral stimulation (Zaretsky et al., 2006). While it is known that injection of muscimol into the POA elicits responses that are similar but not identical to those produced by PGE_2, the role of the DMH in these responses is unknown. I hypothesized that neuronal activity in the DMH mediates the physiological and behavioral responses evoked from the POA. To test this hypothesis, I evaluated the effect of inhibiting neurons in the DMH by bilateral microinjection of muscimol on the increases in heart

rate, arterial pressure, body temperature, locomotor activity, and plasma ACTH elicited by microinjection of muscimol into the POA. I also assessed the expression of Fos, a marker of neuronal activity, in the PVN and the raphe pallidus, two downstream areas known to mediate selected neuroendocrine and autonomic responses.

Effect of pretreatment in the DMH with muscimol on POA-evoked responses

In order to evaluate the role of neuronal activity in the DMH in the initiation of responses evoked from the POA, I microinjected muscimol or vehicle bilaterally into the DMH 5-9 min before the injection of muscimol or vehicle into the POA.

Animals were implanted with telemetric probes for the monitoring of heart rate, arterial pressure, body temperature, and locomotor activity (Sec. 2.3.2). After at least three days of recovery, guide cannulae targeting the POA and DMH (bilateral) were implanted (Sec. 2.3.3). Five days later, femoral arterial lines were implanted for drug delivery and blood sampling (Sec. 2.3.4). Animals were allowed to recover for at least three days before experimentation.

The rats were brought to the testing facilities on the morning of the experiment. Each animal was subjected to two experimental sessions separated by two days of rest. Home cages were placed on telemetric receiver plates, dummy wires were removed from the guide cannulae, and extensions were attached to the arterial catheters. The animals were left undisturbed for at least 2 hr to establish resting baselines.

Using the technique for microinjection described below (Sec. 2.4.5), either 80 pmol of muscimol or vehicle (100 nL total volume) was infused into the right DMH over 30 sec, and one minute later into the left DMH in identical fashion. This dose of muscimol has been previously shown to inhibit effectively stress-induced cardiovascular

and neuroendocrine changes when injected into the DMH (Stotz-Potter et al., 1996). Exactly 5 min after the completion of the second injection into the DMH, either 80 pmol of muscimol or vehicle (100 nL total volume) was injected into the POA. Microinjection of this dose of muscimol into the POA produces robust increases in heart rate, arterial pressure, body temperature, locomotor activity, and plasma ACTH (Zaretsky et al., 2006). Exactly 15 min after the microinjection into the POA, a blood sample was collected from the arterial catheter for the analysis of plasma ACTH (Sec. 2.4.4). The animal was then left undisturbed as telemetric monitoring continued for at least 60 min. The animals were returned to LARC after the first experimental session. For the second experimental session, the injectate into the POA was held constant for a given animal, while the injection of muscimol or vehicle into the DMH was switched. Order effect was controlled by randomly assigning an equal number of animals to each sequence.

In preparation for the analysis of the expression of Fos in the PVN and raphe pallidus, animals were deeply anesthetized with pentobarbital (65 mg/kg, i.a.) 90 min after the final microinjection of the second experimental session. Perfusion and *in situ* fixation of brain tissue commenced within 2 min of establishing anesthesia (Sec. 2.4.6).

Effect of acute microinjection of muscimol into the DMH on established responses evoked from the POA

The previous experiments examined the effect of prior bilateral microinjection of muscimol into the DMH on the physiological and behavioral responses evoked by subsequent microinjections of muscimol into the POA. I also examined the role of the DMH in sustaining physiological and behavioral responses already evoked from the POA. In these latter experiments, animals received bilateral microinjections of muscimol

or vehicle into the DMH during the period of increased heart rate, arterial pressure, body temperature, and locomotor activity caused by microinjection of muscimol into the POA.

The animals were implanted with telemetric probes, femoral arterial catheters, and guide cannulae in a manner identical to that employed in the previous experiment (Sec. 2.3.2, Sec. 2.3.3, Sec. 2.3.4) and allowed to recover for three full days before experimentation. As in the previous experiment, each animal was subjected to two experimental sessions separated by two days of rest where the injection into the POA was held constant and the treatment for the DMH was reversed. Order effect was controlled by randomly assigning an equal number of animals to each sequence.

After establishing resting baselines, either 80 pmol of muscimol or vehicle (100 nL total volume) was infused into the POA over a 30 sec period (Sec. 2.4.5). Five minutes later, 80 pmol of muscimol or vehicle (100 nL total volume) was microinjected into the DMH bilaterally as previously described. The animals were monitored for an additional 60 min before being returned to LARC at the end of the first experimental session. After the second session, animals were perfused and the brains removed and fixed for histological analysis to verify the sites of injection (Sec. 2.4.6).

2.2.2 Effect of microinjection of muscimol into the DMH on the activation of the HPA axis evoked by systemic administration of IL-1β

The previous experiments sought to implicate the DMH in the regulation of a wide variety of POA-evoked responses, including activation of the HPA axis. In the next set of experiments, I tested the hypothesis that neurons in the DMH are activated by the systemic administration of IL-1β and that this activation plays a role in the stimulation of the HPA axis following systemic injections of IL-1β. IL-1β is thought to act as an

endogenous mediator in the activation of the HPA axis following an infectious or inflammatory challenge. Not only do circulating levels of IL-1β increase after bacterial infection or peripheral administration of LPS (Johnson et al., 2002, Givalois et al., 1994), but systemically administered IL-1β produces dose-dependent increases in circulating ACTH and glucocorticoids (Turnbull et al., 1998; Dunn & Chuluyan, 1992; Besedovsky et al., 1986). The POA has been implicated in the induction of the HPA axis produced by elevations in circulating IL-1β. Lesions or inhibition of prostaglandin activity in the POA produce marked attenuations in the activation of the HPA axis evoked by systemically administered IL-1β (Katsuura et al., 1990).

I evaluated the effect of microinjections of muscimol into the DMH on plasma ACTH and corticosterone, as well as on Fos expression in the PVN following intra-arterial administration of IL-1β. In addition, the expression of Fos in the DMH was quantified after injections of IL-1β.

Time course of the increase in plasma ACTH evoked by systemic administration of IL-1β

Before microinjection studies examining the role of the DMH in IL-1β-induced increases in HPA axis activity could be designed, I had to determine the time course for the effect of systemic administration of IL-1β on plasma ACTH in conscious rats. The results of this experiment were used to determine the appropriate time for blood sampling in subsequent studies.

Five days after the implantation of femoral arterial catheters for drug delivery and blood sampling (Sec. 2.3.4), animals were brought to the testing rooms where they were

weighed for proper dosing. After attaching extensions to arterial catheters, the animals were left undisturbed for at least 2 hours.

I began experimental sessions by taking a blood sample for determination of baseline ACTH (Sec. 2.4.4). A freshly prepared solution of IL-1β (1 ug/kg/mL) or vehicle (1 mL/kg) was then immediately injected intra-arterially, followed by 0.35 ml of sterile normal saline to flush the catheter and replace lost blood volume. A review of the literature suggests that this was the lowest intravascular dose of IL-1β producing reliable increases in plasma ACTH and corticosterone in rats and is a dose of an order of magnitude less than required to produce plasma concentrations of IL-1β that are comparable to those observed in patients with sepsis (Katsuura et al., 1990). Blood samples were taken 30, 60, and 90 min after injection of IL-1β or vehicle. After the final blood sample, animals were returned to LARC.

Each animal was subjected to injections of both IL-1β and vehicle, separated by three days. Order effect for responses to injections of IL-1β or vehicle was controlled by randomly assigning an equal number of subjects to both sequences. After the second experimental session, animals were euthanized with an overdose of pentobarbital (100 mg/kg; i.a.) and cervical dislocation.

Effect of systemic administration of IL-1β on the expression of Fos in the DMH and PVN

Systemic administration of IL-1β in rats results in increased expression of Fos, a protein product of the early-immediate gene c-fos and widely used marker of neuronal activation, in the PVN, the site of neurons directly responsible for the activation of the HPA axis (Schiltz & Sawchenko, 2007; Buller et al., 1998; Callahan & Piekut, 1997;

Veening et al., 1993). I hypothesized that the systemic administration of IL-1β results in the activation of neurons in the DMH, and that the latter is required for the excitation of neurons in the PVN and the resulting stimulation of the HPA axis. To test this hypothesis, I quantified the expression of Fos in the region of the DMH and PVN in rats sacrificed 90 min after the intra-arterial injection of IL-1β or vehicle, a time chosen based on published reports investigating the expression of Fos after intravascular administration of IL-1β (Schiltz & Sawchenko, 2007). Based on the peak increase in levels of plasma ACTH from the previous time course study, blood samples were taken at 0 and 30 min to measure plasma ACTH and corticosterone, the primary glucocorticoid in rats.

Five days after the implantation of femoral arterial catheters (Sec. 2.3.4), animals were brought to the testing rooms where they were weighed for proper dosing. After attaching extensions to arterial catheters, animals were left undisturbed for at least 2 hours.

Rats were randomly assigned to receive intra-arterial injections of IL-1β or vehicle. An initial blood sample was taken for determination of baseline ACTH (Sec. 2.4.4). A solution of IL-1β (1 ug/kg/mL) or vehicle (1 mL/kg) was immediately injected intra-arterially as described previously. The animals were left undisturbed except for a blood sample taken 30 min after injection of IL-1β or vehicle.

Animals were deeply anesthetized with pentobarbital (65 mg/kg, i.a.) 90 min after injection of IL-1β or vehicle. Perfusion and *in situ* fixation of brain tissue as described above commenced within 2 min of establishing anesthesia (Sec. 2.4.6).

Effect of acute microinjection of muscimol into the DMH on activation of the HPA axis evoked by systemic injection of IL-1β

The two previous experiments characterized the effect of the systemic administration of IL-1β on the HPA axis and the activity of neurons in the DMH and PVN. To assess directly the role of neuronal activity in the DMH in the activation of the HPA axis evoked by systemic administration of IL-1β, I performed microinjection experiments in conscious rats receiving intra-arterial injections of IL-1β similar to those used in the investigation of POA-evoked responses. This experiment assessed the effect of acute microinjection of muscimol into the DMH on the increase in plasma ACTH and expression of Fos in the PVN following intra-arterial administration of IL-1β.

A bilateral guide cannula was targeted to the DMH (Sec. 2.3.3). After 5 days of recovery, animals were implanted with femoral arterial lines for drug delivery and blood sampling (Sec. 2.3.4). The animals were allowed to recover for at least three days before experimentation.

While all rats received systemic injections of IL-1β, animals were randomly assigned to receive central microinjection of either muscimol or vehicle into the DMH. The animals were left undisturbed for at least two hours before a blood sample was taken for determination of baseline ACTH (Sec. 2.4.4). A freshly prepared solution of IL-1β (1 ug/kg/mL) was then immediately injected intra-arterially in identical fashion to the previous experiment. Approximately two minutes later, 80 pmol of muscimol or vehicle (100 nL total volume) was microinjected into the right DMH, followed one minute later by an identical microinjection into the left DMH (Sec. 2.4.5). Based on the results of the time course study described above, I withdrew blood samples 15 and 30 min after the

injection of IL-1β, and then I left the animals undisturbed for the duration of the experiment.

Exactly 90 min after the injection of IL-1β, animals were deeply anesthetized with pentobarbital (65 mg/kg, i.a.). Perfusion and *in situ* fixation of brain tissue for analysis of the expression of Fos in the PVN commenced within 2 min of establishing anesthesia (Sec. 2.4.6).

Effect of pretreatment in DMH with muscimol on the activation of the HPA axis evoked by systemic injections of IL-1β

The previous experiment employed microinjections of muscimol into the DMH *shortly after* the administration of IL-1β. To address the possibility that the effects of IL-1β were initiated in the short time prior to muscimol reaching the neurons in the DMH, I redesigned the experiment so that the bilateral microinjection of muscimol occurred *5 min before* the intra-arterial administration of IL-1β. In addition to plasma ACTH and the expression of Fos in the PVN, circulating levels of corticosterone were determined.

Guide cannulae were targeted to the DMH (Sec. 2.3.3) and arterial catheters were implanted (Sec. 2.3.4) in a fashion identical to that employed in the previous experiment. The animals were allowed to recover for at least three days before any experimentation.

Animals were randomly assigned to receive a systemic injection of IL-1β and a central microinjection of either muscimol or vehicle or to serve as negative controls, receiving systemic and central injections of vehicle. After a two hour baseline period, a blood sample was taken for baseline ACTH and corticosterone (Sec. 2.4.4). Five minutes later, muscimol (80 pmol/100 nL) or vehicle was microinjected bilaterally into the DMH as described previously (Sec. 2.4.5). Five minutes later, IL-1β (1 μg/kg/mL) or vehicle

was administered intra-arterially as described in the previous experiment. Blood sampling and perfusion of the brain were identical to that employed in the previous experiment.

2.2.3 Role of the DMH in the activation of the HPA axis elicited by the systemic administration of LPS

To explore further the role of the DMH in the activation of the HPA axis evoked by peripheral inflammatory signals, I performed microinjection experiments in a similar fashion to those already described in conscious rats treated with systemic injections of the bacterial endotoxin LPS. Systemic administration of LPS causes elevations in several inflammatory cytokines including IL-1β, and these endogenous molecules are thought to mediate the LPS-induced activation of the HPA axis. Therefore, I hypothesized that neuronal activity in the DMH plays a role in the stimulation of the HPA axis evoked by systemic administration of LPS. To test this hypothesis I examine the effect of acute microinjections of muscimol into the DMH on the levels of plasma ACTH and expression of Fos in the PVN after intra-arterial injections of LPS.

Time course of the increase in plasma ACTH evoked by systemic administration of LPS

Before designing microinjection experiments, I determined the time course for the increase in plasma ACTH following systemic administration of LPS in conscious rats in a time course study similar to that for IL-1β previously described. Results of this experiment were used to determine the appropriate time for microinjections and blood sampling.

Five days after the implantation of femoral arterial catheters for drug delivery and blood sampling (Sec. 2.3.4), animals were brought to the testing rooms where they were weighed for dosing and then left undisturbed for at least two hours. A blood sample was then taken to determine the baseline plasma levels of ACTH (Sec. 2.4.4). A newly prepared solution of LPS (10 µg/kg/mL; *Escherichia coli* 0111:B4) or vehicle (1 mL/kg) was then immediately injected intra-arterially. Blood samples were taken 30, 60, 90 and 120 min after injection of LPS or vehicle.

Each animal was subjected to injections of both LPS and vehicle, separated by three days, and order effect was control by randomly assigning an equal number of subjects to both sequences. After the second experiment, animals were sacrificed with an overdose of pentobarbital (100 mg/kg; i.p.) and cervical dislocation.

Effect of acute microinjection of muscimol into the DMH on activation of the HPA axis evoked by systemic injection of LPS

Rats were implanted with bilateral guide cannulae targeting the DMH (Sec. 2.3.4) and femoral arterial lines (Sec. 2.3.4) as in previous experiments. After a three day recovery period, animals were brought to the testing facilities and left undisturbed for at least two hours.

While all rats received systemic injections of LPS, animals were randomly assigned to receive microinjection of either muscimol or vehicle into the DMH. A baseline blood sample was taken prior to the intra-arterial injection of LPS (10 µg/kg/mL). Sixty minutes later, muscimol (80 pmol/100 nL) or vehicle was microinjected bilaterally into the DMH as described previously (Sec. 2.4.5). According to previous studies, the effects of muscimol when microinjected into the DMH in this

fashion last for at least 40 to 60 minutes. To ensure that the effects of muscimol were present at the time of blood sampling, I microinjected the drug 60 min after the administration of LPS, 30 minutes prior to the peak increase in plasma ACTH. Blood was sampled 75 and 90 min after the injection of LPS.

Animals then were deeply anesthetized with pentobarbital (65 mg/kg, i.a.) 120 min after the injection of LPS. Perfusion and *in situ* fixation of brain tissue for analysis of Fos expression in the PVN commenced within 2 min of establishing anesthesia (Sec. 2.4.6).

2.3 Surgical Procedures

All surgeries were designed as survival procedures. Therefore, proper anesthesia was maintained, sterile conditions were observed, and post-operative care was given to ensure the well-being and rapid recovery of the animal. Only one surgery was performed per day on a given animal, and all animals were allowed to recover at least three days between procedures.

2.3.1 Anesthesia

A cocktail of ketamine/xylazine (80 mg/kg ketamine, 11.5 mg/kg xylazine; i.p.; supplement as needed) was used for all procedures requiring anesthesia. This anesthetic cocktail has been shown to produce relatively little cardiovascular or thermoregulatory depression compared to other commonly employed laboratory anesthetics (Wixson et al., 1987a, b, c, d). Adequate anesthesia was achieved within minutes of administration and confirmed by observing a lack of response to pinching of the tail. Following completion of the surgery, animals were placed in their home cages on a heated plate so that body

temperature could be supported until recovery from the anesthesia. I allowed rats undergoing multiple surgical procedures to recover for at least three days before being re-anesthetized.

2.3.2 Implantation of telemetric probes

In some experiments, telemetric probes were implanted to permit monitoring of physiological parameters by radio transmission in the undisturbed, conscious animal. The parameters measured by telemetry in the current experiments were heart rate, arterial pressure, body temperature, and locomotor activity.

After the rat was anesthetized, hair was removed from the abdomen and left inguinal area with an electric shaver. The exposed skin was wiped down with a Betadine solution. A 3 cm incision was made in the abdominal skin, and the underlying abdominal muscle layer was opened with scissors along the linea alba to expose the peritoneal space. A telemetric probe (TL11M2-C50-PXT, Data Sciences Int.) that had been sterilized with Cidex solution (Advanced Sterilization Products) and flushed clean with sterile saline was inserted into the peritoneal space. A 2 cm incision was made in the inguinal skin. A guide wire was then pushed through the inferior peritoneal wall so that the catheter of the probe could be threaded into the inguinum for implantation in the femoral artery.

Using cotton swabs and dissecting instruments, the femoral vein/artery/nerve bundle was separated from the surrounding fascia and fat layer. Care was taken to minimize pressure or trauma to the femoral nerve. Using a spinal cord hook instrument, a 1 cm length of the femoral artery was isolated. To prevent constriction of the artery during manipulation, I applied a 2% lidocaine solution directly to the artery. The artery was lifted slightly from the inguinum to occlude blood flow, and an incision was made

half way through the short axis. Using microretractors, I inserted the catheter of the probe into the incision and threaded approximately 5 cm into the femoral artery so that the tip came to rest in the abdominal aorta.

After fixing the catheter to the artery with suture, I closed the inguinal incision with 3-0 suture. During closure of the abdominal muscle layer with 3-0 suture, I securely fixed the probe to the inside of the abdominal wall. The abdominal skin was then closed with 3-0 suture. Recovery from anesthesia was ensured before animals were returned to LARC.

2.3.3 Placement of chronic guide cannulae targeting specific brain areas

Chronically implanted guide cannulae permit the microinjection of drug solutions into specific brain regions days to weeks after the surgical procedure, allowing animals to recover before experimentation. The procedure for the implantation of a chronic guide cannula has been developed and extensively used in our laboratory (Shekhar et al., 1990; De Novellis et al., 1995; Stotz-Potter et al., 1996a; Stotz-Potter et al., 1996b; Bailey & DiMicco, 2001; Zaretsky et al., 2006). The technique was modified slightly as dictated by the experimental design, and these modifications will be discussed as needed.

The rat was anesthetized as described above and hair was shaved from the top of head and nape of the neck. After applying Betadine to the exposed skin, I fixed the animal in a sterile stereotaxic apparatus (Kopf) via metal bars firmly pressed into the bony processes of the ear canals. This created an imaginary line between the two ear bars, known as the interaural line, later used as a reference line to appropriately determine the position of the head in space. Another bar was fixed behind the incisors of the animal, while the nose of the animal was pressed down and held firmly against that bar.

This incisor bar was set at either 3.3 mm below or 5.0 mm above the interaural line, determining the vertical-horizontal angle of the skull and thus the angle at which the guide cannula would enter the brain at time of implantation. The choice of angle was dependent on the experimental design and will be noted later.

Using a scalpel blade, a 1 cm incision was made in the skin of the head to expose the cranial aspect of the skull. The underlying connective tissue and fascia covering the bone was removed. Cotton swabs wet with a hydrogen peroxide solution were used to clear the skull of any remaining tissue. The hydrogen peroxide was quickly rinsed off afterwards with sterile saline. Bregma was identified as the point of intersection between the frontal-sagittal and coronal suture lines. All coordinal measurements were expressed relative to bregma. To increase the contrast between the suture lines and the surrounding bone, I applied additional hydrogen peroxide. After they were dried with cotton swabs, the suture lines become a more brilliant white. Bregma was then marked by slightly pressing the tip of a 23 ga needle into the bone tissue.

The brain areas of interest for microinjection and placement of guide cannulae were the DMH and POA. Initial determination of the coordinates for these two areas was determined using an established atlas of the rat brain (Paxinos & Watson, 2007). Geometric calculations were then used to determine the precise coordinal directions for the angled insertion of guides when needed. Some amount of trial and error was necessary to adjust the coordinates so that guides were reliably placed in the desired area.

A sterilized 26 ga single or double guide cannula (Plastics One, Inc.) was mounted in the arm of the stereotaxic device. The arm was moved until the tip of the

cannula rested at bregma, just touching the surface of the skull. After noting the position on the stereotaxic device, I moved the cannula away from the surgical field.

A Dremel drill with a carbide bur (Miltex) was used to bore a 3-5 mm diameter hole in the skull at the approximate position where the guide would enter the brain. Light downward pressure was used while drilling to prevent damage to the underlying brain parenchyma. Saline was applied to the skull, as needed, to prevent the drill bit from becoming too hot and scorching the skull. Circular motions were used so that the underlying dura remained intact after the hole was made. Using the same drill bit, additional small holes were placed in the frontal and parietal bones for the insertion of anchoring stainless steel screws (Plastics One, Inc.).

The guide cannula was then placed over the exposed brain according to the determined coordinates (Table 1). The tip of the cannula was lowered until it just touched the dura matter. A 33 ga needle was used to make a small incision in the dura so that the cannula could be lowered into the brain tissue. The cannula was then lowered appropriately until the tip of the guide cannula was 1 mm above the desired site of injection. This placement prevents any damage to the surrounding parenchyma and supporting tissues at the area of interest by the guide cannula until the day of experimentation when the seated injector descends beyond the tip of the guide cannula.

In microinjection experiments examining responses evoked from the POA, three guide cannulae were implanted during a single surgery. With cannulae mounted in the stereotaxic device 10° away from the sagittal plane and the incisor bar initially set at 3.3 mm below the inter-aural line, two guide cannulae were targeted to the left and right DMH (-3.2 mm AP, ±1.9 mm ML, -7.2 mm DV). Vetbond tissue adhesive (3M, Inc.)

Table 1

Target	Angle	Incisor bar	Coordinates		
			AP	ML	DV
POA	10°	+5.0 mm	+1.9 mm	+1.9 mm	-6.9 mm
DMH(single cannula)	10°	-3.3 mm	-3.2 mm	±1.9 mm	-7.2 mm
DMH(double cannula)	0°	-3.3 mm	-3.2 mm	±0.5 mm	-7.8 mm

Table 1. Coordinates relative to bregma in the anterior-posterior (AP), medial-lateral (ML), and dorsal-ventral (DV) planes used to implant guide cannulae in the preoptic area (POA) and dorsomedial hypothalamus (DMH). The angle of the stereotaxic arm and location of the incisor bar are relative to the sagittal plane and the inter-aural line, respectively.

and dental acrylic (Lang Dental Manufacturing Co., Inc) were added to the posterior aspect of the exposed skull to firmly fix the guides targeting the DMH. The incisor bar was then moved to +5.0 mm above the inter-aural line. A single guide was placed in the left side of the POA (+1.9 mm AP, +1.9 mm ML, -6.9 mm DV) and secured with Vetbond and dental acrylic.

In microinjection experiments examining the activation of the HPA axis by systemically administered IL-1β or LPS, a bilateral double guide cannula was targeted to the DMH. Injections could be more reliably targeted to both sides of the DMH with the use of a bilateral cannula because of the inherently greater technical error associated with the implantation of two individual guides as described above. This reduced the number of animals lost because of misplaced site of injection. The arm of the stereotaxic device was positioned vertically (0° from the sagittal plane) and the incisor bar was set at 3.3 mm below the interaural line. The guide cannula was targeted to the DMH (-3.2 mm AP, ±0.5 mm ML, -7.8 mm DV). The ML position was adjusted so that the right and left sides of the double cannula were equidistant from the sagittal sinus. The guide cannula was then fixed firmly with Vetbond and dental acrylic.

After the acrylic was set, a dummy wire was inserted into the guide cannula to prevent clogging before microinjections. A plastic cap was placed over the dummy wire and cannula, and the animal was returned to its home cage on a heated plate until recovery from anesthesia.

2.3.4 Placement of chronic femoral arterial catheter

Chronic catheters were placed in the femoral arteries in order to administer drug solutions systemically and to obtain arterial blood samples during experiments. With proper post-operative care, catheters remained patent up to three weeks after placement.

Catheters were made by inserting a 6 cm piece of Teflon tubing (0.015 in ID, Small Parts, Inc.) approximately 1 cm into a 20 cm piece of Tygon tubing (0.02 in ID, Small Parts, Inc). These catheters were then sterilized overnight with UV light before implantation.

After induction of anesthesia, I removed the hair from the right inguinal area and nape of the neck with an electric shaver. The exposed skin was then wiped down with a Betadine solution. A 2 cm incision was made in the right inguinal skin. Using cotton swabs and dissecting instruments, I separated the femoral vein, artery, and nerve bundle from the surrounding fascia and fat layer. Care was taken to minimize trauma to the femoral nerve. Using a spinal cord hook instrument, I isolated a 1 cm length of the femoral artery. To prevent constriction of the artery during manipulation, I applied a 2% lidocaine solution directly to the artery. The artery was lifted slightly from the inguinum to occlude blood flow, and an incision was made half way through the short axis. The catheter was filled with sterile saline with a 23 ga needle and syringe. Using microretractors, I inserted the Teflon leader of the catheter and threaded it 5 cm into the femoral artery so that the tip came to a rest in the abdominal aorta. The catheter was flushed with saline before being capped with a solid steel stylet. Suture thread was used to fix the catheter in the artery.

A 1cm incision was made at the nape of the neck between the shoulder blades. A guide wire was inserted into the incision and tunneled subcutaneously to the right inguinum. The Tygon portion of the catheter was routed through the guide wire back to the nape of the neck. The catheter was fixed with 3-0 suture to the skin of the neck while closing the surgical incision. The inguinal incision was also closed with 3-0 suture. Finally, a jacket (Kent Scientific Corp.) was placed on the animal in order to hold the catheter in place. The animal was then returned to its home cage on a heated plate until recovery from anesthesia.

2.3.5 Post-operative care

All animals received post-operative care following survival surgeries as mandated by the protocols of IACUC. Proper post-operative care promoted the well-being of the animals and greatly enhanced the likelihood that each animal would be healthy at the time of experimentation. Animals deemed unhealthy following surgical procedures were excluding from experiments and euthanized.

Post-operative care began immediately following the surgical procedure. Rats received analgesia (buprenorphine, 0.02 mg/kg, s.c.) while recovering on a warming plate to minimize heat loss. Animals were returned to LARC only after recovery from anesthesia. Wet rat chow was placed inside the cage for the first week following surgery to facilitate feeding and promote weight maintenance.

