ELECTROPHYSIOLOGICAL CHARACTERIZATION OF CHRONIC STRESS-INDUCED SENSITIZATION OF NORADRENERGIC NEURONS OF THE LOCUS COERULEUS

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Chronic stress exposure can produce sensitization of norepinephrine release in the terminal fields of locus coeruleus (LC) neurons. The present studies explore the potential localization and mechanism underlying the sensitized response of LC neurons in rats following chronic exposure to cold (2 weeks; 5°C).

Single unit recordings of LC neurons in halothane-anesthetized rats were used to compare the effect of intraventricular administration of corticotropin releasing hormone (CRH; 0.3-3.0μg) in control and previously cold-exposed rats. The CRH-evoked increase in LC neuron activity was enhanced following chronic cold exposure, without alteration in basal activity. The enhanced activation was only apparent at higher doses of CRH, resulting in an increased slope of the dose-response relationship for CRH in previously cold-exposed rats. It is concluded that the sensitization of CRH-evoked norepinephrine release in cold-exposed rats is accompanied by sensitization of LC neuron activity. We hypothesized that the response of LC neurons to multiple excitatory inputs is enhanced.

Using *in vitro* intracellular recordings, we subsequently examined whether CRH exerts a direct effect on LC neurons, and which ionic currents and second messenger systems are likely affected by CRH. It was demonstrated that CRH dose-dependently increases the firing rate of LC neurons through a direct (TTX-insensitive) mechanism by

decreasing a potassium conductance via adenylate cyclase and protein kinase A. The CRH-evoked activation of LC neurons is, at least in part, mediated by CRH_1 receptors.

In subsequent *in vitro* experiments using intracellular recordings, the electrophysiological properties of LC neurons were compared between control and cold-exposed rats. We observed that the excitability and input resistance of LC neurons was enhanced in slices from cold-exposed rats. In addition, the accommodation of spike firing was reduced and there was a strong trend toward a reduction of the post-activation inhibitory period. These data demonstrate that the stress-induced sensitization of LC neurons is, at least in part, maintained *in vitro* and suggest that alterations in electrophysiological properties of LC neurons contribute to the chronic stress-induced sensitization of central noradrenergic function observed *in vivo*. Furthermore, these data suggest that an alteration in auto-inhibitory control of LC activity is involved in chronic stress-induced alterations.

PREFACE

Organization of the thesis

The present thesis is comprised of a general introductory chapter, followed by three chapters each representing a manuscript, and a concluding chapter with a general discussion. The three manuscripts included in this thesis are listed below:

Chapter 2: Jedema HP, Finlay JM, Sved AF, Grace AA (2001) Chronic cold exposure potentiates CRH-evoked increases in electrophysiologic activity of locus coeruleus neurons. *Biological Psychiatry* 49:351-359.

Chapter 3: Jedema HP, Grace AA (*Submitted*) The effect of corticotropin-releasing hormone on noradrenergic neurons of the locus coeruleus recorded *in vitro*.

Chapter 4: Jedema HP, Grace AA (*In Press*) Chronic exposure to cold stress alters electrophysiological properties of locus coeruleus neurons recorded *in vitro*. *Neuropsychopharmacology*.

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To Nic and Holly.

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LIST OF ABBREVIATIONS

aCSF	artificial cerebrospinal fluid
ACTH	adrenocorticotropin hormone
ADC	adenylate cyclase
ADHD	attention-deficit hyperactivity disorder
AHP	afterhyperpolarization
cAMP	cyclic adenosine mono-phosphate
CeA	central nucleus of amygdala
CNS	central nervous system
CRH	corticotropin releasing hormone
CSF	cerebrospinal fluid
DβH	dopamine-β-hydroxylase
ENK	enkephalin
FR	firing rate
GABA	γ-amino-butyric acid
GAL	galanin
GC	glucocorticoid
GIRK	G-protein-coupled inward rectifying potassium channel
HPA	hypothalamus-pituitary-adrenal
ICV	intracerebroventricular
ISI	inter-spike interval
LC	locus coeruleus
mPFC	medial prefrontal cortex
NE	norepinephrine
NPY	neuropeptide Y
OCD	obsessive compulsive disorder
OX	orexin
PAI	post activation inhibition
PGi	nucleus paragigantocellularis
PKA	protein kinase A
PrH	nucleus prepositus hypoglossi
PTSD	post-traumatic stress disorder
SOM	somatostatin
SP	substance P
TH	tyrosine hydroxylase
TTX	tetrodotoxin
VIP	vaso-active intestinal peptide

1 GENERAL INTRODUCTION

1.1 Clinical significance of norepinephrine and stress: a brief history.

As an element of the autonomic nervous system, norepinephrine (NE), originally referred to as "adrenin", has long been recognized as a critical neurotransmitter in the response to stress (Cannon, 1939). In subsequent studies NE was found in the central nervous system (CNS), although it was first hypothesized that "sympathin" originated from cerebral vasomotor nerves (Von Euler, 1946). In 1954, Marthe Vogt first hypothesized a role for NE independent of the sympathetic vasomotor nerves (Vogt, 1954). Her hypothesis was based on the uneven distribution of sympathin in the brain, the drug-induced depletion of sympathin from enriched areas in the brain, and the dissociation between drug-induced depletions in the adrenal gland and hypothalamus. In subsequent work, it has been found that the NE system in the CNS was exquisitely sensitive to stress exposure (Stanford, 1993; Zigmond et al., 1995) and reviewed in section 1.5). Based on clinical findings of abnormalities in NE in the human CNS, central NE has been implicated in mood and anxiety disorders (Charney et al., 1995). Furthermore, classical as well as newer antidepressants exert their effect, at least in part, by modulation of noradrenergic activity in the CNS, providing additional support for noradrenergic dysfunction in the pathophysiology of mood and anxiety disorders (Grant and Weiss, 2001). It has been noted that stress exposure can precipitate or exacerbate symptoms of mood and anxiety disorders. This interaction of CNS NE with mood and anxiety disorders and stress exposure has driven the research on NE that started in the 1960's and continues today. Given the alterations in central NE system following chronic

stress exposure, many chronic protocols have been studied in the hope of elucidating their mechanism and their potential relevance for pathological changes in mood and anxiety disorders. Interestingly, following chronic stress exposure behavioral alterations have been reported which are reminiscent of the alterations observed in patients afflicted with mood and anxiety disorders. Moreover, behavioral alterations evoked by chronic stress coincide with alterations in central noradrenergic function. Therefore, in the present work, I will further examine alterations in the central noradrenergic system following chronic stress exposure.

1.2 Anatomy of the locus coeruleus system

Since the initial demonstration of formaldehyde vapor-induced fluorescence cell groups in the brain stem and midbrain (Dahlstrom and Fuxe, 1964), the locus coeruleus (LC) has been intensely studied as the largest norepinephrine (NE) containing cell group in the central nervous system (Moore and Bloom, 1979; Pfister and Danner, 1980; Foote et al., 1983; Moore and Card, 1984). The LC proper in the rat, located at the lateral edge of the floor of the fourth ventricle, contains the densely-packed somata of approximately 1600 neurons per hemisphere (Swanson, 1976). Cytoarchitectonically, the LC can be divided in a dorsal and ventral division, with the largest number of predominantly fusiform neurons densely-packed in the dorsal LC and approximately 200 neurons in the ventral division (Swanson, 1976). Based on morphological heterogeneity among somata, LC neurons can also be divided in at least two groups distinguished by their dendritic orientations (Shimizu and Imamoto, 1970; Swanson, 1976; Pfister and Danner, 1980; Cintra et al., 1982).

LC neurons send projections throughout the entire neuraxis (Foote et al., 1983). Unmyelinated axon collaterals of individual neurons project to functionally and architectonically distinct brain regions such as the thalamus, hypothalamus, amygdala, hippocampus, cerebellum, and the entire cortical mantle (Moore and Bloom, 1979; Foote et al., 1983; Moore and Card, 1984), making the LC the most widely projecting cell group in the central nervous system (Aston-Jones et al., 1995). Despite the relatively low number of neurons, the LC provides the source of approximately two third of all NE in the central nervous system and it is the sole source of NE in the cortex and hippocampus (Nakamura and Iwama, 1975; Levitt and Moore, 1978; Ader et al., 1980; Loy et al., 1980; Nagai et al., 1981a, b; Room et al., 1981; Loughlin et al., 1982). Despite their highly divergent projections there is a topographical organization of LC neurons within the LC (Loughlin et al., 1986a; Loughlin et al., 1986b; Grzanna and Fritschy, 1991). Neurons in the ventral LC predominantly project to the spinal cord, while LC efferents to the hippocampus are located in the dorsal LC. The neocortical projections of LC neurons are also topographically organized (Waterhouse et al., 1983). Originally the noradrenergic innervation of the cortex was reported to be largely non-synaptic or "en passage" (Beaudet and Descarries, 1978; Seguela et al., 1990), leading to the proposal that noradrenergic neurotransmission mainly occurred in a "volume transmission" mode (Zoli et al., 1998). However, more recent ultrastructural data generated from serial sections demonstrate that the noradrenergic afferents to the cortex , cerebellum, and subcortical structures form (small) synaptic contacts (Olschowka et al., 1981; Papadopoulos et al., 1989; Parnavelas and Papadopoulos, 1989; Papadopoulos and Parnavelas, 1990; Paspalas and Papadopoulos, 1999).

In contrast to the widespread and diffuse character of LC projections, LC afferents were previously thought to be restricted to input from the brainstem nuclei paragigantocellularis (PGi) and prepositus hypoglossi (Aston-Jones et al., 1986; Aston-Jones et al., 1991b). However, Golgi and dopamine-ß-hydroxylase (DßH) studies had demonstrated that LC dendrites extend far outside of the nucleus proper (Swanson, 1976; Grzanna and Molliver, 1980; Pfister and Danner, 1980; Cintra et al., 1982; Shipley et al., 1996). Similarly, we found fluorescent processes extending as far as 1 mm away from individual LC neurons filled with Lucifer Yellow (figure 1), which could be traced well into the pericoerulear area (Jedema and Grace, 1999). Consequently, more recent studies have demonstrated additional afferent input to these extranuclear dendrites (Luppi et al., 1995; Zhu and Aston-Jones, 1996; Van Bockstaele et al., 1999; Aston-Jones et al., 2001), which is consistent with pioneering studies by Cederbaum and Aghajanian (Cedarbaum and Aghajanian, 1978a). The trans-synaptic tracing techniques that have recently become available should provide a very useful tool for obtaining additional insight in afferents to extranuclear LC dendrites (Aston-Jones and Card, 2000).

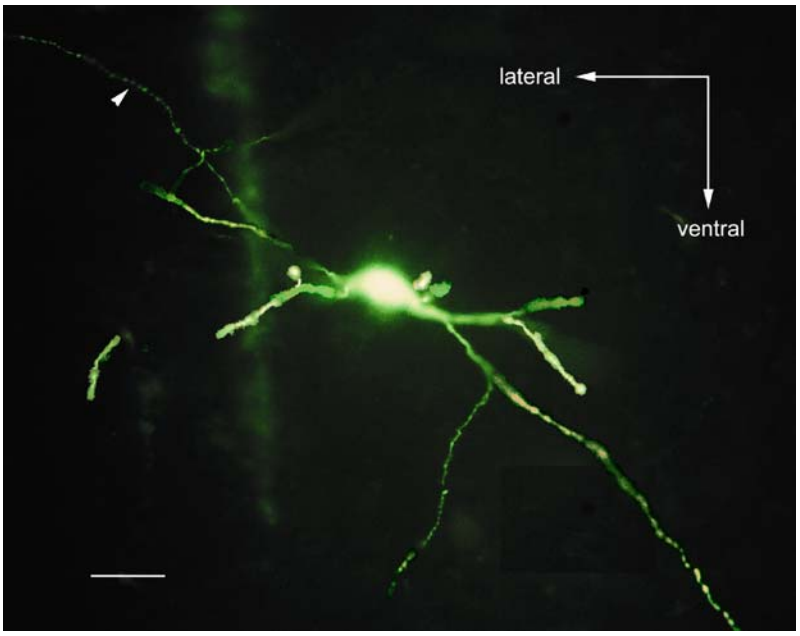

Figure 1 LC neuron filled with Lucifer Yellow following *in vivo* intracellular recording

Montage of LC neuron filled with Lucifer Yellow. Arrow indicates presumed axon. Note varicose axon and dendrites. Processes originating from both poles of the fusiform-shaped cell body extended as far as 960 µm away. Scale bar = 30 µm.

1.3 Neurochemistry

NE is synthesized from the amino acid tyrosine (Udenfriend, 1966). DßH and the rate limiting enzyme tyrosine hydroxylase (TH) are essential biosynthetic enzymes specific for the synthesis of (nor)epinephrine and catecholamines, respectively, and the presence of these enzymes is often used as a marker for the noradrenergic phenotype of LC neurons. All neurons in the LC of the rat contain enzymes unique to the NE biosynthesis and are therefore presumed to be noradrenergic (Swanson, 1976; Grzanna and Molliver, 1980), although the rostral pole of the LC consists of a more heterogeneous population of neurons which includes non-noradrenergic neurons. The LC of primate and rabbit is similarly homogeneous to that of the rat (Hubbard and Di Carlo, 1973; Freedman et al., 1975; Redmond, 1987; Caffe, 1994), while in the cat, guinea pig, and opossum noradrenergic neurons are interdigitated with non-noradrenergic neurons (Leger and Hernandez-Nicaise, 1980; Martin et al., 1982; Foote et al., 1983; Jones and Beaudet, 1987). In addition to NE, many neurons in the rat LC colocalize the neuropeptides galanin (GAL) and/or neuropeptideY (Melander et al., 1986; Holets et al., 1988; Austin et al., 1990). Slightly different patterns of co localization have been described in the cat (Fung et al., 1994).

The adrenergic receptors which mediate the effect of noradrenergic neurotransmission, can be categorized in two major classes (α,β) which can be further divided into multiple sub-classifications (Bylund et al., 1994; Aantaa et al., 1995; Wozniak et al., 1995). All adrenergic receptors are metabotropic receptors, with individual receptor subtypes using different second messenger systems (Duman and Nestler, 1995; Wozniak et al., 1995). LC neurons express only mRNA for α_2 receptors,

which exert an important role on LC neurons as autoreceptors (Nicholas et al., 1993). Ultrastructurally, α_{2A} and α_{2C} receptors have been localized to noradrenergic terminals as well as dendrites (Lee et al., 1998a, b). Physiological experiments also support an important role for α_2 receptors in LC neurons, whereas α_1- or β-receptor stimulation does not affect LC neuron activity (Aghajanian et al., 1977; Cedarbaum and Aghajanian, 1978b; Aghajanian and VanderMaelen, 1982; Williams et al., 1985). Stimulation of α_2 receptors causes a G-protein-mediated hyperpolarization of LC neurons through the opening of potassium channels and the reduction of NE release (Aghajanian and VanderMaelen, 1982; Williams et al., 1985; Abercrombie et al., 1992). Even though α_2 receptor stimulation decreases adenylate cyclase (ADC) activity (Beitner et al., 1989), the opening of potassium channels involves a direct action of the G-protein on the channel and does not involve diffusible second messengers (Grigg et al., 1996; Arima et al., 1998). Once NE is released it can be taken up by the NE transporter (NET), which plays an important role in the clearance of NE from the extracellular space (Barker and Blakely, 1995; Blakely and Bauman, 2000).

1.4 Electrophysiology

Despite the morphological distinctions between subclasses of LC neurons, their electrophysiological features appear quite homogeneous. Both in *in vivo* and *in vitro* preparations, LC neurons exhibit a characteristic tonic pattern of spontaneous activity (~1-3 Hz) with action potentials exhibiting long duration action potentials followed by a prominent afterhyperpolarization (AHP) (Aghajanian and VanderMaelen, 1982; Williams et al., 1984). Electrotonic coupling among neurons is thought to contribute to the

synchronization of spontaneous activity between LC neurons although the prevalence of electrotonic coupling in the LC of adult rats is unclear (Christie et al., 1989; Christie and Jelinek, 1993; Ishimatsu and Williams, 1996; Usher et al., 1999). The pacemaker activity of LC neurons is likely an intrinsic property because the spontaneous activity persists in *in vitro* slice preparations and in acutely dissociated neurons (Williams et al., 1984; Arima et al., 1998). Furthermore, blockade of synaptic activity by tetrodotoxin (TTX) does not disrupt the spontaneous discharge of calcium-spikes in these neurons (Williams et al., 1984). In anesthetized subjects, LC neurons are mainly activated by noxious stimuli (Cedarbaum and Aghajanian, 1976) and the stimulation-evoked excitation of LC neurons is followed by a period of quiescence, or post-activation inhibition (PAI), which has been attributed to the activation of calcium-activated potassium channels and autoreceptor-mediated collateral inhibition (Cedarbaum and Aghajanian, 1978b; Andrade and Aghajanian, 1984; Ennis and Aston-Jones, 1986; Osmanovic and Shefner, 1993). The electrophysiological activity of LC neurons is influenced by a multitude of neurotransmitter systems which is consistent with a more diverse input to LC neurons than was once hypothesized (Aston-Jones et al., 1986). Thus, glutamate (Ivanov and Aston-Jones, 1995), substance P (SP) (Koyano et al., 1993), vaso-active intestinal peptide (VIP) (Wang and Aghajanian, 1990), acetylcholine (ACH) (Egan and North, 1986), and orexin (OX) (Horvath et al., 1999; Ivanov and Aston-Jones, 2000) depolarize or increase the firing rate (FR) of LC neurons, whereas (nor)epinephrine (Williams et al., 1985), γ-amino-butyric acid (GABA) (Shefner and Osmanovic, 1991), enkephalin (ENK) (Williams and North, 1984), somatostatin (SOM) (Inoue et al., 1988), NPY (Illes and Regenold, 1990), and GAL (Pieribone et al., 1995) hyperpolarize or decrease LC FR. A

physiological role for these neurotransmitters is hypothesized based on immunoreactivity for these transmitters in LC afferents and the presence of appropriate receptors within the LC (Aston-Jones et al., 1995).

The net effect of NE on post-synaptic targets is often referred to as neuromodulatory, resulting in an increased signal-to-noise ratio of other afferents inputs (Woodward et al., 1991; Waterhouse et al., 1998b). For example, at doses lower than those necessary to directly influence the firing rate of the post-synaptic neuron, NE potentiates the effect of GABA on Purkinje cells in the cerebellum (Moises et al., 1983; Sessler et al., 1989), increases the glutamate-or current-evoked excitability of spinal motoneurons (White et al., 1991), and hippocampal (Harley, 1991), and cortical pyramidal neurons (Waterhouse et al., 1998a). The proposed neuromodulatory role for NE is further supported by the wide spread and divergent projections of the LC system, the slow axonal conduction velocity of LC neurons (Aston-Jones et al., 1985), and the fact that adrenergic receptors exert their effect via metabotropic signaling cascades. In awake rats, cats, and primates, the activity of LC neurons varies with the behavioral state of the animal (Aston-Jones and Bloom, 1981b; Rasmussen et al., 1986; Grant et al., 1988). LC neurons respond to multimodal sensory stimuli (Foote et al., 1980; Aston-Jones and Bloom, 1981a; Valentino and Foote, 1988) and the stimulus-evoked activation of LC FR is most profound in cases where the stimulus disrupts ongoing behavior and elicits an orienting response (Aston-Jones and Bloom, 1981a; Grant et al., 1988). Based on these observations a role for LC neurons in attention and arousal has been hypothesized (Cole and Robbins, 1992; Robbins, 1997; Aston-Jones et al., 2000; Berridge, 2001).

1.5 NE system and the response to stress

Numerous studies have demonstrated the excitatory effect of acute stress exposure on central noradrenergic function (for review see (Stanford, 1995; Zigmond et al., 1995). For example, in response to acute exposure to stressors, Fos expression in LC neurons is increased (Ceccatelli et al., 1989; Pezzone et al., 1993; Passerin et al., 2000). In addition, following acute exposure, NE tissue levels are decreased in multiple brain regions, presumably as a result of an enhanced release of NE and an increased NE turnover, which is defined as the ratio of NE metabolites and NE (Maynert and Levi, 1964; Thierry et al., 1968; Zigmond and Harvey, 1970; Korf et al., 1973; Kvetnansky et al., 1977; Nakagawa et al., 1981; Roth et al., 1982; Irwin et al., 1986). Furthermore, NE efflux as measured with *in vivo* microdialysis, is increased in response to acute stress exposure (Abercrombie et al., 1988; Rossetti et al., 1990; Cenci et al., 1992; Nakane et al., 1994). Electrophysiological experiments in awake cats and primates have demonstrated that noxious or stressful stimuli are most effective in increasing LC FR (Rasmussen and Jacobs, 1986; Abercrombie and Jacobs, 1987; Grant et al., 1988; Aston-Jones et al., 1991a). Finally, acute stress exposure increases the capacity for NE synthesis of LC neurons by increasing the amount or activity of essential enzymes for NE biosynthesis (Nisenbaum and Abercrombie, 1992; Serova et al., 1999; Chang et al., 2000). In addition to LC activation elicited by stressful stimuli, some behavioral responses to stress exposure can be mimicked by electrical stimulation of the LC, while lesions of the LC prevent emotional responses to threats in non-human primates (Redmond, 1987), further implicating a role for the LC in an organism's behavioral response to stress exposure.

Even though from the literature described above it is clear that a variety of stressful stimuli increases the LC FR and NE release and synthesis, more complex alterations of

noradrenergic function occur following repeated or prolonged exposure to stressors (for review see (Abercrombie et al., 1992; Stanford, 1993; Stanford, 1995; Zigmond et al., 1995). Thus, chronic exposure to cold or repeated exposure to foot shock, tail shock, or restraint does not decrease and often increases baseline tissue NE levels (Ingenito and Bonnycastle, 1967; Thierry et al., 1968; Bhagat, 1969; Adell et al., 1988), although the baseline extracellular levels of NE as measured by microdialysis are unaltered following chronic exposure to some of these stressors (Nisenbaum et al., 1991; Gresch et al., 1994; Terrazzino et al., 1995). The foot shock-evoked depletion of tissue NE levels observed in control rats does not occur following repeated foot shock exposure (Zigmond and Harvey, 1970; Irwin et al., 1986). Furthermore, the NE turnover in hypothalamus, hippocampus, and brainstem-mesencephalon is enhanced following repeated exposure to foot shock (Thierry et al., 1968; Irwin et al., 1986; Anisman and Zacharko, 1990) and NE tissue levels are further depleted by exposure to a novel stressor following repeated restraint (Adell et al., 1988). More recently, microdialysis studies have extended these results by demonstrating that NE efflux is enhanced following chronic stress exposure (Nisenbaum et al., 1991; Pacak et al., 1992; Gresch et al., 1994). It has been suggested the alterations in NE tissue levels, NE turnover, and efflux are at least in part a consequence of alterations in the capacity to synthesize NE, because the amount or activity of TH and DßH is increased following repeated or chronic stress exposure (Gordon et al., 1966; Thoenen, 1970; Zigmond and Harvey, 1970; Melia and Duman, 1991; Melia et al., 1992; Serova et al., 1999). Furthermore, the increase in TH mRNA or tyrosine hydroxylation in response to exposure to an (novel) acute stressor is enhanced

following repeated stress exposure (Nisenbaum et al., 1991; Nisenbaum and Abercrombie, 1992; Rusnak et al., 2001).

Repeated or chronic stress exposure has been reported to enhance the basal FR of LC neurons in some studies (Pavcovich et al., 1990; Mana and Grace, 1997), while others report no significant change of basal FR (Simson and Weiss, 1988; Curtis et al., 1995). Even though these discrepancies could be attributed to differences in methodology or differences in type, duration, frequency, or intensity of stress exposure, more consistent results have been obtained examining the effect of evoked LC FR following the exposure to these different chronic stress paradigms. Thus, prior exposure to tail shock, foot shock, and cold enhance the evoked increase in LC FR (Simson and Weiss, 1988; Curtis et al., 1995; Mana and Grace, 1997).

