AN ABSTRACT OF THE DISSERTATION OF

Emma Coddington for the degree of Doctor of Philosophy in Zoology presented on June 8, 2004.

Title: Neuroendocrine Mechanisms that Regulate Context-Dependent Behavioral Responses to Acute Stress.

Abstract approved: Redacted for privacy

Frank L. Moore

In nature, animals survive threats by responding physiologically and behaviorally in ways that are appropriate to the situation. Which responses are appropriate depend on the animals' surroundings and current physiological and behavioral state. This dissertation investigated the neuroendocrine mechanisms that control context-dependent behavioral responses to stress by focusing on the contrasting actions of corticosterone and vasotocin and using an amphibian model (roughskin newt, *Taricha granulosa*). These two hormones were selected because, in *Taricha*, the stress steroid corticosterone (CORT) suppresses and the peptide vasotocin (VT) enhances male reproductive behaviors.

Behavioral studies confirmed that CORT administration suppresses clasping and that this CORT-induced suppression of clasping is blocked by previous experience of courtship clasping or exposure to VT. Other studies revealed that CORT administration does not suppress clasping behavior by suppressing appetitive responses to visual or olfactory sexual stimuli.

Another series of studies tested the hypothesis that the endocannabinoid system acts on medullary neurons and that the endocannabinoid system is activated by CORT. Behavioral studies revealed that stress-induced and CORT-induced suppression of clasping was blocked by pretreatment with a cannabinoid (CB1) receptor antagonist. Single-unit recording from medullary neurons that control clasping revealed a similar pattern. Spontaneous activity and sensory responsiveness of medullary neurons was suppressed by CORT; this effect was blocked by prior treatment of a cannabinoid antagonist. These studies indicate that

endocannabinoid signaling occurs downstream in the temporal sequence of events initiated by acute stress and CORT.

Given that CORT and VT interact to alter behavior and medullary neuron activity, and that endocannabinoids mediate CORT-induced suppression of these measures, we predicted that cannabinoids interfere with VT signaling. Behavioral studies indicated that cannabinoid agonist suppresses clasping, but electrophysiological studies revealed mixed responses by medullary neurons. When administered sequentially in behavioral and electrophysiological studies, VT-induced enhancement of clasping behavior and neuronal activity was blocked by prior treatment with a cannabinoid agonist.

Together these studies provide evidence that CORT and VT interact, and the temporal nature of their interaction transduces context. One mechanism by which endocrine signals can interact to produce appropriate behavioral responses is by the action of retrograde signaling of endocannabinoids.

Neuroendocrine Mechanisms that Regulate Context-dependent Behavioral Responses to Acute Stress

by

Emma Coddington

A DISSERTATION

Submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of

Doctor of Philosophy

Presented June 8, 2004

Commencement June 2005

Doctor of Philosophy dissertation of Emma Coddington presented on June 8, 2004.

APPROVED:

Redacted for privacy

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Emma Coddington

ACKNOWLEDGEMENTS

Graduate School has been an incredible period of personal and scientific growth for me, and there are many people who have contributed to this part of my life. I gratefully acknowledge my major professor, Dr. Frank Moore. His open door invited conversation at any time and all aspects of my career have benefited by his example and advice; my sincerest thanks to Frank for his unfailing patience and confidence in me. I would also like to thank Dr James D. Rose, who not only provided the expertise, patience and intellectual support for all of the electrophysiology studies, but he engaged, challenged and encouraged me as an individual. I look forward to continuing work with Frank and Jim.

I would like to acknowledge all the faculty members of Zoology, some of whom were part of my committee and many who were not. A big thank you to my committee, Bob Mason, Lynne Houck and Larry Ryan who have at different times challenged, encouraged, and supported me through this process of doctoral studies. Thank you to Barb Taylor, for microscope time and providing uncompromising criticism of presentations and concepts. Thank you to the Houck/Arnold world for allowing me to join their lab meetings for the year that I was figuring out my own project and life, and for engaging me in the intellectual planning component of the salamander pheromone project.

A number of post-docs have been mini-mentors to graduate students and I feel especially grateful to have interacted with a number from our and others labs. Thank you to Joanne Chu for her friendship and essentially mentoring me through the extremely difficult period of finding a project and finding my feet in the lab and in the department. Thank you to Rick Thompson and Joanne Chu for our continued exchange of ideas. Thank you to Dave Hollis who not only gelled out lab, but brought incredible capacity to laugh as well as technical finesse in the lab. And thank you to Weezie Mead for her intellectual and moral support.

The interactions with graduate students and other fulltime member of zoology labs have made my tenure at OSU an incredible learning experience. I especially thank Deborah Lutterschmidt, Kate Semsar, and Christine Lewis for their incredible superwomen strength, the hours of conversation about our projects and science in general, and guidance towards a common goal of maturing into contributing members of our scientific research and teaching community. Special thanks also goes to my lab mates who are not only awesome people, but

have been incredibly supportive and have helped made my work what it is today; Eliza Walthers, Brian Searcy, Simon Evans, Sam Bradford, and Martha Baugh.

Many many many newts came through my nets on their way into and out of behavioral and other experiments. My humble thanks to their lives. And thanks also to the small group of intrepid newt catchers and newt care people who made all the experiments possible and fun to participate in; Garret Woodman, Eric Brown, Jed Stokes, Christina Oaks, Jen Walters, Michelle van Warmerdam, Felicity Shaw, and Ellery Engman. A special thanks to Garrett for the effort above and beyond his duties, such as the many hours of shared all night video shoots of newt porn in an effort to capture spermatophore deposition on tape (which we never actually accomplished!). And a special thanks to Sam Bradford who built the infamous Willamette Valley Red Light District – dark room for behavioral experiments.

A huge thank you to the Zoology department for the constant financial support with teaching stipends and help attending the Woods Hole course that turned my world around. Thank you also for the amazing teaching and lecturing experiences that have helped shape my teaching ethic. The support of the Zoology staff can never be overestimated – their guidance, help and patience all the way through this process have been incredible. Thank you.

I would like to thank my amazing family for their support of me moving halfway around the world to pursue my dreams; Dad for the belief, Mum for her unconditional love, James for the courage, and Patrick for the dreaming. And thanks to my grandmother and grandfather who have given unwavering support and encouragement. My sincerest thanks to my family here; Keri Taff, Marcia Macomber and the Brown Family for their welcome of me into their family in Corvallis, their support, long conversations and laughter, and excellent river trips which have reinforced my soul. And finally, but most importantly a mini-thanks to "the" Eric T. Brown – without him I would never have finished this process, with him I am more than I ever could be on my own. He rocks.

CONTRIBUTION OF AUTHORS

Chapter II is reproduced with the written permission of Elsevier Science Publishers B.V. and the journal *Hormones and Behavior*. I conducted the design, experiments and analysis in the laboratory of Dr. Frank L. Moore. Dr. Moore provided interpretive guidance and editorial comments for manuscript in preparation.

The studies conducted in Chapter III were conducted and analyzed by me in the laboratory of Dr. Frank Moore. Dr. Moore provided advice throughout this experiment and editorial comments for preparation of this paper for submission.

The behavioral component of the studies in Chapter IV and V were conducted in the laboratory of Dr. Frank Moore. I conducted the design, experiments and analysis of the behavioral experiments in the laboratory of Dr. Frank L. Moore. The electrophysiology studies were conducted in the laboratory of Dr. James D. Rose. Christine Lewis provided me with technical training necessary for the performance of the surgeries and single-unit recording studies. Computer work with SPIKE II to filter each spike and monitor the individual spike's frequency over the course of each experiment was done by Christine Lewis. I conducted the counting and analysis of neuron measures. Both the manuscripts have been prepared for submission with the guidance and editorial comments from Dr.s Moore and Rose.

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CHAPTER I: GENERAL INTRODUCTION

An animal's survival and reproductive success relies on its ability to respond appropriately when confronted with an immediate threat. Most wild animals have a large repertoire of defensive behaviors. Which alternative actions are expressed depends on the animal's current situation. For example, an adult Killdeer without young will respond defensively by flying away from a potential predator, whereas a nesting Killdeer with young is more likely to present broken-wing displays and attract the attention of a potential predator away from the nest (Ehrlich, Dobkin, and Wheye, 1988). A lone male newt is more likely to respond defensively by actively swimming away from an approaching person than is a male newt that is engaged in amplectic clasping with a female. Thus, appropriate behavioral responses to threatening situations depend on the animal's context – its current surrounding environment and its physiological and behavioral states. The corresponding neuroendocrine mechanisms underlying these context-dependent behavioral responses are poorly understood. My doctoral dissertation investigated neuroendocrine mechanisms that regulate male reproductive behaviors in an amphibian model, the roughskin newt (*Taricha granulosa*). Specifically, I investigated the neuroendocrine control of context-dependent behavioral responses to acute stress.

BACKGROUND

Animal Model

The animal studied in this project is the roughskin newt, *Taricha granulosa.* This amphibian species is an established research model for studying neuroendocrine mechanisms that underlie behaviors. A critical advantage of *Taricha* for the current project was that males exhibit extremely robust courtship displays and behavioral responses to stress. The courtship and mating sequence can extend over a relatively long period of time, sometimes days. During this period of time males follow a stereotypical series of behaviors. Prior to finding a female, males aggregate along the edges of permanent ponds at inlet sites, presumably waiting for sexually attractive females to enter. Males will first orient towards movement and then visually identify the moving object. If

this object appears appropriate in size and shape then the males approach. Males then appear to use the scent of pheromone(s) at this stage in the sequence to determine whether this moving object is a conspecific female that is reproductively active. A sexually attractive female typically has mature ovaries and has not yet mated with another male. The male will capture a sexually attractive conspecific in a strong amplectic clasp, which involves the male embracing the female by encircling her with both fore- and hind-limbs. Pairs stay in this amplectic clasp from anywhere between 4 to 48 hrs. Presumably, during this period both the male and female assess the quality of the mate, and if both become receptive to the other, then the male will release the female from the clasp, deposit a spermatophore, which includes a sperm containing package, on the substrate in front of the female. He then leads her over the spermatophore, for her to pick the sperm cap with her cloaca. At this point, male *Taricha* re-engage the female in a clasp. Females remain in the pond to lay fertilized eggs, one at a time, on plant substrates. Prior research has led to an understanding of how various hormones enhance and acute stress suppresses reproductive behaviors in male *Taricha.*

Stress and the Hypothalamic-Pituitary-Adrenal Axis

There has been endless debate over how to define the term *stress*. In current literature *stress* can refer to anything from cellular processes, such as induction of heat-shock proteins, to psychological processes, such as chronic anxiety. This broad spectrum reflects varied perspectives from diverse scientific disciplines and even the perspectives of the general public. For the purposes of my thesis I will limit my discussion to acute stress and propose a limited definition for the term: *acute stress* refers to the experience of immediate stimuli that perturbs an animal's homeostatic physiology. A stressor, then, would be the stimuli experienced, and the stress response refers to the resultant physiological and behavioral shifts. Within the context of this thesis, the stressor stimuli is males placed in a clear dry plastic container (8 x 3 x 3 cm) and tumbled by hand 180° sideways every 0.5s for a specified period of time (5 - 15 min).

The hypothalamic-pituitary-adrenal (HPA) axis is the major hormonal system responsible for maintaining homeostatic balance in response to stress, and the hormones of this system are also responsible for mediating the behavioral responses that occur as a result of experiencing acute stress. A stressor can initiate the neuroendocrine HPA cascade beginning with the release of corticotropin releasing hormone (CRH) from hypothalamic parvocellular paraventricular nuclei. This neurotransmitter in turn regulates

the release of adrenocorticotropin hormone (ACTH) from the anterior pituitary into the blood stream. The action of ACTH on cortical cells of the adrenal gland results in the release of corticosterone, a steroid-derived hormone, into the blood stream. The corticosteroids exert peripheral effects on metabolic, immune and reproductive functions, and also complete the HPA loop by feeding back to the pituitary and hypothalamus to negatively regulate the release of ACTH and CRH.

Each of these principle hormones, in addition to functioning in this HPA axis, also functions to alter central nervous system activity ultimately affecting behavior. The behavioral effects of ACTH are predominantly mediated via feedback to the HPA axis. However, CRH and CORT are known to influence behaviors by interacting directly with neural pathways; both hormones directly effect locomotion, arousal, motor function, feeding, and reproduction.

Regulatory Action of Corticosterone on Courtship Behaviors

The roughskin newt, *Taricha granulosa*, has been used to examine the neuroendocrine mechanisms regulating the physiological and behavioral responses to acute stress. Males respond to handling stress with a significant elevation in plasma corticosterone (CORT) titers and a decrease in courtship clasping behaviors (Moore and Miller, 1984). If the synthesis of CORT is inhibited with metyrapone, the stress- induced suppression of *Taricha* courtship is blocked (Moore and Miller, 1984). Administration of CORT rapidly and strongly inhibits male courtship behaviors, specifically inhibiting amplectic clasping behaviors (Moore and Miller, 1984; Orchinik, Murray, and Moore, 1991). Corticosterone administration also rapidly suppresses activity in medullary neurons that respond to clasp-triggering cloacal stimulation (Evans, Moore, and Murray, 1998; Rose, Kinnaird, and Moore, 1995; Rose, Moore, and Orchinik, 1993). Thus, stress-induced suppression of *Taricha* clasping is due to the rapid action of CORT.

The stress-induced physiological and behavioral responses exhibited by *Taricha* are also observed in many other vertebrates. Acute stress is known to inhibit courtship behaviors in many vertebrates, such as decreased territorial marking and ultrasonic courtship vocalizations of male mice (Lumley, Sipos, Charles, Charles, and Meyerhoff, 1999), suppressed estrus in female mice (Marchlewska-Koj, Pochron, Galewicz-Sojecka, and Galas, 1994), decreased lordosis behavior in rats (Hulse and Coleman, 1983), and suppressed primate sexual behaviors (Habib, Weld, Rice, Pushkas, Champoux, Listwak, Webster, Atkinson, Schulkin, Contoreggi, Chrousos, McCann, Suomi, Higley, and Gold,

2000). Furthermore, acute stress has also been shown to rapidly elevate CORT levels in a variety of vertebrates. For example, birds (Heiblum, Arnon, Gvaryahu, Robinzon, and Snapir, 2000), rats (Graessler, Kvetnansky, Jezova, Dobrakovova, and Van Loon, 1989), frogs (Coddington and Cree, 1995; Licht, McCreery, Barnes, and Pang, 1983), and reptiles (Moore, Green, and Mason, 2001; Moore, Thompson, and Marler, 1991) respond to an acute threat with measurable increases in plasma CORT titers. Correlatively, high plasma corticosteroid concentrations have been shown to modify behavioral responses of birds (Breuner, Greenberg, and Wingfield, 1998; Silverin, 1986; Wingfield and Silverin, 1986), rats (Haller, Halasz, Makara, and Kruk, 1998; Sandi, Venero, and Guaza, 1996), hamsters (Hayden-Hixson and Ferris, 1991) and shrews (Schiml and Rissman, 1999).

Regulatory Action of GABA on Courtship Behaviors

There is evidence to suggest that the suppressive action of CORT on reproductive behaviors of *Taricha* involves the neurotransmitter γ-aminobutyric acid, GABA. This neurotransmitter functions primarily as an inhibitory neurotransmitter, binding to GABA receptors on the post-synaptic membranes of target neurons. Binding of the $GABA_A$ agonist, muscimol, has been shown to inhibit clasping behavior of *Taricha* (Boyd and Moore, 1990). Conversely, the administration of a $GABA_A$ antagonist, bicuculline, blocks the inhibitory action of CORT on clasping behaviors or *Taricha* (Boyd and Moore, 1990), which indicates that CORT functionally inhibits clasping behaviors by upregulating GABA signaling.

Sexual behaviors of other vertebrates have also been shown to be regulated by activity of $GABA_A$ receptors. For example, female preference for the odors of male mice was inhibited by CORT and this action of CORT was blocked by treatment with a $GABA_A$ antagonist, bicuculline (Kevaliers & Ossenkopp, 2001). Administration of a GABA agonist in the Ventral Tegmental Area (VTA) of the female rat attenuates incidence of the sexual receptive posture lordosis. Male rabbit sexual behaviors are fully suppressed by $GABA_A$ (THIP) and significantly inhibited by $GABA_B$ (baclofen) agonists (Paredes *et al.*, 1998).

The inhibitory action of GABA can be modulated either postsynaptically by drugs or steroids that allosterically interact with the GABA receptors or presynaptically by retrograde signal molecules that reduce the likelihood of GABA release. A novel class of retrograde signal molecules that function to regulate GABA release has recently been discovered: endogenous cannabinoids, termed "endocannabinoids" (Kreitzer and Regehr,

2001a; Wilson and Nicoll, 2001). These endocannabinoids are synthesized and released by non-vesicular means upon demand and travel in a retrograde fashion to bind to cannabinoid receptors located on presynaptic terminals. Activation of the cannabinoid receptors results in decreased excitability of the presynaptic neuron, reduced GABA release, and therefore removal of inhibitory input to the post-synaptic neuron (for review see: (Wilson and Nicoll, 2002). Given that GABA is a component of the signaling pathway initiated by CORT in *Taricha*, we were interested to determine whether cannabinoids are an integral component of stress-induced inhibition of clasping.

Regulatory Action of Vasotocin on Courtship Behaviors

Another hormone that regulates clasping behaviors in *Taricha* is vasotocin (VT), the non-mammalian homologue of vasopressin (VP). In *Taricha*, administration of VT increases the incidence of male clasping behaviors; whereas, administration of VT antagonists suppresses these behaviors (Moore and Miller, 1983; Moore and Zoeller, 1979). Behavioral studies have found that VT administration enhances appetitive responses of males to visual and olfactory sexual stimuli (Thompson and Moore, 2000). Consistent with these behavioral effects, VT administration enhances the firing rates of medullary neurons that respond to clasp-generating tactile stimulation (Rose, Marrs, and Moore, 1998; Rose et al., 1993).

VT/VP has been shown to affect social behaviors in many vertebrates (for review of systems see: (Goodson and Bass, 2001); mammals: (Young, Lim, Gingrich, and Insel, 2001); Birds: (Panzica, Aste, Castagna, Viglietti-Panzica, and Balthazart, 2001); teleosts: (Bass and Grober, 2001); amphibians; (Deviche and Moore, 1987; Emerson and Boyd, 1999; Iwata, Toyoda, Yamamoto, and Kikuyama, 2000; Moore, Wood, and Boyd, 1992)).

Vasotocin Interacts with Corticosterone to Regulate Sensorimotor Processing in Taricha

Based on prior behavioral and electrophysiological studies in *Taricha*, administration of CORT inhibits and VT enhances clasping behavior and medullary neuron sensory responsiveness. However, electrophysiology studies reveal an interesting and complex interaction between CORT and VT (Rose et al., 1995). The administration of CORT alone typically decreases the responses by medullary neurons to cloacal stimulation; whereas, VT typically enhances the responses of the same group of sensory-responsive neurons. However, if VT is administered prior to CORT, then the CORT-

induced suppression of firing by medullary neurons is blocked. Thus, pre-treatment with VT appears to reverse the effects of CORT on neuronal activity. The question remained, what is the nature of this interaction?

The temporal framework within which CORT exerts its effects are robust and highly predictable; onset of the CORT effect on neuronal activity and amplectic clasping behavior occurs in a time window of 2-5 min following injection, and continues for 40-60 min (Rose, Moore, and Orchinik, 1993). This temporal pattern of CORT effect suggests to us that there is additional neurotransmitter signaling downstream in the sequence of events initiated by CORT. The temporal pattern for the inhibitory effects of CORT in *Taricha* match the onset and duration of endocannabinoid retrograde signaling effects in rats discovered through slice preparations of hippocampus (Wilson and Nicoll, 2001), corticostriatum (Ronesi, Gerdeman, and Lovinger, 2004), hypothalamus (Di *et al.*, 2003), and cerebellum (Kreitzer and Regehr, 2001).

The Endogenous Cannabinoid System

Only recently has evidence supporting the existence of an endocannabinoid system come to light. First, endogenously synthesized compounds with cannabimimetic properties have been discovered in animal tissues, anandamide and 2-arachidonylglycerol (2-AG). And second, cannabimimetic drugs and endogenous compounds bind with high affinity to selective membrane receptors (Elphick and Egertova, 2001). Until these two discoveries, the salient paradigm explaining why *Cannabis sativa* preparations (hashish, marijuana, bhang) exerted their effects was primarily due to disruption of membrane integrity and thereby altering neuronal and cellular function. This hypothesis had merit since the active ingredient of *Cannabis*, Δ^9-tetrahydrocannabinol (THC), is a small lipophilic compound that could insert into the membrane in much the same manner as cholesterol, thereby changing membrane fluidity, and therefore membrane properties. However, this hypothesis is now discarded in light of discovery of endogenous receptors and signaling compounds.

Since the signaling compounds anandamide and 2-AG were discovered in brain tissue several other cannabimimetic compounds have been discovered to date, including palmitoyl-ethanolamide (Priller, Briley, Mansouri, Devane, Mackie, and Felder, 1995), oleamide (Bisogno, Sepe, De Petrocellis, Mechoulam, and Di Marzo, 1997), docosatetraenoyl-ethanolamide, and do-homo-γ-linolenoyl-ethanolamide (for review see; (Felder and Glass, 1998). These fatty-acid derivative compounds bear little resemblance

to THC beyond being small lipophilic compounds, and are not stored, as classical neurotransmitters are, rather they are synthesized and released by neurons in response to depolarization and consequent Ca^{2+} influx (Felder, Joyce, Briley, Mansouri, Mackie, Blond, Lai, Ma, and Mitchell, 1995). The sub-cellular site of cannabinoid production is not yet known as the biosynthetic enzymes are still being characterized (Cadas, di Tomaso, and Piomelli, 1997; Desarnaud, Cadas, and Piomelli, 1995; Di Marzo, Fontana, Cadas, Schinelli, Cimino, Schwartz, and Piomelli, 1994). What is known about the biosynthetic process of these *N*-acyl-ethanolamines is that anandamide is synthesized de novo from arachidonic acid and ethanolamide (Cadas et al., 1997; Desarnaud et al., 1995; Di Marzo et al., 1994). Or, both anandamide and 2-AG can be synthesized from remodeling of a novel membrane-derived phospholipid, di-arachidonoyl-phosphatidylcholine (*N*-ArPC) (Cadas et al., 1997). Anandamide production is Phospholipase D sensitive and 2-AG synthesis is phospholipase C sensitive and can be synthesized from diacylglycerol (DAG) (Cadas et al., 1997).

The first receptor isolated, CB_1 receptor, is now known to be distributed throughout the central and parts of the peripheral nervous system with a greater abundance than most other known G-protein coupled receptors (Sanudo-Pena, Strangman, Mackie, Walker, and Tsou, 1999; Tsou, Brown, Sanudo-Pena, Mackie, and Walker, 1998). A second cannabinoid receptor, CB_2, has been cloned and is primarily distributed in the periphery, and has been implicated in the effects of cannabinoids on immune function (Galiegue, Mary, Marchand, Dussossoy, Carriere, Carayon, Bouaboula, Shire, Le Fur, and Casellas, 1995). There is also accumulating evidence that there is a third cannabinoid binding site (CB_3) in mammalian brains, but not amphibian brains, since brains of $CB_1^{-/-}$ knock-out mice still show significant although reduced levels of binding of the synthetic cannabinoid agonist, (^{3}H)WIN 55212-2 (Breivogel, Griffin, Di Marzo, and Martin, 2001).

The sequence and secondary structure of the CB_1 and CB_2 cannabinoid receptors is consistent with all other G-protein coupled receptors, comprising of seven hydrophobic transmembrane domains (Felder and Glass, 1998; Felder et al., 1995). Cultured neuroblastoma cells and brain tissue respond to cannabimimetics by inhibition of cAMP accumulation. This activity is blocked by pertussis toxin (PTX) , indicating that cannabinoid receptors are coupled to $G_{i/o}$ subtype of G-proteins. Cannabinoids can also enhance K^+-currents via G-protein mediated mechanisms that are also PTX-sensitive. Another cellular effect of cannabimimetics is to inhibit both *N*- and *Q*-type Ca^{2+} currents. This inhibition is via direct coupling of receptor-activated G_o-proteins with ion channels,

and is also PTX sensitive. Direct evidence of G-protein coupling with cannabinoid receptors has been determined by agonist-stimulated (^{35}S)GTPγS binding to slide-mounted sections and isolated brain membranes. This non-hydrolysable analog of GTP indicates successful coupling of a receptor to its activated G-protein and is considered so sensitive that it is commonly used to determine agonist efficacy for the cannabinoid receptor (Felder et al., 1995)

The activation of cannabinoid receptors and their G-proteins functionally results in decreased probability of neurotransmitter release from presynaptically located vesicles. However, it was not until recently that the exact synaptic mechanism whereby cannabinoids achieve this effect was elucidated (Wilson and Nicoll, 2001). More than a decade ago, studies in hippocampal pyramidal cells (Pitler and Alger, 1992; Pitler and Alger, 1994) observed that brief depolarization of these neurons could transiently suppress inhibitory input by GABAergic synaptic events. Since this sequence of events requires depolarization of the post-synaptic neuron and this leads to functional disinhibition, the authors termed this sequence of events Depolarization-induced Suppression of Inhibition (DSI). At the time these authors could not explain how the post-synaptic neurons were coordinating this disinhibition, however they hypothesized the existence of a retrograde signal which would be released by the postsynaptic neuron, travel backwards across the synapse to suppress release of the neurotransmitter from axons of the presynaptic neuron. Wilson and Nicoll (2001) determined that depolarization of hippocampal pyramidal cells results in the release of endocannabinoids which then bind to pre-synaptic CB_1 receptors on GABAergic terminals, decreasing inhibitory input by those GABAergic synapses.

Since the report by Wilson and Nicoll (2001), a number of labs have shown that cannabinoids function to decrease GABAergic inhibitory input at a number of different sites in the vertebrate brain, for example, cerebellum (Kreitzer and Regehr, 2001a), amygdala (Marsicano, Wotjak, Azad, Bisogno, Rammes, Cascio, Hermann, Tang, Hofmann, Zieglgansberger, Di Marzo, and Lutz, 2002b) and hypothalamus Di et al. (2003). Consistent with this concept, a number of groups have co-localized CB_1 receptor to the terminals of GABAergic neurons (Cesa, Mackie, Beltramo, and Franzoni, 2001; Coutts, Anavi-Goffer, Ross, MacEwan, Mackie, Pertwee, and Irving, 2001; Hoffman, Riegel, and Lupica, 2003; Katona, Rancz, Acsady, Ledent, Mackie, Hajos, and Freund, 2001; Salio, Cottone, Conrath, and Franzoni, 2002; Tsou et al., 1998).

Evidence is also growing in support of the hypothesis that endocannabinoids might also function as a retrograde regulator of Glutamatergic function (Cesa et al., 2001;

Di, Malcher-Lopes, Halmos, and Tasker, 2003; Hoffman et al., 2003; Kreitzer and Regehr, 2001a; Robbe, Alonso, Duchamp, Bockaert, and Manzoni, 2001). While in the hippocampus it was determined that cannabinoids only functioned to affect GABAergic signaling, in the cerebellum GABAergic and glutamatergic synapses are affected by cannabinoids. Glutamatergic synapses are involved in a process called DSE, Depolarization-induced Suppression of Excitation, analogous to DSI. Recent studies have shown that CB_1 antagonists blocked cerebellar DSI and DSE, while CB_1 agonists mimicked and suppressed these effects (Kreitzer and Regehr, 2001b; Ohno-Shosaku, Tsubokawa, Mizushima, Yoneda, Zimmer, and Kano, 2002). Furthermore, cannabinoid suppression of vesicular release of neurotransmitter is Ca^{2+}- dependent. These studies are consistent with the hypothesis that endocannabinoids function in a retrograde fashion to decrease the probability of synaptic release of neurotransmitters from presynaptic axon terminals during DSI and DSE. However, cannabinoids do not just function to regulate DSE or DSI.

Endocannabinoid synthesis can also be triggered by activation of group I metabotropic glutamate receptors (mGluRs). Metabotropic GluRs are located almost exclusively on postsynaptic membranes and activation of these mGluRs results in suppression of neurotransmitter release from presynaptic neurons (Varma, Carlson, Ledent, and Alger, 2001). CB_1 antagonists block the mGluR mediated suppression of presynaptic release (Varma et al., 2001). Furthermore, in contrast to DSE and DSI, this suppression of presynaptic input is not mediated by Ca^{2+}. These results are consistent with the hypothesis that there are multiple mechanisms that ultimately activate retrograde cannabinoid signaling.

Functional Effects of Endocannabinoids on Nociceptive Pathways

Endocannabinoids have been implicated in the functional regulation of a number of brain systems. Cannabinoid action at the hippocampus and neocortex is believed to regulate memory and learning; action at basal ganglia and cerebellum regulates motor coordination; action in the hindbrain and spinal cord regulating analgesia. Analgesic properties of *Cannabis sativa,* for instance, have been utilized since ancient times (Iversen, 2000), and endocannabinoids are now thought to participate in the endogenous analgesic system. One of the primary sites of action for cannabinoid nociceptive function is the brainstem and spinal cord.

Although receptors on primary nociceptive afferents probably play a role in cannabinoid mediated analgesia, the most important mechanism seems to be modulation of descending inhibitory inputs from the brainstem to spinal nociceptive neurons. This brainstem circuit has been determined primarily in the rat and comprises the rostral ventral medulla (RVM) and the midbrain periaquaductal gray (PAG), both of which contain CB_1 receptor immunoreactivity (Sanudo-Pena et al., 1999; Tsou et al., 1998). Stimulation of either area results in analgesia (Cui, Feng, McAdoo, and Willis, 1999; Tavares and Lima, 2002), as does microinjection of CB_1 agonists (Lichtman, Cook, and Martin, 1996). In contrast, administration of CB_1 antagonists cause hyperalgesia (Cui et al., 1999), implying that tonic release of cannabinoids regulates nociception via CB_1 receptor. Recent in vitro work has shown that cannabinoids function to disinhibit descending spinal pathways by reducing GABAergic release from presynaptic terminals of local interneurons in the RVM (Jennings, Vaughan, and Christie, 2001) and PAG (Vaughan, Connor, Bagley, and Christie, 2000). As is observed in hippocampus and cerebellar signaling, CB_1 action appears to be entirely presynaptic. Taken together these results suggest that cannabinoids function at brainstem sites to regulate nociception by disinhibiting PAG and RVM. Given the diversity of functions that RVM serves, it seems reasonable to predict that cannabinoids might act here to also functionally alter the circuits regulating clasping behavior.

Endogenous Cannabinoid System regulation of Hypothalamic-Pituitary-Adrenal Axis

The cannabinoid system has also been shown to interact directly with HPA activity. Administration of CB_1 agonists to rats' results in increased HPA activity (Gonzalez, Bisogno, Wenger, Manzanares, Milone, Berrendero, Di Marzo, Ramos, and Fernandez-Ruiz, 2000; Manzanares, Corchero, and Fuentes, 1999), and the pretreatment with a specific CB_1 antagonist, SR141716A, blocks these cannabinoid effects (Manzanares et al., 1999). Administration of high concentrations of THC to rats stimulates ACTH release within 20 min and CORT secretion within 30-60 min in rats (Puder, Weidenfeld, Chowers, Nir, Conforti, and Siegel, 1982; Zuardi, Teixeira, and Karniol, 1984). The stimulation of CORT release by THC does not occur in hypophysectomized rats (Puder et al., 1982). Furthermore, direct ICV administration of THC results in increased ACTH release from the pituitary (Manzanares et al., 1999). Taken together these findings suggest that the cannabinoids are most likely functioning to increase HPA activity by increasing CRH release from the hypothalamus which in turn

up-regulates the release of ACTH from the pituitary. The timeframe of this cannabinoid effect is considered long: 20-60 min.

The timeframe within which CORT affects courtship clasping behaviors of *Taricha* is considered rapid: onset of effect within 2 min and lasting for 20-40 min. Rapid actions of CORT and the synthetic glucocorticoid dexamethasone (s – min) have recently been documented using whole cell recordings in slice preparations of the hypothalamus from rat brains (Di et al., 2003). Binding of glucocorticoids to membrane binding sites on parvocellular neurosecretory cells in the paraventricular nucleus (PVN) increases the synthesis and release of endocannabinoids from these cells. The newly released endocannabinoids then travel in a retrograde direction to bind to CB_1 receptors on presynaptic glutamate terminals, leading to inhibition of glutamate release onto the PVN neuron. Ultimately, the glucocorticoid-induced increase in endocannabinoid activity leads to decreased PVN neuronal activity and hormone secretion. Given that CORT-induced suppression of clasping also occurs within a rapid time frame it seems likely that CORT maybe regulating clasping via upregulation of endocannabinoid signaling.

Endogenous Cannabinoid System regulation of Hypothalamic-Pituitary-Gonad Axis

Earlier studies determined that chronic administration of cannabinoids had detrimental effects on the endocrine physiology of reproduction. Most evidence supported the idea that the cannabimimetics mediated this effect indirectly by action on sites in the central nervous system, primarily the medial basal hypothalamus (de Miguel, Romero, Munoz, Garcia-Gil, Gonzalez, Villanua, Makriyannis, Ramos, and Fernandez-Ruiz, 1998). Although it is the general consensus that the primary site of cannabinoid action on reproductive hormone secretion is most likely at the level of the central nervous system, direct effects of cannabis and cannabimimetics on gonads have also been reported. THC and cannabinoid agonists have been shown to inhibit steroidogenesis of cultured follicular cells (Reich, Laufer, Lewysohn, Cordova, Ayalon, and Tsafriri, 1982; Treinen, Sneeden, and Heindel, 1993; Zoller, 1985; Zoller, Quealy Buono, Carr, Ninke, and Vegso, 1987), granulosa cells (Adashi, Jones, and Hsueh, 1983; Lewysohn, Cordova, Nimrod, and Ayalon, 1984), and testes (Dalterio, Badr, Bartke, and Mayfield, 1982; Dalterio and Bartke, 1979; Dalterio, Bartke, and Burstein, 1977). THC-induced inhibition of testosterone synthesis has also been observed as reductions in plasma testosterone concentrations (Dalterio et al., 1977), suppression of spermatogenesis, and reduction in testis weight (Dixit, Gupta, and Agrawal, 1977; Harmon, Locke, Aliapoulios, and

MacIndoe, 1976; Patra and Wadsworth, 1990; Patra and Wadsworth, 1991). Furthermore, studies on testicular cultures revealed a biphasic response to cannabinoids, low doses being stimulatory, while high doses were inhibitory (Dalterio, Bartke, and Mayfield, 1983). Taken together, these studies show that chronic exposure to cannabinoids interferes with endocrine physiology of reproduction by directly interfering with steroidogenesis at the gonad and indirectly interfering with releasing and tropic hormone production at the hypothalamus. These mechanisms of long-term effects of administered cannabinoids are of importance within the context of assessing human-related reproduction problems associated with chronic drug use. However, these mechanisms are insufficient to explain the role of endocannabinoids in courtship behaviors, the mechanisms of which are occurring within much shorter and defined timeframe (s – min).