The status of the animals was checked daily until the final day of experimentation. Surgical incisions were evaluated for signs of infection or significant inflammation. While antibiotics were available, no animals in these studies required treatment. Lower limb mobility was assessed daily for signs of femoral nerve damage. Arterial catheters

were flushed daily with heparinized saline to maintain patency. Dummy wires were moved in and out of the guide cannulae to prevent clogging of the guide as well as to habituate the animal to this manipulation before microinjection experiments. The weight of the animal was checked at least every third day. Persistent inflammation, progressive weight loss, clogged guide cannulae or catheters, lower limb paralysis, or abnormal behavioral patterns warranted exclusion and euthanasia.

2.4 Experimental techniques

Since all experiments in these studies were performed on conscious, freely moving rats, the experimental techniques were designed to minimize external and environmental disturbances during the experiments.

2.4.1 Testing facilities

Two isolated, temperature-controlled (23 ± 2°C) rooms were used for all experiments. The rooms were equipped with video cameras and telemetric receivers linked to computers just outside so that animals could be monitored during experiments without entering the rooms. All animals were placed in the testing room in their home cages several hours before any experiment to minimize the stress from a novel environment.

2.4.2 Drugs

Stock preparations of drugs were prepared prior to the day of experimentation. These solutions were immediately stored at -20°C until the morning of the experiments.

Artificial cerebrospinal fluid

Artificial cerebrospinal fluid (122 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 20 mM NaH_2PO_4, 11 mM $C_6H_{12}O_6$ in deionized water) was used as the diluent to prepare the necessary solutions of muscimol for microinjection. It was also used as the vehicle control for all microinjection experiments. The solution was sterilized with a 22 μm sterile filter (Millipore) and stored at -20°C in sterilized Eppendorf tubes for up to one year. Osmolality of the final solution was approximately 300 mOsm/L.

Muscimol

Stock solutions of muscimol (Sigma, 80 mM in aCSF) were stored at -20°C for up to one year. On the day of the experiment, this stock solution was reconstituted and immediately diluted to a final concentration of 0.8 mM in aCSF.

Phosphate buffered saline containing bovine serum albumin

Phosphate buffered normal saline (0.01 M) containing 0.1% bovine serum albumin was prepared as the diluent for IL-1β solutions and as the vehicle control for peripheral injections. The BSA-containing PBS solution was passed through a 22 μm sterile filter (Millipore) and stored at 4°C for up to a week.

Interleukin-1β

Lyophilized recombinant human IL-1β (R&D Systems) was reconstituted to a final concentration of 1 μg/mL in BSA-containing PBS. Preparations of IL-1β were stored at -20°C for less than one month before use or disposal if unused.

Lipopolysaccharide (LPS)

Stock solutions of LPS from *Escherichia coli* 0111:B4 (Sigma, 200 μg/mL in sterile 0.9% normal saline) were stored at -20°C for up to six months. On the day of

experiments, this solution was diluted with sterile normal saline to a final concentration of 10 μg/mL.

2.4.3 Telemetric monitoring

Telemetric monitoring of heart rate, arterial pressure, body temperature, and locomotor activity was performed with a Dataquest telemetry system (Data Sciences Int.). Data was transmitted via radio signal from the implanted probe (TL11M2-C50-PXT, Data Sciences Int.) to a receiver plate (RPC-1, Data Sciences Int.) placed under the home cage of the animal. Data was transferred immediately via data cable and written to the hard drive of a personal computer placed outside of the testing room. Dataquest software was used to create a real-time, minute-by-minute visualization of recorded parameters, displayed on video monitors during experiments.

2.4.4 Blood sampling

Arterial blood samples were taken during experiments for the analysis of ACTH and corticosterone. A 2 ft extension of PE-50 tubing filled with sterile normal saline was connected to the arterial catheter fixed to the rat. This extension was fixed completely outside of the home cage, leaving enough slack to allow the animal to move freely around the entire cage. At the time of blood sampling, the metal stylet was removed to allow blood to flow freely through the open catheter and extension. A 23 ga needle and syringe containing approximately 60 μL of an EDTA/aprotinin solution were used to collect 0.35 mL of blood. Blood volume was replaced by the injection of normal saline. Collected blood was immediately transferred to chilled Eppendorf tubes and centrifuged (6,000 x g) for 45 sec. Separated plasma was stored at -80°C until needed for analysis.

2.4.5 Microinjection of drug solutions

The current technique for central microinjection of solutions in conscious, freely-moving animals has been developed and refined in our lab over the past several years. A 33 ga microinjector (Plastics One, Inc.) was connected to a 10 μL syringe (Hamilton) with Teflon tubing (ID 0.12 mm, OD 0.65 mm; Bioanalytic Systems). Just prior to the injection, the microinjector was inserted into the guide cannula of the conscious animal and fixed in placed with a modified plastic dust cap (Plastics One, Inc.). An infusion pump (KD Scientific) was used to inject 100 nL of solution over a 30 sec period. The microinjector was left in place for an additional 60 sec to prevent the backflow of solution up the cannula track. After the microinjector was removed from the guide cannula, successful microinjection was verified by visualizing the flow of solution within seconds of reactivating the infusion pump. Any deviations from this general description of the technique for microinjections, such as multiple microinjections in the same animal, will be discussed later as needed.

2.4.6 Perfusion and fixation of brain tissue

Transcardial perfusion was used for *in situ* fixation of brain tissue. Animals were deeply anesthetized with sodium pentobarbital (65 mg/kg, i.a.) at the end of the experiment. A 60 ml syringe was placed approximately 3 feet above the animal and connected to a 14 ga blunt needle by plastic tubing. The pleural cavity was opened, and a small incision was quickly made at the apex of the heart. The needle was threaded through the left ventricle and clamped with hemostats to the very proximal portion of the ascending aorta. Rats were perfused with 40 mL of 0.9% normal saline containing 15,000 U/I heparin sulfate over a time of approximately 30 sec followed by 100 mL of an

ice-cold solution containing 3% paraformaldehyde and 1% acrolein in 0.1 M phosphate buffer (pH 7.4) over 10 min. After dissection from the calvarium, brains were post-fixed in 3% paraformaldehyde in 0.1 M phosphate buffer at room temperature for 60 min. To facilitate cryoprotection, brains were then transferred into 20% sucrose in 0.01 M PBS and stored overnight at 4°C. Brains were then frozen on dry ice and stored at -80°C until further processing.

2.5 Analysis of brain tissue and blood samples

All brains and blood samples were stored at -80°C until the day of processing. All tissues were processed immediately upon thawing, and refreezing to -80°C was avoided.

2.5.1 Preparation of brain sections for histology and immunohistochemistry

Brains were taken from -80°C and stored at -20°C for at least 1 hr. Serial coronal sections were then cut on a cryostat and transferred into vials containing 0.01 M PBS (pH 7.4). Sections for immunohistochemical processing were cut at either 25 or 30 μm thickness, while sections used for verification of site injection were cut at 45 μm. Sections were stored in 0.01 M PBS at 4°C. Within 2 days, sections were either further processed or transferred into an ethylene glycol/glycerol-based freezing solution and stored at -20°C.

2.5.2 Verification of site of microinjection

To facilitate identification of the precise sites where solutions were injected into the brain, all final drug and vehicle solutions contained fluorescent-embedded, polystyrene microspheres (5% v/v, Molecular Probes). The fluorescent microspheres

were added to the injectate at the time of the experiment and presumably concentrated at the site of injection until microscopic evaluation.

Within 2 days of being cut, 45 μm sections containing the site of injection as indicated by the tracts of guide cannulae were mounted on glass slides. The slides were then cover slipped using Vectashield hard set for fluorescence (Vector) and stored at 4°C. Using a microscope equipped with filters for the detection of fluorescence (Leica), the location of intense fluorescence was identified as the site of injection based on the atlas of Paxinos and Watson (2007).

2.5.3 Immunohistochemical visualization of Fos protein

To determine the relative activation of early-immediate gene, c-fos, I performed immunohistochemical quantification of its protein product, Fos. The protocol used in the current study for the detection of Fos was developed and refined by Dr. Sumit Sarkar in our laboratory. The antibody against Fos (Calbiochem) has been used extensively in functional neuroanatomical research (Singru et al., 2007; Zhang et al., 2003; Zhang et al., 2000) and its immunological specificity for Fos has been demonstrated (Honore et al., 2000).

Brain sections were washed 3 times in 0.01 M PBS and then immersed in 1% sodium borohydride in deionized water for 25 min. The sections were rinsed several times in alternating PBS and deionized water until they were free from visible bubbles. Treatment with 0.5% H_20_2 for 12 min was used to eliminate endogenous peroxidase activity. After rinsing 3 times in PBS, the sections were incubated in 0.5% Triton-X-100 in 0.01 M PBS for 30 min to improve antibody penetration. The sections were then placed in 10% normal horse serum (Sigma) in 0.01 M PBS for 20 min to reduce

nonspecific binding. The sections were incubated in polyclonal rabbit antiserum against Fos (1:20,000, Calbiochem) for 2-3 days at 4°C.

The sections were again washed 3 times in PBS before being incubated in biotinylated goat anti-rabbit IgG (1:400, Jackson Immunoresearch) for 3 hr. After washing 3 times in PBS, the sections were incubated in avidin-biotin-peroxidase complex (ABC Elite Kit, Vector Laboratories) for 60 min. The sections were washed 3 times in PBS followed by a brief wash in 0.05 M Tris-buffer (pH 7.8). Staining was developed in freshly prepared Tris-buffer solution containing 0.025% diaminobenzidine tetrachloride, 0.06% nickel-ammonium sulfate, and 0.0027% H_20_2 for approximately 2 min to yield dark purple color in reacting nuclei while minimizing nonspecific background staining. The color reaction was stopped by dilution of the section in Tris buffer.

The sections were mounted on charged slides and left to air dry for 24-48 hr. After dehydration in increasing concentrations of ethanol, the sections were immersed in 4 changes of Histosol (National Diagnostics). The slides were coverslipped in DPX-mountant (Fluka) and allowed to dry at least overnight before analysis.

2.5.4 Radioimmunoassay for quantification of plasma ACTH

A double antibody radioimmunoassay was used to quantify ACTH (Li et al., 1993). A rabbit polyclonal antiserum (IgG Corp.) that recognizes the 5-18 segment of ACTH was used in the assay. Standards were created from the serial dilution of purchased ACTH (1-39; Bachem). ^{125}I-ACTH (Diasorin) was used as the radioactive competitor. Goat anti-rabbit γ-globulin (Calbiochem) was used to precipitate antigen-antibody complex.

After they were thawed, the samples of plasma were diluted appropriately in order to place content in the linear range of the calibration curve (approx. 1-10 pg ACTH/tube). Duplicates of samples and standards were incubated in ACTH antiserum (1:30,000) at 4°C for 24 hr. Reconstituted ^{125}I-ACTH was added and incubated overnight at 4°C. Anti-rabbit γ-globulin (1:50) was added and incubated at 4°C for an additional 24 hr.

After adding 1.5 mL of ice-cold 0.01 PBS (pH 7.6), I centrifuged the samples (2000 x g) at 4°C for 20 min. After aspiration of the supernatant, an additional 1.5 mL of PBS was added, and centrifugation was repeated for 16 min. After removing the supernatant, I determined the radioactivity present in the precipitate with a gamma counter (Cobra II, Packard). Concentrations were then calculated by comparison to the determined radioactivity of the known standards.

2.5.5 Radioimmunoassay for quantification of plasma corticosterone

Corticosterone was measured with a double antibody ^{125}I RIA kit (MP Biomedicals). The assay was performed as directed by the manufacturer. Plasma was diluted (1:200) with steroid diluent. Calibration standards against which samples were compared were reconstituted. ^{125}I-corticosterone was added to samples and standards prior to the addition of rabbit anti-corticosterone serum. The tubes were vortexed and incubated at room temperature (22-25°C) for 2 hr. Goat anti-rabbit γ-globulin was added to precipitate antigen-antibody complex. The tubes were centrifuged (1000 x g) for 15 min, followed by aspiration of the supernatant. Precipitate was counted on a gamma counter (Cobra II, Packard). Concentrations were then calculated by comparison to the determined radioactivity of the known standards.

2.6 Data Analysis

All data were reported as mean ± S.E.M. The level of significance was set at $p<0.05$ for all statistical tests.

Baseline values for heart rate, arterial pressure, body temperature and locomotor activity were calculated from the average of 20 min prior to the first microinjection. The change from baseline was determined by subtracting the baseline from a 20 min averaged value that included the peak response for that parameter. Within-group differences were analyzed with paired t-tests, while between-group differences were analyzed by one-way ANOVA with Bonferroni post-hoc tests.

Between-groups differences in plasma ACTH for experiments employing a single blood sample were analyzed by one-way ANOVA with Bonferroni post-hoc tests. For serial blood samples, within- and between-group differences in plasma ACTH and corticosterone were by analyzed by repeated measures ANOVA with Bonferroni post-hoc tests.

Values for the expression of Fos in the regions of PVN and the DMH represent the mean number of neurons staining positive for Fos in either three 25 μm coronal sections at 125 μm intervals (PVN) or two 30 μm coronal sections at 120 μm (DMH); seven 25 μm coronal sections at 125 μm intervals were used in the analysis of the raphe pallidus. Differences in the quantified expression of Fos were analyzed by either unpaired t-test or one-way ANOVA with Bonferroni post-hoc tests.

3.0 RESULTS

These experiments examined the role of neurons in the DMH in the regulation of acute inflammatory responses and responses evoked form the POA, focusing particularly on the HPA axis. First, I evaluated the effect of microinjections of muscimol, a neuronal inhibitor, into the DMH on physiological and behavioral responses evoked from the POA. I then evaluated the effect of similar microinjections on neuroendocrine responses elicited by systemic administration of either IL-1β or LPS.

3.1 Effect of microinjection of muscimol into the DMH on responses evoked from the POA

Microinjection of muscimol into the POA has been shown to elicit tachycardia, hypertension, hyperthermia, and activation of the HPA axis, responses that mimic the acute phase response, as well as an increase in locomotor activity (Zaretsky et al., 2006). I tested the hypothesis that neuronal activity in the DMH is necessary in the physiological and behavioral responses evoked from the POA by inhibiting neurons in the DMH with muscimol either *prior to* or *after* the injection of muscimol into the POA.

3.1.1 Prior microinjection of muscimol into the DMH

To evaluate the role of neuronal activity in the DMH in the increases in heart rate, arterial pressure, body temperature, locomotor activity, plasma ACTH, and expression of Fos in the PVN and RP evoked from the POA, I microinjected muscimol or vehicle bilaterally into the DMH shortly before the injection of muscimol or vehicle into the POA. The site of microinjection was confirmed by post-mortem analysis (Fig. 1-3). The approximate location of injections into the POA for all animals receiving pretreatment

Figure 1

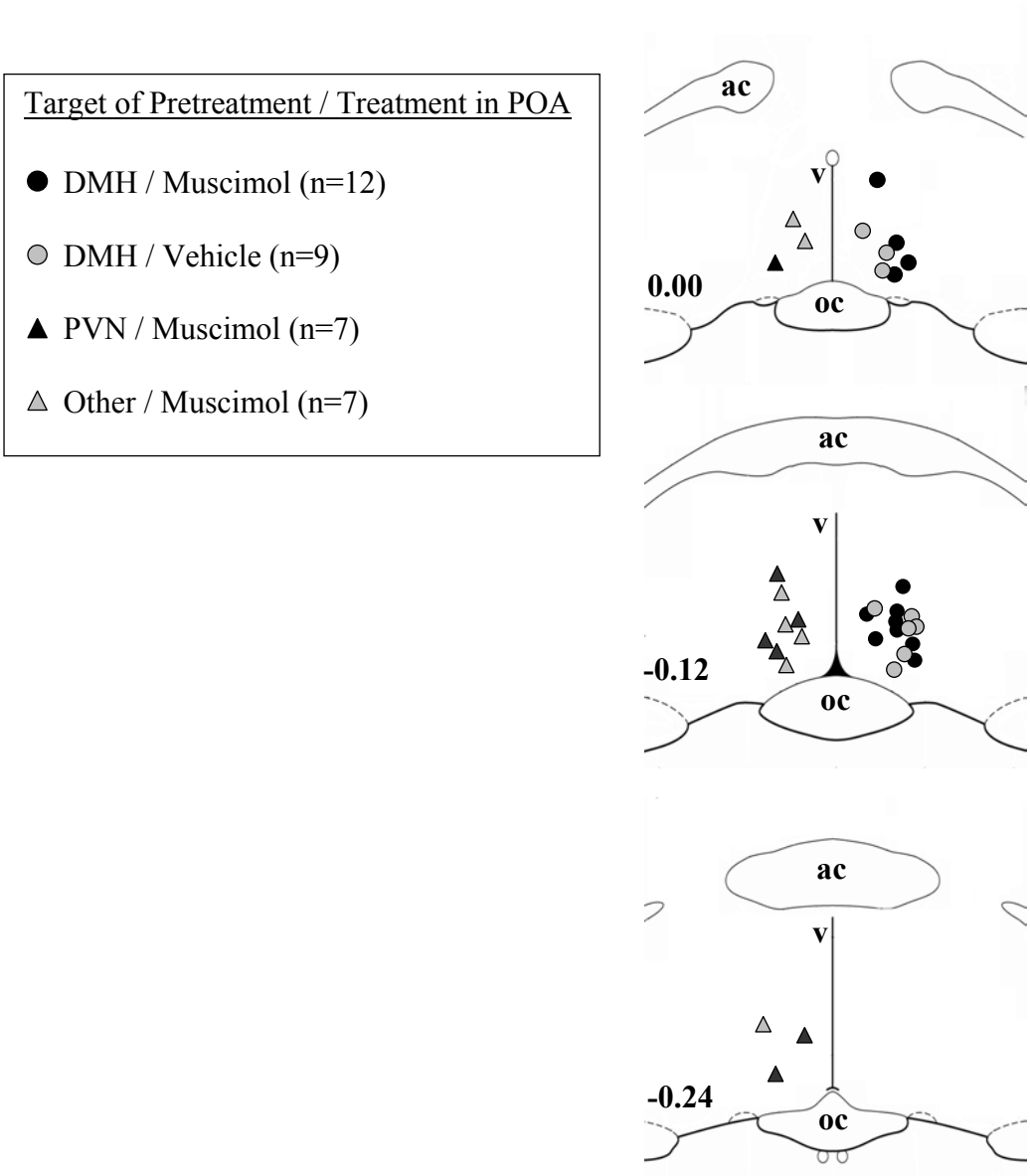

Figure 1. Schematic coronal sections adapted from the atlas of Paxinos and Watson (2007) illustrating approximate location of intrapreoptic injections in animals with DMH-targeted cannulae receiving injections of muscimol (closed circles) or vehicle (grey circles) into the POA and for animals with cannulae targeted outside the DMH within the borders of the PVN (black triangles) or in other adjacent areas (grey triangles) receiving injections of muscimol into the POA. Numbers indicate the distance in millimeters from bregma. Abbreviations: anterior commissure (ac); optic chiasm (oc); third ventricle (v).

Figure 2

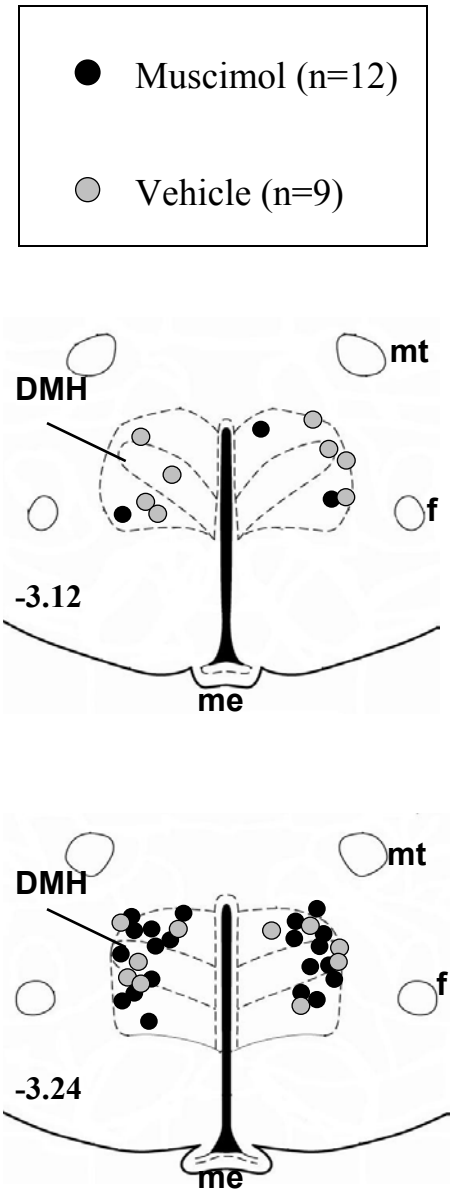

Figure 2. Schematic coronal sections adapted from the atlas of Paxinos and Watson (2007) illustrating approximate location of intra-DMH injections in animals receiving microinjection of muscimol (closed circles) or vehicle (grey circles) into the POA. Numbers indicate the distance in millimeters from bregma. Abbreviations: dorsomedial hypothalamus (DMH); fornix (f); mammillothalamic tract (mt); median eminence (me).

Figure 3

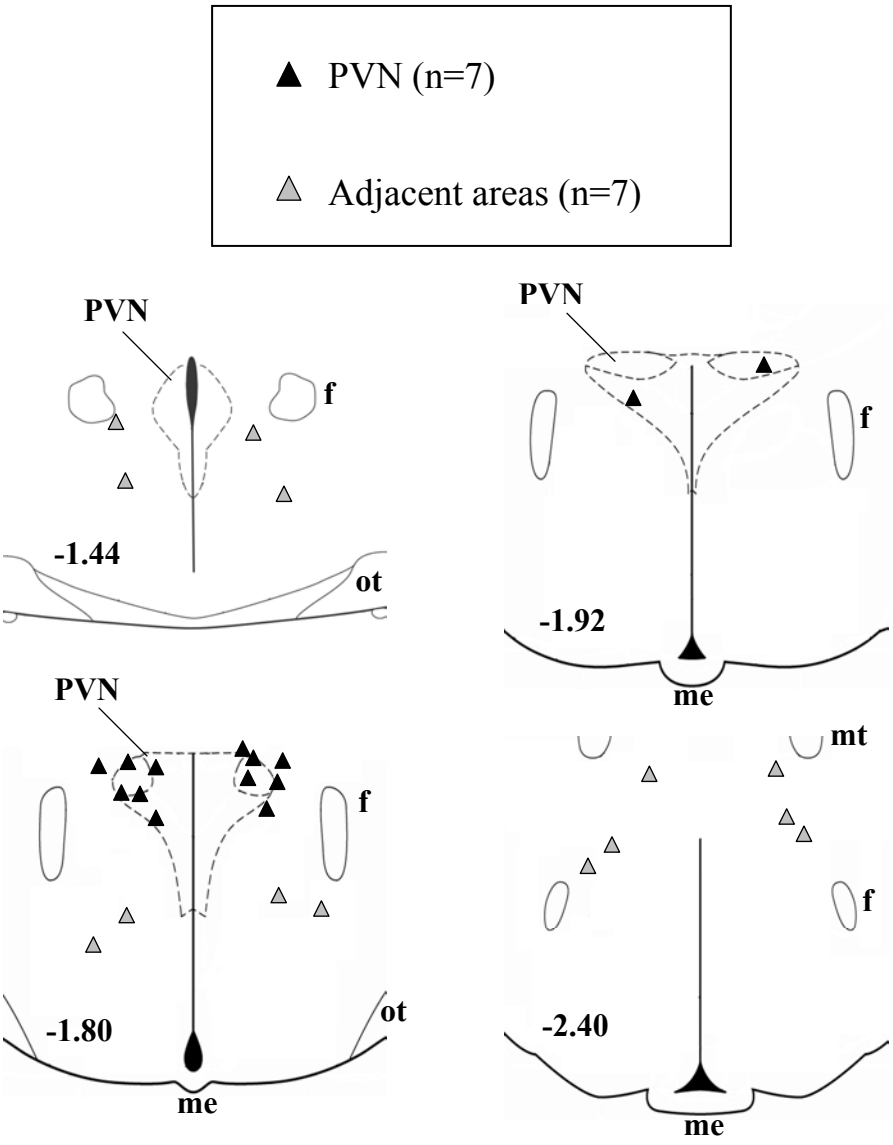

Figure 3. Schematic coronal sections adapted from the atlas of Paxinos and Watson (2007) illustrating approximate location of site of injections in animals with cannulae targeted outside the DMH within the borders of the PVN (black triangles) or in other adjacent areas (grey triangles) receiving injections of muscimol into the POA. Numbers indicate the distance in millimeters from bregma. Abbreviations: fornix (f); mammillothalamic tract (mt); median eminence (me); optic tract (ot); paraventricular nucleus (PVN).

into the DMH, PVN, or other adjacent sites is depicted in Fig. 1. Fig. 2 illustrates the approximate location of injections that were targeted to the DMH. No difference in the location of injections within the DMH was apparent between animals that received muscimol or vehicle in the POA. Lastly, the approximate location of injections that were targeted to either the PVN or other adjacent sites is demonstrated in Fig. 3. Microinjections were considered outside the DMH only if both left and right injections were outside the borders of the dorsomedial hypothalamic nucleus as defined by the atlas of Paxinos and Watson (2007).

Effect on autonomic and behavioral responses

There were no significant differences in basal heart rate, arterial pressure, body temperature, or locomotor activity in any group prior to microinjections (one-way ANOVA, Table 2).

Animals receiving microinjections of vehicle into both the DMH and POA (n=9) displayed modest increases in heart rate, arterial pressure, body temperature, and locomotor activity (Fig. 4-7). Rats pretreated with vehicle in the DMH after microinjection of muscimol into the POA (n=12) exhibited immediate and dramatic increases in heart rate, arterial pressure, body temperature, and locomotor activity, replicating previously published findings (Zaretsky et al., 2006). Maximal increases in heart rate, arterial pressure, and locomotor activity were reached approximately 10 min after injection of muscimol into the POA. Body temperature increased more slowly, reaching a maximum approximately 20 min after injection of muscimol into the POA. The mean maximal increases in heart rate, arterial pressure, body temperature, and locomotor activity (+142 ± 8 beats/min, +15 ± 2 mmHg, +1.8 ± 0.1°C, and +12 ± 2

Table 2

Treatment: (POA / DMH) Microinjection into DMH:	**HR** (beats/min)	**BP** (mmHg)	**BT** (°C)	**LA** (counts/min)
Vehicle / Vehicle (n=9)	340 ± 7	128 ± 3	37.7 ± 0.2	1.3 ± 0.5
Muscimol / Vehicle (n=12)	356 ± 7	117 ± 4	37.4 ± 0.2	0.6 ± 0.2
Muscimol / Muscimol (n=12)	351 ± 8	117 ± 3	37.6 ± 0.2	1.7 ± 0.5
Vehicle / Muscimol (n=9)	340 ± 8	125 ± 5	37.5 ± 0.2	0.6 ± 0.4
Microinjection outside DMH				
Muscimol / Vehicle (n=14)	359 ± 9	132 ± 6	37.9 ± 0.3	0.5 ± 0.1
Muscimol / Muscimol (n=14)	350 ± 7	131 ± 6	37.8 ± 0.2	0.4 ± 0.2

Table 2. Mean (±SEM) baseline heart rate (HR), arterial pressure (BP), body temperature (BT), and locomotor activity (LA) in conscious rats just prior to microinjections into the POA and DMH as indicated. There were no significant differences for any parameter among any of the groups (one-way ANOVA; $p>0.05$).