1.6 CRH and response to stress

The neuropeptide corticotropin releasing hormone (CRH) also plays an important role during stress-exposure. First, CRH is the primary activator of adrenocorticotropin hormone (ACTH) release from the anterior pituitary resulting in activation of the hypothalamus-pituitary-adrenal (HPA)-axis during stress-exposure (Vale et al., 1981). In addition, CRH is localized to many areas outside the hypothalamus (Swanson et al., 1983; Sakanaka et al., 1987), where it also plays an important role in an organism's response to stress exposure (Owens and Nemeroff, 1991; Heinrichs et al., 1995). The LC in particular is an important extrahypothalamic site of action of CRH (Valentino et al., 1993; Valentino et al., 1998), and local injection of CRH into the LC results in alterations in behavior similar to those elicited by stress exposure (Butler et al., 1990). The LC

receives CRH immunoreactive afferents from the dorsal cap of the hypothalamus, the central nucleus of the amygdala (CeA), the PGi, and Barrington's nucleus (Valentino et al., 1992; Van Bockstaele et al., 2001). Despite co-localization of catecholamines with multiple neuropeptides, CRH does not colocalize with TH in LC neurons (Valentino et al., 1992). Intraventricular (ICV) or intra-coerulear CRH administration potently increases the FR of LC neurons and NE release in terminal regions (Valentino et al., 1983; Valentino and Foote, 1988; Emoto et al., 1993a; Smagin et al., 1995; Curtis et al., 1997; Finlay et al., 1997). Following cold exposure and adverse early life experiences CRH levels in the LC increase (Chappell et al., 1986; Ladd et al., 2000). Furthermore, several studies have demonstrated an essential role of CRH in the activation of LC neurons in response to hypovolemia (Page et al., 1993), colon distention (Lechner et al., 1997), restraint (Emoto et al., 1993b; Smagin et al., 1996; Smagin et al., 1997), and cold exposure (Melia and Duman, 1991).

1.7 Cold exposure as a stressor

Even though exposure to a variety of stressors can cause alterations in central noradrenergic function, it is clear that exposure to different stressors results in stressor-specific response patterns (Herman and Cullinan, 1997; Pacak and Palkovits, 2001). Therefore, different stressors are not necessarily interchangeable (Jedema et al., 1999; Dayas et al., 2001). Because this work will focus on the effects of chronic cold exposure, the impact of chronic cold exposure on multiple physiological parameters including central noradrenergic function is discussed in more detail.

Prolonged exposure to cold has been used as a laboratory stressor for decades (Cannon, 1939; Maynert and Levi, 1964). Even though some of the early physiological experiments were performed in cats, the majority of the data on cold exposure as a stressful stimulus has been obtained in rats. Acute exposure to cold causes multiple alterations in physiology, some of which return to baseline following prolonged exposure. For example, cold exposure initially reduces weight gain, however, following approximately one week of exposure to cold, weight gain returns to levels similar to control rats (Folk Jr., 1974). In addition, cold exposure increases plasma NE levels although NE levels appear to decline with continued exposure (Benedict et al., 1979). It is not clear whether plasma NE levels remain elevated with prolonged exposure, although this may be hypothesized based on the increased capacity for biosynthesis of NE in the adrenal following chronic cold exposure (Fluharty et al., 1983; Stachowiak et al., 1986; Baruchin et al., 1990), and the increased urinary NE-metabolite levels (Leduc, 1961; Ostman-Smith, 1979). Additional alterations that persist following chronic cold exposure include increased basal and evoked adrenocorticotropin hormone (ACTH) levels (Vernikos et al., 1982; Uehara et al., 1989) as well as increased plasma and pituitary prolactin levels (Jobin et al., 1975). In addition, chronic cold exposure increases basal and evoked plasma corticosterone and causes adrenal hypertrophy (Daniels-Severs et al., 1973; Vernikos et al., 1982).

Changes in the central nervous system that have been reported following chronic cold exposure include an increased basal and evoked firing rate of the basolateral nucleus of the amygdala, a reduction in evoked firing rate of the central nucleus of the amygdala, and a reduction of the number of spontaneously active dopamine neurons in VTA in

combination with increased burst firing of the dopamine neurons that continue to be spontaneously active (Correll et al., 2001; Moore et al., 2001; Correll et al., 2002). Examination of the interactions of these cold-induced alterations with those in the central noradrenergic system (described below) has only recently been started (Ramsooksingh et al., 2001).

1.8 Sensitization of LC neurons following chronic cold exposure

LC neurons do not exhibit an immediate strong Fos-activation in response to cold exposure as they do in response to noxious stimuli (Miyata et al., 1995; Baffi and Palkovits, 2000; Passerin et al., 2000). Nevertheless, some investigators demonstrate a modest increase in Fos in LC following acute or chronic cold exposure, while others do not (Miyata et al., 1995; Baffi and Palkovits, 2000). An enhanced increase in evoked NE efflux in the hippocampus and medial prefrontal cortex (mPFC) and LC spike firing have been demonstrated following chronic cold exposure (Nisenbaum et al., 1991; Abercrombie et al., 1992; Gresch et al., 1994; Finlay et al., 1997; Mana and Grace, 1997; Jedema et al., 1999; Ramsooksingh et al., 2001). We have referred to these alterations following chronic cold exposure as stress-evoked sensitization of LC neurons. The development of stress-evoked sensitization is characterized by an enhanced activation of LC neurons in response to tail shock, sciatic nerve stimulation, or ICV CRH (Nisenbaum et al., 1991; Abercrombie et al., 1992; Gresch et al., 1994; Finlay et al., 1997; Mana and Grace, 1997; Jedema et al., 1999; Ramsooksingh et al., 2001). In contrast, baseline levels of NE release or spike firing of LC neurons are minimally affected in cold-exposed rats.

Consistent with the enhanced activity of LC neurons following chronic cold exposure, levels of TH mRNA and TH activity are increased in the LC (Gordon et al., 1966; Thoenen, 1970; Zigmond et al., 1974; Richard et al., 1988; Seiple et al., 1997) and the increase in tyrosine hydroxylation evoked by exposure to a novel stressor is enhanced (Nisenbaum and Abercrombie, 1992).

The increase of NE efflux in the hippocampus in response to hippocampal elevations in potassium concentrations was also reported to be enhanced following chronic cold exposure. Based on these data it was hypothesized that the sensitization of NE efflux was a terminal phenomenon (Nisenbaum and Abercrombie, 1993). This was further supported by the demonstration that the response to hippocampal infusion of the autoreceptor agonist, clonidine was augmented (Nisenbaum and Abercrombie, 1993). However, subsequent studies found no enhancement of NE efflux in response to local potassium elevations or local infusion of amphetamine (Finlay et al., 1997). In addition, an enhanced increase in NE efflux evoked by ICV administration of CRH was found, suggesting that the sensitization of NE efflux was not just a terminal phenomenon (Finlay et al., 1997). Consistent with this hypothesis, it has been found that the increase of LC firing rate in response to sciatic nerve stimulation is enhanced following chronic cold exposure (Mana and Grace, 1997). Therefore, I will continue to refer to the alterations observed following chronic cold exposure as sensitization of LC neurons, which will include sensitization of NE release.

Two weeks of cold exposure is necessary to elicit an enhanced NE efflux in response to subsequent tail shock exposure, and longer exposure to cold does not lead to further enhancement of the sensitization (Finlay et al., 1997). Furthermore, the

sensitization of NE efflux is treatment-dependent, since one week of cold exposure followed by 2 weeks at room temperature does not result in sensitization of NE efflux (Finlay et al., 1997). Similar to these neurochemical data, the increase in spike firing of LC neurons evoked by sciatic nerve stimulation was enhanced following 2weeks, but not 1 week, of cold exposure (Mana and Grace, 1997). Finally, the sensitization of LC neurons is a lasting phenomenon because the enhanced LC spike firing in response to sciatic nerve stimulation (Mana and Grace, 1997; Ramsooksingh et al., 2001) persists for at least 10 days following the discontinuation of 2 weeks of cold exposure (Ramsooksingh et al., 2002).

1.9 Research objectives

In the present work, I sought to further examine some fundamental questions regarding the stress-induced sensitization of LC neurons. Studies were designed to explore the potential localization and potential mechanisms underlying sensitization of NE release in chronically stressed rats along the following specific aims:

1] To determine the effect of chronic cold-exposure on electrophysiological activation of LC neurons in response to ICV CRH.

Based on the neurochemical evidence of sensitized NE release in response to CRH and the hypothesis that sensitization of NE release is not simply a terminal phenomenon (Finlay et al., 1997), the electrophysiological response to different doses of CRH was determined in control and previously cold-exposed rats. It was hypothesized that the

sensitization of NE release in response to CRH following chronic cold exposure would be accompanied by an enhanced increase of electrophysiological activity of LC neurons.

2] To determine potential mechanism of CRH activation of LC neurons *in vitro*

2A] To determine whether CRH activates LC neurons directly or via presynaptic modulation of afferent input

Based on the ultrastructural evidence indicating that CRH-immunoreactive (ir) terminals make synaptic appositions with TH-ir dendrites as well as non-TH-ir terminals in the pericoerulear area, the effect of CRH on LC neurons was characterized in brain slices containing the LC using intracellular recordings. In addition, the persistence of any CRH-evoked effects on LC neurons was examined following the blockade of synaptic activity using TTX. It was hypothesized that CRH would activate LC neurons *in vitro* via a direct (TTX-insensitive) mechanism.

2B] To determine potential pathways mediating the CRH-evoked activation of LC neurons.

Using inhibitors of the adenylate cyclase (ADC)-cAMP-protein kinase A (PKA)-signaling cascade, it was examined whether specific blockade of steps in this second messenger cascade could prevent the CRH-evoked effect on LC neurons. It was hypothesized that the CRH-evoked activation of LC neurons would be mediated by a second messenger mechanism involving ADC and PKA.

3] To determine the effect of chronic cold-exposure on the activation of LC neurons by excitatory inputs *in vitro*.

Based on the enhanced activation of LC neurons in response to multiple excitatory inputs, the persistence of stress-induced sensitization of NE neurons was examined in the *in vitro* slice preparation. It was hypothesized that the stress-induced sensitization of LC neurons of LC neurons would persist *in vitro*.

2 CHRONIC COLD EXPOSURE POTENTIATES CRH-EVOKED INCREASES IN ELECTROPHYSIOLOGICAL ACTIVITY OF LOCUS COERULEUS NEURONS.

2.1 INTRODUCTION

Dysfunction of noradrenergic neurons of the LC has long been implicated in the pathophysiology of psychiatric disorders such as anxiety, panic disorder and depression (Aston-Jones et al., 1994; Asnis and Van Praag, 1995; Bremner et al., 1996; Anand and Charney, 1997; Wong et al., 2000). In support of this view, studies of patients afflicted with these disorders have demonstrated increased urinary, plasma, or cerebrospinal fluid (CSF) levels of NE or its primary metabolite (reviewed by (Charney et al., 1995; Schatzberg and Schildkraut, 1995), and a blunted growth hormone and plasma catechol response to the adrenergic agonist, clonidine (Matussek et al., 1980; Matussek and Laakmann, 1981; Nutt, 1989; Charney et al., 1992). In addition, recent imaging studies suggest that patients with post-traumatic stress disorder (PTSD) exhibit an enhanced activation in prefrontal, temporal, parietal, and orbitofrontal cortices in response to a challenge administration with the adrenergic antagonist, yohimbine (Bremner et al., 1997). The involvement of noradrenergic systems in anxiety and depression is further supported by the efficacy of NE uptake inhibitors as antidepressant drugs for the treatment of these psychiatric disorders (Barker and Blakely, 1995).

Acute exposure to a stressor transiently increases the discharge rate of noradrenergic neurons (Abercrombie and Jacobs, 1987), resulting in an increase in the release of NE (Abercrombie et al., 1988). Furthermore, chronic exposure to stress can alter the response of LC neurons to subsequent presentations of a stressor (for review see (Stanford, 1993; Zigmond et al., 1995). For example, we found that rats previously

exposed to cold (5°C) for 2-4 weeks exhibit a greater increase in extracellular NE in hippocampus and mPFC in response to acute tail shock (Nisenbaum et al., 1991; Gresch et al., 1994; Finlay et al., 1997; Jedema et al., 1999), as well as greater activation of LC neuron discharge rate in response to sciatic nerve stimulation (Mana and Grace, 1997). We have referred to this phenomenon as stress-induced sensitization of NE neurons.

Previous exposure to chronic cold also enhanced the increase of NE release in response to ICV administration of CRH (Finlay et al., 1997). CRH, a neuropeptide first discovered to function in the pituitary as the primary initiator of ACTH release (Vale et al., 1981; Rivier et al., 1983), also exists in many extrahypothalamic structures in the brain including the LC (Swanson et al., 1983; Owens and Nemeroff, 1991; Morin et al., 1999). In the LC and peri-coerulear area, CRH immunoreactive terminals contact TH immunoreactive dendrites (Van Bockstaele et al., 1996a, 1998a; Van Bockstaele et al., 1999). CRH administered either ICV or locally into the LC increases the electrophysiological activity of LC neurons and the release of NE from nerve terminals (Valentino et al., 1983; Smagin et al., 1995; Curtis et al., 1997; Finlay et al., 1997; Page and Abercrombie, 1999). CRH is known to play a role in the response to stress (for review see (Dunn and Berridge, 1990; Valentino et al., 1993; Koob, 1999). It has been demonstrated that CRH levels in the LC increase during stressful events (Chappell et al., 1986). In addition, the increase in electrophysiological activity of LC neurons in response to some stressful stimuli can be prevented by pre-treatment with CRH antagonists (Valentino et al., 1991; Page et al., 1993; Smagin et al., 1997). Prior exposure to stress can also alter CRH-evoked changes in electrophysiological activity of LC neurons (Curtis

et al., 1995; Conti and Foote, 1996; Curtis et al., 1999), suggesting that CRH also may play a role in the adaptation of LC neurons to chronic or repeated stress exposure.

In the present study we examined whether ICV CRH elicits an enhanced activation of LC neuron electrophysiological activity in rats previously exposed to cold. We used single unit extracellular recording to compare the increase in electrophysiological activity produced in LC neurons in response to different doses of CRH in both control and previously cold-exposed rats.

2.2 METHODS AND MATERIALS

2.2.1 Animals

Upon arrival, male Sprague-Dawley rats (Hilltop, Scottdale, PA) weighing 150-175 g were singly housed in hanging stainless steel cages in a colony room maintained at an ambient temperature of 23°C. Throughout the experiments, lights were maintained on a 12 hr light/dark cycle (lights on at 08.00 a.m.), with food (Laboratory rodent diet 5001, PMI Feeds, St. Louis, MO) and water available *ad libitum*. All rats were housed in the colony room for 5-10 days prior to any treatment. All protocols were approved by the Animal Care and Use Committee at the University of Pittsburgh and were in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals*.

2.2.2 Cold exposure

Rats were randomly assigned to a control or a cold-exposed group (n=25 and 23, respectively). Control rats were housed in the same colony room where they had been housed previously, for an additional 14 days. Given that the effects of chronic cold

exposure on adrenal TH are more profound in shaved than unshaved rats (Fluharty et al., 1983), the body fur of rats in the cold-exposed group was shaved from the rump to the forelimbs, immediately prior to cold exposure. These rats were singly housed in hanging stainless steel cages in a cold room maintained at an ambient temperature of 5°C, where they remained undisturbed for 14 days. Cold-exposed rats appeared healthy and they continued to eat and increase their body weight. Nevertheless, cold-exposed rats gained weight at a reduced rate, such that at the time of electrophysiological recording a slight but significant reduction in body weight of cold-exposed rats was observed compared to control rats (302 $\pm$ 6 vs 333 $\pm$ 5 g respectively; $t(46)$=-4.280, $p<0.001$). Rats were removed from the cold room the afternoon prior to the experiment, and housed overnight in a colony room maintained at an ambient temperature of 23°C, thereby maintaining a protocol similar to that used previously for microdialysis experiments studying sensitization of NE release (Nisenbaum et al., 1991; Gresch et al., 1994; Finlay et al., 1997; Jedema et al., 1999).

2.2.3 Electrophysiological Recording

Single-unit activity was recorded from neurons in the LC of rats weighing 245-380g. Rats were anesthetized with 5% halothane (Halocarbon Laboratories, River Edge, NJ) in O_2 and placed in a stereotaxic apparatus (David Kopf, Tujunga CA) with the nose pointing downward at an angle of ±15° from the horizontal plane (differential of DV coordinates of bregma and lambda was 3.0 mm). During the remainder of the experiment, anesthesia was maintained with 1.5-2.5% halothane in O_2 using a vaporizer (Matrix Medical Inc., Orchard Park, NY). Core temperature was maintained at 37°C using a heating pad (Fintronics VL-20F, New Haven, CT). Holes were drilled in the skull

overlying the lateral ventricle and the LC. A 33G stainless steel cannula was implanted into the lateral ventricle (-1.0mm AP, ±1.4mm ML with respect to bregma, and 5.3 mm DV from dorsal brain surface). The cannula was connected via PE tubing to a syringe for intraventricular drug infusion. In some experiments a second aCSF-filled cannula was placed in the lateral ventricle in the opposite hemisphere, in an attempt to minimize ventricular pressure fluctuations and stabilize recording conditions. Intraventricular administration of CRH was chosen to allow comparison with the neurochemical studies performed previously (Finlay et al., 1997). Glass electrodes (Omegadot, 2mm; WPI, New York, NY) pulled using a vertical puller (Narishige PE-2, Tokyo, Japan) were filled with 2M NaCl/2% Pontamine Sky Blue (impedance 6-12 MΩ). Electrodes were positioned in the LC (-3.5mm AP, ±1.1mm ML with respect to lambda, and -5.0-6.0 mm DV from dorsal brain surface) using a hydraulic microdrive (Kopf model 640). LC neurons were tentatively identified based on well-established criteria including spike waveform, firing pattern, and response to paw compression (Cedarbaum and Aghajanian, 1978a; Foote et al., 1983; Mana and Grace, 1997). Signals from electrodes were amplified using a high-impedance headstage amplifier connected to a amplifier/window discriminator (Fintronics WDR 420). Electrophysiological activity was monitored using an audio monitor (Grass AM-8, West Warwick, RI) and a storage oscilloscope (Hitachi V134, Brisbane, CA). In addition, data was monitored and analyzed on-line using a data acquisition board (Microstar Labs™, Bellevue, WA) interfaced with a Windows based PC and custom designed software (Neuroscope®).

Rat/human CRH (RBI, Natick, MA) was dissolved to a concentration of 1.0, 0.33, or 0.11 μg/μl in artificial cerebrospinal fluid (aCSF) containing 145 mM NaCl, 2.7 mM

KCl, 1.0 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.1% bovine serum albumin, and 0.3 mM ascorbate. Aliquots were stored at –80°C for up to 3 weeks. When stable, well-isolated recordings of single LC neurons were achieved, basal activity was monitored for a minimum of 5 min. Subsequently, CRH was infused through the cannula into the lateral ventricle ipsilateral to the recording site at a flow rate of 1.0 µl/min using a 10-µl Hamilton syringe and a syringe pump (KDS-1; KD Scientific, Boston, MA). Depending on the dose, a 2.7 µl aliquot (0.3 µg) or a 3.0 µl-aliquot (1.0 or 3.0 µg) of CRH was infused. Control infusions consisted of 3.0 µl vehicle (aCSF) infused at 1.0 µl/min. Because of the long duration of LC activation following CRH administration, the response to CRH was tested on only one cell per rat, and the effect of only a single dose of CRH was tested in each rat. The single-unit activity was recorded for a minimum of 12 min (up to 45 min) following infusion. In cases where a post-infusion recording could not be maintained for 12 minutes, baseline data was included in the analysis provided that the histological criteria (see below) were met.

2.2.4 Histology

Following each experiment, the location of the recording site was marked by iontophoretic ejection of Pontamine Sky Blue from the tip of the electrode using –30 µA constant current delivered for 30 min. In addition, a Fast Green solution was infused through the cannula in the lateral ventricle. Each rat received an overdose of anesthetic, and was decapitated. The brain was removed and the presence of dye in the third ventricle close to the optic chiasm was taken as evidence that the infusion cannula had been located in the lateral ventricle. Subsequently, brain tissue was post-fixed in 10% buffered formalin solution (Fisher scientific; Pittsburgh, PA), sectioned into 40 µm thick

slices and stained with cresyl violet. Only data from subjects with recording sites within the LC (figure 2) and cannula placement in the lateral ventricle were included in the data analysis.

2.2.5 Data analysis

Basal activity of LC neurons was quantified by determining 1] the basal FR, expressed as the average number of spikes per second, and 2] the percentage of spikes occurring within bursts. The onset of a burst was defined as two spikes occurring with an interspike interval (ISI) equal to or less than 80 msec, and the termination of a burst was defined as a subsequent ISI exceeding 160 msec, as previously described (Grace and Bunney, 1984; Mana and Grace, 1997). To determine the effect of CRH on individual LC neurons, sliding FR averages were calculated using 20 consecutive 1-sec bins. The maximal response to CRH was defined as the difference between the peak value of these sliding FR averages and the basal FR of the neuron. The time of maximal response was defined as the 1 sec bin during which this maximal increase in FR was first attained.

Basal activity (FR and spikes fired in bursts) in control rats and cold-exposed rats was compared using independent *t*-tests. Comparison of the maximal response to CRH was performed using a 2-way ANOVA with group and dose as factors, followed by planned *t*-tests. Multiple regression analysis was performed to compare the slope of the log-transformed dose response curve of the cold-exposed and control group.

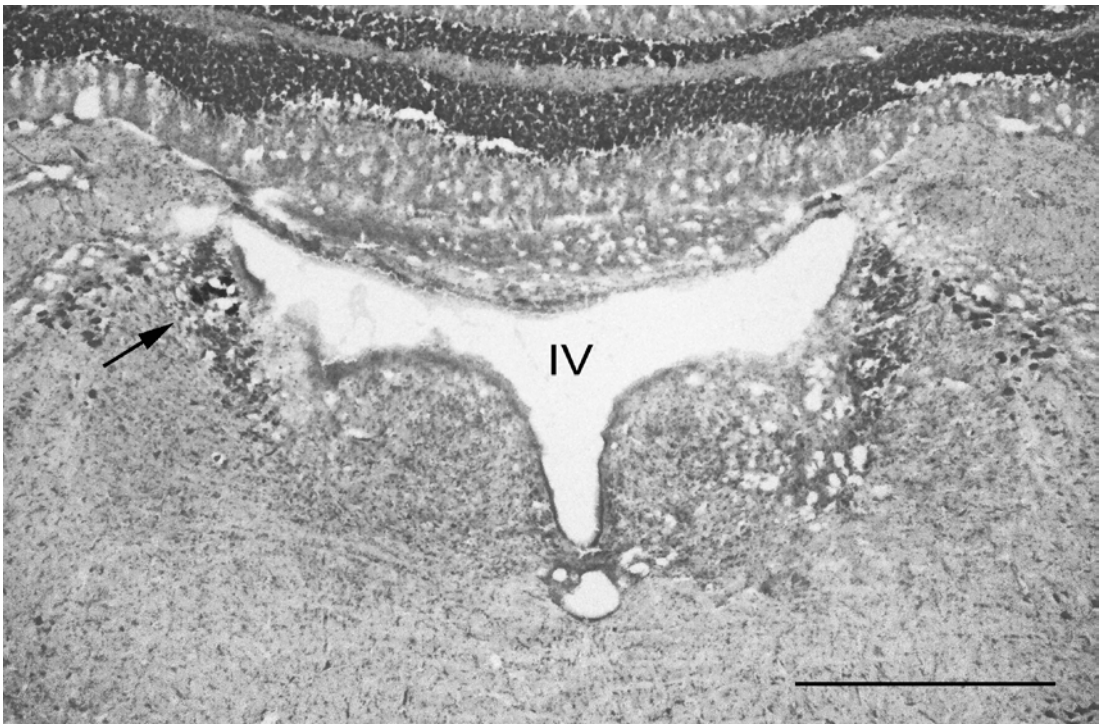

Figure 2 Localization of recording electrode in the LC

Photomicrograph of a 40 μm thick coronal section of a rat brain sectioned at the level of the LC and stained with cresyl violet. Tissue damage dorsal to the LC and iontophoretic injection of Pontamine Sky Blue (dark area; arrow) indicate the location of the recording electrode. IV: 4^{th} ventricle. Dorsal portion of the section is at the top of the photomicrograph. Scale bar indicates 1 mm.

