MODULATION OF LOCUS COERULEAR NEURONAL ACTIVITY BY THE CENTRAL NUCLEUS OF THE AMYGDALA

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The limbic and central noradrenergic systems are sensitive to stress and demonstrate pathophysiology in individuals with mood and anxiety disorders. Neurons from the central nucleus of the amygdala (CeA) form synapses onto the dendrites of noradrenergic neurons of the locus coeruleus (LC) in the rostrolateral peri-coerulear area. The CeA-LC pathway is thought to contain GABA and corticotropin-releasing hormone (CRH), both of which have opposing effects on LC activity. The current study further characterized the CeA-LC pathway *in vivo* by examining extracellular electrophysiological LC activity during electrical and pharmacological manipulation of the CeA in halothane-anesthetized rats.

The majority of LC neurons exhibited an excitatory response to electrical CeA stimulation, with a small group of neurons responding with pure inhibition or antidromic activation. Pharmacological activation of the CeA confirmed excitatory responses to electrical stimulation, whereas pharmacological inactivation of the CeA had no effect on LC activity. Excitatory responses to electrical CeA stimulation were partially attenuated following ventricular infusions of the CRH antagonist, D-Phe-CRH, but were completely attenuated following infusions of the excitatory amino acid antagonist, kynurenic acid. Ventricular administration of kynurenic acid during electrical CeA stimulation also revealed an inhibitory period that simultaneously occurs with excitatory responses.

This study confirms previous findings suggesting that the CeA partially mediates excitatory responses via CRH. These findings further suggest that there is a glutamatergic component to

excitatory responses following activation of the CeA. In addition, these data suggest that upon activation, the CeA provides a simultaneous inhibitory drive to LC neurons.

Collectively, these data provide additional support for an excitatory limbic input to neurons of the LC. The present data also suggest that the CeA may selectively modulate LC activity across its projection sites via the simultaneous inhibitory drive from the CeA. Activation of the CeA-LC pathway, particularly during stress responses, may be critical for noradrenergic modulation of cortical and limbic areas involved in attention, emotional learning, and responses to stressful stimuli.

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PREFACE

The completion of this thesis and this chapter in my life would not have been possible without the generous mentorship, support, and encouragement from many people, who I would like to acknowledge and to whom I extend my deepest gratitude and appreciation. As I reflect on the last 8 years at the University of Pittsburgh, by merely giving me an opportunity, many of these people have opened doors that I did not know existed, and this has allowed me to expand my thinking and to be successful in and out of the laboratory.

I would like to thank my committee members, Drs. Anthony Grace, Susan Sesack, and Linda Rinaman. As an undergraduate student in the Department of Neuroscience, Susan and Linda, both inspiring scientists in their respective fields, challenged my thinking simply by asking questions. Their insight and experience as expressed through their questions, though difficult at times, have helped me to refine the interpretation of the present work. I would like to especially thank Susan Sesack for her support and imparting her trust and confidence in my character and abilities as a student and scientist. Susan has followed my research as an undergraduate by serving as the second reader for my senior thesis, which was the beginning of the work presented in this thesis. I appreciate her frank and decisive but always fair way of dealing with tough questions and situations. I thank Linda Rinaman for bringing to my attention the GK-12 Fellowship, an opportunity that has been a rich source of inspiration outside of the laboratory. Along those lines, Linda has been supportive of my future goals and directions by allowing me to complete coursework outside of this

degree. I thank Tony for giving me the opportunity to continue working in his lab as a graduate student. I especially appreciate the freedom and independence he has given me in pursuing scientific questions that were outside of his primary research interests in addition to allowing me the opportunity and time to explore my interests in foreign languages and GK-12 Education as I completed the research towards this thesis.

My success and accomplishments in the laboratory would not have been achieved without the mentorship, encouragement, and support of colleagues with whom I have worked in Tony's lab. Under the supervision of Dr. Holly Moore, at the time a post-doctoral researcher, I initially started my work in Tony's lab as work study student during the fall semester of my sophomore year. Naive about Tony, his accomplishments, and what it means to do scientific research, I eagerly told Holly that I wanted to do research regardless of its difficulty. For me, research was a way to make the most of my time as an undergraduate at Pitt. As I reflect on the experience and knowledge gained, I have no regrets. I thank Holly for seeing and having faith in my abilities before I knew I had them by assigning me a project well beyond what I could manage as a sophomore, but always teaching, encouraging and supporting every accomplishment, even the smallest.

After participating in the GK-12 Fellowship during my second year as a graduate student, I finally realized how difficult it is to mentor students, to challenge their abilities, yet encourage them to always achieve their best. Dr. Hank Jedema graciously rose to this challenge and embodies, at least in my mind, what it means to be a mentor. While trying to accomplish his own academic endeavors and working through the difficulties his research presented to him as a graduate student and subsequent post-doctoral researcher in Tony's lab, he has not only successfully taught me the techniques of *in vivo* extracellular electrophysiological recordings of LC neurons, which are not trivial, but has also taught me how to ask good scientific questions, critically think about science, and challenge data, including my own. Hank sets high standards for himself and works to the best of his

abilities with utmost dedication, and I believe he expects others to do the same. These qualities often leave me calling Hank my harshest critic, but never in a negative way, as I welcomed his challenges, for they served as encouragement and motivation for me to always achieve my best. Hank has set an example of what it means to be a mentor, and I am privileged and honored to have worked under his guidance and with such a scientist.

While working in Tony's lab for the past 7 years, I have had the opportunity to work with a group of talented scientists, technicians, and staff within the department, who have supported my work and made dealing with the difficulties of research easier or more bearable. I thank Drs. Pauline Belujon and Daniel Lodge for reading preliminary versions of this thesis and offering suggestions for improvement. I thank Nicole MacMurdo, Christy Smolak, and Brian Lowry for their excellent technical support. I thank past and present graduate students and post-doctoral researchers in Tony's lab, all of whom contributed to my research in some way. I also thank Andrea Abt for her friendship, advice, and encouragement even long after she served as my undergraduate academic advisor.

As a student at Pitt, I led two academic lives: budding neuroscientist and student of language. My professors and instructors within the German department, namely Drs. John Lyon, Beverly Harris-Schenz, Sabine von Dirke, Elizabeth Wylie-Ernst, and Zsuzsa Horvath, taught me to appreciate subjects and ideas outside the world of science. This experience led to my studies of the Polish language under the guidance and encouragement of Dr. Oscar Swan in the Slavic department. While my training as a scientist has taught me to think critically, my German and Polish studies have taught me to think globally and have been instrumental in defining my future academic goals and realizing my natural ability to learn foreign languages.

For the past two years, I was fortunate to participate in the GK-12 fellowship, during which I had the opportunity to work with a group of talented professors, graduate students of other natural

science disciplines, and GK-12 teachers, all of whom are just as passionate about science as they are about teaching. This project attempted to build a partnership between the scientists of academia and GK-12 educators by combining multiple talents and skills to improve science education and inspire future scientists. Dr. Jennifer Cartier, a talented and visionary educator, has been instrumental in achieving this goal and teaching me how use my skills as a scientist outside of the lab, as well as how to think about "Big Ideas" in science. Her mentorship and the GK-12 experience has helped me to become a better scientist in that I am more confident to contextualize my small thesis project into a bigger picture.

Though much of my academic success can be attributed to the mentorship of the above mentioned academics, I am indebt to my close friends for their love, support and encouragement as I pursue and achieve my goals. I thank all of my closest friends, but I am most grateful for the friendships I have shared with Patrick Anderson, Kelly Keslar, and Kenneth Urban. They have not only shared my interests outside of the lab, but also lifted my spirits with their humor, love for food, and unfaltering advice and support when life at times became too stressful. I also thank the evening swimmers from Team Pittsburgh Master's swim club, many of which are competitive swimmers and triathletes. In re-learning how to swim with this group of eclectic and multitalented people, I have become a better person in a number of ways. I am humbled by their athletic endeavors that often leave me inspired to keep setting new goals.

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1.0 INTRODUCTION

Mood and anxiety disorders, e.g. post-traumatic disorder, panic disorder, generalized anxiety disorder, and major depression, often have detrimental effects on the daily functioning of about 25% of the American population (Kessler et al., 2005; Merikangas et al., 2007). The prevalence of these disorders among all populations has driven in a wealth of clinical research that has provided evidence for abnormalities in the biological and neurological processes underlying the symptoms of these disorders. A combination of clinical, imaging and post mortem studies in individuals with mood and anxiety disorders have indicated pathophysiology in both the limbic and central noradrenergic systems, which contribute to the symptoms observed in these mood and anxiety disorders.

Imaging studies of individuals who suffer from mood and anxiety disorders suggest abnormal hyperactivity in the amygdala, a limbic circuit nucleus involved in emotional regulation and processing. Baseline amygdala activity in patients suffering from depression, as measured by positron emission tomography (Drevets et al., 1999) or magnetic resonance imaging (Drevets et al., 2002), is greater than in control subjects and it is positively correlated with the severity of depression. In studies that evaluated emotional processing to negative affect words, depressed individuals had increased activity in the amygdala, as measured by MRI, and this increased activity was sustained throughout the non-emotional processing trials (Siegle et al., 2002). Furthermore, there was exaggerated amygdala responsivity to fearful facial expressions in patients suffering from post-traumatic stress disorder (Rauch et al., 2000; Shin et al., 2005; Williams et al., 2006). Increased

amygdala activity is also associated with feelings of anxiety (Adolphs et al., 2001; Anand and Shekhar, 2003; Baxter and Murray, 2002). In individuals with mood and anxiety disorders, this abnormal activity in the amygdala may correlate with impairments in processing and responding to emotional information.

The symptoms observed in mood and anxiety disorders are not completely attributed to pathophysiology of the amygdala and limbic system. Clinical studies have reported abnormalities in central norepinephrine system in the human central nervous system (CNS) of individuals with mood and anxiety disorders, and this suggests a role for the central noradrenergic system in these disorders (Anand and Charney, 1997; Anand and Charney, 2000; Asnis and Van Praag, 1995; Aston-Jones et al., 1994; Bremner et al., 1996; Charney et al., 1995; Wong et al., 2000). Like the amygdala, clinical studies suggest that central noradrenergic activity, in comparison to normal subjects, is increased in individuals suffering from mood and anxiety disorders. In these individuals, there are increases in urinary, plasma, or cerebrospinal fluid (CSF) levels of norepinephrine or its primary metabolite (Charney et al., 1995; Schatzberg and Schildkraut, 1995) and a blunted plasma catechol response to the adrenergic agonist clonidine (Charney et al., 1992; Matussek et al., 1980; Matussek and Laakmann, 1981; Nutt, 1989). When examining the dysfunctions observed in the central noradrenergic system in mood and anxiety disorders, the site of interest in clinical research has been a rostral pons nucleus, the locus coeruleus (LC), which contains the majority of NE-containing cell bodies in the CNS (Foote et al., 1983; Moore and Bloom, 1979; Moore and Card, 1984; Pfister and Danner, 1980). Post mortem human studies examining the LC from patients who suffered from mood and anxiety disorders have focused their attention on examining abnormalities in NE-specific proteins, such as tyrosine hydroxylase (TH), the rate limiting enzyme for catecholamines, and alpha2-noradrenergic autoreceptors. In patients diagnosed with major depression (Zhu et al., 1999) and suicide victims (Ordway et al., 1994a), elevated levels of TH were observed in the LC. There was

also an increased agonist binding to noradrenergic alpha2 autoreceptors in the LC from suicide victims, which implies an increased auto-inhibitory control of the LC in depressed patients (Ordway et al., 1994b). Pharmacological treatments for major depression and anxiety disorders modulate central noradrenergic activity and transmission thus providing additional evidence for dysfunction of the noradrenergic system in the pathophysiology of mood and anxiety disorders (Barker and Blakely, 1995; Grant and Weiss, 2001; Thase et al., 1997; Thase et al., 2002).

Clinical research has not only identified pathology in both the amygdala and the central noradrenergic system, but also indicates that stress plays a critical role in the manifestation of mood and anxiety disorders. Exposure to situations or environments that are aversive and stressful contributes to onset, development, and reoccurrence of mood and anxiety disorders, e.g., post-traumatic disorder, panic disorder, generalized anxiety disorder, and major depression (Bremner et al., 1996; Kessler, 1997; Southwick et al., 1999; Sullivan et al., 1999). Since both the central noradrenergic system (Stanford, 1993; Stanford, 1995; Zigmond et al., 1995) and the amygdala (Akirav et al., 2001; Dayas et al., 2001; Rosen et al., 1998b) are sensitive to stressful stimuli and have pathophysiology in mood and anxiety disorders, understanding the interactions between the limbic and central noradrenergic systems may provide important insights into the circuitry involved in stress responses that precipitate and exacerbate the symptoms of mood and anxiety disorders.

1.1 THE STRESS RESPONSE

Whether in the clinic or basic science laboratories, the term "stress" is often used imprecisely to describe a wide range of conditions and environments and to characterize the consequences that arise from being in these environments. Most often, consequences of exposure to "stressful"

environments and conditions are negatively perceived and have the potential to be maladaptive for the normal functioning of an organism. In order to encompass the multitude of conditions and consequences of stress, it can be vaguely defined as a threat to the maintenance of homeostasis of an organism (Day, 2005; Pacak and Palkovits, 2001). The result of stress exposure is not limited to changes in an organism's behavior when responding to a threat or to stressful stimuli. A "stress response", another term that is also often used ambiguously, involves a combination of changes in neuroendocrine, cardiovascular, immune, and autonomic processes observed upon presentation or exposure to stressful stimuli and contexts (Kopin, 1995). The "stress response" is classically thought of as the activation of the hypothalamic-pituitary-adrenal (HPA) axis. However, research has shown that the stress responses is not limited to activation of the HPA axis, but also involves activation of various brain regions and circuits, and this differential activation of brain regions depends on the type of stress and its severity and duration (Dayas et al., 2001; Morrison, 2001; Pacak, 2000; Pacak and Palkovits, 2001).

1.2 ACUTE STRESS ACTIVATES NORADRENERGIC NEURONS OF THE LOCUS COERULEUS

The locus coeruleus is the major norepinephrine containing nucleus in the CNS (Foote et al., 1983; Moore and Bloom, 1979; Moore and Card, 1984; Pfister and Danner, 1980) that sends its projections throughout the entire neuroaxis (Foote et al., 1983). Although it is a small cell group comprised of roughly 1,600 neurons per hemisphere (Swanson, 1976), the LC projects widely (Aston-Jones et al., 1995) to innervate distinct brain regions such as the cerebellum, thalamus, and the paraventricular hypothalamus, which is one of the main output nuclei partially involved in the

stress response (Cunningham and Sawchenko, 1988; Foote et al., 1983; Moore and Bloom, 1979; Moore and Card, 1984; Sawchenko and Swanson, 1981, 1982). The LC also innervates limbic areas such as the hippocampus, cortex, and the amygdala (Asan, 1998; Aston-Jones et al., 1999; Delfs et al., 2000; Foote et al., 1983; Jones and Moore, 1977; Kobayashi et al., 1975; Mason, 1979; Moore and Bloom, 1979; Moore and Card, 1984; Room et al., 1981). Whereas the hippocampus and cortex receives the sole source of noradrenergic innervation from the LC (Levitt and Moore, 1978; Loy et al., 1980; Nagai et al., 1981a, b; Nakamura and Iwama, 1975), subnuclei of the amygdala, such as the basolateral nucleus of the amygdala (BLA; Asan, 1998) and bed nucleus of the stria terminals (BNST; Aston-Jones et al., 1999; Delfs et al., 2000) receive partial noradrenergic innervation from the LC. Within the amygdala, the BLA receives the majority of LC innervation in comparison to the central nucleus of the amygdala (Asan, 1998), whereas the BNST, which is considered to be a nucleus of the extended amygdala, receives a minor LC input (Aston-Jones et al., 1999; Delfs et al., 2000). With its diffuse projections, particularly to cortical and limbic areas, the LC has the ability to initiate and maintain limbic and cortical arousals as well as modulate other neural systems. Thus any changes in NE levels throughout the brain will likely result in an overall change in behavior and emotional states (Aston-Jones et al., 1986; Thase et al., 2002).

The diffuse projections of LC neurons to a variety of neural systems suggest that the LC is involved in a multitude of functions that modulate behavioral and emotional state. Although it has been suggested that the LC is critical in maintaining arousal and attention (Aston-Jones et al., 2000; Berridge, 2001; Robbins, 1997), the LC is also particularly sensitive to stress such that acute stress exposure and presentation of noxious stimuli result in activation of the LC neurons (Stanford, 1993; Stanford, 1995; Zigmond et al., 1995) as measured by a variety of techniques, such as immunohistochemistry, microdialysis, and electrophysiology. Subcutaneous injection of capsaicin and intraperitoneal injection of hypertonic salt solution (Ceccatelli et al., 1989), immobilization stress

(Ceccatelli et al., 1989; Chen and Herbert, 1995; Dayas et al., 2001; Dayas et al., 1999; Ma and Morilak, 2004) as well as footshock (Passerin et al., 2000; Pezzone et al., 1993) results in increased Fos expression in LC neurons. Correlating with increased LC activation following stress exposure, extracellular levels of NE in the hippocampus or cortex, as measured with *in vivo* microdialysis, is increased in response to immobilization stress or intermittent tailshock (Abercrombie et al., 1988; Cenci et al., 1992; Nakane et al., 1994; Rossetti et al., 1990). Nitroprusside-induced hypotensive stress increases neuronal activity of LC neurons of both anesthetized and unanesthetized rats (Curtis et al., 2002; Curtis et al., 1993; Page et al., 1993; Valentino et al., 1991; Valentino and Wehby, 1988). In awake cats and primates, electrophysiological experiments have shown an increased neuronal activity of the LC in response to noxious or stressful stimuli (Abercrombie and Jacobs, 1987; Aston-Jones et al., 1991a; Grant et al., 1988; Rasmussen and Jacobs, 1986). In anesthetized rats, indirect stimulation of the contralateral sciatic nerve through footshock, a noxious stimulus, also increases LC neuronal activity (Ennis et al., 1992; Mana and Grace, 1995). Noradrenergic LC activity following acute stress exposure was also measured by examining the activity of NE-specific proteins. Acute stress exposure increases the amount or activity of tyrosine hydroxylase, the rate limiting enzyme for NE synthesis, and dopamine beta-hydroxylase, the enzyme that converts dopamine into norepinephrine; thus increasing the capacity for NE synthesis in LC neurons (Chang, 2002; Melia and Duman, 1991; Nisenbaum and Abercrombie, 1992; Serova et al., 1999).

1.3 ROLE OF CRH IN STRESS-INDUCED ACTIVATION OF LC NEURONS

The neuropeptide corticotropin-releasing hormone (CRH) plays an important role in mediating responses to stress exposure. CRH was initially discovered as an excitatory neuropeptide released

from the hypothalamus and initiates adrenocorticotropin release from the pituitary in the endocrine response to stress (Vale et al., 1981). A number of studies examining stress-induced activation of LC neurons suggest that CRH may be mediating this activation, which provides an extrahypothalamic site of action of CRH during stress responses (Valentino et al., 1998; Valentino et al., 1993). Increased extracellular NE release in the prefrontal cortex following immobilization stress (Nakane et al., 1994) can be attenuated by CRH antagonist administration in the LC (Emoto et al., 1993; Nakane et al., 1994; Shimizu et al., 1994; Smagin et al., 1996; Smagin et al., 1997). Nitroprusside-induced hypotensive stress activates LC neurons, and this activation is attenuated following coerulear or ventricular administration of CRH antagonists (Page et al., 1993; Valentino et al., 1991; Valentino and Wehby, 1988). Coerulear CRH administration resulted in increased behaviors typically elicited by stress exposure (Butler et al., 1990). Together, these data suggest that CRH may be mediating LC activation following acute stress exposure.

Consistent with previous studies examining stress-induced activation of LC neurons via CRH, ventricular or coerulear administration of CRH results in increased LC neuronal activity (Jedema et al., 2001; Valentino and Foote, 1988; Valentino et al., 1983). Correlating with CRH-induced activation of LC neuronal activity, CRH administration increased extracellular NE efflux in the hippocampus and prefrontal cortex as measured by microdialysis (Finlay et al., 1997; Page and Abercrombie, 1999; Smagin et al., 1995). CRH-induced activation of LC neuronal activity is attenuated by both ventricular (Curtis et al., 1994) and coerulear administration (Curtis et al., 1997b) of CRH antagonists. This is further correlated with attenuation of CRH-induced extracellular NE increase in the hippocampus by coerulear CRH antagonist administration (Page and Abercrombie, 1999). These *in vivo* electrophysiological and microdialysis studies examining CRH-mediated activation of LC activity and extracellular NE efflux, respectively, in areas where the LC provides the sole source of noradrenergic innervation, are consistent with *in vitro* studies examining CRH-

mediated activation of LC neurons (Curtis et al., 1994; Jedema and Grace, 2004), which is also attenuated by bath application of CRH antagonists (Curtis et al., 1994; Jedema and Grace, 2004). These *in vitro* studies further suggest that the LC is an extrahypothalamic site of action for CRH during stress responses.

Localization of CRH receptors on LC neurons strongly suggest that CRH directly activates LC neurons following both CRH administration and acute stress exposure. Audioradiographic and immunohistochemical studies show that CRH can directly activate LC neurons via CRH receptors that are localized on LC neurons and their dendrites (Chen et al., 2000; De Souza and Insel, 1990; Fox et al., 2002; Millan et al., 1986; Primus et al., 1997; Reyes et al., 2006; Reyes et al., 2008; Sanchez et al., 1999; Sauvage and Steckler, 2001; Xu et al., 2004). Ultrastructural evidence shows that CRH receptors that are localized on LC neurons and its dendrites are of the CRH1 receptor subtype (Fox et al., 2002; Reyes et al., 2006; Reyes et al., 2008; Sauvage and Steckler, 2001). This is consistent with mRNA for the CRH1 receptor subtype within the LC, which was determined using reverse transcription polymerase chain reaction (Reyes et al., 2006). *In vitro* studies examining electrophysiological LC activity, which severs connectivity between the LC and its afferents, have demonstrated that CRH directly activates LC neurons in the presence of TTX (Jedema and Grace, 2004). These findings suggest that CRH can directly activate LC neurons rather than by modulating LC afferents.

In addition to the LC, several immunohistochemical studies have demonstrated multiple extrahypothalamic sites within the CNS for CRH-containing neurons, and these sites also innervate the LC. Combined immunohistochemical and anatomical tract-tracing studies have demonstrated that the peri-coerulear area is rich with CRH-positive axon terminals (Valentino et al., 1992; Van Bockstaele et al., 1996b) and brainstem regions, such as the nucleus paragigantocellularis, prepositus hypoglossi, and Barrington's nucleus (Valentino et al., 1992), innervate and provide the LC with a

source of CRH. In addition to the CRH-containing brainstem LC afferents, CRH-containing limbic regions, such as the central nucleus of the amygdala (CeA), which is the primary output nucleus of the amygdala (Lechner and Valentino, 1999; Sakanaka et al., 1986, 1987; Van Bockstaele et al., 1998; Wallace et al., 1992) and the bed nucleus of the stria terminalis (Van Bockstaele et al., 1999) form synapses onto dendrites of LC neurons in the peri-coerulear area, where the majority of CRH innervation occurs (Van Bockstaele et al., 2001). Although both of these limbic areas contain CRH and innervate LC neurons, ultra-structural evidences shows that only neurons of the CeA and not the BNST form CRH-positive synapses onto dendrites of the LC (Van Bockstaele et al., 1998; Van Bockstaele et al., 1999). While all of these CRH-containing brain regions that innervate the LC may be involved in CRH-mediated activation of the LC following exposure to acute stressors, the CeA is of particular interest for this study, since it provides the LC with a source of CRH and may contribute to the stress-induced activation of LC neurons that is thought to be mediated by CRH. In addition, clinical studies have shown abnormal amygdalar activity in individuals with mood and anxiety disorders in comparison to control subjects (Drevets, 1999; Drevets et al., 2002; Rauch et al., 2000; Siegle et al., 2002; Williams et al., 2006) and this abnormal overactivity within the amygdala may have an abnormal influence of downstream structures (Gilboa et al., 2004).

1.4 THE CENTRAL NUCLEUS OF THE AMYGDALA

The amygdala is a complex of forebrain nuclei with separate yet highly integrated functions. Each nucleus within the amygdalar complex has distinct neurochemical and cytoarchitectonic features as well as anatomical connections. Among these nuclei of the amygdala are the basolateral nucleus (BLA) and the central nucleus of the amygdala (CeA). Of these two nuclei, the CeA is the focus of

this study as it provides a source of CRH to the LC (Van Bockstaele et al., 1998) and may partially mediate stress-induced activation of LC neurons (Curtis et al., 2002).

Immunohistochemical studies have demonstrated that neurons of the CeA contain the inhibitory transmitter, γ-aminobutyric acid (Ben-Ari et al., 1976; Sun and Cassell, 1993; Sun et al., 1994). Consistent with this, neurons of the CeA contain high levels of the immunohistochemical marker for GABA, glutamic acid decarboxylase (GAD), the enzyme which synthesizes GABA from glutamic acid (Ben-Ari et al., 1976; Oertel et al., 1983; Sun and Cassell, 1993; Sun et al., 1994; Veinante et al., 1997). Using in situ hybridization, there was substantial mRNA for GAD isoforms 65 and 67 in neurons of the CeA (Day et al., 1999). Unlike the projection neurons of the BLA, which are glutamatergic (McDonald, 1982b, 1985, 1992, 1996; McDonald and Betette, 2001; McDonald and Pearson, 1989), projection neurons of the CeA that target the brainstem contain GABA (Sun et al., 1994). Although the CeA is often considered to be a GABAergic nucleus, immunohistochemical studies have shown that neurons of the CeA contain a variety of neuropeptides, such as neurotensin, enkephalin, somatostatin, substance P, cholecystokinin, vasoactive peptide, and galanin (Cassell et al., 1986; Gray and Magnuson, 1987a, b; Oertel et al., 1983; Palkovits, 2000; Veinante et al., 1997). Furthermore, immunohistochemical studies have identified the CeA as an extrahypothalamic source of CRH (Gray and Magnuson, 1987b; Lechner and Valentino, 1999; Sakanaka et al., 1986, 1987; Swanson et al., 1983; Thompson and Cassell, 1989; Van Bockstaele et al., 1998).

With respect to its cytoarchitectonic properties, the CeA is more striatal-like, in comparison to the BLA which is more similar to cortical structures (Carlsen and Heimer, 1988; McDonald, 1982a; McDonald, 1992; McDonald and Pearson, 1989). Based on cytoarchitectonic features, the CeA is divided into three subdivisions, the medial, lateral, and capsular divisions (McDonald, 1982a). Like the BLA, CeA is comprised of both projection and local circuit interneurons with numerous

topographically organized intrinsic connections between its subdivisions (Jolkkonen and Pitkanen, 1998; Pitkanen and Amaral, 1994; Pitkanen et al., 1997). Extensive interconnectivity exists between the different subdivisions of the CeA as well as reciprocal connections between the CeA and BLA (Jolkkonen and Pitkanen, 1998; Pitkanen et al., 1997).

The BLA and CeA have distinct functions within the amgydalar complex based on afferent and efferent connections between the BLA and CeA and different brain regions. The BLA is thought to integrate sensory information (Davis, 1994; Davis and Whalen, 2001; LeDoux, 2000) received from sensory areas, sensory association cortex, and thalamic nuclei (Armony et al., 1997; Arnault and Roger, 1990; Campeau and Davis, 1995; Cassell and Wright, 1986; Edeline et al., 1993; Farb and Ledoux, 1999; Li et al., 1996; Mascagni et al., 1993; McDonald, 1998; Romanski et al., 1993; Rosen et al., 1992; Shi and Cassell, 1997, 1998a, b, 1999; Turner and Herkenham, 1991; van Vulpen and Verwer, 1989). The CeA is thought to be the primary output nucleus of the amygdala (Davis, 1994; Davis and Whalen, 2001; LeDoux, 2000), since it projects to the hypothalamus and to different nuclei located throughout the brainstem (Gray et al., 1989; Gray and Magnuson, 1987a, b; Jongen-Relo and Amaral, 1998; Petrovich et al., 2001; Petrovich and Swanson, 1997; Saha et al., 2000; Van de Kar and Blair, 1999; Veening et al., 1984). Through its efferent connections to the CeA (Pitkanen, 1997), the BLA sends integrated sensory information to the CeA, which is thought to influence autonomic, endocrine, and cardiovascular responses (Davis, 1994; Davis and Whalen, 2001; LeDoux, 2000) through its innervation of hypothalamic and brainstem regions (Gray et al., 1989; Gray and Magnuson, 1987a, b; Jongen-Relo and Amaral, 1998; Petrovich et al., 2001; Petrovich and Swanson, 1997; Saha et al., 2000; Veening et al., 1984).

1.5 THE ROLE OF THE CENTRAL NUCLEUS OF THE AMYGDALA IN THE STRESS RESPONSE

Like the LC, previous studies have shown that the CeA is involved in responses to a variety of stressful or noxious stimuli (Dayas et al., 2001) Psychological stressors, such as swim stress (Dayas et al., 2001), and immobilization stress activate neurons of the CeA as measured by Fos expression (Campeau et al., 1991; Honkaniemi, 1992) or expression of other immediate early genes (Rosen et al., 1998a). In rats that were exposed to physiological stressors, such as nitroprusside-induced hypotension (Curtis et al., 2002), hemorrhage and immune challenge (Dayas et al, 2001), and noise (Dayas et al, 2001), there were significant increases in Fos expression in a large number of neurons in the CeA in comparison to naïve rats. In anesthetized rats, electrophysiological neuronal activity of CeA neurons is increased following presentation of a noxious stimulus such as footshock (Correll et al., 2005). Several lesion studies have examined the role of the CeA in activation of the HPA axis during a stress response (for reviews, (Gray and Bingaman, 1996; Herman and Cullinan, 1997; Roozendaal et al., 1997). Lesions of the CeA disrupt adrenocorticotropin hormone (ACTH) and corticosterone responses to stressors, such as immobilization stress (Beaulieu et al., 1986; Beaulieu et al., 1987; Prewitt and Herman, 1994; Roozendaal et al., 1991b; Van de Kar et al., 1991) and unavoidable footshock (Roozendaal et al., 1991a).

In addition to involvement in the stress response to a variety of aversive or stressful stimuli as evaluated through Fos expression in the CeA or measurement of neuroendocrine and autonomic responses to acute stressors following lesions of the CeA, the CeA is also critically involved in the expression of behaviors and neuroendocrine and autonomic responses observed during fear conditioning (Davis, 1994, 1997; Davis and Whalen, 2001; LeDoux, 2000). Fear conditioning involves the pairing of a conditioned stimulus, such as a tone or a light, with an unconditioned

stimulus, such as an electric shock (Brown et al., 1951). Chemical or electrolytic lesions of the CeA blocked expression of fear-potentiated startle (Davis et al., 1993; Falls and Davis, 1995; Gentile et al., 1986; Helmstetter, 1992; Hitchcock and Davis, 1991; Iwata et al., 1986; Kapp et al., 1979; Kim and Davis, 1993; Sananes and Campbell, 1989; Weisz et al., 1992; Zhang et al., 1986). Lesions of the CeA disrupt conditioned freezing (Iwata et al., 1986), conditioned increases in arterial pressure (Iwata et al., 1986), fear-potentiated startled (Campeau and Davis, 1995), and other conditioned responses (Gentile et al., 1986; Helmstetter, 1992). Consistent with this, blocking glutamate transmission within the CeA with infusions of the glutamate receptor antagonists into the CeA prevented expression of fear-potentiated startle (Davis et al., 1993; Walker and Davis, 1997).

Due to its projections to brainstem and hypothalamic areas that modulate neuroendocrine and autonomic processes, the CeA has the potential to influence changes in autonomic and neuroendocrine processes that are involved in the stress responses (Dayas et al, 2001) and fear conditioning (Davis and Whalen, 2001). Although the CeA is known to be a GABAergic brain region, neurons of the CeA also synthesize the neuropeptide CRH (Gray et al, 1987b; Lechner et al, 1999; Sakanaka et al, 1986, 1987; Swanson et al, 1983; Van Bockstaele et al, 1998), and it is suggested that changes in autonomic and endocrine processes during the stress response may be mediated by CRH (Gray and Bingaman, 1996; Roberts, 1992). Combined immunohistochemical and anatomical tract-tracing studies have shown that the CRH-positive neurons in the CeA project to regions in the brainstem that modulate neuroendocrine and autonomic processes, such as the parabrachial nucleus (Moga and Gray, 1985), dorsal vagal complex (Veening et al., 1984), the midbrain central gray (Gray et al., 1989), and the LC (Sakanaka et al, 1986; Swanson et al, 1983; Van Bockstaele et al, 1996a; Van Bockstaele et al, 1998; Wallace et al, 1992). Given the involvement of both the CeA and LC in mood and anxiety disorders and that both regions are activated by acute stressors, with evidence suggesting

that CRH mediates stress-induced activation of LC, this study examines the pathway between the CeA and LC, particularly with respect to CRH.