Figure 4

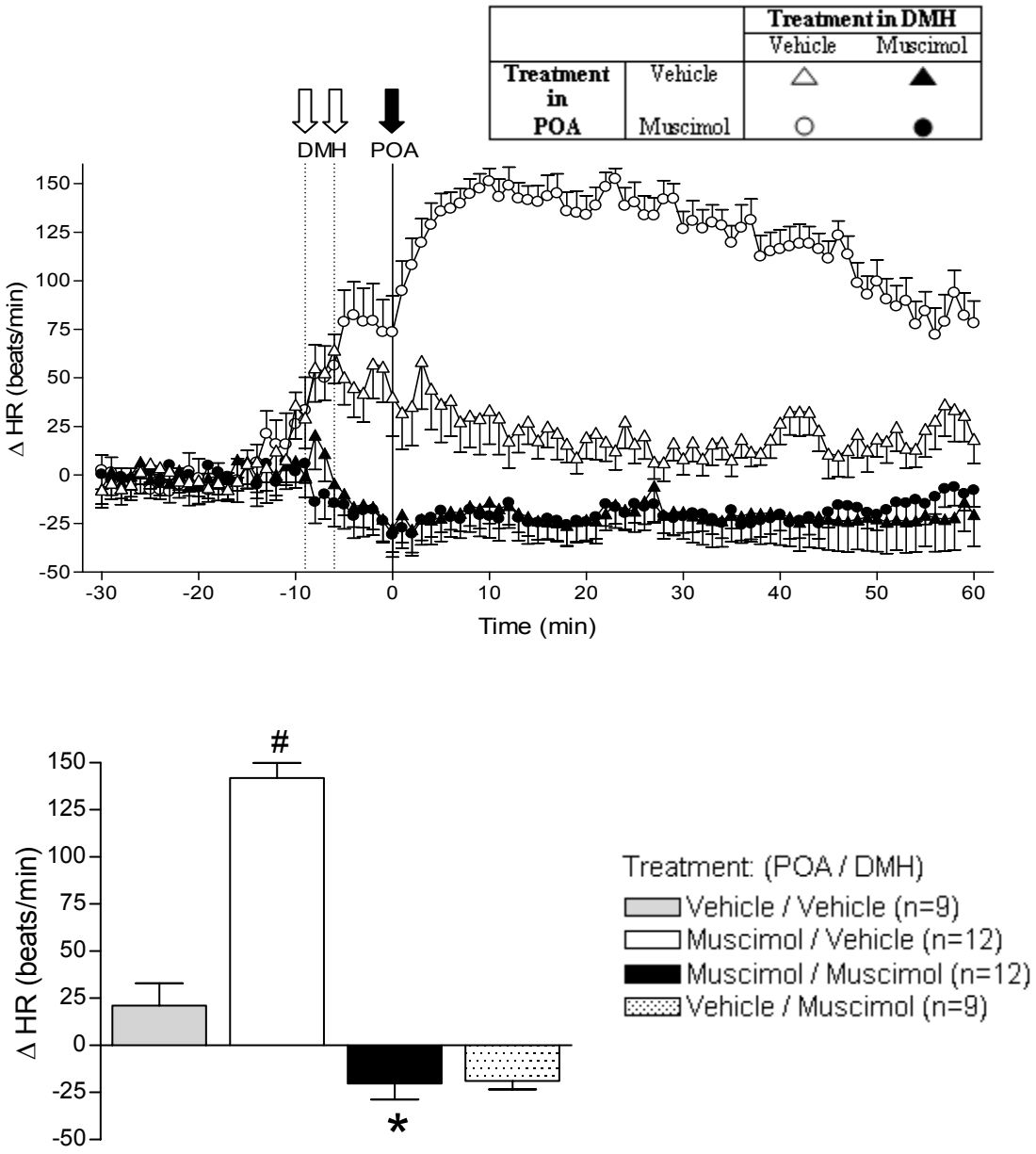

Figure 4. (Top) Mean (±SEM) heart rate (HR) after bilateral microinjection of muscimol or vehicle into the DMH (open arrows) and microinjection of either muscimol or vehicle into the POA (closed arrow). (Bottom) Mean heart rate changes from baseline (±SEM) averaged over a time interval that encompassed the maximal response elicited by microinjection of the substances (10 to 29 min). (#) Significantly greater than corresponding values for vehicle/vehicle controls; (*) significantly less than corresponding values for animals pretreated with vehicle receiving muscimol in the POA (one-way ANOVA with Bonferroni post-hoc tests; $p<0.05$).

Figure 5

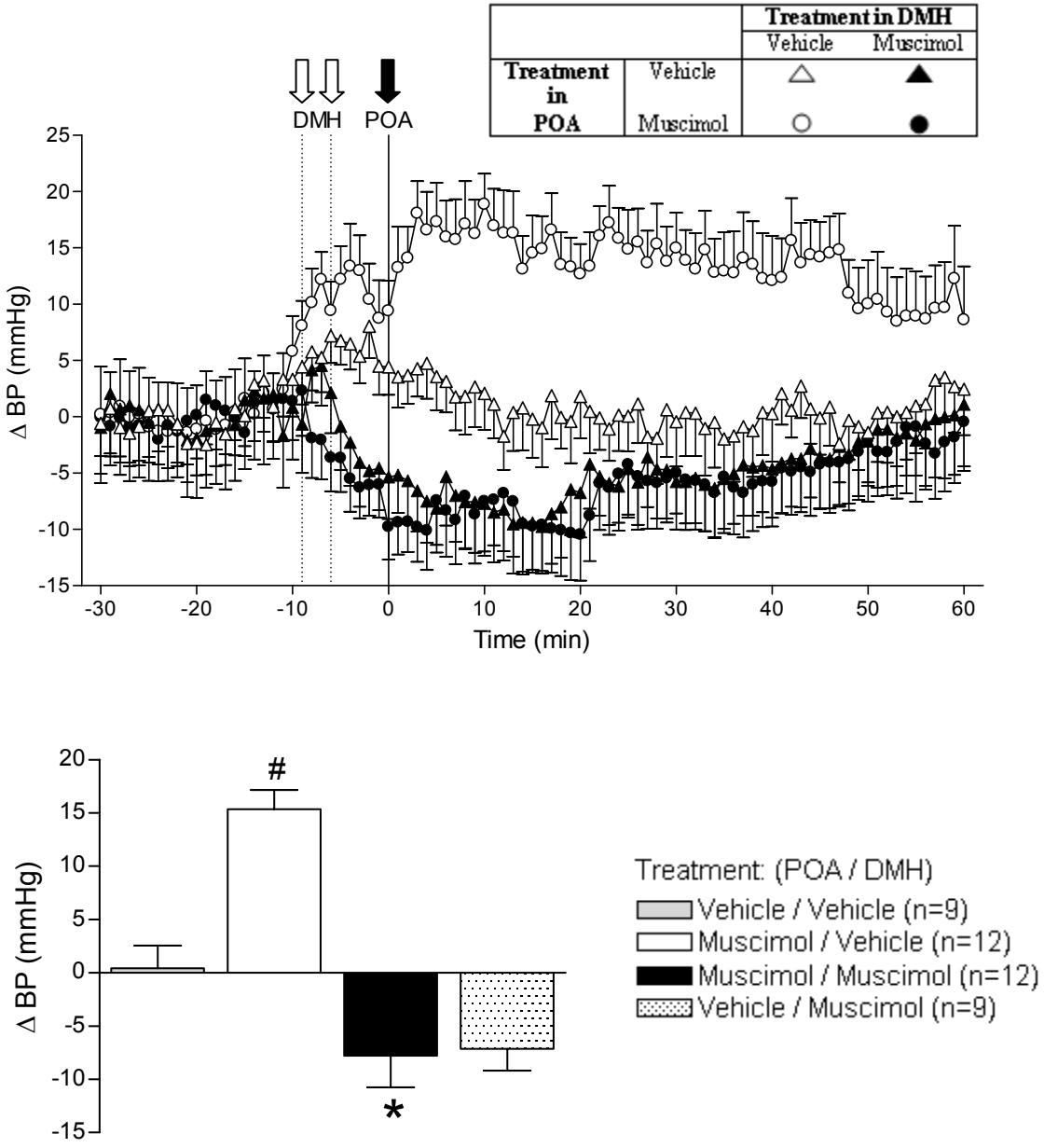

Figure 5. (Top) Mean (±SEM) arterial pressure (BP) after bilateral microinjection of muscimol or vehicle into the DMH (open arrows) and microinjection of either muscimol or vehicle into the POA (closed arrow). (Bottom) Mean arterial pressure changes from baseline (±SEM) averaged over a time interval that encompassed the maximal response elicited by microinjection of the substances (10 to 29 min). (#) Significantly greater than corresponding values for vehicle/vehicle controls; (*) significantly less than corresponding values for animals pretreated with vehicle receiving muscimol in the POA (one-way ANOVA with Bonferroni post-hoc tests; $p<0.05$).

Figure 6

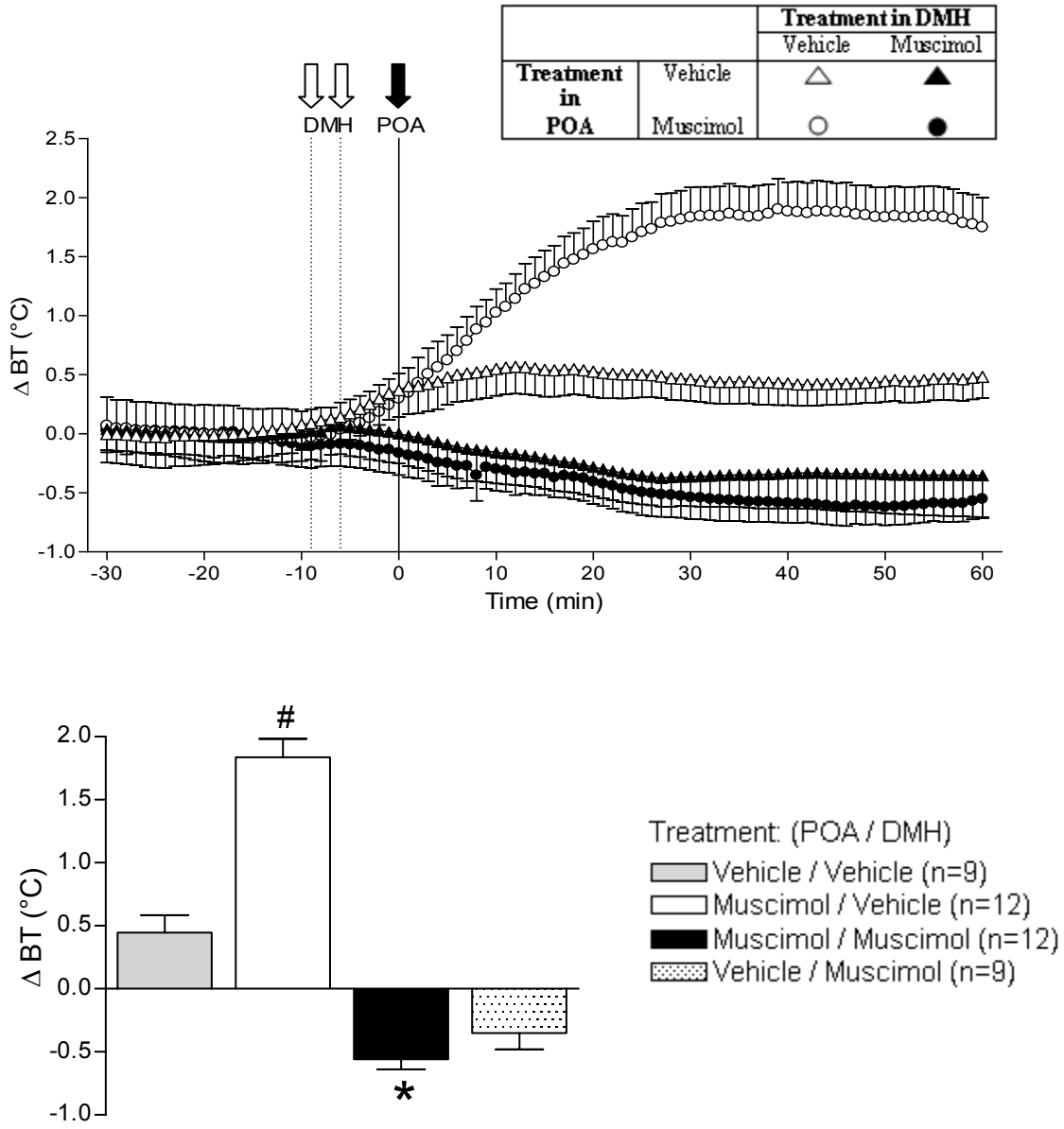

Figure 6. (Top) Mean (±SEM) body temperature (BT) after bilateral microinjection of muscimol or vehicle into the DMH (open arrows) and microinjection of either muscimol or vehicle into the POA (closed arrow). (Bottom) Mean body temperature changes from baseline (±SEM) averaged over a time interval that encompassed the maximal response elicited by microinjection of the substances (25 to 44 min). (#) Significantly greater than corresponding values for vehicle/vehicle controls; (*) significantly less than corresponding values for animals pretreated with vehicle receiving muscimol in the POA (one-way ANOVA with Bonferroni post-hoc tests; $p<0.05$).

Figure 7

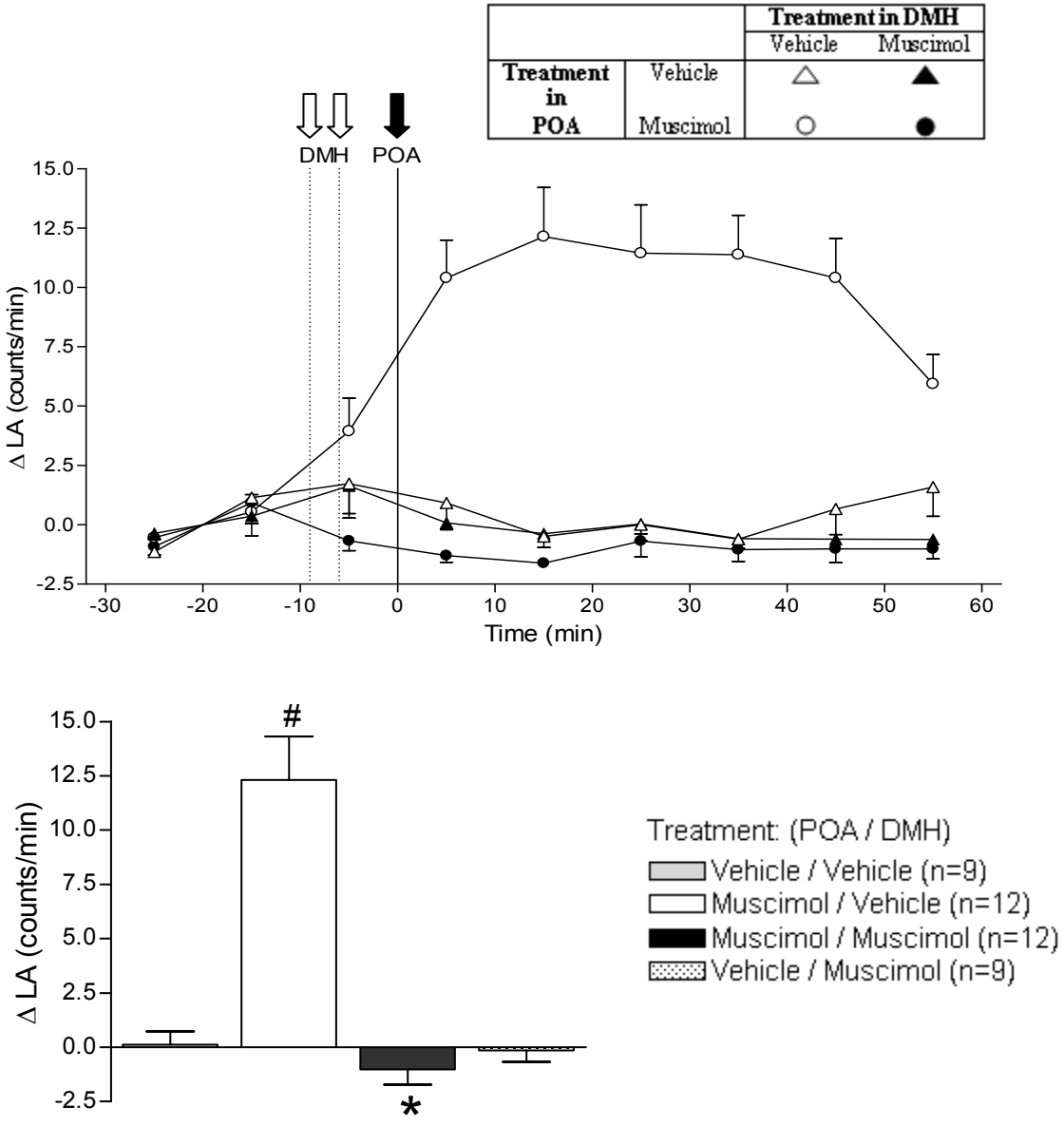

Figure 7. (Top) Mean (±SEM) locomotor activity (LA) after bilateral microinjection of muscimol or vehicle into the DMH (open arrows) and microinjection of either muscimol or vehicle into the POA (closed arrow). (Bottom) Mean locomotor activity changes from baseline (±SEM) averaged over a time interval that encompassed the maximal response elicited by microinjection of the substances (10 to 29 min). (#) Significantly greater than corresponding values for vehicle/vehicle controls; (*) significantly less than corresponding values for animals pretreated with vehicle receiving muscimol in the POA (one-way ANOVA with Bonferroni post-hoc tests; $p<0.05$).

counts/min, respectively; Fig. 4-7) in these animals were significantly greater than the corresponding increases in animals receiving microinjections of only vehicle (+21 ± 12 beats/min, 0 ± 2 mmHg, +0.4 ± 0.1°C, 0 ± 1 counts/min).

Rats pretreated with muscimol in the DMH prior to injection of vehicle into the POA (n=9) exhibited maximal changes in heart rate, arterial pressure, and body temperature (-19 ± 4 beats/min, -7 ± 2 mmHg, -0.4 ± 0.1°C; Fig. 4-7) that tended to be lower than baselines. Locomotor activity did not change from baseline in these animals. With the exception of locomotor activity, these maximal changes were also significantly less than corresponding levels from control animals receiving injections of vehicle into both the DMH and the POA.

Animals microinjected with muscimol in the POA after pretreatment of the DMH with muscimol exhibited significantly lower mean increases in heart rate, arterial pressure, body temperature, and locomotor activity than those in rats pretreated with vehicle (-20 ± 9 beats/min, -8 ± 3 mmHg, -0.6 ± 0.1°C, -1 ± 1 counts/min versus +142 ± 8 beats/min, +15 ± 2 mmHg, +1.8 ± 0.1°C, and +12 ± 2 counts/min; n=12; Fig. 4-7). These changes were similar to those seen after microinjection of muscimol into the DMH in rats receiving vehicle in the POA.

The anatomical specificity of the effect of muscimol in the DMH on the increases in heart rate, arterial pressure, body temperature, and locomotor activity elicited from the POA was addressed by microinjecting muscimol into brain areas just outside of the DMH (Fig. 3). When vehicle was injected into areas outside the DMH, microinjection of muscimol into the POA (n=14) evoked mean increases in heart rate, arterial pressure, body temperature, and locomotor activity (+132 ± 14 beats/min, +10 ± 4 mmHg, +1.5 ±

Figure 8

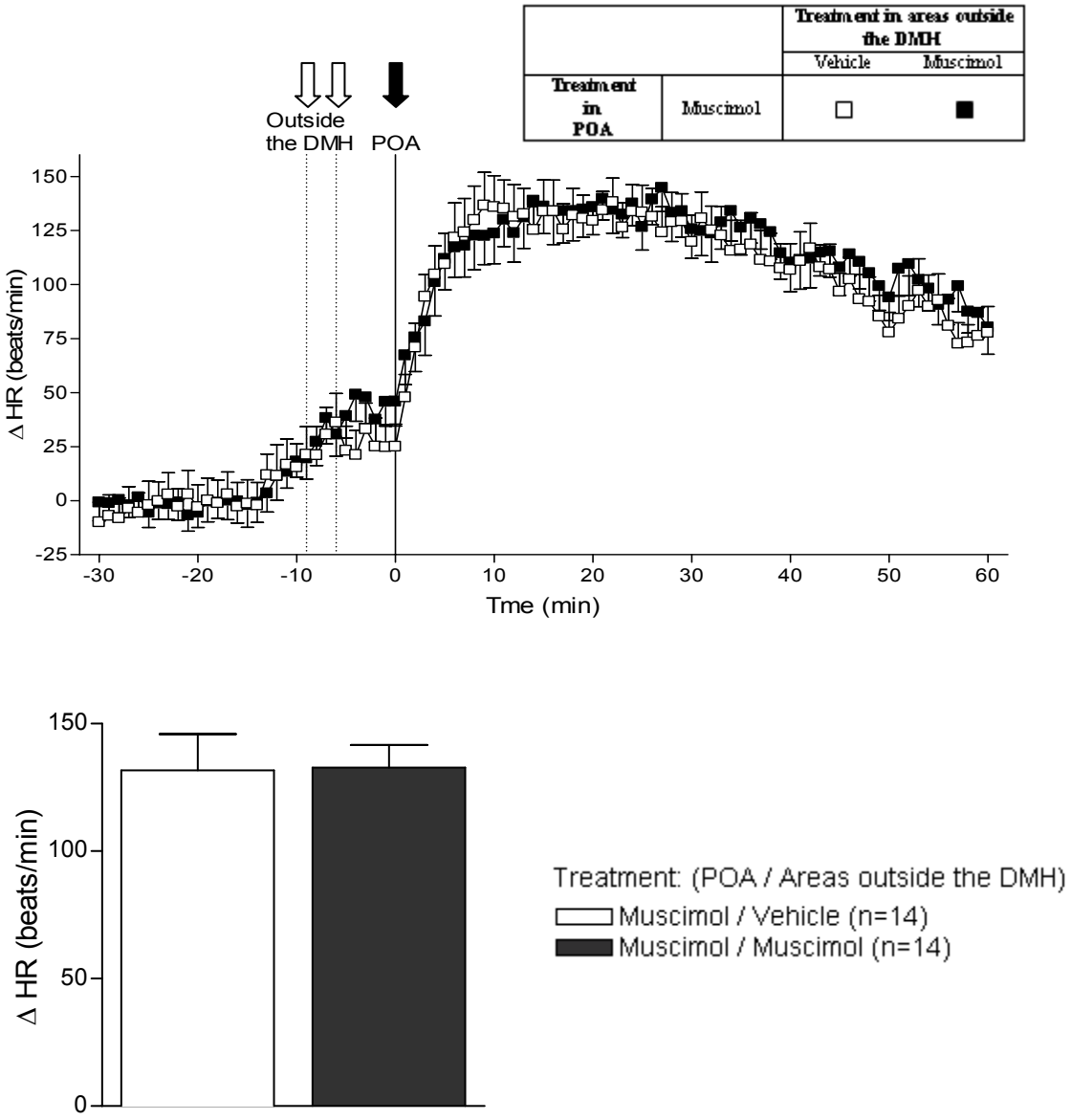

Figure 8. (Top) Mean (±SEM) heart rate (HR) after microinjection of either muscimol (closed squares) or vehicle (open squares) into brain regions outside the DMH open arrows) in rats microinjected with muscimol into the POA (closed arrow). (Bottom) Mean heart rate changes from baseline (±SEM) averaged over a time interval that encompassed the maximal response elicited by microinjection of the substances (10 to 29 min). No significant difference noted (paired t-test; $p>0.05$).

Figure 9

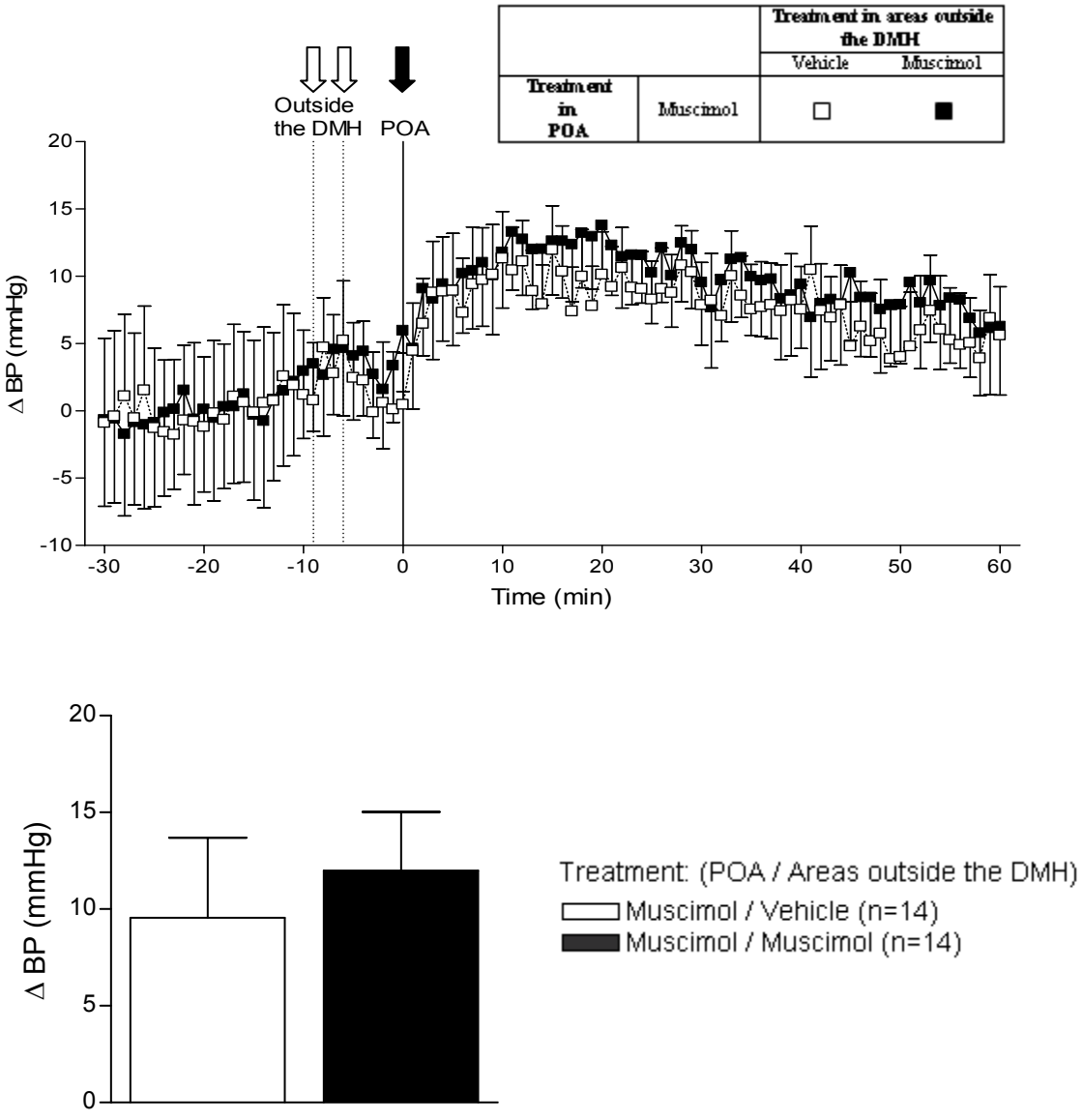

Figure 9. (Top) Mean (±SEM) arterial pressure (BP) after microinjection of either muscimol (closed squares) or vehicle (open squares) into brain regions outside the DMH (open arrows) in rats microinjected with muscimol into the POA (closed arrow). (Bottom) Mean arterial pressure changes from baseline (±SEM) averaged over a time interval that encompassed the maximal response elicited by microinjection of the substances (10 to 29 min). No significant difference noted (paired t-test; $p>0.05$).