2.3 RESULTS

Basal electrophysiological activity of LC neurons was similar in control and cold-exposed rats (figure 3). The basal FR in control rats of 1.52 ± 0.12 Hz did not differ from the FR in cold exposed rats of 1.67 ± 0.21 Hz. (n=25 and 23, respectively; $t(46)=0.63$). A small proportion of LC spikes occurred in bursts (for definition see 2.2 *METHODS*), and this value was also similar for control and cold-exposed animals (1.58 ± 0.45 vs 2.07 ± 0.57 % respectively; $t(46)=0.70$). In both groups, the vast majority (>95%) of bursts occurred as doublets, while on occasion triplets were observed.

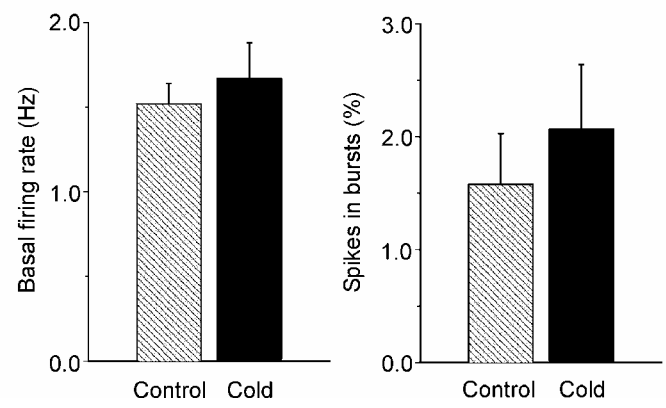

Figure 3 Basal discharge characteristics of LC neurons in cold-exposed and control rats

The basal discharge rate in control rats was similar to that in cold-exposed (14 days at 5°C) rats (1.52 ± 0.12 Hz [$n = 25$] and 1.67 ± 0.21 Hz [$n = 23$], respectively). The percentage of spikes occurring in bursts was also similar in control and cold-exposed rats (1.58% ± 0.45% vs. 2.07% ± 0.57%, respectively).

Intraventricular administration of CRH increased LC FR (figure 4). Following a brief delay, presumably due to the time required for diffusion of the peptide from the injection site, LC firing gradually increased to reach a maximum approximately 10 min following CRH injection in both the control and cold-exposed rats [time post-infusion to peak response 643 ± 40 (n=18) and 623 ± 61 sec (n=22), respectively, $t(38)=-0.30$]. The increase in FR often lasted until the end of the recording session, as much as 45 min following the start of the infusion of CRH. Therefore, it was not feasible to maintain the recording until the neuron had returned to its pre-drug level of activity.

The increase in FR was dose-dependent in control and cold exposed rats (figure 5). Higher doses resulted in higher drug-evoked maximal increases in FR [main effect of dose $F(3,32)=27.2$, $p<0.01$]. Comparison of the dose response curves for control and previously cold-exposed rats demonstrated that prior cold exposure enhanced the CRH-evoked increase in FR at the higher dose but not at the lowest doses, as evidenced by a significant group x dose interaction [$F(3,32)=3.6$, $p<0.05$]. Comparison at individual dose levels indicated a significant difference in response to CRH between control and cold-exposed rats at the 3.0 μg dose ($t(9)=3.8$, $p<0.01$). Multiple regression analysis of the log-transformed dose response curves of the cold-exposed and the control group indicated a significant difference in slope (2.566 ± 0.386 and 1.117 ± 0.399, respectively; $t(33)=-2.54$, $p<0.05$). Therefore, cold exposure did not result in a simple leftward shift of the dose-response curve, but rather in a change in the slope of the dose-response curve.

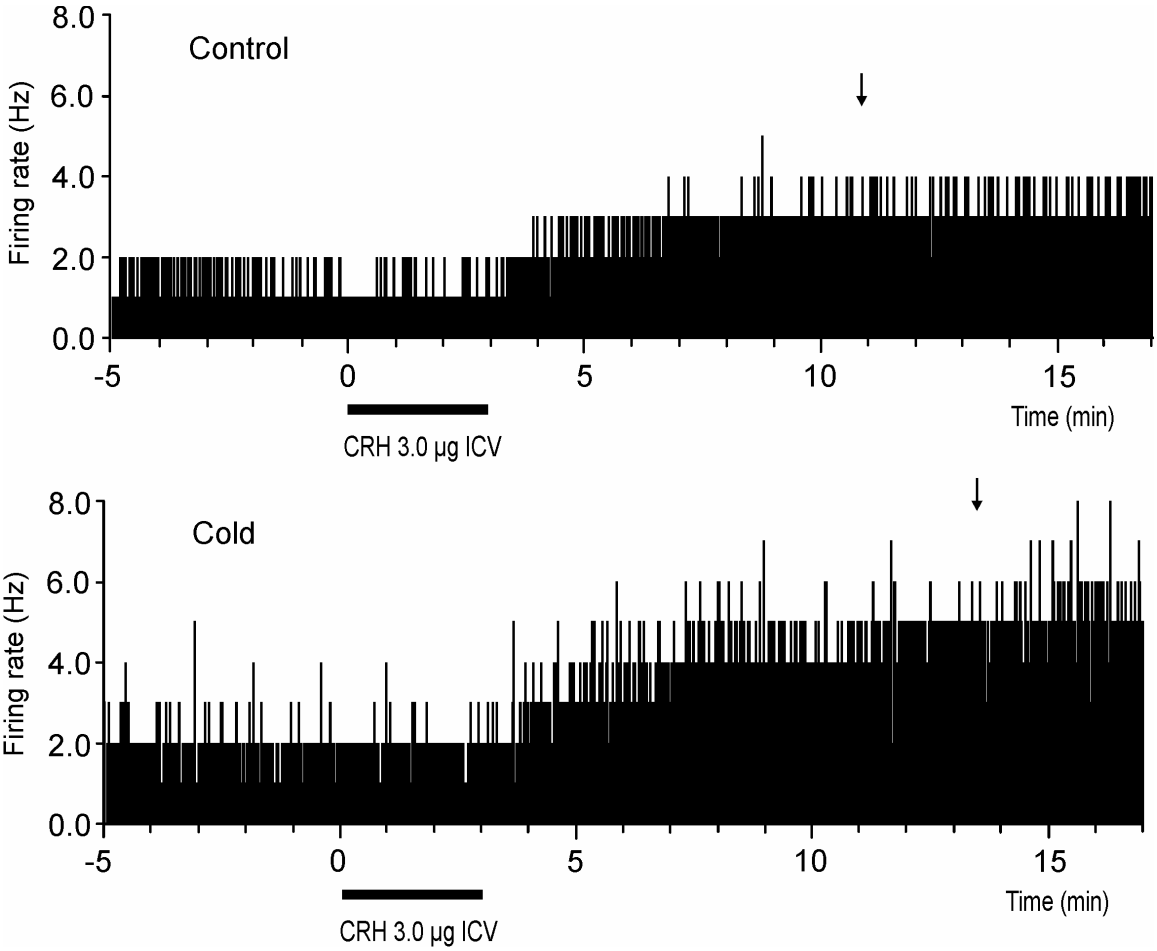

Figure 4 Representative firing rate histograms of a control and a cold-exposed rat

In the control rat the FR increased from 1.2 Hz to 3.0 Hz following CRH administration (3.0µg ICV; indicated by the horizontal bar). In the cold-exposed (14 days at 5°C) rat the FR increased from 1.6 Hz. to 5.2 Hz following the same dose of CRH. Arrows indicate the time point of the maximal response.

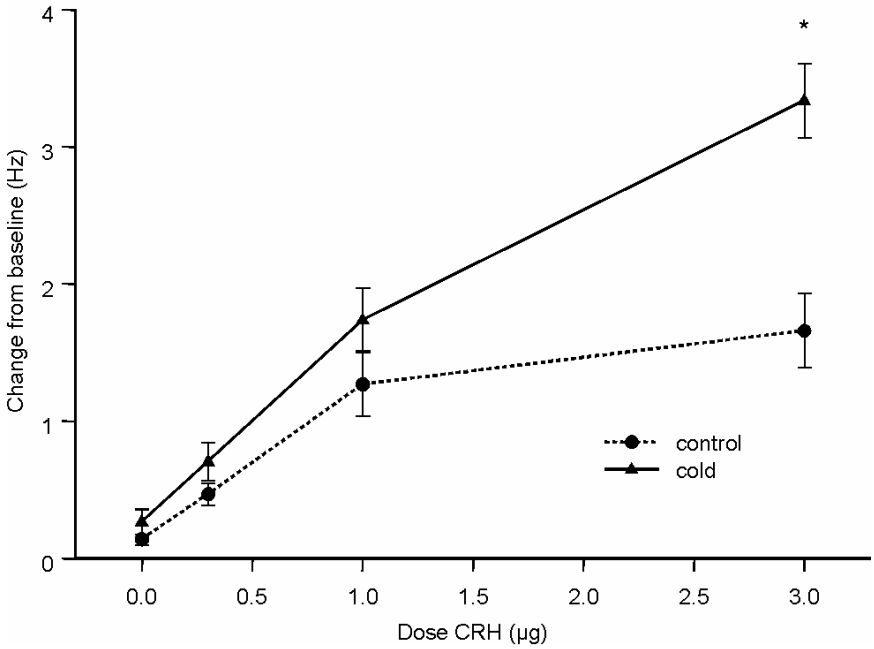

Figure 5 Dose response curve for CRH in control and cold-exposed rats

The response of LC neurons to intraventricular administration of CRH following chronic cold exposure (14 days at 5°C) was significantly greater than that in control rats. The maximal change from baseline to a single intraventricular dose of CRH was similar in both groups at low doses of CRH, but was larger in previously cold-exposed rats at higher doses [$F(3,32)=3.6$, $p<0.05$; n=3-8 rats per dose per group].

* Significantly different from respective dose in control group $p<0.01$

2.4 DISCUSSION

The present study demonstrates that cold exposure for 2 weeks enhances the electrophysiological response of LC neurons to CRH in a dose-dependent manner, supporting the view that the sensitization of NE release in response to CRH in cold-exposed rats (Finlay et al., 1997) may be accompanied by sensitization of electrophysiological activation of LC neurons. The response of control rats to ICV CRH was comparable in magnitude, onset latency and duration to previously published data (Valentino et al., 1983; Conti and Foote, 1995; Curtis et al., 1995; Borsody and Weiss, 1996; Conti and Foote, 1996). In contrast, previously cold-exposed rats exhibited an enhanced response to CRH, even though the change in basal activity of LC neurons was minimal. This finding is in correspondence with our neurochemistry experiments demonstrating an enhanced NE efflux in response to 3.0 μg CRH without alterations of basal NE levels in cold-exposed rats (Finlay et al., 1997). Interestingly, the CRH-evoked increase in both NE efflux (Finlay et al., 1997) and LC discharge rate (present study) in response to 3.0 μg of CRH was approximately twice as great in cold-exposed rats than in control rats.

Removing rats from the cold room and housing them overnight at room temperature may itself trigger adaptive changes to the noradrenergic system. However, our previous studies suggest that continuous cold exposure is responsible for the observed changes in the responsivity of LC neurons. First, cold exposure for 1 week followed by overnight housing at room temperature does not result in sensitization of NE release, whereas cold exposure for two weeks followed by overnight housing at room temperature does (Finlay et al., 1997). Second, intermittent exposure to cold for 4 hrs per day with repeated

overnight housing at room temperature for a total period of two weeks does not result in sensitization of NE release (Jedema et al., 1999).

In the present study, basal activity (FR and bursting) of LC neurons in cold-exposed rats did not differ from control rats. In contrast, a previous study reported a slight, but significant increase in FR and bursting in cold-exposed rats (Mana and Grace, 1997). A difference in the method of anesthesia (chloral-hydrate in the previous study vs. halothane in the present study) may have contributed to this discrepancy. However, the basal FR observed in the previous study is within the distribution of values obtained in the present study, using a larger number of subjects. Nevertheless, the finding of enhanced responsivity of LC neurons under stimulus-evoked conditions is consistent across the two studies.

We found a slight but significant decrease in body weight of cold-exposed rats at the time of experimentation (302± 6 vs 333± 5 g). However, there was no correlation between the magnitude of the CRH-evoked increase in LC activity and body weight at any dose level in either group. For example, the difference in increase of LC activity between cold and control rats at the 3.0 µg dose of CRH was 1.6-fold, whereas the difference in body weight at this dose level was less than 7% (315 vs 295 g).

An augmented CRH-evoked activation of LC neuron FR in cold exposed rats may, in part, underlie the sensitized CRH-evoked NE efflux reported previously (Finlay et al., 1997). Results of several studies suggest that ICV CRH acts directly within the LC (Valentino and Wehby, 1988; Valentino et al., 1993). The effect of ICV CRH can be mimicked by direct administration of CRH into the LC (Curtis et al., 1997) and can be blocked by administration of a CRH antagonist directly into the LC (Smagin et al., 1995;

Curtis et al., 1997). Therefore, the sensitization of CRH-evoked NE release following cold exposure may occur in part due to an altered responsiveness of LC neurons at the cell body level.

The present data show that following chronic cold exposure there is a significant increase in the responsiveness of LC neurons to a higher dose but not the lower doses of CRH. This effect differs from the results of studies demonstrating that swim stress or repeated foot shock stress results in a leftward shift of the dose-response curve for CRH while decreasing the maximum CRH-evoked response (Curtis et al., 1995; Curtis et al., 1999). However, previous studies from our laboratory suggest that the impact of chronic stress on LC neurons varies as a function of the nature and the pattern of stress exposure. For example, it is known that neither repeated intermittent cold exposure nor repeated intermittent foot shock for 2 weeks results in sensitization of NE release (Finlay et al., 1997; Jedema et al., 1999). An increase in the slope of the dose-response curve for CRH following cold exposure suggests that the altered response of cold-exposed rats is not a result of increased affinity of CRH receptors or increased effectiveness of its second messenger cascade. Indeed, previous studies reported a lack of change in affinity or receptor number following swim stress or repeated foot shock (Curtis et al., 1995; Curtis et al., 1999). A change in clearance of CRH or a change in the interaction with CRH binding protein could underlie the present observation of increased CRH-evoked activation of LC neurons following chronic cold exposure.

Alterations in the responsiveness to CRH are however, not the only change observed following chronic cold exposure. In a previous study on rats exposed to cold, a marked increase in the response of LC neurons to sciatic nerve stimulation was observed

with only a minor increase in basal activity (Mana and Grace, 1997). Sciatic nerve stimulation increases LC activity via glutamatergic inputs to the LC (Ennis et al., 1992) and this excitation is insensitive to local administration of CRH antagonists (Valentino et al., 1991). Therefore, the present study in combination with data from Mana & Grace (Mana and Grace, 1997), suggest a general increased responsivity of LC neurons to excitatory inputs in rats exposed to cold. The fact that the response of LC neurons to excitatory input mediated by glutamate and CRH may be enhanced without major alterations of basal activity suggests that previous cold exposure may alter either the intrinsic properties of LC neurons or their regulation by a feedback loop. In addition to potentially enhancing activation within feedforward loops, it is possible that inhibitory feedback loops could be altered following chronic stress exposure, such as the direct feedback by NE acting on dendritic α_2 autoreceptors or feedback loops involving the GABA/benzodiazepine (BZD) receptor complex or enkephalin (Van Bockstaele, 1998). Previous data suggest that α_2 receptor regulation of LC neurons is increased following chronic cold exposure (Nisenbaum and Abercrombie, 1993; Mana and Grace, 1995a, b) and repeated immobilization (Pavcovich et al., 1990); although see (Pavcovich and Ramirez, 1991), arguing against a decreased α_2 receptor-mediated feedback inhibition contributing to sensitization of LC neurons. An alteration in the interaction between the GABA/BZD complex and LC neurons following chronic cold stress has been reported (Finlay et al., 1995; Mana and Grace, 1995a), such that reduced GABA-ergic inhibition of LC neurons occurs under both basal and stress-evoked conditions. Given the tonic GABA-ergic inhibition of LC neurons (Ennis and Aston-Jones, 1989; Kawahara et al., 1999), additional alterations to the LC system must be hypothesized to account for the

observation that basal LC FR and release are not altered by chronic cold exposure. Opioids directly inhibit LC activity (Williams and North, 1984; Aghajanian and Wang, 1987; Williams et al., 1988), and appear to exert their effect on LC neurons under stressful conditions (Abercrombie and Jacobs, 1988). However, no studies have reported on the modulation of opioid-LC interaction following repeated or chronic stress. Changes in intrinsic properties of LC neurons following chronic stress may also contribute to stress-induced sensitization. For example, alterations in electrotonic coupling between LC neurons (Christie and Jelinek, 1993), or alterations in input resistance or calcium-activated potassium conductance could underlie the development of stress-induced sensitization.

2.5 CONCLUSION & CLINICAL IMPLICATIONS

In summary, the present studies demonstrate that CRH-evoked LC activation is enhanced following chronic exposure to cold stress. Together with previous studies, these data suggest a general increased responsiveness of the noradrenergic LC system to excitatory input following chronic stress. Studies in humans have implicated enhanced activity of central NE neurons in mood and anxiety disorders (Aston-Jones et al., 1994; Bremner et al., 1996; Bremner et al., 1997; Southwick et al., 1997a; Southwick et al., 1997b; Maes et al., 1999; Wong et al., 2000). Other physiological changes associated with mood and anxiety disorders are also present in rats chronically exposed to cold. For example, both clinical anxiety disorders and cold exposure are associated with increased basal heart rate (Leduc, 1961; Barney et al., 1980; Orr et al., 1997), sympathetic activation (Leduc, 1961; McFall et al., 1990), and tyrosine hydroxylase levels in LC

(Nisenbaum et al., 1991; Nisenbaum and Abercrombie, 1992; Ordway et al., 1994). In addition, alterations in CRH-LC interactions have been implicated in both anxiety disorders (Aston-Jones et al., 1994; Weiss et al., 1994) and following cold exposure (Finlay et al., 1997), present study). Therefore, the paradigm of continuous cold exposure in rats may represent a unique experimental model to study the mechanisms underlying the enhanced activation of LC function following chronic stress and its relation to anxiety and mood disorders.

3. THE EFFECT OF CORTICOTROPIN-RELEASING HORMONE ON NORADRENERGIC NEURONS OF THE LOCUS COERULEUS RECORDED *IN VITRO*

3.1 INTRODUCTION

Since the initial discovery of CRH as the primary initiator for the release of ACTH from corticotropes (Vale et al., 1981), it has become clear that CRH is also located at many extrahypothalamic sites (Swanson et al., 1983; Potter et al., 1994), where it plays an important role in the behavioral response to stress (Dunn and Berridge, 1990; Owens and Nemeroff, 1991; Valentino et al., 1993; Heinrichs et al., 1995).

The major source of norepinephrine (NE) in the central nervous system, the LC, receives CRH afferents from Barrington's nucleus, the central nucleus of the amygdala, the brainstem nuclei PGi and PrH, and the dorsal cap of the paraventricular nucleus of the hypothalamus (Valentino et al., 1992; Van Bockstaele et al., 1999). Some CRH afferents to LC preferentially target the rostromedial and dorsolateral pericoerulear regions, whereas other afferents target the LC proper (Valentino et al., 1992; Van Bockstaele et al., 2001). Furthermore, CRH terminals contact LC dendrites as well as presynaptic terminals, thereby providing a neuroanatomical substrate for an interaction between CRH and noradrenergic activity throughout the entire forebrain (Van Bockstaele et al., 1996a, 1998a; Van Bockstaele et al., 1999).

CRH administration either ICV or locally into the LC increases the FR of LC neurons and NE release in terminal fields of LC neurons (Valentino et al., 1983; Valentino and Foote, 1988; Smagin et al., 1995; Finlay et al., 1997; Jedema et al., 2001). CRH immunoreactivity in the LC is increased by cold exposure (Chappell et al., 1986) and CRH is functionally important for activation of the LC during exposure to several

stressors (cold, hypovolemia, and immobilization) (Valentino and Wehby, 1988; Melia and Duman, 1991; Valentino et al., 1991; Smagin et al., 1995; Smagin et al., 1996; Curtis et al., 1997).

CRH receptors have also been demonstrated in the LC (Millan et al., 1986; De Souza and Insel, 1990; Primus et al., 1997; Sanchez et al., 1999; Chen et al., 2000) and a recent study suggests that these CRH receptors are of the CRH_1 receptor subtype (Sauvage and Steckler, 2001). CRH receptor mRNA has been detected in LC neurons of primates (Sanchez et al., 1999), although it has been difficult to detect in rodents (Potter et al., 1994; Chalmers et al., 1995; Chalmers et al., 1996; Van Pett et al., 2000). Given the problems detecting CRH mRNA in LC neurons of rats, a preferential presynaptic neuromodulatory role for CRH innervation of the LC could be hypothesized, although very recent ultrastructural evidence for CRH_1 receptors on LC neurons has been reported (Fox et al., 2002).

In addition to the site at which CRH acts to affect LC neurons, the mechanism underlying the CRH activation of LC neurons is unknown. In hippocampus CRH is thought to inhibit a calcium-activated potassium current ($I_{K(Ca)}$) (Aldenhoff et al., 1983; Siggins et al., 1985), while in the amygdala CRH activates a calcium current (I_{Ca}) and inhibits $I_{K(Ca)}$ (Rainnie et al., 1992; Yu and Shinnick-Gallagher, 1998). In corticotropes, CRH activates I_{Ca} and a hyperpolarization-activated current (I_H), in addition to inhibition of an inward rectifying current ($I_{K(IR)}$) (Kuryshev et al., 1996a, b; Kuryshev et al., 1997).

All CRH receptors subtypes cloned and characterized to date are G-protein coupled receptors linked to ADC (Litvin et al., 1984; Chen et al., 1986; Battaglia et al., 1987; De Souza, 1995). In some studies inhibition of PKA inhibited the effect of CRH (Kuryshev

et al., 1995; Bundey and Kendall, 1999; Thiel and Cibelli, 1999; Cibelli et al., 2001). It has been hypothesized that CRH activates LC neurons in a similar manner as vasoactive intestinal peptide (VIP) (Alreja and Aghajanian, 1991a; Nestler et al., 1999), which has been demonstrated to increase LC FR by increasing a TTX-insensitive sodium current via ADC activation, resulting in a cAMP-mediated activation of PKA (Wang and Aghajanian, 1990).

In the present studies we have characterized the effect of local application of CRH on the electrophysiological activity of LC neurons in horizontal brain stem slices of rats using *in vitro* intracellular recording techniques.

3.2 METHODS

3.2.1 Animals

Male Sprague-Dawley rats (Hilltop, Scottdale, PA) were housed singly in hanging stainless steel cages in a colony room maintained at an ambient temperature of 23°C. Throughout the experiments, lights were maintained on a 12 hr light/dark cycle (lights on at 08.00 a.m.), with food (Laboratory rodent diet 5001, PMI Feeds, St. Louis, MO) and water available *ad libitum*. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh and were in accordance with the USPHS *Guide for the Care and Use of Laboratory Animals*.