1.6 THE CEA-LC PATHWAY MAY BE INVOLVED IN MODULATING RESPONSES TO ACUTE STRESSORS

Anatomical tract-tracing studies examining the innervation of the LC have identified a specific spatial arrangement of the inputs within the LC. It was previously thought that innervation of the LC was restricted to brainstem regions, such as the PGi, prepositus hypoglossi, and Barrington's nucleus (Aston-Jones et al, 1986; Aston-Jones et al, 1991b; Chiang and Aston-Jones, 1993; Valentino et al, 1992). However, Golgi staining and immunohistochemical staining for dopamine-beta-hydroxylase showed that dendrites of the LC extend beyond the densely packed nucleus into what is known as the peri-coerulear area (Cintra et al., 1982; Grzanna and Molliver, 1980; Pfister and Danner, 1980; Shipley et al., 1996; Swanson, 1976). Subsequent studies have identified limbic afferents of LC, such as the prefrontal cortex (Aston-Jones et al., 1991b; Zhu and Aston-Jones, 1996), the BNST (Van Bockstaele et al., 1999) and the CeA (Sakanaka et al., 1986; Van Bockstaele et al., 1996a; Wallace et al., 1992), that innervate dendrites of LC neurons in the peri-coerulear area. The peri-coerulear area can be further divided into the rostrolateral and rostromedial peri-coerulear areas (Shipley et al., 1996), which are differentially innvervated by limbic areas. The prefrontal cortex innervates LC dendrites in the rostromedial peri-coerulear area (Aston-Jones et al., 1991b; Zhu and Aston-Jones, 1996), whereas amygdalar inputs such as the BNST (Van Bockstaele et al, 1999) and the CeA (Van Bockstaele et al, 1996a; Van Bockstaele et al, 1998) innervate LC dendrites localized in the rostrolateral peri-coerulear area. It was suggested that brainstem afferents that innervate the LC

proper may convey information of immediate physiological survival value, i.e, the "systemic" stressors, while limbic afferents that innervate LC neuron dendrites in the peri-coerulear area may modulate LC responses to stressful stimuli (Herman and Cullinan, 1997).

Combined anatomical tract-tracing and immunohistochemical studies have suggested that the CeA may modulate LC activity by providing an excitatory limbic input to LC neurons. Not only do neurons of the CeA form synapses onto dendrites of LC neurons in the rostrolateral peri-coerulear area, but these synapses are CRH-positive (Van Bockstaele et al., 1998). This is consistent with ultra-structural studies showing that CRH-positive neurons of the CeA form putative asymmetric (excitatory) synapses onto TH-positive dendrites of LC (Van Bockstaele et al., 1998). Although this may suggest that the GABAergic projection from the CeA is separate from the CRH-containing projection, it has been demonstrated that CRH co-localizes with GABA in terminals within the dendritic field of LC neurons (Valentino et al., 2001) in addition to neurons of the CeA (Veinante et al., 1997). Older studies using combined anterograde tract-tracing from the CeA and immunohistochemical staining for CRH show that neurons from the CeA provides a source of CRH to the dendritic areas of the LC (Sakanaka et al, 1986; Wallace et al, 1992). Lesions of the CeA decreased the density of CRH-positive fibers in the rostrolateral peri-coerulear area (Sakanaka et al., 1986), an area rich with CRH-positive terminals (Van Bockstaele et al, 1996b). Furthermore, studies using combined retrograde tract-tracing from the LC and immunohistochemical staining for CRH show that CRH-containing CeA neurons target the LC and are located in the more medial areas of the CeA (Thompson et al, 1989; Van Bockstaele et al, 1998). Although combined immunohistochemical and anatomical tract-tract studies have demonstrated that CRH-positive neurons of the CeA form synapses onto TH-positive dendrites of the LC, there is no ultra-structural evidence to date that demonstrates that GABAergic neurons of the CeA form synaptic contacts with dendrites of LC neurons.

While anatomical tract-tracing and immunohistochemical studies have provided evidence for modulation of LC activity by the CeA that is excitatory, subsequent electrophysiological studies have examined the modulation of LC activity by the CeA. It is thought that the CeA mediates hypotension-induced LC activation through CRH since LC neurons were not activated by nitroprusside-induced hypotensive stress in animals with electrolytic lesions of the CeA (Curtis et al., 2002). Subsequent electrophysiological studies more directly characterized the CeA-LC pathway. In anesthetized rats, electrical stimulation of the CeA resulted in activation of LC neurons, and this activation was attenuated following both ventricular and coerulear administration of CRH antagonists (Bouret et al., 2003). Taken together, previous studies examining the CeA-LC pathway suggest that the CeA provides an excitatory input to LC neurons and activation of this pathway may be activating LC neurons during exposure to acute stressors. Activation of the CeA-LC pathway may result in noradrenergic modulation of areas that are innervated by LC neurons. Therefore, together with other monoamines, NE innervation arising from the LC has the ability to modulate limbic and cortical arousal as well as modulate other neural systems, which suggests that any changes in NE levels throughout the brain as a result of LC activation will likely result in a modulation of behavior and emotional states (Aston-Jones et al, 2000; Aston-Jones et al, 1996; Thase et al, 2002).

1.7 RESEARCH OBJECTIVES

Studies examining stress-induced activation of the LC have suggested that this activation is mediated in part by CRH. Furthermore, previous studies have shown that the CeA, which is also activated by stress and aversive stimuli, provides a source of CRH to LC neurons. Ultra-structural evidence from combined anatomical tract-tracing and immunohistochemical studies along with electrophysiological

studies examining the CeA-LC pathway suggested that the CeA provides an excitatory limbic input to LC neurons and that this activation is mediated by CRH. Together these data suggests that the CeA modulates LC activity and may contribute to stress-induced LC activation via CRH during responses to stressful stimuli.

Given that neurons of the CeA contain a combination of excitatory neuropeptides and inhibitory neurotransmitters and peptides, namely CRH and GABA, this present work characterized the CeA-LC pathway using a combination of electrophysiological and pharmacological techniques in an anesthetized rodent preparation. Studies were designed to confirm results from previous work (Bouret et al., 2003) and further characterize how the CeA may modulate LC activity following both electrical and pharmacological manipulation of the CeA along the following specific aims:

1. **To determine the effects of electrical and pharmacological manipulation of the CeA on LC activity.**

A. **Since neurons of the CeA contain the inhibitory neurotransmitter, GABA, as well as various peptides, all of which have either inhibitory or excitatory effects on LC activity, what is the effect of electrical stimulation of the CeA on LC activity?**

It is hypothesized that electrical activation of the CeA using a variety of stimulation parameters will result in both excitatory and inhibitory responses in LC neurons. Excitatory responses are expected since neurons of the CeA that directly form synapses on LC neurons will release CRH onto LC neurons, thereby mediating excitatory responses. *In vivo* CRH administration increases electrophysiological activity of LC neurons and *in vitro* electrophysiological studies show direct activation of LC neurons in the presence of TTX following bath application of CRH. These observations correlate with increased extracellular NE release at terminal sites as a result of CRH

administration. Previous studies examining the CeA-LC pathway in anesthetized rats have shown that electrical stimulation of the CeA results in excitatory responses.

In addition to excitatory responses, it is hypothesized that electrical stimulation of the CeA will result in pure inhibitory responses, since projection neurons of the CeA contain GABA and neuropeptides that depress LC activity. Electrophysiological studies have shown that bath application of GABA *in vitro* decreases electrophysiological LC activity (Shefner and Osmanovic, 1991). LC activity is also depressed following administration of the following neuropeptides, which are contained in neurons of the CeA (Cassell et al, 1986): enkephalin (Travagli et al., 1995), neurotensin, somatostatin, and galanin (Olpe and Steinmann, 1991).

B. What are the effects of pharmacological activation and inactivation of the CeA on LC activity?

To confirm responses as a result of electrical stimulation of the CeA, responses to pharmacological activation of the CeA will be characterized. It is hypothesized that pharmacological activation of the CeA will result in both excitatory and inhibitory responses of LC neurons, which are hypothesized to be mediated by the same mechanisms underlying excitatory and inhibitory responses following electrical stimulation of the CeA.

In addition, *in vivo* LC activity is under tonic inhibitory control (Ennis and Aston-Jones, 1989; Kawahara et al, 1999; Pudovkina et al, 2001) that is thought to be mediated by local circuit GABAergic interneurons (Aston-Jones et al, 2004). Local application of GABAergic antagonists increases LC firing rate (Ennis and Aston-Jones, 1989a) and NE release both in the medial PFC and locally within the LC (Kawahara et al, 1999; Pudovkina et al, 2001). Sources of tonic inhibitory control may be intrinsic via GABAergic interneurons within the dendritic fields of the LC (Aston-

Jones et al, 2004) or extrinsic (Van Bockstaele, 1998; Van Bockstaele and Chan, 1997). Since projection neurons of the CeA provides a source of GABA (Sun et al., 1994), pharmacological inactivation of the CeA will determine if the CeA may provide an extrinsic source of tonic inhibitory control of LC neurons.

2. To determine whether CRH mediates the excitatory responses of LC neurons following electrical train stimulation of the CeA. Does CRH mediate the excitatory responses following CeA stimulation?

Previous studies have shown that electrical stimulation of the CeA results in excitatory responses in the majority of LC neurons and this can be attenuated following ventricular and intra-LC administration of the peptidergic CRH antagonist, alpha-helical CRH. To confirm this observation, the effects of ventricular administration of the CRH antagonist, D-Phe-CRH, on excitatory responses following high-frequency high-intensity electrical train stimulation of the CeA were examined. It is hypothesized that D-Phe-CRH will attenuate excitatory responses following electrical train stimulation of the CeA.

Bouret et al suggested that excitatory responses to train stimulation were partially attenuated following ICV and intra-coerulear infusions of the peptidergic antagonist alpha-helical CRH. This suggested that the CeA activates the LC via other brain regions and/or mechanisms. To further evaluate whether excitatory responses to train stimulation of the CeA were mediated by CRH, and determine if glutamatergic afferents contribute to the excitatory responses as a result of electrical stimulation of the CeA, the effects of the glutamatergic antagonist, kynurenic acid, on excitatory responses to train stimulation were examined. It is hypothesized that excitatory responses to train stimulation will not be attenuated following ventricular administration of kynurenic acid.

2.0 METHODS

2.1 ANIMALS

Experiments were performed using 117 male Fisher 344 rats (Hilltop, Scottsdale, PA) (200-300 g) that were doubly housed in hanging stainless steel cages in a colony room maintained at 23 °C. Lights were maintained on a 12 hour light-dark cycle (lights on at 7 am), and food (Laboratory rodent diet 5001, PMI Feeds, St. Louis, MO) and water were available ad libitum. All protocols were approved by the Animal Care and Use Committee of the University of Pittsburgh and were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

2.2 MATERIALS

D-Phe$_{12}$-Nle$_{21,38}$alpha-Me-Leu$_{37}$-CRH$_{(12-41)}$ (D-Phe-CRH; Bachem) was dissolved to a concentration of 3 μg or 12 μg/3 μl in artificial cerebral spinal fluid (aCSF; in mM : 124 NaCl, 1.8 KCl, 1.2 KH_2PO_4, 1.0 $MgSO_4$, 20.0 $NaCHO_3$, 1.25 glucose, 0.3 ascorbic acid, and 0.1% bovine serum albumin) and acidified with 0.5 uL 30% acetic acid. D-Phe-CRH was used in this experiment rather than alpha-helical CRH, since it was reported that D-Phe-CRH is more potent in the LC by 100-fold *in vivo* (Curtis et al., 1994). This difference in potency *in vivo* was attributed to differences in peptide

binding distribution following ICV infusion, such that the effective concentration that reaches the LC for alpha-helical CRH was less than that for D-Phe-CRH (Curtis et al., 1994).

Kynurenic acid (KYN; Sigma) was first dissolved in 30 μl 10 N NaOH solution to a final concentration of 50 mM in aCSF. (±)-alpha-amino-3-hydroxy-4-methyl-isoxazole-4-proprionic acid hydrobromide (AMPA; Sigma) was dissolved in distilled water and further diluted to a final concentration of 0.24 mM with Dulbecco's Phosphate Buffered Saline (dPBS; Sigma). Muscimol and baclofen (Sigma) were dissolved in dPBS to a final concentration of 0.2 μg/0.5 μl.

2.3 SURGERY

Rats were anesthetized with 5% halothane (Halocarbon Laboratories, River Edge, NJ) in O_2 and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). The nose was pointed downward at 15° from the horizontal plane with a 3.0-mm differential of dorsal-ventral (DV) coordinates of bregma and lambda. For the remainder of the experiments, anesthesia was maintained with 1.5-2.5% halothane in O_2 using a vaporizer (VIP 3000, Matrix Medical, Orchard Park, NY) to maintain suppression of the limb compression withdrawal reflex. Core temperature was maintained between 36-38 °C using a heating pad (VL-20F, Fintronics, New Haven, CT).

Glass recording electrodes (2.0 mm; World Precision Instruments, Inc., Sarasota, FL) were made using a vertical puller (PE2, Narishige, Tokyo, Japan) and filled with 2.0 M NaCl and 2% Pontamine Sky Blue (impedance 6-10 MΩ). Using a hydraulic microdrive (model 640, Kopf), recording electrodes were positioned in the LC (-3.5 mm anterior-posterior [AP], 1.1 mm medial-

lateral [ML] with respect to lambda, and 5.5-6.8 mm DV from dorsal brain surface) (Paxinos and Watson, 2005).

Local or intracerebroventricular (ICV) infusions of drugs were made though 33-gauge cannulae (Plastics One). The cannulae extended beyond the 26-gauge guide cannulae by 1.0 mm and were connected via PE10 tubing (Plastics One) to a 100-µl Hamilton syringe (Fisher, Pittsburgh, PA) for local or ICV infusions. The syringe was attached to a syringe pump (Bioanalytical Systems, West Lafayette, IN). The flexible tubing was filled with drugs prior to the implantation of the cannula and cannula guide in the contralateral lateral ventricle (-1.0 mm AP, 1.4 mm ML with respect to bregma, and 5.3 mm DV from dorsal brain surface) or the CeA (-4.8 mm AP, 4.4 mm ML with respect to bregma, and 5.1-5.3 mm DV from the dorsal brain surface).

To electrically stimulate the CeA, a concentric bipolar electrode (Model NEX-100; Kopf) was lowered into the CeA (-4.8 mm AP, 4.4 mm ML with respect to bregma, and 5.1 - 5.3 mm DV from the dorsal brain surface) (Paxinos and Watson, 2005). The concentric bipolar electrode was connected to a current isolation unit (PSIU6 Grass) and a stimulator (S88 Grass).

2.4 ELECTROPHYSIOLOGY

LC neurons were identified based on well-established criteria including spike waveform, spontaneous firing, and response to paw and tail compression (Cedarbaum and Aghajanian, 1978a; Curtis et al., 2002; Foote et al., 1983). Signals were amplified using a high-impedance headstage connected to an amplifier/window discriminator (Fintronics). Electrophysiological activity was monitored using an audio monitor (AM6 Grass, Quincy, MA) and a storage oscilloscope (V134, Hitachi, Japan). Data were digitized and monitored online using a data acquisition board (Microstar

Labs, Bellevue, WA) connected to a Windows-based personal computer and custom-designed software (Neuroscope).

Once isolated, the basal activity of single LC neurons was recorded for 3 minutes. For a subset of neurons, extracellular single-unit LC neuronal activity was recorded during single-pulse or train stimulation (5 pulses; 10 Hz) of the CeA (300-900 μA, 300 μsec, 0.4 Hz, 75 trials). For a second subset of neurons, extracellular single-unit LC neuronal activity was recorded during both single-pulse and train stimulation (5 pulses; 20 Hz) of the CeA (900 μA, 300 μsec, 0.25-0.4 Hz, 75 trials), before and for approximately 50 minutes after an ICV infusion of 3 μl of aCSF, 3 or 12 μg/3 μl D-Phe-CRH, or 50 mM/3 μl KYN at a rate of 1 μl/min. LC activity was recorded for 3 minutes before, during and 5 minutes after an ICV infusion and for approximately 2 minutes every 7 minutes between train stimulation of the CeA.

Spontaneous LC activity was recorded for 3 minutes prior, during and for 7-10 minutes following a microinfusion of 0.25 mM AMPA in 0.25 or 0.5 μl dPBS, 0.2 μg muscimol and 0.2 μg baclofen in 0.5 μl dPBS, or 0.5 μl of dPBS into the CeA. Only one ICV or intra-CeA infusion was made per rat.

2.5 EUTHANASIA AND HISTOLOGY

At the end of each experiment, the location of the recording site was marked by an iontophoretic ejection of Pontamine Sky Blue from the tip of the recording electrode using -26 μA constant current delivered for 30-40 min. The electrical stimulation site in the CeA was marked by passing 300 μA for 10 sec across the stimulating electrode. The microinfusion sites within the CeA or the

lateral ventricle were marked by infusion of 1 μl Pontamine Sky Blue or 3 μl Fast Green dye, respectively.

Each rat received an overdose of chloral hydrate and was perfused transcardially with cold isotonic saline (Sigma) followed by 4% paraformaldehyde (Sigma) in 0.1 M phosphate buffer (Sigma). The rat was decapitated and the brain was removed. The presence of Fast Green dye in the third ventricle near the optic chiasm was evidence that the infusion cannula was in the lateral ventricle. The brain was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer. Following fixation, the brain was cryoprotected with 25% sucrose (Sigma) in 0.1 M phosphate buffer, sectioned into 60-μm thick coronal slices, and stained with cresyl violet.

Stained sections were examined microscopically to confirm the location of the recording and stimulating electrodes and cannulae lowered into the CeA with respect to the stereotaxic atlas of Paxinos and Watson (2005). Only data from animals with recording sites in the LC (**figure 1A**), electrical activation sites in the CeA (**figure 1B**), the presence of Pontamine Sky Blue in the CeA (**figure 1C**), and the presence of Fast Green dye in the third ventricle near the optic chiasm were included in the data analysis.

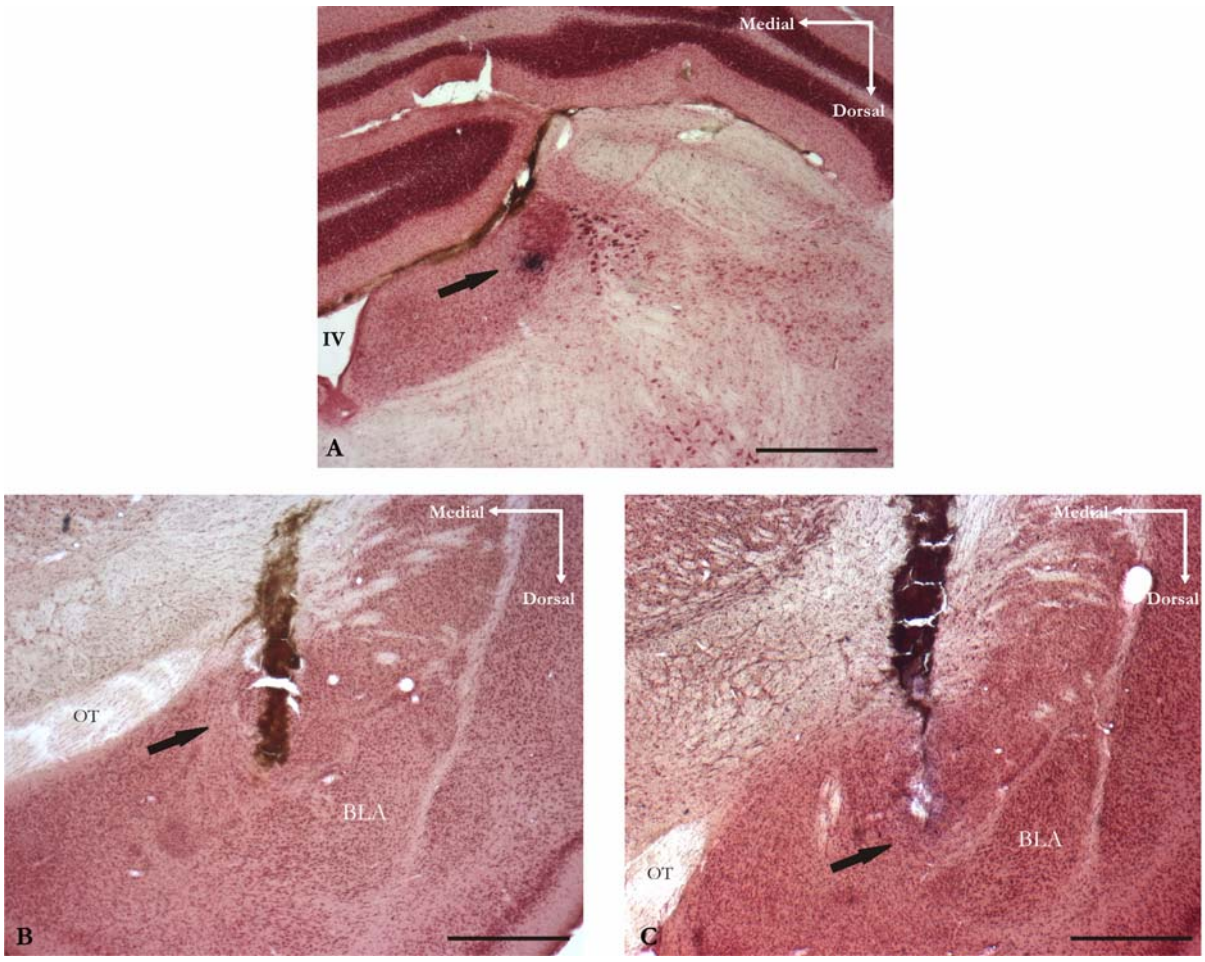

Figure 1. Recording sites in the LC and activation sites in the CeA in Nissl-stained coronal sections.

A) The photomicrograph of the LC shows a representative placement of the tip of the recording electrode in the LC (arrow) made by ejection of Pontamine Sky Blue at the termination of recording. **B)** The photomicrograph of the CeA shows a representative placement of the stimulating electrode in the CeA (arrow). **C)** The photomicrograph of the CeA shows a representative placement of the cannula in the CeA. Following termination of recording, the CeA was marked by injecting 1 μL of dye (15 μL/hr) through the cannula (arrow). Scale bar = 500 μm. IV = Fourth ventricle. OT = optic tract. BLA = basolateral nucleus of the amygdala

2.6 DATA ANALYSIS

2.6.1 Effects of electrical stimulation of the CeA on LC activity.

To determine the effects of single-pulse or train stimulation of the CeA on LC activity, peri-stimulus time histograms (PSTH) were generated (5-ms bins for excitation; 25-ms bins for inhibition) for 75 trials. For cells exhibiting an excitatory response, sliding averages of spike probability (spikes/bin) were calculated using 3 consecutive 5-ms bins. Baseline spike probability was expressed as the average number of spikes/bin prior to electrical stimulation of the CeA. A neuron was considered to be excited when a bin exceeded baseline spike probability by 2.5 standard deviations within 50 ms of single-pulse stimulation of the CeA or within 50 ms of each stimulus of the train. The latency for the peak spike probability in excitatory responses to single-pulse or train stimulation of the CeA was defined as the bin that expressed the maximum number of spikes within 50 ms of stimulus in single-pulse stimulation or within 50 ms of each stimulus of the train and expressed as milliseconds (ms). In contrast, cells demonstrating an inhibition were characterized by at least 3 consecutive 25-ms bins that were less than 80% of the baseline spike probability within 1000 ms following single-pulse or the onset of train stimulation of the CeA. The average onset and offset of an inhibitory period was the first and last bin, respectively, of consecutive series of 3 25-ms bins that was less than 80% of baseline; data are expressed as milliseconds (ms). The duration of inhibition was the difference between the offset and onset of inhibition and expressed as milliseconds (ms). To quantify inhibition, spike probability was calculated from a given period that was determined by the average onset and duration of inhibition. Although not sufficient to eliminate othrodromic activation with a fixed-latency, antidromic activation was operationally defined as spikes elicited at a fixed latency and could follow high frequency stimulation (20 Hz) of the CeA.

Train stimulation of the CeA resulted in 3 types of excitatory responses: acute, late or multiple. An acute excitatory response was defined as the presence of at least 1 bin that exceeded baseline spike probability by 2.5 standard deviations following only the first stimulus of the train. A late excitatory response was defined by the presence of at least 1 bin that exceeded baseline spike probability by 2.5 standard deviations following the second-fifth stimuli of the train, but not following the first stimulus of the train. A multiple excitatory response was defined by the presence of at least 1 bin that exceeded baseline spike probability by 2.5 standard deviations following the first stimulus and any of the remaining stimuli of the train.

2.6.2 Effects of ICV infusions and intra-CeA microinfusions on spontaneous and evoked LC activity.

All data are normalized to the average spike probability obtained prior to ICV drug infusions, and are shown and analysed as percent change in spike probabilities. To examine spontaneous LC activity, sliding firing rate (FR) averages were calculated using 10 consecutive 1-sec bins. The maximal response to an intra-CeA microinfusion of dPBS, 60 and 120 pmol AMPA, or 0.2 μg muscimol/0.2 μg baclofen was defined as the difference between the peak value of sliding FR averages following microinfusion and the basal FR of the neuron. The time of maximal response was defined as the 1-sec bin during which the maximal increase in FR was first attained with respect to the onset of the microinfusion.

Onset and offset of the increase in FR following microinfusions of dPBS, 60 and 120 pmol AMPA, or 0.2 μg muscimol/0.2 μg baclofen in the CeA was defined as the first and last bin, respectively, that was 10% greater than baseline FR. Duration of the increases in LC firing rate as a

result of microinfusions of drug into the CeA was the difference between the offset and onset of the increase FR.

2.6.3 Statistics.

For all data exhibiting a normal distribution, the following statistics tests were used to test significance: Chi-square test, t-test, paired t-test, 1-way ANOVA, or 1-way ANOVA with repeated measures followed by the Tukey or Holm-Sidak test, with type of excitation following 20-Hz train stimulation of the CeA, time or drug as between-subject or within-subject factor. Multiple comparisons were analyzed using a 2-way ANOVA followed by the Holm-Sidak test, with type of excitation following 20-Hz train stimulation of the CeA, drug, or stimulation type, as the between-subject factor and time or current intensity as the within-subject factor. If data did not follow a normal distribution, a Wilcoxon Signed Rank test, Mann-Whitney Rank Sum Test, 1-way ANOVA on Ranks or Kruskal-Wallis 1-way ANOVA on Ranks followed by Dunn's test were used with stimulation type or drug as the between-subject factor. Significance was set as a p value less than 0.05. Data that are not significant are denoted as NS.

3.0 RESULTS

3.1 EFFECTS OF ELECTRICAL STIMULATION OF THE CEA ON LC ACTIVITY

3.1.1 Effects of single-pulse stimulation of the CeA on LC activity.

LC activity from 184 neurons was recorded during single-pulse stimulation of the CeA (900 μA, 300 μsec, 0.4 Hz, 75 trials) to determine the effects of electrical activation of the CeA on LC neuronal activity. Of these neurons, 49% (90 neurons) responded with an excitation within 50 ms of the stimulus, 32% (59 neurons) responded with an inhibition within 1000 ms of the stimulus, 8% (15 neurons) were antidromically activated, and 11% (20 neurons) did not respond to single-pulse stimulation of the CeA.

To determine if LC responses to single-pulse stimulation of the CeA at 900 μA were due to high current intensities, LC activity from 16 neurons was recorded using lower intensities (0-600 μA) of single pulse stimulation of the CeA (300 μsec, 0.4 Hz, 75 trials). Of these neurons, 44% (7 neurons) responded with an excitation within 50 ms of the stimulus, 38% (6 neurons) responded with an inhibition within 1000 ms of the stimulus, and 3 neurons did not respond to single-pulse stimulation of the CeA at 900 μA.

3.1.2 Excitatory responses to single-pulse stimulation of the CeA.

The majority of LC cells (49%) recorded during single-pulse stimulation of the CeA at 900 μA responded with an excitatory response (n = 90/184; **figure 2**). The spike probability within 50 ms of the stimulus was two times greater than baseline spike probability (**figure 2B and table 1**; Wilcoxon Signed Rank test, $p<0.05$). The average latency of the peak spike probability of excitation was 24.1 ± 1.4 ms from the onset of the stimulus. Excitation was followed by an inhibitory period (post-excitation inhibition) that had an average onset of 94 ± 6 ms with respect to onset of single-pulse stimulation and lasted 236 ± 15 ms. During the post-excitation inhibitory period, single-pulse stimulation of the CeA exhibited a 36% decrease from baseline spike probability when tested between 100-350 ms from the onset of stimulation (**table 1**). Spike probability during the post-excitation inhibitory period between 100-350 ms from the onset of stimulation was significantly less than baseline spike probability (**figure 2C and table 1**; paired t-test, $p<0.05$).

A current-response relationship was examined for excitatory responses to single-pulse stimulation of the CeA (n = 7/16; **figure 3**). Increasing current intensities resulted in significant increases in spike probability within 50 ms of the stimulus during single-pulse stimulation of the CeA with respect to baseline spike probability (**figure 4A**; 2-way RM ANOVA, $p<0.05$). Baseline spike probability did not vary significantly at each current intensity tested (**figure 4A**; 2-way RM ANOVA, Holm-Sidak, NS). The spike probability within 50 ms of a 0-μA (control) or 300-μA stimulus was not significantly different from their respective baseline spike probabilities (**figure 4A**; 2-way RM ANOVA, Holm-Sidak, NS). Higher current intensities (600 and 900 μA) during single-pulse stimulation of the CeA resulted in spike probabilities within 50 ms of the stimulus that were significantly greater than their respective baseline spike probability (**figure 4A**; 2-way RM ANOVA, Holm-Sidak, $p<0.05$). In addition, increasing current intensities during single-pulse stimulation of

the CeA resulted in significant increases in post-excitation inhibition (**figure 4B**; 2-way RM ANOVA, $p<0.05$). In the neurons that showed an excitatory response to single-pulse CeA stimulation at 900 μA (n = 7/16; **figure 3D**), post-excitation inhibition had an average onset of 104 ± 25 ms following the onset of stimulation and lasted 211 ± 40 ms. During the post-excitation inhibitory period, the spike probability between 150-250 ms following lower current intensity single-pulse stimulation (0 and 300 μA) was not significantly different from their respective baseline spike probabilities (**figure 4B**; 2-way RM ANOVA, Holm-Sidak, NS). Single-pulse CeA stimulation at higher current intensities (600 and 900 μA) resulted in significantly decreased spike probability during the post-excitation inhibitory period between 150-250 ms following the onset of the stimulus in comparison to their respective baseline spike probabilities (**figure 4B**; 2-way RM ANOVA, Holm-Sidak, $p<0.05$).

Table 1. Excitatory responses to electrical stimulation of the CeA at 900 μA

			single-pulse (n = 90/184)	**10-Hz train** (n = 9/11)	**20-Hz train** (n = 129/184)
Excitation	Spike probability (spikes/50 ms)	Baseline	5.5 ± 0.3	5.1 ± 1.0	5.7 ± 0.2
		S1	13.3 ± 1.0*	13.3 ± 3.6*	11.5 ± 0.7*
		S2	-	9.0 ± 2.2	10.6 ± 0.7*
		S3	-	6.3 ± 1.5	9.6 ± 0.7*
		S4	-	4.9 ± 1.2	9.9 ± 0.7*
		S5	-	5.7 ± 1.3	10.8 ± 0.7*
Post-excitation inhibition	Onset (ms)†		94 ± 6	494 ± 9	261 ± 8
	Duration (ms)		236 ± 15	267 ± 33	518 ± 20
	Spike probability	Period†	100-300 ms	500-750 ms	300-800 ms
		Baseline	27.4 ± 1.5 spikes/200 ms	25.51 ± 4.18 spikes/250 ms	56.7 ± 2.5 spikes/500 ms
		Inhibition††	17.9 ± 1.5 spikes/200 ms*	7.9 ± 2.0 spikes/250 ms*	23.5 ± 2.3 spikes/500 ms*
		% BL†	64 ± 3	37 ± 12	40 ± 3

† with respect to onset of electrical stimulation of the CeA
†† spike probability within the period from onset of electrical CeA stimulation
* $p<0.05$, compared to baseline spike probability

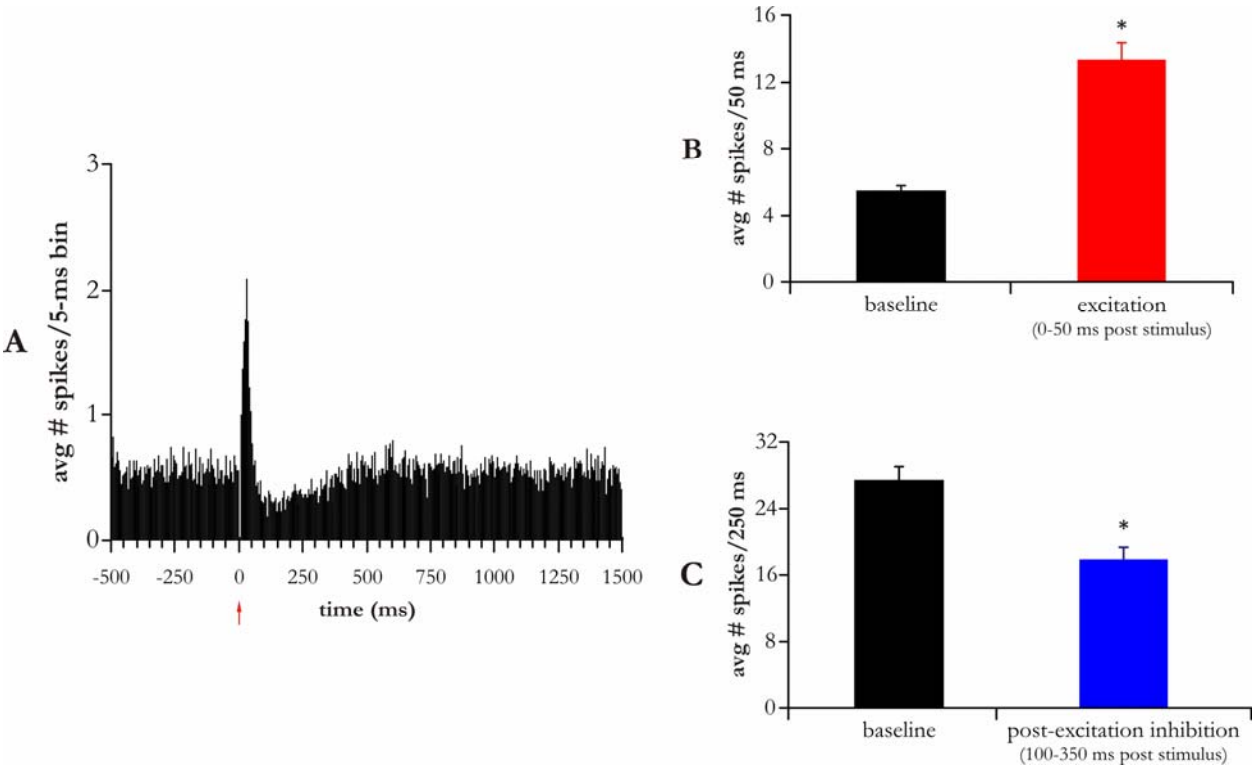

Figure 2. Excitatory responses to single-pulse CeA stimulation.