Figure 10

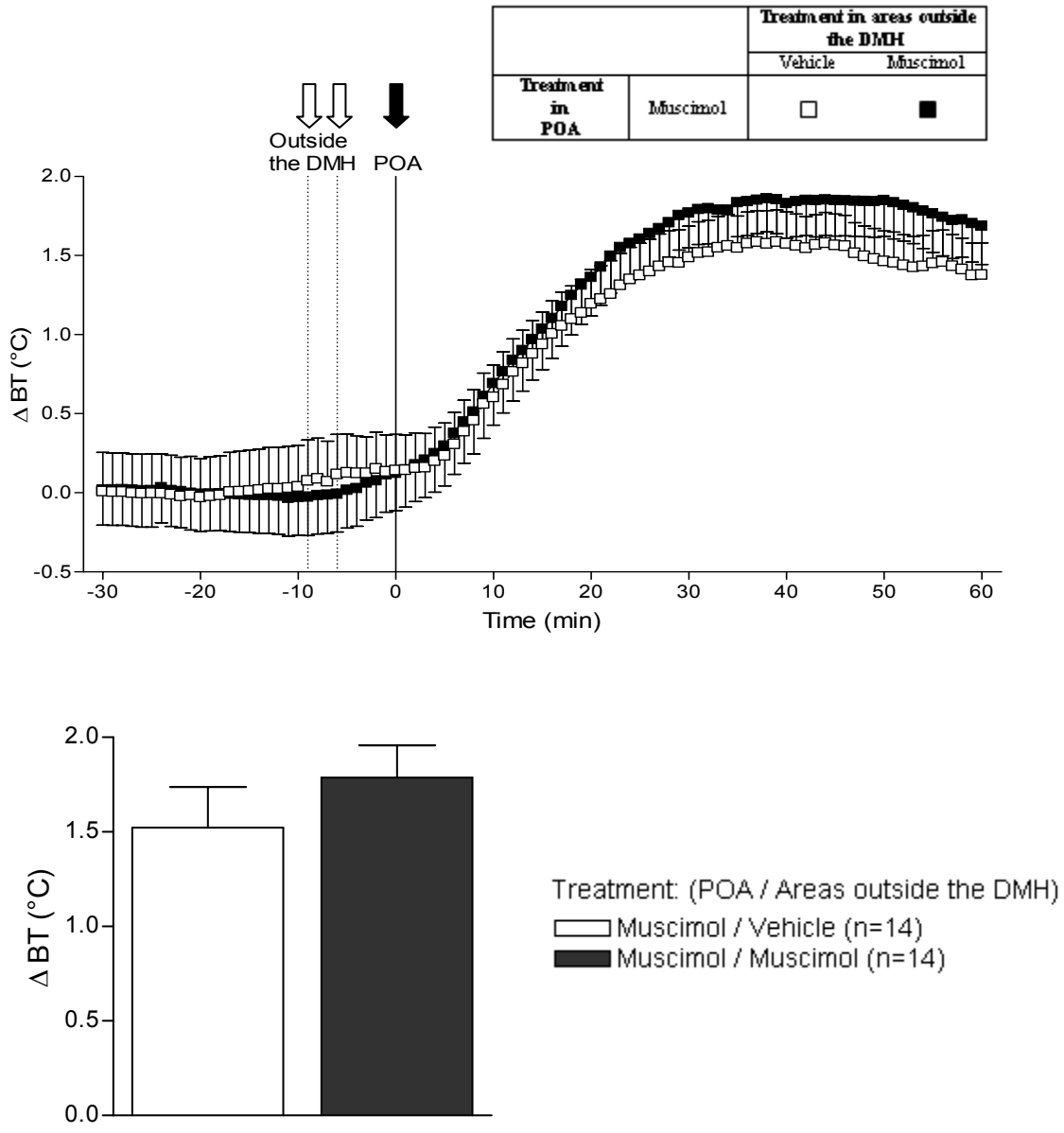

Figure 10. (Top) Mean (±SEM) body temperature (BT) after microinjection of either muscimol (closed squares) or vehicle (open squares) into brain regions outside the DMH (open arrows) in rats microinjected with muscimol into the POA (closed arrow). (Bottom) Mean body temperature changes from baseline (±SEM) averaged over a time interval that encompassed the maximal response elicited by microinjection of the substances (25 to 44 min). No significant difference noted (paired t-test; $p>0.05$).

Figure 11

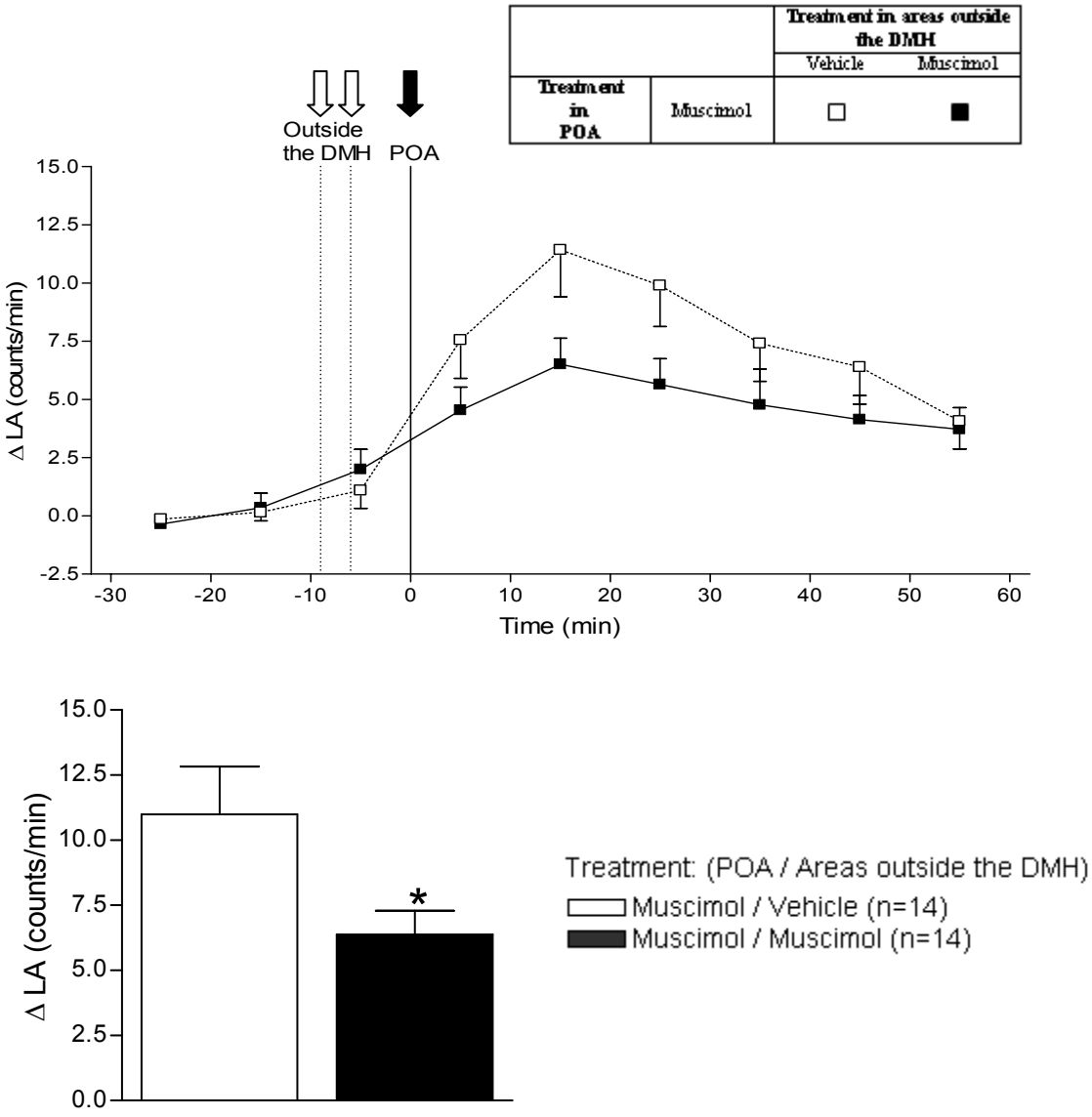

Figure 11. (Top) Mean (±SEM) locomotor activity (LA) after microinjection of either muscimol (closed squares) or vehicle (open squares) into brain regions outside the DMH (open arrows) in rats microinjected with muscimol into the POA (closed arrow). (Bottom) Mean locomotor activity changes from baseline (±SEM) averaged over a time interval that encompassed the maximal response elicited by microinjection of the substances (10 to 29 min). (*) Significantly different from corresponding values after microinjection of vehicle into the DMH (paired t-test; $p<0.05$).

0.2°C, +11 ± 2 counts/min; Fig. 8-11) that were similar to those seen when vehicle was injected into the DMH (Fig. 4-7). With the exception of locomotor activity, these increases were not significantly different from those seen when muscimol was microinjected into areas outside the DMH (+133 ± 9 beats/min, +12 ± 3 mmHg, +1.8 ± 0.2°C, +6 ± 1 counts/min, n=14).

Effect on activation of the hypothalamic-pituitary-adrenal axis

The concentration of ACTH in the plasma of blood samples taken 15 min after microinjection into the POA was determined as a measure of HPA axis activity. Animals pretreated with vehicle in the DMH displayed significantly greater levels of plasma ACTH 15 min after the microinjection of muscimol into the POA (390 ± 65 pg/mL; n=12) than those seen after the microinjection of vehicle into the POA (72 ± 15 pg/mL; n=9; Fig. 12), supporting published data (Zaretsky et al., 2006). Rats microinjected with vehicle into the POA displayed similar plasma levels of ACTH regardless of pretreatment of the DMH with vehicle or muscimol (n=9), levels similar to those seen in unstressed rats (Anseloni et al., 2005; Faraday et al., 2005).

Compared to pretreated with vehicle, microinjection of muscimol into the DMH significantly attenuated the increase in plasma levels of ACTH 15 min after microinjection of muscimol into the POA (97 ± 30 pg/mL versus 390 ± 65 pg/mL, n=12; Fig. 12). Rats pretreated with muscimol at sites outside the DMH but within the borders of the PVN also had levels of plasma ACTH 15 min after the injection of muscimol in the POA that were significantly lower than those of rats pretreated with vehicle (168 ± 43 pg/mL versus 728 ± 120 pg/mL; n=7; Fig. 13). Interestingly, microinjection of muscimol into the POA evoked significantly greater increases in plasma ACTH in animals

Figure 12

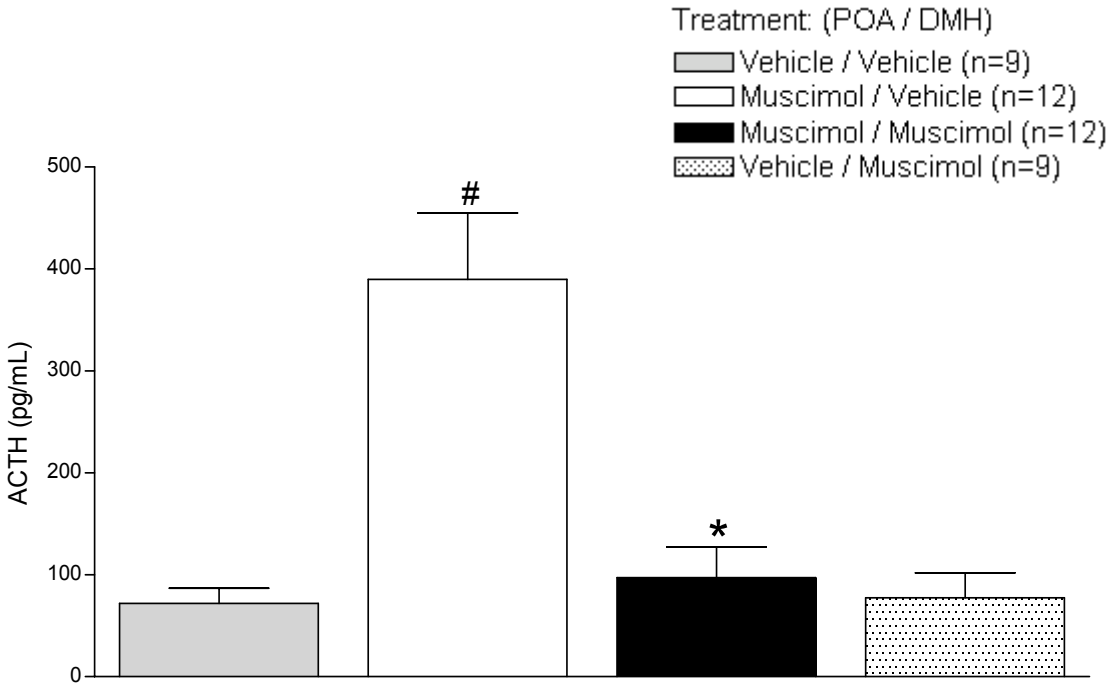

Figure 12. Mean (±SEM) plasma levels of ACTH in blood samples taken 15 min after microinjection of muscimol or vehicle into the POA in rats pretreated with bilateral microinjections of either muscimol or vehicle in the DMH. (#) Significantly greater than corresponding value in vehicle/vehicle controls; (*) significantly less than corresponding value in rats pretreated with vehicle receiving microinjection of muscimol in the POA (one-way ANOVA with Bonferroni post-hoc tests; $p<0.05$).

Figure 13

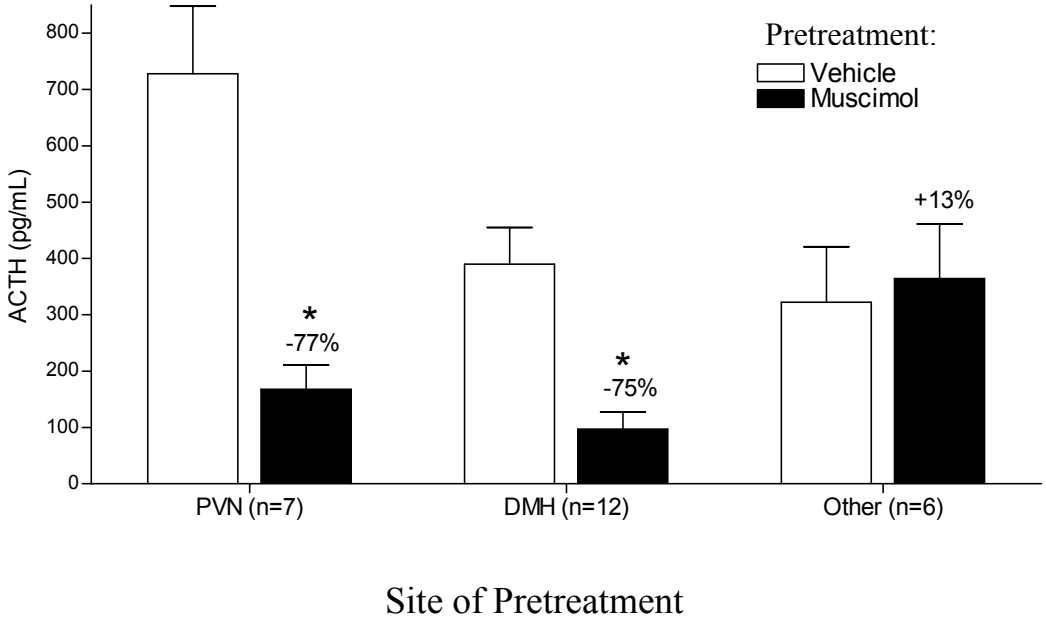

Figure 13. Mean (±SEM) plasma levels of ACTH in blood samples taken 15 min after microinjection of muscimol into the POA in rats pretreated with bilateral microinjections of muscimol or vehicle into the PVN (left), DMH (middle), or other adjacent areas (right). (*) Significantly less than corresponding pretreatment with vehicle (paired t-tests; $p<0.05$).

receiving vehicle in the PVN compared to those receiving vehicle in the DMH (728 ± 120 pg/mL versus 390 ± 65 pg/mL). However, the magnitude of the muscimol-induced suppression figured as the percent of the increase in plasma ACTH seen after vehicle treatment was nearly identical regardless of whether microinjections were targeted to the DMH or PVN (-75% versus -77%). However, when microinjections were targeted to adjacent hypothalamic sites between or just outside the DMH and PVN, increases in plasma ACTH seen after the injection of muscimol into the POA were equivalent after pretreatment with either muscimol or vehicle (364 ± 97 pg/mL versus 322 ± 98 pg/mL, n=6).

Effect on expression of Fos

Neurons positive for Fos, a commonly employed marker for neuronal activation, were observed in all animals regardless of treatment (Fig. 14). In rats pretreated with vehicle in the DMH, the numbers of Fos-positive neurons in the parvocellular (pPVN) and magnocellular (mPVN) regions of the PVN and in the raphe pallidus of rats receiving microinjections of muscimol into the POA were significantly greater than the numbers seen in rats receiving the microinjection of vehicle into the POA (Muscimol (n=3): pPVN-630 ± 7 neurons, mPVN-296 ± 12 neurons, raphe pallidus-140 ± 26 neurons; Vehicle (n=4): pPVN-133 ± 18 neurons, mPVN-52 ± 9 neurons, raphe pallidus-42 ± 9 neurons; Fig. 15). Animals pretreated with muscimol in the DMH exhibited significantly fewer neurons positive for Fos in the pPVN, mPVN, and raphe pallidus after injection of muscimol into the POA (pPVN-130 ± 16 neurons, mPVN-52 ± 10 neurons, raphe pallidus-43 ± 9 neurons; n=4) compared to animals pretreated with vehicle. In fact, the expression of Fos in the PVN and raphe pallidus observed after the pretreatment with

Figure 14

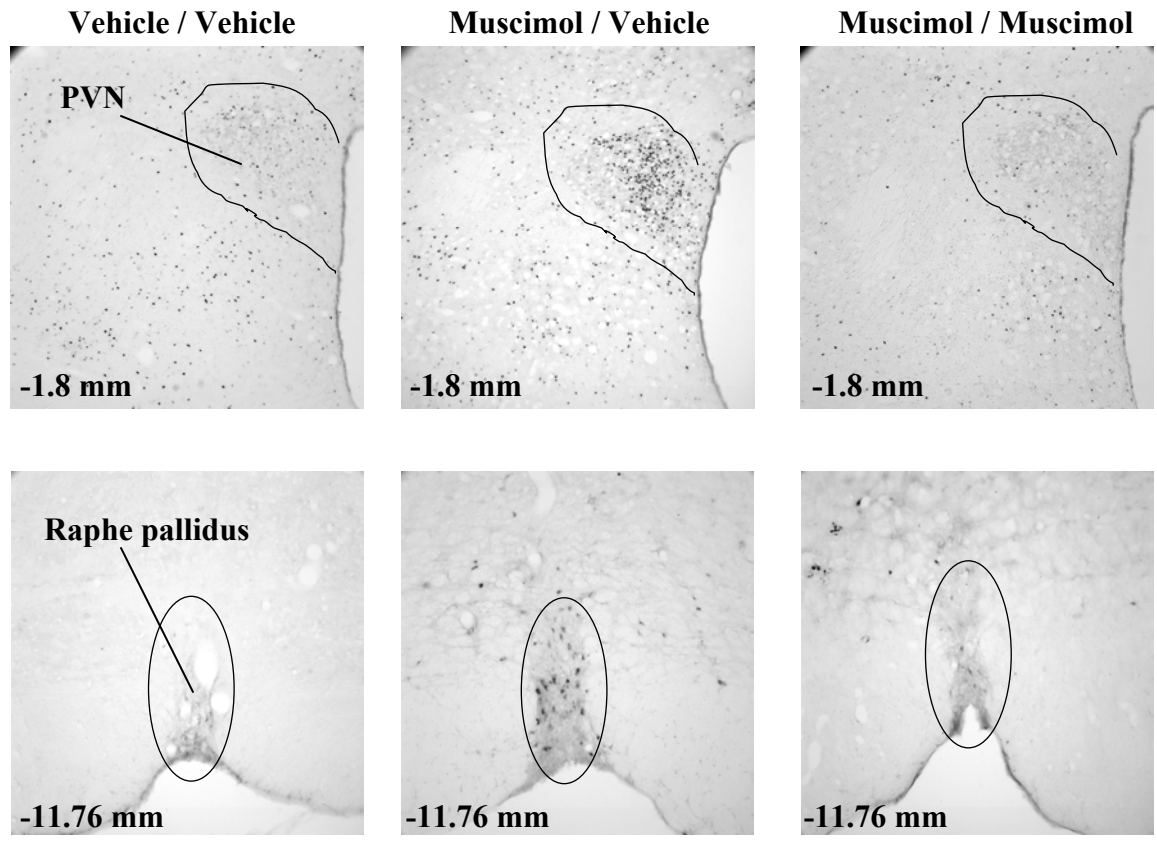

Figure 14. Representative 25 μm sections demonstrating the extent of Fos expression in the paraventricular nucleus of the hypothalamus (PVN) and the raphe pallidus in rats sacrificed 90 min after microinjections into the DMH and POA. Sections are from rats treated with (1) microinjection of vehicle in both the POA and the DMH (vehicle/vehicle; left), (2) microinjection of muscimol into the POA after microinjection of vehicle bilaterally into the DMH (muscimol/vehicle; middle), and (3) microinjection of muscimol into the POA after microinjection of muscimol bilaterally into the DMH (muscimol/muscimol; right). Numbers indicate distance in millimeters from bregma.

Figure 15

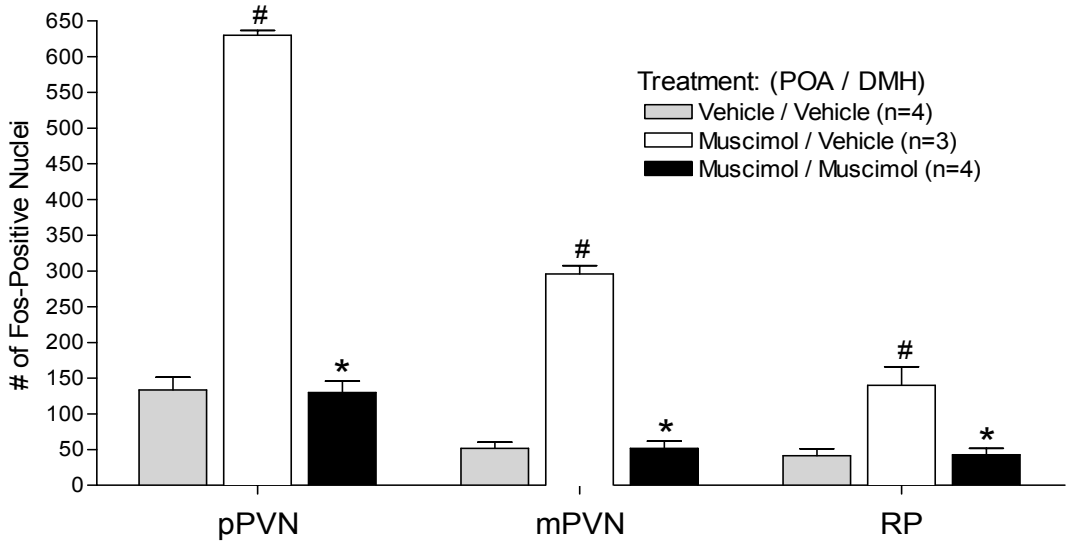

Figure 15. Mean (±SEM) number of nuclei positive for Fos in the parvocellular (pPVN) and magnocellular (mPVN) regions of the paraventricular nucleus of the hypothalamus and raphe pallidus (RP) in rats sacrificed 90 min after microinjections into the DMH and POA. Treatments (POA/DMH) displayed are vehicle/vehicle (grey), muscimol/vehicle (white), and muscimol/muscimol (black). Three 25 µm coronal sections at 125 µm intervals were used for analysis. (#) Significantly greater than corresponding value in vehicle/vehicle controls; (*) significantly less than corresponding value in rats pretreated with vehicle receiving muscimol in the POA (one-way ANOVA with Bonferroni post-hoc tests; $p<0.05$).

muscimol showed no difference from controls receiving vehicle in both the DMH and the POA.

3.1.2 Acute microinjection of muscimol into the DMH

The previous experiments examined the effect of *prior* bilateral microinjection of muscimol into the DMH on physiological and behavioral responses evoked by subsequent microinjections of muscimol into the POA. To examined the role of the DMH in sustaining ongoing physiological and behavioral responses evoked from the POA, I microinjected muscimol bilaterally into the DMH during the period of increased heart rate, arterial pressure, body temperature, and locomotor activity caused by microinjection of muscimol into the POA. The site of microinjection was confirmed by post-mortem analysis (Fig. 16).

Effect on autonomic and behavioral response

There were no significant differences in basal heart rate, arterial pressure, body temperature, or locomotor activity prior to any treatment (Table 3). After the microinjection of muscimol into the POA, all animals displayed nearly immediate increases in heart rate, arterial pressure, body temperature, and locomotor activity (Fig. 17-20). These increases were either sustained or continued to develop after bilateral microinjection of vehicle into the DMH. In contrast, microinjection of muscimol into the DMH was followed by an immediate reversal of the increases in heart rate and arterial pressure, a slightly delayed reversal of the increases in body temperature, and a clear blunting of the effect on locomotor activity. Thus, mean maximal changes from baseline heart rate, arterial pressure, body temperature, and locomotor activity were significantly less after injection of muscimol into the DMH compared with those seen after vehicle

Figure 16

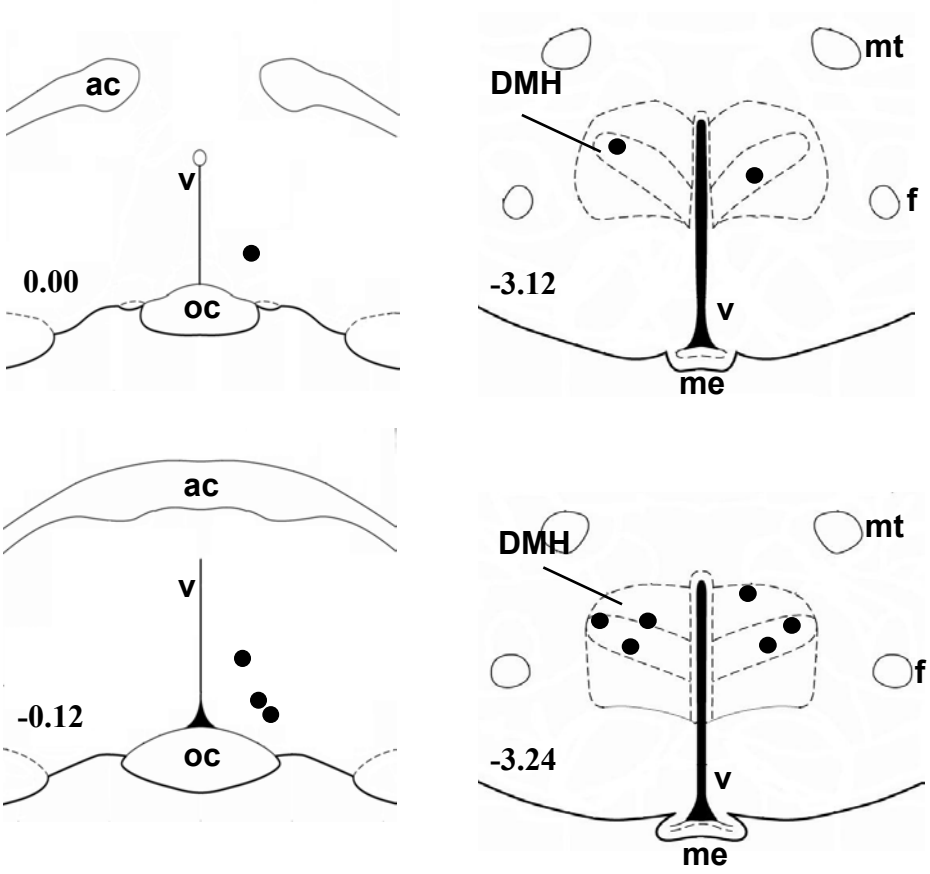

Figure 16. Schematic coronal sections adapted from the atlas of Paxinos and Watson (2007) illustrating approximate locations of sites of injection into the POA (left) and DMH (bilateral, right). Numbers indicate the distance in millimeters from bregma. Abbreviations: anterior commissure (ac); dorsomedial hypothalamus (DMH); fornix (f); mammillothalamic tract (mt); median eminence (me); optic chiasm (oc); paraventricular nucleus (PVN).