3.2.2 Tissue preparation

Rats (180-300g) were anesthetized with chloral hydrate (400 mg/kg, i.p.) and perfused through the ascending aorta with an ice-cold, oxygenated (low Na/high sucrose) perfusion solution (1.9 mM KCl, 1.2 mM Na_2HPO_4, 6 mM $MgCl_2$, 33 mM $NaHCO_3$, 20

mM glucose, 229 mM sucrose saturated with 95% O_2/5% CO_2) (Aghajanian and Rasmussen, 1989). Following decapitation, the brain was rapidly removed, placed in cold perfusion solution and 300 μm thick horizontal slices containing the LC were prepared using a DSK Microslicer (Ted Pella, Redding, CA). Tissue was transferred to cold, oxygenated aCSF (124 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 2.4 mM $CaCl_2$, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, 10 mM glucose saturated with 95% O_2/5% CO_2). After a recovery period of a minimum of 60-90 min, sections were transferred to a temperature-controlled recording chamber (RC-22C; Warner Instrument Corp., Hamden, CT) where they were superfused with oxygenated aCSF at a flow rate of 0.8-1.5 ml/min at 35°C.

3.2.3 Electrophysiology

Intracellular recordings were obtained from neurons in the LC that were initially identified by their location within the trans-illuminated slice. Electrodes were filled with Biocytin (2% biocytin in 2-3M $KC_2H_3O_2$ or 2M KCl) and had an impedance of approximately 50-100 MΩ. Putative LC neurons were identified based on a regular pattern of low frequency discharge of long duration action potentials, as described in previous studies (Andrade et al., 1983; Williams et al., 1984; Ivanov and Aston-Jones, 1995) and a minimal spike amplitude of 65mV. Electrodes were connected to the headstage (HS-2A) of an Axoclamp-2A amplifier (Axon Instruments, Union City, CA) in current clamp mode. The output of the amplifier was displayed and analyzed using an A/D-board (Microstar Labs, Bellevue, WA) and customized data-analysis software (Neuroscope). Following determination of basal activity for 3-10min, CRH was locally applied by pressure ejection using a Picospritzer II (General Valve, Parker Hannifin Corp; Fairfield NJ) from a calibrated glass pipette positioned in the bath above the slice

(tip size 10-20µm ID, approximately 400-600µm from recording site, ejection pressure 50-75psi). For each neuron multiple doses of CRH were administered in order to determine the "sensitivity" of the neuron to CRH, which appeared to be a function of the responsivity of the individual neuron tested and the proximity of the CRH pipette to the neuron.

3.2.4 Drugs

Rat/human, CRH obtained from RBI (Natick, MA) or Bachem (Torrance, CA) was dissolved to a concentration of 1µg/µl in aCSF containing 0.1% bovine serum albumin and 0.3mM ascorbate. Generally, it was necessary to acidify the solution using 1µl of a 30% acetic acid solution. D-Phe-CRH (12-41) and α-helical CRH were obtained from Bachem. CP154,526 was a gift from Pfizer Inc. (Groton, CT). A stock solution of CP154,526 was made by dissolving the compound in either 0.1M HCl or in aCSF containing 10% DMSO. The stock solution was subsequently diluted to final concentration using aCSF. The final DMSO concentration in the buffer was equal to or less than 0.1%. Apamin and SQ22,536 were obtained from Calbiochem (La Jolla, CA). Rp-cAMPS was obtained from Tocris Cookson Inc. (Ellisville, MO). Tetrodotoxin (TTX) and all other compounds were obtained from Sigma (St. Louis, MO). All drugs were dissolved in aCSF and bath-applied at the concentration mentioned, with the exception of the PKA-inhibitor Rp-cAMPS, which was applied intracellularly via the recording electrode (10mM). The exchange from aCSF to drug-containing aCSF was achieved using a switch valve (UpChurch Scientific; Oak Harbor, WA). Following switchover it required approximately 45 sec for the drug-containing solution to reach the recording chamber and 2-3 min before stabilization of the drug effect.

Biocytin was injected into the recorded cell for histological verification. The activity and responsiveness of only one neuron per slice was determined.

3.2.5 Immunocytochemistry

The location of the recorded neurons was microscopically verified to be within the LC (figure 6) following immunocytochemical processing for TH and biocytin as previously described (Jedema and Grace, *In Press*). Briefly, tissue sections were post-fixed in 4% paraformaldehyde or 10% buffered formalin solution, and following a freeze-thaw procedure and overnight incubation in a 1% Triton X-100 solution, they were incubated in avidin-biotin peroxidase complex (ABC; 1:200; Vectastain Elite kit; Vector Laboratories). The peroxidase reaction was visualized by incubating the sections in 0.022% diaminobenzidine, 0.3% $NiSO_4(NH_4)_2SO_4$ and 0.003% hydrogen peroxide for 6-7 min. Following incubation in blocking solution containing 3% normal goat serum, 0.4% Triton X-100, and 1% bovine serum albumin, sections were incubated overnight in blocking solution containing a monoclonal antibody raised in mouse against TH (1:10,000; Chemicon), followed by incubation in secondary antibody (BA2001; 1:200; Vector), which was then visualized using an ABC and a peroxidase reaction.

3.2.6 Data Analysis

The membrane potential was determined from the change of potential following withdrawal from the neuron. Action potential threshold was defined as the potential where the greatest change in slope of the membrane potential occurred, which was determined from the second order derivative of membrane potential waveform. Amplitude of action potential and afterhyperpolarization (AHP) were measured relative to this threshold, and the action potential duration was measured at the level of threshold. The average rate of repolarization was defined as the ratio of the magnitude of individual

AHP and ISIs. To determine the effect of CRH on the action potential waveform, the characteristics for all action potentials occurring during a 5 sec window around the time point of maximal CRH-evoked activation were averaged for each neuron and compared to the average of the same number of action potentials occurring immediately prior to CRH administration using paired *t*-tests. The FR was calculated as the sliding average of the number of spikes per second occurring during a 10 second window starting 4 seconds prior and ending 5 seconds following the one second bin for which it was calculated (Jedema et al., 2001). CRH-evoked increases in FR were expressed as the maximal increase in FR within 30 sec following CRH administration. We observed that this increase in FR above baseline was fairly constant and independent of "basal" FR by altering FR by hyperpolarizing and depolarizing current injection. The effect of drug application is expressed as a percentage (±SEM) of the CRH-evoked effect in that neuron under non-drug conditions. The CRH-evoked effect was the average of the CRH effect before drug application and, whenever possible, after drug washout. CRH-evoked depolarization was measured as the maximal change in membrane potential within 60 sec following CRH administration. The reversal potential of the CRH-evoked depolarization for each neuron was estimated by linear regression of the relationship between the maximal CRH-evoked depolarization versus membrane potential. The average basal activity calculated for the LC neurons in the present study includes the basal activity of some control LC neurons examined in another manuscript (Jedema and Grace, *In Press*).

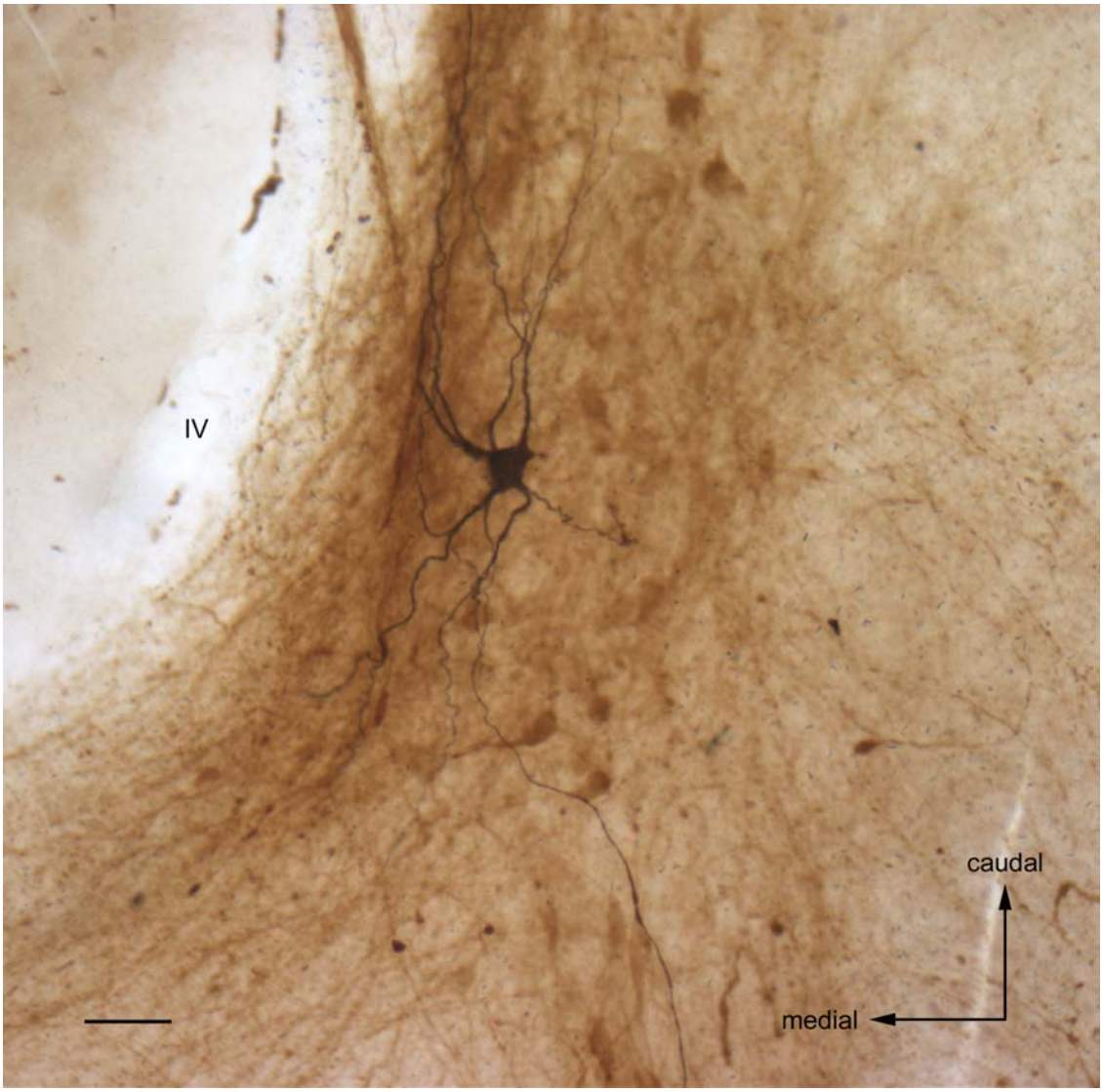

Figure 6 Identification and morphology of LC neurons

An LC neuron stained with biocytin (black) following intracellular recording in a 300µm thick horizontal slice of the rat brainstem. Immunoreactivity for tyrosine hydroxylase (brown) in somata outlines the locus coeruleus. Immunoreactive processes illustrate the extensive arborization of LC neurons outside the nucleus proper.

Scale bar = 50 µm; IV: fourth ventricle

3.3 RESULTS

3.3.1 Basal activity of LC neurons

The vast majority of LC neurons recorded *in vitro* with potassium acetate electrodes were spontaneously active (53/57) with an average basal firing rate (FR) of 2.2±0.2 Hz. Hyperpolarizing current injection caused an amplitude-dependent deflection of the membrane potential. The relationship between the magnitude of the current injection and the resulting membrane potential deflection, which demonstrated inward rectification, could be well-described by a quadratic function (R^2=0.97±0.01, n=19) (figure 7). The input resistance obtained from the y-intercept of the derivative of this function was 81±5 MΩ.

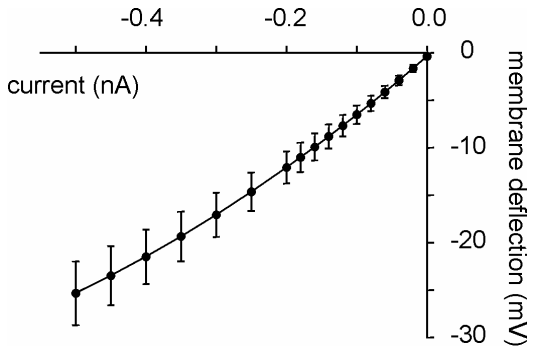

Figure 7 Input resistance of LC neurons

Plotting the deflection of the membrane potential versus the amplitude of hyperpolarizing current injected to elicit them revealed a non-linear current-voltage relationship (inward rectification). The input resistance calculated from the slope of the curve at the zero-current level is 81±5 MΩ (n=19).

3.3.2 Effect of CRH on spontaneous activity

Administration of CRH (average dose 143±15ng) transiently increased the FR of 51 out of 53 neurons (figure 8). The increase was dose-dependent with an average increase of 0.7±0.1 Hz occurring approximately 14±1 sec after ejection of CRH from the pipette, and the CRH-evoked activation subsided by 90-120 sec (following the termination of the CRH ejection). The time course of CRH-evoked activation of LC neurons observed in this study is remarkebly similar to the time course of CRH-evoked activation of anterior pituitary cells (Watanabe and Orth, 1987). LC neurons showed little if any short-term desensitization in their response when pulses of CRH were administered repeatedly at ~3min intervals. Similar results were obtained with electrodes filled with potassium chloride suggesting that chloride currents are unlikely to be involved in the effect of CRH on LC neurons. Vehicle ejections (acidified aCSF with 0.1 BSA and 0.3mM ascorbate) did not cause an activation of LC FR (data not shown). These findings are consistent with the existing evidence of an excitatory effect of CRH on LC neurons observed *in vivo* (Valentino et al., 1983; Valentino et al., 1991; Valentino et al., 1993; Conti and Foote, 1995; Curtis et al., 1997; Pavcovich and Valentino, 1997; Jedema et al., 2001). Because CRH was administered by local application, it is difficult to estimate the concentration of CRH at the receptor site, especially since the drug is washed out rapidly by fresh buffer entering the recording chamber. Based on an average volume of 143 nl per CRH administration, one would estimate a more than 1000-fold dilution in recording chamber (200 µl), which would result in an estimated CRH concentration of approximately 150 nM. The concentration of half-maximal activation of CRH_1 and CRH_2 receptors by r/hCRH has been reported to be in the low nanomolar range (Lovenberg et al., 1995).

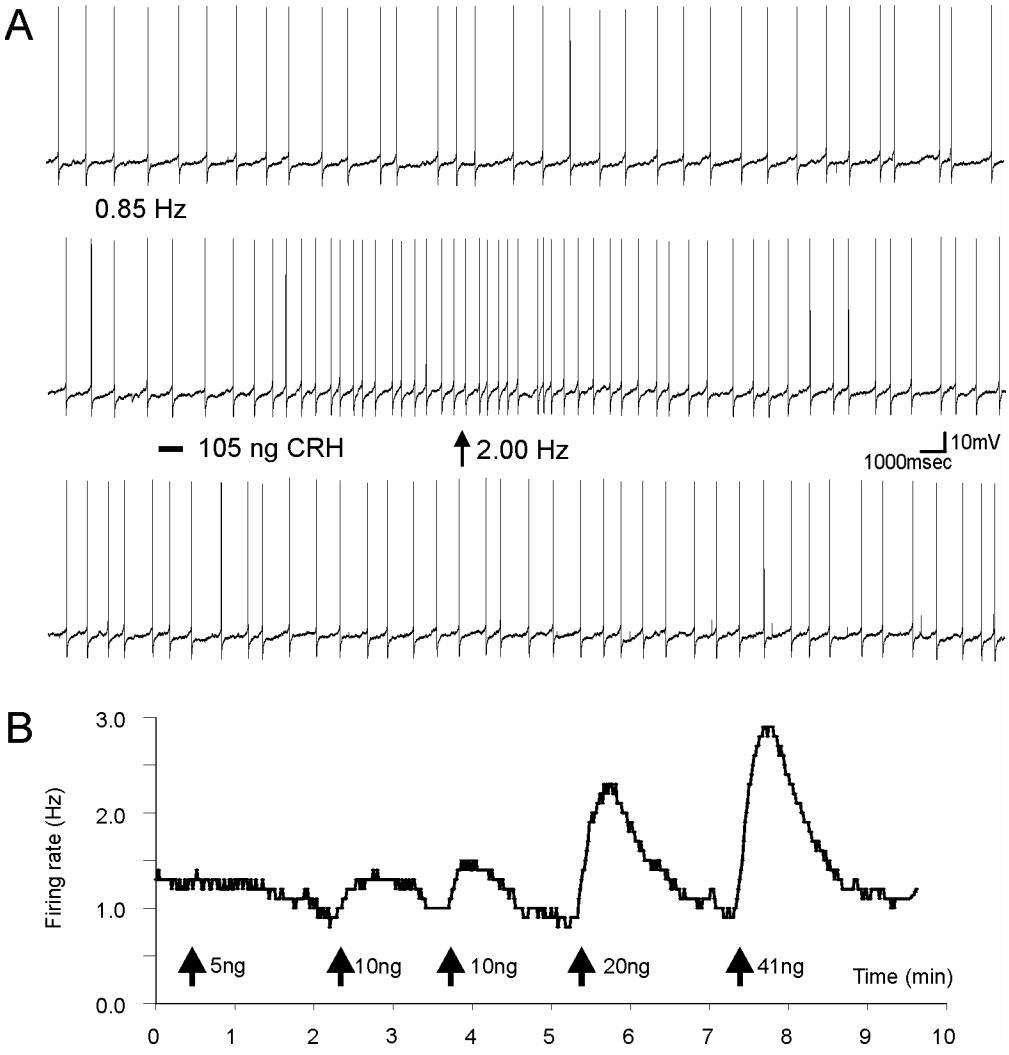

Figure 8 CRH increases the firing rate of spontaneously active LC neurons

(**A**) The average baseline firing rate (FR) of this spontaneously active LC neuron was 0.85 Hz. CRH (105ng) was ejected in the bath approximately 400 μm from the recording site (horizontal bar). A maximum FR of 2.00 Hz was reached at 12 sec (arrow) following CRH ejection. (**B**) Plotting the firing rate of another neuron over time demonstrates that repeated CRH administration causes an increase in firing rate that is transient and dose-dependent. The maximal activation in this LC neuron occurs ~15 sec after CRH ejection and the firing rate has returned to baseline levels within 90-120 sec.

In previous studies 15-500 nM of CRH has been used for electrophysiological experiments on corticotropes (Kuryshev et al., 1996a, b; Kuryshev et al., 1997) and neurons in the amygdala and hippocampus (Aldenhoff et al., 1983; Siggins et al., 1985; Aldenhoff, 1986; Rainnie et al., 1992; Yu and Shinnick-Gallagher, 1998). In studies on an LC-like cell line (CATHa), 500nM of CRH was necessary to achieve a maximal effect on neurite outgrowth and cAMP accumulation (Bundey and Kendall, 1999; Thiel and Cibelli, 1999; Cibelli et al., 2001). Therefore, the doses of CRH used in the present study may be estimated to be within the range of those used in previous *in vitro* studies.

3.3.3 Effect of CRH on action potential characteristics

There was no difference in the action potential waveform immediately prior to and at the point of maximal CRH-evoked activation (table 1). However, the rate of repolarization of the membrane potential following action potential discharge was faster after CRH administration, resulting in a more rapid depolarization to spike threshold (table 1; figure 9). Given that CRH increases LC FR, it was indeed expected that CRH affects conductances that are active during the period between action potentials rather than conductances that are only active during action potential discharge.

Table 1: Action potential characteristics before and during CRH

Characteristics of spontaneously occurring action potentials recorded during a 5sec epoch of maximal CRH-evoked activation compared to the same number of action potentials recorded immediately prior to CRH administration (two pulses for each neuron) in 10 LC neurons. (* $p < 0.05$ versus control)

	Control	CRH
Threshold (mV)	-45.9±2.1	-45.7±2.1
Amplitude (mV)	71.7±1.2	71.3±1.2
Duration (msec)	2.0±0.1	2.0±0.1
AfterHyperPolarization (mV)	20.4±0.5	20.3±0.6
Inter-Spike Interval (msec)	585±114	421±84 *
Rate of repolarization (mV/sec)	55±7	74±9 *

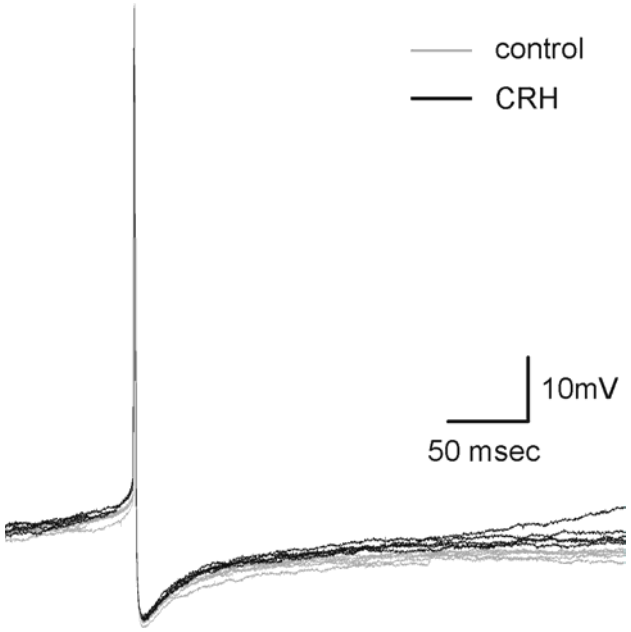

Figure 9 CRH increases the rate of repolarization without affecting the action potential waveform of spontaneously occurring action potentials

An overlay of 5 action potential waveforms at the time point of maximal CRH-evoked activation (black) demonstrates that the waveform is similar to the waveform of 5 action potentials from the same neuron immediately prior to CRH ejection (gray). At the time of maximal CRH-evoked activation the rate of repolarization following action potential discharge is faster leading to a more rapid return to action potential threshold and consequently an increase in the firing rate of the neuron.

3.3.4 Effect of CRH following blockade of synaptic activity

When synaptic activity in the slice was prevented by bath application of 2μM TTX, the spontaneous discharge of action potentials was changed, resulting in the discharge of putative calcium spikes (Williams et al., 1984). In most cases (4/6) the neurons would cease their spontaneous activity, although calcium spike discharge activity could be re-instated by a small amount of intracellular depolarizing current injection (20-250 pA). In two cases these calcium spikes continued to occur spontaneously. Administration of CRH (same doses as prior to TTX) in the presence of TTX caused a transient increase in calcium spike activity with a similar time course as in the same cell tested prior to TTX perfusion. This was observed in 6 out of 6 LC neurons. (figure 10). The fact that the increase in FR persisted following blockade of synaptic activity by TTX demonstrates that the effect of CRH *in vitro* is largely mediated via a direct action onto LC neurons, and that fast sodium currents are likely not to be a major contributor to the excitatory effect of CRH. The fact that no post synaptic potentials were observed in our slices before and during CRH administration is consistent with CRH exerting its strong excitatory effect via a direct action on LC neurons.

3.3.5 Effect of CRH on hyperpolarized LC neurons

When hyperpolarizing current was delivered through the recording electrode to prevent spike discharge, the administration of CRH caused a transient depolarization (average depolarization: 2.9 ±0.5 mV) with a similar time-course as the CRH-evoked increase in FR (time point of maximal depolarization: 17±1sec after CRH) (figure 11).

