NORADRENERGIC INPUTS TO THE BED NUCLEUS OF THE STRIA TERMINALIS CONTRIBUTE TO THE NEURAL AND BEHAVIORAL EFFECTS OF THE ANXIOGENIC DRUG YOHIMBINE

by

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The alpha-2 adrenoceptor antagonist yohimbine (YO) increases transmitter release from NA terminals, activates the hypothalamic-pituitary-adrenal axis, and is anxiogenic. We have shown that YO dose-dependently activates Fos expression in the BNST and PVN, inhibits food intake, and supports conditioned flavor avoidance (CFA). Recruitment of NA inputs to the BNST may underlie these neural and behavioral effects of YO. To test this, NA inputs to the BNST were lesioned by microinjecting saporin toxin conjugated to DBH antibody (DSAP). Ten to 14 days post-surgery, food-deprived DSAP and sham rats were given i.p. YO (5.0 mg/kg) or vehicle. YO significantly inhibited food intake in both sham and DSAP rats. The second experiment examined the ability of YO to support CFA. In a two-bottle choice test, DSAP and sham rats significantly avoided drinking water containing flavors previously paired with YO treatment. The third experiment examined YO-enhanced anxiety on the elevated plus maze (EPMZ). The ability of YO to increase anxiety-like behavior was attenuated in DSAP rats. Finally, DSAP and sham rats were injected with YO or vehicle and perfused 90–120 minutes later. Brain sections were processed to reveal lesion extent and Fos activation. YO activated significantly fewer BNST and CRH-positive PVN neurons in DSAP rats compared to sham controls. DBH immunolabeling in the BNST and medial parvocellular PVN also was depleted in DSAP rats. The NST and VLM contained significantly fewer NA neurons in DSAP rats compared to sham controls; however, YO

activated similar proportions of the NA neurons that remained. We conclude that NA neurons innervating the BNST collateralize to innervate the medial parvocellular PVN, and that these NA inputs are necessary for YO to activate Fos within the BNST and PVN. Additionally, these inputs contribute to the YO-induced anxiety-like behaviors on the EPMZ. However, the NA inputs to the BNST are unnecessary for YO to inhibit food intake or support CFA, suggesting that other neural pathways are sufficient for these responses.

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PREFACE

I would like to dedicate this work to my parents Gholam and Azame Banihashemi. Their support, hard work and sacrifices have made it possible for me to pursue higher education. I would also like to thank my committee. Despite those who wish to see you fail, they remind me there are those who are there only to see you succeed. I would like to acknowledge and thank Joy Balcita-Pedicino, Victoria Maldovan, Jen-Shew Yen, Atara Marzouk, and Teresa Cox for their expert technical assistance.

1. INTRODUCTION

1.1. Aims and hypotheses

Noradrenergic (NA) projections from the nucleus of the solitary tract (NST) and ventrolateral medulla (VLM) to the bed nucleus of the stria terminalis (BNST) are implicated in stress and anxiety. For example, NA inputs to the BNST are activated by anxiogenic treatments such as precipitated opiate withdrawal (Aston-Jones, Delfs et al. 1999; Delfs, Zhu et al. 2000). The alpha-2 adrenoceptor antagonist yohimbine (YO) increases transmitter release from NA terminals, activates the hypothalamic-pituitary-adrenal (HPA) axis, and is anxiogenic (File 1986; Kiem, Barna et al. 1995). We have recently shown that YO (5 mg/kg BW, i.p.) activates Fos in NA [(i.e. dopamine beta-hydroxylase (DbH)-positive)] neurons in the NST, VLM, and locus coeruleus (LC). YO also induces Fos in the central nucleus of the amygdala (CeA), BNST, and in corticotropin-releasing hormone (CRH)-positive neurons in the paraventricular nucleus of the hypothalamus (PVN) (Myers, Banihashemi et al.). This is consistent with other reports using the same dose of YO (Tsujino, Sano et al. 1992; Singewald and Sharp 2000; Singewald, Salchner et al. 2003). We also have shown that YO (5 mg/kg BW, i.p.) inhibits food intake and supports conditioned flavor avoidance (Myers, Banihashemi et al.). Recruitment of NA inputs to the BNST may underlie these neural (Fos responses) and behavioral effects of YO.

Aim 1: To determine if YO activates NA inputs to the BNST.

Hypothesis 1: YO will induce Fos expression in BNST-projecting NA neurons.

Aim 2: To determine if the NA input to the BNST is necessary for YO's neural and behavioral effects.

Hypothesis 2.1: NA inputs to the BNST are necessary for YO to induce Fos expression in the BNST and PVN. BNST NA lesions will attenuate YO-induced Fos responses in BNST and PVN.

Hypothesis 2.2: BNST NA lesions will attenuate behavioral responses to YO including YO-induced anorexia, conditioned flavor avoidance (CFA), and anxiety-like behavior on the elevated plus maze (EPMZ).

1.2. Background

1.2.1. Emotions: stress, anxiety and viscerosensory feedback

Emotions are subjective feelings that are always associated with physiological states expressed through changes in the autonomic nervous system. In 1884, William James proposed that emotions may represent the perceptual consequences of visceral feedback. He stated, "What kind of an emotion of fear would be left if the feeling neither of quickened heart-beats nor of shallow breathing, neither of trembling lips nor of weakened limbs, neither of goose-flesh nor of visceral stirrings, were present, it is quite impossible for me to think… I say that for us emotion dissociated from all bodily feeling is inconceivable (James 1890)."

Damasio has extended this idea by proposing that emotions are central representations of internal stimuli or body states. In his "somatic marker" hypothesis, Damasio explains his view of how these representations are necessary for rational decision-making (Damasio 1999). He proposes that decision-making is strongly influenced by recollections of body/visceral states associated with previous experiences. If a memory of the result of a previous experience was negative, and was therefore associated with an aversive body state, a decision which may lead to a similar body state would be avoided (Damasio 1999). Damasio's somatic marker hypothesis is

embedded in lay language. For instance, the phrase “gut feeling” indicates a “pre-rational insight” or “emotional intelligence” associated with visceral states (Mayer, Naliboff et al. 2000).

As William James suggested, without accompanying physiological changes, emotions, as we know them, would not exist. Evidence for this is that individuals with pure autonomic failure (PAF) or peripheral denervation of autonomic neurons who do not have the ability to mount autonomic responses to emotional processing perform worse in emotional attribution tasks in which they have to predict the subjective emotional

states of others (Heims, Critchley et al. 2004). Furthermore, PAF patients have less conditioning-related activity in the amygdala than healthy subjects (Critchley, Mathias et al. 2002). Additionally, individuals with cervical or high thoracic spinal lesions, who receive less visceral feedback, have blunted feelings of fear (Hohmann 1966). Some of these patients report that although they do experience anger, their anger has a cognitive quality and lacks intensity or “heat” (Hohmann 1966). Additionally, stimulating autonomic responses can produce emotions associated with that body state. For example, drugs that activate the sympathetic nervous system can produce subjective feelings of anxiety (Charney, Heninger et al. 1983).

1.2.1.1. The stress response

A stressor is anything in the environment, real, perceived, or anticipated, that threatens an organism’s homeostasis. The stress response is the collection of physiological adaptations that deal with the threat and ultimately reestablish homeostasis (Sapolsky 2003). The stress response is characterized by autonomic, neuroendocrine and behavioral factors. The autonomic component ultimately leads to the release of norepinephrine (NE) and epinephrine from the adrenal medulla. The neuroendocrine component includes the HPA axis, which begins with the PVN and ultimately leads to the release of glucocorticoids from the adrenal cortex. These

responses lead to the mobilization of stored energy and the deactivation of nonessential activities like digestion and reproduction. These are adaptive responses when faced with a potentially dangerous situation.

1.2.1.2. Anxiety, fear and stress

Anxiety is a subjective emotional state sometimes associated with a threatening stimulus that can be characterized by its accompanying set of physiological symptoms including hyperarousal, tachycardia, HPA activation, nausea, etc. Anxiety and fear are terms often used synonymously. They are referred to as being triggered by "unconditioned stimuli" that are innately anxiety- or fear-inducing but are also triggered in response to "conditioned" or learned stimuli (Walker and Davis 1997). Stress and anxiety are highly interrelated because the physiological response to anxiety is a stress response. The stress response and the subjective feeling of anxiety are influenced by visceral feedback. For instance, palpitations and GI discomfort produce viscerosensory feedback that can provoke anxiety and stress responses. Drugs such as beta blockers, which reduce these peripheral symptoms, alleviate anxiety and have been shown to improve the performance of public speakers or artists (Argyropoulos, Sandford et al. 2000).

1.2.1.3. Anxiolytics and arousal: cholinergic and noradrenergic systems

Anxiolytic drugs include benzodiazepines and some noradrenergic antagonists. Benzodiazepine receptor (BZR) agonists, such as chlordiazepoxide, enhance GABA-gated chloride flux at the GABA/BZR receptor complex and are anxiolytic. These BZR agonists also impair cognitive processing. In contrast, BZR antagonists have anxiogenic actions and enhance certain cognitive processes. BZR antagonists stimulate basal cortical acetylcholine (ACh)

release and enhance autonomic reactions to anxiogenic stimuli. It is thought that basal forebrain cortical cholinergic activity may contribute to cognitive aspects of anxiety by promoting attentional processing of threat-associated stimuli. Attentional dysregulation is thought to be a critical aspect of anxiety disorder as anxious individuals are thought to attend more to threat-related stimuli than normal individuals. Thus, anxiolytics are thought to be therapeutically effective because of their attention-reducing properties (Bernston, Sarter et al. 2003).

The central NA system also has been implicated in attention/arousal, particularly the LC, which has widespread NA projections to subcortical and cortical areas (Southwick, Bremner et al. 1999). Additionally, the NA input to the amygdala has been strongly implicated in emotional memory (Clayton and Williams 2000; Williams, Disheng et al. 2000). Pharmacological agents that decrease central and peripheral NA signaling have some anxiolytic effects; for instance, alpha2-adrenergic agonists have been shown to decrease arousal and intrusive memories in post-traumatic stress disorder (PTSD) patients (Argyropoulos, Sandford et al. 2000).

Thus, the cortical cholinergic system has been hypothesized to be an essential part of the forebrain circuitry mediating general attentional functions, while activation of central NA systems may be required for viscerosensory feedback recruitment of attention/arousal and responses to emotionally salient, stressful or affective stimuli (Bernston, Sarter et al. 2003).

1.2.2. Role of noradrenergic projections in stress responses

The NST is the major central visceral sensory relay. It is the primary recipient of visceral sensory information from the vagus, glossopharyngeal, and facial nerves and also the spinal cord (spino-solitary tract) (Kalia and Sullivan 1982). The cell bodies of vagal afferents lie in the nodose ganglion, the peripheral processes of which innervate many major bodily organs (Loewy 1990). Another region of importance is the VLM which is best known for its role in

cardiovascular regulation. Both NST and VLM send NA inputs to other areas of the brain that are known to be involved in stress responses such as the LC, parabrachial nucleus (PBN), CeA, BNST, and PVN (Fig. 1-1). NA projections to these regions also originate in the LC and have been implicated in arousal (Fig. 1-1). However, there is increasing evidence that the NST and VLM have a more predominant role in physiological stress responses than the LC. In animal models, NA inputs from NST and VLM to forebrain regions have been implicated in emotional memory, and endocrine and behavioral responses to various stressors, including glucoprivation, immune challenge, and drug withdrawal.

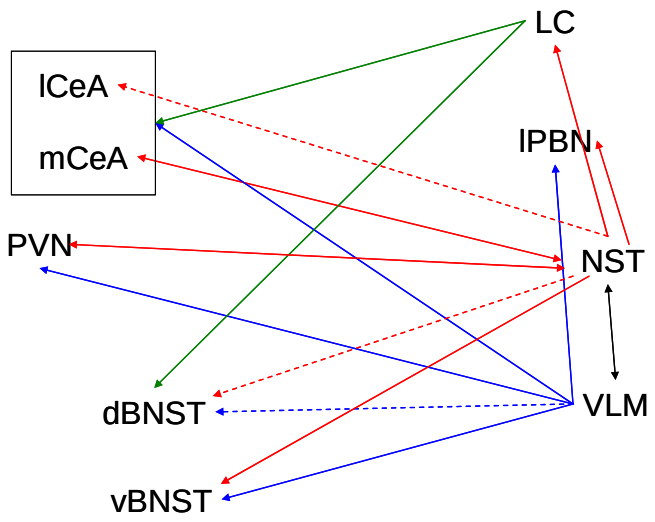

Figure 1-1 Ascending noradrenergic projections from NST, VLM, and LC. Double headed arrows indicate reciprocal projections. lBNST = lateral BNST, dBNST = dorsal BNST, lCeA = lateral CeA, mCeA = medial CeA, lPBN = lateral PBN

1.2.2.1. Emotional memory

Heightened arousal and emotional memory are very adaptive because they enable the organism to learn to avoid potentially dangerous situations or noxious stimuli. The NA system has been shown to play an essential role in arousal and encoding memory for emotionally salient events. Increases in NA neuron firing rate in the LC have been linked to alertness, stress and

fear activate the NA system, and stress-related increases in NE have been seen in targets of brainstem NA regions, such as the amygdala and the BNST (Pacak, McCarty et al. 1995; Southwick, Bremner et al. 1999).

Studies have shown that posttraining peripheral administration of epinephrine improves memory retention and increases extracellular NE in limbic regions such as the LC and the amygdala (Williams, Disheng et al. 2000). This memory enhancing effect of epinephrine is blocked by peripherally-acting beta antagonist, inactivation of the NST, NA antagonists into the NST, lesions of the amygdala, or beta antagonist into the amygdala (Clayton and Williams 2000; Williams, Disheng et al. 2000). Studies have shown that memory is significantly improved during retention tests after posttraining intra-NST beta agonist or epinephrine, both of which significantly increased NE in the amygdala (Clayton and Williams 2000; Williams, Disheng et al. 2000). Because epinephrine does not cross the blood brain barrier, it is thought that systemic epinephrine enhances memory by activating peripheral beta adrenergic receptors on the vagus nerve which terminate in the NST (Southwick, Bremner et al. 1999). According to this scenario, the NST then releases NE in the amygdala and other targets (Southwick, Bremner et al. 1999).

1.2.2.2. Glucoprivation stress

A study by Ritter and colleagues demonstrated the involvement of NA inputs to the PVN in the behavioral and neuroendocrine responses to glucoprivation stress (Ritter, Watts et al. 2003). In this study, specific lesions of NE and epinephrine neurons (DbH-containing) were made using DSAP (an antibody against DbH conjugated to saporin toxin). DSAP binds to vesicular DbH when the vesicle is exposed to the synaptic cleft during transmitter exocytosis. The enzyme-antibody-toxin complex is internalized by vesicle membrane endocytosis and retrogradely transported to the cell body. Once inside the cell body, saporin toxin inactivates

ribosomes, halting protein synthesis, thereby producing cell death (Madden, Ito et al. 1999). Glucoprivation was induced by metabolic blockade of glucose uptake with 2-deoxyglucose (2DG), or by administering a hypoglycemic dose of insulin. DSAP lesions of the NA inputs to the PVN blocked the increase in food intake normally induced by glucoprivation stress. DSAP lesions of the PVN also attenuated the corticosterone (CORT) response to 2DG and insulin. Additionally, DSAP lesions of the PVN attenuated the cFos mRNA response to 2DG (Ritter, Watts et al. 2003). As the LC does not project directly to the medial parvocellular PVN (Jones and Yang 1985), these results suggest that the ascending NA projections from the NST and VLM to the PVN are required for the behavioral and endocrine responses to glucoprivation stress.

1.2.2.3. Immune stress

Ascending NA projections have also been implicated in stress responses to an immune challenge. Immune challenge can be experimentally induced by direct intravenous injection of cytokines, such as interleukin-1 beta (IL-1). Ericcson et al. found that discrete unilateral knife cuts of ascending catecholaminergic projections from the medulla attenuated the Fos response to systemic IL-1 administration, as well as the increase in CRH mRNA in the ipsilateral PVN. These findings indicate that medullary catecholaminergic projections to the PVN are involved in mediating PVN activation in response to an immune challenge (Ericsson, Kovacs et al. 1994).

1.2.2.4. Drug withdrawal

Ascending NA projections to forebrain regions have also been implicated in stress responses to drug withdrawal. The aversive nature of drug withdrawal has been experimentally measured by conditioned place aversion, in which rats tend to avoid an environment previously paired with precipitated withdrawal. In the following study, 6-OHDA was used to lesion the

ventral NA bundle (VNAB), comprising axons of neurons in the NST and VLM. Aston-Jones and colleagues showed that VNAB lesions significantly attenuated opiate withdrawal-induced place aversion in morphine-dependent animals compared to controls (Delfs, Zhu et al. 2000). They also showed that lesions of the dorsal NA bundle, which originates in the LC, had no significant effect on opiate withdrawal-induced place aversion (Aston-Jones, Delfs et al. 1999; Delfs, Zhu et al. 2000). These studies demonstrate that the NA projections originating in the NST and VLM, and not the LC, play a role in mediating the aversiveness of drug withdrawal.

NA projections to forebrain regions have also been implicated in stress-induced reinstatement of drug seeking, an animal model of drug relapse. In this paradigm, rats that are trained to self-administer drugs undergo extinction sessions in which drugs are substituted with saline. Then, to test stress-induced reinstatement of extinguished drug taking, rats are subjected to footshock. Shaham and colleagues found that clonidine, an alpha-2 receptor agonist which decreases neurotransmitter release from NA terminals, injected systemically or into the lateral or fourth ventricles blocks footshock-induced reinstatement of heroin seeking. However, this effect was not seen when clonidine or its polar analogue was injected bilaterally into the LC. Thus, systemically or intraventricularly administered clonidine may be acting on NA systems originating in the caudal brainstem NST and VLM to block stress-induced reinstatement. Additionally, 6-OHDA lesions of the VNAB significantly attenuated footshock-induced reinstatement of heroin seeking (Shaham, Highfield et al. 2000). These data suggest that activation of the ascending NA projections from NST and VLM, and not the LC, are heavily involved in stress-induced reinstatement of drug seeking.

In summary, NA projections to limbic forebrain regions have been implicated in emotional memory, as well in responses to various physical stressors, including glucoprivation, immune challenge, and drug withdrawal.

1.2.3. Role of the NA inputs to the BNST in Stress Responses

There is increasing evidence implicating the NA projections from the NST and VLM to the BNST in mediating autonomic, neuroendocrine, and behavioral responses to stress or anxiety-inducing stimuli. The BNST is tactically positioned to mediate such responses. In addition to receiving its primary NA input from the NST and VLM (TerHorst, Boer et al. 1989; Phelix, Liposits et al. 1992; TerHorst and Streefland 1994; Terenzi and Ingram 1995), it is also interconnected with the amygdala (Dong, Petrovich et al. 2001) and lateral PBN (Alden, Besson et al. 1994) and sends a dense projection to the medial parvocellular division of the PVN (Dong, Petrovich et al. 2001), which contains CRH-positive neurons that comprise the apex of the HPA axis (Fig. 1-2).

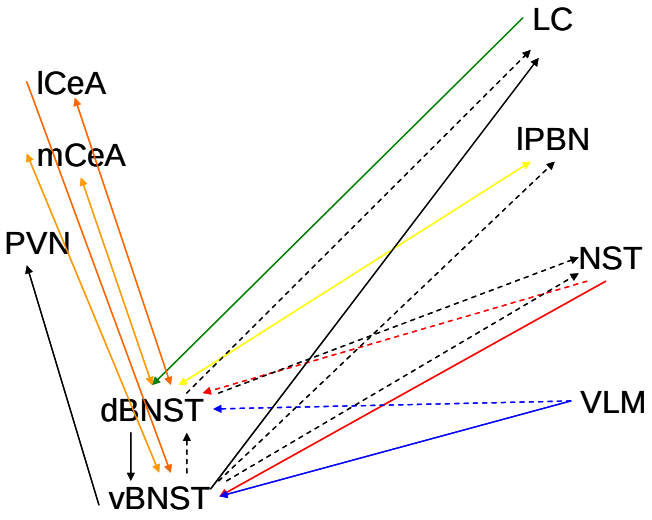

Figure 1-2 Projections from dorsal and ventral BNST subnuclei. Double headed arrows indicate reciprocal projections. Sparse projections are represented by dashed lines and moderate to heavy projection by solid lines.

1.2.3.1. Drug withdrawal

Several studies have implicated the BNST in drug withdrawal and stress-induced drug seeking. It has been reported that chronic morphine treatment and precipitated withdrawal increase extracellular NE levels in the ventral BNST (Fuentealba, Forray et al. 2000). Thus, NE release in the BNST could modulate its involvement in drug withdrawal.

Aston-Jones and colleagues have clearly demonstrated the involvement of the BNST in neural and behavioral responses to drug withdrawal. First, they found that opiate withdrawal markedly increased Fos-positive neurons in the ventrolateral and dorsolateral BNST. This effect was diminished by systemic treatment with a beta antagonist prior to withdrawal precipitation. Additionally, opiate withdrawal increased Fos in BNST-projecting, TH-positive NST and VLM neurons (Aston-Jones, Delfs et al. 1999; Delfs, Zhu et al. 2000). Thus, the NA projection from the NST and VLM to the BNST is activated by withdrawal.

This projection also appears to be involved in behavioral responses to withdrawal. Bilateral microinjection of a cocktail of beta-1 and beta-2 antagonists or alpha-2 agonists (the latter decreases NE release) into the BNST remarkably reduced conditioned-place aversion in a dose-dependent manner (Aston-Jones, Delfs et al. 1999; Delfs, Zhu et al. 2000). These data indicate that NA released endogenously into the BNST promotes withdrawal-induced aversion.

1.2.3.2. Drug relapse

The BNST has been implicated in drug relapse, which is fitting due to its described involvement in mediating the aversiveness of drug withdrawal. A series of studies by Stewart and colleagues implicate the BNST in stress-induced reinstatement of drug seeking. In this paradigm, rats trained to self-administer cocaine are removed from the drug for 5 days and are then given extinction and reinforcement tests. This group has previously shown that

intracerebroventricular (icv) corticotropin-releasing factor (CRF) receptor antagonist blocks footshock-induced reinstatement of drug seeking in cocaine-trained rats (Erb, Shaham et al. 1998). They then isolated these effects to the BNST when they showed that CRF receptor antagonist in the BNST, and not the amygdala, completely blocked footshock-induced reinstatement of cocaine seeking. Furthermore, CRF injections into the BNST, and not the amygdala, are sufficient to produce reinstatement of cocaine-seeking (Erb and Stewart 1999). These results suggest that CRF acting in the BNST is critical for footshock-induced reinstatement of cocaine seeking.

These investigators implicated the NA inputs to the BNST in stress-induced reinstatement of cocaine-seeking when they found that bilateral infusion of a cocktail of beta-1 and beta-2 receptor antagonists into the BNST attenuate footshock-induced reinstatement of cocaine seeking. Interestingly, a complete block of footshock-induced reinstatement was seen with beta antagonist infusions into the CeA. These results suggest that NE interactions with CRF neurons in both regions are critical for stress-induced relapse of drug seeking (Leri, Flores et al. 2002).

1.2.3.3. Immobilization stress

As mentioned above, drug dependency and withdrawal cause increased levels of NE in the BNST (Fuentealba, Forray et al. 2000). Additionally, Pacak and colleagues have shown that a two hour immobilization stress causes increased levels of NE in the lateral BNST during the first 30 minutes of immobilization that remain elevated for 90 minutes after the termination of immobilization (Pacak, McCarty et al. 1995). This result was later confirmed by Cecchi and colleagues who found increased NE release in the lateral BNST during 30 minute immobilization stress. This study also examined the effects of intra-BNST NA antagonists on social interaction

and EPMZ behaviors to a 5 minute immobilization. They found that intra-BNST alpha-1 receptor antagonist or a cocktail of beta-1 and beta-2 antagonists blocked the stress-induced reduction in open-arm exploration on the elevated plus maze but not the reduction in social behavior. Additionally, intra-BNST alpha-1 antagonist reduced plasma adrenocorticotropic hormone (ACTH) following 30 minute immobilization stress (Cecchi, Khoshbouei et al. 2002). These data suggest that stress-induced increases in NE in the BNST act on alpha receptors to mediate the stress-induced ACTH release and anxiety-like behavior on the EPMZ.

1.2.4. Yohimbine

YO is an indolealkylamine alkaloid obtained from the bark of Pausinystalia yohimbine, which grows in tropical West Africa and the Congo (Linden, Vellman et al. 1985). YO is primarily an antagonist of alpha-2 adrenergic receptors, which are found in the central and peripheral nervous systems and are located pre- and post-synaptically. Pre-synaptically, the alpha-2 receptor is an autoreceptor which normally inhibits the synaptic release of NE and other co-stored transmitters. Thus, YO's antagonistic action on presynaptic autoreceptors increases the release of endogenous transmitter and co-stored peptides from NA terminals. YO's effect at postsynaptic alpha-2 receptors reduces the ability of endogenous NE to decrease cAMP and intracellular [Ca^{2+}], while leaving unopposed the stimulating actions of NE on other postsynaptic receptor subtypes.

YO acts peripherally to produce sympathomimetic effects, which are relayed to the CNS via viscerosensory pathways. YO also acts centrally by crossing the blood-brain barrier. Therefore, the physiological and behavioral effects of systemic YO likely involve both peripheral and central nervous system actions.

Systemic YO increases neural firing activity in the LC (Aghajanian and VanderMaelen 1982) and promotes increased levels of extracellular NA in several terminal areas implicated in stress and anxiety responses, including the medulla, hypothalamus, CeA, and BNST (Szemeredi, Komoly et al. 1991; Pacak, Palkovits et al. 1995; Forray, Bustos et al. 1997; Khoshbouei, Cecchi et al. 2002). Systemic administration of YO mimics several symptoms commonly associated with anxiety (i.e., hyperarousal, tachycardia, HPA activation, autonomic dysfunction, and nausea) (Charney, Heninger et al. 1983; Linden, Vellman et al. 1985; Southwick, Bremner et al. 1999).

1.2.4.1. YO-induced Fos expression

It has been reported that YO (5 mg/kg) induces Fos expression in brainstem regions such as the LC, lateral PBN, NST and VLM, and forebrain regions such as the BNST, CeA, BLA, and PVN (Tsujino, Sano et al. 1992; Singewald, Salchner et al. 2003).

1.2.4.2. YO-induced anxiety-like behaviors

YO has been shown to inhibit food intake, produce conditioned place aversion, decrease social interaction, and promote anxiety-like behavior on the elevated plus maze. Callahan et al. found that YO (4.6 mg/kg) significantly reduced food intake 3 and 6 hours after i.p. administration in genetically obese and lean mice (Callahan, Beales et al. 1984).

Sandra File has used a place-conditioning paradigm to examine the aversive nature of YO. Following YO administration, rats were confined to one side of a two-compartment chamber. Rats were confined to the alternate side of the chamber after vehicle administration. Results showed rats preferred the side of the chamber that had been previously associated with vehicle administration; thus, YO produced a conditioned place avoidance (File 1986).

Additionally, YO (1.25 and 2.5 mg/kg) has been reported to cause a significant reduction in social interaction in rats. This effect is thought to be mediated by the action of YO on alpha-2 receptors because of its reversal by clonidine and its failure to be reversed by a BZR agonist (Pellow, Chopin et al. 1985).

YO has been reported to produce anxiety-like behavior on the EPMZ. The EPMZ is an apparatus consisting of four elevated arms, of which two opposing arms are open and the other two are closed. This model, which has high validity for use with rats, is based on rats' innate fear of open, elevated arms (Hogg 1996). As a result, rats allowed to freely explore the maze tend to avoid the open arms and remain in the closed arms. The EPMZ has commonly been used to examine the effects of anxiolytic drugs, which increase the number of entries into and time spent on open arms. Anxiogenic drugs tend to produce the opposite result (Cruz, Frei et al. 1994).

YO (2.5 mg/kg) has been reported to reduce the total number of arm entries, the percentage of entries onto the open arms, and the percentage of time spent on the open arms in rats (Baldwin, Johnston et al. 1989; Johnston and File 1989). A separate study reported that although YO did not affect closed arm entries, it did significantly decrease the open to total time ratio by approximately half compared to the control group (Khoshbouei, Cecchi et al. 2002). This effect is thought to be mediated by alpha-2 and dopamine receptors since it was reversed by both clonidine and apomorphine, a DA receptor agonist (Johnston and File 1989).

1.2.4.3. YO: clinical research

YO (5 mg/kg) produces a stimulatory effect on ACTH secretion in rats (Kiem, Barna et al. 1995). Additionally, YO increases subjective anxiety, blood pressure, 3-Methoxy-4-Hydroxyphenylglycol (MHPG, a NE metabolite), and cortisol in healthy human subjects

(Charney, Heninger et al. 1983; Charney, Woods et al. 1989; Gurguis, Vitton et al. 1997). YO-induced increases in plasma MHPG and CORT were seen in patients with generalized anxiety disorder (Charney, Woods et al. 1989).

The use of YO during clinical studies has provided evidence to support the hypothesis that the NA system is hyperactive in PTSD. PTSD patients experience panic attack and flashbacks in response to YO, as well as greater increases in heart rate, blood pressure, and plasma MHPG than controls. PTSD subjects also have an exaggerated acoustic startle when YO-treated compared to placebo, which was not seen in controls (Gurguis, Vitton et al. 1997; Southwick, Bremner et al. 1999). These data suggest that the NA system is hyperactive in PTSD patients, which is exacerbated by YO, leading to exaggerated physiological and behavioral responses to stress.

1.3. SUMMARY

In summary, NA projections to the BNST have been implicated in behavioral and/or endocrine responses to various stressors, including drug withdrawal and immobilization stress. Additionally, the NA input to the BNST has been implicated in stress-induced conditioned avoidance and anxiety-like behavior on the EPMZ. The present work focuses on the role of the NA input to the BNST in neural and behavioral responses to YO. We chose YO because of its aforementioned sympathomimetic, neuroendocrine, and behavioral effects indicate that it provides a pharmacological model for inducing stress and anxiety responses in rats.

We hypothesized that YO will activate BNST-projecting NA neurons, as they are activated by other anxiogenic treatments such as opiate withdrawal (Aston-Jones, Delfs et al. 1999; Delfs, Zhu et al. 2000). We hypothesized that NA inputs to the BNST are necessary for YO to induce Fos expression in the BNST and PVN, and that NA BNST lesions will attenuate

YO-induced Fos expression in these regions. Additionally, we hypothesized that NA inputs to the BNST are necessary for YO-induced anorexia, conditioned flavor avoidance (CFA), and anxiety-like behavior on the elevated plus maze (EPMZ), and consequently, that BNST NA lesions will attenuate these behavioral responses to YO.

A portion of this work is currently in press in the Journal of Comparative Neurology. This manuscript is presented in its entirety in the following chapter of this thesis. My contribution to this manuscript includes the study examining the YO-induced recruitment of viscerosensory inputs to the BNST and the studies examining YO-induced anorexia and conditioned flavor avoidance induced by a low dose of YO (1 mg/kg).

Portions of this work have also been presented in abstract form [Experimental Biology (FASEB) Annual Meeting 2004, Washington, D.C.; Society for Neuroscience Annual Meeting 2004, San Diego, CA; Neuroendocrinology of Stress Workshop 2005, San Diego, CA; Society for the Study of Ingestive Behaviors 2005, Pittsburgh, PA].

2. THE ANXIOGENIC DRUG YOHIMBINE ACTIVATES CENTRAL VISCEROSENSORY CIRCUITS IN RATS

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2.1. ABSTRACT

Systemic administration of the α_2-adrenoceptor antagonist yohimbine (YO) activates the HPA stress axis and promotes anxiety in humans and experimental animals. We propose that visceral malaise contributes to the stressful and anxiogenic effects of systemic YO, and that YO recruits brainstem NA and peptidergic neurons that relay viscerosensory signals to the hypothalamus and limbic forebrain. To begin testing these hypotheses, the present study explored dose-related effects of YO on food intake, conditioned flavor avoidance (CFA), and Fos immunolabeling in rats. Systemic YO (5.0 mg/kg BW, i.p.) inhibited food intake, supported CFA, and increased Fos immunolabeling in identified NA neurons in the ventrolateral medulla, nucleus of the solitary tract, and locus coeruleus. YO also increased Fos in the majority of corticotropin releasing hormone-positive neurons in the paraventricular nucleus of the hypothalamus. YO administered at 1.0 mg/kg BW did not inhibit food intake, did not support CFA, and did not increase Fos immunolabeling. Retrograde neural tracing demonstrated that neurons activated by YO at 5.0 mg/kg BW included medullary and pontine neurons that project to the central nucleus of the amygdala and to the lateral bed nucleus of the stria terminalis, with the latter region receiving comparatively greater input by Fos-positive neurons. We conclude that YO produces anorexigenic and aversive effects that correlate with activation of brainstem viscerosensory inputs to the limbic forebrain. These findings invite continued investigation of

how central viscerosensory signaling pathways interact with hypothalamic and limbic regions to influence interrelated physiological and behavioral components of anxiety, stress, and visceral malaise.

2.2. INTRODUCTION

Stress exacerbates and contributes to the development of psychiatric disorders such as anxiety, depression, post-traumatic stress disorder, panic disorder, and eating disorders (Smith, Kling et al. 1989; Charney and Deutch 1996; Southwick, Bremner et al. 1999; Gerra, Zaimovic et al. 2000; Brambilla 2001; Nandi, Meguid et al. 2002). Noradrenergic (NA) systems play an important role in shaping stress responses to real or perceived challenges and threats, and excessive central NA signaling is implicated in stress- and anxiety-related disorders in humans (Charney, Woods et al. 1989; Nutt 1990; Nandi, Meguid et al. 2002).

Hyperarousal, tachycardia, HPA activation, and nausea are some key symptoms commonly associated with anxiety that can be effectively mimicked by systemic administration of yohimbine (YO) and other drugs that increase NA signaling (Matilla, Seppala et al. 1988; Charney, Woods et al. 1989; Southwick, Bremner et al. 1999; Malcolm, Camilleri et al. 2000; Corre, Palmer et al. 2004). YO is an antagonist of pre- and postsynaptic α_2-adrenergic receptors. Its antagonistic effect at presynaptic autoreceptors potentiates the release of endogenous transmitter from NA nerve terminals, while its effect at postsynaptic receptors reduces the ability of endogenous NA to decrease cAMP and intracellular [Ca^{2+}] while leaving unopposed the stimulatory postsynaptic effects of NA that are mediated by other adrenoceptor subtypes. YO acts peripherally as a sympathomimetic, the consequences of which are relayed to the brain along viscerosensory pathways (Gurguis, Vitton et al. 1997). YO is highly lipophilic and crosses

the blood-brain barrier; thus, its physiological and behavioral effects after systemic administration involve both peripheral and CNS actions (Szemeredi, Komoly et al. 1991).