A) The cumulative PSTH plots the average number of the spikes of all neurons (n = 90/184) that showed an excitatory response within 50 ms of single-pulse stimulation. Excitation was typically followed by an inhibitory period that lasted approximately 240 ms. **B)** Evoked spike probability evoked within 50 ms of the stimulus was two times and significantly greater than baseline (Wilcoxon Signed Rank test, $p<0.05$). **C)** Spike probability during post-excitation inhibition was significantly decreased by 36% in comparison to baseline (paired t-test, $p<0.05$).

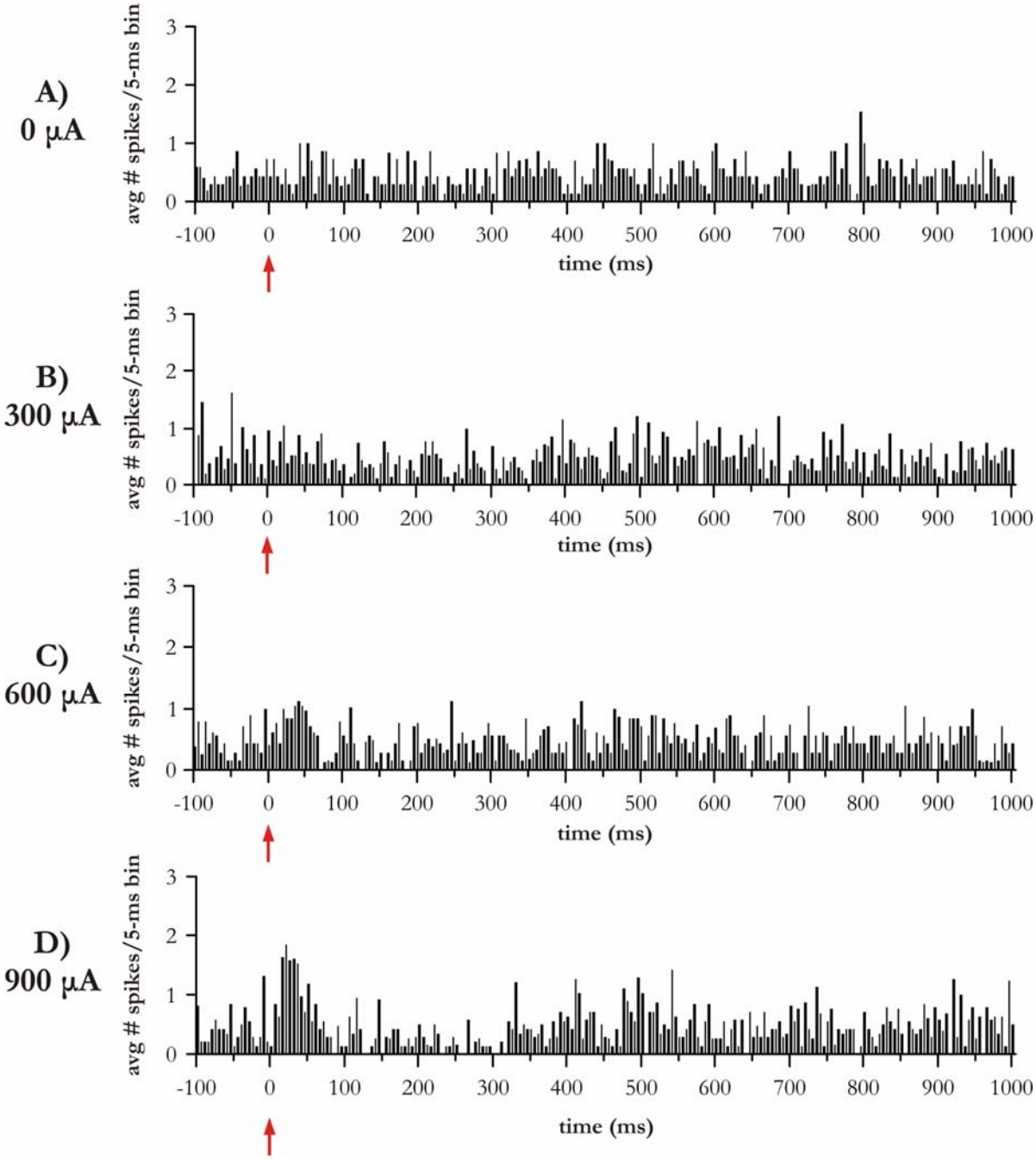

Figure 3. Effects of increasing current intensity on spike probability in excitatory responses to single-pulse stimulation of the CeA.

Cumulative PSTHs depict the average number of the spikes of all neurons (n = 7/16) that showed an excitatory response within 50 ms of single-pulse stimulation **(D)** at 900 µA. **(A-C)** LC activity from neurons that showed an excitatory response to single-pulse stimulation at 900 µA was also recorded at lower current intensities.

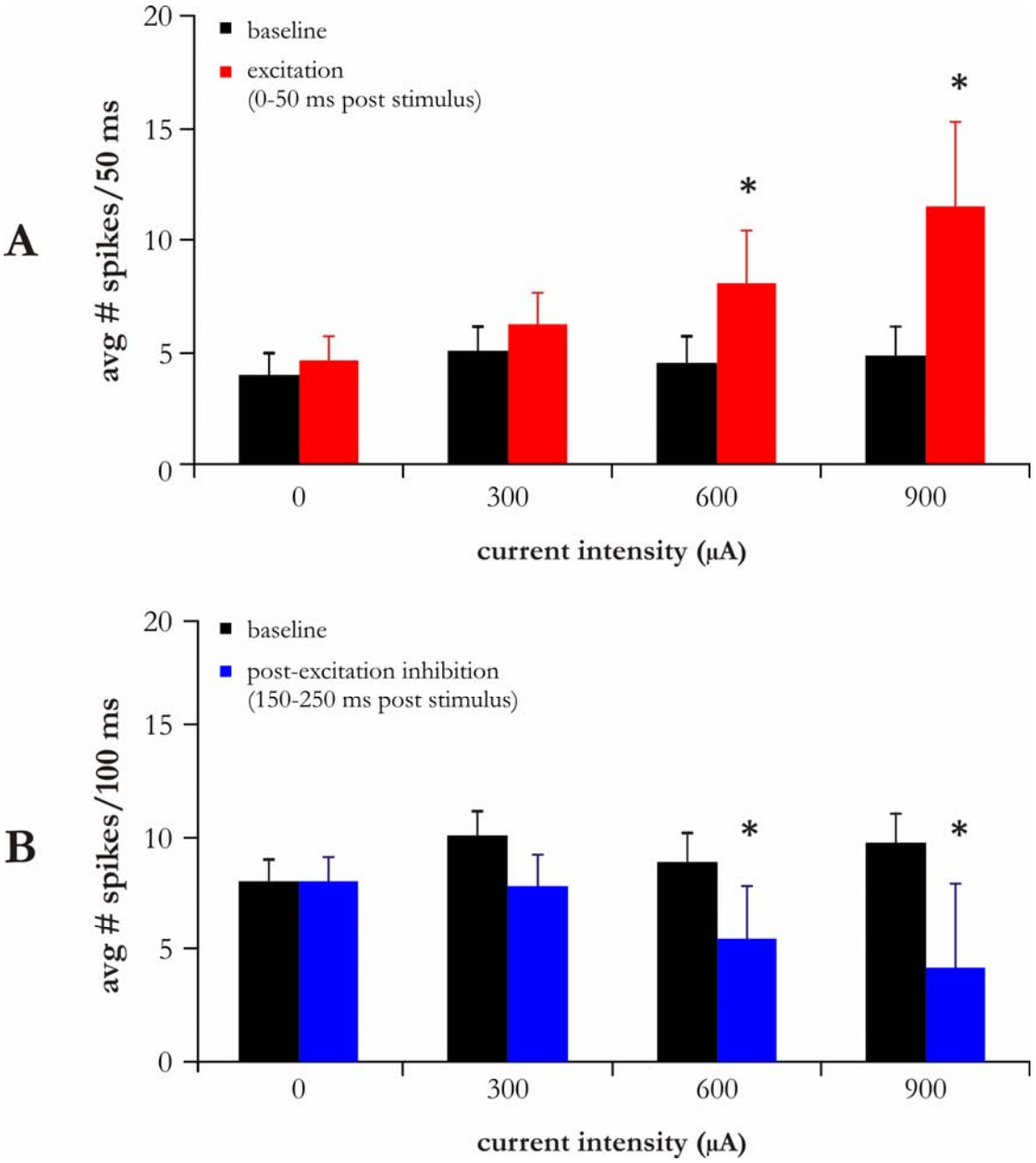

Figure 4. Current-response relationship for excitatory responses to single-pulse stimulation of the CeA.

A) For excitatory responses, higher current intensities during single-pulse stimulation of the CeA resulted in significant increases in evoked spike probability in comparison to baseline (n = 7; 2-way RM ANOVA, Holm-Sidak, $p<0.05$), whereas **(B)** spike probability during post-excitation inhibition was significantly less in comparison to baseline (2-way RM ANOVA, Holm-Sidak, $p<0.05$).

3.1.3 Inhibitory responses to single-pulse stimulation of the CeA.

In addition to excitatory responses, inhibitory responses were observed in approximately one-third of LC neurons recorded during single-pulse stimulation of the CeA in 32% (59/184) of the neurons tested (**figure 5A**). The inhibitory period following CeA stimulation had an average onset of 81 ± 8 ms, which was similar to the average onset of post-excitation inhibition following excitatory responses to single-pulse CeA stimulation (94 ± 6 ms). The average duration of the inhibitory period was 226 ± 16 ms. Inhibitory responses to CeA stimulation showed a 40% decrease in spike probability with respect to baseline between 50-250 ms from the onset of stimulation, which was significantly less than baseline spike probability (**figure 5B and table 3**; 1-way RM ANOVA, Tukey, $p<0.05$).

Similar to the excitatory responses, a current-response relationship was examined for inhibitory responses to single-pulse stimulation of the CeA (n = 6/16; **figure 6**). In neurons that showed an inhibitory response at 900 μA (**figure 6D**), the inhibition had an average onset latency of 121 ± 15 ms and lasted 192 ± 46 ms. Increases in current intensities resulted in significant decreases in spike probability between 100-200 ms following the onset of stimulation in comparison to baseline spike probability (**figure 7**; 2-way RM ANOVA, $p<0.05$). There were no significant differences between the baseline spike probabilities at each current intensity tested (**figure 7**; 2-way RM ANOVA, Holm-Sidak, NS). The spike probability between 100-200 ms following the onset of a lower intensity stimulus (0 and 300 μA) was not significantly different from their respective baseline spike probability (**figure 7**; 2-way RM ANOVA, Holm-Sidak, NS). Higher current intensities (600 and 900 μA) during inhibitory responses to single-pulse CeA stimulation resulted in significantly decreased spike probability between 100-200 ms following the onset of the stimulus in comparison to their respective baseline spike probabilities (**figure 7**; 2-way RM ANOVA, Holm-Sidak, $p<0.05$).

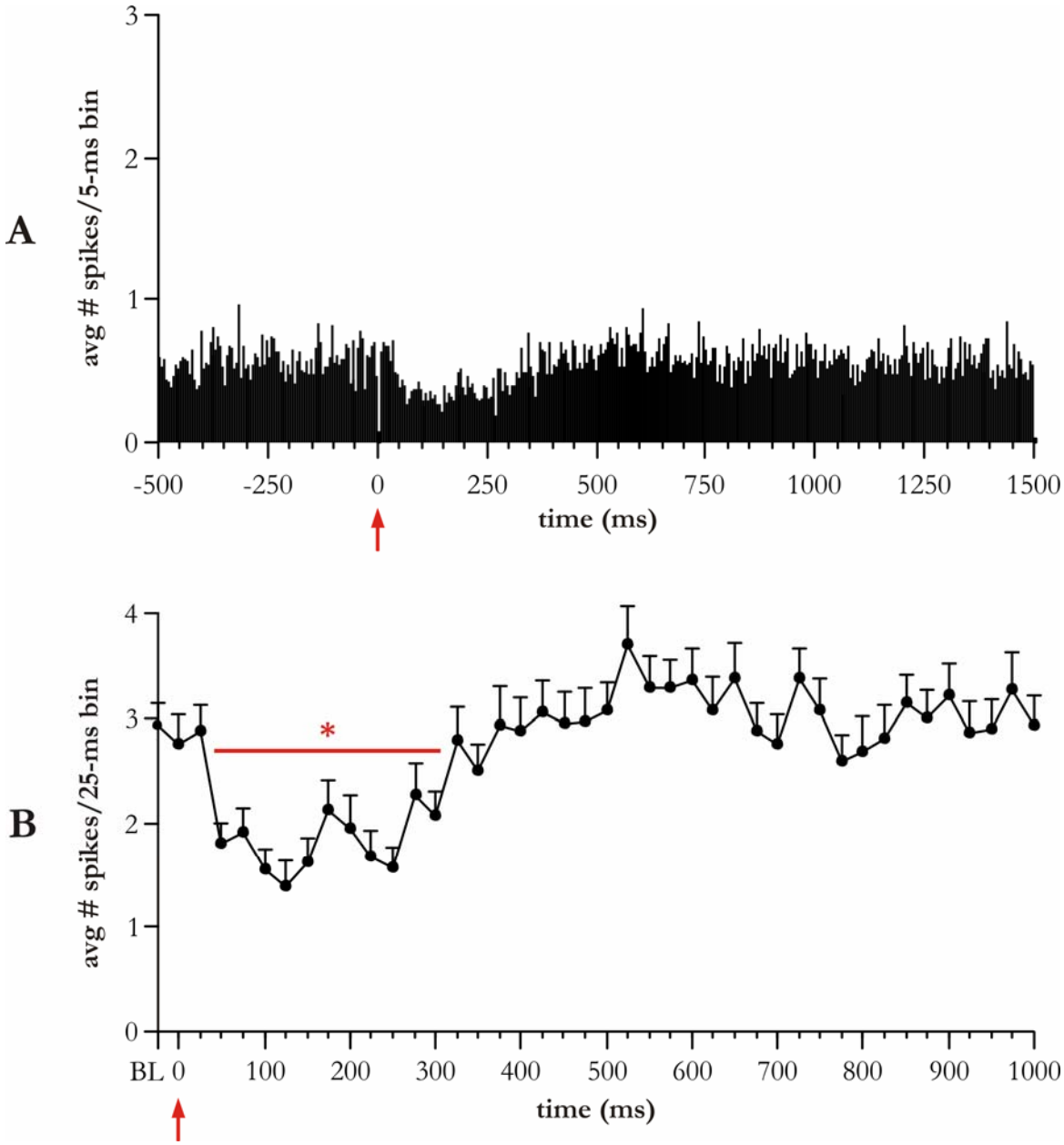

Figure 5. Pure inhibitory responses to single-pulse stimulation of the CeA.

A) The cumulative PSTH plots the average number of the spikes of all neurons (n = 59/184) that showed a pure inhibitory response within 1000 ms of single-pulse stimulation at 900 μA. **B)** Inhibitory responses showed a significant decrease in spike probability between 50-250 ms from the onset of stimulation in comparison to baseline (1-way RM ANOVA, Tukey, $p<0.05$).

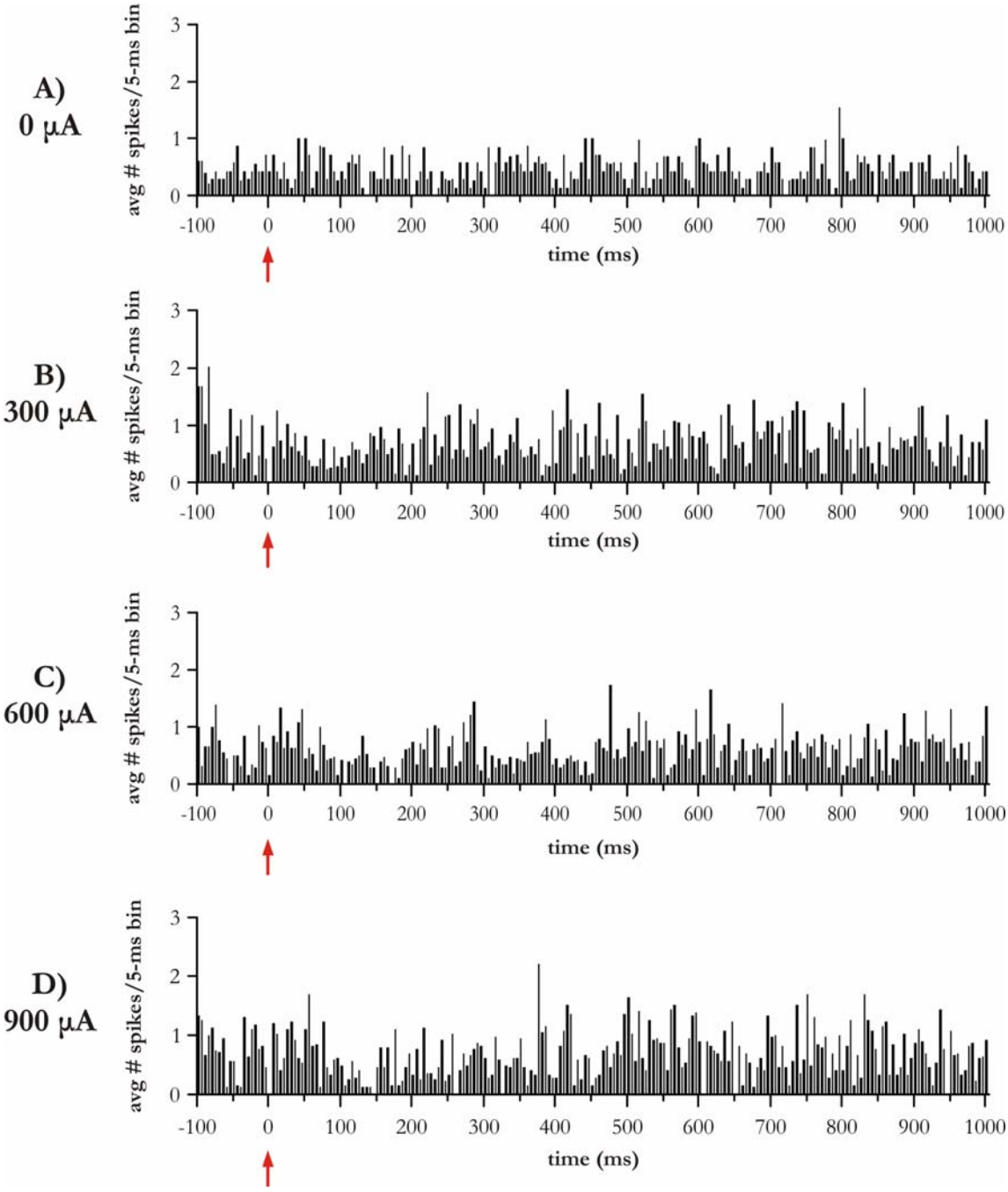

Figure 6. Effects of increasing current intensity on spike probability in inhibitory responses to single-pulse stimulation of the CeA.

Cumulative PSTHs at increasing current intensities plot the average number of the spikes of all neurons (n = 6/16) that showed a pure inhibitory response within 1000 ms of single-pulse stimulation **(D)** at 900 μA. **(A-C)** LC activity from neurons that showed a pure inhibitory response to single-pulse stimulation at 900 μA was also recorded at lower current intensities.

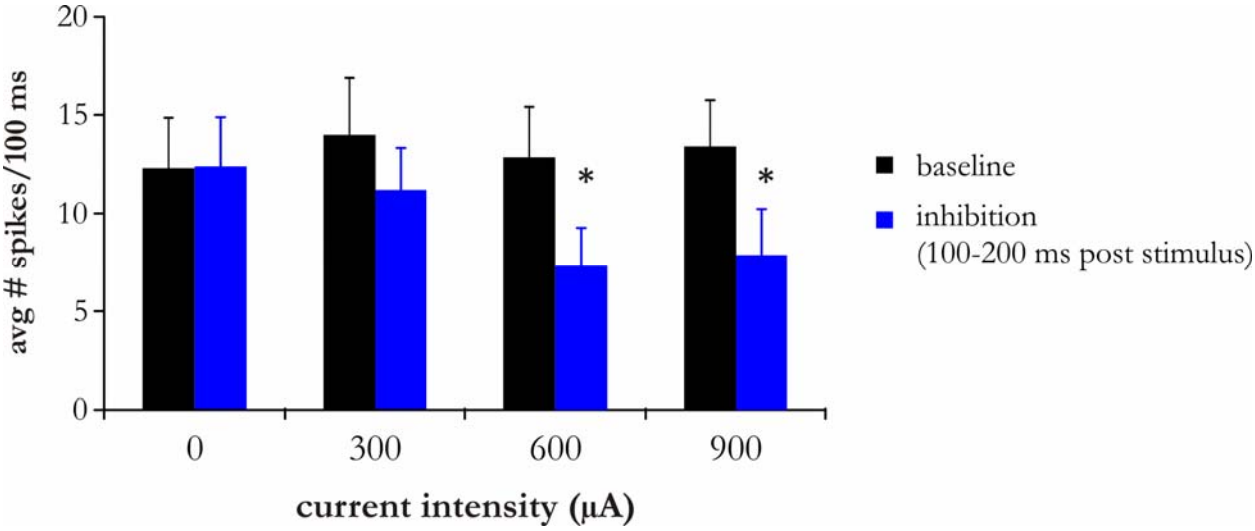

Figure 7. Inverse current-response relationship for inhibitory responses to single-pulse stimulation of the CeA.

Higher current intensities during single-pulse stimulation of the CeA resulted in significant decreases in spike probability during the inhibitory period in comparison to baseline (n = 6; 2-way RM ANOVA, Holm-Sidak, $p<0.05$).

3.1.4 Antidromic activation in response to single-pulse and 20-Hz train stimulation of the CeA.

Single-pulse and train stimulation (5 pulses; 20 Hz) of the CeA resulted in antidromic activation of 8% LC neurons (n = 15/184; **figure 8**). Latencies of antidromic activation were between 10-55 ms from the onset of the stimulus (**figure 8C**). The majority of antidromically activated LC neurons (n = 11/15) showed shorter latencies between 10-30 ms from stimulus, whereas longer latencies of 40 and 55 ms were observed in 4 antidromically activated LC neurons (**figure 8C**).

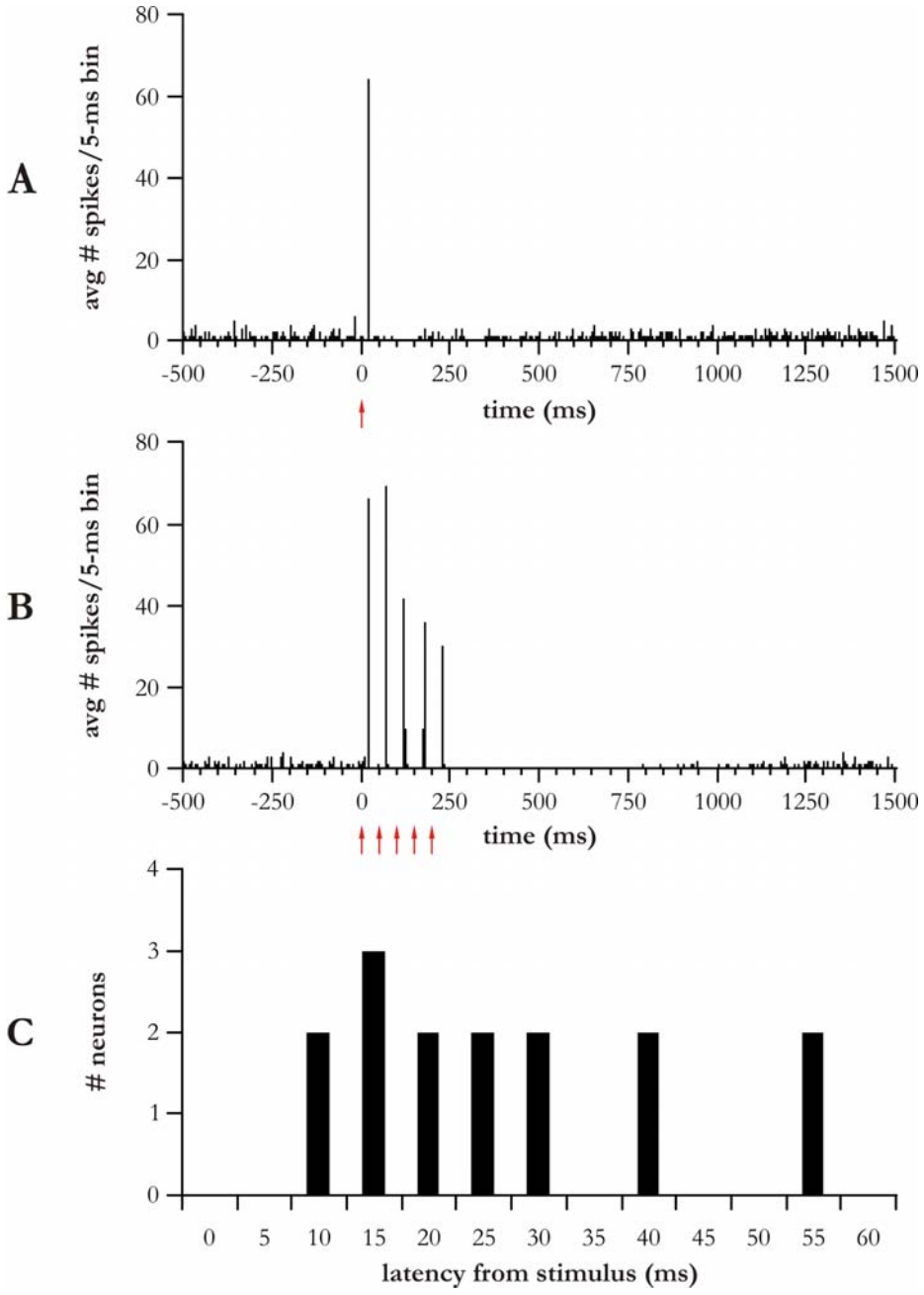

Figure 8. Electrical stimulation of the CeA resulted in antidromic activation of 8% of LC neurons.

PSTHs plot the average number of spikes from a representative neuron responding with antidromic activation to **(A)** single-pulse and **(B)** 20-hz train stimulation of the CeA at 900 μA. Unlike excitatory responses to single-pulse stimulation CeA stimulation, antidromic responses have a fixed latency and **(B)** followed high-frequency train stimulation. **C)** The majority of antidromic responses had latencies between 10-30 ms from the stimulus.

3.1.5 Excitatory responses to 10-Hz train stimulation of the CeA.

Given studies showing that peptidergic components of cotransmission are typically released at high frequencies (Bean et al., 1989; Bean and Roth, 1991), the response of 11 LC neurons to electrical train stimulation (5 pulses; 10 Hz) of the CeA (900 μA, 300 μsec, 0.4 Hz, 75 trials) was tested (**figure 9D**). In response to train stimulation of the CeA at 900 μA, 82% of LC neurons (n = 9/11) responded with an excitation during the train, while the 2 remaining neurons responded with an inhibition within 1000 ms of the onset of train stimulation. To determine whether the LC responses to 10-Hz train stimulation of the CeA at 900 μA were due to high current intensities, LC activity was also recorded during lower intensities (0-600 μA) of train stimulation (5 pulses at 10 Hz, 300 μsec, 0.4 Hz, 75 trials; **figure 9A-C**).

A current-response relationship was examined only for excitatory responses to 10-Hz train stimulation of the CeA. Increasing current intensities resulted in increasing spike probability during the train with respect to baseline spike probability (**figure 10A**; 2-way RM ANOVA, $p<0.05$). There were no significant differences between the baseline spike probabilities at each current intensity tested (**figure 10A**; 2-way RM ANOVA, Holm-Sidak, NS). There were no significant differences in spike probability following the stimuli of the train at the lower current intensities (0-600 μA) in comparison to their respective baseline spike probabilities (**figure 10A**; 2-way RM ANOVA, Holm-Sidak, NS). The spike probability following a 900-μA train was significantly greater than baseline spike probability (**figure 10A**; 2-way RM ANOVA, Holm-Sidak, $p<0.05$).

A current-response relationship was examined for individual stimuli of the train. Increasing current intensities had the largest effect on spike probability at the first stimulus of the train (**figure 10B**; 2-way RM ANOVA, $p<0.05$). There were no significant differences in the spike probability following any stimuli of the train at lower current intensities (0-600 μA) in comparison to their

respective baseline spike probabilities (**figure 10B**; 2-way RM ANOVA, Holm-Sidak, NS). At 900 μA, the spike probability following the first stimulus of the train was significantly greater than baseline spike probability (**figure 10B & table 1**; 2-way RM ANOVA, Holm-Sidak, $p<0.05$), whereas the spike probability following the remaining stimuli was not significantly different from baseline (**figure 10B**; 2-way RM ANOVA, Holm-Sidak, NS).

Similar to excitatory responses to single-pulse stimulation of the CeA, an inhibitory period followed excitatory responses to 10-Hz train stimulation of the CeA at 900 μA (**figure 9D**). This post-excitation inhibitory period had an average onset of 494 ± 9 ms with respect to onset of train stimulation and lasted 267 ± 33 ms (**table 1**). During the post-excitation inhibitory period, there was an approximate 63% decrease in spike probability with respect to baseline between 500-700 ms following the onset of the train, which was significantly less than baseline spike probability (**figure 11 and table 1;** 2-way RM ANOVA, Holm-Sidak, $p<0.05$). A current-response relationship was examined for the post-excitation inhibitory period that occurred following excitatory responses to 10-Hz train stimulation of the CeA. Similar to single-pulse stimulation of the CeA, there was a current-response relationship in the post-excitation inhibition following 10-Hz train stimulation of the CeA (**figure 11**; 2-way RM ANOVA, $p<0.05$). At 0 μA, the spike probability between 500-700 ms following the onset of train stimulation was not significantly different from baseline spike probability (**figure 11**; 2-way RM AVOVA, Holm-Sidak, NS). In contrast, at all current intensities tested (300-900 μA), the spike probability between 500-700 ms following the onset of train stimulation was significantly less than baseline spike probability (**figure 11**; 2-way RM ANOVA, Holm-Sidak, $p<0.05$).