Endogenous Cannabinoid System regulation of Courtship Behaviors

In humans, marijuana has historically been considered an aphrodisiac, and has been self-reported in surveys of college age students to enhance the sexual experience (Bills and Duncan, 1991). Eighty-one percent (N=97) of marijuana users between the ages of 23-38, reported enhanced sexual pleasure (Halikas, Weller, Mose, 1982), yet, in this same study 39% of the males reported that the duration of intercourse was increased or variably increased. Rodent studies suggest that when the physical act of sexual performance is examined, exogenous cannabinoids consistently increase latencies to mount, intromit, and ejaculate (Corcoran et al, 1974; Merari et al., 1973; Murphy et al., 1994). Consistent with these studies, Soderstrom et al. (2000) observed a dose dependent inhibition of *Taricha* courtship clasping behaviors in response to levonantradol, a cannabinoid agonist. The mechanism by which cannabinoids affect sexual performance of vertebrates has yet to be identified.

Interpretation of cannabinoid effects on courtship behaviors is often compounded by cannabinoids general sedative effects observable as suppressed locomotor activity. Many studies observing an inhibition of courtship behaviors also observe a concurrent decrease in locomotor behaviors. For example, the courtship clasping behaviors of male *Taricha* were inhibited by levonantradol; however, the locomotion of these males was also inhibited by 50% (Soderstrom *et al.*, 2000).

***Taricha* CB_1 Receptor Sequence and Pharmacology**

We have confidence that synthetic ligands used to examine cannabinoid effects on clasping or stress responses in *Taricha* are binding to specific receptors. The *Taricha* CB_1 receptor has been cloned, sequenced, and expressed (Soderstrom, Leid, Moore, and Murray, 2000). The predicted amino acid sequence of the *Taricha* CB_1 receptor has a high degree of similarity when compared with other species; *Taricha* shares 84% sequence identity with rat, mice and human CB_1 receptors. Furthermore, several functional features of the *Taricha* CB_1 receptor sequence are shared with other species: a) Dual sites for N-linked glycosylation in N-terminal extracellular domain, b) potential PKC posphorylation sites, c) Lysine in transmembrane domain III important for interaction with bicyclic classes of cannabinoid agonists, d) leucine and alanine pair in C-terminus of the third intracellular loop which has been implicated in G_s interaction. The *Taricha* CB_1 receptor was stably expressed in a CHO cell line and functionally detected using adenylate cyclase assays. Treatment with the CB_1 agonist levonantradol resulted in a 21.5% decline in forskolin-stimulated (25 μM) adenylate cyclase activity. Radioligand binding studies using (^{3}H)CP-55940 identified a single binding site in brain membranes (K_D = 6.5 nM, B_{max}= 1,853 fmol/mg of protein) with a rank order affinity that was consistent with mammalian species: CP55940 (K_D = 3.8 nM) > levonantradol (13.0) > WIN55212-2 (25.7) >> anandamide (1,665) ≈ anandamide + 100 μM phenylmethylsulfonyl fluoride (2,398). Thus essential groundwork has been laid prior to the present study on behavioral and electrophysiological effects of cannabinoids.

Summary of Background

The following is a summary of the critical information highlighted from the previous Background section.

- Acute stress-induced suppression of *Taricha* clasping behavior is mediated by rapid actions of elevated plasma CORT.
- In *Taricha,* CORT suppresses clasping behavior by rapidly suppressing neuron activity in the rostroventral medulla and spinal cord.
- In *Taricha,* GABA antagonists block CORT-induced suppression of clasping; therefore GABA signaling is downstream of the events initiated by CORT.
- The neuropeptide VT enhances courtship clasping behaviors of male *Taricha,* in part, by enhancing appetitive responses to sexual visual and olfactory stimuli, as well as enhancing sensorimotor responses to sexual somatosensory stimuli.

- In *Taricha*, CORT blocks VT-induced enhancement of medullary neuron sensorimotor responses to clasp-generating somatosensory stimuli.
- The temporal pattern of CORT/VT interaction in *Taricha* neuronal activity matches the timing of endocannabinoid signaling effects in rodent neural systems (hippocampus, cerebellum, amygdala, striatum, and hypothalamus).
- In rodents and amphibians, CB_1 receptors consistently located on the nerve terminals of GABAergic neurons
- In rodents, endocannabinoids known to have an analgesic function at the level of the hindbrain and spinal cord, by disinhibiting nociceptive pathways from GABA input.
- In *Taricha,* administration of cannabinoid agonist suppresses courtship clasping.
- Functional CB_1 receptors are expressed in the central nervous system, including the rostroventral medulla, of *Taricha.*

THE PROBLEM

When confronted with an immediate threat, an animal's survival is contingent upon engaging the appropriate behavioral response. In nature, the appropriate behavioral response depends on the animal's current physiological and behavioral context. The neuroendocrine mechanisms that regulate context-dependent behaviors are, as yet, undescribed. In *Taricha*, evidence suggests that context-dependent behavioral responses to acute stress result from an interaction between CORT and VT, and that endocannabinoids are involved in mediating these context-dependent behavioral responses. This dissertation project investigated the nature of the CORT/VT interaction.

HYPOTHESES AND OBJECTIVES

The primary objective of this thesis was motivated by the observation that appropriate behavioral responses must be determined by current physiological and behavioral state or context, and that seasonal fluctuation in hormones was insufficient to account for the behavioral responses being made on a second-by-second or minute-by-minute basis. The major objective of the studies described herein, therefore, was to

characterize the neuroendocrine basis to context-dependent behavioral responses to acute stress.

The first hypothesis, the subject of Chapter 2, was that the behavioral experience of courtship and/or administration of VT modulate the context-dependent effect of CORT on clasping behavior. Prior studies performed with *Taricha*, consistently showed that CORT suppressed, while VT enhanced, clasping behavior and the activity of medullary neurons that control clasping. One electrophysiological study by Rose et al. (1995) reveals an intriguing interaction between this steroid and peptide, such that if VT was applied to the medulla prior to CORT, then the CORT-induced suppression of medullary neuron activity was blocked. Given that the behavioral effect of CORT or VT alone corresponds to the effect of these hormones on medullary neurons, we predicted that the interaction observed electrophysiologically should reveal a similar pattern behaviorally. Chapter 2 describes the behavioral studies performed to examine whether the effect of CORT on sex behavior of male *Taricha* depends on the behavioral context, and whether the mechanism for this effect might involve vasotocin VT.

The second hypothesis related to the specific sensory modality by which CORT affected courtship behavior. Prior research has shown that the VT-induced enhancement of courtship behaviors in male *Taricha* is in part due to VT enhancing the appetitive responses to stimuli that are processed by individual sensory modalities, visual, olfactory and somatosensory (Thompson and Moore, 2000). Furthermore, several studies have consistently shown that CORT suppresses neuronal and behavioral responses to somatosensory stimulation of the male cloacal area, the artificial stimulation of which models somatosensory events during courtship (Lewis and Rose, 2003; Rose et al., 1995; Rose et al., 1998). Therefore, Chapter 3 investigates whether CORT modifies appetitive responses of male *Taricha* to visual and/or olfactory sensory sexual stimuli.

The third hypothesis, expanding on the overall objective to determine the neuroendocrine nature of context-dependent behavioral responses, was to determine whether stress-induced suppression of courtship behavior requires signaling of endocannabinoids downstream in the temporal sequence of events initiated by CORT. This hypothesis was motivated by the observations that CORT-induced suppression of medullary and behavioral measures was blocked by the prior experience of clasping or administered VT and that this interaction effect had a strict temporal requirement. The timing of prior VT administration and the duration of subsequent VT and/or CORT effects suggested that another neurochemical was involved in the interaction. We proposed that a

likely neurochemical was the signaling of endocannabinoids. Chapter 4 reports behavioral and electrophysiological studies performed that determine that endocannabinoids are involved in transducing CORT effects in male *Taricha*.

As a corollary to the third hypothesis, I tested whether the behavioral and neural effect of VT is blocked by the administration of a cannabinoid agonist. This was a reasonable hypothesis because the previous set of experiments suggested that endocannabinoid signaling is indeed required downstream in the temporal events initiated by CORT, and the behavioral and electrophysiological studies consistently showed that prior treatment with CORT blocks the VT-induced enhancement of clasping. If endocannabinoid signaling is downstream of CORT, then it follows that administration of a cannabinoid agonist would block VT-induced enhancement of clasping. This hypothesis is addressed in Chapter 5.

SIGNIFICANCE

Together the studies in this thesis provide strong evidence that behavioral responses to acute stress are context-dependent, and context is transduced by fluctuations in endocrine signals, such as, in the case of *Taricha*, CORT and VT. Finally, this thesis provides the first documented evidence that signaling of endocannabinoids regulates the ultimate effect of CORT and VT upon behavior. The nature of CORT/VT interaction and the role of endocannabinoids are placed within the context of sensory input and behavioral output. This offers a unique perspective that has not been attempted before and is made possible by the use of the *Taricha* clasping behavior as a model. Recently a number of valid models have emerged examining retrograde signaling of endocannabinoids and their action at primary neurons of the hippocampal (Wilson and Nicoll, 2001), amygdala (Katona et al., 2001), and cerebellar (Kreitzer and Regehr, 2001a) formations. While these elegant models have revealed unique signaling qualities of endocannabinoids at the cellular level, they are too far removed from stimulus input and motor output to ask direct questions about behavioral effects of endocannabinoids.

There are a small number of excellent models examining endocannabinoid action at behavioral level – feeding (Gomez, Navarro, Ferrer, Trigo, Bilbao, Del Arco, Cippitelli, Nava, Piomelli, and Rodriguez de Fonseca, 2002; Rodriguez de Fonseca, Navarro, Gomez, Escuredo, Nava, Fu, Murillo-Rodriguez, Giuffrida, LoVerme, Gaetani, Kathuria,

Gall, and Piomelli, 2001), and extinction of fear memories (Marsicano, Wotjak, Azad, Bisogno, Rammes, Cascio, Hermann, Tang, Hofmann, Zieglgansberger, Di Marzo, and Lutz, 2002a). These models offer interesting analysis of endocannabinoid action on behaviors, but have not been linked to the neural components involved. In other words, there is ample evidence that supports the hypothesis that behaviors are regulated by endocannabinoid action; however, no direct link has yet been made between the cellular responses and changes in natural behaviors.

In order to approach this type of question, we require a model system that allows us to examine the role of endocannabinoids at each level of sensory integration and motor output. Such a model system for understanding the role of endocannabinoids and behavior will serve the same purpose as the giant squid axon for conduction properties of neurons, the Mauthner reticulospinal neuron for brain stem regulation of locomotion, and contemporary models such as hippocampal and striatial examination of LTP and LTD. Qualities that are required of an outstanding model system are: 1) neural components well characterized both anatomically and physiologically; 2) behavioral outputs that are a direct function of identifiable neural processing; 3) quantifiable sensory input that engage the same neural processes. Our model system, *Taricha granulosa*, meets all of the requirements of a model necessary to causally link behavior with nervous system.

For example, male *Taricha* exhibit an extremely robust and quantifiable sex behavior: the amplectic clasp. This clasping behavior is relatively simple and can be viewed as a stereotyped response that is triggered by somatosensory input, generated by spinal circuits, and modulated by descending input from rostromedial medullary neurons in the brainstem (for a review of this circuit see: Rose and Moore, 2002; Coddington n.d., Chapter 5). There are three basic levels to the regulation of clasping: 1) Primary sensory afferents which detect and relay somatosensory input, classically activated by tactile stimulation of the cloaca; 2) Non-reticulospinal interneurons (referred to as medullary neurons in this thesis) which process this information; 3) Reticulospinal neurons which also process and principally relay sensorimotor information back down the spinal cord to effector muscles of the limbs. We can identify each level anatomically and physiologically (Rose et al. 1995; 1998; and review 2002). We can monitor the primary sensory afferent response to somatosensory stimulation. We can monitor the interneuron and reticulospinal neuron sensorimotor response to sensory stimulation as well as motor output. And we have successfully used this system to begin to determine how neuromodulatory influences of vasotocin enhance sex behavior, and how the stress

steroid, corticosterone, non-genomically inhibits sex behavior. Thus our model system is ideal to ask questions about how endocannabinoids might influence behavioral circuits.

Current evidence supports the hypothesis that endocannabinoids function primarily as modulators of cellular processes, modulating the excitatory and inhibitory input to a primary output neuron. They do not work in isolation as a regular neurotransmitter, rather, endocannabinoids function by gain-setting the principle neuron for reception of excitatory or inhibitory input. The behavioral function of endocannabinoids can only be revealed when examined within the context of a well defined neuroendocrine system. In the *Taricha* model, VT enhances, while CORT inhibits clasping behavior. Their action converges onto the same neural system controlling clasping output, the rostromedial medullary neurons. We conceptualize the medullary circuits to function as coincidence detectors, the mode of output (to clasp or not to clasp) determined by the precise timing and composition of presynaptic input. Furthermore, we hypothesize that these coincidence detectors do not passively absorb input, rather, they must be actively modulating their own input. This concept is not without precedence, the phenomenon of LTP, LTD, DSI, and DSE all require the active participation of the principle neuron. In each case the principle neuron acts as a coincidence detector, and rapidly regulates its own input by release of retrograde signals. In the *Taricha* model the principle neurons, rostroventral medullary neurons, receive a variety of stimulatory and inhibitory input, for example, from somatosensory sources (cloacal stimulation), and endocrine sources (VT or CORT). The precise temporal as well as spatial combination of these inputs results in the precise pattern of output by rostromedial medullary neurons, thereby regulating clasping behavior.

If rostromedial medullary neurons are truly behaving as coincidence detectors, we predict that these neurons might regulate their own input by use of a retrograde signal. The only retrograde signaling molecules that have been discovered to behave within a temporal framework of seconds to minutes are endocannabinoids. Therefore, we hypothesize that cannabinoids influence behavioral output by regulating the functional effects of sensory inputs, such as acute stress, and endocrine inputs, such as VT and CORT. We predict that endocannabinoids may be playing a pivotal role in transducing internal and external environmental stimuli onto neural systems that control the behavioral output of the sexual behavior of amplectic clasping.

CHAPTER 2: NEUROENDOCRINOLOGY OF CONTEXT-DEPENDENT STRESS RESPONSES: VASOTOCIN ALTERS THE EFFECT OF CORTICOSTERONE ON AMPHIBIAN BEHAVIORS

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ABSTRACT

The ability of an animal to respond with appropriate defensive behaviors when confronted with an immediate threat can affect its survival and reproductive success. The neuroendocrine regulation of alternative behavioral responses to a threatening or stressful situation is poorly understood. In the roughskin newt (*Taricha granulosa*), previous studies have shown that exogenous corticosterone (CORT) rapidly blocks, while vasotocin (VT) enhances, sex behavior (amplectic clasping behaviors). Electrophysiology studies have shown that the CORT-mediated inhibition of medullary neurons involved in the male *Taricha* sex behavior is eliminated by the prior administration of VT. To test whether VT modulates the context-dependent effect of CORT on courtship behavior of *Taricha*, we administered VT or Veh at 60 min, and CORT or Veh at 5 min before presentation of a female. Consistent with past studies, we found that administration of CORT alone inhibited clasping. However, VT pre-treatment consistently increased the incidence of clasping, even in males that were subsequently administered with CORT. Considering these results and the potential that performance of clasping behaviors themselves might cause an increase in endogenous VT, we tested whether performance of clasping for 60 min prior to administration of CORT altered the behavioral response to CORT. Results show that males performing clasping prior to CORT administration all continued to clasp. In contrast, CORT administration before females were introduced consistently inhibited clasping behavior. Our results suggest that the neuroendocrine state currently experienced by an animal will alter the processing and the final behavioral response to CORT or an acute threat.

INTRODUCTION

An animal's survival and reproductive success relies on its ability to respond appropriately when confronted with an immediate threat. Most wild animals have a large repertoire of defensive behaviors that are used in response to a perceived threat. Which of the alternative behaviors is expressed depends on the animals' current situation. For example, an adult Killdeer without young will respond defensively by flying away from a potential predator; whereas a nesting Killdeer with young is more likely to present broken-wing displays and attract the attentions of a potential predator away from the nest (Ehrlich, Dobkin, and Wheye, 1988). A lone male newt is more likely to respond defensively by actively swimming away from an approaching person, than is a male newt that is engaged in amplectic clasping with a female (pers. Obs.). Thus, appropriate behavioral responses to threatening situations depend on the animals' context – its surrounding environment and its physiological and behavioral states. The corresponding neuroendocrine mechanisms underlying these context-dependent behavioral responses are poorly understood.

The roughskin newt, *Taricha granulosa*, has been used to examine the neuroendocrine mechanisms regulating the physiological and behavioral responses to threats, regularly referred to as stress responses. In this amphibian, males respond to handling stress with a significant elevation in plasma corticosterone (CORT) titers and a decrease in courtship behaviors (Moore and Miller, 1984). Administration of CORT rapidly and strongly inhibits male courtship behaviors, specifically inhibiting amplectic clasping behaviors (Moore and Miller, 1984; Orchinik et al., 1991). Corticosterone administration also rapidly suppresses activity in medullary neurons that respond to clasp-triggering cloacal stimulation Rose (Rose et al., 1995; Rose et al., 1998; Rose et al., 1993). If the synthesis of CORT is inhibited with the administration of metyrapone the behavioral response to stress, inhibition of *Taricha* courtship, is blocked (Moore and Miller, 1984). Thus, the stress-induced inhibition of clasping in *Taricha* is due to the rapid action of CORT.

The physiological and behavioral responses exhibited by *Taricha* are also observed in many other vertebrates. Acute stress is known to inhibit courtship behaviors in many vertebrates, for example, decreased territorial marking and ultrasonic courtship vocalizations of male mice (Lumley et al., 1999), suppressed estrus in female mice (Marchlewska-Koj et al., 1994), decreased lordosis behavior in rats (Hulse and Coleman, 1983), and suppressed primate sexual behaviors (Habib et al., 2000). Furthermore, acute

stress has also been shown to very rapidly elevate CORT levels in a variety of vertebrates. For example, birds (Heiblum et al., 2000), rats (Graessler et al., 1989), frogs (Coddington and Cree, 1995; Licht et al., 1983), and reptiles (Moore et al., 1991) respond to an acute threat with measurable increases in plasma CORT titers. High plasma Corticosteroid concentrations have been shown to modify behavioral responses of birds (Breuner et al., 1998; Silverin, 1986; Wingfield and Silverin, 1986), rats (Haller et al., 1998; Sandi et al., 1996), hamsters (Hayden-Hixson and Ferris, 1991)and shrews (Schiml and Rissman, 1999). While, exogenous CORT has been shown to rapidly inhibit the female's preferences for the odor of males within 10 min of administration (Kavaliers and Ossenkopp, 2001).

Another hormone that regulates clasping behaviors in *Taricha* is vasotocin (VT), the non-mammalian homologue of vasopressin. This hormone has been shown to affect social behaviors in many vertebrates. For review of systems: (Goodson and Bass, 2001); mammals: (Young et al., 2001); birds (Panzica et al., 2001); teleosts (Bass and Grober, 2001); amphibians (Emerson and Boyd, 1999; Iwata et al., 2000; Moore, 1992; Moore et al., 1992). In *Taricha*, administration of VT agonists increases the incidence of male clasping behaviors; whereas, administration of VT antagonists suppresses these behaviors (Moore and Miller, 1983; Moore and Zoeller, 1979). Behavioral studies found that VT administration enhances appetitive responses of males to visual and olfactory sexual stimuli (Thompson and Moore, 2000). Consistent with these behavioral effects, VT administration enhances the firing rates of medullary neurons that respond to clasp-generating tactile stimulation (Lewis and Rose, 2002; Rose et al., 1995).

These previous studies clearly show that an injection of CORT inhibits and VT enhances amplectic-clasping behaviors. Electrophysiology studies have revealed an interesting and complex interaction between these two hormones (Rose et al., 1995). The administration of CORT alone typically decreases the responses by medullary neurons to cloacal stimulation; whereas, VT typically enhances the responses of the same group of sensory-responsive neurons. However, if VT is administered prior to CORT, then there is an overall potentiation of firing by medullary neurons in response to clasp-generating tactile stimulation. Thus, pre-treatment with VT appears to reverse the effects of CORT on neuronal activity.

This interaction between VT and CORT observed in the medulla suggested to us that these two hormones might interact in a similar manner to affect whole animal behavioral responses. Therefore, we hypothesized that in male *Taricha* pre-treatment with

VT will decrease the inhibitory effect of CORT administration on the clasping behaviors. Furthermore, considering that the performance of clasping behaviors might cause increases in endogenous VT activity, we also hypothesized that prior exposure to clasping would decrease the inhibitory effects of CORT of clasping behaviors.

Consistent with past studies, we found that administration of CORT alone inhibited clasping behaviors. However, VT pre-treatment or prior performance of clasping consistently increased the incidence of clasping, even in males that were subsequently administered with CORT. Thus, the stress hormone, CORT, can elicit different behaviors within an individual. The results of this study may add to our understanding of neuroendocrine regulation of context-dependent stress responses.

MATERIALS AND METHODS

Animals

Sexually active adult newts (*Taricha granulosa*) were collected locally during their breeding season (March, 2000) from permanent ponds in the Coastal Range of Benton Co., Oregon. Newts were housed in an environmentally controlled room with natural photoperiod (13L:11D) and temperature (average 13°C). Males collected from the perimeters of ponds using a dip-net were held together for 24 – 48 h in tanks of decholorinated water and fed each evening an excess mixture of red worms and beef liver. Males weighed, on average, 14 g. Females were captured during migrations to breeding ponds in pit-traps placed along drift-fences. They were housed individually for 24 -72 hrs in small containers with damp moss, leaf-litter, and an abundant supply of red worms and crickets. These collecting strategies allowed for capture of males in breeding condition and the capture of unmated females that are sexually attractive to males (Moore, 1978; Propper, 1989).

Behavioral Testing

Individual males were tested in circular tanks (27 cm diameter) filled to a depth of 6 cm with dechlorinated water. Testing tanks were kept in low-light conditions within a specified area (3m X 3m) enclosed by a curtain of black plastic hung from the ceiling to the floor. Males were transferred from holding tanks into testing tanks at least 30 min prior to any injections and behavioral testing. Behaviors were monitored and recorded using a low-light video camera. Incidence of clasping and time spent clasping were

determined from videotape. Incidence of clasping was analyzed as proportion of males observed clasping at 15, 30, and 60 min after a female was added to the arena.

Preliminary Experiment: Determination of CORT dose required to inhibit clasping

Because *Taricha's* sensitivity to CORT varies seasonally, a dose-response study was run to determine the appropriate dose of CORT to be used with *Taricha* during the breeding season. Of the different doses (0, 2, 11, 20, 30, or 40 μg CORT / 0.1 ml) the only dose of CORT that reliably inhibited male clasping behavior was 40 μg / 0.1 ml.

Injections

Newts were injected intraperitoneally (i.p.) with 40 μg CORT / 0.1 ml or 0.1 ml vehicle alone, and 100 μg VT / 0.1 ml or 0.1 ml vehicle alone. CORT and VT were purchased from Sigma-Aldrich Co. (St Louis, MO). CORT solution (40 μg / 0.1 ml) was prepared with 99.8% Amphibian Ringers and 0.2% DMSO (Dimethyl sulfoxide; Sigma-Aldrich, MO), and stored at 4 °C. Vehicle to control for CORT effect was prepared simultaneously with 99.8% Amphibian Ringers and 0.2% DMSO. VT solution (100 μg/0.1 ml) was prepared with Amphibian Ringers in silicone-coated glassware (Sigmacoat, Sigma-Aldrich, MO), divided into 1 ml aliquots, snap frozen in dry ice, and stored at -80°C until use. A vehicle control solution (Amphibian Ringers) was prepared simultaneously and stored in the same manner as VT stock.

Experiment 1: Vasotocin Pre-treatment

This experiment examined whether prior exposure to VT might modulate the effect of CORT on clasping behavior. Male newts were randomly assigned to one of four treatment groups. All four treatments were run simultaneously, with 4 males/treatment being assessed per block. A total of three blocks were run with N=12 for each treatment. The four treatments were: VT followed by CORT (VT/CORT), VT followed by vehicle (VT/veh), vehicle followed by CORT (veh/CORT), and vehicle followed by vehicle (veh/veh). Males were randomly assigned to test tanks at time 0. At 30 min all males received an i.p. injection of either VT or vehicle, and at 90 min all males received an i.p. injection of either CORT or vehicle. Five min after the second injection one female was placed in each tank, and behavioral observations began immediately and lasted for one hour.

Experiment 2: Sexual Experience Pre-treatment

This second experiment examined whether immediate previous sexual experience (the performance of amplectic clasping behavior) alters the effect of CORT on the incidence of amplectic clasping. A total of two blocks (Each containing 4 males randomly assigned to each treatment group) were run, giving a total group size of 8 males per treatment. The four treatments were: 1 hour of prior clasping experience before receiving CORT (CLASP/CORT), 1 hour of prior clasping experience before receiving vehicle (CLASP/veh), no prior clasping experience before receiving CORT (NO CLASP/CORT), and no prior clasping experience before receiving vehicle (NO CLASP/veh). All four treatments were run simultaneously, with 4 males/treatment being assessed per block. In order to avoid confounding problems with endogenous circadian rhythms of CORT, the treatments were organized such that all males received the injection of CORT (or vehicle) at the same time (at 90 min) and behaviors were observed during the same 30 min (95 – 125 min).

Statistical Analysis:

Non-parametric statistical tests were used as data from Experiment 1 and 2 were skewed and variances were heterogeneous. A significant difference among treatments in 'Time spent Clasping' was determined using Kruskal-Wallis (KW) non-parametric analysis of variance ($\alpha = 0.05$), and differences between pairs of treatment groups were determined using Dunn's Multiple Comparisons Test ($\alpha = 0.05$). Percentage of males in each treatment observed clasping at 15, 30, and 60 min were expressed as a proportion and then transformed using arcsine$\sqrt{}$, a standard transformation function applied to proportion data. This allowed us to compare within treatment (repeated measures from individuals at times 15, 30, and 60min) as well as between treatments using Friedman's non-parametric analysis of variance with repeated measures ($\alpha = 0.05$). If the non-parametric ANOVA's revealed a significant effect of treatment or time, then differences between pairs of treatment groups or time periods were determined using Mann-Whitney pair-wise comparisons ($\alpha = 0.05$).

RESULTS

Experiment 1: Vasotocin Pre-treatment

Pre-treatment with VT appears to counteract the suppressive effect of CORT administration on male clasping behaviors. Overall, treatment significantly influenced the time males spent clasping (Kruskal Wallis (KW) = 32.38, $p<0.0001$) (Figure 2.1a). Pair-wise analysis revealed that VT/CORT males spent approximately the same amount of time clasping as veh/veh males ($p > 0.05$), and a significantly longer amount of time clasping than veh/CORT males ($p<0.05$). VT/CORT males spent significantly less time clasping than VT/veh males ($p < 0.01$) (Figure 2.1a). There was a significant difference in the proportion of males clasping within all of the treatments over time (at 15, 30, 60 min)(Friedman's = 29.52, $p=0.0019$). Figure 2.1b shows that the proportion of VT/CORT males clasping increased over time (Friedman's = 6.0, $p = 0.0278$); whereas, proportion of veh/CORT males clasping decreased over time (Friedman's = 6.0, $p = 0.0278$). No changes in time spent clasping were observed in the other treatments (Figure 2.1).

Experiment 2: Sexual Experience Pre-treatment

Pre-treatment with immediate sexual experience also appears to counteract the suppressive effect of CORT administration on male clasping behavior (Figure 2.2). Treatment significantly influenced the time males spent clasping (KW = 19.74, $p=0.0002$). Pair-wise analysis revealed that males who had been clasping with a female for 1 hour prior to administration of CORT (CLASP/CORT) spent the same amount of time clasping as the vehicle-injected males (CLASP/veh, NO CLASP/veh) ($p>0.05$) (Fig 2.2). In contrast, NO CLASP/CORT males spent significantly less time clasping compared to all of the other treatment groups (NO CLASP/veh, CLASP/CORT, or CLASP/veh) ($p<0.05$). There were no differences in the proportion of males observed to be clasping across time within each treatment group (Friedman's = 19.2, $p > 0.05$) (Figure 2.2). NO CLASP/CORT treatment resulted in significantly lower proportions of males observed clasping at 15 min (KW = 6.857, $p< 0.0001$) and 60 min (KW= 6.237, $p= 0.0095$) compared to CLASP/CORT or CLASP/veh males. The percent of experienced males observed clasping after CORT injection was 100% at 15 min and 60 min after hormone administration (Fig 2.2). In contrast, the percentage of experienced males observed clasping after vehicle injection declined from 100% at 15 min to 60% at 60 min after hormone administration (Fig 2.2).

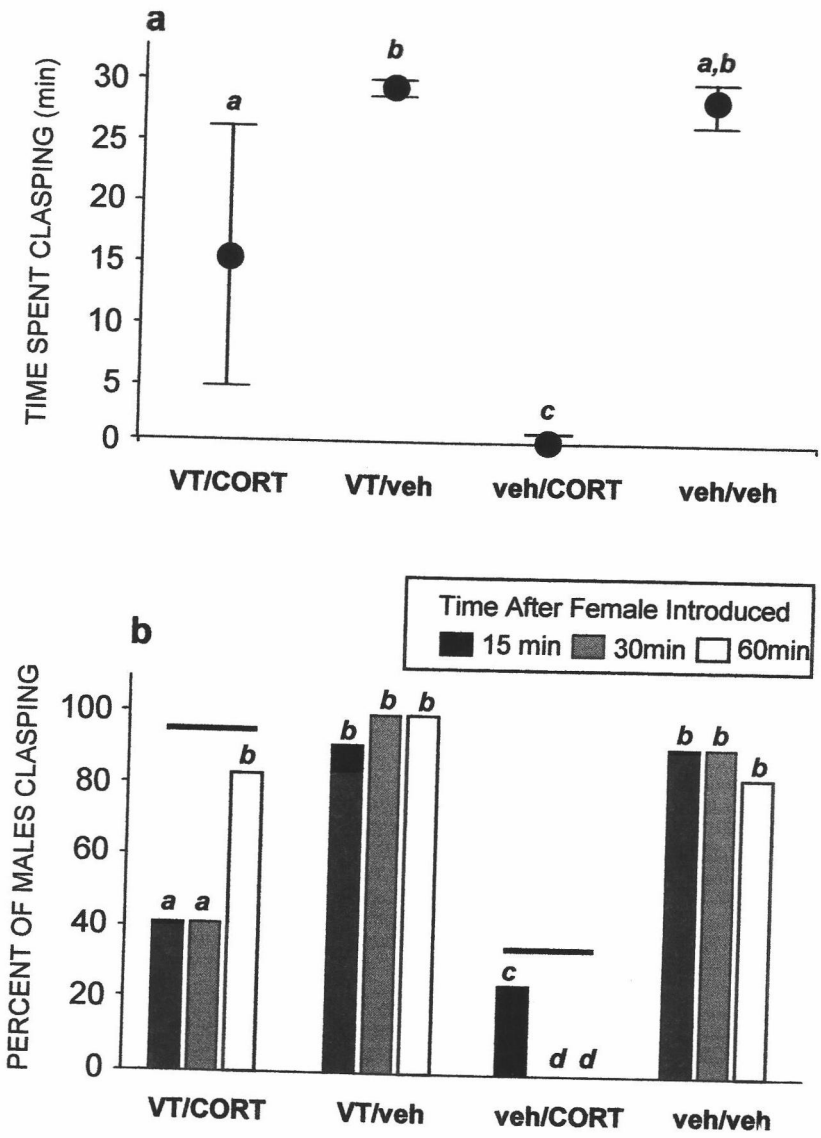

Figure 2.1. Proportion and Median time spent clasping by males after receiving pre-treatment and hormone treatment. Males were pre-treated with VT or vehicle, and then 1 hour later received an injection of CORT or vehicle. Thus, there were a total of 4 treatment groups assessed. **a.** Time spent clasping is presented as median ± 1st quartile for each treatment group. **b.** Proportion of males observed clasping at 15, 30, 60 min after the female is introduced. Bar's indicate significant difference between times within treatment group ($p < 0.05$). Different letters indicate significant differences between all treatments at each time observation ($p < 0.05$).