In humans, systemic YO elevates plasma catechol and cortisol levels, promotes nausea, increases subjective feelings of anxiety, increases startle responses, and can induce panic attacks (Charney, Heninger et al. 1983; Matilla, Seppala et al. 1988; Charney, Woods et al. 1989; Charney and Deutch 1996; Gurguis, Vitton et al. 1997; Stine, Southwick et al. 2002). YO also increases plasma catechol and ACTH levels and promotes behavioral signs of anxiety in rats and mice (Callahan, Beales et al. 1984; Pellow, Chopin et al. 1985; Szemeredi, Komoly et al. 1991; Wada and Fukada 1991; Kiem, Barna et al. 1995; Tanaka, Yoshida et al. 2000; White and Birkle 2001; Khoshbouei, Cecchi et al. 2002; Schroeder, Schiltz et al. 2003). Multiple brain regions are implicated in these behavioral and physiological responses, and systemic YO increases immunolabeling of the immediate-early gene product, Fos, in these regions (Bing, Stone et al. 1992; Tsujino, Sano et al. 1992; Stone, Zhang et al. 1993; Singewald and Sharp 2000; Singewald, Salchner et al. 2003).

The generally similar physiological and behavioral effects of YO in humans and animals makes this agent a useful experimental tool for bringing together clinical and preclinical findings relevant to the neural underpinnings of stress, visceral malaise, and anxiety, which appear to be highly interrelated. Shared neural mechanisms may include altered signaling within viscerosensory circuits that impinge upon the hypothalamus and limbic forebrain. However, the extent to which various central components of these neural circuits overlap or are necessary for stress, malaise, and anxiety is unclear. To address this issue, the present study explored dose-related effects of YO on two interoceptively influenced behaviors (i.e., feeding and conditioned flavor avoidance), and correlated these behavioral effects with YO-induced recruitment of

identified NA neurons in the medulla and pons, corticotropin releasing hormone (CRH)-positive neurons in the paraventricular nucleus of the hypothalamus (PVN), and ascending inputs to the central nucleus of the amygdala (CeA) and bed nucleus of the stria terminalis (BNST).

2.3. Materials and Methods

2.3.1. Subjects

Adult male Sprague-Dawley rats (250-330 g BW; Harlan Laboratories) were housed singly in stainless steel cages in a controlled environment (20-22ºC, 12:12 hr light:dark cycle; lights on at 0700 hr) with ad libitum access to water and pelleted chow (Purina 5001), except as noted. Rats were acclimated to daily handling for at least one week before experiments. All experimental protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

2.3.2. Experiment 1: Deprivation-Induced Food Intake

Food was removed from cages 2 hr before lights out. Twenty-four hours later, food-deprived rats (n=16) were injected i.p. with 2.0 ml of 0.15M NaCl vehicle alone, or vehicle containing YO (Sigma-Aldrich) at a dose of either 1.0 or 5.0 mg/kg BW. Rats received a pre-weighed measure of pelleted chow 30 min after i.p. injection (i.e., 1.5 hr before lights out). Cumulative food intake was recorded after 30 min, 60 min, and 15 hr of food access (i.e., 60 min, 90 min, and 15.5 hr post-injection), with intake amounts corrected for spillage. After a subsequent 48 hr period of *ad libitum* chow access, the 24 hr food deprivation and feeding test was repeated in a crossover design in which rats that received one of the two YO doses in the first test now received vehicle treatment, and rats that received vehicle treatment in the first test

now received one of the two YO doses. Thus, each rat served as its own control for determining the effect of YO on food intake (n=8 for each YO dose).

Data analysis. The effect of YO on deprivation-induced food intake was expressed in each rat as % suppression of intake compared to intake after vehicle treatment at each time point. Group data were analyzed by repeated measures ANOVA with YO dose and time as independent measures. When f values indicated significant overall main effects of dose and time on cumulative food intake, ANOVAs were followed up with selected comparisons of interest using planned t-tests. Differences were considered significant when $P < 0.05$.

2.3.3. Experiment 2: Conditioned Flavor Avoidance (CFA)

A sensitive two-bottle choice paradigm (Deutsch and Hardy 1977) was used to determine whether rats avoid consuming water that contains flavors that were previously paired with YO treatment. Separate groups of rats were used to determine the effects of each YO dose (i.e., 1.0 and 5.0 mg/kg BW; n=6 and n=10, respectively) on CFA. Flavor exposure during CFA training and testing was conducted near the end of the light cycle of the photoperiod, between 1500 and 1700 hr. Rats were acclimated for two days to i.p. injection of vehicle (0.15M NaCl; 2.0 ml) prior to the start of the CFA experiment.

Rats were water deprived for 22 hr. Half of the rats in each group were then presented with almond-flavored tap water to drink from a graduated tube, and the others with banana-flavored water (both 0.5% McCormick brand flavor extract). The left-right position of the drinking tube on each cage was switched after 15 min, with intake recorded at 15 and 30 min time points. Thirty minutes after the end of this initial flavor exposure session, all rats were injected i.p. with 2.0 ml 0.15M NaCl vehicle. Plain water was returned 30 min later, and rats had *ad libitum* water access for the next 24 hr. Rats were then water deprived again for 22 hr,

followed by presentation of the alternate flavor to drink for 30 min, with bottle positions switched after 15 min. Thirty minutes after the end of this second flavor exposure session, rats were injected i.p. with 2.0 ml of vehicle containing YO at a dose of either 1.0 or 5.0 mg/kg BW. Plain water was returned 30 min later, and rats had *ad libitum* water access for 24-48 hr. Rats were finally water deprived again for 22 hr, and then given 30 min simultaneous access to two bottles of water, one containing the vehicle-paired flavor and the other containing the YO-paired flavor. The volume consumed from each bottle was recorded at 15 min, bottle positions were switched, and cumulative intake was recorded at 30 min. Rats then were returned to *ad libitum* water access.

Data analysis. Flavor preference ratios displayed by each rat during the two-bottle choice test were determined by dividing the volume consumed from each bottle (i.e., vehicle-paired flavor vs. YO-paired flavor) by the total volume consumed from both bottles in 30 min. Individual preference ratios were averaged within each treatment group to obtain group preference ratios (mean ± SE) for intake of vehicle-paired flavors relative to YO-paired flavors. Outcomes indicating mean flavor preference ratios that did not differ significantly (i.e., close to 50%:50%) were interpreted as an absence of CFA, whereas outcomes indicating significantly shifted preference ratios (e.g., 70%:30%) were interpreted as evidence for conditioned avoidance of the flavor represented by the lower value in the ratio. Student's t-test was used to determine whether differences in preference for vehicle-paired vs. YO-paired flavors were statistically different, with significance set at $P < 0.05$.

2.3.4. Experiment 3: Activation of central Fos expression

In a terminal study, rats were injected i.p. with YO at doses of 0 (n = 6), 1.0 (n = 6), or 5.0 (n = 10) mg/kg BW in 2.0 ml 0.15M NaCl vehicle during the light cycle of the photoperiod,

between 1200 and 1500 hr. Rats were left undisturbed in their home cages for 90-120 min after injection, then were anesthetized with sodium pentobarbital (Nembutal, 100 mg/kg BW, i.p) and transcardially perfused with a brief saline rinse followed by 500 ml of fixative [4% paraformaldehyde in 0.1 M phosphate buffer with lysine and sodium metaperiodate (McLean and Nakane 1974). The 90-120 min post-treatment survival window was selected based on evidence that the inhibitory effect of YO on cumulative food intake in Experiment 1 was greatest at the 30 min time point and reduced but still significant at the 60 min time point (i.e., 60 and 90 min post-injection; see *Results*). Fos immunolabeling generally peaks 60-90 min after the onset of significant neural stimulation, and persists at high levels for at least 60 min more even when neural stimulation is transient (Morgan and Curran 1991; Hoffman, Smith et al. 1993). Thus, rats were perfused during a 30 min portion of the temporal window that corresponded to presumably peak Fos immunolabeling. Brains were post-fixed overnight at 4˚C, then blocked and cryoprotected in 20% sucrose prior to sectioning.

Neural tracer injections. Additional rats (n = 25) received microinjections of retrograde neural tracer into the CeA or lateral BNST (procedure described below) one week before i.p. injection of 2.0 ml 0.15M NaCl vehicle or YO (5.0 mg/kg BW). Rats were anesthetized and perfused with fixative 90-120 min after i.p. injection, as described in the preceding section. None of the tracer-injected rats received YO at the lower 1.0 mg/kg BW dose, because the lower dose did not significantly activate neurons in medullary or pontine regions that provide direct neural input to the CeA or BNST (see Results).

CeA injections. Rats were anesthetized by halothane inhalation (Halocarbon Laboratories; 1-3% in oxygen) and mounted into a stereotaxic frame in the flat-skull position. Two retrograde tracers were used for CeA microinjections (n=14): Fluorogold (FG,

Fluorochrome, Inc; 2.0% in 0.15 M NaCl) and cholera toxin, beta subunit (CTb, List Biological Laboratories; 0.25% in 0.15 M NaCl). Rats received FG injections targeted to the left CeA and CTb injections targeted to the right CeA. The dual tracer injection procedure increased the chance of obtaining at least one accurate tracer injection site in each rat. A 1.0 µl Hamilton syringe filled with either FG or CTb was attached to the stereotaxic arm. CeA coordinates (2.2 mm posterior, 4.0 mm lateral, and 8.2 mm ventral to Bregma) were selected based on a standard rat brain atlas (Paxinos and Watson 1997). The syringe was lowered into the brain and left in place for 5 min prior to injection. FG (50 nl) or CTb (100-150 nl) was delivered by pressure over a 1-2 minute period. The syringe was left in place for an additional 7 min after each injection to reduce tracer diffusion up the needle tract. The skin was closed with stainless steel clips, a topical anesthetic (2% lidocaine) was applied to the incision site, and rats were returned to their home cages after recovery from anesthesia. Rats were given 7-10 days to recover from surgery and allow sufficient time for retrograde tracer transport before YO or vehicle treatment and perfusion, as described above.

BNST injections. FG (50 nl) was injected by pressure into the left lateral BNST (0.28 mm posterior, 2.8 mm lateral, and 7.6 mm ventral to bregma) in a separate group of halothane-anesthetized rats (n=11). A subset of these rats (n=8) received a contralateral injection of CTb (100 nl) into the right BNST using the same microinjection coordinates. The injecting needle was angled 10 degrees laterally from vertical in order to avoid passing through the lateral ventricle and septum en route to the BNST. Other details regarding surgical procedures and post-surgical treatment are similar to those described for CeA tracer injections.

Histology and immunocytochemistry. Coronal 35 µm-thick tissue sections were cut from the caudal extent of the medullary dorsal vagal complex through the rostral extent of the

corpus callosum using a freezing microtome. Sections were collected serially in six adjacent sets and stored at –20°C in cryopreservent (Watson, Wiegand et al. 1986). Sections were removed from storage and rinsed for 1 hr in buffer (0.1 M sodium phosphate, pH 7.4) prior to immunocytochemical procedures. Antisera were diluted in buffer containing 0.3% Triton-X and 1% normal donkey serum. Biotinylated secondary antisera (Jackson Immunochemicals) were used at a dilution of 1:500.

Tissue sections were processed for immunocytochemical localization of Fos protein using a rabbit polyclonal antiserum (1:50,000; provided by Dr. Philip Larsen, Denmark) and Vectastain Elite ABC immunoperoxidase reagents (Vector Laboratories). The specificity of this antibody for Fos has been reported (Rinaman and Stricker 1997). Sections were processed using a nickel sulfate-intensified DAB reaction to generate a blue-black reaction product in the nuclei of Fos-positive cells. Adjacent sets of Fos-labeled tissue sections were processed for immunoperoxidase localization of cytoplasmic CRH (rabbit anti-CRH, 1:15,000; Penninsula) and the NA synthetic enzyme, dopamine beta hydroxylase (mouse anti-DbH, 1:30,000; Chemicon), using non-intensified DAB to generate a brown cytoplasmic reaction product. In tracer-injected rats, Fos-labeled tissue sections were processed for immunoperoxidase localization of FG (rabbit anti-FG: Chemicon; 1:30,000) or CTb (goat anti-CTb; List Biological Laboratories; 1:50,000). DAB was used to generate a brown reaction product at the amygdala and BNST FG and CTb injection sites, and in the cytoplasm of retrogradely labeled neurons.

After immunocytochemical processing, tissue sections were mounted onto Superfrost Plus microscope slides (Fisher Scientific), allowed to dry overnight, dehydrated and defatted in graded ethanols and xylene, and coverslipped using Cytoseal 60 (VWR).

Quantification of Fos activation. Dual immunoperoxidase-labeled tissue sections were analyzed with a light microscope to determine the number and proportions of phenotypically identified Fos-positive neurons. Criteria for counting a neuron as DbH-positive, CRH-positive, or retrogradely labeled (i.e., CTb- or FG-positive) included the presence of brown cytoplasmic immunoreactivity and a visible nucleus. Phenotypically identified neurons were considered Fos-positive if their nucleus contained blue-black immunolabel, regardless of intensity, and Fos-negative if their nucleus was unlabeled.

DbH-positive neurons and retrogradely labeled neurons were counted bilaterally in each region at 40x magnification. Quantitative analysis of Fos immunolabeling of DbH-positive NA neurons and retrogradely labeled neurons was limited to a subset of brainstem regions that provide direct input to the CeA and/or BNST, including the pontine locus coeruleus (LC; location of the A6 cell group), the lateral parabrachial nucleus (PBN), the nucleus of the solitary tract (NST; location of the A2/C2 cell groups), and the ventrolateral medulla (VLM; location of the A1/C1 cell groups).

NST (A2/C2 cell groups) and VLM (A1/C1 cell groups): Counts of DbH-positive or retrogradely-labeled NST and VLM neurons were grouped according to 3 rostrocaudal levels defined with respect to the area postrema (AP): (1) sections caudal to the AP (cNST and cVLM; A2 and A1 cell groups, respectively); (2) sections through the level of the AP (mNST and mVLM; A2/C2 and A1/C1 cell groups, respectively); and (3) sections rostral to the AP (rNST and rVLM; C2 and C1 cell groups, respectively). Counts in rostral sections were discontinued at the level at which the NST moves laterally away from the floor of the fourth ventricle. Counts of retrogradely labeled NST and VLM neurons and the proportions that were Fos-positive were summed across sections at each rostrocaudal level. Counts of Fos- and DbH-positive NST and

VLM neurons were summed at each rostrocaudal level and then averaged across the number of sections analyzed to obtain mean counts per section.

In a preliminary study, DbH-positive neurons with visible nuclei were counted bilaterally within the NST and VLM in six representative cases with optimal immunolabeling and tissue quality (vehicle controls, n = 3; YO 5.0 mg/kg, n=3). In each case, counts were grouped by rostrocaudal level as described in the preceding paragraph, and then averaged across sections counted to derive the mean number of DbH-positive NST and VLM neurons per section at each rostrocaudal level. As expected, there was little variability across animals in the number of DbH-positive neurons counted within the NST or VLM at each level, and no significant effect of treatment. Thus, counts obtained in these six rats were averaged to provide the following estimates of the number of DbH-positive neurons per section (mean ± SE): cNST, 30.2 ± 1.8; mNST, 66.7 ± 3.4; rNST, 38.3 ± 2.2; cVLM, 26.3 ± 1.5; mVLM, 28.1 ± 1.1; and rVLM, 27.8 ± 2.0. These values were used to estimate the proportions of NA neurons within the NST and VLM that were Fos-positive within each treatment group.

***LC (A6 cell group)*:** Counts of DbH-positive or retrogradely labeled LC neurons that were double labeled for Fos were summed bilaterally and averaged over 2 tissue sections spaced 210 m apart that contained the greatest number of Fos-positive neurons within the LC.

PBN. Counts of retrogradely labeled neurons within the lateral PBN and the proportion expressing Fos were summed unilaterally and averaged over two sections spaced 210 µm apart that contained the highest density of retrograde labeling ipsilateral to the CeA or BNST tracer injection site.

***Hypothalamic CRH neurons*.** The number of CRH-positive PVN neurons and the proportion of these that were Fos-positive in each rat was documented at 100x magnification

using oil immersion with the assistance of Stereo Investigator X-Y plotting software (MicroBrightField, Inc). CRH-positive neurons in the PVN were summed bilaterally and averaged in each rat across two sections spaced 210 m apart that contained the highest density of CRH neural labeling. In each rat, Fos-positive (i.e., double labeled) neurons were expressed as a percentage of the total number of CRH-positive neurons counted.

Statistical analyses. Statistical comparisons of the numbers and/or proportions of phenotypically identified neurons that were Fos-positive were made using one-, two-, and three-way ANOVA with experimental treatments (i.e., YO dose and/or retrograde tracer injection site) and brain region as independent variables. When f values indicated significant main effects and/or interactions among experimental variables, ANOVAs were followed up with Fisher's least significant difference protected *t*-tests for multiple post hoc comparisons. Differences were considered statistically significant when $P < 0.05$.

Photography and image preparation. Tissue sections were photographed using a color digital camera attached to the microscope. Digital image files were imported into Adobe Photoshop for cropping and assembly of figures. Some color images were transformed to grayscale. Brightness and contrast were adjusted for consistency, but images were not otherwise altered.

2.4. RESULTS

2.4.1. Experiment 1: Deprivation-Induced Food Intake

Rats in low- and high-dose YO groups consumed equivalent amounts of rat chow after 24 hr food deprivation and i.p. vehicle injection (Table 2-1). Two-way repeated measures ANOVA revealed significant main effects of YO dose [$F(2,31) = 8.79$; $P = 0.001$] and time [$F(2,64) =$

1227.79; $P < 0.0001$] on deprivation-induced food intake, with no significant interaction between dose and time. Compared to food intake after vehicle treatment, intake after 5.0 mg/kg YO was suppressed by approximately 40% after 30 min of food access (i.e., 60 min post-injection), and by approximately 27% after 60 min of food access ($P < 0.05$ at each time point; Table 2-1, Fig. 2-1). Conversely, the lower 1.0 mg/kg YO dose did not suppress food intake at any time (Table 2-1, Fig. 2-1). The anorexigenic effect of 5.0 mg/kg YO diminished overnight and was not significant at the final 15 hr time point (Table 2-1, Fig. 2-1).

2.4.2. Experiment 2: Conditioned Flavor Avoidance (CFA)

Rats in both CFA experimental groups consumed equivalent volumes of novel almond- and banana-flavored water when these were initially offered in 30 min training exposure sessions [1.0 mg/kg group (n=6), average fluid intake 14.8 ± 0.7 ml, range 9-16 ml; 5.0 mg/kg group (n=10), average fluid intake 15.5 ± 0.5 ml, range 12-17 ml]. There were no significant differences in the volumes of almond- or banana-flavored water consumed by either group, and no significant intake difference between the two exposure days.

Results from the two-bottle choice tests revealed that rats avoided consuming flavors paired previously with the 5.0 mg/kg dose of YO, but did not avoid consuming flavors paired with the 1.0 mg/kg dose (Fig. 2-2). In the 5.0 mg/kg dose group (n=10), fluid intake in the 30 min choice test consisted of 9.6 ± 1.4 ml of the flavor previously paired with i.p. vehicle treatment, and 4.8 ± 1.1 ml of the flavor previously paired with YO ($P < 0.05$), evidence for a relatively strong CFA response to YO-paired flavors (i.e., ~69%:31% preference ratio). Conversely, in the 1.0 mg/kg dose group (n=6), fluid intake in the 30 min choice test consisted of 10.7 ± 1.3 ml of vehicle-paired flavors and 9.0 ± 1.7 ml of YO-paired flavors ($P = 0.36$, difference not significant; ~55%:45% preference ratio).

2.4.3. Experiment 3: Central Fos Immunolabeling

NST (A2/C2 cell groups): Two-way repeated measures ANOVA revealed a significant main effect (between subjects) of YO dose on Fos immunolabeling within the NST [$F(2,22) = 31.66$, $P < 0.0001$], with significantly more NA (i.e., DbH-positive) NST neurons double labeled for Fos in rats after treatment with YO at 5.0 mg/kg compared to rats treated with vehicle or with YO at 1.0 mg/kg (Fig. 2-3). Within subjects, there was a significant effect of rostrocaudal level (cNST, mNST, rNST) [$F(2,46) = 17.46$, $P < 0.0001$] and a significant interaction between treatment group and rostrocaudal level [$F(4,46) = 8.38$, $P < 0.0001$] in the average number of Fos-positive NA neurons. Post hoc *t* comparisons revealed that significantly more NA neurons at each rostrocaudal level of the NST were Fos-positive in rats treated with YO at 5.0 mg/kg compared to rats treated with vehicle or YO at 1.0 mg/kg (Fig. 2-3). Low and statistically similar numbers of NA neurons expressed Fos labeling in rats that received vehicle or the 1.0 mg/kg dose of YO; the only exception was a small but significant increase in Fos-positive NA neurons within the rNST in rats treated with 1.0 mg/kg YO (Fig. 2-3). The estimated proportions of DbH-positive neurons that also were Fos-positive within each NST region are indicated in Figure 2-3 by the number above each bar. Figure 2-4 shows representative photomicrographs of Fos immunolabeling in the mNST in rats after vehicle treatment (Fig. 2-4A) or after 5.0 mg/kg YO (Fig. 2-4D).

VLM (A1/C1 cell groups): Two-way repeated measures ANOVA revealed a significant main effect (between subjects) of YO dose on Fos immunolabeling within the VLM [$F(2,22) = 39.11$, $P < 0.0001$], with significantly more NA (i.e., DbH-positive) VLM neurons double labeled for Fos at each rostrocaudal level in rats treated with YO at 5.0 mg/kg compared to rats treated with vehicle or YO at 1.0 mg/kg (Fig. 2-3). Within subjects, there was a significant

effect of rostrocaudal level (cVLM, mVLM, rVLM) [$F(2,44) = 3.70$, $P = 0.034$] but no significant interaction between treatment group and rostrocaudal level in the average number of NA neurons per section expressing Fos immunolabeling. Low and statistically similar numbers of VLM NA neurons were Fos-positive in rats that received vehicle or the 1.0 mg/kg dose of YO. The estimated proportions of DbH-positive neurons expressing Fos immunolabeling within each VLM region are indicated in Figure 2-3 by the number above each bar. Figure 2-4 shows representative photomicrographs of Fos labeling in the mVLM in rats after vehicle treatment (Fig. 2-4C) or after 5.0 mg/kg YO (Fig. 2-4D).

***LC (A6 cell group)*:** One-way ANOVA revealed a significant main effect (between subjects) of YO dose on the number of LC neurons expressing Fos immunolabeling [$F(2,20) = 93.73$, $P < 0.0001$], with significantly more NA neurons double labeled for Fos in rats treated with YO at 5.0 mg/kg compared to rats treated with vehicle or the 1.0 mg/kg dose ($P < 0.05$ for each comparison). Relatively few Fos-positive neurons were present within the LC of rats treated i.p. with vehicle (5.1 ± 2.1 neurons per section; n = 6) or in rats treated with 1.0 mg/kg YO (13.6 ± 5.0 neurons per section; n = 6). The difference between these two groups in Fos immunolabeling was not significant ($P = 0.17$). Conversely, the 5.0 mg/kg dose of YO produced a marked increase in the average number of DbH-positive LC cells that were Fos-positive (163.3 ± 15.8 neurons per section; n = 10). Figure 2-4 shows representative photomicrographs of Fos immunolabeling within the LC in rats after vehicle treatment (Fig. 2-4E) or after treatment with 5.0 mg/kg YO (Fig. 2-4F). The proportion of NA neurons in the LC that were double labeled for Fos could not be accurately determined due to the high density of DbH immunolabeling and the resulting difficulty in distinguishing individual neurons and in visualizing unlabeled (i.e., Fos-negative) cell nuclei.

Hypothalamic CRH neurons. An average total of 521 ± 14.7 (mean ± SE) CRH-immunopositive PVN neurons were counted bilaterally across two tissue sections in each rat. There was no significant effect of treatment group on CRH-positive cell counts. One-way ANOVA revealed a significant main effect of treatment (0, 1.0, or 5.0 mg/kg YO) in the proportion of CRH neurons that expressed Fos immunolabeling [$F(2,20) = 215.9$, $P < 0.0001$], with a significantly larger proportion of CRH neurons labeled for Fos in rats treated with YO at 5.0 mg/kg (i.e., ~ 73%) compared to the low and statistically similar proportions of CRH-positive neurons double labeled for Fos in rats treated with vehicle (~ 3%) or treated with YO at 1.0 mg/kg (~ 2%) (Fig. 2-5). Figure 2-6 shows representative photomicrographs of Fos immunolabeling in CRH-positive PVN neurons in rats after treatment with vehicle (Fig. 2-6A) or YO (5.0 mg/kg; Fig. 2-6B).

Fos immunolabeling in other brain regions. In rats treated with the higher 5.0 mg/kg dose of YO, Fos expression was activated in many brain regions in addition to those described above, consistent with two earlier reports in which the same YO dose and administration procedure was used (Singewald and Sharp 2000; Singewald et al., 2003). These additional activated regions included the lateral PBN (see Figs. 2-8 and 2-9), lateral subdivision of the CeA (Fig. 2-6D), and lateral BNST (Fig. 2-6F). Conversely, Fos labeling in these regions was sparse or absent in rats treated with vehicle (Fig. 6C, E) or with 1.0 mg/kg YO (not shown).

CeA and BNST tracer injection sites and distribution of retrograde labeling

CeA injection sites. Analysis of tracer injection sites revealed that 12 injections were accurately targeted to the center of the CeA (5.0 mg/kg YO, n=8: vehicle controls, n=4). FG produced the most effective tracer injection sites (see Fig. 7A), as judged by the quality of retrograde labeling within the brainstem (described below). Amygdala regions adjacent to the

CeA (i.e., basolateral, basomedial, and medial nuclei) were included within the outer boundaries of CeA-centered injection sites to varying degrees. These extra-CeA regions reportedly do not receive direct neural input from the NST or VLM (Myers and Rinaman, 2002; Petrov 1993; Zardetto-Smith, 1990; Zardetto-Smith, 1995). In agreement with those reports, in the present study very few or no retrogradely labeled neurons were found within the NST or VLM in rats with tracer injection sites that excluded the CeA but included the medial, basomedial, and/or basolateral amygdala. Conversely, neural tracer diffusing to extra-CeA sites could contribute to the retrograde labeling observed within the LC after CeA injections, because a similar pattern and extent of LC neural labeling was observed in the present study in rats with tracer injection sites that were centered in the CeA or in surrounding amygdalar regions.

BNST injection sites. FG produced the most effective BNST injection sites (see Fig. 7B) and retrograde labeling (described below), similar to results with CeA injections. By comparison, retrograde brainstem labeling was suboptimal after CTb tracer injections into the lateral BNST; thus, the CTb retrograde labeling data were excluded from analysis. In addition, one of the BNST FG injections inexplicably produced very little retrograde labeling within the brainstem. This case also was excluded from analysis. Thus, nine cases were quantitatively analyzed for treatment-induced Fos expression in BNST-projecting neurons (5.0 mg/kg YO, n=5: vehicle controls, n=4).

One FG injection site missed the lateral BNST but included the anterodorsal, anteroventral, and reticular thalamic nuclei, and part of the lateral globus pallidus. Essentially no retrograde labeling was seen in the NTS or VLM in this case, and very little retrograde labeling was seen in the LC (i.e., only 1-3 labeled cells per section). Modest PBN retrograde labeling was observed in this case, whereas labeling was much more prominent in rats with tracer

injection sites centered in the lateral BNST. Several other regions adjacent to the BNST and involved to varying degrees in tracer injection sites could have contributed to retrograde labeling within the pons and medulla; these include the medial and lateral preoptic areas, lateral hypothalamus, substantia innominata, and nucleus accumbens.

Retrograde labeling after CeA and BNST injections. As expected, there were no significant differences in the distribution or amount of retrograde labeling in rats that received vehicle or YO treatment prior to sacrifice (see Tables 2-2 and 2-3). Numerous retrogradely labeled neurons were present within the NST, VLM, PBN, and LC after CeA-centered (Fig. 8; Table 2-2) or BNST-centered (Fig. 9; Table 2-3) tracer injections. The distribution and number of retrogradely labeled brainstem neurons in rats with CeA tracer injections was consistent with a recent report from our laboratory (Myers and Rinaman 2005). Unilateral CeA and BNST tracer injections produced bilateral retrograde labeling in the NST, VLM, LC, and PBN, but with an ipsilateral predominance relative to the side of tracer injection. YO treatment activated Fos expression in the lateral but not the medial PBN; thus, retrogradely labeled neurons in the medial PBN were not included in quantitative analyses.

Prior reports indicate that the LC provides very little direct input to the ventrolateral BNST. In the present study, LC labeling was highly variable in rats with BNST tracer injections, ranging from 38-160 labeled neurons in each case. The amount of retrograde LC labeling was greatest in rats with FG injection sites that included the dorsolateral BNST and/or the substantia innominata, consistent with known LC projection targets (Jones and Yang 1985).

Activation of brainstem neurons projecting to the CeA and BNST

CeA: Two-way repeated measures ANOVA revealed a significant main effect (between subjects) of treatment group in the proportion of CeA-projecting brainstem neurons activated to

express Fos [$F(1,11) = 53.44$, $P < 0.001$], with YO (5.0 mg/kg) activating significantly larger proportions of neurons in the NST, VLM, LC, and lateral PBN compared to vehicle treatment (Table 2-2; Fig. 8). Within subjects, there was a significant effect of brainstem region (NST, VLM, LC, PBN) on YO-induced activation of CeA-projecting neurons [$F(3,30) = 5.89$, $P < 0.001$], but no significant interaction between treatment group and brainstem region. No significant trends in the number of CeA-projecting neurons or the proportion activated to express Fos were observed at different rostro-caudal levels of the NST or VLM (Table 2-2). Figure 10 shows representative photomicrographs of Fos expression by CeA-projecting neurons in the NST, VLM, LC, and lateral PBN in rats after YO treatment.

BNST: Two-way repeated measures ANOVA revealed a significant main effect (between subjects) of treatment group on Fos expression by BNST-projecting brainstem neurons ($F(1,8) = 58.08$, $P = 0.001$), with YO (5.0 mg/kg) activating a significantly larger proportion of neurons in the NST, VLM, LC, and lateral PBN compared to vehicle treatment (Table 2-3; Fig. 9). Within subjects, there was a significant effect of brainstem region (NST, VLM, LC, PBN) on YO-induced activation of BNST-projecting neurons [$F(3,27) = 5.95$, $P = 0.0042$], but no significant interaction between treatment group and brainstem region. Further analysis revealed that the treatment effect on Fos activation was restricted to BNST-projecting neurons within the NST and VLM. A trend towards increased Fos activation in BNST-projecting LC and PBN neurons was apparent in rats after YO treatment (Table 2-3), but did not reach statistical significance in either region (i.e., $P = 0.064$ for the LC and $P = 0.057$ for the PBN).

ANOVA revealed significant main effects (within subjects) of rostro-caudal level in the number of BNST-projecting neurons counted within the NST [$F(2,18) = 12.63$, $P = 0.0005$] and VLM [$F(2,18) = 17.96$, $P < 0.0001$], with the largest numbers of retrogradely labeled neurons

located in the mNST and cVLM (Table 2-3). Post-hoc t-tests confirmed a significant effect of YO to activate Fos at each rostro-caudal level of the NST and VLM ($P < 0.05$ for each region at each level). Within subjects, there was no significant effect of rostro-caudal level on the proportion of BNST-projecting NST neurons activated to express Fos. However, there was a significant effect of rostro-caudal VLM level ($F(2,18) = 16.68$, $P = 0.0002$), with both the cVLM and mVLM containing higher proportions of activated retrogradely labeled neurons compared to the rVLM ($P < 0.05$ for each comparison; Table 2-3). Figure 9 shows representative photomicrographs of Fos expression by BNST-projecting neurons in the NST, VLM, LC, and lateral PBN in rats after YO treatment.

Comparisons of retrograde labeling and YO-induced recruitment of CeA-projecting vs. BNST-projecting neurons. Two-way ANOVA revealed significant main effects of tracer injection site [i.e., CeA vs. BNST; $F(1,10) = 13.1$, $P = 0.0018$] and brainstem region [i.e., 3 NST levels, 3 VLM levels, PBN, and LC; $F(7,147) = 110.3$, $P < 0.0001$] on the average number of retrogradely-labeled neurons (see Tables 2-2 and 2-3). There also was a significant interaction between injection site and brainstem region in the number of retrogradely-labeled neurons [$F(7,147) = 18.7$, $P < 0.0001$]. BNST tracer injections produced significantly more retrograde labeling within the NST and VLM compared to labeling in those regions after CeA tracer injections ($P < 0.05$ for each within-region comparison), whereas the PBN and LC contained statistically similar numbers of retrogradely labeled neurons after CeA or BNST tracer injections.

Three-way ANOVA revealed significant main effects of tracer injection site [$F(1,20) = 13.98$, $P = 0.0016$], treatment group [i.e., vehicle vs. YO; $F(1,20) = 57.7$, $P < 0.0001$], and brainstem region [$F(7,147) = 12.75$, $P < 0.0001$] in the proportions of retrogradely labeled

neurons activated to express Fos. There also were significant interactions between tracer injection site and brainstem region [$F(7,147) = 19.69$, $P < 0.0001$], between treatment group and brainstem region [$F(7,147) = 6.46$, $P < 0.0001$], and a three-way interaction between all three factors [$F(7, 147) = 3.47$, $P = 0.002$] in the proportions of retrogradely labeled neurons activated to express Fos. Vehicle treatment induced Fos in relatively low and statistically similar proportions of CeA- and BNST-projecting neurons within each brainstem region. YO treatment activated statistically similar proportions of BNST- and CeA-projecting neurons within the LC (i.e., ~32-38%; Tables 2-2 and 2-3), and similar proportions of BNST- and CeA-projecting neurons within the lateral PBN (i.e., ~21-26%; Tables 2-2 and 2-3). Conversely, YO activated significantly greater proportions of BNST-projecting neurons within the cNST and within the VLM at all three rostro-caudal levels compared to activation of CeA-projecting neurons in the same regions ($P < 0.05$ for each within-region comparison; see Tables 2-2 and 2-3).

2.5. DISCUSSION

The present study provides new evidence that YO administered systemically at a dose of 5.0 mg/kg BW significantly inhibits food intake, supports conditioned flavor avoidance, and increases Fos immunolabeling in central viscerosensory circuits. Fos-positive circuit components included medullary and pontine NA neurons, hypothalamic CRH-positive neurons, and neurons in viscerosensory brainstem nuclei that project directly to the CeA and lateral BNST.