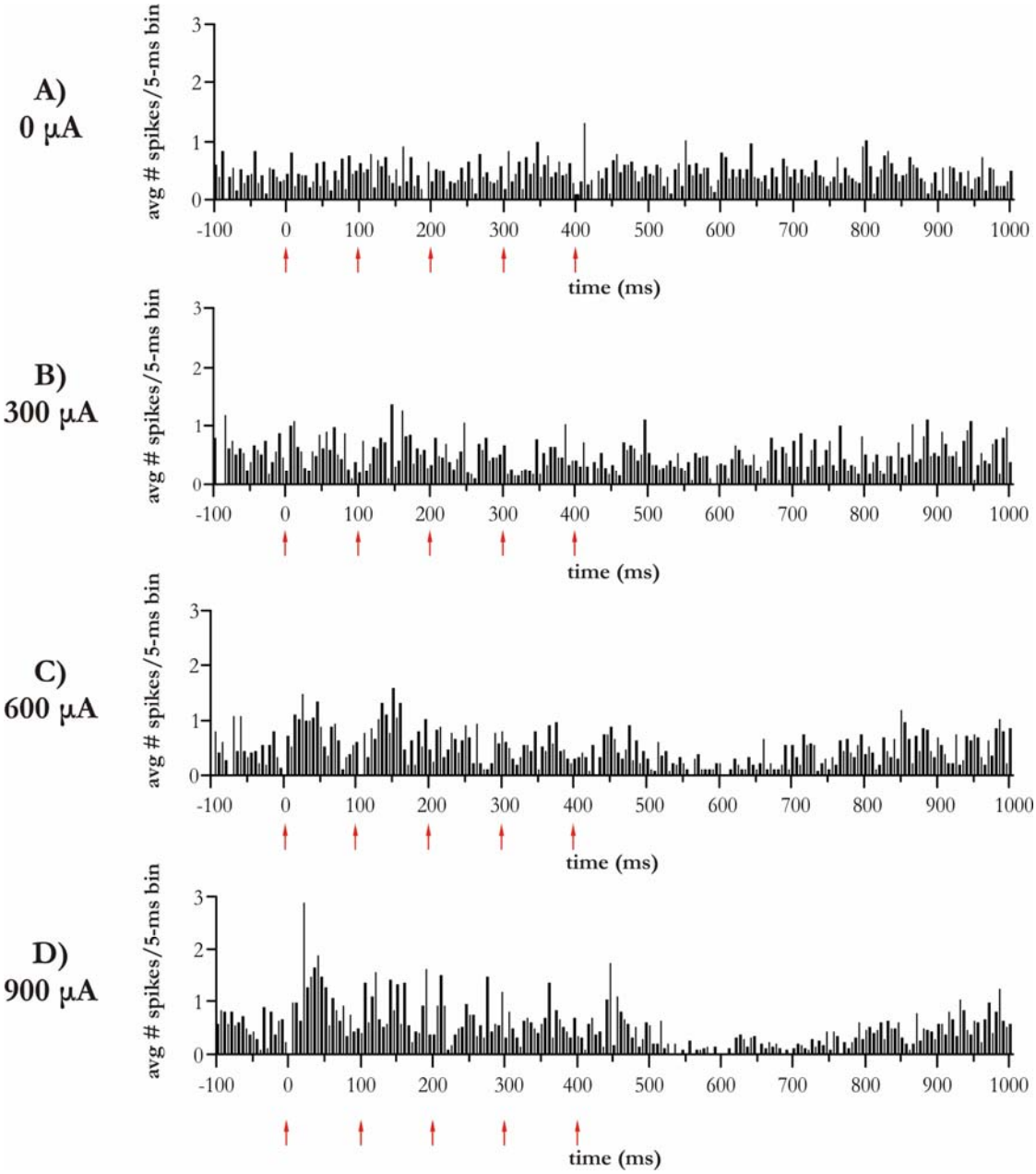

Figure 9. Effects of increasing current intensity on spike probability in excitatory responses to 10-Hz train stimulation of the CeA.

Cumulative PSTHs at increasing current intensities plot the average number of the spikes of all neurons (n = 9/11) that showed an excitatory response to 10-Hz train stimulation **(D)** at 900 µA. **(A-C)** LC activity from neurons that showed an excitatory response to 10-Hz train stimulation at 900 µA was also recorded at lower current intensities. Increasing the current intensity during 10-Hz train stimulation of the CeA resulted in increased evoked activity with an enhanced post-excitation inhibitory period.

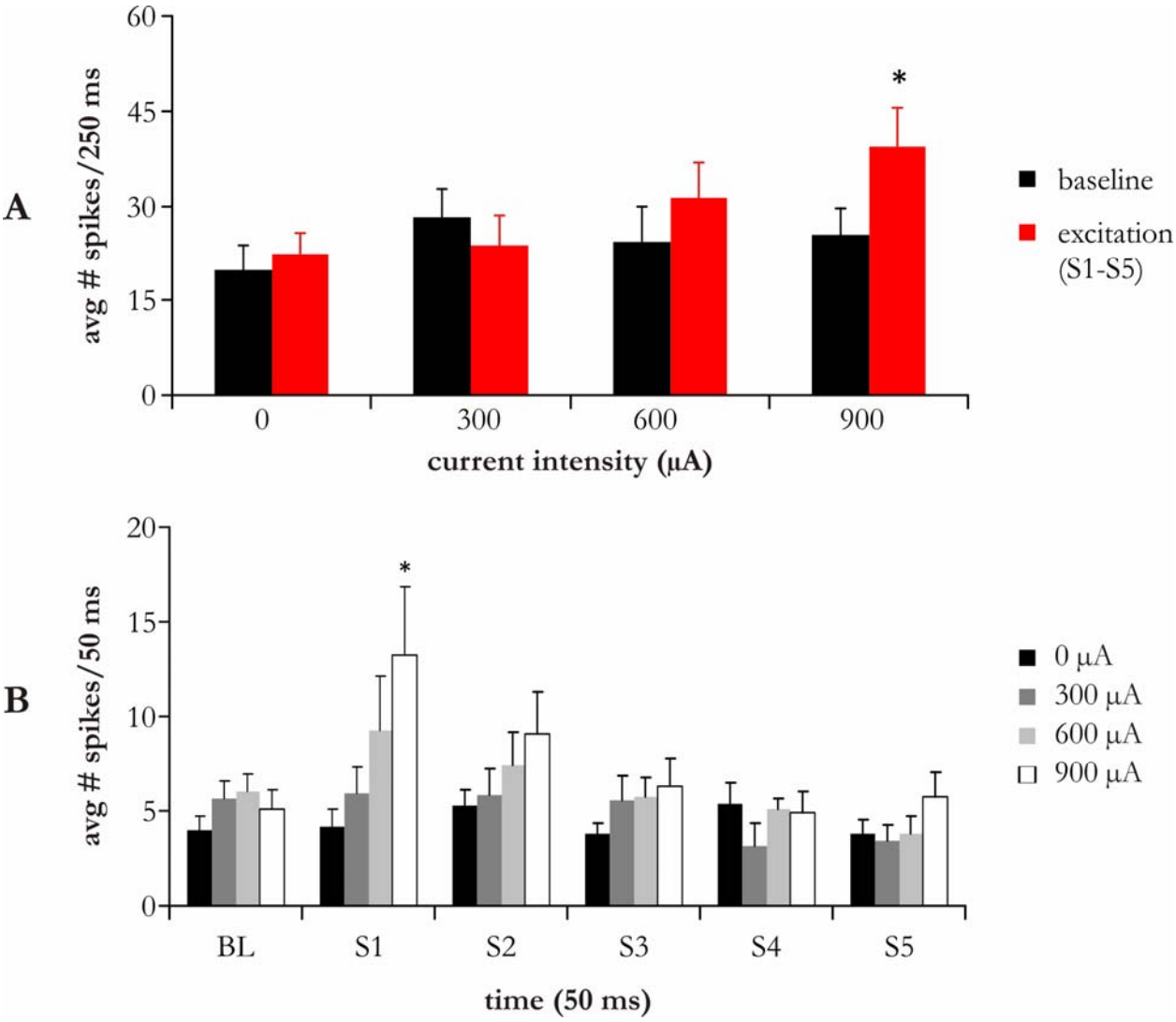

Figure 10. Direct current-response relationship for excitatory responses to 10-Hz train stimulation of the CeA.

A) Increasing the current intensity resulted in increased evoked activity with significant effects with respect to baseline occurring at 900 μA (n = 9; 2-way RM ANOVA, Holm-Sidak, $p<0.05$). **B)** Only the first stimulus of 10-Hz train stimulation at 900 μA elicited a significantly greater spike probability as compared to baseline (n = 9; 2-way RM ANOVA, Holm-Sidak, $p<0.05$).

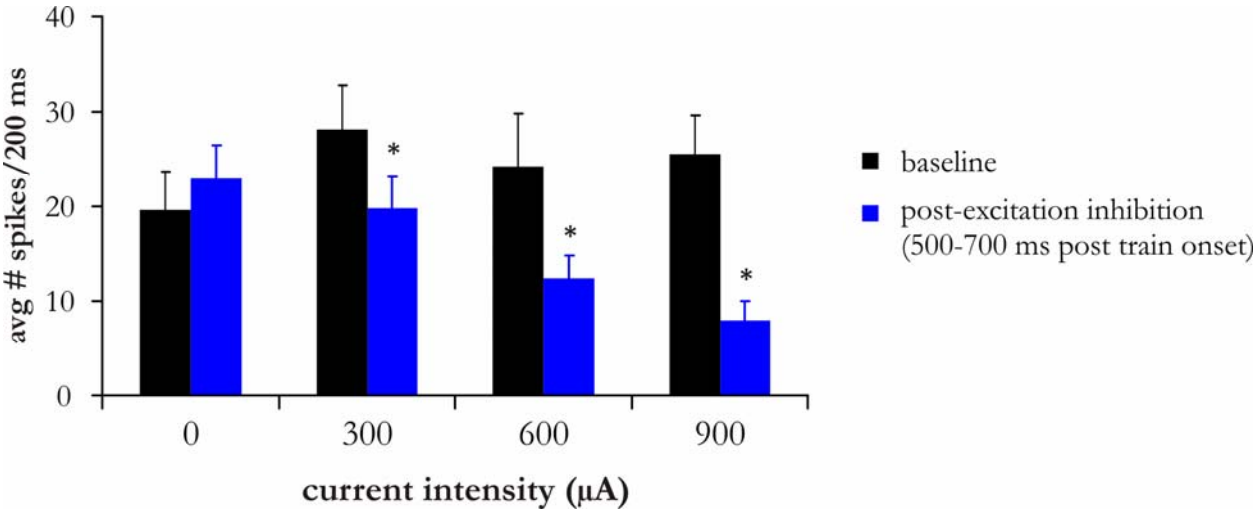

Figure 11. Inverse current-response relationship for post-excitation inhibition in response to 10-Hz train stimulation of the CeA.

Increasing the current intensity during 10-Hz train stimulation resulted in significant decreases in spike probability with respect to baseline during the post-excitation inhibitory period (n = 9; 2-way RM ANOVA, Holm-Sidak, $p<0.05$).

3.1.6 Effects of 20-Hz train stimulation of the CeA on LC activity.

Since 10-Hz train stimulation of the CeA at high current intensities did not significantly increase LC spike probability following each stimulus of the train, the effects of 20-Hz train stimulation (5 pulses) of the CeA (900 μA, 300 μsec, 0.25 Hz, 75 trials) on LC activity were examined in 184 neurons. Of these neurons, 74% (129 neurons) responded with an excitation within 250 ms from the onset of the train, 21% (38 neurons) responded with an inhibition within 1000 ms from the onset of the train, and 2 neurons did not respond to train stimulation of the CeA.

3.1.7 Excitatory LC responses to 20-Hz train stimulation of the CeA.

Following 20-Hz train stimulation of the CeA at 900 μA, 74% of neurons responded with an excitation within 250 ms from the onset of the train (n = 129/184; **figure 12A**). Unlike 10-Hz train stimulation at 900 μA, in neurons that responded with an excitation within 250 ms from the onset of 20-Hz train stimulation at 900 μA, spike probabilities following each stimulus of the train were significantly greater than baseline spike probability (**figure 12B and table 1**; Friedman RM ANOVA on ranks, Tukey, $p<0.05$). Similar to single-pulse CeA stimulation at 900 μA, the average latency of the peak spike probability of excitation for each stimulus was 22.5± 1.0 ms, 20.7 ± 1.0 ms, 21.2 ± 1.0 ms, 20.2 ± 1.0 ms, and 21.4± 1.1 ms from the onset of each stimulus.

An inhibitory period followed excitatory responses to 20-Hz train stimulation, which was consistent with excitatory responses following both single-pulse and 10-Hz train stimulation. This post-excitation inhibitory period had an average onset of 261 ± 8 ms from the onset of train stimulation. There was a 60% decrease in spike probability with respect to baseline during the post-excitation inhibit, which was significantly reduced in comparison to baseline spike probability (**figure 12C and table 1**; paired t-test, $p<0.05$).

Three different types of excitatory responses resulted from 20-Hz train stimulation of the CeA: acute, late, and multiple excitatory responses (**figure 13**). A small population of LC neurons (14%; n = 18/129) responded with an acute excitatory response, which is characterized by an excitation following only the first stimulus of the train **(figure 13A)**. In acute excitatory responses, spike probability following the first stimulus of the train was significantly greater than baseline spike probability (**figure 14 and table 2**; 2-way RM ANOVA, Holm-Sidak, $p<0.05$), whereas the spike probability following the remaining stimuli was not significantly different from baseline spike probability (**figure 14 and table 2**; 2-way RM ANOVA, Holm-Sidak, NS). In contrast, a larger

population of LC neurons responded with a late excitatory response (n = 37/129; **figure 13B**), which is characterized by an excitation occurring during the later stimuli of the train (2^{nd}-5^{th} stimuli). In late excitatory responses, the spike probability following only the later stimuli of the train (2^{nd}-5^{th} stimuli) was significantly greater than baseline spike probability (**figure 14 and table 2**; 2-way RM ANOVA, Holm-Sidak, $p<0.05$), whereas spike probability following the first stimulus of the train was not significantly different than baseline spike probability (**figure 14 and table 2**; 2-way RM ANOVA, Holm-Sidak, NS). The majority of excitatory responses to 20-Hz train stimulation were multiple excitatory responses (57%; n = 74/129), which are characterized by an excitation following the first and any of the remaining stimuli of the train (**figure 13C**). Multiple excitatory responses have combined properties of acute and late excitatory responses. In these responses, the spike probability following all stimuli of the train were significantly greater than baseline spike probability (**figure 14 and table 2**; 2-way RM ANOVA, Holm-Sidak, $p<0.05$). Overall, 20-Hz train stimulation of the CeA resulted in a greater magnitude of excitation following individual stimuli in multiple excitatory responses in comparison to acute and late excitatory responses.

Table 2. Excitatory responses to 20-Hz train stimulation of the CeA at 900 μA

			Acute (n = 18/129)	**Late** (n = 37/129)	**Multiple** (n = 74/129)
Excitation	Spike probability (spikes/50 ms)	Baseline	6.0 ± 1.1	5.6 ± 0.4	5.6 ± 0.3
		S1	10.7 ± 1.8*	6.1 ± 0.6	14.5 ± 1.0*
		S2	3.6 ± 1.0	8.4 ± 0.8*	13.4 ± 1.0*
		S3	3.2 ± 0.9	8.5 ± 1.0*	11.7 ± 1.1*
		S4	2.7 ± 0.8	8.6 ± 0.9*	12.3 ± 1.0*
		S5	4.3 ± 1.0	8.7 ± 0.8*	13.5 ± 1.0*
Post-excitation inhibition	Onset (ms) †		167 ± 32	282 ± 17	273 ± 6
	Duration (ms)		414 ± 45	445 ± 44	579 ± 23
	Spike probability (spikes/500 ms)	Baseline	59.5 ± 11.1	56.4 ± 4.1	56.2 ± 2.8
		Inhibition††	44.0 ± 11.1*	29.0 ± 3.7*	15.9 ± 1.8*
		% BL	69 ± 8	50 ± 5	28 ± 3

† with respect to onset of 20-Hz train stimulation
†† spike probability within 300-800 ms from onset of 20-Hz train stimulation
* $p<0.05$, compared to baseline spike probability

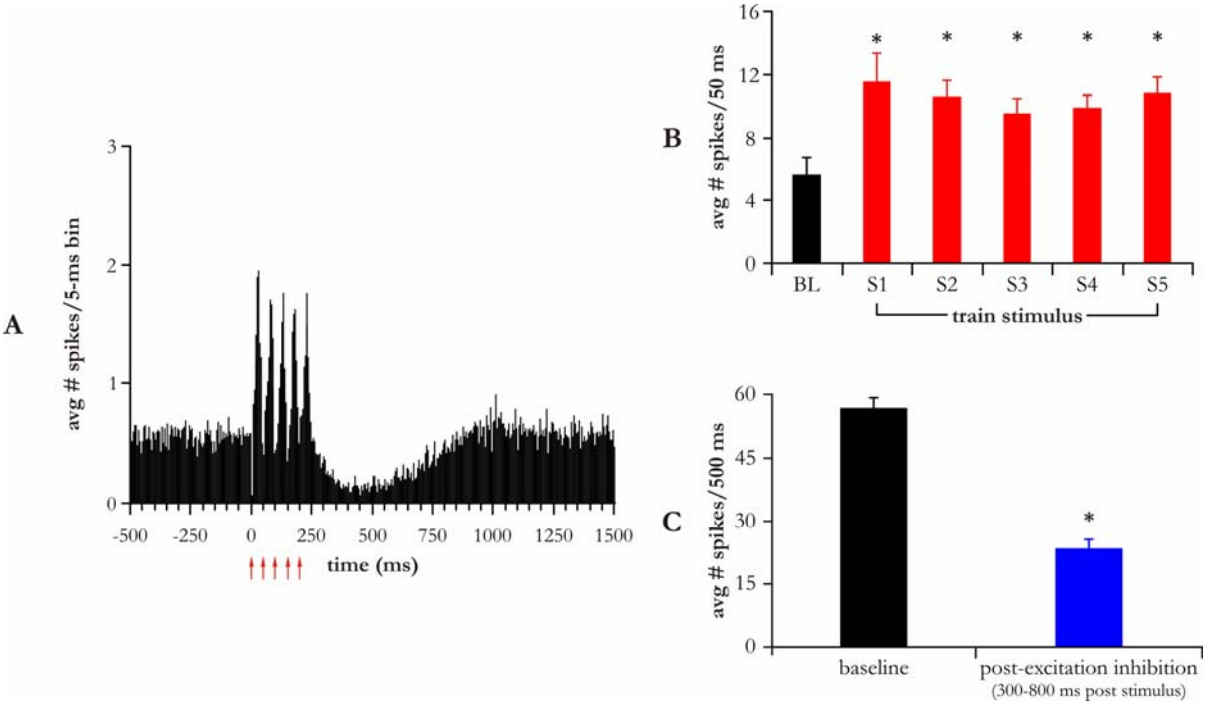

Figure 12. Excitatory responses to 20-Hz train stimulation of the CeA.

A) Cumulative PSTH plots the average number of the spikes of all neurons (n = 129/184) that showed an excitatory response during 20-Hz train stimulation of the CeA. Excitation as followed by an enhanced post-excitation inhibitory period. **B)** Spike probability during the train was significantly greater than baseline (1-way RM ANOVA, Holm-Sidak, $p<0.05$), whereas spike probability during **(C)** post-excitation inhibition was significantly less than baseline (paired t-test, $p<0.05$).

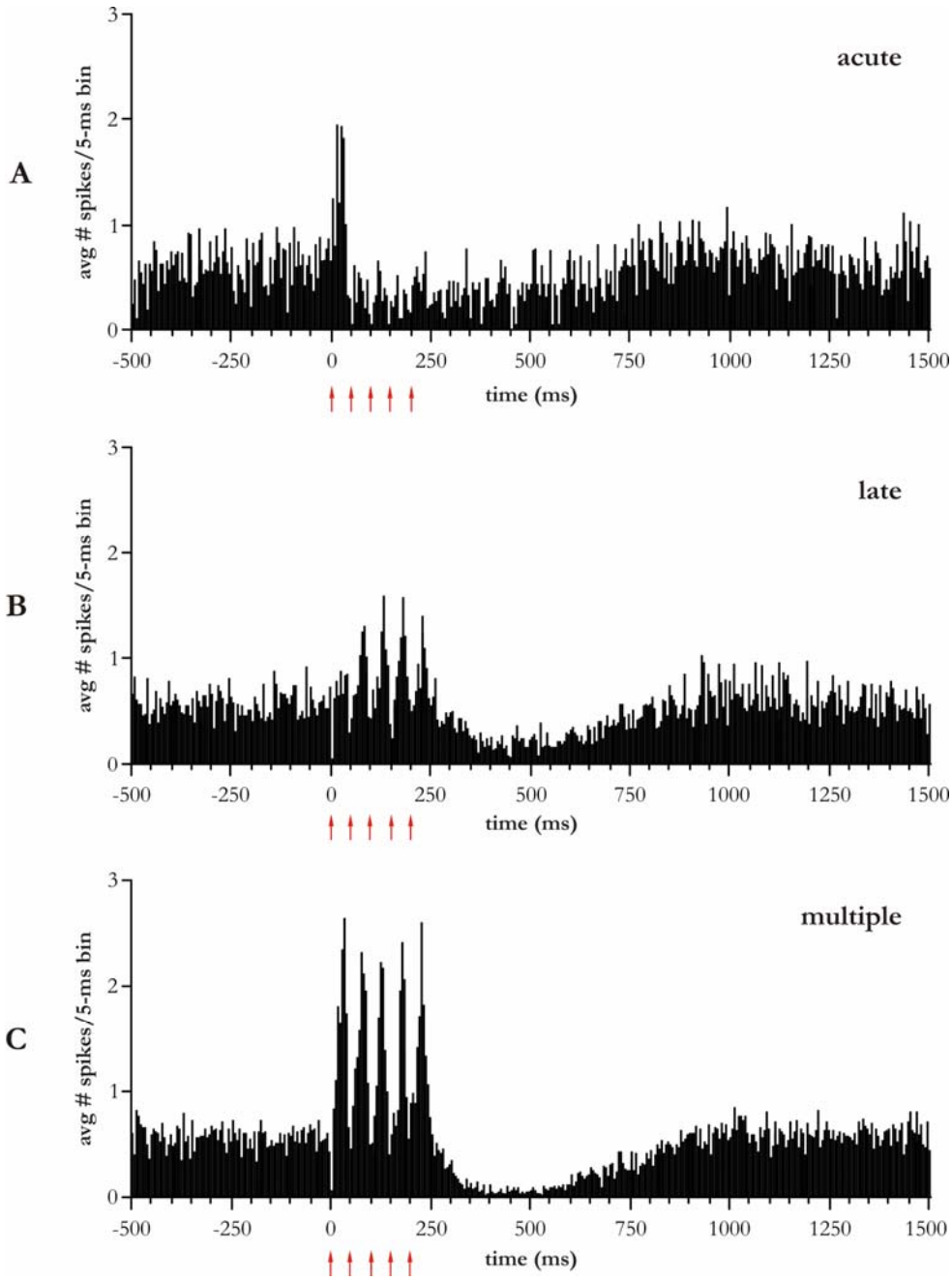

Figure 13. Three types of excitatory responses to 20-Hz train stimulation of the CeA.

Cumulative PSTHs plot the average number of the spikes for the three types of excitatory responses to 20-Hz train stimulation of the CeA. **A)** Acute excitatory responses (n = 18/129) occurred following the first stimulus of the train, whereas **(B)** late excitatory responses (n = 37/129) occurred during the later stimuli of the train. **C)** Multiple excitatory responses (n = 74/129) showed an excitatory response throughout the entire train. All types of excitatory responses were followed by post-excitation inhibition.

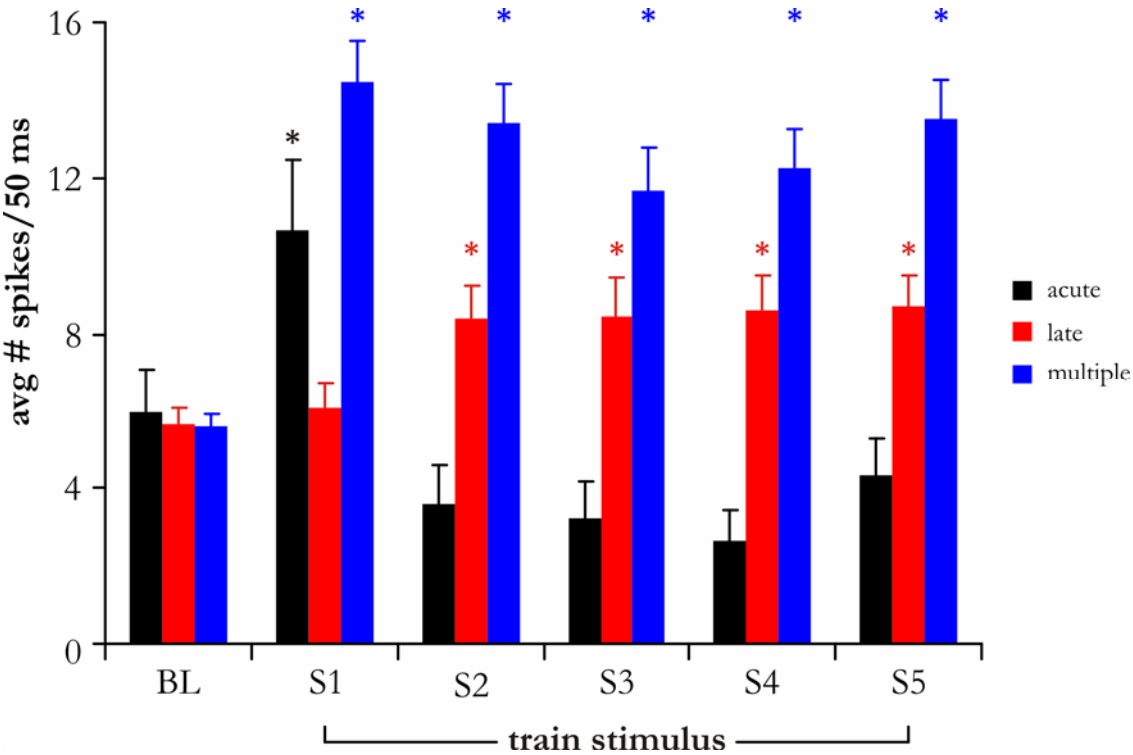

Figure 14. Evoked activity in the three different types of excitatory responses to 20-Hz train stimulation.

Acute excitatory responses showed significant increases in evoked activity only following the first stimulus of the train in comparison to baseline (n = 18; 2-way ANOVA, Holm-Sidak, $p<0.05$), whereas late excitatory responses showed significant increases in evoked activity during the later stimuli of the train in comparison to baseline (n = 37; 2-way ANOVA, Holm-Sidak, $p<0.05$). Multiple excitatory responses showed significant increases in evoked activity following each stimulus of the train in comparison to baseline (n = 74; 2-way ANOVA, Holm-Sidak, $p<0.05$).

Post-excitation inhibition followed all three types of excitatory responses to 20-Hz train stimulation of the CeA (**figure 13**). Acute excitatory responses were followed by a post-excitation inhibition than had an average latency of 167 ± 32 ms from the onset of train stimulation, which was significantly earlier than the latencies for post-excitation inhibition following late and multiple excitatory responses (**figure 15A and table 2**; Kruskal-Wallis 1-way ANOVA on Ranks, Dunn's, $p<0.05$). The latency for post-excitation inhibition from the onset of train stimulation following

both late and multiple excitatory responses was not significantly different (**figure 15A and table 2**; Kruskal-Wallis 1-way ANOVA on Ranks, Dunn's, NS). The duration of the inhibitory period following acute and late excitatory responses was not significantly different from each other (**figure 15A and table 2**; 1-way ANOVA, Holm-Sidak, NS). In contrast, the duration of the inhibitory period that followed multiple excitatory responses was significantly longer than the inhibitory period that followed both acute and late excitatory responses (**figure 15A and table 2**; 1-way ANOVA, Holm-Sidak, $p<0.05$).

For all types of excitatory responses, the spike probability during post-excitation inhibition (300-800 ms following the onset of train stimulation) was significantly reduced in comparison to their respective baseline spike probability (**figure 15B and table 2**; 2-way RM ANOVA, Holm-Sidak, $p<0.05$). There were no significant differences between the baseline spike probabilities for each excitatory response (**figure 15B and table 2**; 2-way RM ANOVA, Holm-Sidak, NS). In acute excitatory responses, the spike probability between 300-800 ms from the onset of the train was reduced by about 31% from baseline spike probability. In comparison to acute excitatory responses, late excitatory responses were followed by a greater, but not significant post-excitation inhibition (**figure 16 and table 2**). In comparison to acute and late excitatory responses, the magnitude of the post-excitation inhibition was dramatically and significantly reduced by 72% from baseline spike probability (**figure 16 and table 2**; 1-way ANOVA on Ranks, Dunn's, $p<0.05$).

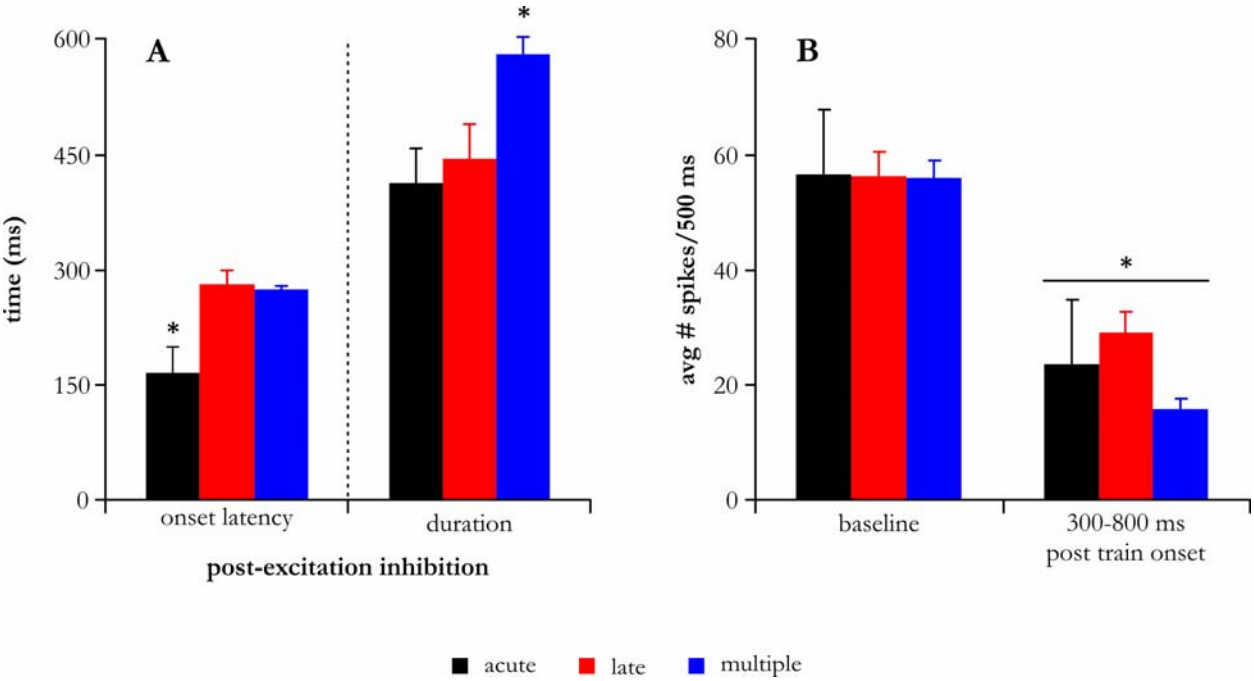

Figure 15. Post-excitation inhibition following all types of excitatory responses to 20-Hz train stimulation.

A) Post-excitation inhibition following acute excitatory responses had a significantly earlier onset than late and multiple excitatory responses (Kruskal-Wallis 1-way ANOVA on Ranks, Dunn's, $p<0.05$). The duration of post-excitation inhibition following multiple excitatory responses was significantly longer than that following acute and late excitatory responses (1-way ANOVA, Holm-Sidak, $p<0.05$). **B)** The spike probability during post-excitation inhibition was significantly less than baseline for all types of excitatory responses (2-way RM ANOVA, Holm-Sidak, $p<0.05$).

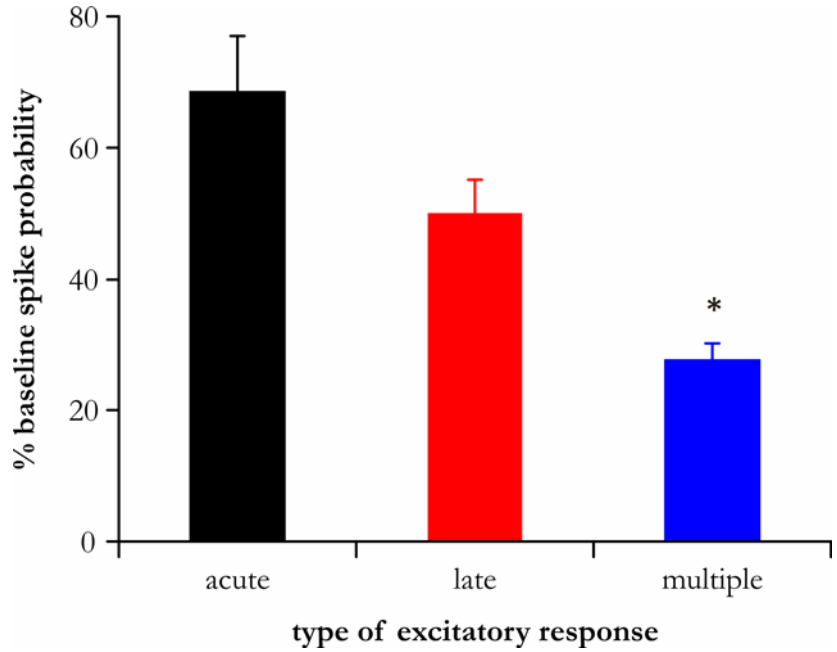

Figure 16. Magnitude of post-excitation inhibition following all types of excitatory responses to 20-Hz train stimulation.

Spike probably as a percentage of baseline during post-excitation inhibition in multiple excitatory responses were significantly less than that in acute and late excitatory responses (1-way ANOVA on Ranks, Dunn's, $p<0.05$).