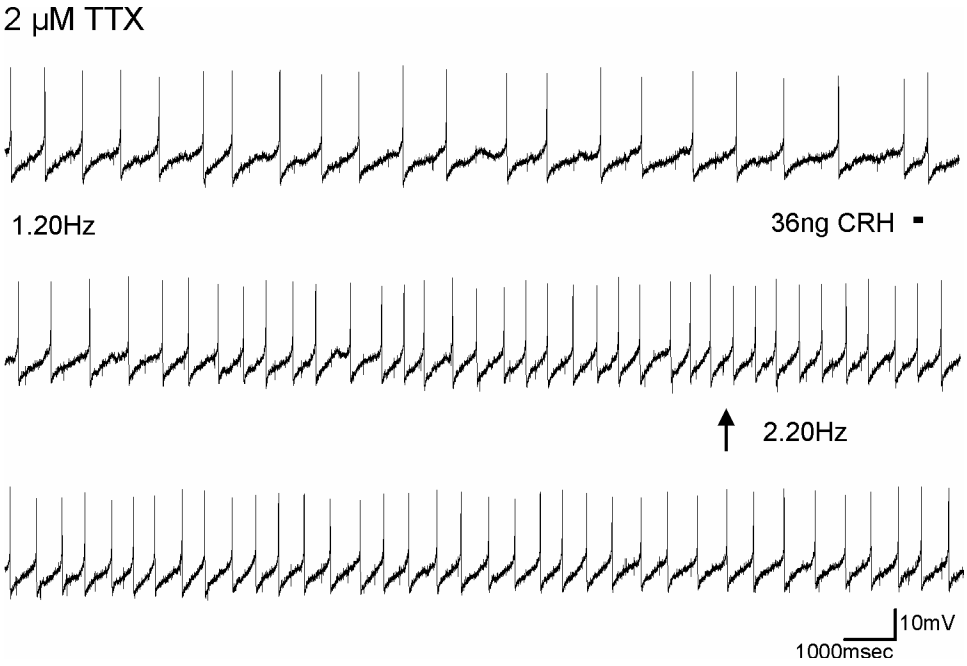

Figure 10 CRH increases the discharge rate of putative calcium spikes in LC neurons in the presence of TTX

When synaptic activity is prevented by bath application of TTX (2 μM), LC neurons continue to discharge calcium spikes. CRH administration (36ng, horizontal bar) increased the calcium spike rate of this spontaneously active LC neuron from 1.20 to 2.20 Hz at 16 sec following CRH ejection. CRH increased the calcium spike rate in 6/6 neurons.

The magnitude of the depolarization was voltage-dependent with a reversal potential of –115±8 mV (n=20). In some experiments, repeated brief hyperpolarizing current steps (10-20msec, 100-300pA, 1Hz) were applied in addition to the constant hyperpolarizing current in order to estimate changes in input resistance. An increase in input resistance was noticed that coincided with the CRH-evoked depolarization (figure 11). A depolarization of similar magnitude evoked by reducing the amplitude of constant hyperpolarizing current injected did not change the input resistance estimate (Data not shown). The CRH-evoked depolarization also persisted in the presence of TTX (not shown).

3.3.6 Inhibition of potassium conductances

To determine whether the increase in FR was mediated by calcium-activated potassium conductances (K_{Ca}), the effect of bath application of apamin (200nM) was examined. Apamin caused a small reduction in the amplitude of the AHP but the CRH-evoked increase in FR persisted during bath application of apamin in 3/3 neurons (figure 12A). The effect of blockade of the inward-rectifying potassium (K_{IR}) conductances on the CRH-evoked increase in FR was examined using bath application of $BaCl_2$ (100μM). $BaCl_2$ application increased the spontaneous FR of LC neurons in all neurons tested. The current-voltage relationship of LC neurons during $BaCl_2$ application was linear indicating that the K_{IR} conductances were effectively blocked (figure 12B). The CRH-evoked increase in FR persisted during bath application of $BaCl_2$ in 5 out of 5 neurons (figure 12B).

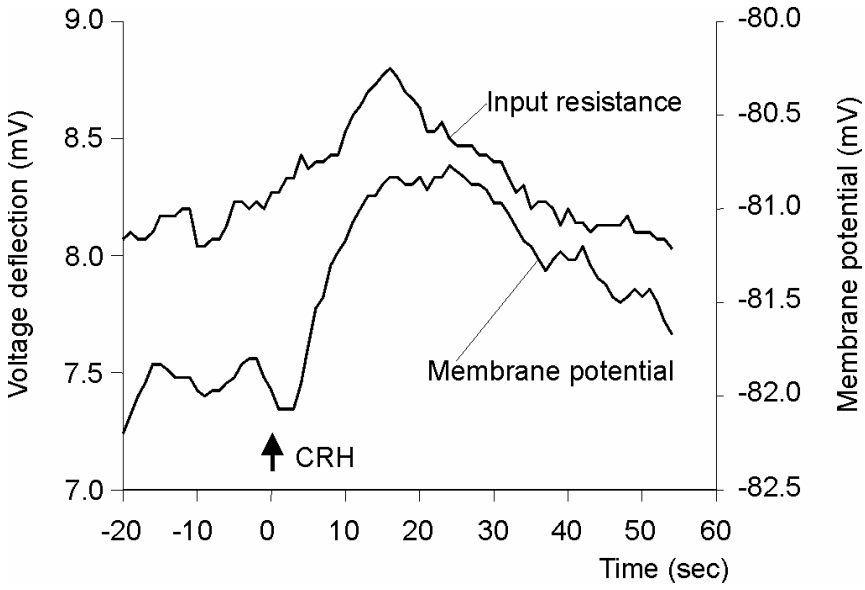

Figure 11 CRH depolarizes the membrane of LC neurons in the absence of action potential discharge

When action potential discharge is prevented by constant hyperpolarizing current delivered through the recording electrode, CRH ejection causes depolarization of the membrane potential. The magnitude of voltage deflections caused by repeated current steps (0.3nA, 10msec, 1 Hz) increases during the CRH-evoked depolarization, indicating an increase in input resistance. A current-evoked depolarization of the same magnitude as that caused by CRH did not change the input resistance.

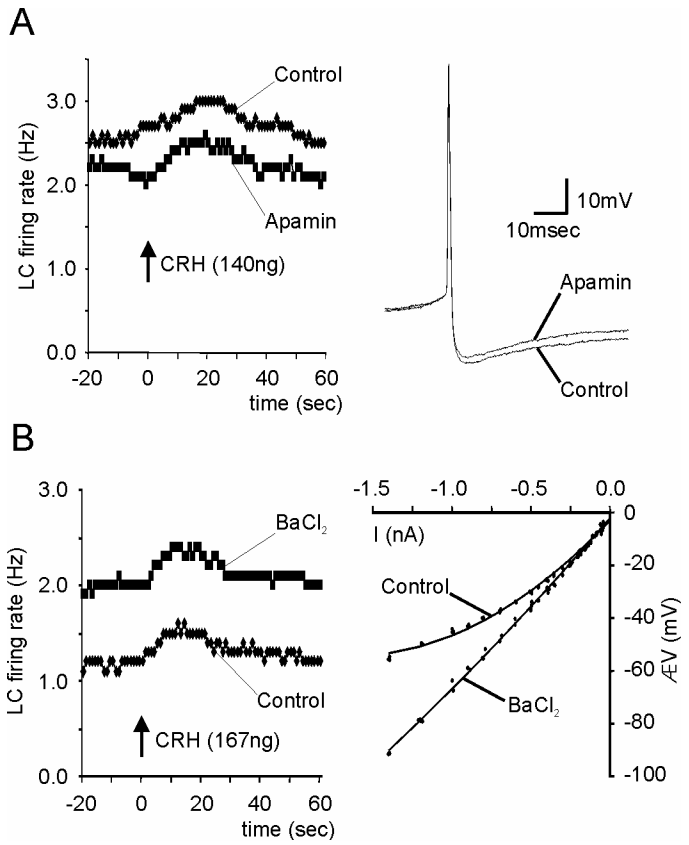

Figure 12 The CRH-evoked increase in firing rate persists in the presence of blockade of several potassium conductances

(**A**) Bath application of a blocker of calcium-activated potassium conductances, apamin (200nM), does not affect the CRH-evoked increase in LC firing rate. The effectiveness of apamin blockade on the after hyperpolarization is evident in an overlay of the action potential waveform obtained before and during apamin administration. (**B**) Bath application of a blocker of inward rectifying potassium conductances, $BaCl_2$ (100μM), increases the spontaneous firing rate of LC neurons but does not affect the CRH-evoked increase in LC firing rate. The effectiveness of barium in blocking inward-rectifying potassium conductances is evident from a comparison of the current-voltage plots obtained before and during barium administration.

3.3.7 Effect of CRH following PKA inhibition

LC neurons recorded with electrodes containing the specific PKA-inhibitor Rp-cAMPS (10mM) exhibited normal electrophysiological characteristics immediately following penetration by the electrode. The spontaneous activity of these neurons gradually decreased over a 5-10min time period and eventually ceased. When CRH was applied during the first 5 min following penetration of the cell, an increase in FR was still observed that exhibited a similar time course as observed with standard potassium-acetate containing electrodes (figure 13). However, the response of the neurons recorded with electrodes containing the PKA-inhibitor subsequently decreased until, at time points of ~5 min or greater, CRH was no longer capable of producing an increase in the firing rate of the neurons, even at substantially higher concentrations of CRH (n=3). Similarly, in one experiment using bath application of the cell-permeable ADC inhibitor, SQ22536 (1μM), the neuron ceased firing and the CRH-evoked depolarization was greatly reduced (data not shown).

3.3.8 Effect of CRH antagonists

Bath application of the CRH antagonist D-Phe-CRH (0.1-1 μM) caused a transient increase in basal FR in 5/7 LC neurons. Nevertheless, the magnitude of the CRH-evoked increase in FR was reduced to 64±12% by the administration of D-Phe-CRH (figure 14). Local application of D-Phe-CRH via pressure ejection also increased the spontaneous discharge activity. The increase in FR elicited by local D-Phe-CRH was transient and had a similar time course as the CRH-evoked activation (figure 14). A similar effect was observed for local application of another peptidergic CRH antagonist, α-helical CRH. Despite the fact that local administration of the antagonist increased basal FR, it reduced

the absolute magnitude of the CRH-evoked activation (to 77±11%; n=3). Bath application of the specific CRH1 receptor antagonist CP154,526 (1-100μM) reduced the magnitude of the effect of CRH without alterations of basal firing rate (figure 14).

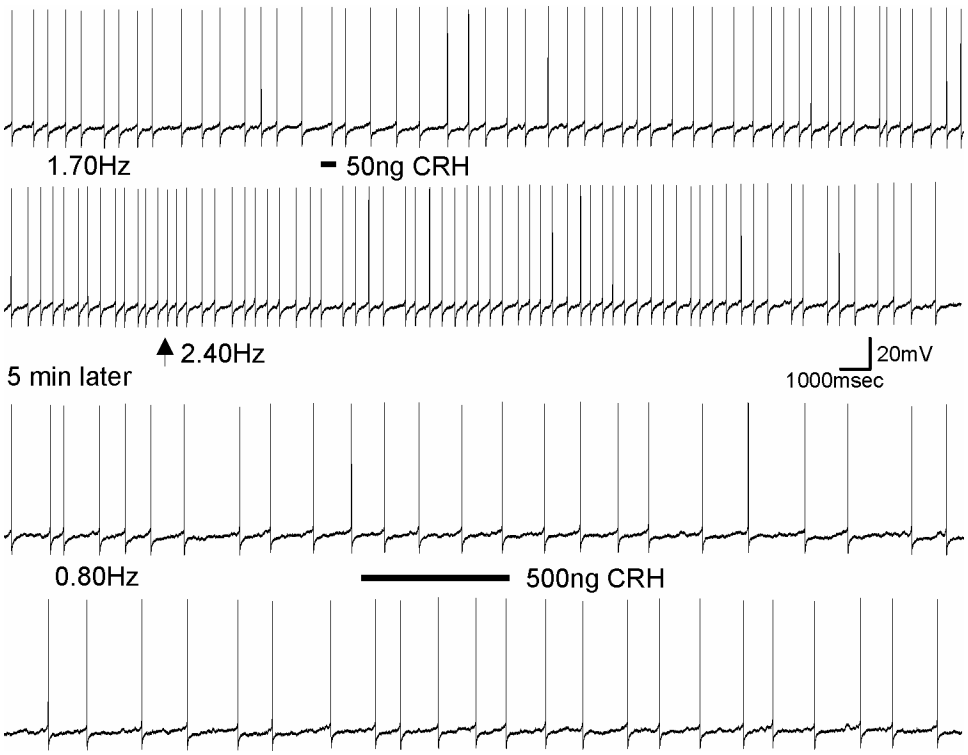

Figure 13 The CRH-evoked increase in firing rate is prevented by intracellular administration of the PKA inhibitor, Rp-cAMPS

(*Top two traces*) Within the first 5 min of impalement of the neuron with an electrode containing Rp-cAMPS (10mM), CRH administration (50ng) increased LC neuron firing rate. (*Bottom two traces*) Diffusion of Rp-cAMPS into the cell decreases the spontaneous FR. Five minutes following the initial activation by CRH, administration of a 10-fold higher dose of CRH no longer increases LC firing rate.

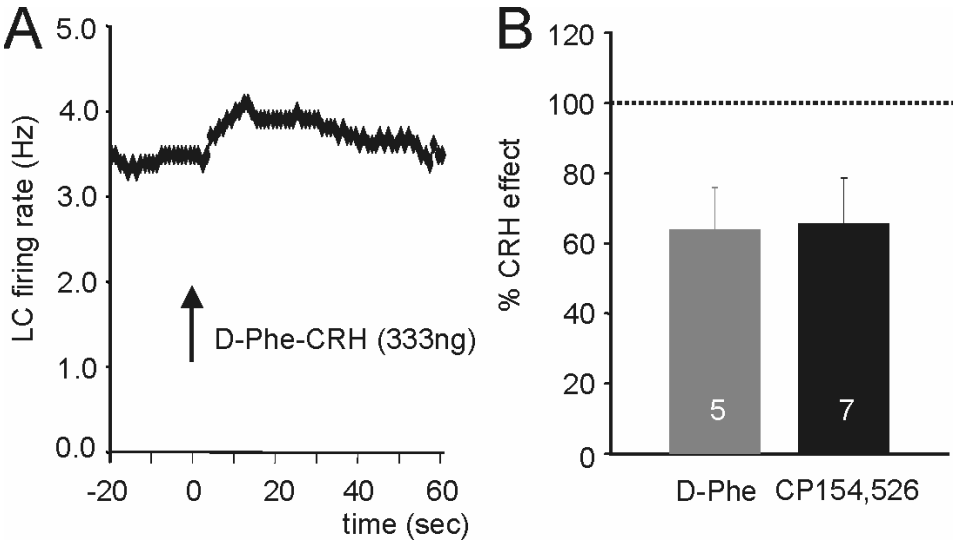

Figure 14 The magnitude of CRH-evoked increase in firing rate is reduced by CRH antagonists

(**A**) Local administration of D-Phe-CRH increases LC firing rate with a time course similar to the CRH-evoked activation of LC neurons. (**B**) Although bath application of the CRH antagonist D-Phe-CRH (1μM) also increased the spontaneous firing rate of LC neurons, it reduced the magnitude of the effect of CRH. Bath application of CP154,526 (1-100μM) reduced the effect of CRH without increasing the basal firing rate. The numbers on each bar indicate the number of neurons contributing to the average.

3.4 DISCUSSION

3.4.1 Site of action of CRH on LC neurons

Valentino and co-workers first demonstrated that CRH increases LC FR *in vivo* (Valentino et al., 1983) and have long hypothesized that CRH acts within the LC based on the fact that 1] CRH applied into the LC exerts a more potent effect than ICV CRH and 2] the effect of ICV-administered CRH and certain stressors can be blocked by infusion of a CRH antagonist into the LC (Valentino and Wehby, 1988; Valentino et al., 1991; Smagin et al., 1995; Curtis et al., 1997; Lechner et al., 1997; Smagin et al., 1997). In the present experiments we clearly demonstrate that CRH can activate LC neurons directly even when synaptic activity is prevented. The possibility of a direct action of CRH on LC neurons is further supported by ultrastructural evidence for synaptic contacts between LC dendrites and CRH immunoreactive terminals and by the presence of CRH1 receptors on LC neurons (Van Bockstaele et al., 1996a, 1998a; Van Bockstaele et al., 1999; Fox et al., 2002). The present data demonstrating a clear direct excitatory effect of CRH on LC neurons *in vitro* do not, however, rule out the possibility of additional presynaptic actions of CRH *in vivo*. Others have argued that CRH would increase presumed NE efflux through an indirect mechanism (Palamarchouk et al., 2000), although several alternative interpretations of these results may account for their findings. For example, based on the caudal injection sites in LC in their study, CRH would have to diffuse a much greater distance to its receptor if one considered the point of CRH innervation in pericoerulear area (Van Bockstaele et al., 1996a, 1998a; Van Bockstaele et al., 1999), compared to glutamate, which would be expected to act on its receptors at any

site along the dendritic tree (Van Bockstaele and Colago, 1996). In addition, faster diffusion characteristics for glutamate based on lower molecular weight (Nicholson and Tao, 1993), and a very short half-life for glutamate in the extracellular space due to an efficient uptake system (Danbolt, 2001) could further contribute to the difference in time course of glutamate and CRH-evoked NE efflux. The fact that in the present experiments the CRH-evoked activation reaches a maximum within approximately 15sec following CRH administration further suggests that differences in diffusion kinetics underlie the difference in time course of NE efflux that these authors observed.

3.4.2 Potential mechanism of CRH action: Potassium conductances

Consistent with a CRH-evoked increase in FR of LC neurons, CRH administration depolarized LC neurons when spike discharge was prevented by hyperpolarizing current injection. The reversal potential of the CRH-evoked depolarization was more hyperpolarized than the reversal potential for potassium predicted by the Nernst equation (-88mV at 35˚C and $[K^+]_o$=6.2mM; (Egan et al., 1983), and was accompanied by an increase in input resistance. The combination of an increase in input resistance and a reversal potential of –115 mV suggests that the depolarizing effect of CRH is largely mediated by a decrease in potassium conductances. The fact that CRH likely acted on sites electrotonically distant to the soma could account for the discrepancy between the reversal potential for the CRH-evoked depolarization and that for potassium. LC neurons in general, and LC neurons in horizontal slices in particular, are known not to be electrotonically compact (Ishimatsu and Williams, 1996; Travagli et al., 1996). Given that a large part of the CRH innervation of LC neurons occurs in the peri-coerulear area, i.e. distal to the soma, one would predict that the reversal potential of an effect measured

at the somatic recording site would overestimate the reversal potential of an event that occurs at electrotonically distal sites. However, as reported previously (Brown et al., 1971), it is also possible that other conductances with a positive reversal potential are contributing to the CRH-evoked depolarization, resulting in a reversal potential more negative than that for potassium. Thus, the closing of an inward cation conductance in addition to the opening of a potassium conductance has been suggested for the enkephalin- and muscarine-mediated hyperpolarization of LC neurons (Shen and North, 1992a; Alreja and Aghajanian, 1994), whereas the opposite has been suggested for the substance P-mediated depolarization of LC neurons (Shen and North, 1992b; Koyano et al., 1993). Therefore, the present data suggest that a potassium conductance carries the majority of the CRH-evoked response, but cannot exclude contribution of other conductances in the CRH-evoked depolarization.

The effect of CRH on LC neurons could not be blocked by the specific blocker for $I_{K(Ca)}$, apamin. The concentration of apamin used was effective in reducing the amplitude of the AHP and has been demonstrated to be effective in blocking a $I_{K(Ca)}$ current involved in the intermediate component of the afterhyperpolarization in LC neurons (Osmanovic et al., 1990; Osmanovic and Shefner, 1993). The blockade of $I_{K(IR)}$ did not prevent the effect of CRH on LC neurons even though barium clearly blocked the inward rectification. These data suggest that CRH modulates LC neuron activity via a different type of potassium conductance than has been suggested for corticotropes and neurons in hippocampus and amygdala (Aldenhoff et al., 1983; Siggins et al., 1985; Aldenhoff, 1986; Rainnie et al., 1992; Kuryshev et al., 1997).

Several neurotransmitters influence LC activity via modulation of potassium conductances. Thus, NPY, GAL, SOM, ENK and NE all reduce LC activity by increasing an inward rectifying potassium conductance (Williams et al., 1985; Inoue et al., 1988; Williams et al., 1988; Illes et al., 1993; Pieribone et al., 1995), while orexin/hypocretin, substance P (SP), and VIP increase LC activity by decreasing a potassium conductance (Wang and Aghajanian, 1990; Koyano et al., 1993; Ivanov and Aston-Jones, 2000). Based on occlusion experiments, it has been suggested that ENK, NE, SP, SOM, and NPY affect the same inward rectifying potassium conductance, even though their effect is mediated by different receptors (Aghajanian and Wang, 1987; Illes and Regenold, 1990; Velimirovic et al., 1995). It has been reported that SP receptors colocalize with Kir2.2 potassium channels in LC neurons (Stonehouse et al., 1999). Future experiments will have to address whether the main effect of CRH is also achieved by affecting this potassium conductance.

3.4.3 CRH effects are mediated by second messenger cascade

The excitatory effect of CRH, established immediately following penetration of the neuron, was prevented by the intracellular PKA inhibitor, Rp-cAMPS. Rp-cAMPS treatment also blocked spontaneous spike discharge of the neuron, as was previously shown following PKA inhibition (Alreja and Aghajanian, 1991b), although action potentials could still be elicited by depolarizing current injection. Similarly, extracellular application of a membrane permeable ADC inhibitor blocked the spontaneous activity of the neuron and reduced the CRH-evoked depolarization. These data suggest that the effect of CRH on the electrophysiological activity of LC neurons *in vitro* is completely mediated by activation of the ADC-cAMP-PKA second messenger cascade, consistent

with observations of the effect of Rp-cAMPS on the effect of CRH in a CATHa cell line (Bundey and Kendall, 1999). Since the binding site for cyclic nucleotides is not very specific and is affected by cAMP analogs (Ingram and Williams, 1996; Bois et al., 1997), direct effects of Rp-cAMPS on cyclic nucleotide-gated channels should also be considered. The fact that intracellular inhibition of the second messenger system coupled to CRH receptors blocks the CRH-evoked activation of LC neurons is again consistent with a direct action of CRH on LC neurons.

3.4.4 Antagonists of CRH receptors

The effect of CRH administration was reduced by local or bath application of the CRH receptor antagonist D-Phe-CRH, although a complete blockade of the effect of CRH was not observed. Since D-Phe-CRH is a competitive antagonist, local application of small volumes of agonist in high concentration may have effectively displaced a sufficient amount of antagonist to elicit some excitatory effect. In addition, we noted a significant activation of LC neurons by local or bath application of the antagonist alone, and the time course of activation by local antagonist administration was similar to that of the CRH-evoked activation. Such data are consistent with a partial agonist action of D-Phe-CRH, perhaps similar to the effect of higher concentrations of D-Phe-CRH reported for dorsal raphe neurons (Kirby et al., 2000) but not in the LC (Curtis et al., 1994; Curtis et al., 1999). However, *in vivo* a partial agonist effect of D-Phe-CRH in the LC could potentially be masked by a low baseline CRH tone. Partial agonist effects of α-helical CRH have been reported previously *in vitro* (Rainnie et al., 1992; Yu and Shinnick-Gallagher, 1998; Smart et al., 1999) and *in vivo* (Menzaghi et al., 1994; Borsody and Weiss, 1996). Regardless of their potential partial agonist effect, the CRH antagonists

reduced the magnitude of the CRH-evoked increase in LC FR. Given that very recently a novel CRH receptor has been characterized in catfish (Arai et al., 2001), the possibility of a novel type of CRH receptor should be considered (Bittencourt and Sawchenko, 2000; Van Pett et al., 2000), although previous *in vivo* studies suggest that the receptor mediating the effect of CRH in LC can be blocked by D-Phe-CRH (Curtis et al., 1994; Curtis et al., 1997). The receptor subtype-specific antagonist CP154,526 did not cause a change in basal FR, but due to the poor solubility of this compound in aqueous physiological solutions it was not possible to determine whether the effect of CRH could be completely prevented by this antagonist. Nevertheless, at the doses used, CP154,526 reduced the effect of CRH suggesting that CRH affects LC neurons *in vitro* at least in part via CRH_1 receptors. This finding is consistent with recent evidence demonstrating CRH_1 receptor immunoreactivity in LC (Sauvage and Steckler, 2001; Fox et al., 2002) and evidence demonstrating that systemic CP154,526 could reduce the increase in LC FR evoked by ICV CRH (Schulz et al., 1996).