There are limitations associated with using Fos as a marker of neurons and circuits that respond to and/or mediate the effects of experimental treatments (Morgan and Curran 1991). Neural transcription of the immediate early gene *c-fos* is effected through calcium-dependent pathways that are not activated by all of the signals that a neuron receives. For example, Fos

immunolabeling does not identify neurons whose activity is inhibited, even though such inhibition may be a critical feature of overall circuit activity. In addition, some neurons may not increase *c-fos* transcription or manifest increased Fos immunolabeling even when demonstrably activated. Nevertheless, a treatment-related increase in Fos immunolabeling within an identified neural population that is Fos-negative under control conditions can be interpreted as evidence that the neurons within that population experienced an increase in intracellular calcium levels in conjunction with the experimental treatment. It is reasonable to say that such Fos-positive neurons were "activated" as a direct or indirect consequence of the treatment.

The two YO doses used in this study were based on previous reports of the drug's effects on anxiety-related behavior and its ability to increase central Fos immunolabeling in rats and mice (Szemeredi, Komoly et al. 1991; Wada and Fukada 1991; Bing, Stone et al. 1992; Tsujino, Sano et al. 1992; Stone, Zhang et al. 1993; Kiem, Barna et al. 1995; Tanaka, Yoshida et al. 2000; White and Birkle 2001; Khoshbouei, Cecchi et al. 2002; Schroeder, Schiltz et al. 2003; Shepard, Bossert et al. 2004). However, in the majority of studies only a single YO dose was used, and none attempted to correlate the drug's effects on behavior with its effects on Fos expression. Doses of 1.0 mg/kg BW and lower have been reported to increase extracellular NA levels and to promote anxiety-like behavior in rats when administered intravenously, and/or when combined with other stressors or conditions such as drug withdrawal, immobilization, exposure to the elevated-plus maze, hypertension, or prenatal stress (Szemeredi, Komoly et al. 1991; Tanaka, Yoshida et al. 2000; White and Birkle 2001; Shepard, Bossert et al. 2004). However, our findings indicate that in the absence of such conditions, a 1.0 mg/kg BW dose of YO administered i.p. is by itself subthreshold for inhibiting food intake, supporting CFA, or increasing central Fos immunolabeling. Conversely, the 5.0 mg/kg dose of YO produced

significant effects on all three parameters. Prior reports of Fos expression after YO treatment did not report the effects of doses lower than 5.0 mg/kg BW; thus, our findings provide new evidence for a correlation between YO-induced behavioral effects and YO-induced recruitment of brainstem, hypothalamic, and limbic forebrain regions that may mediate these effects.

YO-induced anorexia and CFA: evidence for malaise

The anxiogenic effect of YO is evidenced in rats by its ability to decrease social interaction and exploration, support conditioned place avoidance, enhance startle, and reduce open-arm exploration in the elevated-plus maze (Pellow, Chopin et al. 1985; File 1986; Bhattacharya, Satyan et al. 1997; White and Birkle 2001; File and Seth 2003). The present study provides new evidence that YO also reduces food intake in food-deprived rats, consistent with a previous report in mice (Callahan, Beales et al. 1984). The time course of the anorexigenic effect of YO is consistent with its known half-life in rats after systemic administration (Hubbard, Pfister et al. 1988). We also report that a dose of YO that inhibits food intake (i.e., 5.0 mg/kg BW) can serve as the unconditioned stimulus for the formation of CFA. This new finding complements a previous report that YO supports conditioned place avoidance in rats (File 1986).

The ability of YO to support CFA demonstrates that YO is aversive to rats, and is consistent with the view that YO is anxiogenic. However, the data also are consistent with evidence in humans that YO generates nausea and malaise (Linden, Vellman et al. 1985; Matilla, Seppala et al. 1988). Chemical agents and treatment conditions often are defined as "nauseogenic" or malaise-inducing in rats based on their ability to both inhibit food intake and support conditioned taste aversion (CTA) (Reilly 1999; Sakai and Yamamoto 1999; Parker 2003). CTA in rats is operationally defined as conditioned rejection reactions (e.g., gapes, chin rubs, purposeful expelling) during involuntary exposure to a conditioned taste stimulus.

Unconditioned stimuli such as lithium chloride that support CTA also support CFA, but the converse is not always true (Parker 2003). Thus, treatments that are nauseogenic also are aversive, but treatments that are aversive are not always nauseogenic.

The aversive and potentially nauseogenic effect of YO in rats may represent an important component of its ability to activate the HPA stress axis and to promote anxiety-like behavior. We recently reported that rats exhibit conditioned avoidance of flavors previously paired with exposure to trimethylthiazoline (TMT), an odiferous component of fox feces that serves as an unconditioned stimulus for stress and anxiety responses in rats (Myers and Rinaman 2005). The magnitude of CFA response to YO-paired flavors in the present study is remarkably similar to the magnitude of CFA response to TMT-paired flavors (Myers and Rinaman 2005), suggesting that the treatments are equivalently aversive. It will be of interest to determine whether YO and/or TMT exposure support CTA, as this would provide more direct evidence that the treatments are nauseogenic in addition to being aversive and, apparently, anxiogenic. In this regard, it is relevant that anxiety and nausea/malaise occur concurrently after bacterial endotoxin or cytokine challenge in humans and rodents (Lacosta, Merali et al. 1999; Yirmiya, Pollak et al. 2000; Nava and Carta 2001; Kusnecov and Goldfarb 2005), and in rats the "sickness behavior" associated with these challenges is accompanied by recruitment of central neural circuits (Ericsson, Kovacs et al. 1994; Elmquist and Saper 1996; Elmquist, Scammell et al. 1996) that overlap extensively with circuits recruited by TMT exposure (Myers and Rinaman 2005) and YO (present report).

Phenotypic characterization of neurons recruited by YO treatment

Several reports have described the central distribution of Fos-positive neurons in rats after YO treatment (Bing, Stone et al. 1992; Tsujino, Sano et al. 1992; Stone, Zhang et al. 1993;

Singewald and Sharp 2000; Singewald, Salchner et al. 2003). The present results are consistent with those earlier studies, all of which used a 5.0 mg/kg BW dose of YO administered i.p.. Our results provide new evidence that neurons activated by YO include significant proportions of medullary and pontine NA neurons. Activated neurons also include the majority (i.e., ~73%) of CRH-positive neurons within the medial parvocellular PVN, comprising the central apex of the HPA axis. This result was predicted by evidence that the same dose of YO produces large increases in plasma ACTH concentration in rats (Kiem, Barna et al. 1995). The stimulatory effect of systemic YO on the HPA axis is blocked by central administration of an α_2-adrenoceptor agonist (Kiem, Barna et al. 1995), implicating central NA signaling mechanisms.

Systemic YO increases neural firing activity in the LC (Aghajanian and VanderMaelen 1982) and promotes increased levels of extracellular NA in several terminal areas implicated in stress and anxiety responses, including the medulla, hypothalamus, CeA, and BNST (Szemeredi, Komoly et al. 1991; Pacak, Armando et al. 1992; Forray, Bustos et al. 1997; Khoshbouei, Cecchi et al. 2002). LC neurons of the A6 cell group provide widespread input to cortical and many subcortical brain regions, including the CeA, and likely contribute importantly to hyperarousal after YO treatment; however, NA inputs to the PVN and ventral BNST arise primarily from neurons located within the NST and VLM (Sawchenko and Swanson 1982; Terenzi and Ingram 1995). Our Fos data indicate that YO activates significant proportions of NA neurons within all three brainstem regions. The proportion of medullary NA neurons activated after YO treatment ranged from approximately 18% to 26% of DbH-positive neurons in the NST (A2/C2 cell groups) and from approximately 51% to 66% of those in the VLM (A1/C1 cell groups).

Central Fos activation after systemic YO likely involves direct effects at pre- and postsynaptic α_2-adrenoceptors in NA terminal regions within the medulla, pons, hypothalamus,

and limbic forebrain. YO-induced recruitment of hypothalamic and limbic forebrain neurons also could contribute to the activation of brainstem neurons, and vice-versa. The PVN, CeA, and lateral BNST have well characterized descending projections to hindbrain NA cell groups, and these projections contribute to activation of medullary NA neurons during stress (Petrov, Krukoff et al. 1993; Li, Ericsson et al. 1996; Dayas and Day 2001; Buller, Dayas et al. 2003; Dayas, Buller et al. 2004). An additional source of central neural activation after systemic YO treatment likely arises from viscerosensory feedback subsequent to the peripheral sympathomimetic effects of the drug, which significantly alters cardiovascular and gastrointestinal functions (Malcolm, Camilleri et al. 2000; Corre, Palmer et al. 2004). Interestingly, systemic YO in humans dose-dependently alters colonic and rectal compliance curves, producing increased tone and sensations of pain and urgency during phasic distension (Malcolm, Camilleri et al. 2000). Potentially relevant viscerosensory feedback signals travel along neural pathways that synapse in the NST, VLM, and lateral PBN en route to the hypothalamus and limbic forebrain, and Fos activation was observed in all of these regions in rats after YO treatment.

YO-induced recruitment of viscerosensory inputs to the CeA and BNST

There is growing evidence that NA inputs to the amygdala and BNST play a critical role in integrating behavioral and visceral responses to stress and anxiety, and are important for conditioned learning and the encoding of memories for emotionally salient stimuli (Clayton and Williams 2000; McGaugh 2000; Williams, Disheng et al. 2000). The present study demonstrates that significant proportions of CeA- and BNST-projecting neurons in the medulla and pons are activated by a behaviorally effective dose of YO. We did not determine the chemical phenotypes of retrogradely labeled neurons in this study; however, previous reports indicate that the large

majority of NST, VLM, and LC neurons that project to the hypothalamus and/or limbic forebrain are NA neurons (Woulfe, Hrycyshyn et al. 1988; Riche, DePommery et al. 1990; Woulfe, Flumerfelt et al. 1990; Zardetto-Smith and Gray 1990; Petrov, Krukoff et al. 1993; Roder and Ciriello 1993; Roder and Ciriello 1994; Terenzi and Ingram 1995; Zardetto-Smith and Gray 1995; Aston-Jones, Delfs et al. 1999; Delfs, Zhu et al. 2000). YO treatment also activated moderate proportions of BNST- and CeA-projecting neurons in the lateral PBN, although YO-induced activation of PBN inputs to the BNST was variable and did not achieve statistical significance. Activation of lateral PBN inputs to the CeA is consistent with the finding that YO supports CFA, a type of learning that requires the PBN, the CeA, and the connections between them (Lasiter and Glanzman 1985; Yamamoto, Shimura et al. 1994; Reilly 1999).

Although YO activated significant numbers and proportions of brainstem neurons projecting to the CeA and to the BNST, recruitment of NST and VLM inputs to the BNST was especially robust. As discussed above, the large majority of BNST-projecting neurons within the NST and VLM are NA neurons. Prior studies have strongly implicated NA inputs to the lateral BNST as underlying the aversive effects of stressors such as drug withdrawal, immobilization, and footshock (Pacak, McCarty et al. 1995; Shepard, Bossert et al. 2004). Of particular interest is evidence that NA inputs to the BNST contribute to the ability of YO and other experimental stressors to reinstate drug seeking in rat models of relapse (Aston-Jones, Delfs et al. 1999; Delfs, Zhu et al. 2000; Shaham, Highfield et al. 2000; Shepard, Bossert et al. 2004). Similar neural mechanisms may underlie stress-induced provocation of relapse in drug-addicted humans, in whom YO induces opioid withdrawal-like symptoms and drug craving (Stine, Grillon et al. 2001; Stine, Southwick et al. 2002).

In summary, we report that the ability of systemic YO to inhibit food intake and support conditioned avoidance behavior in rats is correlated with increased Fos immunolabeling in brainstem NA neurons, hypothalamic CRH neurons, and ascending inputs to limbic forebrain regions that play key roles in stress and emotional learning. These results do not demonstrate causal relationships between neural activation patterns and behavior after YO treatment, but they do highlight several key regions that should be targeted in continued investigation of the functional organization of neural circuits that contribute to physical and psychological features of anxiety and malaise. In particular, our results provide a foundation on which to build experiments to determine which circuit components are necessary and sufficient for various behavioral and physiological aspects of stress, anxiety, and visceral malaise, and the extent to which these components are functionally redundant or unique.

Other acknowledgements

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Table 2-1 Dose-related effect of YO to inhibit feeding in rats after 24 hr food deprivation followed by acute vehicle or YO treatment.

	Cumulative Food Intake (g)		
			overnight
Treatment *(n)*	**30 min**	**60 min**	**(14-15 hr)**
vehicle *(8)*	5.8 ± 0.3	6.6 ± 0.3	28.4 ± 0.9
YO 1.0 mg/kg BW *(8)*	5.2 ± 0.3	6.7 ± 0.4	27.9 ± 1.2
vehicle *(8)*	6.2 ± 0.3	8.8 ± 0.5	25.8 ± 0.9
YO 5.0 mg/kg BW *(8)*	3.7 ± 0.4*	6.3 ± 0.4*	24.6 ± 0.7

* significantly less ($P < 0.05$) compared to intake by the same rats after vehicle treatment (2.0 ml 0.15M NaCl, i.p.)

Table 2-2 YO-induced activation of Fos in brainstem neurons projecting to the CeA.

Tracer injection site	**Treatment**	**Brainstem region**	**Total # of retrogradely labeled neurons *(mean ± SE)***	**% double-labeled for Fos *(mean ± SE)***
CeA *(vehicle, n=4; YO 5.0 mg/kg BW, n=8)*	vehicle	cNST	22.5 ± 2.0	8.6 ± 7.6
	YO		23.9 ± 2.7	28.3 ± 5.9*
	vehicle	mNST	25.8 ± 4.9	5.4 ± 5.4
	YO		29.9 ± 5.0	35.8 ± 7.3*
	vehicle	rNST	22.3 ± 3.2	5.0 ± 3.2
	YO		22.6 ± 3.7	55.4 ± 3.2*
	vehicle	**NST total**	**70.5 ± 9.8**	**8.3 ± 5.0**
	YO		**76.4 ± 8.6**	**38.3 ± 2.9***
	vehicle	cVLM	29.5 ± 1.7	9.7 ± 4.7
	YO		22.4 ± 3.9	53.3 ± 4.9*
	vehicle	mVLM	24.2 ± 0.9	5.1 ± 1.9
	YO		19.8 ± 5.2	40.0 ± 10.5*
	vehicle	rVLM	16.5 ± 5.4	0 ± 0
	YO		15.0 ± 2.1	15.1 ± 5.4*
	vehicle	**VLM total**	**70.2 ± 4.8**	**6.4 ± 3.0**
	YO		**57.2 ± 9.5**	**37.0 ± 5.1***
	vehicle	**LC**	**85 ± 16.1**	**6.9 ± 2.6**
	YO		**66.3 ± 7.3**	**32.2 ± 4.0***
	vehicle	**latPBN**	**132.3 ± 9.7**	**8.1 ± 0.7**
	YO		**116.7 ± 12.3**	**21.4 ± 1.8***

*, significantly greater than the proportion of CeA-projecting neurons expressing Fos in the same region after vehicle (0.15M NaCl) treatment ($P < 0.05$).

Table 2-3 YO-induced activation of Fos in brainstem neurons projecting to the BNST.

Tracer injection site	**Treatment**	**Brainstem region**	**Total # of retrogradely labeled neurons *(mean ± SE)***	**% double-labeled for Fos *(mean ± SE)***
BNST *(vehicle, n=4; YO 5.0 mg/kg BW, n=5)*	vehicle	cNST	55.5 ± 6.7[#]	8.7 ± 3.1
	YO		35.4 ± 6.8[#]	49.2 ± 6.5*[@]
	vehicle	mNST	77.5 ± 7.6[#]	3.5 ± 1.5
	YO		91.8 ± 24.2[#]	42.2 ± 9.1*
	vehicle	rNST	29.8 ± 4.7	1.25 ± 1.25
	YO		30.4 ± 4.0	53.2 ± 5.2*
	vehicle	**NST total**	**162.8 ± 6.2[#]**	**4.7 ± 1.7**
	YO		**157.6 ± 30.6[#]**	**46.5 ± 6.7***
	vehicle	cVLM	76.8 ± 12.7[#]	11.6 ± 4.0
	YO		79.4 ± 6.4[#]	77.0 ± 4.8*[@]
	vehicle	mVLM	36.3 ± 10.1[#]	10.4 ± 4.0
	YO		59.0 ± 17.0[#]	80.4 ± 4.0*[@]
	vehicle	rVLM	26.3 ± 14.5[#]	3.0 ± 1.8
	YO		31.4 ± 5.7[#]	52.3 ± 8.4*[@]
	vehicle	**VLM total**	**139.4 ± 33.8[#]**	**10.5 ± 3.6**
	YO		**169.8 ± 24.5[#]**	**72.6 ± 5.8*[@]**
	vehicle	**LC**	**96.0 ± 25.2**	**2.1 ± 1.0**
	YO		**85.6 ± 8.7**	**37.6 ± 17.9**
	vehicle	**latPBN**	**134.8 ± 11.6**	**3.4 ± 2.5**
	YO		**122.0 ± 25.7**	**26.1 ± 9.2**

*, significantly greater than the proportion of BNST-projecting neurons expressing Fos in the same region after vehicle (0.15M NaCl) treatment ($P < 0.05$).

[#], significantly greater than the number of CeA-projecting neurons in the same region ($P < 0.05$); see Table 2 for CeA data.

@, significantly greater than the proportion of CeA-projecting neurons expressing Fos in the same region after YO treatment ($P < 0.05$); see Table 2 for CeA data.

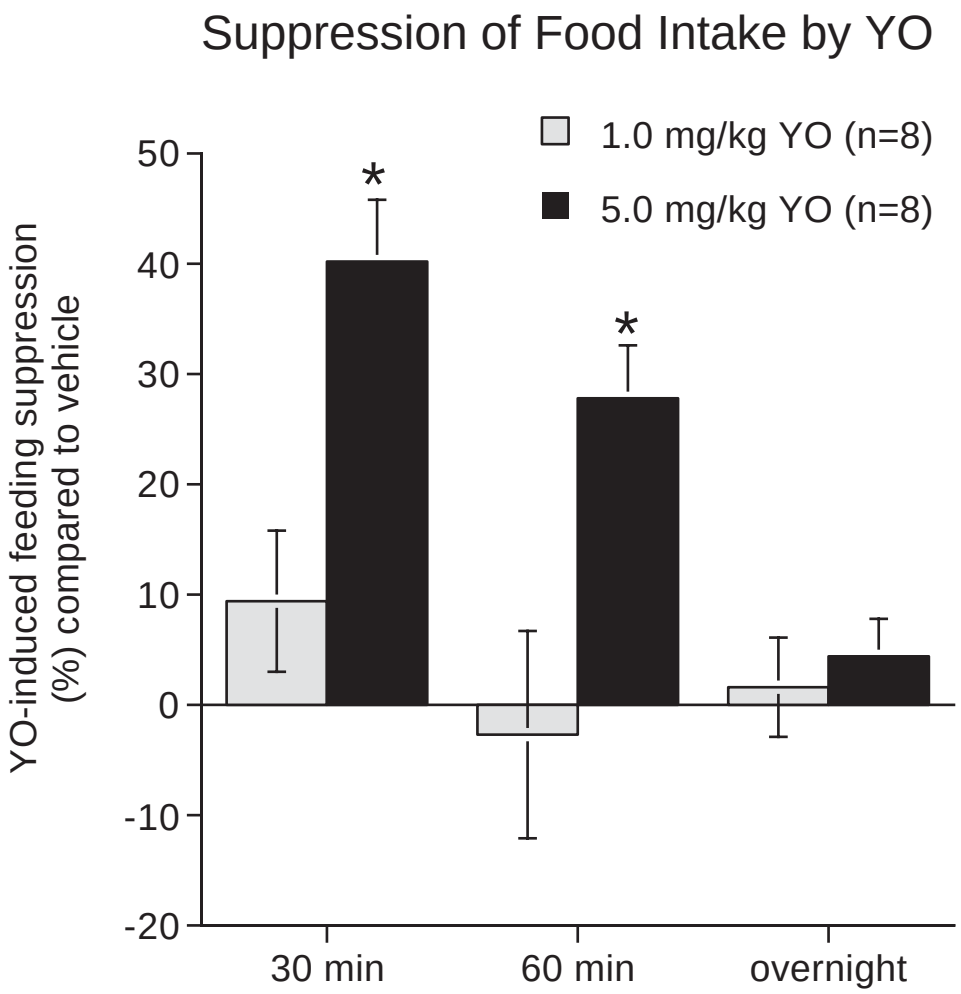

Figure 2-1 Food intake at three time points in rats after 24 hr food deprivation followed by YO treatment (1.0 or 5.0 mg/kg BW, i.p.).

The degree of feeding suppression after each dose of YO is expressed relative to the amount consumed by the same rats after 24 hr food deprivation followed by vehicle treatment (0.15M NaCl, i.p.). *, significantly suppressed compared to intake at the same time point after vehicle treatment ($P < 0.05$).

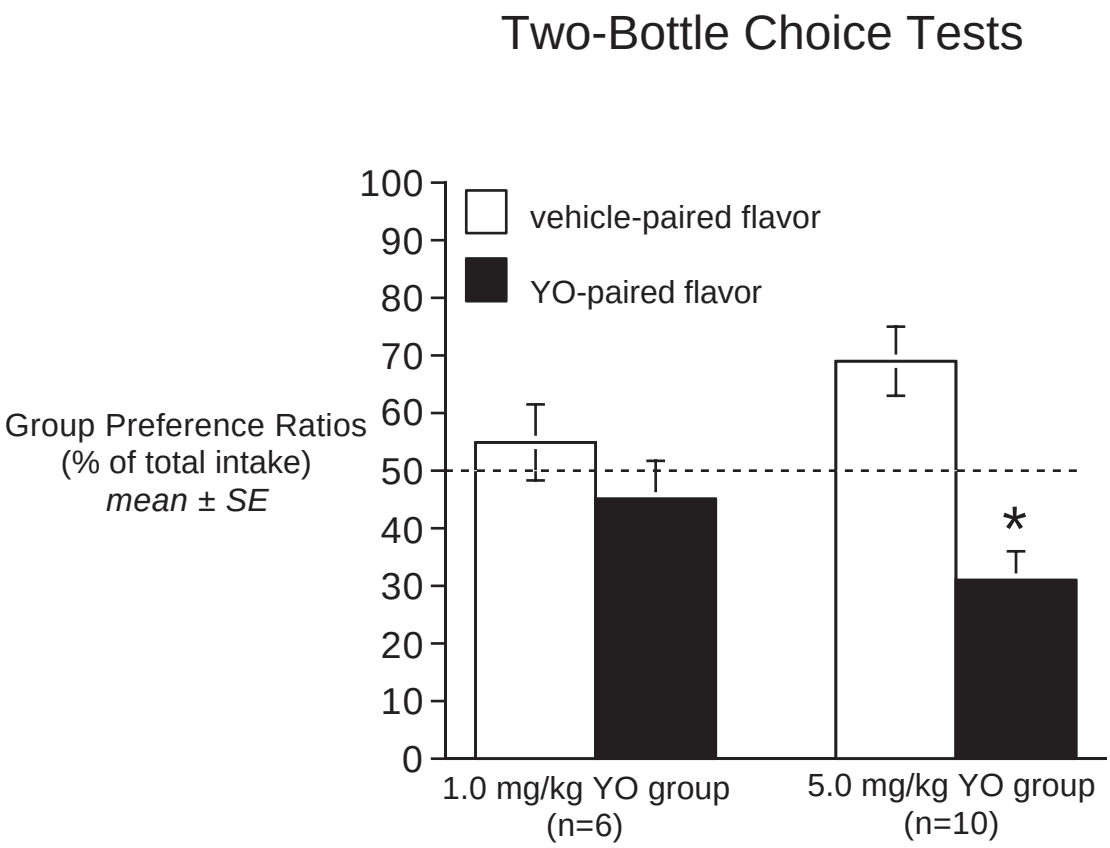

Figure 2-2 Average group preference ratios for novel flavors in 2-bottle choice tests after flavors were previously paired with vehicle treatment (0.15M NaCl, i.p.) or with YO (1.0 or 5.0 mg/kg BW, i.p.).

Results from two experimental cohorts are shown. Dashed line indicates expected preference ratio of 50%:50% with no effect of flavor pairing condition. *, significantly lower preference for YO-paired flavors compared to vehicle-paired flavors ($P < 0.05$).

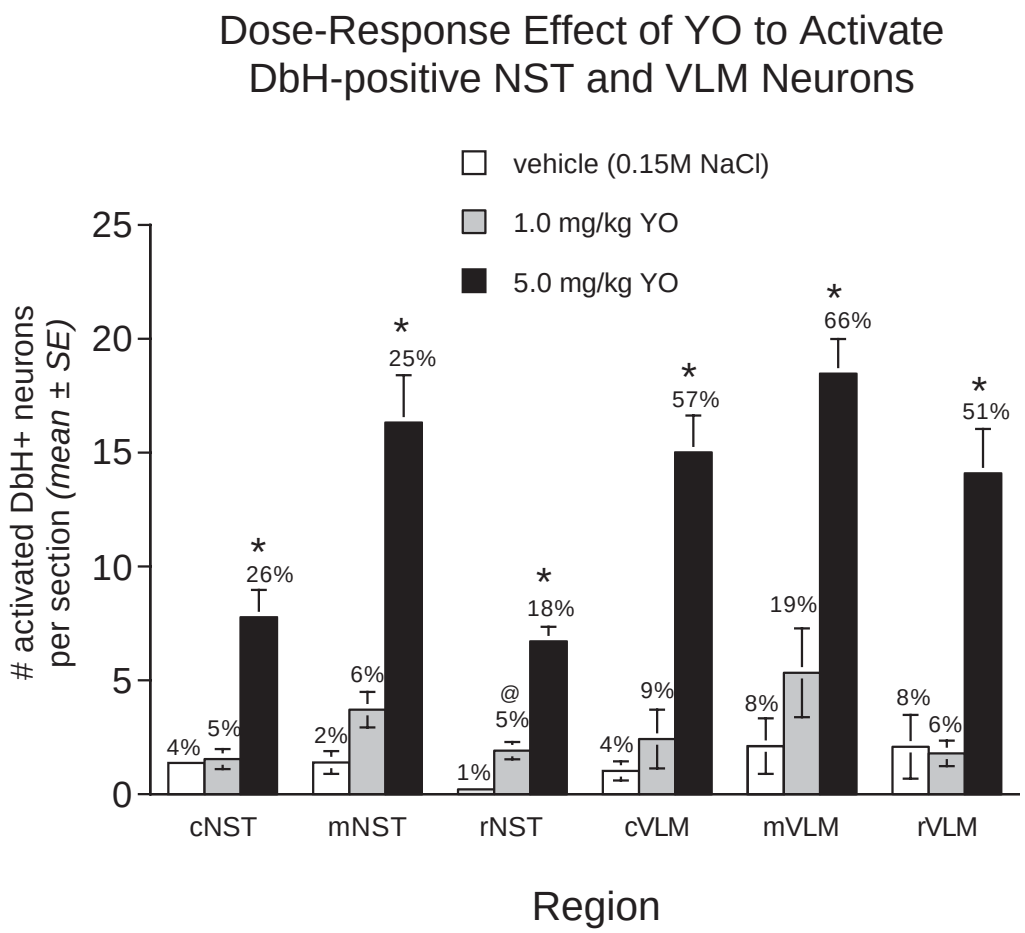

Figure 2-3 Treatment-induced activation of NA (i.e., DbH-positive) neurons within the NST and VLM at three rostrocaudal levels (c, caudal to the area postrema (AP); m, at the level of the AP; r, rostral to the AP).

Values represent the average number of activated neurons per tissue section within each treatment group. *, significantly greater compared to activation after vehicle or YO at the lower 1.0 mg/kg BW dose ($P < 0.05$ for each comparison). $^{@}$, significantly greater compared to activation after vehicle treatment ($P < 0.05$). The % values above each bar indicate the estimated proportion of NA neurons expressing Fos.

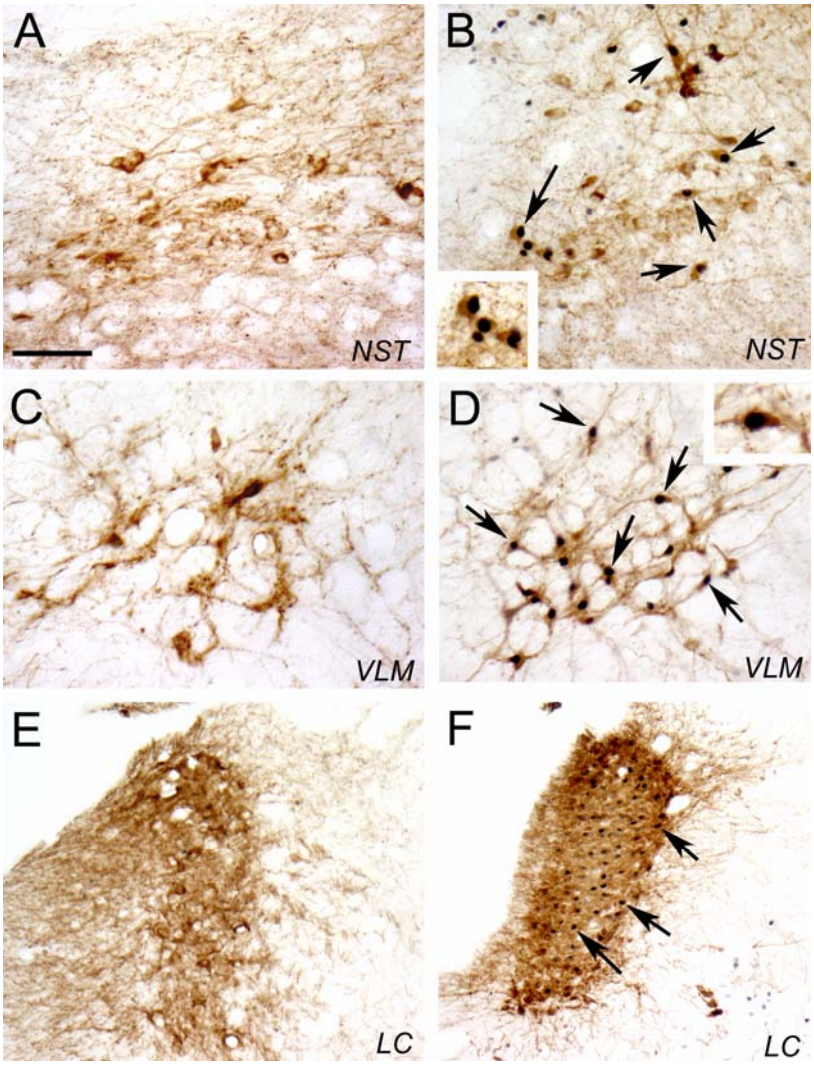

Figure 2-4 Representative color photomicrographs depicting dual immunoperoxidase labeling of Fos (black nuclear label) and DbH (brown cytoplasmic label) within the mNST (A,B), mVLM (C,D), and LC (E,F) in rats after i.p. administration of 0.15M NaCl vehicle (A,C,E) or YO at a dose of 5.0 mg/kg BW (B,D,F).Arrows point out some of the NA (i.e., DbH-positive) neurons activated after YO treatment. Insets show double-labeled neurons at a higher magnification. Scale bar = 200 μm, applies to all main panels.

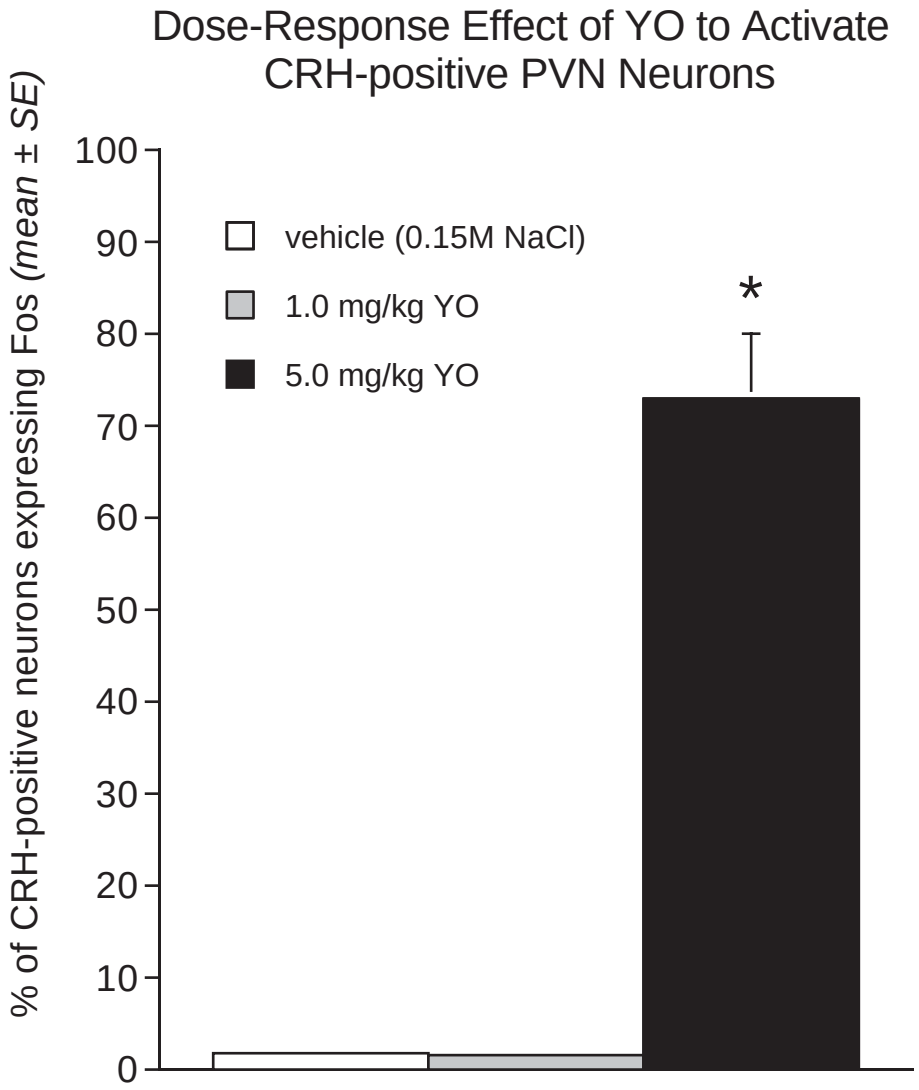

Figure 2-5 Treatment-induced activation of CRH-positive PVN neurons in rats after i.p. administration of 0.15M NaCl vehicle or YO at doses of 1.0 or 5.0 mg/kg BW. *, significantly greater compared to activation after vehicle or the lower dose of YO ($P < 0.05$ for each comparison).