3.1.8 Inhibitory LC responses to 20-Hz train stimulation of the CeA.

In addition to excitatory responses, 21% of LC neurons (n = 38/184 neurons) responded with a pure inhibitory response following 20-Hz train stimulation of the CeA (**figure 17A**). Inhibition had an average onset of 97±17 ms from the onset of train stimulation and lasted 403 ± 34 ms. During the inhibitory period, there was an 80% decrease in spike probability between 100-400 ms from the onset of train stimulation with respect to baseline (**table 3**). Spike probability between 100-275 ms and 350-400 ms from the onset of train stimulation was significantly less than baseline spike probability (**figure 17B and table 3**; 1-way RM ANOVA, Tukey, $p<0.05$).

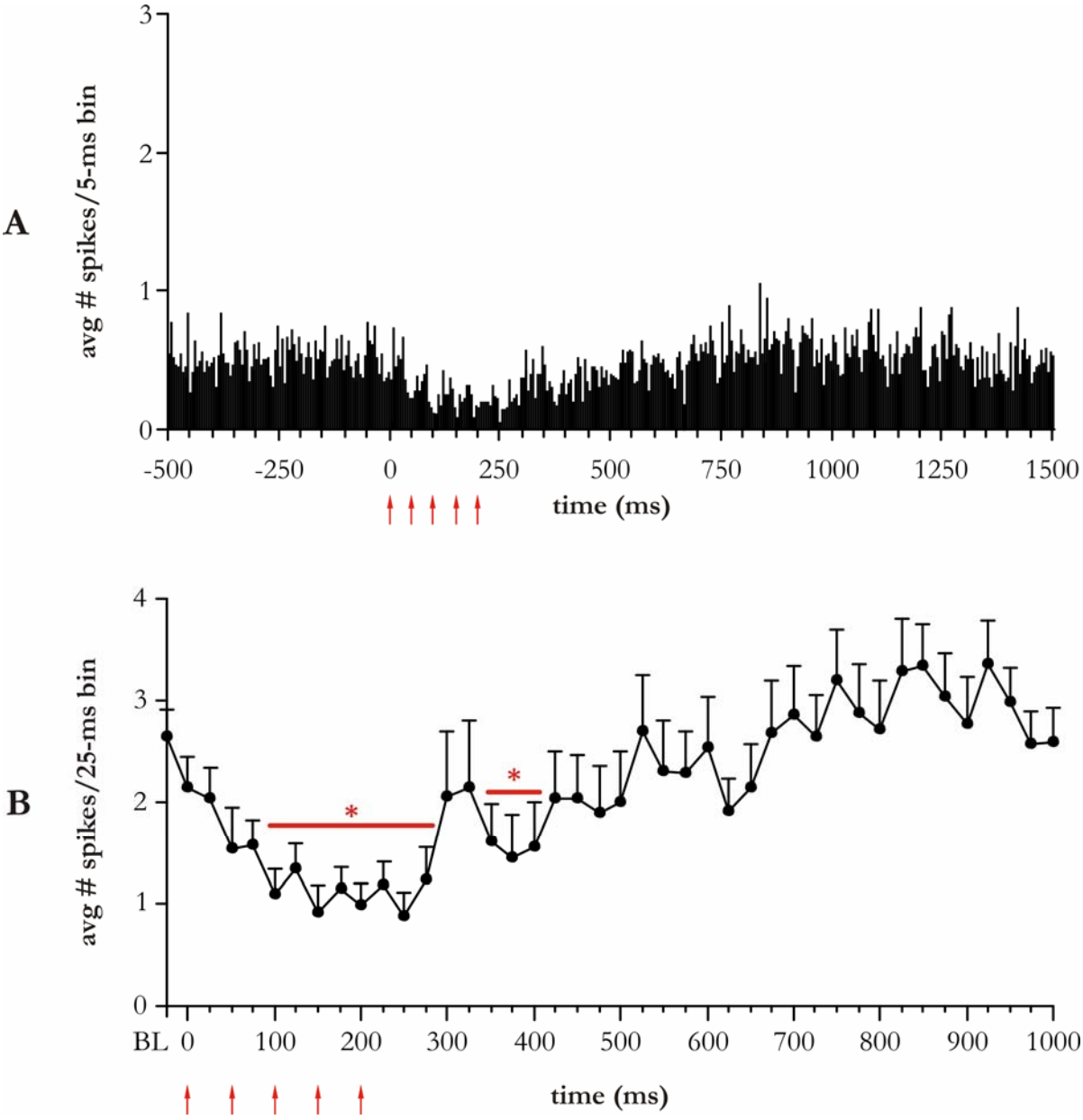

Figure 17. Pure inhibitory responses to 20-Hz train stimulation of the CeA.

A) The cumulative PSTH plots the average number of the spikes of all neurons (n = 38/184) that showed a pure inhibitory response within 1000 ms of the onset of train stimulation 900 μA. **B)** Inhibitory responses showed a significant decrease in spike probability between 100-275 ms and 350-400 ms from the onset of train stimulation in comparison to baseline (1-way RM ANOVA, Tukey, $p<0.05$).

Table 3. Inhibitory responses to electrical stimulation of the CeA at 900 μA

			Single-pulse (n = 59/184)	**20-Hz train** (n = 38/184)
Inhibition	Onset (ms)†		81 ± 8	97 ± 17
	Duration (ms)		226 ± 16	403 ± 34
	Spike probability	Period†	50-250 ms	100-400 ms
		Baseline	23.0 ± 1.6 spikes/200 ms	31.6 ± 3.2 spikes/300 ms
		Inhibition††	13.9 ± 1.4 spikes/200 ms*	5.5 ± 0.6 spikes/300 ms*
		% BL†	60 ± 4	20 ± 2

† with respect to onset of electrical stimulation of the CeA
†† spike probability within the period from onset of CeA stimulation
* $p<0.05$, compared to baseline spike probability

3.1.9 Single-pulse vs. train stimulation of the CeA.

There were significant differences between single-pulse and high-frequency train stimulation of the CeA at 900 μA. There were significantly more neurons responding with an excitation and significantly fewer neurons responding with an inhibition or were unresponsive to 20-Hz train stimulation (n = 2/184) compared to single-pulse stimulation (**figure 18**; Chi-square, $p<0.05$). The magnitude of the excitation as a percentage of baseline following single-pulse stimulation was not significantly different than that following individual stimuli of 10-Hz train stimulation (**figure 19**; 1-way RM ANOVA, NS) or 20-Hz train stimulation (**figure 19**; 1-way RM ANOVA, NS). Although the later stimuli of 10-Hz train stimulation did not result in spike probability that significantly exceeded baseline (**figure 10B**), there were no significant differences in the magnitude of the excitation as a percentage of baseline following individual stimuli of 10-Hz train stimulation in comparison to 20-Hz train stimulation (**figure 19**; 2-way RM ANOVA, NS).

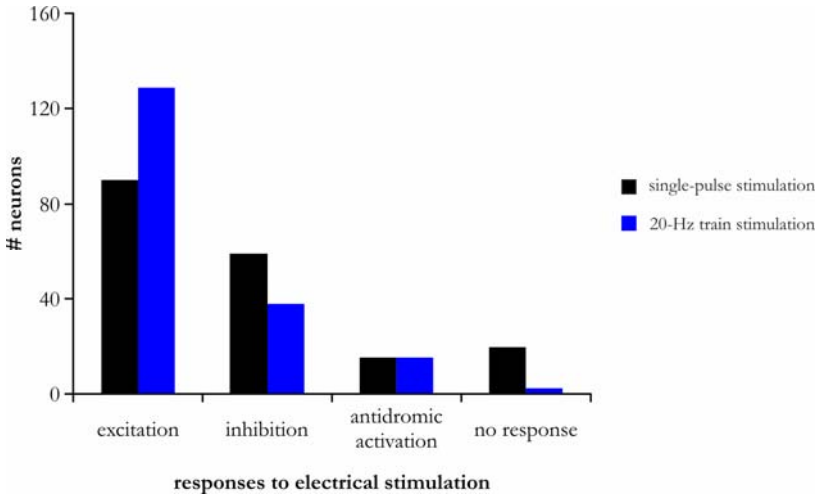

Figure 18. Reponses to single-pulse and 20-Hz train stimulation of the CeA.

There were significantly more neurons responding with an excitation and significantly fewer neurons responding with an inhibition or were unresponsive to 20-Hz train stimulation in comparison to single-pulse stimulation of the CeA (Chi-square, $p<0.05$).

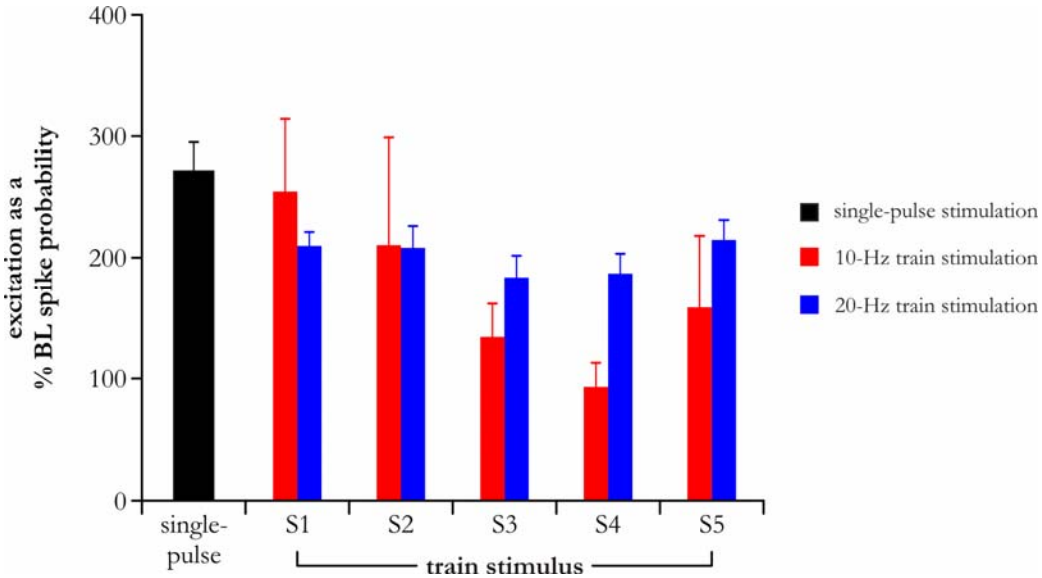

Figure 19. Excitatory responses to single-pulse and train stimulation of the CeA.

Although high-frequency 20-Hz train stimulation of the CeA resulted in an excitatory response following all stimuli of the train in the majority of excitatory responses, the magnitude of the response was not significantly different than that of single-pulse stimulation of the CeA (1-way RM ANOVA, NS).

There were significant differences between the different types of electrical stimulation of the CeA in the duration and magnitude of the inhibitory period following excitatory responses. There were no significant differences between single-pulse and 10-Hz train stimulation in the duration of post-excitation inhibition (**figure 20A**; 1-way ANOVA on Ranks, Dunn's, NS). In contrast, post-excitation-inhibition as a result of 20-Hz train stimulation was significantly longer than that as a result of both single-pulse or 10-Hz train stimulation (**figure 20A and table 1**; 1-way ANOVA on Ranks, Dunn's, $p<0.05$). The magnitude of post-excitation inhibition as a percentage of baseline between 100-300 ms following single-pulse stimulation was greater but not significantly different that between 500-750 ms following the onset of 10-Hz train stimulation (**figure 20B and table 1**; 1-way ANOVA on Ranks, Dunn's, NS). The post-excitation inhibitory period as a percentage of baseline between 300-800 ms following the onset of 20-Hz train stimulation was not significantly different to that following 10-hz train stimulation (**figure 20 and table 1**; 1-way ANOVA on Ranks, Dunn's, NS), but significantly less than that following single-pulse stimulation (**figure 20 and table 1**; 1-way ANOVA on Ranks, Dunn's, $p<0.05$).

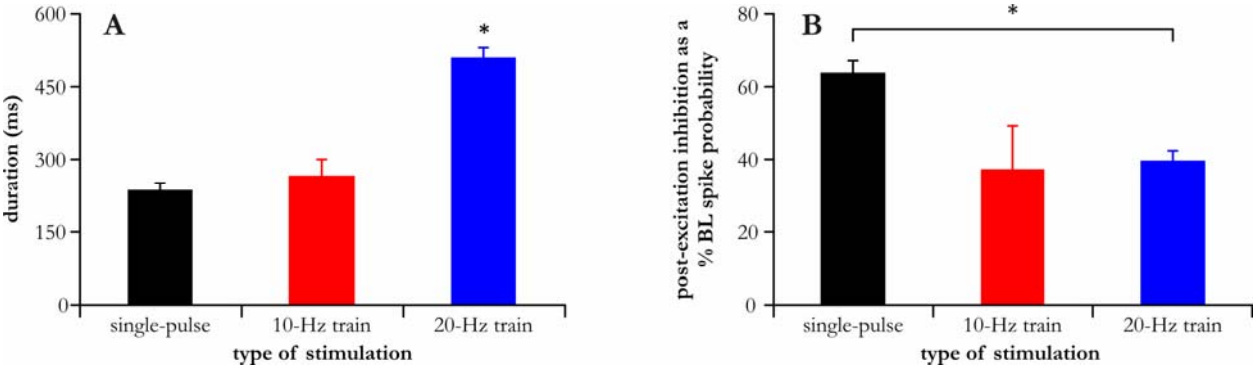

Figure 20. Inhibition following excitatory responses to single-pulse and train stimulation.
A) Post-excitation inhibition was significantly longer following 20-Hz train stimulation in comparison to single-pulse and 10-Hz train stimulation (1-way ANOVA on Ranks, Dunn's, $p<0.05$). **B)** The magnitude of post-excitation following excitatory responses to 20-Hz train stimulation was significantly enhanced in comparison to single-pulse stimulation (1-way ANOVA on Ranks, Dunn's, $p<0.05$).

There were also significant differences between types of electrical stimulation in the onset, duration, and magnitude of pure inhibitory responses to electrical stimulation. There were no significant differences in the onset of inhibition between single-pulse and 20-Hz train stimulation (**figure 21A and table 3**; Mann-Whitney Rank Sum Test, NS). Pure inhibitory responses as a result of 20-Hz train stimulation were significantly longer than pure inhibitory responses following single-pulse stimulation (**figure 21A and table 3**; Mann-Whitney Rank Sum Test, $p<0.05$). Inhibition as a result of 20-Hz train stimulation exhibited an approximate 80% decrease from baseline spike probability between 100-400 ms, and this decrease was significantly greater following 20-Hz train stimulation than for inhibitory period between 50-250 ms following single-pulse stimulation (**figure 21B and table 3**; Mann-Whitney Rank Sum Test, $p<0.05$).

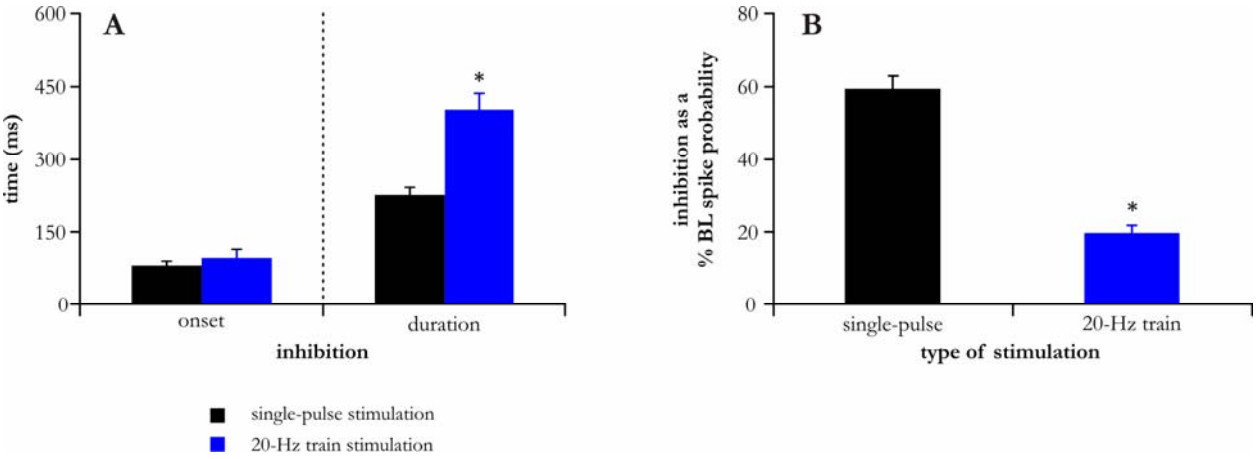

Figure 21. Pure inhibitory responses to single-pulse and 20-Hz train stimulation.

A) In comparison to single-pulse stimulation, pure inhibitory responses were significantly longer following 20-Hz train stimulation (Mann-Whitney Rank Sum Test, $p<0.05$) with no significant differences in onset (Mann-Whitney Rank Sum Test, NS). **B)** Train stimulation resulted in a significantly enhanced pure inhibitory response in comparison to single-pulse stimulation (Mann-Whitney Rank Sum Test, $p<0.05$).

3.1.10 Responses to electrical stimulation of the CeA with respect to the location of stimulating electrode within the CeA and recording electrode within the LC.

To determine if electrical stimulation of different regions of the CeA preferentially produces a specific response to either single-pulse or train stimulation of the CeA, responses to both single-pulse (**figure 22**) and 20-Hz train stimulation (**figure 23**) were correlated with the placement of the stimulating electrode within the CeA with respect to 1) the different divisions of the CeA (CeC: capsular CeA, CeM: medial CeA, CeL: lateral CeA) as defined by Paxinos and Watson, 2005, or 2) the entire anterior-posterior axis of the CeA in increments of 0.24 mm. In response to single-pulse CeA stimulation, the distribution of excitatory and inhibitory responses, antidromic activation or no response was not systematically dependent on the placement of the stimulating electrode 1) within

the different subdivisions of the CeA (**figure 22A-D**; Chi-square, NS) or 2) along the entire anterior-posterior axis of the CeA (**figure 22E-H**; Chi-square, NS). Similar to single-pulse stimulation of the CeA, the distribution of the different types of excitatory responses (acute, late, and multiple) and inhibitory responses to 20-Hz train stimulation of the CeA was not significantly dependent on the placement of the stimulating electrode in the CeA 1) within the different subdivisions of the CeA (**figure 23A-D**; Chi-square, NS) or 2) along the entire anterior-posterior axis of the CeA (**figure 23E-H**; Chi-square, NS).

To determine if specific responses to either single-pulse or train stimulation of the CeA are preferentially distributed within the LC, responses to both single-pulse (**figure 22**) and 20-Hz train stimulation (**figure 23**) were correlated with the placement of the recording electrode within the LC along its entire anterior-posterior axis in increments of 120 μm. The distribution of excitatory and inhibitory responses, antidromic activation, or no response to single-pulse CeA stimulation was not significantly dependent on the placement of the recording electrode along the entire anterior-posterior axis of the LC (**figure 22I-L**; Chi-square, NS). Similar to single-pulse stimulation of the CeA, the distribution of acute, late, and multiple excitatory responses and inhibitory responses to 20-Hz train stimulation was not dependent on the placement of the recording electrode along the entire anterior-posterior axis of the LC (**figure 23I-L**; Chi-square, NS).

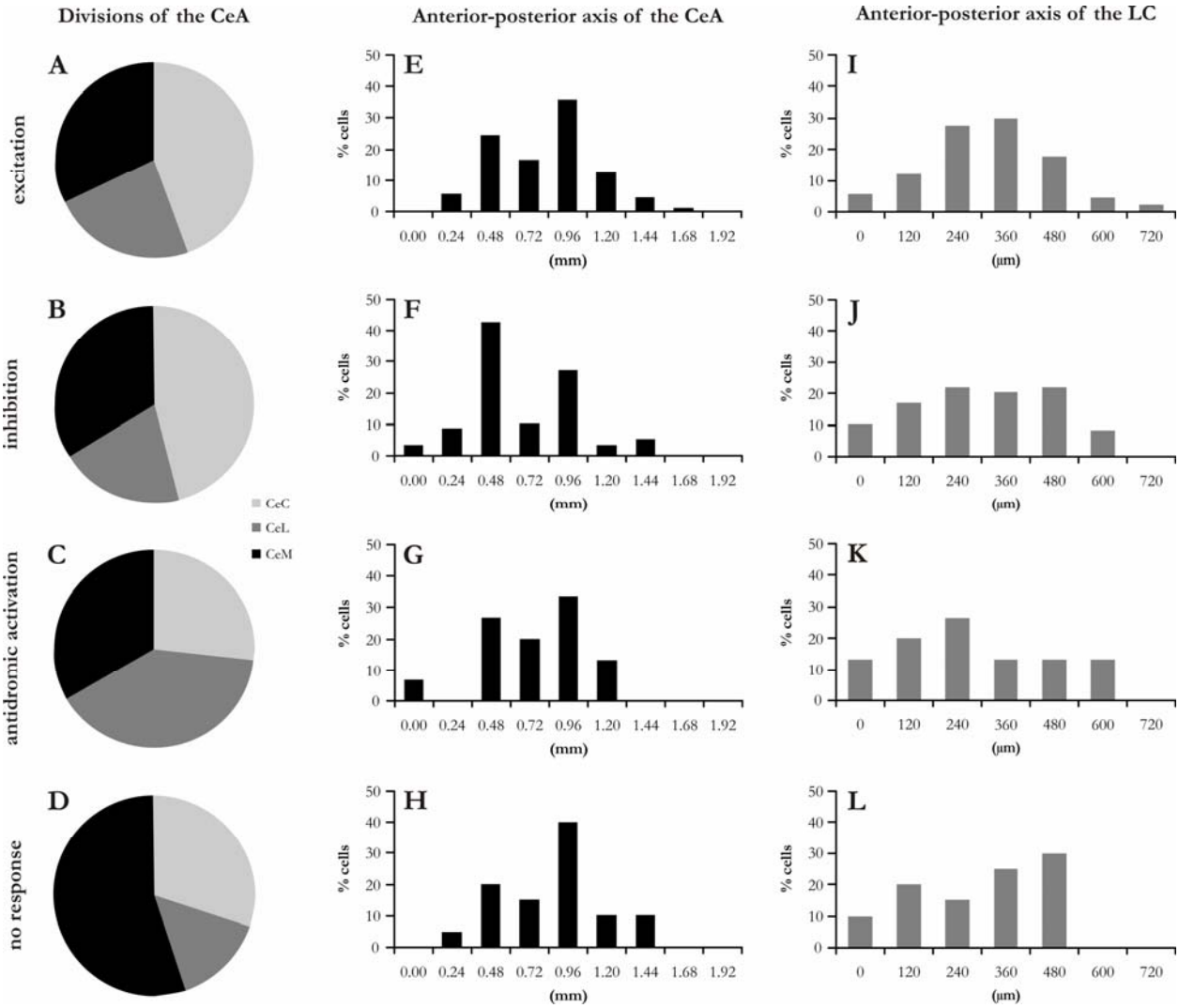

Figure 22. Responses to single-pulse stimulation of the CeA with respect to the location of stimulating and recording electrodes.

All responses to single-pulse stimulation were not correlated to the location of the stimulating electrode in the different subdivisions (**A-D**; Chi-square, NS) or anterior-posterior axis of the CeA (**E-H**; Chi-square, NS), and location of the recording electrode in the anterior-posterior axis of the LC (**I-L**; Chi-square, NS).

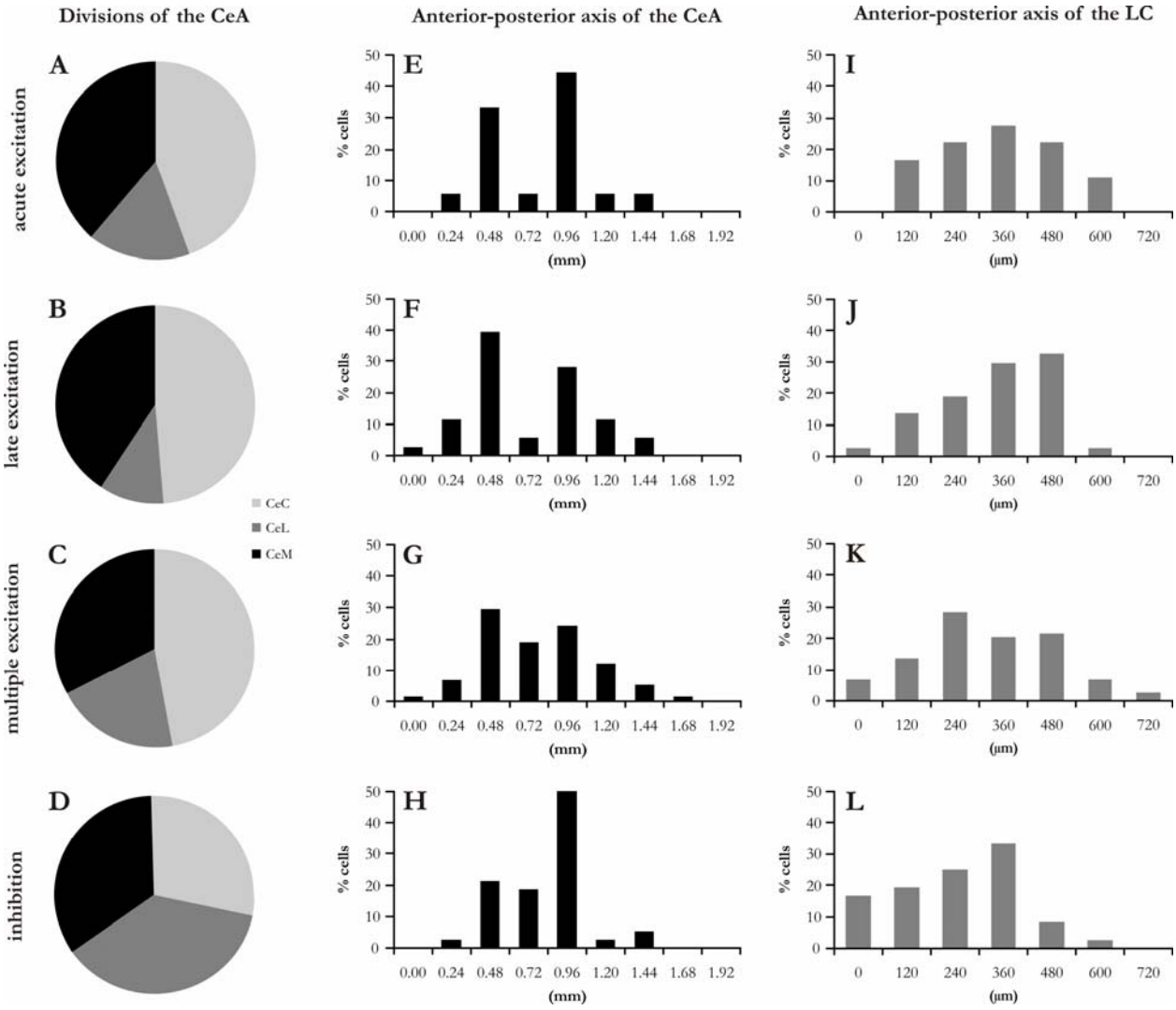

Figure 23. Responses to 20-Hz stimulation of the CeA with respect to the location of stimulating and recording electrodes.

All responses to single-pulse stimulation were not correlated to the location of the stimulating electrode in the different subdivisions (**A-D**; Chi-square, NS) or anterior-posterior axis of the CeA (**E-H**; Chi-square, NS), and location of the recording electrode in the anterior-posterior axis of the LC (**I-L**; Chi-square, NS).

3.2 EFFECTS OF ICV ANTAGONIST INFUSIONS ON SPONTANEOUS AND EVOKED LC ACTIVITY

3.2.1 Spontaneous LC activity.

To determine the effect of ICV infusions of CRF and glutamate antagonist on spontaneous LC activity, LC activity was recorded prior, during, and following ICV infusions of drugs. LC activity prior to ICV drug infusions were not significantly different between aCSF (n = 7), 3 μg D-Phe-CRH (n = 4), 12 μg D-Phe-CRH (n = 7), or 50 mM KYN (**figure 24A**; 1-way ANOVA, NS). Overall, there were no significant effects of ICV drug infusions on spontaneous LC activity over time (**figure 24B**; 2-way RM ANOVA, NS).

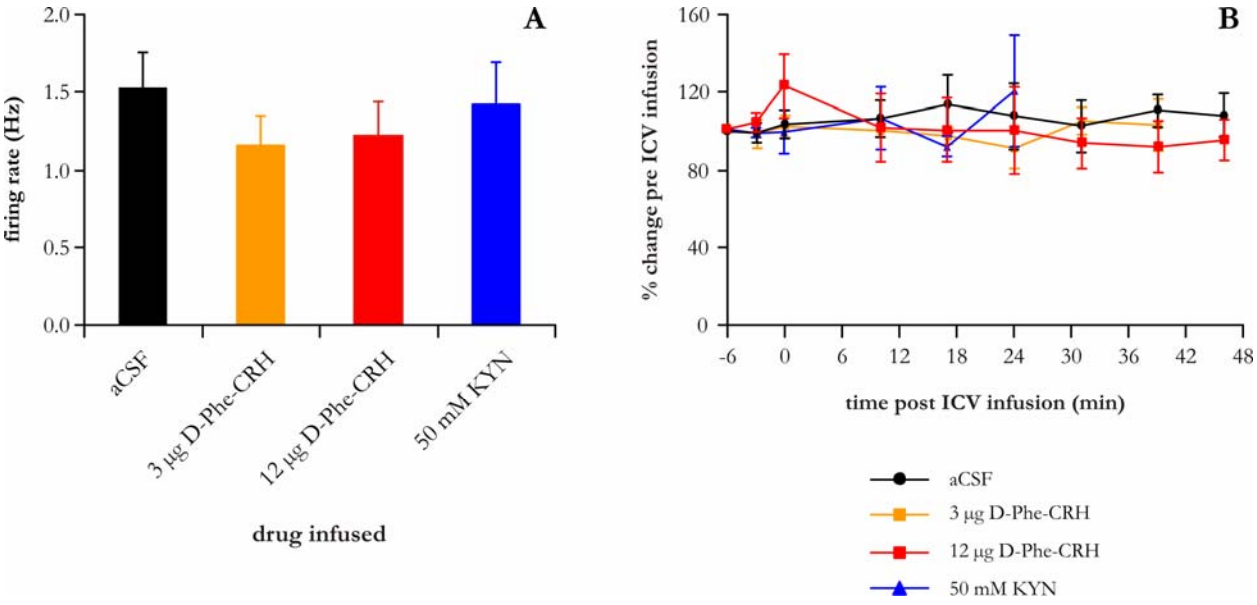

Figure 24. Spontaneous LC activity prior to and following ICV drug infusions.

3.2.2 Evoked LC activity.

Since the majority of LC responses to 20-Hz train stimulation of the CeA were excitatory responses (n = 129/184), and within those, multiple excitatory responses (n = 74/129), the effects of ICV infusions of excitatory transmitter antagonists were examined only on multiple excitatory LC responses following 20-Hz train stimulation of the CeA (**figure 25A-D**). LC activity was recorded during 20-Hz train stimulation of the CeA prior to and approximately every 7 minutes for approximately 50 minutes after an ICV of aCSF aCSF (n = 7), 3 μg D-Phe-CRH (n = 4), 12 μg D-Phe-CRH (n = 7), or 50 mM KYN (n = 6). To determine if multiple excitatory responses to 20-Hz train stimulation of the CeA were similar between drug groups prior to ICV drug infusions, the evoked spike probability, which is the spike probability elicited within 250 ms from the onset of the train stimulation, and spike probability during post-excitation inhibition was compared to baseline probability between drug groups (**figure 26**). There were no significant differences in either baseline spike probability (**figure 26A-B**), evoked spike probability (**figure 26A**), and spike probability during post-excitation inhibition (300-800 ms from onset of stimulation; **figure 26B**) between drug groups prior to ICV drug infusions (2-way RM ANOVA, NS). Spike probability within 250 ms of the onset of train stimulation was significantly greater than baseline spike probability (**figure 26A and table 4**; 2-way RM ANOVA, Holm-Sidak, $p<0.05$), whereas spike probability during post-excitation inhibition was significantly less than baseline spike probability for each drug (**figure 26B and table 4**; 2-way RM ANOVA, Holm-Sidak, $p<0.05$**)** for each drug prior to ICV drug infusions.