Table 3

Treatment to DMH	**HR** (beats/min)	**BP** (mmHg)	**BT** (°C)	**LA** (counts/min)
Muscimol (n=4)	340 ± 7	128 ± 3	37.7 ± 0.2	1.3 ± 0.5
Vehicle (n=4)	356 ± 7	117 ± 4	37.4 ± 0.2	0.6 ± 0.2

Table 3. Mean (±SEM) baseline heart rate (HR), arterial pressure (BP), body temperature (BT), and locomotor activity (LA) prior to microinjection of muscimol into the POA in conscious rats treated acutely with microinjections of muscimol or vehicle into the DMH. There were no significant differences in any parameter between the groups (paired-t-test; $p>0.05$).

Figure 17

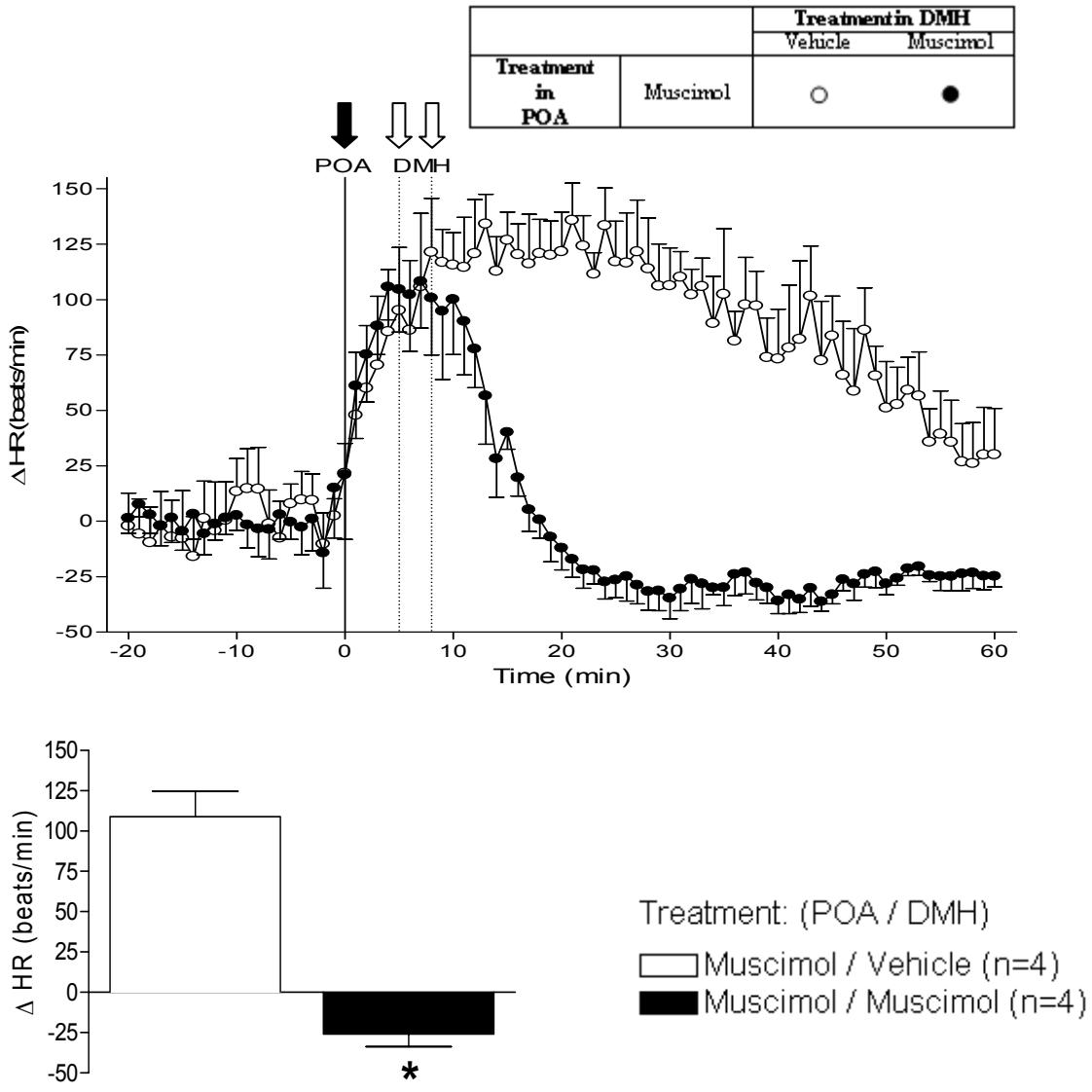

Figure 17. (Top) Mean (±SEM) heart rate (HR) after microinjection of muscimol into the POA (closed arrow) and acute microinjections of either muscimol (closed circles) or vehicle (open circles) bilaterally into the DMH (open arrows). (Bottom) Mean heart rate changes from baseline (±SEM) over a time interval that encompassed the maximal response evoked by microinjection of the substances (20 to 39 min). (*) Significantly different from corresponding value after microinjection of vehicle into the DMH (paired t-test; $p<0.05$).

Figure 18

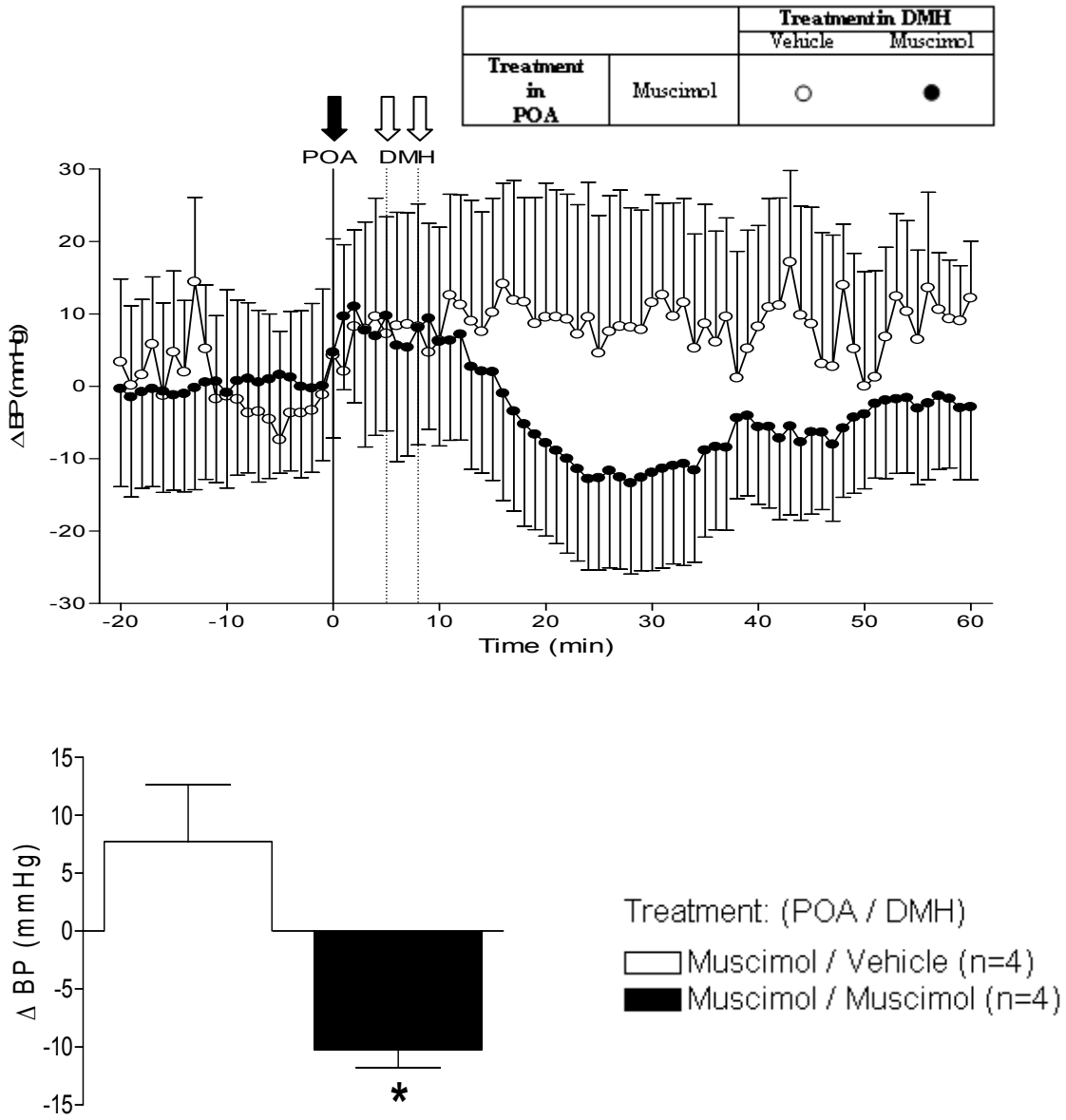

Figure 18. (Top) Mean (±SEM) arterial pressure (BP) after microinjection of muscimol into the POA (closed arrow) and acute microinjections of either muscimol (closed circles) or vehicle (open circles) bilaterally into the DMH (open arrows). (Bottom) Mean arterial pressure changes from baseline (±SEM) over a time interval that encompassed the maximal response evoked by microinjection of the substances (20 to 39 min). (*) Significantly different from corresponding value after microinjection of vehicle into the DMH (paired t-test; $p<0.05$).

Figure 19

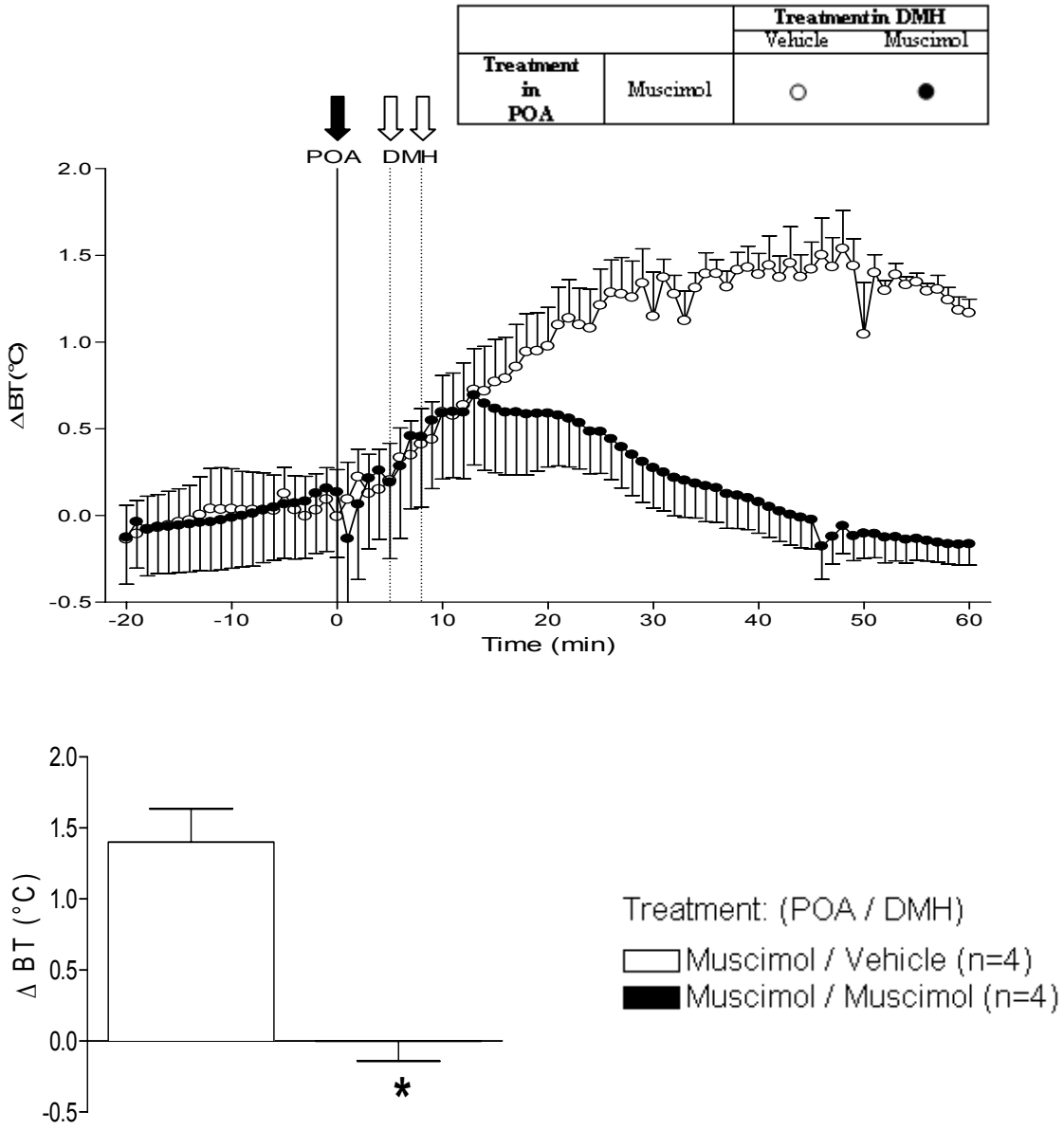

Figure 19. (Top) Mean (±SEM) body temperature (BT) after microinjection of muscimol into the POA (closed arrow) and acute microinjections of either muscimol (closed circles) or vehicle (open circles) bilaterally into the DMH (open arrows). (Bottom) Mean body temperature changes from baseline (±SEM) over a time interval that encompassed the maximal response evoked by microinjection of the substances (35 to 54 min). (*) Significantly different from corresponding value after microinjection of vehicle into the DMH (paired t-test; $p<0.05$).

Figure 20

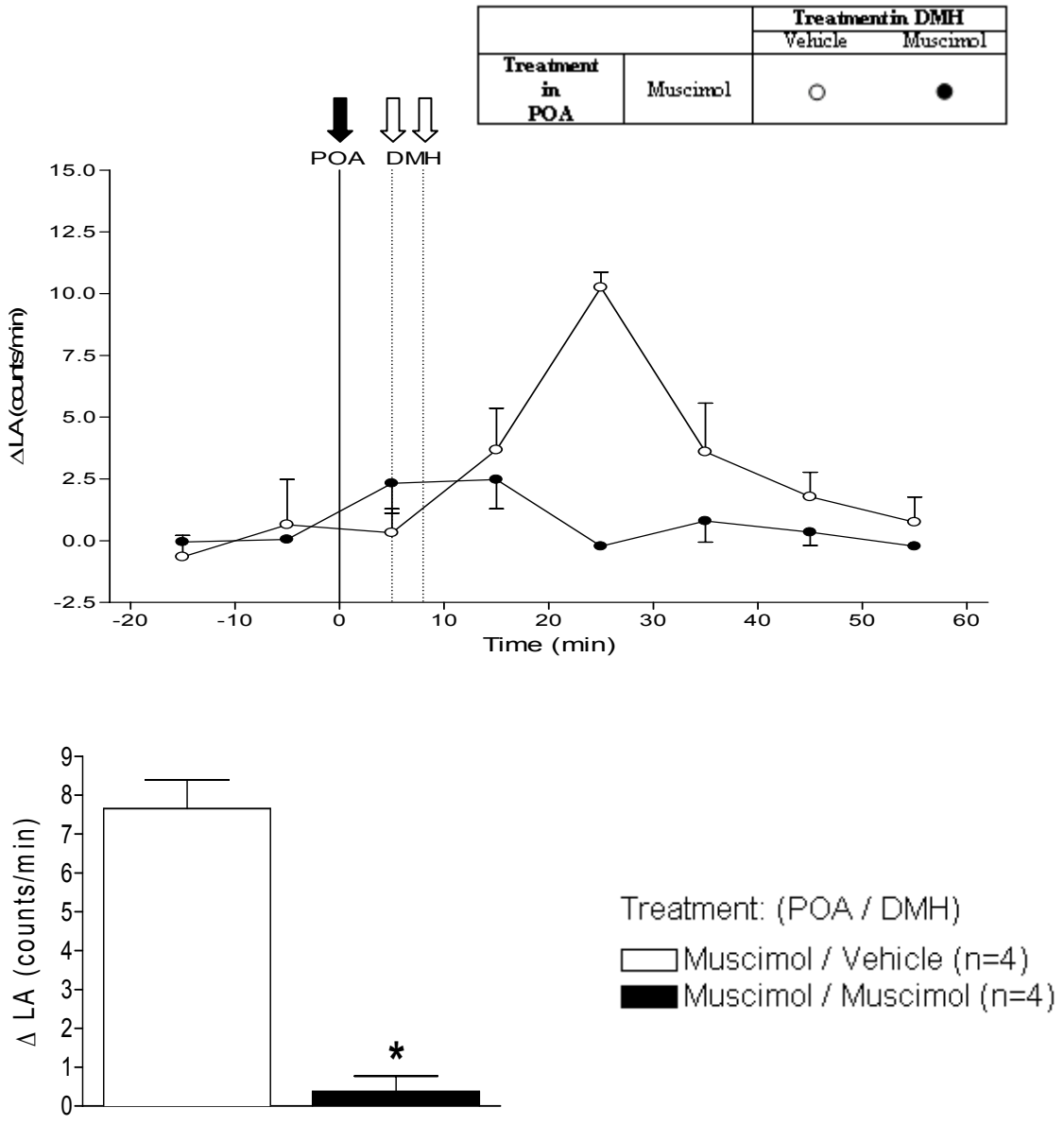

Figure 20. (Top) Mean (±SEM) locomotor activity(LA) after microinjection of muscimol into the POA (closed arrow) and acute microinjections of either muscimol (closed circles) or vehicle (open circles) bilaterally into the DMH (open arrows). (Bottom) Mean locomotor activity changes from baseline (±SEM) over a time interval that encompassed the maximal response evoked by microinjection of the substances (20 to 39 min). (*) Significantly different from corresponding value after microinjection of vehicle into the DMH (paired t-test; $p<0.05$).

(-26 ± 8 beats/min, -10 ± 2 mmHg, 0 ± 0.1°C, 0 ± 1 counts/min versus +109 ± 16 beats/min, +8 ± 5 mmHg, +1.4 ± 0.2°C, +8 ± 1 counts/min; n=4).

3.2 Effect of microinjection of muscimol into the DMH on the activation of the HPA axis evoked by systemic administration of IL-1β

The previous experiments explored the hypothesis that neurons in the DMH mediate a wide variety of responses evoked from the POA, including activation of the HPA axis. The POA has been implicated as a brain region through which inflammatory cytokines might act to produce a number of effects, including the activation of the HPA axis. One such cytokine is IL-1β which is known to stimulate the HPA axis. However, the role of neurons in the DMH in the activation of the HPA axis evoked by systemic administration of IL-1β has never been investigated. In the next set of experiments, I examined the effect of microinjections of muscimol bilaterally into the DMH on the increases in plasma levels of ACTH and corticosterone, and expression of Fos in the region of the PVN evoked by systemic administration of IL-1β.

3.2.1 Increase in plasma ACTH evoked by systemic administration of IL-1β

Before examining the role of neuronal activity in the DMH in the activation of the HPA axis evoked by systemically administered IL-1β, I determined the time course of the increase in plasma ACTH in conscious rats injected intra-arterially with IL-1β (1 μg/kg). Blood samples from arterial catheters were taken immediately prior to and at various times after the injection of IL-1β or vehicle. The results of this experiment were used to determine the appropriate time of blood sampling in future studies.

There was no significant difference in plasma ACTH prior to administration of IL-1β or vehicle (30 ± 18 pg/mL versus 59 ±19 pg/mL; n=4; Fig. 21), and at no point after injection of vehicle did animals exhibit a significant change from this baseline. However, after injection of IL-1β, plasma ACTH rapidly increased to reach a maximal level at the first post-injection blood sample (30 min), replicating published findings (Katsuura et al., 1990). Compared to vehicle, injection of IL-1β evoked significantly higher levels of ACTH at 30, 60, and 90 min after administration.

3.2.2 Change in the expression of Fos evoked by systemic injection of IL-1β

To test the hypothesis that systemic administration of IL-1β activates neurons in the DMH, I examined the expression of Fos in the DMH in rats sacrificed 90 min after the intra-arterial injection of IL-1β or vehicle. In addition, the expression of Fos in the PVN and plasma levels of ACTH and corticosterone were determined. Values for Fos represent the mean number of neurons staining positive for Fos per animal in two 30 μm coronal sections at 120 μm intervals in the DMH and PVN as defined by the atlas of Paxinos and Watson (2007).

Baseline levels of plasma ACTH and corticosterone prior to intra-arterial injection were similar in animals receiving IL-1β or vehicle (Fig. 22). Compared to controls (n=5), animals receiving injections of IL-1β (n=6) exhibited significantly greater plasma concentrations of ACTH (1162 ± 209 pg/mL after IL-1β versus 45 ± 5 pg/mL after vehicle) and corticosterone (344 ± 17 ng/mL after IL-1β versus 75 ± 31 ng/mL after vehicle) 30 min after injection. As was seen with the levels of plasma ACTH and corticosterone, a significantly greater number of neurons staining positive for Fos in the parvocellular and magnocellular regions of PVN were seen in rats sacrificed 90 min after

Figure 21

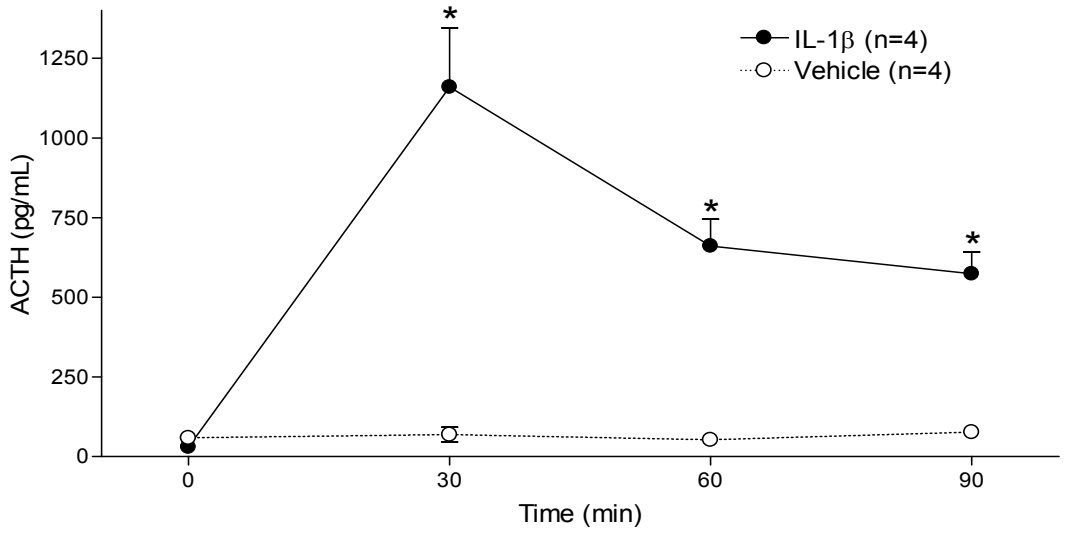

Figure 21. Mean (±SEM) plasma levels of ACTH immediately before and at various times after intra-arterial administration of IL-1β (1 μg/kg) or vehicle. (*) Significantly greater than corresponding levels after vehicle (repeated measures ANOVA with Bonferroni post-hoc tests; $p<0.05$).

Figure 22

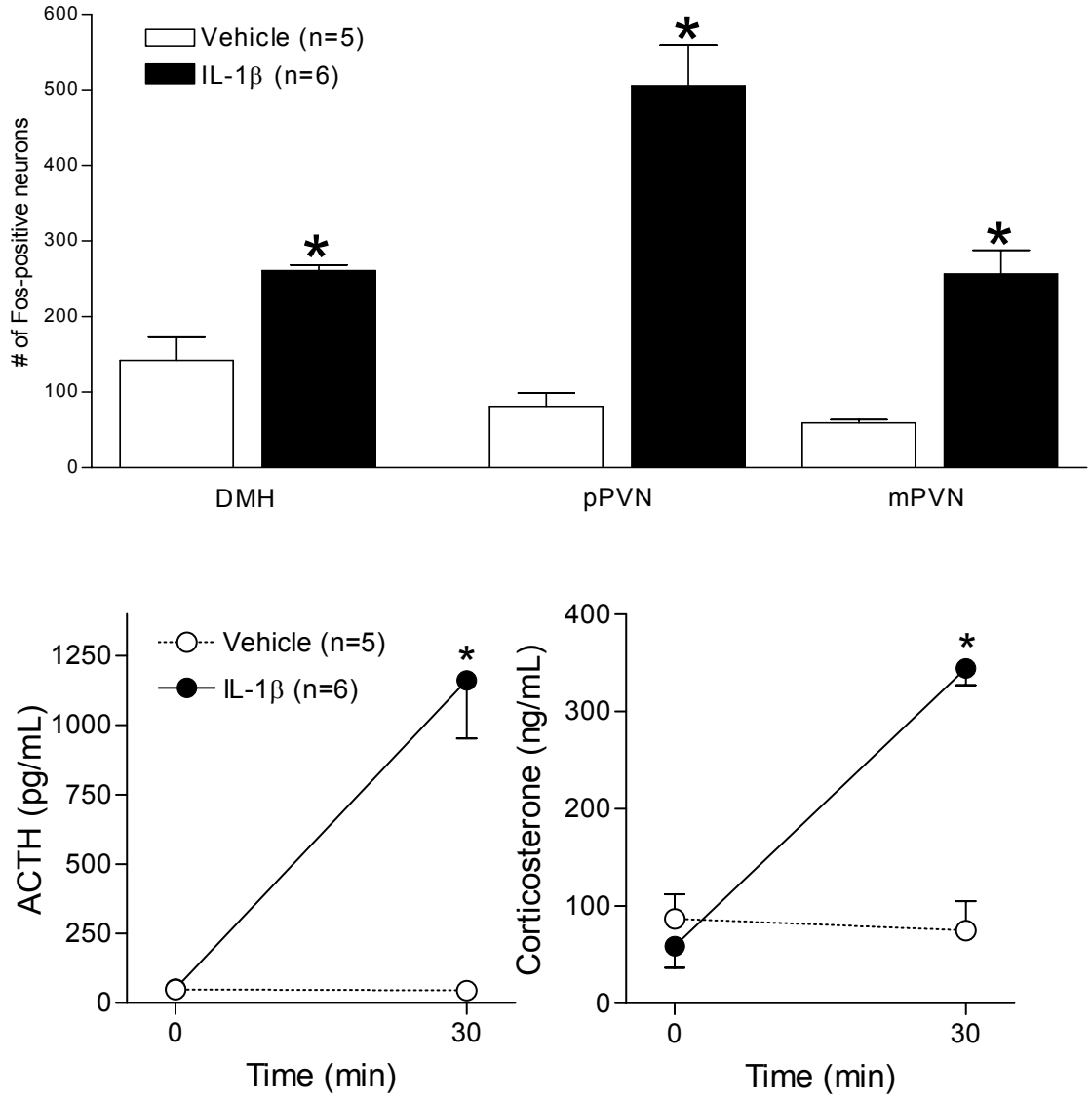

Figure 22. (Top) Mean (±SEM) number of neurons positive for Fos in the dorsomedial hypothalamus (DMH) and parvocellular (pPVN) and magnocellular (mPVN) regions of the paraventricular nucleus of the hypothalamus in rats sacrificed 90 min after intra-arterial administration of either IL-1β (closed) or vehicle (open). (Bottom) Corresponding Mean (±SEM) plasma levels of ACTH prior to and after the intra-arterial administration of IL-1β or vehicle. (*) Significantly greater than corresponding levels in rats treated with vehicle (paired t-test; $p<0.05$).

the administration of IL-1β than in rats receiving similar injections of vehicle (IL-1β: pPVN-506 ± 54 neurons, mPVN-256 ± 31 neurons; Vehicle: pPVN-81 ± 18 neurons, mPVN-59 ± 5 neurons; Fig. 22), replicating previous data (Schiltz & Sawchenko, 2007). Analysis of the DMH revealed a significantly greater number of neurons staining positive for Fos in rats sacrificed 90 min after the intra-arterial administration of IL-1β compared to animals receiving injection of vehicle (260 ± 7 neurons after IL-1β versus 142 ± 31 neurons after vehicle; Fig. 22).