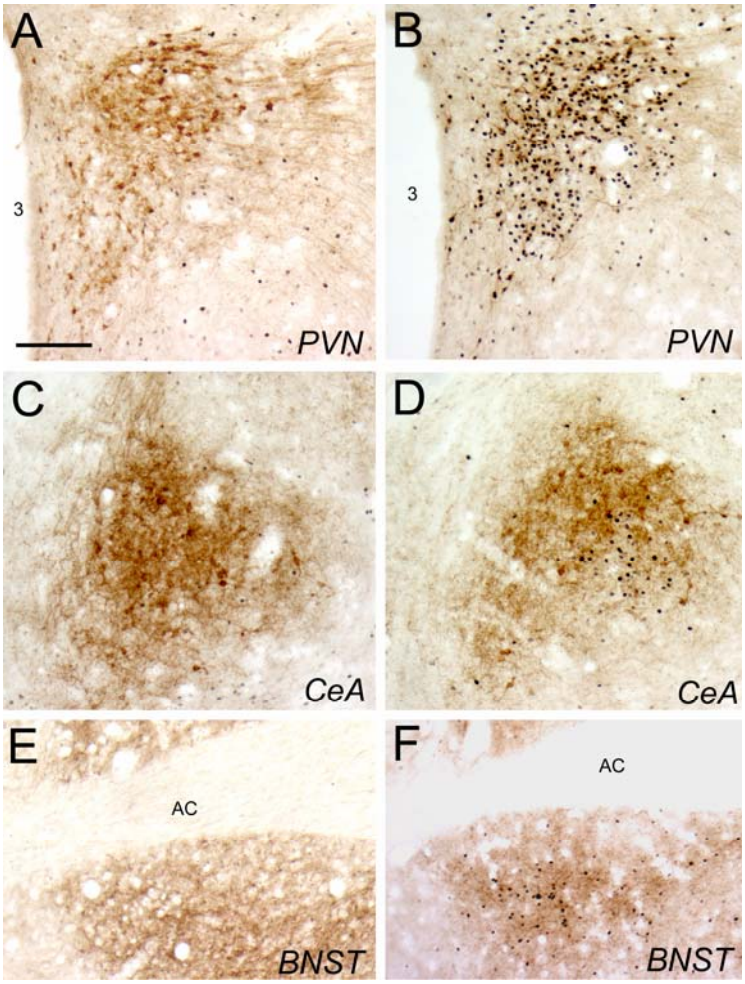

Figure 2-6 Representative color photomicrographs depicting dual immunoperoxidase labeling of Fos (black nuclear label) and CRF (brown cytoplasmic label) within the PVN (A,B), CeA (C,D), and ventrolateral BNST (E,F) in rats after i.p. administration of 0.15M NaCl vehicle (A,C,E) or YO at a dose of 5.0 mg/kg BW (B,D,F). 3, third ventricle; AC, anterior commissure. Scale bar = 250 µm, applies to all panels.

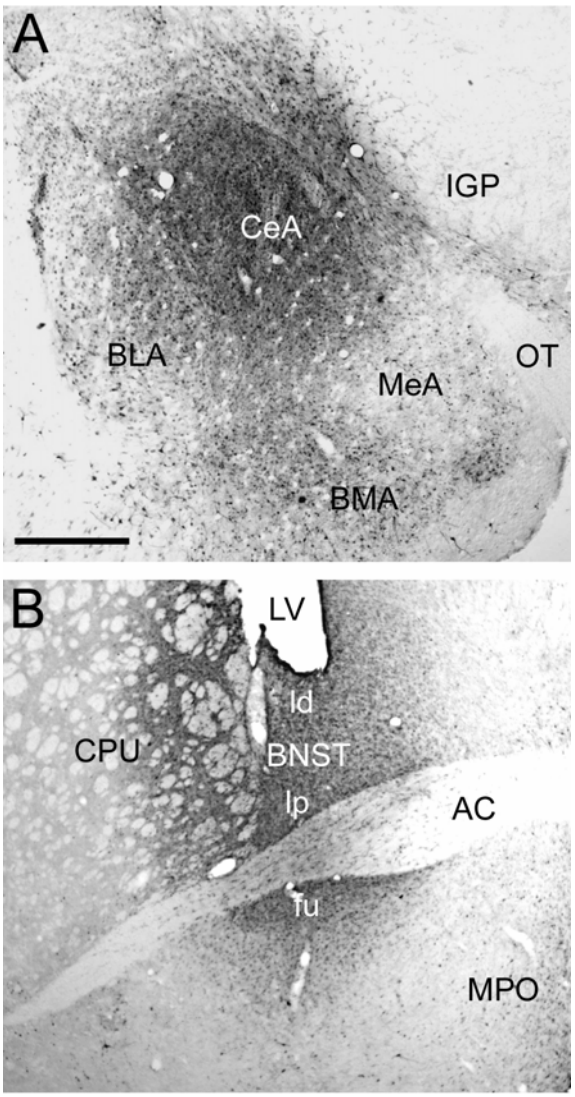

Figure 2-7 Representative examples of FG tracer injection sites (dark immunolabeling) centered in the CeA (A) and BNST (B). AC, anterior commissure; BLA, basolateral amygdala; BMA, basomedial amygdala; CPU, caudate putamen; fu, fusiform BNST; IGP, internal globus pallidus; ld, laterodorsal BNST; lp, lateral posterior BNST; LV, lateral ventricle; MeA, medial amygdala; MPO, medial preoptic area; OT, optic tract. Scale bar = 300 µm, applies to both panels.

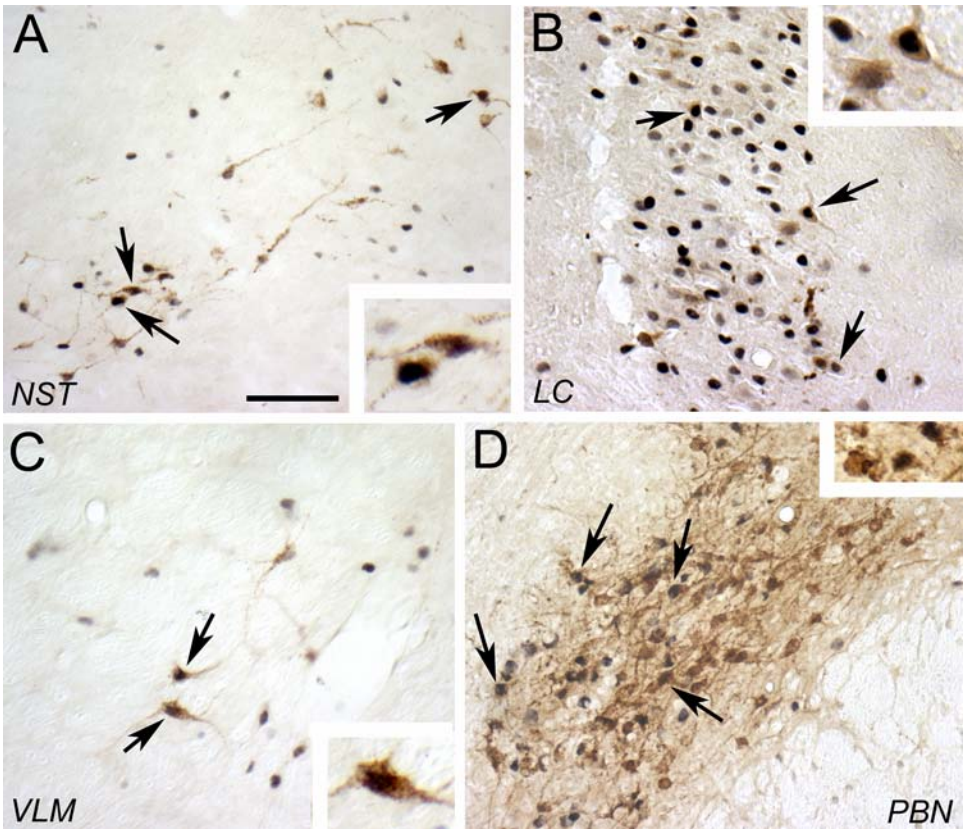

Figure 2-8 Representative color photomicrographs depicting dual immunoperoxidase labeling of Fos (black nuclear label) and FG (brown cytoplasmic label) within the mNST (A), LC (B), mVLM (C) and lateral PBN (D) in a rat with a CeA-centered FG tracer injection and subsequent i.p. administration of YO (5.0 mg/kg BW, i.p.). Arrows point out some of the activated retrogradely-labeled neurons visible within each region. Insets show double-labeled neurons at a higher magnification. Scale bar = 300 μm, applies to all main panels.

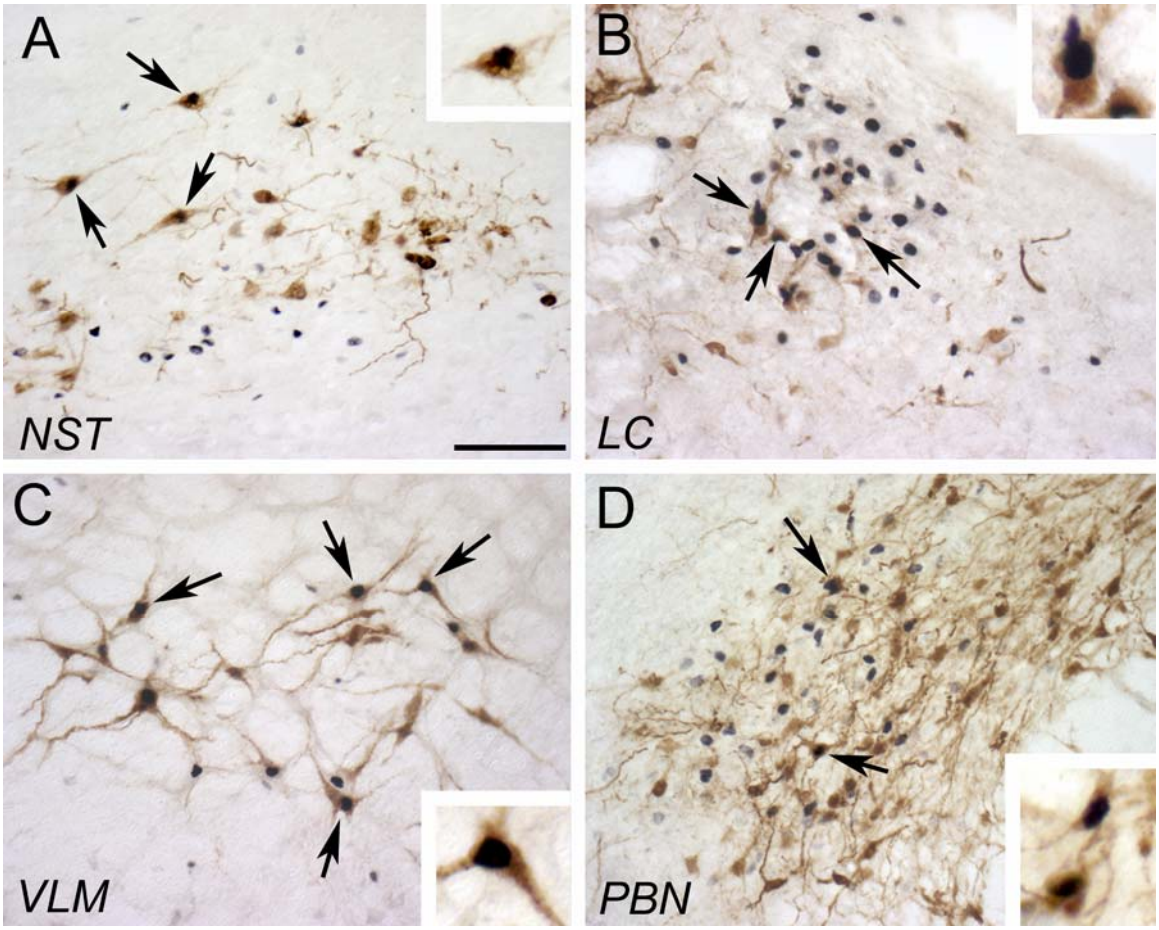

Figure 2-9 Representative color photomicrographs depicting dual immunoperoxidase labeling of Fos (black nuclear label) and FG (brown cytoplasmic label) within the mNST (A), LC (B), mVLM (C) and lateral PBN (D) in a rat with a BNST-centered FG tracer injection and subsequent i.p. administration of YO (5.0 mg/kg BW, i.p.). Arrows point out some of the activated retrogradely-labeled neurons visible within each region.

3. MATERIALS AND METHODS

3.1. Rationale

Emerging evidence implicates the NA input to the BNST in anxiety and stress. As discussed in chapter two, the NA input to the BNST shows a more robust YO-induced activation in comparison to the NA input to the amygdala. Thus, the BNST was chosen as the target site to examine whether its NA input is necessary for YO's neural and behavioral effects. In order to test this, DSAP (an antibody against DbH conjugated to saporin toxin) was used to specifically lesion NA inputs to the BNST. DSAP binds to vesicular DbH when the vesicle is exposed to the synaptic cleft during transmitter exocytosis. The enzyme-antibody-toxin complex is internalized by vesicle membrane endocytosis and retrogradely transported to the cell body. Once inside the cell body, saporin toxin inactivates ribosomes, leading to a halt in protein synthesis, which after 1-2 weeks produces cell death (Madden, Ito et al. 1999; Rinaman 2003; Ritter, Watts et al. 2003).

3.2. DSAP Injections

The same surgical protocol and microinjection coordinates described in chapter two for neural tracer injections were used to inject DSAP (Advanced Targeting Systems; 11 ng delivered in 50nl) or 0.15M NaCl (sham, 50nl) bilaterally into the ventrolateral BNST. The syringe was left in place for an additional 10 minutes after each injection to reduce diffusion up the needle tract. DSAP rats were given 10-14 days to allow adequate time for DSAP to prevent the synthesis of new proteins and cause cell death before commencing any behavioral experiments (see below). Sham rats recovered for an equivalent amount of time.

3.3. Drug Preparation

Animals designated to receive YO treatment were weighed prior to test days. YO was dissolved in 0.15M NaCl by vortexing for 5 minutes, and then filtered to remove particulate residue (MillexHV 0.45 micrometer). The YO solution was made based on average body weight. Injection volume was adjusted so each rat would receive the exact dose for its body weight (YO 5 mg/kg).

3.4. Activation of Central Fos Expression

During terminal experiments, DSAP (n=31) and sham rats (n=14) were given i.p. injections of ~2.0 ml 0.15M NaCl vehicle or YO (5.0 mg/kg BW) during the light cycle, between 1100 and 1400 hour. Rats were left undisturbed in their home cages for 90-120 min after i.p. injection, then were anesthetized with sodium pentobarbital (Nembutal, 100 mg/kg BW, i.p.) and transcardially perfused with a brief saline rinse followed by 500 ml of fixative (4% paraformaldehyde in 0.1 M phosphate buffer). Brains were post-fixed in situ overnight at 4˚C, then removed from the skull, blocked and cryoprotected in 20% sucrose prior to sectioning.

3.4.1. Histology and immunocytochemistry

Coronal 35 µm-thick tissue sections were cut from the caudal extent of the medullary dorsal vagal complex through the rostral extent of the corpus callosum using a freezing microtome. Sections were collected serially in six adjacent sets and stored at -20°C in cryopreservent (Watson, Wiegand et al. 1986). Sections were removed from storage and rinsed for 1 hour in buffer (0.1 M sodium phosphate, pH 7.4) prior to immunocytochemical procedures. Antisera were diluted in buffer containing 0.3% Triton-X and 1% normal donkey serum. Biotinylated secondary antisera (Jackson Immunochemicals) were used at a dilution of 1:500.

Tissue sections were processed for immunocytochemical localization of Fos protein using a rabbit polyclonal antiserum (1:50,000; provided by Dr. Philip Larsen, Denmark) and Vectastain Elite ABC immunoperoxidase reagents (Vector Laboratories). The specificity of this antibody for Fos has been reported (Rinaman and Stricker 1997). Sections were processed using a nickel sulfate-intensified DAB reaction to generate a blue-black Fos reaction product in the nuclei of activated cells. Adjacent sets of Fos-labeled tissue sections were processed for immunoperoxidase localization of cytoplasmic CRH (rabbit anti-CRH, 1:15,000; Penninsula) and the NA synthetic enzyme, dopamine beta hydroxylase (mouse anti-DbH, 1:30,000; Chemicon), using non-intensified DAB to generate a brown cytoplasmic reaction product. After immunocytochemical processing, tissue sections were mounted onto Superfrost Plus microscope slides (Fisher Scientific), allowed to dry overnight, dehydrated and defatted in graded ethanols and xylene, and coverslipped using Cytoseal 60 (VWR).

3.4.2. Quantification of Fos activation

Dual immunoperoxidase-labeled tissue sections were analyzed with a light microscope to determine the number and proportions of phenotypically identified neurons activated to express Fos. Criteria for counting a neuron as DbH-positive or CRH-positive included the presence of brown cytoplasmic immunoreactivity and a visible nucleus. Phenotypically identified neurons were considered Fos-positive (i.e., activated) if their nucleus contained blue-black immunolabel, regardless of intensity, and Fos-negative if their nucleus was unlabeled.

In a subset of sham (n=4) and DSAP (n=4) rats selected for completeness of lesion in both NST and VLM, Fos expression was quantified in the ventrolateral BNST, dorsolateral BNST and CeA at 20 x magnification using CRH labeling as a boundary. Fos expression was also quantified in CRH neurons in the medial parvocellular PVN at 40 x magnification. Fos was

quantified in available sections through each region and then divided by the total number of amygdalar, BNST, or PVN nuclei counted.

In DSAP and sham animals, DbH-positive neurons were counted bilaterally at 60x magnification in NST and VLM. Counts of DbH-positive NST and VLM neurons were grouped according to 3 rostrocaudal levels defined with respect to the area postrema (AP): (1) sections caudal to the AP (cNST and cVLM; A2 and A1 cell groups, respectively); (2) sections at the level of the AP (mNST and mVLM; A2/C2 and A1/C1 cell groups, respectively); and (3) sections rostral to the AP (rNST and rVLM; C2 and C1 cell groups, respectively). Counts of DbH-positive and activated DbH-positive NST and VLM neurons were summed at each rostrocaudal level and then averaged across the number of sections analyzed to obtain mean counts per section. These averages per section were then used to generate percent activation per section.

3.4.3. Statistical analyses

Statistical comparisons of the numbers and/or proportions of phenotypically identified neurons expressing Fos were made using two- and three-way ANOVA [surgery (DSAP vs sham) x treatment (YO vs saline vehicle) x region (rostrocaudal level). When f values indicated significant main effects and/or interactions among experimental variables, ANOVAs were followed up with planned t-comparisons of interest or Fisher's LSD tests. Differences were considered statistically significant when $P < 0.05$.

3.5. Deprivation-Induced Food Intake

Food was removed from cages @ 1530 hour (3.5 hours before the onset of the dark cycle). Twenty-three and a half hours later @ 1500 hour, food-deprived rats were injected i.p. with 2.0 ml of 0.15M NaCl vehicle alone, or vehicle containing YO (Sigma-Aldrich; 5.0 mg/kg BW).

Thirty minutes after i.p. injection (1530 hour) food was returned. Rats received pre-measured amounts of pelleted chow in order to record cumulative food intake at 30 minutes (1600 hour), 60 minutes (1630 hour), and 18 hours (0930 hour). Paper towels were folded along two sides and positioned beneath each cage to collect spillage. After a subsequent 48 hr period of ad libitum access to chow, the 24 hr food deprivation and feeding test was repeated in a counterbalanced design in which rats received the alternate treatment. Thus, each rat (DSAP n=9, sham n=7) served as its own control for determining the effect of YO on food intake.

3.5.1. Data analysis

The effect of YO on deprivation-induced food intake was analyzed by three-way ANOVA with surgery, treatment, and time as the independent variables. When f values indicated significant overall main effects of surgery, treatment, or time on cumulative food intake, the ANOVA was followed by Fisher's protected LSD tests. Differences were considered significant when $P < 0.05$.

3.6. Conditioned Flavor Avoidance

A two-bottle choice paradigm (Deutsch and Hardy 1977) was used to determine whether DSAP rats (DSAP n= 14, sham n=7) avoid flavors previously paired with YO treatment. Flavor exposure during CFA training and testing was conducted near the end of the light cycle of the photoperiod, between 1500 and 1700 hr. Rats were acclimated for two days to i.p. injection of vehicle (0.15M NaCl; 2.0 ml) prior to the start of the CFA experiment. Rats also were acclimated to water deprivation and to the graduated drinking tube used during the experiment. The CFA study was conducted in two different environments. One cohort of rats was removed from their home cages for flavor exposure and two-bottle choice tests and then returned; another

cohort of rats remained in automated feeding chambers which they had inhabited for several weeks. These different environments did not affect the formation of the CFA (see Results).

Following 24 hour water deprivation, rats were given 30 minute access to one of two novel-flavored waters; approximately half of the rats in each group were presented with almond-flavored tap water to drink from a graduated drinking tube, and the others with vanilla-flavored tap water (0.5% McCormick flavor extract). The left-right position of the bottle on each cage was switched after 15 minutes, with intake recorded at 15 and 30 minute time points. Thirty minutes after the end of flavor exposure, all rats were injected i.p. with 2.0 ml 0.15M NaCl vehicle. Water was returned 30 minutes later with ad libitum access for the next 24 hours. Rats were then water deprived again for 24 hours and then given the alternate flavor to drink for 30 minutes, with bottle positions switched after 15 minutes. Thirty minutes after the end of the second flavor exposure, rats were injected i.p. with YO (5.0 mg/kg BW). Water was returned 30 minutes later with ad libitum access for 1-4 days. Rats were finally water deprived again for 24 hours, and then given 30 minute simultaneous access to two bottles, one containing almond-flavored water and the other containing vanilla-flavored water. The volume of each flavor consumed was recorded at 15 minutes, bottle positions were switched, and cumulative intake was recorded at 30 minutes. Rats then were returned to ad libitum water access.

3.6.1. Data analysis

Flavor preference ratios displayed by each rat during the two-bottle choice test were determined by dividing the volume consumed from each bottle (i.e., vehicle-paired flavor vs. YO-paired flavor) by the total volume consumed from both bottles in 30 minutes. Individual preference ratios were averaged within each surgical and treatment group to obtain group preference ratios (mean ± SE) for i.p. vehicle-paired flavors relative to i.p. YO-paired flavors.

Outcomes indicating mean flavor preference ratios that did not differ significantly (i.e., close to 50%:50%) were interpreted as an absence of CFA, whereas outcomes indicating significantly shifted preference ratios (e.g., 70%:30%) were interpreted as evidence for conditioned avoidance of the flavor represented by the lower value in the ratio. Student t-tests were used to determine whether group preference ratios for vehicle-paired and YO-paired flavors were statistically different, with significance set at $P < 0.05$.

3.7. Elevated Plus Maze

The elevated plus maze used in this study was elevated 100 cm; it had four white laminate arms (10 x 50 cm each) situated in a cross. Two opposing arms were enclosed by walls 50 cm high; the two remaining arms had a 3 cm clear plastic rim to help prevent the animals from falling off. One to several days prior to surgery, rats were exposed to the elevated plus maze for 5 minutes. Sixteen to 17 days after surgery, DSAP (n=7) and sham (n=8) rats underwent 3 acclimation days prior to the first test day. During acclimation days, rats experienced handling, transport, and mock injections (i.p. injection with the blunt end of a syringe) following the same time course as on the test day, without placement on the elevated plus maze. Rats were removed from their home cages and placed into individual cages at 0930 or 1030 hour and transported to a waiting room adjacent to the testing room. Rats were left undisturbed in this room for 30 minutes. Then, rats were taken individually to an adjacent room and injected with either YO (5 mg/kg BW, i.p.) or vehicle. Injections were staggered every 10 minutes in order to avoid excessive activity and resulting noise during behavioral tests. Rats were brought back to the initial room; thirty minutes after i.p. injection each rat was transported to the testing room and placed on the elevated plus maze for 5 minutes. A digital video camera was positioned to record behavior on both open arms and number of entrances into the open and closed arms. When the 5

minute test was complete, rats were transported back to the initial area. The elevated plus maze was cleaned with Quatricide PV (germicidal detergent and deodorant) after each rat. When all tests were complete rats were returned to their home cages. Rats underwent another acclimation prior to the second test day. The second test day was 5 days subsequent to the first test day, in which the same procedure was used in a counterbalanced design with rats receiving the alternate treatment. The video taped behaviors were scored off line. This study was also performed using surgically intact animals to determine the effects of a lower dose of YO (1 mg/kg BW, i.p.) using the same protocol as above.

3.7.1. Scoring criteria

For an entrance into an arm the animal must have crossed into the arm with all four paws. Similarly, for an exit out of an arm the animal must have brought all four paws out of the arm. For example, if an animal brings its head and forepaws out of a closed arm, it is not counted as an exit and time in the closed arm continues to accrue. Time spent in the 10 x 10 central square was not counted as time spent in either arm. Additionally, open arm head dipping, in which the animal dips its head over the ledge of the open arm, open arm endpoint forays, in which the animal travels to the end of the open arm, and fecal boli were counted.

3.7.2. Data analysis

The effect of YO on anxiety-like behavior on the elevated plus maze was analyzed by two-way ANOVA with surgery and treatment as the independent variables. Dependant variables included open arm time, open arm entries, closed arm entries, closed arm time, open arm head dips, open arm endpoint forays, and fecal boli. When f values indicated significant overall main

effects of surgery or treatment on any of the above variables, the ANOVA was followed by Fisher's protected LSD tests. Differences were considered significant when $P < 0.05$.

3.8. Correlations of Behavior and Lesion Extent

Behavior in each paradigm was correlated with the average number of DbH-positive neurons per section remaining in the NST, VLM, and/or the sum of the NA neurons per section in both regions using simple regression analyses and regression ANOVA. Correlation coefficients were regarded as significant when $P<0.05$.

4. RESULTS

4.1. DbH immunolableing

DSAP rats displayed nearly complete loss of DBH immunolabeling in the BNST (Fig. 4-1) and medial parvocellular PVN, whereas the lateral magnocellular PVN appeared to be unaffected (Fig. 4-2). DBH immunolabeling appeared to be slightly reduced in the CeA (data not shown) but not in the PBN (Fig. 4-3B). DSAP rats also displayed moderate loss of DbH-positive neurons in the LC (Fig. 4-4).

4.2. Central Fos Expression

Completely randomized one-way ANOVA confirmed that DSAP significantly reduced YO-induced Fos activation in ventral [$F(1,6) = 15.0449$, $P=0.0082$] but not dorsal BNST (Fig. 4-1, 4-5 and 4-6). DSAP also significantly reduced YO-induced Fos activation in mpPVN and in mpPVN CRH neurons compared to sham controls [$F(1,6) = 14.4071$, $P=0.009$] (Fig. 4-2, 4-7 and 4-8). However, DSAP did not significantly decrease YO-induced Fos in CeA (Fig. 4-3A and 4-9) and did not appear to reduce YO-induced Fos in PBN (Fig. 4-3B), although the latter was not subjected to quantitative analysis.

4.3. DbH immunolabeling and Fos expression in NST and VLM

In general, the NST and VLM contained significantly fewer NA neurons, including fewer activated NA neurons in DSAP rats compared to sham controls (Fig. 4-10); however, YO activated similar proportions of the NA neurons that remained (see below). The average number of DbH -positive neurons per section in the NST were positively correlated those in the VLM in both DSAP and sham animals (r=0.8733, [$F(1,43)=138.1322$;$P<0.0001$]) (Fig. 4-11).

4.3.1. NST

4.3.1.1. Average DbH-positive neurons/section

Two-way completely randomized ANOVA showed a significant effect of treatment between subjects [F(1,44)=13.5065;P <0.0007] on the average number of DbH -positive neurons per section through the NST. Fisher's LSD revealed that this effect was restricted to the sham YO group which had a significantly higher average number of DbH-positive neurons per section compared to the sham saline group (P<0.01) (Fig. 4-12). This is likely due to visible nuclei being part of the criteria for identifying DbH-positive neurons and Fos-positive nuclei being more visible because of the blue-black immunolabel (See materials and methods).

Two-way completely randomized ANOVA revealed a significant effect of surgery [F(1,44)=165.9447;P<0.0001] on the average number of DbH-positive neurons per section. DSAP rats in both treatment groups had significantly fewer DbH-positive neurons per section compared to their respective sham controls (P<0.01) (Fig. 4-12). Two-way completely randomized ANOVA displayed a significant interaction between surgery and treatment [F(1,44)=5.7648;P=0.021]. It is possible that the ANOVA detected a significant interaction between surgery and treatment because of the greater number of DbH-positive neurons in the sham YO group.

4.3.1.2. Average activated NA neurons/section

Two-way completely randomized ANOVA revealed a significant effect of treatment [$F(1,44)=40.5719$;$P<0.0001$] on the average number of activated NA neurons per section. YO induced a significant increase in the average number of activated NA neurons per section in both sham and DSAP rats ($P<0.01$ for both surgical groups) (Fig. 4-13).

Two-way completely randomized ANOVA showed a significant effect of surgery [$F(1,44)=38.0576$;$P<0.0001$] on the average number of activated NA neurons per section. DSAP attenuated the YO-induced increase in activation ($P<0.01$) (Fig. 4-13). Two-way completely randomized ANOVA displayed a significant interaction between surgery and treatment [$F(1,44)=6.7937$;$P=0.0127$]. Additionally, simple regression analysis showed a positive correlation between the average number of NA neurons per section in NST and the average number of activated NA neurons per section ($r=0.8027$, [$F(1,43)$;$P<0.0001$]).

4.3.1.3. Percent activation of NA neurons

Two-way completely randomized ANOVA revealed a significant effect of treatment [$F(1,44)=38.4499$;$P<0.0001$)] but not surgery [$F(1,44)=2.6199$;$P=0.1132$] on the proportion of NA neurons activated. YO induced a significant increase in the proportion of NA neurons activated compared to saline-treated controls in both surgical groups ($P<0.01$) (Fig. 4-14). There was no significant interaction between surgery and treatment. Thus, YO increased the proportion of NA neurons activated to a similar extent in both sham and DSAP rats.

4.3.2. NST: 3 rostrocaudal levels

4.3.2.1. Average DbH -positive neurons/section

Three-way repeated measures ANOVA displayed a significant effect of surgery between subjects [$F(1,44) = 163.2117$, $P<0.0001$] on the average number of DbH -positive neurons per

section of the NST. DSAP rats in both treatment groups had significantly fewer DbH-positive neurons than sham rats in both treatment groups (P<0.01) at all 3 rostro-caudal levels of the NST (Fig. 4-15). Three-way repeated measures ANOVA also showed a significant difference between treatment groups [F(1,44) = 4.6657, P = 0.0367) in the average number of DbH - positive neurons per section, in which YO-treated sham rats had a higher number of DbH-positive neurons per section than the vehicle-treated sham rats at the AP level of the NST (P<0.01) (Fig. 4-15). There was no significant interaction between surgery and treatment. There was a significant effect within subjects of rostro-caudal level on the average number of DbH-positive neurons per section [F(2,90) = 93.7013, P <0.0001). The cNST had fewer DbH-positive neurons in each of the 4 groups (P<0.05). There was a significant interaction within subjects between surgery and rostro-caudal level ([F(2,90) = 5.123, P = 0.008), between treatment and rostro-caudal level [F(2,90) = 7.6942, P = 0.0009], and between surgery, treatment and rostro-caudal level [F(2,90) = 6.301, P = 0.0028].

4.3.2.2. Average activated NA neurons per section

Three-way repeated measures ANOVA displayed a significant effect of treatment between subjects [F(1,44)=78.952, P <0.0001] on the average number of activated (Fos-positive) DbH-positive/NA neurons per section. Fisher's LSD test showed that YO significantly increased the average number of activated NA neurons per section in sham rats (P<0.01) at all rostro-caudal NST levels and in DSAP rats at the mNST and rNST (P<0.01), but not the cNST (Fig. 4-16).

Three-way repeated measures ANOVA revealed a significant effect between subjects of surgery [F(1,44) = 51.5226, P<0.0001) on the average number of activated NA neurons per section. DSAP rats had significantly fewer average activated NA neurons per section compared

to their respective sham controls (saline $P<0.05$, YO $P<0.01$) at all rostrocaudal levels of the NST (Fig. 4-16). There also was a significant interaction between surgery and treatment [$F(1,44) = 6.0878$, $P = 0.0179$]. There was a significant effect of rostro-caudal NST level on the average number of activated NA neurons per section [$F(2,90) = 18.4287$, $P < 0.0001$]. Within the YO-treated sham rats, the mNST had a greater number of activated NA neurons than the cNST ($P<0.01$), but was not significantly different from the rNST. Within YO-treated DSAP rats, the cNST had least activated NA neurons ($P<0.01$). There was no significant interaction between surgery and level. There was a significant interaction between treatment and level [$F(2,90) = 8.6861$, $P = 0.0004$], but no significant interaction between surgery, treatment and level [$F(2,90) = 2.4348$, $P = 0.0939$].

4.3.2.3. Percentage of activation of NA neurons

Three-way repeated measures ANOVA showed a significant effect of treatment [$F(1,44)=128.0619$;$P < 0.0001$)] on the proportion of NA neurons activated of NA neurons. YO induced a significantly higher percent activation compared to saline-injected controls in both surgical groups at all rostro-caudal NST levels ($P<0.01$ for both sham and DSAP) (Fig. 4-17).

Three-way repeated measures ANOVA revealed a significant effect of surgery [$F(1,44)=9.1637$;$P=0.0043$) on the proportion of NA neurons activated in the NST. Fisher's LSD tests showed that this effect was confined to the cNST, in which DSAP rats had higher percentages of activation compared to their respective sham controls ($P<0.01$ for both treatment groups) (Fig. 4-17). However, there was no significant interaction between surgery and treatment. Thus, YO had a similar effect on the proportion of NA neurons activated in both sham and DSAP rats. There was a significant within subjects effect of rostro-caudal level on the proportion of NA neurons activated [$F(2,90)=153.9548$;$P < 0.0001$]. This effect was confined to

the cNST which had the greatest percent activation compared to mNST and rNST in both YO-treated sham and DSAP rats. There was a significant interaction between surgery and rostro-caudal level on the proportion of NA neurons activated [$F(2,90)=17.5989$;$P<0.0001$]. There was no significant interaction between treatment and rostro-caudal level [$F(2,90)=2.5929$;P 0.0809) or between surgery, treatment and rostro-caudal level [$F(2,90)=1.1954$;P 0.3078].

4.3.3. VLM

4.3.3.1. Average DbH -positive neurons per section

Two-way completely randomized ANOVA showed a significant effect of surgery [$F(1,44)=181.646$;$P<0.0001$], but no significant effect of treatment [$F(1,44)=2.3557$;$P=0.1325$)] on the average number of DbH -positive neurons per section

DSAP rats in both treatment groups had significantly fewer average DbH-positive neurons per section compared to their respective sham controls ($P<0.01$) (Fig. 4-12). Two-way completely randomized ANOVA displayed no significant interaction between surgery and treatment.