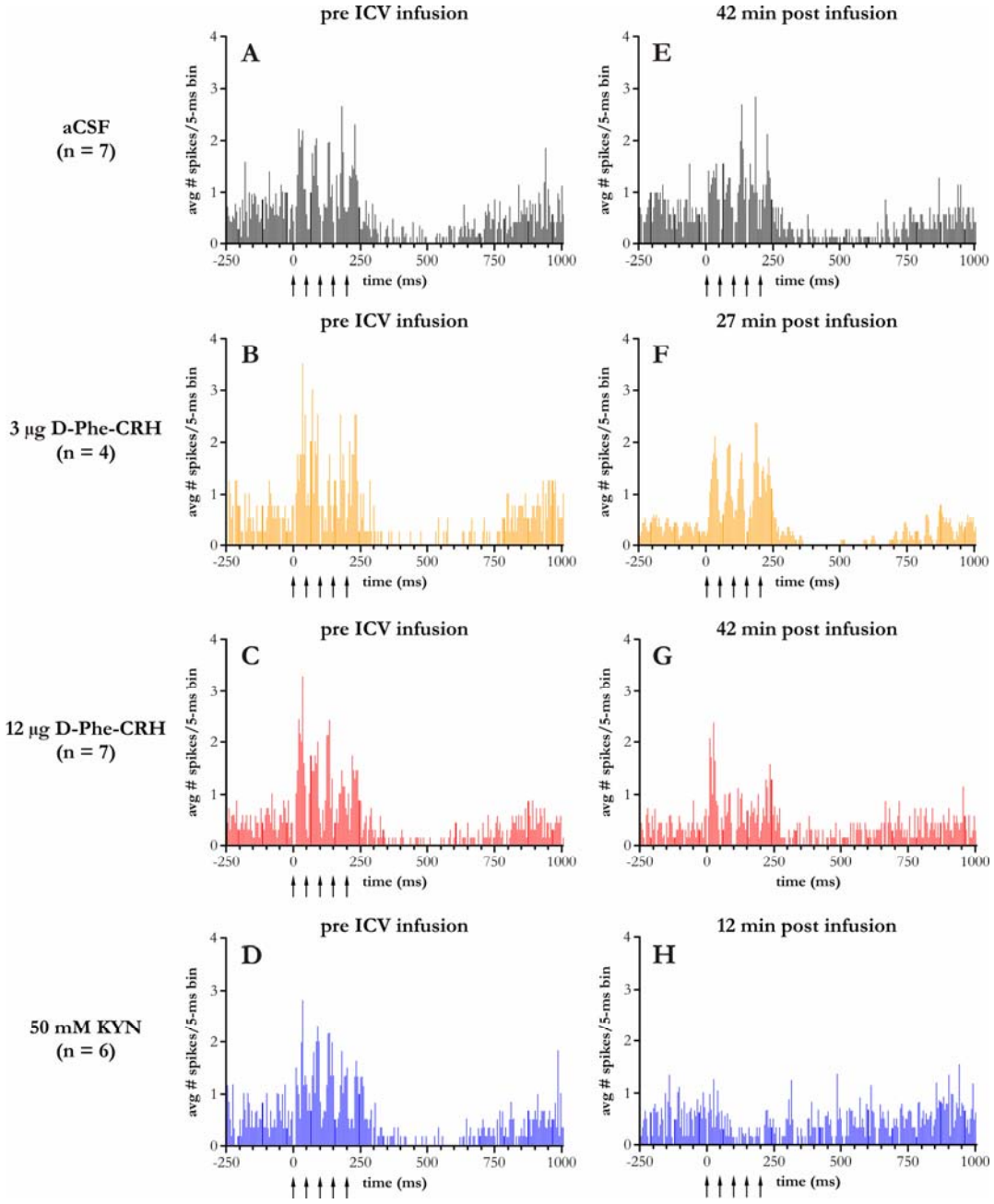

Figure 25. Effects of ICV drug infusions on multiple excitatory responses to 20-Hz train stimulation of the CeA.

A-D) Cumulative PSTHs depict multiple excitatory responses to 20-Hz train stimulation of the CeA prior to ICV drug infusions of **(A)** aCSF, **(B)** 3 µg D-Phe-CRH, **(C)** 12 µg D-Phe-CRH or **(D)** 50 mM KYN. **E-H)** Cumulative PSTHs depict spike activity as a result of 20-Hz train stimulation of the CeA at different time points following ICV drug infusions of **(E)** aCSF, **(F)** 3 µg D-Phe-CRH, **(G)** 12 µg D-Phe-CRH or **(H)** 50 mM KYN.

Table 4. Evoked activity prior to ICV infusions

			aCSF (n = 7)	3 µg D-Phe-CRH (n = 4)	12 µg D-Phe-CRH (n = 7)	50 mM KYN (n = 6)
Excitation	Spike probability (spikes/250 ms)	Baseline	34.1 ± 5.6	27.9 ± 2.8	23.1 ± 4.5	28.5 ± 6.4
		Evoked‡	55.6 ± 4.9*	63.3 ± 4.4*	57.4 ± 8.0*	56.4 ± 11.3*
Post-excitation inhibition	Onset (ms)†		271 ± 18	281 ± 14	286 ± 14	342 ± 15
	Duration (ms)		600 ± 80	519 ± 52	536 ± 56	354 ± 60
	Spike probability (spikes/500 ms)	Baseline	68.2 ± 11.1	55.8 ± 5.5	46.2 ± 9.0	57.1 ± 12.8
		Inhibition††	21.4 ± 7.7*	11.0 ± 7.5*	11.5 ± 5.4*	13.1 ±4.0*
		% BL	27 ± 7	20 ± 13	23 ± 7	24 ± 6

‡ spike probability during the train (S1-S5)
† with respect to onset of 20-Hz train stimulation of the CeA
†† spike probability within 300-800 ms from onset of 20-Hz train stimulation of the CeA
* $p<0.05$, compared to baseline spike probability

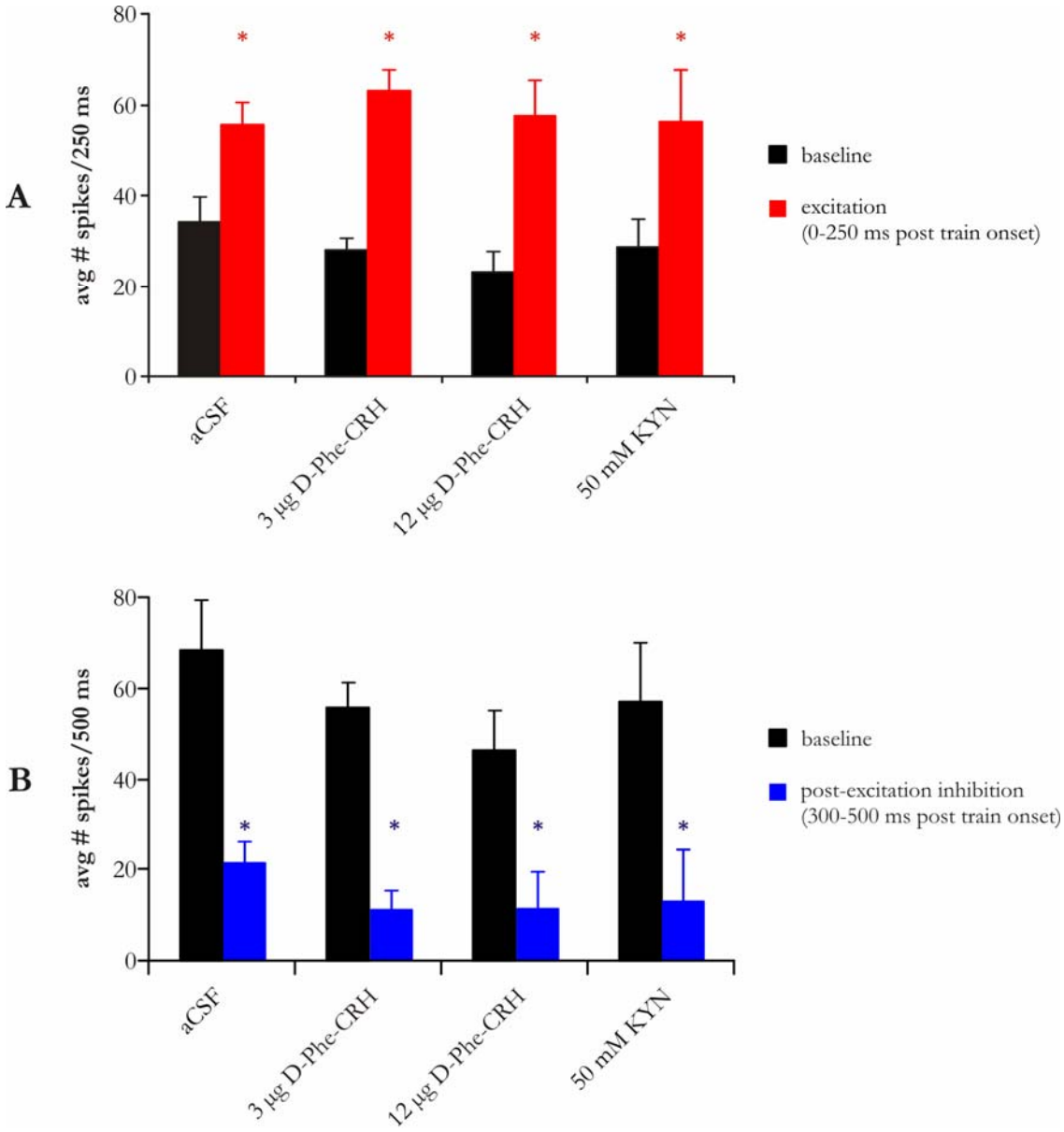

Figure 26. Spike activity from multiple excitatory responses as a result of 20-Hz train stimulation of the CeA prior to ICV drug infusions.

A-B) There were no significant differences in the spike probability between drug groups during **(A-B)** baseline, **(A)** 20-Hz train stimulation, or **(B)** post-excitation inhibition (300-500 ms following onset of train stimulation) (2-way RM ANOVA, NS). **A)** Evoked spike probability from all drug groups was significantly greater than baseline, whereas **(B)** spike probability during post-excitation inhibition was significantly less than baseline (2-way RM ANOVA, Holm-Sidak, $p<0.05$).

ICV infusions rather than intra-LC infusions of drugs were made since the LC is below the 4th ventricle, and drugs infused into the ventricle would diffuse through the LC and its dendrites in the rostrolateral peri-coerulear area, where neurons from the CeA synapse onto dendrites of LC neurons. To determine if multiple excitatory responses to train stimulation of the CeA were mediated by CRH, the effects of ICV infusions of the peptidergic CRH antagonist, D-Phe-CRH, were examined on multiple excitatory responses to train stimulation of the CeA. Two doses of D-Phe-CRH (3 and 12 μg) were infused in the ventricle in order to determine a dose-response relationship on the effect of D-Phe-CRH on multiple excitatory responses to train stimulation of the CeA. Since D-Phe-CRH has similar properties to CRH (Curtis et al., 1994), it was expected to exert its effects on spontaneous and evoked LC activity for an extended period of time following ICV infusions (Curtis et al., 1994; Curtis et al., 1997b; Jedema et al., 2001; Valentino and Curtis, 1991a; Valentino et al., 1983); thus LC activity following train stimulation of the CeA was recorded every 7 minutes for approximately 50 minutes following ICV infusions of aCSF and both doses of D-Phe-CRH. It was reported that excitatory responses to train stimulation were only partially attenuated following ICV (3-4 μg) and intra-coerulear (1 μg) infusions of the peptidergic antagonist alpha-helical CRH (Bouret et al., 2003). This suggested that the CeA activates the LC via other brain regions and/or mechanisms. To further evaluate whether multiple excitatory responses to 20-Hz train stimulation of the CeA were mediated by CRH, and determine if glutamate afferents play a role in mediating excitatory responses, the effects of the glutamatergic antagonist, KYN, on multiple excitatory responses to 20-Hz train stimulation were examined. Since an ICV infusion of KYN exerts its effects on evoked LC activity shortly after the infusion (Ennis and Aston-Jones, 1988; Ennis et al., 1992; Valentino et al., 1991), LC activity following train stimulation of the CeA was recorded every 7 minutes for approximately 25 minutes following ICV infusions.

ICV infusions of 12 μg D-Phe-CRH or 50 mM KYN significantly attenuated the excitatory response to train stimulation in comparison to aCSF (**figure 27**). The time course for the effects of 12 μg D-Phe-CRH and 50 mM KYN on excitatory responses to train stimulation of the CeA were distinct and consistent with previous findings (**figure 25E-H**). More specifically, 12 μg D-Phe-CRH exerted significant effects only 42 minutes following the infusion, whereas effects of 50 mM KYN occurred almost immediately (**figure 27A**; 2-way RM ANOVA, Holm-Sidak, $p<0.05$). Following high doses of D-Phe-CRH, spike probability during the train was attenuated over time with a significant decrease approximately 42 minutes following the infusion (**figure 27A**; 2-way RM ANOVA, Holm-Sidak, $p<0.05$). At 42 minutes, the spike probability during the train following ICV infusions of 12 μg D-Phe-CRH was attenuated to baseline spike probability (**figure 27C**; 2-way RM ANOVA, Holm-Sidak, NS), but significantly less than the spike probability evoked during the train following ICV infusions of aCSF (**figure 27C**; 2-way RM ANOVA, Holm-Sidak, $p<0.05$). At 12 minutes, the spike probability during the train following ICV infusions of 50 mM KYN was significantly attenuated in comparison to aCSF (**figure 27D**; 2-way RM ANOVA, Holm-Sidak, $p<0.05$), but was not significantly different from baseline (**figure 27D**; 2-way RM ANOVA, Holm-Sidak, $p<0.05$). Neither low doses of D-Phe-CRH (3 μg; **figure 27B**) nor aCSF had a significant effect on the excitatory responses to train stimulation of the CeA (**figure 27**; 2-way RM ANOVA, Holm-Sidak, NS).

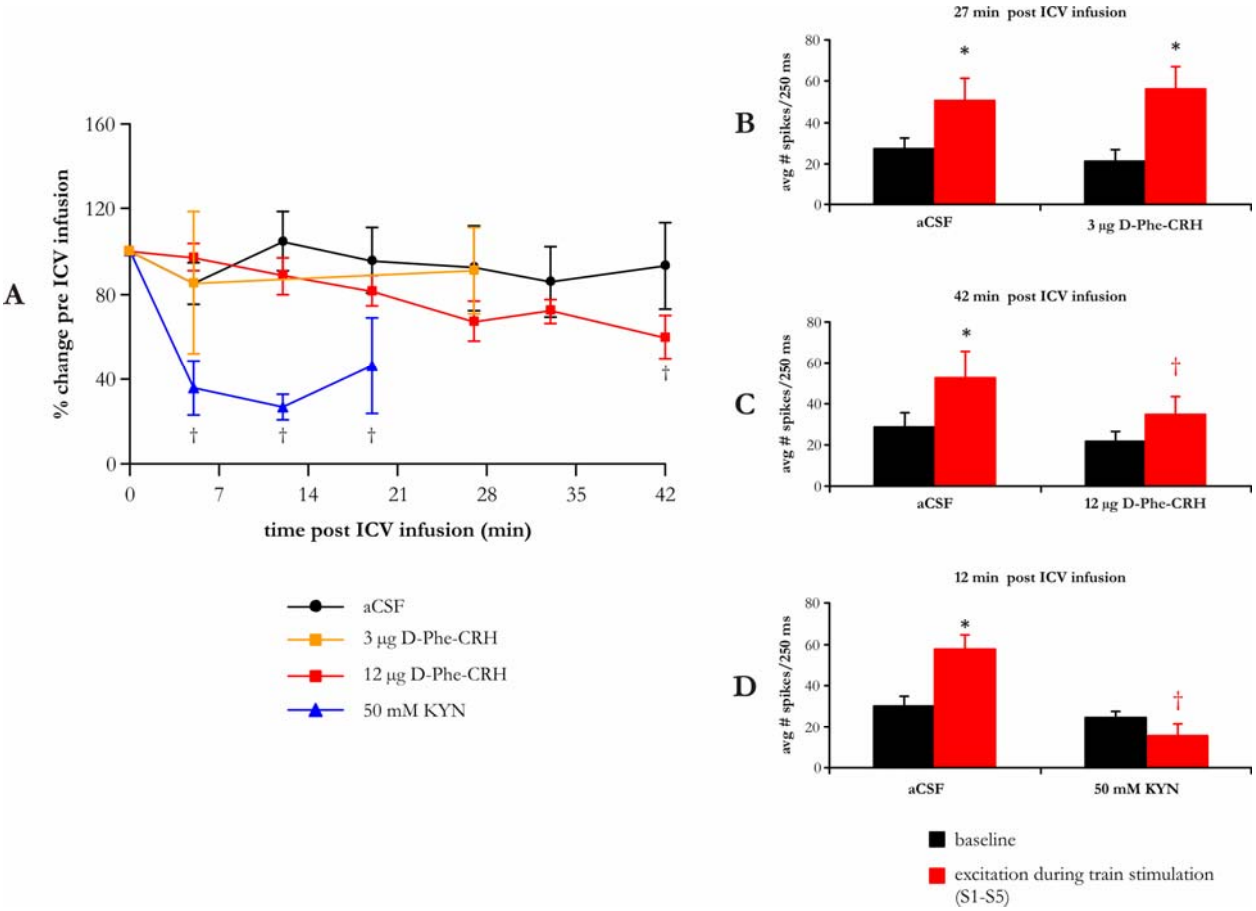

Figure 27. Effects of ICV infusions on evoked spike activity during 20-Hz train stimulation of the CeA.

A) ICV infusions of drugs significantly exerted its effects on evoked spike probability during train stimulation (S1-S5) at different time points following the infusion. Spike probability as a percentage of spike probability prior to ICV infusions during all stimuli of train stimulation was significantly attenuated 42 minutes following ICV infusions of 12 µg D-Phe-CRH or 12 minutes following ICV infusions of 50 mM KYN in comparison to aCSF (2-way RM ANOVA, Holm-Sidak, $p<0.05$). **B)** ICV infusions of 3 µg D-Phe-CRH did not attenuate evoked activiuty during all stmuli in the train in comparison to baseline or aCSF (2-way RM ANOVA, Holm-Sidak, NS). Evoked spike probability was significantly attenuated **(C)** 42 minutes following ICV infusions of 12 µg D-Phe-CRH or **(D)** 50 mM KYN in comparison to aCSF (2-way RM ANOVA, Holm-Sidak, $p<0.05$). * = $p<0.05$, compared to baseline; † = $p<0.05$, compared to aCSF

Excitatory responses to train stimulation of the CeA comprised two components: early and late. The early excitatory component was an excitatory response following the first stimulus of the train, whereas the late excitatory component occurred following the later stimuli of the train (2^{nd}-5^{th} stimuli). ICV drug infusions had differential effects on both the late and early excitatory components following train stimulation of the CeA. The early excitatory component was not significantly attenuated by any manipulation (**figure 28**; 2-way RM ANOVA, Holm-Sidak, NS). Although the spike probability during the early excitatory component was decreased but not significantly following 50 mM KYN in comparison to aCSF (**figure 28D**; 2-way RM ANOVA, Holm-Sidak, NS), it was similar to baseline spike probability 12 minutes following the infusion (**figure 28D**; 2-way RM ANOVA, Holm-Sidak, NS). Overall, in comparison to the early excitatory component, the late excitatory component was significantly attenuated by ICV infusions of drug (**figure 29A**). Neither a low dose of D-Phe-CRH nor aCSF significantly attenuated the late excitatory component (**figure 29A-B**; 2-way ANOVA, Holm-Sidak, NS). In contrast to the early excitatory component, the late excitatory component was significantly attenuated by 12 μg D-Phe-CRH and 50 mM KYN in comparison to aCSF (**figure 29A**; 2-way RM ANOVA, $p<0.05$). More specifically, 12 μg D-Phe-CRH exerted a significant effect 42 minutes following infusion, whereas the effects of 50 mM KYN occurred almost immediately (**figure 29A**; 2-way RM ANOVA, Holm-Sidak, $p<0.05$). The late excitatory component was not completely blocked by 12 μg D-Phe-CRH at 42 minutes following ICV infusions. High doses of D-Phe-CRH resulted in a maximum 44% reduction in spike probability during the late excitatory component (**figure 29A**). At 42 minutes following ICV infusions of D-Phe-CRH, the spike probability during the late excitatory component was not significantly different from baseline spike probability (**figure 29C**; 2-way RM ANOVA, Holm-Sidak, NS), but in comparison to aCSF, it was significantly attenuated (**figure 29C**; 2-way RM ANOVA, Holm-Sidak, $p<0.05$). In contrast, 50 mM KYN had a much greater effect on the late excitatory

component and completely abolished excitatory responses to train stimulation (**figure 29A**). In comparison to a high dose of D-Phe-CRH, 50 mM KYN resulted in a maximum 80% reduction in spike probability during the late excitatory component (**figure 29A**) that was significantly less than both baseline spike probability and aCSF (**figure 29D**; 2-way RM ANOVA, Holm-Sidak, $p<0.05$).

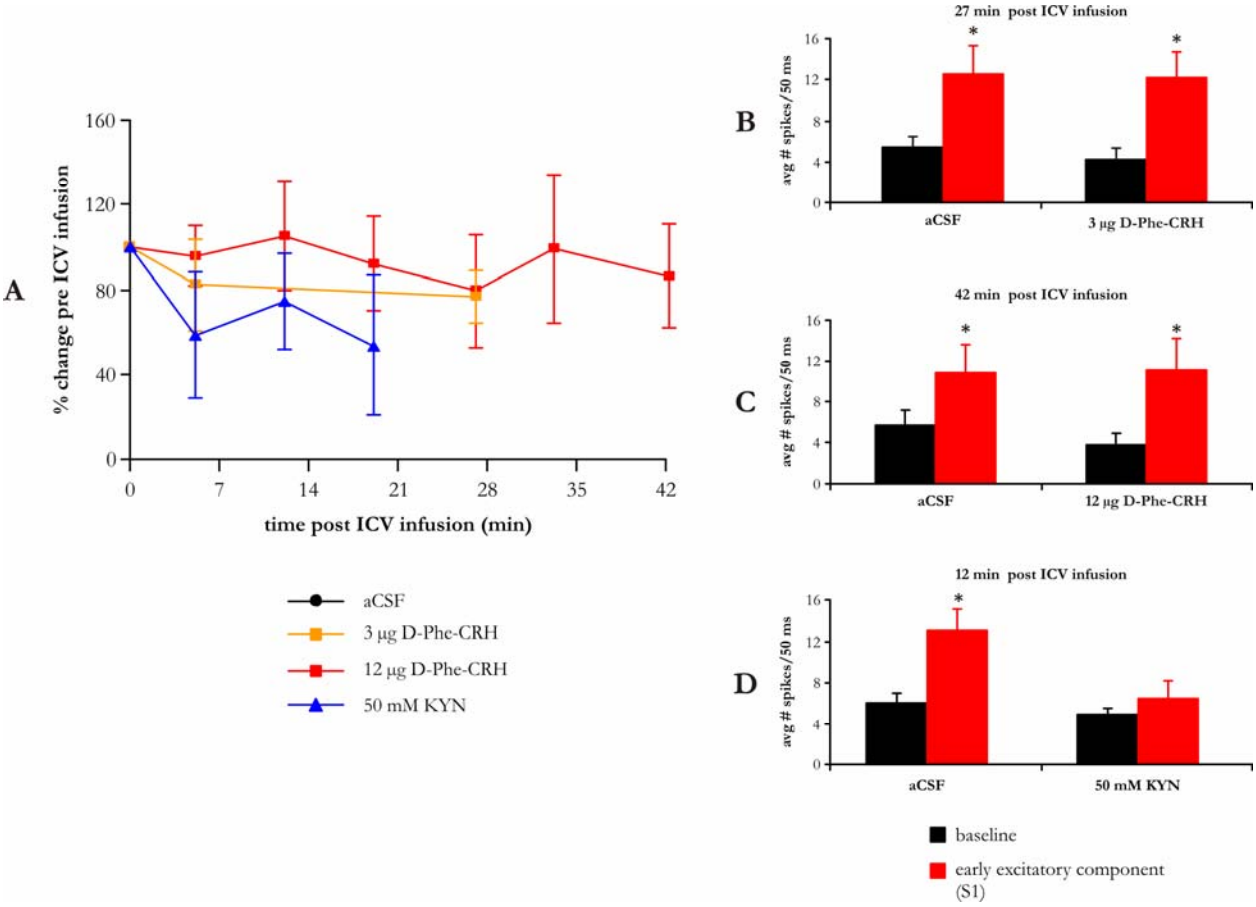

Figure 28. Early excitatory component during 20-Hz train stimulation following ICV infusions of drugs.

A-D) The early excitatory component was not significantly attenuated by any manipulation (2-way RM ANOVA, Holm-Sidak, NS). **B-C)** Neither dose of D-Phe-CRH nor **(D)** 50 mM KYN attenuated the spike probability following S1 of the train in comparison to aCSF (2-way RM ANOVA, NS). **B-C)** Evoked spike probability remained significantly greater than baseline for both doses of D-Phe-CRH (2-way RM ANOVA, Holm-Sidak, $p<0.05$), whereas **(D)** evoked spike probability following 50 mM KYN was reduced in comparison to baseline, but not significantly attenuated in comparison to aCSF (2-way RM ANOVA, Holm-Sidak, NS). * = $p<0.05$, compared to baseline

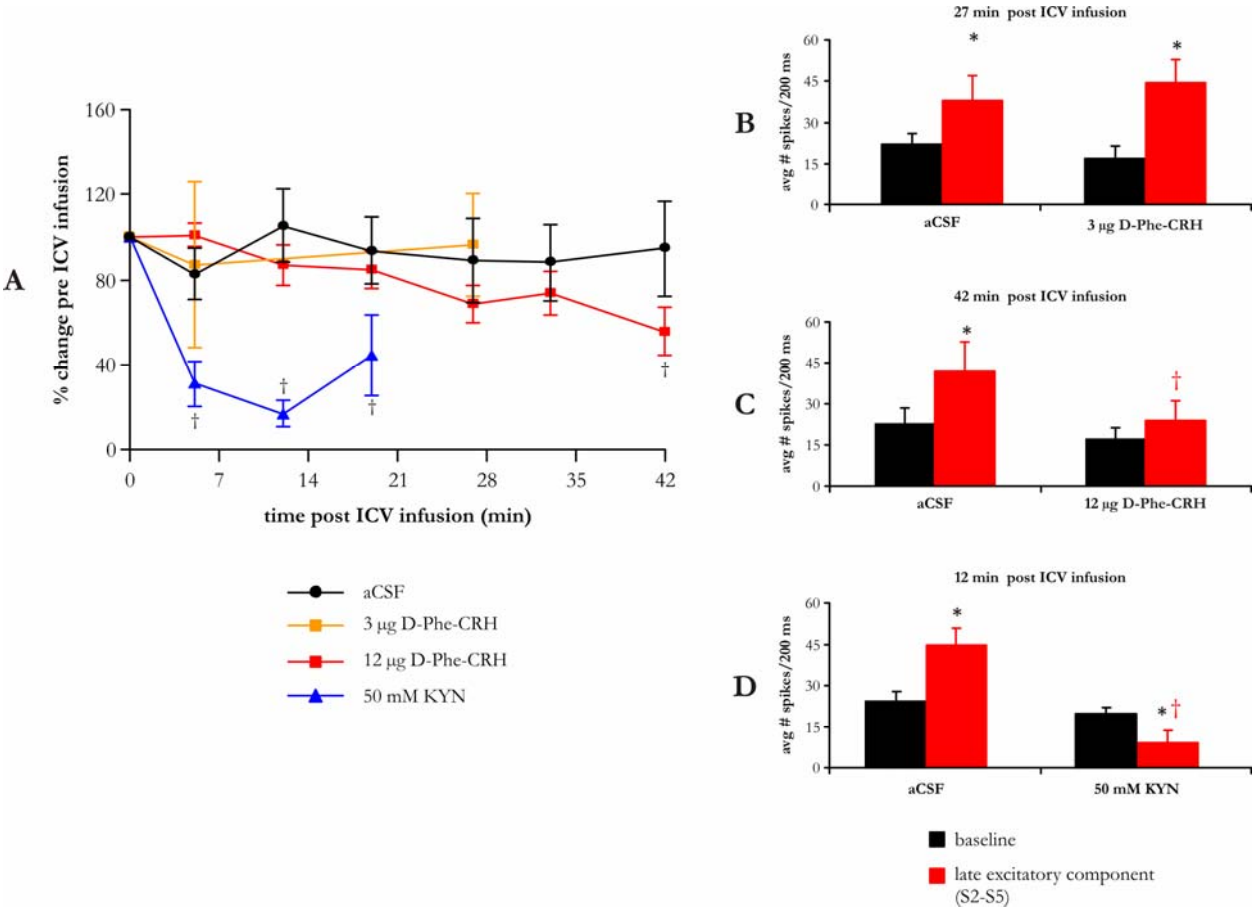

Figure 29. Late excitatory component during 20-Hz train stimulation following ICV infusions of drugs.

A) Spike probability as a percentage of spike probability prior to ICV infusions during S2-S5 of train stimulation was significantly attenuated 42 minutes following ICV infusions of 12 µg D-Phe-CRH or 12 minutes following ICV infusions of 50 mM KYN in comparison to aCSF (2-way RM ANOVA, Holm-Sidak, $p<0.05$). **B)** Evoked spike probability was not significantly attenuated 27 minutes following ICV infusions of 3 µg D-Phe-CRH in comparison to aCSF (2-way RM ANOVA, Holm-Sidak, NS), thus remained significantly greater than baseline (2-way RM ANOVA, Holm-Sidak, $p<0.05$). ICV infusions of **(C)** 12 µg D-Phe-CRh or **(D)** significantly attenuated evoked spike probability during S2-S5 in comparison to aCSF (2-way RM ANOVA, HolmSidak, $p<0.05$). Evoked spike probability following 50 mM KYN was significantly less than baseline (2-way RM ANOVA, Holm-Sidak, $p<0.05$). * = $p<0.05$, compared to baseline; † = $p<0.05$, compared to aCSF

In contrast to 50 mM KYN, neither aCSF nor either doses of D-Phe-CRH had any significant effect on onset, duration, or magnitude of spike probability of the post-excitation inhibition that occurred between 300-800 ms following the onset of train stimulation (**figure 30 and table 4**; 2-way RM ANOVA, NS). The spike probability during post-excitation inhibition following infusions of aCSF (**figure 30B-D**) and both doses of D-Phe-CRH (**figure B-C**) was significantly less than baseline spike probability (**table 4**; 2-way RM AVOVA, Holm-Sidak, $p<0.05$). In contrast, spike probability during post-excitation inhibition following infusions of 50 mM KYN was not significantly different than baseline (**figure 30D and table 4**; 2-way RM AVOVA, Holm-Sidak, NS). ICV infusions of KYN not only resulted in a complete blockage of excitatory responses to 20-Hz train stimulation of the CeA but also revealed an underlying short-onset (129 ± 44 ms) short-duration (221 ± 52 ms) inhibitory period following train stimulation (**figure 31**). ICV infusions of 50 mM KYN significantly reduced spike probability expressed as a percentage of baseline spike probability between 125-375 ms following the onset of the train in comparison to aCSF (**figure 31A**; 2-way RM ANOVA, Holm-Sidak, $p<0.05$). At the maximal effect of 50 mM KYN, the inhibitory period between 125-375 ms was 49% of baseline spike probability (**figure 31A**). Spike probability during this underlying inhibitory period following infusions of 50 mM KYN was significantly less than baseline and less than that following aCSF infusions (**figure 31B**).

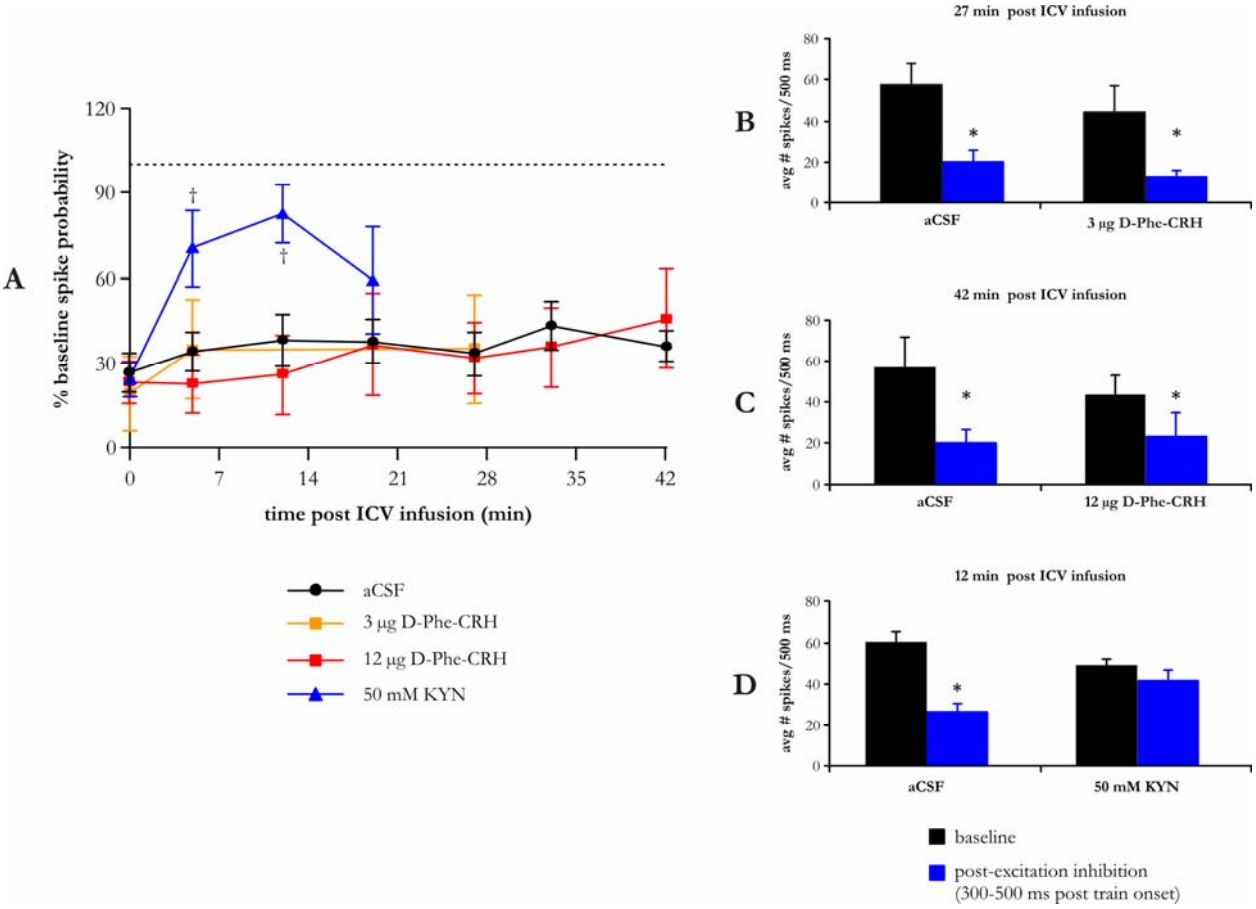

Figure 30. Effects of ICV infusions on post-excitation inhibition following excitatory responses to 20-Hz train stimulation.