3.2.3 Acute microinjection of muscimol into the DMH

To examine the role of neuronal activity in the DMH in the increases in plasma ACTH and expression of Fos in the PVN evoked by systemic administration of IL-1β, I microinjected muscimol bilaterally into the DMH approximately *two min after* the intra-arterial injection of IL-1β in conscious rats. The site of microinjection was confirmed by post-mortem analysis (Fig. 23).

Baseline levels of plasma ACTH were similar prior to the microinjection of muscimol or vehicle into the DMH (Fig. 24). Rats microinjected with muscimol into the DMH (n=8) did not exhibit significantly different levels of plasma ACTH 15 min after the administration of IL-1β compared to animals receiving microinjection of vehicle (n=7). However, a significantly lower level of plasma ACTH was observed in muscimol-treated animals at 30 min than that seen in vehicle-treated animals (460 ± 83 pg/mL after muscimol versus 1110 ± 93 pg/mL after vehicle). The number of neurons expressing Fos in the regions of the pPVN and mPVN in rats sacrificed 90 min after the intra-arterial administration of IL-1β was significantly less in animals subsequently microinjected with muscimol into the DMH compared to microinjection of vehicle (Muscimol: pPVN-384 ±

Figure 23

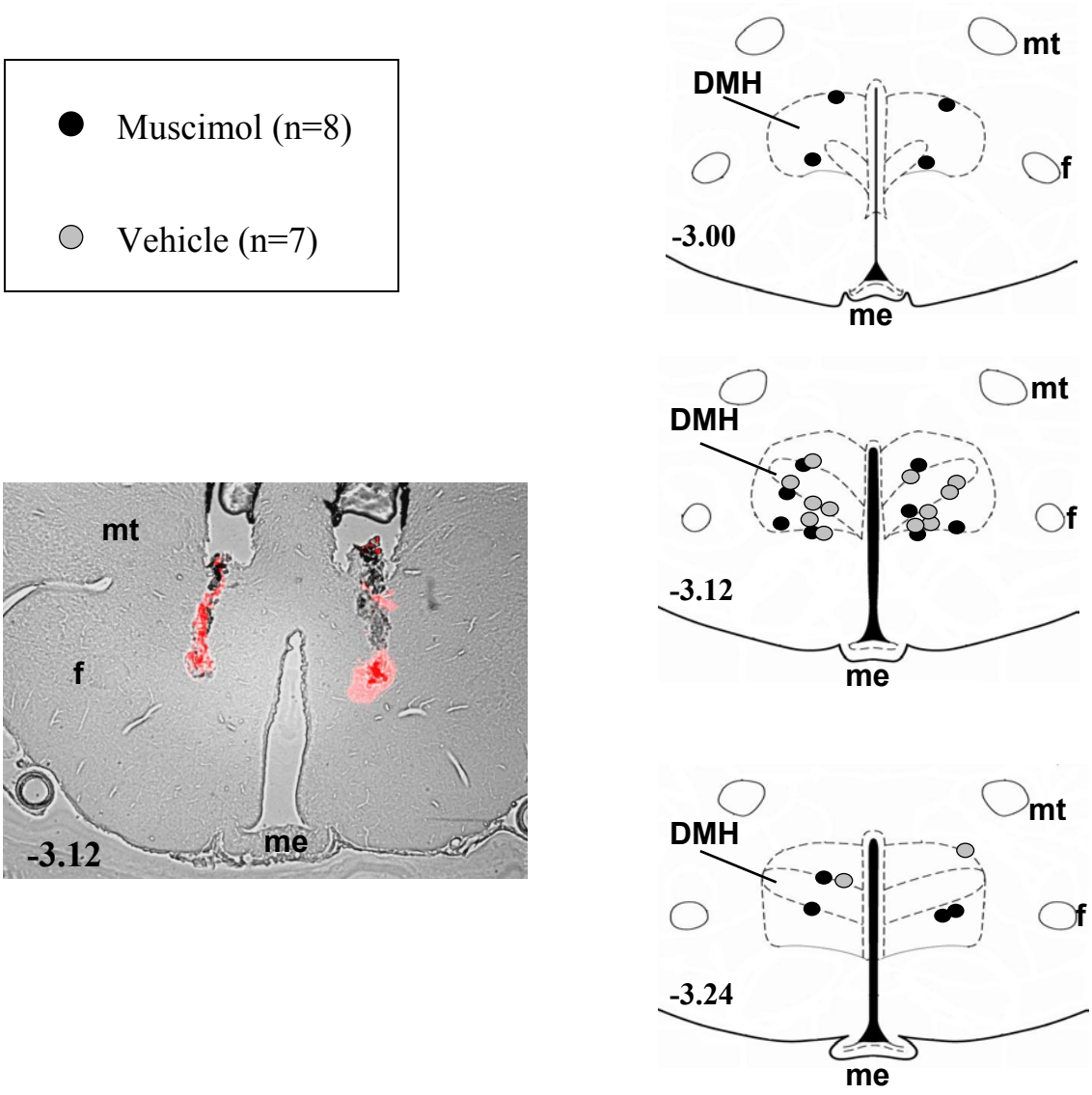

Figure 23. (Right) Schematic coronal sections adapted from the atlas of Paxinos and Watson (2007) illustrating approximate location of sites of injection of muscimol (closed circles) or vehicle (grey circles) into the DMH in rats approximately two minutes after intra-arterial administration of IL-1β. (Lower left) Photomicrograph of coronal section at the level of the DMH demonstrating visualization of fluorescent microspheres. Numbers indicate the distance in millimeters from bregma. Abbreviations: dorsomedial hypothalamus (DMH); fornix (f); mammillothalamic tract (mt); median eminence (me).

Figure 24

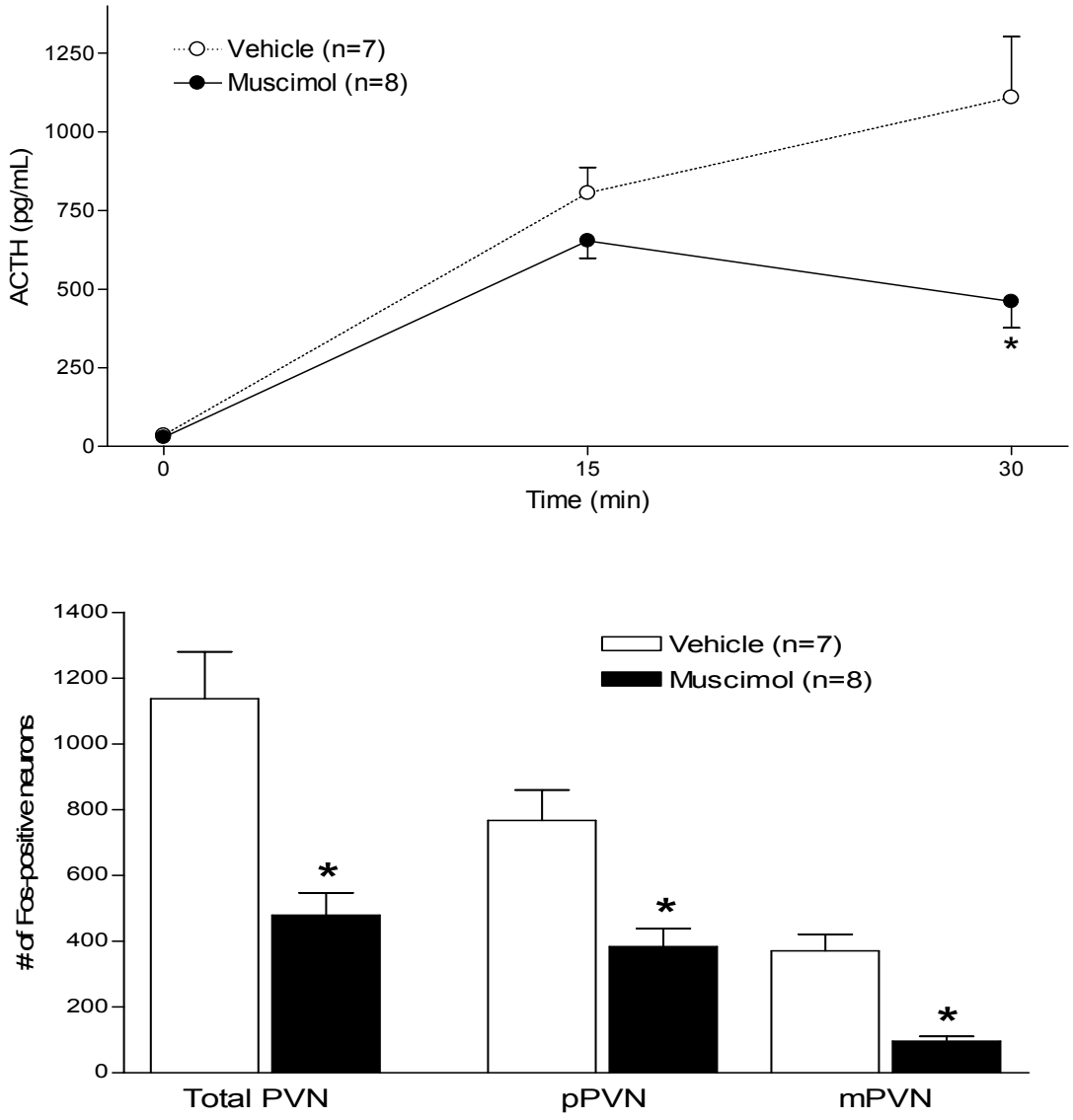

Figure 24. (Top) Mean (±SEM) plasma levels of ACTH immediately before and at 15 and 30 min after intra-arterial administration of IL-1β followed approximately two minutes later by microinjection of either muscimol (closed circles) or vehicle (open circles) into the DMH. (Bottom) Mean (±SEM) number of neurons positive for Fos in the parvocellular (pPVN) and magnocellular (mPVN) regions of the paraventricular nucleus of the hypothalamus in rats sacrificed 90 min after intra-arterial administration of IL-1β followed approximately two minutes later by microinjection of either muscimol (black) or vehicle (white) into the DMH. (*) Significantly less than corresponding levels after vehicle (repeated measures ANOVA with Bonferroni post-hoc tests (top) or unpaired t-tests (bottom); $p<0.05$).

56 neurons, mPVN-96 ± 14 neurons; Vehicle: pPVN-767 ± 92 neurons, mPVN-371 ± 51 neurons; Fig. 24).

3.2.4 Prior microinjection of muscimol into the DMH

In the previous experiment, muscimol was injected in to the DMH approximately *two min after* the administration of IL-1β. To address the possibility that the effects of IL-1β were initiated in the short time between administration of IL-1β and the subsequent microinjection of muscimol, I redesigned the experiment so that bilateral microinjection of muscimol into the DMH preceded the administration of IL-1β by five minutes. As in the previous experiment, I determined the concentration of plasma ACTH, as well as circulating levels of corticosterone, and the expression of Fos in the region of the PVN. Another control receiving microinjection and intra-arterial injection of vehicle was added to the design. The site of microinjection was confirmed by post-mortem analysis (Fig. 25).

No significant differences in baseline levels of plasma ACTH were noted in any group prior to the microinjections (Fig. 26). While no significant change in plasma ACTH from baseline levels was seen at any time point after the intra-arterial administration of vehicle in rats pretreated with vehicle in the DMH (n=6), a slight but significant increase in plasma corticosterone from baseline was noted 15 min later (repeated measures ANOVA with Bonferroni post-hoc test; $p<0.05$). Fifteen and 30 min after the intra-arterial administration of IL-1β, rats exhibited greater levels of plasma ACTH and corticosterone compared to animals injected with vehicle regardless of pretreatment (Fig. 26). Animals pretreated with muscimol in the DMH (n=6) exhibited increased plasma levels of ACTH that were similar to those in rats pretreated with vehicle

Figure 25

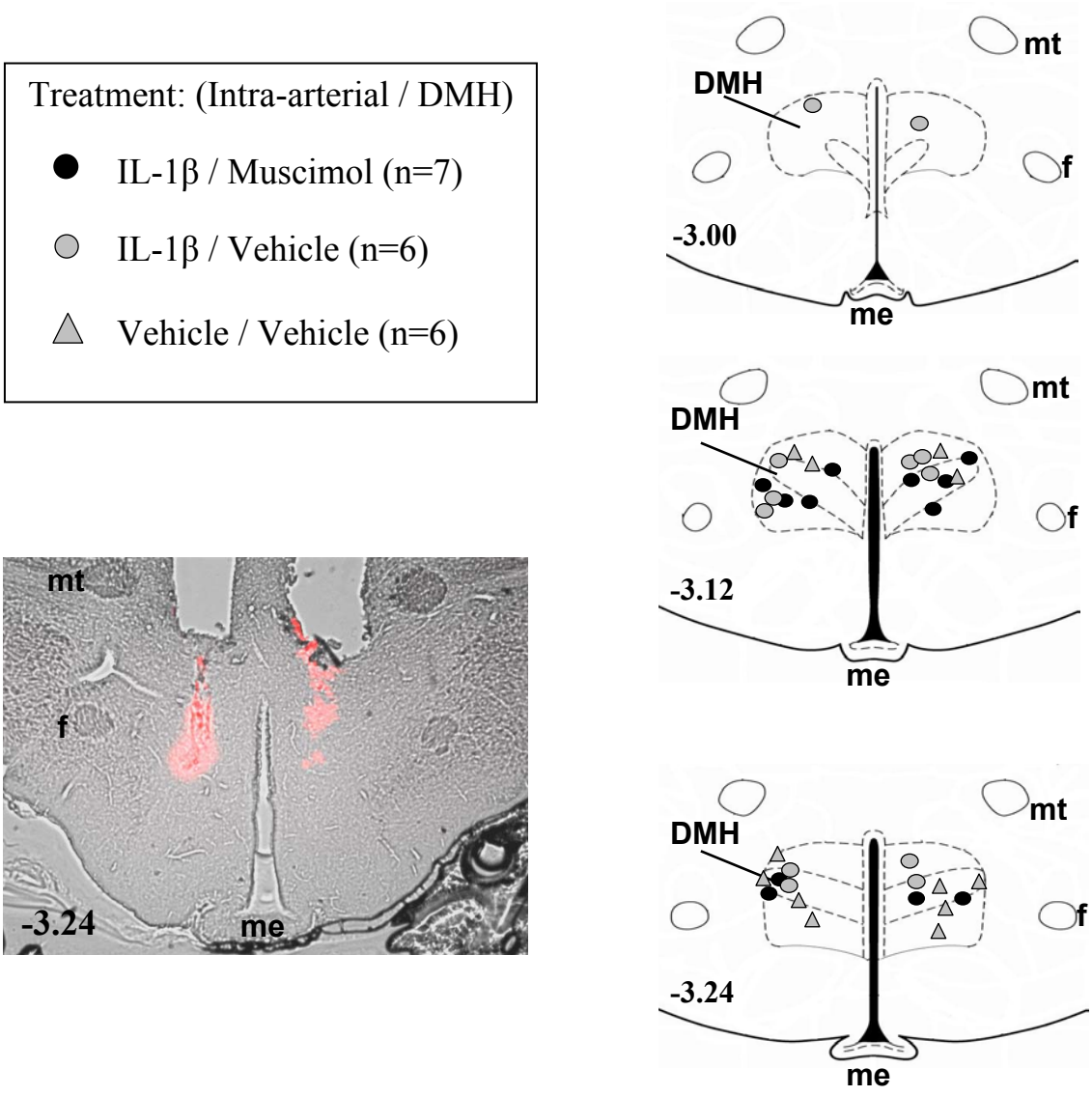

Figure 25. Schematic coronal sections adapted from the atlas of Paxinos and Watson (2007) illustrating approximate locations of sites of injection of muscimol (closed circles) or vehicle (grey circles) into the DMH in rats five minutes prior to intra-arterial administration of IL-1β. Sites of injection for vehicle/vehicle controls also displayed (grey triangles). (Left lower) Photomicrograph of coronal section at the level of the DMH demonstrating visualization of fluorescent microspheres. Numbers indicate the distance in millimeters from bregma. Abbreviations: dorsomedial hypothalamus (DMH); fornix (f); mammillothalamic tract (mt); median eminence (me).

Figure 26

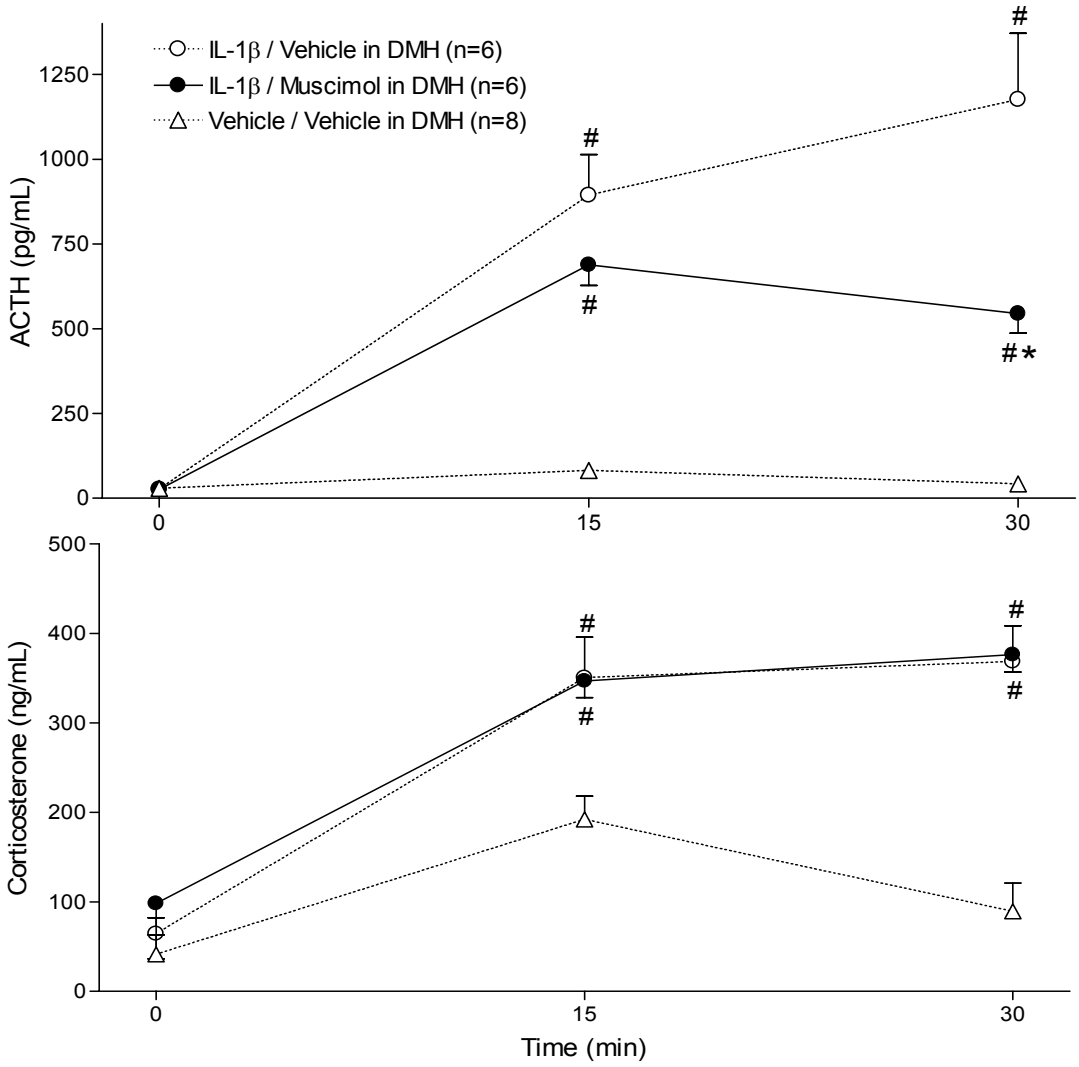

Figure 26. Mean (±SEM) plasma levels of ACTH (top) and corticosterone (bottom) before (0) and 30 and 60 min after intra-arterial administration of IL-1β in rats pretreated with microinjections of either muscimol (closed circles) or vehicle (open circles) into the DMH. Corresponding levels for vehicle/vehicle controls also displayed (open triangles). (#) Significantly greater than corresponding levels from vehicle/vehicle controls; (*) significantly less than corresponding levels after administration of IL-1β in rats pretreated with vehicle (repeated measures ANOVA with Bonferroni post-hoc tests; $p<0.05$).

(n=6) 15 min after the administration of IL-1β, but levels were significantly lower in muscimol-treated animals at 30 min (545 ± 58 pg/mL after muscimol versus 1176 ± 196 pg/mL after vehicle). Interestingly, plasma corticosterone did not differ at either time point after the administration of IL-1β regardless of pretreatment. In rats sacrificed 90 min after the intra-arterial administration of IL-1β, the number of Fos-expressing neurons in the regions of the pPVN and mPVN was significantly less in animals pretreated with microinjection of muscimol in the DMH than in animals pretreated with microinjection of vehicle (Muscimol: pPVN-301 ± 33 neurons, mPVN-116 ± 23 neurons; Vehicle: pPVN-519 ± 16 neurons, mPVN-259 ± 20 neurons; Fig. 27). However, the expression of Fos in the PVN observed in rats pretreated with muscimol was still significantly higher than that seen in vehicle/vehicle-treated control animals (pPVN-83 ± 9 neurons, mPVN-61 ± 9 neurons).

3.3 Effect of microinjection of muscimol into the DMH on the activation of the HPA axis evoked by systemic administration of LPS

Systemic administration of the bacterial endotoxin LPS causes activation of the HPA axis possibly through the induction of circulating IL-1β as well as other cytokines. To examine further the role of the DMH in the activation of the HPA axis evoked by peripheral inflammatory signals, I evaluated the effect of acute microinjections of muscimol into the DMH on the increases in plasma ACTH and expression of Fos in the PVN elicited by intra-arterial administration of LPS.

Figure 27

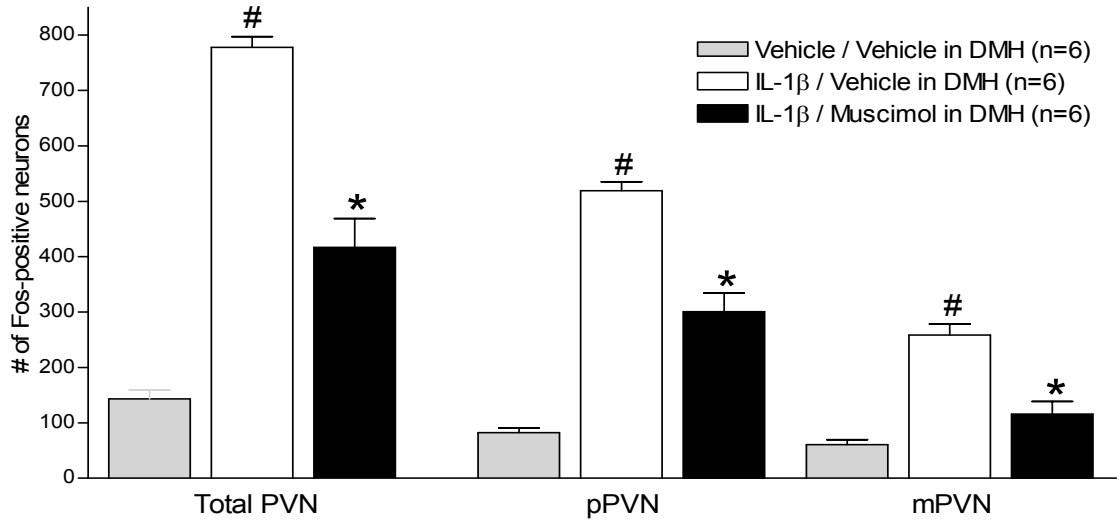

Figure 27. Mean (±SEM) number of neurons positive for Fos in the parvocellular (pPVN) and magnocellular (mPVN) regions of the paraventricular nucleus of the hypothalamus in rats pretreated with microinjections of muscimol or vehicle into the DMH sacrificed 90 min after intra-arterial administration of either IL-1β or vehicle. (#) Significantly greater than corresponding counts from vehicle/vehicle controls; (*) significantly less than corresponding counts after administration of IL-1β in rats pretreated with vehicle (one-way ANOVA with Bonferroni post-hoc tests; $p<0.05$).

3.3.1 Increase in plasma ACTH evoked by systemic administration of LPS

Before any experiments employing microinjections of muscimol into the DMH, I injected LPS intra-arterially in conscious rats to determine the time course of the increase in plasma ACTH. These results were used to determine the time of microinjections and blood sampling in the subsequent experiment.

Prior to injection, there was no significant difference in plasma ACTH between animals receiving LPS or vehicle (40 ± 11 pg/mL versus 46 ± 11 pg/mL; n=5; Fig. 28), and at no point did animals receiving injections of vehicle exhibit a significant change from this baseline. Animals injected with LPS exhibited an increase in plasma ACTH that was significantly greater than that of rats treated with vehicle 60 min after injection. The maximal increase in plasma ACTH was observed 90 min after injection of LPS.

3.3.2 Acute microinjection of muscimol into the DMH

To examine the role of neuronal activity in the DMH in the increases in ACTH and expression of Fos in the PVN evoked by systemic administration of LPS, I microinjected muscimol bilaterally into the DMH *60 min after* the intra-arterial injection of LPS in conscious rats. Thus, muscimol was microinjected 30 min before the peak LPS-evoked increase in plasma ACTH as determined by the previous experiment. This time was chosen to ensure that muscimol was effective in the DMH during the peak response as explained previously (Sec. 2.2.3). The site of microinjection was confirmed by post-mortem analysis (Fig. 29).