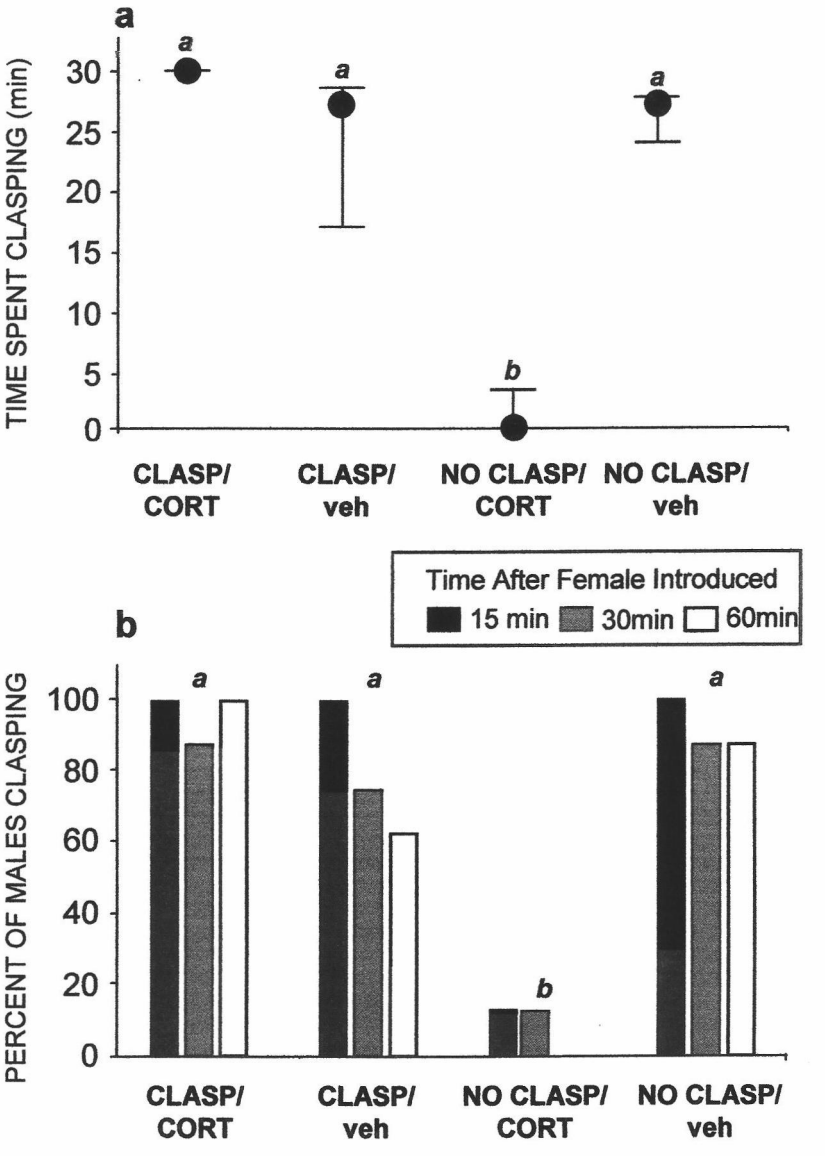

Figure 2.2. Proportion of males clasping and Time spent clasping after females were introduced. Males were pre-treated with clasping a female for 1 hour or no clasping, and were then treated with CORT or vehicle. Thus, there were a total of 4 treatment groups assessed. Time spent clasping is presented as median ± 1st quartile for each treatment group. **a.** Time spent clasping during 30 min after males received pre-treatment and hormone treatment. **b.** Percentage of males in each treatment group that were observed clasping at 15, 30, and 60 min after the female was introduced. Treatments with different letters indicate significant difference between treatments ($p<0.05$). There were no differences across time within any of the treatment groups.

DISCUSSION

Our study is the first to show that the behavioral or physiological state can modify the effects of CORT administration on sexual behavior. Administration of CORT alone will block male clasping behaviors, however, this effect of CORT can be modified by previous exposure to clasping or VT. That CORT administration can elicit a variety of behaviors within one individual is consistent with the observation that animals respond to potential threats with context-dependent behavioral responses. Our results suggest that one of the neuroendocrine mechanisms regulating context-dependent behavioral outcomes involve interactions between VT and CORT.

CORT Inhibits Clasping Behavior

Our study confirms that administration of CORT blocks male courtship clasping. Past research has shown that handling stress rapidly elevates endogenous CORT levels and concomitantly blocks courtship clasping behaviors of *Taricha* (Moore and Miller, 1984). Furthermore, administration of CORT alone robustly and rapidly decreases the incidence of male *Taricha* clasping (Moore and Miller, 1984; Orchinik et al., 1991). Electrophysiological studies indicate that tactile stimulation of the cloaca is necessary to initiate clasping and it appears that one function of CORT is to inhibit the medullary neurons responsive to cloacal tactile stimulation Rose (Rose et al., 1995; Rose et al., 1998). Thus, in *Taricha,* acute stress results in elevated CORT levels, which in turn influences sensorimotor coordination of clasping behaviors. Corticosterone-mediated behavioral responses to stress are a phenomenon observed throughout vertebrate taxa (see Introduction for discussion of literature).

VT Enhances Clasping Behavior

In contrast to the effects of CORT, administration of VT alone resulted in a higher incidence of *Taricha* clasping than any of the other treatment groups from Experiment I. These results are consistent with past studies carried out with *Taricha,* which have found that i.p. and intracereboventricular (icv) injections of VT increased the incidence of clasping (Moore and Miller, 1983). This effect appears to be specific since an injection of VT antagonist decreases incidence of clasping (Moore and Miller, 1983). More recent detailed behavioral studies have revealed that VT enhances appetitive responses to visual and olfactory sexual stimuli (Thompson and Moore, 2000).

The behavioral effects of VT in *Taricha* support the general hypotheses developed in mammals, that VT, and the mammalian homologue VP, functions to regulate social behaviors. VT and VP are known to enhance social behaviors such as scent marking in hamsters (Albers and Rawls, 1989), parental behavior in voles (Wang, Liu, Young, and Insel, 2000), courtship and sperm transfer in another salamander (Iwata et al., 2000), pair-bonding and mate choice in voles (Young et al., 2001), aggressive behaviors in humans (Coccaro, Kavoussi, Hauger, Cooper, and Ferris, 1998) and mice (Compaan, Buijs, Pool, De Ruiter, and Koolhaas, 1993), frog calling (Burmeister, Somes, and Wilczynski, 2001; Marler, Boyd, and Wilczynski, 1999; Marler, Chu, and Wilczynski, 1995), sexual advertisement vocalizations of a teleost (Goodson and Bass, 2000; Goodson and Bass, 2001), and territorial courtship behavior of a teleost (Semsar and Godwin, 2003; Semsar, Kandel, and Godwin, 2001). For reviews see Bass and Grober (2001), Goodson and Bass (2001a), Insel and Young (2001), Moore (1987), Moore (1992b), Young et al. (2001)

Pretreatment with VT Blocks the Suppressive Effects of CORT

In male *Taricha* pretreated with VT, CORT administration had no observable effects on male clasping behaviors. These results are noteworthy because, in the absence of VT pretreatment, CORT administration potently suppresses amplectic clasping behaviors in *Taricha*. We conducted Experiment 1 because Rose et al. (1995) found that in *Taricha* the suppressive effects of CORT administration on firing rates of medullary neurons can be blocked by pretreatment with VT. Therefore, our behavioral observations are consistent with electrophysiological data (Rose et al., 1995). These results, showing that a behaviorally active neuropeptide can override the inhibitory effects of a stress hormone on reproductive behaviors, have not been reported previously.

A recently published experiment with green treefrogs (*Hyla cinerea)* tested the effects of VT and CORT administration on calling behaviors (Burmeister et al., 2001). This experiment found that VT injections in treefrogs increased the probability of calling compared to vehicle-injected controls. But because control frogs had low levels of calling, any inhibitory effects of CORT administration on calling were not measurable. (As far as we know, there are no reports of frog calling being inhibited by acute CORT administration.). Nevertheless, this study with green treefrogs is significant because it found that an injection of CORT could block the stimulatory effects of VT on calling. The apparent discrepancy in the results with treefrogs and our results in Experiment 1 with *Taricha*, we suspect, relates to differences in the temporal relationship between the VT

and CORT injections. Treefrogs received concurrent injections of VT and CORT; whereas, *Taricha* received VT 60 min prior to the CORT injection. Based on the electrophysiological studies by Rose et al. (1995), if *Taricha* were given concurrent icv injections of VT and CORT, we would predict that the CORT injection would suppress the stimulatory effects of VT.

Prior Performance of Clasping Behaviors Block CORT Inhibition

In Experiment 2, the suppressive effects of CORT on amplectic clasping were completely blocked in male *Taricha* that were performing clasping behaviors prior to receiving CORT administration. Therefore in both experiments, the performance of clasping for 60min prior to CORT injection and receiving an injection of VT 60 min prior to CORT injection resulted in attenuating the suppressive effects of CORT on clasping behaviors. Our results suggest to us that the performance of clasping behaviors might stimulate the secretion of endogenous VT, and that this elevation in VT might counteract the suppressive effects of CORT on reproductive behaviors. The suggestion that the performance of a behavior might stimulate the VT system is consistent with the suggestion that the performance of mating behaviors by male Prairie voles activates the VP system (Young et al., 2001).

Context-Dependent Stress Responses

Results from Experiments 1 and 2 seem to be consistent with the natural history of *T. granulosa*. During the breeding season, single males in the near shore shallow waters typically will quickly retreat to deeper water in response to a potential predator; males that are engaged in amplexus typically do not disengage with the female to facilitate retreat. This is one example of where the behavioral state of the male, single versus in amplexus, influences the expression of defensive behaviors to a perceived threat. Many other examples are in the literature. The aggressive behavioral response of Mockingbirds to song playback varies according to breeding context (Logan, 1988). The predator avoidance response of voles to an owl call is either to freeze or flee, depending previous behavioral experiences Eilam (Eilam, Dayan, Shamgar, Schulman, Shefer, and Hendrie, 1999). The probability that Capuchin monkey will choose novel food items increases when in a social situation (Visalberghi and Fragaszy, 1995). Shyness and boldness of pumpkinseed fish are context specific behaviors and not individual personality traits

expressed across different situations (Coleman and Wilson, 1998). Thus animals from a variety of taxa express specific types of behavioral responses to perceived threats.

One explanation for the variations in defensive behavioral responses to a given treat is that stress hormones exert different behavioral effects depending on the animals' physiological or behavioral state. If true, then, we would expect to find experiments with variable effects of acute administration of CORT on reproductive behaviors. A few studies report that CORT administration rapidly suppresses reproductive behaviors, for example, in newts Moore (Moore and Miller, 1984) and female mice (Kavaliers and Ossenkopp, 2001). Many more studies suggest that high plasma CORT concentration can enhance reproductive behaviors. An injection of corticosteroids facilitates lordosis behavior of estrogen-primed rats (Kubli-Garfias, 1990) and display of sexual receptivity (tail-wagging) in female musk shrews (Schiml and Rissman, 1999), while blockade of corticosteroid synthesis in female musk shrews decreases reproductive behavior displays. A number of species also have high plasma corticosteroid levels coincident with the onset of courtship and mating behaviors including *Bufo marinus* (Orchinik, Licht, and Crews, 1988), *Triturus carnifex* (Zerani and Gobbetti, 1993), musk shrews (Schiml and Rissman, 1999), boars (Liptrap and Raeside, 1983) (Liptrap and Raeside, 1983) and rams (Borg, Esbenshade, and Johnson, 1991; Borg, Esbenshade, Johnson, Lunstra, and Ford, 1992). Perhaps a rise in corticosteroid levels in sexually primed animals can enhance reproductive behaviors.

The ability of an individual animal to respond with appropriate behaviors to a potential threat is essential to its survival and reproductive fitness. Animals have a repertoire of different behavioral responses and which one is expressed depends on the animals' environmental, physiological, and behavioral state. In this way, these are context-dependent behavioral responses. This study provides evidence, for the first time in any species, that a peptide that enhances reproductive behaviors (VT) can block the suppressive the effects of a stress hormone (CORT). This finding suggests to us that the neuroendocrine regulation of context-dependent behavioral responses to acute stress can be explained in part by the actions of VT on neuronal responses to corticosteroids.

ACKNOWLEDGEMENTS

Special thanks must go to the two students who were fellow intrepid newt catchers and helped run the behavioral tests; Jen Walters and Kristina Oaks.

CHAPTER 3: CORTICOSTERONE SUPPRESSES AMPHIBIAN COURTSHIP BEHAVIOR BY MODIFYING SPECIFIC SENSORIMOTOR PROCESSING PATHWAYS

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ABSTRACT

Stress steroids are known to have profound effects on behavior of all vertebrates. What is not understood is how these steroids alter behavioral patterns. Prior research has shown that courtship behaviors in male roughskin newts (*Taricha granulosa*) are suppressed by corticosterone (CORT). Furthermore, CORT is known to suppress neuronal and behavioral responses to somatosensory stimulation of the male's cloaca area, the artificial stimulation of which models somatosensory events during courtship. The question remains whether CORT suppresses courtship by causing changes in sensorimotor processing of visual or olfactory sensory processing. To address this question, we investigated the effects of CORT on the appetitive behavioral responses of male *Taricha* to specific olfactory and visual sexual stimuli. These experiments found that 100% of vehicle-injected controls engaged in courtship, spent significantly more time in close proximity to pheromone-scented pads than unscented pads ($P<0.0001$), and spent significantly more time in close proximity to clear glass beakers containing a female than to an empty beaker ($P=0.03$). Although CORT administration suppressed courtship in 90% of the males, CORT-injected newts were not significantly different from vehicle-injected newts for the measures of attraction to pheromone-scented pads ($P=0.51$) or to glass beakers containing a female newt ($P=0.56$). These experiments suggest that CORT does not suppress courtship behavior by acting on the sensorimotor processing of either olfactory or visual sexual stimuli. Therefore, the suppressive effects of CORT on newt courtship are best explained by CORT acting on the sensorimotor processing pathway associated with somatosensory sensory input and clasping motor output.

INTRODUCTION

Stress steroids are known to have profound effects on behavior of all vertebrates; the neural mechanisms by which these hormones control behavior are not well understood. An amphibian, the roughskin newt, *Taricha granulosa*, has been utilized to determine the mechanisms underlying hormone action on behaviors.

The behaviors most studied in *Taricha* are associated with courtship events. The courtship sequence performed by male *Taricha* involves a series of events that are guided by cues from the female. The female cues are detected by male *Taricha* using at least three different sensory modalities: visual, olfactory, somatosensory. A male must first locate a potential mate, which is accomplished by responding to moving objects in their visual field. In other species of salamanders, the visual stimulus of a female is an important sexual cue (Roth, 1987). Once a male *Taricha* has visually located a moving object, for the mating sequence to progress the male must assess the nature of the moving object: species, sex, sexual attractivity. Females are assessed using olfactory cues; in *Taricha* the female emits an attraction pheromone(s) (Thompson, Tokar, Pistohl, and Moore, 1999). A male responds to a sexually attractive female by embracing her dorsal aspect with hind and forelimbs, an embrace that can last for hours (Propper, 1991). At this stage in the sequence, mechanical pressure applied against the male's cloaca initiates the clasp reflex, and continued somatosensory stimuli will favor maintenance of the clasp. The clasp reflex is controlled by motor neurons and sensory afferents located in the spine. This reflex arc is regulated by descending influence from medullary neurons in the hindbrain, and hormones act at both sites, spinal and hindbrain, to influence clasping behavior (Lewis and Rose, 2003; Rose and Moore, 1999; Rose and Moore, 2002).

Electrophysiological and behavioral studies have revealed that the neural peptide vasotocin, VT, robustly enhances courtship clasping. Intra-cerebroventricular and intraperitoneal administration of VT increase the incidence of courtship clasping by males, and administration of VT antagonists suppresses these behaviors (Moore and Miller, 1983; Moore and Zoeller, 1979). VT-induced enhancement of behavior is also observed in animals from all vertebrate taxa examined, where VP or VT has been shown to affect social behaviors in many vertebrates. For review of systems: (Goodson and Bass, 2001); amphibians (Emerson and Boyd, 1999; Iwata et al., 2000; Moore, 1992; Moore et al., 1992); teleosts (Bass and Grober, 2001); birds (Panzica et al., 2001); and mammals: (Young et al., 2001).

In *Taricha*, VT enhances courtship clasping by acting on sensorimotor processing of all three sensory modalities. Electrophysiology studies reveal that sensorimotor processing of

somatosensory information is appreciably enhanced by VT (Lewis and Rose, 2002; Rose et al., 1995). The output of medullary neurons is directly related to the incidence of clasping (Rose et al., 1998), and VT robustly enhances sensory responsiveness and spontaneous activity of these neurons. Behavioral studies show that VT also acts by enhancing appetitive responses to visual and olfactory stimuli (Thompson and Moore, 2000). Behavioral assays determine the role of vasotocin on appetitive responses to visual and olfactory sexual stimuli. In *Taricha*, the visual cue of a female and olfactory pheromonal cue emitted by a female function as releasing stimuli eliciting courtship behaviors in this species. In the behavior studies, the female in a beaker is used as the visual cue, to which males spend longer time in proximity to and investigating the stimulus (Thompson and Moore, 2000). The non-volatile non-peptidergic pheromone(s) emitted by the female can be collected and applied to pads, to which the males will respond by spending a longer amount of time investigating and even clasping the pheromone-scented models (Thompson et al., 1999). Thus, the electrophysiological and behavioral evidence suggest that VT enhances courtship clasping by enhancing sensorimotor processing of at least three independent sensory modalities.

The courtship clasping behavior of male *Taricha* is also modified very rapidly and noticeably by the action of the stress steroid corticosterone (CORT). In males, acute stress suppresses clasping by elevating endogenous levels of the glucocorticoid, CORT. If synthesis of CORT is blocked by metyropone, then acute stress does not suppress courtship. Furthermore, administration of CORT robustly and rapidly suppresses male courtship (Moore and Miller, 1984). The behavioral effect of CORT is transduced by cellular mechanisms that allow for rapid relay of information. Membrane-associated G-protein coupled binding sites for CORT have been characterized (Orchinik, Moore, and Rose, 1994; Orchinik, Murray, Franklin, and Moore, 1992; Orchinik et al., 1991). Autoradiography and electrophysiology studies suggest that these putative receptors are located throughout the central nervous system of *Taricha*, including medullary and spinal cord regions (Lewis and Rose, 2003; Orchinik et al., 1994; Rose et al., 1993).

Although a membrane binding site has not been identified in other vertebrates, the rapid physiological and behavioral responses to CORT exhibited by *Taricha* are observed in many species. The experience of acute stress very rapidly elevates CORT levels in frogs (Coddington and Cree, 1995; Licht et al., 1983), reptiles (Moore et al., 2001; Moore et al., 1991), birds (Heiblum et al., 2000), and rats (Graessler et al., 1989). High titers of CORT modify behavioral responses of hamsters (Hayden-Hixson and Ferris, 1991), shrews (Schiml

and Rissman, 1999), mice (Kavaliers and Ossenkopp, 2001), rats (Haller et al., 1998; Sandi et al., 1996), and birds (Breuner et al., 1998; Silverin, 1986; Wingfield and Silverin, 1986).

Whereas it is not known whether CORT affects the processing of visual or olfactory sensory stimuli, in *Taricha* CORT suppresses courtship clasping by depressing neural processing of somatosensory processing. CORT suppresses the spontaneous activity and sensory responsiveness of medullary and spinal neurons involved in generating and regulating the output of the clasp reflex (Rose et al., 1993). Furthermore, CORT fully blocks the VT-induced enhancement of sensorimotor processing of somatosensory information, as revealed by electrophysiological recordings from medullary neurons. A recent study reported that the VT-induced enhancement of clasping could be blocked by prior treatment with CORT (Coddington and Moore, 2003). Given that VT influences courtship behaviors by acting on at least three sensory modalities and that CORT interacts with VT to influence courtship behaviors, these data suggest that CORT may also modify courtship behaviors by influencing sensorimotor responses to sensory stimuli from other sensory modalities, such as visual and olfactory.

To understand the mechanisms by which hormones modify behaviors, we asked whether CORT suppresses courtship clasping behavior by suppressing the processing of stimuli from multiple sensory modalities. Specifically, we hypothesized that CORT suppresses clasping by diminishing responses to visual and/or olfactory sexual sensory stimuli. We tested this hypothesis using behavioral assays developed by Thompson et al. (2000). We predicted that, if CORT did suppress processing of multiple sensory modalities then appetitive responses towards a sexual visual stimulus and a sexual olfactory stimulus would be diminished. We also developed another behavioral test to verify independently whether the appetitive responses of male *Taricha* towards females were affected by CORT administration.

METHODS

Animals

Sexually active adult male newts (*Taricha granulosa*) were collected locally from the perimeters of permanent ponds in the Coast Range (Benton Co., Oregon) using dip-nets. The newts were held in community tanks supplied with continuously flowing, aerated dechlorinated water and fed an excess of chopped earthworms. Newts were housed in an environmentally controlled room with natural photoperiod and temperature (average 13°C). Males weighed, on average, 14 ± 0.5 g. Females were captured during migration to breeding

ponds by hand and were housed together in terrariums (12 / tank) with damp moss and leaf-litter at one end and water in the other. Females to be used in olfactory experiments were kept overnight for one night and fed before collecting skin secretions. Those to be used in CORT dose-response and visual experiments were maintained in breeding condition, and therefore attractive to the males, by injections of prolactin (0.5 IU / 0.1 ml / newt) administered intraperitoneally (i.p.) every second day for the duration of their stay in captivity (2 weeks -2 months). During this time the females were fed an abundant supply of chopped earthworms. These collecting and maintenance protocols allowed for capture and maintenance of unmated females that were sexually attractive to males (Moore, 1978; Propper, 1989).

Hormones Administered

Newts were i.p. injected CORT / 0.1 ml or 0.1 ml vehicle. CORT (Sigma-Aldrich Co., St Louis, MO) solution (40 μg / 0.1 ml) was prepared with 99.8% Amphibian Ringers and 0.2% DMSO (Dimethyl sulfoxide; Sigma-Aldrich, MO), and stored at 4 °C. Vehicle to control for CORT effect was prepared simultaneously with 99.8% Amphibian Ringers and 0.2% DMSO. All injections were prepared ahead of time, and coded by another person, before being injected "blind" by Emma Coddington in order to remove any bias towards hormone effect.

Behavior Experiments

All behavioral testing was performed between 1400 and 2000 h on males collected recently in the prior 24 – 48 h period. Each male was tested only once. All tests were performed in low-light conditions within a specified arena (3m X 3m) enclosed by a curtain of black plastic hung from the ceiling to the floor. Individual males were transferred from community tanks into separate testing tanks 30 min prior to any injections and behavioral testing. Estimates of time spent in the vicinity of the test-stimulus was determined later from video tape, recorded with a low-light video camera.

Behaviorally Relevant CORT Dose

From prior studies we knew that i.p. administration of CORT suppresses clasping behavior (Moore 1984 Orchinik 1991). In order to determine the effect of CORT on other behaviors associated with clasping we needed to confirm that the dose of CORT to be used was relevant to this behavioral paradigm. The appropriate dose of CORT to be used in the olfactory and visual sensory experiments was determined by performing two dose-response

experiments testing CORT-induced suppression of clasping behavior. The first test was conducted in early mating season (February, 2001), and the second test during the height of the breeding season (March, 2001). The early experiment tested the effect of 0, 20, or 40 μg/newt CORT on incidence of clasping by males (N = 8 / treatment group). The second tested the effect of 0, 2, 11, 20, 30, 40 μg/newt CORT on incidence of clasping by males (N = 10 / treatment group). For detailed methods of this behavioral test see Coddington and Moore (2003).

CORT effect on Behavioral Responses to Visual Sexual Stimuli

This behavioral testing paradigm was developed by (Thompson et al., 2000) to test the longer acting effects of vasotocin, and was modified here to test the rapid actions of CORT. Males were tested in a large black circular cattle tank (d = 100 cm , h = 14 cm), filled to a depth of 6 cm with dechlorinated water. The visual stimulus was a female *Taricha* placed in a 500 ml glass beaker, in this way the male could not be exposed to any pheromonal cues. A white circular area (d = 12 cm) was demarked by fixing water-proof paper to the base of the tank, and the 500 ml glass beaker (d = 8 cm) filled to 6 cm depth was placed in the center of the white circle. Two centimeters of white area extended beyond the edges of the beaker on all sides. Males were placed in the tank (outside the beaker) for 30 min to acclimatize before receiving an i.p. injection of CORT or VEH. Eight minutes after injection, a female was placed in the glass beaker, or for control Emma's hand swirled the water in the beaker for approximately 5 s. Each male was tested only once and with a unique female stimulus each time. Out of 30 min of recording, the total amount of time the male spent with his head in the white area (close proximity) was analyzed from video tape.

CORT effect on Behavioral Responses to Olfactory Sexual Stimuli

Males were individually tested in circular tanks (d = 27cm) filled to a depth of 6 cm with dechlorinated water. The floor of each tank was marked with eight triangles of equal area. Once acclimatized to the test tank (30 min), each male received either CORT or VEH. Eight min after receiving hormone, one scented piece of craft foam pad (thickness 0.4 mm) the same size as one of the eight triangles drawn on the floor, was attached to the base of the tank. The foam pad was scented with either female sex pheromones (see below) or 5% ethanol water. Each male was tested with a unique scented foam pad. Time spent with snout on the foam was monitored for 30 min after being placed in the testing tank. Behaviors were monitored and recorded using a low-light video camera. These behavioral procedures were

developed from Thompson et al. (2000), who showed that sexually active male newts (but not females) were attracted to glass beakers that contain a female.

Female skin secretions were collected in an aluminum pan (9 x 11 x 4 inches), the base of which was covered with triangular foam pads and 10 ml of 5% ethanol / female. Twenty sexually attractive non-mated females were placed into the dish, covered, and placed at 10 °C for 24 h. The females were usually moving very slowly by the time the collection procedure was completed. They were immediately removed and placed into dechlorinated water to recover. The foam pads were stored for no longer than 24 h at 4 °C before experiments were performed.

CORT effect on Affiliative Behavior towards Conspecifics of Opposite and Same Sex

To assess whether CORT inhibits clasping behavior by decreasing a male's general interest in females, we injected males with CORT or VEH i.p. and then recorded the time a male spent with it's snout on or against the body of either a male or female newt. Males were tested in circular tanks (d = 27 cm), acclimatized for 30 min before i.p. injection of CORT or VEH, and then two females or two males were introduced to the arena. Time in proximity to females or males was monitored during the 30 min immediate after the conspecifics were introduced to the tank.

Statistical Analysis

Non-parametric analysis of variance (Kruskal Wallis tests; KW) tests were used because data from all experiments were skewed and variances were heterogeneous ($\alpha = 0.05$). Comparisons among groups were evaluated with Dunn's Multiple Comparisons Test ($\alpha \leq 0.05$).

RESULTS

Behaviorally Relevant CORT Dose

Clasping behavior is suppressed by CORT when administered to males in the early (KW=12.16, P=0.0023; Fig 3.1A) and middle (KW = 40.5, P < 0.0001 (Figure 3.1B) periods of the breeding season. Clasping by males receiving CORT early in the breeding season (Jan-Feb) was significantly suppressed by 20 μg/newt or 1.4 mg/kg (D = 10.5, P < 0.01 (Figure 3.1A). A higher dose of CORT, 40 μg/newt (2.67 mg/kg) suppressed fewer males than did the 20 μg/newt dose. Clasping by males receiving CORT in the middle of the breeding season

(March) was only suppressed at the highest of doses, 40 μg/newt ($D = 24.0$, $P < 0.05$) (Figure 3.1B).

CORT effect on Behavioral Responses to Visual Sexual Stimuli

CORT did not decrease time spent in proximity to a female visual stimulus. Treatment significantly affected time males spent in the proximity of the visual stimulus (KW = 8.882, P = 0.0309) (Figure 3.2). VEH males spent a significantly longer amount of time in proximity of sexual visual stimulus compared with CORT males with an empty beaker ($D = 12.72$, $P < 0.05$). No other comparisons were significantly different from each other. Although it is of interest that one CORT male spent over 150 s investigating the female, and in fact was crawling the sides of the beaker.

CORT effect on Behavioral Responses to Olfactory Sexual Stimuli

Treatment significantly effected time males spent with snout or head on the pad (KW = 26.4, $P < 0.0001$). Males were consistently attracted to pads scented with female pheromones over control pads (Figure 3.3), which confirmed that males are attracted to female scented pads. There was no difference in the time spent investigating pheromone scented pads by males injected with CORT or Veh ($D = 2.20$, $P > 0.05$). Likewise, males receiving VEH or CORT spent comparable amounts of time investigating the control pads ($D = 4.67$, $P > 0.05$).

CORT effect on Affiliative Behavior towards Conspecifics of Opposite and Same Sex

In this experiment, treatment did have a significant effect on the amount of time males spent with snout on conspecific's body (KW = 19.46, P = 0.0002) (Figure 3.4). Males receiving CORT injections spent a significantly longer period of time investigating females than males ($D = 13.8$, $P < 0.05$). Likewise, males receiving VEH spent a significantly longer period of time investigating females than males ($D = 15.4$, $P < 0.05$). Hormone treatment (CORT vs VEH) did not alter the amount of time males investigated males ($D = 6.4$, $P > 0.05$), or females ($D = 8.0$, $P > 0.05$). There was a significant difference between VEH – injected males investigating females compared to CORT-injected males investigating males ($D = 21.8$, $P < 0.001$).

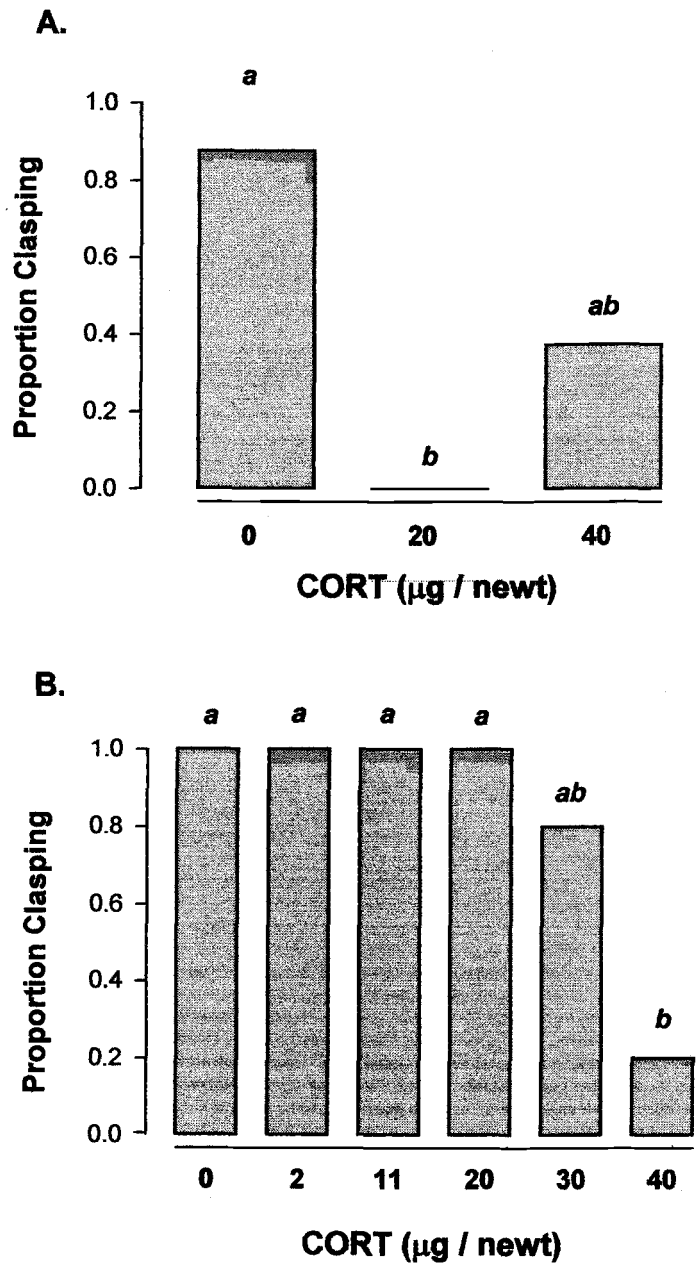

Figure 3.1. CORT-induced inhibition of clasping behavior is subject to seasonal variation in sensitivity to CORT administration. Proportion of male's clasping **A.** during the early breeding season, N=8/tmt group (February 2001), and **B.** during the height of the breeding season, N=10/tmt group (Early March, 2001). Nonparametric ANOVA revealed a significant effect of CORT concentration on proportion of clasping males in early season (KW=12.12, P=0.0023) and late season (KW=40.5, P<0.0001). Different letters above each group indicate significant difference from other groups as determined by Dunns Multiple Comparison Test (all were at least $P \leq 0.05$).

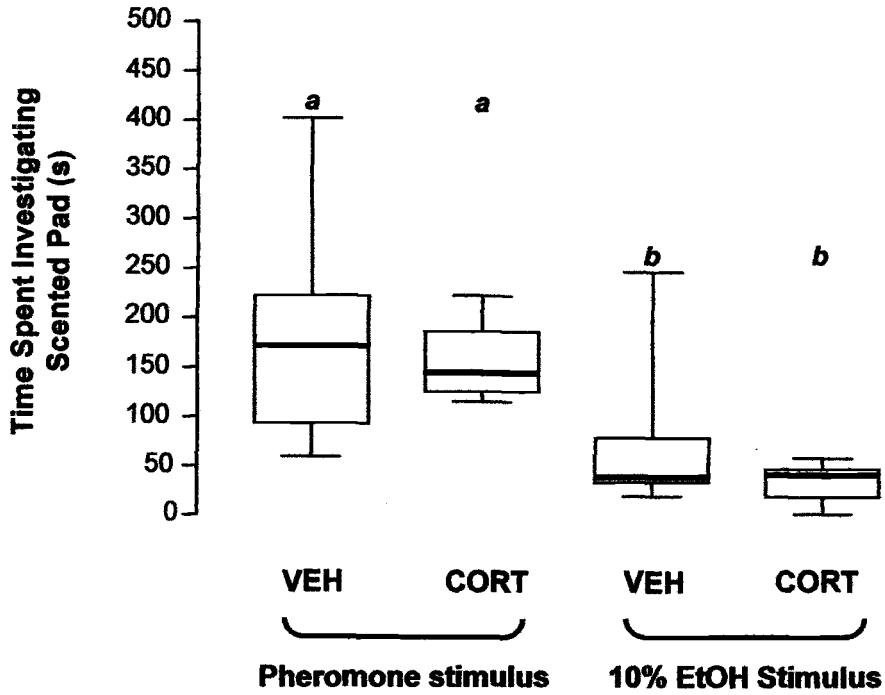

Figure 3.2. The effect of corticosterone (40 μg/newt) on a behavioral response to olfactory stimuli. Data are presented in a Box and Whisker plot format with median = central bar, 25th and 75th percentile = box, range of data = whiskers. Nonparametric ANOVA revealed a significant effect of treatment (KW = 26.40, $P < 0.0001$). Different letters above each group indicate significant difference from other groups as determined by Dunns Multiple Comparison Test (all were at least $P \leq 0.01$). Pheromone stimulus was collected by keeping 24 sexually attractive females on testing pads in 10 ml water for 24 hrs. Water stimulus was a parallel set of testing pads kept in 10 ml of water for 24 hrs. To ensure that there was no residual olfactory trace of the former test individual, each arena was washed with biodegradable aquarium cleaner and then sprayed with bleach in between each test individual.