4.3.3.2. Average activated NA neurons per section

Two-way completely randomized ANOVA revealed a significant effect of treatment [$F(1,44)=12.4963$;P=0.001] on the average number of activated NA neurons per section in the VLM. This effect was confined to sham rats, in which YO significantly increased the average number of activated NA neurons per section in the VLM (Fig. 4-13). Two-way completely randomized ANOVA showed a significant effect of surgery [$F(1,44)=28.1512$;P <0.0001) on the average number of activated NA neurons per section in the VLM. The YO-induced increase in activated NA neurons was blocked by the DSAP lesion ($P<0.01$) (Fig. 4-13). DSAP rats treated with saline also had significantly fewer average Fos-positive NA neurons per section than the sham saline group ($P<0.05$) (Fig 4-13). There was no significant interaction between surgery

and treatment [$F(1,44)=3.6564$;$P=0.0629$]. Additionally, simple regression analysis displayed a positive correlation between the average number of NA neurons per section in VLM and the average number of activated NA neurons per section (r=0.7991, [$F(1,43)$;$P<0.0001$]).

4.3.3.3. Percent activation of NA neurons

Two-way completely randomized ANOVA showed a significant effect of treatment [$F(1,44)= 20.299$; $P<0.0001$) but not surgery [$F(1,44)=0.1469$;$P=0.7035$)] on the proportion of NA neurons activated in the VLM. YO induced significant increases in percent activation of NA neurons in both surgical groups ($P<0.01$) (Fig. 4-14). There was no significant interaction between surgery and treatment.

4.3.4. VLM: 3 rostrocaudal levels

4.3.4.1. Average DbH-positive neurons/section

Three-way repeated measures ANOVA revealed no significant effect of treatment [$F(1,44)=0.7815$;$P=0.3818$] on the average number of DbH-positive VLM neurons per section and no significant interaction between surgery and treatment.

Three-way repeated measures ANOVA showed a significant effect of surgery [$F(1,44)=386.2169$;$P<0.0001$] on the average number of DbH-positive neurons per section. DSAP significantly reduced the number of DbH-positive neurons per section compared to sham controls ($P<0.01$) at all rostro-caudal VLM levels (Fig. 4-18). There was a significant effect of rostro-caudal level on the average DbH-positive neurons per section [$F(2,90)=106.2148$;$P<0.0001$] in which the rVLM had higher average numbers of DbH-positive neurons per section. There was a significant interaction between surgery and rostro-caudal level [$F(2,90)=4.1985$;$P=0.0184$), treatment and rostro-caudal level [$F(2,90)=4.3524$;$P=0.016$] and surgery, treatment and rostro-caudal level [$F(2,90)=3.3623$;$P=0.0395$).

4.3.4.2. Average activated NA neurons per section

Three-way repeated measures ANOVA revealed a significant effect of treatment between subjects [$F(1,44)=28.7736; P < 0.0001$) on the average number of activated NA neurons per section. YO produced significant increases in activated NA neurons in sham rats at all rostrocaudal VLM levels ($P<0.01$) and in DSAP rats in the rVLM ($P<0.01$) (Fig. 4-19).

Three-way repeated measures ANOVA displayed a significant effect of surgery between subjects [$F(1,44)=56.6874; P < 0.0001$) on the average number of activated NA neurons per section in the VLM. DSAP rats had fewer activated NA neurons compared to respective sham controls at all rostrocaudal levels of the VLM (sham saline cVLM $P<0.05$, mVLM and rVLM $P<0.01$; sham YO $P<0.01$) (Fig. 4-19). There was a significant interaction between surgery and treatment [$F(1,44)=4.6482; P=0.037$]. There was a significant effect of rostro-caudal level [$F(2,90)=13.9246; P < 0.0001$) on activated NA neurons. The rVLM had a higher average number of activated NA neurons than the cVLM and mVLM in YO-treated DSAP rats and the cVLM in YO-treated sham rats. There was no significant interaction between surgery and rostro-caudal level, between treatment and rostro-caudal level, or between surgery, treatment and rostro-caudal level.

4.3.4.3. Percentage of activation of NA neurons

Three-way repeated measures ANOVA displayed a significant effect of treatment [$F(1,44)=54.9688; P < 0.0001$)] but no significant effect of surgery on the proportion of NA neurons activated [$F(1,44)=0.3283; P=0.5698$). YO provoked significantly higher percentages of activation in sham (mVLM $P<0.05$; cVLM and rVLM $P<0.01$) and DSAP ($P<0.01$) surgical groups at all rostrocaudal VLM levels (Fig. 4-20). There was no significant interaction between surgery and treatment and no significant effect of rostro-caudal level within subjects

[F(2,90)=0.2735;P=0.7614). There was no significant interaction between surgery and rostro-caudal level, between treatment and rostro-caudal level, between surgery, treatment and rostro-caudal level.

4.4. Deprivation-Induced Food intake

Three-way repeated measures ANOVA showed a significant between subjects effect of treatment [F(1,32)=30.9362;P=0.0001] but not surgical group [F(1,32)=0.2334;P=0.6326] on cumulative food intake (g). YO significantly inhibited food intake in sham and DSAP rats at 30 and 60 minutes (P<0.01) (Fig. 4-21). Three-way repeated measures ANOVA revealed a significant effect of time within subjects [F(2,66)=1942.4264;P<0.0001] and a significant interaction between surgery and time [F(2,66)=3.6867; P=0.0311]. Sham rats recovered their cumulative food intake by 18 hours whereas DSAP rats did not (DSAP saline vs. DSAP YO, P<0.0106) (Fig. 4-22).

4.5. Conditioned Flavor Avoidance

Two-way repeated measures ANOVA showed a significant effect of treatment [F(1,21)=20.9776;P=0.0002] but not surgery [F(1, 20)=0.4879;P=0.4933] on preference ratio. In both sham and DSAP rats, the preference ratio for the YO-paired flavor was significantly less than that for the saline-paired flavor (sham P<0.0055, DSAP P<0.0001) (Fig. 4-23). Given that in both surgical groups the preference was approximately 70%:30% in favor of the vehicle-paired flavor, YO supported a CFA in sham and DSAP rats.

4.6. Elevated Plus Maze

4.6.1. YO (1 mg/kg)

One way ANOVA displayed no significant effect of treatment between groups (saline vs YO 1 mg/kg) on open arm entries [$F(1,8)=0.2547;P=0.6293$], open arm time [$F(1,8)=0.0106;P=0.921$], closed arm entries [$F(1,8)=3.2881;P=0.1127$)], or closed arm time [$F(1,8)= 0.0158;P=0.9034$)] (Figs. 4-24 and 4-25).

4.6.2. YO (5 mg/kg)

Two-way ANOVA revealed a significant effect of treatment on open arm entries [$F(1,8)= 0.0158$; $P=0.9034$)], closed arm entries [$F(1,8)= 0.0158$; $P=0.9034$)], closed arm time [$F(1,8)= 0.0158$; $P=0.9034$)], and fecal boli [$F(1,8)= 0.0158$; $P=0.9034$)]. Fisher's LSD test revealed that YO significantly decreased the number of open arm entries in DSAP rats ($P<0.01$) and decreased the number of in closed arm entries in sham and DSAP rats ($P<0.05$ for both surgical groups) (Fig. 4-26). Additionally, YO significantly increased closed arm time and fecal boli in sham but not DSAP rats ($P<0.05$ for both measures) (Figs. 4-27 and 4-28). Despite the confinement of YO-induced increases in closed arm time and fecal boli to the sham control group, two-way ANOVA revealed no significant effect of surgery between subjects on any of the measured behaviors. Additionally, there was no correlation between the average number of DbH-positive neurons per section in the NST, VLM, or the sum of both regions on any of the measured behaviors (Fig. 4-29).

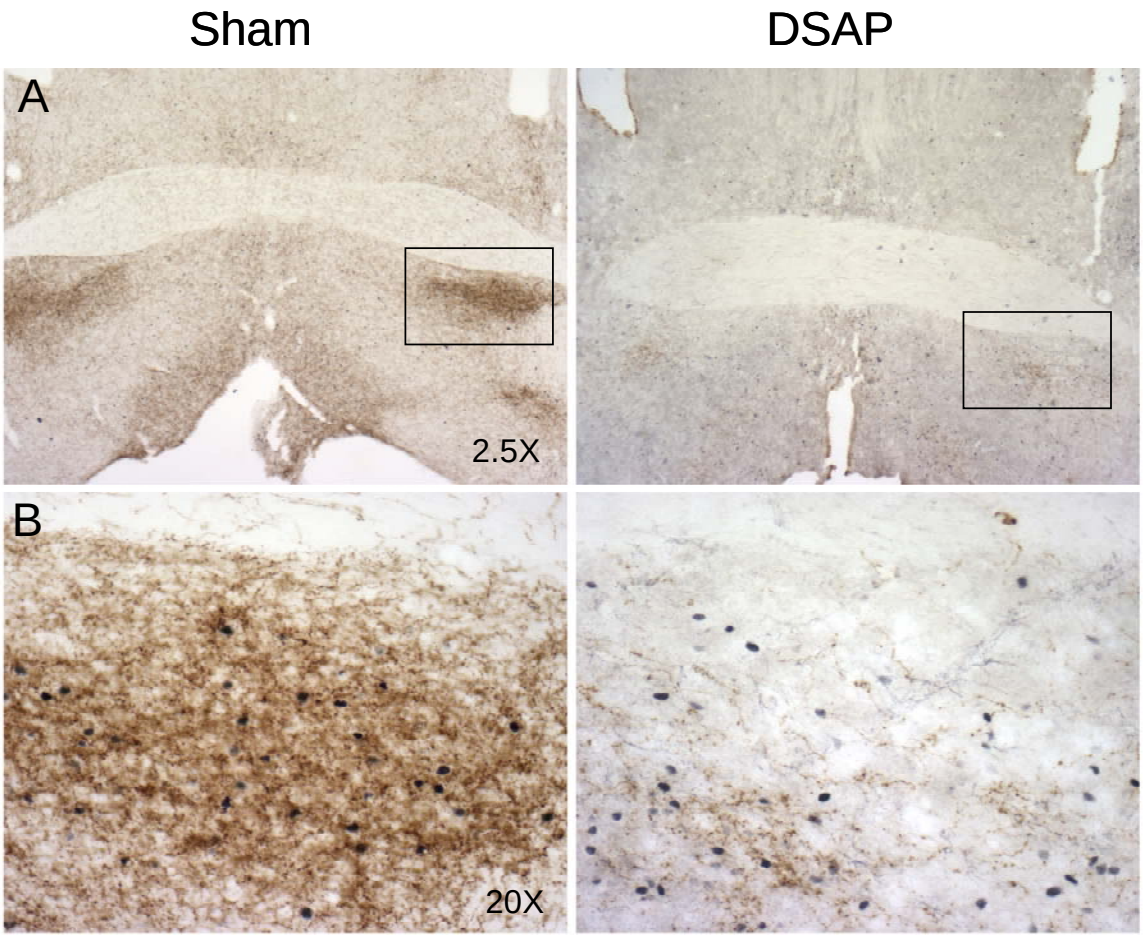

Figure 4-1 DSAP reduced NA terminals and YO-induced Fos expression in ventral BNST.

BNST: A. Representative color photomicrographs depicting dual immunoperoxidase labeling of DbH (brown label) and Fos (black nuclear label) in a YO-treated sham and DSAP rat. B. Higher magnification of boxed region in 4-1A (above).

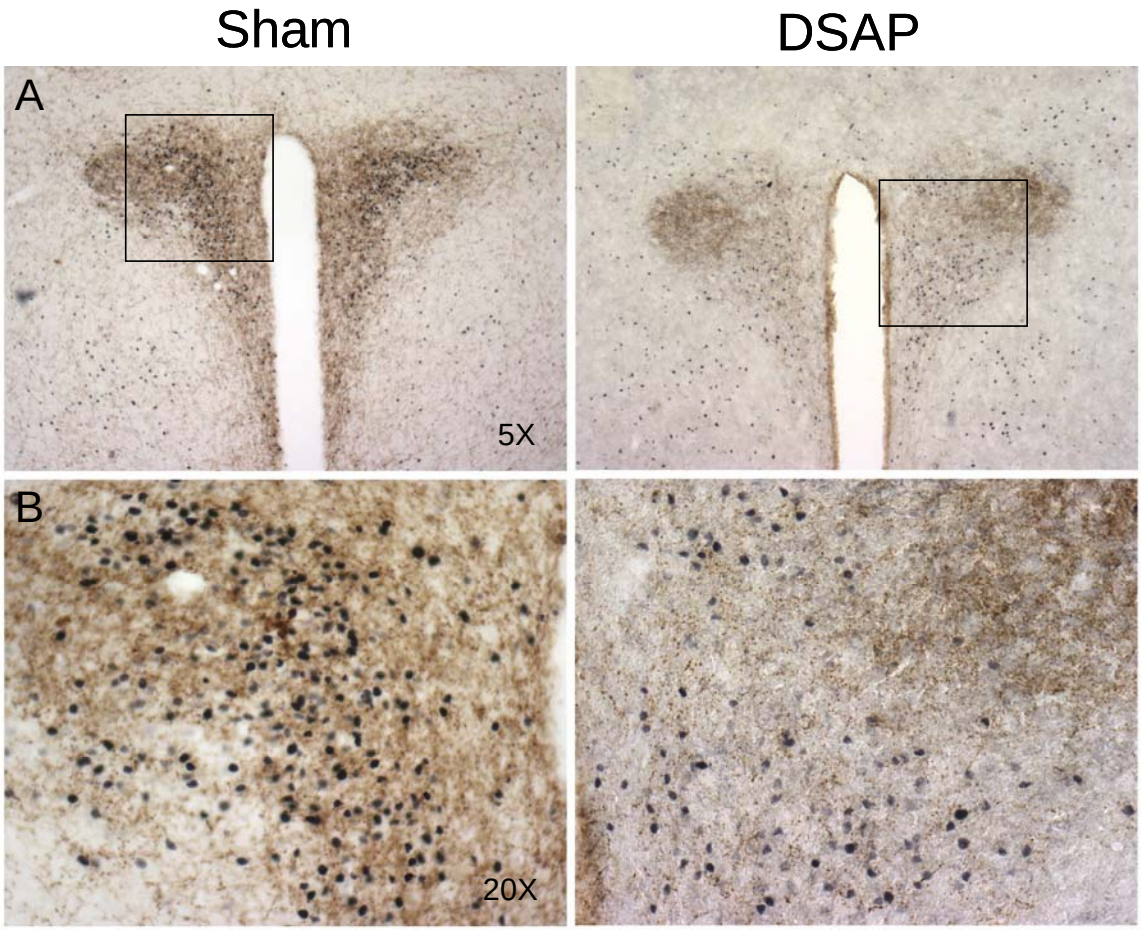

Figure 4-2 DSAP decreased NA terminals and YO-induced Fos activation in the medial parvocellular PVN.

PVN: A. Representative color photomicrographs depicting dual immunoperoxidase labeling of DbH (brown label) and Fos (black nuclear label) in a YO-treated sham and DSAP rat. B. Higher magnification of boxed region in 4-2A (above).

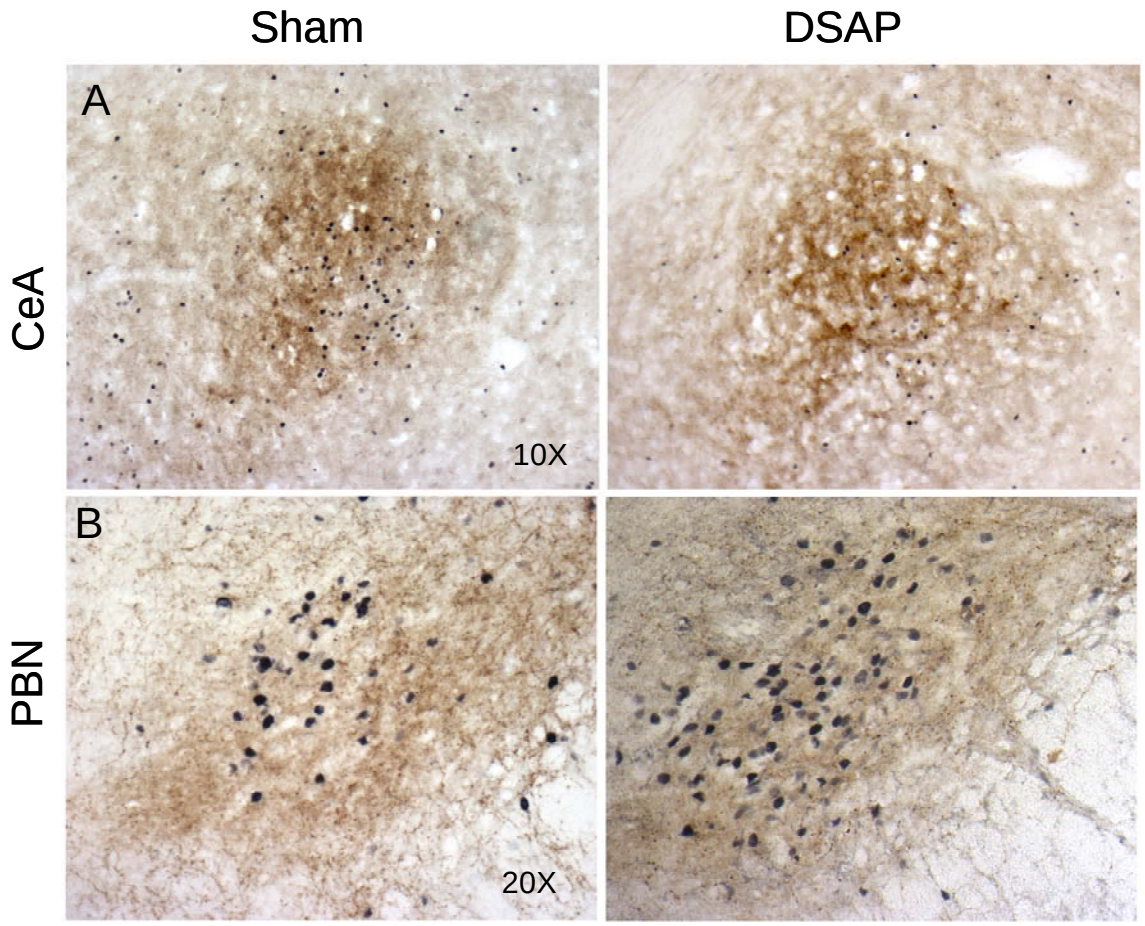

Figure 4-3 DSAP did not decrease YO-induced Fos expression in CeA or PBN.

A. CeA: Representative color photomicrographs depicting dual immunoperoxidase labeling of CRH (brown label) and Fos (black nuclear label) in a YO-treated sham and DSAP rat. B. PBN: Representative color photomicrographs depicting dual immunoperoxidase labeling of DbH (brown label) and Fos (black nuclear label) in a YO-treated sham and DSAP rat.

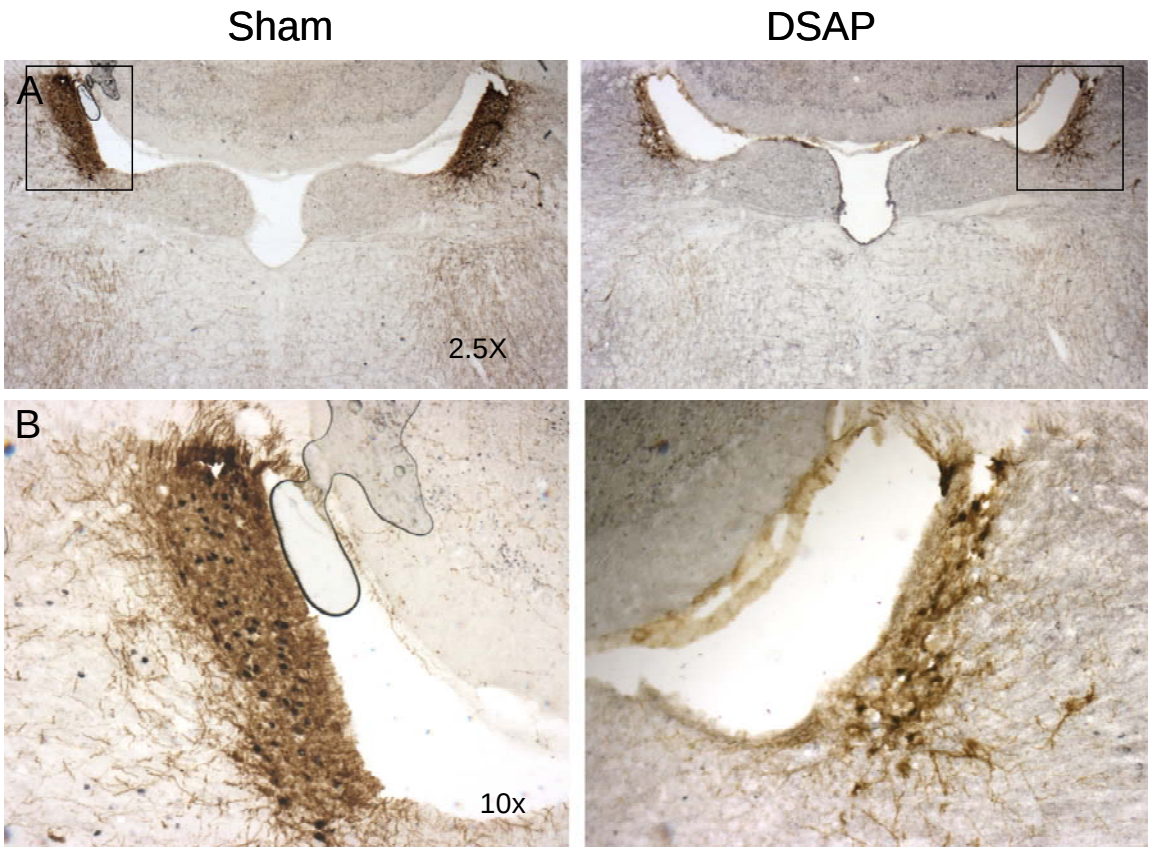

Figure 4-4 DSAP reduced NA neurons in the LC.

LC: A. Representative color photomicrographs depicting dual immunoperoxidase labeling of DbH (brown label) and Fos (black nuclear label) in a YO-treated sham and DSAP rat. B. Higher magnification of boxed region in 4-4A (above).

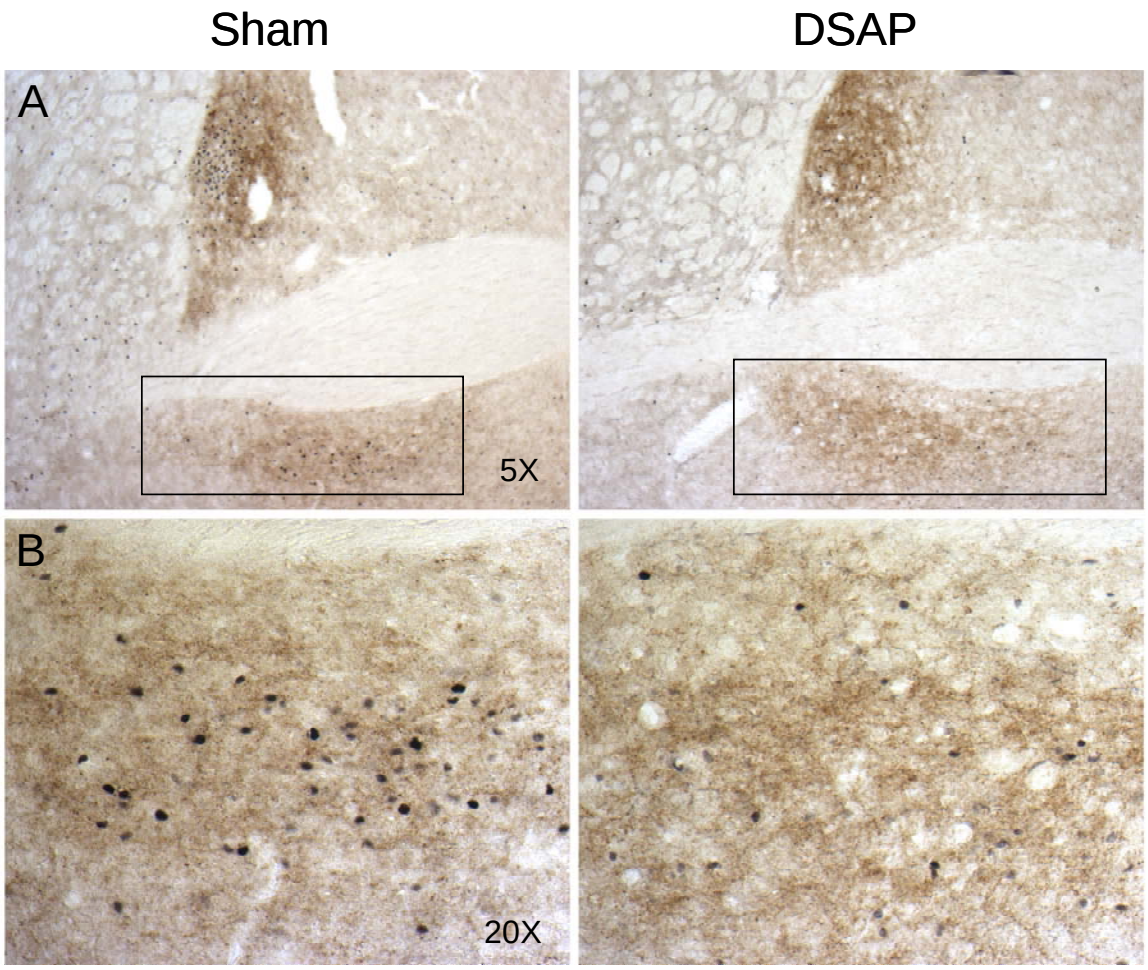

Figure 4-5 DSAP decreased YO-induced Fos activation in ventral BNST.

BNST: A. Representative color photomicrographs depicting dual immunoperoxidase labeling of CRH (brown label) and Fos (black nuclear label) in a YO-treated sham and DSAP rat. B. Higher magnification of boxed region in 4-5A (above).

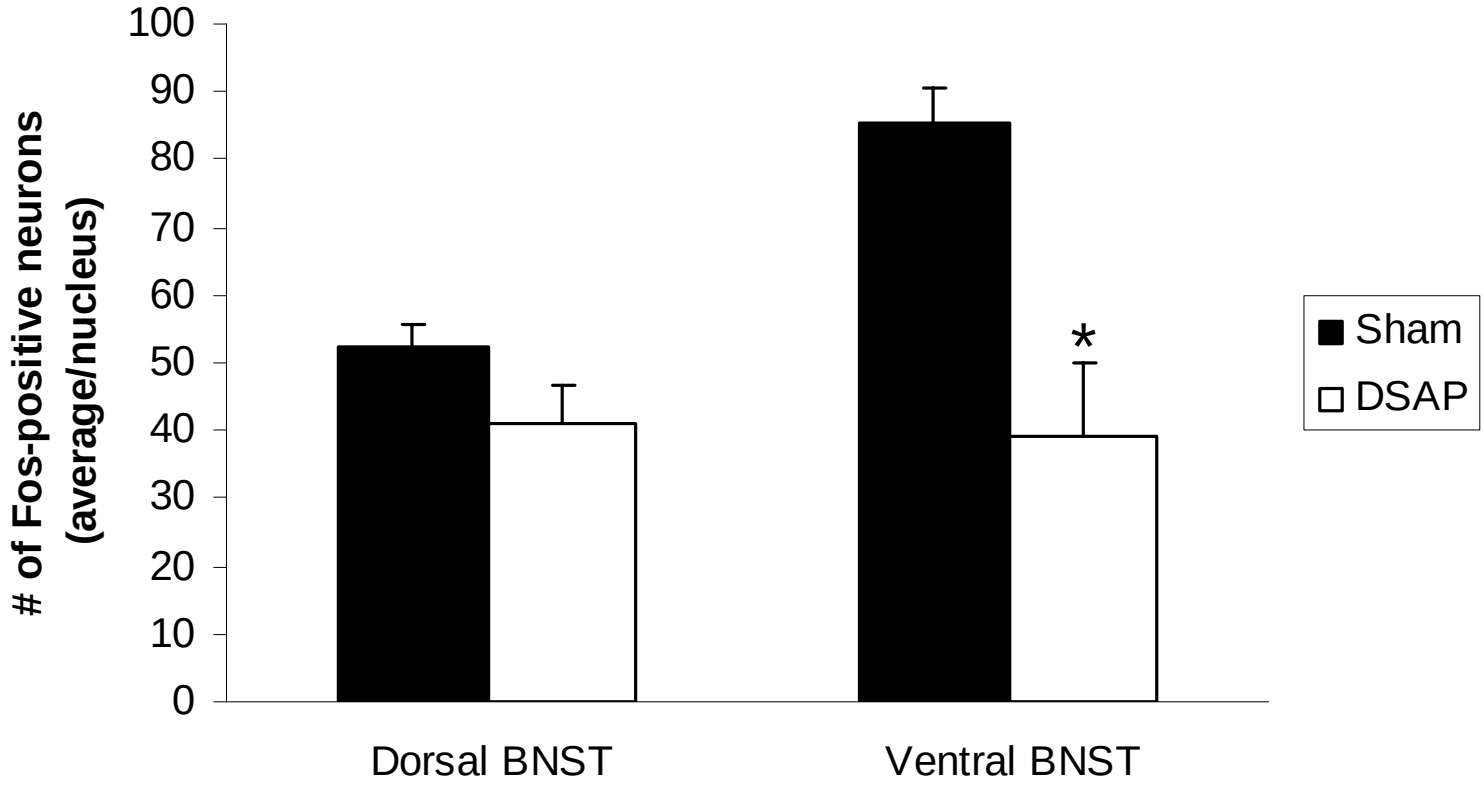

Figure 4-6 DSAP significantly reduced YO-induced Fos activation in ventral [$F(1,6) = 15.0449$, $P=0.0082$] but not dorsal BNST (sham n=4, DSAP n=4).

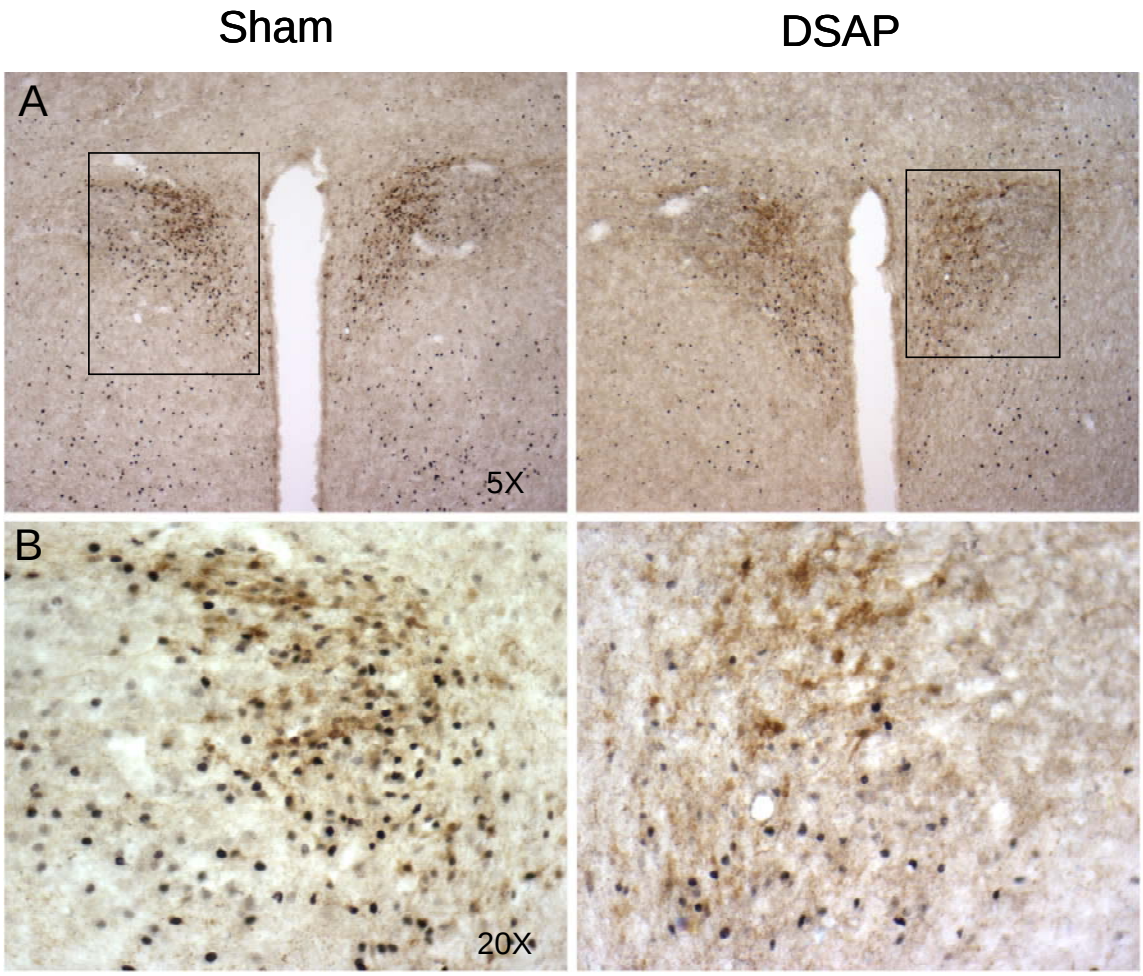

Figure 4-7 DSAP decreased YO-induced Fos expression in CRH neurons of the PVN.

PVN: A. Representative color photomicrographs depicting dual immunoperoxidase labeling of CRH (brown cytoplasmic label) and Fos (black nuclear label) in a YO-treated sham and DSAP rat. B. Higher magnification of boxed region in 4-7A (above).