A) Spike probability as a percentage of baseline spike probability during post-excitation inhibition (300-500 ms post train onset) was significantly increased following ICV infusions of 50 mM KYN (2-way ANOVA, Holm-Sidak, $p<0.05$) but not following ICV infusions of either dose of D-Phe-CRH (2-way ANOVA, Holm-Sidak, NS) in comparison to aCSF. Spike probability during post-excitation inhibition remained significantly less than baseline following ICV infusions of both doses of **(B-C)** D-Phe-CRH and **(B-D)** aCSF (2-way ANOVA, Holm-Sidak, $p<0.05$). **D)** Spike probability during post-excitation inhibition was not significantly less than baseline following ICV infusions of 50 mM KYN (2-way ANOVA, Holm-Sidak, NS). * = $p<0.05$, compared to baseline; † = $p<0.05$, compared to aCSF

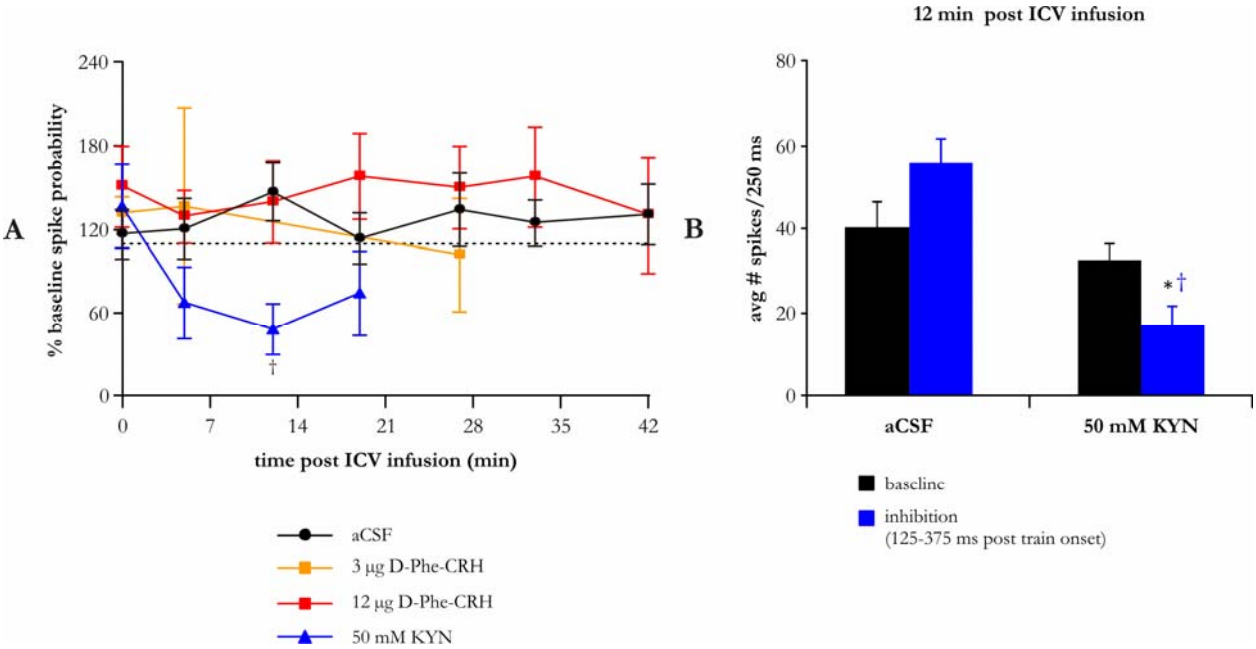

Figure 31. 50 mM KYN reveals underlying inhibitory period during 20-Hz train stimulation of the CeA.

A) Spike probability expressed as a percentage of baseline spike probability between 125-375 ms following the onset of the train was significantly reduced 12 minutes following ICV infusions of 50 mM KYN in comparison to aCSF (2-way RM ANOVA, Holm-Sidak, $p<0.05$). **B)** Spike probability during the underlying inhibitory period following ICV infusions of 50 mM KYN was significantly less than baseline (2-way RM ANOVA, Holm-Sidak, $p<0.05$) and aCSF (2-way RM ANOVA, Holm-Sidak, $p<0.05$). * = $p<0.05$, compared to baseline; † = $p<0.05$, compared to aCSF

3.3 EFFECTS OF PHARMACOLOGICAL ACTIVATION AND INACTIVATION OF THE CEA ON LC ACTIVITY

3.3.1 Effects of pharmacological activation of the CeA on LC activity.

Given the effects of 50 mM KYN on the multiple excitatory responses to electrical train stimulation, the effects of local pharmacological stimulation of the CeA via infusion of the glutamatergic agonist, AMPA, were examined to ensure that excitatory responses to train stimulation of the CeA were a result of activating neurons within the CeA and not fibers of passage. Consistent with the observed excitatory responses to electrical stimulation of the CeA, 120 pmol AMPA infusions within the CeA (n = 7) significantly increased LC activity in comparison to dPBS infusions within the CeA (n = 8) (**figures 32A and 33A**; 1-way ANOVA, $p<0.05$). Although not significant, there was a dose-response relationship for the maximal increase in FR following intra-CeA infusions of increasing doses of AMPA (60 pmol and 120 mpol) (**figure 32A**; 1-way ANOVA, NS). The maximal increase in FR following 60 and 120 pmol AMPA intra-CeA infusions occurred at 132 ± 38 s and 137 ± 35 s, respectively, from the onset of the infusion. Intra-CeA infusions of 60 and 120 pmol AMPA resulted in LC activity that was at least 10% greater than baseline activity for 149 ± 33 s and 147 ± 61 s, respectively.

3.3.2 Effects of pharmacological inactivation of the CeA on LC activity.

To determine if spontaneous activity within the CeA provides a tonic drive to the LC, the effects of chemical inactivation of the CeA on LC activity were examined by recording LC activity following intra-CeA infusions of a combination of the $GABA_A$ and $GABA_B$ agonists, muscimol and baclofen,

respectively. Inactivation of the CeA (n = 8) had no effect on the maximum increase in LC activity in comparison to intra-CeA infusions of dPBS (n = 8) (**figures 32A and 33B**; 1-way ANOVA, NS). To determine if inactivation of the CeA had any long-term effects on LC activity, LC activity was recorded for approximately 30 minutes following an intra-CeA infusion of 0.2 μg mus/0.2 μg. There were no significant effects of CeA inactivation (n = 7) on LC activity over time in comparison to infusions of dPBS win the CeA (n = 5) (**figure 32B**; 2-way RM ANOVA, NS).

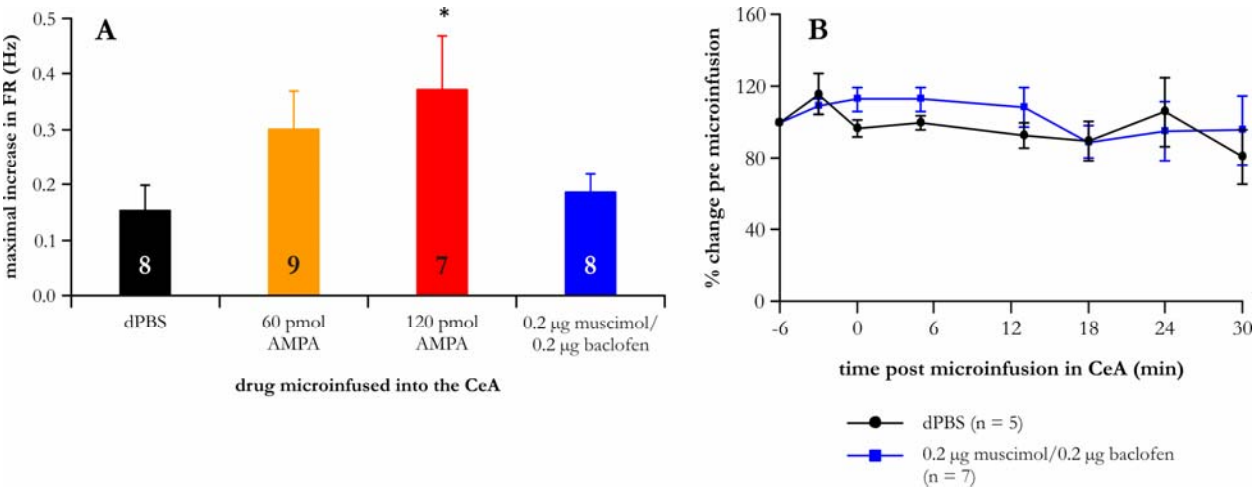

Figure 32. Effects of pharmacological activation and inactivation of the CeA on LC activity. **A)** Pharmacological activation of the CeA with higher doses of AMPA significantly increased LC firing rate (1-way ANOVA, $p<0.05$), whereas lower doses of AMPA or inactivation of the CeA with 0.2 μg muscimol/0.2 μg baclofen did not significantly alter LC activity in comparison to intra-CeA infusions of dPBS (1-way ANOVA, NS). Number in each drug group is indicated in the bar. **B)** Pharmacological inactivation of the CeA had no significant long term effects on LC activity in comparison to dPBS (2-way RM ANOVA, NS).

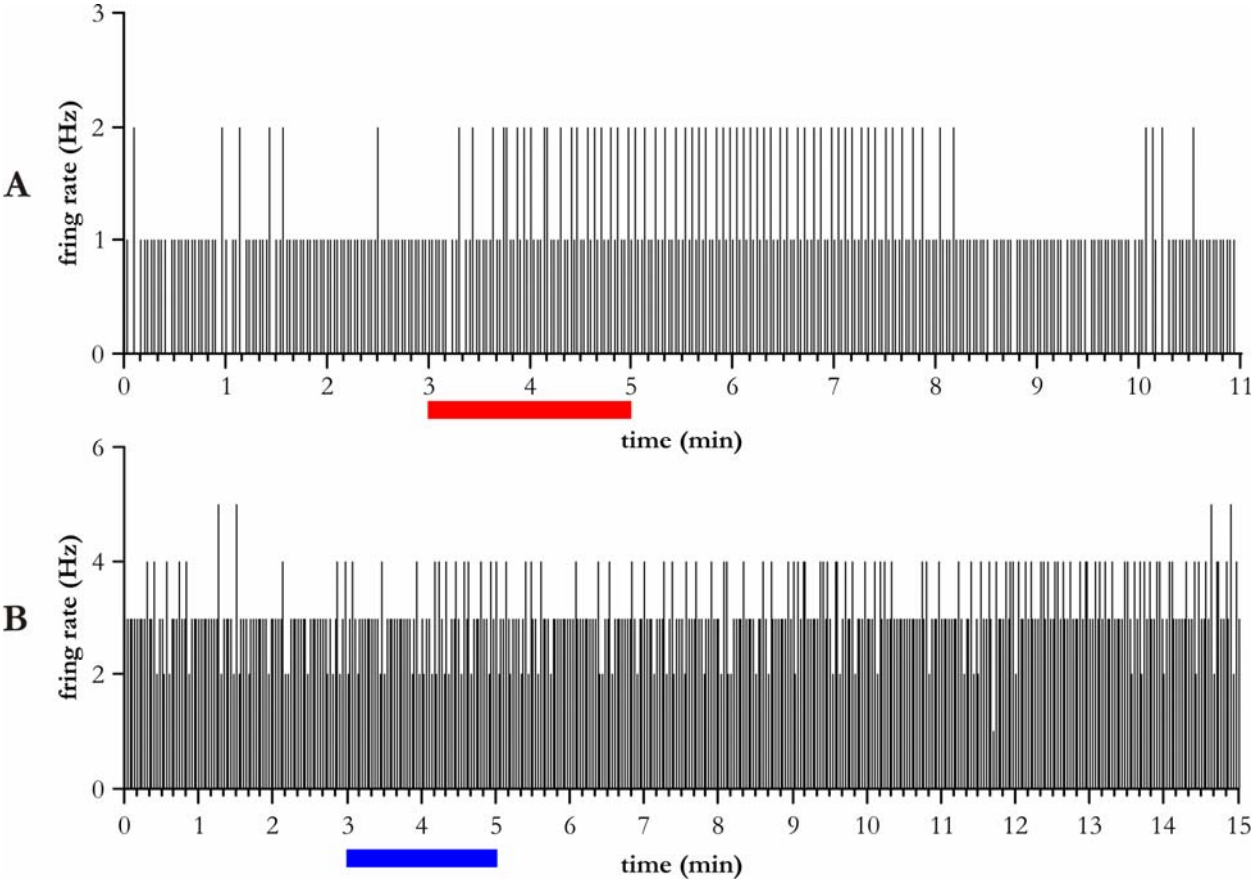

Figure 33. Representative firing rate histograms of LC activity following pharmacological manipulation of the CeA.

A) Pharmacological activation of the CeA increased LC firing rate for approximately 4 minutes. The CeA was pharmacologically activated with 120 pmol AMPA infused over 2 minutes (red bar). There was a 50% peak increase in firing rate, which occurred approximately 1 minute from the start of the infusion. **B)** Pharmacological inactivation of the CeA with 0.2 μg muscimol/0.2 μg baclofen infused over 2 minutes (blue bar) has no significant effects on LC activity.

4.0 DISCUSSION

Stress exposure has maladaptive consequences for an organism, which can precipitate and exacerbate the symptoms of mood and anxiety disorders. Clinical studies have demonstrated that there is a pathophysiology in the amygdala and the locus coeruleus in individuals with mood and anxiety disorders. More specifically, clinical studies suggest that both of these structures are hyperactive in individuals with mood and anxiety disorders, which may contribute to the symptoms observed in these disorders. Furthermore, studies have shown that stressful stimuli or stress exposure activates the complex of nuclei that comprise the amygdala in addition to the locus coeruleus. It is hypothesized that CRH mediates the stress-induced activation of the LC. Although the amygdala is comprised of several nuclei, the central nucleus of the amygdala is most relevant to this study, since neurons of the CeA directly contact the dendrites of the noradrenergic neurons of the LC and provide a source of CRH to the LC. Therefore, it is hypothesized that activation of the CeA-LC pathway may modulate stress responses by contributing to the stress-induced CRH-mediated activation of the LC.

Since both CRH and GABA are colocalized in neurons of the CeA (Veinante et al., 1997), both of which have opposing effects on LC activity, this study examined and further characterized the CeA-LC pathway using electrophysiological and pharmacological techniques. The current results confirm previous findings (Bouret et al., 2003) that activation of the CeA results in excitatory responses in the majority of LC cells, with inhibitory and antidromic activation being the other responses evoked by electrical stimulation of the CeA. Furthermore, excitatory responses as a result

of electrical stimulation of the CeA may be partially attenuated by the peptidergic antagonist, D-Phe-CRH. This current study also extends previously reported findings about the CeA-LC pathway by suggesting that there is a glutamatergic component in the excitatory responses following electrical stimulation of the CeA and that inhibition simultaneously occurs during excitatory responses to electrical stimulation of the CeA.

4.1 LC RESPONSES TO ELECTRICAL STIMULATION OF THE CEA

Electrical stimulation of the CeA evoked four response types in the LC: excitation followed by inhibition, pure inhibition, antidromic activation or no response. Consistent with previous findings (Bouret et al., 2003), the majority of the responses to both single-pulse and train stimulation of the CeA were excitatory, with 70% of neurons responding with an excitation following 20-Hz train stimulation in comparison to single-pulse stimulation. Excitatory responses to both single-pulse and train stimulation were followed by a long lasting inhibitory period. Though fewer in numbers in comparison to excitatory responses, pure inhibitory responses to both single-pulse and even fewer to train stimulation of the CeA were also observed, in contrast to previous findings that report a single pure inhibitory response to electrical stimulation of the CeA (Bouret et al., 2003). This inconsistency may be due the criteria used to determine if a neuron responded with an excitation. Furthermore, consistent with previous findings (Bouret et al., 2003), antidromic activation was observed in a small percentage of neurons. Finally, a small population of neurons did not respond to single-pulse CeA stimulation, and this proportion was greatly reduced following train stimulation.

Anatomical and immunohistochemical studies have shown that neurons of the CeA contain CRH (Sakanaka et al., 1986, 1987; Van Bockstaele et al., 1998) and form asymmetric excitatory

synapses onto TH-positive dendrites of LC neurons in the rostrolateral peri-coerulear area (Van Bockstaele et al., 1996a; Van Bockstaele et al., 1998). Intracereboventricular or local application of CRH increases LC neuronal activity, which is correlated with increased levels of extracellular NE in the hippocampus and prefrontal cortex measured by microdialysis (Curtis et al., 1997b; Finlay et al., 1997; Jedema et al., 2001; Page and Abercrombie, 1999; Smagin et al., 1995; Valentino and Foote, 1988; Valentino et al., 1983). Moreover, studies show that CRH directly activates LC neurons in the presence of TTX *in vitro* (Jedema and Grace, 2004), demonstrating that the effect is mediated directly on LC neurons. CRH-induced activation of LC neurons and increases in extracelluar NE can be attenuated by administration of CRH antagonists D-Phe-CRH and alpha-helical CRH (Curtis et al., 1994; Curtis et al., 1997b; Page and Abercrombie, 1999). Previous electrophysiological findings showed that alpha-helical CRH attenuated evoked LC activity following electrical stimulation of the CeA (Bouret et al., 2003). Taken together, CRH in the CeA-LC pathway appears to mediate at least some of the excitatory responses following electrical stimulation of the CeA.

Excitatory responses to both single-pulse and train stimulation were typically followed by an inhibitory period called post-excitation inhibition. Post-excitation inhibition was first observed following activation of LC neurons by noxious stimuli in anesthetized subjects (Cedarbaum and Aghajanian, 1976) and is consistent with subsequent studies that observed an inhibitory period that followed glutamate-mediated excitation as a result of stimulation of the nucleus paragigantocellularis (Ennis and Aston-Jones, 1988) and indirect stimulation of the sciatic nerve (Ennis et al., 1992; Mana and Grace, 1997). This post-excitation inhibitory period following stimulation of the sciatic nerve, nucleus paragigantocellularis and presentation of noxious stimuli has been attributed to intrinsic LC mechanisms, such as activation of calcium-activated potassium channels and lateral inhibition mediated by alpha2 noradrenergic autoreceptors (Andrade and Aghajanian, 1984a; Andrade and Aghajanian, 1984b; Cedarbaum and Aghajanian, 1978a; Ennis and Aston-Jones, 1986). It is thought

that post-excitation inhibition in LC neurons occurs to modulate spike activity following activation (Cedarbaum and Aghajanian, 1976, 1978a; Ennis and Aston-Jones, 1986).

In addition to excitatory responses, there was a small percentage of LC cells responding with a pure inhibitory response to both single-pulse and train stimulation of the CeA. Although the precise source of inhibition is not known, it may be mediated by a combination of GABA and inhibitory neuropeptides released directly from neurons in the CeA that synapse onto the dendrites of LC neurons (Cassell et al., 1986) and possibly via indirect GABAergic input into the LC (Van Bockstaele et al., 1996a; Van Bockstaele et al., 1998). Immunohistochemical studies show high levels of immunoreactivity for GABA and glutamic acid decarboxylase (GAD), the enzyme which catalyzes the syntheses of GABA from glutamic acid, within the CeA (Ben-Ari et al., 1976; Sun and Cassell, 1993; Sun et al., 1994); Sun & Cassell, 1993). In addition, projection neurons of the CeA that target the brainstem contain GABA (Sun et al., 1994). Electrophysiological studies have shown that GABA application onto LC neurons *in vitro* decreases electrophysiological LC activity (Shefner and Osmanovic, 1991) and inhibition as a result of electrical stimulation of the GABAergic afferent, prepositus hyperglossi, can be blocked by local and iontophoretic application of the GABAA antagonist, bicuculline (Ennis and Aston-Jones, 1989a). In addition to containing the fast inhibitory neurotransmitter, GABA, the CeA also contains neuropeptides other than CRH that have inhibitory effects on LC activity. Neurons of the CeA contain neuropeptides, such as neurotensin, enkephalin, somatostatin, substance P, cholecystokinin, vasoactive peptide (Cassell et al., 1986) and galanin (Palkovits, 2000). Electrophysiological studies have shown that enkephalin (Travagli et al., 1995), neurotensin, somatostatin, and galanin depress LC activity (Olpe and Steinmann, 1991). In addition to directly inhibiting LC activity, ultrastructural evidence suggests that the CeA may also inhibit the LC via indirect GABAergic inputs. CRH-positive neurons from the CeA form asymmetric synapses onto TH-negative neurons within the rostrolateral peri-coerulear area (Van Bockstaele et al., 1998).

These TH-negative neurons within the rostrolateral peri-coerulear area may be local GABAergic interneurons that modulate LC activity through inhibition following activation of its afferents (Aston-Jones et al., 2004). Taken together, the pure inhibitory responses and inhibitory period following excitatory responses to electrical CeA stimulation may be a result of CeA directly inhibiting LC neurons via GABA alone or in conjunction with inhibitory neuropeptides, or indirectly via activation of local interneurons within the rostrolateral peri-coerulear area.

Combined with intrinsic mechanisms within the LC that inhibit LC activity immediately following activation, the inhibitory period that follows activation as a result of electrical stimulation of the CeA may be directly mediated by CeA. The occurrence of pure inhibitory responses following electrical stimulation of the CeA, which have a similar onset latency and duration as the inhibitory period that follows excitatory responses, indicate that the CeA may be simultaneously mediating the inhibitory period that immediately follows activation. Combined with present findings, immunohistochemical and tract-tracing findings suggest that the CeA provides an inhibitory input to the LC via GABA released directly from CeA neurons forming synapses onto LC neurons or indirectly by activating GABAergic interneurons within the peri-coerulear area. Present data do not indicate the precise source of inhibition, thus it is unclear whether mechanisms within the LC alone or combined with direct CeA input mediate the inhibitory period following excitatory responses to electrical stimulation of the CeA.

A small population of LC neurons was antidromically activated by CeA stimulation. These neurons responded to single-pulse stimulation and followed high-frequency train stimulation of the CeA with fixed latencies. This was consistent with previous electrophysiological findings showing a small percentage of LC neurons that were antidromically activated following electrical stimulation of the CeA (Bouret et al., 2003). The majority of the latencies of antidromic responses were between 10-25 ms from the onset of the stimulus, which was consistent with previous findings (Bouret et al.,

2003). Together, these electrophysiological findings are consistent with anatomical tract-tracing studies confirming that the CeA receives a small LC input (Cedarbaum and Aghajanian, 1978b; Jones and Moore, 1977; Petrov et al., 1993; Usunoff et al., 2006).

4.1.1 Single-pulse vs. train stimulation of the CeA.

Present findings show differences in LC responses to electrical stimulation of the CeA with respect to stimulation parameters, such as increasing current intensity and use of high-frequency repeated stimulation. At the highest intensity, the majority of LC neurons responded with an excitatory response to both single-pulse and train stimulation of the CeA, which was consistent with previous findings (Bouret et al., 2003). For excitatory responses, the average latency to the peak of the excitation occurs between 20-25 ms from the onset of single-pulse or individual stimuli of train stimulation. Although Bouret et al reported an average onset latency of 11.7 $\pm$ 0.7 ms, but did not report the average peak latency, it can be estimated from PSTHs of individual neurons that the average peak latency occurred approximately 20-25 ms from the onset of the stimulus, which is consistent with the present findings.

The present findings indicate a relationship between current intensity and the magnitude of evoked or inhibited activity with respect to baseline for both single-pulse and 10-Hz train stimulation of the CeA. For excitatory responses, higher current intensities (600-900 μA) in comparison to lower current intensities (0-300 μA) resulted in greater evoked activity during the excitatory period following the stimuli with respect to baseline activity, with the greatest effect occurring at the highest intensity. For pure inhibitory responses and during the inhibitory period following excitatory responses, there was an inverse relationship between current intensity and spike probability during the inhibitory period as a result of single-pulse and 10-Hz train stimulation of the

CeA. This suggests that increasing current intensities during electrical stimulation of the CeA activated more fibers that resulted in either increased evoked activity or increased inhibition during both pure inhibitory responses and the inhibitory period following excitatory responses.

In addition to the effects of higher current intensities on evoked and inhibited activity as a result of electrical stimulation, the high-frequency repeated stimulation resulted in enhanced activation of LC neurons. Overall, train stimulation of the CeA (10- and 20-Hz) resulted in a greater proportion of neurons responding with an excitation in comparison to single-pulse CeA stimulation. There was also a significant reduction in the number of neurons responding with an inhibitory response or not responding to 20-Hz train stimulation in comparison to single-pulse CeA stimulation. These observations were consistent with previous observations that showed a greater percentage of neurons responding with an excitatory response to 200-Hz train stimulation in comparison to single-pulse stimulation of the CeA (Bouret et al., 2003). Furthermore, train stimulation resulted in three types of excitatory responses, two of which comprised the majority of excitatory responses. Both late and multiple excitatory responses showed enhanced activation during the later stimuli of the train. Previous studies have shown that high-frequency train stimulation facilitates peptide release (Bean et al., 1989; Bean and Roth, 1991). Since the CeA not only contains the fast inhibitory transmitter GABA (Ben-Ari et al., 1976; Sun and Cassell, 1993; Sun et al., 1994), but also the excitatory neuropeptide, CRH (Sakanaka et al., 1986, 1987; Van Bockstaele et al., 1998), high-frequency stimulation of the CeA may have facilitated release of CRH resulting in a greater number of neurons responding with an excitatory responses to train stimulation of CeA in comparison to single-pulse CeA stimulation. In addition, activation during later stimuli of the train in late and multiple excitatory responses may be due to facilitated CRH release during the later stimuli of the train as a result of high-frequency repeated stimulation. Present findings suggest that

increased activation of the CeA with high-frequency stimulation at high current intensities increased the likelihood of an LC neuron responding with an excitation.

The present findings also indicate differences in the magnitude of evoked activity following individual stimuli of the train at different frequencies. The activity evoked with train stimulation at 10-Hz was over two times greater than baseline only after the first stimulus, with remaining stimuli not evoking activity exceeding baseline. In contrast, 20-Hz train stimulation resulted in evoked activity that exceeded baseline throughout the entire train. If the excitatory response to 20-Hz train stimulation is indeed due to facilitated peptide release, these data suggest that a 10-Hz train is not adequate to effectively facilitate peptide release from the CeA, and increasing the train frequency possibly enhanced CRH release from the CeA during later stimuli of the train, thus resulting in an excitatory response throughout the 20-Hz train. However, it should be noted that the magnitude of the excitatory responses following individual stimuli of a 20-Hz train was not greater than the magnitude of the excitation following single-pulse CeA stimulation or following the first stimulus of a 10-Hz train, which suggests that a maximum excitatory response to CeA stimulation was attained. Based on PSTHs for individual neurons during 200-Hz train stimulation in previously reported findings (Bouret et al., 2003), the magnitude of the excitatory response does not appear greater than those observed during 20-Hz train stimulation. Taken together, this suggests that increasing the train frequency increases the magnitude and likelihood of an LC neuron responding with an excitatory response; however, a train frequency that is greater than 20 Hz may not necessarily result in further enhancement of the evoked activity during train stimulation.

High-frequency stimulation of the CeA not only resulted in enhanced activation of LC neurons, but also resulted in inhibition, both pure inhibition and inhibition that followed excitatory responses. Both types of inhibition were significantly longer with a significant reduction in spike activity with respect to baseline. Furthermore, acute excitatory responses, which occurred in a

minority of excitatory responses to 20-Hz train stimulation, showed an excitation only during the early part of the train. There are several mechanisms that may account for this finding, Thus, repeated stimulation may have facilitated both GABA and inhibitory peptide release from neurons of the CeA during the later stimuli of the train. Enhanced activation of the CeA may also have resulted in increased CRH-mediated activation of local GABAergic interneurons in the rostrolateral peri-coerulear area. A combination of these mechanisms may have accounted for a prolonged inhibitory period that showed greater reduction in spike activity with respect to baseline activity and may have contributed to the absence of excitation in acute excitatory responses during the later stimuli of the train.

Immunohistochemical studies have shown that CRH is more prominent to the lateral and medial divisions of the CeA (Cassell et al., 1986), based on previously made delineations (McDonald, 1982a). Consistent with these findings, CRH-containing CeA neurons that innervate the LC are located in the medial division of the CeA (Thompson and Cassell, 1989; Van Bockstaele et al., 1998). Given these immunohistochemical findings, placement of the stimulating electrode within the CeA may account for the enhanced activation of LC neurons and different types of excitatory responses following train stimulation of the CeA. However, the present findings indicate that excitatory, inhibitory, antidromic, and absence of responses to both single-pulse and train stimulation of the CeA were not dependent on the placement of the stimulating electrode within the CeA. High current intensity and current spread within the CeA may preclude observing a relationship between responses to electrical stimulation of the CeA and location of the stimulating electrode within the CeA. Responses to electrical stimulation were not dependent on the placement of the recording electrode within the LC. This may be due to methodology in that extracellular recordings of LC activity were made within the LC proper, while CRF-containing CeA neurons provide direct synaptic input to dendrites of LC neurons in the rostrolateral peri-coerulear area, which is located

outside of the recording area (Van Bockstaele et al., 1996a; Van Bockstaele et al., 1998). Although spatial relationship between location of the cell body and its dendrites is still currently unknown, present findings indicate that there is no topographical organization of responses within the LC.

The present findings indicate that electrical stimulation of the CeA resulted in both excitatory and inhibitory responses in LC neurons. Increased activation of the CeA either by increasing the current intensity or use of high-frequency repeated stimulation resulted in enhanced activation of LC neurons that was followed by a prolonged enhanced inhibitory period. These general observations about excitatory responses to electrical stimulation were consistent with those previously reported (Bouret et al., 2003), although stimulation parameters, criteria for excitatory responses, and characteristics of excitatory responses to electrical stimulation of the CeA were different than those used and/or observed in this study. Although increased activation of the CeA through high-frequency stimulation resulted in fewer inhibitory responses, these responses were prolonged and enhanced. Taken together, the present findings indicate that the CeA provides both an excitatory and inhibitory drive to LC neurons, and that increasing current intensity and high-frequency stimulation can greatly affect overall responses to stimulation.

4.2 MECHANISMS MEDIATING EXCITATORY RESPONSES TO ELECTRICAL STIMULATION OF THE CEA

Previous immunohistochemical, anatomical tract-tracing, and electrophysiological studies strongly suggest that CRH mediates the excitatory responses that occur in a majority of LC neurons following electrical stimulation of the CeA. This present study furthered confirmed that CRH partially mediates the excitatory responses following electrical train stimulation, and is the first to

suggest that excitatory responses to electrical stimulation of the CeA may be partially mediated by the excitatory amino acid, glutamate. Furthermore, pharmacological manipulations during excitatory responses to electrical stimulation of the CeA unmasked an underlying inhibitory period that occurs simultaneously with excitatory responses to train stimulation of the CeA.

4.2.1 Effects of ICV infusions on spontaneous LC activity.

Spontaneous LC activity remained unchanged during and following ICV infusions of both doses of D-Phe-CRH and KYN in comparison to aCSF. This is consistent with previous studies showing that ICV infusions of D-Phe-CRH do not modulate spontaneous LC activity *in vivo* (Curtis et al., 1994; Curtis et al., 1997b; Menzaghi et al., 1994), although it was reported that D-Phe-CRH has partial agonist effects on spontaneous LC activity *in vitro* (Jedema and Grace, 2004). Similar to D-Phe-CRH, KYN had no effect on spontaneous LC activity, which was consistent with previous findings (Ennis and Aston-Jones, 1988; Valentino et al., 1991). Taken together, this suggests that LC inputs containing either CRH or glutamate do not tonically modulate LC neurons in anesthetized rats. Furthermore, spontaneous LC activity was recorded between train stimulation of the CeA for approximately 50 minutes. Present findings indicate that previous train stimulation of the CeA does not affect spontaneous LC activity over long periods of time, which suggests that the CeA does not tonically modulate LC activity.