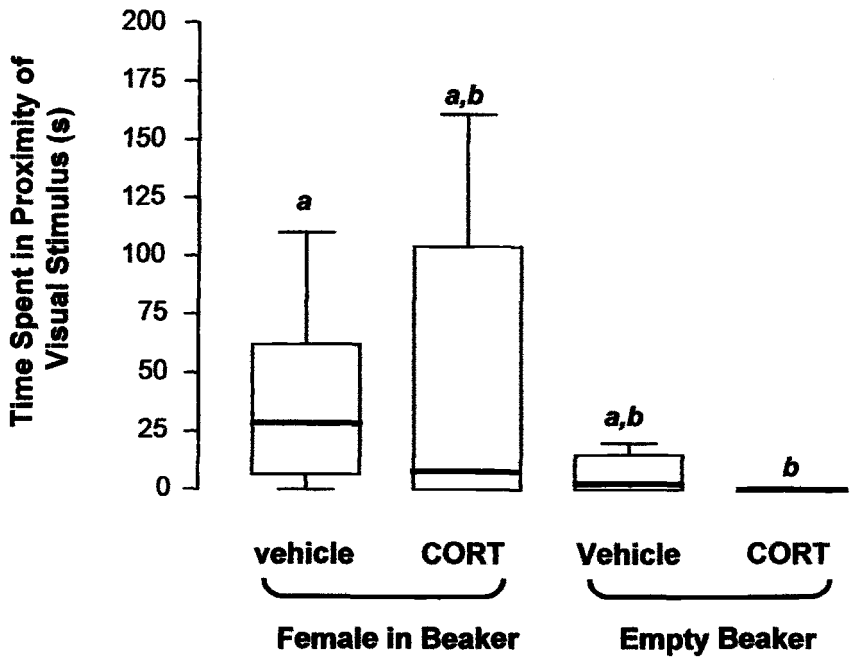

Figure 3.3. The effect of corticosterone (40 μg/newt) on the behavioral response to visual stimuli. Data are presented in a Box and Whisker plot format with median = central bar, 25th and 75th percentile = box, range of data = whiskers. Nonparametric ANOVA revealed a significant effect of treatment (KW = 8.882, P= 0.031). Different letters above each group indicate significant difference from other groups as determined by Dunns Multiple Comparison Test ($P \leq 0.05$). Visual stimulus was created by placing a female newt in a 1 L beaker of water placed in the center of a circular arena (d = 1 m). No visual stimulus was an empty 1 L beaker of water in the arena. To ensure that there was no residual olfactory trace of the former test individual, each arena was washed with biodegradable aquarium cleaner and then sprayed with bleach in between each test individual. The sand used in each test was removed and baked at high T for 4 hr in between each test individual.

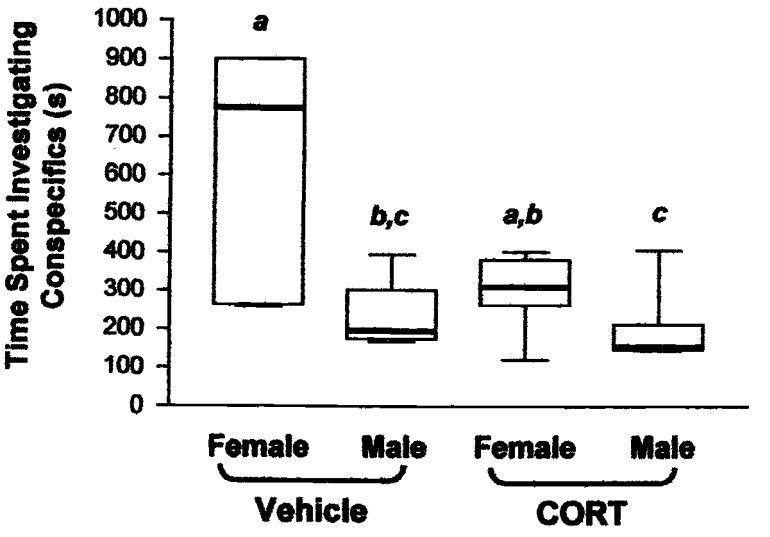

Figure 3.4. CORT does not inhibit male *Taricha* clasping behavior by decreasing the interest the males have in the females. Investigation of conspecific was defined as time spent with snout on conspecific's torso or head region. Data are presented in a Box and Whisker plot format with median = central bar, 25th and 75th percentile = box, range of data = whiskers. N=10/group. Nonparametric ANOVA revealed a significant effect of treatment (KW=19.46, $P = 0.0002$). Different letters above each group indicate significant difference from other groups as determined by Dunns Multiple Comparison Test ($P \leq 0.05$).

DISCUSSION

The current study investigated whether CORT suppresses courtship clasping by suppressing sensorimotor responses to multiple sensory modalities. We specifically tested whether appetitive behavioral responses to sexual visual and olfactory stimuli were diminished by CORT. To determine whether CORT suppresses courtship clasping by altering behavioral response to a sexual visual stimulus, we measured the amount of time a male spent in close proximity to a beaker containing a female. This study confirmed prior findings (Thompson and Moore, 2000) that males were attracted to the visual stimulus of a female newt in the beaker. Treatment with CORT did not suppress this attraction. Therefore, we conclude that the sensorimotor processing of sexual visual information is not suppressed by CORT. We can find no other report of glucocorticoid effects on behavioral responses to visual stimuli.

Personal observation and experience suggest that *Taricha granulosa* does not discriminate well based on visual cues. In nature, male *Taricha* will orient and swim towards anything that moves inside and outside of the pond, including researchers, presumably waiting for sexually attractive females to migrate and enter the pond. It seems that, like the toad, orienting towards a moving object in *Taricha's* visual field can be considered the first step in a successful mating strategy. That CORT injected males spent as much time in close proximity to the sexual visual stimulus as VEH injected males suggests that CORT does not suppress courtship clasping by inhibiting orientation or approach to a moving visual stimulus. These negative results are in contrast to the positive effect of VT in the same behavioral testing paradigm, where administration of VT enhances appetitive responses to visual stimuli (Thompson and Moore, 2000).

We also tested whether CORT-induced suppression of clasping behavior involves changes in behavioral responses to a sexual olfactory stimulus. This experiment confirmed prior work (Thompson and Moore, 2000), that males are attracted to pads scented with sex pheromones collected from females. Male *Taricha* spend a significantly longer period of time investigating pheromone-scented pads than control-pads. The injection of CORT did not effect time that males spend investigating this olfactory stimulus. Again, these negative data are in contrast to the positive effect of VT on male *Taricha* using the same olfactory testing paradigm, where administration of VT enhances appetitive responses to olfactory stimuli (Thompson and Moore, 2000).

To date, there are no other studies examining the effect of glucocorticoids or acute stress on male behavioral responses to olfactory stimuli. However, the preference for male odors by female mice is rapidly inhibited by CORT (Kavaliers and Ossenkopp, 2001). Given that the

behavioral testing paradigms have been validated for newts (Thompson and Moore, 2000; Thompson et al., 1999) and mice (Kavaliers and Ossenkopp, 2001), differences between studies could be due to a number of reasons. Difference in glucocorticoid effects on behavioral responses to sexual attractant could be due to differences between sexes, species, or physiological context (See Coddington n.d. Chapter 2).

In the final experiment males consistently spent a significantly longer total time investigating conspecific females than males. CORT-injected males investigate females for significantly longer period of time than conspecific males. CORT, compared to VEH, administration did reduce the total amount of time males spent with females. This was most likely due to VEH injected males clasping females and therefore biasing the total time for this specific group. Nevertheless, that CORT-injected males continue to investigate females suggests that males' interest and motivation to clasp is not suppressed by CORT administration.

Taken together, all the results from this study consistently indicate that CORT does not suppress appetitive behavioral responses to olfactory or visual cues. In contrast, male *Taricha* appetitive responses to olfactory and visual stimuli, using the same behavioral testing paradigms, are enhanced by the administration of VT (Thompson and Moore, 2000). Because VT also enhances sensory responsiveness to somatosensory stimulation of the cloacal region (Lewis and Rose, 2002; Rose et al., 1995; Rose and Moore, 2002), Thompson and Moore (2000) concluded that VT influences behaviors by acting on at least three sensory modalities. Thompsons (2000) study also indicates that VT could be acting to enhance general motivational or arousal state. The same cannot be said for the action of CORT.

Electrophysiological studies show that CORT acts on medullary and spinal neurons to suppress clasping in response to somatosensory stimulation of the cloaca. Somatosensory (mechanical) stimulation of the cloaca is necessary to initiate clasping and one function of CORT is to inhibit the spinal and medullary neurons responsive to cloacal stimulation (Rose and Moore, 1999; Rose et al., 1993). Spinally transected newts retain a strong clasp response that is rapidly and robustly inhibited by CORT (Lewis and Rose, 2003). The clasp in spinal newts persists past the termination of clasp-eliciting stimulus. This extended clasp is due to the severing of descending projections from reticulospinal medullary neurons (Aronson and Noble, 1945; Hutchison, 1963; ten Donkelaar, 1989) which appear to be primarily inhibitory to the maintenance of the clasp. In freely moving newts, reticulospinal neurons fire in response to the onset (initiation) and offset (termination) of clasp-eliciting stimuli (Rose et al., 1998).

Together, these electrophysiology studies have led to the generation of a model describing a brainstem-spinal control of clasping (Rose, 2000). In the model, a hypothetical spinal cord clasp generator controls the expression, in response to cloacal stimulation, and the maintenance of limb position during clasping. As used here, the term "Generator" is descriptive and does not refer to central pattern generators. In the natural setting cloacal stimulation would occur when the male initially grasps the female and then later as courtship progresses by the male pulling the female towards his ventrum in rhythmic bilateral contraction of his hind limbs around the female. In electrophysiology studies we simulate this type of stimulation by applying light pressure to the cloaca with a small probe, delivering a constant pressure for 2-3 s. As described by the model (Rose, 2000), descending influence from reticulospinal neurons provides input to the spinal cord, regulating onset and offset of the clasp. Evidence indicates that reticulospinal neurons are controlled partially by afferent input from the cloaca. Removal of higher brain influence by forebrain-ablating males reveals that full maintenance and responses to onset and offset of clasp-generating stimuli are retained (Lewis and Rose, 2002). The sensory responsiveness, as measured by neuronal activity and generation of clasp, of forebrain ablated and spinally transected males are consistently suppressed by CORT (Lewis and Rose, 2003; Rose et al., 1993). Thus, in *Taricha*, there is strong evidence that one way in which elevated CORT levels suppress clasping behaviors is by acting at the level of the hindbrain and spinal cord to suppress the reflexive clasping response triggered by somatosensory stimulation.

In conclusion, the findings of this study suggest that CORT suppresses courtship clasping by specifically suppressing sensorimotor processing of clasp-generating somatosensory stimuli, and not by suppressing visual or olfactory processing. Furthermore, CORT-treated males remain motivated and interested in sexually attractive females. Based on past studies, we infer that acute stress suppresses courtship clasping by elevating CORT and thereby modifying behavioral responses to specific somatosensory stimuli.

CHAPTER 4: THE ENDOGENOUS CANNABINOID SYSTEM CONTROLS BEHAVIORAL RESPONSES TO ACUTE STRESS.

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ABSTRACT

Behavioral responses to acute threats have been identified in all major groups of vertebrates. While glucocorticoids are instrumental in the chronic disease states developed after experiencing prolonged stress, there are many reports of glucocorticoids initiating immediate behavioral responses to direct threats or acutely stressful situations. The ability of an animal to respond appropriately when faced with an immediate threat is critical to survival. The neuroendocrine regulation of behavioral responses to immediate threats is not well understood. However, an amphibian, *Taricha granulosa*, provides an ideal model to examine these neuroendocrine pathways. When threatened, male *Taricha* suppress reproductive clasping behavior. Exposure to a threat results in elevation of endogenous corticosterone titers, which in turn suppresses the brainstem neural circuitry regulating the clasp behavior. The activity of the medullary neurons directly determines sensorimotor responses and clasping behavior output. Here, we demonstrate that stress-induced suppression of reproductive behavior requires signaling of endocannabinoids downstream in the temporal sequence of events initiated by CORT. The clasping behavior of males is not suppressed by CORT when pretreated with the cannabinoid antagonist, AM 281. Similarly, measurements of medullary neuron excitability, spontaneous and sexual stimulus-induced activity are rapidly and robustly suppressed by CORT alone. However, if a male is pretreated with AM 281, CORT no longer suppresses neuron activity. Placed in the context of an animal model system that integrates behavior with neural control and endocrine regulation, the broader functional significance of endocannabinoid signaling becomes understandable as an integral part of other signaling and neuroendocrine processes. The conservative nature of cannabinoid CB1 receptors and retrograde signaling mechanisms suggests that the neuroendocrine pathway outlined in this study may be a fundamental feature of behavioral responses to acute stress.

In the wild, animals typically respond to a threat, an acutely stressful stimuli, by suppressing conspicuous reproductive behaviors and remaining sexually inactive for minutes after the potential threat have receded. For example, when a person approaches a pond with calling frogs, a marsh with singing wrens, or a meadow with bugling bull elk, these animals respond by suppressing their advertising displays and remaining silent. Consistent with these observations of wild animals, laboratory studies have shown that acute stress inhibits reproductive behaviors in frogs (Burmeister et al., 2001), salamanders (Moore and Miller, 1984), mice (Lumley 1999 (Lumley et al., 1999; Marchlewska-Koj et al., 1994), rats (Hulse and Coleman, 1983), and primates (Habib et al., 2000). The underlying hormonal and neurochemical signals that function to suppress an animal's propensity to display reproductive behaviors in response to detected threats, or acute stress, are poorly understood.

Research with an amphibian, roughskin newt (*Taricha granulosa*), has focused on the neuroendocrine mechanisms that control behavioral responses to acute stress (Orchinik et al., 1994; Rose and Moore, 1999). Male *Taricha* exposed to an acute stressor are less likely to engage in amplectic clasping (Moore and Miller, 1984), a component of newt courtship behavior where the male embraces the female from the dorsal aspect with fore and hind limbs. Acute stress results in elevated plasma elevated plasma corticosterone (CORT), a steroid hormone secreted by adrenal glands, which in turn acts to suppress amplectic clasping behavior (Moore and Miller, 1984). An injection of CORT rapidly and potently suppresses amplectic clasping behavior. Furthermore, metyrapone treatment, which inhibits the synthesis and release of CORT, can block the stress-induced inhibition of amplectic clasping. At the level of neural circuitry, amplectic clasping behavior is generated by spinal circuits that respond to pressure applied to the cloacal region (a sexually relevant sensory stimulus), and is regulated by descending input from medullary neurons activating motor neurons (Figure 4.1) (Rose and Moore, 1999). Single-unit electrophysiology studies have revealed that CORT acts to suppress clasp-regulating neurons in the medulla (Rose et al., 1995; Rose et al., 1998; Rose et al., 1993) (Figure 4.1). The temporal framework within which CORT exerts its effects are robust and highly predictable; onset of the CORT effect on neuronal activity and amplectic clasping behavior occurs in a time window of 2-5 min following injection, and continues for 40-60 min (Rose et al., 1993). The temporal pattern of the CORT effect suggests to us that there are neurotransmitter mechanisms downstream in a sequence of events initiated by CORT.

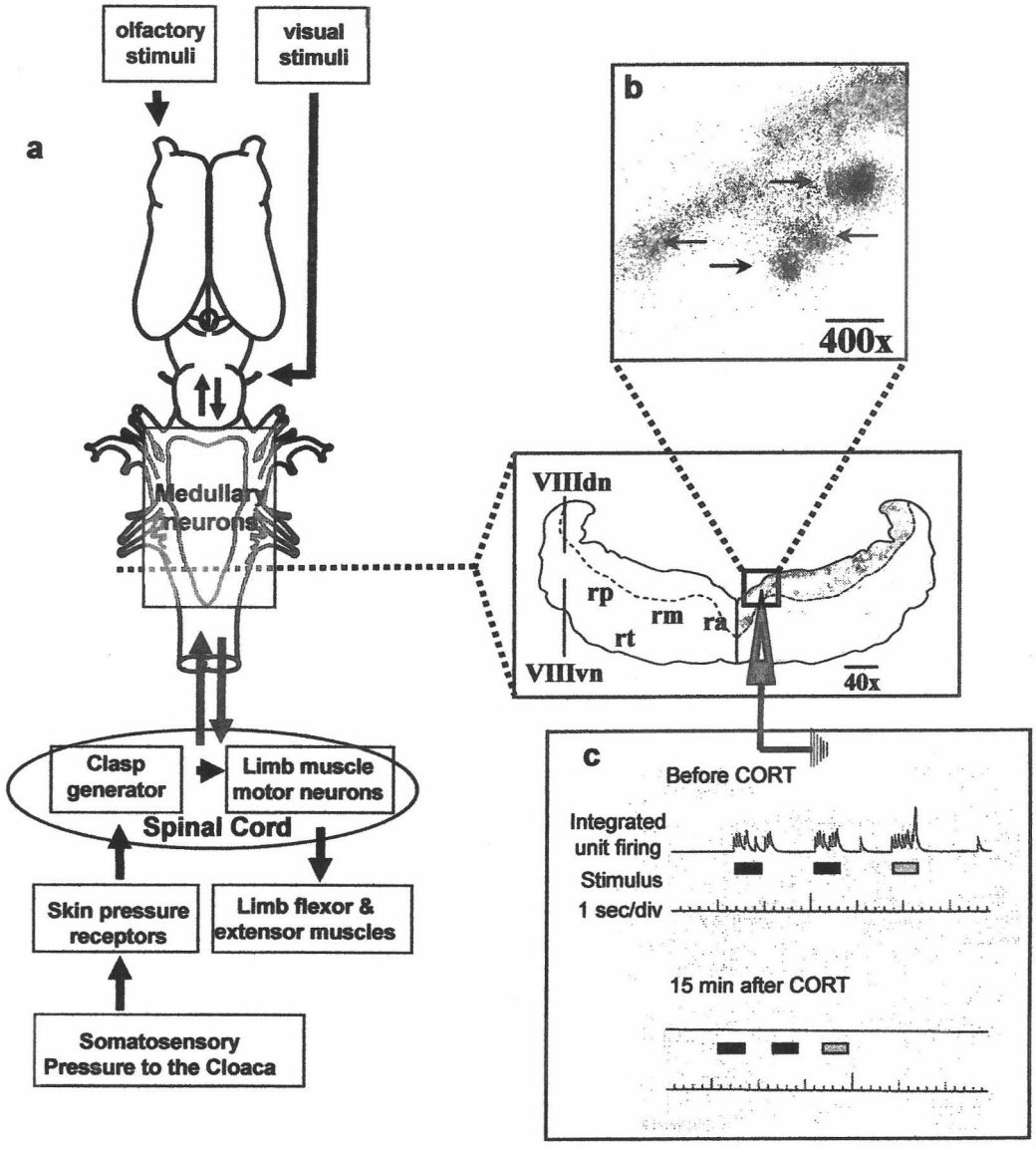


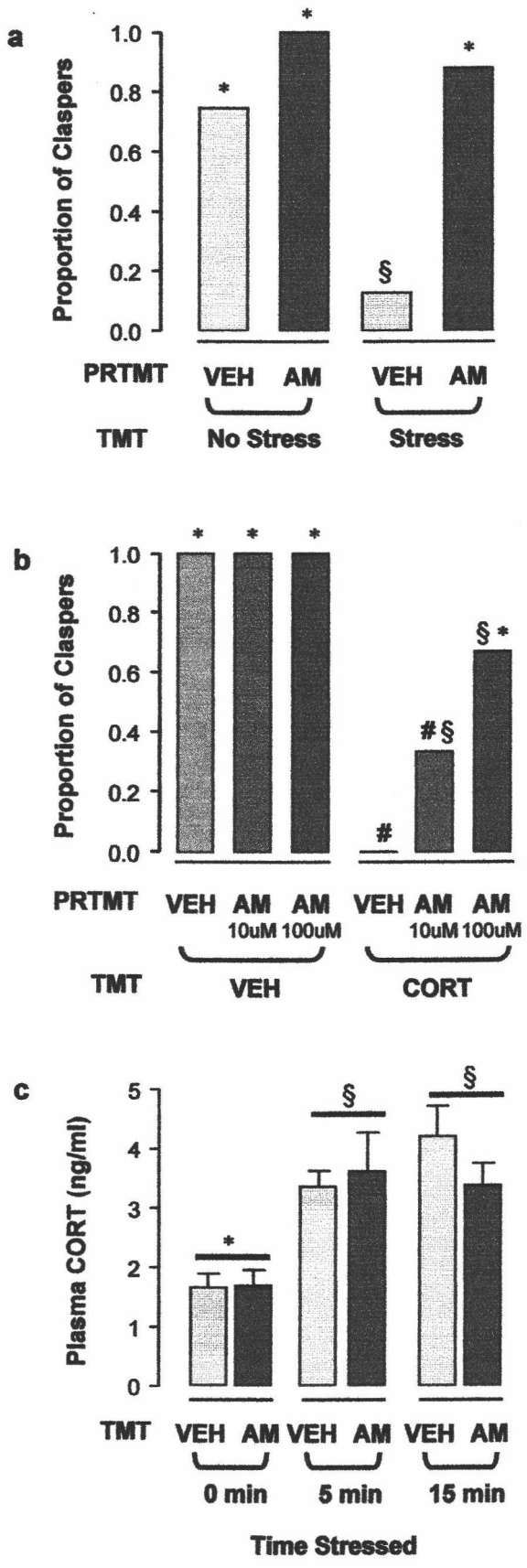
Figure 4.1. Schematic illustration of principal neural systems controlling clasping in *Taricha*. **a** Visual and olfactory sensory input is hypothesized to prepare the system for clasp-triggering stimuli. Mechanical pressure applied to the cloaca triggers clasp-generating processes in the spinal cord, resulting in excitation of flexor motor neurons, and presumably inhibition of extensor motor neurons, and consequently flexion of limb muscles. The spinal clasp generator receives descending influences from the brain, principally from medullary neurons that control clasp onset, termination, and adjustments to clasp coordination and strength. Neural activity elicited in the spinal circuits also ascends to the medulla, producing mainly an increase in firing of medullary neurons and a mixture of increasing and decreasing in firing of reticulospinal neurons. Collectively, the responses of medullary neurons are closely associated with the onset, maintenance, and offset of clasping, allowing us to observe hormone effects on this sensorimotor system. **b** A light microscope photograph of CB1 receptor labeling in the same region that electrophysiological recordings are made, as revealed by in situ hybridization using newt specific cRNA probes (Hollis, Coddington, Moore, in preparation). Abbreviations are as follows: VIII dn, dorsal nucleus of the eighth nerve; VIII vn, ventral nucleus of the eighth nerve; rp, parvocellular reticular nucleus; rt, reticular nuclei; rm, middle reticular nucleus; ra, raphe nucleus. **c** A representative output of a single medullary neuron in response to cloacal pressure, recorded using single-unit extracellular electrodes (Rose et al., 1993).

We specifically tested the hypothesis that stress-induced suppression of sex behavior requires signaling of endocannabinoids downstream in the temporal sequence of events initiated by CORT. This hypothesis was based on five observations. First, hypothalamic slice preparations with rats show that the inhibitory effect of corticosteroids on the hypothalamic-pituitary-adrenal (HPA) axis is mediated by retrograde signaling of endocannabinoids (Di et al., 2003). Second, the temporal pattern of the inhibitory effects of CORT in *Taricha* match the onset and duration of endocannabinoid retrograde signaling effects in rats using slice preparations of hippocampus (Wilson and Nicoll, 2001), corticostriatum (Ronesi et al., 2004), hypothalamus (Di et al., 2003), and cerebellum (Kreitzer and Regehr, 2001a; Kreitzer and Regehr, 2001b). Third, *Taricha* express functional cannabinoid type 1 (CB1) receptors that are structurally and pharmacologically similar to mammalian CB1 receptors; these receptors therefore bind synthetic and presumably endogenous cannabinoids with high affinity (Soderstrom et al., 2000). Fourth, mRNA for CB1 receptors are located in the region of the hindbrain known to regulate clasping behavior, as resolved from electrophysiological recording of rapid responses to CORT in the rostral ventral medulla (Figure 4.1). Fifth, behavioral studies show that an injection of the CB agonist CP-55940 suppresses amplectic clasping behavior in *Taricha* in a dose dependent manner (Coddington, n.d.; Soderstrom et al., 2000).

If stress-induced inhibition of amplectic clasping behavior is mediated by elevated endocannabinoid activity, then we would expect newts pre-treated with a CB antagonist to continue to clasp even when exposed to the same acute stress. To test this prediction, sexually active male *Taricha* were pre-treated with an intraperitoneal injection of CB1 antagonist AM 281 (100 μM) or VEH 15 min prior to exposure to a standardized acute stressor (see methods for detail). Eight to ten min after exposure to the acute stress, males were individually presented with two sexually active females and tested for the incidence and latency of clasping for 30 min. In VEH-injected controls, the incidence of clasping was significantly reduced by exposure to the standardized stressor ($KW = 25.2$, $P < 0.0001$) (Figure 4.2a), as expected from earlier studies (Moore and Miller, 1984). The stress-induced suppression of clasping was blocked in males pre-treated with AM 281, which indicates that the endocannabinoid system is involved in mediating this behavioral response to stress. Note that, in this experimental system the effects of CB antagonist appear to be independent of effects at the level of the HPA axis because plasma levels of CORT were elevated to similar titers in VEH - and AM 281-treated males (Figure 4.2c).

Figure 4.2. CORT-induced suppression of clasping is blocked by pretreatment with CB antagonist AM 281. Bar shading indicates treatment with AM 281. Different symbols indicate significant difference from all other treatment groups. **a** The effect of acute stress on the proportion of males clasping. Males were pretreated with 100 μM AM 281 or VEH 15 min before receiving standardized acute stressor (see Methods). Afterwards, males were placed individually with 2 females and incidence of clasping was determined in the following 30 min. N = 16 males per treatment group. **b** The effect of CORT on the proportion of males observed clasping. Males were pretreated with AM 281 (10 or 100 μM) or VEH 15 min before receiving CORT (115 nM). After 5 min males were placed individually with 2 females and incidence of clasping was determined in the following 30 min. N = 16 males per treatment group. Data for **a** and **b** were analyzed by nonparametric ANOVA (Kruskal-Wallis) to determine overall significance of treatment effect, and then pairwise comparisons were made using Dunn's multiple comparison test. **c**, Plasma levels of CORT in male *Taricha* are elevated in response to the standardized acute stressor, and pretreatment with CB antagonist does not block the release of adrenal CORT. Males were pretreated with 100 μM AM 281 or VEH 15 min before receiving standardized acute stressor for 0, 5 or 15 min (N = 8 per treatment group; see Methods). After which, males were sacrificed, blood collected, and plasma analyzed by RIA (see Methods). RIA analysis was performed by Dr. David Hess, Oregon Health Sciences University, Oregon. Data are presented as mean ± standard error. Number of *Taricha* are given in parentheses. Plasma levels of CORT were analyzed using a Two-way ANOVA followed by corrected Bonferroni *t*-tests for pairwise comparisons. Bars indicate no difference due to hormone treatment ($F_{1,42}$= 0.275, P = 0.604), however, there was a significant effect of Stress treatment ($F_{2,42}$= 14.593, $P < 0.001$). Different symbols above bars indicate significant difference ($P < 0.0001$) as compared with 0 min Time Stress.

Figure 4.2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To determine whether endocannabinoids are involved in the CORT-induced suppression of courtship clasping, we pre-treated sexually active male *Taricha* with an intraperitoneal injection of CB antagonist AM 281 (100 μM) 15 min prior to treatment with i.p. CORT (114 nM). Then, to be comparable to the acute stress test, males were individually presented with two sexually active females 8-10 min after CORT administration. In males pretreated with VEH, administration of CORT significantly reduced the incidence of clasping (Figure 4.2b), which is consistent with previous studies (Moore and Miller, 1984; Orchinik et al., 1991). We observed a significant dose-dependent increase in the incidence of clasping in CORT-treated males that were pretreated with CB antagonist (Figure 4.2b). These results support the hypothesis that endocannabinoid signaling occurs downstream in the sequence of events initiated by CORT. Taken together, results from both behavioral studies support the hypothesis that, in this species, the neuroendocrine pathway controlling stress-induced suppression of courtship behaviors involves the detected threat triggering increases in CORT, which in turn enhance endocannabinoid activity and activation of CB1 receptors.

We predicted that the nature of endocannabinoid regulation of clasping behavior will be revealed by examining medullary neurons in situ in the rostroventral medulla. This hypothesis is supported by several lines of evidence. First, medullary neurons respond to clasp-generating stimulation of the cloaca and are readily monitored in situ using single-unit neurophysiological recordings; mechanical pressure applied to the cloacal region results in increased spike frequency of medullary neurons in a one-to-one fashion (Rose et al., 1995) (Figure 4.1). Second, prior studies have confirmed that the sensory responsiveness of these neurons, measured as peak spike frequency, is directly related to the generation and maintenance of clasp (Lewis and Rose, 2002). Third, CORT affects medullary neurons by decreasing spontaneous discharge and sensory responsiveness to clasp-generating stimulation of the cloaca (Rose et al., 1995). Fourth, slice preparation of rat hippocampus (Wilson and Nicoll, 2001) and cerebellum (Kreitzer and Regehr, 2001a; Kreitzer and Regehr, 2001b) reveal that endocannabinoid signaling occurs in a retrograde fashion within a limited spatial area. Fifth, antagonism of endocannabinoid signaling disrupts behavioral responses to acute stress and medullary neurons regulate the clasp behavior.

Given the above observations, we hypothesized that endocannabinoids function to modulate medullary neuron excitability, thereby facilitating the expression of appropriate behavioral responses to acute stress and elevated CORT. If true, then we would expect that pretreatment with CB antagonist, applied directly to the medullary surface, would block the CORT-induced suppression of medullary neuron spontaneous or sensory-induced activity. To

test this prediction we used single-unit recording of medullary neurons in non-anaesthetized immobilized male newts. We monitored two parameters of neuron excitability: spontaneous activity and stimulus-induced activity (also referred to as sensory responsiveness). Spontaneous activity of medullary neurons was defined as the total number of spikes occurring within a 10 s period, 90 s after each stimulus-induced trial. Stimulus-induced activity was produced by applying pressure to the cloacal region, which simulates somatosensory stimulation that occurs when a male newt clasps, and to the exterior forelimb, a non-sexual control sensory stimulus. In this manner, neuronal responses to initiation of pressure (onset) and termination of pressure (offset) was monitored at three-minute intervals during the baseline, pretreatment and treatment periods. Pretreatment period corresponded to the first stable 9-15 min of the experiment in the absence of hormonal treatment. We then delivered the First hormone (AM 281 or VEH control) directly onto the medullary surface 9 min prior to administration of the second hormone (CORT or VEH control) by intraperitoneal injection. The doses of AM 281 (1 μM) and CORT (64 nM) were consistent with concentrations that are effective in modulating behavior and neurophysiology in other vertebrate species (Cosenza, Gifford, Gatley, Pyatt, Liu, Makriyannis, and Volkow, 2000; Soderstrom and Johnson, 2001; Soderstrom et al., 2000). Neuronal activity was analyzed in two different ways: change in the frequency of firing, and change in the number of neurons that increase or decrease due to hormone treatment. Each measurement was normalized to each neuron's own pre-hormone period (baseline).

The magnitude of changes that we observed in medullary neuron activity following hormone treatment regimes was revealed when analyzing the change in frequency of firing of both parameters of neuron excitability, spontaneous activity and stimulus-induced activity. The spontaneous activity of medullary neurons during pre-hormone period averaged 10 – 13 spikes/10 s window, or 1 Hz. We observed no significant change in spontaneous activity as a result of VEH compared with AM 281 ($U = 133$, $P = 0.097$), which indicated that AM 281 alone is not altering the activity of medullary neurons. In contrast, AM/CORT resulted in a 26% increase in median spontaneous activity, which was significantly higher than the other treatment regimes (Figure 4.3a, Table 4.1). This increase suggests that blocking of endocannabinoid signaling results in CORT eliciting a stimulatory effect on unitary activity. This reversal of CORT effects with pretreatment of CB antagonist is similar to the neuronal response that pretreatment with VT has on CORT effects (Rose *et al.*, 1995). The pre-hormone response magnitude of medullary neurons to onset of cloacal stimuli was a peak mean frequency of 15-30 spikes (0.2 s Bin). There was no change in response due to AM 281,

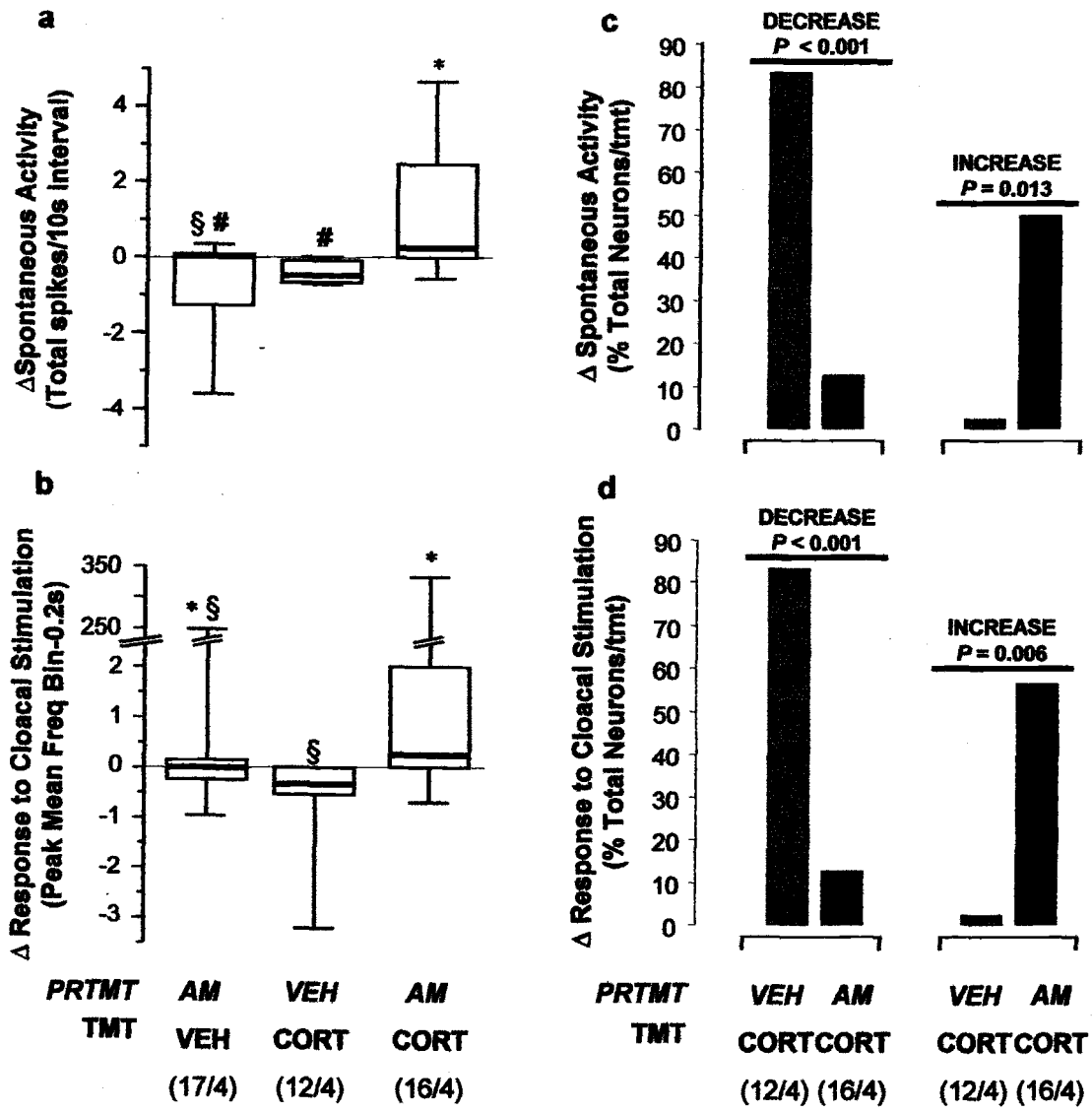

Figure 4.3. Sensory responses to spontaneous activity and onset of cloacal stimulation of medullary neurons were significantly affected by hormone treatment regimes. **a** and **b** Quantitative change in spontaneous activity (**a**; KW = 15.9; $P = 0.0004$) and sensory response to onset of cloacal stimulation (**b**; KW = 14.94; $P = 0.0006$) (see text for methods). Data from **a** and **b** were analyzed using non-parametric ANOVA (Kruskal-Wallis) followed by Dunn's Multiple Comparison Test for pairwise comparisons (Prism). Results are expressed as median (bar) ± 25th and 75th percentile (box); whiskers show the range of the data. Different symbols indicate significant difference compared to all other treatments as determined by Dunn's Multiple Comparison Test. **c** and **d** Comparison of the proportion of neurons observed to decrease or increase in spontaneous activity (**c**; $P < 0.001$) and sensorimotor response to onset of cloacal stimulation (**d**; $P < 0.001$) as a result of hormone treatment. To determine overall significance proportion data from **c** and **d** were analyzed first using Fishers Exact Test, and then pairwise comparisons were made using *z*-tests (SigmaStat). Probability of significance of pairwise comparisons is given above each pair. Total number of neurons / number of *Taricha* are given in parentheses, e.g., 12 neurons total from 4 newts were given VEH/CORT treatment regime.