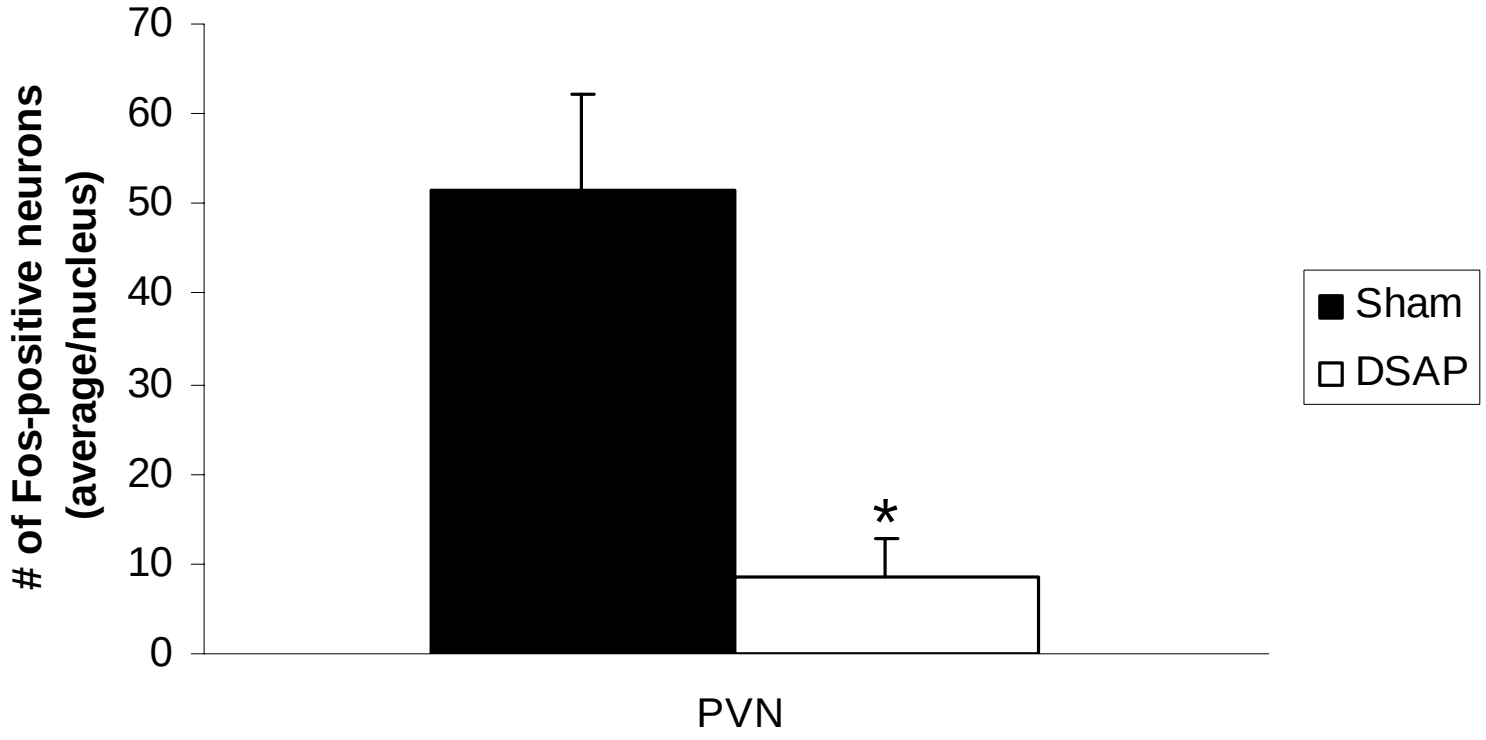

Figure 4-8 DSAP significantly decreased YO-induced Fos activation in medial parvocellular PVN CRH neurons compared to sham controls [$F(1,6) = 14.4071$, $P=0.009$] (sham n=4, DSAP n=4).

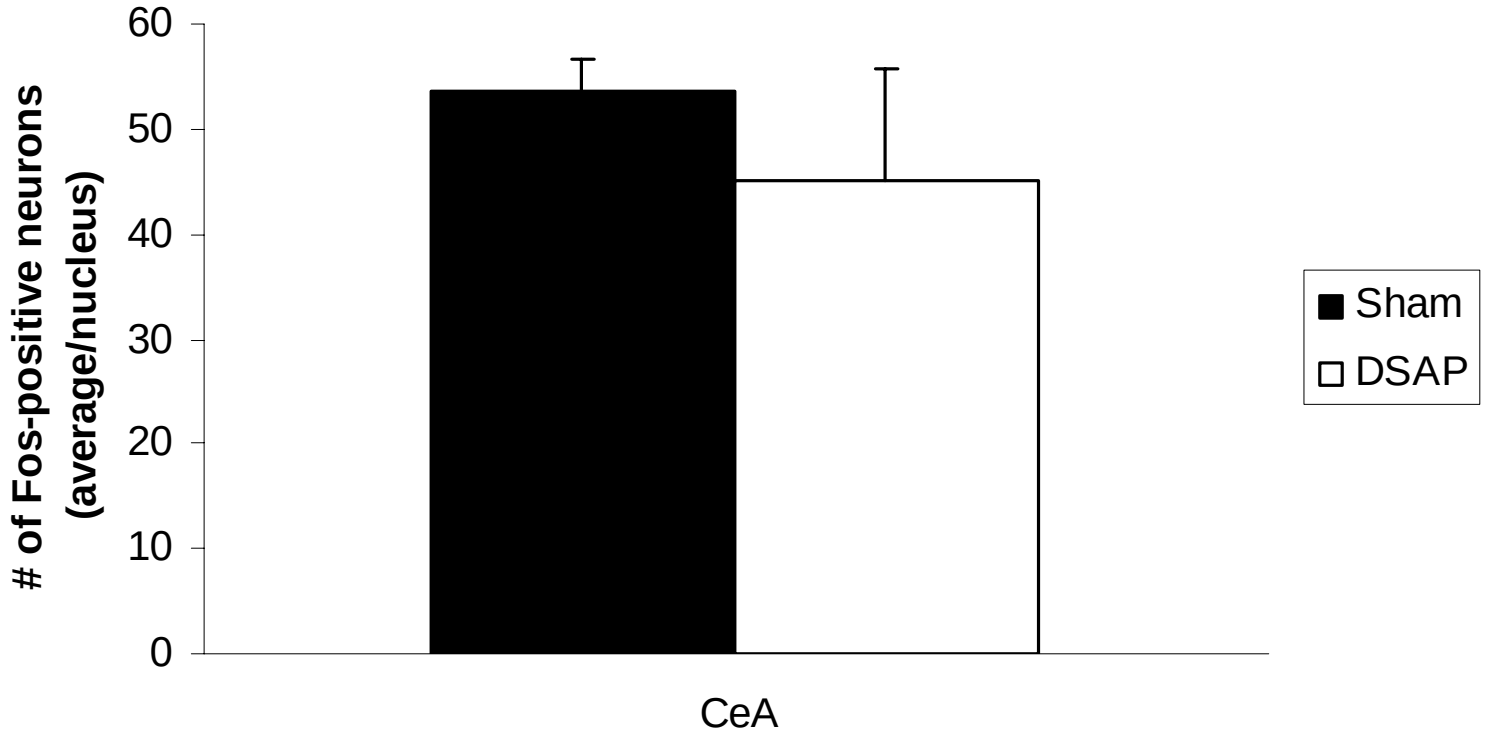

Figure 4-9 DSAP did not significantly decrease YO-induced Fos expression in CeA (sham n=4, DSAP n=4).

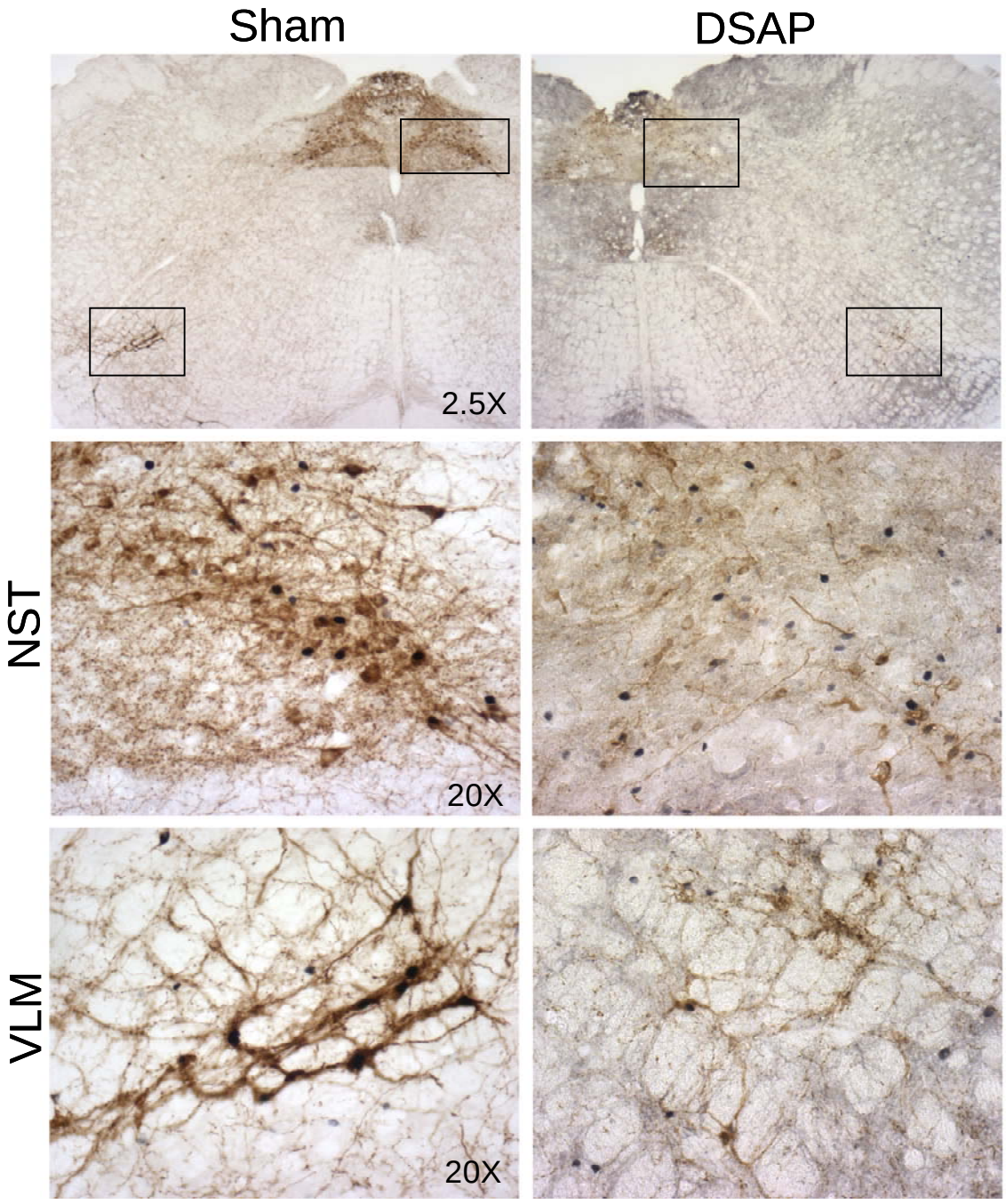

Figure 4-10 DSAP reduced NA neurons in the NST and VLM.

NST and VLM: Representative color photomicrographs depicting dual immunoperoxidase labeling of DbH (brown cytoplasmic label) and Fos (black nuclear label) in a YO-treated sham and DSAP animal.

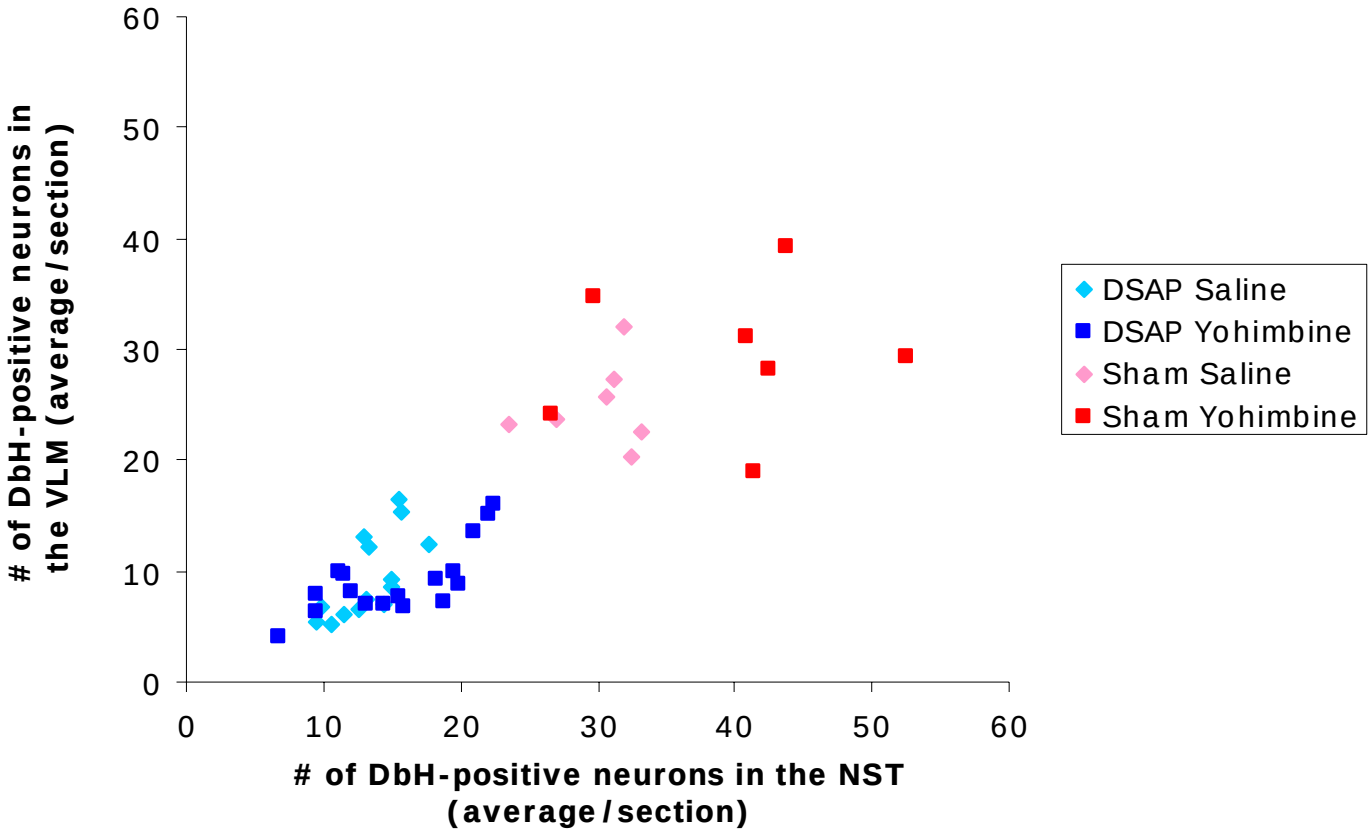

Figure 4-11 DSAP rats have fewer DbH-positive neurons in the NST and VLM than sham rats. The average DbH - positive neurons per section in the NST and VLM in DSAP and sham animals was positively correlated (r=0.8733, [$F(1,43)=138.1322$;$P<0.0001$]).

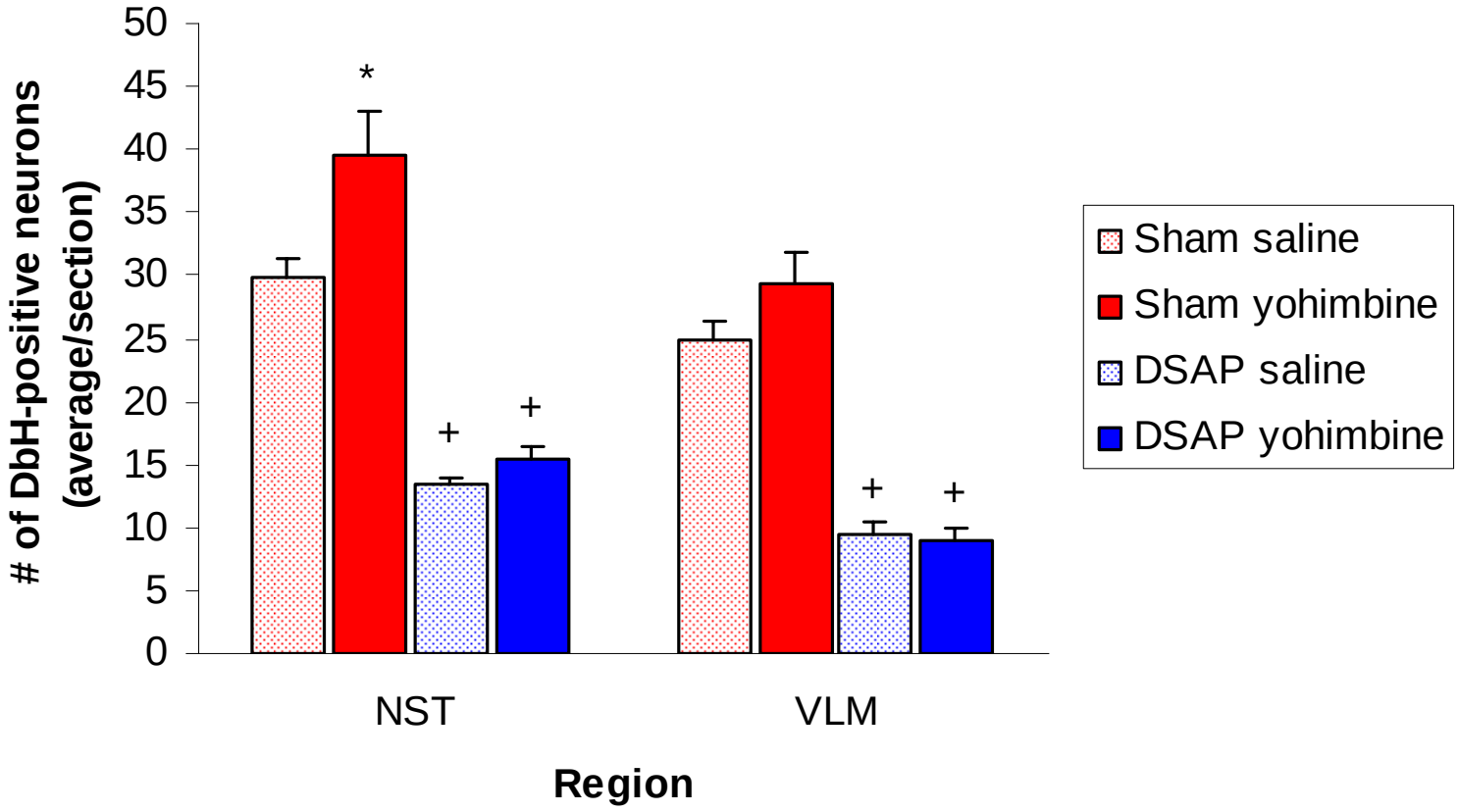

Figure 4-12 DSAP rats have significantly fewer DbH-positive neurons than sham rats in the NST and VLM.

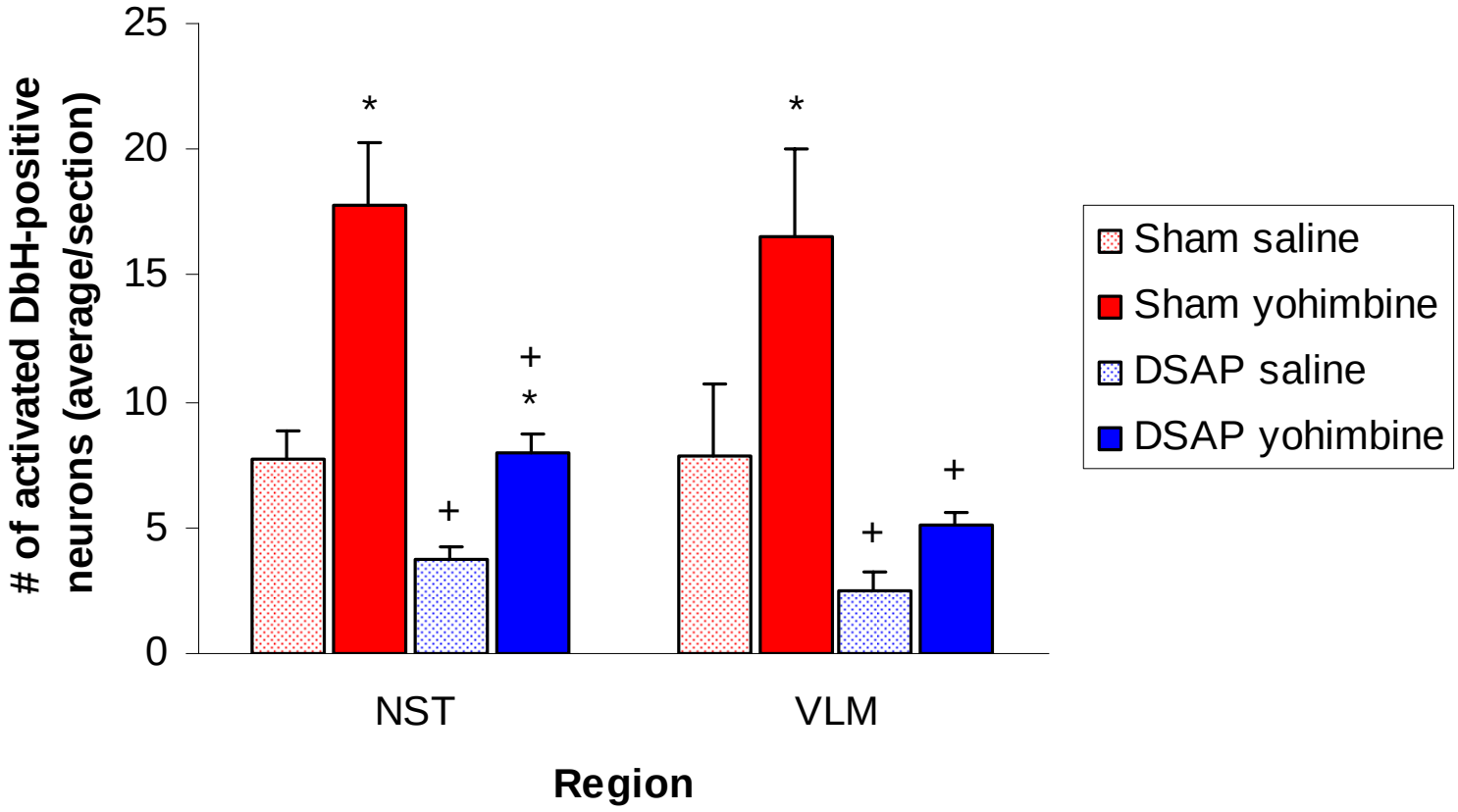

Figure 4-13 YO increased the number of activated NA neurons in the sham rats in the NST and VLM and in DSAP rats in the NST. However, DSAP rats had significantly fewer activated DbH-positive neurons than sham rats in the NST and VLM.

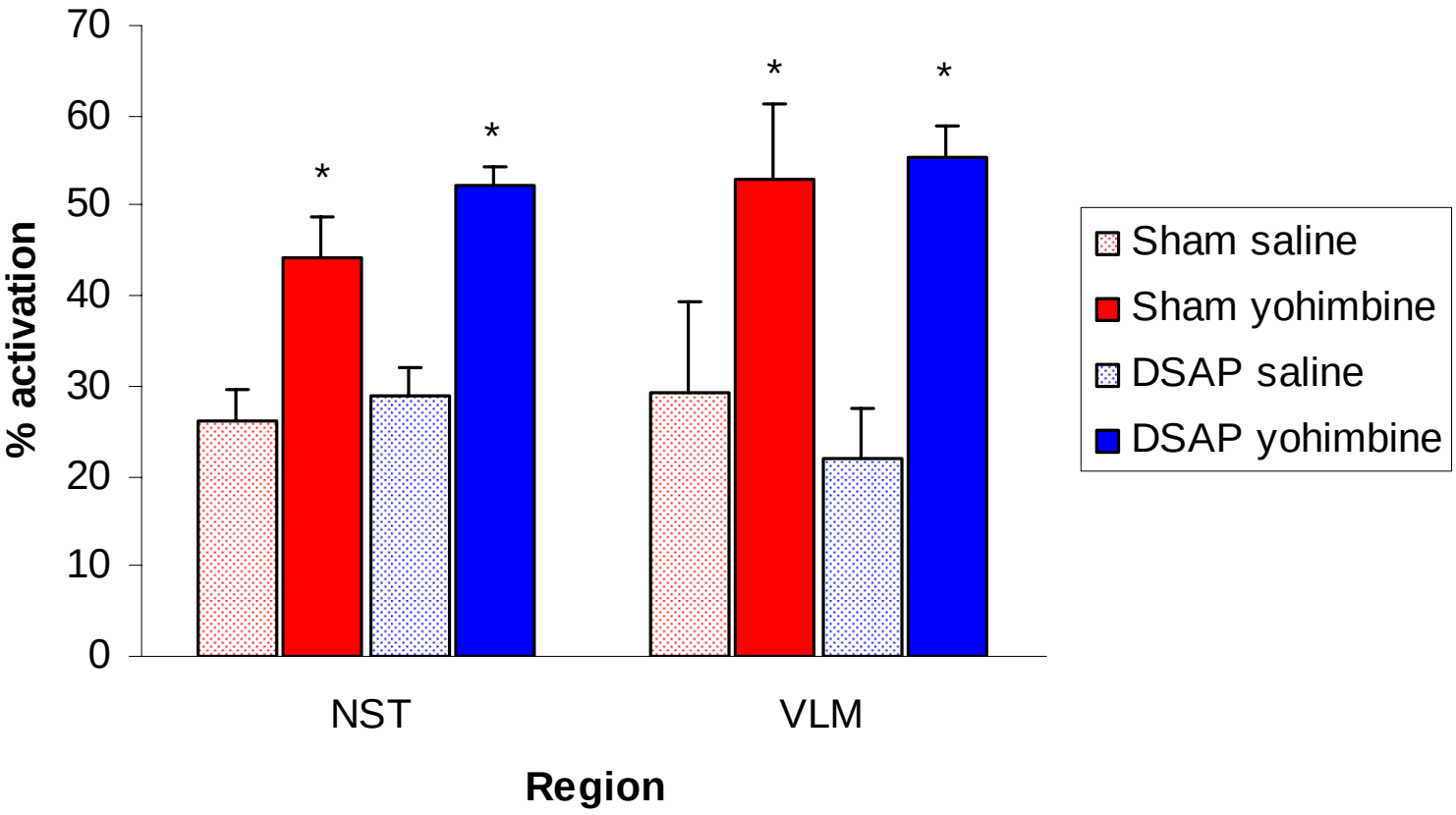

Figure 4-14 YO increased the percent activation to a similar extent in sham and DSAP rats in the NST and VLM.

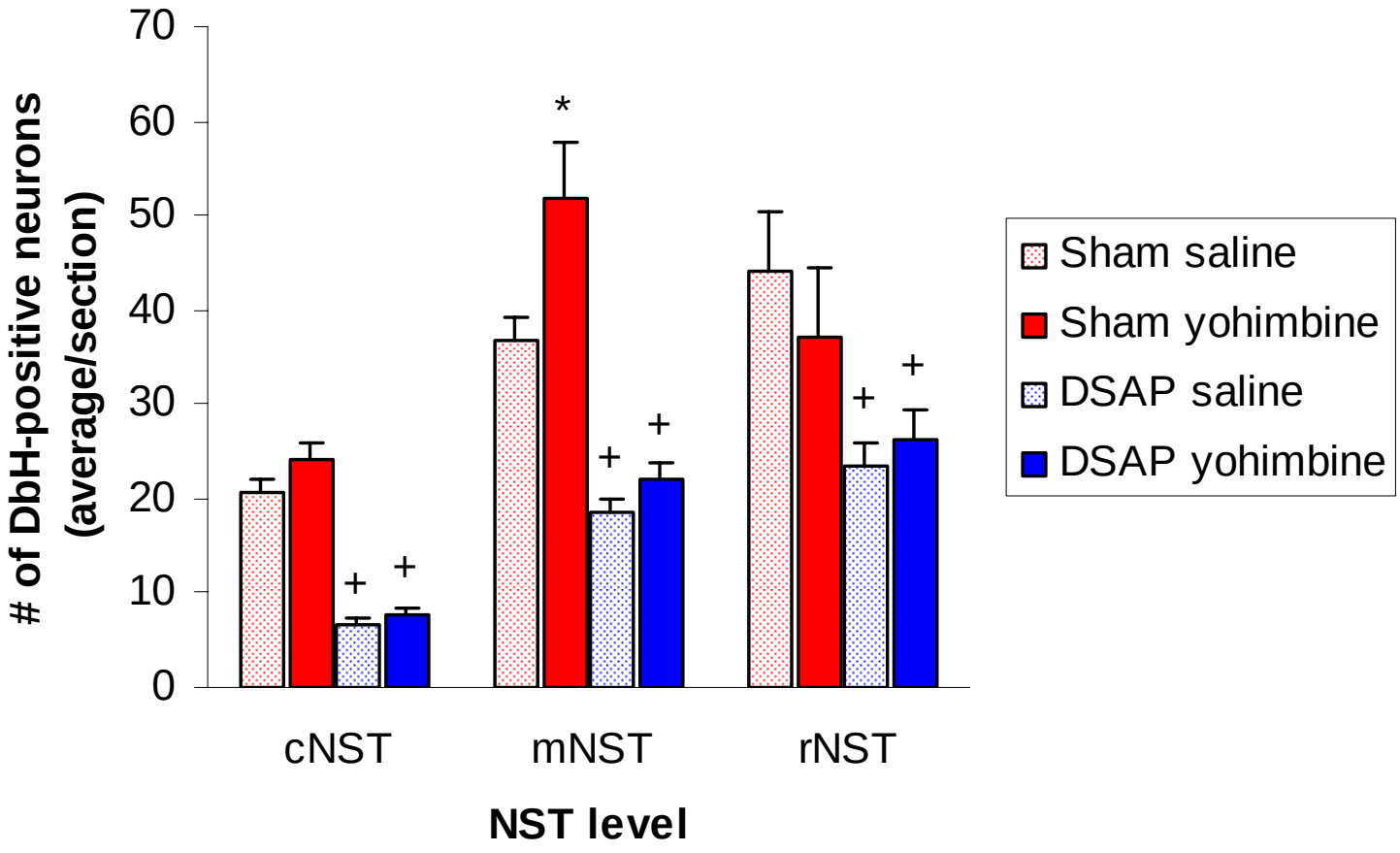

Figure 4-15 DSAP rats in both treatment groups had significantly fewer NA neurons than their respective controls ($P<0.01$) at all 3 rostro-caudal levels of the NST. (* indicates significance ($P<0.05$) within group, + indicates significance ($P<0.05$) when compared to respective between group control)

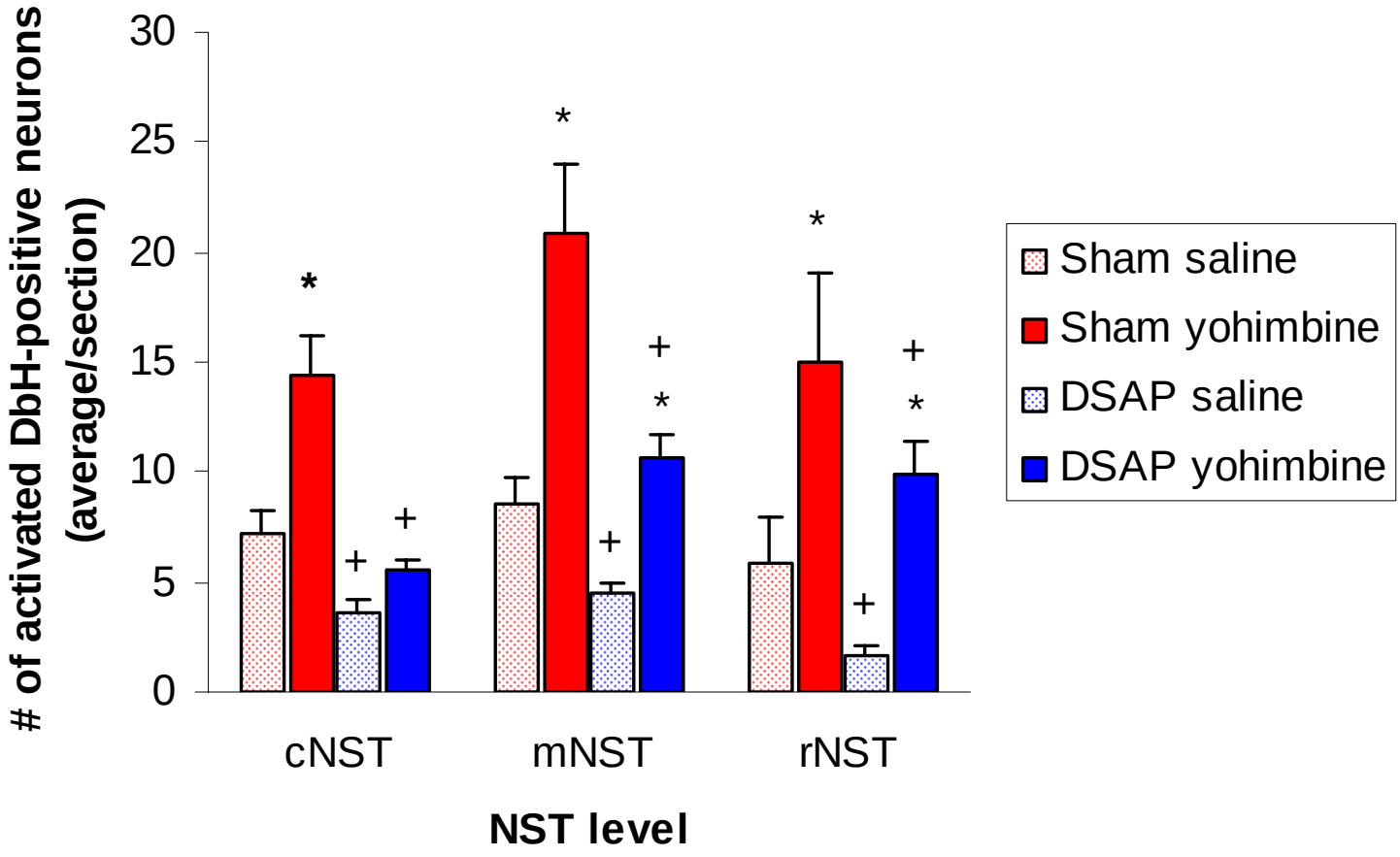

Figure 4-16 DSAP rats had significantly fewer activated (Fos-positive) NA neurons than their respective sham controls at all rostrocaudal NST levels.