4.2.2 Effects of ICV infusions of CRH antagonists on evoked LC activity.

LC activity evoked by 20-Hz train stimulation of the CeA was significantly attenuated by D-Phe-CRH. When compared to aCSF, high doses of D-Phe-CRH attenuated excitatory responses over

time with a significant decrease occurring approximately 42 minutes following infusions. ICV infusions of aCSF did not attenuate the excitatory response to train stimulation, which suggests that the attenuation of evoked activity during train stimulation following ICV administration of D-Phe-CRH is not a consequence of high-frequency train stimulation or CRH depletion from neurons of the CeA that innervate dendrites of LC neurons. Ultra-structural evidence shows that CRH-positive terminals of CeA neurons form asymmetric synapses onto TH-positive dendrites of LC neurons (Van Bockstaele et al., 1998) in the rostrolateral peri-coerulear area (Van Bockstaele et al., 1996a). Previous electrophysiological findings showed that alpha-helical CRH attenuated evoked activity following electrical stimulation of the CeA (Bouret et al., 2003). Hypotensive stress-induced LC activation was proposed to be as a result of CeA activation, since LC neurons were not activated as a result of hypotensive stress in animals with electrolytic lesions of the CeA (Curtis et al., 2002). It was also suggested that LC activation as a result of hypotensive stress is mediated in part by CRH released from neurons of the CeA (Curtis et al., 2002). Previous and present data strongly suggest that multiple excitatory responses as a result of electrical stimulation of the CeA are partially mediated by CRH released from neurons of the CeA that innervate LC neurons.

Although D-Phe-CRH significantly attenuated excitatory responses throughout the entire train, its most prominent effects occurred during the later stimuli of the train. Train stimulation of the CeA increased the probability that an LC neuron would respond with an excitation in comparison to single-pulse stimulation of the CeA, and it elicited significantly more excitatory responses than pure inhibitory responses, with the majority of cells exhibiting an excitation during the later stimuli of the train. This is consistent with the idea that neuropeptide release requires high-frequency repeated stimulation (Bean et al., 1989; Bean and Roth, 1991). Since the majority of responses to train stimulation were excitatory and D-Phe-CRH attenuated the excitation during the later stimuli, this suggests that CRH mediates the late component of excitatory responses to train

stimulation of the CeA. These data also suggest that late excitatory responses, which show a late onset excitation, may be mediated by CRH as a result of high-frequency stimulation of the CeA.

D-Phe-CRH did not attenuate the excitatory response following the first stimulus of the train. The differential effects of D-Phe-CRH on multiple excitatory responses indicate these responses are comprised of multiple components and display characteristics of both acute and late excitatory responses to train stimulation, with the late excitatory component being mediated by CRH. Bouret et al also suggested that excitatory responses to train stimulation of the CeA were comprised of multiple components, since ICV and intra-coerulear infusions of alpha-helical CRH partially attenuated the early component of the excitatory response to train stimulation, leaving the late component unaffected. This inconsistency between the present and previous findings may be attributed to differences in stimulation parameters, particularly with respect to the frequency of train stimulation. Attenuation of the early excitatory component, as previously described, in response to 200-Hz train stimulation suggests that 200-Hz train stimulation of the CeA may have facilitated an earlier CRH release from the CeA in comparison to 20-Hz train stimulation, which may have mediated the early excitatory response as described by Bouret et al (2003).

Present findings indicate differences in the efficacy of high and low doses of D-Phe-CRH as well as a time course with respect to ICV administration on attenuating excitatory responses to train stimulation of the CeA. Although examined only 27 minutes following the infusion, evoked activity during train stimulation of the CeA was not attenuated by low doses of D-Phe-CRH. In contrast, high doses of D-Phe-CRH only partially attenuated the excitatory response during the later stimuli of the train over time with significant effects occurring after approximately 42 min after the infusion. Since neurons of the CeA innervate LC neurons at their dendrites located in the rostrolateral peri-coerulear area and not at the LC somata, where inputs such as the PGi and prepositus hypoglosi form their synapses (Aston-Jones et al., 1986; Aston-Jones et al., 1991b; Chiang and Aston-Jones,

1993a; Valentino et al., 1992), and the majority of CRH receptors are located in the peri-coerulear area (Fox et al., 2002), significant time is required for D-Phe-CRH, a peptide, to diffuse through the dendritic regions of the LC and block CRH receptors localized to these dendrites. This is consistent with unpublished data indicating that evoked activity as a result of CeA simulation was attenuated approximately 30-40 minutes following ICV infusions of alpha-helical CRH (Susan Sara, personal communication). In addition to the long diffusion rate throughout the dendritic regions of the LC, a possible mechanism that may account for the effects of the CRH antagonists long after its infusion in the both the previous (Bouret et al., 2003) and present studies may be attributed to CRH-binding protein (Behan et al., 1989; Potter et al., 1991). In general, it has been hypothesized that binding proteins sequester ligand activity or deliver the ligand to the target tissue (Kemp et al., 1998; Mendel et al., 1989). CRH-binding protein attenuates the ACTH-releasing activity of CRH (Linton et al., 1990) in addition to inactivating (Linton et al., 1990) or clearing (Woods et al., 1994) extracellular CRH in the periphery. Since the CRH antagonist D-Phe-CRH is considered to be an analogue of CRH, it is possible that CRH-binding protein may be inactivating the antagonist before it blocks CRH receptors in the dendritic regions of the LC. Thus a low dose of D-Phe-CRH has no effect on evoked activity as a result of train stimulation of the CeA and partial attenuation of excitatory responses by high doses of D-Phe-CRH occur long after the infusion.

Alternatively, evoked spike activity that was not attenuated by high doses of D-Phe-CRH may be a result of disinhibition of local GABAergic interneurons within the rostrolateral peri-coerulear area. Ultrastructural evidence shows that CRH-positive neurons from the CeA form asymmetric synapses onto TH-negative neurons within the rostrolateral peri-coerulear area (Van Bockstaele et al., 1996a; Van Bockstaele et al., 1998). These TH-negative neurons may be local GABAergic interneurons that are hypothesized to regulate LC activity during activation (Aston-Jones et al., 2004). In the presence of CRH, activation of the CeA may result in activation of these

interneurons via CRH; therefore these interneurons have the potential to contribute to the inhibitory period that follows activation in response to electrical stimulation of the CeA. When CRH receptors localized on LC neurons are blocked, CRH released from neurons of the CeA cannot directly excite LC neurons, which is what the present findings suggest occurs following high doses of D-Phe-CRH. In addition to blocking direct activation of LC neurons by CRH released from the CeA, D-Phe-CRH administration may have blocked CRH receptors localized to interneurons, therefore preventing CRH-mediated activation of local interneurons. This would result in CeA inhibition of these interneurons, resulting in disinhibition of LC neurons during train stimulation of the CeA in the presence of D-Phe-CRH. This is further supported by previous studies showing doses comparable to the low dose of D-Phe-CRH used in this study attenuated CRH-mediated activation of LC neurons (Curtis et al., 1994; Curtis et al., 1997b).

4.2.3 Effects of ICV infusions of glutamatergic antagonists on evoked LC activity.

The differential effects of D-Phe-CRH on excitatory responses suggested that the excitatory responses evoked by train stimulation of the CeA consist of multiple components, with CRH partially mediating the late excitatory component. This further suggested that the multiple excitatory responses are mediated by multiple mechanisms, one of them being CRH. The excitatory responses that were not completely attenuated by D-Phe-CRH are thought to be mediated by the excitatory amino acid, glutamate, since application of glutamate and activation of glutamatergic afferents activate LC neurons.

ICV infusions of KYN attenuated excitatory responses elicited during train stimulation of the CeA, and had a more profound effect than D-Phe-CRH. The early excitatory component was attenuated to baseline levels following KYN infusions, but was not attenuated by D-Phe-CRH. In

contrast, KYN completely blocked the late excitatory component, while D-Phe-CRH only partially attenuated this component of the excitation. Attenuation of the excitatory response during train stimulation of the CeA following ICV infusions of KYN suggest that, in addition to CRH, glutamate partially mediates these excitatory responses. Without citing immunohistochemical evidence, it was suggested that the CeA contains glutamate (Davis, 1994). Furthermore, in axon terminals within the peri-coerulear area, CRH preferentially co-localizes with glutamate rather than GABA (Valentino et al., 2001). Although the origin of axon terminals showing co-localization of glutamate and CRH is unknown, it is unlikely that excitatory responses following electrical stimulation of CeA is a result of neurons from the CeA co-releasing glutamate with CRH onto dendrites of LC neurons. Glutamatergic vesicular transporters (VGLUT) serve as immunohistochemical markers for glutamate, thus identifying glutamatergic cell groups within the CNS. Although it has been demonstrated that the cell bodies of neurons in the CeA do not express immunoreactivity for VGLUT1, VGLUT2 (Fremeau et al., 2001; Poulin et al., 2006), and VGLUT3 (Poulin et al., 2008), it was demonstrated that the glutamatergic transporters are localized to excitatory synapses (Bellocchio et al., 1998; Fremeau et al., 2001) rather than in the cell bodies of neurons. Together, these data suggested that the CeA may still provide its targets with a potential source of glutamate. However, in situ hybridization studies have shown that neurons of the CeA do not express mRNA for vGLU1 (Fremeau et al., 2001) and vGLUT2 (Fremeau et al., 2001; Hur and Zaborszky, 2005). This evidence suggests that neurons of the CeA do not synthesize VGLUT protein and further suggest that neurons of the CeA do not contain or use glutamate as an excitatory transmitter. Given the lack of immunohistochemical evidence for glutamate localization in neurons of the CeA, this suggests that the CeA may be activating LC neurons indirectly via glutamatergic afferents.

A variety of excitatory amino acid afferents converge onto LC neurons (Aston-Jones et al., 1991b) and it is likely that glutamate mediates LC activation following stimulation of these

glutamergic inputs. It is unlikely that these glutamatergic afferents, such as the medial prefrontal cortex (Jodo et al., 1998), paragigantocellularis (Ennis and Aston-Jones, 1988), and Barrington's nucleus (Page et al., 1992), contribute to the excitatory responses as a result of CeA stimulation since the latencies of the maximal excitation as a result of stimulation of prefrontal cortex, paragigantocellularis and Barrington's nucleus are similar to those observed in this study. Although the paragigantocellularis provides a source of both CRH and glutamate to the LC (Valentino et al., 1992) and mediates LC activation as a result of indirect sciatic nerve stimulation (Chiang and Aston-Jones, 1993b; Ennis et al., 1992) and opiate withdrawal (Akaoka & Aston-Jones), it is unlikely that activation of the LC as a result of the CeA is mediated by the paragigantocellularis. Thus, CRH antagonist administration does not alter PGi-mediated LC activation as a result of sciatic nerve stimulation (Curtis et al., 1994; Valentino and Curtis, 1991b) or opiate withdrawal (Valentino and Wehby, 1989), both of which are mediated by glutamate released from the PGi (Akaoka and Aston-Jones, 1991; Ennis et al., 1992; Rasmussen and Aghajanian, 1989). Taken together, these data suggest that the CeA may modulate an unknown glutamatergic input into the LC, particularly during the early excitatory component of the responses to train stimulation.

Another possible mechanism involved in excitatory responses to CeA stimulation may involve CRH-mediated modulation of glutamate receptors. There is ultrastructural evidence showing dendrites in the rostrolateral peri-coerulear area receiving convergent glutamatergic and CRH inputs (Valentino and Van Bockstaele, 2001). At least within the dopaminergic ventral tegmental area, electrophysiological data suggest that CRH can potentiate glutamatergic NMDA receptors via the CRH2 receptor subtype (Ungless et al., 2003). The CRH1 receptor subtype is more prevalent in the LC than the CRH2 receptor subtype (Fox et al., 2002; Sauvage and Steckler, 2001); nonetheless, CRH modulation of converging glutamate inputs at dendrites of LC neurons in the rostrolateral peri-coerulear area may be a potential mechanism to mediate excitatory responses to CeA

stimulation. The present findings indicate that KYN completely attenuates excitatory responses during the late excitatory component, while D-Phe-CRH only partially attenuates evoked activity. Although it was demonstrated that CRH directly excites LC neurons *in vitro* in the presence of TTX (Jedema and Grace, 2004), it was not demonstrated in the presence of excitatory amino acid antagonists. If CRH modulation of glutamate receptors is a potential mechanism underlying excitatory responses to CeA activation, together with ultrastructural findings, these present findings suggest that CRH activation may be attenuated once glutamate receptors are blocked by KYN.

4.3 THE CEA ACTIVATES WHILE SIMULTANEOUSLY INHIBITING LC NEURONS

A high dose of the CRH antagonist, D-Phe-CRH, and the glutamatergic antagonist, KYN, revealed that glutamate, together with CRH, may mediate excitatory responses during train stimulation of the CeA. In addition, attenuation of excitatory responses by KYN revealed a short-onset, short-latency inhibitory period that occurs simultaneously during the excitatory responses to train stimulation of the CeA. Furthermore, the presence of an underlying inhibitory period during excitatory responses is consistent with the CeA directly mediating the inhibitory period that immediately follows activation of LC neurons following electrical stimulation of the CeA. In colchicine-treated rats, it has been shown that CRH co-localizes with GABA in neurons of the CeA (Veinante et al., 1997). Using in situ hybridization, there was substantial coexistence of mRNA for both CRH and GAD isoforms 65 and 67 in neurons of the CeA (Day et al., 1999). Within the rostrolateral peri-coerulear area, a small percentage of CRH-positive terminals were also dual labeled for GABA (Valentino et al., 2001). These present electrophysiological findings are consistent with immunohistochemical and in situ

hybridization studies suggesting that the inhibitory period underlying excitatory responses following electrical stimulation of the CeA may be mediated by both CRH and GABA simultaneously released from neurons of the CeA that directly innervate LC neurons.

Immunohistochemical and in situ hybridization studies showing co-localization of GABA and CRH strongly suggest that GABA mediates the inhibitory period underlying the excitatory response following train stimulation of the CeA. Given that neurons of the CeA contain neuropeptides that have inhibitory effects on LC neurons, the underlying inhibition may also be mediated by these neuropeptides together with GABA as a result of facilitated peptide release during high-frequency repeated stimulation. It has been shown that the peptide enkephalin is contained in neurons of the CeA (Cassell et al., 1986), co-localizes with GABA in axon terminals within the LC (Van Bockstaele, 1998; Van Bockstaele and Chan, 1997), and decreases LC activity (Travagli et al., 1995). High-frequency stimulation of the CeA may have facilitated enkephalin release, thus mediating the underlying inhibitory period. Although immunohistochemical evidence strongly suggests that inhibitory responses may be mediated by multiple inhibitory transmitters, it should be noted that the present data do not provide direct evidence regarding whether the inhibitory period is mediated by GABA alone or in conjunction with inhibitory neuropeptides released by projection neurons of the CeA following electrical stimulation.

The presence of an underlying inhibitory period during excitatory responses to train stimulation of the CeA may be a mechanism by which the CeA modulates the magnitude of the excitatory response observed in the majority of LC neurons. This is consistent with the present findings, in which later stimuli of a 10-Hz train failed to elicit spike activity that exceeded baseline, whereas 20-Hz train stimulation of the CeA evoked excitatory responses throughout the entire train. Differences in evoked activity during the train may be due to the frequency of the train, in that a slower frequency failed to facilitate CRH release that would result in excitatory responses during the

later stimuli of the train. Alternatively, the inhibitory period that simultaneously occurs during excitatory responses to train stimulation may have prevented LC neurons from responding with an excitatory response during the latter stimuli of a 10-Hz train. In addition, the lack of response of some LC neurons to single-pulse stimulation of the CeA may be due to LC neurons simultaneously receiving excitatory and inhibitory input from the CeA, thus resulting in spike activity that is not significantly greater or less than baseline activity following electrical CeA stimulation. However, increased activation of the CeA as a result of high-frequency repeated stimulation resulted in more neurons responding with an excitatory response, with fewer pure inhibitory responses and nonresponsive neurons. The presence of an inhibitory period that occurs simultaneously with excitatory responses and pure inhibitory responses to activation of the CeA suggests that the CeA provides an extrinsic mechanism to regulate overall LC output as a result of activation of the CeA. CeA-mediated inhibition of LC neurons may modulate the magnitude of the excitation and the number of LC neurons that are excited based on the level of CeA activation. This has functional implications during behaviors that activate the CeA-LC pathway.

4.4 EFFECTS OF PHARMACOLOGICAL ACTIVATION OF THE CEA

The present findings indicate that electrical stimulation of the CeA results in excitatory responses in the majority of LC neurons. ICV infusions of D-Phe-CRH and KYN, antagonists for CRH and glutamate, respectively, differentially attenuated excitatory responses to train stimulation of the CeA, and further suggested that the CeA may activate LC neurons via both CRH and glutamate. Alternatively, attenuation of excitatory responses to electrical stimulation of the CeA by kynurenic acid and the absence of immunohistochemical evidence for glutamate localization in neurons of the

CeA together suggest that excitatory responses to electrical train stimulation of the CeA may be mediated by activation of fibers of passage. Electrical stimulation of sites immediately adjacent to the CeA, such as the ventral striatum and ventral hippocampus, did not result in either excitatory or inhibitory responses in LC neurons (Bouret et al., 2003) and suggested the specificity of the CeA in mediating the excitatory responses. However, high current intensity and current spread within the CeA and to the output pathway of the bed nucleus of the stria terminalis (BNST), which lies in close proximity to the CeA, suggests that the BNST may mediate the excitatory responses as a result of electrical stimulation of the CeA. Anatomical tract-tracing studies and ultra-structural evidence suggest that the BNST-LC pathway is inhibitory since terminals of BNST neurons form symmetric synapses onto neurons that were both TH-positive and TH-negative in the rostrolateral peri-coerulear area and also do not provide a significant source of CRH to the LC (Van Bockstaele et al., 1999). Inconsistent with the ultra-structural evidence that strongly indicates that the BNST provides inhibitory input to LC neurons, unpublished electrophysiological data suggest that electrical stimulation of the BNST results in excitatory responses in LC neurons (Jedema & Grace, unpublished findings). This further suggests that excitatory responses as a result of electrical stimulation of the CeA may partially be a result of stimulating fibers originating in the BNST.

In order to further characterize the specificity of CeA-mediated activation of LC neurons, pharmacological activation of the CeA with the excitatory amino acid agonist, AMPA, resulted in increases in LC firing rate. Presumably, infusions of AMPA within the CeA would activate AMPA receptors on cell bodies and dendrites of CeA neurons, resulting in an activation of neurons in the CeA that innervate LC neurons. Increases in LC firing rate as a result of AMPA infusions in the CeA is consistent with present and previous findings (Bouret et al., 2003) showing that electrical stimulation of the CeA results in excitatory responses in LC neurons. AMPA infusions in the CeA tended to increase LC firing rate in a dose-dependent manner, which is consistent with the direct

current-response relationship observed for excitatory responses as a result of electrical stimulation of the CeA. Increased LC firing rate following AMPA infusions in the CeA indicates that the excitatory responses following CeA electrical stimulation is not mediated by activation of fibers of passage through the CeA, possibly those originating from the BNST. These pharmacological results indicate that activation of LC neurons following AMPA infusions into the CeA may be mediated directly by neurons of the CeA or possibly indirectly by CeA-activated excitatory afferents to the LC.

Given that CRH is localized in CeA terminals arising from neurons of the CeA that form synapses on dendrites of LC neurons (Van Bockstaele et al., 1998), and that excitatory responses to electrical train stimulation of the CeA are partially mediated by CRH, increases in LC activity following pharmacological activation of the CeA are thought to be at least partially mediated by CRH. AMPA infusions in the CeA resulted in increases that were long in duration with a late occurrence in the maximal increase in LC activity from the onset of the infusion. This observation is consistent with previous findings showing a slow-onset long-lasting activation of LC neurons as a result of intra-coerulear and ICV CRH administration (Curtis et al., 1997a; Curtis et al., 1994; Jedema et al., 2001; Valentino et al., 1983; Valentino and Wehby, 1988). Pharmacological activation of the CeA resulted in increased LC activity that was low in magnitude. This is consistent with present findings suggesting that CeA may also be providing an inhibitory drive via GABA alone or together with inhibitory neuropeptides to LC neurons while simultaneously increasing LC activity via CRH and possibly glutamate.

4.5 THE CEA DOES NOT PROVIDE A SOURCE OF TONIC INHIBITION TO LC ACTIVITY

LC activity *in vivo* is under tonic inhibitory control of GABAergic neurons (Ennis and Aston-Jones, 1989b; Kawahara et al., 1999; Pudovkina et al., 2001). Local application of GABAergic antagonists increases LC firing rate (Ennis and Aston-Jones, 1989b) and NE release both in the medial PFC and locally within the LC (Kawahara et al., 1999; Pudovkina et al., 2001). Tonic inhibition is mediated by GABAA receptors (Ennis and Aston-Jones, 1989b; Kawahara et al., 1999) with a small contribution of GABAB receptors (Kawahara et al., 1999; Shefner and Osmanovic, 1991). Sources of tonic inhibitory control may be intrinsic via GABAergic interneurons within the dendritic fields of the LC (Aston-Jones et al., 2004) or extrinsic (Van Bockstaele, 1998; Van Bockstaele and Chan, 1997). It was hypothesized that the CeA would provide an extrinsic source of tonic inhibitory control of LC neurons, since the CeA provides a source of GABA to the LC (Sun et al., 1994). Inactivation of the CeA by combination of the GABAA agonist, muscimol, and the GABAB agonist, baclofen, showed that the CeA does not provide an extrinsic source of tonic inhibitory control of the LC at least in the anesthetized rat. This is consistent with the slow firing rate of projection neurons within the CeA in anesthetized rats (Correll et al., 2005). This further suggests that the CeA-LC pathway may be important only during behaviors that activate the CeA, particularly following exposure to stressful or aversive stimuli.

4.6 METHODOLOGICAL CONSIDERATIONS

Combined electrical and pharmacological activation of the CeA strongly suggests that the CeA provides an excitatory input to LC neurons. ICV administration of D-Phe-CRH and KYN suggests that the CeA activates LC neurons through multiple mechanisms that involve both CRH and glutamate. These findings are, in part, consistent with previously reported findings from immunohistochemical, anatomical tract-tracing, and electrophysiological studies. This study contributes to the current literature by further demonstrating that the CeA provides an excitatory input to LC neurons and providing insight into the mechanisms underlying these excitatory responses.

In order to investigate possible mechanisms underlying these excitatory responses, drugs were administered into the ventricle rather than by local microinjections into the rostrolateral peri-coerulear area, where neurons of the CeA form synapses onto LC dendrites. Drug infusions into the ventricle have the potential to affect other pathways by which the CeA modulates LC activity. Therefore, although strongly suggestive, this study still does not provide conclusive evidence that CRH, which is released from neurons of the CeA that innervate dendrites of LC neurons, mediates the excitatory responses as a result of CeA activation. It does, however, provide preliminary evidence that the CeA modulates the LC via multiple mechanisms, and possibly through an indirect pathway, which is evidence that was not previously elucidated from combined immunohistochemical and anatomical tract-tracing studies that investigated the CeA-LC pathway. Based on present findings, intracoerulear infusions of drugs that attenuate excitatory responses as a result of CeA stimulation still would not provide evidence for direct activation of the LC by the CeA.

The present data, along with immunohistochemical and anatomical tract-tracing studies, further reinforce the importance of local inhibitory interneurons within the rostrolateral peri-

coerulear area, particularly with respect to activation by CRH, in modulating LC activity during CeA activation. Furthermore, present findings suggest that the CeA modulates LC neurons through inhibition while simultaneously activating LC neurons. Whether this inhibitory response is mediated directly by GABA alone or in conjunction with inhibitory neuropeptides released from neurons of the CeA innervating LC neurons, or indirectly via inhibitory interneurons, is unclear. Nonetheless, this inhibitory period may be critical in modulating LC activity during behaviors that activate the CeA-LC pathway.

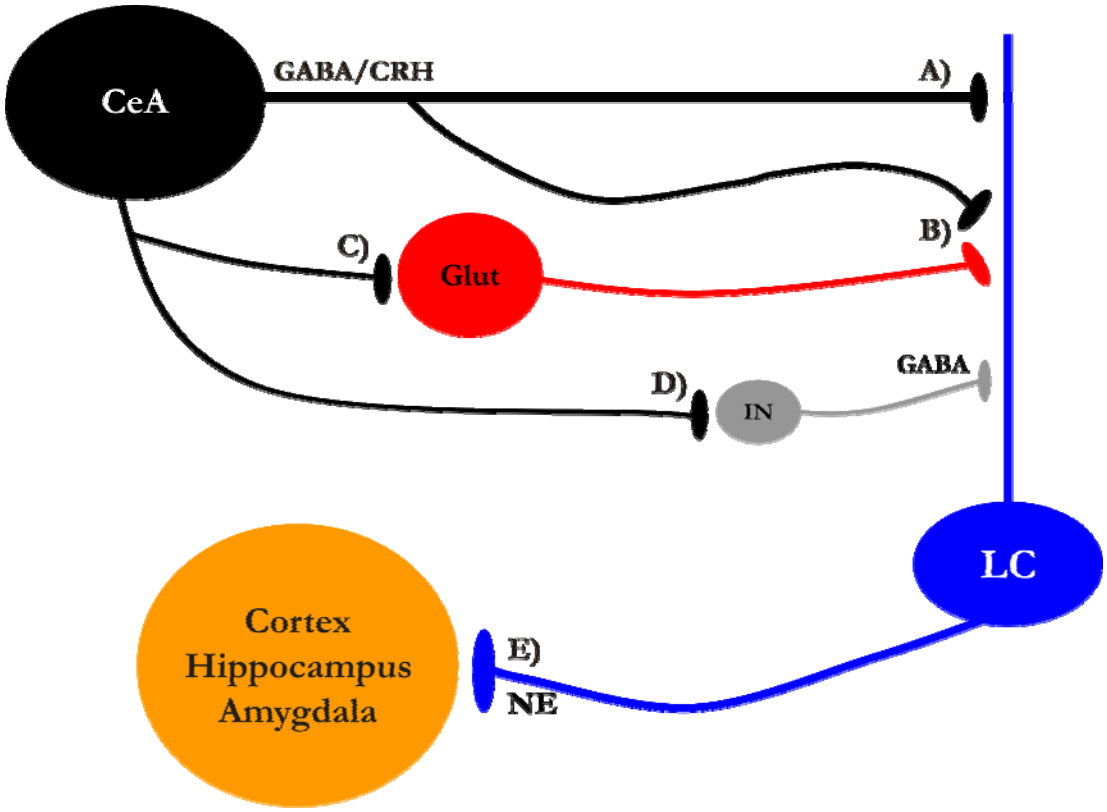

Figure 34. The CeA may modulate LC neurons via multiple mechanisms.

A) Activation of LC neurons following activation of the CeA appears to be mediated in part by CRH likely originating from the direct projection from the CeA. During LC activation, neurons of the CeA may provide a simultaneous inhibitory drive to LC neurons via GABA. The CeA may also increase LC activity by **(B)** modulating glutamate receptors localized on dendrites of LC neurons within the rostrolateral peri-coerulear area via CRH or **(C)** modulating glutamatergic LC afferents via CRH. **D)** The CeA may modulate LC neurons via GABAergic interneurons localized within the rostrolateral peri-coerulear area. **E)** Increases in LC activity following activation of the CeA may be important for noradrenergic modulation of affective learning, arousal and attention, and responses to aversive stimuli.

4.7 FUNCTIONAL SIGNIFICANCE OF THE CEA-LC PATHWAY

Several studies have indicated that the LC is sensitive to stress exposure in that some stressors result in the activation of LC neurons (Stanford, 1993; Stanford, 1995; Zigmond et al., 1995). Furthermore, several studies have shown that stress-induced activation of the LC is mediated by CRH. The present data, along with previous findings, indicate that the CeA provides an excitatory input to LC neurons, and that this excitation may be mediated by CRH. These findings suggest that stress-induced activation of the LC, which is thought to be mediated by CRH, may be a result of activation of the CeA-LC pathway during exposure to stressful stimuli, given that the CeA is also activated following presentation of stressful stimuli.

Several studies have also suggested that acute physiological and psychological stressors differentially activate distinct nuclei of the amygdala, such as the CeA and medial amygdala (Dayas et al., 2001; Dayas et al., 1999; Dayas and Day, 2002). Although both physiological and psychological stressors significantly activate the CeA and medial amygdala as measured by Fos expression (Dayas et al., 2001), physiological stressors, such as hemorrhage and immune challenge, preferentially activate the CeA (Dayas et al., 2001), while psychological stressors, such as swim stress and restraint activate the medial amygdala (Dayas et al., 2001). Observations from multiple studies have suggested that the CeA mediates LC activation as a result of nitroprusside-induced stress, which can be thought of as physiological stressor (Curtis et al., 2002). In animals with electrolytic lesions of the CeA, LC neurons failed to activate following nitroprusside-induced stress (Curtis et al., 2002), a stressor known to activate LC neurons via CRH (Page et al., 1993; Valentino et al., 1991; Valentino and Wehby, 1988) in addition to neurons of the CeA (Curtis et al., 2002). Although these studies suggest that amygdala nuclei are preferentially activated following different types of stress exposure, this does not completely eliminate any role the CeA may have in modulating activation of LC

neurons in response to psychological stressors, since these stressors still significantly activated neurons of the CeA in comparison to naïve rats (Dayas et al., 2001). Thus, immobilization stress-induced increases in extracellular NE release in the prefrontal cortex, which is a correlate of LC neuronal activation and is attenuated following administration of CRH antagonists (Emoto et al., 1993; Nakane et al., 1994; Shimizu et al., 1994; Smagin et al., 1996; Smagin et al., 1997), may be modulated by the CeA, given that the CeA is also activated following immobilization stress and provides a direct source of CRH to the LC.

Although the CeA is activated following presentation of both acute physiological and psychological stressors, several studies have also demonstrated that the CeA is preferentially involved in conditioned fear rather than unconditioned fear (Davis et al., 1994; Davis and Whalen, 2001; LeDoux, 2000; Walker and Davis, 1997). For example, it has been shown that the CeA preferentially mediates expression of conditioned fear, while the bed nucleus of the stria termianalis (BNST) preferentially mediates expression of unconditioned fear (Endres et al., 2005; Fendt et al., 2005; Muller and Fendt, 2006; Walker and Davis, 1997). Following contextual fear conditioning, there was a significant increase in Fos expression in neurons of the LC cells as compared to non-conditioned rats (Ishida et al., 2002). This suggests that activation of the CeA during fear conditioning may have contributed to the increased Fos expression in LC neurons during responses to conditioned fear (Davis and Whalen, 2001; Ishida et al., 2002)

It was suggested the spatial arrangement of LC afferents would result in differential regulation of LC activity (Herman and Cullinan, 1997). Brainstem afferents, such as the paragigantocellularis, prepositus hypoglossi, and Barrington's nucleus, that innervate the LC proper would convey information of immediate physiological survival value, i.e, the "systemic" stressors, while limbic afferents that innervate the dendrites located in the peri-coerulear area would modulate LC responses to stressful stimuli (Herman and Cullinan, 1997). Since the CeA provides an excitatory

limbic input to the LC by forming synapses onto LC dendrites in the peri-coerulear area (Van Bockstaele et al., 1996a; Van Bockstaele et al., 1998), it was hypothesized that the CeA-LC pathway may modulate LC responses to footshock, which is mediated by the PGi, a glutamatergic brainstem LC afferent contacting somata of the LC. Although footshock, a noxious stimulus, activates not only LC neurons, but also CeA neurons in anesthetized rats (Correll et al., 2005), inactivation of the CeA does not affect footshock-induced LC activation in anesthetized rats (Ramsooksingh and Grace, unpublished findings). This may be due to the characterization of footshock as a noxious stimulus rather than an acute stressor and/or use of an anesthetized preparation in examining the role of the CeA-LC pathway in modulating LC responses to aversive stimuli.

Together with the previous findings, the present findings provide support for the CeA not only as an excitatory limbic LC afferent, but as an afferent that is positioned to potently modulate other stimulatory drive of this nucleus. This has implications for noradrenergic modulation of LC-innervated brain regions that are involved in processes regulating affect, learning, and memory, particularly during exposure to stressful stimuli and/or fear conditioning (Aston-Jones et al., 1996). Studies have described the function of NE as means of gating information (Aston-Jones and Cohen, 2005; Devilbiss and Waterhouse, 2000), since NE has decreased spontaneous activity, while simultaneously either increasing or decreasing evoked activity in the same neurons (Devilbiss and Waterhouse, 2000; Foote et al., 1975; Segal and Bloom, 1974, 1976; Waterhouse et al., 1998a; Waterhouse et al., 1980; Waterhouse et al., 1984). Increases in LC activity via activation of the CeA-LC pathway may result in increased NE release throughout the entire neuroaxis, thereby altering the signal-to-noise ratio in regions that receive noradrenergic LC innervation and process sensory stimuli, such as the BLA, hippocampus, and cortex. Within the BLA and hippocampus, increases in NE releases as a result of activation of the CeA-LC pathway may modulate gating of sensory information (Waterhouse et al., 1998b) and modulate learning and memory processes involved in

fear conditioning (McGaugh et al., 1996). It has been suggested that the LC is critical in maintaining arousal and attention (Aston-Jones et al., 2000; Berridge, 2001; Robbins, 1997), thus increased LC activation during fear conditioning may result in increased vigilance and signal detection during states of fear and exposure to stressful stimuli (Davis and Whalen, 2001). By biasing the LC either towards simultaneous activation via a CRF-mediated facilitation, or to a selective activation via GABAergic inhibition, the CeA may be positioned to selectively modulate the extent of this parallel activation across LC projection sites.

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