Table 1: Effects of antagonizing endocannabinoid signaling on CORT-induced inhibition of clasp-regulating medullary neurons

	Spontaneous Activity	Sensory Response to *CLOACAL* Stimuli		Sensory Response to *FORELIMB* Stimuli	
		ONSET	OFFSET	ONSET	OFFSET
Comparison of all Treatments	KW = 14.4, $P = 0.0007$	KW = 14.94, $P = 0.0006$	KW = 13.42, $P = 0.0012$	KW = 7.217, $P = 0.0271$	KW = 13.73, $P = 0.001$
AM/vehicle *VS* Vehicle/CORT	n.s.	n.s.	n.s.	n.s.	$^*P < 0.05$
AM/CORT *VS* AM/VEH	$^*P < 0.05$	n.s.	n.s.	n.s.	$^*P < 0.01$
AM 281/CORT *VS* Vehicle/CORT	$^*P < 0.001$	$^*P < 0.001$	$^*P < 0.001$	$^*P < 0.05$	n.s.

Nonparametric ANOVA, Kruskal-Wallis tests, applied to all treatments are shown with KW-statistic and *P* values. Dunn's multiple comparisons test *P*-values for comparison among all treatments are denoted with an asterisk. See Figure 3 for details on doses, sample size, and statistics.

as compared with vehicle ($U = 166$, $P = 0.4108$). AM/CORT treatment significantly increased the sensory responsiveness to onset (0.26, or 26% increase) and offset (0.765, 76.5%) of cloacal stimuli when compared with VEH/CORT (Figure 4.4B, Table 4.1). Sensory responsiveness to onset and offset of forelimb stimuli was also significantly affected by hormone treatment regimes (Table 4.1). The baseline pre-hormone response of a single unit to onset of forelimb stimuli was 15-20 spikes / 0.2 s Bin. Pretreatment with AM 281 compared with VEH did not alter the median change in response (Mann-Whitney U = 192, $P = 0.8774$). AM/CORT treatment significantly decreased the response to onset but not offset of forelimb stimulation (Table 4.1). Overall, pretreatment with a CB antagonist completely blocks, if not reverses, CORT-induced suppression of spontaneous and sensory-induced activity of clasp-regulating medullary neurons. These findings support the hypothesis that CORT initiates the release of endocannabinoids which then act to suppress medullary neuronal circuits that regulate clasping behavior.

When monitoring the change in the number of neurons that increase or decrease firing following hormone treatment we found that systemic CORT administration, in doses that suppress amplectic clasping, suppressed both measurements of medullary neuron excitability. CORT-induced suppression of medullary neuron activity had a rapid onset (within 2-6 min) and lasted at least 30 min. Of the twelve neurons examined for effects of CORT (as compared with VEH) 83.3 % decreased in spontaneous activity ($z = 3.279$, $P = 0.001$), 83.3 % decreased in response to onset of cloacal stimulation ($z = 3.712$, $P < 0.001$), 66.6% decreased in response to offset of cloacal stimuli ($z = 2.068$, $P = 0.038$), 75 % decreased in response to onset of forelimb stimuli ($z = 2.825$, $P = 0.005$), and 58 % decreased in response to offset of forelimb stimuli ($z = 3.712$, $P < 0.001$). Overall, exogenous CORT rapidly and robustly suppressed medullary neuron spontaneous and sensory-induced activity. The strong CORT-induced suppression of medullary neuron excitability was completely masked, if not reversed, by the pretreatment with the CB antagonist AM 281. When compared with VEH/CORT, AM/CORT treatment significantly reduced the number of neurons decreasing in spontaneous activity and in response to onset of cloacal stimulation to only 16.6% (Figure 4.4C, Table 4.2). In contrast, significantly more neurons increased in spontaneous activity (50 %), response to onset (56 %) and offset (69 %) of cloacal stimulation when AM/CORT was applied (Figure 4.4C, Table 4.1). The differences observed in response to forelimb stimulation were not as dramatic as for cloacal stimulation. Whereas VEH/CORT regime consistently resulted in a majority of neurons decreasing in response to onset (83.3 %) and offset (83.3 %) of forelimb stimulation, pretreatment with AM 281 failed to recover a significant number of neurons from decreasing

FIGURE 4.4. Traces of extracellular recordings from two representative single neuron units. The newt from which neuron **a** was recorded, was treated with VEH followed by CORT, while the newt from which neuron **b** was recorded, was treated with AM 281 followed by CORT. The Y-axis shows the mean spike frequency (0.2s bin). For neurons **a** and **b**, the bottom trace illustrates a full experimental trial starting at time 0 min and extending out to 45 min. To fully illustrate how the sensory responsiveness of neurons changes due to drug manipulation there are three periods enlarged; at pretreatment, after vehicle (VEH) or AM 281 (AM), and after CORT. Application of stimulus, pressure to the cloaca (red bar) or forelimb (blue bar), is indicated by the bars below the traces. The length of the bar indicates the time the pressure was applied from initiation (onset) to termination (offset). Time of drug delivery is indicated as arrows below the trace.

Figure 4.4a

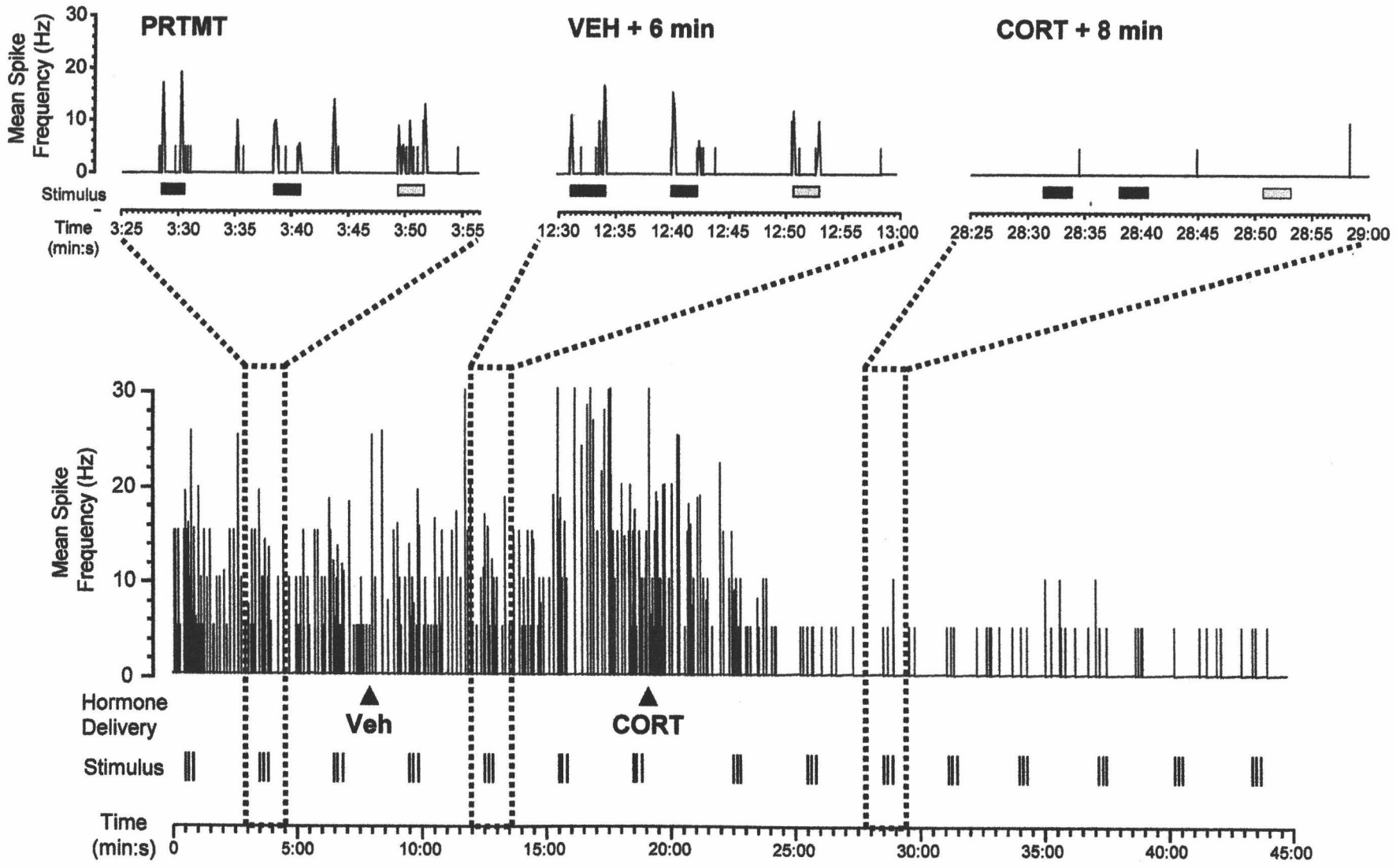
PRTMT
VEH + 6 min
CORT + 8 min
Mean Spike Frequency (Hz)
30
20
10
0
Stimulus
Time (min:s)
3:25
3:30
3:35
3:40
3:45
3:50
3:55
12:30
12:35
12:40
12:45
12:50
12:55
13:00
28:25
28:30
28:35
28:40
28:45
28:50
28:55
29:00
Hormone Delivery
Veh
CORT
0
5:00
10:00
15:00
20:00
25:00
30:00
35:00
40:00
45:00

Figure 4.4b

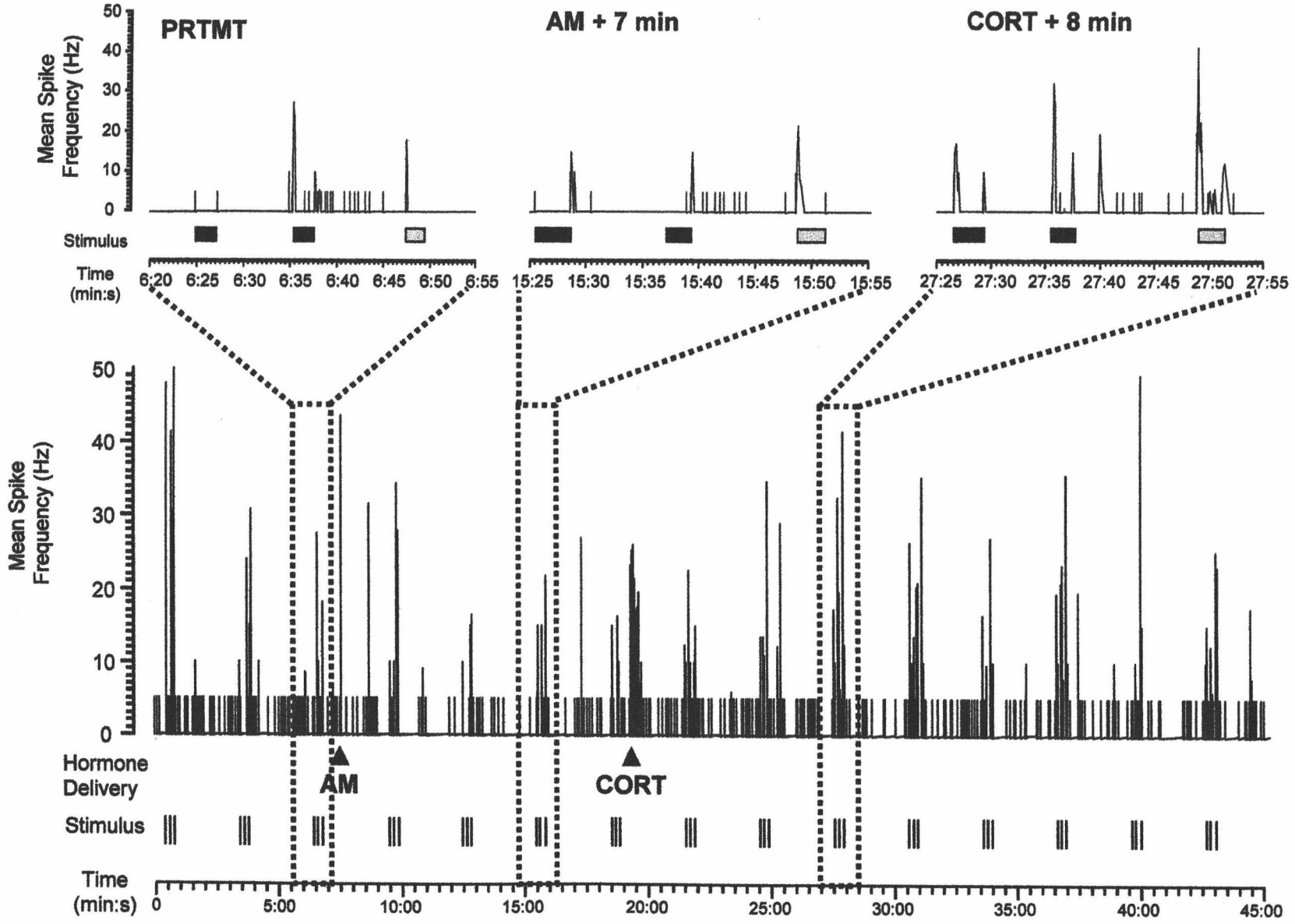
PRTMT
AM + 7 min
CORT + 8 min
Mean Spike Frequency (Hz)
Stimulus
Time (min:s)
6:20 6:25 6:30 6:35 6:40 6:45 6:50 6:55
15:25 15:30 15:35 15:40 15:45 15:50 15:55
27:25 27:30 27:35 27:40 27:45 27:50 27:55
Hormone Delivery
AM
CORT
0 5:00 10:00 15:00 20:00 25:00 30:00 35:00 40:00 45:00

Table 2: The proportion of medullary neurons exhibiting an increase or decrease in activity or responsiveness due to hormone treatments.

	Spontaneous Activity		Sensory Response to *CLOACAL* Stimuli				Sensory Response to *FORELIMB* Stimuli			
			ONSET		OFFSET		ONSET		OFFSET	
	Increase	**Decrease**	**Increase**	**Decrease**	**Increase**	**Decrease**	**Increase**	**Decrease**	**Increase**	**Decrease**
AM/veh *VS* Veh/CORT	n.s.	$P = 0.029$	n.s.	n.s.	n.s.	n.s.	n.s.	$P = 0.029$	n.s.	$P = 0.005$
AM/CORT *VS* AM/Veh	$P = 0.014$	n.s.	n.s.	n.s.	$P = 0.025$	n.s.	n.s.	n.s.	n.s.	n.s.
AM/CORT *VS* Veh/CORT	$P = 0.013$	$P < 0.001$	$P = 0.006$	$P < 0.001$	$P < 0.001$	$P = 0.011$	n.s.	n.s.	n.s.	$P = 0.003$

Comparisons of proportions were made using pair-wise z – tests. P values for pair-wise comparisons are shown. A stringent quantitative criterion was used to assess effects of hormone on unit sensory responses and activity levels; In order for a unit's response to cloacal stimulus to be designated as increased or decreased by hormone action, the peak firing rate during the repeated applications of that stimulus had to be uniformly higher or lower than the responses to a comparable series of stimulus applications at baseline prior to the administration of the hormone. See Figure 3 for details on doses.

in response to onset of forelimb stimulation when AM/CORT was applied. Response to offset of forelimb stimulation, in contrast, was recovered when AM/CORT was applied (Table 4.2). That the proportion of neurons exhibiting a CORT-induced suppression in activity and sensory responsiveness is blocked and in most cases reversed by the pretreatment with a CB antagonist suggests that CORT-induced inhibition of clasp-generating circuits requires signaling of endocannabinoids downstream in the temporal events initiated by acute stress and elevated CORT.

Behavioral and electrophysiological experiments show that, in this species, the neuroendocrine pathway controlling stress-induced suppression of reproductive behaviors involves the following steps: a detected threat elicits an increase in plasma CORT; in turn, CORT increases endocannabinoid activity at CB1 receptors, and endocannabinoids suppress medullary neuron responses to sexually relevant somatosensory information. This neuroendocrine cascade may be involved in regulating the behavioral response to acute threats of other vertebrates such as frogs, rodents or primates, all of which exhibit acutely suppressed reproductive behaviors when presented with an immediate threat (Burmeister et al., 2001; Habib et al., 2000; Hulse and Coleman, 1983; Lumley et al., 1999). The CB1 receptor is prevalent and highly expressed with a conservative gene/peptide sequence and pharmacological binding profile in many different species of vertebrates (Elphick and Egertova, 2001) and cannabinoid actions in invertebrate species (Salzet and Stefano, 2002). It is clear that endocannabinoid signaling is an important basic process, but it has been mechanistically examined only in truncated preparations where the broader functional significance is very difficult to validate. The newt model allows analysis from cellular to behavioral levels with a validated understanding of function across multiple levels of organization. When viewed in this way, the function of endocannabinoid signaling becomes understandable as an integral part of other neuroendocrine signaling processes.

METHODS

Animals

Sexually active adult male newts (*Taricha granulosa*) were collected during the height of the breeding season (February - March, 2002 - 03) for use in behavior experiments, and adult male newts were collected in April 2002 at the end of the breeding season for use in electrophysiology experiments. Males were collected locally from the perimeters of permanent ponds in the Coast Range (Benton Co., Oregon) using dip-nets, and were held in community tanks supplied with continuously flowing, aerated and dechlorinated water and fed an excess of chopped earthworms. Newts were housed in an environmentally controlled room with natural photoperiod (14L:10D) and temperature (average 13°C). Males weighed, on average, 12 ± 0.5 g. Females were captured during migration to breeding ponds by hand. Females were housed together in terraria (12 / tank) with damp moss and leaf-litter at one end and water in the other. They were maintained in breeding condition, and therefore attractive to the males, by injections of prolactin (0.5 IU / 0.1 ml / newt) administered intraperitoneally every second day for the duration of their stay in captivity (maximum 2 months), during which time the females were fed an abundant supply of chopped earthworms. For the electrophysiology experiments, adult males were housed in Laramie, WY, in community tanks supplied with continuously flowing, aerated water and fed chopped beef heart. Room lighting was regulated to simulate the natural photoperiod. The experiments were conducted in April and May 2003.

Hormones Administered

CORT (Sigma-Aldrich Inc., St. Louis, MO, USA) was solubolized in 2% DMSO (dimethyl sulfoxide; Sigma-Aldrich), 8% EtOH and 90 % Amphibian Ringers for intraperitoneal administration of 40 μg/0.1 ml /newt (2.6 mg / kg) for behavioral studies. Electrophysiological studies were performed towards the end of the breeding season when male *Taricha* are more sensitive to CORT. Therefore, a smaller dose of CORT was injected, 22 μg / 0.1 ml / newt (1.46 mg / kg). AM 281 (Tocris Cookson Inc., Ellisville, MO, USA) was solubolized in 2% DMSO + 98% Amphibian Ringers solution for intraperitoneal injection of 0.5 or 5 μg /0.1 ml / newt (0.03 mg / kg or 0.3 mg / kg) for behavioral studies. For electrophysiological studies, 5 μl of 100 nM AM 281 in 0.1% DMSO solution was applied directly to the medullary surface. From prior studies it is known that *Taricha* express a functional CB1 receptor which binds ligands with a rank order affinity consistent with the profile observed for mammalian species (Soderstrom et al., 2000).

Behavior Experiments

All behavioral testing was performed between 1400 and 2000 h on males collected within a 24 – 48 h period. Males were tested individually in circular tanks (27 cm diameter) filled to a depth of 6 cm with dechlorinated water, maintained in low-light conditions within a specified arena (3m X 3m) enclosed by a curtain of black plastic hung from the ceiling to the floor. Individual males were transferred from holding tanks into separate testing tanks 30 min prior to any injections. In the experiment where two injections were given, injections were separated in time by 10 min. Thirty min after the second injection, two females were introduced to each male. Incidence of clasping and latency to clasp was determined during the experiment, and the estimate of activity was determined later from video tape, recorded with a low-light video camera. Incidence of clasping was analyzed as proportion of males observed clasping at 15 min after a female was added to the arena. An estimate of activity was determined by counting the number of lines crossed within a 3 min time period before and after hormone treatment. To induce standardized, acute stress, males were placed in a clear dry plastic container (8 cm x 3 cm x 3 cm) and tumbled by hand 180° forward and 180° sideways every 0.5 s for 15 min (unless otherwise specified).

Electrophysiology

Surgery & animal preparation

Newts were anesthetized by immersion in 0.1% MS-222 for 40 – 75 min to allow for surgical exposure of the caudal brainstem and placement of recording electrodes. The head was stabilized by securing a small stainless steel screw to the most rostral aspect of the skull, and by dental cementing of the screw to the surface via a steel rod. After surgery, males were partially immersed in well water (T = 16-17 °C). Each male's torso and tail were covered with cellulose paper to keep the skin moist and facilitate transcutaneous respiration while it's eyes were covered with damp cotton to remove any stimulation by light or movement. General anesthesia depresses the central nervous system activity in amphibians. Therefore, post-surgery, males were removed from the anesthetic and newts were immobilized with gallamine triethiodide (0.2 – 0.6 ml of a 2% solution / newt) which specifically blocks transmission at the neuromuscular junction. Data collection was not begun until sufficient time (approximately 2-3 hours) had elapsed to allow the newt to recover from the anesthetic. A concern about this experimental protocol is that the newts might be distressed, and therefore have elevated CORT levels. While it is not feasible to repeatedly draw blood from newts to

determine CORT levels during experiments such as these, the following observations indicated that the newts were not distressed by pharmacological immobilization or recording procedures. First, when the effect of the myoneural blocker was permitted to wane periodically, little movement occurred in the absence of sensory stimulation to the skin and there was little evidence of discharge indicative of 'fictive movement'. Second, if these males were distressed and therefore experiencing a substantial elevation of CORT, the circulating hormone would have attenuated or completely masked the CORT-induced inhibition of sensorimotor responses and spontaneous activity of the medullary neurons. Our results suggest that this was not the case.

Recording Procedures

Individual neurons were recorded extracellularly with tungsten microelectrodes by means of conventional amplification and displayed on a digital oscilloscope. Most neurons observed expressed low basal levels of spontaneous activity and responded to sensory input to both the forelimb and cloaca. Cloacal and forelimb stimuli consisted of applying constant pressure to the cloaca then 20 s later the forelimb, for 3 seconds each, with a modified force transducer, allowing us to monitor the onset, duration, and offset of the stimulus. Each stimulation bout (cloaca + forelimb) was applied every 3 min for the duration of the experiment. The intensity of the cloacal stimuli would have been sufficient to elicit reflexive clasping behavior in nonimmobilized newts. This tactile stimulation is considered below the threshold for activating nociceptive effects based on behavioral observations of untreated newts when presented with the equivalent stimulus. At least three stimulation bouts were given before the pretreatment hormone was applied. Then at least three stimulation bouts were delivered before the treatment hormone was applied. After the application of the treatment hormone, up to 14 stimulation bouts were given.

Analysis of Electrophysiological Data

The unit activity, pressure transducer signals, and a voice narrative were recorded on video tape by means of a pulse-code modulation device (Neurodata Neurorecorder). At all recording sites, spikes were recorded from more than one neuron. The spikes from individual neurons were identified with a template matching program (SPIKE II). In order to assess the stability of unit isolation during application of solutions to the medullary surface or i.p. injections of gallamine, single traces and averages of multiple spikes were acquired on a

digital oscilloscope throughout the duration of the experiment. Hard copies were printed and scrutinized for any variation on spike amplitude or overall shape.

The spontaneous activity and sensory responses of neurons were analyzed from chart records showing integrated firing rates, as well as stimulus pressure transducer signals. The sensorimotor responsiveness to onset and offset of cloacal and forelimb stimulation (a control tactile sensory input) was monitored every three minutes during the baseline (before hormone administration), and during the two hormone treatments. Responsiveness was recorded as the absolute peak firing rate that corresponded to the time of onset and offset (± 0.2 s) of cloacal and forelimb stimulation. Spontaneous activity of medullary neurons was also monitored throughout the experiments as a measure of overall unit excitability. This activity was defined as the total number of spontaneous spikes observed for each unit in a 10s time window at 90s after each stimulation bout. To account for individual unit and newt differences we determined the mean of three stimulus bouts at baseline, three after pretreatment, and four after treatment, and then calculated the difference between treatment and pretreatment, normalizing to individual unit baseline.

As in previous studies (Rose et al., 1995; Rose et al., 1998; Rose et al., 1993), the responses of medullary neurons to sensory stimuli tended to be very prominent and often occurred against a background of little to nil spontaneous discharge. As a consequence of this feature, we also applied a stringent quantitative criterion to assess the direction of hormone effects on unit sensory responses and activity levels. In order for a unit's response to a given stimulus to have been designated as increased or decreased by hormone action, the peak firing rate during the repeated applications of the stimulus had to be uniformly higher or lower, respectively, than the responses to a comparable series of stimulus bouts prior to the administration of the hormone. Units that did not fit either increase or decrease criteria were designated no change. In this manner, we can allow for differences in the variability of response parameters among individual neurons. To specifically determine whether pretreatment with cannabinoid antagonist blocks the CORT-induced inhibition of neuron responses and activity, we compared the proportion of neurons decreasing or increasing in response or activity under the two treatment regimes, CP/VT and VEH/VT, using *z*-tests (SYSTAT Inc.).

To specifically determine whether CORT inhibited each parameter we compared the proportion of neurons that increased or decreased after VEH to the proportion of neurons after CORT using *z*-tests (SIGMASTAT Inc.). Then, to determine whether pretreatment with a CB antagonist blocks the CORT-induced inhibition of neuron responses or spontaneous activity,

we compared the proportion of neurons decreasing or increasing in response or activity under two treatment regimes, AM/CORT and VEH/CORT, using *z*-tests. Figures 4.3c and d illustrate analyses comparing the number of neurons increasing or decreasing in their spontaneous activity or capacity to respond to onset of cloacal stimuli. Table 4.2 shows the statistical results from all other comparisons.

To determine an overall sense of the magnitude of change occurring to the function of these neurons as a result of hormone treatment we also statistically analyzed absolute numbers from parameters monitored by recording the absolute numbers of total firing (spontaneous activity, SA) and peak firing (response to stimuli), and then determined the mean of three stimulus bouts at baseline, three after pretreatment, and four-five after treatment, normalized the mean of pretreatment and treatment by dividing each by their individual baseline, and then subtracted treatment from pretreatment. Effects of CB antagonist compared to VEH were determined using Mann Whitney U test. Overall differences among treatments were determined by performing non-parametric ANOVA (Kruskal-Wallis) on the difference between normalized pretreatment and treatment for each parameter measured. The data shown in Figures 3a and b illustrate the changes to the spontaneous activity and sensorimotor responses to onset of cloacal stimulation, as these are representative of the overall pattern of changes observed for all measurements. Statistical results from all measurements, including sensorimotor responses to offset of cloacal stimulation and onset and offset of forelimb stimulation, are shown in Table 1. These experiments included internal controls; the comparison of hormone effect on neuron parameters to the baseline of each neuron.

Plasma Assays

Immediately following the standardized acute stress, males were sacrificed and blood collected from the carotid arteries into tubes containing 3% heparin (Sigma-Aldrich). Samples were kept on ice until centrifuged at 12000 g for 15 min. Plasma was collected and stored at -80°C. RIA assays were performed by Dr. David Hess, Oregon Health Sciences University, Oregon. H_2O blank = 6.1 pg, intraassay % CV = 5.7, interassay % CV = 10.6.

CHAPTER 5: CANNABINOID AGONIST BLOCKS THE ENHANCING EFFECTS OF VASOTOCIN ON SEX BEHAVIORS AND NEURONAL ACTIVITY IN THE HINDBRAIN OF AN AMPHIBIAN.

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ABSTRACT

Appropriate behavioral responses to threatening situations depend on an animal's environmental, physiological and behavioral context. Previous studies in *Taricha* have shown that context can be defined by the endocrine milieu of the neuropeptide vasotocin (VT) and the stress steroid corticosterone (CORT). Courtship behaviors are enhanced by VT and suppressed by CORT. However, these two hormones interact such that pretreatment with VT blocks CORT's suppressive effects, and pretreatment with CORT blocks VT's stimulatory effects on courtship. We investigated the neurochemical mechanisms that mediate this VT-CORT interaction, specifically addressing the question of whether an endogenous cannabinoid system is involved. The interaction between VT and CORT occurs within at least one defined spatial area, the medulla, and has an important temporal component such that the effect of each hormone is contingent upon which hormone arrives first. Our behavioral and electrophysiological studies are consistent with the hypothesis that the behavioral effects of VT-CORT interactions are transduced by an endocannabinoid signaling system. These studies provide the first link between a natural behavior and endocannabinoid function. The unique ability of endocannabinoids to function as neuroendocrine coincidence detectors provides a novel mechanism of endocrine regulation.

INTRODUCTION

Behavioral effects of the neuropeptide vasotocin (VT) and its mammalian homologue vasopressin (VP) have been extensively studied in a wide variety of vertebrates. A large body of literature documents the diverse effects of exogenous VT/VP upon a variety of social behaviors (Goodson and Bass, 2001), and describes the neuroanatomical distributions of VT/VP containing cells (Goodson and Bass, 2001; Moore and Lowry, 1998). Nevertheless, the neural mechanism by which VT/VP functions to regulate behaviors has remained to be established. Studies using the roughskin newt, *Taricha granulosa*, allow analysis across multiple levels with a validated understanding of VT function. We utilized the newt model to test the hypothesis that the behavioral effects of VT are state-dependent, and that one of the mechanisms by which this occurs is by cannabinoid action at cannabinoid (CB_1) receptors.

Prior studies in male *Taricha* have shown that VT influences clasping behavior. Systemic or intracerebroventricular (ICV) injection of VT increases the incidence of clasping behaviors in male *Taricha* (Moore and Miller, 1984). ICV injections with anti-VP serum or V1 receptor antagonists decrease the incidence of clasping behaviors in sexually active newts (Moore and Miller, 1984). Furthermore, the levels of VT in cerebrospinal fluid, dorsal preoptic area, optic tectum, and ventral infundibular nucleus correlate with a male's behavioral state (Zoeller and Moore, 1988). Single-unit recordings made in the rostroventral medulla reveal that VT administration enhances the spontaneous activity and sensory responsiveness of medullary neurons (Rose et al., 1995).

The behavioral effect of VT is determined by the current behavioral and neuroendocrine state of the animal. Corticosterone (CORT) administration, 10 min prior to VT, blocks the VT-induced enhancement of clasping behavior (Coddington and Moore, 2003). These behavioral data are consistent with electrophysiology studies examining the hindbrain neural-circuitry regulating clasping; prior administration of CORT blocks the robust VT-induced enhancement of medullary neuron excitability (Rose et al., 1995). The suppressive effects of CORT occur by upregulating signaling of endogenous cannabinoids (eCB). Pretreatment of male *Taricha* with a CB_1 receptor antagonist, AM 281, blocks CORT-induced suppression of clasping behavior and sensory responsiveness of medullary neurons (Coddington, n.d.). Studies with rats show a similar phenomenon. Whole-cell patch-clamp recordings in acute hypothalamic slice preparations revealed that CB_1 receptor antagonists AM 281 and AM 251 blocked the reduction in glutamate release induced by dexamethasone (a synthetic glucocorticoid) (Di et al., 2003). These studies with *Taricha* and rats suggest that endogenous cannabinoid signaling occurs downstream in the temporal events initiated by

glucocorticoids. Given that elevated CORT blocks VT-induced enhancement of clasping and medullary neuron sensory responsiveness, and that the CORT-induced suppression of clasping is blocked by CB_1 receptor antagonists, we hypothesized that administration of a CB receptor agonist, CP-55940, will block the VT-induced enhancement of clasping and the medullary neurons regulating clasping.