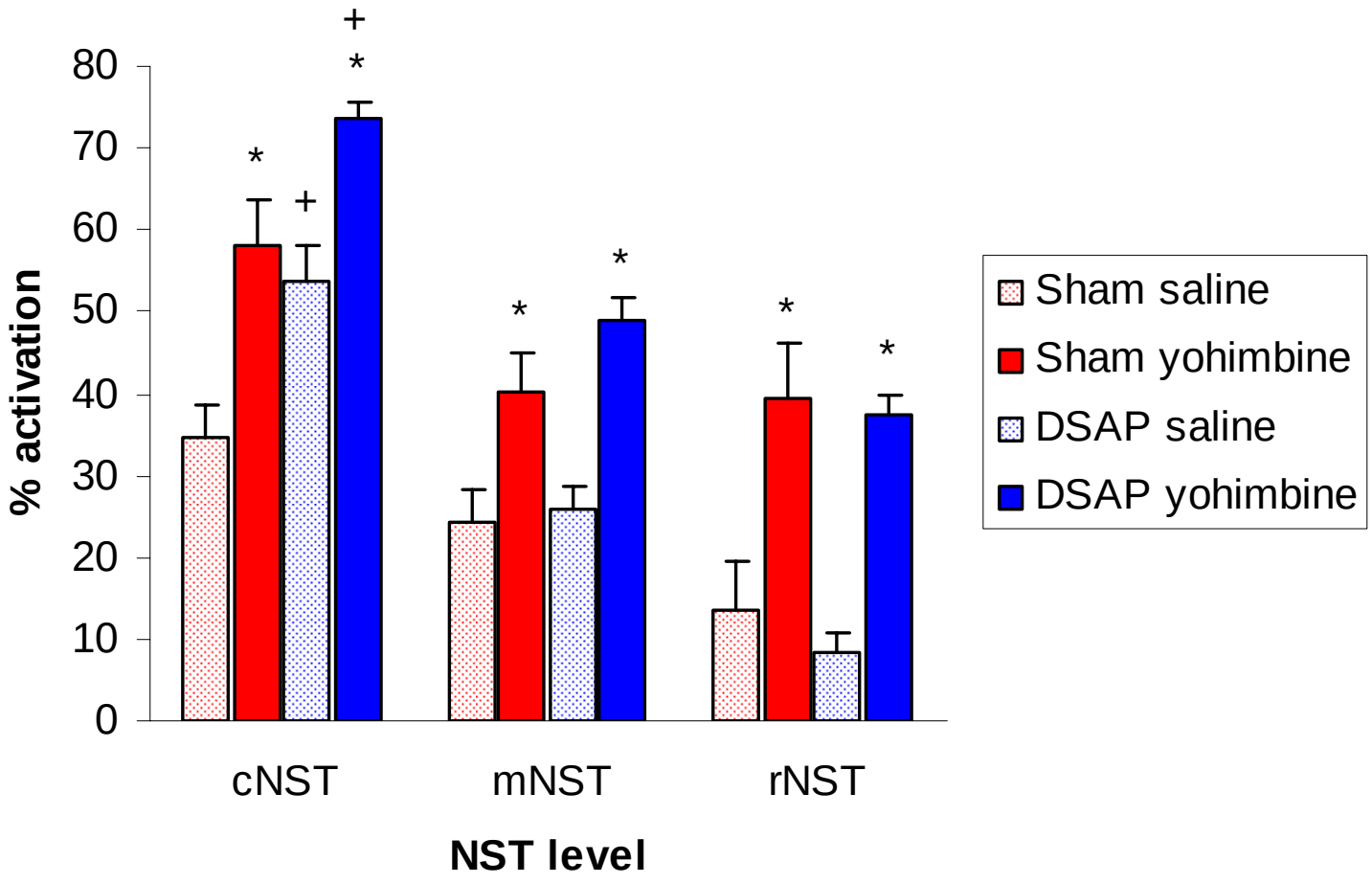

Figure 4-17 YO-treated rats had significantly higher percent activation than within group controls at all rostrocaudal NST levels. However, DSAP rats had significantly higher percent activations than their respective sham controls at the cNST level.

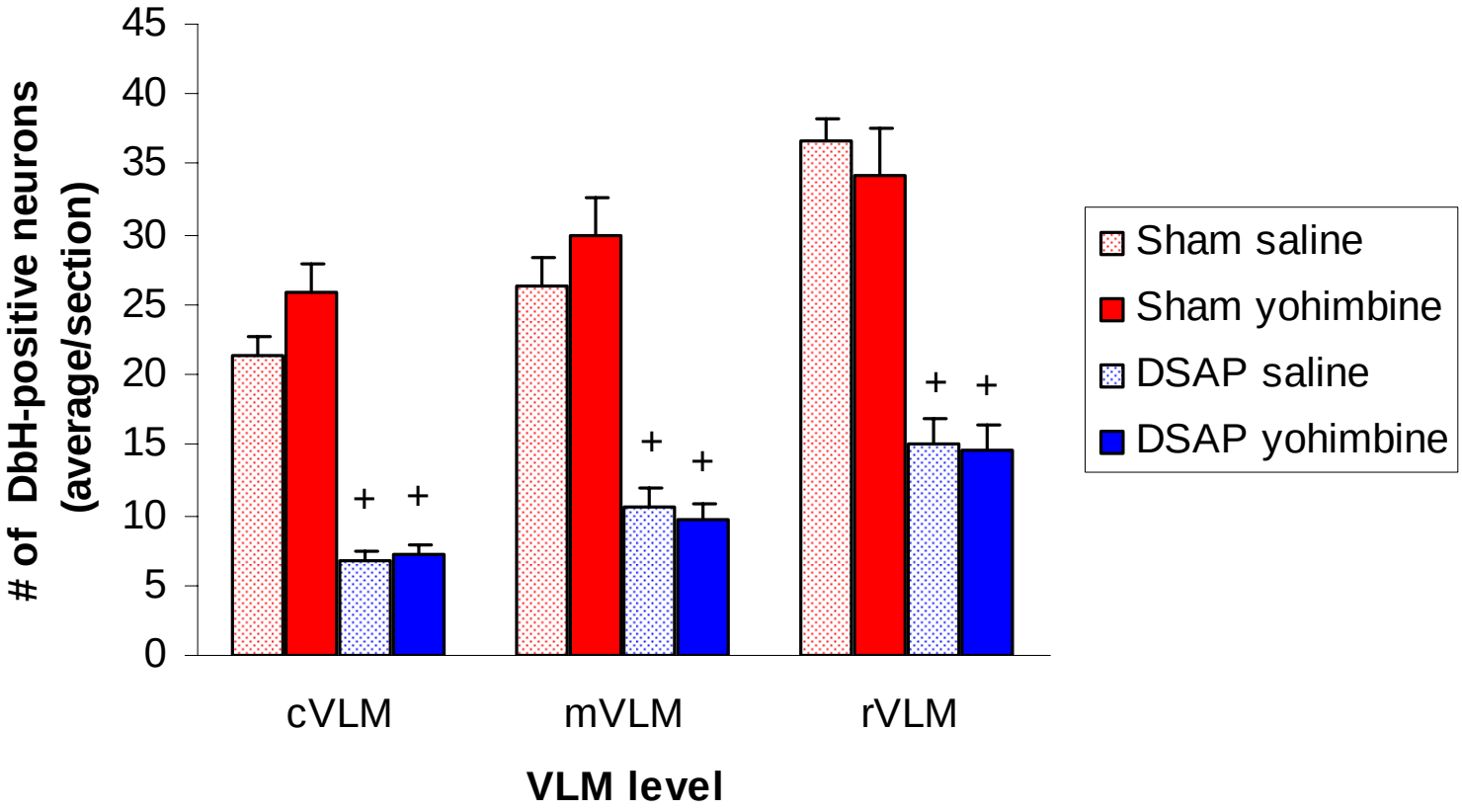

Figure 4-18 DSAP rats have significantly fewer DbH-positive neurons than sham rats at all rostrocaudal VLM levels.

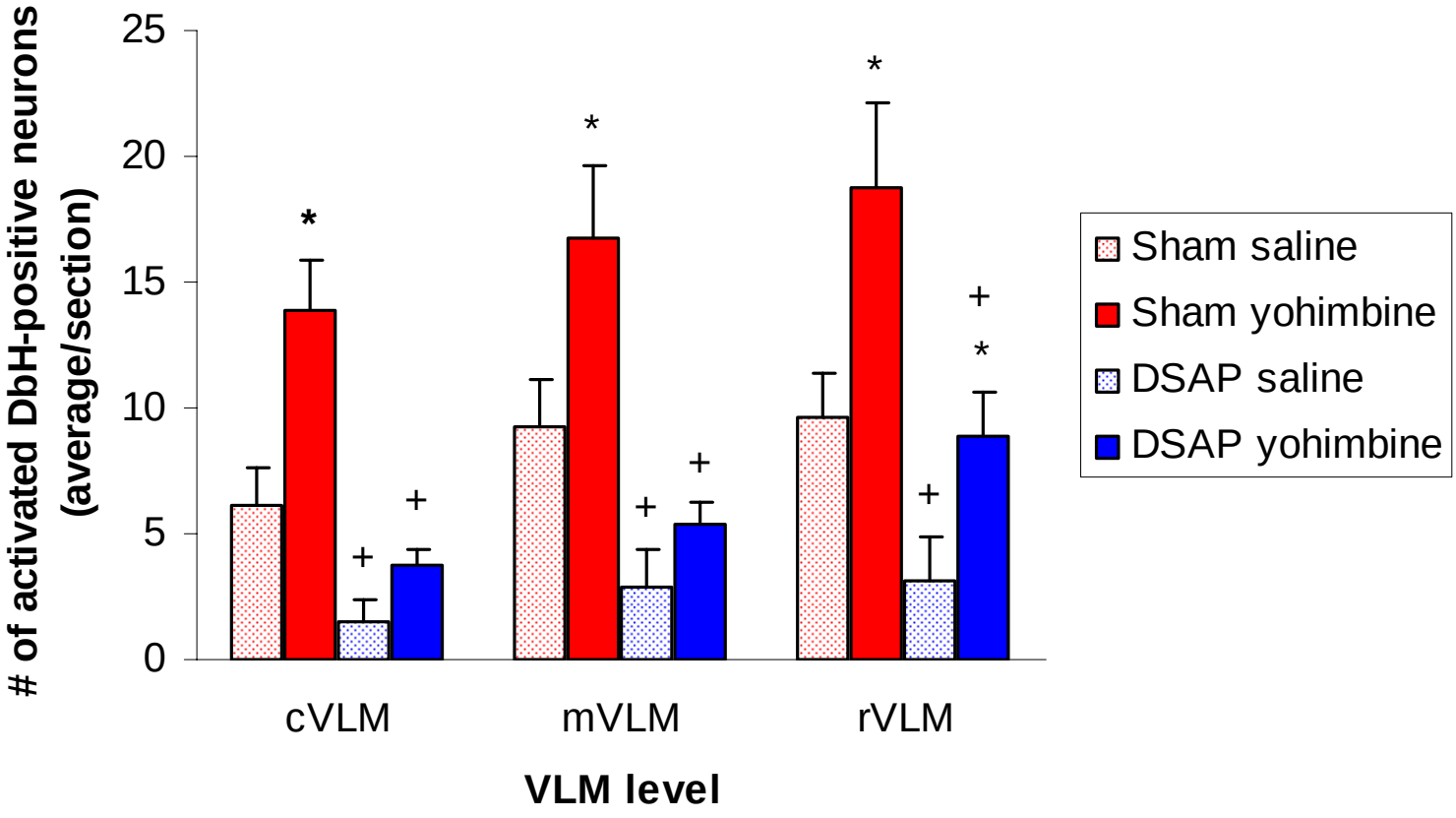

Figure 4-19 YO significantly increases the number of activated NA neurons in sham rats at all rostrocaudal VLM levels. DSAP rats have significantly fewer activated NA neurons than their respective sham controls at all rostrocaudal VLM levels.

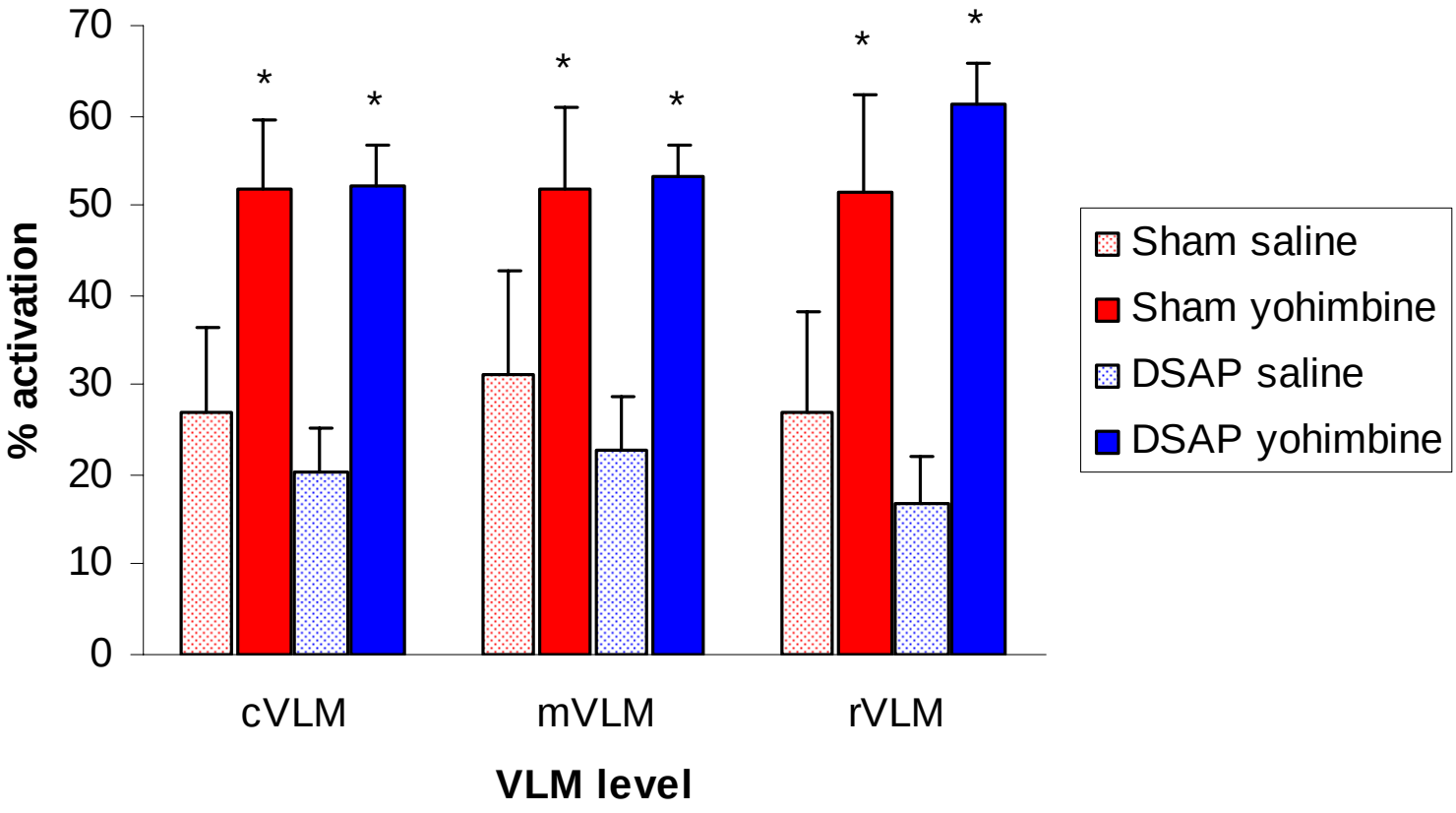

Figure 4-20 YO significantly increases percent activation in sham and DSAP rats to a similar extent at all rostrocaudal VLM levels.

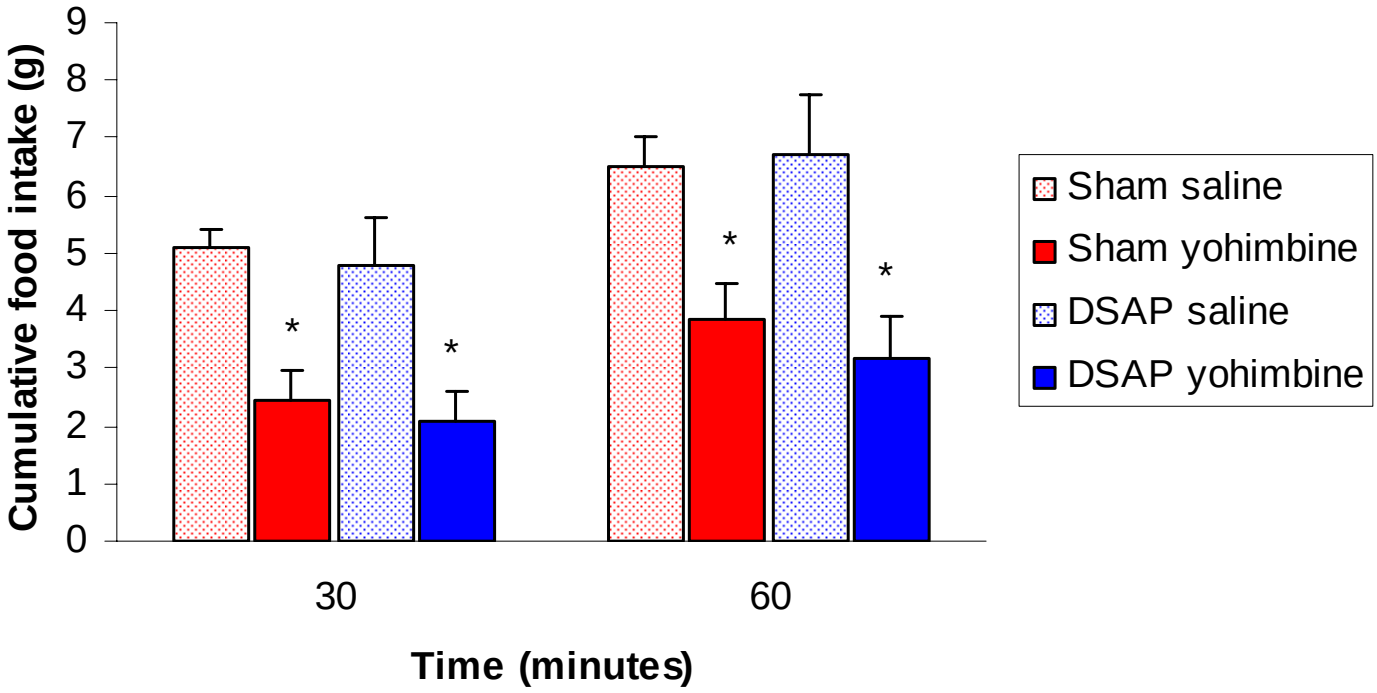

Figure 4-21 YO significantly inhibits deprivation-induced food intake at 30 and 60 minutes to a similar extent in both sham and DSAP rats.

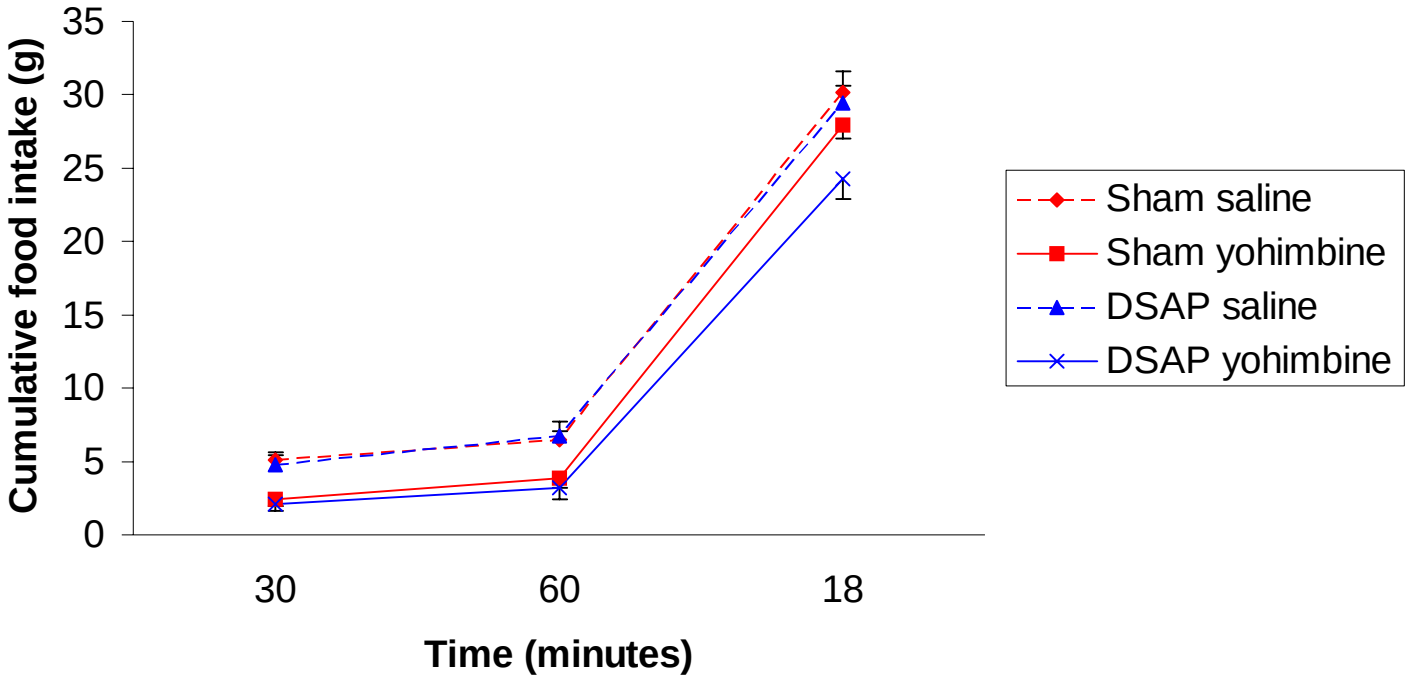

Figure 4-22 Sham rats recovered their cumulative food intake by 18 hours whereas DSAP rats did not.

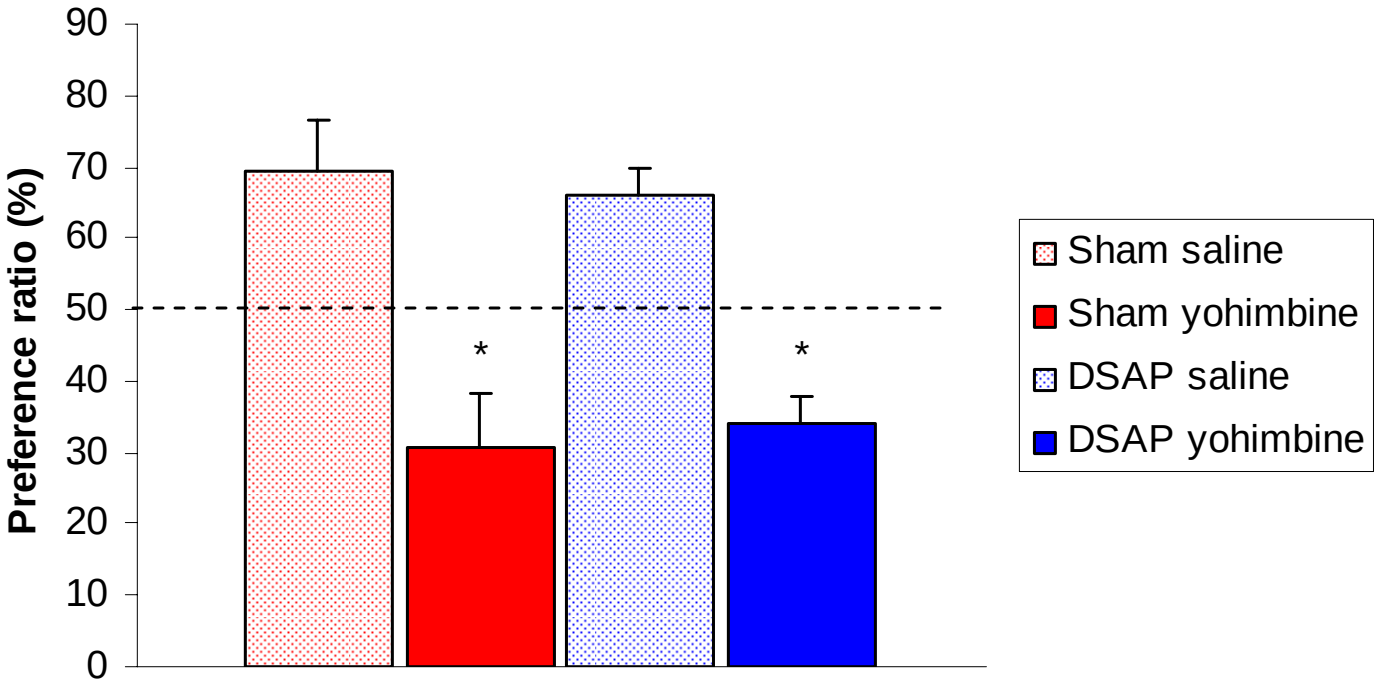

Figure 4-23 Sham and DSAP rats significantly avoided a flavor previously paired with YO in a two-bottle choice test.

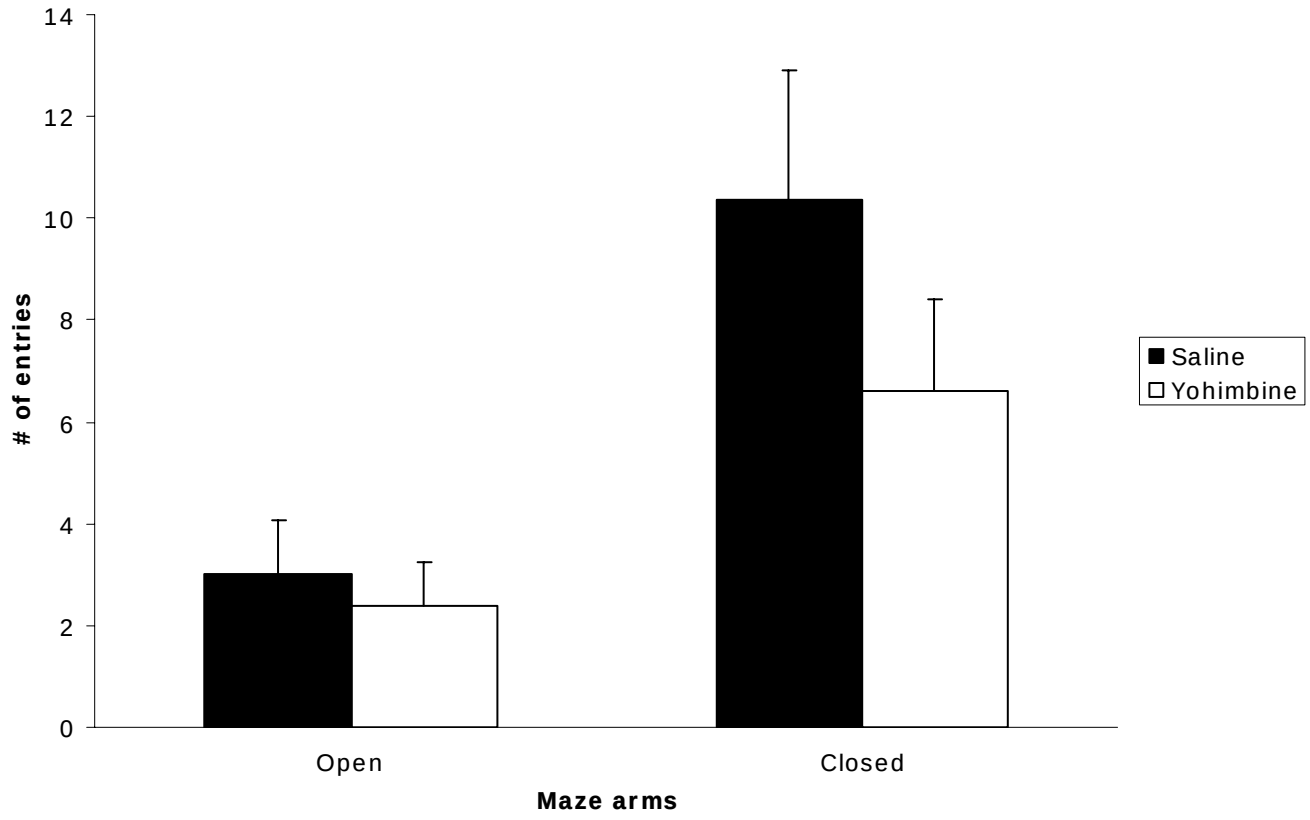

Figure 4-24 YO (1 mg/kg BW, i.p.) did not significantly alter open or closed arm entries on the EPMZ.

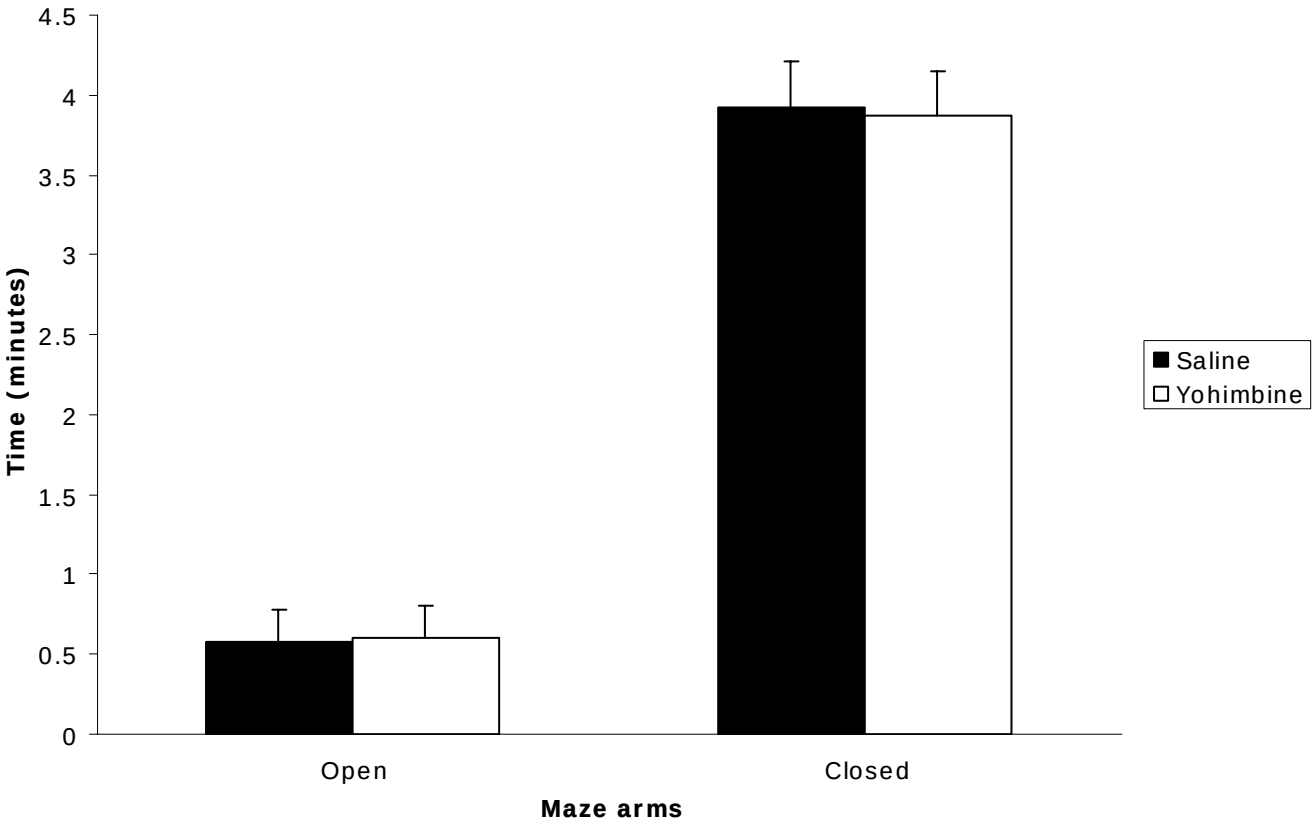

Figure 4-25 YO (1 mg/kg BW, i.p.) did not alter the amount of time spent on the open or closed arms of the EPMZ.

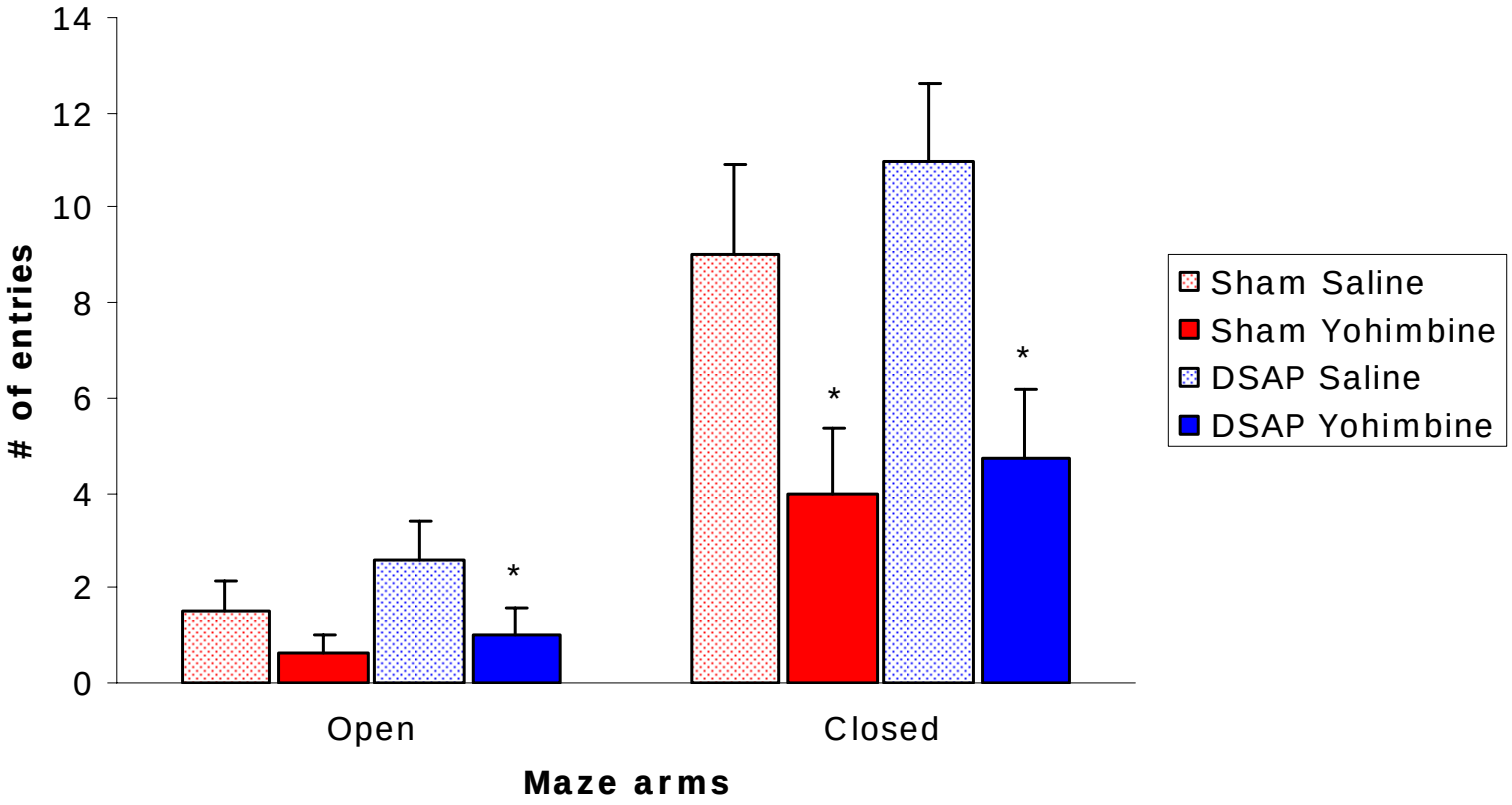

Figure 4-26 YO (5 mg/kg) significantly decreased the number of open arm entries in DSAP rats ($P<0.01$) and in closed arm entries in sham and DSAP rats ($P<0.05$) for both surgical groups).