3.4.5 Relevance to stress-related psychiatric disorders

The noradrenergic system of the LC has long been implicated in the pathophysiology of mood and anxiety disorders (Charney et al., 1995; Wong et al., 2000). Increased CSF levels of CRH, which primarily reflect CRH activity at extrahypothalamic sites (Kalin et al., 1987), have been reported in several studies of patients with major depression, PTSD, obsessive compulsive disorder (OCD), and Tourettes's Syndrome (see (Arborelius et al., 1999) for review). Furthermore, chronic treatment with different types of antidepressants alters the interaction between CRH and LC neurons (Curtis and Valentino, 1994). Therefore, the direct link between the CRH and noradrenergic systems

reported in the present study further supports the notion that the interaction between these transmitters systems plays an important role in the pathophysiology of mood and anxiety disorders.

4 CHRONIC EXPOSURE TO COLD STRESS ALTERS ELECTROPHYSIOLOGICAL PROPERTIES OF LOCUS COERULEUS NEURONS RECORDED *IN VITRO.*

4.1 INTRODUCTION

The neurons of the LC, tightly clustered at the floor of the fourth ventricle, provide the major source of NE in the central nervous system via their projections throughout the entire neuraxis (Foote et al., 1983; Moore and Card, 1984). NE released from LC neurons is thought to play an important role in attention and arousal (Robbins, 1984; Aston-Jones et al., 1999; Berridge, 2001) and alterations in central noradrenergic function have been implicated in the pathophysiology of multiple mood and anxiety disorders (Charney et al., 1990; Aston-Jones et al., 1994; Charney et al., 1995; Bremner et al., 1996; Wong et al., 2000).

The noradrenergic system is postulated to play a primary role in an organism's response to stress (Stanford, 1995). Thus, it is well-established that acute stress exposure can increase the discharge activity and NE release from noradrenergic LC neurons (Korf et al., 1973; Abercrombie and Jacobs, 1987; Abercrombie et al., 1988). Furthermore, chronic exposure to stress can alter the response of LC neurons to subsequent stress exposure (for reviews see (Stanford, 1993; Stanford, 1995; Zigmond et al., 1995). For example, the activity of the rate-limiting enzyme in the synthesis of NE, TH, is increased in the LC following chronic exposure to cold, social stress, social isolation, or repeated exposure to restraint, or foot shock (Zigmond et al., 1974; Stone et al., 1978; Angulo et al., 1991; Nisenbaum et al., 1991; Watanabe et al., 1995; Rusnak et al., 1998). Furthermore, the stress-evoked turnover of NE is enhanced following repeated exposure to foot shock or restraint (Thierry et al., 1968; Irwin et al., 1986; Adell et al., 1988;

Anisman and Zacharko, 1990), and the evoked increase in LC spike firing is enhanced following chronic exposure to cold, repeated exposure to foot or tail shock but not to white noise (Simson and Weiss, 1988; Curtis et al., 1995; Conti and Foote, 1996; Mana and Grace, 1997; Jedema et al., 2001). We have focused on cold exposure as a chronic stressor because the alterations in central noradrenergic function that occur in rats following chronic cold exposure resemble those observed in humans afflicted with mood and anxiety disorders (Aston-Jones et al., 1994; Charney et al., 1995; Wong et al., 2000). Thus, in addition to the alterations in TH (Zigmond et al., 1974; Nisenbaum et al., 1991; Melia et al., 1992) and evoked LC spike firing (Mana and Grace, 1997; Jedema et al., 2001) described above, chronic exposure to cold also increases the evoked release of NE from nerve terminals in the hippocampus or mPFC (Nisenbaum et al., 1991; Gresch et al., 1994; Finlay et al., 1997; Jedema et al., 1999) and the increased responsivity of the LC noradrenergic system persist for many days following removal from the cold (Ramsooksingh et al., 2001). We have referred to this phenomenon as stress-induced sensitization of NE neurons.

Given that sciatic nerve stimulation activates LC neurons via a mechanism mediated by excitatory amino acids (Ennis et al., 1992) and is *not* mediated by CRH (Valentino et al., 1991), we hypothesized that either: 1] cold exposure leads to sensitization of multiple pathways that activate LC neurons, or 2] that the LC neurons themselves are more responsive to excitatory input in general following chronic cold exposure (Jedema et al., 2001). If the sensitized response occurs at the level of the LC neuron itself, then the enhanced responsiveness could be maintained in the *in vitro* brain slice preparation.

The present experiments used intracellular recording techniques to examine whether the sensitization of NE neurons observed previously *in vivo* is also present *in vitro* in brainstem slices containing the LC. Specifically, we examined both basal electrophysiological properties and excitability of LC neurons in slices obtained from control rats and rats previously exposed to cold. In addition, we compared the morphology of the recorded neurons from both groups.

4.2 METHODS

4.2.1 Animals

Male Sprague-Dawley rats (Hilltop, Scottdale, PA) weighing 50-100g at the time of arrival were housed singly in hanging stainless steel cages in a colony room maintained at an ambient temperature of 23°C. Throughout the experiments, lights were maintained on a 12 hr light/dark cycle (lights on at 08.00 a.m.), with food (Laboratory rodent diet 5001, PMI Feeds, St. Louis, MO) and water available *ad libitum*. All rats were housed in the colony room for 5-10 days prior to any treatment. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh and were in accordance with the USPHS *Guide for the Care and Use of Laboratory Animals*.

4.2.2 Cold Exposure

Rats (135-200g) were randomly assigned to a control or a cold-exposed group. Control rats were housed singly in hanging stainless steel cages in a colony room maintained at an ambient temperature of 23°C for 2 weeks. The effects of chronic cold exposure on adrenal TH are more profound in shaved than unshaved rats (Fluharty et al., 1983) despite adequate thermal homeostasis (Moore et al., 2001). Therefore, the body fur

of rats in the cold exposure group was shaved from the rump to the forelimbs immediately prior to cold exposure. These rats were housed singly in hanging stainless steel cages in a cold room maintained at an ambient temperature of 5°C, where they remained undisturbed for 2 weeks. Cold-exposed rats were removed from the cold room the afternoon prior to the experiment and housed overnight in a colony room maintained at an ambient temperature of 23°C, thereby maintaining a protocol similar to that used previously for studying stress-induced sensitization of NE neurons (Nisenbaum et al., 1991; Gresch et al., 1994; Finlay et al., 1997; Mana and Grace, 1997; Jedema et al., 2001).

4.2.3 Tissue preparation

Rats (190-300g) were anesthetized with chloral hydrate (400 mg/kg, i.p.) and perfused through the ascending aorta with an ice-cold, oxygenated (low Na/high sucrose) perfusion solution (1.9 mM KCl, 1.2 mM Na_2HPO_4, 6 mM $MgCl_2$, 33 mM $NaHCO_3$, 20 mM glucose, 229 mM sucrose saturated with 95% O_2/5% CO_2) (Aghajanian and Rasmussen, 1989). Following decapitation, the brain was rapidly removed, placed in cold perfusion solution and 300 μm thick horizontal slices containing the LC were prepared using a DSK Microslicer (Ted Pella). Tissue was transferred to cold, oxygenated aCSF (124 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 2.4 mM $CaCl_2$, 1.3 mM $MgSO_4$, 26 mM $NaHCO_3$, 10 mM glucose saturated with 95% O_2/5% CO_2) (Lavin and Grace, 1998). After a recovery period of a minimum of 60-90 min, sections were transferred to the recording chamber where they were superfused with oxygenated aCSF at a flow rate of 0.8-1.5 ml/min at 35°C.

4.2.4 Electrophysiology

Intracellular recordings were obtained from neurons in the LC that were tentatively identified by their location within the trans-illuminated slice. Electrodes were filled with biocytin (2% biocytin in 2-3M $KC_2H_3O_2$) and had an impedance of approximately 50-100 MΩ. Putative LC neurons were identified based on a regular pattern of low frequency discharge of long duration action potentials, as described in previous studies (Andrade et al., 1983; Williams et al., 1984; Ivanov and Aston-Jones, 1995). Input resistance was determined from current-voltage relationships using hyperpolarizing current steps (0.02-0.4nA, 200msec, 0.2Hz). Following recording of basal activity for 3-10min, small depolarizing current pulses (0.05-0.4nA; 500msec, 0.5 Hz, 4 current steps at each intensity with different intensities delivered at 20-30 sec intervals) were applied through the recording electrode in order to examine the neuronal excitability. At the end of each recording session, biocytin was ejected into the recorded cell for histological verification. Only one neuron per slice was examined.

4.2.5 Immunocytochemistry

Sections were stored in 4% paraformaldehyde or 10% buffered formalin solution for a minimum of 1 week. Sections were rinsed several times in phosphate buffer (PB; 0.1M; pH 7.2-7.4) or tris-buffered saline solution (TBS; 0.1M; pH 7.6) before and between the following steps. Following a freeze-thaw procedure using decreasing concentrations of cryoprotectant and overnight incubation in a 1% Triton X-100 in TBS solution, tissue sections were incubated in avidin-biotin peroxidase complex (ABC; 1:200; Vectastain Elite kit; Vector Laboratories) in TBS. The peroxidase reaction was visualized by incubating the sections in 0.022% diaminobenzidine (DAB), 0.3% $NiSO_4(NH_4)_2SO_4$ and 0.003% hydrogen peroxide in TBS for 6-7 min. The peroxidase

reaction was terminated by several rinses in TBS. Subsequently, the tissue was processed for TH-immunoreactivity. Following incubation for 30 min in blocking solution (BS) containing 3% normal goat serum, 0.4% Triton X-100, and 1% bovine serum albumin in TBS, pH 7.6, sections were incubated overnight in BS containing a monoclonal antibody raised in mouse against TH (1:10,000; Chemicon). Following rinses in TBS, tissue was incubated in secondary antibody (1:400; BA2001, Vector Laboratories) in BS for 30 min, rinsed in TBS, and incubated in ABC. The peroxidase reaction was visualized by incubation in 0.022% DAB and 0.003% hydrogen peroxide in TBS for ~5 min. Finally, sections were mounted on gelatin-coated glass slides, dehydrated through increasing concentrations of alcohols followed by xylene, and coverslipped, before verification that the location of the recorded neuron was indeed within the LC.

4.2.6 Data Analysis:

Stable basal activity of LC neurons was recorded for a minimum period of 3 min. Data for all action potentials for each individual neuron were averaged over the baseline period, and these values for each neuron were averaged for each treatment group. FR was expressed as the average number of spikes discharged per second. The average membrane potential was estimated from the reading of the Vm displayed on the amplifier within the first 30 sec of the penetration of the neuron. The action potential threshold was defined as the potential at which the greatest change in slope of the membrane potential occurred, which was determined from the second order derivative of the membrane potential waveform. The amplitude of the action potential and afterhyperpolarization were measured relative to this threshold, and the action potential duration was measured at the level of threshold. Statistical comparison of all parameters of basal activity between LC neurons from control and cold-exposed rats was performed using a *t*-test for

independent samples. The excitability of LC neurons in response to current injection was defined as the differential of the firing rate occurring during a depolarizing current step and the basal FR for a specific neuron. Statistical comparison was performed by Greenhouse-Geisser corrected ANOVA using group as factor and current intensity as a repeated measure. We expressed accommodation as the differential of the increase in instantaneous firing (reciprocal of ISI) above basal firing rate between the first two and last two action potentials during the train evoked by depolarizing current injection in order to account for any confounding effect due to differences in basal firing rate. The duration of the PAI was expressed as the ISI between the last action potential during and the first action potential following a 0.4nA current step. Statistical comparison of accommodation and PAI were performed using a *t*-test for independent samples. All statistical comparisons were performed using SPSS for Windows v10.1 (Chicago, IL) and the α-level was set to 0.05.

4.3 RESULTS

4.3.1 Morphology of LC neurons

Recordings were made from 66 spontaneously active neurons from 29 control and 17 cold-exposed rats. The majority of these neurons (44 out of 66) was filled with biocytin following the recording session and subsequently processed for double labeling immunocytochemistry against biocytin and TH. In all cases in which a cell was filled with biocytin, it was surrounded by TH-immunoreactive somata and processes (figure 15)

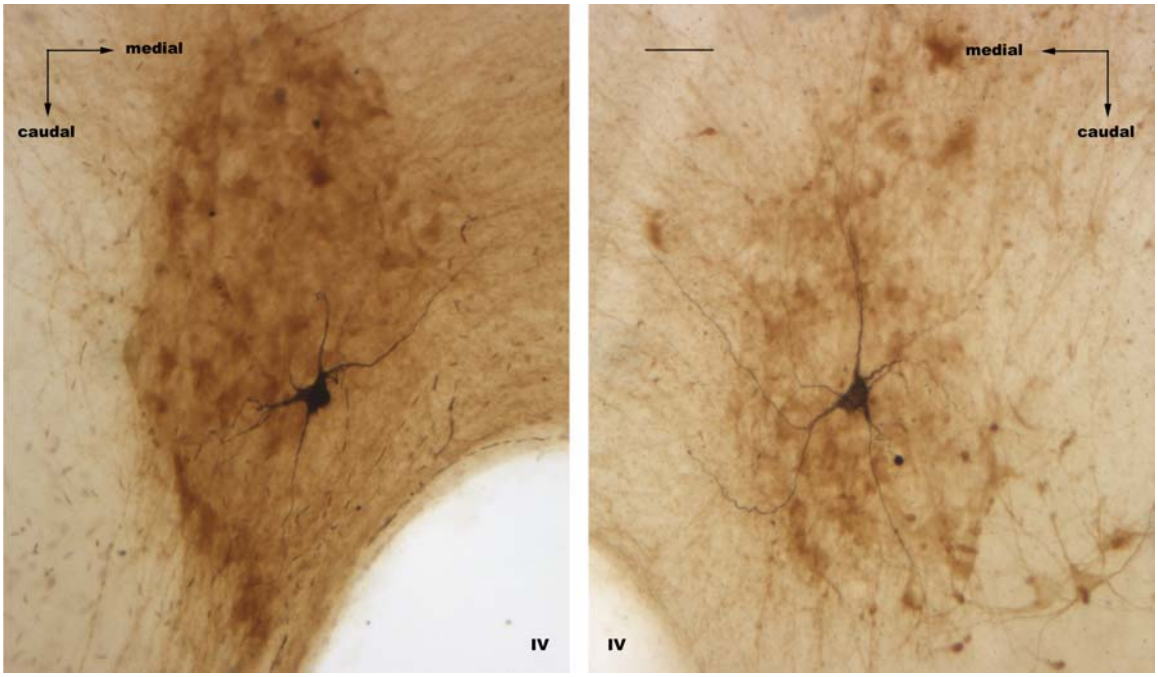

Figure 15 Identification and morphology of LC neurons recorded *in vitro*

(*left*) A representative example of an LC neuron filled with biocytin (black) in a horizontal brainstem slice obtained from a control rat. Immunoreactivity for tyrosine hydroxylase (brown) outlines the area of the locus coeruleus.

Representative examples of LC neurons from a control (*left*) and cold-exposed rat (*right*) demonstrate that LC neurons from control and cold-exposed rats have a similar morphology. LC neurons in slices from control rats had an average soma size of 26±1 μm with an average of 5.7±0.2 primary dendrites emanating from its perimeter (n=33), while LC neurons from in slices from cold-exposed rats had an average soma size of 25±2μm with an average of 5.6±0.4 primary dendrites emanating from its perimeter (n=11). Scale bar represents 50 μm for both photomicrographs.

demonstrating that the recorded neuron was indeed within the LC. The morphology of the biocytin-immunoreactive neurons was consistent with that of identified LC neurons based on previous anatomical studies (Swanson, 1976; Groves and Wilson, 1980b; Cintra et al., 1982) as well as that of LC neurons stained intracellularly in *in vivo* or *in vitro* preparations (Aghajanian and Vandermaelen, 1982; Travagli et al., 1996; Jedema and Grace, 1999): a disc-shaped or multipolar soma with 4-9 processes emanating from its outer edge. There were no gross morphological differences between LC neurons from previously cold-exposed rats and control rats. LC neurons in slices from control rats had an average soma size of 26±1 μm with an average of 5.7±0.2 primary dendrites emanating from its perimeter (n=33), while LC neurons from in slices from cold-exposed rats had an average soma size of 25±2 μm with an average of 5.6±0.4 primary dendrites emanating from its perimeter (n=11).

4.3.2 Basal discharge activity

The spontaneous discharge frequency of LC neurons in slices from control and cold-exposed rats was not significantly different (2.15±0.19Hz vs 2.81±0.55 Hz; n=43 and 23, respectively). There were no differences in any of the action potential waveform characteristics between slices from control and cold-exposed rats (table 2). No spontaneous post-synaptic potentials (excitatory or inhibitory) were observed.

4.3.3 Hyperpolarizing current injection

Hyperpolarizing current injection (duration 200-300msec) through the recording electrode resulted in an amplitude-dependent deflection of the steady-state membrane

Table 2: Action potential waveform characteristics of control and cold-exposed LC neurons

Action potential waveform characteristics (±SEM) for spontaneous action potentials in LC neurons in slices obtained from control and previously cold-exposed rats (n=14 for each group). No between-group differences were observed.

	Control	Cold
Resting membrane potential (mV)	-58.2 ± 2.0	-56.0 ± 1.4
Action potential threshold (mV)	-49.4 ± 2.5	-50.2 ± 1.9
Action potential amplitude (mV)	73.2 ± 2.0	75.3 ± 1.3
Afterhyperpolarization (mV)	19.0 ± 0.6	20.1 ± 0.9
Action potential duration (msec)	2.2 ± 0.1	2.1 ± 0.1

potential (figure 16). Neurons from both cold-exposed and control rats exhibited inward rectification of the conductance, which is characteristic for LC neurons (Williams et al., 1984). The current-evoked steady-state membrane potential deflection was greater in LC neurons from cold-exposed rats [$F(1,304)=9.101$, $p=0.007$) compared to control rats (figure 17). The difference in membrane potential deflection between cold-exposed and control neurons varied linearly with the intensity of current injection in the range tested (-0.5 to 0.0 nA). The input resistance as determined from the instantaneous slope of the steady-state current-voltage relationship was greater in neurons from cold-exposed rats compared to control rats [$F(1,304)=4.948$, $p=0.038$), and this effect was independent of the amplitude of the current injection (figure 17).

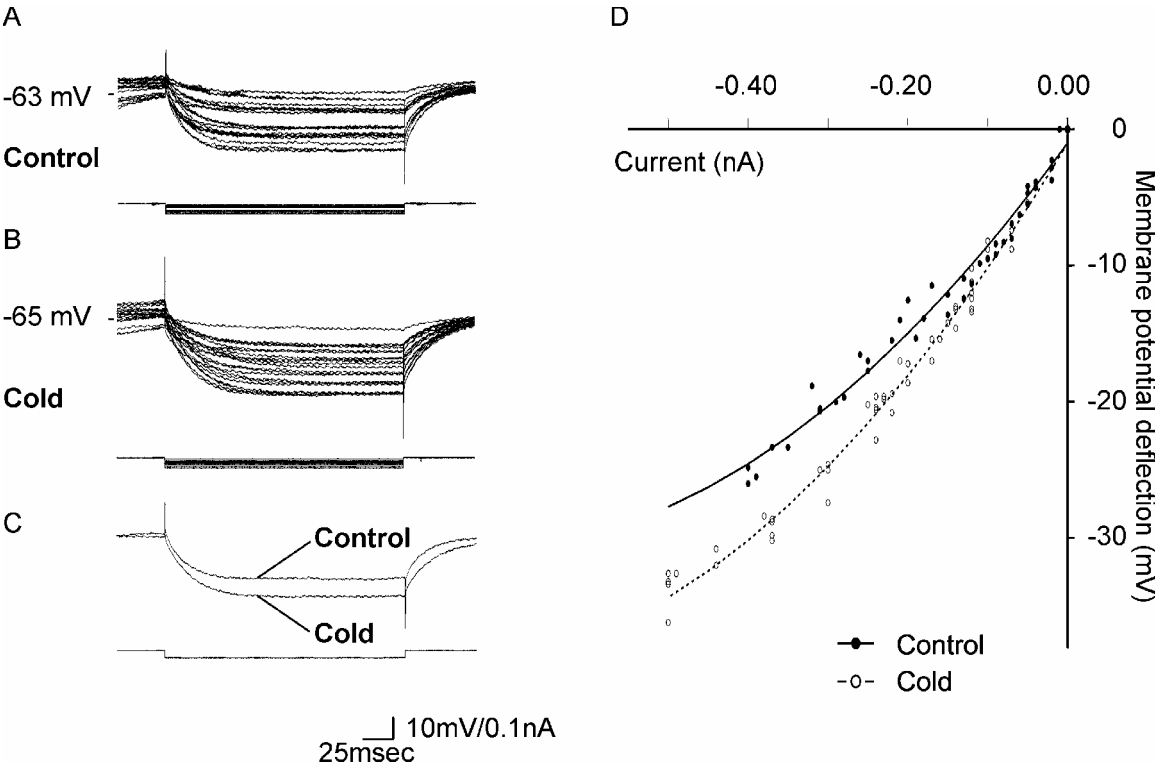

Figure 16 Current-voltage relationship of LC neurons from a control and a cold-exposed rat

Hyperpolarizing current steps (0-0.5nA, 200msec) delivered through the recording electrode caused a deflection of the membrane potential in LC neurons from a control (**A**) and cold-exposed rat (**B**). **C**) Overlay of traces from a neuron of a control and cold-exposed rat shows the larger deflection in the neuron of the cold-exposed rat. The values plotted in the current-voltage plot (**D**) were acquired at membrane steady-state, which was more than 125 msec following the initiation of the current pulse. The steady-state deflection of the membrane potential varied with the amplitude of current injection in a non-linear manner indicative of inward rectification. The slope resistances at the zero-current level calculated from the (quadratic) regression line were 81 MΩ and 98 MΩ for the control and cold-exposed rat, respectively.