That synthetic cannabinoids are binding to specific cannabinoid receptors in the *Taricha* brain has been established in prior studies (Soderstrom et al., 2000). Radioligand binding studies using (^{3}H)CP-55940 identified a specific binding site in *Taricha* brain membranes. This binding site was pharmacologically characterized to bind ligands consistent with that reported in mammalian species; the rank order of affinity was CP-55940 > levonantradol > WIN 55212-2 >> anandamide. The *Taricha* CB_1 receptor was cloned and its mRNA was found to be highly expressed in the *Taricha* brain. Consequently, the amphibian CB_1 receptor is very similar in density, ligand binding affinity, ligand binding specificity, and in amino acid sequence to mammalian CB_1. Therefore, we have confidence that the synthetic ligands we apply to *Taricha* are binding to specific CB_1 receptors.

The overall goal was to determine the function of endocannabinoid signaling in the context an integrated neuroendocrine behavioral system. We used synthetic ligands to address the following principle objectives. First, to determine whether exogenous administration of a cannabinoid agonist suppresses clasping behavior, and whether pretreatment of males with cannabinoid agonist blocks VT-induced enhancement of clasping behavior. To date there have been no reports on the role of cannabinoids in regulating the actions of VT or VP in any animal species. Second, to obtain a preliminary characterization of the neurophysiological actions of a cannabinoid agonist on medullary neurons engaged in processing somatosensory information relevant to clasping. While cellular mechanisms of endocannabinoid signaling have been extensively studied in mammalian neural systems, these investigations have been limited to truncated in vitro preparations where the broader functional significance is very difficult to validate. The newt model provides for analysis from behavioral to cellular levels, with a solid understanding of function across multiple levels of organization. Third, to determine whether pretreatment with CB receptor agonist suppresses VT-induced enhancement of spontaneous and sensory-associated activity.

METHODS

The original research reported herein was performed in accordance with the U.S. Public Health Service's *Guide to the Care and Use of Laboratory Animals.*

Animals

Sexually active adult male newts (*Taricha granulosa*) were collected towards the end of the breeding season (April, 2002 - 03) for use in behavior experiments and other males were collected in June 2002 (out of breeding condition) for use in electrophysiology experiments. *Taricha* were collected locally from the perimeters of permanent ponds in the Coast Range (Benton Co., Oregon) using dip-nets, and were held in community tanks supplied with continuously flowing, aerated dechlorinated water and fed an excess of chopped earthworms. Newts were housed in an environmentally controlled room with natural photoperiod (14L:10D) and temperature (average 13°C). Males weighed, on average, 14 ± 0.5 g. Females were captured by hand during migration to breeding ponds. Females were housed together in terraria (12 / tank) with damp moss and leaf-litter at one end and water in the other. They were maintained in breeding condition (and therefore attractive to the males) by injections of prolactin (0.5 IU / 0.1 ml / newt) administered intraperitoneally every other day for the duration of their stay in captivity (2 weeks - 2 months). Females were provided with an abundant supply of chopped earthworms. These collecting and maintenance protocols allowed for capture of males still in breeding condition (but at the tail end of their breeding season) and the capture of unmated females that were sexually attractive to males (Propper, 1991). For the electrophysiology experiments, adult males were housed in Laramie, WY, in community tanks supplied with continuously flowing, aerated water and fed chopped beef heart and earthworms. Room lighting was regulated to simulate the natural photoperiod. The experiments were conducted in July, 2002.

Behavioral Testing

All behavioral testing was performed between 1400 and 2000 h on males collected recently within the prior 48 h period. Individual males were tested in circular tanks (27 cm diameter) filled to a depth of 6 cm with dechlorinated water. These testing tanks were kept in low-light conditions within a testing arena (3m X 3m) enclosed by a curtain of black plastic hung from the ceiling to the floor. Individual males were transferred from holding tanks into separate testing tanks 30 min prior to any injections and behavioral testing. In experiments involving one injection, females were introduced to the arena 30 minutes after the injection.

In experiments with two injections, injections were separated in time by 10 min, and then the females were introduced 30 min after the second injection. Incidence of clasping and time spent clasping was recorded by a trained observer who was blind to which males received each treatment. Locomotor activity was determined from video tape, recorded with a low-light video camera. Incidence of clasping reflects the proportion of males observed clasping at 15 min after females were introduced.

Hormones Administered

Behavioral experiments used VT (0 or 100 μg / 0.1 ml / newt) and CP-55940 (0, 5, 10, 50 μg / 0.1 ml / newt which corresponds to 0, 130, 260, 1230 μM). (Arg8)-VT was purchased from Bachem Bioscience Inc. (King of Prussia, PA, USA). VT solution was prepared with amphibian Ringers solution in silicone-coated glassware (Sigmacoat, Sigma-Aldrich), divided into 1 ml aliquots, snap frozen in dry ice, and stored at -80°C until use. A different vehicle control solution (amphibian Ringers) for VT effect was prepared simultaneously and stored in the same manner as VT stock. CP-55940 was purchased from Tocris Cookson Ltd. (Bristol, UK). CP-55940 solution was prepared with 98% amphibian Ringers and 2% DMSO (dimethyl sulfoxide; Sigma-Aldrich, St Louis, MO), and stored at 4 °C. Vehicle to control for CP-55940 effects was prepared simultaneously with 98% amphibian Ringers and 2% DMSO. Electrophysiology experiments applied VT (1 dose = 80 ng/ 4μl Amphibian Ringers) and CP-55940 (100nM, 0.1% DMSO) directly to the fourth ventricle and were therefore applied at lower concentrations.

Behavior Experiments

CP-55940 dose response

To determine whether a cannabinoid agonist that binds CB_1 receptors affects courtship behaviors, a dose response experiment was performed using CP-55940. Based on preliminary experiments behavioral responses at four doses were examined; 0, 5, 10, 50 *μg* / 0.1 ml / newt (0, 130, 260, or 1230 *μM*). A total of 16 males were examined per treatment.

CP-55940 co-administered with VT

To determine whether cannabinoids blocks the VT-induced enhancement of clasping behavior we co-administered a mixture of CP-55940 (5 μg / 0.1 ml / newt = 130 *μM*; 2% DMSO) with VT (100 μg / 0.1 ml Ringers / newt). Control groups were CP-55940 alone, VT

alone, or vehicle (2% DMSO in Amphibian Ringers). A total of 20 males were examined per treatment.

CP-5540 Administered 10 min prior to VT

Based on results from a prior electrophysiology study, the VT-induced enhancement of medullary neuron activity was most noticeably blocked by CORT when CORT was administered at least 10 min prior to VT (Rose et al., 1995). Therefore, we separated the administration of the cannabinoid agonist, CP-55940 (5 μg / 0.1 ml Ringers / newt = 130 μM; 2% DMSO) from VT (100 μg / 0.1 ml Ringers / newt) by 10 min. A total of 80 males were assigned to one of four treatment groups, as follows (pretreatment/treatment): VEH/VEH, CP/VEH, VEH/VT, and CP/VT.

Statistical Analysis

Non-parametric statistical tests were used because data were skewed and variances were heterogeneous. The "time spent clasping" data were analyzed using Kruskal-Wallis (KW) non-parametric analysis of variance ($\alpha = 0.05$) and, after finding significant differences with KW tests, comparisons among groups were evaluated with Dunn's Multiple Comparisons Test ($\alpha \leq 0.05$).

Electrophysiology Experiments

Surgery & animal preparation

Newts were anaesthetized by immersion in 0.1% MS-222 for the duration of the surgery, 40 - 75 min. The caudal brainstem was exposed for placement of recording electrodes. The head was stabilized by securing a small stainless steel screw to the most rostral aspect of the skull, and dental cementing the screw to the surface via a steel rod. After the surgery was complete males were partially immersed in well water (T= 16-17 °C). Their torso and tail were covered with cellulose paper to keep the skin moist and facilitate transcutaneous respiration, while their eyes were covered with dampened cotton wool to block visual stimuli. General anesthesia depresses the central nervous system activity in amphibians. Therefore, post-surgery, males were removed from the anesthetic and newts were immobilized with gallamine triethiodide (0.2 – 0.6 ml of a 2% solution / newt, intraperitoneal injection), which specifically blocks transmission at the neuromuscular junction. Data collection was not begun until sufficient time (approximately 2-3 hours) had elapsed to allow

the newt to recover from the anesthetic. Prior studies indicate that these experimental procedures do not cause physiological stress or elevated plasma CORT (Rose et al., 1995).

Recording Procedures

Extracellular recordings from individual neurons were made with tungsten microelectrodes, amplified and displayed on a digital oscilloscope. Spontaneously active neurons were recorded as well as neurons that responded to tactile sensory pressure of the forelimb and cloaca. Most neurons observed expressed low basal levels of spontaneous activity, and responded to sensory input to both the forelimb and cloaca. Cloacal and forelimb stimuli consisted of applying constant pressure to the cloacal region then 20 s later the forelimb, for 3 seconds each, with a modified force transducer, allowing us to monitor the onset, duration, and offset of neuronal responses to somatosensory stimuli. Each stimulation bout (cloaca + forelimb) was applied every 3 min for the duration of the experiment. Prior studies determined that this somatosensory stimulation elicits reflexive clasping behavior in nonimmobilized newts (Rose and Moore, 1999; Rose et al., 1993). This tactile pressure is considered below the threshold for activating nociceptive effects based on behavioral observations of newts when presented with the equivalent stimulus. At least three stimulation bouts were given before the first treatment hormone (VEH or CP-55940), and then at least three stimulation bouts were delivered before the second treatment (VEH or VT). Up to 14 stimulation bouts were given after the application of the second treatment.

Analysis of Electrophysiological Data

The unit activity, pressure transducer signals, and a voice narrative were recorded on video tape by means of a pulse-code modulation device (Neurodata Neurorecorder). At most recording sites, spikes were recorded from more than one neuron. The spikes from individual neurons were identified with a template matching program (SPIKE II). In order to asses the stability of unit isolation during application of solutions to the medullary surface or i.p. injections of gallamine, single traces and averages of multiple spikes were acquired on a digital oscilloscope throughout the duration of the experiment. Hard copies printed and scrutinized for any variation on spike amplitude, or overall shape.

The spontaneous activity and sensory responses of neurons were analyzed from chart records showing integrated firing rates, as well as stimulus pressure transducer signals. The sensorimotor responsiveness to onset and offset of cloacal and forelimb pressure (a control tactile sensory input) was monitored every three minutes during the baseline (before hormone

administration), and two hormone treatments, and was recorded as the absolute peak firing rate that corresponded to the time of onset and offset (± 0.2 s) of cloacal and forelimb pressure. Spontaneous activity of medullary neurons was also monitored throughout the experiments as a measure of overall unit excitability; and was defined as the total number of spontaneous spikes observed for each unit in a 10s time window at 90s after each stimulation bout. To account for individual unit and newt differences we determined the mean of three stimulus bouts at baseline, three after pretreatment, and four after treatment, and then calculated the difference between treatment and pretreatment, normalizing to individual unit baseline. Overall effects of hormone manipulation on spontaneous activity and sensory responsiveness to onset and offset of cloacal and forelimb pressure were determined using Mann Whitney U tests.

As in previous studies (Rose et al., 1995; Rose et al., 1993), the responses of medullary neurons to sensory stimuli tended to be very prominent and often occurred against a background of little to nil spontaneous discharge. As a consequence of this feature, no quantitative response criterion was required for identification of sensory responses. A stringent quantitative criterion was employed, however, for the assessment of hormone effects on unit sensory responses and activity levels. In order for a unit's response to a given stimulus to have been designated as increased or decreased by hormone action, the peak firing rate during the repeated applications of the stimulus had to be uniformly higher or lower, respectively, than the responses to a comparable series of stimulus bouts prior to the administration of the hormone. Unit's that did not fit either increase or decrease criteria were designated no change. To specifically determine whether pretreatment with cannabinoid antagonist blocks the CORT-induced inhibition of neuron responses and activity we compared the proportion of neurons decreasing or increasing in response or activity under the two treatment regimes, CP/VT and VEH/VT, using *z*-tests (SYSTAT Inc.).

RESULTS

Behavior Experiments

CP-55940 dose response

Courtship clasping was inhibited by intraperitoneal administration of the synthetic cannabinoid CP-55940 in a dose-dependent manner (KW = 13.49, $P = 0.0037$) (Figure 5.1A). Dunn's Multiple Comparison test revealed that the incidence of males clasping was significantly reduced at the highest dose (1.23 mM) where only 18.75% males were observed clasping compared to 81.5% males clasping with control injection ($P<0.05$). Consistent with these data, latency to engage in clasping was also significantly inhibited in a dose dependent manner by CP-55940 (F = 4.16, $P = 0.0096$), and Bonferroni's multiple comparisons test revealed that the highest dose was significantly different compared to control group ($P< 0.05$) (Figure 5.1B). Locomotor activity measures of were also significantly reduced with increasing dose of CP-55940 (F = 2.977, $P= 0.0385$). Bonferroni's multiple comparison analysis revealed that the only treatment group that resulted in a significantly different outcome compared with control was 0.26 mM dose ($P < 0.05$) (Figure 5.1C).

CP-55940 co-administered with VT

The proportion of males observed clasping significantly differed with hormone treatment (KW = 30.24, $P < 0.0001$). Dunn's multiple comparison test revealed that co-administration of CP&VT ($P < 0.01$) and administration of CP ($P < 0.01$) significantly reduced the number of males observed clasping compared with VT treatment (Figure 5.2A). Latency to engage in clasping was also significantly inhibited by hormone treatment (KW = 26.65, $P < 0.0001$), and the treatments CP&VT ($P < 0.01$) and CP ($P < 0.01$) significantly increased latency to engage in clasping with respect to VT treatment (Figure 5.2B). Activity was not affected by hormone treatments (F = 0.068, $P = 0.4599$).

CP-5540 Administered 10 min prior to VT

When 10 min separated of the administration of CP and VT, we observed a significant effect of Pretreatment-Treatment (PreTMT/TMT) upon proportion of clasping males observed (KW = 43.83, $P < 0.0001$) and latency to engage in clasping (KW = 14.4, $P = 0.0024$) (Figure 5.3). Dunn's multiple comparison test revealed that the number of males observed clasping after receiving Veh-Veh, CP-Veh or CP-VT were not significantly different from each other. A quarter of the males receiving Veh-Veh were observed clasping, and even fewer males were observed clasping when treated with CP-Veh (0 %) or CP-VT (4%). In

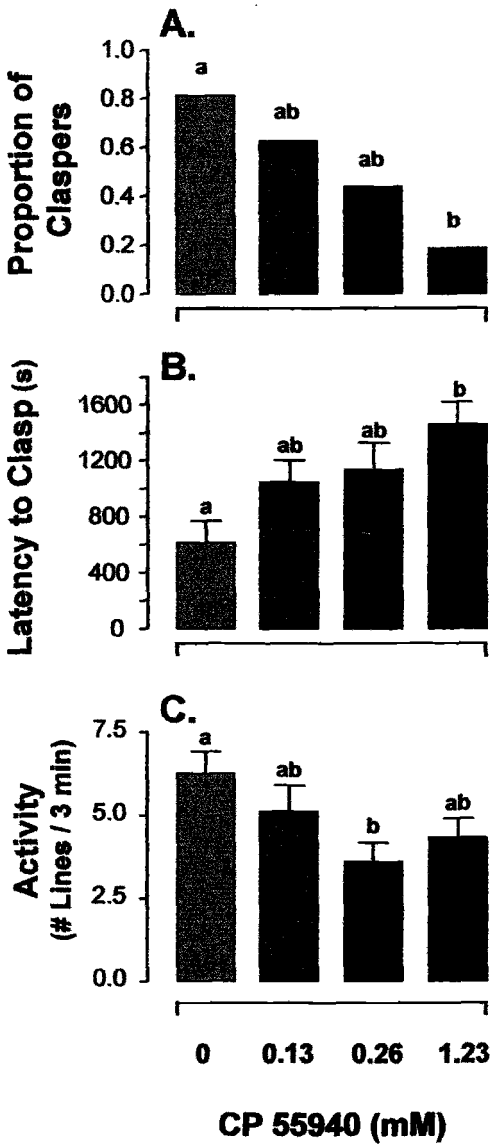


Figure 5.1. Courtship behaviors are inhibited by intraperitoneal administration of synthetic cannabinoid, CP-55940, in a dose dependent manner. Males were tested during the height of the breeding season, early March, to ensure high incidence of clasping. Data are given as either proportion or mean ± se. Different letter's indicate significant difference, α=0.05. **A** Incidence of males clasping during the 40 min period after receiving CP-55940, reported as proportion of males observed clasping (N=16/tmt). **B** Latency to initiate a successful clasp is determined as the time between females being introduced to the test arena and time the male initiates clasping. **C** Effect of CP-55940 on locomotor activity of males was estimated by counting number of lines crossed in a 3 min time frame 7 min after CP-55940 administration, before females were introduced.

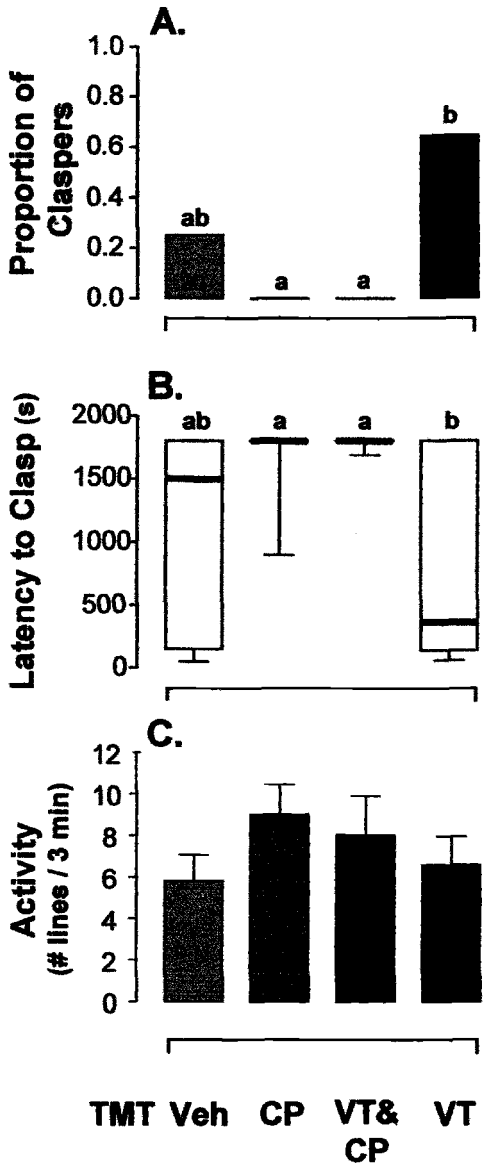


Figure 5.2. The enhancing effect of VT on courtship behaviors is blocked by co-administration of CP-55940. Males were tested towards the end of the breeding season, April 2001, when the incidence of clasping is low and the enhancing effect of VT can be observed. Data had heterogeneous variances and are therefore shown as proportion or box and whisker plots of median (thick bar), interquartiles (box), and ranges (whiskers). **A** Incidence of males clasping during the 40 min period after receiving hormone treatment, reported as proportion of males observed clasping (N=20/tmt). **B** Latency to initiate a successful clasp is determined as the time between females being introduced to the test arena and the time male initiates clasping. **C** Effect of hormone treatment on activity of males was estimated by the difference in the number of lines crossed in a 3 min time frame before and 7 min after hormone treatment, before females were introduced.

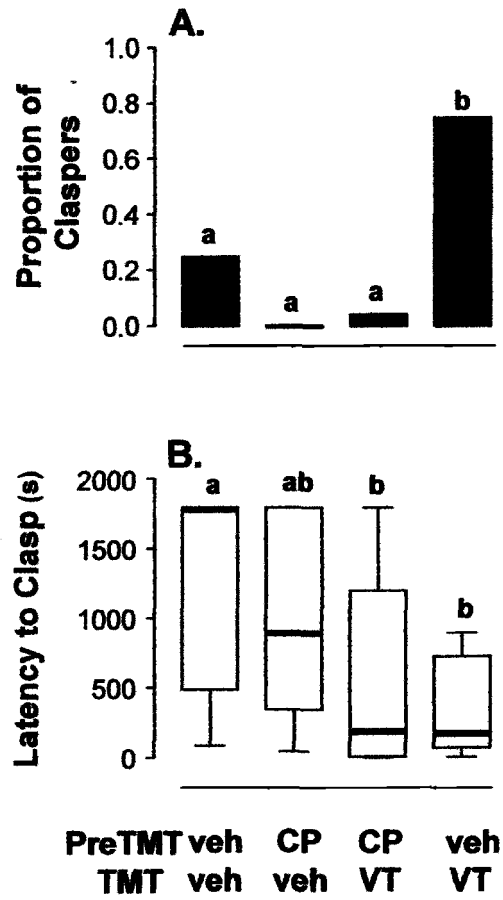


Figure 5.3. The enhancing effect of VT on courtship behaviors is blocked by the pretreatment of CP-55940. This experiment was designed to incorporate the time component of hormone effects. Males were tested towards the end of the breeding season, April 2002. Data had heterogeneous variances and are therefore shown as proportion or box and whisker plots of median (thick bar), interquartiles (box), and ranges (whiskers). **A** Incidence of males clasping during the 40 min period after receiving hormone treatment, reported as proportion of males observed clasping (N=24/tmt). **B** Latency to initiate a successful clasp is determined as the time between females being introduced to the test arena and the time male initiates clasping.

contrast, 75 % of males receiving Veh-VT were observed clasping, which was significantly greater than those in groups Veh-Veh ($P < 0.05$), CP-Veh ($P < 0.01$), or CP-VT ($P < 0.01$) (Figure 5.3).

Electrophysiology Experiments

Recorded Neurons and Their Locations

All experiments were performed using mature male *Taricha.* The findings reported here are based on recordings from 35 sensory-responsive medullary interneurons, 14 of which were tested with VEH followed by VT and 21 of which were tested CP-55940 followed by VT. The neurons were all recorded for at least 15 min prior to the first hormone administration to assure stability of spike and then for another 9 min after the first treatment (Pretreatment) was administered, and a further 60 min after the second treatment (Treatment) administration. In this manner we can determine effects of the first hormone compared with baseline, and the effects of combined treatments, VEH/VT or CP/VT with respect to baseline. The locations of the medullary neurons were determined based on placement of the electrode and are shown in Figure 5.4. We deliberately targeted neurons more medially located in zones corresponding to less specialized reticular regions of the medulla where prior studies have identified neurons responsive to corticosterone (Rose et al., 1995; Rose et al., 1998), vasotocin (Lewis and Rose, 2002; Rose et al., 1995) and the cannabinoid antagonist AM 281 (Coddington, n.d.).

Activity level and Sensory Responses of Medullary Neurons

All medullary neurons were responsive to at least one parameter of sensory stimuli, by virtue of the method used to locate them. Of the neurons examined under the CP/VT treatment regime 19/21 neurons responded to onset of cloacal pressure, 18/21 neurons responded to offset of cloacal pressure, 19/21 responded to onset of forelimb pressure, 20/21 responded to offset of forelimb pressure. Of the neurons examined under the Veh/VT treatment regime 14/14 responded to onset of cloacal pressure, 13/14 to offset of cloacal pressure, 13/14 to onset of forelimb stimuli, and 14/14 to offset of forelimb stimuli. In contrast, sixteen of the 21 neurons examined for CP/VT and 13 of the 14 neurons examined for VT effects showed spontaneous activity.

The general behavior of the medullary neurons prior to any hormone treatment is illustrated in Figure 5.5A and 5.6A and is elaborated by the following observations during 15 min at baseline. The median frequency of spontaneous spikes observed at baseline was 1-1.33

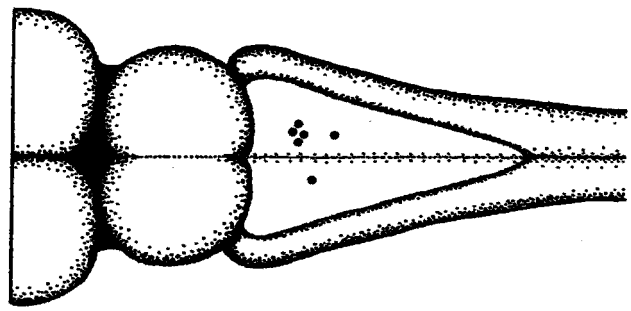


Figure 5.4. Diagram of dorsal surface of the *Taricha* medulla. The dots located around the midline of the RVM depict the locations of recorded neurons. For clarity, the cerebellum has been omitted from the diagram.

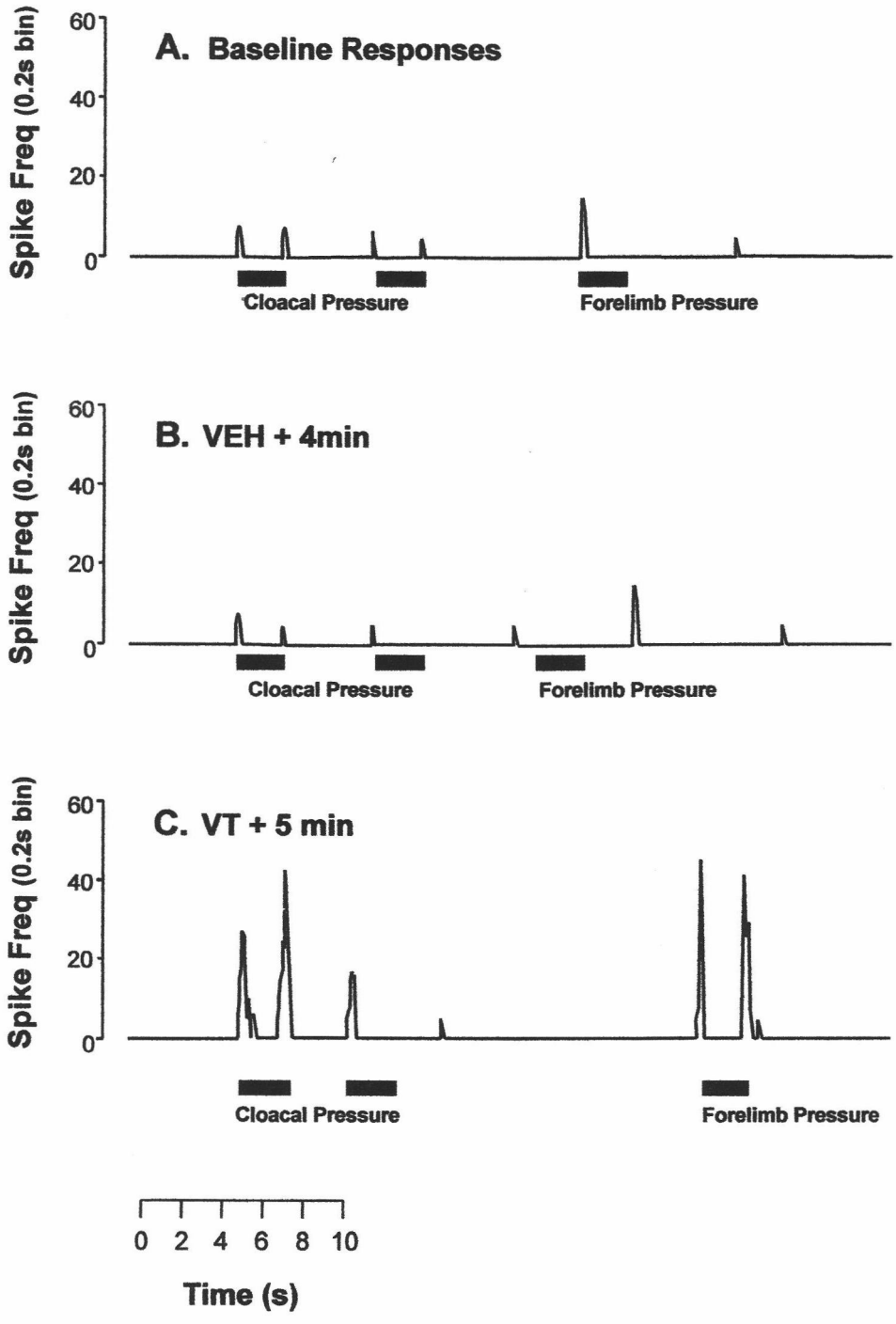


Figure 5.5. Responses of one medullary interneuron to cloacal and forelimb pressure and the facilitation of these responses by VT. Each of the traces **A – C** shows the integrated activity from a single medullary interneuron. **A** Baseline frequency of spikes elicited by application of cloacal or forelimb pressure. **B** Frequency of spikes elicited by the same sensory input 4 min after application of VEH to the medullary surface. **C** Frequency of spikes elicited by the same type of sensory input is profoundly increased by 5 min after application of VT to the medullary surface. There was a total of 9 min separating the delivery of VEH then VT.

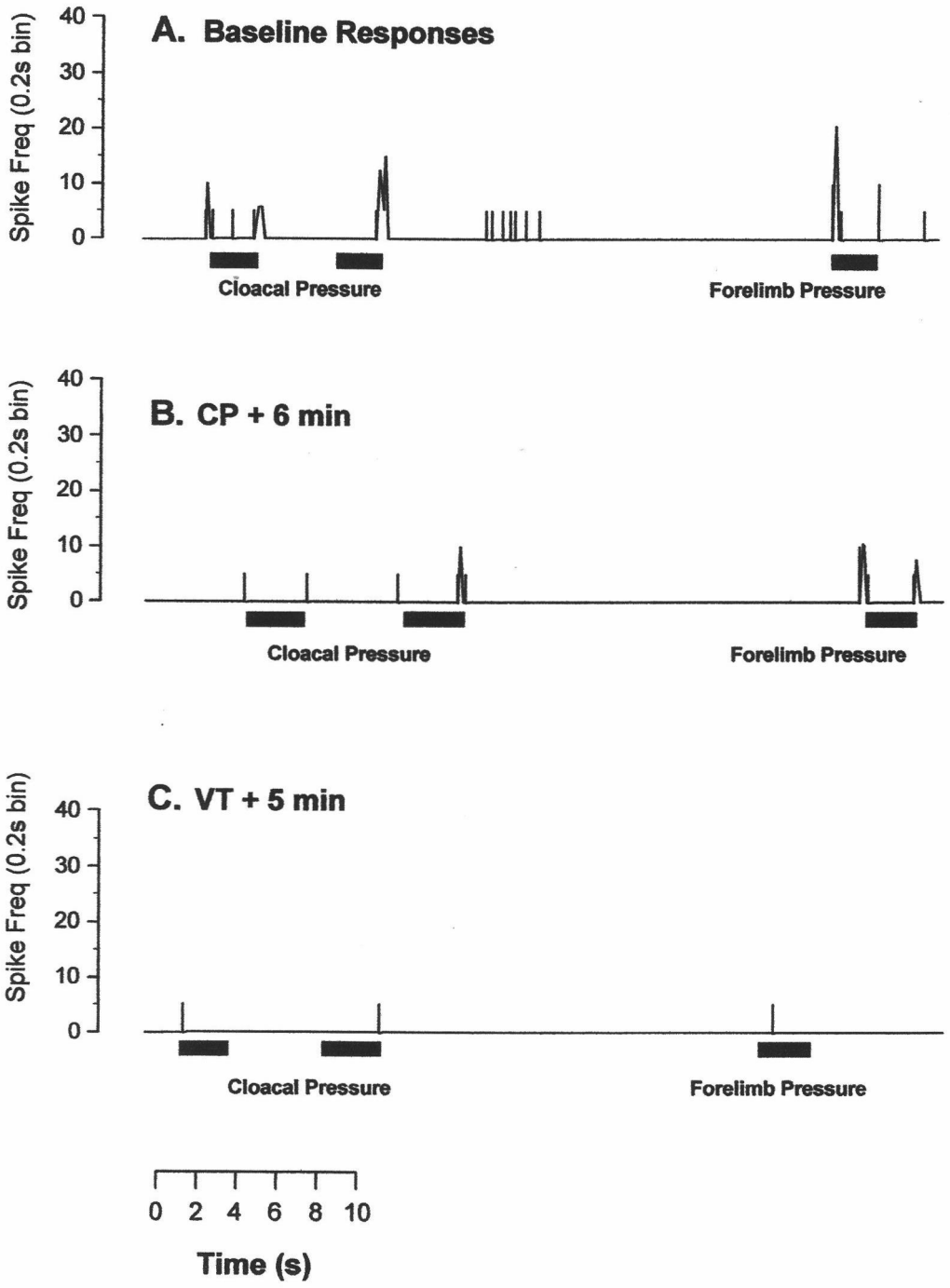


Figure 5.6. Responses of a medullary interneuron to cloacal and forelimb pressure and the blockade of VT-facilitated enhancement by pretreatment with the cannabinoid agonist CP55, 940. Each trace **A-C** shows the integrated activity from a single medullary interneuron. **A** Baseline frequency of spikes elicited by application of cloacal or forelimb pressure. **B** Frequency of spikes elicited by the same type of sensory input 5 min after CP-55940 was delivered directly to the medullary surface. **C** Frequency of spikes elicited by the same sensory input was reduced in this interneuron 5 min after medullary application of VT. As in the VEH/VT treatment regime, CP-55940 was delivered 9 min prior to VT.

spikes/10s, or 0.1 Hz. The Peak Spike Frequency generated in response to onset of cloacal pressure was 7-14 / 0.2s bin (35-70 Hz), response to offset of cloacal pressure was 9-9.33 / 0.2 s bin (45-46.7 Hz), to onset of forelimb pressure was 6.7 – 8.7 / 0.2 s bin (33.5-43.5 Hz), and to offset of forelimb pressure was 4.2 – 7 / 0.2 s bin (21-35 Hz).

Effect of VEH on Activity and Sensorimotor Responsiveness of Medullary Neurons

The application of VEH directly to the medullary surface resulted in little to no obvious changes in neuron spontaneous or sensory-induced activity (Figure 5.5B). To test this observation we analyzed the paired results during 15 min of Baseline and 9 min of VEH (100nM) from the same neuron using Wilcoxon Matched Pairs Test. This type of paired analyses tests whether the median difference in the entire population is different from zero, zero being no difference between baseline and hormone pretreatment (VEH). Wilcoxon Matched Pairs Test confirmed that VEH did not significantly alter any of the parameters monitored (Figure 5.7A-E).

Consistent with the Wilcoxin Matched Pairs Test, we observe that a majority of the neurons do not exhibit a change in any activity parameter after delivery of VEH (Figure 5.7F). At most 10% neurons increased in spontaneous activity, or 20% of neurons decreased in response to offset of cloacal stimulation. A vast majority (70-85%) of neurons did not change the level of spontaneous or sensory-induced activity.