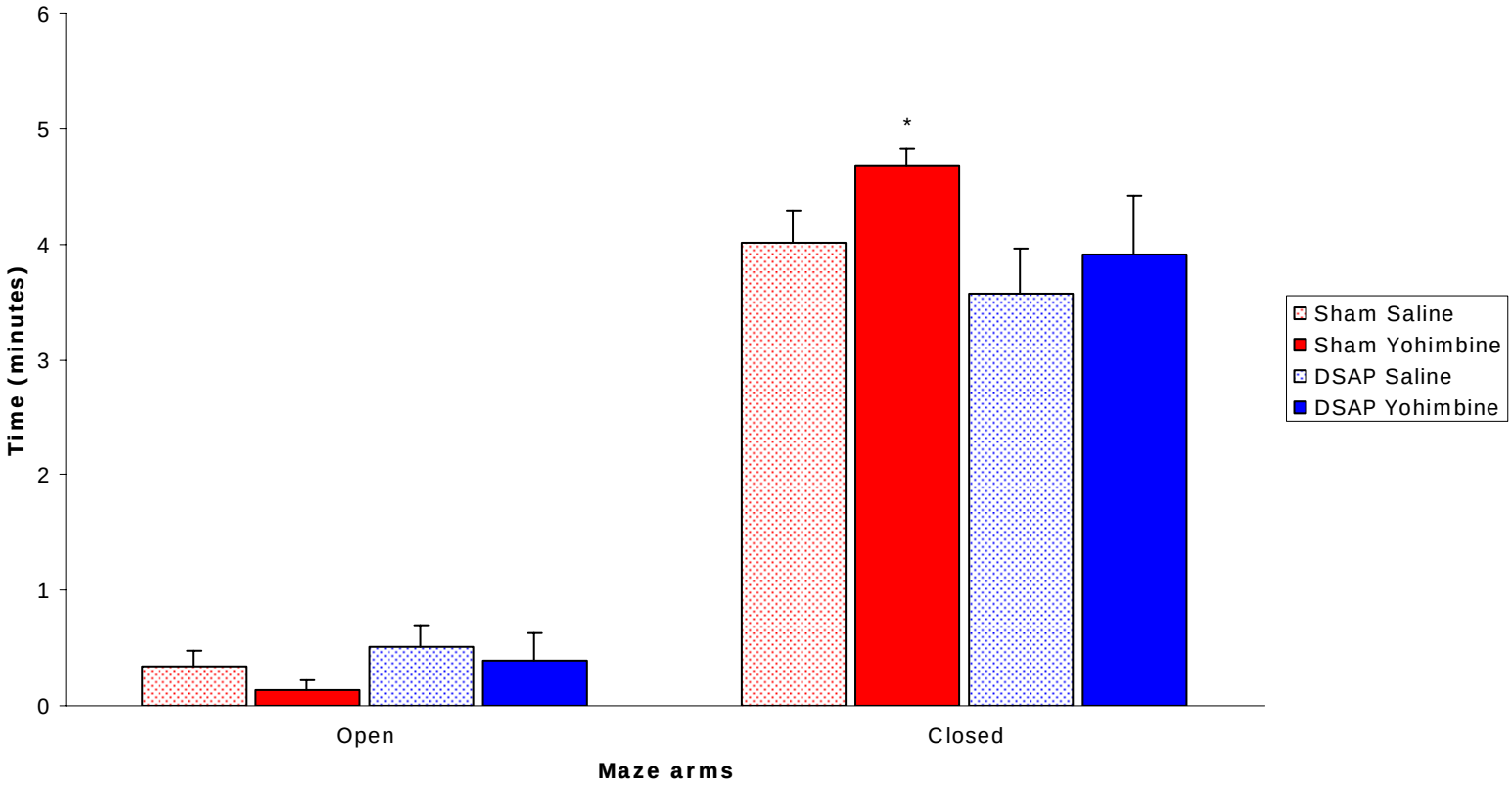

Figure 4-27 YO (5 mg/kg) significantly increased closed arm time in sham but not DSAP rats (P<0.05).

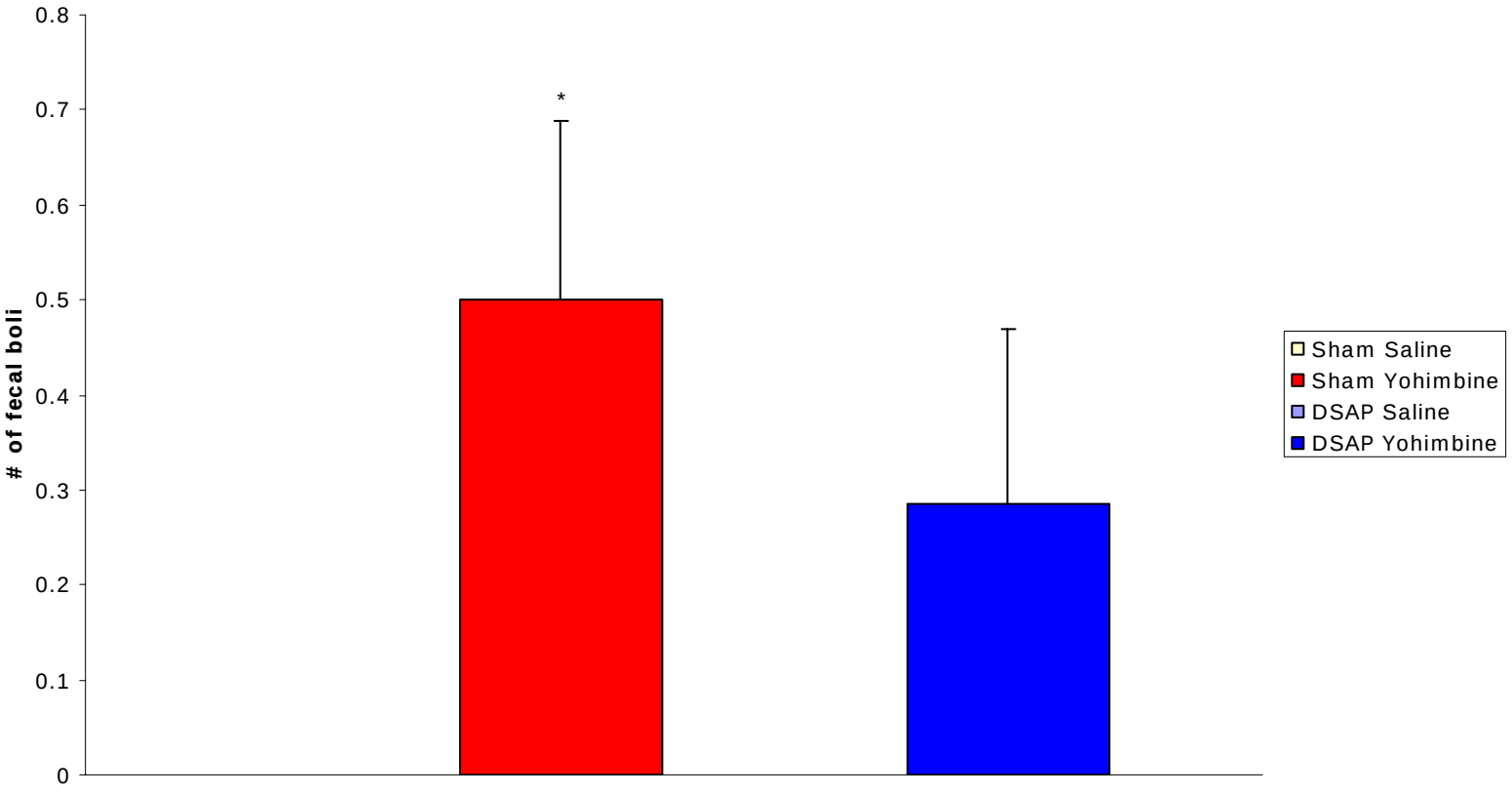

Figure 4-28 YO (5 mg/kg) significantly increased fecal boli in sham but not DSAP rats ($P<0.05$).

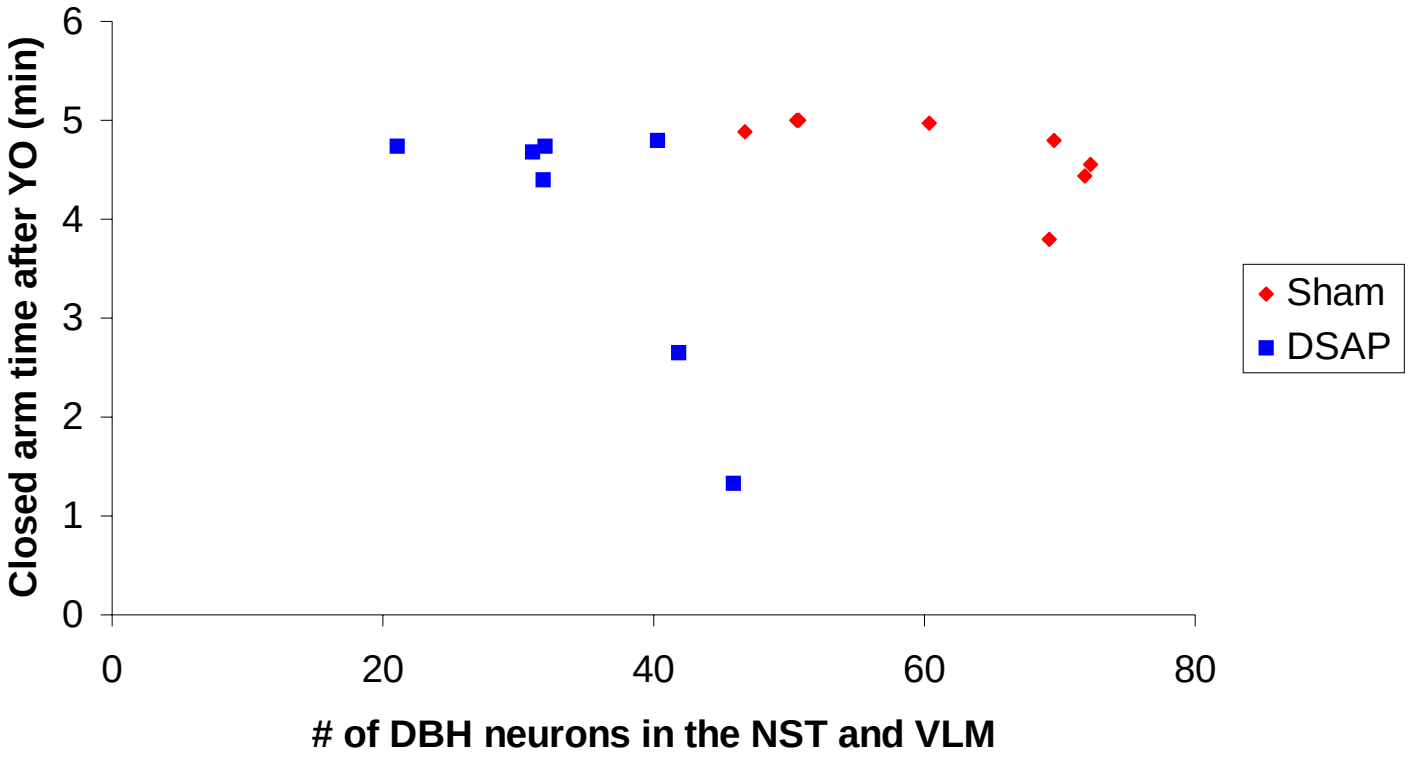

Figure 4-29 There was no correlation between the number of average DbH neurons per section in the NST, VLM, or sum of the two on closed arm time after YO.

5. DISCUSSION

The findings presented in this document provide new evidence that the NA input to the BNST is necessary for YO-induced Fos expression in the BNST and PVN, and that NA inputs to the BNST contribute to the ability of YO to alter behavior in the EPMZ. However, the NA input to the BNST appears to be unnecessary for YO-induced Fos expression in the CeA or PBN, or for YO-induced anorexia or CFA.

5.1. Anatomical Effects of DSAP Lesion

BNST DSAP lesions produced a nearly complete loss of NA terminals in the BNST and mpPVN, indicating that a subset of NA neurons in the NST and/or VLM that project to the BNST collaterize to also innervate the medial parvocellular PVN. A NA collateral projection from the VLM to the PVN and BNST has been reported based on findings obtained using a less direct approach (Woulfe, Hrycyshyn et al. 1988). Woulfe et al. used a dual retrograde tracer technique to examine the collateral axonal projections from the A1 region to the PVN and BNST. Although these investigators did not quantify the extent of collateralization, it appeared far less extensive than what we would predict based on our findings. Additionally, the present study shows that NA terminals in the magnocellular PVN remain largely intact in BNST DSAP rats, demonstrating that the projection to the magnocellular PVN arises from a different subpopulation of neurons within the NST and/or VLM. BNST DSAP lesions also leave the CRH-rich regions of the dorsal lateral and ventral lateral BNST and PVN CRH neurons intact, supporting the specificity of DSAP for NA terminals and neurons. The specificity of DSAP as a NA lesioning agent has been demonstrated previously (Madden, Ito et al. 1999; Rinaman 2003; Ritter, Watts et al. 2003).

A NA collateral axonal projection from the VLM to the CeA and the BNST has also been shown (Roder and Ciriello 1994) which may account for the slight loss of NA terminals in the CeA (data not shown). The NA input to the PBN appears to be untouched by the BNST DSAP lesion, indicating that the population of NA NST and/or VLM neurons projecting to the BNST is completely separate from the population projecting to PBN.

5.2. Neural effects of DSAP lesion

The present work shows that removal of the NA input to the BNST significantly attenuated the YO-induced increase in Fos expression in the ventral, but not the dorsal BNST. Little is known regarding the actions of NE within the BNST. A recent study performed in slice preparation in mouse tissue reported differential actions of NE in dorsal and ventral BNST. These investigators found that NE produces a decrease in excitatory transmission in the ventral BNST that appears to be mediated primarily by the alpha-2a adrenergic receptor (Egli, Kash et al. 2005). In the dorsal BNST, NE has been reported to produce decreases as well as increases in excitatory transmission (Egli, Kash et al. 2005). However, the organization of the circuitry and actions of NE may not be readily extended to those in rat; although, a similar finding that NE produces decreases in firing rate in BNST was reported in rats (Casada and Dafny 1993). Furthermore, application of NE is not necessarily similar to endogenously released NE because of its release with co-stored transmitters which have been shown to modulate its effects (Khoshbouei, Cecchi et al. 2002).

NA inputs to the BNST predominantly terminate in the ventral lateral subdivision, and have been reported to synapse on CRH neurons as well as galanin- and enkephalin-immunoreactive neurons (Woulfe, Flumerfelt et al. 1990; Phelix, Liposits et al. 1992; Kozicz 2001; Kozicz 2002). It has been shown that stress leads to NE release, presumably from

brainstem NST and VLM, and Fos expression in the BNST (Ericsson, Kovacs et al. 1994; Pacak, McCarty et al. 1995; Fuentealba, Forray et al. 2000; Cecchi, Khoshbouei et al. 2002). Additionally, YO, which potentiates the release of endogenous transmitter from NA nerve terminals, promotes increased levels of extracellular NA and increased Fos expression in medulla, hypothalamus, CeA, and BNST (Tsujino, Sano et al. 1992; Khoshbouei, Cecchi et al. 2002; Singewald, Salchner et al. 2003). Thus, if NE does indeed decrease excitatory transmission in ventral BNST, it may lead to a local disinhibition that would in turn lead to Fos expression. Nonetheless, based on previous data showing the increase in YO-induced NE and Fos expression in BNST, we would predict that removal of NA input to the BNST would lead to an attenuation of YO-induced Fos expression in the ventral BNST as was seen in the present study.

In the dorsal BNST NE has been reported to produce decreases and increases in excitatory transmission. The increases in excitatory transmission appear to be mediated by beta-2 and alpha-2a receptors (Egli, Kash et al. 2005). It has also been reported that the dorsal BNST is under tonic GABAergic inhibition (Egli and Winder 2003). Thus, YO-induced Fos expression could be produced by a direct action on neurons that respond to NE with an increased transmission or by disinhibition of local dorsal BNST neurons. This disinhibition could allow these neurons to respond to other inputs, i.e. PBN, CeA (Alden, Besson et al. 1994; Dong, Petrovich et al. 2001). The dorsal BNST receives NA input primarily from the LC (Jones and Yang 1985), in which there is some NA cell loss in BNST DSAP animals. However, removal of the NA input to the BNST does not significantly alter YO-induced Fos expression in dorsal BNST compared YO-injected sham controls. Thus, YO-induced Fos expression in the dorsal BNST is not likely to be mediated by NA inputs. YO-induced Fos expression in the dorsal

BNST could be influenced by the CGRP-positive input from the PBN, which projects heavily to the dorsal lateral BNST (Alden, Besson et al. 1994), GLP-1 inputs from the NST (Merchenthaler, Lane et al. 1999), or inputs from the CeA (Dong, Petrovich et al. 2001).

BNST DSAP lesions attenuated the YO-induced Fos response in CRH neurons of the PVN. Stress produces NE release and Fos expression in the PVN (Myers, Banihashemi et al.; Ericsson, Kovacs et al. 1994; Pacak, Palkovits et al. 1995). The ventral BNST/fusiform subnuclei has a dense projection to the medial parvocellular PVN (Dong, Petrovich et al. 2001). Approximately half of the PVN-projecting BNST neurons are GABAergic (Cullinan, Herman et al. 1993). Thus, if NE produces an inhibitory effect on firing rate within the ventral BNST (Egli, Kash et al. 2005), the BNST's inhibitory influence on the PVN, and therefore the HPA axis maybe regulated by disinhibition from NST and VLM NA neurons (See schematic below). Additionally, collateral projections have been shown to arise from VLM neurons to both BNST and PVN (Woulfe, Hrycyshyn et al. 1988). These collaterals may disinhibit the PVN via the BNST pathway while simultaneously exciting the PVN (Fig. 5-1). Thus, the BNST DSAP lesion removes not only the "inhibitory" NA input to the BNST but also the "excitatory" NA input to the PVN, leaving intact the GABAergic input from the ventral BNST/fusiform nucleus to the PVN. This remaining GABAergic inhibition of the PVN is consistent with the marked reduction of YO-induced Fos expression in the PVN in BNST DSAP animals as seen in the present study.

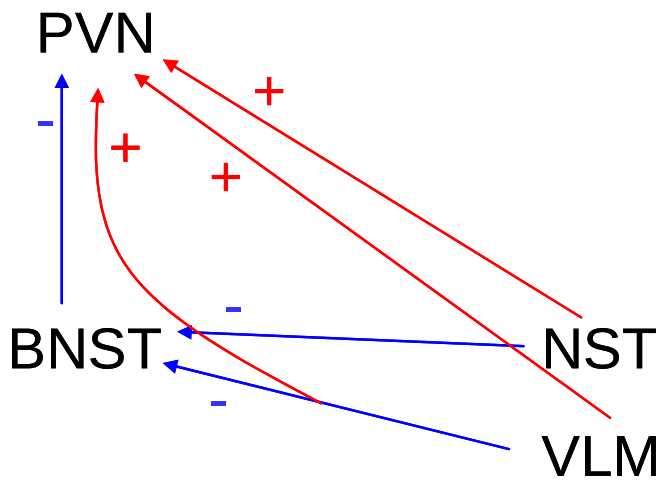

Figure 5-1 Disinhibition of PVN via NA input to BNST and excitation of PVN via direct NA input.

Our results are consistent with findings that unilateral ibotenic acid lesions of the ventral BNST reduced IL-1 beta-induced increases in Fos bilaterally in the BNST and in CRH neurons of the PVN (Crane, Buller et al. 2003). This lesion also led to a reduction in IL-1-induced increases in plasma ACTH (Crane, Buller et al. 2003). We would predict that the attenuation in Fos expression in CRH neurons of the PVN seen after YO administration in DSAP lesioned rats would also produce an attenuation of YO-induced increases in plasma ACTH.

DSAP lesions produced a significant loss of DbH-positive neurons in the NST and VLM. The loss of DbH-positive NST and VLM neurons was directly correlated with a significant attenuation of the YO-induced increase in Fos-positive NA neurons in both regions. However, YO activated similar proportions of DbH-positive NST and VLM neurons in sham and DSAP rats. This demonstrates that in DSAP rats, a majority of the remaining DbH-positive neurons are still activated by YO. This could be a result of a direct action of YO on those neurons or remaining neural pathways, i.e. descending pathways from limbic forebrain regions, such as medial CeA and dorsal BNST (VanderKooy, Koda et al. 1984).

DSAP lesions did not significantly alter YO-induced Fos expression in the CeA. Despite a slight denervation of the NA input to the CeA, the intact YO-induced Fos response in this region could be evidence that the NA projection to CeA is still functionally intact, as NA inputs to the CeA are activated by viscerosensory stimuli (Myers and Rinaman 2002). An alternative explanation is that YO-induced Fos expression in the CeA does not require NA inputs to the BNST. YO-induced Fos expression in CeA could also be mediated by GLP-1 inputs from NST or CGRP input from the PBN. DSAP lesions did not appear to alter DbH-positive terminals or YO-induced Fos expression in the PBN.

5.3. DSAP lesion effects on YO-induced anorexia

Although there is limited evidence that the BNST is involved in food intake, its descending projections to the dorsal vagal complex [area postrema (AP), NST, dorsal motor nucleus of the vagus] (Gray and Magnuson 1987) may implicate it in parasympathetic functions. Electrical stimulation of the BNST has been shown to produce increases in gastric motility and to modulate medullary taste responses (Hermann, McCann et al. 1990; Smith, Ye et al. 2005). However, our results show that the NA input to the BNST is not necessary for the anorexic effect of YO. Ritter et al. have reported that DSAP lesions of the PVN are not required for CCK-induced anorexia (Ritter, Bugarith et al. 2001). As the present study shows many of the NA neurons innervating the PVN also innervate the BNST. Additionally, it has been shown that DSAP lesions of the NST attenuate anorexia induced by CCK administration and that the number of DbH-positive neurons in the NST was positively correlated with the loss of CCK-induced anorexia (Rinaman 2003). Considered together, it is likely that local NA brainstem circuits are necessary and sufficient for anorexia induced by anxiogenic treatments. Similar to the present study, DSAP lesions of the NST produced an attenuation of the Fos response to CCK

in the PVN, but not the CeA (Rinaman 2003). Interestingly, NST DSAP lesions produced a nearly complete loss of the DbH terminals in the PBN, but CCK-induced Fos expression within PBN was not altered. An alternative source of input that may mediate stressor-related Fos expression in the PBN is the AP, a circumventricular organ implicated in the integration of viscerosensory information (Shapiro and Miselis 1985). Considered together, these data suggest that the ascending NA inputs to the BNST, the CeA or the PBN are not necessary for YO-induced anorexia and that this response may be mediated by local brainstem circuits.

5.4. Role of the BNST in Conditioned versus Unconditioned Fear Responses: Implications for DSAP Lesion Effects on Conditioned Flavor Avoidance

There is a continuing discussion in the literature regarding the involvement of the BNST in fear conditioning. Despite the similarities between the amygdala and the BNST in afferents, efferents, neuropeptide content, and HPA responses to stimulation described above, some evidence demonstrates a separation in function. Evidence to support this functional dissociation was found using two startle paradigms, fear-potentiated startle (FPS) and light-enhanced startle (LES). LES, which is an unconditioned fear paradigm, is the increased acoustic startle elicited in the presence of light, which is naturally aversive to rats. FPS, which is a conditioned fear paradigm, is the increased acoustic startle elicited in the presence of a conditioned stimulus previously paired with shock. Michael Davis and colleagues found that chemical lesions of the BNST using the AMPA receptor antagonist NBQX eliminated LES but not FPS, whereas lesions of the CeA eliminated FPS but not LES (Walker and Davis 1997). Based on these results, Davis suggested that the BNST is involved in unconditioned fear and anxiety responses, whereas the CeA is involved in conditioned fear responses (Walker and Davis 1997).

This hypothesis was supported when the same group used CRH-enhanced startle, i.e., increased acoustic startle after intracerebral administration of CRH. CRH-enhanced startle can

be considered an unconditioned paradigm in which CRH is the unconditioned stimulus. NMDA lesions of the BNST or intra-BNST infusion of CRH antagonist blocked CRH-enhanced startle. NMDA lesions of the amygdala did not block CRH-enhanced startle; however, amygdala-lesioned animals did show a blockade of FPS (Lee and Davis 1997).

Further support for this dissociation between amygdala and BNST came from studies with the unconditioned stimulus trimethylthiazoline (TMT), an odiferous component of fox feces, which elicits freezing behavior in rats. Fendt et al. found that muscimol inactivation of the BNST but not the amygdala significantly blocked TMT-induced freezing (Fendt, Endres et al. 2002). These data suggest that the BNST is involved in unlearned fear, whereas the amygdala is not.

Other evidence suggests that the separation of function between the BNST and the amygdala may not be so clear. There is evidence that the BNST is involved in some conditioned fear responses. Gray and colleagues used a conditioned stress procedure in which rats received a 1mA footshock at the end of a 10 min period in a chamber for 3 days. On the fourth day, the rats are placed in the chamber for 10 minutes and receive no shock. Thus, this procedure is a contextual conditioning procedure, in which the footshock is the unconditioned stimulus and the chamber becomes the conditioned stimulus. Rats with ibotenic acid lesions of the lateral BNST displayed an attenuated ACTH and CORT response to the conditioned stress but not to a 20 minute immobilization stress (Gray, Piechowski et al. 1993). This study suggests that the BNST is involved in contextual conditioned fear, which may be mediated by inputs from the ventral subiculum to the BNST (Cullinan, Herman et al. 1993).

A recent study by Sullivan and colleagues supports these findings. In this study, HPA and freezing responses to cue and context conditioning were examined. These paradigms

consisted of conditioning and testing phases. The conditioning phase of the cue conditioning paradigm consisted of the pairing of an auditory cue or tone with a footshock in a chamber. During the testing phase, the tone is presented in a novel chamber, distinct from the conditioning chamber in structure, scent, color, and lighting. Thus, learned behavioral or endocrine responses should be evoked by the cue, and not by the context. The context conditioning paradigm simply pairs the footshock with the chamber or context and then places the animal in the same chamber for testing. Electrolytic lesions of the CeA attenuated conditioned freezing and CORT responses to both cue and context, whereas BNST lesions attenuated freezing and CORT responses to context only (Sullivan, Apergis et al. 2004). These results suggest that the BNST is necessary for behavioral and HPA responses to contextual conditioned fear, but not to cue-conditioned fear.

Further complicating this debate, a recent study suggests that the BNST mediates both conditioned and unconditioned fear responses. Schweimer et al. found that bilateral intra-BNST injections of clonidine (which decreases neurotransmitter release from NA terminals) disrupt the acquisition and expression of both FPS and LES (Schweimer, Fendt et al. 2005). Thus, the NA input to the BNST may mediate conditioned as well as unconditioned fear responses. These authors hypothesize that there is a neurochemical rather than a neuroanatomical dissociation between conditioned fear and anxiety. This hypothesis is supported by the demonstration that local infusion of clonidine blocks TMT-induced NE release within the BNST as well as attenuates TMT-induced freezing behavior (Fendt, Siegl et al. 2005). Thus, the NA innervation of the ventral BNST may be important for behavioral expression of unlearned fear.

Thus, although the CeA's important role in conditioned learning has long been known (Walker and Davis 1997), there is evidence that the BNST is also involved in conditioned

responses which may be mediated by NA inputs (Schweimer, Fendt et al. 2005). One of the paradigms used in the present work is conditioned flavor avoidance (CFA), in which an animal avoids a flavor previously paired with a noxious stimulus. Thus, CFA is a "conditioned" or learned response. It has previously been shown that the formation of a CFA requires the PBN projection to the CeA (Lasiter and Glanzman 1985; Sakai and Yamamoto 1999). Therefore, as may have been anticipated by unaltered YO-induced Fos expression in both the PBN and the CeA in DSAP rats, the present report indicates that NA inputs to the BNST are not necessary for YO-induced CFA.

This result was not consistent with our initial hypothesis that DSAP lesions would attenuate YO-induced CFA. This hypothesis was in part based on evidence that intra-BNST NA antagonists reduced conditioned-place aversion to precipitated opiate withdrawal, suggesting that NA in the BNST promotes withdrawal-induced aversion (File 1986; Aston-Jones, Delfs et al. 1999; Delfs, Zhu et al. 2000). Evidence that YO and opiate-withdrawal both produce conditioned place aversion (File 1986) suggests a common anxiogenic or aversive effect of both stressors. Thus, we hypothesized that NA in the BNST would similarly promote YO-induced aversion and that lesioning the NA input to the BNST would attenuate this behavior.

Interestingly, intra-BNST beta antagonists did not reduce the expression of somatic signs of opiate withdrawal, such as teeth chattering, eye twitching and wet dog shakes, which may involve other NA targets (Aston-Jones, Delfs et al. 1999; Delfs, Zhu et al. 2000). Thus, it is possible that somatic effects of YO administration involve other NA targets and therefore were not attenuated by the BNST DSAP lesion. Thus, the viscerosensory feedback produced by this dose of YO lead to intact YO-induced anorexia and CFA.

5.5. DSAP Lesions Effects on YO-induced Anxiety-like Behavior on the Elevated Plus Maze

The EPMZ model is based on rats' innate fear of open, elevated arms and light. As a result, rats tend to avoid the open arms and remain in the closed arms. The EPMZ has commonly been used to examine the effects of anxiolytic drugs, which increase the number of entries into and time spent on open arms. Anxiogenic drugs tend to produce the opposite result (Cruz, Frei et al. 1994). YO has been reported to enhance anxiety-like behavior on the EPMZ in rats (Baldwin, Johnston et al. 1989; Johnston and File 1989; Khoshbouei, Cecchi et al. 2002). This effect is thought to be mediated in part by alpha 2 adrenoceptors as evidenced by its reversal with clonidine (Johnston and File 1989). Additionally, NA inputs to the BNST may modulate stress-induced anxiety like behavior on the EPMZ based on a study by Cecchi and colleagues. They reported that intra-BNST alpha 1 receptor antagonist or a cocktail of beta 1 and beta 2 antagonists blocked the stress-induced reduction in open-arm exploration on the elevated plus maze. Based on these results, we anticipated that BNST DSAP lesions would attenuate YO-induced anxiety-like behavior on the EPMZ.

In the present study, a behaviorally effective dose of YO (5 mg/kg BW, i.p.) tended to decrease open and closed arm entries in sham and DSAP rats, suggesting that YO was reducing the locomotion of these animals. This could be a result of a nauseogenic effect of YO, which has been previously reported in humans (Linden, Vellman et al. 1985). Thus, YO appears to be inducing sickness behavior on the EPMZ in sham and DSAP rats, which is reflected in the inability of the lesion to reduce the anorexic or conditioned aversion effects of YO.

YO (5 mg/kg i.p.) had no significant effects on open arm time which is consistent with evidence that rats tend to spend a majority of time in the closed arms during baseline conditions (Cruz, Frei et al. 1994). YO induced a significant increase in closed arm time on the EPMZ that

was restricted to sham controls. Thus, the DSAP lesion attenuated the increase in closed arm time after YO. However, this effect was not correlated with the number of DbH-positive neurons in the NST or VLM. Thus, in intact animals, perhaps it is not NE release in the BNST that is mediating the aversiveness of YO but the interaction of NE with co-stored peptides, which are also removed with the BNST DSAP lesion. However, the BNST DSAP lesion was not effective in significantly increasing time spent in the open arms. Additionally, the finding that YO significantly increased fecal boli in sham but not DSAP rats is consistent with a previous report in which NA antagonists injected into the BNST significantly reduced stress-induced defecation (Cecchi, Khoshbouei et al. 2002). Thus, the DSAP lesion attenuated YO-induced anxiety-like behaviors, in particular the YO-induced increase in closed arm time. However, the DSAP lesion by itself does not have an anxiolytic effect demonstrated by its inability to increase open arm time under baseline conditions or after YO.

A potential limitation with the experimental design employed in this study could be that the animals underwent two test days spaced 5 days apart in which treatments were counterbalanced. A subset of the animals who received YO on the first test day may have associated the environment of the EPMZ with that aversive experience. This association may have increased the animal's baseline anxiety-like behavior on the second test day, thus reducing the apparent effectiveness of the lesion. As such, a significant order effect of treatment was not seen in this study (data not shown) but may alter the animals' behavior in a subtle capacity.

Another potential variable is the anxiogenic nature of the maze itself. In an effort to reduce baseline anxiety in the maze and thereby promote exploration, the animals used in the present study were exposed to the maze prior to surgery. However, further studies are necessary to determine if an alternative experimental design employing the EPMZ as the anxiogenic

stimulus would show a greater effect of the lesion in attenuating anxiety-like behavior. For instance, a more extensive acclimation to the maze or enticement with a palatable food reward may increase exploration on the maze.

5.6. Summary and conclusions

As discussed above, Michael Davis' theory that the BNST is involved in unconditioned fear and anxiety responses, whereas the CeA is involved in conditioned fear responses (Walker and Davis 1997) is prevalent in the literature today and has served as a guide for many studies. Another hypothesis, posed by Schweimer and colleagues, suggests that the NA input to the BNST may mediate both conditioned and unconditioned fear responses (Schweimer, Fendt et al. 2005). The results of the present study showing that NA inputs to the BNST are necessary for YO-induced Fos expression in the BNST and PVN, and thus presumptive HPA responses, and that these inputs contribute to YO-induced enhancement of anxiety like behavior on the EPMZ support the view that the NA input to the BNST contributes to unconditioned fear and anxiety responses. Additionally, the finding that the NA input to the BNST is unnecessary for YO-induced CFA and YO-induced Fos expression in the CeA supports the view that the CeA mediates conditioned responses to stress.

There is increasing evidence that NA inputs to the BNST are involved in physiological and behavioral responses to aversive stimuli, including opiate withdrawal, immobilization stress, and TMT exposure. The present study is the first to examine the role of the NA input to the BNST in the neural and behavioral responses to the anxiogenic drug YO, using the specific immunotoxin DSAP. We conclude that the NA input to the BNST is necessary for YO-induced Fos expression in the BNST and PVN, evidenced by a marked decrease YO-induced Fos expression in DSAP rats. Despite these neural findings, the NA input to the BNST appears to be

unnecessary for YO-induced anorexia or CFA, which may be mediated by remaining neural pathways including the CeA and PBN. Interestingly, BNST DSAP lesions did attenuate the YO-induced increase in closed arm time. Thus, we conclude that the NA input to the BNST contributes to anxiogenic effects of YO on the EPMZ. Further studies are necessary to examine the role of the NA input to the BNST in response to other anxiogenic treatments.

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