Baseline levels of plasma ACTH were similar prior to the microinjection of muscimol or vehicle into the DMH (Fig. 30). After the administration of LPS, animals microinjected with muscimol into the DMH (n=8) did not exhibit plasma levels of ACTH

Figure 28

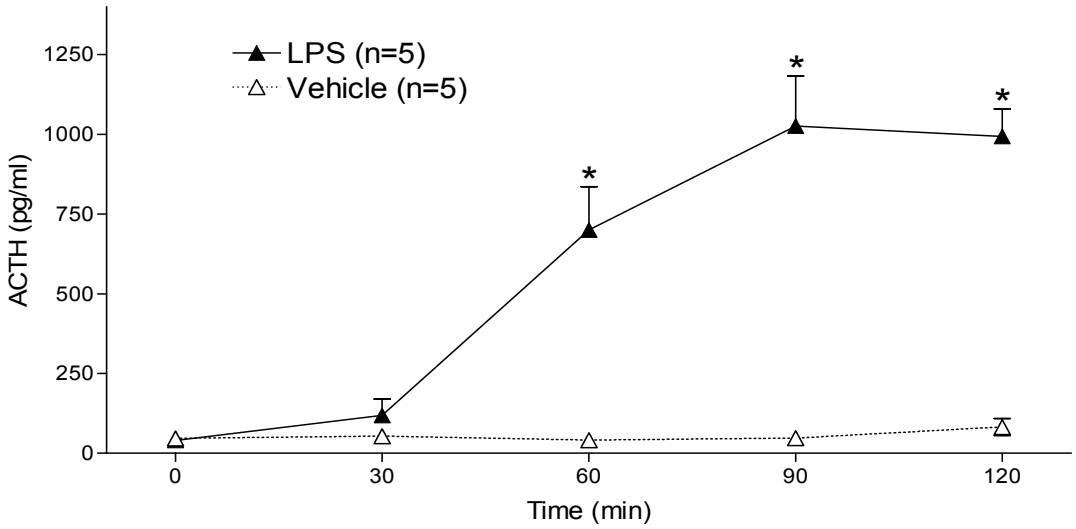

Figure 28. Mean (±SEM) plasma levels of ACTH immediately before and at various times after intra-arterial administration of LPS (10 μg/kg) or vehicle. (*) Significantly greater than corresponding levels after vehicle (repeated measures ANOVA with Bonferroni post-hoc tests; $p<0.05$).

Figure 29

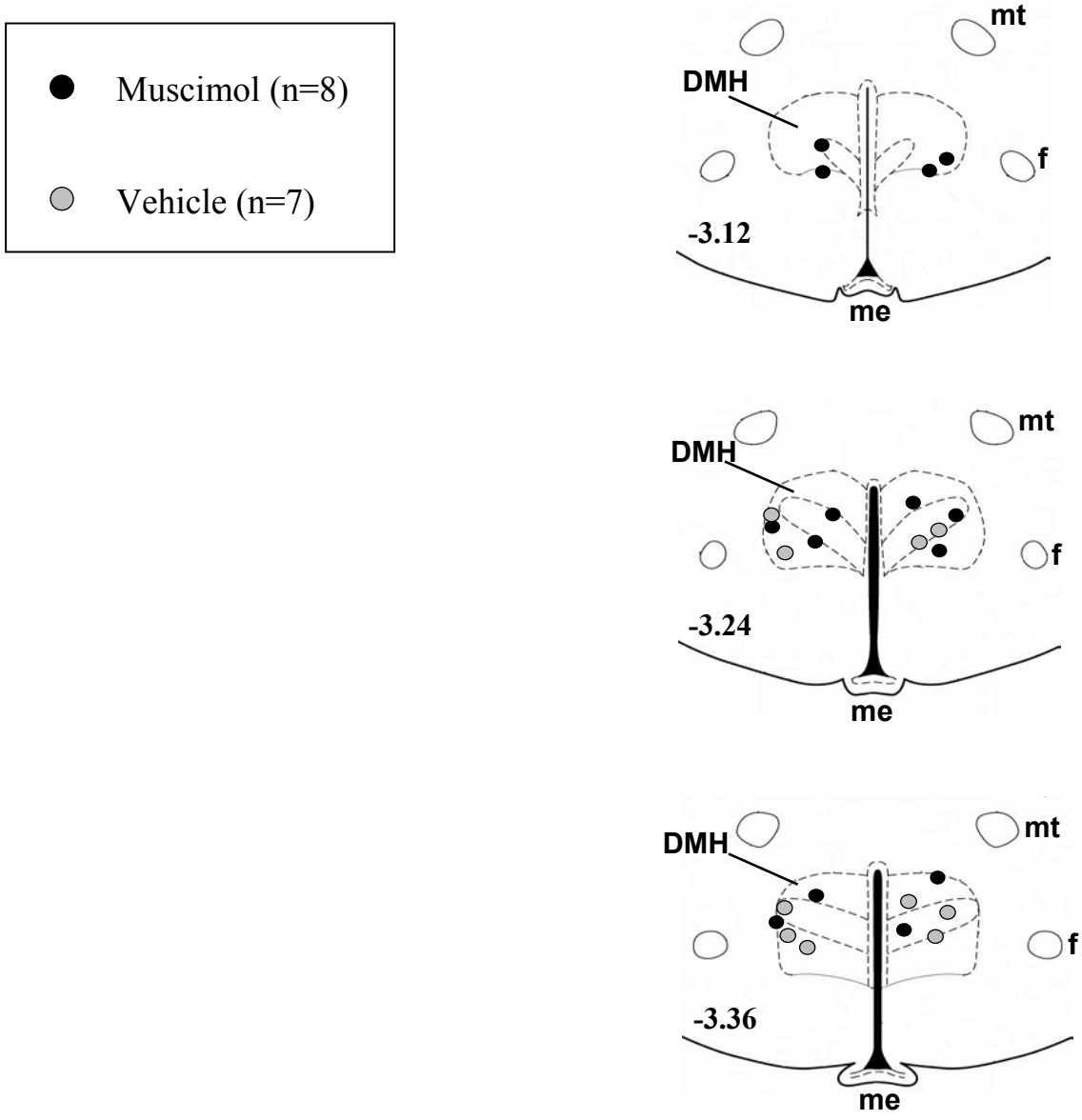

Figure 29. Schematic coronal sections adapted from the atlas of Paxinos and Watson (2007) illustrating approximate locations of sites of injection of muscimol (closed circles) or vehicle (grey circles) into the DMH in rats approximately two minutes after intra-arterial administration of LPS. Numbers indicate the distance in millimeters from bregma. Abbreviations: dorsomedial hypothalamus (DMH); fornix (f); mammillothalamic tract (mt); median eminence (me).

Figure 30

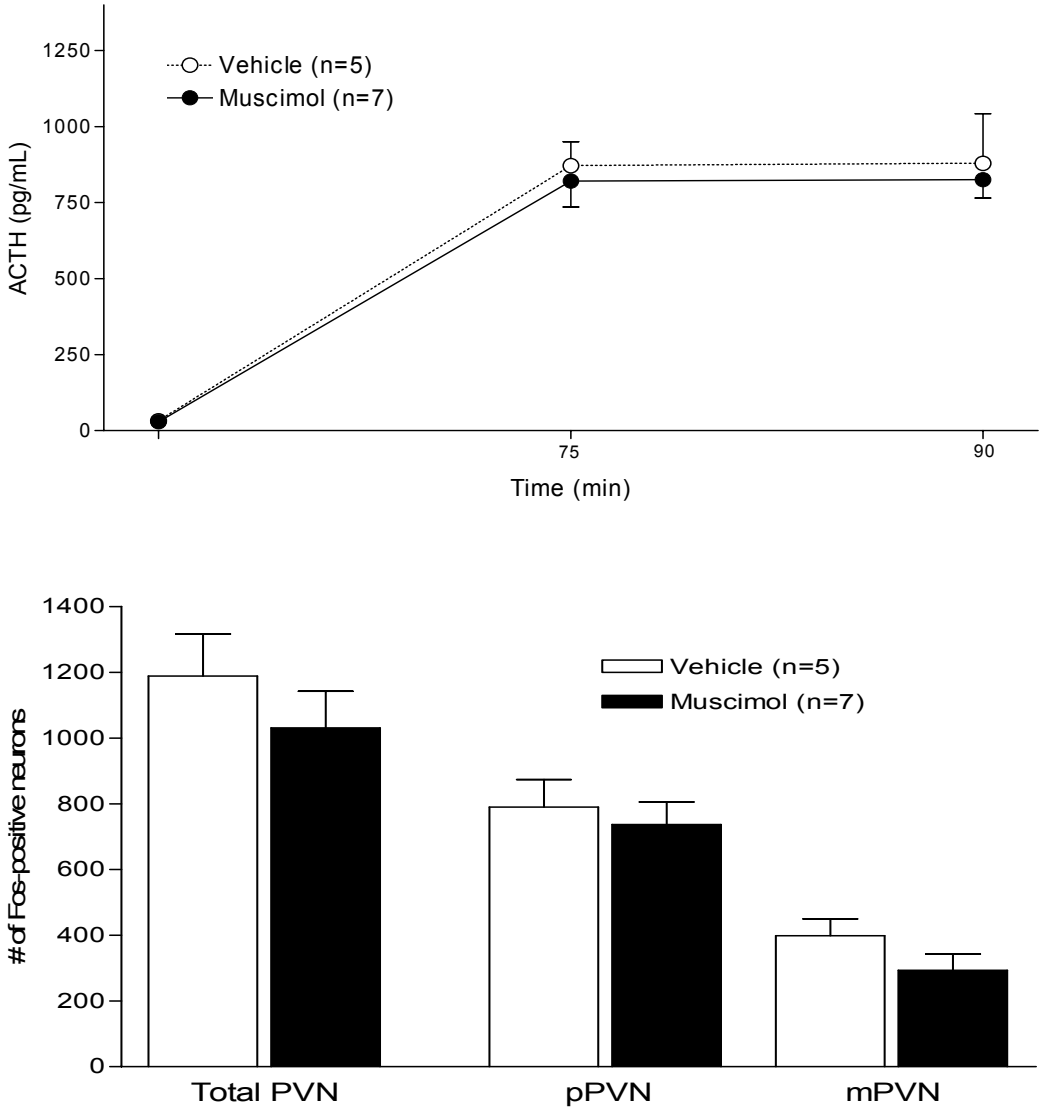

Figure 30. (Top) Mean (±SEM) plasma levels of ACTH immediately before and at 75 and 90 min after intra-arterial administration of LPS followed approximately two minutes later by microinjection of either muscimol (closed circles) or vehicle (open circles) into the DMH. (Bottom) Mean (±SEM) number of neurons positive for Fos in the parvocellular (pPVN) and magnocellular (mPVN) regions of the paraventricular nucleus of the hypothalamus in rats sacrificed 120 min after intra-arterial administration of LPS followed approximately two minutes later by microinjection of either muscimol (black) or vehicle (white) into the DMH.

that were significantly different from those receiving microinjection of vehicle (n=7) at any time point. Also, no difference was seen in the number of Fos-expressing neurons in any region of the PVN in rats sacrificed 120 min after the intra-arterial administration of LPS regardless of the treatment in the DMH (Fig. 30).

4.0 DISCUSSION

The goal of my studies was to clarify the role of neuronal activity in the DMH in responses evoked from the POA and by the systemic administration of IL-1β or LPS. Specifically, I used microinjections of muscimol, a $GABA_A$-agonist and neuronal inhibitor, into the DMH to elucidate the role of neurons in this region in (1) the physiological and behavioral responses evoked by microinjection of muscimol into the POA (Sec. 4.1) and (2) the activation of the HPA axis evoked by intra-arterial administration of inflammatory cytokine IL-1β or bacterial endotoxin LPS (Sec. 4.2). The results of these experiments indicate that (1) neurons in the DMH mediate varied responses evoked from the POA, including the activation of the HPA axis, and (2) neurons in the DMH contribute to the activation of the HPA axis evoked by systemically administered IL-1β. However, the results suggest that injection of inflammatory mediators may also activate the HPA axis through mechanisms that do not involve neuronal activity in the DMH and are possibly independent of the POA.

4.1 Role of neuronal activity in the DMH in responses evoked from the POA

To assess the role of the DMH in physiological and behavioral responses evoked from the POA, I examined the effect of inhibiting neuronal activity in the DMH in conscious rats on the responses to microinjections of muscimol into the POA, which elicited dramatic increases in heart rate, arterial pressure, body temperature, locomotor activity, and plasma ACTH, as previously reported (Zaretsky et al., 2006). Previously unknown, the expression of Fos in the PVN and raphe pallidus was also increased after injection of muscimol into the POA. Prior bilateral inhibition of the DMH completely

prevented the increase in all of these responses evoked from the POA. In addition, microinjection of muscimol into the DMH while these responses were ongoing caused rapid and nearly complete reversal of the increases in all parameters measured. Together the results suggest that a variety of physiological responses evoked from the POA in conscious rats are wholly dependent on neuronal activity in the DMH.

Microinjection of muscimol into the POA of conscious rats elicited marked increases in heart rate, arterial pressure, body temperature, and locomotor activity (Fig. 4-7), replicating previous findings (Osborne et al., 1993; Osborne et al., 1994; Osborne & Kurosawa, 1994; Osaka, 2004; Ishiwata et al., 2005; Zaretsky et al., 2006). All of these responses were apparent within minutes of the injection of muscimol into the POA. Maximal increases were reached within 10 min of injections for every parameter except body temperature for which the maximal increase was modestly delayed, occurring approximately 20 min later than that for heart rate, arterial pressure, and locomotor activity. Since these responses were evoked by the injection of a neuronal inhibitor, the POA appears to exert tonic inhibitory tone on downstream brain centers associated with these effects. In support of this, PGE_2 which produces similar autonomic responses when injected into the POA is thought to inhibit EP_3-receptor expressing neurons in the POA, most of which are GABAergic (Nakamura et al., 2002). Tonic GABAergic inhibition appears to exist in several specific brain regions associated with sympathetically mediated tachycardia, hypertension, and thermogenesis. One of these areas is the DMH, where disinhibition with the $GABA_A$-antagonist BMI induces all these effects as well as increased locomotor activity (Soltis & DiMicco, 1991; DeNovellis et al., 1995; Bailey & DiMicco, 2001; Zaretskaia et al., 2002). However, the source of the inhibitory tone to the

DMH remains unclear. The POA which sends efferents to the DMH (Thompson & Swanson, 1998) is a major source of GABAergic neurons in the brain (Okamura et al., 1990). Thus, the finding that either inhibition of the POA or disinhibition of the DMH produces an identical pattern of physiologic responses suggests that the POA may represent the source of inhibitory tone to the DMH.

As noted above, microinjection of muscimol into the POA evoked a significant increase in arterial pressure. This increase was similar to that reported in a recent study employing conscious rats (Zaretsky et al., 2006). In contrast, Osborne and Kurosawa previously reported that perfusion of the POA with muscimol had no significant effect on arterial pressure (Osborne & Kurosawa, 1994). A possible explanation for this discrepancy is that the latter study was performed in halothane anesthetized rats, and anesthetics are known to have confounding affects on cardiovascular responses (Wang et al., 1999). In particular, halothane has been recognized to decrease norepinephrine-induced vascular smooth muscle contraction (Stadnicka et al., 1993), thereby obscuring the interpretation of sympathetically-mediated cardiovascular responses under these conditions. Osborne and Kurosawa even reported that the POA-evoked tachycardia they observed could be attenuated by increasing the depth of the anesthesia (Osborne & Kurosawa, 1994), supporting the idea that the use of anesthetic was a confounding variable in that study.

Pretreatment of the DMH with muscimol completely abolished the increases in heart rate, arterial pressure, body temperature, and locomotor activity evoked by microinjection of muscimol into the POA. This finding suggests that these varied responses evoked from the POA are all mediated through the activation of neurons in the

DMH. Previous studies demonstrated that chemical inhibition of neurons in the DMH attenuates tachycardia, hypertension, and hyperthermia evoked by microinjection of PGE_2 into the POA in anesthetized rats (Zaretskaia et al., 2003; Madden & Morrison, 2004; Nakamura et al., 2005). However, my work represents the first study to examine all of these responses evoked from the POA, with the addition of locomotor activity and activation of the HPA axis, in the same conscious animal. Inactivation of neurons in the DMH prevents air jet stress-induced tachycardia and hypertension (Soltis & DiMicco, 1992; Stotz-Potter et al., 1996a,b). My results suggest that neuronal activity in the DMH mediates the integrated response to a variety of stressors, including those that act through the POA. It is possible that emotional stressors may signal through the POA to activate the DMH. However, no direct evidence supporting this hypothesis is known.

Injection of muscimol into areas outside the DMH failed to suppress autonomic responses evoked from the POA. Owing to the widely held belief that the PVN represents the principal hypothalamic site for the integration of autonomic activity, it could be argued that the effects attributed to the inhibition of the DMH were a consequence of muscimol spreading to act upon neurons in the PVN. However, microinjections of muscimol targeted to areas adjacent to but outside the DMH including the PVN itself (Fig. 8-10) did not prevent the increases in heart rate, arterial pressure, and body temperature evoked by microinjection of muscimol into the POA. Microinjection of muscimol into these same areas attenuated the increase in locomotor activity (Fig. 11), although not to the extent as did similar microinjections into the DMH. The reason for this apparent discrepancy may be related to differing sensitivity of neurons in the DMH to muscimol. Thus, low concentrations of muscimol diffusing to the DMH from injection

sites adjacent to the region may have been sufficient to produce modest suppression of the activity of neurons mediating locomotor changes without affecting those mediating autonomic responses. If this were the case, then an association between the magnitude of the suppression of locomotor activity and the proximity of the site of injection to the DMH would be anticipated. However, examination of the data from individual experiments revealed no such relationship. Alternatively, muscimol may have suppressed the activity of neurons in the areas adjacent to the DMH that contribute to behavioral responses evoked from the POA, although to a lesser degree than neurons within the DMH. Orexins are neuropeptides synthesized by hypothalamic neurons that are thought to play a role in arousal and the induction of locomotor activity (Estabrooke et al., 2001; Kiyashchenko, et al., 2002). While the site of action of endogenous orexins remains unclear, neurons in the PVN represent a possible site mediating orexin-induced locomotor activity. Intracerebroventricular administration of orexin induces the expression of Fos in neurons of the PVN (Edwards et al., 1999), and direct injection of orexin into the PVN elicits an increase in locomotor activity (Kiwaki et al., 2004). It is possible that POA-evoked increases in locomotor activity may be mediated by the release of orexin from hypothalamic neurons to act on neurons in the PVN or elsewhere. Interestingly, perfusion of the POA with muscimol elicits the increased expression of Fos in orexin neurons in the lateral hypothalamic area (Satoh et al., 2004). Muscimol injected into adjacent areas outside the DMH including the PVN itself may suppress locomotor activity by inhibiting orexin-activated neurons in the PVN or in areas between the PVN and the DMH, thus explaining my results. Alternatively, muscimol may be acting on neurons in the lateral hypothalamus to prevent POA-evoked activation of orexin neurons.

Acute bilateral inhibition of the DMH during the period of increased heart rate, arterial pressure, body temperature, and locomotor activity evoked by injection of muscimol into the POA produced rapid and nearly complete suppression of these responses (Fig. 17-20), although the suppression of body temperature was slightly delayed relative to other responses. The design of this experiment was similar to that of a previously published study testing the effect of acute microinjections of muscimol into the DMH on the tachycardia and hyperthermia elicited by intrapreoptic microinjection of PGE_2 in anesthetized rats (Zaretskaia et al., 2003). In that study, unilateral injection of muscimol into the DMH produced an immediate reduction in the tachycardia and hyperthermia evoked from the POA. My experiments demonstrated a greater suppression in POA-evoked tachycardia and hyperthermia than that reported previously. However, as noted above, the latter study involved unilateral microinjection of muscimol into the DMH. Bilateral microinjections of muscimol into the DMH are reportedly necessary to prevent tachycardia induced by experimental stress (Anderson & DiMicco, 1990), and thus it is likely that bilateral inhibition of the DMH is necessary for complete suppression of tachycardia and hyperthermia evoked from the POA. In fact, Madden and Morrison demonstrated greater suppression of the tachycardia, hypertension, and hyperthermia evoked by PGE_2 in anesthetized rats by employing bilateral microinjections of muscimol into the DMH (Madden & Morrison, 2004).

Zaretskaia and colleagues reported an immediate reduction in the hyperthermia evoked by microinjection of PGE_2 into the POA with unilateral injection of muscimol into the DMH (Zaretskaia et al., 2003). In contrast, the suppression of increases in body temperature by bilateral inhibition of the DMH in the current study occurred more

slowly, appearing approximately 10 min after microinjections. While inherent differences in the thermogenic processes evoked by PGE_2 and muscimol might explain this difference in the suppression of body temperature, another explanation exists. The previous study employing microinjection of PGE_2 into the POA was performed in urethane-anesthetized rats that were kept on heating pads until 10 min before experimental protocols began. Inhibiting thermogenic mechanisms at the level of the DMH may have a greater effect on body temperature in anesthetized animals that are not continually heated by an external source than in the conscious, freely moving animals with intact and operational thermoregulatory mechanisms that were used in the present experiments.

Pretreatment of the DMH with muscimol lowered basal heart rate, arterial pressure, and body temperature in animals treated with vehicle in the POA (Fig. 4-6). A similar decrease in basal heart rate evoked by bilateral injection of muscimol into the DMH has been previously described (Lisa et al., 1989). The depressive effects of inhibiting the DMH could suggest that tonic neuronal activity in the DMH is important to the maintenance of basal heart rate, arterial pressure, and body temperature in conscious animals. Alternatively, the reduction in baseline heart rate, arterial pressure, and body temperature may represent muscimol-induced suppression of the low level of neuronal activity in the DMH caused by the mild stress related to the experimental setting. Habituation of the animals to the experimental setting and protocol could resolve this issue. However, the relatively small effect on baselines cannot account for the magnitude of the reduction in responses evoked from POA by pretreatment with muscimol in the DMH. Because locomotor activity was nearly absent in the unstressed rats prior to

microinjections, the effect of microinjection of muscimol into the DMH on baseline locomotor activity could not be detected or assessed (Fig. 7).

Increased locomotor activity evoked by muscimol in the POA was completely abolished by inhibiting the DMH (Fig. 7, Fig. 20). Previous studies have shown that the injection of BMI into the DMH produces an 'anxiety-like' response characterized by an increase in locomotor activity (Shekhar & DiMicco, 1987; Shekhar et al., 1987), suggesting that locomotor activity-inducing pathways in the DMH are under tonic inhibition. The POA represents a possible source of this tonic inhibition on locomotor activity. Endogenous GABAergic systems in the POA have been implicated in cold-induced thermogenic responses. The application of the $GABA_A$-antagonist BMI to the POA prevented the thermogenic response to cold-stress in anesthetized (Osaka, 2004) and conscious rats (Ishiwata et al., 2005). Interestingly, locomotor activity may significantly contribute to the thermogenic response evoked by cold stress in rats (Girardier et al., 1995). A direct relationship between the similar increases in locomotor activity evoked by chemical manipulation of the DMH and POA has never been tested. The results of my studies suggest that the locomotor response to cold-stress thought to be evoked by endogenous GABA in the POA and possibly contributing to thermogenesis is dependent on neuronal activity in the DMH.

Bilateral inhibition of the DMH prevented the increase in plasma ACTH evoked by muscimol in the POA (Fig. 12). The microinjection of muscimol into the POA produces rapid activation of the HPA axis as indicated by the increase in the plasma levels of ACTH (Zaretsky et al., 2006), a finding replicated for the first time in these experiments. Similar increases in plasma ACTH have been evoked by intrapreoptic

injections of PGE_2 (Morimoto et al., 1989; Katsuura et al., 1990; Watanabe et al., 2000; Zaretsky et al., 2006). However, to date no studies have examined the role of the DMH in the POA-evoked increases in plasma ACTH or activation of the HPA axis. In the current study, rats receiving vehicle for all microinjections exhibited plasma levels of ACTH that were similar to those reported previously for unstressed Sprague-Dawley rats (Anseloni et al., 2005; Faraday et al., 2005). In rats pretreated with bilateral microinjections of muscimol into the DMH, plasma levels of ACTH 15 minutes after intrapreoptic injection of muscimol were no different from those in vehicle/vehicle control animals, suggesting that POA-evoked increases in plasma ACTH are entirely dependent on neuronal activity in the DMH.

Interestingly, pretreatment of the DMH with muscimol did not have a significant effect on baseline plasma levels of ACTH. In contrast to its apparent role in supporting basal heart rate, arterial pressure, and body temperature, this observation suggests that activity of neurons in the DMH does not contribute to basal activity of the HPA axis in these conscious rats. This finding appears to conflict with published evidence that supports the role of the DMH in mediating the normal circadian pattern of the HPA axis. The release of vasopressin from DMH-projecting neurons originating in the suprachiasmatic nucleus is an important inhibitor of basal levels of ACTH and corticosterone (Kalsbeek et al., 1996a,b). It is the diurnal pattern of activity and release of vasopressin from neurons in suprachiasmatic nucleus (Gillette & Reppert, 1987; Kalsbeek, 1995) that likely mediates the activity of the HPA axis through the DMH (Kalsbeek et al., 1996a,b). However, my experiments were performed during the first half of the light cycle when the inhibitory tone to the HPA axis is highest (Kalsbeek,

1996b). Since basal levels of ACTH were presumably suppressed by endogenous circadian inhibitory mechanisms at the time of my experiments, it is not unexpected that inhibition of neuronal activity in the DMH with muscimol failed to decrease plasma ACTH.

Microinjection of muscimol into the PVN, the site of CRF-containing neurons representing the final common pathway in the activation of the HPA axis, also resulted in a reduction in the plasma level of ACTH evoked by muscimol in the POA. Compared to vehicle, injections of muscimol into either the PVN or DMH caused similar reductions in plasma levels of ACTH evoked by the injection of muscimol into the POA (77% versus 75%, respectively, Fig. 13). Because of this, it could be argued that the effect of microinjection of muscimol into the DMH may have resulted from spread or diffusion of the drug to neurons in the PVN. However, two points would argue against this suggestion. First, microinjections of muscimol into adjacent sites outside the DMH and PVN, most of which were closer to the PVN, did not result in an attenuation of the increase in plasma ACTH evoked by muscimol in the POA (Fig. 13). Second, injection of muscimol into the PVN does not suppress the cardiovascular effects of experimental stress as does similar injection of muscimol into the DMH (Stotz-Potter et al., 1996). Therefore, it is unlikely that the dose and volume of muscimol (80 pmol/100 nL) used in this study were large enough to allow the diffusion of drug from the DMH to the PVN in a concentration capable of inhibiting these neurons. Interestingly, plasma levels of ACTH 15 min after injection of muscimol into the POA were significantly greater in rats receiving vehicle injections into the PVN than in rats receiving muscimol in the DMH. It may be that the effect of microinjection of vehicle into the PVN alone produced modest

stimulation of neurons in the region and so enhanced the POA-evoked increases in plasma ACTH.