Effect of combined treatment with VEH followed by VT

We calculated the change in all the parameters due to combined treatment using the following equation:

Equation 1. $$\Delta A = \frac{T - PT}{B}$$

Where ΔA is the change in any Activity parameter (spontaneous or sensory-induced activity), median number of total spikes (spontaneous activity) or peak spike frequency (sensory responses) after both hormones are delivered (T), after the first hormone is administered (PT), or at baseline (B). This calculation allowed us to internally control for individual neuron phenotypic differences while assessing for hormone effects.

VT administration produced rapid and pronounced effects on the medullary neurons. Within 1-5 min of topical administration, VT increased the level of spontaneous activity by 2-fold (Figure 5.9A), and responses to sensory input by 2-3 fold (Figure 5.5A-C, 5.9B-E).

Figure 5.7. Comparison within the VEH/VT treatment regime to determine whether the application of VEH significantly altered activity from baseline using Wilcoxon matched pairs test, a nonparametric test that compares two paired groups (VEH vs Baseline). **A** Illustrates spontaneous activity and **B – E** sensory-induced activity; **B** response to onset of cloacal pressure, **C.** response to offset of cloacal pressure, **D** response to onset of forelimb pressure, and **E** response to offset of forelimb pressure. Box and Whisker plots show the median total spike or median peak spike frequency, with 75^{th} and 25^{th} percentile and ranges in the data. The median total spikes or peak spike frequency at baseline and after VEH for each individual neuron (1 pair) is connected by different color lines to illustrate the pattern of individual neuron responses. A total of 14 neurons from 2 animals were exposed to VEH/VT regime and used in these comparisons. There were no significant differences between VEH and baseline for any of the activity parameters monitored. **F** The percentage of neurons changing their activity responses after delivery of VEH.

Figure 5.7

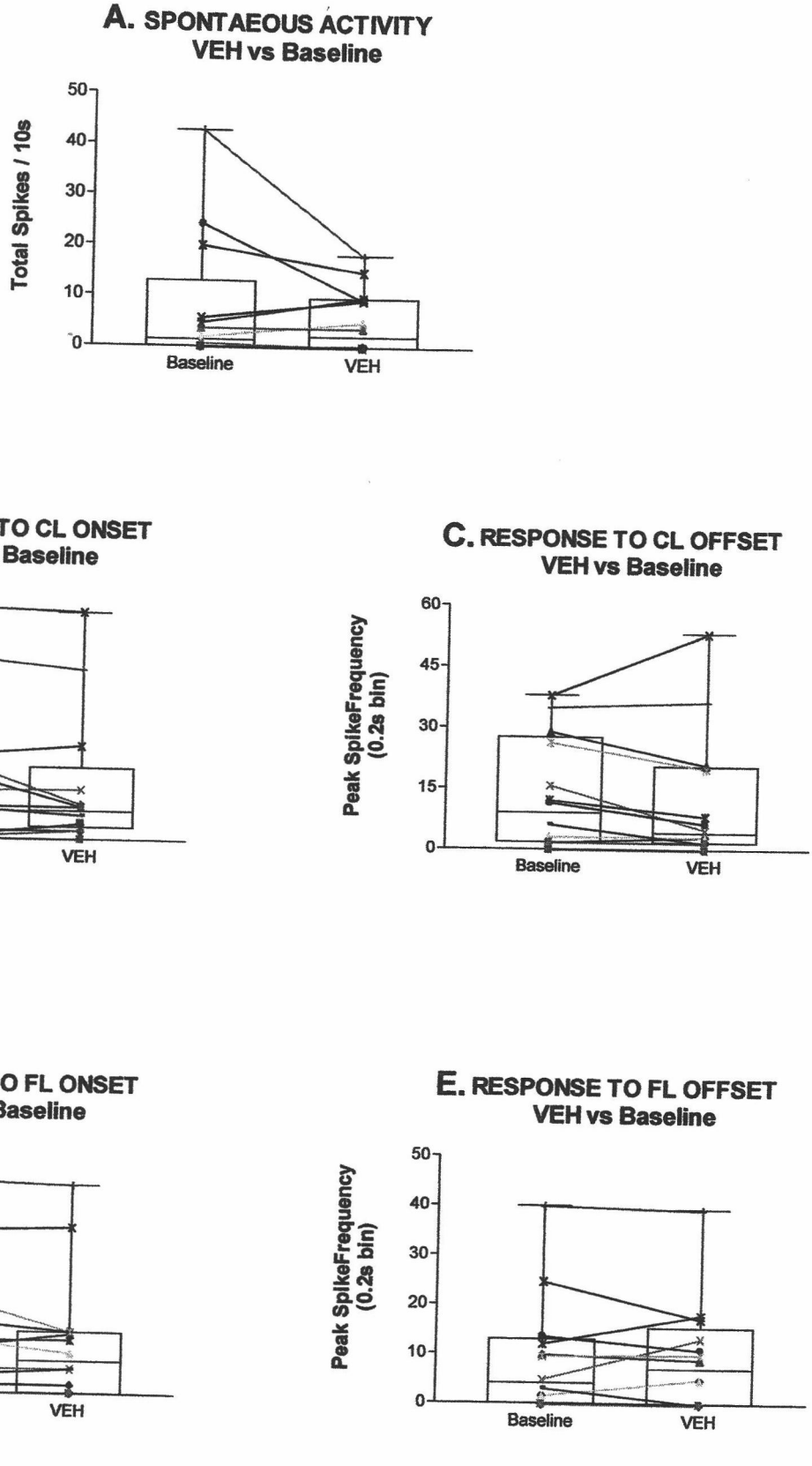

A. SPONTAEOUS ACTIVITY
VEH vs Baseline
Total Spikes / 10s
50
40
30
20
10
0
Baseline
VEH
B. RESPONSE TO CL ONSET
VEH vs Baseline
Peak SpikeFrequency (0.2s bin)
50
40
30
20
10
0
Baseline
VEH
C. RESPONSE TO CL OFFSET
VEH vs Baseline
Peak SpikeFrequency (0.2s bin)
60
45
30
15
0
Baseline
VEH
D. RESPONSE TO FL ONSET
VEH vs Baseline
Peak SpikeFrequency (0.2s bin)
50
40
30
20
10
0
Baseline
VEH
E. RESPONSE TO FL OFFSET
VEH vs Baseline
Peak SpikeFrequency (0.2s bin)
50
40
30
20
10
0
Baseline
VEH

Figure 5.7

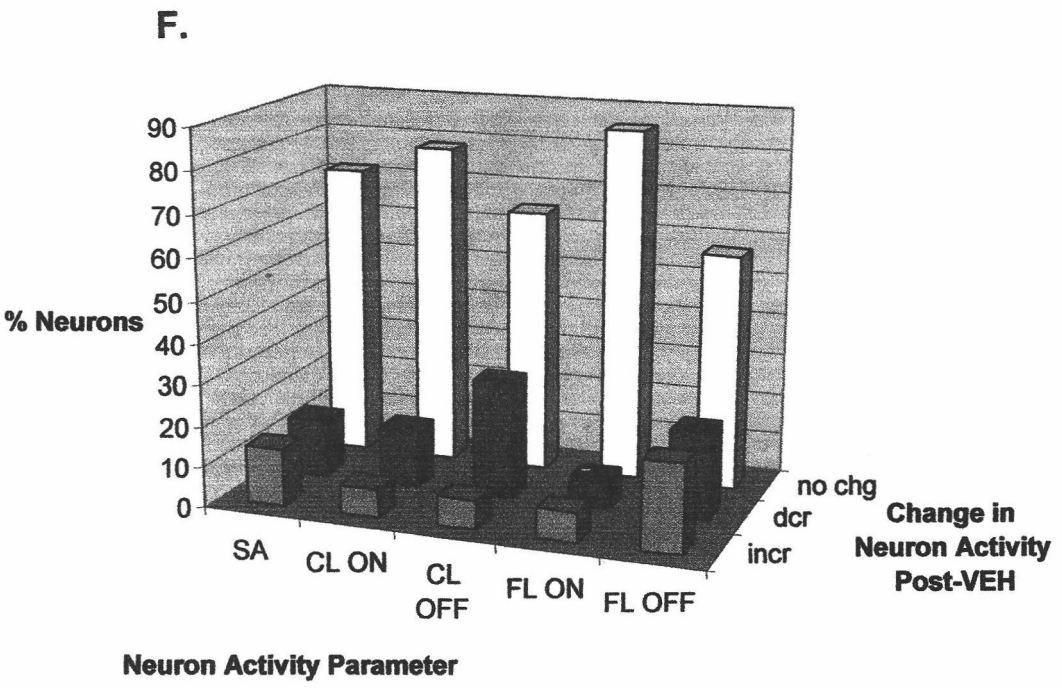

F.
% Neurons
90
80
70
60
50
40
30
20
10
0
SA
CL ON
CL OFF
FL ON
FL OFF
Neuron Activity Parameter
no chg
dcr
incr
Change in Neuron Activity Post-VEH

Figure 5.8. Comparison within the CP/VT treatment regime to determine whether the application of CP significantly altered activity form baseline using Wilcoxon matched pairs test (CP vs Baseline). **A** illustrates spontaneous activity and **B – E** sensory-induced activity; **B** response to onset of cloacal pressure, **C.** response to offset of cloacal pressure, **D** response to onset of forelimb pressure, and **E** response to offset of forelimb pressure. Box and Whisker plots show the median total spike or median peak spike frequency, with 75^{th} and 25^{th} percentile and ranges in the data. The median total spikes or peak spike frequency at baseline and after CP for each individual neuron (1 pair) is connected by different color lines to illustrate the pattern of individual neuron responses. A total of 21 neurons from 4 animals were exposed to CP/VT regime and used in these comparisons. Level of significant difference between baseline and CP is indicated by the number of stars above the VEH/VT column; * ≤ 0.05. **F** The percentage of neurons changing their activity responses after delivery of CP-55940.

Figure 5.8

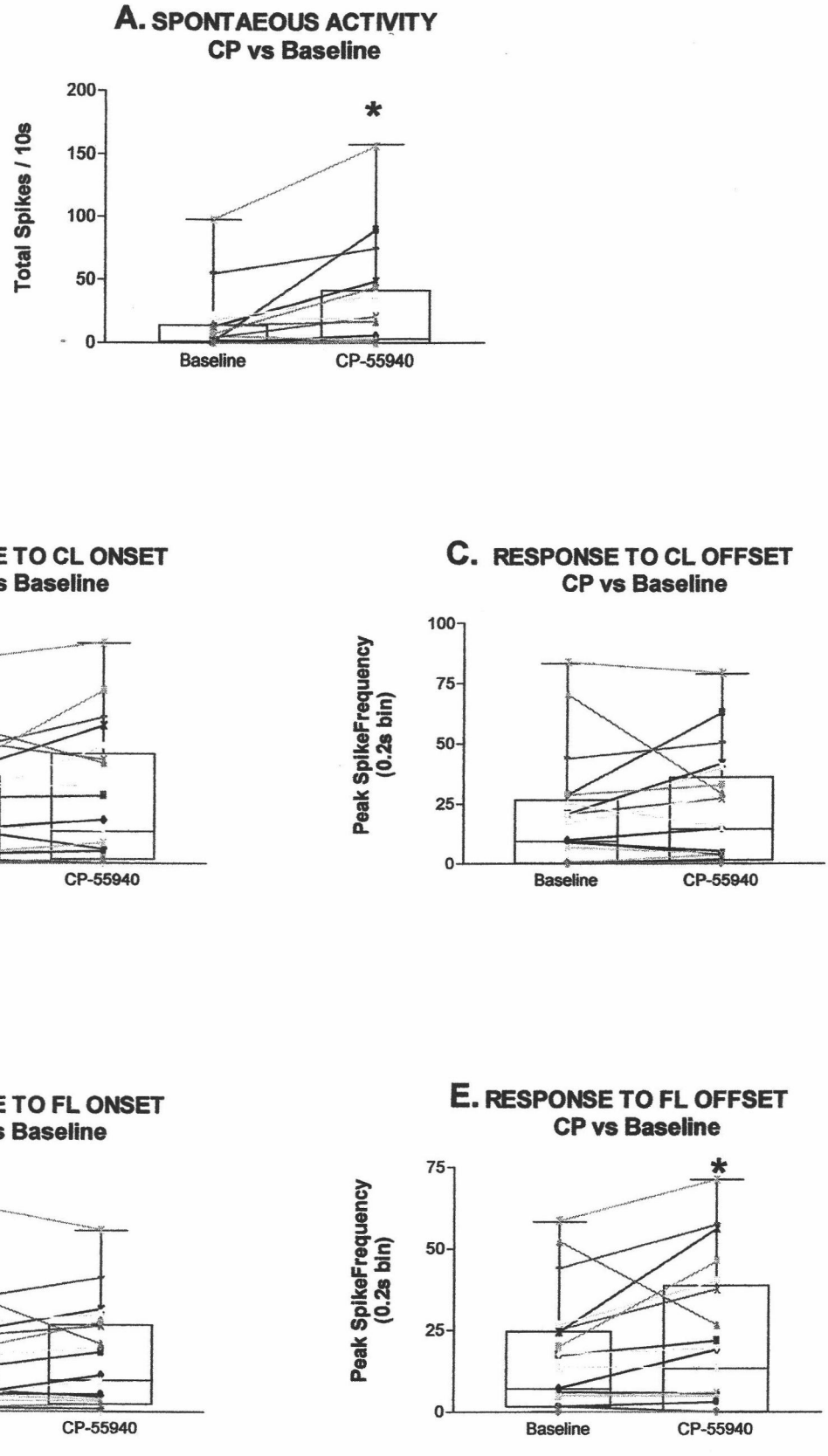

A. SPONTAEOUS ACTIVITY
CP vs Baseline
Total Spikes / 10s
200
150
100
50
0
*
Baseline
CP-55940
B. RESPONSE TO CL ONSET
CP vs Baseline
Peak SpikeFrequency (0.2s bin)
100
75
50
25
0
Baseline
CP-55940
C. RESPONSE TO CL OFFSET
CP vs Baseline
Peak SpikeFrequency (0.2s bin)
100
75
50
25
0
Baseline
CP-55940
D. RESPONSE TO FL ONSET
CP vs Baseline
Peak SpikeFrequency (0.2s bin)
120
100
80
60
40
20
0
Baseline
CP-55940
E. RESPONSE TO FL OFFSET
CP vs Baseline
Peak SpikeFrequency (0.2s bin)
75
50
25
0
*
Baseline
CP-55940

Figure 5.8

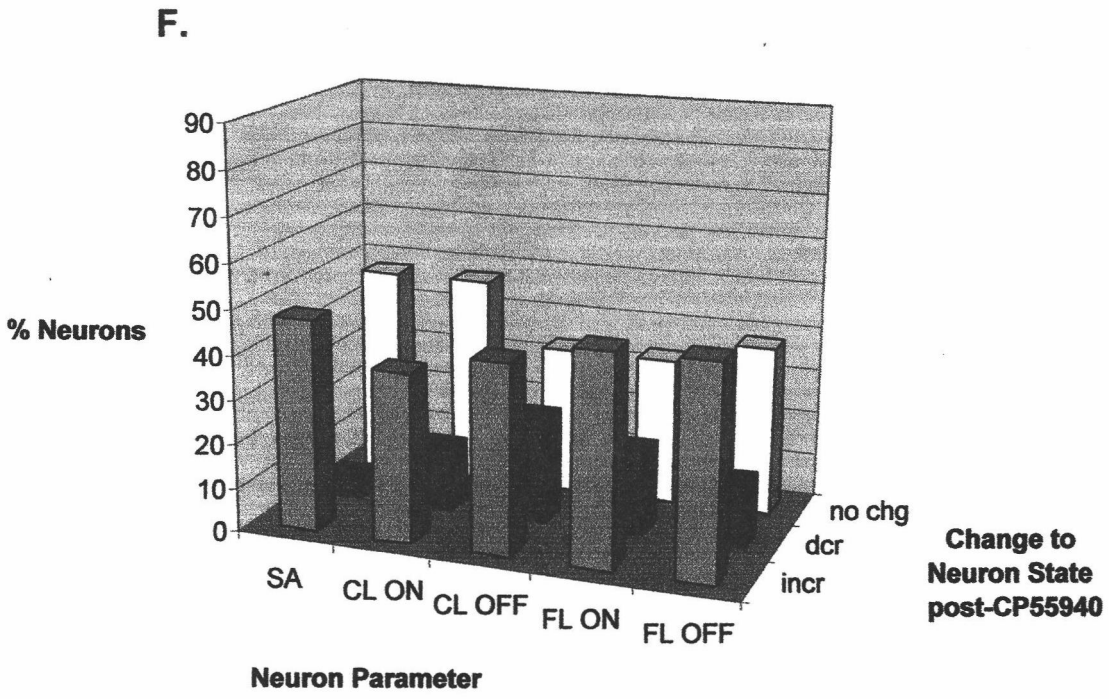
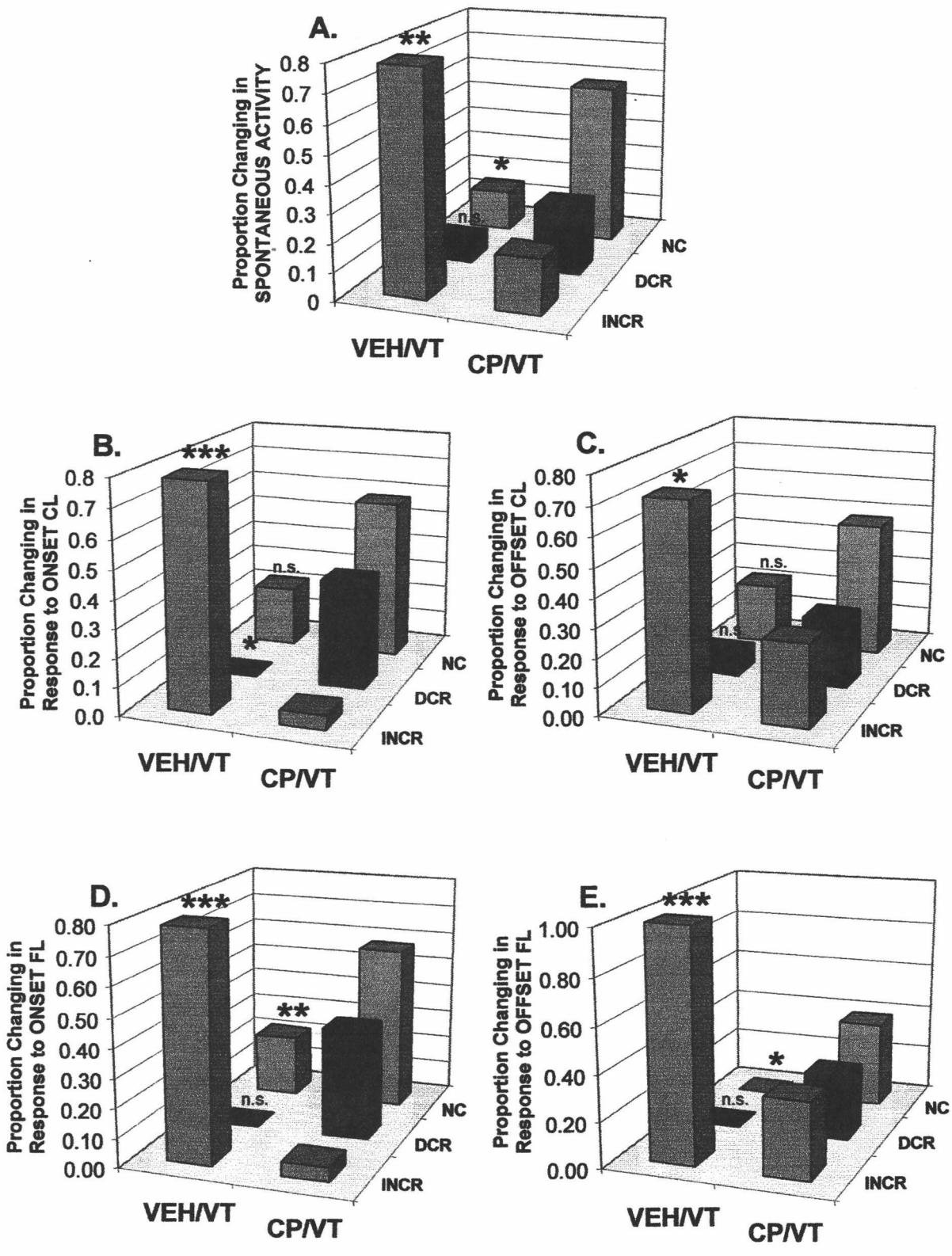

F.
90
80
70
60
50
40
30
20
10
0
% Neurons
SA
CL ON
CL OFF
FL ON
FL OFF
Neuron Parameter
no chg
dcr
incr
Change to
Neuron State
post-CP55940

Figure 5.9. The proportions of medullary interneurons that increased, decreased or were not changed in activity after delivery of hormones to the medullary surface. **A** Illustrates the change in spontaneous activity and **B – E** the change in sensory-induced activity; **B** response to onset of cloacal pressure, **C.** response to offset of cloacal pressure, **D** response to onset of forelimb pressure, and **E** response to offset of forelimb pressure. The direction of change, increase (**INCR**) / decrease (**DCR**) / no change (**NC**), is represented along the z axis. The delivery of the hormones (x axis) was separated in time; the first hormone was applied (VEH or CP-55940) 15 min after baseline recording, the second hormone was applied (VT) 9 min after the first. All interneuron parameters were monitored throughout the experiment. A total of 21 neurons from 4 animals were exposed to CP/VT regime, and 14 neurons from 2 animals were exposed to VEH/VT regime. Statistical comparison was made between the two hormone treatment regimes (VEH/VT vs CP/VT) at each level of change (**INCR, DCR,** or **NC**) using *z*-tests, which are specific χ^2 tests for comparison of two proportions. Level of significant difference between treatment regimes is indicated by the number of stars above the VEH/VT column; * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, n.s. ≥ 0.05.

Figure 5.9


A.
Proportion Changing in SPONTANEOUS ACTIVITY
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
**
*
n.s.
NC
DCR
INCR
VEH/VT
CP/VT
B.
Proportion Changing in Response to ONSET CL
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
***
n.s.
*
NC
DCR
INCR
VEH/VT
CP/VT
C.
Proportion Changing in Response to OFFSET CL
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
*
n.s.
NC
DCR
INCR
VEH/VT
CP/VT
D.
Proportion Changing in Response to ONSET FL
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
***
**
n.s.
NC
DCR
INCR
VEH/VT
CP/VT
E.
Proportion Changing in Response to OFFSET FL
1.00
0.80
0.60
0.40
0.20
0.00
***
*
n.s.
NC
DCR
INCR
VEH/VT
CP/VT

Typically, the changes in spontaneous activity returned to baseline levels within 15-20 min. In contrast, VT's enhancing effects on sensory responsiveness peaked within 10-15 min, but were still visible for as long as 30 min after VT was administered.

Monitoring the number of neurons exhibiting a change in spontaneous or sensory-induced activity revealed that a majority of the neurons responded to VT by increasing in activity. Of the fourteen neurons examined for VT effects, 79% neurons increased spontaneous discharge and response to onset of cloacal pressure (Figure 5.9A and B, respectively), while 100% neurons increased in response to offset of forelimb pressure (Figure 5.9E). In contrast, very few neurons decreased in activity due to VT application; 7% of neurons decreased in spontaneous activity, but none decreased in response to onset of cloacal pressure or onset and offset of forelimb pressure.

Effects of Cannabinoid Agonist on Activity and Sensorimotor Responsiveness of Medullary Neurons

CP-55940 application did not produce a consistent change in sensory responsiveness; some neurons exhibited a decrease in sensory responsiveness as shown in Figure 5.6A-B, others exhibited either an increase or no change. Wilcoxon Matched Pairs Test (pairing baseline to CP within the same neuron) suggested that CP-55940 did not affect sensory responses to sexual stimuli but did alter the spontaneous discharge of neurons. CP-55940 failed to affect the response to onset ($P = 0.15$) or offset ($P = 0.23$) of cloacal stimuli, or onset of forelimb stimuli (0.15) (Figure 5.8B – D). CP-55940 did significantly increase the spontaneous activity ($W = -84.0$, $P = 0.0151$) and sensory responsiveness to offset of forelimb stimulus ($W=-76.0$, $P = 0.032$) (Figure 5.8A and E respectively).

The statistical results from the Wilcoxon test reveals strong patterns; however, if an equal number of neurons are responding to CP-55940 delivery in both directions then there is the potential to miss individual neuron responses. In this study individual neurons responded to CP-55940 application (Figure 5.8F) in a very different manner to the response to VEH (Figure 5.7F). Where VEH application resulted in very few neurons changing, CP-55940 resulted in 38-47% neurons increasing in all activity parameters. Only 5% neurons decreased in spontaneous activity after CP-55940 delivery, 23% neurons decreased in response to offset of cloacal pressure, but 15-20% neurons decreased in response to all other sensory input (Figure 5.8F). The remaining 33-47% neurons did not change in their activity levels.

Effect of combined treatment with CP followed by VT

Observation of the real-time traces (Figure 6A-C) revealed that the rapid pronounced enhancement usually associated with VT delivery to the medulla was noticeably absent. We calculated the change in neuron spontaneous and sensory-induced activity due to CP/VT treatment regime using Equation 1. In contrast to VEH/VT treatment regime, a vast majority of neurons treated with CP/VT did not change in any of the parameters monitored (Figure 5.9A-E). Of the 21 neurons examined, the percent of neurons exhibiting increased spontaneous activity dropped to 19%, and the percent of neurons with increased sensory responsiveness dropped to as low as 5% at onset of cloacal pressure (Figure 5.9B).

A comparison between VEH/VT and CP/VT treatment regimes

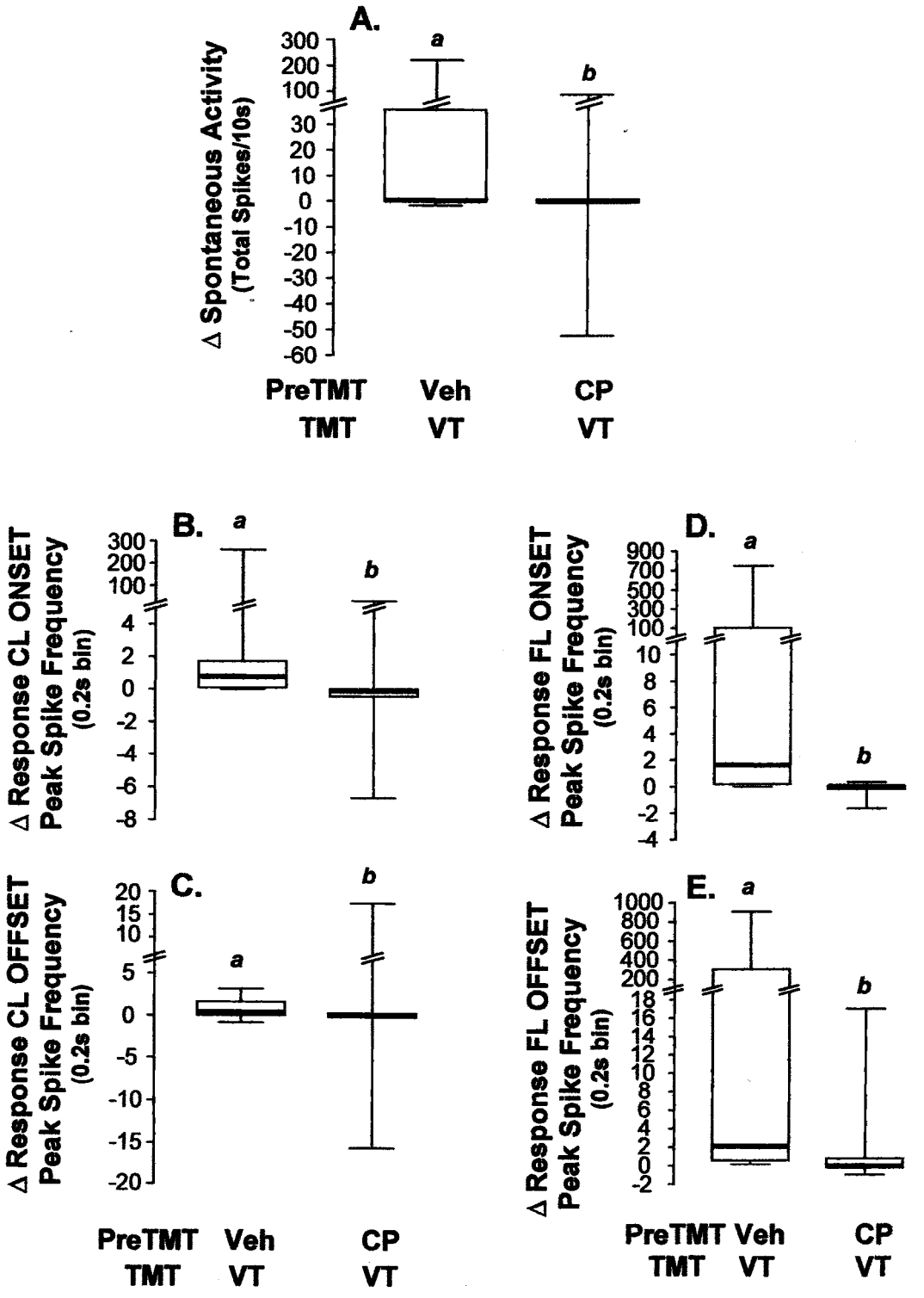
Statistical analysis of the change in the proportion of neurons that increase or decrease confirms that the treatments produced pronounced shifts in the overall patterns of excitability. The proportion of neurons increasing due to the hormone treatment VEH/VT, in comparison to neurons receiving CP/VT, is significantly higher in all neuronal parameters that we measured (Figure 5.9A-E). Additionally, there were also a significantly higher proportion of neurons that decreased in response to onset of cloacal pressure in the CP/VT treatment regime compared to VEH/VT (Figure 5.9B). For the most part, however, the majority of neurons receiving CP/VT failed to change in there activity levels. For example, when compared to VEH/VT, the number of neurons not changing after CP/VT treatment was significantly higher when examining spontaneous activity (Figure 5.9A), and responses to onset (Figure 5.9D) and offset (Figure 5.9E) to forelimb pressure.

A comparison of the change in the frequency of firing of medullary interneurons that were treated with Veh/VT to CP/VT revealed a significant reduction in excitability due to the pretreatment of CP-55940 in all parameters of neuron excitability that were monitored (Figure 5.10A-E). The level of spontaneous activity medullary neurons exhibit was significantly lower when treated with CP/VT compared to Veh/VT ($U = 96.00$, $P = 0.025$; Figure 5.10A). An even greater difference was observed in the response to onset of cloacal pressure, which was significantly enhanced, by a median factor of 0.77, when neurons were treated with Veh/VT compared to CP/VT (decreases by a median factor of - 0.09) ($U = 24.00$, $P < 0.0001$; Figure 5.10B). When comparing the hormone treatment effects on response to offset of cloacal stimuli ($U = 89.00$, $P = 0.0264$; Figure 5.10C), onset of forelimb stimuli ($U = 20.50$, $P < 0.0001$; Figure 5.10D), and offset of forelimb stimuli ($U = 48.00$, $P = 0.0005$ Figure 5.10E),

all medullary neuron parameters were significantly lower in CP/VT compared to Veh/VT treatments.

Figure 5.10. The change in spontaneous and sensory-induced activity of medullary interneurons was significantly affected by hormone treatment regime. **A** The frequency of spikes spontaneously generated by medullary interneurons increase dramatically with the application of VT. Pretreatment with cannabinoid agonist, CP-55940, significantly blocked the VT-induced enhancement (Two-tailed Mann-Whitney U = 96.00, $P = 0.025$). **B** and **C** The frequency of spikes generated by medullary interneurons in response to **B** onset and **C** offset of tactile pressure on the cloaca is rapidly enhanced by application of VT. However, pretreatment with cannabinoid agonist, CP-55940, significantly blocks the VT-induced enhancement of onset (One-tailed Mann-Whitney U = 24.00, $P < 0.0001$) and offset (One-tailed Mann-Whitney U = 89.00, $P = 0.246$). **D** and **E** The frequency of spikes generated by medullary interneurons in response to **D** onset and **E** offset of tactile pressure on the forelimb is rapidly enhanced by application of VT. However, pretreatment with cannabinoid agonist, CP-55940, significantly blocks the VT-induced enhancement of onset (One-tailed Mann-Whitney U = 20.50, $P < 0.0001$) and offset (One-tailed Mann-Whitney U = 48.00, $P = 0.0005$). Data from all parameters had heterogeneous variances and are therefore shown as proportion or box and whisker plots of median (thick bar), interquartiles (box), and ranges (whiskers).

Figure 5.10


A.
Δ Spontaneous Activity (Total Spikes/10s)
300
200
100
30
20
10
0
-10
-20
-30
-40
-50
-60
a
b
PreTMT TMT
Veh VT
CP VT
B.
Δ Response CL ONSET Peak Spike Frequency (0.2s bin)
300
200
100
4
2
0
-2
-4
-6
-8
a
b
D.
Δ Response FL ONSET Peak Spike Frequency (0.2s bin)
900
700
500
300
100
10
8
6
4
2
0
-2
-4
a
b
C.
Δ Response CL OFFSET Peak Spike Frequency (0.2s bin)
20
15
10
5
0
-5
-10
-15
-20
a
b
E.
Δ Response FL OFFSET Peak Spike Frequency (0.2s bin)
1000
800
600
400
200
18
16
14
12
10
8
6
4
2
0
-2
a
b
PreTMT TMT
Veh VT
CP VT

DISCUSSION

This study found that the administration of a cannabinoid agonist, when given 10 min prior to a VT injection, blocks VT-induced enhancement of clasping behavior. In vivo single-unit recordings of medullary neurons that regulate the motor control of clasping revealed that application of cannabinoid agonist also blocks the VT-induced enhancement of medullary neuron activity and sensory responsiveness. This is the first report of cannabinoids interacting with the behavioral or electrophysiological effect of VT or VP in any species. We propose that the site of action for the cannabinoid/VT interaction is on CB_1 receptors located on neurons in the rostroventral medulla based on the following evidence. The results from behavioral and electrophysiological experiments in the present study consistently show that CORT blocks VT-induced enhancement effects. Cannabinoid antagonist AM 281 blocks the CORT-induced inhibition of medullary neurons in the same preparation (Coddington, n.d.), and VT action on the medulla facilitates clasping in roughskin newts (Lewis and Rose, 2002). Finally, CB_1 receptors have been localized to the rostroventral medulla region of *Taricha* (Hollis, Coddington, and Moore, in preparation). We propose that the mechanism, by which cannabinoids interfere with VT-induced enhancing effects on clasping, is by acting at the level of the rostroventral medulla to block VT's ability to enhance sensory processing.