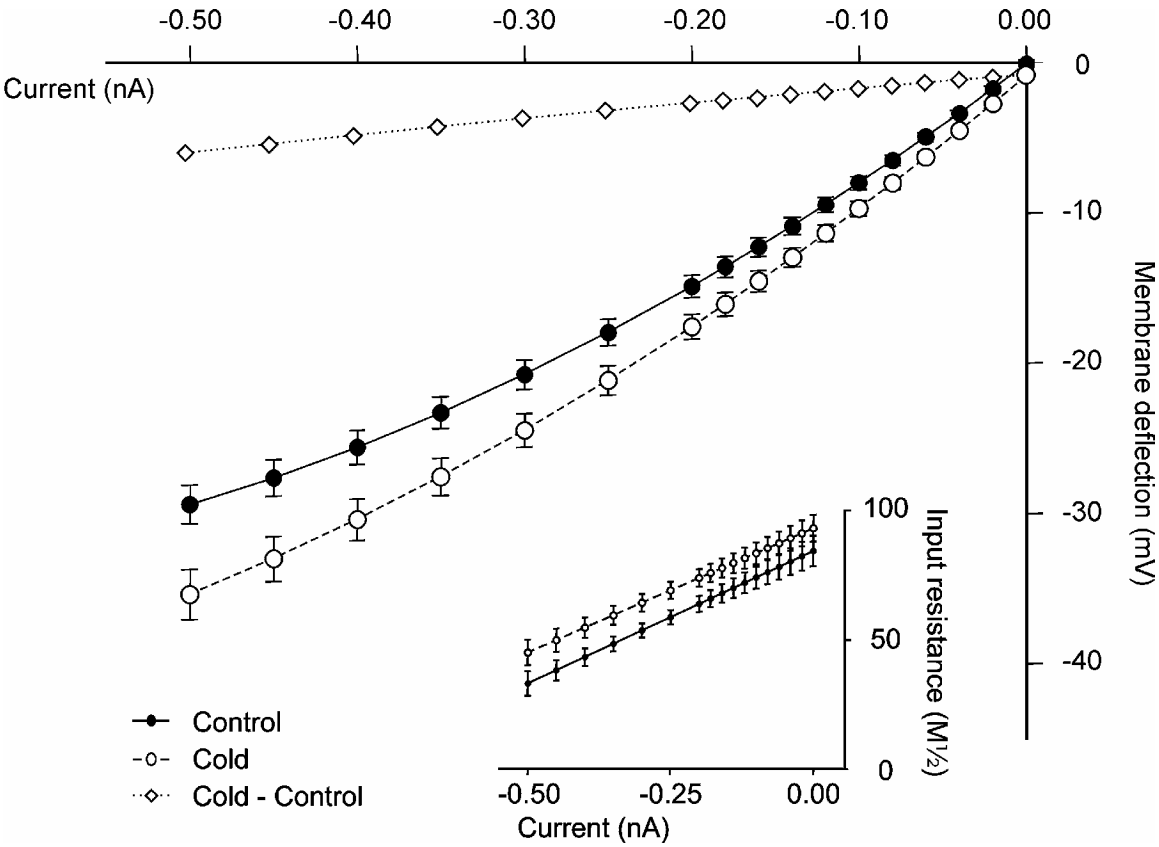

Figure 17 Input resistance of LC neurons from control and cold-exposed rats

Plotting the amplitude of hyperpolarizing current steps (200-300msec) against the resultant steady-state deflection of the membrane potential for all neurons revealed that LC neurons from cold exposed rats exhibit a larger deflection than control rats. The differential in voltage deflection between cold-exposed and control rats varies with the amplitude of current injection in a linear manner. (*Inset*) The slope resistance of neurons of cold-exposed rats is larger compared to control. The average slope resistance at the zero-current level is 84±6 and 93±5 MΩ for control (n=12) and cold-exposed rats (n=9), respectively.

4.3.4 Depolarizing current injection

In response to intracellular depolarizing current injection (duration 500msec), an amplitude-dependent increase in the discharge activity above basal activity was observed, with larger current steps eliciting larger increases in discharge activity for neurons from both control and previously cold-exposed rats (figure 18). Neurons from cold-exposed rats showed a progressively larger increase in activation compared to neurons from control rats as evidenced by a significant group by current interaction [$F(7,44)=3.849$, $p=0.044$].

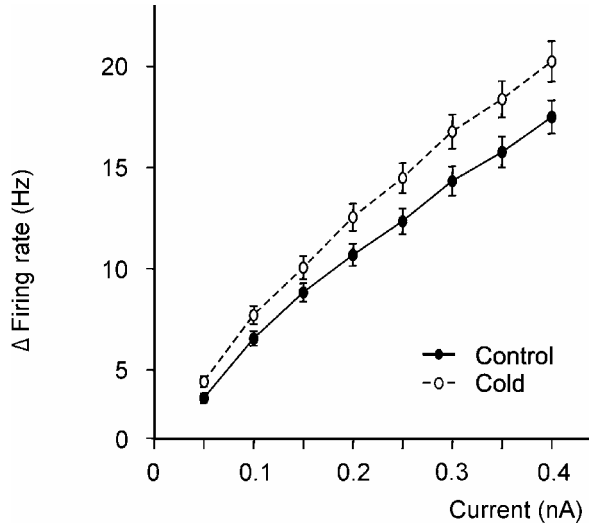

Figure 18 Excitability of LC neurons from control and cold-exposed rats

In response to an intracellular depolarizing current step (500msec), LC neurons from cold-exposed rats exhibited a larger increase in firing rate above baseline compared to control rats [$F(7,44)=3.849$, $p=0.044$; n= 20 and 26 respectively].

When sufficient depolarizing current was injected through the electrode, multiple spikes could be elicited with progressively longer ISI's; a process referred to as accommodation. The distribution of spikes during a current step, adjusted to evoke the same number of spikes above baseline, was different between neurons from cold-exposed and control rats. Specifically, when spike trains elicited by current steps adjusted to evoke six spikes were compared, the accommodation was significantly less in neurons from cold-exposed rats compared to control rats (figure 19). Thus, neurons from cold-exposed rats exhibited a smaller decrease in instantaneous firing rate above baseline (inverse of ISI minus basal FR) during the current step compared to neurons obtained from control rats. The instantaneous firing rate above baseline for the last spike pair during the current step was 49±2% of that for the first spike pair in neurons from cold-exposed rats versus 44±1% in control rats [$t(36)= 2.328$; $p= 0.026$] (Fig.6). The PAI, or time necessary to resume firing following a depolarizing current step eliciting the same number of spikes, appeared to be shorter in neurons from cold-exposed rats compared to those from controls (2227±359 msec versus 3151±360 msec; n= 16 and 20, respectively; figure 20); however, this difference was not significant ($p=0.069$) due to the large between-subject variation within both treatment groups.

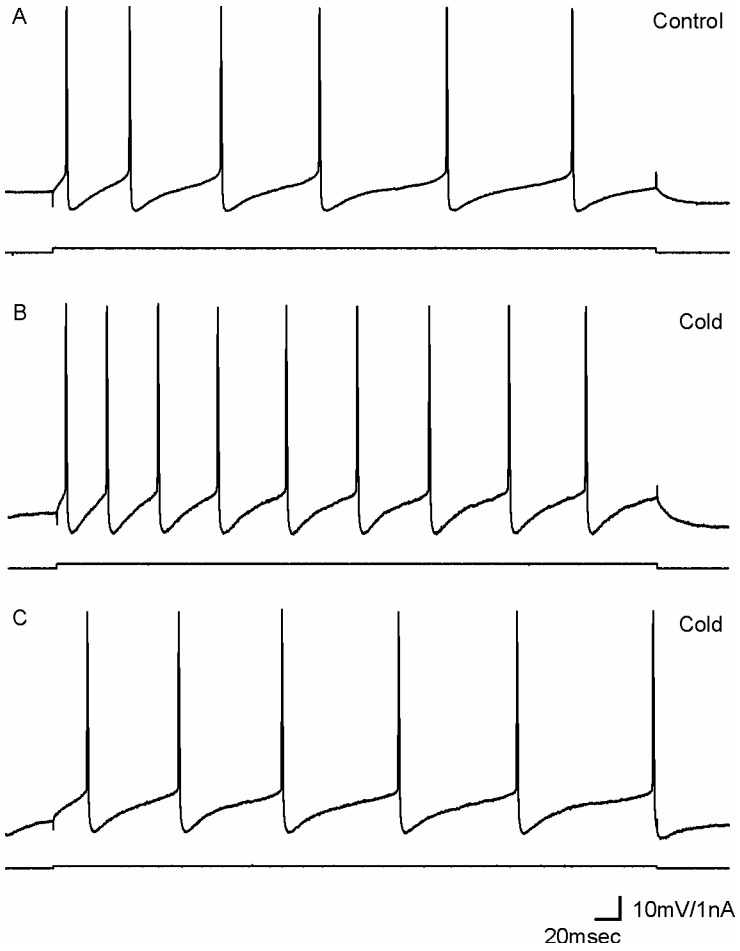

Figure 19 Example of trains of action potentials of LC neurons from control and cold-exposed rats evoked by sustained depolarizing current injection

In response to an intracellular depolarizing current step of equal amplitude (0.2nA), LC neurons from cold-exposed rats (**B**) discharged more action potential than control neurons (**A**). When the amplitude of current injection was adjusted to achieve the same level of excitability (i.e. the same number of action potentials), the neurons from the cold-exposed rat (**C**) exhibit less accommodation of spike firing than neurons from control rats. Basal firing rates were 2.5 and 2.6 Hz for the neurons from the control and cold-exposed rat, respectively.

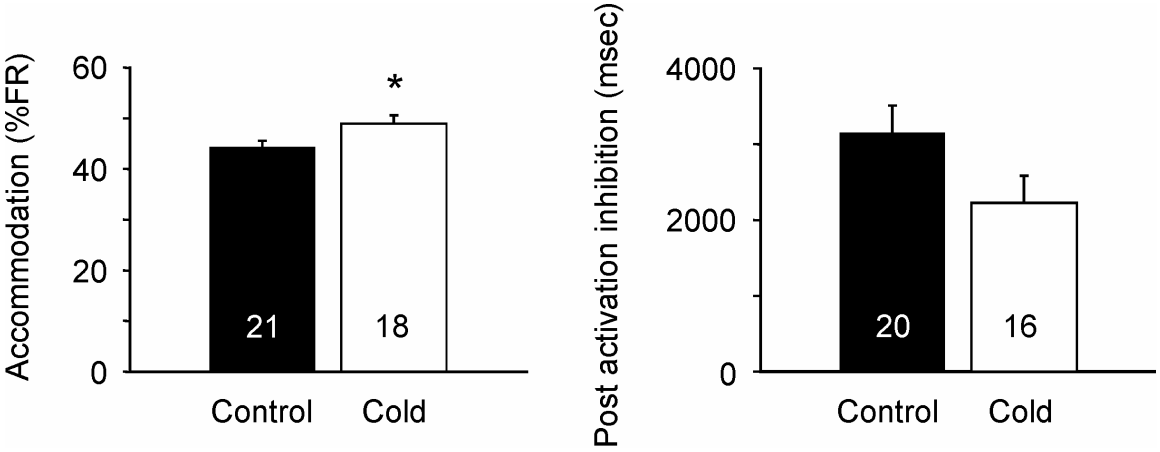

Figure 20 Evoked spike train characteristics of LC neurons from control and cold-exposed rats

(*Left*) In response to a depolarization of the membrane, LC neurons discharged trains of action potentials that exhibited accommodation, which is observed as a decrease in instantaneous firing rate above baseline from the first to the last spike during a current step (expressed as percentage of initial instantaneous firing rate). LC neurons in slices from control rats showed a larger decrease in instantaneous firing rate: instantaneous firing rate at the last spike over the first spike in control neurons was significantly less compared to neurons from cold-exposed rats. (*Right*) LC neurons typically show a post-activation period of inhibition. The duration of inhibition appeared shorter in neurons from cold-exposed rats, although this difference was not statistically significant as a result of large between-subjects variation within groups.

4.4 DISCUSSION

The present data demonstrate that, following chronic cold-exposure, LC neurons recorded *in vitro* exhibit an increased excitability without major alterations in basal firing rate. These data extend those of previous *in vivo* experiments demonstrating stress-induced sensitization of NE neurons (Nisenbaum et al., 1991; Gresch et al., 1994; Finlay et al., 1997; Mana and Grace, 1997; Jedema et al., 1999; Jedema et al., 2001). The fact that an increased excitability without alteration of basal activity is found in the present *in vitro* experiments, in which presynaptic input does not play a significant role, supports the hypothesis that the sensitization of NE neurons observed *in vivo* is, at least in part, mediated by an alteration of the LC neurons themselves (Jedema et al., 2001).

4.4.1 Chronic cold exposure

Chronic cold exposure, as used in the present study, is a complex stressor consisting of shaving, relocation to another room, return to a room at room temperature prior to testing, etc. We feel that the cold exposure itself is the most likely cause for the alterations in central noradrenergic function that is studied. Thus, we demonstrated that shaving followed by intermittent cold-exposure or continuous cold-exposure for only one week does not result in sensitization of NE release or electrophysiological activity of LC neurons (Finlay et al., 1997; Mana and Grace, 1997; Jedema et al., 1999), suggesting that neither shaving, room changes nor removal from the cold room are responsible for the sensitization of NE neurons. Nevertheless, the possibility exists that an interaction of cold exposure and other factors is required to cause sensitization of LC neurons.

4.4.2 Basal discharge activity

The basal discharge activity of LC neurons in slices from both control and previously cold-exposed rats is similar to that observed in our *in vivo* experiments (Mana and Grace, 1997; Jedema et al., 2001). In addition, the values obtained for basal LC activity in slices from control rats were consistent with previously reported values from other *in vitro* experiments (Andrade et al., 1983; Williams et al., 1984; Ivanov and Aston-Jones, 1995). Furthermore, the action potential waveform characteristics of spontaneously occurring action potentials of LC neurons from both control rats and cold-exposed rats were similar to each other and they were completely consistent with those reported previously (Andrade et al., 1983; Williams et al., 1984; Travagli et al., 1996).

4.4.3 Hyperpolarizing current injection

The amplitude of the steady-state membrane potential deflection produced in response to hyperpolarizing current injection increased with the current injection amplitude in a non-linear manner, which could be well described by a quadratic function. LC neurons show a clear inward rectification as previously described (Williams et al., 1984; Williams et al., 1988). This rectification is thought to be a result of the inward rectifying properties of potassium conductances (Williams et al., 1984; Williams et al., 1988; Grigg et al., 1996). The input resistance in control slices was lower than the majority of previously published findings. However, it should be noted that in the present study brainstem slices were used that were sectioned in the horizontal plane. It has been reported that the plane in which slices are sectioned greatly influences the number of remaining dendrites and the input resistance of LC neurons (Travagli et al., 1996), with horizontal sections allowing the greatest preservation of the dendritic arbor. Indeed, the

input resistance obtained in the present study in horizontal slices of control rats is similar to the values for LC neurons in horizontal slices estimated from the publication by Travagli and co-workers (Travagli et al., 1996). In addition, the input resistance in our slice experiments more closely approximate previous findings for input resistance of LC neurons obtained *in vivo* (Aghajanian and VanderMaelen, 1982; Jedema and Grace, 1999).

The membrane potential deflection produced in neurons from cold-exposed rats was significantly greater when compared to control rats. This difference in membrane potential deflection varied linearly with the amplitude of current injection, which would suggest that a change in non-rectifying conductances underlies the observed difference between neurons from cold-exposed and control rats. Although the amplitude of hyperpolarization did not extend into the voltage range where inward rectification is most prominent (Williams et al., 1988), inward rectification was evident in the present recordings in both control and cold-exposed preparations (i.e. figures 16-17). The difference in input resistance between LC neurons from control and cold-exposed rats was independent of the amplitude of the hyperpolarizing current injected throughout the range of hyperpolarizing current steps tested, further indicating that the difference between neurons from cold-exposed and control rats was not caused by alteration of conductances exhibiting rectification in the voltage range tested.

4.4.4 Depolarizing current injection

A higher level of excitability was observed in LC neurons obtained from cold-exposed rats when compared to control rats, which was most apparent with larger amplitudes of depolarization. This was likely due, at least in part, to a change in membrane conductance since the input resistance of LC neurons from cold-exposed rats

was greater compared to control rats. The fact that higher amplitudes of current injection will evoke a greater number of action potentials, in combination with an increase in the input resistance, suggests that a decrease in potassium or chloride conductances is involved in the mechanism underlying the enhanced excitability that we have observed in neurons from chronically stressed rats. The chloride conductance evoked by GABA administration, however, is known to exhibit a strong outward rectification (Osmanovic and Shefner, 1990), suggesting that this conductance is not responsible for the observed differences between neurons from cold-exposed and control rats.

In addition to an increase in excitability, LC neurons from cold-exposed rats exhibited a smaller decrease in instantaneous firing rate over baseline (i.e. less accommodation) during a 500 msec "burst" evoked by depolarizing current injection. Nonetheless, there was no significant difference in the amplitude of the AHP between control and cold-exposed groups. Differences in AHP duration, on the other hand, were difficult to evaluate in spontaneously active LC cells, because the membrane potential increases in a continuous manner from the AHP into the depolarizing ramp that precedes the next action potential discharge. Even though only voltage clamp recordings will enable us to resolve which currents are altered by chronic cold exposure, one potential alteration that could contribute to a change in AHP duration could be the apamin-sensitive calcium-activated potassium conductance that plays an important role during the middle phase of the AHP as well as in the accommodation observed in LC neurons (Osmanovic et al., 1990; Osmanovic and Shefner, 1993). Apamin was reported to cause these changes in AHP and accommodation without changing the spontaneous firing rate or membrane potential of the majority of the LC neurons (Osmanovic et al., 1990). In a

few cases we tested the excitability of control neurons during bath application of apamin and observed an increase in excitability similar to what we observed in neurons of cold-exposed rats (Jedema and Grace, unpublished observations). Even though a reduction in a calcium-activated potassium conductance may potentially account for the increase in excitability, it is unlikely that this would explain the increased membrane potential deflection in response to hyperpolarizing current, given the requirement for calcium influx.

Alterations in autoreceptor function following chronic stress exposure that might result in changes in auto-inhibition have been described *in vivo* (Pavcovich et al., 1990; Pavcovich and Ramirez, 1991; Nisenbaum and Abercrombie, 1993; Flugge, 2000). Alpha-2 receptor activation causes a G-protein-mediated hyperpolarization of LC neurons via the opening of potassium channels (Aghajanian and VanderMaelen, 1982; Williams et al., 1985; Arima et al., 1998). This hyperpolarization is accompanied by a decrease of the input resistance, and the current-voltage relationship of this conductance is fairly linear (Williams et al., 1985). However, the role for α-adrenergic autoreceptors in the auto-inhibition of LC neurons *in vitro* is controversial (Andrade and Aghajanian, 1984; Ivanov and Aston-Jones, 1995), and it is unclear to what extend the basal activity of LC neurons *in vitro* is influenced by tonic α_2-receptor activation. Regardless, whether it is an α_2 receptor effect on potassium conductances or a calcium-activated potassium conductance, an alteration in auto-inhibition is a likely candidate that may contribute to the differences that we have observed *in vitro* between LC neurons from control and cold-exposed rats. It remains to be determined whether the difference in excitability observed in the present experiments can completely account for the enhanced excitability

observed in our *in vivo* experiments, or whether alterations in LC afferent input also play a role. Additional alterations to afferent input are suggested by recent *in vivo* studies from our laboratory demonstrating a prolonged or secondary excitation in addition to an enhanced excitation of LC neurons in cold-exposed rats in response to sciatic nerve stimulation (Mana and Grace, 1997; Ramsooksingh et al., 2001).

4.4.5 Conclusion & clinical implications

In summary, the present studies demonstrate an enhanced evoked activity of LC neurons following chronic cold exposure. Studies in humans have implicated an enhanced central noradrenergic activity in mood and anxiety disorders (Charney et al., 1990; Aston-Jones et al., 1994; Charney et al., 1995; Bremner et al., 1996; Wong et al., 2000). Given the proposed role for central NE in arousal and attention (Robbins, 1984; Aston-Jones et al., 1999; Berridge, 2001), an enhanced central noradrenergic activation could underlie the increased response to stress observed in post-traumatic stress disorder or the increased distractibility reported for attention deficit hyperactivity disorder (ADHD). Given other parallels between mood and anxiety disorders in humans and alterations in rats following cold exposure, the paradigm of continuous cold exposure and the resultant sensitization of LC neuron responses may represent a unique experimental model to study potential mechanisms involved in the alterations underlying these disorders. The fact that the changes in excitability occur *in vitro*, largely in absence of synaptic input, suggests that alterations integral to the LC neurons themselves underlie the enhanced noradrenergic function in cold exposed rats, and perhaps could contribute to the altered central noradrenergic function in mood or anxiety disorders observed in humans.

5 GENERAL DISCUSSION

The studies described in the previous chapters aim to further elucidate the mechanism(s) underlying chronic stress-induced sensitization of NE neurons. First, we determined that the enhanced increase of NE efflux in the mPFC evoked by ICV administration of CRH observed in chronically cold-exposed rats was accompanied by an enhanced increase of LC FR. The enhanced response to ICV CRH was apparent at higher doses, but not a lower doses of CRH, leading to a steeper slope of the dose-response relationship. Based on these data, it was hypothesized that sensitization of NE efflux is not solely a terminal phenomenon, as originally thought (Nisenbaum and Abercrombie, 1993), and that the electrophysiological response of LC neurons to multiple excitatory stimuli is enhanced (Jedema et al., 2001).

It has been questioned whether CRH has a direct action on LC neurons based on the difficulty to demonstrate CRH receptor mRNA in LC neurons in the rat (Chalmers et al., 1996). Because ICV administration of CRH is likely to increase LC firing rate via an action within the LC (Curtis et al., 1997), the effect of CRH on LC neurons recorded *in vitro* was subsequently characterized. Based on experiments demonstrating that the CRH-evoked activation of LC neurons recorded *in vitro* persists following blockade of synaptic activity and that intracellular inhibition of the seconds messenger cascade for CRH receptors inhibited the CRH-evoked activation, it was concluded that CRH has direct effects on LC neurons (Jedema and Grace, *Submitted*).

Because the response of LC neurons to multiple excitatory stimuli is enhanced, it was hypothesized that alterations in feedback pathways recruited following activation of LC neurons or alterations of intrinsic properties of LC neurons would, at least in part,

underlie the sensitization of LC neurons observed *in vivo* (Jedema et al., 2001). It was further hypothesized that, if alterations of intrinsic properties of LC neurons were involved in the mechanism underlying sensitization of LC neurons *in vivo*, these alterations could persist in the *in vitro* slice preparation (Jedema and Grace, *In Press*). In subsequent *in vitro* experiments it was demonstrated that the excitation of LC neurons evoked by intracellular current injection was enhanced following chronic cold exposure (Jedema and Grace, *In Press*). The findings summarized above have been discussed in the previous chapters. In the following paragraphs I will elaborate on the interpretations of these findings and discuss potential underlying mechanisms in further detail.

5.1 Basal activity of LC neurons

Baseline LC electrophysiological activity following chronic cold exposure was similar to control both in our *in vivo* and *in vitro* studies. Despite the discrepancy of these findings with the only report on LC firing rate following cold exposure (Mana and Grace, 1997), they are consistent with neurochemical data demonstrating similar baseline extracellular NE levels following chronic cold exposure in hippocampus and PFC (Nisenbaum et al., 1991; Gresch et al., 1994; Finlay et al., 1997; Jedema et al., 1999), and the observation that basal levels of tyrosine hydroxylation were similar to control following chronic cold exposure (Nisenbaum et al., 1991; Nisenbaum and Abercrombie, 1992). The slight but significant increase in baseline FR of LC neurons observed in the one previous study (Mana and Grace, 1997) may reflect differences in methodology or the fact that the basal firing rates of LC neurons in that study were not all independent of each other. Alternatively, the slight increase in basal firing rate may indicate that there is

an increase of LC FR along a continuum of activity that is simply more profound at higher (evoked) levels of activity. In this regard, it should be pointed out that across all studies using chronic cold exposure, basal levels of NE efflux or LC FR were higher, albeit in most cases not significantly, in all (Nisenbaum et al., 1991; Gresch et al., 1995; Mana and Grace, 1997; Jedema et al., 1999; Jedema et al., 2001; Jedema and Grace, *In Press*), but one study (Finlay et al., 1997). Furthermore, exposure to other chronic stressors, like tail shock, foot shock or restraint, increases baseline LC FR, although again these increases do not reach statistical significance in some cases (Weiss and Simson, 1988; Pavcovich et al., 1990; Curtis et al., 1995). Indeed, if the chronic stress-evoked sensitization of LC neurons is a consequence of alterations within LC neurons as we proposed (Jedema and Grace, *In Press*), one would have to hypothesize that this altered process is not at all involved in the spontaneous activity of LC neurons for this alterations not to affect basal NE efflux or LC FR.

5.2 Evoked activity of LC neurons

A clearly consistent finding across different studies in both anesthetized and awake rats following chronic cold exposure as well as different stressors is the enhanced evoked activity of LC neurons in response to excitatory stimuli (Simson and Weiss, 1988; Nisenbaum et al., 1991; Gresch et al., 1994; Curtis et al., 1995; Finlay et al., 1997; Mana and Grace, 1997; Jedema and Grace, 1999; Jedema et al., 2001). The fact that a sensitized response is present *in vitro* demonstrates that at least some of the physiological alterations underlying chronic stress-induced sensitization are maintained in the slice and are localized within LC neurons or their proximity. In addition, the enhanced response of

LC neurons to excitatory stimulation can not be an acute consequence of sensitization that occurs in the HPA axis or sympathetic nervous system following chronic stress exposure (Vernikos et al., 1982; Konarska et al., 1989). However, as discussed below (Section 5.4.3 and 5.5), chronic upregulation of ACTH and corticosterone could lead to long term alterations in channels or receptors within the LC that then lead to a sensitized response of LC neurons to subsequent stimulation. Furthermore, alterations in other brain regions may further contribute to the sensitization of LC neurons observed *in vivo*.