The current study indicates for the first time that intrapreoptic injection of muscimol also increases Fos expression, a commonly used marker for neuronal activation, in the PVN and the raphe pallidus (Fig. 15). Neuronal activity in the PVN and raphe pallidus is thought to mediate POA-evoked neuroendocrine and autonomic responses, respectively. Intrapreoptic doses of PGE_2 that evoke tachycardia, hyperthermia, and increased levels of plasma ACTH also elicit increased immunoreactivty for Fos in both the PVN and raphe pallidus (Scammell et al., 1996; Nakamura et al., 2002). However, prior to my experiments there has been no evidence for altered neuronal activity in the PVN or raphe pallidus in animals resulting from microinjection of muscimol into the POA. Intrapreoptic injection of muscimol increased the expression of Fos in both the pPVN and the mPVN (Fig. 14). Interestingly, Scammell and colleagues reported that intrapreoptic injections of PGE_2 evoked increased expression of Fos in the PVN that was restricted predominantly to the parvocellular region of the nucleus (Scammell et al., 1996). This difference in the regional expression of Fos may be a consequence of the population of neurons in the POA that are responsive to muscimol versus PGE_2. Muscimol is thought to inhibit virtually all mammalian neurons, while PGE_2 most likely acts on a subpopulation of neurons in the POA possessing EP_3 receptors, the principal receptor for PGE_2 in the POA (Nakamura et al., 1999; 2000).

Bilateral inhibition of the DMH completely abolished the increased expression of Fos in the PVN and raphe pallidus elicited by muscimol in the POA (Fig. 15). Identical

injections of muscimol into the DMH have been reported to prevent stress-induced expression of Fos in the PVN (Morin et al., 2001). However, involvement of the DMH in the induction of Fos evoked from the POA has never been demonstrated. In animals receiving muscimol in the DMH, the expression of Fos following injection of muscimol into the POA in both the pPVN and mPVN and the raphe pallidus was no different from that seen in vehicle/vehicle control animals. This finding suggests that neuronal activation in the DMH is essential to POA-evoked activation of the PVN and the raphe pallidus.

Thus, neurons in the DMH appear to mediate a variety of responses evoked from the POA. Increases in heart rate, arterial pressure, body temperature, locomotor activity, plasma ACTH, and Fos-expressing neurons in the PVN and raphe pallidus evoked from the POA were all completely abolished by inhibiting neurons in the DMH. The results suggest that neuronal activity in the DMH may contribute to responses evoked by other stressors that are thought to elicit their responses through the POA such as inflammatory cytokines or bacterial endotoxin. However, to date there are no published reports implicating the DMH in such a role.

4.2 Role of neuronal activity in the DMH in the activation of the HPA axis evoked by systemic administration of IL-1β or LPS

To characterize the role of neuronal activity in the DMH in the activation of the HPA axis evoked by systemic administration of inflammatory agents, I examined the effect of inhibiting neurons in the DMH on the increases in plasma ACTH seen in conscious rats treated with intra-arterial injections of IL-1β or LPS. Systemic

administration of IL-1β or LPS elicited dramatic increases in plasma ACTH and Fos-expressing neurons in the PVN (Fig. 24, 30), as previously reported (Katsuura et al., 1990; Dunn, 1992; Ericsson et al., 1994; Elmquist et al., 1996; Turnbull et al., 1998; Wieczorek et al., 2005). While the DMH has been proposed to mediate activation of the HPA axis evoked by experimental stress in rats (DiMicco et al., 2002), the role of the DMH in inflammatory cytokine-evoked activation of the HPA axis has never been tested. Inhibition of the DMH significantly attenuated the increase in plasma ACTH evoked by intra-arterial administration of IL-1β (Fig. 24, 26) and markedly suppressed the associated increase in expression of Fos in the PVN (Fig. 24, 27). In contrast, inhibition of the DMH failed to prevent LPS-induced increases in plasma ACTH and expression of Fos (Fig. 30). Together these results indicate that neuronal activity in the DMH may contribute to the activation of the HPA axis evoked by systemically administered IL-1β, but that other pathways independent of the DMH must exist for cytokine-mediated activation of the HPA axis.

Intra-arterial injection of IL-1β in conscious rats evoked rapid increases in plasma levels of ACTH that peaked 30 minutes after administration (Fig. 21). Intravascular administration of similar doses of IL-1β is a powerful activator of the HPA axis, although smaller increases in plasma ACTH than were found in the current experiments have been reported previously (Katsuura et al., 1990; Kannan et al., 1996; Turnbull et al., 1998). This difference may be a result of the route of administration used, since in previous studies IL-1β was administered intravenously while in my experiments intra-arterial injection was employed. Supporting the idea that activation of the HPA axis by inflammatory agents is dependent on the route of administration, Givalois and colleagues

reported increases in plasma levels of ACTH evoked by intra-arterial administration of LPS in rats that are similar to those seen in my experiments (Givalois et al., 1994). These increases in plasma ACTH evoked by intra-arterial administration of LPS are greater than those seen with intravenous administration (Turnbull et al., 1998). The mechanisms that account for route-dependent differences have not been resolved. A future study designed to compare the time course of plasma levels of cytokines in the carotid or cerebral arterial system after administration of intravenous and intra-arterial administration of LPS or IL-1β may help to identify factors that could account for these differences.

Intra-arterial administration of IL-1β increased the expression of Fos in the region of DMH (Fig. 22), suggesting that systemically administered IL-1β evokes neuronal activation in the DMH. Increased expression of Fos in the PVN evoked by intravascular administration of IL-1β has previously been described (Veening et al., 1993; Ericsson et al., 1997; Schiltz & Sawchenko, 2007) and was replicated in my experiments. However, no data existed describing the effect of IL-1β on neurons in the DMH. Experimental stress, known to activate neurons in the PVN and increase plasma ACTH (Ceccatelli et al., 1989; Whitnall, 1993; Senba et al., 1993; Stotz-Potter et al., 1996b; Morin et al., 2001), induces the expression of Fos in neurons of the DMH that project to the PVN (Cullinan et al., 1996; Li & Sawchenko, 1998). The neurons in the DMH are thought to be a source of stimulatory input to neurons in the PVN (Boudaba et al., 1997). Microinjection of EAA or BMI into the DMH evokes increases in plasma levels of ACTH (Keim & Shekhar, 1996; Bailey & DiMicco, 2001), possibly through direct projections to areas of the PVN densely populated with CRF-containing neurons (Ter Horst & Luiten, 1987). Therefore, the activation of neurons in the DMH by systemically

administered IL-1β identified for the first time in my experiments may represent one mechanism through which IL-1β activates the HPA axis. Intravascular administration of IL-1β also induces tachycardia and hyperthermia in rats (Dascombe et al., 1988; Bataillard & Sassard, 1994). Activation of neurons in the DMH evokes tachycardia and hyperthermia, and these same neurons are thought to mediate stress-induced increases in heart rate and body temperature (DiMicco et al., 2006). Thus, the increased expression of Fos in the DMH evoked by IL-1β may also reflect activation of sympathoexcitatory neurons mediating cardiovascular and thermogenic responses.

Acute microinjection of muscimol into the DMH suppressed the increase in plasma levels of ACTH and Fos-expressing neurons in the PVN evoked by the administration of IL-1β (Fig. 24), suggesting that neuronal activity in the DMH contributes to the IL-1β-induced activation of neurons in the PVN that regulate the HPA axis. Identical injections of muscimol in the DMH also prevent the increases in Fos-expressing neurons in the PVN evoked by experimental stress (Morin et al., 2001). Thus, the DMH may represent one component of a common neural pathway for the activation of the HPA axis evoked by either stress or inflammatory cytokines. However, the time-dependent nature of the suppression in plasma ACTH indicates that mechanisms involved in the IL-1β-induced activation of the HPA axis may be more complex and that other pathways independent of the DMH are likely to participate. Animals treated with muscimol in the DMH exhibited increases in plasma ACTH 15 minutes after the administration of IL-1β that were similar to those seen in vehicle-treated animals. However, the increase in plasma ACTH 30 min after administration of IL-1β was reduced by more than 50 percent after injection of muscimol into the DMH compared to that seen

after control injection of vehicle. These results suggest that neuronal activity in the DMH participates in the activation of the HPA axis evoked by systemic administration of IL-1β, but that the early activation may not depend on the DMH. However, an inherent flaw in the design of this study was that muscimol was microinjected into the DMH minutes after the administration of IL-1β. Therefore, IL-1β may have been able to stimulate DMH-dependent pathways activating the HPA axis before they were inhibited by the microinjection of muscimol. Only after the DMH was inhibited would further release of ACTH be suppressed and plasma levels decline according to the short-half life of ACTH in the circulation (2-10 min) (Matsuyama et al., 1972). To address this issue, the study was redesigned to inhibit the DMH by microinjection of muscimol prior to administration of IL-1β.

Prior inhibition of the DMH produced attenuation in the IL-1β-induced increases in plasma levels of ACTH and expression of Fos in the PVN that was nearly identical to that seen after acute microinjection of muscimol (Fig. 26, 27). These results suggest that neuronal activity in the DMH may not be involved in the increase in the plasma ACTH seen 15 minutes after administration of IL-1β. Even though pretreatment of the DMH with muscimol significantly attenuated the increase in plasma ACTH 30 minutes after the injection of IL-1β, muscimol-pretreated animals still exhibited significant greater plasma levels of ACTH than were seen in vehicle/vehicle control animals at this time. Likewise in these muscimol-pretreated animals, the expression of Fos in the PVN evoked by IL-1β, which was significantly lower than that in vehicle-pretreated animals, remained significantly greater than that in vehicle/vehicle controls. Control experiments involving the microinjection of muscimol into sites outside the DMH were not performed. Thus,

the partial attenuation in the increase in plasma ACTH evoked by IL-1β could be argued to be due to the effect of muscimol diffusing to the nearby PVN. However, microinjection of the same dose of muscimol into the DMH had no effect on the expression of Fos in the PVN induced by hemorrhage (Morin et al., 2001), suggesting that any effects here are not due to spread to the PVN. Together, these results indicate that neuronal activity in the DMH contributes to the increases in plasma ACTH and neuronal activity in the PVN evoked by intra-arterial administration of IL-1β, but that IL-1β also activates the HPA axis through other pathways that are independent of the DMH.

While inhibition of the DMH caused a partial, but significant reduction in plasma ACTH evoked by intra-arterial administration of IL-1β, this inhibition failed to affect the increase in corticosterone (Fig. 26), the principle glucocorticoid in rats (Webster & Sternberg, 2004). As noted above, administration of IL-1β to muscimol-pretreated animals resulted in plasma levels of ACTH that were significantly greater than those seen in vehicle/vehicle controls and may have been sufficient to produce a maximal release of corticosterone from the adrenal gland. In fact, maximal release of corticosterone occurs at plasma levels of ACTH between 200-300 pg/mL (Kaneko et al., 1981; Keller-wood et al., 1983), much lower than the levels seen in these rats at 30 min (i.e., 545 ± 58 pg/mL). While plasma levels of ACTH above 300 pg/mL do not further increase levels of corticosterone, these levels do prolong the duration of the corticosterone response (Keller-Wood, 1983). By itself, the DMH-mediated suppression of the increase in plasma ACTH evoked by IL-1β that was found to be statistically significant may not be physiologically relevant in terms of the release of corticosterone in the rat.

Microinjection of muscimol into the DMH failed produce a significant reduction in plasma ACTH and expression of Fos in the PVN evoked by intra-arterial administration of LPS (Fig. 30). These results, while providing further support that muscimol microinjected into the DMH in this manner does not directly affect neurons in the PVN, also suggest that neuronal activity in the DMH does not play a critical role in LPS-induced activation of the HPA axis. Thus, LPS, which causes the release of IL-1β, may signal activation of the HPA axis through additional mechanisms as well. Interleukin-1β-deficient mice exhibited normal LPS-induced activation of HPA axis (Fantuzzi & Dinarello, 1996), and IL-1 receptor blockade failed to prevent increases in plasma ACTH and glucocorticoids in humans treated with LPS (Van Zee et al., 1995). Systemic administration of LPS results in the release of several inflammatory cytokines in addition to IL-1β that are capable of activating the HPA axis, including IL-6 and TNF-α (Givalois et al., 1994; Dunn, 2000; Johnson et al., 2002). These cytokines may activate the HPA axis through pathways that do not involve the DMH (Watkins et al., 1995; Turnbull & Rivier, 1999; Conti et al., 2004). Thus, neuronal activity in the DMH may be one of several mechanisms contributing to the activation of the HPA axis by LPS, but no evidence for that role was provided by my results.

To ensure that muscimol was active during the peak increase in plasma ACTH, I microinjected muscimol into the DMH 60 minutes after the administration of LPS (Fig. 28). However, plasma levels of ACTH were already significantly increased 60 minutes after the injection of LPS, suggesting that, at least to some extent, activation of neurons in the PVN had already occurred by this point. Return to baseline levels of Fos expression after a stimulus takes at least several hours (Dragunow & Faull, 1989). Therefore, if LPS

had already activated neurons in the PVN at the time of the microinjection of muscimol into the DMH at 60 minutes, the resulting increase in Fos expression might be unaffected in rats sacrificed 120 minutes after administration of LPS. Consequently, the results of this experiment cannot definitively clarify the role of the DMH in LPS-induced activation of neurons in the PVN.

In summary, the results of these experiments indicate that neurons in the DMH contribute to the activation of the HPA axis evoked by systemic administration of IL-1β in conscious rats. Expression of Fos in the DMH was increased by the systemic administration of IL-1β, an effect that was associated with increases in plasma ACTH, corticosterone, and the expression of Fos in the PVN of the same rats. However, increases in plasma ACTH and the expression of Fos in the PVN evoked by IL-1β were only modestly attenuated 30 minutes after microinjection of muscimol into the DMH, and circulating corticosterone was unaffected in these animals. The results of my studies discussed previously suggest that activation of the HPA axis evoked from the POA is completely dependent on neuronal activity in the DMH. Thus, failure of neuronal inhibition in the DMH to similarly suppress the activation of the HPA axis evoked by administration of IL-1β or LPS may suggest that the more important signaling pathways used by these agents to evoke neuroendocrine responses do not involve the POA.

Despite the anatomical and physiological evidence that support the role of the POA in responses evoked by bacterial endotoxin or inflammatory cytokines, alternative theories exist to explain how cytokines elicit centrally mediated responses. Distinct carrier-mediated transporters capable of chaperoning substances across the blood-brain-barrier have been identified for several inflammatory cytokines including IL-1β (Banks et

al., 1991; Banks & Kastin, 1991; Banks et al., 1995). These transporters could hypothetically increase central levels of cytokines in brain regions thought to be protected from circulating substances by the BBB (Banks et al., 1995). However, it has been argued that the amount entering the brain through this mechanism may not be sufficient to account for the rapid induction of responses evoked by the systemic administration of these cytokines (Watkins et al., 1995). Turnbull & Rivier suggested that transport-mediated entry of cytokines into the brain may play a more significant role when circulating levels of cytokines are elevated for prolonged periods of time, such as in chronic inflammation (Turnbull & Rivier, 1999).

Another theory proposes that cytokines act on neural afferents to evoke physiological responses through ascending central pathways. Vagotomy has been reported to abolish or significantly reduce the febrile response to intraperitoneal administration of LPS (Opp & Toth, 1998) or IL-1β (Watkins et al., 1995a; Hansen & Krueger, 1997; Opp & Toth, 1998). Disruption of vagal afferents also attenuates increases in plasma levels of ACTH and corticosterone and the expression of Fos in the region of the PVN evoked by intraperitoneal administration of LPS (Wan et al. 1994; Wieczorek et al., 2005) or IL-1β (Kapcala et al., 1996; Wieczorek & Dunn, 2006). Increased electrical discharge in vagal afferents or the expression of Fos in primary afferent neurons of the vagus is evoked by intraperitoneal (Goehler et al., 1998), intravenous (Ek et al., 1998), and intra-hepatic portal administration of IL-1β (Niijima, 1996). Binding sites for IL-1β have also been localized to vagal afferents (Goehler et al., 1997; 1999). However, Hansen and colleagues reported that the levels of corticosterone evoked by intraperitoneal administration of various doses of LPS are unaffected by

vagotomy (Hansen et al., 2000), contradicting other published findings (Wieczorek et al., 2005) and suggesting that inflammatory insults may signal through alternative or redundant pathways to activate the HPA axis. Most of these studies employed intraperitoneal administration of IL-1β and LPS, and vagotomy may have significantly different effects on responses evoked by these substances when they are given by different routes (Katsuura et al., 1988; Wan et al., 1994; Goldbach et al., 1997). For example, subdiaphragmatic vagotomy in rats completely blocked the increase in the expression of Fos in the PVN evoked by intra-peritoneal injection of LPS, but had no effect on that elicited by intravenous administration (Wan et al., 1994).

The physiological responses evoked by inflammatory insults may also be the result of increased production of inflammatory cytokines within the CNS. Immunoreactivity for IL-1β and other inflammatory cytokines in several brain areas including the hypothalamus has been reported in animals (Lechan et al., 1990) and humans (Breder et al., 1988). While constitutive expression of cytokines in the CNS is relatively low, peripheral challenge with LPS elicits an increase in hypothalamic expression of IL-1β (Hillhouse & Mosley, 1993), suggesting a possible role for central cytokines in physiological responses evoked by systemic inflammatory insults. Injection of cytokines directly into the cerebral ventricles elicits autonomic and neuroendocrine responses that are similar to those seen with systemic administration, although typically at much lower doses (Rothwell et al., 1996; Turnbull & Rivier, 1999). While it is unclear whether the receptors for cytokines within the brain parenchyma are located on neurons or non-neuronal cells, binding sites and expression of mRNA for IL-1β as well as other cytokines have been identified within the hypothalamus (Hopkins & Rothwell, 1995).

The febrile response to systemically administered LPS is suppressed by intracerebroventricular injection of neutralizing antibodies to IL-1β (Rothwell at et., 1996), and activation of the HPA axis evoked by LPS is abolished by continuous intracerebroventricular perfusion of an IL-1 receptor antagonist (Kakucska et al., 1993). Interestingly, whole-cell patch clamp recordings from the POA indicate that IL-1β decreases the activity of neurons in this area through the presynaptic release of GABA (Tabarean et al., 2006), suggesting that IL-1β may evoke physiological responses by indirectly inhibiting neurons in the POA. Together, these results indicate a role for cytokines, and in particular IL-1β, acting within the brain, possibly at the levels of the POA, to evoke physiological responses to peripheral inflammatory challenges.

In summary, despite the multiple theories describing how circulating cytokines may act on the brain, the POA is likely to play an important role in the generation of central responses evoked by these substances. The POA is a thermoregulatory brain center, and acute phase responses including the induction of fever are thought to depend on signals from this region (Saper & Breder, 1994). Injection of the putative secondary mediator of inflammatory cytokines PGE_2 directly into the POA elicits a pattern of physiological responses that is similar to that seen following systemic injection of inflammatory cytokines. Inhibitors of PGE_2 synthesis, administered systemically or directly into the POA, are powerful suppressors of cytokine-evoked responses. Finally, as described above, the neural pathways exist between the POA and the brain centers necessary to evoke the physiological responses observed following the peripheral administration of inflammatory cytokines.

It is clear from my experiments that neuronal activity in the DMH plays some role in responses evoked by systemic administration of IL-1β. Intra-arterial injections of IL-1β increased the expression of Fos in the DMH, indicating increased neuronal activity in this region. Furthermore, inhibiting neurons in the DMH did significantly attenuate the increase in plasma ACTH and expression of Fos in the PVN evoked by IL-1β. However, the overall physiologic relevance of DMH-dependent mechanisms in responses elicited by IL-1β is not entirely clear from my experiments. It is likely, based on the evidence described above, that multiple central pathways contribute to inflammatory responses, including not only the activation of the HPA axis but also the autonomic responses that characterize these responses. My data indicates that a pathway involving the POA and the DMH should be considered by future investigators when these responses are further described.

4.3 Future directions for study

The results of these studies suggest that physiological and behavioral responses evoked from the POA are dependent on the activation of neurons in the DMH. Unfortunately, only muscimol was employed to evoke responses from the POA. The microinjection of PGE_2 into the POA is known to produce autonomic and neuroendocrine responses similar to those seen with muscimol and is a widely employed model of the acute phase response (Zaretsky et al., 2006). To date little is known about the role of the DMH in the integrative autonomic and neuroendocrine response evoked by PGE_2 in the POA. Three studies in anesthetized rats have suggested that neuronal activity in the DMH is involved in thermogenic and cardiovascular responses evoked by PGE_2

(Zaretskaia et al., 2003; Madden & Morrison, 2004; Nakamura et al., 2005). However, none of these studies examined activation of the HPA axis. Microinjection studies in conscious animals similar to those used in my experiments could better define the role of the DMH in the generation of the acute phase response by examining responses evoked by PGE_2 in the POA.

While the microinjection of PGE_2 or muscimol into the POA in conscious rats produces similar autonomic and neuroendocrine responses, effects on locomotor activity differ substantially (Zaretsky et al., 2006). Muscimol elicits dramatic increases in locomotor activity, while PGE_2 evokes no significant change from baseline levels (Osborne et al., 1993; Zaretsky et al., 2006). The results of these studies are the first to indicate that locomotor activity evoked by muscimol in the POA is dependent on the activity of neurons in the DMH. However, these experiments did not reveal the exact distribution and location of neurons within the DMH that were activated by injection of muscimol into the POA. The expression of Fos in the DMH evoked by microinjections of PGE_2 into the POA has never been reported. Comparison of the distribution of Fos-expressing neurons in the DMH seen after microinjection of PGE_2 into the POA with that seen after similar microinjection of muscimol may reveal a distinct population of neurons in the DMH that are potentially involved in locomotor activity evoked from the POA. Further, the analysis of retrogradely-labeled cells from the DMH co-localized with markers for GABA or prostaglandin receptors could identify distinct neurons in the POA responsible for the difference in locomotor activity evoked by muscimol and PGE_2. While the results of a recent microinjection study by Zaretsky and colleagues revealed no difference in the distribution of sites in the POA responsive to PGE_2 versus those

responsive to muscimol (Zaretsky et al., 2006), a similar study employing microinjection of smaller volume or dose may elucidate previously unknown differences in the distribution of these neurons. Together, these studies would assist in defining neural pathways for stressors thought to evoke locomotor activity through GABAergic mechanisms in the POA, such as the exposure to cold (Girardier et al., 1995; Ishiwata et al., 2005). Hyperactivity evoked by perfusion of muscimol into the POA is associated with an increase in striatal dopamine (Osborne et al., 1993) suggesting the possibility of tonic inhibition from the POA on extrapyramidal dopaminergic systems involved in locomotor activity. Since bilateral inhibition of the DMH prevented the increase in locomotor activity evoked from the POA, similar inhibition of the DMH may prevent the activation of extrapyramidal dopaminergic neurons resulting from the microinjection of muscimol into the POA. Analysis of the expression of Fos in extrapyramidal neurons or the measurement of striatal dopamine evoked by injection of muscimol into the POA in animals treated with muscimol in the DMH could identify a pathway responsible for the induction of locomotor activity evoked from the POA that involves the DMH.

Inhibition of the DMH produced a partial, but significant suppression in the increased plasma level of ACTH evoked by intra-arterial administration of the IL-1β. However, this treatment failed to attenuate the increase in corticosterone in these same animals. With my previous results that demonstrated POA-evoked activation of the HPA axis is dependent on neuronal activity in the DMH, this finding suggests that the DMH, and consequently the POA as well, may not significantly contribute to the increase in circulating glucocorticoids evoked by systemically administered IL-1β. However, this does not exclude the possibility that other components of the acute phase response

evoked by intravascular administration IL-1β such as fever (Dascombe et al., 1988; Murakami et al., 1990) and tachycardia (Dascombe et al., 1988; Bataillard & Sassard, 1994) are mediated by the POA. Cytokines are thought to decrease the firing rate of warm sensitive neurons in the POA to induce febrile responses (Boulant, 2000), and inhibition of neurons in the POA disinhibits neurons in the DMH to evoke thermogenesis and tachycardia (Nakamura et al., 2005; Zaretsky et al., 2006). To date, no studies have investigated the role of the DMH in cytokine-induced thermogenesis or tachycardia. Microinjection studies similar to my experiments would advance our understanding of thermogenic mechanisms associated with infectious and inflammatory processes.

4.4 Summary and significance of this thesis

The goal of my study was to characterize the role of the DMH in responses evoked from the POA and by systemic administration of inflammatory cytokines. I hypothesized that neuronal activity in the DMH was necessary for (1) physiological and behavioral responses evoked by microinjection of muscimol into the POA and (2) activation of the HPA axis evoked by intra-arterial administration of IL-1β. The results of experiments designed to test these hypotheses indicate that neurons in the DMH mediate a variety of responses evoked from the POA, including tachycardia, hypertension, hyperthermia, hyperactivity, and the activation of the HPA axis. This finding suggests that the DMH may participate in autonomic, neuroendocrine, and behavioral responses to a variety of stressors that are thought to involve the POA such as cold stress or systemic challenges with pyrogenic agents. This study was the first to test the role of the DMH in a central response, activation of the HPA axis, evoked by

systemic administration of a specific inflammatory cytokine, IL-1β. Neuronal activity in the DMH significantly contributed to the increase in plasma levels of ACTH evoked by IL-1β, but did not appear to affect corticosterone. While these results suggest that a DMH-dependent pathway is not the primary mechanism for the activation of the HPA axis evoked by IL-1β, they do not exclude the possibility that the DMH significantly contributes to the induction of other components of the acute phase response such as fever evoked by IL-1β. Clarifying the role of the DMH in cardiovascular and thermogenic responses evoked by IL-1β and other inflammatory cytokines will promote our understanding of the mechanisms mediating the acute phase response and may ultimately lead to the development of better treatment strategies for patients with systemic inflammatory disease.

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CURRICULUM VITAE

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IUSM Graduate Student Travel Award (2007)
Greater Midwest Pre-Doctoral Fellowship, American Heart Association (2005-2007)
Indiana Medical Scientist Training Program Scholarship, IUSM (2003-2004; 2007-Present)
Frederick and Anne Taylor Scholarship, IUSM (2002-2003, 2007-Present)
Henry and Hattie Steinmetz Scholarship, IUSM (2002)
Presidential Full Academic Scholarship, University of Indianapolis (1997-2002)

Research Experience:

Hypothalamic mechanisms of fever and thermoregulation (2002-2007). As part of the PhD in Medical Neuroscience under the supervision of Joseph DiMicco, PhD, Indiana University School of Medicine.
Physiologic response to stress following ligation-induced myocardial infarction in rats (2005-2007). Independent project under the supervision of Joseph DiMicco, PhD and Keith March, MD, Indiana University School of Medicine.

Professional Experience:

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Extracurricular Activities and Organizations:

Society for Neuroscience, Member (2004-Present)
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Publications/Dissertation:

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Posters, Oral Presentations, and Attended Conferences:

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J.L. Hunt. "The dorsomedial hypothalamus (DMH) mediates the integrative physiologic response to inhibition of neurons in the preoptic area (POA) of the mammalian

hypothalamus." Oral presentation at the 6th Annual IU School of Medicine MD/PhD Student Retreat, Bloomington, IN (2006)

J.L. Hunt, S. Sarker, D.V. Zaretsky, J.A. DiMicco. "Neurons in the dorsomedial hypothalamus (DMH) mediate activation of the hypothalamic-pituitary-adrenal (HPA) axis evoked from the preoptic area (POA) in conscious rats." Poster presentation at the Society for Neuroscience Meeting, Atlanta, GA (2006)

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