Effect of Vasotocin on Clasping Behaviors and Medullary Neurons

Intraperitoneal injections of VT lead to an increased incidence of clasping and a reduced latency to clasp by male *Taricha.* These results are consistent with past studies (Moore and Miller, 1984; Zoeller and Moore, 1988) . Recent behavioral studies (Thompson and Moore, 2000) revealed that injections of VT to male *Taricha* enhance appetitive responses to olfactory sexual stimuli (female sex pheromones) and to visual stimuli. Other studies suggest that VT injections also enhance responses to somatosensory stimuli (mechanical clasp-generating pressure to the cloacal region) (Rose et al., 1995). Thus it appears that VT acts on at least three different sensory modalities (visual, olfactory, and somatosensory) as part of the peptidic mechanism for controlling behaviors.

Delivery of behaviorally relevant doses of VT to the fourth ventricle produced rapid and profound effects on the medullary neurons. Within 1-5 min of topical administration, 79% neurons increased the level of spontaneous activity and responses to clasp-generating mechanical stimuli. In contrast, very few neurons decreased in spontaneous or sensory-induced activity. All of the neurons examined in this study responded to the onset and offset of clasp-triggering cloacal pressure with strong increases in spike frequency, and these

responses were consistently enhanced 2-3 fold higher with the application of VT. Based on single-unit recording in freely behaving newts, these sensory responses are coincident with onset of cloacal stimuli initiating clasping behavior and the offset of cloacal stimuli terminating clasping (Rose and Moore, 2002).

The neural effects of VT are behaviorally significant given that the medullary neurons examined are an integral component of a neural circuit that is critical to clasping (Rose and Moore, 2002). Mechanical pressure applied to the cloacal region triggers clasp-generating processes in the spinal cord, resulting in excitation of motor neurons innervating flexor muscles, and presumably inhibition of motor neurons innervating the extensor muscles, consequently resulting in flexion of limb muscles. Male *Taricha* with spinal transections retain their capacity to respond to the same clasp-generating mechanical pressure by initiating and maintaining a clasp (Lewis and Rose, 2003), but unlike intact animals, they do not terminate the clasp when somatosensory stimulation of the cloacal region is removed. Motor neurons that generate the clasping response receive descending input from the brain, principally from medullary neurons that regulate clasp onset, termination and adjustments to clasp quality. Neural activity elicited in the spinal circuits also ascends to the medulla, producing mainly an increase in firing of medullary neurons. Forebrain ablation studies established that an intact hindbrain-spinal cord connection is sufficient to initiate, maintain, regulate and terminate a clasp (Lewis and Rose, 2002). Collectively, the responses of medullary neurons are closely associated with the onset, maintenance and offset of clasping, allowing us to observe hormonal effects on this sensorimotor system. It is on these neurons that VT exerts an effect, ultimately leading to a pronounced modulation of the clasp behavior.

The VT-induced enhanced sensory responsiveness and spontaneous activity observed in *Taricha* likely represents a common mechanism by which VT/VP peptides exert their behavioral effects given that this phenomenon is observed in a variety of mammalian neural systems. VP administration has been found to enhance neuronal activity in various locations of other vertebrate central nervous systems: Neurons of the area postrema (Smith, Lowes, and Ferguson, 1994), suprachiasmatic nucleus (Liou and Albers, 1989), septum (Raggenbass, 1987; Joels, 1984), neonatal ventral spinal cord (Oz, Kolaj, and Renaud, 2001), as well as lateral medulla (Sun and Guyenet, 1989). There were a small number of neurons in the present study that exhibited a suppression of spontaneous activity after the delivery of VT; however, we did not observe the kinds of pervasive suppressive effects as described for VP effect on neurons in rat hippocampus (Smock, Albeck, and McMechen, 1991; Smock, Arnold, Albeck, Emerson, Garritano, Burrows, Derber, Sanson, Marrs, Weatherly, and et al., 1992) or

caudate putamen (Castillo-Romero, Vives-Montero, Reiter, and Acuna-Castroviejo, 1993). The latency of VT effects was on the order of seconds to minutes, which is also consistent with VP effects in mammalian systems (all the above papers)

Effect of Cannabinoid Agonist CP-55940 on Clasping Behavior

Systemic delivery of CP-55940 resulted in decreased incidence of clasping and a concomitant increased latency to clasp in a dose-dependent manner. These data are consistent with an earlier study where a systemic injection of the cannabinoid agonist levonantradol inhibited *Taricha* clasping (Soderstrom et al., 2000).

In this behavioral study with *Taricha,* cannabinoids delivered by intraperitoneal injection had access to multiple anatomical sites that might affect clasping behavior. In mammals, exogenous cannabinoids modulate peripheral sensory neuron processing of nociception [Ross, 2004 #391]. Cannabinoid agonists can suppress the excitation of primary sensory afferents and cell bodies in the dorsal root ganglion (DRG). Other studies indicate that activation of CB_1 receptors affects nociceptive and non-nociceptive neurons. Co-localization studies report that only 10-15% of DRG neurons containing CB1 receptor mRNA also contain message for known nociceptive markers (Bridges, Rice, Egertova, Elphick, Winter, and Michael, 2003), and only 5% of CB_1 mRNA is co-localized in trigeminal neurons with nociceptive markers, while 75% co-localized with a marker for myelin, which is an anatomical marker of non-nociceptive neurons (Price, Helesic, Parghi, Hargreaves, and Flores, 2003). Immunocytochemistry studies also reveal that of the neurons in the DRG that contain CB_1 receptors only 36% are found on the small diameter nociceptive primary afferents (Khasabova, Simone, and Seybold, 2002). Considering these observations, it is plausible that intraperitoneal injection of cannabinoid could affect clasping behavior of male *Taricha* by modulating the responsiveness of sensory afferents.

The interpretation of behavioral tests of cannabinoid effects on sexual behavior is confounded because cannabinoids affect *Taricha* arousal state. There were dose-dependent decreases in locomotor activity in newts injected with CP-55940 (current study) or levanontradol (Soderstrom et al., 2000). In both studies, even at the highest doses, no incidence of catalepsy or immobility was observed. This inhibition of *Taricha* activity is consistent with cannabinoid agonist effects on mammals (Martin, Compton, Thomas, Prescott, Little, Razdan, Johnson, Melvin, Mechoulam, and Ward, 1991; Wiley, Balster, and Martin, 1995)

Effect of Cannabinoid Agonist CP-55940 on Medullary Neuron Spontaneous and Sensory-Induced Activity

Direct delivery of the cannabinoid agonist CP-55940 to the fourth ventricle resulted in notable changes in neuronal activity (spontaneous and sensory-induced). Given that delivery of VT results in a consistent directional change observed in behavior and medullary activity, we had predicted that application of cannabinoid agonist would result in a decrease in medullary activity consistent with behavioral effects of the same agonist. However, our data were not as expected because the application of CP-55940 alone increased the frequency of spontaneous and sensory-induced activity in a significant proportion of medullary neurons in our in vivo system.

We suggest that this electrophysiological result is likely due to the removal or dis-coordination of tonic GABAergic inhibitory input based on several lines of evidence. First, anti-nociceptive effects of systemically administered cannabinoid agonist WIN55212-2 are prevented by microinjection of muscimol, a $GABA_A$ receptor agonist, into the rostroventral medulla (Meng, Manning, Martin, and Fields, 1998). Second, anatomical studies consistently report CB_1 receptors on the terminal boutons of GABAergic [Freund, 2003 #332]. Third, the activation of CB_1 receptors reduces the excitability of the presynaptic neurons and ultimately reduces the probability of neurotransmitter release [Ross, 2004 #391]. Fourth, one of the few electrophysiology studies that examine the cannabinoid function in medullary circuits' reports that delivery of cannabinoid agonist WIN55212-2 to brain slice preparation of the medulla reduces GABA release (Jennings et al., 2001).

The current findings of cannabinoid effects in *Taricha* are unique because they are presented within the context of a well characterized neuroendocrine regulated behavioral system. Very few in vivo preparations have been utilized or are available to answer questions about cannabinoid effects on neural circuitry. A number of single-unit recording studies consistently report that systemically administered Δ^9-THC increase the spontaneous activity of dopaminergic neurons in the ventral tegmental area (VTA) (Melis, Gessa, and Diana, 2000; Wu and French, 2000) and substantia nigra pars compacta (SNc) to 120 and 128% of basal levels (Melis et al., 2000). This THC-induced increase was reversed by administration of the CB_1 receptor antagonist SR141716A (French, 1997). The synthetic agonist WIN55212-2 produces increases in these same DA neuron populations (French, Dillon, and Wu, 1997). Another in vivo single unit recording study reported a similar dose-dependent increase in spontaneous activity of nucleus accumbens dopaminergic neurons (D10 population) (Gessa, Melis, Muntoni, and Diana, 1998)and meso-prefrontal dopaminergic neurons (Diana, Melis,

and Gessa, 1998) with systemic administration of cannabinoid agonists, Δ^9-THC, WIN55,212-2, and CP-55940. The authors all interpreted these data to suggest that cannabinoids act through CB_1 receptors to remove inhibitory GABAergic input. Indeed, in vivo brain microdialysis study revealed that Δ^9-THC significantly decreased GABA levels, while increasing glutamate and dopamine levels in the same meso-prefrontal cortex region (Pistis, Ferraro, Pira, Flore, Tanganelli, Gessa, and Devoto, 2002).

In situ hybridization using newt-specific cRNA probes for the CB_1 receptor show specific positive labeling throughout forebrain regions and in the same region the electrodes were placed in the rostroventral medulla (Hollis et al., in preparation). Studies in mammalian systems have revealed that the mechanism by which CB_1 receptors are activated is unusual [Freund, 2003 #332]. Endogenous cannabinoids are released by postsynaptic neurons and travel in a retrograde fashion to bind to CB_1 receptors on presynaptic neurons. Action of cannabinoids at presynaptic CB_1 receptors universally reduces the probability of transmitter release from the presynaptic neuron. In this fashion, the post-synaptic neuron possesses the capacity to modulate input via retrograde signaling with endocannabinoids. Endocannabinoids function primarily in a retrograde fashion throughout the central nervous system, including in the medulla (Jennings et al., 2001; Jennings, Vaughan, Roberts, and Christie, 2003). Therefore, CB_1 receptors are localized in medullary neurons in *Taricha* hindbrain and the retrograde signaling of endocannabinoids could act in this site to modulate somatosensory medullary circuits.

Our electrophysiological data suggest medullary neurons are involved in a medullary endocannabinoid circuit because they respond to direct application of cannabinoid agonist CP-55940. The results from the present study are consistent with the small number of studies investigating cannabinoid action in the hindbrain. Direct micro-injection of cannabinoid agonists WIN55212-2 and HU210 into the rostroventral medulla lengthen tail-flick latencies in rats (Martin, Tsou, and Walker, 1998); the longer the latency to tail-flick in response to a hot plate, the stronger the anti-nociceptive properties of the delivered drug. Furthermore, delivery of $GABA_A$ receptor agonist to rostroventral medulla prevents anti-nociceptive action of systemically administered WIN55,212-2 (Meng et al., 1998). Evidence from whole-cell patch-clamp studies in brain slice preparation support the hypothesis that endocannabinoids function in the medulla by signaling in a retrograde fashion to regulate GABAergic input (Jennings et al., 2001; Jennings et al., 2003).

Cannabinoid Agonist inhibits the VT-induced enhancement of clasping and neuronal activity

This current study is the first report of cannabinoids interacting with the behavioral or electrophysiological effect of VT or VP in any species. Our behavioral experiments revealed that administration of CP-55940 blocked VT-induced enhancement of clasping, without affecting locomotor activity. Our electrophysiological studies showed that the prior treatment of CP-55940 resulted in drastically altered responses to VT. When administered alone, VT enhanced spontaneous and sensory-induced activity in over 75% of neurons. In contrast, prior treatment with CP-55940 resulted in severely reduced neuronal responses to VT, increasing in activity levels by a mere 5 – 15%. Furthermore, the combined treatment of CP/VT increased the number of neurons showing decreased sensory-responsiveness to the onset of clasp-generating stimuli. That pretreatment with CP blocked VT-induced enhancement of neuronal activity is consistent with the behavioral results. Taken together, these data suggest that CP-55940 administration interferes with sensorimotor processing in the neuronal circuits that are activated by sexually relevant somatosensory stimulation.

There are potentially two ways that cannabinoids can interfere with VT enhancement of clasping. Cannabinoids could act by either regulating the release of the endogenous peptide or by suppressing the downstream effects of the released peptide. An in vitro study of rat hypothalamic slice preparation provides evidence to support the idea that cannabinoids regulate release of peptide (Di et al., 2003). Whole cell recordings were made from parvocellular neurons revealing that these neurons release cannabinoids which travel retrograde to inhibit presynaptic input. Single-cell reverse transcription-PCR analysis revealed that a proportion of the parvocellular neurons were vasopressinergic. Therefore, in the rodent hypothalamic paraventricular nucleus, endogenous cannabinoids act to regulate vasopressinergic neuron activity, and ultimately regulate the release of VP. In contrast, our current study provides evidence to support the idea that cannabinoids act by suppressing the downstream effects of the released peptide because administered cannabinoids blocked the enhancing effect of subsequently administered VT.

CONCLUSIONS

Administration of VT to male *Taricha* increases the incidence of clasping. The behavioral effect of VT is reflected in the medullary neuron responses, whose activity is noticeably enhanced by VT. That exogenous VT enhanced behavioral and neural output is consistent with past studies in *Taricha* and other vertebrates. Administration of exogenous

cannabinoid agonist to male *Taricha* reduces the incidence of clasping. However, in contrast to VT neural effects, cannabinoid agonist applied to the medulla results in mixed neuronal responses that most likely reflect cannabinoid agonist binding and altering both glutamatergic and GABAergic release.

The unique result from our study is that exogenous cannabinoid agonist and VT interact to alter a sex behavior and the neuronal regulation of that behavior. VT-induced enhancement of clasping is blocked when male *Taricha* receive a prior injection of cannabinoid agonist. This interaction observed at the level of the whole organism is reflected in the medullary neuron responses; the pronounced VT-induced enhancement of spontaneous and sensory-induced activity is completely blocked, if not reversed, by the prior administration of cannabinoid agonist. Past electrophysiological studies (Lewis and Rose, 2002; Rose et al., 1995; Rose et al., 1998; Rose and Moore, 1999; Rose and Moore, 2002) suggest that the medulla plays an important role in regulating responses to somatosensory stimulation, and ultimately the performance of clasping behavior. Cannabinoids appear to act on medullary neurons altering their response to VT. The current study is unique because it focuses on the actions of the cannabinoid system on neural circuits that regulate sexual behaviors, and is the first study of its kind to assess cannabinoid role in regulating peptidergic effects on behaviors.

CHAPTER 6: CONCLUSIONS

The major findings of this thesis are as follows. First, the experience of courtship clasping and/or the administration of a peptide (VT), that enhances courtship behaviors, block the suppressive effects of a stress hormone, CORT. Second, the suppressive effects of CORT on newt courtship are best explained by CORT acting on the sensorimotor processing pathway associated with somatosensory input and clasping motor output. Third, the neuroendocrine pathway controlling stress-induced suppression of reproductive behaviors involves endocannabinoid activity at CB_1 receptors, suppressing medullary neuron responses to sexually relevant somatosensory information. Fourth, exogenous cannabinoid agonist and VT interact to alter a sex behavior and the neuronal regulation of that behavior, whereby VT-induced enhancement of clasping is blocked when male *Taricha* receive a prior injection of cannabinoid agonist.

Results from the current thesis confirm previous studies of male *Taricha* and consistently show that the administration of CORT alone rapidly suppresses the incidence of courtship clasping. Electrophysiology studies demonstrate that CORT suppresses neuronal and behavioral responses to somatosensory stimulation of the male cloacal area, the artificial stimulation of which models somatosensory events during courtship (Lewis and Rose, 2003; Rose et al., 1995; Rose et al., 1998). Results reported in Chapter 3 of this thesis, indicate that CORT does not alter appetitive responses of males towards visual or olfactory sexual stimuli. This effect of CORT is in contrast to VT which does alter appetitive responses of males towards all three sensory modalities: visual and olfactory (Thompson and Moore, 2000) and somatosensory (Lewis and Rose, 2002; Rose et al., 1995; Rose and Moore, 2002). The suppressive effects of CORT on newt courtship are best explained by CORT acting on the specific sensorimotor processing pathway associated with somatosensory input and clasping motor output.

The study reported in Chapter 2, is the first to show that the behavioral or physiological state modifies the effects of CORT administration on sexual behavior. CORT-induced suppression of clasping is blocked by previous experience of courtship clasping or exposure to VT. These data are consistent with electrophysiological studies showing that prior administration of VT blocks the CORT-induced suppression of the medullary neurons that regulate clasping (Rose et al., 1995). This effect occurs within a discrete and specific temporal arrangement; the temporal pattern of each hormone's arrival codes for context and

therefore determines which behavioral output is most appropriate (Fig 6.1). In this way, a hormone's message has multiple translations. That CORT administration can elicit a variety of behaviors within one individual is consistent with the observation that animals respond to potential threats with context-dependent behavioral responses.

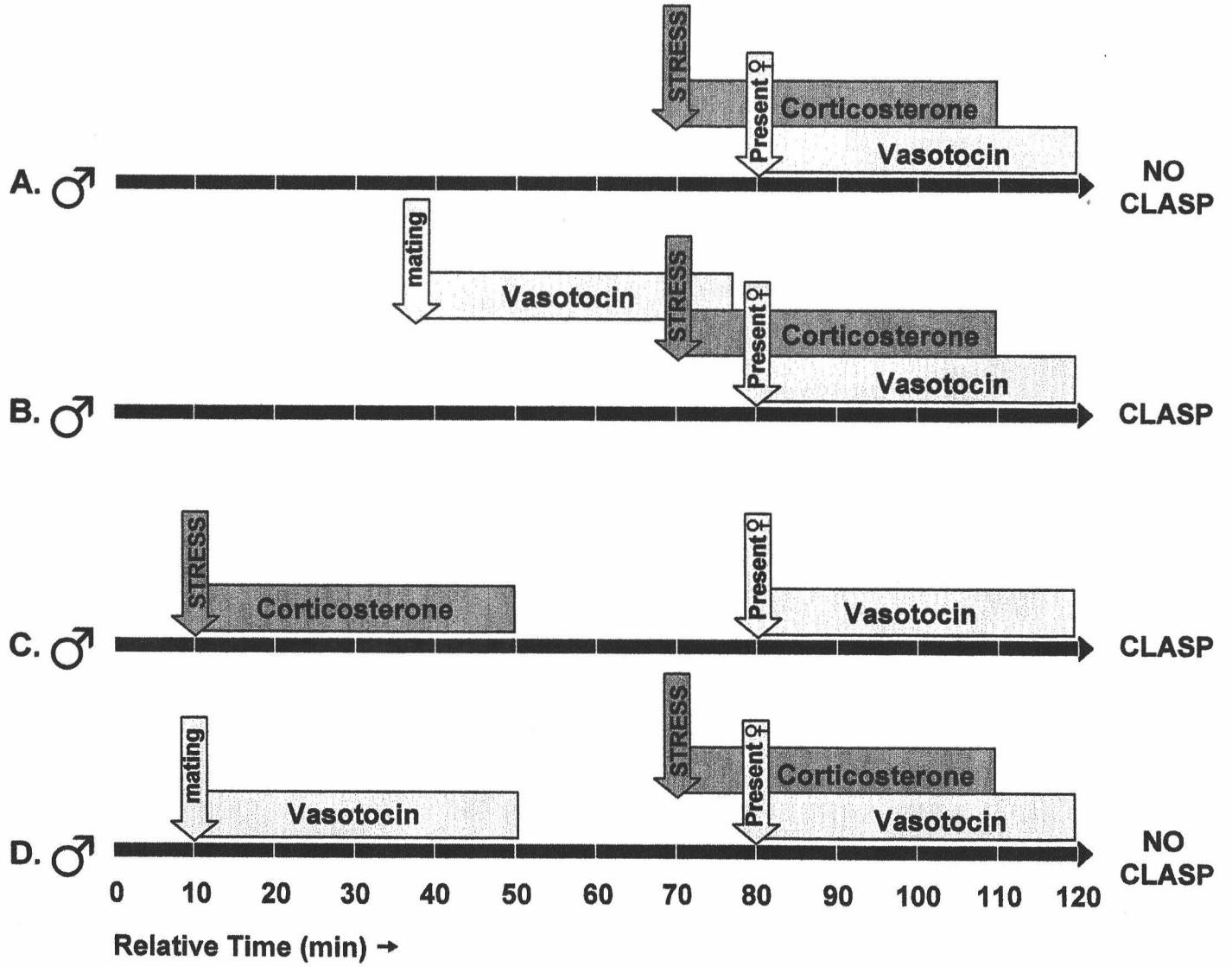
Together the aforementioned studies provide strong evidence that behavioral responses to acute stress are context-dependent, and context is transduced by fluctuations in endocrine signals, in the case of *Taricha*, CORT and VT. The next objective central to this thesis sought to determine the nature of the interaction between VT and CORT. Several observations from research on *Taricha* and rodents lead to the suggestion that endocannabinoids might regulate the interaction between VT and CORT. We hypothesized that endocannabinoids influence behavioral output by regulating the functional effects of endocrine inputs, such as VT and CORT. Results from Chapter 4 and 5, are consistent with this hypothesis.

This thesis supports our correlative hypothesis that stress-induced and CORT-induced suppression of sex behavior requires signaling of endocannabinoids (eCB) downstream in the temporal sequence of events initiated by CORT. This conclusion is based on behavioral and electrophysiological studies in Chapter 4 that showed that cannabinoid CB_1 receptor antagonist, AM 281, blocked stress- and CORT-induced suppression of clasping. That glucocorticoids exert their rapid inhibitory effect via upregulating endocannabinoid signaling has been shown in parvocellular neurons of the PVN hypothalamus (Di et al., 2003). Given this precedent, it seems likely that signaling by endocannabinoids might be a conserved mechanism by which CORT exerts an inhibitory effect.

Behavioral and electrophysiological experiments described in Chapter 5 reveal that exogenous cannabinoid agonist blocks VT-induced enhancement effects. Administration of a cannabinoid agonist similarly blocks VT-induced enhancement of clasping behavior. In vivo single-unit recordings of medullary neurons that regulate the motor control of clasping showed that application of cannabinoid agonist blocks the VT-induced enhancement of medullary neuron activity and sensory responsiveness. This is the first report of cannabinoids interacting with the behavioral or electrophysiological effect of VT or VP in any species. We propose that the mechanism, by which cannabinoids interfere with VT-induced enhancing effects on clasping, is by acting at the level of the rostroventral medulla to block VT's ability to enhance sensory processing.

Figure 6.1. Time line illustrating temporal requirement for CORT and VT interaction and the resultant behavioral outcome. In this figure a behavioral experience is represented by vertical arrows, with the particular experience highlighted in that arrow. The resultant endocrine change is illustrated in the connected horizontal bars. The length of the horizontal bars represents the amount of time that the hormone remains elevated after an experience. Note that the time CORT remains elevated has been verified in a number of different behavioral and electrophysiological experiments. The time that VT remains elevated after experiencing clasping is estimated based on the length of time VT effect is apparent when applied directly to medulla (Rose et al., 1995; Coddington dissertation Chapter 5). **A** and **B** illustrate scenarios that have supporting data from work in this thesis and by others (Rose and Moore, 2002). **C** and **D** are predicted outcomes if the experience of acute stress (elevated CORT) or mating (elevated VT) is too far separated in time from the immediate situation.

Figure 6.1

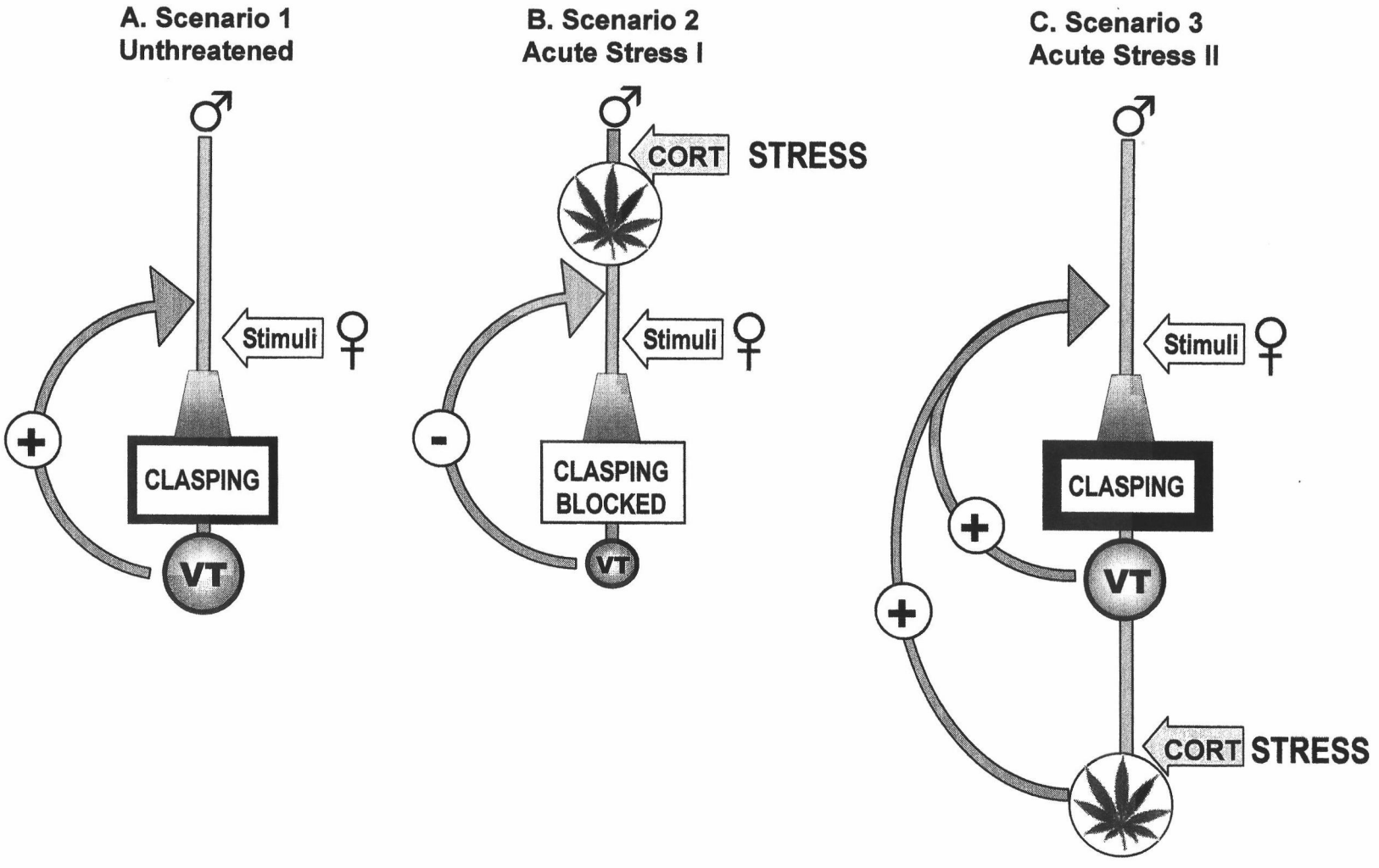

STRESS
Present ♀
Corticosterone
Vasotocin
A. ♂
NO CLASP
mating
Vasotocin
STRESS
Present ♀
Corticosterone
Vasotocin
B. ♂
CLASP
STRESS
Corticosterone
Present ♀
Vasotocin
C. ♂
CLASP
mating
Vasotocin
STRESS
Present ♀
Corticosterone
Vasotocin
D. ♂
NO CLASP
0 10 20 30 40 50 60 70 80 90 100 110 120
Relative Time (min) →

Taken together the behavioral and electrophysiological results are consistent with the hypothesis that endocannabinoids mediate the interaction between VT and CORT. I propose that the site of action for the VT/CORT interaction is on neurons in the rostroventral medulla based on the following evidence: The results from behavioral and electrophysiological experiments (described in Chapter 2 and prior studies) consistently report that CORT blocks VT-induced enhancement effects. Cannabinoid antagonist blocks the CORT-induced inhibition of medullary neurons in the same preparation (Chapter 4), and VT action on the medulla is blocked by prior treatment with cannabinoid agonist (Chapter 5). Finally, CB_1 receptors have been localized to the rostroventral medulla region of *Taricha* (Hollis, Coddington, and Moore, in preparation).

Based on data from this thesis I have developed a model illustrating the predicted endocrine cascade that would occur to a male *Taricha* under three different scenarios (Figure 6.2A-B). The first scenario, Figure 6.2A, illustrates the endocrine cascade that would occur to an unthreatened *Taricha*, where the male recognizes a sexually attractive conspecific and engages her in an amplectic clasp. His experience of courtship clasping would result in an increased release of endogenous VT, and would in turn provide positive feedback to the medullary circuits regulating the clasp. Under increased exposure to VT, the medullary circuits would enhance excitatory descending input to the clasp motor centers in the spinal cord. In scenario 2 and 3, the experience of acute stress would elevate endogenous levels of CORT and to increased signaling by endocannabinoids in the rostroventral medulla. In scenario 2 (Figure 6.2B), if the acute stress is experienced prior to detecting a female then mating is suppressed due to endocannabinoids interfering with processing of somatosensory stimuli. Note that, according to data from Chapter 3, the male would still exhibit appetitive behaviors towards the female's visual and olfactory cues, but would not respond to somatosensory input required to initiate clasping. Under this second scenario, if there is an elevation of VT, it is occurring after CORT and endocannabinoid elevation. Based on data from Chapter 2 and 4 and prior studies (Rose et al., 1995), if CORT elevation precedes VT elevation then CORT-induced suppression of clasping and medullary neuron activity is potentiated. However in the third scenario (Figure 6.2C), if the acute stress is experienced after he has engaged with the female then mating is predicted to be enhanced. In this third scenario, VT is elevated prior to stress-induced elevation of CORT. According to results in Chapter 2 and Rose et al. (1995), the combination of VT followed by CORT is predicted to potentiate the VT-induced enhancement of medullary neuron activity. Based on the current thesis, we also predict that if VT elevation occurs prior to stress-induced elevation of

Figure 6.2. The predicted endocrine cascades that would occur in a male *Taricha* under three different scenarios. The vertical bar represents the temporal sequence of events and hormone changes as they occur from the beginning (top), including feed back in first and third scenarios. **A. Scenario 1 Unthreatened:** Illustrates the endocrine cascade of an unthreatened male. **B. Scenario 2 Acute Stress I:** The endocrine cascade if the male experiences stress-induced elevation of CORT prior to encountering a female. **C. Scenario 2 Acute Stress II:** The endocrine interactions if the male experiences clasping prior to stress-induced elevation of CORT. Sensory or hormonal inputs are represented by horizontal arrows. Elevated endocannabinoid signaling is represented by cannabis leaf within a circle. Processing of sensory, hormone, and neural inputs is represented by Δ within a circle. Clasping output is indicated in a box below processing symbol; the thickness of the box indicates the predicted intensity of clasping. Feedback by various hormones or endocannabinoids is indicated with loop arrows.

Figure 6.2


A. Scenario 1
Unthreatened
Stimuli
CLASPING
VT
+
B. Scenario 2
Acute Stress I
CORT
STRESS
Stimuli
CLASPING
BLOCKED
VT
-
C. Scenario 3
Acute Stress II
Stimuli
CLASPING
VT
+
+
CORT
STRESS

endocannabinoid signaling, then VT-induced enhancement of clasping and activity of medullary neurons is potentiated.

The behavioral function of endocannabinoids is revealed when examined within the context of the well-defined neuroendocrine system of *Taricha*. Current evidence from in vitro studies in rats and mice supports the hypothesis that endocannabinoids function primarily as modulators of cellular processes, modulating the excitatory (Glutamate) and inhibitory (GABA) input to a primary output neuron. They do not work in isolation as a regular neurotransmitter, rather, function by gain-setting the principle neuron for reception of excitatory or inhibitory input. In the *Taricha* model, VT enhances, while CORT inhibits clasping behavior. Their action converges onto the same neural system controlling clasping output, the rostromedial medullary neurons. We conceptualize the medullary circuits to function as coincidence detectors, the mode of output (to clasp or not to clasp) determined by the precise timing and composition of presynaptic input. Furthermore, we hypothesize that these coincidence detectors do not passively absorb input; rather, they must be actively modulating their own input as principle neurons of hippocampus and cerebellum do during processes such as LTP, LTD, DSI, and DSE. In each case the principle neuron acts as a coincidence detector, and rapidly regulates its own input by release of retrograde signals. In the *Taricha* model the principle neurons, rostroventral medullary neurons, receive a variety of stimulatory and inhibitory input, for example, from somatosensory sources (cloacal stimulation), and endocrine sources (VT or CORT). The precise temporal as well as spatial combination of these inputs results in the precise pattern of output by rostromedial medullary neurons. These rostromedial medullary neurons, therefore, function as coincidence detectors utilizing endocannabinoids to modulate their own input, thereby regulating clasping behavior.

In order to function as a dynamic coincidence detector the medullary neuron must be able to strengthen (LTP) or weaken (DSI, DSE, LTD) activity at a synapse. This feature results in the capacity to weight incoming information; the impact of any given input will vary with the state of the neuron and the sum of all the synaptic inputs. Behavioral biologists have long known that behavioral responses can be context-specific, and neurobiologists understand that neuronal responses can be state-specific. For example, neurons in escape circuits well respond differently when an insect is walking than when it is flying. Given that the neural state of a circuit will vary with behavioral state of the animal, it follows that synaptic events such as DSE, DSI, LTP or LTD that are mediated by endocannabinoid retrograde signaling contribute to context-specific behaviors. The data from this thesis are

consistent with the prediction that endocannabinoids play a pivotal role in transducing internal and external environmental stimuli onto neural systems that control the behavioral output of clasping behavior.

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