It has been demonstrated that in LC neurons, PKA activity and basal and evoked ADC activity are increased following 5-days of cold exposure as well as following chronic opiate treatment (Melia et al., 1992; Nestler et al., 1999). In addition, it has been demonstrated that ADC and PKA activity are important for the spontaneous pacemaker activity and VIP-evoked inward current in LC neurons (Wang and Aghajanian, 1990; Alreja and Aghajanian, 1991a, b). Based on these data it was hypothesized that the observed upregulation of PKA and ADC might underlie the enhanced excitability of LC neurons following chronic stress exposure or chronic opiate treatment (Nestler et al., 1999). However, our findings of an increased excitability combined with an increased input resistance of LC neurons following chronic cold exposure suggest that there is also a contribution of an inhibition of other (outward) conductances to the alterations observed following chronic cold.

The enhanced response of LC neurons *in vitro* was evoked by current injection, thus by-passing any receptors that mediate a pharmacological excitation. These data indicate that stress-induced sensitization of NE neurons is not dependent on enhanced sensitivity of one or more excitatory receptors. Based on the steeper slope of the dose-response

relationship for CRH, we hypothesized that stress-induced sensitization of NE neurons was a consequence of an alteration in a feedback circuit (Jedema et al., 2001). Given that this feedback circuit is maintained *in vitro*, this feedback circuit is expected to be a local circuit and is likely inhibitory, based on the presence of GABA-ergic interneurons on the border of the LC area (Van Bockstaele and Chan, 1997). Based on these findings, it could be hypothesized that stress-induced sensitization of NE neurons is a consequence of a reduction of the effect of local GABA-ergic interneurons or auto-inhibition of LC neurons.

5.3 GABA-ergic inhibition of LC neurons

In vivo the LC is under tonic inhibitory control of GABA-ergic neurons (Ennis and Aston-Jones, 1989; Kawahara et al., 1999; Pudovkina et al., 2001). GABA-ergic neurons, both from extrinsic sources as well local interneurons innervate the LC (Van Bockstaele and Chan, 1997; Van Bockstaele, 1998). GABA-ergic inhibition of LC neuronal FR and NE release is largely mediated by $GABA_A$ receptors with a small contribution of $GABA_B$ receptors (Shefner and Osmanovic, 1991; Kawahara et al., 1999). The $GABA_A$ receptor complex also contains the BZD binding site. Alterations in $GABA_A$ receptor binding and pharmacology as well as alterations in BZD receptor binding and expression have been described following chronic stress exposure (Acosta et al., 1993; Deutsch et al., 1994; Orchinik et al., 1995; Orchinik et al., 2001). In addition, alterations in $GABA_A$ and BZD receptors have been described in the LC following adverse early life experiences (Caldji et al., 2000a; Caldji et al., 2000b). However, these changes are generally small and variable (Deutsch et al., 1994). Nevertheless, a reduction of GABA-ergic tone under

baseline conditions in cold-exposed rats can be concluded from a microdialysis study demonstrating that intraperitoneal injection of diazepam does not reduce baseline NE levels as in control rats (Finlay et al., 1995). However, the response to acute stress exposure in the control rats was not reduced by benzodiazepine administration, suggesting that GABA-mediated inhibition does not play a major role in the modulation of evoked NE release (Finlay et al., 1995). Since the effect of sensitization of LC neurons is most prominent under evoked conditions, this would argue against a reduction in GABA-ergic function underlying stress-induced sensitization of LC neurons. On the other hand, the foot shock evoked activation of CeA neurons is reduced following chronic cold exposure (Correll et al., 2002), which may lead to a reduced inhibition during foot shock based on the GABA-ergic nature of CeA afferents to the LC (Cassell et al., 1999). Furthermore, the response of LC neurons to CeA stimulation, which is typically inhibitory in control rats, is greatly reduced in cold-exposed rats (Ramsooksingh et al., 2001). Given that CeA stimulation typically results in a general suppression of LC activity rather than a time-locked discreet inhibition (Ramsooksingh et al., 2001), perhaps the reduction in evoked activation of CeA neurons and the reduced response of LC neurons to CeA stimulation might contribute to the sensitization of LC neurons by influencing the late component of the excitation of LC neurons evoked by sciatic nerve stimulation (Ramsooksingh et al., 2002). Even though these alterations in interaction between different brain regions may further contribute to stress-induced sensitization of LC neurons *in vivo*, only a potential decrease of function of local GABA-ergic interneurons or $GABA_A$ receptors can be involved in the enhanced excitation that was observed *in vitro* (Jedema and Grace, *In Press*). It is not clear whether there is any

GABA-ergic tone present *in vitro* or whether there is any functionally important GABA-ergic activity of interneurons in the LC as a result of an increase in the activity of LC neurons.

5.4 Auto-inhibition of LC neurons

5.4.1 Auto-inhibition

Auto-inhibition of LC neurons mediated by α_2 receptor has been extensively debated and the resolution of this issue has been hampered by (nor)adrenergic innervation of the LC from extrinsic sources (Hökfelt et al., 1984; Milner et al., 1989; Kachidian et al., 1990; Pieribone and Aston-Jones, 1991; Shipley et al., 1996; Van Bockstaele et al., 1996b; Van Bockstaele et al., 1998b). In addition to extrinsic (nor)adrenergic innervation, the presence of axon collaterals (Shimizu and Imamoto, 1970; Swanson, 1976; Cintra et al., 1982) and dendro-dendritic contacts (Shimizu and Imamoto, 1970; Sladek and Parnavelas, 1975; Shimizu et al., 1979; Groves and Wilson, 1980a, b; Milner et al., 1989; Van Bockstaele et al., 1996b; Van Bockstaele et al., 1998b) have been reported in the LC. It has been suggested that auto-inhibition of LC neurons *in vivo* occurs via NE release from feedback axon-collaterals or from dendrites (Aghajanian et al., 1977; Cedarbaum and Aghajanian, 1978b; Ennis and Aston-Jones, 1986). In contrast, it has been suggested that auto-inhibition *in vitro* does not involve NE release, but that it is a consequence of activation of a calcium-activated potassium conductance (Andrade and Aghajanian, 1984). The plane of section used for preparation of slices for *in vitro* recording influences the preservation of axon collaterals or dendrites (Travagli et al., 1996) and based on the

morphology of LC neurons and the orientation of their dendritic arborization (Swanson, 1976; Cintra et al., 1982; Travagli et al., 1996; Jedema and Grace, 1999), it would be expected that in preparations using coronal sections many of these processes potentially involved in auto-inhibition would be severed. Both *in vivo* and *in vitro* studies have demonstrated that administration of α_2-receptor antagonists increases basal FR of LC neurons (Cedarbaum and Aghajanian, 1976; Simson and Weiss, 1987; Ivanov and Aston-Jones, 1995; Simson, 2001). The increase in basal firing rate observed *in vivo* and *in vitro* following administration of α_2-receptor antagonists suggests the presence of a constant adrenergic tone, which would be consistent with NE-mediated inhibition. Furthermore, *in vivo* administration of the auto-receptor antagonist idazoxan greatly increases the excitability of LC neurons in response to glutamate, nicotine and sensory stimulation (Simson and Weiss, 1987; Simson, 2001). Similarly, bath application of yohimbine or idazoxan enhances the increase in FR evoked by local administration of glutamate *in vitro* (Ivanov and Aston-Jones, 1995). These data suggest that even *in vitro*, NE is being released from LC neurons leading to auto-inhibition of their own activity.

5.4.2 Stress-induced alterations in auto-inhibition

Several chronic stress-induced alterations of α_2 receptor function have been reported previously. Thus, α_2 receptor binding and α_{2A} mRNA in LC are decreased in tree shrews following exposure to chronic social stress (Flugge, 1996; Meyer et al., 2000) although more recently this same group failed to replicate its own binding data (Flugge et al., 2001). In rats, α_2 receptor binding in the LC is decreased following adverse early life experiences (Ladd et al., 2000; Liu et al., 2000). In addition, rats chronically exposed to tail shock exhibit a decreased sensitivity to the α_2 receptor antagonist idazoxan (Simson

and Weiss, 1988). In contrast, a paradoxical increase in sensitivity to the α_2 receptor agonist clonidine has been reported for NE release in the hippocampus following chronic cold exposure (Nisenbaum and Abercrombie, 1993). In addition, repeated immobilization has been reported to increase the sensitivity to clonidine (Pavcovich et al., 1990), while an increased sensitivity to clonidine was reported following acute exposure to foot shock (Pavcovich and Ramirez, 1991). Even though these apparent discrepant alterations in α_2 receptors may be hard to reconcile, it should be noted that a change in α_2 receptor-mediated auto-inhibition could also occur without alterations in the number or affinity of receptors. Alterations in the efficacy of the coupling of the receptor to the intracellular effector enzymes or alterations within the second messenger pathways can functionally impact on auto-inhibition. For example, a decrease in α_2 receptor function can be caused by a decrease in the amount or activity of the G_i protein coupling the α_2 receptor to its effector, whether that is a potassium channel or the enzyme ADC. Many different types of regulators of G-protein-signaling (RGS) have been described which can dramatically alters G_i protein efficacy (Hepler, 1999). Agonist binding typically results in the dissociation of the G-protein into $G_{i\alpha}$ and $G_{i\beta\gamma}$ subunits. The $G_{i\alpha}$ subunit subsequently activates ion channels or enzymes like ADC. More recently a signaling role for the $G_{i\beta\gamma}$ subunit has also been recognized (Hepler, 1999). Termination of the channel opening or enzyme activation occurs through the endogenous GTP-ase activity of the $G_{i\alpha}$ subunit leading to the reunion of an $G_{i\alpha}$ and a $G_{i\beta\gamma}$ subunit. It has been demonstrated that RGS is necessary for normal kinetics of G protein-coupled inward rectifying potassium (GIRK) channel-inactivation (Doupnik et al., 1997). Furthermore, RGS4, which is preferentially

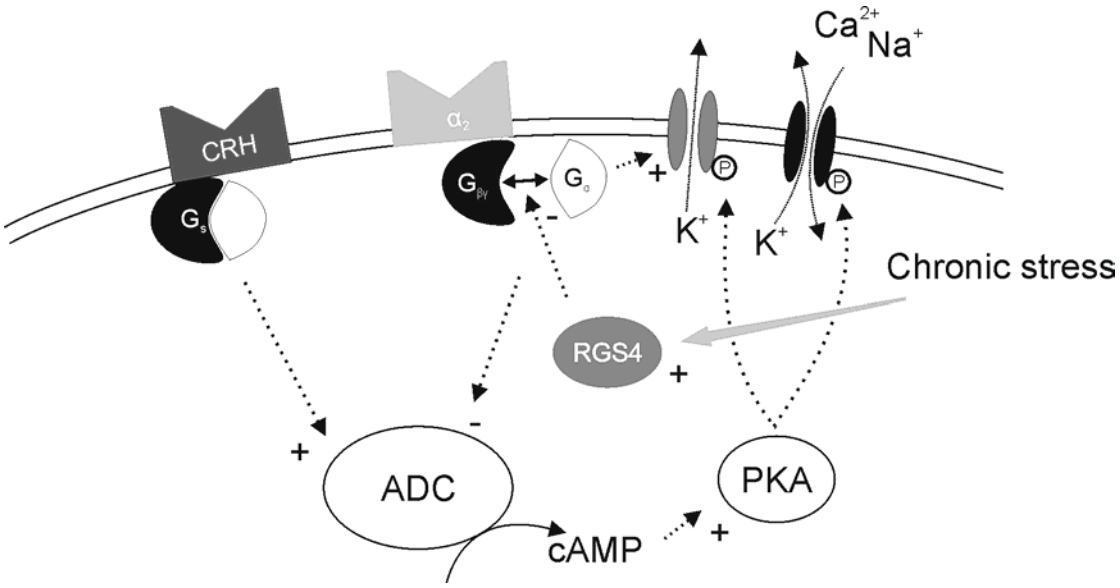

Figure 21 Potential alterations to second-messenger systems after chronic cold

Chronic stress exposure increases RGS4, which can result in increase of the endogenous GTP-ase activity of $G_{i\alpha}$ leading to a more rapid termination of α_2 receptor stimulation, thereby reducing its effect. In addition, this could lead to a disinhibition of PKA activity and an enhancement of receptor stimulation that involves this second messenger cascade.

expressed in the brain, has been demonstrated to greatly increase the GTP-ase activity of the $G_{i\alpha}$ subunit activated by α_{2A} receptor stimulation (Cavalli et al., 2000), thereby greatly reducing the effect of α_{2A} receptor stimulation (figure 21). In addition, the effect of stimulation of metabotropic receptors, which exert their effect through the PKA second messenger cascade, such as CRH receptors, would be hypothesized to be even further enhanced. Interestingly, RGS4 levels in LC neurons are high (Gold et al., 1997) and these levels are further increased by 10days of chronic unpredictable stress (Ni et al., 1999). Thus, chronic and variable stress exposure may effectively reduce the efficacy of α_{2A} receptor stimulation and perhaps other metabotropic neurotransmitter receptors that are coupled to G_i or G_q. The stress induced increase in RGS4 levels is highly specific for LC neurons, since in all other brain regions RGS4 levels are unaffected with exception of the paraventricular nucleus of the hypothalamus (PVN), where levels are decreased by chronic stress exposure (Ni et al., 1999).

5.5 Potassium channel function

In addition to chronic cold-evoked alterations in the number of autoreceptors or the efficacy of their signaling cascade, alterations in the number or function of the ion channels mediating their effect can influence neuronal excitability in a similar manner. Based on the linear current-voltage relationship of the differential voltage deflection between neurons from control and cold-exposed rats and the strong trend toward a reduction of the duration of the PAI, we hypothesized that a change in potassium conductance is most likely underlying the differences observed between both groups (Jedema and Grace, *In Press*). An alteration in potassium conductance could very well be

a consequence of an alteration of (auto)receptor number or function as described in the previous section.

Although alterations in potassium channel function *per se* have not been described following chronic stress exposure, recently it has been demonstrated that hypophysectomy greatly reduces the excitability of adrenal chromaffin cells via alteration of BK potassium channels (Lovell and McCobb, 2001). Furthermore, low levels of ACTH or cAMP analogs have been demonstrated to greatly reduce the expression of voltage gated potassium channels (Kv1.4) and consequently $I_{K(A)}$ in adrenal cortical cells (Enyeart et al., 2000). As mentioned previously, following chronic cold exposure basal and evoked ACTH levels are enhanced (Vernikos et al., 1982). Thus, a similar alteration of the function of potassium channels that are expressed in LC neurons could lead to an enhanced excitability.

5.6 Role of glucocorticoids

Glucocorticoid (GC) receptors are present in many brain regions, and they are present in high amounts in the LC (Harfstrand et al., 1986). Given the fact that GC act following translocation to the nucleus through modulation of gene transcription, alterations in LC function resulting from alterations in GC signaling would be expected to persist for some time in an *in vitro* preparation. GC are thought to exert an inhibitory function on noradrenergic activity. Consistent with an inhibitory effect of GC on LC function is the fact that adrenalectomy increases the basal FR of LC neurons. This increase presumably occurs via an increase of CRH release in the LC since the increase in FR is prevented by intra-LC administration of a CRH antagonist (Pavcovich and

Valentino, 1997). The increase in LC FR evoked by sciatic nerve stimulation or carbachol is not altered following adrenalectomy, but the effects of hypotensive challenge or low doses of CRH were enhanced (Pavcovich and Valentino, 1997). Following chronic cold exposure the basal and evoked levels of GC are enhanced (Vernikos et al., 1982), which would be expected to result in a reduction rather than a sensitization of noradrenergic function. Furthermore, following chronic cold exposure CRH levels in the LC are enhanced (Chappell et al., 1986), and the response to exogenous CRH is enhanced (Jedema et al., 2001). These data are not consistent with a primary role for an alteration in GC affecting CRH levels to underlie the stress-induced sensitization of LC neurons. On the other hand, GC mimic the effect of chronic stress on RGS4 levels in LC neurons (Ni et al., 1999), which would be expected to reduce the autoinhibitory function of α_{2A} receptor stimulation (Cavalli et al., 2000). As described above, a decreased α_{2A} receptor function is consistent with the alterations observed in LC neurons following chronic cold exposure.

In addition to the effect of GC on RGS4 levels in the LC, chronic stress levels of GC affect the levels of $GABA_A$ receptor subunits mRNA in hippocampus (Orchinik et al., 1995) and alter the pharmacology of $GABA_A$ receptor function in hippocampus (Orchinik et al., 2001). Whether similar chronic GC-evoked alterations in $GABA_A$ receptors subunits occur within the LC area is unknown.

5.7 The role of CRH

Previously, it was clearly demonstrated that CRH can increase the electrophysiological activity of LC neurons and NE release at the terminals of LC

neurons (Valentino et al., 1983; Smagin et al., 1995; Curtis et al., 1997). These data of CRH evoked activation of LC neurons were extended by the present work by the demonstration that CRH can directly increase the FR of LC neurons *in vitro* and by the demonstration that the dose-response relationship for the electrophysiological activation of LC neurons by CRH is altered following chronic cold exposure.

The observation that CRH continues to activate LC neurons in the presence of TTX demonstrates that the blockade of impulse-dependent neurotransmitter release does not prevent CRH from exerting its excitatory action on LC neurons and suggest a direct action of CRH on receptors on LC neurons *in vitro*. However, the experiment in TTX does not rule out the possibility that CRH also affects LC activity by modulating presynaptic afferents to LC neurons *in vivo.* However, other than the presence of CRH receptors in the LC without CRH receptor mRNA in LC neurons, there is currently no evidence available to support this notion. As indicated in sections 3.1 and 3.4.1 preliminary ultrastructural evidence has been obtained demonstrating the presence of CRH receptors on the surface of LC neurons (Fox et al., 2002). The experiment in TTX also does not rule out the possibility that CRH acts through stimulation of impulse-independent neurotransmitter release from presynaptic terminals afferents that form synapses onto LC neurons. Nevertheless, I do not believe that CRH is acting through such a mechanism *in vitro*. First, neither before nor during CRH administration was there any evidence of post-synaptic potentials that would be indicative of impulse-independent neurotransmission. Nevertheless, it should be acknowledged that such post-synaptic potentials could be too small to detect at the soma when they occur at the distal dendrites. Second, blockade of the intracellular signaling cascade reported to mediate the effect of

CRH receptors, completely inhibited the CRH-evoked increase in FR of LC neurons. Given that the blockade of the intracellular signaling cascade occurred intracellularly, it would not be expected to influence the effect of CRH on any potential impulse-independent neurotransmitter release that might subsequently affect LC activity. Nevertheless, the possibility still exists that CRH were to act through stimulation of impulse-independent release of a neurotransmitter that subsequently influenced LC activity through the stimulation of a metabotropic receptor that exerted its effect via a signaling cascade involving PKA. An experiment in which the depolarization evoked by CRH administration were recorded in a low calcium-high magnesium buffer would address the uncertainty left by the experiment of CRH administration in the presence of TTX.

5.8 Functional implications

It has been clearly demonstrated that the evoked LC spike firing rate is enhanced following chronic cold exposure (Mana and Grace, 1997; Jedema et al., 2001; Jedema and Grace, *In Press*) and that the increased excitability of LC neurons is translated in an enhanced release of NE in the terminal regions (Nisenbaum et al., 1991; Gresch et al., 1994; Finlay et al., 1997; Jedema et al., 1999). The functional outcome of this enhanced NE release is less clear. NE released in terminal areas exerts its function on multiple subclasses of post-synaptic α- and β receptors. Chronic foot shock or restraint-induced decreases in α_1 function and β receptor density have been reported presumably mediated by alterations in by GC (Stone and Platt, 1982; Stone, 1987). In addition, a reduction in cortical α_2 receptor function following chronic GC treatment has been observed (Duman

et al., 1986). It is unknown whether the density or efficacy of adrenergic receptors is affected by chronic cold-exposure, but given the modulatory role of GC on these adrenergic receptors and the elevated GC levels following chronic cold exposure it is likely that the effect of NE on its targets is altered following chronic cold exposure. Given the opposing effects of different adrenergic receptor subtypes on behavior (see below), the final outcome of these hypothesized adrenergic receptor alterations is difficult to predict.

Under normal conditions, NE has complex effects on the performance in tasks requiring focused attention. This has led to the hypothesis that the dose-response relationship of task performance as a function of NE concentration follows a Yerkes-Dodson relationship (i.e. an inverted U-shape) (Usher et al., 1999; Arnsten, 2000). Whereas low levels of NE were associated with drowsiness, moderate levels of NE improved task performance via high-affinity α_2 receptors (Arnsten et al., 1996; Franowicz and Arnsten, 1999), whereas higher levels of NE resulted in an increased false-alarm rate via lower affinity α_1 receptors (Birnbaum et al., 1999; Mao et al., 1999). Assuming that the enhanced release of NE also takes place in situations with more selective, low levels of stimulation of NE release in target areas rather than massive NE release, which is expected to occur during stress exposure, task performance would be differently affected by enhanced NE release dependent on whether low or high levels of NE are present. Thus, under low basal levels of NE stimulation one may hypothesize an improvement of task performance in sensitized rats, whereas under high levels of NE stimulation a deterioration of task performance could be hypothesized. If, on the other hand, NE levels are selectively enhanced in sensitized rats during high levels of LC activity and NE

release, a further deterioration of task performance would be expected following chronic stress exposure.

There is limited behavioral data on rats following chronic cold exposure. The locomotor activity of cold-exposed rats in response to exposure to shock is enhanced without alterations of basal locomotor activity (Moore, Jedema, Seiple, Finlay; unpublished observations). In addition, rats previously exposed to chronic cold spent less time in the open arms of an elevated plus maze than naive control rats (Seiple et al., 1997). Similar data have recently been obtained in cold-exposed mice (Hata et al., 2001). A decrease of time spend in open arms of the elevated plus maze is often interpreted as evidence for an increased level of anxiety, given that anxiogenic drugs decrease and anxiolytic drugs increase the amount of time rodents spend in the open arms of the elevated plus maze (File, 2001). Therefore, the available data suggest that chronic cold exposure may increase anxiety in rats.

Abnormalities in noradrenergic function have long been associated with a number of mood and anxiety disorders (Charney et al., 1995; Bremner et al., 1996; Wong et al., 2000). Thus, noradrenergic dysfunction has been implicated in depression (monoamine hypothesis of depression)(Schatzberg and Schildkraut, 1995), as well as in PTSD (Aston-Jones et al., 1994; Southwick et al., 1999), panic disorder (Charney et al., 1995), OCD, and ADHD (Solanto, 1998; Biederman and Spencer, 2000; Berridge, 2001). For example, recent imaging studies of PTSD patients demonstrated an increased activity in multiple cortical areas in response to the α_2 receptor antagonist yohimbine (Bremner et al., 1997). Furthermore, the anxiogenic response to yohimbine was enhanced in PTSD patients (Bremner et al., 1997). Thus, the increased anxiety in cold-exposed rats, perhaps as a

consequence of, or at least in combination with the alterations of noradrenergic function following chronic cold exposure, may indicate that chronic cold exposure of rats can be a useful model to study the hypothesized pathophysiology of the noradrenergic system in mood and anxiety disorders. Therefore, the alterations observed in electrophysiological properties of LC neurons reported in the present work may further aid to elucidate the pathological changes in noradrenergic function in patients with mood and anxiety disorders.

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